Quantitative risk assessment of norovirus and adenovirus for the use of reclaimed water to irrigate lettuce in Catalonia

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Abstract

Wastewater is an important resource in water-scarce regions of the world, and its use in agriculture requires the guarantee of acceptable public health risks. The use of fecal indicator bacteria to evaluate safety does not represent viruses, the main potential health hazards. Viral pathogens could complement the use of fecal indicator bacteria in the evaluation of water quality. In this study, we characterized the concentration and removal of human adenovirus (HAdV) and norovirus genogroup II (NoV GII), highly abundant and important viral pathogens found in wastewater, in two wastewater treatment plants (WWTPs) that use different tertiary treatments (constructed wetland vs conventional UV, chlorination and Actiflo® treatments) for a year in Catalonia. The main objective of this study was to develop a Quantitative Microbial Risk Assessment for viral gastroenteritis caused by norovirus GII and adenovirus, associated with the ingestion of lettuce irrigated with tertiary effluents from these WWTPs. The results show that the disease burden of NoV GII and HAdV for the consumption of lettuce irrigated with tertiary effluent from either WWTP was higher than the WHO recommendation of 10^{-6} DALYs for both viruses. The WWTP with constructed wetland showed a higher viral reduction on average (3.9 and 2.8 logs for NoV GII and HAdV, respectively) than conventional treatment (1.9 and 2.5 logs) but a higher variability than the conventional WWTP. Sensitivity analysis demonstrated that the input parameters used to estimate the viral reduction by treatment and viral concentrations accounted for much of the model output variability. The estimated reductions required to reach the WHO recommended levels in tertiary effluent are influenced by the characteristics of the treatments developed in the WWTPs, and additional average reductions are necessary (in WWTP with a constructed wetland: 6.7 and 5.1 logs

for NoV GII and HAdV, respectively; and in the more conventional treatment: 7 and 5.6 logs). This recommendation would be achieved with an average quantification of 0.5 genome copies per 100 mL in reclaimed water for both viruses. The results suggest that the analyzed reclaimed water would require additional treatments to achieve acceptable risk in the irrigation of vegetables with reclaimed water.

Keywords: Quantitative Microbial Risk Assessment, Wastewater Treatment Plant, water reuse, Norovirus, Adenovirus, Crop irrigation

Abbreviations

DALYs: Disability-Adjusted Life Years

FIB: Fecal indicator bacteria

HAdV: Human adenovirus

NoV: Norovirus

NoV GII: Norovirus genogroup II

Pppy: per person per year

QMRA: Quantitative Microbial Risk Assessment

qPCR: Quantitative PCR

q(RT)PCR: qPCR and RT-qPCR

RT-qPCR: Real-time quantitative PCR

SMF: skimmed milk flocculation

WHO: World Health Organization

WWTP: Wastewater Treatment Plant

1. Introduction

Reuse of wastewater for agricultural irrigation is being implemented widely because water scarcity is reported in nearly all river basins in the Mediterranean area. Wastewater is often a reliable year-round source of water, and it contains necessary nutrients for plant growth. For example, Spain uses 71% of its total volume of reclaimed water for agricultural irrigation (Iglesias et al., 2010). Reclaimed water is also used for urban, industrial, recreational and environmental activities. Wastewater needs to be treated to produce reclaimed water to be used for irrigation (EU, 2016; Sanz and Gawlik, 2014). The use of reclaimed water in Spain is regulated under the Real Decreto 1620/2007. This regulation sets the minimum acceptable safety limits for each type of use in Spain, including agricultural irrigation. These limits include the levels of intestinal nematode eggs, *Escherichia coli*, suspended solids and turbidity (Boletin Oficial del Estado, 2007), but this regulation does not include addressing the acceptable levels of viruses. Food crops irrigated with untreated or poorly treated water are a main source of viruses in outbreaks associated to fresh vegetables (Gerba et al., 2018).

The control of the microbiological quality of reclaimed water in wastewater treatment plants (WWTPs) is currently based on the levels of fecal indicator bacteria (FIB), which include fecal coliforms, *Escherichia coli* and enterococci. However, bacterial indicators are poorly related to the presence of human enteric viruses (Petterson et al., 2001). FIB behave differently than enteric viruses in wastewater and aquatic environments, where these bacteria are more susceptible to water treatments and environmental conditions than enteric viruses (McMinn et al., 2017). Among the pathogen groups found in wastewater, viruses

present the greatest risk because they generally occur in much greater concentrations and have a much greater infectivity (i.e. higher probability of infection with a given exposure), than bacteria and parasitic protozoa (Gerba et al., 2018). Viruses have been associated with outbreaks via irrigated fresh produce (Chatziprodromidou et al., 2018) and the risk of illness from viruses is 10 – 10000 times greater than that from bacteria at a similar level of exposure (Haas et al., 1993). For that reason, the evaluation of reclaimed water systems with only FIB underestimates the public health risk of enteric viruses.

The most effective means of consistently ensuring safety in the agricultural application of wastewater is through the use of a comprehensive risk assessment and risk management approach that encompasses all steps in the process from waste generation to the treatment and use of wastewater to product use or consumption (WHO, 2006). Quantitative Microbial Risk Assessments (QMRAs) generate an understanding of the risks associated with water reclamation, by characterizing the pathogen occurrence in wastewater and evaluate how well these pathogens are controlled by the wastewater treatment system (and follow-up control measures in irrigation, farming and food processing practice). The pathogen dose that consumers are exposed to in a particular scenario is translated into probabilities of infection and illness. These can be compared against a tolerable disease burden. Disability-Adjusted Life Years (DALYs) are the recommended metric in the WHO guidelines for the overall community health burden, and the tolerable recommended value is 10^{-6} DALY loss per person per year (pppy) (WHO, 2006).

