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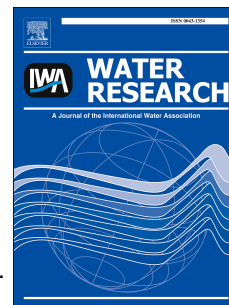
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Viral indicators for wastewater contamination

Human enteric viruses

dsDNA — Adenovirus

dsDNA — Polyomavirus

ssRNA — Aichi virus

Human waste associated viruses

ssRNA — Pepper mild mottle virus

ssRNA — Type II/ III FRNA phage

dsDNA — *Bacteroides* phages

dsDNA — CrAssphage

Viral indicator suitability

	Adenovirus	Polyomavirus	Aichi virus	Pepper mild mottle virus	Type II/ III FRNA phage	Bacteroides phages	CrAssphage
Easy detection	Highly	Somewhat	Somewhat	Somewhat	Highly	Somewhat	?
Present in human faeces	Highly	Somewhat	Somewhat	Somewhat	Somewhat	Highly	?
Abundant in wastewater	Highly	Somewhat	Somewhat	Highly	Somewhat	Highly	Highly
Resistant to wastewater treatment	Highly	Not	?	?	Not	Highly	?
Persistent in the aquatic environment	Highly	Somewhat	?	?	?	?	?
Global distribution	Highly	Highly	Highly	Highly	Highly	Highly	Highly

Legend: ■ Highly appropriate ■ Somewhat appropriate □ Not appropriate ? Limited data

1   Viral indicators for tracking domestic wastewater contamination in the aquatic

2   environment

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Abstract

Waterborne enteric viruses are an emerging cause of disease outbreaks and represent a major threat to global public health. Enteric viruses may originate from human wastewater and can undergo rapid transport through aquatic environments with minimal decay. Surveillance and source apportionment of enteric viruses in environmental waters is therefore essential for accurate risk management. However, individual monitoring of the >100 enteric viral strains that have been identified as aquatic contaminants is unfeasible. Instead, viral indicators are often used for quantitative assessments of wastewater contamination, viral decay and transport in water. An ideal indicator for tracking wastewater contamination should be (i) easy to detect and quantify, (ii) source-specific, (iii) resistant to wastewater treatment processes, and (iv) persistent in the aquatic environment, with similar behaviour to viral pathogens. Here, we conducted a comprehensive review of 127 peer-reviewed publications, to critically evaluate the effectiveness of several viral indicators of wastewater pollution, including common enteric viruses (mastadenoviruses, polyomaviruses, and Aichi viruses), the pepper mild mottle virus (PMMoV), and gut-associated bacteriophages (Type II/III FRNA phages and phages infecting human *Bacteroides* species, including crAssphage). Our analysis suggests that overall, human mastadenoviruses have the greatest potential to indicate contamination by domestic wastewater due to their easy detection, culturability, and high prevalence in wastewater and in the polluted environment. Aichi virus, crAssphage and PMMoV are also widely detected in wastewater and in the environment, and may be used as molecular markers for human-derived contamination. We conclude that viral indicators are suitable for the long-term monitoring of viral contamination in freshwater and marine environments and that these should be implemented within monitoring programmes to provide a holistic assessment of microbiological water quality and wastewater-based epidemiology, improve current risk management strategies and protect global human health.

2

1. Introduction

1.1 Waterborne enteric viruses

Waterborne diarrheal diseases account for approximately 4 billion cases annually, resulting in 2 million deaths, most of which occur in children under five (WHO, 2010). A significant proportion of these illnesses are caused by enteric viral infections (Ramani and Kang, 2009). Enteric viruses are transmitted via the faecal-oral route and the most important route of transmission is direct contact with infected individuals (Katayama and Vinje, 2017). Nonetheless, most enteric viruses are persistent in environments affected by domestic wastewater discharge and are often associated with waterborne outbreaks (Gibson, 2014; Kauppinen et al., 2018; Sekwadi et al., 2018). Wastewater often receives treatment prior to release into the environment, although traditional wastewater treatment methods can be relatively ineffective at removing enteric viruses (Kitajima et al., 2014; Qiu et al., 2015; Sidhu et al., 2017b). In developing countries, many areas lack adequate sanitary infrastructure and wastewater treatment facilities and hence faecal matter contaminates the environment and drinking water sources (Bain et al., 2014). Furthermore, large volumes of untreated wastewater may also be discharged via combined sewer overflows (CSOs) during heavy rainfall events and via dry water overflows for example during snowmelt, tidal infiltration or system failures and blockages (Ahmed et al., 2020). These events enable the direct entry of enteric pathogens into the environment (Fong et al., 2010), where people in direct or indirect contact with contaminated waters may be at risk of acquiring viral infections (Sinclair et al., 2009). Enteric viruses are readily transported in environmental waters and can adsorb to solid matter present in the water column or accumulate in sediment (Hassard et al., 2016). Subsequently, they may also be taken up by filter feeding aquatic animals such as bivalve shellfish that are harvested for human consumption (Landry et al., 1983; Lowther et al., 2012). Furthermore, wastewater is often used for irrigation in countries experiencing freshwater shortage, and hence, enteric viruses may directly contaminate fruit and salad vegetables and result in foodborne outbreaks (Bosch et al., 2016; Chatziprodromidou et al., 2018; Jasim et al., 2016).

Enteric viruses usually cause gastroenteritis lasting for 2-5 days. In some cases, the infection results in respiratory, neural or epidermal symptoms, or remains asymptomatic (Table 1). The viruses most often

4

76 associated with gastroenteritis are members of the *Picornaviridae*, *Caliciviridae*, *Reoviridae* and

77 *Adenoviridae* families (Table 1). For example, noroviruses (family *Caliciviridae*) are responsible for a high

78 proportion of gastroenteritis infections globally, with 685 million cases and approximately 200,000 deaths

79 (CDC, 2016; Katayama and Vinje, 2017), resulting in a total direct cost of US$4.2 billion to the healthcare

80 system and US$60.3 billion in associated societal costs per year (Bartsch et al., 2016). Rotaviruses (family

81 *Reoviridae*) and group F mastadenoviruses (AdVs) (family *Adenoviridae*) are the main causative agents of

82 gastroenteritis amongst infants and young children (Desselberger and Gray, 2009; Jiang, 2006).

83 Noroviruses, hepatitis A virus (family *Picornaviridae*) and AdVs are the most common viral pathogens

84 associated with waterborne and water-associated foodborne outbreaks and infection may result in serious

85 illness, e.g. acute hepatitis (Bellou et al., 2013; Harris et al., 2006; Jiang, 2006; Parshionikar et al., 2003;

86 Sinclair et al., 2009).

87 Rotaviruses, enteroviruses, sapoviruses, astroviruses, Aichi virus (AiV) and hepatitis E virus are also often

88 shown to be associated with wastewater contamination. For example, in Maharashtra state, India in 2017,

89 a rotavirus B outbreak with a 22.8% attack rate (i.e. new cases/number of people) was sourced from

90 contaminated wells used for drinking water (Joshi et al., 2019). In addition, several viral gastroenteritis

91 outbreaks linked to sewage-contaminated drinking water containing AdV, noro- sapo-, astro-, rota-, and

92 enteroviruses have been reported (Kauppinen et al., 2019; Maunula et al., 2009; Rasanen et al., 2010). The

93 largest viral waterborne outbreak affecting approximately 80,000 people in Kanpur, India was associated

94 with hepatitis E virus (Naik et al., 1992). The surveillance of enteric viral illnesses can be challenging, as

95 many of the enteric viral outbreaks are unreported as the symptoms are often subclinical (Cortez et al.,

96 2017; Koff, 1992; Li et al., 2017; Matson et al., 1993; Sakai et al., 2001; Zaoutis and Klein, 1998).

97 Over the last decade, both newly discovered viruses and known viruses that had previously not been

98 associated with wastewater have been found in environmental waters (Table 1). Human polyomaviruses

99 (PyVs) and papillomaviruses were first discovered in the 1970s and 1950s, respectively, however, they have

100 only recently been found in the faeces and urine of infected individuals (Knowles, 2006; Rachmadi et al.,

101 2016). Some PyVs, including BKPyV, WUPyV, KIPyV, MCPyV and JCPyV have been detected at high

102 concentrations (up to 10^8 genome copies (gc)/l) in wastewater, river and seawater and sediment, in

103 swimming pools and in tap water (Di Bonito et al., 2017; Dias et al., 2018; Farkas et al., 2018a; Fratini et al.,

104 2014; Hamza and Hamza, 2018; Rachmadi et al., 2016). As these viruses are commonly asymptomatic in

105 healthy individuals, the route of transmission is not yet clear, however, waterborne infections are likely

106 (Fratini et al., 2014).

107 Bocaviruses (family *Parvoviridae*), causing respiratory tract infections and gastroenteritis, were first

108 described in 2005 (Allander et al., 2005). They have since been found in untreated and treated wastewater

109 at concentrations of 10^3-10^5 genome copies (gc)/l (Hamza et al., 2017; Iaconelli et al., 2016; Myrmel et al.,

110 2015), however, their prevalence in environmental water has not been explored. The torque teno virus

111 (family *Anelloviridae*), which causes gastroenteritis has also been found in wastewater and in polluted river

112 waters. Similar to bocaviruses, torque teno virus concentrations are considerably lower (up to 10^6 gc/l) than

113 the concentrations of other, more common enteric viruses (10^4-10^9 gc/l) (Hamza et al., 2011; Haramoto et

114 al., 2008). Human picobirnaviruses (family *Picobirnaviridae*) have also been detected in wastewater

115 (concentration range: 10^3 – 10^6 gc/l) and in contaminated rivers with variable prevalence (Adriaenssens et

116 al., 2018; Hamza et al., 2011; Symonds et al., 2009). In addition, recent comparative genomics analysis has

117 suggested that picobirnaviruses are bacteriophages, likely associated with mammalian gut bacteria

118 (Krishnamurthy and Wang, 2018). Genomes or partial genomes of circoviruses (family *Circoviridae*) and

119 cardioviruses (family *Picornaviridae*) along with enveloped viruses (coronaviruses, influenza virus) have also

120 been found in wastewater (Bibby and Peccia, 2013; Blinkova et al., 2009; Ng et al., 2012). Enveloped viruses

121 degrade in water rapidly (Gundy et al., 2009; Lebarbenchon et al., 2011), hence, human infections from

122 waterborne corona- and influenza viruses (e.g. SARS-CoV-2) are unlikely.

