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Concentration of Norovirus during Wastewater Treatment and Its Impact on Oyster Contamination

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The concentrations of *Escherichia coli*, F-specific RNA bacteriophage (FRNA bacteriophage), and norovirus genogroup I (NoV GI) and norovirus genogroup II (NoV GII) in wastewater were monitored weekly over a 1-year period at a wastewater treatment plant (WWTP) providing secondary wastewater treatment. A total of 49 samples of influent wastewater and wastewater that had been treated by primary and secondary wastewater treatment processes (primary and secondary treated wastewater) were analyzed. Using a real-time reverse transcription-quantitative PCR (RT-qPCR), the 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. The mean NoV concentrations in wastewater during the winter period (January to March) ($n = 12$) were 0.82 (NoV GI) and 1.41 (NoV GII) log units greater than the mean concentrations for the rest of the year ($n = 37$). The mean reductions of NoV GI and GII during treatment were 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 the concentrations of NoV GI and GII detected in oysters 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.

Norovirus (NoV) is the most common cause of outbreaks of acute gastroenteritis in Ireland (9) and is the major cause of acute nonbacterial gastroenteritis in adults worldwide (33). In general, NoV causes mild illness involving diarrhea and vomiting, although symptoms can be more severe in vulnerable groups such as the elderly (33). NoV is spread by the fecal-oral route and has been demonstrated to be highly infectious particularly in closed settings, such as schools, hospitals, nursing or care homes, cruise ships, and domestic residences (16, 20, 33). The NoV genus comprises nonenveloped, positive-sense RNA viruses of the family *Caliciviridae*. The norovirus genus is genetically diverse and is divided into 5 different genogroups based on the sequence similarity of the capsid protein (24). Each genogroup has a different 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 (6, 31, 45).

NoV can be shed in large numbers (up to 10^8 viruses g^{-1}) in the feces of infected individuals (27) and can continue to be excreted for up to 2 weeks after resolution of symptoms (39). NoV is, therefore, commonly present in municipal wastewater (11, 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 (28). Such contamination occurs because bivalve molluscan shellfish such as oysters are filter feeders and can accumulate microorganisms particularly when grown in sewage-contaminated water (28). Oysters can become contaminated with NoV in this manner and have been linked to numerous outbreaks of gastroenteritis in consumers (1, 13, 43, 51). This public health problem is recognized 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 (13, 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 appropriate environmental regulations such as the Shellfish Waters Directive (2006/113/EC) (17). Authorities must endeavor to ensure that shellfish harvested from these areas comply with the relevant bacterial standards. Wastewater treatment can be considered 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 fecal indicator organisms such as *Escherichia coli* has been extensively studied, and comprehensive data on the removal of such organisms through wastewater treatment exist (32, 50). Similarly, the survival of fecal indicator organisms in the marine environment is well described (7). Therefore, it is possible to accurately predict the likely microbiological impact of a wastewater treatment plant (WWTP) discharge on a shellfishery in terms of fecal bacteria, allowing the likelihood of compliance with the regulatory limits to be determined. Data from previous studies generally indicate that the concentrations of enteric viruses may be reduced to a lesser extent than those of bacteria during the wastewater treatment processes (19, 22); however, lim-

