Presence of human noro- and adenoviruses in river and treated wastewater, a longitudinal study and method comparison

Leena Maunula, Kirsi Söderberg, Heli Vahtera, Veli-Pekka Vuorilehto, Carl-Henrik von Bonsdorff, Maria Valtari, Tuula Laakso and Kirsti Lahti

ABSTRACT

Norovirus (NoV) is one of the most common causative agents of waterborne gastroenteritis outbreaks. The main objective of the study was to determine the presence of human NoVs in river water and in treated wastewater (TW) released into the river. During a one-year survey in 2007/2008, NoVs were detected in 30.8% of river samples (20/65), and 40.5% of TW samples (17/45) with a real-time reverse transcription-PCR assay. NoVs were present in the river water in the winter and spring, coinciding with the NoV epidemiological peak in the community and the presence of NoVs in TW. Later in 2009, the concentration method used, pre-filtration with a Waterra filter combined with filtration through a negatively charged membrane, was evaluated against glass wool filtration and freeze-drying for the detection of adenoviruses in river water. The virus amounts measured varied greatly depending on the virus concentration method. The continued monitoring in the spring of 2009 also revealed that the average concentration of noro- and adenoviruses in TW was 2.64×10^3 and 1.29×10^4 pcr units per mL, respectively. No correlation between the presence of viruses and *Escherichia coli* was found. These results may be useful for risk assessment studies.

Key words | adenovirus, natural water, norovirus, raw water, real-time PCR, RT-PCR

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INTRODUCTION

Human noroviruses (NoVs) cause gastroenteritis with vomiting in children and adults. Disease episodes occur throughout the year, with a high epidemic peak in the winter months in countries in the northern hemisphere (Green 2007). Human enteric adenoviruses, types 40 and 41, which belong to group F, also cause mild gastroenteritis, with vomiting, mild fever and dehydration (Uhnoo *et al.* 1990), and they are mainly diagnosed in children. Other adenoviruses, among them type 31 (group A) and 52 (group G), have been linked to gastroenteritis cases (Filho *et al.* 2007; Jones *et al.* 2007). In adults, the pathogenic nature of adenoviruses is less clear than in children, since asymptomatic shedding can occur for months, even years (Langley 2005).

Both noro- and adenoviruses transmit easily from person to person, and although huge amounts of NoVs are excreted into sewage in winter as well as a lesser amount in autumn, more or less constant levels of adenoviruses are present in sewage throughout the year (Katayama *et al.* 2008). The efficiency of wastewater treatment plants in inactivating viruses depends on the one hand on treatment techniques and on the other hand on the stability of the particular viruses. Treated wastewater (TW) still containing possibly viable viruses is then discharged to natural water sources. The contamination of natural waters by enteric viruses has been reported in many countries (Wyn-Jones *et al.* 2010).

In natural waters enteric viruses can survive for prolonged periods, their half-life being dependent on several parameters, such as temperature, sunlight and the water matrix (Gerba 2007). In groundwater the persistence of

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noro- and adenoviruses has been reported to be more or less equal when measured by reverse transcriptase polymerase chain reaction (RT-PCR) or PCR (Charles *et al.* 2009). Infective adenoviruses persisted for about 1 year, whereas the genomes of noro- and adenoviruses could be detected for 1.6–2 years. In surface water viruses are likely to become inactivated more rapidly than in groundwater (Gerba 2007; Bae & Schwab 2008), but it is known that adenoviruses are quite resistant to UV light and thus to sunlight (Hijnen *et al.* 2006). It has also become obvious that only a few viruses are needed for infection, especially in the case of NoVs, which can spread from person to person as well as via food and water.

While the levels of hygienic indicator bacteria in many natural waters are monitored regularly, the burden of human enteric viruses, such as noro- and adenoviruses, usually remains unknown. One of the reasons for the scarcity of viral burden data so far is the lack of infectivity assays for detection of many human diarrhea-causing enteric viruses. As it is today still practically impossible to cultivate human NoVs in cell cultures in the laboratory, genome amplification has been a well-received technique for showing the presence of these viruses in environmental samples. Also, enteric adenoviruses are challenging to grow in cell culture. Integrated cell-culture-nested PCR has increased the sensitivity of virus detection (Chapron *et al.* 2000). The introduction of real-time PCR/RT-PCR has enabled quantitative measurement in genome amplification assays.

