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1 **Title:**

2 A new understanding of somatic coliphages belonging to the *Microviridae* family in urban
3 wastewater

4

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18

19

20 **ABSTRACT**

21 Somatic coliphages (SC) and F-specific RNA coliphages (FRNAPH) have been included in
22 regulations or guidelines by several developed countries as a way of monitoring water safety
23 and the microbiological quality of shellfish harvesting waters. SC are highly diverse in their
24 morphology, size and genome. The *Microviridae* family contains three genera of phages
25 (*Alphatrevirus*, *Gequatrovirus*, and *Sinsheimervirus*), all having a capsid of similar morphology
26 (icosahedral) and size (25-30 nm in diameter) to that of common pathogenic enteric viruses.
27 Three PCR assays specific for each genus of *Microviridae* were designed to study these phages
28 in raw and treated wastewater (WW) in order to gain knowledge about the diversity and
29 prevalence of *Microviridae* among SC, as well as their inactivation and removal during WW
30 treatments. Among the four wastewater treatment plants (WWTPs) monitored here, two
31 WWTPs applied disinfection by UV light as tertiary treatment. First, we noticed that
32 *Microviridae* represented 10 to 30 % of infectious SC in both raw and treated WW.
33 *Microviridae* appeared to behave in the same way as all SC during these WW treatments. As
34 expected, the highest inactivation, at least 4 log₁₀, was achieved for infectious *Microviridae*
35 and SC in both WWTPs using UV disinfection. PCR assays showed that the highest removal of
36 *Microviridae* reached about 4 log₁₀, but the phage removal can vary greatly between WWTPs
37 using similar treatments. This work forms the basis for a broader evaluation of *Microviridae*
38 as a viral indicator of water treatment efficiency and WW reuse.

39

40

41 **Keywords:** Somatic coliphages; *Microviridae*; Wastewater; Viral pollution; Bacteriophages

42

43 **1. Introduction**

44 Waterborne enteric viruses are a major threat to global public health. These viruses
45 are transmitted by the faecal-oral route and most of them are persistent in the environment
46 (Kotwal and Cannon, 2014). Metagenomic studies have demonstrated the abundance and
47 diversity of enteric viruses in the human gastrointestinal tract (Reyes et al., 2012), as well as
48 in urban wastewater (WW) (Aw et al., 2014; Cantalupo et al., 2011; Petrovich et al., 2019).
49 Enteric viruses may be discharged in aquatic environments depending on both the treatments
50 applied in WWTP and their initial concentration. Viral contamination of environmental waters
51 has been involved in drinking waterborne outbreaks (Moreira and Bondelind, 2017),
52 foodborne outbreaks due shellfish consumption such as oysters (Bellou et al., 2013), and
53 direct human contaminations during recreational activities (Sinclair et al., 2009). It is therefore
54 important to monitor viral pollution in environmental waters for more effective viral hazard
55 management. However, the direct detection of pathogenic enteric viruses in environmental
56 waters remains difficult owing to irregular sampling, the small volume of water analysed, the
57 temporal variability, and the low viral content outside of epidemic peaks. For this reason, F-
58 specific RNA coliphages (FRNAPH) and somatic coliphages (SC) have been included in
59 regulations or guidelines by numerous developed countries as a way of monitoring water
60 quality (US EPA, 2006; US EPA, 2015; European Commission, 2020; Health Canada, 2019) and
61 ensuring the safety of shellfish (ASQAP, 2022; US FDA, 2019). The main advantages of FRNAPH
62 and SC are their high prevalence and concentration in urban WW – with between 5 and 8 log₁₀
63 plaque forming units (PFU)/L (Blanch et al., 2006; Cole et al., 2003; Farkas et al., 2020;
64 Gourmelon et al., 2007; Hartard et al., 2015; Havelaar et al., 1990; Korajkic et al., 2020) and
65 the standardised methods used, which allow a fast and accurate quantification of the number
66 of infectious particles (ISO 10705-1, 1995; ISO 10705-2, 2001). Furthermore, the effectiveness

67 of the removal of SC and FRNAPH during WW treatments has been reported to be similar to
68 that of pathogenic enteric viruses (Carducci et al., 2009; Martín-Díaz et al., 2016). SC have been
69 shown to be more abundant than FRNAPH, excepted in some situations such as reclaimed
70 water, in which UV is the main factor in viral inactivation (Jofre et al., 2016).

71 SC are non-enveloped phages whose taxonomy has recently been modified. The three
72 families of tailed phages *Myoviridae*, *Podoviridae*, and *Siphoviridae* have been removed from
73 the phage taxonomy proposed by the International Committee on Taxonomy of Viruses (ICTV)
74 because they are not monophyletic (Turner et al., 2021). Now, these phages belong to the
75 class of *Caudoviricetes* and the terms myovirus, podovirus, and siphovirus have been
76 suggested for publications to indicate their morphology (Turner et al., 2021). Due to their high
77 genomic diversity, there are not currently any available methods for the quantification of SC
78 genomes. It is therefore interesting to focus on one specific family; the SC belonging to the
79 *Microviridae* family are morphologically closer to the main enteric pathogenic viruses in terms
80 of capsid shape (icosahedral) and size (around 25 nm) than the other SC. They are organised
81 into three genera: *Alphatrevirus*, *Gequatrovirus* and *Sinsheimervirus*; the coliphages $\alpha 3$, G4,
82 and $\varphi X174$ were chosen as representative of these genera. A previous study, using
83 transmission electron microscopy on infectious phages, observed that *Microviridae* were less
84 removed by a full-scale membrane bioreactor (MBR) than the other SC (Purnell et al., 2015).
85 The authors suggested that the highest susceptibility of the tailed SC to MBR could be due to
86 adsorption to solids and/or damage within the MBR process. Since only *Microviridae* were
87 observed among infectious SC in treated WW, the viral morphology of SC may be an important
88 factor in their removal by MBR membranes (Purnell et al., 2015). It has also been shown that
89 the removal of $\varphi X174$ genomes after WW treatment is similar to that of some other

90 pathogenic viruses commonly found in WW like the adenovirus or polyomavirus (Sidhu et al.,
91 2017).

92 First, this work aimed to study the quantity and diversity of *Microviridae* compared to
93 SC as a whole in raw and treated wastewater. The second objective was to evaluate the
94 inactivation and removal of these *Microviridae* during different WW treatments. To do this,
95 new PCR assays were developed for each genus of *Microviridae*. For the purposes of this study,
96 the three genera will be named α 3-like, G4-like and ϕ X174-like phages, for *Alphatrevirus*,
97 *Gequatrovirus* and *Sinsheimervirus*, respectively. The PCR assays were carried out on raw and
98 treated WW samples to quantify the number of genomes present as well as to genotype the
99 infectious PFUs particles to estimate the abundance of *Microviridae* as a proportion of all SC.

