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Executive summary

Contamination of bivalve shellfish with norovirus is recognised as a significant human health risk associated with faecally polluted shellfish. Norovirus is a human pathogen spread by the faecal-oral route and therefore contamination of the marine environment with human sewage is expected to be a major route of contamination of shellfish. Studies using real-time PCR have indicated a significant reduction of enteric virus levels by typical sewage treatments. However, the presence of norovirus in treated wastewater samples (as determined by RT-PCR) is well documented and this may provide a significant contamination risk for nearby harvesting areas - particularly during times of high levels of community infection.

This project aimed to contribute to the understanding of the risks to shellfish production posed by norovirus in sewage. The project tested regular samples of influent and effluent wastewater from a typical large municipal secondary sewage treatment works (STW), with potential impacts on several shellfish production sites, over an approximately 2-year period.

Both GI and GII norovirus were detected in all samples tested. Levels of GII norovirus were significantly higher than GI and ranged up to >1 million detectable genome copies/ml in crude influent and up to 64,000 copies/ml in treated final effluent. For GII levels a significant winter-spring seasonality was observed, with higher levels recorded between December and May than between June and November. This seasonality is similar to that seen for laboratory reports of norovirus illness reported to national surveillance. Average log reductions of GI and GII norovirus from crude to final effluent samples were 1.26 and 1.64 respectively.

Overall, this study highlights the potential for municipal STWs, if situated upstream of shellfish production areas, to contaminate the shellfish grown in such areas.

Acknowledgements

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1.0 Introduction

Contamination of bivalve shellfish with norovirus from human faecal sources is recognised as a major human health risk (Lees, 2000). Previous published and informal studies examining norovirus in UK oysters have revealed the presence of viral RNA in a significant proportion of oysters and other shellfish species taken from inshore waters in the UK (Lowther *et al*., 2008; Lowther *et al*., 2010). These viruses are spread by the faecal-oral route and contamination of the marine environment and the shellfish that grow within it can therefore be expected to occur principally via the sewerage system (Lees, 2000, Pommepuy *et al*., 2004). Studies using real-time PCR have indicated a significant reduction of enteric virus levels by typical sewage treatments (Carducci *et al*., 2009, Nordgren *et al*., 2009). However the presence of norovirus in treated wastewater samples as determined by RT-PCR is well documented (Carducci *et al*., 2009, da Silva *et al*., 2007, Myrmel *et al*., 2006, Nordgren *et al*., 2009) and, particularly during times of high levels of community norovirus illness, treated wastewater may provide a significant contamination risk for nearby harvesting areas.

This project aimed to contribute to the understanding of the risks to shellfish production posed by norovirus in sewage through regular testing of influent and effluent samples from a typical large secondary sewage treatment works (STW).

2.0 Aims and objectives

This small project aimed to determine the prevalence, typical levels and seasonality of norovirus in influent and treated wastewater from a single STW with secondary treatment (serving a population of >100,000, and with potential impacts on several shellfish production sites [5-10km downstream]), in order to investigate the effectiveness of treatment for removal of viruses and the potential of treated wastewater to contaminate shellfish production areas. This works utilised:-

- preliminary treatment using 6mm mechanical and 12mm hand raked screens plus grit removal using Detritors
- primary treatment using settlement in circular tanks
- secondary treatment using an activated sludge plant
- final settlement in circular tanks

3.0 Methods

3.1 Collection of wastewater samples

One litre samples of screened crude wastewater and treated final effluent from the STW were taken on an approximately fortnightly basis between June 2009 and May 2011 (total of 41 pairs of samples). Screened crude samples were taken from a sampling point immediately downstream of the screens and Detritors. Final effluent samples were taken from the designated Environment Agency sampling point immediately downstream of the final settlement tanks. Samples were dispatched to the laboratory for analysis on the day of sampling.

