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1 # Concentration of SARS-CoV-2 from large volumes of raw wastewater is
2 enhanced 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: first, as an early-warning system for the spread of the virus in the
26 community, second, to find hotspots of infection, and third, to aid in the early detection
27 and follow-up of circulating virus variants.

28 Although detection of SARS-CoV-2 in raw wastewater may be prompted with good
29 recoveries during periods of high community prevalence, in the early stages of
30 population outbreaks concentration procedures are required to overcome low viral
31 concentrations. Several methods have become available for the recovery of SARS-
32 CoV-2 from raw wastewater, generally involving filtration. However, these methods
33 are limited to small sample volumes, possibly missing the early stages of virus
34 circulation, and restrained applicability across different water matrices. The aim of this
35 study was thus to evaluate the performance of three methods enabling the
36 concentration of SARS-CoV-2 from large volumes of wastewater: i) hollow fiber
37 filtration using the inuvai R180, with an enhanced elution protocol and polyethylene
38 glycol (PEG) precipitation; ii) PEG precipitation; and iii) skimmed milk flocculation. The
39 performance of the three approaches was evaluated in wastewater from multiple
40 wastewater treatment plants (WWTP) with distinct singularities, according to: i)
41 effective volume; ii) percentage of recovery; iii) extraction efficiency; iv) inhibitory
42 effect; and v) the limits of detection and quantification (The inuvai R180 system had
43 the best performance, with detection of spiked controls across all samples, average
44 recovery percentages of 64% for SARS-CoV-2 control and 68% for porcine epidemic
45 diarrhea virus (PEDV), with low variability.

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46 The inuvai R180 enables the scalability of volumes without negative impact on the
47 costs, time for analysis, and recovery/inhibition. Moreover, hollow fiber filters favor the
48 concentration of different microbial taxonomic groups. Such combined features make
49 this technology attractive for usage in environmental waters monitoring.

50

51 **Keywords:** SARS-CoV-2; methods performance and evaluation; wastewater;
52 wastewater-based epidemiology

53

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54 ## 1. Introduction

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

72 Existing methods for the recovery of viruses were primarily developed for the detection
73 of nonenveloped viruses. Knowledge gaps concerning the recovery efficiencies of
74 enveloped viruses, such as SARS-CoV-2, remain. A study by Haramoto *et al*. (2009)
75 showed recovery efficiencies to be largely different for both types of viruses, with
76 methods performing better for the recovery of nonenveloped viruses. Blanco *et al*.
77 (2019) determined similar recovery efficiencies using precipitation with 20%
78 polyethylene glycol (PEG) following glass wool concentration for enveloped

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79 (Transmissible gastroenteritis virus (TGEV)) and nonenveloped viruses (Hepatitis A
80 virus (HAV)). A recent study by Ahmed *et al*. (2020) showed recovery efficiencies
81 varying between 26.7 and 65.7% for murine hepatitis virus (MHV) in raw wastewater
82 with very disparate recovery rates, even for similar methods, for this SARS-CoV-2
83 surrogate. Data using porcine epidemic diarrhea virus (PEDV) and aluminum
84 flocculation-based concentration demonstrated recovery efficiencies of 11 and 3% for
85 raw and treated wastewater, respectively (Randazzo *et al*., 2020).

86 Despite scarce information on diagnostic performance, SARS-CoV-2 RNA has been
87 detected globally in raw wastewater with different approaches. Reported methods
88 included ultrafiltration (Bertrand *et al*., 2021; Medema *et al*., 2020), ultracentrifugation
89 (Wurtzer *et al*. 2020), PEG precipitation (Chavarria-Miró *et al*., 2020; La Rosa *et al*.,
90 2020), aluminum flocculation (Randazzo *et al*., 2020), skimmed milk flocculation (Philo
91 *et al*., 2021), and filtration through an electronegative membrane (Gonzalez *et al*.,
92 2020; Haramoto *et al*., 2020).

