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Implementation of a sensitive method to assess high virus retention performance of low-pressure reverse osmosis process

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Abstract

Human enteric viruses are important etiological agents of waterborne diseases. Environmental waters are usually contaminated with low virus concentration requiring large concentration factors for effective detection by (RT)-qPCR. Low pressure reverse osmosis is often used to remove water contaminants, but very few studies focused on the effective virus removal of reverse osmosis treatment with feed concentrations as close as possible to environmental concentrations, and principally relied on theoretical virus removal. The very low viral concentrations usually reported in the permeates (i.e. at least 5 log of removal rate) mean that very large volumes of water need to be analysed to have sufficient sensitivity and assess the process efficiency. This study evaluates two methods for the concentration of adenoviruses, enteroviruses and MS2 bacteriophages at different viral concentrations in large (<200 L) and very large (>200 L) volumes. The first method is composed of two ultrafiltration membranes with low molecular weight cut-offs while the second method primarily relies on adsorption and elution phases using electropositive-charged filters. The recovery rates were assessed for both methods. For the ultrafiltration-based protocol, recovery rates were similar for each virus studied: 80 % on average at high virus concentrations (10^6 - 10^7 viruses L^{-1}) and 50 % at low virus concentrations (10^3 - 10^4 viruses L^{-1}). For the electropositive-charged filter-based method, the average recoveries obtained were about 36 % for ADV 41, 57 % for CV-B5 and 1.6 % for MS2. The ultrafiltration-based method was then used to evaluate the performance of a low-pressure reverse osmosis lab-scale pilot plant. The retentions by reverse osmosis were similar for all studied viruses and the validated recovery rates applied to the system confirmed the reliability of the concentration method. This method was effective in concentrating all three viruses over a wide range of viral concentrations. Moreover, the second concentration method using electropositive-charged filters was studied, allowing the filtration of larger volumes of permeate from a semi-industrial low-pressure reverse osmosis pilot plant. This reference method was used because of the inability of the UF method to filter volumes on the order of one cubic metre.

Keywords: Virus concentration method, enteric viruses, large volume concentration, ultrafiltration, adsorption-elution, low pressure reverse osmosis.

Abbreviations: ADV: adenovirus; CCMV: cowpea chlorotic mottle virus; EV: enterovirus; HBV: hepatitis B virus; LRV: logarithmic reduction value; LPRO: low pressure reverse osmosis; MW: molecular weight; MWCO: molecular weight cut-off; RR: recovery rates; (RT)-qPCR: reverse transcription-quantitative polymerase chain reaction; TPP: tripolyphosphate; UF: ultrafiltration; USEPA: U.S. environmental protection agency

45 1. Introduction

46 Microbial pollution of water is a threat to human health. The discharge of untreated or insufficiently
47 treated wastewater into the aquatic environment can lead to microbial pollution of water resources used
48 for drinking water production, hence the need to control this threat. Indeed, various viral infections can
49 be transmitted through consumption or contact with water contaminated by pathogenic microorganisms
50 (Gibson, 2014). The detection of viral pathogens is of great importance for water safety (USEPA, 2001)
51 because they are smaller and more resistant to treatment, unlike bacteria and protozoa (Alberts, 2002).

52 There are different treatment processes for the production of drinking water, including membrane-based
53 processes whose performance has been widely demonstrated for the elimination of a number of
54 microorganisms (Bodzek *et al.*, 2011). Recent studies reported ultrafiltration-based process efficiency
55 dependent on the virus concentration to remove from fresh water (Jacquet *et al.*, 2021) or sea water
56 (Taligrot *et al.*, 2022). For feed concentration close to that found in the environment (10^3 - 10^4 virus
57 copies L⁻¹) removal of 1.5-2.5 log have been reported for ultrafiltration (UF) (Jacquet *et al.*, 2021). For
58 low-pressure reverse osmosis (LPRO), only high viral concentration studies ($> 10^{10}$ copies L⁻¹) have
59 been able to calculate retentions as shown by Mi *et al.* (2004) with 6-log reduction. The absence of a
60 sensitive method for detecting and quantifying viruses in permeates did not allow for an accurate
61 evaluation of retention performance at lower concentrations. In this context, to overcome this limitation,
62 the development of a method allowing the analysis of large volumes with low virus concentrations was
63 required.

64 Over the last decade, different viral concentration procedures have been studied for different viruses,
65 viral concentrations and types of water, allowing a significant development of environmental virology
66 (Hamza and Bibby, 2019). Although some single-step concentration methods are used, most viral
67 concentration methods consist of two steps: a primary concentration step leading to small volumes
68 (around 200 mL) and then to hundreds microliters after second concentration step (Bofill-Mas and
69 Rusiñol, 2020). For most studies, the methods are cumbersome, time-consuming and a large proportion
70 of the virus particles present are lost during concentration. Furthermore, for the same concentration
71 method or virus used, the recovery rates (RR) obtained can differ from one study to another. Indeed,
72 Polaczyk *et al.* (2008) and Wu *et al.* (2013) studied the concentration with the same hollow-fibre
73 ultrafilters of drinking water spiked with MS2 and obtained recoveries between 67 and 80 % and values
74 exceeding 120 %, respectively. Similarly, Winona *et al.* (2001) and Olszewski *et al.* (2005) obtained
75 recoveries ranging from 20-60 % and above 70 % respectively after concentrating a few litres of surface
76 water contaminated with bacteriophage PP7, through hollow-fibre ultrafilters. However, a large number
77 of studies have shown that low cut-off UF is effective in concentrating viruses such as adenovirus,
78 poliovirus, echovirus, bacteriophage, etc. (Cooksey *et al.*, 2019; Gibson and Schwab, 2011; Holowecky
79 *et al.*, 2009; Olszewski *et al.*, 2005; Rhodes *et al.*, 2011; Winona *et al.*, 2001). Nevertheless, these
80 membranes are not suitable for concentrating large water volumes (> 100 L) due to the membrane
81 fouling and its loss of integrity (Olszewski *et al.*, 2005).

