Quantification of human enteric viruses as alternative indicators of fecal pollution to evaluate wastewater treatment processes

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We investigated the potential use and quantitation of human enteric viruses in municipal wastewater samples of Winnipeg (Manitoba, Canada) as alternative indicators of contamination and evaluated the processing stages of the wastewater treatment plant. During the fall 2019 and winter 2020 seasons, samples of raw sewage, activated sludge, effluents, and biosolids (sludge cake) were collected from the North End Sewage Treatment Plant (NESTP), which is the largest wastewater treatment plant in the City of Winnipeg. DNA (Adenovirus and crAssphage) and RNA enteric viruses (Pepper mild mottle virus, Norovirus genogroups GI and GII, Rotavirus Astrovirus, and Sapovirus) as well as the *uidA* gene found in *Escherichia coli* were targeted in the samples collected from the NESTP. Total nucleic acids from each wastewater treatment sample were extracted using a commercial spin-column kit. Enteric viruses were quantified in the extracted samples via quantitative PCR using TaqMan assays.Overall, the average gene copies assessed in the raw sewage were not significantly different (p-values ranged between 0.1023 and 0.9921) than the average gene copies assessed in the effluents for DNA and RNA viruses and *uidA* in terms of both volume and biomass. A significant reduction (p-value ≤ 0.0438) of Adenovirus and Noroviruses genogroups GI and GII was observed in activated sludge samples compared with those for raw sewage per volume. Higher GCNs of enteric viruses were observed in dewatered sludge samples compared to liquid samples in terms of volume (g of sample) and biomass (ng of nucleic acids). Enteric viruses found in gene copy numbers were at least one order of magnitude higher than the *E. coli* marker *uidA*, indicating that enteric viruses may survive the wastewater treatment process and viral-like particles are being released into the aquatic environment. Viruses such as Noroviruses genogroups GI and GII, and Rotavirus were detected during colder months. Our results suggest that Adenovirus, crAssphage, and Pepper mild mottle virus can be used

confidently as complementary viral indicators of human fecal pollution.

1 **Quantification of human enteric viruses as alternative indicators of fecal pollution to**

2 **evaluate wastewater treatment processes**

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20   **ABSTRACT**

21   We investigated the potential use and quantitation of human enteric viruses in municipal

22   wastewater samples of Winnipeg (Manitoba, Canada) as alternative indicators of contamination

23   and evaluated the processing stages of the wastewater treatment plant. During the fall 2019 and

24   winter 2020 seasons, samples of raw sewage, activated sludge, effluents, and biosolids (sludge

25   cake) were collected from the North End Sewage Treatment Plant (NESTP), which is the largest

26   wastewater treatment plant in the City of Winnipeg. DNA (Adenovirus and crAssphage) and

27   RNA enteric viruses (Pepper mild mottle virus, Norovirus genogroups GI and GII, Rotavirus

28   Astrovirus, and Sapovirus) as well as the *uidA* gene found in *Escherichia coli* were targeted in

29   the samples collected from the NESTP. Total nucleic acids from each wastewater treatment

30   sample were extracted using a commercial spin-column kit. Enteric viruses were quantified in

31   the extracted samples via quantitative PCR using TaqMan assays.

32   Overall, the average gene copies assessed in the raw sewage were not significantly different (p-

33   values ranged between 0.1023 and 0.9921) than the average gene copies assessed in the effluents

34   for DNA and RNA viruses and *uidA* in terms of both volume and biomass. A significant

35   reduction (p-value ≤ 0.0438) of Adenovirus and Noroviruses genogroups GI and GII was

36   observed in activated sludge samples compared with those for raw sewage per volume. Higher

37   GCNs of enteric viruses were observed in dewatered sludge samples compared to liquid samples

38   in terms of volume (g of sample) and biomass (ng of nucleic acids). Enteric viruses found in

39   gene copy numbers were at least one order of magnitude higher than the *E. coli* marker *uidA*,

40   indicating that enteric viruses may survive the wastewater treatment process and viral-like

41   particles are being released into the aquatic environment. Viruses such as Noroviruses

42   genogroups GI and GII, and Rotavirus were detected during colder months. Our results suggest

43   that Adenovirus, crAssphage, and Pepper mild mottle virus can be used confidently as

44   complementary viral indicators of human fecal pollution.

46   **INTRODUCTION**

47   The human fecal waste present in raw sewage (RS) contains pathogens that can cause numerous

48   diseases. This can have a huge negative impact to public, aquatic health, and the economy

49   (Stachler, et al., 2017). Wastewater treatment plants (WWTPs) serve as protective barriers

50   between communities and the environment by reducing the organic matter present in wastewater.

51   Water quality is currently assessed using traditional markers such as coliforms and *Escherichia*

52   *coli*, leaving other microbes such as viruses largely unexplored. The North End Sewage

53   Treatment Plant (NESTP) in Winnipeg, Manitoba handles approximately 70% of the city's

54   wastewater treatment, serving over 400,000 people (City of Winnipeg, Water and Waste

55   Department, 2020). The treatment process at the NESTP first involves RS undergoing primary

56   treatment to remove solids. During the next treatment cycle, activated sludge (AS), a

57   heterotrophic cocktail of bacteria and protozoa, degrades organic matter present in solid waste.

58   The activated sludge (also known as biological treatment or secondary treatment) is the most

59   widely used process around the world to treat municipal wastewater (Racz et al., 2010; Scholz,

60   2016), and its use will likely continue due to its low cost and high efficiency. After the biological

61   treatment, wastewater is UV-disinfected and discharged as effluents (EF) into the river (City of

62   Winnipeg, Water and Waste Department, 2020). Approximately 200 million liters of EF are

63   discharged per day (City of Winnipeg, Water and Waste Department, 2020).

64   The main indicator of biological contamination used in wastewater treatment screening is *E. coli*,

65   a fecal coliform bacterium (Hood et al., 1983). It is present in the gut of humans and warm-

66   blooded animals, and widely used as the main indicator of fecal pollution during the wastewater

67   treatment process. Nevertheless, the use of only fecal bacteria indicator in wastewater excludes

68   other possible pathogen groups present, such as human enteric viruses. Targeting these viruses in

69   EF could be an alternative method to monitor the wastewater treatment process. Within this

70   context, Dutilh et al. (2014) targeted the DNA crAssphage genome in a human fecal sample.

71   With further bioinformatics testing, it was predicted that the crAssphage genome is highly

72   abundant, having been identified in 73% of human fecal metagenomes surveyed (Dutilh, et al.,

73   2014). The dynamics of crAssphage as human source marker has recently been explored in

74   fecally polluted environment (Ballesté et al., 2019; Wu et al., 2020). In terms of RNA viruses,

75   Pepper mild mottle virus (PMMV), a single-stranded RNA plant virus, has been identified highly

76   abundant in feces as a surrogate indicator to assess microbial quality for food, water and

77   wastewater (Zhang et al., 2006; Symonds et al., 2018).

78   In the present study, samples of RS, AS, EF, and biosolids/sludge cake (SC) from the NESTP

79   were collected (during fall 2019 and winter 2020) to investigate the potential of quantitating

80   human enteric viruses in wastewater samples as complementary indicators of contamination to

81   evaluate the processing stages of wastewater treatment. DNA enteric viruses in this study include

82   human Adenovirus (AdV) and cross-assembly phage (crAssphage), while RNA enteric viruses

83   include PMMV, Noroviruses (NoV) of the genogroups GI and GII, Astrovirus (AstV), Sapovirus

84   (SaV), and Rotavirus (RoV). We also studied the presence of a molecular marker for *E. coli*, the

85   *uidA* gene, in the samples collected from the NESTP. An overview of the workflow is illustrated

86   in *Fig. 1*.

87

88   **MATERIALS AND METHODS**

89   **Sample Collection.** A liter of RS, AS, EF, and 1 kg of SC were collected from the NESTP

90   during each sampling event. Each sample was sealed in a 1-L sterile polyethylene container lined

91   with a sterile plastic bag. Samples were collected on October 22nd, 2019 (Event 1) and November

92   28th, 2019 (Event 2) in the fall season. In the winter season, samples were collected on December

93   18th, 2019 (Event 3) and February 6th, 2020 (Event 4). SC samples were collected earlier in the

94   day during Events 3 and 4. All samples were kept at 4°C and processed within 24 hours of

95   collection.

