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Exploring the Resistance of Adenovirus to Uv Disinfection

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Exploring the Resistance of Adenovirus to UV Disinfection

by

Sarah Bounty

B.S., University of New Hampshire 2009

A thesis submitted to the Faculty of the Graduate School of the University of Colorado in partial fulfillment of the requirements for the degree of:

Master of Science

Department of Civil, Environmental, and Architectural Engineering

2012

This thesis entitled:

Exploring the Resistance of Adenovirus to UV Disinfection

written by Sarah Bounty

has been approved for the

Department of Civil, Environmental, and Architectural Engineering

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Karl G. Linden

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James P. Malley, Jr.

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Kevin M. McCabe

June 18, 2012

The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.

Abstract

Sarah Bounty (M.S. Environmental Engineering)

Exploring the resistance of adenovirus to UV disinfection

Directed by Professor Karl G. Linden

Ultraviolet (UV) light is an attractive disinfectant for water due to its lack of byproduct formation and ability to disinfect some organisms that are resistant to chlorine, such as *Cryptosporidium parvum*. However recent regulations by the USEPA have limited the use of UV disinfection for virus disinfection of groundwater supplies and raised the required dose for inactivation of viruses in surface water supplies. The reasoning behind these restrictions is the well documented resistance of one particular virus to UV disinfection. Adenovirus has changed the regulations regarding UV disinfection because it has been observed to require up to four times the dose of almost all other known pathogens to achieve 4-log (99.99%) inactivation. .

Although its resistance to traditional monochromatic low pressure (LP) UV lamps is well known, adenovirus has been observed to be more susceptible to disinfection by polychromatic medium pressure (MP) lamps. The reason behind this has been investigated and it has been hypothesized that the polychromatic wavelengths emitted by MP lamps have the potential to disrupt the virus particle via different mechanisms than LP UV lamps. This idea was investigated further in this thesis, by the use of a polymerase chain reaction (PCR) assay to quantify damage to DNA and the use of an enzyme linked immunosorbent assay (ELISA) to examine damage to capsid proteins following both types of UV radiation.

In addition to studying the mechanism of action of different types of UV light, there is an interest in developing methods to improve the disinfection potential of adenovirus with the use of UV light. In this thesis the addition of H_2O_2 to LP UV exposure was shown to increase the inactivation potential over LP UV alone. This combination is an advanced oxidation process (AOP) and produces hydroxyl radicals. These radicals are non-specific oxidants and can be used to degrade chemical contaminants at high UV doses. The doses typically used in UV disinfection are one to two orders of magnitude lower than typical AOP doses. Even with the low dose UV and low levels of H_2O_2, hydroxyl radicals were sufficiently produced to cause enhanced disinfection. This provides a potential tool for systems with LP UV in place to increase their inactivation credit for adenovirus without changing the type of UV lamp used.

Contents

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1. Introduction

Adenovirus

Adenovirus is a virus with 52 different serotypes that infects humans. Depending on the serotype a variety of symptoms can manifest themselves including gastrointestinal disease, respiratory infection, eye infection, and, in severe cases, pneumonia. It has been detected in water samples from drinking water sources in many locations. Due to its ability to be transferred through water and its relative persistence in the environment, this virus has gained particular interest from a water treatment perspective.

Adenovirus is the second leading cause of gastroenteritis in children (Crabtree et al, 1997). It has also been shown to be transferred through the fecal-oral route, making it of concern in water treatment. Outbreaks of adenovirus from contaminated water in swimming pools have been observed (Mena and Gerba, 2009). Three outbreaks of gastroenteritis in Europe may also be linked to the virus, although there were several different viruses detected in the inadequately disinfected water that led to the outbreaks (Mena and Gerba, 2009). Adenovirus has been detected in raw wastewater, and in surface waters all over the world. Due to the ubiquity of the virus it is essential that drinking water treatment systems have adequate disinfection processes in place to inactivate adenovirus.

The virus has been shown to be disinfected relatively easily by free chlorine, with a CT close to 0.2 mg-min/L for 4-log inactivation (Baxter et al., 2007). Unlike most pathogens, adenovirus has been shown to have significant resistance to inactivation by UV light. A dose (or fluence) of 40 mJ/cm^2 has been shown to be sufficient to provide 4-log inactivation of many viruses

including coxsackievirus, echovirus, and poliovirus (Gerba et al., 2002) but has only been shown to achieve 1-log inactivation of adenovirus (Baxter et al., 2007).

The fact that adenovirus has displayed such resistance to UV disinfection has been noted by regulators. The US EPA sets regulations in this country regarding disinfection of drinking water. In the Long Term 2 Enhanced Surface Water Treatment Rule (LT2ESWTR) the required UV dose is set at 186 mJ/cm^2 to account for the resistance of adenovirus (USEPA 2006a). The Groundwater Rule also addresses the adenovirus resistance by declaring standalone UV an inadequate disinfection process for all viruses in groundwater supplies (USEPA 2006b). In addition, adenovirus has appeared on the Contaminant Candidate List (CCL) several times, and is on the current version of CCL3 (USEPA 1998, 2005, 2009). These regulations have shown how significant the resistance of adenovirus is to the field of UV disinfection. In order to use UV disinfection effectively and safely, it is important to understand how adenovirus is disinfected by this method and how the process can be used most effectively against adenovirus.

Adenovirus structure

Adenoviruses are icosahedral, nonenveloped, double stranded DNA viruses. The virus is made up of a protein capsid which contains the double stranded linear DNA. The similarity in structure between adenovirus genetic material and that of its host cells (mammalian) cannot be ignored. Viruses can have a variety of genetic structure which can be composed of DNA or RNA, which then can be single or double stranded, and further can be linear or circular. Due to mechanisms that exist within mammalian cells to repair their own damaged DNA and the fact that adenovirus has the same structure to its genetic material as the host cell the inadvertent repair of UV damaged adenoviral DNA is possible. This phenomenon has been observed by the use of a repair deficient cell line in the quantification of adenovirus treated with LP and MP UV

(Guo et al., 2010). Adenovirus treated with LP UV appeared just as susceptible as when treated with MP UV when the cell culture quantification took place in a cell line that was deficient in the necessary repair mechanisms.

Adenovirus infectious cycle

The lifecycle of adenovirus is a stepwise process with the end goal of reproducing. Adenovirus must enter the host cell in order to cause infection. In order to enter the cell it utilizes the proteins that make up its capsid. This stage of the virus lifecycle has been extensively studied, due to the fact that this virus is used in gene therapy applications (Seth, 1999). The virus is able to bind to the cell through the Coxsackie Adenovirus Receptor (CAR). Once it has identified the CAR it uses its fiber and penton proteins that are part of its capsid to attach to this receptor (Walters et al., 2002).

After attachment the virus is able to gain entry into the cell through an endosome. Endosomes exist for the purpose of transporting essential molecules across the cell membrane. In this case, a virus is brought in by the cell. Immediately following its entry into the cell the virus particle begins to shed its protein capsid. After it utilizes the endosome to enter the cell the virus works toward the nucleus, possibly facilitated by cellular microtubules. When it reaches the nucleus it is able to inject its DNA into the nucleus (Seth, 1999). After injection into the nucleus the DNA is copied using the cell machinery in place for the host cell's own replication. This copied DNA holds the code for production of proteins that make up the virus and in this way the virus can be replicated by the cell. Once it has replicated the viruses are able to exit the cell through the same receptor they entered and go on to infect more cells (Walters et al., 2002)

UV Disinfection

Ultraviolet (UV) light falls on the electromagnetic spectrum between 200 and 400 nm. The waves of light that fall in this category have been used for drinking water disinfection for more than a century. The light is absorbed by the microorganisms that exist in water supplies and can potentially cause disease in humans. This method of disinfection is a physical process, causing disinfection by the physical transfer of energy to structures within the microbe as contrasted with other chemical disinfection methods such as chlorine which require the addition of chemical agents to disrupt the structure of a cell or virus. UV disinfection has an advantage over chemical disinfection due to the lack of byproduct formation.

The first law of photochemistry is the requirement that light must be absorbed to cause a chemical change. This law applies for UV disinfection, as with all UV photochemistry. The absorbance spectrum of DNA overlaps with the output of UV lamps used for disinfection with a peak absorbance of DNA occurring at 260 nm and the output of a low pressure (LP) mercury vapor lamp at 253.7 nm.

Damage to nucleic acids by LP UV light is primarily through the formation of cyclobutane pyrimidine dimers (CPDs) (Cadet et al., 1986). The production of these photoproducts inhibits the replication of the organism's genetic material. If an organism cannot replicate its genome it does not have the ability to cause infection.

In addition to DNA, UV light is also absorbed by other molecules that make up microorganisms. UV light is absorbed by the amino acids that make up proteins.

Beer Lambert Law

Some light is attenuated as the light travels through a media to reach microbes. Light passing through an absorbing media, such as water, follows the Beer-Lambert law, which is as follows:

$$\log A = log \frac{I_0}{I} = \alpha \times z \times c \qquad \text{(Equation 1-1)}$$

with A as absorbance, I as the intensity of the light passing through the water, I_0 is the intensity of the light entering the water, α is the molar absorption coefficient (M^{-1} cm^{-1}), z is the pathlength or depth of the sample (cm) and c is the concentration of dissolved constituents (M).

UV Transmittance (UVT) is a parameter used to describe the percentage of light that passes through the water. This is related to absorbance by the following equation.

$$UVT = 100 \times 10^{-A} \qquad \text{(Equation 1-2)}$$

Biological Effects of UV Exposure

The biological effects of UV exposure are reviewed in great detail by Harm, 1980. This is summarized below in relation to the work presented in this thesis. As mentioned previously, UV light is absorbed by nucleic acids and proteins of biological molecules The nucleotide bases of DNA are chromophores that absorb light in the UV range. UV absorption is a function of the chemical structure of a molecule and requires an unsaturated bond to absorb light. The absorption of UV by nucleotide bases produces photoproducts, the most common of which are the cyclobutane pyrimidine dimers (CPDs) . The production of these photoproducts within the organism's DNA makes reproduction impossible. If a pathogen is not able to reproduce it is unable to cause infection, even if the individual organism remains metabolically active. Due to the relatively high absorbance of UV light by DNA with a peak around 260 the damage to the nucleic acids is thought to be the main mechanism of action for UV disinfection.

Proteins also absorb light in the UV range. The absorption of proteins is lower than that of nucleic acids, except at wavelengths below 230 nm. The absorption spectrum of individual proteins is directly related to the sequence of amino acids that make up the protein, in the same

way that the absorbance of a DNA molecule is related to the sequence of bases. Very few amino acids contain the unsaturated bonds necessary to be a chromophore. These amino acids include tryptophan, tyrosine, phenylalanine, cysteine, and histidine.

Types of UV lamps

The two types of UV lamps used in disinfection of water are low pressure (LP) mercury vapor lamps and medium pressure (MP) lamps. These two different types of lamp have different outputs of light due to their differing components. LP lamps are monochromatic with an output at 253.7 nm and MP lamps are polychromatic and have an output of light with varying intensity from wavelengths between 185 and 300 nm. The relative emissions of the two types of UV lamp are shown in Figure 1-1.

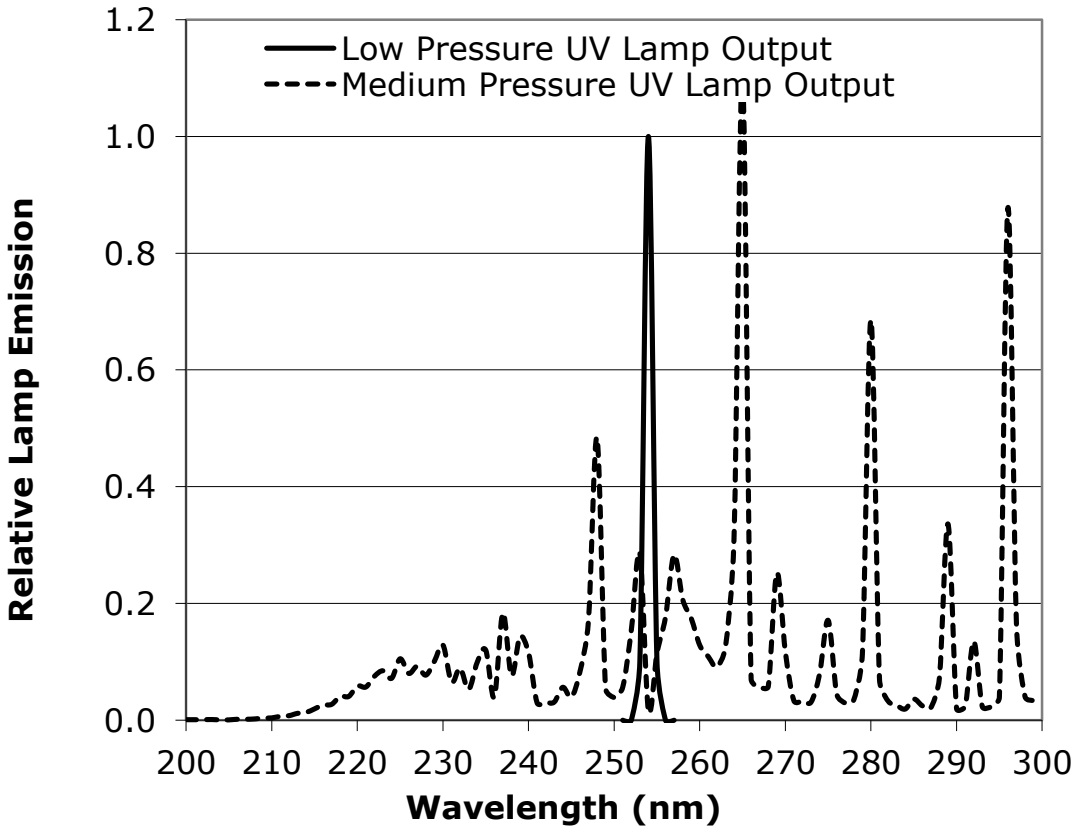

Figure 1-1: Relative emission spectra of LP and MP UV lamps.

Experimental set up of UV exposures

A collimated beam apparatus is typically used for laboratory scale study of UV disinfection. The set up can be seen in Figure 1-2 below. The sample is in a circular petri dish and constantly stirred during exposure. Further details on the experimental procedure can be found in the materials and methods sections of chapters 2-4.

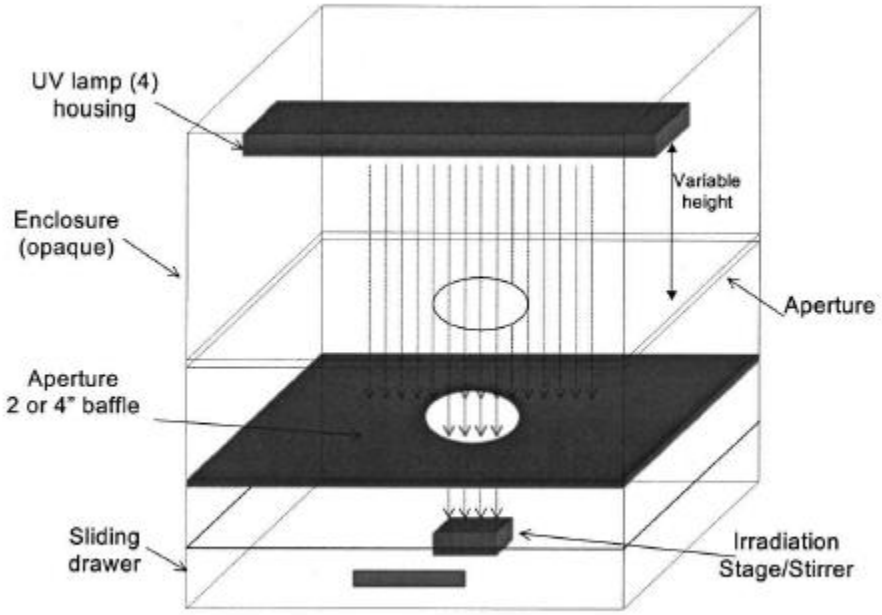

Figure 1-2: UV collimated beam set up (Bolton and Linden, 2003).

