One year monitoring of norovirus in a sewage treatment plant in Rio de Janeiro, Brazil

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ABSTRACT

Norovirus (NoV) is one of the most important aetiological agents of acute gastroenteritis both in developed and developing countries. NoV is shed in high concentrations by infected persons and contaminates recreational and drinking water through sewage discharge into the environment. The aim of this study was to determine the prevalence, genotypes and removal ratio of NoV by PCR, seminested-PCR and quantitative PCR (qPCR) assays in a sewage treatment plant in Rio de Janeiro city, Brazil, during one year of surveillance. NoV was detected in 7 (15%), 14 (29%) and 28 (58%) samples using PCR, seminested-PCR and qPCR, respectively. The mean removal ratio for the activated sludge process was 0.6 $\log_{10}$ for NoV genogroup I (GI) and 0.32 $\log_{10}$ for NoV genogroup II (GII). The peak NoV concentration was detected in the coldest months, with 53,300 genomic copies/litre. Nucleotide sequencing and phylogenetic analysis revealed that five strains clustered with GI strains and six with GII strains. This study demonstrates that NoV spreads into the environment despite the sewage treatment process and remains a source of waterborne outbreaks of acute gastroenteritis.

Key words | norovirus, quantitative PCR, sewage, treatment efficiency

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INTRODUCTION

Noroviruses (NoV) are the leading cause of nonbacterial outbreaks of acute gastroenteritis worldwide; they infect children and adults in both developed and developing countries. NoV is transmitted through the faecal-oral route by the consumption of contaminated water or food (Green *et al.* 2001). The virus has a non-enveloped capsid and possesses a positive single strand RNA genome of 7.7 kb with three open reading frames (ORF). ORF1 codifies a polyprotein that, after cleavage, gives rise to RNA-dependent RNA polymerase and viral protease. ORF2 encodes the major capsid protein, and ORF3 a minor capsid protein (Hardy 2005). NoV is classified in the Caliciviridae family, belonging to the *Norovirus* genus. Five genetically diverse genogroups (GI–V) have been described, with GI, GII and GIV infecting humans with 8, 17 and 1 genotypes, respectively (Zheng *et al.* 2006). In surveillance studies of acute gastroenteritis caused by NoV around the world, GII has been described as the most prevalent genogroup; however, the GI distribution remains largely unknown.

NoV is shed from patients with a median peak of 9.5×10^{10} genomic copies/g faeces in sewage (Atmar *et al.* 2008). Previous studies have determined the removal efficiency of the treatment process performed in sewage treatment plants (STP) for enteric viruses, and only showed small titre reductions when comparing influent vs. effluent samples (Haramoto *et al.* 2008; He *et al.* 2008). Owing to the lack of removal efficiency, enteric viruses can contaminate different types of environmental water, such as rivers and

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underground waters, resulting in an important risk for waterborne outbreaks of acute gastroenteritis.

The microbiological quality of different types of water is defined by the determination of total and faecal coliform levels present; however there is a scientific consensus that there is no correlation between bacterial indicators and viral contamination (Pusch *et al.* 2005; Carducci *et al.* 2008). This highlights the need for creating new regulations to determine which parameters must be accessed in order to certify good virological quality in water samples.

The detection of NoV is performed by molecular methods such as qualitative or quantitative PCR (qPCR) because of the lack of an efficient *in vitro* cell culture assay. Recently, a new approach was developed to culture viruses; however this methodology is in the early stage of development and needs to be tested with a broad range of NoV strains (Straub *et al.* 2007). The first qPCR for NoV quantification was described by Kageyama *et al.* (2003). The sensitivity of this assay permits the detection of up to 10 genomic copies/reaction, and is more sensitive than qualitative PCR. Several studies in environmental virology have used this technique, and the high sensitivity allowed for the detection of NoV at the low titres frequently found in those samples (Haramoto *et al.* 2006; Katayama *et al.* 2008).

The aim of this study was to determine the prevalence and circulating genotypes of NoV present in a STP in Rio de Janeiro city, Brazil, from January to December 2005, as well as the removal efficiency for that plant of enteric viruses, with NoV as a model.

