ACCEPTED MANUSCRIPT

# The application of bacteriophages as novel indicators of viral pathogens in wastewater treatment systems

**Edgard Dias[1,2], James Ebdon[2] and Huw Taylor[2]**

[1] Department of Sanitary and Environmental Engineering, Faculty of Engineering, Federal University of Juiz de Fora, Juiz de Fora – MG, Brazil, 36.036-330

[2] The Environment and Public Health Research Group (EPHReG), School of Environment and Technology, University of Brighton, Brighton, UK, BN2 4GJ

edgard.dias@ufjf.edu.br, je3@brighton.ac.uk, h.d.taylor@brighton.ac.uk

## Abstract

Many wastewater treatment technologies have been shown to remove bacterial pathogens more effectively than viral pathogens and, in aquatic environments, levels of traditional faecal indicator bacteria (FIB) do not appear to correlate consistently with levels of human viral pathogens. There is, therefore, a need for novel viral indicators of faecal pollution and surrogates of viral pathogens, especially given the increasing importance of indirect and direct wastewater reuse. Potential candidates include bacteriophages (phages) and the study described here sought to elucidate the relationship between three groups of phages (somatic coliphages (SOMPH), F RNA coliphages (F RNAPH) and human-specific phages infecting *B. fragilis* (Bf124PH) – enumeration using double layer agar technique) and viral pathogens (human adenovirus (HuAdV) and norovirus (NoV) – enumeration using molecular methods) through full-scale municipal wastewater treatment processes. FIB (faecal coliforms (FC) and intestinal enterococci (ENT) – enumeration using membrane filtration) were also monitored. Samples were collected every fortnight, during a twelve-month period, at each stage of four full-scale wastewater treatment plants (WWTP) in southern England (two activated sludge

(AS) and two trickling filter (TF) plants) (n = 360 samples). FIB and SOMPH were consistently found in all samples tested, whereas F RNAPH, Bf124PH and HuAdV were less frequently detected, especially following AS treatment. The detection rate of NoV was low and consequently discussion of this group of viruses is limited. Concentrations of SOMPH and FIB were statistically higher (p value < 0.05) than concentrations of F RNAPH, Bf124PH and HuAdV in raw wastewater. FIB were more effectively removed than phages in both systems. Removal rates of HuAdV were similar to those of phages at the secondary treatment stage of both systems. In TF systems, HuAdV were removed at the same rate as F-RNAPH, but at lower rates than SOMPH and Bf124PH. The findings suggest that phages (in particular SOMPH) are better indicators of the fate of viral pathogens in WWTP than existing FIB and that these organisms may have a useful role to play in future sanitation safety planning.

**Key words:** human viral pathogens, phages, faecal indicator bacteria, reuse, risk, sanitation safety planning.

## 1. INTRODUCTION

Waters polluted with faecal material may contain a wide variety of viruses originating from the human gastro-intestinal tract (enteric viruses). It is estimated that over one hundred viral species of enteric origin are present in municipal wastewaters, many of which are capable of causing illnesses in humans (Bosch, 1998; Tchobanoglous *et al.*, 2014). Viruses that cause waterborne diseases include noroviruses (NoV) and human adenoviruses (HuAdV). NoV are responsible for outbreaks of acute gastroenteritis in children and adults worldwide (Victoria *et al.*, 2010; Sima *et al.*, 2011; WHO, 2011). Human adenoviruses (HuAdV) can cause a wide range of diseases, including respiratory, ocular, gastroenteric and other infections (Kuo *et al.*, 2010; Sidhu *et al.*, 2012).

NoV are small (38-40 nm in diameter) round structured viruses, with a non-enveloped capsid and a positive single-strand RNA genome (Liu *et al.*, 2007; Victoria *et al.*, 2010). Sima *et al.* (2011) have reported that NoV are shed at high titre in faeces during the acute phase of the infection and for three weeks after symptoms have subsided, reaching concentrations of $10^{11}$ viral particles per gramme of faeces (Atmar, 2010). As a consequence, NoV can be detected in high concentrations in domestic wastewater (van den Berg *et al.*, 2005; Haramoto *et al.*, 2008; Katayama *et al.*, 2008; Eftim *et al*., 2017). HuAdV are medium-sized (90-100 nm diameter) viruses, with a non-enveloped capsid and a linear double-stranded DNA genome (Jiang, 2006; Hewitt *et al.*, 2013). HuAdV are shed in human faeces at concentrations of up to $10^{11}$ viral particles per gramme of faeces (Fields *et al.*, 2007). Thus, their presence is commonly reported in raw wastewater, final effluents and aquatic environments (Kuo *et al.*, 2010; Hewitt *et al.*, 2011).

As recognised by the UN Sustainable Development Goal 6 Target 3 (UN-Water, 2016), wastewater reuse makes more water available for drinking and other uses and can reduce impacts on water-related ecosystems. However, a matter of considerable societal concern is the potential risk to human health associated with human contact with waterborne pathogenic microorganisms present in wastewater. More specifically, evidence suggests that waterborne viral pathogens are inadequately removed from existing wastewater treatment systems and that bacterial indicators used to assess water quality fail to detect their presence accurately (USEPA, 2015).

Conventional wastewater treatment technologies were chiefly developed with the aim of removing organic matter and suspended solids rather than of removing, or inactivating pathogenic microorganisms (OFWAT/DEFRA, 2006). Although some degree of pathogen reduction occurs during these treatment processes, they have been shown to be much more effective at removing bacterial pathogens than viral pathogens, which are smaller in size, simpler in structure and tend to be more persistent in the environment (Grabow, 2001; Sinton *et al.*, 2002; Diston *et al.*, 2012). In addition, levels of traditional faecal indicator bacteria

(FIB) – e.g., *Escherichia coli* and intestinal enterococci – do not appear to correlate consistently with levels of human water- and excreta-borne viral pathogens (Baggi *et al.*, 2001; Espinosa *et al.*, 2009; Jurzik *et al.*, 2010; Morens *et al.*, 2010) in treated wastewaters.

In response, bacteriophages (phages), which are viruses capable of infecting bacteria, have been proposed as potential novel viral indicators (Ebdon *et al*., 2012; McMinn *et al*., 2014). The three groups of phages most commonly used for water quality monitoring are F-specific and somatic coliphages, as well as phages that infect host-specific *Bacteroides* spp. (Grabow, 2001). Phages are considered to be better predictors of human enteric virus persistence and environmental behaviour than traditional FIB because they have a similar composition, morphology, structure, size and site of replication (Grabow, 2001; Sinton *et al.*, 2002; Diston *et al.*, 2012). In addition, the incidence and survival of phages in aquatic environments have also been reported to resemble those of human viruses more closely than the traditional bacterial indicators commonly used (Lin and Ganesh, 2013). Furthermore, evidence has shown that phages may be associated with gastrointestinal illness (Griffith *et al*., 2016).

Whilst FIB and phages are consistently found in raw and treated municipal wastewater (Kay *et al*., 2008; Carducci *et al*., 2009; Wu *et al*., 2011; De Luca *et al*., 2013), the detection of human enteric viruses tends to vary according to the number of infected individuals in the population using the sewers, with high detection and/or concentrations in some cases (Aw and Gin, 2010; Kuo *et al*., 2010; Wolf *et al*., 2010; Sidhu *et al*., 2012) and low detection and/or concentrations in others (Victoria *et al*., 2010; Hewitt *et al*., 2011). In addition, it has been observed that FIB are more effectively removed than phages (and, more importantly, viral pathogens) during wastewater treatment (Rose *et al*., 2004; Ottoson *et al*., 2006; Ebdon *et al*., 2012; Flannery *et al*., 2012; Purnell *et al*., 2015; Purnell *et al*., 2016). Furthermore, several studies have reported no correlations between levels of pathogens and indicator organisms in wastewater at various stages of treatment (Rose *et al*., 2004; Wu *et al*., 2011; Flannery *et al*., 2012). Therefore, further research is needed to evaluate the use of phages

as indicators of enteric viruses during wastewater treatment. Ideally this research should enumerate a variety of phage groups, FIB and human enteric viruses in the influent and effluent of each treatment step of full-scale wastewater treatment facilities and calculate the $\log_{10}$ removal rates achieved (USEPA, 2015).

A systematic review of the literature on the use of coliphages as potential faecal indicator organisms that was recently carried out on behalf of the US Environmental Protection Agency (USEPA) suggested that coliphages are likely to be a better indicator of viruses within faecal contamination than currently-used FIB (i.e., enterococci and *E. coli*) and that these phages may be a better surrogate for specific viruses than FIB in WWTP effluent (USEPA, 2015). It is within the context of a growing need for more effective faecal indicators and surrogates that this research was established, with the aim of investigating the concentrations and removal rates of viral pathogens, phages and FIB at each treatment step of two of the most widely applied wastewater treatment processes (activated sludge and trickling filters). The aim of the present study was to investigate whether phages better reflect the fate of viral pathogens in AS and TF systems than FIB.

