Centre for Environment
Fisheries & Aquaculture
Science

Human noroviruses in shellfish waters: sources, distribution and relationships with *E. coli*

Summary of Defra funded project SEPF/WT0996 – Contamination of shellfish waters with human noroviruses: environmental risk factors and management options, 2013

Summary

- Study highlights the importance of storm overflows in NoV contamination of shellfish beds.
- Provides evidence that the activated sludge treatment process (modified Ludzack-Ettinger (MLE) at this study site is effective in reducing NoV titre in sewage.
- Provides evidence that an activated sludge treatment (MLE) followed by UV disinfection can deliver average total NoV (GI+GII) and *E. coli* reductions of 2.9$\log_{10}$ and 5.2$\log_{10}$, respectively.
- Helps to quantify the impact of NoV in shellfish waters by showing that total NoV levels in oysters can exceed 1,000 copies/g over distances of more than 7km from the pollution sources in an estuary impacted by a high number of storm water spills.
- Indicates that distance from sources of human faecal pollution, rainfall and river flows are significant risk factors for NoV contamination of shellfish waters.
- Shows that, on an individual sample basis, there is low correlation between levels of *E. coli* and NoV in oysters.
- Demonstrates successful application of the piloted sampling and analysis approaches used in this study and justifies continuation of the project to examine further shellfishery scenarios.

Project aims

There is currently insufficient evidence to evaluate the potential impact of a NoV standard on the protection of shellfish waters and future sewerage infrastructure investment.

To help satisfy the above policy requirement, Defra funded this investigation into the fate and behaviour of NoV within a shellfish water catchment.
This report details the findings from year one (phase I pilot study). Further reports will detail findings from years 2 and 3 of the study (phase II).

The overall objectives of the study are:

- To quantify NoV inputs from sewage discharges and rivers to the shellfish water.
- To evaluate the environmental dispersion of NoV in the shellfish water.
- To characterise the environmental conditions driving NoV contamination of shellfish.
- To compare concentrations of *E. coli* with levels of NoV in shellfish.

defra Environment Agency UKWIR FOOD STANDARDS AGENCY

Study site

The field experiments were carried out in a shallow, macro-tidal estuary draining a catchment with low levels of urbanisation (<7%) on the south coast of England.

Native oysters (*O. edulis*) have been classified in this harbour under food hygiene regulations since 1992. Currently, there are production areas classified at B and C grades under Regulation (EC) No 854/2004. The estuary has also been designated under the SWD since 1999. Data from the SWD monitoring indicates that the microbial quality of surface waters improved from 2006 to 2011.

There are two designated bathing waters near the harbour entrance which typically achieve higher (passing Guideline faecal indicator bacteria standards) water quality standards. Under the WFD, the harbour is classified as a 'heavily modified water body' and, as such, it is required to achieve 'Good Ecological Potential' by 2015.

The water company operates a combined sewerage network serving approximately 40,000 people in the catchment. The shellfish water catchment comprises 28 water company discharges of which 17 discharge directly to the estuary. The most significant discharges to the estuary were improved during the 'AMP4' (2005–2010) programme of water company waste water improvements. Further improvements to continuous and intermittent discharges will be implemented during 'AMP6' (2015–2020).

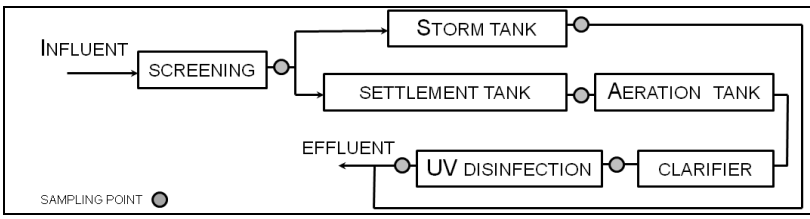
The harbour receives freshwater inputs from a river and a number of streams. Flows in these watercourses are dominated by the base-flow component, although they can increase substantially after exceptional rainfall.

Tidal flows in the harbour channels are generally bi-directional although wind-driven currents may complicate flow patterns to some extent. Wave energy inside the harbour is generally low.

Sampling programme

Sewage and freshwater samples

Sewage samples (250ml each) representative of the different stages of the treatment process (Figure 1) were collected at the main sewage treatment works (STW) discharging to the estuary on nine occasions from 12 October 2012 to 05 March 2013. These samples were collected in the morning (09:00–11:30) to coincide with higher flow rates through the works and prior to harbour sampling. Freshwater samples were collected from the main river on those same occasions. Samples were transported in cool boxes to Weymouth Laboratory for *E. coli* and NoV testing within 24h of collection.

