Supporting information

Norovirus dynamics in wastewater discharges and in the recipient drinking water source: Long-term monitoring and hydrodynamic modelling

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The supporting information contains twelve pages (S1-S12), two figures (Figures S1 and S2), and six tables (Tables S1-S6). The supporting information provides details regarding the sampling programme, norovirus analyses, hydrodynamic modelling, measured microbial concentrations in wastewater, and simulated microbial concentrations in source water.

Sampling

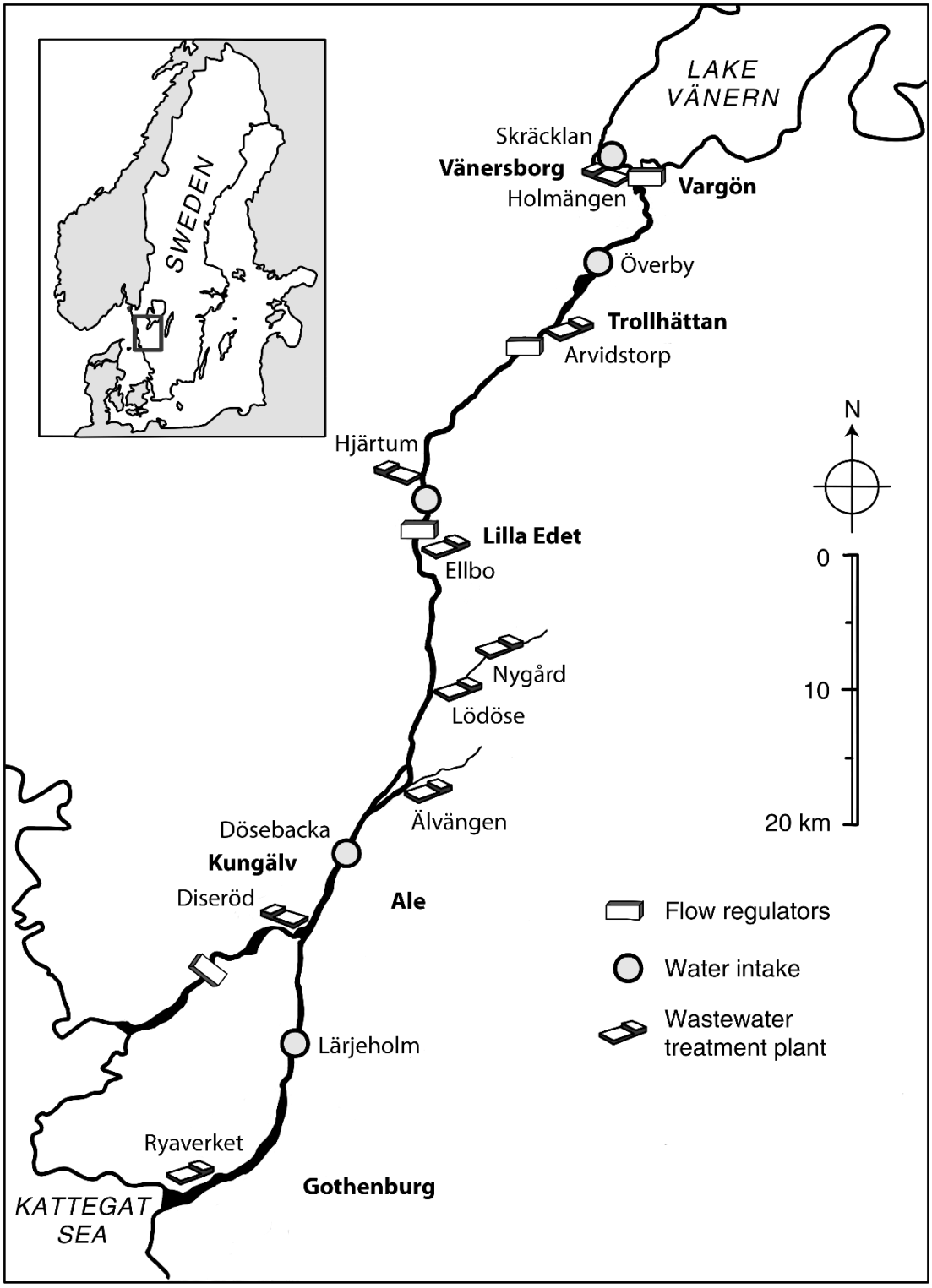

Figure S1 Map of the river Göta älv between Lake Vänern and the strait Kattegat. The map shows the location of the wastewater treatment plants, water intakes, and hydropower stations (flow regulators).

