

## Quantification of human adenovirus and norovirus in river water in the north-east of France

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1 **Quantification of human adenovirus and norovirus in river water in the north-east of**  
2 **France**

3

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18

19 **Abstract**

20 Human adenoviruses (HAdVs) are a major cause of infection and have been proposed as viral  
21 indicators of water quality. Human noroviruses (NoV) are the main cause of viral acute  
22 gastroenteritis. Quantitative data on the environmental prevalence of both viruses are needed.  
23 The genomes of HAdVs, enteric adenovirus type 41 (HAdV41) and noroviruses of  
24 genogroups I and II (NoV GGI and GGII) were quantified over a six-month period in a river  
25 located in north-eastern France. The samples were collected downstream from the discharge  
26 of a wastewater treatment plant. The viruses were concentrated using a glass wool method  
27 and the viral genomes were quantified using digital droplet PCR (ddPCR). All river water  
28 samples (15/15) were positive for the genomes of HAdVs, HAdV41, NoV GGI and NoV  
29 GGII. Concentrations of HAdVs, HAdV41 and NoV GII genomes were similar and HAdV41  
30 represented ~80% of HAdVs. Infectious HAdVs were quantified in these samples using an  
31 integrated cell culture - quantitative PCR method (ICC-qPCR); they were detected in 93%  
32 (14/15) and quantified in 53% (8/15) of the samples. Thus, infectious HAdVs represented  
33 0.3% to 12.2% of total HAdV particles detected by ddPCR. Infectious HAdV41 particles  
34 were found in 73% (11/15) of the samples. This common presence of pathogenic enteric  
35 viruses underlines the impact of wastewater discharge on quality of surface waters and may  
36 constitute a threat for human health. The relative abundance of genome of HAdV41  
37 underlines the need for studies focusing on the specific detection of its infectious forms along  
38 water cycle.

39

40

41 **Keywords:** Adenovirus type 41; Norovirus; River water; Genome; Infectivity; ICC-qPCR;  
42 Quantification

43

## 44 **Introduction**

45 HAdVs are a major cause of infections which can be fatal for immunocompromised  
46 individuals. They are associated with a wide range of clinical illnesses including upper and  
47 lower respiratory diseases, conjunctivitis, cystitis and gastroenteritis. All HAdVs are excreted  
48 in faeces for an extended period of time even if diarrhoea is not present (Mena and Gerba  
49 2009; Rodriguez-Lazaro et al. 2012). A recent study has shown the role of intestinal  
50 lymphocytes as a reservoir for HAdV persistence and reactivation, whereas the intestinal  
51 epithelium is the main site of viral proliferation preceding dissemination in  
52 immunocompromised patients (Kosulin et al. 2016). HAdVs are non-enveloped viruses  
53 characterized by a double-stranded DNA genome. They belong to the family *Adenoviridae* of  
54 the genus *Mastadenovirus* and are divided into seven species (A to G). Ninety genotypes are  
55 currently proposed by the Human Adenovirus Working Group ([hadv.wg.gmu.edu](http://hadv.wg.gmu.edu); update July  
56 2018).

57 HAdVs have been proposed as a viral indicator for water quality (Bofill-Mas et al.  
58 2010; La Rosa et al. 2010; Wyn-Jones et al. 2011; Rusiñol et al. 2015; Rames et al. 2016;  
59 Farkas et al. 2018). Indeed, these viruses reach at least the six following criteria: i) presence  
60 from wastewater to surface water, ii) lack of seasonality in wastewater, iii) high resistance to  
61 ultraviolet disinfection, iv) higher abundance relative to other enteric viruses, v) availability  
62 of molecular methods and culture assays, and vi) human host specificity (Rames et al. 2016).  
63 HAdVs seem to be highly persistent in the environment and may reflect the behaviour of  
64 persistent enteric viruses (Farkas et al. 2018). Eight viral types of HAdVs belonging to A, C  
65 and F species have been reported mainly in water (Ogorzaly et al. 2015). The most prevalent  
66 HAdV type detected in wastewater and surface water is enteric type HAdV41 which causes  
67 diarrhoea (Rames et al. 2016). Thus, HAdV41 genome was detected in 92% to 96% of  
68 wastewater samples positive for HAdVs in Italy (Iaconelli et al. 2017a) and Japan (Haramoto  
69 et al. 2007), and in 100% of the hospital wastewater samples in Tunisia (Ibrahim et al. 2018).

70 HAdV41 was also detected in 100% of the wastewater samples positive for HAdVs in  
71 Luxembourg (n=8) with a relative abundance estimated to more than 80% using next-  
72 generation sequencing (Ogorzaly et al. 2015). This enteric type is also highly prevalent in  
73 surface water; it was detected in 94% to 100% of river water samples positive for HAdVs in  
74 Luxembourg (Ogorzaly et al. 2015), Italy (La Rosa et al. 2017) and Japan (Haramoto et al.  
75 2007). HAdV41 represented 12 to 77% of overall HAdVs in lake samples collected at  
76 recreational beaches in the USA (Xagorarakis et al. 2007). The frequent presence of HAdV41  
77 in recreational hot springs in Taiwan has also been recently described (Shih et al. 2017).

