**Removal properties of human enteric viruses in a pilot-scale membrane bioreactor (MBR)
process**

Takayuki Miura¹, Satoshi Okabe¹, Yoshihito Nakahara^{2#}, and Daisuke Sano^{1*}

Each author's affiliation and mailing address:

¹ Division of Environmental Engineering, Faculty of Engineering, Hokkaido University, North 13,
West 8, Kita-ku, Sapporo, Hokkaido 060-8628, Japan

² Aqua Technology Administration, Mitsubishi Rayon Co. Ltd., 1-1-1, Marunouchi, Chiyoda-ku,
Tokyo, 100-8251, Japan

[#] Current affiliation

Wellthy Corporation, 4-8-1 Kojimachi, Chiyoda-ku, Tokyo, 102-0083, Japan

*Corresponding author

Address: Division of Environmental Engineering, Faculty of Engineering, Hokkaido University,
North 13, West 8, Kita-ku, Sapporo, Hokkaido, 060-8628, Japan. Tel/fax: +81-11-706-7597,
E-mail: dsano@eng.hokudai.ac.jp

Abstract

In order to evaluate removal properties of human enteric viruses from wastewater by a membrane bioreactor (MBR), influent, anoxic and oxic mixed liquor, and membrane effluent samples were collected in a pilot-scale anoxic-oxic MBR process for 16 months, and concentrations of enteroviruses, norovirus GII, and sapoviruses were determined by real-time PCR using murine norovirus as a process control. Mixed liquor samples were separated into liquid and solid phases by centrifugation, and viruses in the bulk solution and those associated with mixed liquor suspended solids (MLSS) were quantified. Enteroviruses, norovirus GII, and sapoviruses were detected in the influent throughout the sampling period (geometrical mean, 4.0, 3.1, and 4.4 log copies/mL, respectively). Enterovirus concentrations in the solid phase of mixed liquor were generally lower than those in the liquid phase, and the mean log reduction value between influent and anoxic mixed liquor was 0.40 log units. In contrast, norovirus GII and sapovirus concentrations in the solid phase were equal to or higher than those in the liquid phase, and higher log reduction values (1.3 and 1.1 log units, respectively) were observed between influent and anoxic mixed liquor. This suggested that enteroviruses were less associated with MLSS than norovirus GII and sapoviruses, resulting in lower enterovirus removal in the activated sludge process. Enteroviruses and norovirus GII were detected in the MBR effluent but sapoviruses were not in any effluent samples. When MLSS concentration was reduced to 50–60% of a normal operation level, passages of enteroviruses and norovirus GII through a PVDF microfiltration membrane were observed. Since rejection of viruses by the membrane was not related to trans-membrane pressure which was monitored as a parameter of membrane fouling, the results indicated that adsorption to MLSS plays an important role in virus removal by an MBR, and removal properties vary by viruses reflecting different adsorptive behavior to MLSS. Our observations suggested that sapoviruses are more associated with MLSS and removed more efficiently than enteroviruses and norovirus GII.

48 **Keywords**

49 Enterovirus; norovirus; sapovirus; MBR; mixed liquor suspended solids; adsorption

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52 **1. Introduction**

53 A membrane bioreactor (MBR) is a promising key technical element for municipal or domestic
 54 wastewater reclamation with many advantages such as high quality effluent, compactness, and
 55 short start-up time. Wastewater effluent discharge is the major source of human enteric viruses
 56 detected in natural waterways (Ueki et al., 2005), and waterborne diseases caused by enteric viruses
 57 are serious concerns in both developed and developing countries worldwide (Arvelo et al., 2012;
 58 Giammanco et al., 2014). Therefore, virus removal in wastewater treatment processes continues to
 59 receive attention due to the epidemiologically significant fact that pathogenic viruses in wastewater
 60 are highly diverse (Lodder and de Roda Husman, 2005).

61 Noroviruses (NoVs) and sapoviruses (SaVs), members of the family *Caliciviridae*, are
 62 major etiological agents of acute viral gastroenteritis. They are genetically diverse, and NoV
 63 genogroup I (GI), GII, and GIV and SaV GI, GII, GIV, and GV infect humans. Their virions are
 64 noneveloped and composed of icosahedral capsid with the diameter of 27–40 nm and a linear,
 65 positive-sense, single-stranded RNA genome (Green, 2007). Enteroviruses (EVs) belong to the
 66 family *Picornaviridae* and include coxsackieviruses, echoviruses, polioviruses, and the numbered
 67 enteroviruses. Members of the *Picornaviridae* are noneveloped viruses with a 30-nm diameter
 68 icosahedral capsid and a linear, positive-sense, single-stranded RNA genome (Racaniello, 2007).
 69 Enterovirus 71 and coxsackieviruses A6 (CVA6) and A16 are the major causative agents of hand,
 70 foot, and mouth disease (HFMD), and a growing epidemic of CVA6 infections has been reported
 71 worldwide (Shimizu and Nakashima, 2014). Recently, numerous large outbreaks of HFMD have
 72 occurred in the Asia-Pacific region (Ji et al., 2012), and infectious diseases caused by EVs, as well

73 as NoVs and SaVs, are public health concerns.