123 1.2 Viral indicators for wastewater contamination

124 Over 100 types of human enteric viruses are known to be common water pollutants (Melnick, 1984) and

125 with novel and emerging strains, the number is increasing. Due to the diversity of human pathogenic

126 viruses in the environment, surrogates and indicators are often used to investigate the fate and transport

127 of pathogenic strains in the environment. An indicator may be suitable for a broad assessment of

128   wastewater and drinking water treatment efficiency and for studying pathogen abundance, persistence,

129   adsorption and transport in the aquatic environment. Furthermore, quantitative monitoring of viral

130   indicators can provide useful data for microbial source tracking, transport modelling and risk assessment.

131   Traditionally, faecal indicator bacteria (FIB; including coliform bacteria, *Escherichia coli, Enterococcus* and

132   *Streptococcus* spp.) have been used to determine levels of faecal contamination in water. However, it has

133   been shown that bacteria are significantly less resistant to wastewater treatment and less persistent in the

134   environment than enteric viruses (Fong et al., 2005; Kim et al., 2009; Lin and Ganesh, 2013; Prez et al.,

135   2015; Sidhu et al., 2017a; Staley et al., 2012). Consequently, FIB are poor indicators of viral infection risk

136   and this suggests that current water quality monitoring programmes based solely on FIB are inadequate.

137   Ideally, a good viral indicator for wastewater-contamination assessment should have similar inactivation

138   and retention to the target pathogens and should be present in wastewater and in wastewater-

139   contaminated environments throughout the year. That would enable continuous monitoring and inform on

140   the level of contamination and the probability of pathogen presence. Furthermore, an indicator with

141   constant levels in wastewater may serve as a proxy for population size when wastewater-based

142   epidemiology is used to estimate the proportion of infected people during a viral outbreak or pandemic,

143   e.g. COVID-19 (Xagoraraki and O'Brien, 2020). Additionally, it should be source-specific to distinguish

144   between animal- and human-derived pollution (Scott et al., 2002). Some enteric viruses associated with

145   wastewater (as listed in Table 1) have potential to be used as indicators, however, not all of those viruses

146   fulfil these requirements. Influenza viruses, coronaviruses, circoviruses and papillomaviruses have been

147   detected at high concentrations in wastewater but not in polluted environments, which may be due to their

148   rapid decay in water. Furthermore, some enteric viruses (e.g. astrovirus, rotavirus, torque teno virus and

149   hepatitis E virus; Table 1) may be zoonotic, hence their presence in the environment may be a result of e.g.

150   agricultural activities instead of human waste. Hepatitis A and E viruses are abundant in less economically

151   developed countries, however, they are only responsible for sporadic outbreaks in more developed regions

152   (Bosch et al., 2016). Further, enteroviruses, noroviruses and sapoviruses show clear seasonality with peaks

153   either in the summer (enteroviruses) or during the winter (noroviruses and sapoviruses) in temperate

154   climates. Hence, these viruses are not found in wastewater and in the contaminated environment at all

155 times of the year (Farkas et al., 2018a; Nino Khetsuriani et al., 2006; Pons-salort et al., 2018; Prevost et al.,

156 2015). Human AdVs, PyVs and AiVs are frequently found in wastewater and in polluted environments

157 without any distinct seasonality, hence their utility as effective faecal indicators have been suggested

158 (Kitajima and Gerba, 2015; Rachmadi et al., 2016; Rames et al., 2016).

159 Bacteriophages infecting bacteria associated with the human gut are also common in wastewater. Somatic

160 coliphages (phages infecting *E. coli*) and F-specific RNA bacteriophages (FRNAP; phages infecting bacteria

161 through the F-pili) are commonly used to assess wastewater contamination. However, as not all strains

162 exclusively associate with human bacteria, they should be used with caution. Bacteriophages infecting

163 *Bacteroides* spp. also have the potential to indicate wastewater contamination. Amongst these phages are

164 a newly discovered group of viruses called crass-like phages. The type genome, crAssphage *sensu stricto*

165 (metagenome-assembled genome), belongs to the normal gut virome, having co-evolved with humans

166 (Dutilh et al., 2014; Edwards et al., 2019). Since the discovery of the first crAssphage genome, more crass-

167 like sequences have been found and one phage has been isolated. However, their genomic diversity is large

168 and the crAssphage *sensu stricto* and the isolated crass-like phage do not belong to the same genus

169 (Shkoporov et al., 2018). As the taxonomy of crass-like phages remains to be established, we refer to

170 crAssphage as a group of viruses with nucleotide similarity to the crAssphage *sensu stricto* described by

171 Dutilh et al. (2014) and quantified by Stachler et al. (2017).

172 Interestingly, a plant virus, the pepper mild mottle virus (PMMoV; family *Virgaviridae*), has also been

173 shown to be associated with human wastewater and found in polluted surface and groundwater and in

174 drinking water (Symonds et al., 2018). The primary source of PMMoV in human excreta is through

175 consumption of peppers (*Capsicum* spp.) and food products containing peppers that are contaminated with

176 the virus (Zhang et al., 2005). PMMoV is suggested to be a useful indicator for wastewater contamination

177 (Kitajima et al., 2018b; Symonds et al., 2018), however, its shape and size (17 x 300 nm rod-shaped capsid)

178 differs from other pathogenic viruses with icosahedral capsids and hence its fate and behaviour in the

179 environment may be different.

8

180 In this review, we evaluated the practicality of a set of human-waste associated viruses as indicators for

181 wastewater contamination of the aquatic environment (Table 2). We have extracted data from 127

182 individual studies to assess the usefulness of viral indicators by addressing specific aspects. The data

183 collected on viral concentrations in wastewater and environmental receiving waters are presented in

184 Tables S1-6, while the corresponding wastewater treatment log removal rates for each virus are presented

185 in Table S7. Together, we used this information to assess the ranges of virus abundance and distribution in

186 global aquatic systems. We included human wastewater-associated viruses, which are often present in

187 wastewater at high concentration without seasonality. We considered enteric viruses, (human AdVs, PyVs

188 and AiVs), PMMoV and human gut bacteria-associated bacteriophages, including FRNAP infecting *E. coli*

189 (specifically genogroups II and III), and bacteriophages of human gut commensal *Bacteroides* spp. (including

190 crAssphage). For evaluation, we used the following criteria:

191 1. Ease of detection and quantification

192 2. Human waste association

193 3. Presence in wastewater at high concentrations

194 4. Resistance to wastewater treatment

195 5. Persistence in the aquatic environment

196 6. Global distribution and temporal stability

197 2. Data collection

198 We collected viral concentration data published in peer-reviewed journal articles since 2005 (Tables S1-7;

199 Figure 1). Articles were identified via Google Scholar in September 2018 – October 2019 using the following

200 keywords: 'wastewater adenovirus', 'wastewater polyomavirus', 'wastewater Aichi virus', 'crAssphage',

201 'wastewater pepper mild mottle virus', 'wastewater AND ("F-specific RNA" OR F+ OR "FRNA" OR "male

202 specific") AND *phage AND genogroup' and 'wastewater *Bacteroides* bacteriophage''. The Google Scholar

203 search included these terms or part of them in the whole text, hence enabling the identification of studies

204 on the aquatic environment where wastewater contamination was assessed using the target viruses. The

205 studies were screened based on the title and abstract and initially 243 papers were selected.

9

206 The assessment of enteric viruses, PMMoV and crAssphage concentrations usually involved the

207 concentration of large volumes of water (1-10 l), and the efficiency of those procedures may therefore

208 affect the outcomes. Hence, studies where viral concentrations were not determined or sample process

209 recovery efficiency and/or quantitative PCR (qPCR) performance was not addressed were excluded from

210 the study. Studies where sample process recovery was <10% were also excluded. After the quality screen,

211 127 peer-reviewed research papers were included in the review (Table 2). Viral concentration data were

212 classified by water type (untreated and treated wastewater, surface freshwater, groundwater and

213 seawater) and the detection rates (i.e. positive samples / all samples x 100%), mean/median concentrations

214 and/or minimum-maximum concentrations were extracted (Figure 2, Table S1-S6). In most studies only the

215 mean/median and/or minimum-maximum concentrations were reported, hence further meta-analysis was

216 not performed. Virus removal rates reported during wastewater treatment processes were also retrieved

217 (Figure 3; Table S7). Additionally, the primers and probes used for the qPCR detection and quantification of

218 viruses have also been summarised (Table S8).

219 3.   Evaluation of viral indicators

220 3.1   Criterion 1: Ease of detection and quantification

221 For the accurate detection of low viral titres, environmental samples are often concentrated prior to virus

222 detection. Ultracentrifugation, ultrafiltration, adsorption/elution and flocculation are often used for the

223 concentration of water samples, and their effectiveness and limitations have been reviewed previously

224 (Barardi et al., 2012; Bofill-Mas and Rusiñol, 2020; Cashdollar and Wymer, 2013; Haramoto et al., 2018;

225 Ikner et al., 2012). The efficiency of viral recovery depends on the type of concentration method used, the

226 sample type and the virus type. Hence, viruses that can be easily and reproducibly recovered using simple

227 concentration methods should be used as indicator viruses.

228 Many approaches are available for the detection and quantification of viruses in environmental samples,

229 including PCR and isothermal amplification of target genes, microfluidics, metagenomics, biosensors,

230 microarrays and culturing-based techniques, as reviewed recently (Bonadonna et al., 2019; Farkas et al.,

10

231 2020; Hamza and Bibby, 2019). Many of the emerging approaches show great potential to detect low

232 concentrations of viruses in difficult matrices (Dhar and Lee, 2018; Farkas et al., 2020; Gyawali et al.,

233 2019b), however, to date they have not been implemented in the monitoring of viral contamination in the

234 aquatic environment.

235 In most studies, enteric viruses and proposed indicators were detected and quantified using real-time

236 quantitative PCR (qPCR)-based approaches (Girones et al., 2010; Haramoto et al., 2018), which are rapid,

237 easy, and cheap methods enabling strain-level detection. For instance, targeting different regions of the

238 hexon capsid protein gene, all human AdVs or only enteric AdVs (AdV genogroup F) can be quantified

239 (Table S1 and S8). qPCR can be easily multiplexed, enabling the simultaneous detection of 2-5 viral targets

240 (Ahmed et al., 2019a; Farkas et al., 2017b; Jiang et al., 2014; Lee et al., 2016; Montazeri et al., 2015). Hence,

241 it is widely used for the analysis of the level and spread of viral contamination in the aquatic environment

242 (Staggemeier et al., 2017). More recently, digital PCR approaches, enabling absolute quantification without

243 relying on standards, have also been used for the estimation of viral counts in wastewater and in

244 environmental waters (Ishii et al., 2014; Jumat et al., 2017; Kishida et al., 2014; Sedji et al., 2018). These

245 methods are also efficient, sensitive and often provide more accurate results that qPCR (Ishii et al., 2014;

246 Kishida et al., 2014).

247 The primers and probes repeatedly used in environmental studies for the detection and quantification of

248 the potential indicator viruses with qPCR, reverse transcription (RT) qPCR, and dPCR, are listed in Table S8.

