

1 **HUMAN-SPECIFIC PHAGES INFECTING *ENTEROCOCCUS* HOST STRAIN**
2 **MW47: ARE THEY RELIABLE MICROBIAL SOURCE TRACKING MARKERS?**

3

4 Purnell, S.E., Ebdon, J.E., Wilkins, H and Taylor, H.D.

5

6 Environment and Public Health Research Group, Centre for Aquatic Environments, School of
7 Environment and Technology, University of Brighton, Cockcroft Building, Lewes Road,
8 Brighton, BN2 4GJ, United Kingdom

9

10 *Corresponding author phone: +44 1273 641344; e-mail: S.E.Purnell@Brighton.ac.uk

11

12 Running head: Reliability of MW47 phages for MST

13

14 **Abstract**

15 **Aim:** The aim of this study was to determine the morphological diversity and environmental
16 survival of human-specific phages infecting *Enterococcus faecium* host strain MW47, to
17 support their use as microbial source tracking (MST) markers.

18 **Methods and Results:** Twenty phages capable of infecting strain MW47 were propagated
19 and their morphologies determined using transmission electron microscopy (TEM), which
20 revealed that a heterogeneous group of phages was able to infect strain MW47. Three distinct
21 morphologies from two different families (*Myoviridae* and *Siphoviridae*) were observed. *In*
22 *situ* inactivation experiments were subsequently conducted to determine their environmental
23 persistence.

24 **Conclusion:** The findings revealed a statistically significant link between morphology and
25 the rate of inactivation, with phages belonging to the *Myoviridae* family demonstrating more
26 rapid inactivation in comparison to those belonging to the *Siphoviridae* family.

27 **Significance and Impact of Study:** The results suggest that whilst *Enterococcus* MW47
28 phages appear to be a potentially valuable MST tools, significant variations in the persistence
29 of the different phages mean that the approach should be used with caution, as this may
30 adversely affect the reliability of the approach, especially when comparing MW47 phage
31 levels or presence across different matrices (e.g. levels in sediments or shellfish). This
32 highlights the importance of elucidating the ecological characteristics of newly proposed
33 MST markers before they are used in full-scale MST investigations.

34

35 **Keywords**

36 Bacteriophages, Viruses, Diversity, Markers, Microbial Source Tracking, Enterococcus,
37 TEM, Inactivation

38

39 **1. Introduction**

40 Faecal indicator organisms (FIO) are currently used to assess the hygienic quality of water
41 and are present in the faeces of both human and non-human animals. Their detection offers
42 no insight into the source(s) of environmental contamination, which would support a water
43 quality monitoring strategy and provide more effective preventive measures. Many human
44 waterborne pathogens are only found in human faeces, whereas important zoonotic disease
45 agents (e.g. *Cryptosporidium*) are associated with specific non-human faecal sources (Soller
46 *et al.*, 2010). Determining the source of faecal pollution in raw drinking water provides
47 valuable information on potential risks to human health. Microbial source tracking (MST)
48 techniques, which make use of microbial populations that are specific to particular faecal
49 sources, can support risk assessments by providing information on which pathogens are
50 present in samples.

51

52 The field of MST has advanced rapidly during the past twenty five years and the available
53 literature on possible techniques is extensive (Boehm *et al.*, 2013). Many researchers have
54 suggested that no single MST technique may be able to satisfy all requirements in all
55 situations and instead a ‘toolbox’ approach combining several methods is needed (Gourmelon
56 *et al.*, 2010). This is due to a number of factors, such as differences in size, morphology,
57 environmental resistance, shedding rate, inactivation rate and geographical distribution of
58 different proposed markers and pathogens (Diston *et al.*, 2012; Duran *et al.*, 2003; Ebdon *et al.*,
59 *et al.*, 2012). Therefore, there remains a need for a greater understanding of the ecological
60 characteristics of candidate MST markers (Vogel *et al.*, 2007; Plummer *et al.*, 2009;
61 Gourmelon *et al.*, 2010). For instance, studies have shown that enteric viruses survive longer
62 in the environment than traditional bacterial faecal indicators, such as *Escherichia coli*
63 (Nasser and Oman, 1999; Moce-Llivina *et al.*, 2005). Bacteriophages (phages) have been
64 proposed as alternative indicators that may better predict risks to human health associated
65 with enteric viruses. The detection and enumeration of phages such as those infecting
66 *Bacteroides* spp. and the subgroups of F-specific RNA coliphages have been successfully
67 used to discriminate human from non-human faecal contamination of the environment (Jofre
68 *et al.*, 1986; Gourmelon *et al.*, 2010; Ebdon *et al.*, 2012). Ratios of somatic coliphages (non-
69 specific indicators of faecal contamination) to phages of *Bacteroides thetaiotaomicron* GA17
70 (human-specific indicators of faecal contamination) have also been shown to provide a
71 method for discriminating human and non-human samples (Muniesa *et al.*, 2012). Another
72 group of phages which have received growing attention in recent years are those of
73 *Enterococcus* spp. (Bonilla *et al.*, 2010; Santiago-Rodríguez *et al.*, 2010; Purnell *et al.*, 2011;
74 Vijayavel *et al.*, 2014; Verçosa and Moreira, 2016; Wangkahad *et al.*, 2017). Initial
75 investigations into the use of these phages have shown them to be promising MST
76 candidates, with some displaying high specificity to particular human and non-human faecal

77 sources (Purnell *et al.*, 2011; Santiago-Rodriguez *et al.*, 2010; Santiago-Rodriguez *et al.*,
78 2013).

