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Prevalence of norovirus and factors influencing virus concentrations during one year in a full-scale wastewater treatment plant

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ABSTRACT

Norovirus (NoV) is a leading cause of acute gastroenteritis and is often spread via wastewater contamination. Little is known about how the wastewater treatment process affects norovirus, and which factors influence virus concentrations. To investigate this, we collected wastewater samples monthly during one year at eight different key sites at the municipal wastewater treatment plant in Gothenburg, Sweden. Virus particles were concentrated using ultracentrifugation, viral RNA was subsequently extracted, and transformed into cDNA by reverse transcription. The quantification was performed with real-time PCR assays for NoV genogroups I (GGI) and II (GGII), respectively. We found seasonal changes of NoV genogroups, with the highest concentration of NoV GGII during the winter months, and the highest concentration of NoV GGI during the summer months. Virus transmission in wastewater was more stable for NoV GGI, with NoV GGII demonstrating larger seasonal peaks. Virus reduction took place at similar rates in the primary settling, and in the activated sludge in combination with the secondary settling. Different physicochemical parameters and incoming virus concentrations were correlated to reduction of NoV between different treatment sites. This study gives new information about NoV transmission and virus reduction in a wastewater treatment plant.

1. Introduction

Norovirus (NoV) is the leading cause of nonbacterial, acute gastroenteritis in adults, causing numerous outbreaks worldwide (Hedlund et al., 2000; Inouye et al., 2000; Fankhauser et al., 2002; Lopman et al., 2003). NoVs are non-enveloped, positive-sense RNA viruses belonging to the *Caliciviridae* family. They constitute a genetically diverse group of viruses, and can be further subdivided into five genogroups, where NoV genogroup I (GGI) and NoV genogroup II (GGII) contain most strains that infect humans (Zheng et al., 2006). Of these two genogroups, GGII is the most common, causing

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75 to >90% of all norovirus related outbreaks (Fankhauser et al., 2002; Gallimore et al., 2007). The norovirus virion is robust, and well adapted for survival in the environment outside the host, and to withstand different treatment processes (Duizer et al., 2004; Rzezutka and Cook, 2004). NoV spread via the fecal-oral route and are frequently found in different environmental settings such as wastewater (Pusch et al., 2005; Haramoto et al., 2006; da Silva et al., 2007; La Rosa et al., 2007), shellfish (Formiga-Cruz et al., 2002; Jothikumar et al., 2005), and drinking water (Boccia et al., 2002; Vainio and Myrmel, 2006). Numerous studies have shown that enteric viruses are present in high levels in wastewater, even after the treatment process (Laverick et al., 2004; van den Berg et al., 2005; Haramoto et al., 2006; da Silva et al., 2007; Katayama et al., 2008). The degree of faecal contamination is often assessed by monitoring the number of indicator microorganisms in the wastewater. However, commonly used bacterial indicators are unreliable in terms of viral contamination, and often no correlation between levels of enteric bacteria and enteric viruses has been found (Haramoto et al., 2006; Ottoson et al., 2006).

Epidemiological studies of norovirus have often focused on the winter months, when norovirus gastroenteritis is more common (Lodder and de Roda Husman, 2005; Gallimore et al., 2007). However, to understand the noroviral transmission mechanisms in the community, it is important to investigate NoV prevalence during a complete year. There are some studies describing NoV concentrations in wastewater during one year in Japan and the Netherlands (van den Berg et al., 2005; Haramoto et al., 2006; Katayama et al., 2008), but the understanding of noroviral transmission in the environment, including the summer months is to a large extent unclear.

Previous studies on enteric viruses in wastewater usually describe only virus concentrations from the influent and effluent water, and physicochemical parameters are often not considered in the investigation (Lodder and de Roda Husman, 2005; Pusch et al., 2005; da Silva et al., 2007). However, this approach fails in understanding which processes of the wastewater treatment plant (WWTP) are important for reduction of norovirus. To study this, wastewater from many different key sites in the treatment process needs to be investigated, and the viral data must be related to physicochemical parameters in the WWTP.

The aim of this study was to investigate the variation and reduction of NoV GGI and GGII in a full-scale municipal WWTP in Sweden during one year. Furthermore, we wanted to elucidate which parameters and factors in the treatment process affect the viral concentration and reduction, by analyzing NoV GGI and GGII from eight different key sites in the treatment process, and relating them to physicochemical parameters.

2. Materials and methods

2.1. *The wastewater treatment plant and sampling of wastewater*

The wastewater samples were collected as grab samples on the first Tuesday of every month during one year, from October 2005 until September 2006, in the municipal wastewater treatment plant (WWTP) Ryaverket, Gothenburg, Sweden. The samples were collected at eight different sites (Fig. 1).