96

97   **Ultrafiltration of Wastewater Samples.** Each wastewater treatment sample (RS, AS, and EF),

98   including Millipore Milli-Q water as a negative control, was first filtered via a funnel and

99   cheesecloth to remove any solid waste or debris. Next, 140 mL of each wastewater sample was

100 concentrated using an ultrafiltration method with Centricon Plus-70 filter units of 30 KDa

101 molecular-weight cutoff (Millipore Corporation, Billerica, MA, USA). The ultrafiltration process

102 used a sterile glass pipette, where 70 mL of each wastewater sample was added into their

103 correspondingly labeled sample filter cup pre-assembled with the filtrate collection cup. Each

104 assembly was then sealed with a cap. The Centricon Plus-70 assemblies were placed into a

105 swinging bucket rotor and centrifuged at 3000 x g for 30 minutes at 20°C. Subsequently, the

106 filtrate was discarded, and the remaining 70 mL of the samples was added into their

107 correspondingly labeled sample filter cup pre-assembled with the filtrate collection cup. Samples

108 were spun at the same speed and temperature for 45 minutes. After centrifugation, the sample

109 filter cup was separated from the filtrate collection cup. The concentration collection cup was

110 then turned upside down and placed on top of the sample filter cup. The device was carefully

111 inverted and placed into the centrifuge. Centricon Plus-70 filter units were centrifuged at 800 x g

112 for 2 minutes at 20°C. After this step, the concentrated sample was collected from the

113 concentration cup via a micropipette. The final volume was measured for each wastewater

114 sample. If needed, 10 mM Tris-HCl, pH 8.5 buffer (Qiagen Sciences, Maryland, MD) was added

115 to the concentrate to make up a total volume of 250 μL. If the final volume of the concentrate

116 was over 250 μL, Tris buffer was not added. Aliquots containing 250 μL were made and stored

117 at 4°C and processed within 24h.

118

119 **Sludge Cake Preparation for Ultrafiltration.** To remove cells and virus particles from the SC

120 samples, a 1X phosphate-buffered solution (PBS) with 0.15M NaCl, 0.05% Tween-20, and pH

121 7.5 was used. Approximately 30 g of SC sample per sampling event (Events 3 and 4) was

122 collected and divided into six Falcon tubes for each event (~5-6 g per tube). Approximately 30

123 mL of PBS was added to each tube. The Falcon tubes filled with SC samples were homogenized

124 at constant agitation for 15 minutes at 2500 rpm in a vortex mixer. These tubes were then

125 centrifuged at a speed of 4500 x g for 50 minutes. The supernatant from each tube was

126 subsequently recovered and transferred to a new sterile Falcon tube. For each sample event, 140

127 mL of supernatant was used for ultrafiltration as described previously.

128

129 **Nucleic Acid (DNA/RNA) Extraction and Fluorometric Assessment.** Once the final volume

130 of concentrate was collected from each wastewater sample, the sample was pretreated with

131 InhibitEX buffer (Qiagen Sciences, Maryland, MD) as indicated by the manufacturer. Then,

132 QIAamp MinElute virus spin kit (Qiagen Sciences, Maryland, MD) was used to extract total

133 nucleic acids from each wastewater sample as per the manufacturer's instructions, which

134   included the use of Qiagen Protease and carrier RNA (Qiagen Sciences, Maryland, MD).

135   Samples were eluted in 75 μL of Buffer AVE (Qiagen Sciences, Maryland, MD), quantified, and

136   stored at -80°C for downstream processes. The nucleic acid concentration and purity were

137   assessed using Qubit dsDNA high sensitivity and RNA assay kits in a Qubit 4 fluorometer

138   (Invitrogen, Carlsbad, CA, USA). Qubit results can be found in Supplementary Materials (*Table*

139   *S1*).

140

141   **Quantitative PCR Primers, Probes, and gBlocks Gene Fragments.** *Table 1* summarizes the

142   primers and probes used in this study. Forward and reverse primers listed in *Table 1* were used in

143   the Primer-BLAST tool to extract gene target regions (Ye, et al., 2012). Extracted regions were

144   then uploaded to the Geneious software to verify oligonucleotide sequences associated to the

145   flanking regions and probe. The generated sequences were sent to Integrated DNA Technologies

146   (IDT, Inc., Coralville, Iowa, USA) to generate the desired gBlocks constructs. IDT manufactured

147   all the primers used for quantitative PCR (qPCR) and quantitative reverse transcription PCR

148   (RT-qPCR), as well as the probes Ast-P, Ring1a.2, and Ring 2.2 *(Table 1)*. However, probes

149   SaV124TP, SaV5TP, Tampere NSP3, AdV-P, PMMV-Probe, and 056P1 were manufactured by

150   Life Technologies (Carlsbad, CA, USA).

151

152   **Quantitative PCR Assays.** Taqman Environmental Master Mix 2.0 (Life Technologies,

153   Carlsbad, CA, USA) was used for qPCR assays involving DNA enteric viruses and *uidA*.

154   Taqman Fast Virus 1-Step Master Mix (4X) (Life Technologies, Carlsbad, CA, USA) was used

155   for RNA enteric viruses via RT-qPCR. Each 10 μl qPCR and RT-qPCR reaction contained 500

156   nM of each of the forward primer and the reverse primer and 250 nM of its designated probe

157   when targeting both DNA and RNA viruses. Five μl of Environmental Master Mix was utilized

158   in each qPCR reaction for targeting DNA viruses, while 2.5 µl of 4x Fast Virus Master Mix was

159   used in each RT-qPCR reaction for targeting RNA viruses. The *uidA* qPCR reaction consisted of

160   5 µl of Environmental Master Mix, 400 nM of each primer, and 100 nM of probe. All qPCR and

161   RT-qPCR reactions used 2 µl of template.

162   Each qPCR and RT-qPCR reaction were performed in triplicate on an ABI QuantStudio 5 PCR

163   system (Applied Biosystems, Foster City, CA, USA). The DNA enteric viruses (AdV and

164   crAssphage) and *uidA* were subjected to the following conditions: 50.0°C for 2 minutes and

165   95.0°C for 10 minutes followed by 40 cycles of 95.0°C for 15 seconds and 60.0°C for 1 minute.

166   The RNA enteric viruses (SaV, RoV, AstV, GI and GII NoV, PMMV) were subjected to the

167   following conditions: 50.0°C for 5 minutes and 95.0°C for 20 seconds followed by 40 cycles of

168   95.0°C for 3 seconds and 60.0°C for 30 seconds. Raw qPCR and RT-qPCR output files can be

169   found on GitHub (https://git.io/J8VJ6).

170

171   **Assessment of Ultrafiltration for Viral Recovery Efficiency.** Armored RNA (Asuragen, Inc.,

172   Austin, TX, USA), an artificial virus packed with a 1000-bp single-stranded fragment and

173   encapsulated in a protein coat, was used to assess recovery efficiency of the ultrafiltration

174   method employed herein. We spiked in 16000 copies of Armored RNA into 7.5 mL of

175   representative RS, AS, and EF samples from the NESTP. For the SC sample, 1.25 g of solid SC

176   was dissolved in 7.5 mL of PBS 1x then homogenized by vortexing at 2500 rpm for 15 minutes

177   and centrifuged at 4500 x g for 50 minutes. The supernatant was transferred to a new Falcon tube

178   to be undergoing the same treatment as the RS, AS, and EF samples. The 7.5-mL MilliQ

179   negative control also spiked with 16000 copies of Armored RNA. These five samples were first

180   filtered through cheesecloth. 0.5 mL was aliquoted from each filtrate for subsequent assessment

181   of recovery efficiency. The remaining volumes were subject to ultrafiltration using the Amicon

182   Ultra-15 Centrifugal Filter Unit (Millipore Corporation, Billerica, MA, USA). Again, 0.5 mL of

183   each flowthrough was stored for efficiency evaluation. Nucleic acid extraction of the retentate

184   was performed in a manner similar to that described above. The final elution volume was 30 μL.

185   Primers (381F: 5'- AGCCTGTCAATACCTGCACC-3' and 475R: 5'-

186   CACGCTTAGATCTCCGTGCT-3'), and probe (420P: 5' Cy5-

187   AGAGTATGAGAGGTCGACGA-TAO 3') were designed using Primer design tool of Geneious

188   Prime version 2021.1.1 (https://www.geneious.com) and targeted a 95-bp region within the

189   Armored RNA genome. This targeted 95-bp fragment was sent to Integrated DNA Technologies

190   (IDT, Inc., Coralville, Iowa) to synthetize a gBlock construct. Serial dilutions of this synthetic

191   fragment were used to generate standards and quantify gene copy numbers (GCNs) of Armored

192   RNA via RT-qPCR. DNA quantification using the same 95-bp fragment was also performed via

193   qPCR. Standards, samples, and non-template controls were run in triplicate.