79

80 A potential limitation of certain phage-based MST techniques, particularly those which
81 involve the detection of a wide range of phages (such as somatic coliphages) can be the
82 differential survival rates of the various phages that are capable of infecting the host strain.
83 Phages exhibiting different morphologies have been shown also to differ with respect to their
84 survival rates (and hence abundance) in the non-gut environment, which can hinder the
85 interpretation of MST (Muniesa *et al.*, 1999). Previous studies have also shown that the
86 predominant phages observed in wastewaters and other waterbodies recently contaminated by
87 faeces may differ markedly from those observed in waterbodies exposed to environmental
88 factors such as UV light, desiccation, and elevated temperatures and those that have
89 undergone drinking water treatment processes (Dee and Fogleman, 1992; Lasobras *et al.*,
90 1997; Muniesa *et al.*, 1999). In order to provide consistent source tracking information
91 throughout the water cycle, candidate MST markers must demonstrate consistent responses to
92 environmental stressors (Diston *et al.*, 2012), which are more likely among homogenous
93 groups of phages that demonstrate consistent ecological behaviour and environmental
94 survival characteristics (Queralt *et al.*, 2003). For this reason it is important to assess the
95 morphological homogeneity of phages infecting host strains prior to their widespread uses as
96 MST tools.

97

98 *Enterococcus* phages have been examined by transmission electron microscopy (TEM).
99 According to Ackerman (2007), the majority of these have been demonstrated to belong to
100 tailed phage families, predominantly the family *Siphoviridae*. These phages have shown
101 varying host ranges. Whilst some phages only appear to be able to infect their original hosts,

102 others appear to be capable of infecting all *Enterococcus* species (Paisano *et al.*, 2004;
103 Ramírez *et al.*, 2006; Son *et al.*, 2010; Vinodkumar *et al.*, 2011). Although the majority of
104 *Enterococcus* phages observed are members of the *Siphoviridae* family, authors have also
105 reported tail-less phages that are capable of infecting such strains (Bachrach *et al.*, 2003; Fard
106 *et al.*, 2010). Santiago-Rodríguez *et al.* (2010) proposed phages infecting an *Enterococcus*
107 *faecalis* host strain as potential surrogates of enteric viruses in studies of recreational waters
108 as they were more resistant to primary and tertiary wastewater treatments and their survival
109 abilities in fresh and marine waters were comparable to somatic coliphages. The data
110 indicated that various phage populations were able to infect the *E. faecalis* host strain. Initial
111 *in vitro* survival experiments also demonstrated that the phages shared similar survival
112 characteristics (Santiago-Rodriquez *et al.*, 2010; 2013). The authors observed the presence of
113 both non-tailed and tailed phages, though their findings suggested that the majority of these
114 phages belonged to the *Siphoviridae* family.

115

116 Purnell *et al.* (2011) isolated an *Enterococcus faecium* host strain (MW47) that demonstrated
117 high specificity to human faecal sources. Phages of MW47 were detected in both raw and
118 treated wastewaters and have not to date been detected in non-human faeces. Whilst the
119 specificity of these phages indicates their strong potential as MST markers, the degree of
120 morphological diversity and their survival characteristics have not been explored. This study
121 elucidates ecological characteristics of these phages that are essential to assessing suitabilities
122 as MST tools. The study described here presents new knowledge of the morphological
123 diversity and survival characteristics of human-specific phages capable of infecting *E.*
124 *faecium* host strain MW47.

125

126 **2. Materials and Methods**

127 **2.1 Phage enumeration**

128 Phages infecting *E. faecium* (MW47) were enumerated using previously described double
129 agar-layer and spot test plaque assay methods (Adams, 1959; Jofre *et al.*, 1986; Purnell *et al.*,
130 2011). Tryptone soya broth (TSB) (Oxoid, Fisher Scientific, UK) was used as the growth
131 medium for host strain MW47. Tryptone soya agar (TSA) was used as the solid medium for
132 all double agar-layer and spot test plaque assays. The concentrations of agar in top and
133 bottom layers used were the same as those reported elsewhere (ISO 10705/2) (Anon, 2001).
134 Briefly, when performing double agar-layer assays, 1 ml of each sample was added to 1 ml of
135 exponentially growing host strain, and 2.5 ml of semi-solid agar (TSAss). The resulting
136 suspension was mixed briefly using a Whirlimixer™ (Fisher Scientific, UK) and poured onto
137 previously prepared TSA (Oxoid, Fisher Scientific, UK) in 90 mm diameter Petri-plates.
138 Once the top layer had solidified, plates were inverted and incubated at 37 °C (± 2 °C) for 18-
139 24 h. To perform spot test assays, double agar-layer plates were prepared as described above,
140 with 1 ml of exponentially growing host strain and 2.5 ml of TSAss without addition of
141 sample and were poured onto previously prepared 90 mm TSA agar-layer Petri-plates. Once
142 solidified, 10 μ l of phage lysates and dilutions thereof (1:10) were spotted onto the top agar,
143 being careful to label each spot, so that they could be identified following incubation. The
144 drops were left to air dry before plates were inverted and incubated at 37 °C (± 2 °C) for 18-24
145 h. Following incubation, circular ‘zones of lysis’ in the confluent lawn were expressed as
146 Plaque Forming Units (PFU) per ml of sample.

147

148 **2.2 Phage isolation, purification and concentration**

149 Samples (n=5) of untreated municipal wastewater were collected for isolation of phages
150 capable of infecting *E. faecium* MW47 from the influent of a biological wastewater treatment
151 works (WWTW) situated in South East England, UK (population equivalent 37,327). Well-

152 distributed plaques enumerated by the double agar-layer method were picked at random for
153 isolation to avoid plaque morphology bias with the intention of characterising twenty phages
154 from the host bacterium MW47. Phages were numbered MW47- 1 to MW47- 20 for all
155 subsequent analyses.

