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Virus Type-Specific Removal in a Full-Scale Membrane Bioreactor Treatment Process

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Abstract :

We investigated removal of noroviruses, sapoviruses, and rotaviruses in a full-scale membrane bioreactor (MBR) plant by monitoring virus concentrations in wastewater samples during two gastroenteritis seasons and evaluating the adsorption of viruses to mixed liquor suspended solids (MLSS). Sapoviruses and rotaviruses were detected in 25% of MBR effluent samples with log reduction values of 3- and 2-logs in geometric mean concentrations, respectively, while noroviruses were detected in only 6% of the samples. We found that norovirus and sapovirus concentrations in the solid phase of mixed liquor samples were significantly higher than in the liquid phase ($P < 0.01$, t test), while the concentration of rotaviruses was similar in both phases. The efficiency of adsorption of the rotavirus G1P[8] strain to MLSS was significantly less than norovirus GI.1 and GII.4 and sapovirus GI.2 strains ($P < 0.01$, t test). Differences in the adsorption of viruses to MLSS may cause virus type-specific removal during the MBR treatment process as shown by this study.

Keywords : Norovirus, Sapovirus, Rotavirus, MBR, Mixed liquor suspended solids, Adsorption

Introduction

Contaminated food and water is a major pathway for the transmission of infectious disease and poses a global health issue, with viruses being one of the most frequently reported infectious agents (Havelaar et al., 2015; Semenza et al., 2016). Human enteric viruses causing gastroenteritis or hepatitis are excreted in stools and vomitus from infected individuals at high concentrations, and wastewater effluent is a major source of viruses in water environments. Understanding the mechanisms of viral elimination through wastewater treatment processes may lead to improvements in effluent quality and help to prevent further distribution and transmission of viruses (Uyttendaele et al., 2015). Membrane bioreactors (MBRs) show promise in improving municipal and domestic wastewater treatment; they have many advantages including the production of high quality effluent, compactness, and short start-up times. However, virus removal through MBR treatment processes requires further study, as pathogenic viruses in sewage are highly diverse (Furtak et al., 2016) and considering the paucity data on human virus rejection available. Human gastroenteritis viruses are among the most frequently detected in sewage. Noroviruses (NoVs) and sapoviruses (SaVs) are members of the *Caliciviridae* family, and are important etiological agents of acute viral gastroenteritis. These viruses are non-enveloped, have icosahedral capsids that are 27−40 nm in diameter, and have linear, positive-sense, single-stranded RNA genomes. They are genetically diverse, and NoV genogroups I (GI), GII, and GIV, and SaV GI, GII, GIV, and GV, infect humans (de Graaf et al., 2016; Oka et al. 2006). Rotaviruses (RVs) belonging to the family *Reoviridae* are the major cause of viral gastroenteritis in children. RVs are non-enveloped with triple-layered icosahedral capsids that are approximately 100 nm diameter, and they have double-stranded segmented RNA genomes. Based

58 on the RNA sequences encoding VP7 (G: glycoprotein) and VP4 (P: proteinase-
59 sensitive protein), group A RVs are classified into G and P genotypes (Estes and
60 Greenberg, 2013).

61 Virus removal through MBR treatment processes has been investigated
62 using bacteriophages as culturable models of human enteric viruses. Two main
63 elimination pathways have been identified: (1) adsorption of viruses to mixed liquor
64 suspended solids (MLSS), and (2) rejection of the viruses by the membrane (Wu et
65 al., 2010; Marti et al., 2011). The two pathways combine to produce significant log
66 reductions, and large differences in virus concentrations between the membrane
67 influent and effluent samples (Purnell et al., 2016). However, differences in the
68 behavior of bacteriophages and human enteric viruses through MBR processes have
69 been observed, including variations in viral removal efficiencies through the two
70 major removal pathways (Ottoson et al., 2006; Chaudhry et al., 2015b). Different
71 removal efficiencies have also been reported for adenoviruses, enteroviruses, and
72 NoVs, which belong to different viral families (Ottoson et al., 2006; Simmons et al.,
73 2011; Purnell et al., 2016). We previously investigated removal of NoVs and SaVs
74 in full- and pilot-scale MBR plants and found that SaVs were more efficiently
75 removed than NoVs, although they have a similar structure, morphology, and size
76 (Sima et al., 2011; Miura et al., 2015). The surface characteristics of viruses, such as
77 electrostatic charge and hydrophobicity of viral capsid proteins, play a significant
78 role in viral adsorption (Michen and Graule, 2010; Armanious et al., 2016), and may
79 be responsible for the different removal efficiencies. However, the underlying
80 mechanisms that cause virus removal efficiency to vary are not yet fully understood,
81 and further investigations may assist in enhancing virus elimination through the
82 MBR process.

In this study, we hypothesized that the differing adsorption of viruses to MLSS would result in diverse removal efficiencies through the MBR process. To test this hypothesis, we determined the concentration of NoVs, SaVs, and RVs in influent and effluent samples collected from a full-scale anoxic-oxic MBR process over two gastroenteritis seasons (from October 2013 to May 2015). Furthermore, we collected oxic mixed liquor samples, as well as influent and effluent samples, and quantified viruses in the bulk liquor samples and those associated with suspended solids separately. To confirm the monitoring results, adsorption and removal experiments were also conducted using NoV, SaV, and RV strains and oxic mixed liquor samples.

