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# Prevalence of common enteric viruses in municipal wastewater treatment plants and their health risks arising from wastewater reuse

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## ABSTRACT

Enteric viruses are known to be prevalent in municipal wastewater, but information on the health risks arising from wastewater reuse is limited. This study quantified six common enteric viruses in raw wastewater and determined the effectiveness of different secondary and tertiary treatment processes at reducing their abundances in three full-scale wastewater treatment plants in China. In the raw wastewater, polyomavirus BK and norovirus GII (Nov GII) exhibited the highest abundance among the detected DNA and RNA viruses, respectively, with concentrations >5 $\log_{10}$ copies/L. Viruses in the raw wastewater were mainly removed by the secondary treatment processes, with log reduction values ranging from 1 to 2. The tertiary treatment processes of both chlorination and ultraviolet irradiation facilitated the additional reduction of viruses. The quantitative microbial risk assessment was applied to estimate the health risks of adenovirus (Adv) and Nov GII when reusing the treated wastewater for irrigation of public green spaces and crops. Estimated disability-adjusted life years of Adv and Nov GII for both reuses were higher than the risk threshold ($10^{-6}$) required by the WHO in the actual scenarios. More effective treatment technologies should be implemented to remove viruses for safe reuse of the treated wastewater.

**Key words:** enteric virus, health risk, QMRA, reuse, wastewater treatment

## HIGHLIGHTS

- Secondary and tertiary treatment processes can remove viruses from wastewater.
- Health risk induced by Adv and Nov GII for the reuse of treated wastewater is higher than the required threshold.
- More effective technologies should be applied to remove viruses for safe reuse of the treated wastewater.

## 1. INTRODUCTION

Wastewater reuse has been widely practiced for decades worldwide due to the shortage of water resources. Globally, wastewater is widely used in agriculture irrigation, landscape irrigation, and industrial purposes (Wang *et al.* 2017). It has been reported that up to 18 million hectares of land are irrigated with untreated wastewater (Verbyla *et al.* 2016), having serious human health implications for both farmers and consumers. In China, given the serious water scarcity and water pollution, water reuse is recognized as an integral part of the water and wastewater management scheme. Thus, the Chinese Government has launched nationwide efforts to optimize the use of reclaimed water (Wang *et al.* 2017; Dou & Zhu 2018). The quantity of annual recycled and reused wastewater in China reached $3.5 \times 10^9$ $m^3$ in 2013 (Wang *et al.* 2017). However, the health risks induced by human pathogens, especially human viruses, during wastewater reuse have been neglected, and few technical standards governing reclaimed water use have been developed thus far.

Municipal wastewater treatment plants (WWTPs) are important hubs of urban water cycling that receive wastewater from drainage systems. Thus, WWTPs have been considered a great reservoir for human pathogens, including bacteria, protozoa and viruses (Lu *et al.* 2015; Huang *et al.* 2018; Farkas *et al.* 2020). Over 100 types of human viruses have been detected in raw wastewater (Qiu *et al.* 2015), a large proportion of which are enteric viruses excreted with human stool and urine (Zhong *et al.* 2007; Aoki *et al.* 2010). Enteric viruses, including noroviruses

(Nov), adenovirus (Adv), astrovirus, enteroviruses (EV), and rotavirus (RV), are primarily transmitted via the fecal–oral route, by either person-to-person contact or ingestion of contaminated food or water (Gibson 2014; Castro-Mayorga *et al.* 2016). These enteric viruses, most of which are associated with acute gastroenteritis (Rodriguez-Lazaro *et al.* 2012), present a serious threat to public health for their presumed low infection dose; prolonged (3–4 weeks) and asymptomatic periods of shedding; and excellent environmental stability (Gibson 2014; Kotwal & Cannon 2014). The occurrence and abundance of enteric viruses in wastewater have been monitored in many countries including Japan (Katayama *et al.* 2008), the UK (Campos *et al.* 2016), and the USA (Schmitz *et al.* 2016). However, while WWTPs are effective in reducing viral abundance (Kitajima *et al.* 2014; Schmitz *et al.* 2016), information is limited regarding the health risks associated with the reuse of this treated wastewater.

Owing to the substantial hazards of enteric viruses within recycled wastewater, their subsequent spread in the environment should be controlled as much as possible. The abundance of enteric viruses in raw wastewater is reduced in WWTPs via secondary and tertiary treatment processes (Montazeri *et al.* 2015). However, how much reduction of virus is needed for safe recycled water is still unclear (Gerba *et al.* 2017). In the USA, California has the most comprehensive water reuse regulations, requiring a 5-log reduction in viruses after the postsecondary treatment prior to unrestricted use of recycled water in irrigation and a 12-log reduction prior to potable reuse via groundwater recharge (Chaudhry *et al.* 2015). In the case of recycled water used for irrigation, a 6- to 7-log reduction of viruses after the treatment has been suggested by the WHO (Sano *et al.* 2016). In China, there are no regulations limiting the abundance of enteric viruses, while tolerant concentrations of fecal coliforms (FCs) are specified (Chinese Standard 2010). Recently, quantitative microbial risk assessment (QMRA) has been developed and applied to reasonably evaluate virus-related health risks arising from water reuse (Chhipi-Shrestha *et al.* 2017; Simhon *et al.* 2020). QMRA estimates the pathogen dose to which the consumer is exposed, calculates the probability of infection or disease, and finally provides the value of disability-adjusted life years (DALYs) caused by viral infection (Owens *et al.* 2020). Owing to the common consumption of raw vegetables in many regions, a great number of studies have only considered the health risks caused by exposure to viruses resulting from the agricultural application of wastewater effluent (Moazeni *et al.* 2017). However, little attention has been paid to public green space irrigation (Chhipi-Shrestha *et al.* 2017), which is one of the most important wastewater reuse strategies in many cities of China.