194   Thermal cycling reactions were performed at 50°C for 5 minutes, followed by 45 cycles at 95°C

195   for 10 seconds and 60°C for 30 seconds on a QuantStudio 5 Real-Time PCR System (Life

196   Technologies, Carlsbad, CA, USA). For RNA assays, each 10-μl RT-qPCR mixture consisted of

197   2.5 μL 4X TaqMan Fast Virus 1-Step Master Mix (Life Technologies, Carlsbad, CA, USA), 400

198   nM each primer, 200 nM probe, and 2.5 μl of template, as well as ultrapure DNAse/RNAse free

199   distilled water (Promega Corporation, Fitchburg, WI, USA). For DNA assays, 5.0 μL Master

200   Mix was used.

201

202   **Assessment of Gene Copy Numbers by Volume and Biomass.** Gene copy numbers (GCNs)

203   were expressed in terms of sample (per mL or g of sample) and biomass (per ng of DNA or

204 RNA). GCNs per mL of sample were calculated as previously described by Ritalahti et al.

205 (2006). When calculating GCNs per mL of sample, the final volume recovered after filtering 140

206 mL of wastewater sample was used in the formula. For the SC samples, the mass of SC collected

207 was used in the formula to produce results in GCNs per g of sample.

208

209 **Collection of Metadata for Sampling Events.** To perform Principal Component Analysis

210 (PCA) and Spearman's rank correlation analysis for EF samples, metadata pertinent to the

211 sampling events was retrieved. Water quality parameters obtained from the NESTP were

212 combined with their October 2019 monitoring data (City of Winnipeg, Water and Waste

213 Department, 2019) to complete some of the missing fields. For each value not found in either

214 document, data interpolation was performed by taking an average of the corresponding values for

215 the days before and after the sampling event. In addition, the Government of Canada's historical

216 weather database was utilized to obtain the mean temperature on the sampling dates and the total

217 precipitation over three days before each sampling event (hereafter referred to as "precipitation")

218 (Environment and Climate Change Canada, 2021). The values for all parameters were

219 transformed using $\log_{10}$, except for precipitation due to the presence of zero values. These

220 variables were used with $\log_{10}$-transformed GCNs per mL sample for AdV, crAssphage, PMMV,

221 and *uidA* (targets with quantifiable qPCR and RT-qPCR readings for all replicates across all

222 events) as input for downstream analyses (PCA and Spearman's rank correlation analysis).

223

224 **Data Handling, Statistical Analysis, and Data Visualization.** Various applications were

225 employed to process data at different steps of the pipeline. Input data, such as output from the

226 quantitative PCR instrument, was subjected to manual formatting and cleaning in Microsoft

227   Excel, which was also used to calculate GCNs per mL or g sample and per ng nucleic acid.

228   GCNs and metadata were transformed using $\log_{10}$ function for analysis. GCNs across sampling

229   events were pooled, and then comparisons were conducted across treatment.

230   R (R Core Team, 2021) and its integrated development environment RStudio (RStudio Team,

231   2021) as well as Statistical Analysis System (SAS, version 9.4 for Windows) were utilized to

232   further process the data and perform statistical analyses and output visualizations. These

233   operations included general linear models and multiple comparison procedures using Tukey's

234   tests, PCA (corresponding biplots were created using the package *ggbiplot* version 0.55 (Vu,

235   2011)), and Spearman's correlation matrix using the package *Hmisc* version 4.5-0 (Harrell Jr.,

236   2021). The package *reshape2* version 1.4.4 (Wickham, 2020) was used to reformat these

237   correlation matrices to enhance compatibility with other data-handling tools. Information about

238   other packages is provided in Supplementary Materials (*Table S2*). The R script used for analysis

239   can be found on GitHub (https://git.io/J8VUl). Additional t-tests were conducted to compare

240   water quality parameters between influent (RS) and discharges (EF).

241   Another software involved in data visualization was Tableau. Specifically, it was used to

242   generate boxplots for GCNs per mL or g sample and per ng nucleic acid, as well as the heatmap

243   representing the above-mentioned Spearman correlation matrix.

244   For all tests, a p-value of 0.05 was assumed to be the minimum level of significance.

245

246   **RESULTS**

247   From our assessment of the sample processing method used in this study, the recovery

248   efficiencies of Armored RNA as measured by RT-qPCR were between 14.03% and 15.94% for

249   RS, 2.63-4.36% for AS, 12.36-18.74% for EF, and 2.40-5.45% for SC. Meanwhile, DNA

250 recovery efficiencies were 32.48-40.87% (RS), 20.96-45.22% (AS), 14.14-20.15% (EF), and

251 23.41-68.42% (SC).

252 The GCN values for the DNA and RNA viruses and *uidA* were transformed into $\log_{10}$ form.

253 These values were run through a general linear model Tukey-Kramer analysis, and the means of

254 each wastewater processing stage for each target were analyzed. The GCNs were expressed in

255 terms of volume (mL) or weight (g) of sample and biomass (ng of nucleic acids). The result for

256 the GCN representing triplicate values from the corresponding sampling event was visualized as

257 a circle in the box plots. We followed cut-off Ct values established by the Molecular

258 Microbiology & Genomics Team at the British Columbia Centre for Disease Control (2017a,

259 2017b). With these values, the presence of DNA and RNA viral gene copies and *uidA* in the

260 Milli-Q water (negative control) samples across all Events 1-4 were determined to be negative.

261 The boxplots in *Figs. 2-6* indicate the minimum, first quartile, median, third quartile, and

262 maximum of the GCNs of each wastewater treatment sample across all events.

263 Overall, the average GCNs of the DNA and RNA enteric viruses assessed in RS, EF and SC

264 were significantly and consistently higher (p<0.05) compared to AS in terms of both volume and

265 biomass (*Fig. 2-3, S1* and *S2*). Average values of AdV ranged from 6.5 (AS) to 370.3 (SC)

266 GCNs per ml or g of sample (*Fig. 2A*), while that RS and EF had on average 33.1 and 38.5

267 GCNs per ng DNA (*Fig. 2B*). Moreover, crAssphage ranged from 264 (AS) to 65,388 (SC)

268 GCNs per ml or g of sample (*Fig. 2C*). Except for AS, crAssphage values in terms of biomass

269 were unaltered across wastewater processes (*Fig. 2D*). In terms of RNA viruses, PMMV were

270 observed in higher values (p-value ≤ 0.0001) in SC compared to other treatments (*Fig. 3*).

271 Average values for SC were 6,478.1 copies per g and 189.2 copies per ng of sample. Similar to

272 GCNs of AdV and crAssphage, lower values were observed in AS. On the other hand, values for

273   *uidA* per volume were not significant (p-value $\geq$ 0.3716), we observed in higher and significant

274   (p$\leq$0.0273) numbers of *uidA* per biomass in RS and EF compared to SC (*Fig. 4*). No other

275   significant differences were detected for *uidA*. For all the aforementioned targets, there was a

276   relatively higher number of gene copies observed in the EF across all events compared to AS

277   samples.

278   NoV GI and GII were also targets for our study. Boxplots of their GCNs across the different

279   wastewater stages and events 1-4 can be found in Supplementary Materials (*Fig. S1*). Norovirus

280   GI was below RT-qPCR detection limits for all samples (RS, AS, and EF) during the events 1

281   and 2 (Fall season). Values were only detected during events 3 and 4 (Winter season). When

282   detected NoV GI GCNs were higher (p-value < 0.0001) in SC, EF and RS compared to AS.

283   Values ranged between 0.4 (AS) to 51.8 (SC) per ml or g of sample, while that in terms of

284   biomass NoV GI GCNs were observed between 0.05 (AS) and 2.1 (EF). In addition, NoV GII

285   GCNs for all samples collected in Event 2 and AS samples in Events 3 and 4 (Winter season)

286   were also below the detection limits (*Fig. S1*). Among the quantifiable samples, statistically

287   significant GCN differences in terms of volume/mass and biomass were calculated for the pairs

288   of AS-EF (p-values were 0.0129 and 0.0117, respectively), AS-RS (p-value=0.0223 per volume),

289   and AS-SC (p-value < 0.0001 for both). No other significant differences were detected among

290   treatments for GCNs of NoV GII.

291   RoV gene copies across the various wastewater treatment stages from Event 1 to 4 were also

292   examined. The boxplots illustrating these results in terms of both sample and biomass can be

293   found in the Supplementary Materials (*Fig. S2*). RoV GCNs were only detected for wastewater

294   samples collected during Events 3 and 4. Higher values per volume and biomass were detected in

295   SC compared to the other treatments. RoV values ranged from 0.9 (AS) to 26.8 (SC) and 0.08

296   (AS) to 0.8 in terms of volume/mass and biomass, respectively. RoV GCNs were higher (0.0186)

297   in EF per volume and biomass compared to AS. Looking at the EF-SC pair, the mean GCNs

298   differed significantly in terms of volume/mass (p-value < 0.0001) but not biomass (p-value =

299   0.8510). No other significant differences were detected for RoV per volume/mass or biomass.

