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Assessment of wastewater derived pollution using viral monitoring in two estuaries

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ABSTRACT

Human wastewater-derived pollution of the environment is an emerging health risk that increases the number of waterborne and foodborne illnesses globally. To better understand and mitigate such health risks, we investigated the prevalence of faecal indicator bacteria, *Escherichia coli*, and indicator virus (crAssphage) along with human and animal enteric viruses (adenoviruses, noroviruses, sapoviruses, hepatitis E virus) in shellfish and water samples collected from two shellfish harvesting areas in the UK. Human noroviruses were detected at higher detection rates in oyster and water samples compared to mussels with peaks during the autumn-winter seasons. Human enteric viruses were sporadically detected during the warmer months, suggesting potential introduction by tourists following the relaxation of COVID-19 lockdown measures. Our results suggest that viral indicators are more suitable for risk assessment and source tracking than *E. coli*. The detection of emerging hepatitis and sapoviruses, support the need for comprehensive viral monitoring in shellfish harvesting areas.

1. Introduction

Estuarine environments provide a wealth of economic, social and natural benefits which include employment, food, habitation and recreation. Marine aquaculture is a growing industry producing 33.6 million tonnes annually with approximately 50 % of the production related to bivalve shellfish (FAO, 2022). Shellfish are a sustainable source of nutrients including proteins, vitamins and minerals and hence there is a growing demand on shellfish production, resulting in an increase in coastal areas used for harvesting (Venugopal and Gopakumar, 2017; Wijsman et al., 2018). However, as bivalve shellfish are filter-feeders, it is important to grow and harvest shellfish in clean water bodies.

Over 50 % of the world's population lives within 100 km of the coast and anthropogenic activities cause substantial impacts on the health of estuarine and ocean ecosystems. Domestic wastewater is a major source of pollution because it often contains pathogens which may affect the health of people using water bodies that receive discharges for either recreation or consuming seafood. Human pathogens, especially enteric viruses, often survive wastewater treatment and hence viable viruses can enter the coastal environment via discharge. Furthermore, during heavy rainfall events, untreated wastewater also enters the aquatic environment via storm overflows designed to prevent the overload of wastewater treatment plants. Hence human pathogens are often present in the estuarine and marine environment, including shellfish harvesting areas.

There are over 100 sewage-derived viruses that can cause waterborne illnesses (Bosch, 1998). Caliciviruses, such as human noroviruses (NoVs) and sapoviruses (SaVs), are the most common cause of viral gastroenteritis, including food- and waterborne infections, globally (Katayama and Vinje, 2017). Hepatitis A and E viruses (HAV and HEV) are also considered emerging and re-emerging waterborne pathogens (Lemon and Walker, 2019). To date, most of the monitoring efforts and environmental studies have been focusing on NoVs and HAV and little is known about the abundance of other pathogenic viruses in the marine environment.

Due to the large number of viral pathogens and the difficulty in detecting them, faecal indicator organisms (often found in the gut microbiome) are often used to assess bathing water quality and shellfish hygiene. Most countries have been relying on *Escherichia coli* as a universal indicator and classify their water bodies and shellfish based on its levels. *E. coli* is easy to detect and quantify using rapid culturing

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techniques, however, it may not be suitable as an indicator for viral contamination due to its different transport and survival patterns (Bazzardi et al., 2014a; Burkhardt and Calci, 2000; Chung et al., 1998; Love et al., 2010). Furthermore, *E. coli* is found in the gut of many animal species, hence its presence in water may not indicate solely human pollution.

As an alternative, the suitability of several viral indicators for human pathogenic viruses have been assessed (Farkas et al., 2020; Lin and Ganesh, 2013; McKee and Cruz, 2021). Coliphages are easy to detect and quantify, however, as they associate with coliform bacteria, they may originate from animal sources. CrAssphages are a newly-discovered group of viruses that associates with human gut bacteria, *Bacteroides* spp. They are found in 14–36 % of the human population and detected in untreated and treated wastewater and in the polluted aquatic environment globally (Farkas et al., 2020; Honap et al., 2020; Sabar et al., 2022). Human adenoviruses (AdVs) are enteric viruses usually causing asymptomatic infections in otherwise healthy individuals. They are also found in wastewater and receiving waters at high concentrations and have also been recommended to be used as faecal indicators in water (Farkas et al., 2020; Rames et al., 2016), however, their use in bivalve shellfish is yet to be explored. Different species of AdVs infect other vertebrates, including cattle and sheep and hence they may be suitable to indicate faecal pollution of water of agricultural source (Staggemeier et al., 2015; Wolf et al., 2010).

The aim of this study was to assess animal and human originated faecal pollution in two shellfish harvesting areas in England (Camel Estuary) and Wales (Menai Strait). While both sites were in rural areas affected by seasonal tourism, the Camel site is a ria estuary whereas the Menai Strait is an open marine environment with river input. We collected oyster, mussel and water samples and tested them for *E. coli*, NoV, SaV, HEV, HAV, crAssphage and Human AdV F and C (AdVF and AdVC), atadenoviruses (AtAdV, infecting sheep, cattle, deer and goats) and ovine AdV (OAdV, infecting sheep and cattle). The physico-chemical properties and nutrient levels of water samples were also measured to assess the relationship between these parameters of water quality and viral pollution.

2. Methods and materials

2.1. Sampling sites and sample collection

2.1.1. The Camel Estuary

The Camel site is a ria estuary with an approximate 6 km^2 intertidal area (Buck, 1993), located in Cornwall, England (Fig. 1). The estuary is situated in a rural area with considerable agricultural activities, including livestock farming and wild animal grazing. During the summer, the area is frequently visited by tourists (approximately 18,000 each summer). The area is affected by human wastewater pollution via

