

Quantitative real-time PCR of enteric viruses in influent and effluent samples from wastewater treatment plants in Italy

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Summary. The prevalence of enteric viruses in wastewater, the efficacy of wastewater treatments in eliminating such viruses, and potential health risks from their release into the environment or by recycling of treated wastewaters, are very important issues in environmental microbiology. In this study we performed a quantitative TaqMan real-time PCR (polymerase chain reaction) analysis of enteric viruses on samples of influents and effluents from 5 wastewater treatment plants in and around Rome. Three epidemiologically important, waterborne enteric viruses were analyzed: adenoviruses, enteroviruses and noroviruses (GI and GII) and compared to classical bacterial indicators of fecal contamination. The concentration of adenoviruses was the highest, in both raw and treated waters. Mean values in influents were ranked as follows: adenovirus > norovirus GI > norovirus GII > enterovirus. In effluents, the ranking was: adenovirus > norovirus GI > enterovirus > norovirus GII. Removal efficiencies ranged from 35% (enterovirus) to 78% (norovirus GI), while removal efficiency for bacterial indicators was up to 99%. Since molecular quantification does not necessarily indicate an actual threat to human health, we proceeded to evaluate the infectivity of enterovirus particles in treated effluents through integrated cell culture and real-time PCR. Infectivity assays detected live virions in treated water, pointing to potential public health risks through the release of these viruses into the environment. A better understanding of viral presence and resistance to sewage purification processes have the potential of contributing to the effective management of risks linked to the recycling of treated wastewater, and its discharge into the environment.

Key words: norovirus, adenovirus, enterovirus, sewage, real-time quantitative PCR assays.

Riassunto (*Determinazione quantitativa mediante real-time PCR di virus enterici in campioni di acque reflue affluenti ed effluenti da impianti di trattamento dei liquami in Italia*). La prevalenza dei virus enterici nei liquami urbani, la loro rimozione durante i trattamenti di depurazione delle acque reflue, e rischi potenziali per la salute legati alla contaminazione degli ambienti idrici o al riutilizzo delle acque depurate, sono argomenti di grande interesse in microbiologia ambientale. In questo studio abbiamo stimato la concentrazione di virus enterici, in campioni di reflui grezzi e depurati di 5 impianti di trattamento di reflui urbani localizzati in Roma e dintorni, utilizzando metodologie quantitative molecolari (TaqMan real-time PCR). Sono stati analizzati tre gruppi di virus enterici responsabili di epidemie di origine idrica (adenovirus, enterovirus e norovirus GI/GII), parallelamente ai classici indicatori batterici di contaminazione fecale. Le concentrazioni virali più elevate sono state rilevate per gli adenovirus, sia nei reflui non depurati sia in quelli trattati. Nei reflui grezzi sono state osservate le seguenti concentrazioni medie: adenovirus > norovirus GI > norovirus GII > enterovirus; negli effluenti depurati: adenovirus > norovirus GI > enterovirus > norovirus GII. L'efficienza di rimozione virale variava dal 35% (enterovirus) al 78% (norovirus GI), mentre per gli indicatori batterici raggiungeva il 99%. Poiché i metodi molecolari non forniscono indicazioni circa l'infettività dei virus rilevati, si è proceduto a valutare la capacità di replicazione delle particelle virali (limitatamente agli enterovirus) nei liquami depurati utilizzando un sistema integrato colture cellulari/real-time PCR. L'analisi ha rilevato particelle virali infettive, indicando potenziali rischi per la salute pubblica legati alla contaminazione dei corpi idrici recettori. Una migliore comprensione della resistenza dei virus ai processi di depurazione è importante per la gestione dei rischi legati al riutilizzo delle acque reflue trattate, o alla loro immissione nell'ambiente.

Parole chiave: norovirus, adenovirus, enterovirus, liquami, PCR quantitativa real-time.