Table S1 Overview of the long-term monitoring project. The samples of source water and wastewater were collected at drinking water and wastewater treatment plants in Vänersborg (V), Trollhättan (T), Lilla Edet (L), Kungälv (K), and Gothenburg (G). At wastewater treatment plants in V, T, and L, both incoming and treated wastewater was sampled, while in G only treated wastewater was sampled. The samples were analysed for NoV (a), *E. coli* (b), and somatic coliphages (c).

	Drinking water treatment plant					Wastewater treatment plant			
Date	V	T	L	K	G	V	T	L	G
08 Jun 2011		a,b,c	a,b,c		a,b,c		a,b	a	
22 Jun 2011		b	b		b		a,b,c	a,c	
06 Jul 2011		a,b,c	a,b,c		a,b,c		a,b,c	a,c	
20 Jul 2011		b	b		b		a,b	a,c	
03 Aug 2011		a,b,c	a,b		a,b,c		a,b,c	a,c	
17 Aug 2011		b	b		b		a,b,c	a	
31 Aug 2011		a,b,c	a,b,c		a,b,c		a,b,c	a,c	
14 Sep 2011			b		b		a,b,c	a,c	
28 Sep 2011		a	a,b		a,b		a	a	
12 Oct 2011		b	b		b		a,b,c	a,c	
26 Oct 2011	a,c	a,b,c	a,b,c		a,b,c	a	a,b,c	a,c	
09 Nov 2011		b	b		b		a,b,c	a,c	
23 Nov 2011	a	a,b	a,b,c		a,b	a	a,b	a	
07 Dec 2011		b	b		b		a,b,c	a,c	
21 Dec 2011	a,c	a,b,c	a,b,c	a,c	a,b	a	a,b,c	a,c	a,c
04 Jan 2012		b	b		b		a,b,c	a,c	
18 Jan 2012	a,c	a,b,c	a,b,c	a,c	a,b,c	a	a,b,c	a,c	a,c
01 Feb 2012		b	b		b		a,c	a,c	
15 Feb 2012	a,c	a,b,c	a,b,c	a,c	a,b,c	a	a,b,c	a,c	a
29 Feb 2012		b	b		b		a,b,c	a,c	
14 Mar 2012	a,c	a,b,c	a,b,c	a,c	a,b,c	a	a,b,c	a,c	a
28 Mar 2012		b	b		b		a,b,c	a,c	
11 Apr 2012	a,c	a,b,c	a,b,c	a,c	a,b,c	a	a,b,c	a,c	a,c
25 Apr 2012		b	b				a,b,c	a,c	
09 May 2012	a,c	a,b,c	a,b,c	a,c	a,c	a	a,b,c	a,c	a,c
23 May 2012		b	b		b		a,b,c	a,c	
05 Jun 2012	a,c	a,b,c	a,b,c	a,c	a,b,c	a	a,c	a,c	a,c

Norovirus analyses

Enrichment from source water

Source water samples were processed the day after sampling. NoV enrichment was a three step procedure with two different types of filters and a centrifugal microconcentrator device.[1] Source water samples (4.5 L) were passed through a 47 mm non-charged 2 µm glass fibre filter (Millipore, Billerica, MA). A 2.5 M $MgCl_2$ solution was added to the eluate, reaching a final concentration of 25 mM, to simulate the flocculation process in a treatment plant. The thoroughly mixed solution was passed through a 47 mm HA mixed-cellulose ester filter (Millipore). Virus particles fixed on the surface of the filter were rinsed with 200 mL 0.5 mM H_2SO_4. Virus was eluted with a back flush of 15 mL 50 mM KH_2PO_4, 1 M NaCl, 0.1% Triton X-100 (v/v), and pH 9.2 buffer. The eluate was recovered in a tube containing 200 µL 100 mM H_2SO_4 and 33 × Tris-EDTA buffer for neutralization. The eluate was added to a Centriprep Concentrator YM-50 kDa (Millipore) and centrifuged for 30 min at 4000 × *g* at 4 °C. The resulting 200 µL concentrate was mixed with 100 µL RLT buffer (Qiagen, Hilden, Germany) and then subjected to RNA extraction.