82 Other methods exist, such as adsorption-elution, which has been widely studied to concentrate samples
83 on the order of one cubic metre or more. Also, electronegative filters can be used, in particular for the
84 concentration of coronavirus (Rusiñol *et al.*, 2020) norovirus (El-Senousy *et al.*, 2013), adenovirus (Li
85 *et al.*, 2010) but also electropositive filters such as NanoCeram® for the concentration of norovirus
86 (Hennechart-Collette *et al.*, 2022), poliovirus (Falman *et al.*, 2019), adenovirus and enterovirus (Ikner
87 *et al.*, 2011; Miao *et al.*, 2019; Prevost *et al.*, 2015), echovirus (Ikner *et al.*, 2011), T1 and PP7
88 bacteriophage (Francy *et al.*, 2013) and MS2 bacteriophage (Nasser *et al.*, 2021). In addition,
89 NanoCeram® filters are used in the United States Environmental Protection Agency's reference method
90 for the detection of norovirus and enterovirus in high volumes (1,800 L) of groundwater (Cashdollar *et al.*,
91 2013; USEPA, 2014). However, the studies reported variable recovery rates, similar to the UF
92 concentration, and only investigated the recovery of specific viruses. In addition, viruses in the

93 environment can interact with other viruses, other microorganisms and natural organic matter (Fiksdal
94 and Leiknes, 2006), which may then influence virus recovery rates.

95 This study aimed to implement a method to concentrate and evaluate virus removal in large permeate
96 volumes from LPRO adapted to lab-scale and semi-industrial scale pilots. Two protocols were tested,
97 (i) a 2-step UF method for concentrating volumes of less than 200 L and (ii) a 3-step protocol using
98 adsorption/elution as a primary concentration step. This second protocol was used because of the
99 inability of the UF method to filter volumes greater than 200 L (Olszewski *et al.*, 2005). These
100 concentration methods allow the assessment of the removal efficiency of the LPRO process, enabling
101 to work above detection limits using feed viral concentrations that closely resemble those found in the
102 environment. For this evaluation, three viruses with different physical characteristics, including size,
103 were studied in order to bring out a potential steric effect on recovery efficiency. Recovery rates at
104 various virus concentrations that are found in waters were estimated for adenovirus type 41 (ADV 41),
105 coxsackievirus-B5 (CV-B5) and MS2 bacteriophage, a widely used viral surrogate in the literature
106 (Dawson *et al.*, 2005). The first method using UF membranes to concentrate viruses was applied to the
107 permeates of a lab-scale LPRO pilot plant whereas the second method based on electropositive-charged
108 filters was used for the permeate of a semi-industrial scale LPRO pilot plant. For each case, the
109 concentrations in the feed, permeate and retentate were evaluated to assess the viral material balances
110 and to estimate the removal rates of LPRO processes. The viral concentration in the permeate was
111 calculated by applying the recovery rates of the different concentration methods.

112

113 2. Material and methods

114

2.1 Virus preparation

115 ADV 41 (#ATCC VR-930), CV-B5 (#ATCC VR-185) were cultivated on monolayer cultures of 293A
116 cells (#ATCC CRL-1573) in Dulbecco's Modified Eagle Medium (#R70507, Invitrogen) supplemented
117 with 2 % fetal calf serum (#30-2021, Sigma-Aldrich) at 37 °C with 5 % CO₂. MS2 phages (#ATCC
118 15597-B1) were cultivated on K12hFr *E. coli* cells at 37 °C in Luria-Bertani medium. The supernatants
119 of eukaryotic cell cultures and bacterial cultures were recovered after cell lysis and mixed with
120 chloroform (10 % v/v) and clarified by centrifugation 8,000 xg for 15 min at 4 °C. Supernatants were
121 then collected. Viral stocks about 10¹⁰ copies were aliquoted, titrated by (RT)-qPCR as detailed in 2.1.2,
122 and stored for a few weeks at -80 °C until use.

123

124 2.1.1 Virus properties

125 Three different viruses were used in this study: ADV 41, CV-B5 and MS2 phage. The main
126 characteristics of these viruses are presented in Table A.1. All viruses present isoelectric point (pI) in
127 the same range, below the value of neutral pH and are therefore negatively charged in the experimental
128 conditions of this study. They also present a similar shape with an icosahedral capsid. It is noticeable
129 that CV-B5 and MS2 phage have very similar characteristics: they are both single-stranded RNA viruses
130 in the same size range (30 and 27 nm respectively) with similar molecular weight (MW), while ADV
131 41 is a double-stranded DNA virus with a size approximately three times larger than the two others (90
132 nm) and it also presents a larger MW.

133 *Table A.1: ADV 41, CV-B5 and MS2 phage characteristics (ElHadidy et al., 2013; Jacquet et al., 2021; Michen and Graule,*
134 *2010)*

| Virus | Size (nm) | pI | DNA/RNA virus | MW (× 10 ³ Da) | Shape |
|--------|-----------|-----|---------------------|---------------------------|---|
| ADV 41 | 90 | 2-6 | Double-stranded DNA | 150-180 | Icosahedral capsid, non-enveloped virus |
| CV-B5 | 30 | 3.6 | Single-stranded RNA | 8-9 | |

135

136 **2.1.2 Viral nucleic acid extraction and quantification**

137 Viral nucleic acids from concentrates were extracted using MagNA Pure Compact Acid Isolation Kit
 138 on a MagNA Pure compact system (Roche Applied Science, Bâle, Switzerland) and according to the
 139 manufacturer's instructions. A blank control was carried out in each extraction cycle. Nucleic acids were
 140 eluted into a 100 µL volume allowing titration in duplicate. Each reaction for each virus was carried out
 141 with 10 µL of nucleic acid samples, 5 µL of master mix TaqMan® Fast Virus1-Step (Life Technologies,
 142 Carlsbad, CA), and 5 µL of mix containing primers and probes. Specific primers and probes designed
 143 for MS2 bacteriophage, CV-B5 (Enterovirus) and ADV 41 (Adenovirus F) are presented in Table A.2
 144 with final concentrations per reaction (Prevost *et al.*, 2015).