74 Removal mechanisms of enteric viruses in an MBR process have been investigated using
75 bacteriophages as indicators in bench or pilot studies. Based on the variation of phage removal
76 efficiencies observed in different MBRs, membrane pore size is considered to be one of the factors
77 affecting phage removal in bench-scale MBRs (Wu et al., 2010). In a pilot-scale MBR that had
78 been operated over a long period of time, however, differences in membrane pore size (0.04–0.2
79 μm) did not significantly affect removal of indigenous phages (Hirani et al., 2010). Removal of
80 seeded phage MS2 was different from indigenous phages (Hirani et al., 2010), and association of
81 phages with mixed liquor suspended solids (MLSS) and physically irreversible membrane fouling
82 (gel layer) that can be removed by chemical cleaning affected the phage removal (Hirani et al.,
83 2010; Marti et al., 2011). In bench studies, although significance of membrane fouling on phage
84 removal has been demonstrated (Lu et al., 2013), phage concentration was efficiently reduced also
85 in an activated sludge process (Wu et al., 2010). Phages adsorbed to activated sludge were not only
86 removed as waste sludge but also degraded by biomass (Wu et al., 2010). Meanwhile, knowledge
87 on human enteric virus removal in an MBR process is very limited. Variation of EV removal
88 efficiency was different from bacteriophages in a one-year pilot study (Ottoson et al., 2006), and it
89 is still not known that the factors that affect phage removal make similar contributions to human
90 enteric virus removal, and why removal efficiencies vary by viruses.

91 Surface electrostatic charge plays a major role in viral adsorptive behavior (Michen and
92 Graule, 2010). Coxsackieviruses and echoviruses, which are prevalent EVs in sewage and
93 environmental water (Chen et al., 2008; Iwai et al., 2006; Zheng et al., 2013), have isoelectric
94 points (pI) of 4.0–6.4 depending on types and strains, while NoVs and SaVs have pI of 5.5–6.0
95 (Michen and Graule, 2010; Wang et al., 2012). It has been demonstrated that EVs have different
96 adsorptive behavior to activated sludge ranging from 67–99.8% adsorption (Gerba et al., 1980). In
97 fact, different removal efficiencies have been reported for adenoviruses, EVs, NoVs, and SaVs in

full-scale MBR processes based on the monitoring studies up to 9 months (Kuo et al., 2010; Sima et al., 2011; Simmons et al., 2011). Consequently, we speculate that different interfacial characteristics of viruses cause different adsorptive behavior to MLSS, resulting in different virus removal properties in an MBR process. Understanding the virus removal properties will lead to an improved water quality management in wastewater treatment, and this must be elucidated.

In this study, we investigated removal properties of EVs, NoV GII, and SaVs in a pilot-scale anoxic-oxic (AO) MBR process treating real domestic wastewater over 16 months. We hypothesized that different adsorptive behavior of viruses to MLSS would result in the different removal properties in an AO MBR process. Thus, anoxic and oxic mixed liquor samples were collected, as well as influent and MBR effluent samples, and viruses in the bulk solution and those associated with MLSS were separately quantified by real-time PCR using murine norovirus (MNV) as a process control. Additionally, MLSS concentration was reduced to 50–60% of a normal operation level, and any change in virus removal performance was investigated. We also monitored trans-membrane pressure (TMP) as a parameter of membrane fouling and evaluated its contribution to enteric virus removal. Based on our observations, removal properties of EVs, NoV GII, and SaVs in an AO MBR process and their seasonal trends are discussed. This is the first study to investigate the removal properties of EVs, NoV GII, and SaVs in an MBR process for over a year in relation to virus association with MLSS.

2. Material and methods

2.1. Sample collection

Samples were collected from a pilot-scale anoxic-oxic (AO) membrane bioreactor (MBR) process treating a pre-screened real domestic wastewater (**Figure 1**). The total volume of the AO MBR process was 1.5 m³, and the oxic tank (0.75 m³) was equipped with a submerged hollow-fiber

123 PVDF membrane module with a nominal pore size of 0.4 μm . A total surface area of the membrane
 124 was 10 m^2 . The membrane flux was set at 0.4–0.8 m^3/d with an intermittent suction of 7 min on and
 125 1 min off. The hydraulic retention time in the whole process was 7.2 h, and the solid retention time
 126 (SRT) was 15 d. The mixed liquor suspended solids (MLSS) concentration in the oxic tank was
 127 maintained, and the excess sludge in the oxic tank was returned to the anoxic process at the return
 128 sludge recycle ratio of 200%. Wastewater samples (1 L each) were collected from four points in the
 129 AO MBR process (**Figure 1**). In total 16 sets of influent, anoxic and oxic mixed liquor, and MBR
 130 effluent samples were collected monthly from December 2010 to March 2012. Trans-membrane
 131 pressure (TMP) was recorded as a parameter of membrane fouling when the samples were collected.
 132 Influent and effluent quality parameters during the study period are listed in **Table 1**. Additionally,
 133 to evaluate the effect of MLSS concentration on virus removal in the oxic tank (i.e., activated
 134 sludge and membrane filtration processes), three additional sets of samples were collected 1–3 days
 135 after the MLSS concentration was reduced to 50–60% of an normal operation level in the oxic tank
 136 (**Table 2**). The samples were transported to the laboratory and stored at -20°C until processed.

