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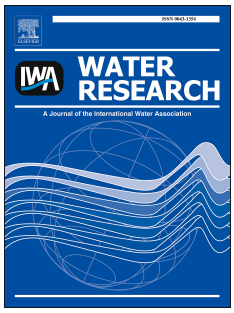
Water Research

DOI:
10.1016/j.watres.2020.115926

Published: 15/08/2020

Peer reviewed version

Journal Pre-proof



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PII: S0043-1354(20)30463-2

DOI: https://doi.org/10.1016/j.watres.2020.115926

Reference: WR 115926

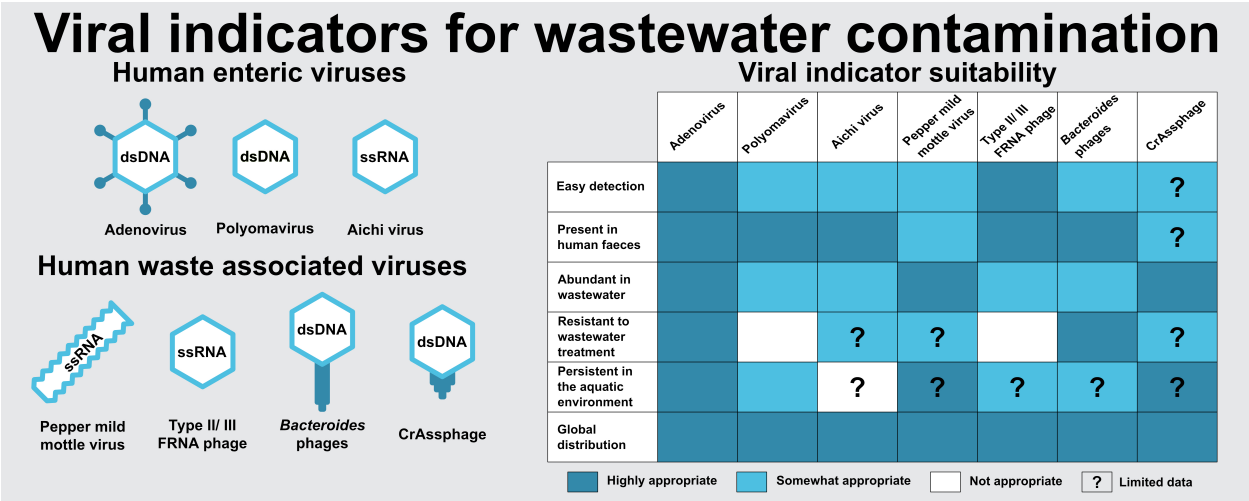
To appear in: *Water Research*

Received Date: 22 November 2019

Revised Date: 7 May 2020

Accepted Date: 8 May 2020

Please cite this article as: Farkas, K., Walker, D.I., Adriaenssens, E.M., McDonald, J.E., Hillary, L.S., Malham, S.K., Jones, D.L., Viral indicators for tracking domestic wastewater contamination in the aquatic environment, *Water Research* (2020), doi: https://doi.org/10.1016/j.watres.2020.115926.



Viral indicators for tracking domestic wastewater contamination in the aquatic 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.

Keywords: gastroenteric viruses; environmental sampling; viral indicators; sewage contamination; risk assessment

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

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

concentrations (up to 10^8 genome copies (gc)/l) in wastewater, river and seawater and sediment, in swimming pools and in tap water (Di Bonito et al., 2017; Dias et al., 2018; Farkas et al., 2018a; Fratini et al., 2014; Hamza and Hamza, 2018; Rachmadi et al., 2016). As these viruses are commonly asymptomatic in healthy individuals, the route of transmission is not yet clear, however, waterborne infections are likely (Fratini et al., 2014).