93 In the present study, we evaluated the efficiency of SARS-CoV-2 recovery from raw
94 wastewater using three concentration methods: i) a newly developed hollow-fiber filter,
95 inuvai R180 (inuvai, a division of Fresenius Medical Care), with an improved elution
96 protocol; ii) PEG precipitation; and iii) skimmed milk flocculation. The inuvai R180 filter
97 has a large membrane area (1.8 m^2) and a fiber inner diameter of 220 μm, allowing
98 for the concentration of large volumes of water, including wastewater, without
99 problems such as clogging or compromising of the membrane structure. The
100 performance of the three methods was compared in aged raw wastewater according
101 to several characteristics, including: i) effective volume tested; ii) frequency and
102 consistency of detection; iii) percentage of recovery; iv) extraction efficiency; v)
103 inhibitory effect on reverse transcription-qPCR (RT-qPCR); and vi) concentration

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104 information (including, Limit of Detection (LoD) and Limit of Quantification (LoQ)). This
105 study benchmarks new and old methodologies for the detection of SARS-CoV-2 from
106 raw wastewater for WBE applications.
107

108 ## 2. Materials and Methods

109 ### *2.1. SARS-CoV-2 control*

110 SARS-CoV-2 control (nCoV-ALL-Control plasmid, Eurofins Genomics, Germany) was
111 seeded into raw wastewater samples collected from five different WWTP in Portugal
112 (as described below), following quantification by reverse transcription digital PCR (RT-
113 dPCR) using two assays from the Charité protocol (Corman *et al*., 2020): E_Sarbecco
114 and RdRp assays (Supplementary Table S1). Following absolute quantification (as
115 described below), a stock solution with the concentration of 2.27 x 10^4 genome copies
116 per liter (GC/L) final concentration of wastewater (as measured for the E_Sarbecco
117 assay) was prepared in DNase/RNase free water. The same stock was used for all
118 experiments described below.
119

120 ### 2.2. Porcine Epidemic Diarrhea Virus (PEDV) strain and cell lines

121 Porcine Epidemic Diarrhea Virus (PEDV) strain CV777 (kindly provided by Dr. Gloria
122 Sanchez, IATA-CSIC) is an enveloped virus from the genus *Alphacoronavirus* and
123 member of the *Coronaviridae* family, responsible for the porcine epidemic diarrhea.
124 PEDV was propagated in Vero cell line (ATCC CCL-81, LGC Standards). Briefly, Vero
125 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco),
126 supplemented with 100 units/mL of penicillin (Lonza), 100 units/mL of streptomycin
127 (Lonza), and 10% heat-inactivated fetal bovine serum (Biological Industries). Cells
128 were cultured in T175 flasks at 37 (± 1) ºC under 5 % CO_2. For infection with PEDV,

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129 cells were grown in T25 flasks and inoculated with 100 µL of viral stock. At 2h post
130 infection, DMEM supplemented with 0.3% tryptose phosphate broth, 100 units/mL of
131 penicillin (Lonza), 100 units/mL of streptomycin (Lonza), and 10 µg/µL trypsin, was
132 added to the flasks. Flasks were then incubated at 37 (± 1) ºC in 5% CO_2 for 4 days.
133 PEDV were recovered following three cycles of freeze/thawing and centrifugation at
134 1,100 xg for 10 min. Quantification was performed by RT-dPCR as described on
135 section 2.5 using the primers and probes from Supplementary Table S1 (Zhou *et al.*,
136 2017), following nucleic acid extraction as described on section 2.4. After absolute
137 quantification by RT-dPCR (as described below), a stock solution was prepared in
138 DNase/RNase free water to obtain a PEDV final concentration of 1.21 x 10^4 GC/L in
139 wastewater. The same stock was used in all experiments described below.
140

141 *2.3. Wastewater sample preparation*

142 Twenty-four-hour composite samples were collected, on two separate rounds, from
143 five wastewater treatment plants (WWTP) in Portugal (Serzedelo, Gaia, Alcântara,
144 Beirolas and Guia). The first round comprised samples collected between April 27 and
145 May 8, 2020 (n = 8; n = 2 for Serzedelo, Gaia and Beirolas; n = 1 for Alcântara and
146 Guia) and the second round comprised samples collected between July 6-10, 2020 (n
147 = 8; n = 2 for Serzedelo, Gaia and Guia; n = 1 for Alcântara and Beirolas). In each
148 round, the samples were transported to the laboratory, refrigerated and within eight
149 hours of collection. Samples collected in April-May were seeded with SARS-CoV-2
150 control whereas samples collected in July were seeded with PEDV. Raw wastewater
151 samples were kept at 37 (± 1) ºC for seven days to ensure that the levels of SARS-
152 CoV-2 RNA, if and where existing, decreased substantially prior to analysis. SARS-