Before gene amplification is performed, the viruses in a water sample have to be concentrated into smaller liquid volumes. Several techniques, such as filtration through glass wool, positively or negatively charged disk filters or cartridges, the use of glass powder, flocculation, polyethylene glycol (PEG) precipitation, ultrafiltration and ultracentrifugation have been applied, as reviewed earlier (Ueki *et al.* 2005; Wyn-Jones 2007; Bosch *et al.* 2008). The most popular virus adsorption-elution (VIRADEL) methods are based on adsorption of viruses present in water to a solid matrix, from which they are then eluted into a smaller liquid volume. For several decades, glass wool has been used as an adsorption support for the concentration of enteroviruses (Vilagines *et al.* 1988). An alternative option has been to use commercial filters, especially for filtration of drinking water samples (as reviewed by Maunula 2007), but also of natural waters (Fuhrman *et al.* 2005). While the filtration of drinking water is usually straightforward, that of surface water is more challenging owing to a higher turbidity due to clay, algae, microbes and different organic and inorganic compounds. Several consecutive concentration steps, such as PEG precipitation after filtration, may be necessary for further volume reduction.

Waterborne outbreaks, small or large scale, caused mainly by NoVs in drinking water occur annually in Finland (Maunula *et al.* 2005). In this study, we monitored water of the River Vantaa, serving as a secondary source for drinking water, as well as recipient of effluents from three municipal wastewater treatment plants situated along the river, for the presence of human NoVs and/or adenoviruses. Data about bacterial indicators present in the river water was compared with that of viruses. First, a one-year survey was conducted (March 2007–February 2008) and water and TW were surveyed for the occurrence of human NoVs. A simple method of a pre-filter and negatively charged membrane combined with real-time gene amplification was applied for river water. Later, monitoring was continued in a three-month survey (between February and May 2009), and then, besides genogroup 2 NoV concentrations, those of human adenoviruses were determined in order to increase the likelihood of finding viruses in the samples. This time river water and TW were analysed in parallel with other concentration methods, glass wool and freeze-drying, to evaluate their suitability for monitoring viruses in naturally contaminated waters.

MATERIALS AND METHODS

Description of the River Vantaa

The river passes through the most densely populated area in the metropolitan district of southern Finland (about 1.5 million inhabitants) and ends in the Baltic Sea in Helsinki (Figure 1). Several smaller towns are situated along the river, and the wastewater effluents of some municipal wastewater treatment plants are discharged into the river. The river offers an important recreational element for inhabitants, and it is a secondary water source for the water works for drinking water production in the Helsinki

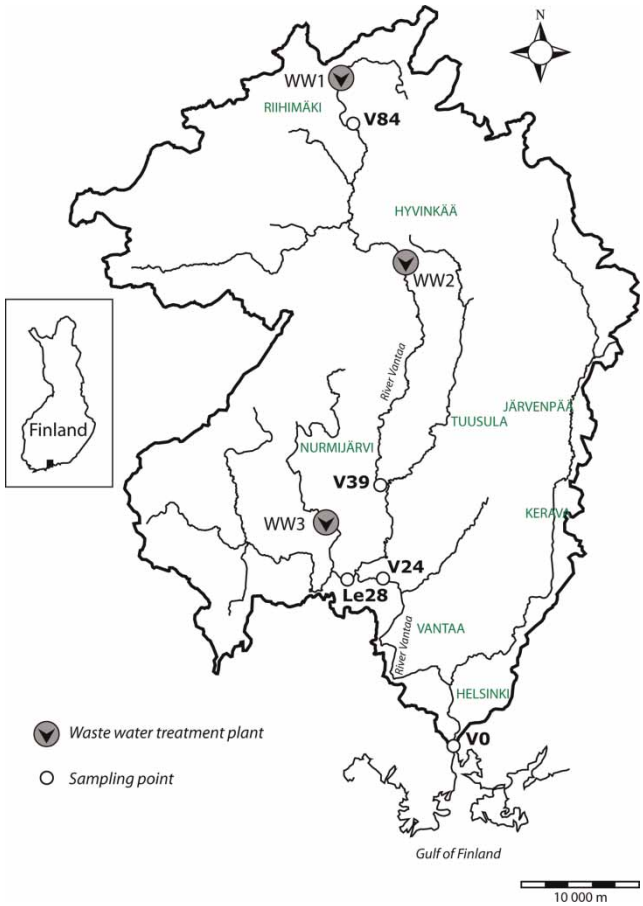

Figure 1 | River Vantaa and sampling sites (V84, V39, V24, Le28 and V0) and the location of the wastewater treatment plants (WW1, WW2 and WW3).

metropolitan area. The catchment area is 1,680 km^2, and the mean flow in 2007 was 17 m^3/s (range < 2.0 to 140 m^3/s). The clay catchment of the river (38%) causes turbidity that increases downstream from 5 to 52 FTU. In 2007, the electrical conductivity of the river water, mean value 32 mS/m, was the highest in the upper part of the river owing to wastewater discharge. Tributaries dilute the water at the river mouth, at which the mean value of electrical conductivity was 20 mS/m. In July and August over 75% of upstream river water may be TW.

