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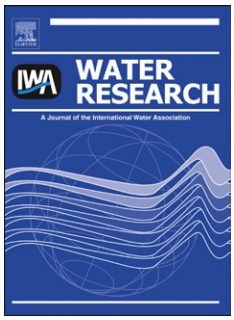

High occurrence of Hepatitis E virus in samples from wastewater treatment plants in Switzerland and comparison with other enteric viruses

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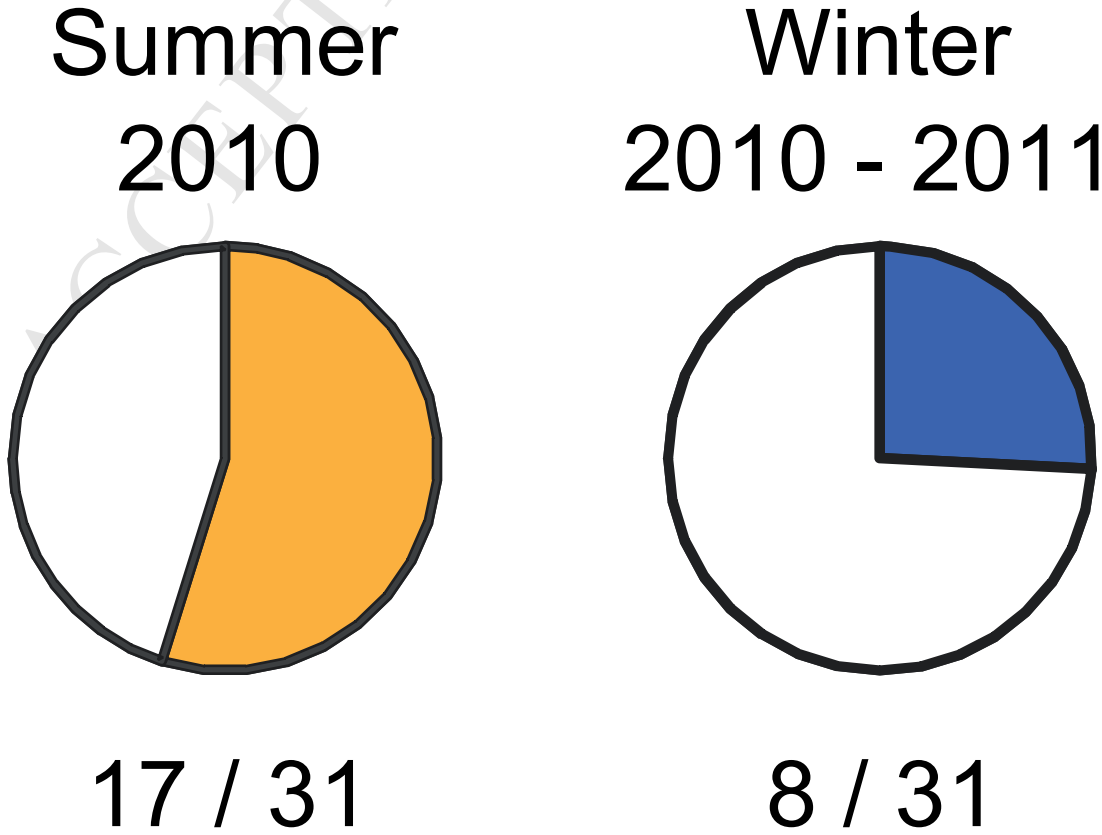
Seasonal occurrence of Hepatitis E vir



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Highlights

► Hepatitis E virus (HEV) was searched in raw and treated wastewater in Switzerland.

► HEV was found frequently in influent wastewater, but was undetectable in effluent.

► HEV occurrences in wastewater are more frequent in summer (seasonal pattern).

► HEV found in wastewater does not seem to be produced by swine.

► HEV frequencies and concentration are lower than those of adenovirus and norovirus.

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High occurrence of Hepatitis E virus in samples from wastewater treatment plants in Switzerland and comparison with other enteric viruses.

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Abstract

Hepatitis E virus (HEV) is responsible for many enterically transmitted viral hepatitides around the world. It is currently one of the waterborne diseases of global concern. In industrialized countries, HEV appears to be more common than previously thought, even if it is rarely virulent. In Switzerland, seroprevalence studies revealed that HEV is endemic, but no information was available on its environmental spread. The aim of this study was to investigate –using qPCR– the occurrence and concentration of HEV and three other viruses (norovirus genogroup II, human adenovirus-40 and porcine adenovirus) in influents and effluents of 31 wastewater treatment plants (WWTPs) in Switzerland. Low concentrations of HEV were detected in 40 out of 124 WWTP influent samples, showing that HEV is commonly present in this region. The frequency of HEV occurrence was higher in summer than in winter. No HEV was detected in WWTP effluent samples, which indicates a low risk of environmental contamination. HEV occurrence and concentrations were lower than those of norovirus and adenovirus. The autochthonous HEV genotype 3 was found in all positive samples, but a strain of the non-endemic and highly pathogenic HEV genotype I was isolated in one sample, highlighting the possibility of environmental circulation of this genotype. A porcine fecal marker (porcine adenovirus) was not detected in HEV positive samples, indicating that swine are not the direct source of HEV present in wastewater. Further investigations will be necessary to determine the reservoirs and the routes of dissemination of HEV.

Keywords

Hepatitis E, HEV, norovirus, adenovirus, wastewater, qPCR

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1. Introduction

Many waterborne diseases, like gastroenteritis or hepatitis, are caused by viruses and are a major threat to public health (Bosch et al., 2008). Human viruses such as adenovirus type 40 (HAdV-40) and noroviruses (NoV) genogroup I (GGI) and genogroup II (GGII) are commonly found in wastewater due to fecal excretion. Wastewater is treated physically, chemically, and biologically in wastewater treatment plants (WWTPs) in order to eliminate or reduce contaminants before the release of environmentally safe water. Fecal pollution of environmental water is a major health concern since environmental waters are used for drinking water supply and food production. Moreover, released viruses might reach diverse food items such as vegetables, fruits and raw shellfish (Bosch et al., 2008). Some viruses, like HAdV-40 and NoV, are good fecal indicators for evaluating the microbiological quality of environmental water, since they are excreted in high concentrations and are persistent in environmental water (Roslev and Bukh, 2011). Furthermore, viruses can be used to track the sources of fecal contamination (Roslev and Bukh, 2011). It is possible to distinguish between human and animal sources of pollution, since many human and animal viruses have a very narrow host spectrum. For example, HAdV-40, bovine adenovirus (BAdV) and porcine adenovirus (PAdV) are good indicators for determining the source of fecal contamination (Hundesa et al., 2006).