Among the viruses of fecal origin that are present in reclaimed water, norovirus (NoV) is the main cause of viral gastroenteritis in people of all ages worldwide and is replacing rotavirus as the predominant gastrointestinal pathogen in children. This virus is often found

in wastewater and selected as reference virus in QMRAs in a broad variety of scenarios, including exposure to irrigated crops (Allende and Monaghan, 2015; Barker, 2014; Mara and Sleigh, 2010; Mok et al., 2014; Owusu-Ansah et al., 2017; Sales-Ortells et al., 2015). Previous epidemiological studies have demonstrated that NoV genogroup II (NoV GII), including the genotypes GII.2, GII.3, GII.4, and GII.6, is the main cause of endemic persistence and recent large outbreaks of gastroenteritis. Furthermore, another genotype, the GII.P17-GII.17 virus, emerged in 2013 and is spreading as fast as GII.4 (Kobayashi et al., 2016).

Another virus transmitted by contaminated food and water is human adenovirus (HAdV), which is highly prevalent and resistant to sewage treatment (Adefisoye et al., 2016; Calgua et al., 2013b; Grøndahl-Rosado et al., 2014). This virus has been recommended as an indicator for human fecal contamination in water (Albinana-Gimenez et al., 2009; Pina et al., 1998; Rusiñol et al., 2015; Wyn-Jones et al., 2011). However, little scientific information is available about the transmission of HAdV through vegetables. HAdVs can cause an array of clinical diseases, including conjunctivitis, gastroenteritis, myocarditis, and pneumonia (Ghebremedhin, 2014). However, HAdVs and NoV rarely cause serious illness or death although infants and people with weakened immune systems or existing respiratory or cardiac disease are at higher risk of developing severe disease. Nevertheless, the high prevalence of both viruses could make them suitable ‘indicator viruses’; adequate control of these viruses in a water reclamation system implies that other enteric viruses are also controlled.

This study characterizes the HAdV and NoV GII viral concentrations in reclaimed water based on q(RT)PCRs and removal by tertiary wastewater treatment. The main objective of

this study was to develop a Quantitative Microbial Risk Assessment for viral gastroenteritis caused by norovirus GII and adenovirus, associated with the ingestion of lettuce irrigated with reclaimed water. We use a mathematical approach that models the variability of the viral load before and after treatment and its reduction in WWTPs. Moreover, we assess the health risk associated with the consumption of lettuce irrigated with reclaimed water from two WWTPs with different tertiary treatments: conventional with flocculation, UV, chlorine, and a constructed wetland. We also evaluate the use of these viruses as indicators of virus control in reclamation systems.

2. Methods

2.1. Study site description

Two WWTPs located in the northeast of Spain were selected. WWTP 1 was designed to treat wastewater from two million inhabitants with a flow capacity of 420,000 m^3/day. WWTP 2 was designed to treat wastewater from 112,000 inhabitants with a flow capacity of 30,000 m^3/day. Both WWTPs have conventional primary and secondary treatments that consist of sedimentation and activated sludge. WWTP 1 has a tertiary treatment, with a design capacity of 3.25 m^3/s, that consists of chlorination, flocculation (Actiflo®) and low-pressure UV lamp treatment. WWTP 2 introduces 10% of the secondary treatment water into a constructed wetland that is located next to the WWTP as tertiary treatment. The constructed wetland comprises a single cell with an elongated shape and a surface area of 1 ha. It was planted with an amalgam of *Phragmites australis* and *Typha latifolia*. The wetland has planted shallow zones (water depth between 0.3 and 0.4 m), unplanted deep zones (water depth of 1.5 m), and a small island (surface area of 550 m^2). In both WWTPs, part of the reclaimed water is used by local people to irrigate the vegetables of small farms;

157 in the case of WWTP 2 in addition to the tertiary effluent studied, a small volume of the
158 treated water is chlorinated before use, but this chlorinated water has not been evaluated in
159 this study.

160 **2.2. Sampling, concentration and molecular quantification:**

161 For both WWTPs, monthly samples were taken of raw sewage, after secondary treatment
162 and after tertiary treatment for one year, the samples were collected in each sampling site
163 approximately at the same hour during the morning. At each site, 500 mL and 10 L of raw
164 and treated wastewater, respectively, were collected. Viruses in these samples were
165 concentrated using the skimmed milk flocculation (SMF) method for raw (Calgua et al.,
166 2013a) and treated water (Calgua et al., 2008). Viral nucleic acids were extracted using a
167 QIAmp Viral RNA kit (Qiagen, Inc., Valencia, CA) following the manufacturer's
168 instructions. Samples were tested for the viral pathogens HAdV (Hernroth et al., 2002) and
169 NoV GII (Kageyama et al., 2003) using real-time qPCR and RT-qPCR, respectively.
170 Undiluted and 10-fold diluted samples of the nucleic acid extracts were analyzed in
171 duplicate, including the concentrates from negative control buckets. The q(RT)PCR assays
172 of negative control buckets and four non-template controls were evaluated to demonstrate
173 that the reaction mix itself did not produce fluorescence. The virus standards were prepared
174 using synthetic gBlocks® Gene Fragments (IDT®) and quantified with a Qubit®
175 fluorometer (Thermo Fisher Scientific). Ten-fold dilutions were used to prepare samples
176 with concentrations ranging from 10^0 to 10^7 copies per reaction. The MS2 virus was spiked
177 into and monitored in all the samples as a control to ensure the efficacy of the laboratory
178 procedure.