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153 CoV-2 control and PEDV were seeded at concentrations of 2.27 x 10^4 GC/L and 1.21
154 x 10^4 GC/L, respectively (quantified as described previously).
155 Seeded raw wastewater samples were aliquoted and concentrated using three
156 methods: (i) hollow fiber with the newly developed inuvai R180 filters (inuvai, a division
157 of Fresenius Medical Care, Germany) followed by PEG precipitation (method 1); (ii)
158 direct PEG precipitation (method 2); and (iii) skimmed-milk flocculation (method 3). All
159 methods were tested using the same initial volume of wastewater (1-L) for a more
160 accurate comparison.
161 Method 1 employed the use of hollow fiber filters: 1-L of raw wastewater was filtered
162 through inuvai R180 filters using a peristaltic pump with a flow rate of 250 mL/min. The
163 elution was performed in three steps: (i) air forward push using 60 mL of air; (ii)
164 backflush with 250 mL of elution buffer (1× PBS with 0.01% NaPP and 0.01% Tween
165 80/0.001% antifoam) at a flow rate of 140-280 mL/min; and (iii) forward flush using 50
166 mL of elution buffer. The final elution volume was 300 mL. Samples were further
167 concentrated by precipitation with 20% (w/v) PEG 8000 overnight (Blanco *et al*., 2019).
168 Samples were centrifuged at 10,000 ×g for 30 min, the supernatant discarded, and the
169 pellet resuspended in 5 mL 1× PBS, pH 7.4.
170 Method 2 used PEG precipitation: 20% PEG 8000 was added directly to 1-L of raw
171 wastewater, with overnight precipitation followed by centrifugation as described above
172 for method 1. Method 3 employed skimmed milk flocculation, performed in accordance
173 with Calgua *et al*. (2008). Briefly, a pre-flocculated solution of 1% (w/v) skimmed milk
174 pH 3.5 was prepared in artificial seawater. The solution of skimmed milk was then
175 added to a final concentration of 0.01% (w/v) to 1-L of previously acidified raw
176 wastewater (pH 3.5). Samples were stirred for 8h at room temperature and flocs were
177 allowed to sediment for another 8h. Supernatant was carefully removed without

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178 disturbing the sediment. The final volume (approximately 500 mL) was centrifuged at
179 7,000 ×g for 30 min at 12 ºC. The supernatant was carefully discarded, and the pellet
180 resuspended in 0.2 M phosphate buffer at pH 7.5 to a final volume of 5 mL. All
181 concentrates were stored at – 80 (± 10) ºC until further analysis.
182

183 *2.4. Nucleic acid extraction*

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

193 $$Extraction\ efficiency\ (\%) = \frac{Total\ MNV\ copies\ recovered}{Total\ MNV\ copies\ seeded} \times 100 \text{ (Eq. 1).}$$

194

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

197 *2.5. Absolute quantification by RT-dPCR*

198 RT-dPCR was used to determine the exact concentration of SARS-CoV-2 and PEDV
199 spiked controls. Controls were amplified using the AgPath-ID One-Step RT-PCR kit
200 (Thermo Fischer Scientific) with the set of primers and probe described on
201 Supplementary Table S1 (PEDV; E_Sarbecco and RdRP assays). The 15 μL reaction
202 mixture consisted of 7.5 μL of 2× RT-PCR buffer, 0.6 μL of 25× RT-PCR enzyme mix,