145

Table A.2: (RT)-qPCR mix composition

| Virus | Oligonucleotide | Sequence | Final concentration (nM) | Target |
|-------------------|-----------------|--|--------------------------|--------------------|
| Adenovirus F | ADV_F_F102 | CACCGATACGTACTTCAG | 600 | Hexon |
| | ADV_F_R231 | GCGCACTTTGTAAGARTA | 900 | |
| | ADV_F_Ps160 | Yakima Yellow-CACGATGTAACCACAGACAGG-BHQ1 | 200 | |
| Enterovirus | EV_F453 | GCCCCTGAATGCG | 900 | 5'UTR |
| | EV_R583 | TGTCACCATAAGCAGY | 900 | |
| | EV_P536 | FAM-CCAAAGTAGTCGGTTC-NFQ MGB | 100 | |
| MS2 Bacteriophage | MS2_F632 | GTCGCGGTAATTGGCGC | 100 | Maturation protein |
| | MS2_R708 | GGCCACGTGTTTTGATCGA | 300 | |
| | MS2_P650 | FAM-AGGCGCTCCGCTACCTTGCCCT-BHQ1 | 300 | |

146 All reactions were performed with a ViiA™ 7 real-time PCR system (Life Technologies, Carlsbad, CA).
 147 The thermal cycling profile was one step of reverse transcription at 50 °C for 5 min, one step of initial
 148 denaturation at 95 °C for 20 s, 45 cycles of 5 s denaturation at 95 °C and 40 s annealing/extension at
 149 60 °C. PCRs were performed simultaneously for DNA and RNA viruses due to the duration of the
 150 reverse transcription step. Each (RT)-qPCR assay included positive and negative amplification controls.
 151 Calibration ranges were carried out beforehand for each of the viruses in order to quantify the number
 152 of viruses present in each sample. Limits of detection and quantification of the (RT)-qPCR method were
 153 respectively estimated at 10 and 15 copies reaction⁻¹. Results reported for each sample were means of
 154 duplicate. The raw amplification data were collected with ViiA™ 7 software (Life Technologies,
 155 Carlsbad, CA).

156

157 **2.2 Low-volume concentration method (< 200 L) for lab-scale pilot**158 **2.2.1 Concentration method**

159 The concentration method was carried out using hollow fibre UF cartridges (Inuvai R180, Fresenius
 160 Medical Care, Germany) and membrane-based UF (Vivaspin 20 MWCO 10kDa, Sartorius), both in
 161 polyethersulfone (PES), according to the manufacturer's instructions. The characteristics of the two
 162 membranes used are presented in Table A.3. This method can handle volumes up to 200 L.

163

Table A.3: Characteristics of the membranes used for concentration of low volumes

| Model, manufacturer | Membrane type (composition) | MWCO (Da) | Final volume | Membrane area | Concentrate recovery |
|-------------------------------|-----------------------------|-----------|--------------|--------------------|----------------------|
| R180, Inuvai | UF (PES) | 18,000 | 200 mL | 1.8 m ² | Backwash |
| Vivaspin 20, Sartorius | | 10,000 | 200 µL | 6 cm ² | Membrane lysis |

164 The concentration was then carried out in two steps: (i) Filtration using Inuvai R180 cartridge system
165 which is itself divided in three phases: filter preparation, sample filtration and the recovery of retained
166 virus. The cartridge was first filled with a 0.05 % sodium tripolyphosphate solution for conditioning the
167 membrane and chasing the air from the cartridge, according to the manufacturer's instructions. Using
168 sodium tripolyphosphate imparts a negative charge to the filter fibres, increasing repulsion between
169 negatively charged viruses and the filter surface to minimize adhesion (Polaczyk *et al.*, 2008). Then the
170 sample was concentrated by filtration through the cartridge using a peristaltic pump set to a maximal
171 flow rate of 15 Lh⁻¹. The retentate containing the retained viruses was eluted by backwashing (in the
172 opposite direction of filtration) using an elution solution. This first step allows up to 200 L to be
173 concentrated into a first elution volume of approximately 200 mL. (ii) The 200 mL recovered were then
174 concentrated using Vivaspin 20 concentration devices by centrifugation at 4000 g at 4°C, until a volume
175 of 200 µl was obtained. The virus concentrates were then directly lysed on the membrane of the Vivaspin
176 20 concentrators by adding 200 µL of lysis buffer (#19076, ATL buffer, QIAGEN). The nucleic acids
177 were recovered by pipetting and then purified.

178 In order to evaluate this method, a viral spiking of a known concentration was performed in a free-virus
179 water sample (noted as reference sample). The sample was then thoroughly homogenised, concentrated
180 and then quantified by (RT)-qPCR. It is important to note that the recovery rate is the efficiency of the
181 concentration method and is calculated using the number of viruses recovered at the end of the
182 concentration and the number of viruses introduced into the reference sample (Equation A.1).

$$183 \quad RR (\%) = \frac{\text{Number of virus genomes recovered after the concentration}}{\text{Initial number of virus genomes in the reference suspension}} \times 100 \quad \text{Eq. (A.1)}$$

184 The concentration tests were carried out at two different virus concentrations to see the potential effect
185 of concentration on recovery rates of the method. For this study, the experiments were performed at high
186 and low concentrations, respectively 10⁶-10⁷ viruses L⁻¹ and 10³-10⁴ viruses L⁻¹. The higher
187 concentration was selected for experiment because of the feed concentrations used in the filtrations
188 planned in the LPRO laboratory-scale pilot plant, where the removal rate had to exceed 5 log (Comerton
189 *et al.*, 2005; Mi *et al.*, 2004). The lower concentration was chosen as it was as close of the expected
190 concentrations in the LPRO pilot permeate. Additionally, it corresponds approximately to the freshwater
191 concentration that has been tested for viral concentration studies (D'Ugo *et al.*, 2016; Wyn-Jones *et al.*,
192 2011).