249 In general, hydrolysis probe-based assays were predominantly used for viral detection. The specificity and

250 sensitivity of the primer and probe sets had been assessed (empirically or *in silico*) using a set of target and

251 non-target sequences and dilution series and shown to be adequate for the quantification of the target

252 sequences (Barrios et al., 2018; Chehadeh and Nampoory, 2013; Dumonceaux et al., 2008; Goh et al., 2009;

253 Gröndahl et al., 1999; Heim et al., 2003; Hernroth et al., 2002; Jothikumar et al., 2005; Kitajima et al., 2013;

254 Ko et al., 2005; McQuaig et al., 2009; Ogorzaly and Gantzer, 2006; Pal et al., 2006; Pang et al., 2012; Prevost

255 et al., 2015; Rusiñol et al., 2015; Stachler et al., 2017; van Maarseveen et al., 2010; Wolf et al., 2010, 2008;

256 Xagoraraki et al., 2007). The high detection rates (Figure 2; Table 2) also suggest that the primer and probe

257 sets were suitable for the sensitive detection of the target viruses. Nonetheless, the specificity of the sets

258 should be revised frequently in order to assure that novel strains are detected.

259 PCR-based approaches however have some disadvantages. qPCR and especially RT-qPCR are often inhibited

260 by organic substances, e.g. polyphenolic compounds, found in environmental samples (Ahmed et al., 2015;

261 Farkas et al., 2017a; Girones et al., 2010; Matheson et al., 2014). Therefore, the use of DNA viruses as

262 indicators (e.g. AdV, PyV, crAssphage) for wastewater-derived viral contamination may be more feasible

263 than the use of RNA viruses (AiV, PMMoV, FRNAP) due to the more robust molecular detection of DNA

264 targets (Farkas et al., 2017a; Hata et al., 2011). A major disadvantage of all PCR-based viral detection

265 approaches is that they do not give any indication on the infectivity of the target, and hence often

266 overestimate viral concentration and human health risks (Knight et al., 2013). Detecting segments of

267 indicator genes is helpful for the evaluation of the magnitude of faecal contamination and source tracking,

268 nonetheless, an ideal indicator should be capable of being cultured *in vitro* to enable the direct assessment

269 of viral infectivity and decay in wastewater and in the aquatic environment.

270 In the studies subject to this review, enteric viruses, crAssphage and PMMoV were predominantly detected

271 and quantified using qPCR-based assays. However, in a few studies plaque assay or integrated cell culture-

272 qPCR (ICC-qPCR) were used for AdV detection (Table S1). The combination of cell culture and qPCR

273 detection of viral replication enabled the detection of infectious viruses, which grew slowly and/or failed to

274 produce cytopathic effects. Using this approach, the time required for infectivity analysis has been reduced

275 from one week to two days, enabling rapid detection. Overall, qPCR/dPCR gave 1-5 $\log_{10}$ higher AdV

276 concentrations than plaque assay and ICC-qPCR due to the presence of damaged virus particles and free

277 viral DNA derived from degraded viruses in the environmental samples (Fongaro et al., 2015, 2013; Hamza

278 et al., 2011; Hewitt et al., 2011; Rigotto et al., 2010; Rodríguez et al., 2013; Sassoubre et al., 2012; Sedji et

279 al., 2018). The higher concentrations detected using ICC-qPCR compared to the traditional culturing assays

280 suggest that using qPCR-based quantification of cultured viruses is more sensitive and hence more reliable

281 in environmental settings (Fongaro et al., 2013; Sedji et al., 2018). PyVs and AiV are also culturable,

282 however, the propagation process is time consuming (2-4 and 4-6 weeks, respectively) and often

283 inconclusive (Reuter et al., 2011; Seehafer et al., 1978), hence, these approaches have not been adapted to

284 environmental studies. ICC-qPCR-based approaches may be suitable for assessing the infectivity of these

285 viruses in the environment, however, to date no ICC-qPCR assays have been developed for these targets.

286 The disadvantage of any culturing-based assay for enteric virus detection is the need for specific equipment

287 (e.g. CO_2 incubator), environment (BSL2 or BSL3) and staff for the maintenance of specific cell lines, which

288 may not be available in routine monitoring laboratories.

289 FRNAP are easy to culture and readily form plaques on a lawn of the host bacterium, which is usually the

290 WG49 strain of *Salmonella typhimurium* or *Escherichia coli* HS(pFamp)R (USEPA, 2001). Higher volumes (up

291 to 100 ml) of samples or concentrates are typically used for culture-based assays than for qPCR (a few

292 microliters of nucleic acid extract) and so culturing can be more sensitive than direct qPCR. However, this

293 method will produce plaques of a range of different strains that cannot be differentiated based on

294 morphology. Therefore, to identify and quantify specific FRNAP genogroups, it is necessary to use

295 genogroup-specific molecular detection methods. Such methods include RT-PCR analysis of plaques

296 (Haramoto et al., 2015, 2012) and 1-day ICC-RTqPCR (Hartard et al., 2017), most-probable number assays

297 (Hata et al., 2016) or *in-situ* plaque membrane hybridisation techniques (Flannery et al., 2013).

298 Many *Bacteroides*-associated phages are also culturable using appropriate hosts, including *Bacteroides*

299 strains GB-124, RYC2056, GA17 and ARABA-84, with double-layer agar method to quantify the number of

300 plaque forming units (pfu). However, the assay is more challenging than the plaque assay for FRNAP as

301 culturing *Bacteroides spp.* require anaerobic conditions, which may not be available in most laboratories.

302 The recent isolation and *in vitro* maintenance of phage ΦCrAss001 infecting *Bacteroides* sp. indicates that

303 plaque assays for this type of phage may be used in future environmental studies (Shkoporov et al., 2018).

304 It is important to mention, though, that the crAssphage qPCR assay (Table S7) does not detect ΦCrAss001,

305 as this phage is reported to belong to a different genus.

306 In order to estimate viral decay where no *in vitro* infectivity assay is routinely available (e.g. hepatitis E

307 virus, noro- and sapovirus), capsid integrity assays can be performed based on the assumption that an

308 intact virus particle is infectious. Capsid integrity can be assessed by the elimination of free viral nucleic

309 acids using enzymatic treatment, such as DNase, RNase (Fongaro et al., 2013) and intercalating dye pre-

310 treatment (Prevost et al., 2016) or by capturing only the intact virus particles using immunomagnetic

311 separation (IMS) (Haramoto et al., 2010). PCR-based enumeration following integrity assays show lower

312 viral concentrations than direct qPCR, as the free viral nucleic acids are eliminated. However, as the intact

313 virus particles may be damaged and hence non-infectious, these approaches may still overestimate viral

314 counts (Fongaro et al., 2013; Walker et al., 2019). Nonetheless, integrity assays are valuable tools for

315 estimating the number of viral particles in environmental samples and their use may improve viral risk

316 assessment.

317 3.2   Criterion 2: Human waste association

318 Viruses, such as AdV, PyV and AiV strains (Table S1-S3), which specifically infect humans, are logical choices

319 for indicators for human faecal contamination. Using these viruses and their corresponding animal

320 associated strains, the source of contamination (e.g. human vs wildlife, livestock, etc.) can be assessed. For

321 example, Staggemeier et al. (2015) used SYBR Green qPCR for the detection and quantification of AdVs in

322 water and sediment samples by distinguishing human, bovine, porcine, canine and avian AdV genome

323 sequences based on their melting temperature. Human and porcine AdVs, bovine PyV and porcine

324 circovirus have also been used to assess the level of agriculture-related and human sewage-associated

325 contamination in recreational, groundwater and drinking water (Fongaro et al., 2015; Garcia et al., 2012;

326 Rusiñol et al., 2014). In the studies evaluated in this review, the AdV qPCR assays targeted all human AdV

327 groups (A-G), the most common groups (A-F) or specific groups (C and F; Table S1). All of these groups are

328 human-specific, demonstrating that waterborne infections of AdV F (predominantly type 41) are the most

329 prevalent in wastewater and in the aquatic environment (Bofill-Mas et al., 2010; Chigor and Okoh, 2012;

330 Fong et al., 2010; Fumian et al., 2013; Haramoto et al., 2007; Hewitt et al., 2011; Iaconelli et al., 2017;

331 Ibrahim et al., 2018; Lun et al., 2019; Myrmel et al., 2015; Ogorzaly et al., 2015; Shih et al., 2017). The most

332 common PyVs associated with wastewater are the JC and BK strains (Table S2), however, MC PyV is also

333 found in wastewater and in wastewater-contaminated water (Di Bonito et al., 2014; Rusiñol et al., 2015).

334 All known human AiV (group A and B) have been found in human wastewater (Table S3). These viruses are

335 highly human-specific and have not been found to associate with animal diseases. The JC and BK strain

336 have not been found in animal waste (McQuaig et al., 2009), whereas to date, animal waste samples have

337 not been tested for human AiV.

338 PMMoV has been found at high concentrations in domestic wastewater (raw and treated) and in

339 wastewater-polluted environments and shown to correlate well with other human markers (*Bacteriodes*

340 HF183, PyV) (Kitajima et al., 2018b; Symonds et al., 2018, 2016) implying it associates with human waste.

341 Nonetheless, the primary source of the virus are bell and chilli peppers, with the suggestion that it should

342 not be used as a faecal indicator nearby to commercial pepper plant production areas. qPCR assays

343 targeting PMMoV show high sensitivity, however, the viruses were also detected in avian, bovine and dog

344 faeces at low concentrations (Gyawali et al., 2019a; Hamza et al., 2011; Rosario et al., 2009) suggesting that

345 animals may also access pepper as a food source. Furthermore, it has been suggested that PMMoV is more

346 abundant in faeces and in wastewater where more pepper products are consumed (Symonds et al., 2018),

347 therefore the prevalence of PMMoV should be further investigated.