## 2. MATERIAL & METHODS

### *2.1. Wastewater treatment sites and samples collection*

Four wastewater treatment plants (WWTP) were used to obtain a comprehensive dataset of wastewater quality parameters to describe treatment operation and efficacy over a period of twelve continuous months. The four WWTP were located in southern England, UK, and included secondary biological treatment in the form of activated sludge (AS) and trickling filters (TF). The two TF treatment plants included ‘settlement ponds’ as a tertiary treatment step, whereas one AS treatment plant included sand filters as a tertiary treatment step; the other AS treatment plant did not include any tertiary treatment. The scale of the monitored WWTP ranged from ‘small’ (5,000 p.e.) to ‘medium’ (45,000 p.e.). Samples were collected every fortnight from June 2013 to May 2014 (inclusive), resulting in a total of 24 sampling

132 occasions and 360 samples. On each sampling occasion, a one-litre volume of each sample
133 was collected in pre-sterilised (autoclaved at 121ºC for 15 minutes) polyethylene bottles,
134 stored in cooler boxes at approximately 4ºC and transported to the laboratory for further
135 analysis within 4 h. At all sites, four different samples were collected on each occasion: (i)
136 raw wastewater (RW); (ii) primary effluent (immediately after the primary sedimentation
137 tanks) (PST); (iii) secondary effluent (immediately after the secondary sedimentation tanks)
138 (SST); and (iv) final effluent (after the tertiary treatment systems) (FE).

139

140 ***2.2. Enumeration of indicator organisms***

141 Faecal coliforms (FC) and intestinal enterococci (ENT) were enumerated (presumptive
142 counts) following the protocols described in ISO 9308-1 (BSI, 2009) and ISO 7899-2 (BSI,
143 2000), respectively. For both bacterial groups, samples were filtered through 0.45 µm
144 cellulose nitrate membrane filters (Sartorius, Göttingen, Germany) and then incubated on
145 selective agar at specific temperatures: membrane incubation on M-FC agar (Merck
146 Millipore, Darmstadt, Germany) at 44±2ºC for 24±2 h for FC; and on Slanetz and Bartley
147 agar (Merck Millipore, Darmstadt, Germany) at 37±2ºC for 44±2 h for ENT. Concentrations of
148 FIB were expressed as colony-forming units per 100 mL (cfu.100mL$^{-1}$).

149 The three groups of phages commonly used in water quality monitoring were analysed in the
150 present study and were detected and enumerated as follows: somatic coliphages (SOMPH)
151 were enumerated according to ISO 10705-2 (BSI, 2001) using the host strain *E. coli* WG-5;
152 F-RNA coliphages (F-RNAPH) were enumerated according to 10705-1 (BSI, 2002) using the
153 host strain *S. typhimurium* WG-49; and phages infecting *B. fragilis* (Bf124PH) were
154 enumerated according to ISO 10705-2 (BSI, 2003) using the host strain *B. fragilis* GB-124. In
155 order to increase sensitivity, the method was modified (as described by Vijayavel *et al.*
156 (2010)) to process 5 mL rather than 1 mL of secondary effluent (SST) and final effluent (FE)
157 from the AS systems on the final twelve sampling dates. Concentrations of phages were
158 expressed as plaque-forming units per 100 mL (pfu.100mL$^{-1}$).

159

160 ***2.3. Molecular detection and enumeration of viral pathogens***

161 NoV and HuAdV were chosen as the pathogens of interest in this study because they are
162 responsible for a range of disease in humans and are commonly found in municipal
163 wastewater. A description of the methods used for their detection and enumeration is
164 presented as follows: Once samples had been collected and transferred to the laboratory, a
165 10-mL volume of each sample, with 5% glycerol (v/v) added, was stored at -20ºC until
166 processed. In order to increase the sensitivity of the method, this volume was increased to
167 50 mL, with 5% glycerol (v/v) added, for samples of secondary (SST) and final (FE) effluent
168 from both AS and TF systems for the final 16 sampling occasions. The elution and
169 concentration methods used for the preparation of samples prior to the enumeration of viral
170 pathogens were selected from a range of methods previously tested by Dias (2016). In brief,
171 before processing, samples were allowed to thaw at 4ºC. The 10 mL samples were
172 transferred to 50-mL sterile polypropylene centrifuge tubes (Fisherbrand, Loughborough, UK)
173 and viruses were eluted using 2.5 mL of glycine buffer 2.0 M, pH 9.5 (1:0.25, v/v). The 50 mL
174 samples were transferred to 100-mL sterile polyethylene containers (Plastiques Gosselin,
175 Borre, France) and the viruses were eluted using 12.5 mL of glycine buffer 2.0 M, pH 9.5
176 (1:0.25, v/v). Samples were stirred rapidly in an orbital shaker for 30 min at 300 rpm and then
177 filtered through 0.22 µm polyethersulfone Millex-GP syringe filter units (Merck Millipore,
178 Darmstadt, Germany) in order to remove bacteria and other suspended material.
179 Subsequently, samples were concentrated using Amicon Ultra-15 centrifugal filters units
180 (50 kDa molecular weight cut-off) (Merck Millipore, Darmstadt, Germany) and centrifuged at
181 5,000 *g* at 4ºC for 10 min to obtain a final volume of less than 500 µL. Multiple centrifugation
182 steps were applied to the 50-mL samples. The final volume was made up to 500 µL with
183 phosphate buffer solution (PBS) and stored at 4ºC before nucleic acids were extracted. The
184 preparation methods used were tested for their recovery of SOMPH, and a recovery rate of
185 21% was recorded. This recovery rate was then used to calculate the concentrations of NoV

and HuAdV. After the preparation steps, viral DNA and RNA were extracted from samples using the commercial kits QIAamp Fast DNA Stool Mini Kit and QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), respectively, according to the manufacturers' instructions. Both DNA and RNA extracts were then stored at -80ºC until further processing within six months.

Prior to RT-qPCR assay, samples were allowed to thaw at 4ºC. All qPCR assays were performed using a Qiagen Rotor-gene Q (Qiagen, Hilden, Germany). '", 'no template' and 'internal extraction' controls were used in every assay run. HuAdV RT-qPCR was carried out by amplifying the hexon gene using the commercial primer and probe set Adenovirus Type F and G genesig® Advanced Kit (PrimerDesign, Southampton, UK), according to the manufacturer's instructions. NoV G1 RT-qPCR was carried out by amplifying the Norovirus GI capsid protein gene, whereas NoV G2 RT-qPCR was carried out by amplifying Norovirus GII RNA dependent RNA polymerase gene, both using the commercial primer and probe set Norovirus Genogroups 1 and 2 genesig® Advanced Kit (PrimerDesign, Southampton, UK), according to the manufacturer's instructions. Primers and probes for both HuAdV and NoV qPCR assays were designed by the manufacturer (PrimerDesign, Southampton, UK). The primers present 100% homology with all reference sequences included in the NCBI database and therefore these kits are considered to have very broad detection profiles. For HuAdV, each sample (5 µL) was prepared with a 15 µL reaction mix, containing 10 µL PrecisionPLUS™ 2x qPCR MasterMix, 1 µL Adv F+G primer/probe mix, 1 µL internal extraction control primer/probe mix and 3 µL RNAse/DNAse free water. For NoV G1 and G2 detection, each sample (5 µL) was prepared with a 15 µL reaction mix, containing 10 µL PrecisionTM OneStep 2x qRT-PCR MasterMix, 1 µL RNA-pol primer/probe mix, 1 µL internal extraction control primer/probe mix and 3 µL RNAse/DNAse free water. Thermal conditions for HuAdV consisted of enzyme activation for 2 min at 95ºC, followed by 50 cycles of denaturation for 10 s at 95ºC and data collection for 60 s at 60ºC. NoV detection followed the same thermal conditions, with the addition of a prior reverse transcription stage of 10 min at

42ºC before enzyme activation. No inhibition control was performed. Concentrations of viral pathogens were expressed as copies per 100 mL (copies.100mL$^{-1}$).

### *2.4. Data analysis*

For statistical analysis, the data were divided into two groups: one group comprising the data collected from the two TF plants; and a second group comprising the data collected from the two AS plants. It is relevant to mention that non-detects were not included in the statistical analyses performed. The unpaired t-test (ranked t-test) and one-way analysis of variance (ANOVA on ranks) were applied to the ranked data. The unpaired two-sample t-test was applied to ranked data in order to compare: (i) AS and TF systems in terms of the concentrations of the microorganism at each treatment step; (ii) AS and TF systems in terms of the removal rates of the microorganism at each treatment step. ANOVA on ranks and Tukey's statistics were applied to compare the following: (i) the concentrations of microorganisms (at each treatment step of both AS and TF systems); (ii) the removal rates of microorganisms (at each treatment step of both AS and TF systems); the removal rates at the primary, secondary and tertiary treatment steps (for each microorganism in both AS and TF systems). In addition, the non-parametric Spearman's rank correlation test was used to check correlations between concentrations and removal rates of different microorganisms at different treatment steps of the AS and TF systems. All statistical tests were performed using a significance level of 5% ($\alpha = 0.05$) with the aid of Minitab version 17.1.0 (Minitab Inc, Pennsylvania, USA).