100

101 **2. Materials and methods**

102 2.1. Water samples

103 Influent and effluents of urban WW from four different French WWTPs were selected
104 to consider the variability of urban WW. In this study, we will refer to them as WWTP-1,
105 WWTP-2, WWTP-3, and WWTP-4 (Figure 1A). Influent samples were collected at the WWTPs'
106 entry point and effluent samples were collected just before environmental discharge. All
107 samples were collected during 24h periods using automated samplers. The characteristics of
108 the WWTPs (location, treatment capacity, type of WW, automated sampler, steps
109 implemented to treat WW) are described in Table 1. Between 8 and 12 independent samples
110 of influent and effluent WW were collected in each WWTP with a total of 38 influent samples
111 and 34 effluent samples (see Supplementary Table S1). For WWTP-1 and WWTP-2, samples
112 were collected between September 2021 and April 2022 and between April 2022 and May

113 2022, respectively. For WWTP-3 and WWTP-4, samples were collected between September
114 2021 and June 2022.

115

116 2.2. Production, quantification, and genotyping of infectious somatic coliphages

117 Three representative *Microviridae* strains, ϕ X174, α 3, and G4-like, belonging
118 respectively to the *Sinsheimvirus*, *Alphatrevirus* and *Gequatrovirus* genera, were selected
119 for the development of specific real-time PCR methods. Referenced strains were used for the
120 ϕ X174 phage (ATCC 13706) and α 3 phage (HER 536, Félix d'Hérelle Reference Center for
121 Bacterial Viruses from the University of Laval, Québec, Canada), whereas the G4-like phage
122 was isolated from wastewater and fully sequenced (as described below). Each phage was
123 separately replicated and produced according to the ISO 10705-2 standard method (ISO
124 10705-2, 2001) with minor adjustments (the chloroform step was not carried out and the
125 bacterial host used was *Escherichia coli* CN13, which is also known as WG5 (ATCC 700078).
126 Phage suspensions were then centrifuged ($6000 \times g$ for 20 min at 4 °C) and supernatants were
127 filtered through 0.22- μ m sterile syringe filters made of hydrophilic polyethersulfone (Cytiva,
128 USA). Initial concentrations of viral stocks were determined using the ISO 10705-2 standard
129 method (NF EN ISO 10705-2, 2001). Only infectious SC can be replicated using the *E. coli* CN13
130 bacterial strain, since the absence of F-pilus on the surface of the bacterial strain prevents the
131 replication of infectious FRNAPH. Concentrations of phage suspensions were about 10^{12}
132 PFU/mL for the α 3 and G4-like phages, and about 10^8 PFU/mL for ϕ X174 phages. The phage
133 suspensions were stored at 4 °C for up to two months.

134 Infectious SC were enumerated in duplicate in each WW sample using the ISO 10705-
135 2 standard method as described above. After enumeration, plates containing 10 to 70 PFUs
136 per sample were selected for genotyping. All the PFU of each plate were then individually

137 recovered using sterile loops and resuspended in 1 mL of PBS-5% Glycerol by vortexing for 20
138 sec. The total PFU analysed for the four WWTP reached 1,001 for influents and 664 for
139 effluents (Table S1). Samples were then stored at -20 °C before DNA extraction for up to two
140 weeks.

141

142 2.3. Viral DNA extraction

143 Viral DNA extraction was directly performed on one mL of WW. Two mL of NucliSENS®
144 lysis buffer (BioMérieux, France) were added to the sample, followed by vortexing and
145 incubation at room temperature for 10 min. Samples were then stored at -20 °C for up to
146 seven days or immediately extracted using NucliSENS® magnetic extraction reagents on
147 NucliSENS® MiniMAG (BioMérieux, France) according to the manufacturer's
148 recommendations. A negative control (water) was included in each run of extraction. The
149 extracted DNA was eluted in 100 µL of elution buffer, purified with a OneStep PCR Inhibitor
150 Removal Kit (Zymo Research, Ozyme, France) according to the manufacturer's
151 recommendations. Briefly, a column was prepared prior to use with a solution provided in the
152 kit. The matrix of the column has been designed for efficient removal of polyphenolic
153 compounds, humic and fulvic acids, tannins, etc. The sample was transferred on the matrix
154 and recovered by centrifugation. The samples were stored at -80 °C for up to one month
155 before PCR analyses.

156 The individual plaques recovered in PBS-5% Glycerol were heated at 95°C for 5 min at 500 rpm
157 to extract DNA (ThermoMixer comfort, Eppendorf, France). Then, the genotype of each plaque
158 was determined by qPCR as described below. All plaques containing a mix of genotypes were
159 removed from the analysis. This represented 2% of the total number of plaques collected over
160 all samples from the four WWTPs (see Supplementary Table S1).

161

162 2.4. Design of primers and TaqMan probes

163 The primers and probes were designed using Primer3Plus and the genomic sequences
164 available on Genbank (in 2021, when the primers were designed) for the three genera of the
165 *Microviridae* family. Using BLAST alignment for nucleotides, the identity ranged from 98.9%
166 to 99.9% (coverage: 100%) between ϕ X174 sequence with accession number NC_001422.1
167 and the 114 other complete sequences of *Sinsheimervirus*. The identity ranged from 84.0% to
168 100% (coverage: 84% to 100%) between α 3 sequence with accession number NC_001330.1
169 and the 14 other *Alphatrevirus* sequences. It ranged from 91.3% to 100% (coverage: 99% to
170 100%) between G4 sequence with accession number NC_001420.2 and the 19 other
171 *Gequatrovirus* sequences. The previously described PCR system for ϕ X174 detection (Dika et
172 al., 2015) matched with only 63% of the 115 sequences of *Sinsheimervirus*, but this result could
173 be improved to 86% by degenerating one nucleotide on the reverse primer at position 1727
174 on NC_001422.1. The system with the degenerated reverse primer was used for ϕ X174-like
175 phage detection and quantification. The primers and probes were located in the replicase
176 gene for α 3-like phages and in the hypothetical Protein A for G4-like phages because of the
177 relatively small amount of sequence variation encountered in those areas. BLAST alignments
178 showed that these two PCR systems detected all the complete sequences of their genus
179 disponible on Genbank. The specificity for each genus could also be estimated at 100% for the
180 three PCR assays. An additional validation was done by testing DNA from α 3, G4-like, and
181 ϕ X174 with each PCR detection assay. The specifications of the oligonucleotides are given in
182 Supplementary Table S2.