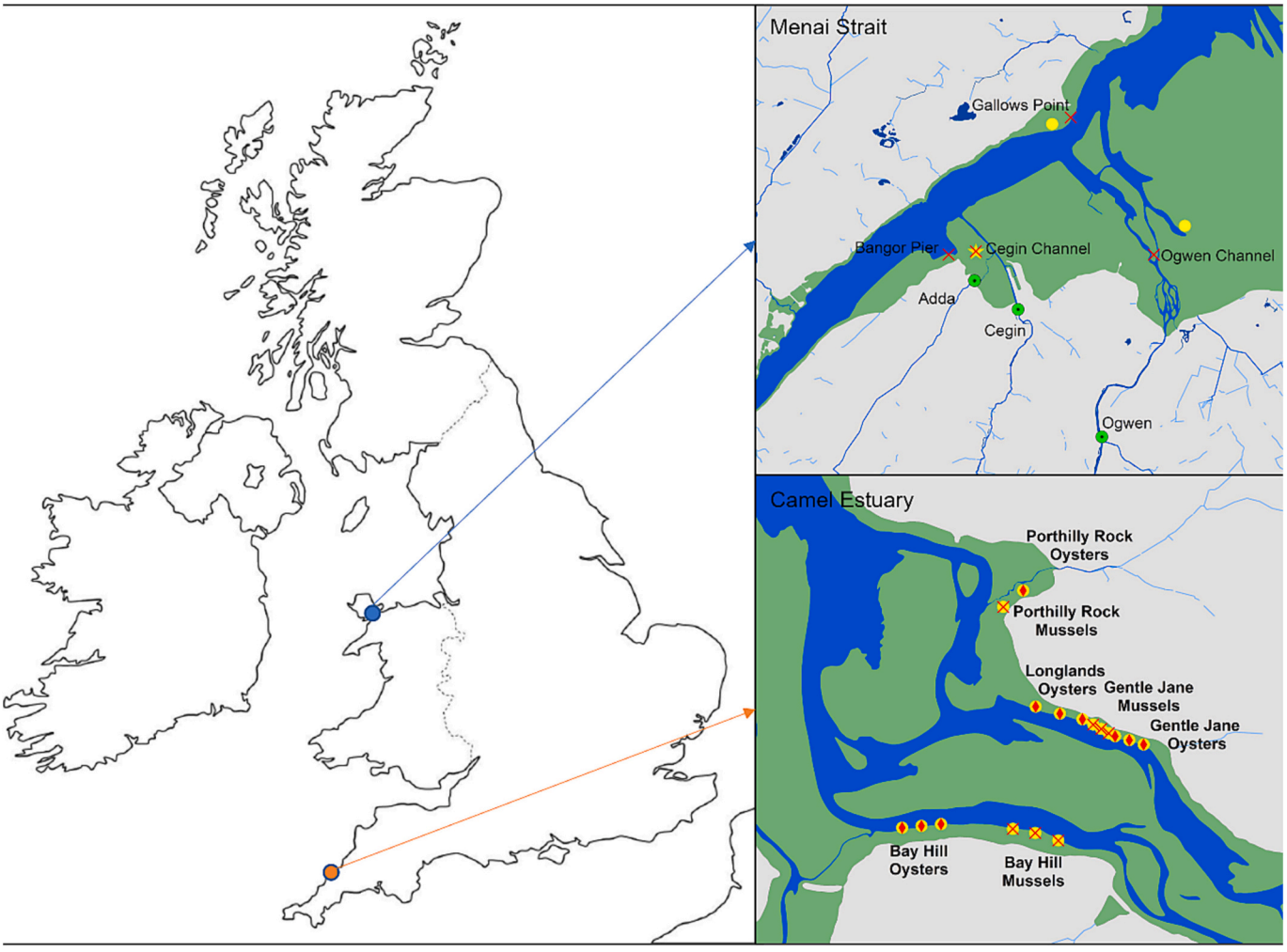

Fig. 1. Sampling site locations at the Camel estuary (Cornwall, England) and at the Menai Strait (North Wales). Area shaded green is intertidal. Red crosses show mussel sites. Red diamonds show oyster sites. Yellow circles show seawater sites sampled for *E. coli*. The yellow star (Cegin Channel) shows the site at which high-volume seawater samples were also collected for virological analysis. Green circles show river water sites. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

treated effluent discharge, CSOs and possible waste dump from recreational boats (CEFAS, 2015). The local wastewater treatment plants serving a population of approximately 37,000 inhabitants utilises UV disinfection as tertiary treatment. No data on the volume of CSO discharges is available. The estuary has been used for bivalve shellfish harvesting for centuries and currently, blue mussels (*Mytilus edulis*) and pacific oysters (*Magallana* (=*Crassostrea*) *gigas*) are harvested commercially. The area was classified as 'B' based on *E. coli* levels during the study.

Mussel and oyster samples were collected between the 2nd June 2019 and 11th April 2021 from seven shellfish beds along the Camel Estuary fortnightly. For larger beds, samples were pooled from three stations (Fig. 1). Seawater samples were also collected from each station. Sampling was paused between 2nd March and 15th August 2020 due to COVID-19 lockdown measures. Altogether, 153 oyster and 114 mussel samples were collected. The samples were sent to the laboratory chilled within 24 h of collection and processed immediately upon arrival.

2.1.2. Menai Strait Mussel and Oyster Fishery Order

The Menai Strait (East) Mussel and Oyster Fishery Order is an 8 km^2 region situated at the eastern end of the Menai Strait, which is a 30-km tidal channel between mainland Wales and the Isle of Anglesey (Fig. 1). The site is affected by freshwater input via three main rivers (River Ogwen, River Cegin, and River Adda), human wastewater input via treated and untreated wastewater discharge points originated from the city of Bangor (population of 17,300) and smaller towns surrounding the Strait and agricultural runoff (CEFAS, 2013). The local wastewater treatment plant has a catchment of approximately 26,000 and the plant also utilises UV treatment. No data on the volume of CSO discharges is available. The area has a changing human population due to tourism from spring to autumn and the approximately 11,000 students studying at the local university. Similar to the Camel Estuary, the area has been used for harvesting shellfish, mainly blue mussels. Within the fishery order there are six classification zones, in 2023 five had long-term 'B' classification and one had a 'seasonal A/B' classification.

For this study, mussel, river water and seawater samples were collected weekly between 10th January and 22nd November 2022. Mussels were collected at four sampling points corresponding with Representative Monitoring Points (RMPs) used in Official Control Regulations (Fig. 1). Mussels were harvested from cages which were refilled with mussels from the Menai Strait regularly at least a week prior to each sampling event. Seawater was collected from designated Shellfish Water Monitoring Points. The Cegin Channel mussel RMP corresponds to one such shellfish water monitoring point. At this site, additional 10 L water samples were collected for virus recovery (Fig. 1). River water samples were collected from each main river (Adda, Ogwen and Cegin). River water samples were collected during low tide to ensure no tidal water was collected. Samples were transported to the laboratory chilled and processed within 24 h upon collection.