193

194 **2.2.2 Backwash composition**

195 The addition of different surfactants in the backwash composition can influence virus recovery
196 efficiency, as tripolyphosphate (TPP), glycine or tween 80 (Hill *et al.*, 2005; Mendez, 2004; Morales-
197 Morales *et al.*, 2003; Sharma *et al.*, 1985; USEPA, 2001). Indeed, using sodium tripolyphosphate can
198 decrease bacterial adhesion (Hill *et al.*, 2005; Sharma *et al.*, 1985). Moreover, Hill *et al.* (2005) showed
199 that a backflushing step using glycine or beef extract has improved virus recovery from hollow fiber UF
200 membranes. Also, USEPA (2001) recommended using glycine in viral elution of a negatively-charged
201 membrane. For the Tween 80, which is a non-ionic surfactant, it is commonly used in microbiological
202 techniques where the minimization of adhesion or desorption of microbes from surfaces is desired
203 (Mendez, 2004; Morales-Morales *et al.*, 2003). The composition (0.5 % TPP, 0,1 % tween 80, 0,1 %
204 antifoam and 0,05 %) has been chosen according to the manufacturer's recommendations and the
205 literature to maximise the recovery of viruses.

206

207 **2.3 High-volume concentration method (> 200 L) for semi-industrial scale pilot**

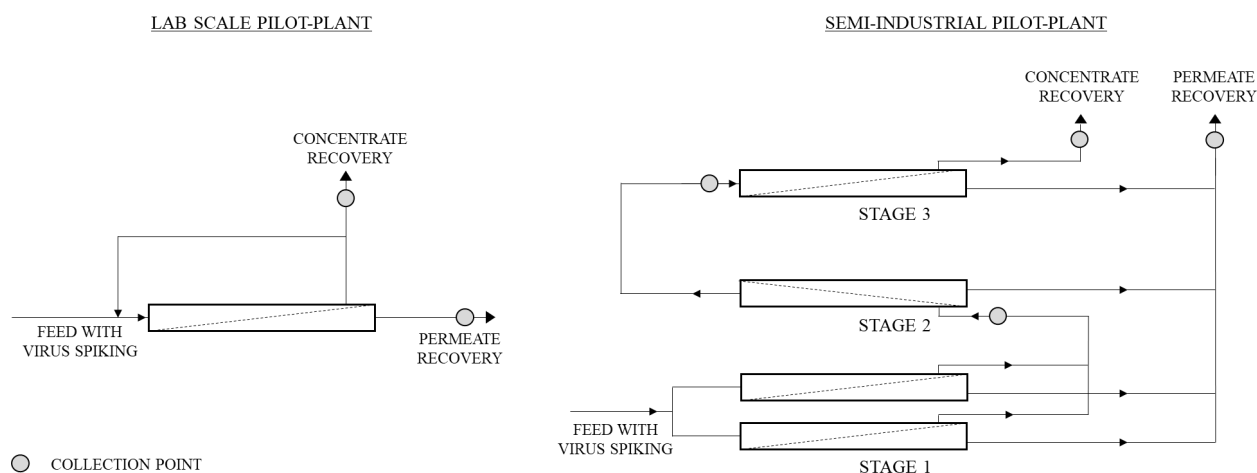
208 The water production rate by the semi-industrial scale pilot did not allow the use of the primary
209 concentration method by UF. In these conditions, a second method more adapted to the filtration of very
210 large volumes was used. This method has already been developed in the laboratory and is derived from
211 the 1615 USEPA method for the detection of norovirus and enterovirus in environmental samples. This

212 alternative concentration method was carried out using an electropositive adsorption-elution filter
 213 (NanoCeram® VS2.5-5, Argonide) with a pore size, an extensive surface area and an isoelectric point
 214 of approximately 2 μm , 500 $\text{m}^2 \text{g}^{-1}$ and 8-9, respectively (Ikner *et al.*, 2011). This electropositive filter
 215 is made of microglass filaments coated with nanoalumina fibres. It can therefore be used to concentrate
 216 the entire permeate sample (5 m^3), which was recovered during filtration on an LPRO semi-industrial
 217 pilot plant. The concentration method is composed of 3 steps including adsorption/elution on the
 218 electropositive membrane, organic flocculation, and ultracentrifugation. The samples were filtered on
 219 NanoCeram® filters using a peristaltic pump at a 100 L h^{-1} flow rate. The filter holder was then emptied
 220 and filled with 450 mL of an elution solution (TPP 0.1 %, Beef extract 1 %, Tween80 0.1 %, Antifoam
 221 0.1 %, Glycine 0.05 M), adjusted extemporaneously to a 9.5 pH. The filter holder was incubated for
 222 1 hour on ice. The eluate was then collected by inverted flow. The eluate was flocculated for 1 hour at
 223 ambient temperature by adjusting the pH of the solution to 3.5 with 3 M HCl solution and gentle stirring.
 224 After flocculation, centrifugation (1 h, 4000 xg and 4 $^\circ\text{C}$) was performed and the resulting supernatant
 225 was removed. The pellet was taken up in 8 mL of Na_2HPO_4 (0.15 M, pH = 7.4) by vortexing and
 226 pipetting. To clarify the sample, centrifugation (10 min, 4000 xg) was performed. The samples were
 227 transferred onto 1 mL of a 40 % sucrose cushion. The tubes were then ultra-centrifugated for 2 h at
 228 150,000 g at 4 $^\circ\text{C}$. The supernatant was discarded, and pellet re-suspended in 500 μL . The samples were
 229 then adjusted to 1 mL with a 40 % sucrose solution and total nucleic acids were extracted and eluted
 230 into a 100 μL elution buffer as indicated in 2.1.2.