Normalization and statistical analysis of censored data (results below the detection limit) with zero, or with a proportion of the detection limits (e.g., 1/2 or $1/(\sqrt{2})$) is an approach that has been widely applied in other studies (McCall et al., 2014; Wangkahad et al., 2016). This approach has also been applied when dealing with non-detects in real-time quantitative PCR (qPCR) data (McCall et al., 2014). However, certain issues have been observed when using such an approach. Firstly, the inclusion of non-detects in datasets has been shown to produce significant biases in subsequent data analysis (Helsel, 2012; McCall et al., 2014;

239 Wangkahad et al., 2016). With regards to qPCR data, although qPCR is one of the most
240 widely used techniques to measure viral pathogens in water, lack of standardization for
241 preparation techniques (elution and concentration steps) as well as for the subsequent
242 molecular detection of pathogens is still problematic (Persing, 2004; USEPA, 2015). In
243 addition, the possibility of inhibition during the amplification steps is another issue to be
244 aware of, especially when dealing with wastewater, which typically contains a complex
245 cocktail of compounds (Hedman and Radstrom, 2013). These issues could, potentially,
246 adversely alter the qPCR results. Consequently, although the inclusion of censored data
247 within the dataset may increase the sample size and, consequently, result in more significant
248 statistical relationships, these results may not necessarily reflect reality. What's more, the
249 exclusion of non-detects from subsequent data analysis is likely if anything to result in an
250 overestimation of the mean concentrations of viral pathogens in the samples, which would be
251 a more conservative (cautious) approach, as it represents a 'worst-case' scenario. As such,
252 this approach could help to more effectively determine the suitability of bacteriophages as
253 surrogates of viral pathogens in WWTP.

254

255 **3. RESULTS AND DISCUSSION**

256 Table 1 presents detection rates and mean concentrations of the microorganisms studied,
257 whereas Table 2 presents the removal rates of the microorganisms studied at the primary
258 ($\pi_{prim}$), secondary ($\pi_{sec}$) and tertiary ($\pi_{tert}$) treatment steps of AS and TF systems. Overall
259 removal rates ($\pi_{overall}$), which are the total removal rates obtained from all three treatment
260 steps (primary, secondary and tertiary) combined, were also computed.

261

262 **Table 1 –** Detection rates and mean ± standard deviation (SD) for the concentrations of all
263 microorganisms monitored at each treatment step of both types of treatment system

264

**Table 2 –** Mean ± standard deviation (SD) of removal rates ($log_{10}$) of all microorganisms monitored at each treatment step (primary, secondary and tertiary) of both types of treatment system

### *3.1. Detection and quantitation of microorganisms*

FIB and SOMPH were detected in 100% of the samples tested, including secondary and tertiary (final) effluent samples of both AS and TF systems (Table 1). In contrast, whilst F-RNAPH and Bf124PH were consistently found in samples from TF systems, as well as in raw wastewater and primary effluent samples from the AS systems, the detection rate of both groups of phages was lower in SST and FE samples from AS systems.

High detection rates (> 80%) for FIB and SOMPH have been reported in the literature (Kay *et al.*, 2008; Carducci *et al.*, 2009; Wu *et al.*, 2011; De Luca *et al.*, 2013). Despite the high removal rates of FC (6.8 $log_{10}$) and SOMPH (5.3 $log_{10}$) in a MBR system in the UK, Purnell *et al.* (2015; 2016) reported the occasional presence of FIB and SOMPH in the MBR product. *Bacteroides* spp. phages, which were consistently detected in the raw wastewater of this study, have also consistently been detected in raw municipal wastewater in previous studies in the UK (Ebdon *et al.*, 2012; Purnell *et al.*, 2015; Purnell *et al*., 2016) and in Austria (Mayer *et al*., 2016). Purnell *et al.* (2015) detected both F-RNAPH and Bf124PH in all raw wastewater samples analysed, but did not detect them in MBR product. It is then perhaps not surprising that in this study, given that AS systems were shown to remove phages more effectively than TF systems, that the detection rates of F-RNAPH and Bf124 were lower in the AS effluents.

With regard to viral pathogens, the detection rate of HuAdV was considerably lower in all cases when compared with FIB and phages. In AS systems, the detection rate of HuAdV decreased gradually through the treatment process: 56.3% in RW, 55.3% in PST, 23.9% in SST and 8.7% in FE samples (Table 1). Conversely, in TF systems the detection rate of

HuAdV ranged between 46.8% and 55.3% in RW, PST and SST samples, and in FE samples the detection rate was slightly higher (72.9%) (Table 1). The detection rate of NoV G1 and NoV G2 was lower than 20% in all treatment steps of both AS and TF systems. As a consequence of the low detection rate, discussion of the results for both NoV G1 and NoV G2 is necessarily limited.

Other studies reported that HuAdV have been consistently (>80%) detected in both raw wastewater and final effluent (Aw and Gin, 2010; Kuo *et al.*, 2010; Wolf *et al.*, 2010; Hewitt *et al.*, 2011; Hewitt *et al.*, 2013; Mayer *et al*., 2016). However, Ebdon *et al.* (2012) reported a similar detection rate of HuAdV to this study in UK raw municipal wastewater (58%), and that the detection rate was observed to reduce through the treatment processes. The detection rate of NoV G1 and NoV G2 in raw wastewater varies considerably according to other studies: below 10% in Brazil (Victoria *et al.*, 2010), between 40 and 80% in New Zealand (Hewitt *et al.*, 2011), to above 80% also in New Zealand (Wolf *et al.*, 2010).

Recently, much effort has been expended on the development of molecular techniques to detect and quantify viral pathogens (Heim *et al.*, 2003; Choi and Jiang, 2005; Jothikumar *et al.*, 2005; Trujillo *et al.*, 2006; Le Guyader *et al.*, 2009; Wolf *et al.*, 2010; Sidhu *et al.*, 2012). Although availability and affordability of molecular methods (i.e., RT-qPCR) for the detection and enumeration of human enteric viruses have increased in recent years, it is important to state that molecular techniques present issues associated with levels of detection (sensitivity), infectivity of viruses, complexity, timeliness and costs of analytical methods. In addition, lack of standardisation for sample preparation (elution and concentration methods) and molecular techniques are also problematic (Persing, 2004; USEPA, 2015). Furthermore, RT-qPCR methods may also present inhibition of the amplification steps because of the presence of certain substances, especially when analysing wastewater samples (Hedman and Radstrom, 2013). Therefore, the issues associated with molecular methods may explain the variations observed in detection rates for viral pathogens in the present study, especially for NoV.

In both AS and TF systems, the concentration in RW samples of FC (6.6-6.7 $log_{10}$ cfu.100mL$^{-1}$) was significantly higher than the levels of ENT (5.8 $log_{10}$ cfu.100mL$^{-1}$) and SOMPH (5.9-6.1 $log_{10}$ pfu.100mL$^{-1}$), followed by HuAdV (4.4-4.5 $log_{10}$ copies.100mL$^{-1}$), and then Bf124PH and F-RNAPH (3.5-3.8 and 3.2-3.3 $log_{10}$ pfu.100mL$^{-1}$, respectively) (ANOVA on ranks; p-value < 0.0001). Mean levels of NoV G1 and G2 in RW samples ranged from 3.4 to 4.7 $log_{10}$ copies.100mL$^{-1}$.

Similar concentrations of FIB in municipal raw wastewater are reported in the literature related to studies performed in the UK (Kay *et al.*, 2008; Purnell *et al.*, 2015; 2016) and in Italy (Carducci *et al.*, 2009; De Luca *et al.*, 2013). In contrast, the levels of phages observed in RW samples in this study were about 1.0 $log_{10}$ lower than those reported by Purnell *et al.* (2015) elsewhere in the UK; Aw and Gin (2010) also reported greater concentrations of F-RNAPH in RW in a study performed in Singapore, and the levels found by De Luca *et al.* (2013) in Italy were considerably higher (8.5 $log_{10}$ pfu.100mL$^{-1}$). Similar concentrations of SOMPH and *Bacteroides* spp. phages in untreated wastewater to those reported here were observed by Aw and Gin (2010) in Singapore and Ebdon *et al.* (2007) in the UK, respectively. The concentrations of HuAdV observed here in raw wastewater were similar to those reported in studies performed in Singapore (Aw and Gin, 2010) and in New Zealand (Hewitt *et al.*, 2011), but were lower than the levels reported by other studies performed in other parts of the world: Italy (Carducci *et al.*, 2009); USA (Kuo *et al.*, 2010); Australia (Sidhu *et al.*, 2012); and New Zealand (Wolf *et al.*, 2010; Hewitt *et al.*, 2013). Similar concentrations of NoV G1 and G2 in untreated wastewater to those reported here are also reported in the literature: New Zealand (Hewitt *et al.*, 2011); and Ireland (Flannery *et al.*, 2012). From the results obtained from this study and the literature, it appears that SOMPH and FIB are the investigated indicator organisms detected at the highest concentrations in raw wastewater, at levels higher than those observed for HuAdV. In contrast, it has been reported that F-RNAPH and Bf124PH are detected in raw wastewater at concentrations lower than those of SOMPH and FIB, and relatively similar to those of HuAdV (Purnell *et al.*, 2016).