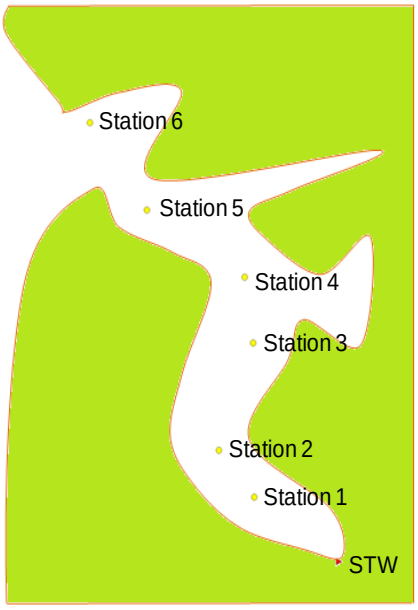


◄ **Figure 1** Schematic of sampling points in the sewage treatment works.

Shellfish samples

Two bags containing 11kg of native oysters each were initially deployed at 2 sentinel stations in the upper reaches of the shellfish water (station 1) and near the harbour entrance (station 5) on 12 October 2012 (Figure 2). The oysters had been harvested from a different estuary and depurated at an approved plant prior to the experiments.

Each sampling station consisted of an oyster bag and an iron chain of 10–15kg attached to one of end of a rope and a mooring buoy attached to the other end of the same rope (Figure 3).



▲ **Figure 2** Schematic of sampling stations.

▲ **Figure 3** Sampling station.

Oyster sampling commenced 1 week after deployment of bags until 12 November 2012. For a subsequent experiment, the quantities of oysters per bag were doubled and a further 4 bags were deployed. These bags contained native oysters harvested locally. The depurated oysters at stations 1 and 5 were discarded and local oysters also placed in these bags.

Five sampling campaigns were carried out under wet weather conditions and one under dry weather. Water temperature and salinity in surface waters were recorded at the time of sampling using a portable conductivity meter WTW Cond 197i.

Microbiological methods

Concentrations of *E. coli* were quantified in oysters using the most probable number method [1]. Levels of human NoV (genogroups I and II) were quantified in oysters using the method described by Lees and CEN WG6 TAG4 [2]. Levels of the virus were quantified in sewage and freshwater using the adsorption-elution method described by Lowther [3]. The preparation of wastewater concentrates followed the procedure developed by Cross [4] as modified by Puig et al. [5]. These methods employ polymerase chain reaction (PCR) to target genogroup I (GI) and genogroup II (GII) which cause the vast majority of human NoV infections.

Additional data

Levels of NoV quantified in native oysters from an oyster bed in the study site from May 2009 to April 2011 were obtained under permission from the FSA Food Standards Agency to complement the study. Notifications of sewage spills and additional information on sewage spill frequency and duration, flows, estimated sewage residence time and measured applied UV dose for the STW were supplied by the water company Total daily rainfall and river flow data recorded at two stations representative of the catchment were provided by the Environment Agency.

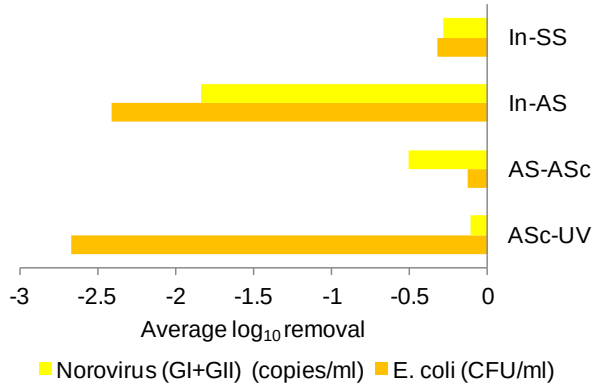
Results and significant findings

Removal of E. coli *and norovirus during the sewage treatment process*

Norovirus was consistently detected in influent sewage with all samples positive for GII. Total NoV levels (GI+GII) in settled storm water were not significantly (Mann-Whitney test; U=15; p>0.02) different from those in influent samples.

Overall, the STW delivered average reductions (screened influent to final discharged treated effluent) of 5.2log$_{10}$ and 2.9log$_{10}$ for *E. coli* and total NoV, respectively.

