CONCENTRATION OF NOROVIRUS DURING WASTEWATER TREATMENT AND THE IMPACT ON OYSTER CONTAMINATION

John Flannery, Sinéad Keaveney, Paulina Rajko-Nenow, Vincent O'Flaherty* and William Doré

Marine Institute, Rinville, Oranmore, Co. Galway, Ireland.

**Microbial Ecology Laboratory, Microbiology, School of Natural Sciences, National University of Ireland, Galway, Ireland*

Running headline: norovirus reduction during waste water treatment

Correspondence to:

Bill Doré

Marine Institute,

Rinville,

Oranmore,

Co. Galway,

Ireland

Tel. +353 91 387224

Fax. +353 91 387201

Email: bill.dore@marine.ie

ABSTRACT

Concentrations of *E. coli*, FRNA bacteriophage, norovirus genogroup I (NoV GI) and II (NoV GII) in wastewater were monitored weekly over a one-year period at a wastewater treatment plant (WWTP) providing secondary treatment. A total of 49 samples of influent, primary and secondary-treated wastewater were analyzed. Using a real-time RT-qPCR, mean NoV GI and NoV GII concentrations detected in effluent wastewater were 2.53 and 2.63 $\log_{10}$ virus genome copies 100 ml^{-1} respectively. Mean NoV concentrations in wastewater during the winter period (January to March inclusive) (n=12) were 0.82 (NoV GI) and 1.41 (NoV GII) log units greater than mean concentrations for the rest of the year (n=37). The mean reduction of NoV GI and GII during treatment was 0.80 and 0.92 log units respectively with no significant difference detected in the extent of NoV reductions due to season. No seasonal trend was detected in the concentrations of *E. coli* or FRNA bacteriophage in wastewater influent and showed mean reductions of 1.49 and 2.13 log units respectively. Mean concentrations of 3.56 and 3.72 $\log_{10}$ virus genome copies 100 ml^{-1} for NoV GI and GII respectively were detected in oysters sampled adjacent to the WWTP discharge. A strong seasonal trend was observed and concentrations of NoV GI and GII detected in oyster were correlated with concentrations detected in the wastewater effluent. No seasonal difference was detected in concentrations of *E. coli* or FRNA bacteriophage detected in oysters.

Keywords: norovirus, wastewater treatment**,** seasonality, oysters

INTRODUCTION

Norovirus (NoV) is the most common cause of outbreaks of acute gastroenteritis in Ireland (11) and is the major cause of acute non-bacterial gastroenteritis in adults worldwide (33). In general NoV causes mild illness involving diarrhoea and vomiting although symptoms can be more severe in vulnerable groups such as the elderly (33). NoV is spread by the faecal oral route and has been demonstrated to be highly infectious particularly in enclosed settings such as schools, hospitals, care homes, cruise ships and domestic residence (17, 20, 33). The NoV genus comprises non-enveloped, positive-sense RNA viruses of the family *Caliciviridae*. The genus norovirus is genetically diverse and is divided into 5 different genogroups based on the sequence similarity of the capsid protein (24). Each genogroup has a varying number of genotypes; NoV genogroup I (NoV GI) and NoV genogroup II (NoV GII) contain the majority of NoV genotypes that have been implicated as causing illness in humans (45). NoV GII, and in particular variants of the NoV GII genotype 4, are most commonly associated with human illness in clinical and community outbreaks (8, 31, 45).

NoV can be shed in large numbers (up to 10^8 viruses g^{-1}) in the faeces of infected individuals (28) and can continue to be excreted for up to two weeks post-resolution of symptoms (39), NoV is, therefore, commonly present in municipal wastewater (13, 25, 36). The discharge of municipal wastewater into aquatic environments is practiced throughout the world and the link between wastewater discharge and the contamination of bivalve molluscan shellfish is well established (29). Such contamination occurs because bivalve molluscan shellfish such as oysters are filter feeders and can accumulate micro-organisms particularly when grown in sewage contaminated water (29). Oysters can become contaminated with NoV in this manner

and have been linked to numerous outbreaks of gastroenteritis in consumers (1, 15, 43, 51). This public health problem is recognised worldwide and sanitary regulations based on bacterial standards exist to control the risk. However, despite compliance with the existing bacterial standards, NoV contaminated oysters continue to cause illness on an ongoing basis (15, 51). The environmental discharge of wastewater is also closely regulated to limit this impact. In Europe, designated sensitive marine sites such as shellfish harvesting areas are protected under the appropriate environmental regulations such as the Shellfish Waters Directive (2006/113/EC) (2). Authorities must endeavour to ensure that shellfish harvested from designated shellfisheries comply with the relevant bacterial standards. Wastewater treatment can be considered to be a significant control point to limit the extent of microbial contamination of the marine environment and achieve compliance with both food safety and environmental bacterial standards. The impact of wastewater treatment on faecal indicator organisms such as *Escherichia coli* (*E. coli*) has been extensively studied and comprehensive data exists on the removal of such organisms through wastewater treatment (32, 50). Similarly, the survival of faecal indicator organisms in the marine environment is well described (9). Therefore it is possible to accurately predict the likely microbiological impact of a wastewater treatment plant (WWTP) discharge on a shellfishery in terms of faecal bacteria allowing the likelihood of compliance with the regulatory limits to be determined. Data from previous studies generally indicate that concentrations of enteric viruses may be reduced to a lesser extent than bacteria during the wastewater treatment processes (19, 22); limited data, however, exists concerning the extent of NoV removal during WWTP treatment. The lack of such data is primarily due to the absence of a reliable culture system for NoV and has lead to the use of viral indicator organisms. FRNA bacteriophage of the family *Leviviridae* have been used as

surrogates for enteric viruses in wastewater (22) and in shellfish (16). The detection of NoV in environmental samples using molecular techniques has traditionally been difficult because of the relatively low target concentrations involved and the inhibitory substances present in such samples (27). Recently, robust real-time RT-qPCR procedures has been used for the quantitation of NoV in shellfish (27) and wastewater (13, 36). da Silva et al., (13) monitored wastewater effluents to assess the removal of NoV during different wastewater treatment processes using real-time RT-qPCR and found that all processes studied reduced the NoV concentrations discharged into receiving waters. Nordgren et al., (36) monitored the concentrations of NoV in wastewater effluents over a one year period and found that NoV GII demonstrated a seasonal trend with greater concentrations detected in the winter months. In addition, both NoV GI and GII reductions during wastewater treatment were similar. However, no quantitative studies assessing the reduction of NoV through a WWTP and subsequent transmission to shellfish are present in the available literature. The aim of this study was to evaluate the reduction of NoV GI and NoV GII through a WWTP providing secondary treatment and to evaluate the impact of the discharge on the concentrations of NoV in oysters adjacent to the outfall.

