The application of phage-based faecal pollution markers to predict the concentration of adenoviruses in mussels (*Mytilus edulis*) and their overlying waters

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Running head: Bacteriophages as surrogates of viral pathogens

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42 **Abstract**
43 **Aim:** This study set out to determine whether phage-based indicators may provide a 'low-tech' alternative to
44 existing approaches that might help maintain the microbial safety of shellfish and their overlying waters.
45 **Methods and Results:** Mussels and their overlying waters were collected biweekly from an estuary in southeast
46 England over a two-year period (May 2013 - April 2015) (n= 48). Levels of bacterial indicators were determined
47 using membrane filtration and most probable number methods and those of bacteriophages were determined by
48 direct plaque assay. The detection of adenovirus was determined using real-time polymerase chain reaction. The
49 results revealed that somatic coliphages demonstrated the most significant correlations with AdV F and G in
50 mussels (rho=0.55) and overlying waters (rho=0.66), followed by GB124 phages (rho=0.43) while *E. coli* showed
51 no correlation with AdV F and G in mussels.
52 **Conclusion:** This study demonstrates that the use of somatic coliphages and GB124 phages may provide a better
53 indication of the risk of adenovirus contamination of mussels and their overlying waters than existing bacterial
54 indicators.
55 **Significance and impact of study:** Phage-based detection may be particularly advantageous in low-resource
56 settings where viral infectious disease presents a significant burden to human health.
57
58 Keywords: Adenoviruses, somatic coliphages, microbial indicators, human health, shellfish, surrogates, policy
59
60 **Introduction**
61 Adenoviruses are medium-sized (60-90 nm), non-enveloped icosahedral viruses containing linear, non-segmented
62 double-stranded DNA, belonging to the family *Adenoviridae*. They have a worldwide distribution and they are
63 ubiquitous in environments contaminated by human faeces or sewage. And as a result, they are most commonly
64 transmitted through the faecal-oral route, i.e., through ingestion of faecally-contaminated food or water (Pond
65 2005). They represent the largest non-enveloped viruses and are usually stable to chemical or physical agents and
66 adverse pH conditions, allowing for prolonged survival in the natural environment, particularly water bodies
67 (Thurston-Enriquez *et al*. 2003). Adenoviruses have also been proposed as viral faecal indicators because of their
68 stability and prevalence throughout the year (Girones *et al*. 2013; Rodriguez-Manzano 2014).
69
70 One gram of faeces may contain ten million viruses, one million bacteria, 1,000 parasite cysts and 100 parasite
71 eggs (UNICEF 2008) and faecally-polluted waters to which humans may come into contact and faecally-polluted
72 shellfish that are consumed therefore present a hazard (potential risk) to human health. Many pathogens of faecal
73 origin (and human health significance) may contaminate the environment and food chain unless barriers to their
74 environmental transmission (such as water and wastewater treatment) are sufficiently robust. Several practices,
75 such as sanitation, hygiene and surveillance activities to monitor the microbiological quality of water and food by
76 environmental and public health protection agencies, support efforts to control the environmental transmission of
77 human enteric diseases. Several terms have been used to describe the microbial tools used in these monitoring
78 exercises, including 'microbial indicator', 'index organism', 'faecal indicator' and 'surrogate organism' (Ashbolt
79 *et al*. 2001; Sinclair *et al*. 2012). *E. coli,* faecal coliforms and intestinal enterococci represent the classic bacterial
80 indicators of the presence of faecal pollution (faecal indicator bacteria) in the environment.
81
82 There are, however, limitations to the use of traditional faecal indicator bacteria to predict the presence of enteric
83 pathogens and they appear to have limited or no predictive value for many important human viral pathogens, such
84 as enterovirus, norovirus, hepatitis A virus (Jofre 1992, Muniain-Mujika *et al*. 2003) and parasite cysts. Critically,
85 Dore *et al*. (2003) and Griffin *et al*. (2003) suggested that shellfish may meet *E. coli* standards for human
86 consumption but still contain infectious doses of human enteric viruses that can cause gastroenteritis.
87
88 Human enteric viruses are found in the gastrointestinal tract and the group that causes gastroenteritis infects the
89 epithelial cells (DiCaprio *et al*. 2013). Large numbers of these viruses are excreted in the faeces and urine of
90 infected humans (Melnick and Gerba 1980). Indiscriminate discharge of sewage into surface waters is the most
91 conspicuous channel through which these viruses contaminate the environment (Pouillot *et al*. 2015), where their
92 low infectious dose may present a human health hazard (Fong and Lipp 2005). Their commonest route of
93 transmission is faecal-oral route, principally through the consumption of contaminated drinking water and

