

**VIRUS CONCENTRATION BY CROSSFLOW MEMBRANE
FILTRATION: EFFECT OF HYDRODYNAMIC CONDITIONS
AND MEMBRANE PROPERTIES**

By

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ABSTRACT
**VIRUS CONCENTRATION BY CROSSFLOW MEMBRANE
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Crossflow membrane filtration is emerging as a concentration method for the recovery and detection of viruses in large volumes of water. To increase virus recovery, the membrane is usually “blocked” with a proteinaceous solution or a chemical dispersant. This study explores a novel approach using controlled and rapid (<1 h) layer-by-layer adsorption of polyelectrolytes to form an anti-adhesive, sacrificial layer on the membrane surface. Membranes coated with polyelectrolyte multilayers (PEMs) can ensure up to 2-fold higher pre-elution recoveries of P22 bacteriophage than membranes blocked with calf serum (CS). Calculations of virus-membrane interaction energies confirm that the higher recoveries with PEM-coated membranes are due to higher charge and hydrophilicity relative to the CS-blocked counterpart. The pre-elution recovery with PEM-coated membranes at high cross flow rates is >70%, which might eliminate the need for elution and backflushing steps, thus reducing both the time required for pathogen concentration and the volume of the final sample. Operating the filter at high transmembrane pressures and crossflow rates leads to a significant increase in the concentration rate; for example, for CS-blocked membranes the initial rate of $5.8 \pm 0.3 \text{ L}/(\text{min} \cdot \text{m}^2)$ was achieved without affecting P22 recovery; this flux value is almost 4 times higher than values reported in the literature. In addition, the effects of crossflow and high permeate flow rate on the recovery and removal of infective virus from DI and surface water are evaluated using the CS-blocked membrane. An increase in the average permeate flux (up to 5-fold for DI water and 2-fold for

surface water over values reported in literature) does not affect the recovery or removal of infective P22. Finally, the performance of the anti-adhesive PEM membrane coatings with complex water matrices is evaluated. The data are only preliminary as only a small number of filtrations have been performed but results seem to indicate that PEM-coated membranes can maintain their advantage over CS-blocked membrane in terms of P22 pre-elution recovery when concentrating viruses from MBR effluent. When challenged by MBR effluent, PEM-coated membranes also appear to be more resistant to fouling than their CS-blocked counterparts. With surface water however, no difference was observed.

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ABBREVIATIONS

AFM	atomic force microscopy
BE	beef extract
BSA	bovine serum albumin
CHI	chitosan
CN	cellulose nitrate
CS	calf serum
DE	diatomaceous earth
DI	deionized
DLVO	Derjaguin, Landau, Verwey and Overbeek
E1	echovirus 1
FLOC	flocculation
HA	hyaluronic acid
HAdV	human adenovirus
HAV	hepatitis A virus
HE	heparin
HSA	human serum albumin
IEP	isoelectric point
J_{cf}	crossflow rate
J_p	permeate flux
LRV	log removal

MBR	membrane bioreactor
MNoV	murine norovirus
MWCO	molecular weight cut off
NaPP	sodium polyphosphate
NOM	natural organic matter
NoV	norovirus
PAA	poly(acrylic acid)
PAAm	polyacrylamide
PAH	poly(allylamine hydrochloride)
PAN	polyacrylonitrile
PDADMAC	poly(dimethyldiallylammonium)
PEG	poly(ethylene glycol)
PEI	polyethyleneimine
PEM	polyelectrolyte multilayer
PGA	poly(L-glutamic acid)
PLL	poly(L-lysine)
PSU	polysulfone
PV	poliovirus
QCM	quartz crystal microbalance
RV-A	group A rotavirus
SW	sewage
TW	tap water
XDLVO	extended Derjaguin, Landau, Verwey and Overbeek

Chapter 1. Introduction

1.1 Waterborne pathogen collection and quantification: importance and challenges

1.1.1 Waterborne disease outbreaks

Waterborne diseases are infections transmitted through contact with or consumption of contaminated waters. The main etiological agents are bacteria, protozoa and viruses, and the main reservoirs of concern are recreational waters and surface and groundwater used as sources of drinking water. Progress in drinking water treatment and regulation since the beginning of the 20th century [1] led to a decrease in the occurrence of waterborne disease outbreaks from exposure to contaminated drinking water. As a result, such outbreaks have become relatively uncommon in the U.S. and other developed countries [2]. For example, the Center for Disease Control listed on average 18 disease outbreaks associated with drinking water per year for the 1999-2008 period in the United States (actual outbreak occurrences might have been higher as it has been suggested that only 10% to 50% of outbreaks are actually reported [1]). These outbreaks resulted in an average of about 1,000 sick individuals and 2 deaths per year [3-7]. Since 2000, approximately 15% of the outbreaks for which the etiological agent was identified have been caused by virus contamination [4-7]. In contrast, occurrences of waterborne disease outbreaks caused by exposure to contaminated recreational water in the U.S. are on the rise from an average of 31 outbreaks resulting in about 1,200 illness per year for the 1999-2004 period to an average of 53 outbreaks resulting in about 4,600 illness per year for the 2005-2008 period [3, 8-11]. Virus-associated outbreaks accounted for 3% to 10% of the events for which the

etiological agents has been identified, with norovirus being the most prevalent viral etiological agent identified. A recent review of all published literature on virus-associated waterborne disease outbreaks also found norovirus to be the largest cause of outbreaks with adenoviruses and echoviruses coming in second and third position, respectively [12]. In swimming pools, where almost half of outbreaks occurred, inadequate disinfection was often the culprit. In lakes and ponds, the second largest affected recreational type of water, water quality surveillance is the most effective means of preventing outbreaks [12].

1.1.2 Virus detection: Needs, challenges and current methods

Detection of waterborne pathogens is essential because: i) although deficiencies of the water treatment system can be minimized, zero risk cannot be achieved [13] and the consequences of one incident can be very large such as in the cases of the 2000 Walkerton, ON outbreak that affected over 2,000 people and killed 6 [14]; or the 1993 Milwaukee outbreak where more than 400,000 were affected [1, 15, 16]. And as the number of people more susceptible to infection (individuals with weak immune systems) increases [2], the extent of these consequences can be expected to increase. ii) The detection of pathogen in recreational water is a key factor in preventing outbreaks through this route of exposure [12, 17]. iii) As direct and indirect water reuse increases [18, 19], the potential for drinking water contamination increases and so does the need for monitoring the microbiological quality of water. This is especially true for viruses as several studies have shown that viruses were more resistant than bacteria with respect to environmental conditions and other stressors including disinfection [2]. Infective viruses have been found in 5% of groundwater samples taken from 35 different states

[20] and were also found in drinking water that met bacteriological standards and contained an adequate level of chlorine [1, 2, 21].

The methods currently employed to concentrate and quantify pathogens in water are complex and time consuming. The first difficulty is that there is currently no approved method to simultaneously collect and concentrate all microorganisms [22, 23]. The VIRADEL (VIRus Adsorption-ELution) method, which is the EPA-approved method of virus collection, involves the filtration of a large sample (up to 1,000 L for drinking water) with a microfilter either positively charged [24-33] or negatively charged [26, 30, 32, 34-36]. Positively charged filters are usually favored as the use of negatively charged ones requires the acidification of the sample at a pH of 3.5 and sometimes the addition of multivalent cation salts [32, 36], which might affect virus integrity and infectivity [37]. During filtration, viruses are adsorbed by the filter (mostly through electrostatic interactions) and are later eluted using a basic 1.5% to 6% beef extract (BE) solution and/or 0.05 M glycine solution. If further volume reduction is required, this step can be followed by flocculation to precipitate viruses [32]. The method greatly differs from methods of bacteria collection and protozoa collection. The former usually involves collection of bacteria at the surface of a microfiltration membrane [38, 39] while the latter includes depth filtration followed by elution with a detergent solution and centrifugation [40].

Table 1.1: Virus recovery using the VIRADEL method

Virus	Water ¹	Flocculation step	Sample amendment	Filter (+/-) ² / Treatment ³	Elution	Recovery (%)	Ref.
coliphage	TW	no	NA	double 1-MDS (+)	3% BE ⁶ , pH 9	12 ± 6	[25]
coliphage	TW	no	NA	CN ⁴ (-)/PEI	3% BE, pH 9	44 ± 8	[25]
coliphage	TW	no	NA	DE ⁵ (-)/Nalco	3% BE, pH 9 – drop by drop	103 ± 7	[25]
coliphage	SW	no	NA	DE (-)/Nalco	3% BE, pH 9 – drop by drop	71 ± 15	[25]
coliphage	SW	no	NA	double 1-MDS (+)	3% BE, pH 9	39 ± 5	[25]
coliphage	SW	no	NA	DE (-)/Nalco	3% BE, pH 9	47 ± 15	[25]
coliphage	SW	no	NA	Double 1-MDS (+)	3% BE, pH 9.5	14	[30]
coliphage	SW	no	NA	50 S Zeta Plus (+)	3% BE, pH 9.5	14	[30]
coliphage	SW	no	pH 3.5/ AlCl ₃	Filterite (-)	3% BE, pH 9.5	17	[30]
coliphage	sea	no	Prefiltered	cellulose ester	1.5% BE, pH 9	27	[37]

Table 1.1 (cont'd)

MS2	TW	no	NA	DE (-)/FeCl ₃ + AlCl ₃	3% BE pH 9	53 ± 12	[41]
PV1	TW	no	NA	Nanoceram (+)	1.5 % BE/0.05M Gly ⁷ pH 9	51 ± 26	[27]
PV1	TW	no	NA	1-MDS (+)	1.5 % BE/0.05M Gly pH 9	67 ± 6	[27]
PV1	TW	no	NA	MK (+)	3% BE/0.05M Gly pH 9.5	101 ± 57	[28]
PV1	TW	no	NA	50 S Zeta Plus (+)	0.3% BE/.05 M Gly, pH 9.5	56 ± 8	[29]
PV1	sea	yes	pH 3.5/ AlCl ₃	epoxy fiberglass (-)	6% BE pH 10.5	36 ± 5	[34]
PV3	TW	yes	NA	glass wool	3% BE, 0.5M Gly, PH 9.5	98 ± 24	[42]
HNoV	TW	no	25 mM MgCl ₂	cellulose esters (-)	0.5 mM H ₂ SO ₄ /1mM NaOH	55 to 104	[26]
HNoV	river	no	25 mM MgCl ₂	cellulose esters (-)	0.5 mM H ₂ SO ₄ /1mM NaOH	11 to 18	[26]
HNoV	TW	no	NA	1-MDS (+)	3% BE pH 9	14 to 46	[26]

Table 1.1 (cont'd)

HAdV	TW	yes	NA	glass wool	3% BE, 0.5M Gly, PH 9.5	28 ± 14	[42]
HAdV	GW	yes	NA	glass wool	3% BE, 0.5M Gly, PH 9.5	8 ± 4	[42]
HAdV	TW	no	NA	MK (+)	1% BE, 0.25 N Gly, pH 9.5	0.007 to 0.02	[24]

¹ type of water: TW: tap water, SW: sewage, GW: groundwater

² filter surface charge

³ filter pretreatment

⁴ CN: cellulose nitrate

⁵ DE diatomaceous earth

⁶ BE: beef extract

⁷ Gly: glycine

The VIRADEL method also suffers, in some cases, from poor recovery efficiencies and large standard deviations (Table 1.1) [23]. For example, Borrego et al. observed that less than 35% of the indigenous *Escherichia coli* phages present in tap water spiked with sewage were adsorbed on the Virosorb 1-MDS filter (the only electropositive filter suggested by EPA for the VIRADEL method), and less than 12% were recovered by elution [25]. Low elution recovery number were also observed from Abdelzaher et al. who only eluted up to 35% of the coliphage spiked in seawater and adsorbed on a negatively charged cellulose membrane [37]. The method is also very sensitive to water quality. Haramoto et al. showed that recovery of norovirus (NoV) spiked in surface water was 80% less than the recovery observed when NoV was spiked in deionized water [26]. This leads to unacceptably low recoveries for certain kinds of viruses such as adenovirus for which recovery as low as 0.02% has been reported [24]. Several groups have investigated other positively charged filters as alternatives to Virosorb microfilters, because of Virosorb's high cost and vulnerability with respect to clogging by colloids [25, 27, 28, 30, 31, 43]. The MK filter was less efficient (lower recovery and greater variability) [28], while Zeta Plus 50S, which seems as efficient as Virosorb to recover virus from various type of water, was not available as a cartridge and thus limited to smaller water volumes [30, 31]. Nanoceram, a cheaper positively charged filter was found to be as efficient as Virosorb [27]. Negatively charged filters made of diatomaceous earth (DE) treated with cationic polymer solution (such as Nalco 7111) showed improved coliphage recovery from sewage and sewage spiked tap water samples [25] when compared with Virosorb filters, but unless a drop by drop elution was performed, coliphage recovery was still less than 50% for sewage effluent samples (data not available for tap water samples).

Other methods such as ultracentrifugation [44, 45], centrifugal ultrafiltration [46], flocculation-re-dissolution-ultrafiltration [47], and tangential filtration have also been investigated (Table 1.2). Tangential filtration (also known as crossflow filtration) has emerged as the most promising alternative to VIRADEL [24, 48-54] due to its ability to concentrate large volumes of water relatively fast. Centrifugation-based methods are still of interest for secondary concentration purposes. The crossflow filtration method has been shown to give significantly better results than the VIRADEL method [48] and can also be used to collect and concentrate bacteria and protozoa [55-61]. Crossflow ultrafiltration also presents the advantage of maintaining virus integrity as, ideally, virions remain suspended in the retentate throughout the concentration process. At the same time, low molecular weight solutes, which can potentially inhibit qPCR detection, can pass through the membrane and are not concentrated [22]. In practice, hollow fiber modules have been the most commonly used membrane configuration [48, 50, 51, 60, 61, 65, 66]. These filters have the advantage of a high surface area to volume ratio allowing practical transport of the concentration device to the filtration site if needed and filtration of large volumes of water in a relatively short amount of time. They can also be reusable, thus making the procedure more cost-effective.

Table 1.2: Virus recovery by methods other than crossflow filtration or VIRADEL

Virus	Water ¹	Method	Recovery (%)	Ref.
RV-A	SW	Ultracentrifugation	34-60	[45]
Qbeta	TW	Electronegative membrane vortex ²	26-67	[62]
HAdV 2	eluates	Centrifugal filtration	33 ± 14	[63]
PV1	eluates	Centrifugal filtration	95 ± 5	[63]
Viruses ³	sea	ultracentrifugation	76%	[64]
Viruses ³	sea	flocculation	22%	[64]

¹ type of water: TW: tap water, SW: sewage

² i) Metallic cations are added to water samples. ii) Samples are then filtered on electronegative microporous membranes. iii) Membranes are then vortexed in presence of an elution buffer. iv) the elution buffer is centrifuged to separate membrane debris from viruses in suspension

³ enumerated by epifluorescence and consequently species were not identified (other virus were enumerated by plaque assay or qPCR)

Nevertheless, crossflow filtration also requires that the membrane surface be covered or “blocked” by a layer of proteins (beef extract, calf serum, bovine serum albumin) or a chemical dispersant (NaPP) [52, 57, 58] to improve recovery. Protein-blocked membranes might be prone to contamination during storage and transport [57]. In addition, values and reproducibilities of recoveries of certain viruses such as HAdV remain low (Table 1.3) [24].

Table 1.3: Virus recovery by crossflow filtration

	Virus	Water¹	Membrane	Blocking	Recovery (%)	Ref.
Phages	MS2	DIW	15-20 kDa PSU	no blocking	4.5 ± 2.9	[58]
	MS2	DIW + E-coli	15-20 kDa PSU	no blocking	29.5 ± 7.1	[58]
	MS2	DIW + E-coli	15-20 kDa PSU	3 % BE	54.3 ± 23.8	[58]
	MS2	TW	15-20 kDa PSU	0.1 % NaPP	53 ± 13	[61]
	MS2	TW	15-20 kDa PSU	5% CS	108 ± 16	[57]
	MS2	TW	15-20 kDa PSU	5% CS	120 ± 22	[56]
	T1	surface water	50 kDa PAN	5% CS	73 ± 17	[60]
	PP7	surface water	50 kDa PAN	5% CS	62 ± 5	[60]
Animal-viruses	MNoV	DIW	15-20 kDa PSU	no blocking	2.0 ± 1.5	[58]
	MNoV	DIW + E-coli	15-20 kDa PSU	no blocking	5.3 ± 6.2	[58]
	MNoV	DIW + E-coli	15-20 kDa PSU	3 % BE	63.3 ± 29.7	[58]
	NoV	surface water	ND	0.1% BSA	<0.95	[24]
	HAdV	surface water	ND	0.1 % BSA	3.18 – 6.02	[24]
	E1	TW	15-20 kDa PSU	0.1% NaPP	49 ± 15	[61]
	PV1	DIW	10 kDa	no blocking	16.9 - 17.8	[52]
	PV1	DIW	10 kDa	3% BE	49.6 – 76.8	[52]

¹ type of water: DIW: deionized water, TW: tap water, SW: sewage

1.2 Polyelectrolyte multilayer films as anti-adhesive recoverable membrane coatings

1.2.1 Polyelectrolyte multilayer (PEM) films

PEM films consist of alternating layers of polycations and polyanions. They can form by the simple alternating adsorption of oppositely charged polyelectrolytes on a substrate (layer by layer assembly) through electrostatic interactions [67]. The formation of PEM films through H-bonding has also been shown [68]. By choosing different polyelectrolytes to build the film and by varying the conditions of polyelectrolyte deposition (e.g., pH and ionic strength of the polyelectrolyte solution), films with a wide range of surface charge, hydrophilicity, swellability, and stiffness [69-74] have been prepared. PEM films have been deposited onto porous supports to prepare nanofiltration membranes with unusually large water permeabilities [75-78] but also as anti-adhesive coatings on filtration membranes [79, 80] or other surfaces [68, 72, 81-90], to decrease the adsorption of one or several proteins [68, 72, 80-83, 91, 92], selected mammalian cells [68, 72, 84-88], or selected bacteria [81, 84, 89, 90].

1.2.2 Anti-adhesive performance of PEMs

Meier-Haack and Muller [83, 91] achieved a 10-fold decrease in the amount of human serum albumin (HSA) irreversibly adsorbed on a polypropylene microfiltration membrane during filtration by coating the membrane with a PEM composed of poly(dimethyldiallylammonium) (PDADMAC) and poly(acrylic acid) PAA; coating with the PEM increased HSA rejection but did not impair the filtration flux. Mendelsohn et al. showed that murine fibroblast cell adhesion

on a substrate was completely eliminated by coating the surface with ten poly(allylamine hydrochloride) (PAH)/PAA bilayers. Yang et al. achieved the same result with only one bilayer of PAA/polyacrylamide (PAAm), and the film anti-adhesive properties were preserved for more than a month [68, 72]. Lichter et al. have demonstrated that a PAH/PAA coating on a titanium alloy also decreased bacterial adhesion (*Staphylococcus epidermis* and *Escherichia coli*) by almost two orders of magnitude [89].

PEMs built using natural polypeptides and polysaccharides have been of particular interest in the field of biomedicine. Richert et al. used hyaluronic acid (HA) and chitosan (CHI) to form PEM films that decreased rat chondrocyte adhesion by more than 90% and bacterial adhesion (*E. coli*) by approximately 80% [84]. Poly(L-lysine) (PLL)/alginate films and heparin(HE)/CHI films also demonstrated anti-adhesive properties for fibroblast cells and *E. coli*, respectively [86, 90].

The mechanisms involved in the adhesion of proteins, mammalian cells and bacteria are not fully understood; however, we do know that although proteins play a large role as promoters in mammalian cell and bacteria adhesion, there is not always a correlation between protein adhesion to a surface and mammalian cell or bacteria adhesion to the same surface [72]. Protein adhesion onto PEM films is mostly regulated by electrostatic forces. In conditions where proteins are negatively charged they tend to adsorb a lot more onto a PEM that contains a polycation as the outermost layer and vice versa. In addition pH and ionic strength strongly affect this adsorption behavior by altering the protein and polyelectrolyte charge densities and screening those charges, respectively [72, 80, 81, 83, 91]. When both the protein and the top

layer have the same charge, protein adsorption is low unless the pH is close to the protein and top polyelectrolyte layer's pI, where electrostatic repulsion is low enough that hydrophobic interaction can play a role [91]; as expected in that case, increasing the ionic strength had no effect on the adsorption of proteins [82]. The effect of the charge density of the PEM top layer on adhesion can be of two types. As mentioned earlier, increasing the charge density of the PEM top layer can increase electrostatic repulsion and attraction. In addition, it can also affect the conformation of the layer [74]. At higher charge density, polyelectrolyte chains adopt a flatter conformation and form thinner layer while at lower charge density layers tend to be thicker and loopier [74]. If a condensed high charge density layer is deposited on top a loopier one, it is possible for some loops and segment from the layer before last to reach the PEM surface and affect adsorption behavior [90]. This phenomenon might explain observations by Muller et al. who used a strong polyanion with a high negative charge density, instead of weak polyanions, to build the PEM's outermost layer and saw an increase in the adsorption of the negatively charged human serum albumin (HSA) [91]. Muller et al. speculated that in the case of PEMs containing strong polyanions, strong attraction between the protein's positively charged segment (although HSA's net charge is negative, some segments are positively charged) and the polyanion lead to a reorientation of the protein to favor binding between the two. In the case of PEMs containing weak polyanions, attractive interactions between the protein's positive segments and the polyanions are not strong enough to orient the protein to allow binding and thus the repulsion between the protein's negatively charged domains and the polyanions dominate the interaction [91]. Salloum et al. also found that the use of more swellable PEM films decreased significantly the amount of protein adsorbed by the surface compared to other PEMs, including those charged similarly to the sorbing protein. In two other studies, however, no correlation between PEM

swelling and protein adsorption was found [72, 82].

The PEM film's ability to swell was also correlated with its ability to resist adhesion of mammalian cells and bacteria [68, 72, 84]. In fact, poor swellability and stiffness [87, 89], which both depend on the amount of cross-linking between the polycations and the polyanions [72, 87, 88, 92], are the only two properties of the PEM film that could be correlated with the adhesion of bacteria or mammalian cells. Neither the nature (polyanion or polycation) of the outermost layer, the surface charge density, the roughness, the wettability, the conformation of the layer (flat and smooth or thick and loopy) nor the interaction energy (estimated using the Lifshitz-van der Waals acid-base approach described by van Oss [93]) of PEM film was shown to impact cell attachment [72, 89].

To explain the correlation between cell adhesion and swellability, Mendelsohn et al. hypothesized that in solution the water rich structure of the film does not allow cell's adhesion proteins to adapt a configuration supporting cell adhesion. The environment "looks" more like water than a surface to the cell, which is why cell adhesion does not occur [72]. Lichter et al. showed that a 100-fold increase in elastic modulus resulted in approximately a 100-fold decrease in bacterial adhesion and speculated that as the surface become stiffer, bacteria fimbriae interactions with the surface are altered. More precisely, the fimbriae retraction after collision with the surface is slower with a stiffer surface because there is more mechanical resistance to the retraction; this additional time of contact between the bacteria and the surface would enhance bacterial adhesion [89].

1.2.3 PEM films as sacrificial layers

When appropriately constructed, a PEM can be disassembled by way of exposure to high [69, 94], neutral [95, 96] or low [97] pH, higher ionic strength [98-100], a specific solvent [101] or lower [102] or higher [103] temperature. These properties of PEM have been exploited to create micropatterned [95], porous [100] or even self-standing [98] PEM films. For example Dubas was able to create a free standing PSS/PDADMAC multilayer by depositing 20 bilayer of PSS/PDADMAC on top on 20 bilayer layers of PAA/PDADMAC and exposing the film to a salt concentration higher than 0.6 M. In these conditions PAA/PDADMAC dissociates while PSS/PDADMAC remains stable [98]. Sacrificial PEM layers have been considered for the controlled delivery of drugs [101], the development of easy to clean surfaces [97] and the control of membrane fouling during membrane filtration [69]. In that latter study, a PEM was deposited on a UF membrane to create a nanofiltration membrane; once fouled the PEM layer was sacrificed allowing the almost complete removal of both the fouling layer and PEM from the UF membrane surface. The PEM layer could be regenerated to create a new nanofiltration membrane [69].

1.3 Virus interaction with inert surfaces: Methods, challenges and current knowledge

1.3.1 Virus surface characterization

To understand virus interaction with surfaces, one must first gather information on the virus' own surface properties. The surface of a virion (virus particle) consists of a protein shell, called capsid and, in the case of so called enveloped viruses, of a lipid bilayer enveloping the

protein shell. Sequencing techniques permit the determination of the composition of the protein shell and theoretical surface properties (e.g. isoelectric point). Such theoretical values, however, do not always accurately predict values measured in experiments [104]. Experimental determination of virus surface properties is thus necessary. Such characterization requires that virions be separated from protein and host cell debris present in the virus growth medium. Purification methods include differential centrifugation and equilibrium gradient centrifugation. In the differential centrifugation method, high and low speed centrifugation are used alternatively to separate soluble components, large macromolecules and cell debris from virions [105]. In the equilibrium gradient centrifugation method, the suspensions of viruses is introduced into a tube where solutions of increasing density have been layered on top of each other. During the centrifugation process virions migrate to the layer corresponding to their density [106, 107]. Other methods have also been used; these include precipitation followed by centrifugation to recover the pellet [108], centrifugal filtration with multiple washings [109], multiple dialysis [110], or successive microfiltration and ultrafiltration using membranes of various molecular weight cut-off (MWCO) values [111].

Once purified, virions can be characterized. The isoelectric point (IEP), a crucial property that indicates if the particle will be positively or negatively charged at a given pH, is determined by analyzing the behavior of the particles in an electric field under different pH conditions. Two methods have been frequently employed: isoelectric focusing (IEF) and determination of the electrophoretic mobility (EM) by light scattering (ELS) or a microscopy-based method [112]. In IEF, particles are subjected to an electric field and migrate through a gradient of increasing pH. When the particles reach the pH zone corresponding to its IEP the

particle stops. In EM analysis, the electrophoretic mobility, which is the ratio of the migration velocity to the electric field, is measured at various pH values by light scattering or microscopic methods. The EM is used to calculate the zeta-potential for each pH and the pH at which the zeta-potential changes sign is the IEP.

