Interlaboratory comparative study to detect potentially infectious human enteric viruses in influent and effluent waters

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Abstract

Wastewater represents the main reusable water source after being adequately sanitized by wastewater treatment plants (WWTP). In this sense, only bacterial quality indicators are usually checked to this end, and human pathogenic viruses usually escape from both sanitization procedures and controls, posing a health risk on the use of effluent waters. In this study, we evaluated a protocol based on aluminium adsorption-precipitation to concentrate several human enteric viruses, including norovirus genogroup I (NoV GI), NoV GII, hepatitis A virus (HAV), astrovirus (HAstV), and rotavirus (RV), with limits of detection of 4.08, 4.64, 5.46 log genomic copies/L, 3.31, and 5.41 log PCR units (PCRU)/L, respectively. Furthermore, the method was applied in two independent laboratories to monitor the presence of NoV GI, NoV GII, and HAV in effluent and influent waters collected from five WWTPs at two different sampling dates. Concomitantly, a viability PMAxx-RT-qPCR was applied to all the samples to get information on the potential infectivity of both influent and effluent waters. The range of the titers in influent waters for NoV GI, NoV GII, RV and HAstV was 4.80-7.56, 5.19-7.31 log genomic copies/L, 5.41-6.52, and 4.59-7.33 log PCRU/L, respectively. In effluent waters, the titers ranged between 4.08-6.27, 4.64-6.08 log genomic copies/L, <5.51, and 3.31-5.58 log PCRU/L. Moreover, the viral titers detected by viability RT-qPCR showed statistical differences with RT-qPCR alone, suggesting the potential viral infectivity of the samples despite some observed reductions. The proposed method could be applied in ill-equipped laboratories, due to the lack of a requirement for a specific apparatus (i.e., ultracentrifuge).

Keywords: Foodborne viruses, viability RT-qPCR, sewage, effluent water.

Introduction

Water represents the primary production source for almost all agricultural and industrial processes. Given the limitation of water resources and the global climate changes, many concerns have been raised in the last decades to reduce its use and develop strategies to reuse it. In this sense, wastewater has been pointed out as the main reusable water source, and wastewater treatment plants (WWTPs) are the most common systems used for wastewater management worldwide, where influent waters are treated and effluent waters are intended to be reused (Becerra-Castro et al. 2015; Haramoto et al. 2018). Thereby, wastewater discharge to the environment or its reuse after sanitization procedures poses a great issue, given the increasing public health risk of human infections and illnesses associated with viral contamination of drinking water, coastal waters (i.e., shellfish), irrigation waters (i.e., vegetables and berries) and recreational activities (e.g., swimming). In fact, human enteric viruses are at high concentrations in faeces excreted by infected people and, hence, can be present in untreated and treated wastewater (Sano et al. 2016).

While physical and chemical parameters, together with microbiological indicators (i.e., fecal indicator bacteria (FIB) and *Escherichia coli* counts) have been widely used to assess water quality and its foreseeable use, the presence of human enteric viruses has not been routinely considered to this purpose by legal authorities. The presence of human enteric viruses in effluent waters has been well documented (Sano et al. 2016; Gerba, et al. 2018), posing a public health risk-related concern and questioning the efficiency of WWTP in virus removal (National Research Council, 2012; Verbyla and Mihelcic, 2015). Among others, the viruses most commonly detected in influent and effluent waters include adenovirus (AdV), enterovirus (EV), hepatitis A and E viruses (HAV and HEV), norovirus (NoV), sapovirus (SaV), astrovirus (HAstV), and rotavirus A (RV) (Ashbolt 2015). To our best knowledge, only the USA included caliciviruses, a

73 virus family that comprises the genus NoV and SaV, in the list of water contaminants
74 that need to be regulated (EPA 2016).
75 Despite the agreement between the scientific community and governments on
76 controlling the viral population in various types of waters, a state of uncertainty lays on
77 the analytical methods for quantification and, thus, on reduction levels required to
78 ensure minimal risk to the exposed population (WHO 2017; Gerba et al. 2018). It is
79 evident that methodologies to concentrate and quantify human enteric viruses in
80 environmental waters need to be improved to finally design suitable water reclamation
81 systems. Cell-culture methods have been used for a long time to detect infectious
82 enteric viruses in water samples. However, these methods are impaired by the low
83 levels of environmental contamination, the availability of a single cell-culture system
84 for each targeted virus, and by the absence of reliable cell-culture assays for some
85 viruses (Hamza et al. 2011; Condit 2013; Gerba et al. 2018). Alternatively, molecular
86 detection methods have emerged as rapid, sensitive, and reliable tools for enteric virus
87 detection and quantification in water samples (Katayama et al. 2008; Simmons and
88 Xagoraraki 2011; Farkas et al. 2018). However, despite the huge progress in viral
89 detection due to the development of molecular assays based on real-time polymerase
90 chain reaction (qPCR), water concentration procedures are cumbersome, and most of
91 them require special equipment (Nordgren et al. 2009). Additionally, molecular-based
92 methods cannot discriminate between inactivated and potentially infectious viruses.
93 Thus, the use of viability markers has been incorporated into qPCR-based methods for
94 assessing infectivity of enteric viruses in several types of water samples in the last years
95 (Parshionikar et al. 2010; Kim et al. 2011; Coudray-Meunier et al. 2013; Prevost et al.
96 2016; Randazzo et al. 2016; López-Gálvez et al. 2018; Randazzo et al. 2018a,b).

To overcome these issues, in this study, a rapid and user-friendly protocol based on aluminium adsorption-precipitation previously approved by the Standard Methods Committee (2011) was evaluated to recover, detect and quantify NoV GI, NoV GII, and HAV in influent and effluent water. Moreover, the limits of detection were determined for the above mentioned viruses as well as for HAstV and RV in effluent waters. Ultimately, an interlaboratory study involving two laboratories was designed to validate the proposed method. To reduce the uncertainty and control the factors influencing virus estimation, the following variables were included: (i) two different sampling dates (November 2017 and May 2018); (ii) five WWTP facilities applying different reclaiming treatments and (iii) at least two sampling points (influent and effluent waters) for each WWTP. In addition, to obtain information on the potential infectivity of the samples, a viability treatment previously optimized for water samples (Randazzo et al. 2016; López-Gálvez et al. 2018; Randazzo et al. 2018a,b) was run in parallel for all the samples.