Hepatitis E is a waterborne disease responsible for over 50% of acute viral hepatitis cases in endemic countries (Dalton et al., 2008; Meng, 2010). The disease is caused by the hepatitis E virus (HEV), which is a non-enveloped positive-strand RNA virus (Dalton et al., 2008; Meng, 2010). HEV infection in humans can be caused by 4 genotypes (GI, GII, GIII and GIV) resulting in a single serotype (Dalton et al., 2008; Meng, 2010). Epidemics occur in countries with poor sanitation systems (Asia, Africa, Middle East and Mexico) and are due to GI and GII (Dalton et al., 2008; Meng, 2010). GI is a hyper-virulent genotype, responsible for most of the large outbreaks(Dalton et al., 2008; Meng, 2010; Bose et al., 2011). Furthermore, GI strongly affects pregnant woman by

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causing fulminant hepatic failure, which can lead to the death of both mother and child (Bose et al., 2011). For a long time, HEV was considered non-endemic in industrialized countries as only sporadic travel-associated cases were reported (Purcell and Emerson, 2008). However, the increasing number of autochthonous cases and the high seroprevalence reported in certain countries indicated that HEV is actually endemic to these countries (Purcell and Emerson, 2008). These autochthonous cases are due to GIII in most industrialized countries and to GIV in Eastern Asia (Purcell and Emerson, 2008; Lewis et al., 2010; Colson et al., 2012). Whereas GI and GII are restricted to humans, GIII and GIV have a wider host range within mammals and their main reservoir is suspected to be pigs and wild boar (Lewis et al., 2010; Meng, 2010; Rose et al., 2011; Wacheck et al., 2012). Hepatitis E has received ever more attention in recent years and is now considered an emerging problem. Its success in spreading may illustrate weaknesses in water management systems or food processes related to pork.

Studying the occurrence of enteric pathogens in influents at WWTP provides an efficient overview of the presence of these pathogens in the population. HEV has been detected in WWTPs in France (Clemente-Casares et al., 2003), Italy (La Rosa et al., 2010) and Spain (Clemente-Casares et al., 2009; Rodriguez-Manzano et al., 2010). The presence of the non-endemic GI in wastewater was recently reported in Spain and Italy (Clemente-Casares et al., 2009; La Rosa et al., 2010). HEV seroprevalence rates in populations from industrialized countries are usually relatively low (i.e. ranging from 1% to 5%) in comparison to those in developing countries, where rates from 15% to 60% have been reported (Dalton et al., 2008). Seroprevalence rates exceeded 20% in some regions within the USA (Thomas et al., 1997; Meng et al., 2002) and Japan (Li et al., 2000), showing that seroprevalence rates can reach locally unexpected higher values. However, comparison of seroprevalence between regions is problematic due to a lack of standardised serological tests (Bendall et al., 2010). In Switzerland, two blood donor studies reported HEV seroprevalence of 3.2% and 4.9% respectively (Lavanchy et al., 1994; Kaufmann et al., 2011).

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Furthermore, 26 cases of asymptomatic HEV seroconversion were recorded in a cohort of 667 workers including 332 WWTP workers in 5 years (Tschopp et al., 2009). Since these infections were asymptomatic, it was hypothesized that the workers were infected by the low pathogenic HEV GIII. However, neither the genotype involved in these seroconversions, nor the source of infection, could be determined accurately.

The present study investigated the occurrence and the concentration of HEV in the influents and effluents of 31 WWTPs located in the same area as the above mentioned cohort study (Jeggli et al., 2004; Tschopp et al., 2009). The objectives were to assess the environmental circulation of HEV in Switzerland and to determine whether HEV GI is present in wastewater. As points of comparison, the occurrence and concentration of two human viruses, HAdV-40 and NoV-GGII, were assessed. PAdV, a porcine fecal marker, was searched in order to evaluate whether any detected HEV might be of porcine origin.

2. Materials and methods

2.1. Sampling site selection

Thirty-one municipal WWTPs were selected within the Canton of Zurich in Switzerland (about 1.39 million inhabitants; 1,729 km^2). All WWTPs comprise a cleaning and an activated sludge step ("Zurich WWTP website," 2013). The selection was made using the following criteria. First, WWTPs where a seroconversion in workers had been ascertained in the recent cohort study on hepatitis E incidence (Tschopp et al., 2009) were included. Second, the WWTP servicing Zurich's international airport was included because international travelling increases the probability of the occurrence of genotypes GI and GII. Third, WWTPs where occupational hygiene measurements had been taken in a previous study (Oppliger et al., 2005; Daneshzadeh Tabrizi et al., 2010) were included. Finally, further WWTPs were selected to represent a well-balanced sample of the whole canton. The final sample included 6 very large (> 50,000 inhabitants and inhabitant-equivalents),

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12 large (10,000–50,000 inhabitants and inhabitant-equivalents) and 13 small WWTPS (2,000–10,000 inhabitants and inhabitant-equivalents). Very small WWTPs (< 2,000 inhabitants and inhabitant-equivalents) were not included, but there was always a larger WWTP in the same area. A total of 247 pig farms housing about 43,000 pigs were recorded in the Canton of Zurich ("Federal Office of Statistics," 2013). These WWTPs treat only household sewage and farmers are not allowed to use these sewer systems to eliminate animal sewage. The processes used to eliminate animal sewage are diverse (production of biogas, spreading on fields as a fertilizer...).

2.2. Sample collection

Both in 2010 and 2011, we collected one summer sample (defined as June to August) and one winter sample (defined as November to January) from each WWTP. Each seasonal collection campaign lasted four weeks. At each WWTP, 24-hour composite samples of both influent and effluent were collected in parallel using sterile plastic bottles. The 248 samples collected were stored at 4°C for up to 12 h, then frozen at -20°C and stored at -80°C for no more than 40 days. Before concentration, samples were allowed to slowly liquefy at 4°C.

2.3. Generation of standard curve, calculation of virus concentration, and controls

Standards were prepared from plasmids (pGEM-T cloning vector, Promega, Switzerland) containing corresponding PCR products. DNA was quantified by spectrophotometry using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Switzerland), and 10-fold serial dilutions, ranging from 10^6 to 1 genome equivalent (GE) copies/µL, were prepared for each plasmid. The sets of serial dilutions were used to confirm the specificity and the efficiency of the assays, to generate the standard curves, and to establish the limits of quantification (LOQ) and the limits of detection (LOD). Based on these standard curves and dilution calculations, all qPCR assays were converted from GE copies/reaction to GE copies/L.