183

184 2.5. Digital droplet PCR

185 The digital droplet PCR (ddPCR) technology was used to quantify the genomes of each
186 *Microviridae* genus directly in the WW samples. It was carried out using a ddPCR supermix for
187 Probes (no dUTP) kit with a QX200 ddPCR system (Bio-Rad). Each reaction contained a final
188 concentration of 1 X Supermix, 900 nM of each primer, 130 nM of the ϕ 174-like probe, or 230
189 nM of the α 3-like and G4-like probes (Eurogentec, Belgium) and molecular-grade water.
190 Template DNA (5.5 μ L) was added for a final reaction volume of 22 μ L. Droplet generation was
191 performed according to the manufacturer's instructions (BioRad). The resulting droplets were
192 then transferred to a Thermal Cycler for PCR amplification using the following parameters with
193 a ramp rate of 70% between each step: 95 °C for 10 min, 40 cycles of 94 °C for 30 sec and 55
194 °C for 1 min, followed by 98 °C for 10 min and a final step at 4 °C for 30 min. The droplets were
195 kept at 4 °C up to 12 hours before analysis using the QX200 Droplet Reader (BioRad).

196 The limit of quantification (LOQ) of the ddPCR assay was of three positive droplets for
197 20,000 total droplets. By modelling with a Poisson distribution, this represented a
198 concentration \sim 50 genome copies (GC)/100 μ L of extracted DNA. As only one mL of WW was
199 analysed, the LOQ was estimated to be \sim 4.7 \log_{10} GC/L for these experiments.

200

201 2.6. Real-time PCR

202 The real-time PCR technique was used to genotype the PFU recovered from the plates
203 for each *Microviridae* genus. For influents samples, a total of 1,001 PFU were genotyped. The
204 number of genotyped PFU per WWTP varied between 218 (WWTP-1) and 283 (WWTP-3). For
205 effluent samples, a total of 664 PFU were genotyped. The number of genotyped PFU per
206 WWTP varied between 110 (WWTP-4) and 269 (WWTP-2). The detailed data are given in
207 Supplementary Table S1. The reactions were performed using the TaqMan Universal PCR
208 Master Mix (ThermoFisher Scientific). In accordance with the manufacturer's

209 recommendations, each reaction was performed in a 25 μ L reaction mixture containing 5 μ L
210 of template DNA and 15 μ L of 1 \times TaqMan mix, 800 nM of each primer and 200 nM of the
211 probe, and molecular grade water (Sigma). The amplification was carried out on a CFX96
212 Touch Real-Time PCR System (BioRad) using the following parameters: 50 $^{\circ}$ C for 2 min, 95 $^{\circ}$ C
213 for 10, min, followed by 45 cycles of 95 $^{\circ}$ C for 15 sec and 60 $^{\circ}$ C for 1 min. Infectious phage
214 genotyping was performed using the extracted phage plaque DNA and was considered
215 positive when Cq was below 30 because a PFU is expected to contain a high quantity of
216 genome. This limit corresponded to $\sim 5 \log_{10}$ GC/PFU.

217

218 2.7. Isolation and sequencing of environmental G4-like phage

219 A phage belonging to G4-like phages was isolated from an influent sample collected at
220 WWTP-1 during SC enumeration and genotyping of PFU, amplified on *E. coli* CN13 and
221 quantified at 2×10^{13} GC/mL before sequencing analysis. Several steps were then applied to
222 obtain a sufficient concentration of purified phage DNA for sequencing analysis. First, this
223 phage was amplified on a plate; then the top agar layer was separated from the other layer
224 starting from the edge of the plate using sterile forceps and transferred to 200 mL of PBS,
225 homogenised by vortexing and then slowly agitated during 4 hours at room temperature. After
226 centrifugation at $2,520 \times g$ for 10 min at 4 $^{\circ}$ C, the supernatant was filtered through 5- μ m and
227 0.22- μ m filters. The filtrate was ultra-centrifuged at room temperature, at $53,900 \times g$, for 7
228 hours (Beckman Optima XE-90; Rotor type 19), and the resulting pellet was resuspended in 4
229 mL of PBS. Before sequencing, the nucleic acids were extracted using NucliSENS[®] magnetic
230 extraction reagents on NucliSENS[®] MiniMAG (BioMérieux, France) and treated with DNase-I
231 to remove bacterial DNA. The bacterial DNA removal was validated using a 16S PCR method
232 (Muyzer et al., 1993; Nadkarni et al., 2002). As SC are DNA phages, the treatment with DNase

233 resulted in a 3 log₁₀ decrease in phage DNA, but 2 × 10¹³ GC/mL were still present in the
234 sample. The phage was sequenced using next generation sequencing (NGS) (Microsynth AG,
235 Balgach, Switzerland). Briefly, the second-strand DNA was synthesised with DNA polymerase
236 I, an Illumina TruSeq DNA library was prepared and the sequencing was performed using
237 Illumina MiSeq 1 Mio reads (2x250 bp runs, v2). After demultiplexing and trimming of Illumina
238 adaptor residuals, the assembled sequence was obtained using Unicycler v0.5.0. The sequence
239 is available on GenBank with accession number OR492646. BLAST analysis showed between
240 92.1% and 96.7% identity (coverage: 100%) with the 20 other complete sequences of G4-like
241 phages, 77% identity (coverage: 33%) with the NC_001422.1 sequences (*Escherichia* phage
242 phiX174, complete genome) and between 71.1% and 75.8% identity (coverage: 24% to 32%)
243 with the 15 complete sequences of α3-like phages.

244

245 2.8. Statistical analysis

246 All the statistical tests were performed using GraphPad-PRISM software (version
247 9.5.0). The α level was fixed at 0.05. All data were analysed for single outliers using the Grubbs
248 test. After removing the detected single outliers, the normality of the data sets was assessed
249 using the Shapiro-Wilk test. All data were independent of each other. So, we used the Kruskal-
250 Wallis test followed by a post-hoc Dunn multiple pairwise comparison test to compare more
251 than two samples. A Mann-Whitney test was used to compare two samples. Asterisks were
252 used to indicate significant differences: * for p-value ≤ 0.05; ** for p-value ≤ 0.01; *** for p-
253 value ≤ 0.001, and **** for p-value ≤ 0.0001.

254

255 3. Results

256 3.1. Assessment of the presence and diversity of *Microviridae* in raw wastewater

257 Our first aim was to assess the presence and diversity of the three genera of
258 *Microviridae* in raw WW by detecting their corresponding infectious particles and viral
259 genomes. Thirty-eight influent samples from four WWTP locations in two different regions of
260 France were analysed (Figure 1A and Supplementary Table S1).