2. Materials and Methods

2.1. Viral stocks

Feces positive for NoV GI, NoV GII and HAstV (courtesy of Dr. Buesa from Hospital Clínico Universitario, University of Valencia, Spain) were resuspended (10%, wt/vol) in phosphate-buffered saline (PBS) containing 2 M $NaNO_3$ (Panreac), 1% beef extract (Conda), and 0.1% Triton X-100 (Fisher Scientific) (pH 7.2), vortexed and centrifuged at 1,000 × g for 5 min. The supernatant was stored at −80 °C in aliquots.

The cytopathogenic HM-175 strain of HAV (ATCC VR-140), the human RV strain Wa (ATCC VR-2018) and mengovirus (CECT 100000) were propagated in FRhK, MA-104 and HeLa cell monolayers, respectively. Semipurified stocks were thereafter produced

121 in the same cells by low-speed centrifugations of infected cell lysates (3,000 x g for 20
122 min).

123 **2.2. WWTPs and sample collection**

124 Influent and effluent waters were collected from five different municipal wastewater
125 treatment plants located in Valencia (Spain), a region with high population density
126 where agriculture and food processing are important economic activities (Table 1). For
127 each WWTP, grab samples were taken from the flow at the influent and effluent in
128 November 2017 and May 2018. Water samples were immediately placed on ice, sent to
129 laboratories and immediately processed or, alternatively, stored at −80°C.

130 **2.3. Comparison of virus concentration method in influent waters**

131 Initially, the performance of two different concentration methods was evaluated in five
132 influent water samples by an ultracentrifugation-based protocol (Method A) and by an
133 aluminium hydroxide adsorption-precipitation procedure (Method B). For method A, 35
134 mL of influent water were centrifuged at 140,000 g for 2 h 30 min at 4 °C. The pellet
135 was incubated on ice for 30 min with 5 ml of 0.25 mol/L glycine buffer (pH 9.5) and
136 then the solution neutralized with 5 mL of PBS 2X. Suspended solids were removed by
137 centrifugation at 12,000 g for 15 min, and, viruses from supernatant were recovered by
138 ultracentrifugation at 229,600 g for 1 h at 4°C and, finally eluted in 1 mL of PBS
139 (Rodríguez-Díaz et al. 2009).

140 For method B, 35 mL of influent water were adjusted to pH 6.0 and $Al(OH)_3$ precipitate
141 formed by adding 1 part 0.9N $AlCl_3$ solution to 100 parts of sample. The pH was
142 readjusted to 6.0 and sample mixed slowly for 15 min at room temperature. Then,
143 viruses were collected by centrifugation at 1,700 x *g* for 20 min. The pellet was
144 resuspended into 1.75 mL of 3% beef extract pH 7.4 and samples were shacked for 10
145 min at 150 rpm. The concentrate was recovered by centrifugation at 1,900 x *g* for 30

146 min and the pellet resuspended in 1 mL of PBS ("Standard Methods For the
147 Examination of Water and Wastewater," 2011). Moreover, to determine the efficacy of
148 the procedures, and thus validate the results, water samples were spiked with approx.
149 10^6 PCRU of MgV as process control as suggested by the ISO 15216-1:2017 (2017)
150 guidelines and Gerba et al. (2018). Experiments were performed in triplicate.

151 **2.4. Detection limit of enteric viruses in effluent waters**

152 Effluent water samples were concentrated by Method B and the limit of detection of
153 each viruses was determined. Experiments were performed in triplicate using 200 mL of
154 effluent water samples previously tested negative for the viruses under study. Water
155 samples were artificially inoculated with different concentrations of viral inocula
156 (approximately 6, 5, 4 and 3 log genomic copies/L) and concentrated according to
157 Method B described in Section 2.3. The limit of detection ($LoD_{95\%}$) was calculated for
158 each virus according to Wilrich and Wilrich (2009).

159 **2.5. Interlaboratory comparison study of influent and effluent WWTP waters**

160 An interlaboratory study involving two laboratories was designed to validate the
161 proposed method. Thus, Method B was applied by Lab1 and Lab2 to determine NoV GI
162 GII and HAV levels in influent and effluent water samples collected in November 2017
163 and May 2018 from five WWTPs. The two laboratories independently concentrated and
164 analyzed single samples and RT-qPCRs were run in duplicate. Moreover, to obtain
165 information on the potential infectivity of the samples a viability RT-qPCR was run in
166 parallel for all the samples by both laboratories. In addition, to obtain further
167 information on enteric virus population present in influent and effluent waters, Lab1
168 screened all influent and effluent waters for RV and HAstV by both RT-qPCR and
169 viability RT-qPCR. For viability RT-qPCR a previously optimized protocol was applied
170 (Randazzo et al. 2016; López-Gálvez et al. 2018; Randazzo et al. 2018b). In brief, the

171 photoactivatable dye PMAxx™ (Biotium) was added to concentrated samples at 50 µM
172 together with 7.7 mmol/L Triton 100-X (Fisher-Scientific) and incubated in the dark at
173 room temperature for 10 min at 150 rpm. Then, samples, in DNA LoBind 1.5 mL tubes
174 (Eppendorf), were exposed to photo-activation using a photo-activation system (Led-
175 Active Blue, GenIUL) for 15 min and RNA was extracted as described in Section 2.6.

176 **2.6. RNA extraction and RT-qPCR**

177 RNA from water sample concentrates was extracted using the NucleoSpin® RNA virus
178 kit (Macherey-Nagel GmbH & Co.) according to the manufacturer's instructions
179 including the Plant RNA Isolation Aid (Ambion) pretreatment as previously described
180 (Randazzo et al. 2016; Randazzo et al. 2018b). Primers, probes and reverse
181 transcription-qPCR (RT-qPCR) conditions used in this study are listed in Table S1.
182 RT-qPCRs were carried out in 96-well plates using the RNA UltraSense One-Step
183 quantitative RT-PCR system (Invitrogen SA) with a half-scale modification of the
184 manufacturer's protocol and the LightCycler 480 (Roche Diagnostics at Lab1)
185 or QuantStudio 5 (Applied Biosystems at Lab2) instruments. Each viral RNA was
186 analyzed at least in duplicate. Undiluted and ten-fold diluted RNA was tested to
187 determine inhibition of the RT-qPCRs. Different controls were used: positive and
188 negative extraction and RT-qPCR controls, and MgV as a whole process control spiked
189 prior to concentration, and detected in downstream RT-qPCR (Gerba et al. 2018).

190 **2.5. Virus quantification**

191 Standard curves were determined by the use of the Public Health England (PHE)
192 Reference Materials for Microbiology for NoV GI (batch number 0122-17), NoV GII
193 (batch number 0247-17) and HAV (batch number 0261-2017) while standard curves for
194 RV, MgV and HAstV were generated by amplifying 10-fold serial dilutions of viral
195 suspensions in quintuplicates and calculating the numbers of PCR units (PCRU).

