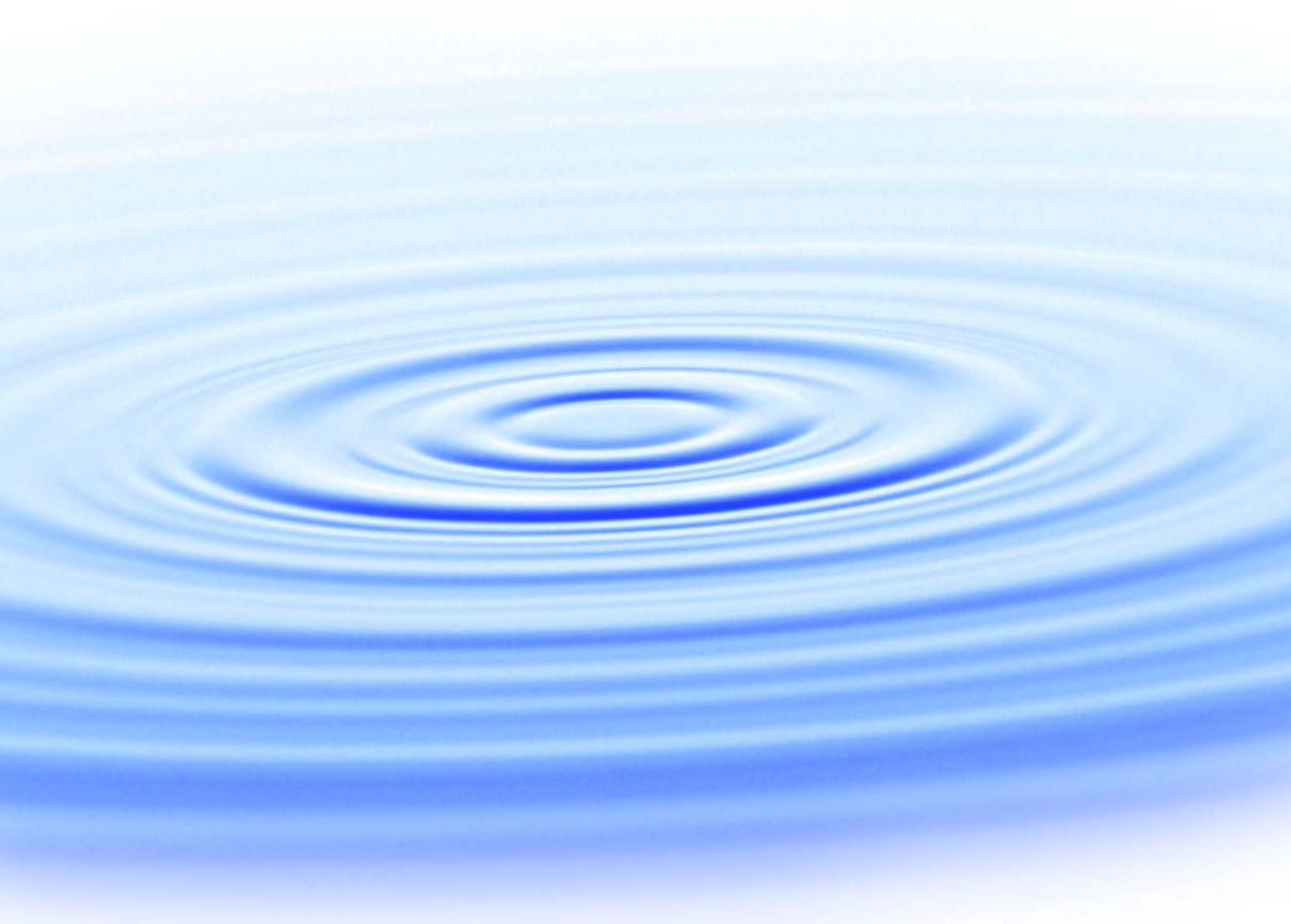
Enhanced Disinfection of Adenoviruses with UV Irradiation



WateReuse Research Foundation

Enhanced Disinfection of Adenoviruses with UV Irradiation

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The mission of the WateReuse Research Foundation is to conduct and promote applied research on the reclamation, recycling, reuse, and desalination of water. The Foundation's research advances the science of water reuse and supports communities across the United States and abroad in their efforts to create new sources of high quality water for various uses through reclamation, recycling, reuse, and desalination while protecting public health and the environment.

The Foundation sponsors research on all aspects of water reuse, including emerging chemical contaminants, microbiological agents, treatment technologies, reduction of energy requirements, concentrate management and desalination, public perception and acceptance, economics, and marketing. The Foundation's research informs the public of the safety of reclaimed water and provides water professionals with the tools and knowledge to meet their commitment of providing a reliable, safe product for its intended use.

The Foundation's funding partners include the supporters of the California Direct Potable Reuse Initiative, Water Services Association of Australia, Pentair Foundation, and Bureau of Reclamation. Funding is also provided by the Foundation's Subscribers, water and wastewater agencies, and other interested organizations.

Enhanced Disinfection of Adenoviruses with UV Irradiation

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Acronyms

AD	adenovirus
ATCC	American Type Culture Collection
CPE	cytopathic effect
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DPD	N-Diethyl-E-Phenylenediamine (reagent used for determining chlorine residual)
ELISA	enzyme-linked immunosorbant assay
LP	low pressure
LP UV	low pressure ultraviolet
MAV	murine (or mouse) adenovirus
MAV-1	mouse adenovirus type 1
MAV-2	mouse adenovirus type 2
MEM	Modified Eagles's Medium
MP	medium pressure
MPN	most probable number
MP UV	medium pressure ultraviolet
MR	morbidity ratio
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
RNA	ribonucleic acid
QPCR	quantitative real time polymerase chain reaction
USDA	United States Department of Agriculture
US EPA	United States Environmental Protection Agency
UV	ultraviolet

Foreword

The WateReuse Research Foundation, a nonprofit corporation, sponsors research that advances the science of water reclamation, recycling, reuse, and desalination. The Foundation funds projects that meet the water reuse and desalination research needs of water and wastewater agencies and the public. The goal of the Foundation's research is to ensure that water reuse and desalination projects provide sustainable sources of high-quality water, protect public health, and improve the environment.