78 Environmental data on HAdVs have been mainly obtained using molecular methods  
79 only; this led to the absence of information about the infectious status of HAdVs in  
80 environmental samples (Girones et al. 2010; Ogorzaly et al. 2015). Various methods could be  
81 combined with molecular methods to favour the detection of infectious enteric viruses. Most  
82 of them are based on the increased permeability of the capsid to intercalating dyes such as  
83 ethidium monoazide (EMA) or propidium monoazide (PMA) (Prevost et al. 2016).  
84 Intercalating dyes may be useful for the discrimination between infectious and non-infectious  
85 viruses such as HAdVs if the inactivation treatment alters the capsid with an increase in its  
86 permeability (Leifels et al. 2016). This approach could also be applied for the monitoring of  
87 viruses for which cell culture method could not be easily applied on environmental samples or  
88 does not exist yet (Prevost et al. 2016).

89 Infectious HAdVs can be quantified using cell culture methods but long periods of time  
90 are required for HAdVs to produce cytopathic effects (Rames et al. 2016). The integrated cell  
91 culture-quantitative polymerase chain reaction (ICC-qPCR) approach combines cell culture  
92 with qPCR. This approach does not require the observation of cytopathic effects and allows  
93 the reduction of the incubation period down to one to three days even for environmental  
94 samples (Rodriguez et al. 2013; Fongaro et al. 2015; Ogorzaly et al. 2015). Two analytical  
95 approaches are described for HAdVs based on the detection of either viral DNA (Greening et

96 al. 2002) or viral mRNA (Ko et al. 2003) in host cells. Viral DNA detection is easily  
97 performed, but it requires monitoring of the increase in initial genome copy numbers over  
98 time thus reflecting viral replication upon infection (Ogorzaly et al. 2013). The presence of  
99 viral mRNA in host cells is evidence of viral infection, but mRNA detection involves a  
100 reverse transcription step and an enzymatic digestion of contaminating DNA, whose  
101 efficiency has to be checked. Moreover, the variation in mRNA concentration during the virus  
102 replication cycle and the susceptibility of such molecules to degradation under some  
103 conditions require the use of housekeeping genes as cell-based positive control for mRNA  
104 expression (Rodriguez et al. 2013). Another technical aspect of major importance in the use of  
105 the ICC-qPCR method for the detection of infectious HAdVs in environmental samples  
106 concerns the cell line used. Indeed, the 293 cell line, also called HEK293 or Graham 293  
107 (G293), seems to be the most suitable for the detection of a wide diversity of HAdVs  
108 occurring in water samples, especially HAdV41 (Ogorzaly et al. 2015). A549 and BGM cell  
109 lines are also frequently used for the detection of infectious HAdVs using ICC-qPCR  
110 approach (Greening et al. 2002; Lee et al. 2004; Lee et al. 2005; Wyn-Jones et al. 2011;  
111 Fongaro et al. 2013; Fongaro et al. 2015), but their use may result in a lack of detection of  
112 enteric adenoviruses types 40 and 41 (Wyn-Jones et al. 2011). The infection of permissive  
113 cells with serial dilutions of a known HAdVs suspension in parallel with the sample allows  
114 the quantification, and not only the detection, of infectious HAdVs. Whereas the ICC-qPCR  
115 assay is currently used for quantifying infectious HAdVs in laboratory strains (Gerrity et al.  
116 2008; Li et al. 2009; Ryu et al. 2015), very few studies have applied it for the quantification  
117 of infectious HAdVs in environmental water samples (Fongaro et al. 2013; Rodriguez et al.,  
118 2013; Fongaro et al., 2015).

119 Although river waters are commonly used for production of drinking water or culture  
120 irrigation, data are still limited concerning the concentrations of HAdVs in such samples. The  
121 occurrence and concentrations of HAdVs have been compared to those of other pathogenic

122 enteric viruses (*e.g.* noroviruses, enteroviruses) but mainly in wastewaters (Rames et al.  
123 2016). Moreover, data are lacking on the relative abundance of infectious HAdVs.  
124 Comparison between HAdVs and other pathogenic enteric viruses, in particular noroviruses,  
125 in terms of total particles and data about concentrations of infectious HAdVs are needed for  
126 their evaluation as viral indicators of water quality.

127 In this context, the present study aimed to improve the knowledge about HAdVs in  
128 environmental samples. First, the genomes from all types of HAdVs, enteric type HAdV41,  
129 NoV GGI and NoV GGII were quantified in 15 river water samples in order to give elements  
130 in the evaluation of HAdVs as potential indicators of viral contamination. Second, a one-day  
131 ICC-qPCR assay allowing the quantification of infectious HAdVs was applied on the same  
132 river samples. This method allowed us to estimate the relative abundance of infectious  
133 HAdVs among total HAdV particles. Third, the prevalence of infectious HAdV41 was also  
134 investigated in these river water samples.

135

## 136 **Materials and methods**

### 137 **Cell line and virus**

138 Human embryonic kidney cell line 293A (Thermo Fisher Scientific, R705-07) was used in  
139 this study between passages 15 and 30. Cell line 293A is a sub-clone of 293 cells with a rather  
140 flat morphology which strongly adheres to conventional culture surfaces. They were cultured  
141 in Dulbecco's modified Eagle's medium (DMEM) containing a high glucose concentration  
142 and Glutamax (Gibco). This medium was supplemented with 5% of foetal bovine serum (FBS,  
143 Gibco) and 1% of non-essential amino acids (NEAA, Gibco).