Hydrophobicity is another surface characteristic essential for understanding virion interactions with surfaces. When particles are immersed in an aqueous solution, the free energy of cohesion of water drives particles to agglomerate to minimize their surface area exposed to water, this phenomenon is called hydrophobic attraction. Sometimes, this attractive force is attenuated or reversed when interaction between particles and water are preferred to interaction between particles; this component of the total free energy is called hydrophilic repulsion [113]. If hydrophobic repulsion prevails, the material seems to repel water and this property is called hydrophobicity. Van Oss and Giese [114] formally defined the hydrophobicity of a material i based on the interfacial free energy of interaction between two objects of the same material i when immersed in water: ΔG_{iwi} , which is expressed in terms of free energy per unit surface area and can be determined using the following equation:

$$\Delta G_{iwi} = -2 \left(\sqrt{\gamma_i^{LW}} - \sqrt{\gamma_w^{LW}} \right)^2 - 4 \left(\sqrt{\gamma_i^+ \gamma_i^-} + \sqrt{\gamma_w^+ \gamma_w^-} - \sqrt{\gamma_i^+ \gamma_w^-} - \sqrt{\gamma_i^- \gamma_w^+} \right) \quad (1.1)$$

γ_w^{LW} , γ_w^+ and γ_w^- are the surface tension parameters of water (the “+”, “-“ and “LW” superscripts correspond to the electron acceptor, the electron donor and the Lifhsitz-van der Waals components of the surface tension, respectively), and γ_i^{LW} , γ_i^+ and γ_i^- are the surface tension parameters of the material i .

When ΔG_{iwi} is negative, there is a cohesive attraction between the two objects and the material is considered hydrophobic. The magnitude of ΔG_{iwi} can be interpreted as the degree of hydrophobicity [114]. If ΔG_{iwi} is positive the material is hydrophilic. This definition is not yet adopted by the entire scientific community. Often a surface is considered hydrophilic if it is “wetted” by water that is if the water contact angle is smaller than 90° , which does not necessarily corresponds to a positive ΔG_{iwi} . For example dry biological surfaces typically have a negligible γ_i^+ and a γ_i^{LW} of approximately 40 mJ/m^2 ; for such surfaces the transition from hydrophilic to hydrophobic corresponds to a water contact angle of approximately 60° (equations 1.1 and 1.2).

The surface tension parameters of a given surface can be determined using the extended Young equation (1.2) after measuring the contact angle of three probe liquids with known surface tension parameters γ .

$$(1 + \cos\theta)\gamma_\ell^{TOT} = 2 \left(\sqrt{\gamma_s^{LW} \gamma_\ell^{LW}} + \sqrt{\gamma_s^+ \gamma_\ell^-} + \sqrt{\gamma_s^- \gamma_\ell^+} \right) \quad (1.2)$$

In these equations, θ is the contact angle for a given probe liquid and the subscripts s and ℓ correspond to the solid surface and the liquid probe, respectively. In the case of viruses [115-117] and nanoparticles in general [118, 119], contact angle measurements can be done by depositing layers of particles on a clean surface and determining the contact angles using the sessile drop method. The roughness of the deposited layer, which can be independently

determined by atomic force microscopy (AFM), and remnant contamination will affect the measured contact angle following equations 1.3 and 1.4, respectively:

$$\cos\theta_{apparent} = r \cos\theta_{theory} \quad (1.3)$$

$$\cos\theta_{apparent} = F_1 \cos\theta_1 + F_2 \cos\theta_2 \quad (1.4)$$

where θ is the contact angle, r the roughness and F_i the fractional amount of each material present in the layer. It should be noted that in two out of the three studies where the contact angle of a virus was measured [115, 116], the virus suspension was not purified before formation of the virus layer. The measured contact angles were probably significantly affected by the presence of bacterial debris and broth components within the virus deposit. Alternatively, virus hydrophobicity has also been estimated qualitatively by hydrophobic interaction chromatography [120].

1.3.2 Quantifying interactions between viruses and a surface

It is possible to directly measure the interfacial energy of interaction between a surface and a particle using AFM [121-130]. In AFM, a surface is scanned by a tip, which is a part of a flexible cantilever. The position of the cantilever depends on both surface morphology and forces between the cantilever tip and the surface. In force spectroscopy, the vertical movements of the cantilever, proportional to interaction forces, are recorded while the surface is approached and moved away from the tip [131]. A single microparticle composed of silica [121, 123, 125], alumina [121], polystyrene [121, 122], zirconia [124], lactose [127] or a bacterial spore [126], can be glued to a tipless cantilever to study the particle's interaction with various surfaces.

Cantilevers with tips have also been modified with nanoparticles: for example, Vakarelski et al. [130] fabricated a cantilever tip terminated by a single gold nanoparticle; Das et al [129] glued a nanoparticle agglomerate on a cantilever tip and Attinti et al. [117] opted to coat the cantilever tip with nanoparticles. Others chose to deposit microparticles [128] or nanoparticles [132, 133] on the surface scanned by the cantilever, and to functionalize the cantilever tip with molecules of interest such as humic acid [128].

In the field of virology, AFM has mostly been used to image viruses and investigate virus interactions with a host cell or other surfaces through imaging. For example, AFM was used to visualize the pattern formed by phages assembled on a surface and to quantify the number of viruses deposited and/or the presence of aggregates [134-138]). AFM was also used to quantify the binding force between macromolecules present at the surface of a virus capsid and other surfaces by functionalizing the AFM tip with the macromolecules of interest [139]. A few studies used AFM to quantify interaction forces between viruses and surfaces. Attinti et al. [117] studied virus-sand interaction using a virus-coated tip and Liu et al. [140] measured the force of interaction between a tip functionalized with anti-hemagglutinin monoclonal antibody and a single influenza virus deposited on a glass slide.

Virus interactions with surfaces such as clay or NOM-coated substrates have also been investigated using Quartz Crystal Microbalance (QCM) gravimetry [141-146]. By measuring very small changes in the mass of material deposited on the crystal surface, QCM enables rigorous quantification of the mass of viruses deposited over time and can be used to study the deposition kinetics. Other methods for studying virus-surface interactions include total internal

reflection fluorescence microscopy (TIRF), which has been used to study virus adsorption kinetics [147], other fluorescence microscopies [115], transmission electron microscopy (TEM) [148], and methods that employ radioactively labeled viruses [149]. Finally, in many studies the interaction of a virus with surfaces was evaluated by measuring the difference in the concentration of the virus in suspension (measured by traditional methods such as plaque assay) before and after the suspension is exposed to a surface [116, 120, 150-161]. It should be noted, however, that loss of infectivity or particle aggregation during virus interaction with the surface can affect the results of this test when an infectivity assay is selected as the detection method.

Several authors employed the DLVO (Derjaguin, Landau, Verwey and Overbeek) [115, 116, 117, 141, 142, 144, 156, 157, 162-165] and XDLVO (extended DLVO) [115, 116, 117, 156, 157] theories to describe virus attachment to surfaces. The DLVO theory predicts surface-surface interaction by taking into consideration Lifshitz-van der Waals (U^{LW}) and electrostatic double layer interactions (U^{EL}). In the XDLVO model, Lewis acid-base forces are also considered (U^{AB}). Only one study [115] determined the virus surface tension parameters γ^{LW} , γ^+ and γ^- , which allow for the rigorous determination of the Lifshitz-van der Waals and Lewis acid-base interactions; however these viruses were not purified. All but the study by Chattopadhyay et al. [116], computed van der Waals interaction based on the Hamaker method [166]:

$$U^{vanderwaals} = -\frac{A_1 32\pi p}{6h} \left[1 + \frac{14h}{\lambda} \right]^{-1} \quad (1.5)$$

where r_p is the virion radius, h the distance between the virion and the surface, λ the characteristic wavelength of the interaction and A_{132} the Hamaker constant for the particle-water-surface system. Most of these studies (7 out of 10) [117, 141, 142, 144, 157, 162, 165] used Hamaker constants based on Murray and Park's [167] estimation of the poliovirus–water-clay system which itself was based on the Hamaker constant of the polystyrene–water-clay system and the assumption that polystyrene and poliovirus shared similar dielectric properties. By following this approach, these studies did not take into account the potential specificity of each virus surface for the determination of the van der Waals interactions; however, it is true that most biological surfaces have a γ^{LW} of about 40 mJ/m²; as a consequence van der Waals interactions between viruses and surface might not differ greatly from one virus species to another [111]. Three studies [156, 163, 164] used the Lifshitz theory [168], which links the substance relative permittivity and refractive index to the Hamaker constant, to determine the virus Hamaker constant.

Three studies computed the Lewis acid-base interactions [117, 156, 157] to use the XDLVO model. They did so using Yoon's empirical approach [169], which is described in the following equation:

$$U^{AB} = -\frac{K_{123}T}{6h} \text{ with } \log K_{123} = a \left(\frac{\cos\theta_1 + \cos\theta_2}{2} \right) + b \quad (1.6)$$

where T is the temperature, h the separation distance, θ_1 and θ_2 the water contact angles of the virus and the surface and a and b are system specific constants. Yoon's approach only requires the measurement of the water contact angle rather than the 3 liquid probe contact angles necessary in van Oss' theoretical approach [113]. Bergendahl and Grasso found that Yoon's

empirical approach and van Oss's theoretical approach (described in chapter 2) gave similar trends in terms of which interactions were the most attractive; however, calculated values for Lewis acid base interaction energies were quite different; and for one system Lewis acid base interactions were found to be attractive using Yoon's approach but repulsive using the van Oss rigorous theoretical approach. In Yoon's approach, variations in water contact angles are used to calculate variations in hydrophobic interactions whereas variations in contact angles are also due to changes in Lifshitz van der Waals interactions. In van Oss' approach, measuring the contact angle of a completely apolar liquid probe (diiodomethane for example) allows the determination of the Lifshitz van der Waals interaction term independently from the Lewis acid-base term. Additionally, measuring contact angles of two polar liquid probes with different electron donor and electron acceptor characteristics allows the determination of these two parameters for the surface studied [113].

Other models have been used to estimate hydrophobic interaction. Chattopadhyay et al. [116], who defined hydrophobic interaction the same way as van Oss and Giuse [112] (i.e. hydrophobic interaction is the sum of van der Waals and Lewis acid-base interactions), used the empirical method developed by Neumann et al. [170] to estimate the hydrophobic interaction between bacteriophages and clay. Neumann's method predicts interfacial tensions between surfaces using only water contact angles [170]; however, several authors have demonstrated that Neumann's empirical approach is flawed [113, 171]. The hydrophobic interaction term used by Lee et al. did not include the van der Waals interaction and was estimated using an empirical model usually applied to determine protein hydrophobic energy [164].

1.3.3 Current knowledge

Sorption of viruses to various surfaces is the subject of numerous studies. Already in 1975 Bitton reviewed more than 50 studies looking at virus adsorption to materials such as activated carbon, nitrocellulose, glass, clays, sands, polyelectrolytes, iron oxides, ion exchange resins and membrane filters [172]. Viruses sorb to a wide range of surfaces including positively charged surfaces such as alumina and cationic polyelectrolytes and negatively charged surfaces such as nitrocellulose, glass, or anionic polymers. Adsorption, especially on negatively charged surfaces, depended on the ionic strength [161, 172]. Indeed high salt concentrations decreased the thickness of the double-layer, allowing negatively charged virus to get close enough to the surface for attractive forces such as van der Waals forces to act. Localized interactions between the positively charged fraction of the virus protein coat and a negatively charged surface could also explain the attachment of viruses to such surfaces [172]. The presence of proteinaceous material such as bovine albumin or bovine serum or natural organic matter hindered virus adsorption on activated carbon, clay, sands, anion exchange resin and membrane filters [160, 172-174]. Some authors suggested these proteinaceous materials competed with virus for the surface adsorption sites [172]; other authors have mentioned the possibility of the thick layer of organic matter on the surfaces increasing steric repulsion between viruses and the surface [174].

It was observed in those earlier studies that not all viruses exhibited similar adsorption behavior; however, only few reports considered viruses' surface properties and morphologies and how they correlated with the adsorption behavior [175, 176]. It was later found that viruses present a wide range of surface properties and thus a range of sorption behaviors [112, 154].

Michen et al. [112] reviewed 48 studies where the isoelectric point of 104 viruses was measured. The reported IEPs ranged from 1.9 to 8.4 but were mostly below 7, and depended not only on the species but also on the strain. Several viruses (such as poliovirus, mengovirus and coxsackievirus) had 2 IEPs, probably due to a change of conformation of the viral capsid at a certain pH. Viruses also vary widely in terms of their hydrophobicity [116, 117, 120]. An example of a very hydrophilic virus is phiX174 with a water contact angle between $26 \pm 2^\circ$ [117] and $42 \pm 6^\circ$ [116]. An example of a hydrophobic virus is T2 with a water contact angle of $96 \pm 9^\circ$ [116].

The IEP of viruses was a determining factor in virus adsorption indicating the importance of electrostatic interactions for virus attachment to surface [142, 144, 150, 151, 159, 173]. Hydrophobic interactions have also been shown to be important in the attachment of MS2 [116, 151, 153, 156, 157, 173], a hydrophilic virus. In some cases, interaction with hydrophobic surfaces disrupted the virus protein coat and lead to virus disassembly [147]. As ionic strength increases and repulsive electrostatic interactions decrease, the hydrophobic interaction becomes increasingly important [151].

Other factors that seem to influence virus adsorption include the virus size (higher surface areas present more sorption sites) [114,157], virus morphology (the presence of spikes, fibers, tails, extruding protein loops) [116, 172, 177], and the specific interaction with divalent cations especially Ca^{2+} which can form bridges between functional groups on the virus and on the surface [141, 143, 144].

In several studies DLVO theory failed to predict virus attachment behavior [144, 165], probably because of the importance of hydrophobic interaction in virus adsorption to surfaces. Also representing viruses as hard particles in models predicting double layer interactions is not always accurate [178]. Although the XDLVO model predicted the virus attachment experimentally observed in some studies well [116, 157, 164], others found discrepancies between the predicted and experimentally observed virus behavior [117, 156].

1.4 Dissertation overview

This work aims at improving virus recovery and concentration by crossflow filtration. In chapter 2 we designed highly controllable anti-adhesive membrane coatings to replace the traditional protein based blocking layer and assessed their performance in terms of virus recovery and concentration rate, under different filtration conditions that include filtration rates more than double the highest rate reported in the literature. In chapter 3, the effect of high crossflow and high permeate flow rate, which are necessary to hasten the filtration but might affect virus infectivity, on recovery and removal of infective virus from DI and surface water are evaluated using the CS-blocked membrane. The effect of those filtration conditions on the stability of the blocking layer is also investigated. In chapter 4, we looked at the performance of the anti-adhesive PEM membrane coatings with complex water matrices.

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Chapter 2. Anti-adhesive membrane films for virus concentration and recovery from water prior to detection

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2.1 Introduction

Efficient pathogen detection in drinking and recreational water is vital for preventing disease outbreaks. Because waterborne pathogens are present in low concentrations, a fast and reliable concentration step is crucial for their detection. Crossflow filtration with hollow fiber membranes, an emerging technique for concentrating various waterborne pathogens [1-13] including viruses [7-13], offers several advantages over traditional pathogen concentration methods (see below). First, membranes with an appropriate pore size simultaneously concentrate microbial pathogens ranging from protozoa to bacteria to viruses [2, 3, 5, 6] without concentrating molecular toxins and low molecular weight qPCR inhibitors [14]. Second, crossflow decreases membrane fouling and results in increased concentration rates [15]. Third, in contrast to filtration in a dead-end geometry, crossflow minimizes virus-membrane contact so more viruses remain suspended in the retentate throughout the concentration process, facilitating virus recovery [14].

The EPA-approved VIRADEL (VIRus ADsorption-ELution) method [16] for virus concentration includes filtration of a water sample through a positively [7, 16-18] or negatively

[16, 18, 19] charged microfilter. During the filtration, viruses adsorb onto the filter, and elution occurs in a high pH solution containing 1.5 to 6% beef extract and/or 0.05 M glycine (Fig. 2.1). If necessary, addition of a flocculant to the eluate can precipitate viruses to further reduce volume [16]. The VIRADEL method often gives low or poorly reproducible recoveries [20]. For example, Virosorb 1-MDS, the only electropositive VIRADEL filter recommended by EPA, adsorbed less than 35% of the indigenous *Escherichia coli* phages in sewage-spiked tap water, and the eluate contained only 12% of the phages [17]. The VIRADEL method is also sensitive to various water constituents; indeed, norovirus recovery was 92% from deionized (DI) water but only 19% from surface water [18]. In addition, highly adhesive viruses such as human adenovirus (HAdV) [21], give recoveries as low as 0.02% from surface water [7], and norovirus recoveries can also be low [7, 22]. To increase recovery, several studies explored ultracentrifugation [23, 24], centrifugal ultrafiltration (UF) [25], and flocculation-re-dissolution-UF [26]; however, these methods are impractical for large volume samples.

Crossflow filtration with a “blocked” membrane (see below) gives recoveries equal to or better than those with VIRADEL for a range of viruses in various types of water [7, 11]. Additionally, the large membrane surface area in hollow fiber cartridges and the use of crossflow to mitigate fouling [15] allow rapid concentration of high-volume samples [2, 4, 6, 11, 12, 27]. Covering or “blocking” the membrane surface with a layer of proteins (beef extract [10, 13], calf serum [13], bovine serum albumin [13]) or glycine [10] is crucial to improve virus recovery during crossflow filtration (Fig. 2.1).

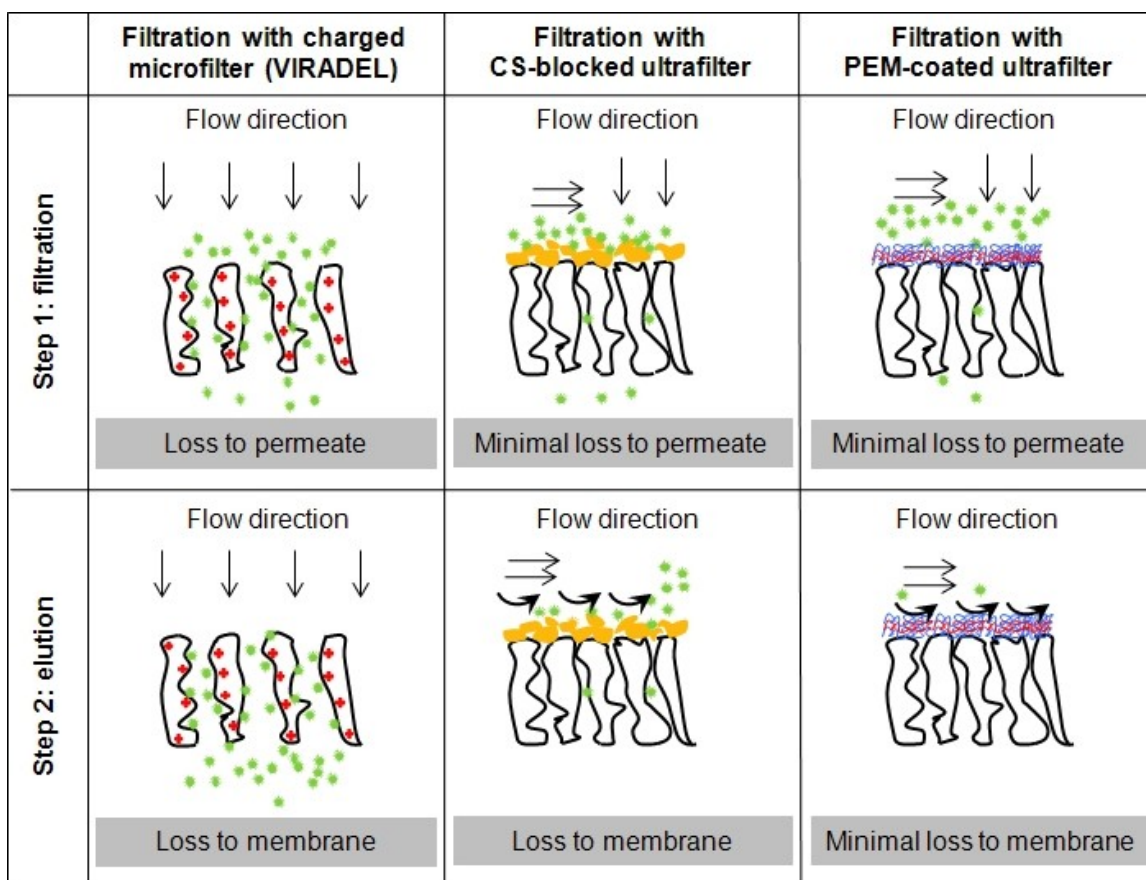


Figure 2.1: Comparison of the proposed virus pre-concentration approach based on polyelectrolyte multilayer coatings (right) with other current methods.

For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation)

These blocking procedures usually employ complex mixtures of macromolecules of various molecular weights to cover virus adsorption sites and possibly minimize virus passage through the membrane filter [2, 3, 5, 7, 11, 28]. Although the term “blocking” has also been used to describe treatment with small molecular weight compounds such as sodium polyphosphate (e.g., 600 Da [3, 6]) and glycine (75 Da), their effect is most likely limited to preventing adsorption of viruses to the membrane surface and does not include true pore

blockage. The blocking step can be time consuming (e.g., overnight deposition of a protein layer), and a membrane blocked with proteins might be prone to contamination during storage and transport [3]. In addition for certain viruses such as HAdV recovery values remain low and variable [7]

To overcome these challenges we propose coating UF filters with rationally designed polyelectrolyte multilayer (PEM) films [29, 30]. PEM deposition is in effect controlled membrane blocking that employs alternating adsorption of two oppositely charged polymers. PEMs can form quickly (in less than 1 h) and reproducibly [31]. Several studies described anti-adhesive PEM coatings on various surfaces [32-35], including membrane filters [36, 37], to decrease the adsorption of selected proteins [32, 33, 36, 37], mammalian cells [32, 34], or bacteria [34, 35]. Although PEMs were employed to create ordered virus monolayers [38, 39], no studies examined virus adhesion to PEM-modified surfaces or applied PEMs to prevent virus adsorption or to enhance recovery for virus concentration and detection.

In addition to low and poorly reproducible recoveries, the long duration of the sample concentration step remains a bottleneck in virus detection. For example, concentrating 100 L of tap water to 400 mL takes 2 h with a hollow fiber cartridge, and further volume reduction to a few mL [6] for qPCR analysis requires another 1 to 3 h [6]. Because virus concentrations of less than 1 genomic copy per 100 L of water represent a significant risk of illness [40], virus monitoring often requires testing up to 1,000 L for drinking water [16]. Increasing transmembrane pressure can, in principle, increase filtration rate; however, this may exacerbate membrane fouling, alter virus recovery and offset the gains in the permeate flux. To overcome

this limitation we operated the membrane at a high transmembrane pressure while simultaneously increasing the retentate crossflow rate to maintain a high permeate flux while limiting fouling to a manageable level.

In summary, this work aims to improve virus concentration by membranes. We designed highly controllable anti-adhesive membrane coatings and assessed their performance in terms of virus recovery and concentration rate. We also evaluated how crossflow rate and permeate flux affect virus rejection, recovery, and rate of concentration with CS-blocked and PEM-coated membranes. The anti-adhesive membrane coating required less than an hour to deposit and increased pre-elution virus recovery up to 2-fold.

2.2 Materials and Methods

2.2.1 Reagents

Lysozyme (from chicken egg white), ethylenediaminetetraacetic acid (EDTA), calf serum, heparin (sodium salt), chitosan (medium molecular weight), diiodomethane and sodium polyphosphate (NaPP) were purchased from Sigma-Aldrich. Tween 80 (Fisher), tryptic soy broth (Becton, Dickinson) and glycerol (J.T. Baker) were used as received.

2.2.2 Bacteriophage

Bacteriophage P22 (provided by Prof. Joan B. Rose, MSU) is a non-enveloped, double stranded DNA phage that has been used to understand dsDNA virus assembly [41-44] and as a surrogate for human viruses to understand their fate in the environment [45, 46]. P22 has an icosahedral head with a diameter of 60 nm and a short tail [47]. P22 stock was grown by inoculating *Salmonella enterica* serovar Typhimurium LT2 (also provided by Prof. Rose) in tryptic soy broth. After incubation bacterial host cells were lysed by adding 0.1 mL of lysozyme (50 mg/mL) and 0.75 mL of EDTA (0.5 M). The culture was then centrifuged for 10 min at 4000 rpm and the supernatant was filtered through a 0.45 µm cartridge filter. P22 stock suspension ($\sim 3 \cdot 10^{10}$ plaque forming units per mL) was stored at 4°C. One liter of water was spiked with 1 mL of P22 stock on the day of the filtration.

2.2.3 Bacteriophage characterization

To characterize the surface properties of P22 particles, they had to be separated from other components present in the stock, i.e. tryptic soy broth components and host bacteria cell debris. The purification protocol was modified from Mylon et al. [48]. First, 150 mL of P22 stock solution was filtered through a 0.1 µm membrane (Nucleopore, Whatman) using a stirred Amicon 8050 dead-end UF cell (Millipore) to remove molecules and debris larger than 100 nm. The permeate volume was then reduced 5- fold by filtration through a 50 kDa polyethersulfone membrane (Omega, Pall) in an Amicon 8200 filtration cell. At this point the cell was connected to a feed tank containing 1 L of 10 mM KCl. This solution was passed through the 50 kDa

membrane to wash away molecules smaller than 50 kDa such as broth components, until only 30 mL remained in the cell. The particle size distribution in this purified stock was measured by dynamic light scattering at pH 6.. The purified P22 stock was then filtered through a 30 kDa polyethersulfone membrane (Omega, Pall) to deposit the equivalent of ~ 9 layers of virus. Contact angles of 3 probe liquids (deionized water, glycerol, diiodomethane) with these layers were measured using the sessile drop method (FTÅ 200 contact angle analyzer, First Ten Angstroms) to determine the surface tension parameters of P22 phage particles (see section 2.2.12). The ζ -potential of P22 was determined based on electrophoretic mobility using phase analysis light scattering (ZetaPALS, Brookhaven Instrument Corporation) at pH 6¹, using purified P22 washed with 1 mM KCl (instead of 10 mM KCl used for particle sizing and contact angle).

2.2.4 Crossflow concentration apparatus

Fig. 2.2 shows the schematic of the crossflow concentration unit. A high-pressure peristaltic pump (model 621 CC, Watson-Marlow) drew the solution from the pressurized feed tank to the membrane filtration cell (CF042, Sterlitech), where the membrane surface area exposed to the feed was 41 cm². The permeate was collected in an Erlenmeyer flask positioned on an electronic mass balance (Adventurer Pro AV8101C, Ohaus) interfaced with a computer. The transmembrane pressure varied from ~ 0.5 bars (7 psi) to ~ 2.8 bars (40 psi). An inline flowmeter (101-7, McMilan) recorded the crossflow velocity.

¹ It should be noted that the ζ -potential of P22 determined with this method is a good descriptor of P22 surface potential only if P22 is a soft particle, which has not been verified here.