MATERIALS AND METHODS

Wastewater Treatment Plant and Wastewater Sampling

The WWTP studied treated wastewater from a population equivalent (P.E.) of 91600 and received an average daily volume of incoming wastewater of 45000 m^3. Preliminary treatment at the plant provided screening and grit removal. This was followed by treatment with a conventional activated sludge system including primary settlement, aeration and final settlement. The final effluent was discharged into the sea through a 400 m long outfall pipe at a depth of 10 m.

One litre, 24-hour composite samples of influent and final effluent were taken on a weekly basis. In addition, a one litre grab sample of wastewater was collected following primary treatment. All wastewater samples were collected in polyethylene bottles and transported under ambient temperatures to the laboratory within one hour of collection. Wastewater sampling commenced in June 2009 and ended in May 2010 (n=49).

Concentration procedure for wastewater sample NoV analysis

A conventional filter adsorption-elution method was used for the concentration of wastewater samples and was based on previously described methods (6, 26). Four hundred microlitres of 2.5M $MgCl_2$ (Sigma-Aldrich, United Kingdom) was added to a single, 40 ml sample volume of wastewater to obtain a final concentration of 25 mM $MgCl_2$. The sample was then adjusted to between pH 3.5 and pH 6.0 with 1M HCl (Sigma-Aldrich) and mixed on a rocking platform for 45 minutes. The sample was then passed through a glass fibre pre-filter (Millipore, Billerica, MA) placed directly on a bacteriological membrane filter (0.45 µm pore size and 90mm diameter; Millipore) attached to a plastic magnetic filter holder (Pall, Port Washington, NY).

The filters were then washed once using 25 ml 0.14M NaCl and dried of excess wash solution prior to placing the bacteriological membrane filter in 4 ml 50 mM glycine-NaOH buffer pH 9.5 and shaking at 500 rpm for 20 min. The virus eluate was transferred to a tube containing 100 µl of 1 M HCl (pH 1.0) followed by centrifugation using an Amicon® Ultra-4 centrifugal filter unit (Millipore) at 4000 x g for 10 min. The filter unit was washed in 550 µl of molecular biology grade water and the virus concentrate (>500 µl) stored at -20°C prior to RNA extraction.

Oyster sampling

Oysters from a batch previously demonstrated to be free from microbial contamination (*E. coli*, FRNA bacteriophage and NoV) were suspended in mesh bags 1m below the water surface directly above the WWTP outfall. Oysters were deployed at the outfall for one month before sampling commenced. Samples of 24 oysters were collected each week and transported to the laboratory within 2 hours under chilled conditions (<15°C). Each week oyster samples were collected five days before the wastewater samples were collected. Oyster sampling commenced in July 2009 and ended in May 2010 (n=38).

Preparation of oyster samples for *E. coli*, FRNA bacteriophage and NoV analysis

Upon receipt into the laboratory any dead or open oysters not responding to percussion were discarded. Oyster samples were analyzed for *E. coli* and FRNA bacteriophage within 24 hours of receipt using previously published methods (4, 5). For *E. coli* and FRNA bacteriophage analysis, 10 oysters were thoroughly cleaned under running potable water, the meat and intravalvular fluid was homogenized using a blender and diluted 1:3 with 0.1% (w/v) neutralised bacteriological peptone (Oxoid,

Cambridge, U.K.) (3). For FRNA bacteriophage analysis, 50 ml of the diluted homogenate was centrifuged at 2000 x g for 10 min and the supernatant retained for testing.

For NoV analysis a further 10 oysters were opened and the hepatopancreas from each oyster was dissected and was finely chopped. Two grams of oyster hepatopancreas was weighed to which 2 ml of 100 µg ml^{-1} Proteinase K solution (30 Umg^{-1}; Sigma-Aldrich) was added. Fifty microlitres of Mengo virus strain MC_0 was added at this stage as an internal positive control (IPC) virus controlling for the virus extraction efficiency similar to that described by Costafreda et al., (12). The sample was then incubated at 37°C with shaking at 150 rpm for 1 hour followed by incubation at 60°C for 15 min. The sample was then centrifuged at 3000 × g for 5 min and the supernatant was retained for RNA extraction. The homogenates were either stored at 4°C prior to RNA extraction within 24 hours, or stored at -80°C where RNA extraction was undertaken within 1 month.

***E. coli* enumeration in wastewater and bivalve molluscan shellfish**

Appropriate log dilutions of influent and effluent wastewater samples respectively and diluted shellfish homogenates were assayed for *E. coli* using a standardized five-tube three-dilution most probable number (MPN) method (5). This procedure is the mandatory method used in Europe to classify shellfish harvesting areas. The diluted wastewater and homogenates were inoculated into 10 ml volumes of minerals modified glutamate broth MMGB (CM0607, Oxoid) and were incubated at 37°C for 24 ± 2 hours. The presence of *E. coli* was subsequently confirmed by subculturing tubes indicating acid production onto TBX agar (CM0945, Oxoid) at 44°C for 24 ± 2

hours. The limit of detection (LOD) of the assay was an MPN of 20 *E. coli* 100 g^{-1} and 20 *E. coli* 100 ml^{-1} for shellfish and wastewater samples respectively.

FRNA bacteriophage enumeration in wastewater and bivalve molluscan shellfish

The diluted wastewater samples and shellfish homogenate was analyzed for FRNA bacteriophage using a standardized procedure (4) that uses the *Salmonella enterica* serovar Typhimurium WG49 host (21). *S.* Typhimurium has been genetically engineered by the inclusion of an F-pili producing plasmid and has been shown to reliably select for FRNA bacteriophage and demonstrate negligible interference from somatic bacteriophage (22). Briefly, to 2.5 ml of molten 1% tryptone yeast-extract glucose agar held at 45°C, was added 1 ml volumes of appropriately diluted sample and 1ml of host culture (>10^6 cfu ml^{-1}). This mixture was poured onto 2 % tryptone yeast-extract glucose agar plates and incubated overnight at 37°C. Characteristic plaques were counted and each plaque was assumed to originate from one FRNA bacteriophage. The results were expressed as the number of plaque-forming units (pfu) $100g^{-1}$. The LOD of the assay for shellfish and wastewater samples was 30 pfu 100 g^{-1} and 10 pfu 100 ml^{-1}, respectively.