An Operating Plan guides the Foundation's research program. Under the plan, a research agenda of high-priority topics is maintained. The agenda is developed in cooperation with the water reuse and desalination communities including water professionals, academics, and Foundation subscribers. The Foundation's research focuses on a broad range of water reuse and desalination research topics including:

- Defining and addressing emerging contaminants, including chemicals and pathogens
- Determining effective and efficient treatment technologies to create 'fit for purpose' water
- Understanding public perceptions and increasing acceptance of water reuse
- Enhancing management practices related to direct and indirect potable reuse
- Managing concentrate resulting from desalination and potable reuse operations
- Demonstrating the feasibility and safety of direct potable reuse

The Operating Plan outlines the role of the Foundation's Research Advisory Committee (RAC), Project Advisory Committees (PACs), and Foundation staff. The RAC sets priorities, recommends projects for funding, and provides advice and recommendations on the Foundation's research agenda and other related efforts. PACs are convened for each project to provide technical review and oversight. The Foundation's RAC and PACs consist of experts in their fields and provide the Foundation with an independent review, which ensures the credibility of the Foundation's research results. The Foundation's Project Managers facilitate the efforts of the RAC and PACs and provide overall management of projects.

This research investigated the UV inactivation of adenovirus, comparing two common UV technologies—low and medium pressure lamps—and two methods of adenovirus infectivity assays—cell culture and a mouse animal model. The data illustrated that the polychromatic medium pressure (MP) UV lamps were much more effective than low pressure (LP) UV lamps, and the doses required for virus inactivation were much lower than those in the United States Environmental Protection Agency (USEPA) regulations. There was no difference in the inactivation of adenoviruses when assayed in a cell culture model as compared to a mouse animal model, indicating that existing cell culture data appear to be representative of in vivo infectivity models.

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Executive Summary

There are questions pertaining to the applicability of mammalian cell lines serving as a realistic surrogate for describing human pathogenic adenovirus inactivation after ultraviolet (UV) disinfection. Adenoviruses are double-stranded deoxyribonucleic acid (DNA) viruses that are thought to be repaired by the mammalian cell lines used for their recovery after disinfectant exposure. The repair of viral DNA may or may not actually occur in-vivo, yet there has been no research to our knowledge answering this very important question. The possibility for viral DNA repair by cell culture lines may result in an overestimation of the resistance of adenoviruses. This would then result in an underestimation of the efficacy of UV disinfection technologies. If adenoviruses are significantly less resistant to UV technologies than currently thought, this would have significant ramifications in the water treatment industry because the US EPA's current regulations around UV disinfection indicate that viruses are extremely resistant to UV and require a very high UV dose application for effective control. These regulations are based on the previous work on adenoviruses performed using LP UV technology and mammalian cell culture assays. This previous research has shown low reduction of adenoviruses at commonly applied doses of UV for disinfection of water and wastewater. This previous work has raised caution flags to regulatory bodies, resulting in consideration of increasing UV dose requirements to levels that would essentially eliminate UV as an economically feasible water treatment technology for small systems that require virus disinfection, as well as make UV a potentially costly technology for water reclamation utilities that need to have control over waterborne viruses.

At the time of this proposed research, it was reported by the investigators involved in this project that UV systems using lamps that emit polychromatic light, such as medium pressure (MP) UV lamps, were much more effective than LP for inactivation of adenoviruses as assayed in cell culture. With this fresh information, the investigators developed a study to incorporate two common, yet different, UV technologies, and two methods for assaying infectivity of adenoviruses after UV disinfection to better understand some of the issues in the UV disinfection of adenoviruses.

All the data for adenovirus inactivation have come from using a cell culture infectivity assay, yet these data have never been compared to animal infectivity studies. No research has been conducted to determine if human adenoviruses are repaired in-vivo after UV disinfection; however, there are other adenovirus types that cause similar disease in other animal species. Infection has been shown in mice that are exposed to murine adenovirus. As this virus is commercially available and mice models are relatively inexpensive compared to larger mammals such as goats and swine, this animal model was proposed for study to understand how an in vivo model compares to a cell culture in vitro model for assessing the inactivation of adenoviruses with UV light.

Research Objectives

In this study, we used animal adenoviruses that are closely related to human adenoviruses to determine if there were differences between cell culture and animal models of infectivity while evaluating UV disinfection effectiveness. Therefore, the objectives of this research were to investigate the use of a polychromatic medium pressure UV source, in comparison to LP UV light, for inactivation of a number of adenovirus types, and compare the results of the conventional cell culture assays to those using an animal infectivity method.

Results and Conclusions

Important insights into the comparison of the use of cell culture infectivity and animal infectivity for assessing the effectiveness of UV for disinfection of viruses were uncovered in this research. For LP UV light at 254 nm, which mainly causes damage to the viral genome, there was almost no difference in the UV dose response of adenoviruses when assayed in cell culture or an animal model. Both models also proved once again that MP UV light was much more effective than LP UV light for inactivation of adenoviruses. What was not evident from these data is that the use of an animal model provided a different outcome or interpretation of the UV inactivation of adenoviruses compared to cell culture. Although this study was not comprehensive enough to prove specifically that animal models were similar to cell culture models for assessing infectivity of viruses, the evidence presented herein certainly points to this likelihood and provides more confidence in cell culture results as being representative of what would be expected in an animal infectivity case.

Chapter 1

Introduction

This chapter provides a brief overview of adenoviruses, UV disinfection, and the underlying rationale for the research project. A comprehensive literature review concerning this information has been previously published and can be referred to for additional details (Eischeid et al., 2011).

1.1 Background

Enteric and respiratory viruses, such as adenoviruses, are prevalent around the world and are primarily spread through fecal-oral and respiratory channels (Sattar et al., 2002; Wadell, 1984; Strauss and Strauss, 2002). Immunocompromised individuals are the most vulnerable to adenovirus-caused diseases with infections resulting in a 50% fatality rate (Wadell, 1984). Infants and young children are also at high risk as adenoviruses have been attributed as a major cause (second only to rotaviruses) of gastroenteritis (Crabtree et al., 1997; Rux and Burnett, 1999). Healthy individuals with mild infections may also be at increased risk of severe disease because some virus species cause persistent infection and may be shed for several years in these susceptible populations (Sattar et al., 2002).

