

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

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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-Rodriquez *et al.*, 2010; Santiago-Rodriquez *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 ( $\pm 2$  °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  $\mu$ 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 ( $\pm 2$  °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 Na<sub>2</sub>HPO<sub>4</sub>, 22 mmol KH<sub>2</sub>PO<sub>4</sub>,  
161 85.5 mmol NaCl, 1 mmol MgSO<sub>4</sub>, 0.1 mmol CaCl<sub>2</sub>) 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<sup>-1</sup> to 10<sup>-8</sup>) using the spot test plaque  
174 assay. The process was repeated until a minimum titre of 10<sup>8</sup> PFU ml<sup>-1</sup> 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. pseudoaerium*, *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<sup>-1</sup>.

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<sup>-1</sup>) 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 (T<sub>0</sub>), and after one (T<sub>24</sub>), two (T<sub>48</sub>), five (T<sub>120</sub>), eight (T<sub>192</sub>) and nine (T<sub>216</sub>) 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 Log<sub>10</sub> units (Log  
217 N<sub>0</sub> / N<sub>t</sub>) of phage numbers of each phage type (MW47-1, -2, -5, -6, -10, -15) before (N<sub>0</sub>) and  
218 after (N<sub>t</sub>) 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<sup>-1</sup>) 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  $5.0 \times 10^8$  and  $1.1 \times 10^{10}$  PFU ml<sup>-1</sup>. 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 ( $1.0 \times 10^3$  PFU  
273  $\text{ml}^{-1}$ ). 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  
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363

## 364 **6. Conflict of interest**

365 No conflict of interest declared.

366

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501 **Tables**

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

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

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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 <sup>a</sup>	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 <sup>7</sup>	N	N	5.4x10 <sup>6</sup>	N	N	N	N	N	N	N
9	N	N	N	1.1x10 <sup>7</sup>	N	N	N	N	N	N	N
10	N	N	N	1.7x10 <sup>6</sup>	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 <sup>8</sup>	N	N	3.7x10 <sup>8</sup>	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 <sup>3</sup>	N	N	N	N	N	N	N	N	N	N

528 <sup>a</sup>N= Phage lysis not detected; DSM= *Deutsche Sammlung* von Mikroorganismen

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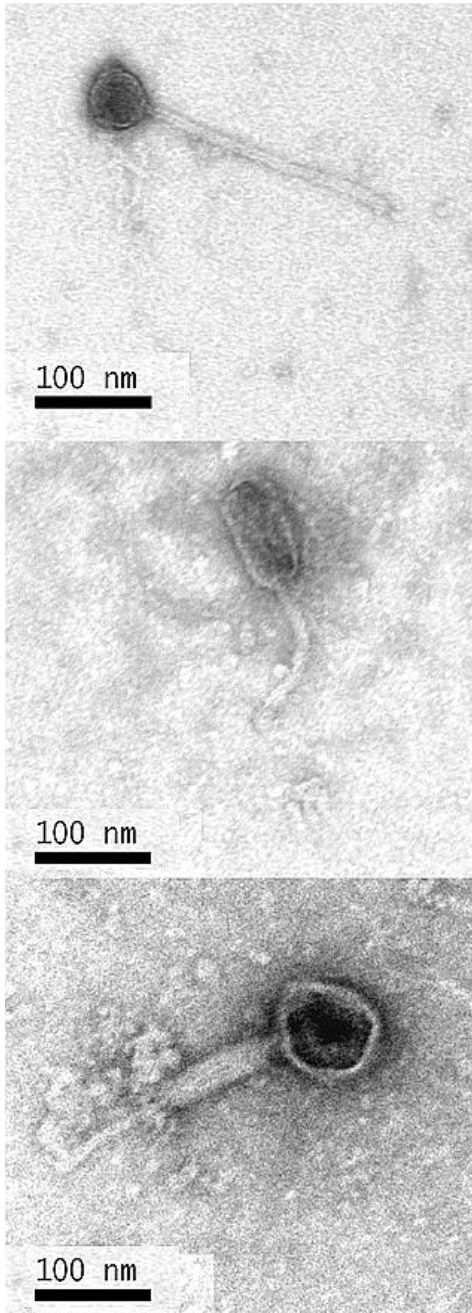
538 **Table 3.** Physico-chemical parameters

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

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574 **Figures**  
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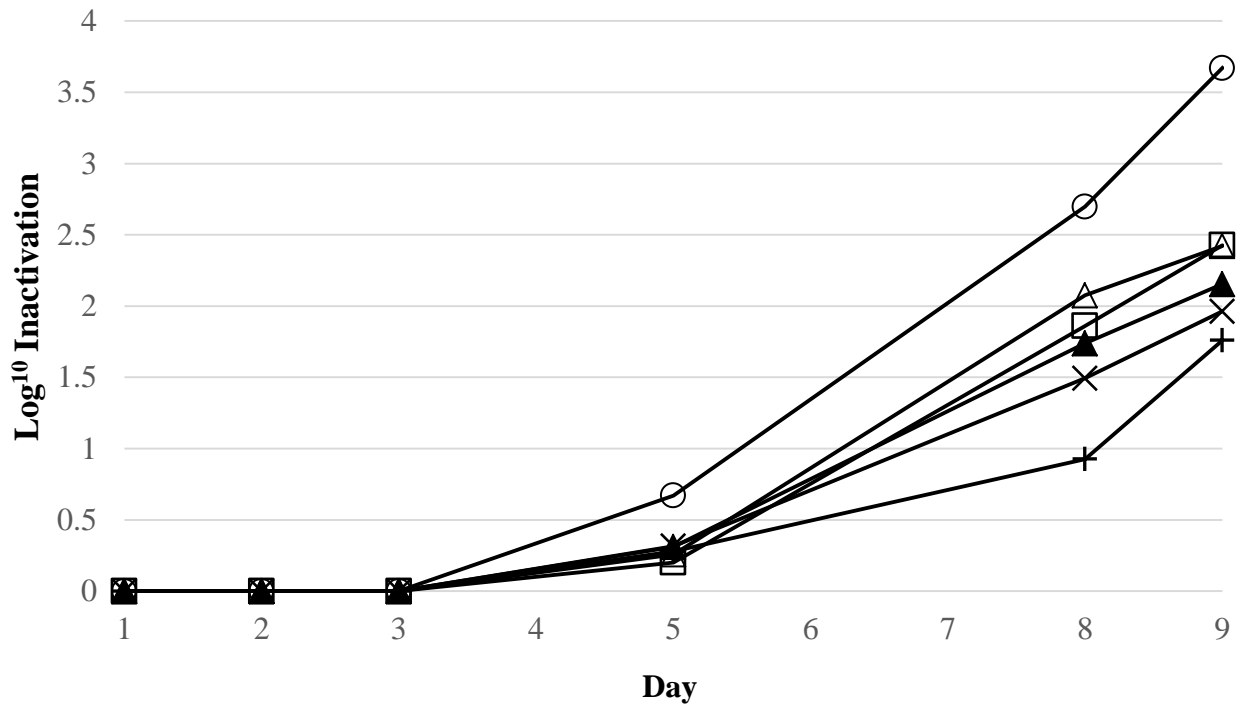


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577 **Figure 1.** Examples of the three major morphologies observed by transmission electron  
578 microscopy (TEM). Top - MW47-1 (*Siphoviridae* with icosahedral capsid); middle - MW47-  
579 5 (*Siphoviridae* with elongated icosahedral capsid); and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

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

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