NoV RNA extraction procedure for shellfish and wastewater extracts

RNA was extracted from 500 µl of wastewater extract or shellfish Proteinase K extract using the NucliSENS® miniMAG® extraction platform and NucliSENS® magnetic extraction reagents (bioMérieux, Marcy l'Etoile, France) following the manufacturer's instructions. Viral RNA was eluted into 100 µl of elution buffer (bioMérieux). A single negative RNA extraction control (using water only) was processed alongside shellfish and wastewater samples to be extracted. The eluted RNA was stored at -80°C until analysis using real-time RT-qPCR was undertaken.

RT-qPCR controls and standards

Plasmids carrying the NoV GI and GII target sequences (supplied by Dr. Francoise S. LeGuyader, Ifremer, Nantes, France) were used to prepare standards for quantitation and controls for determining RT-PCR inhibition. Plasmids were transformed in competent cells to create dsDNA and purified as described by Le Guyader et al., 2009 (27). From the purified dsDNA, single-use aliquots containing 10^5 genome copies μl^{-1} NoV GI and NoV GII were prepared for quantitation in the RT-qPCR. From the dsDNA plasmids, external control (EC) RNA was prepared using the same procedure as Le Guyader et al., 2009 (27) and were divided into single-use aliquots of 10^7 genome copies μl^{-1} for NoV GI and GII for use in determining RT-PCR inhibition. The dsDNA and EC RNA standards were stored at -20°C for a period of less than 6 months at which time a new batch was prepared containing the same concentration.

Determination of NoV GI and GII using one-step RT-qPCR

For NoV GI and NoV GII analysis of wastewater and shellfish samples, duplicate 5µl aliquots of sample RNA was added to adjacent wells of a 96-well optical reaction plate. This was followed by 20 µl of the appropriate one-step reaction mix prepared using RNA Ultrasense one-step RT-qPCR system (Invitrogen, Carlsbad, CA) containing 1 × reaction mix, 500 nM forward primer, 900 nM reverse primer, 250 nM probe, 1 × µl Rox and 1.25 µl of enzyme mix. For NoV GI analysis, previously described primers QNIF4 (13), NV1LCR and probe NVGG1p (48) and for NoV GII analysis, primers QNIF2 (30), COG2R (23) and probe QNIFS (30) were used. In addition, no template controls were included for NoV GI, GII and IPC virus on the same 96-well plate. The plate was incubated at 55°C for 60 min, 95°C for 5 min and

then 45 cycles of 95°C for 15s, 60°C for 1 min and 65°C for 1 min on an AB7500 real-time PCR instrument (Applied Biosystems, Foster City, CA).

To control for the presence of RT-PCR inhibitors 5 µl of sample RNA was added to a further two wells to which 1 µl of EC RNA (10^7 genome copies μl^{-1}) was added. A log dilution series of the NoV GI and GII EC RNA ranging from 10^7 to 10^4 copies μl^{-1} was included on each RT-qPCR run. The mean C_T value obtained for samples that included the EC RNA was used to calculate the quantity of EC RNA detected in the sample which was then used to estimate PCR amplification efficiency which was expressed as a percentage. Wastewater and oyster samples with an amplification efficiency greater than 25% were accepted for inclusion in this study.

For extraction efficiency, samples seeded with the IPC, Mengo virus, were subjected to RT-qPCR for Mengo virus. Twenty microlitres of a one-step reaction mix prepared with the same one-step RT-qPCR system containing the same concentrations of reaction mix, primers, probe, Rox and enzyme mix as was used for NoV analysis. Duplicate 5µl aliquots of sample or extraction control RNA were added to the adjacent wells of the 96-well plate. Forward (Mengo209) and reverse (Mengo110) primers and probe (Mengo147) used were the same as those described by Pinto et al., 2009 (44). The C_T value of the sample was compared to a standard curve obtained by preparing log dilutions from the same batch of Mengo virus as was used to seed samples for analysis, and was subsequently expressed as percentage extraction efficiency. Samples with an extraction efficiency of greater than 1% were accepted for inclusion in this study.

To enable quantification of NoV RNA in copies per µl, a log dilution series of the GI and GII DNA plasmids (range 1×10^0 to 1×10^5 copies per µl) was included in duplicate on each RT-qPCR run. The number of RNA copies in NoV positive samples

was determined by comparing the C_T value to the standard curves. The final concentration was then adjusted to reflect the volume of RNA analyzed and was expressed as detectable virus genome copies g^{-1} hepatopancreas or detectable virus genome copies 100 ml^{-1} wastewater. The LOD for NoV GI and GII was 20 detectable virus genome copies g^{-1} and 25 detectable virus genome copies 100 ml^{-1} for shellfish and wastewater samples respectively.

Calculation of log reductions of *E. coli,* FRNA bacteriophage and NoV through the wastewater treatment process

The reductions by the wastewater treatment process were calculated using the following equation:

Log reductions = $\log_{10} (N_{inf}/N_{eff})$

where: N_{inf} = concentration of microbial parameter (MPN *E. coli* 100 ml^{-1}, FRNA bacteriophage pfu 100 ml^{-1} and NoV genome copies 100 ml^{-1}) detected in influent wastewater; N_{eff} = concentration of microbial parameter (MPN *E. coli* 100 ml^{-1}, FRNA bacteriophage pfu 100 ml^{-1} and NoV genome copies 100 ml^{-1}) detected in primary or secondary treated effluent wastewater. For the samples with negative results (n=2), the log reductions could not be determined; however, the minimum log reductions were estimated by applying a value of the detection limit of the assay. Minitab statistical software version 15 (Minitab Inc., PA, USA) was used for the data analysis whereby all data was initially assessed for normality (Anderson Darling) and then log transformed to achieve a normal distribution.

RESULTS

Concentrations of microbial parameters detected in wastewater

E. coli, FRNA bacteriophage, NoV GI and GII concentrations detected in all influent, post-primary and post-secondary treated effluents are shown in table 1. *E. coli* concentrations ranged from 3.73 to 7.54 $\log_{10}$ MPN 100 ml^{-1} in influent wastewater and underwent a mean log reduction of 1.49 $\log_{10}$ MPN 100 ml^{-1} during the entire treatment process. The mean reduction of FRNA bacteriophage was 2.13 $\log_{10}$ pfu 100 ml^{-1} with mean concentrations of 5.54, 5.46 and 3.41 $\log_{10}$ pfu 100 ml^{-1} detected in influent, primary treated and secondary treated effluent respectively. No correlation was found between concentrations of *E. coli* and FRNA bacteriophage with either NoV GI or Nov GII levels in influent and effluent wastewater ($r< 0.07$ in all instances).