Adenovirus is now considered an emerging human pathogen and has been listed twice on the US EPA's Contaminant Candidate List—first in 1998 then again in 2005 (Nwachcuku and Gerba, 2004; USEPA, 2005; Yates et al., 2006). Disinfection processes, such as UV treatment, are the primary defense for most waterborne viruses (Nwachcuku and Gerba, 2004). However, adenoviruses have exhibited a high resistivity to UV disinfection as compared to all other viruses (Ballester and Malley, 2004; Gerba et al., 2002; Ko et al., 2003; Ko et al., 2005a; Nwachcuku and Gerba, 2004). Because of this resistivity, the Long-Term 2 Enhanced Surface Water Treatment Rule and the 2007 Groundwater Rule of the US EPA have used adenoviruses as the new standard for all viral inactivation. Now a UV dose of 186 mJ/cm^2 is required for 4-log virus inactivation (USEPA, 2006; USEPA, 2003). These new standards have many implications including the potential to render a more costly UV treatment process that is out of reach for some water utilities.

Studies have shown that viral response to UV inactivation can vary owing to size (e.g., larger virions are more resistant), composition (e.g., DNA versus RNA, or single stranded versus double stranded), structure, as well as host cell characteristics (Battigelli et al., 1993; Harm, 1980; Harris et al., 1987; Rauth, 1965; Shin et al., 2005). However, it is unclear how each of these factors interacts to affect a specific virus's sensitivity to UV (Rauth, 1965; Shin et al., 2005). It is, therefore, necessary to gain a deeper understanding of adenoviruses specific response to UV light, which can serve to verify the determination of UV dose requirements and to increase the body of knowledge that can be used for future water treatment regulations.

1.1.1 Adenovirus Biology and Infectious Cycle

Adenoviruses are nonenveloped icosahedral particles that contain approximately 12 different types of proteins. Most of the protein mass is in the capsid, the majority of which is hexon protein; at each vertex of the icosahedron is a penton base or penton complex from which a fiber protein protrudes (Rux and Burnett,1999). The hexon and fiber exist as trimers in the

mature viral particle, whereas the penton base is a pentamer; each is composed of identical subunits (Phillipson, 1983; Rux and Burnett, 1999). Hexon is the dominant capsid protein and there are 240 copies of hexon trimer per virion. Each adenovirus particle has 12 penton bases and 12 molecules of fiber protein extending outward from its surface; the function of the fiber protein is the attachment of viral particles to their host cells (Seth, 1999b). A representation of the infectious cycle is shown in Figure 1.1.

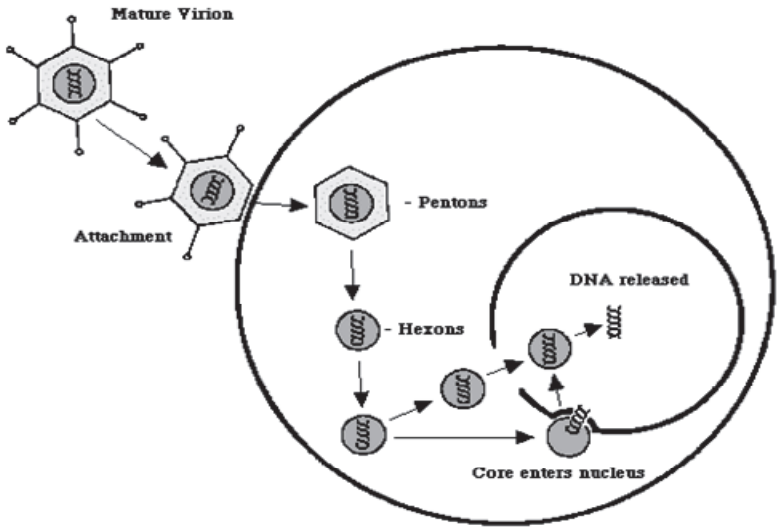

Figure 1.1. Adenovirus infectious cycle.
Source: www.tulane.edu/~dmsander/WWW/335/Adenoviruses.html

For other minor capsid proteins, their structures and locations in the mature virion are less well-understood (Vellinga et al., 2005). In the viral core, the DNA is associated with the major and minor core proteins. The major core protein is by far dominant and is present in more than 1100 copies per virion, whereas there are thought to be approximately 180 copies of the minor core protein (Phillipson, 1983; Rux and Burnett, 1999). Both of the core proteins are rich in positively charged amino acids, which facilitate their association with the negatively charged phosphate backbone of the viral DNA. Viral proteins are an integral part of every step in the process of infection and enable adenoviruses to successfully infect host cells even if their DNA is damaged (Seth, 1999b). Thus, optimal inactivation of adenoviruses requires not only damage to the viral genome, but also damage to the viral proteins that are crucial in the infection of host cells.

UV-induced damage to proteins can take several forms: oxidation of amino acids, crosslinking of the protein with itself or with DNA, breakage or formation of disulfide bridges, or breakdown of the polypeptide chain. Tryptophan, tyrosine, phenylalanine, cysteine, and cystine are the only aromatic amino acids that have absorbance maxima near 280 nm. Whereas MP UV has emissions at and around 280 nm and is most easily absorbed, LP UV can also affect these amino acids, most notably tryptophan, cysteine, and cystine. Breakage of disulfide bonds—which converts cystine amino acid residues to cysteine—also has a high quantum yield at the 254 nm wavelength emitted by LP UV lamps (Jagger, 1967). The effect that different types of damage have on protein function varies according to tertiary structure, disulfide bonds, or changes in individual amino acids.

1.1.2 Variation in UV Disinfection Technology

A significant amount of data has been published on UV inactivation of adenovirus and other viruses using monochromatic LP UV followed by assays of infectivity using cell culture (Ballester and Malley, 2004; Battigelli at el., 1993; Baxter et al., 2007; Gerba et al., 2002; Harris et al., 1987; Ko et al., 2003; Ko et al., 2005a; Meng and Gerba, 1996; Nwachcuku et al., 2005; Shin et al., 2005; Thompson et al., 2003; Thurston-Enriquez et al., 2003a). These studies have shown that 4-log inactivation of adenovirus requires a low pressure UV dose of up to 200 mJ/cm2, whereas 30 to 40 mJ/cm^2 is sufficient to cause 4-log inactivation of other viruses (Gerba et al., 2002; Meng and Gerba, 1996; Shin et al., 2005). It is possible that the higher dose requirement for LP UV inactivation of adenovirus reflects not true resistance, but rather repair of damaged adenoviral DNA in host cells during the cell culture infectivity