156

157 All phages were purified and concentrated by a plate propagation technique that was
158 modified from previously described methods used by Carey-Smith *et al.* (2006) and Fard *et*
159 *al.* (2010). In brief, cores of agar containing plaques were picked using sterile glass Pasteur
160 pipettes and suspended in 200 µl of phage buffer (19.5 mmol Na₂HPO₄, 22 mmol KH₂PO₄,
161 85.5 mmol NaCl, 1 mmol MgSO₄, 0.1 mmol CaCl₂) in microcentrifuge tubes (Fisher
162 Scientific, UK). The phage suspensions were incubated at 4°C overnight to allow diffusion of
163 phage into the buffer. The phage suspensions and dilutions were retested with the double
164 agar-layer method to purify and confirm the presence of phage. This was repeated three times
165 to obtain purified phages. Once purified, 5 ml of phage buffer solution was added to plates
166 with near complete lysis of the host bacterium and left at room temperature for 1 h. The
167 plates were swirled regularly. The liquid and top agar-layer were then scraped into 50 ml
168 centrifuge tubes (Fisher Scientific, UK), mixed briefly using a Whirlimixer™, and left at
169 room temperature for an additional 30 min. Bacterial debris and top agar-layer were removed
170 from the suspension by centrifugation at 3000 g for 20 min. The supernatant was then filtered
171 through a 0.22 µm polyvinylidene difluoride membrane syringe filter unit and stored in light
172 tight glass bottle at 4 °C in the dark (for not longer than seven days). The titre of the
173 suspension was determined by testing ten-fold dilutions (10⁻¹ to 10⁻⁸) using the spot test plaque
174 assay. The process was repeated until a minimum titre of 10⁸ PFU ml⁻¹ was achieved for all
175 phage suspensions. Plaque diameters were measured using a Vernier Caliper (Fisher
176 Scientific, UK).

177

178 **2.3 Transmission electron microscopy (TEM)**

179 All 20 propagated phage were examined by TEM to determine the phage morphologies. In
180 order to view phage under the TEM, the phage suspensions were negatively stained. Uranyl
181 acetate (UA) stain (pH 4-4.5) was used to stain the phage suspensions. One drop (10 µl) of
182 previously prepared high-titre phage suspension was applied to a 200 mesh Formvar/Carbon
183 copper electron microscope grid (Agar Scientific, UK). After 2 min, excess suspension was
184 removed with Whatman No.1 filter paper (Whatman, UK). One drop (10 µl) of UA stain (1
185 % w/v, previously filtered through a 0.22 µm filter unit) was then applied to the grid for 1
186 min. Excess stain was removed again using a new Whatman No. 1 filter paper, and the grid
187 was then left to dry. The grid was kept in a labelled Petri-plate (55 mm) prior to viewing
188 under the TEM (Hitachi-7100) at 100 kV.

189

190 **2.4 Assessment of host range**

191 The phages isolated from *E. faecium* strain MW47 were tested for their abilities to infect
192 other species of the genus *Enterococcus* (including *E. asini*, *E. casseliflavus*, *E. durans*, *E.*
193 *faecalis*, *E. gallinarum*, *E. hirae*, *E. mundti*, *E. pseudoavium*, *E. saccharolyticus*, and *E.*
194 *sulfureus*), and their abilities to infect two other bacterial host strains from genetically
195 different genera, GB-124 (*Bacteroides*) and WG5 (*E. coli*). Host range provides further
196 information on phage diversity and was determined by spot tests of dilutions (10^{-1} to 10^{-8}) of
197 each high titre phage stock suspension on a lawn of each host bacterium. Spot tests were
198 performed in triplicate and reported as mean PFU ml⁻¹.

199

200 **2.5 *In situ* inactivation investigation**

201 River water samples (100ml fresh-water) were collected from the Bevern Stream (a tributary
202 of the River Ouse, UK). The samples were immediately analysed for temperature, pH level,
203 conductivity, salinity, dissolved oxygen, and turbidity using a handheld multi-parameter
204 probe (Aquaread Ltd, UK). *In situ* inactivation experiments were then set up by spiking
205 surface water samples with isolated phages (10^5 PFU ml⁻¹) and placing them into individual
206 dialysis tubes (cut-off 14 kDa), which were then sealed and positioned in the stream at a
207 depth of 15-20 cm below the surface. The site was chosen as being representative of streams
208 found in the catchment in terms of flow characteristics, land use, and underlying geology.
209 The *in situ* inactivation experiments were performed in duplicate using phages belonging to
210 the three distinct morphologies isolated. Phages were enumerated in duplicate, immediately
211 after spiking (T₀), and after one (T₂₄), two (T₄₈), five (T₁₂₀), eight (T₁₉₂) and nine (T₂₁₆) days.
212 Results were reported as mean PFU per ml of sample.

213

214 **2.6 Statistical analysis**

215 Statistical tests were performed using the statistical package IBM SPSS Statistics 20.0, with
216 the significance level set at 5%. Inactivation is presented as the decrease in Log₁₀ units (Log
217 N₀ / N_t) of phage numbers of each phage type (MW47-1, -2, -5, -6, -10, -15) before (N₀) and
218 after (N_t) inactivation processes. Presenting the data in this way allows for comparison with
219 similar studies of inactivation of bacteriophages used in MST. Anderson-Darling normality
220 tests were performed and indicated that data were not-normally distributed. Therefore, non-
221 parametric tests were selected to determine if there were significant differences between the
222 number of phages detected (Wilcoxon signed-rank test), inactivation of phages from different
223 families (Mann-Whitney test) and inactivation of phages with different capsid morphologies
224 (Mann-Whitney test). The P-value and the test conducted are presented in the text where
225 appropriate.

226

227 **3. Results**

228 **3.1 Phage Isolation**

229 In total, 20 single distinct plaques were successfully picked and purified from double agar-
230 layers of host strain MW47 infected with phages from raw (untreated) municipal wastewater
231 obtained from the influent of a wastewater treatment works. All plaques were successfully
232 propagated to a high titre (at least 10^8 PFU ml⁻¹) in accordance with the plate propagation
233 method described in section 2.2. To determine the resulting titre, phage lysates were tested in
234 duplicate using spot test plaque assays. The final twenty phage cultures demonstrated titres of
235 between 5.0×10^8 and 1.1×10^{10} PFU ml⁻¹. During phage isolation and propagation, it was
236 evident that different phages produced plaques of varying size. For example, phages MW47-
237 4, -9, -10, -15 and -19 produced particularly small ‘pin-hole’ sized plaques (<0.2 mm
238 diameter). Although these plaques were small, they were still clear and readable. MW47-15
239 produced the smallest plaques, with a mean diameter of 0.18 mm ($n=10$) and MW47-7
240 produced the largest plaques with a mean diameter of 1.74 mm ($n=10$). A number of the
241 phage isolates producing larger plaques (>1 mm diameter) also exhibited a halo formation
242 around the plaque.