3.2 Detection and quantification of GI and GII norovirus

3.2.1 Preparation of wastewater concentrates

Each wastewater sample was shaken by hand to mix, then separate 20ml volumes were added to each of two polycarbonate centrifuge bottles. A 10µl volume of Mengo virus strain vMC_0 (to act as a process control) was then added to each bottle and the samples were subjected to ultracentrifugation at >150,000 x g and 4ºC for 1 hr using a Beckman LE-80K ultracentrifuge. The supernatants were discarded and the two pellets for each sample combined by stepwise resuspension in a single 2ml volume of glycine buffer (0.25M, pH 9.5). The bottle containing the resuspended pellet was incubated on ice for 20 min to enable viruses to elute then 2ml of cold 2x Phosphate Buffered Saline (PBS) was added. The sample was centrifuged at 12,000 x g and 4°C for 20 min to pellet particulate matter then the supernatant was transferred to a clean bottle and the pellet discarded. A volume of 18ml 1x PBS was added to the tube then this was subjected to ultracentrifugation at >150,000 x g and 4ºC for 1 hr to pellet viruses. Finally, the supernatant was discarded then the pellet resuspended in 1ml 1x PBS. This was transferred to a clean tube and retained at 4ºC for RNA purification and RT-PCR.

3.2.2 Purification of viral RNA

Viral RNA extraction was carried out using NucliSens magnetic extraction technology (BioMerieux). For each wastewater sample a 500µl aliquot of sample concentrate was added to 2ml NucliSens lysis buffer in a 15ml centrifuge tube. In addition for each batch of samples tested a negative extracted control consisting of 500µl water only was also prepared and tested in parallel. Samples and controls were vortexed briefly then incubated at room temperature for 10 min before 50µl magnetic silica was added to each tube and the samples incubated at room temperature for a further 10 min. The tubes were centrifuged at 1,500 x g for 2 min and the supernatants removed by aspiration. The pelleted silica beads were resuspended in 400µl wash buffer 1 then transferred to individually labelled 1.5ml tubes on the MiniMag extraction station. The magnet of the MiniMag was raised to collect the silica beads on the walls of the tubes, then the beads washed for 30 secs using the wash function of the MiniMag. The supernatants were removed by aspiration, then the magnet lowered and the silica beads resuspended with 400µl of wash buffer 1, then washed and the supernatant aspirated as before. The resuspension/wash/aspiration cycle was then repeated using 500µl wash buffer 2 then 500µl wash buffer 3 (wash for 15 secs only). The pelleted silica beads were then resuspended with 100µl of elution buffer. The tubes were capped and transferred to the thermoshaker at 60°C and shaken at 1,400 rpm for 5 min, to allow elution of nucleic acids from the silica beads. After elution the tubes were transferred to a portable magnet to collect the silica beads on the walls of the tubes, then the supernatant (nucleic acid (NA) extract) was transferred to a clean 0.5ml tube and stored at -20°C until required for reverse transcription.

3.2.3 One-step RT-PCR

For each sample or control and both norovirus genogroups three aliquots of 5µl NA extract were added to adjacent wells of a 96-well optical reaction plate and made up to 25µl with (GI or GII) TaqMan reaction mix (final concentration of 1x each Ultrasense reaction mix, Rox reference dye and RNA Ultrasense enzyme mix (Invitrogen), 500nM forward primer, 900nM reverse primer, and 250nM probe.

Positive (dilution series prepared from a known concentration of plasmid carrying a copy of the target sequence) and negative (water only) PCR control materials were also tested. The plate was placed in a Stratagene Mx3005P real-time PCR machine with the following amplification program; 55°C for 60 min, then 95°C for 5 min, followed by 45 cycles of 95°C for 15 secs, 60°C for 1 min and 65°C for 1 min. For analysis, threshold values were set at 0.20 fluorescence units, then threshold cycle (Ct) values were determined using the Mx3005P system software. Unexpected results in any positive or negative extraction or RT-PCR control triggered retesting of any affected samples.

3.2.4 Calculation of extraction efficiency

For each sample two aliquots of 5µl NA extract were added to adjacent wells of a 96-well optical reaction plate and made up to 25µl with Mengo virus-specific TaqMan reaction mix (final concentrations as described above). A dilution series prepared from the Mengo virus process control material was also tested. The plate was placed in a Stratagene Mx3005P real-time PCR machine and amplified using the program described above. The percentage extraction efficiency for each sample was determined by comparing the Ct values for the sample NA extract with those for the Mengo virus dilution series.