Water and wastewater samples

River water and TW were analysed for NoVs in the 1-year survey for noro- and adenoviruses in the continued monitoring. The location of the river and sampling sites are shown in Figure 1. River water was collected from five sampling sites (V84, V39, Le28, V24 and V0) and wastewater effluent from three sampling sites (WW1, WW2 and WW3) representing three major wastewater treatment plants. Sampling sites Le28 and WW3 were situated in a side branch of the river, while the rest were along the main river. All three plants use chemical phosphorus removal, nitrification-denitrification and activated sludge processes. The characteristics of the wastewater treatment plants are presented in Table 1. The sampling frequency was once per month from March 2007 to February 2008 (in total 13 samplings, see Figure 3 for dates) and three times in total between February and May 2009. The river was ice-covered during sampling in March 2007 as well as February and March 2009, whereas winter 2008 was mild and rainy. TW samples were collected over 12 to 24 hours as discharge-based composite samples and river water as grab samples (volumes below).

Concentration methods for water and treated wastewater

For river water, (i) adsorption-elution with a pre-filter and a HA filter, (ii) glass wool, and (iii) freeze-drying methods were applied. In the one-year survey only the first method was used. The wastewater effluents had either: (i) no pre-treatment, or (ii) a 50 mL sample was freeze-dried.

Table 1 | Characteristics of the wastewater treatment plants in 2007

Wastewater treatment plant	Average daily treated volume (m^3 d^{-1})	Hydraulic retention time (h)	Population equivalents	BOD_7 removal %	Total P removal %	Total N removal %
WW1	13,250	21.0	67,100	99	97	74
WW2	13,120	26.8	40,000	99	98	76
WW3	6,300	18.9	28,570	99	98	83

BOD = biochemical oxygen demand.

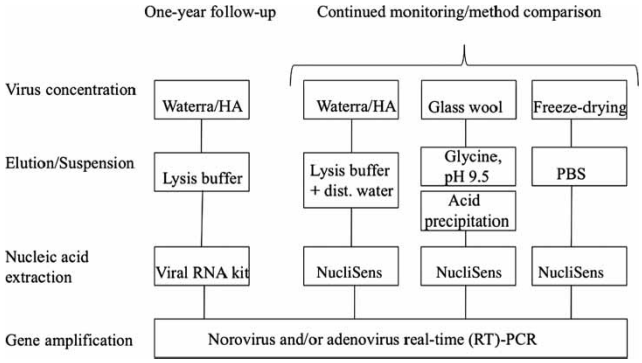

Figure 2 | Schematic of the methods for river water analysis used in the study.

A method schematic is shown in Figure 2 and details of the protocols are described below.

River water: adsorption-elution with a pre-filter and a HA filter

Three litres of water were filtered with an adsorption-elution method using a pre-filter Waterra (FHT-700, Waterra) (Anon 2000) and a negatively charged HA membrane (a mixture of cellulose acetate and nitrate, Millipore, Billerica, MA, diameter 47 mm, pore size 0.45 μm). In the one-year survey viruses were eluted and analysed separately from the pre-filter and the HA filter. Combined results have been presented here for clarity. Glycine buffer, 50 mmol/L, pH 9.5, with 3% beef extract, in volume of 50 mL was used for the elution of viruses from the pre-filter. From the HA filter, viruses were eluted with 1 mL AVL buffer (Qiagen, GmbH, Hilden, Germany) using mechanical shaking for 10 min at room temperature. Eluates were analysed for noro- and/or adenoviruses with a real-time one-step RT-PCR after nucleic acid extraction. In the continued monitoring in 2009, a slightly modified elution procedure was used: the virus was first eluted with 2.5 mL NucliSens lysis buffer (Biomerieux, Boxtel, The Netherlands) from a HA membrane, and the membrane was washed once with 1 mL distilled sterile water. Entire eluate containing water and lysis buffer was used for RNA extraction.

River water: glass wool filtration

River water samples of 3 L, a volume comparable to that used in the HA filtration method, with pH adjustment to 3.5 were filtrated through a glass wool column (725; Rantigny, Saint-Gobain, France) according to Gantzer *et al.* (1997) and Wyn-Jones *et al.* (2010). After elution of the viruses with 200 mL in a 50 mmol/L glycine buffer, pH 9.5 containing 3% (w/v) beef extract, the pH of the eluate was lowered to 3.5. The precipitated virus was collected with centrifugation at 7,000 *g* for 30 min, after which 8 mL PBS (pH 7.2) was added to the pellet. One mL of sample was used for nucleic acid extraction.

Freeze-drying

Freeze-drying, which was applied for river water and TW, was performed for 30–50 mL samples which had been pre-frozen at −80 °C, using a freeze-drying device (Heto DW3, Hallerød, Denmark). After freeze-drying for two days the remnants were suspended in 1 mL PBS and all was used for the extraction of viral nucleic acids.

Direct method

In the direct method, 0.2 mL of TW was used for viral RNA extraction.