Materials and Methods

Sample collection. Samples were collected from a full-scale anoxic-oxic (AO) MBR process treating approximately 1,800 m^3/day of municipal wastewater from a community in northwestern France. The MBR was equipped with a submerged flat-sheet membrane module (Kubota, Japan) and the membrane sheet was made from chlorinated polyethylene with a nominal pore size of 0.4 μm. A total surface area of the membrane was 6,960 m^2 and the membrane flux was set at 14−16 L/m^2/h with trans-membrane pressure (TMP) ranging from 1.3 to 3.6 kPa. The hydraulic retention time in the whole process was approximately 36 h, and the solid retention time (SRT) was 50−180 days. The MLSS concentration in the oxic tank was maintained between 5,000 and 6,000 mg/L, and the excess sludge in the oxic tank was returned to the anoxic process at the return sludge recycle ratio of 125%. The MBR effluent was finally treated with activated carbon (Carboplus^R, Stereau, France).

Twenty-four-hour composite influent and effluent samples, and grab mixed liquor samples (2 L each) were collected from three points in the AO MBR process (**Figure 1**). Eight sets of influent and effluent samples were collected monthly from October 2013 to May 2014, and from October 2014 to May 2015. Additionally eight oxic mixed liquor samples were collected during the second monitoring period as well as influent and effluent quality parameters (as listed in **Table 1)**. The samples were transported to the laboratory on ice and processed within 24 h of collection.

SS, pH, and electrical conductivity. The concentration of suspended solids (SS) was measured according to the standard protocols of the Japan Sewage Works Association. Briefly, a sample was filtered with a glass fiber filter (1 μm pore size, 47 mm diameter, Whatman GF/B, GE Healthcare, France), and the filter was dried in an oven at 105°C for 2 h. SS concentration was calculated from the dry weight of particles trapped by the filter. pH and electrical conductivity (EC) were measured with an HI 98103 meter (Hanna Instruments, Japan) and Cond 340i meter (WTW GmbH, Germany) respectively.

Viral RNA preparation from samples. During the first monitoring period, effluent samples were concentrated from 1 L to 40 mL with cross-flow ultrafiltration (Vivaflow 50, Sartorius, Germany) (Sima et al., 2011). During the second monitoring period, 40 mL of each effluent sample was directly applied to the polyethylene glycol (PEG) precipitation. All influent samples (40mL) were also directly applied to the PEG precipitation.

Oxic mixed liquor samples were centrifuged at 3,000 × *g* for 10 min at 4°C, and separated into pellet (regarded as the solid phase) and supernatant portions

133 (regarded as the liquid phase) (Miura et al., 2015). Viral particles in the solid phase
134 were eluted by vortex mixing for 1-min in 25 mL of phosphate/citric acid buffer
135 with 10% beef extract, followed by centrifugation at 10,000 × *g* for 5 min at 4°C
136 according to the method of the US EPA with minor modifications (U.S. EPA 2003;
137 Miura et al., 2015). After these first steps, 5.4 ± 0.4 log copies of mengovirus vMC_0
138 (MgV, provided by A. Bosch, University of Barcelona, Spain) were inoculated into
139 all pre concentrate or liquid sub-samples. To prevent any loss of MgV by binding to
140 particles and considering that the elution method was proven to be efficient , it was
141 added to the liquid phase (U.S. EPA 2003; Miura et al., 2015; Sima et al., 2011).
142 Then concentration was performed using a PEG precipitation method (Sima et al.,
143 2011). The PEG pellet was suspended in 1 mL of deionized distilled water (DDW)
144 with a vortex mixer and used to extract viral RNA using the NucliSENS kit
145 (bioMérieux, Lyon, France) according to the manufacturer's instructions but with
146 incubation with lysis buffer for 30 min at 56°C instead of 10 min at room
147 temperature (Sima et al., 2011). All washing steps were performed using the
148 NucliSENS easyMAG, and viral RNA was recovered in 100 µL of elution buffer.
149
150 **Quantification of viral genomes.** All viral RNA was amplified using an RNA
151 UltraSense One-Step Quantitative RT-PCR System (Thermo Fisher Scientific, France)
152 and previously reported primers and probes for MgV (Pintó et al., 2009), NoV GI (da
153 Silva et al., 2007; Svraka et al., 2007), NoV GII (Loisy et al., 2005; Kageyama et al.,
154 2003), SaVs (Oka et al., 2006), and RVs (Pang et al., 2011), except one modification
155 was made to the RV probe, which was shortened and modified with MGB (i.e. FAM-
156 TGAGCACAATAGTTAAAAGC-MGB-NFQ). The cycle threshold (C_T) was defined
157 as the cycle at which a significant increase in fluorescence occurred. Undiluted, 10-

158 and 100-fold-diluted RNA extracts were tested and absence of inhibition in real-time
159 RT-PCR was verified for each sample. The number of genome copies in each reaction
160 well was calculated by comparing the C_T value to a standard curve generated from a
161 dilution series of plasmid containing each target region (Sima et al., 2011). Then the
162 virus concentration in the sample was calculated based on the volume of viral RNA
163 analyzed and was reported per liter. A C_T value less than 40 was regarded as positive
164 (Sima et al., 2011), and the detection limits when 40 mL samples were analyzed were
165 900, 400, 490, and 460 copies/L for NoV GI, NoV GII, SaVs, and RVs, respectively
166 (36, 16, 20, and 19 copies/L when 1 L samples were analyzed). Only samples with
167 MgV recovery efficiencies above 1% were considered for quantification (Miura et al.,
168 2016). We used the MgV recovery efficiency as a quality assurance parameter only
169 and did not use to adjust test results (Kazama et al., 2017).