300   In the present study, there was no detection of gene copies for AstV and SaV (Sav1, Sav124, and

301   Sav5) in any of the wastewater samples across all events. In addition, to eliminate the possibility

302   of inhibitors or contaminants such as humic acids, additional qPCR and RT-qPCR tests using

303   bovine serum albumin (data not shown) were conducted with environmental samples (including

304   AS). No significant differences were observed between samples with and without the enzyme.

305   To investigate any potential relationship between collected data for EF samples, PCA was

306   performed with $\log_{10}$-transformed variables. We found that three components (PC1, PC2, and

307   PC3) explained 99.14% of the variance between variables. A summary of the weight of

308   components is included in the Supplementary Materials (*Table S3*). PC1 and PC2 were used to

309   create the biplot in *Fig. 5*. Biplots for PC1 versus PC3 (*Fig. S3*) and PC2 versus PC3 (*Fig. S4*).

310   Water quality parameters associated to UV-final effluents and used for PCA are summarized in

311   Table 2. Physico-chemical and biological wastewater parameters (metadata) are also included in

312   the Supplementary Materials. Water quality parameters available for RS and EF were also

313   compared. Organic pollutants in EF from the NESTP were significantly reduced for BOD (p-

314   value < 0.0001), COD (p-value < 0.0001), sCOD (p-value = 0.0046), TOC (p-value < 0.0001),

315   TP (p-value = 0.0069), TS (p-value = 0.0223), and TSS (p-value < 0.0001). No significant

316   differences were observed between RS and EF for NH_4-N (p-value 0.5006), and TN (p-value

317   0.3312).

318   Overall, based on the biplot of PC1 and PC2, samples from the four events were distinct from

319   one another, as point clusters of the four events can be seen occupying different quadrants. PC1,

320   explaining 54.9% of the observed variance, received a notable and positive contribution from

321   COD, cBOD, BOD, and TOC. Strongly negative contributors to PC1 were mean temperature,

322   grab filtered UVT, NO_x-N, and TS. These observations were supported by subsequent

323   Spearman's rank correlation analysis (*Fig. 6*), as COD, cBOD, BOD, and TOC demonstrated

324   strongly positive correlations with one another (rho ranging between 0.8000 and 0.9487) (p-

325   value < 0.005) and strongly negative correlations with mean temperature, grab filtered UVT,

326   NO_x-N, and TS (rho ranging between -1.000 and -0.8000) (p-value < 0.005). PC2 explained

327   31.9% of the variance between sampling events and showed a strong contribution from

328   crAssphage, *uidA*, and grab temperature. This observation was also supported by the Spearman's

329   rank correlation analysis showing these variables having strongly positive correlation with one

330   another (rho ranging between 0.7169 and 0.9218) (p-value < 0.0100). Additionally, in the biplot,

331   the axes representing *E. coli* and fecal coliform specifically pointed towards the same quadrant,

332   which was reflected in their moderately positive Spearman's coefficient (0.6325) (p-value =

333   0.0273). However, it is worth noting that *uidA* and *E. coli* exhibited a moderately weak negative

334   correlation (rho = -0.3073), although it was not statistically significant (p-value = 0.3313). The

335   two parameters with the strongest contribution against PC2 were grab pH and turbidity, which

336   was illustrated by the strongly positive Spearman's coefficient heatmap (rho = 0.8000) (p-value

337   = 0.0018).

338

339   **DISCUSSION**

340   The ultrafiltration method used in this study was assessed and the recovery efficiencies among

341   all samples for Armored RNA were estimated to be between 2.40-18.74% for RNA. This range

342   was comparable to other methods to concentrate viral particles such as JumboSep (13.38% ±

343   9.11%) or skimmed milk flocculation (15.27% ± 3.32%), spiked-in wastewater samples, and

344   using Armored RNA as internal control (Yanaç et al., 2021). Viral particles may have been

345   sorbed to biosolids present in wastewater samples, which were filtered out during the processing

346   stage. In this context, matrix has a significant effect for recovery of viral particles. When

347   compared to other environmental matrices such as surface water samples, recovery efficiency is

348   higher using ultrafiltration (tangential flow filtration) (32.6% ± 11.81%) and skimmed milk

349   flocculation (42.64% ± 15.12%) (Francis and Uyaguari, unpublished results). Water with high

350   turbidity and amounts of suspended solids tend to saturate filters and impact the recovery of viral

351   particles (Aslan et al., 2011; Karim et al., 2009; Uyaguari-Diaz, et al., 2016). Additionally, the

352   flow-through from ultrafiltration is another potential source of lost nucleic acid.

353   The GCNs were expressed in terms of biomass and volume (except for SC, which was expressed

354   in g of sample). The higher abundance and more stable signal over time of GCNs of AdV and

355   crAssphage (*Fig. 2*) as well as PMMV (*Fig. 3*) relative to the results of other assays make these

356   target more representative for conducting comparisons with *E. coli*. This persistent presence is

357   consistent with various longitudinal studies previously performed (Ballesté, et al., 2019; Farkas,

358   et al., 2018; Farkas, et al., 2019; Hamza et al., 2019; Nour, et al., 2021; Schmitz et al., 2016;

359   Tandukar et al., 2020; Worley-Morse et al., 2019; Wu et al., 2020).

360   A reduction of AdV, crAssphage, PMMV, and *uidA* GCNs was observed consistently in AS

361   samples (*Figs. 2-4*). This could be a result of virus particles and bacterial cells being sorbed to

362   larger fractions of organic matter that had been filtered by cheesecloth early in the sample-

363   handling process or retained in the filtration devices as previously described. It is important to

364   mention that samples were collected within a 2-hour period from RS→AS→EF consecutively

365 within each sampling event. The higher GCNs of viruses and *E. coli* observed in the EF may be

366 associated with the hydraulic retention time (12 hours) in the facility and may not reflect

367 wastewater treatment profiles at the time of collection. In other words, the EF samples may not

368 have been the corresponding RS samples collected earlier. The ideal situation would have seen

369 the former being collected 12 hours after the latter. It is best that similar logistical issues be

370 accounted for in future studies. Other variables to consider are the overflow of sewage from

371 rainy events and fluctuations in mixed liquor-suspended solids (Pérez et al., 2019). In our study,

372 there were 4.6 mm of precipitation for Event 1, which may have affected the results. In the PCA

373 analysis (*Fig. 5*), the vector for precipitation sharply denotes data points representing Event 1,

374 indicating a possible relationship. Precipitation was also found to have positive correlations with

375 grab flow (rho = 0.7746) and raw flow (rho = 0.7746) (*Fig. 6*). Nonetheless, further studies

376 and/or more replications are needed to corroborate the potential link between precipitation and

377 microbial counts.

378 Moreover, the duration of anaerobic sludge digestion is 25 days (City of Winnipeg, Water and

379 Waste Department, 2020). In this context, GCNs of *uidA* in the SC were reduced by anaerobic

380 digestion (*Fig. 4*). This may explain why the gene copies of *uidA* in terms of biomass were lower

381 in SC compared to RS and EF (p-value < 0.0273), but not when compared to AS (p-value =

382 0.0705). The average gene copies across all wastewater stages (RS, AS, and EF) for *uidA* were

383 not significantly different in terms of both volume and biomass. When compared to *uidA*, enteric

384 viruses were found to be at least one order of magnitude more abundant than the *E. coli* marker.

385 Other studies have reported *uidA* in RS at copy numbers between 2 to 4 orders of magnitude

386 higher that in our report (Jikumaru, et al., 2020; Mbanga et al., 2020). In a related study

387 conducted in the NESTP, we observed the same orders of magnitude for *uidA* gene marker

388   across wastewater treatments (Jankowski et al., 2022) as the ones reported here. Although we

389   acknowledge some sample may have been lost during the cheesecloth pre-filtration step, the

390   same order of magnitude observed in both studies reflects true positive values for *uidA* across

391   wastewater treatment processes.

392   GCNs of crAssphage in terms of biomass in SC were significantly higher than AS (p-value =

393   0.0123) (*Fig. 2*). For PMMV, SC samples had significantly more GCNs in terms of volume/mass

394   and biomass than samples from other parts of the wastewater treatment process (p-value ≤

395   0.0030) (*Fig. 3*). Since SC is the by-product of RS and AS using anaerobic digestion, this may

396   indicate that the presence of crAssphage and PMMV was lower in the wastewater being treated

397   in the AS, but higher in the solids. On the other hand, GCNs of AdV in terms of biomass were

398   not significantly different between the AS and SC samples (*Fig. 2B*). Meanwhile, plant viruses

399   such as PMMV remain more stable (in terms of biomass) during these digestion processes

400   (Jumat, et al., 2017).