MATERIALS AND METHODS

Sewage samples

From January to December 2005, 24 influent and 24 effluent composite samples were collected twice a month from a STP located in Rio de Janeiro city, Brazil. The treatment includes secondary aerobic digestion by activated sludge, extended aeration with biological nitrification and denitrification, without chlorination.

Each sample consisted of eight 250 ml portions that were stored in glass bottles and transported to the laboratory, where they were immediately tested for bacterial parameters. Within 24 hours, the samples were processed for viral concentration and stored at −80°C until RNA extraction for viral detection.

Bacterial parameters

Total and faecal coliforms were measured using the Colilert®-18 Quanti-Tray/2000 method (IDEXX Laboratories, Westbrook, Maine) according to the manufacturer's instructions.

Viral concentration, RNA extraction and cDNA synthesis

The viruses were concentrated using an adsorption–elution method with a negatively charged membrane as described previously by Katayama *et al.* (2002). After concentration, the eluate (15 ml) was ultrafiltered using a Centriprep concentrator 50® (Nihon Millipore, Tokyo, Japan) to a final volume of 2 ml.

The samples were clarified by adding 70 μl of Vertrel® (Sigma-Aldrich Corporation, St Louis, Missouri) and centrifuged at 800 × *g* for 10 min. RNA was extracted by using the QIAamp Viral RNA Mini Kit® (QIAGEN, Valencia, California) following the manufacturer's instructions. The cDNA synthesis was performed with random primers pd(N)6 (Amersham Biosciences, Piscataway, New Jersey) at 42°C for one hour and 95°C for 10 minutes as previously described by Victoria *et al.* (2009).

Recovery efficiency of the method for the concentration of norovirus

To determine the recovery efficiency for NoV of this adsorption–elution method followed by the ultrafiltration procedure, 100 μl of a 10% faecal suspension of a GII/4 NoV strain (GenBank accession number DQ997040) previously quantified by qPCR was spiked in raw and treated sewage samples. Negative control without spiking NoV was also tested in order to certify the absence of a natural contamination. All the assays for viral recovery efficiency were carried out in triplicate (independent experiments) for each type of water (raw or treated samples).

PCR, seminested-PCR and qPCR reactions

For NoV detection, three different protocols were carried out. A PCR was performed by using a set of degenerate primers (Mon 431/Mon434) previously used to detect the most common strains of NoV from GI and GII according to the following conditions: denaturation for 3 minutes at 94°C, 40 cycles of 30 seconds at 94°C, 30 seconds at 50°C, one minute at 72°C and a final extension for 10 minutes at 72°C (Beuret *et al.* 2002). A second detection method performed in this study was based on a seminested-PCR and used to discriminate between GI and GII (Boxman *et al.* 2006). The first round of the seminested-PCR was carried out by denaturing the cDNA at 94°C for 5 minutes followed by 40 cycles at 94°C for 30 seconds, 45°C for 30 seconds and 72°C for one minute; the final elongation was at 72°C for 10 minutes. The second round was performed with 35 cycles with the same conditions of the first round of the seminested-PCR. These two PCR protocols use primers targeting the RNA polymerase region on the NoV genome.

The qPCR reactions were performed in duplicate using the ABI 7500 Real-Time PCR System® (Applied Biosystems, Foster City, California) following the manufacturer's instructions. The assay performed for NoV quantification was carried out by using the protocol described by Kageyama *et al.* (2003) that uses primers targeting the ORF1/ORF2 overlap region in the viral genome. The reaction was performed at 50°C for two minutes to activate UNG, followed by an initial denaturation at 95°C for 10 minutes, 45 cycles with denaturation at 95°C for 15 seconds and annealing/extension at 56°C for one minute.

For all amplification procedures, basic precautions were taken to avoid cross-contamination among samples; these included the use of separated rooms for each PCR step and the inclusion of negative (milli-Q water) and positive controls (specific NoV GII and GI positive faecal samples previously tested) in all procedures.

Sequencing and phylogenetic analysis

The amplicons obtained from the seminested-PCR reactions were sequenced using Big Dye® Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems) and ABI Prism 3730 Genetic Analyzer® (Applied Biosystems) as described by Otto *et al.* (2008). BioEdit® Sequence Alignment Editor software was used to edit and align the sequences obtained in this study (Hall 1999). Phylogenetic analyses were carried out by using the MEGA v. 4.0 software package (Tamura *et al.* 2007) with the neighbor-joining method, Kimura 2-parameters model for distance correction and 2,000 bootstrap re-sampling test. Sequences from GI (187 nt) and GII (237 nt) obtained in this study were submitted to GenBank under accession numbers FJ429186 to FJ429197.