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203 800 nM of each primer, 200 nM of probe, 3.63 μL RNase/DNase-free water, and 3 μL
204 of DNA (diluted 4-, 5-, 6- fold). The reaction mixture was then spread over the
205 QuantStudio 3D Digital PCR chip (Thermo Fischer Scientific) and the chips transferred
206 to the QuantStudio 3D Digital PCR thermal cycler. Amplification was performed as
207 follows: i) SARS-CoV-2: 10 min at 45 ºC, 10 min at 96 ºC, 39 cycles of 2 min at 58 ºC
208 and 30 s at 98 ºC, and final elongation step for 2 min at 58 ºC; ii) PEDV: 10 min at 45
209 ºC, 10 min at 96 ºC, 39 cycles of 2 min at 60 ºC and 30 s at 98 ºC, and a final elongation
210 step for 2 min at 60 ºC. Reactions were performed in duplicate, and a non-template
211 control (NTC) was included in each run.
212
213 *2.6. Relative quantification of seeded material in wastewater*
214 Relative quantification of SARS-CoV-2 control, PEDV and MNV-1 was carried out by
215 RT-qPCR on all extracts using the AgPath-ID One-Step RT-PCR kit (Thermo Fischer
216 Scientific). The final volume of 25 μL was composed of 12.5 μL of 2× RT-PCR buffer,
217 1 μL of 25× RT-PCR enzyme mixture, 800 nM of each primer, 200 nM of the probe,
218 6.05 μL RNase/DNase-free water, and 5 μL of RNA. All RT-qPCR reactions were run
219 on undiluted, 4- and 10-fold diluted extracts. RT-qPCR conditions were as follows: i)
220 SARS-CoV-2 control: 10 min at 45 ºC, 10 min at 95 ºC, 45 cycles of 15 s at 95 ºC and
221 1 min at 58 ºC; ii) PEDV and MNV-1: 10 min at 45 ºC, 10 min at 95 ºC, 40 cycles of 15
222 s at 95 ºC and 1 min at 60 ºC. Standard curves, run with each PCR, for SARS-CoV-2
223 control (E_Sarbecco and RdRp assays), PEDV and MNV-1 were prepared in serial
224 10-fold dilutions in RNase/DNase-free water. Positive and NTC controls were also
225 added to each PCR assay. Limits of detection (LoD) and quantification (LoQ) were
226 determined in RNase/DNase-free water. The LoD was considered the lowest
227 concentration of target that could be consistently detected (in more than 95%

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228 replicates tested) (Burd *et al*., 2010) and LoQ, the lowest concentration at which the
229 performance of the method is acceptable, with a coefficient of variation below 35%
230 (Klymus *et al*., 2020).

231
232 *2.7. Recovery efficiency*

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

235

236 $$Recovery\ efficiency\ (\%) = \frac{Total\ nucleic\ acid\ copies\ recovered}{Total\ nucleic\ acid\ copies\ seeded} \times 100 \quad \text{(Eq. 2)}$$

237

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

239

240 *2.8. Quality control*

241 To minimize nucleic acid carry-over and cross-contamination, sampling concentration,
242 extraction procedures and RT-qPCR/RT-dPCR were performed in separate rooms of
243 the laboratory. A process blank and extraction blank were included for each
244 concentration method and each nucleic acid extraction, respectively. As described
245 above, and before spiking, all wastewater samples were aged to decay potentially
246 present SARS-CoV-2 RNA; following aging, all spiked samples were tested in parallel
247 with the corresponding unseeded samples to rule out or estimate the contribution of
248 potentially native SARS-CoV-2 and PEDV.

249

250 *2.9. Data analyses*

251 All data analyses were performed with SPSS Statistics 26 (IBM). Repeated
252 measurement ANOVA was conducted to compare the differences between the

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253 parameters estimated for the three methods. In all cases, p-values < 0.05 were
254 considered statistically significant.
255
256 ## 3. Results and discussion
257 ### *3.1. Quantification of controls*
258 Appropriate quantification of the controls used in spiking experiments and in standard
259 curve for RT-qPCR is extremely important, as it will influence downstream data
260 interpretation. That is why we opted for RT-dPCR, with high precision and sensitivity,
261 for the absolute quantification of controls. Digital PCR works by partitioning a unique
262 sample into thousands of individual reactions running in parallel, being particularly
263 useful for low-abundance targets or targets in complex matrices. Through Poisson
264 statistics, the total number of target molecules is calculated, with no need for external
265 reference standards (Monteiro and Santos, 2017). Several dilutions of SARS-CoV-2
266 control and PEDV, in duplicate, were quantified by RT-dPCR. The concentrations of
267 the initial stocks for SARS-CoV-2 control were 1.94×10^8 GC/µL and 1.00×10^8 GC/µL
268 for E_Sarbecco and RdRp assays, respectively. Concentration of PEDV as
269 determined by RT-dPCR was 1.20×10^8 GC/µL.
270
271 ### *3.2. Method comparison using SARS-CoV-2 and PEDV as surrogates for SARS-*
272 ### *CoV-2*
273 All unseeded wastewater samples were negative for the presence of SARS-CoV-2
274 and PEDV. Samples were chosen in periods with low number of daily COVID-19 cases
275 (mean for entire country, 287 from April 27 to May 8, and 374, between July 6 and 10,
276 2020) (DGS, 2020). All process and extraction blanks were negative.