NoV GI and GII was detected in influent and effluent wastewater on all sampling occasions throughout the sampling period. Mean concentrations of NoV GI and NoV GII detected in influent wastewater were 3.32 and 3.55 $\log_{10}$ genome copies 100 ml^{-1} respectively. Mean concentrations of NoV GI and NoV GII detected in effluent wastewater were 2.53 and 2.63 $\log_{10}$ genome copies 100 ml^{-1}, respectively. NoV GII concentrations in influent wastewater were significantly greater ($p<0.05$) than concentrations of NoV GI and the mean concentrations of NoV GII were 0.23 $\log_{10}$ virus genome copies 100 ml^{-1} higher than NoV GI concentrations.

The mean NoV GI and GII reduction during the entire treatment process was 0.80 and 0.92 $\log_{10}$ virus genome copies respectively. Although the mean $\log_{10}$ reduction achieved throughout the study period was 0.12 greater for NoV GII compared with NoV GI, this difference was not statistically different ($p =0.25$). Mean $\log_{10}$

reductions for all microorganisms ranged from 0.13 (NoV GI) to 0.32 (FRNA bacteriophage) $\log_{10}$ units following primary treatment (Table 1).

Seasonal variation in NoV concentrations

NoV GI and GII concentrations detected in the influent wastewater during the winter period were significantly higher ($p < 0.05$) than during the rest of the year (Table 2). Mean concentrations of NoV GI and GII in the influent wastewater for the period January to March inclusive (n=12) were 0.82 and 1.41 $\log_{10}$ virus genome copies $100ml^{-1}$ greater than mean concentrations for the rest of the year (n=37) respectively. No significant difference was detected in the extent of NoV reductions during treatment due to season and consequently NoV concentrations in the final effluent were also significantly higher ($p < 0.05$) during the January to March period (Table 2) than during the rest of the year. The ratio of NoV GI to GII detected in wastewater also varied by season. Throughout the period January-March 2010, NoV GII concentrations were on average 0.49 $\log_{10}$ higher than NoV GI concentrations in effluent wastewater and 0.63 $\log_{10}$ higher in influent wastewater. The mean difference between NoV GI and GII concentrations at this time of year was highly significant ($p < 0.05$). However, no significant difference was detected between NoV GI and GII concentrations during the rest of the year (April-December 2009). Unlike NoV concentrations, no seasonal trend was detected in the concentration of FRNA bacteriophage or *E. coli* in wastewater influent or effluent.

Oysters

Mean FRNA bacteriophage and *E. coli* concentrations detected in oysters throughout the study period were 4.14 $\log_{10}$ pfu $100\ g^{-1}$ (SD ± 0.64) and 3.22 $\log_{10}$ MPN $100\ g^{-1}$

(SD ± 0.55) respectively. On a sample by sample basis, *E. coli* concentrations in oysters did not correlate with concentrations of NoV GI or GII (r= -0.097; p= 0.57) (r= 0.184; p= 0.26). Similarly, FRNA bacteriophage concentrations did not correlate with NoV GI or GII(r= 0.015; p= 0.93 or r= 0.252; p= 0.127 respectively). Unlike for NoV, no seasonal difference was observed in the concentrations of FRNA bacteriophage and *E. coli* in oysters. Weekly concentrations of NoV detected in oysters and wastewater effluent are shown in Figure 1. Mean NoV GI and GII concentrations detected in oysters over the year long monitoring were 3.53 and 3.73 $\log_{10}$ virus genome copies g^{-1} respectively (Table 3). NoV detected in oyster samples displayed a strong seasonal trend with significantly higher concentrations ($p < 0.05$) in the winter compared with the rest of the year. Mean concentrations of NoV GI and GII detected during the January to March period were 1.31 and 1.65 $\log_{10}$ virus genome copies g^{-1} greater than concentrations detected during the rest of the year respectively. Log concentrations of NoV in oysters were significantly correlated with concentrations detected in effluent wastewater on a weekly basis (NoV GI r =0.48; p <0.05 and NoV GII r =0.68; p <0.05).

DISCUSSION

In this study we detected NoV in wastewater from a WWTP on a weekly basis throughout a year-long monitoring period. The use of real-time RT-qPCR in this study demonstrated that NoV was continuously discharged into the marine environment from the WWTP throughout the year. NoV GI and NoV GII was continuously detected in influent wastewater, demonstrating that both NoV genotypes circulate in the human population throughout the year. Whilst NoV was detected in wastewater year-round, the concentrations of NoV GI and GII increased significantly during the

period January to March. This increase was most pronounced with NoV GII and is consistent with epidemiological reports that generally record a predominance of NoV GII infections occurring at this time of year (35). NoV related gastroenteritis infections in the community are recognised as being strongly seasonal with peak infections observed during the colder winter months (24, 35). During the period January to March 2010, the Health Protection Surveillance Centre in Ireland recorded 1309 cases of NoV infections. Furthermore, 202 cases were recorded in the region where the WWTP investigated in this study is located (11) compared with a total of 60 during the remainder of the year recorded in this area. It is notable that although the majority of NoV infections are generally associated with NoV GII (45), high concentrations of NoV GI were simultaneously detected in wastewater. The fact that there was a seasonal increase in the concentration of NoV GI detected in the wastewater concurrent with increased NoV GII concentrations during this study would appear to be evidence of a simultaneous increase of NoV GI infections in the community during this period. Given this, it is possible that the significance of symptomatic NoV GI infections in the community is under-estimated or alternatively that there is a significant concentration of shedding of NoV GI in the community associated with increased asymptomatic infections occurring at this time of year. The concentrations of NoV detected in the present study differ to those found in a number of previous studies investigating the removal of NoV during wastewater treatment. These have indicated that NoV is often absent in wastewater effluent particularly during the summer months (19, 25). However in a recent year-long study by Nordgren *et al.*, NoV was detected from a WWTP serving a P.E. in excess of 800,000 (36). It may be that the detection of NoV throughout the year during the previous study and our investigation may be related to the size of the population

served by the WWTP. There is likely to be a greater chance of NoV being present in wastewater from WWTPs serving large populations considering that only a relatively small percentage of the population may be shedding NoV during non-epidemic periods.