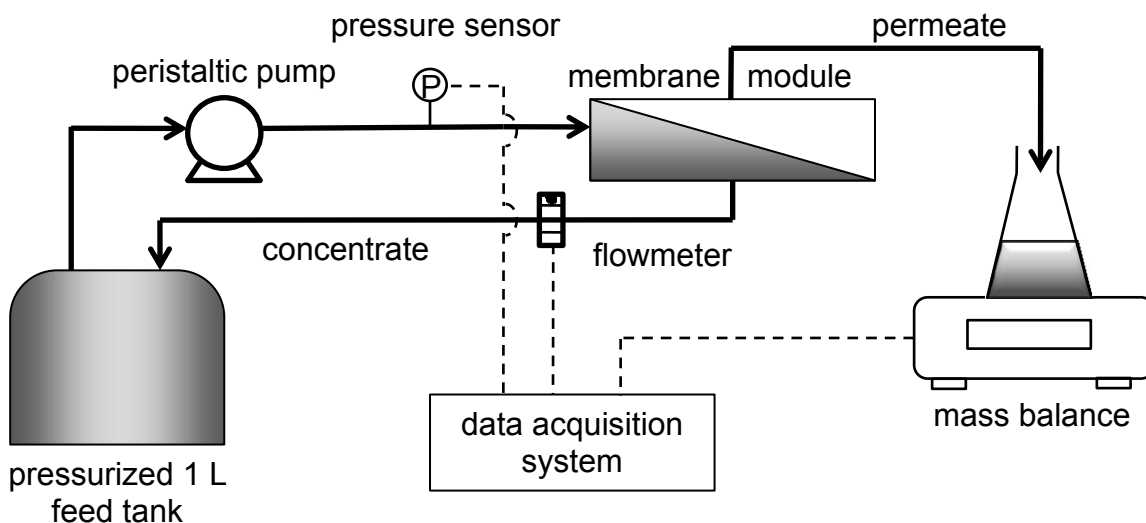


Figure 2.2: Crossflow filtration apparatus

2.2.5 Membrane preparation

Prior to modification, a polyethersulfone (PES) UF membrane (OMEGA 30 kDa, Pall) was submerged for 24 h in 1 N NaOH and thoroughly rinsed with deionized water. The membrane was either blocked with CS or coated with a PEM film. For CS blocking, the membrane was loaded in the crossflow filtration cell, and 500 mL of 5 % CS solution was circulated over the membrane surface for 16 to 18 h with no pressure differential across the membrane. The filtration unit was then rinsed twice with DI water for 10 min. To coat the membrane with a PEM film, the membrane was placed in a custom-made holder and the membrane surface was alternately exposed to HE solution (1 mg/L, pH 5) and CHI solution (1 mg/L, pH 5) with a 1-min rinse with DI water in between. The duration of each exposure was 5 min. The total number of deposited bilayers was 4.5, where the last half bilayer was HE. Both

HE and CHI solutions also contained 0.15 M NaCl. Membranes were prepared immediately prior to filtration.

2.2.6 Membrane characterization

The ζ -potential of the membranes was measured using an electrokinetic analyzer (BI-EKA, Brookhaven Instrument Corp.) at pH 6. Membrane hydrophilicity was characterized by measuring water contact angles using the sessile drop method. At least 3 different membranes were used for each analysis. Contact angles of diiodomethane and glycerol² were also measured to determine the surface tension parameters of the CS-blocked and the PEM-coated membranes. Water permeabilities of coated membranes were determined before and after membrane compaction by measuring the permeate flux at a transmembrane pressure differential of 40 psi.

2.2.7 Concentration procedure

The membranes were first compacted by filtering DI water at 40 psi for 90 min. Filtration of 1 L of the DI water spiked with 1 mL of P22 stock solution was then performed to reduce the volume to 250 mL. The ratio of the crossflow flux to the initial permeate flux, J_{cf} / J_p , ranged from 1,500 to 11,100 (Fig. 2.3). Crossflow flux was regulated from 6,800 to 26,800 L/(min·m²), which corresponded to Reynolds numbers from 400 to 1,700.

²Contact angles were stable for at least 10 s except for glycerol. For glycerol contact angles measured 3 s after droplet deposition were used.

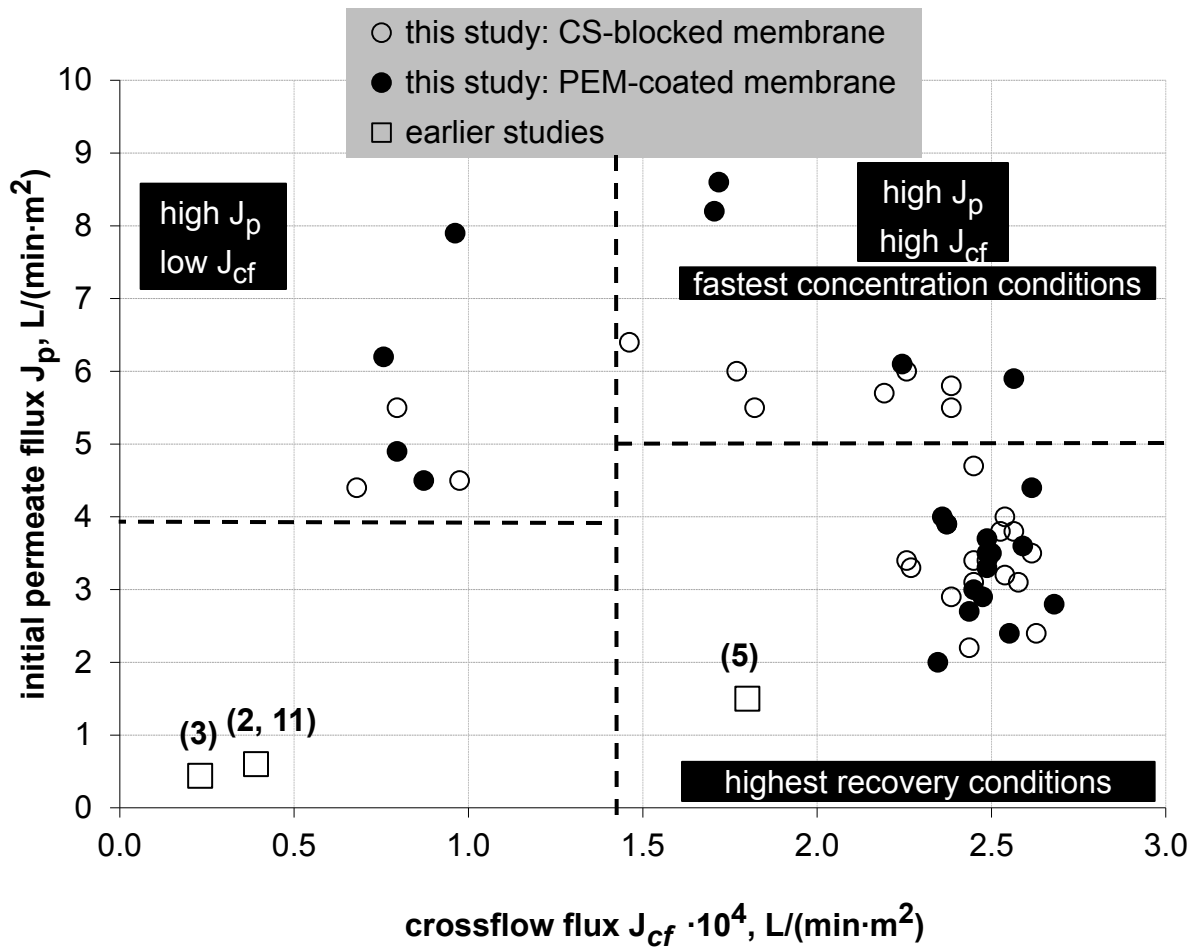


Figure 2.3: Filtration conditions in experiments on P22 preconcentration and recovery using CS-blocked and PEM-coated membranes. Number of tests: n=27 for CS, n=22 for PEM.

Following the filtration, viruses were eluted from the membrane using 0.01% Tween 80, 0.01% NaPP [2, 3]. The elution occurred with the membrane still in the filtration cell and at the same crossflow rate as in the filtration, but with a retentate pressure of only 2.5 psi, which was due to the crossflow. Aliquots of the feed, retentate, permeate and eluate were stored at 4°C. Samples were also taken during compaction with DI water to verify that no contamination was

present before P22 was introduced. A new membrane was used for each filtration and the unit was disinfected with CIDEX (Advanced Sterilization Products) between filtration tests.

2.2.8 Sample analysis

The P22 concentration in each sample was determined by qPCR. Within 24 h of the filtration experiment, MagNA Pure Compact System (Roche) was used to extract P22 DNA from 390 μ L grab samples into 100 μ L eluates. RNA carrier (10 μ L, 1 μ g/ μ L) was added to the 390 μ L samples before extraction to prevent bacteriophage adsorption on the MagNA Pure system tips and magnetic beads. Each extract was then analyzed by qPCR in triplicate following the protocols described by Masago et al.[45] PCR mixtures (20 μ L) contained 5 μ L of extracted DNA sample, 2 μ L each of forward and reverse primers (5 μ mol/ μ L), 0.3 μ L of Taqman Probe (10 μ mol/L), 10 μ L of PCR master mix (LightCycler 480 Probes, Roche) and 0.7 μ L of water. qPCR was performed using the LightCycler 1.5 system (Roche). Primer and probe sequences (Table 2.1), and qPCR conditions were similar to the ones described by Masago et al. [45]. QPCR mixtures were heated at 95°C for 10 min prior to 45 cycles of 95°C for 10 s, 60°C for 20 s and 72°C for 10 s. Finally mixtures were cooled for 30 s at 40°C. To prepare a standard curve linking qPCR crossing-point (CP) values with numbers of P22 DNA copies, P22 DNA standards were prepared following the method described by Xagorarakis et al. [49]. The phage target DNA sequence was amplified by PCR using the same primers that were used for qPCR, and the amplicon was cloned into plasmid vector using the TOPO TA Cloning kit (Invitrogen). Plasmid vector DNA was extracted and purified using QIAprep Spin Miniprep kit (Qiagen) and the DNA concentration was quantified by spectrophotometry using Nanodrop ND-1000 (Thermo Fisher

Scientific). The number of DNA copies per unit volume could then be calculated based on the plasmid vector DNA length of 4,000 base pairs (bp) and assuming an average mass per bp of 650 Da. The purified DNA was serially diluted and the dilutions run by qPCR to develop the standard curve.

Table 2.1: Primer and probe sequence (from Masago et al. [45])

Primer/probe	Sequence
Reverse	CTT AAC AAG CTC TGA CTG CTC ATC A
Forward	CCA TCG CCT GTG ACT CGA T
Taqman Probe	FAM-TCG CAA CGA TGC AGA ACG ACT CG-TAMRA

2.2.9 Data analysis and statistics

P22 log removal (LRV), percentage of pre-elution recovery (r_{pre}), and percentage of post-elution recovery (r_{post}) were calculated using the following equations:

$$LRV = \log \left(\frac{C_f}{C_p} \right), \quad (2.1)$$

$$r_{pre} = \frac{C_r V_r}{C_f V_f} \times 100\%, \quad (2.2)$$

$$r_{post} = \frac{C_r V_r + C_e V_e}{C_f V_f} \times 100\%, \quad (2.3)$$

where C_f , C_r , and C_e are P22 concentrations in feed, retentate, and eluate samples, respectively, and V_f , V_r , and V_e are the volumes of these samples. Virus recovery and virus removal were plotted as a function of the J_{cf} / J_p ratio. The relationships between J_{cf} / J_p , on the one hand, and r_{pre} , r_{post} and LRV, on the other hand, for the PEM-coated membranes and the CS-blocked membranes were statistically analyzed using the regression procedure of the SAS software. An interaction term (between J_{cf} / J_p and the type of membrane) was introduced in the regression model. The software Minitab was used to statistically characterize the distributions of pre-elution and post-elution recoveries.

2.2.10 Determination of the fouling resistance R_f

As we concentrate P22, permeate flux decreases due to fouling of the membrane by virions and other components present in the feed (proteins and bacterial debris). The flow resistance due to fouling at the end of filtration was calculated using the following equation:

$$R_f = R_m \left(\frac{J_0}{J_f} - 1 \right) \quad (2.4)$$

Where R_f is the resistance due to fouling, R_m the resistance of the blocked or coated membrane, J_0 the average permeate flux during the first minute of filtration and J_f is the permeate flux during the last minute of filtration.

2.2.11 Blocking law analysis

Dead-end filtrations of P22-containing feed solution (1 L of DI water spiked with 1 mL of P22 stock) through CS-blocked and PEM-coated membranes were conducted using an unstirred Amicon 8010 ultrafiltration cell connected to a pressurized feed tank. The permeate flux was recorded continuously for 1 hour using an electronic mass balance connected to a computer. For each experiment the membrane was first compacted for 90 min with DI water. Permeate flux data were then obtained and fitted to the linearized equations describing the four fouling mechanisms: complete blocking, standard blocking, intermediate blocking and cake filtration [50]. To assign a fouling mechanism to each portion of the permeate flux dataset, we used the procedure described by Wang et al. [50]. A core portion of the filtration dataset that fitted a given mechanism with a high coefficient of determination ($r^2 > 0.999$ unless otherwise specified) was identified and expanded point by point by evaluating if the point belonged to the 95% prediction interval given by the regression. If it did, the point was added to the core and a new linear regression analysis was performed. For more detail on the procedure, please refer to the paper by Wang et al. [50].

2.2.12 Calculation of the energy of interaction between P22 phage and membrane surfaces using the XDLVO approach

The XDLVO theory predicts the energy of interaction between particles and a surface by taking into consideration Lifshitz-van der Waals (LW), electrostatic double layer (EL) and Lewis

acid-base (AB) forces [51, 52]. The total energy of interaction between a particle and a plane surface can be written as [52]:

$$U^{XDLVO} = U^{LW} + U^{EL} + U^{AB} \quad (2.5)$$

where U^{XDLVO} is the total energy of interaction, U^{LW} is the van der Waals interaction energy, U^{EL} is the electrostatic interaction energy and U^{AB} is the Lewis acid-base interaction energy.

Each of these terms can be expressed as a function of the separation distance (d) using the following equations [52]:

$$U^{LW}(d) = 4\pi \left(\sqrt{\gamma_{\ell}^{LW}} - \sqrt{\gamma_s^{LW}} \right) \left(\sqrt{\gamma_p^{LW}} - \sqrt{\gamma_{\ell}^{LW}} \right) \frac{y_0^2 r_p}{d} \quad (2.6)$$

$$U^{EL}(d) = \pi \epsilon_r \epsilon_0 r_p \left[2\zeta_p \zeta_s \ln \left(\frac{1 + e^{-\kappa d}}{1 - e^{-\kappa d}} \right) + \left(\zeta_p^2 + \zeta_s^2 \right) \ln \left(1 - e^{-2\kappa d} \right) \right] \quad (2.7)$$

$$U^{AB}(d) = 4\pi r_p \lambda \left[\begin{aligned} & \sqrt{\gamma_{\ell}^{+}} \left(\sqrt{\gamma_s^{-}} + \sqrt{\gamma_p^{-}} - \sqrt{\gamma_{\ell}^{-}} \right) + \sqrt{\gamma_{\ell}^{-}} \left(\sqrt{\gamma_s^{+}} + \sqrt{\gamma_p^{+}} - \sqrt{\gamma_{\ell}^{+}} \right) \\ & - \sqrt{\gamma_s^{+} \gamma_p^{-}} - \sqrt{\gamma_s^{-} \gamma_p^{+}} \end{aligned} \right] \exp \left(\frac{y_0 - d}{\lambda} \right) \quad (2.8)$$

where γ_{ℓ}^{LW} , γ_{ℓ}^{+} and γ_{ℓ}^{-} are the surface tension parameters of immersion liquid (the + and - superscripts correspond to the electron acceptor and electron donor components of the surface tension, respectively), γ_p^{LW} , γ_p^{+} and γ_p^{-} are the surface tension parameters of the particle, γ_s^{LW} , γ_s^{+} and γ_s^{-} are the surface tension parameters of the planar surface, r_p is the particle radius, and ζ_p and ζ_s are the zeta potentials of the particle and the surface. The minimum equilibrium cut-off distance, y_0 , was assigned a value of 0.16 nm [53]. For other symbols,

$\varepsilon_r \varepsilon_0$ is the dielectric permittivity of water, κ is the inverse of the Debye length and λ is the characteristic decay length of Lewis acid-base interactions in water (0.6 nm) [53].

The surface tension parameters of the P22 phage and membrane surfaces were determined using the extended Young equation (2.9) after measuring the contact angle of three probe liquids with known surface tension parameters.

$$(1 + \cos \theta) \gamma_\ell^{TOT} = 2 \left(\sqrt{\gamma_s^{LW} \gamma_\ell^{LW}} + \sqrt{\gamma_s^+ \gamma_\ell^-} + \sqrt{\gamma_s^- \gamma_\ell^+} \right) \quad (2.9)$$

$$\gamma^{AB} = 2 \sqrt{\gamma^+ \gamma^-} \quad (2.10)$$

$$\gamma^{TOT} = \gamma^{AB} + \gamma^{LW} \quad (2.11)$$

In these equations, θ is the contact angle for a given probe liquid, and the subscripts s and ℓ correspond to the solid surface and the liquid probe, respectively.

2.2.13 Effect of polyelectrolytes and CS on qPCR analysis

A P22 solution ($\sim 3 \cdot 10^8$ PFU/mL) was prepared by diluting 1 mL of P22 stock with deionized (DI) water. One mL of 0.12 mg/mL solutions of HE, PAA, poly(styrene sulfonate), CHI and PDADMAC, and 1 mL of a 0.24 mg/mL calf serum solution were seeded with 0.1 mL of the P22 solution. The pH of each resulting solution was adjusted to 6.3 (similar to the feed pH during filtration). The final concentration of polyelectrolyte and calf serum in each solution was 0.11 and 0.22 mg/L respectively, which is 100-fold higher than the concentration we would

expect if we would backflush our membrane with 100 ml of solution. The P22 concentration in these samples was determined using the same methodology as for the filtration samples.

2.3 Results and Discussion

2.3.1 PEM selection

As described in Chapter 1 various polyelectrolytes have been used to prepare anti-adhesive coatings. Based on these studies HA/CHI, HA/PAH, HE/CHI and HE/PAH films were deposited on UF membranes. (PAA/PAH films are not highly permeable.) Contact angles of water with the resulting surfaces and water permeability were measured. Based on a limited set of data it appeared that HE based films were more hydrophilic than their HA counterparts.

Additionally we looked at the effect of polyelectrolytes on qPCR analysis. As Fig. 2.4 shows, polycations out of 3 significantly inhibited DNA extraction or qPCR while most polyanions had no effect on qPCR results. Although CHI partially inhibited qPCR, PAH completely annihilated any fluorescence signal and as a consequence P22 could not be detected in samples containing PAH. As a result, CHI was considered a better candidate than PAH and based partly on the results of these inhibition tests and partly on the measured values of water contact angles and water permeabilities of candidate PEMs, we selected (HE/CHI)_{4,5} as one suitable PEM film. Several polyelectrolytes such as PDADMAC or PAA, not considered in these studies, were more compatible than CHI or HE with qPCR and should be considered in future work

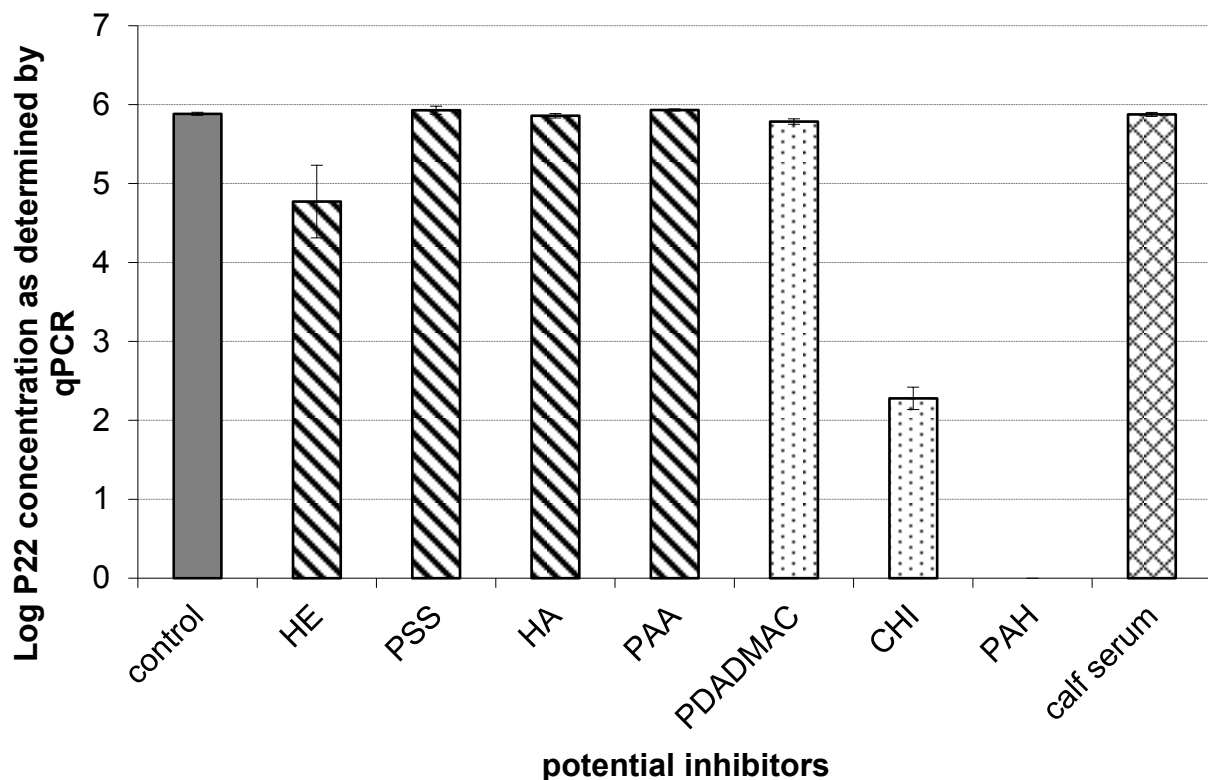


Figure 2.4: Effect of various polyelectrolytes and calf serum on the P22 concentration determined by qPCR. Error bars indicate the standard deviation interval. n=3.

2.3.2 Properties of CS-blocked and PEM-coated membranes and P22 phage

2.3.2.1 Membrane permeability and fouling mechanisms

The DI water permeabilities of PES, CS-blocked PES, and PEM-coated PES membranes after compaction were 280 ± 20 , 90 ± 20 , and 120 ± 30 L/(m²·h·bar), respectively. Thus, the two modification methods yielded membranes of similar permeabilities. We applied blocking laws to P22 dead-end filtration data to interpret how feed components interacted with CS-blocked and

PEM-coated membranes. As Fig. 2.5 shows, for both membranes each of the four mechanisms could fit at least a portion of the dataset. Concurrent fits to different mechanisms indicate either a combination of mechanisms or that the data are insufficient to differentiate between mechanisms. Clearly, however, a cake filtration law provides a high quality fit to plots of flux versus time after filtering 5 mL of virus suspension through PEM-coated membranes, but only after filtering 32 mL through CS-blocked membranes. (Filtration of one mL corresponds to one P22 phage, quantified as DNA copies, deposited per $1 \mu\text{m}^2$ of membrane). The larger volume required to achieve cake filtration with CS-blocked membranes suggests that P22 (or other components not removed from the growth medium by centrifugation) can partially or fully enter pores in the CS layer. For a description of different blocking mechanisms please refer to the study by Wang et al. [50].

2.3.2.2 Surface properties of membranes and P22 phage

Several studies have shown that blocking the membrane with proteins improves virus recovery [3, 10, 28]. While two studies reported that blocking a membrane with proteins had no [4] or a negative [12] effect on virus recovery, most studies used CS or other proteinaceous solutions to block membranes for microorganism concentration by crossflow filtration [2, 3, 5, 7, 10, 11, 13]. .

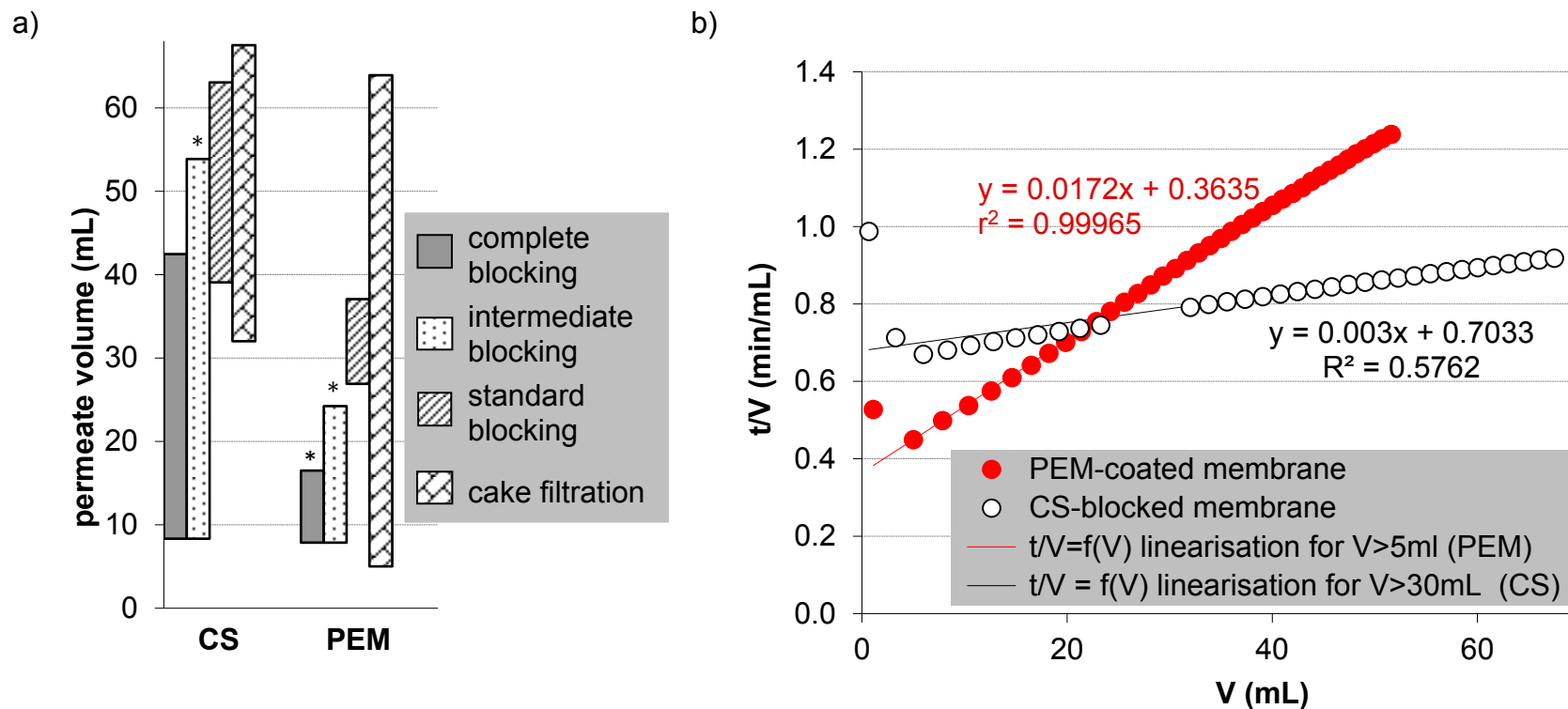


Figure 2.5: Blocking law analysis for fouling of CS-blocked and PEM-coated membranes during filtration of DI water spiked with P22 stock. a) Distributions and contributions of the blocking mechanisms in terms of permeate volume. * indicates that the r^2 value corresponding to fitting that blocking mechanism with the experimental data was > 0.99 . When no * is present, r^2 was > 0.999 . b)

Inverse cumulative flow rate (t/V) as a function of permeate volume V for CS-blocked and PEM-coated membranes.