170

171 **Adsorption and removal experiment.** The oxic mixed liquor samples were used for
172 experiments within 48 h of collection. The MLSS concentration was adjusted to
173 5,500 mg/L with a final volume of 100 mL by discarding the supernatant or by
174 adding effluent sample from the same collection date. Following storage at 4°C, the
175 adjusted mixed liquor sample was left for 30 min to warm up to room temperature
176 (20 ± 1°C). One milliliter of stool mixture containing 5.8 ± 0.2 log copies of NoV
177 GI.1, 6.9 ± 0.7 log copies of NoV GII.4, 7.8 ± 0.1 log copies of SaV GI.2, and 7.6 ±
178 0.4 log copies of RV G1P[8], which were excessively large numbers compared to
179 indigenous viruses, was added and continuously mixed with a magnetic stirrer at
180 250 rpm, at room temperature. After 1, 5, 10, 20, 30, and 60 min of stirring, 3 mL of
181 mixture was collected with a 10-mL syringe and immediately filtered with a 0.45-

μm membrane (Minisart 16533-K, Sartorius, Germany). Viruses contained in the filtrate were considered to be 'un-adsorbed' viruses. After MgV addition, viral RNA was extracted from 1 mL of filtrate using the NucliSENS kit as described above, and log reduction values (LRVs) were calculated using the following equation.

$$\mathrm{LRV} = -\log_{10} (C_{\text{un-adsorbed}} / C_{\text{initial}})$$

$C_{\text{un-adsorbed}}$ represents the virus concentration observed in the filtrate and C_{initial} is the theoretical concentration just after the addition of stool mixture containing viruses. This experiment was performed for the eight oxic mixed liquor samples collected, and mean LRVs were reported for each virus.

To investigate the effect of surface charge on adsorption of viruses to MLSS, we performed an additional sampling of oxic mixed liquor sample in June 2015. The pH of the oxic mixed liquor sample was 7.0 and MLSS concentration was 5,500 mg/L. We adjusted the pH to 4.0 ± 0.1 with HCl or 9.0 ± 0.1 with NaOH and performed the adsorption and removal experiments as described above.

Quality controls. Filter tips and dedicated rooms were used to prevent sample contamination, and sample and data analyses were carried out using the following quality controls.

(i) Wastewater sample analysis. MgV was added to all samples as a process control virus and its recovery efficiency was monitored. Absence of inhibition in real-time RT-PCR was verified for each sample as described above.

(ii) Adsorption and removal experiment. MgV was added to 1 mL of each filtrate just before RNA extraction and the RNA extraction efficiency was monitored. To mimic the viruses in sewage flowing into a bioreactor, we directly added the stool mixture suspended in DDW by vortex mixing to the mixed liquor.

207 Amounts of inoculated viruses were measured in each experiment. Briefly, 100 µL
208 of the same inoculum (stool mixture suspended in DDW) were added to 10 mL of
209 DDW, 3 mL were collected and filtered. Viruses associated with stool material were
210 removed by the filtration and virus concentration detected in the filtrate was
211 considered as the theoretical concentration just after the addition of stool mixture
212 containing viruses ($C_{initial}$). In parallel, 100 µL of the same stool mixture was added
213 to 10 mL of filtered oxic mixed liquor samples, 3 mL were collected and filtered.
214 Virus concentration in the filtrate was compared to that in the filtrate from stool-
215 inoculated DDW, and the inhibitory effect of filtered oxic mixed liquor on RNA
216 extraction was evaluated. Furthermore, virus stability in the filtrate was evaluated by
217 keeping the filtered oxic mixed liquor samples for 24 h at room temperature, and
218 investigating the presence of possible degradation of viruses. In order to evaluate the
219 variability of the experiment, some experiments were performed in triplicate on the
220 same day by three operators filtering the mixed liquor samples simultaneously.
221 (iii) Standard curves. After completion of all real-time RT-PCR runs, all
222 standard curves for each virus were compared, and quality criteria were applied
223 (AFNOR 2011). Only PCR runs in which standard curves with amplification
224 efficiencies of 85 to 110% were kept for quantification. Mean Ct values were
225 calculated for each point of the standard curves and were used to estimate the
226 uncertainty of quantification (the error estimated by variations of Ct values for each
227 standard curve dilution) for NoV GI (0.16 log), NoV GII (0.12 log), SaV (0.20 log)
228 and RV (0.13 log).
229
230 **Statistical analysis.** To compare virus concentrations between sample types, the
231 student's t-test was performed using IBM SPSS Statistics Ver. 19.

232

233 **Results**

234 **Recovery efficiency of the MgV process control.** A total of 16 sets of influent and
235 effluent samples were collected (eight sets in each period). In the first monitoring
236 period (October 2013−May 2014), mean MgV recovery rates were 7% (range:
237 1−30%) for influent and 36% (1−88%) for effluent samples. In the second
238 monitoring period (October 2014−May 2015), mean MgV recovery rates of influent
239 and effluent samples (15% [6−31%] and 29% [4−80%], respectively) were
240 comparable to those of the first year. Liquid phase and solid phase eluate of oxic
241 mixed liquor samples presented similar recovery rates (12% [4−42%] and 31%
242 [4−105%], respectively). All samples gave an MgV recovery rate above 1%, giving
243 confidence in the quantitative detection results.

244 Inhibition was not detected in effluent samples, but 10-fold dilutions of six
245 influent samples were analyzed due to inhibition. Inhibition was not noted for the
246 liquid phase of five mixed liquor samples, but the solid phase was prone to
247 inhibition. The C_T values of undiluted (neat) RNA were used for direct
248 quantification in only two solid phase samples, and those of diluted RNA were used
249 in the other six samples (the dilution factor was thus considered for quantification).