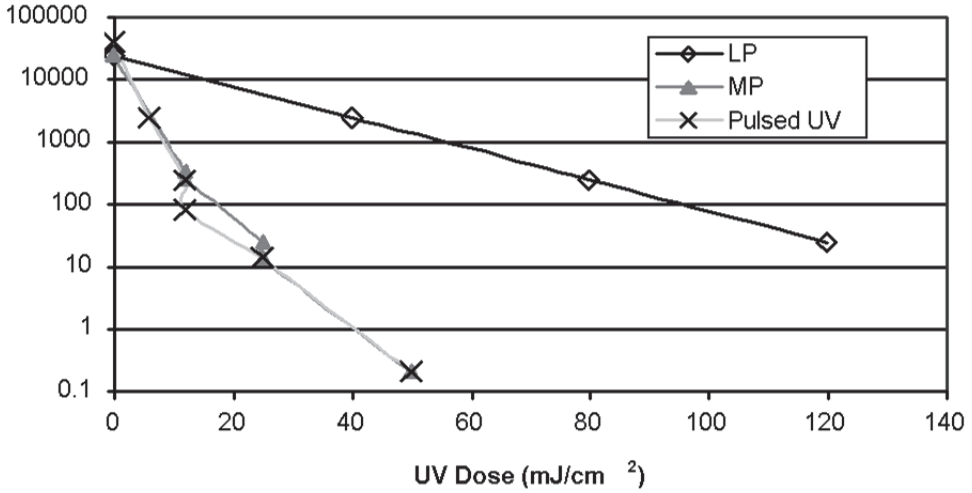

Figure 1.2. Inactivation of adenovirus type 40 under LP, MP, and pulsed UV light.
Note: Y axis is MPN per mL
Source: Linden et al., 2007

assays. LP UV used in the studies described herein is nearly monochromatic at 253.7 nm—very near the 260 nm absorbance maximum of nucleic acids such as deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) that make up the genomes of viruses and other pathogens. It is widely accepted that LP UV inactivates microorganisms by damaging their genomes. Because adenovirus can infect host cells even when its genome is damaged (Seth, 1999a), and because that genome is double-stranded DNA like the genome of the host cell, it follows that the DNA repair machinery of the host cell might recognize and repair damage to the adenoviral genome during standard cell culture infectivity assays. Similar effects in cell culture have likely not been seen in other waterborne viruses because their genomes are single-stranded or composed of RNA (Battigelli et al., 1993; Chang et al., 1985; Gerba et al., 2002; Harris et al., 1987; Meng and Gerba, 1996; Roberts and Hope, 2003; Thurston-Enriquez et al., 2003a) and are therefore not recognized by host cell DNA repair machinery. Furthermore, when irradiated with MP UV or other polychromatic UV sources such as pulsed UV, adenoviruses have been shown to be as susceptible to UV inactivation as other viruses, even in standard cell culture infectivity assays as illustrated in Figure 1.2 (Linden et al., 2007). The emission spectra for LP and MP UV lamps is presented in Figure 1.3. MP UV is

polychromatic—it emits a range of wavelengths including those that are absorbed by both DNA and proteins, so it has the potential to damage the viral coat and core proteins in addition to the genome. Such extragenomic damage appears to play an important role in viral inactivation.

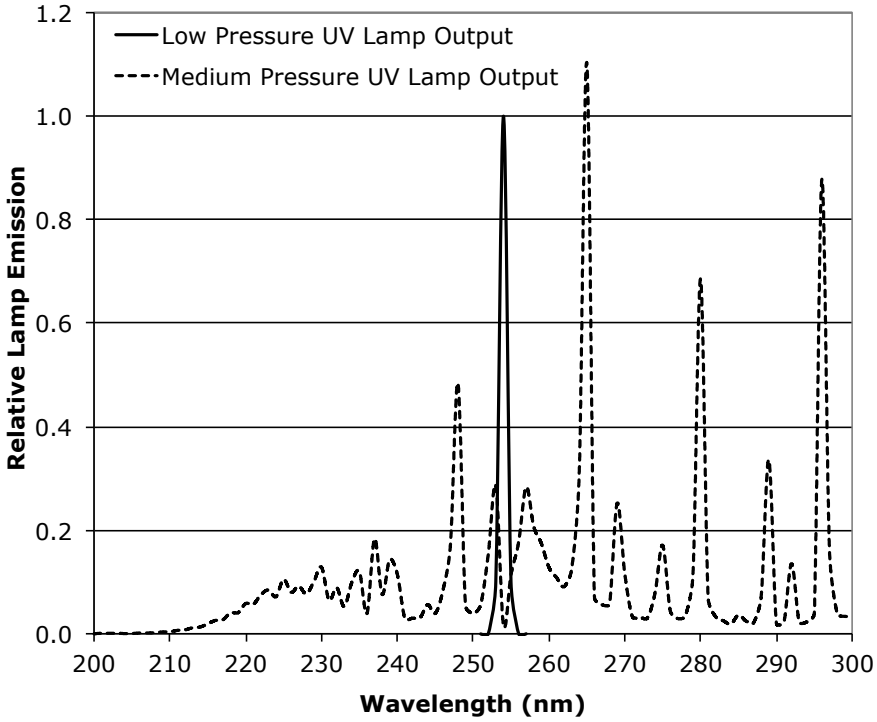

Figure 1.3. Emission spectra of low and medium pressure UV light sources.

1.1.3 Differences in UV Inactivation Among Types of Adenoviruses

Human adenoviruses are classified into six subgroups and more than 50 different types have been identified (Ginsberg, 1999; Strauss and Strauss, 2002; Wadell, 1984). Subgroup C (AD2 and AD5) have been used in gene therapy studies as they can be easily propagated at high concentrations and rarely affect adults (Seth, 1999b; Strauss and Strauss, 2002). Cell culture assays for AD2 have undergone 0.5 to 1 log inactivation at a dose of 40 mJ/cm2 of LP UV. Inactivation increased to 4-log when assays were conducted using cell lines taken from patients with an autosomal recessive disorder known as xeroderma pigmentosum (Day, 1974; Rainbow, 1980). Some minor differences in inactivation have also been observed among adenovirus Types 1, 3, 5 and 6 (Nwachcuku et al., 2005).

Subgroup F (AD40 and AD41) are most likely to be waterborne and cause a high degree of infant death in the third world and hospitalizations in developed countries (Jothikumar et al., 2005; Ko et al., 2003; Ko et al., 2005a; Ko et al., 2005b; Sattar et al., 2002; Wadell, 1984). However, studies performed by the water treatment community have met difficulties propagating these viruses in vitro (Mautner, 1999; Wadell, 1984).