RESULTS

The prevalence and genotypes of NoV were determined in a one year monitoring study in a STP in Rio de Janeiro city, Brazil. A total of 48 samples were collected: 24 influent and 24 effluent. The recovery efficiency for NoV of the adsorption-elution method followed by the ultrafiltration procedure used in this study was 7.8 and 6.4% for raw and treated sewage samples, respectively (data not shown). Comparing PCR, seminested-PCR and qPCR, the most sensitive method for detection of NoV in these sewage samples was qPCR, which detected a total of 28 (58%) positive samples for NoV; of these, 16 were (67%) positive for GII and 1 (4%) for GI in influent samples; and 10 (42%) for GII and 1 (4%) for GI in effluent samples. When seminested-PCR was performed, NoV was detected in 14 (29%) samples, with 8 (33%) positive for GII and 2 (8%) for GI in influent samples and 3 (12%) for GII and 3 (12%) for GI in effluent samples. Two samples were concomitantly positive for both GI and GII genotypes. The PCR detected 6 (25%) samples positive for NoV in influent samples and 1 (4%) in effluent samples (Table 1). For qPCR, the mean titres of NoV GII in influent and effluent samples were 7,290 genomic copies/l ($gc\,l^{-1}$) and 3,470 $gc\,l^{-1}$, respectively. For NoV GI, the values observed were 2,400 $gc\,l^{-1}$ and 643 $gc\,l^{-1}$, respectively. The mean removal ratio for the treatment process was 0.6 $\log_{10}$ for NoV GI and 0.3 $\log_{10}$ for NoV GII. For total and faecal coliforms, the mean removal ratios were 2.5 $\log_{10}$ and 3.2 $\log_{10}$, respectively.

When using qPCR, NoV GII was detected in all influent samples except that collected in January. A peak in concentration was observed during the coldest/driest

Table 1 | Norovirus positive samples detected by PCR, seminested and quantitative PCR (qPCR) in influent and effluent samples from the sewage treatment plant in Rio de Janeiro, Brazil

	Norovirus detection using each methodology				
		Seminested-PCR		QPCR	
Samples (n)	PCR	GI	GII	GI	GII
Influent (24)	6	2	8	1	16
Effluent (24)	1	3	3	1	10
Total (48)	7 (14.6)*	14 (29.2)*		28 (58.3)*	

*Total samples with percentage in parentheses.

months of the year (June and July). Comparatively, when effluent samples were analysed, NoV GII was not detected in a five-month period (Figure 1).

All positive samples for NoV (11 for GII and 5 for GI) detected by seminested-PCR were sequenced and confirmed as NoV by comparison with sequences available in GenBank using the BLAST program. Phylogenetic analyses were carried out for molecular characterization of detected NoV genotypes. The GII strains detected in this study clustered in four different groups (Figure 2). The strains clustered with GII/4 prototype strains presented 100% nucleotide identity and 98% identity with the most similar sequence identified from GenBank; this strain was isolated in Canada in 2005 and belonged to the GII/4 genotype. The sequences from samples 70 and 71 were identical, confirming the presence of the same NoV strain in influent and the effluent samples from the same collection date; together with the sequence from sample 107, these viruses formed a cluster without any reference strain. Strains 70 and 71 were more similar to a strain isolated in Stockholm in 2002 that clustered with GGIIb variants. Strain 105 clustered with a GII/3 reference strain, presenting the highest nucleotide identity (97%) with a strain isolated in Mexico in 2006 from a seawater sample.

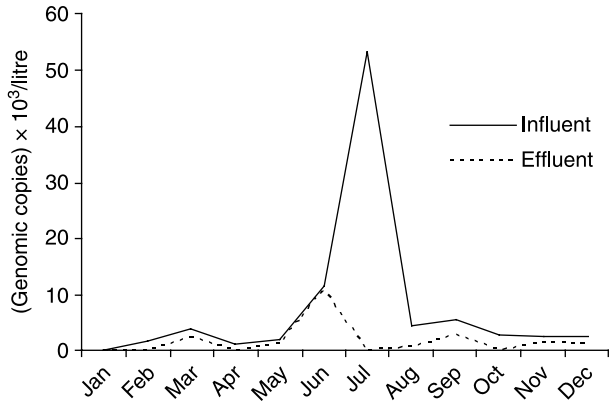

Figure 1 | Average concentration of norovirus genogroup II in influent and effluent samples from January to December 2005 at the sewage treatment plant in Rio de Janeiro, Brazil.