401   The higher presence (p-value < 0.0001) of RoV gene copies in the EF and SC in terms of

402   volume/mass and biomass during the winter season (*Fig. S2* and *Table 2*) may indicate a higher

403   risk of transmission during cold seasons (Atabakhsh et al. 2020), since a greater presence of RoV

404   in EF and SC has been previously found during the colder months of the year (Li, et al., 2011)

405   and little decay occurs at the desiccation step (Sánchez & Bosch, 2016).

406   The negative results of SaV (Sav1, Sav124, and Sav5) across all wastewater treatment stages

407   during the fall and winter season are consistent with Varela et al. (2018) where samples were

408   retrieved from a wastewater treatment plant in Tunisia. Their results did not support the general

409   belief that the peak of detection of SaV occurs during the cold and rainy months of the year.

410   However, quantitative detection of SaV in wastewater and river water in Japan showed an

411   increased concentration of SaV in influents between winter and spring (December to May), but a

412   decrease in SaV concentration during the summer and autumn months (July to October)

413   (Haramoto et al., 2008). Yet another pattern of SaV presence was reported in France, as Sima et

414   al. (2011) found the virus to be readily detected in influents but had no clear variations in

415   numbers over the 9-month (October to June) duration of the study. Similarly, seasonal

416   differences in SaV concentrations were not statistically significant in a 3-year study conducted

417   by Song et al. (2021) in China between 2017 and 2019. As a result, there are other factors that

418   can influence wastewater SaV concentrations. For example, it has been hypothesized that

419   isoelectric point could affect how viruses and their different strains behave in bioreactors (Miura

420   et al., 2015). Monitoring over a time period longer than our current study would likely shed more

421   insight into the seasonal variation in the presence of SaV in wastewater.

422   The gene copies of NoV GI and GII were below the detection limit in many of the AS samples

423   (in terms of both volume and biomass), but still relatively abundant in the other samples (*Fig.*

424   *S1*). A possible explanation for the greatly reduced viral GCNs in AS samples is the high

425   efficiency with which NoV GI and GII are removed, a notion supported by literature (Ibrahim et

426   al., 2020; Kitajima et al., 2014; Schmitz et al., 2016). Furthermore, considering the observation

427   that these viruses were found in abundance in SC samples, another contributing factor could be

428   limitations in the sample collection process, which might not have adequately retrieved the slurry

429   part of the sludge where the viruses are found in greater numbers as they might have sorbed to

430   the larger fractions of the sludge solids. The relative abundance of NoV GI and GII gene copies

431   in RS, EF, and SC during the colder months (December and February) and the absence of NoV

432   GII in RS in November may be due to seasonal variability including intermittences (Guix et al.,

433 2002; Pérez et al, 2019; Sánchez and Bosch, 2016). In this context, the presence of NoV GI and

434 GII gene copies in RS during Events 3 and 4 (December and February) is consistent with a study

435 conducted by Flannery et al. (2012), in which the concentration of NoV GI and GII gene copies

436 in the influents of a wastewater treatment plant were significantly higher during the winter

437 months (January to March). During colder months, we observed mean (ambient) temperatures

438 averaged -17.05 °C (*Table 2*). This seasonal trend is also reflected colloquially through the

439 virus's sobriquet, the winter vomiting bug (Farkas, et al., 2021). Overall, GCNs of NoV GI and

440 GII did not seem to be reduced by the wastewater treatment process (*Fig. S1*).

441 High numbers of AstV gene copies (per liter) in sewage samples from the Greater Cairo area in

442 Egypt were observed at the end of autumn (daily mean temperatures ranged from 10.8 °C to 22.8

443 °C) and during the winter months (daily mean temperatures ranged from 6.8 °C to 17.0 °C) (El-

444 Senousy et al., 2007; Weather Spark, 2022), but the AstV concentrations tended to decrease as

445 temperatures increased. These results are different from our findings where there was no

446 detection of AstV in any of the wastewater treatment stages across all events. Mean (ambient)

447 and grab sample average temperatures during our study-period were -9.5 °C (range 2.7 °C to -

448 17.1 °C) and 13.6 °C (range 12.7 °C to 14.1 °C), respectively (Supplementary Materials and

449 *Table 2*). These results may be due to seasonal variability (Pérez et al., 2019) as well as reflect

450 the pattern of infection within the community under study (Corpuz et al., 2020).

451 Grab filtered UVT being inversely correlated with COD, cBOD, BOD, and TOC is consistent

452 with the widespread use of UV radiation to regulate microbial growth in a variety of medium,

453 including water (Raeiszadeh & Adeli, 2020). Furthermore, it had been suggested that UV is an

454 important influence to the survival of pathogens in wastewater environments, especially in cold

455 weather conditions, such as those found in Manitoba during the surveying period (Murphy,

456 2017). The NESTP uses UV as disinfection treatment and significantly reduces most organic

457 pollutants (RS vs EF). In the present study, we observed an incomplete removal of nitrogen (TN

458 and NH_4-N). Excess of these nutrients is associated to efficiency of the activated sludge process

459 and discharges can be toxic for aquatic organisms and/or cause algal blooms (Templeton &

460 Butler, 2011; Chahal et al., 2016). Moreover, virus-mediated transfer of nitrogen can occur from

461 heterotrophic bacteria such as the pool of microorganisms present in AS to primary producers in

462 aquatic environments (Shelford & Suttle, 2018; Chen et al., 2021). Further studies are needed to

463 evaluate the survival of enteric viruses in these reservoirs by using modification to the biological

464 treatment, the disinfection process and/or physical methods (i.e., filtration methods). Some of

465 these modifications may include fixed bed reactors (Sizirici & Yildiz, 2020), biofilm systems

466 such as membrane bioreactors, biofilters, biofiltration, and carriers (Zhao et al., 2019). Other

467 disinfection processes include the use of chlorine (liquid sodium hypochlorite solution, solid

468 calcium hypochlorite) or newer methods such as ozone (Mezzanotte et al., 2007; Abou-Elela et

469 al., 2012; Collivignarelli et al., 2018). Although microfiltration and ultrafiltration can be used to

470 reduce bacterial and protist pathogens, and enteric viruses (Chahal et al., 2016), membrane

471 foulants and fouling mechanisms occur in WWTP effluents (Nguyen et al., 2010).

472 There is a possibility that viral GCNs quantified in the EF may represent an overestimation of the

473 actual number of infectious viral particles since quantitative PCR detects both infective and non-

474 infective agents and UV treatment influences viral viability (Lizasoain et al., 2017). Thus, the

475 interpretation of these results must be performed with caution. Future studies could incorporate

476 culturable assays for a more complete and accurate evaluation as well as longer time series. On

477 the other hand, it is also possible that the non-enveloped enteric viruses (Corpuz, et al., 2020)

478 studied here survived the wastewater treatment process. Non-enveloped viruses are more

479   resilient than their enveloped counterparts in numerous environmental conditions and water

480   treatment processes (La Rosa et al., 2020). This is because of the latter group's envelope, which

481   contains receptors needed for infection; if the envelope is lysed, infection is not possible (La

482   Rosa et al., 2020). Various publications have noted the resilience of non-enveloped viruses after

483   wastewater treatment (Adefisoye et al., 2016; Campos & Lees, 2014; Farkas, et al., 2019;

484   Fitzgerald, 2015; Fong et al., 2010; Li, et al., 2021; Prevost, et al., 2015; Ruggeri, et al., 2015;

485   Varela, et al., 2018). In this context, we have consistently detected GCNs of AdV, crAssphage,

486   and PMMV in environmental surface waters receiving discharges from the NESTP, two other

487   WWTPs, and other areas radiating away from the WWTPs within the City of Winnipeg (Francis

488   and Uyaguari, unpublished data). Therefore, despite potential factors affecting interpretation, our

489   results still reflect the presence of several non-enveloped enteric viruses in EF and SC samples

490   with reasonable quantitative accuracy. While it is undeniable the key role that WWTPs have

491   played in reducing nutrient loading and maintain water quality, they are not designed to remove

492   all microbes, especially smaller fractions such as viruses, genetic material or micropollutants. In

493   this aspect, unproperly treated effluents or mismanagement of SC, often employed as fertilizers,

494   may represent a route for microbes including pathogens and their genetic material to be

495   transported into downstream waterways or introduced into other settings (i.e.,

496   urban→agricultural, urban→rural).

497

498   **CONCLUSION**

499   Our study's primary goal was to identify human enteric viruses with the potential to become

500   alternative indicators of fecal pollution. Towards that end, we found AdV, crAssphage, and

501   PMMV as more stable viral indicators of water quality due to their quantifiability illustrated in

502   this investigation and the literature. RNA viruses such as NoV GI and GII, and Rotavirus were

503   detected during colder months, while AstV and SaV could not be detected in any of the samples.