NoV and *E. coli* reductions varied between sampling campaigns. All NoV results in final effluent samples were <100 copies/ml.



◄ **Figure 4** Average log$_{10}$ removal of levels of *E. coli* and norovirus at the STW.
In -influent
SS -settled storm
AS -activated sludge (settlement tank)
ASC -activated sludge (post-clarification)
UV -ultra-violet disinfected effluent

Both *E. coli* and NoV concentrations decreased from primary to secondary treated effluent. However, *E. coli* at the end of the activated sludge process remained relatively high whereas NoV titre at this stage was generally in the lower limit of the infectious dose of the virus.

Key findings:

- The activated sludge process operating in this particular STW is effective in reducing *E. coli* and NoV from sewage.
- The average total reduction in *E. coli* (5.21log$_{10}$) meets the requirement of 5.25log$_{10}$ reduction for faecal coliforms in discharges impacting shellfish waters as per EA's UV disinfection policy [6]. *E. coli* reductions were similar to those characteristic of the better performing plants in E&W [7].
- The removal rates for total NoV during activated sludge processes (2.6log$_{10}$) are similar to those found in a selection of STWs in the UK [8].

Influence of performance parameters on norovirus removal

Levels of NoV showed a positive relationship with the residence time of the sewage in the storm tank. NoV removal also increased with applied UV dose. Large differences were detected in NoV titre over relatively small differences in applied UV dose. This suggests that this parameter is critically important in terms of effectiveness of NoV removal and that higher doses will significantly assist NoV reduction during treatment.

These studies used a low number of samples. Acquisition of further STW data will be explored in phase II of the study to permit wider generalisations and modelling.

Inputs of E. coli *and norovirus to the shellfish waters*

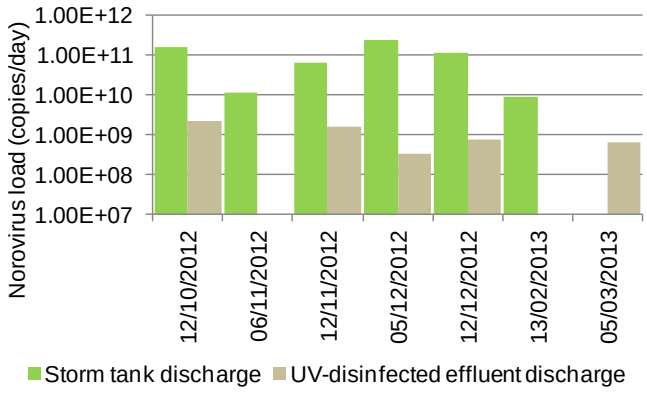
Sewage flows discharged from the storm tank to the recieving water, which by-pass full treatment, from this STW substantially exceed the design standard maximum number of 10 significant spills per annum on average as determined by the EA Discharge Consenting Policy [9]. Measured NoV loadings from the storm tank represented 97.6%–99.9% of the total load from the STW to the shellfish waters. This indicates that intermittent discharges represent the main mechanism of introduction of NoV to the shellfish waters.

Of the five samples of freshwater tested (immediately above the tidal limit upstream of the STW), one was positive for GI and three were positive for GII. None of the samples tested positive for both genogroups. Total NoV (GI+GII) and *E. coli* concentrations ranged from 3 to 178 copies/ml and from 333 to 7,000 CFU/100ml, respectively.

Figure 5 Loadings of norovirus from the STW. ►

Sample taken on 05/03/2013 under dry-weather.
Samples collected on 06/11/2012 and 13/02/2013 were negative for NoV.

Norovirus loadings (copies/day) from the storm tank ranged from $9x10^9$ to $2x10^{11}$. Under similar weather conditions, they were 1–3 orders of magnitude higher than those from the UV-disinfected effluent.



Key findings:

- The low concentrations of NoV in freshwater samples indicate low risk of NoV transmission from diffuse sources and other sewerage-related sources in this catchment upstream of the STW.
- Storm tank discharges represented 97.6%–99.9% of the total loading from the plant. During wet weather, storm tank discharges contributed concentrations of NoV that are much larger than those from the continuous discharge.

Environmental dispersion of E. coli *and norovirus in the shellfish waters*

Depurated oysters (initial NoV content of <100 copies/g) deployed at stations 1 and 5, sampled 23 days later, had total NoV content (GI+GII) of 4,832 and 200 copies/g, respectively. Samples taken at 31 days following deployment had NoV concentrations of 1,793 and 250 copies/g, respectively.