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Three duplex qPCR assays were developed to allow simultaneous detection of viruses: NoV-GGII/RYMV and HEV/RYMV for RNA viruses, and HAdV-40/PAdV for DNA viruses. The reaction efficiencies were measured on serial 10-fold dilution mixtures of 2 virus amplicons cloned in pGEM-T as described for the monoplex assays. Cross-reactivity between the assays in duplex was evaluated by comparing the amplification of the target in single-plasmid solution and in multiple plasmid solution.

2.4. Virus control

To ensure that every sample had been treated appropriately to allow detection of target viruses, we used the Rice Yellow Mottle Virus (RYMV) isolate CI116 as an internal positive control. RYMV is a plant pathogen present mainly in Africa and Asia, but absent from Europe (Kouassi et al., 2005). This virus is very resistant in the environment and is structurally similar to HEV (no envelope, one single-strand RNA with positive polarity). Preliminary experiments showed that seeded RYMV is efficiently recovered from wastewater (data not shown). Virus stock solutions were kindly provided by Jean-Paul Brizard (IRD Montpellier) and were quantified by qPCR. An amount of 2×10^6 GE copies of RYMV was used to spike each sample. The quality of each sample was assessed by the efficient amplification of RYMV. The sample validation threshold was 4×10^5 GE copies of RYMV. Samples with an amplification of spiked RYMV under the threshold were reanalyzed or not considered.

2.5. Virus concentration from water samples

Viruses were concentrated from water samples either by a membrane filtration procedure adapted to HEV (method used in first year) or using a direct polyethylene glycol precipitation (method used in second year). The membrane filtration procedure was based on the Viradel method (Eaton and Franson, 2005). Briefly, 500 mL of cold water samples under agitation were supplemented with 50 mM $MgCl_2$ and adjusted to a pH of 3.5 with HCl. Water was filtered

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through a glass fiber pre-filter (AP20, Millipore, Switzerland) and an electronegative nitrocellulose membrane (HA, Millipore, Switzerland) at 50 mL/min. Filters were washed with cold 0.05 M glycine, 1.5% beef extract, pH=9.5. Filter surfaces were scratched and a bath sonication treatment was applied for 5 min. Eluates were neutralized with diluted HCl and centrifuged at 2500g for 5 min at 4°C. The supernatant was spiked with RYMV and precipitated with PEG as described below. The pellet was resuspended in 460 µL of PBS. Nucleic acids were directly extracted from 140 µL of this suspension.

For the direct precipitation method, influent and effluent water samples were concentrated using polyethylene glycol as described previously (Lewis and Metcalf, 1988) with the following modifications. Briefly, 90 mL water samples were spiked with RYMV and clarified by centrifugation in a swing-bucket rotor at 2500g for 5 min at 4°C. The liquid was carefully recovered without disturbing the pellet and 30 mL of a stock solution of 32% PEG_{8000} and 1.2 M NaCl were added to the recovered liquid. PEG precipitation was achieved by a short, vigorous shaking followed by incubation for 16 h in ice. The solutions were then centrifuged at 10000g for 30 min at 4°C in a fixed-angle rotor. The pellet was drained from most of the supernatant and directly treated with 560 µL of lysis buffer (AVL buffer, Qiagen, Switzerland) to start nucleic acid extraction.

2.6. Evaluation of the efficiency of the virus concentration methods.

The recovery efficiency of the filtration method was evaluated by spiking raw wastewater samples (n=3) with HEV (5×10^5 GE copies). Spiked samples were concentrated by filtration and quantification was performed by qPCR after reverse transcription (RT). Using this approach, the LOQ was established at 5.02×10^4 GE copies/L. The recovery efficiency of the PEG precipitation method was determined by spiking water samples (n=5) with known quantities of HEV (5×10^5 GE

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199 copies) and RYMV (2×10^6 GE copies). Spiked samples were precipitated with PEG and nucleic
200 acids were extracted from the pellets. HEV and RYMV were quantified by qPCR after RT.
201

202 2.7. Extraction of viral nucleic acid

203 RNA and DNA were extracted together from concentrated samples with the QIAamp Viral RNA
204 mini kit (Qiagen, Switzerland) using the manufacturer's protocol. After elution, an additive
205 ethanol precipitation cleaning step was carried out on the samples, using Glycoblue (Ambion,
206 Switzerland) as a co-precipitant. The nucleic acids were finally suspended again in 60 µl of AVE
207 buffer and stored at -20°C until use.
208

209 2.8. Reverse transcription

210 Reverse transcription was carried out using the Superscript III first-strand synthesis system for RT-
211 PCR (Life Technologies, Switzerland) and a mixture of reverse primers priming towards the
212 particular RNA viruses to be detected (Table S1). The 20 µL reaction mix included 10 µL of RNA
213 solution and was prepared as per the manufacturer's protocol, using RNAsin (Promega,
214 Switzerland) as the RNase inhibitor. The reaction was incubated for 60 min at 50°C and heat-
215 inactivated at 70°C for 15 min. The cDNAs were finally diluted to 100 µL with TE 0.1X. No
216 difference of RT efficiency was detected when using a single reverse primer or a mixture of
217 reverse primers in the reaction mix.
218

219 2.9. qPCR assay

220 Each reaction was performed on 5 µL of nucleic acid solution with the qPCR core kit (No ROX, with
221 dUTP, Eurogentec, Switzerland) as per the manufacturer's protocol. All reactions were performed
222 in a RotorGene-3000 (Corbett Research/Qiagen, Switzerland) using the following profile: digestion
223 with uracil-*N*-glycosylase at 50°C for 2 min; initial denaturation at 95°C for 10 min; 45 cycles of 15
224 s denaturation at 95°C; and 30 s annealing and extension at 60°C. Each sample was analyzed in

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triplicate and the corresponding mean was reported. No template controls were included in each run. We followed good laboratory practices strictly and took all necessary standard precautions to prevent PCR contamination (separate working areas and specific material for extraction, preparation and amplification of samples). Quantitative data were obtained with RotorGene software version 6.1.93 and were subsequently analyzed using custom-designed Excel spreadsheets using the standard curve equation as a reference for the quantification. A normalized fluorescence signal (Cq value) was considered to be positive when it was above the threshold for Cq determination defined for the standard curve.

2.10. Nested PCR for detection of HEV GI or HEV GIII

Nested PCR was performed with a set of primers allowing specific amplification of HEV GI (La Rosa et al., 2010). The reverse internal primer was modified to take into account the variability of HEV GI in this region (Table S1). The first reaction was carried out on 5 µL of cDNA in a total volume of 50 µl containing *Pfu* PCR buffer 1× (Promega, Switzerland), 200 µM of each deoxynucleotide (dATP, dCTP, dGTP, dTTP), 0.4 µM of each external primer, and a combination of polymerases–1 U of *Taq* polymerase (Promega, Switzerland) and 0.2 U of *Pfu* polymerase (Promega, Switzerland) to achieve efficient amplification at low error rate. PCR amplification included: an initial denaturation step at 94°C for 1 min; followed by 35 cycles of denaturation at 94°C for 45 s; primer annealing at 50° C for 45 s; and an extension step at 72°C for 1 min; and then a final extension step at 72°C for 5 min. A second round of amplification was performed similarly to the first PCR, using the internal primers and 0.5 µL of the first PCR product. PCR products were identified by electrophoresis in 1.5% agarose gels and stained by ethidium bromide. Positive PCR samples were confirmed by direct sequencing. Strict precautions were taken to avoid cross-contamination, as described above. HEV GIII was detected with the same protocol using 3 GIII-specific primers to allow efficient detection (Table S1).

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3. Results

3.1. Validation of the qPCR assays

With the exception of the qPCR assay to amplify the internal positive control RYMV, the specificity and efficiency of the qPCR assays have been previously described (Table S1 and references therein). We evaluated the ability of the different qPCR assays to efficiently amplify their targets under our conditions. Reaction efficiencies and specificity were confirmed for all qPCR assays (Supplemental Table S2).

Three duplex assays were developed for the detection of viruses: NoV-GGII/RYMV, HEV/RYMV, and HAdV-40/PAdV. Duplex qPCRs showed equivalent reaction efficiencies to the corresponding monoplex qPCR (Supplemental Table S2). Furthermore, no cross-reactivity was observed for any duplex assay combination.

3.2. Evaluation of the membrane filtration and PEG precipitation methods for virus recovery from water samples

The first method, based on a membrane filtration of HEV-spiked samples, showed a mean recovery efficiency of 30% (n=3) and ranged from 12% to 45%. The second method was evaluated based on a direct PEG precipitation of clarified wastewater samples. The recovery efficiency for HEV had a mean of 39% (n= 5) and ranged from 25% to 53%. For RYMV, the recovery efficiency ranged from 58% to 71% with a mean recovery efficiency of 66% (n=5).

As the PEG precipitation method could lead to the concentration of enzymatic inhibitors, the effect of such compounds on PCR and RT efficiency was evaluated. Compared to the spiked distilled water sample, PCR efficiency was reduced to 71% and 81% in influent and effluent water samples respectively (Table S3). RT efficiency was reduced to 79% for influent water samples (Table S4).

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3.3. Occurrence and concentrations of HEV in influent wastewater

HEV was detected in 17 samples from summer 2010, 8 samples from winter 2010-2011, 14 samples from summer 2011 and 1 sample from winter 2011-2012 (Fig. 1). HEV occurrence in summer was significantly higher than in winter (Marascuillo procedure, $p < 0.05$, Fig. 1). The presence of the virus in wastewater was variable since not one WWTP was positive in all 4 successive samplings and the majority of WWTPs had a single occurrence. Only 7 of the WWTPs did not test positively for HEV at all over the two consecutive years. There is no difference of HEV occurrence between the size categories of the WWTP with 44.4% (8/18) of large WWTP (> 10'000 inhabitants) positive compared to 46.1% (6/13) of small WWTP (< 10'000 inhabitants), (Pearson Chi-square = 0.009, df = 1, p = 0.9). The overall HEV concentration in the study was low since values under the LOQ were reported for every sample but one. Therefore, the concentration of HEV in wastewater was only determined in this single sample from summer 2011. A concentration of 7.81×10^4 GE copies/L was found for this sample (Table 1).

3.4. Occurrence and concentrations of human and porcine fecal virus in influent wastewater

NoV-GGII was detected in 30 summer samples and 30 winter samples (Table 1). Quantification was possible for 21 summer samples and their concentrations ranged from 7.40×10^4 to 3.73×10^6 GE copies/L. In 22 winter samples, concentrations ranged from 1.22×10^4 to 9.99×10^5 GE copies/L. HAdV-40 was detected in 30 summer samples and 31 winter samples (Table 1). In 26 summer samples, HAdV-40 concentrations ranged from 1.88×10^4 to 6.67×10^6 GE copies/L. Twenty-four winter samples were quantifiable and showed concentrations ranging from 1.12×10^4 to 1.43×10^6 GE copies/L. The PAdV was not detected in summer, although 2 samples showed traces of the virus in winter.

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3.5. Detection of HEV genotype I in influent wastewater

To determine whether HEV GI is present in wastewater, a GI-specific semi-nested PCR was performed on the HEV positive samples identified by qPCR. Only one sample produced a positive 221-bp PCR band, which was isolated and sequenced. This sample corresponded to a very large WWTP (ARA27, Table 1). The new sequence was submitted to the Basic Local Alignment Search Tool (BLAST) web server (US National Centre for Biotechnology Information) to search for near identical sequences. The result revealed that the most closely related sequence (98% identity) was a HEV genotype I strain isolated from Nepal (Genbank HM641296, Fig. S1). Alignment with the corresponding 221-bp region of selected HEV strains of all genotypes showed that this new sequence belongs to GI group of HEV strains (phylogenetic tree, Fig. 2).

3.6. Occurrence of viruses in WWTP effluents wastewater

We searched for HEV in effluent samples from WWTPs which had HEV positive influent samples (14 summer samples and 1 winter sample). As a control, 10 randomly selected effluent samples for which influent samples were negative for HEV were also included in the analysis. None of these samples was positive for HEV. The NoV-GGII concentrations were evaluated in effluent samples from WWTPs for which quantifiable virus loads were found in influent samples (21 summer samples and 22 winter samples). For most samples, NoV-GGII concentrations from influent to effluent were reduced under the LOQ (1.86×10^4 GE copies/L) (Table 2). Only 2 summer and 3 winter effluent samples were above the LOQ (Table 2). However, traces of NoV-GGII were still detected in 7 summer and 11 winter effluent samples. The HAdV-40 concentrations were evaluated in effluent samples from WWTPs for which quantifiable HAdV-40 loads were found in influent samples (26 summer samples and 24 winter samples). All but 3 effluent samples were positive for HAdV 40 (Table 2). Among these, 13 summer and 5 winter samples displayed quantifiable HAdV-40 levels. (Fig. 3).

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4. Discussion

These results clearly demonstrate the presence of HEV in the Canton of Zurich, as previously hypothesized by studies on HEV seroprevalence (Jeggli et al., 2004; Tschopp et al., 2009). We showed a 32% (40/124) HEV occurrence in WWTP influent samples, with a significantly higher occurrence in summer than in winter. This occurrence is similar to that observed in Spain (Rodriguez-Manzano et al., 2010) and higher than that reported from WWTPs in Italy (La Rosa et al., 2010). However, HEV quantification was only possible for one sample since virus concentrations were too low in all the others. The calculated concentration was in the same range as those found in Spain: 1×10^4 GE copies/L to 1×10^5 GE copies/L (Rodriguez-Manzano et al., 2010).

Untreated wastewater contains many infectious agents and the safety of WWTP workers has been of interest for many years. The study by Tschopp et al. (2009) showed that there was no difference in the rates of HEV seroconversion between workers exposed to wastewater and unexposed workers. Our results confirm that concentrations of HEV circulating in wastewater are quite low compared to concentrations of HAdV-40 and NoV-GII – viruses which were found in nearly all samples. In consequence, under the exposure conditions found in this study WWTP workers' risk of exposure to HEV GIII is likely to be limited and comparable to the risk in the general population. This conclusion is in line with the results of the cohort study carried out in the same region (Jeggli et al., 2004; Tschopp et al., 2009). However, the risk of HEV infection for individuals is difficult to assess since the infectious dose and the routes of transmission are not clearly defined.

The concentration of infectious HEV particles in raw wastewater is probably lower than the concentration of particles detected by qPCR since particles may be damaged by wastewater plant treatments. It is difficult to assess the viability of HEV particles since the virus is refractory to *in*

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352 *vitro* culture methods. However, infectious viral particles can survive wastewater treatment as
353 demonstrated by HAdV and other viruses (Calgua et al., 2011; Simmons and Xagoraraki, 2011). In
354 addition, some particles might not be recovered or might be damaged by the concentration
355 process. New methods with high recovery efficiency, low LOQ and preservation of the particles
356 still need to be developed (Connell et al., 2012). During our study's first year we used the
357 membrane filtration method to concentrate viruses from wastewater samples. However, some
358 influent wastewater samples were significantly turbid or contained particles that clogged the
359 double filter, requiring the continuous intervention of the experimenter. We tested the direct PEG
360 precipitation method to avoid clogging problems and to allow time-efficient processing of the
361 samples. This method, described previously (Lewis and Metcalf, 1988), has been used to
362 efficiently recover viruses from water samples (Aw and Gin, 2010; Tong et al., 2011). Moreover,
363 many virus species can be concentrated at the same using this method. Compared to the
364 membrane filtration method, the direct PEG precipitation method is more adapted to raw
365 wastewater samples, which have high turbidity and variable composition. Our comparison of the
366 2 methods showed that both methods have similar HEV recovery efficiencies. Since the PEG
367 precipitation method was highly more practical than the membrane filtration method, with no
368 interference on the results, we used it during our second year of study.
369
370 The WWTPs included in our study all used activated sludge treatment, but they varied in size,
371 structural organization and location. Our objective was not to determine the virus removal
372 capabilities of WWTPs, but rather to evaluate the possibility of virus release from those WWTPs to
373 environmental water. HEV was not detected in any effluent samples, which is in agreement with
374 the low concentrations detected in influent samples. However, we cannot completely rule out the
375 possibility of HEV release into environmental water since the detection method cannot detect
376 concentrations lower than 6.50×10^4 GE copies/L. Although wastewater treatment processes
377 efficiently reduced the concentrations of NoV-GGII in most samples, the presence of NoV-GGII

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was still detected in 9 summer and 14 winter effluent samples. Furthermore, 2 summer and 3 winter effluent samples showed a NoV-GGII concentration higher than the LOQ (1.86×10^4 GE copies/L). Other studies have reported the frequent release of NoV-GGII in WWTP effluent (Katayama et al., 2008; Hewitt et al., 2011; Simmons and Xagoraraki, 2011). Although concentrations of HAdV-40 were reduced in many of them, the virus persisted in effluent samples. This result is explained by the highly resistant properties of this virus (Thurston-Enriquez et al., 2003).

In this study, we observed that the occurrence of HEV in wastewater is significantly higher in summer than in winter. This seasonal difference could depend on many factors, such as particle stability, environmental conditions or outbreaks. The possible influence of incoming water flow is unlikely, since there is no remarkable difference in flow between the seasons (Head of Zurich WWTPs, pers. comm.). It is noteworthy that medical studies of HEV infection have never revealed a seasonal pattern. Since HEV GIII usually causes an asymptomatic infection, it is possible that most cases of HEV remain not diagnosed. We also found that NoV-GGII and HAdV-40 were present in almost all influent water samples at high, stable concentrations (Table 1), as little variation was observed between the 2 seasons. HAdV-40 is known to be widespread in the European population, where it can cause outbreaks of gastroenteritis, mostly in children during winter. After infection, HAdV-40 excretion by the host can last from months to years (Jiang, 2006) and the consequent lack of a seasonal pattern for this virus in wastewater has been confirmed by several studies (Jiang, 2006; Katayama et al., 2008). NoV-GGII is also common in the European population and is frequently responsible for winter gastroenteritis outbreaks (Glass et al., 2009). Our study in Switzerland clearly shows that NoV-GGII is present in wastewater in both winter and summer, without any noteworthy variation. Other studies have found that NoV-GGII is present in wastewater year-round, with higher concentrations in winter and lower concentrations in summer (Katayama et al., 2008; Nordgren et al., 2009). Since NoV-GII outbreaks mainly occur in