196 **2.7. Statistical analysis**

197 To statistically compare the performance of the evaluated concentration methods, data
198 were subjected to the analysis of variance (ANOVA) to test the impact of the method
199 followed by the Tukey's HSD as post-hoc test to obtain homogenous groups (Table 2).
200 Moreover, to explore the effect of the five variables considered (WWTP, sampling date,
201 influent/ effluent water, use of PMAxx and laboratory facility), ANOVA was applied to
202 the data matrix of quantification values of each targeted virus. To this end, a
203 hypothetical value of half of the detection limit was assigned to viral loads under the
204 detection limit (SEPA, 2008). Furthermore, a principal component analysis (PCA)
205 explored the input matrix based on titers of NoV GI and GII replicates introduced as
206 cases together with the explanatory variables WWTP, type of water (influent or effluent
207 water) laboratory facility (Lab1 and Lab2) and viability marker pre-treatment. Raw data
208 were collected and preliminary analyzed in Excel spreadsheets (Microsoft), statistical
209 data processing was performed using STATISTICA software version 7 (StatSoft Inc.,
210 Tulsa, OK, USA) and graphic constructions executed on R (R Core Team, 2014) by
211 using Scatterplot3d Package (Ligges & Mächler, 2003). In all cases, values of $p < 0.05$
212 were deemed significant.

213 **3. Results and Discussion**

214 **3.1. Comparison of concentration methods for enteric viruses from influent waters**

215 Several protocols for the concentration, detection, and the quantification of human
216 enteric viruses from wastewater have been published (reviewed by Cashdollar and
217 Wymer 2013; Haramoto et al. 2018). The surveillance of water supplies for the presence
218 of enteric viruses requires procedures that have a high likelihood for adaptation to
219 different laboratory facilities around the world. Initially, an ultracentrifuge-based
220 methodology (Method A) (Rodríguez-Díaz et al. 2009), which requires equipment

221 which is not always available at the water analysis laboratories, and an $AlCl_3$-based
222 method (Method B) were compared by using naturally contaminated influent water
223 samples. The titers of NoV GI, NoV GII, HAV, RV, and HAstV from five influent
224 water samples using the two different methods are reported in Table 2, together with
225 MgV recoveries. Viral concentrations ranged between 4.10-4.81 and 4.41-5.66 for NoV
226 GI and between 4.64-5.30 and 4.13-4.94 for NoV GII log genomic copies/L for Method
227 A and B, respectively. HAV was detected in a total of 3 out of 5 samples at levels of
228 <5.46 log genomic copies/L applying Method B, while only two positive samples
229 resulted from Method A. RV titers ranged between 5.19-6.01 and 6.22-6.73 log
230 PCRU/L for Method A and B, respectively. Similarly, concentrations of 5.00-5.65 and
231 5.44-6.00 log PCRU/L were detected for HAstV by Method A and B, respectively.
232 Significant differences ($p<0.05$) were shown for NoV GI, NoV GII, HAV, RV, and
233 HAstV for the compared two methods. In particular, slightly higher NoV GI, RV, and
234 HAstV titers were reported for Method B than Method A. On the contrary, a sharp
235 pattern cannot be defined for NoV GII and HAV, since differences were detected in
236 samples with the lowest viral titers, close to the detection limits, as in the case of IW4
237 and IW5 for NoV GII and IW2 for HAV.
238 The recovery of spiked MgV was also determined, and greater efficiency was detected
239 for Method A (8.04-25.72%) compared to Method B (0.02-4.30%) for all samples
240 (n=5). Only one sample, IW4 concentrated with Method B, did not comply with the
241 recovery efficiency indicated in the ISO 15216-1:2017 to validate viral concentration in
242 bottled water (>1% of MgV recovery).
243 Despite the discordance on MgV recoveries, targeted viral quantifications were similar
244 when comparing the two methods. An explication of such difference between the
245 process control recoveries (MgV) and the similarity of targeted genome titers maybe

246 related with the pH changes in Method B that may interfere with MgV stability. These
247 pH changes may not affect the viral population already present in the water, being these
248 last aggregated among themselves and together with organic material finally resulting
249 protected by pH changes (Gerba et al. 2017). Nevertheless, Table 4 showed that MgV
250 recoveries analysed later reported higher recoveries, most likely due to be more familiar
251 with the procedure. In line with previously reported MgV recoveries in influent waters
252 (Miura et al., 2016).
253 Moreover, when considering concentration methods, several factors (such as the volume
254 of the sample, organic matters and chemicals) can affect RT-qPCR outcomes by
255 inhibiting RNA extractions and amplification steps (Ikner et al. 2011; Ikner et al. 2012;
256 Cashdollar and Wymer 2013; Borgmästars et al., 2017). Thus, ten-fold diluted RNAs
257 were also analyzed to check for inhibitors according to ISO 15216-1:2017, and the
258 results confirmed the absence of interfering substances (data not shown). This approach
259 excludes the possibility of false negative and/or sub-estimation due to complete or
260 partial inhibition of PCRs, confirming the reliability of obtained results, especially in
261 sensitive samples as influent and effluent waters.
262 To sum up, our results suggest that the aluminium-based procedure (Method B) is an
263 alternative concentration method when in lack of an ultracentrifuge or when greater
264 volumes of samples need to be processed, as the case of effluent waters. Taking these
265 reasons together, Method B was further used to determine the $LoD_{95\%}$ in effluent waters
266 and to compare two independent laboratories's outcomes in analyzing naturally
267 contaminated influent and effluent water samples.
268 **3.2. Detection limit of enteric viruses in effluent waters**
269 Low virus levels in effluent water samples are a major analytical challenge, thus the
270 $LoD_{95\%}$ were assessed. Method B resulted in $LoD_{95\%}$ of 4.08, 4.64, 5.46, 3.31, and 5.41