144 Human adenovirus type 2 (HAdV2; HPACC, NCPV#213) was chosen since it is a common  
145 type in environmental water samples and one of the most studied types in lab-scale  
146 experiments. HAdV2 was propagated in 293A cells after three days of culture. The culture  
147 medium was supplemented with 2% FBS for HAdV2 replication. After three to four days,

148 FBS was added at a 20% final concentration just before three freeze/thaw cycles. A  
149 centrifugation ( $2,000 \times g$  for 10 min) was then performed to remove cell debris from the viral  
150 lysate. The supernatant was supplemented with sterile glycerol at a 30% final concentration,  
151 aliquoted (1.2 mL) and stored at  $-80^{\circ}\text{C}$ .

152

### 153 **River water samples**

154 Over a six-month period (January-June 2016), a total of 15 river water samples (20 L) were  
155 collected in the Meurthe River in Maxéville. The samples were collected downstream from  
156 the metropolitan wastewater treatment plant (WWTP) (~250,000 inhabitants, Maxéville-  
157 Nancy) (Fig. 1 in Supplementary material). The wastewater treatment is based on activated  
158 sludge followed by removal of nitrogen and phosphorus. The metropolitan area is equipped  
159 with rainwater collection systems with a retention capacity of  $180,000 \text{ m}^3$  to reduce the effect  
160 of heavy rain events on the operation of the WWTP. The average volume of treated  
161 wastewater discharged by the WWTP is  $\sim 100,000 \text{ m}^3/\text{day}$  and the mean flow of this river  
162 ranges from  $\sim 65.7 \text{ m}^3/\text{s}$  in January to  $\sim 30.6 \text{ m}^3/\text{s}$  in June (mean of data collected from 1960 to  
163 2018, <http://www.hydro.eaufrance.fr/stations/A6941020&procedure=synthese>).

164

165

### 166 **Quantification of infectious HAdVs in laboratory strain using the most probable** 167 **number assay**

168 The Most Probable Number (MPN) assay was performed to determine the infectious titre of  
169 the laboratory strain that was then used for the standard of the ICC-qPCR method. This  
170 method is used for the quantification of infectious enteric viruses (Gassilloud et al 2003;  
171 Ogorzaly et al 2010; Brié et al 2017). In our case, the MPN assay was performed in 96-well  
172 microplaques using 293A cells. Fifty-microlitre portions of successive logarithmic dilutions  
173 of each sample were added to 200  $\mu\text{L}$  of preparation containing  $3 \times 10^4$  293A cells/mL in



174 DMEM containing 2% FBS. Each dilution was seeded in 40 wells. Plates were incubated in  
175 5% CO<sub>2</sub> at 37°C for 11 days and then examined for cytopathic effects. The number of wells  
176 with cytopathic effects were counted and the MPNCU (most probable number of cytopathic  
177 units) was calculated. The final result of each sample analysed was expressed as the geometric  
178 mean of the MPNCU per millilitre. The quantification limit of this method is of 1.5  
179 MPNCU/mL.

180

### 181 **Virus concentration from river water samples**

182 A glass wool method was used for virus concentration, as previously described; this method is  
183 well adapted to the detection of infectious HAdVs using cell culture methods (Wyn-Jones et  
184 al 2011; Ogorzaly et al 2013). Briefly, river samples (20 L) were acidified with 1 M HCl to  
185 pH 3.5 and 10 g of sterile glass wool was used for the filtration column. When the entire  
186 sample had passed through the filter HAdVs were eluted from the glass wool by slow passage  
187 of 200 mL 3% beef extract (w/v) at pH 9.5 in 0.05 M glycine buffer. The eluate was  
188 flocculated by the addition of 1 M HCl to pH 3.5. The protein floc, containing viruses, was  
189 recovered by centrifugation (7,000 × g for 30 min) and dissolved to a final volume of 20 mL  
190 DMEM. The suspension was passed through a combination of pre-filter (5 µm, Sartorius  
191 Minisart) and membranes (1.2 and 0.45 µm, Sartorius Minisart) before freezing at -80°C.

192

### 193 **Genome extraction in river water concentrates**

194 For the quantification of the viral genomes in the river water concentrates, a 1 mL aliquot was  
195 mixed with 2 mL of lysis buffer and incubated at room temperature for 10 min. The sample  
196 was then mixed with 3 mL of phenol/chloroform/isoamyl alcohol (24:5:1; Acros Organics)  
197 and centrifuged (2,000 × g for 5 min). Viral DNA and RNA extraction was then performed on  
198 the supernatant using a NucliSens Magnetic extraction kit (bioMérieux). The elution step was  
199 carried out with 100 µL of the appropriate buffer and the extracted nucleic acids were

200 immediately stored at  $-80^{\circ}\text{C}$ . This kit is well adapted for the extraction of viral genomes and  
201 can be easily combined to the phenol/chloroform/isoamyl alcohol pre-treatment applied on  
202 the water concentrates.