1.1.4 Differences in UV Inactivation Among Types of Viruses

Many studies have been carried out on viruses with single-strand DNA or RNA (Battigelli et al., 1993; Chang et al., 1985; Gerba et al., 2002; Harris et al., 1987; Meng and Gerba, 1996; Thurston-Enriquez et al., 2003a). However, adenovirus contains double-stranded DNA and human repair systems can fix damage more efficiently than with the single-strand viral genomes. Table 1.1 shows the five families of interest (those viruses with double-stranded DNA) and their modes of transmission, the disease they cause, and whether or not they are enveloped. Although human papillomavirus, BK polyomavirus, and JC polyomavirus can be waterborne, the diseases they carry are not typically transmitted by water. Envelopes may affect UV sensitivity such as the herpes viruses, which may be somewhat susceptible to UV disinfection (Cameron, 1973). Overall, pamilloma and polyomaviruses can provide the most insight because of their nonenveloped coat and double-stranded DNA. With cell culture assays in vitro showing that adenoviral DNA is being repaired, it is conceivable that it may also be repaired in vivo in humans.

1.1.5 Mouse Adenovirus as a Model for Human Adenovirus Infection

Like the human adenoviruses, mouse adenoviruses are members of the *Adenoviridae* family of viruses. Human and animal adenoviruses have similar genomes and structure. Mouse adenoviruses have approximately the same naked capsid makeup and size of human adenoviruses. Mouse adenoviruses are approximately 70 nm and human adenoviruses are approximately 80 nm in diameter. Both have icosohedral capsid architecture with fibers projecting from the capsid's vertices. The capsid's hexon protein from mouse adenovirus is antigenically similar to human adenoviruses as evidenced by animal sera that is exposed to human adenovirus hexon protein recognizes mouse adenovirus particles (Larsen et al., 1977).

The mouse adenoviruses contain double-stranded DNA (~30K bp). Human adenovirus types have a similar genome size, ranging range from ~34K bp to ~36K bp (Lauer et al., 2004). There is significant sequence homology of both the DNA polymerase and penton base between mouse adenovirus type 1 and human adenoviruses, specifically adenovirus type 2 and adenovirus type 5. Differences occur in early Region 3, however, among all of the adenoviruses (Dragulev et al., 1991; Flomenberg et al., 1988; Raviprakash et al., 1989). Mouse adenovirus disease has similarities with the human adenoviruses. Mouse adenovirus type 2 infects the gastrointestinal tract and causes wasting syndrome in nude mice. Mouse adenovirus type 1 can result in a range of disease depending on the viral dose and the strain of mouse infected (Guida et al., 1995; Spindler et al., 2001). For MAV-1, the virus infects the brain, spinal cord, and spleen; and death is due to encephalitis or encephalomyelitis (Charles et al., 1998; Kajon et al., 1998). For immunocompetent humans, adenovirus infection is limited to the respiratory tract, gastroenteritis, and conjunctivitis that is due to infection of epithelial cells. For mouse adenoviruses, replication has been shown in endothelial cells (Kajon et al., 1998), macrophages (Kajon et al., 1998) and there is evidence of replication in respiratory epithelial cells (Weinberg et al., 2005). In immunocompromised humans, however, human adenoviruses can cause systemic disease similar to mouse adenovirus type 1 infection (Kampmann et al., 2005; Lukashok and Horwitz, 1999; Russell, 2000). Persistent infections is another similarity between the human and mouse adenoviruses (Garnett et al., 2002; Rowe and Hartley, 1962; Smith et al., 1998;). Because of the similarities between mouse adenoviruses and human adenoviruses, mouse adenoviruses, specifically mouse

adenovirus type 1, have been used extensively as a model for human adenovirus infection (examples include those already cited as well as Lenaerts et al., 2005; Spindler et al., 2001, and many others).

1.1.6 Animal Infectivity in Use for Virus Studies

Although there have been numerous adenovirus infectivity studies with cell culture assays, the important findings through animal studies to determine the infectivity of *Cryptosporidium* and *Giardia* demonstrate a need for animal infectivity studies with adenoviruses. Initially cell culture assays showed *Cryptosporidium* and *Giardia* to be UV resistant; however, eventual animal infectivity assays revealed them to be more sensitive to UV disinfection (Clancy et al., 2000; Craik et al., 2001; Mofidi et al., 2001). Animal infectivity assays are more resource intensive but capture added complexities related to the in vivo environment including cell-matrix interactions that are found in whole animals (Blake and Stacey, 1999; Faubert, 1996). The immune response is also an important factor affecting a host's response to a virus and is carried out at a systematic level as well as a cellular level. The adenovirus evades this response through a variety of means that may not be captured in vitro (Wold et al., 1999). Although the in vitro versus in vivo UV response of *Cryptosporidium* and *Giardia* conflict, other viruses such as bovine ephemeral fever virus have shown similar responses (Murphy et al., 1972). Therefore, additional studies need to be conducted to characterize the in vitro versus in vivo response to UV irradiation of adenovirus.

Table 1.1. Double-Stranded DNA Viruses that Infect Human Hosts

Family	Name	Abbrev.	Envelope	Transmission	Disease
Adenoviridae	subgroups A-F	AD	no	fecal-oral, aerosols	enteritis, diarrhea, respiratory infection
Papillomaviridae	human papillomavirus	HPV	no	aerosols, contact, urine, sexual	genital warts cervical cancer
Polyomaviridae	BK polyomavirus	BKPyV	no	aerosols, contact, urine, sexual	tumors in the immuno-compromised
	JC polyomavirus	JCPyV	no	aerosols, contact, urine, sexual	severe nervous system disease in the immuno-compromised
Herpesviridae	herpes simplex 1	HHV-1, HSV-1	yes	contact	cold sores
	herpes simplex 2	HHV-2, HSV-2	yes	contact	genital ulcers
	human herpesvirus 3 (varicella-zoster)	HHV-3, VSV	yes	aerosols	chicken pox, shingles
	Cytomegalo-virus	CMV	yes	bodily fluids	nervous system
	human herpesvirus 4 (Epstein-Barr)	HHV-4, EBV	yes	contact	mononucleosis, lymphoma
	human herpevirus 6	HHV-6	yes	contact, bodily fluids	"sixth disease"
	human herpesvirus 7	HHV-7	yes	contact	unknown
	human herpesvirus 8	HHV-8	yes	bodily fluids	Kaposi's sarcoma
Poxviridae	smallpox (variola virus)	VARV	yes	contact	smallpox
	molluscum contagiosum	MOCV	yes	contact	lesions