271 log genomic copies/L for NoV GI, NoV GII, HAV, HAstV and RV, respectively,
272 calculated according with Wilrich and Wilrich (2009). Similar limits of detection have
273 been previously reported for NoV GI and GII (around 4 log genome copies/L) applying
274 an ultracentrifugation-based concentration method (Nordgren et al. 2009), suggesting
275 the comparable efficacy of both methods.
276 Lower LoDs could have been reached by concentrating larger water volume, even
277 though thus may impair in coextraction of interfering substances that can affect RT-
278 qPCR outcomes. As example, Hill et al., (2010), concentrating 50 L ground water by
279 ultrafiltration coupled to PEG precipitation, achieved lower LoDs for GI (200-3,000
280 particles/L), but not for GII (1,000-10,000 particles/L), while D'Ugo et al. (2016) was
281 able to detect 4 log genomic copies/L NoV GII and HAV in 50 L river water samples. A
282 wider comparison with other available studies is restricted by the fact that only RT-
283 qPCR detection limits are usually reported, often excluding other molecular process
284 controls (i.e., extraction control) and/or a whole process control (Haramoto et al. 2018).
285 Improvements on the LoD can be also achieved by increasing the volume of concentrate
286 extracted or the volume of the master mix reaction, although the latter will increase the
287 price of the analysis.
288 Viral recoveries of 13.13, 7.52, 0.65, 71.40, and 0.97% were detected for NoV GI, NoV
289 GII, HAV, HAstV, and RV, respectively, by using the aluminium-based method. Given
290 an acceptable level of viral recovery for the whole process of 1% (Haramoto et al.
291 2018), HAV and RV were not recovered efficiently (Table 3). This may be due to the
292 heterogeneity of the RV and HAV suspensions given an estimation that 1 $TCID_{50}$
293 corresponds to 50 genome copies and 1,000 PCRU for HAV and RV, respectively. This
294 would lead to an underestimation of the RV and HAV recoveries. Free RNA molecules
295 and defective particles were detected in the positive control submitted only to the RNA

296 extraction, whereas in water samples, those free RNAs and defective particles were
297 most likely lost during the concentration steps.

298 **3.3. Interlaboratory study for the detection and quantification of potentially**
299 **infectious enteric viruses in influent and effluent waters**

300 The spread of qPCR has allowed the detection of human enteric viruses in
301 environmental waters (Aw and Rose 2012), including those that cannot be detected by
302 routine cell culture. Moreover, monitoring viruses in influent and effluent waters could
303 be an appropriate approach for determining the prevalence, the epidemiology and,
304 finally, the human health risks associated (Sinclair et al. 2008; Prevost et al. 2015;
305 Kazama et al. 2016; Kazama et al. 2017). Thus, to further validate the proposed
306 methodology, influent and effluent water samples were collected at each WWTP (n=5,
307 Table 1) in November 2017 and May 2018 and assayed by two independent laboratories
308 (Lab1 and Lab2) to detect NoV GI, NoV GII and HAV. Moreover, a viability RT-qPCR
309 was run concomitantly to evaluate the potential infectivity of each sample (Table 4).
310 In influent samples, NoV GI ranged between 4.80-6.54 (for Lab1) and 5.29-7.56 log
311 genomic copies/L (for Lab2). In effluent waters, titers were 4.08-6.01 (for Lab1) and
312 4.08-6.27 log genomic copies/L (for Lab2), together with some samples resulting
313 negative. NoV GII varied between 5.19-7.16 (Lab1) and 5.00-7.31 (Lab2) in influent
314 water samples and between 4.64-5.43 (Lab1) and 4.72-6.08 log genomic copies/L
315 (Lab2) in effluent water samples. Only three samples were positive for HAV by Lab1 at
316 quantification values of <5.46 log genomic copies/L.
317 In influent waters, NoV GII showed, on average, slightly greater titers than NoV GI,
318 similar to previous publications reporting ranges from 10^4-10^8 for NoV GI and from
319 10^5-10^9 genomic copies/L for NoV GII (da Silva et al. 2007; Katayama et al. 2008; da

320 Silva et al. 2008; La Rosa et al. 2010; Simmons and Xagoraraki 2011; Kitajima et al.
321 2014; Montazeri et al. 2015; Qiu et al. 2015; Schmitz et al. 2016; Haramoto et al. 2018).
322 For better describing the viral population, Lab1 additionally determined the levels of
323 RV, and HAstV in influent and effluent waters (Figure 1; Table 5). RV titers ranged
324 between 5.51-6.52 log PCRU/L in influent waters resulting below the $LoD_{95\%}$ (<5.51
325 log PCRU/l) in effluent water samples. Similar concentrations (around 4.5 log genomic
326 copies/L) have been previously described in both influent and effluent water samples
327 (Haramoto et al. 2018). Furthermore, levels of HAstV ranging between 4.59-7.33 and
328 3.31-5.58 log PCRU/L were detected in influent and effluent waters, respectively.
329 As a general statement, given an efficiency of the concentration and extraction method
330 below 100%, all of these values should be considered as a conservative estimation of
331 the virus load, being the corrected titer as high as 10^{10} log genomic copies/L (Gerba et
332 al. 2017). Recoveries of spiked whole process control ranged 3.6-40.9% (Lab1) and 3.2-
333 66.6% (Lab2) (Table 4) suggesting that the method was suitable for viral concentration
334 (Haramoto et al. 2018; ISO 15216-1:2017). Overall, previous studies showed similar
335 wide recovery ranges in surface and waste waters by using different viruses as process
336 control such as MgV (Farkas et al. 2018; Miura et al. 2016), PP7 bacteriophage, or AdV
337 (Kundu et al. 2013; Prevost et al. 2015; Barrios et al. 2018). Average recovery
338 percentages ranging from 38 to 49% were obtained in different studies by using
339 adsorption–elution on electro-charged (either positive or negative) filters and
340 summarized in Cashdollar and Wymer (2013)'s review. Regardless, considering that the
341 back-calculation is not recommended (Haramoto et al. 2018), all reported values have
342 not been adjusted in this sense.
343 Comparing viral titers before and after the treatment at WWTP, differences were
344 registered for both NoV GI and GII, as well for RV and HAstV. Reductions were

observed in all samples at different extent with the highest removal rates shown by WWTP 1, 2, and 4 (Figure 1). Other authors reported viral reductions between influent and effluent waters due to WWTP treatments, suggesting a higher persistence of NoV GI compared to GII (Haramoto et al. 2006; da Silva et al. 2007; Nordgren et al. 2009; Haramoto et al. 2015). In contrast, Hewitt and collaborators (2011) found similar NoV GI and GII concentrations in influent and effluent water samples. Our results clearly suggest that reductions are mainly due to the type of reclamation treatments applied at WWTP (secondary vs. tertiary), given the different reductions registered among the WWTPs (Table 1).

In this sense, the determination of virus infectivity in waters is required to assess the real risk of using these waters. Thus, a viability RT-qPCR procedure was applied in parallel in both influent and effluent waters by both laboratories. Even if PMAxx showed to significantly affect the virus titers (Table S2), the high loads of viruses in the PMAxx-treated samples indicate the potential infectivity of detected viruses. Similarly, Gyawali and Hewitt (2018) evaluated the performance of PEMAX-RT-qPCR on influent and effluent water and found a high proportion of potentially infectious NoV GI and GII (i.e., 21 to 89% in influent, and 24 to 59% in effluent).