This study quantified six types of common enteric viruses in the raw and treated wastewater from three full-scale WWTPs in Nanjing, China, so as to determine the effectiveness of the different treatment processes in reducing viral abundances. Moreover, QMRA was used to estimate the human health risk of two viral types (Adv and norovirus GII (Nov GII)) in the treated wastewater to be recycled for public green space irrigation and crop irrigation. This study deepens our understanding of the prevalence of enteric viruses in WWTPs and will help policymakers to develop science-based regulations for the reuse of treated wastewater.

## 2. MATERIALS AND METHODS

### 2.1. Collection of wastewater samples

Wastewater samples were collected from three WWTPs in Nanjing, Jiangsu Province, China, with each WWTP sampled three times from September to December in 2019. Supplementary Material, Table S1 shows the characteristics of the WWTPs. The three plants, which all received domestic wastewater, employed different secondary treatment processes, specifically an oxidation ditch, $A^2/O$ and UNITANK. Plant A utilized ultraviolet radiation as a tertiary treatment, while Plants B and C employed chlorination disinfection via sodium hypochlorite and liquid chlorine, respectively. Wastewater samples collected from the WWTPs included the raw wastewater as well as the treated wastewater following both the secondary and tertiary treatment processes.

The raw wastewater (~5 L/sample) was collected using plastic containers, delivered to the laboratory within 6 h, and then immediately processed for virus concentration upon arrival. The treated wastewater was collected, and the viral fraction from the treated wastewater was concentrated on site using a NanoCeram Virus Sampler (Argonide Corporation) at a rate of approximately 10 L/min (Karim *et al.* 2009). Water was collected until the membrane fouled beyond the point that the flow rate sharply decreased. Supplementary Material, Table S2 shows the volume of the collected effluent samples. Filters were immediately placed on ice and transported to the laboratory within 6 h.

### 2.2. Enrichment of virus-like particles from wastewater

The enrichment of virus-like particles (VLPs) from the raw wastewater was performed via the electronegative filter method (Schmitz *et al.* 2016), with slight modification. Turbid samples were first filtered with a Millipore

filter membrane (0.45 μm pore size) to remove bacteria, protozoa, and some large particles. Next, 2.5 M $MgCl_2$ was added to 2 L of filtered samples (final concentration 25 mM $MgCl_2$). Samples were subsequently passed through an electronegative filter (0.45 μm pore size; catalog no. HAWP-047-00; Millipore, Billerica, MA, USA) attached to a glass filter holder. About 200 mL of 0.5 mM $H_2SO_4$ (pH 3.0) was passed through the filter to remove magnesium ions, while VLPs were directly absorbed to the filter. Finally, 10 mL of 1.0 mM NaOH was used to elute the VLPs into a tube containing 50 μL of 100 mM $H_2SO_4$ and 100 μL of 100× Tris–EDTA buffer (pH 8.0) for neutralization. VLP concentrates were stored at −80 °C until further analysis.

The elution of VLPs from the NanoCeram Virus Sampler filter was conducted with 3% w/v beef extract buffer containing 0.05 M glycine (pH 9.5) (Karim *et al.* 2009). Filters were submerged in 250 mL of elution buffer for 1 min. Next, the inlet and outlet tubes of the filter were connected to peristaltic pump to aid elution for 10 min. A second elution was conducted using the same method as the first elution, except that the submerging time was extended to 15 min. A total of 500 mL of eluate was further concentrated via overnight polyethylene glycol 8,000 precipitation with the final concentration of 120 g/L. After centrifugation for 30 min at 10,000 × g and 4 °C, the supernatant was discarded and the pellets were resuspended in 10 mL of 0.15 M phosphate buffer solution.

### 2.3. Nucleic acid extraction, reverse transcription, and quantitative real-time PCR

Nucleic acids were extracted with the, commercial kit, Allprep® PowerViral® DNA/RNA Kit (Qiagen). DNA was directly used for quantitative real-time PCR (qPCR), while RNA was first reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with random primers prior to quantification. The qPCR was performed on the QuantStudio 3 Real-time System (Applied Biosystems) using DNA or RNA samples as templates to detect DNA viruses (human Adv, polyomavirus BK (BK), and polyomavirus JC (JC)) and RNA viruses (human enterovirus (EV), norovirus GI (Nov GI) and Nov GII). The detailed methods (qPCR mixtures, thermal cycling conditions, and quantification methods) are provided in the Supplementary Material (Text S1, and Tables S3 and S4) and described in a previous report (Schmitz *et al.* 2016). Besides, the recovery efficiency of VLPs during the enrichment process was also taken into consideration for the absolute quantification of viruses (Supplementary Material, Text S1).

### 2.4. Quantitative microbial risk assessment

The health risk of common enteric viruses was assessed by using the QMRA. This method included four steps: hazard identification, exposure assessment, dose–response assessment, and risk characterization.

#### 2.4.1. Hazard identification

Due to limited dose–response assessment data, only Adv and Nov GII were taken into consideration for further health risk assessment. Adv, a double-stranded DNA virus, is widespread in nature and can cause a wide range of infections with a spectrum of clinical manifestations, including in the gastrointestinal tract, respiratory tract, urinary tract, and eyes (Schijven *et al.* 2019). Nov GII, a single-stranded RNA virus belonging to the Caliciviridae family, is a very important cause of gastroenteritis around the world (Gonzales-Gustavson *et al.* 2019).

