1 **Recovery of SARS-CoV-2 from large volumes of raw wastewater is enhanced**
2 **with the inuvai R180 system**

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22 **Abstract**

23 Wastewater-based epidemiology (WBE) for severe acute respiratory syndrome
24 Coronavirus 2 (SARS-CoV-2) is a powerful tool to complement syndromic
25 surveillance. Although detection of SARS-CoV-2 in raw wastewater may be prompted
26 with good recoveries during periods of high community prevalence, in the early stages
27 of population outbreaks concentration procedures are required to overcome low viral
28 concentrations. Several methods have become available for the recovery of SARS-
29 CoV-2 from raw wastewater, generally involving filtration. However, these methods
30 are limited to small sample volumes, possibly missing the early stages of virus
31 circulation, and restrained applicability across different water matrices. The aim of this
32 study was thus to evaluate the performance of three methods enabling the
33 concentration of SARS-CoV-2 from large volumes of wastewater: i) hollow fiber
34 filtration using the inuvai R180, with an enhanced elution protocol and polyethylene
35 glycol (PEG) precipitation; ii) PEG precipitation; and iii) skimmed milk flocculation. The
36 performance of the three approaches was evaluated in wastewater from multiple
37 wastewater treatment plants (WWTP) with distinct singularities, according to: i)
38 effective volume; ii) percentage of recovery; iii) extraction efficiency; iv) inhibitory
39 effect; and v) the limits of detection and quantification. The inuvai R180 system had
40 the best performance, with detection of spiked control across all samples, with average
41 recovery percentages of 68% for porcine epidemic diarrhea virus (PEDV), with low
42 variability. Mean recoveries for PEG precipitation and skimmed milk flocculation were
43 9% and 14%, respectively. The inuvai R180 enables the scalability of volumes without
44 negative impact on the costs, time for analysis, and recovery/inhibition. Moreover,
45 hollow fiber ultrafilters favor the concentration of different microbial taxonomic groups.

46 Such combined features make this technology attractive for usage in environmental
47 waters monitoring.
48
49 **Keywords:** SARS-CoV-2; methods performance and evaluation; wastewater;
50 wastewater-based epidemiology
51

52 **1. Introduction**

53 Surveillance of wastewater for epidemiological purposes has been previously used in
54 public health, with the most important and successful example being the polio
55 eradication program (GPEI, 2021). Given the ongoing Coronavirus disease 2019
56 (COVID-19) pandemic and accumulated reports of the presence of the severe acute
57 respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA in the stools of infected
58 people and in raw wastewater (Gonzalez *et al*., 2020; Medema *et al.*, 2020; Randazzo
59 *et al*., 2020) the use of this matrix as a tool to monitor the emergence, prevalence,
60 molecular epidemiology, and eventual phase out of SARS-CoV-2 in the community
61 was prompted. Wastewater-based epidemiology (WBE) of SARS-CoV-2 has thus
62 been gaining track among scientists, stakeholders, and decision makers throughout
63 the world to complement syndromic surveillance and clinical testing. Several factors
64 impact the detection of SARS-CoV-2 in raw wastewater, particularly in the early stages
65 of virus circulation, including, sample collection (composite vs grab samples; for grab
66 samples, sampling time of the day is also critical) and optimized concentration
67 methods. Although detection of SARS-CoV-2 may be performed directly on raw
68 wastewaters with increased recovery percentages, ultimately optimization of
69 concentration procedures is necessary in the early stages of virus circulation wherein
70 low concentrations are expected (Gonzalez *et al*., 2020). Therefore, cost-effective,
71 rapid, and efficient concentration methods are required for monitoring SARS-CoV-2 or
72 any other pathogen in raw wastewater for the successful deployment of WBE.

73 Existing methods for the recovery of viruses were primarily developed for the detection
74 of nonenveloped viruses. Knowledge gaps concerning the recovery efficiencies of
75 enveloped viruses, such as SARS-CoV-2, remain. A study by Haramoto *et al*. (2009)
76 showed recovery efficiencies to be largely different for both types of viruses, with