Bocaviruses (family *Parvoviridae*), causing respiratory tract infections and gastroenteritis, were first described in 2005 (Allander et al., 2005). They have since been found in untreated and treated wastewater at concentrations of 10^3-10^5 genome copies (gc)/l (Hamza et al., 2017; Iaconelli et al., 2016; Myrmel et al., 2015), however, their prevalence in environmental water has not been explored. The torque teno virus (family *Anelloviridae*), which causes gastroenteritis has also been found in wastewater and in polluted river waters. Similar to bocaviruses, torque teno virus concentrations are considerably lower (up to 10^6 gc/l) than the concentrations of other, more common enteric viruses (10^4-10^9 gc/l) (Hamza et al., 2011; Haramoto et al., 2008). Human picobirnaviruses (family *Picobirnaviridae*) have also been detected in wastewater (concentration range: 10^3 – 10^6 gc/l) and in contaminated rivers with variable prevalence (Adriaenssens et al., 2018; Hamza et al., 2011; Symonds et al., 2009). In addition, recent comparative genomics analysis has suggested that picobirnaviruses are bacteriophages, likely associated with mammalian gut bacteria (Krishnamurthy and Wang, 2018). Genomes or partial genomes of circoviruses (family *Circoviridae*) and cardioviruses (family *Picornaviridae*) along with enveloped viruses (coronaviruses, influenza virus) have also been found in wastewater (Bibby and Peccia, 2013; Blinkova et al., 2009; Ng et al., 2012). Enveloped viruses degrade in water rapidly (Gundy et al., 2009; Lebarbenchon et al., 2011), hence, human infections from waterborne corona- and influenza viruses (e.g. SARS-CoV-2) are unlikely.

1.2 Viral indicators for wastewater contamination

Over 100 types of human enteric viruses are known to be common water pollutants (Melnick, 1984) and with novel and emerging strains, the number is increasing. Due to the diversity of human pathogenic viruses in the environment, surrogates and indicators are often used to investigate the fate and transport 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.

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.

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.,

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

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

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.

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

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.

666 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.

732 Acknowledgements

733 This work was supported by the UK Natural Environment Research Council (NERC) and the Food Standards
734 Agency (FSA) under the Environmental Microbiology and Human Health (EMHH) Programme
735 (NE/M010996/1) and by the Shellfish Centre RD&I operation, part-funded by the EU's West Wales and the
736 Valleys European Regional Development Fund (ERDF) Operational Programme through the Welsh
737 Government. EMA was funded by the Biotechnology and Biological Sciences Research Council (BBSRC); this
738 research was funded by the BBSRC Institute Strategic Programme Gut Microbes and Health BB/R012490/1
739 and its constituent projects BBS/E/F/000PR10353 and BBS/E/F/000PR10356. LSH was supported by a Soils
740 Training and Research Studentship (STARS) grant from the Biotechnology and Biological Sciences Research
741 Council (BBSRC) and Natural Environment Research Council (NE/M009106/1). We thank I. Bertrand
742 (University of Lorraine, France), G. Fongaro, (Federal University of Santa Caterina, Brazil), I. Hamza
743 (National Research Centre, Egypt), A. Hata (University of Tokyo, Japan), M.R. Jumat (King Abdullah
744 University of Science and Technology, Saudi Arabia), M. Rusiñol (University of Barcelona, Spain), T. Sibanda
745 (University of South Africa, South Africa), R. Staggemeier (Feevale University, Brazil), E. Symonds (University
746 of Florida, USA), M. Verani (University of Pisa, Italy), S. Wurtzer (University of Paris, France) for providing
747 data for Table S1-6.

Contributors

KF and DIW conducted the literature search and the collection of data on viral concentrations. All authors contributed to structuring and writing the article.

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Figure 1. Map illustrating the sampling sites where viral indicators have been detected in untreated wastewater (red), treated wastewater (yellow) surface freshwater (blue), groundwater (green), seawater (purple). To zoom in to a particular region visit https://j.mp/2VdQVpY.

Figure 2. Viral concentrations (mean, minimum and maximum values in genome copies (gc) or plaque-forming units (pfu) per litre) extracted from the reviewed studies. (A) All data; (B) Distribution of the data in A grouped by continent. AdV: human mastadenoviruses; PyV: human polyomavirus JC, BK and MC; AiV: human Aichi viruses; PMMoV: pepper mild mottle virus; crAssP: crAssphage; BacP: culturable phages infection Bacteriodes spp.; FRNAP: FRNA phages II and III; WW: wastewater.