### *3.2. WWTP performance*

Although AS and TF treatment systems are not designed with the aim of removing pathogens, some reduction in the concentrations of viral pathogens and indicator organisms were observed through the systems. The results indicate that the AS system are significantly more effective than TF systems at removing FIB and phages (ranked t-test; p-value ≤ 0.005 for $\pi_{overall}$). In addition, in both AS and TF systems, the secondary (biological) treatment stage presented higher removal rates of microorganisms than the primary and tertiary treatment steps (ANOVA on ranks; p-value < 0.0001). This is highly likely to be the result of the contrasting underlying mechanisms that underpin the treatment systems: adsorption of particles onto the biofilm attached to the inert packing medium and subsequent predation by other microorganisms, such as bacteria, protozoa and rotifera in TF systems (Strauss, no date); whereas in AS systems, in addition to predation, particles become attached to the biological floc and consequently transfer to the sludge during settlement (Zhang and Farahbakhsh, 2007; Kuo *et al.*, 2010). Therefore, the removal of enteric microorganisms may be expected to be higher in AS systems, compared with TF systems.

In terms of tertiary treatment, settlement ponds (following TF systems) and sand filters (following AS systems) were shown to be equally effective at removing the microorganism monitored. In general, the tertiary treatment processes contributed to limited removal of viruses and bacteria, with recorded mean removal rates that were lower than 0.60 $\log_{10}$ (Table 1), which is considerably lower than that recorded for other tertiary treatment techniques commonly applied, such as chlorination.

AS systems appear to be capable of producing final effluents of significantly higher quality than TF systems (in terms of concentrations of enteric microorganisms), as can be seen in Table 1. In this study similar concentrations of HuAdV (Aw and Gin, 2010; Kuo *et al.*, 2010; Hewitt *et al.*, 2011), SOMPH (De Luca *et al.*, 2013) and F-RNAPH (Aw and Gin, 2010) in AS secondary effluents to those reported in the literature, whereas higher concentrations have been observed in the literature for FIB (Kay *et al.*, 2008; Flannery *et al.*, 2012; De Luca *et al.*,

2013) and F-RNAPH (Flannery *et al.*, 2012). Considerably lower concentrations of indicator organisms have been reported in MBR product (De Luca *et al.*, 2013; Purnell *et al.*, 2015). With regard to effluents of TF systems, similar levels of FIB and phages to those recorded in this study were reported by Kay *et al.* (2008) and Ebdon *et al.* (2012).

When primary and secondary treatment steps are considered together, similar removal rates for HuAdV are observed to those for the three groups of phages, which were considerably lower than those observed for FIB (Table 2). With regard to overall removal rates, both AS and TF systems removed FIB significantly more effectively than they removed phages, and the removal rates of HuAdV and the three groups of phages were statistically the same (ANOVA on ranks; p-value < 0.0001). An 'ideal indicator' should demonstrate similar survival characteristics to the pathogens in wastewater treatment processes (UKEA, 2002), and, in this study, the removal of phages appeared to indicate the removal of viral pathogens better than the removal of FIB.

### *3.3. Correlations between levels of microorganisms*

Spearman's rank correlation coefficients (rho) between the $\log_{10}$ concentrations of microorganisms were obtained at each treatment step of the AS and TF systems. Overall, in both AS and TF systems, it was observed that concentrations of indicator organisms (FIB and phages) were significantly correlated in raw and treated wastewater samples (p-values < 0.05), with moderate rho values (0.3 to 0.7). In terms of viral pathogens, the only significant correlation observed in AS systems was between the concentrations of HuAdV and Bf124PH in untreated wastewater (rho = 0.506, p-value = 0.007), whereas the levels of HuAdV did not correlate with the concentrations of any other microorganisms in any of the treatment steps of the TF systems.

In a studied performed in Singapore, Aw and Gin (2010) observed significant correlations between levels of SOMPH and HuAdV, and between levels of F-RNAPH and NoV G2 in raw

wastewater samples. However, in general, it has been reported in the literature that indicator organisms tend to correlate positively with each other in wastewaters, treated wastewaters and other aquatic matrices, but little or no correlation between concentrations of indicator organisms and viral pathogens has been reported in untreated and treated wastewater (Rose *et al.*, 2004; Ottoson *et al.*, 2006; Carducci *et al.*, 2009; Flannery *et al.*, 2012).. Despite the fact that no correlations between concentrations of enteric viruses and coliphages were observed by Rose *et al.* (2004) in the US, these authors suggest that the presence or absence of enteric viruses can be predicted by monitoring levels of SOMPH. In a study based on statistical analysis of papers published over a period of 40 years, Wu *et al.* (2011) suggested that total coliforms, coliphages and F-specific coliphages are among the commonly monitored enteric microorganisms that are more likely to correlate positively with pathogens. These authors also suggest that, although no single organism (or group of organisms) can indicate the presence of all pathogens in waters, over the longer term and if the dataset is large enough, FIB and other indicators (i.e., phages) can reliably predict the presence of pathogens.

### *3.4. Novel indicators*

Figure 1presents concentrations and cumulative removal rates of the FIB (FC and ENT datasets combined), phages (SOMPH, F-RNAPH and Bf124PH datasets combined) and viral pathogens (HuAdV dataset) at each treatment step of the AS (Figure 1.A) and TF (Figure 1.B) systems in order to compare the removal of the studied microorganisms through such systems. In AS systems, the recorded removal rates of phages and HAdv were very similar in the primary and secondary treatment steps, both being lower than the removal rates of FIB (Figure 1.A). In TF systems, the removal rates of FIB, phages and HuAdV in the primary treatment step were very similar to one another; removal rates of phages and HAdv were very similar in the secondary treatment step, both being lower than the removal rates of FIB

(Figure 1.B). In the tertiary treatment step of both AS and TF systems, the recorded removal of FIB was higher than that of phages, followed by HAdv (Figure 1).

**Figure 1 –** Concentrations and cumulative removal rates of FIB, phages and viral pathogens at each treatment step of AS (A) and TF (B) systems.

RW= raw wastewater; PST = primary effluent samples; SST = secondary effluent samples; FE = final effluent; Rem =removal.

Figure 2 presents concentrations and cumulative removal rates of SOMPH, F-RNAPH, Bf124PH and HuAdV at each treatment step of the AS (Figure 2.A) and TF (Figure 2.B) systems. The removal rates of the three groups of phages and HuAdV within primary treatment steps were similar to one another in AS systems; in the secondary treatment step, phages demonstrated removal rates 1.0 $\log_{10}$ greater than HuAdV; HuAdV, SOMPH and F-RNAPH demonstrated similar overall removal rates (Figure 2). Levels of SOMPH, F-RNAPH and Bf124PH respectively most closely predicted the removal of HuAdV in primary, secondary and tertiary treatment steps of TF systems (Figure 2).

**Figure 2 –** Concentrations and cumulative removal rates of SOMPH, F-RNAPH, Bf124PH and HuAdV at each treatment step of AS (A) and TF (B) systems.

SOMPH = somatic coliphages; F-RNAPH = F-RNA coliphages; Bf124PH = *B. fragilis* phages; HuAdV = Human Adenovirus Types F & G; RW= raw wastewater; PST = primary effluent samples; SST = secondary effluent samples; FE = final effluent; Rem = removal

In conclusion, FIB were more effectively removed than phages and viral pathogens in the treatment systems studied, and, since HuAdV and phages were removed at similar rates, it appears that phages may better indicate the removal of human viral pathogens in wastewater treatment processes than FIB. In addition, SOMPH were consistently found in raw and treated wastewater, whilst F-RNAPH and Bf124PH were not detected in several

treated effluent samples collected from AS systems. Furthermore, SOMPH were recorded at higher levels, both in comparison with the other phage groups and HuAdV in all treatment steps of the WWTP. Therefore, the results suggest that, of the groups of indicator organisms that are widely used (and, more specifically, of the phage groups currently used), SOMPH appears to be the parameter that best indicates the removal of viral pathogens in AS and TF systems. It is important to stress, however, that no significant correlations were observed between any of the levels and $\log_{10}$ removal rates of viral pathogens and indicator organisms.

## 4. CONCLUSIONS

The principal conclusions and outputs of this study are as follows:

- AS systems were shown to be more effective than TF at removing viral pathogens, traditional FIB and phages.
- In both AS and TF systems, FIB were shown to be more readily removed than phages and viral pathogens. In addition, removal rates of phages were shown to be similar to those of HuAdV.
- It was observed that, whilst indicator organisms correlated positively with one another, they did not appear to correlate with the presence of viral pathogens.
- The results suggest that phages are more useful for indicating the removal of viral pathogens in AS and TF systems than FIB.