Advanced Oxidation Processes (AOPs)

Advanced Oxidation Processes (AOPs) have been studied as mechanisms for the removal of chemical contaminants from water. To a lesser extent, these processes have been explored for their merits as disinfecting processes as well. One goal of this research was to determine the ability of a LP UV/ H_2O_2 AOP to disinfect adenovirus and compare the inactivation kinetics of this process to LP UV alone.

UV/H_2O_2 AOPs rely on the production of hydroxyl radicals (HO●) in order to degrade chemical contaminants in water or react with microbial contaminants. HO● is a highly reactive,

nonspecific oxidant that has the potential to radically alter constituents in the water. HO ● can be produced by exciting H_2O_2 molecules with UV light.

2. Organization and Objectives of Thesis

The goal of this research was to explore the damage that occurs to the adenovirus particle following UV disinfection. By examining the virus on a molecular level after it has been treated by LP and MP UV it was hoped that the difference in cell culture infectivity studies of the virus treated with the two different lamps could be partially explained. The first two chapters of this thesis cover research done to explore the DNA and protein damage to the virus particle after treatment with LP and MP UV. The first assay performed was a polymerase chain reaction (PCR) based assay to examine the damage to the DNA of the virus. This approach has been used previously (Eischeid et al., 2009) and had not shown a difference between the ability of the LP and MP treated adenovirus DNA to be copied by this process. The assay was expanded upon by examining a larger portion of the genome as well as incorporating a direct quantitative measure. The full paper on these results appears in the appendix, as it was submitted to Applied and Environmental Microbiology this year. In addition, chapter 3 describes work regarding an enzyme linked immunosorbent assay (ELISA) that was developed as a way to assess capsid protein integrity by their ability to bind to antibodies. The 4th chapter is a manuscript that was submitted to Water Research on the topic of enhancing the inactivation of adenovirus treated by LP UV with the addition of H_2O_2 to create an advanced oxidation process. Enhanced inactivation was seen with this treatment that suggests a new process for improved inactivation of this virus. The final section of the thesis contains general conclusions as well as suggestions for future study.

3. DNA Damage assessment

Introduction

UV light is known to damage the genetic material of microorganisms. LP UV lamps which produce a monochromatic output at 253.7 nm are thought to derive their effectiveness as a disinfectant from the proximity of the output to the peak absorbance of DNA at 260nm. According to the first law of photochemistry only photons that are absorbed by a molecule can cause a photochemical reaction, so by applying light near the peak absorbance to DNA the most effect can be produced.

Due to the differences observed in quantification of adenovirus with cell culture techniques following disinfection with LP and MP UV it is of interest to explore the molecular mechanism of apparent enhanced inactivation caused by MP UV. One possible explanation for the difference could be differing levels of DNA damage.

In order to test this hypothesis, past study has used polymerase chain reaction (PCR) as a tool to examine DNA damage. The research outlined in this thesis attempted to improve on the methods previously established, and develop a new technique with this method to quantify DNA damage caused by UV light.

PCR is a technique with a wide range of applications for biological research. The process is a chemical reaction that creates copies of a DNA sequence specific to a certain organism or virus in this case. The copies created can be quantified using fluorescent tags (qPCR). By knowing the number of cycles the PCR process achieved the starting amount of DNA fragments can be calculated. The principle behind the use of PCR to detect damage to the genome is that if the DNA is damaged it will not be able to replicate during the PCR cycles. After the PCR process is complete the calculation of DNA will yield only the amount of intact DNA that able to be copied

as part of the PCR process. DNA damaged by UV light will not copy in this process, just as an organism will not be able to replicate its DNA if it has been UV damaged.

Previous study by Anne Eischeid and colleagues (2009) had used a relatively long range PCR of approximately 1,000 base pairs. The entire adenovirus genome is approximately 30-40,000 base pairs, depending on the serotype. The longer the analyzed fragment is the larger and more representative of the entire genome the PCR results will be. For this research several different lengths of DNA fragment were tested to determine the ideal length for this application. The lengths of the fragments tested varied from 1,000-10,000 base pairs.

In addition to the length of genome fragment analyzed the quantitative nature of the assay was improved upon. qPCR, as mentioned previously, is a method used to directly count numbers of DNA fragments as they are copied during PCR. Typical qPCR uses very short fragments of approximately 100-200 base pairs. In order to combine the quantitative nature of the qPCR with the long fragment approach to examine DNA damage a two-step "nested" PCR approach was used. For more detail on this method, please see the manuscript in Appendix A which was submitted to the journal Applied and Environmental Microbiology.

Results

As with previous research, a similar amount of damage to the DNA of adenovirus was observed after treatment with LP and MP UV of the same dose. In Figure 3-1 below the results of cell culture quantification and the results of the PCR assay are shown. Closed circles represent the log reduction observed from cell culture and open circles are the results of the PCR assay. MP and LP results are shown on separate plots, with two different sized fragments shown for the LP lamp. Further description of results can be found in Appendix A.

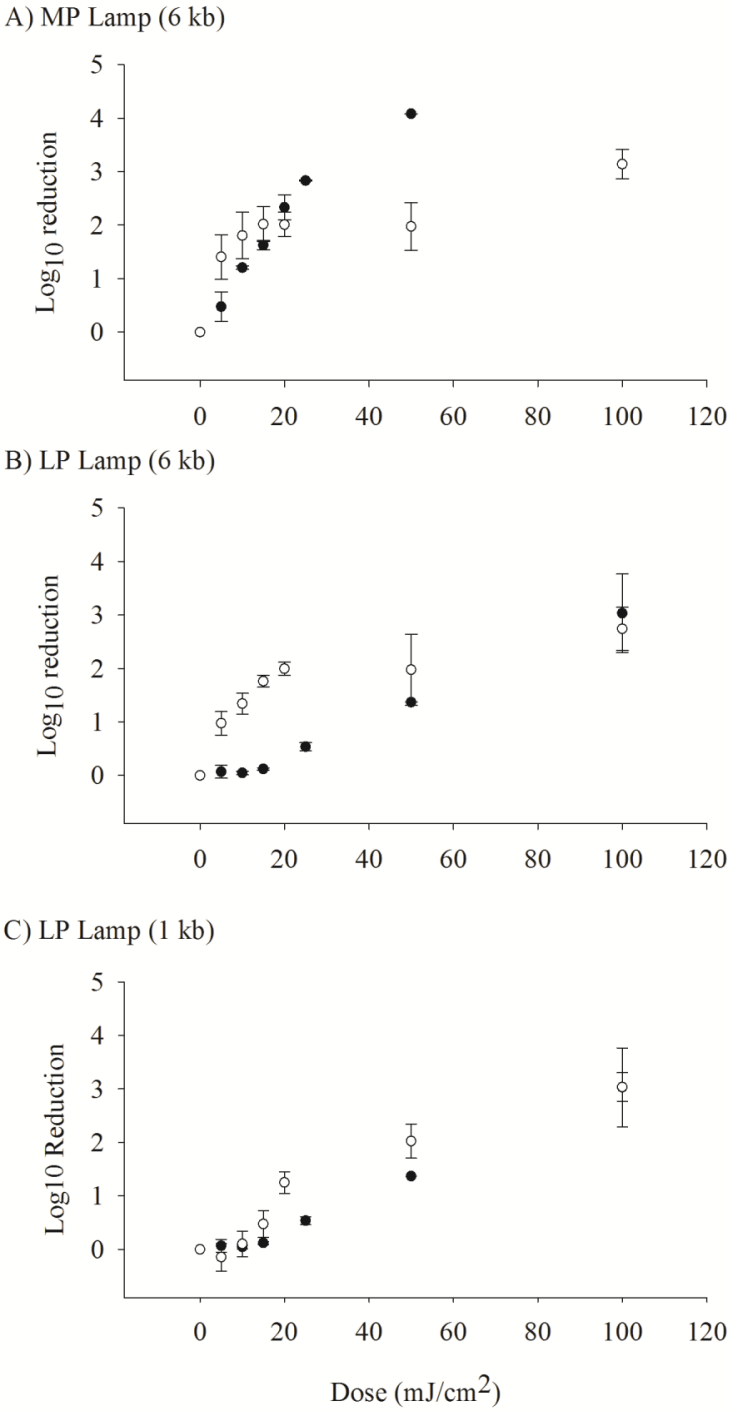

Figure 3-1: Results of PCR Assay to determine DNA damage to UV treated adenovirus. Closed symbols are the results from cell culture infectivity assays and the open symbols are the results of the two step nested PCR approach. Error bars represent one standard deviation from the mean and data are shown for MP inactivation with a 6 kilo base pair fragment (top) and for LP inactivation with a 6 kilo base pair fragment and a 1 kilo base pair fragment.

4. Use of Enzyme Linked Immunosorbent assay to determine capsid protein integrity and attachment efficiency in ultraviolet light treated adenovirus.

Introduction

It has been theorized that medium pressure (MP) ultraviolet (UV) light has the ability to damage proteins in the capsid of adenovirus. When adenovirus is quantified in cell culture after exposure to MP UV it shows greater susceptibility than when exposed to the same dose of LP UV. When DNA damage, thought to be the main inactivation mechanism of UV light, has been examined between samples treated with the two different UV sources there has not been an observed difference to support the difference shown in cell culture studies (Eischeid 2009). This leads to the hypothesis that some other part of the virus must be damaged by the MP UV light; namely the proteins that form the capsid of the virus and are responsible for the virus' attachment and entry into the host cell.

Measurement of protein integrity of adenovirus after treatment with LP/MP UV has been limited in scope thus far. Previously, SDS-PAGE has been used to determine the relative amounts of protein present in the treated virus samples (Eischeid, 2011). Samples of adenovirus treated with LP and MP UV at the same calculated doses had proteins precipitated and then the samples were run on a gel. Bands were quantified using a GelDoc imager. Results indicated that there were decreased levels of certain crucial capsid proteins after treatment with MP UV versus LP UV. In particular three major capsid proteins (hexon, penton, and fiber) showed reduction of more than 80% over untreated controls when treated with MP UV. LP UV also showed reductions in these

proteins, but at about the 40% level. These results influenced the work that took place in this thesis.

Materials and Methods

Adenovirus propagation and cell culture

Briefly, adenovirus serotype 2 (a gift from Dr. Charles Gerba) was propagated in the A549 human lung carcinoma cell line (American Type Culture Collection, Manassas VA) and the cell line was used for all infectivity assays. Cells were grown in Ham's F12K media supplemented with 10 % fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin. Cells were incubated at 37 degrees Celsius with 5% CO2. Replating of stock cells was completed by twice rinsing with Dulbecco's PBS, incubation with 0.25% trypsin/EDTA solution, re-suspension and dilution in new flasks. Cell stocks were split once per week. Media and stock solutions for cell culture and viral propagation were obtained from Invitrogen (Carlsbad, CA).

UV exposure of adenovirus

UV exposures were performed using LP and MP collimated beam apparati. Adenovirus samples were spiked into phosphate buffered saline (PBS) and then exposed to UV light in a stirred batch system. UV doses were determined as described by Bolton and Linden (2003). Briefly, incident irradiance at the surface of the sample was measured using a radiometer (IL1700, SED240/W detector) calibrated at 254 nm. Correction for any nonhomogeneity over the surface of the petri dish was accomplished by the calculation of the petri factor. Average irradiance was calculated by integration of the Beer-Lambert law over the sample depth, taking into account the absorbance of the sample and incident average irradiance. Exposure times to achieve target doses were calculated by dividing the target UV dose by the average UV irradiance. The depth

of sample was 0.5 cm for all exposures. For LP exposures, UV intensity was 1.75 mW/cm2 as measured by the radiometer. Petri factor was 0.97 for all exposures.

MPN quantification of adenovirus

Cell culture infectivity assays were used both to determine the titer of viral stocks and to assay UV/H_2O_2 exposed virus. A549 cells were plated into 24 well plates and allowed to grow to 80% confluence. Cells were introduced to exposed and diluted viral samples and allowed to incubate for one hour, rocked back and forth for a period of one to two minutes every 15 minutes. Following exposure to viral samples the cells were introduced to high glucose DMEM supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 U/mL penicillin, and 100mg/mL streptomycin and allowed to incubate for up to 2 weeks before being scored. Scoring data were entered into a computer program to determine MPN, as previously described (Hurley and Roscoe, 1982). Log inactivation was calculated as $\log_{10}$[(MPN/mL untreated control)/(MPN/mL treated sample)].

ELISA

Enzyme Linked Immunosorbent Assay (ELISA) is a technique often used in clinical applications that relies on the recognition of certain proteins by antibodies specific to the protein. The use of a tagged antibody allows for the colorimetric quantification of protein present.

Indirect Hexon ELISA

The protocol for indirect ELISA comes from abcam (Cambridge, MA) and is described here briefly. Treated and untreated adenovirus diluted in phosphate buffered saline (PBS) was bound directly to 96-well ELISA plates. The plates were sealed with adhesive plastic and incubated overnight at 4 degrees Celsius. The diluent was removed the following day and the plate was rinsed three times with PBS containing 0.05% v/v Tween-20. The wells were blocked by adding 5% w/v bovine serum albumin (BSA) and again covered with adhesive plastic and incubated

overnight at 4 degrees Celsius. The following day the plate was rinsed four times with PBS containing 0.05% Tween. Primary antibody (ab8249 monoclonal mouse anti hexon) diluted was added to each well and the plate was sealed and incubated for two hours at room temperature. The plate was rinsed twice with PBS containing Tween. Secondary antibody was added (ab6790 polyclonal goat anti mouse tagged with alkaline phosphatase) and allowed to incubate for two hours at room temperature. The plate was then rinsed four times with PBS containing Tween. p-Nitrophenyl-phosphate was added to each well and the strength of the yellow color was read with a plate reader at 405 nm after 30-45 minutes of incubation.

Sandwich ELISA with CAR protein

The procedure for the sandwich ELISA was also adapted from the abcam general procedure. The first step of this assay is the binding of the CAR protein to the well. Purified CAR obtained from Novus Biologics was diluted in carbonate buffer and allowed to incubate overnight at 4 degrees Celsius on the sealed plate. The following day the plate was washed with PBS containing 0.05% Tween. The same blocking procedure as in the indirect ELISA was used. The addition of treated and untreated adenovirus was followed by an incubation period of 90 minutes at 37 degrees Celsius. The plate was then rinsed with PBS containing Tween and the detection antibody (ab8249, same as above) was added. The plate was incubated for two hours at room temperature. Four rinses of the plate with PBS containing Tween took place. Secondary antibody (ab6790 polyclonal goat anti mouse tagged with alkaline phosphatase) was added and the plate was again incubated at room temperature, for two hours. The plate was then rinsed four times with PBS containing Tween. p-Nitrophenyl-phosphate was added to each well and the strength of the yellow color was read with a plate reader at 405 nm after 30-45 minutes of incubation.

Results and Discussion

The observed differences in infectivity results obtained from LP and MP UV treated adenovirus prompted the need for further research into the mechanism of disinfection by UV light. Cell culture quantification of adenovirus treated with both types of UV light is shown in Figure 4-1. The adenovirus samples exposed to MP UV appear less resistant to UV disinfection at the same dose levels as the LP UV treated samples. This observation, which has been illustrated in several other studies, leads to the assumption that LP and MP UV are able to affect the adenovirus particle in a different way, leading to the increased effectiveness of MP UV. The goal of this research was to examine the effects of UV light on the proteins of the virus capsid and its ability to interact with the host cell and cause infectivity.