137

138 **2.2. SS, pH, and electrical conductivity**

139 Suspended solids (SS) concentration was measured according to the standard protocols of the Japan
 140 Sewage Works Association (JapanSewageWorksAssociation, 1997). Briefly, a sample was filtrated
 141 with a glass fiber filter (1 μm pore size, 47 mm diameter, Whatman GF/B, GE Healthcare, Japan),
 142 and the filter was dried in an oven at 105°C for 2 h. pH and electrical conductivity (EC) were
 143 measured with respective meters (HM-30P and CM-31P, DKK-TOA, Japan).

144

145 **2.3. Process control virus**

146 An MNV strain S7-PP3, kindly provided by Dr. Yukinobu Tohya (Nihon University, Japan), was
 147 used as a process control virus for indigenous virus recovery from wastewater samples. MNV was

148 prepared using RAW 264.7 cells (ATCC TIB-71) according to the procedure described by Kitajima
149 et al (Kitajima et al., 2010).

150

151 **2.4. Preparation of viral RNA**

152 Ten microliters of MNV stock solution (geometrical mean of 5.7×10^6 copies; geometrical standard
153 deviation of 0.98) were added to 50 mL of all samples. Both the anoxic and oxic mixed liquor
154 samples were gently stirred for 30 min at room temperature, resulting in adsorption of some MNVs
155 to the suspended solids. Then the samples were centrifuged at $9,000 \times g$ for 10 min at 4°C , and the
156 pellet and supernatant were regarded as solid and liquid phases, respectively, in this study. Viral
157 particles in the solid phase were eluted by a vortex mixing in 50 mL of phosphate/citric acid buffer
158 with 10% beef extract and centrifugation at $10,000 \times g$ for 30 min at 4°C according to the method
159 of the US EPA (U.S.EPA, 2003). The eluted viruses, viruses in the liquid phase, influent and
160 effluent water samples were concentrated with the polyethylene glycol (PEG) precipitation method
161 (Lewis and Metcalf, 1988). Briefly, PEG 6000 (Wako Pure Chemical Industries, Japan) and NaCl
162 was added to 50 mL of samples to yield a final concentration of 8.0% (wt/vol) and 2.3% (wt/vol),
163 respectively, and the solution was gently stirred overnight at 4°C . The solution was transferred to a
164 50-mL centrifuge tube and centrifuged at $9,000 \times g$ for 30 min at 4°C . The supernatant was
165 discarded and the pellet was suspended in 5 mL of deionized distilled water with a vortex mixer.

166 Viral RNA was extracted from 200 μL of the virus concentrate using a NucliSENS kit
167 (bioMérieux, Tokyo, Japan) according to the manufacturer's instructions. All washing steps were
168 performed using the NucliSENS miniMAG, and viral RNA was recovered in 50 μL of elution buffer.
169 The extracted RNA was stored at -80°C .

170 The extracted RNA was treated with Recombinant DNase I (Takara Bio Inc., Japan).
171 Twenty microliters of the reaction mixture contained 10 μL of the extracted RNA, 2 μL of DNase I
172 Buffer, 2 μL of Recombinant DNase I, and 0.5 μL of Recombinant RNase Inhibitor (Takara Bio

173 Inc.). Thermal conditions consisted of enzyme reaction at 37°C for 30 min and inactivation of the
174 enzyme at 80°C for 10 min.

175

176 **2.5. Quantification of viral genome**

177 Complementary DNA (cDNA) was obtained through a reverse transcription (RT) reaction using a
178 PrimeScript RT reagent Kit (Takara Bio Inc.). Twenty microliters of the reaction mixture contained
179 4 µL of DNase-treated RNA, 4 µL of PrimeScript Buffer, 1 µL of PrimeScript RT Enzyme Mix I,
180 2.5 µM oligo dT primer, and 20 µM random hexamers. Thermal conditions consisted of RT at 37°C
181 for 15 min and 42°C for 5 min, and inactivation of the enzyme at 85°C for 5 s.

182 The cDNA concentrations of MNV, EVs, NoV GII, and SaVs were determined with the
183 quantitative real-time PCR method using an Applied Biosystems 7500 Real-time PCR System (Life
184 Technologies Japan, Tokyo) and previously reported primers and probes [ENREF 18](#) listed in
185 **Table S1** (Kageyama et al., 2003; Kitajima et al., 2010; Loisy et al., 2005; Monpoeho et al., 2000;
186 Oka et al., 2006). Each 25-µL PCR mixture contained 5 µL of cDNA, 12.5 µL of Premix Ex Taq
187 (Perfect Real Time, Takara Bio Inc.), each primer and probe at the concentrations specified in
188 **Table S1**. PCR conditions consisted of an initial denaturation at 95°C for 30 s, followed by 45
189 cycles of amplification with denaturation at 95°C for 15 s and annealing and extension at 60°C for
190 1 min. All amplification reactions were run in duplicate.