243

244 **3.2 Phage morphology**

245 In order to explore the degree of morphological diversity of the isolated phages, all 20 high-
246 titre phage cultures were viewed using TEM. TEM phage micrographs of the three distinct
247 phage morphologies observed are presented in Figure 1.

248

249 The TEM data revealed all phages have helical tails and thus belong to the order
250 *Caudovirales*. Of the 20 phages viewed by TEM, 18 (90%) had simple non-contractile tails,

251 placing them in the *Siphoviridae* family (Table 1). The remaining two phages (10%), MW47-
252 10 and -15, exhibited tails with a contractile sheath. This tail structure identified these phages
253 as belonging to the *Myoviridae* family. Within the 18 recognised *Siphoviridae* phages, two
254 distinctive capsid morphologies were apparent. Twelve of the phage possessed icosahedral
255 capsids, whereas six possessed elongated icosahedral capsids. Both recognised *Myoviridae*
256 phages possessed icosahedral capsids that appeared to be much larger than the capsids
257 observed for *Siphoviridae* phages.

258

259 **3.3 Assessment of host range**

260 Assessment of host range is an important additional investigation because for these phages to
261 be useful for MST they should be highly specific to their bacterial host (origin). The host
262 ranges of MW47 phages were determined using 11 *Enterococcus* type strains (Table 2), an *E.*
263 *coli* host strain WG5 and a *Bacteroides* host strain GB124. As expected, no phages were
264 shown to be capable of infecting either the *E.coli* WG5 or the human-specific *Bacteroides*
265 GB124. Fifteen of the phage isolates were unable to infect any other *Enterococcus* host type
266 strains (Table 2). Interestingly, no phages of *E. faecium* MW47 were able to infect *E. faecium*
267 type strain DSM 20477. These results provide further evidence that these phages have very
268 narrow host ranges.

269

270 Phages MW47-9, -10, and -20 displayed broader host ranges (Table 2). Phages MW47-9 and
271 -10 were also capable of infecting the *E. faecalis* type strain DSM 20478, and MW47-20 was
272 able to infect *E. asini* DSM 11492, but the phage numbers detected were low (1.0×10^3 PFU
273 ml^{-1}). Phages MW47-8, -15 and -20 displayed the broadest host ranges and were able to
274 infect type strains *E. faecalis* and *E. asini*. Although some phages exhibited broader host
275 ranges, the number of phages detected on the alternative *Enterococcus* host strains was

276 significantly lower than that recorded on host strain MW47 (Wilcoxon signed ranks test,
277 $P < 0.05$). Phages MW47-8, -9, -10, -15, and -20, represent a morphologically diverse group
278 that includes phage from the families *Siphoviridae* and *Myoviridae*, which display both
279 icosahedral and elongated icosahedral capsids. These results indicate variation in host ranges
280 that may be linked to the diversity of phage morphology. *Enterococcus faecalis* is commonly
281 found in human faeces and in raw wastewater. However, *E. asini* is reported to be found
282 exclusively in donkey faeces (de Vaux *et al.*, 1998). In southern England, *E. asini* is less
283 likely to be encountered in inland surface waters. Therefore, it is probable that the primary
284 host of the phages MW47-8, -15 and -20 is *E. faecalis*.

285

286 **3.4 Phage survival**

287 The results demonstrated that phages isolated from MW47 have diverse morphologies.
288 Previous investigations have shown that the inactivation characteristics of phages appear to
289 vary with regard to phage morphology (Muniesa *et al.*, 1999). Although it was not practically
290 feasible to perform natural *in situ* survival experiments on all 20 phages isolated, an
291 investigation into natural survival was successfully conducted on a representative subset of
292 six phages that showed the greatest morphological diversity. The aim of this investigation
293 was to determine whether these phages exhibit varying inactivation rates in surface water as
294 this could impact the future use of host strain MW47 as an MST marker. Four phages
295 belonging to the *Siphoviridae* family - two with icosahedral capsids and two with elongated
296 icosahedral capsids (MW47-1, -2, -5 and -6)- were selected for investigation, alongside both
297 isolated phages of the *Myoviridae* family (MW47-10 and -15). A summary of the observed
298 physico-chemical composition of the Bevern Stream used in these experiments is presented in
299 Table 3. The results of the inactivation experiments for phages MW47-1, -2, -5, -6, -10 and -
300 15 are presented in Figure 2, which demonstrates that limited inactivation of the six phages

301 was observed during the first three days of the experiment. From day three onwards, the
302 *Myoviridae* phage MW47-10 was inactivated to the greatest degree (3.4 log), whereas all
303 other phages (MW47-1,-2,-5,-6 and -15) demonstrated between 1.8 and 2.4 log reductions. It
304 may be important to note that the water temperature during the first three days at the time of
305 sampling was on average 3.1°C lower than on days four to nine (Table 3). Statistical analysis
306 supported observations that the inactivation rates of *Siphoviridae* and *Myoviridae* phages
307 ($P < 0.05$; Mann-Whitney) were significantly different, with greater persistence exhibited by
308 *Siphoviridae* phages. Statistical analysis also revealed that differences between phages with
309 different capsid morphology were not significant ($P = > 0.05$; Mann-Whitney).