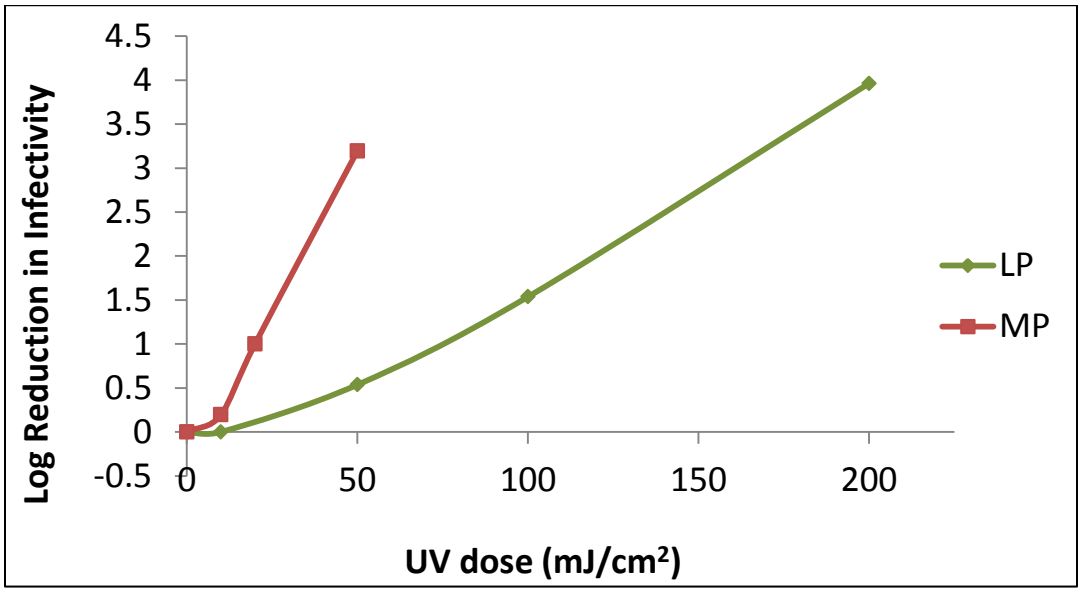

Figure 4-1: Results of cell culture infectivity assays (MPN test) for LP and MP UV treated adenovirus. Log reduction is calculated as log (N/N_0).

This research used a novel approach to determine protein damage to adenovirus exposed to different types of UV light. ELISA is typically used for clinical applications to determine the presence or absence of a virus or protein. Only one other study is known to have utilized ELISA in this type approach with adenovirus. The authors of another study used ELISA to determine

the capsid protein integrity of adenovirus after treatment with chlorine and chloramines (Page et al., 2010). The results of this study indicated that free chlorine reduced by up to 28% the amount of hexon capsid that reacted with anti-hexon antibodies when analyzed with ELISA. The similar approach used in the current study was applied to both MP UV and LP UV treated adenovirus.

There are some difficulties in the use of this technique to study protein integrity. ELISA utilizes tagged antibodies binding to a known antigen in order to give a colorimetric response based on the presence or absence of the antigen. The colorimetric signal strength is directly proportional to the amount of tagged antibody present. Figures 4-2 and 4-3 show the two different ELISA approaches tested in this work. It can be observed that the difference in the two methods is only in the addition of the CAR (coxsackie adenovirus receptor) protein in the sandwich ELISA. The purpose of this was to observe the ability of the virus to attach to this protein before and after treatment with LP and MP UV. This is a critical step to the viral lifecycle. If the virus does not attach to this protein on the host cell it is incapable of entering the cell and causing infection. If the viral structure is damaged in some way by UV light it would not be able to attach to this protein.

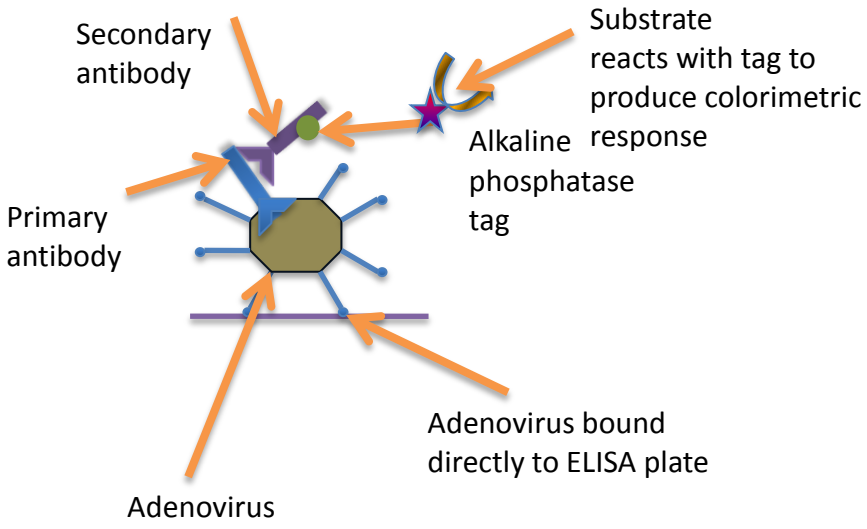

Figure 4-2: Indirect ELISA to hexon protein of adenovirus

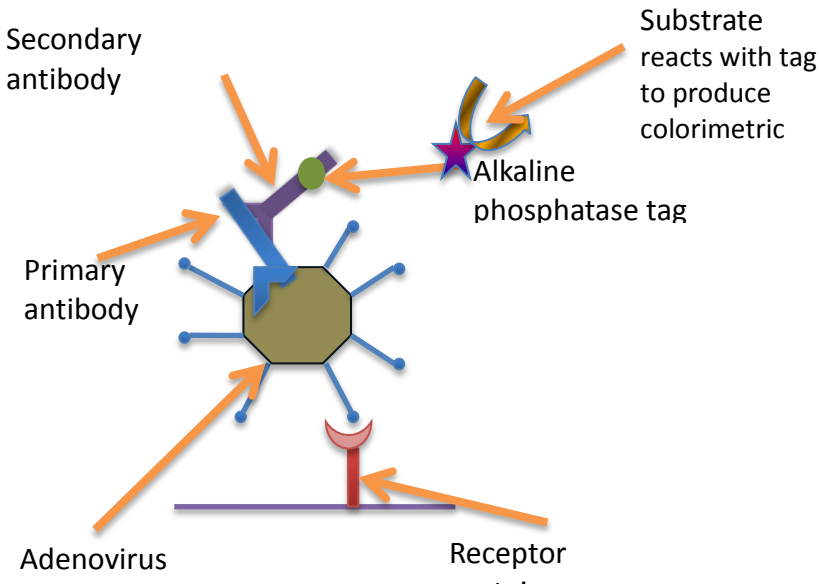

Figure 4-3: Sandwich ELISA with Coxsackie Adenovirus Receptor (CAR).

The colorimetric signal is typically measured for a standard curve of samples, and treated samples are compared to this standard curve to determine the quantity of protein present in the samples tested by ELISA. For this study a standard curve was prepared using the untreated, serially diluted adenovirus. LP and MP UV treated samples' colorimetric signals were compared back to this standard curve to determine the reduction in signal and thus reduction in amount of protein measured by the assay. Results for both the indirect ELISA and the sandwich ELISA are shown below in Figures 4-4 and 4-5.

For the indirect ELISA of the hexon protein, there was little observable difference between the colorimetric signal measured for LP and MP treated adenovirus. An observable reduction in the signal from the advanced oxidation treated adenovirus is shown by the symbols for H_2O_2/LP UV AOP samples. This is an indication that the hydroxyl radicals produced in the advanced oxidation process may be damaging the virus particle via a different mechanism than UV light alone. From this data it would appear that the hexon protein of virus particle

is still intact enough for attachment of the monoclonal hexon antibody even after the virus has been treated with both LP and MP UV light. This is an indication that another mechanism must be responsible for the observed differences in the infectivity results between the two types of UV light.

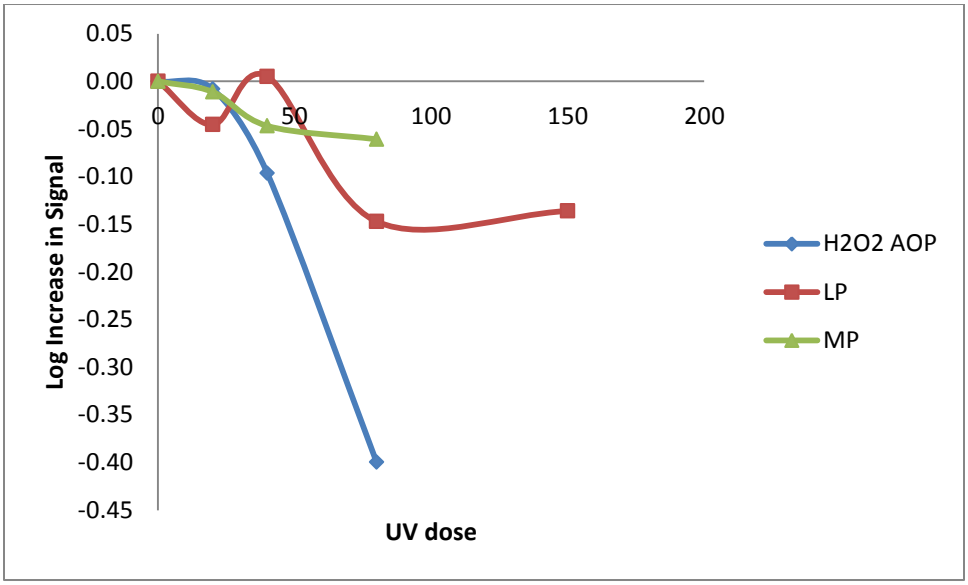

Figure 4-4: Results of Indirect ELISA of hexon protein for LP UV, MP UV, and LP UV/ H2O2 treated adenovirus. Log increase in signal was calculated as log (S/S_0) where S_0 is the colorimetric signal from untreated adenovirus**.**

The results of the sandwich ELISA are shown in Figure 4-5. The purpose of this assay was to determine the ability of the virus to attach to the coxsackie-adenovirus receptor. This attachment must take place for the virus to infect the host cell. The assay utilizes the same primary and secondary antibodies as the indirect hexon ELISA. It appears from these results that there is some reduction in the ability of LP and MP treated virus to bind to the CAR protein. The effect appears to level off. Part of this is due to the limitations of this type of assay. The detection limit of this ELISA (determined from the standard curve of diluted, untreated virus) is approximately a titer of virus of $5x10^5$ IFU/mL. Given that the titer of

virus used in this study was approximately 5x 10^7 IFU/mL, a reduction of 1.5 logs would be very close to the detection limit of the assay. An increased titer of virus may be able to overcome this limitation, but achieving a titer of several orders of magnitude greater would be difficult.

The data show a trend of a sharp decline in the ability of the virus to attach after a relatively small UV dose of 10 mJ/cm^2. For LP UV there is a slight decreasing trend as dose is increased, but for MP UV it is not possible to observe the same effect due to the assay limitation. It would appear that even LP UV affects the ability of adenovirus to attach to the CAR receptor in this assay. MP UV seems to have a stronger effect on the ability of the virus to attach to the CAR protein but the extent of the effect of MP UV on virus attachment is not able to be determined from this data.

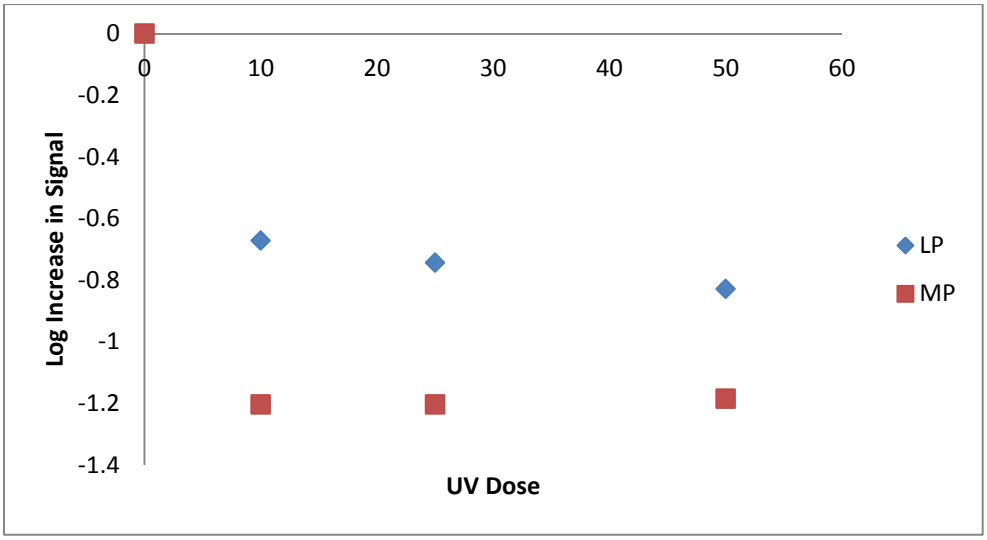

Figure 4-5: Results for LP and MP treated adenovirus from sandwich ELISA. Log increase in signal was calculated as log (S/S_0) where S_0 is the colorimetric signal from untreated adenovirus.

Conclusion

There are a variety of methods for disinfecting water. UV has gained attention for its effectiveness against protozoa, which have historically been a problem for chlorine based disinfection systems. The resistance of adenovirus to LP UV disinfection creates concern over the effectiveness of this method against a pathogen previously thought to be a major public health hazard. Its relative susceptibility to MP UV indicates there is potential for this technology to be effective against adenovirus; however, the mechanism of enhanced inactivation remains unclear. This research helps to show that the hexon capsid protein has a similar response to ELISA after UV treatment by LP and MP lamps. It is possible that the virus is damaged in different ways by the two types of UV lamps, but the hexon protein is able to be identified by the antibody in a similar way after both treatments. Further research should focus on alternative methods for examining proteins, including methods for determining the stage of the viral lifecycle that is impacted by UV disinfection of both LP and MP types.

ELISA is a valuable tool and is useful for quantifying viruses due to its more rapid time scale than traditional cell culture quantification. If infectivity can be related to an ELISA it would aid in determining the efficiency of a disinfection process with regards to a specific virus. Further work could be done to optimize this technique so it could be of use to water practitioners. A rapid ELISA that was well correlated to infectivity could be used for UV validation or testing of a reactor.

5. Advanced Oxidation Disinfection of Adenovirus

Abstract

Adenovirus has consistently been observed to be the most resistant known pathogen to disinfection by ultraviolet light. This has had an impact on regulations set by the United States Environmental Protection Agency regarding the use of UV disinfection for virus inactivation in groundwater and surface water. In this study, enhancement of UV inactivation of adenovirus was evaluated when hydrogen peroxide was added to create an advanced oxidation process (AOP). While 4 log reduction of adenovirus was determined to require a UV dose (UV fluence) of about 200 mJ/cm^2 from a low pressure (LP) UV source (emitting at 253.7 nm), addition of 10 mg/L H_2O_2 achieved 4 log inactivation at a dose of 120 mJ/cm^2. DNA damage was assessed using a novel nested PCR approach, and similar levels of DNA damage between the two different treatments were noted, suggesting the AOP enhancement in inactivation was not due to additional DNA damage. Hydroxyl radicals produced in the advanced oxidation process are likely able to damage parts of the virus not targeted by LPUV, such as attachment proteins, enhancing the UV-induced inactivation. This research sheds light on the inactivation mechanisms of viruses with ultraviolet light and in the presence of hydroxyl radicals and provides a practical means to enhance inactivation of this UV-resistant virus.

Introduction

Adenovirus is a virus that infects humans and can cause a variety of symptoms including respiratory infection, gastroenteritis, conjunctivitis, and pneumonia. Due to its persistence in the environment it has been detected in treated wastewater and drinking water sources worldwide (Crabtree et al., 1997). It is a non-enveloped double stranded DNA virus consisting of a protein capsid encasing the DNA. There are 52 serotypes of which 17 are known to infect humans.

