

## Evaluation of Removal of Noroviruses during Wastewater Treatment, Using Real-Time Reverse Transcription-PCR: Different Behaviors of Genogroups I and II<sup>∇</sup>

Allegra Kyria da Silva,<sup>1</sup> Jean-Claude Le Saux,<sup>2</sup> Sylvain Parnaudeau,<sup>2</sup> Monique Pommepuy,<sup>2</sup> Menachem Elimelech,<sup>1</sup> and Françoise S. Le Guyader<sup>2\*</sup>

Department of Chemical Engineering, Environmental Engineering Program, Yale University, P.O. Box 208286, New Haven, Connecticut 06520-8286,<sup>1</sup> and Laboratoire de Microbiologie, IFREMER, 44311 Nantes Cedex 03, France<sup>2</sup>

Received 27 June 2007/Accepted 2 October 2007

**Noroviruses, an important cause of gastroenteritis, are excreted by infected individuals and are therefore present in wastewater. We quantified norovirus genogroup I (GI) and GII in wastewater at different locations in France and evaluated removal by a range of treatment types, including basic (waste stabilization pond), current industry standard (activated sludge), and state-of-the-art (submerged membrane bioreactor) treatments. Noroviruses were quantified using real-time reverse transcription-PCR (rRT-PCR). Mengovirus was used as a virus extraction control, and internal controls were used to verify the level of GI and GII rRT-PCR inhibition. A total of 161 (81 influent and 79 effluent) samples were examined; GI and GII were detected in 43 and 88% of the influent samples, respectively, and in 24 and 14% of the effluent samples, respectively. Physicians in France report far more cases of GII than GI during outbreaks; thus, the frequent presence of GI was unexpected. The GI influent concentrations were more variable, the peak GI influent concentrations were higher than the peak GII influent concentrations at all four sites (up to  $1 \times 10^9$  and  $6 \times 10^7$  genome copies/liter, respectively), and the average positive influent concentrations of GI were higher than the average positive influent concentrations of GII. The maximum effluent breakthrough concentrations were  $6 \times 10^6$  and  $3 \times 10^6$  genome copies/liter for GI and GII, respectively, indicating that the four treatment systems studied decreased the norovirus contamination load in receiving waters.**

Noroviruses, the leading cause of gastroenteritis worldwide, are extremely genetically diverse (2, 5, 33). Members of the *Caliciviridae* family, they are subdivided into five genogroups (genogroup I [GI], GII, GIII, GIV, and GV), and GI, GII, and GIV have been detected in humans (42). GII has been shown to account for the majority (up to 92%) of reported norovirus gastroenteritis cases, and GI accounts for the large majority of the remaining cases (2, 5, 22). Norovirus infections occur throughout the year, but there is a large annual peak of gastroenteritis during the cold winter months (27). Although the illness is generally self-limiting in otherwise healthy individuals, the high incidence of norovirus cases imposes a high cost on society (24). Besides person-to-person transmission, food contaminated by sewage, such as oysters, berries, or water, has been implicated in outbreaks, although often the source cannot be determined (10, 33, 41). Noroviruses have been shown to be resistant to wastewater treatment (17, 28, 30, 36, 38, 39) and have been detected in wastewater-polluted water, as well as shellfish (19, 20, 26, 36).

Currently, molecular detection is the only method for detection of noroviruses, but their genetic diversity has made genomic detection of these viruses a challenge (1, 2). Recently developed broadly reactive one-step real-time reverse transcription PCR (rRT-PCR) assays have allowed sensitive detection (13, 21, 35, 37), although precise quantification of envi-

ronmental samples is still difficult due to variability in extraction efficiencies, the presence of inhibitory compounds which are copurified during nucleic acid (NA) isolation, and low levels of viral contamination (7, 11, 19).

The focus of this study was to understand norovirus removal in wastewater treatment, since discharged treated or untreated wastewater can impact irrigation, shellfish-growing, recreational, and drinking waters. We used rRT-PCR to examine the relative concentrations of norovirus GI and GII in wastewater at different locations in northwestern France and to evaluate how effectively different types of centralized treatment systems remove these two genogroups. The types of wastewater treatment examined in this study included the most simple (waste stabilization pond [WSP]), the most widely used (activated sludge [AS]), and the current state-of-the-art (submerged membrane bioreactor [MBR]) treatments. The four sites were sampled during the winter months since norovirus concentrations in wastewater were expected to be highest during that period of the year. At two of the sites, sampling continued for a whole year. To avoid false-negative samples, the extraction efficiency and the presence of inhibition were evaluated. The results demonstrated that GI and GII were present at high concentrations in the influents at all four sites during the winter gastroenteritis community outbreaks and that each of the treatment systems studied reduced viral concentrations.