191 The cycle threshold (C_T) was defined as the cycle at which a significant increase in
192 fluorescence occurred. The variation in the real-time PCR assay was generally at most 1 C_T units.
193 When the difference in duplicate wells was $>1.5 C_T$ units, we repeated the real-time PCR assay
194 using triplicate wells or re-extracted viral RNA from the virus concentrate. The number of genome
195 copies in each reaction tube was calculated by comparing the C_T value to a standard curve
196 generated from a dilution series of plasmid containing each target region. Then the virus
197 concentration in the sample was calculated based on the volume of viral RNA analyzed and was

198 reported per milliliter. A C_T value less than 40 was regarded as positive in this study, and the
 199 detection limits were 69, 29, and 40 copies/mL of sample, for EVs, NoV GII, and SaVs,
 200 respectively. The MNV recovery efficiency was not used to adjust the concentrations of indigenous
 201 viruses since the recovery of exogenously-added MNV and indigenous viruses may be different.

202

203 **2.6. Statistical analysis**

204 Pearson's correlation analysis was performed by using IBM SPSS Statistics Ver. 19 to assess the
 205 correlations of virus concentrations between sample types.

206

207

208 **3. Results**

209 **3.1. SS, pH, and EC**

210 Levels of SS, pH, and EC were summarized in **Table 3**. SS concentration in the influent fluctuated
 211 ranging from 36 to 195 mg/L (mean $\pm$ SD = 83 ± 35 mg/L), but pH and EC were stable (7.4 ± 0.4
 212 and 42 ± 5.7 mS/m, respectively). The MLSS concentration in the oxic process ranged from 7,000
 213 to 14,000 mg/L during a normal sampling campaign, which was comparable to other MBR
 214 processes where enteric virus removal was previously investigated (Otto et al., 2006; Sima et al.,
 215 2011). pH and EC in the anoxic mixed liquor (6.4 ± 0.3 and 63 ± 22 mS/m, respectively) were
 216 similar to those in the oxic mixed liquor (6.4 ± 0.2 and 66 ± 25 mS/m, respectively). The MBR
 217 effluent presented low SS (<1.0 mg/L), and pH and EC were stable (7.3 ± 0.4 and 31 ± 4.3 mS/m,
 218 respectively).

219

220 **3.2. Recovery efficiencies of process control virus**

221 To evaluate the recovery efficiencies of viral RNA sample preparation and the inhibitory effect in
 222 RT-PCR, MNV was added to the samples and its recovery rate was monitored. The MNV recovery

rate varied among the sample types but was relatively stable within a sample type. The highest MNV recovery ($72 \pm 26\%$) was achieved for influent samples, whereas the lowest recovery ($16 \pm 8.8\%$) was obtained for MBR effluent samples. This result suggested that the PEG precipitation method could efficiently recover viral particles from the samples containing a certain level of SS as this method is based on flocculation.

The total MNV recovery efficiencies in the anoxic and oxic mixed liquor samples (liquid and solid phases combined) were as high as in the influent samples. As for the anoxic mixed liquor samples, total MNV recovery efficiency was $63 \pm 26\%$, and $59 \pm 25\%$ of added MNV was found in the liquid phase and $4.4 \pm 4.1\%$ in the solid phase. The added MNV was not efficiently adsorbed to MLSS in 30-min incubation even though MNV was detected in all solid phase samples. The total MNV recovery efficiency in oxic mixed liquor samples was similar to that in the anoxic mixed liquor, presenting $71 \pm 26\%$ ($65 \pm 22\%$ and $6.0 \pm 7.7\%$ in the liquid and solid phases, respectively). Significant inhibition in RT-PCR was not observed partly because only a small volume of sample was used for viral RNA sample preparation according to the preliminary experiments. Thus, the indigenous viruses in the collected wastewater samples can be quantified without excessive bias.

3.3. Detection and quantification of EVs, NoV GII, and SaVs

Detection rates varied with the types of samples and viruses, and EVs, NoV GII, and SaVs presented different behavior in the treatment processes. EVs were detected in the influent (detection rate, 68%) throughout the sampling period at a concentration ranging from 1.5×10^2 to 1.2×10^5 copies/mL (geometrical mean of 1.1×10^4 copies/mL) except during the winter months in 2012 (**Table 4** and **Figure 2A**), and seasonal variation of influent concentration was not clearly observed. EV concentrations in the solid phase of anoxic mixed liquor are generally lower than those in the liquid phase (**Figure 2B**), resulting in lower detection rates in the solid phase (47%) compared to the liquid phase (74%, **Table 4**). The mean log reduction value between influent and anoxic mixed