348 Coliphages are commonly used as indicators for faecal viral contamination in water (McMinn et al., 2017).

349 FRNAP genogroups II and III (FRNAP-II and FRNAP-III) have been shown to be associated with human

350 sources, while genogroups I and IV (FRNAP-I and FRNAP-IV) are generally associated with non-human

351 sources (Lee et al., 2011; Stewart-Pullaro et al., 2006). For this reason, several studies have used methods

352 (described in Section 3.1) to distinguish between FRNAP genogroups to determine faecal sources. However,

353 while there does appear to be a general association between genogroups and faecal sources, the bacterial

354 host (*E. coli* expressing F-pili) is not source-specific and there is often overlap between source types for

355 each genogroup (Cole et al., 2003; Harwood et al., 2013).

356 Bacteriophages infecting *Bacteroides*, common human gut bacteria, have also shown potential as indicators

357 for faecal contamination in the environment. The most commonly used strains, which are phages that

358 infect *Bacteroides* BG-124 (BacBG124P), RYC-2056 (BacRYC2056P), GA-17 (BacGA17P) and ARABA-84

359 (BacARABA84P), were shown to be human specific. However, one study detected BacRYC2056P in

15

360 wastewater samples derived from abattoirs, suggesting animal association (Wicki et al., 2015). qPCR based

361 assays targeting crAssphage have shown good human specificity. While some cross-reactivity has been

362 shown with dog, gull, poultry, pig and cattle faeces (Ahmed et al., 2018a; García-Aljaro et al., 2017; Stachler

363 et al., 2017), the levels of crAssphage found in these non-human sources were several orders of magnitude

364 lower than that of human sources. The highest crAssphage concentrations in animal sources were found by

365 García-Aljaro et al. (2017) which may be attributed to pooled samples and/or the use of a different qPCR

366 assay. García-Aljaro et al. (2017) also found that by normalising crAssphage levels against a general faecal

367 indicator (*E. coli*), it was still possible to distinguish between human and non-human sources. Nevertheless,

368 the cross-reactions of the qPCR with animal excreta should be further investigated to assess human

369 specificity.

370 ### 3.3   Criterion 3: Presence in wastewater at high concentrations

371 AdVs, PyV, AiV, human gut-associated bacteriophages and PMMoV are all frequently found in raw sewage

372 and untreated wastewater at high concentrations (Figure 2; Table S1-6). The highest concentrations among

373 the potential indicators were noted for crAssphage with concentrations of $10^{10} - 10^{12}$ gc/l in raw sewage

374 detected in samples taken in Japan. CrAssphage concentrations were lower in wastewater samples taken in

375 the US (Florida; $10^9 - 10^{10}$ gc/l) (Ahmed et al., 2018a) and in the UK (Wales; $10^5 - 10^8$ gc/l) (Farkas et al.,

376 2019). CrAssphage is not currently well characterised and while the current primer and probe set do not

377 align to any recently discovered relatives of crAssphage, it is possible that the qPCR-based detection assay

378 is not specific to a single strain.

379 AdVs were detected at 10^{11} gc/l concentration in wastewater influent, in Pisa, Italy in 2009-2010 (Carducci

380 and Verani, 2013). In other studies using the same primer and probe set (Hernroth et al., 2002), the

381 concentration of AdV was lower, between 10^3 and 10^9 gc/l with the highest concentrations measured in

382 other wastewater treatment plants in Italy (La Rosa et al., 2010), followed by peak concentrations of 10^8

383 gc/l in Rome, Italy (Muscillo et al., 2008), Barcelona, Spain (Bofill-Mas et al., 2006), and in Minas Gerais and

384 Rio de Janeiro, Brazil (Assis et al., 2017). Similar peak concentrations (10^8 gc/l) were observed when AdV

385 groups A-G were targeted in Germany (Hamza et al., 2009a) and in Queensland, Australia (Sidhu et al.,

386   2017b). The highest concentrations of AdVF (10^8-10^{10} gc/l) were observed in the US (Michigan) (Simmons et

387   al., 2011) and in Giza, Egypt (Elmahdy et al., 2019) further verifying that this group of AdV is highly

388   prevalent.

389   PMMoV are also present in wastewater at high concentrations (up to 10^{10} gc/l). The highest PMMoV

390   concentrations were reported in Florida and other states in the US (Rosario et al., 2009) followed by

391   Germany ($10^7 – 10^8$ gc/l) (Hamza et al., 2011), New Zealand (10^7 gc/l) (Gyawali et al., 2019a), Vietnam and

392   the US (Arizona; $10^6 – 10^7$ gc/l) (Kitajima et al., 2014; Kuroda et al., 2015; Schmitz et al., 2016). Slightly

393   lower FRNAP concentrations were noted in wastewater with the FRNAP-II appears to be more prevalent

394   ($10^7 – 10^9$ gc/l) than FRNAP-III ($10^4 – 10^7$ gc/l) (Figure 2). However, more studies are needed to further

395   investigate FRNAP-II/III concentrations in wastewater.

396   Among PyVs, JCV had the highest concentrations (10^7-10^8 gc/l) in wastewater collected in Brazil and Chile

397   (Fumian et al., 2010; Levican et al., 2019), however, it was less prevalent (10^3-10^6 gc/l) in the US (Arizona),

398   Spain and in the UK (Wales) (Bofill-Mas et al., 2006; Farkas et al., 2018a; Kitajima et al., 2014; Rusiñol et al.,

399   2015; Schmitz et al., 2016). BKV and MCV are probably less abundant in wastewater than JCV with

400   concentration ranges of 10^3-10^7 gc/l and 10^4-10^5 gc/l, however, limited surveillance has been done on these

401   viruses in wastewater. AiV has only been sought in untreated wastewater in the USA (Arizona), Vietnam

402   and Nepal (Haramoto and Kitajima, 2017; Kitajima et al., 2014; Kuroda et al., 2015; Schmitz et al., 2016)

403   with concentrations between 10^4 and 10^6 gc/l, and shown to be less prevalent than the other indicators.

404   The detection rate and concentration of AdV, PyV, AiV, FRNAP-II, crAssphage and PMMoV usually exceeded

405   the concentration of noroviruses, sapovirus, enterovirus, astrovirus, rotavirus and hepatitis E virus (Farkas

406   et al., 2019, 2018a, 2018b; Flannery et al., 2013; Fumian et al., 2013; Grøndahl-Rosado et al., 2014a; Hata

407   et al., 2014; Kitajima et al., 2014, 2013; Masclaux et al., 2013; Prevost et al., 2015; Qiu et al., 2015;

408   Simmons et al., 2011). However, in some cases norovirus and rotavirus showed higher concentrations than

409   AdV and PyV (Kaas et al., 2018; Prado et al., 2019).

410   The peak concentrations of cultured phages infecting *Bacteroides* in untreated wastewater was 10^6 pfu/l,

411   however, this number cannot be directly compared with the concentration of other indicators due to the

412 different methods used for detection. The viral infectivity rate (i.e. gc: infective units) of virus may be as

413 high as 1000:1 as determined for AdV (Hewitt et al., 2011), however, the actual infectivity rates for

414 *Bacteroides*-associated phages is yet to be determined.

415 All potential indicator viruses showed high (>90%) detection rates in untreated wastewater, except

416 BacRYC2056P, which was found only in 82% and 38% of the analysed samples. AdVs and PyVs were

417 frequently detected in wastewater from large and small wastewater treatment plants in the Americas,

418 Europe, Asia and Australia (Figure 1; Table 2; Table S1, S2). Prevalence and concentration information for

419 AiV was only reported for untreated wastewater samples from large wastewater treatment plants in the

420 USA (Arizona) and Vietnam (Kitajima et al., 2018a, 2014, 2013; Kuroda et al., 2015; Schmitz et al., 2016).

421 The PMMoV titre was assessed in wastewater samples derived from the USA, Germany, New Zealand and

422 Vietnam (Hamza et al., 2011; Kitajima et al., 2014; Kuroda et al., 2015; Rosario et al., 2009; Schmitz et al.,

423 2016; Symonds et al., 2016). FRNAP-II prevalence in wastewater was only assessed in Japan and Ireland

424 (Flannery et al., 2013; Haramoto et al., 2015, 2012; Lee et al., 2018), while FRNAP-III prevalence in

425 wastewater was only assessed in Japan (Haramoto et al., 2015, 2012; Lee et al., 2018; Setiyawan et al.,

426 2014, 2013). In wastewater, the concentrations of phages associated with *Bacteroides* BG-124 were

427 assessed in the US, Brazil, the UK (England) and Switzerland (E. Dias et al., 2018; Mcminn et al., 2014; Prado

428 et al., 2018; Purnell et al., 2015; Stefanakis et al., 2019; Wicki et al., 2015), BacRYC2056P were investigated

429 in Colombia, the UK, Spain, France, Cyprus, Sweden, Switzerland and Thailand (Costán-Longares et al.,

430 2008; Gomila et al., 2008; Payan et al., 2005; Venegas et al., 2015; Wangkahad et al., 2017; Wicki et al.,

431 2015, 2011), BacGA17P were found in Colombia and several European countries (Casanovas-Massana et al.,

432 2015; Costán-Longares et al., 2008; Gomila et al., 2008; Mayer et al., 2016; Payan et al., 2005; Venegas et

433 al., 2015; Wicki et al., 2015) and BacARABA84P was identified in Switzerland (Wicki et al., 2015, 2011).

434 CrAssphage concentrations were only determined in wastewater in the UK (Wales), Australia and the USA

435 (Florida) (Ahmed et al., 2019a, 2018b; Farkas et al., 2018a). Due to the limited number of studies (Table 2),

436 further testing is necessary to evaluate the prevalence and distribution of AiV, FRNAP-II, FRNAP-III,

437 culturable phages infecting *Bacteroides*, crAssphage and PMMoV in untreated wastewater to assess their

438 usefulness as indicators of wastewater pollution. Current data suggest that all assessed viral indicators, are

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439 present in untreated wastewater at high concentration and hence they are potentially good indicators for

440 wastewater contamination.

441 3.4 Criterion 4: Resistance to wastewater treatment

442 Enteric viruses have been shown to be extremely resistant to traditional wastewater treatment procedures

443 (Figure 3; Table S7). As the removal efficiency varies amongst sites and the type of treatment process,

444 comparative studies have been performed to study the resistance of enteric viruses and potential

445 indicators during wastewater treatment.