Enrichment from wastewater

Wastewater samples were processed on the day of sampling. From each sample, 50 mL was centrifuged at 4300 × *g* for 30 min to remove debris, and then 40 mL of the supernatant was mixed with 10 mL of sterile PEG solution (100 g PEG-8 000, 6 g NaCl in 250 mL water at pH 7.2) under slow rotation overnight at 4 °C.[2] The mixture was then centrifuged at 10 000 × *g* for 90 min at 4 °C, and the supernatant was removed. The pellet was dissolved in RLT buffer in a total volume of 300 µL.

Extraction of RNA and reverse transcription

Total RNA extraction was done in a Biorobot EZ1 (Qiagen) with the EZ1 Virus mini kit v.2.0 (Qiagen), according to the manufacturer's protocol. The sample volume was 300 µL, and the purified RNA was eluted in 60 µL. Potentially inhibiting components were eliminated by the OneStep™ PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA). Purified total RNA was stored at −80 °C until further processing.

First strand cDNA synthesis was done in a total volume of 40 µL, containing 20 µL purified RNA, 8 µL RNase free water, 8 µL 5 × Reaction mix, and 4 µL Maxima™ First Strand cDNA Synthesis RT-qPCR enzyme mix (Thermo Scientific, Stockholm, Sweden) with random N6 primer. Synthesis of cDNA was done at 25 °C for 10 min, followed by 50 °C for 15 min. The reaction was stopped at 85 °C for 5 min, and then the synthesis was cooled to 4 °C. Until further processing, cDNA was stored at −20 °C. RNase free water was used as a negative control.

Detection and quantification by TaqMan real-time PCR

Quantitative real-time PCR was done as a duplex PCR in an ABI 7500 FAST PCR system (Applied Biosystems). Each sample was tested in duplicate for NoV GGI and GGII (Table S2). The thermal cycling conditions are described in Table S2. For the quantification of NoV, 2 µL cDNA was used as a template in a total reaction volume of 25 µL, containing 200 nM of each primer (NV-G1-fwd1b, NV-G1-rev, NV-G2-fwd and COG2R), 200 nM of each TaqMan probe,[3-4] 0.2 µL ROX (50 µM), and 12.5 µL Maxima™ Probe qPCR Master Mix (2X) (Thermo Scientific). A pUC57 plasmid with two inserts (Genscript, Piscataway, NJ), which include the regions of the ORF1-ORF2 junction for NoV GGI and GGII, was used as a standard for quantification. The insert for NoV GGI corresponded to Genebank NCBI JX023285.1 position 5286 to 5383; and the insert for NoV GGII corresponded to AF414417.1 position 485 to 592. Quantification

standards were amplified in duplicate with tenfold dilution series of the plasmid ranging from 10^6 to 10 copies per reaction. For source water, the limit of quantification was 3.8×10^1 g.e. L^{-1}, and the limit of detection was extrapolated to 1.1×10^1 g.e. L^{-1}. For wastewater, the limit of quantification was 1.5×10^4 g.e. L^{-1}, and the limit of detection was extrapolated to 4.5×10^3 g.e. L^{-1}.