The reduction of NoV GI and GII during wastewater treatment was consistent between genogroups irrespective of the initial concentrations of virus present in the influent. This suggests that both genogroups are impacted in a similar manner to one another during the activated sludge treatment process investigated here. Moreover, NoV GI and NoV GII underwent similar reductions, irrespective of the season and NoV was released to the environment with the same seasonal profile as observed for infections in the community. The application of real-time RT-qPCR procedures in this study indicates that mean reductions for NoV GI and NoV GII concentrations of less than one $\log_{10}$ virus genome copy are achieved through a conventional activated sludge WWTP and falls within the range previously reported (36, 40). This limited reduction means that during the winter period, NoV GI and GII were discharged in wastewater effluent at concentrations greater than 3 $\log_{10}$ virus genome copies 100 ml^{-1}. Concentrations recorded post primary treatment for all microbiological parameters in this study indicate that minimal reduction is achieved by this process. In this study, the majority of the reduction achieved for each of the parameters investigated was observed during the activated sludge, secondary treatment process.

Recently a specialised tissue culture system for the detection of NoV has been reported (47). However, this has not been used to investigate NoV concentrations in environmental samples and currently it is not possible to directly investigate the viability of NoV in wastewater effluent. The absence of a reliable tissue culture system has lead to the adoption of virus surrogates for use in inactivation studies (14,

38, 49). FRNA bacteriophage have been proposed as surrogates for enteric viruses in a range of settings including shellfish harvesting areas and wastewater treatment processes (16, 18, 22, 46). The mean $\log_{10}$ FRNA bacteriophage reduction observed during this study was 2.11 $\log_{10}$ which is significantly greater than that observed for NoV and is consistent with other reports (7, 10, 52). We employed a direct agar overlay plaque assay to detect only viable FRNA bacteriophage and this may account for the greater reduction observed over NoV rather than a true difference between the level of reduction for the two viruses. It has been demonstrated that real-time RT-qPCR procedures may detect both infectious and non-infectious virus particles (37, 41, 42). It is possible; therefore, that inactivated NoV may be detected by the real-time RT-qPCR method used here. Therefore, the results from our study and others (13, 25, 36) may overestimate the number of infectious virus present in the final effluent and thus underestimate the reduction of viable viruses and the infectious risk. Pecson et al., (41) found that a 4-log reduction in infectious bacteriophage MS2 when exposed to UV irradiation produced a real-time PCR signal loss of just 0.11 $\log_{10}$. Therefore in this study, it was not possible to determine whether the reductions of NoV are representative of the actual level of NoV reduction that would be observed if a viability assay was used to detect infectious NoV. It is clear that relying solely on real-time PCR to determine the viral reduction during wastewater treatment may be misleading and in the absence of a culture system for NoV, a surrogate culturable virus may provide a better indication of the reduction of infectious viruses throughout wastewater treatment processes. FRNA bacteriophage may prove useful for this purpose until such time that a reliable culture system for NoV or procedures to estimate concentrations of viable NoV become available.

In Ireland, as in the rest of the European Union, *E. coli* is used as the bacterial indicator organism to assess the sanitary quality of bivalve molluscan shellfish. Monthly sampling of the oysters in this study would have showed compliance with a category B harvesting area (<4600 MPN *E. coli* 100 g^{-1} in 90% of samples) meaning that the oysters could be sold for consumption following minimal treatment such as depuration (29). Given the minimal reduction of NoV provided by the WWTP, elevated concentrations of NoV were detected in oysters harvested adjacent to the outfall throughout the year. These concentrations would be consistent with those that have caused illness in consumers (15) and demonstrates the inadequacy of *E. coli* to assess the NoV risk associated with oysters. As alternatives to *E. coli*, FRNA bacteriophage have been proposed as a viral surrogate to indicate the presence of NoV in oysters previously (16, 18) and thus were included in this study. However, no seasonal trend was observed during our study as has been observed by others (34) and oysters were contaminated to consistent concentrations year round and did not demonstrate an increased risk of higher concentrations of NoV being present during the winter months. This questions their suitability of use as an indicator of NoV in oysters. However, it has been proposed that FRNA bacteriophage may provide useful information on the viral contamination of shellfish in areas that are infrequently impacted by sewage rather than in areas undergoing continuous wastewater inputs as studied here (18).

This study provides a comprehensive dataset concerning the concentrations of NoV GI and GII in a WWTP providing secondary treatment and the effect of effluent on NoV concentrations in shellfish. As wastewater treatment is considered an important control in reducing the microbial contamination of aquatic environments to acceptable concentrations, the actual reduction provided by treatment processes has implications

for plant operators and water management agencies. The data from this and other studies (25, 36) demonstrates that conventional wastewater treatment processes cannot be relied upon in isolation to prevent the contamination of the marine environment and thus oysters with NoV as determined using real-time PCR. As yet, methods are not available to differentiate infectious from non-infectious NoV and the detection of NoV in oysters using current procedures may overestimate the infectious risk. It is probable that low concentrations of NoV, as determined using real-time PCR, may not have an impact on consumer health. Therefore, results from widespread general monitoring of oysters need to be placed in context and should be considered to be one element of a more comprehensive risk-based approach to managing NoV contamination in shellfisheries. A more useful approach may be to target at risk harvest areas identified through the use of sanitary surveys and areas known to be at risk of contamination by municipal wastewater to mitigate the risk of NoV contamination from oysters.

ACKNOWLEDGEMENTS

We thank Dr. Soizick Le Guyader for kindly providing us with the NoV GI and GII plasmids used in the RT-qPCR.

This work was funded by the Environmental Protection Agency, Ireland as part of the Science, Technology, Research and Innovation for the Environment (STRIVE) Programme 2007–2013. Project code: 2008-EH-MS-7-53.

REFERENCES

1. **Ang, L. H.** 1998. An outbreak of viral gastroenteritis associated with eating raw oysters. Communicable Disease and Public Health **1:**38-40.
2. **Annonymous.** 2006. Council Directive 2006/113/EC of 12 December 2006 on the quality required of shellfish waters (codified version). p. 14-20. *In* O. J. o. E. C. L. 376 (ed.), vol. L376.
3. **Anonymous.** 2003. ISO 6887-3 Microbiology of food and animal feeding stuffs -- Preparation of test samples, initial suspension and decimal dilutions for microbiological examination -- Part 3: Specific rules for the preparation of fish and fishery products.
4. **Anonymous.** 1995. ISO 10705-1:1995 Water quality -- Detection and enumeration of bacteriophages -- Part 1: Enumeration of F-specific RNA bacteriophages.
5. **Anonymous.** 2005. ISO/TS 16649-3 Microbiology of food and animal feeding stuffs-Horizontal method for the enumeration of B-glucuronidase-positive *Escherichia coli* - Part 3: Most probable number technique using 5-bromo-4-chloro-3-indolyl-B-D-gucuronide.
6. **APHA.** 2005. Standard methods for the examination of water and wastewater, 21 ed. American Public health Association, American Waterworks Association water Environment Federation, Water Environment Federation, Washington.
7. **Arraj, A., B. J., H. Laveran, and O. Traore.** 2005. Comparison of bacteriophage and enteric virus removal in pilot scale activated sludge plants. J. Appl. Bacteriol. **98:**516-524.