### MATERIALS AND METHODS

**Sample collection.** Four different municipal wastewater treatment plants were chosen in order to include different treatment methods and population sizes: a

\* Corresponding author. Mailing address: Microbiology Laboratory, IFREMER BP 21105, 44311 Nantes cedex 03, France. Phone: (33) 2 40 37 40 52. Fax: (33) 2 40 37 40 73. E-mail: sleguyad@ifremer.fr.

<sup>∇</sup> Published ahead of print on 12 October 2007.

TABLE 1. Wastewater treatment sampling sites and sampling design

Treatment system	Year built	Design capacity (inhabitant equivalents)	System flow rate (m <sup>3</sup> /day) (mean $\pm$ SD)	Type of sample <sup>a</sup>	Sampling dates
MBR	2004	26,000	390 $\pm$ 100	24-h composite	December 2005 to March 2006
Small AS	1996	58,000	5,200 $\pm$ 1,680	24-h composite	December 2005 to March 2006
Large AS	1998	600,000	95,300 $\pm$ 34,000	24-h composite	December 2005 to December 2006
WSP	1985	1,200 <sup>b</sup>	290 $\pm$ 180	Grab	December 2005 to December 2006

<sup>a</sup> Influent and effluent samples were collected.

<sup>b</sup> The WSP system is currently being operated over capacity.

gravity-fed serial three-pond WSP system plant, a small conventional AS plant, a large AS plant, and a state-of-the-art MBR plant (Table 1). All four sites are in northwestern France within 200 km of each other. No industrial wastewater is received at any of these wastewater treatment sites. Samples were collected in December 2005, followed by weekly sampling from January through March 2006. At two of the sites (the WSP and large AS sites), biweekly sampling continued through December 2006.

At the WSP system site, grab samples were taken from the flow at the influent and effluent. At the other three sites, 24-h composite samples of influent and effluent waters were taken from automatic samplers. Samples were immediately placed on ice and arrived at the laboratory within 4 h after collection. Samples were processed within 1 week for viral detection or stored at  $-20^{\circ}\text{C}$ .

**Extraction and rRT-PCR controls.** Three separate controls were employed to evaluate the efficiencies of extraction of the samples and the rRT-PCR (Fig. 1).

First, to determine the efficiency of NA extraction, titrated mengovirus (provided by Albert Bosch, Department of Microbiology, University of Barcelona, Barcelona, Spain) was added to each sample prior to virus concentration and NA purification and then detected using rRT-PCR (7). A second important check of the assay was a test to determine inhibition of GI and GII rRT-PCRs. Synthetic short RNA fragments corresponding to the target sequence of the primers and probes were created based on the GI.1 Norwalk and GII.4 Lordsdale strain sequences. In each sequence, a restriction site (BamHI) was included to discriminate the sequence from sample RNA if contamination was suspected. The single-stranded DNA molecules were ordered as purified primers (Sigma-ProLigo, France), amplified using *Pfu* Taq DNA polymerase (Promega, Charbonnier les Bains, France), and then cloned into the pGEM-3Zf(+) vector by overnight ligation at  $16^{\circ}\text{C}$  using T4 DNA ligase. Then both vectors were transformed in *Escherichia coli* and transformant clones were screened. Plasmids were extracted, linearized, and transcribed in vitro using the Promega riboprobe system. After DNase treatment, RNA internal controls were purified and quantified by determining the optical density at 260 nm.