3.2.5 Quantification of norovirus using dsDNA standard curve analysis

On each TaqMan run a log dilution series of dsDNA control corresponding to a range of approx 1 to 10,000 template copies/µl (quantified using spectrophotometry at 260nm) was included. The Ct values from this dilution series were then used to produce a standard curve. For each TaqMan replicate for the samples under test a quantity in copies/µl was determined using the corresponding standard curve. Not detected replicates were ascribed a quantity of zero. The average quantities from the three replicates in each norovirus genogroup-specific TaqMan assay were calculated to give an overall quantity in detectable copies/µl NA extract for that sample and genogroup. This quantity was corrected using the percentage extraction efficiency and converted into a concentration in copies/ml wastewater taking into account the various concentration factors involved in the testing.

3.3 Statistical analysis

All statistical analysis was carried out using Minitab 16 software. Results of all analyses are included in Appendix 2.

4.0 Results

Full results are included in Appendix 1. All crude and final effluent samples tested positive for both norovirus GI and GII, however levels varied widely (Figures 1 & 2). For crude samples GI levels ranged between 48 – 94,541 copies/ml (geometric mean 3,691 copies/ml, 95% CI 2028-6730 copies/ml), while GII levels ranged between 1,399 – 1,141,478 copies/ml (geometric mean 25,504 copies/ml, 95% CI 14,158-45,604 copies/ml). For final effluent samples GI levels ranged between 2 – 18,747 copies/ml (geometric mean 202 copies/ml, 95% CI 108-379 copies/ml), while GII levels ranged between 18 – 64,406 copies/ml (geometric mean 585 copies/ml, 95% CI 315-1,086 copies/ml).

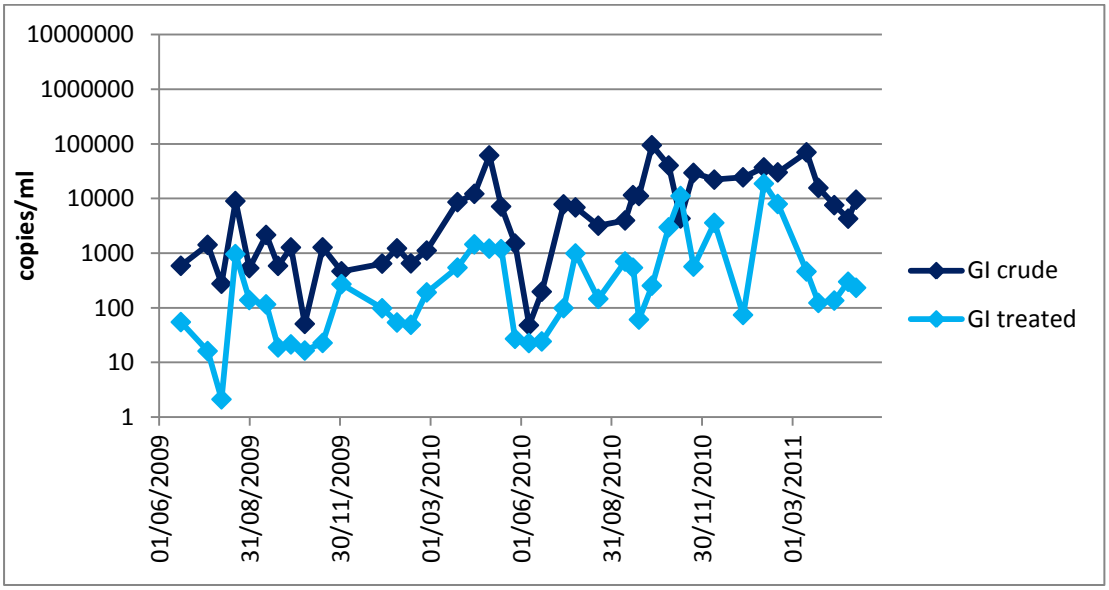

Figure 1: Norovirus GI levels in wastewater samples.