8. **Atmar, R. L., and M. K. Estes.** 2006. The epidemiologic and clinical importance of norovirus infection. Gastroenterol. Clin. N. Am. **35:**275-290.

9. **Bitton, G.** 2005. Wastewater microbiology, vol. 3. Wiley, New Jersey.

10. **Carducci, A., P. Morici, F. Pizzi, R. Battistini, E. Rovini, and M. Verani.** 2008. Study of the viral removal efficiency in an urban waste water treatment. IWA Publishing.

11. **Cloak, F., S. Jackson, P. Garvey, and P. McKeown.** 2010. Surveillance of infectious intestinal (IID), zoonotic and vectorborne disease, and outbreaks of infectious disease: Quarter 1 –2010. Health Protection Surveillance Centre.

12. **Costafreda, M. I., A. Bosch, and R. M. Pintó.** 2006. Development, evaluation, and standardization of a real-time TaqMan reverse transcription-PCR assay for quantification of hepatitis A virus in clinical and shellfish samples. Appl. Environ. Microbiol. **72:**3846-3855.

13. **da Silva, A. K., J. C. Le Saux, S. Parnaudeau, M. Pommepuy, M. Elimelech, and F. S. Le Guyader.** 2007. Evaluation of removal of noroviruses during wastewater treatment, using real-time reverse transcription-PCR: different behaviours of genogroups I and II. Appl. Environ. Microbiol. **73:**7891-7897.

14. **de Roda Husman, A. M., P. Bijkerk, W. Lodder, H. van den Burg, W. Pribil, A. Cabaj, P. Gehringer, R. Sommer, and E. Duizer.** 2004. Calicivirus inactivation by nonionizing (253.7-nanometer-wavelenght [UV]) and ionizing (gamma) radiation. Appl. Environ. Microbiol. **70:**5089-5093.

15. **Doré, B., S. Keaveney, J. Flannery, and P. Rajko-Nenow.** 2010. Management of health risk associated with oysters harvested from a norovirus contaminated area, Ireland, February - March 2010. Euro Surveill. **15:**1-5.

16. **Doré, W. J., K. Henshilwood, and D. N. Lees.** 2000. Evaluation of F-specific RNA bacteriophage as a candidate human enteric virus indicator for bivalve molluscan shellfish. Appl. Environ. Microbiol. **66:**1280-1285.

17. **Estes, M. K., B. V. V. Prasad, and R. L. Atmar.** 2006. Noroviruses everywhere: has something changed? Curr. Opin. Infect. Dis. **19:**467-474.

18. **Flannery, J., S. Keaveney, and W. Dore.** 2009. Use of FRNA bacteriophage to indicate the risk of norovirus contamination in Irish oysters. J. Food Protect. **72:**2358-2362.

19. **Haramoto, E., H. Katayama, K. Oguma, H. Yamashita, A. Tajima, H. Nakalima, and S. Ohgaki.** 2006. Seasonal profiles of human noroviruses and indicator bacteria in a wastewater treatment plant in Tokyo, Japan. Water Sci. Technol. **54:**301-308.

20. **Harris, J., B. Lopman, R. Edmunds, and B. adak.** 2007. Norovirus associated mortality in England and Wales, Third International Calicivirus Conference, Cancun, mexico.

21. **Havelaar, A. H., and W. M. Hogeboom.** 1984. A method for the enumeration of male-specific bacteriophages in sewage. J. Appl. Bacteriol. **56:**439-447.

22. **Havelaar, A. H., M. V. Olphen, and Y. C. drost.** 1993. F-specific RNA bacteriophage are adequate model organisms for enteric viruses in freshwater. Appl. Environ. Microbiol. **59:**2956-2962.

23. **Kageyama, T., S. Kojima, M. Shinohara, K. Uchida, S. Fukushi, F. B. Hoshino, N. Takeda, and K. Katayama.** 2003. Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. J. Clin. Microbiol. **41:**1548-1557.

24. **Karst, S. M.** 2010. Pathogenesis of Noroviruses, Emerging RNA Viruses. Viruses **2:**748-781.

25. **Katayama, H., E. Haramoto, K. Oguma, H. Yamashita, A. Tajima, H. Nakajima, and S. Ohyaki.** 2008. One-year monthly quantitative survey of noroviruses, enteroviruses, and adenoviruses in wastewater collected from six plants in Japan. Water Res. **42:**1441-1448.

26. **Katayama, H., A. Shimasaki, and S. Ohgaki.** 2002. Development of a virus concentration method and its application to detection of enteroviruses and norwalk virus from coastal seawater. Appl. Environ. Microbiol. **68:**1033-1039.

27. **Le Guyader, F. S., S. Parnaudeau, J. Schaeffer, A. Bosch, F. Loisy, M. Pommepuy, and R. L. Atmar.** 2009. Detection and Quantification of Noroviruses in Shellfish. Appl. Environ. Microbiol. **75:**618-624.

28. **Lee, N., M. C. W. Chan, B. Wong, K. W. Choi, W. Sin, G. Lui, P. K. S. Chan, R. W. M. Lai, C. S. Cockram, J. J. Y. Sung, and W. K. Leung.** 2007. Fecal Viral Concentration and Diarrhea in Norovirus Gastroenteritis. Emerg. Infect. Dis **13:**1399-1401.

29. **Lees, D.** 2000. Viruses and bivalve shellfish. Int. J. Food Microbiol. **59:**81-116.

30. **Loisy, F., R. L. Atmar, P. Guillon, P. Le Cann, M. Pommepuy, and F. S. Le Guyager.** 2005. Real-time RT-PCR for norovirus screening in shellfish. J. Virol. Methods **123:**1-7.

31. **Lopman, B., B. Armstrong, C. Atchison, and J. J. Gray.** 2009. Host, weather and virological factors drive norovirus epidemiology: time series analysis of laboratory surveillance data in England and Wales. PLos ONE **4:**1-10.