On the contrary, a recent study by Prevost et al. (2016) demonstrated the suitability of EMA to differentiate infectious and inactivated NoV GI, NoV GII, RV and HAstV in effluent waters from drinking water plants with UV and chlorination treatments in which none of the samples resulted positive following the viability RT-qPCR.

Our results are not surprising when taking into account that (i) in influent water, detected viruses originated from recently excreted faeces, being expected as infectious; (ii) the viability RT-qPCR has been mainly fostered to discriminate heat inactivated viruses, and is still not being optimized and validated with other inactivation techniques,

370 such as chlorination or UV; (iii) altered, non-infectious viruses and their free RNA
371 degrade rapidly, being improbable in their detection (Limsawat and Ohgaki 1997), iv)
372 the water concentration procedure are most likely targets infectious viruses rather than
373 free RNAs or altered capsids.
374 Thus, the infectivity profiles obtained by viability RT-qPCR may reflect either an
375 ineffective virus inactivation at WWTP and/or that the capsid damage by
376 secondary/tertiary water treatments may not be enough to allow viability marker
377 penetration. In this sense, a recent study by López-Gálvez et al. (2018) demonstrated
378 that chlorination with chlorine dioxide (ClO_2) is not able to significantly reduce the
379 NoV GI, NoV GII and HAstV loads in effluent waters, according to previous research
380 by Kingsley and collaborators (2014) that suggested that NoVs are quite resistant to
381 ClO_2. The differences in the final outcomes of such studies are justified by different
382 parameters such as pH, turbidity, temperature, dissolved organic matter, and ionic
383 strength, that all play a key role in inactivation kinetics, and especially in chlorination
384 sanitizing strategies (Carvajal et al. 2017; López-Gálvez et al. 2018).
385 Thus, the effectiveness of viability RT-qPCR should be investigated and validated for
386 different inactivation mechanisms (i.e. UV treatment, chlorination, ozone treatment etc.)
387 for each virus of interest and for each type of water supply. Despite limitations, viability
388 RT-qPCR may improve public health risk evaluations by providing more realistic data
389 sets than RT-qPCR alone.
390 Variation is observed in viral titers independently detected by two laboratories (Table
391 4). Significant roles in determining such differences in measurements could have been
392 played by the different level of expertise in virus analysis among laboratories and
393 homogeneity of the sample. A more robust comparison of the data set generated in this

394 study should take advantage of water quality parameters (Borgmästars et al., (2017),
395 that unfortunately were not available for the tested samples.

396 **3.4. Statistical analysis**

397 The effect of the five variables considered in this interlaboratory comparison study
398 (WWTP, sampling date, influent/ effluent water, use of PMAxx and laboratory facility)
399 tested by ANOVA for each virus is shown in Table S2. The results, indicated as p value,
400 show significant differences within the parameters of WWTP, the type of water
401 (influent/ effluent water) and the use of viability marker (PMAxx) for all the
402 investigated enteric viruses. The effect of the sampling date was significant for RV and
403 HAstV, but not for NoV GI and GII. In this sense, NoVs fluctuation over the seasons
404 has been described by Farkas et al. (2018) and Haramoto et al. (2006), but not always
405 confirmed (Nordgren et al. 2009). Seasonal profiles of human caliciviruses
406 concentration in water environments is not surprising, since higher concentrations in
407 colder months reflect the epidemic period for those viruses. Despite that, Katayama et
408 al. (2008) and Kitajima et al. (2014) reported that constant concentrations of AdV, EVs,
409 and Aichi virus in influent water are without a clear seasonal pattern. Despite of all of
410 that, the results of this study cannot support any robust seasonal pattern conclusion due
411 to the limited number of samples analyzed.
412 Regarding the effect of the laboratory facilities, significant differences were detected
413 only for NoV GI, but not for NoV GII. A PCA was applied to the data matrix of NoV
414 GI and GII titers and the results are shown in Figure 2, Table S3 and S4. The correlation
415 analysis among variables (Table S3 and S4) showed significant relationships and the
416 data matrix were appropriated to be subjected to the PCA to condense the information
417 within factors. Thus, the three main factors, representing up to 72.11 and 73.66% of the
418 total variance for NoV GI and GII, respectively, were plotted in a 3D scatterplot

showing the relationship among them and samples. For NoV GI (Fig. 2A), samples were grouped mainly according to Factor1 and Factor2, correlated with viability treatment, laboratory facilities, and WWTP. Factor3 contributed only marginally to discriminate samples according to the type of water, influent or effluent. For NoV GII (Fig. 2B), all the factors contributed to represent the total variance resulting in a wide spread of samples in the plot. In particular, Factor1 and Factor2 mainly discriminated samples being correlated with WWTP, viability treatment, and type of water.

Finally, the PCA of titers of both NoV GI and GII showed that the main factors that contributed in discriminating samples were correlated to WWTP and type of water.

Conclusions

The demand for rapid and reliable protocols to define virological water quality is increasing due to the spread of alternative uses of regenerated/recycled water given the limitation of water resources. In such a scenario, we tested a rapid and low-cost method based on aluminium hydroxide adsorption-precipitation. We determined the enteric viral pathogen population, including NoV GI, NoV GII, HAV, RV, and HAstV, in influent and effluent water samples. Even MgV recoveries varied greatly across samples, the method, evaluated by an interlaboratory comparative study, provided good mean recovery of the process control virus in both influent and effluent waters. Titers of up to 7 log genomic copies/L were detected for NoV GI and GII in influent waters, in line with previous studies. Moreover, three samples were positive for HAV. Generally, reductions in viral loads were detected in effluent water samples compared to influent waters, posing a health risk concern since the viability pretreatment applied showed their potential viral infectivity.

A PCA analysis applied to NoVs data matrix confirmed the correlation among WWTP, type of water, viability pretreatment, and sample variability. Thus, despite the

limitations, this proposed approach could be useful not only to control virus loads in influent and effluent water samples, but also to evaluate the efficacy of sanitation procedures applied in WWTPs and, thus, to better predict the risk by quantitative microbial risk assessment (QMRA) analysis (Van et al. 2017; Dias et al. 2019). However, further improvements should be considered before adapting the method for routine use such as lowering the limit of detections by analyzing full-scale RT-qPCR and/or validating the viability PMAxx-RT-qPCRs with viral inactivation by UV treatment and chlorination.

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Table 1. Waste water treatment plants (WWTP) used for influent and effluent water sampling.