**Viral extraction and NA purification.** Samples (40 ml) were inoculated with mengovirus at a final concentration of 250 tissue culture-infective doses per ml. The samples were mixed with 10 ml of a 50% polyethylene glycol 6000 solution (Sigma, St. Quentin, France) and rocked overnight at  $4^{\circ}\text{C}$ . After centrifugation for 1.5 h at  $1,500 \times g$  to precipitate viruses, the pellet was suspended in 1 ml of water and NA were extracted and purified using a method adapted from a shellfish analysis method (3). Briefly, viruses were digested with proteinase K (Amresco, Solon, OH) for 30 min at  $56^{\circ}\text{C}$ , and NA were extracted with phenol-chloroform-water (68:18:14; Applied Biosystems, Foster City, CA) and chloroform-isoamyl alcohol (24:1; Sigma, St. Quentin, France). The aqueous phase was precipitated with ethanol and resuspended in water. Cetyltrimethylammonium bromide (Sigma) and sodium chloride were added to the solution to final concentrations of 1.4% (wt/wt) and 0.11 M, respectively. After incubation at room temperature for 15 min, the mixture was pelleted, resuspended in 1 M sodium chloride, and precipitated in ethanol. The final pellet was resuspended in  $100 \mu\text{l}$  of water with  $1 \mu\text{l}$  of RNase inhibitor (Invitrogen, France) and stored at  $-20^{\circ}\text{C}$ .

**Primers and probes.** For GI, forward primer QNIF4 (CGCTGGATGCGNTTCCAT), reverse primer NV1LCR (CCTTAGACGCCATCATCATTTAC) (33) and probe NV1LCpr (6-carboxyfluorescein-TGGACAGGAGAYCGCRA TCT-6-carboxytetramethylrhodamine) (33) were employed; for GII, forward primer QNIF2d (ATGTTCAAGRTGGATGAGRTTCTCWGA) (21), reverse primer COG2R (TCGACGCCATCTTCATTCACA) (13), and probe QNIFS (6-carboxyfluorescein-AGCACGTGGGAGGGGATCG-6-carboxytetramethylrhodamine) (21) were used (Y = C or T; r = A or G; N = A, C, G, or T; and W = A or T). The mengovirus primers and probes were the same as those used by Costafreda et al. (7).

**Construction of quantification standards.** The quantification standards were long RNA fragments mimicking the viral genomes and were kindly provided by Robert Atmar (Baylor College of Medicine, Houston, TX). For GI, the first two open reading frames (nucleotides 146 to 6935) of the Norwalk virus (prototype strain GI.1) were cloned in the pCRII TOPO (Invitrogen) vector. For GII, the sequence between nucleotides 4191 and 5863 of the Houston virus (strain GII.4) was likewise cloned. Then both vectors were transformed in *E. coli*, and transformant clones were screened. Plasmids were extracted, linearized, and transcribed in vitro using the Promega riboprobe system. After DNase treatment, RNA standards were purified and quantified by determining the optical density at 260 nm.

**Amplification conditions.** rRT-PCR was performed with an MX3000 (Stratagene, France) as previously described (21), using the Platinum quantitative RT-PCR ThermoScript One-Step system (Invitrogen, France). Briefly,  $5\text{-}\mu\text{l}$  portions of RNA extract dilutions were combined with  $20 \mu\text{l}$  of a mixture containing  $1 \times$  ThermoScript reaction buffer, 200 nM probe, 200 nM of each primer,  $0.5 \mu\text{M}$

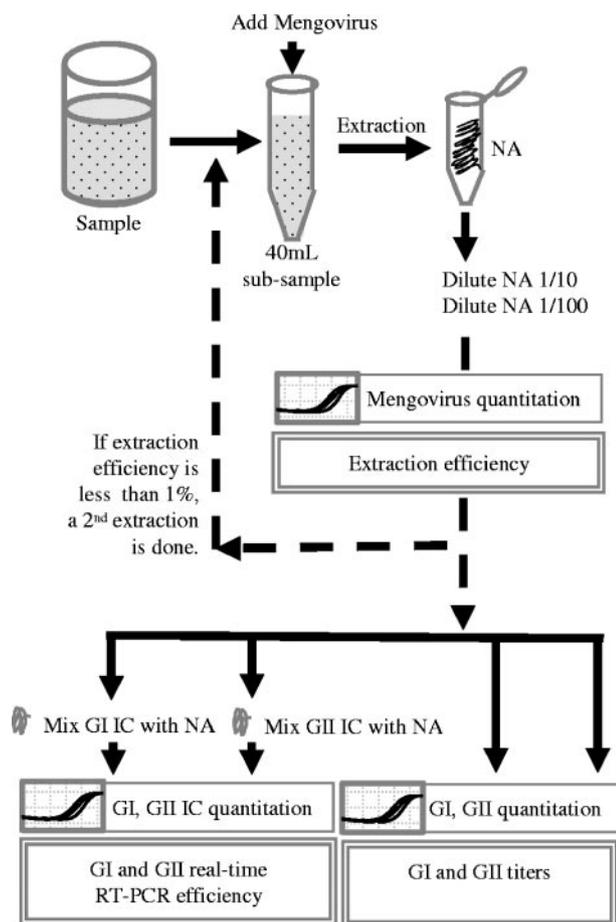


FIG. 1. Control procedure for sample processing and norovirus quantification. IC, internal control; NA, nucleic acid extract. The graphs indicate usage of rRT-PCR. Adapted from the study of Costafreda et al. (7).

