Inactivation of Viruses by Combination Processes of UV and Chlorine

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ABSTRACT
Adenoviruses, the most UV resistant microorganism currently known, are posing concerns in UV treated drinking water. To reduce the risk from adenovirus infection, combination processes of UV and chlorination are attractive. Bacteriophage MS2 and adenovirus 5 (AdV5) were used in this study, and inactivated by low-pressure UV (LPUV) lamp, chlorination, sequential processes (UV-Cl_2 and Cl_2-UV) and a simultaneous process (UV/Cl_2). MS2 was more resistant against chlorine than AdV5, and CT values for 2 log reduction of MS2 and AdV5 were 0.77 and 0.033 mg-min/L, respectively. However, AdV5 was more resistant to UV than MS2 and required a 101 mJ/cm^2 of fluence for 2 log reduction. Compared to the application of UV or chlorine separately, an increasing trend of MS2 inactivation rate was found in the sequential processes, which was statistically significant ($p < 0.05$, ANCOVA). The simultaneous process of UV/Cl_2 for MS2 provided about 2.3 times higher inactivation rate than a summation of inactivation rates by the separate application of either chlorine or UV, even at the same UV fluence rate and the same initial chlorine concentration. The combination processes of UV and chlorine, either sequential or simultaneous application, seemed to be more effective than a standalone process in viral inactivation.

Keywords: chlorine, disinfection, ultraviolet (UV) light, virus, water treatment

INTRODUCTION

Recently, fresh water requirement is increasing more and more due to increasing considerable world population. Also, deterioration of fresh water resource has been severely increased by emerging chemicals and pathogens, which are the results of urbanization, water stress, industrial growth, global warming etc. Pathogens seemed to be an importance for health issue because it could easily spread through water bodies. Thus, effective technologies to control pathogens in drinking water are required for water treatment, and many processes have been successfully used to inactivate pathogens in drinking water treatment; including Ultraviolet light (UV). UV disinfection process has been received a lot of success to inactivate water-borne pathogenic microorganisms, even though low fluence (dose) was effective to inactivate wide range of pathogens including cyst and some viruses (Clancy *et al*., 2000; Zimmer *et al*., 2003; Friedler and Gilboa, 2010). However, some studies revealed that some microbial species, especially viruses, have high UV resistance, and adenoviruses are the most resistant microorganism that are currently known (Gerba *et al*., 2002; Thurston-Enriquez *et al*., 2003).

Adenoviruses (AdV) are non-enveloped icosahedral-shaped viruses with normal size of 70 – 100 nm, having double stranded DNA and protecting protein capsid. AdV are currently known to have 57 serotypes, and each serotype provides different human diseases such as a respiratory infection caused by AdV5 and, an eye infection caused by AdV2. Interestingly, there are only 2 serotypes, AdV40 and AdV41 that cause human

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enteric diseases. Many studies detected AdV in water bodies including; surface water, seawater, treated wastewater, and ground-water (Pina *et al*., 1998; Jiang, 2006; Haramoto *et al*., 2007; Gibson and Schwab, 2011; Kishida *et al*., 2012). As a result, if UV technology is introduced for water treatment, drinking water consumers might be at risk for enteric disease caused by adenoviruses.

A coupling of UV with chlorine is an interesting solution of the adenoviruses issue. Typically, a sequential process of UV followed by chlorine is applied in water treatment plants, namely; UV is applied for primary disinfection and chlorine is added to maintain residual chlorine. A study of Ballester and Malley (2004) showed that the sequential process was a good option dealing with AdV2. However, Lee and Shin (2011) reported that there were only additive effects of UV and chlorine because the inactivation rates did not statistically increase in the sequential process (UV-Cl_2) compared to chlorination alone.

Meanwhile, there are other options for a coupling of UV and chlorine, namely, a sequential process of chlorine followed by UV (Cl_2-UV) and a simultaneous application of UV and chlorine (UV/Cl_2). The Cl_2-UV is not very common because of the concerns on the decomposition of chlorine by UV radiation, but a pre-chlorination treatment is occasionally followed by UV treatment in practice. The simultaneous process is considered as an advanced oxidation process (AOP) because hydroxyl radicals (OH•) and other oxidant is produced and chemically decompose wide range of pollutants (Wang *et al*., 2012; Ibánez *et al*., 2013). UV/Cl_2 as an AOP has been investigated only in the last few years, and information of this process is still limited. Thus, these two options could be alternative options for adenoviruses control in water treatments.