The WWTP is designed for biological nitrogen removal utilizing pre-denitrification in a non-nitrifying activated sludge system and post-nitrification in a trickling filter. The WWTP receives wastewater from nearly 830,000 person equivalents, with an average daily incoming water volume of ~350,000 m^3 (~4 m^3/s). The system has a hydraulic retention time of 8 h, and the activated sludge system has a solid retention time of 2–4 days. During primary settling, heavy particles are removed. The activated sludge contains high levels of biomass and is divided into two phases: an anaerobic phase, where denitrification occurs, and an aerobic phase for decomposition of organic material. During secondary settling, sludge and phosphorous aggregates are removed, and the sludge is collected and pumped to the primary settling. After the secondary settling, ~50% of the water goes out into the recipient water, and the rest goes back into circulation via the nitrifying trickling filter. Sludge is extracted from the primary settlers and digested in completely mixed mesophilic

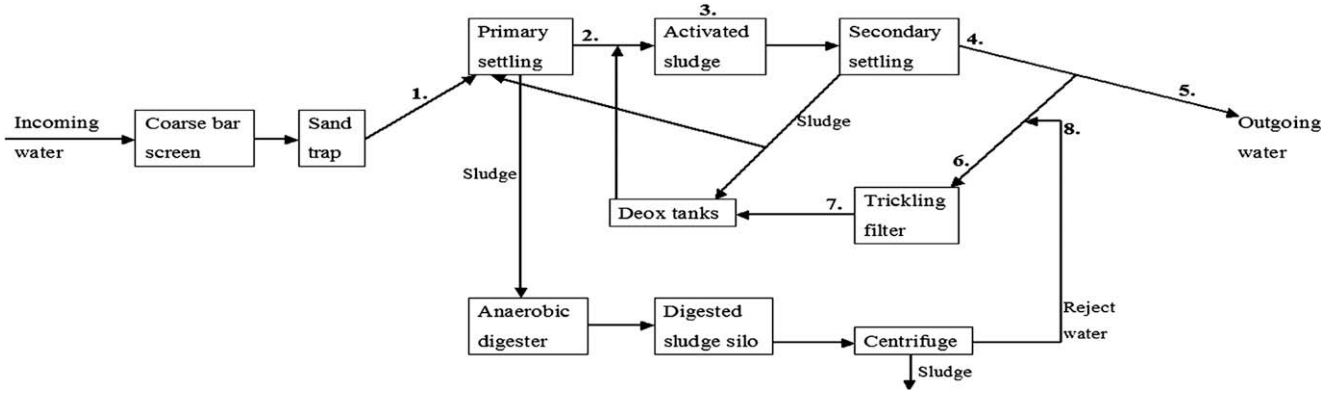

Fig. 1 – Sampling sites in the municipal WWTP Ryaverket, Gothenburg, Sweden. 1. Incoming water, 2. After primary settling, 3. Activated sludge, 4. After secondary settling, 5. Outgoing water, 6. Before trickling filter, 7. After trickling filter, and 8. Reject water. Modified from Börjesson et al. (2009).

anaerobic digesters with a retention time of 20–30 days. The digested sludge is centrifuged and the reject water is returned to the WWTP (Fig. 1). All samples were collected in plastic bottles in volumes of 500 ml and stored at 4 °C until further use.

2.2. Measurement of physicochemical parameters in the wastewater

Physicochemical parameters were measured in incoming and outgoing water for all sampling months, as part of the routine at Gryaab laboratory, Ryaverket, Gothenburg, Sweden. The analyses were performed according to Swedish standards (suspended solids, SS-EN 872; electrical conductivity, SS-EN 27888:1993; pH, SS 028122) and International Standards Organization (total Nitrogen, ISO 11905 and 13395). COD was measured using LANGE COD cuvette test LCK 114 (HACH LANGE LTD, Manchester, UK). REDOX and temperature was determined online using electrode measurements.

2.3. Concentration of virus using ultracentrifugation

14 ml of the wastewater sample was put into a 15 ml falcon tube and centrifuged at $3000 \times g$ at 4 °C for 10 min. The supernatants were poured into a 13.5 ml Ultra-Clear™ ultracentrifuge tube (Beckman-Coulter, Stockholm, Sweden) and centrifuged at $180{,}000 \times g$ for 2 h. The supernatants were discarded and the pellet redissolved in 500 μl RNAse free water. The suspension was subsequently aliquoted and stored at −80 °C until RNA extraction.

2.4. RNA extraction

RNA was extracted from 140 μl of the viral suspension (corresponding to approximately 3.5 ml of wastewater) using QIAamp viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. De-ionized water was always used as a negative control, and NoV from clinical samples was used as a positive control. RNA was eluted in 60 μl RNase free water containing 0.04% sodium azide (AVE buffer; QIAGEN, Hilden, Germany) and stored in aliquots at −80 °C until further use. In order to investigate the virus recovery, we spiked wastewater from different sampling points and MilliQ water with the same amount of NoV, and run in parallel with the described assay.