TABLE 2. Efficiency of extraction

Treatment type	Sample type	No. of samples with the following extraction efficiencies <sup>a</sup> :			Total no. of samples
		Poor (<1%)	Acceptable (1–10%)	Good (>10%)	
WSP	Influent	1 <sup>b</sup>	7	20	28
	Effluent	0	1	26	27
Small AS	Influent	0	0	12	12
	Effluent	0	0	12	12
Large AS	Influent	1 <sup>b</sup>	4	24	29
	Effluent	0	2	26	28
MBR	Influent	0	1	11	12
	Effluent	1	3	8	12
Total	Influent	2 (2) <sup>c</sup>	12 (15)	67 (83)	81
	Effluent	1 (1)	6 (8)	72 (91)	79

<sup>a</sup> Samples with poor extraction efficiencies (less than 1%) were reextracted.  
<sup>b</sup> Although the extraction efficiency was poor, the sample was not rejected because GI and/or GII values were positive.  
<sup>c</sup> The numbers in parentheses are percentages.

Rox reference dye 50X, 0.5 µl of a ThermoScript Plus/Platinum *Taq* enzyme mixture, and 2 U of RNase inhibitor (Applied Biosystems, France). RT was carried out for 30 min at 50°C, and denaturation was carried out for 5 min at 95°C, followed by 45 cycles of PCR amplification (denaturation at 95°C for 15 s, annealing and extension at 60°C for 1 min).

Separate subsamples of each NA suspension were analyzed for mengovirus, RT-PCR efficiency (with GI and GII internal controls), and detection of GI and GII (1/10 and 1/100 dilutions of the NA extract). A negative amplification control (water) and positive amplification controls (GI and GII reference strains) were included in each amplification series.

**Quantification.** The cycle threshold (*C<sub>T</sub>*) was the cycle at which a significant increase in fluorescence occurred in rRT-PCR (i.e., when the fluorescence became distinguishable from the background). The extraction efficiency was evaluated by comparing the *C<sub>T</sub>* value for the mengovirus-positive amplification control with the *C<sub>T</sub>* value for a sample and was classified as poor (<1%), acceptable (1 to 10%), or good (>10%). To calculate the rRT-PCR efficiency, the *C<sub>T</sub>* value of a sample mixed with the GI or GII internal control was compared to the *C<sub>T</sub>* value of the internal control mixed only with RNA-free sterile water,

and then the efficiency was classified in the same three categories (poor, acceptable, and good). High titers of the internal controls (10<sup>7</sup> RNA copies) were used to avoid competition in samples that were otherwise positive for GI or GII. These steps allowed identification of samples which required reextraction and provided assurance that samples with no GI or GII signal were negative (values below the limit of detection for the assays) and not simply inhibited. Samples that required reextraction were extracted a second time using the method that was used for the first extraction.

To determine the amount of GI and GII present, the number of RNA copies present in each positive sample was estimated by comparing the sample *C<sub>T</sub>* value to standard curves. The final concentration was then adjusted based on the dilution factor used.

RESULTS

Over the study period, 160 (81 influent and 79 effluent) samples were collected and analyzed. At the four wastewater treatment plants the following numbers of influent and effluent samples were collected: at the WSP plant, 28 and 27 samples, respectively; at the small AS plant, 12 and 12 samples, respectively; at the large AS plant, 29 and 28 samples, respectively; and at the MBR plant, 12 and 12 samples, respectively.

**Extraction efficiencies.** Samples having an unacceptable extraction efficiency (<1%; 22 of the 160 samples studied [14%]) were reextracted. After reextraction, three samples still had a poor extraction efficiency (Table 2). The one effluent sample from the MBR site was rejected, and the two samples (influent samples from the WSP and large AS sites) were kept, since they were positive for either GI or GII. For all four sites, the majority (87%) of the samples had good (>10%) extraction efficiency.

