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ORIGINAL ARTICLE

Human enteric viruses in a wastewater treatment plant: evaluation of activated sludge combined with UV disinfection process reveals different removal performances for viruses with different features

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Significance and Impact of the Study: This study demonstrates that an activated-sludge wastewater treatment plant with UV disinfection reduces to levels below the detection limit those single-stranded RNA viruses as noroviruses and astroviruses and reach significant lower levels of rotaviruses and adenoviruses after the complete treatment process.

Keywords
disinfection, environmental, ultraviolet applications, viruses, wastewater treatment.

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Abstract

This study assess the quality of wastewater through the detection and quantification of important viruses causing gastroenteritis at different stages of the wastewater treatment process in an activated-sludge wastewater treatment plant with ultraviolet disinfection. Ten sampling events were carried out in a campaign along a period of 18 months collecting wastewater samples from the influent, after the activated-sludge treatment, and after the final disinfection with UV radiation. Samples were concentrated through ultracentrifugation and analysed using retro-transcription, PCR and real time quantitative PCR protocols, for detection and quantification of Group A Rotavirus (RVA), Human Astrovirus (HAstV), Norovirus Genogroup II (NoV GII) and Human Adenovirus (HAdV). HAdV (100%), NoV GII (90%), RVA (70%) and HAstV (60%) were detected in influent samples with concentration from 1·4 (NoV GII) to 8·0 (RVA) $\log_{10}$ gc l^{-1}. Activated-sludge treatment reached well quality effluents with low organic material concentration, although nonstatistical significant differences were registered among influent and postactivated sludge treatment samples, regarding the presence and concentration for most viruses. All post-UV samples were negative for NoV GII and HAstV, although RVA and HAdV were detected in 38% and 63% of those samples respectively, with concentration ranging from 2·2 to 5·5 and 3·1 to 3·4 $\log_{10}$ gc l^{-1}.

Introduction

Gastroenteritis is an important disease worldwide affecting mainly children under five years of age (Lanata *et al.* 2013). Gastroenteric viruses are important pathogens among the gastroenteritis causing agents(Oude Munnink and van der Hoek 2016), and since they normally replicate efficiently in the gastrointestinal tract, each gram of stool can arise an amount of 10^{13} viral particles (Carter 2005; Bosch *et al.* 2008). Both symptomatic and asymptomatic

persons shed daily a huge concentration of viruses to the sanitary network. Thereby, the wastewater is one of the most concentrated source of enteric viruses in the environment and contamination of other environmental water matrixes as rivers, groundwater, or lakes is possible when sanitary network is damaged or when wastewater is discharged into the environment without a previous treatment, or with an inefficiently performed treatment (Fong and Lipp 2005).

Sporadic cases and gastroenteritis outbreaks are frequently associated to Group A Rotavirus (RVA), Human Astrovirus (HAstV), Norovirus Genogroup II (NoV GII) and Human Adenovirus (HAdV), which are also important cause of water-related gastroenteritis (Bosch *et al.* 2008).

Group A Rotavirus belongs to the *Rotavirus* genus, subfamily *Sedoreovirinae*, family *Reoviridae* (ICTV, 2017) and has a segmented double-stranded RNA genome surrounded by a nonenveloped three-layered icosahedral capsid. Based on nucleotide sequence of the two outer capsid proteins genes (VP7 and VP4) RVA is classified in several G and P types (Estes and Greenberg 2013). Human Adenovirus comprises seven species (A to G) that belong to the *Mastadenovirus* genus in the *Adenoviridae* family (ICTV (International Committee on Taxonomy of Viruses) 2017) and comprises virions without envelope, with a double-stranded DNA genome of about 36 kb contained in a 90 nm in diameter icosahedral capsid, with fibres projecting from the vertices (Berk 2013). Besides being second to RVA as a leading cause of childhood gastroenteritis worldwide (Rames *et al.* 2016) thanks to the HAdV-41 genotype (a member of specie F), different HAdV are associated to different symptoms as respiratory diseases or conjunctivitis, among others (Lion 2014).