310

311 **4. Discussion**

312 The results from this study present an important and timely insight into the diversity and
313 environmental survival of human-specific phages capable of infecting *Enterococcus* host
314 MW47. Transmission electron microscope (TEM) analysis revealed that a heterogeneous
315 group of phages were able to infect host strain MW47. Three distinct morphologies were
316 observed from two different families (*Myoviridae* and *Siphoviridae*). This highlights that
317 phages infecting this enterococcal host strain appear to be morphologically more
318 heterogeneous than F-RNA phages and those infecting *Bacteroides* spp. (Queralt *et al.*,
319 2003). According to previous inactivation studies that focused on the role that somatic
320 coliphage morphology plays in wastewater and faecally-polluted surface waters in which a
321 wide variety of phages exists, *Myoviridae* phages are often initially shown to be the
322 predominant family (Muniesa *et al.*, 1999). However, following wastewater treatment in
323 engineered systems or inactivation in the natural environment, these proportions have been
324 shown to change, with *Siphoviridae* phages becoming dominant (Lasobras *et al.*, 1997;
325 Muniesa *et al.*, 1999). The results reported here support the findings of other studies that have

326 shown phages of the *Siphoviridae* family to demonstrate greater resistance to inactivation
327 than other phage families ($P < 0.05$, Mann-Whitney), including *Myoviridae* (Duran *et al.*,
328 2003). *Siphoviridae* may be more resistant to inactivation because they can incorporate into
329 the host genome (lysogenic) and may be released by lysogenic bacteria, whereas *Myoviridae*
330 are primarily lytic (lysis causing) (Muniesa *et al.*, 1999). There also may be differences in the
331 ability of the phages to form plaques, because of some phages going into the prophage state
332 (latent). The differences observed in the morphology of phages capable of infecting
333 *Enterococcus* strain MW47 do have implication for their potential use in MST studies and it
334 is these differences that prompted the investigation into the inactivation of phage of different
335 morphologies capable of infecting host strain MW47 that is reported here.

336

337 A significantly statistical link was established between inactivation rate and phage
338 morphology. In comparison with phages of the family *Siphoviridae*, MW47-10 and MW47-
339 15 (both members of the *Myoviridae* family) demonstrated more rapid inactivation. The
340 results of this study suggest that some cautions should be exercised in interpreting
341 *Enterococcus* phage-lysis results from MST studies, especially when different sample types
342 are being compared because observed variance in phage counts could be the result of a
343 disparity in inactivation rates rather than actual differences in contamination level. Whilst
344 only phages capable of infecting host strain MW47 were assessed in this study, it may be that
345 other *Enterococcus* phages that infect host strains of importance to MST also comprise
346 heterogeneous families. Further investigation of these phages is warranted. This study has
347 demonstrated that, whereas *Enterococcus* phages continue to demonstrate potential as a
348 valuable MST tool, they are heterogeneous and demonstrate varying inactivation rates in the
349 environment. This issue needs to be considered when interpreting findings of MST studies
350 that use phage-lysis of host-specific *Enterococcus* spp. as a way to identify sources of faecal

351 contamination, particularly when samples from different environmental matrices are being
352 compared. It also highlights the importance of gaining a better understanding of the
353 ecological characteristics of newly proposed markers. In conclusion, the research outlined
354 here indicates that whilst there may be certain benefits to using *Enterococcus* phages over
355 FRNA or *Bacteroides* phages for MST purposes (simpler growth requirements, lower cost
356 media, and higher resistance to wastewater treatment), the use of FRNA and *Bacteroides*
357 phage for MST studies may still offer a more credible source of information on pollution
358 sources because of the significantly lower heterogeneity of these phage groups.

359

360 **5. Acknowledgements**

361 The authors would like to acknowledge Southern Water for providing access to Scaynes Hill
362 wastewater treatment works for sample collection.

363

364 **6. Conflict of interest**

365 No conflict of interest declared.

366

367 **7. References**

368 Ackermann, H. W. (2007). 5500 Phages examined in the electron microscope. *Arch Virol*,
369 152 (2), pp. 227-243.

370

371 Adams, M. H. (1959). *Bacteriophages*. New York: Interscience Publishers.

372

373 Anon. (2001). ISO 10705-1, Water quality - Detection and enumeration of bacteriophages -
374 Part 1: enumeration of F-specific RNA bacteriophages. International Organisation for
375 Standardisation, Geneva, Switzerland.

376

377 Bachrach, G. Leizerovici-Zigmond, M. Zlotkin, A. Naor, R. and Steinberg, D. (2003).
378 Bacteriophage isolation from human saliva. *Lett Appl Microbiol*, 36 (1), pp. 50-53.

379

380 Boehm, A.B. Van De Werfhorst, L.C. Griffith, J.F. Holden, P.A. Jay, J.A. Shanks, O.C.
381 Wang, D. and Weisberg, S.B. (2013). Performance of forty-one microbial source tracking
382 methods: A twenty-seven lab evaluation study. *Water Res*, 47 (18), pp. 6812-6828.

383

384 Bonilla, N. Santiago, T. Marcos, P. Urdaneta, M. Domingo, J. S. and Toranzos, G. A. (2010).
385 Enterophages, a group of phages infecting *Enterococcus faecalis*, and their potential as
386 alternate indicators of human faecal contamination. *Water Sci Technol*, 61 (2), pp. 293-300.

387

388 Carey-Smith, G. V. Billington, C. Cornelius, A. J. Hudson, J. A. and Heinemann, J. A.
389 (2006). Isolation and characterization of bacteriophages infecting *Salmonella spp.* *FEMS*
390 *Microbiol Lett*, 258 (2), pp. 182-186.

391

392 De Vaux, A. Laguerre, G. Divies, C. and Prevost, H. (1998). *Enterococcus asini* sp. nov.
393 isolated from the caecum of donkeys (*Equus asinus*). *Int J Syst Bacteriol*, 48, pp. 383-387.

394

395 Dee, S.W. and Fogleman, J.C. (1992). Rates of inactivation of waterborne coliphages by
396 monochloramine. *Appl Environ Microbiol*, 58, pp. 3136-3141.

397

398 Diston, D. Ebdon, J.E. and Taylor, H.D. (2012). The effect of UV-C radiation (254 nm) on
399 candidate microbial source tracking phages infecting a human-specific strain of *Bacteroides*
400 *fragilis* (GB-124). *J Water Health*, 10 (2), pp. 262-270.