2.2. Physico-chemical analyses of water samples

Water turbidity was measured using the Oakton T-100 Turbidity meter (Oakton Instruments, USA) and salinity was measured with a HI-98319 marine salinity tester (Hanna Instruments, USA) according to the manufacturers' instructions. Data was expressed as nephelometric turbidity units (NTU) for turbidity and practical salinity units (PSU) for salinity. Phosphate (PO_4), total nitrate/nitrite (NO_x), nitrite (NO_2), and ammonium ($NH4^+$) were measured using an AutoAnalyzer (SEAL Analytical, USA) according to manufacturer's instructions (technical notes G-175-96, G-384-08, G-173-96, G-327-05).

2.3. E. coli quantification

For *E. coli* enumeration in shellfish, a most probable number (MPN) method (ISO 16649-3 2016) with some minor modifications was used. Briefly, the flesh of 10 oysters or 15 mussels were homogenised and mixed with 2 ml of 0.1 % bacteriological peptone water per gram of shellfish flesh, followed by the creation of a dilution series made by adding 80 ml of peptone water to 20 ml of sample to reach 10×, 100× and 1000× dilutions. The dilutions were aliquoted to five tubes with minerals modified glutamate broth and incubated at 37 ± 1 °C for 24 ± 2 h. After incubation, the tubes with yellow solution were considered positive and those were then subcultured on tryptone bile X-glucuronide (TBX) agar at 44 ± 1 °C for 21 ± 3 h. The plates with blue/green colonies were considered positive. The number of positive tubes at each dilution was then used to calculate the MPN of *E. coli* per 100 g of the sample using the calculation tool created by Jarvis et al. (2010).

E. coli was quantified in river and seawater samples following the ISO 9308-1:2014 standard. Seawater sample volumes of 500 ml and 100 ml, and river sample volumes of 10 ml, 1 ml and 0.1 ml were filtered through 0.45 μm cellulose nitrate filters (Sartorius, Germany) equilibrated with sterile 0.1 % peptone water. The membrane filters were then incubated on Chromogenic Coliform Agar at 37 °C for 22 h. The dark blue/violet colony-forming units were counted and *E. coli* concentrations were calculated as:

$$CFU/100\,ml = (c/V)*100 \quad (1)$$

where CFU is colony-forming units, c is the sum of dark blue/violet colonies and V is the volume of sample filtered.

2.4. Virus concentration

2.4.1. Concentration of surface water for viral detection

Viruses were precipitated from 10 l river and seawater samples using the skimmed milk method described previously (Calgua et al., 2013). In brief, sample conductivity and pH were adjusted to 1.5 mS and 3.5, respectively. Then, skimmed milk was added in form of 1 % pH 3.5 artificial seawater solution to reach the final concentration of 0.01 %. Samples were then stirred for 8 h and then settled for 8 h at room temperature. The supernatant was removed, and the remaining concentrate was centrifuged at 10,000 ×*g* for 30 min at 4 °C. The pellet was then resuspended in 10 ml PBS prior to RNA/DNA extraction.

2.4.2. Virus extraction from shellfish

To elute viruses from shellfish, 15 mussels and 10 oysters were processed according to the ISO 15216-2:2019 standard. First, the digestive tissue from the animals were extracted and mixed. Then, 2 g of the digestive tissue was mixed with 2 ml 3 U/ml proteinase K solution at 37 °C for 60 min followed by enzyme inactivation at 60 °C for 15 min. The liquid phase was separated using centrifugation and was subject to RNA/DNA extraction. Selected shellfish digestive tissue samples were spiked with approximately 10^5 genome copies (gc) of process control virus, the murine norovirus (MNV) prior to sample process to discover virus recovery rates. For positive control, the same aliquot of MNV used for spiking was subject to RNA extraction.

2.5. Viral RNA/DNA extraction and quantification

Viral RNA and DNA were coextracted from 0.5 ml of concentrates using the Nuclisens MiniMag® Nucleic Acid Purification System (BioMerieux, France). The final volume of the eluent was 0.1 ml.

Viral nucleic acids were quantified using RT-qPCR and qPCR on the QuantStudio Flex 6 system (Applied Biosystems, USA). We used a dilution series of plasmid standards incorporating the target sequences for quantification (Farkas et al., 2017). Each sample/standard was run in duplicates and each plate contained two negative controls to assess contamination. Samples were tested for NoV GI, GII, SaV, HAV, HEV and the process control virus MNV in two triplex, one-step RT-qPCR assays as described previously (Farkas et al., 2017; Kitajima et al., 2010). For the samples from the Camel estuary, the RNA Ultrasense 1-step qRT-PCR

system (Applied Biosystems, USA) was used for the detection of NoVGI, NoVGII, HAV, HEV, SaV and MNV (Farkas et al., 2017). For the Menai Strait samples, the TaqMan Virus Fast 1-step qRT-PCR mix (Applied Biosystems, USA) was used to quantify NoVGI and NoVGII (Farkas et al., 2022). For human mastadenovirus C and F, ovine adenovirus and atadenovirus a quadruplex qPCR assay was used with existing primer and probe sequences (Wolf et al., 2010). Unfortunately, no qPCR standards for HEV and SaV were available for the Menai Strait study and hence those viruses were not targeted in those samples. However, we included a singleplex assay for crAssphage for the samples collected at the Menai Strait (Farkas et al., 2019; Stachler et al., 2017). The 20 µl reaction mix targeting DNA viruses contained 1× QuantiNova Probe PCR mix with low ROX (Qiagen, Germany), 12.5 pmol primers, 6.25 pmol probes, 1 µg bovine serum albumin (BSA) and 2 µl of samples/controls. Amplification was carried out using the following conditions: 95 °C for 2 min, then 40 cycles of 95 °C for 15 s, 59 °C (adenoviruses) or 60 °C (crAssphage) for 1 min. The assay efficiency was 90–110 %. The primer, probe and target sequences along with qPCR conditions are detailed in Table S1.