Due to the ability of adenovirus to persist in the environment and its presence in drinking water sources, it is of concern for drinking water treatment (Enriquez et al., 1995). Disinfection of adenovirus is easily accomplished by free chlorine (Thurston-Enriquez et al., 2002). Study of adenovirus disinfection by low pressure (LP) ultraviolet (UV) light (emitting at 253.7 nm) has shown the virus to be the most resistant known pathogen to this type of disinfection (Nwachuku et al., 2005; Gerba et al. 2002; Meng and Gerba, 2002). Adenovirus' resistance has impacted the regulations regarding UV disinfection of drinking water (Yates et al., 2006). The Long Term 2 Enhanced Surface Water Treatment Rule (LT2ESWTR) regulates virus disinfection with UV light based on the high dose (186 mJ/cm^2) observed to be required for 4-log inactivation of adenovirus (USEPA, 2006a) under LP UV. In addition, the Groundwater Rule does not explicitly allow the use of UV for virus inactivation credit in groundwater treatment due to the lack of an existing surrogate organism for validation up to and beyond 186 mJ/cm^2 (USEPA, 2006b). Due to the extreme resistance of adenovirus to UV disinfection and resulting regulations, many systems may rule out the use of this technology for their disinfection process (Yates et al., 2006). Therefore it is useful to explore ways of enhancing UV technology to make it more effective against adenoviruses and subsequently more accessible to utilities wishing to implement UV technology-based solutions for virus disinfection.

Advanced oxidation processes (AOP) are typically used to remove organic contaminants and taste and odor causing compounds from drinking water. This process typically uses high doses of UV light in combination with H_2O_2 to produce hydroxyl radicals. Hydroxyl radicals are a powerful oxidant that can chemically transform organic molecules. Mamane et al. (2007) indicated enhancement of MS2 bacteriophage (a surrogate organism for viruses during UV validation testing) disinfection when exposed to hydroxyl radicals and identified the need for

further investigation into germicidal UVC in combination with H_2O_2 to produce hydroxyl radicals and enhance viral disinfection. Additional studies have examined the effect of hydroxyl radicals produced during a UV/TiO_2 process on bacteria and phage for the disinfection of *E. coli* by hydroxyl radicals (Cho et al., 2004; Cho et al., 2005). From these past studies it has been suggested that viruses such as MS2 are inactivated in the presence of hydroxyl radicals. However, there has not been study into the inactivation kinetics of human viruses such as adenovirus in the presence of hydroxyl radicals. The goal of this study was to examine the effects of hydroxyl radicals produced by UV/H_2O_2 advanced oxidation on adenovirus inactivation under disinfection level UV doses, with the goal of enhancing UV-based inactivation of this virus.

Materials and Methods

UV Irradiation and Experimental Design

UV exposures were performed using a LP UV quasi-collimated beam apparatus. Adenovirus samples were spiked into phosphate buffered saline (PBS) and then exposed to UV light in a stirred batch system. UV doses were determined as described by Bolton and Linden (2003). Briefly, incident irradiance at the surface of the sample was measured using a radiometer (IL1700, SED240/W detector) calibrated at 254 nm. Correction was made for any nonhomogeneity of UV light over the surface of the petri dish (Petri Factor). Average irradiance was calculated by integration of the Beer-Lambert law over the sample depth, taking into account the absorbance of the sample and incident irradiance. Exposure times to achieve target doses were calculated by dividing the target UV dose by the average UV irradiance. The depth of sample was 0.5 cm for all exposures. Incident UV irradiance was 1.75 mW/cm^2. The Petri Factor was 0.97 for all exposures.

Propagation of Stock Cells and Virus

Briefly, adenovirus was propagated in an A549 human lung carcinoma cell line (American Type Culture Collection, Manassas VA) and the cell line was used for all infectivity assays. Cells were grown in Ham's F12K media supplemented with fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin. Cells were incubated at 37 degrees Celsius with 5% CO_2. Replating of stock cells was completed by twice rinsing with Dulbecco's PBS, incubation with 0.25% trypsin/EDTA solution, re-suspension and dilution in new flasks. Cell stocks were split once per week. Media and stock solutions for cell culture and viral propagation were obtained from Invitrogen (Carlsbad, CA).

Cell culture infectivity assays were used both to determine the titer of viral stocks and to assay the UV and UV/H_2O_2 exposed virus. A549 cells were plated into 24 well plates and allowed to grow to 80% confluence. Cells were introduced to exposed and diluted viral samples and allowed to incubate for one hour, rotated every 15 minutes. Following exposure to viral samples the cells were introduced to high glucose DMEM supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 U/mL penicillin, and 100mg/mL streptomycin and allowed to incubate for up to 2 weeks before being scored. Scoring data were entered into a computer program to determine MPN, as previously described (Hurley and Roscoe, 1982). Log inactivation was calculated as $\log_{10}$[(MPN/mL untreated control)/(MPN/mL treated sample)].

Hydroxyl Radical Concentration Determination

An OH radical probe compound, *para*-chlorobenzoic acid (*p*CBA) was used to indirectly determine the concentration of hydroxyl radicals. The steady state OH radical concentration was determined following methods described in Mamane et al. (2007). Briefly, *p*CBA at a concentration of 0.15 mg/L (150 ppb) was dissolved into PBS samples containing adenovirus at an approximate titer of 10^6 MPN/mL. Hydrogen peroxide was added at a concentration of 10

mg/L and samples were exposed to LP UV for the doses used in the infectivity analyses. *p*CBA is degraded by hydroxyl radicals with a pseudo first order rate constant (k_{OH}*p*CBA) of 5 x 10^9 M^{-1} s^{-1}. *p*CBA concentration was determined using liquid chromatography with UV detection (Agilent 1200 HPLC) for the different UV doses, and steady state hydroxyl radical concentration was determined from the degradation of *p*CBA in the samples as previously described (Rosenfeldt and Linden 2007). In addition, the approximate steady state hydroxyl radical concentration for a variety of model waters was estimated using a model based on Glaze et al., (1995).

Most Probable Number (MPN) Calculation for Virus Titer

Virus titer was determined by plating virus samples in 24-well plates with 80% confluent A549 cells with identical samples plated in rows of six wells and different dilutions in each row. The most probable number was determined after 14 days of incubation from the wells showing cytopathic effect (CPE). A positive score was given if the well showed evidence of CPE when viewed with an inverted microscope. CPE is visibly determined by the rounding up and detachment of cells. The most probable number calculation is described in detail in Hurley and Roscoe (1982). MPN was determined using the MPN Calculator available online at i2workout.com.

Measurement and Quenching of H_2O_2

H_2O_2 was measured using the I_3^- method described by Klassen et al. (1994). Residual H_2O_2 was quenched using bovine catalase (Sigma Aldrich, St. Louis MO) before microbial or chemical analyses (Liu et al., 2003).

Results and Discussion

OH radical concentration

The hydroxyl radical concentration exposed to adenoviruses was determined from Equation 1 using data from *p*CBA degradation

$$OH_{ss} = \frac{k_{exp}}{k_{OH,p\mathrm{CBA}}} \qquad \text{(Equation 4-1)}$$

where k_{exp} is the slope of the plot of degradation of *p*CBA (ln [*p*CBA/*p*CBA$_0$]) as a function of time, and $k_{OH,pCBA}$ is equal to 5 x 10^9 M^{-1} s^{-1}. A steady state hydroxyl radical concentration of approximately 4 x 10^{-13} M was reached when 10 mg/L of H_2O_2 was present in the UV exposed working solution of adenovirus.

Cell Culture Infectivity Results

Results from the cell culture infectivity studies with and without peroxide are shown in Figure 5-1. 4-log reduction of adenoviruses under LP UV required a UV dose of 206 mJ/cm^2. The log reduction of adenovirus in the presence of H_2O_2 showed significantly increased effectiveness. 4-log reduction of adenovirus was achieved with a dose of 122 mJ/cm^2 (with 10 mg/L H_2O_2). T-tests were performed on the data and significant differences between exposures with and without peroxide were observed for UV doses above 40 mJ/cm^2 ($p<0.05$). A control of virus with 10 mg/L H_2O_2 and no UV treatment was also evaluated and no significant inactivation was achieved by the H_2O_2 alone (data not shown).

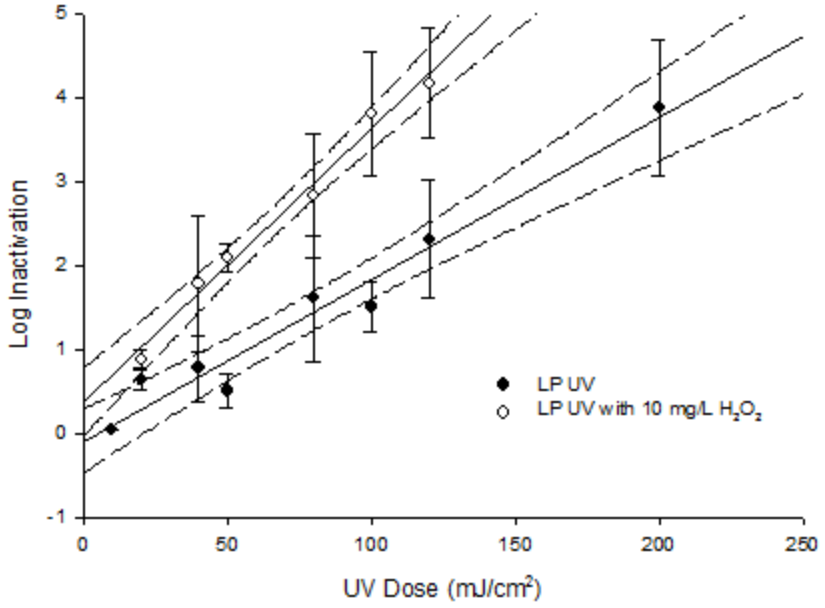

Figure 5-1: Log reduction of adenovirus 2 after treatment with UV and with UV/H_2O_2. Results are from a series of 4 independent experiments. Error bars on points represent one standard deviation. Dashed lines are the 95% confidence intervals for the regression analysis performed with SigmaPlot.

Figure 5-2 illustrates the log inactivation of adenovirus specifically due to the OH radical exposure, where OH radical CT is calculated as the steady state hydroxyl radical concentration multiplied by the time of UV exposure (M-s). The OH radical induced log inactivation is calculated as the difference in inactivation between the samples exposed to UV with hydrogen peroxide and the UV-exposed samples without the addition of hydrogen peroxide. Based on the data in in Figure 2, the rate constant for inactivation of adenovirus by hydroxyl radicals was calculated to be 4.6 x 10^{10} $M^{-1}s^{-1}$. This rate constant was calculated as the slope of the linear regression of the data. Linear regression indicated a correlation with an r^2 value equal to 0.77.

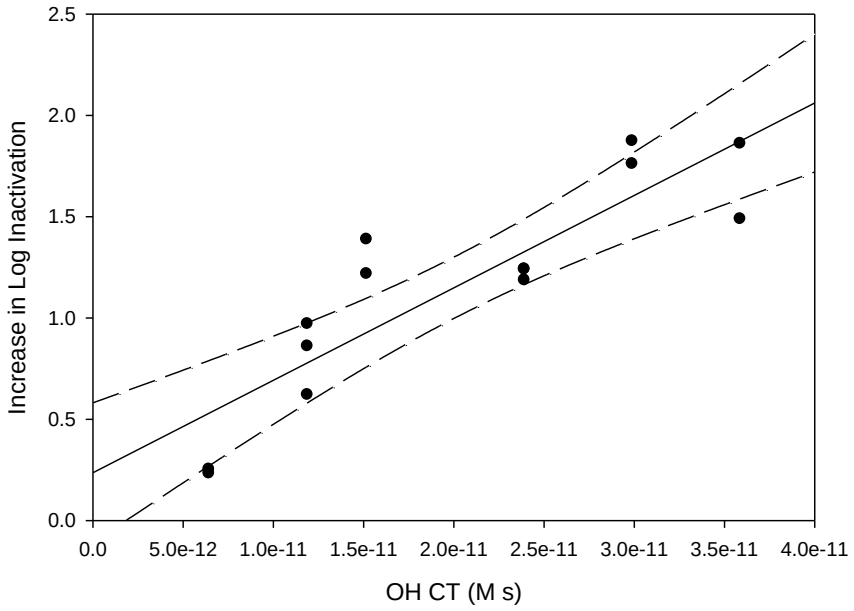

Figure 5-2: Log inactivation of adenovirus due to OH radical inactivation, for various exposures (CT levels) of hydroxyl radicals. The OH_{ss} was 4 x 10^{-13} M for these exposures. Dashed lines represent the 95% confidence on the regression performed by SigmaPlot software. OH radical CTs were generated in solution with 10 mg/L H_2O_2 and UV doses of 20, 40, 50, 80, 100, and 120 mJ/cm^2.

Mechanism of Enhanced Inactivation

To gain insight into the mechanism of disinfection by hydroxyl radicals and why disinfection may be enhanced in the presence of peroxide, the damage to the adenovirus genome was assessed using a polymerase chain reaction (PCR) assay on UV alone and AOP treated virus samples. LP UV mainly targets the genome of viruses due to its monochromatic output at 253.7 nm, close to the peak absorbance of DNA at 260 nm. The UV light primarily creates dimers between adjacent pyrimidine bases within the genetic code that inhibit replication and prevent the virus from reproducing and subsequently causing infection. Difference in DNA damage in the presence of hydroxyl radicals compared to UV alone would indicate a mechanism of inactivation related to the effect of OH radicals on the genetic material. No difference would indicate the enhanced inactivation is related to some other mechanism.

The PCR assay used in this study incorporates a two-step process (based on Rodriguez et al., 2012). The first PCR step is used to create many exact copies of a large fragment of the adenovirus genome. The use of PCR to detect level of UV damage to adenovirus has been shown previously in Eischeid et al. (2009). The ability of the large fragment of DNA to copy by PCR is dependent on the segment of viral DNA being left intact, undamaged after UV treatment of the virus, much as the ability of the virus' DNA to replicate during infection is dependent on it being left undamaged from UV exposure. UV damaged DNA will not replicate during the PCR process. During this first step a short sequence "tag" is attached to all copies created from the larger fragment. The second step to the PCR uses a quantitative (qPCR) step to quantify the small sequence "tags" attached to successful copies. The number of "tags" quantified directly relates to the amount of intact DNA (undamaged by UV) present in the original sample. The higher the number of tags successfully replicated and copied, the lower the level of nucleic acid damage that was inflicted by UV. The results of the DNA damage assay are shown in Figure 4. The log reduction in intact DNA was calculated as the $-\log(N/N_0)$ with N being the number of intact DNA fragments in the sample and N_0 being the number of fragments intact in the untreated control. There was no significant difference observed in the DNA damage between the UV treated samples and the UV/H_2O_2 treated samples, indicating the mechanism of enhanced inactivation by OH radicals was not likely DNA damage-based and another mechanism must have caused the differences in results observed in the cell culture infectivity assays.