401

402 Duran, A. E. Muniesa, M. Moce-Llivina, L. Campos, C. Jofre, J. and Lucena, F. (2003).
403 Usefulness of different groups of bacteriophages as model micro-organisms for evaluating
404 chlorination. *J Appl Microbiol*, 95 (1), pp. 29-37.

405

406 Ebdon, J.E., Sellwood, J., Shore, J., and Taylor, H.D. (2012). Detection of *Bacteroides* (GB-
407 124) phages as a surrogate for pathogenic human enteric viruses. *Environ Sci Technol*. 46 (2),
408 1163–1169.

409

410 Fard, R. M. N. Barton, M. D. and Heuzenroeder, M. W. (2010). Novel Bacteriophages in
411 *Enterococcus spp.* *Curr Microbiol*, 60 (6), pp. 400-406.

412

413 Gourmelon, M. Caprais, M. P. Mieszkin, S. Marti, R. Wery, N. Jarde, E. Derrien, M. Jadas-
414 Hecart, A. Communal, P. Y. Jaffrezic, A. and Pourcher, A. M. (2010). Development of
415 microbial and chemical MST tools to identify the origin of the faecal pollution in bathing and
416 shellfish harvesting waters in France. *Water Res*, 44 (16), pp. 4812-4824.

417

418 Jofre, J. Bosch, A. Lucena, F. Girones, R. and Tartera, C. (1986). Evaluation of *Bacteroides*-
419 *fragilis* bacteriophages as indicators of the virological quality of water. *Water Sci Technol*, 18
420 (10), pp. 167-173.

421

422 Lasobras, J. Muniesa, M. Frias, J. Lucena, F. and Jofre, J. (1997). Relationship between the
423 morphology of bacteriophages and their resistance in the environment. *Water Sci Technol*,
424 35, pp. 129-132.

425

426 Moce-Llivina, L. Lucena, F. and Jofre, J. (2005). Enteroviruses and bacteriophages in bathing
427 waters. *Appl Environ Microbiol*, 71 (11), pp. 6838-6844.

428

429 Muniesa, M. Lucena, F. and Jofre, J. (1999). Study of the potential relationship between the
430 morphology of infectious somatic coliphages and their persistence in the environment. *J Appl*
431 *Microbiol*, 87 (3), pp. 402-409.

432

433 Muniesa, M. Payan, A. Moce-Llivina, L. Blanch, A. R. and Jofre, J. (2009). Differential
434 persistence of F-specific RNA phage subgroups hinders their use as single tracers for faecal
435 source tracking in surface water. *Water Res*, 43 (6), pp. 1559-1564.

436

437 Muniesa, M. Lucena, F. Blanch, A. R. Payan, A. and Jofre, J. (2012). Use of abundance ratios
438 of somatic coliphages and bacteriophages of *Bacteroides thetaiotaomicron* GA17 for
439 microbial source identification. *Water Res*, 46(19), pp. 6410-6418.

440

441 Nasser, A. M. and Oman, S. D. (1999). Quantitative assessment of the inactivation of
442 pathogenic and indicator viruses in natural water sources. *Water Res*, 33 (7), pp. 1748-1752.

443

444 Paisano, A. F. Spira, B. Cai, S. and Bombana, A. C. (2004). In vitro antimicrobial effect of
445 bacteriophages on human dentin infected with *Enterococcus faecalis* ATCC 29212. *Oral*
446 *Microbiol Immunol*, 19 (5), pp. 327-330.

447

448 Plummer, J. D. and Long, S. C. (2009). Identifying sources of surface water pollution: A
449 toolbox approach. *J Am Water Works Assoc*, 101 (9), pp. 75-88.

450

451 Purnell, S. E. Ebdon, J. E. and Taylor, H. D. (2011). Bacteriophage lysis of *Enterococcus*
452 host strains: A tool for microbial source tracking? *Environ Sci Technol*, 45 (24), pp. 10699-
453 10705.

454

455 Queralt, N. Jofre, J. Araujo, R. and Muniesa, M. (2003). Homogeneity of the morphological
456 groups of bacteriophages infecting *Bacteroides fragilis* strain HSP40 and strain RYC2056.
457 *Curr Microbiol*, 46 (3), pp. 163-168.

458

459 Ramírez, B. Centrón, D. Ramírez, M. S. and Lopardo, H. (2006). Isolation and
460 characterization of lytic bacteriophages of *Enterococcus* spp. *Int Congr Ser*, 1289, pp. 162-
461 164.

462

463 Santiago-Rodriguez, T. M. Davila, C. Gonzalez, J. Bonilla, N. Marcos, P. Urdaneta, M.
464 Cadete, M. Monteiro, S. Santos, R. Domingo, J. S. and Toranzos, G. A. (2010).
465 Characterization of *Enterococcus faecalis*-infecting phages (enterophages) as markers of
466 human faecal pollution in recreational waters. *Water Res*, 44 (16), pp. 4716-4725.

467

468 Santiago-Rodriguez, T. M. Marcos, P. Monteiro, S. Urdaneta, M. Santos, R. and Toranzos, G.
469 A. (2013). Evaluation of *Enterococcus*-infecting phages as indices of faecal pollution. *J*
470 *Water Health*, 11 (1), pp. 51-63.

471

472 Soller, J. A. Schoen, M. E. Bartrand, T. Ravenscroft, J. E. and Ashbolt, N. J. (2010).
473 Estimated human health risks from exposure to recreational waters impacted by human and
474 non-human sources of faecal contamination. *Water Res*, 44 (16), pp. 4674-4691.

475

476 Son, J. S. Jun, S. Y. Kim, E. B. Park, J. E. Paik, H. R. Yoon, S. J. Kang, S. H. and Choi, Y. J.
477 (2010). Complete genome sequence of a newly isolated lytic bacteriophage, EFAP-1 of
478 *Enterococcus faecalis*, and antibacterial activity of its endolysin EFAL-1. *J Appl Microbiol*,
479 108 (5), pp. 1769-1779.