Nucleic acid extraction and real-time RT-PCR assays

In the one-year survey in 2007, the viral RNA was extracted using a QIAamp Viral RNA kit (Qiagen GmbH, Hilden, Germany). In the continued monitoring in 2009, total nucleic acid extraction that also extracted adenovirus DNA from all the samples was performed with a NucliSens magnetic extraction kit (Biomerieux Boxtel, The Netherlands). Volumes used are indicated in the context of the concentration methods. With both methods the final nucleic acid was eluted using 60 μl elution buffer.

For the NoV analysis, a one-step real-time RT-PCR with primers and a FAM-TAMRA-labelled TaqMan probe was performed using a QuantiTect Probe RT-PCR kit (Qiagen), as previously reported (Loisy *et al.* 2005; Maunula *et al.* 2009). For the adenovirus analysis, a real-time PCR with primers and the TaqMan probe in hexon gene (JTVXF, JTVXR and JTVXP catching adenoviruses A–F)

described by Jothikumar *et al.* (2005) were used. LightCycler Software 3.5 (Roche Applied Sciences) was used for the gene amplification of NoVs, and a Rotorgene RG-3000 (Corbett/Qiagen, Australia) real-time PCR cycler was used for adenoviruses.

Two high-titre patient faecal samples, one positive for NoV GII.4 and one for adenovirus, were used to create a standard curve. PCR units were used in calculations, since the exact genome copies were unknown for the control samples. Tenfold dilution series of viral nucleic acid were tested by gene amplification and the highest positive dilution was determined to contain one PCR unit. The detection limit of both noro- and adenovirus assays was thus 1 unit per 5 μl of extracted nucleic acids monitored in one PCR reaction.

For all samples, nucleic acids were always tested in PCR at least as neat and dilution 1:10 to reduce the inhibition level. For TW, further dilutions were performed to determine the end dilution for noro- and/or adenovirus. The viral load in the sample was the mean of interpolated values for each dilution with volume adjustment. Negative controls for each step, virus concentration, nucleic acid extraction and PCR, were included to reveal possible laboratory contamination.

NoV GII.4 genotyping

For GII.4 genotype screening, samples positive in the GII RT-PCR were run in a real-time RT-PCR using a SYBR green I PCR kit (Qiagen) and specific primers were designed to detect GII.4 NoVs (F1 5′-act ctc tgt gca ctc tcc gaa gt-3′ and *R*2 5′-gct ttg ctg tca act tct ctg g-3′). The PCR running conditions using Rotorgene RG-3000 PCR cycler were 95 °C 15 min, 50 cycles with 95 °C 15 s, 60 °C 35 s and 72 °C 35 s (fluorescence measured). The cDNA was prepared according to the method described previously (Maunula *et al.* 1999). A melting curve analysis was performed, and samples with a melting temperature of 79 ± 0.5 °C were regarded as positive.

Water quality analyses

The number of *Escherichia coli* in the river and in the TW was measured with the Colilert Quantitray (IDEXX) and turbidity according to the EN-ISO 7027 standard method. Conductivity of river water was analysed according to EN-ISO 27888.

Statistical analysis

Pearson's correlation coefficients between the number of *E. coli* and viruses were counted from log-transformed data.

RESULTS

One-year survey of NoVs: river water

Water filtration through pre-filter Waterra and negatively charged HA membrane was applied for river water from five sampling sites along the river. During the one-year survey period from March 2007 to February 2008, the presence of NoVs could be shown in Vantaa river water mainly during the winter months, between December and April (Table 2a). Genogroup II NoVs were detected significantly more often than genogroup I NoVs. The number of NoV positive samples ranged from 3 to 5 per 13 annual samples depending on the sampling point; in total 20/65 (30.8%) water samples contained NoVs. During the early autumn a period of several months occurred with no NoVs in the water collected from any of the five sampling points.

One-year survey of NoVs: treated wastewater

TW from three plants located along the river was also monitored for viruses as a possible source of virus contamination. In the TW NoVs were detected in all months with the exception of September, October and November (Table 2b). Taken together, NoV RNA was detected in 17/42 (40.5%) of the samples. The presence of NoV genotype GII.4 could be determined in several river and TW samples when genotype GII.4 specific primers were used in a real-time RT-PCR (data not shown). Both the river water and TW contained indicator bacteria *E. coli* throughout the year (Figure 3). While the number of *E. coli* in the TW varied from 2.3×10^1 to 1.2×10^3 per mL, noroviral RNA loads exceeding those numbers were measured several times between March and May, and between October and December 2007.