## ACKNOWLEDGEMENTS

The authors would like to thank the Brazilian National Council for Scientific and Technological Development (CNPq) for funding the PhD studies of Edgard Dias, and

475 Southern Water Services Ltd for its cooperation and support in ensuring access to the
476 municipal WWTP.

477

ACCEPTED MANUSCRIPT

## REFERENCES

ARRAJ, A., BOHATIER, J., LAVERAN, H. & TRAORE, O., 2005. Comparison of bacteriophage and enteric virus removal in pilot scale activated sludge plants. Journal of Applied Microbiology, 98 (2), 516-524.

ATMAR, R. L., 2010. Noroviruses: State of the Art. Food and Environmental Virology, 2 (3), 117-126.

AW, T. G. & GIN, K. Y. H., 2010. Environmental surveillance and molecular characterization of human enteric viruses in tropical urban wastewaters. Journal of Applied Microbiology, 109 (2), 716-730.

BAGGI, F., DEMARTA, A. & PEDUZZI, R., 2001. Persistence of viral pathogens and bacteriophages during sewage treatment: lack of correlation with indicator bacteria. Research in Microbiology, 152 (8), 743-751.

BOSCH, A., 1998. Human enteric viruses in the water environment: a minireview. International microbiology: the official journal of the Spanish Society for Microbiology, 1 (3), 191-196.

BSI - British Standard Institute, 2000. BS EN ISO 78999-2: Water quality - Detection and enumeration of intestinal enterococci - Part 2: Membrane filtration method. London: BSI.

BSI - British Standard Institute, 2001. BS EN ISO 10705-2: Water quality - Detection and enumeration of bacteriophages - Part 2: Enumeration of somatic coliphages. London: BSI.

BSI - British Standard Institute, 2002. BS EN ISO 10705-1: Water quality - Detection and enumeration of bacteriophages - Part 1: Enumeration of F-specific RNA bacteriophages. London: BSI.

BSI - British Standard Institute, 2003. BS ISO 10705-4: Water quality - Detection and enumeration of bacteriophages - Part 4: Enumeration of bacteriophages infecting Bacteriodes fragilis. London: BSI.

BSI - British Standard Institute, 2009. BS EN ISO 9308-1: Water quality - Detection and enumeration of Escherichia coli and coliform bacteria - Part 1: Membrane filtration method. London: BSI.

CARDUCCI, A., BATTISTINI, R., ROVINI, E. & VERANI, M., 2009. Viral Removal by Wastewater Treatment: Monitoring of Indicators and Pathogens. Food and Environmental Virology, 1 (2), 85-91.

CHO, H. B., LEE, S. H., CHO, J. C. & KIM, S. J., 2000. Detection of adenoviruses and enteroviruses in tap water and river water by reverse transcription multiplex PCR. Canadian Journal of Microbiology, 46 (5), 417-424.

DE LUCA, G., SACCHETTI, R., LEONI, E. & ZANETTI, F., 2013. Removal of indicator bacteriophages from municipal wastewater by a full-scale membrane bioreactor and a conventional activated sludge process: Implications to water reuse. Bioresource Technology, 129 (0), 526-531.

DIAS, E. H. O., 2016. Bacteriophages as surrogates of viral pathogens in wastewater treatment processes. Unpublished thesis (PhD), University of Brighton.

DISTON, D., EBDON, J. E. & TAYLOR, H. D., 2012. The effect of UV-C radiation (254 nm) on candidate microbial source tracking phages infecting a human-specific strain of Bacteroides fragilis (GB-124). Journal of Water and Health, 10 (2), 262-270.

EBDON, J. E., SELLWOOD, J., SHORE, J. & TAYLOR, H. D., 2012. Phages of Bacteroides (GB-124): a novel tool for viral waterborne disease control? Environmental Science & Technology, 46 (2), 1163-1169.

EFTIM, S. E., HONG, T., SOLLER, J., BOEHM, A., WARREN, I., ICHIDA, A., NAPPIER, S. P., 2017. Occurrence of norovirus in raw sewage – A systematic literature review and meta-analysis. Water Research, 111, 366-374.ESPINOSA, A. C., ARIAS, C. F., SANCHEZ-COLON, S. & MAZARI-HIRIART, M., 2009. Comparative study of enteric viruses, coliphages and indicator bacteria for evaluating water quality in a tropical high-altitude system. Environmental Health, 8:49, 1-10.

FIELDS, B. N., KNIPE, D. M. & HOWLEY, P. M., 2007. Fields Virology, Philadelphia, Lippincott-Raven.

FLANNERY, J., KEAVENEY, S., RAJKO-NENOW, P., O'FLAHERTY, V. & DORE, W., 2012. Concentration of norovirus during wastewater treatment and its impact on oyster contamination. Applied and Environmental Microbiology, 78 (9), 3400-3406.

FUMIAN, T. M., LEITE, J. P., ROSE, T. L., PRADO, T. & MIAGOSTOVICH, M. P., 2011. One year environmental surveillance of rotavirus specie A (RVA) genotypes in circulation after the introduction of the Rotarix(R) vaccine in Rio de Janeiro, Brazil. Water Research, 45 (17), 5755-63.

GRABOW, W. O. K., 2001. Bacteriophages: Update on application as models for viruses in water. Water Sa, 27 (2), 251-268.

GRIFFITH, J. F., WEISBERG, S. B., ARNOLD, B. F., CAO, Y., SCHIFF, K. C., COLFORD JR, J, M., 2016. Epidemiologic evaluation of multiple alternate microbial water quality monitoring indicators at three California beaches. Water Research, 94, 371-381.

HARAMOTO, E., KATAYAMA, H., PHANUWAN, C. & OHGAKI, S., 2008. Quantitative detection of sapoviruses in wastewater and river water in Japan. Letters in Applied Microbiology, 46 (3), 408-413.

HEDMAN, J. & RADSTROM, P., 2013. Overcoming inhibition in real-time diagnostic PCR. Methods in Molecular Biology, 943, 17-48.

HEIM, A., EBNET, C., HARSTE, G. & PRING-AKERBLOM, P., 2003. Rapid and quantitative detection of human adenovirus DNA by real-time PCR. Journal of Medical Virology, 70 (2), 228-239.

HELSEL, D. R., 2012. *Statistics for censored environmental data using Minitab and R*, New Jersey, John Wiley & Sons.

HEWITT, J., GREENING, G. E., LEONARD, M. & LEWIS, G. D., 2013. Evaluation of human adenovirus and human polyomavirus as indicators of human sewage contamination in the aquatic environment. Water Research, 47 (17), 6750-6761.

HEWITT, J., LEONARD, M., GREENING, G. E. & LEWIS, G. D., 2011. Influence of wastewater treatment process and the population size on human virus profiles in wastewater. Water Research, 45 (18), 6267-6276.

JIANG, S. C., 2006. Human Adenoviruses in water: Occurrence and health implications: A critical review. Environmental Science & Technology, 40 (23), 7132-7140.

JOTHIKUMAR, N., CROMEANS, T. L., HILL, V. R., LU, X., SOBSEY, M. D. & ERDMAN, D. D., 2005. Quantitative real-time PCR assays for detection of human adenoviruses and identification of serotypes 40 and 41. Applied and Environmental Microbiology, 71 (6), 3131-6.

JURZIK, L., HAMZA, I. A., PUCHERT, W., UEBERLA, K. & WILHELM, M., 2010. Chemical and microbiological parameters as possible indicators for human enteric viruses in surface water. International Journal of Hygiene and Environmental Health, 213 (3), 210-216.

KATAYAMA, H., HARAMOTO, E., OGUMA, K., YAMASHITA, H., TAJIMA, A., NAKAJIMA, H. & OHGAKI, S., 2008. One-year monthly quantitative survey of noroviruses, enteroviruses, and adenoviruses in wastewater collected from six plants in Japan. Water research, 42 (6-7), 1441-1448.

KAY, D., CROWTHER, J., STAPLETON, C. M., WYER, M. D., FEWTRELL, L., EDWARDS, A., FRANCIS, C. A., MCDONALD, A. T., WATKINS, J. & WILKINSON, J., 2008. Faecal indicator organism concentrations in sewage and treated effluents. Water Research, 42 (1–2), 442-454.

KUO, D. H., SIMMONS, F. J., BLAIR, S., HART, E., ROSE, J. B. & XAGORARAKI, I., 2010. Assessment of human adenovirus removal in a full-scale membrane bioreactor treating municipal wastewater. Water Research, 44 (5), 1520-1530.

LE GUYADER, F. S., PARNAUDEAU, S., SCHAEFFER, J., BOSCH, A., LOISY, F., POMMEPUY, M. & ATMAR, R. L., 2009. Detection and quantification of noroviruses in shellfish. Applied and Environmental Microbiology, 75 (3), 618-624.

LIN, J. & GANESH, A., 2013. Water quality indicators: bacteria, coliphages, enteric viruses. International Journal of Environmental Health Research, 23 (6), 484-506.