480

481 Vinodkumar, C. S. Srinivasa, H. Basavarajappa, K. G. Geethalakshmi, S. and Bandekar,
482 Nitin (2011). Isolation of bacteriophages to multi-drug resistant enterococci obtained from
483 diabetic foot: A novel antimicrobial agent waiting in the shelf? *Indian J Pathol Microbiol*, 54
484 (1), pp. 90-95.

485

486 Verçosa and Moreira (2016). Detecção de bacteriófagos de cepas hospedeiras de enterococos
487 como uma ferramenta para rastreamento de fontes de poluição fecal. In: VII Congresso
488 Brasileiro de Gestão Ambiental Campina Grande. 24 Nov, 2016.

489

490 Vijayavel, K. Byappanahalli, M. N. Ebdon, J. Taylor, H. Whitman, R. L. and Kashian, D.R.
491 (2014). Enterococcus phages as potential tool for identifying sewage inputs in the Great
492 Lakes region. *J Great Lakes Res*, 40 (4). pp. 989-993.

493

494 Vogel, J. R. Stoeckel, D. M. Lamendella, R. Zelt, R. B. Domingo, J. W. S. Walker, S. R. and
495 Oerther, D. B. (2007). Identifying faecal sources in a selected catchment reach using multiple
496 source-tracking tools. *J Environ Qual*, 36 (3), pp. 718-729.

497

498 Wangkahad, B. Mongkolsuk, S. and Sirikanchana, K. (2017). Integrated multivariate analysis
499 with non-detects for the development of human sewage source-tracking tools using
500 bacteriophages of *Enterococcus faecalis*. *Environ Sci Technol*. 51 (4), pp 2235–2245

501 **Tables**

502 **Table 1.** Morphology of phages isolated from MW47

Phage ID (MW47-)	Capsid morphology	Family	
1	Icosahedral	<i>Siphoviridae</i>	503
2	Elongated icosahedral	<i>Siphoviridae</i>	504
3	Icosahedral	<i>Siphoviridae</i>	505
4	Icosahedral	<i>Siphoviridae</i>	506
5	Elongated icosahedral	<i>Siphoviridae</i>	507
6	Icosahedral	<i>Siphoviridae</i>	508
7	Elongated icosahedral	<i>Siphoviridae</i>	509
8	Elongated icosahedral	<i>Siphoviridae</i>	510
9	Elongated icosahedral	<i>Siphoviridae</i>	511
10	Icosahedral	<i>Myoviridae</i>	512
11	Icosahedral	<i>Siphoviridae</i>	513
12	Icosahedral	<i>Siphoviridae</i>	514
13	Icosahedral	<i>Siphoviridae</i>	515
14	Icosahedral	<i>Siphoviridae</i>	516
15	Icosahedral	<i>Myoviridae</i>	517
16	Elongated icosahedral	<i>Siphoviridae</i>	518
17	Icosahedral	<i>Siphoviridae</i>	519
18	Icosahedral	<i>Siphoviridae</i>	520
19	Icosahedral	<i>Siphoviridae</i>	520
20	Icosahedral	<i>Siphoviridae</i>	520

521

522

523

524

525

526

527 **Table 2.** Host range of phages capable of infecting enterococcal host strain MW47

ID	<i>Enterococcus</i> type strains (DSM No.)										
	<i>asini</i> (20681)	<i>cassel.</i> (20680)	<i>durans</i> (20633)	<i>faecalis</i> (20478)	<i>faecium</i> (20477)	<i>gallin.</i> (24841)	<i>hirae</i> (20160)	<i>mundti</i> (4838)	<i>pseudo.</i> (5632)	<i>sacchar.</i> (20726)	<i>sulphur.</i> (6905)
1	N ^a	N	N	N	N	N	N	N	N	N	N
2	N	N	N	N	N	N	N	N	N	N	N
3	N	N	N	N	N	N	N	N	N	N	N
4	N	N	N	N	N	N	N	N	N	N	N
5	N	N	N	N	N	N	N	N	N	N	N
6	N	N	N	N	N	N	N	N	N	N	N
7	N	N	N	N	N	N	N	N	N	N	N
8	6.1x10 ⁷	N	N	5.4x10 ⁶	N	N	N	N	N	N	N
9	N	N	N	1.1x10 ⁷	N	N	N	N	N	N	N
10	N	N	N	1.7x10 ⁶	N	N	N	N	N	N	N
11	N	N	N	N	N	N	N	N	N	N	N
12	N	N	N	N	N	N	N	N	N	N	N
13	N	N	N	N	N	N	N	N	N	N	N
14	N	N	N	N	N	N	N	N	N	N	N
15	4.7x10 ⁸	N	N	3.7x10 ⁸	N	N	N	N	N	N	N
16	N	N	N	N	N	N	N	N	N	N	N
17	N	N	N	N	N	N	N	N	N	N	N
18	N	N	N	N	N	N	N	N	N	N	N
19	N	N	N	N	N	N	N	N	N	N	N
20	1.0x10 ³	N	N	N	N	N	N	N	N	N	N