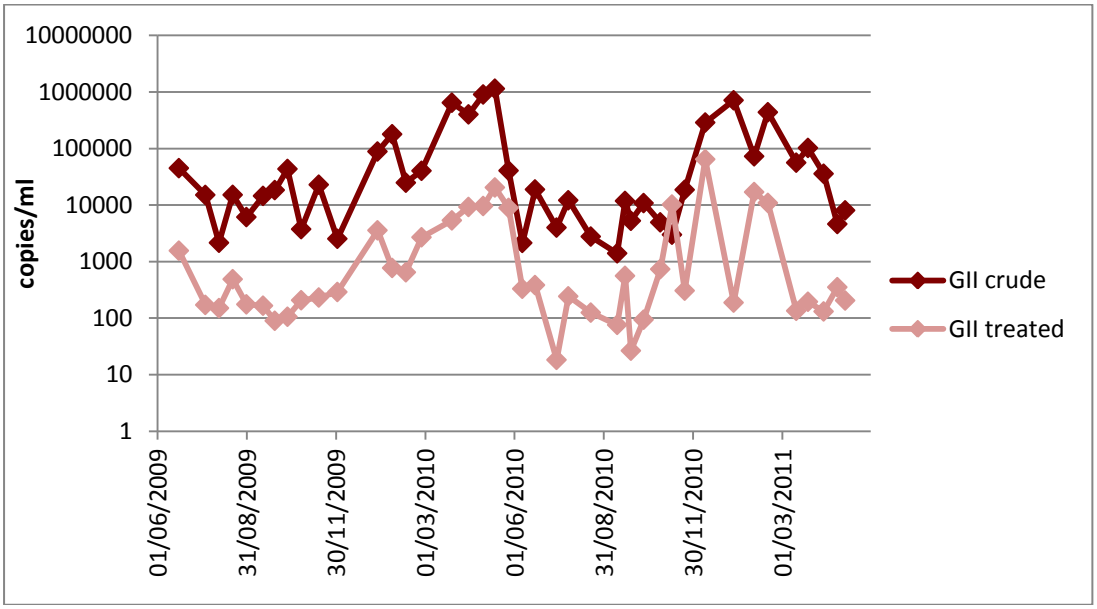

Figure 2: Norovirus GII levels in wastewater samples.

In all but one pair of samples lower levels of both norovirus GI and GII were found in final effluent compared with crude wastewater. Analysis of $\log_{10}$ transformed results for each pair of samples using the Wilcoxon signed ranks test indicated that, for both GI and GII, within pairs, results for crude wastewater were significantly higher than for final effluent (p=0.000 for both genogroups). Average log reductions from crude to effluent were 1.26 (range -0.41 to 2.57, 95% CI for mean 1.05-1.47) and 1.64 (range -0.53 to 3.58, 95% CI for mean 1.41-1.87) for GI and GII respectively. Application of the Wilcoxon test to GI and GII reductions for each set of samples indicated that within each set reductions in GII were significantly greater than reductions in GI (p=0.000).

Levels of norovirus GII were higher than GI in the majority of samples; analysis of $\log_{10}$ transformed results for each sample using the Wilcoxon test indicated that this difference was significant (mean difference=0.650 [95% CI 0.461 - 0.839], p=0.000). This difference was also found to be significant if only crude (mean difference=0.838 [95% CI 0.557 – 1.119], p=0.000) or only final effluent samples (mean difference=0.461 [95% CI 0.210 - 0.712], p=0.002) were included.

Levels of GII norovirus displayed a distinct winter-spring peak, with highest levels recorded between December and May. For crude wastewater, samples taken during this period averaged 1.043 logs higher than samples taken during June to November. Analysis of the two sets using the Kruskal-Wallis test revealed this difference was significant (p=0.000). Similarly for final effluent, samples taken during December and May averaged 0.907 logs higher than those from June to November; these two sets were also found to be significantly different using the Kruskal-Wallis test (p=0.002). GI norovirus did not display the same pronounced seasonality. However, the lowest levels were again observed during the summer months.