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ited data concerning the extent of NoV removal during WWTP treatment exist. The lack of such data is primarily due to the absence of a reliable culture system for NoV and has led to the use of viral indicator organisms. F-specific RNA bacteriophage (FRNA bacteriophage) of the family *Leviviridae* have been used as surrogates for enteric viruses in wastewater (22) and in shellfish (14). 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 (29). Recently, robust real-time reverse transcription-quantitative PCR (RT-qPCR) procedures have been used for the quantitation of NoV in shellfish and wastewater (11, 36). da Silva et al. (11) 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 1-year period and found that NoV GII demonstrated a seasonal trend with greater concentrations detected in winter. 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 aims of this study were to evaluate the reduction of NoV GI and NoV GII through a WWTP providing secondary wastewater 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 (PE) of 91,600 and received an average daily volume of incoming wastewater of 45,000 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-liter, 24-hour composite samples of influent and final effluent were taken on a weekly basis. In addition, a 1-liter grab sample of wastewater was collected following primary wastewater treatment. All wastewater samples were collected in polyethylene bottles and transported under ambient temperatures to the laboratory within 1 h 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 (15, 26). Four hundred microliters of 2.5 M $MgCl_2$ (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 a pH between pH 3.5 and pH 6.0 with 1 M HCl (Sigma-Aldrich) and mixed on a rocking platform for 45 min. The sample was then passed through a glass-fiber prefilter (Millipore, Billerica, MA) placed directly on a bacteriological membrane filter (filter with a pore size of 0.45 μm and diameter of 90 mm; Millipore) attached to a plastic magnetic filter holder (Pall, Port Washington, NY). The filters were then washed once using 25 ml of 0.14 M NaCl and drained of excess wash solution prior to placing the bacteriological membrane filter in 4 ml of 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 4,000 $\times$ *g* for 10 min. The filter unit was washed in 550 μl of molecular biology-grade water, and the virus concentrate (>500 μl) was 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 1 m below the water surface directly above the WWTP outfall. Oysters were deployed at the outfall for 1 month before sampling commenced. Samples of 24 oysters were collected each week and transported to the laboratory within 2 h under chilled conditions (<15°C). Each week, oyster samples were collected 5 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 analyses. Upon receiving oysters in the laboratory, any dead oysters or open oysters not responding to percussion were discarded. Oyster samples were analyzed for *E. coli* and FRNA bacteriophage within 24 h of receipt using previously published methods (3, 4). For *E. coli* and FRNA bacteriophage analyses, 10 oysters were thoroughly cleaned under running potable water, and the meat and intravalvular fluid were homogenized using a blender and diluted 1:3 with 0.1% (wt/vol) neutralized bacteriological peptone (Oxoid, Cambridge, United Kingdom) (2). For FRNA bacteriophage analysis, 50 ml of the diluted homogenate was centrifuged at 2,000 $\times$ *g* for 10 min, and the supernatant was retained for testing.

For NoV analysis, a further 10 oysters were opened, and the hepatopancreas from each oyster was dissected and finely chopped. Two grams of oyster hepatopancreas was weighed, and 2 ml of 100-μg ml^{-1} proteinase K solution (30 U mg^{-1}; Sigma-Aldrich) was added to the oyster hepatopancreas. Fifty microliters 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. (10). The sample was then incubated at 37°C with shaking at 150 rpm for 1 h followed by incubation at 60°C for 15 min. The sample was then centrifuged at 3,000 $\times$ *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 h or stored at −80°C prior to RNA extraction within 1 month.

***E. coli* enumeration in wastewater and bivalve molluscan shellfish.** Appropriate log dilutions of influent and effluent wastewater samples and diluted shellfish homogenates were assayed for *E. coli* using a standardized five-tube three-dilution most probable number (MPN) method (4). 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 h. The presence of *E. coli* was subsequently confirmed by subculturing tubes showing acid production onto TBX agar (CM0945; Oxoid) at 44°C for 24 ± 2 h. The limits of detection (LODs) of the assay were 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 samples were analyzed for FRNA bacteriophage using a standardized procedure (3) that uses the *Salmonella enterica* serovar Typhimurium WG49 host (21). *S.* Typhimurium has been genetically engineered by the inclusion of an F-pilus-producing plasmid and has been shown to reliably select for FRNA bacteriophage and demonstrate negligible interference from somatic bacteriophage (22). Briefly, 1-ml volumes of appropriately diluted sample and 1 ml of host culture (>10^6 CFU ml^{-1}) were added to 2.5 ml of molten 1% tryptone-yeast extract-glucose agar held at 45°C. 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 PFU 100 g^{-1}. The LODs of the assay for shellfish and wastewater samples were 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

TABLE 1 Concentrations of *E. coli*, FRNA bacteriophage, and NoV GI and GII wastewater treatment stages and associated reductions[a]