Source: Eischeid et al., 2011

1.2 Project Justification

There are questions pertaining to the applicability of mammalian cell lines serving as a realistic surrogate for describing human pathogenic adenovirus inactivation after UV disinfection. Adenoviruses are double-stranded DNA viruses that are thought to be repaired by the mammalian cell lines used for their recovery after disinfectant exposure. The repair of viral DNA may or may not actually occur in vivo, yet there has been no research to our knowledge answering this very important question. The possibility for viral DNA repair by cell culture lines may result in an overestimation of the resistance of adenoviruses. This would then result in an underestimation of the efficacy of UV disinfection technologies. If adenoviruses are significantly less resistant to UV technologies than currently thought, this would have enormous ramifications in the water treatment industry because the US EPA's current regulations around UV disinfection indicate that viruses are extremely resistant to UV and require a very high UV dose application for effective control. These regulations are based on the previous work on adenoviruses performed using LP UV technology and mammalian cell culture assays. This previous research has shown little to no reduction of adenoviruses at commonly applied doses of UV for disinfection of water and wastewater. This work raised caution flags to regulatory bodies, resulting in consideration of increasing UV dose requirements to levels that would essentially eliminate UV as an economically feasible water treatment technology for small systems that require virus disinfection, as well as make UV a potentially costly technology for water reclamation utilities that need to have control over waterborne viruses.

No research has been conducted to determine if human adenoviruses are repaired in-vivo after UV disinfection; however, there are other adenovirus types that cause similar disease in other animal species. Infection has been shown in mice that are infected with murine adenovirus. As this virus is commercially available and mice models are relatively inexpensive compared to larger mammals such as goats and swine, this animal model was proposed for study to answer the question of the possibility of adenoviral DNA repair in-vivo when water containing adenovirus has been UV irradiated.

1.3 Research Objectives

At the time of the proposed work, all existing published data on adenovirus UV inactivation was performed with monochromatic LP UV irradiation coupled with cell culture infectivity and indicated extreme resistivity compared to all other known pathogens. The objectives of this research were to investigate the use of both monochromatic LP UV light and a polychromatic MP UV source for inactivation of a number of adenovirus types and compare the results of the conventional cell culture assays to those using an animal infectivity method.

Chapter 2

UV Inactivation of Human Respiratory and Enteric Adenoviruses Along with Murine Adenovirus Using Both Low and Medium Pressure UV with Cell Culture Assay

2.1 Objectives

The research objectives of this task were (1) to evaluate the UV inactivation of both respiratory and enteric adenoviruses using both LP and MP UV systems, (2) to compare the UV inactivation of murine and human adenoviruses, and (3) to assess the impact of reuse water matrices on the inactivation of adenoviruses.

2.2 Approach Methods

All procedures (viral propagation, concentration, purification, and cell culture assay) were based on previously published protocols (e.g., Thurston-Enriquez et al., 2003a, 2003b) and are summarized in the following sections.

2.2.1 Virus Propagation

The human cell line A549 was used for growth of viral stocks and for cell culture infectivity assays for human adenovirus. Murine (mouse) adenovirus type 2 (MAV-2) was propagated using CMT-93 cells. Human adenovirus type 2 was initially prepared using two different methods. The first method was polyethylene glycol (PEG) precipitation followed by chloroform extraction, which resulted in a viral stock suspended in phosphate buffered saline (PBS). The second method was direct chloroform extraction of the viral supernatant from cell culture and resulted in a viral stock suspended in cell culture medium. Some of each type of stock was stored for longer term use at -80 °C. The second method required less researcher time and fewer materials, and it resulted in viral stock of a higher titer according to cell culture infectivity assays, which would lend itself better to dilution. Cell culture medium, however, contains numerous amino acids and small molecules, which may absorb UV and affect experiment results.

All viruses were grown in a serum-free medium that was chosen based on what is typically used in growing adenovirus rather than what is typically used to grow A549 cells. The decision to use serum-free medium was based on the assumption that differences between LP and MP UV inactivation of adenovirus are likely caused by differences in their effects on viral proteins that are present in fetal bovine serum.

2.2.2 Bench-Scale UV Irradiation

Each of the human pathogenic adenoviruses under study was exposed to two to three doses of LP and MP UV irradiation in replicate experiments. UV dosimetry was determined based on standardized methods developed by Linden and colleagues (Bolton and Linden, 2003; Linden

and Darby, 1997). All doses from polychromatic UV systems were calculated on a germicidal weighting based on the DNA absorbance spectrum to be consistent with previously published research and data for other microbes. A range of UV doses were used to study the LP UV and MP UV efficacy covering up to 4-log inactivation.

Three different types of reclaimed water from participating utility partners were chosen based on a range of physical and chemical parameters. Specifically, UV absorbance scans (emphasizing a range of absorbance in the wavelengths <240 nm), turbidity, and total organic carbon were used. Waters received from the three utilities (Pinellas County, FL; Orange County, CA; L.A. County, CA) were sterile-filtered and irradiated with high doses (1200 mJ/cm^2) of LP UV to break down any chlorine compounds present, and chlorine removal was confirmed using the DPD method. Filtered, irradiated waters were used for 100-fold dilution of adenovirus types 2, 4, and 5. Absorbance scans of each water were taken (1) after filtration before high-dose UV treatment, (2) after high-dose LP UV and filtration but before addition of virus, and (3) after addition of virus. The scan of the water before UV with no virus added is presented in Figure 2.1. It was identical to all the other scans (after UV and with viruses added), so only this one is shown here.