446 In this study, 24 studies comparing the virus removal efficiency of different wastewater treatment

447 processes were evaluated (Figure 3). Fifteen of these studies exclusively used qPCR and RT-qPCR for the

448 quantitative analysis of viral concentrations. As discussed in Section 3.1, these molecular techniques give

449 no indication on the infectivity state of the viruses and hence may overestimate infective viral titres in

450 untreated and treated wastewater and other environmental samples. This was demonstrated by Flannery

451 et al. (2013a) whose data showed that while infectious FRNAP-II in UV-treated effluent was approximately

452 2.3 $\log_{10}$ less than influent, only a 0.54 $\log_{10}$ reduction was found when using RT-qPCR alone. Most of this

453 reduction in FRNAP-II infectivity occurred during the secondary treatment stage (1.69 $\log_{10}$ reduction), but

454 the type of secondary treatment used in that study was not specified. In a study by Lee et al. (2018), an

455 activated sludge process (a form of secondary treatment) resulted in 2.1 and 3.1 $\log_{10}$ reductions of

456 infectious FRNAP-II and FRNAP-III, respectively. RT-qPCR analysis of the same samples showed $\log_{10}$

457 reductions of 1.6 and 2.5 for FRNAP-II and FRNAP-III, respectively. The differences between infectious virus

458 and genome removal were not significant. These studies therefore reached conflicting conclusions with the

459 former showing that infectivity studies are vital and the latter showing that they are unnecessary. It is

460 possible that the activated sludge process used by Lee et al. (2018) resulted in physical removal of viruses,

461 while the process used by Flannery et al. (2013a) inactivated the viruses without physically removing them

462 from the treated water. This highlights the importance of including the specific mechanisms used within a

463 sewage treatment process when reporting such data, as it is not clear whether the secondary treatment

464 processes in the two studies shared any mechanistic similarities.

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465 Low removal rates have been reported for BacGB124P and BacGA17P during wastewater treatment. In

466 most studies, the removal of these phages was ≤ 2 $\log_{10}$, regardless of the treatment method used (Dias et

467 al., 2018; Mayer et al., 2016; Prado et al., 2018; Stefanakis et al., 2019), except for one study showing >5.6

468 $\log_{10}$ removal of BacGB124P when disk filtration and chlorination was used as tertiary treatment (Prado et

469 al., 2018).

470 Data obtained from a wide range of qPCR-based viral quantification studies have shown limited removal of

471 AdV, PyV, AiV, crAssphage and PMMoV during wastewater treatment. Activated sludge treatment and

472 biofiltration, without further treatment resulted in 0.6-1.9 and 0.3-3.0 $\log_{10}$ removal of AdV and PyV,

473 respectively (Figure 3; Table S7). Tertiary treatment processes resulted in an additional 1-3.5 $\log_{10}$ removal

474 of AdV and PyV with membrane bioreactors coupled with additional chlorination, filtration and UV

475 treatment being the most efficient method for viral removal (Qiu et al., 2018; Rusiñol et al., 2015; Simmons

476 et al., 2011). Furthermore, AdVs have been shown to be more resistant to UV treatment than poliovirus,

477 rotavirus, caliciviruses and hepatitis A virus (Hijnen et al., 2006). However, laboratory-scale studies suggest

478 that AdVs are more susceptible to chlorine treatment than enteroviruses and caliciviruses (Cromeans et al.,

479 2010; Kahler et al., 2010; Thurston-Enriquez et al., 2005, 2003). Interestingly, significant differences were

480 found for the removal of PyV strains. MCV was found to be the most resistant to treatment followed by JCV

481 and BKV (Rusiñol et al., 2015).

482 Fewer studies evaluated the removal of AiV, crAssphage and PMMoV, than the removal of AdVs, PyVs and

483 phages. Overall, AiV showed 1-3 $\log_{10}$ reduction during secondary and 1-2 log reduction during tertiary

484 wastewater treatment (Kitajima et al., 2018a, 2014, 2013; Schmitz et al., 2016). The removal of crAssphage

485 was also in the range of 1.0-1.2 $\log_{10}$ during secondary wastewater treatment (Farkas et al., 2019),

486 however, crAssphage removal has not been assessed during tertiary treatment yet. The current data

487 suggests that PMMoV is stable during secondary treatment and chlorination, which results in <2 log

488 reduction (Symonds et al., 2018). Larger PMMoV removal (>4 log) was only observed using

489 electrocoagulation and Bardenpho (aerobic/anaerobic multi-reactor) technologies (Schmitz et al., 2016;

490 Symonds et al., 2015). Further research is needed to evaluate the reduction of PMMoV during UV

491 treatment and other wastewater treatment procedures to evaluate its usefulness as an indicator. The

492 major advantage of crAssphage and PMMoV is that their concentrations are usually high in wastewater,

493 and hence the efficiency of their removal can be easily monitored. Nonetheless, their infectivity and decay

494 have not been investigated due to the current lack of i*n vitro* culturing-based methods.

495 In studies where the removal of indicator viruses was compared with the removal of common pathogenic

496 enteric viruses, indicator viruses showed similar or less removal than the pathogens (Carducci and Verani,

497 2013; Farkas et al., 2018a; Kitajima et al., 2014; Prado et al., 2019; Rusiñol et al., 2015; Schmitz et al., 2016).

498 Furthermore, a meta-analysis on the efficiency of secondary wastewater processes showed that activated

499 sludge treatment resulted in 0.20 – 2.18 $\log_{10}$ reduction of rotavirus, and norovirus GI and GII, whereas

500 biofiltration resulted in higher removal (1.52 – 4.30 $\log_{10}$) of norovirus GII and enteroviruses (Sano et al.,

501 2016). These removal rates are higher than the removal rates determined for the indicators reviewed here

502 suggesting that the indicators can represent the removal of the most resistant viruses. However, three

503 studies showed higher removal rates of BKV and JCV than norovirus, sapovirus, enterovirus and rotavirus

504 (Farkas et al., 2018a; Fumian et al., 2013; Schmitz et al., 2016) suggesting that PyVs are less resistant than

505 the pathogenic viruses and hence should be used with caution as an indicator. Current data shows that

506 AdV, AiV, FRNAPII/III, crAssphage and PMMoV may be suitable for the assessment of wastewater

507 treatment processes.

508 As different viruses have varying reactions to wastewater treatment processes, the use of multiple

509 indicators is recommended. For indicators other than bacteriophages reviewed here, the exclusive use of

510 molecular detection and quantitation is a major limitation in understanding enteric virus removal. Hence,

511 combinations of infectivity studies and molecular assays should be performed for viruses that can be

512 cultured *in vitro* in order to assess viral survival during wastewater treatment.

513 3.5   Criterion 5: Persistence in the aquatic environment

514 Many of the studies that have been conducted to estimate viral persistence in natural waters have relied

515 solely on qPCR-based quantification. While the reliance on qPCR data alone may lead to overestimations of

516 infectious viral persistence, its use is nonetheless important especially when considering unculturable

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517 enteric viruses. When measuring persistence of viral indicators in the aquatic environment, researchers

518 should therefore be clear whether they are studying the persistence of a viral signal (for example nucleic

519 acids detected by qPCR) or the infectivity of viruses (for example by culture).

520 3.5.1 Indicators in surface freshwater

521 Most research has focused on the occurrence and survival of indicator viruses in surface water. When

522 quantified in surface freshwater bodies (lakes, rivers, streams, etc.) by qPCR, these viral indicators (e.g. AiV,

523 AdV, JCV, PMMoV) are typically detected at up to 4 $\log_{10}$ higher concentrations than common enteric

524 pathogenic viruses, e.g. norovirus, enterovirus and rotavirus (Hata et al., 2014; Jurzik et al., 2010; Rusiñol et

525 al., 2015; Sassi et al., 2018). All indicator concentrations in river water correlated with the distance of

526 sampling point from the source of contamination (wastewater treatment plant), with significantly higher

527 concentrations occurring near the wastewater treatment plant than further downstream or upstream

528 (Ebdon et al., 2007; Farkas et al., 2018a; Prevost et al., 2015; Rusiñol et al., 2015; Sassi et al., 2018; Sibanda

529 and Okoh, 2012; Tandukar et al., 2018; Venegas et al., 2015; Wangkahad et al., 2017). Comparative studies

530 showed that PMMoV occurred at higher concentration than AdV, AiV and PyV in surface water bodies in

531 the USA (Arizona: 10^3-10^6 gc/l and Colorado: 10^4-10^5 gc/l), Germany (10^4-10^5 gc/l) and Vietnam (10^4-10^6

532 gc/l)(Betancourt et al., 2014; Hamza et al., 2011; Kuroda et al., 2015; Sassi et al., 2018). The concentration

533 of AdV and AiV (up to 10^4 gc/l) were similar in river water collected in the USA (Colorado) and Japan

534 (Betancourt et al., 2014; Hata et al., 2014; Sassi et al., 2018), whereas AdV was more prevalent than PyV in

535 river water samples collected in Spain (76% vs 48% detection rates), the UK (Wales: 88% vs 65%), Japan

536 (61% vs 11%) and Germany (79% vs 59%) (Albinana-Gimenez et al., 2009; Farkas et al., 2018a; Haramoto et

537 al., 2010; Jurzik et al., 2010; Rusiñol et al., 2015). However, detection rates and concentrations of AdV and

538 JCV were similar in highly polluted rivers close to the wastewater discharge points near Barcelona, Spain

539 (100%, 10^3-10^4 gc/l) and Rio de Janeiro, Brazil (100%, 10^2-10^5 gc/l) (Calgua et al., 2013). Taken together, our

540 analysis shows that these indicator viruses are present in wastewater-polluted surface freshwater at high

541 concentrations, which enables the accurate detection of the viruses and the comparative analysis of the

542 rate of pollution (Crank et al., 2019; Zhang et al., 2019).

543   Culturable bacteriophages were also present in surface freshwater (Figure 2; Table 2). FRNAP-II were

544   detected most frequently with concentrations up to 10^6 pfu/l followed by FRNAP-III and phages infecting

545   *Bacteroides* (up to 10^5 pfu/l). FRNAP-II concentrations correlated with AdV concentrations in river water in

546   France (Ogorzaly et al., 2009) and with AdV, norovirus, astrovirus and rotavirus in tropical freshwater

547   samples in Singapore (Vergara et al., 2015). To date, no comparative studies have been done to compare

548   enteric viruses and phages infecting *Bacteroides* spp. in surface freshwater. More research is essential on

549   the prevalence of culturable human gut associated phages to assess their usefulness as indicators.