#### 2.4.2. Exposure assessment

In this study, probabilistic distributions were used to model the concentrations of Adv and Nov GII in the raw wastewater ($C_{raw}$), the reduction by secondary treatment ($R_t$), and disinfection treatment and concentrations of the two viruses in the final effluent ($C_{final}$) (Teunis *et al.* 2009; Gonzales-Gustavson *et al.* 2019). Gamma distribution and beta distribution were applied to fit concentrations of enteric viruses in the raw wastewater and the reduction rate for secondary and tertiary treatment processes via maximum likelihood estimation (Gonzales-Gustavson *et al.* 2019). The treated water can be used for various urban purposes based on its quality and intended applications, including the irrigation of public green spaces and crops. When the treated water was reused for the irrigation of public green spaces, the daily dose of exposed viruses ($d_{\text{public irrigation}}$) was calculated by the following equation:

$$d_{\text{public irrigation}} = C_{\text{raw}} \times R_t \times 10^{(-R)} \times (\text{Aero} + \text{Cont}) \times \frac{1}{1000} \quad (1)$$

where $C_{raw}$ is the concentration of viruses in raw sewage (copies/L), $R_t$ is the reduction of viruses due to the wastewater treatment process in WWTPs, and $R$ is the reduction of viruses before usage. Aero and Cont are the exposure amounts of water via direct inhalation by aerosol and ingestion by plant contact (mL).

When the treated water was reused for crop irrigation, the daily dose of viruses on the vegetables ingested by consumers was calculated by the following equation:

$$d_{\text{crop irrigation}} = C_{\text{raw}} \times R_t \times 10^{(-R_f - R_{t-s} - R_{\text{wash}})} \times \text{Consumption} \times \frac{1}{1000} \quad (2)$$

where $R_f$, $R_{t-s}$, and $R_{\text{wash}}$ are the reduction of viruses in the field, during the transport and storage, and due to washing, respectively, Consumption is the daily amount of wastewater ingested by daily consumption of lettuce (mL). Table 1 lists the associated input exposure factors mentioned above (Chhipi-Shrestha *et al.* 2017; Gonzales-Gustavson *et al.* 2019).

### 2.4.3. Dose–response assessment

Following previous studies (Teunis *et al.* 2009; Viau *et al.* 2011; Teunis *et al.* 2016), Supplementary Material, Table S5 shows the dose–response models used to describe the relationship between exposure and the probability of infection. The daily probability of illness ($P_{\text{ill}}$) was obtained by multiplying the daily probability of infection by the conditional probability of illness given infection ($P_{\text{ill|inf}}$), as listed in Supplementary Material, Table S5. The yearly probability of disease was then calculated using the following equation:

$$P_{\text{ill annual}} = 1 - \prod_{1}^{\text{Fre}} (1 - \text{Random}(P_{\text{ill}})) \quad (3)$$

where Random ($P_{\text{ill}}$) is a random sample from the distribution of $P_{\text{ill}}$, and Fre is the exposure frequency.

### 2.4.4. Risk characterization

Risk characterization was carried out by integrating the hazard identification, exposure assessment and dose–response assessment. The final risk was expressed in disease burden, i.e., disability-adjusted life years (DALYs) per year per person, which was calculated as follows:

$$\text{DALYs} = P_{\text{ill annual}} \times \text{DBPC} \times f_s \quad (4)$$

where DBPC is the disease burden per case (DALY/year), and $f_s$ is the proportion of the population susceptible to the disease (Supplementary Material, Table S5).

## 2.5. Statistical analyses

Log reduction values (LRVs) are used to quantitatively describe the reduction efficiency of viruses and define with the following equation:

$$\text{LRV} = \log_{10} \frac{N_0}{N} \quad (5)$$

**Table 1** | Exposure factors for different water uses

| Models inputs | Notations | Units | Distribution[a] |
|---|---|---|---|
| Public green space irrigation | | | |
| Inhalation by aerosol | Aero | mL | Uniform (0.09, 0.11) |
| Ingestion by plant contact | Cont | mL | Uniform (0.9, 1.1) |
| Reduction before usage | $R$ | $\log_{10}$ units | Uniform (1.1, 1.3) |
| Exposure frequency (per year) | Fre | | Uniform (81, 99) |
| Crop irrigation | | | |
| Ingestion by daily consumption of lettuce | Consumption | mL | Uniform (4.5, 5.5) |
| In-field reduction of surface virus | $R_f$ | $\log_{10}$ units | Uniform (1, 2) |
| Reduction in viruses during transport and storage | $R_{t-s}$ | $\log_{10}$ units | Uniform (0, 1) |
| Reduction in surface viruses due to washing | $R_{\text{wash}}$ | $\log_{10}$ units | PERT (0.1, 1, 2) |
| Exposure frequency (per year) | Fre | | Uniform (63, 77) |

[a]Parameters for uniform distribution, uniform (min, max) and PERT distribution, PERT (min, mode, max).

where $N_0$ is the abundance of viruses in the raw wastewater and $N$ is the abundance of viruses in the treated wastewater.