77 methods performing better for the recovery of nonenveloped viruses. Blanco *et al*.
78 (2019) determined similar recovery efficiencies using precipitation with 20%
79 polyethylene glycol (PEG) following glass wool concentration for enveloped
80 (Transmissible gastroenteritis virus (TGEV)) and nonenveloped viruses (Hepatitis A
81 virus (HAV)). A recent study by Ahmed *et al*. (2020) showed recovery efficiencies
82 varying between 26.7 and 65.7% for murine hepatitis virus (MHV) in raw wastewater
83 with very disparate recovery rates, even for similar methods, for this SARS-CoV-2
84 surrogate. Data using porcine epidemic diarrhea virus (PEDV) and aluminum
85 flocculation-based concentration demonstrated recovery efficiencies of 11 and 3% for
86 raw and treated wastewater, respectively (Randazzo *et al*., 2020).
87 Despite scarce information on diagnostic performance, SARS-CoV-2 RNA has been
88 detected globally in raw wastewater with different approaches. Reported methods
89 included ultrafiltration (Bertrand *et al*., 2021; Medema *et al*., 2020), ultracentrifugation
90 (Wurtzer *et al*. 2020), PEG precipitation (Chavarria-Miró *et al*., 2020; La Rosa *et al*.,
91 2020), aluminum flocculation (Randazzo *et al*., 2020), skimmed milk flocculation (Philo
92 *et al*., 2021), and filtration through an electronegative membrane (Gonzalez *et al*.,
93 2020; Haramoto *et al*., 2020). For an effective environmental surveillance to be put in
94 place, not only for SARS-CoV-2 but also for potential future pandemics involving
95 enveloped virus, it is paramount to have validated methods. Nonetheless,
96 comparisons between published methods are difficult as they differ in many aspects
97 including: i) seeding controls; ii) concentration methods; iii) extraction methods; iv)
98 diagnostic and quantification molecular assays and genome targets; v) and mostly,
99 the accepted performance levels. Some publications only mention the recovery
100 efficiency (Ahmed *et al*., 2020; McMinn *et al*., 2021), others mention the recovery
101 efficiency and the LoD but not LoQ (Gonzalez *et al*., 2020; Randazzo *et al*., 2020;

102 Pérez-Cataluña *et al*., 2021), some mention LoQ but not LOD (LaTurner *et al*., 2021),
103 while other studies show all data performance, including LoD, LoQ and recovery
104 percentages (Philo *et al*., 2021). Additionally, different studies calculate the LoD and
105 LoQ differently. The information collected from different studies should inform
106 laboratories on method performance.
107 In the present study, we evaluated the efficiency of SARS-CoV-2 recovery from raw
108 wastewater using three concentration methods: i) a newly developed hollow-fiber
109 ultrafilter, inuvai R180 (inuvai, a division of Fresenius Medical Care), with an improved
110 elution protocol; ii) PEG precipitation; and iii) skimmed milk flocculation. The
111 performance of the three methods was compared in raw wastewater according to
112 several characteristics, including: i) effective volume tested; ii) frequency and
113 consistency of detection; iii) percentage of recovery; iv) extraction efficiency; v)
114 inhibitory effect on reverse transcription-qPCR (RT-qPCR); and vi) concentration
115 information (including, Limit of Detection (LoD) and Limit of Quantification (LoQ)).
116 These three methodologies were chosen primarily because they allow for the
117 concentration of large volumes of water which is of extreme relevance at the early
118 stages of the epidemiological curve and because they allow for the concentration of
119 different types of water, including treated wastewater and environmental waters,
120 important when analyzing the entire SARS-CoV-2 urban water cycle. This study
121 benchmarks new and old methodologies for the detection of SARS-CoV-2 from raw
122 wastewater for WBE applications.
123

124 ## 2. Materials and Methods

125 ### 2.1. PEDV strain and cell lines

126 PEDV strain CV777 (kindly provided by Dr. Gloria Sanchez, IATA-CSIC) is an
127 enveloped virus from the genus *Alphacoronavirus* and member of the *Coronaviridae*
128 family, responsible for the porcine epidemic diarrhea. PEDV was propagated in Vero
129 cell line (ATCC CCL-81, LGC Standards). Briefly, Vero cells were grown in Dulbecco's
130 Modified Eagle's Medium (DMEM; Gibco), supplemented with 100 units/mL of
131 penicillin (Lonza), 100 units/mL of streptomycin (Lonza), and 10% heat-inactivated
132 fetal bovine serum (Biological Industries). Cells were cultured in T175 flasks at 37 (±
133 1) ºC under 5 % CO_2. For infection with PEDV, cells were grown in T25 flasks and
134 inoculated with 100 µL of viral stock. At 2h post infection, DMEM supplemented with
135 0.3% tryptose phosphate broth, 100 units/mL of penicillin (Lonza), 100 units/mL of
136 streptomycin (Lonza), and 10 µg/µL trypsin, was added to the flasks. Flasks were then
137 incubated at 37 (± 1) ºC in 5% CO_2 for 4 days. PEDV were recovered following three
138 cycles of freeze/thawing and centrifugation at 1,100 xg for 10 min. Quantification was
139 performed by reverse transcription digital PCR (RT-dPCR) as described on section
140 2.5 using the primers and probes from Supplementary Table S1 (Zhou *et al*., 2017),
141 following nucleic acid extraction as described on section 2.4. After absolute
142 quantification by RT-dPCR (as described below), a stock solution was prepared in
143 DNase/RNase free water to obtain a PEDV final concentration of 1.21 x 10^4 GC/L in
144 wastewater. The same stock was used in all experiments described below.
145