261 The three specific genera of *Microviridae* were detected using three specific PCR assays
262 designed for α 3-like, G4-like and ϕ X174-like phages. The specificity of the PCR assays,
263 evaluated using BLAST alignments (see above in 2.4), was confirmed by the absence of cross-
264 reactivity when testing DNA from α 3, G4-like, and ϕ X174 with each PCR detection system.
265 Both qPCR and ddPCR methods could be used to quantify the *Microviridae* genomes in
266 environmental samples. For convenience, the qPCR method was used for determine which of
267 the three genera of *Microviridae* the plaques of infectious SC were made up of, while the
268 ddPCR method was used for the direct quantification of the genome copies in WW samples.

269 Infectious SC were detected using the standardised PFU method (NF EN ISO 10705-2,
270 2001) in all the WW samples. The mean concentrations of infectious SC in influents reached
271 $7.1 \pm 0.5 \log_{10}$ PFU/L (WWTP-1), $7.4 \pm 0.2 \log_{10}$ PFU/L (WWTP-2), $8.1 \pm 0.4 \log_{10}$ PFU/L (WWTP-
272 3) and $8.0 \pm 0.6 \log_{10}$ PFU/L (WWTP-4) (Figure 1B), with the SC concentrations in WWTP-1
273 being significantly lower than those observed in WWTP-3 (p-value = 0.0009) and WWTP-4
274 (p-value = 0.0064). After genotyping each plaque from each sample, the results allowed us to
275 determine which of the three *Microviridae* genera (or other SC) each PFU was made up of (see
276 Supplementary Figure S1B). The mean concentration of infectious *Microviridae* for each site
277 varied from $5.1 \pm 2.4 \log_{10}$ PFU/L (WWTP-1) to $7.4 \pm 0.5 \log_{10}$ PFU/L (WWTP-4) (Figure 1B). As
278 observed for infectious SC, the concentration of infectious *Microviridae* was significantly lower
279 at WWTP-1 than at WWTP-3 (p-value = 0.0031) and WWTP-4 (p-value \leq 0.0001). From these
280 values, we estimated that infectious *Microviridae* make up on average between $12 \% \pm 9 \%$

281 (WWTP-1) and $30\% \pm 14\%$ (WWTP-4) of total infectious SC in influents. If we take into
282 account all four WWTPs, the mean concentrations of infectious *Microviridae* of each genus
283 varied from $2.6 \pm 3.4 \log_{10}$ PFU/L for G4-like phages to $4.0 \pm 3.2 \log_{10}$ PFU/L for $\alpha 3$ -like phages
284 (data not shown). When analysing each site separately (Figure 1C), the only significant
285 difference was a lower proportion of G4-like phages than $\alpha 3$ -like phages in influents from
286 WWTP-2 (p-value = 0.0168). Nevertheless, the wide distribution of the concentration values
287 with negative samples frequently observed for at least one genus may hide the differences
288 between genera.

289 The mean concentration of *Microviridae* genomes varied from $7.6 \pm 0.3 \log_{10}$ GC/L
290 (WWTP-1) to $8.1 \pm 0.3 \log_{10}$ GC/L (WWTP-3) (Figure 1B). As observed for the infectious phages,
291 WWTP-1 had a lower concentration of *Microviridae* genomes than WWTP-3 (p-value = 0.0149)
292 and WWTP-4 (p-value = 0.0288). If we take into account all four WWTPs, the mean
293 concentrations of each genus varied from $6.7 \pm 0.6 \log_{10}$ GC/L for G4-like phages to 7.6 ± 0.4
294 \log_{10} GC/L for $\alpha 3$ -like phages (data not shown). When analysing each site separately (Figure
295 1D), the concentrations of $\alpha 3$ -like genomes were significantly higher than those of G4-like
296 genomes at all WWTPs (p-values from ≤ 0.0001 for WWTP-2 and -4 to 0.0316 for WWTP-1).
297 The concentrations of the $\phi X174$ -like genomes were in between those of the two other
298 genera, with no significant differences. These original genome data may be more
299 representative of the real proportions of $\alpha 3$ -like, G4-like and $\phi X174$ -like phages than those
300 obtained for PFUs as it was observed less variability for any given site for the genome
301 parameter than what we observed for the infectious parameter.

302

303 3.2. Comparison of the behaviour of *Microviridae* and somatic coliphages during wastewater
304 treatments

305 For the four same WWTPs, 34 effluent samples were collected in parallel with influent
306 samples and analysed using the same methods to determine the concentrations of infectious
307 SC and *Microviridae* and *Microviridae* genomes at the end of WW treatment (see
308 Supplementary Table S1 and Supplementary Figure S1). From influent and effluent
309 concentrations, the removal of infectious SC and *Microviridae* as well as the removal of
310 *Microviridae* genomes could be estimated and expressed as a \log_{10} reduction (Figure 2A).

311 A reduction of $1.9 \pm 0.3 \log_{10}$ and $2.9 \pm 0.2 \log_{10}$ was observed for infectious SC at
312 WWTP-1 and -2. WWTP-3 and -4 achieved a reduction of $4.2 \pm 0.4 \log_{10}$ and $3.9 \pm 0.5 \log_{10}$,
313 respectively, with both sites doing tertiary disinfection treatment based on UV light (Figure 2A
314 and Table 1). The reduction in infectious SC was statistically higher in WWTP-3 and -4 than in
315 WWTP-1 (p -value ≤ 0.0001 for WWTP-3 and p -value = 0.0002 for WWTP-4). No significant
316 difference was observed between WWTP-2 and the three other WWTPs.

317 The reduction in infectious *Microviridae* followed a similar trend to that observed for
318 all SC (Figure 2A), but higher standard deviation values were noticed for *Microviridae* than for
319 SC. The reduction of infectious *Microviridae* reached $0.5 \pm 2.5 \log_{10}$ at WWTP-1 and 3.1 ± 1.5
320 \log_{10} at WWTP-2, while average reductions of $4.0 \pm 3.6 \log_{10}$ and $5.6 \pm 1.9 \log_{10}$ were observed
321 at WWTP-3 and WWTP-4, respectively. As observed for all SC, the reduction in infectious
322 *Microviridae* was significantly lower at WWTP-1 than at WWTP-3 (p -value = 0.0042) and
323 WWTP-4 (p -value ≤ 0.0001). Based on the concentration values of infectious particles
324 (Supplemental Figure 1), the average proportions of infectious *Microviridae* were estimated
325 to be between $17 \% \pm 17 \%$ (WWTP-3) and $32 \% \pm 16 \%$ (WWTP-2) of all infectious SC in
326 effluent samples. Overall, except for WWTP-1 where infectious *Microviridae* were significantly
327 less reduced than infectious SC (p -value = 0.0088), similar behaviour was observed between

328 infectious SC and *Microviridae* during WW treatments, even when tertiary UV treatment was
329 carried out.