Controls of virus enrichment, RNA extraction, reverse transcription, and PCR reactions

To control virus enrichment and RNA extraction steps,[5] the samples were spiked with murine norovirus 1 solution (MNV-1, median tissue culture infective dose TCID50 ~ 10^7 mL^{-1}; Swedish University of Agricultural Sciences, Department of Biomedical Sciences and Veterinary Public Health, Uppsala, Sweden). Before enrichment, the source water and the wastewater samples were spiked with 90 µL and 5 µL MNV-1 solution, respectively. For process control, 1 L of tap water spiked with 80 µL MNV-1 solution was handled in the same way as the source water samples; and 50 mL tap water spiked with 5 µL MNV-1 was handled in the same way as the wastewater samples. Inhibition control of the reverse transcription step was done with Alien Reference RNA VIC QRT-PCR Detection Kit (Agilent, La Jolla, CA), according to the manufacturer's instructions. To rule out PCR inhibition and to optimise PCR performance, tick-borne encephalitis virus (TBEV, inactivated, strain K23, Encepur®, Chiron Vaccines, Marburg, Germany) was used.

For the amplification of MNV-1 and of TBEV in a duplex PCR, the following primers and probes were used (Table S2): MNV fwd, MNV rev, F-TBEV 1 SC, R-TBEV 1 SC, MNV-probe, TBEV-probe SC. The reaction volume was 25 µL, containing 300 nM MNV primer, 100 nM MNV probe, 200 nM TBEV primer and probe, 2 µL 1:100 dilution of TBEV cDNA synthesis, 0.2 µL ROX (50 µM), and 12.5 µL Maxima™ Probe qPCR Master Mix (2X) (Thermo Scientific). The thermal cycling conditions are described in Table S2. The individual filtration performance on a per sample basis was estimated from the quantification cycle (Cp) value of the MNV-1 PCR.

The process control MNV-1 showed that the virus recovery rate varied between 1.2 % and 23 % with a mean of 18 % for source water, and between 0.1 % and 31 % with a mean of 5 % for wastewater. Inhibition control of the reverse transcription step showed no inhibition. The PCR inhibition control TBEV showed no inhibition.

Table S2 Primers and probes for real-time qPCR assays*

Target virus	Primer/probe name	Sequence (5' - 3')	Target region	Amplicon size [bp]	Reference
NoV GGI	NV-G1-fwd1b	CGY TGG ATG CGN TTC CAT GA	ORF1-ORF2 junction region	88	Nordgren et al. 2008[3]
	NV-G1-rev	GTC CTT AGA CGC CAT CAT C			Nordgren et al. 2008[3]
	G1-probe	[6FAM][i]-AGA TYG CGR TCY CCT GTC CA-[BHQ1][ii]			Kageyama et al. 2003[4]
NoV GGII	NV-G2-fwd	ATG TTY AGR TGG ATG AGR TTY TC	ORF1-ORF2 junction region	67	Nordgren et al. 2008[3]
	COG2R	TCG ACG CCA TCT TCA TTC ACA			Kageyama et al. 2003[4]
	G2-probe	[JOE][iii]-TGG GAG GGC GAT CGC AAT CT-[BHQ1]			Kageyama et al. 2003[4]
MNV	MNV fwd	TTG GGA ACA TGG AGG TTC AR	capsid region of ORF2	88	Persson 2013[6]
	MNV rev	GGR AAA TAG GGT GGT ACA AGG			Persson 2013[6]
	MNV-probe	[6FAM]-CCA +CCT +TGC +CAG +CAG T-[BHQ1]			Persson 2013[6]
TBEV	F-TBEV 1 SC	GGG CGG TTC TTG TTC TCC	3' non-coding region	68	Schwaiger et al. 2003[7]
	R-TBEV 1 SC	ACA CAT CAC CTC CTT GTC AGA CT			Schwaiger et al. 2003[7]
	TBEV-probe SC	[JOE]-TGA GCC ACC ATC ACC CAG ACA CA-[BHQ1]			Schwaiger et al. 2003[7]

*Thermal cycling conditions:

- NoV GGI/GGII: 95 °C, 10 min; (95 °C,15 s; 56 °C[iv], 60 s) x 50 cycles
- MNV/TBE: 95 °C, 10 min; (95 °C,15 s; 60 °C[iv], 60 s) x 45 cycles

[i]6FAM=6-Carboxyfluorescein; [ii]BHQ1= Black Hole Quencher 1; [iii]JOE=4-5-Dichloro carboxyfluorescein; mixed bases Y=C/T, N=A/C/G/T, R=A/G denote degenerate bases (IUP code); + denotes Locked Nucleic Acid (LNA bases); [iv]fluorescence data acquisition for FAM and JOE signal

Hydrodynamic modelling

Since the river is regulated by the hydropower stations, the modelling domain was divided into three stretches according to the locations of the hydropower stations: Vargön – Trollhättan; Trollhättan – Lilla Edet; Lilla Edet – the Gothenburg harbour. The simulations for the different stretches of the river were run separately.