2.5. Reverse transcription

Briefly, 20 μl of extracted RNA was mixed with 50 pmol of $Pd(N)_6$ (GE-Healthcare, Uppsala, Sweden), denatured at 97 °C for 5 min, and quickly chilled on ice for 2 min, followed by addition of one Illustra Ready-To-Go™ RT-PCR bead (GE-Healthcare, Uppsala, Sweden), and RNase free water to a final volume of 50 μl. De-ionized water was always used as a negative control, and the positive control from the extraction step was also processed in parallel with the other samples. The reverse transcription (RT) reaction was then carried out for 90 min at 42 °C, to produce the complementary DNA (cDNA), later used for real-time PCR. The produced cDNA was then stored at −20 °C. Using spiked wastewater from all the eight sampling sites, a slight RT inhibition was detected in "activated sludge", "reject water", "incoming water" and "after primary settling". We found no RT inhibition in wastewater from the remaining four sampling sites. To compensate for the inhibition, these samples were diluted 1:10 before the RT reaction, and run in parallel with undiluted samples in order to account for a possible inhibition.

2.6. LUX real-time PCR assay and calculation of norovirus concentrations

4 μl of cDNA was used in triplicates for quantification of the cDNA from the RT reaction using a real-time PCR assay described previously (Nordgren et al., 2008), running 45 cycles. The primers in this assay (NVG1f1b: 5′-CGY TGG ATG CGN TTC CAT GA-3′; NVG1rlux: 5′-GAT GAG TCC TTA GAC GCC ATC ATC-3′; NVG2flux1: 5′-GAR AAA TGT TYA GRT GGA TGA GRT TYTC-3′; COG2R: 5′-TCG ACG CCA TCT TCA TTC ACA-3′) target the ORF1-ORF2 junction, and distinguish between NoV GGI and NoV GGII respectively. By using a standard curve, constructed after a serial dilution of plasmids with inserted viral cDNA from clinical NoV strains GGI.4 and GGII.4 respectively (Nordgren et al., 2008), the number of viral genomes in the real-time PCR reaction was determined, thus allowing us to estimate the number of norovirus particles per litre of wastewater for respective genogroup. The lowest detection level of norovirus in the real-time PCR assay is theoretically ~1–3 genes per PCR reaction which corresponds to ~$1\text{–}3 \times 10^4$ genome equivalents (g.e.) l^{-1} H_2O. Quantification of concentrations lower than 5×10^4 g.e. l^{-1} H_2O is less accurate due to less linearity of the real-time PCR standard curve close to the detection limit. No inhibition in the real-time PCR reaction was noted when diluting cDNA, inhibition was accounted for by using 1:10 RNA dilution as described in the reverse transcription section above.

2.7. Calculation of norovirus reduction and concentrations

Norovirus reduction was determined as the quotient between the $\log_{10}$ transformation of virus concentration before treatment, and the $\log_{10}$ transformation of virus concentration after treatment at the respective sites. When NoV was under the detection limit after a given treatment site, a hypothetical value of 5×10^3 g.e. l^{-1} H_2O (which corresponds to approximately half of the detection limit) was given for use in the correlation statistics, and measurement of average reduction (Swedish Environmental Protection Agency, 2008). The geometrical mean was used to describe the average virus concentrations. The sum of NoV GGI and NoV GGII is denoted Total NoV in the text.

2.8. Statistics

Arithmetical and geometrical means and standard deviations were determined by the one-sample *t*-test (GraphPad Prism 5, GraphPad Software Inc., San Diego, CA). Statistical differences between NoV concentrations were determined with the unpaired *t*-test. Correlation between physicochemical parameters, NoV concentrations and NoV reduction was

determined with Kendall's tau-*b* test using two-tailed significance calculations (SPSS 14.0, SPSS Inc., Chicago, IL).

3. Results

3.1. Virus recovery

Using NoV spiked wastewater samples and comparing the yield with spiked MilliQ water, we observed that the recovery of virus, measured by real-time PCR, varied between 25 and 55% as compared to the clean water. No significant difference between different types of wastewater was noted, although wastewater with more particle density generally yielded slightly fewer viruses.