5.0 Discussion

This project aimed to determine the levels of norovirus RNA in crude and final effluent wastewater samples from a single STW with secondary treatment over an approximately 2-year period. Both GI and GII norovirus were detected in all samples tested. The levels detected in crude influent samples were comparable to those reported from raw sewage sampled at a plant serving a population of ~300,000 in Toyama, Japan (Iwai *et al*., 2009) although the peak levels reported in this study were 1-2 logs higher. In both studies levels of GII norovirus were higher than GI, reflecting the clinical situation where the overwhelming majority of norovirus outbreaks are caused by GII strains (Kroneman *et al*., 2008). The strong winter-spring seasonality in norovirus levels exhibited here is similar to that usually observed with infections in the community in the temperate Northern hemisphere and closely corresponds to the distribution of lab reports of norovirus infections in England and Wales received by the HPA over the study period (see e.g. http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/Norovirus/)

The average log differences in detectable levels between crude and final effluent were 1.26 and 1.64 (corresponding to 94.5% and 97.7% reductions) in GI and GII norovirus respectively. By comparison an average log reduction of 1.5 logs has been reported for the municipal treatment works in Gothenburg, Sweden (Nordgren *et al*., 2009). By the nature of PCR however, it is not possible to ascertain to what extent

reductions in virus infectivity may have occurred, as the method does not discriminate between infectious and non-infectious virus. Previous studies have demonstrated that certain treatments can reduce levels of infectious virus in water without significantly impacting PCR detection (Gasilloud *et al*., 2003). It is therefore possible that the treatment regime at the STW investigated here produced greater reductions in infectivity than the numbers for PCR detection reported.

In conclusion, this study demonstrates the continuous presence of norovirus (both GI and GII) in both crude and final effluent wastewater from a single, large municipal STW in the UK. The average reduction in PCR detectable norovirus genome copies from crude to final effluent was 1-2 logs (90-99%). Final discharged effluent at this STW was found to routinely contain significant levels of norovirus. This highlights the potential for municipal STWs, if situated upstream of shellfish production areas, to contaminate the shellfish grown in such areas. It further highlights the potential of the methods applied to demonstrate and monitor this risk.

This study examined a single large STW and the results may not therefore be directly relevant for other sewage discharges of which there are many different forms, ranging from untreated storm overflows to tertiary treated (disinfected) effluents. There are also a variety of sizes of effluents from septic tanks for individual dwellings through to major STWs such as studied here. Sewage discharges may also vary in the levels of norovirus they contain due to the population served, for example hospitals and closed institutions may discharge high levels. Finally the point of discharge and the circulation and dilution prior to impacting shellfish beds will also be significant in relation to norovirus risk. It is apparent that the methods employed in this study are capable of significantly improving analysis of the contributions of these various norovirus risk factors for shellfish production areas and a similar but larger study examining a number of different types of STW and other discharges would potentially prove very useful and could help focus food safety risk management measures. Considering the large and ongoing investment in sewage infrastructure to improve the health status of UK shellfisheries, information on how best to control norovirus risks could be seen as critical information to inform such investment decisions.

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Appendix 1: wastewater sample results

	Detectable virus genome copies/ml			
	Crude		Final effluent	
Sampling date	GI	GII	GI	GII
23/06/2009	588	44878	55	1558
20/07/2009	1424	15076	16	171
03/08/2009	276	2152	2	150
17/08/2009	8993	15130	962	487
31/08/2009	533	6118	139	175
17/09/2009	2157	14498	116	166
29/09/2009	586	18374	19	90
12/10/2009	1275	43420	21	106
26/10/2009	51	3744	17	207
13/11/2009	1279	22890	23	231
02/12/2009	466	2530	271	290
12/01/2010	645	88393	98	3566
27/01/2010	1226	178802	54	770
10/02/2010	652	24826	49	646
26/02/2010	1117	40282	192	2689
29/03/2010	8599	644658	544	5348
15/04/2010	12201	401137	1452	9232
30/04/2010	61554	902515	1212	9601
12/05/2010	7157	1141478	1185	20441
26/05/2010	1500	40551	27	8897
09/06/2010	48	2138	23	330
22/06/2010	197	18842	24	384
14/07/2010	7827	3989	99	18
26/07/2010	6884	12127	989	242
18/08/2010	3185	2768	146	125
14/09/2010	3978	1399	704	76
22/09/2010	11543	11811	543	559
28/09/2010	11130	5259	61	27
11/10/2010	94541	10828	254	94
28/10/2010	40296	4941	2974	734
09/11/2010	4316	2985	11139	10199
22/11/2010	29507	18589	568	306
13/12/2010	22178	287619	3592	64406
11/01/2011	24456	712128	74	188
01/02/2011	37118	72689	18747	16950
15/02/2011	30109	437721	7949	10910
16/03/2011	70089	56126	463	135
28/03/2011	15659	102268	123	195
13/04/2011	7572	35773	136	131
27/04/2011	4285	4612	300	352
05/05/2011	9537	8045	233	205