504   Regular monitoring of these organisms can be useful complements to current methods for

505   assessing wastewater treatment processes including seasonal viruses. Wastewater surveillance of

506   SARS-CoV-2 during the current pandemic has demonstrated the utility of early warning tools.

507   Such vigilance could be a helpful tool to assist public health efforts in the event of a viral

508   outbreak.

509   Our study indicated that enteric viruses may have survived the wastewater treatment process and

510   viral-like particles are possibly being released into the aquatic environment. Therefore, in

511   addition to such methods as UV radiation (which is currently used in the NESTP and was shown

512   in our study to be inversely correlated with biological parameters), we also suggest that WWTPs

513   consider implementing modifications and/or additions (disinfection processes) to their workflow

514   to reduce the number of viral particles being released into the aquatic environment.

515

516   **ABBREVIATIONS**

517   **AdV:** Adenovirus

518   **AS:** activated sludge

519   **AstV:** Astrovirus

520   **BOD:** biochemical oxygen demand

521   **cBOD:** carbonaceous biochemical oxygen demand

522   **COD:** chemical oxygen demand

523   **EF:** effluents

524   **GCN:** gene copy number

525   **NESTP:** North End Sewage Treatment Plant

526   **NH$_4$-N:** ammonium-nitrogen

527   **NoV:** Norovirus

528   **NO$_x$-N:** nitrogen oxides - nitrogen

529   **PCA:** Principal Component Analysis

530   **PMMV:** Pepper mild mottle virus

531   **PO$_4$-P:** orthophosphate as phosphorus

532   **RoV:** Rotavirus

533   **RS:** raw sewage

534   **qPCR:** quantitative PCR

535   **RT-qPCR:** quantitative reverse transcription PCR

536   **SaV:** Sapovirus

537   **SC:** sludge cake

538   **sCOD:** soluble chemical oxygen demand

539   **TN:** total nitrogen

540   **TOC:** total organic carbon

541   **TP:** total phosphorus

542   **TS:** total solids

543   **TSS:** total suspended solids

544   ***uidA*:** β-d-glucuronidase gene

545   **WWTP:** wastewater treatment plant

546 **AUTHOR CONTRIBUTIONS**

547 AG performed the experiments, analyzed the data, prepared the figures and tables, and wrote and

548 reviewed the drafts of the manuscript.

549 TL performed the validation experiments, analyzed the data, prepared the figures and tables, and

550 wrote and reviewed the drafts of the manuscript.

551 PJ performed the experiments, prepared table and reviewed the drafts of the manuscript.

552 KY performed the validation experiments and reviewed the drafts of the manuscript.

553 QY contributed the analysis tools and reviewed the drafts of the manuscript.

554 MUD designed the experiments, provided lead guidance during the experiments and analyses,

555 contributed the analysis tools, analyzed the data, prepared the figures and tables, and wrote and

556 reviewed the drafts of the manuscript.

557 All authors read and approved of the final manuscript.

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566

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Table 1. Primers and probes used in the present study.

Target	DNA or RNA	Primer/ Probe	Sequence (5'-3')	Genomic Target	References
Adenovirus 40/41	DNA	AdV-F	GCC TGG GGA ACA AGT TCA G	Hexon	Molecular Microbiology & Genomics Team, British Columbia Centre for Disease Control, 2017a
		AdV-R	ACG GCC AGC GTA AAG CG		
		AdV-P (Probe)	NED-ACC CAC GAT GTA ACC AC-MGB-NFQ		
Astrovirus	RNA	Ast-F	AAG CAG CTT CGT GAR TCT GG	Junction of polymerase and capsid	Molecular Microbiology & Genomics Team, British Columbia Centre for Disease Control, 2017a
		Ast-R	GCC ATC RCA CTT CTT TGG TCC		
		Ast-P (Probe)	Cy5-CAC AGA AGA GCA ACT CCA TCG CAT TTG-Tao-IBDRQ		
crAssphage	DNA	056F1	CAG AAG TAC AAA CTC CTA AAA AAC GTA GAG	Genomic base pair region: 14731 bp-14856 bp	Stachler, Akyon, Carvalho, Ference, & Bibby, 2018
		056R1	GAT GAC CAA TAA ACA AGC CAT TAG C		
		056P1	FAM-AAT AAC GAT TTA CGT GAT GTA AC		
Escherichia coli	DNA	784F	GTG TGA TAT CTA CCC GCT TCG C	*uidA*	Frahm and Obst, 2003
		866R	AGA ACG GTT TGT GGT TAA TCA GGA		
		EC807 (Probe)	FAM-TCG GCA TCC GGT CAG TGG CAG T-BHQ1		
GI	RNA	COG1F-flap	AATAAATCATAACGYTGGATGCGNTTYCATGA	Norovirus GI	Kageyama, et al., 2003; Wang, et al., 2019
		COG1R-flap	AATAAATCATAACTTAGACGCCATCATCATTYAC		
		Ring1a.2 (Probe)	6-FAM- AGATYGCGR/ZEN/ TCYCCTGTCCA -IBFQ		Molecular Microbiology & Genomics Team, British Columbia Centre for Disease Control, 2017b
GII	RNA	COG2F-flap	AATAAATCATAACARGARBCNATGTTYAGRTGGAT GAG	Norovirus GII	Kageyama, et al., 2003; Wang, et al., 2019
		COG2R-flap	AATAAATCATAATCGACGCCATCTTCATTCACA		
		Ring 2.2 (Probe)	JOE - TGGGAGGGY/ZEN/ GATCGCAATCT - IBFQ		Molecular Microbiology & Genomics Team, British Columbia Centre for Disease Control, 2017b
Pepper Mild Mottle Virus	RNA	PMMV-FP	GAG TGG TTT GAC CTT AAC GTT TGA	1878 bp-1901 bp[a] and 1945 bp-1926 bp[a]	Rosario, Symonds, Sinigalliano, Stewart, & Breitbart, 2009
		PMMV-RP	TTG TCG GTT GCA ATG CAA GT		
		PMMV-Probe	FAM-CCT ACC GAA GCA AAT G-MGB-NFQ		
Rotavirus Type A	RNA	Tampere NSP3-F	ACC ATC TWC ACR TRA CCC TCT ATG AG	Non-structural Protein 3	Zeng, et al., 2008
		Tampere NSP3-R	GGT CAC ATA ACG CCC CTA TAG C		
		Tampere NSP3 (Probe)	VIC-AGT TAA AAG CTA ACA CTG TCA AA-MGB-NFQ		
Sapovirus	RNA	SaV1F	TTG GCC CTC GCC ACC TAC	Junction of polymerase and capsid	Oka, et al., 2006
		SaV5F	TTT GAA CAA GCT GTG GCA TGC TAC		
		SaV124F	GAY CAS GCT CTC GCY ACC TAC		
		SaV1245R	CCC TCC ATY TCA AAC ACT A		
		SaV124TP (Probe)	FAM-CCR CCT ATR AAC CA-MGB-NFQ		
		SaV5TP (Probe)	FAM-TGC CAC CAA TGT ACC A-MGB-NFQ		

[a]Corresponding nucleotide position of GenBank accession number M81413 (PMMoV strain S)

Table 2. UV-treated final effluent water quality parameters measured in the North End Sewage Treatment Plant.^

Parameters	Sampling Events				Mean	SD
	Oct-22-2019 (#1)	Nov-28-2019 (#2)	Dec-18-2019 (#3)	Feb-6-2020 (#4)‡		
BOD (mg/L)	19.5†	13	18	26	19.13	4.64
cBOD (mg/L)	4	5	7	7	5.8	1.5
COD (mg/L)	51.5†	66	94	89	75.13	17.25
sCOD (mg/L)	25	51	68	63	51.8	19.2
E. coli (counts/100 ml)	60	60	90	1080	322.5	437.5
Fecal coliforms (counts/100 ml)	100	20	40	640	200	295.3
Grab Temperature (°C)	13.4	14.1	14.1	12.7	13.6	0.7
Mean (Ambient) Temperature (°C)	2.7	-6.6	-17.0	-17.1	-9.5	9.5
NH_4-N (mg/L)	5.9	26.3	34.3	34.9	25.4	13.5
NO_x-N (mg/L)	7.5	5.3	3.3	2.1	4.5	2.4
pH	7.12	6.81	6.79	6.99	6.93	0.14
PO4-P (mg/L)	1.3	2.6	1.2	1.0	1.5	0.7
Precipitation (mm)*	4.8	0	1	1.6	1.85	1.8
TN (mg/L)	15.4†	40.7	49.8	50.5	39.1	14.22
TOC (mg/L)	19.8†	21.2	29.6	34.4	26.25	6.02
TP (mg/L)	1.67†	3.43	1.69	1.69	2.12	0.76
TS (mg/L)	1,065	886	818	598	841.8	193
TSS (mg/L)	19.5†	6	10	18	13.38	5.58
Turbidity (NTU)	12.25†	4.2	6.3	9.2	7.99	3.03
grab filtered UVT (%)	56.7	52.1	45.8	46.9	50.4	4.4

1 BOD, biochemical oxygen demand; cBOD, Carbonaceous Biochemical Oxygen Demand; COD,
2 chemical oxygen demand; sCOD, soluble chemical oxygen demand; NH_4-N, ammonium-nitrogen;
3 NO_x-N, nitrogen oxides – nitrogen; PO_4-P, orthophosphate; TN, total nitrogen; TP, total phosphorus;
4 TOC, total organic carbon; TS, total solids; TSS, total suspended solids; UVT, Ultraviolet
5 transmittance.
6 * Cumulative amount of rainfall over three days.
7 †Parameters measured the day before and the day after were averaged and used to estimate
8 parameters of sample date.
9 ^ modified from Jankowski et al., 2022.