250

251 **Detection of NoVs, SaVs, and RVs in sewage samples.** Influent and effluent
252 samples were collected over two years, covering two gastroenteritis seasons (from
253 October to May). Indeed, NoVs (GI and GII combined) and SaVs were detected in
254 all influent samples (except one, which did not contain NoVs), and RVs were
255 detected in 12 out of 16 collected samples. Geometric mean concentrations of
256 positive samples were around 10^5 RNA copies/L for SaVs and RVs, while NoV

257 levels were about 1-log higher (**Table 2**). In effluent samples, differences were
258 observed among viruses. NoVs were detected in only 6% of the samples, compared
259 to 25% for both SaVs and RVs, suggesting more efficient removal of NoVs
260 compared to SaVs and RVs. The decrease in geometric mean concentration was
261 about 3-log units for SaVs and 2-log units for RVs, which means that removal
262 efficiency of SaVs and RVs in the AO MBR process was more than 3-log and 2-log
263 units, respectively. Main removal mechanisms such as adsorption to MLSS, or
264 rejection by the membranes, are common to the three viruses, but the extent of
265 removal by each mechanism may vary.

266 Following the first year's observations, to further investigate removal of the
267 three viruses, oxic mixed liquor samples were collected during the second
268 monitoring period (i.e. from October 2014 to May 2015) in addition to influent and
269 effluent samples. NoV GI, NoV GII, and SaV levels in the solid phase of mixed
270 liquor samples were significantly higher than those in the liquid phase ($P < 0.01$, t-
271 test, **Figure 2**), indicating that NoVs and SaVs from influent were efficiently
272 adsorbed to MLSS in the bioreactor. In contrast, RV concentrations between liquid
273 and solid fractions were comparable, indicating that RVs were poorly associated
274 with MLSS. Although the contribution of recycled sludge to the virus concentration
275 in solid phase is unknown, the accumulation of viruses was not observed in the
276 bioreactor, suggesting that some parts of them were degraded or discharged from the
277 bioreactor as waste sludge (Wu et al., 2010; Sima et al., 2011).

278

279 **Adsorption and removal experiment.** To test the hypothesis that differences in the
280 adsorption of viruses could influence removal through the MBR process, we
281 performed a bench-scale adsorption and removal experiment. Mean RNA extraction

efficiency of MgV, which was added to each filtrate just before RNA extraction, was 117% (range: 73−190%) and no significant difference in the efficiency was observed between the inoculum and each sample type (i.e. 1, 5, 10, 20, 30, and 60 min post inoculation).

The concentrations inoculated into beakers were checked before each experiment and were 3.8 ± 0.2, 4.9 ± 0.7, 5.8 ± 0.1 and 5.6 ± 0.4 $\log_{10}$ RNA copies/mL of NoV GI, NoV GII, SaV, and RV respectively. The concentrations of viruses recovered from the filtered oxic mixed liquor were 3.7 ± 0.2, 4.7 ± 0.4, 5.4 ± 0.2 and 5.4 ± 0.8 $\log_{10}$ RNA copies/mL for NoV GI, NoV GII, SaV, and RV respectively, and the inhibitory effect of filtered oxic mixed liquor on RNA extraction (i.e. insufficient lysis of viral particles or capture and purification of nucleic acids by magnetic silica beads) was not observed. Stability of viral particles in the filtered oxic mixed liquor at 20°C over 24 h was checked in two different experiments and no variation in concentration was observed (data not shown). These controls indicate that the decrease of virus concentrations related to the time course in the experiments is linked to adsorption to MLSS. Another important control was to evaluate variability in the adsorption experiments. For three experiments (April, May, June 2015), the adsorption experiment was done in triplicate, at the same time by three operators. Standard deviations (SD) observed for the triplicate experiments were significantly smaller than SD observed for the eight different experiments ($P < 0.01$, t-test), except for NoV GI which showed similar variability between and within experiments ($P = 0.18$).

The NoV GI.1 strain was efficiently adsorbed to MLSS and virus concentration in the bulk solution was reduced by 0.6 ± 0.1 log after 1 min of mixing and decreased by 1.5 log after 60 min (**Figure 3**). Adsorption of the NoV

307 GII.4 strain was similar with that of GI.1 strain; where the LRV was 0.7 ± 0.3 and
308 increased to 1.6 ± 0.4 after 60 min of mixing (**Figure 3**). The SaV GI.2 strain was
309 also efficiently adsorbed to MLSS and the LRV was 1.2 ± 0.4 after 60 min of
310 stirring. In contrast, the RV G1P[8] strain was less adsorbed to MLSS and the LRV
311 (0.3 ± 0.2) was significantly lower than those of NoV GI.1, NoV GII.4, and SaV
312 GI.2 strains after 1 min of mixing ($P < 0.01$, t-test, **Figure 3**). The difference
313 between the RV G1P[8] strain, and the NoV GI.1 and GII.4 strains, was statistically
314 significant up to 30 min of mixing ($P < 0.01$, t-test). After 60 min of stirring, the
315 LRV was 0.9 ± 0.5 for the RV G1P[8] strain. The results observed in the bench-scale
316 experiments were consistent with those in the full-scale MBR plant and confirmed
317 that RVs were less efficiently adsorbed to MLSS and less efficiently removed
318 compared to NoVs and SaVs.
319
320 **Influence of pH.** The pH is known to have an influence on viral adsorption to
321 particles and an additional adsorption experiment was performed using pH-modified
322 oxic mixed liquor samples. For the NoV GI.1 and RV G1P[8] strains, removal due to
323 the adsorption to MLSS was not impacted by modification to pH 9, whereas NoV
324 GII.4 and SaV GI.2 strains were less adsorbed to MLSS at pH 9. The acidic pH 4
325 had an impact for all four viral strains and the LRVs were double those at pH 7
326 (**Table 3**). The SaV GI.2 strain was removed particularly efficiently from the bulk
327 solution at pH 4, as well as the RV G1P[8] strain.
328
329 **Discussion**
330 The need for clean water is increasing, both for drinking water and food production
331 (Uyttendaele et al., 2015). Indeed, viral contamination of foods is clear, with