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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 filters (method 1) enabled the filtration of 2.5 – 5-L of raw wastewater.
283 Increasing the initial volume of sample with the inuvai R180 filters would conduct to an
284 increment of the effective volume assayed from 2.2 mL to 5.5 – 11 mL without further
285 increases in the concentration time, the concentrate volume, costs for analysis, and
286 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 SARS-CoV-2 control and PEDV were used to compare concentration recoveries. The
299 highest average percentage of recovery was obtained with the inuvai R180 system at
300 64% (± 6%) for SARS-CoV-2 control and 68% (± 7%) for PEDV, with global recoveries
301 varying between 50 and 82% (Fig. 1A).

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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 Recovery using SARS-CoV-2 control was similar for PEG and skimmed milk (4% (±
305 2%)). There were statistically significant differences in the lower recovery percentage
306 of PEG and skimmed milk compared to inuvai R180 ($F(1, 3) = 14.94$, $p = 0.03$ for
307 PEDV and $F(1, 3) = 171.7$, $p = 0.006$ for SARS-CoV-2 control).
308
309 The inuvai R180 was the single method that consistently led to nucleic acid detection
310 in all samples. Concentration using PEG and skimmed milk led to the detection of
311 PEDV in 50% of the samples, while detection of SARS-CoV-2 control was attained in
312 38% and 63% of the samples, respectively.
313 The method using the inuvai R180 system led to detection by RT-qPCR of the highest
314 mean concentration of genome copies, for both targets: 8.98 and 4.25 GC/reaction for
315 SARS-CoV-2 and PEDV, respectively. Concentration with PEG (1.19 and 0.21
316 GC/reaction for SARS-CoV-2 and PEDV, respectively) and skimmed milk (1.74
317 GC/reaction for SARS-CoV-2 and 0.28 GC/reaction for PEDV) showed similar results
318 (Fig. 1B).
319 Our recovery values using the inuvai R180 system were similar to those reported for
320 MHV, while enabling an increase in the filtration volume (Ahmed *et al*., 2020). For PEG
321 precipitation and skimmed milk flocculation the recoveries were slightly higher than
322 those reported by Philo *et al*. (2021). The authors used a concentration of 14% (w/v)
323 of PEG compared to 20% (w/v) PEG in our study. The use of higher concentrations of
324 PEG, although implying increased costs, has been shown to increase the recovery of
325 enveloped viruses from 31% to 51% (Blanco *et al*., 2019). In our study, recovery values
326 for PEG precipitation were higher than those reported by Pérez-Cataluña *et al*. (2021)

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327 when using similar nucleic acid extraction method (spin column). McMinn *et al*. (2021)
328 developed a method for the recovery of coronavirus from raw wastewater also using
329 hollow fibers as a primary concentration approach, followed by Concentrating Pipette
330 Select^{TM} (CP Select^{TM}), reporting overall recovery values for human coronavirus OC43
331 of 22%. Differences in recovery between our study and that of McMinn *et al*. (2021)
332 may be attributed to the filter that used in our study (inuvai R180 vs Rexeed), coupled
333 with an enhanced elution strategy with three steps that we adopted, and/or to the
334 secondary concentration protocol. The inuvai R180 filter has a reduced nominal pore
335 size (≤ 5.5 nm with a correspondent cut-off ≤ 18.8 Kda) compared to the Rexeed 15S,
336 which has a more open pore structure. Additionally, the filter used in our study has a
337 larger membrane area (1.8 m^2 for inuvai R180 vs 1.5 m^2 for Rexeed S15) and larger
338 fiber inner diameters (220 µm for inuvai R180 vs 185 µm for Rexeed S15). In addition
339 to the optimized elution and secondary concentration protocols, such features might
340 help justify the differences registered in the recovery efficiencies of our study and
341 McMinn *et al*. (2021).

342 The extraction efficiency using MNV as proxy averaged 70% (±19%) for inuvai R180
343 protocol. Extraction efficiencies for PEG precipitation and skimmed milk flocculation
344 averaged 50% (±15%) and 36 (±13%), respectively.