2.6. Data analysis

Initial data analysis and quality control was carried out using the QuantStudio real-time PCR software v1.7 (Applied Biosystems, USA). The viral concentrations were expressed as gc/µl nucleic acid extract and transformed to gc/l or gc/g digestive tissue as follows:

$$\frac{(RNA\ concentration\ in\ eluent \times volume\ of\ RNA\ eluent)}{Volume\ of\ extracted\ concentrate} \times \frac{Concentrate\ volume}{Total\ sample\ volume} \quad (2)$$

Viral detection rates were calculated as

$$\frac{Number\ of\ positive\ samples}{Number\ of\ all\ samples\ tested} \times 100\% \quad (3)$$

The MNV recovery rates were calculated by dividing the MNV concentrations observed in samples by the concentrations noted in the extraction positive controls.

Kruskal-Wallis test was performed to assess differences in physicochemical and microbiological parameters of water and shellfish samples among sites. Spearman correlation was also performed to investigate correlation between parameters. All statistical analyses were performed using SPSS V27 (IBM, USA).

3. Results

3.1. Quality control

To estimate viral recovery rates, selected mussel and oyster samples collected at the Camel Estuary along with associated controls were spiked with known quantities of MNV. The control virus was recovered from all samples with higher than 1 % recovery, as required by the ISO 15216:2019 standard, with 94 % of the samples having higher than 10 % recoveries. The mean recoveries were 62 % ($n = 75$) and 50 % ($n = 56$) in oyster and mussel samples, respectively.

The sample process and PCR negative controls were negative throughout the study. The qPCR standard curves were fit in the general requirements of −3.1 - -3.3 slope, and 90 %–110 % efficiency. R^2 values were above 0.9.

3.2. Water and shellfish quality parameters

Overall, *E. coli* was detected in most samples, with only seven shellfish samples being negative ($n = 383$) and all water samples being positive ($n = 200$). Higher concentrations of *E. coli* were observed in the shellfish samples collected at the Camel Estuary compared to those from the Menai Strait with mussels retaining more *E. coli* than oysters (Fig. 2). The Camel Estuary shellfish samples had higher overall *E. coli* concentrations than the mussel samples collected at Menai Strait. At the Camel Estuary, the oyster samples collected at Porthilly Rocks had significantly higher *E. coli* concentrations than the mussels collected at the Ball Hill and Gentle Jane locations. No significant differences in *E. coli* levels in shellfish samples collected at the Menai Strait was observed.

Similarly, the water samples from the Camel Estuary had higher levels of *E. coli* than the seawater samples, but lower concentrations than the river water samples from Menai Strait (Fig. 2). At the Camel Estuary, no significant differences in the *E. coli* levels in water among sites were observed, however, the bacterial titres in shellfish samples showed significant differences ($p = 0.011$). Opposite trends were observed in the water samples collected at the Menai Strait where the water samples had significantly different *E. coli* levels ($p < 0.001$) between river and seawater samples and between Rivers Cegin and Adda.

Water turbidity was much higher in the samples collected at the Camel Estuary than those from Menai Strait. The samples collected at River Ogwen had significantly lower turbidity levels ($p < 0.001$) than the Ogwen Channel samples (Table 1). As expected, salinity levels, measured in the Menai Strait samples, were the highest in seawater samples, followed by Rivers Adda, Cegin and Ogwen and the differences were significant ($p < 0.001$) among the river sites and between seawater and river water samples.

The NO_x levels were consistently high at the Camel sites, whereas significant differences ($p < 0.001$) were observed at the Menai Strait river samples with River Adda having the highest levels, followed by Rivers Cegin and Ogwen (Table 1). The NO_2 levels were consistent among the Camel Estuary sites whereas higher levels were observed at the River Adda than at the other Menai Strait river sites (Table 1). In

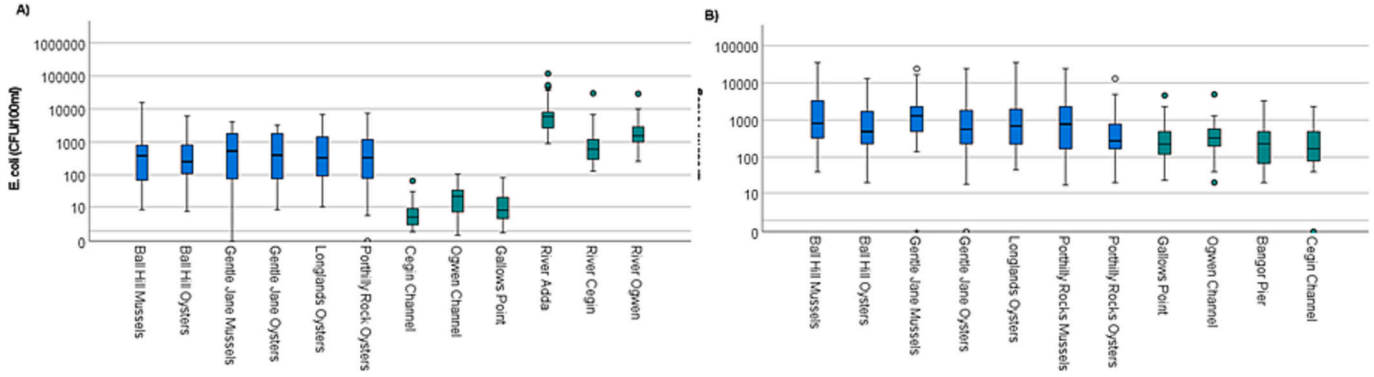

Fig. 2. (Alternative to Table 1) *E. coli* concentrations in A) water and B) shellfish samples at the Camel Estuary (blue bars) and the Menai Strait (green bars). *E. coli* concentrations are expressed at most probable number (MPN)/100 g shellfish flesh, whereas in water samples, colony-forming units (CFU)/100 ml units were applied.

Table 1
Summary of water temperature, turbidity, salinity and nutrient levels (mean ± standard error) at the sampling sites at the Camel Estuary and the Menai Strait. NTU: nephelometric turbidity units; PSU: practical salinity units.