Analysis of variance (ANOVA) was applied to determine whether viral abundances and their reductions were significantly different among the tested samples, and Tukey's test was used for *post-hoc* analysis. A Monte Carlo simulation of 200,000 iterations was used for the QMRA. Spearman's rank correlation coefficient tests were performed to determine the associations between input parameters and output values of DALYs to describe the sensitivity of input parameters in the QMRA. By changing the input of the LRVs by steps of 0.01 in the QMRA models, we obtained a series of mean DALYs and then used linear regression to build the correlation between the LRV and the mean DALY. All statistical analyses were performed on the R platform (Team RC 2019).

## 3. RESULTS

### 3.1. Abundance of common enteric viruses in WWTPs

The qPCR was used to determine the abundance of six common enteric viruses (three DNA viruses and three RNA viruses). All six viruses were identified in the raw wastewater, secondary-treated effluent, and tertiary-treated effluent throughout the sampling period in all three Chinese WWTPs (Table 2). Among the DNA viruses, BK exhibited the highest abundance, ranging from 5.38 ± 0.39 to 5.86 ± 0.10 $\log_{10}$ copies/L in the raw wastewater. The mean concentrations of Adv and JC were both >4 $\log_{10}$ copies/L in the raw wastewater from the three WWTPs. In the effluent wastewater, the abundances of Adv and JC were <3 $\log_{10}$ copies/L with the exception of JC in Plant A, while the mean concentrations of BK were still >3 $\log_{10}$ copies/L in Plants A and B. Among the RNA viruses, Nov GII, with a mean concentration of >5 $\log_{10}$ copies/L, was more prevalent than Nov GI with a concentration of about 4 $\log_{10}$ copies/L in the raw wastewater ($P < 0.05$). However, their concentrations were similar in the effluent wastewater. EV had mean concentrations of >4 $\log_{10}$ copies/L in the raw wastewater from all three WWTPs, and its concentration decreased to 2.86 ± 0.34, 2.45 ± 0.24, and 2.47 ± 0.22 $\log_{10}$ copies/L in the effluent wastewater from Plants A, B, and C, respectively.

To estimate the effect of sampling time and location on the concentrations of the six virus types, ANOVA following two-way factors was performed to obtain the *P*-values (Supplementary Material, Table S6). No significant differences were observed for the concentrations of EV among the different sampling time points, whereas the concentrations of other viruses showed significant differences among the three WWTPs and the three sampling time points (each $P < 0.05$). Similarly, the viruses had significantly different concentrations among the three WWTPs and the three sampling times for the effluent wastewater (each $P < 0.05$), with the exception JC (Supplementary Material, Table S6).

### 3.2. Reduction of common enteric viruses by secondary and tertiary treatments

All six viruses decreased after the secondary and tertiary treatments (Figure 1), and the concentrations of FCs showed the similar changes (Supplementary Material, Figure S1). LRVs of the six virus types mostly ranged from 1 to 2 after the secondary treatment. After the tertiary treatment, most viruses exhibited an LRV >2, except Nov GI, which might be due to its relatively lower concentration in raw wastewater. The ANOVA and Tukey's test indicate that the three secondary treatment processes (oxidation ditch, $A^2/O$, and UNITANK) resulted in different reductions of all six virus types, except EV ($P < 0.05$) (Figure 1). For example, the LRV of BK in Plant C, which used the UNITANK process,

**Table 2** | Concentrations of common enteric viruses ($\log_{10}$ copies/L) in raw wastewater (RW), effluent of secondary treatment (ES), and effluent of tertiary treatment (ET) in the three WWTPs

| WWTP | Wastewater | Adenovirus (Adv) | Polyomavirus BK (BK) | Polyomavirus JC (JC) | Norovirus G I (Nov GI) | Norovirus G II (Nov GII) | Enterovirus (EV) |
|---|---|---|---|---|---|---|---|
| Plant A | RW | 4.75 ± 0.25 | 5.86 ± 0.10 | 4.74 ± 0.26 | 3.87 ± 0.13 | 5.82 ± 0.19 | 4.66 ± 0.10 |
| | ES | 3.04 ± 0.31 | 3.85 ± 0.27 | 3.39 ± 0.20 | 3.22 ± 0.12 | 4.04 ± 0.21 | 3.04 ± 0.16 |
| | ET | 2.39 ± 0.47 | 3.62 ± 0.23 | 3.12 ± 0.14 | 3.09 ± 0.08 | 3.85 ± 0.14 | 2.64 ± 0.34 |
| Plant B | RW | 4.41 ± 0.41 | 5.38 ± 0.39 | 4.45 ± 0.22 | 4.00 ± 0.15 | 5.41 ± 0.49 | 4.04 ± 0.29 |
| | ES | 3.35 ± 0.23 | 3.94 ± 0.23 | 2.93 ± 0.15 | 3.34 ± 0.17 | 4.74 ± 0.25 | 2.98 ± 0.28 |
| | ET | 2.48 ± 0.35 | 3.51 ± 0.10 | 2.75 ± 0.12 | 2.90 ± 0.17 | 2.60 ± 0.24 | 2.45 ± 0.24 |
| Plant C | RW | 4.40 ± 0.24 | 5.63 ± 0.18 | 4.67 ± 0.20 | 4.07 ± 0.18 | 5.87 ± 0.36 | 4.43 ± 0.45 |
| | ES | 2.61 ± 0.20 | 2.50 ± 0.18 | 2.87 ± 0.09 | 3.02 ± 0.20 | 4.29 ± 0.71 | 3.04 ± 0.27 |
| | ET | 1.29 ± 0.10 | 1.61 ± 0.24 | 2.35 ± 0.09 | 2.58 ± 0.13 | 3.28 ± 0.51 | 2.47 ± 0.22 |