Locally sourced un-depurated oysters deployed at all 7 study stations and allowed to acclimatise for a period of 23 days exhibited similar NoV levels over a significant distance across the oyster fishery, decreasing only $0.6\log_{10}$ at station 5 (9.7km) near the harbour entrance (Figure 6). Maximum *E. coli* concentrations were within class B range at all stations.

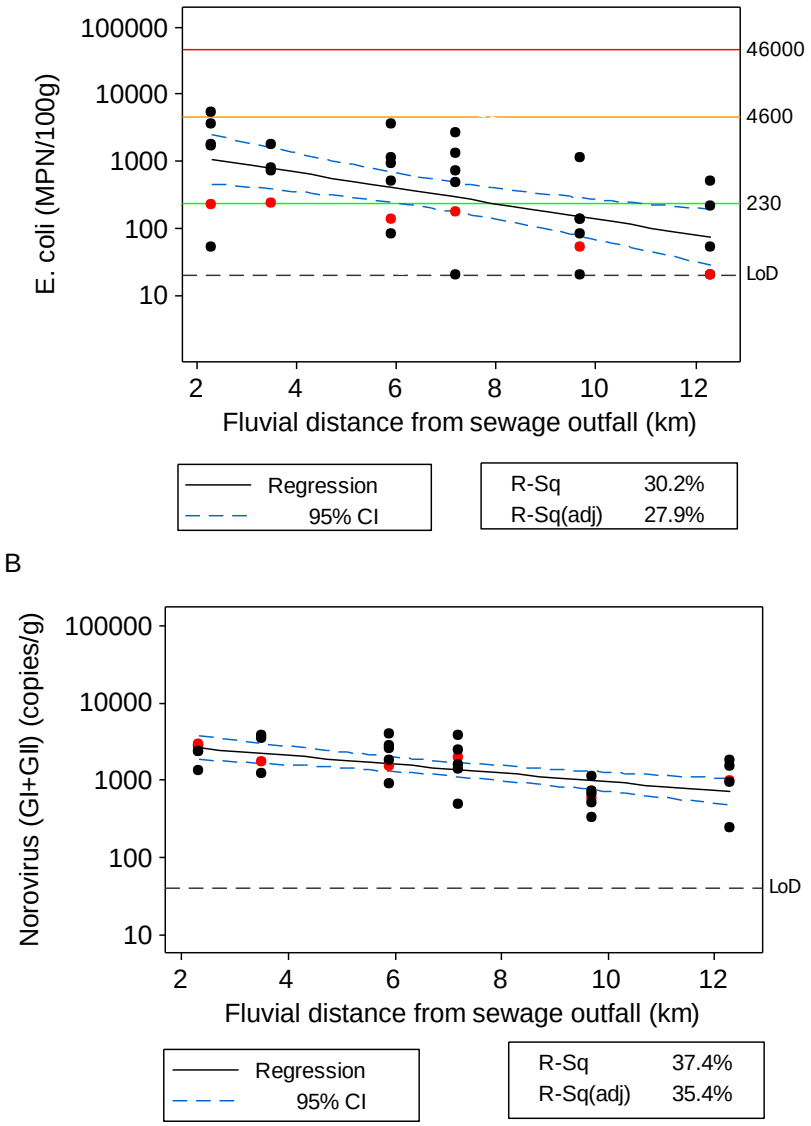
Concentrations of *E. coli* were significantly (Kruskal-Wallis test; H=18.81; p=0.002) different between sampling campaigns. In contrast, NoV concentrations between sampling occasions were not significantly different. NoV was consistently detected in oysters at all stations during the study period. All of the oyster samples were positive for both genogroups.

The gradient of NoV in oysters obtained in this study site was less evident than that detected in mussels from Dunedin-Otago Peninsula (New Zealand) (≈9km to reduce $1\log_{10}$) an open coast environment with deeper bathymetric profile [10, 11].

This information contributes to understanding of the likely zone of impact of NoV contamination in oysters following release from point source discharges however further work is needed to test these results in a range of other environments.

Key findings:

- The geographical extent of NoV contamination could be very significant considering the potentially long residence times of the water and relatively small areas associated with commercial oyster beds.
- The detection of NoV concentrations in oysters above 1,000 copies/g in the lower reaches of the harbour could be associated with reservoirs of the virus in sediments/bottom fauna and/or the combined impact of other sewage discharges. This will be further investigated during phase II of the study.