Some of these studies suggest that treating the membrane with beef extract or CS solutions minimizes viral adsorption to the membrane [10, 13]; however, to our knowledge all evidence for such an effect is indirect and based on higher values for virus recovery. Previous studies did not consider the specific interactions between the virus and the membrane.

To quantify the anti-adhesiveness of the CS layer, we measured the surface energy (Table 2.2) and charge of the CS-blocked membrane. Based on literature values of the ζ -potential and isoelectric point, at least 2 out of the 4 viruses were negatively charged [55, 56] in studies showing that CS or beef extract increased recovery [10, 13, 54]. (Isoelectric points for the other 2 viruses are not known.) Therefore, we expected that CS renders the membrane surface more hydrophilic and negatively charged to make it more anti-adhesive; however, CS-blocked membranes were slightly positively charged at pH 6 ($\zeta = 3 \pm 2$ mV) and not more hydrophilic ($\theta_w = 66 \pm 13^\circ$) than the polyethersulfone support ($\theta_w = 67 \pm 3^\circ$). In fact, the CS-blocked membranes were hydrophobic (see Table 2.2) according to van Oss and Giese's definition of hydrophobicity (a negative free energy of interfacial interaction when immersed in water; see Chapter 1) [57]. Although the IEP of BSA, the main component of CS, is 4.7 so the protein would have a slight negative charge at pH 6, the CS also includes components other than BSA that might preferentially deposit and be responsible for the slightly positive charge of the CS-blocked surface. Thus, although CS can prevent virus attachment to the UF support membrane by occupying adsorption sites, negatively charged viruses might still adsorb onto CS surface during filtration.

Table 2.2 Measured contact angles, calculated surface energy parameters and free energy of interfacial interaction when immersed in water for P22 phage, CS-blocked membranes and PEM-coated membranes.

	Surface energy parameters (mJ/m ²)					ΔG_{iwi}	Contact angle (°)		
Probe liquid	γ^{LW}	γ^+	γ^-	γ^{AB}	γ^{TOT}	(mJ/m ²)			
Ultrapure water	21.8	25.5	25.5	51.0	72.8				
Glycerol	34.0	3.9	57.4	30.0	64.0				
Diiodomethane	50.8	0.0	0.0	0.0	50.8				
Material							H ₂ O	Glycerol	Diiodo- methane
P22	44	3	57	27	71	25	49 ± 8	75 ± 6	30 ± 2
CS-blocked membrane	41	0.004	17	0.5	42	-24	66 ± 13	64 ± 2	37 ± 3
PEM-coated membrane	42	0.07	53	4	46	36	36 ± 3	55 ± 4	34 ± 5

This could suggest that CS blocking improves recovery by limiting virus penetration into the membrane but not by minimizing virus adhesion. Facile elution of viruses attached to the CS might also enhance recovery. In contrast, the PEM-coated membranes were negatively charged at pH 6 ($\zeta = -7 \pm 3$ mV) and considerably more hydrophilic ($\theta_w = 36 \pm 3^\circ$) than the CS-blocked membranes. Given that P22 phages were negatively charged at pH 6 ($\zeta = -17 \pm 1$ mV) and hydrophilic ($\theta_w = 49 \pm 8^\circ$), we expect the PEM to resist P22 adsorption. As a precedent in this area, Hill et al. [3] and Polaczyk et al. [6] evaluated the efficiency of a 15-min blocking of 15-20 kDa UF membrane with a 600 Da NaPP solution. Although surface charges were not measured, they hypothesized that deposition of the polyanion NaPP increased electrostatic repulsion between the membrane and microorganisms. NaPP “blocking” was faster than overnight CS-blocking and resulted in similar recovery for MS2 bacteriophage [3, 6]. To further quantify the propensity of P22 to adhere to the CS-blocked and PEM-blocked membranes, we calculated the energy of phage-membrane interactions.

2.3.2.3 Virus-membrane interaction energies

The XDLVO theory predicted secondary minima in the energy of the virus-membrane interaction at separations of 4.3 nm and 10.9 nm for the CS-blocked and PEM-coated membranes, respectively (Fig. 2.6). Due to attractive van der Waals and electrostatic interactions between CS-blocked membranes and P22, the secondary minimum (-2.6 kT) could be enough for some P22 to reversibly attach to the membrane surface despite not being able to overcome the repulsive barrier (500 kT) [58]. The depth (-0.6 kT) of the secondary minimum in the PEM-P22 interaction energy was less than the average thermal energy of particles, indicating

that attachment of P22 to this membrane was not efficient. This XDLVO analysis only takes into account macroscopic phenomena, and adhesion of P22 to either surface could also occur through microscopic attraction to discrete electron acceptor sites on the membrane surfaces [53] as well as through non-XDLVO interactions.

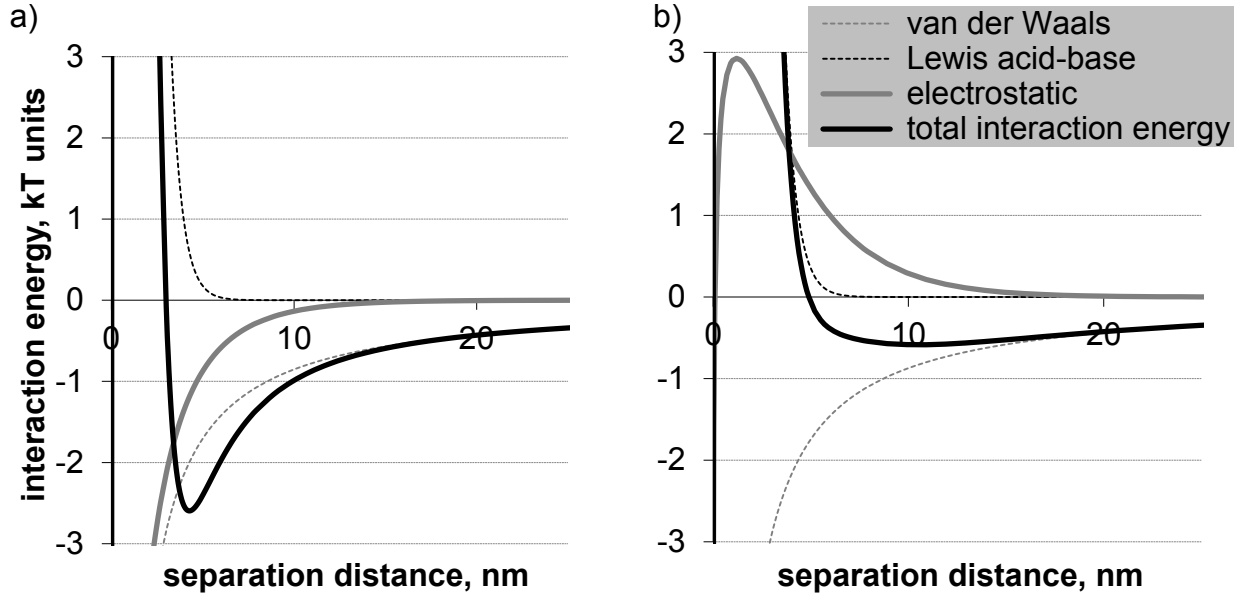


Figure 2.6: XDLVO energy profiles for the interaction of P22 with a) CS-blocked membranes and b) PEM-coated membranes at pH = 6 and in 10 mM KCl.

2.3.3 Virus recovery with PEM-coated and CS-blocked membranes

2.3.3.1 Pre-elution recovery

Figure 2.7 shows P22 recoveries in the retentate as a function of the J_{cf} / J_p ratio. The J_{cf} / J_p ratio is important as it influences the transport of viruses to and, possibly, across the

membrane [59]. Generally, if permeate flow increases, increased concentration polarization leads to both more particle adhesion to the membrane and more particle passage through the membrane. In contrast, increasing the crossflow velocity decreases concentration polarization and facilitates particle removal from the membrane surface through shear-induced back-transport[15]; however, it has also been shown that, in conditions typical of crossflow ultrafiltration, the minimum in particle back-transport is obtained for particle diameters near 100 nm [60]. It is therefore possible that for P22 phage with a diameter of approximately 60 nm crossflow has little or no effect on the net depositional flux of viruses. Pre-elution recovery with CS-blocked membranes ranged from 22% to 51%. These values are comparable to results of a previous study with CS-blocked UF membranes where pre-elution recoveries were $38 \pm 22\%$ for T1 phage and $45 \pm 55\%$ for PP7 phage [5]; we should note, however, that this prior study employed surface water that could foul the membrane significantly [9]. When concentrating bacteriophage MS2 from tap water, Hill et al. [3] reported pre-elution recoveries of $84 \pm 13\%$. This high recovery probably stemmed from the relatively simple composition of the water samples combined with the addition of 0.1% NaPP, a negatively charged chemical dispersant thought to enhance charge repulsion between the membrane and viruses by altering the viruses' ζ -potential [6].

P22 pre-elution recovery with PEM-coated membranes ranged from 32 to 85%. Over the entire range of filtration conditions (Fig. 2.7), PEM-coated membranes statistically outperformed CS-blocked membranes in terms of P22 pre-elution recovery, but the improvement was most pronounced at higher J_{cf} / J_p ratios. We attribute the observed higher pre-elution to the PEM's anti-adhesiveness stemming from its negative charge [30] and hydrophilicity [29].

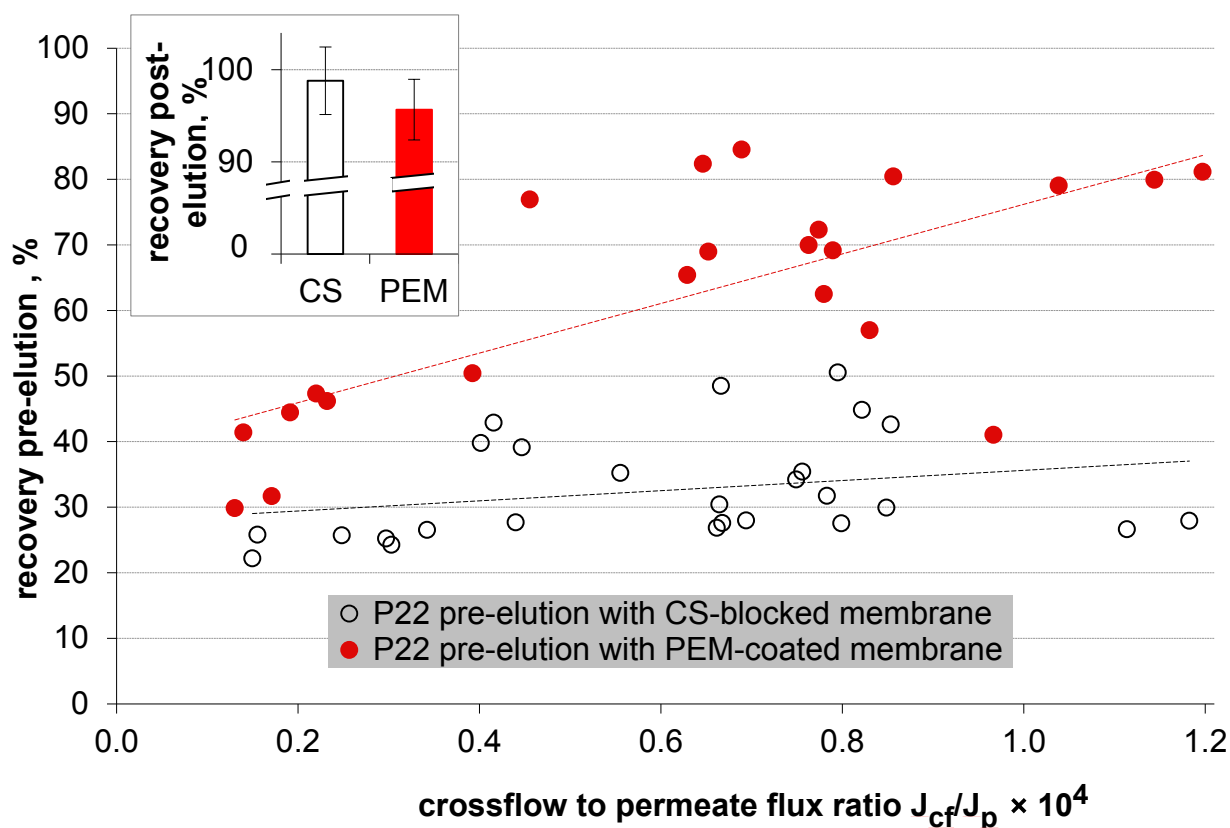


Figure 2.7: Pre-elution and post-elution (see inset) recovery of P22 from DI water. Because there was no significant dependence of post-elution recovery on the value of J_{cf} / J_p , only the average recovery and the 90% confidence interval are reported.

Number of experiments: $n = 26$ for CS-blocked membranes and $n = 22$ for PEM-coated membranes.

2.3.3.2 Post-elution recovery and potential of PEMs as sacrificial coatings

Post-elution recovery of P22 did not differ significantly between CS-blocked ($99 \pm 11\%$) and PEM-coated membranes ($96 \pm 9\%$). These values for post-elution recovery were within the

range typically reported for crossflow filtration of bacteriophages [2, 5, 6, 11-13, 28]. For example, Hill et al. [2] recovered $86 \pm 13\%$ of the Φ X174 phage and $120 \pm 22\%$ of MS2 phage from a 100 L tap sample containing 0.01% NaPP. The high post-elution recovery with both CS-blocked and PEM-coated membranes may reflect the simplicity of the water matrix or weak adhesion of P22 to surfaces. We expect that with more adhesive viruses, recovery should decrease, and the competitive advantage of the PEM as a more anti-adhesive surface will increase. When 10-L surface water samples were concentrated by crossflow filtration followed by elution, Albinana-Gimenez et al. [7] observed post-elution recoveries of only 18.7% for polyomavirus, 5.06% for adenovirus and less than 0.95% for norovirus. Anti-adhesive properties of PEM-coated membranes, evidenced by high pre-elution recoveries of P22, might help to improve the recovery of such “sticky” and difficult to concentrate human viruses.

Treating the PEM film as a sacrificial layer and removing it from the support using a backflush could also increase post-elution recovery. Such removal will not affect the supporting UF membrane and should help to recover surface-associated viruses. After the backflush, a new PEM layer can be deposited at the surface of the same support [61]. To implement such a procedure, however, electrolytes of the dissolved PEM should minimally interfere with qPCR. Tests with select polyelectrolytes indicate that although some polyanions and most polycations interfere with qPCR analysis; there are polyanions and polycations that, at the concentration expected in backflush samples, do not inhibit qPCR or DNA extraction (see Fig. 2.4). Indeed adding PSS, HA, PAA or PDADMAC to P22 samples did not affect the count of P22 by qPCR when compared to the control sample where P22 was suspended in DI water.

2.3.3.3 Reproducibility

Blocking with CS is inherently poorly reproducible, so we expected the PEM-coated membranes to show more reproducible recoveries than CS-blocked membranes. We performed 15 filtrations under conditions that should lead to the highest recoveries (“low J_p ; high J_{cf} ” domain) with CS-blocked membranes, and 14 filtrations under the same conditions with PEM-coated membranes. Figure 2.8 illustrates the distribution of pre-elution and post-elution recoveries under these conditions for both membrane types. The interquartile range³ (IQR, also called midspread) for pre- and post-elution recoveries were comparable for both types of membranes. IQRs for pre-elution recovery distributions are 15% and 16% for CS-blocked membranes and PEM-blocked membranes respectively, whereas IQRs for post-elution recovery distributions were lower, 10% and 9%, respectively for CS-blocked and PEM-coated membranes. We conclude that there was no significant difference in recovery reproducibility between the two types of membranes.

³The interquartile range is the difference between the upper and lower quartiles of the cumulative distribution curve.

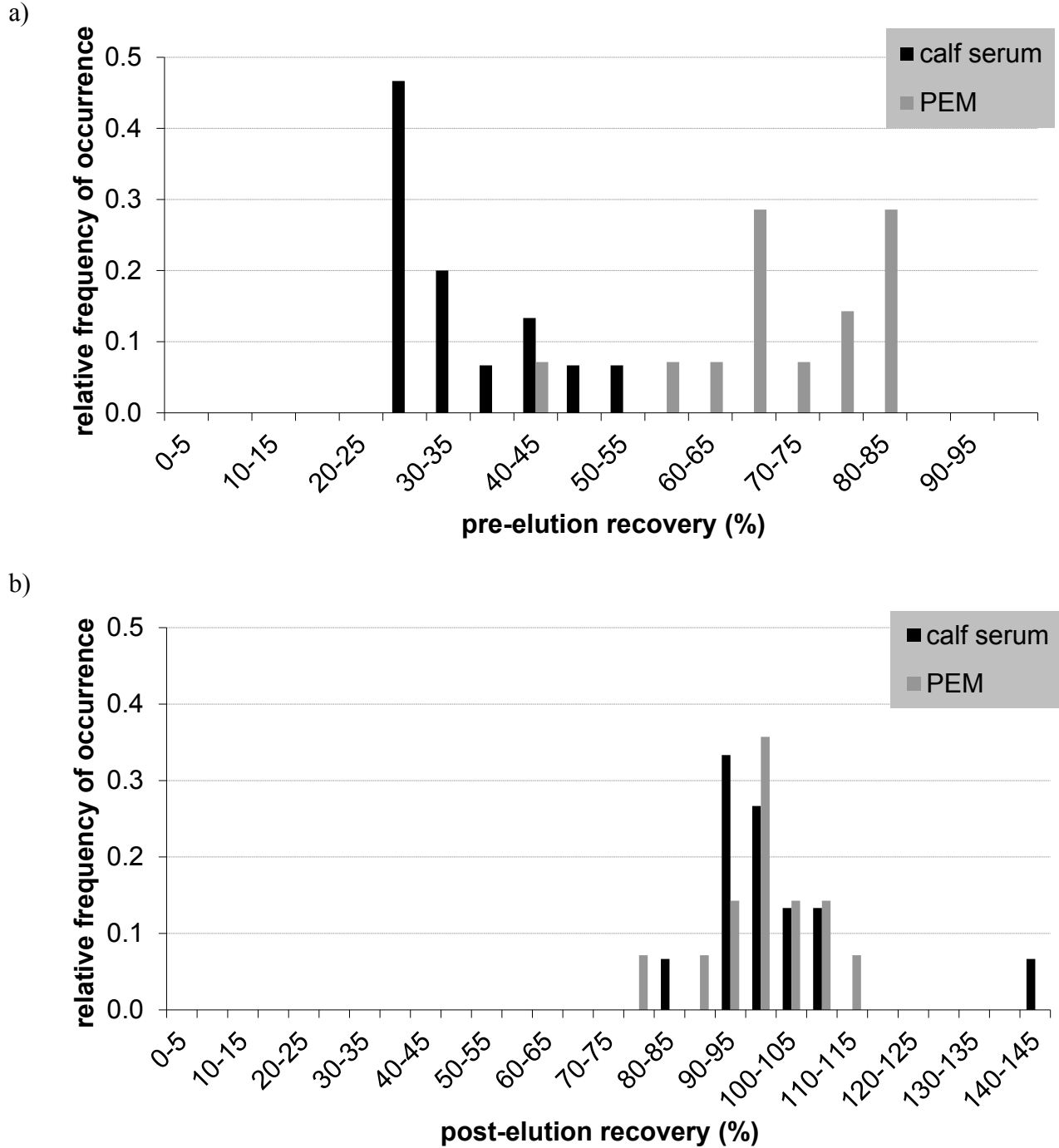


Figure 2.8: Relative frequency of occurrence of P22 (a) pre-elution and (b) post-elution recoveries from DI water under the operational conditions optimized to maximize P22 recovery ($J_{cf} / J_p > 6,400$). Number of experiments: $n=15$ for CS-blocked membranes and $n=14$ for PEM-coated membranes.

2.3.4 Virus log removal

LRV values varied in the 3.9 ± 1.2 range for CS-blocked membranes and in the 4.1 ± 1.1 range for PEM-coated membranes (Fig. 2.9). There was no significant difference between the two types of membranes and no significant influence of J_{cf} / J_p on virus removal.

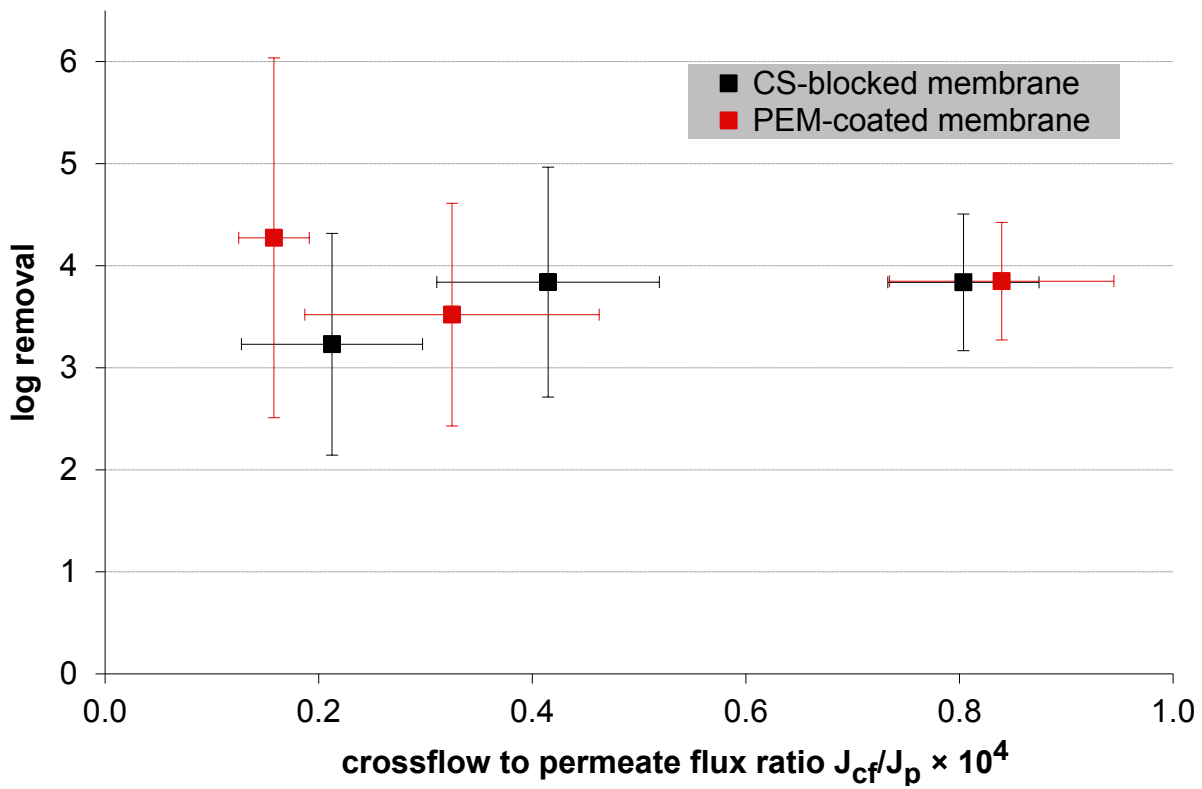


Figure 2.9: P22 removal from DI water by CS-blocked and PEM-coated membrane at various

J_{cf} / J_p .

2.3.5 Effect of operational conditions on virus concentration

2.3.5.1 Operational conditions and flux

Figure 2.3 illustrates the range of operational conditions employed in this work. The J_p values are the permeate fluxes averaged over the first minute of filtration. For experiments conducted at lower transmembrane pressures (Fig. 2.3; “low J_p ” domain), plotted J_p values also represent the average J_p as minimal or no fouling occurred in these tests. In experiments run at higher transmembrane pressures (Fig. 2.3; two “high J_p ” domains) fouling did occur, especially for PEM-coated membranes (Fig. 2.10), so average permeate fluxes were lower than the plotted initial values of J_p (Fig. 2.11 shows average rather than initial fluxes). For comparison, Fig. 2.3 and Fig. 2.11 also show J_p values from earlier studies where crossflow membrane filtration was used to recover viruses and where values of both J_p and J_{cf} were available (see Appendix A).

Compared to earlier studies [2, 3, 5, 6, 11, 13], we evaluated a relatively large range of operational conditions by varying both J_p (2.0 to 8.6 (L/min·m²)) and J_{cf} (6,800 to 26,800 (L/min·m²)). This allowed us to assess the virus concentration performance of CS-blocked and PEM-coated membranes under conditions optimal for either highest virus recovery or fastest sample concentration (Fig. 2.7). The highest recovery conditions belong to the “low J_p ; high

J_{cf} ” domain, while the fastest concentration conditions correspond to the “high J_p ; high J_{cf} ” domain (Fig. 2.3 and 2.11).

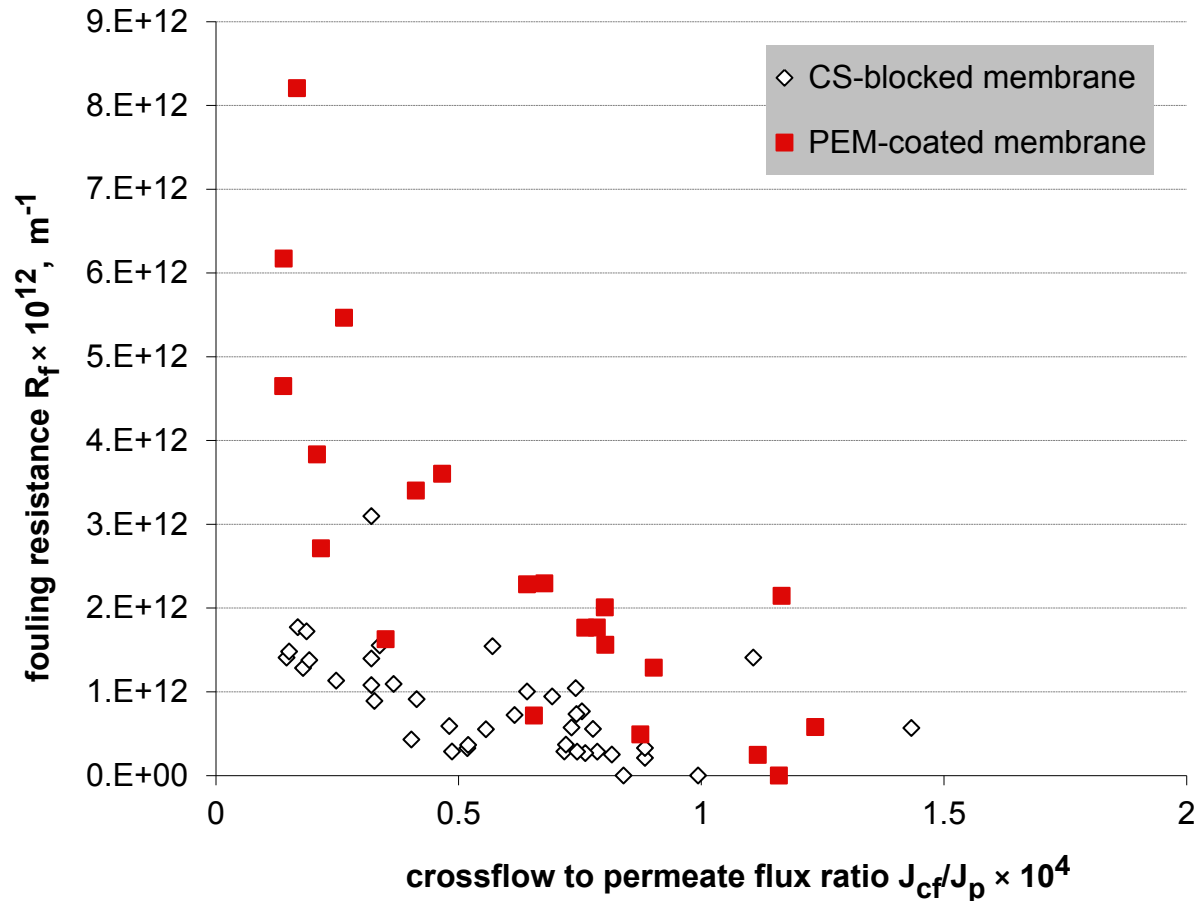


Figure 2.10: Observed fouling resistance (averaged over the duration of experiment) for CS-blocked and PEM-coated membranes during the concentration of 1L of DI water seeded with P22 to 250 mL at various crossflow to permeate flow ratios.