345 Detection of SARS-CoV-2 control and PEDV using the inuvai R180 system was
346 consistently achieved with the 1/4-fold dilution, while for undiluted spiked samples,
347 only 38% could be detected without inhibition. PEG precipitation was the single
348 method that detected both targets from undiluted samples, although inhibition still
349 occurred (as evidenced subsequently by testing the 4- and 10-fold dilution). As for the
350 skimmed milk concentration method, detection in undiluted concentrates was found
351 for 75% of the samples, although inhibition still occurred (as measured by the

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352 dilutions). These results indicate that inhibitory effects exerted upon RT-qPCR could
353 be confirmed for the three methods under comparison.
354 Overall, our results showed that the inuvai R180 system coupled with an improved
355 elution protocol is highly suitable for the detection of SARS-CoV-2 and PEDV,
356 exhibiting the highest percentage of detection and mean recovery value. Additionally,
357 this method also showed greater extraction efficiency and larger volume processing
358 without increased cost or time for downstream analyses. Furthermore, the
359 performance of the inuvai system showed consistency across raw wastewater
360 samples from different catchments / WWTP, including the Serzedelo WWTP, which is
361 highly impacted by industrial effluents (tannery industry) and therefore an extremely
362 complicated matrix to work with altogether, a result corroborated by the Pan-European
363 Umbrella study (Gawik *et al.*, 2021). In the Umbrella study, raw wastewater samples
364 from different European countries were collected and sent for analysis in a centralized
365 laboratory. In parallel, the same samples were also analyzed in each country for
366 comparison of results. The centralized European laboratory was unable to recover
367 SARS-CoV-2 RNA from Serzedelo raw wastewater presenting low recovery
368 percentages (0.1%) and lower concentrations of crAssphage compared to the other
369 samples analyzed. The same sample, analyzed by our group and using the inuvai
370 R180 system, was positive for SARS-CoV-2 and the concentration of crAssphage was
371 3-log above that detected by the centralized laboratory. These results demonstrate the
372 difficulty of working with this raw wastewater, highlighting the need to test method
373 performance in raw wastewater from different origins.
374
375 ### 3.3. *RT-qPCR efficiency*

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376 After establishing the inuvai R180 system as gold-standard for primary concentration,
377 the efficiency of the relative quantification method (RT-qPCR) was assessed by
378 calculating the LoD and LoQ for the E_Sarbecco and RdRp assays using SARS-CoV-
379 2 control. Fig. 2 displays the subset of points from the standard curve to determine the
380 LoD and LoQ.
381 The LoD was 3.99 GC and 5.52 GC per reaction for the E_Sarbecco and RdRp
382 assays, respectively. This corresponded to a method LoD of 2.73 x 10^3 GC/L for
383 E_Sarbecco and 3.79 x 10^3 GC/L for RdRp using the inuvai R180 system.
384 As for the LoQ, the results were 66 GC and 178 GC per reaction for the E_Sarbecco
385 and RdRp assays, respectively. This corresponded to a method LoQ of 4.56 x 10^4
386 GC/L for E_Sarbecco and 1.22 x 10^5 GC/L for RdRp assay.
387 The LoD obtained in our study were inferior to those obtained by Philo *et al*. (2021).
388 Pérez-Cataluña *et al*. (2021) reported similar LoD for E_Sarbecco assay, while also
389 presenting method-dependence LoD. Gonzalez *et al*. (2020), testing the CDC assay
390 (N1, N2, and N3), reported different theoretical limits of detection depending on the
391 RT-qPCR assay used but the LoD were similar to those obtained in our study. A
392 comparison between the performance of our method (evaluated through LoD and
393 LoQ) and the method reported by McMinn *et al*. (2021) would have been useful, given
394 that the authors have also used hollow-fiber filters for primary concentration, but such
395 parameter information is missing on the former report. In fact, information on LoQ is
396 missing from most publications with very few exceptions, such as LaTurner *et al*.
397 (2021) who, while testing five distinct concentration methods, reported LoQ ranging
398 from 2.76 x 10^5 to 8.39 x 10^6 GC/L. Philo *et al*. (2021) calculated their LoQ in nuclease-
399 free water to be 100 gene copies per reaction for all CDC assays.
400

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401 ## 4. Conclusions

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. In this study, the inuvai R180 system with improved three-step
409 elution protocol was selected for monitoring SARS-CoV-2 in raw wastewaters. Such
410 system is attractive as it enables the concentration of large volumes of raw
411 wastewater, while also being useful to concentrate larger volumes of samples from
412 other origins, such as treated wastewater, environmental waters and drinking water.
413 This feature enables handling a single concentration method across different water
414 types without sensitivity loss, increasing costs or time for analysis, while also allowing
415 a less challenging result comparison.