248 liquor (the liquid and solid phases combined) was 0.40 log units. A significant correlation was
 249 observed between the concentrations in the influent and in the anoxic mixed liquor samples (**Table**
 250 **S2**). Pearson's correlation coefficients were 0.61 for the liquid phase samples ($p < 0.05$, $N = 13$) and
 251 0.92 for the solid phase samples ($p < 0.01$, $N = 9$). However, a large variation was observed in the
 252 difference between the liquid and solid phase concentrations (**Figure 2B**). Concentrations in the
 253 oxic mixed liquor samples were weakly correlated with the influent concentration, and strongly
 254 followed those in the anoxic mixed liquor (correlation coefficient of 0.93, $p < 0.01$, $N = 13$, **Table S2**).
 255 No significant difference was observed between the concentrations in the anoxic and oxic mixed
 256 liquor samples, because almost all the EVs were rejected by the membrane and the mixed liquor
 257 was recycled. EVs were detected in the MBR effluent samples only twice in March and April 2011
 258 (1.2×10^3 and 6.1×10^2 copies/mL, respectively, **Figure 2A**). Although EVs were found in the oxic
 259 mixed liquor samples from June to December 2011 (**Figure 2C**), they were never detected in the
 260 MBR effluent in this period. Two of the three additional MBR effluent samples were positive for
 261 EVs, when the MLSS concentration was reduced to half of the normal operation concentration
 262 (**Table 2**), suggesting that MLSS may affect the EV removal by the membrane. Log removal
 263 efficiency of EVs in the whole process (i.e., comparing the influent and MBR effluent
 264 concentrations) ranged from >0.3 to >3.2 assigning the detection limit (69 copies/mL) to negative
 265 samples (**Table S5**). When EV was detected in the MBR effluent, mean removal efficiency was 1.6
 266 ± 0.4 log units ($N = 4$).

267 NoV GII was detected in 95% of the influent samples and 26% of the other respective
 268 samples including the MBR effluent (**Table 4**). Seasonal variation of NoV GII concentration in the
 269 influent was weakly observed, with the maximum concentration up to 10^4 copies/mL from
 270 December 2010 to February 2011 and the minimum concentration in August ($<10^2$ copies/mL,
 271 **Figure 2D**). The geometrical mean of influent concentrations was 1.2×10^3 copies/mL, which was
 272 approximately 1-log lower than that of EVs. Concentrations of NoV GII in the anoxic mixed liquor

273 samples were also lower than those of EVs, resulting in a number of negative samples except the
 274 first winter months (**Figure 2E**). The mean log reduction value between influent and anoxic mixed
 275 liquor (the liquid and solid phases combined) was 1.3 log units, which was larger than that of EVs.
 276 A significant correlation was not observed in concentrations between the influent and the anoxic
 277 mixed liquor (**Table S3**), which was different from EVs. Concentrations in the oxic mixed liquor
 278 samples were similar to those in the anoxic mixed liquor (correlation coefficient of 0.92, $p < 0.05$,
 279 $N=5$). NoV GII concentrations in the solid phase were generally equal to those in the liquid phase
 280 (**Figure 2E and 2F**), which was inconsistent with the case of EVs. NoV GII was found twice in the
 281 MBR effluent samples collected in winter months. It is notable that all three additional MBR
 282 effluent samples were positive for NoV GII at the concentration ranging from 4.5×10^1 to 1.1×10^2
 283 copies/mL (**Figure 2D**). Log removal efficiency of NoV GII in the whole process ranged from >0.2
 284 to >3.4 (**Table S5**), and mean value was 1.3 ± 0.8 log units when it was detected in the MBR
 285 effluent ($N=5$).

286 SaVs were detected in 68% of the influent samples, 37% of the liquid and solid phases of
 287 the anoxic mixed liquor, 42% of the liquid and solid phases of the oxic mixed liquor, and none of
 288 the MBR effluent (**Table 4**). The influent samples presented high SaV concentrations up to 10^5
 289 copies/mL (geometrical mean of 2.7×10^4 copies/mL) except those from September 2011 to
 290 January 2012 (**Figure 2G**). Influent concentrations showed weak seasonal variation as was
 291 observed for NoV GII. Despite the detection rates and concentrations comparable to EVs in the
 292 influent, SaVs were less frequently found in the anoxic and oxic mixed liquor samples and never
 293 detected in the MBR effluent. SaV concentrations in the solid phase were generally higher than
 294 those in the liquid phase (**Figure 2H and 2I**), which was different from the cases of EVs and NoV
 295 GII. As observed for NoV GII, SaV concentrations in the anoxic and oxic mixed liquor samples did
 296 not follow those in the influent, and the mean log reduction value between influent and anoxic
 297 mixed liquor was 1.1 log units. Unlike with EVs and NoV GII, no correlation was observed

298 between the anoxic and oxic mixed liquor concentrations (**Table S4**). Log removal efficiency of
 299 SaVs in the whole process ranged from >1.3 to >4.1 (**Table S5**).

300 During the study period, TMP ranged from 3 to 20 kPa (mean $\pm$ SD = 8.2 ± 6.1 kPa,
 301 **Table S5**), and variation of membrane fouling degree was observed. Passages of EVs and NoV GII
 302 through the PVDF microfiltration membrane were observed regardless of the membrane fouling
 303 degree (TMP range, 4–20 kPa, **Table S5 and S6**). The membrane module was replaced once at the
 304 end of January 2011 (9 days before the sample collection in February) and was cleaned with
 305 sodium hypochlorite solution once in April (43 days before the sample collection in May), which
 306 had no impact on all virus concentrations in the MBR effluent.

