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

Winterbourn, James; Clements, Katie; Lowther, James A.; Malham, Shelagh; McDonald, James; Jones, David

Water Research

DOI:
10.1016/j.watres.2016.09.002

Published: 15/11/2016

Peer reviewed version

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

James B. Winterbourn[a,*], Katie Clements[b], James A. Lowther[c], Shelagh K. Malham[b], James E. McDonald[d], Davey L. Jones[a]

[a] *School of Environment, Natural Resources & Geography, Bangor University, Bangor, Gwynedd, LL57 2UW, UK*

[b] *School of Ocean Sciences, Bangor University, Bangor, Gwynedd, LL59 5AB, UK*

[c] *CEFAS, The Nothe, Barrack Road, Weymouth, Dorset, DT4 8UB, UK*

[d] *School of Biological Sciences, Bangor University, Bangor, Gwynedd, LL57 2UW, UK*

*Corresponding author: Environment Centre Wales, Bangor University, Deiniol Road, Bangor, Gwynedd, LL57 2UW, UK.

E-mail address: benwinterbourn@gmail.com (Ben Winterbourn)

ABSTRACT

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

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

1. Introduction

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

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

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

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

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

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

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

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

2. Materials and methods

2.1. Site selection

The offshore submarine sewage outfall pipe at Kinmel Bay, North Wales (53.336901N, 3.569200W; Fig. 1), which serves a total population equivalent of 77,953 people, was selected for this study. The discharge is consented for up to 38,860 m^3 d^{-1} with a dry weather flow not exceeding 15,941 m^3 d^{-1}. Sewage released from the outfall receives only primary and secondary treatment (activated sludge) prior to discharge. Previous studies have indicated that similar activated sludge wastewater treatment plants (WWTP) may achieve reductions for NoV GI and GII concentrations of less than one log_{10} genome copy (Flannery et al., 2012; Nordgren et al., 2009). In addition to treated effluent, under high flow conditions (i.e. stormflow) there are periods when storm water is discharged untreated into marine waters via this outfall, however, no such events were recorded during the duration of this trial. In compliance with EU bathing water quality standards at proximate beaches, the outfall discharges into coastal waters of Liverpool Bay at 4 km offshore, in 6.9 m of water at Lowest Astronomical Tide. The conditions reported here are typical of many other discharge points around the European coastline. We hypothesized that these conditions could result in a significant release and persistence of potential human pathogens in marine waters. This site was also chosen as shellfish are commercially farmed on a large scale near the study area with the harvested product exported to a range of European countries.

2.2. Sampling regime and shellfish biosentinels

This study exploited the potential of the common (or blue) edible mussel *Mytilus edulis* (L.) to accumulate virions and bacterial cells from shellfish growing waters. *Mytilus edulis* were collected 50 km away from the study site. To minimize variability associated with growing conditions, animals were collected via a single short trawl (<5 m) of broadcast-cultivated animals from a commercial bed with a long term EU designation of Class B (i.e. 230-4600 *E. coli* CFU per 100 g of flesh) and which has a history of low level NoV contamination. The animals were washed, size graded (>45 mm) and 200 animals randomly selected for baseline enumeration of NoV and *E. coli* at time zero (T_0). Ten 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 vMC_0 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

each NoV genogroup-specific qRT-PCR assay, average quantities from three replicates giving overall quantity in detectable genome copies g^{-1} digestive gland (gc g^{-1}). Extraction efficiency and RT-PCR efficiency/inhibition were assessed using Mengovirus vMC_0 and RNA external controls, respectively. Retesting was undertaken according to action thresholds for extraction and RT-PCR efficiencies of 1% and 25% respectively or due to failed positive/negative PCR controls. No adjustment for losses during processing or RT-PCR inhibition was made (uncorrected). This system was in agreement with the principles outlined in the draft Technical Specification developed by the joint CEN/ISO working group for standardization of methods for detection of viruses in foodstuffs (Lees and CEN WG6 TAG4, 2010).