416 For an effective environmental surveillance to be put in place, not only for SARS-CoV-
417 2 but also for potential future pandemics involving enveloped virus, it is paramount to
418 have validated methods. Nonetheless, comparisons between published methods are
419 difficult as they differ in many aspects including: i) seeding controls; ii) concentration
420 methods; iii) extraction methods; iv) diagnostic and quantification molecular assays
421 and genome targets; v) and mostly, the accepted performance levels. Some
422 publications only mention the recovery efficiency (Ahmed *et al*., 2020; McMinn *et al*.,
423 2021), others mention the recovery efficiency and the LoD but not LoQ (Gonzalez *et*
424 *al*., 2020; Randazzo *et al*., 2020; Pérez-Cataluña *et al*., 2021), some mention LoQ but
425 not LOD (LaTurner *et al*., 2021), while other studies show all data performance,

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426 including LoD, LoQ and recovery percentages (Philo *et al.*, 2021). Additionally,
427 different studies calculate the LoD and LoQ differently. The information collected from
428 different studies should inform laboratories on method performance. A 'one size fits
429 all' approach, that is having a single standardized method worldwide for the
430 concentration of SARS-CoV-2, may not be the best approach. This was demonstrated
431 with the Umbrella study (Gawik *et al.*, 2021), due to several issues, including: (i)
432 laboratories already have their own preferred methods with performances studied; (ii)
433 the methods may not be useful for application in less economically developed
434 countries; (iii) or simply because it is difficult to get a hold of laboratory
435 materials/equipment (as it was the case of ultrafiltration filters or ultracentrifuges).
436 Nonetheless, standards as to what should be asked in terms of method performance
437 should be established so that laboratories could gather all the information about the
438 methods to make a more informed choice. Wastewater surveillance has the potential
439 to prevent the occurrence of new outbreaks (Peiser, 2020), and to help understand
440 changes in the pandemic trends. Effective methods, with performance specifications
441 detailed, are paramount for wastewater surveillance to be applied in accurately
442 describing the transmission of SARS-CoV-2 in the community. This study expands the
443 knowledge on analytical methods introducing a method with robust performance for
444 SARS-CoV-2 detection in wastewater and establishing a step forward for the global
445 application of WBE not only for this pandemic but also in future health crisis as the
446 established protocol is modular for different taxonomic groups.
447

448 **CrediT authorship contribution statement**

449 **Sílvia Monteiro**: conceptualization, methodology, software, validation, formal analysis, investigation,
450 writing – original draft, writing – review and editing, visualization. **Daniela Rente**: investigation. **Mónica**

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457 **Declaration of Competing Interest**

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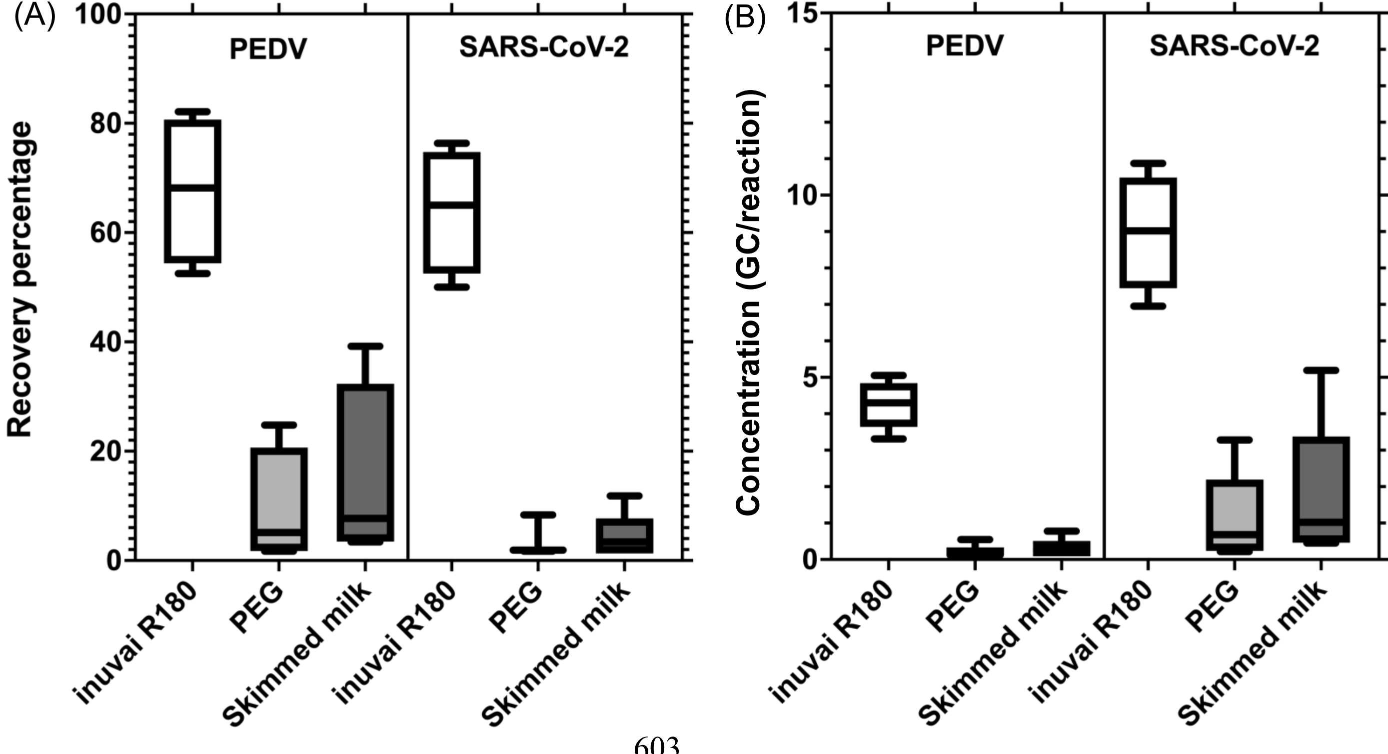