Appendix 2: Statistical analysis

Crude vs Treated
Wilcoxon Signed Rank Test: diff crude-treated GI

```
Test of median = 0.000000 versus median not = 0.000000

                           N for   Wilcoxon         Estimated
                      N   Test  Statistic      P     Median
diff crude-treated GI  41     41      857.0  0.000      1.263
```

Wilcoxon Signed Rank Test: diff crude-treated GII

```
Test of median = 0.000000 versus median not = 0.000000

                            N for   Wilcoxon         Estimated
                       N   Test  Statistic      P     Median
diff crude-treated GII  41     41      860.0  0.000      1.635
```

GI vs GII reductions
Wilcoxon Signed Rank Test: GI redn - GII redn

```
Test of median = 0.000000 versus median not = 0.000000

                         N for   Wilcoxon         Estimated
                    N   Test  Statistic      P     Median
GI redn - GII redn  41     41      137.0  0.000    -0.4248
```

GI vs. GII
Wilcoxon Signed Rank Test: GI-GII all

```
Test of median = 0.000000 versus median not = 0.000000

               N for   Wilcoxon         Estimated
          N   Test  Statistic      P     Median
GI-GII all  82     82      555.0  0.000    -0.6276
```

Wilcoxon Signed Rank Test: GI-GII crude

```
Test of median = 0.000000 versus median not = 0.000000

                 N for   Wilcoxon         Estimated
            N   Test  Statistic      P     Median
GI-GII crude  41     41      100.0  0.000    -0.8551
```

Wilcoxon Signed Rank Test: GI-GII treated

```
Test of median = 0.000000 versus median not = 0.000000

                   N for   Wilcoxon         Estimated
              N   Test  Statistic      P     Median
GI-GII treated  41     41      189.0  0.002    -0.4623
```

December – May vs. June – November

Kruskal-Wallis Test: GI crude versus Period

```
Kruskal-Wallis Test on GI crude

Period    N  Median  Ave Rank      Z
Dec-May  19   3.934      24.5   1.75
Jun-Nov  22   3.418      18.0  -1.75
Overall  41              21.0

H = 3.07  DF = 1  P = 0.080
```

Kruskal-Wallis Test: GII crude versus Period

```
Kruskal-Wallis Test on GII crude

Period    N  Median  Ave Rank      Z
Dec-May  19   4.946      29.2   4.05
Jun-Nov  22   4.053      14.0  -4.05
Overall  41              21.0

H = 16.42  DF = 1  P = 0.000
```

Kruskal-Wallis Test: GI treated versus Period

```
Kruskal-Wallis Test on GI treated

Period    N  Median  Ave Rank      Z
Dec-May  19   2.433      24.8   1.88
Jun-Nov  22   2.029      17.7  -1.88
Overall  41              21.0

H = 3.54  DF = 1  P = 0.060
```

Kruskal-Wallis Test: GII treated versus Period

```
Kruskal-Wallis Test on GII treated

Period    N  Median  Ave Rank      Z
Dec-May  19   3.430      27.4   3.16
Jun-Nov  22   2.279      15.5  -3.16
Overall  41              21.0

H = 10.01  DF = 1  P = 0.002
```