The initial conditions in the river were defined by the surface elevation. The upstream boundary conditions for different stretches were defined by the water flow through the hydropower stations. The downstream boundary conditions for different stretches were defined by the surface elevation at the hydropower stations and the Gothenburg harbour. The model accounted for the inflow to the river from the tributaries with catchment areas greater than 50 km^2 (tributaries Bastån, Stallbackaån, Slumpån, Gårdaån, Lärjeån, Grönån, Säveån, Hältorpsån and Mölndalsån). On the land boundary the normal component of velocity was set to zero. The horizontal and vertical eddy viscosities were simulated using Smagorinsky and Log law formulations, respectively. The bed resistance was described by a constant roughness height of 0.05 m. The model was run with default parameterisation.

Validation of the hydrodynamic model was done by comparing the simulated and measured water surface elevation at the Lärjeholm water intake. To include different hydrodynamic conditions, validation was done on four periods, which were selected based on the water flow in the river during 2011: low and stable (1 – 31 May), low and varying (15 Jun – 16 Jul), high and stable (25 Aug – 25 Sep), as well as high and varying (10 Nov – 11 Dec). The validation showed that the model described the measured data well – the Pearson correlation coefficient was 0.99. The absolute mean difference between the simulated and measured water surface elevation was 0.03 m. This absolute mean difference and its standard deviation were 11 % and 14 % of the standard deviation of the measured data, respectively.[8]

The initial conditions regarding the microbial concentrations in the river were set as zero concentration. The model output regarding the microbial concentrations from the upstream stretches was used as input for the downstream stretches.

Table S3 Wastewater treatment plants located along the river Göta älv between Lake Vänern and the Lärjeholm water intake (Figure S1).

Municipality	WWTP	People	Treated wastewater (10^3 m^3 $year^{-1}$)
Vänersborg	Holmängen	27000	4900
Trollhättan	Arvidstorp	49000	10000
Lilla Edet	Nygård [a]	500	33
Lilla Edet	Lödöse	1600	160
Lilla Edet	Hjärtum	380	52
Lilla Edet	Ellbo	5780	824
Ale	Älvängen	6200	1136
Kungälv	Diseröd	1200	130

[a] Treated wastewater is discharged into the Gårdaån tributary.

Table S4 Input data for the water quality model: microbial concentrations in treated wastewater.

Microorganism	WWTP	n	median	95 %
E. coli, CFU L^{-1}	Arvidstorp (Trollhättan)	25	3.70E+05	1.97E+06
	Combined dataset	25	3.70E+05	1.97E+06
Somatic coliphages, PFU L^{-1}	Arvidstorp (Trollhättan)	24	1.05E+05	7.68E+05
	Ellbo (Lilla Edet)	24	2.40E+04	2.20E+05
	Combined dataset	53	7.00E+04	5.16E+05
NoV GGI, g.e. L^{-1} [a]	Combined dataset warm period	30	2.34E+05	3.96E+08
	Combined dataset cold period	40	3.18E+05	2.15E+07
NoV GGII, g.e. L^{-1} [a]	Combined dataset warm period	30	2.37E+05	1.68E+08
	Combined dataset cold period	40	2.35E+06	8.33E+07

[a] For wastewater, the limit of quantification (LOQ) for NoV was 1.5×10^4 g.e. L^{-1}, and the limit of detection (LOD) was extrapolated to 4.5×10^3 g.e. L^{-1}. When the NoV genome concentration was above the LOD, but below the LOQ, the geometric mean (8.2×10^3 g.e. L^{-1}) of the LOD and LOQ was used for calculations. When the NoV genome concentration was below the LOD, the value for LOD was used for calculations.