The sequences belonging to GI clustered with two different sets of strains. Samples 56 and 57 were detected on the same collection date in both influent and effluent samples; they presented 99% nucleotide identity. These samples were more similar to a strain isolated in Japan, with 97% of identity in the nucleotide sequence, and clustered with the Southampton (GI/2) reference strain. The sewage strains 103, 104 and 110 were identical and presented 95% nucleotide identity with a strain isolated in the USA. They did not cluster with any reference strain (Figure 3).

DISCUSSION

To our knowledge this is the first study performed in Brazil to detect NoV in sewage samples collected from a STP. The prevalence, genotypes, seasonality and removal ratio of NoV were determined. The use of an adsorption–elution method for concentration of enteric viruses from environmental water samples followed by viral detection with molecular methods such as qualitative and quantitative PCR have been previously applied successfully (Haramoto *et al.* 2006; Silva *et al.* 2007; Villar *et al.* 2007; Miagostovich *et al.* 2008). Katayama *et al.* (2002, 2008) described the adsorption–elution method and obtained satisfactory results for detection of NoV in six STPs with an activated sludge process in Japan. The NoV recovery efficiency of 7.8 and 4.6% for raw and treated sewage samples, respectively, was similar to the astrovirus recovery of 4.2 and 4.3% for untreated and treated sewage samples, respectively, obtained in a previous study (Guimarães *et al.* 2008). Three different approaches for NoV detection were compared for sewage samples. The qPCR using TaqMan® technology presented a higher sensitivity when compared with both PCR and seminested-PCR; however, none of these assays determined the infective capacity of the viral particles. The primary advantage of using real time PCR is