307

308

309 **4. Discussion**

310 In this study, the removal performance of human enteric viruses, EVs, NoV GII, and SaVs, of a
 311 pilot-scale AO MBR process was investigated in relation to virus association with MLSS for over a
 312 year. We observed different removal properties among viral families or genera in the activated
 313 sludge and membrane filtration processes, and rejection of viruses by the membrane was not related
 314 to the membrane fouling degree. When the MLSS concentration in the oxic tank equipped with a
 315 submerged membrane module was reduced to 50–60% of a normal operation level, passages of
 316 EVs and NoV GII through the membrane were observed. The results suggest that virus adsorption
 317 to activated sludge plays a significant role in virus removal by a PVDF microfiltration membrane.

318 EV concentrations in the solid phase of mixed liquor were generally lower than those in
 319 the liquid phase (**Figure 2B**). In contrast, NoV GII and SaV concentrations in the solid phase were
 320 equal to or higher than those in the liquid phase (**Figure 2E and 2H**), and higher log reduction
 321 values (1.3 and 1.1 log units, respectively) were observed between influent and anoxic mixed liquor
 322 compared to EVs (0.40 log units). The different behavior of EVs and caliciviruses (i.e., NoV GII

and SaVs) cannot be explained by the environmental stability of each virus, since it has been demonstrated that NoVs are more persistent than EVs (coxsackievirus B1 and polioviruses) in environmental water (Bae and Schwab, 2008; Charles et al., 2009). Although there is no report of SaV stability in environmental water, SaVs and human NoV showed similar resistance to heat and chlorine inactivation (Wang et al., 2012), and it is likely that they are similarly more stable than EVs in environmental water. Therefore, our observations indicate that EVs were less associated with activated sludge than caliciviruses resulting in the lower EV removal in the activated sludge process. Simmons et al. reported a similar observation in a full-scale MBR plant, where EV concentration increased in the membrane influent (mixed liquor) sample compared to the plant influent due to returning sludge, whereas NoV GII was significantly reduced in the activated sludge process by more than 2 log units (Simmons et al., 2011). Sima et al. investigated NoV concentrations in oxic mixed liquor and waste sludge samples and deduced the degradation of NoVs in the activated sludge process since buildup of NoVs was not observed in the reactor with SRT of 20–30 d (Sima et al., 2011), which also supports our observations. It is highly likely that NoV GII and SaVs, which were adsorbed to MLSS more efficiently than EVs, were degraded by biomass and/or removed as waste sludge in the pilot-scale AO MBR process.

Surface charge and hydrophobicity play a significant role in the adsorptive behavior of viruses to solid surfaces (Gerba, 1984), and coxsackieviruses and echoviruses have presented the different adsorption efficiencies (67–99.8%) to activated sludge (Gerba et al., 1980). Although there is no report of calicivirus adsorptive behavior to activated sludge, NoV GI and GII presented different adsorptive properties to particles in untreated wastewater (da Silva et al., 2008). Therefore, we speculate that different pI of EV, NoV, and SaV strains is one of the factors contributing to the different behavior of EVs and caliciviruses observed in the reactors. However, further study would have to be conducted to determine i) distribution of viral types or strains, ii) the net surface charges of viruses at pH 5.8–7.2 in the anoxic and oxic mixed liquor samples (**Table 3**), iii) the effect of

348 virus-particle association in untreated wastewater on viral adsorptive behavior to activated sludge.

349 In the present study, we hypothesized that an association with MLSS would play an
 350 important role in virus removal both in activated sludge and membrane filtration processes, and
 351 samples were collected after the MLSS concentration was reduced. In those additional MBR
 352 effluent samples, EVs and NoV GII were simultaneously detected (**Figure 2A and D**). The
 353 passages of EVs and NoV GII were not related to the membrane fouling degree (TMP range, 4–20
 354 kPa, **Table S5**), and the replacement and chemical cleaning of membrane module did not change
 355 the virus removal performance. These results suggest that biofilm accumulation (called as dynamic
 356 membrane) is not the dominant mechanism for the removals of EVs, NoV GII and SaVs, but MLSS
 357 concentration can be the important parameter. However, SaVs were not detected in the
 358 MLSS-reduced MBR effluent samples, and this leaves the possibility that the association with
 359 MLSS may not be the dominant removal mechanism for all enteric viruses. Since the additional
 360 sampling was conducted only in winter months, continuous investigations are required to confirm
 361 our observations.

362 Interestingly, EVs were not detected in the routinely collected MBR effluent samples
 363 from June to November 2011, although they were found in the influent and mixed liquor samples at
 364 high concentrations (**Figure 2A–C**). This observation could be possibly explained by the following
 365 two reasons. One is seasonal variation in the amount and composition of extracellular polymeric
 366 substances (EPS) and soluble microbial products (SMPs) in mixed liquor and membrane foulants
 367 due to temperature change (Gao et al., 2013; Ma et al., 2013; Miyoshi et al., 2009). The other
 368 reason is seasonal variation in prevalent EV types. The removal efficiency of EV from wastewater
 369 is thought to be dependent on the types and specific strains of EV. Soga-Momoki et al. reported that
 370 certain types of coxsackieviruses, echoviruses, and enteroviruses causing HFMD, herpangina, or
 371 respiratory illness were prevalent between June and November based on five year surveillance
 372 records in Japan (Soga-Momoki, 2009). Interactions between certain EV types and EPS or SMPs in

373 mixed liquor must be investigated in the future.