WWTP	Flow (m^3/day)	Inhabitants equivalent	Secondary treatment	Tertiary treatment
1	31,690	151,692	Coagulation, flocculation and phosphorus compounds elimination	UV disinfection
2	9,087	45,523	Coagulation and flocculation	None
3	23,718	81,340	Coagulation, flocculation and phosphorus compounds elimination	None
4	4,119	23,381	Coagulation, flocculation and nitrogen compounds elimination	UV disinfection
5	36,427	118,102	Coagulation and flocculation	None

Table 2. Comparison of concentration methods for enteric virus detection and quantification by RT-qPCR in influent water samples.

Sample	Method A						Method B					
	Ultracentrifugation method						Aluminium-based method					
	(log genome copies/L)						(log genome copies/L)					
	MgV recovery (%)	NoV GI	NoV GII	HAV	RV	HAstV	MgV recovery (%)	NoV GI	NoV GII	HAV	RV	HAstV
IW1	8.04	4.81 ± 0.38[a]	4.68 ± 0.11[a]	<5.46[a]*	6.01 ± 0.00[a]	5.65 ± 0.04[a]	4.14	5.34 ± 0.09[a]	4.89 ± 0.20[a]	<5.46[a]*	6.49 ± 0.10[b]	6.00 ± 0.03[b]
IW2	13.27	4.36 ± 0.06[a]	5.27 ± 0.06[b]	n	5.75 ± 0.09[a]	5.65 ± 0.11[a]	1.58	4.41 ± 0.49[a]	4.94 ± 0.07[a]	<5.46[a]*	6.73 ± 0.23[b]	5.97 ± 0.00[b]
IW3	25.72	4.59 ± 0.00[a]	4.73 ± 0.08[b]	<5.46[a]*	5.19 ± 0.13[a]	5.00 ± 0.37[a]	4.30	5.15 ± 0.0[b]	4.13[a]	<5.46[a]*	6.22 ± 0.14[b]	5.62 ± 0.09[b]
IW4	9.86	4.10 ± 0.12[a]	4.64*	n	5.65 ± 0.10[a]	5.01[a]	0.02	5.66 ± 0.23[b]	n	n	6.43 ± 0.30[b]	5.44 ± 0.03[b]
IW5	8.54	4.27 ± 0.22[a]	5.30 ± 0.00[b]	n	N/A	N/A	3.30	5.48[b]	4.30 ± 0.05[a]	n	N/A	N/A

*Limit of detection; n, negative; N/A, data no available.

Each value represents the average of triplicates. Within each sample and virus, different letters denote significant differences between treatments ($p < 0.05$).

Table 3. Detection limit of human enteric viruses in effluent waters using the aluminium-based method

Spiked virus	Levels of inocula					
	Direct inoculum		10-fold diluted inoculum	100-fold diluted inoculum	1000-fold diluted inoculum	$LoD_{95\%}$[b]
	Log genome copies/L	Positive/total numbers of samples Recovery (%)[a]	Positive/total numbers of samples	Positive/total numbers of samples	Positive/total numbers of samples	Log genome copies/L
NoV GI	≈1.0 X 10^6	4/4 (13.13)	4/4	2/4	0/4	4.08
NoV GII	≈1.0 X 10^6	4/4 (7.52)	4/4	1/4	0/4	4.64
HAV	≈1.0 X 10^6	4/4 (0.65)	4/4	1/4	0/4	5.46
RV	≈1.0 X 10^7	4/4 (0.97)	4/4	4/4	1/4	5.41
HAstV	≈1.0 X 10^6	4/4 (71.40)	4/4	3/4	0/4	3.31

[a], percentage of recovered titer with respect to initial inoculum.

[b], $LoD_{95\%}$, limit od detection calculated according to Wilrich and Wilrich (2009).

Table 4. Comparative detection of potentially infectious enteric viruses in influent and effluent water samples by two independent laboratories.

Waste Water Treatment Plant	Sampling date	Influent/ Effluent Water	PMAxx/Triton pretreatment	MgV Recovery (%)		NoV GI (log genome copies/L)		NoV GII (log genome copies/L)		HAV (log genome copies/L)	
				Lab1	Lab2	Lab1	Lab2	Lab1	Lab2	Lab1	Lab2
WWTP 1	Nov 2017	IW	-	15.33	47.95	5.41	6.28 ± 0.02	7.01	6.73 ± 0.04	-	-
			+	14.29	34.73	5.29 ± 0.02	6.17 ± 0.05	6.71 ± 0.02	6.33 ± 0.05	-	-
		EW	-	15.29	4.08	4.80 ± 0.04	<4.08*	4.89 ± 0.04	-	-	-
			+	20.45	3.87	4.88	<4.08*	4.97 ± 0.23	-	-	-
	May 2018	IW	-	23.89	55.64	6.01 ± 0.07	6.53 ± 0.06	6.57 ± 0.02	6.90 ± 0.10	<5.46*	-
			+	21.72	45.63	5.85 ± 0.04	6.32 ± 0.21	6.45 ± 0.05	6.38 ± 0.16	-	-
		EW	-	8.06	3.21	<4.08*	<4.08*	<4.64*	-	-	-
			+	6.04	8.41	<4.08*	-	4.74	-	-	-
WWTP 2	Nov 2017	IW	-	5.12	22.7	5.02 ± 0.09	6.22 ± 0.06	6.78 ± 0.02	6.58 ± 0.00	-	-
			+	5.30	66.64	5.16 ± 0.16	5.29	7.08 ± 0.57	7.11 ± 0.06	-	-
		EW	-	17.60	45.71	<4.08*	-	4.88 ± 0.11	4.74 ± 0.20	-	-
			+	24.38	30.55	<4.08	-	4.86 ± 0.12	-	-	-
	May 2018	IW	-	28.39	34.67	6.20 ± 0.01	6.51 ± 0.02	6.44 ± 0.06	6.81 ± 0.20	-	-
			+	24.44	31.21	5.93 ± 0.00	6.27 ± 0.00	6.21 ± 0.13	6.23 ± 0.11	-	-
		EW	-	23.07	13.84	<4.08*	<4.08*	<4.64*	-	<5.46*	-
			+	16.68	38.85	<4.08*	-	<4.64*	-	-	-
WWTP 3	Nov 2017	Urban IW	-	6.30	33.10	6.15 ± 0.08	7.35 ± 0.15	7.16 ± 0.14	7.02 ± 0.04	-	-
			+	17.96	65.8	6.08 ± 0.11	7.56 ± 0.03	7.05 ± 0.13	7.31 ± 0.05	-	-
		Industrial IW	-	8.34	39.71	5.71 ± 0.25	6.27 ± 0.21	5.43 ± 0.60	5.77 ± 0.06	-	-
			+	6.85	46.89	5.39	6.09	4.69	6.08 ± 0.47	-	-
		EW	-	12.23	14.56	5.81 ± 0.08	5.39 ± 0.20	5.76 ± 0.14	5.00 ± 0.02	-	-
			+	24.51	29.04	5.62 ± 0.10	5.48 ± 0.03	5.44 ± 0.37	5.36 ± 0.05	-	-
	May 2018	Urban IW	-	3.66	32.00	-	5.15 ± 0.90	-	5.44	-	-
			+	3.94	43.35	6.01 ± 1.36	4.33	-	4.97	-	-
		Industrial IW	-	8.54	8.81	-	-	5.19	-	-	-
			+	8.98	16.46	-	-	-	-	-	-