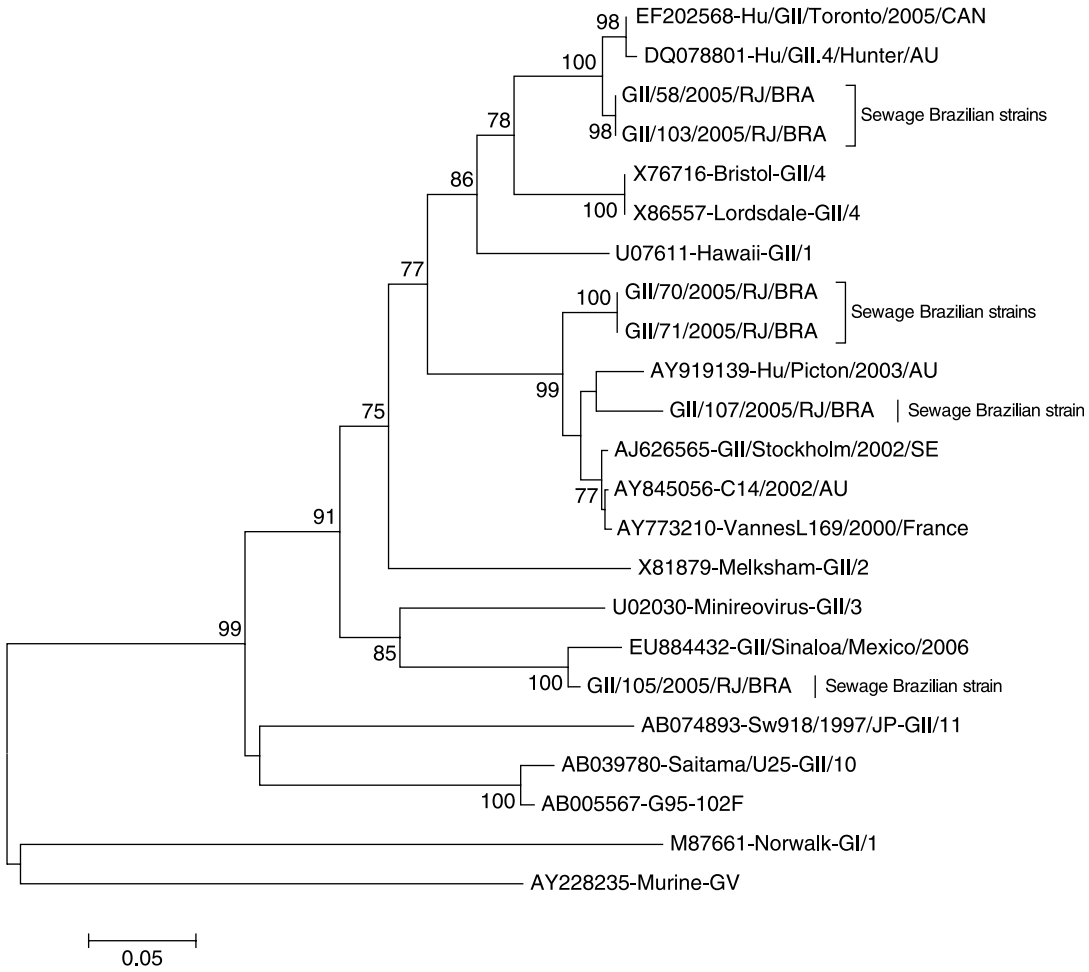

Figure 2 | Phylogenetic tree of 237 nucleotides within the polymerase region of norovirus genogroup II (GII) strains collected from a sewage treatment plant in Rio de Janeiro in 2005 and strains obtained from GenBank. Reference strains are indicated with the Genbank accession number followed by genogroup/genotype (in bold). Bootstrap values higher than 70% are given for each node.

that it permits the quantification of viral particles in the sample; this is currently the only way to achieve NoV quantification, since there is no efficient *in vitro* cell culture technique for these viruses (Straub *et al.* 2007). The qPCR protocol used in this study was described by Kageyama *et al.* (2003) for detection of NoV in stool specimens, and similarly to the present study, they observed a higher sensitivity (10 genomic copies/qPCR reaction) when compared with the nested PCR used in the study. The data obtained here highlight the importance of selecting the correct molecular detection method for NoV.

A seasonal profile was observed for the quantification of NoV GII from influent samples, with the peak of virus load in July. This was in accord with previous studies suggesting a higher prevalence of NoV in winter months in environmental samples (Haramoto *et al.* 2006; Silva *et al.* 2007; Katayama *et al.* 2008). Sewage samples were positive for NoV in all periods analysed. However, during the surveillance of NoV in stool samples from patients in three public hospitals in the city of Rio de Janeiro carried out by our laboratory in 2005, NoV was not detected in January and November (data not shown). The absence of clinical cases can be explained by NoV excretion by asymptomatic individuals with resulting contamination of the environment as previously described by Okabayashi *et al.* (2008), or by the location of the STP and the hospitals in different regions of the city. Effluents contaminated with NoV can spread to recreational and drinking waters, providing a