146 *2.2. Wastewater sample preparation*

147 Twenty-four-hour composite samples were collected between July 6-10, 2020, from
148 five wastewater treatment plants (WWTP) in Portugal (*n* =8; *n* = 2 for Serzedelo, Gaia
149 and Guia; *n* = 1 for Alcântara and Beirolas). The samples were transported to the
150 laboratory, refrigerated, and processed within eight hours of collection. Samples were

151 seeded with PEDV at a concentration of 1.21 x 10^4 GC/L (quantified as described
152 below).
153 Seeded raw wastewater samples were aliquoted and concentrated using three
154 methods: (i) hollow fiber with the newly developed inuvai R180 ultrafilters (inuvai, a
155 division of Fresenius Medical Care, Germany) followed by PEG precipitation (method
156 1); (ii) direct PEG precipitation (method 2); and (iii) skimmed-milk flocculation (method
157 3). The inuvai R180 ultrafilter has a large membrane area (1.8 m^2) and a fiber inner
158 diameter of 220 µm, allowing for the concentration of large volumes of water, including
159 wastewater, without problems such as clogging or compromising of the membrane
160 structure. All methods were tested using the same initial volume of wastewater (1-L)
161 for a more accurate comparison.
162 Method 1 employed the use of hollow fiber ultrafilters: 1-L of raw wastewater was
163 filtered through inuvai R180 ultrafilters using a peristaltic pump with a flow rate of 250
164 mL/min. The elution was performed in three steps: (i) air forward push using 60 mL of
165 air; (ii) backflush with 250 mL of elution buffer (1× PBS with 0.01% NaPP and 0.01%
166 Tween 80/0.001% antifoam) at a flow rate of 140-280 mL/min; and (iii) forward flush
167 using 50 mL of elution buffer. The final elution volume was 300 mL. Samples were
168 further concentrated by precipitation with 20% (w/v) PEG 8000 (Sigma, Portugal)
169 overnight (Blanco *et al*., 2019). Samples were centrifuged at 10,000 ×g for 30 min, the
170 supernatant discarded, and the pellet resuspended in 5 mL 1× PBS, pH 7.4.
171 Method 2 used PEG precipitation: 20% PEG 8000 was added directly to 1-L of raw
172 wastewater, with overnight precipitation followed by centrifugation as described above
173 for method 1. Method 3 employed skimmed milk flocculation, performed in accordance
174 with Calgua *et al*. (2008). Briefly, a pre-flocculated solution of 1% (w/v) skimmed milk
175 pH 3.5 was prepared in artificial seawater. The solution of skimmed milk was then

176 added to a final concentration of 0.01% (w/v) to 1-L of previously acidified raw
177 wastewater (pH 3.5). Samples were stirred for 8h at room temperature and flocs were
178 allowed to sediment for another 8h. Supernatant was carefully removed without
179 disturbing the sediment. The final volume (approximately 500 mL) was centrifuged at
180 7,000 ×g for 30 min at 12 ºC. The supernatant was carefully discarded, and the pellet
181 resuspended in 0.2 M phosphate buffer at pH 7.5 to a final volume of 5 mL. All
182 concentrates were stored at – 80 (± 10) ºC until further analysis.

183

184 *2.3. Nucleic acid extraction*

185 Nucleic acid extraction was conducted using the QIAamp Fast DNA Stool mini kit
186 (QIAGEN, Germany) from 220 μL of PEDV stock or concentrated raw wastewater
187 samples according to the manufacturer's instructions, recovering the nucleic acids in
188 a final volume of 100 μL. Recovery efficiency for extraction was evaluated using
189 Murine Norovirus 1 (MNV-1), added to the concentrates, as an extraction control. MNV
190 was quantified using the assay described by Baert *et al.*, 2008. Primers and probe
191 information is provided on Supplementary Table S1. The extraction efficiency was
192 calculated as

193

194 $Extraction\ efficiency\ (\%) = \frac{Total\ MNV\ copies\ recovered}{Total\ MNV\ copies\ seeded} \times 100$ (Eq. 1).

195

196 Following extraction, samples were stored at -30 (± 5) ºC until further processing.

197

198 *2.4. Absolute quantification by RT-dPCR*

199 RT-dPCR was used to determine the exact concentration of SARS-CoV-2 control
200 (nCoV-ALL-Control plasmid, Eurofins Genomics, Germany) and PEDV. Controls were