374 It is notable that the removal property of SaVs in the membrane filtration process was
 375 different from EVs and NoV GII since SaVs were not detected in any MBR effluent samples
 376 (**Figure 2G**). Although SaVs and NoVs are in the same family *Caliciviridae*, there may be different
 377 adsorptive properties to activated sludge or membrane foulants. It has been shown that human
 378 NoVs bind to histo-blood group antigens (HBGAs), which are considered susceptibility factors and
 379 cell attachment factors in NoV infections (Shanker et al., 2011), but SaVs do not recognize HBGAs
 380 (Shirato-Horikoshi et al., 2007). Different HBGA-binding properties or different adsorptive
 381 behavior in response to solution chemistry have been reported depending on NoV genotypes or
 382 strains, which is probably attributed to a different arrangement of amino acid residues on the capsid
 383 surface (da Silva et al., 2011; Shirato et al., 2008). We previously demonstrated specific adsorption
 384 of NoV particles to enteric bacteria via HBGA-like substances contained in the extracellular region
 385 (Miura et al., 2013). If mixed liquor or membrane foulants contain virus-specific adsorbents
 386 produced by bacteria (e.g., HBGA-like EPS for NoVs), this could also be a reason for the different
 387 virus removal properties in wastewater treatment processes. In fact, it has been reported that NoV
 388 GII is more efficiently removed in conventional wastewater treatment processes than NoV GI (da
 389 Silva et al., 2007; Hata et al., 2013; Nordgren et al., 2009).

390 The removal efficiencies of EVs (>0.3 to >3.2 log units), NoV GII (>0.2 to >3.4 log
 391 units), and SaVs (>1.3 to >4.1 log units) observed in this study were within the range of previously
 392 reported values. Ottoson et al. reported mean log removals of 1.79 ± 0.55 for EVs and 1.14 ± 0.88
 393 for NoVs in the pilot-scale MBR with a 0.4 μm pore size membrane (Ottoson et al., 2006). Sima et
 394 al. reported log removals of 0.9 to >6.8 for NoVs and >1.7 to >4.1 for SaVs in the full-scale MBR
 395 with a 0.45 μm pore size membrane (Sima et al., 2011). Since concentrations of human enteric
 396 viruses in MBR effluent samples are frequently below their detection limits, log removal efficiency
 397 has been calculated by using the detection limit of each assay (Sima et al., 2011; Simmons et al.,

2011). Therefore, log removal efficiency greatly varies depending on the influent virus concentrations and the detection limit of effluent samples, making it difficult to compare removal efficiencies among viruses or studies. In the present study, difference of log removal efficiencies in relation to size of viruses was not clearly observed partly because the difference between EVs (30 nm) and caliciviruses (27–40 nm) is not obvious. Simmons et al. reported greater removal of human adenoviruses (90–100 nm) compared to EVs and NoV GII in a membrane filtration process, and virus size may affect removal efficiency; however, higher membrane influent concentration was also observed for human adenoviruses (Simmons et al., 2011). When virus concentration in a treated wastewater sample is below the detection limit, accurate estimation of the removal efficiency is needed to evaluate the factors affecting the virus removal efficiency (Kato et al., 2013). Furthermore, different recovery efficiencies of indigenous viruses between influent and effluent samples can also affect the estimation of removal efficiency. If the recovery rate of MNV is equal to that of indigenous viruses, the total removal efficiency could be lower than the estimated values due to the lower MNV recovery in the MBR effluent ($16 \pm 8.8\%$) compared to the influent ($72 \pm 26\%$).

We used the PEG precipitation method to concentrate viral particles in all the wastewater samples with MNV as a process control. MNV recovery efficiencies were stable in each sample type and allowed us to quantify indigenous viruses without excessive bias. Sima et al. also applied the PEG precipitation method for wastewater samples using mengovirus as a process control (Sima et al., 2011). They reported recovery efficiencies of less than 10% for the mixed liquor samples (the liquid and solid phases combined) collected in an aeration tank (Sima et al., 2011), whereas MNV recovery in the oxic mixed liquor samples was $71 \pm 26\%$ in the present study. This difference could be explained by different interfacial characteristics between mengovirus and MNV. Virus recovery from environmental samples and quantitative genome detection are still challenging because of low concentrations of human enteric viruses and the presence of inhibitory substances (Miura et al.,

2011). In order to improve the assay sensitivity, we must sample higher volumes of water samples, efficiently concentrate, and extract RNA.

We for the first time presented different removal properties of EVs, NoV GII, and SaVs in the pilot-scale AO MBR process in relation to virus association with MLSS. Rejection of viruses by the dynamic membrane was not the dominant removal mechanism, but the association with MLSS was significant for the removal of EVs, NoV GII, and SaVs. This indicates that appropriate control of MLSS concentration can contribute to efficient virus removal in an MBR process. In this study, we selected EVs, NoV GII, and SaVs, which are the most important waterborne human pathogenic viruses of public health concern. The selected viruses are all non-enveloped and single-stranded RNA viruses of similar size (27–40 nm), and wastewater samples were collected for 16 months with only one summer period. Human enteric viruses contained in wastewater are highly diverse, and circulating virus strains vary from year to year. Since each virus removal property may be different, further studies are needed to accumulate data on other enteric virus removal in an MBR process and to improve the reclaimed wastewater quality.