231

232 2.4 Application: pilot plants and filtrations

233 For the lab-scale pilot plant, experiments were performed in batch mode at a constant permeate flow
 234 rate and in continuous mode at a constant transmembrane pressure on semi-industrial pilot plant. The
 235 experiments were performed on each pilot with the same virus spiking, corresponding to a virus-feeding
 236 concentration of approximately 10^6 copies L^{-1} . They were performed in quadruplicate and triplicate
 237 respectively for the lab-scale and the semi-industrial pilot plants. The pilots and their main
 238 characteristics are presented in Fig. A.1 and Table A.4, **Erreur! Source du renvoi**
 239 **introuvable.** respectively. During the filtrations, the overall permeate of the process was recovered as
 240 well as the retentate at each sampling point for both pilot plants. The retentate samples were analysed
 241 directly due to their high concentrations and the permeate samples only were concentrated and analysed
 242 by (RT)-qPCR. To determine the efficiency of the LPRO process (i.e. logarithmic reduction value), it is
 243 important to verify the viral mass balances beforehand using the number of viruses spiked (inlet) and
 244 the sum of the number of viruses recovered in permeate and retentate (outlet). The logarithmic reduction
 245 value (LRV) was then calculated using Equation A.2.



246
$$LRV = \log \left(\frac{\text{Number of virus genomes in the feed}}{\text{Number of virus genomes recovered in the permeate}} \right) \quad \text{Eq. (A.2)}$$

247 *Fig. A.1. Simplified flowsheets of the lab-scale and semi-industrial LPRO pilot plants*

248 *Table A.4: Main characteristics of the laboratory and semi-industrial pilot plants*

| | Lab-scale pilot plant | Semi-industrial pilot plant |
|---|---|---|
| Permeate flowrate (L min⁻¹) | 0.90 | 100 |
| Membrane area and manufacturer | 1 membrane of 2.5 m ² from CSM | 24 membranes of 8 m ² from Toray |
| Operating mode (# of membranes) | Single-stage with recirculation (1) | Multistage without recirculation (24) |
| Feed water | Ultrafiltered groundwater | |

249

250 2.5 Statistical analysis

251 Statistical tests were carried out using a non-parametric paired t-test, corresponding to the Wilcoxon
 252 test, to compare two dependent groups for which the normality assumption does not hold and the sample
 253 size was too small. The calculation of the *p*-value which must be greater than the significance level
 254 (defined at 5 %) is used to validate the absence of significant differences. All the statistical analyses and
 255 graphics were performed using GraphPad Prism 9.5 (GraphPad Software, San Diego, CA, USA).

256

257 3. Results

258 3.1 Efficiency of the low-volume concentration method

259 3.1.1 Effect of the viral concentration on the method's recovery rate

260 The results of the concentration experiments obtained for three replicates at high and low concentrations,
 261 respectively 10⁶-10⁷ virus copies L⁻¹ and 10³-10⁴ virus copies L⁻¹ were compared for each virus. It is
 262 important to note that the recovery rates between each replicate were not statistically different with *p*-
 263 values ranging from 0.41 to 0.92 showing the repeatability of the concentration method. Average
 264 recovery rates and associated standard deviations are listed in Table A.5. The average retention rates are
 265 at least 50 %, regardless of the concentration and virus used. This indicated that the concentration
 266 method, although using UF membranes, can be used for this study, due to its very low MW and constant
 267 cut-off in comparison with the virus size. The comparison of recovery rates at low and high
 268 concentrations obtained no statistically significant difference (*p*-value>0.25) for each virus which
 269 induces that the concentration did not affect the recovery rate for this range of virus concentration.

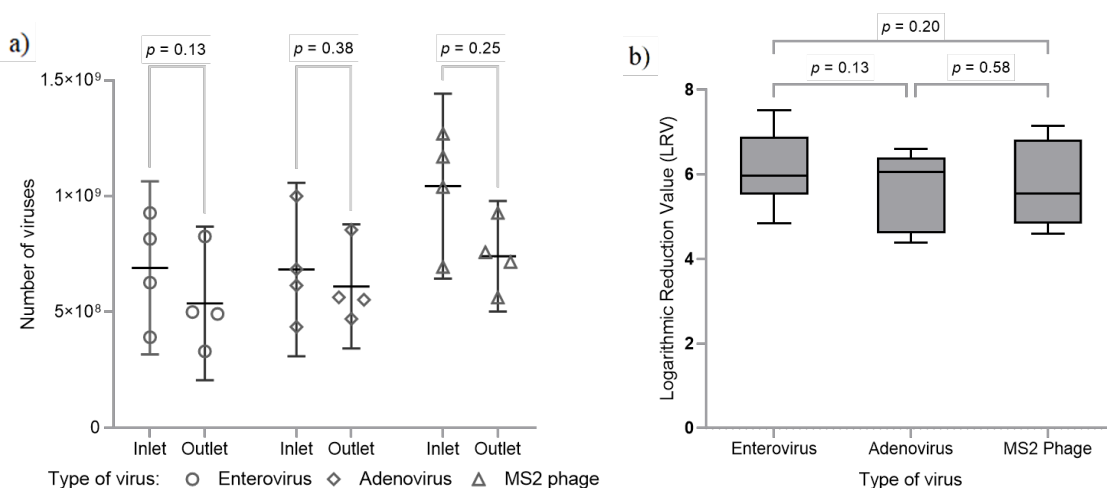
270 *Table A.5: Recovery rates of the low-volume concentration method of ADV 41, CV-B5 and MS2 phage with backwash*
 271 *composition 4*

| Virus | Low concentration (10 ³ -10 ⁴ virus copies L ⁻¹) | | High concentration (10 ⁶ -10 ⁷ virus copies L ⁻¹) | |
|------------------|---|---------------------------|--|---------------------------|
| | Average UF recovery rate (%) | Standard deviation (%) | Average UF recovery rate (%) | Standard deviation (%) |
| ADV 41 | 50 | 2 | 84 | 14 |
| CV-B5 | 54 | 6 | 83 | 14 |
| MS2 phage | 55 | 12 | 109 | 15 |