Site		Sample type (n)	Temperature (°C)	Turbidity (NTU)	Salinity (PSU)	NO_x (μmol/l)	NO_2 (μmol/l)	PO_4 (μmol/l)	NH_3 (μmol/l)
Camel Estuary	Ball Hill mussels site	Water (38)	n.t.	119.6 ± 31.8	n.t.	111.8 ± 14.3	1.1 ± 0.2	0.8 ± 0.1	4.4 ± 0.6
	Ball Hill oysters site	Water (37)	n.t.	89.4 ± 21.7	n.t.	91.6 ± 11.8	0.9 ± 0.1	0.7 ± 0.1	4.0 ± 0.4
	Gentle Jane mussels site	Water (40)	n.t.	113.1 ± 28.1	n.t.	151.6 ± 22.5	1.1 ± 0.1	3.7 ± 2.8	5.0 ± 0.5
	Gentle Jane oysters site	Water (37)	n.t.	100.7 ± 21.0	n.t.	131.8 ± 21.1	1.1 ± 0.1	3.9 ± 2.7	5.2 ± 0.6
	Longlands	Water (34)	n.t.	107.7 ± 23.3	n.t.	137.6 ± 15.6	1.3 ± 0.1	2.1 ± 0.6	6.1 ± 0.9
	Porthilly Rock	Water (36)	n.t.	101.6 ± 27.2	n.t.	104.3 ± 16.7	0.7 ± 0.1	1.6 ± 1.0	4.0 ± 0.5
	Cegin Channel	Seawater (33)	n.t.	3.9 ± 0.8	31.5 ± 0.2	<0.213	<0.03	0.4 ± 0.1	1.8 ± 0.1
	Gallows Point	Seawater (33)	n.t.	3.3 ± 0.6	31.4 ± 0.2	<0.213	<0.03	0.3 ± 0.0	1.1 ± 0.1
	Ogwen Channel	Seawater (32)	n.t.	4.5 ± 1.4	30.2 ± 1.3	<0.213	<0.03	0.5 ± 0.0	1.2 ± 0.2
	River Adda	River water (35)	14.3 ± 0.4	1.9 ± 0.3	3.1 ± 0.6	150.1 ± 7.1	4.0 ± 0.6	4.2 ± 0.2	11.3 ± 1.5
	River Cegin	River water (35)	13.6 ± 0.5	1.9 ± 0.6	0.6 ± 0.1	114.3 ± 5.2	0.6 ± 0.1	2.0 ± 0.2	5.1 ± 0.6
	River Ogwen	River water (35)	12.8 ± 0.6	0.1 ± 0.7	<0.01	30.2 ± 2.9	0.4 ± 0.1	1.5 ± 0.2	2.2 ± 0.2

most cases, the NO_x/NO_2 levels were too low to measure in the Menai Strait seawater samples, hence no statistical comparisons were performed. At the Camel Estuary, significant differences were observed in PO_4 levels ($p < 0.001$), whereas NH_3 showed little variation ($p = 0.586$). At the Menai Strait, the difference in these parameters between sea and river sites and between Rivers Ogwen and Adda were significant ($p < 0.001$).

3.3. Human pathogenic RNA viruses

Noroviruses, SaV and HEV viruses were detected in shellfish samples, however, some virus detection was site-specific. Norovirus GI and NoVGII were frequently detected at both the Camel Estuary and at the Menai Strait, however, at low concentrations. Norovirus GI was not detected in the mussel samples collected at Ogwen Channel (Menai Strait) and in the seawater sample from the Cegin Channel (Menai Strait). Overall, NoVGII was more frequently detected than NoVGI except in the river water samples taken at River Cegin (Menai Strait) (Table 2 and Fig. 3).

Noroviruses were more frequently detected in oysters and in water samples compared to mussels (Table 2 and Fig. 3). However, the NoV concentrations were generally low, small peaks in titres and high detection rates were observed at the Camel site samples late August 2019 and between November 2019 and February 2020 and in January–February 2021 indicating high viral abundance. Noroviruses were most abundant in the Menai Strait between March and May 2022 and September–October 2022 (Figs. S2–3). High detection rates for NoVGI were observed in mussel and water samples in September–October 2022, however, the high NoVGII detection rates in water during the same time period were not reflected in mussel samples. No differences in viral concentrations were observed among the sites at the Camel Estuary nor at the sites at the Menai Strait.

Sapovirus and HEV were only detected in samples from the Camel Estuary between August 2020 and August 2021 (Table 2 and Figs. 3, S1). The detection of these viruses was sporadic and at low concentration, therefore, no distinct patterns or peaks were observed.

3.4. Indicator DNA viruses

Only samples collected at the Menai Strait were tested for crAssphage. Overall, water samples had higher crAssphage detection rates than the mussel samples (Table 2 or Figs. 3–4). Site-specific differences were also noted for the water samples with River Adda having the highest crAssphage concentrations ($p < 0.001$). No seasonal patterns were observed in any of the sample types (Figs. S2–3).

All four AdVs were detected in the shellfish samples from the Camel Estuary with high detection rates and concentrations. Adenovirus F and AtAdV were the most abundant viruses, followed by AdVC and OAdV (Table 2 and Fig. 3). No significant differences in AdV concentrations were detected between sampling sites and shellfish types. At the Camel

Table 2
Detection rates (D), calculated as in Eq. (3) of norovirus GI, GII (NoVGI, NoVGII), sapovirus (SaV), and hepatitis A and E viruses (HAV and HEV), crAssphage (crAss), adenovirus F and C (AdVF and AdVC), atadenovirus (AtAdV) and ovine adenovirus (OAdV) in mussels, oysters and water samples collected at the Camel Estuary and the Menai Strait. n.t.: not tested.