reported viral outbreaks now outnumbering bacterial outbreaks (Semenza et al., 2016). Several factors have contributed to this observation, including improved outbreak identification, viral pathogen detection, and regulations to control bacteria, but one important causative factor is contamination of environmental waterways with human enteric viruses (Campos et al., 2017; Jones et al., 2017). A sewer system collects pathogens from the surrounding community and as a consequence sewage is considered to represent the microbiome of human populations (Newton et al., 2015). These microorganisms, from healthy or sick people, are eliminated with varying efficiencies in wastewater treatment processes, depending on their physical properties. Environmental stability of non-enveloped viruses was identified as significant issue that may contribute to viral transmission (Geoghegan et al., 2016). Thus, it is imperative to improve wastewater treatment and to understand key parameters that may contribute to viral elimination. This approach will also assist with the development of guidelines to manage risks relating to wastewater reuse (Sano et al., 2016). However despite huge interest of improving wastewater treatment, knowledge on how viruses are removed are still scarce. This lack of data may be due to the lack of reliable method to detect and quantify human enteric viruses from such difficult samples that contain a huge diversity of microorganisms and inhibitory compounds impeding molecular approaches. Even if recent developments demonstrated the capacity to cultivate infectious NoV and RV on human intestinal organoids (Ettayebi et al., 2016; Saxena et al., 2016), up to now, only molecular methods such as real time RT-PCR can be used to study sewage samples. This approach has several limits that can be overcome by quality controls introduced at different steps of the samples analysis. Indeed detection of free RNA is unlikely after applying concentration steps based on PEG precipitation as it selects

357 particles (Lee et al., 1981; Atmar et al., 1995). This method combining elution and
358 precipitation was applied several times to analyze shellfish samples implicated in
359 outbreaks (Thebault et al., 2013). Additional controls such as taking into account the
360 presence of PCR inhibitors or applying criteria to eliminate deviations from the
361 standard curves allowed reliable real-time RT-PCR-based virus quantification in a
362 complex matrix (Le Mennec et al., 2016).

363 Microorganisms, especially viruses, tend to cluster in sewage and the
364 MBR process has been demonstrated to be efficient for the elimination of small
365 particles (Sima et al., 2011; Miura et al., 2015; Purnell et al., 2016). During a field
366 study aiming at evaluating the efficiency of a new wastewater treatment plant we
367 observed a poor reduction of RV concentrations compared to NoVs and SaVs
368 between influent and effluent. Although RV removal in an MBR process has not
369 been previously reported, the lower LRV of RVs compared to NoVs and SaVs has
370 been reported in conventional activated sludge processes (Qiu et al., 2015; Kitajima
371 et al., 2014). To determine if this result was reproducible, monitoring was repeated
372 for a second period and the difference between RV behavior and that of NoV and
373 SaV was even larger. Concentrations of viruses adsorbed to MLSS (solid phase) and
374 those contained in the bulk solution (liquid phase) showed the largest difference for
375 NoV GI, followed by SaVs and NoV GII, whereas no difference was observed for
376 RVs. To further investigate the adsorption efficiency of these different viruses, we
377 set up bench-scale adsorption experiments, that demonstrated a significantly less
378 adsorption of the RV G1P[8] strain to MLSS than other viruses, confirming the field
379 observations.

380 In the present study, a first approach of virus type-specific removal in a
381 full-scale AO MBR process was investigated to evaluate virus association with

MLSS. Indeed, after the observation that some viruses seemed to be more efficiently eliminated than others, and considering that viruses in sewage are either aggregated or attached to particles (da Silva et al., 2008), we compared the binding capacity of four strains that are representative of NoV GI and GII, SaV, and RV. Our study clearly demonstrates that the viruses display different behaviors, with efficient binding of both NoV strains to MLSS, and incomplete binding of the RV strain after 1 hour. Demonstrating that virus adsorption to MLSS contributes to the overall efficiency of viral removal in an MBR process may not be surprising but these different behaviors may explain differences in removal efficiency observed between NoV GI and GII (da Silva et al., 2007).

Surface charge and hydrophobicity play a significant role in the adsorption of viruses to solid surfaces as demonstrated for coxsackieviruses and echoviruses having different adsorption efficiencies (67−99.8%) to activated sludge (Gerba et al., 1980; Gerba et al., 1984). NoV GI and GII have different adsorption efficiencies to particles in untreated wastewater, and their binding capacity depends on pH and solution chemistry (da Silva et al., 2008; da Silva et al., 2011). Therefore, we speculated that the surface charge may be the key factor modulating the different behaviors observed in the monitoring and bench-scale experiments, as different isoelectric points (pI) were reported for NoV GI.1 (4.3−6.0) and GII.4 (4.2−6.9) (Michen and Graule, 2010; Samandoulgou et al., 2015), porcine SaV Cowden (5.4) (Wang et al., 2012), and simian RV SA11 (8.0) (Michen and Graule, 2010). Although the net surface charges of viral particles of four different strains in the oxic mixed liquor samples (pH, 6.9−7.4) were not measured in this study, their binding behaviors changed after pH adjustment of oxic mixed liquor samples. The LRVs decreased for NoV GII.4 and SaV GI.2 strains at pH 9 as they may gain more

407 negative charges compared to NoV GI.1 and RV G1P[8] strains. In contrast, at pH 4,
408 the LRVs increased for all four strains, in accordance with their theoretical gain of
409 positive charges. This result suggests that surface charge influences viral adsorption
410 to MLSS.