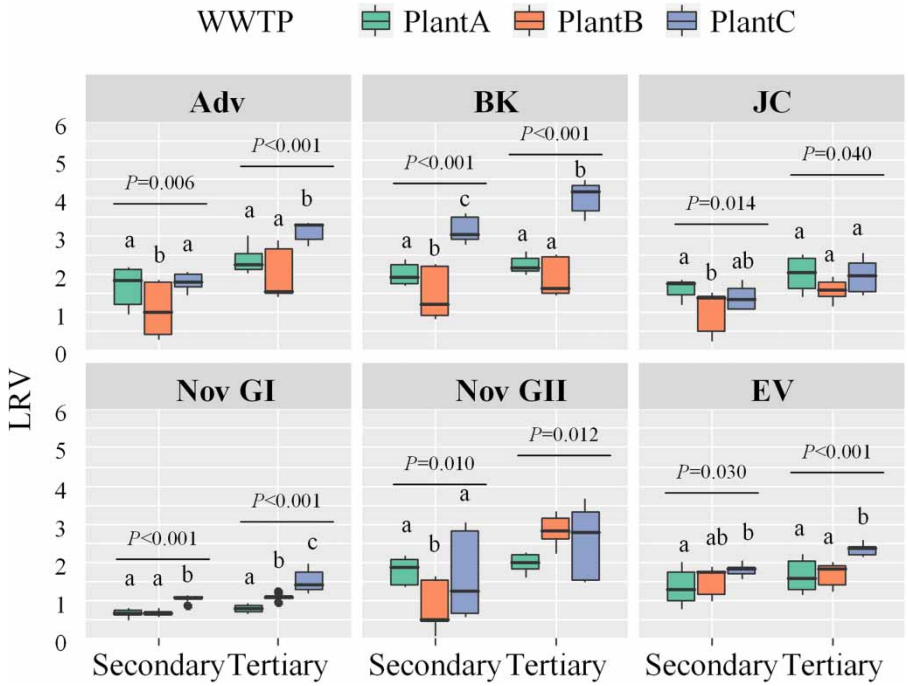


**Figure 1** | Log reduction values of the enteric viruses in the secondary and tertiary treatment processes in the three WWTPs. *P*-value indicates the effect of different treatment processes employed in the three different WWTPs on the reduction of viruses. Lower-case letters above the boxes indicate results of *post-hoc* analysis with Tukey's test. Boxes with the different lower-case letters are statistically different ($P < 0.05$).

reached 3.13 ± 0.32, while its LRV in Plant A, which used an oxidation ditch, was 2.01 ± 0.27. For Adv, BK, JC, and Nov GII, the WWTP with the oxidation ditch showed higher LRVs than the WWTP with $A^2/O$. Similar differences were also observed after the tertiary treatment using three different disinfectants ($P < 0.05$). Generally, higher LRVs for the viruses were achieved in Plants B and C, which adopted chlorination disinfection, than Plant A adopting ultraviolet radiation. ANOVA also revealed that the six viruses showed significantly different LRVs under the same treatment process ($P>0.05$, Supplementary Material, Table S7).

### 3.3. Health risk of Adv and Nov GII arising from different wastewater reuses

Estimated concentrations of enteric viruses in raw wastewater and reduction rates related to the treatment process were obtained with fitted parameters (Supplementary Material, Table S8) and used as inputs for the QMRA models. When the effluent wastewater of the three WWTPs was reclaimed for crop irrigation, the average daily exposure doses were $2.7 \times 10^{-4}$–$6.1 \times 10^{-3}$ copies and $9.3 \times 10^{-3}$–$9.1 \times 10^{-2}$ copies for Adv and Nov GII, respectively (Supplementary Material, Figure S2). In contrast, if the wastewater was recycled for public irrigation, the average daily dose of the two viruses was much higher, reaching $1.8 \times 10^{-3}$–$4.2 \times 10^{-2}$ copies for Adv and $6.4 \times 10^{-2}$–$6.3 \times 10^{-1}$ copies for Nov GII.

Considering exposure frequencies for different reuse scenarios and the morbidity of different viruses, we further obtained the yearly probability of disease for infection by Adv and Nov GII (Supplementary Material, Figure S3). The yearly probability of disease was much lower for crop irrigation than that for public irrigation. Moreover, disease via Nov GII infection showed a higher yearly probability than that infected by Adv. We further considered the disease burden and the proportion of the population susceptible to the disease to obtain the DALYs, in which case we could compare the health risk of the two viruses under a uniform criterion (Figure 2). The DALYs of both viruses were much higher than the WHO-recommended threshold of $10^{-6}$, irrespective of whether the treated wastewater was reused for public or crop irrigation. For public irrigation, the mean DALY values were $3.3 \times 10^{-4}$–$3.4 \times 10^{-2}$ for Adv and $4.0 \times 10^{-4}$–$5.4 \times 10^{-4}$ for Nov GII. For crop irrigation, the DALY values decreased to $2.9 \times 10^{-4}$–$6.1 \times 10^{-3}$ for Adv and $7.8 \times 10^{-5}$–$3.9 \times 10^{-4}$ for Nov GII. Although Adv had relatively lower values for the daily exposure dose and yearly probability of disease, it exhibited significantly higher DALYs than Nov GII due to the greater disease burden of Adv.