NoV GII belong to the *Norovirus* genus from the *Caliciviridae* family and comprises virions with a nonenveloped icosahedral capsid surrounded a single positive-stranded RNA genome of approximately 7 kb (Green 2013; ICTV 2017). NoV account for >90% of viral epidemic gastroenteritis worldwide (Patel *et al.* 2009) and for a 17% of the acute gastroenteritis sporadic cases in developing countries (Nguyen *et al.* 2017), meaning almost one out of every six hospitalizations because of acute diarrhoea in children younger than 5 years of age in Latin America (O'Ryan *et al.* 2017). Genogroup II is the most frequently associated with the disease among the three well-known genogroups infecting human -GI, GII and GIV- (Vinjé 2015). HAstVs comprise the viral species *Mamastrovirus 1* (*Mamastrovirus* genus from the *Astroviridae* family) and are associated up to 9% of cases of acute nonbacterial diarrhoea in children worldwide. Although the infection is more common in children younger than 2 years old, the age of infection is highly variable (from newborns to over five-years-old children) (Bosch *et al.* 2014; ICTV 2017). With a star-like appearance when virions are visualized by electron microscopy, HAstVs are small, nonenveloped viruses, with an icosahedral capsid and a single positive-stranded RNA genome of approx. 7 kb in length (Méndez and Arias 2013).

Despite the lack of studies describing in depth the impact of NoV and HAdV in the health of Uruguayan children, all the above-mentioned viruses have been reported as acute gastroenteritis causing agents in Uruguayan paediatric population, with incidences according the expected for RVA (37%) and HAstV (10%). Moreover, a wide dispersion as well as a wide genetic diversity in the Uruguayan environment has been recently reported for these viral pathogens (Victoria *et al.* 2014, 2016; Lizasoain *et al.* 2015; Tort *et al.* 2015; Varela *et al.* 2015; Lopez *et al.* 2017). This information raises the need to evaluate the wastewater treatment process existing in the country to assure viral removal from wastewater and reach effluent of good quality.

Wastewater treatment plants (WTP) could achieve the removal up to approx. 7 $\log_{10}$ units of viral genomic copies per litre ($\log_{10}$ gc l^{-1}) along the wastewater treatment process (Xagoraraki *et al.* 2014). However, viruses are able to resist to various kinds of treatments, being detected in the final effluent (Francy *et al.* 2012; Hata *et al.* 2013; Kitajima *et al.* 2014).

Viral contamination of diverse water environmental matrixes must be avoided to preserve the health of population who has contact with them. An accurately performed wastewater treatment that assures virus removal is essential to minimize the dissemination of enteric viruses into the environment and the subsequent transmission to humans.

This study evaluates the presence and concentration of important gastroenteric viruses during wastewater treatment process in a WTP located in Uruguay. This facility, performs an activated-sludge treatment as secondary treatment and the final effluent is disinfected with UV radiation previous its discharge into a natural course of water.

Results and discussion

In this work, we have studied the quality of the wastewater along the treatment process evaluating if differences in detection rates and concentration levels for viruses among different sites of sampling, are statistically significant, as an indirect approximation to know the WTP efficiency.

As Fig. 1 shows, all viruses were detected in influent and postactivated sludge samples, but when post-UV samples were analysed, nor HAstV neither NoV GII were detected, which indicates a statistically significant removal

for these viruses from the influent up to the final UV treated effluent ($P = 0{\cdot}0128$ and $P = 0{\cdot}0002$ respectively). Nevertheless, RVA and HAdV were detected in 38 and 63% of post-UV samples respectively.

Viral surrogate (PP7 bacteriophage) detection resulted in the 100% of samples, indicating that viral concentration, nucleic acid extraction and cDNA synthesis were carried out correctly (data available upon request).

The genomic quantification of viruses (expressed as $\log_{10}$ gc l^{-1}) performed in positive samples, revealed concentrations ranging from 1·4 (NoV GII) to 8·0 (RVA) in influent samples, from 1·8 (NoV GII) to 5·5 (RVA) in postactivated sludge samples, and from 2·2 (RVA) to 5·5 (RVA) in post-UV effluent samples (Fig. 2).

When comparing with influent samples, lower concentrations of RVA and HAdV were detected in post-UV samples, meaning that RVA and HAdV particles are discharged into the natural watercourse that receive the final effluent. The viral decay (calculated as Log Removal Values) for RVA and HAdV due to the complete treatment, resulted in values of 1·1 to 2·5 and 1·6 to 2·1 respectively, which means a decay between 90 and 99 % in the populations of RVA and HAdV (Hai *et al.* 2014). Nevertheless, these values could be strongly biased since were calculated among few set of paired samples (influent and post-UV samples collected in the same sampling event) which were positive and effectively quantified for these viruses. Studies with larger samplings could be more precise regarding this information.