2.4. Quantification of E. coli and coliforms in mussels

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

2.5. Statistical analysis

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

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

2.6. Hydrodynamic modelling

The Danish Hydraulic Institute (DHI) MIKE21 AD/HD hydrodynamic and water quality model was used to describe the dispersion of the effluent plume from the offshore outfall (DHI, 2003; DHI, 2011; Ekebjærg and Justesenu, 1991; Siegle et al., 2007). We chose this model due to its extensive use for simulating hydrodynamics, water quality, wave dynamics and related processes in UK coastal areas (Babu et al., 2005; Davies et al., 2009; Williams et al., 2014). The model is also used as part of the Bathing Water Compliance Assessment undertaken by Intertek Energy and Water Consultancy Services for this stretch of coastline on behalf of Welsh Water. The model had a resolution of 45 × 45 m and encompassed 600 × 400 such cells. The model simulation was undertaken for a 3 day period, run under a calm wind scenario, with a model time step of 60 s and an output timestep of 10 min. The model predicted the effluent plume dispersal of a 1 m^3 s^{-1} discharge released continuously over 12 h at a concentration typical of crude sewage (1×10^6 pathogen units l^{-1}). No microbiological decay rate was used in the model to describe loss of cell viability, instead it was run as a conservative microbiological pollutant. We considered this appropriate for our purposes as NoV exhibits moderate persistence in UK coastal waters (Dancer et al., 2010). The sum concentration of pathogen in each grid cell over the model run was recorded and graphically presented (i.e. total number of pathogen units predicted to pass through a cell over a model run). Therefore the measure is an amalgamation of all the modelled timesteps and does not denote a moment in time. The summed concentration for specific model cells (i.e. where our experimental moorings were located) was 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 (ΣGI + GII
254 2255 ± 154 gc g^{-1}) was significantly higher than for three adjacent sites to the North (ΣGI + GII 329
255 ± 84 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 ± 166 CFU 100 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 ± 1764 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 (ΣGI + 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

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

4. Discussion

4.1. Spatial patterns of NoV accumulation in mussels

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

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

4.2. NoV GI and GII accumulation ratios in mussels

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

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

4.3. Spatial patterns of faecal indicator bacteria accumulation in mussels

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

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

4.4. Implications for human health

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

5. Conclusions

Our research has five key conclusions:

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

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

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

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

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

Acknowledgements

This study was funded by Bangor Mussel Producers, Welsh Water and the European Social Fund via the KESS Scholarships programme. We would like to thank Richard Dannatt and Samantha Mullan (Intertek) for assistance with model interpretation and Paul Henderson, Fergus O'Brien and Roger Cragg (Welsh Water Ltd) for outfall information, flow data and permission to use the model. We also thank Ian Harris (Bangor University) for help with the GIS and Gwynne Parry-Jones (Bangor University) and Trevor Jones (Bangor Mussel Producers) for boat time and skippering.

References

Anon, 2004. Corrigendum to regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. Official Journal of the European Union L226, 83-127.

Anon, 2013. ISO TS 15216-1:2013 Microbiology of food and animal feed: Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR, Part 1:

428 Method for quantification International Organization for Standardization. International
429 Organization for Standardization, Geneva, Switzerland.
430 Asahina, A.Y., Lu, Y., Wu, C., Fujioka, R.S., Loh, P.C., 2009. Potential biosentinels of human waste
431 in marine coastal waters: Bioaccumulation of human noroviruses and enteroviruses from
432 sewage-polluted waters by indigenous mollusks. J. Virol. Meth. 158, 46-50.
433 Babu, M.T., Vethamony, P., Desa, E., 2005. Modelling tide-driven currents and residual eddies in the
434 Gulf of Kachchh and their seasonal variability: A marine environmental planning perspective.
435 Ecol. Model. 184, 299-312.
436 Bagordo, F., Grassi, T., Idolo, A., Serio, F., Gabutti, G., De Donno, A., 2013. Rotavirus occurrence
437 in shellfish with low levels of *E. coli.* Food Environ. Virol. 5, 169-175.
438 Calgua, B., Mengewein, A., Grunert, A., Boffil-Mas, S., Clemente-Casares, P., Hundesa, A., Wyn-
439 Jones, A.P., López-Pila, J.M., Girones, R. 2008. Development and application of a one-step
440 low cost procedure to concentrate viruses from seawater samples. J. Virol. Meth. 153, 79-83.
441 Campos, C.J.A., Acornley, R., Morgan, O.C., Kershaw, S., 2013a. Trends in the levels of *Escherichia*
442 *coli* in commercially harvested bivalve shellfish from England and Wales, 1999-2008. Mar.
443 Pollut. Bull. 67, 223-227.
444 Campos, C.J.A., Kershaw, S.R., Lee, R.J., 2013b. Environmental influences on faecal indicator
445 organisms in coastal waters and their accumulation in bivalve shellfish Estuar. Coast. 36, 834-
446 853.
447 Campos, C.J.A., Avant, J., Gustar, N., Lowther, J., Powell, A., Stockley, L., Lees, D.N., 2015. Fate
448 of Human Noroviruses in Shellfish and Water Impacted by Frequent Sewage Pollution
449 Events. Environ. Sci. Technol. 49, 8377−8385
450 Cormier, J., Gutierrez, M., Goodridge, L., Janes, M., 2014. Concentration of enteric virus indicator
451 from seawater using granular activated carbon. J. Virol. Meth. 196, 212-218.
452 Cormier, J., Janes, M., 2016. Concentration and detection of hepatitis A virus and its indicator from
453 artificial seawater using zeolite. J. Virol. Meth. 235, 1-8.

Clements, K., Giménez, L., Jones, D.L., Wilson, J., Malham, S.K., 2013. Epizoic barnacles act as pathogen reservoirs on shellfish beds. J. Shellfish Res. 32, 1-6.

Dancer, D., Rangdale, R.E., Lowther, J.A., Lees, D.N., 2010. Human Norovirus RNA persists in seawater under simulated winter conditions but does not bioaccumulate efficiently in Pacific Oysters (*Crassostrea gigas*). J. Food Protec. 73, 2123-2127.

Davies, S., Mirfenderesk, H., Tomlinson, R., Szylkarski, S., 2009. Hydrodynamic, water quality and sediment transport modeling of estuarine and coastal waters on the Gold Coast Australia. J. Coast. Res. 56, 937-941.

De Donno, A. Grassi, T., Bagordo, F., Idolo, A., Serio, F., Gabutti, G., 2012. Detection of viruses in coastal seawater using *Mytilus Galloprovincialis* as an accumulation matrix. Food Environ. Virol. 4, 81-88.

Da Silva, A.K., Le Saux, J.C., Parnaudeau, S., Pommepuy, M., Elimelech, M., Le Guyad, F.S., 2007. Evaluation of removal of noroviruses during wastewater treatment, using real-time Reverse Transcription-PCR: Different behaviors of genogroups I and II. Appl. Environ. Microbiol. 73, 7891-7897.

DHI, 2003. MIKE 21: Coastal Hydraulics and Oceanography, Hydrodynamic Module. Scientific Documentation. DHI Software, Hørsholm, Denmark.

DHI, 2011. MIKE 21 & MIKE 3 FLOW MODEL FM Hydrodynamic and Transport Module Scientific Documentation. DHI Software, Hørsholm, Denmark.

Diez-Valcarce, M., Kokkinos, P., Soderberg, K., Bouwknegt, M., Willems, K., de Roda-Husman, A., von Bonsdorff, C.H., Bellou, M., Hernandez, M. Maunula, L., Vantarakis, A., Rodríguez-Lázaro, D., 2012. Occurrence of human enteric viruses in commercial mussels at retail level in three European countries. Food Environ. Virol. 4, 73-80.