Similarly, significant differences of DALYs were observed among the three WWTPs in this study ($P < 0.05$). The health risk of Adv arising from wastewater reuse was lowest for Plant C and highest for Plant B. In

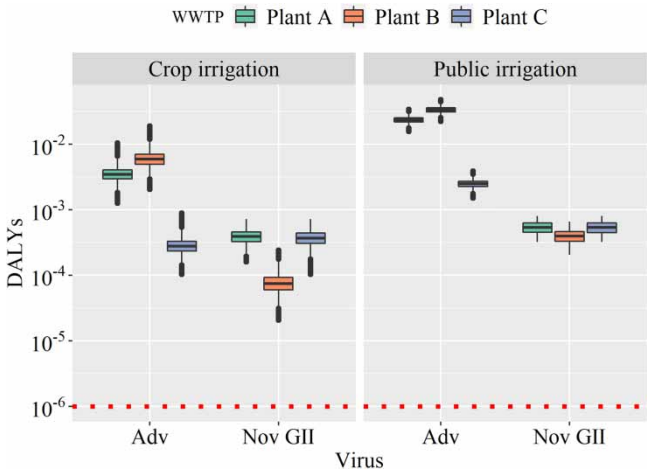


**Figure 2** | Boxplot of DALYs for different reuses of the tertiary-treated effluent using Adv and Nov II as virus indicators in the three WWTPs. Red dotted lines indicated acceptable DALYs with the value of $10^{-6}$ recommended by the WHO. The *y*-axis representing DALYs was log10 transformed.

comparison, the health risk of Nov GII was lowest for Plant B and highest for Plant A. Furthermore, sensitivity analysis demonstrated that the disease burden and proportion of the population susceptible to the disease were the most sensitive input parameters affecting the results of DALYs in the three WWTPs (Figure 3), regardless of

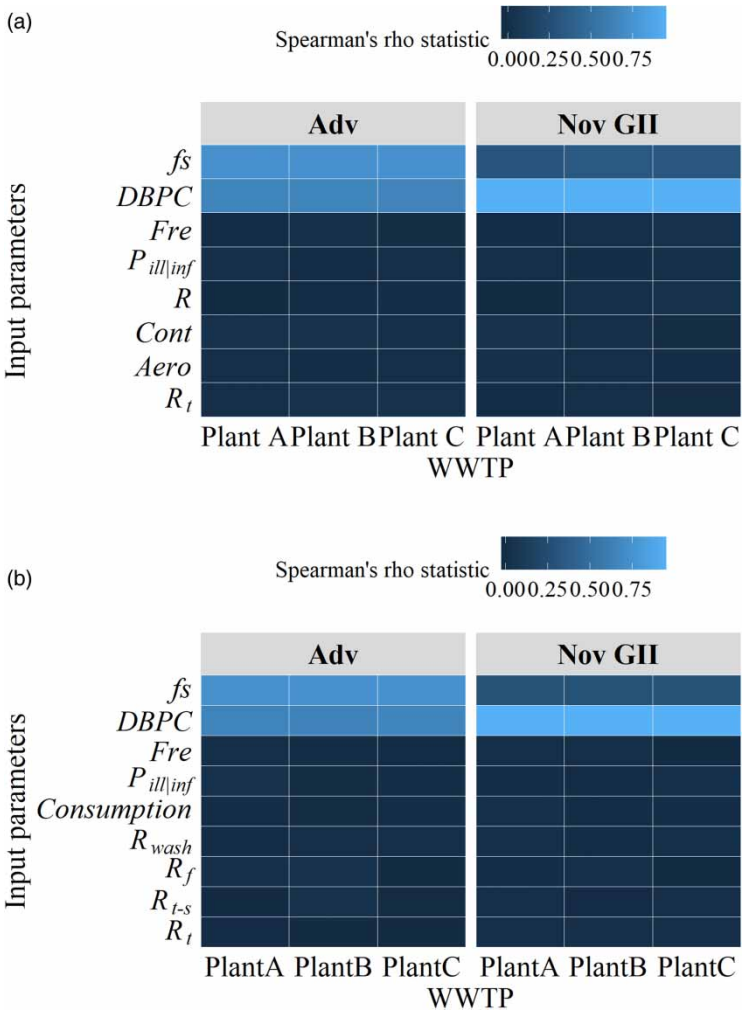


**Figure 3** | Heatmaps for the median estimates of the Spearman's rank correlation between the input parameters and DALYs for public irrigation (a) and crop irrigation (b) in the three WWTPs.

virus type and reuse scenario. With the aid of models, we determined the regression equation between the DALYs and LRV for the treatment processes in the WWTPs (Supplementary Material, Table S9 and Figure S4) in order to obtain the required LRV in the WWTPs to meet the requirement of $10^{-6}$ DALYs, as proposed by the WHO (Victor *et al.* 2008). If the treated wastewater is reused for crop irrigation, the LRVs for Adv and Nov GII should reach 5.51–5.87 and 4.60–4.97, respectively (Table 3), while higher LRVs should be reached for both Adv and Nov GII (Table 3) to meet the reuse requirements for public irrigation.