411 MLSS mostly consists of microorganisms and extracellular polymeric
412 substances (EPS) that contain both hydrophilic and hydrophobic sites on their
413 structure (Nguyen et al., 2012). Selective adsorption of organic pollutants in water
414 was demonstrated in the hydrophobic parts of EPS (Späth et al., 1998), and
415 hydrophobicity of the surface capsid protein may also be different among viruses.
416 Phage fr, which displayed the highest number of hydrophobic amino acids on the
417 external capsid surface compared to bacteriophage MS2 or ΦX174, presented the
418 highest log removal due to attachment to biomass (Chaudhry et al., 2015a). As for
419 RVs, the outer capsid spike protein VP4 conformationally changes into VP8* and
420 VP5* proteins after proteolytic cleavage by trypsin. This cleavage is required for RV
421 cell attachment (Denisova et al., 1999), and the hydrophobic part of VP5* is not
422 exposed prior to the cleavage (Kim et al., 2010). Thus, hydrophobic interaction
423 between RVs and MLSS may be weaker compared to the other three strains, which
424 can also explain the less efficient adsorption of RVs to MLSS. In addition to the
425 nonspecific interactions due to surface charge and hydrophobicity, specific
426 interactions between capsid proteins and MLSS can also contribute to viral removal
427 through the MBR process. For example, interaction with bacterial histo-blood group
428 antigens (HBGAs) resulted in genotype-dependent removal of NoVs in a
429 microfiltration process and A-like antigens were contained in the EPS region of
430 MLSS (Amarasiri et al., 2016). Interactions between the NoV protruding domain
431 and HBGAs, present on some bacteria or shellfish tissues, seem to play a role in

persistence of viral particles under stringent conditions such as heat stress or seawater environment (Miura et al., 2013, Li et al., 2015, Le Guyader et al., 2012). The effect of hydrophobicity on viral adsorption to MLSS, and the specific interaction between viral particles and HBGAs in MLSS, must be investigated in future studies.

In our study, both in situ and experimental bench-scale adsorption showed different behaviors among the main human gastroenteritis viruses, even if some limitations have to be considered. An unexpected observation was that NoVs were removed more efficiently than SaVs in the present study, which is inconsistent with our previous observation in France (Sima et al., 2011) and Japan (Miura et al., 2015). These discrepancies show that the virus removal process is complex and may be impacted by many parameters. Differences in viral genotypes or strains circulating in human populations, which vary from year to year, may partly explain our data and re-inforce the need to obtain more observations from different area of the world with clarifying the major genotypes detected in influent, mixed liquor, and MBR effluent samples. Other possible explanations include seasonal variations in the amount and composition of EPS and soluble microbial products (SMPs) in mixed liquor, due to changes in activity of activated sludge bacteria of temperature dependence (Ma et al., 2013) and possibly in composition of gut microbiome of human populations (Davenport et al. 2014; Newton et al., 2015), and their contributions in virus removal are challenging issues in further studies.

In conclusion, we investigated the removal of NoVs, SaVs, and RVs in a full-scale MBR plant during two gastroenteritis seasons and found that NoV removal was most efficient followed by SaVs and RVs. RV G1P[8] strain did not adsorb to MLSS as much as NoV GI.1, NoV GII.4 and SaV GI.2 strains in membrane

457 filtration experiments, confirming the observation in the full-scale process. Our
458 work showed that virus adsorption to MLSS contributes to the different removal
459 properties in the AO MBR process. Water quality will be a challenging issue for
460 human health in the upcoming decades. With an increasingly globalized and
461 industrialized society, pathogens can move rapidly through human populations and
462 wastewater treatment needs to be efficient to eliminate different pathogens.
463 Knowledge of the rejection of human viral pathogens from sewage is becoming
464 increasingly important. A better understanding on mechanisms will provide some
465 tools to eliminate emerging viruses, during wastewater treatment processes, essential
466 key to prevent virus distribution in environmental waters and limiting outbreaks.
467
468
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693

694 **Table 1**. Influent and effluent quality for the full-scale MBR plant

Parameter	Units	Influent		MBR effluent	
		Mean ± SD (range)	N	Mean ± SD (range)	N
pH	–	7.5 ± 0.1 (7.2–7.7)	15	7.5 ± 0.1 (7.3–7.8)	14
EC	µS/cm	740 ± 180 (580–1080)	15	590 ± 110 (460–870)	14
SS	mg/L	160 ± 77 (92–320)	9	2.3 ± 1.0 (2.0–5.0)	9
BOD	mg/L	90 ± 50 (43–190)	9	2.9 ± 0.3 (2.0–3.0)	9
COD	mg/L	270 ± 140 (150–590)	9	17 ± 2.3 (15–22)	9
TOC	mg/L	64 ± 34 (35–130)	7	7.0 ± 0.8 (5.8–8.0)	7
T-N	mg/L	32 ± 16 (18–67)	9	<2	9
NH_4-N	mg/L	22 ± 11 (12–46)	9	0.17 ± 0.16 (0.03–0.45)	9
T-P	mg/L	4.3 ± 1.9 (2.6–8.6)	9	0.53 ± 0.45 (0.26–1.7)	9
PO_4-P	mg/L	2.4 ± 1.2 (1.3–4.9)	9	0.50 ± 0.43 (0.21–1.60)	9
Cl^-	mg/L	95 ± 34 (57–140)	9	100 ± 32 (75–170)	9
Total coliforms	log CFU/100 mL	6.9 ± 0.3 (6.3–7.4)	9	<1–20	9
E. coli	log CFU/100 mL	6.3 ± 0.4 (5.7–6.8)	9	<1	9