5. Conclusions

EVs, NoV GII, and SaVs were detected in the influent wastewater samples throughout the sampling period at geometrical mean concentrations of 4.0, 3.1, and 4.4 log copies/mL, respectively, and behavior of enteric viruses in the pilot-scale AO MBR treatment processes was different among viruses. Our work suggests important implications as follows.

- EV types or strains contained in the collected samples were less associated with MLSS, which may explain the lower concentrations in the solid phase of mixed liquor than the liquid phase and the limited EV removal in the activated sludge process compared to NoV GII and SaVs. Removal efficiencies of EVs in the whole process ranged from >0.3 to >3.2 log units.

- NoV GII was highly associated with MLSS and was removed efficiently, resulting in the lower detection rate in mixed liquor samples. Removal efficiencies of NoV GII in the whole process ranged from >0.2 to >3.4 log units.
- SaVs were highly associated with MLSS, which may explain the efficient reduction in the activated sludge process and the complete rejection by the membrane. Removal efficiencies of SaVs in the whole process ranged from >1.3 to >4.1 log units.
- Rejection of viruses by the membrane was not related to TMP which was monitored as a parameter of membrane fouling, and dynamic membrane was not the dominant removal mechanism for EVs, NoV GII, and SaVs in the pilot-scale AO MBR treatment processes.
- When MLSS concentration was reduced to 50–60% of a normal operation level, passages of EVs and NoV GII through a PVDF microfiltration membrane were observed; suggesting that virus adsorption to MLSS plays an important role in virus removal by an MBR.

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611

1 **Table 1.** Influent and effluent quality of the pilot-scale anoxic-oxic MBR process.

Parameter	Units	Mean $\pm$ SD (range)	
		Influent	MBR effluent
BOD	mg/L	80 $\pm$ 33 (8.1–214)	0.9 $\pm$ 1.4 (0–7.5)
COD	mg/L	71 $\pm$ 34 (39–243)	4.5 $\pm$ 1.5 (3.3–11)
TOC	mg/L	<100	<10
T-N	mg/L	26 $\pm$ 5.9 (13–52)	11 $\pm$ 4.5 (4.8–33)
T-P	mg/L	3.0 $\pm$ 1.0 (1.4–7.6)	1.6 $\pm$ 1.0 (0.2–6.3)

2

3

4 **Table 2.** Change of MLSS concentrations for the collection of additional samples.

Sample	Concentration change			Sample collection	
	Date	MLSS [mg/L]		Date	MLSS [mg/L]
		Before	After		
Additional 1	Dec. 13, 2010	13,000	6,600	Dec. 16, 2010	7,000
Additional 2	Jan. 25, 2011	9,000	4,500	Jan. 26, 2011	4,800
Additional 3	Jan. 31, 2012	15,500	9,900	Feb. 1, 2012	9,900

5

Table 3. Levels of SS, pH and EC in wastewater samples.

	Mean $\pm$ SD (range)			
	Influent	Anoxic mixed liquor	Oxic mixed liquor	MBR effluent
SS [mg/L]	83 $\pm$ 35 (36–195)	Not measured	8,700 $\pm$ 2,200 (4,800–14,000)	<1.0
pH	7.4 $\pm$ 0.4 (6.8–8.4)	6.4 $\pm$ 0.3 (5.8–6.9)	6.4 $\pm$ 0.2 (6.0–7.2)	7.3 $\pm$ 0.4 (6.4–8.3)
EC [mS/m]	42 $\pm$ 5.7 (23–47)	63 $\pm$ 22 (36–96)	66 $\pm$ 25 (36–110)	31 $\pm$ 4.3 (21–37)

Table 4. Detection rates of viruses in wastewater samples.

Virus	Detection rate [%]				
	Influent	Anoxic		Oxic	
		Liquid	Solid	Liquid	Solid
EVs	68	74	47	74	47
NoV GII	95	26	26	26	26
SaVs	68	37	37	42	42

Figure 1

1
2 **Figure 1.** Scheme of the pilot-scale anoxic-oxic MBR process and sampling points (1)–(4).
3 Pre-screened domestic wastewater was fed as an influent, and the bioreactors consisted of the
4 anoxic and oxic tanks. The oxic tank was equipped with a submerged hollow-fiber PVDF
5 membrane module.

Figure 2

1
2 **Figure 2.** Concentrations of EVs (A–C), NoV GII (D–F), and SaVs (G–I) in wastewater samples. Black squares, influent; white squares,
3 MBR effluent; grey diamonds, solid phase of anoxic mixed liquor; white diamonds, liquid phase of anoxic mixed liquor; grey triangles,
4 solid phase of oxidic mixed liquor; white triangles, liquid phase of oxidic mixed liquor; dashed line, detection limit; ND, not detected.

Different removal properties of viruses in an MBR process