In this study, we first applied either chlorination or low-pressure UV (LPUV) lamp disinfection to *Escherichia coli* (*E. coli*), bacteriophage MS2, bacteriophage Qβ, and AdV5, as a standalone process. Subsequently, bacteriophage MS2 was further tested with sequential processes (UV-Cl_2 and Cl_2-UV) and a simultaneous process (UV/Cl_2). We focused MS2 as a surrogate virus in this study, because MS2 has been widely used for UV researches and reactor validation at water treatment plants. The UV Disinfection Guidance Manual in the United States (USEPA, 2006) proposes MS2 as a surrogate virus, because MS2 has the highest UV resistance among bacteriophages (WRF, 2010) and therefore allows experiment at high UV fluence. Moreover, MS2 is easy to handle in laboratories routinely. The objective of this study is to investigate the effectiveness of combining chlorine and UV, in comparison with that of separate application, in the viewpoint of inactivation rate constants.

MATERIALS AND METHODS

Stock preparation and infectivity assay of bacteriophages

MS2 or Qβ stock solution was prepared according to method described by Shin and Sobsey (2008) with a modification. A 1 mL of *E. coli* K12 A/λ (F^+) and 0.1 mL MS2 suspension were spiked into 9 mL of Lennox L broth base (LB broth) (Invitrogen, USA), and was incubated at 37°C for overnight. A 0.2 mL of $CaCl_2$ (1 M) was also added into the tube containing MS2 and host cell to increase adsorption ability on host cell. The suspension containing active MS2 was filtered through 0.2 μm membrane filter

(Advantec, USA) in order to remove bacterial debris, and then filtrate was mixed with an equal volume of chloroform, followed by centrifugation at 4,000 g for 30 minutes at 4°C. Supernatant was removed and the previous step was repeated again to minimize culture media. The supernatant was further purified by centrifugal ultrafiltration (Ultracel® YM-50, Millipore, USA), and was re-suspended in 1/15 M phosphate buffer solution (pH ≈ 7.2 ± 0.2) and then it was filtered and dispersed through polycarbonate filters 0.2 μm pore size pretreated with 0.1% Tween® 80 solution (Sigma-Aldrich®, USA) in water. Stock solution was kept at –80°C before analysis. The infectivity assay of MS2 with an initial concentration of 10^5 – 10^6 pfu/mL was done by double agar technique using *E. coli* K12 A/λ (F^+) as a host cell (Adams, 1959). A 3 mL of bottom agar layer was added and made hard following with 1 mL of serial 10-fold dilution sample and 3 mL of upper agar layer. The plaque was counted after 1 day incubation.

Stock preparation and infectivity assay of Adenovirus 5

AdV5 stock solution was also prepared according to method described by Shin and Sobsey (2008). AdV5 was propagated using A549 cell line (ATCC, CCL 185). A549 cells were cultivated in a 75 cm^2 tissue cell culture flask with nutrient mixture F-12 Ham or KF-12 Ham media (Sigma-Aldrich®, USA) containing of 2 % fetal bovine serum (Sigma-Aldrich®, USA), antibiotic-antimycotic (Gibco®, USA) and amphotericin B (Sigma-Aldrich®, USA). A 1 mL of AdV5 stock was inoculated on A549 cells and incubated for a week, when A549 cells attained 80 – 90% of confluence. Subsequently, a freeze-thaw treatment of infected cells was done 3 times, which was then followed by the same step as MS2 stock solution preparation. The infectivity assay of AdV5 with an initial concentration of 10^4 – 10^5 pfu/mL was done, when A549 cells attained 80 – 90%. The A549 monolayer cell was washed out by 0.01 M PBS (–), which used only for tissue washing and 0.25% trypsin (Sigma-Aldrich®, USA). A549 cells were diluted into 40 mL of KF-12 Ham media, and grew in 6-well plates for 4 days. Thereafter, AdV5 was inoculated on confluent cells in 6-well plates and incubated for 1.5 – 2 hours with gentle rocking every 15 minutes in order to allow virus attachment. Then, the inoculated cells were overlaid by a mixture of an Eagle's minimum essential medium 2 (MEM2) (Nissui, Japan) and agar EPI (Nacalai tesque, Japan). Cytophatic effect (CPE) was confirmed to be complete in 8 – 10 days, which allowed to count number of plaques to measure the concentration of AdV5.

Stock preparation and cultivation of *E. coli* IFO3301

A 1 mL of *E. coli* K12 IFO3301 was incubated in a 9 mL of LB broth at 37°C for 6 hours, and was mixed with an equal volume of a 50 % of glycerol, and then was stored at –80°C. Cultivation of *E. coli* was done following method describes by APHA (1995). Incubated *E. coli* in LB broth was concentrated by centrifuge at 6,000 rpm for 10 minutes. Concentrated *E. coli* cell was rinsed with PBS 3 times before use. Samples containing *E. coli* were cultivated in Chromocult coliform agar (Merck, USA). The initial concentration of *E. coli* for UV and chlorination experiments was about 10^5 – 10^6 cfu/mL.

Chlorination procedure

Sodium hypochlorite (NaOCl, 5% analytical grade, Chameleon reagent-Kishida, Japan) was diluted in PBS to make a concentration of 250 mg/L chlorine stock solution. Free chlorine concentration was measured by using *N*, *N*-dimethyl-*p*-phenylenediamine

(DPD) colorimetric method (APHA, 1999) by DR840 spectrophotometer (HACH, USA). All glassware was soaked in a 100 mg/L chlorine solution overnight, rinsed with Milli-Q water 5 times and baked at 140°C for 24 hours.

Chlorine disinfection experiments were done as a batch reactor using chlorine demand-free glassware prepared as described above. A 100 mL of mixture between PBS and microbial stock solution was placed into a 200 mL glassware and then continuously stirred by a magnetic spin bar. Theoretical amount of chlorine stock solution was spiked into the sample to make the initial concentration, or the injection rate of chlorine in the working solution at 1 mg/L, 0.15 mg/L and 0.1 mg/L as free chlorine for MS2, AdV5 and *E. coli* sample, respectively. Subsequently, the solution was sampled from the reaction beaker at desired contact time up to 4 minutes, 1 minute and 40 seconds for MS2, *E.* coli and AdV5, respectively. A 10 mL of chlorinated sample was removed to measure free chlorine concentration and a 1 or 2 mL was immediately quenched by 50 µL of 0.3 M sterile sodium thiosulfate for microbial measurement. CT values for chlorination experiments were calculated by determination of area under graph between residual chlorine (mg/L) and time (minute).

UV disinfection procedure

The bench scale LPUV collimated beam apparatus consisted of a 15 watts low-pressure UV Lamp (GL15, TOSHIBA, Japan) was adopted to conduct UV irradiation at 254 nm. Measurement of fluence rate (irradiance) at the surface of the solution was done by using a radiometer (UVR-2 UD25, TOPCON). UV absorbance at 254 nm of sample without or with sodium thiosulfate was 0.003 and 0.357 cm^{-1}, respectively. Thus, the surface fluence rate and UV absorbance were used to calculate the incident fluence rate following the protocol of Bolton and Linden (2003). Subsequently, fluence was obtained as the product of the incident fluence rate and UV exposure time, and the fluence was altered by changing the exposure time.

In disinfection procedure, a 35 mL of diluted microbial stock solution was placed into a petri dish with 8.5 cm diameter providing 0.617 cm of sample depth. The petri dish was under collimated beam system and continuously stirred by a magnetic bar for UV inactivation experiment. The sample was taken from the petri dish at desired exposure time in order to measure microbial concentrations in the sample.

Experimental protocol for combination processes

Three combination processes were tested with MS2 including; sequential UV followed by chlorine (UV-Cl_2), sequential chlorine followed by UV (Cl_2-UV) and a simultaneous of UV and chlorine (UV/Cl_2). In UV-Cl_2 process, samples containing MS2 were exposed to LPUV at 23, 46 and 69 mJ/cm^2 to achieve 1, 2 and 3 log inactivation, respectively, which were then chlorinated with an initial chlorine concentration of 1 mg/L. The sample was collected at a desired contact time and immediately mixed with sterile sodium thiosulfate to quench residual chlorine. In Cl_2-UV process, there were two procedures for this process. First, samples were chlorinated with an initial chlorine concentration of 1 mg/L to achieve 1, 2 and 3 log inactivation, respectively, and were immediately mixed with sterile sodium thiosulfate to quench the residual chlorine. The dechlorinated samples were further treated by subsequent LPUV, and the samples were collected at a desired exposure time, this procedure was expressed as Cl_2-deCl_2-UV in

later discussion. Another procedure was done as same as the method above except for the dechlorination procedure. Namely, the chlorinated samples were further expose to UV without adding quenching agent. The samples were then collected and immediately mixed with sterile sodium thiosulfate at a desired contact time, and the procedure was expressed as Cl_2-UV. For UV/Cl_2 process, samples were injected with chlorine solution at the same time of starting LPUV exposure, which were collected at a desired contact time to be immediately mixed with sterile sodium thiosulfate. All experiments in this study were performed with 3 independent experiments at 20 ± 1°C.

Statistical analysis

Inactivation rates, or slopes for the log-linear regression lines of inactivation profiles determined by the least squares method, were compared by analysis of covariance (ANCOVA) using SPSS® statistic version 17 software.

RESULTS AND DISCUSSION

Microbial inactivation by UV or chlorine

Inactivation profiles of Qβ, MS2, AdV5 and *E. coli* by either UV or chlorine are shown in Fig. 1, error bars indicate standard deviation of triplicate experiments. Inactivation profiles of all microbes in both processes in this study followed typical first-order inactivation kinetics. In UV treatment (Fig. 1a), AdV5 was the most resistant against UV and a 101 mJ/cm^2 was required for 2 log inactivation, which was very high because a typical fluence requirement in water treatment plants is around 40 mJ/cm^2 (NSF/ANSI, 2012). The fluence was higher than other studies reporting 64 mJ/cm^2 (Nwachuku *et al*., 2005) for 2 log inactivation of AdV5. However, a study of Guo *et al*. (2010) showed a wide range of fluence required for 2 log inactivation, which was 26 to 76 mJ/cm^2. The result of the study clearly explained that difference of host cells using for infectivity assay affected log inactivation of AdV5. In this study, a different host cell was used compared to the studies and resulted higher fluence requirement for 2 log inactivation of AdV5. MS2 was more sensitive to UV than AdV5 based on the inactivation rates, and a required fluence for 2 log inactivation of MS2 was around 48 mJ/cm^2, which was similar to a previous study by Sommer *et al*. (2001). *E.coli* was most susceptible to UV and only a 4 mJ/cm^2 could achieve around 2 log inactivation. Meanwhile in chlorination (Fig. 1b), *E. coli* was most resistant and the CT value for 2 log inactivation (0.025 mg-min/L) was similar to a previous study (Hoff, 1986). AdV5 was very sensitive to chlorination and a CT value for 2 log inactivation was only 0.033 mg-min/L, which was lower than another study reporting 0.11 mg-min/L of CT value (Baxter *et al*., 2007). The difference may be due to the different experimental conditions such as pH and temperature, because we worked at pH 7.2 at 20°C while Baxter's study was done at pH 8.5 at 5°C. However, it was similar with a study by Cromeans *et al*. (2010) conducted at similar pH but targeting different serotypes (AdV2). As for MS2, a 0.77 mg-min/L of CT value was required for 2 log inactivation, which was comparable with a study by Clevenger *et al*. (2007) at similar experimental condition (pH 7, 22°C). AdV5 was around 23 times more sensitive to chlorination than MS2, based on the inactivation rates.

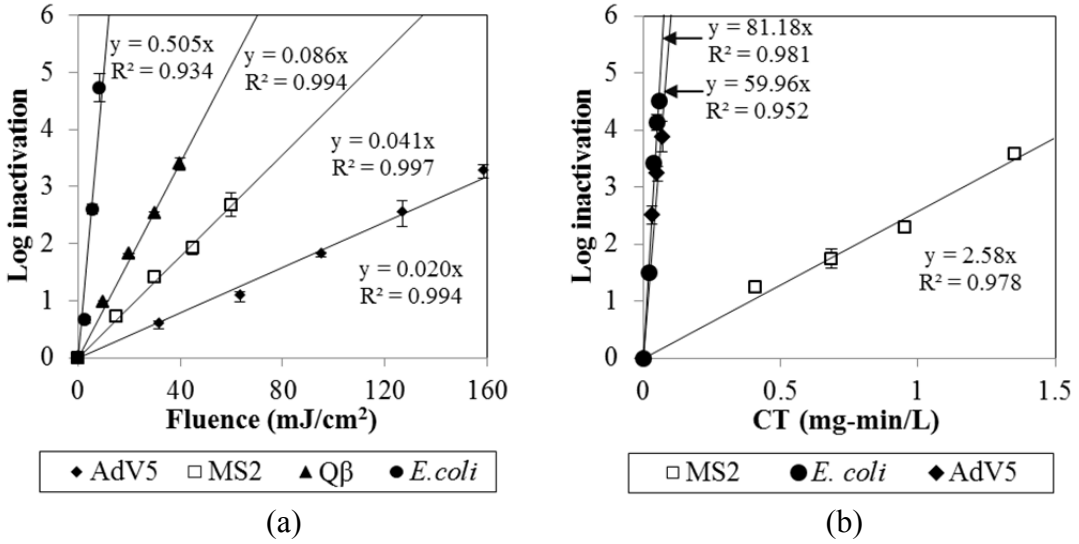

Fig. 1 - Inactivation profiles of bacteria and viruses by (a) UV and (b) Chlorine.

MS2 inactivation by a sequential process of UV followed by chlorination (UV-Cl_2)

This sequential process (UV-Cl_2) is a practical process adopted in water treatment plants. UV is used for pre-disinfection, and chlorine is added to provide residual chlorine in UV treated water before pumping into water distribution system. Samples were pretreated by UV at typical fluences applied in water treatment plants (23, 46, and 69 mJ/cm^2 to achieve 1, 2 and 3 log inactivation, respectively), and the UV treated samples were chlorinated afterward as shown in Fig. 2. Pretreatment with UV could increase inactivation rates in the subsequent chlorination, and higher fluences provided higher inactivation rates. The increase of inactivation rates was statistically significant (p < 0.05, ANCOVA). Accordingly, synergistic effects of UV and chlorine were observed in this study. Possible mechanism providing enhanced chlorination efficacy might be the damage in viral capsid caused by UV. Viral capsid consists of protein. It was reported that protein absorbed UV at 254 nm and caused cleavage of peptide bonds (Jagger, 1967; Harm, 1980). Thus, it is expected that viral capsid got damaged by UV light and became more susceptible in subsequent chlorination. The results of this study were contrary to study by Lee and Shin (2011) showing an additive effect in a sequential process (UV-Cl_2) using AdV2 at pH 8 at 5°C. The discrepancy may be due to the difference in experimental conditions and microbial species, and further study is needed to understand the mechanisms working in UV-Cl_2 process.

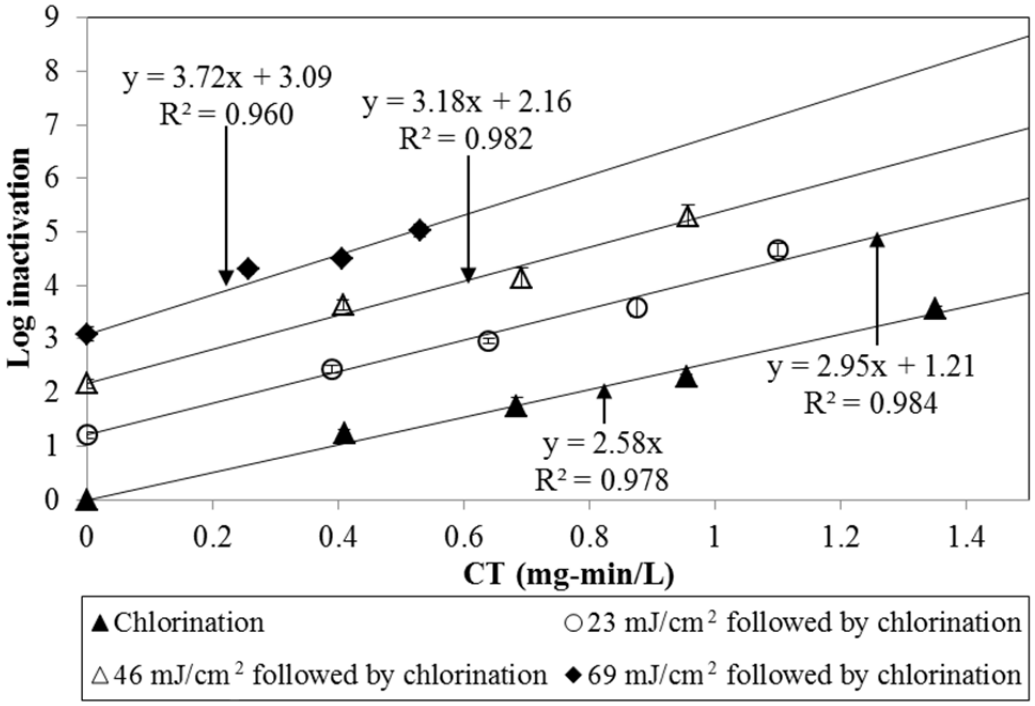

Fig. 2 - Inactivation profiles of sequential process of UV-Cl_2.

MS2 inactivation by a sequential process of chlorination followed by UV (Cl_2-deCl_2-UV)

The sequential process (Cl_2-UV) is not practically applied in water treatment plants because there are concerns about chlorine decay due to photo-degradation by UV. However, pre-chlorination is applied in some cases to control bacteria and algae that may cause problem of subsequent treatment units. Accordingly, Cl_2-UV combination has some significance in practice. Fig. 3a shows that the sequential process of Cl_2-deCl_2-UV also provided synergistic effects on MS2 inactivation rates in subsequent UV, even though low CT values were applied in advance ($p < 0.05$, ANCOVA). The increase in inactivation rates were enhanced, when higher CT values were applied. The effects of quenching agent, sodium thiosulfate, were also tested as shown in Fig. 3b, showing no significant difference in the inactivation rates ($p > 0.05$, ANCOVA). Therefore, the observed synergistic effect was attributable to chlorination. It is assumed that viral envelop and capsid might be damaged by chlorine and the virus became malfunction to attach host cell, and subsequently more sensitive against UV (Page *et al*., 2010). Thus, if UV treatment is applied after pre-chlorination, the synergistic effects may be expected.

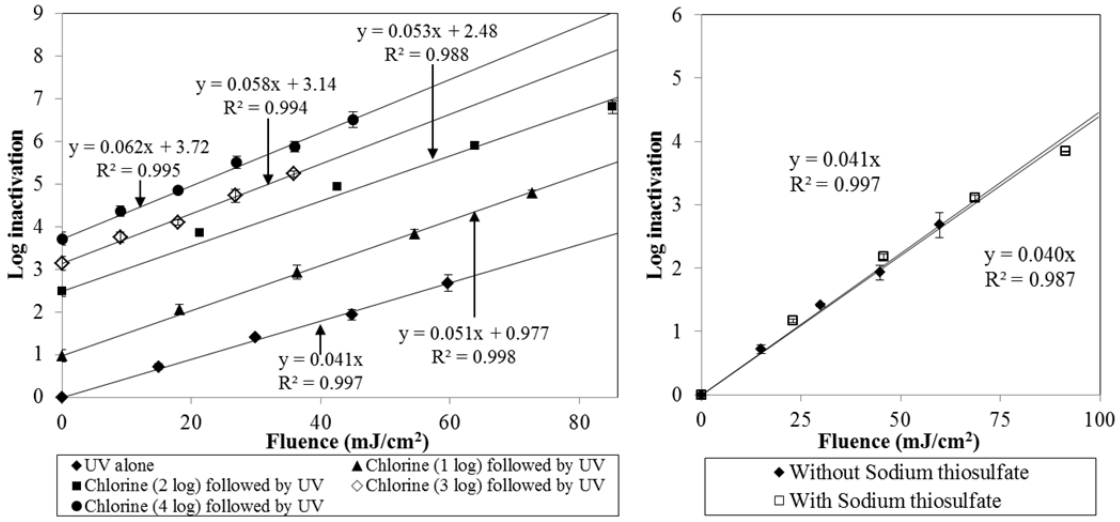

Fig. 3 - (a) Inactivation profiles of sequential process of Cl_2-deCl_2-UV
(b) Effects of sodium thiosulfate ($Na_2S_2O_3$) on MS2 inactivation rates.

MS2 inactivation by simultaneous process of UV/Cl_2

Inactivation profiles of both chlorination alone and UV alone were compared to an inactivation profile of simultaneous process (UV/Cl_2) along with the time for treatment (Fig. 4). The incident fluence and initial free chlorine concentration (1 mg/L) were the same in sequential and simultaneous processes. Surprisingly, when evaluated the time-based, a huge synergistic effect was occurring in simultaneous process, and inactivation rate was 0.09 s^{-1}, which was about 2.3 times higher than the sum of inactivation rates (dot line) by separate application of either UV or chlorine alone as shown in Fig 4. This synergy might be due to advanced oxidation process (AOP). Normally, AOP in UV/Cl_2 is expected to occur at high chlorine dose (8 mg/L) and high UV fluences (250 – 1,000 mJ/cm^2) (Watts *et al*., 2012), but according to this study, active species such as hydroxyl radical (OH•) and chlorine radical (Cl•) might be produced at UV fluences and chlorine concentrations that are practically applied at water treatment plants.

The sequential process of Cl_2-UV could be concluded as a kind of simultaneous process because chlorine presents in water when UV light is exposed. Therefore, AOP might also be occurring in the sequential process of Cl_2-UV. As shown in Fig. 5, the inactivation rate of the sequential process of Cl_2-UV provided higher inactivation rate (0.11 s^{-1}) than the simultaneous process of UV/Cl_2 (0.09 s^{-1}), and the difference was statistically significant ($p < 0.05$, ANCOVA). Moreover, the inactivation rate of sequential Cl_2-UV (0.11 s^{-1}) was even much higher than summation of inactivation rates of, either UV or chlorine alone (0.04 s^{-1}).

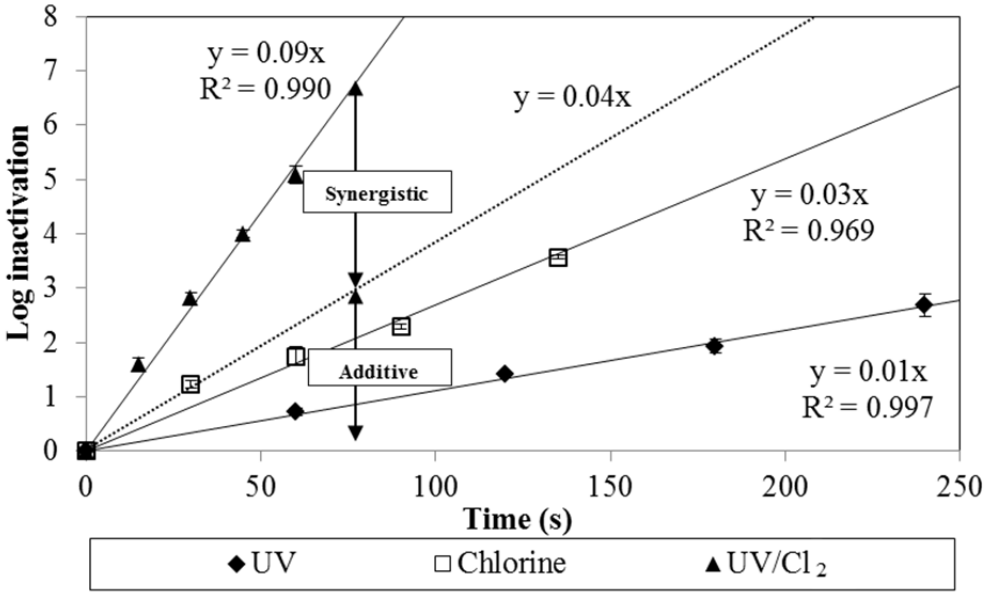

Fig. 4 - Inactivation profiles of simultaneous process of UV/Cl_2.

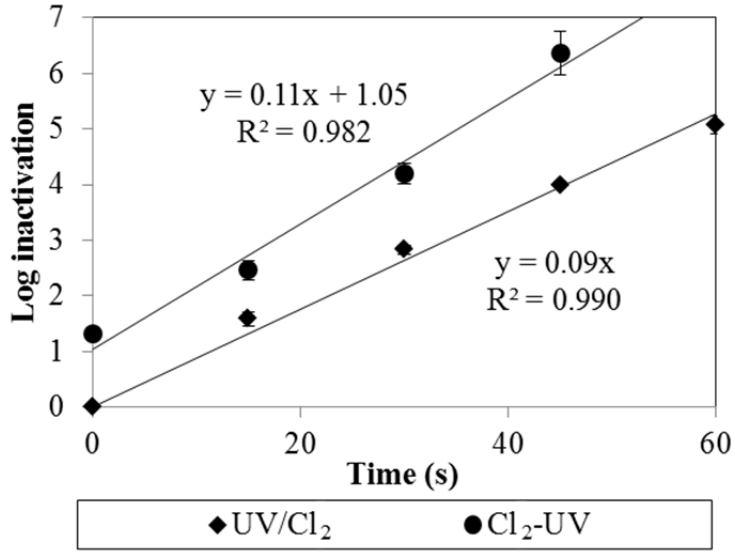

Fig. 5 - Comparison of inactivation profiles between $UV/Cl_2$and Cl_2-UV.