330 The removal of *Microviridae* particles was estimated using genome quantification data
331 (Figure 2A). The highest mean removal of *Microviridae* particles was $4.2 \pm 2.1 \log_{10}$ for WWTP-
332 2 and was significantly higher than the reduction of about $2.0 \log_{10}$ obtained at the three other
333 WWTPs (p-values = 0.0029, 0.0092, and 0.0417, respectively). Despite both applying similar
334 steps for the entire wastewater treatment, WWTP-1 and -2 showed a significant difference (p-
335 value = 0.0029) in effectiveness in removing *Microviridae* particles (about $2 \log_{10}$). Even if the
336 treatments applied in these two WWTPs were similar, they may differ in their conditions of
337 application. The $4.0 \log_{10}$ reduction obtained for infectious *Microviridae* and SC at WWTP-3
338 and -4 was not observed for the *Microviridae* genomes detected with a single target.

339 Few differences were found between the three genera of *Microviridae*. For infectious
340 *Microviridae* (Figure 2B), we did not observe a reduction in the concentration of G4-like
341 phages at WWTP-1 and WWTP-2 because infectious G4-like phages were more frequently
342 detected in effluents than in influents in these two WWTPs. Thus, for these two WWTPs, G4-
343 like phages represented only 11.5% and 8.9% of infectious *Microviridae* in influents, whereas
344 they represented 56.8% and 63.5% of infectious *Microviridae* in effluents (Table S1). This led
345 to the only significant difference observed between the three genera for infectious
346 *Microviridae* with a higher removal of α 3-like phages than G4-like phages at WWTP-2
347 (p-value = 0.0251). If we take into account all the data, irrespective of the WW treatment
348 method used, there was similar reductions of infectious particles for all three genera (p-value
349 > 0.05). Regarding genome data (Figure 2C), the only difference between genera was observed
350 at WWTP-3, where the decrease in α 3-like phages was higher than those observed for G4-like
351 phages (p-value = 0.0063) and ϕ X174-like phages (p-value = 0.0126). If we take into account

352 all the data, there was significantly higher reduction of α 3-like phages than ϕ X174-like phages
353 during WW treatments (p-value = 0.0186). Moreover, the higher genome concentrations of
354 α 3-like phages relative to G4-like phages in all influent samples were no longer visible in the
355 effluent samples (see Supplementary Figure 1C). Despite the variability of the data, especially
356 for infectious *Microviridae*, our results suggest that the removal of α 3-like phages during WW
357 treatments was slightly more efficient than for the two other genera (G4-like and ϕ X174-like).

358

359 **4. Discussion**

360 This study aimed to increase knowledge about SC belonging to the *Microviridae* family
361 in WW, and to better understand the effect of WW treatments on their inactivation and
362 removal. PCR assays were developed here for the quantification of the three genera of
363 *Microviridae*, which is the only remaining recognized family in the SC (Turner et al., 2021). The
364 design of these PCR assays was done by using the complete sequences available on Genbank
365 which are more numerous for ϕ X-174-like genus than the other genera. Three PCR assays
366 were necessary since this family is too genetically diverse to create a single PCR assay that can
367 target all its members. A previous study proposed a PCR assay that only targeted the ϕ X-174-
368 like genus (Lee, 2009) and did not detect all *Microviridae* or compare the three genera of
369 *Microviridae* between each other. By conducting our PCR assays on PFUs, we also quantified
370 infectious *Microviridae* in WW, which provided information on both the removal and
371 inactivation of *Microviridae* by WW treatments. Raw and treated WW samples were collected
372 in four French WWTPs using tertiary treatments based either on lamellar settling for the
373 removal of phosphorous and residual suspended solids or on UV disinfection.

374 All influent samples were positive for infectious SC using the standardised method with
375 values ranging from 7.1 to 8.2 log₁₀ PFUs per litre. A meta-analysis of infectious SC in raw WW

376 has previously shown a significant difference in the mean density distributions between
377 geographic regions (Nappier et al., 2019). Nevertheless, our values were consistent with mean
378 concentration values ranging from 5.1 to 9.7 log₁₀ PFUs per litre previously reported for raw
379 WW (Blanch et al., 2006; Chacón et al., 2020; Jofre et al., 2021; Kelmer et al., 2023; Levantesi
380 et al., 2010; Lucena et al., 2004; Muniesa et al., 1999; Purnell et al., 2015; Truchado et al.,
381 2021; Zhang and Farahbakhsh, 2007). Moreover, a mean concentration value of 7.1 log₁₀ PFUs
382 per litre had been previously obtained for WWTP-1 featured in this study (Lucena et al., 2004).

383 The reduction in infectious SC between influents and effluents reached 2 and 3 log₁₀ in
384 WWTP-1 and -2, respectively. These WWTP included tertiary treatment for phosphorous
385 removal based on flocculation with FeCl₃ and lamellar setting. About 2.7 log₁₀ removal of
386 infectious SC was observed in a WWTP applying similar treatment including flocculation (n=14
387 samples) (Fu et al., 2010). This tertiary treatment was estimated to eliminate about 0.5 to 0.8
388 log₁₀ of infectious SC (Fu et al., 2010; Gantzer et al., 1998; Lucena et al., 2004). The reduction
389 in infectious SC between influents and effluents reached 4 log₁₀ in WWTP-3 and -4. It can be
390 considered that the difference of 1 to 2 log₁₀ observed between WWTP-1 and -2 on one hand
391 and WWTP-3 and -4 on the other hand was due to UV disinfection because it was the main
392 difference between these WWTPs. Similarly, Korajkic and colleagues (2022) observed that UV
393 light treatment improved infectious SC reduction by 1 to 2 log₁₀. About 5 log₁₀ reduction of
394 infectious SC was observed in two WWTPs using UV or chlorine as tertiary treatment
395 (Truchado et al., 2021).

396 The three PCR assays developed here allowed us to measure the genome
397 concentrations of each *Microviridae* genus and the concentrations of infectious *Microviridae*.
398 The *Microviridae* genomes were systematically detected in raw WW and their concentrations
399 were between 7.6 and 8.1 log₁₀ GC per litre. By using the PCR assay designed by Lee (2009),

400 the genome of the ϕ X-174-like genus was detected at 6 to 7 log₁₀ GC per litre of raw WW in
401 Australia (Sidhu et al., 2018, 2017). We observed a predominance of the α 3-like genome
402 compared to the G4-like genome in raw WW, indicating differences in the initial
403 concentrations of the three genera of *Microviridae*. Infectious *Microviridae* were also
404 systematically detected in raw WW and they represented between 12 % and 30 % of all
405 infectious SC (21% by considering the 1,001 PFU from all influents samples together).
406 Moreover, we analysed all PFU from plates containing between 10 and 70 PFU in order to
407 have more chance to detect phages present at low frequency. Our percentage values for
408 *Microviridae* were considerably higher than those observed by Muniesa et al., (1999), who
409 detected no more than 2 % of *Microviridae* among infectious SC in raw WW. However, these
410 authors analysed plates containing no more than 20 PFU. This low quantity of PFU per plate
411 may have contributed to the low detection of *Microviridae* as suggested by previous study
412 performed on bacterial diversity (Bianchi and Bianchi, 1982). Metagenomic approaches
413 previously applied to WW gave rates that were closer to ours, with *Microviridae* representing
414 19 % to 37 % of total SC in raw sewage (Cantalupo et al., 2011; Aw et al., 2014).