		EW	-	8.78	13.73	<4.08*	<4.08*	-	-	-	-
			+	6.63	30.75	<4.08*	-	-	-	-	-
WWTP 4	Nov 2017	IW	-	9.91	29.69	6.54 ± 0.20	6.38 ± 0.25	6.59 ± 0.54	6.80 ± 0.06	-	-
			+	6.37	34.29	5.92 ± 0.08	6.53	6.28 ± 0.74	7.08 ± 0.03	-	-
		EW	-	6.29	6.14	-	-	-	-	-	-
			+	27.11	4.64	-	-	-	-	-	-
	May 2018	IW	-	19.72	51.47	5.91 ± 0.18	6.28 ± 0.05	6.02 ± 0.05	5.75 ± 0.10	-	-
			+	7.80	48.85	5.81 ± 0.15	6.39 ± 0.08	5.80 ± 0.06	5.36 ± 0.18	-	-
		EW	-	13.32	11.53	<4.08*	<4.08*	-	-	-	-
			+	11.51	39.88	<4.08*	-	-	-	-	-
WWTP 5	Nov 2017	IW	-	15.51	8.85	4.86 ± 0.25	5.96	5.76 ± 0.39	6.10	-	-
			+	17.26	3.58	4.80	-	5.69 ± 0.25	-	-	-
		EW	-	40.92	8.96	4.78	-	4.65 ± 0.35	-	<5.46*	-
			+	19.76	16.03	-	-	<4.64*	-	-	-
	May 2018	IW	-	39.27	22.43	5.86 ± 0.14	6.46 ± 0.20	5.76 ± 0.44	6.21 ± 0.10	-	-
			+	36.53	7.57	5.58 ± 0.02	5.9	5.85 ± 0.08	5.19 ± 0.02	-	-
		EW	-	10.74	10.23	4.08 ± 0.11	-	4.72 ± 0.03	4.72	-	-
			+	7.54	23.82	<4.08*	-	4.79 ± 0.29	-	-	-

*, <Limit of detection; -, negative.

Each value represents the average and the standard deviation (log genome copies/L ± SD) of RT-qPCRs technical duplicates of a single concentrated sample.

Table 5. Detection of potentially infectious enteric viruses in influent and effluent waters by Lab1.

Waste Water Treatment Plant	Sampling date	Influent Water / Secondary Effluent	PMAxx	MgV Recovery (%)	RV (log PCRU/L)	HAstV (log PCRU/L)
WWTP 1	Nov 2017	IW	-	15.33	-	6.07
			+	14.29	-	-
		EW	-	15.29	-	-
			+	20.45	<5.41	4.72
	May 2018	IW	-	23.89	5.52 ± 0.02	5.05 ± 0.11
			+	21.72	-	-
		EW	-	8.06	<5.41	-
			+	6.04	<5.41	-
WWTP 2	Nov 2017	IW	-	5.12	5.91 ± 0.02	7.38 ± 0.14
			+	5.30	5.47 ± 0.11	7.14 ± 0.03
		EW	-	17.60	<5.41	4.68
			+	24.38	<5.41	4.37
	May 2018	IW	-	28.39	5.75 ± 0.15	5.44 ± 0.01
			+	24.44	-	-
		EW	-	23.07	<5.41	<3.31*
			+	16.68	<5.41	<3.31*
WWTP 3	Nov 2017	Urban IW	-	6.30	5.43	7.46 ± 0.21
			+	17.96	<5.41	7.21 ± 0.02
		Industrial IW	-	8.34	5.43 ± 0.09	6.81 ± 0.01
			+	6.85	<5.41	6.40 ± 0.02
		EW	-	12.23	<5.41	5.58 ± 0.04
			+	24.51	<5.41	5.29 ± 0.01
	May 2018	Urban IW	-	3.66	5.45	4.59 ± 0.13
			+	3.94	-	-
		Industrial IW	-	8.54	-	5.25 ± 0.17
			+	8.98	-	-

		EW	-	8.78	<5.41	3.94 ± 0.11
			+	6.63	<5.41	-
WWTP 4	Nov 2017	IW	-	9.91	5.64 ± 0.04	7.33 ± 0.15
			+	6.37	5.47	7.00 ± 0.23
		EW	-	6.29	-	-
			+	27.11	-	-
	May 2018	IW	-	19.72	<5.41	5.87 ± 0.05
			+	7.80	-	-
		EW	-	13.32	-	3.74
			+	11.51	-	-
WWTP 5	Nov 2017	IW	-	15.51	6.52 ± 0.05	6.79 ± 0.12
			+	17.26	6.03 ± 0.05	6.48 ± 0.05
		EW	-	40.92	<5.41	5.42 ± 0.02
			+	19.76	<5.41	-
	May 2018	IW	-	39.27	6.22 ± 0.03	5.56 ± 0.03
			+	36.53	-	-
		EW	-	10.74	<5.41	4.34 ± 0.03
			+	7.54	<5.41	-

*, <Limit of detection; -, negative.

Each value represents the average and the standard deviation (log genome copies/L ± SD) of RT-qPCRs technical duplicates of a single concentrated sample.

Figure 1. Overview of detected enteric viruses in influent and effluent waters. Box plots show median concentrations (log genome copies/L) with the 25-th and 75-th percentile values of NoV GI (blue), NoV GII (orange), RV (green), HAstV (yellow) in influent (dark colors) and effluent (light colors) waters from five different wastewater treatment plants as detected by Lab1.