Site		Sample type (n)	NoVGI	NoVGII	SaV	HEV	HAV	CrAss	AdVF	AdVC	OAdV	AtAdV
Camel Estuary	Ball Hill	Mussels (38)	13 %	18 %	15 %	8 %	0 %	n.t.	61 %	37 %	3 %	55 %
	Ball Hill	Oysters (38)	15 %	22 %	10 %	7 %	0 %	n.t.	59 %	33 %	3 %	64 %
	Gentle Jane	Mussels (41)	14 %	27 %	11 %	2 %	0 %	n.t.	63 %	33 %	3 %	60 %
	Gentle Jane	Oysters (41)	11 %	23 %	9 %	0 %	0 %	n.t.	65 %	33 %	10 %	68 %
	Longlands	Oysters (38)	22 %	24 %	5 %	0 %	0 %	n.t.	67 %	31 %	10 %	62 %
	Porthilly Rock	Mussels (35)	8 %	18 %	13 %	3 %	0 %	n.t.	50 %	36 %	8 %	53 %
	Porthilly Rock	Oysters (36)	13 %	23 %	15 %	10 %	0 %	n.t.	51 %	32 %	8 %	65 %
Menai Strait	Gallows Point	Mussels (25)	4 %	12 %	n.t.	n.t.	n.t.	12 %	24 %	0 %	4 %	40 %
	Ogwen Channel	Mussels (26)	4 %	15 %	n.t.	n.t.	n.t.	35 %	15 %	0 %	4 %	50 %
	Bangor Pier	Mussels (33)	18 %	21 %	n.t.	n.t.	n.t.	61 %	18 %	0 %	3 %	39 %
	Cegin Channel	Mussels (32)	16 %	19 %	n.t.	n.t.	n.t.	38 %	25 %	0 %	0 %	47 %
	Cegin Channel	Seawater (23)	0 %	9 %	n.t.	n.t.	n.t.	78 %	22 %	0 %	4 %	26 %
	River Adda	River water (34)	21 %	41 %	n.t.	n.t.	n.t.	100 %	24 %	0 %	0 %	29 %
	River Cegin	River water (34)	19 %	12 %	n.t.	n.t.	n.t.	97 %	18 %	0 %	12 %	18 %
	River Ogwen	River water (34)	27 %	30 %	n.t.	n.t.	n.t.	97 %	12 %	0 %	9 %	38 %

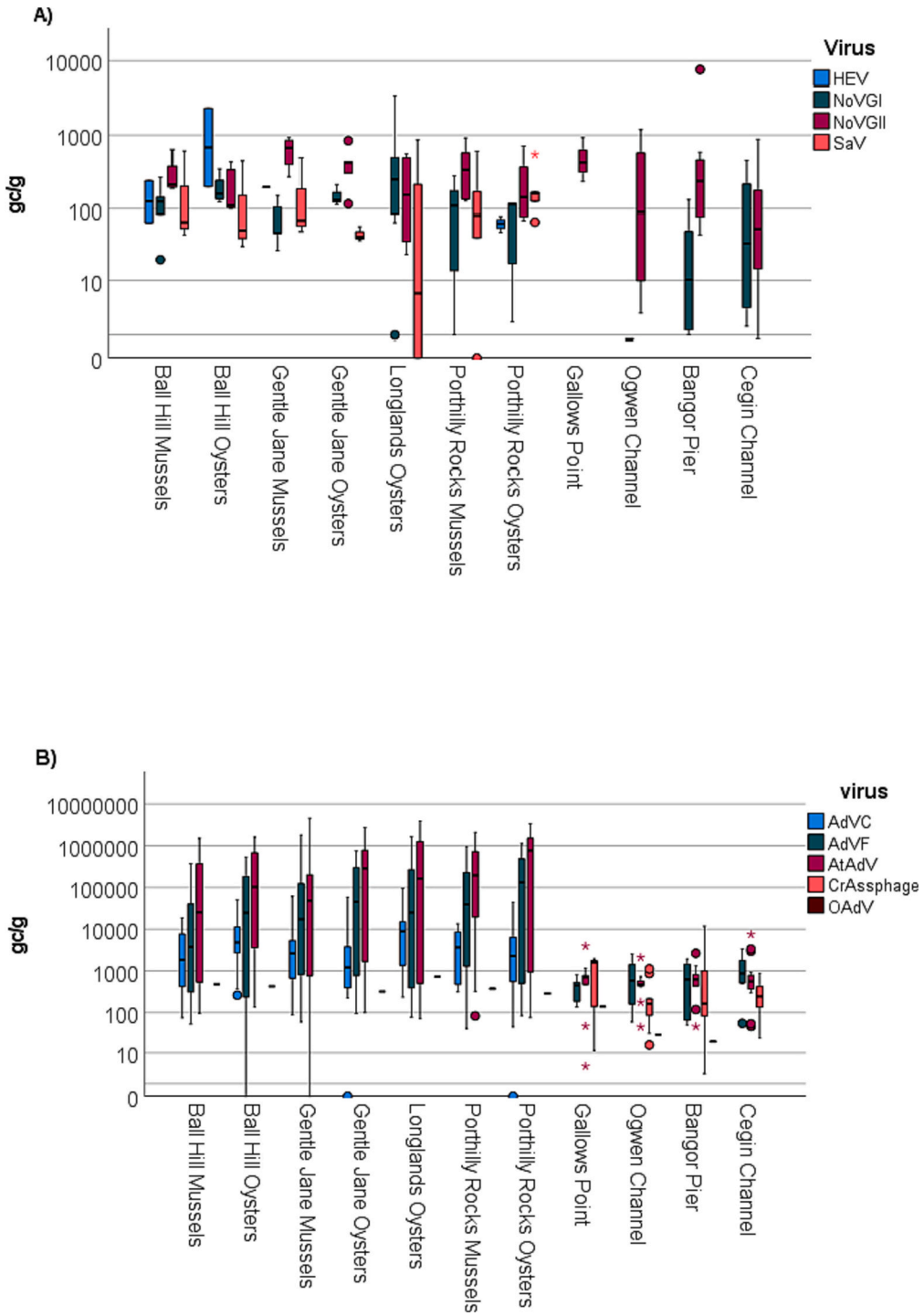

Fig. 3. Concentration of human pathogenic viruses (A) and indicator viruses (B) detected in bivalve shellfish.

Estuary, human AdVF and AdVC and AtAdV were detected more frequently and at higher concentrations in the period of August 2020 to August 2021 than during the period of May 2019 to March 2020, whereas OAdV was only detected between June 2019 and January 2020 (Fig. S1). No other seasonal patterns were observed for any of the AdVs. Atadenoviruses were slightly more abundant in oyster samples than in mussels, whereas no such pattern was observed for the other AdVs (Table 2 and Figs. 3, S1).

In comparison, the detection of the AdVs was more sporadic with low concentrations at the Menai Strait (Table 2). Similar to the Camel Estuary samples, AtAdV and AdVF were the most abundant adenoviruses, followed by OAdV, whereas AdVC was not detected in any of the samples. At the Menai Strait, the detection rates observed in water samples were similar to those in mussel samples, except for AtAdV, which was more abundant in mussels than in water. No site-specific differences were observed for either sample type. No distinct seasonal patterns were noted for any of the AdVs (Figs. S2–3).