Bacterium or virus	Log_{10} concn or log_{10} reduction in concn				
	Mean concn ± SD (range) in influent wastewater	After primary treatment		After final treatment	
		Mean concn ± SD (range)	Mean reduction ± SD	Mean concn ± SD (range)	Mean reduction[b] ± SD
E. coli	6.54 ± 0.59 (3.73–7.54)	6.38 ± 0.51 (4.54–7.38)	0.16 ± 0.64	5.06 ± 0.58 (3.54–6.20)	1.49 ± 0.63
FRNA bacteriophage	5.54 ± 0.51 (3.87–6.82)	5.23 ± 0.55 (3.41–5.96)	0.32 ± 0.55	3.41 ± 0.77 (2.00–5.84)	2.13 ± 0.76
NoV GI	3.32 ± 0.64 (2.05–4.76)	3.17 ± 0.71 (1.62–4.57)	0.13 ± 0.64	2.53 ± 0.57 (1.26–4.06)	0.80 ± 0.49
NoV GII	3.55 ± 0.89 (1.81–5.34)	3.40 ± 0.84 (1.46–5.51)	0.14 ± 0.65	2.63 ± 0.71 (1.51–4.08)	0.92 ± 0.76

[a] A total of 49 samples of influent wastewater and effluent wastewater after primary and secondary wastewater treatments (settlement) were studied.
[b] The reduction shown is the total reduction provided by the entire treatment process.

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 along with shellfish and wastewater samples. 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 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 double-stranded DNA (dsDNA) and purified by the method of Le Guyader et al. (29). 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. External control (EC) RNA was extracted from the dsDNA plasmids, prepared by the same procedure, the method of Le Guyader et al. (29), and 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 GII analyses of wastewater and shellfish samples, duplicate 5-μl aliquots of sample RNA were added to adjacent wells of a 96-well optical reaction plate. Twenty microliters 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, 0.5 μl Rox, and 1.25 μl of enzyme mix was then added to each well. For NoV GI analysis, previously described primers QNIF4 (11) and NV1LCR and probe NVGG1p (48) were used. For NoV GII analysis, primers QNIF2 (30) and COG2R (23) and probe QNIFS (30) were used. In addition, no-template controls were included for NoV GI and GII and IPC virus on the same 96-well plate. The plate was incubated at 55°C for 60 min and 95°C for 5 min, and then 45 cycles of PCR, with 1 cycle consisting of 95°C for 15 s, 60°C for 1 min, and 65°C for 1 min, were performed in 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 threshold cycle (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 and Mengo virus were subjected to RT-qPCR for Mengo virus. Twenty microliters of a one-step reaction mix was 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 5-μl 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 Pintó et al. (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 the value 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 NoV GI and GII DNA plasmids (ranging from 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 expressed as the number of detectable virus genome copies g^{-1} hepatopancreas or number of detectable virus genome copies 100 ml^{-1} wastewater. The LODs for NoV GI and GII were 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 reduction = $\log_{10}(N_{inf}/N_{eff})$ where N_{inf} is the concentration of microbial parameter (MPN *E. coli* 100 ml^{-1}, number of FRNA bacteriophage PFU 100 ml^{-1}, and number of NoV genome copies 100 ml^{-1}) detected in influent wastewater and N_{eff} is the concentration of microbial parameter (MPN *E. coli* 100 ml^{-1}, number of FRNA bacteriophage PFU 100 ml^{-1}, and number of NoV genome copies 100 ml^{-1}) detected in effluent wastewater treated by primary or secondary wastewater treatment processes. 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 for the detection limit of the assay. Minitab statistical software version 15 (Minitab Inc., State College, PA) was used for the data analysis whereby all data were initially assessed for normality (Anderson-Darling test) and then log transformed to achieve a normal distribution.