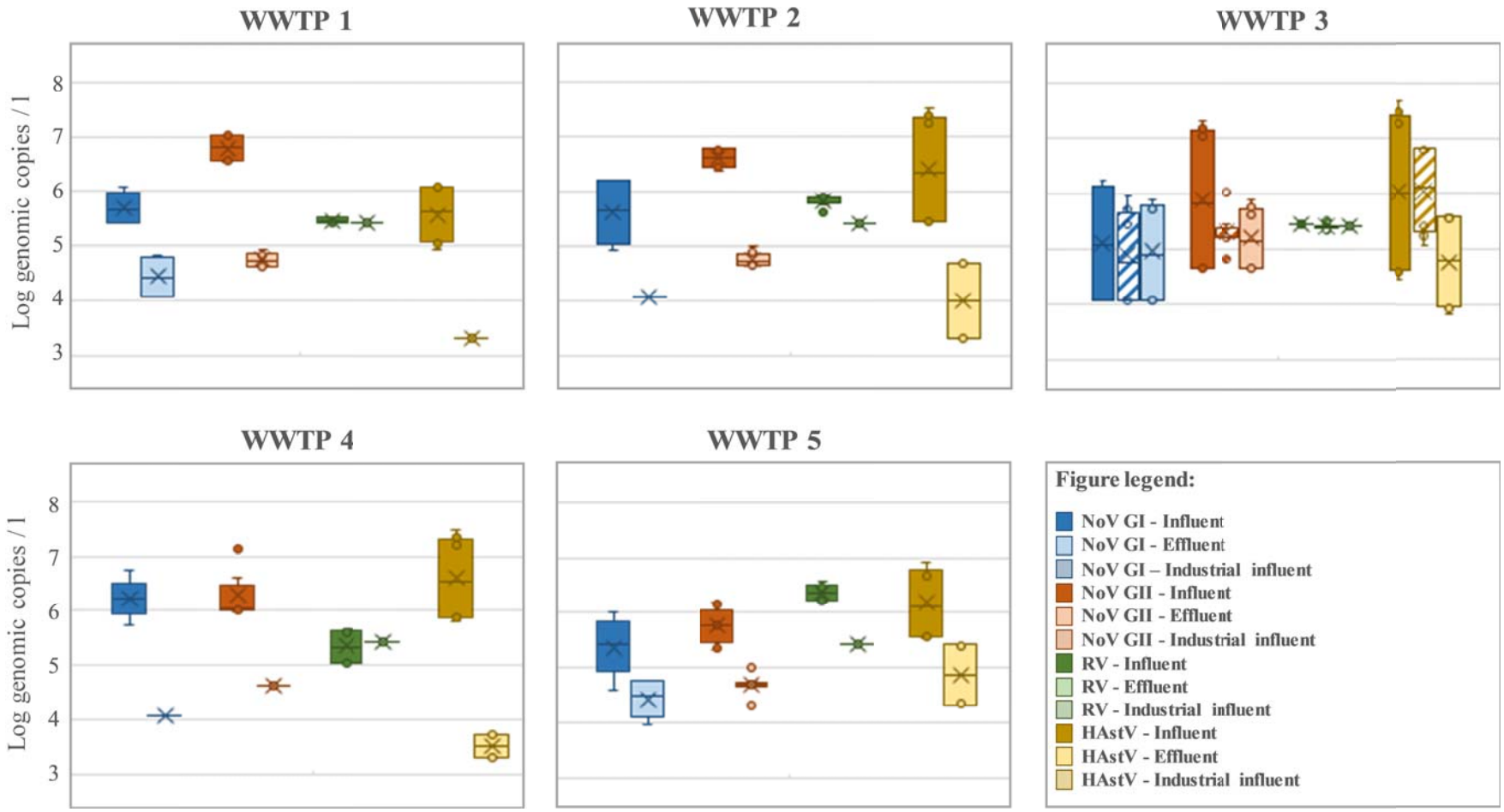

Figure 2. Scatterplots show relationship between Factors and samples based on principal component analysis of the detected titers of NoV GI (A) and NoV GII (B) in influent and effluent water samples by two independent laboratory facilities.

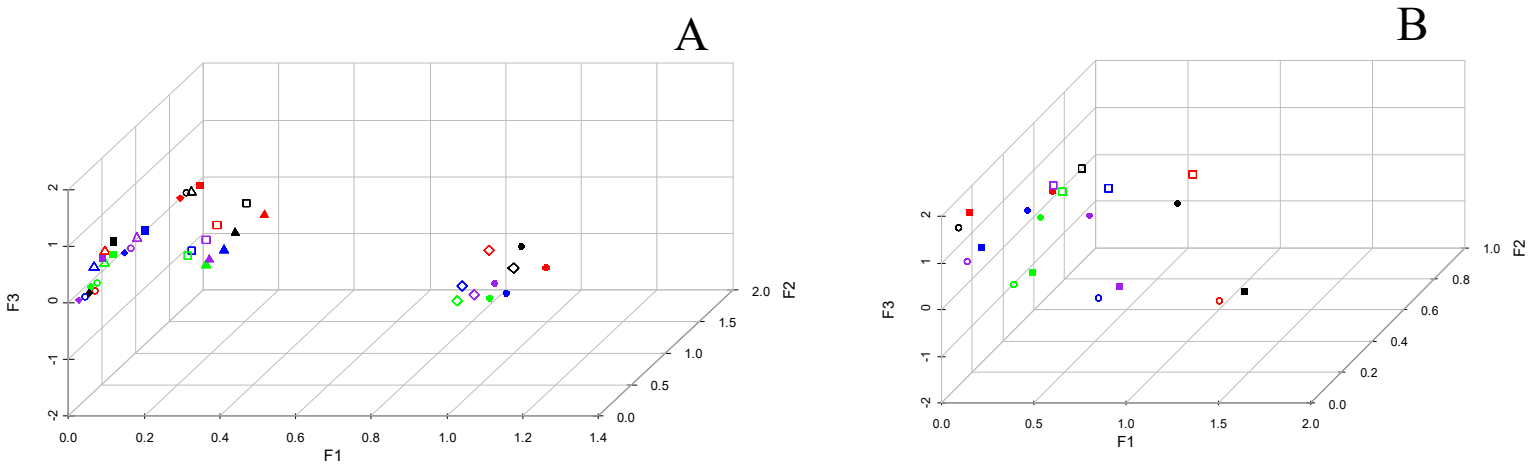

Figure legend: Influent (■, □, ♦, ◊) and effluent (●, ○, ▲, Δ) water samples tested by Lab1 (■, □, ●, ○) and Lab2 (♦, ◊, ▲, Δ) with (non-solid symbols) and without (solid symbols) viability marker pre-treatment. Symbols are coloured according to waste water treatment plant (WWTP1 in red, WWTP2 in blue, WWTP3 in green, WWTP4 in yellow, WWTP5 in black).

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