Table 2 | Presence of NoVs (genogroup GI and GII) in 3-litre samples of river water (a) or in TW (b) (NoV concentration in PCR units per mL) from March 2007 to February 2008

Sampling site	III/2007	IV	V	VI	VII	VIII	IX	X	XI	XII	I/2008	II	Total
a)													
V84	0/2 GII	1/2 GII	1/1 GI	1/1 GII	nd	0/1	0/1	0/1	0/1	1/1 GII	1/1 GII	0/1	5/13
V39	1 GI, 2 GII/2	0/2	0/1	0/1	nd	0/1	0/1	0/1	0/1	0/1	1/1 GII	0/1	3/13
Le28	1/2 GII	1/2 GII	0/1	1/1 GII	nd	0/1	0/1	0/1	0/1	1/1 GII	0/1	0/1	4/13
V24	1/2 GII	0/2	0/1	1/1 GII	nd	0/1	0/1	0/1	0/1	1/1 GII	0/1	1/1 GII	4/13
V0	1/2 GII	0/2	0/1	0/1	nd	1/1 GII	0/1	0/1	0/1	1/1 GII	1/1 GII	0/1	4/13
Total	5/10	2/10	1/5	3/5		1/5	0/5	0/5	0/5	4/5	3/5	1/5	20/65
b)													
WW1	1/1 GI, GII (5.7×10^2, 2.1×10^3)	1/2 GI,GII (2.4×10^3, 2.1×10^2)	0/2	0/1	0/1	0/1	0/1	0/1	0/1	1/1 GII (2.1×10^2)	1/1 GII (2.1×10^2)	1/1 GII (1.4×10^2)	5/14
WW2	1/1 GI, GII (1.3×10^3, 1.0×10^4)	2/2 GI,GII (3.9×10^4, 3.6×10^4)	0/2	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	1/1 GII (3.0×10^2)	4/14
WW3	1/1 GI, GII (1.4×10^2, 4.4×10^3)	2/2 GII (1.0×10^4)	1/2 GII (5.5×10^3, 7.1×10^2)	0/1	0/1	0/1	0/1	1/1 GII (2.3×10^4)	1/1 GII (1.8×10^4)	1/1 GII (1.4×10^1)	0/1	1/1 GII (3.2×10^2)	8/14
Total	3/3	5/6	1/6	0/3	0/3	0/3	0/3	1/3	1/3	2/3	1/3	3/3	17/42

The mean of turbidity values for V84, V39, Le28, V24 and V0 were 20.5, 16.5, 41.7, 28.0 and 62.5 FTU, respectively.
Limit of detection is 3.33 pcr units (pcr detectable units) per L river water.
Limit of detection is 10 pcr units per mL TW.
nd = not done.

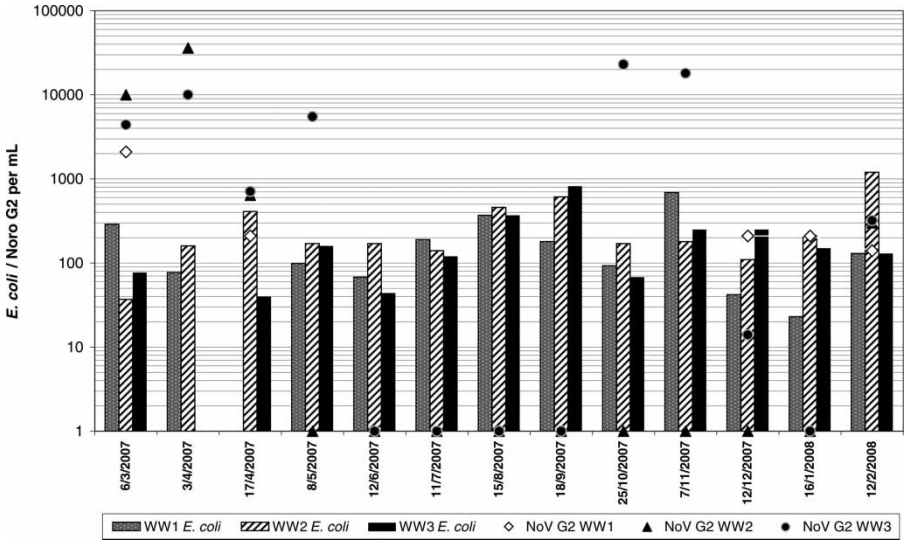

Figure 3 | Concentrations of *E. coli* (per mL, bars) and genogroup GII noroviruses (pcr units per mL, symbols) in TW obtained from three wastewater treatment plants between March 2007 and February 2008.

Continued monitoring and method comparison: noro- and adenoviruses in river water

In 2009, the survey was continued; the river water and TW were analysed for NoV genogroup II and adenoviruses three times during February, March and May. In addition to filtration through the HA membrane after pre-filtration, glass wool and freeze-drying were applied for the water in parallel after sampling from four sites along the river.

This time NoVs were not detected in river water samples with any of the virus concentration methods (data not shown), but all with the exception of one sample (11/12) contained adenoviruses (Table 3). Using the glass wool method, 9/11 adenovirus-positive samples were detected (versus 8/12 with HA and 4/12 with the freeze-drying method). The mean concentration of adenoviruses was 5.39×10^2 and 1.17×10^5 pcr units/L in river water samples in March 2009 with the HA and glass wool methods, respectively. PCR inhibition was not obvious for any of the methods when nucleic acids were tested as neat and dilution 1:10 in PCR.