3.2. Seasonal variation of NoV concentrations in the wastewater treatment process

The NoV concentrations during one year, in the eight different sampling sites for each genogroup are shown in Table 1. NoV GGII shows higher levels in all sites during the winter months, while NoV GGI shows higher levels during the summer months. The incoming concentrations of NoV GGII ranged between 3×10^4 and 1×10^7 g.e. l^{-1} H_2O (average 4.1×10^5), and the incoming concentration of NoV GGI ranged between 1×10^4 and 2×10^6 g.e. l^{-1} H_2O (average 3.2×10^5) during the year. During the winter months (Jan–Mar), however, the average NoV GGII concentration was 6.2×10^6 g.e. l^{-1} H_2O, as compared to 2.7×10^5 g.e. l^{-1} H_2O for NoV GGI ($p < 0.05$). During summer months (Jun–Aug), the average NoV GGII concentration was only 1.3×10^5 g.e. l^{-1} H_2O, compared to 1.2×10^6 g.e. l^{-1} H_2O for NoV GGI. The difference in concentration between the seasons was significant ($p < 0.05$) for NoV GGII, but not for NoV GGI. Accordingly, NoV GGII in the outgoing water was detected mainly during the winter months (average 1.2×10^5 g.e. l^{-1} H_2O), and NoV GGI exhibited the highest outgoing concentration during the summer months (Fig. 2). Moreover, NoV GGI shows a smaller variation in virus concentrations than NoV GGII in different sampling sites (Fig. 3). The range of NoV GGI concentration in incoming water is $\sim 2 \log_{10}$ units, whereas the range of NoV GGII concentration is $\sim 3 \log_{10}$ units (Figs. 2 and 3).

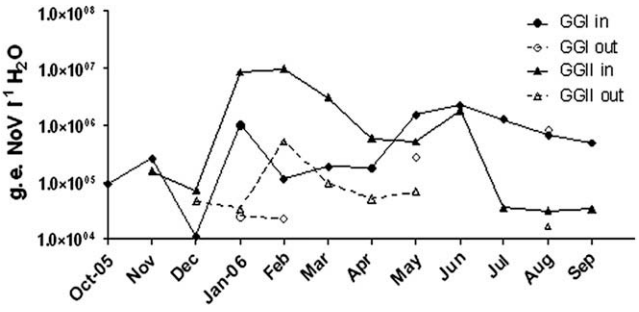

Fig. 2 – Norovirus concentrations in incoming and outgoing wastewater from Ryaverket WWTP during one year, as determined by real-time PCR.

In activated sludge, norovirus was only detected during three months, October 2005, December 2005 and Januray 2006, and only NoV GGI was found during all these three months with high concentrations in January 2006 (Table 1). NoV GGII, however, was only found in activated sludge in low concentrations in January 2006. In the water from the reject pumps, we only detected NoV GGII levels in January 2006, when the incoming concentration of NoV GGII was high, and high levels

Table 1 – Concentration of NoV GGI and NoV GGII at the eight different sampling sites during one year.[a,b]

Sampling site	Oct 2005	Nov 2005	Dec 2005	Jan 2006	Feb 2006	Mar 2006	Apr 2006	May 2006	Jun 2006	Jul 2006	Aug 2006	Sep 2006
Incoming water	9.2	26	1.1	100	11	18	17	150	220	120	67	49
	u.d[c]	16	7.2	840	940	300	58	50	180	3.6	3.2	3.3
After primary	3.8	8.1	5.0	29	3.3	29	u.d	87	u.d	73	20	u.d
settling	370	u.d	7.4	2900	39	170	5.6	3.3	2.2	1.4	u.d	u.d
Activated sludge	2.9	u.d	5.5	18	u.d	u.d	u.d	u.d	u.d	u.d	u.d	u.d
	u.d	u.d	u.d	2.2	u.d	u.d	u.d	u.d	u.d	u.d	u.d	u.d
After secondary	u.d	u.d	u.d	2.2	11	u.d	u.d	10	u.d	u.d	49	u.d
settling	500	u.d	2.8	u.d	26	5.6	8.5	3.3	u.d	u.d	u.d	u.d
Outgoing water	u.d	u.d	u.d	2.5	2.2	u.d	u.d	27	u.d	u.d	79	u.d
	u.d	u.d	4.6	3.4	51	9.6	5.0	6.7	u.d	u.d	1.7	u.d
Before trickling	u.d	u.d	u.d	1.7	5.6	u.d	u.d	19	u.d	u.d	60	7.8
filter	500	u.d	u.d	u.d	35	42	6.0	u.d	u.d	u.d	u.d	u.d
After trickling	u.d	u.d	u.d	5.0	3.3	u.d	u.d	14	u.d	u.d	40	9.7
filter	u.d	u.d	20	u.d	26	6.9	6.0	u.d	u.d	u.d	1.4	u.d
Reject water	u.d	u.d	u.d	u.d	u.d	u.d	u.d	u.d	u.d	u.d	7.8	u.d
	u.d	u.d	u.d	840	u.d	u.d	u.d	u.d	u.d	u.d	u.d	u.d

a The values are given in genome equivalents per litre water $\times 10^{-4}$.
b The concentrations above are of NoV GGI, and concentrations below are of NoV GGII.
c Under detection limit, $< \sim 10{,}000$ genome equivalents per litre water.