528 ^aN= Phage lysis not detected; DSM= *Deutsche Sammlung* von Mikroorganismen

529

530

531

532

533

534

535

536

537

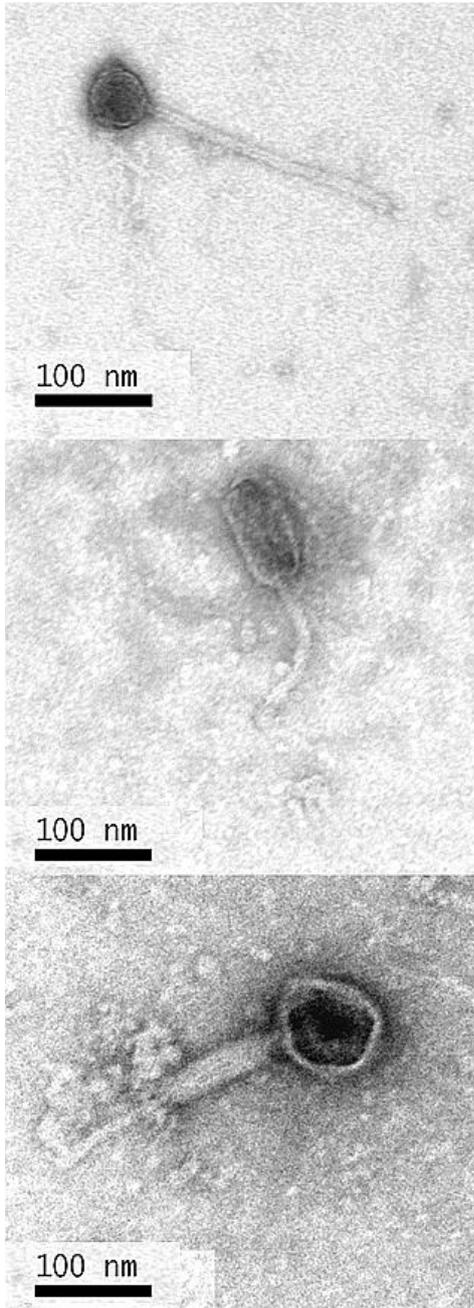
538 **Table 3.** Physico-chemical parameters

	Day						Mean
	1	2	3	5	8	9	
Temperature (°C)	11.7	10.3	11.9	12.4	14.3	16.7	13.5
Turbidity (NTU)	31.2	36.7	24.0	22.1	30.9	29.8	29.4
pH	7.9	8.1	7.8	8.0	8.0	8.0	8.0
DO (mg/l)	9.0	10.9	9.3	8.9	9.8	8.4	9.4
EC (uS/cm)	553.0	472.0	599.0	629.0	744.0	712.0	637.9
TDS (mg/l)	359.0	306.0	389.0	408.0	483.0	462.0	414.0
SAL (ppt)	0.3	0.2	0.3	0.3	0.4	0.4	0.3

539 °C = degrees Celsius, NTU = Nephelometric Turbidity Unit, mg/l = milligrams per litre,
 540 uS/cm = microsiemens per centimetre, ppt= part per thousand.

541
 542
 543
 544
 545
 546
 547
 548
 549
 550
 551
 552
 553
 554
 555
 556
 557
 558
 559
 560
 561
 562
 563
 564
 565
 566
 567
 568
 569
 570
 571
 572
 573

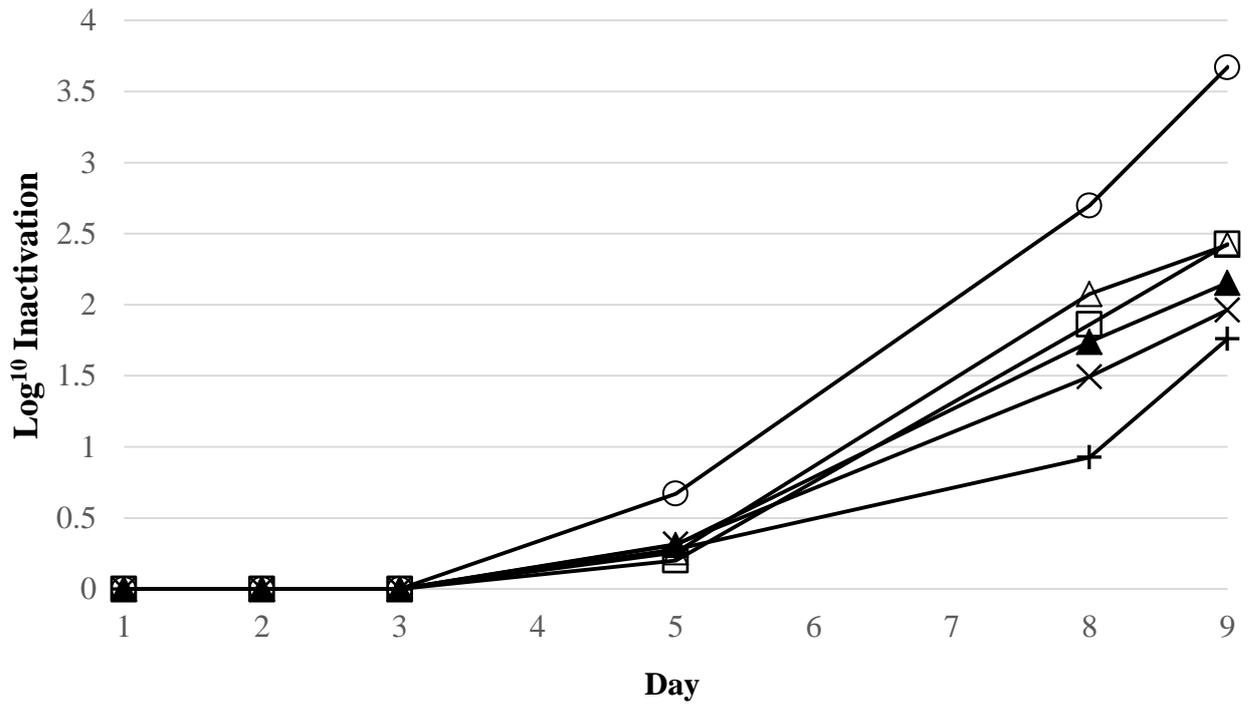
574 **Figures**
575



576

577 **Figure 1.** Examples of the three major morphologies observed by transmission electron
578 microscopy (TEM). Top - MW47-1 (*Siphoviridae* with icosahedral capsid); middle - MW47-
579 5 (*Siphoviridae* with elongated icosahedral capsid); and bottom - MW47-10 (*Myoviridae* with
580 icosahedral capsid).

581



582

583 **Figure 2.** Log₁₀ inactivation of the six phage morphologies: (□) MW47-1, (▲) MW47-2, (+)
 584 MW47-5, (X) MW47-6, (O) MW47-10, (Δ) MW47-15

585