INTRODUCTION

Enteric viruses are largely excreted through fecal matter, and current methods of sewage treatment do not always effectively remove these organisms [1].

Most treated wastewater, as well as untreated sewage, flow into the water environment and have the potential to impact agricultural, recreational and drinking waters. Currently, bacterial indicators such as fecal

coliforms and enterococci are commonly used to assess water quality. These bacteria are simple and inexpensive to monitor, but have been shown to be less than ideal as indicators of fecal pollution. In fact, enteric human-pathogenic viruses are generally more resistant than enteric bacteria to current methods of wastewater treatment [2, 3]. Different studies having found that, despite treatment, enteric viruses persist in high levels in wastewaters [4-7], proposed the use of a viral indicator of wastewater contamination. Specifically, enteroviruses (EVs) and adenoviruses (AdVs) have been suggested as indicators for monitoring the human fecal contamination of water and for determining the efficacy of disinfection treatments [8]. The monitoring of wastewater treatment plants (WTP) may prove a suitable approach for the study of circulating viruses in their respective service areas [9], and the persistence of such viruses in treated effluents.

The objective of the present study was to assess, in both raw and treated sewages, the concentration of three epidemiologically important, waterborne enteric viruses, AdVs, EVs and noroviruses (NoV GI and NoV GII), to gain insight into the infectious potential of viral contamination of treated sewage, and to obtain a rough estimate the efficacy of disinfection treatments. For the sake of reference, we also tested and quantified two enteric bacteria commonly used as fecal indicators, *Escherichia coli* and enterococci.

EVs are single-stranded RNA, non-enveloped viruses. Symptoms of EV infection include mild upper respiratory illness, febrile rash, aseptic meningitis, pleurodynia, encephalitis, acute flaccid paralysis, paralytic poliomyelitis, gastroenteritis, myocarditis, pericarditis and diabetes [10, 11].

Human AdVs are double-stranded DNA, non-enveloped viruses, with 52 serotypes grouped into six species, from A to F. Symptoms of AdV infection include gastroenteritis, pharyngitis, pneumonia, conjunctivitis, and meningoencephalitis [12].

NoVs, the most commonly identified cause of acute nonbacterial gastroenteritis in all age groups, contain a single-stranded, positive-sense RNA genome [13, 14]. These viruses are members of the Caliciviridae family, and are classified into five genogroups, GI-GV [15].

Each group of viruses was quantified using an appropriate TaqMan real-time PCR (or RT-PCR) assay. Real-time PCR has been demonstrated to be a powerful tool for nucleic acid quantification in water environments, thanks not only to its sensitivity and specificity, but also to the fact that, relative to other quantitative tools, it is quick and easy to use.

The quantitative molecular test is unable to discriminate between viable and inactivated virions, so that the presence of a large quantity of viral particles does not necessarily indicate an actual threat to human health. Therefore, we evaluated the infectivity of EVs in treated effluents through integrated cell culture and real-time PCR (ICC-RT-PCR), a

method combining the sensitive conventional quantitative PCR with an infectivity assay.

METHODS

Sample collection

Five different municipal WTPs were chosen, all situated in central Italy, within 30 km of each other. The WTPs in question are conventional activated sludge plants which receive waters from urban areas. Wastewater treatment typically includes grid separation, primary sedimentation, secondary biological treatment and final disinfection with chlorine, before effluents are discharged into the water environment. Daily flows in these plants range from 4800 to 750 000 cubic meters, and design capacity from 60 000 to 1 100 000 population equivalents. No industrial wastewater is treated in any of these facilities.

A total of 50 grab samples were collected (25 inflows and 25 outflows, 50 mL each). Sampling was carried out monthly, from May to September 2007. Samples were collected in the morning on sunny days, stored in sterile 50 mL Falcon tubes kept in thermal bags at 4 °C, and delivered to the laboratory, where they were processed within 24 hours.