415 The analysis of raw and treated WW with our PCR assays showed the level of removal
416 of *Microviridae*, whereas PCR assays on PFUs provided a relative information about the rate
417 of *Microviridae* inactivation. PCR assays allow the quantification of both infectious and non-
418 infectious *Microviridae* and several states of viral particles (infectious, non-infectious with
419 intact capsid, non-infectious with altered capsid) can be present and detected using PCR
420 methods in environmental samples (Randazzo et al., 2018; Wurtzer et al., 2021). For the two
421 WWTPs that used tertiary settling treatments, the reduction in *Microviridae* genomes (2 to 4
422 log₁₀) was greater than the reduction in infectious *Microviridae* (0.5 to 3 log₁₀), showing that
423 physical removal of *Microviridae* particles predominated over inactivation. This could be due

424 to the adhesion of a part of *Microviridae* phages to suspended matter leading to their removal
425 with these particulates during flocculation and lamellar settling. The study of the surface
426 properties of *Microviridae*, especially their relative hydrophobicity as performed for other
427 phages (Dika et al., 2015), could contribute to the understanding of this phenomenon. On the
428 contrary, for tertiary treatment using UV disinfection, the reduction in infectious *Microviridae*
429 (4 to 5 log₁₀) was greater than the reduction in *Microviridae* genomes (2 log₁₀). This was
430 expected, because viral inactivation is due to genomic lesions during UV disinfection (Beck et
431 al., 2016; Pecson et al., 2011; Simonet and Gantzer, 2006), but damage due to UV are often
432 limited to some parts of the genome and the use of a single genome target is commonly no
433 sufficient to evaluate such a treatment (Young et al., 2020). As we targeted only a small part
434 of *Microviridae* genome (~100 nucleotides), these phages were still detected by PCR whereas
435 they were inactivated. These results showed that UV treatment is highly efficient for the
436 inactivation of *Microviridae* and that PCR methods using only a single target cannot be used
437 to evaluate this tertiary treatment.

438 Overall, similar reductions were observed for infectious *Microviridae* and SC, and the
439 proportion of infectious *Microviridae* was shown to be between 10% and 30% of total
440 infectious SC in both raw and treated WW. This suggested similar behaviour for un-tailed
441 *Microviridae* and tailed SC exposed to these WW treatments. A link between the phages'
442 morphology and the efficiency of WW treatment has been previously described for a full-scale
443 MBR (Purnell et al., 2015). These authors only detected infectious *Microviridae* in treated WW
444 using transmission electronic microscopy; this was explained by tailed SC's higher adsorption
445 of solids and/or susceptibility to damage within the MBR process. The specific monitoring of
446 each *Microviridae* genus performed in our work suggested that α3-like genus might be more

447 sensitive to WW treatments than other *Microviridae*, but this point requires further
448 investigation taking account of the frequency of each genus.

449

450 **5. Conclusion**

451 The PCR approach developed in the present study showed that the specific monitoring
452 of *Microviridae* genomes and infectious particles is possible in raw and treated WW; it could
453 also be applied to other environmental samples. In the WWTPs studied here, these phages
454 appeared to behave similarly to all SC. Moreover, the size and capsid morphology of
455 *Microviridae* are closer to common pathogenic enteric viruses than other SC; this could be a
456 tremendous advantage for the evaluation of WW treatment. Thus, further studies focusing on
457 *Microviridae* may contribute to the improvement of WW treatment and reuse and,
458 consequently, human health risk management.

459

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467

468 **COMPETING INTERESTS**

469 The authors declare no competing interest.

470

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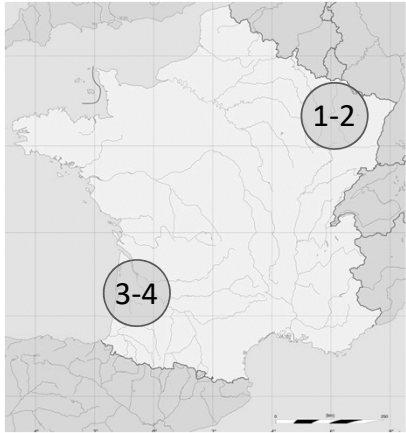
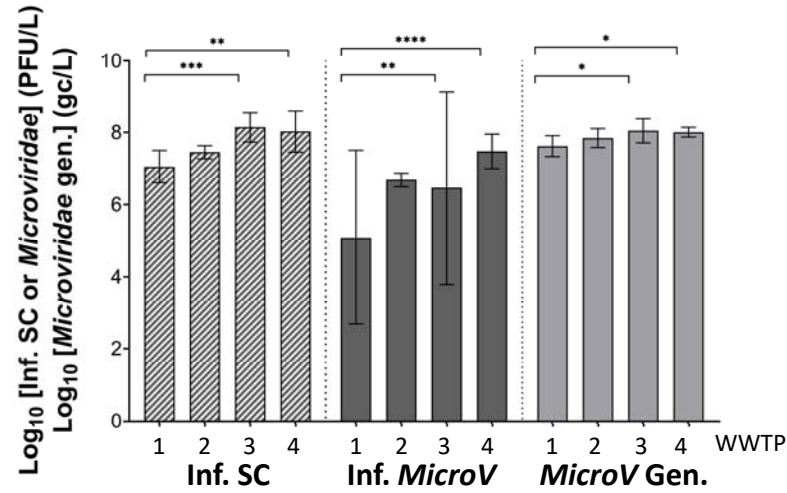
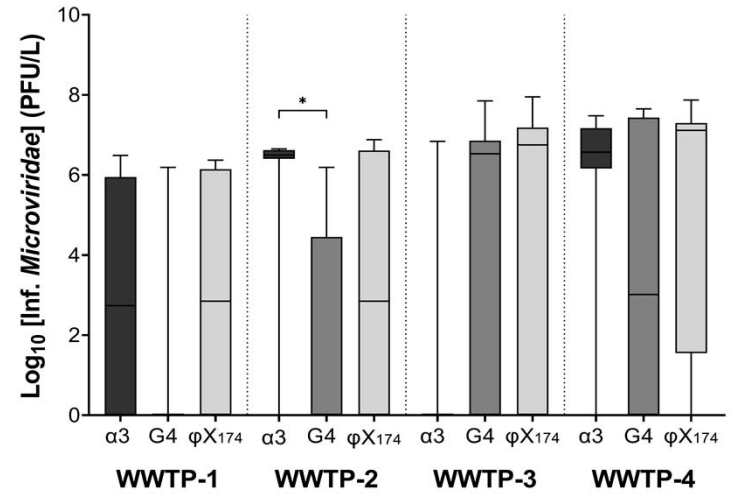
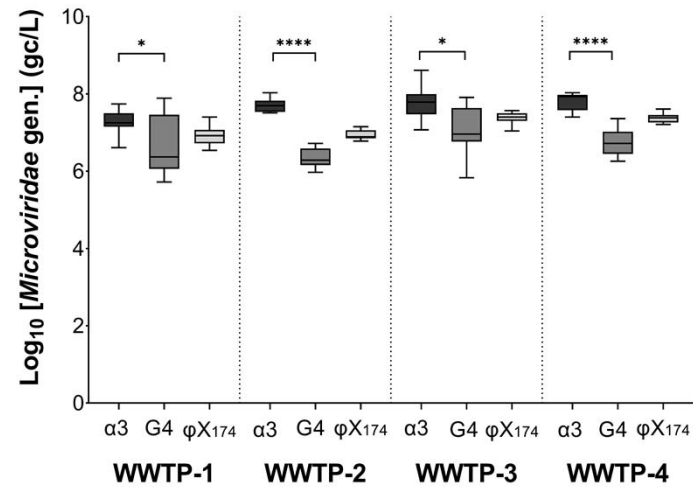
A**B****C****D**