94 shellfish (Lipp and Rose 1997). Symptoms of viral gastroenteritis include diarrhoea, vomiting, abdominal pains
95 and nausea (Bosch 2010). Some infected individuals fail to show any symptoms but nevertheless constantly shed
96 the virus in their faeces and urine and the existence of these asymptomatic carriers can complicate epidemiological
97 studies of human disease transmission (Pina *et al*. 1998).
98
99 Advances in culture-independent molecular-based approaches to monitoring the sanitary quality of shellfish,
100 through amplification and quantification of viral nucleic acids by real-time polymerase chain reaction (qPCR) or
101 reverse transcription polymerase chain reaction (RT-qPCR), have aided the rapid detection of pathogenic human
102 viruses (Campos and Lees 2014). These molecular methods demonstrate high levels of sensitivity and specificity
103 (Pina *et al*. 1998) but remain relatively expensive option, thus, less likely to be taken up in low-resource settings.
104 The methods are also unable to distinguish readily between infective and non-infective viral particles (Bosch
105 2010).
106
107 Bacteriophages, such as somatic coliphages, F-RNA coliphages and phages infecting *Bacteroides fragilis* have
108 been suggested as potentially useful indicators of viral contamination (IAWPRC 1991; Pina *et al*. 1998; Hernroth
109 *et al*. 2002; Ebdon *et al*. 2012; Jofre *et al*. 2014, Pouillot *et al*. 2015). This is because they have several
110 characteristics in common with enteric viruses. For instance, they are found in high concentrations in sewage,
111 they are non-latent and their cell sizes, shapes, morphology, physiochemistry and surface chemistry are all similar
112 to those of enteric viruses (IAWPRC 1991). They also exhibit similar patterns of persistence and do not replicate
113 in the environment except where there is a suitable bacterial host at sufficient concentration. Phages are easier and
114 less expensive to enumerate in the laboratory and they pose little or no risk to the health of higher organisms
115 (Tufenkji and Emelko 2011). Phages of host-specific strains of enteric bacteria may also represent useful targets
116 for microbial source tracking (MST) and have successfully been used to particularly identify human sources of
117 faecal pollution (Ebdon *et al*. 2007).
118
119 Fifty seven immunologically distinct human adenovirus serotypes have been identified and have been divided
120 into seven groups (A–G), based on their physical, chemical and biological properties (Jones *et al*. 2007). Some
121 reported cases of human adenoviral infection caused by various serotypes include conjunctivitis, pharyngitis,
122 pneumonia, acute and chronic appendicitis, bronchiolitis, haemorrhagic cystitis, nephritis, acute respiratory
123 diseases and keratitis. Only human adenovirus F serotypes 40 and 41, and adenovirus G serotypes 52, however,
124 have been strongly associated with gastroenteritis (Pond 2005). Furthermore, adenovirus F serotypes 40 and 41
125 have been associated with acute gastroenteritis and responsible for up to 20 % of the cases of diarrheal disease
126 globally in children (Dey *et al*. 2011). Infections are usually mild or self-limiting but can be fatal in
127 immunocompromised individuals (Jiang 2006).
128 The aim of this study was to examine the distribution pattern of faecal indicator bacteria, bacteriophages and AdV
129 F and G in *M. edulis* and their overlying waters harvested from a site that could best be described as a 'Class B'
130 harvesting area according to the EU shellfish hygiene classification criteria (Regulation (EC) No. 854/2004) (CEU
131 2004). This may be considered an essential first 'hazard evaluation' step in the chain of effective human health
132 risk management practices of shellfish harvesting areas. The objective of this study was to examine the
133 applicability of phage-based pathogen surrogates to predict the concentration of AdV F and G in *M. edulis* and
134 their overlying waters. This objective was based on the widely-accepted principle that phages are (and will for
135 some time be) easier and cheaper to detect and enumerate than viral pathogens (Hernroth *et al*. 2002; Ebdon *et al*.
136 2012; Jofre *et al*. 2014) and the proposition that using phages to predict the risk of AdV F and G in *M. edulis* and
137 their overlying waters could be of immense benefit to human health protection.
138

139 **Materials and methods**

140 **Sampling site and collection of samples**

141 The sampling site was situated in the village of Piddinghoe, on the estuary of the River Ouse, which is the second
142 largest river in the county of East Sussex in southern England, draining an area of 396 km^2 to its tidal limit. At
143 mid-tide, the river has a depth of approximately three to four metres at its deepest point, about eight metres at high
144 tide and less than one metre at low tide with a fluctuating salinity of between 0.6 and 16 ppt. This estuarine site is