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Table S5. F-specific coliphages in wastewater and in the aquatic environment

772 Table S6. *Bacteroides*-associated phages in wastewater and in the aquatic environment

773 Table S7. Indicator virus removal rates during wastewater treatment processes

774 Table S8. qPCR primers and probes used for the detection of viral indicators

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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

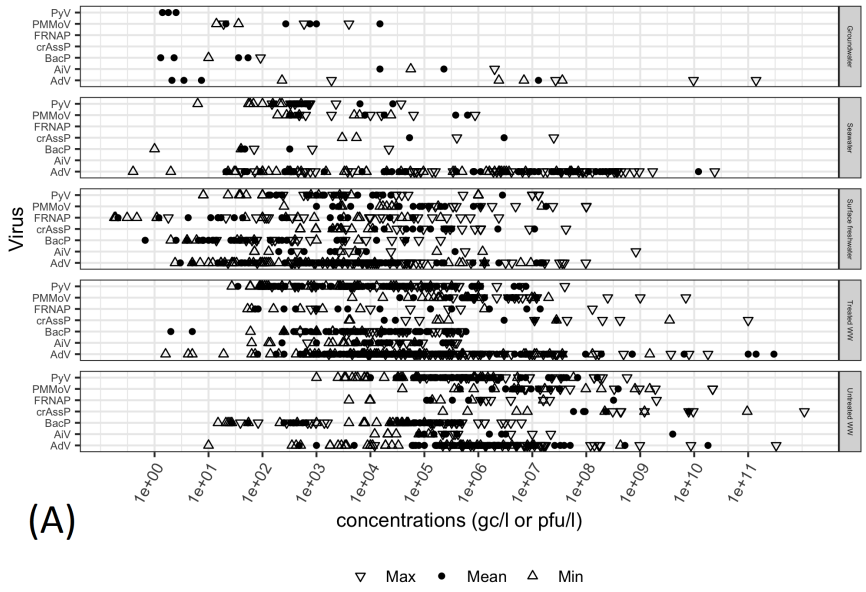
*May be asymptomatic in otherwise healthy individuals

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	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)	$1x10^{1}$ – $3x10^{11}$	$1x10^{3}$ – $6x10^{8}$	$1x10^{4}$ – $4x10^{6}$	$3x10^{5}$ – $2x10^{10}$	$4x10^{3}$- $2x10^{9}$	$1x10^{1}$ – $6x10^{6}$	$2x10^{5}$ – $1x10^{12}$
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 – $2x10^{10}$	1 – $1x10^{7}$	$7x10^{1}$ – $8x10^{8}$	$1x10^{1}$ – $8x10^{8}$	0.2-$2x10^{6}$	1 – $2x10^{5}$	$1x10^{3}$ – $3x10^{7}$
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