32. **Lucena, F., A. E. Duran, A. Morón, E. Calderón, C. Campos, C. Gantzer, S. Skraber, and J. Jofre.** 2004. Reduction of bacterial indicators and bacteriophages infecting faecal bacteria in primary and secondary wastewater treatments. J. Appl. Microbiol. **97:**1069-1076.

33. **Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe.** 1999. Food-related illness and death in the United States. Emerg. Infect. Dis **5:**607-625.

34. **Miossec, L., F. Le Guyader, D. Pelletier, L. Haugarreau, M. P. Caprais, and M. Pommepuy.** 2001. Validity of Escherichia coli, enterovirus, and F-specific RNA bacteriophages as indicators of viral shellfish contamination. J. Shellfish Res. **20:**1223-1227.

35. **Mounts, A., T. Ando, M. P. G. Koopmans, J. S. Bresee, J. Noel, and R. I. Glass.** 2000. Cold weather seasonality of gastroenteritis associated with norwalk-like viruses. J. Infect. Dis. **181 Supplement 2:**S284-S287.

36. **Nordgren, J., A. Matussek, A. Mattsson, L. Svensson, and P. E. Lindgren.** 2009. Prevalence of norovirus and factors influencing virus concentrations during one year in a full-scale wastewater treatment plant. Water Res. **43:**1117-1125.

37. **Nuanualsuwan, S., and D. O. Cliver.** 2003. Capsid functions of inactivated human picornaviruses and feline calicivirus. Appl. Environ. Microbiol. **69:**350–357.

38. **Nuanualsuwan, S., T. Mariam, S. Himathongkham, and D. O. Cliver.** 2002. Ultraviolet Inactivation of feline calicivirus, human enteric viruses and coliphages. Photochem. Photobiol. **76:**406-410.

39. **Okhuysen, P. C., X. Jiang, L. Ye, P. C. Johnson, and M. K. Estes.** 1995. Viral Shedding and Fecal IgA Response after Norwalk Virus Infection. J. Infect. Dis. **171:**556-569.

40. **Ottoson, J., A. Hansen, B. Bjorlenius, H. Norder, and T. A. Stenstrom.** 2006. Removal of viruses, parasitic protozoa and microbial indicators in conventional and membrane processes in a wastewater pilot plant. Water Res. **40:**1449-1457.

41. **Pecson, B. M., M. Ackermann, and T. Kohn.** 2011. Framework for using quantitative PCR as a nonculture based method to estimate virus infectivity. Envir. Sci. Tech. **45:**2257-2263.

42. **Pecson, B. M., L. V. Martin, and T. Kohn.** 2009. Quantitative PCR for determining the infectivity of bacteriophage MS2 upon inactivation by heat, UV-B radiation, and singlet Oxygen: advantages and limitations of an enzymatic treatment to reduce false-positive results. Appl. Environ. Microbiol. **75:**5544-5554.

43. **Perret, K., and G. Kudesia.** 1995. Gastroenteritis associated with oysters. Communicable Disease Report Reviews **5:**153-154.

44. **Pintó, R. M., M. I. Costafreda, and A. Bosch.** 2009. Risk Assessment in Shellfish-Borne Outbreaks of Hepatitis A. Appl. Environ. Microbiol. **75:**7350–7355.

45. **Siebenga, J. J., E. Duizer, and M. Koopmans.** 2010. Norovirus epidemiology. Caister Academic Press, Norfolk, UK.

46. **Skraber, S., L. Ogorzaly, K. Helmi, A. Maul, L. Hoffman, H. M. Cauchie, and C. Gantzer.** 2009. Occurrence and Persistence of enteroviruses,

noroviruses and F-specific RNA phages in natural wastewater biofilms. Water Res.

47. **Straub, T. M., R. A. Bartholomew, C. O. Valdez, N. B. Valentine, A. Dohnalkova, R. M. Ozanich, C. J. Bruckner-Lea, and D. R. Call.** 2011. Human norovirus infection of Caco-2 cells grown as a three-dimensional tissue structure. J. Water Health **9:**225-240.

48. **Svraka, S., E. Duizer, H. Vennema, E. de Bruin, B. van de Veer, B. Dorresteijn, and M. Koopmans.** 2007. Etiological role of viruses in outbreaks of acute gastroenteritis in The Netherlands from 1994 through 2005. J. Clin. Microbiol. **45:**1389-1394.

49. **Tree, J. A., M. R. Adams, and D. N. Lees.** 1997. Virus inactivation during disinfection and wastewater by chlorination and UV irradiation and the efficacy of F+ bacteriophage as a viral indicator. Water Sci. Technol. **35:**227-232.

50. **Wery, N., C. Monteil, A.-M. Pourcher, and J.-J. Godon.** 2009. Human-specific fecal bacteria in wastewater treatment plant effluents. Water Res.**:**1-11.

51. **Westrell, T., D. Dusch, Ethelberg, J. Harris, M. Hjertqvist, N. Jourdan-da-Silva, A. Koller, A. Lenglet, M. Lisby, and L. Vold.** 2010. Norovirus outbreaks linked to oyster consumption in the United Kingdom, Norway, France, Sweden and Denmark 2010. Euro Surveill. **15:**8-11.

52. **Zhang, K., and K. Farahbakhsh.** 2007. Removal of native coliphages and coliform bacteria from municipal wastewater treatment processes: implications to water reuse. Water Res. **41:**2816-2824.

Figure 1. Concentrations of NoV GI (A) and NoV GII (B) detected in oysters and effluent wastewater. Concentrations of NoV GI (○) and NoV GII (□) detected in oysters are expressed as $\log_{10}$ genome copies g^{-1} oyster hepatopancreas and concentrations of NoV GI (●) and NoV GII (■) in effluent are expressed as $\log_{10}$ genome copies 100 ml^{-1}. Dashed lines indicate the limit of detection for shellfish (——) and wastewater analysis (– –).