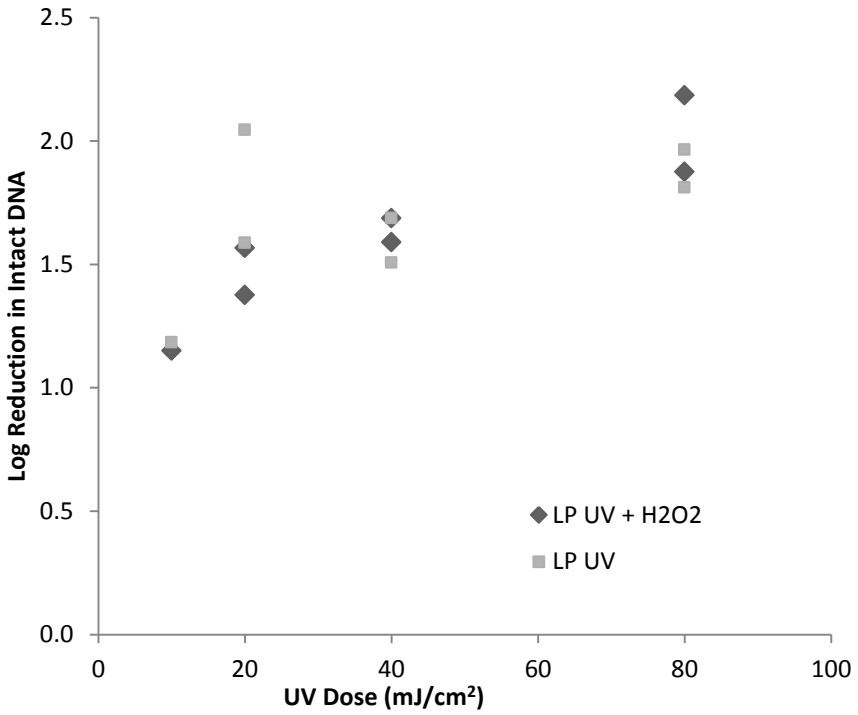

Figure 5-3: Evaluation of the DNA damage to adenovirus nucleic acids under UV alone and UV-based AOP conditions, as measured by a two-step quantitative PCR assay. T-tests were performed on the paired data and no statistically significant difference was observed ($p \geq 0.29$).

Further study is necessary to elucidate the mechanism that enhances disinfection of adenoviruses by OH radical treatment. It is likely that the hydroxyl radicals damage a different part of the virus structure than the LP UV damaged DNA. For instance, proteins of the capsid are important to the lifecycle of the virus and without these intact the virus is unable to infect cells and replicate itself. It is possible that these capsid proteins may be damaged by the hydroxyl radicals produced by the combination of UV light and H_2O_2. Future study into the integrity of capsid proteins is necessary to determine the mechanism of inactivation by hydroxyl radicals.

Practical Impacts of OH Radical Enhanced UV Inactivation

In order to determine how this process may perform in natural waters, a steady state hydroxyl radical production model (Glaze et al., 1995) was used to predict the formation of OH radicals under various water disinfection scenarios. This model incorporates the OH radical scavenging potential of natural waters into its calculation for OH_{ss}. The model was tested for 5 and 10 mg/L H_2O_2 over a range of UV doses using water quality parameters from three field waters. Results of this modeling effort are shown in Table 5-1. The OH radical CT values calculated from the OH_{ss} model were used to predict the expected enhancement in inactivation of adenovirus under UV disinfection conditions at H_2O_2 concentrations of both 5 and 10 mg/L, using the rate constant generated from the data in Figure 5-2, with the results illustrated in Figure 5-4.

Table 5-1: Model predictions of OH radical exposure (CT) formed in different water sources under UV doses commonly used in UV disinfection.

Water Type	**pH**	**Alkalinity (mg CaCO3/L)**	**TOC (mg-C/L)**	**% UVT 254 nm**	**OH Radical Exposure (M s) CT**		
					UV 20[1]	**UV 40[1]**	**UV 80[1]**
Groundwater	7.4	280.6	1.29	97%	7.14E-12	1.43E-11	2.85E-11
Surface Water 1	8.0	37.6	5.43	81%	4.96E-12	9.92E-12	1.98E-11
Surface Water 2	6.7	40.4	2.76	85%	7.95E-12	1.59E-11	3.18E-11

[1]Numbers represent the UV doses in mJ/cm^2. OH radical CT reported is calculated based on 10 mg/L H_2O_2 levels

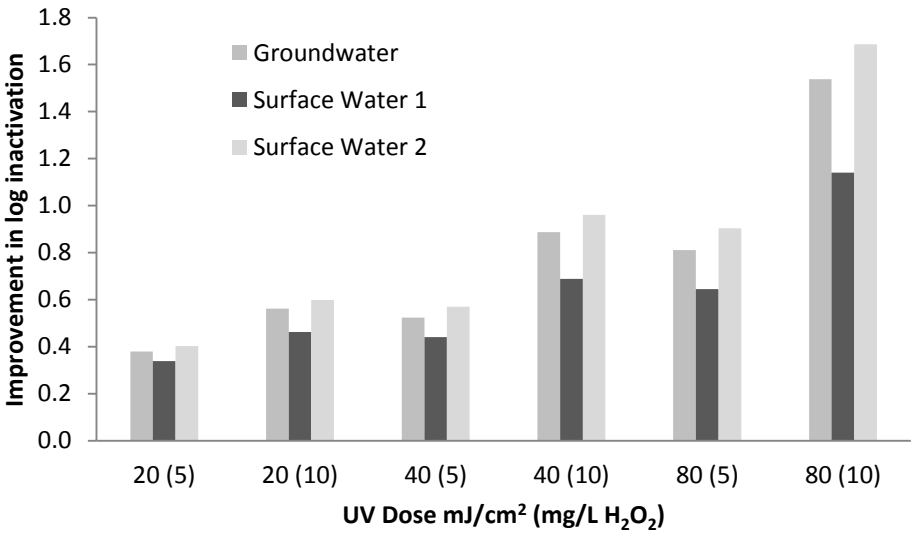

Figure 5-4: Improvement in log inactivation of adenovirus over LP UV alone, with the addition of 5 or 10 mg/L H_2O_2. Values in parentheses on the x-axis indicate the mg/L of H_2O_2 for each dose condition.

For the waters tested, the addition of 5 mg/L H_2O_2 to a LP UV disinfection process can enhance inactivation of adenoviruses by 0.44 to 0.57 log at a dose of 40 and 0.65 to 0.90 log at a dose of 80 mJ/cm^2. With addition of 10 mg/L H_2O_2, the UV disinfection process can be enhanced by 0.69 to 0.96 log at a dose of 40 and 1.1 to 1.7 log at a dose of 80 mJ/cm^2 for inactivation of adenoviruses. While water quality plays some role due to OH radical scavenging, the improvements for inactivation are evident across the water qualities examined and these results could be extended to any water quality of interest.

Conclusions

UV inactivation of adenovirus can be enhanced by addition of hydrogen peroxide during disinfection, due to the formation of OH radicals. While OH radicals improved virus disinfection, the increased inactivation was not due to higher levels of DNA damage, based on

data from a novel qPCR-based DNA damage assay. Therefore, this study adds to the mechanistic-based understanding of AOP based disinfection.

A steady-state OH radical model was used to calculate expected enhanced inactivation of adenovirus with the addition of H_2O_2 to an existing UV disinfection system. The increased inactivation due to OH radicals varied with the water quality characteristics and the amount of H_2O_2 added, but was significant, reaching up to 1.7 log at a dose of 80 mJ/cm^2 with 10 mg/L H_2O_2.

This study illustrates a means to enhance traditional LP UV disinfection technology, thus obtaining disinfection credit for viruses at lower than regulatory doses. It provides water systems that currently may not consider use of UV disinfection for virus inactivation (i.e. groundwater systems) a possible strategy to install UV disinfection and gain virus credit at lower UV doses or enhance their existing inactivation levels without increasing the size of their UV disinfection system.

Future study should focus further on the mechanisms of inactivation by OH radicals. For instance, determination of the extent of damage to capsid proteins of the virus will be beneficial to furthering the fundamental understanding of the effects of UV light and hydroxyl radicals on viruses.

6. Conclusions

This research helps to provide insight into the fundamental effects of UV light in the disinfection of adenovirus, as well as introduce a possible treatment technique for improving disinfection credit from LP UV. The more that is understood about the basic effects of UV disinfection on a micro and nano-scale, the more this technology can be used in efficient ways. The work described here should be used to guide future research projects in this field.

PCR

A two-step nested PCR method was developed to quantitatively assess a long segment of the adenovirus genome. The first step of the PCR copied a segment of adenovirus DNA between 1,000- 10,000 base pairs. The second step used qPCR to quantify by fluorescence the amount of large copies that were successfully copied by the first step. If the DNA had been damaged by UV it would not be able to be copied in the first step of the PCR, which means this method was able to assess the amount of DNA damage overall by both MP and LP UV. The PCR study confirmed earlier results showing levels of DNA damage, as measured by ability of the DNA fragment to copy during PCR, to adenovirus are similar between LP and MP UV. The assay used in this thesis improved upon earlier methods. It also provides a more rapid test for quantifying UV damage to adenovirus compared to cell culture infectivity assays. A similar approach could be developed for other viruses, which often also have time and labor intensive cell culture infectivity assays or no assay for infectivity at all.

ELISA

ELISA is a technique often applied in a clinical setting to quantify levels of proteins using antibodies specific to the protein of interest. Using this approach, the method was tested and demonstrated no reduction in hexon capsid protein integrity by LP or MP UV, but some reduction when the virus was treated with LP UV and H_2O_2. Further testing should be done to

achieve a greater sample size. In addition a sandwich ELISA approach was used to test the ability of the virus to bind to the coxsackie-adenovirus receptor which is the binding site of the virus on the host cell. Reduction in the signal of bound virus was observed after both LP and MP treatment, however it did not appear to increase with increasing dose. It would appear that the sensitivity of this assay may not be strong enough for determining the potential for damage to capsid proteins of viruses. Further examination of this topic should focus on improvement of the sensitivity of the assay. Increasing the titer of the virus sample would likely result in increased sensitivity, but this study was limited by the amount of virus able to be grown in the laboratory. Another way to increase the sensitivity may be to use a sandwich ELISA with two different antibodies to the hexon protein that recognize different epitopes. The results that were gathered appear to indicate that the capsid hexon protein is not damaged by MP or LP UV in a way that makes it unrecognizable by the antibody used. Using antibodies to different epitopes of the protein may provide breadth to the data that is able to be gathered by this method.

Advanced Oxidation Treatment of Adenovirus

Adenovirus was exposed to LP UV combined with the addition of 10 mg/L of H_2O_2. This combination is known to produce hydroxyl radicals, which are nonspecific oxidants. Previous study has shown enhanced inactivation of MS2 in the presence of LP UV and H_2O_2 over LP UV alone (Mamane, 2007). Cell culture infectivity results for adenovirus showed that improvement in the inactivation of adenovirus was possible in the presence H_2O_2 and LP UV light compared to UV light alone. The production of hydroxyl radicals by this process appears to enhance inactivation of adenovirus. This process has the potential to provide increased inactivation credit for adenovirus for LP UV systems that add H_2O_2.

Future Work

The research conducted as part of this thesis attempted to gain further insight into the resistance of adenovirus to disinfection by UV light, and how to increase susceptibility of adenovirus to disinfection with UV. Many questions still remain for future research.

Advanced Oxidation Disinfection of Adenovirus

Advanced Oxidation processes that produce hydroxyl radicals have the potential to be a powerful disinfectant tool to enhance inactivation by LP UV light. Further work should focus on the mechanism of disinfection. Molecular study on the damage caused to the virus particle is necessary to confirm the hypothesis that proteins necessary for virus entry into the cell are targeted and damaged by the hydroxyl radicals. This type of disinfection could provide a tool for systems with LP UV in place to meet the disinfection credit for adenovirus with only the addition of a small amount of hydrogen peroxide. Real world systems should be tested to determine if the model predictions for hydroxyl radical production and enhanced disinfection of adenovirus scale up from the bench to full-scale.

Molecular Work to Determine Effect of LP/MP UV

It is of interest to the field to determine which part of the adenovirus lifecycle is inhibited by MP UV light. The apparent increased susceptibility of adenovirus to polychromatic MP UV light is an interesting phenomenon that merits further study to determine its explanation. Future study should attempt to isolate specific steps of adenovirus lifecycle to determine which steps may be inhibited. PCR techniques could be applied to treated adenovirus after it has been introduced to cell culture. This may provide insight into the repair of DNA after introduction to cell culture. Another assay that could be performed would be immunofluorescence assays after the virus has been introduced to cell culture to determine the levels of proteins that are being produced after infection; specifically after the virus DNA has reached the cell nucleus. The comparison of this

data between adenovirus treated with LP and MP UV would show whether or not one of the types of UV light inhibits the ability of the virus to reach this step in its lifecycle. A similar approach has been used in the past to examine the effects of LP UV alone on adenovirus (Sirikanchana, 2008).

The potential repair of DNA of adenovirus treated with UV light is an occurrence that has been studied by Guo et al. (2010). In addition, the apparent repair of nucleic acids in phage has been observed previously (Dulbecco, 1952). Further exploration of this topic should include evaluating the repair potential of virus in a mammalian host (in vivo) to support the results obtained in vitro. This work is necessary to determine if cell culture results are indicative of how UV treated virus responds in a host organism. Other double stranded DNA viruses should be evaluated for their repair potential after UV treatment.

There is certainly more work to be done to determine the exact mechanism of enhanced inactivation of adenovirus under MP UV light, and in the presence of LP UV with H_2O_2. Other viruses with similar structure to adenovirus should also be evaluated for their resistance to disinfection by LP UV. Polyomaviruses and papillomaviruses are other double stranded DNA viruses that may be of interest for future study. Effective use of UV technology for disinfection of water is dependent on the furthering of the knowledge of the mechanisms of inactivation that it provides.

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Appendix A. Long-Range Quantitative PCR for determining inactivation of adenovirus-2 caused by Ultraviolet Light.

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Abstract

An extra-long range quantitative PCR (LR-qPCR) was developed for estimating genome damage to adenovirus caused by UV irradiation. The objective was to use the LR-qPCR as a rapid method to determine advs inactivation by UV. The LR-qPCR consisted of two steps: a long-range PCR (up to 10kb fragment length) and a real-time (q)PCR for quantifying the products of the first PCR. We evaluated the LR-qPCR with adenovirus irradiated with medium pressure (MP, polychromatic wavelength emission) and low pressure (LP, monochromatic emission at 254nm) mercury vapor UV lamps and compare the results with results obtained with cell culture infectivity assays. No difference was observed when using fragments lengths of 10 kb and 6 kb for LR-qPCR. When comparing the results obtained with LR-qPCR using a fragment of 6kb performed better for estimating DNA damage at doses between 0 to 20 mJ/cm^2 and a 1kb performed better for doses between 20 to 100 mJ/cm^2. The results of LR-qPCR analyzing a fragment of 6kbs were similar to the results obtained with infectivity assays for adenovirus exposed to MP UV lamps. In the case of adenovirus irradiated with LP lamps, the results obtained with LR-qPCR with shorter fragment size (1kb) were similar to the reduction of viral infectivity. In conclusion, the LR-qPCR can be used for estimation of DNA damage caused by UV in adenovirus. In addition, results obtained with LR-qPCR were related with reduction in viral infectivity.

INTRODUCTION

Adenovirus (Adv) is the most resistant known human pathogen to UV disinfection(12, 20). In addition, Adv is commonly found in wastewater (8, 16, 18) and contaminated water bodies (2, 10, 22) and can remain infectious after long periods in the water (13, 15). Since UV based disinfection systems have become popular and more accessible this extreme resistance of Advs to UV disinfection is of interest to the water treatment industry, and additionally is the reason Adv is used as a viral model for validating UV systems (12). Water treatment regulations typically require a disinfection system to achieve 4-log inactivation of viruses, so for UV disinfection, utilities are required to meet this 4-log level using Advs. This requirement limits the use of UV water treatment to systems that can achieve very high doses (up 186 mJ/cm^2) when using low pressure (LP) lamps. Use of polychromatic medium pressure (MP) UV systems has been shown to enhance the inactivation of adenoviruses, lowering the dose necessary to achieve a level of inactivation compared to LP UV.

UV irradiation inactivates microorganisms by damaging the DNA, inhibiting the replication of DNA and transcription of DNA into mRNA, essential steps for the viability of microbes including viruses. There are two different UV systems commonly used. One is based on a monochromatic (low pressure (LP) mercury lamp) UV light source that emits at a wavelength of 254 nm and the second is based on polychromatic, medium pressure (MP) mercury lamp UV light source that emits UV irradiation between 200 and 400 nm. The peak of absorption of DNA is 260 nm, therefore monochromatic (LP lamp) UV radiation of 254nm mainly targets the microorganisms' DNA. Alternatively, polychromatic (MP lamp) UV systems have the potential

of targeting proteins and other essential macromolecules that are important to the viability of the microbe, due to the polychromatic nature of the UV emission. The calculation of germicidal dose has been standardized for LP and MP UV lamps making it possible to compare the performance of both UV systems (4). For example, the inactivation and the production of DNA damage in *E. coli* is similar between MP and LP UV systems (6). Interestingly, in the case of Adv, MP lamps are more effective than LP UV systems for inactivating the virus. For example in Adv 2, the dose required for 4-log reduction using MP lamps is between 30 to 50 mJ/cm^2 but the dose required for the same reduction using LP lamps is between 120 to 180 mJ/cm^2 (12). Despite the difference in viral inactivation observed between LP and MP UV systems evaluated using the cell culture infectivity assay, the damage caused in the Adv DNA is very similar (7). Moreover, the use of a cell line deficient in DNA repair mechanisms for infectivity assay has shown similar sensitivity of Adv to both UV lamps (9), suggesting that the extreme resistance observed when using LP UV lamps could be the result of host DNA repair mechanisms and the possibility that somehow MP UV emissions inhibits DNA repair. Therefore, the quantitation of DNA damage in Adv could provide a useful tool for the determination of viral inactivation, bypassing the bias produced when using a cell culture approach.

Polymerase chain reaction (PCR) has been used previously to analyze DNA damage (3) because PCR needs an intact DNA strand for the progression of the polymerase during the reaction. The effectiveness of PCR to determine DNA damage will depend in the length of DNA that is analyzed, in which a PCR analyzing long fragments will have more provability to detect damage in the DNA than PCR analyzing a short fragment (17). Real-time (q) PCR is commonly used for the quantitation of DNA and the detection of viruses, but usually qPCR assays are based on the amplification of very short fragments (<140 bp) because the use of long fragments for RT-PCR

will reduce efficiency of the reaction. This study describes the use of a nested approach based in a very long range (LR) PCR (up to 10kb) and a qPCR targeting the LR-PCR products for the quantitation of undamaged DNA of Adv 2. In addition, we demonstrated the use of this technique in Adv 2 exposed to LP and MP UV lamps and compare the results to those obtained with cell culture. It was hypothesized that the reduction in undamaged DNA determined by analyzing a long DNA fragment will be similar or higher than the inactivation of viruses as determined by cell culture assays. To our knowledge, this is the first time that this combined use of Long range PCR and qPCR have been employed for determining UV damage in Adv.

MATERIALS AND METHODS

Adenovirus (Adv) 2 stock and cell culture assay: The Adv-2 stock was provided by Dr. Charles P. Gerba, University of Arizona and propagated in A549 cells (ATCC **CCL-185)**. The viral stock was cleaned by chloroform extraction and concentrated by polyethylene glycol precipitation. Adv 2 samples were titrated using a plaque assay. Briefly, confluent monolayers of A549 cells in 6-well plates were infected with Adv 2 samples in ten-fold dilutions and incubated for one hour at 37^0C and 5 % CO_2. After allowing the viruses to attach to the cells, sample was removed by aspiration and a layer of 1 X complete MEM (2% of heat inactivated fetal bovine serum, 25mM $MgCl_2$ and 0.5% of agarose) was applied to the infected monolayer. The cell culture assays were incubated for 7 days at 37^0C and 5% CO_2. After incubation, the overlying media was removed and the cells monolayer was covered with 1mL of crystal violet solution and incubated for 30 minutes. The crystal violet was removed by aspiration and viral induced plaques were observed under a white lamp.

UV exposure: The low pressure (253.7 nm) mercury lamp and medium pressure lamp collimated beam systems used in this study were described previously (5). Viral sample absorption 400 nm to 200 nm (bio100, Varian), UV beam homogeneity, sample depth, and UV lamp irradiance were used for the calculation of UV doses for monochromatic and polychromatic UV lamps as described previously (4). Sample exposures were performed in petri dishes covered with quartz lids and slowly agitated.

Long-Range PCR: For long-range (LR) PCR, primers were designed using the Adv 2 sequence published in NCBI (accession number 56160492). The desired characteristics of the primers were: GC content over 50% and melting temperature around 65°C. The GeneAmp XL PCR kit (Applied Biosystem) was used for the LR PCR and the reactions were carryout using HotStart reaction tubes (Molecular BioProducts). The lower PCR mixture consisted of 6 µL of 3.3 X LX PCR buffer, 1.2 mM of MgCl, 200 µM of each dNTP, 0.3 µM of each primer and PCR grade water for a volume of 20 µL. The lower PCR mixture was aliquoted into the HotStart tubes, and then the tubes were heated at 90°C for 1 minute and cooled down to 4°C until the wax layer hardened. Then, 25 µL of the upper PCR mixture was aliquoted into the HotStart tubes. The upper PCR mixture consisted of 9µL of 3.3X buffer, 2 units of rTth DNA polymerase and water up to a volume of 25µL. Five microliters of purified DNA were used. The LR PCR conditions were: an initial denaturation step of 95°C for 1 minute, two-step cycles of 94°C for 15 seconds and annealing-extension step of 65°C for 10 minutes, then a final extension step of 73°C for 10 minutes. For initial assessment of primers, 30 cycles in the PCR program were used.

Combined use of LR-PCR and real-time (q)PCR (LR-qPCR): For the quantitation of undamaged DNA targets, a nested approach was designed in which a first step LR PCR was performed on the DNA and then a real-time (q)PCR was used for the quantitation of the LR PCR

product. For first step, an adapter was added to the 5' end of the forward primer, this adapter sequence served as the forward primer for the real-time PCR. In addition, the LR PCR step only consisted of 10 cycles. A successful LR PCR will provide the complementary sequence necessary for the forward primer used in the qPCR. After the first LR PCR step, the PCR products were digested with nuclease S1 to remove primers and single-stranded overhands from LR-PCR products. For the nuclease digestion, 20 units of Nuclease S1 (Promega), 5µL of 10X digestion buffer (promega) and water were added to 40µL of LR PCR product for a total volume of 50µL. The enzyme digestion was incubated at 37°C for 30 minutes and immediately after incubation, the PCR products were cleaned with the QIAquick PCR purification kit (Qiagen) as described by the manufacturer.

For the q PCR, the Quantitect probe PCR kit was used (Qiagen). The qPCR mixture consisted of 12.5 µL of 2X Quantitect master mix, 5 µM of each forward and reverse primers, 2µM of dual-labeled probe, 3 µL of digested LR PCR product and water for a reaction volume of 25 µL. The cycling conditions were as follows: an initial denaturation step of 95°C for 15 minutes, then 40 cycles of 94°C for 15 seconds and 60°C for 1 minute. The primers for the real-time are described in Table 1 and consisted of the adapter primer as forward primer, the reverse primer Ad 2 17.9 and the dual-labeled DNA probe LRAD2. The product size of the real-time PCR is 139 bp. The q PCR assays were performed on a MiniOpticon™ Real-Time PCR detection system (Bio-Rad). For the calibration of the real-time PCR, a fragment of the Adv genome of 10kb was amplified using the primers forward ad 2 17.7 + ADT and reverse Ad2 27.8 as described before. The PCR product was purified with the QIAquick PCR cleaning kit, the concentration was determined by reading the absorption at 260 nm (Bio100, Varian) and purified fragment was corroborated by gel electrophoresis. The molar concentration was determined in

the sample and number of copies was determined from the moles of the fragment purified using Avocadro's number. Ten-fold dilutions of the purified fragment were used to calibrate the cycle threshold (Ct) value obtained with the real-time PCR with gene copies.

Real-time (q)PCR for hexon gene of Adv: The qPCR for the hexon gene of Adv described previously by Jothikumar et al (11) was used. The target region of this PCR assay is inside the region targeted for the long-range PCR assays developed in the present study. The PCR mixture consisted of 12.5 µL of 2X quantitect probe mix (Qiagen), 5 µM of each primer (JTVXF and JTVXR), 2 µM of dual-labeled DNA probe (JTVX_P), 3 µL of DNA target and water for a final volume of 25 µL. The cycling temperatures were: an initial denaturation step of 95°C for 15 minutes, and 40 cycles of 94°C for 15 seconds and 60°C for 1 minute. The reactions were carried out in the same PCR system described previously. The PCR was calibrated using the DNA control described previously.

Statistical analysis: The log of gene copies were calculated using the linear equation obtained from the calibration curve described previously (log10 copies = - (0.326X Ct value) + 11.8, r^2 0.998). Gene copies were calculated from the following equation: gene copies =$10^{(\log 10 \text{ copies})}$. Log reduction was calculated from the following equation: log reduction = $\log_{10}$ copies (d0) - $\log_{10}$ copies (dX). Were d0 was used for samples not exposed to UV and dX was for samples exposed to a specific dose of UV (5, 10 15….mJ/cm^2). For calculating the amount of damage in specific DNA fragment the poison equation described previously by Ayala et al (3) was used (damage/ fragment size (kb) = - ln (copies d0/copies dX). Analysis of variance was used to determine the statistical effect of Lamp, UV dose and fragment sizes have on the reduction of undamaged DNA.

RESULTS

Adenovirus (Adv) UV inactivation: The inactivation rates of Adv 2 was determined using LP UV lamps and MP UV lamps quantified using a cell culture infectivity assay. From the dose response curves, the dose required for 4 log reduction of advs 2 by MP UV lamps was 48 mJ/cm^2 and for LP UV lamps was 120 mJ/cm^2 .

Primer design and long range PCR: Using a published sequence of the Adv 2, primers were designed for long-range PCR with the main goal of analyzing the largest fragment possible. Set of primers were designed to analyze the region between the 17.8 kb to 27.8 kb of the Adv 2 genome. Three primer sets were designed, increasing fragment sizes in three steps: first step of 1.1 kb in size, second of 6 kb, and the largest fragment of 10 kb in size (Figure 1 A). The primers for this step wise approach and for the q PCR are described in Table 1. The primer sizes varied from 20 bp to 29 bp. The GC composition of the primer sequences ranged from 55.2% to 75% with melting temperatures ranging from 63.0 to 65.2^{0}C. The adapter sequence was optimized for real-time PCR (Table 1). This sequence was added to the 5' end of the forward primer Ad 2 17.8 and the modified forward primer was labeled "ad 2 17.8 + ADT". Figure 1-B shows the amplification of the 10 kb fragment of the Adv 2 genome with and without the adapter sequence in the forward primer. In addition, the figure shows that the addition of the adapter did not affected the amplification of such large fragment of the genome.

In order to demonstrate the difference in the use of 1 kb fragment size versus using 10 kb fragment size in detecting damage on Adv DNA, viruses were irradiated under monochromatic UV light and DNA damage was analyzed using two different fragment length with PCR. The amplification of 10 kb fragment was inhibited at lower doses compared to the amplification of

1kb fragment as demonstrated in the reduced amount of PCR product observed in EtBr stained agarose gel electrophoresis (Figure 2).

Combined use of LR-PCR and Real-time PCR: A real-time (q) PCR method was designed for the quantitation of the product of LR-PCR. This q PCR used the adapter as forward primer and targeted the first 139 base pairs of the amplified fragment. The efficiency of the Real-time PCR assay when calibrated with the DNA control was 98.1 % and the lower detection limit was 1 copy per PCR reaction. The nuclease S1 digestion was added to remove single stranded overhands that could result in the quantification of damaged DNA or incomplete PCR products t. Different concentrations of nuclease S1 were used but higher concentrations of nuclease resulted in the degradation of the LR-PCR product. By purifying the PCR products with the QIAquick cleaning kit, PCR products are removed from the suspension containing the nuclease and primers.

Figure 3 shows the results of the combined use of LR-PCR with Real-time PCR for the quantification of undamaged DNA using fragment sizes of 10 kb and 6 kb. Up to 1.2 log reduction was observed when analyzing 10kb fragment at the relatively low doses used (from 0 to 20mJ/cm$^{2)}$ and up to 2-log$_{10}$ reduction when analyzing a 6kb fragment at the same doses. Differences in the performance of both primers in amplifying a portion of 10kb and 6kb were that more consistent amplifications were obtained when using fragment size of 6 kb for LR PCR. However, both fragment sizes show more reduction in undamaged DNA when the virus is exposed to low doses of MP UV light in contrast to the reduction observed with LP UV lamp. Although, different between lamps were not statistically significant at those lower doses. At a dose of 15mJ/cm^2 similar reduction of undamaged DNA was observed with both UV light sources. Figure 4 compares the quantification of undamaged DNA when using target regions of 6

kb versus 1 kb for the LR-PCR with viruses exposed from 0 to 100 mJ/cm^2 of UV irradiation. The maximum log reduction observed was 3-logs at the maximum dose. When analyzing a fragment of 6kb the assay was more sensitive at low doses than when analyzing DNA fragments of 1kb. For example, when analyzing a DNA fragment of 6kb, a two-log reduction was observed at 20mJ/cm^2 but after that only one additional log reduction was obtained when the UV dose was increased to 100mJ/cm^2 (Figure 5-a). Alternatively, a steady increase of log reduction was observed when analyzing a DNA fragment of 1 kb (Figure 4-b). Interestingly, higher reduction in undamaged DNA was observed at lower doses when using 1kb fragment using a polychromatic UV source in contrast to the monochromatic UV source ($p=0.03$ for 5mJ/cm^2 and $p=0.01$ for 10mJ/cm^2). However, this pattern change was at higher doses where more reduction in the undamaged DNA was observed with the monochromatic UV source($p<0.001$ for doses higher than 20mJ/cm^2). There was no apparent reduction in the quantification of DNA copies when quantifying the hexon gene copies of the sample (Figure 4-c). This Real-Time PCR assay targeted a region that is inside the region used for the LR-PCR, and the amplified fragment size was 98 bp.

Performance of the LR-qPCR: For determining the performance of the combined long-range approach for the quantification of different concentration of target DNA, unexposed adenovirus 2 was diluted and DNA was extracted from each dilution as described previously. The figure 5 A shows that the cycle-threshold (Ct) value obtained between with the LR qPCR at each target concentration was consistently 6 cycles less than the values obtained with direct qPCR. Both PCR have similar slope of 3.0 for copies(x) vs Ct value(y). In addition, these decreases in Ct values correspond to an increase of 100 copies per each initial DNA copies from the sample after the first LR PCR step. Figure 5B demonstrate that amplification efficiency of LR range PCR is

similar at each concentration of target DNA, demonstrating the quantitative nature of the combined use of LR PCR with qPCR (LR-qPCR).

Determining DNA damage (lesions): Lesions on the DNA strand were calculated for Advs irradiated with UV Light (Figure 6). The maximum number of lesions that each PCR approach can detect is about 6 lesions per specific fragment size (6kb or 1 kb). When analyzing a fragment size of 6kb, up to 5 lesions/ 6 kb were detected at 20 mJ/cm^2, but then only 6 lesions/6 kb were observed at a dose of $100mJ/cm^2$ (Figure 6 a.). In the case of analyzing a fragment size of 1 kb, the relationship between dose and DNA lesion was more linear and the maximum number of lesions was observed at UV doses of $100mJ/cm^2$ (Figure 6 b). Moreover, the use of a 1kb fragment reveals that LP UV lamp creates more damage in the DNA at higher doses than MP lamps. Conversely, at lower doses MP UV lamps seem to create more damage in the DNA than LP UV lamps.