201 amplified using the AgPath-ID One-Step RT-PCR kit (Thermo Fischer Scientific) with
202 the set of primers and probes described on Supplementary Table S1 (PEDV;
203 E_Sarbeco and RdRP assays). The 15 µL reaction mixture consisted of 7.5 µL of 2×
204 RT-PCR buffer, 0.6 µL of 25× RT-PCR enzyme mix, 800 nM of each primer, 200 nM
205 of probe, 3.63 µL RNase/DNase-free water, and 3 µL of DNA (diluted 4-, 5-, 6- fold).
206 The reaction mixture was then spread over the QuantStudio 3D Digital PCR chip
207 (Thermo Fischer Scientific) and the chips transferred to the QuantStudio 3D Digital
208 PCR thermal cycler. Amplification was performed as follows: i) SARS-CoV-2: 10 min
209 at 45 ºC, 10 min at 96 ºC, 39 cycles of 2 min at 58 ºC and 30 s at 98 ºC, and final
210 elongation step for 2 min at 58 ºC; ii) PEDV: 10 min at 45 ºC, 10 min at 96 ºC, 39
211 cycles of 2 min at 60 ºC and 30 s at 98 ºC, and a final elongation step for 2 min at 60
212 ºC. Reactions were performed in duplicate, and a non-template control (NTC) was
213 included in each run.
214
215
216 *2.5. Relative quantification of seeded material in wastewater*
217 Relative quantification of SARS-CoV-2, PEDV and MNV-1 was carried out by RT-
218 qPCR on all extracts using the AgPath-ID One-Step RT-PCR kit (Thermo Fischer
219 Scientific). The final volume of 25 µL was composed of 12.5 µL of 2× RT-PCR buffer,
220 1 µL of 25× RT-PCR enzyme mixture, 800 nM of each primer, 200 nM of the probe,
221 6.05 µL RNase/DNase-free water, and 5 µL of RNA. All RT-qPCR reactions were run
222 on undiluted, 4- and 10-fold diluted extracts. RT-qPCR conditions were as follows: i)
223 SARS-CoV-2 control: 10 min at 45 ºC, 10 min at 95 ºC, 45 cycles of 15 s at 95 ºC and
224 1 min at 58 ºC; ii) PEDV and MNV-1: 10 min at 45 ºC, 10 min at 95 ºC, 40 cycles of 15
225 s at 95 ºC and 1 min at 60 ºC. Standard curves, run with each PCR, for SARS-CoV-2

control (E_Sarbeco and RdRp assays), PEDV and MNV-1 were prepared in serial 10-fold dilutions in RNase/DNase-free water. Positive and NTC controls were also added to each PCR assay. Positive and NTC controls were also added to each PCR assay. Limits of detection (LoD) and quantification (LoQ) were determined in RNase/DNase-free water. The LoD was considered the lowest concentration of target that could be consistently detected (in more than 95% replicates tested) (Burd *et al.*, 2010) and LoQ, the lowest concentration at which the performance of the method is acceptable, with a coefficient of variation below 35% (Klymus *et al.*, 2020).

2.6. Recovery efficiency

The mean recovery efficiency of PEDV for each method was calculated using the copies quantified by RT-qPCR as follows (Eq. 2):

$$Recovery\ efficiency\ (\%) = \frac{Total\ nucleic\ acid\ copies\ recovered}{Total\ nucleic\ acid\ copies\ seeded} \times 100 \quad \text{(Eq. 2)}$$

The mean and standard deviation for each method were also calculated.

2.7. Quality control

To minimize nucleic acid carry-over and cross-contamination, sample concentration, extraction procedures and RT-qPCR/RT-dPCR were performed in separate rooms of the laboratory. A process blank and extraction blank were included for each concentration method and each nucleic acid extraction, respectively. All spiked samples were tested in parallel with the corresponding unseeded samples to rule out or estimate the contribution of potentially native PEDV.

251 *2.8. Data analyses*

252 All data analyses were performed with SPSS Statistics 26 (IBM). Kruskal-Wallis test
253 was conducted to compare the differences between the parameters estimated for the
254 three methods and pairwise comparison was performed with Dunn's test. In all cases,
255 P-values < 0.05 were considered statistically significant.

256

257 3. Results and discussion

258 *3.1. Quantification of controls*

259 Appropriate quantification of the controls used in spiking experiments and in standard
260 curve for qPCR is extremely important, as it will influence downstream data
261 interpretation. That is why we opted for RT-dPCR, with high precision and sensitivity,
262 for the absolute quantification of controls. Digital PCR works by partitioning a unique
263 sample into thousands of individual reactions running in parallel, being particularly
264 useful for low-abundance targets or targets in complex matrices. Through Poisson
265 statistics, the total number of target molecules is calculated, with no need for external
266 reference standards (Monteiro and Santos, 2017). Several dilutions of SARS-CoV-2
267 control and PEDV, in duplicate, were quantified by RT-dPCR. The concentrations of
268 the initial stocks for SARS-CoV-2 control were 1.94×10^{8} GC/µL and 1.00×10^{8} GC/µL
269 for E_Sarbeco and RdRp assays, respectively. Concentration of PEDV as determined
270 by RT-dPCR was 1.20×10^{8} GC/µL.