**rRT-PCR efficiencies.** The rRT-PCR efficiencies were generally good (>10%), indicating that the diluted NA extracts were not too inhibited to allow detection of targets (Table 3). Of the 160 samples tested, 8 exhibited poor (<1%) rRT-PCR efficiency for GI and 8 exhibited poor rRT-PCR efficiency for GII (5% of the samples in each case). Most of these low-efficiency samples were rejected as false-negative samples; the exceptions were two samples (an influent sample and an efflu-

TABLE 3. Efficiency of rRT-PCR for norovirus GI and GII

Treatment type	Sample type	No. of samples with the following rRT-PCR efficiencies:						Total no. of samples
		GI			GII			
		Poor (<1%)	Acceptable (1%–10%)	Good (>10%)	Poor (<1%)	Acceptable (1%–10%)	Good (>10%)	
WSP	Influent	2 <sup>a</sup>	6	20	0	1	27	28
	Effluent	4 <sup>b</sup>	4	19	4	0	23	27
Small AS	Influent	0	1	11	0	0	12	12
	Effluent	0	0	12	0	0	12	12
Large AS	Influent	0	1	28	0	0	29	29
	Effluent	0	0	28	0	1	27	28
MBR	Influent	1	1	10	0	0	12	12
	Effluent	1	0	11	4	0	8	12
Total	Influent	3 (4) <sup>c</sup>	9 (11)	69 (85)	0 (0)	1 (1)	80 (99)	81
	Effluent	5 (6)	4 (5)	70 (89)	8 (10)	1 (1)	70 (89)	79

<sup>a</sup> Although the rRT-PCR efficiency was poor, one of the two samples was kept, since both GI and GII values were positive.  
<sup>b</sup> Although the rRT-PCR efficiency was poor, one of the four samples was analyzed, since the GI values were positive.  
<sup>c</sup> The numbers in parentheses are percentages.

TABLE 4. Influent and effluent samples positive for norovirus GI and GII

Treatment type	No. of positive samples/no. tested			
	GI		GII	
	Influent	Effluent	Influent	Effluent
WSP	7/27	7/24	22/28	5/23
Small AS	6/12	3/12	12/12	0/12
Large AS	13/29	6/28	25/29	5/28
MBR	8/11	2/11	12/12	0/8
Total	34/79 (43) <sup>a</sup>	18/75 (24)	71/81 (88)	10/71 (14)

<sup>a</sup> The numbers in parentheses are percentages.

ent sample from the WSP site) whose corresponding GI values were positive. At the MBR site, samples with poor rRT-PCR efficiencies were inhibited both for GI and GII, although additional samples were inhibited only for GII. At the WSP site, two samples were inhibited for both GI and GII rRT-PCRs, while two samples were inhibited only for GI rRT-PCR and two other samples were inhibited only for GII rRT-PCR.

**Detection of GI and GII.** For the samples that passed the control checks for extraction and rRT-PCR, GI was detected in about one-half (43%) and GII was detected in most (88%) of the influent samples obtained during the study period (Table 4). GI was present in 24% of the effluent samples, and GII was present in 14% of these samples. The small AS and MBR plants removed enough GII so that the levels were below the limit of detection for all 12 weeks studied.

Based on our protocol and factoring in the amount of NA analyzed, the sensitivity of our assay was  $5 \times 10^3$  and  $2 \times 10^2$  genome copies/liter for GI and GII, respectively.

In the first period of the study, when samples were collected from all four sites, the influent samples taken in December 2005 from the small AS, large AS, and MBR plants were all positive for GII, and the concentrations were as high as  $9 \times 10^5$  genome copies/liter (Fig. 2). Starting in January and continuing through April, GI and GII were detected at all four sites at different influent concentrations (up to  $1 \times 10^9$  and  $6 \times 10^7$  genome copies/liter, respectively). All four sites were determined to be positive for GII for each week (with the exception of a single sample from the large AS site), while the results for GI in the influents from all of the sites fluctuated between negative and elevated concentrations erratically.

The concentrations of GI and GII in effluent samples were up to  $6 \times 10^6$  and  $3 \times 10^6$  genome copies/liter, respectively. Between December and March, it appeared that the MBR and small AS plants were slightly more effective than the large AS and WSP plants for decreasing GI and GII norovirus contamination, as the concentration of GII was below the detection limit for all weeks studied, whereas GI was present in the effluents from these two plants occasionally. However, the difference between the sewage treatment methods is not statistically significant, based on a 95% confidence interval, due to the limited number of samples.