550   3.5.2   Indicators in seawater

551   As for freshwater environments, similar viral indicator trends have been observed in coastal waters where

552   wastewater contamination is present. PMMoV was present at higher concentrations (10^2-10^5 gc/l) than PyV

553   (10^2 gc/l) in coastal water at Miami, Florida (Symonds et al., 2016) and crAssphage was also present at

554   higher concentrations (10^3-10^5 gc/l) than AdV (10^2-10^4 gc/l) and JCV (10^2-10^3 gc/l) in seawater collected at

555   Conwy, Wales (Farkas et al., 2018a). AdV also had higher concentrations than PyV in seawater collected at

556   Rio de Janeiro and Santa Caterina, Brazil (10^2-10^5 gc/l vs 10^1-10^3 gc/l), Florianopolis, Brazil (10^3-10^7 gc/l vs

557   <10 gc/l), North Wales (10^2-10^4 gc/l vs 10^2-10^3 gc/l), and Catalonia, Spain (10^1-10^5 gc/l vs 10^0-10^2 gc/l) (Dias

558   et al., 2018; Farkas et al., 2018b; Moresco et al., 2012; Rusiñol et al., 2015). CrAssphage, PMMoV and AdV

559   and PyV are usually present up to 4 $\log_{10}$ higher concentrations in seawater than hepatitis A virus, norovirus

560   and sapovirus (Dias et al., 2018; Farkas et al., 2018b; Fongaro et al., 2015; Moresco et al., 2012; Rusiñol et

561   al., 2015; Symonds et al., 2018), however, one study found that the concentration of indicators and

562   norovirus GII, rotavirus and sapovirus were similar, approx. 10^4 gc/l, in seawater collected at the Tahiti

563   coast (Kaas et al., 2018). BacGB124P, BacRYC2056P and BacGA17P were also found in seawater at

564   concentrations up to 10^4 pfu/l (Olalemi et al., 2016), however, these concentrations were not compared

565   with enteric viruses. To date, FRNAP-II/III concentrations have not been measured in seawater samples.

566   Based on the data reviewed here, PMMoV, crAssphage and AdV are suitable markers for wastewater

567   contamination in seawater.

568 3.5.3 Indicators in groundwater

569 Very few studies have evaluated the concentration of enteric viruses and viral indicators in groundwater.

570 AdV, JCV, AiV, PMMoV, BacGB124P and BacARABA84P were detected in polluted groundwater in the USA

571 (Arizona and Colorado) and Vietnam at very low concentrations (Albinana-Gimenez et al., 2009; Betancourt

572 et al., 2014; Kuroda et al., 2015), hence the concentrations cannot be compared (Figure2; Table 2). Future

573 studies may include the efficient concentration of high volumes (> 100 l) of groundwater to accurately

574 determine viral concentrations and the associated risks.

575 3.5.4 Persistence of indicators in water

576 Understanding how long pathogenic and indicator viruses survive in the environment is crucial for accurate

577 risk assessment and management. The mechanisms and factors influencing viral decay, such as virus type,

578 temperature, microbial activity, pH, water type/conductivity, UV/sunlight radiation and the presence of

579 solid/organic matter, have been assessed (Jin and Flury, 2002; Rzeżutka and Cook, 2004; Verbyla and

580 Mihelcic, 2015). Many studies have shown that enteric viruses are more stable in the aquatic environment

581 than traditional indicators, such as coliform bacteria and coliphages (El-Senousy et al., 2014; Fattal et al.,

582 1983; Keswick et al., 1982; Muscillo et al., 2008; Ogorzaly et al., 2010; Wait and Sobsey, 2001).

583 FRNAP are easily cultured, and their persistence in surface waters has been studied in both surface

584 freshwaters and seawater (Hata et al., 2016; Muniesa et al., 2009; Ravva and Sarreal, 2016; Yang and

585 Griffiths, 2013). In general, FRNAP-I has been found to be the most persistent followed by FRNAP-II, FRNAP-

586 III and then FRNAP-IV. Using simulated sunlight, Flannery et al. (2013 b) studied the effect of solar radiation

587 on the persistence of FRNAP-II and norovirus in seawater. The reductions in RT-qPCR detectable viruses

588 was similar for norovirus and FRNAP-II under both summer and winter sunlight conditions. However, it took

589 between 81% and 88% longer for a 90% reduction in RT-qPCR detectable FRNAP-II than for infectious

590 FRNAP-II. This highlights again the need to consider infectivity when studying viral persistence in the

591 environment. Brion et al. (2002) also studied the survival of different FRNAP genogroups in surface water.

592 Environmental isolates of FRNAP-II had the highest variability in survival between isolates, while FRNAP-III

593 had the lowest variability in survival between isolates. They concluded that FRNAP-III is suited to

594 determining whether there had been recent contamination to a water body by human faeces. In contrast,

595 FRNAP-II was more suited to indicating contamination by distant or sporadic human source contamination.

596 Due to its easy molecular detection and relatively straightforward *in vitro* culturing, the survival of AdV has

597 also been well-studied. AdVs have shown 1-2 $\log_{10}$ reduction in infectivity in raw and sterilised groundwater

598 and surface water over 120-180 days (Ogorzaly et al., 2010; Rigotto et al., 2011). In seawater, the decrease

599 of viral infectivity was more rapid than in groundwater, with 1.2-1.4 $\log_{10}$ AdV reductions in 28 days

600 (Enriquez et al., 1995) and sunlight significantly enhancing degradation at a rate of at least 2 $\log_{10}$ reduction

601 per day (Liang et al., 2017). AdVs were more stable in groundwater and surface water than poliovirus,

602 rotavirus and hepatitis A virus (El-Senousy et al., 2014; Enriquez et al., 1995). Ogorzaly et al. (2010) showed

603 that AdV persists for longer in ground water than both MS2 (FRNAP-I) and GA phages (FRNAP-II). This

604 difference in survival and persistence was greatly increased with an increase in temperature from 4°C to

605 20°C. These studies highlight the stability of AdV compared to other viruses, however, these studies were

606 conducted in laboratory experiments and the viral stability may differ in field conditions.

607 PyV has also been shown to be as resistant to sunlight in seawater as AdV (Ahmed et al., 2019b; Liang et al.,

608 2017), however, the monitoring experiments detailed in Section 3.5.2 suggest that PyV degrades in water

609 more rapidly than AdV. In contrast, crAssphage proved to be as persistent as AdV and PyV in coastal

610 bathing water (Ahmed et al., 2019b). The temporal decay of AiV, PMMoV and culturable bacteriophages

611 infecting *Bacteroides* is not yet known and should be investigated and compared with AdV decay to

612 determine their usefulness as indicators. The comparison of the mechanisms of decay of PMMoV and the

613 other viral indicators would be especially interesting due to the differences in the structure of the virions

614 (tubular vs. icosahedral).

615 3.6   Criterion 6: Global distribution and temporal stability

616 All potential indicator viruses reviewed here have been detected in environmental waters, wastewater or

617 stool samples of individuals in Asia, Europe, Australia, Africa and the Americas, highlighting the global

618 distribution of these viruses (Cinek et al., 2018; Fratini et al., 2014; Friedman et al., 2009; Guido et al., 2016;

619 Jofre et al., 2014; Kitajima and Gerba, 2015; Rames et al., 2016; Schaper et al., 2002; Symonds et al., 2018).

620 In the reviewed studies, the indicator viruses were detected and quantified in 31 countries (including 10 US

621 states), with the majority of studies conducted in the US, Brazil, Western Europe, Japan and Australia

622 (Figure 1; Table 2). While the available data suggest that these viruses are distributed globally, very limited

623 information on enteric and indicator virus quantities is available from developing countries (e.g. India,

624 Northern Asia and most African countries) (Table 2). To date, none of these viruses have been studied in

625 water from Antarctica, where they could point towards contamination of pristine areas by research

626 scientists, or long-distance dispersal.

627 During long-term monitoring surveys, in the Katsura River to the west of Kyoto, Japan, FRNAP-II was shown

628 to have very limited seasonality, with similar levels in the winter and summer months (Hata et al., 2016).

629 However, in a tributary of the Uji River to the South of Kyoto, FRNAP-II was detected only during winter.

630 FRNAP-III was also found to be more prevalent during winter at both sites, a trend also observed in effluent

631 from Johkasou effluent by Setiyawan et al. (2013). Culturable phages infecting *Bacteroides* showed no

632 seasonal changes in their concentration in river water in the UK (Ebdon et al., 2007) and in wastewater

633 collected in seven US states (McMinn et al., 2014) and in Brazil (Prado et al., 2018).

634 In the studies reviewed, crAssphage, AdV and PyV showed no seasonal changes in concentrations in

635 untreated and treated wastewater, river and seawater samples (Carducci and Verani, 2013; Farkas et al.,

636 2018a, 2018b; Fumian et al., 2013; Iaconelli et al., 2017; Masclaux et al., 2013; Qiu et al., 2015; Rusiñol et

637 al., 2015; Schmitz et al., 2016). AiV and PMMoV also showed stable titres in treated and untreated

638 wastewater over a year (Iaconelli et al., 2016; Kitajima et al., 2014; Myrmel et al., 2015; Schmitz et al.,

639 2016), however, peak concentrations for AiV were noted in wastewater in Japan during winter and spring

640 (Kitajima et al., 2013). PMMoV showed no seasonality in river water either (Haramoto et al., 2013; Rosario

641 et al., 2009). Higher AdV concentrations were observed in treated wastewater collected in Wales during

642 summer than in winter and spring, which was most likely due to dry weather and a transient increase in

643 population due to tourism in the summer months (Farkas et al., 2018b). Furthermore, higher AdV

644 concentrations were detected in untreated wastewater in Norway during January-March compared to the

645 concentrations observed during April-December (Myrmel et al., 2015). The prevalence of AdV was also

646 higher during autumn-winter than during the spring-summer in wastewater collected in Egypt (Elmahdy et

647 al., 2019). Similarly, AdVs were detected at low concentrations during the summer and autumn months in

648 river water samples collected in Japan and in Germany, respectively (Hamza et al., 2009b; Kishida et al.,

649 2012), probably due to dry weather conditions. Overall, these findings suggest that the indicators are

650 detectable and quantifiable throughout the year, which enables the continuous evaluation of wastewater

651 contamination. The current data imply that precipitation has more effect on viral loads than temporal

652 changes in the number of infections. Nonetheless, this should be further investigated by comparing

653 epidemiological data, viral loads in wastewater and precipitation over several years.

654 In terms of fine-scale temporal variability, the effect of rainfall on virus concentrations in surface water is

655 variable. On one hand, a decrease in virus concentrations in surface water has been reported due to

656 dilution of the water body (Grøndahl-Rosado et al., 2014b). In contrast, a number of studies have shown

657 association between precipitation and elevated enteric viral concentrations in water (Ebdon et al., 2007;

658 Wicki et al., 2015). In regions with combined sewers, much of this increase in contamination is likely due to

659 the additional wastewater input via CSOs and storm water drainage.