Relationship of DNA damage and viral infectivity: The inactivation of Adv DNA was compared with reduction in viral infectivity after exposure to UV irradiation. For this comparison, the LR-qPCR analyzed 6kb of the advs genome. In the case of inactivation of advs by MP UV lamps, the reduction in viral infectivity follows closely the DNA damage at low doses (figure 7a). In the case of LP lamps, the reduction in viral infectivity was significantly lower than the amount of DNA damage observed, especially at lower doses. When a dose of 15 mJ/cm^2 was applied using a LP UV lamp, the reduction in advs DNA was 1.7 $\log_{10}$ but the reduction in viral infectivity was only 0.2 $\log_{10}$. Interestingly, when analyzing shorter fragments (1.1kb, figure 7C), the PCR results follows the reduction of viral infectivity for advs exposed to LP UV lamps.

DISCUSSION

The combined use of a long-range (LR) PCR with qPCR for determining damage on DNA was successfully demonstrated. This approach has the advantage of being quantitative and able to detect undamaged genome at very low concentrations (Figure 5). Similar approach was described for determining the inactivation of parvovirus with amotosalen and UV in palettes (1). In this study, reduction in PCR signal was related to the determination of viral infectivity. The difference between both methods is that in our method an adapter sequence was added in the LR PCR. This adapter sequence is complementary to the forward primer of the qPCR. In addition, a nuclease S1 degradation step was used to remove all single strand overhand that could result from incomplete PCR products. Both steps were added to ensure that the qPCR only quantify complete DNA products from the first step. In theory, this approach should be able to detect damage in any of DNA strands of the advs genome and damage in only one strand will reduce the amount of template by a factor of two. Eischeid et al. (7) also used PCR for determining DNA damage caused by UV irradiation by amplifying a region of advs genome of 1kb and determining the amount of PCR product using gel electrophoresis. The combined use of LR PCR and qPCR has advantages over previous approaches in that it allows for the accurate quantification of PCR product over a larger concentration range than with gel electrophoresis. In addition, as demonstrated in figure 5, the LR-qPCR allows for the detection of low concentrations of DNA. Although both methods used different regions in the adenovirus genome, the results were very similar when analyzing a fragment size of 1 kb.

As discussed previously, the probability of the PCR to find damage in the genome increases with the length of the fragment analyzed (14) and this principle has been demonstrated in RNA viruses such as poliovirus (19), bacteriophage MS2 (19), and norovirus (21). Therefore, the

length of genome fragment analyzed will determine the sensitivity of the LR PCR to damage caused by UV light. When analyzing different fragment sizes, PCRs using genome fragments of 10kb and 6kb were useful for DNA damage at low UV doses up to 15 mJ/cm^2. Interestingly, the use of 1kb for the analysis show less sensitivity to UV damage but demonstrates a linear response to a wider range of UV doses ($15mJ/cm^2$ to $100mJ/cm^2$). As calculated from the data presented here, the maximum average lesions per fragment size is independent of length and is constant to an average of 6 lesions per fragment. This may suggest that this PCR assays could be adjusted to a specific range of UV doses, using long fragments when determining inactivation at low doses is necessary and shorter fragments when higher levels of inactivation is observed at higher doses.

The calculation of germicidal dose for MP UV lamp sources is based on the DNA absorbance weighted dose normalized to 254 nm (4), therefore, it is expected that the DNA damage would be similar for both UV lamps. Previous studies on DNA damage in adenovirus also confirmed similar result for both types of UV lamps (7). Interestingly, our results and results obtained by Eischeid et al. (7) demonstrated that at higher doses LP lamps tend to cause more damage to the advs genome than MP lamps. LP lamps emit UV irradiance monochromatically at a wavelength of 254 nm which is close to the peak adsorption of DNA (260nm). Therefore, DNA may absorb more efficiently the energy irradiated from LP lamps than from MP lamps at high doses because the DNA could adsorb more efficiently the energy irradiated from LP lamps. However, MP lamp emissions create more damage to DNA at low doses indicating the possibility that the damage created is different from the types of damage observed from LP Lamps. Previous studies in DNA damage in irradiated E. coli demonstrated that formation of pyrimidine dimers was similar for MP and LP lamps (6). The difference between using PCR to using a pyrimidine dimer based

endonuclease sensitive site assay is that PCR will detect any damage on DNA that makes the template DNA unamplifiable. Therefore, PCR is sensitive to broader types of DNA damage, beyond the dimers. It has been suggested before that MP lamps could damage the DNA differently because E. coli irradiated with light from MP lamps did not photoreactivate as efficiently as those exposed to LP lamps (23). There are multiple types of DNA damage other than the formation of cyclobutane pyrimidine dimers, including pyrimidine pyrimidone photoproducts, Dewar isomers and single strand breaks that can be caused by broadband UV irradiation. Therefore determining the differences in types of damage that may be caused by MP lamps is an interesting area that still needs to be studied.

The results of LR-qPCR were compared with plaque assays results for advs 2. When analyzing a fragment of the advs genome of 6 kb, reduction in undamaged DNA closely follows reduction in viral infectivity. For the advs exposed to UV irradiation from MP lamps, the results of infectivity and LR-PCR were similar up to 2-log reduction. In the case of advs irradiated with LP UV lamps, reduction in undamaged DNA is much greater than reduction in viral infectivity (figure 7b). For example at a dose of 15 mJ/cm^2, 2-log reduction was observed in undamaged DNA but only 0.2 logreduction in infectivity was observed. However, if shorter fragment of genome was analyzed (1kb), reduction in undamaged genome as determined with LR-qPCR then follows reduction in viral infectivity. Pecson et al. (14) demonstrated that it is possible to relate results obtained with PCR to viral infectivity when the reduction in PCR signal is calibrated with reduction in viral infectivity. From the data presented here, when using MP lamps the amount of damage observed when 2-log reduction in viral infectivity was calculated to an average of 0.8 lesions/kb of genome and for 4-log reduction was calculated to average 2 lesions/kb. In the case of advs irradiated with LP UV lamp, 3.7 lesions/kb was calculated for a reduction in viral

infectivity of 2-$\log_{10}$ and 7.1 lesions/kb was calculated for 4 $\log_{10}$ reduction in viral infectivity. Therefore using the mathematical approach described by Pecson et al. (14)and the LR-qPCR described here, it could be possible to determine the performance of UV disinfection system in inactivating advs-2 without the need for cell culture. This would drastically reduce the time need for disinfection results since infectivity assays for advs-2 can take up to 7 days when using plaque assay or 14 days when using $TCID_{50}$ and the results with the LR-qPCR can be obtained in one day. One other advantage is that as sample control could be done by using direct real-time PCR (0.1 kb fragment size) to estimate total genomes copies (either damaged or undamaged) and LR-qPCR can be used estimate the undamaged portion of the genome present in the same sample. Therefore, the calculation in genome damage could be performed in each sample.

In conclusion, the Long range q PCR is an alternative to cell culture assays for determining inactivation of adenovirus-2 caused by UV irradiation.

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Table 1. Primers designed for long range PCR and Real-Time PCR

Name	Orientation	Bases	Sequence	GC Content (%)	Tm (°C) [a]	Position [b]	Frag. size (bp)[c]
AD2 For 17.8	Forward	20	CGG CGG TAT CCT GCC CCT CC	75	65.2	17,822-17,842	
Adapter (APT)	Forward	20	CAC GGA GAG ATG GCT ATG CG	60	57.9		
Ad2 17.8 kbp +ADT	Forward	40	CAC GGA GAG ATG GCT ATG CGC GGC GGT ATC CTG CCC CTC C	67.5	73.6		
AD2 rev 18.9	Reverse	28	CGT AGG TGC CAC CGT GGG GTT TCT AAA C	57.1	64.3	18,969-18,996	1174

AD2 rev 23.8	Reverse	29	CGA AGA AGA CTC GTC ACA AGA TGC GCT GG	55.2	63.9	23,887-23,915	6093
Ad2-rev 27.8	Reverse	29	CGC TCT GCC TCT CCA CTG GTC ATT CAG TC	58.6	65	27,893-27,929	10107
Ad2 17.9 R	Reverse	19	CAA GCG AGC GTG AGA CTC C	63.2	58.3	17,961-17,979	119[d]
LRAD2-p[e] 17.8	Forward	25	FAM/TTG CAT CCG TGG CCT TGC AGG CGC A/BHQ	64	69.6	17,884-17,908	

[a] melting temperature at 50 mM of NaCl

[b] Position of the primer in the adenovirus 2 genome (NCBI accession number 56160492)

[c] Size of the long-range PCR product using the primer "ad 2 17.8" as forward primer with its respective reverse primer.

[d.] Size of the Real-Time PCR product using the "ADT" primer as forward primer.

[e] The dual-labeled DNA probed used for real-time PCR contains the FAM chromophore in the 5' end and a black-hole quencher in the 3' end

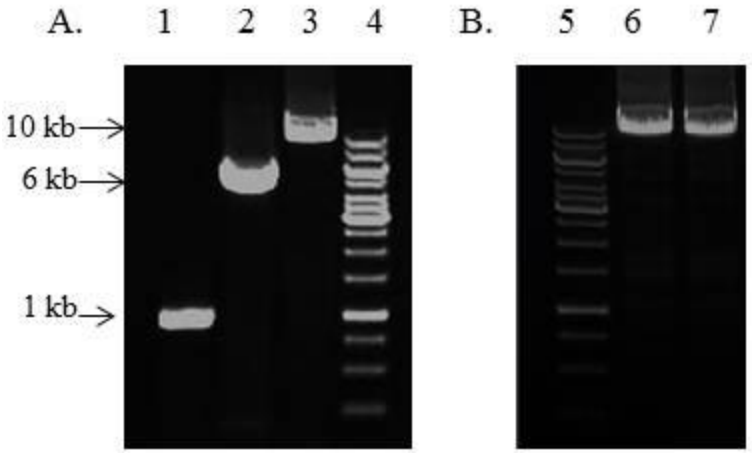


Figure 1. PCR DNA products of the Long Range PCR using the genome of adenovirus 2 with different product sizes (A) and with/out the adapter on the forward primer (B). The PCR products were visualized on .0.8% agarose gel with EtBr at concentration of 50µg/mL. The lanes are: 1) 1 kb 2) 6kb and 3) 10 kb long range PCR products. Lanes 6 and 7 are the PCR product of without and with adapter on the forward primer. Lanes 4 and 5 are the 1kb step DNA ladder. For these LR-PCR assays, the amplification was carried out for up to 30 cycles.

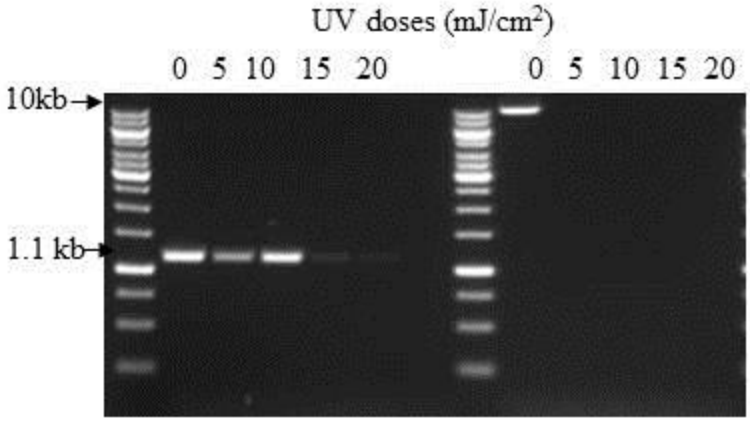


Figure 2. Comparing the amplification of two LR-PCRs targeting genome regions of 1.1 kb and 10 kb in size of adenovirus exposed to different doses of monochromatic (LP) UV lamp. The PCR products were visualized on 0.8% agarose gel with EtBr at a concentration of 50µg/mL. For these LR-PCR assays the amplification was carried out for up to 20 cycles

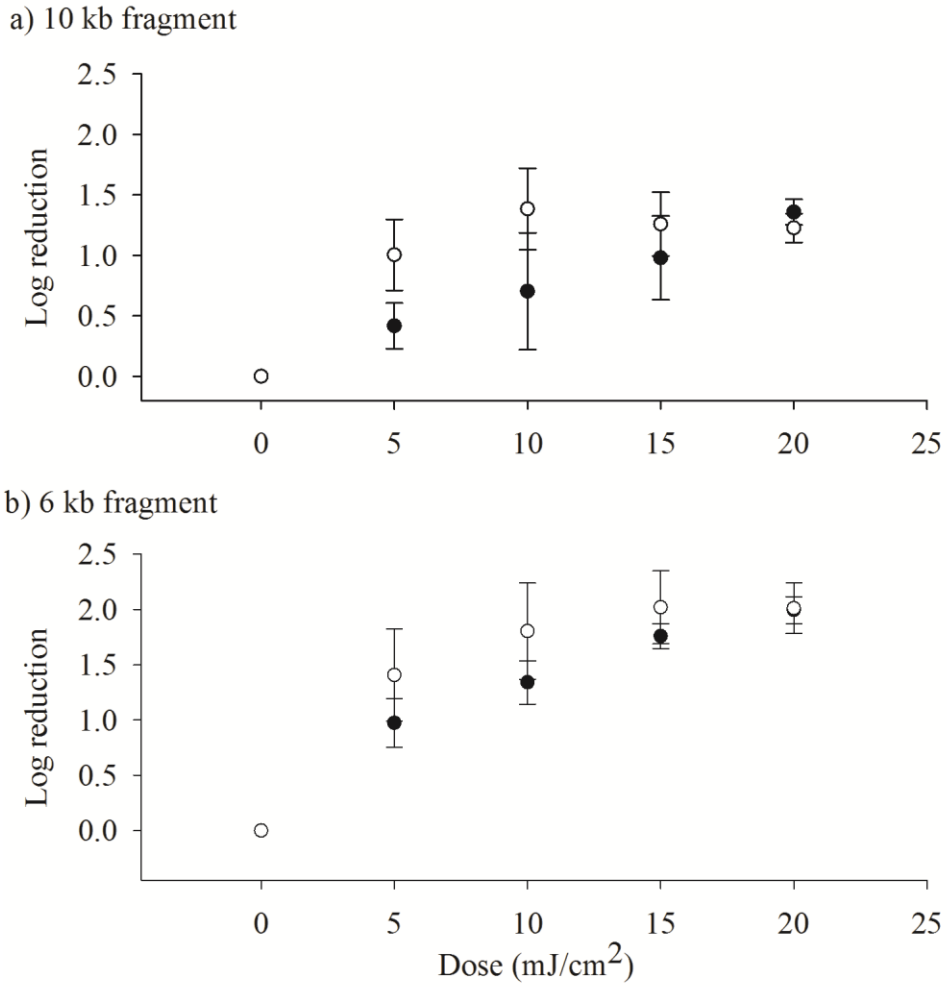


Figure 3. Comparing the use of 10 kb (a) and 6 kb (b) sizes of targeted regions in the quantitation on undamaged DNA by the combined LR-PCR and Real-Time PCR in adenovirus 2 exposed to UV irradiation. Open circles are for viruses exposed to polychromatic (MP) UV lamps and closed circles were samples exposed to monochromatic (LP) UV lamps. Log reduction was calculated with the following formula: log reduction = $\log_{10}$ dose 0 - Log_{10} dose X. The bars are standard deviation (n =4)