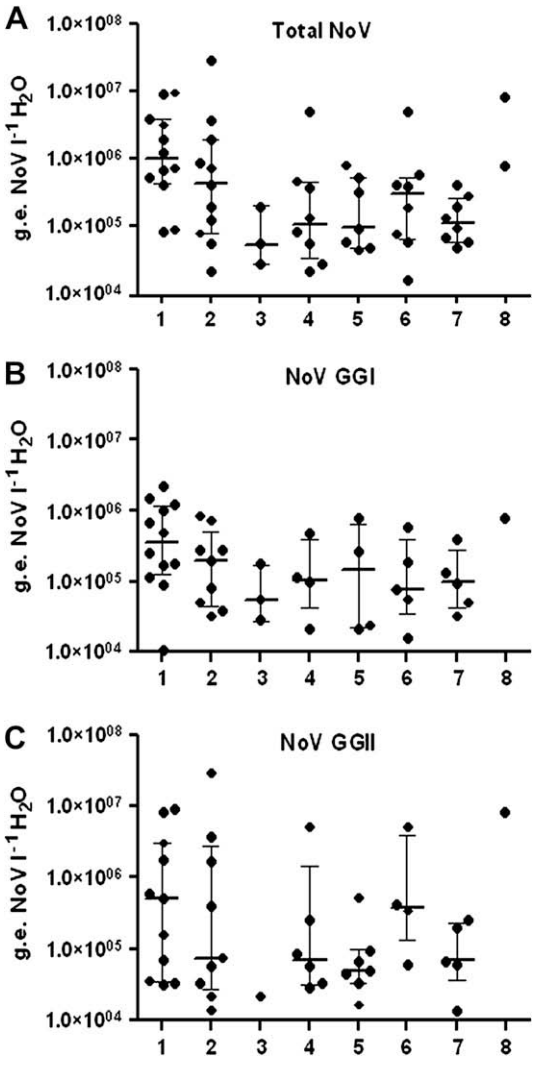

Fig. 3 – Concentration of NoV at all sample sites during on year for total NoV (A), NoV GGI (B), and NoV GGII (C). The outlying lines represent the inter-quartile range which contains 50% of the values; the line across indicates the median. The numbers indicate sample sites which are as follows: 1. Incoming water, 2. After primary settling, 3. Activated sludge, 4. After secondary settling, 5. Outgoing water, 6. Before trickling filter, 7. After trickling filter, and 8. Reject water.

of NoV GGI in August 2006, when the incoming concentration of NoV GGI was high (Table 1).

3.3. *Virus reduction of NoV GGI and NoV GGII between different sites in the wastewater treatment process*

The reduction between incoming and outgoing water was on average 1.5 $\log_{10}$ units (Table 2). The reduction ratio was largely the same between the two genogroups. The virus concentration is reduced in the primary settling (average 0.7 $\log_{10}$ units) and in the activated sludge in combination with the secondary settling (average 0.9 $\log_{10}$ units). The trickling filter exhibited a limited reduction for the few occasions that the remaining virus was detected in the influent to the trickling filters (Table 2). When measuring reduction only where quantification data is available both before and after treatment, we observe smaller reductions and a different pattern between the genogroups, with GGI being reduced less than GGII (Table 2).

3.4. *NoV reduction is affected by physicochemical parameters and concentration of NoV in the incoming water*

The reduction of NoV in the WWTP varied between months (Table 1, Fig. 3). We investigated the reduction in relation to incoming virus concentrations and to different physicochemical parameters, measured continuously in the WWTP. We found a correlation between reduction of total NoV, NoV GGI and NoV GGII, with incoming concentrations of NoV GGI and NoV GGII respectively (Table 3). We furthermore found a negative correlation between the incoming water flow and reduction of both genogroups, particularly for NoV GGI. Also a higher conductivity was associated with a higher reduction, mainly in the activated sludge in combination with secondary settling. Reduction of total nitrogen was associated with reduction of NoV, particularly for NoV GGII.

3.5. *Incoming and outgoing concentrations of NoV are affected by inflow of water and conductivity in the wastewater treatment plant*

The correlation between incoming and outgoing concentrations of NoV, inflow of water and conductivity is shown in Table 4. We note that the incoming concentration of NoV GGI

Table 2 – Reduction ratios[a,b] of NoV genome equivalents between different treatment sites.