660 CSOs discharge largely untreated (screened, partially settled or untreated) wastewater into the

661 environment. This almost certainly results in higher numbers of enteric viruses being discharged into

662 receiving waters than would otherwise be the case from fully treated sewage effluents (Fong et al., 2010;

663 Hata et al., 2014). Furthermore, relatively few CSOs have spill duration monitoring and almost none have

664 microbiological or chemical monitoring requirements. Therefore, in most cases the input of contamination

665 can only be monitored via the surveillance of wastewater-derived contaminants in water bodies.

4. Conclusions and future research

667 The viruses reviewed here have all been shown to have potential to indicate wastewater-derived pollution

668 in the aquatic environment (Table 3). Due to their wide distribution, they may be implemented in water

669 quality risk assessments worldwide. The major advantage of enteric viral indicators (AdV, PyV, AiV) is that

670 they are human specific, hence their use as indicators enables us to track human-derived contamination

671 exclusively. In addition, crAssphages and other phages, which infect commensal bacteria associated with

672 human gut, and PMMoV, which is a plant virus accumulating in the human gut due to the consumption of

673 infected plant-derived food, are also associated primarily with domestic wastewater contamination. A

674 major advantage of phages and PMMoV is that they are not infectious to humans and hence their detection

675 and culturing in the laboratory pose no risk of infection to the operators. FRNAP-II and FRNAP-III have also

676 been shown to be useful in determining human sources of viral contamination due to their prevalence in

677 human waste. However, due to the non-specific nature of their natural *E. coli* hosts, it is not certain what

678 the reason is for the specific prevalence in human waste relative to FRNAP-I and FRNAP-IV. As such, it is

679 unclear how well these can be applied globally and indeed how stable that relationship is.

680 The viruses reviewed here can be easily detected by qPCR-based methods, however, no such assay has

681 been developed yet for culturable phages infecting *Bacteroides* spp. When using molecular methods, DNA

682 viruses (AdV, PyV and crAssphage) may be easier and more affordable to monitor than RNA viruses (AiV,

683 PMMoV, FRNAP). The infectivity of FRNAP can be easily studied using a simple and rapid plaque assay.

684 Furthermore, the infectivity state of AdV can also be monitored using ICC-qPCR. Infectivity assays are also

685 available for PyV, AiV and crAssphage, however, the usefulness of those assays in environmental setting

686 have not been critically evaluated. Furthermore, there are emerging technologies, such as isothermal

687 amplification, biosensors and microfluidics approaches, which may be useful for the routine monitoring of

688 viruses in the environment (Farkas et al., 2020). In some cases, these may offer the potential for near real-

689 time reporting of viral concentrations in water, however, their applicability still needs to be critically

690 evaluated from a scientific, practical and economic perspective. This is particularly the case for *in situ*

691 devices where biofouling, cross-reactivity and sensor drift represent major problems when translating

692 technologies developed in the laboratory to the field (Lin and Li, 2020).

693 Here, the review of global studies suggests that AdVs, AiV, FRNAP-II, FRNAP-III, crAssphage and PMMoV are

694 detected more frequently and at high concentrations in wastewater and within polluted water bodies than

695 the other indicators reviewed. PyVs are also present in wastewater at high concentrations, however, they

696 are less prevalent in the environment than AdV (Albinana-Gimenez et al., 2009; Bortagaray et al., 2019;

697 Dias et al., 2018; Haramoto et al., 2010; Moresco et al., 2012), suggesting rapid degradation. Similarly,

698 while FRNAP-II and FRNAP-III are initially found at high concentrations in wastewater, it is likely that they

699 degrade more rapidly in the environment than AdV. The concentration of BacGB124P, BacGA17P and

700 BacARABA84P was also high in wastewater and have been found in wastewater polluted environments,

701 however, only a limited number of studies have been conducted to date on viral decay prompting the need

702 for more research in this area.

703 Based on their ease of detection, high concentrations in wastewater and environmental persistence, our

704 review suggests that AdVs are the most useful viral indicators of wastewater contamination. However, AiV,

705 crAssphage and PMMoV also show potential. More research is essential to evaluate the usefulness of these

706 viruses and indicators. Future research should therefore focus on:

707 (i)   Careful monitoring of the association of crAssphage and PMMoV with non-human
708   contamination.

709 (ii)   Monitoring the concentration and persistence of AiV, crAssphage and PMMoV in the aquatic
710   environment, especially in groundwater and in seawater. The effect of extreme weather events
711   on viral concentrations should also be investigated.

712 (iii) Development of a simple and rapid standard operating procedure for concentrating and
713   detecting viruses from water to facilitate the accurate detection of selected indicator virus(es).

714 (iv)   The development of multiplex qPCR assays to simultaneously detect a panel of the best
715   markers, potentially tailored to differences in geographical diversity (particularly for PMMoV).

716 (v)   Critical evaluation and application of new and emerging rapid approaches for viral surveillance.

717 (vi)   Survival and maintenance of infectivity monitoring of AiV, crAssphage and PMMoV in
718   wastewater and in the water environment. For that, the usefulness of infectivity assays for
719   these viruses should be developed and evaluated.

720 (vii) Undertake comprehensive field campaigns in areas where data is not available (e.g. Africa, Asia,
721   Oceania) to validate the use of viral indicators as an effective way to monitor wastewater
722   pollution.

723   (viii)   Use these viral indicators to validate current mathematical models which predict viral dispersal

724   and which are used for risk assessment purposes.

725   (ix)   Better establish the relationship between viral indicators and wastewater pollution to enable

726   the development of legislative standards for viral contamination of waterbodies.

727   A greater understanding of the fate and behaviour of these viruses will allow them to be routinely

728 implemented for water quality monitoring and for viral risk assessment. With a standardised protocol for

729 the detection and quantification of proposed indicators, viral contamination can be efficiently addressed by

730 regulators and hence the number of waterborne and foodborne viral diseases can be reduced, ultimately

731 enhancing global human health.

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Contributors

748

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Table 1. Human pathogenic viruses detected in the aquatic environment

| Family	Genus	Virus types found in water	Structure			Symptoms	Zoonotic	Reference
			Capsid	Genome	Size			
Adenoviridae	*Mastadenovirus*	Mastadenovirus A-F	Icosahedral	dsDNA	70-90 nm	Gastroenteritis*, respiratory illness, ear infection, conjunctivitis	No	King et al., 2009
Anelloviridae	*Alphatorquevirus*	Torque teno virus	Icosahedral	ssDNA	30 nm	Unknown, hepatitis*	Yes	King et al., 2009
Astroviridae	*Mamastrovirus*	Astrovirus	Icosahedral	ssRNA+	28-30 nm	Gastroenteritis	Potentially	De Benedictis et al., 2011; King et al., 2009
Caliciviridae	*Norovirus*	Norovirus GI, GII	Icosahedral	ssRNA+	35-40 nm	Gastroenteritis	No	King et al., 2009
	Sapovirus	Sapovirus GI, GII				Gastroenteritis	No	King et al., 2009
Circoviridae	*Circovirus*	Human-associated circovirus	Icosahedral	ssDNA	15-25 nm	Unknown*	No	Breitbart et al., 2017
Hepeviridae	*Orthohepevirus*	Hepatitis E virus type 1-4	Icosahedral	ssRNA+	27-34 nm	Acute hepatitis*	Yes	Purdy et al., 2017
Papillomaviridae	various	assorted papillomaviruses	Icosahedral	dsDNA	55 nm	Genital tract infection, cancer*	No	Van Doorslaer et al., 2018
Parvoviridae	*Bocavirus*	Human bocavirus type 1-4	Icosahedral	ssDNA	22 nm	Gastroenteritis and respiratory disease	No	King et al., 2009
Picornaviridae	*Kobuvirus*	Aichivirus A-B	Icosahedral	ssRNA+	30-32 nm	Gastroenteritis*	No	Zell et al., 2017
	Cosavirus	Cosavirus A				Gastroenteritis*	No	
	Enterovirus	Coxsackievirus B Enterovirus A-D Poliovirus type 1-3				Gastroenteritis, mild meningitis, encephalitis, myelitis, myocarditis, conjunctivitis*	No	
	Hepatovirus	Hepatitis A virus				Gastroenteritis, hepatitis	No	
Polyomaviridae	*Alpha-polyomavirus*	MC polyomavirus	Icosahedral	dsDNA	40-45 nm	Cancer*	No	Moens et al., 2017
	Beta-polyomavirus	BK polyomavirus JC polyomavirus	Icosahedral	dsDNA	40-45 nm	Respiratory, urinary tract and skin infection, cancer*	No	Moens et al., 2017
Reoviridae	*Reovirus*	Rotavirus A	Icosahedral	dsRNA	60-80 nm	Gastroenteritis	Potentially	Cook et al., 2004; King et al., 2009

*May be asymptomatic in otherwise healthy individuals

Table 2. Number of reviewed studies for each indicator at each region.

		North America	South America	Africa	Europe	Asia	Oceania	Global detection rate
AdV	Raw wastewater	7	2	2	13	3	5	94% (772/823)
	Treated wastewater	10	7	2	13	3	3	86% (1223/1436)
	Surface freshwater	4	5	4	7	4	0	65% (835/1283)
	Groundwater	1	2	0	1	1	0	65% (40/62)
	Seawater	2	6	0	4	0	0	60% (229/381)
	Total	13	16	5	20	5	4	76% (3099/3985)
PyV	Raw wastewater	5	5	1	7	2	3	93% (542/581)
	Treated wastewater	6	5	1	6	2	2	68% (608/892)
	Surface freshwater	1	2	0	6	2	0	52% (326/631)
	Groundwater	0	0	0	1	0	0	48% (10/21)
	Seawater	4	0	0	2	2	1	24% (83/350)
	Total	9	7	1	10	4	3	63% (1569/2475)
AiV	Raw wastewater	5	0	0	0	4	0	91% (92/101)
	Treated wastewater	5	0	0	1	3	0	74% (184/250)
	Surface freshwater	2	0	0	1	3	0	33% (77/236)
	Groundwater	1	0	0	0	0	0	55% (26/47)
	Seawater	0	0	0	0	0	0	NA
	Total	6	0	1	6	0		60% (379/634)
PMMoV	Raw wastewater	6	0	1	1	2	1	100% (110/110)
	Treated wastewater	6	0	1	1	2	0	99% (135/137)
	Surface freshwater	2	0	0	1	4	0	87% (278/319)
	Groundwater	1	0	0	0	0	0	72% (18/25)
	Seawater	1	0	0	0	0	1	55% (45/82)
	Total	7	0	1	1	5	1	87% (586/673)
Bacteroides phages	Raw wastewater	2	2	0	14	2	0	97% (531/549)
	Treated wastewater	0	1	0	8	1	0	75% (911/1216)
	Surface freshwater	2	1	0	4	1	0	66% (280/427)
	Groundwater	0	0	0	3	0	0	38% (48/127)
	Seawater	0	0	0	3	0	0	42% (43/102)
	Total	3	2	0	19	2	0	72% (1741/2421)
FRNAP (II/III)	Raw wastewater	0	0	0	1	3	0	73% (96/132)
	Treated wastewater	0	0	0	1	4	0	81% (219/270)
	Surface freshwater	0	0	0	2	5	0	59% (375/634)
	Groundwater	0	0	0	0	1	0	0% (0/10)
	Seawater	0	0	0	0	0	0	NA
	Total	0	0	0	3	8	0	66% (690/1046)

Table 3. Summary on how the reviewed viruses meet the criteria for wastewater indicator.