695

696

Table 2. Detection of viruses in influent and effluent samples[a]

Sampling period	Sample type	No. of samples	Mean extraction efficiency [%]	NoVs		SaVs		RVs	
				No. of positive	GMC	No. of positive	GMC	No. of positive	GMC
Oct 2013 −May 2014	Influent	8	6.7	7	1.4×10^6	8	1.8×10^5	6	3.2×10^5
	Effluent	8	36	1	2.7×10^3*	3	5.5×10^2	3	3.2×10^3
Oct 2014 −May 2015	Influent	8	15	8	2.7×10^6	8	5.1×10^5	6	6.0×10^5
	Effluent	8	29	0	−	1	3.4×10^2*	1	1.3×10^3*
Total	Influent	16	11	15	2.0×10^6	16	3.0×10^5	12	4.4×10^5
	Effluent	16	32	1	2.7×10^3*	4	4.9×10^2	4	2.5×10^3

[a] A total of eight sets of influent and effluent samples were collected in each period. Concentrations are expressed as the GMC (geometric mean concentration of all positive samples) of RNA copies/L, or detected concentration when only one sample was positive (*).

701 **Table 3**. Log reduction values of viruses from the liquid phase of mixed liquor
702 samples with different pH values[a]

pH	Reaction time [min]	Log reduction value			
		NoV GI.1	NoV GII.4	SaV GI.2	RV G1P[8]
9	1	1.3	0.4	0.6	1.4
	30	1.6	0.7	0.4	1.3
	60	1.8	0.8	0.4	1.1
7	1	0.7	0.7	0.8	0.9
	30	1.7	1.3	1.0	1.5
	60	1.4	1.6	1.1	1.8
4	1	1.4	1.3	1.9	1.6
	30	>2.3[b]	2.7	3.1	4.1
	60	>2.3[b]	2.5	3.4	>4.5[b]

703 [a] Adsorption and removal experiment was performed two times and the mean log
704 reduction value was reported.
705 [b] Log reduction value was calculated assigning the detection limit (31 and 23
706 copies/mL for NoV GI.1 and RV G1P[8], respectively).
707

FIGURE LEGENDS

Figure 1. Scheme of the full-scale anoxic-oxic MBR process and sampling points (1)−(3).

Figure 2. Geometric mean concentrations of NoV GI, NoV GII, SaVs, and RVs in positive wastewater samples collected from October 2014 to May 2015. The detection limits were 900, 400, 490, and 460 copies/L for NoV GI, NoV GII, SaVs, and RVs, respectively. The error bars represent the standard deviations of the means and the numbers in parentheses represent the numbers of positive samples. **t-test, $P < 0.01$.

Figure 3. Log reduction values of NoV GI.1, NoV GII.4, SaV GI.2, and RV G1P[8] strains from mixed liquor liquid fractions. Mean log reduction values were calculated from eight independent measurements and the error bars represent the standard deviations.

Figure 1

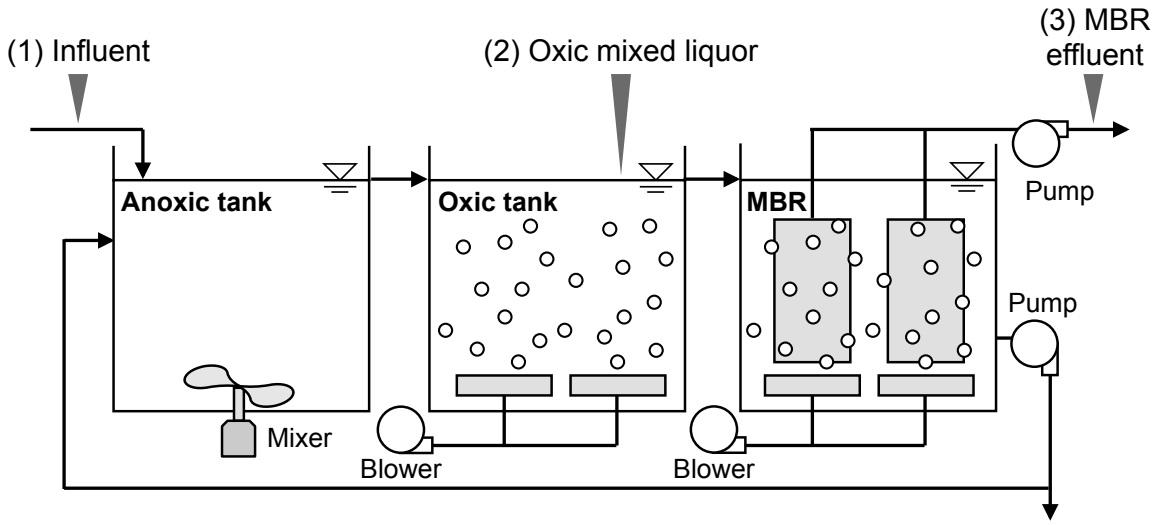

(1) Influent
(2) Oxic mixed liquor
(3) MBR effluent
Anoxic tank
Oxic tank
MBR
Pump
Pump
Mixer
Blower
Blower