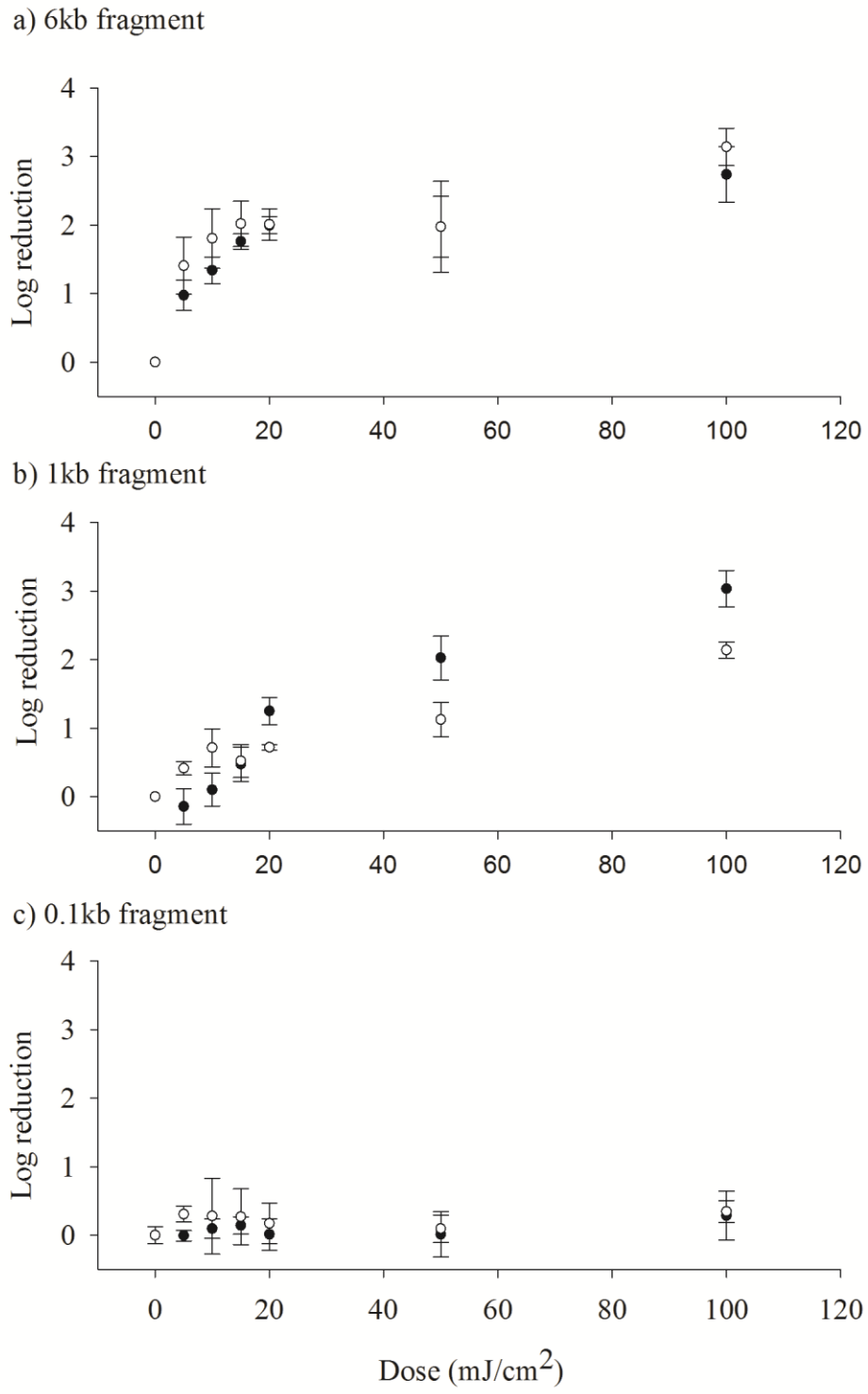


Figure 4. Comparing the use of 6 kb (a) and 1 kb (b) of targeted regions in the quantitation of undamaged DNA by the combined LR-PCR and Real-Time PCR in adenovirus 2 exposed to UV

irradiation. The results obtained for 0.1 kb target region (c) were obtained by a direct quantitation of the adenovirus genome using the real-time PCR that targets the hexon gene described previously(11). Open circles are for viruses exposed to polychromatic (MP) UV lamps and closed circles were samples exposed to monochromatic (LP) UV lamps. Log reduction was calculated with the following formula: log reduction = $\log_{10}$ dose 0 - $\log_{10}$ dose X. The bars are standard deviation (n=4).

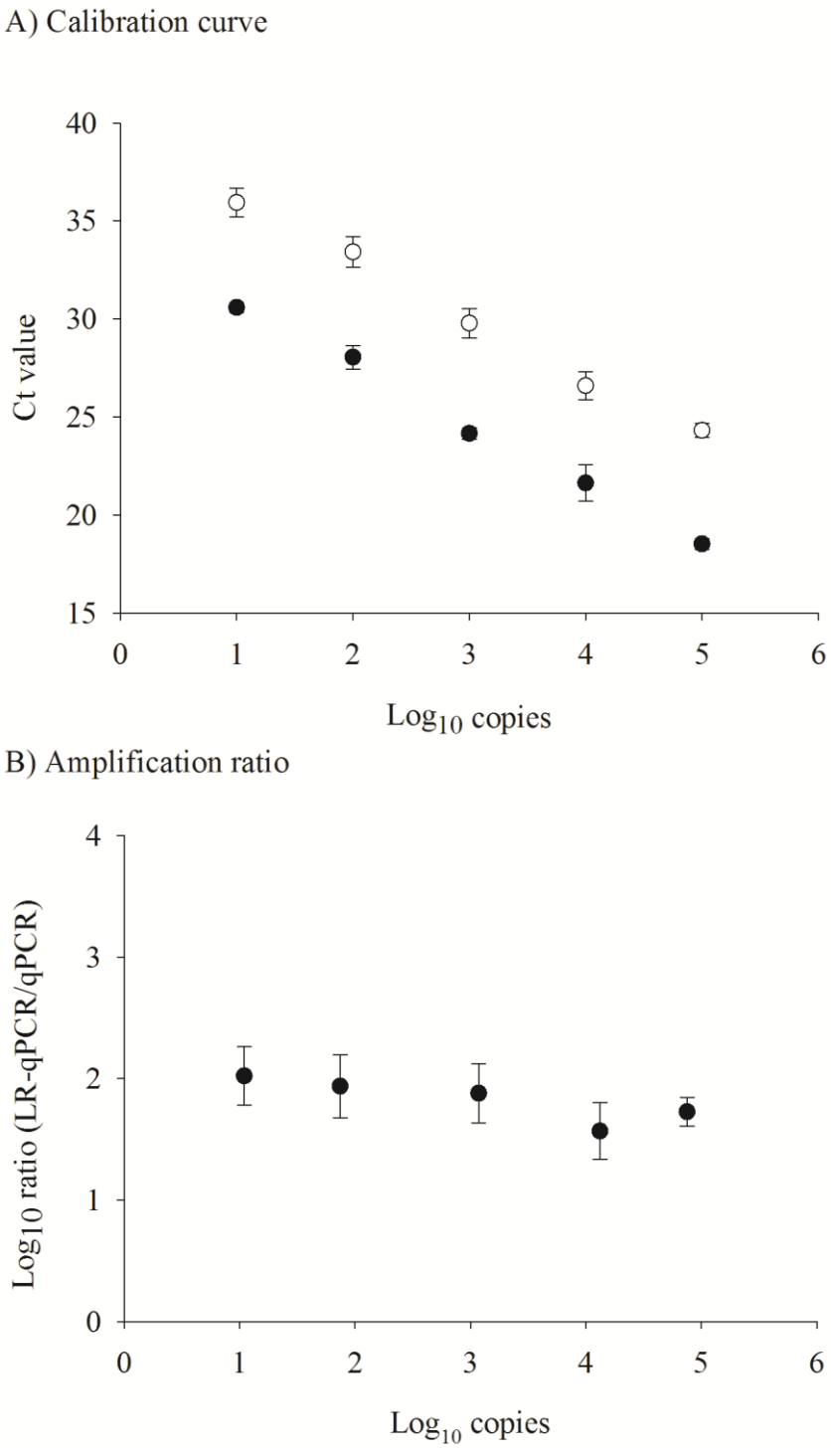


Figure 5. Performance of the combined LR-qPCR approach in quantifying adenovirus DNA. The fragment size for LR-qPCR (closed circles) was 6kb compared with direct qPCR (open circles) that was 0.1 kb. Figure A shows the cross-threshold (Ct) values obtained using LR-qPCR at

different concentrations of target advs 2 genome. The figure B shows the amplification ratio of the LR PCR at different concentrations of advs 2 genome. Bars are standard deviation (n=3).

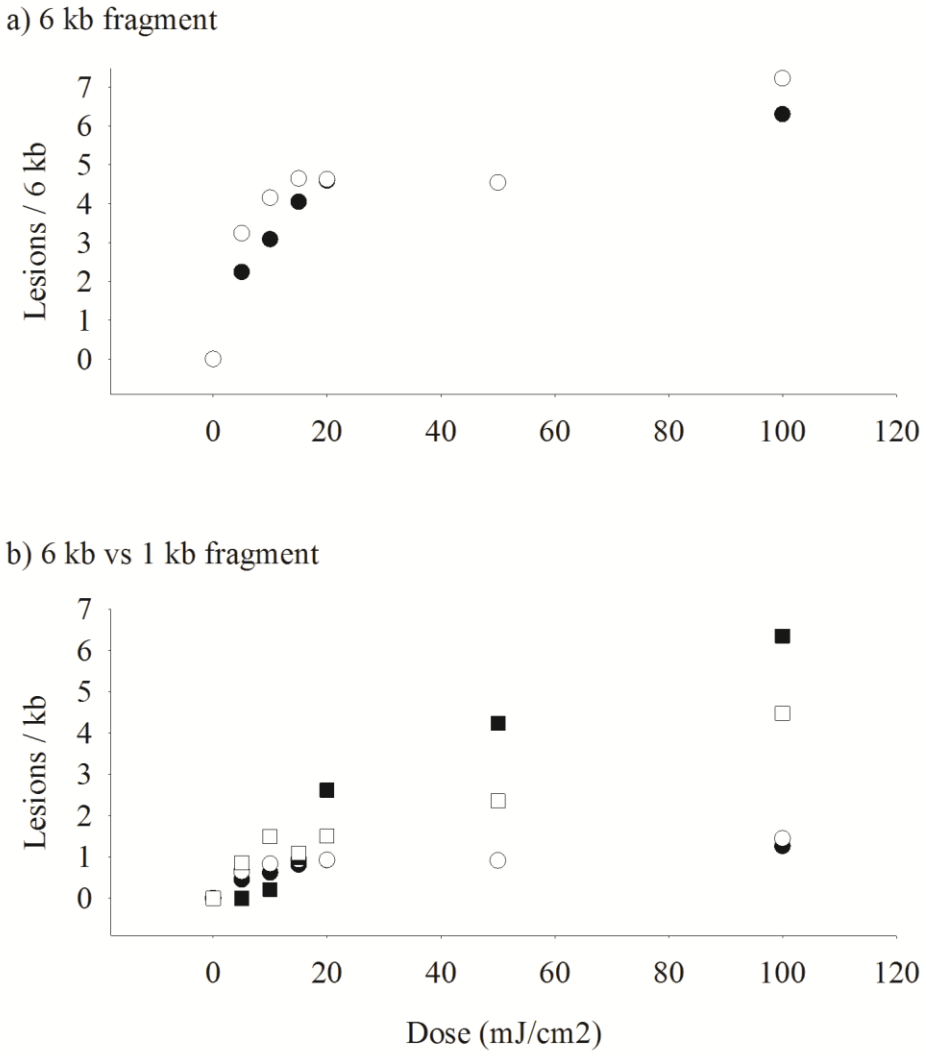
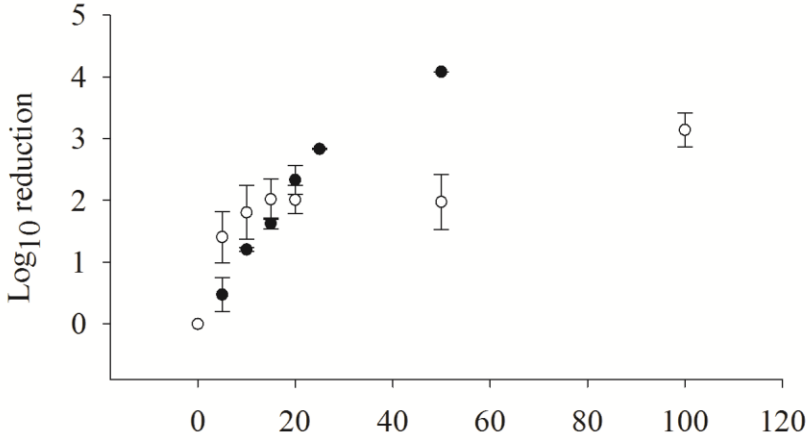


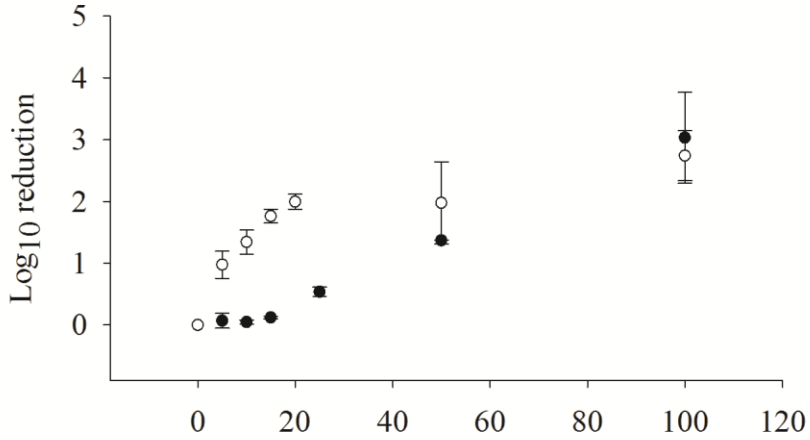
Figure 6. DNA lesions observed when using a target region of 6 kb and 1 kb of adenovirus 2 as a function of UV dose. A) DNA lesions when using a target region of 6kb for the LR-PCR and determined by lesions per 6 kb fragment. B) Comparing the results obtained when using target region of 6 kb versus 1 kb target region for calculating DNA lesions per 1 kb fragment of adenovirus genome. The lesions were calculated using the average of 4 runs. Open symbols were used for polychromatic UV lamps and closed symbols were used for monochromatic UV lamps. Circles were used for analysis that were performed using 6 kb as target region for the LR-

PCR and squares were used for analysis that were performed using 1kb as target region for LR-PCR.

A) MP Lamp (6 kb)



B) LP Lamp (6 kb)



C) LP Lamp (1 kb)

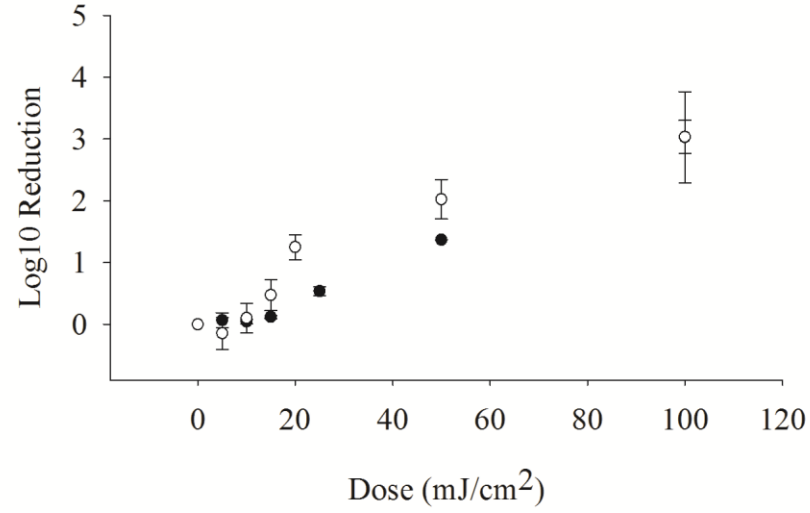


Figure 7. Inactivation of adenovirus 2 by UV irradiation from MP and LP lamps determined by genome damage (open circles) and plaque assays (close-circles). The fragment of the advs genome analyzed was 6kb for figures A and B and 1kb for figure C. Bars are standard error (n=3)