	Incoming water/ outgoing water	Incoming water/after primary settling	After primary settling/ after secondary settling	Before/after trickling filter
Total NoV	1.5 ± 0.3 ($n = 12$)	0.7 ± 0.2 ($n = 12$)	0.9 ± 0.3 ($n = 11$)	0.1 ± 0.1 ($n = 7$)
	1.0 ± 0.3 ($n = 7$)	0.6 ± 0.2 ($n = 11$)	0.4 ± 0.2 ($n = 8$)	0.1 ± 0.1 ($n = 7$)
NoV GGI	1.4 ± 0.2 ($n = 12$)	0.7 ± 0.3 ($n = 12$)	0.9 ± 0.3 ($n = 9$)	0.0 ± 0.1 ($n = 5$)
	0.7 ± 0.3 ($n = 4$)	0.2 ± 0.1 ($n = 9$)	0.3 ± 0.4 ($n = 4$)	0.0 ± 0.1 ($n = 5$)
NoV GGII	1.2 ± 0.2 ($n = 11$)	0.8 ± 0.2 ($n = 11$)	0.7 ± 0.4 ($n = 10$)	0.3 ± 0.2 ($n = 3$)
	1.2 ± 0.3 ($n = 6$)	0.7 ± 0.3 ($n = 8$)	0.3 ± 0.3 ($n = 6$)	0.3 ± 0.2 ($n = 3$)

a Reduction average expressed in $\log_{10}$ units + SE.
b Above: reduction average using half detection limit as an estimate for virus quantity after a given treatment process when NoV concentration is under the detection limit. Below: reduction average between sites that both have quantification data.

Table 3 – Correlation coefficients between reduction of norovirus in different treatment sites and physiochemical parameters.

Norovirus reduction between treatment sites	Incoming concentration of NoV GGI	Incoming concentration of NoV GGII	Inflow of water[a]	Conductivity[b]	Reduction of total nitrogen
Incoming water/outgoing water					
Total NoV	0.5** ($n=12$)	0.2 ($n=11$)	−0.4* ($n=12$)	0.3 ($n=12$)	0.2 ($n=12$)
NoV GGI	0.5** ($n=12$)	0.1 ($n=11$)	−0.4* ($n=12$)	0.2 ($n=12$)	0.1 ($n=12$)
NoV GGII	0.2 ($n=11$)	0.6** ($n=11$)	−0.3 ($n=11$)	0.2 ($n=11$)	0.4 ($n=11$)
Incoming water/after primary settling					
Total NoV	0.1 ($n=12$)	0.0 ($n=11$)	0.2 ($n=12$)	0.0 ($n=12$)	−0.3 ($n=12$)
NoV GGI	0.3 ($n=12$)	0.1 ($n=11$)	0.1 ($n=12$)	0.1 ($n=12$)	−0.2 ($n=12$)
NoV GGII	0.1 ($n=11$)	0.1 ($n=11$)	0.2 ($n=11$)	−0.1 ($n=11$)	−0.1 ($n=11$)
After primary settling/after secondary settling					
Total NoV	0.2 ($n=11$)	0.2 ($n=10$)	−0.4 ($n=11$)	0.4 ($n=11$)	0.3 ($n=11$)
NoV GGI	0.2 ($n=9$)	−0.1 ($n=8$)	−0.4 ($n=9$)	0.1 ($n=9$)	0.2 ($n=9$)
NoV GGII	0.2 ($n=9$)	0.2 ($n=8$)	−0.3 ($n=9$)	0.4* ($n=9$)	0.5* ($n=9$)

*$p < 0.10$.
**$p < 0.05$.
a Measured as $m^3 s^{-1}$.
b Measured as $mS m^{-1}$.

is significantly correlated to inflow, the less inflow the higher concentration of NoV GGI. No such correlation exists for NoV GGII. Furthermore, we detected a trend towards a higher concentration of NoV GGII in outgoing water if the incoming concentration is high, which is not the case for NoV GGI (Table 4). We also note that higher outgoing concentration of NoV is associated with higher inflow, particularly for NoV GGII.

4. Discussion

We found a variation of NoV GGI and NoV GGII at all sites during the 12-month period investigated, with the highest concentrations of NoV GGI in summer, and NoV GGII in winter. The increase of NoV GGII in winter was expected, since during the winter months most clinical cases of norovirus gastroenteritis in Sweden are reported, and the same pattern has also been described elsewhere (Haramoto et al., 2006; Katayama et al., 2008). We detected an increase of NoV GGI concentrations in the beginning of May 2006, which then remained in higher concentrations than NoV GGII throughout the summer (Fig. 2). In contrast to this, previous studies have reported NoV GGII to be more prevalent than NoV GGI in wastewater (Lodder and de Roda Husman, 2005; Haramoto et al., 2006; Katayama et al., 2008), with lower concentrations of both genogroups during summer (van den Berg et al., 2005; Haramoto et al., 2006; Ottoson et al., 2006; da Silva et al., 2007). Our results may reflect epidemiological patterns in the community, with the emergence of NoV GGI strains during the summer. The reasons for this increase need to be further investigated, especially since the summer is ''low season'' for clinically reported norovirus gastroenteritis. Possibly, the emerging NoV GGI strains give rise to less severe or asymptomatic infections as compared to the NoV GGII strains circulating during winter time. Since the vast majority of NoVs is clinical disease belong to GGII (Fankhauser et al., 2002; La

Table 4 – Correlation coefficients between incoming and outgoing concentrations of norovirus and physiochemical parameters in the wastewater treatment plant.