Continued monitoring and method comparison: noro- and adenoviruses in treated wastewater

In the TW NoV RNA and adenovirus DNA were consistently detected (Table 4). Adenovirus and NoV assays were performed either directly or after freeze-drying a 50 mL sample. Both viruses were detected in all nine samples after freeze-drying, whereas direct analysis detected NoVs in eight and adenoviruses in six samples. On the other hand, generally higher viral loads of mean values 2.64×10^3 and 1.29×10^4 pcr units/mL for noro- and adenoviruses, respectively, were measured with the direct method. The levels of viruses in the effluents fluctuated so that more adenoviruses than NoVs were found in February and March, whereas NoV concentrations were higher in May (Figure 4). There were no significant correlations between the number of *E. coli* and noro- ($r = -0.05$, $n = 22$) or adenoviruses ($r = 0.35$, $n = 17$) in TW. No correlation was found between the number of noro- and adenoviruses ($r = 0.34$, $n = 11$), either.

DISCUSSION

In this study noro- and adenoviruses were found in both river water and TW. As the annual occurrence of viral findings often coincided, the latter is likely one of the sources increasing the viral load in the river. The results suggest that a portion of the enteric viruses had passed through wastewater plants in which secondary wastewater treatment with denitrification was used. Here and elsewhere (Jothikumar *et al.* 1993; Gantzer *et al.* 1998; Lodder &

de Roda Husman 2005) it has become obvious that viruses in effluents enter environmental waters and cause a risk if the water is consumed without appropriate treatment to remove or inactivate the viruses. In 2005, van den Berg *et al.* (2005) estimated that 10^2–10^3 PCR-detectable units of NoVs per L of treated sewage was discharged into the surface water in The Netherlands.

One-year survey of NoVs: river water

The presence of NoVs in Finnish surface waters was reported in 2004 (Hörman *et al.* 2004). At that time a NoV analysis developed for drinking water was applied to surface waters, and often difficulties arose owing to the clogging of the membrane during the filtration of water with higher turbidity. In the method for natural waters presented here no clogging occurred when the pre-filter or glass wool was applied, and several litres of water could be filtered in the procedure.

In the one-year survey, the noroviral load in the river water was determined by a real-time RT-PCR in which c_q values of 35–40, corresponding to a low number of PCR units, were consistently obtained. Since quantitative results that are so close to the detection limit for a real-time PCR have increased uncertainty and because of the low virus recovery of the water filtration method (Fong *et al.* 2005), the quantities of NoV RNA present in the river water were not calculated. Also, the values might have been affected by changes in the amplification efficiency of the PCR due to changes in the quality of the river water, as the average turbidity of the sampling sites ranged from 16 to 52 FTU. In European countries, the presence of NoVs in river water was first reported in the United Kingdom (Laverick *et al.* 2004) and The Netherlands (Lodder & de Roda Husman 2005). Recently, in Germany, Jurzik *et al.* (2010) found genogroup II NoVs in 25.7% of the water samples in the Ruhr and Rhine Rivers, which is comparable with the NoV frequency in our study. In a large European study, 9.4% of the recreational water samples including river water contained NoVs (Wyn-Jones *et al.* 2010).

One-year survey of NoVs: treated wastewater

In our study the viral loads in TW varied from negative to 10^4 pcr units/mL (that is 10^7 pcr units/L). The positive

Table 3 | Concentrations of human adenoviruses (pcr units per L) found in river water in spring 2009 using three parallel virus concentration methods

	February 2009			March 2009			May 2009		
	HA	Glass wool	Freeze-drying	HA	Glass wool	Freeze-drying	HA	Glass wool	Freeze-drying
V84	<3.33	6.15×10^5	<200	3.33×10^0	3.02×10^5	3.36×10^6	3.56×10^3	1.06×10^5	6.66×10^2
V39	<3.33	nd	1.00×10^4	3.33×10^0	7.29×10^4	<200	4.36×10^2	<26.70	<330
V24	1.20×10^4	2.86×10^4	6.98×10^4	1.50×10^3	3.64×10^4	<200	<4.00	6.40×10^2	<330
V0	1.14×10^4	3.45×10^5	<200	6.50×10^2	5.62×10^4	<200	<4.00	<26.70	<330

Limits of detection for HA, glass wool and freeze-drying are 3.33–4.00, 26.70 and 200–330 pcr units per L river water, respectively.
nd = not done.