Measured concentrations of norovirus and faecal indicators in wastewater

Table S5 Measured concentrations (Log_{10} transformed) of faecal indicators and norovirus (NoV) genome in wastewater at the wastewater treatment plants in Vänersborg (V), Trollhättan (T), Lilla Edet (L), and Gothenburg (G).

	Place	N [a]	Mean	St. dev.
Incoming wastewater				
E. coli, Log_{10} (CFU L^{-1})	T	26 (26)	6.55	1.22
Somatic coliphages, Log_{10} (PFU L^{-1})	T	23 (23)	5.99	0.46
	L	22 (22)	5.90	0.56
NoV GGI, Log_{10} (g.e. L^{-1}) [b]	V	9 (9)	6.67	1.06
	T	27 (27)	6.79	1.07
	L	20 (27)	5.50	1.40
NoV GGII, Log_{10} (g.e. L^{-1}) [b]	V	9 (9)	7.98	0.82
	T	24 (27)	7.36	1.57
	L	17 (27)	5.79	1.74
Treated wastewater				
E. coli, Log_{10} (CFU L^{-1})	T	25 (25)	5.52	0.62
Somatic coliphages, Log_{10} (PFU L^{-1})	T	24 (24)	5.08	0.39
	L	24 (24)	4.28	0.61
	G	5 (5)	4.92	0.41
NoV GGI, Log_{10} (g.e. L^{-1}) [b]	V	9 (9)	6.26	1.08
	T	22 (27)	5.64	1.42
	L	11 (27)	4.66	1.33
	G	7 (7)	5.32	0.61
NoV GGII, Log_{10} (g.e. L^{-1}) [b]	V	9 (9)	7.24	0.84
	T	24 (27)	6.31	1.37
	L	17 (27)	5.05	1.24
	G	7 (7)	5.87	0.52

[a] number of samples above the LOD (total number of samples)

[b] For wastewater, the limit of quantification (LOQ) for NoV was 1.5×10^4 g.e. L^{-1}, and the limit of detection (LOD) was extrapolated to 4.5×10^3 g.e. L^{-1}. When the NoV genome concentration was above the LOD, but below the LOQ, the geometric mean (8.2×10^3 g.e. L^{-1}) of the LOD and LOQ was used for calculations. When the NoV genome concentration was below the LOD, the value for LOD was used for calculations.