LIU, J., WU, Q. & KOU, X., 2007. Development of a virus concentration method and its application for the detection of noroviruses in drinking water in China. Journal of Microbiology, 45 (1), 48-52.

MAYER, R.E., BOFILL-MAS, S., EGLE, L., REISCHER, G.H., SCHADE, M., FERNANDEZ-CASSI, X., FUCHS, W., MACH, R.L., LINDNER, G., KIRSCHNER, A., GAISBAUER, M., PIRINGER, H., BLASCHKE, A.P., GIRONES, R., ZESSNER, M., SOMMER, R. & FARNLEITNER, A.H., 2016. Occurrence of human-associated Bacteroidetes genetic source tracking markers in raw and treated wastewater of municipal and domestic origin and comparison to standard and alternative indicators of faecal pollution. Water Research, 90, 265-276

MCCALL, M. N., MCMURRAY, H. R., LAND, H., ALMUDEVAR, A., 2014. On non-detects in qPCR data. *Bioinformatics*, 30, 2310-2316.

McMINN, B. R., KORAJKIC, A. & ASHBOLT, N. J., 2014. Evaluation of Bacteroides fragilis GB-124 bacteriophages as novel human-associated faecal indicators in the United States. Letters in Applied Microbiology, (1):115-121.

MORENS, D. M., FOLKERS, G. K. & FAUCI, A. S., 2010. The challenge of emerging and re-emerging infectious diseases. Nature, 463 (7277), 122-122.

OFWAT/DEFRA - The Water Services Regulation Authority / Department for Environment Food and Rural Affairs, 2006. The development of the water industry in England and Wales. UK: Ofwat & Defra.

OTTOSON, J., HANSEN, A., BJORLENIUS, B., NORDER, H. & STENSTROM, T. A., 2006. Removal of viruses, parasitic protozoa and microbial indicators in conventional and membrane processes in a wastewater pilot plant. Water Research, 40 (7), 1449-1457.

PERSING, D. H., 2004. Molecular microbiology: diagnostic principles and practice, Washington DC, ASM Press.

PURNELL, S., EBDON, J., BUCK, A., TUPPER, M. & TAYLOR, H., 2015. Bacteriophage removal in a full-scale membrane bioreactor (MBR) - Implications for wastewater reuse. Water Research, 73, 109-117.

PURNELL, S., EBDON, J., BUCK, A., TUPPER, M. & TAYLOR, H., 2016. Removal of phages and viral pathogens in a full-scale MBR: Implications for wastewater reuse and potable water. Water Research, 100, 20-27.

ROSE, J. B., FARRAH, S. R., HARWOOD, V. J., LEVINE, A. D., LUKASIK, J., MENENDEZ, P. & SCOTT, T. M., 2004. Reduction of Pathogens, Indicator Bacteria, and Alternative Indicators by Wastewater Treatment and Reclamation Processes, IWA Publishing, Water Environment Research Foundation (WERF).

SIDHU, J. P., AHMED, W. & TOZE, S., 2012. Sensitive detection of human adenovirus from small volume of primary wastewater samples by quantitative PCR. Journal of Virological Methods, 187 (2), 395-400.

SIMA, L. C., SCHAEFFER, J., LE SAUX, J. C., PARNAUDEAU, S., ELIMELECH, M. & LE GUYADER, F. S., 2011. Calicivirus removal in a membrane bioreactor wastewater treatment plant. Applied and Environmental Microbiology, 77 (15), 5170-5177.

SINTON, L. W., HALL, C. H., LYNCH, P. A. & DAVIES-COLLEY, R. J., 2002. Sunlight Inactivation of Fecal Indicator Bacteria and Bacteriophages from Waste Stabilization Pond Effluent in Fresh and Saline Waters. Applied and Environmental Microbiology, 68 (3), 1122-1131.

STRAUSS, M., no date. Health (Pathogen) Considerations Regarding the Use of Human Waste in Aquaculture. Available at http://www.eawag.ch/forschung/sandec/publikationen/wra/dl/human_waste_use_health__pathogen__risks_in_aquaculture.pdf (Accessed: 28 September 2015).

SYMONDS, E. M. & BREITBART, M., 2014. Affordable Enteric Virus Detection Techniques Are Needed to Support Changing Paradigms in Water Quality Management. CLEAN - Soil, Air, Water, n/a-n/a.

TCHOBANOGLOUS, G., ABU-ORF, M., BURTON, F. L., BOWDEN, G., METCALF, EDDY, STENSEL, H. D., PFRANG, W. & EDUCATION, M.-H., 2014. Wastewater Engineering: Treatment and Resource Recovery, McGraw-Hill Education.

TRUJILLO, A. A., MCCAUSTLAND, K. A., ZHENG, D. P., HADLEY, L. A., VAUGHN, G., ADAMS, S. M., ANDO, T., GLASS, R. I. & MONROE, S. S., 2006. Use of TaqMan real-time reverse transcription-PCR for rapid detection, quantification, and typing of norovirus. Journal of Clinical Microbiology, 44 (4), 1405-1412.

UKEA - United Kingdom Environment Agency, 2002. The Microbiology of Drinking Water (2002) - Part 1 - Water Quality and Public Health. UK: UKEA.

UN-Water, 2016. *Water and Sanitation Interlinkages across the 2030 Agenda for Sustainable Development*. Geneva.

USEPA - United Kingdom Environment Agency, 2015. Review of coliphages as possible indicators of fecal contamination for ambient water quality. 820-R-15-098. Office of Water, Washington, DC

VAN DEN BERG, H., LODDER, W., VAN DER POEL, W., VENNEMA, H. & DE RODA HUSMAN, A. M., 2005. Genetic diversity of noroviruses in raw and treated sewage water. Research in Microbiology, 156 (4), 532-540.

VICTORIA, M., GUIMARAES, F. R., FUMIAN, T. M., FERREIRA, F. F., VIEIRA, C. B., SHUBO, T., LEITE, J. P. & MIAGOSTOVICH, M. P., 2010. One year monitoring of norovirus in a sewage treatment plant in Rio de Janeiro, Brazil. Journal of Water and Health, 8 (1), 158-165.

VIJAYAVEL, K., FUJIOKA, R., EBDON, J. & TAYLOR, H., 2010. Isolation and characterization of Bacteroides host strain HB-73 used to detect sewage specific phages in Hawaii. Water Research, 44 (12), 3714-3724.

WANGKAHAD, B., MONGKOLSUK, S., SIRIKANCHANA, K, 2016. Integrated multivariate analysis with nondetects for the development of human sewage source-tracking tools using bacteriophages of Enterococcus faecalis. *Environmental Science and Technology*, 51, 2235-2245

WHO - World Health Organization, 2011. Guidelines for Drinking Water Quality. 4th ed. Geneva: WHO.

WOLF, S., HEWITT, J. & GREENING, G. E., 2010. Viral multiplex quantitative PCR assays for tracking sources of fecal contamination. Applied and Environmental Microbiology, 76 (5), 1388-1394.

654 WU, J., LONG, S. C., DAS, D. & DORNER, S. M., 2011. Are microbial indicators and pathogens
655 correlated? A statistical analysis of 40 years of research. Journal of Water and Health, 9 (2), 265-
656 278.
657 XUE, B., JIN, M., YANG, D., GUO, X., CHEN, Z., SHEN, Z., WANG, X., QIU, Z., WANG, J., ZHANG,
658 B. & LI, J., 2013. Effects of chlorine and chlorine dioxide on human rotavirus infectivity and
659 genome stability. Water Research, 47 (10), 3329-3338.
660 ZANETTI, F., DE LUCA, G., SACCHETTI, R. & STAMPI, S., 2007. Disinfection efficiency of peracetic
661 acid (PAA): Inactivation of coliphages and bacterial indicators in a municipal wastewater plant.
662 Environmental Technology, 28 (11), 1265-1271.
663 ZHANG, K. & FARAHBAKHSH, K., 2007. Removal of native coliphages and coliform bacteria from
664 municipal wastewater by various wastewater treatment processes: implications to water reuse.
665 Water Research, 41 (12), 2816-2824.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

**Table 1 –** Detection rates and mean ± standard deviation (SD) for the concentrations of all microorganisms monitored at each treatment step of both types of treatment system.