Figure 1: Quantification of viral infectious particles and genome copies in wastewater influents. (A) Localisation of the four WWTP in France. (B) Concentration values of infectious SC (grey pattern), infectious Microviridae (dark grey) and Microviridae genome copies (light grey) before WW treatments (bars represent the mean with SD for each condition). (C) Concentration values of infectious particles for the three genera of Microviridae in the influent (Box and whiskers representing the mean and the maximum and minimum values). (D) Concentration values of the genome of the three genera of Microviridae in the influent (Box and whiskers representing the mean and the maximum and minimum values). The LOQ of the three ddPCR assays in WW was between 4.7 log₁₀ GC/L. For all panels, Kruskal-Wallis statistical test was performed: stars represent significative differences (* = p ≤ 0.05; ** = p ≤ 0.01; *** = p ≤ 0.001 and **** = p ≤ 0.0001). In Figure 1B, the four WWTP were compared between each other and the WWTP were tested independently from each other for 1C and 1D.

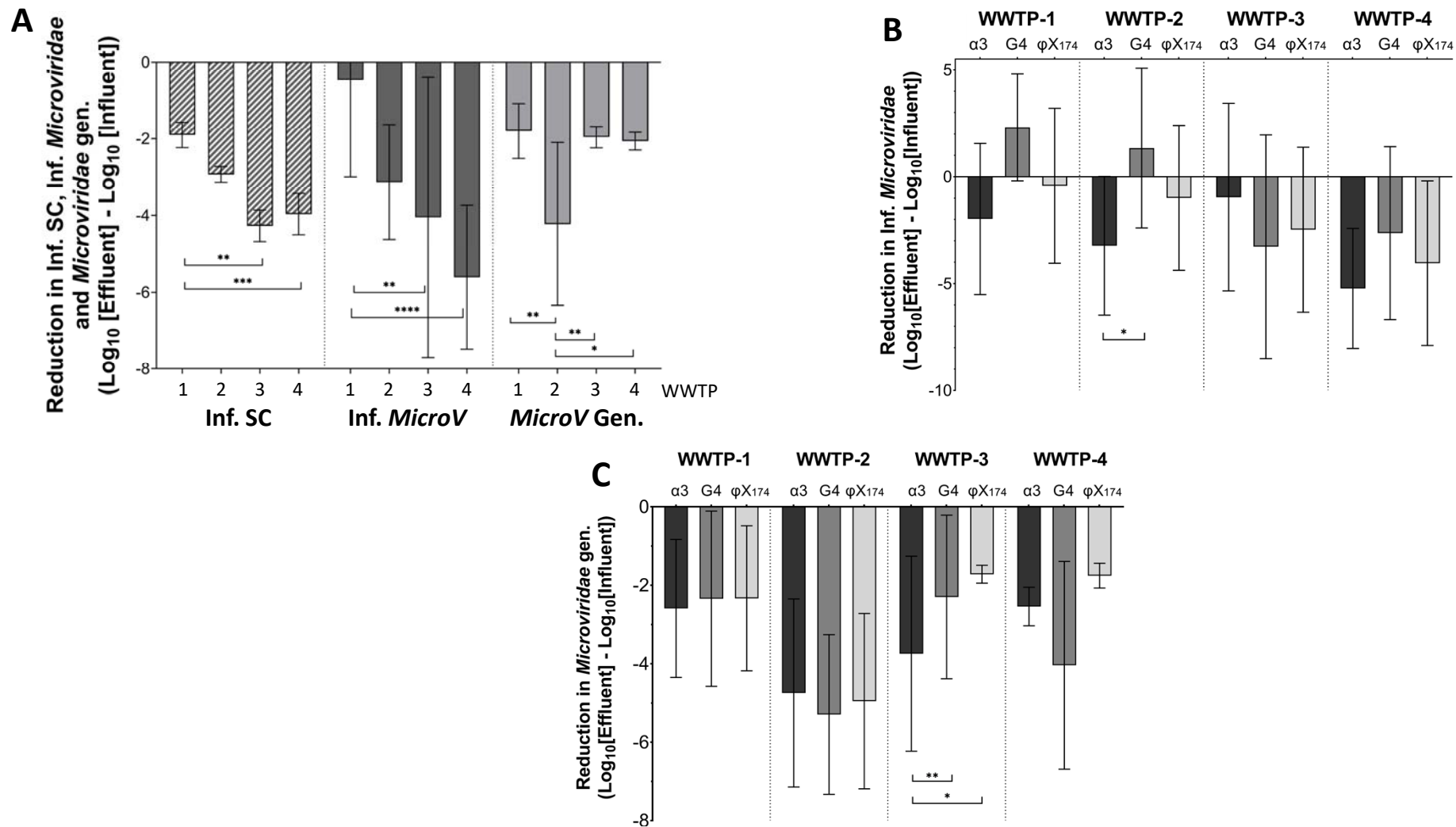
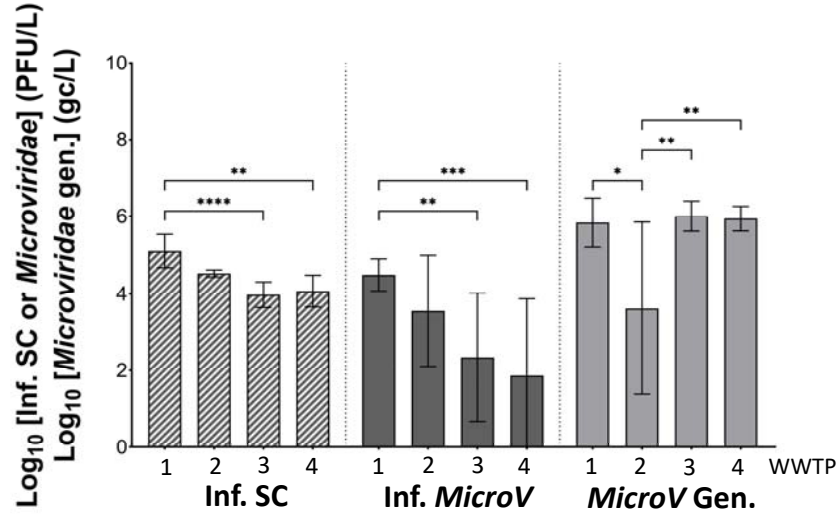
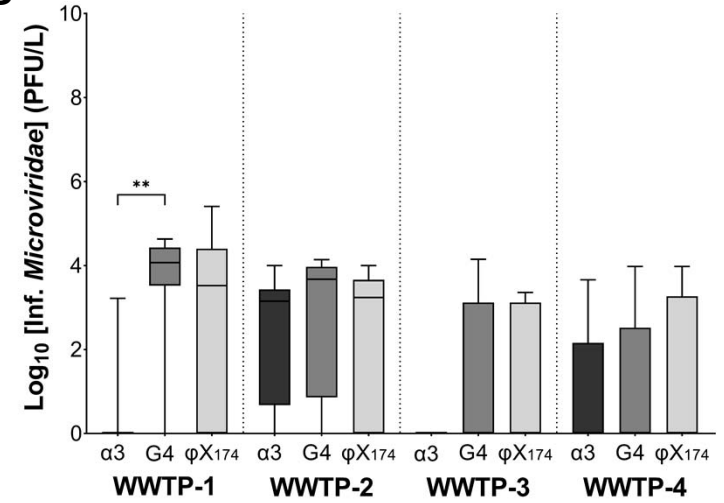
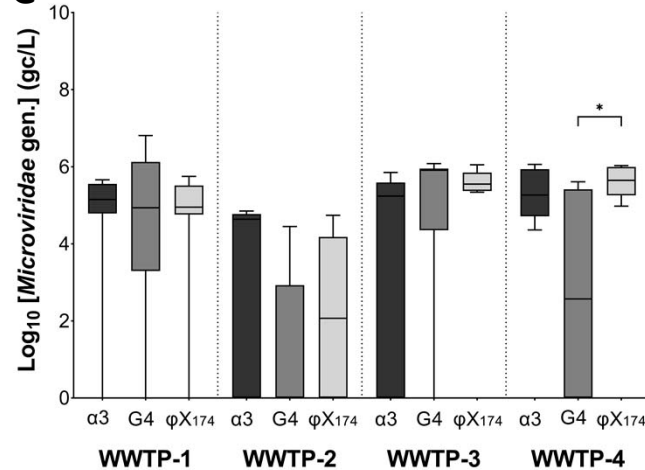