2.3. Quantitative microbial risk assessment

The QMRA was constructed for lettuce consumption patterns to determine the DALYs following the steps suggested by the WHO guidelines (WHO, 2016) as described in the following paragraphs.

2.3.1. Problem formulation:

There is the need to evaluate the risk associated to water reuse in the irrigation of edible raw vegetables. The QMRA study will facilitate evidence base manager decision for the selection of suitable water treatments to produce irrigation water of acceptable microbiological quality when used with a vegetable such as lettuce. The reference pathogens HAdV and NoV GII were selected to provide a model to describe the viral risk of waterborne transmission through contaminated vegetables. HAdV is a double-stranded DNA virus that belongs to the *Adenoviridae* family. NoV is a single-stranded RNA that belongs to the *Caliciviridae* family. Both viruses were chosen because they are a very important cause of gastroenteritis illness in Catalonia; additionally, they are commonly found in water, are resistant to environmental degradation and differ in their sensitivity to water treatment processes such as UV light exposure (Hijnen et al., 2006; Rusiñol et al., 2015, 2014).

2.3.2. Exposure assessment:

The values reported by q-PCR correspond to number of viruses per volume unit (see Table S1, raw data expressed in GC/100mL). We used the probabilistic distributions described previously (Teunis et al. 1999,2009) in order to model: 1) the number of viruses in raw sewage, 2) the virus reduction, and 3) the number of viruses in treated water. The approach described by Teunis et al. allows an unequal number of samples before and after treatment

to be used with the advantage of including zero counts in the model. Concretely, function g in equation 1 specify the distribution in raw sewage:

$$C_{raw} = g(n, V|r, \lambda) = \frac{\Gamma(n+r)}{n! \times \Gamma(r)} \times \frac{(\lambda \times V)^n}{(1+\lambda \times V)^{n+r}} \quad (1)$$

where n is the number of viruses in a volume V (i.e. 100 mL) of raw sewage and λ and r are the scale and shape parameters of the gamma distribution, respectively.

Indeed, after a suitable transformation of the parameters r and lambda, this distribution can be written equivalently with the more familiar form of a negative binomial distribution, see Teunis for further details. Teunis propose that virus reduction (π_t) due to water treatment will follow a Beta distribution while the number of viruses after treatment *Ceff* follow the distribution described in equation (2):

$$h(k, W|\lambda, \rho, \alpha, \beta) = (\lambda \times W)^k \frac{\Gamma(r+k)}{k!\Gamma(r)} \times \frac{\Gamma(\alpha+\beta) \times \Gamma(\alpha+k)}{\Gamma(\alpha) \times \Gamma(\alpha+\beta+k)} \times_2 F_1(k+r, \alpha+k, \alpha+\beta+k, -\lambda \times W) \quad (2)$$

where *k* is the number of viruses in a volume W (i.e. 100 mL) of water after treatment, α and β are the shape parameters of the Beta distribution (π_t), which expresses the reduction in the number of viruses due to the treatment, and ${}_2F_1$ is the Gaussian hypergeometric function. The parameters were estimated by maximum likelihood following the method described by Teunis et al. (Teunis et al., 1999 and 2009) for unpaired samples.

Based on the suggestion of previous studies, the viral enumeration data were also corrected in the assessment to account for viral loss during the concentration procedure (Petterson et al., 2015). The concentration was corrected with a Beta distribution, with recoveries previously described specifically for the SMF. For HAdV data was previously described with an average recovery of 66% (Table 1) (Gonzales-Gustavson et al., 2017), and for NoV

GII, we used data from a previous study where 8 water samples were spiked showing an average recovery of 41% (Unpublished results). The recoveries when testing 50 mL raw sewage samples have been evaluated also in previous studies in the laboratory and presented equivalent results (Calgua et al., 2013b).

The scenario modeled in this study involved the consumption of lettuce irrigated with tertiary-treated water. This vegetable was chosen because lettuce potentially protects viruses from light and desiccation, thus enhancing pathogenic persistence (Petterson et al., 2001). Moreover, leafy greens, such as lettuce, are prone to contamination with pathogens as they have large surface areas, are grown in close proximity to soil, are irrigated intensively and are mainly consumed raw (De Keuckelaere et al., 2015). This paper considered only overhead sprinkler irrigation because it is the method used in the field. The transfer of viruses to lettuce by irrigation was described in a previous study (Mok and Hamilton, 2014), and its stochastic description was used here.

The in-field virus decay (Rs) and the inactivation that occurs during storage and transport (Rt) were included in the analysis based on a previous study with HAdV and MS2 (Carratalà et al., 2013) and assumed to be between 1 and 2 $\log_{10}$ in the period between the last irrigation and harvesting and between 0 and 1 $\log_{10}$ during dark storage and transport. Additionally, lettuce washing reduces virus concentrations between 0.1 and 2 $\log_{10}$ and was described here with a PERT distribution (Mok et al., 2014). To estimate the level of exposure, we assumed the daily rate of lettuce consumption in Spain to be lognormal distributed based on the national census of Spain, which described the per capita Spanish consumption of lettuce (Aecosan, 2015). Finally, the daily dose of viruses on lettuce

surfaces (d_s) ingested by consumers in the area where the lettuce irrigated with reclaimed water had been sold was calculated by:

$$d_{NoV} = C_{raw} \times \pi_t \times 10^{(-R_s - R_t - R_{wash})} \times V_{surf} \times \frac{1}{\pi_{rec}} \times I \quad (3)$$

where C_{raw} is the concentration in raw sewage per mL, π_t is the reduction in the number of viruses due to the treatment, V_{surf} represents the clinging of viruses to the lettuce, R_s is the reduction in the number of viruses on the surface due to UV light and high temperatures in the field, R_t is the reduction in the number of viruses between harvest and consumption, R_{wash} is the reduction in the number of surface viruses due to washing with water, π_{rec} is the recovery factor of the concentration method (SMF) and I is the amount of lettuce ingested per day. The general fitting parameters for the probability distributions are shown in Table 1.