272

273 **3.1.2 Application of the method to assess LPRO performance for virus retention on a lab-scale**
 274 **pilot plant**

275 The UF-based method was used to concentrate permeates from LPRO lab-scale pilot plant of
 276 approximately 160 L volume. It must be noted that viruses were detected and quantified in all the
 277 samples (permeate and retentate). First, the viral mass balances of the filtrations are presented in Fig.
 278 A.2a. No statistical differences (p -value $> 0,05$) were found between the virus number inlet (i.e. in feed)
 279 and outlet (i.e. in permeate and retentate) of the LPRO process for each virus. Assuming that recovery
 280 with UF devices was comparable, this result implies that all the viral particles injected into the pilot
 281 were found in the retentate and permeate. The mass balances of each virus were therefore validated (i.e.
 282 the low-volume concentration method) demonstrating the reliability of the experiments. The viral
 283 retention efficiency of the lab-scale pilot plant could then be determined and is presented in Fig. A.2b.
 284 For the three viruses studied, the average LRVs were not statistically different with 6.1, 5.7, and 5.7 log
 285 for CV-B5, ADV 41 and MS2 respectively, but the range of experimental LRVs was from 4.5 to 7.1
 286 log. It could be explained by the fact that the average LRV are calculated with all values including the
 287 values obtained during the concentration rise from the feed value to the recirculation loop concentration.



288
 289 *Fig. A.2. Validation of the low-volume concentration method: a) Viral mass balance and b) Retention rate for different*
 290 *viruses (CV-B5, ADV 41, and MS2 phage) on a LPRO lab-scale pilot plant*

291
 292 **3.2 Efficiency of the high-volume concentration method**

293 **3.2.1 Effect of the viral concentration on the method recovery rate**

294 Preliminary tests were conducted on the UF concentration method and showed that, at constant
 295 pressures, the permeate flow rate decreased to almost zero during the filtration process, indicating
 296 membrane fouling. But when the volume treated exceeded approximately 200 L, the Inuvai membrane
 297 lost its integrity, as evidenced by a 20-fold increase in permeability, suggesting a membrane fibre break.
 298 Due to the loss of integrity and fouling of the Inuvai membrane, it was not possible to process volumes
 299 exceeding 200 L. To overcome this limitation, concentration by electropositive filters was used. The
 300 results of the concentration experiments obtained for the different concentrations (10^5 and 10^6 copies L^{-1})
 301 were compared for each virus and presented in Fig. A.3. The recovery rates between each replicate
 302 were not statistically different with p -values ranging from 0.28 to 0.72 showing the repeatability of the
 303 concentration method. In addition, there was no statistically significant difference (p -value > 0.25)
 304 between virus concentration for any type of virus which induces that the concentration did not affect the
 305 recovery rate for this range of virus concentration. The average recovery rates for ADV 41 and CV-B5
 306 were respectively around 40 and 55 % but this rate was low for MS2 (around 1.6 %).

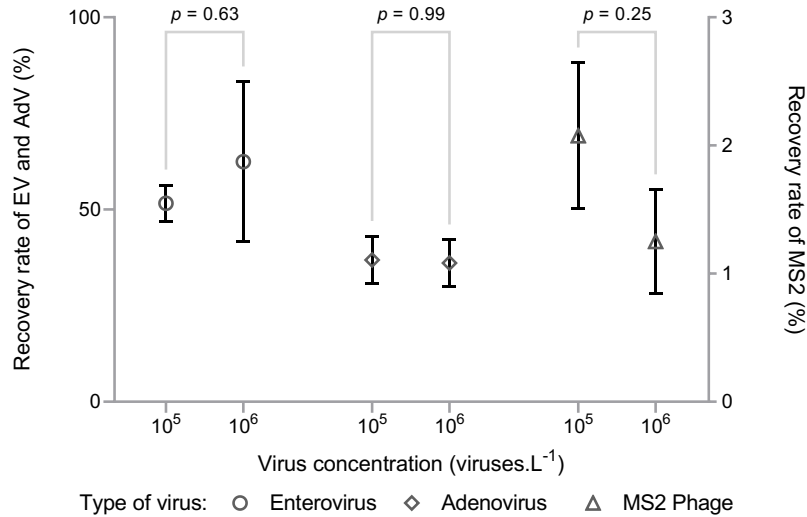


Fig. A.3. Variation of NanoCeram® recovery rates for the high-volume concentration method for different types of virus (CV-B5, ADV 41, and MS2 phage) at two different concentrations

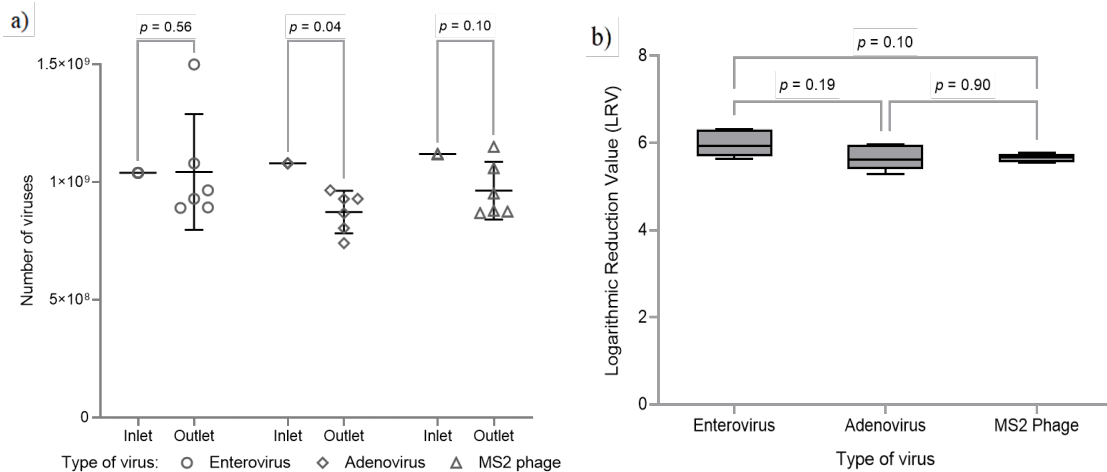
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3.2.2 Application of the method to assess LPRO performance for virus retention on a semi-industrial pilot plant