RESULTS

Concentrations of microbes detected in wastewater. The concentrations of *E. coli*, FRNA bacteriophage, and NoV GI and GII detected in all influent wastewater and effluents after primary and secondary wastewater treatments 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 and effluent after primary and secondary wastewater treatment, respectively. No correlation was found between the concentrations of *E. coli* and FRNA bacterio-

TABLE 2 Concentrations of NoV GI and GII in influent and effluent wastewater by season

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

[a] A total of 37 samples were analyzed for samples collected from April to December (April-Dec.), and a total of 12 samples were analyzed for samples collected from January to March (Jan.-Mar.).

phage 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. The 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. The 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 the 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 reductions during the entire treatment process were 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 winter were significantly higher ($P < 0.05$) than during the rest of the year (Table 2). The mean concentrations of NoV GI and GII in the influent wastewater in January to March ($n = 12$) were 0.82 and 1.41 $\log_{10}$ virus genome copies 100 ml^{-1} greater than the 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 the season, and consequently, NoV concentrations in the final effluent were also significantly higher ($P < 0.05$) during the period from 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 from January to March in 2010, NoV GII concentrations were on average 0.49 $\log_{10}$ unit higher than NoV GI concentrations in effluent wastewater and 0.63 $\log_{10}$ unit 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 to 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. The mean FRNA bacteriophage and *E. coli* concentrations detected in oysters throughout the study period were 4.14 $\log_{10}$ PFU 100 g^{-1} (standard deviation [SD] of 0.64 $\log_{10}$ PFU 100 g^{-1}) and 3.22 $\log_{10}$ MPN 100 g^{-1} (SD of 0.55 $\log_{10}$ MPN 100 g^{-1}), respectively. On a sample-by-sample basis, the *E. coli* concentrations in oysters did not correlate with the concentrations of NoV GI ($r = -0.097$; $P = 0.57$) or GII ($r = 0.184$; $P = 0.26$). Similarly, FRNA bacteriophage concentrations did not correlate with NoV GI ($r = 0.015$; $P = 0.93$) or GII ($r = 0.252$; $P = 0.127$). 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 Fig. 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. The mean concentrations of NoV GI and GII detected during the period from January to March were 1.31 and 1.65 $\log_{10}$ virus genome copies g^{-1} greater than the 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 (for NoV GI, $r = 0.48$ and $P < 0.05$; for NoV GII, $r = 0.68$ and $P < 0.05$).

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 were continuously detected in influent wastewater, demonstrating that both NoV genotypes circulate in the human population throughout the year. While NoV was detected in wastewater year-round, the concentrations of NoV GI and GII increased significantly during the period from January to March. This increase was most pronounced for 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 recognized as being strongly seasonal, with peak infections observed during the colder winter months (24, 35). During the period from January to March 2010, the Health Protection Surveillance Centre in Ireland recorded 1,309 cases of NoV infections. Furthermore, 202 cases were recorded in the region where the WWTP investigated in this study is located (9) 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 underestimated 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 from those found in a number of previous studies investigating