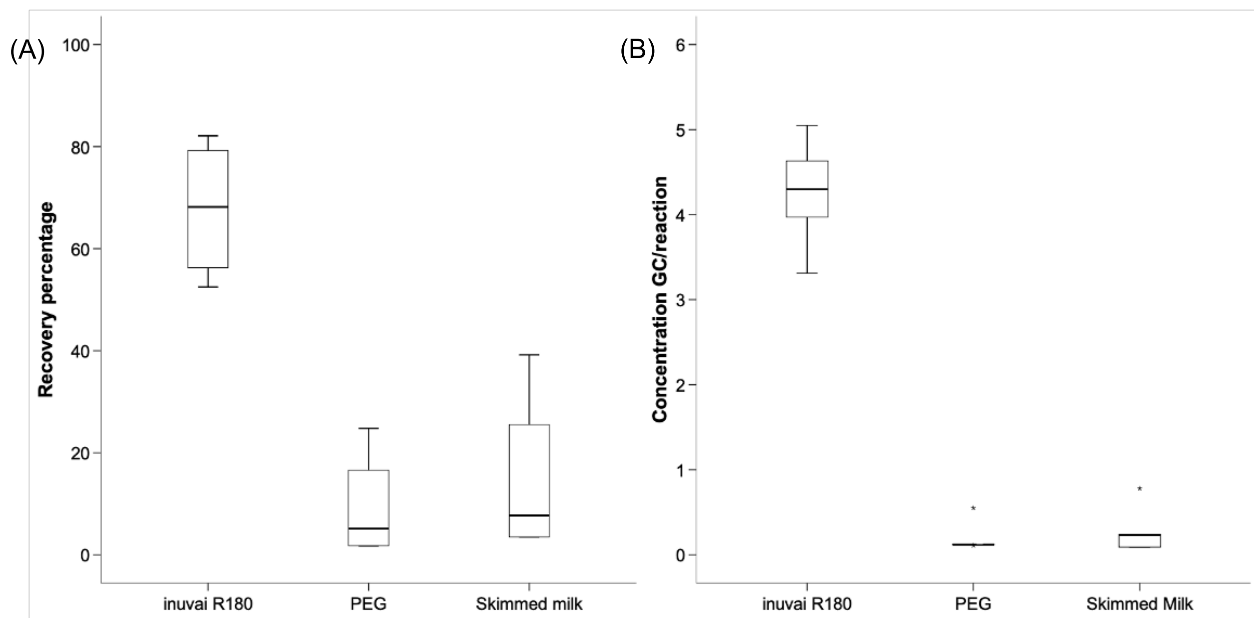
271

272 *3.2. Method comparison using PEDV as surrogates for SARS-CoV-2*

273 All unseeded wastewater samples were negative for the presence of PEDV. Samples
274 were chosen in a period with low number of daily COVID-19 cases (mean for entire

275 country, and 374, between July 6 and 10, 2020) (DGS, 2020). All process and
276 extraction blanks were negative.
277 The effective volume tested within each method was the same (2.2 mL): all methods
278 started with the same initial volume (1-L) of wastewater, followed by concentration
279 steps prior to extraction and sediment resuspension in 5 mL of elution buffer; samples
280 tested across the three methods were extracted using the same extraction protocol,
281 and the same volumes and dilutions were analyzed by RT-qPCR. Nonetheless, the
282 inuvai R180 ultrafilters (method 1) enabled the filtration of 2.5 – 5-L of raw wastewater.
283 Increasing the initial volume of sample with the inuvai R180 ultrafilters would conduct
284 to an increment of the effective volume assayed from 2.2 mL to 5.5 – 11 mL without
285 further increases in the concentration time, the concentrate volume, costs for analysis,
286 and RT-qPCR inhibition. On the other hand, increasing the volume of filtration in the
287 skimmed milk flocculation method (and therefore, theoretically, increasing the effective
288 volume assayed; method 3) would imply an increase of skimmed milk and artificial
289 seawater, as well as of HCl to adjust the pH; the volume of concentrated matter and,
290 therefore, of the concentrate would also increase, leading to a decrease in the
291 efficiency of extraction and an increase of inhibitory effects on RT-qPCR. Additionally,
292 increasing the processing volume would require the acquisition of larger volume
293 sample containers, which would also take up more space in the laboratory.
294 Concomitantly, increasing the processing volume when using solely PEG precipitation
295 (method 2) implicates increasing substantially the volume to be centrifuged, which
296 increases the time spent in the concentration step and the costs due to the usage of
297 larger amounts of PEG.
298