The NanoCeram® filters were used to concentrate permeates from LPRO semi-industrial pilot plant of 800 L. It must be noted that viruses were detected and quantified in all the samples (permeate and retentates). The recovery rates of the filtrations are presented in Fig. A.4a. Statistically, no significant differences were found between the virus number inlet and outlet of the LPRO process for enterovirus and MS2 phage. However, the mass balances for adenovirus were not validated, even though there was a small significant difference (below 20 % and p -value close to 5 %) between the number of viruses at the inlet and the average number of viruses recovered at the outlet. The viral retention efficiency of the lab-scale pilot plant for the three viruses was still calculated and is presented in Fig. A.4b. For the three viruses studied, the global LRVs were not statistically different with reductions of 6.0, 5.7, and 5.7 log for CV-B5, ADV 41 and MS2 respectively, but the range of experimental LRVs was from 5.2 to 6.5 log.

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Fig. A.4. Validation of the high-volume concentration method: a) Viral mass balance and b) Retention rate for different viruses (CV-B5, ADV 41, and MS2 phage) on a LPRO semi-industrial pilot plant

327 **4. Discussion**

328 The yields obtained for each concentration method could be used to assess the viral risk at low viral
329 concentrations. First, the method using UF membranes achieved high recovery rates of at least 50 % for
330 each virus. It could be stated that the use of three surfactants in the backwash solution could increase
331 the surface tension of the membrane and facilitated the removal of viruses retained during filtration more
332 easily (Hill *et al.*, 2005; Mendez, 2004; USEPA, 2001). Nonetheless, incomplete recovery rates could
333 result from virus adsorption on UF membranes, reducing the efficiency of Inuvai membrane backwash
334 and Vivaspin membrane lysis. It is imperative to address the issue to enhance overall recovery rates.

335 Then, for this UF-based method, it would appear that for ADV 41, CV-B5 and MS2 recovery rates were
336 higher at 10^6 - 10^7 viruses L^{-1} than 10^3 - 10^4 viruses L^{-1} . There may be two phenomena involved in the
337 retention of viruses on the Inuvai membrane that may affect the overall recovery of the method. First,
338 this could be explained by a phenomenon of aggregation of the viruses with higher concentrations
339 (Jacquet *et al.*, 2021). Second, at low concentrations, when viruses are not aggregated, it could be
340 explained by the fact that viral capsids can undergo elastic and bending deformations when pressure is
341 applied (Arkhipov *et al.*, 2009; Buenemann and Lenz, 2007; Roos *et al.*, 2007). Roos *et al.* (2007)
342 showed in indentation experiments on Φ 29 proheads and cowpea chlorotic mottle virus (CCMV)
343 capsids, that a reversible linear regime could be observed when the indentation did not exceed 20-30 %
344 of capsid height. Arkhipov *et al.* (2009) demonstrated that hepatitis B virus (HBV) capsids exhibited
345 irreversible deformations without showing signs of fatigue after multiple shallow indentations. Thus,
346 when the concentration was of 10^3 - 10^4 viruses L^{-1} , it would be possible that some viruses were poorly
347 or not aggregated and could cross the membrane by this deformation phenomenon. On the contrary,
348 when the concentration became very high, the viruses could be aggregated, and were easier to retain.
349 However, this hypothesis cannot be validated in this study, as the recovery rates at these concentration
350 ranges have no statistically significant difference. In fact, the retention mechanisms between virus
351 concentration and UF are complex (Jacquet *et al.*, 2021; Taligrot *et al.*, 2022).

352 For the method using NanoCeram® filters, adsorption-elution of adenovirus, enterovirus was
353 comparable to that found for other electropositive filters (Chang *et al.*, 1981; Karim *et al.*, 2009; Prevost
354 *et al.*, 2015). Prevost *et al.* (2015) used this concentration method to concentrate water spiked with
355 viruses (3 experiments with 10 L of surface water and 3 experiments with 10 L of wastewater treatment
356 plant effluents). Recovery rates ranging from 18 to 42 % have been obtained for ADV 41 and from 57
357 to 83 % for the coxsackievirus B3. Karim *et al.* (2009) found recovery efficiencies of CV-B5 from 100
358 L samples of seeded tap water around 27 %, using NanoCeram® filter. Chang, Farrah and Bitton (1981)
359 obtained recovery rates of about 44 % for coxsackievirus B3 from 19 L of activated sludge effluent
360 using positively charged filters (Zeta Plus 30S). The difference in recovery between different studies of
361 coxsackievirus, adenovirus could be due to the difference in virus type and water matrix. For the low
362 recovery rates obtained for MS2, they were still consistent with some studies finding recovery
363 efficiencies of less than 10 % for viruses with an electrical iso-point close to MS2. Deboosere *et al.*
364 (2011) obtained overall recovery efficiencies of the influenza virus ranging from less than 0.01 % to
365 7.89 % with NanoCeram® filters. The influenza virus used in this study had an isoelectric point around
366 4, similar to MS2 (Michen and Graule, 2010). Similarly, Francy *et al.* (2013) noted recovery efficiencies
367 below 1 % for the Avian influenza virus. In addition, previous studies have indicated that pH can affect
368 virus recovery during concentration (Haramoto and Katayama, 2013). In particular, Sobsey and Glass,
369 (1980) showed that adsorption of poliovirus by electropositive filters is similar between pH 3.5 and 7.5,
370 but that above pH 7.5, adsorption efficiency decreases sharply. Thus, pH, virus interactions and
371 interfering substances could be the factors that resulted in poor MS2 recovery rates in the current study
372 (Deboosere *et al.*, 2011; Francy *et al.*, 2013). On the other hand, Ikner *et al.* (2011) showed for
373 NanoCeram® filters, retention rates ranging from 0.5 to 89 % for the MS2 bacteriophage depending on
374 the composition of the elution solution. Therefore, it would be possible to increase the recovery of
375 NanoCeram® filters for MS2 in this study, however a different elution solution could affect the recovery