## 4. DISCUSSION

The quantification of enteric viruses in the wastewater was the first and the most fundamental step in the assessment of health risks with QMRA. Considering the relatively low levels of virus in the wastewater, especially in the effluent after the tertiary treatment process, enrichment is essential before the quantification. In this study, we adapted the method with an electronegative filter for the enrichment of VLPs in the raw sewage (Katayama *et al.* 2008; Schmitz *et al.* 2016). However, this method could only handle with the samples with small volumes; thus, it could not be applied to enrich VLPs in the effluent after secondary and tertiary treatments because of extremely low virus levels. We turned to another enrichment method with the NanoCeram Virus Sampler filter which could concentrate a larger volume of water to meet the detection limit though much more costly and time-consuming (Pang *et al.* 2012; Cashdollar & Wymer 2013). Neglection of the recovery efficiency during enrichment would cause the relatively low abundance of viruses and thus led to underestimate the health risk. In the present study, we used recovery efficiency obtained from spiking experiments to adjust the absolute quantification of viruses to get comparatively accurate results. Besides, the recovery efficiency of viruses during nucleic acid extraction, reverse transcription, and qPCR (extraction–RT–qPCR efficiency) was another factor that might underestimate virus abundances. However, the recovery efficiency during the process was very close to 100% according to a previous study, in which murine norovirus was used as sample process control to determine the efficiency (Schmitz *et al.* 2016). Thus, in the present study, the quantification of viruses was not adjusted with extraction–RT–qPCR efficiency.

All six viruses are commonly detected in WWTPs around the world. In the present study, polyomavirus BK, which is known to cause hemorrhagic cystitis in immunocompromised patients that receive bone marrow transplantation (Ahasan *et al.* 2019), showed a high prevalence in the raw wastewater. Urine is considered the main excretion mechanism by infected patients (Zhong *et al.* 2007). Similar BK concentrations (5–6 $\log_{10}$ copies/L) were also observed in wastewater from two WWTPs in Japan (Kitajima *et al.* 2014). The abundance of Adv in the untreated wastewater was also similar to the data reported in Arizona (USA) (Schmitz *et al.* 2016) and London (UK) (Purnell *et al.* 2016), but was lower than that in California (Chaudhry *et al.* 2015).

Additionally, we found that Nov GII was the dominant RNA virus during the autumn and winter in the raw wastewater from the WWTPs and showed a significantly higher abundance than Nov GI. Different prevalences of Nov GI and Nov GII have also been found in some WWTPs located in England (Campos *et al.* 2016), while WWTPs in the USA (Schmitz *et al.* 2016) and Japan (Katayama *et al.* 2008) did not show any difference between the noroviruses. In China, Nov GII, which can cause acute gastroenteritis, is the most predominant genotype infecting humans (Ao *et al.* 2017). It has been reported that 556 norovirus outbreaks occurred between October 2016 and September 2018, with 81.2% of all norovirus outbreaks typed as GII.2[P16] (Jin *et al.* 2020). Between

**Table 3** | Mean of the best fit distributions of LRV in the tertiary effluent by each virus in actual scenario and the required LRV for reuse proposed by the WHO ($10^{-6}$ DALYs)

| | | | Required LRV for reuse | |
|---|---|---|---|---|
| **Virus** | **WWTP** | **LRV in the actual scenario** | **Public irrigation** | **Crop irrigation** |
| Adv | Plant A | 2.27 | 6.84 | 5.87 |
| Adv | Plant B | 1.96 | 6.57 | 5.61 |
| Adv | Plant C | 3.16 | 6.46 | 5.51 |
| Nov GII | Plant A | 1.99 | 5.86 | 4.83 |
| Nov GII | Plant B | 2.98 | 5.59 | 4.60 |
| Nov GII | Plant C | 2.46 | 6.05 | 4.97 |

2012 and 2016, more than half of the outbreaks were reported to occur during November and December (Jin *et al.* 2020). High viral loads can usually be detected in the stool samples of infected patients, and these viruses can subsequently enter WWTPs by the urban drainage system (Strubbia *et al.* 2019). Although the duration of symptoms induced by norovirus infection usually ranges from 1 to 3 days, the average period in which norovirus is excreted in the stool is 14.3 days (Aoki *et al.* 2010).

This study indicates that the secondary treatment plays an important role in the reduction of viruses from all the three WWTPs, which agrees with previous studies (Francy *et al.* 2012; Campos *et al.* 2016). However, the different secondary treatments, namely oxidation ditch, $A^2/O$ and UNITANK, were found to have significantly different LRVs for the viruses. Many factors associated with the secondary treatment may affect the removal of viruses, including adsorption to suspended particles, biological predation within the microbial community, and deactivation by solar light or enzymes (Katayama *et al.* 2008; Chaudhry *et al.* 2015). Moreover, the mixed liquid suspended solids and hydraulic retention time of the system can exert a great influence on the reduction of virus (Katayama *et al.* 2008; Chaudhry *et al.* 2015). Even so, due to the lack of information regarding the removal mechanisms of viruses, it is difficult to determine the contribution of different external factors to the reduction efficiency. Thus, further optimization of operation parameters to improve virus reduction by the secondary treatment process remains challenging.

Disinfection is commonly adopted as a tertiary treatment process in municipal WWTPs to further prevent the spread of human pathogens (Gerba & Pepper 2019). In this study, we found that ultraviolet disinfection provided limited reduction of most tested viruses when compared with chlorination disinfection, which is consistent with a previous study conducted in Canada (Qiu *et al.* 2015). Although ultraviolet irradiation technology has been widely used for wastewater disinfection due to its safety and economic benefits, viral reduction rates highly depend on the quality of the wastewater, especially with respect to turbidity (Hijnen *et al.* 2006; Gray *et al.* 2014). Compared with ultraviolet disinfection, which mainly targets nucleic acids and renders the genome non-replicable, chlorination disinfection can simultaneously damage genomes and proteins, and inhibit host-cell recognition/binding (Wigginton *et al.* 2012). In addition, previous studies have also shown that ultraviolet disinfection is more effective in reducing bacterial pathogens than virus, especially Adv (Gray *et al.* 2014; Song *et al.* 2016). Thus, a greater ultraviolet irradiation dose is needed to meet the demand of virus reduction.