	Incoming concentration of NoV GGII	Outgoing concentration of NoV GGI	Outgoing concentration of NoV GGII	Inflow of water[a]	Conductivity[b]
Incoming concentration					
NoV GGI	−0.1 ($n=11$)	0.2 ($n=12$)	−0.2 ($n=12$)	−0.5** ($n=12$)	−0.1 ($n=12$)
NoV GGII	1.0	0.0 ($n=11$)	0.4* ($n=11$)	0.1 ($n=11$)	0.2 ($n=11$)
Outgoing concentration					
NoV GGI	0.0 ($n=11$)	1.0	0.3 ($n=12$)	0.1 ($n=12$)	0.0 ($n=12$)
NoV GGII	0.4* ($n=11$)	0.3 ($n=11$)	1.0	0.4* ($n=12$)	−0.1 ($n=12$)

*$p < 0.10$.
**$p < 0.05$.
a Measured as $m^3 s^{-1}$.
b Measured as $mS m^{-1}$.

Rosa et al., 2007; Bucardo et al., 2008; Kroneman et al., 2008), the stable transmission of NoV GGI which we observed in wastewater is indeed intriguing, and there is need for further studies to explain this phenomenon.

The incoming concentrations of NoVs ranged between 1×10^4 and 1×10^7 g.e. l^{-1} H_2O, which are somewhat higher but comparable with previous studies. These studies have described NoV concentrations in incoming wastewater ranging between 1.7×10^2 and 1.9×10^6 g.e. l^{-1} H_2O in WWTPs in Japan, the Netherlands and Germany (Pusch et al., 2005; van den Berg et al., 2005; Haramoto et al., 2006). We found a reduction of total norovirus concentration between the outgoing and incoming water of in average 1.5 $\log_{10}$ units (Table 1, Fig. 3), and other studies have described reduction levels ranging from 1.0 to 2.7 $\log_{10}$ units (van den Berg et al., 2005; Haramoto et al., 2006; Ottoson et al., 2006). Primary treatment and treatment in a conventional, non-nitrifying activated sludge system reduced the norovirus content with 0.7 and 0.9 $\log_{10}$ units respectively. A subsequent nitrifying trickling filter typically reduced norovirus further, how much however was difficult to estimate due to a large number of influent samples below the detection limit. The variation in reduction was high, with reduction levels ranging from 0 to 1.6 $\log_{10}$ units for NoV GGI, and between 0.2 and 2.4 $\log_{10}$ units for NoV GGII. For NoV GGI in particular, there were many occasions when no virus was detected in the effluent which gives an underestimation of reduction levels. We asserted this by using half the detection limit as an estimate for virus concentrations when it was under detection limit after treatment sites (Swedish Environmental Protection Agency, 2008). The reduction levels then range between 0 and 2.6 $\log_{10}$ units for NoV GGI, and 0.2 and 2.6 $\log_{10}$ units for NoV GGII.

A report from a WWTP in Japan, describes reduction levels that are almost constant throughout the year, whereas a WWTP in the Netherlands demonstrated more variation regarding reduction levels (van den Berg et al., 2005; Haramoto et al., 2006). We investigated the correlation between reduction of NoV, physicochemical parameters, and incoming concentrations of NoV. We observed that higher incoming concentration of virus correlated to higher reductions for both genogroups (Table 3). We furthermore observed that higher inflow was associated with less reduction (Table 3). This negative correlation could be related to the fact that low flows give less dilution and thus higher NoV concentrations creating a higher potential for reduction. A similar correlation between good nitrogen removal and low flow may explain the correlation between nitrogen removal and removal of virus (Table 3). Grab samples were extracted at approximately the same time at the collecting locations of the WWTP, making it important to consider the retention times in the system. The area served by the WWTP is very large with runtimes of 0–24 h causing the typical diurnal variation of influent wastewater to be limited. The ammonium concentration of the wastewater, which is a good indicator of human activity, varies very little around the clock (Ann Mattsson, personal communication). We therefore assume that the maximum retention time (up to 8 h between incoming and outgoing water) would not have a big impact on the reduction calculation. During a long rain event, as is often the case at the Rya WWTP, the effluent wastewater is also diluted so the reduction will be correctly calculated despite the effect of dilution. However, during transient conditions at the beginning or end of a rain event, the dilution will not be equal in influent and effluent. This could be one explanation to the spread of reduction results in the data. This is a problem inherent of studies in full-scale wastewater systems including stormwater.