◄ **Figure 6** Relationships between levels of *E. coli* or NoV in native oysters and distance from the STW sewage outfall.
Linear models: (A) $\log_{10}$ *E. coli* (MPN/100g)=3.296-0.1166*fluvial distance (Km). (B) $\log_{10}$ norovirus (GI+GII) (copies/g)=3.542-0.05694*fluvial distance (km); R^2=37.4%.
Reference lines indicate thresholds for classification of shellfish production areas: class A–230 *E. coli*/100g; class B–4600 *E. coli*/100g; class C–46000 *E. coli*/100g. Red dots represent dry weather samples. LoD - limit of detection.

A gradient of *E. coli* (MPN/100g) contamination across the harbour was detected with geometric mean levels decreasing from 990 at station 1 to 69 at station 7.
NoV titres (copies/g) decreased with increasing distance from the STW outfall; mean total NoV concentrations decreased from 2,314 at station 1 to 963 at station 7. Maximum total NoV concentrations exceeded 1,000 at all stations.

Factors influencing norovirus contamination of shellfish

Rainfall

Significant correlations were found at stations 1, 3, 4 and 5 between NoV in oysters and rainfall either on the day of sampling or the total rainfall for 7 days prior to sampling.

Sewage spills

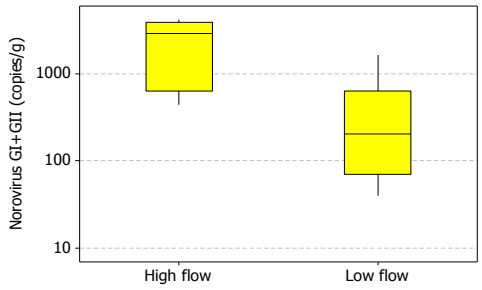
The larger variation of NoV results across the harbour occurred 30 days after deployment of oyster bags and coincided with a high number of spill events.

During dry weather, the concentrations of NoV in oysters from the deeper stations (5, 7) were not substantially different from those during wet weather.

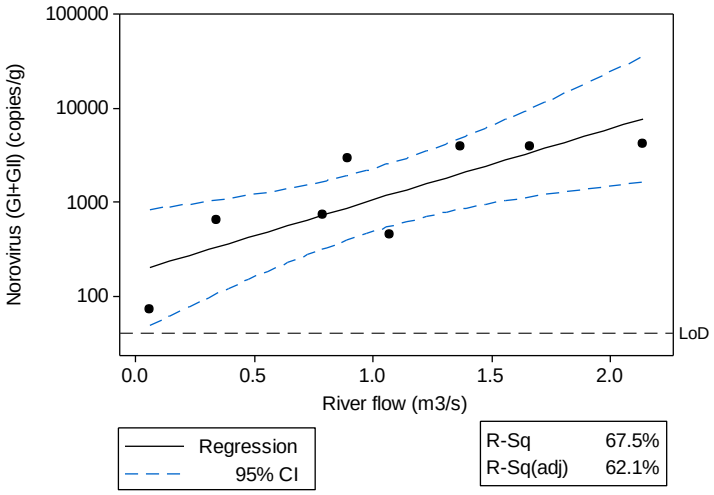
River flows using the FSA surveillance data

Total NoV (GI+GII) levels in oysters sampled during high river flow conditions were significantly (Mann-Whitney test; U=12.7; p<0.002) higher than those sampled during low-flows. Median NoV levels increased 1$\log_{10}$ during high-flow conditions (Figure 7). Significant correlations were found between levels of NoV and of *E. coli* in native oysters and river flows both on the day of sampling and 7 days prior to sampling.

Figure 8 shows that on a year round basis river flows account for 67% of the variation of NoV results. However, there are very few data points in the model to support the increase in NoV titre over the range of 0.06–2.140m^3/s.



▲ Figure 7 Boxplot of NoV levels in native oysters under high- and low-flows.



▲ Figure 8 Relationship between NoV levels in native oysters and river flows. Linear model: $\log_{10}$ norovirus (copies/g)=2.260+0.7595*river flow (m^3/s).

Data points under no flow conditions removed from the dataset to study the variation of NoV levels when freshwater inputs impacted the oyster beds.