| Org. | Sample | Activated sludge | | Trickling filter | |
|---|---|---|---|---|---|
| | | No. positives / No. samples (%) | Mean±SD | No. positives / No. samples (%) | Mean±SD |
| FC | RW | 48/48 (100%) | 6.63±0.64 | 48/48 (100%) | 6.70±0.55 |
| | PST | 47/47 (100%) | 6.31±0.57 | 48/48 (100%) | 6.52±0.45 |
| | SST | 48/48 (100%) | 4.10±0.58 | 46/46 (100%) | 5.04±0.29 |
| | FE | 22/22 (100%) | 3.17±0.60 | 45/45 (100%) | 4.43±0.52 |
| ENT | RW | 47/47 (100%) | 5.80±0.42 | 48/48 (100%) | 5.84±0.55 |
| | PST | 47/47 (100%) | 4.91±1.05 | 47/47 (100%) | 5.67±0.30 |
| | SST | 47/47 (100%) | 3.26±0.49 | 45/45 (100%) | 3.89±0.34 |
| | FE | 22/22 (100%) | 2.61±0.46 | 48/48 (100%) | 3.46±0.63 |
| SOMPH | RW | 48/48 (100%) | 5.94±0.52 | 48/48 (100%) | 6.05±0.51 |
| | PST | 46/46 (100%) | 5.65±0.50 | 46/46 (100%) | 5.94±0.45 |
| | SST | 47/47 (100%) | 3.85±0.39 | 48/48 (100%) | 5.42±0.47 |
| | FE | 22/22 (100%) | 3.45±0.42 | 48/48 (100%) | 5.20±0.45 |
| F-RNAPH | RW | 44/45 (97.8%) | 3.33±0.85 | 45/46 (97.8%) | 3.23±0.89 |
| | PST | 41/46 (89.1%) | 3.11±0.92 | 47/48 (97.9%) | 3.27±0.87 |
| | SST | 22/48 (45.8%) | 1.91±0.69 | 46/47 (97.9%) | 3.17±0.63 |
| | FE | 5/23 (21.7%) | 1.88±0.27 | 48/48 (100%) | 3.01±0.59 |
| Bf124PH | RW | 44/44 (100%) | 3.52±0.82 | 45/47 (95.7%) | 3.81±0.67 |
| | PST | 44/47 (93.6%) | 3.36±0.84 | 47/47 (100%) | 3.84±0.73 |
| | SST | 35/48 (72.9%) | 1.79±0.72 | 46/47 (97.9%) | 3.36±0.78 |
| | FE | 9/23 (39.1%) | 1.81±0.70 | 44/47 (93.6%) | 3.16±0.76 |
| HuAdV | RW | 27/48 (56.3%) | 4.52±0.85 | 22/47 (46.8%) | 4.42±1.32 |
| | PST | 26/47 (55.3%) | 4.39±0.72 | 24/47 (51.1%) | 4.43±0.70 |
| | SST | 11/46 (23.9%) | 3.01±0.91 | 26/47 (55.3%) | 3.97±0.78 |
| | FE | 2/23 (8.7%) | 2.34±0.73 | 35/48 (72.9%) | 4.09±0.96 |
| NoV G1 | RW | 5/45 (11.1%) | 3.37±1.42 | 5/44 (11.4%) | 3.41±1.50 |
| | PST | 1/46 (2.2%) | 2.03±* | 4/46 (8.7%) | 3.11±1.03 |
| | SST | 1/46 (2.2%) | 4.38±* | 5/48 (10.4%) | 1.44±0.72 |
| | FE | 4/22 (18.2%) | 4.60±2.45 | 5/46 (10.9%) | 1.84±0.61 |
| NoV G2 | RW | 6/46 (13.0%) | 3.51±2.16 | 10/47 (21.3%) | 4.72±1.71 |
| | PST | 8/47 (17.0%) | 3.65±1.75 | 5/48 (10.4%) | 5.20±1.69 |
| | SST | 4/47 (8.5%) | 4.54±1.08 | 8/46 (17.4%) | 2.64±1.25 |
| | FE | 1/46 (2.2%) | 5.87±* | 7/48 (14.6%) | 2.19±0.78 |

FC = faecal coliforms; ENT = intestinal enterococci; SOMPH = somatic coliphages; F-RNAPH = F-RNA coliphages; Bf124PH = *B. fragilis* phages; HuAdV = Human Adenovirus Types F & G; Nv G1 = noroviruses genogroup 1; NoV G2 = noroviruses genogroup 2; RW= raw wastewater; PST = primary effluent samples; SST = secondary effluent samples; FE = final effluent.

* Not calculated because of insufficient data.

ACCEPTED MANUSCRIPT

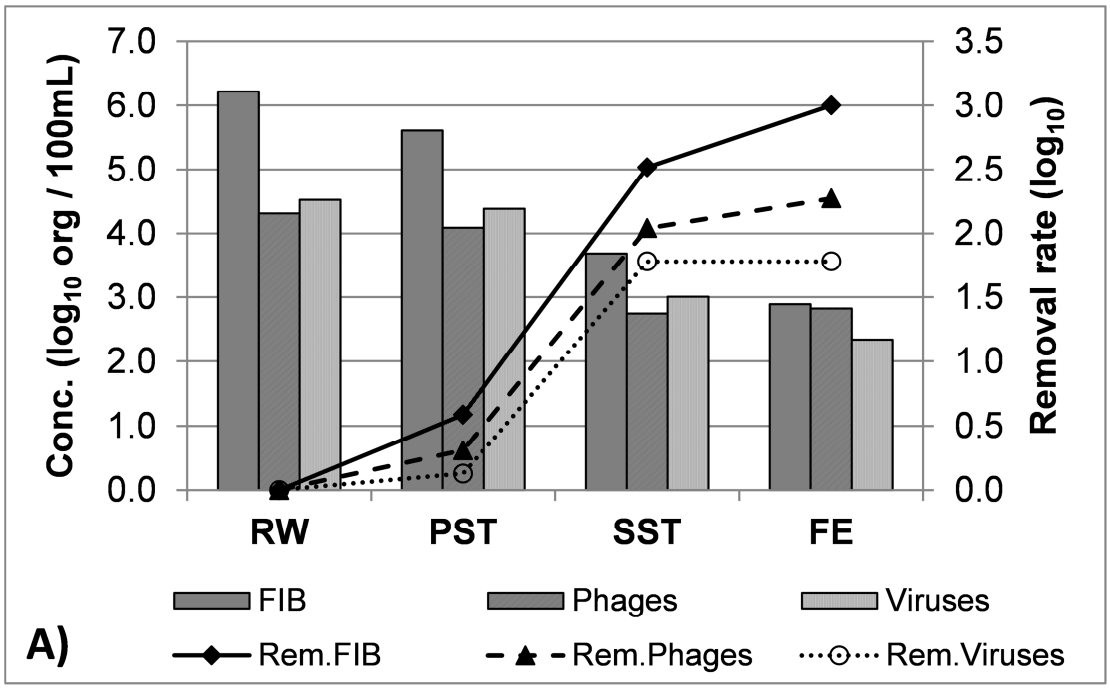
**Table 2 –** Mean ± standard deviation (SD) of removal rates ($log_{10}$) of all microorganisms monitored at each treatment step (primary, secondary and tertiary) of both types of treatment system.

| Org. | Treatment step | Activated sludge | Trickling filter |
|---|---|---|---|
| | | Mean±SD | Mean±SD |
| FC | $\pi_{prim}$ | 0.33±0.61 | 0.18±0.33 |
| | $\pi_{sec}$ | 2.21±0.68 | 1.47±0.36 |
| | $\pi_{tert}$ | 0.58±0.31 | 0.58±0.54 |
| | $\pi_{overall}$ | 3.59±0.69 | 2.26±0.87 |
| ENT | $\pi_{prim}$ | 0.85±0.92 | 0.16±0.55 |
| | $\pi_{sec}$ | 1.64±0.95 | 1.77±0.41 |
| | $\pi_{tert}$ | 0.39±0.43 | 0.42±0.49 |
| | $\pi_{overall}$ | 3.10±0.71 | 2.38±1.02 |
| SOMPH | $\pi_{prim}$ | 0.33±0.44 | 0.10±0.41 |
| | $\pi_{sec}$ | 1.77±0.46 | 0.54±0.35 |
| | $\pi_{tert}$ | 0.26±0.30 | 0.21±0.25 |
| | $\pi_{overall}$ | 2.42±0.69 | 0.84±0.42 |
| F-RNAPH | $\pi_{prim}$ | 0.33±0.57 | 0.01±0.45 |
| | $\pi_{sec}$ | 1.60±0.72 | 0.07±0.57 |
| | $\pi_{tert}$ | 0.39±0.44 | 0.16±0.36 |
| | $\pi_{overall}$ | 2.26±0.82 | 0.23±0.75 |
| Bf124PH | $\pi_{prim}$ | 0.27±0.63 | -0.09±0.53 |
| | $\pi_{sec}$ | 1.75±0.64 | 0.52±0.43 |
| | $\pi_{tert}$ | 0.08±0.35 | 0.19±0.37 |
| | $\pi_{overall}$ | 2.00±1.19 | 0.60±0.69 |
| HuAdV | $\pi_{prim}$ | 0.13±0.84 | 0.15±1.29 |
| | $\pi_{sec}$ | 1.65±0.92 | 0.37±0.62 |
| | $\pi_{tert}$ | * | -0.27±1.05 |
| | $\pi_{overall}$ | * | 0.17±1.07 |