Figure 2: Variation in genome and infectious viral particles concentrations after WWTP treatments. (A) Log₁₀ reduction of infectious SC (grey pattern), infectious *Microviridae* (dark grey) and *Microviridae* genome (light grey) after WW treatments (bars represent the mean with SD for each condition). (B) Log₁₀ reduction of infectious *Microviridae* after WW treatments for each WWTP (bars represent the mean with SD for each condition). (C) Log₁₀ reduction of the *Microviridae* genome copies after WW treatments for each WWTP (bars represent the mean with SD for each condition). For all panels, Kruskal-Wallis statistical test was performed: stars represent significant differences (* = p ≤ 0.05; ** = p ≤ 0.01; *** = p ≤ 0.001 and **** = p ≤ 0.0001). In Figure 2A, the WWTP were compared between each other and the WWTP were tested independently from each other for 2B and 2C.

Table 1: Characteristics of the four WWTP

	WWTP-1	WWTP-2	WWTP-3	WWTP-4
Location	Grand Est region (north-east of France)	Grand Est region (north-east of France)	Nouvelle Aquitaine region (south-west of France)	Nouvelle Aquitaine region (south-west of France)
Capacity (inhabitant- equivalents)	400,000	440,000	150,000	135,000
Type of WW	Urban	Urban	Urban	Urban
	Residential	Residential and industrial ^a	Residential	Residential
	Combined sewer system	Combined sewer-system	Separative sewer-system	Separative sewer-system
Automated sampler	Hach Lange Bühler	Endress Hauser (ASP2000)	Endress Hauser (Liquistation CSF48)	Endress Hauser (Liquistation CSF48)
	Refrigerated	Refrigerated	Refrigerated	Refrigerated
	Dry weather: 60 mL / 250-300 m ³ Wet weather: 60 mL / 350-450 m ³	60 mL / 200 m ³	50 mL / 50 m ³	50 mL / 50 m ³
Pre-treatment	Screening Removal of sand, oil, and grease			
Treatment-I	Primary settling tank			
Treatment-II	Biological filtration Secondary settling tank			
Treatment-III	Phosphorous treatment by flocculation with FeCl ₃ , sand and polymer, and lamellar settling			UV

^a: industrial WW reached about 42,000 inhabitant-equivalents in terms of pollution.

A**B****C**

Supplementary Figure 1: Concentration of the genome and infectious viral particles after WW treatment. (A) Concentration values of infectious SC (grey pattern), infectious *Microviridae* (dark grey) and *Microviridae* genome copies (light grey) after WWTP treatments (bars represent the mean with SD for each condition). **(B)** Concentration values of infectious particles for the genera of *Microviridae* in the effluents (Box and whiskers representing the mean and the maximum and minimum values). **(C)** Concentration values of the genome of the three genera of *Microviridae* in the effluents (Box and whiskers representing the mean and the maximum and minimum values). The LOQ of the three ddPCR assays in WW was $\sim 4.7 \log_{10}$ GC/L. For all panels, Kruskal-Wallis statistical test was performed, stars represent significant differences (* = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$ and **** = $p \leq 0.0001$). In Figure S1A, the four WWTP were compared between each other and the WWTP were tested independently from each other for S1B and S1C. For ddPCR, the LOQ was $5.0 \log_{10}$ gc/L.