Bathymetry and tides

Levels of total NoV in oysters at stations 5 (-6.8m) and 7 (-9.8m) were significantly (Kruskal-Wallis test; H=14.21; p=0.014) different from those in the remaining stations. Higher levels of *E. coli* were detected during flood tides. This is unusual in estuaries where the ebb usually imports headwater stream contaminated waters and feed downstream areas with faecal indicator bacteria [12]. This may be due to the *E. coli* bioaccumulation lag time following persistent pollution exposure.

Key findings:

- Rainfall enhances connectivity between sources of sewage pollution and the estuary by activating pollutant flow paths and increasing the capacity for NoV transport throughout the hydrological system.
- Tidal flushing is insufficient to dilute NoV contamination impacting the oyster beds at this study site.
- The different environmental influences on *E. coli* and NoV could be explained by the known differential speed of removal of the two organisms with viruses being removed much more slowly than bacteria (weeks rather than hours). Thus NoV levels are unlikely to be significantly reduced during a 6 hour tidal cycle even if the incoming flood tide water is of high microbial quality.

Relationship between E. coli *and norovirus*

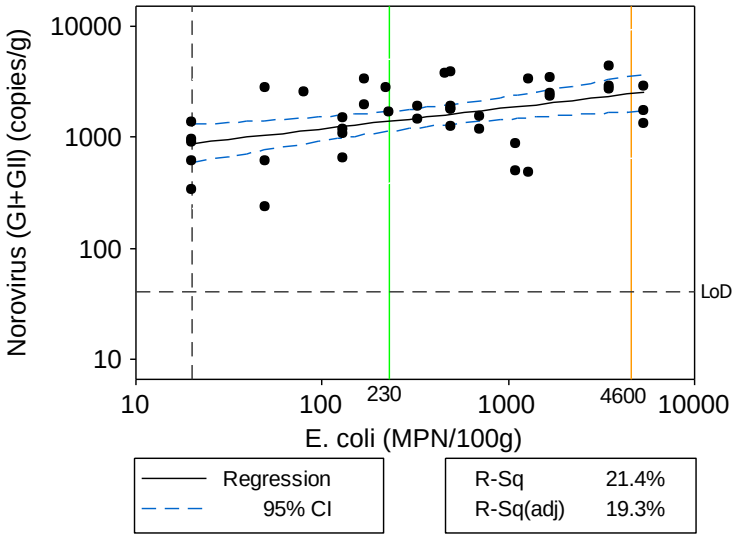
NoV in oysters showed a tendency to increase with *E. coli* levels although the spread of results around the regression line is also evident (Figure 9). A very significant proportion of NoV results lie above the 1,000 NoV level, particularly those concerning stations closer to the sewage outfall (1–3) which are associated with significant variation in *E. coli* results.

The range of NoV results corresponding to *E. coli* levels below the class A threshold (230 MPN/100g) was 233–3,347 copies/g.

Figure 9 Relationship between levels of *E. coli* and NoV. ►

Linear model: $\log_{10}$ norovirus (copies/g)=2.695+0.1900*$\log_{10}$ *E. coli* (MPN/100g). Reference lines indicate thresholds for classification of shellfish production areas under Regulation (EC) No 854/2004: class A–230 *E. coli*/100g; class B–4,600 *E. coli*/100g. LoD - limit of detection (40 copies/g norovirus and 20 MPN/100g *E. coli*).

The model showed good fit to the data (p=0.003). The R^2 indicates that $\log_{10}$ *E. coli* levels account for 21% of the $\log_{10}$ variation of total NoV in oysters.



Key findings:

- The low predictive value of *E. coli* could be associated with the sewage spills impacting the shellfish water.
- High (500–1,000 copies/g) or very high (1,000–10,000 copies/g) NoV titres were detected in samples with *E. coli* levels within the class B classification upper threshold (≤4,600 MPN/100g).

Recommendations

- This pilot study presents data on the relative contributions of storm water and the treated discharge to NoV contamination of oysters along a 12km stretch of an estuary. Further studies are recommended for phase II which will assist in broadening understanding of the relationship between sewage plume dilution and NoV contamination at this STW and investigating additional STWs, to attempt to develop generic models.
- The data presented on the zone of impact of NoV in the environment assists understanding but needs to be further explored in a wider range of coastal/estuary settings during phase II to build confidence in their wider applicability and predictive capability.
- The finding of occasional significant NoV contamination in more distant sampling stations raises the potential issue of environmental reservoirs or sinks for NoV. It is recommended that this is further explored during phase II.
- The data presented in this study can be used to assist development of strategies for assessment and management of NoV risks in shellfish waters. It is recommended that further complementary studies are undertaken during phase II to help ensure general applicability.