Figure 1

Figure 1. Graphical abstract of workflow.
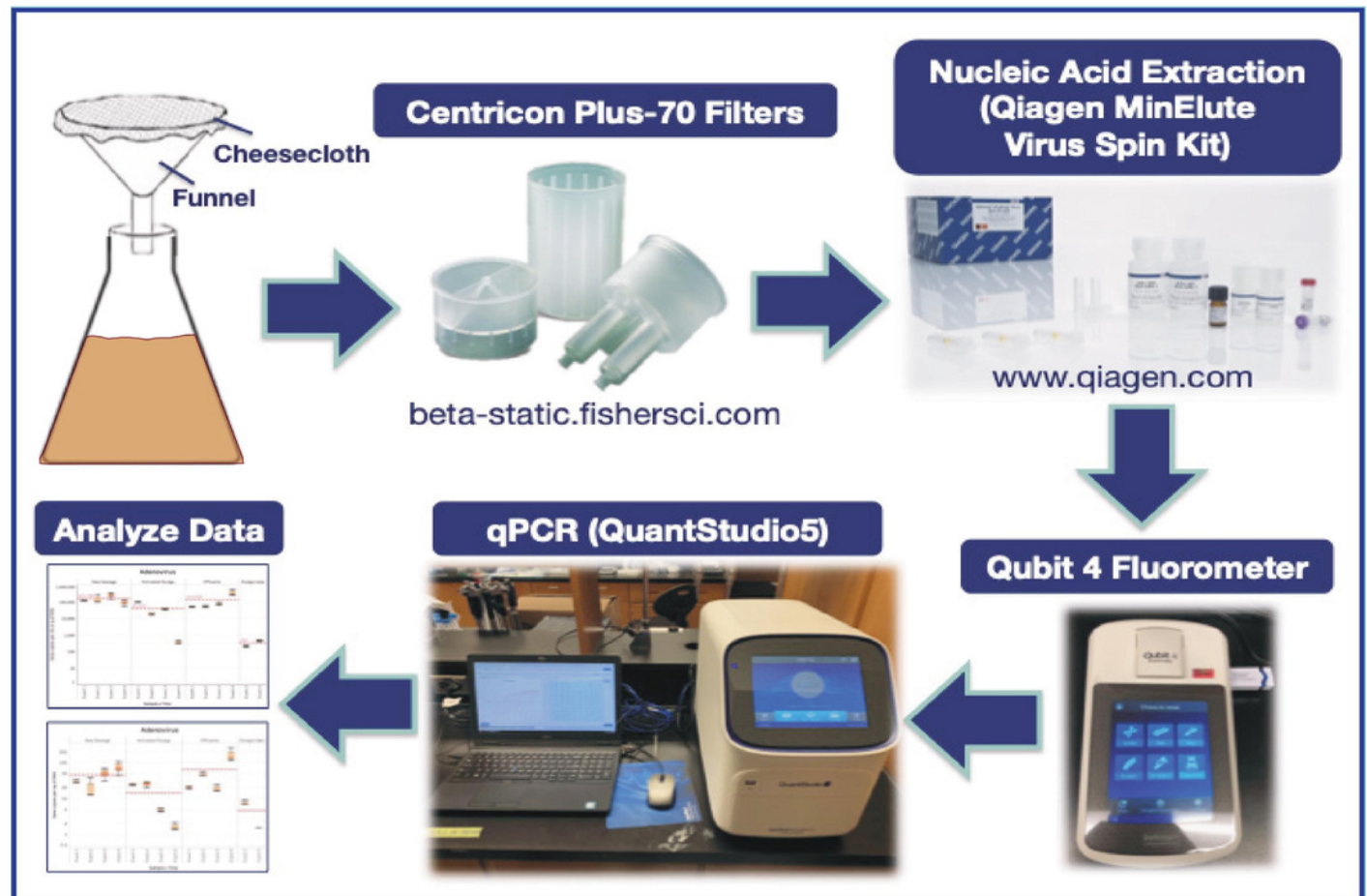
Figure 2

Figure 2. Box plots of the number of gene copies of DNA enteric viruses across each wastewater stage throughout Events 1-4.

Figures 2A and 2C visualize the number of gene copies per mL or g of sample, while *Fig. 2B* and *Fig. 2D* visualize the number of gene copies per ng of DNA. In *Fig. 2A,* Fig. 2C, and *Fig. 2D* these quantities were $\log_{10}$-transformed for aesthetic purposes. The unit for the SC in *Fig. 2A* and *Fig. 2C* is gene copies per g of sample. Means with different letters indicate significant differences at the 0.05 level across treatments.
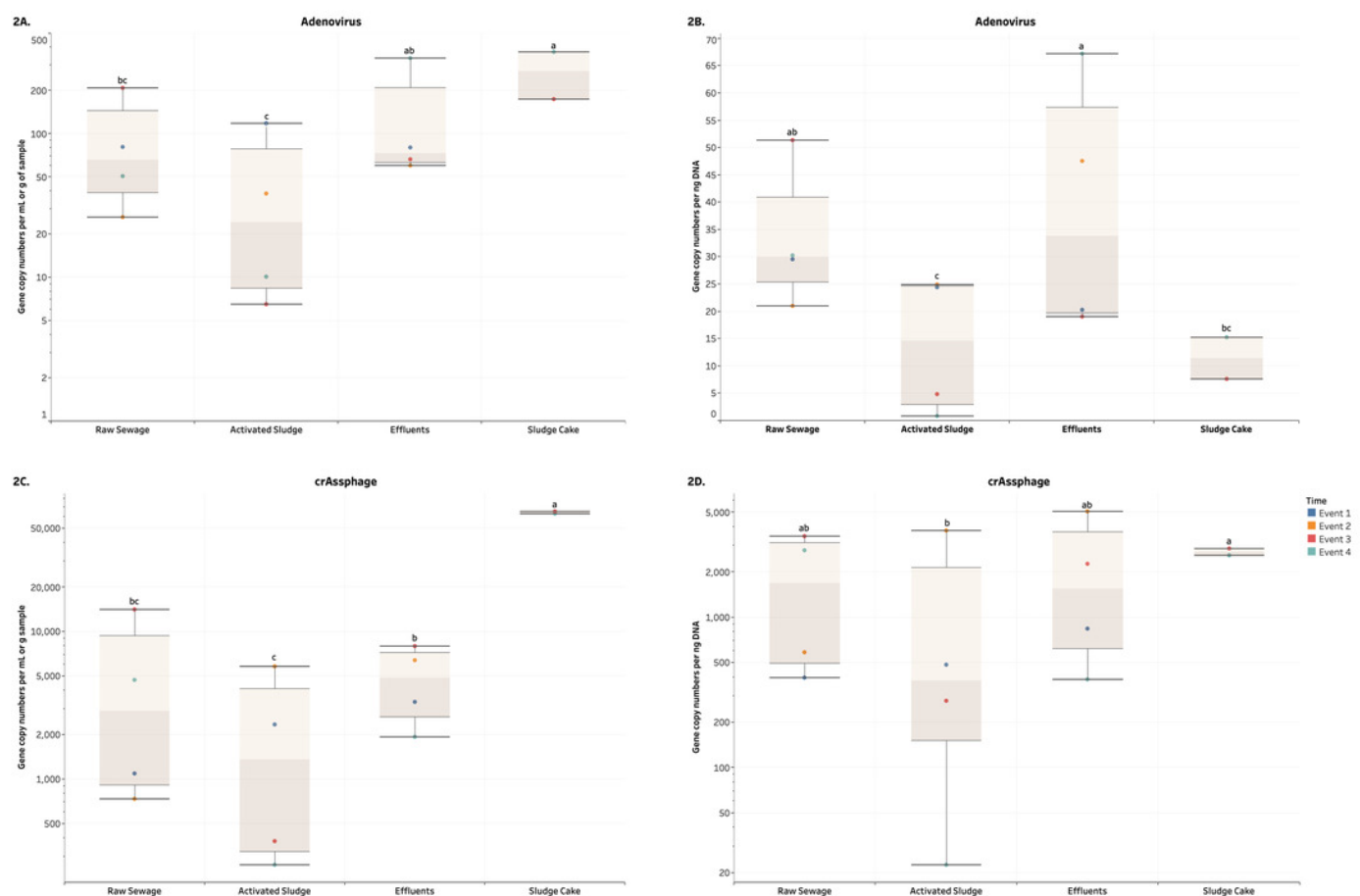
Figure 3

Figure 3. Box plots of the number of genes copies of PMMV across each wastewater stage throughout Events 1-4.