203

#### 204 **Genome quantification in river water concentrates using digital droplet PCR**

205 The digital droplet PCR approach (dd-PCR) was performed for the quantification of the  
206 genomes of HAdVs, HAdV41, NoV GGI and NoV GGII in river water concentrates. For  
207 HAdVs, the assay described by Hernroth et al. (2002) using degenerated primers (AdF and  
208 AdR) and a TaqMan probe (Ad/ACDEF) was used. This assay is based on the amplification  
209 of the hexon gene and recognizes most HAdVs (Bofill-Mas et al. 2006). For HAdV41, the  
210 assay based on the amplification of the fibre protein was designed in our laboratory. The  
211 TaqMan MGB probe was: 5'-FAM CTT CGC CTT CAA AGT GC-MGB NFQ-3' (position  
212 30415-30431 on AdV41 sequence, KY316161.1) and the primers were: forward 5'-CGC  
213 CCC ACT TAA TGC TGA CA-3' (position 30387-30406), reverse primer 5'-TCC ACT  
214 AGT CCA AGA GGT GCA-3' (position 30432-30452). Primer and probe sequences were  
215 subsequently confirmed for specificity using BLAST. NoV GGI and NoV GGII genomes  
216 were quantified using primers and probes described in ISO/TS Standard 15216-1:2013  
217 (International Organization for Standardization, 2013). Amplifications were performed in a 20  
218  $\mu\text{L}$  reaction mixture containing 5  $\mu\text{L}$  of template DNA for HAdVs and HAdV41 or RNA for  
219 NoV GGI and GGII, and 15  $\mu\text{L}$  of ddPCR<sup>TM</sup> Supermix for Probes (BIO-RAD) for HAdVs  
220 and HAdV41 and One-Step RT-ddPCR<sup>TM</sup> Kit for Probes (BIO-RAD) for NoV GGI and GGII.  
221 The reaction mix contained 0.9  $\mu\text{M}$  of each primer, 0.225  $\mu\text{M}$  of probe for HAdVs and 0.3  
222  $\mu\text{M}$  of probe for NoV. The samples were placed in the droplet generator using 70  $\mu\text{L}$  of  
223 generator oil. The resulting picolitre droplet emulsions (40  $\mu\text{L}$ ) were transferred to a Veriti  
224 96-Well Thermal Cycler (Applied Biosystems). Viral DNA amplification was performed  
225 using the following conditions: 10 min hold at  $95^{\circ}\text{C}$ , 40 cycles of  $94^{\circ}\text{C}$  for 30 s then  $55^{\circ}\text{C}$  for

226 60 s and finally a 10 min hold at 98°C with a ramp rate set at 70% between each step. For  
227 viral RNA amplification, the reverse transcription was performed with 60 min hold at 50°C.  
228 After amplification the plate was transferred to QX100™ Droplet Reader (BIO-RAD) using  
229 QuantaSoft™ Software (BIO-RAD) to measure the number of positive and negative droplets  
230 based on fluorescence amplitude. Each sample was analysed undiluted and after a 10-fold  
231 dilution. Negative and positive controls were included in each experiment to ensure no  
232 contamination and good reproducibility of the extractions and PCR assays. The genome  
233 concentrations obtained with this method were given in genome copies (gc) per unit volume.

234

### 235 **Quantification of infectious HAdVs using ICC-qPCR method**

236 For the ICC-qPCR assay the river water samples were analysed concomitantly with a  
237 10-fold dilution series of the laboratory strain HAdV2 used as standard in order to quantify  
238 infectious HAdVs. The standard was made with concentrations ranging from  $10^5$  to 10  
239 MPNCU/mL. A 1 mL volume of standard or river water sample was inoculated per well of  
240 six-well plates (Nunc™) containing two-day-old 293A cells, with a growth area of 9.6 cm<sup>2</sup>. A  
241 negative control was included in all our experiments, consisting in the inoculation of cells  
242 with 1 mL of DMEM medium. After inoculation, plates were incubated for 2 h at 37°C with  
243 5% CO<sub>2</sub> for adsorption of viral particles. Afterwards, the inoculum was removed, and the  
244 cells were washed twice with 2 mL of DMEM. Finally, 2 mL of DMEM containing 2% of  
245 FBS and supplemented with a 1% final concentration of an antibiotic-antimycotic solution  
246 (10,000 units/mL of penicillin, 10,000 units/mL of streptomycin and 25 µg/mL of  
247 amphotericin B; Gibco) were added to each well. For the wells marked as  $T_0$  viral DNA was  
248 immediately extracted. The wells marked as  $T_{24h}$  were incubated at 37°C with 5% CO<sub>2</sub> for one  
249 day, before viral DNA extraction which was performed using QIAamp viral RNA mini kit  
250 (Qiagen). This kit allows the extraction of viral DNA in particular from infected cell cultures  
251 (Ogorzaly et al. 2013, 2015). The culture medium was discarded from each well by aspiration

252 and the 293A cells - potentially infected by viruses - were covered with 1 mL of lysis buffer  
253 (AVL buffer + Carrier RNA) for 15 min. Viral nucleic acids were eluted in 100 µL of the  
254 appropriate buffer, according to manufacturer's instructions. The extracts were immediately  
255 stored at -80°C. Quantification of the HAdV and HAdV41 genomes in the cell culture  
256 samples was performed by a generic real-time PCR system using the same primers and probes  
257 as those described for ddPCR. Amplifications were performed in a 25 µL reaction mixture  
258 containing 10 µL of template DNA and 15 µL of TaqMan Universal MasterMix (Applied  
259 Biosystems) containing 0.9 µM of each primer and 0.225 µM of TaqMan probe. Temperature  
260 cycles were performed in CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD).  
261 PCR amplification was performed at 95°C for 10 min, followed by 45 cycles of 15 s at 95°C  
262 and 60 s at 55°C. Negative and positive controls were included in each experiment to ensure  
263 no contamination and good reproducibility of the extractions and real-time PCR steps.  
264 Samples were considered positive for infectious HAdVs if a decrease in their quantification  
265 cycle (Cq) values was observed between  $T_0$  and  $T_{24h}$ . Three wells were used for each dilution  
266 of the standard (one  $T_0$  and two  $T_{24h}$ ). For each environmental sample, four wells were each  
267 infected with 1 mL of concentrate (one  $T_0$  and three  $T_{24h}$ ). The concentrations obtained with  
268 this method were given in equivalent Most Probable Number of Cytopathic Unit (eqMPNCU)  
269 per millilitre. The quantification limit of this method was determined prior to the analysis of  
270 the river water samples.