At the two sites which were surveyed for the entire year, the influent samples showed high GI and GII concentrations during the winter months (December through March). After the winter season, the GI concentrations decreased in May and the GII concentrations decreased steadily through the spring and

summer months (April through September). The frequencies of GI breakthrough and GII breakthrough in effluents were similar.

## DISCUSSION

In this study we evaluated samples over a range of sites during a 1-year period, which gave a broad picture of the norovirus presence and removal in sewage treatment plants, using quantitative data. Some interesting results emerged, including the presence of GI in the influents, the steady concentration of GII in the influents, and the difference in the frequency of effluent breakthrough between GI and GII. The first quality control measure required for analysis of complicated environment samples is assurance of successful virus extraction (7, 11). Only 14% of the samples exhibited initially low extraction efficiencies and thus needed to be reextracted. Mengovirus was used as a broad check for extraction success to avoid false-negative samples, which was needed in order to be confident of the quality of the results. It was also important to verify the extent of inhibition of each sample to avoid false-negative results. Monitoring the extent of inhibition using an internal control has been found to be valuable for stool (32), water (11, 16), and shellfish (7, 19) samples. The observation that only one genogroup was inhibited in some samples confirmed that inhibitors do not affect the targets' primers and probes equivalently. However, overall, the difference between the rRT-PCR efficiencies for GI and GII was not statistically significant, based on a 95% confidence interval. Two of the eight samples inhibited for GI rRT-PCR were still positive for GI, implying that there may have been variability in the quantity and nature of the inhibitors present and in the susceptibility of the target sequences.

A wide range of viral concentration techniques for water samples have been proposed that include concentration from larger samples, which is essential for some waters with low levels of contamination, such as drinking or seawater (16, 17, 31, 35, 38). However, a recent study by Gregory et al. (11) demonstrated that concentration of viruses from a larger sample may be counterproductive for certain sample types, as inhibitors are coconcentrated. A detailed analysis of inhibition was outside the scope of this study, and thus we chose to employ the same sample size (40 ml) and extraction method for all sites and sample types. Until now, there have been few quantitative data available for either GI or GII of norovirus and even less data comparing GI and GII; most studies have focused on GII or on only the presence or absence of GI and GII (16, 20, 28, 31). Furthermore, as each assay differs slightly, the sensitivities of detection vary from study to study, making it difficult to precisely compare results from one study to another (1). In studies employing rRT-PCR, primer design largely determines the sensitivity of the assay (7, 37, 40). Previously described rRT-PCR assays for GI were not sensitive with shellfish or water samples (18, 30), even though sequences were obtained. In our assay, the GI primers and probe were selected to increase the sensitivity to strains circulating in Europe (33).

The influent GI and GII concentration ranges for all four sites seem to have been the same, indicating epidemiological similarity in the population, regardless of the size of the town.