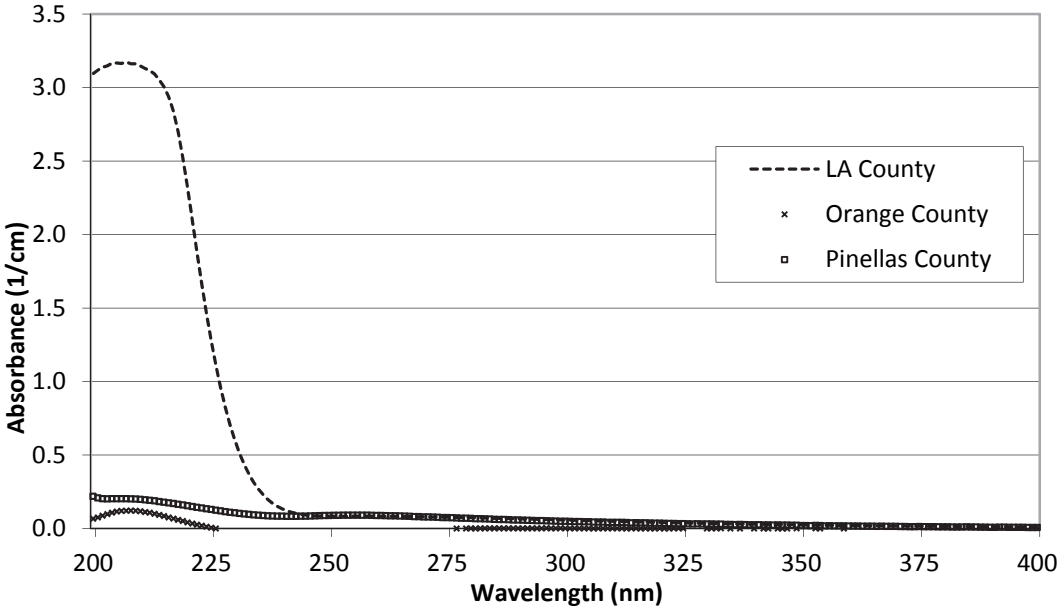

Figure 2.1. Absorbance spectra of the reuse waters used in the virus experiments (after 0.22 um filtration, before 1200 mJ/cm^2 LP UV irradiation, no virus).

2.2.3 Mammalian Cell Culture Assay

Adenovirus presence and concentration in UV-irradiated water was determined using mammalian cell culture assays. Details of the original procedure used for the cell culture infectivity assays had to be modified in order to minimize contamination and to improve assay reliability. Originally, assays were performed in 24 well plates as described by Thurston-Enriquez et al. (2003a), but the procedure was modified for use in flasks. A549 or PLC/PRF5 cells were plated into 25 cm^2 flasks at a density of 3–3.5 x 10^5 cells per flask in complete DMEM (high-glucose DMEM, 10% FBS, 2 mM L glutamine, 100 units/ml penicillin, 100 ug/ml streptomycin, and either 0.25 or 2.5 ug/ml amphotericin B) and allowed

to grow for two days at 37 ^{o}C and 5% CO_2. At least three different dilutions of virus and at least three different flasks per dilution were inoculated onto cells. Cells and viruses were incubated at 37 ^{o}C for up to three weeks before being scored. Flasks were scored as positive or negative for cytopathic effects and scoring data were entered into a computer program, which calculates most probable number (MPN/ml) as described by Hurley and Roscoe (1983). The results of these assays were compared to the results of previous experiments conducted by Linden and Thurston to ensure that results from the two different laboratories were similar.

2.3 Results

2.3.1 Absorbance Spectra of Media for Viral Preps and Natural Water

Absorbance spectra were obtained for both types of viral stocks along with several natural waters for comparison. Figure 2.2 shows the absorbance spectra of viral suspensions prepared by either (1) PEG precipitation and chloroform extraction, resulting in virus suspended in PBS or (2) chloroform extraction of cell culture supernatant, resulting in virus suspended in cell culture medium. The viral suspension in culture medium was used at a 1:10 dilution, because it will be diluted at least that much prior to UV experiments. PBS alone is shown as a reference. Although the virus in cell culture medium does have a higher UV absorbance at wavelengths between approximately 210 nm and 235 nm, the UV absorbance of the virus in PBS is also relatively high in this region. Absorbance in this low wavelength region is not expected to affect the results of LP UV experiments because LP UV lamps do not emit light at these wavelengths. Wavelengths from 210 to 235 nm account for approximately 15% of the emissions of a MP UV lamp, but according to previous results (also carried out in PEG precipitated/chloroform extracted PBS suspension), these wavelengths may be most effective for inactivation of adenovirus.

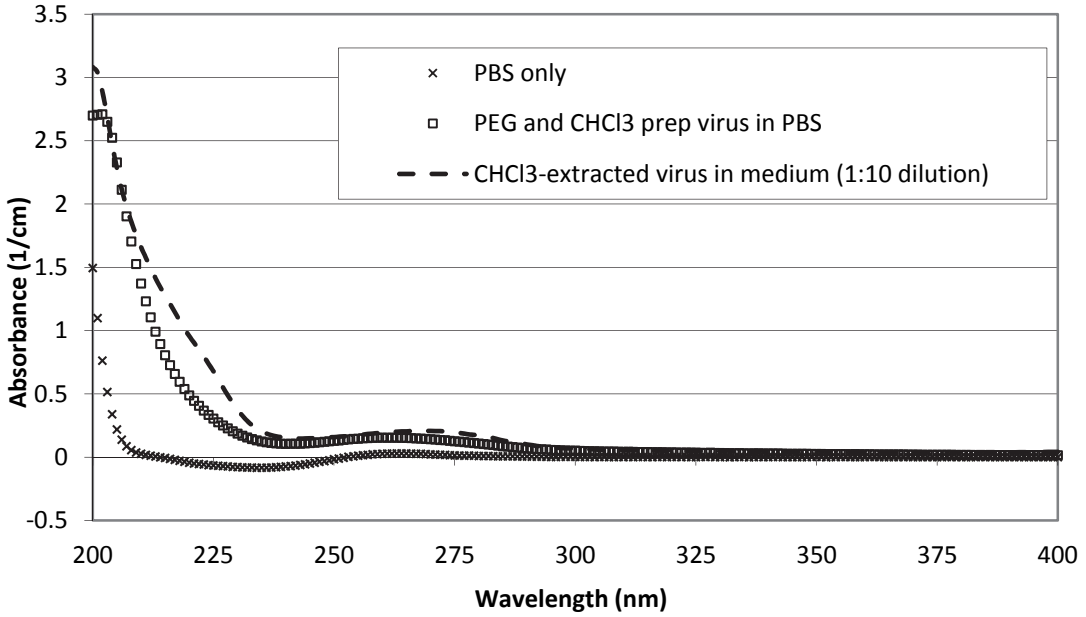

Figure 2.2. Absorbance spectra of viral stocks.