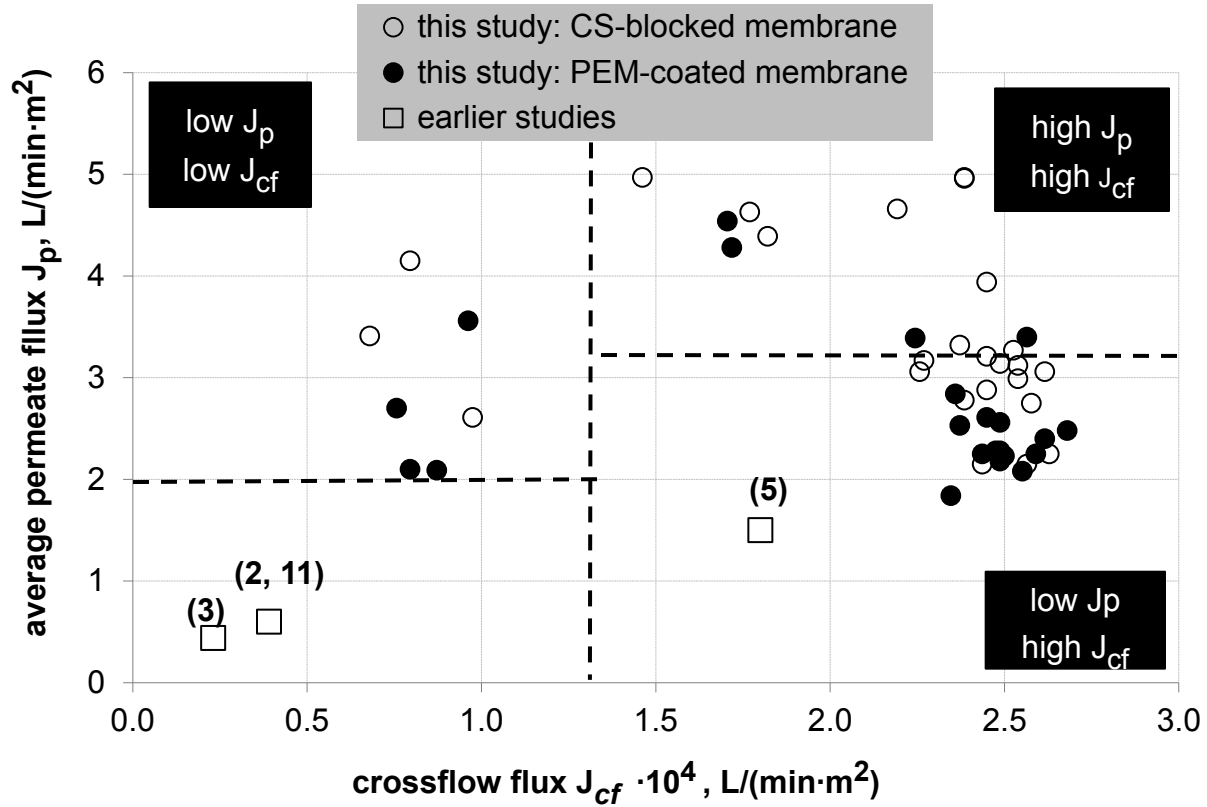


Figure 2.11: Filtration conditions: average permeate flux and crossflow flux, used for P22 recovery with CS-blocked membrane and PEM-coated membranes. $n=27$ for CS, $n=22$ for PEM. In contrast to Fig. 2.3, this graph presents values of permeate flux *averaged* over the duration of filtration experiment rather than over the first minute.

Under “high J_p ; high J_{cf} ” conditions where J_{cf} was in the $20,400 \pm 3,600$ $L/(min \cdot m^2)$ range, fouling decreased the concentration rate; for the CS-blocked membrane, the initial J_p (averaged over the first minute of filtration) was 5.8 ± 0.3 $L/(min \cdot m^2)$, whereas the J_p averaged over the length of the filtration was 4.9 ± 0.3 $L/(min \cdot m^2)$. Similarly, for PEM-coated membranes, under the fastest concentration conditions the initial J_p was 7.2 ± 1.4

L/(min·m²) and the average J_p was 3.9 ± 0.6 L/(min·m²). Thus the concentration rates were similar for CS-blocked and PEM-blocked membranes, but the PEM may show somewhat more fouling due to a higher initial flux.

With polysulfone membranes of similar MWCO (30 kDa) blocked with bovine serum, Hill et al. [2, 3, 11] obtained permeate flow rates between 0.4 and 0.6 L/(min·m²) with tap water samples at a transmembrane pressure of ~ 13 psi. Morales-Morales et al.[5] reported a J_p of 1.5 L/(min·m²) at a transmembrane pressure of 12 psi for surface and tap water samples with a 50 kDa polyacrylonitrile membrane also blocked with CS. By increasing both the transmembrane pressure and cross-flow rate we achieve significantly higher (~ 3-fold) rates of concentration. The comparison is not direct, however, because of different feed water matrices that were spiked by viruses and different concentration ratios: 4.9 mL of feed per cm² of membrane surface for surface/tap water in the study by Morales-Morales et al. [5], 5.5 mL/cm² for tap water in the study by Hill et al. [2] versus 18.3 mL/cm² for DI water in this study.

2.3.5.2 Effect of J_{cf} / J_p on virus recovery

P22 pre-elution recovery by CS-blocked membranes did not significantly increase with an increase in J_{cf} / J_p (Fig. 2.7). Others looked briefly at the dependence of virus recovery on hydraulic conditions [8]. Belfort et al. noticed no significant difference in poliovirus recovery by filtration through a non-blocked cellulose acetate membrane when tripling the crossflow rate; the

observation, however, was based on only one filtration performed at a lower crossflow rate [8]. More recently, Polaczyk et al. [6] observed no statistical difference in recoveries of MS2 phage and echovirus 1 when operating a membrane blocked with NaPP at two different permeate flow rates; however, in their study, the permeate flow rate and crossflow rate were linked, and as a consequence the J_{cf} / J_p ratio changed only from 5,800 to 6,500.

In contrast, the pre-elution recovery of P22 with PEM-coated membranes significantly increased with an increase in J_{cf} / J_p . The recovery is $55 \pm 15\%$ for the fastest concentration conditions (“high J_p ; high J_{cf} ” domain) and $71 \pm 11\%$ for the highest recovery conditions (“low J_p ; high J_{cf} ” domain). This effect of J_{cf} / J_p on virus recovery is expected. At higher J_p , the force driving the virus toward the membrane is higher. In addition at higher J_{cf} / J_p there is more fouling as seen in Fig. 2.10. Fouling can conceal, at least partially, the membrane surface and its anti-adhesive properties which decreased pre-elution recovery as seen in Fig 2.12. Such high recoveries may render the elution step unnecessary. Indeed this level of recovery is comparable to what might be obtained by other virus concentration methods that involve elution. Eliminating the need for elution has several advantages. Time is gained and the volume of the concentrated sample is smaller. In addition, all viruses are recovered pre-elution. These viruses interacted less with the membrane surface than viruses recovered by elution and as a consequence their integrity is better maintained [62]. Post-elution recovery for both types of membrane is not affected by J_{cf} / J_p .

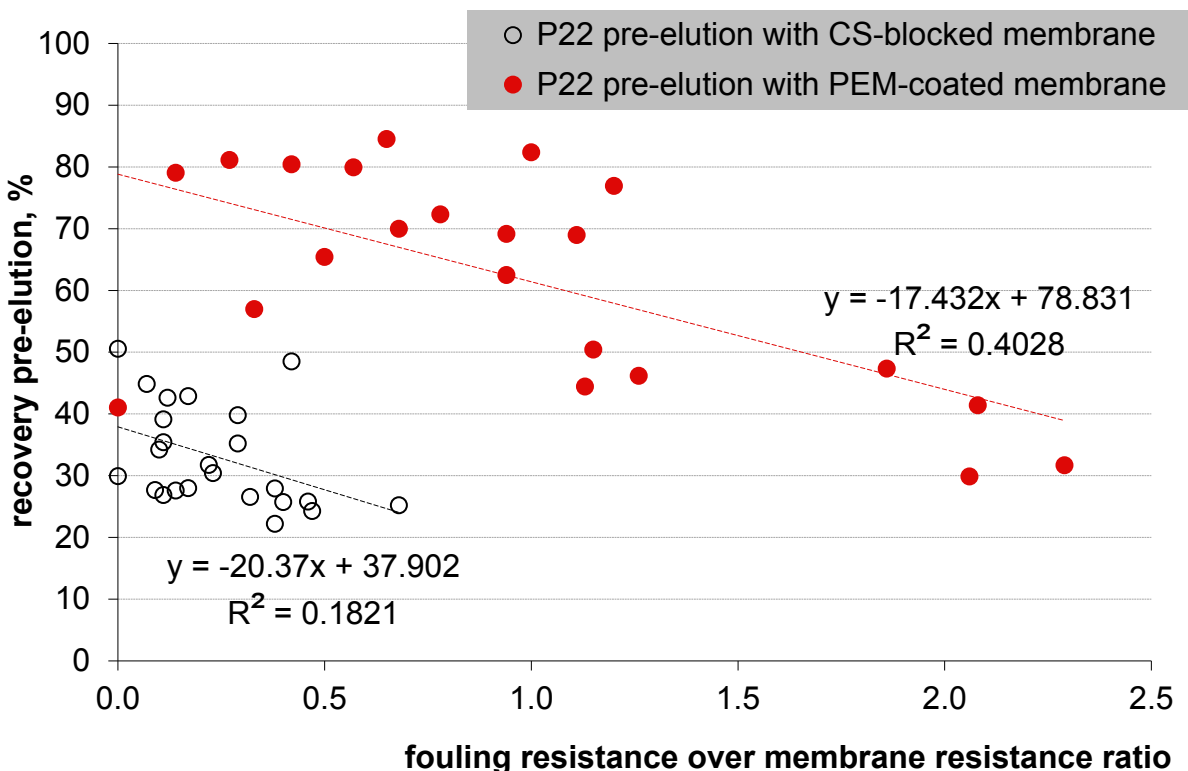


Figure 2.12: Pre-elution recovery of P22 from DI water as a function of the fouling layer resistance to membrane resistance ratio.

2.3.6 Potential for practical applications

Preparation of membranes with a large surface area is critical for successfully scaling up the proposed sample concentration approach [63, 64]. PEMs have already been deposited on flat sheet membranes [65], tubular membranes [66], and hollow fibers [67]. The proposed approach could be scaled-up by depositing a PEM using alternating circulation of cationic and anionic polyelectrolyte solutions through a standard hollow fiber cartridge [2, 3, 5, 6, 11]. Elution, back-flushing, and coating removal and regeneration could occur in situ by flushing the cartridge with appropriate solutions. Other components of a scaled-up portable system would include a back-

pressure valve for generating and controlling the transmembrane pressure and a bypass valve for regulating the crossflow rate. When appropriate, the proposed PEM-based approach can be combined with the addition of chemical dispersant, such as NaPP, as described in several earlier studies [2, 3, 5, 6, 11] to further reduce virus attachment to the membrane surface and improve recovery.

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Chapter 3. Effect of flow conditions and water composition on the removal and recovery of infective bacteriophage by crossflow filtration with CS-blocked membranes

3.1 Introduction

As mentioned in Chapter 1, efficient virus detection in drinking and recreational water is vital for preventing disease outbreaks and this requires an efficient concentration method. The ideal concentration/quantification method should be able to differentiate infectious viruses from non-infectious ones [1] as only the former are a concern in terms of public health, and quantification of viral nucleic acid is not necessarily a good indicator of the presence of infectious virus [2]. It is thus important that the concentration method used does not affect virus infectivity.

Crossflow filtration is emerging as a concentration method for the collection, concentration and quantification of viruses in large volumes of water [3-9], but has the potential to disrupt virus infectivity. Indeed, a decrease in infective virus titer in the retentate of crossflow filtration units has been observed in several studies [9-12] but others did not observe such trends [13]. In two studies, virus aggregation, which affects virus infectivity assays, was initially considered a culprit but eventually ruled out as the reason for the observed loss of infectivity [11, 12]. In some studies increasing ionic strength was found to correlate with virus loss of infectivity in tap water [11, 12], while others, using more complex water samples did not observe any correlation between sample conductivity (an approximate measure of ionic strength) and loss of infectivity

[9]. Virus morphology, the presence of hydrophobic surfaces in the concentration device, and the pumping-induced shear stress, have also been mentioned as possible factors contributing to the loss of viral infectivity during crossflow filtration [10, 11]. Another factor that might influence infectivity assays, and thus recovery of infective viruses by crossflow filtration, is the presence of natural organic matter or cations which have been shown to affect virus aggregation [14]. Finally, changes in filtration conditions such as crossflow rate and permeate flow rate translate into changes in the forces applied to the viruses and, therefore, might affect virus infectivity, virus aggregation, or both.

In Chapter 2, we showed that it was possible to increase the initial permeate flux through CS-blocked membranes by up to 4 times (over the values reported in the literature) without affecting P22 recovery as determined by qPCR. In this Chapter we will look at the effect of filtration conditions and water matrix on the removal and the pre- and post-elution recoveries of infective bacteriophage P22 by CS-blocked membranes. In Chapter 2 we also showed that increasing J_{cf} / J_p increased P22 pre-elution recovery by PEM-coated membranes, whereas for CS-blocked membrane, increasing J_{cf} / J_p had no effect on P22 recovery or removal. This was contrary to our expectation. Indeed decreasing J_p should decrease concentration polarization and lead to less particle adhesion to the membrane [15]. In addition, increasing the crossflow rate can decrease concentration polarization [15] and has been shown to decrease passage of virus through membrane [15, 16]; however, this effect of crossflow might be minimal for viruses as their size corresponds to a minimum in Brownian and shear-induced particle back-transport from the membrane surface [17]. It is possible that the stable P22 removal at increasing J_{cf} / J_p is a result of two mutually counterbalancing effects: 1) a partial loss of integrity of the

CS layer at high crossflow and 2) a decrease in the net flux of viruses towards the membrane surface at higher J_{cf} / J_p . To determine which scenario is operative we assessed the integrity of the CS layer under various filtration conditions.

3.2 Material and Methods

3.2.1 Reagents

Lysozyme (from chicken egg white), ethylenediaminetetraacetic acid (EDTA), calf serum, sodium polyphosphate (NaPP), and dextran standard (25 kDa) were purchased from Sigma-Aldrich. Tween 80 was purchased from Fisher and tryptic soy broth from Becton, Dickinson.

3.2.2 Water samples

Deionized (DI) water was supplied by a commercial ultrapure water system equipped with a terminal 0.2 μm capsule microfilter (PolyCap, Whatman Plc., Sanford, ME). Ten liters surface water samples were collected from Lake Lansing (Lansing, MI) as grab samples in May and September 2009 and in February 2010, and filtered through a 0.45 μm membrane on the day of collection. Samples were kept at 4°C until used. Surface water samples were used as early as the day following their collection and within 4 months of their collection. Before use, the surface water container was agitated to resuspend any settled particles.

The total organic carbon (TOC) content of filtered Lake Lansing samples was determined using a Total Organic Carbon analyzer (1010, O I Analytical, USA). Each sample was analyzed in triplicate. Calibration standards and blanks were run for each set of measurements.

3.2.3 Bacteriophage

Bacteriophages were cultured as described in Chapter 2, section 2.2.2.

3.2.4 Membrane preparation, crossflow concentration apparatus and concentration procedure

The CS-blocked membranes were prepared as described in Chapter 2 section 2.2.5. Membranes were first compacted for 90 min at 40 psi with DI water. The filtration apparatus and concentration procedure are described in Chapter 2 sections 2.2.4 and 2.2.7. Elution was performed with non-spiked surface water amended with 0.01% Tween 80 and 0.01% NaPP. The filtration conditions used in this study are shown in Fig. 3.1.

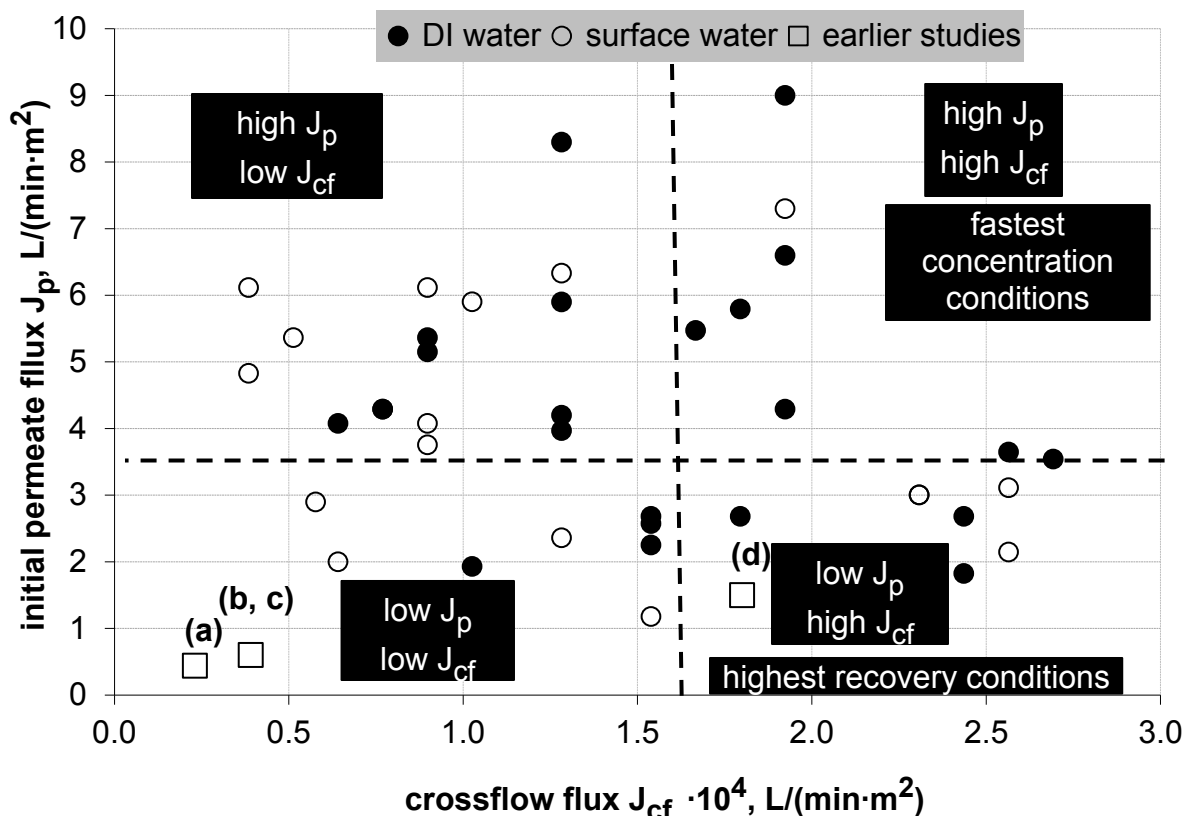


Figure 3.1: Filtration conditions in experiments on infective P22 concentration and recovery from DI water and surface water using CS-blocked membranes. Number of tests: $n=23$ for DI water, $n=18$ for surface water. Earlier studies: a: Hill et al. [18], b: Hill et al. [19], c: Hill et al. [5], d: Morales-Morales et al. [20].

3.2.5 Sample analysis

The P22 concentration in each sample was determined by plaque assay using the double agar layer procedure within 24 h of the filtration experiment. Ten-fold serial dilutions of each sample were prepared. The bacterial host, *S. typhimurium*, was grown to log phase in tryptic soy broth and added to a 1% soft agar solution, which was inoculated with 1 mL dilution aliquots and poured on top of an agar plate. Plates were inoculated overnight at 37°C. Plaques (clear zone of

infected bacterial cells) were then counted and this count was used in combination with the dilution factor to determine P22 concentration in plaque forming unit (PFU)/mL in the sample. Plaque assays were run in triplicate for each dilution of each sample. Samples from 3 filtrations corresponding to 3 different filtration conditions (low J_p - low J_{cf} ; low J_p - high J_{cf} and high J_p - low J_{cf}) were also stored at -80°C in presence of glycerol (10%) to be later analyzed by qPCR. The procedure for qPCR analysis is described in Chapter 2 section 2.2.8.

P22 log removal (LRV) percentage of pre-elution recovery (r_{pre}), and percentage of post-elution recovery (r_{post}) were calculated using the equations (1), (2) and (3) from Chapter 2. Data were compared with the Student t-test. $P < 0.05$ was considered to show statistical significance. A Bonferroni correction was performed when multiple comparison were done.

3.2.6 Calf serum layer stability test

The stability of the calf serum layer during filtration was tested in the filtration unit described in section 2.2.4. CS-blocked membranes were prepared and compacted as described in section 2.2.5. The membranes were then used to filter a 20 mg/L aqueous solution of 25 kDa dextran using a transmembrane pressure of approximately 40 psi. The crossflow rates was increased from 1,500 L/(min·m²) to 22,000 L/(min·m²), which corresponds to J_{cf} / J_p ratios from 900 to 8,900. Each crossflow rate was maintained for 5 min. and permeate samples were collected before and after the crossflow rate was increased. Retentate samples were also collected several times during the test to monitor dextran concentration. Concentrations of

dextran in the retentate and in the permeate were determined using the total organic carbon analyzer. Each sample was analyzed in triplicate. Calibration standards and blanks were run for each set of measurements. Rejection (R) of dextran at a given time was determined using equation (3.1):

$$R(t) = \left(1 - \frac{C_p(t)}{C_r(t)} \right) \cdot 100\% \quad (3.1)$$

where C_p and C_r are, respectively, dextran concentrations in the permeate and retentate at that given time. C_r at any given time was determined using linear interpolation of retentate concentration values measured at several times during each experiment

The stability of the calf serum layer during elution was also tested. The test was similar to the one described above except that pressure was only applied to collect samples. CS layer stability during filtration was tested in triplicate, only one test was performed to determine the CS layer stability during elution.

3.3 Results and Discussion

3.3.1 Effect of filtration conditions on the recovery and removal of infective P22

A preliminary test was performed to determine if infective viruses were lost due to the adhesion to the components of the filtration unit, other than the membrane or due to loss of infectivity. We showed that circulating 1 L of DI water spiked with P22 ($1.3 \cdot 10^6$ PFU/mL) for 3 h in the filtration unit, with no membrane in place and no pressure applied, did not affect P22

counts in the infectivity assay indicating that there was no loss of infectivity due to contact with components of the concentration unit as some have observed [11] or that this loss of infectivity was coincidentally offset by virus disaggregation.

Figure 3.2 shows the recovery and removal of infective P22 from DI water and surface water by CS-coated membrane under various filtration conditions. At the pre-elution level, there was no effect of the filtration conditions on the recovery of infective P22 from either DI water or surface water. At the post-elution level, although the recovery of infective P22 from DI water in the least favorable conditions (high J_p , low J_{cf}) seemed lower on average than under other conditions, a t-test analysis showed that the difference was not significant. In the case of surface water, again no effect of filtration conditions on infective P22 recovery is observed.

Recovery of infective P22 from DI water or surface water was not affected by filtration conditions. As mentioned in Chapter 2, a similar trend has been observed by others [21, 22], however the scope of their studies was limited in terms of the number of filtrations performed [21] or range of J_{cf} / J_p ratios explored [21]. The trend is counterintuitive as it was expected that increasing J_{cf} / J_p would decrease concentration polarization and fouling (Fig. 3.3), which should increase pre-elution recovery as virions have a lesser chance to be trapped near the membrane.