Concentration of viruses and RNA extraction

Viruses were concentrated as previously described [16]. Briefly, samples were centrifuged at $3000 \times g$ at 4 °C for 10'. Supernatants were centrifuged at $200\,000 \times g$ for 2 h. The pellets were resuspended in 1 mL of phosphate-buffered saline and the suspension aliquoted and stored at -80 °C until genome extraction.

To assess virus recovery, we spiked 50 mL of distilled water with 1.91×10^8 genome copies (GC) of Echovirus 2 (Cornelis strain, american type culture collection, ATCC, VR-32) and computed the ratio of GCs after/before concentration by real-time PCR.

RNA and DNA were isolated from 100 µl of viral pellet using the NucliSens miniMAG (BioMérieux Italia, Florence, Italy) nucleic acid isolation kit, based on the Boom nucleic acid extraction method [17] in combination with magnetic silica beads. A NucliSens miniMag extractor (BioMérieux Italia, Italy) was used to collect and wash the magnetic silica particles, and to recover nucleic acids from the magnetic beads. Viral genomes were eluted from the silica in 100 µl elution buffer and stored in aliquots at -80 °C until use.

Bacteriological assays

Upon arrival, samples were aliquoted and analyzed. For *E. coli*, microtiter plates (BioRAD, Milan, Italy) were used, following the manufacturer's protocol "Miniaturized method for the enumeration of *E. coli* in surface and waste waters MUG/EC". Similarly, for enterococci, microtiter plates were used following the manufacturer's protocol "Miniaturized method for the enumeration of enterococci in surface and waste waters MUD/SF". Both these protocols are in

accordance with ISO procedures (ISO 9308-3, 1998, ISO-7899-1, 1998).

In both cases, bacterial counts, corresponding to the number of fluorescent wells, were expressed in Most Probable Number (MPN) (Table 1), in accordance with ISO 8199.

Quantification standard

We performed an absolute quantization to determine the number of viral GCs contained in the samples, using an external calibration curve generated by 10-fold serial dilutions (10^8 - 10^1) of a standard consisting of a linearized plasmid DNA for AdVs, and of synthetic ssRNA strands for EVs and NoVs GI and GII. The standards were diluted in nuclease-free water containing carrier yeast tRNA (100 ng/ μ l).

The quantification standards for NoV GI and GII, previously constructed by our team, were 762- and 591-nucleotide ssRNA strands, respectively, containing a partial sequence coding for RNA-dependent RNA polymerase [18]. Quantitative analysis for NoVs was performed on the highly conserved region between ORF1 and ORF2, using genogroup-specific assays, able to amplify 96- and 98-bp fragments, for genogroup I and II respectively, following a slightly modified version [18] of the TaqMan assay described by Jothikumar and collaborators [19].

The calibration curve used for AdV quantization was a *HindIII* linearized plasmid prepared from a recombinant pCR4TOPO vector containing part of the hexon gene [20]. TaqMan real-time PCR was performed using the assay described by Hernroth and collaborators [21], with a minor modification [20], which amplify a 69-bp fragment of the conserved region of the hexon gene.

The standard RNA for EV quantification was prepared by our team as follows: a BamHI220-670 fragment (451-bp) from a pVR10⁶ plasmid [22] containing the whole Poliovirus 1 genome was cloned into the pGEM-4Z vector. The recombinant plasmid was purified using Qiagen minipreps (Qiagen, Hilden, Germany). Having obtained the DNA standard, we then proceeded to construct the RNA standard using RiboMAX large scale RNA production systems-T7 (Promega Italia, Milan, Italy) following the manufacturer's instructions. A 513-nucleotide ssRNA was T7-transcribed and quantified using the ND-1000 instrument (Nanodrop, Wilmington, DE, USA). The concentrations of ssRNA were then converted into copy number using the following equation: $[GC/mL = C/MW * 6.02 * 10^{23}]$, where C is RNA concentration ($C = g/mL$), and MW is the molecular weight. Quantitative PCR was performed according to the assay described by Donaldson and collaborators [23], an assay based on TaqMan probe hydrolysis, amplifying 192 bp of the 5' untranslated region.

Table 2 shows the list of primers and PCRs used in this study.

Real-time PCR

Real-time PCRs were carried out in an ABI-PRISM 7000 sequence detection instrument (Applied

Biosystems, Monza, Italy) on a duplicate set using 10 μ l of the extracted genome. Reactions were carried out in disposable optical 96-well PCR plates with flat tops and printed grids (Labcon, Petaluma, CA, USA) in a 25 μ l mixture using a SensiMix DNA kit for AdVs, and a SensiMix One-Step RNA kit (Quantace, London, UK) for EVs and NoVs.

For AdVs, the reaction mixture was heated at 50 °C for 2 min and then at 95 °C for 10 min; activation was followed by a 40-cycle, two step process, each cycle consisting of denaturation at 95 °C for 15 s and annealing or extension at 60 °C for 1 min. For EVs and NoVs, the reaction mixture was initially incubated at 42 °C for 45 min to transcribe RNA. Because of the extreme sensitivity of the quantitative PCR, we paid particular attention to the prevention of contamination: separate rooms were used for the preparation or mixing of reagents, sample processing, and gel electrophoresis; reagents and samples were stored in separate rooms; and the equipment used in each room was not used in other areas. Negative PCR mixture controls, and extraction controls were systematically used.

Curves were generated by the Sequence Detection System software, plotting cycle threshold (Ct) against the number of viral GCs. Run acceptability was defined as a correlation coefficient (R²) > 0.98 and a slope between -3.6 and -3.1, corresponding to reaction efficiencies between 90 and 110%, according to the equation: Efficiency = $10^{(-1/slope)} - 1$ [24].

To verify the suitability of environmental samples for amplification (absence of inhibitors), negative samples were retested, with a calibrated stock of RNA or DNA standard (see above) added to the real-time mixture.

Statistical analysis

Reproducibility of real time PCR was estimated by assessing the intra- and interassay variability (*i.e.* between identical experiments performed in duplicate on the same day, and experiments performed on different days). Percent coefficient of variation (% CV) was used to quantify the variation of Ct values across replicates of standard curve dilutions (Reed *et al.* 2002).

Quantification data were exported into a Microsoft Excel file for subsequent statistical analysis.

Percent removal was calculated as follows: $R (\%) = (MPC_{out} - MPC_{in} / MPC_{in}) * 100$, where MPC_{out} stands for mean pathogen concentration in effluent samples, and MPC_{in} stands for mean pathogen concentration in influent samples. Mean is intended as arithmetic mean for bacteria and mean trimmed 20% for viruses. Additionally, maximum and minimum values were calculated.

Cell culture analysis and sequencing for human enteroviruses

The infectivity of EVs in treated water samples was analyzed using human rhabdomyosarcoma (RD) cells. After concentration and ultracentrifugation, 200 μ L of viral suspension were purified by chloroform extraction, and inoculated into a 25 cm² tissue

culture flask. The inoculum was discarded and cells were overlaid with 4 mL of maintenance medium (Dulbecco's Modified Eagle Medium, DMEM). Cell cultures were incubated for 5 days in 5% CO₂ at 37 °C and observed daily by optical microscope. An echovirus 2 strain (VR-32, ATCC, USA) was used as a positive control; uninfected cells served as negative controls. The incubation and cell culture facilities were physically separated from the PCR facility. After 5 days, cell culture flasks were scored for the presence of CPE and then frozen and thawed three times. Two-hundred microliters of the supernatant were used to extract viral nucleic acids using the NucliSens miniMAG (BioMérieux Italia, Italy), and the RNA was resuspended in a final volume of 100 µl of elution buffer. Ten microliters of purified viral nucleic acids were used as template in real-time PCR amplifications, to measure the increment in GC counts after cell culture, as described above.

The products obtained through ICC-RT-PCR were then analyzed to confirm the presence of EV, and possibly gain additional information regarding the type of EV found. This was done by running PCR amplicons (expected size 192 bp) on 2% agarose gels containing ethidium bromide (0.5 µg/mL) and visualizing them on a UV transilluminator. Some representative positive samples were purified and sequenced in a capillary automatic sequencer (ABI PRISM 310 Genetic Analyzer, Applied Biosystems, Italy) using previously described protocols [16].

RESULTS

Concentration efficiency

The efficiency of the method of concentration was evaluated by real-time PCR using distilled water spiked with echovirus 2 (in triplicate) and computing the ratio of GC after/before concentration. Out of 5.4 × 10⁸/reaction of spiked virus, 1.9 × 10⁸ GC/reaction (SD = 7.3 × 10⁷) were recovered, giving a yield of 35.4% ± 3.3%.

Bacteriological results

Quantitative bacteriological data is presented in Table 1. For each microorganism tested, the table reports mean, minimum and maximum values for influents and effluents, as well as percent removal. A two-log reduction, or more, in viable bacterial

counts was observed in effluent waters as compared to influents. The mean removal of viable bacterial particles in treated sewage was 98.9% and 98.5% for *E. coli* and enterococci, respectively.

Real-time PCR results

Virus concentrations in sewage samples were determined by quantitative real-time PCR, as described above. Slope values ranged from -3.2 to -3.5 indicating a high PCR efficiency in these experiments (> 90%). Intra- and interassay variability showed an overall good reproducibility (coefficient of variation < 5%).

AdVs were found in the highest concentrations in both raw and treated waters (up to 9.8 × 10⁸ GC/mL and up to 4.9 × 10⁸ GC/mL, respectively, see Table 2). The viruses found in the lowest concentrations were EVs (2.4 × 10⁶ GC/mL) in inflows, and NoVs GII (9.9 × 10⁵ GC/mL) in outflows. Mean values (trimmed mean) in raw samples were ranked as follows: AdV > NoV GI > NoV GII > EV, while in treated waters, AdV > NoV GI > EV > NoV GII (Figure 1).

Considering the three groups of viruses together, the total GC count was 4.8 × 10⁷ in influents, and 1.2 × 10⁷ in effluents, for a total removal of 74%. In an overall analysis of removal efficiency, based on mean values across months, the viruses most efficiently removed were NoV GI (78.4%); while EVs were the least efficiently removed (34.6%).

Considering sample-pairs, it was noted that some effluent samples were completely cleared of certain viruses (GCs in outflows not detected): 37.5% of effluent samples were free of NoV GII, 53.3% were free of NoV GI, 8.3% were free of EVs, and 11.1% were free of AdVs. The majority of effluent samples were only partially cleared, each to a different extent. Moreover, in a few, rare cases, outflows were found to be more contaminated than inflows.

The viruses most frequently found in samples tested by real-time PCR were EVs (96% in influent, and 84% in effluent samples) and AdVs (96% in influent, and 76% in effluent samples). A smaller proportion of samples were positive for NoVs GI and GII (92% and 72% at the point of entry and 68% and 40% at the point of exit, respectively). The simultaneous presence of more than one virus was the rule: 64% of influent samples (against 28% of effluent samples) were positive for all tested viruses simultane-

Table 1 | Quantification of viruses and bacterial indicators in wastewater treatment plants

Statistics	Total		NoV GII		NoV GI		Enterovirus		Adenovirus		E. Coli		Enterococci	
	IN	OUT	IN	OUT	IN	OUT	IN	OUT	IN	OUT	IN	OUT	IN	OUT
Mean	4.80E+07	1.20E+07	2.60E+06	9.90E+05	9.30E+06	2.00E+06	2.40E+06	1.60E+06	3.30E+07	7.60E+06	2.10E+07	2.20E+05	2.9E+06	4.40E+04
Max			5.70E+08	5.60E+07	1.60E+08	1.80E+08	6.80E+06	6.50E+06	9.80E+08	4.90E+08	8.30E+07	1.50E+06	7.70E+06	3.20E+05
Min			6.20E+04	7.80E+02	1.90E+06	1.80E+06	1.10E+05	2.00E+05	4.60E+05	3.40E+05	1.10E+06	5.80E+01	8.40E+05	8.10E+05
Removal	-74.00%		-62.60%		-78.40%		-34.60%		-77.10%		-98.90%		-98.50%	

Data are expressed in GC/mL for viruses and MPN/100mL for bacteria. Mean is intended as arithmetic mean for bacteria and mean trimmed 20% for viruses.

Table 2 | Primers and PCRs used in this study

PCR ID	Primer ID	Sequence (5' > 3')	Product length (bp)	
494	1080-f	CWTACATGCACATCKCSGG	69	Adenovirus
	1081-r	CRCGGGCRAAYTGACCAG		
	1082-p	fam-CCGGGCTCAGGTACTCCGAGGCGTCCT -tamra		
377	1064-f	GGCCCCTGAATGCGGCTAAT	192	Enterovirus
	1065-r	CACCGGATGGCCAATCCAA		
	1066-p	fam-CGGACACCCAAAGTAGTCGGTTCCG-tamra		
500	1315-f	GCCATGTTCCGTTGGATG	96	Norovirus GI
	1316-r	TCCTTAGACGCCATCATCAT		
	1377-p	fam-TGTGGACAGGAGATCGCAATCTC -tamra		
581	1380-f	CAAGAGYCAATGTTYAGRTGGATGAG	98	Norovirus GII
	1319-r	TCGACGCCATCTTCATTACA		
	1379-p	joe-TGGGAGGGCGATCGCAATCT-tamra		

ously; 28%, in both raw and treated samples, were positive for three viruses; and 8% of samples were positive for 2 viruses at the point of entry (28% at the point of exit). None of the influent samples were positive for only one virus (16% in outflows), and none were completely negative.

Enterovirus infectivity and sequence analysis

Nine (out 25) effluent samples showed signs of cytopathic effect on RD cells; GC/liter were calculated before and after cell culture and the exponential viral growth was confirmed by GC counts in cellular lysates (at least 4 log). Real-time positive samples were confirmed by sequence analysis of the 192-bp amplicon and characterized as EV of the B species (accession numbers from FN397854 to FN397859).

DISCUSSION

Disposal of inadequately treated wastewater is one of the main sources of pathogenic contamination

of the water environment. Due to their highly infectious nature, viruses are of particular public health concern. Indeed, they are believed to be the major cause of disease contracted through direct contact with sewage, and are responsible for gastroenteritis, hepatitis, respiratory illness, and other health problems. Among the wide range of viruses excreted in human waste, three groups of enteric viruses – EVs, AdVs, and NoVs – have attracted considerable attention due to their epidemiological significance as waterborne pathogens [25-27, 13, 8].

The objective of the present study was to assess the concentration of these viruses in inflows and outflows, to gain some insight into the infectious potential of viral contamination in treated wastewater, and to obtain a rough estimate of the efficacy of the purification process. Molecular methods of virus quantification were used for this purpose. In particular, we used TaqMan real-time PCR assays which have the advantage of providing rapid, accurate, sensitive and quantitative detection. For AdVs