PyV
PMMoV
FRNAP
crAssP
BacP
AiV
AdV
Virus
Groundwater
Seawater
Surface freshwater
Treated WW
Untreated WW
1e+00
1e+01
1e+02
1e+03
1e+04
1e+05
1e+06
1e+07
1e+08
1e+09
1e+10
1e+11
concentrations (gc/l or pfu/l)
(A)
Max
Mean
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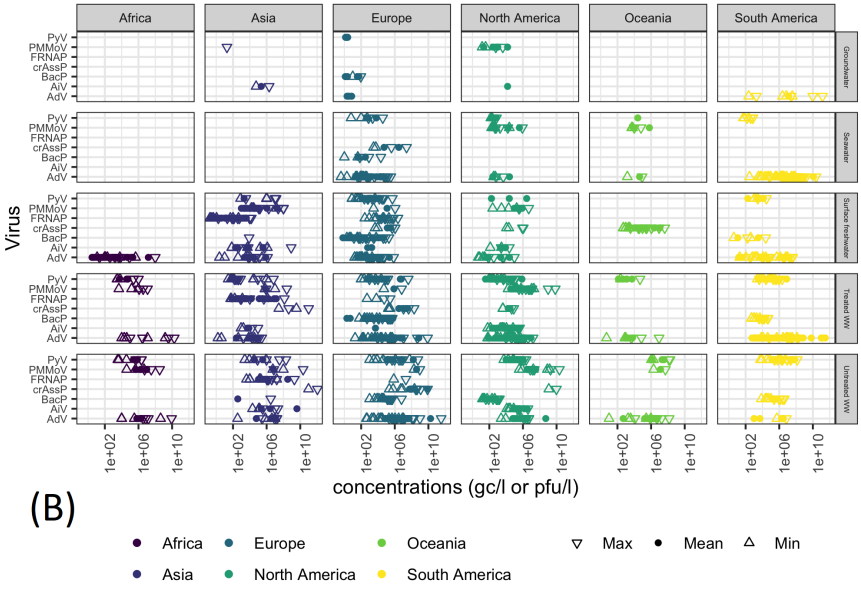
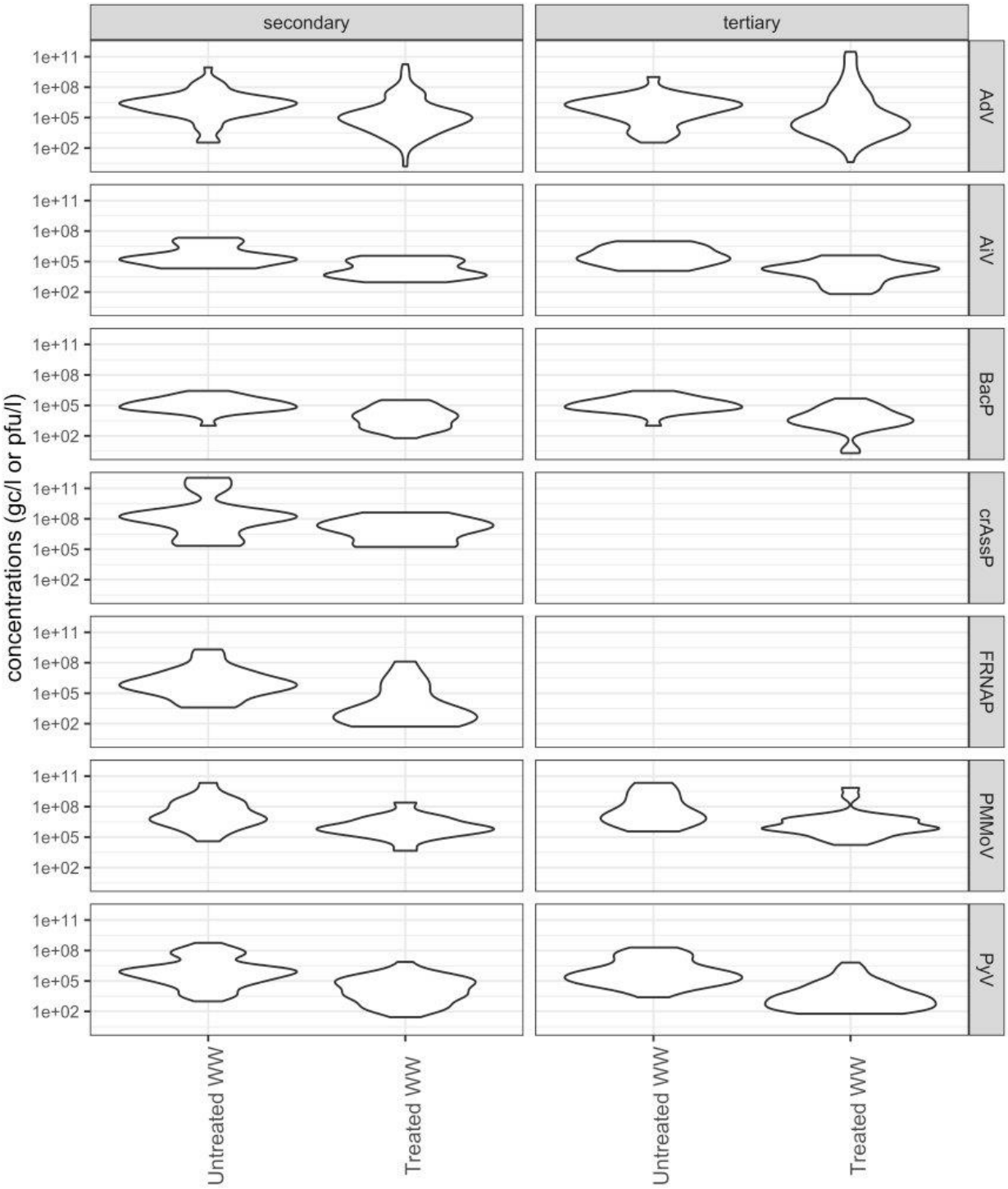

Africa
Asia
Europe
North America
Oceania
South America
PyV
PMMoV
FRNAP
crAssP
BacP
AiV
AdV
Virus
Groundwater
Seawater
Surface freshwater
Treated WW
Untreated WW
1e+02
1e+06
1e+10
concentrations (gc/l or pfu/l)
(B)
Africa
Asia
Europe
North America
Oceania
South America
Max
Mean
Min


secondary
tertiary
AdV
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crAssP
FRNAP
PMMoV
PyV
1e+11
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concentrations (gc/l or pfu/l)
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Treated WW

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: