Applicability of infectivity assay for the quantification of infectious human adenovirus genotype 5 in UV-irradiated wastewater

Surapong Rattanakul and Kumiko Oguma

ABSTRACT

The use of infectivity assays in domestic wastewater samples is limited because of the concerns around cytotoxicity to host cells, thus, the UV inactivation efficiency of human adenoviruses (HAdVs) in wastewater remains unclear. In this study, a human adenovirus genotype five (HAdV-5) host cell line (A549 cells) was incubated with wastewater at varied dilutions from 1:1.5 to 1:4 (the ratio of wastewater to a mixture of wastewater and the cell culture medium) and the cytotoxicity was assessed by the cell morphology and viability. No change was observed in either cell viability or morphology in comparison with control samples, even at lowest dilution of 1:1.5, indicating the dilution allowed infectivity assays. The minimal degree of dilution to avoid cytotoxicity may differ with different water matrix. Consequently, the technique was applied to quantify spiked HAdV-5 after the UV disinfection of wastewater. A significant increase in UV disinfection efficiency was noted in wastewater and hydroxyl radicals (OH•) produced by the photosensitization of dissolved organic matter were suggested to be responsible for the enhancement. This study indicated that dilution can be a simple solution to avoid cytotoxicity, and UV inactivation may be enhanced in wastewater due to OH• radicals produced by UV radiation.

Key words | adenovirus, cytotoxicity, hydroxyl radicals, infectivity assay, UV disinfection

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HIGHLIGHTS

- Applicability of infectivity assay for wastewater samples was confirmed.
- Dilution suppressed cytotoxicity to viral host cell.
- Enhanced human adenovirus inactivation in wastewater by UV was observed.
- Dissolved organic matter involved in hydroxyl radicals formation under UV exposure.

INTRODUCTION

Human adenoviruses (HAdVs) are non-enveloped and double-stranded DNA viruses with a diameter of approximately 90–100 nm. They have 85 recently identified genotypes, which are classified into seven species (A–G), principally on the basis of phylogenetic calculations of informative viral proteins and genomic organization. Clinically, HAdVs are causative agents of various diseases, such as gastroenteritis (HAdV-40 and -41), epidemic keratoconjunctivitis (HAdV-4 and -7), pharyngoconjunctival fever (HAdV-3 and -14), acute hemorrhagic cystitis (HAdV-11 and -21), and hepatitis (HAdV-2 and -5) (Van *et al.* 1992; CDC 2017). Several studies have reported the detection of HAdVs in water bodies, including rivers, lakes, water resources, and sewage, with a mean value of

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3.87×10^4 genome copies/mL (Bofill-Mas *et al.* 2006; Park *et al.* 2010; Kishida *et al.* 2012). The first water-related outbreak of HAdV infection was reported in 1953, known as the Greeley epidemic. The outbreak resulted in a total number of 206 cases, with symptoms of conjunctivitis, keratitis, muscle pain, and fever after attending a local swimming pool (Cockburn 1953). Lately, numerous adenovirus outbreaks associated with recreation water have been reported since the cell culture technique became available (Martone *et al.* 1980; White & Fenner 1994; van Heerden *et al.* 2005). Three adenovirus outbreaks associated with drinking water were also documented as a result of inadequate disinfection (Kukkala *et al.* 1997; Villena *et al.* 2003; Divizia *et al.* 2004). Owing to public health implications and their prevalence, HAdV was listed on the Contaminant Candidate List 4 (CCL4) for a fourth time by the U.S. Environmental Protection Agency (EPA) (USEPA 2016). Therefore, to prevent HAdV contamination and transmission through water bodies, effective control methods in drinking water and wastewater treatment plants are needed.

Chlorination is a conventional method for the control of pathogens, including HAdVs, at wastewater treatment plants, and helps to reduce the deterioration of natural water. To estimate disinfection efficiency, the number of infectious virus particles before and after treatment needs to be determined. Typically, an analysis of clean water samples (such as lab-grade water, buffered saline, or drinking water) can be easily performed by infectivity assays, but wastewater is a challenge. Cytotoxicity, the quality of being toxic to cells, caused by some components in the wastewater, can result in cell lysis or genome damage, and this situation can cause failure of the infectivity assays. Quantitative polymerase chain reaction (qPCR)-based techniques are instead applied to wastewater samples and can effectively be used with a high accuracy for a wide range of water qualities (Hata *et al.* 2013; Prevost *et al.* 2015). However, the application of simple qPCR assays for the quantification of infectious HAdVs in chlorinated wastewater may not be suitable because it has shown a wide range of correlation between infectivity loss and genome damage which depended on the primer sets and target genes used (Page *et al.* 2010; Gall *et al.* 2015). As chlorine species can degrade a viral capsid and subsequently genome, the application of ethidium monoazide (EMA) and propidium monoazide (PMA) coupled with qPCR as PMA/EMA-PCR assays to chlorine-inactivated HAdVs have been suggested rather than the simple qPCR assays and yet have not been able to perfectly assess the infectivity loss (Leifels *et al.* 2015, 2019).

Ultraviolet (UV) light disinfection has become a popular method for both water and wastewater treatment plants, because it yields no disinfection by-products that can cause adverse effects on human health or on aquatic life, as chlorination does (USEPA 2006; Reckhow *et al.* 2010). Nonetheless, the estimation of the UV disinfection efficiency of wastewater treatments by qPCR-based techniques is questionable, similar to chlorination. The UV inactivation mechanism primarily generates thymine dimers in the viral genome at random locations, and application of the simple qPCR assays covering a small section of HAdV genome revealed no correlation between the number of genomic damage sites and HAdV inactivation during UV treatment (Bosshard *et al.* 2013). A long-range qPCR assay which covers almost the entire HAdV genome showed great agreement between genome reduction and infectivity loss (Eischeid *et al.* 2009; Rodríguez *et al.* 2013; Beck *et al.* 2014), but procedures seemed to be complicated in practice. As UV primarily targets viral genomes, damage to viral capsid was subtle and the application of PMA/EMA-PCR assays to UV-inactivated HAdVs also failed to estimate the number of infectious HadVs, as reported by Sangsanont *et al.* (2014) and Leifels *et al.* (2015). Additionally, integrated cell culture PCR or ICC-PCR assay, an alternative qPCR-based technique, was shown to be a more reliable and accurate method to allow for an estimation of infectious HAdVs, but still it provided a wider range of detection (Ryu *et al.* 2015).

Although qPCR-based techniques have received a lot of attention recently, they seem not to be effective for UV-irradiated samples. Therefore, the old-school method, an infectivity assay, seems to be the best choice to estimate infectious HAdVs after UV disinfection. In this study, we investigated and verified the applicability of an infectivity assay by applying to quantification of HAdV-5 in UV-irradiated wastewater. The HAdV-5 was chosen to be a surrogate genotype for human adenoviruses because it has been well-characterized and can be cultured in the laboratory easily.

MATERIALS AND METHODS

The virus strain, cell line, and infectivity assay

Bacteriophage MS-2

Bacteriophage MS-2 (ATCC® 15597-B1™) and its responsive host, *Escherichia coli* C-3000 (ATCC® 15597™) were purchased from the American Type Culture Collection (ATCC, USA) and the infectivity assays were performed by using a single layer agar technique (USEPA 2018). The number of bacteriophage MS-2 is reported in plaque-forming units per mL (PFU/mL). Bacteriophage MS-2 was studied because it is widely used as a surrogate for enteric viruses including human adenoviruses and it is highly resistant to UV disinfection (USEPA 2006; Boczek *et al.* 2016).

Human adenovirus genotype 5

Human adenovirus genotype 5, HAdV-5 (ATCC® VR-5™), and its host cells, the A549 cell line (ATCC® CCL-185™), were purchased from the ATCC. Infectivity assays of HAdV-5 were performed using the A549 cell line. The cells were cultured in six-well cell culture plates (Iwaki, Japan) with the KF-12 Ham medium (Sigma-Aldrich, USA) supplemented with 2% of fetal bovine serum, an antibiotic-antimycotic solution (100×) (Gibco, USA), and 250 μg/mL of amphotericin B at 37 °C in a 5% CO_2 incubator for approximately 4 days. When the monolayer of the A549 cells achieved 80–90% confluence, the cells were inoculated with 1 mL samples containing HAdV-5, overlaid with 1.25% EPI agar supplemented with Eagle's minimum essential medium (Nissui, Japan), and incubated again for 1 week at 37 °C and 5% CO_2. The results of the cytopathic effects on the cell line allowed us to determine the density of the infectious HAdV-5 particles in the samples after staining with 0.3% neutral red, and the number of HAdV-5 particles is reported in the unit of PFU/mL.

Water sample preparation

Phosphate buffered saline

Phosphate buffered saline or PBS (1/15 mol/L pH 7.2) was prepared by mixing PBS powder (FUJIFILM Wako Pure Chemical, Japan) into 1 L of DI water and sterilized in an autoclave.

Wastewater from plant A

To investigate apparent effects of constituents in domestic wastewater on cytotoxicity and virus inactivation efficiency, raw wastewater samples in dry weather were used because they contained high concentrations of those constituents and also represented the worst scenario for assessing a minimal degree of dilution to prevent cytotoxicity. Ten liters of raw wastewater with an average influent of 14,510 m^3/d were collected at a domestic wastewater treatment plant located near Tokyo, Japan. The wastewater treatment plant area is 44,625 m^2 and it receives wastewater produced from 37,238 inhabitants. The samples were allowed to settle for 30 min to remove grit, sand, and large suspended solids. Then, 500 mL of the supernatant (referred to as pretreated wastewater) was either directly used for the HAdV-5 UV disinfection experiments or was filtered through a 0.2 μm cellulose acetate membrane (Advantec, Japan) for cytotoxicity testing, in order to prevent bacterial and fungal contamination. Raw wastewater samples were collected three times or batches on different dates. Only the first two batches of the wastewater samples were used for the UV experiments, and all three batches underwent physico-chemical analysis.

Wastewater from plant B

Five liters of wastewater were collected in dry weather from a domestic wastewater treatment plant in Bangkok, Thailand. The wastewater treatment plant area is 14,400 m^2 with a maximum capacity of 65,000 m^3/d, and it receives wastewater from a service area (44 km^2) with 177,000 inhabitants. The wastewater samples were pretreated by allowing settlement of suspended solid for 30 min and the supernatant was subsequently exposed to UV for 2 min to inactivate indigenous bacteriophage. The pretreated wastewater was further used for either the UV disinfection experiments of bacteriophage MS-2 or the investigation of hydroxyl radical production. Three batches of raw wastewater were collected and analyzed.

Drinking water

After the chlorination process, 5 L of treated drinking water was collected three times, independently, at a water treatment plant in Tokyo, Japan, and the samples were dechlorinated by aeration for 24 h at 20 °C, until the residual chlorine was not detectable by a colorimetric method (HACH DR900, HACH®, USA). Next, the dechlorinated drinking water was either employed for UV disinfection experiments or filtered through a 0.2 μm cellulose acetate membrane for cytotoxicity testing. The water sample characteristics of raw wastewater and drinking water samples are presented in Table 1.

Measurement of hydroxyl radical production

A probe compound, *para*-chlorobenzoic acid or *p*CBA (Merck, USA) was chosen to investigate photochemical formation of hydroxyl radicals (OH•) in wastewater under UV exposure because of its high reactivity with OH• radicals. A *p*CBA stock solution of 100 μM was prepared in ultrapure water, and further mixed into either wastewater samples or ultrapure water to obtain working solution with an initial concentration of 2.4 μM. Samples containing *p*CBA were exposed to UV light with various exposure times, and then were further collected and filtered through a 0.2 μm membrane filter. *p*CBA concentration was analyzed by high-performance liquid chromatography (Water Alliance™, USA) installed with a photodiode array detector and a Vertisep™ AQS C-18 column (4.6 × 150 mm) with a 5 μm particle size. A mobile phase for the *p*CBA detection included 50% of orthophosphoric acid (pH 2.3) and 50% of methanol at 1 mL/min of flow rate. Decrease of *p*CBA concentration indicated an indirect formation of OH• radicals and the steady-state concentration of OH• radicals was calculated from the following equation because of its short lifetime:

$$d[pCBA]/dt = -k_{OH\bullet,pCBA}[pCBA][OH\bullet]_{ss}$$

where, $k_{OH\cdot,pCBA}$ is the rate constant of reaction between *p*CBA and OH• radicals ($5.2 \times 10^9\ M^{-1}s^{-1}$), [*p*CBA] is the concentration of *p*CBA (M) and $[OH\cdot]_{ss}$ is the steady-state concentration of OH• radicals (M).

Cytotoxicity tests

To investigate the effects of wastewater strength (dilution) on the A549 cell line, filtered wastewater samples were diluted with the KF-12 Ham medium supplemented with nutrients at various dilutions (wastewater:a mixture of wastewater and the culture medium), including 0:1 (control), 1:4, 1:2, 1:1.5, and 1:1 (no dilution). Next, the diluted wastewater samples were incubated with 90% confluent A549 cells at 37 °C and 5% CO_2 for 2 h. After that, the diluted wastewater samples were discarded, and the

Table 1 | Characteristics of drinking water and of raw domestic wastewater

		Raw domestic wastewater ($n = 3$)	
Characteristic	**Drinking water ($n^c = 3$) concentration ± S.D.[a]**	**Plant A concentration ± S.D.**	**Plant B concentration ± S.D.**
pH	7.90 ± 0.06	7.62 ± 0.07	7.84 ± 0.08
UV_{254} absorbance (cm^{-1})	0.002 ± 0.001	1.19 ± 0.17	0.258 ± 0.002
TOC (mg/L as C)	0.88 ± 0.04	N/A	N/A
Chloride (mg/L as Cl^-)	11.15 ± 0.51	N/A	N/A
Nitrate (mg/L as NO_3^-)	3.58 ± 0.11	N/A	N/A
BOD_5 (mg/L)	N/A	186 ± 21.2	32.4 ± 5.6
TSS (mg/L)[b]	N/A	225.5 ± 31.59	42.6 ± 3.97

N/A, not applicable.
[a]Standard deviation.
[b]TSS was measured before settling.
[c]number of samples.

cells were washed twice with a sterile phosphate buffer, which was replaced by the KF-12 Ham medium with incubation for 0, 6, 12, and 24 h. After each designated incubation period, the cell morphology was examined under an inverted microscope equipped with a camera (Toshiba, Japan), so as to evaluate the cell appearance and shape. Namely, the cells were examined thoroughly in culture wells, and photographs were taken at the center of the culture wells as a representative. A Trypan blue exclusion assay was employed for the investigation of cell viability. This assay allows researchers to determine the number of viable cells in a cell suspension, and can be performed by detaching A549 cells with 0.25% trypsin (Sigma-Aldrich, USA); mixing the cell suspension with an equal volume of a 0.4% Trypan blue dye solution prepared in 1/15 M PBS, pH 7.2 (Sigma-Aldrich, USA); loading the mixture into a hemocytometer; and then counting the numbers of both blue-stained cells (nonviable cells) and unstained cells (viable cells), sequentially. The cytotoxicity test was not performed on treated drinking water, because there were no toxic effects on A549 cells in the preliminary experiments (data not shown).

HAdV-5 infectivity assay

Infectivity assays were performed by filtering wastewater samples containing HAdV-5 through a 0.2 μm cellulose acetate membrane to remove suspended solids and bacteria. By using the cellulose acetate membrane, the retention of a non-enveloped virus (e.g., HAdV) is negligible (Field 1974; Pierre *et al.* 2010). Subsequently, the filtered samples were diluted with suitable dilutions investigated in the cytotoxicity tests to suppress the cytotoxicity effects on A549 cells, and then the infectivity assays performed as described above.

Virus recovery

Spike-and-recovery experiments were performed in water samples with a stock of either HAdV-5 (2×10^8 PFU/mL) or bacteriophage MS-2 (3.5×10^{11} PFU/mL), before UV inactivation experiments. Briefly, the pretreated wastewater, drinking water samples and PBS prepared as described above were spiked with an aliquot of either HAdV-5 or bacteriophage MS-2, thoroughly mixed, and collected to measure recovered HAdV-5 or bacteriophage MS-2 by the infectivity assays. Recovery efficiencies were estimated according to a recent study (Li *et al.* 2019).

The UV inactivation experiments

Samples for the UV inactivation experiment were prepared by adding the same stock of either HAdV-5 or bacteriophage MS-2 into pretreated wastewater, drinking water samples and PBS, in order to obtain a final concentration of approximately 10^6 and 10^8 PFU/mL for HAdV-5 and bacteriophage MS-2, respectively. Two collimated beam systems were used for UV inactivation experiments because of a difference in locations of wastewater treatment plant.

For HAdV-5, 35 mL of 16 wastewater samples and six drinking water samples from each batch were then exposed to UV light by placing the samples 51 cm below a collimated beam system consisting of a 15-Watt low-pressure UV lamp (Hitachi, Japan). The incident fluence rate at 254 nm on the surface of a sample was 0.278 mW/cm^2, as measured by means of a radiometer (UVR-2 UD25, TOPCON, Japan). These data were corrected for water, reflection, Petri, and divergence factor, thus resulting in an average fluence rate of 0.261 mW/cm^2 for drinking water and 0.132 mW/cm^2 for wastewater, according to the standard method (Bolton *et al.* 2015). The samples were collected at designated exposure time points so as to measure the concentration of HAdV-5 with infectivity assays. A negative control experiment for each type of sample, namely, drinking water or wastewater without added HAdV-5 was conducted in parallel, so as to confirm that the plaque formation was neither attributable to other viruses nor indigenous adenoviruses that may be present in drinking water and wastewater samples. Based on the result, there were no plaques observed, indicating that in the volume analyzed without concentration, no indigenous adenoviruses were present in either the drinking water or wastewater samples.

Besides HAdV-5, protocol for UV inactivation experiments of bacteriophage MS-2 were similar to HAdV-5 except for the use of 16-Watt low-pressure UV lamp (JuGuangUV, China) in a second collimated beam system. Average fluence rates of 0.608 and 0.520 mW/cm^2 for PBS and wastewater, respectively, after adjustment using the same protocol, were acquired for the second collimated beam system.

Modeling of inactivation kinetics and statistics

To determine the inactivation constants of different types of water samples, the Chick–Watson model (Chick 1908; Watson 1908) was chosen, which can be described as follows:

$$\mathrm{Log}_{10}(N_t/N_0) = -kF$$

where, N_0 is the number of HAdV-5 or bacteriophage MS-2 particles at time zero (PFU/mL), N_t is the number of HAdV-5 or bacteriophage MS-2 particles at exposure time t (PFU/mL), k is the inactivation rate constant (cm^2/mJ), and F is the UV fluence (mJ/cm^2).

Analyses of variance (ANOVA) and covariance (ANCOVA) were applied to identify the differences in A549 cell viability after different incubation periods and HAdV-5 or bacteriophage MS-2 inactivation rate constants in different water samples, respectively, where p-value less than 0.05 indicated a significant difference.

RESULTS AND DISCUSSION

Toxicity of wastewater to A549 cells

The A549 cells were first exposed to a series of pretreated wastewater dilutions, and their viability was determined after different incubation periods, as shown in Figure 1. It was found that the cell viability rates at any dilution were not significantly different ($p > 0.05$, ANOVA) from the cell viability of the control samples containing no wastewater for all of the incubation periods tested in this study. This result indicated that the dilution of wastewater even at a low dilution of 1:1.5 suppressed the cytotoxic effects of some constituents of wastewater, and resulted in a high cell viability of approximately 85% after 0–12 h incubation. The high viability was suggestive of the integrity of A549 cells, as can be confirmed by their appearance under a microscope (Figure 2). The cell shape and distribution on the culture plates for all of the diluted pretreated wastewater samples were similar to those of the control samples, except for the no-dilution sample. The results were consistent with previous studies reporting that cytotoxicity to mammalian cells decreased with higher dilutions of organic contents in wastewater (Dong *et al.* 2016).

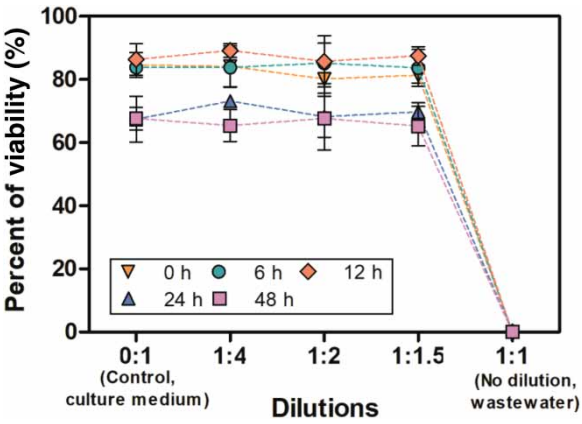

Figure 1 | A549 cell viability after exposure to wastewater at different dilutions for different incubation periods. The bars indicate the standard deviation of the triplicate experiments.

Moreover, similar results were noted in the samples with longer incubation periods, namely, incubation from 24 to 48 h. Nevertheless, the extension of the incubation time lowered the rate of cell viability to ~70%, and there might be a few possible explanations for this phenomenon. For example, when the incubation periods were prolonged beyond 24 h, nutrients could be used up and become deficient, resulting in cell death (McLean *et al.* 1997; Battaglia-Hsu *et al.* 2009). The senescence or aging of mature cells during the culture can also affect cell viability.

The exposure of A549 cells to raw pretreated wastewater (dilution = 1:1) immediately changed the shape of the cells to a circular one, as shown in Figure 2, at 0 h. This adverse change was clearly produced by constituents of wastewater and caused the death of A549 cells, because their viability dropped to zero (Figure 1). Subsequently, when the raw pretreated wastewater-exposed A549 cells were incubated further, they completely disappeared through a cytolysis process where only the cell debris remained, and no viable cells were observed. In conclusion, our results clearly showed that raw pretreated wastewater had toxic effects on the A549 cells, but these effects can be easily suppressed by the dilution of wastewater with the culture medium, at least with a dilution of 1:1.5. The chemical species inducing cytotoxicity should be investigated in future studies.

To apply this technique in the infectivity assays for future studies, an important factor that must be considered is wastewater characteristics. The reported dilution in this

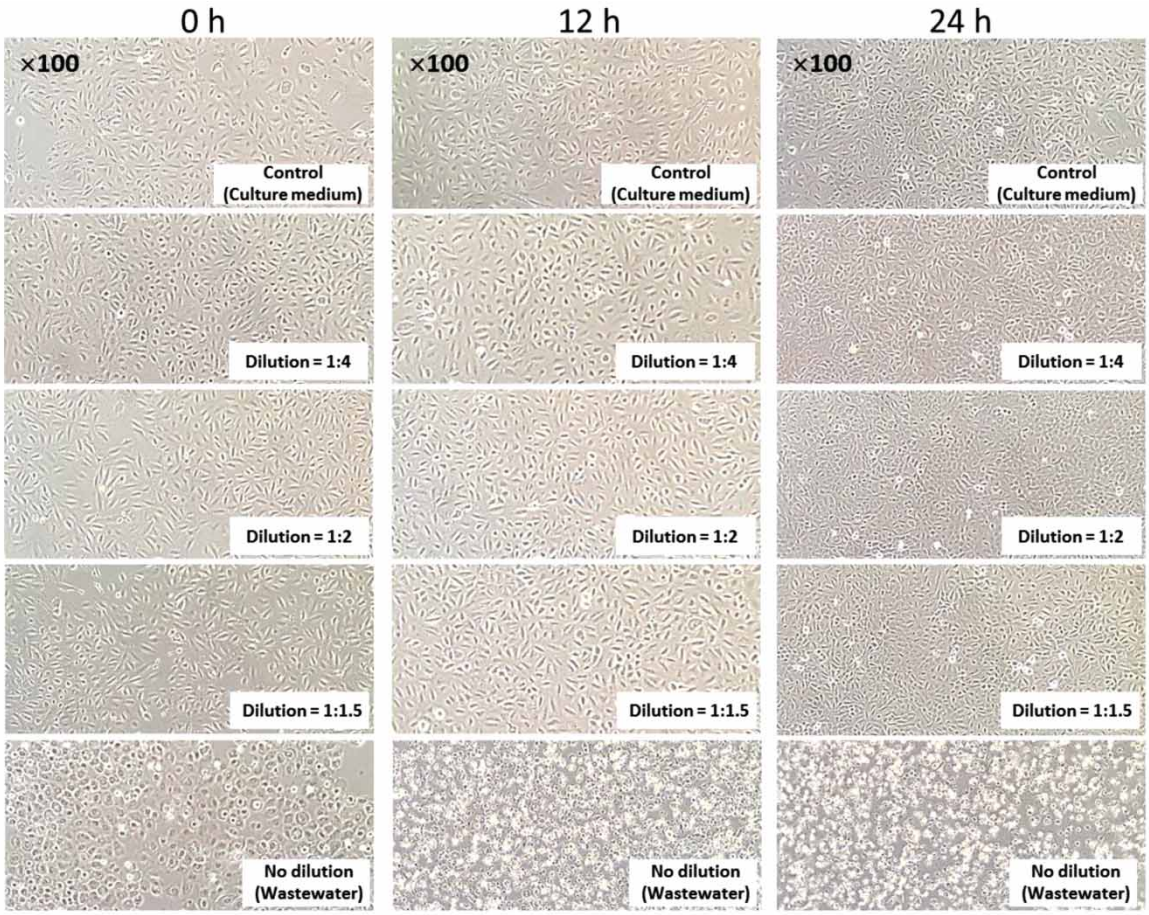

Figure 2 | A549 cell morphology after exposure to wastewater at different dilutions for different incubation periods.

study (1:1.5) may not work, as the strength of the constituents in wastewater from different locations could vary, as well as the sensitivity of different cell lines. However, further works dealing with similar or lower levels of wastewater strength using A549 cell line could apply the dilution level reported in this study as a starting point for assessing a suitable dilution needed in the infectivity assays.

HAdV-5 recovery

In this study, the recovery efficiencies of HAdV-5 for pretreated wastewater and drinking water samples were 93.75% and 97.91%, respectively. The high recovery efficiencies obtained in the recent work were consistent with past publications reporting a high recovery of infectious HAdV-5 in different types of water samples (Li *et al.* 2010; Turnbull *et al.* 2019), and this indicated that water matrices caused an insignificant impact on HAdV-5 inactivation.

UV inactivation of HAdV-5 and bacteriophage MS-2

HAdV-5

According to Figure 3, the inactivation profiles of HAdV-5 in all types of water samples followed the first-order inactivation kinetics of Chick (1908) and Watson (1908). The linear inactivation profiles without a shoulder region were suggestive of a homogeneous population of HAdV-5, with similar levels of UV sensitivity. Moreover, in this study, aggregation or clumps of HAdV-5 particles facilitating viral recombination were negligible because of no tailing (Mattle & Kohn 2012).

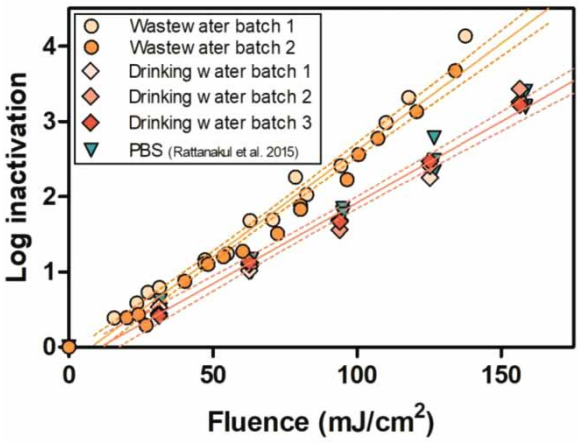

Figure 3 | Human adenovirus genotype five (HAdV-5) inactivation profiles of different water samples. Dashed lines show 95% confidence intervals.

Considering the inactivation rate constants obtained via the Chick–Watson model, the values for drinking water samples and wastewater samples were 0.022 and 0.028 cm^2/mJ, respectively, as shown in Table 2. The inactivation rate constant in the drinking water in this study was statistically not different ($p > 0.05$, ANCOVA) from that in (PBS pH 7.2) investigated in our previous study (Rattanakul *et al.* 2015). The reported value was consistent with the results of a previous report (Baxter *et al.* 2007), which showed an HAdV-5 inactivation rate constant of 0.023 cm^2/mJ in groundwater samples.

These results revealed that water samples with a low organic matter content (drinking water and groundwater) had no effect on the UV inactivation of HAdV-5, as in PBS. Moreover, a low value of the inactivation rate constant (0.022 cm^2/mJ) indicated a high level of UV light resistance, where a fluence higher than 186 mJ/cm^2 was required for over four-log inactivation of HAdV-5. This is because HAdV-5 can employ host cellular machinery to repair UV-induced lesions in its DNA (Eischeid *et al.* 2009). This finding raises a concern about drinking water treatment plants that rely solely on UV disinfection at a practical fluence of 40 mJ/cm^2, especially application of UV disinfection in small systems (Dotson *et al.* 2012; NSF/ANSI 2012).

Considering the wastewater samples, there was no difference in the inactivation rate constants among the different batches of wastewater samples collected on different dates. Nonetheless, a comparison of the inactivation rate constants between drinking water and pretreated wastewater indicated that the UV inactivation of HAdV-5 was significantly more effective in wastewater than in drinking water about 27% (ANCOVA; $p < 0.05$). Unlike the clean water samples, the pretreated wastewater samples contained a variety of components, and some may be involved in this increase, for example, solid particles. Namely, suspended solids in wastewater may help to remove HAdV-5 from the liquid to solid phase; this change can result in a higher inactivation. On the contrary, adenovirus is non-enveloped, and its attachment ability is lower compared with an enveloped virus covered with a sticky layer of a lipid (Ye *et al.* 2016). Thus, adsorption probably contributed less to the increase in UV inactivation in this study, and it is supported by a foregoing study reporting that the presence of suspended solids in wastewater showed no significant effects on HAdV removal (Yin *et al.* 2015).

Table 2 | Human adenovirus genotype five (HAdV-5) and bacteriophage MS-2 inactivation rate constants for UV treatment

	Inactivation rate constant, $k \pm$ S.D.[a] (cm^2/mJ)	
Type of water sample	**HAdV-5**	**Bacteriophage MS-2**
Domestic wastewater	0.028 ± 0.001 ($n^b = 32$)	0.040 ± 0.002 ($n = 26$)
Drinking water	0.022 ± 0.001 ($n = 18$)	N/A
PBS (Rattanakul *et al.* 2015)	0.021 ± 0.001	0.034 ± 0.001 ($n = 11$)

PBS, phosphate-buffered saline; N/A, not applicable.
[a]Standard deviation (S.D.).
[b]Number of samples.

Bacteriophage MS-2

To confirm the increment of viral inactivation efficiency by UV as in HAdV-5, UV inactivation experiments with bacteriophage MS-2 in plant B wastewater samples were performed. The recovery of MS-2 in wastewater samples was 11.84%, which was in an acceptable range following the USEPA method (USEPA 2018), and results of bacteriophage MS-2 inactivation by UV are shown in Figure 4. Although there were differences in wastewater characteristic and viral type, UV inactivation efficiency in wastewater was 17.65% higher than in PBS significantly

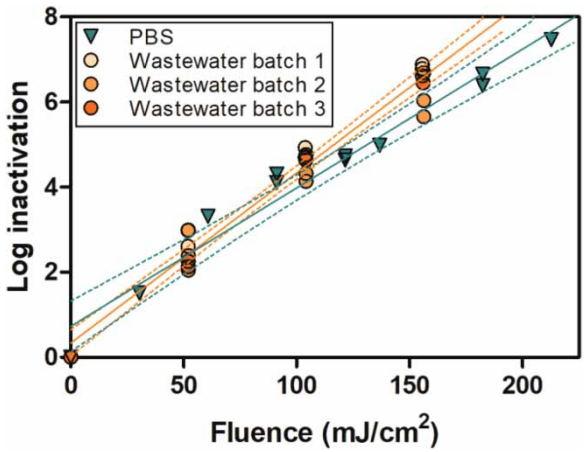

Figure 4 | Bacteriophage MS-2 inactivation profiles of PBS and wastewater samples. Dashed lines show 95% confidence intervals.

($p < 0.05$, ANCOVA) based on inactivation rate constants (Table 2). The results supported that some constituents even in effluent-like wastewater samples (low BOD_5) can enhance the virus inactivation under UV exposure. Also, because bacteriophage MS-2 and HAdV are non-enveloped, suspended solids could also play a negligible role in viral inactivation mechanism. Therefore, an important factor enhancing virus inactivation efficiency can be the organic contents in wastewater, especially dissolved organic matter (DOM) because it was the major composition in pretreated wastewater.