Figure 3A visualizes the number of gene copies per mL or g of sample, while *Fig. 3B* visualizes the number of gene copies per ng of DNA. Both quantities were $\log_{10}$-transformed for aesthetic purposes. The unit for the SC in *Fig. 3A* is gene copies per g of sample. Means with different letters indicate significant differences at the 0.05 level across treatments.
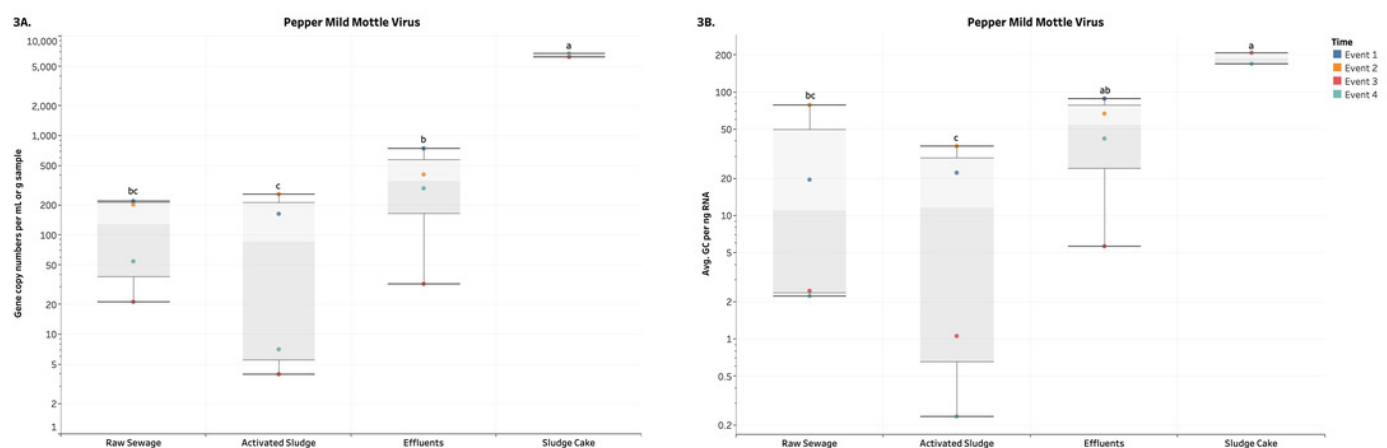
Figure 4

Figure 4. Box plots of the number of gene copies of *uidA* across each wastewater stage throughout Events 1-4.

Figure 4A visualizes the number of gene copies per mL or g of sample, while *Fig. 4B* visualizes the number of gene copies per ng of DNA. Both quantities were $\log_{10}$-transformed for aesthetic purposes. The unit for the SC in *Fig. 4A* is gene copies per g of sample. Means with different letters indicate significant differences at the 0.05 level across treatments.
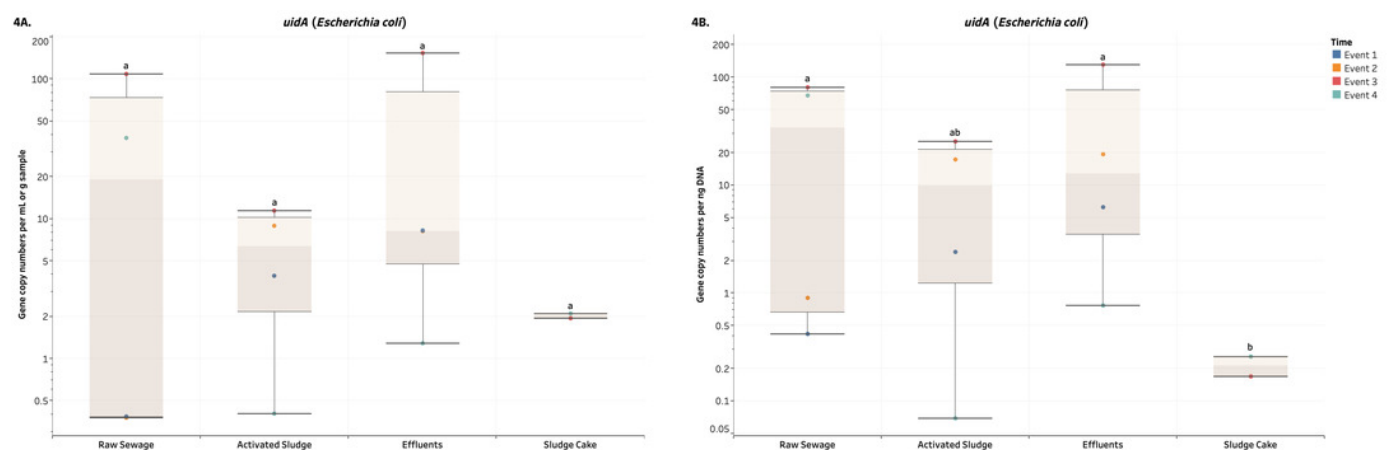
Figure 5

Figure 5. Principal Component Analysis of $\log_{10}$-transformed EF parameters, PC1 versus PC2.

The only variable not $\log_{10}$-transformed was precipitation due to presence of zero values.
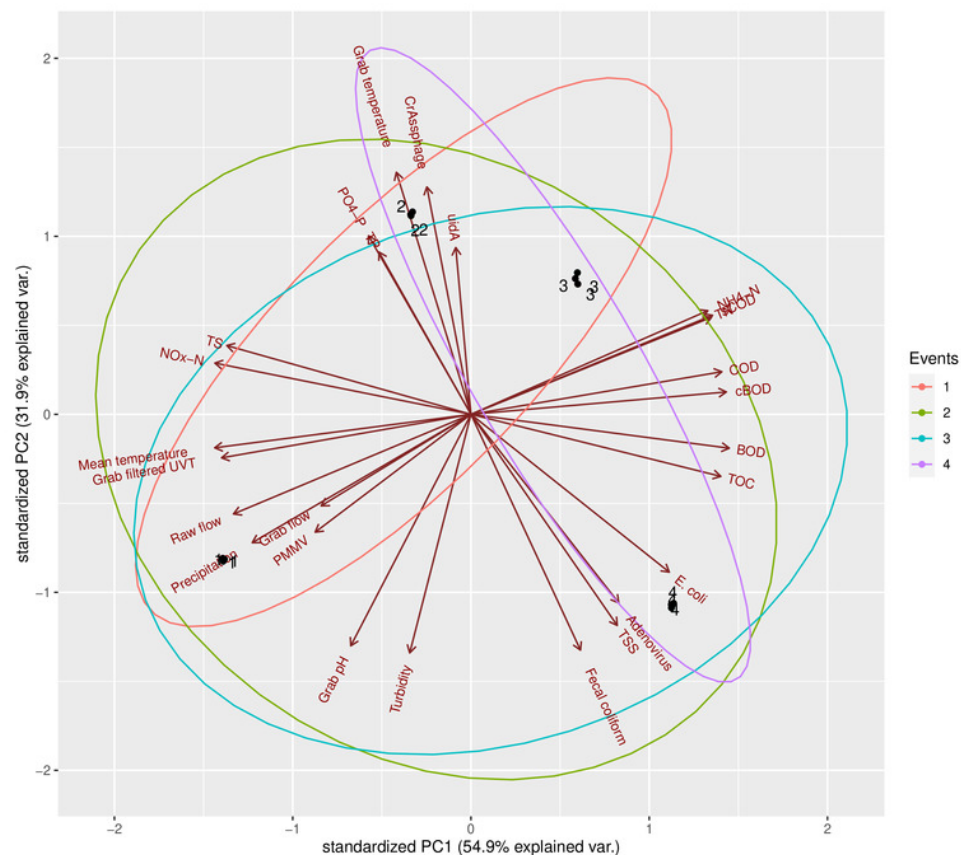
Figure 6

Figure 6. Heatmap showing Spearman's rank correlation analysis between parameters collected for EF sampling events.