Table 4 | Concentrations of genogroup II NoVs and human adenoviruses (pcr units per mL) in TW collected in spring 2009, determined by two parallel methods

		NoV GII		Adenovirus	
Wastewater	**Month/Year**	**Directly**	**Freeze-drying**	**Directly**	**Freeze-drying**
WW1	II/09	1.60×10^3	1.69×10^2	<50	1.08×10^3
WW2	II/09	3.00×10^2	5.86×10^2	<50	8.80×10^2
WW3	II/09	<50	4.72×10^2	2.59×10^4	8.89×10^2
WW1	III/09	4.50×10^2	1.71×10^2	1.45×10^3	1.24×10^3
WW2	III/09	3.00×10^2	1.32×10^2	5.63×10^4	9.33×10^2
WW3	III/09	7.50×10^2	8.06×10^1	1.70×10^4	3.93×10^2
WW1	V/09	1.80×10^3	1.39×10^2	<50	8.03×10^1
WW2	V/09	1.00×10^4	2.90×10^2	5.20×10^3	1.36×10^2
WW3	V/09	8.60×10^3	6.61×10^3	9.95×10^3	3.18×10^2
Mean value		2.64×10^3	9.61×10^2	1.29×10^4	6.61×10^2

Limits of detection for direct and freeze-drying are 50 and 1 pcr units per mL TW, respectively.

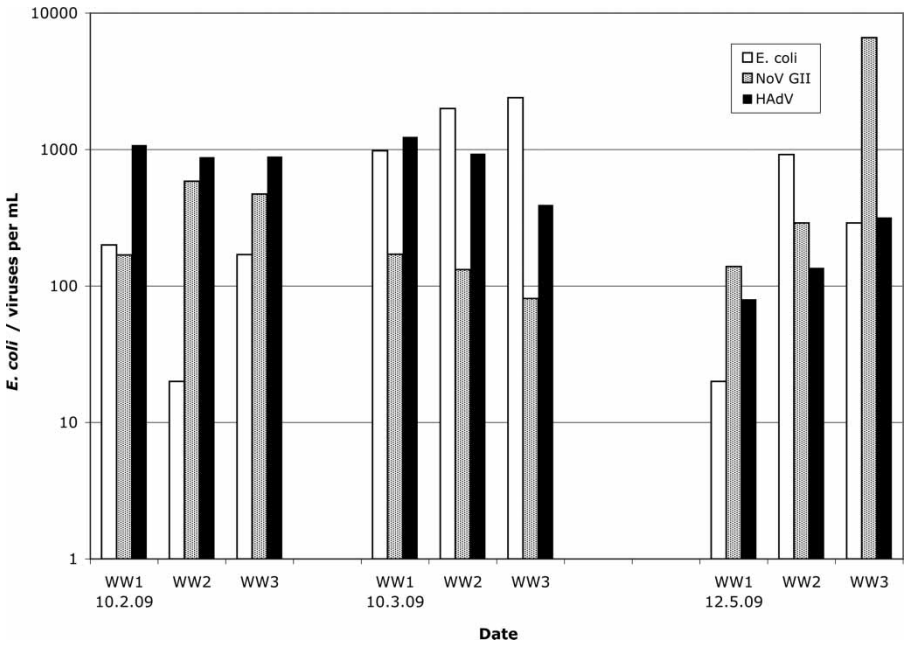

Figure 4 | Concentrations of *E. coli* (per mL), genogroup GII noroviruses and adenoviruses (pcr units per mL) in TW (after freeze-drying) obtained from three wastewater treatment plants between February and May 2009.

values were generally higher than those reported in several other studies (Lodder & de Roda Husman 2005; Pusch *et al*. 2005; van den Berg *et al*. 2005). The lower viral load (a maximum of 10^4 pcr units/L) in effluents obtained in a Japanese study (Katayama *et al*. 2008) may be due to the chlorination of wastewater, whereas in our study the treatment process did not include any disinfection steps. In Finland disinfection is not a general practice in wastewater treatment. In a Swedish study, water was analysed for NoVs before and after several successive treatment steps in one wastewater treatment plant. In that study, viral loads of 10^4–10^5 gene equivalents/L in effluents were

found (Nordgren *et al.* 2009), agreeing with the Japanese study. In addition to the difference in the efficiency of virus removal in these sewage treatment plants or seasonal changes to their efficiency, the methods used for the concentration of viruses are reflected in the results as well. In our study, the virus loss in the direct assay was limited to the nucleic acid extraction procedure.

NoVs were present in TW more often in winter than in summer. The same seasonal profile of NoVs in sewage effluents was also obvious in Japan (Katayama *et al.* 2008), whereas in Sweden, the highest levels of genogroup I NoVs were detected in summer (Nordgren *et al.* 2009). The coincidence of viruses in TW and river water suggests a rapid transfer of a portion of the viruses from human excreta via sewage treatment plants to the river water. Ueki *et al.* (2005) deduced that TW is an essential link in the circulation of NoVs between humans and oysters in seawater. During the study period, an emerging genotype, the GII.4-2006b (Minerva) variant NoV, was the most prevalent clinical strain, and it caused a significant increase in NoV outbreaks (Kanerva *et al.* 2009). It was shown in this study that genotype GII.4 NoVs were present in the river water and TW, but a more detailed characterization of all virus genotypes or variants was not performed.