3.5. Correlation between viral and bacterial concentrations and physico-chemical parameters

Weak positive correlation was observed between NoV genotypes,

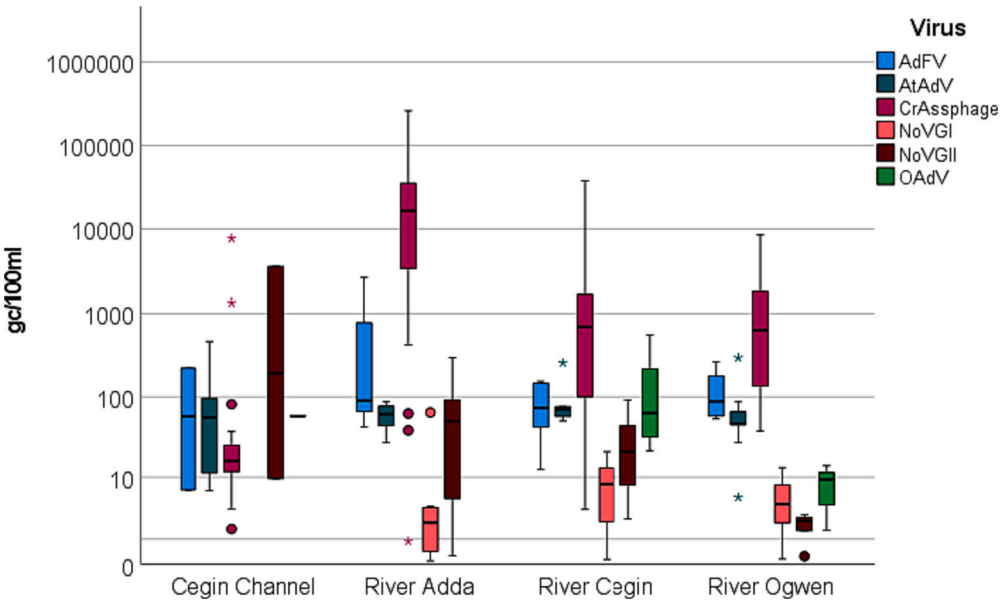

Fig. 4. Concentration of viruses detected in seawater samples collected at the Cegin Channel representative monitoring point in the Menai Strait (East) Mussel and Oyster Fishery Order, and river water from three rivers discharging into the Menai Strait.

SaV, HEV, human AdVs and AtAdV in the shellfish samples collected at the Camel Estuary (Table S2). *E. coli* showed weak negative correlation with OAdV and did not correlate with the other viruses. Adenovirus F and OAdV also showed weak negative correlation. No significant correlation between viral and *E. coli* levels were observed in the mussel samples collected at the Menai Strait (Table S3), probably due to small sample size and low positivity rates.

In the river water samples collected at the Menai Strait, nutrients (NO_x, NO_2, PO_4, NH_3) levels showed strong correlations and they also showed moderate correlation to *E. coli* and crAssphage levels (Table S4). While salinity strongly correlated with OAdV levels, it showed weak-moderate negative correlation with crAssphage and *E. coli*. However, *E coli* and crAssphage negatively correlated with OAdV, these correlations were not significant (Table S4). CrAssphage also showed weak correlation with water temperature. No significant correlation was observed between AdVs and NoVs probably due to low positivity rates.

4. Discussion

4.1. Quality control

The skimmed milk-based water concentration method has been widely used for the recovery of human and animal enteric viruses in environmental water samples with recovery rates of 15–95 % (Borgmästars et al., 2021; Calgua et al., 2013, 2008; Gonzales-Gustavson et al., 2017). Due to the robustness of the method, its efficiency was not assessed in this study. Most of the shellfish samples spiked with process control virus had >10 % recoveries, and similarly high recoveries were noted using this method before (Farkas et al., 2018; Lowther et al., 2018; Zhang et al., 2020), suggesting that the method successfully recovered the target viruses. The qPCR and RT-qPCR assay has also been validated previously, suggesting high sensitivity and minimal inhibition (Farkas et al., 2022, 2018, 2017; Wolf et al., 2010). Therefore, assay sensitivity and specificity were not evaluated in the current study.

4.2. Human virus abundance in shellfish harvesting areas

In this study, we conducted extensive monitoring of human enteric viruses and viral indicators in shellfish and water to assess wastewater pollution in two shellfish harvesting areas in the UK. While both sampling sites are in rural areas with agricultural activities and seasonal tourism, their hydrogeology is different. Due to the differences in the environment, sampling was also slightly altered between sites. While at the Camel Estuary, water sampling was limited to the shellfish beds, at the Menai Strait water samples were also collected from the rivers entering the Strait (Fig. 1).

Overall, the results showed high abundance of all targeted viruses, except HAV, in all sample types. Human NoVs were detected frequently albeit at low concentrations. In the Camel Estuary, higher NoV detection rates were observed in oysters than in immediately adjacent mussels. Differences in the uptake and accumulation of oysters and mussels have been observed previously, however, most studies suggested opposite trends (Dirks et al., 2021; Maekawa et al., 2007; Sarmento et al., 2020; Tian et al., 2007). Exposure to point source plumes can vary at fine spatial scales which may explain the results. Further studies would be required to explore the factors affecting viral bioaccumulation in different bivalve shellfish. In the Menai Strait, NoV detection rates were higher in river water samples compared to mussels. The observed differences in viral detection in water vs in mussels may be due to the differences in sampling locations. Three out of four water samples tested for viruses were collected from rivers where the microbial contamination from agricultural activities and from domestic wastewater are less diluted than in the receiving seawater. The low *E. coli* concentrations in seawater vs river water samples also suggest rapid dilution in the Menai Strait.

The NoV abundance peaked in the autumn-winter, in agreement with previous studies and the clinical observation of NoVs being winter pathogens (Bazzardi et al., 2014b; Flannery et al., 2013; Kambhampati et al., 2023; Li et al., 2023). Interestingly, NoVs were also detected in shellfish in the spring and summer, outside their usual season. These observations coincide with increased number of visitors in the area during Easter, end of COVID-19 lockdown measures and summer holidays, suggesting that tourists may introduce NoV to the local communities, resulting in high infections rates and subsequent detection of NoV in the aquatic environment polluted with domestic wastewater.