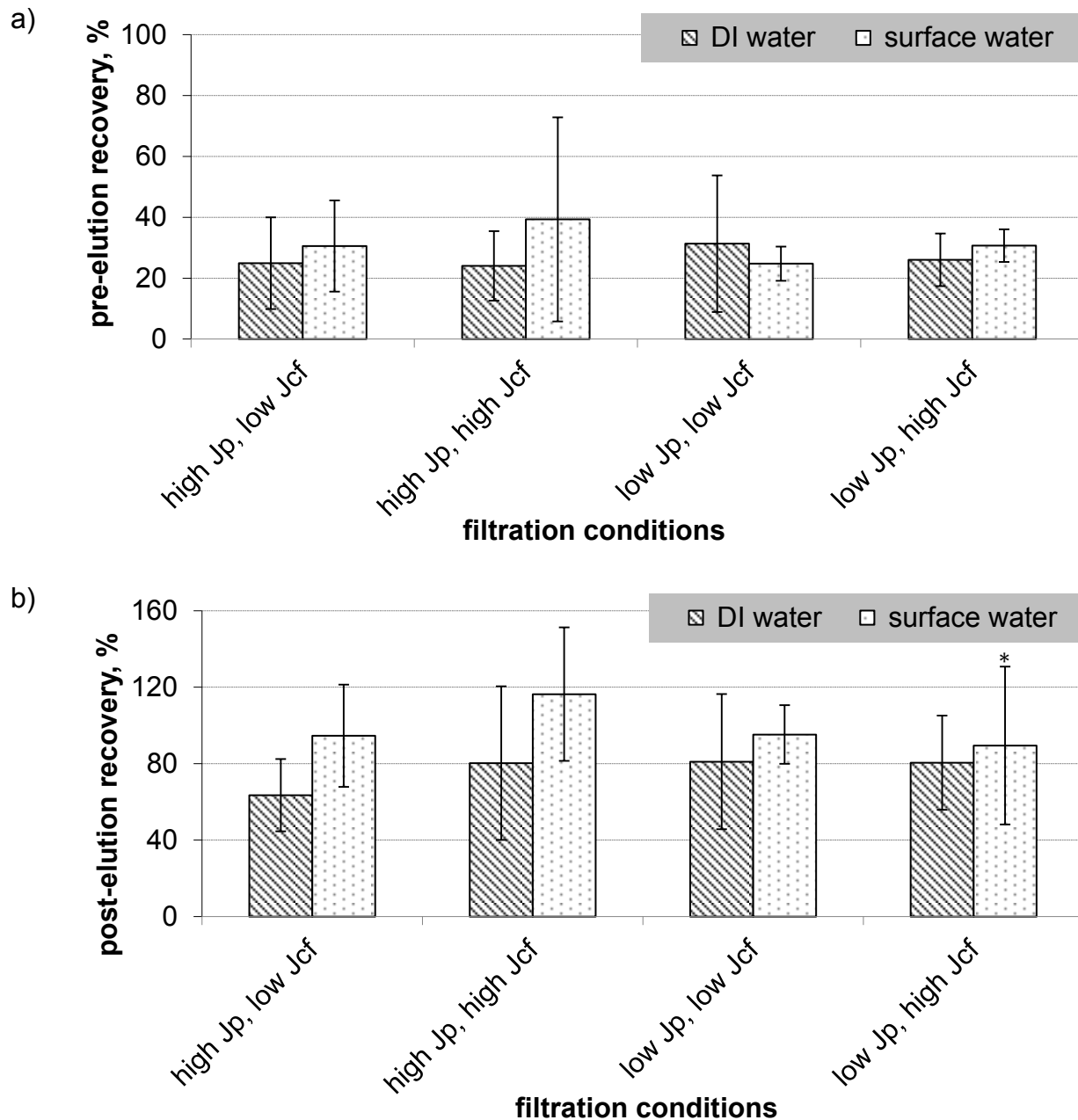
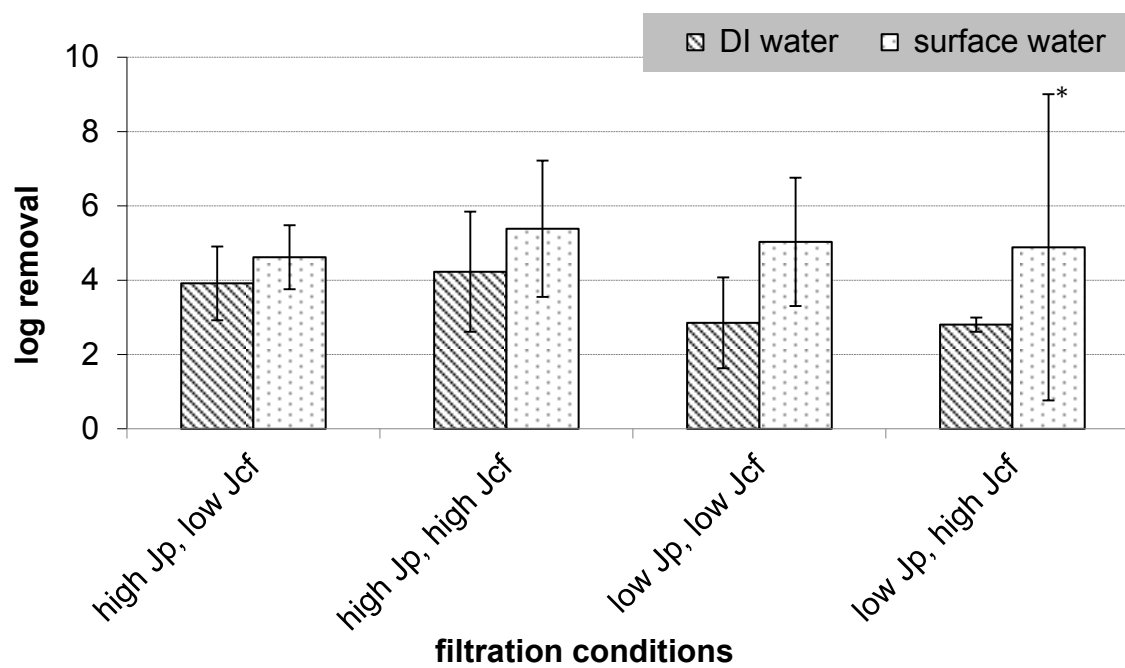


Figure 3.2: Effect of crossflow filtration on a) pre-elution recovery, b) post-elution recovery, c) removal of infective P22 by crossflow filtration with CS-blocked membrane. Error bar indicates 90% confidence interval except for the bars marked with *, for which the standard deviation was used as only 2 filtration tests were performed.

Figure 3.2 (cont'd)

c)



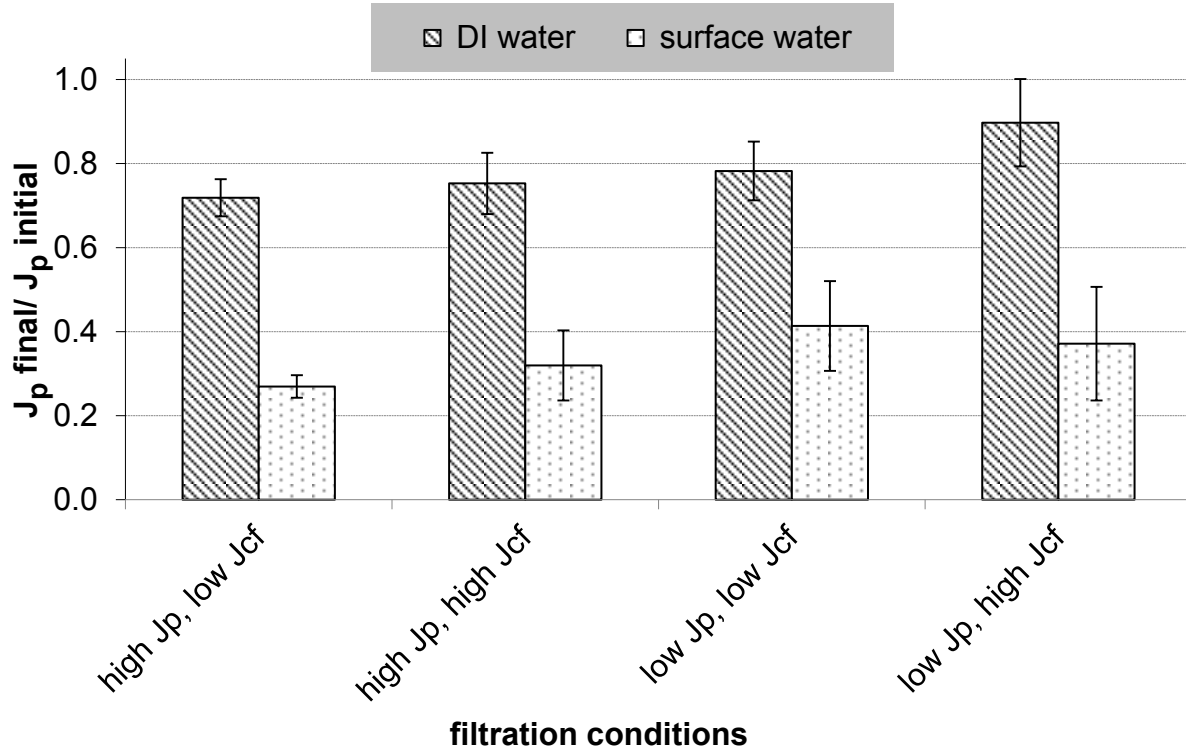


Figure 3.3: Effect of crossflow filtration conditions on fouling, measured by the ratio of final over initial permeate flux for DI water and surface water. Error bar indicates the 90% confidence interval.

For microfiltration (MF) membranes it was shown that increasing the J_{cf} / J_p ratio leads to an increase in the removal of MS2 and Q β phages [16] and that higher J_p and stirring increase removal of poliovirus [23]. In the present study, however, changing filtration conditions had no effect on P22 removal from DI water or surface water. In the case of surface water, the formation of a thicker fouling layer preventing virus passage at lower J_{cf} / J_p might have opposed the effect of increased concentration polarization and helped maintaining removal levels as high as at higher J_{cf} / J_p . Others have observed a positive influence of fouling or cake formation on virus removal during low-pressure membrane filtration [24, 25]; however this

mechanism cannot explain the absence of effect observed for removal from DI water or recovery from DI water and surface water.

3.3.2 qPCR vs. plaque assay

As reported in Chapter 2, no significant effect of J_{cf} / J_p on P22 pre and post-elution recoveries or removal (as determined by qPCR) by CS-blocked membrane was observed. In this Chapter we report the absence of such trends observed for infective P22 as well. In addition, the average removal and pre-elution recoveries of infective P22 observed in this study were not significantly different than the average removal and recoveries based on qPCR analysis as reported in Chapter 2 for CS-blocked-membranes; however, post-elution recovery of infective virus was significantly lower than recovery measured by qPCR ($74 \pm 31\%$ and $99 \pm 11\%$ respectively).

Data from the three filtrations for which samples were analyzed by both qPCR and plaque assay, confirm this observation (Fig. 3.4). Similar elution and removal recovery were observed, except at the post-elution level where a significant difference was detected at high J_p , low J_{cf} . If this observation is confirmed to be statistically significant, it would indicate that, under high J_p , low J_{cf} , viruses recovered during elution have either lost infectivity (for example due to interaction with the membrane during the filtration step or because of the additive -Tween 80 and NaPP- used for elution) or have aggregated. Since NaPP is often used as a dispersant when concentrating various pathogens including viruses [20], the decrease in viral count observed by plaque assay was most probably due to the loss of P22 infectivity rather than

aggregation of the phage. This could be confirmed by measuring the particle size in the retentate and eluate; however, particle size analysis requires higher virus titer than the one that we had in our filtrations and concentrating those samples was not an option as any concentration could affect the aggregation state of the virus suspension.

Another difference between results obtained by qPCR and plaque assay is the reproducibility of virus recovery and removal data. Standard deviations of pre-elution recoveries, post-elution recoveries and LRV values obtained by qPCR (8%, 11% and 1.2 respectively) were lower than standard deviations obtained in plaque assays (17%, 31% and 1.4 LRV respectively). This might be explained by a greater standard deviation associated with the plaque assay method compared to the qPCR method (as seen in Fig. 3.4), which is due, in part, to the fact that plaque assay was performed on 3 different grab samples while qPCR was performed in triplicate on one grab sample.

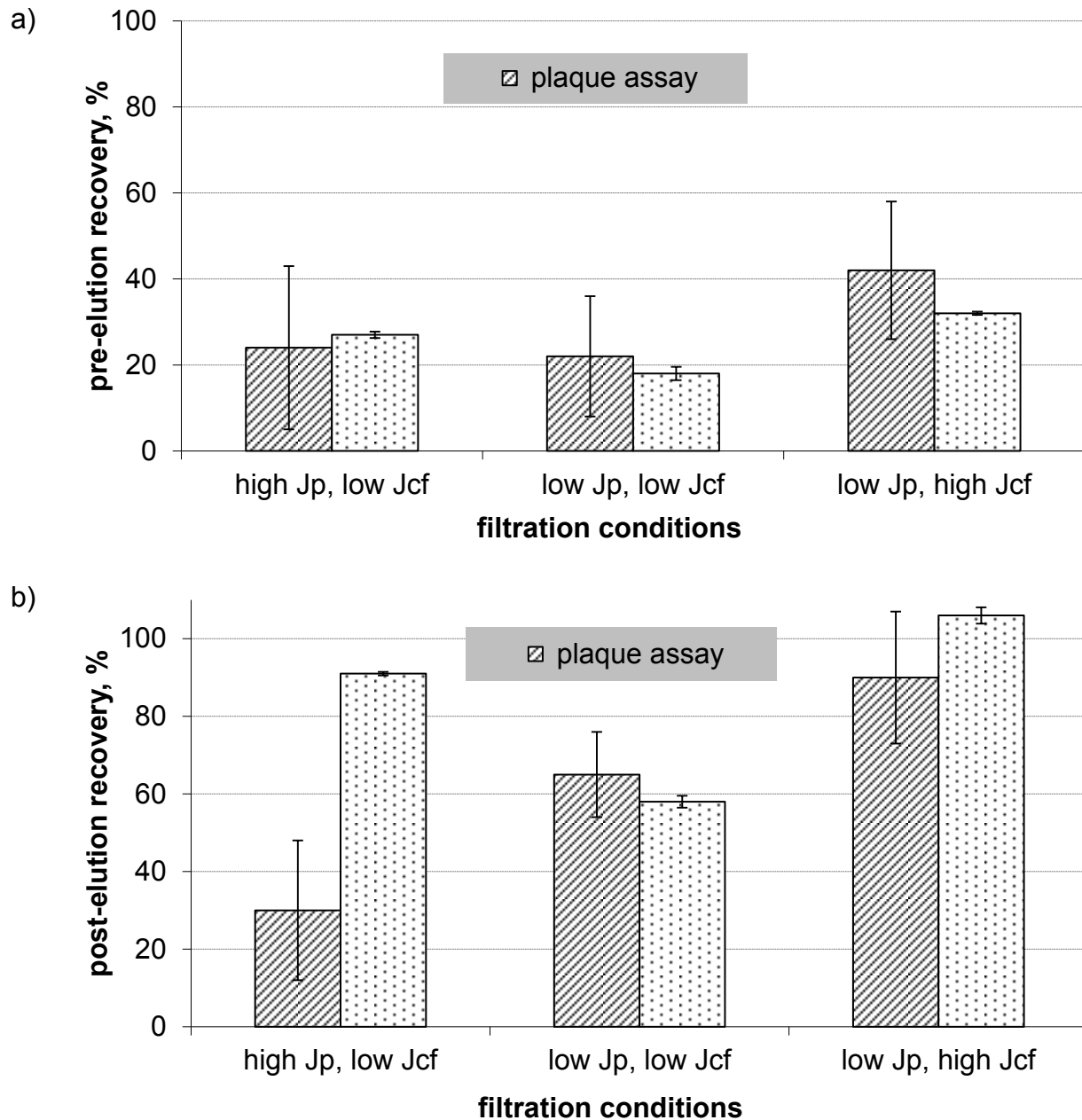
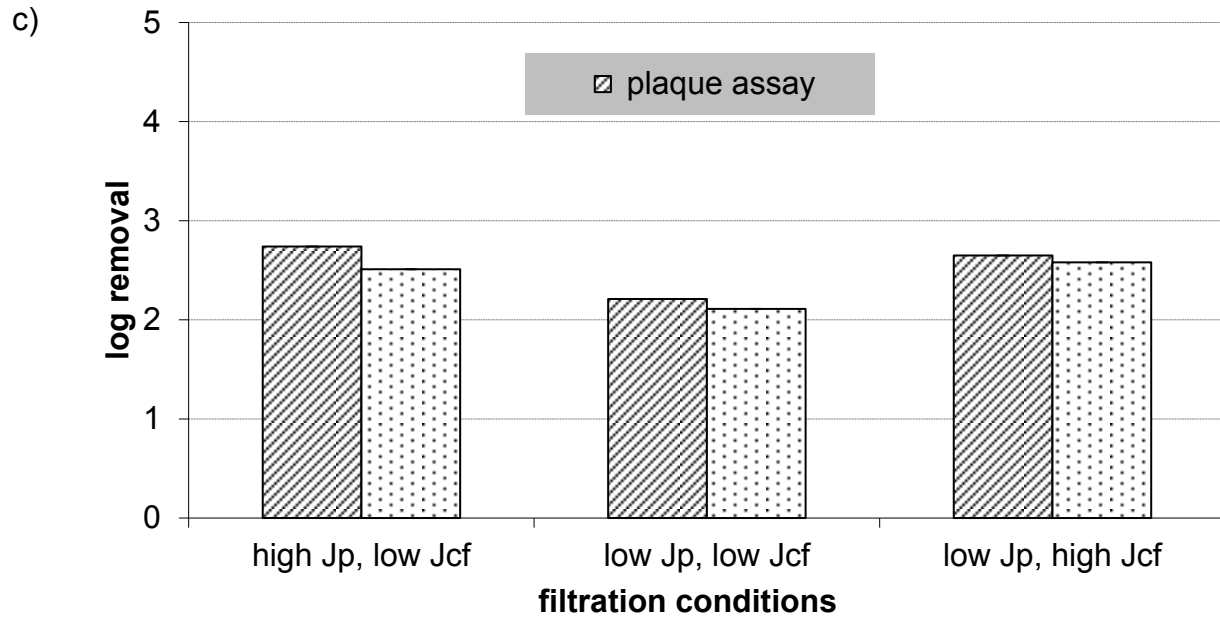


Figure 3.4: Effect of analysis method on P22 a) pre-elution recovery, b) post-elution recovery, c) removal, by crossflow filtration with CS-blocked membrane. One filtration was run for each set of conditions. The error bar indicates the standard deviation associated with the experimental method.

Figure 3.4 (cont'd)



3.3.3 DI water vs. surface water

As Fig. 3.2 demonstrates, at the pre-elution level there was no significant difference between in the recovery of infective P22 from surface water and from DI water. At the post-elution level, average recovery from surface water was consistently higher than recovery from DI water, however, the difference is not statistically significant. We did not expect post-elution recovery from surface water to be higher than post-elution recovery from DI water, especially under high J_p - low J_{cf} . In these conditions, virus can get trapped in the fouling layer, which is more important for surface water than for DI water (Fig 3.3).

3.3.4 Effect of filtration condition on CS layer integrity

To explain the absence of influence of J_{cf} / J_p on the recovery and removal of infective P22 and P22 DNA, we hypothesized that the calf serum layer was disintegrating at higher J_{cf} / J_p values thus offsetting the benefit of lower concentration polarization and fouling. However, as seen in Fig. 3.5, 25 kDa dextran rejection by CS-blocked membrane was not affected by filtration conditions, indicating that the layer integrity is maintained. The test was performed at 40 psi and, as a consequence, J_{cf} / J_p values higher than 8,000 were not tested (although those conditions were used in P22 filtration tests).

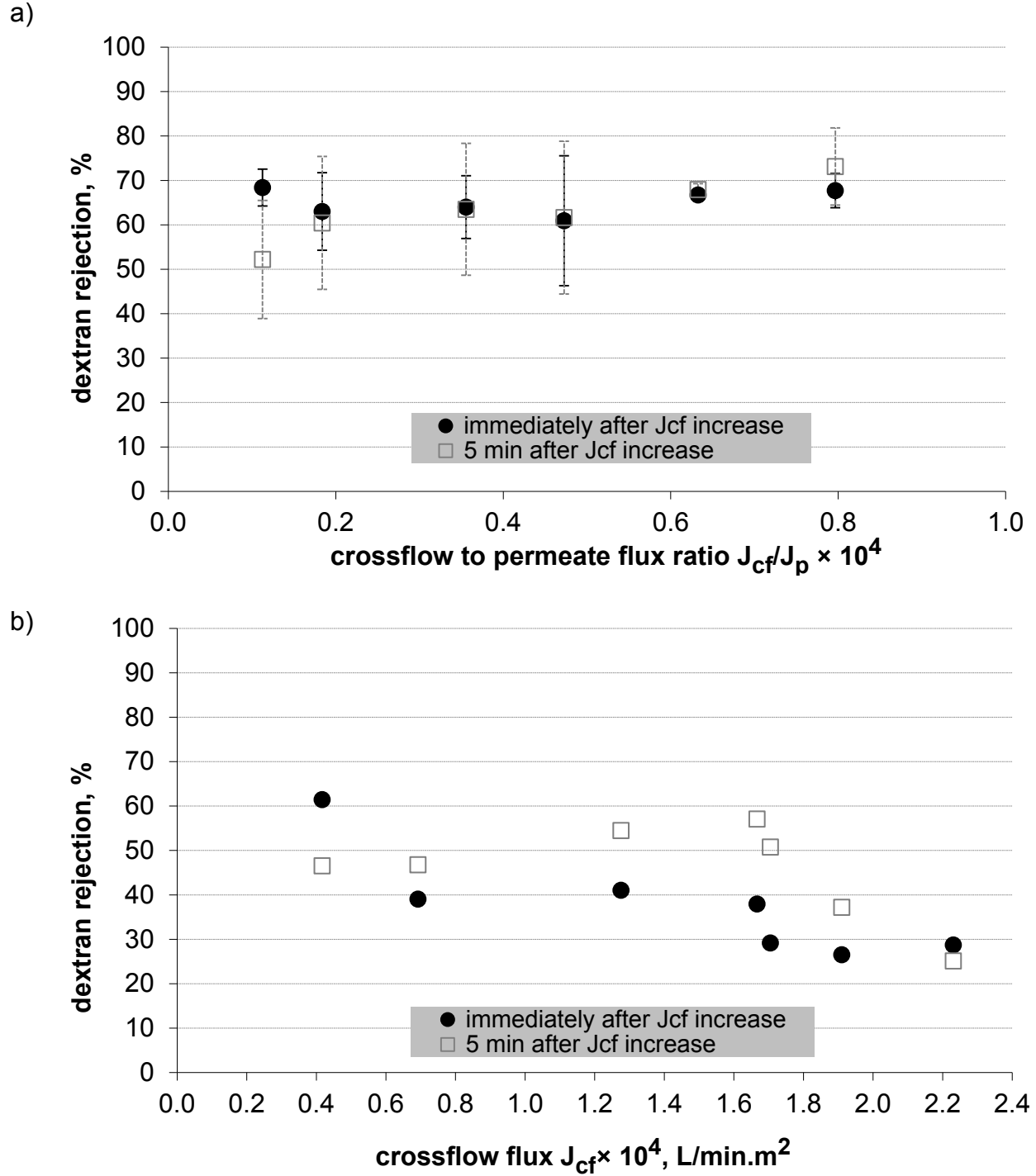


Figure 3.5: Dextran rejection by CS-blocked membrane under increasing J_{cf} / J_p during a) filtration and b) elution. $n=3$ for filtration, error bar indicates standard deviation; $n=1$ for elution.

During elution, dextran rejection decreased as J_{cf} increased, indicating that the layer integrity was compromised. (Additional testing should be performed to confirm this trend as only one elution test was performed). We do not expect this phenomenon, if confirmed, to affect P22 recovery. On the contrary, stripping the layer off the membrane may be an effective approach for recovering those viruses that penetrated that layer during filtration. It is possible that when submitted to the pressure of 20 psi, the calf serum layer also loses integrity, which would balance the effect of decreasing the permeate flux in terms of virus adhesion to the membrane. In addition, increasing crossflow only has a minimal effect on decreasing concentration polarization of P22 [17]. Together, these phenomena could explain the lack of improvement in P22 pre-elution recovery or removal at increasing J_{cf} / J_p . However the calf serum layer stability at 20 psi should be assessed to confirm this hypothesis.

3.3.5 Conclusions

Permeate flux and crossflow rate were found to have no statistically significant effect on the recovery and removal of infective P22. The average permeate flux could be increased up to 5-fold for DI water and 2-fold for surface water over values reported in the literature without affecting the recovery or removal of infective P22. However, virus infectivity and/or aggregation did seem to be affected by crossflow filtration. Specifically, under high J_p - low J_{cf} conditions, viruses recovered at the post-elution stage seemed to have lower infectivity or be aggregated, which might be due to interactions with the membrane surface or the presence of additive at the elution stage.

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Chapter 4. Virus recovery and concentration from complex water matrices using PEM-coated membranes: Preliminary assessment

4.1 Introduction

In Chapter 2 we showed that PEM-coated membranes show promising results for the recovery of viruses present in DI water; however, viruses often need to be concentrated from more complex matrices: tap water, groundwater, surface water as well as water treated for reuse applications. It has been shown that increasing water complexity can affect virus recovery by crossflow filtration [1]. For example, Belfort et al. showed that poliovirus recovery decreased from 77% with DI water to 52% with tap water. In at least one study, however, water complexity did not affect virus recovery as there was no significant difference in infective MS2 and PRD1 recovery between drinking and surface water [2].

Even for the same type of water, variation in water composition can greatly affect virus recovery [3, 4]. T1 phage recovery from surface water from 6 different locations varied from 34% to 75% [3], and PP7 recoveries from storm water from 21 different locations varied from 10% to 98 % when assessed by qPCR, and from 25% to 90% when assessed by plaque assay. However, since filtrations were not replicated it is not possible to conclude whether the variations were due to differences in the water composition or to an inherent irreproducibility of the combination of the recovery and detection methods. In a study on virus recovery from drinking water from 8 different locations, Hill et al. [5] did perform replicate measurements and found no statistically significant correlations between the 7 parameters tested (pH, turbidity,

alkalinity, conductivity, iron concentration, TOC, and DOC) and recoveries of MS2 or Φ X174 phages.

Water composition can affect both concentration and quantification steps. The formation of a cake layer may prevent virus passage through the membrane [6, 7] or conceal a membrane's anti-adhesive properties with respect to a virus. Virus surface properties also depend on the solution makeup (such as ionic strength, presence of divalent cations and dissolved NOM). In addition, materials present in the water might affect the detection method [8], especially in the case of highly concentrated samples. This is why it is important to evaluate the concentration and detection methods with a wide range of water compositions.

In this Chapter the performance of PEM-coated membrane in recovering P22 from MBR effluent and surface water is evaluated and compared to the performance of CS-blocked membrane. The effect of adjusting TOC levels in samples before DNA extraction is assessed so that we can determine if the observed effects of water complexity on recovery or removal occurred at the concentration or detection steps. In addition, since the possibility of virion entrapment at the membrane surface increases with water complexity, the effect of adding a backflush step after elution on final P22 recovery is studied for surface water samples.

4.2 Materials and Methods

4.2.1 Reagents

The reagents used in this chapter were the same as those described in corresponding sections of previous Chapters.

4.2.2 Water samples

A 25 L membrane bioreactor (MBR) effluent sample was collected from the Traverse City wastewater treatment plant (Traverse City, MI) as a grab sample in August 2011. A 10 L surface water sample was collected from Lake Lansing (Lansing, MI) as a grab sample in September 2011, and filtered through a 0.45 μm membrane on the day of collection. Samples were kept at 4°C until used. Water samples were used as early as the day following their collection and within 1 months of their collection. Before use, the surface water container was agitated to resuspend any settled particles.

4.2.3 Water quality testing.

Total organic carbon (TOC) contents of MBR effluent sample and filtered Lake Lansing water were determined using a Total Organic Carbon analyzer (1010, O I Analytical, USA). To determine how filtration affected the TOC content of retentate and permeate samples, filtrations of MBR effluent and surface water were performed with a PEM-coated membrane and a CS-

blocked membrane in conditions similar to the ones used in virus concentration tests (except for the filtration of MBR effluent with a CS-blocked membrane which was performed at 40 psi instead of 20 psi), and TOC analysis was performed on feed, retentate and permeate samples. Each sample was analyzed at least in triplicate. Calibration standards and blanks were run for each set of measurements.

4.2.4 Bacteriophage

Bacteriophages were cultured as described in Chapter 2, section 2.2.2.

4.2.5 Membrane preparation, crossflow concentration apparatus and concentration procedure

The PEM-coated membranes and CS-blocked membranes were prepared as described in Chapter 2 section 2.2.5. The filtration apparatus and concentration procedure are described in Chapter 2 sections 2.2.4 and 2.2.7. For both types of water, filtrations at high J_{cf} and low J_p (i.e., “highest recovery conditions”) were performed at least in triplicate. For the MBR effluent, PEM performance at high J_{cf} and high J_p was also evaluated. Filtration conditions used in this study are shown in Fig. 4.1. Elution was performed with 500 mL of permeate amended with 0.01% Tween 80 and 0.01% NaPP. Filtrations with surface water included an additional backflush step after elution. To backflush the membrane, it was flipped in the filtration cell so that the membrane skin, coated by PEM or blocked with CS, was on the permeate side. The backflush was performed by filtering 100 mL of permeate through the membrane at the applied transmembrane pressure of approximately 40 psi.