Continued monitoring and method comparison: noro- and adenoviruses in river water

The methods comparison studies revealed clearly how much the measured amount of viruses in a water sample depended on the virus concentration and extraction method used. The membrane filtration method applied in the one-year survey turned out to be somewhat less efficient in detecting adenoviruses in water than glass wool, but more efficient than the freeze-drying method. Generally glass wool also gave the highest values of viral loads, but not always. Based on the results of all methods we can conclude that 11 water samples truly contained adenoviruses. Taking this assumption we deduce that we missed one positive with the glass wool method, three with HA and seven with freeze-drying.

Our experience was that establishment of the glass wool 'in-house' method takes somewhat more time than applying commercial disk membranes. The glass wool material suitable for virus capture is not necessarily easily available. On the other hand, glass wool has the capacity to bind more virus particles than disk membranes, and is more comparable to filter cartridges in that respect. For all applications, an efficient elution of viruses from the adsorptive matrix has been found to be challenging. An increase in the volume of the elution buffer also increases the virus recovery from the matrix, but a second concentration step is then needed. The use of the direct lysis of viruses from disk membranes with nucleic acid extraction lysis buffer (Beuret 2003; Schultz *et al.* 2011) makes the procedure convenient, and no further concentration is needed. Sulfuric acid and sodium hydroxide have also successfully been used in the elution of enteric viruses from negatively charged membranes in large volumes of freshwater (Haramoto *et al.* 2004), but handling these hazardous chemicals in a laboratory cannot be recommended.

The optimal conditions for concentrating noro- and adenoviruses may not necessarily be identical, but might depend on their physical and chemical characteristics, such as the isoelectric point of the virus particle. In a Japanese publication, different virus recoveries were obtained when water was artificially contaminated either by noro- or by poliovirus (Haramoto *et al.* 2009), although the same method was used.

The occurrence of enteric adenoviruses in river water has been reported in several continents, such as in Africa (van Heerden *et al.* 2005) and in North (Fong *et al.* 2005) and South America (Rodriguez-Diaz *et al.* 2009). Wyn-Jones *et al.* (2010) reported a mean value of >3000 genome copies of adenoviruses per L of water in European recreational waters; Albinana-Gimenez *et al.* (2009) measured about 10^4 genome copies per L in a Spanish river water; and Jurzik *et al.* (2010) 5.7×10^1 and 7.3×10^5 gene equivalents per L in Germany. These values are in a similar range to those found in the River Vantaa, with values up to 10^4 pcr units per L.

Continued monitoring and method comparison: noro- and adenoviruses in treated wastewater

In a US study, 10^3–10^4 adenovirus particles per L were measured in a membrane bioreactor wastewater effluent (Kuo *et al.* 2010). In another recently published North American study, Fong *et al.* (2010) found up to 4×10^5

adenoviruses per L using a real-time gene amplification assay in a tertiary, chlorinated effluent. In our study the values obtained were generally higher with up to 10^4 pcr units per mL, which may partly be a result of different sample treatment. It has to be taken into account that both noro- and adenoviruses were monitored in the water with a gene amplification assay that does not reveal the number of infectious viruses in the sample. The presence of infectious adenoviruses in wastewater effluents, however, has been verified by cell culture as well (Rodriguez-Diaz *et al*. 2009).

The microbial quality of the water of the River Vantaa is monitored regularly for levels of faecal indicator bacteria. Thus, in this study it was possible to analyse viral and indicator bacteria levels in river water as well as in TW samples for possible correlation of numbers. In line with other studies (Jurzik *et al*. 2010), no correlation between bacterial and viral loads, or between noro- and adenoviral loads, was detected in TW in winter and spring, but *E. coli* was always present when viruses were found in the sample.

In this study we have been able to show that even in a sparsely populated country, human pathogenic enteric viruses, noro- and adenoviruses, exist in natural waters. The link between burden of enteric viruses in river water and in TW seems obvious. Although the techniques for measuring exact quantities of viruses, especially infectious viruses, still need much improvement, several feasible methods already exist for virus detection in water. The monitoring of viruses in drinking and other water sources is becoming both more rapid and more cost-beneficial. When implemented, whether for monitoring adenoviruses, as suggested by Wyn-Jones *et al*. (2010), or for NoVs, it may be used to prevent waterborne viral outbreaks.

CONCLUSIONS

In this study NoVs were present in the river water mainly during the winter and spring months, but not in summer and autumn. NoVs were also present in TW when they were found in river water, suggesting it as a source of viral contamination.

It became evident that the methods used for viral analyses have a high impact on the results obtained. In particular, quantification of viruses varies depending on the methods and probably because of changes in the composition of water from time to time.

In the light of the method comparison, we can conclude that during the one-year survey the number of virus-containing samples we obtained with the pre-filter/HA method were under-estimated, since the glass wool method proved to be somewhat more sensitive. As compensation for this, values obtained by PCR probably exceeded the number of infectious viruses.

Monitoring of human pathogenic viruses in relevant natural waters such as rivers passing through densely populated areas should be considered.

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