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404 the cold season and the typical shedding time is up to 8 weeks, further investigations are required
405 to understand the dynamics of NoV-GGII persistence in population.
406
407 In industrialized countries, most cases of HEV infection are due to the autochthonous zoonotic
408 GIII and GIV variants whose reservoir might be swine (Lewis et al., 2010; Meng, 2010; Rose et al.,
409 2011; Wacheck et al., 2012). In theory, swine manure is kept completely separate from
410 wastewater, but hypothetical dysfunctions or accidental contaminations cannot be absolutely
411 eliminated. Absence of the porcine fecal marker (i.e. PAdV) in our HEV-positive wastewater
412 samples indicates that HEV was unlikely excreted by swine.
413
414 Medical cases of HEV GI are not frequent in Europe since this genotype is non-endemic to the
415 region. However, the present study did detect GI in one sample, showing that its occurrence in
416 wastewater, although very rare, is still possible. Interestingly, studies in non-endemic Italy and
417 Spain, also showed the presence of GI in wastewater (Clemente-Casares et al., 2009; La Rosa et
418 al., 2010). Overall, these results show that GI can be detected in wastewater produced in
419 industrialized countries. It is assumed that GI released in wastewater is due to people who have
420 recently travelled to a GI-endemic country. This assumption is confirmed by the alignment of our
421 detected GI sequence–with a HEV strain originating from Nepal (Fig. 2 and S1). However, it is not
422 known if HEV GI present in wastewater can spread into the environment and infect a new host.
423 Follow-up studies are necessary, especially in the light of unknown reservoirs for HEV in
424 industrialized countries.
425
426

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5. Conclusions

- HEV is present frequently but at low concentrations in raw wastewater in the Canton of Zurich in Switzerland, indicating that HEV is common in the population of the area studied.
- There was no evidence of HEV release from WWTPs into environmental water.
- HEV frequency depends on the season, with higher frequencies of HEV detection in summer. The seasonal character of HEV occurrence has not been previously described and requires further investigation to understand its causes.

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436 **Author contributions**

437 FGM conceived the experiments and analyzed the data. DG organized and carried out the
438 sampling. FGM and DSB performed the research. FGM, AO and PH designed the project and wrote
439 the manuscript.

440

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446 cloned DNA, and Stephanie Héritier and Dennis Thonney (WWTP Lausanne) for their help in this
447 project. We also very much thank the workers and the head of the Canton of Zurich's sewage
448 plants for their constant support.

449

450 **Appendix. Supplementary material**

451 Table S1 – List of primers used in this study

452 Table S2 – qPCR assay characteristics

453 Table S3 – Detection of PCR inhibitors by measurement of the amplification efficiency of spiked
454 DNA in WWTP water samples and in distilled water samples

455 Table S4 – Detection of PCR inhibitors by measuring the amplification efficiency of a spiked DNA in
456 WWTP samples and in distilled water samples

457 Figure S1 – Alignment of the sequence of WWTP influent sample GI with the closest match in
458 NCBI

459

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578 **Figure legends**

579 Fig. 1 – Results of HEV detection in influent samples. Selected WWTPs were randomly labeled
580 with an identifier ranging from ARA01 to ARA31. A total of 31 influent samples were analyzed for
581 each season. Different letters indicate significant differences ($p < 0.05$) between seasonal
582 proportions according to multiple comparisons of proportions (Marascuillo procedure).

583

584 Fig. 2 – Phylogenetic tree of HEV strains. Analysis was based on a 221-bp region of HEV genome.
585 The new sequence GI from a positive WWTP influent (named "WWTP influent sample") and a
586 subset of HEV sequences of all genotypes were included in this analysis. Sequences were aligned
587 using MAFFT (Katoh and Standley, 2013) and the corresponding unrooted phylogenetic tree was
588 generated with Jalview (Waterhouse et al., 2009). Known sequences are labeled with Genbank
589 accession, country of origin, genotype, and start and final positions used for the alignment. The
590 location of the new sequence GI in the phylogenetic tree is indicated with an arrow.

591

592 Fig. 3 – Concentration of HAdV-40 in influent and effluent water samples. Results are expressed as
593 $\log_{10}$ virus GE per liter. Box plots are generated with influent and effluent concentrations from
594 WWTPs where both influent and effluent samples were quantifiable (WWTPs in summer n = 13,
595 WWTPs in winter n = 5).

596

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Table 5 – Detection and concentration of viruses in influent samples.

Sampling sites	HEV		NoV-GGII		HAdV-40		PAdV	
	summer	*winter*	*summer*	*winter*	*summer*	*winter*	*summer*	*winter*
ARA01			1.96×10^5	+	2.67×10^4	5.62×10^5		
ARA02	+		3.79×10^5	8.78×10^4	7.50×10^5	1.17×10^6		
ARA03	+		3.86×10^5	3.36×10^5	5.18×10^5	1.43×10^6		
ARA04			4.10×10^5	9.99×10^5	1.46×10^5	5.40×10^5		
ARA05	+		1.73×10^5	1.16×10^5	1.88×10^4	3.83×10^4		
ARA06	+		6.62×10^5	9.99×10^4	+	+		
ARA07	+		+	5.13×10^4	1.78×10^6	+		
ARA08			+	4.09×10^5	1.00×10^5	4.79×10^5		
ARA09			1.35×10^5	1.97×10^5	3.06×10^4	+		
ARA10	+		+	+	1.67×10^5	1.96×10^4		+
ARA11	+		6.50×10^5	1.28×10^5	1.39×10^5	4.78×10^5		
ARA12		+	+	+	4.21×10^4	2.19×10^4		
ARA13	+		7.40×10^4	5.83×10^4	+	3.66×10^5		
ARA14	+		5.60×10^5	2.82×10^5	5.33×10^5	4.07×10^5		
ARA15				1.24×10^4	3.63×10^5	2.52×10^5		
ARA16	+		1.77×10^5	7.83×10^4	+	7.65×10^5		
ARA17	+		1.53×10^5	+	4.45×10^5	1.96×10^4		
ARA18			+	2.63×10^4	7.55×10^4	1.95×10^5		
ARA19			8.75×10^4		2.06×10^4	1.56×10^4		
ARA20	+		+	+	1.45×10^5	+		
ARA21			1.83×10^5	2.91×10^4	6.67×10^6	+		
ARA22			3.06×10^5	3.63×10^4	8.56×10^5	1.48×10^5		
ARA23			3.73×10^6	+		+		
ARA24			1.16×10^5	5.88×10^4	3.07×10^4	1.15×10^5		
ARA25			+	1.22×10^4	+	6.15×10^5		+
ARA26	+		3.26×10^5	1.23×10^5	1.78×10^6	1.23×10^5		
ARA27	7.81×10^4		5.20×10^5	2.39×10^4	8.04×10^5	4.27×10^4		
ARA28			8.46×10^5	+	7.35×10^5	2.77×10^4		
ARA29			+	+	5.07×10^4	3.11×10^4		
ARA30			1.47×10^5	1.90×10^4	2.11×10^5	1.12×10^4		
ARA31			+	7.42×10^4	1.24×10^6	+		
mean	–	–	4.86×10^5	1.48×10^5	6.80×10^5	3.28×10^5	–	–
LOQ	6.50×10^4		1.86×10^4		9.27×10^3		5.67×10^3	

+: positive qPCR signal under the limit of quantification (LOQ). Blanc: no detection. Values are expressed in GE.L-1

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Table 6: Efficiency of the WWTP processes on virus concentration reduction.