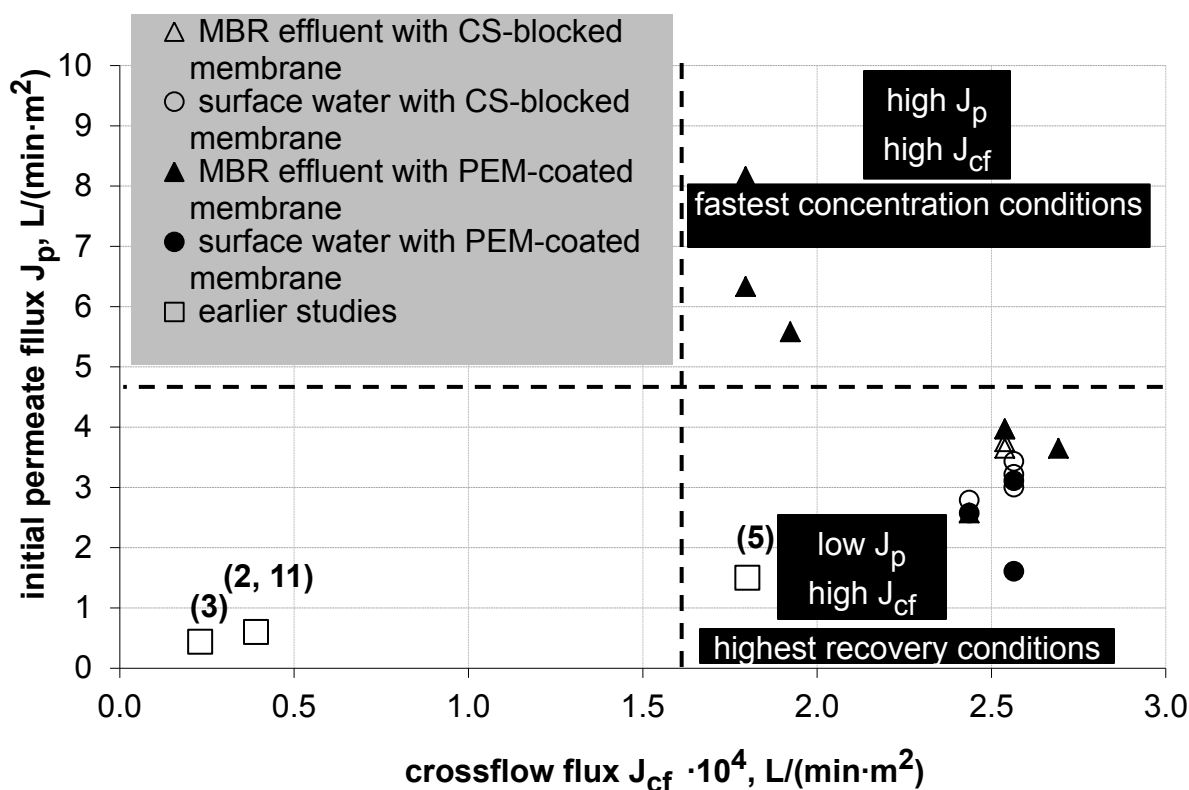


Figure 4.1: Filtration conditions in experiments on P22 concentration and recovery from MBR effluent and surface water using CS-blocked membranes and PEM-coated membrane. Number of tests: $n=9$ for MBR effluent (3 with CS-blocked membranes, 6 with PEM-coated membranes), $n=7$ for surface water (3 with CS-blocked membranes, 4 with PEM-coated membranes).

4.2.6 Sample analysis

P22 concentration in feed, retentate, permeate, eluate and backflush samples were measured by qPCR as described in Chapter 2 section 2.2.8. For comparison, all samples except the one from filtration tests performed in “fastest concentration” conditions were also run as described in Chapter 2 section 2.2.8 with the exception that prior to DNA extraction, retentate and feed samples were diluted with PCR grade water (Roche) so that TOC levels in those

samples matched TOC levels in permeate samples. Values of P22 recovery and removal from MBR effluent and surface water samples were compared to corresponding values recorded in an experiment that used DI water and was run in similar filtration conditions (data from Chapter 2). Data were compared using the Student t-test. $P < 0.1$ was considered to show statistical significance. A Bonferroni correction was performed when multiple comparisons were done.

4.3 Results and Discussion

4.3.1 Water quality

Results from TOC analysis are presented in Table 4.1. TOC levels in surface water, 9.0 ± 0.2 ppm, are slightly higher than TOC levels in MBR effluent, 7.3 ± 1.0 ppm. TOC from surface water seemed to be better recovered than TOC from MBR effluent by both CS-blocked and PEM-coated membranes. (The experiment should be replicated to determine if the difference is statistically significant.) When surface water was filtered through CS-blocked membranes, the combined TOC values of the permeate and the retentate exceeded the TOC values measured in the feed. This could either be due to calf serum shed by the blocking layer to the retentate stream, or the result of an experimental error. Additional experiments are necessary to identify which explanation is valid.

Table 4.1: MBR effluent and surface water TOC levels

Type of water	Membrane	Sample (Sample volume)	TOC level (ppm)	TOC rejection	TOC recovery
MBR effluent	CS-blocked	Feed (1L)	8.0	13 %	27 %
		Retentate (250 mL)	8.5		
		Permeate (750 mL)	7.0		
	PEM-coated	Feed (1L)	6.6	18 %	28 %
		Retentate (250 mL)	7.2		
		Permeate (750 mL)	5.4		
Surface water	CS-blocked	Feed (1L)	8.8	5%	41%
		Retentate (250 mL)	15.5		
		Permeate (750 mL)	8.4		
	PEM-coated	Feed (1L)	9.1	19%	36%
		Retentate (250 mL)	13.7		
		Permeate (750 mL)	7.4		

4.3.2 P22 recovery and removal from MBR effluent and surface water samples

We expected that the higher TOC levels in the retentate samples would enhance P22 DNA extraction in retentate sample compared to P22 extraction from samples with lower TOC (feed and permeate). Indeed the presence of organic matter in samples has been shown to increase P22 extraction by MagNA Pure Compact system hypothetically by blocking adsorption sites on the surfaces of various system components such as tips or magnetic particles to prevent

the loss of P22 DNA due to adsorption (see Appendix B). As a consequence, any observed increase in recovery could be due to either a more efficient collection/concentration step or only a more efficient DNA extraction/detection step. For this reason, TOC levels in feed and retentate were adjusted to the TOC level in the permeate by dilution with PCR grade water. As can be seen in Fig. 4.2, except for the recovery of P22 from MBR effluent with PEM-coated membranes, recoveries obtained from TOC-adjusted qPCR analyses were on average lower than when TOC levels were not adjusted, however, the difference was not significant.

Under the “highest recovery” conditions the pre-elution recovery of P22 by PEM-coated membranes was lower on average for MBR effluent and surface water than for DI water but the difference was not statistically significant. Lower pre-elution recovery recoveries may result from the “masking” of the anti-adhesive properties of the membrane by the organic fouling layer, from the entrapment of viruses within this fouling layer, or from both of these phenomena (Fig. 4.2). The post-elution recovery by PEM membranes was not significantly affected by the type of water. Increasing the permeate flux did not significantly affect the pre-elution or post-elution recovery of P22 from MBR effluent using PEM membranes (Fig. 4.3).

For CS-blocked membrane, pre-elution and post-elution recoveries were not significantly different for DI water, MBR effluent and surface water.

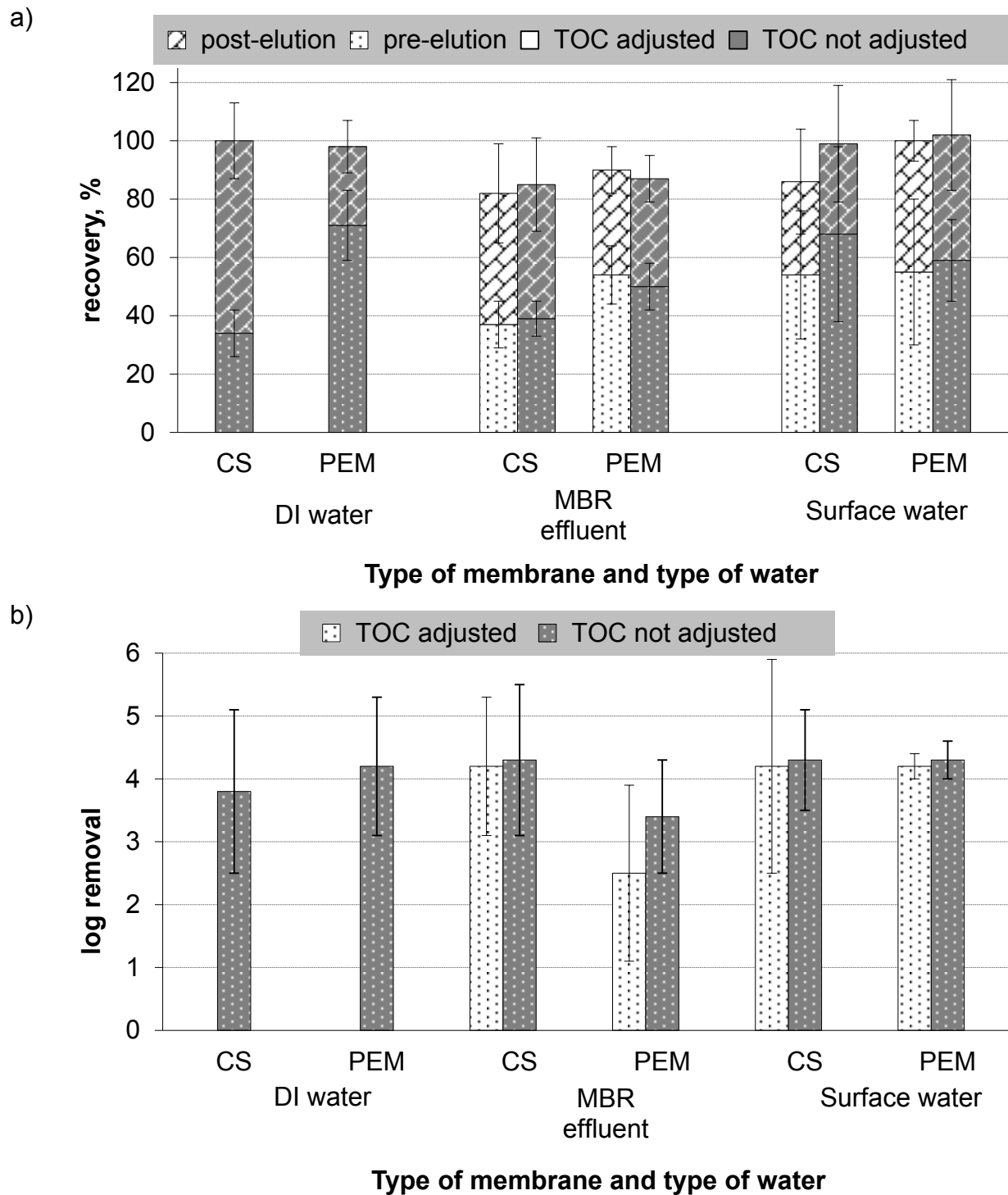


Figure 4.2: a) P22 recovery and b) removal from DI water (data from Chapter 2), MBR effluent and surface water with CS-blocked and PEM-coated membranes under “highest recovery” conditions. Error bars indicate standard deviation.

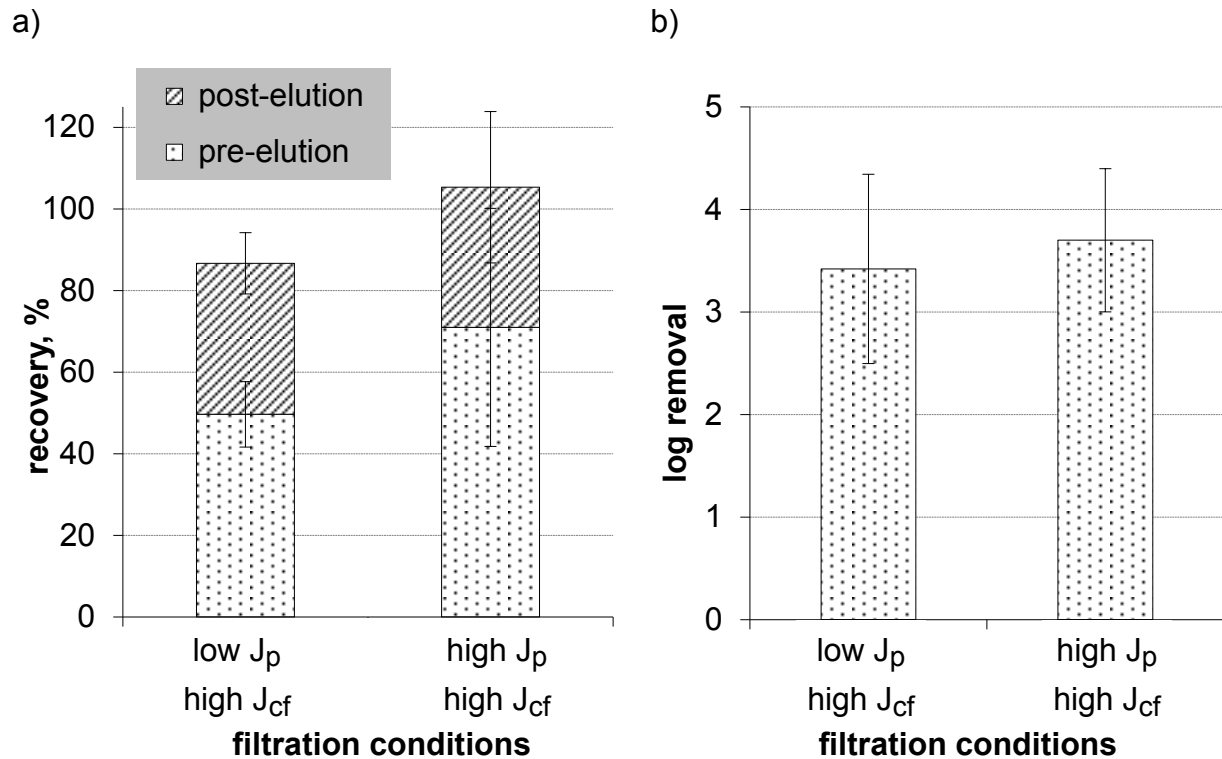


Figure 4.3: a) P22 recovery and b) removal from MBR effluent with PEM-coated membrane at high and low permeate fluxes. Error bars indicate standard deviation. Data reported here corresponds to samples where TOC was not adjusted.

Although pre-elution recovery from MBR effluent by PEM-coated membranes was noticeably higher than by CS-blocked membrane, the difference was not significant. No difference was observed for surface water. At the post-elution level there was no difference between CS-blocked membranes and PEM-coated membranes for both types of water, which is also what was observed for DI water and was attributed to the weak adhesion of P22 to surfaces compared to other viruses. P22 removal was not significantly affected by the type of membrane or the type of water.

4.3.3 Effect of backflushing on P22 recovery from surface water.

The fouling layer can entrap virions and render them less recoverable by elution. In that case adding a backflushing step can help recover viruses entrapped in the fouling layer. We performed backflushing after tests on the filtration of surface water spiked with P22. For these tests, we already showed (Table 4.2) that post-elution recoveries were not significantly lower than post-elution recoveries from DI water which means that the loss of viruses due to entrapment was probably minimal. It is thus not surprising that backflushing did not significantly increase P22 recovery. For all 6 filtrations where a backflush was performed, the post-backflush recovery was only marginally (less than 1%) higher the post- elution recovery.

4.3.4 Fouling resistance of CS-blocked and PEM-coated membrane

Figure 4.4 shows the ratio of final to initial permeate flux. This ratio is a measure of the amount of fouling occurring during filtration. Initial and final permeate flux were averaged over the first and last minute of filtration, respectively. For DI water experiments the permeate flux reduction was higher for PEM-coated membranes than for CS-blocked membranes. For MBR effluent, the trend seemed to be reversed and PEM-coated membranes seemed to experience less fouling under both “highest recovery” and “fastest filtration” conditions (the difference was only significant under “fastest filtration” conditions) compared to CS-blocked membranes. Fouling resistance of the PEM-coated membranes stemmed from its higher hydrophilicity compared to CS--blocked membranes (see Chapter 2). For surface water, the amount of fouling observed for

CS- blocked membranes was higher on average than for PEM-coated membrane, but the difference was not statistically significant.

4.3.5 Conclusion

Due to the small number of filtrations performed the data presented here are only preliminary. It was not possible to observe significant difference between PEM-coated membrane and CS-blocked membrane in complex water matrices. However, PEM-coated membranes seemed to maintain their advantage in terms of P22 pre-elution recovery when concentrating viruses from MBR effluent. PEM-coated membranes also seemed to be more resistant to fouling. In experiments with surface water, however, no difference was observed. Additional filtration tests are needed to validate these trends.

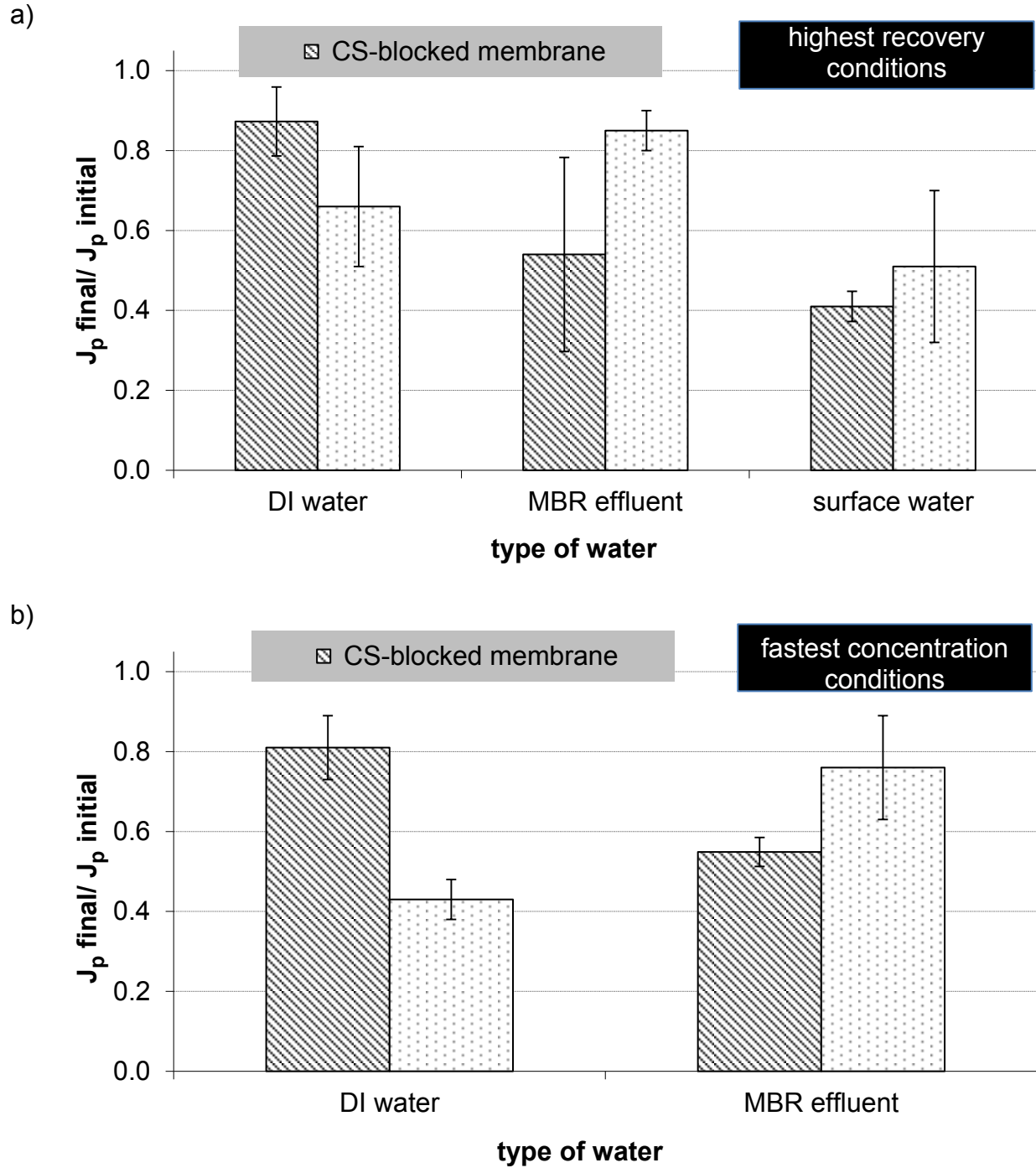


Figure 4.4: Fouling observed for CS-blocked membrane and PEM-coated membrane during filtration tests performed to recover P22 from DI water, MBR effluent and surface water under a) “highest recovery” conditions and b) “fastest filtration” conditions.

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Chapter 5. Suggested future research

Chapters 2 and 4 describe results of what is to our knowledge the first study of PEM membranes' ability to remove and concentrate viruses. The membranes showed promising performance in terms of virus removal and pre-elution recovery; the latter was attributed to the negative charge and hydrophilicity of the PEM surface that resulted in unfavorable virus-PEM interactions. Our findings regarding the performance of PEM-coated membrane and calf serum-blocked membrane are summarized in Table 5.1.

A large number of experiments was necessary to evaluate the effect of crossflow and permeate flow rates on virus recovery. To make such screening feasible, we chose to work with the bacteriophage P22, which is a non-pathogenic virus. This choice was later proved be not optimal as relatively high post-elution recovery of P22 could be achieved by an existing method (using CS-blocked membranes). We expect that in the case of more adhesive viruses, such as adenovirus, the competitive advantage of the anti-adhesive PEM surface will be more evident. Specifically we expect the post-elution recovery obtained with PEM-coated membranes to be significantly higher than the one obtained with CS-blocked membranes. Thus, we suggest that PEM membranes are evaluated with adhesive, difficult-to-recover viruses. We also propose that these tests include backflushing the membrane to recover viruses that do attach to the surface.

The performance of PEM-coated membranes in recovering viruses from complex water was evaluated with only three filtration tests for each water matrix (MBR effluent and surface water). Additional tests should be performed to confirm or disprove the advantage of PEM-

coated membrane over CS-blocked membranes in terms of recovery and/or reproducibility at the post-elution stage. Using a backflushing step instead of the elution step should also be evaluated as a possibility to improve reproducibility. The polyelectrolytes used in this study inhibited P22 infectivity and as a consequence the performance of PEM-coated membranes for the recovery of infective virus was not investigated. Future work should consider alternative PEM coatings that would not inhibit infectivity assay; in this context, multilayer formed through H-bonding rather polyelectrolyte interaction are an interesting option. Finally the PEM-based approach should be evaluated in a scaled-up system with a reusable, high surface area hollow fiber cartridge to concentrate large-volume samples. As both membrane surface area and sample volume will be increased proportionally, we expect that PEM-coated membrane performance will be maintained.

The role of surface properties such as hydrophobicity, charge, roughness and stiffness in determining virus adhesion needs to be better understood. We suggest experimenting with carefully selected bacteriophages that present a wide range of surface properties and using a QCM-D instrument to rigorously quantify bacteriophage adhesion to various PEMs. Both PEM-coated surfaces, prepared using various polyelectrolytes combinations as well as various pH and ionic strength, and phage surfaces would need to be thoroughly characterized to determine their surface tension parameters and surface charge. In addition, atomic force microscopy would need to be used to characterize PEMs in terms of their roughness, swelling and stiffness. Based on the comparison of results of QCM-D tests, results of AFM measurements, and computed XDLVO energies of virus-PEM interactions, conclusions may be drawn on the factors determining virus adhesion to surfaces.

Table 5.1: Virus recovery performance of PEM-coated membrane and calf serum-blocked membrane at the pre-elution (r_{pre}), post-elution (r_{post}) and coating removal - through elution or backflush with an adequate solution - ($r_{sacrificial}$) stages. Note: + and ++ indicate that we expect the membrane well and very well, respectively. – indicates that we expect the membrane to perform poorly and ? indicates that additional experiments need to be performed to confirm our assumption

		r_{pre}	r_{post}	$r_{sacrificial}$
PEM-coated membrane	infective virus	+ ?	- ?	-
	total virus	++	+ ¹	+ ²
calf serum-blocked membrane	infective virus	-	-	?
	total virus	-	+ ¹	?

¹ P22-specific inhibition

² If PEM used does not inhibit qPCR

APPENDICES

Appendix A. Determination of crossflow and permeate fluxes used by Morales-Morales et al. [1] and Hill et al. [2-4]

Morales-Morales et al. [1] reported concentrating pathogen from a 10 L sample using a permeate flow of 300 mL/min and a crossflow rate of 3600 mL/min in a Microza hollow fiber cartridge with 0.2 m^2 membrane surface (model (AHP-1010)). The permeate flux obtained by dividing the permeate flow by the membrane surface area was $1.5 \text{ L}/(\text{min} \cdot \text{m}^2)$. Pall Corporation, Microza's manufacturer, reports on its website [5] that a crossflow rate of 724 L/h corresponds to a velocity of 1 m/s. For a 3,600 mL/min crossflow rate the velocity was thus 0.3 m/s, so the fluid volumetric flux is $0.3 \text{ m}^3/(\text{m}^2 \cdot \text{s})$ (or $18,000 \text{ L}/(\text{min} \cdot \text{m}^2)$) [6].

In 2005, Hill et al. [2] reported concentrating pathogen from 10 L tap water samples with a permeate flow of 800 mL/min and a crossflow rate of 900 mL/min using a Fresenius Hemaflow F80A hollow fiber filter with a membrane surface of 1.8 m^2 . Again by dividing the permeate flow by the membrane surface area gives a permeate flux of $0.44 \text{ L}/(\text{min} \cdot \text{m}^2)$. Ronco et al. [7] reported that the Hemaflow F80A cartridge contains 12,288 fibers, each with an inner diameter of $200 \text{ }\mu\text{m}$ corresponding to a total crossflow cross-sectional area of $3.86 \cdot 10^{-4} \text{ m}^2$. Thus, a crossflow rate of 900 mL/min corresponds to a crossflow volumetric flux of $2,300 \text{ L}/(\text{min} \cdot \text{m}^2)$.

In 2007 and 2009, Hill et al. [3, 4] used a Fresenius F200NR hollow fiber cartridge with a crossflow rate of 1,700 mL/min and a permeate flow rate of 1,200 L/min. The membrane surface area was 2 m^2 , which corresponds to a permeate flux of $0.6 \text{ L}/(\text{min} \cdot \text{m}^2)$. The Fresenius F200NR was also described by Ronco et al. [7] The cartridge contained 13,824 fibers, each with an inner diameter of $200 \text{ }\mu\text{m}$, which corresponded to a total cross-sectional area of $4.24 \cdot 10^{-4} \text{ m}^2$. We thus determined that a crossflow rate of 1,200 L/min corresponds to a crossflow volumetric flux of $3,900 \text{ L}/(\text{min} \cdot \text{m}^2)$.