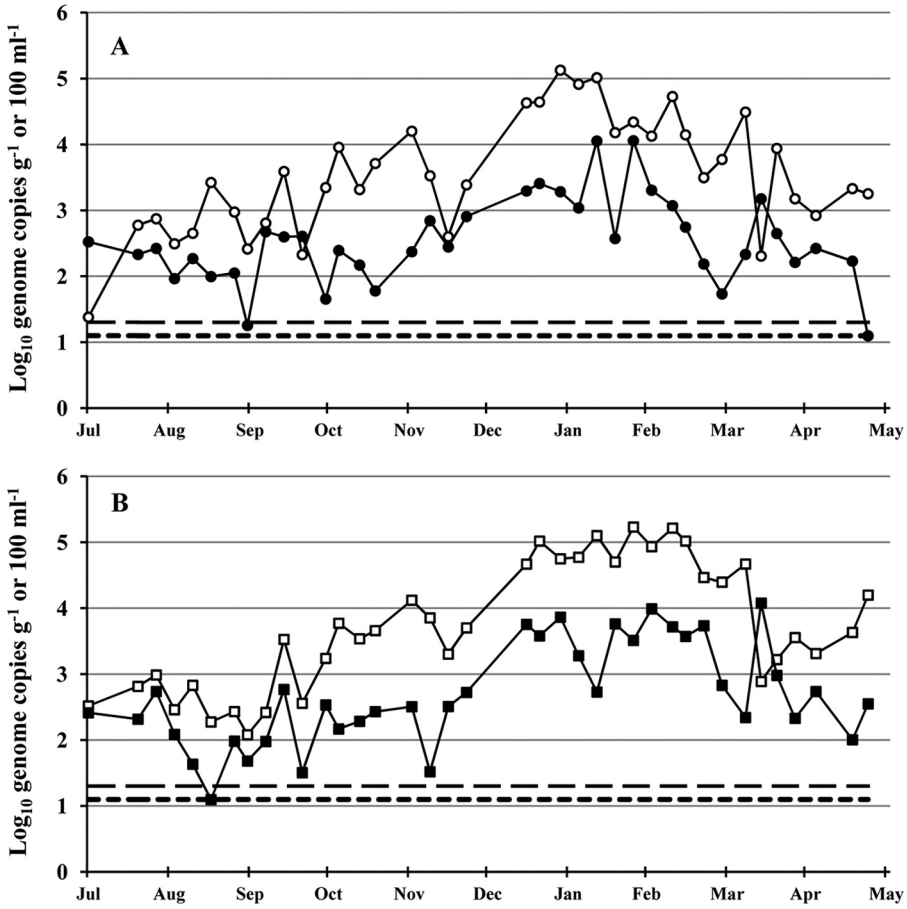

FIG 1 Concentrations of NoV GI (A) and NoV GII (B) detected in oysters and effluent wastewater samples. The concentrations of NoV GI (○) and NoV GII (□) detected in oysters are expressed as $\log_{10}$ genome copies g^{-1} oyster hepatopancreas and the concentrations of NoV GI (●) and NoV GII (■) in effluent wastewater samples are expressed as $\log_{10}$ genome copies 100 ml^{-1}. The two lines in each graph indicate the limits of detection for shellfish (bottom line) and wastewater analysis (top line).

the removal of NoV during wastewater treatment. These studies 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. (36), NoV was detected from a WWTP serving a PE in excess of 800,000. 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 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 nonepidemic 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 into 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 after 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 specialized tissue culture system for the detection of NoV was reported (47). However, this has not been used to investigate NoV concentrations in environmental samples, and at this time, it is not possible to directly investigate the viability of NoV in

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

Months (no. of samples)	Mean concn ($\log_{10}$ virus genome copies g^{-1}) ± SD	
	NoV GI	NoV GII
All (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

wastewater effluent. The absence of a reliable tissue culture system has led to the adoption of virus surrogates for use in inactivation studies (12, 38, 49). FRNA bacteriophages have been proposed as surrogates for enteric viruses in a range of settings, including shellfish harvesting areas and wastewater treatment processes (14, 18, 22, 46). The mean $\log_{10}$ FRNA bacteriophage reduction observed during this study was 2.11 $\log_{10}$ unit, which is significantly greater than that observed for NoV and is consistent with other reports (5, 8, 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 noninfectious 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 other studies (11, 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-unit reduction in infectious bacteriophage MS2 when exposed to UV irradiation produced a real-time PCR signal loss of just 0.11 $\log_{10}$ unit. 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 were 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 (<4,600 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 (13) and demonstrates the inadequacy of *E. coli* to assess the NoV risk associated with oysters. As an alternative to *E. coli*, FRNA bacteriophages have previously been proposed as a viral surrogate to indicate the presence of NoV in oysters (14, 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 the oysters were contaminated to consistent concentrations year-round and did not demonstrate an increased risk of higher concentrations of NoV during the winter months. These findings cast doubt on their suitability for 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 data set 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) demonstrate 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 by using real-time PCR. As yet, methods are not available to differentiate infectious from noninfectious 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 as 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.

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