299 PEDV was used to compare concentration recoveries. The highest average
300 percentage of recovery was obtained with the inuvai R180 system at 68% (± 11%) for
301 PEDV, with recoveries varying between 52 and 82% (Fig. 1A).
302 PEG precipitation had the lowest percentage of recovery for PEDV (9% (± 5%)).
303 Recovery with skimmed milk performed only slightly better (14% (± 8%)) (Fig. 1A).
304 There were statistically significant differences in the recovery percentage of PEG and
305 skimmed milk compared to inuvai R180 (KW = 15.989, df = 2, $p < 0.05$), although no
306 difference was observed between skimmed milk flocculation and PEG precipitation (p
307 < 0.05).
308 The inuvai R180 was the single method that consistently led to nucleic acid detection
309 in all samples. Concentration using PEG and skimmed milk led to the detection of
310 PEDV in 50% of the samples.
311

312 

313

314 **Fig. 1.** Performance of concentration methods for the detection of PEDV from raw wastewater. Percentage of
315 recovery obtained in each method (A). copy numbers of viral genome copies detected by RT-qPCR in each method
316 (B). Asterisks represent outliers.

317

318 The method using the inuvai R180 system led to detection by RT-qPCR of the highest
319 mean genome copies, 4.25 GC/reaction for PEDV, respectively. Concentration with
320 PEG (0.21 GC/reaction) and skimmed milk (0.28 GC/reaction) showed similar results
321 (Fig. 1B).
322 Our recovery values using the inuvai R180 system were similar to those reported for
323 MHV, while enabling an increase in the filtration volume (Ahmed *et al*., 2020). For PEG
324 precipitation and skimmed milk flocculation the recoveries were slightly higher than
325 those reported by Philo *et al*. (2021). The authors used a concentration of 14% (w/v)
326 of PEG compared to 20% (w/v) PEG in our study. The use of higher concentrations of
327 PEG, although implying increased costs, has been shown to increase the recovery of
328 enveloped viruses from 31% to 51% (Blanco *et al*., 2019). In our study, recovery values
329 for PEG precipitation were higher than those reported by Pérez-Cataluña *et al*. (2021)
330 when using similar nucleic acid extraction method (spin column). McMinn *et al*. (2021)
331 developed a method for the recovery of coronavirus from raw wastewater also using
332 hollow fibers as a primary concentration approach, followed by Concentrating Pipette
333 Select™ (CP Select™), reporting overall recovery values for human coronavirus OC43
334 of 22%. Differences in recovery between our study and that of McMinn *et al*. (2021)
335 may be attributed to the ultrafilter that used in our study (inuvai R180 vs Rexeed),
336 coupled with an enhanced elution strategy with three steps that we adopted, and/or to
337 the secondary concentration protocol. The inuvai R180 ultrafilter has a reduced
338 nominal pore size (≤ 5.5 nm with a correspondent cut-off ≤ 18.8 Kda) compared to the
339 Rexeed 15S, which has a more open pore structure. Additionally, the ultrafilter used
340 in our study has a larger membrane area (1.8 m^2 for inuvai R180 vs 1.5 m^2 for Rexeed
341 S15) and larger fiber inner diameters (220 µm for inuvai R180 vs 185 µm for Rexeed

342 S15). In addition to the optimized elution and secondary concentration protocols, such
343 features might help justify the differences registered in the recovery efficiencies of our
344 study and McMinn *et al*. (2021). Table 1 summarizes the strong and weak points for
345 the different methods used to concentrate SARS-CoV-2 from raw wastewater,
346 including the ones tested in this manuscript.

347

348 **Table 1**

349 Operational and theoretical advantages and disadvantages of different concentration methods including those
350 tested in this study

Concentration method	Advantages	Disadvantages
inuvai R180 system	• Concentrate viruses from both solid and liquid phase • Concentration of large volumes of raw wastewater (up to 5-L depending on the turbidity) without increase in time and cost for analysis • Concentration of samples in the field • Multiple samples processed at the same time if several filtration apparatuses are available • Easy to store and transport • Low probability of clogging in volumes up to 3-L (particularly using volumes of just up to 1-L) • Possible application to other water matrices, with higher sample volumes, without further cost or time increase	• Time consuming • Requires hollow-fiber ultrafilters
PEG precipitation	• Concentrate viruses from both solid and liquid phase • Concentration of large volumes of raw wastewater (e.g. 1-L) • Only equipment necessary is centrifuge • Relatively inexpensive	• Time consuming • Cannot be applied in the field • Analysis of larger volumes implies increases in time for analysis, reagent costs (PEG particularly), and larger centrifuges
Skimmed milk flocculation	• Concentrate viruses from both solid and liquid phase • Concentration of large volumes of raw wastewater, without time increase • Only equipment necessary is centrifuge • Possible application to other water matrices, with higher sample volumes, without further cost or time increase	• Time consuming • pH adjustment required • Cannot be applied in the field • Analysis of larger volumes implies increases in time for analysis, reagent costs (PEG particularly), and larger centrifuges
Ultrafiltration	• Rapid (depends on the turbidity of the water) • Only equipment necessary is a centrifuge	• Concentration of viruses only from the liquid phase • Concentration volumes extremely variable • Clogging occurs for samples with high turbidity