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Appendix B. Addition of carrier RNA to sample: effect of DNA extraction/detection

QPCR analysis of sample of P22 spiked in DI water and extracted using Roche's MagNA Pure resulted in higher CP value than expected. This phenomenon (lower CP value for DNA material extracted from "clean samples" using MagNA Pure compared to environmental samples) had also been observed by Dr. Kelvin Wong when he was working in Dr. Xagoraki's lab and was attributed to the adsorption of virions and DNA to the magnetics beads and other components of the MagNA Pure instrument. The solution developed by Dr. Wong was to add carrier RNA to the sample before the extraction step. Carrier RNA had been used previously to increase DNA extraction efficiency [1]. In Table A.1 we show the results of tests we performed to determine the optimal amount of carrier RNA to add to filtration samples.

Carrier RNA (obtained from Qiagen) solutions were prepared at a concentration of 1 mg/mL in PCR grade water or at a concentration of 0.01 mg/mL in viral lysis buffer (buffer AVL from Qiagen) and amount ranging from 5 μ L to 15 μ L of carrier RNA solution were added to 385 μ L P22 in DI water samples. Samples were extracted using MagNA Pure instruments and qPCR was run in triplicate as described in Chapter 2 section 2.2.8.

Table B.1: Effect of carrier RNA addition on CP value obtained by qPCR

	Tube number				
Volume introduced (μL)	1	2	3	4	6
sample	385	385	385	385	385
Carrier RNA in water (1 mg/mL)	NA	NA	10	NA	NA
Carrier RNA in buffer (0.01 mg/mL)	5	10	NA	15	NA
PCR grade water	10	5	5	NA	NA
total	400	400	400	400	400
qPCR results (mean CP value ± stdev)	19.81 ± 0.22	19.66 ± 0.01	19.18 ± 0.03	19.63 ± 0.12	23.62 ± 0.02

The lowest CP value, corresponding to the highest P22 concentration detected, was obtained for tube number 3 (which corresponds to the addition of 10 μL of carrier RNA at 1 mg/mL in qPCR grade water). Ten microliters of carrier RNA (1 mg/mL) were added to all samples used in this study before DNA extraction.

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Appendix C. Compaction behavior of PEM-coated membranes and CS-blocked membranes.

Permeate flux data were averaged over the first and last minute of compaction and over the first minute of filtration in order to look at possible difference of behavior during compaction and decompression (between the compaction stage and the filtration stage) of the membrane. Results are shown in Fig. C.1. There is no significant difference of behavior between CS-blocked membrane and PEM-coated membrane.

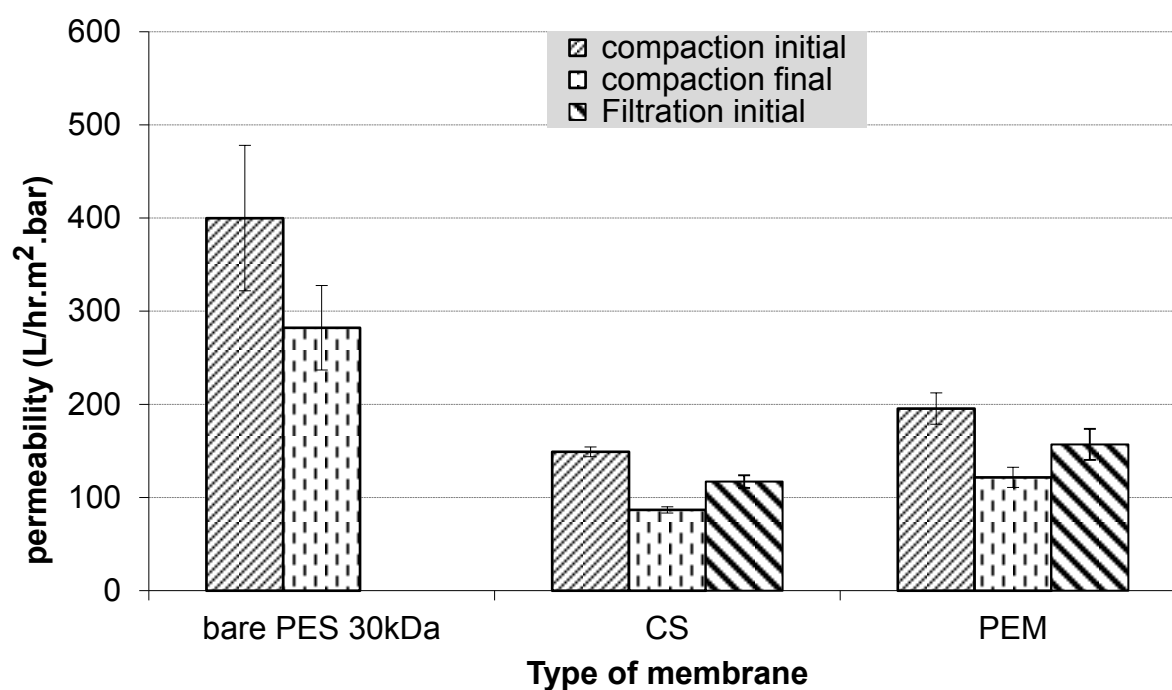


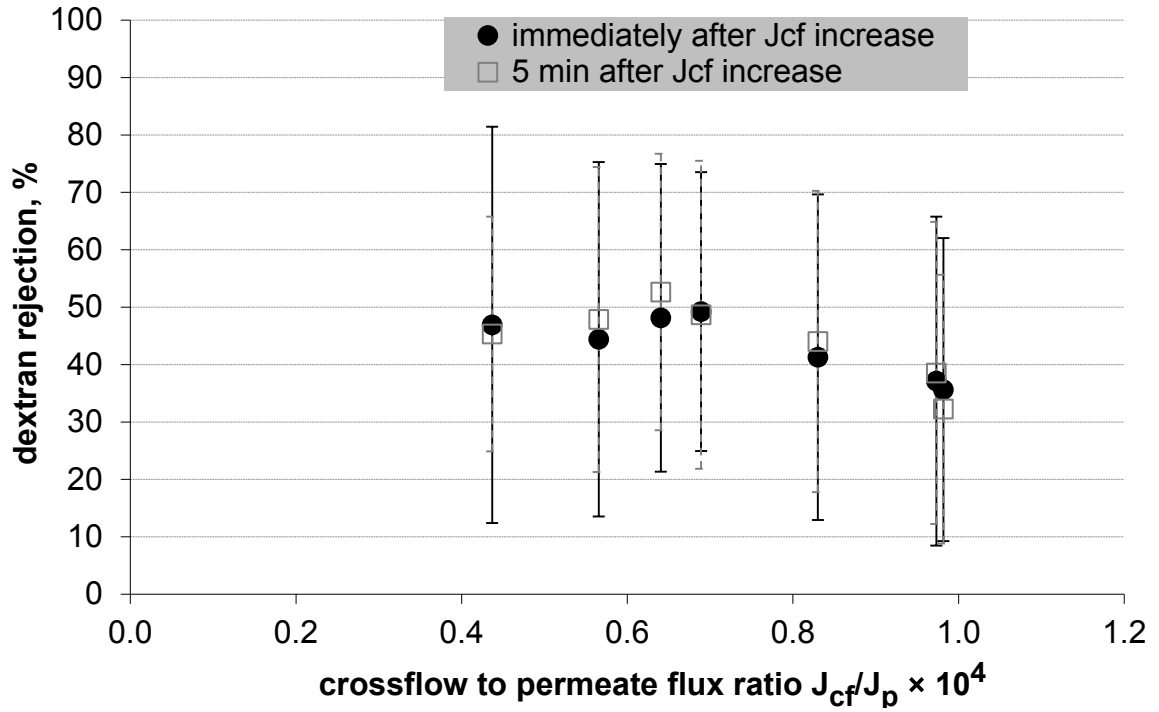
Figure C.1: Compaction and decompaction behavior of bare PES membranes, CS-blocked membranes and PEM-coated membranes. Error bar indicates the 95% confidence intervals.

Number of test: $n = 3$ for bare PES membrane, $n = 72$ for CS-blocked membrane and $n = 37$ for PEM coated membrane

Appendix D. PEM layer stability during filtration and elution.

The stability of the PEM layer during filtration was tested as described in section 3.2.6. The test was performed in triplicate for filtration and elution conditions. On Fig D.1 we can see that 25 kDa dextran rejection by (HE/CHI)_{4.5} was highly variable and was only approximately 50% on average (whereas CS-blocked membrane rejected approximately 60 to 70 % of 25 kDa dextran). A higher molecular weight dextran might have been a better probe to assess the stability of (HE/CHI)_{4.5}. The PEM stability seems to be maintained at low J_{cf} / J_p however, for J_{cf} / J_p value higher than 7,000 rejection of 25 kDa dextran seems to decrease. During elution, dextran rejection dramatically decreased even at the lowest crossflow rate used in these experiments which indicates that (HE/CHI)_{4.5} probably lose integrity during elution.

a)



b)

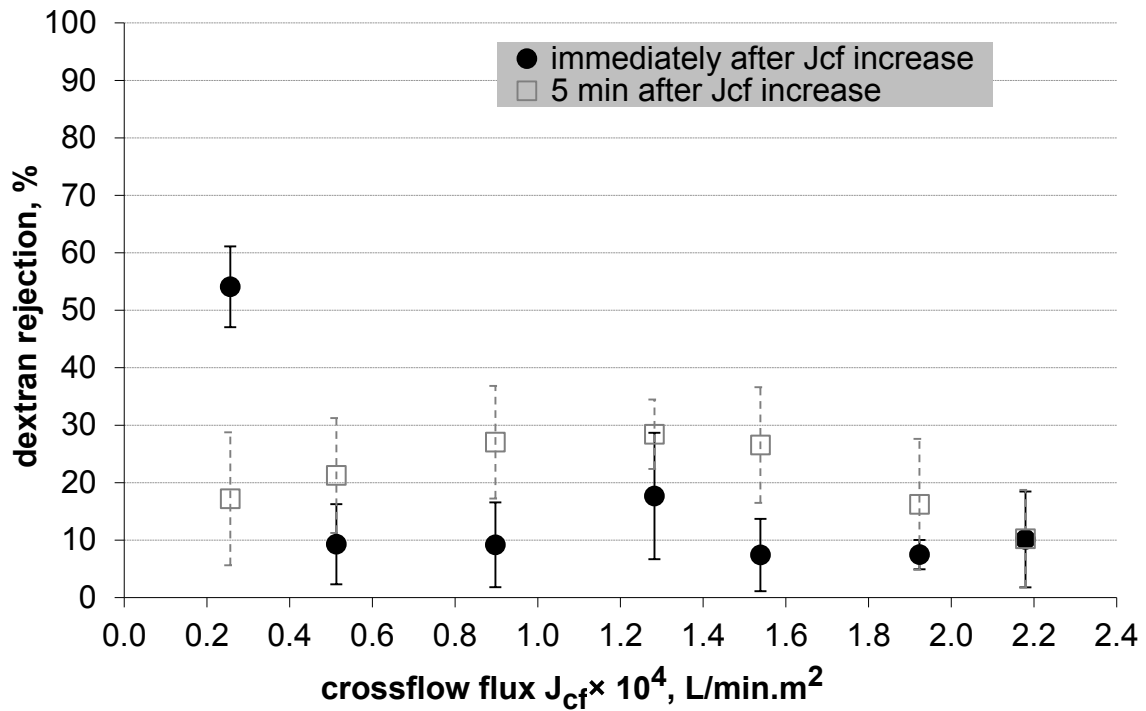


Figure D.1: Dextran rejection by PEM-coated membrane under increasing J_{cf} / J_p during a)

filtration and b) elution. $n=3$ for filtration and elution, error bar indicates standard deviation.

Appendix E. Preliminary work on visualization of virus adhesion on surfaces

As mentioned in Chapter 1 it is possible to visualize virions and aggregates deposited on surfaces using AFM [1-5]. Aside from Pereira et al. study [5], viruses were deposited on clean and smooth surface such as silicon or mica. In Pereira study, dengue virus adsorption on silicon wafers and protein covered silicon wafers. On those they were observed aggregated and isolated particles with height of approximately 10 to 15 nm which the authors considered to be hemagglutinins particles secreted by dengue virus, and 25 to 30 nm which the authors considered to be dengue particles with a collapsed lipid envelope [5].

In this preliminary study (PDADMA/PSS)_{4.5} were deposited on mica support (polyelectrolyte solution were prepared at a polyelectrolyte concentration of 0.02M and a NaCl concentration of 0.5M). The PEM-coated supports were then dipped for 30 min. in P22 suspension. To prepare this suspension, P22 stock was purified by centrifugal filtration at 4000 rpm using Amicon Ultra 4 centrifugal filter device with a 100,000 kDa cellulose membrane (Millipore). The virions retained by the membrane were resuspended in KCl 0. M. P22 concentration was approximately 10^9 PFU/mL. The supports were then dried and analyzed by AFM using the Nanoscope IV Multimode SPM from Veeco in tapping mode at room temperature using silicon cantilever with NSC15 tip from Mikromasch.

The obtained AFM topographic images are shown in Fig. E.1

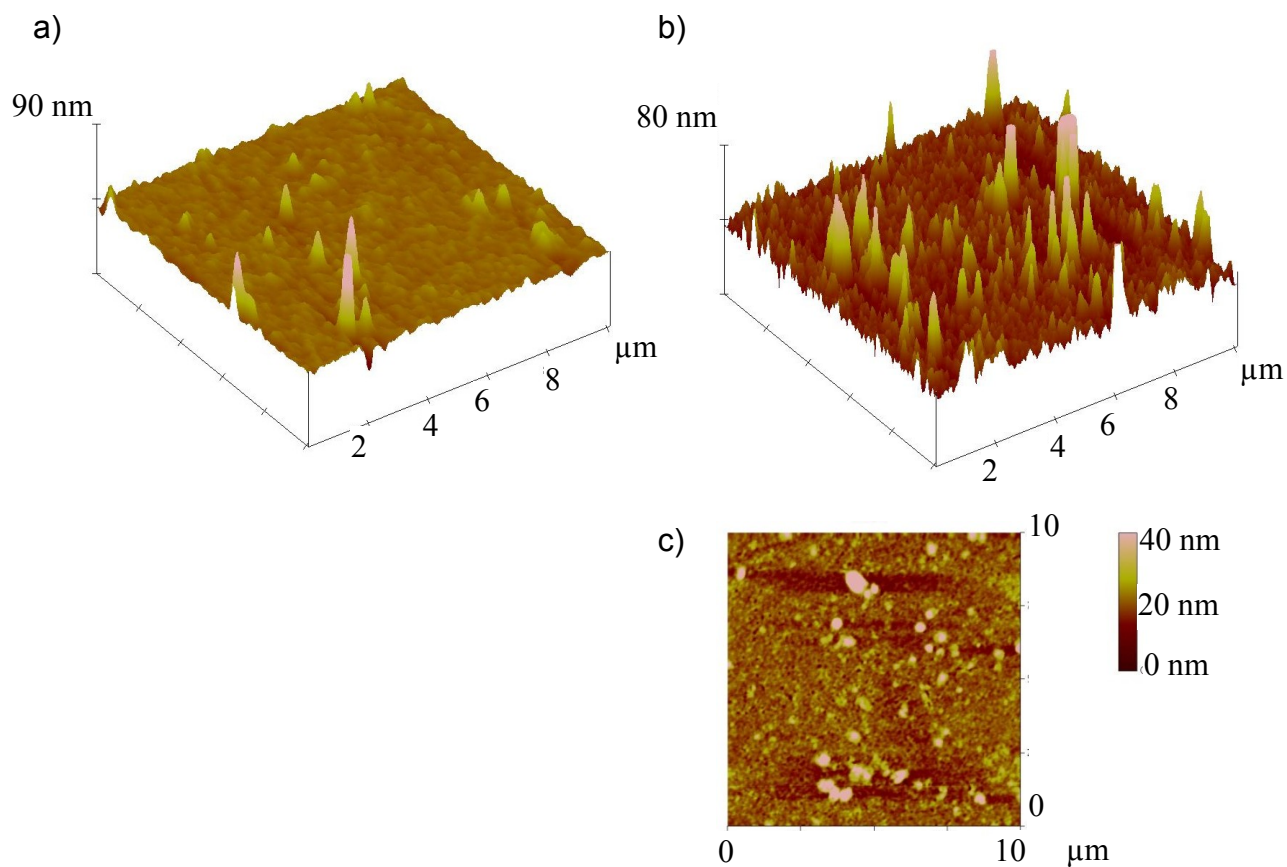


Figure E.1: AFM topographic images of a) (PDADMA/PSS)_{4.5} on gold substrate b) and c)

(PDADMA/PSS)_{4.5} on mica dipped in P22 suspension

In Fig. E.1, image b which is the (PDADMA/PSS)_{4.5} dipped in P22 suspension scan, shows more high structure (up to 80 nm) than image a which is the scan of a (PDADMA/PSS)_{4.5} which could be attributed to P22 particles (~60 nm). However, it is not possible to make a direct comparison as the layers were not deposited on similar surface.

Another potential method to visualize virus is epifluorescence [4]. Epifluorescence has been used to enumerate virions from water samples [4, 6-11]. Viruses are recovered on a filter which is strained with SYBR Green, DAPI or Yo-Pro (fluorescent dyes that specifically bind to DNA). The virus can then be visualized and enumerated by epifluorescence microscopy.

In this preliminary study a serial dilution of P22 suspensions was prepared. Ten milliliters from each dilution was filtered with 0.02µm anodisc filter from Whatman backed with a 0.45 µm cellulose ester filter. The filters were then dried and following Noble 2001 protocols a drop of staining solution (0.25% SYBR Green I gel stain from Invitrogen) was placed on the filter for 15 min, the drop was then removed and the filter dried. The filter was mounted on a glass slide with a drop of anti-fading solution (0.1% p-phenylenediamine in a mixture of 50% glycerol and 50% phosphate buffer saline) between the sample and the cover slip. Samples were frozen until analyzed using a Zeiss Pascal laser scanning confocal microscope with a 488 nm illumination. The emission was detected through a long pass 505 nm filter.

In addition to the serial dilution (PSS/PAH)_{4,5} and (PSS/PAH)₄ were deposited on anodisc filters which were then dipped for 30 min in P22 suspension ($\sim 10^8$ PFU/mL). The filters were then dried and stained as described above.

Results are shown in Fig. E.2 and E.3.

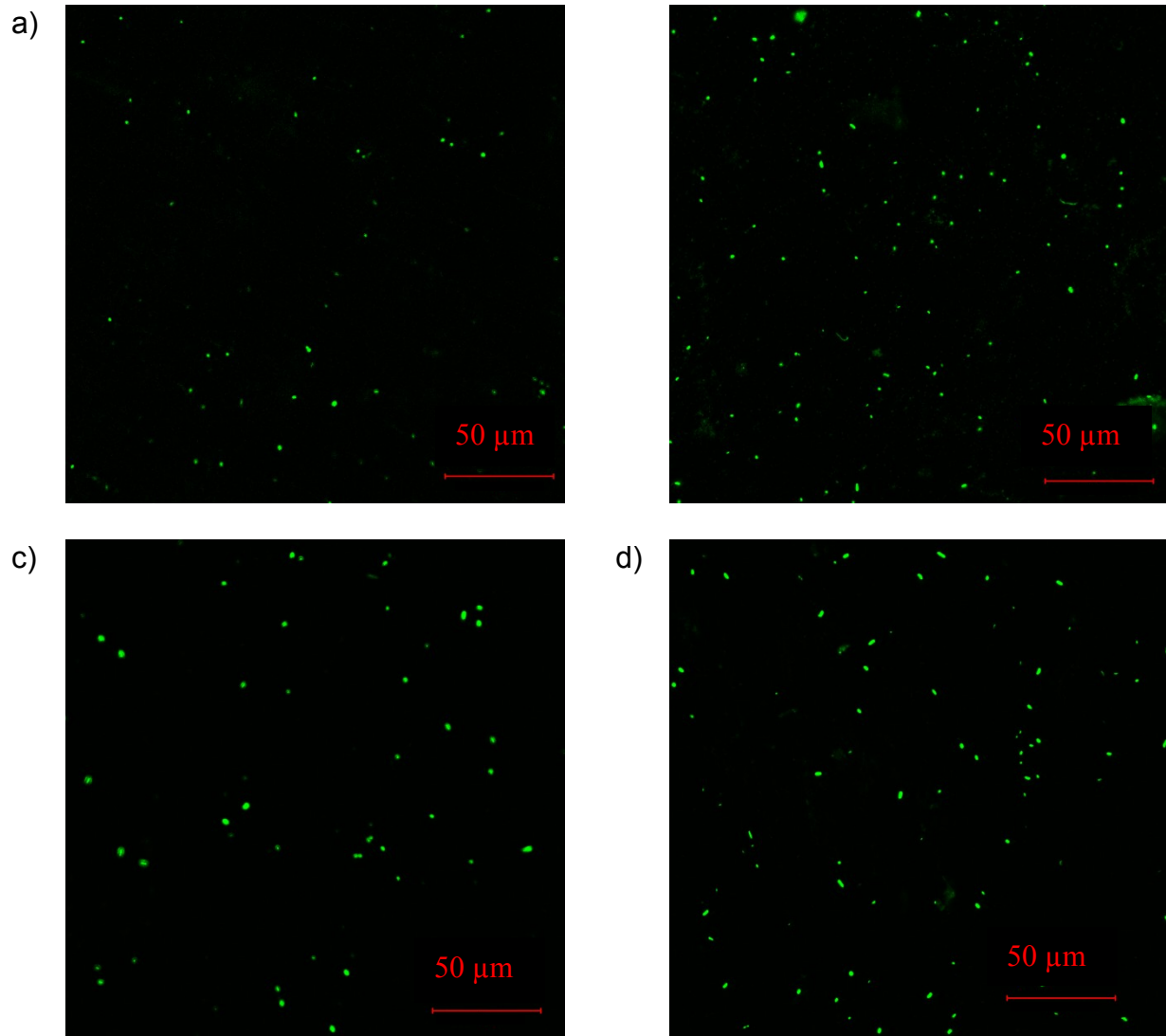


Figure E.2: Fluorescence images of serial dilution samples. a) corresponds to the 1:1000 dilution, b) to the $1:10^4$ dilution, c) to the $1:10^5$ dilution and d) to the $1:10^6$ dilution

Pictures were taken at $\times 40$ magnification

There does not seem to be any correlation between the number of fluorescent spots and the dilution factor. One possibility is that focusing was done on viral aggregates and that the rest of the virions would be visible at a different focal plan.

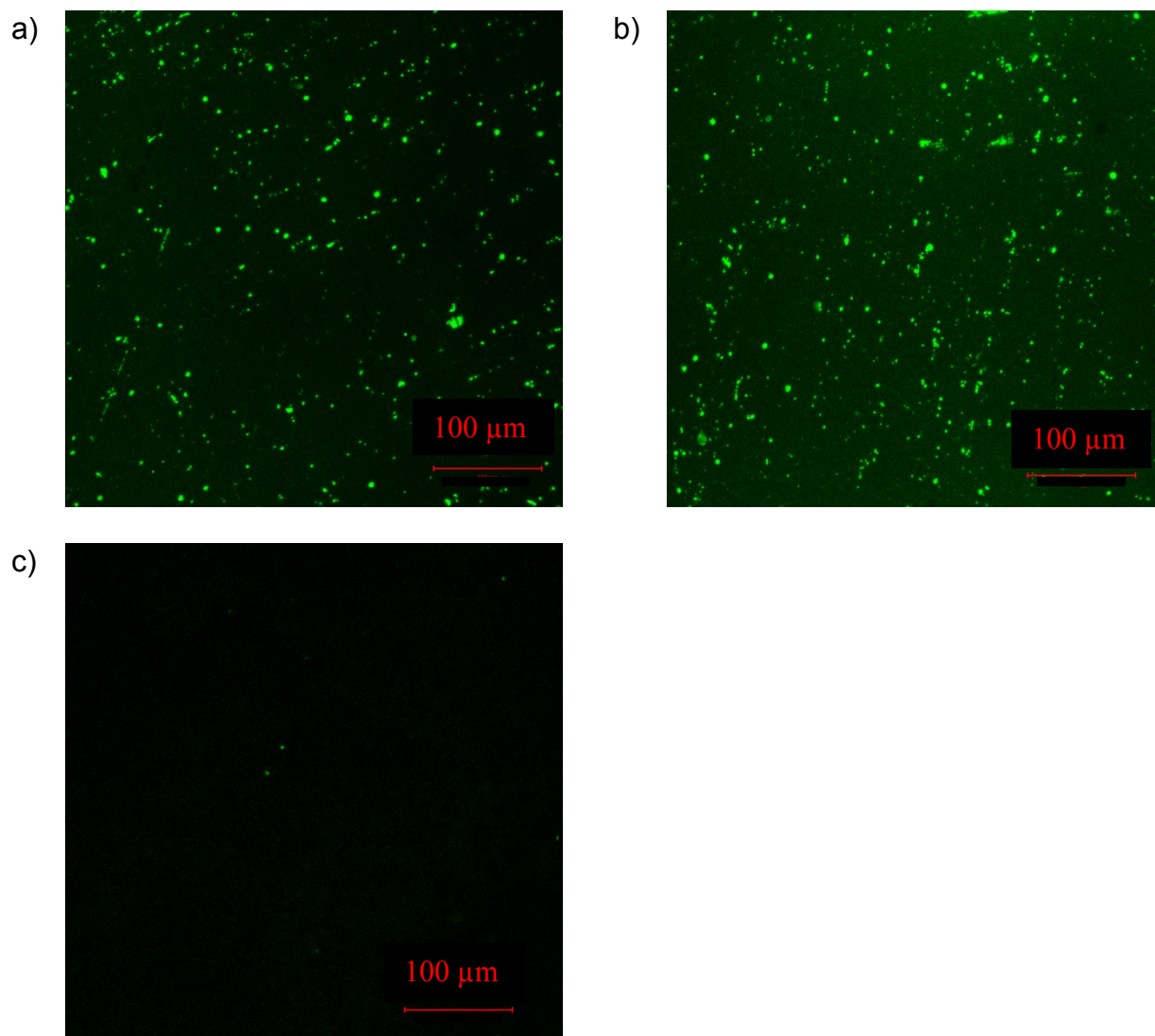


Figure E.3: Fluorescence images of a) (PSS/PAH)₄-coated ; b) (PSS/PAH)_{4.5}-coated and c) bare anodisc filters dipped in P22 suspension.

Pictures were taken at $\times 20$ magnification

Fluorescent spots are visible on both PEM-coated anodisc but barely any are visible on the bare anodisc filter. Unfortunately, because no controls were performed it is not possible to say if these fluorescent spot are attributable to P22 particles or caused by agglomerates of polyelectrolyte itself.

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