Simulated concentrations of norovirus and faecal indicators in source water

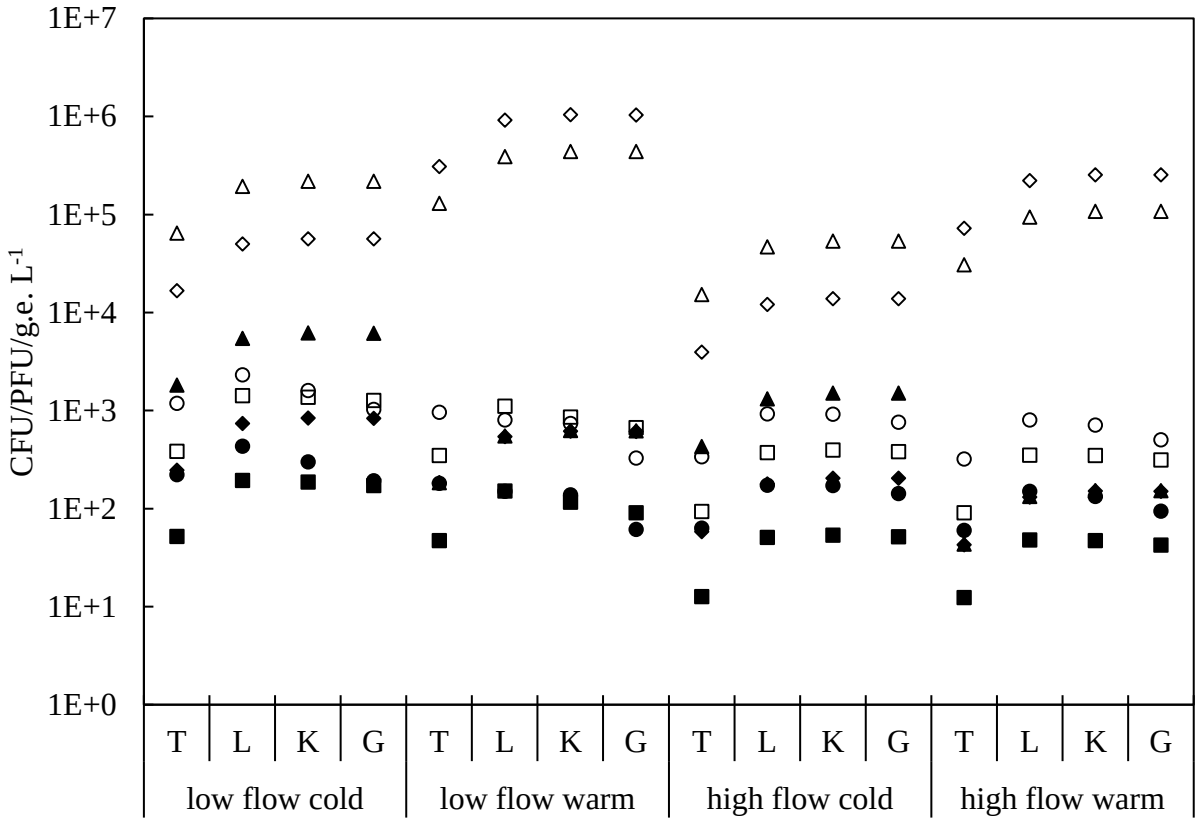

Figure S2 Modelling results: Concentrations of *E. coli* (circles), somatic coliphages (squares), norovirus GGI (diamonds) and GGII (triangles) at the water intakes in Trollhättan (T), Lilla Edet (L), Kungälv (K), and Gothenburg (G). Filled symbols represent the baseline scenario, and hollow symbols represent the worst case scenario.

Table S6 Sensitivity analysis: measured and simulated concentrations (Log_{10} transformed) of norovirus (NoV) genome in source water at the drinking water treatment plants in Trollhättan (T), Lilla Edet (L), Kungälv (K), and Gothenburg (G).

Place	Measured [a]		Simulated – no decay		Simulated – decay [b]	
	N [c]	Minimum – Maximum [b]	Minimum – Maximum Baseline	Minimum – Maximum Worst case	Minimum – Maximum Baseline	Minimum – Maximum Worst case
NoV GGI, Log_{10} (g.e. L^{-1})						
T	1 (14)	<1.04 – 2.69	1.63 – 2.39	3.59 – 5.49	1.61 – 2.37	3.59 – 5.43
L	2 (14)	<1.04 – 3.75	2.12 – 2.87	4.08 – 5.96	2.07 – 2.82	4.07 – 5.80
K	3 (7)	<1.04 – 3.78	2.18 – 2.92	4.14 – 6.02	2.10 – 2.83	4.12 – 5.72
G	0 (14)	<1.04	2.18 – 2.92	4.14 – 6.01	2.05 – 2.79	4.10 – 5.62
NoV GGII, Log_{10} (g.e. L^{-1})						
T	4 (14)	<1.04 – 3.79	1.64 – 3.26	4.18 – 5.11	1.62 – 3.24	4.18 – 5.05
L	4 (14)	<1.04 – 3.77	2.12 – 3.74	4.67 – 5.59	2.08 – 3.68	4.65 – 5.42
K	1 (7)	<1.04 – 3.72	2.18 – 3.79	4.73 – 5.64	2.10 – 3.70	4.70 – 5.35
G	5 (14)	<1.04 – 3.72	2.18 – 3.79	4.73 – 5.64	2.06 – 3.66	4.69 – 5.24

[a] When the concentration was below the LOD, the value for LOD was used for calculations. For NoV in source water, the LOD was extrapolated to 1.1×10^{1} g.e. L^{-1}, i.e. 1.04 Log_{10} units.

[b] In these simulations, the decay of NoV was described in the same way as for somatic coliphages.

[c] number of samples above the LOD (total number of samples)

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