Insufficient reduction of viruses is an important cause of high health risks associated with wastewater reuse. Though the utilization of recycled water is encouraged in China, little attention has been paid to the health risks induced by virus contamination during the recycling. A standard for landscape irrigation using recycled water (GB/T 25499-2010) was released in 2010 (Chinese Standard 2010). However, the only guideline concerning pathogens is FCs, with a threshold of 200 CFU/L for unrestricted access green spaces, such as those of public parks, campuses or communities. It is a more restricted standard when compared with the Discharge Standard of Pollutants for Municipal WWTPs (Chinese Standard 2002). In the present study, FCs were not detected in the final effluent of Plants B and C, while the mean FC concentration in Plant A was 291 CFU/L, which is slightly higher than the guideline for irrigation (Supplementary Material, Figure S1). Notwithstanding, the effluent from the three WWTPs did not satisfy the WHO-recommended threshold from the point of view of health risks. Indeed, the LRVs for Adv and Nov GII should be higher than 6 and 5, respectively. Thus, advanced treatment processes, such as membrane filtration, should be supplemented in WWTPs for recycling water in order to obtain further viral reduction. Direct ultrafiltration using membranes with nominal molecular weight cutoffs of 1–100 kDa can effectively remove viruses through size exclusion. For example, an LRV of $>4$ was achieved using a membrane with a nominal molecular weight cutoff of 1 kDa (Shirasaki *et al.* 2017). One study conducted in Canada also found that after secondary treatment processes, membrane ultrafiltration with a nominal pore size of 0.04 μm could achieve a log reduction of $4.85 \pm 0.59$ for Adv and $4.55 \pm 0.66$ for Nov (Qiu *et al.* 2015).

Different DALYs of the viruses associated with public irrigation and crop irrigation indicate that the proper reuse of recycled water is important for controlling the health risk. When the recycled water was reused for public irrigation, higher DALYs were observed when compared with crop irrigation. Although people are exposed to a larger volume of water, the number of viruses attached to lettuce might be greatly reduced in the field and may undergo additional slight reduction because of storage and washing (Yates *et al.* 1987; Beuchat 2002; Li *et al.* 2015). In contrast, people are more directly exposed to the viruses via ingestion and plant contact when the recycled water is used for irrigation of public green spaces (Page *et al.* 2014; Simhon *et al.* 2020). Furthermore, consumption of raw leafy vegetables is uncommon in Chinese dietary habits. Thus, the DALYs caused

by crop irrigation may be overestimated. By comparison, a common reuse scheme implemented in China is to irrigate public green spaces and recharge into rivers and groundwater (Yi *et al.* 2011).

Besides, Adv showed to have higher DALYs than Nov II in the sampling period for the different reuse scenarios. As the outbreaks of Nov II usually occur during September and December (Jin *et al.* 2020), the abundance and the induced health risk of Nov II might reach the peak in this period. However, Adv does not share the same prevalence periodicity with Nov II since the outbreaks of Adv can take place in any season and there is no time specificity for its infections (James *et al.* 2007; Biggs *et al.* 2018; Killerby *et al.* 2019). Thus, temporal variation of the abundance of viruses in WWTPs should be deeply understood for effective control of the induced health risk.

We conducted a detailed evaluation on the health risks of viruses in treated wastewater for recycling based on a QMRA framework; however, this study still had some limitations, especially regarding dose–response models and input parameters concerning disease burden. The variation of dose–response models and their parameters were not considered, which might cause epistemic uncertainty of the DALYs (Donald *et al.* 2011). Thus, we adopted updated models of Adv and Nov GII dose–responses proposed by Teunis *et al.* (2008, 2016), which have been used in many published studies (Poma *et al.* 2019; Schijven *et al.* 2019). Additionally, sensitivity analysis showed that the disease burden and proportion of the population susceptible to the disease had the greatest impact on the DALY results. Owing to the lack of related studies in China, the disease burden distributions were sourced from studies conducted in Canada and Australia (Gibney *et al.* 2014; Chhipi-Shrestha *et al.* 2017). Thus, further studies on the input parameters for exposure assessment, dose–response models, and risk characterizations should be carried out to increase the accuracy and reduce epistemic uncertainty of QMRA results.

## 5. CONCLUSIONS

This study investigated the abundance of common enteric viruses in raw wastewater and the effectiveness of different secondary and tertiary treatment processes in reducing viral abundances in three full-scale WWTPs in the same city of China. Polyomavirus BK and Nov GII exhibited the highest abundances among the detected DNA and RNA viruses, respectively, with concentrations $>5$ $\log_{10}$ copies/L in the raw wastewater. Viruses in the raw wastewater were mainly removed by the secondary treatment processes in the three WWTPs, and the log reduction values ranged from 1 to 2. Tertiary treatment processes including chlorination disinfection and ultraviolet disinfection could provide additional reduction of viruses. When the recycled water was used for public or crop irrigation in actual scenarios, the estimated DALYs were above the WHO-recommended threshold of $10^{-6}$ for both Adv and Nov GII. Thus, more effective treatment technologies must be implemented to remove viruses to meet the health requirements proposed by the WHO.

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## DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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