Criterion	AdV	PyV	AiV	PMMoV	FRNAP (II/III)	Culturable *Bacteroides* phages	CrAssphage
Methods used for detection in environmental samples	qPCR; ICC-qPCR; culturing	qPCR	qRT-PCR	qRT-PCR; plant infectivity assay	qRT-PCR; culturing	culturing	qPCR
Human association	Human-specific	Human-specific	Human-specific	Human waste and agricultural sites	Primarily human gut-associated	Primarily human gut-associated, have been found in animal faeces at low titres	Primarily human gut-associated, have been found in animal faeces at low titres
Concentration in wastewater (gc/l)	$1\times10^1 - 3\times10^{11}$	$1\times10^3 - 6\times10^8$	$1\times10^4 - 4\times10^6$	$3\times10^5 - 2\times10^{10}$	$4\times10^3 - 2\times10^9$	$1\times10^1 - 6\times10^6$	$2\times10^5 - 1\times10^{12}$
Log_{10} removal during wastewater treatment	$0.2 - 5.5$ (n=500)	$0.3 - 4.2$ (n=407)	$0.8 - 2.7$ (n=72)	$0 - 2.7$ (n=106)	$0.1 - 3.1$ (n=172)	$0.5 - 5.6$ (n=304)	$1 - 1.2$ (n=39)
Concentration in the aquatic environment (gc/l)	$4 - 2\times10^{10}$	$1 - 1\times10^7$	$7\times10^1 - 8\times10^8$	$1\times10^1 - 8\times10^8$	$0.2 - 2\times10^6$	$1 - 2\times10^5$	$1\times10^3 - 3\times10^7$
Global distribution and temporal stability	Detected in clinical samples globally; limited seasonal variations	Detected in clinical samples globally; limited seasonal variations	Detected in clinical samples globally; limited seasonal variations	Detected in clinical samples globally; limited seasonal variations	Detected in clinical samples globally; limited seasonal variations	Detected in clinical samples globally; limited seasonal variations	Detected in clinical samples globally; limited seasonal variations

(A)

(B)
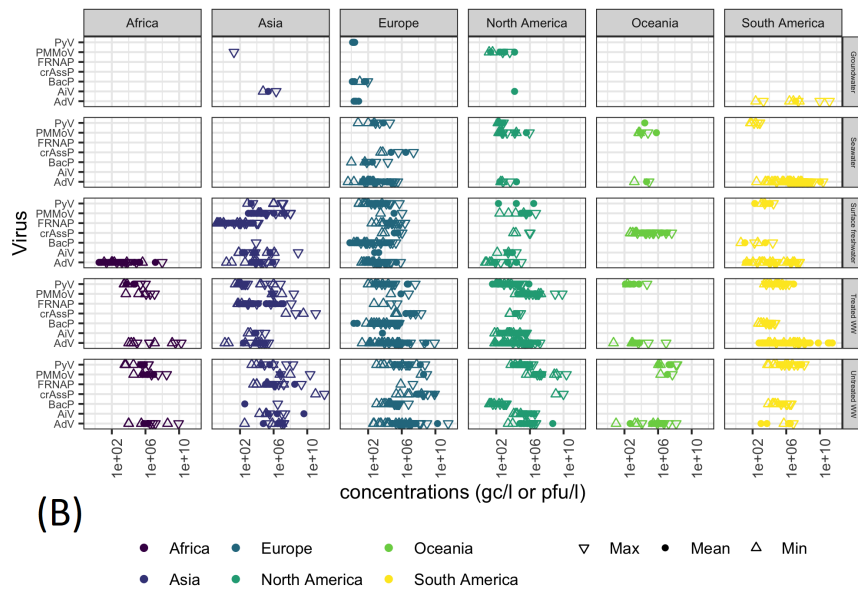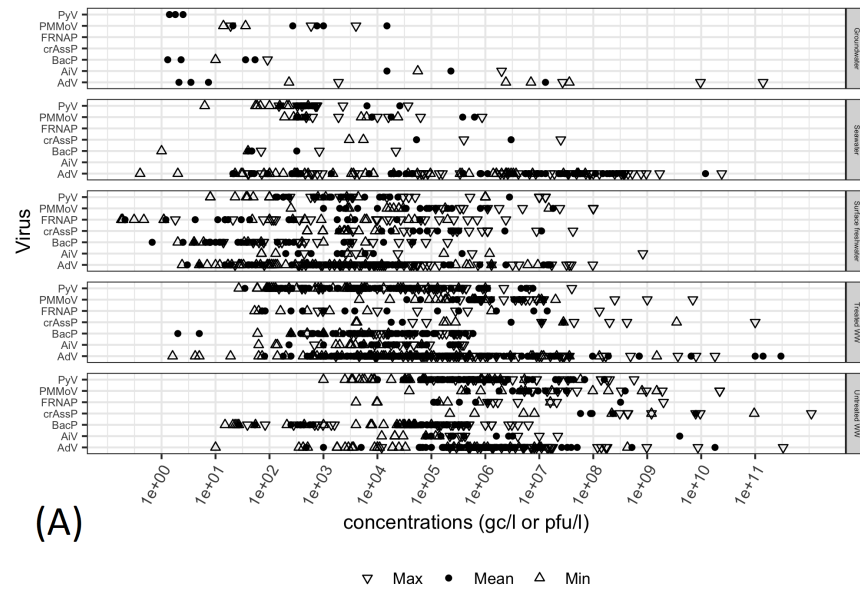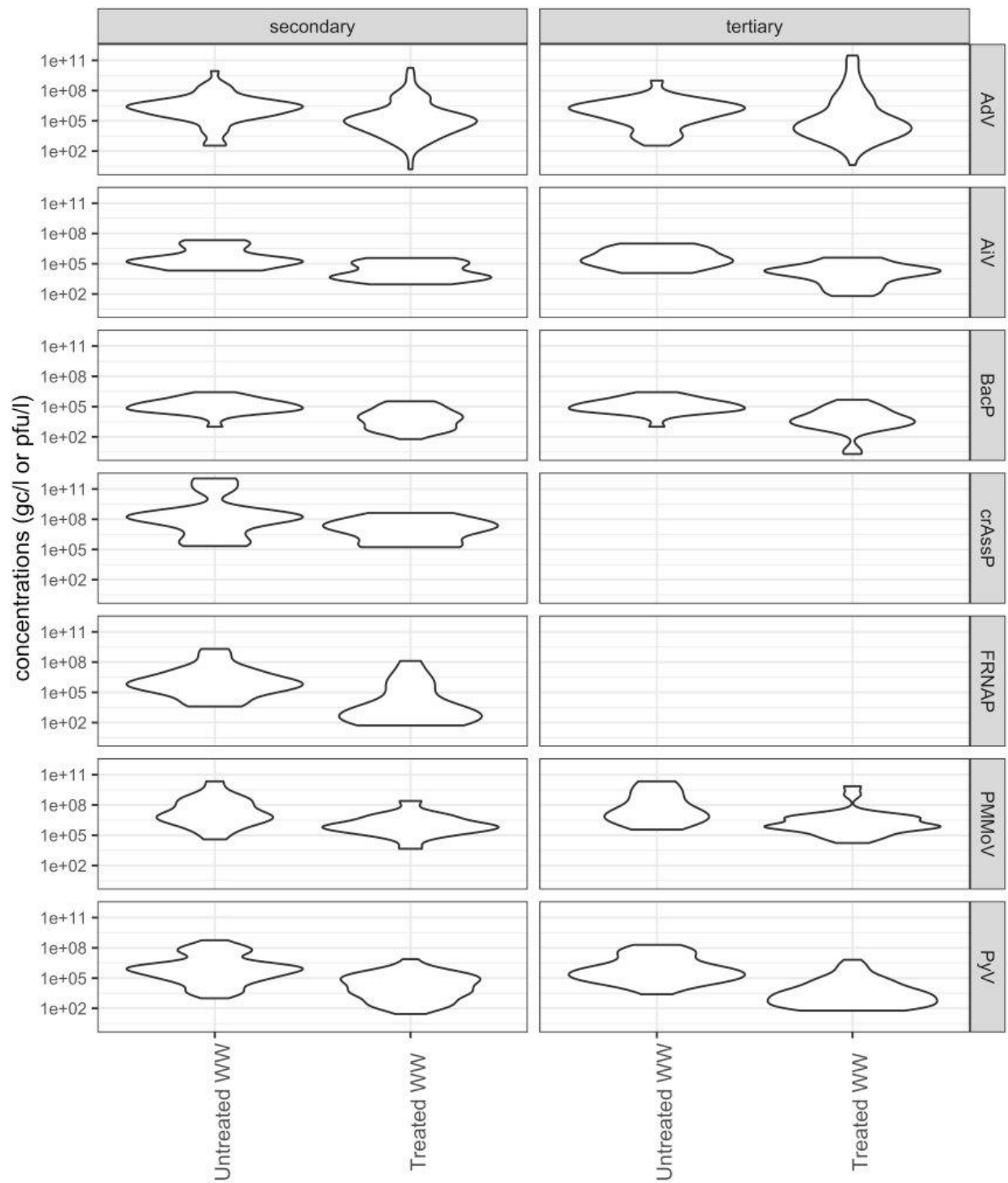
Highlights

- Human mastadenoviruses are robust indicators for human-associated pollution in water

- *Bacteroides*-associated phages and crAssphage are promising indicators

- Multiple indicators should be used to assess wastewater treatment efficiency

- Survival and abundance of indicator viruses should be further assessed

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: