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1 **Use of *Mytilus edulis* biosentinels to investigate spatial patterns of norovirus and faecal**
2 **indicator organism contamination around coastal sewage discharges**

3

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16

17 **ABSTRACT**

18 Bivalve shellfish have the capacity to accumulate norovirus (NoV) from waters contaminated with
19 human sewage. Consequently, shellfish represent a major vector for NoV entry into the human food
20 chain, leading to gastrointestinal illness. Identification of areas suitable for the safe cultivation of
21 shellfish requires an understanding of NoV behaviour upon discharge of municipal-derived sewage
22 into coastal waters. This study exploited the potential of edible mussels (*Mytilus edulis*) to accumulate
23 NoV and employed the ISO method for quantification of NoV within mussel digestive tissues. To
24 evaluate the spatial spread of NoV from an offshore sewage discharge pipe, mesh cages of mussels
25 were suspended from moorings deployed in a 9 km² grid array around the outfall. Caged mussels
26 were retrieved after 30 days and NoV (GI and GII), total coliforms and *E. coli* enumerated. The
27 experimentally-derived levels of NoV GI and GII in mussels were similar with total NoV levels
28 ranging from 7×10^1 to 1.6×10^4 genome copies g⁻¹ shellfish digestive gland (Σ GI + GII). NoV spread
29 from the outfall showed a distinct plume which matched very closely to predictions from the tidally-
30 driven effluent dispersal model MIKE21. A contrasting spatial pattern was observed for coliforms
31 (range 1.7×10^2 to 2.1×10^4 CFU 100 g⁻¹ shellfish tissue) and *E. coli* (range 0 to 1.2×10^3 CFU 100
32 g⁻¹ shellfish tissue). These data demonstrate that hydrodynamic models may help inform effective
33 exclusion zones for bivalve harvesting, whilst coliform / *E. coli* concentrations do not accurately
34 reflect viral dispersal in marine waters and contamination of shellfish by sewage-derived viral
35 pathogens.

36

37 *Keywords:* Food safety; Marine pollution; Risk assessment; Viral contamination; Wastewater
38 treatment plant.

39 1. Introduction

40 The overall global burden of human disease caused by sewage pollution of coastal waters has
41 been estimated at 4 million lost person-years annually (Moore et al., 2013). Within this, consumption
42 of bivalve molluscan shellfish contaminated with norovirus (NoV) derived from human faeces
43 represents a well-established human health risk (Lees, 2000; Malham et al., 2014). According to the
44 European Food Safety Authority (EFSA), production of shellfish in areas which are not faecally
45 contaminated represents the most effective control measure for NoV (EFSA Panel on Biological
46 Hazards, 2012). However, achieving this goal represents a major challenge to the shellfish industry
47 due to the vast number of wastewater discharges along the European coastline and the traditional co-
48 location of shellfish harvesting areas around estuaries and coastal communities where sewage
49 contamination is most apparent (Fleming et al., 2006; Paraskevas et al., 2002). Although significant
50 improvements have been made in the microbiological quality of coastal waters in Europe (Campos et
51 al., 2013), in some regions this is being hampered by the increased pressure on the wastewater
52 infrastructure (due to a rise in human population and extreme weather events which are increasing
53 the volumes of untreated sewage being released into coastal waters; Matthiessen and Law, 2002;
54 Stapleton et al., 2008). The introduction of exclusion zones around sewage discharges preventing
55 shellfish harvesting is being considered in Europe and elsewhere, however, their delineation and
56 social acceptability remains difficult, particularly if a quasi-zero risk of contamination is required
57 (Dunn et al., 2014; Fitzgerald, 2014).

58 Traditionally, bacteria including coliforms and enterococci have been used to estimate the
59 level of faecal contamination of water and / or shellfish (Oliveira et al., 2011; Pancorbo and Barnhart,
60 1992), and may be referred to collectively as Faecal Indicator Bacteria (FIB). In Europe, *Escherichia*
61 *coli* is adopted as the traditional indicator of faecal (sewage) contamination in shellfish and is used
62 for risk assessment and management purposes (Anon, 2004). However, studies have indicated that *E.*
63 *coli* or total coliforms provides a relatively poor indicator of the potential risk of contracting illness
64 from a wide range of human pathogenic organisms (Ferguson et al., 1996; Griffin et al., 2001; Majori

65 et al., 1984). Reasons for this poor correlation include the different environmental persistence of
66 coliforms relative to viruses, protozoa and other bacteria in marine water, and differences in their
67 spatial and temporal discharge patterns (Fong and Lipp, 2005). In addition, *E. coli* may be introduced
68 to the environment from agricultural livestock making it a poor indicator of point-source, human-
69 derived wastewater discharges (Campos et al., 2013). Therefore, *E. coli* and NoV may originate from
70 different sources, be conveyed into the marine environment via alternate routes, may be susceptible
71 to different stresses, and may be differentially accumulated by shellfish. The current faecal indicator
72 approach may underestimate the risk from human viruses which are introduced from inadequately-
73 or un-treated wastewater (De Donno et al., 2012; Fong and Lipp, 2005; Griffin et al., 1999).

74 Methods for direct recovery and concentration of enteric viruses from coastal waters include
75 adsorption to and elution from charged membranes or particles, and ultrafiltration and flocculation
76 approaches (Katayama et al., 2002; Cormier et al., 2014; Calgua et al., 2008). Complications include
77 the need for large sample volumes and difficulties in removing PCR-inhibitory substances originating
78 from the marine environment. Of the methods available, the best choice may depend upon specific
79 PCR-inhibitory compounds present in samples from different locations, and the target virus
80 (Rodriguez et al., 2012). Recently, streamlined processes giving high recoveries of Hepatitis A Virus
81 from seawater using zeolite have been described and other studies have been able to report on the
82 presence and levels of enteric viruses recovered directly from coastal waters using flocculation
83 (Cormier et al., 2016; Kaas et al., 2016). However, direct recovery of viruses from environmental
84 waters can only provide a snapshot in time. This may limit our understanding of viral pollutant flow
85 in areas subject to intermittent discharges and/or complex tidal regimes.

86 Bivalve shellfish have been shown to efficiently accumulate viral particles (Asahina et al.,
87 2009; De Donno et al., 2012; Nenonen et al., 2008) and sensitive quantitative methods which detect
88 NoV genomes in molluscan shellfish using molecular techniques (PCR) exist (Anon, 2013; Lees and
89 CEN WG6 TAG4, 2010). This offers the potential to use shellfish as an integrator of NoV pollution
90 within both marine and estuarine environments. NoV levels bioaccumulated in oysters experimentally

91 placed at several locations within an estuary impacted by sewage discharges have recently been
92 presented (Campos et al., 2015). Due to their fixed location, shellfish can be employed to provide a
93 spatial map of viral pollutant flow from point source wastewater discharges. Further, due to their
94 fixed location, they can be employed to provide a spatial map of viral pollutant flow from point source
95 wastewater discharges.

96 The position and dilution of wastewater effluent plumes has been determined using
97 approaches such as bacterial, bacteriophage or dye tracing (Hammerstein et al., 2015). More recently,
98 hydrodynamic models have been used to predict the spatial and temporal patterns of contamination
99 originating from coastal discharges (Dunn et al., 2014). Such models have been parameterized to
100 predict microbial concentrations and the potential for shellfish exposure (Gourmelon et al., 2010;
101 Muhammetoglu et al., 2012; Riou et al., 2007). Validation of these models, however, remains critical
102 if they are to be adopted for risk assessment purposes and coastal zone management (Gourmelon et
103 al., 2010).

104 The aim of this study was to improve our understanding of NoV behaviour upon discharge of
105 sewage into coastal waters. Our first objective was to derive and compare the spatial contamination
106 patterns for NoV genogroups one and two (GI and GII), *E. coli* and total coliforms about a long sea
107 wastewater outfall. Our second objective was to compare these field-derived spatial contamination
108 patterns with those predicted from a tidally-driven effluent dispersal model. In lieu of EFSA advice
109 to produce shellfish in waters which are not faecally contaminated and considering that FIB may be
110 a poor indicator of sewage-derived viral contamination, the specific intentions were a) to detect any
111 differences in the spatial contamination pattern for NoV, which might not be captured by the FIB
112 approach, and b) to determine whether hydrodynamic models may offer greater potential for
113 prediction of NoV contamination and designation of shellfish harvesting exclusion zones.

114

115 **2. Materials and methods**

116 *2.1. Site selection*

117 The offshore submarine sewage outfall pipe at Kinnel Bay, North Wales (53.336901N,
118 3.569200W; Fig. 1), which serves a total population equivalent of 77,953 people, was selected for
119 this study. The discharge is consented for up to 38,860 m³ d⁻¹ with a dry weather flow not exceeding
120 15,941 m³ d⁻¹. Sewage released from the outfall receives only primary and secondary treatment
121 (activated sludge) prior to discharge. Previous studies have indicated that similar activated sludge
122 wastewater treatment plants (WWTP) may achieve reductions for NoV GI and GII concentrations of
123 less than one log₁₀ genome copy (Flannery et al., 2012; Nordgren et al., 2009). In addition to treated
124 effluent, under high flow conditions (i.e. stormflow) there are periods when storm water is discharged
125 untreated into marine waters via this outfall, however, no such events were recorded during the
126 duration of this trial. In compliance with EU bathing water quality standards at proximate beaches,
127 the outfall discharges into coastal waters of Liverpool Bay at 4 km offshore, in 6.9 m of water at
128 Lowest Astronomical Tide. The conditions reported here are typical of many other discharge points
129 around the European coastline. We hypothesized that these conditions could result in a significant
130 release and persistence of potential human pathogens in marine waters. This site was also chosen as
131 shellfish are commercially farmed on a large scale near the study area with the harvested product
132 exported to a range of European countries.

133

134 2.2. Sampling regime and shellfish biosentinels

135 This study exploited the potential of the common (or blue) edible mussel *Mytilus edulis* (L.)
136 to accumulate virions and bacterial cells from shellfish growing waters. *Mytilus edulis* were collected
137 50 km away from the study site. To minimize variability associated with growing conditions, animals
138 were collected via a single short trawl (<5 m) of broadcast-cultivated animals from a commercial bed
139 with a long term EU designation of Class B (i.e. 230-4600 *E. coli* CFU per 100 g of flesh) and which
140 has a history of low level NoV contamination. The animals were washed, size graded (>45 mm) and
141 200 animals randomly selected for baseline enumeration of NoV and *E. coli* at time zero (T_0). Ten
142 replicate samples of 10 animals were analyzed for NoV and 10 replicate samples of 50 g shellfish

143 flesh for total coliforms and *E. coli*. Seventy eight batches containing 35 live animals were then placed
144 in individual net bags (300 × 300 mm). Six net bags were then placed in each of 13 plastic cages to
145 allow collection of one net bag from each cage at six time points of ~30 d interval. Cages were placed
146 in triplicate at 13 independent points in a diamond-shaped array around the wastewater outfall (Fig.
147 1). The cages were suspended at a sea depth of 1 m by attaching them to a plough anchored Polyform
148 A3 buoy. The individual sample points were separated by 1 km in x and y dimensions. The cages
149 were deployed in March when NoV community outbreaks were close to maximal (PHE, 2016) and
150 the first samples were recovered 30 d later in April, 2012.

151

152 2.3. *Quantification of norovirus in mussels*

153 NoV quantification in mussel digestive tissue was determined by quantitative reverse-
154 transcription PCR (qRT-PCR) in accordance with the approved method of the European Committee
155 for Standardization (CEN) (Lees and CEN WG6 TAG4, 2010; Lowther et al., 2012a). Briefly, tissue
156 homogenates were prepared by Proteinase K digestion of a 2 g aliquot of pooled digestive glands
157 dissected from 10 animals and after the addition of Mengovirus vMC₀ as an extraction control. RNA
158 extraction was performed with a Nuclisens miniMAG[®] and magnetic extraction reagents
159 (bioMérieux Inc., Durham, NC) following the manufacturer's protocol. The positive controls were
160 derived from homogenates prepared as per the samples but after addition of 1 Lenticule[®] disc of
161 Norovirus Reference Material for each genogroup (Public Health England, London, UK) to ten
162 digestive glands. The animals used for the positive controls originated from extra mesh bags placed
163 within the experimental cages. One-step qRT-PCR for Mengovirus (extraction control) and for both
164 NoV genogroups, including plate layout, and reaction mixes, were performed exactly as described by
165 Lowther et al. (2012a) except for the genogroup II assay where TAMRA was used as the quencher.
166 The thermocycler used was an Applied Biosystems 7900HT (Life Technologies Ltd, Paisley, UK).
167 The use and treatment of a suite of qRT-PCR controls and all quantification steps also followed the
168 same methods of Lowther et al. (2012a). Three aliquots of extracted RNA per sample were tested in

169 each NoV genogroup-specific qRT-PCR assay, average quantities from three replicates giving overall
170 quantity in detectable genome copies g^{-1} digestive gland (gc g^{-1}). Extraction efficiency and RT-PCR
171 efficiency/inhibition were assessed using Mengovirus vMC₀ and RNA external controls, respectively.
172 Retesting was undertaken according to action thresholds for extraction and RT-PCR efficiencies of
173 1% and 25% respectively or due to failed positive/negative PCR controls. No adjustment for losses
174 during processing or RT-PCR inhibition was made (uncorrected). This system was in agreement with
175 the principles outlined in the draft Technical Specification developed by the joint CEN/ISO working
176 group for standardization of methods for detection of viruses in foodstuffs (Lees and CEN WG6
177 TAG4, 2010).

178

179 2.4. Quantification of *E. coli* and coliforms in mussels

180 Culture methods were used for determination of bacterial Colony Forming Units (CFU) in
181 line with the European Union Shellfish Water Directive (EU, 2006). Bacterial colony forming units
182 were enumerated from shellfish flesh by direct plating onto selective agar as described in Clements
183 et al. (2013). Briefly, mussel samples were washed with sterile seawater to remove any residual
184 sediment, debris and encrusting organisms before swabbing with 100% methanol to remove the shell
185 surface biofilm. Samples were left for approximately 15 min to allow the methanol to fully evaporate.
186 Mussels were opened aseptically and 50 g of flesh and intra-valvular fluid was obtained. Samples
187 were homogenized for 60 s at $10,000 \text{ rev min}^{-1}$ using a Bamix[®] blender (Seal Rock Enterprises Ltd.,
188 Bishops Stortford, UK). From the resulting homogenate, 200 μl was plated onto Brilliance[®] selective
189 agar (#CM0956; Oxoid Ltd, Basingstoke, UK) to determine *E. coli* and coliform counts. All plates
190 were inverted and incubated at 37°C and bacterial CFU enumerated after 24 h.

191

192 2.5. Statistical analysis

193 To ensure our data are comparable with survey data generated by the UK government National
194 Reference Laboratory (Lowther et al., 2012a), samples returning “not detected” results for a particular

195 NoV genogroup were assigned a score of 20 gc g⁻¹ for that genogroup (half the limit of detection;
196 LOD). Samples giving positive results below the limit of quantification (LOQ; 100 gc g⁻¹) were
197 assigned a score of 50 gc g⁻¹. Statistical analysis was carried out using SPSS Statistics v20 (IBM
198 Corp., Armonk, NY) while geostatistical analysis was carried out in ArcGIS v9.3.1 (ESRI Inc.,
199 Redlands, CA) using the spline method in the Spatial Analyst toolbox.

200

201 2.6. Hydrodynamic modelling

202 The Danish Hydraulic Institute (DHI) MIKE21 AD/HD hydrodynamic and water quality
203 model was used to describe the dispersion of the effluent plume from the offshore outfall (DHI, 2003;
204 DHI, 2011; Ekebjærg and Justesenu, 1991; Siegle et al., 2007). We chose this model due to its
205 extensive use for simulating hydrodynamics, water quality, wave dynamics and related processes in
206 UK coastal areas (Babu et al., 2005; Davies et al., 2009; Williams et al., 2014). The model is also
207 used as part of the Bathing Water Compliance Assessment undertaken by Intertek Energy and Water
208 Consultancy Services for this stretch of coastline on behalf of Welsh Water. The model had a
209 resolution of 45 × 45 m and encompassed 600 × 400 such cells. The model simulation was undertaken
210 for a 3 day period, run under a calm wind scenario, with a model time step of 60 s and an output
211 timestep of 10 min. The model predicted the effluent plume dispersal of a 1 m³ s⁻¹ discharge released
212 continuously over 12 h at a concentration typical of crude sewage (1 × 10⁶ pathogen units l⁻¹). No
213 microbiological decay rate was used in the model to describe loss of cell viability, instead it was run
214 as a conservative microbiological pollutant. We considered this appropriate for our purposes as NoV
215 exhibits moderate persistence in UK coastal waters (Dancer et al., 2010). The sum concentration of
216 pathogen in each grid cell over the model run was recorded and graphically presented (i.e. total
217 number of pathogen units predicted to pass through a cell over a model run). Therefore the measure
218 is an amalgamation of all the modelled timesteps and does not denote a moment in time. The summed
219 concentration for specific model cells (i.e. where our experimental moorings were located) was
220 extracted and used as a predictor of relative exposure to contaminants originating from the plume.

221

222 **3. Results**

223 *3.1. Baseline microbiological contaminant levels*

224 Baseline levels for NoV GI and GII, *E. coli* and coliforms in mussels used to stock the
225 experimental cages at T_0 are shown in Table 1. Overall, the levels of NoV GII were very similar
226 between the replicate batches (CV = 15.9%) with the levels being approximately 60 times higher than
227 those of NoV GI. In 8 out of 10 replicates, NoV GI could only be detected at levels which were below
228 the LOQ while NoV GI was not detected in one out of the ten replicates. The concentration of *E. coli*
229 in the shellfish flesh was low, represented 12% of the total coliforms and had a high variability
230 between the replicate batches (CV = 128%).

231

232 *3.2. Norovirus and bacterial levels in mussels after 30 days*

233 After 30 d (April) all moorings remained *in-situ* and the mussels (51.5 ± 0.2 mm, 98.0%
234 survival) from 11 of 13 sites contained quantifiable levels of NoV GI and GII, both showing a distinct
235 spatial pattern. After 60 d (May) only 2 and 3 of 12 remaining moorings provided samples with NoV
236 levels above the method limit of quantification for GI and GII, respectively. As the summer
237 progressed, NoV remained mostly below quantifiable levels. We therefore present the spatial pattern
238 derived for the initial 30 d deployment period.

239 After being deployed around the wastewater outfall for 30 d, NoV GI levels significantly
240 increased from the T_0 baseline value of 52 ± 6 gc g⁻¹ to 1990 ± 619 gc g⁻¹ when averaged across all
241 sites ($P < 0.05$). In contrast, across the sampling array, mean NoV GII levels decreased slightly from
242 the T_0 baseline value of 3311 ± 167 gc g⁻¹ to 1990 ± 851 gc g⁻¹ after 30 d, although this was not
243 statistically significant due to the variability across samples. If the point directly above the outfall is
244 omitted, the levels of GI and GII in the mussels were highly correlated across all the samples ($r^2 =$
245 0.98 ; $P < 0.001$). Within the sampling array, significant spatial variation in NoV GI and GII levels in
246 the mussels was apparent (Fig. 2); mussels either accumulated or eliminated NoV depending on their

247 situation. Overall, both NoV GI and GII showed much greater dispersion to the East and West and
248 symmetry about the outfall. NoV GI decreased with distance in all directions from the outfall (7825
249 gc g^{-1}), however, for NoV GII, the highest contamination levels (9958 gc g^{-1}) were observed at the
250 most Easterly sample point, 2 km to the East of the outfall (7954 gc g^{-1}). For both NoV genogroups,
251 levels in the shellfish declined more rapidly to the North and South of the outfall than to the East and
252 West. However, significantly higher NoV contamination was observed South of the outfall (onshore)
253 than to the North. The mean concentration for three adjacent sites South of the outfall ($\Sigma\text{GI} + \text{GII}$
254 $2255 \pm 154 \text{ gc g}^{-1}$) was significantly higher than for three adjacent sites to the North ($\Sigma\text{GI} + \text{GII}$ 329
255 $\pm 84 \text{ gc g}^{-1}$) for both GI and GII (t-test $P = 0.005$ and $P = 0.019$ respectively).

256 *E. coli* contamination of shellfish flesh increased in the samples collected directly over the
257 outfall (approximately 3-fold from the T_0 value of 400 ± 163 to $1167 \pm 166 \text{ CFU } 100 \text{ g}^{-1}$) and
258 decreased to undetectable levels at 5 sites (Fig. 2). The total coliform content of the mussels increased
259 approximately 6-fold when placed directly over the outfall (3400 ± 670 at T_0 to $20,833 \pm 1764 \text{ CFU}$
260 100 g^{-1} at 30 d) and decreased at all but four sites where there was no significant change. Total
261 coliforms and *E. coli* concentrations were also highly correlated across all sites ($r^2 = 0.82$; $P < 0.001$).
262 For *E. coli* and coliforms the spatial contamination pattern around the outfall were slightly different.
263 *E. coli* was detected at highest levels directly over the outfall, but was not detected within the transect
264 to the West nor the North of the outfall, being skewed East and towards the shore. Total coliforms
265 were also detected at highest levels over the outfall, and also showed a skewed distribution East and
266 slightly towards shore, but were detected at all sites. Correlation between total coliform and total NoV
267 ($\Sigma\text{GI} + \text{GII}$) concentrations was weakly significant ($r^2 = 0.43$; $P < 0.01$). *E. coli* did not correlate
268 significantly with NoV levels ($r^2 = 0.28$, $P > 0.05$).

269

270 3.3. Comparison of experimental results with hydrodynamic model predictions

271 Our data failed the assumptions for regression analysis, but Spearman's rank-order correlation
272 coefficients (r_s) and their significance were calculated between the model prediction for water

273 concentrations and experimentally derived levels of NoV, *E. coli* and total coliforms in shellfish tissue
274 (Table 2). Both NoV GI and GII showed strong correlations with model predictions, which were
275 highly significant. However, neither *E. coli* nor total coliforms showed any significant correlation
276 with the model predictions. Experimentally-derived levels found in the shellfish tissues were plotted
277 and compared with predicted relative concentrations according to the model for North-South and
278 West-East transects passing over the outfall (Fig. 3). The relative values predicted by the model were
279 normalized to the values found directly above the outfall for each measure. Overall, NoV (GI and
280 GII) results showed very good agreement with the model simulations. To the West of the outfall, and
281 particularly for GII, predictions and experimentally-derived levels matched very closely while to the
282 East there were some differences. Slightly higher levels than those predicted by the model were also
283 found 1 km to the South of the outfall for both NoV GI and GII. The model overestimated the relative
284 levels for *E. coli* and total coliforms both to the East and to the West of the outfall (Fig. 3). However,
285 higher levels than the model would predict were found to the South (onshore) of the outfall.

286

287 **4. Discussion**

288 *4.1. Spatial patterns of NoV accumulation in mussels*

289 This field-based study investigated the spatial accumulation of NoV and FIB around an
290 offshore coastal discharge originating from a large municipal WWTP. The low levels of NoV GI in
291 the biosentinel mussels used to stock the experiment allowed us to obtain clear spatial patterns of
292 contamination around the outfall after a 30 d period. A period of 23 d has been considered sufficient
293 for transplanted oysters to stabilize and represent *in situ* background levels (Campos et al., 2015).
294 Higher initial levels of NoV GII in the mussels used to stock the experiment were observed to either
295 increase at some sites, or decline at others, revealing a similar pattern. This suggests that the levels
296 after 30 d are representative of contamination *in situ*, depending upon relative exposure to the effluent
297 plume during a peak period of NoV community incidence (PHE 2016). Furthermore, spatial
298 contamination patterns for GI and GII NoV were highly correlated. A peak NoV GII concentration

299 observed 2 km East of the outfall could indicate a secondary contamination source (e.g. River Clwyd)
300 impacting this location. The most contaminated sites by either NoV genogroup all occupy the East-
301 West transect through the center point of the array, over the outfall, and concentrations declined
302 steeply with distance both to the North and South. This finding was expected due to the reversing
303 East- and Westerly currents during ebb and flow, and is in visual agreement with hydrodynamic
304 model predictions for the same sewage discharge plume. It coincides with a strong correlation
305 between model predictions and experimentally-derived levels for both NoV GI and GII. In the future,
306 we expect that this type of correlation can be used to predict potential NoV levels using summed or
307 average effluent dilutions as predicted by hydrodynamic models. This would greatly help the
308 generation of tools for determining shellfish production exclusion zones around other outfalls for
309 which a hydrodynamic model is available (e.g. a zone where mussels may be expected to accumulate
310 >1000 NoV gc g^{-1}). Such an approach would have clear benefits over arbitrary proximity-based
311 zoning as detailed by Fitzgerald (2015) and Silva et al. (2011).

312

313 *4.2. NoV GI and GII accumulation ratios in mussels*

314 Baseline measurements made at the start of the experiment (T_0) showed a much greater
315 abundance of NoV GII relative to the amount of NoV GI present in the mussels (GI:GII ratio = 0.016
316 ± 0.001). This ratio is highly consistent with NoV outbreaks and presence within the wider
317 community measured during the same time (monthly Mar-Apr mean GI:GII ratio = 0.016 ± 0.005 ;
318 mainly associated with GII.4; PHE, 2016). Interestingly, however, after being deployed around the
319 outfall for 30 d, levels of GI in mussels markedly increased becoming similar to NoV GII levels
320 across all samples (GI:GII ratio = 0.98 ± 0.15). Due to access issues, effluent samples of wastewater
321 were not available for analysis. However, factors known to affect the ratio of GI:GII ratio in
322 wastewater and shellfish include: (i) prevalence of GI:GII infection in the community, (ii) their
323 differential resistance to water treatment processes, (iii) differences in biotic and abiotic degradation
324 in seawater, and (iv) differential accumulation and subsequent loss from shellfish tissues. The ratio

325 of NoV GI:GII ratio has remained relatively stable in the human population over a long time (PHE,
326 2016). Although there is a possibility of a high community prevalence of NoV GI infection during
327 the study period, there is strong evidence to suggest that the other three factors contributed to the
328 preferential accumulation of GI in our shellfish. Firstly, Da Silva et al. (2007) and Rajko-Nenow et
329 al. (2013) both present data to suggest that NoV GI is more resistant to WWTP processes than NoV
330 GII. Secondly, in terms of environmental persistence, NoV GI may be more stable in the water
331 environment than GII (Lysén et al., 2009). Thirdly, it has been shown that NoV GI may accumulate
332 more efficiently and strongly in oysters and mussels than NoV GII (Langlet et al., 2015; Ventrone et
333 al., 2013). In addition, NoV GII accumulates at sites in shellfish where it might be more susceptible
334 to being destroyed (Maalouf et al., 2010; Maalouf et al., 2011). Lastly, a depuration study by Polo et
335 al. (2014) showed that GI showed greater retention in mussel tissue when exposed to clean seawater.
336 Taken together, this also correlates with the finding that NoV GI is more frequently encountered in
337 shellfish-related NoV outbreaks (LeGuyader et al., 2012). Low levels (below LOQ) of both GI and
338 GII in most samples collected in and after May (data not presented) is not surprising given the widely
339 recognized seasonality of NoV incidence in the community and detection in shellfish (Lowther et al.,
340 2012a).

341 342 4.3. Spatial patterns of faecal indicator bacteria accumulation in mussels

343 In contrast to NoV, no significant agreement was found between the measured concentrations
344 of *E. coli* or coliforms in mussels and the modelled effluent plume exposure. Furthermore, whilst *E.*
345 *coli* correlated with total coliforms and NoV GI correlated strongly with NoV GII, no significant
346 correlation was found between *E. coli* and NoV. Indeed, NoV GI and GII were detected in mussels
347 at very high concentrations at sites at which *E. coli* was not detected, notably to the West of the
348 outfall. We are aware that the tidal current was flowing to the East at the time of sampling and
349 therefore mussels to the West are likely to have been less recently exposed to the effluent plume. This
350 is consistent with evidence that FIB are indicators of recent faecal contamination but NoV can persist

351 for weeks in shellfish tissue (Johne et al., 2011). The water is deeper to the West of the outfall and a
352 differential effect of water depth upon NoV / FIB behavior is also plausible given potential association
353 with particles and related sedimentation / resuspension phenomena. Importantly, all cages were
354 suspended at 1 m below the surface rather than on the seabed. Conversely, FIB were detected at sites
355 at which NoV was not detected, with the distribution of FIB being somewhat more skewed towards
356 the shore. We hypothesize that secondary non-point sources, which may be of animal origin, affect
357 this pattern. Therefore, this study suggests that FIB indicate the presence of faecal contamination but
358 may not accurately reflect persistent contamination by viral pathogens associated with human-sewage
359 effluent.

360

361 *4.4. Implications for human health*

362 The regulations for the commercial sale of shellfish in Europe are solely based on
363 concentrations of *E. coli* in shellfish flesh. All the mussels in this study recovered from around the
364 WWTP outfall after exposure for 30 d would be deemed Class B (<4600 *E. coli* 100 g⁻¹). After
365 depuration in an approved facility this would permit them to be sold on the open market. Based on
366 current evidence it is clear that current depuration practices would have been inadequate at removing
367 NoV from our shellfish (Polo et al., 2014; Sharp et al., 2016).

368

369 **5. Conclusions**

370 Our research has five key conclusions:

371 1. Outfalls dispensing effluent of this type (secondary treated wastewater) are common and
372 result in a significant environmental release of NoV during outbreaks in the human population. This
373 can result in high levels of NoV accumulation in shellfish. Investment in wastewater treatment
374 technology could reduce the level of risk in shellfisheries and recreational waters impacted by sewage
375 discharges.

376 2. Mussels with intrinsically low NoV loads can be used as effective bio-sentinels for NoV
377 pollution in marine waters. As viruses appear to be more persistent in shellfish tissue than some FIB,
378 they may provide a more integrated pollution signal. It is also likely that they can be used to
379 simultaneously evaluate the prevalence of a wide range of human pathogenic viruses in marine waters
380 (Bagordo et al., 2013; Diez-Valcarce et al., 2012). It should be noted, however, that a reliance on
381 NoV alone may provide a poor indicator of other viral pathogens and we recommend the introduction
382 of multi-viral standards for evaluating the potential contamination of recreational waters and shellfish
383 harvesting areas.

384 3. It is clear that current shellfish hygiene regulations based on *E. coli* alone are inadequate to
385 protect the human population from consuming shellfish contaminated with high loads of viral
386 pathogens. The mussels recovered here contained NoV levels up to 1.6×10^4 gc g⁻¹, while in
387 comparison, the human infective dose for NoV is very low (≥ 18 viral particles; Hall, 2012). While
388 we cannot confirm that all the NoV contained in our mussels remained infective to humans, from a
389 risk assessment perspective it is safest to assume that there is some infection potential. Further, there
390 is recent evidence to show that the amount of genome copies detected in shellfish is generally
391 proportional to risk (Lowther et al., 2012b). While adequate cooking may eliminate the risk of
392 contracting NoV, there are many instances where the product is eaten raw or partially cooked or
393 where cross contamination can occur during food preparation (Flannery et al., 2014). We conclude
394 therefore that viral standards are required for shellfish destined for human consumption.

395 4. Methods for the quantitative recovery of viruses from marine waters have improved but
396 water samples can still provide only snapshots of information from potentially complex tidal systems.
397 Their low abundance and ephemeral nature also limits their ability to assess risk. This is limiting the
398 introduction of viral surveillance measures for bathing waters. Mussel biosentinels therefore offer a
399 cost-effective way of measuring microbiological pollution, integrated over a time period, particularly
400 in recreational waters. In this scenario, mussels could be easily deployed on buoys at the perimeter
401 of the bathing zone and sent for routine analysis.

402 5. Mathematical hydrodynamic models offer great potential in the delineation of shellfish
403 harvesting exclusion zones, especially where contamination arises from point source discharges, as
404 per this study. However, more work is needed to validate and improve these models from a viral risk
405 assessment perspective. Part of this needs to include validation for a range of viruses including those
406 which can be assessed for infectivity, and for a range of scenarios (e.g. estuarine/coast typologies)
407 and receptors (beaches vs shellfisheries) and to encompass the full range of environmental conditions
408 (e.g. storms, seasonal). In order to parameterize models, studies should make direct comparison
409 between viral concentrations in shellfish biosentinels and in effluent released during the period. Based
410 on this study, we conclude that mussel biosentinels offer a cost effective way of validating these
411 models.

412

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420

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607 **Figure legends**

608 **Fig. 1.** Map showing the location of the municipal wastewater treatment plant and its offshore
609 discharge point around which an array of biosentinels cages containing mussels were placed in a 1
610 km diamond grid.

611
612 **Fig 2.** Experimentally measured and modelled concentrations of microbiological contaminants in
613 water and biosentinel shellfish in response to an offshore discharge of wastewater. Panel A shows the
614 predicted plume of a conservative microbiological pollutant released from the offshore discharge
615 point into the coastal water. Model simulations were undertaken with MIKE21. Panels B-E show
616 experimentally-derived spatial patterns of NoV GI (Panel B), NoV GII (Panel C), *E. coli* (Panel D)
617 and total coliforms (Panel E). The maps for Panels B-E were derived from the amount of indicator
618 organism accumulated in the mussel biosentinels. For NoV GI and GII, contours represent detectable
619 genome copies g^{-1} of digestive gland. Total coliforms and *E. coli* contours represent CFU $100 g^{-1}$
620 shellfish flesh and intravalvular fluid. The scale of all Panels is the same.

621
622 **Fig. 3.** Direct comparison of experimentally measured and modelled concentrations of four
623 microbiological indicators in shellfish in response to an offshore discharge of wastewater. The graphs
624 represent either the West-East or North-South transects shown in Figure 1. Bars represent the
625 experimental data and dotted lines show the predicted relative concentrations extracted from the
626 hydrodynamic model and normalized to the experimentally-derived value for the sampling point
627 located directly over the outfall.