478 Dunn, R.J.K., Zigic, S., Shiell, G.R., 2014. Modelling the dispersion of treated wastewater in a
479 shallow coastal wind-driven environment, Geographe Bay, Western Australia: implications for
480 environmental management. Environ. Monit. Assess. 186, 6107-6125.
481 Ekebjærg, L., Justesenu, P., 1991. An explicit scheme for advection-diffusion modelling in two
482 dimensions. Comp. Meth. Appl. Mech. Engineer. 88, 287-297.
483 EFSA Panel on Biological Hazards, 2012. Scientific Opinion on Norovirus (NoV) in oysters:
484 methods, limits and control options, Available at: www.efsa.europa.eu/efsajournal.
485 EU, 2006. Directive 2006/113/EC of the European Parliament and of the council of 12th December
486 2006 on the quality required of shellfish waters. European Union, Brussels, Belgium.
487 Ferguson, C.M., Coote, B.G., Ashbolt, N.J., Stevenson, I.M., 1996 Relationships between indicators,
488 pathogens and water quality in an estuarine system. Water Res. 30, 2045-2054.
489 Fitzgerald, A., 2015. Review of approaches for establishing exclusion zones around sewage discharge
490 points; Desk study to inform consideration of the possible introduction of exclusion zones as
491 a control for norovirus in oysters. FS513404 Aquatic Water Services Limited. Wadebridge,
492 UK.
493 Flannery, J. Keaveney, S., Rajko-Nenow, P., O'Flaherty, V., Doré, W., 2012. Concentration of
494 norovirus during wastewater treatment and its impact on oyster contamination. Appl. Environ.
495 Microbiol. 78, 3400-3406.
496 Flannery, J., Rajko-Nenow, P., Winterbourn, J.B., Malham, S.K., Jones, D.L., 2014. Effectiveness of
497 cooking to reduce Norovirus and infectious F-specific RNA bacteriophage concentrations in
498 *Mytilus edulis*. J. Appl. Microbiol. 117, 564-571.
499 Fleming, L.E., Broad, K., Clement, A., Dewailly, E., Elmir, S., Knap, A., Pomponi, S.A., Smith, S.,
500 Gabriele, H.S., Walsh, P., 2006. Oceans and human health: Emerging public health risks in the
501 marine environment. Mar. Pollut. Bull. 53, 545-560.

502 Fong, T.T., and Lipp, E.K., 2005. Enteric viruses of humans and animals in aquatic environments:
503 health risks, detection, and potential water quality assessment tools. Microbiol. Molec. Biol.
504 Rev. 69, 357-371.
505 Gourmelon, M., Lazure, P., Hervio-Heath D., Le Saux, J.C., Caprais, M.P., Le Guyader, F.S.,
506 Catherine, M., Pommepuy, M., 2010. Microbial modelling in coastal environments and early
507 warning systems: useful tools to limit shellfish microbial contamination. pp 297-318. *In* Safe
508 Management of Shellfish and Harvest Waters (Eds. Rees, G., Pond, K., Kay, D., Bartram, J.,
509 Santo Domingo, J.), IWA Publishing, London, UK.
510 Griffin, D.W. Gibson, C.J., Lipp, E.K., Riley, K., Paul, J.H., Rose, JB., 1999. Detection of viral
511 pathogens by reverse transcriptase PCR and of microbial indicators by standard methods in the
512 canals of the Florida Keys. Appl. Environ. Microbiol. 65, 4118-4125.
513 Griffin, D.W., Lipp, E.K., McLaughlin, M.R., Rose, J.B., 2001. Marine recreation and public health
514 microbiology: Quest for the ideal indicator. Bioscience 51, 817-825.
515 Hall, A.J., 2012. Noroviruses: The perfect human pathogens? J. Infect. Dis. 205, 1622-1624.
516 Hammerstein, H.C., Work, P.A., Frischer, M.E., 2015. Bacteriophage tracer for fecal contamination
517 sources in coastal waters. Water Qual. Expos. Health 7, 15-26.
518 Johne, R., Pund, R.P., Schrader, C., 2011. Experimental accumulation and persistence of norovirus,
519 feline calicivirus and rotavirus in blue mussels (*Mytilus edulis*). Arch. Lebensmittelhyg. 62,
520 129-135.
521 Kaas, L., Gourinat, A., Urbès, F., Langlet, J. 2016. A 1-year study on the detection of human enteric
522 viruses in new Caledonia. Food Environ. Virol. 8, 46-56.
523 Katayama, H., Shimasaki, A., Ohgaki, S., 2002. Development of a virus concentration method and
524 its application to detection of enterovirus and Norwalk virus from coastal seawater. Appl.
525 Environ. Microbiol. 68, 1033-1039.
526 Langlet, J., Kaas, L., Greening, G., 2015.Binding-based RT-qPCR assay to assess binding patterns of
527 noroviruses to shellfish. Food Environ. Microbiol. 7, 88-95.