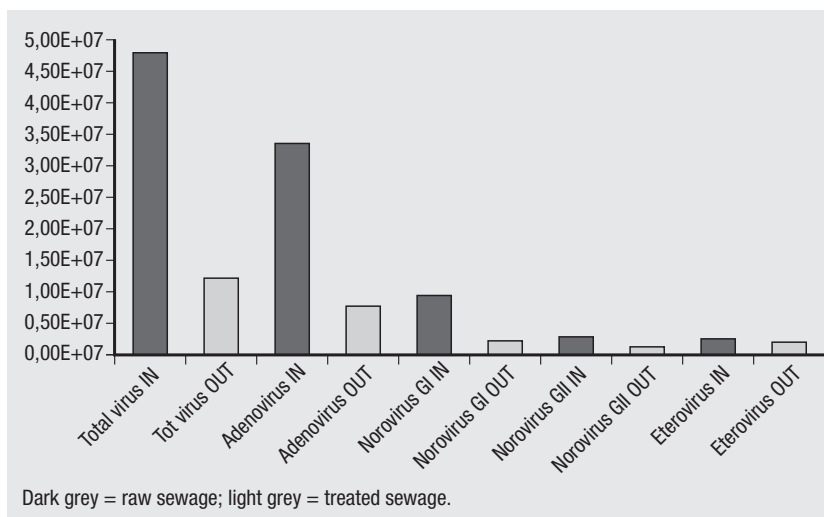


Fig. 1 | Mean genome copy counts in influent and effluent samples in five wastewater treatment plants in and around Rome.

we used a broad range TaqMan PCR assay previously shown to be suitable for quantitative determination of viral DNA from all adenovirus Species (A-F), in both environmental and clinical samples [20]. The presence of AdVs was common in both influent and effluent samples (96%, and 76%, respectively). Moreover, of all tested viruses, AdVs had the highest concentrations in both raw and treated waters. Overall, the concentration of AdVs was higher than that of all other viruses combined. This finding is in agreement with a 2008 study [28], reporting AdV concentrations to be the highest of all enteric viruses tested, as well as with earlier studies [29, 30]. The higher concentrations of AdVs throughout the present study may be due to the fact that AdV, being the only DNA virus, is thus also the most thermostable. Indeed, different studies have shown AdVs to survive longer than fecal indicator bacteria both in sewage and in the environment, and to be very resistant to UV light [31-34]. AdV concentrations reported in the present study are slightly higher than those previously reported [35], and may be due to a higher prevalence of infection in the population.

For NoV quantification we used a real-time RT-PCR method previously tested in clinical samples, as well as in estuarine, seawater and sewage water samples [18]. NoVs were found to be less prevalent than AdVs: 92% and 68% of samples were positive for GII and 72% and 40% for GI in influents and effluents, respectively. Higher counts for GI as compared to GII were detected in both influent and effluent samples. The viral titers obtained in this study are consistent with previously reported levels [7, 4, 36], despite the different assay used.

The higher concentrations of NoV GI relative to GII is seemingly at odds with epidemiological data on the occurrence of NoV outbreaks [37, 38] and sporadic cases [16], mostly attributable to NoV GII. This finding, suggesting a significant presence of GI in human populations, has already been described by others, both in France [7] and in Sweden [36], and implies the need for further studies to address possible differences in the pathogenic potential of different genogroups, their stability in water environments, and their ability to evade the human immune system.

The quantification of EVs was performed using an assay based on TaqMan probe hydrolysis technology in the most conserved region of the enteroviral genome, which has sufficient genetic variability to enable identification of the different enteroviral types [23]. The proportion of samples positive for EVs was the highest (96% and 84% in influents and effluents, respectively), but their concentrations were the lowest. This may indicate that EVs are excreted more consistently, even in cases of asymptomatic infection, while other enteric viruses are mostly associated with outbreaks and therefore excreted less consistently, but at greater concentrations.

To sum up, mean viral genome concentrations were ranked as follows: in influent samples, AdV > NoV GI > NoV GII > EV, and in effluents, AdV > NoV

GI > EV > NoV GII. Removal efficiencies ranged from 35% (EV) to 78% (NoV GII). Considering all groups of viruses together, the overall removal efficiency was 74%. These data are in agreement with previous studies showing that enteric viruses are present in high levels not only in raw, but also in treated wastewaters [4, 5, 39, 7].