Influence of reactive oxygen species on virus inactivation

The photosensitization of DOM can be expected under UV exposure which involves the formation of reactive oxygen species (ROS), such as superoxide ($HO_2^{-}\bullet/O_2^{-}\bullet$), hydroxyl radicals (OH•), and singlet oxygen ($^1O_2$) from excited triplet state DOM (^{3}DOM*) in natural water (Zarifiou *et al.* 1984; Zhang *et al.* 2014), and these ROS have been reported to cause virus inactivation in various studies. Among the ROS species, OH• radicals are the most important species because they are ubiquitous in water environments and highly reactive towards organic pollutants including viruses (Mopper & Zhou 1990; Sun *et al.* 2015). In the recent work, without UV exposure as a control condition, there was no reduction of *p*CBA in ultrapure water and wastewater samples throughout the experimental period (data not shown). Similarly, *p*CBA concentration in ultrapure water was constant after being exposed to a fluence of 184.2 mJ/cm^2 as shown in Figure 5, and it implied no OH• radicals formation in the absence of photosensitizers or DOM. Surprisingly, decreasing profiles of *p*CBA in non-filtered wastewater samples were observed as happened in filtered wastewater samples, and this suggested the production of OH• radicals in wastewater samples under UV exposure. Based on mathematical calculation, average concentration of $[OH\bullet]_{ss}$ in non-filtered and filtered wastewater samples were 2.40×10^{-14} and 4.07×10^{-14} M, respectively, and a subtle difference can be from scavenging of OH• radicals by suspended solids in non-filtered samples. The DOM as a major content in pretreated wastewater, although reported as biochemical oxygen demand received in 5 days or BOD_5 (Table 1) could have undergone photosensitization, and subsequently formed OH• radicals to inactivate viruses which resulted in higher inactivation efficiency. This finding was consistent with the results of Dong & Rosario-Ortiz (2012) and Rosado-Lausell *et al.* (2013), in which OH• radicals were detected in water containing Suwanee River natural organic matter (SRNOM), Loire NOM, and effluent organic matter (EfOM) under a solar simulator system with UV spectrum, and OH• radicals contributed significantly to MS-2 inactivation. Besides excited DOM, the presence of several Fe(III) complexes in pretreated wastewater may possibly contribute to OH• radicals formation through the photo-Fenton processes and this should be further investigated in future studies.

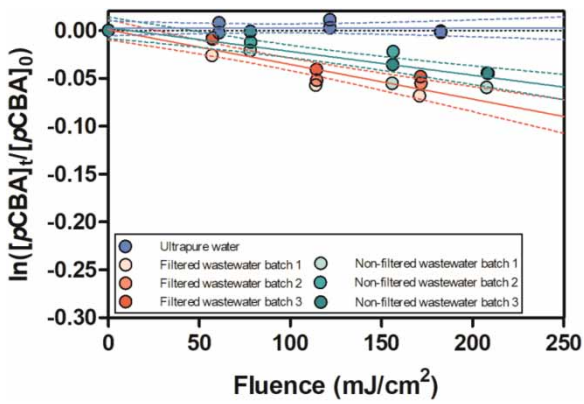

Figure 5 | Reduction profiles of *para*-chlorobenzoic acid (*p*CBA) in ultrapure water and wastewater following UV treatment. Dashed lines show 95% confidence intervals.

Based on the results in this study, although the increment of UV inactivation efficiency of viruses was noted, required fluence for achieving four-log inactivation of UV-resistant virus. e.g.. HAdVs in wastewater, was still higher than practical fluence applied in wastewater treatment plants. Hence, the application of UV disinfection as a standalone process to control HAdVs in wastewater may not be an adequate choice because of high energy consumption, and other disinfection methods such as chlorination and ozonation should be appraised. The use of these chemical disinfectants was proven to achieve higher inactivation efficiencies of HAdVs in water (Wolf *et al.* 2018; Prado *et al.* 2019); however, a major concern about chemical disinfectants is the ecotoxicity of either the disinfection by-products (DBPs) or the free residual oxidants that may be present in treated wastewater (Delacroix *et al.* 2013; Yang *et al.* 2015). Therefore, combination processes of UV and chemical disinfectants (i.e., UV/Cl_2, UV/chloramine, and UV/ClO_2) as a multi-barrier approach could be better options to balance the concerns on ecotoxicity and human health risk as they have been shown to be effective for the inactivation of HAdVs and the control of DBPs formation (Bounty *et al.* 2012; Rattanakul *et al.* 2015; Wang *et al.* 2019; Tain *et al.* 2020).

CONCLUSIONS

This recent work is the first to declare evidence of the toxicity of wastewater on A549 cells, and the infectivity assays were verified to be applicable for HAdV-5 spiked wastewater samples. However, a minimal degree of dilution is needed in order to evaluate case by case, because of the differences of wastewater characteristics and cell lines. The results of UV inactivation in different types of water suggest that the presence of dissolved organic matter in wastewater induced OH• radicals formation which involved a higher inactivation efficiency of viruses.

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DATA AVAILABILITY STATEMENT

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

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