		• Each unit is expensive, and several may be necessary for samples with high turbidity • Cannot be applied in the field
Electronegative membranes	• Concentrates viruses from liquid and solid phase • Rapid (depending on the turbidity of the sample) • Can be performed onsite • Only equipment required is a filtration unit and a pump • Multiple samples processed at the same time if several filtration apparatuses are available • The membrane is easily stored and transported to the laboratory	• Clogging occurs for samples with high turbidity • Low volumes assessed • Upscalling to 90-mm membranes requires the use of expensive filtration units • Extra care is needed to minimize the risks of contamination, requiring washing and cleaning filtration units
Aluminum chloride	• Rapid • Concentrates viruses from both solid and liquid phase • Concentration of large volumes of raw wastewater	• $AlCl_3$ highly hygroscopic, must be kept under the right conditions • Necessary to form $Al(OH)_3$ precipitate properly • pH adjustment is required • Cannot be applied in the field • Requires the use of an orbital shaker, a relatively expensive equipment

351

352 The extraction efficiency using MNV as proxy averaged 70% (±19%) for inuvai R180
353 protocol. Extraction efficiencies for PEG precipitation and skimmed milk flocculation
354 averaged 50% (±15%) and 36 (±13%), respectively. RNA extraction was conducted
355 using QIAamp Fast DNA Stool mini kit (QIAGEN, Germany) that although being used
356 for DNA extraction, can efficiently co-extract RNA. The reagents provided by the
357 manufacturer as well as the columns provided are not specific for DNA. The handbook
358 for the kit indicates that RNase should be used to remove RNA from the final eluate.
359 Detection at a high rate demonstrated for MNV showed high efficiency, demonstrating
360 therefore the application of this DNA extraction kit for the co-extraction of RNA.
361 Detection of PEDV using the inuvai R180 system was consistently achieved with the
362 1/4-fold dilution, while for undiluted spiked samples, only 38% could be detected
363 without inhibition. PEG precipitation was the single method that detected both targets
364 from undiluted samples, although inhibition still occurred (as evidenced subsequently
365 by testing the 4- and 10-fold dilution). As for the skimmed milk concentration method,

366 detection in undiluted concentrates was found for 75% of the samples, although
367 inhibition still occurred (as measured by the dilutions). These results indicate that
368 inhibitory effects exerted upon RT-qPCR could be confirmed for the three methods
369 under comparison. Difference between concentration using the combined R180
370 system (including elution step and PEG concentration) versus simply using PEG
371 concentration may arise from the elution buffer used and high performance of the
372 ultrafilter itself. Also, co-concentration of PCR inhibitors occurred for all three methods,
373 although with a better extraction recovery using MNV obtained for the combined R180
374 system, which indicates that concentration using only PEG and skimmed milk
375 potentiated higher co-concentration of inhibitors. Although the ultrafilters concentrated
376 both liquid and solid phases and possible inhibitors were co-concentrated, many might
377 have been directly eliminated during the filtration process and other may not have
378 been eluted during the elution procedure. Therefore, one of the major driving forces
379 for the different results, particularly when comparing both PEG-based methods, might
380 have been the co-concentration of inhibitors.
381 Overall, our results showed that the inuvai R180 system coupled with an improved
382 elution protocol is highly suitable for the detection of PEDV, used as a surrogate for
383 SARS-CoV-2 in this study, exhibiting the highest percentage of detection and mean
384 recovery value. Additionally, this method also showed greater extraction efficiency and
385 larger volume processing without increased cost or time for downstream analyses.
386 Furthermore, the performance of the inuvai system showed consistency across raw
387 wastewater samples from different catchments / WWTP, including the Serzedelo
388 WWTP, which is highly impacted by industrial effluents (tannery industry) and
389 therefore an extremely complicated matrix to work with altogether, a result
390 corroborated by the Pan-European Umbrella study (Gawik *et al.*, 2021). In the