Our removal efficiency results are in agreement with other studies, suggesting that even properly working wastewater treatment systems remove only about 20-80% of enteric viruses [40-42], viruses which can easily reach water sources and become a serious human health concern. The removal of bacterial indicators, on the other hand, was much more efficient (up to 98.9%). Our data thus confirm both the high removal efficiency for bacterial indicators, and the absence of correlation between the removal of enteric bacteria and that of enteric viruses, as widely described in literature [42-44].

It should be noted, however, that our samples, collected once a month, were grab, rather than composite samples (this, by the way, explains the fact that some samples were more contaminated at the point of exit). Therefore, our results, rather than being considered representative of the actual removal efficiency at the WTP, should instead be regarded as merely giving a general idea of the amount of viral contamination at the points of entry and exit. Another limit of the study is in the collection period (5 months, from May to September), not sufficient for evaluating seasonal changes in viral concentrations, especially for noroviruses, responsible for gastroenteritis in winter months. However, recent studies have shown an increase in spring and summer peak of NoV outbreaks of gastroenteritis since 2002 [37], and an increase of NoV concentration in WTPs throughout the summer [36]. A detailed analysis of seasonal changes in viral concentrations was, however, out of the scope of the present work.

The ability to rapidly detect and quantify viruses in environmental samples using real-time PCR represents a considerable advancement holding great potential for environmental applications. Nevertheless, the interpretation, in terms of health implications, of the presence of viral genome detected by PCR remains difficult due to the fact that molecular methods do not distinguish between infectious and non-infectious viral particles. We therefore proceeded to evaluate the infectivity of viruses in treated effluents through ICC-RT-PCR. This approach allowed us to overcome most of the disadvantages associated with both conventional cell culture and direct PCR assays, reducing the time needed for the detection of infectious viruses. We chose to apply this method to EVs, because, of the three groups of viruses tested in this study, they are the only group that can grow efficiently on cell lines. The culturing of AdVs (particularly enteric types 40 and 41) is more problematic, and no simple cell culture system is available for NoVs at present, despite recent efforts [45]. The ICC-real-time PCR method provided results within 4-5 days of sew-

age-sample collection. Viral growth was confirmed by an exponential increment of GC counts in cellular lysates. The results revealed that treated water still contains infectious human EVs (36% of samples) and thereby represents a potential health hazard.

As for the other enteric viruses studied, the frequent detection of viral genome in sewage effluents suggests that treated sewage may represent a source of environmental contamination with potentially infectious viruses. Indeed, previous studies have detected infectious human AdVs in tertiary-treated and ultraviolet-disinfected wastewaters, confirming the relative resistance of the AdV isolates [46].

In recent years, the detection and molecular characterization of pathogenic human viruses in urban sewage have been extensively used to get information on circulating viruses in given populations throughout the world; however to the best of our knowledge, no previous investigations have specifically addressed and compared viral concentrations in WTPs for different epidemiologically important, waterborne enteric viruses, using quantitative assays.

Given the considerable burden of viral gastrointestinal disease, and in the absence of costly epidemiological monitoring systems, virological monitoring of sewage flowing into a WTP may represent an inexpensive and efficient approach that would allow for an ongoing follow-up on the occurrence of pathogenic viruses in the plant's service area. The monitoring of effluent samples, on the other hand, may contribute to the understanding of viral removal through wastewater treatment and to the prevention of disease.

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CONCLUSIONS

This work confirms the frequent occurrence of enteric viral genomes in sewage effluents, suggesting that treated sewage may represent a source of environmental contamination with potentially infectious viruses. Of all tested viruses, AdVs had the highest concentrations in both raw and treated waters, confirming the relevance of evaluating these viruses as possible indicators of viral contamination of water.

The real-time PCR has been demonstrated to be a powerful tool for rapid determination of enteric viruses in environmental samples and represents a considerable advancement in pathogen quantification in aquatic environments.

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Conflict of interest statement

There are no potential conflicts of interest or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

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