271

## 272 **Statistical analysis**

273 The statistical analysis was performed using IBM SPSS Statistics version 23.0 Software. A  
274 Wilcoxon signed rank test, a Spearman rank correlation test and linear regression tests were  
275 performed to compare the viral genome concentrations of HAdVs, HAdV41, NoV GGI and  
276 NoV GGII in the river water concentrates.

277

## 278 **Results**

279

### 280 **Quantification of viral genomes in the river water samples**

281 The pH and the temperature of the river water samples were measured prior to virus  
282 concentration; the mean pH value was  $8.2 \pm 0.4$  and the mean water temperature was  
283  $12.0 \pm 3.5^\circ\text{C}$ . The cumulative precipitation two days prior to sampling reached  $4.1 \pm 4.2$  mm  
284 (Table 1 in Supplementary material).

285 Prior to the quantification of infectious HAdVs in the river water samples, the genome  
286 concentrations of HAdVs and HAdV41 were determined using ddPCR method (Fig. 1). The  
287 15 samples were positive for these two targets with mean concentrations reaching  
288  $3.6 \times 10^3 \pm 4.4 \times 10^3$  gc/L for HAdVs and  $2.9 \times 10^3 \pm 2.9 \times 10^3$  gc/L for HAdV41. The  
289 concentrations ranged from  $1.1 \times 10^2$  gc/L (S-14 [Sample 14]) to  $1.4 \times 10^4$  gc/L (S-11) for  
290 HAdVs and from  $1.3 \times 10^2$  gc/L (S-14) to  $9 \times 10^3$  gc/L (S-9 and S-11) for HAdV41. The  
291 genome concentrations of NoV GGI and NoV GGII were also evaluated in these samples  
292 (Fig. 1). The mean concentrations reached  $6.1 \times 10^2 \pm 7.4 \times 10^2$  gc/L for NoV GGI and  
293  $3.7 \times 10^3 \pm 3.2 \times 10^3$  gc/L for NoV GGII. The lowest genome concentrations were  
294 systematically observed for NoV GGI (P-value = 0.001, Wilcoxon signed rank test). The  
295 genome concentrations obtained with undiluted and 10-fold diluted samples suggested that  
296 our ddPCR assays were not impacted by inhibitory substances. These samples had been  
297 collected between January and June, but no difference in the concentration was noticed  
298 between the winter and spring periods for any virus. Similarly, no relation was observed  
299 between the cumulative precipitation and the genome concentrations for any virus.

300 In order to assess correlation between these viruses, Spearman rank and linear  
301 regression analyses were performed on these data (Table 2 in Supplementary material). The  
302 strongest correlation was observed between HAdVs and HAdV41 with an  $r_s$  value of 0.943  
303 (P-value < 0.01) and an  $R^2$  value of 0.94 (Fig. 3a). The comparison between the genome

304 concentrations of HAdVs and HAdV41 showed that the enteric type HAdV41 represented  
305 ~80% of HAdVs detected in these river water samples. A strong correlation was also  
306 observed between NoV GGI and NoV GGII and between HAdV41 and NoV GGII with  $r_s$   
307 values reaching 0.844 (P-value < 0.01) and 0.808 (P-value < 0.01), respectively. Moreover,  $R^2$   
308 values of 0.59 and 0.61 were found between HAdV41 and NoV GGII (Fig. 3b) and between  
309 NoV GGI and NoV GGII (Fig. 3c), respectively. The  $R^2$  value reached 0.50 between HAdVs  
310 and NoV GGII. These results suggested an interrelation between NoV GGII and HAdV41 in  
311 these river water samples.