Human SaV and HEV were sporadically detected in mussels and oysters, whereas HAV was not detected. This is the first study where SaV was seen in shellfish in the UK. These viruses were detected exclusively in the summer of 2021, shortly after COVID-19 lockdown measures were lifted, suggesting that tourism may be responsible for the increased

number of cases. Furthermore, HEV can be a zoonotic disease with domestic and wild swine being hosts. Pigs are farmed within the Camel catchment. Therefore, it is possible that the HEV detected in shellfish was due to animal-associated outbreaks.

Human AdVC and AdVF were also detected in this study without seasonal patterns in their concentrations. No difference in detection rates between sample types (i.e., oysters, mussels, water) were observed, similar to previous findings (Olalemi et al., 2016). Adenovirus F was frequently detected in water and shellfish at both sites, probably due to its high abundance in the population and its high persistence in the aquatic environment, established previously (Aslan et al., 2011; Nagarajan et al., 2022). However, AdVF abundance was higher at the Camel Estuary than at the Menai Strait which may be due to differences in population densities.

In contrast, AdVC was sporadically detected only in the shellfish samples collected at the Camel Estuary, even though it is a common cause of viral respiratory diseases in children younger than five years and can be shed in faeces for years after infection (Garnett et al., 2002). Previous research suggested AdVC was highly prevalent in polluted water in Brazil with 52–93 % detection rates (Olalemi et al., 2016; Staggemeier et al., 2017), whereas the virus was less prevalent in shellfish (7 %) and river water (0 %) samples collected from less polluted areas in New Zealand (Wolf et al., 2010), similar to our findings. The lower detection rates or non-detections of AdVF and AdVC at the Menai Strait compared to the Camel Estuary suggests that the latter site was more prone to domestic wastewater contamination during the study period, especially August 2020 onward.

4.3. Viral indicators and microbial source tracking

CrAssphage was detected in almost all river water samples and was less prevalent in seawater and in mussels, similar to previous studies exploring crAssphage abundance in polluted water (Ballesté et al., 2019; Farkas et al., 2019; García-Aljaro et al., 2017; Ward et al., 2020). These findings are consistent with domestic wastewater input to the rivers entering the Menai Strait. The mussel samples collected from Bangor Pier at the Menai Strait had the highest crAssphage detection rates, suggesting that this site was more polluted than the other areas. Interestingly, crAssphage had higher detection rates and concentrations in shellfish and in water than AdVs. That may be due to its higher overall abundance in wastewater, as observed in other studies (Farkas et al., 2019; Wu et al., 2023). Also, some data suggest that crAssphage may associate with farmed animals, however, at a considerably lower degree than with humans (Ahmed et al., 2018; García-Aljaro et al., 2017; Malla et al., 2019; Stachler et al., 2017). Therefore, the farming of sheep and cattle in the study catchment area could make some contribution to crAssphage concentrations in water. The concentrations and detection rates were slightly lower for AdVF than for CrAssphage. AdVF is exclusively associated with humans, therefore indicating human faecal pollution and valuable as a tool for wastewater source tracking in the aquatic environment.

Atadenoviruses were also abundant in water and shellfish samples at both sampling locations throughout the study periods. Interestingly, AtAdV was more prevalent in shellfish samples than in water, as observed in the samples taken at the Menai Strait sites, suggesting that it is more persistent in shellfish tissue than other AdVs or crAssphage, hence, it may be used as an indicator for animal-derived faecal pollution. Adenoviruses are also extremely stable during wastewater treatment and in the aquatic environment, which further support their use for pollution assessment (Farkas et al., 2020; Okoh et al., 2010; Rames et al., 2016). Our data suggest that OAdV and AdVC were less prevalent at the two study sites than AtAdV and AdVF, hence they may not be as useful indicators than the latter two.

No significant correlation between *E. coli* levels and viral targets was noted in shellfish, similar to previous findings (Bazzardi et al., 2014b; Burkhardt and Calci, 2000; Chung et al., 1998; Winterbourn et al., 2016). Interestingly, Atadenoviruses titres also correlated well with AdVF, SaV and HEV, concentrations in shellfish samples collected at the Camel Estuary, suggesting that both animal and human wastewater pollution appear at the same time in that area, probably due to rainfall events (Hundesa et al., 2006; Wolf et al., 2010). In water samples, *E. coli* levels correlated with crAssphage and nutrient levels suggesting that the source of contamination was similar and predominantly human-related. Water salinity showed positive correlation with OAdV, however, only a few samples were positive for OAdV, and more observations would be necessary to draw conclusions. Salinity negatively correlated with *E. coli*, suggesting rapid dilution of the bacteria in seawater. No further correlations were observed probably due to low positivity rates.

This study focused on wastewater pollution in two shellfish harvesting areas in the UK and revealed significant health risks associated with the presence of human enteric viruses in shellfish and water. We detected NoVs and emerging viral pathogens, SaV and HEV, in bivalve shellfish, which may pose a health risk if oysters and mussels are consumed raw. Furthermore, NoVs were also frequently detected in water samples, that can result in waterborne illnesses. These viruses were not only detected during the cold months but during the summer period as well, potentially due to visitors in the areas. Increased population densities in the summers combined with international travel of residents and tourists can introduce viruses to communities which subsequently affects the aquatic environment. In order to reduce the health risks, more holistic environmental surveillance is needed including human pathogens and faecal indicators.

Using human and animal associated AdVs and crAssphage, we determined that the source of contamination in the Camel and the Menai Strait was a mixture of human and animal sources related to agricultural activities and wildlife populations. As *E. coli* may associate with all of these sources, it's use as an indicator may overestimate the magnitude of human-derived pollution in the aquatic environment. Therefore, we propose the use of a combination of human-specific viral indicators (e.g. crAssphage and AdVF) for tracking domestic wastewater contamination in water and shellfish as suggested previously (Gyawali et al., 2021). These indicators are more stable than bacterial proxies, hence better reflect the fate and survival of human enteric viruses in the aquatic environment. However, further studies are necessary to better understand the prevalence of potential indicators in the environment and their bioaccumulation in shellfish.

CRediT authorship contribution statement

Kata Farkas: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Finn Mannion:** Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Rees Sorby:** Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Ben Winterbourn:** Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing. **Susan Allender:** Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Charlie G.M. Gregory:** Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Phoebe Holding:** Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Jamie M. Thorpe:** Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Shelagh K. Malham:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Lewis Le Vay:** Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare no competing interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2024.116081.

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