391 Umbrella study, raw wastewater samples from different European countries were
392 collected and sent for analysis in a centralized laboratory. In parallel, the same
393 samples were also analyzed in each country for comparison of results. The centralized
394 European laboratory was unable to recover SARS-CoV-2 RNA from Serzedelo raw
395 wastewater presenting low recovery percentages (0.1%) and lower concentrations of
396 crAssphage compared to the other samples analyzed. The same sample, analyzed by
397 our group and using the inuvai R180 system, was positive for SARS-CoV-2 and the
398 concentration of crAssphage was 3-log above that detected by the centralized
399 laboratory. These results demonstrate the difficulty of working with this raw
400 wastewater, highlighting the need to test method performance in raw wastewater from
401 different origins.
402 Data from our study demonstrates the importance of validating concentration
403 procedures using seeded controls. Although other studies have tested the efficiency
404 of concentration and extraction methods, this study showed the stability of the inuvai
405 R180 system for the recovery of seeded controls in raw wastewater from WWTP with
406 different composition particularities, including effluents from the tannery industry. A
407 single concentration method may not necessarily be ideal to be used in waters from
408 different backgrounds. The inuvai R180 system with improved three-step elution
409 protocol was selected for monitoring SARS-CoV-2 in raw wastewaters and has been
410 applied extensively for WBE for SARS-CoV-2 (Monteiro *et al.*, 2022). This system is
411 attractive as it enables the concentration of large volumes from different types of water,
412 including raw and treated wastewater, drinking water, surface and bathing waters.
413 Such feature enables the use of a single concentration method across different water
414 types without loss in sensitivity, increasing costs or time for analysis, with a less
415 challenging comparison of results. Moreover, a 'one size fits all' approach, that is

416 having a single standardized method worldwide for the concentration of SARS-CoV-
417 2, may not be the best approach due to several issues, including: (i) laboratories
418 already have their own preferred methods with performances studied; (ii) the methods
419 may not be useful for application in less economically developed countries; (iii) or
420 simply because it is difficult to get a hold of laboratory materials/equipment (as it was
421 the case of ultrafiltration ultrafilters or ultracentrifuges). Nonetheless, standards as to
422 what should be asked in terms of method performance should be established so that
423 laboratories could gather all the information about the methods to make a more
424 informed choice. Wastewater surveillance has the potential to prevent the occurrence
425 of new outbreaks (Peiser, 2020), and to help understand changes in the pandemic
426 trends. Effective methods, with performance specifications detailed, are paramount for
427 wastewater surveillance to be applied in accurately describing the transmission of
428 SARS-CoV-2 in the community. This study expands the knowledge on analytical
429 methods introducing a method with robust performance for SARS-CoV-2 detection in
430 wastewater and establishing a step forward for the global application of WBE not only
431 for this pandemic but also in future health crisis as the established protocol is modular
432 with application to different taxonomic groups and water types.
433
434
435 *3.3. RT-qPCR efficiency*
436 After establishing the inuvai R180 system as gold-standard for primary concentration,
437 the efficiency of the relative quantification method (RT-qPCR) was assessed by
438 calculating the LoD and LoQ for the E_Sarbeco and RdRp assays.
439 The LoD was 3.99 GC and 5.52 GC per reaction for the E_Sarbeco and RdRp assays,
440 respectively. This corresponded to a method LoD of 2.73 x 10^3 GC/L for E_Sarbeco

441 and 3.79 x 10^3 GC/L for RdRp using the inuvai R180 system. As for the LoQ, the
442 results were 66 GC and 178 GC per reaction for the E_Sarbeco and RdRp assays,
443 respectively. This corresponded to a method LoQ of 4.56 x 10^4 GC/L for E_Sarbeco
444 and 1.22 x 10^5 GC/L for RdRp assay.
445 The LoD obtained in our study were inferior to those obtained by Philo *et al*. (2021).
446 Pérez-Cataluña *et al*. (2021) reported similar LoD for E_Sarbeco assay, while also
447 presenting method-dependence LoD. Gonzalez *et al*. (2020), testing the CDC assay
448 (N1, N2, and N3), reported different theoretical limits of detection depending on the
449 RT-qPCR assay used but the LoD were similar to those obtained in our study. A
450 comparison between the performance of our method (evaluated through LoD and
451 LoQ) and the method reported by McMinn *et al*. (2021) would have been useful, given
452 that the authors have also used hollow-fiber ultrafilters for primary concentration, but
453 such parameter information is missing on the former report. In fact, information on LoQ
454 is missing from most publications with very few exceptions, such as LaTurner *et al*.
455 (2021) who, while testing five distinct concentration methods, reported LoQ ranging
456 from 2.76 x 10^5 to 8.39 x 10^6 GC/L. Philo *et al*. (2021) calculated their LoQ in nuclease-
457 free water to be 100 gene copies per reaction for all CDC assays.
458
459 4. Conclusions
460 • The newly developed inuvai R180 ultrafilter, combined with a new elution
461 method and PEG precipitation allowed for a higher percentage of recovery of
462 PEDV, chosen as a surrogate for SARS-CoV-2.
463 • The inuvai R180 system performed at a high level for the detection of PEDV
464 even in more difficult wastewater matrices, including those with a high input of
465 industrial effluents

466 - The RT-qPCR targets have different sensitivity suggesting the choice of
467 molecular targets for detection is crucial
468 - Uniformization of the adequate description of methods performance is
469 necessary.

470

471 **Declaration of Competing Interest**

472 The authors declare that they have no known competing financial interests or personal relationships
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474

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489

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