312

### 313 **Validation of the ICC-qPCR assay on HAdV2 suspension**

314 The ICC-qPCR assay was performed with 10-fold serial dilutions of the HAdV2 laboratory  
315 strain (n = 17) ranging from  $10^5$  to 10 MPNCU/mL which were tested in duplicate in each  
316 assay. At  $T_0$ , all samples were positive (*i.e.* Cq value < 45) for concentrations ranging from  
317  $10^5$  to  $10^3$  MPNCU/mL, whereas only four assays out of 17 were positive for  
318  $10^2$  MPNCU/mL. For 10 MPNCU/mL, all tests were negative (*i.e.* Cq value  $\geq$  45) at  $T_0$  (data  
319 not shown). The Cq value at  $T_0$  showed the quantity of HAdVs bound to cells just after their  
320 inoculation. Between  $T_0$  and  $T_{24h}$ , a decrease in the Cq value was noticed for concentrations  
321 ranging from  $10^5$  to 10 MPNCU/mL, reflecting the replication of viral DNA in the host cells.  
322 When no infectious HAdVs were inoculated onto the cells, samples were negative at  $T_0$  and  
323  $T_{24h}$  (data not shown). The Cq values obtained at  $T_{24h}$  were represented as a function of the  
324 concentration of infectious HAdV2 inoculated onto cells (Fig. 1); a linear response was  
325 obtained from  $10^5$  to 10 MPNCU/mL. The mean Cq values ranged from 17.3  
326 ( $10^5$  MPNCU/mL) to 30.3 (10 MPNCU/mL). The slope of this ICC-qPCR standard curve was  
327 -3.33, with a squared correlation coefficient ( $R^2$ ) of 0.9981. An increase in the Cq value was  
328 observed between  $T_0$  and  $T_{24h}$  in 15 assays out of 17 at a concentration of 10 MPNCU/mL  
329 which evidenced that this ICC-qPCR method allowed the quantification of infectious HAdVs

330 with a quantification limit of 10 eqMPNCU/mL. Thus, we could expect a quantification limit  
331 of 10 eqMPNCU/L of river water.

332 The ICC-qPCR method was then compared to the MPN method on the basis of HAdV2  
333 suspensions exposed to different experimental conditions. A titration was performed with  
334 both methods just after production for one suspension. Concentrations of infectious HAdVs  
335 reached  $1.1 \times 10^{10}$  eqMPNCU/mL and  $4.5 \times 10^9$  MPNCU/mL with the ICC-qPCR and MPN  
336 methods, respectively. Thus, the concentration obtained with the ICC-qPCR method was  
337 twice that obtained with the MPN assay. The titrations performed with two suspensions after  
338 storage at  $-80^\circ\text{C}$  for several weeks showed concentrations of  $8.3 \times 10^8$  eqMPNCU/mL  
339 (suspension 1) and  $1.5 \times 10^9$  eqMPNCU/mL (suspension 2) using ICC-qPCR method and of  
340  $6.9 \times 10^8$  MPNCU/mL (suspension 1) and  $2.0 \times 10^9$  MPNCU/mL (suspension 2) using the  
341 MPN method. For these two suspensions, the concentrations detected with the ICC-qPCR  
342 method reached 120% and 75% of those observed with the MPN method.

343

#### 344 **Quantification of infectious HAdVs in the river water samples**

345 Infectious HAdVs were then quantified in the 15 river samples using the ICC-qPCR assay.  
346 From the  $C_q$  values obtained at  $T_0$  and  $T_{24h}$ , 14 samples (93%) were considered as positive for  
347 infectious HAdVs. Among them, eight samples (53%) contained between  $1.5 \times 10^1$  and  
348  $4.1 \times 10^2$  eqMPNCU/L and the other six were below the quantification limit (Fig. 4). When  
349 quantifiable, infectious HAdVs represented between 0.3% and 12.2% of total HAdVs.

350 Infectious HAdV41 could not be specifically quantified using our ICC-qPCR assay  
351 since it was based on a standard curve obtained with HAdV2. However, the quantification of  
352 the HAdV41 genome in infected cells at  $T_{0h}$  and  $T_{24h}$  allowed us to determine the number of  
353 samples positive for infectious HAdV41. Thus, an increase in the concentration of HAdV41  
354 genome was observed in infected cells between  $T_0$  and  $T_{24h}$  for 11 samples (73%); they were  
355 marked with an asterisk in Fig. 4. Infectious HAdV41 particles were detected in the eight

356 samples in which infectious HAdVs were quantified and in three out of the six samples  
357 containing infectious HAdVs below the quantification limit.

358

## 359 **Discussion**

360 The present work aimed to contribute to the evaluation of HAdVs as viral indicators of  
361 water quality. For this purpose, comparison between the genome concentrations of human  
362 NoV GGI and GGII, which are the main cause of viral acute gastroenteritis, and HAdVs were  
363 performed in river water samples characterised by a recent faecal contamination. Infectious  
364 HAdVs were also quantified in our river water samples using an ICC-qPCR method. Indeed,  
365 the detection of viral genome in environmental samples does not provide information about  
366 the infectivity (Girones et al. 2010), whereas this information is still of major importance in  
367 the evaluation of the human health risk.

368 The virus particles were concentrated from river water using a glass wool method with  
369 pre-acidification. This concentration method was chosen due to the relatively high volume of  
370 river water analysed (20 L). Moreover, a mean recovery of 57.1% (range 34.2% - 78.2%)  
371 (Wyn-Jones et al. 2011) has previously been obtained with surface water samples including  
372 samples from the same river than our study. This concentration method has been previously  
373 shown to allow the detection of infectious HAdVs (Wyn-Jones et al. 2011; Ogorzaly et al.  
374 2013). Nevertheless, the pH range (pH 4-9) used in primary concentration methods based on  
375 adsorption-elution using electronegative or electropositive filters such as glass wool method  
376 and also in concentration methods based on organic flocculation and celite may interfere with  
377 the detection of infectious viruses (Rames et al. 2016). Indeed, viral inactivation and  
378 aggregation may occur with such concentration methods (Rames et al. 2016; Gerba and  
379 Betancourt, 2017). Hollow fibre ultrafiltration (HFUF) is also adapted to large volume  
380 samples and used for primary concentration, but entrapment of viruses on the filter surface



381 and inhibition of molecular methods from the use of sodium polyphosphate have been  
382 described with this method (Rames et al. 2016).