		NoV-GGII		HAdV-40	
		summer	winter	summer	winter
Number of WWTPs with a quantifiable virus charge (influent)[1]		21	22	26	24
Detection results in the corresponding effluent samples	No detection	12	8	0	3
	Detection below LOQ[2]	7	11	13	16
	Detection above LOQ	2	3	13	5

[1]Only WWTPs with an influent concentration of virus higher than LOQ are considered.

[2]LOQ = limit of quantification

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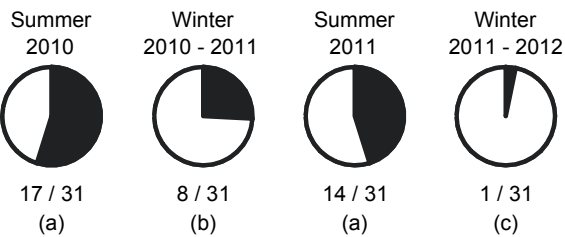

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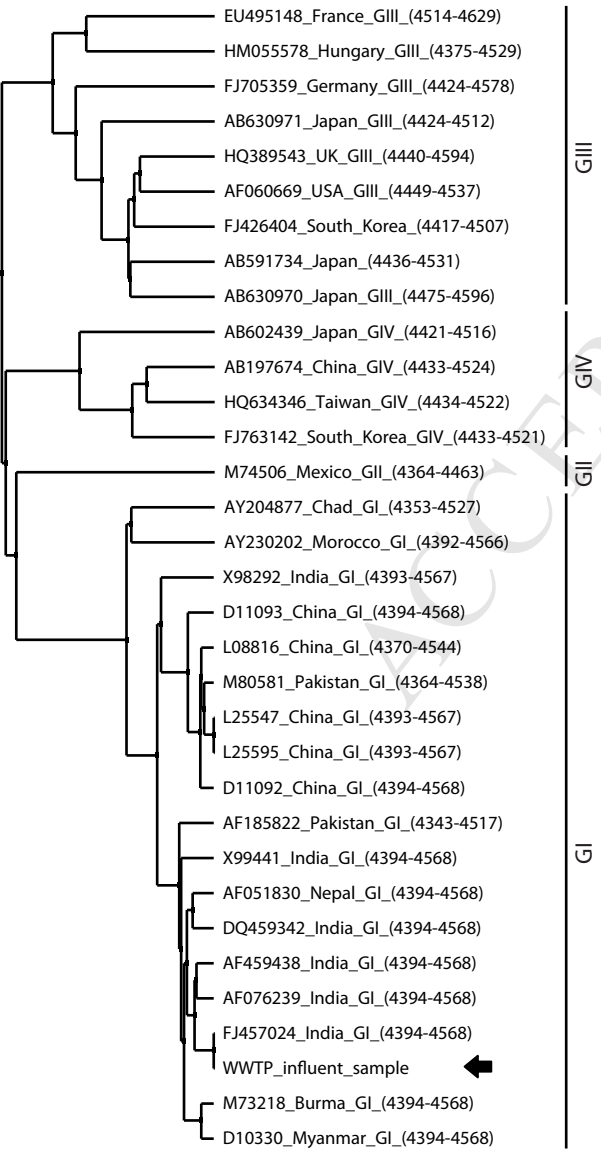

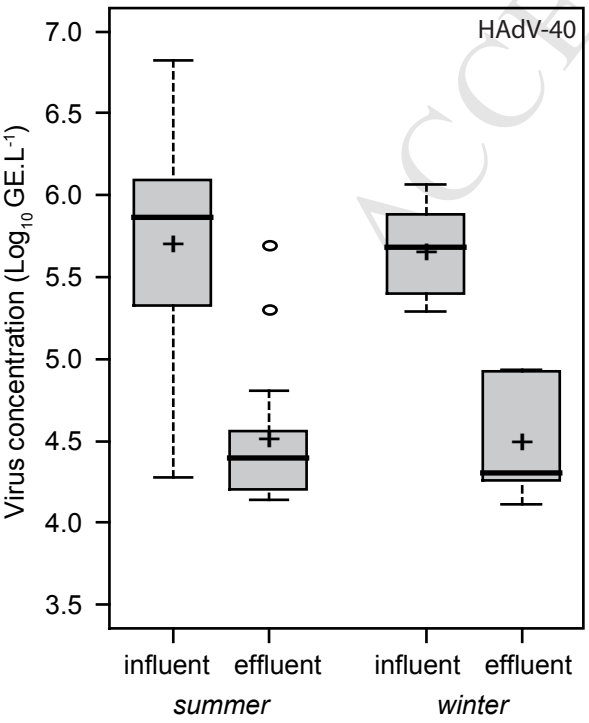

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Table S1 – List of primers used in this study