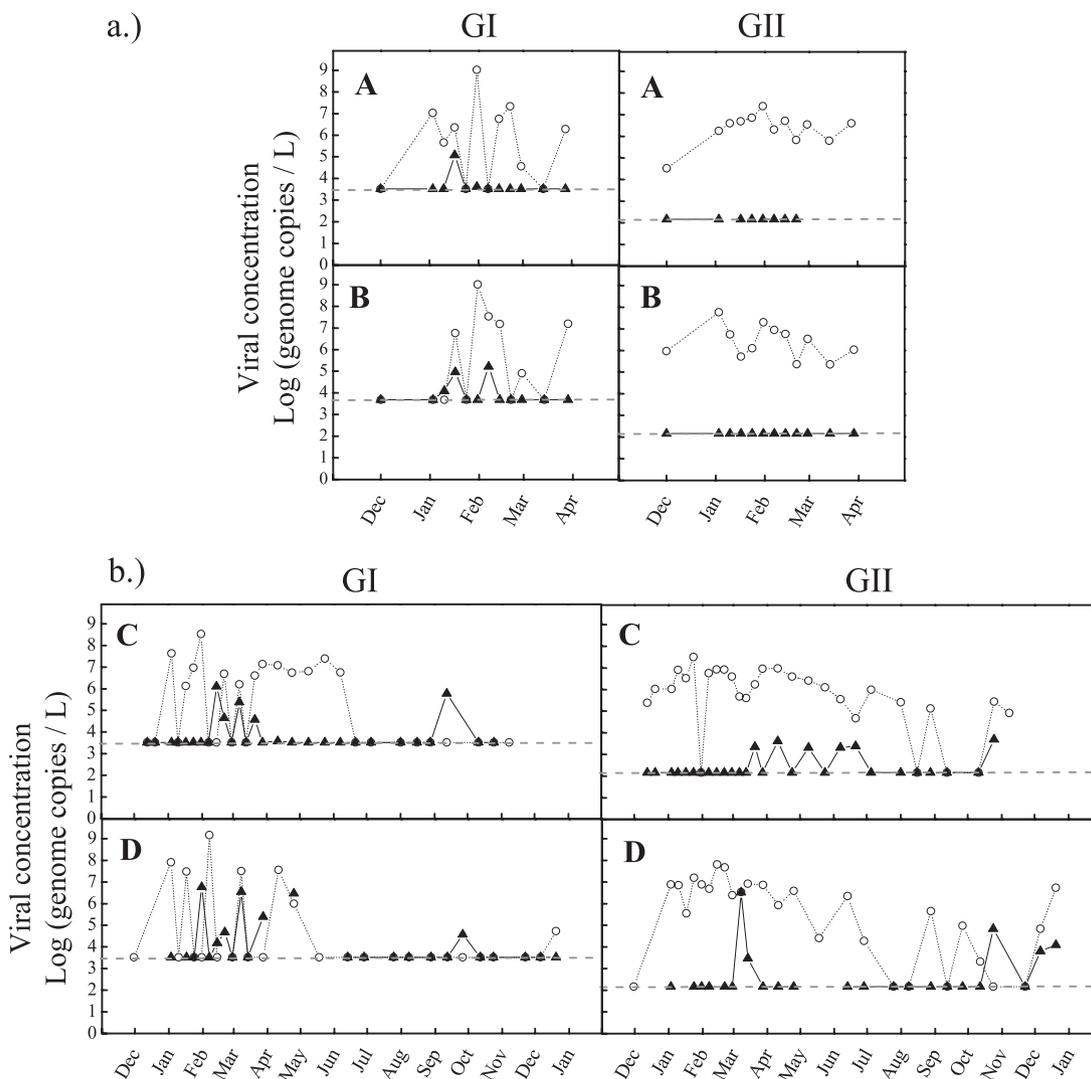


FIG. 2. Concentrations of norovirus GI and GII in influents and effluents. The concentrations of GI and GII are expressed in  $\log_{10}$  genome copies/liter for the four wastewater treatment plants studied during the periods from (a) December 2005 to March 2006 (MBR plant [A] and small AS plant [B]) and (b) December 2005 to December 2006 (large AS plant [C] and WSP plant [D]). The dashed lines indicate limits of detection. ○, influent; ▲, effluent.

The similarity of influent viral concentrations for all sites allowed comparison of the treatment methods. The high influent concentrations of GII starting in January 2006 at all four sites were expected, since clinical and epidemiological data show that GII is responsible for up to 80 to 90% of norovirus clinical cases worldwide (2). Before the winter community outbreaks were declared by the French medical surveillance network, influent samples were positive for GII at three of the four sites, and the concentrations were around  $10^5$  genome copies/liter. Thus, wastewater samples indicated that the population was infected by norovirus before the regional reporting network of physicians reported higher-than-normal numbers of sick patients. The viral titers obtained for GII in this study correspond to levels reported previously: around  $10^7$  and  $10^5$  copies/liter in the influent and effluent of a wastewater treatment plant in England (17), respectively, approximately  $10^6$  and  $10^5$  PCR-detectable units/liter for influent and effluent in The Nether-

lands (20), respectively, and  $10^6$  and  $10^5$  copies/liter in influent and effluent in Germany (30), respectively, despite different PCR (SYBR green, endpoint dilution, and Taqman) assays.

Usually, norovirus cases tend to disappear during summer months; however, in 2006 two new GII variants appeared, which were responsible for an unusual number of cases all over Europe (<http://www.eufoodborneviruses.co.uk/>). The delay in peak concentrations in influents compared to the outbreak peak concentrations may indicate a time lag between the shedding of viruses and their arrival in the influents of the wastewater treatment plants. Additionally, patients have been shown to shed noroviruses in stools for up to 2 weeks after infection (29), so that during an outbreak, the peak cumulative shedding of viruses should occur after the population starts to recover from symptoms.