383 The genomes of HAdVs and HAdV41 were detected in all 15 river water samples and  
384 their concentrations ranged between 2 and 4 logs gc/L. Such a high proportion of positive  
385 samples is in accordance with previous studies performed on the same river (Ogorzaly et al.  
386 2009, 2013); prevalence of HAdVs about 90% has also been reported in surface water in other  
387 countries (Hamza et al. 2009; Garcia et al. 2012; Ye et al. 2012; Calgua et al. 2013a; Vieira et  
388 al. 2016; Farkas et al. 2018). The limitation of our sampling campaign to six months, the  
389 exclusion of the fall and early winter season and the lack of heavy rain or snow event within  
390 the two days prior to sampling may have contributed to the absence of strong variation  
391 between the viral genome concentrations. In a recent study performed on the river Ruhr  
392 (Germany) between July and September 2015, the authors observed that enhanced  
393 precipitation in combination with an increased flow rate were associated with an increase in  
394 the concentration of HAdVs (Mackowiak et al. 2018). HAdVs are not known to show seasonal  
395 differences in their excretion resulting in lack of seasonality in raw wastewater (Rames et al.  
396 2016). In a study performed in the river Ruhr with 184 samples collected between May 2012  
397 and June 2013, no significant difference in the percentage of positive samples for HAdVs  
398 genome was observed between the bathing season (92.5%, n=80) and outside the bathing  
399 season (88.5%, n=104), suggesting a lack of seasonality of this enteric virus (Timm et al.  
400 2016). Nevertheless, seasonal occurrence has been described in environmental waters in some  
401 cases (Rames et al. 2016). The discharge of treated wastewater from a large urban area  
402 (~250,000 inhabitants) upstream from the sampling site had probably contributed to the high  
403 prevalence and concentrations observed in our study. Overall, considerable variations in  
404 prevalence and concentrations of HAdVs have been reported across studies. Indeed, 16% to  
405 100% of surface water samples have been previously shown to be positive for HAdVs with  
406 concentrations ranging from 1 to 7 logs gc/L (Bofill-Mas et al. 2013; Iaconelli et al. 2017a).

407 The high proportion of the enteric type HAdV41 (80%) observed in our river water samples is  
408 in accordance with the few studies performed on surface water (Xagorarakis et al. 2007;  
409 Haramoto et al. 2007; Bofill-Mas et al. 2010; Ogorzaly et al. 2015; La Rosa et al. 2017). The  
410 use of the next-generation sequencing (NGS) technology which seems to be the most relevant  
411 method to study the diversity of HAdVs in environmental waters has recently shown that  
412 HAdV41 was the most abundant type in complex environmental matrices with high  
413 concentrations of HAdVs such as wastewater (Ogorzaly et al. 2015; Iaconelli et al. 2017b).

414 All 15 river water samples were also positive for the genomes of NoV GGI and NoV  
415 GGII and the concentrations of NoV GGII were similar to those observed for HAdVs and  
416 HAdV41. In a recent study performed on the Seine River (France), HAdVs, NoV GGI and  
417 NoV GGII genomes have been detected in 93%, 88% and 92% of the samples (n = 100),  
418 respectively, and the impact of the wastewater treatment plant effluents on the viral  
419 contamination of the river has been clearly shown (Prevost et al. 2015). Overall, considerable  
420 variations between HAdVs and NoV have also been reported across studies. Highly different  
421 prevalence has been described in some studies (Wyn-Jones et al. 2011; Corsi et al. 2014;  
422 Vieira et al. 2016), whereas similar prevalence has been reported in others (Kishida et al.  
423 2012; Prevost et al. 2015; Iaconelli et al. 2017a; Jones et al. 2017; La Rosa et al. 2017).  
424 Differences in prevalence between sampling sites have also been reported in the same study  
425 (Calgua et al., 2013a). Studies comparing the persistence of viral RNA and DNA are still  
426 needed (Bertrand et al., 2012), but similar concentrations for HAdV and NoV GGII genomes  
427 seem more likely to occur in case of a recent and / or high viral contamination.

428 The ICC-qPCR method used in the present work allowed the quantification of  
429 infectious HAdVs in one day with a sensitivity of 10 MPNCU/L of river water. A linear  
430 relationship between infectious dose and viral genome in infected cells at  $T_{24h}$  was observed  
431 between  $10^5$  and  $10^7$  MPNCU/L. A few studies have quantified infectious HAdVs naturally  
432 occurring in environmental waters and only a small part of them have used the ICC-qPCR