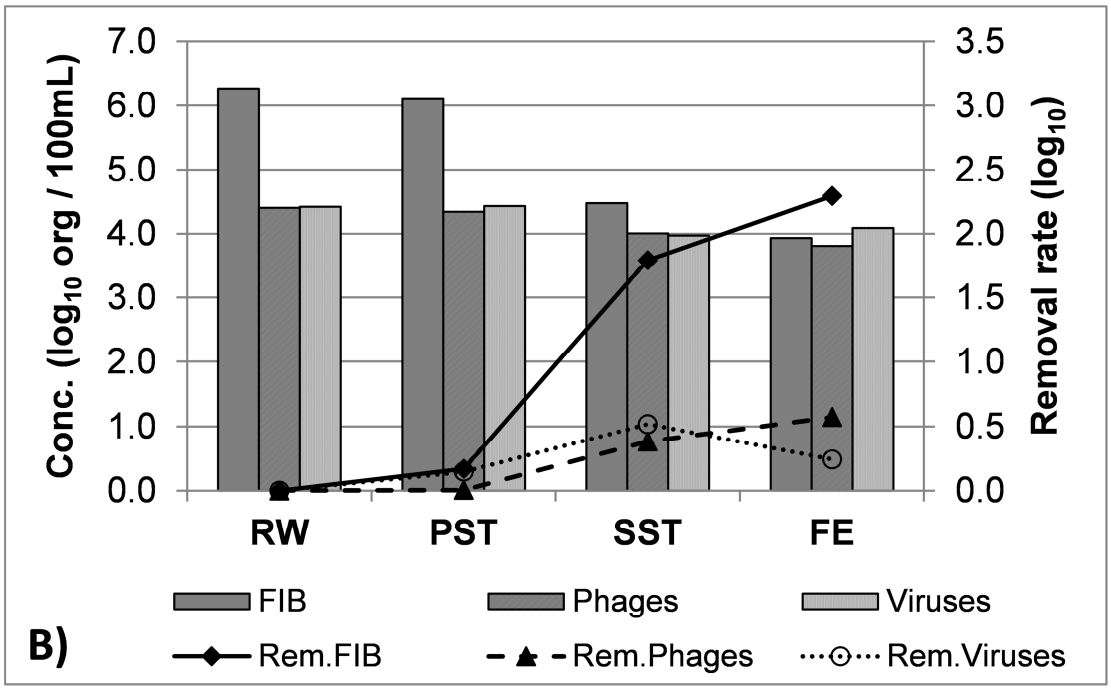
FC = faecal coliforms; ENT = intestinal enterococci; SOMPH = somatic coliphages; F RNAPH = F-RNA coliphages; Bf124PH = *B. fragilis* phages; HuAdV = Human Adenovirus Types F & G; $\pi_{prim}$ = efficacy of preliminary and primary treatment; $\pi_{sec}$ = efficacy of secondary treatment; $\pi_{tert}$ = efficacy of tertiary treatment; $\pi_{overall}$ = overall efficacy.

* Not calculated because of insufficient data.

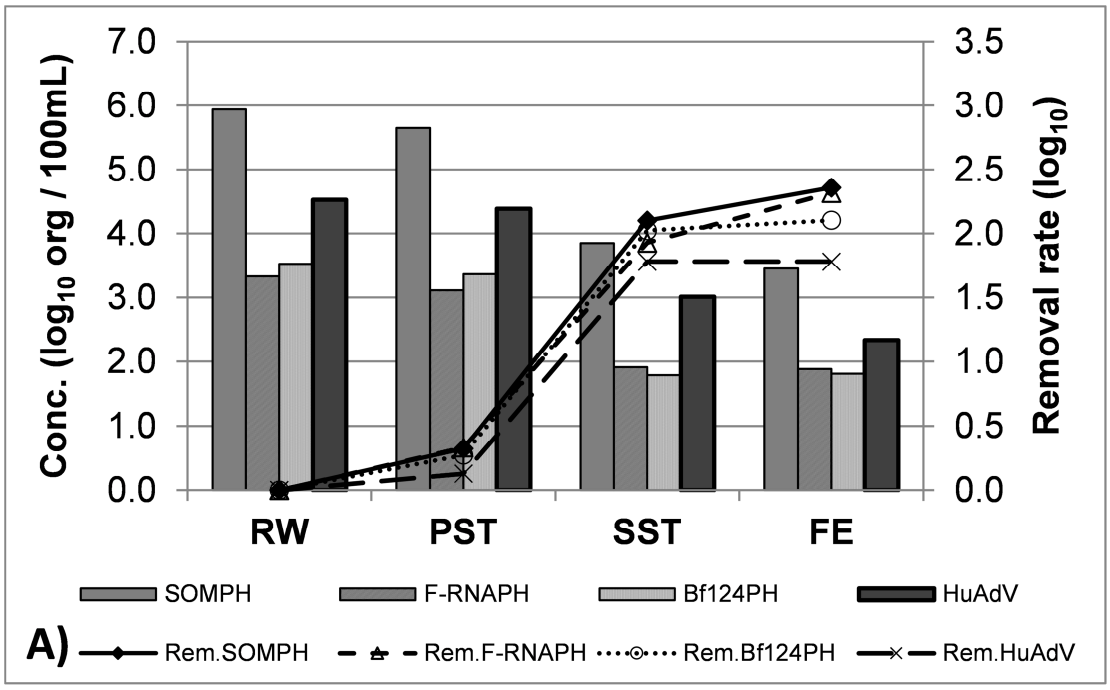
ACCEPTED MANUSCRIPT



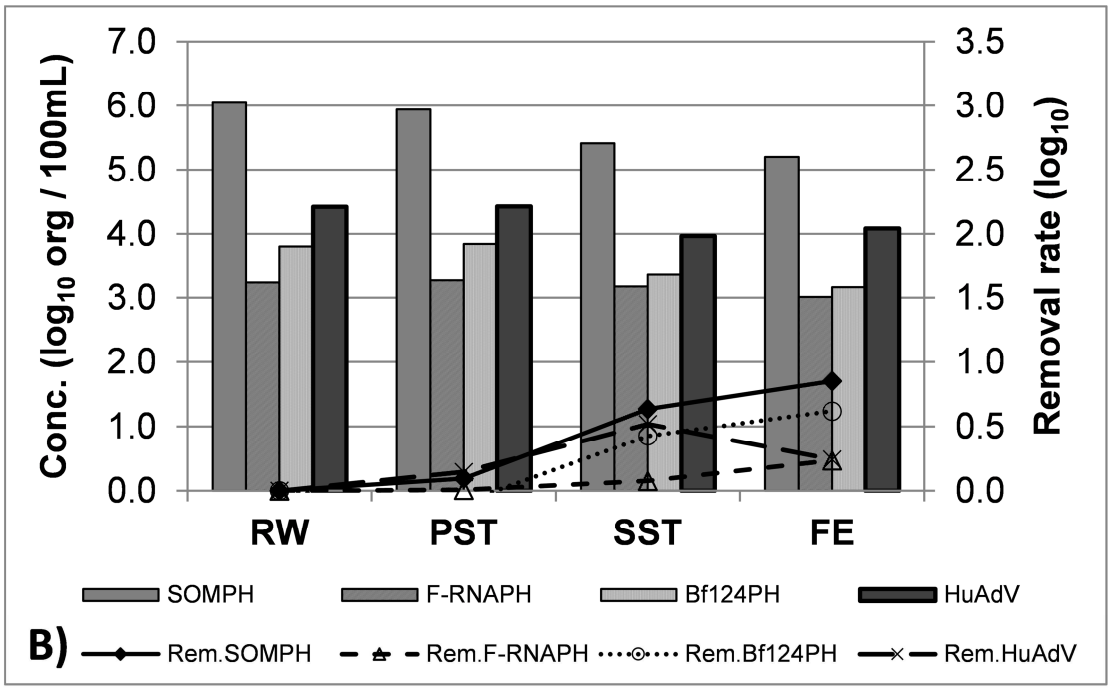

ACCEPTED MANUSCRIPT




ACCEPTED MANUSCRIPT




ACCEPTED MANUSCRIPT




ACCEPTED MANUSCRIPT

Department of Sanitary and Environmental Engineering (ESA)
Faculty of Engineering, Federal University of Juiz de Fora (UFJF)
Juiz de Fora, MG, Brazil
36.036-330

ACCEPTED MANUSCRIPT

February 25th, 2017

To the Editors of Water Research

**Submission of a new manuscript**

Please find attached our submission of a manuscript entitled: **Bacteriophages as surrogates of viral pathogens in wastewater treatment systems**. Authors: Dr. Edgard Dias; Dr. James Ebdon; and Prof. Huw Taylor.

Highlights:

- FIB were more readily removed than phages and viral pathogens in all WWTP monitored
- Removal rates of phages were shown to be similar to those of human adenovirus
- Phages likely to better indicate the removal of viral pathogens in WWTP than FIB
- Phages as surrogates of viral pathogens in WWTP may support safe wastewater reuse

We look forward to hearing from you.

Yours sincerely,

**Dr. Edgard Dias**
Dep. Sanitary and Environmental Engineering
Federal University of Juiz de Fora

ACCEPTED MANUSCRIPT