528 Le Guyader, F.S., Atmar, R.L., Le Pendu, J., 2012. Transmission of viruses through shellfish: when
529 specific ligands come into play. Curr. Opin. Virol. 2, 103-110.
530 Lees, D., 2000. Viruses and bivalve shellfish. Int. J. Food Microbiol. 59, 81-116.
531 Lees, D. and CEN WG6 TAG4, 2010. International standardisation of a method for detection of
532 human pathogenic viruses in molluscan shellfish. Food Environ. Virol. 2, 146-155.
533 Lowther, J.A., Gustar, N.E., Powell, A.L., Hartnell, R.E., Lees, D.N., 2012a. Two-year systematic
534 study to assess norovirus contamination in oysters from commercial harvesting areas in the
535 United Kingdom. Appl. Environ. Microbiol. 78, 5812-5817.
536 Lowther, J.A. Gustar, N.E., Hartnell, R.E., Lees, D.N., 2012b. Comparison of norovirus RNA levels
537 in outbreak-related oysters with background environmental levels. J. Food Protec. 75, 389-
538 393.
539 Lysén, M., Thorhagen, M., Brytting, M., Hjertqvist, M., Andersson, Y., Hedlund, K.O., 2009. Genetic
540 diversity among food-borne and waterborne norovirus strains causing outbreaks in Sweden.
541 J. Clin. Microbiol. 47, 2411-2418.
542 Maalouf, H., Zakhour, M., Le Pendu, J., Le Saux, J.C., Atmar, R.L., Le Guyader, F.S., 2010.
543 Distribution in tissue and seasonal variation of norovirus genogroup I and II ligands in oysters.
544 Appl. Environ. Microbiol. 76, 5621-5630.
545 Maalouf, H., Schaeffer, J., Parnaudeau, S., Le Pendu, J., Atmar, R.L., Crawford, S.E., Le Guyader,
546 F.S., 2011. Strain-dependent norovirus bioaccumulation in oysters. Appl. Environ. Microbiol.
547 77, 3189-3196.
548 Majori, L., Campello, C., D'Alessandro, F., Berzero, R., Bovo, F., Guarineri, G., 1984. *Vibrio*
549 *cholerae* non-O1 e *Vibrio parahaemolyticus* in acque e mitili della Laguna di Venezia: studio
550 ecologico e relativi problem igienico-sanitari. Ig. Mod. 82, 436-455.
551 Malham, S.K., Rajko-Nenow, P., Howlett, E., Tuson, K.E., Perkins, T.L. Pallett, D.W., Wang, H.,
552 Jago, C.F., Jones, D.L., McDonald, J.E., 2014. The interaction of human microbial pathogens,