The dose-response models for HAdV were developed based on infectious particles, while the data in this study are qPCR-based. An additional parameter was therefore included to estimate the dose of infectious HAdV (eq. 4): the ratio of infectious particles to genome copies (GC) detected by qPCR (R_{inf}) was between 1 and 2 logs of difference and describe with a Uniform distribution based on information published previously (Gonzales-Gustavson et al., 2017; Rames et al., 2016). For NoV, both dose-response data and wastewater data are RT-qPCR-based, so no correction was needed.

$$d_{HAdV} = C_{raw} \times \pi_t \times 10^{(-R_s - R_t - R_{wash} - R_{inf})} \times V_{surf} \times \frac{1}{\pi_{rec}} \times I \quad (4)$$

2.3.3. Health effects/dose-response assessment

Dose-response models describe the relationship between exposure and the probability of infection and illness. For NoV, the models described by Teunis et al., 2008 were used. They

described two models, one for aggregated NoV and one for non-aggregated NoV. We used the dose-response model without aggregation, assuming that WWTPs efficiently eliminated aggregates (eq. 5):

$$P_{inf}(d_s|\alpha,\beta) = 1 - {}_1F_1(\alpha, \alpha+\beta, -d_s) \quad (5)$$

where ${}_1F_1$ is the Kummer confluent hypergeometric function, α and β are the maximum likelihood estimates for non-aggregated NoV with values of 0.04 and 0.05, respectively, and d_s is the dose (Teunis et al., 2008).

The dose-response model described by Teunis et al. (Teunis et al., 2016) was used for HAdV. Only oral inoculation was considered; equation 5 was used, and maximum likelihood estimates for HAdV by the oral inoculation route were 5.11 and 2.8 for α and β, respectively.

The probability of illness given infection ($P_{ill|inf}$) considered in this study was a fixed value described in the literature: 0.5 (Kundu et al., 2013) and 0.7 (Atmar et al., 2014) for HAdV and NoV, respectively. The daily probability of illness (P_{ill}) was calculated by multiplying the probability of infection (P_{inf}) by the conditional probability of illness given infection.

To estimate the annual risk, we consider multiple exposure events to occur randomly in the period when farmers irrigate crops with the effluent during dry months (214 days per year) (Sales-Ortells et al., 2015). The annual probability of illness was estimated using equation

$$P_{ill\ annual} = 1 - \prod_1^{214}\left(1 - \mathrm{Random}(P_{ill})\right) \quad (6)$$

where $\mathrm{Random}(P_{ill})$ is a random sample from the distribution of P_{ill} (Karavarsamis and Hamilton, 2010).

290 *2.3.4. Risk characterization:*

291 Risk characterization was carried out by combining all the information of the problem
292 formulation, exposure assessment and dose-response assessment. We translated the
293 probability of illness into DALYs (pppy) as an annual disease burden output. We estimated
294 the DALYs as:

295 $$DALY = P_{ill\ annual} \times DBPC \times f_s \quad (7)$$

296 where $P_{ill\ annual}$ is the annual probability of illness per virus, DBPC is the disease burden
297 (DALYs per case) and f_s is the proportion of the population susceptible to the disease.
298 Since there is no disease burden estimation for either HAdV or NoV in Catalonia, we
299 evaluated two values used previously: a) a mix of Spanish and Dutch parameters (Sales-
300 Ortells et al., 2015); and b) Canadian parameters (Chhipi-Shrestha et al., 2017).

301 A Monte Carlo simulation of 2 x 10^5 iterations was used. Probability distributions were
302 used for most input parameters, and when distributions were fitted to available data sets,
303 parameters were determined using maximum likelihood fitting and chi-squared goodness of
304 fit statistics. All modeling and analyses were conducted in Mathematica 11® (Wolfram
305 Research, 2017). For all model scenarios, 95% quantile was calculated using the percentile
306 method. The sensitivity analysis was performed following two complementary approaches:
307 a. the Spearman correlation of each input parameter was determined with the daily
308 probability of illness as the output parameter (Vose, 2008), and b. the Fourier Amplitude
309 Sensitivity Test (FAST) which estimates the contribution of different inputs to the variance
310 of the output (Cukier et al., 1973).

3. Results

The results of the measured concentrations of HAdV and NoV GII in raw sewage and secondary and tertiary effluent, including the number of positive samples, are described in Table 2. The virus concentrations in the tertiary effluent of each WWTP and in a joint model were estimated using the measured virus concentrations in the raw sewage, and an assessment of the reduction due to secondary treatment, and of the total reduction (secondary and tertiary treatments together) using the differences between the virus concentration in raw sewage and after treatment; distributions were fitted to the data by maximum likelihood estimation (Table 3) and compared with the likelihood ratio test.

The deviances (-2*log-likelihood) in raw sewage showed that the concentration of NoV GII was the same in both WWTPs (p-value 0.408) and that the HAdV concentration was higher in WWTP 1 (p-value 0.037) than WWTP 2. Moreover, the viral concentrations after secondary treatment were also the same in both WWTPs (p-values of 0.072 and 0.287 for HAdV and NoV GII, respectively), but the concentrations after both secondary and tertiary treatments were higher in WWTP 1 for both viruses (p-values 0.009 and 0.04 for HAdV and NoV GII, respectively). This result means that the wetland removed more of both viruses than the conventional tertiary disinfection.

Maximum likelihood estimates for the best fit of the HAdV and NoV GII concentration data described the raw concentrations and concentrations that had been reduced due to the whole treatment (Table 3). The mean $\log_{10}$ reduction of HAdV and NoV GII concentration due to secondary treatments for the two WWTPs was 1 (95% confidence intervals: 0.4, 3.1) and 1.3 (0.7, 3.5), respectively. The log transformations of both viruses in the Beta

distribution that describes the whole treatment efficiency are represented in Figure 1 to demonstrate the differences between the WWTPs in terms of removal of each virus.

The estimation of the concentrations of both viruses in both tertiary effluents, as well as the main steps of the risk characterization, including dose and the probability of illness, infection and DALYs estimates in terms of viruses and WWTPs, are summarized in Table 4. The limited efficiency of virus removal by tertiary treatments results in the disease burden in all the evaluated cases not satisfying the guideline value established by the WHO (10^{-6} DALYs per year).

Sensitivity analysis suggests that the reduction in viral concentration due to treatment, the viral concentration in raw sewage and virus ingestion were the most sensitive parameters that impact the probability of illness and burden of disease (see supplementary material Tables S2-S5 for details).

With the models fully developed, we estimated the virus concentration in tertiary effluent and the virus log reduction necessary to reach the acceptable DALYs recommended by the WHO, which defines the required efficiency of each WWTP. The maximum tolerable concentration was 0.5 GC/100 mL for both viruses when reclaimed water is used for the irrigation of fresh vegetables. The current treatment performance and the log reduction necessary to achieve the WHO recommendation is provided in table 5.

4. Discussion

In this study, the concentrations of HAdV and NoV GII were quantified monthly for one year in two WWTPs and analyzed to characterize the viral concentrations in raw sewage and treated effluents. The changes in viral concentrations by the two WWTPs, both with

conventional secondary treatments but different tertiary treatments, were compared. The virus concentrations found in raw sewage were similar to those of other raw sewage in Mediterranean areas (Calgua et al., 2013b; Iaconelli et al., 2017) and worldwide, evaluated with the same method of quantification, q(RT)PCR (Campos et al., 2016; Grøndahl-Rosado et al., 2014; Hata et al., 2013). The variations in the concentrations observed during the year showed more variability in the concentrations observed of noroviruses compared to adenoviruses, with a tendency, as expected, to present lower levels of noroviruses in the warmer months.

Although q(RT)PCRs overestimate infectious virus concentrations because they do not differentiate between infectious and non-infectious viral particles, and hence may also underestimate treatment efficacy, q(RT)PCRs are the method of choice to quantify viruses in water because they are very efficient in detecting viruses. Moreover, q(RT)PCR is currently the only method available to quantify NoV with reasonable accuracy and precision (Gerba et al., 2017).

The reductions due to secondary treatment observed in both WWTPs were in the expected range found for other WWTPs that use the same treatment including active sludge (Campos et al., 2016; Hata et al., 2013; Sales-Ortells et al., 2015; Sano et al., 2016). However, the reductions in virus concentration by the whole treatment differed between WWTPs. The wetland in WWTP2 was more efficient in reducing virus concentrations, especially with NoV GII, although the large variability in the treatment results and the surface area required to treat the water makes this process difficult to apply for virus control in large volumes. The variability in the efficacy of the wetlands may be related to the diversity of environmental conditions that could affect the virus stability in the wetlands; some of them

are related to the presence of suspended materials and animals, and differences in irradiation and temperature during the year. In addition, the quantification of tertiary treatment effectiveness in WWTP2 showed lower viral loads in the effluent, with several negative samples detected throughout the year of evaluation. For that reason, the simulated distributions of the reduction by treatment showed longer right tails than those observed in WWTP 1. The higher relative variability observed in WWTP2 suggest that this plant would require even more reduction that described in table 5 to reach 10^{-6} DALYs to guarantee acceptable values in a high number of water samples tested. Little information is available about virus removal in treatment wetlands, but lower reductions values were found in the literature than those reported in this study: approximately 2 logs of reduction were observed for coliphages (including somatic, F+ and MS2 coliphages). However, the reductions in WWTP2 for NoV GII was similar to the previously observed removal of enterovirus (4 logs) (Barrett et al., 2001; Kadlec and Wallace, 2009).

Otherwise, the more complex treatment in WWTP 1 (UV, chlorination and Actiflo®) yielded a lower reduction in concentration of both viruses than WWTP 2, but a better control of variability in the process. This comes with higher energy costs. The reduction in virus concentration by the whole treatment process in WWTP 1 was slightly lower than previously reported in the United Kingdom and Italy (Campos et al., 2016; Iaconelli et al., 2017), but similar to the reduction described in Japan (Hata et al., 2013). Treatments with activated sludge, chlorination and sand filtration achieve approximately 3 to 5 logs of reduction in *E. coli*. However, the viral reduction with the same treatments would be between 1 and 3.5 logs, which means that WWTPs are not efficient enough to address viral reductions in water (Hata et al., 2013; Ottoson et al., 2006; Petterson and Stenström, 2015;

401 Sano et al., 2016). Fecal coliform bacteria are much more readily inactivated by free
402 chlorine in comparison to more persistent viruses and protozoa (Ashbolt et al., 2001). Other
403 known tertiary treatments, such as Actiflo®, are recognized to reduce coliphage loads
404 between 1 and 3 logs under experimental conditions, but the reduction depends on several
405 factors such as the wastewater quality and sensitivity of the target microorganisms to the
406 treatment (Mok et al., 2014).

407 Adequate characterization of pathogen concentrations and removal by treatment is essential
408 for making appropriate risk assessments. Mathematical models have thus been developed to
409 address this problem and produce a better approach by combining measured virus
410 concentrations before and after treatment (Teunis et al., 1999, 2009). Microbial monitoring
411 before and after treatment is the most direct way to assess treatment efficacy (Smeets et al.,
412 2010), and these methods have been recommended in QMRA analysis (WHO, 2016). The
413 input and output samples were considered unpaired in this study because sampling the same
414 body of water before and after the treatment process is complicated. The virus
415 quantifications were used to establish a distribution of values that described the
416 concentrations of viruses in raw sewage and the treatment efficacy for viruses by WWTPs.
417 These distributions allow the incorporation of variability in virus concentration in sewage
418 and removal by treatment. These distributions were combined within a QMRA framework
419 recommended by WHO for the irrigation of vegetables with reclaimed water (WHO, 2016,
420 2006).

421 Most of the other parameters used in exposure assessment for the irrigation of vegetables in
422 this study have been described in previous risk assessment studies. However, our study
423 includes modifications that we consider important for describing the correct dose. We used

measured concentrations of HAdV and NoVGII in raw sewage and of treated waters to establish virus concentration and removal. Another modification was to include the recovery efficiency of the SMF concentration method (Gonzales-Gustavson et al., 2017). Virus recovery rates from concentration procedures and molecular methods can be quite low, resulting in underestimations of the true concentration by 1 to 3 orders of magnitude (Mok and Hamilton, 2014; Petterson et al., 2015). The recovery efficiency of a model needs to account for all steps of the concentration method. The advantage of including recovery stochastically is that these values vary between samples. However, the recovery of SMF concentration of viruses in water showed low variability (Gonzales-Gustavson et al., 2017). As a consequence, sensitivity analysis demonstrated that this factor had little impact on the risk assessment.

Another main component of QMRA is the dose-response model, which describes the relation between the dose and the likelihood of infection or illness outcomes. The choice of dose-response model can have a large impact on the overall determination of risk. Several dose-response models are available (Van Abel et al., 2017). Although some publications used the Beta-Poisson approximation and an exponential for NoV and HAdV, respectively, we chose the recently published HAdV model, which has the advantage of been established specifically for oral inoculation (Teunis et al., 2016), in contrast with the exponential dose-response model used for inhalation. The latter method was based on a respiratory HAdV strain, which limits its use in QMRA studies for enteric HAdV (Ashbolt, 2015). In addition, the hypergeometric dose-response function for NoV may include the effects of viruses that are aggregated or not, which is important because in environmental samples, this virus may be in different aggregation states. The Beta-Poisson model might not accurately

447 approximate the dose-response function when little information is available (Teunis et al.,
448 2008; Teunis and Havelaar, 2000). However, the assumption that aggregation occurs is less
449 applicable for treated water since treatment processes remove large particles more
450 effectively than small particles. Therefore, we selected the model without aggregation.
451 Additionally, models that include the effects of aggregation tends to yield a lower
452 probability of infection than models that do not include it, particularly at lower doses,
453 thereby potentially underestimating the risk (Mcbride, 2014; Van Abel et al., 2017).

454 To estimate DALYs, we used parameters described previously by Sales-Ortells et al.
455 (Sales-Ortells et al., 2015) as the years lived with disability plus the years of life lost; these
456 values describe a mix of values from Catalonia and the Netherlands due to a lack of
457 available information (Sales-Ortells et al., 2015). The results were similar to the parameters
458 of disease burden per case and susceptibility fraction described in research from Canada
459 (Chhipi-Shrestha et al., 2017) (data not shown). The Canadian parameters were used in this
460 study to estimate DALYs for HAdV because no disease burden parameters for this virus in
461 Catalonia are available. In our model, the immunity to NoV infections is not relevant to
462 modifying the proportion of susceptible individuals and the proportion of secretor-negative
463 members of the Hispanic population was considered negligible (approximately 2%) (Van
464 Abel et al., 2017).

465 The QMRA results demonstrate that the systems fail to achieve the actual recommendation
466 by the WHO of 10^{-6} DALYs pppy in both WWTPs and with both viruses. Both WWTPs
467 therefore failed to meet the threshold for acceptable risk levels, indicating that the virus
468 removal capacities of these treatments were insufficient and that additional treatments must
469 be considered before reclaimed water can be safely used to irrigate lettuce.

One of the main problems in QMRA studies is the lack of information available to establish a distribution to describe the concentration of microorganisms and the reduction in the WWTPs. Unfortunately, methods to quantify viruses after treatment often yield negative results or values that are below the limits of quantification because of their low sensitivity and the need of testing high volumes of water for accuracy. Negative results for FIB do not mean that viruses were completely removed neither (De Keuckelaere et al., 2015; Mok et al., 2014; Petterson and Ashbolt, 2016; Schijven et al., 2011; WHO, 2016).

Since sewage and secondary and tertiary effluents are not routinely tested for viruses, the occurrence of human enteric viruses in water remains largely unknown unless an outbreak is reported, and the samples that are usually collected seldom demonstrate the viral origin (Gibson, 2014; Gorchev and Ozolins, 2011). Quantification of HAdV, a DNA virus present in sewage year-round in all geographical areas could be a suitable tool for validation of treatment plants and the monitoring of reclaimed water for reuse in agriculture determining whether WWTPs are efficient enough to satisfy the WHO Guidelines. SMF is a very easy and efficient method to concentrate viruses, the current limits of detecting HAdV and NoV GII with this method are 28.6 and 291 GC/100 mL. Considering the results obtained in the QMRA study showing that the safe concentration of both HAdV and NoV GII in reclaimed water used to irrigate lettuce is 0.5 GC/100mL, further concentration methods are needed to achieve this sensitivity. Our study shows that these assays can be used in field evaluations of the concentrations of HAdV and NoV GII in sewage and of the removal efficacy of secondary and tertiary treatment processes, thus providing a foundation of evidence to assess the safety of reclaimed water systems for food crop irrigation and for the required virus removal to provide water safe for unrestricted irrigation.

493 The health risk associated with the consumption of lettuce irrigated with tertiary-treated
494 effluent from two WWTPs, considering NoV GII and HAdV, has been estimated based on
495 the quantification of realistic viral loads in the treatment. The results suggest that HAdV
496 could be used as reference pathogen to validate WWTP treatments as it shows similar risk
497 values as NoV GII.

498 5. Conclusions

499 To assess the health risk associated with reclaimed water, we used a stochastic QMRA
500 model to estimate the annual disease burden from the consumption of lettuce irrigated with
501 tertiary-treated water from two different WWTPs. Major findings are:

502 • High concentrations of NoVGII and HAdV were present in sewage.
503 • The virus removal from two WWTPs that applied either wetland or conventional
504 tertiary treatment with UV, chlorination and Actiflo® differed, with the wetland
505 treatment giving better reductions (3.9 and 2.8 logs for NoV GII and HAdV,
506 respectively) than the conventional treatment (1.9 and 2.5 logs), but with more
507 variation than the conventional treatment.
508 • Neither WWTP with tertiary treatment, on average, met the threshold of $\leq 10^{-6}$.
509 DALY pppy for an acceptable level of risk for irrigation of lettuce for HAdV and
510 NoV GII quantified by q(RT)PCR.
511 • Sensitivity analysis showed that virus reduction due to whole treatment, virus
512 concentration in raw sewage and ingestion of lettuce were major inputs influencing
513 the variability in the risk assessment.
514 • Additional virus reductions are necessary for both WWTPs to reach the WHO
515 Guideline: in the WWTP with constructed wetland: the total removal of 6.7 and 5.1

logs for NoV GII and HAdV is required, respectively; and in conventional treatment: a total removal of 7 and 5.6 logs.

- This report is the first description of a QMRA assay developed with HAdV regarding the irrigation of vegetables, showing approximately similar health risk as observed for NoV GII, even in the wetland-treated samples.
- The quantification of HAdV could be a suitable control measure in validation and monitoring programs for WWTPs producing reclaimed water for water reuse.

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Figure 1: Best fit of probability density functions of virus log reduction (eq. 2) from raw to tertiary treatment in NoV GII (left) and HAdV (Right) concentrations in WWTP 1 (light gray) and WWTP 2 (dark gray). Mean values are represented with dashed lines and its respective color for the WWTP.

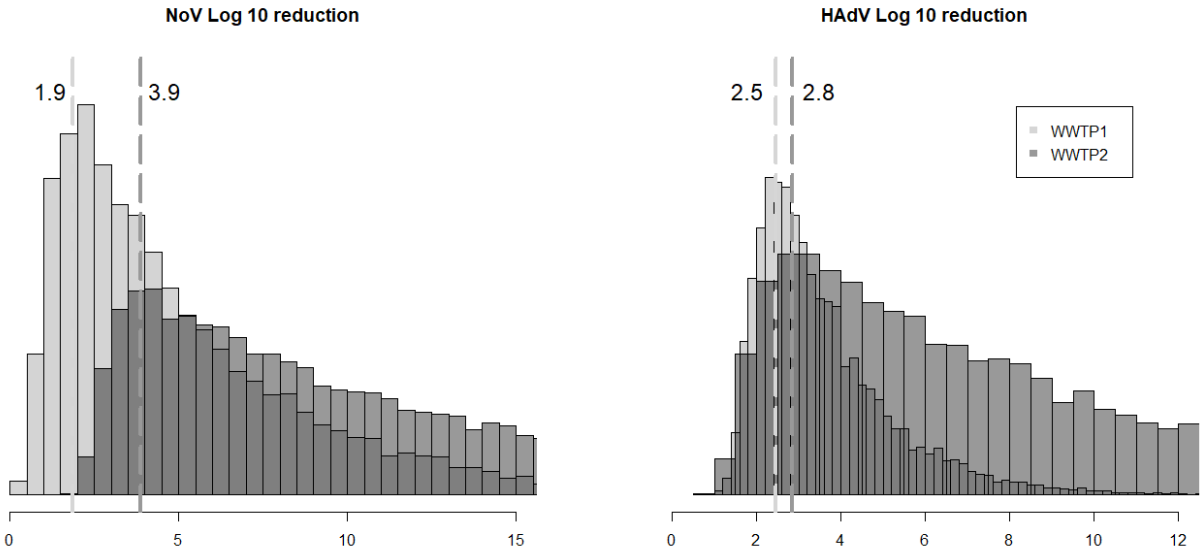

Table 1: Exposure assessment inputs, units, distributions and parameter values, and references

Model inputs	Notation	Units	Distribution	Source
Recovery HAdV	π_{rec}	proportion	Beta (52.62, 27.07)	(Gonzales-Gustavson et al., 2017)
Recovery NoV GII	π_{rec}	proportion	Beta (161, 235)	Unpublished data
Water that clings to lettuce surface through sprinkler irrigation	V_{surf}	ml/g	Lognormal3 (-4.57, 0.5, 0.006)	(Mok and Hamilton, 2014)
In-field reduction of surface virus	R_s	$\log_{10}$ units	Uniform (1, 2)	(Carratalà et al., 2013)

Reduction in viruses during transport and storage	R_t	$\log_{10}$ units	Uniform (0, 1)	(Carratalà et al., 2013)
Reduction in surface viruses due to washing	R_{wash}	$\log_{10}$ units	PERT (0.1, 1, 2)	(Mok et al., 2014)
Daily consumption of lettuce	Ing	g pppd	Lognormal (20.72, 26.35) (inf=0, sup=120)	(Aecosan, 2015)

Distribution parameters for Beta distribution (shape parameter α, shape parameter β); Lognormal3 (meanlog, sdlog, threshold); PERT (min, mode, max); Uniform (min, max).

Table 2: Observed concentrations of HAdV and NoV GII (genome copies (GC)/100 ml) in each WWTP and by type of water (see supplementary materials Table S1 for complete database).

Virus (samples)	Water	WWTP 1			WWTP 2		
		+	Mean[a]	sd	+	Mean[a]	sd
HAdV (12)	Raw sewage	12	1.98×10^5	3.15×10^5	12	6.72×10^4	7.04×10^4
	Secondary	10	2.06×10^4	3.55×10^4	12	9.62×10^3	2.54×10^4
	Tertiary	9	4.30×10^2	5.66×10^2	4	7.70×10^1	2.36×10^2
NoV GII (12)	Raw sewage	12	5.17×10^6	8.88×10^6	12	2.30×10^6	3.67×10^6
	Secondary	10	3.17×10^5	8.86×10^5	9	6.32×10^4	9.11×10^4
	Tertiary	5	1.65×10^4	2.36×10^4	3	8.22×10^1	1.80×10^2

(+) Number of positive samples; (a) mean (GC/100 ml) based on the total number of samples.

Table 3: Maximum likelihood Negative Binomial and Beta distributions parameters fitted to reported HAdV and NoV GII count concentrations (genome copies/100 ml) in raw samples and after full treatment from both WWTPs.

Virus	WWTP	Raw sewage parameters		Reduction from raw to tertiary treatment	
		r	λ	α	β
HAdV	1	0.92	2.16×10^{5}	0.26	7.56×10^{1}
	2	1.24	5.42×10^{4}	0.06	4.22×10^{1}
NoV GII	1	0.46	1.02×10^{7}	0.10	7.41×10^{0}
	2	0.34	5.86×10^{6}	0.05	3.73×10^{2}

Table 4: Mean and 95 percentile results of the QMRA for the irrigation of lettuce with tertiary-treated water of two WWTPs using HAdV and NoV GII data as virus indicators.

			HAdV		NoV GII	
	Outputs	Unit	Mean	95%	Mean	95%
WWTP1	Concentration after tertiary treatment	GC/ml	6.70×10^{0}	3.30×10^{1}	6.45×10^{2}	2.83×10^{3}
	Concentration at consumption	virus/g	2.33×10^{-5}	8.64×10^{-5}	5.90×10^{-2}	1.60×10^{-1}
	Dose	pppd	4.51×10^{-4}	1.15×10^{-3}	1.14×10^{0}	1.59×10^{0}
	Daily Probability of infection	pppd	2.86×10^{-4}	7.45×10^{-4}	3.90×10^{-2}	3.52×10^{-1}
	Daily probability of disease	pppd	1.45×10^{-4}	3.73×10^{-4}	2.80×10^{-2}	2.47×10^{-1}
	Yearly probability of disease	pppy	3.06×10^{-2}	7.01×10^{-2}	9.97×10^{-1}	9.99×10^{-1}
	DALYs	DALYs/year	1.44×10^{-3}	3.31×10^{-3}	1.94×10^{-3}	2.00×10^{-3}
WWTP2	Concentration after tertiary treatment	GC/ml	9.40×10^{-1}	4.30×10^{0}	2.50×10^{0}	5.40×10^{0}
	Concentration at consumption	virus/g	3.27×10^{-6}	7.60×10^{-6}	2.31×10^{-4}	2.53×10^{-4}
	Dose	pppd	6.27×10^{-5}	6.95×10^{-5}	5.02×10^{-3}	1.87×10^{-3}
	Daily Probability of infection	pppd	4.02×10^{-5}	4.49×10^{-5}	1.11×10^{-3}	8.25×10^{-4}
	Daily probability of disease	pppd	1.98×10^{-5}	2.30×10^{-5}	7.75×10^{-4}	5.78×10^{-4}
	Yearly probability of disease	pppy	4.23×10^{-3}	1.19×10^{-2}	1.53×10^{-1}	3.82×10^{-1}
	DALYs	DALYs/year	2.09×10^{-4}	5.87×10^{-4}	2.99×10^{-4}	7.47×10^{-4}

pppd: per person per day; pppy: per person per year; GC: genome copies

808 Table 5: Mean of the best fit distributions of reductions in tertiary effluent by each virus in

809 actual scenario and required reductions to reach suggestions of WHO (10^{-6} DALYs).

WWTP	Virus	Actual	To reach 10^{-6} DALYs
1	HAdV	2.5	5.6
	NoV GII	1.9	7
2	HAdV	2.8	5.1
	NoV GII	3.9	6.7

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