Figure 2

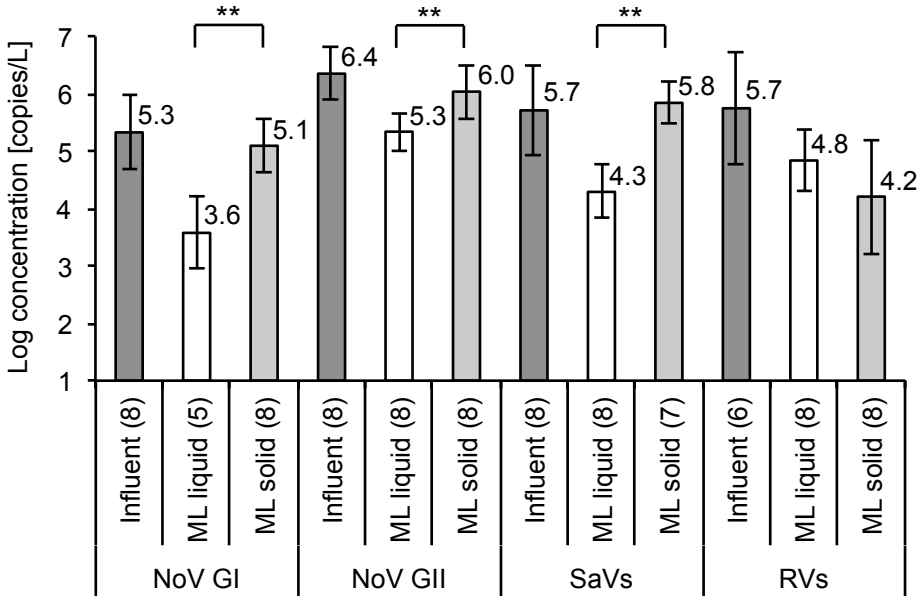
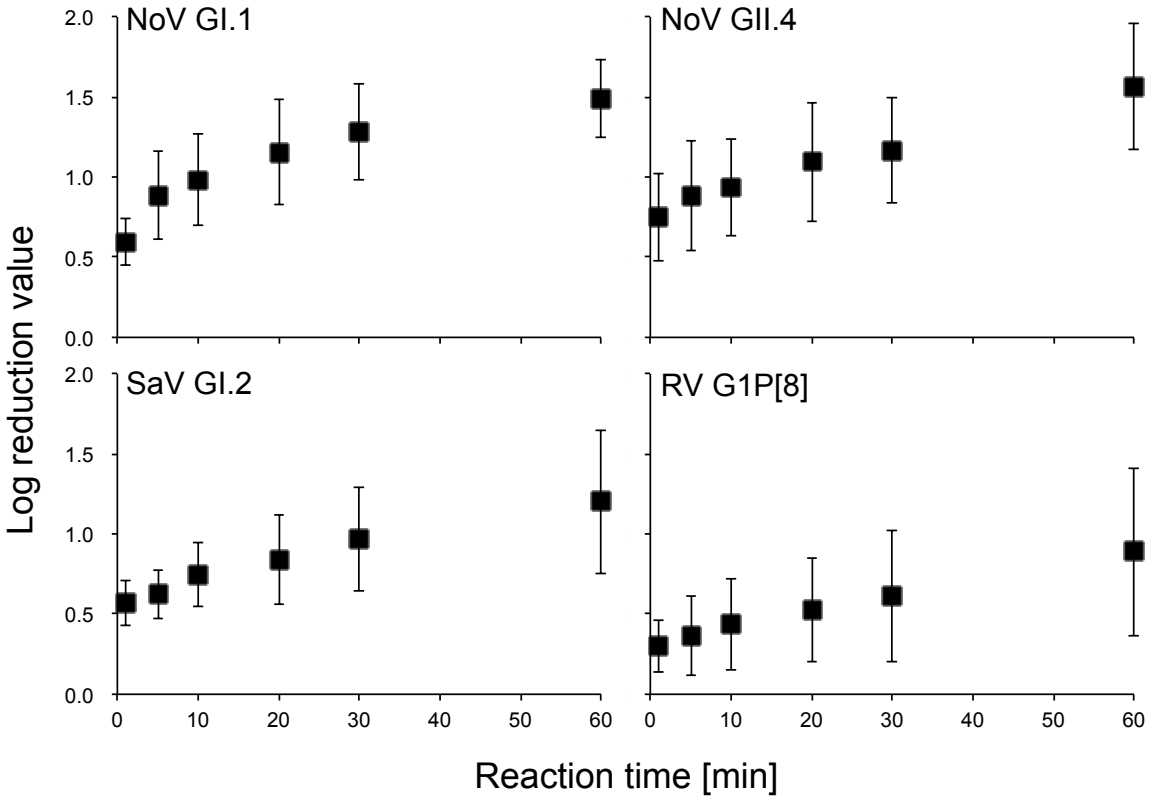

Log concentration [copies/L]
7
6
5
4
3
2
1
**
**
**
5.3
3.6
5.1
6.4
5.3
6.0
5.7
4.3
5.8
5.7
4.8
4.2
Influent (8)
ML liquid (5)
ML solid (8)
Influent (8)
ML liquid (8)
ML solid (8)
Influent (8)
ML liquid (8)
ML solid (7)
Influent (6)
ML liquid (8)
ML solid (8)
NoV GI
NoV GII
SaVs
RVs

Figure 3


NoV GI.1
NoV GII.4
SaV GI.2
RV G1P[8]
Log reduction value
Reaction time [min]
0.0
0.5
1.0
1.5
2.0
0
10
20
30
40
50
60