145 affected by fluctuating faecal input from biologically treated municipal wastewater (e.g., at Scaynes Hill, about
146 24.32 km upstream from the sampling site) and by diffuse pollution from animal rearing (mainly cattle and sheep).
147
148 Sampling activities were carried out every two weeks over a consecutive period of twenty-four months. On each
149 sampling occasion, a grab sample of approximately one litre of overlying water was collected in a pre-sterilised
150 plastic bottle in accordance with standard protocol (Anon. 2012). Mussels with an approximate length (six to ten
151 centimetre) and weight (35 to 41 g) were collected by hand-picking into transparent polyethylene bags.
152 Approximately 1800 mussels were sampled in total (i.e., 30 – 40 mussels were analysed on each occasion
153 depending on the size of individual mussel). Mussels and overlying water samples were transported to the
154 laboratory in the dark at approximately 4 ^{o}C and processed immediately (within less than one hour).
155
156 **Enumeration of faecal indicator bacteria**
157 The concentrations of *E. coli,* faecal coliforms and intestinal enterococci in overlying waters were determined
158 using the membrane filtration method (ISO 9308-1, ISO 7899-2) (Anon. 2000b; 2000c). The membrane filters
159 were placed on freshly prepared selective media (*m*-FC, *m*-Ent, MLSA and TBX). Agar plates were incubated at
160 37 ^{o}C for 48 hours (*m*-Ent), 37 ^{o}C for 24 hours (MLSA) and 44 ^{o}C for 24 hours (*m*-FC, TBX), and colonies were
161 counted, calculated and expressed as colony-forming units (CFU) 100 ml^{-1} of water. Similarly, the concentrations
162 of *E. coli,* faecal coliforms and intestinal enterococci in *M. edulis* were determined using the most probable
163 number method (MPN) (ISO 16649-3) (CEFAS 2011). Briefly, ten to fifteen *M. edulis* were rinsed in cold water,
164 then cut open to remove their flesh and intravalvular fluids, 0.1 % peptone solution (one ratio two) was added and
165 homogenised using a stomacher (400 Lab System) and shaker (Stuart Scientific Ltd). A master mix (M) was
166 prepared by adding 30 ml shellfish homogenate to 70 ml 0.1 % peptone solution and a dilution (N) was then made
167 from the master mix. Five test tubes containing ten ml each of double-strength Mineral Modified Glutamate Broth
168 (dsMMGB) and ten test tubes containing ten ml each of single-strength Mineral Modified Glutamate Broth
169 (ssMMGB) were prepared. Ten millilitres of M (equivalent to one gram of tissue per tube and 10^{0} dilutions) was
170 inoculated into the five test tubes containing ten ml dsMMGB. One millilitre of M (equivalent to 0.1 g of tissue
171 per tube and 10^{-1} dilutions) were inoculated into the five test tubes containing ten ml ssMMGB. One millilitre of
172 N (equivalent to 0.01 g of tissue per tube and 10^{-2} dilutions) were inoculated into the remaining five test tubes
173 containing ten ml ssMMGB. Inoculated test tubes were incubated at 37 ^{o}C for 24 hours and were then examined
174 for acid production. Test tubes with yellow colouration throughout the medium were sub-cultured onto freshly
175 prepared selective media (*m*-FC, *m*-Ent and TBX). Agar plates were incubated at 37 ^{o}C for 24 hours (*m*-Ent) and
176 44 ^{o}C for 24 hours (*m*-FC, TBX), and colonies were counted, and expressed as most probable number (MPN) 100
177 g^{-1} of shellfish flesh and intravalvular fluid.
178
179 **Enumeration of bacteriophages**
180 The concentrations of somatic coliphages, F-RNA coliphages and bacteriophages infecting *B. fragilis* GB124 in
181 *M. edulis* and their overlying waters were determined by direct plaque assay using a standardised double-agar
182 method (ISO 10705) (Anon. 2000a; 2001a; 2001b). Briefly, approximately 50 ml of overlying waters were filtered
183 through a 0.22 µm cellulose nitrate filter (Millipore). Similarly, on each occasion, ten to fifteen *M. edulis* were
184 rinsed in cold distilled water and then cut open to remove their flesh and intravalvular fluids. Their digestive
185 glands were separated carefully from the flesh, and chopped finely to ensure proper mixing. 0.25 mol L^{-1} glycine
186 (pH 9.5) was then added to two grams of the digestive gland (one ratio five). The mixture was placed on a shaker
187 (Stuart Scientific Ltd) for approximately 20 mins, and subsequently centrifuged (Heraeus megafuge 16R) at 2000
188 *g*, 4 ^{o}C for 15 mins. The supernatant was filtered through a 0.22 µm cellulose nitrate filter (Millipore). The required
189 media (MSA, TYGA, BPRMA), semi-solid agar (ssMSA, ssTYGA, ssBPRMA) and broth (MSB, TYGB,
190 BPRMB) were prepared according to the manufacturer's specifications. Bacterial host strains (*E. coli* WG5,
191 *Salmonella typhimurium* WG49, and *B. fragilis* GB124) were grown to ensure confluent lawns for phage
192 detection. One millilitre of sample (filtered water or shellfish glands) was added to one millilitre of bacterial host
193 strain and 2.5 ml of semi-solid agar in a 10 ml vial (Sterilin). The mixture was vortexed and poured onto a solid
194 agar plate, swirled gently for even distribution and allowed to solidify. All inoculated plates were incubated
195 appropriately and plaques (clear zones of lysis) were counted and expressed as plaque-forming units (PFU) 100
196 ml^{-1} of water or 100 g^{-1} of shellfish digestive gland.

197 **Concentration of viral particles and nucleic acids extraction**
198 The basic steps adopted for quantification of viral pathogens in *M. edulis* and their overlying waters were sample
199 concentration, nucleic acid extraction and molecular detection, as described by Harwood *et al*. (2013). AdV F and
200 G were concentrated from 300 ml of overlying river water filtered through 0.45 µm membrane cellulose nitrate
201 filters. Magnesium chloride (five mol L^{-1} $MgCl_2$) was prepared by dissolving 475 g in 1000 ml of distilled water,
202 then filter-sterilised through 0.22 µm membrane cellulose nitrate filters. Prepared $MgCl_2$ was added to the water
203 samples in the filter unit before filtration to increase viral recovery by facilitating and enhancing virus attachment
204 to the filters (Mendez *et al*. 2004). In brief, six ml of five mol L^{-1} $MgCl_2$ was added aseptically to 300 ml of sample
205 to achieve a final concentration of 0.1 mol L^{-1} $MgCl_2$ before filtration. The filters were stored at -80 ^{o}C until
206 nucleic acid extraction. Again, ten to fifteen mussels to be analysed were cleaned with sterile running water and
207 placed in a stainless steel shucking tray. Using a shucking knife and wearing a chain-mail shucking glove, *M.*
208 *edulis* were removed from their shells. The digestive glands were separated from the flesh and intravalvular fluid.
209 The digestive glands have been shown to bio-accumulate viruses more effectively than other parts of the organism,
210 as demonstrated in numerous studies (Jothikumar *et al*. 2005; Le Guyader *et al*. 2009; Pinto *et al*. 2009; Westrell
211 *et al*. 2010; Iizuka *et al*. 2010; Baker *et al*. 2011; Bosch *et al*. 2011; Lowther *et al*. 2012; Trajano Gomes Da Silva
212 2013). Digestive glands were therefore chopped finely to expose the content and stored in centrifuge tubes at -80
213 ^{o}C until nucleic acid extraction.
214
215 The frozen filters were equilibrated at room temperature and the surface was scraped using a fresh scalpel blade
216 (Fisher Scientific) into a 1.5 ml microcentrifuge tube. Viral nucleic acids were extracted using the QIAamp
217 MinElute Virus Spin Kit (Qiagen GmbH) according to the manufacturer's instruction. The nucleic acid extracts
218 were stored at -80°C until quantification. Similarly, the frozen glands were equilibrated at room temperature and
219 200 µl of the finely chopped glands was placed in a 1.5 ml microcentrifuge tube. Viral nucleic acids were extracted
220 using the QIAamp MinElute Virus Spin Kit (Qiagen GmbH) according to the manufacturer's instruction. The
221 nucleic acid extracts were stored at -80°C until quantification.
222
223 **Detection of AdV F and G by qPCR**
224 AdV F and G were enumerated by quantitative real-time polymerase chain reaction (qPCR) (Ogorzaly *et al*. 2009)
225 on QIAGEN Rotor-Gene® Q thermocycler using PrimerDesign™ genesig AdV F and G Hexon gene Advanced
226 kit (Primerdesign UK) following the manufacturer's protocol. The components of the kit were reconstituted in
227 RNase/DNase-free water, and then vortexed to ensure complete resuspension. AdV F and G had a sequence
228 accession number of KC632648.1, anchor nucleotide position of 790 and context sequence length of 190.
229 Thermocycling steps were as follows: enzyme activation (95 ^{o}C for two minutes), 50 cycles of denaturation (95
230 ^{o}C for ten seconds) and data collection (60 ^{o}C for 60 seconds). Data were analysed using Rotor-Gene 2.1.0.9
231 software with a threshold fluorescence value of 1.000. Standards were prepared, serially diluted and quantified to
232 make standard curves following the manufacturer's protocol. The highest concentration of AdV F and G standard
233 was 2×10^5 copies μl^{-1}. Standard curve was run in triplicate and the 'pooled' standard curve was then used to
234 relate quantification cycles to copy numbers and quantity of AdV F and G in samples. Again, all data were
235 analysed with the comprehensive Rotor-Gene® Q software, which enables quantification and enhances data
236 security.
237
238 **Statistical analysis**
239 Data were transformed to $\log_{10}$, then examined using general descriptive statistics and checked for normality using
240 the skewness and kurtosis statistic, Shapiro–Wilk normality test and corresponding normal probability plots.
241 Further analyses were undertaken using Statistical Package for Social Sciences (SPSS) Version 20.0, and all data
242 were subjected to the Spearman's rank non-parametric two-tailed correlation analysis (with test of significance at
243 0.01 and 0.05) to determine whether there were positive correlations between the concentration of AdV F and G
244 and those of bacterial and viral indicators.
245
246
247
248

249 **Results**

250 **Detection of faecal indicator bacteria**

251 The concentration of *E. coli* in *M. edulis* ranged from 2.43 to 4.27 log_{10} MPN 100 g^{-1} of shellfish flesh and
252 intravalvular fluid and in overlying waters the concentration ranged from 1.55 to 4.00 log_{10} CFU 100 ml^{-1}. The
253 concentration of faecal coliforms in *M. edulis* ranged from 2.52 to 4.30 log_{10} MPN 100 g^{-1} of shellfish flesh and
254 intravalvular fluid and in overlying waters the concentration ranged from 1.56 to 4.16 log_{10} CFU 100 ml^{-1}.
255 Similarly, concentration of intestinal enterococci in *M. edulis* ranged from 2.23 to 3.97 log_{10} MPN 100 g^{-1} of
256 shellfish flesh and intravalvular fluid and in overlying waters ranged from 0.99 to 3.50 log_{10} CFU 100 ml^{-1} (Table
257 1).

258

259 **Detection of bacteriophages**

260 The concentration of somatic coliphages in *M. edulis* ranged from 3.43 to 5.36 log_{10} PFU 100 g^{-1} of digestive
261 gland (mean = 4.60 ± 0.52 log_{10} PFU 100 g^{-1}) and 2.00 to 4.02 log_{10} PFU 100 ml^{-1} in overlying waters (mean =
262 3.01 ± 0.54 log_{10} PFU 100 ml^{-1}). The concentration of F-RNA coliphages in *M. edulis* ranged from 0 to 3.82 log_{10}
263 PFU 100 g^{-1} of digestive gland (mean = 1.84 ± 1.62 log_{10} PFU 100 g^{-1}) and 0 to 2.30 log_{10} PFU 100 ml^{-1} in
264 overlying waters (mean = 0.60 ± 0.95 log_{10} PFU 100 ml^{-1}). The concentration of bacteriophages infecting
265 *Bacteroides fragilis* (GB124) in *M. edulis* ranged from 0 to 5.29 log_{10} PFU 100 g^{-1} of digestive gland (mean =
266 1.79 ± 1.64 log_{10} PFU 100 g^{-1}) and 0 to 3.35 log_{10} PFU 100 ml^{-1} in overlying waters (mean = 0.67 ± 1.13 log_{10}
267 PFU 100 ml^{-1}). Fourteen shellfish samples (31 %) and seven overlying water samples (16 %) presented positive
268 for both F-RNA coliphages and bacteriophages infecting *Bacteroides fragilis* (GB124). However, all the samples
269 (100 %) of shellfish and overlying water were positive for somatic coliphages (Table 1). Statistically significant
270 elevated levels of somatic coliphages, F-RNA coliphages and bacteriophages infecting *Bacteroides fragilis*
271 (GB124) in *M. edulis* compared with their overlying waters.

272

273 **Detection of AdV F and G**

274 Concentration of AdV F and G in *M. edulis* ranged from 0 to 2.94 log_{10} genome copies 100 g^{-1} of digestive gland
275 (mean = 0.43 ± 0.85 log_{10} genome copies 100 g^{-1}) and in overlying waters ranged from 0 to 1.34 log_{10} genome
276 copies 100 ml^{-1} (mean = 0.22 ± 0.35 log_{10} genome copies 100 ml^{-1}). The limit of detection for AdV F and G was
277 10 detectable virus genome copies 100 ml^{-1} and 100 g^{-1} for overlying waters and shellfish samples, respectively.
278 Nine shellfish samples (27 %) and eleven overlying water samples (24 %) presented positive for AdV F and G
279 (Table 1). There were statistically significant elevated levels of AdV F and G in *M. edulis* compared with levels
280 recorded in their overlying waters.

281

282 **The relationship between bacteriophages and AdV F and G**

283 The relationships between the concentrations of bacteriophages and those of AdV F and G in *M. edulis* and
284 overlying waters were analysed using a two-tailed Spearman's rank correlation at two levels of significance ($P <$
285 0.01 and $P < 0.05$) representing 99 % and 95 % confidence interval, respectively (Table 2). Levels of AdV F and
286 G in overlying waters showed a positive correlation with those in *M. edulis* (rho = 0.58, $P < 0.01$). Similarly,
287 levels of AdV F and G in overlying waters showed positive correlations with levels of somatic coliphages in both
288 overlying waters (rho = 0.66, $P < 0.01$) and *M. edulis* (rho = 0.48, $P < 0.05$). Importantly, levels of AdV F and G
289 in *M. edulis* showed a positive correlation with levels of somatic coliphages in *M. edulis* (rho = 0.55, $P < 0.01$).
290 Levels of somatic coliphages in overlying waters showed a positive correlation with those in *M. edulis* (rho =
291 0.64, $P < 0.01$). Levels of AdV F and G in overlying waters also showed positive correlations with levels of
292 bacteriophages infecting *B. fragilis* (GB124) in both overlying waters (rho = 0.43, $P < 0.05$) and *M. edulis* (rho =
293 0.49, $P < 0.05$).

294

295 Levels of somatic coliphages in overlying waters, furthermore, showed positive correlations with levels of
296 bacteriophages infecting *B. fragilis* GB124 in *M. edulis* (rho = 0.41, $P < 0.05$). Levels of somatic coliphages in
297 *M. edulis* showed a positive correlation with levels of bacteriophages infecting *B. fragilis* GB124 in *M. edulis* (rho
298 = 0.53, $P < 0.01$). Levels of F-RNA coliphages in overlying waters showed a positive correlation with those in *M.*
299 *edulis* (rho = 0.52, $P < 0.01$). Levels of bacteriophages infecting *B. fragilis* GB124 in overlying waters showed a
300 positive correlation with levels of AdV F and G in *M. edulis* (rho = 0.53, $P < 0.01$).

Discussion

This study investigated the distribution pattern of faecal indicator bacteria, bacteriophages and AdV F and G in *M. edulis* and their overlying waters, and examined whether the enumeration of bacteriophages may offer a way to predict, manage and prevent gastroenteritis caused by adenovirus contamination of mussels consumed by humans. The Shapiro-Wilk's normality test ($P > 0.05$) (Shapiro and Wilk 1965; Razali and Wah 2011) and a visual inspection of histograms, normal Q-Q plots and box plots showed that levels of *E. coli*, faecal coliforms, intestinal enterococci and somatic coliphages in both overlying waters and *M. edulis* were approximately normally distributed, whereas the levels of F-RNA coliphages, GB124 phages and AdV F and G appear not to have been normally distributed (Cramer, 1998; Cramer and Howitt 2004; Doane and Seward 2011) (Table 3). Evidently, the normality tests demonstrated a variable pattern of occurrence and distribution of faecal indicator bacteria, bacteriophages and AdV F and G in *M. edulis* and their overlying waters during the study. The difference in distribution patterns were clearly influenced by the non-detection of F-RNA coliphages, GB124 phages and AdV F and G on several sampling occasion. It is worthy of note that the statistical dataset on the varying levels of faecal indicator bacteria and bacteriophages (against which the levels AdV F and G were compared) were in broad agreement with the findings of Wyer *et al*. (2012). These authors suggested that statistical models based on adequate goodness of fit may be used to predict the probability of the presence of human adenovirus from routine surveillance of European waters for faecal coliforms.

In this study, the Spearman's rank correlation co-efficient demonstrated the bacterial indicators: *E. coli* (rho = -0.11), faecal coliforms (rho = 0.07), and intestinal enterococci (rho = 0.03) to have far less predictive value for the presence of AdV F and G in *M. edulis* (Table 4) compared with somatic coliphages (Table 2). Although, correlation analysis revealed that *E. coli* (rho = 0.57, $P < 0.01$), faecal coliforms (rho = 0.53, $P < 0.01$) and intestinal enterococci (rho = 0.45, $P < 0.05$) presented positive correlations with AdV F and G in overlying waters, these were lower than those observed using somatic coliphages (rho = 0.66, $P < 0.01$). It is interesting to note that the findings from this study contrasted with those observed by Ogorzaly *et al*. (2009) who examined the relationship between F-specific RNA phage genogroups, faecal pollution indicators and human adenoviruses in river water (that was prone to faecal pollution from anthropogenic activities that included animal rearing activities involving approximately 112,000 cows, 7,500 sheep and about 4,100 pigs) in the northeast of France, and demonstrated positive correlations between the concentration of human adenovirus and *E. coli* (rho = 0.513), enterococci (0.616), somatic coliphages (rho = 0.593) and F-RNA phage genogroup II (rho = 0.493) (Ogorzaly *et al*. 2009). This observed difference may be the result of differences in the source of the faecal pollution and the distance from the source. The difference may also be as a result of geographical variation, stability of the indicator organisms, physiochemical, hydrological and meteorological factors etc. (Formiga-Cruz *et al*. 2003).

AdV F and G were detected on several sampling occasions in overlying waters and *M. edulis* during the study period. GB124 phages were not detected on ten sampling occasions in overlying waters and on two sampling occasions in *M. edulis*, F-RNA coliphages were not detected on twelve sampling occasions in overlying waters and on six sampling occasions in *M. edulis*, whereas somatic coliphages were detected in all cases. In the samples that tested positive for AdV F and G, all investigated phages were also consistently detected at varying concentrations. However, these phages were also detected in some samples that tested negative for AdV F and G (Figure 1). These findings are consistent with other studies (Haramoto *et al*. 2007; Ogorzaly *et al*. 2009) that have demonstrated the wide spatial distribution of human adenovirus genome in faecally-impacted aquatic environments.

Phages were also detected in overlying waters and in *M. edulis* that presented negative results for AdV F and G (i.e., were below the detection limit of 1 PFU ml^{-1} or g^{-1}). Total concentrations of 32.01 log_{10} PFU 100 ml^{-1} somatic coliphages, 2.00 log_{10} PFU 100 ml^{-1} GB124 phages, and 4.30 log_{10} PFU 100 ml^{-1} F-RNA coliphages were detected in overlying waters presenting negative results for AdV F and G. Again, 52.05 log_{10} PFU 100 g^{-1} somatic coliphages, 12.00 log_{10} PFU 100 g^{-1} GB124 phages, and 17.68 log_{10} PFU 100 g^{-1} F-RNA coliphages were detected in *M. edulis* presenting negative results for AdV F and G. Again, a number of these samples may reasonably be described as 'false negatives', because, of all the AdV F and G negative samples, GB124 phages were detected on two sampling occasions in overlying waters and on eight sampling occasions in *M. edulis*, F-RNA coliphages

353 were detected on four sampling occasions in overlying waters and on 12 sampling occasions in *M. edulis*, whereas
354 somatic coliphages were detected in all cases. This shows the volume of samples which are positive for phages
355 but negative for AdV F and G. In general, somatic coliphages were detected at significantly higher levels than
356 other groups of phages (i.e., GB124 phages and F-RNA coliphages) in overlying waters and *M. edulis* presenting
357 both positive and negative results for AdV F and G. Also, bacteriophages (especially somatic coliphages) and
358 faecal indicator bacteria were detected at significantly higher levels in AdV F and G positive samples compared
359 with samples that were negative for AdV F and G (Figure 1). These findings further demonstrate the abundance
360 of somatic coliphages in faecally-polluted environments (Grabow 2004).
361
362 This work is applicable to other types of shellfish species, though different shellfish species may demonstrate
363 varying abilities to bioaccumulate microbial indicators or pathogens. This may be the result of differences in the
364 physiology of the shellfish or the pathogen, the rate of metabolism in the shellfish, the duration of exposure of the
365 shellfish to the pathogen or its source, the exposure dose and other factors, such as salinity and temperature which
366 all serve to influence filtration rates within the shellfish (Olalemi *et al*. 2016).
367
368 The current hygiene classifications for shellfisheries, which are based on levels of faecal indicator bacteria, do not
369 appear to offer an accurate indication of risk of viral contamination. There is a clear need to target alternative
370 indicators to maintain and improve the microbial safety of shellfish that are harvested for human consumption. In
371 this study, the enumeration of bacteriophages by a simple phage-lysis method was demonstrated to provide a
372 better indication of the risk of adenovirus contamination in *M. edulis* and their overlying waters than is provided
373 by the faecal indicator bacteria prescribed in EU Directives. Some studies have demonstrated the ability of
374 bacteriophages infecting *Bacteroides fragilis* GB124 to predict the presence of norovirus in mussels (Trajano
375 Gomes Da Silva 2013), and mussels and oysters (Olalemi 2015). Despite the fact that enumeration of somatic
376 coliphages better predicted the risk of viral pathogens than GB124 phages, this study demonstrates for the first
377 time the ability of the human-specific *Bacteroides fragilis* GB124 phages to predict the presence of human AdV
378 F and G in *M. edulis*. These findings support the use of bacteriophages as alternative faecal indicators and effective
379 surrogates of AdV F and G in *M. edulis* and their overlying waters. This is consistent with the findings of other
380 recent studies that have demonstrated the usefulness of bacteriophages as effective surrogates of enteric viral
381 contamination. Incorporating this relatively simple tool into shellfish safety management and planning, especially
382 as a component of routine monitoring of shellfish and their overlying waters would provide improved human
383 health protection.
384
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389
390 **Conflict of interest**
391 No conflict of interest declared.
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581
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583

584 **Table 1: Mean concentration of AdV F and G, bacteriophages and faecal indicator**
585 **bacteria in *M. edulis* and overlying waters**

Microorganisms	*M. edulis*		Overlying waters	
	Mean ± SD	Range (% +)	Mean ± SD	Range (% +)
Adenovirus F and G	0.43 ± 0.85	0.00-2.94 (27%)	0.22 ± 0.35	0.00-1.34 (24%)
Somatic coliphages	**4.60 ± 0.52**	**3.43-5.36 (100%)**	**3.01 ± 0.54**	**2.00-4.02 (100%)**
F-RNA coliphages	1.84 ± 1.62	0.00-3.82 (31%)	0.60 ± 0.95	0.00-2.30 (16%)
GB124 phages	1.79 ± 1.64	0.00-5.29 (31%)	0.67 ± 1.13	0.00-3.35 (16%)
E. coli	3.37 ± 0.51	2.43-4.27 (100%)	2.75 ± 0.76	1.55-4.00 (100%)
Faecal coliforms	3.60 ± 0.50	2.52-4.30 (100%)	2.96 ± 0.78	1.56-4.16 (100%)
Intestinal enterococci	3.10 ± 0.47	2.23-3.97 (100%)	2.46 ± 0.74	0.99-3.50 (100%)

595
596 **Key:** Enteric viruses mean Log_{10} genome copies 100 ml^{-1} or 100 g^{-1} digestive gland ± standard
597 deviation; Phages mean log_{10} PFU 100 ml^{-1} or 100 g^{-1} digestive gland ± standard deviation; Faecal
598 bacteria mean log_{10} MPN $100g^{-1}$ shellfish flesh and intravalvular fluid or CFU 100 ml^{-1} of overlying
599 waters ± standard deviation; Range (Minimum-Maximum); (% +) – Percentage of occurrence. Figure
600 in **bold** – Most abundant parameter in both *M. edulis* and overlying waters.
601

602 **Table 3: Skewness and Kurtosis, significance values for the Shapiro-Wilk normality tests based on log_{10} transformed concentration values**
603 **in *M. edulis* and their overlying waters.**
604
605

606 Microorganisms	Overlying waters Skewness (SE=0.472)	Kurtosis (SE=0.918)	Shapiro-Wilk (*p* value)	*M. edulis* Skewness (SE=0.472)	Kurtosis (SE=0.918)	Shapiro-Wilk (*p* value)	Normal distribution
608 *E. coli*	0.146	-1.001	0.300	-0.287	-0.699	0.251	Yes
609 Faecal coliforms	-0.238	-1.073	0.256	-0.539	-0.369	0.226	Yes
610 Intestinal enterococci	-0.521	-0.859	0.138	-0.353	-0.807	0.219	Yes
611 Somatic coliphages	-0.242	-0.834	0.403	-0.480	-0.406	0.518	Yes
612 F-RNA coliphages	1.013	-1.015	0.000	-0.239	-1.957	0.000	No
613 GB124 phages	1.393	0.630	0.000	0.083	-1.218	0.000	No
614 Adenovirus F and G	2.005	3.860	0.000	2.436	5.382	0.000	No

615
616
617 Key: SE = Standard error
618

619 **Table 2: Significant Spearman's rank correlation between bacteriophages and AdV F**
620 **and G in *M. edulis* and overlying waters (n=48)**

	Water AdV	Water SomC	Water F-RNA	Water GB124	Mussel AdV	Mussel SomC	Mussel F-RNA	Mussel GB124
Water AdV	1.00							
Water SomC	**0.66****	1.00						
Water F-RNA	0.17	0.19	1.00					
Water GB124	0.43*	0.25	0.18	1.00				
Mussel AdV	**0.58****	0.40	-0.04	**0.53****	1.00			
Mussel SomC	0.48*	**0.64****	0.20	0.32	**0.55****	1.00		
Mussel F-RNA	0.11	0.03	**0.52****	0.27	0.05	0.09	1.00	
Mussel GB124	0.49*	0.41*	-0.18	0.36	0.34	**0.53****	-0.04	1.00

621
622 **Key:** SomC – Somatic coliphages; F-RNA – F-RNA coliphages; GB124 – GB124 phages;
623 AdV – Adenovirus F and G; ** – **Correlation is significant at the 0.01 level (2-tailed)**; * –
624 Correlation is significant at the 0.05 level (2-tailed)
625

626 **Table 4: Significant Spearman's rank correlation between faecal indicator bacteria and**
627 **AdV F and G in *M. edulis* and overlying waters (n=48)**

	Water AdV	Water *E. coli*	Water FC	Water Ent.	Mussel AdV	Mussel *E. coli*	Mussel FC	Mussel Ent.
Water AdV	1.00							
Water *E. coli*	**0.57****	1.00						
Water FC	**0.53****	**0.97****	1.00					
Water Ent.	0.45*	**0.71****	**0.73****	1.00				
Mussel AdV	**0.58****	0.10	0.07	0.12	1.00			
Mussel *E. coli*	0.33	**0.75****	**0.72****	**0.55****	-0.11	1.00		
Mussel FC	**0.48****	**0.84****	**0.84****	**0.65****	0.07	**0.90****	1.00	
Mussel Ent.	0.16	0.38	0.38	0.39	0.03	**0.60****	**0.59****	1.00

628
629 **Key: FC –** Faecal coliforms; Ent. – Intestinal enterococci; AdV – AdV F and G; ** –
630 **Correlation is significant at the 0.01 level (2-tailed);** * – Correlation is significant at the 0.05
631 level (2-tailed)
632