433 method in such a context. Two studies carried out in Brazil have observed high concentrations  
434 of infectious HAdVs in groundwater, surface water (*i.e.* lagoon) and spring source water, but  
435 also in chlorinated water from a network distribution system using ICC-RT-qPCR (Fongaro et  
436 al. 2013, 2015). Thus, the authors have reported concentrations ranging from  $10^2$  to  $10^3$  gc/L  
437 of infectious HAdVs in 60% (3/5) to 80% (4/5) of groundwater samples and from  $10^2$  to  $10^5$   
438 gc/L of infectious HAdVs in 83% (5/6) of water samples from a network distribution system  
439 (post chlorination). However, infectious HAdVs constituted  $\leq 0.12\%$  of total HAdVs in all  
440 these samples (Fongaro et al. 2015). Using ICC-RT-qPCR, Rodriguez et al. (2013) have  
441 detected infectious HAdVs at concentrations ranging between  $10^1$  and  $10^3$  mRNA-Infectious  
442 Units/L in sewage (n = 4) in the USA. The ratios between total and infectious HAdVs ranged  
443 from 11/1 to 26/1 for three samples containing HAdV-F species only. One sample showed a  
444 ratio of 381/1 and contained HAdVs belonging to A, E and F species. This meant that the first  
445 three samples contained between 3.8% and 9.1% of infectious HAdVs, whereas the last  
446 sample contained 0.2% of infectious HAdVs. Our concentrations ( $10^1$  to  $10^3$  eqMPNCU/L)  
447 and percentages (0.3% to 12.2%) of infectious HAdVs were similar to those observed in  
448 sewage (Rodriguez et al. 2013) but different from those detected by Fongaro et al. (2013,  
449 2015). The delay between viral contamination and sampling may significantly impact the  
450 percentages of infectious HAdVs. The cell lines may also contribute to these discrepancies  
451 concerning the percentages of infectious HAdVs. Indeed, Fongaro et al. (2013, 2015) have  
452 used A549 cells, whereas Rodriguez et al. (2013) have used G293 cells. As discussed in the  
453 introduction, several studies have shown that A549 cells do not allow the detection of  
454 HAdV41 which is highly frequent in environmental samples. In the present study, infectious  
455 HAdV41 were found in 73% of the river water samples (11/15). Only two studies have  
456 described the detection or quantification of infectious HAdV41 in environmental waters and  
457 were performed on sewage. Infectious HAdV41 have been detected in all sewage samples in  
458 Luxembourg (n = 4) (Ogorzaly et al. 2015) and in the USA (n = 4) (Rodriguez et al. 2013).

459 Above, the possibility of an inactivation of virus particles during the concentration step has  
460 been discussed. Rames et al. (2016) have reported in their review a comparison of HAdVs  
461 concentrations in raw and treated wastewater using culture-based and qPCR methods for  
462 different concentration methods; the percentages of infectious HAdVs ranged from <0.01% to  
463 10%. Thus, our percentages of infectious HAdVs (0.3% to 12.2%) suggested that the glass  
464 wool method does not impact more deeply the infectivity of virus particles than PEG  
465 (polyethylene glycol) precipitation or ultracentrifugation. Overall, further studies on  
466 concentration methods adapted to large volume of surface water samples and considering both  
467 the recovery rates and the preservation of virus infectivity are still needed. These studies  
468 should focus on HAdV41. Intercalating dye treatment prior to qPCR, in particular EMA-  
469 qPCR, seems to be a valuable method to evaluate capsid integrity in water matrices. It could  
470 be a rapid, relatively inexpensive and easy to use tool in the evaluation of the effect of  
471 concentration methods on the virus integrity (Leifels et al. 2016; Prevost et al. 2016). For  
472 ICC-qPCR approach, studies should take into account that specific cell lines such as 293  
473 should be used and that HAdV41 might have a longer multiplication cycle than other HAdV  
474 types (Siqueira-Silva et al. 2009).

475

## 476 **Conclusions**

477 Our work shows that not only HAdV genomes but also infectious particles can be frequent in  
478 river water in Europe. Indeed, infectious HAdVs reached more than 10 infectious particles/L  
479 in more than 50% of the 15 samples. HAdV41 genome was highly predominant in these river  
480 samples and a correlation between HAdV41 and NoV GGII genomes was observed. The  
481 presence of these two enteric viruses should be studied in relation with effluent discharge in  
482 particular for rivers with a strong anthropic pressure. Infectious HAdV41 was detected with a  
483 frequency higher than 70%. This predominance of HAdV41 genome over other HAdV types  
484 and the high prevalence of infectious HAdV41 in environmental waters underline the need for

485 further studies focusing on the specific quantification of infectious particles of HAdV41. Such  
486 data are needed to improve the knowledge on this enteric type and its potential as a viral  
487 indicator of water quality.

488

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494

#### 495 **Conflict of interest**

496 The authors declare that they have no conflict of interest.

497

498

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## Figure legends

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667

668 **Figure 1.** Genome concentrations of HAdVs, HAdV41, NoV GGI and NoV GGII in the river  
669 water samples ( $n = 15$ ) using ddPCR.

670

671 **Figure 2.** Standard curve of the ICC-qPCR assay generated from the range of dilutions of  
672 HAdV2 ( $n = 17 \times 2$ ).

673

674 **Figure 3.** Relation between the concentrations of (a) HAdVs and HAdV41, (b) NoV GGII  
675 and HAdV41, and (c) NoV GGII and NoV GGI in the 15 river water samples.

676

677 **Figure 4.** Concentrations of HAdV genomes and infectious HAdVs in the river water samples  
678 ( $n = 15$ ) using ddPCR and ICC-qPCR, respectively. Samples positive for infectious HAdV41  
679 are notified with an asterisk. ND: not detected. NQ: not quantified.\*: water samples positive  
680 for infectious HAdV41.

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### 683 **Supplementary material**

684 **Figure 1.** Sampling point in the river Meurthe in the metropolitan area of Nancy. Black  
685 arrow: discharge of treated wastewater in the river. Red star: sampling point in the river

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