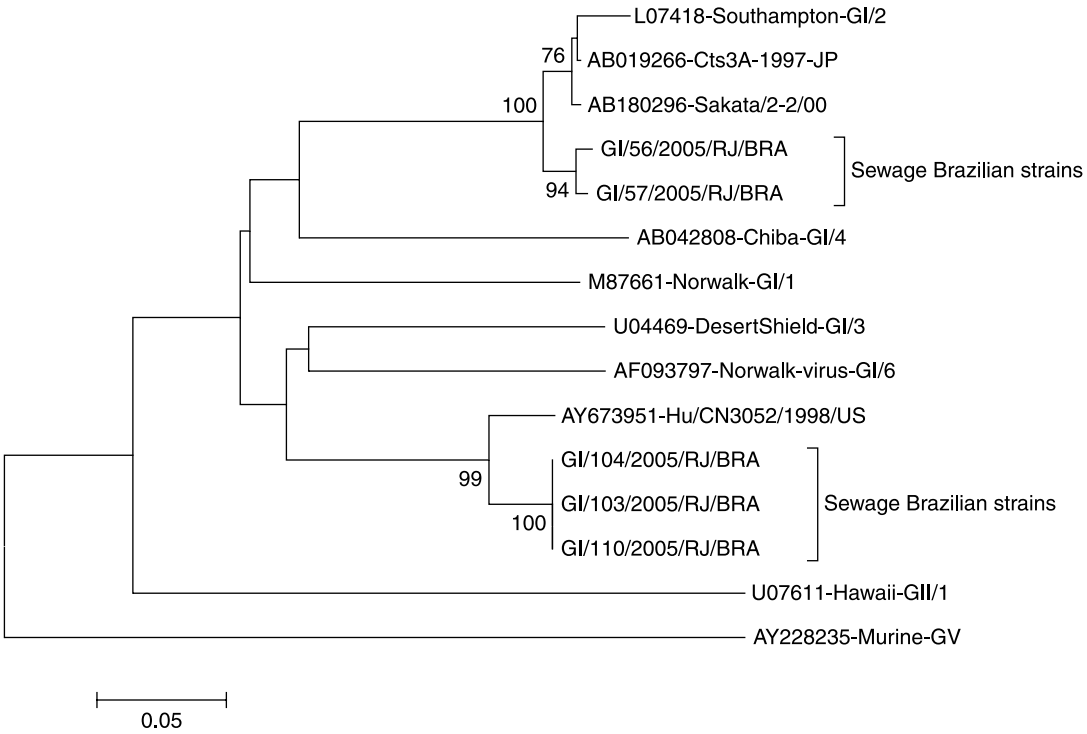

Figure 3 | Phylogenetic tree of 187 nucleotides within the polymerase region of norovirus genogroup I (GI) strains collected from a sewage treatment plant in Rio de Janeiro in 2005 and strains obtained from GenBank. Reference strains are indicated with Genbank accession number followed by genogroup/genotype (in bold). Bootstrap values higher than 70% are given for each node.

source for acute gastroenteritis outbreaks in the vicinity of the STP.

The removal of different types of microorganism was determined for the STP analysed in this study; this STP uses a primary sedimentation and biological treatment with activated sludge. The removal ratio for bacterial indicators was nearly six times higher than for NoV removal; these findings are in agreement with previous studies that indicate a low removal of enteric viruses from STPs (Berg *et al.* 2005; Haramoto *et al.* 2008). NoV GII was detected year-round and was more prevalent than GI, as previously described in both clinical and environmental trials worldwide (Berg *et al.* 2005; Lodder & de Roda Husman 2005; Victoria *et al.* 2007; Patel *et al.* 2008). Chan *et al.* (2006) observed that the viral load of NoV GII is 100-fold higher than that of GI strains in stool specimens of patients with acute gastroenteritis, and suggested that this higher GII viral load facilitates the transmission from infected to susceptible persons. It is likely that this higher transmission rate is responsible for the higher prevalence of GII worldwide.

Shin & Sobsey (2008) suggested that a water chlorination process could improve the inactivation of most enteric viruses, including NoV, present in sewage following the recommendation of the United States Environmental Protection Agency (USEPA Guidance Manual 1989).

Several strains from GI and GII were detected in both influent and effluent samples collected from the STP, indicating co-circulation of these strains in the population of Rio de Janeiro city. A previous study performed in this city using stool specimens from hospitalized children with acute gastroenteritis in 2004 indicated that numerous strains circulated in this set of patients (Victoria *et al.* 2007). Similar results were observed in a study conducted in the Netherlands, which detected 11 different NoV genotypes in sewage samples and up to four different NoV genotypes in a single sample (Berg *et al.* 2005). In the present study, the same NoV strain sequences were detected twice in influent and effluent samples collected on the same date, confirming the resistance of the virus to the sewage treatment process. The seminested-PCR assay permitted the

detection of GII and GI strains in the same sample; this detection was confirmed by sequencing.

The NoV discharged in STP effluents can reach recreational or oyster culture waters. Since recombination is a frequent mechanism of evolution in NoV biology (Bull *et al.* 2007; Nayak *et al.* 2008), the consumption of these waters or oysters containing two strains belonging to different genogroups could be a source of recombination events that could lead to worldwide outbreaks of acute gastroenteritis.

CONCLUSIONS

This study demonstrates the presence of different genotypes of NoV in sewage samples collected in a STP, and highlights that viral contamination can persist after sewage treatment performed with primary sedimentation followed by biological treatment. It is therefore important to consider the risk for local population health concerning outbreaks of waterborne acute gastroenteritis.

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