GI was detected in the influents at all four wastewater treatment plants over the study period at high maximum concen-

trations (up to  $10^9$  genome copies/liter), even though physicians in France, like physicians in other countries worldwide (2, 4, 23, 41), rarely report GI cases in outbreaks (5). The high influent titers suggest that GI circulates in the human population significantly, even if it is not reported by physicians. Physicians see only a small percentage of the infected population (the most severe cases), which may represent a population subset self-selected by genetic or other susceptibility factors that influence the severity of symptoms and thus may influence public health assessments (2, 9, 23). The data for wastewater samples may reflect the presence of strains that circulate more widely in the population and as such may be a powerful and useful tool for public health surveillance (6, 12). Influent samples began to be positive for GI at the same time as the declared outbreak in week 1 of 2006 at three of the four sites, and the concentrations were around  $10^7$  copies/liter. In a Norwegian study, GI was detected in 56% of raw sewage samples (compared to 81% of the samples for GII) (28). Different GI clusters were also detected in Dutch sewage compared to sequences circulating in the population (20, 38) or in Italian sewage (16). It is also interesting that the appearance of GI in the influent was more erratic than the appearance of GII; the estimated concentrations varied widely from week to week and were very high during the first week in February. To our knowledge, there is no other study which quantified GI in wastewater over time, and thus it is not possible to ascertain whether the spiky, sporadic GI influent contamination pattern that we observed is typical or unique to this time period or location. We believe that the GI influent pattern was not an artifact of the assay used (sample size) since GII did not exhibit this pattern.

While all four treatment plants reduced GI and GII concentrations, the small AS and MBR plants showed slightly better performance than the WSP and large AS plants. MBR technology operates through biological treatment as well as physical separation of particles, so it was surprising to find two treated samples positive for GI. The Kubota microfiltration membranes employed in this system have a nominal pore size of 0.4  $\mu\text{m}$ , 10 times the size of noroviruses. However, under operating conditions the membrane surfaces are coated with biomacromolecules and biofilms which allow the membranes to remove smaller particles. Since GI appeared in the effluent two times, this implies that the MBR is not an absolute barrier for the passage of viruses. Breakthrough of noroviruses with the three wastewater treatment systems studied was reported for 31% of treated samples in Norway during a 2-year study (28). Likewise, in Japan during two annual winter gastroenteritis outbreaks, eight of nine treated wastewater samples tested were positive for GI or GII noroviruses (36). In each of these studies, the wastewater treatment technologies used were biological treatment, including conventional AS or WSP, which are designed for organic and nutrient removal and sometimes provide disinfection of bacterial contamination but mechanistically are not expected to eliminate viruses. The mechanisms for virus removal in biological wastewater treatment are unknown, although broadly, removal can be attributed to adsorption and settling to the sludge phase, inactivation, or microbial predation (15).

GI was detected slightly more frequently in treated sewage, although the difference between GI breakthrough and GII breakthrough is not statistically significant. Higher GI influent

values could account for the more frequent breakthrough of GI than of GII. Alternatively, GI may be more resistant to breakdown during wastewater treatment. The fact that GI is more often implicated in food- or water-related outbreaks than GII may also be evidence in favor of higher resistance (4, 14, 18, 25). Although few data on the resistance of norovirus strains to environmental conditions (8) are available, there are known differences between GI and GII with regard to specific biological interactions, implying that there are differences at the capsid surface (34). An analysis of GI and GII contents in the sludge phase (separated from wastewater prior to the final effluent) may show whether there is differential concentration of GI or GII in the solid or particulate phase.

It is significant that all four wastewater treatment systems performed quite similarly with regard to norovirus removal; each of the systems studied improved the quality of the water being discharged. The occasional presence of both GI and GII in effluents implies that both genotypes should be monitored when water quality near wastewater outfalls is considered.

Water quality is increasingly an important public health problem. Improved methods for wastewater analysis may be useful tools for monitoring microbial pathogens circulating in the population in order to complement traditional public health methods.

#### ACKNOWLEDGMENTS

This work was funded by IFREMER (France) and the United States National Science Foundation through a graduate research fellowship for Allegra Kyria da Silva.

We especially thank Estelle Le Quintrec, Larissa Haugarreau, Joanna Krol, Julien Schaeffer, Cecile Le Menec, Richard Casseron, Senuwa Gnanvo, and the operators of the wastewater treatment plants for their technical assistance and sampling.

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