553 particulate material and nutrients in estuarine environments and their impacts on recreational
554 and shellfish waters. Environ. Sci. Process. Impact. 16, 2145-2155.
555 Matthiessen, P., Law, R.J., 2002. Contaminants and their effects on estuarine and coastal organisms
556 in the United Kingdom in the late twentieth century. Environ. Pollut. 120, 739-757.
557 Moore, M.N., Depledge, M.H., Fleming, L., Hess, P., Lees, D., Leonard, P., Madsen, L., Owen, R.,
558 Pirlet, H., Seys, J., Vasconcelos, V., Viarengo, A., Marine Board-ESF Working Group on
559 Oceans and Human Health, 2013. Oceans and Human Health (OHH): A European perspective
560 from the Marine Board of the European Science Foundation (Marine Board-ESF). Microb.
561 Ecol. 65, 889-900.
562 Muhammetoglu, A. Yalcin, O.B., Ozcan, T., 2012. Prediction of wastewater dilution and indicator
563 bacteria concentrations for marine outfall systems. Mar. Environ. Res. 78 53-63.
564 Nenonen, N.P. Hannoun, C., Horal, P., Hernroth, B., Bergström, T., 2008. Tracing of norovirus
565 outbreak strains in mussels collected near sewage effluents. Appl. Environ. Microbiol. 74,
566 2544-2549.
567 Nordgren, J. Matussek, A., Mattsson, A., Svensson, L., Lindgren, P.E., 2009. Prevalence of norovirus
568 and factors influencing virus concentrations during one year in a full-scale wastewater
569 treatment plant. Water Res. 43, 1117-1125.
570 Oliveira, J., Cunha, A., Castilho, F., Romalde, J,L., Pereira, M.J., 2011. Microbial contamination and
571 purification of bivalve shellfish: Crucial aspects in monitoring and future perspectives - A mini-
572 review. Food Control 22, 805-816.
573 Pancorbo, O.C., Barnhart, H.M., 1992. Microbial pathogens and indicators in estuarine environments
574 and shellfish - critical need for better indicator(s) of human-specific fecal pollution. J. Environ.
575 Health 54, 57-63.
576 Paraskevas, P.A., Giokas, D.L., Lekkas, T.D., 2002. Wastewater management in coastal urban areas:
577 the case of Greece. Water Sci. Technol. 46, 177-186.

578 PHE, 2016. PHE Weekly national norovirus and rotavirus report: Summary of surveillance of
579 norovirus and rotavirus, 17th March 2016. Public Health England, London, UK.
580 Polo, D., Alvarez, C., Diez, J., Darriba, S., Longa, A., Romalde, J.L., 2014. Viral elimination during
581 commercial depuration of shellfish. Food Control 43, 206-212.
582 Rajko-Nenow, P. Waters, A., Keaveney, S., Flannery, J., Tuite, G., Coughlan, S., O'Flaherty, V.,
583 Doré, W., 2013. Norovirus genotypes present in oyster and effluent from a wastewater
584 treatment plant during the seasonal peak of infections in Ireland, 2010. Appl. Environ.
585 Microbiol. 79, 2578-2587
586 Riou, P., Le Saux, J.C., Dumas, F., Caprais, M.P., Le Guyader, S.F., Pommepuy, M., 2007. Microbial
587 impact of small tributaries on water and shellfish quality in shallow coastal areas. Water Res.
588 41, 2774-2786.
589 Rodríguez, R.A., Thie, L., Gibbons, C.D., Sobsey, M.D., 2012. Reducing the effects of environmental
590 inhibition in quantitative real-time PCR detection of adenovirus and norovirus in recreational
591 seawaters. J. Virol. Meth. 181, 43-50.
592 Siegle, E., Huntley, D.A., Davidson, M.A., 2007. Coupling video imaging and numerical modelling
593 for the study of inlet morphodynamics. Mar. Geol. 236, 143-163.
594 Silva, C., Ferreira, J.G., Bricker, S.B., DelValls, T.A., Martin-Diaz, M.L., Yanez, E., 2011. Site
595 selection for shellfish aquaculture by means of GIS and farm-scale models, with an emphasis
596 on data-poor environments. Aquaculture 318, 444-457.
597 Stapleton, C.M., Wyer, M.D., Crowther, J., McDonald, A.T., Kay, D., Greaves, J., Wither, A.,
598 Watkins, J., Francis, C., Humphrey, N., 2008. Quantitative catchment profiling to apportion
599 faecal indicator organism budgets for the Ribble system, the UK's sentinel drainage basin for
600 Water Framework Directive research. J. Environ. Manage. 87, 535-550.
601 Ventrone, I., Schaeffer, J., Ollivier, J., Parnaudeau, S., Pepe, T., Le Pendu, J., Le Guyader, F.S., 2013.
602 Chronic or accidental exposure of oysters to norovirus: is there any difference in
603 contamination? J. Food Protec. 76, 505-509.

Williams, J.J., Esteves, L.S., Conduche, T., Barber, P., Tindle, A., 2014. Using combined modelling approaches to improve coastal defence design: a case study at Hopton, UK. J. Coast. Res. 70, 18-23.

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 indictor
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.