Incoming concentrations of NoV GGI are negatively correlated to inflow of water (Table 4), probably due to dilution effects. This pattern was not seen for NoV GGII, which could be due to the fact that NoV GGII is more seasonal dependent than NoV GGI thus disguising the effect of dilution. NoV GGII demonstrated high winter peaks when clinical cases were more common, making it difficult to detect a decrease of concentration due to a higher inflow of wastewater. Since NoV GGI shows a more stable pattern during the year (Figs. 2 and 3B), it is easier to detect decreases of concentration because of higher inflow. We also found a trend towards higher outgoing concentrations for NoV GGII when incoming concentrations are high (Table 4), which is not the case for NoV GGI, something which could be due to that outgoing concentrations of NoV GGI had to be estimated at eight occasions.

Using spiked wastewater samples and comparing to clean water with the described assay, we found that 25–55% of NoV was recovered from the wastewater samples as compared from clean water. We found no significant differences between wastewater types, although wastewater with higher density of particles generally yielded fewer viruses. Since we accounted for inhibition by RNA dilution, it is likely that this difference is mainly due to the viral attachment to particles in the wastewater, thus present in the pellet after the first low speed centrifugation. Also a potential clogging of the membrane used for RNA extraction by the remaining wastewater particles could account for part of the lesser yield. However, since the concentrations in the WWTP varied logarithmically, we believe that this difference of yield in and between samples is of small concern.

The noroviruses were detected in incoming water during the whole year investigated. In particular NoV GGI showed a stable concentration levels (Figs. 2 and 3). Several reports describe outbreaks of NoV GGI related to contaminated food, such as mollusks, or bathing water (Sartorius et al., 2007; Nenonen et al., 2008). These stable levels of NoV GGI in wastewater during the whole year, emphasizes the importance of reducing NoV in WWTPs disposing wastewater into systems where norovirus has a potential impact on public health in order to prevent such outbreaks. Indicator bacteria are often used to control water quality, but some previous reports show no correlation between reduction of indicator bacteria and viruses (Rose et al., 2004; Haramoto et al., 2006; Ottoson et al., 2006). In this study the average reduction ratio of NoV, 1.5 $\log_{10}$, was slightly above the yearly average reduction of coliform bacteria and *Escherichia coli*, which are on average 1.2 and 1.0 $\log_{10}$ units, respectively (Börjesson et al., unpublished). Whether reduction of indicator bacteria correlated to reduction of NoV was not possible to address due to lack of reduction data for indicator bacteria for the days when NoV was measured. There is an ongoing debate about finding a reliable viral indicator, and many enteric viruses or bacteriophages have been suggested, such as adenovirus and somatic coliphages (Contreras-Coll et al., 2002; Carducci et al.,

2008; Katayama et al., 2008). We observed stable transmission of NoV GGI, especially considering the mentioned dilution effects. This observation together with the many described NoV GGI outbreaks related to mollusks and bathing water, indicates a potential for NoV GGI to be used as an indicator for enteric virus contamination and reduction.

In the activated sludge NoV was mostly not detected, even when concentrations were high in other sampling sites (Table 1). According to our experiments with spiked wastewater samples, RT inhibitors are removed using a 1:10 dilution of the RNA extract. In a recent report, (Sano et al., 2004), virus-binding proteins isolated from bacterial cultures in activated sludge were described. These proteins were able to adsorb viral peptides with high affinity in an experiment with an affinity column. An explanation for the low detection of NoV in activated sludge might be that the NoV present are firmly attached to such proteins, and therefore not detected with our assay. However, when using spiked activated sludge samples, we did not find any significant difference in recovery as compared to samples from other collecting point in the WWTP. A further explanation could be protozoan predation, which would be less optimal when using spiked samples in a laboratory environment as compared to the actual situation in the WWTP. Furthermore, when diluting the RNA 1:10 to account for the inhibition, it is possible that the NoV is diluted under the detection limit.

Anaerobic sludge digestion for 20–30 days on most occasions reduced norovirus to levels below the detection limit in the reject water indicating good reduction in the digestors. However, on one occasion for each strain norovirus was detected in the reject water and then at high levels (Table 1). Further studies with repeated sampling of reject water, would be useful in order to determine if there is really a break-through of virus and under which conditions. Other feasible explanations include sampling problems or the possibility of short cuts in the mixed reactor.

5. Conclusions

We found that norovirus is present in wastewater throughout the year, not only during the winter, but also during summer. This correlated to a change of genogroups, possibly due to emerging circulations of new genotypes after the winter outbreaks. We found a different behavior of NoV GGI and NoV GGII, with a more stable transmission for NoV GGI. Primary treatment and treatment in a conventional, non-nitrifying activated sludge system reduced the norovirus content by about a factor 30, and physicochemical parameters correlated with NoV reduction. This study extends previous knowledge of NoV in a WWTP, with new information that could be used for improvement of treatment processes regarding virus removal.

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