Virus	Primer or probe name	Other name	Sequence (5'-3')	Orientation	Position[1]	Amplicon size	Reference
NoV-GGII	Noro-fwd	JJV2F	CAAGAGTCAATGTTTAGGTGGATGAG	+	5003–5028	98 bp	Jothikumar et al 2005
(RNA virus)	Noro-rev	COG2Ra	TCGACGCCATCTTCATTCACA	-	5100–5080		
	Noro-prob	RING2-TPa	(FAM-)TGGGAGGGCGATCGCAATCT(-TAMRA)	+	5048–5067		
HEV	HEV-fwd	JVHEVF	GGTGGTTTCTGGGGTGAC	+	5285–5301	70 bp	Jothikumar et al 2006
(RNA virus)	HEV-rev	JVHEVR	AGGGGTTGGTTGGATGAA	-	5354–5337		
	HEV-prob	JVHEVP	(FAM-)TGATTCTCAGCCCTTCGC(-TAMRA)	+	5308–5325		
RYMV	RYMV-F2		CTTCAACGGGCTCCAGTG	+	3540–3557	80 bp	this study
(RNA virus)	RYMV-R2		AGCGGCCAGGTGTTAGAAG	-	3619–3601		
	RYMV-prob2		(YYE-)GGATATCTGGGACGGTTCCT(-BHQ1)	+	3571–3590		
HAdV-40	HAdV-F	JTVXF	GGACGCCTCGGAGTACCTGAG	+	18895–18915	96 bp	Jothikumar et al 2005
(DNA virus)	HAdV-R	JTVXR	ACIGTGGGGTTTCTGAACTTGTT	-	18990–18968		
	HAdV-prob	JTVXP	(FAM-)CTGGTGCAGTTCGCCCGTGCCA(-BHQ1)	+	18923–18944		
PAdV	PAdV3-F	VTB1-PoAdV3f	CCTCAACAACCTCATTGATACC	+	20574–20595	144 bp	Wolf et al 2010
(DNA virus)	PAdV3-R	VTB1-PoAdV3r	CTTGCAGTAGCGGCCGT	-	20718–20702		
	PAdV3-prob	VTB1-PoAdV3probe	(YYE-)TACGGCCTGCGCTACCGCTCCCA(-BHQ1)	+	20668–20690		
HEV (GI)	nestedG1ext-fwd	1661	TTAYGGKGATGCCTTTGATGACACC	+	4329–4353	302 bp	Adapted from
(RNA virus)	nestedG1ext-rev	1662	TRATAACGGCCATRTTCCAGACAGTATTCC	-	4630–4601		La Rosa et al 2010
	nestedG1int-fwd	1663	TGTTTGAGAATGACTTTTCTGAGTTTGAYT	+	4394–4423	175 bp	
	nestedG1int-rev	1664	TTCCAAAACCCTCGCAGYGAC	-	4568–4548		
HEV (GIII)	nestedG3ext-fwd	1669	GGYGACGCYTATGAGGAGT	+	4360–4378	298 bp	Adapted from
(RNA virus)	nestedG3ext-rev	1670	GCTATRATYGCCATRTTCCA	-	4658–4639		La Rosa et al 2010
	nestedG3int-rev	1672	AGAGACTCCTTCGGSGCYTG	-	4580–4561	220 bp	

[1] Genbank accession numbers: NoV-GGII no. X86557; HEV no. AF082843 (sHEV); RYMV no. L20893; HAdV no. AC_000008 (HAdV5); PAdV no. AB026117 (PAdV3); HEV / GI no. M73218; HEV no. AF082843 (sHEV)

[2] in combination with nestedG3ext-fwd (semi-nested PCR)

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Table S2 – qPCR assay characteristics

	2nd target (duplex qPCR)	efficiency	formulae	Cq threshold for LOQ	LOQ in water (GE copies/L)	Cq threshold for LOD
HEV	RYMV	93%	Cq = -3.501 × log(conc) + 41.032	35.08	6.50×10^4	42.00
NoV-GGII	RYMV	97%	Cq = -3.385 × log(conc) + 39.609	36.25	1.86×10^4	42.00
HAdV-40	PAdV	102%	Cq =-3.272 × log(conc) + 39.748	34.00	9.27×10^3	42.00
PAdV	HAdV-40	97%	Cq = -3.394 × log(conc) + 40.180	34.00	5.67×10^3	42.00
RYMV	HEV	99%	Cq =-3.350 × log(conc) + 41.886	36.00	9.36×10^4	42.00
RYMV	NoV-GGII	96%	Cq = -3.411 × log(conc) + 42.168	36.00	8.70×10^4	42.00

These LOQ values apply only to samples treated by direct PEG precipitation.

Table S3 – Detection of PCR inhibitors by measuring the amplification efficiency of a spiked DNA in WWTP water samples and in distilled water samples

	Quantification cycle (±SD)	Calculated concentration ($GE.L^{-1}$)	Range ($GE.L^{-1}$)	% efficiency
Influent water extracted sample (n=10)	29.6 ± 0.3	1.88×10^3	$1.38 \times 10^3 – 2.55 \times 10^3$	81%
Effluent water extracted sample (n=10)	29.8 ± 0.5	1.65×10^3	$6.63 \times 10^2 – 2.22 \times 10^3$	71%
Distilled water sample (n=8)	29.3 ± 0.3	2.31×10^3	$1.94 \times 10^3 – 3.09 \times 10^3$	

The spiked DNA is a RYMV PCR fragment cloned in pGEM-T. Range is defined by the minimal and the maximal calculated concentrations.

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Table S4 – Detection of PCR inhibitors by measuring the amplification efficiency of a spiked DNA in WWTP samples and in distilled water samples

	Quantification cycle (±SD)	Calculated concentration ($GE.L^{-1}$)	Range ($GE.L^{-1}$)	% efficiency
Influent water extracted sample + spiked RNA mix (n=4)	26.0 ± 0.2	6.20×10^4	$5.93 \times 10^4 – 6.33 \times 10^4$	79%
Elution buffer + spiked RNA mix (n=4)	25.6 ± 0.1	7.87×10^4	$7.53 \times 10^4 – 8.16 \times 10^4$	

The spiked DNA is a RYMV PCR fragment cloned in pGEM-T. Range is defined by the minimal and the maximal calculated concentrations.

Figure S1 – Alignment of the sequence of WWTP influent sample G1 with the closest match in NCBI Blast

```
Hepatitis E virus isolate 5-05-gt1 polymerase (P) gene, partial cds
Sequence ID: gb|HM641296.1|     Length: 326     Number of Matches: 1

Range 1: 152 to 326
Score     Expect          Identities     Gaps          Strand
307 bits(166)             2e-80          172/175(98%)  0/175(0%)  Plus/Plus

Query  1    TGTTTGAGAATGACTTTTCTGAGTTTGATTCCACCCAGAATAATTTCTCTCTAGGCCTTG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  152  TGTTTGAGAATGACTTTTCTGAGTTTGATTCCACCCAGAATAATTTCTCTCTAGGCCTTG  211

Query  61   AGTGTGCTATTATGGAGGAGTGCGGGATGCCGCAGTGGCTCATCCGTTTGTATCACCTTA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  212  AGTGTGCTATTATGGAGGAGTGCGGGATGCCGCAGTGGCTCATCCGTTTGTATCACCTTA  271

Query  121  TAAGGTCTGCGTGGATCTTGCAGGCCCCGAAGGAGTCACTGCGAGGGTTTTGGAA  175
            |||||||||||||||||||||||||||||| ||||| ||||| |||||
Sbjct  272  TAAGGTCTGCGTGGATCTTGCAGGCCCCGAAGGAGTCTCTGCGGGGGTTCTGGAA  326
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