Table 1. Mean $\log_{10}$ concentrations of *E. coli*, FRNA bacteriophage and NoV GI and GII wastewater treatment stages and associated mean log reductions

	Wastewater Treatment Stage				
	Influent	Post primary settlement		Post final settlement	
n = 49	Concentration ± SD	Concentration ± SD	Reduction ± SD	Concentration ± SD	Reduction[a] ± SD
E. coli	6.54 ± 0.59	6.38 ± 0.51	0.16 ± 0.64	5.06 ± 0.58	1.49 ± 0.63
	(3.73-7.54)	(4.54-7.38)		(3.54-6.20)	
FRNA	5.54 ± 0.51	5.23 ± 0.55	0.32 ± 0.55	3.41 ± 0.77	2.13 ± 0.76
bacteriophage	(3.87-6.82)	(3.41-5.96)		(2.00-5.84)	
NoV GI	3.32 ± 0.64	3.17 ± 0.71	0.13 ± 0.64	2.53 ± 0.57	0.80 ± 0.49
	(2.05-4.76)	(1.62-4.57)		(1.26-4.06)	
NoV GII	3.55 ± 0.89	3.40 ± 0.84	0.14 ± 0.65	2.63 ± 0.71	0.92 ± 0.76
	(1.81-5.34)	(1.46-5.51)		(1.51-4.08)	

[a] The reduction shown is the total reduction provided by the entire treatment process

Table 2. Mean $\log_{10}$ concentrations of NoV GI and GII in influent and effluent wastewater by season.

		Mean concentration ($\log_{10}$ virus genome copies 100 ml^{-1}) ± SD	
NoV genogroup	Season	Influent	Effluent
GI	April-Dec.[a]	3.12 ± 0.55	2.32 ± 0.68
	Jan.-Mar.[b]	3.94 ± 0.49	3.06 ± 0.55
GII	April-Dec.[a]	3.20 ± 0.71	2.27 ± 0.39
	Jan.-Mar.[b]	4.61 ± 0.41	3.53 ± 0.65

[a] 37 samples were analyzed during this period

[b] 12 samples were analyzed during this period

Table 3. Mean $\log_{10}$ NoV concentrations in oysters grouped by season

	Mean concentration ($\log_{10}$ virus genome copies g^{-1}) ± SD	
Season (n)	NoV GI	NoV GII
All data (38)	3.53 ± 0.87	3.73 ± 0.55
April-Dec. (26)	3.12 ± 0.68	3.21 ± 0.56
Jan.-Mar. (12)	4.43 ± 0.50	4.86 ± 0.54

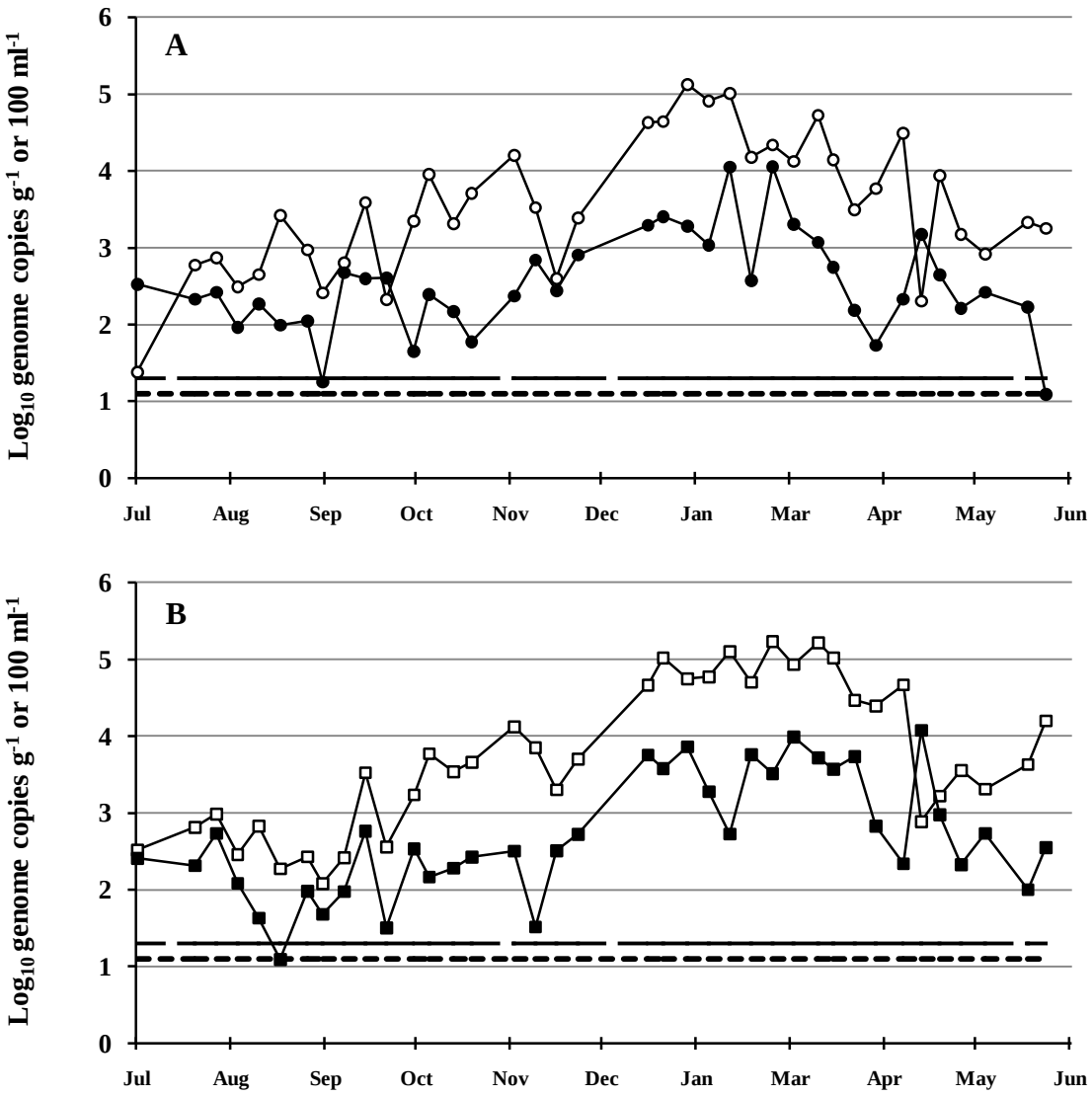
A
B
Log10 genome copies g-1 or 100 ml-1
0
1
2
3
4
5
6
Jul
Aug
Sep
Oct
Nov
Dec
Jan
Mar
Apr
May
Jun

Figure 1. Concentrations of NoV GI (A) and NoV GII (B) detected in oysters and effluent wastewater. Concentrations of NoV GI (○) and NoV GII (□) detected in oysters are expressed as $\log_{10}$ genome copies g^{-1} oyster hepatopancreas and concentrations of NoV GI (●) and NoV GII (■) in effluent are expressed as $\log_{10}$ genome copies 100 ml^{-1}. Dashed lines indicate the limit of detection for shellfish (— —) and wastewater analysis (– –).