Public health implications

- It is important to note that the relative NoV risks identified for settled storm and continuous treated sewage discharges may not be necessarily applicable to discharges in different shellfish water catchments across England and Wales. This will be further studied for other areas during phase II of this project.
- Development of coastal management policy to ensure appropriate separation of sources of NoV pollution and vulnerable shellfisheries is also important for the sustainable future of the aquaculture industry. This will particularly be the case if the European Union introduces legislative standards for norovirus contamination in shellfish.
- This study was carried out during the period of peak prevalence of NoV in the community according to NoV outbreak monitoring data supplied by the Health Protection Agency [13]. A possible limitation of the environmental monitoring data presented is that the detection of NoV genomes by PCR techniques does not provide information about the infectivity of the pathogen. This approach is however the only method of detection available since there is no efficient culture system for this pathogen.
- The PCR genome copies detected in oyster samples from this study site are consistent with those found in samples associated with outbreaks in England and Wales [14].

References

[1] ISO, 2005. ISO TS 16649-3. Microbiology of food and animal feeding stuffs. Enumeration of β-glucuronidase positive *Escherichia coli* – part 3: Most Probable Number technique using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide acid. International Organization for Standardization, Geneva.
[2] Lees, D.N., CENWG6 TAG4. 2010. International standardisation of a method for detection of human pathogenic viruses in molluscan shellfish. *Food and Environmental Virology* 2: 146–155.
[3] Lowther, J. 2011a. Investigation into the levels of norovirus in influent and treated wastewater samples from a sewage treatment works. Cefas report to the Food Standards Agency, Contract FS235003 (P01009), Variation.
[4] Cross L.J. 2004. The development and application of quantitative methods for norovirus detection in sewage effluents and seawater. PhD Thesis of the School of Biomedical & Molecular Sciences, University of Surrey.
[5] Puig, M., Jofre, J., Lucena, F., Allard, A., Wadell, G., Girones, R. 1994. Detection of adenoviruses and enteroviruses in polluted waters by nested PCR amplification. *Applied and Environmental Microbiology* 60(8):2963–2970.
[6] Environment Agency, 2010. Water discharge permitting: disinfection of wastewater. Operational Instruction 347_09, Issued 30/06/2010.
[7] Environment Agency, 2002. Efficacy and effects of wastewater disinfection. Phase 5 Review of wastewater UV disinfection performance in England and Wales. R&D Technical Report P2-036/TR.
[8] Palfrey, R., Harman, M., Moore, R. 2011. Impact of waste water treatments on removal of noroviruses from sewage. WRc R&D Technical Report WT0924/TR to Defra.
[9] Environment Agency, 2003. Consenting guidance to achieve the requirements of the Shellfish Waters Directive (Microbial Quality). Environment Agency Water Quality Consenting Guidance 169_01 Issued 30/01/2003.
[10] Greening, G. 2007. NLRC shellfish safety following sewage spills. Institute of Environmental Science & Research Ltd. Envirolink report No. 162: NLRC20, May 2007.
[11] Greening, G., Lewis, G.D. 2007. FRST Programme C03X0301 Safeguarding environmental health and market access for NZ foods. Objective 2: virus prevalence in shellfish. Stakeholder report: virus prevalence in New Zealand Shellfish.
[12] Campos, C.J.A., Kershaw, S.R., Lee, R.J. 2013. Environmental influences on faecal indicator organisms in coastal waters and their accumulation in bivalve shellfish. *Estuaries and Coasts* 36:834–853.
[13] Health Protection Agency, 2013. Health protection report, Volume 7, No 10, 8 March 2013.
[14] Lowther, J.A., Gustar, N.E., Hartnell, R.E., Lees, D.N. 2012. Comparison of norovirus RNA levels in outbreak-related oysters with background environmental levels. *Journal of Food Protection* 75(2): 389–393.

Acknowledgements

Without the assistance of the many people from regulatory agencies and a member of the shellfish industry this study would not have been possible. To protect the anonymity of the study area, I will not name you individually, but I thank you very much for your input.

For additional information contact:

Carlos Campos
Cefas, Barrack Road, The Nothe, Weymouth, Dorset DT48UB, UK