376 of CV-B5 and ADV 41. It is thus essential to ensure sufficient sensitivity of the concentration method
377 for the studied viruses.

378 By applying the concentration methods to LPRO permeates from different scales (laboratory and semi-
379 industrial) pilots, mass balances were all validated by the absence of significant statistical differences,
380 except for adenovirus at the semi-industrial scale. This difference could be explained by an adsorption
381 phenomenon of the adenoviruses on the LPRO membrane (Gerba, 1984). As the mass balances were
382 almost all validated, the retention rates were calculated using the low concentrations obtained in the
383 permeates (4.52×10^{-2} virus copies L⁻¹ and 4.23 virus copies L⁻¹, respectively for semi-industrial and
384 laboratory scale). The LRVs obtained were similar for both scales: around 6 log. This is consistent as
385 the same membranes and feed concentration were used. Furthermore, the results were in agreement with
386 those found in different studies (Comerton *et al.*, 2005; Mi *et al.*, 2004; Ohkouchi and Ase, 2020;
387 Patterson *et al.*, 2012; Pype *et al.*, 2016; Surawanvijit *et al.*, 2015) using small membrane area (i.e. small
388 volume). In all these studies, the viral removal obtained for the different viruses, as MS2, were around
389 6, in particular for Mi *et al.* (2004) with LRVs around 6.4 and Comerton *et al.* (2005) and Ohkouchi and
390 Ase (2020) with LRVs above 7. However, the retention was not total while dense membranes were used
391 and are ideally supposed to allow only the solvent to pass through. LPRO therefore appears to be a
392 complex process requiring further studies to understand the mechanisms of virus passage. The process
393 is still effective for virus removal with LRVs of at least 5, whatever the feed concentration. This enables
394 to consider the use of this process for water treatment to highly reduce the viral risk (Włodarczyk and
395 Kwarciak-Kozłowska, 2020).

396 Through these applications, the UF concentration method allows similar retention of the three viruses
397 studied simultaneously for volumes of water produced by RO up to 200 L. However, its use is limited
398 by the volume and the quality of the sample to be concentrated, which can lead to fouling and loss of
399 Inuvai membrane integrity (Olszewski *et al.*, 2005). The electropositive filter concentration method
400 appeared to be suitable for the concentration of the three viruses studied, present simultaneously in very
401 large volumes that could not be treated by the UF concentration method.

402 In addition, it is current knowledge that adenoviruses have been detected in several aquatic environments
403 around the world, including wastewater, drinking water, the ocean, and rivers (Allard and Vantarakis,
404 2019). These are responsible for several recreational epidemics of waterborne disease (Sinclair *et al.*,
405 2009). Many studies have suggested that adenoviruses could be used as an indicator for viral pathogens
406 (Verani *et al.*, 2019) and even the microbial water quality (Wyer *et al.*, 2012). Both methods used in this
407 study have been validated for ADV 41 concentration. Among adenoviruses, the use of ADV 41 as an
408 index of microbial quality could be another potential application of this work (in addition to the
409 efficiency assessment of water treatment processes). Indeed, the methods would then allow the
410 concentration of different volumes depending on the type of water to be treated and their viral
411 concentration.

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413 5. Conclusion

414 For the first time, this study has developed a method to concentrate and evaluate virus removal in large
415 permeate volumes from LPRO adapted to lab-scale and semi-industrial scale pilots. Respectively, a
416 protocol based on two UF steps was developed for concentrating volumes of less than 200 L and a 3-
417 steps protocol using adsorption/elution as primary concentration step was also developed for higher
418 volume. The following main conclusions can be drawn:

- 419 • Low-volume concentration method using UF membranes was validated and thus allowed to
420 quantify the lab-scale process efficiencies with feed concentration close to that found in the
421 environment. Concentration with UF could not be used at semi-industrial scale due to excessive

422 fouling and loss of integrity of the Inuvai membrane when the volumes treated exceeded 200 L.
423 High-volume concentration method using Nanoceram® filters was also validated, which have
424 allowed to quantify the semi-industrial process efficiencies with feed concentration close to that
425 found in the environment. Validation of these methods has enabled to quantify the operation of
426 the LPRO process on two scales, for three different viruses.

- 427 • As expected, the LPRO LRVs (around 6 log) were higher than those obtained through UF
428 filtrations (between 3 to 4 log) due to a lower MW cut-off (Jacquet *et al.*, 2021; Mi *et al.*, 2004).
429 However, viral retention was not total, although dense membranes were used. These results
430 open interesting perspectives for LPRO processes used in drinking water production. For
431 example, with these analysis methods, different protocols will be tested to understand why dense
432 membranes do not retain all micrometric viruses.
- 433 • The yields of concentration methods open up perspectives for the use of adenovirus as an
434 indicator of microbial quality. NanoCeram® filters deserve further studies, especially on the
435 different interactions between virus, filter, and elution solution. This work is in progress, and
436 the results will be the subject of forthcoming papers.

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