These results support the assumption that viral capsid may be damaged by chlorine at the first step, and the virus may get more sensitive to UV, as discussed earlier. Also, residual chlorine concentration after 1 minute of UV exposure time was 0.47 ± 0.01 mg/L and 0.50 ± 0.01 mg/L for Cl_2-UV and UV/Cl_2 processes, respectively. Namely, it was confirmed that residual chlorine was still remaining in samples after Cl_2-UV and UV/Cl_2, contrary to the concerns on chlorine degradation by UV exposure. Thus, the sequential Cl_2-UV and simultaneous UV/Cl_2 would be practically possible in water treatment plants.

Apart from inactivation rates, total time requirements to achieve 4 log inactivation of viruses for each treatment process are shown in Table 1. It should be noted that 4 log inactivation of viruses is a common requirement for water disinfection (Chevrefils *et al*., 2006; USEPA, 2006). As far as MS2 are concerned, the total treatment time in sequential process of UV-Cl_2 was increased when higher log inactivation was achieved by pretreatment with UV. This was simply because MS2 was rather resistant to UV as shown in Fig. 1. Meanwhile, the total treatment time in sequential process of Cl_2-deCl_2-UV was decreased when longer chlorine contact times were applied as pretreatment. The results also support the assumption that chlorine could make MS2 become more sensitive to UV. Meanwhile, total treatment time was considerably decreased in the simultaneous process of UV/Cl_2 for 4 log inactivation of MS2, which might be possibly caused by AOP. The simultaneous process of Cl_2-UV required shorter total treatment time compared to either, the sequential processes (Cl_2-deCl_2-UV and UV-Cl_2) and standalone processes. Therefore, this study suggested simultaneous processes were the best option for MS2 inactivation. To expand this outcome to AdV, effects of sequential and simultaneous application of UV and chlorine to AdV are to be examined in the further study.

Table 1 - Total time requirement for 4 log inactivation of MS2 in treatment processes.

Time (min)	Cl_2	UV	Cl_2-deCl_2-UV					UV-Cl_2			UV/Cl_2
			*Log inactivation by pretreatment (Cl_2)					*Log inactivation by pretreatment (UV)			
			1	2	3	4	**1	1	2	3	
Total treatment	2.47	6.06	5.29	4.03	3.29	2.47	1.18	3.26	3.97	5.02	0.76
Pretreatment	N.A.	N.A.	0.62	1.23	1.85	2.47	0.62	1.51	3.03	4.54	N.A.

* For example, in the column of "Log inactivation 2" for Cl_2-deCl_2-UV, 2 log inactivation by chlorination required chlorine contact time for 1.23 min, followed by UV exposure time for 2.8 min, to achieve 4 log inactivation at the end of sequential treatment requiring 4.03 min in total.

**Time requirement of the sequential process of Cl_2-UV without dechlorination procedure. Namely, 1 log inactivation was achieved by chlorination which was directly subjected to UV exposure without quenching chlorine. In other experimental conditions for sequential Cl_2-deCl_2-UV, chlorine was quenched before UV exposure (see MATERIALS AND METHODS for details).

N.A. = Not applicable

CONCLUSIONS

In this study, both sequential processes of UV-Cl_2 and Cl_2-deCl_2-UV provided synergistic effects on MS2 inactivation compared to a standalone process. Also, the simultaneous process (UV/Cl_2 or Cl_2-UV) was the most effective combination process to inactivate MS2 because huge synergy was observed based on time-based inactivation rates. Preceding chlorination before UV exposure seemed to be better than simultaneous application of UV and chlorine because it enhanced the sensitivity of bacteriophage MS2 in subsequent UV treatment. Finally, the simultaneous processes were interesting because advanced oxidation process (AOP) might be occurring, even at practical chlorine doses and UV fluences.

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