Unfortunately, we have not performed viral isolation in cell culture experiments to determine the viability of the detected viral particles, especially after the UV treatment. This kind of disinfection process aims to inactivate the viral particles by affecting the capsid forming proteins or the viral genome by different mechanisms (Wigginton and Kohn 2012). Despite a previous study reported that genomic copies of RVA and HAdV detected in post-UV samples correspond to noninfective viral particles as a result of the UV inactivation (Qiu *et al.* 2015), most of studies carried out upon other facilities reported infectious ones (Thompson *et al.* 2003; Rodríguez *et al.* 2008; Simmons and Xagoraraki 2011). Probably, this is a consequence of the high UV fluence requirements for RVA and HAdV inactivation (Hijnen *et al.* 2006).

Nevertheless, since our quantitative PCR detects both infective and defective viral particles, and considering the UV light effect upon viral viability, the concentration values at the final effluent are not at all indicative of infectious viral particles and the results should be taken with caution, avoiding overestimation of the amount of infective particles in the sample.

Viral detection percentages and concentration values of samples obtained before and after the UV treatment in our study, were different among them. These results are in disagree with Qiu *et al.* (2015) who reported similar detection rates and viral concentration values in this paired samples set. If well the amount of UV radiation that is applied at each WTP directly impacts on the viruses concentration levels (Hijnen *et al.* 2006), in this case, the UV radiation dose applied in the WTP studied by Qiu *et al.* (2015) is similar to the UV radiation dose applied in the WTP studied here. However, as viruses

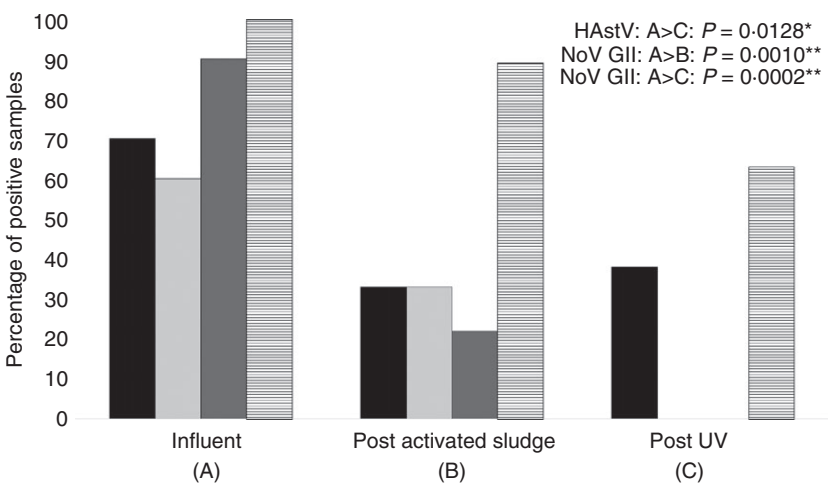

Figure 1 Detection of enteric viruses in different stages of the wastewater treatment process in an Activated Sludge Wastewater Treatment Plant with UV disinfection. RVA: Group A Rotavirus (black bars). HAstV: Classical Human Astrovirus (light–grey bars). NoV GII: Norovirus Genogroup II (dark–grey bars). HAdV: Human Adenovirus (striped bars). On the upper right side are shown *P* values obtained in a Test of Proportion for those cases in which the presence of a virus is significantly different in a stage of the wastewater treatment process comparing with a subsequent stage. *P* values under 0·05 and 0·001 indicate significant differences with 95% and 99% confidence respectively. HAstV: A > C: $P = 0{\cdot}0128$*; NoV GII: A > B: $P = 0{\cdot}0010$**; NoV GII: A > C: $P = 0{\cdot}0002$***.

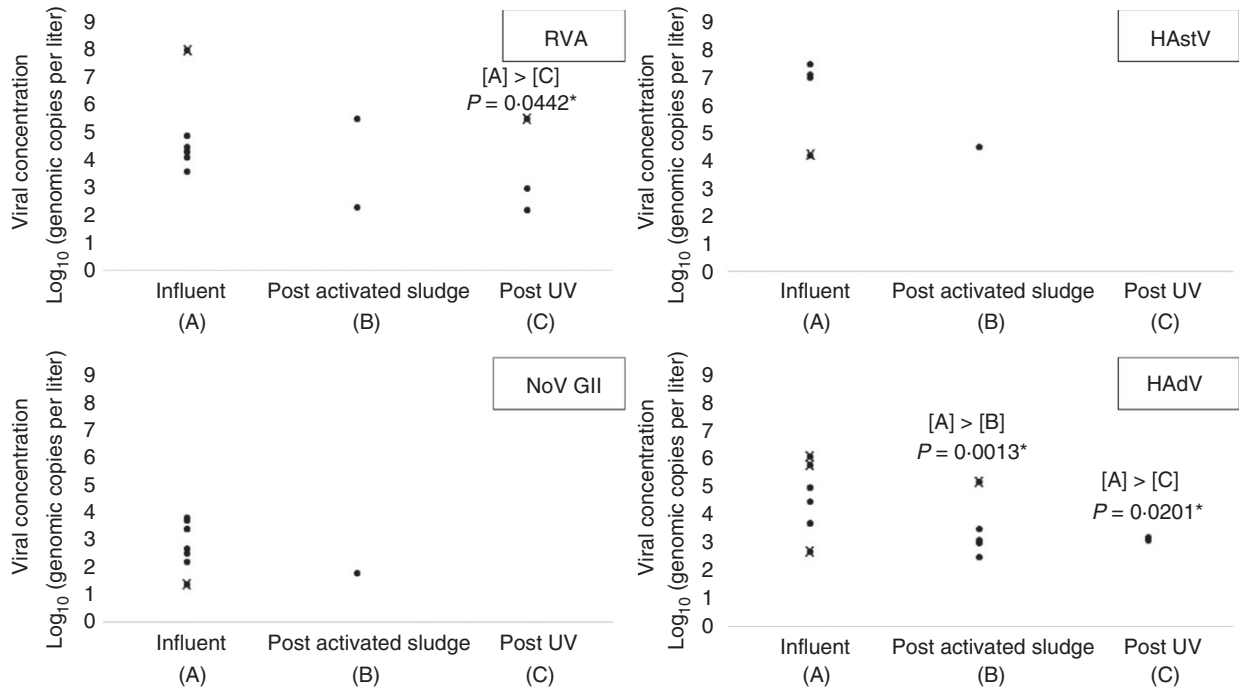

Figure 2 Viral concentration values for enteric viruses in different stages of the wastewater treatment process in an Activated Sludge Wastewater Treatment Plant with UV disinfection. RVA: Group A Rotavirus. HAstV: Classical Human Astrovirus. NoV GII: Norovirus Genogroup II. HAdV: Human Adenovirus. A: influent samples. B: postactivated sludge samples. C: post-UV samples. Viral concentration is expressed as $\log_{10}$ of genomic copies per litre. Statistically significant difference ($P < 0{\cdot}05$) between the underlying distributions of the viral concentration in A–B, B–C or A–C was evaluated in a nonparametric Wilcoxon–Mann–Whitney test. Outlier values, which were not considered for the statistical test, are represented as crossed out black circles.

could aggregate with organic material present in sewage, a shield effect in where viruses protect itself from UV inactivation is possible, mainly when the effluent carry over particulate organic material resulted from an inefficiently performed activated-sludge process. As consequence, UV inactivation of viral particles is compromised (Templeton *et al.* 2005). Interestingly, physicochemical parameters measured at WTP (Table S1) indicate high-quality effluents with low levels of organic and particulate material after the activated-sludge treatment (Uruguay. *Rules for preventing environmental pollution through the control of waters,* 1979), which would contribute to minimize the shield effect by which viruses protected itself from UV light. Unfortunately, the study carried out by Qiu *et al.* (2015) does not report physicochemical data of wastewater, and the comparison with our data is not possible. Nevertheless, other factors such as different population size, geographical location, or indeed, different sampling methods chosen in each study, could be influencing the different UV radiation efficiencies among different studies.

The differences in detection rate and concentration values before and after the activated-sludge treatment process in this study was statistically significant only for NoV GII ($P = 0{\cdot}0010$) and HAdV ($P = 0{\cdot}0103$) respectively. Nevertheless, when influent and post-UV effluent are compared, the WTP appear as an efficient facility-removing virus since HAstV and NoV GII were not detected in the final effluent, despite being detected in the 60 and 90% of the influent samples respectively. Furthermore, although RVA and HAdV were not completely removed, their concentration at post-UV effluent were significant lower than in influent ($P = 0{\cdot}0442$ and $0{\cdot}0201$ respectively), which also is important in terms of viral impact mitigation in the environment. This data indicates that the complete treatment process at the WTP is needed to reach a significant virus removal. Also indicates a differential treatment efficiency depending on the virus since those single stranded RNA viruses (HAstV and NoV GII) were easier to removal than HAdV (a DNA virus) or RVA (a segmented double stranded RNA virus), which is in agreement with previous studies (Meng and Gerba 1996; Calgua *et al.* 2014; Qiu *et al.* 2015).

Although up to date, the Uruguayan legislation does not consider virus concentration limits to evaluate water matrixes quality, additional efforts to assure even lower RVA and HAdV concentration values at final effluent will provide an increasing safety when effluents are dumped

into the natural watercourse. The results presented here encourage the implementation of similar facilities in other regions of the country in which wastewater treatment systems are lacking yet.

Materials and methods

Wastewater treatment plant

The studied WTP (32°22′30·3″S 54°10′35·9″W) is located in a city of the Eastern region of Uruguay which influent represent approximately 35 000 inhabitants with an average flow exclusively of domestic wastewater of approximately 8000 m^3 per day (reaching peaks of 15 000 m^3 per day). In the WTP, an activated sludge with extended aeration treatment is followed by a disinfection process with monochromatic low-pressure UV lamps (UV-C, $\lambda = 254$ nm) submerged in a channel through which effluent passes only once time receiving a radiation dose higher than 20 mWs cm^{-2} before being discharged into a natural course of water.

Samples collection

Wastewater samples of 42 ml were obtained along 10 sampling events in a 20-month campaign (September 2011 to April 2013) and a periodicity of approximately two months. In each event, samples were taken at three different stages of the treatment in the WTP. Twenty-seven samples were obtained along the sampling campaign (influent samples [A] = 10, postactivated sludge samples [B] = 9 and post-UV disinfection samples [C] = 8). Three samples were not collected due to logistic drawbacks.

Viral concentration and PP7 phage used as an internal process control (IPC)

The viral concentration was performed using ultracentrifugation protocol according to Pina *et al.* (1998) with modifications added by Fumian *et al.* (2010). Previously, 500 μl of viral surrogate PP7 bacteriophage (Rajal *et al.* 2007) were inoculated in each sample as an Internal Process Control (IPC).

Nucleic acid extraction and cDNA synthesis

Two hundred microlitres of 1X PBS was added to the concentrated sample to obtain a final volume of 400 μl to carry out the nucleic acid extraction using the guanidinium/silica method (Boom *et al.* 1990) obtaining a final volume of purified nucleic acid extract of 40 μl. For the RNA viruses, cDNA synthesis was carried out using random hexamer primers (SBS Genetech™ Beijing, China) and *SuperScript-II™ Reverse Transcriptase Enzyme* (Invitrogen™, Carlsbad, CA, USA) according to the manufacturer's instructions.

Virus detection and quantification

Group A Rotavirus, HAstV, NoV GII and HAdV were detected by previously described qualitative PCRs protocols (Table S2), as well as the IPC, which conventional PCR detection was carried out before to perform the screening for the enteric viruses.

For RVA detection, two multiplexed nested-PCR generating different fragments whose size depend on the amplified G and P type were carried out according World Health Organization (2009). Consequently, a positive result for VP4 and / or VP7 genes was considered indicative of the presence of the virus.

All amplified samples were visualized in agarose gel electrophoresis (AMRESCO® Radnor, PA, USA) stained with "GoodView™ Nucleic Acid Stain (SBS Genetech) with a trans-illuminator (Cleaver Scientific Ltd., Warwickshire, UK) and the FOTODYNE Incorporated FOTO/Analyst® Express (Fotodyne Incorporated, Hartland, WI, USA) system was used for the photo-documentation.

Positive samples were processed later for viral quantification by real time TaqMan® PCR using the SensiMix II™ Probe Kit (Bioline Reagents Ltd, Taunton, MA, USA) in a Rotor Gene Q instrument (Qiagen®, Hilden, Germany), following the manufacturer's instructions and previously described protocols (Table S2).

The decay of viruses from influent up to the end of the process was evaluated in terms of Log Removal Values (LRV) using virus concentration values of paired samples of influent and post-UV, as reviewed by Hai *et al.* (2014).

Statistical analysis

To evaluate the treatment process in the WTP, a Test of proportions was performed, testing if the presence of each virus has the same proportion within each stage of the process (sites A, B and C). *P*-values under 0·05 and 0·001 indicate significant differences with 95 and 99% confidence respectively.

The nonparametric Wilcoxon–Mann–Whitney test was carried out to compare quantitative data from viral concentration between influent, postactivated sludge and post-UV samples, for each virus. Differences were considered statistically significant if the obtained *P* value was lower than 0·05. Outlier values of the viral concentration at each sampling point were excluded. This analysis was performed with STATA 12.0 software (Statacorp LP, College Station, TX).

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Conflict of Interest

No conflict of interest declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Averages values of physicochemical parameters measured in influent (A) and in postactivated sludge (B) samples.

Table S2. Primers, probes and protocols used in this study to perform the molecular detection or quantification of PP7, Group A Rotavirus (RVA), Classical Human Astrovirus (HAstV), Norovirus Genogroup II (NoV GII), and Human Adenovirus (HAdV) from wastewater samples.