603

604 **Fig. 1.** Performance of concentration methods for the detection of PEDV and SARS-CoV-2 control from raw
605 wastewater. Percentage of recovery obtained in each method (A). log transformed concentration of viral genome
606 copies detected by RT-qPCR in each method (B). The inuvai R180 system presented the highest average
607 percentage of recovery and concentration, followed by PEG precipitation and skimmed milk flocculation.

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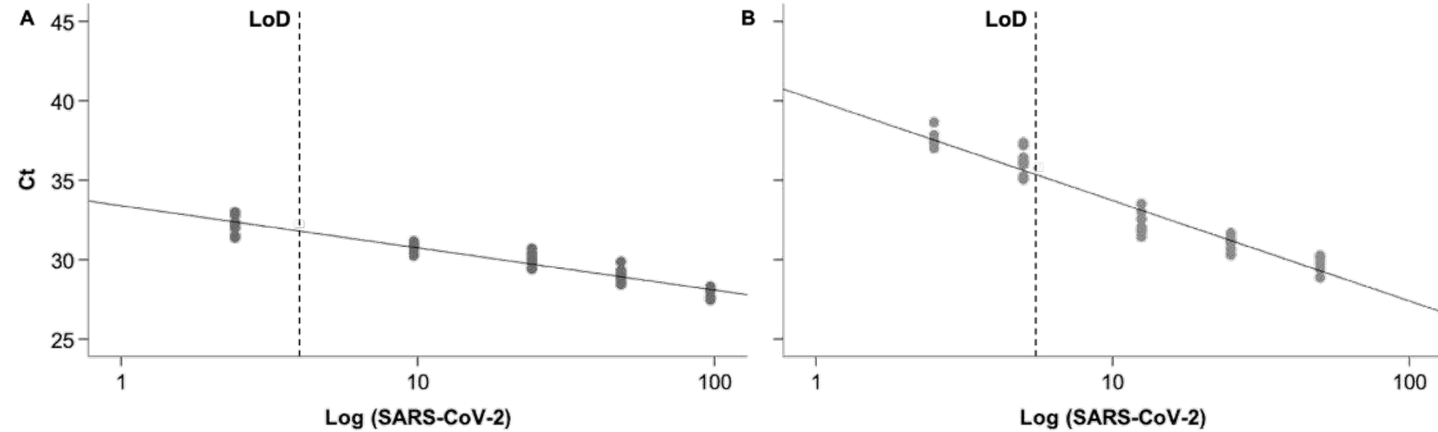

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611 **Fig. 2.** LoD for SARS-CoV-2 relative quantification assays. Subset of standard curve points used to determine the
612 smallest concentration of SARS-CoV-2 detected by E_Sarbecco assay at a 95% confidence level (A). Curve to
613 determine the smallest concentration of SARS-CoV-2 detected by RdRp assay at a 95% confidence level (B).