Figure 2.3 shows the absorbance spectra for several natural waters, including both drinking waters and reuse waters. Spectra are shown for each water alone and for each water with cell culture medium at a 1:10 dilution. The absorbance spectrum of the virus in PBS (shown in Figure 2.2) represents the best case scenario in terms of achieving low UV absorbance in experimental samples, so it is also shown in Figure 2.3 (dashed line) as a reference. The only samples here that have absorbance that is significantly higher than that of the virus in PBS are the reuse waters with cell culture medium added. Whereas it is helpful to compare the UV absorbance of the culture media and reuse waters, in practice, culture media will not actually be added to reuse waters so this increase in absorbance will not affect UV disinfection of adenovirus in the field.

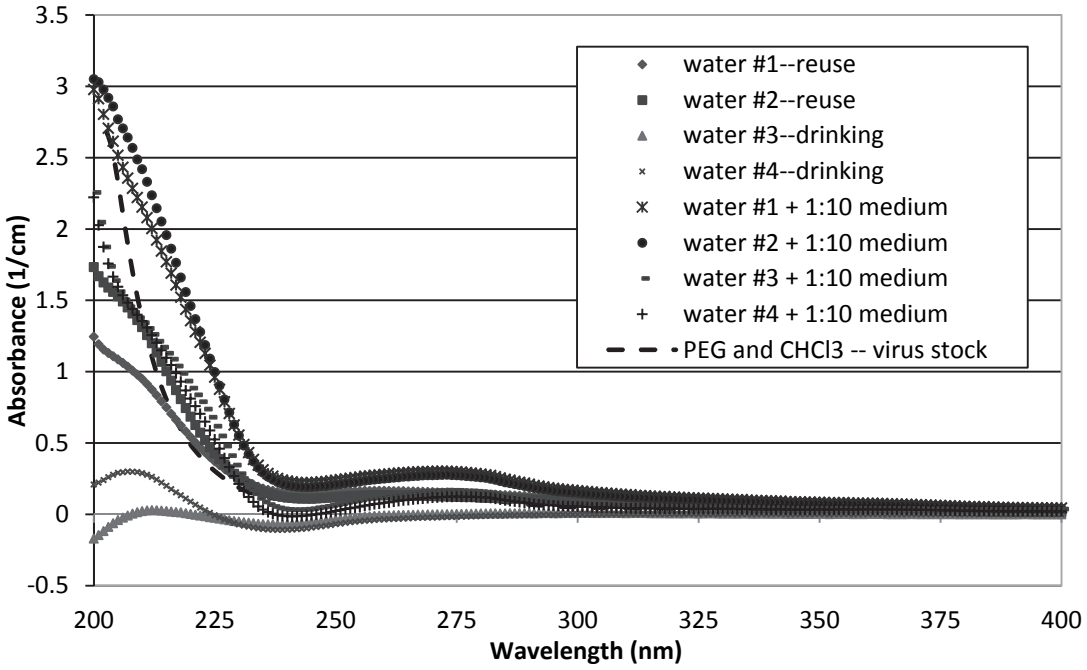

Figure 2.3. Absorbance spectra of natural waters.

On the basis of the collected absorbance data, it was determined that PEG-precipitated adenovirus stock in PBS was the most appropriate method and was therefore used for all experiments. This viral stock preparation was also found to work best for polymerase chain reaction (PCR) assays that will be described in Chapter 3.

2.3.2 LP and MP UV Disinfection

Bench scale assays for LP and MP UV inactivation of AD40 and MAV-2 were conducted. Results are shown in Figure 2.4 and Table 2.1.

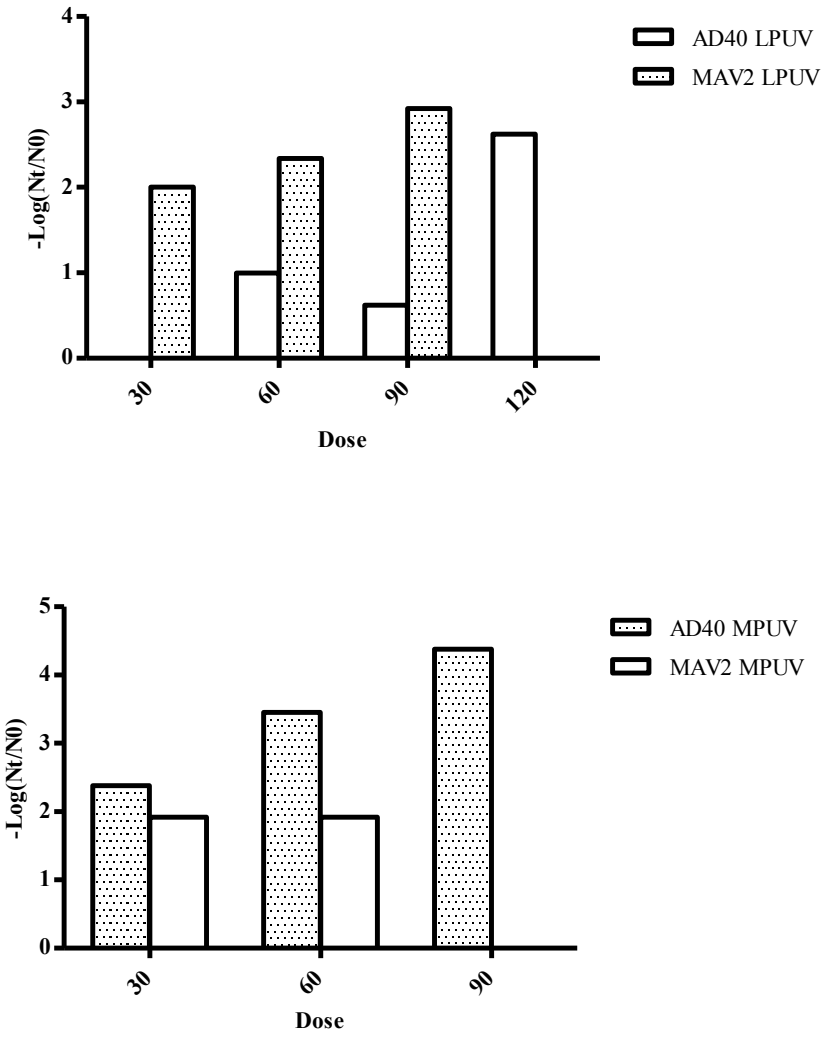

Figure 2.4. Comparison of AD40 and MAV-2 inactivation by LP UV and MP UV (based on one disinfection experiment).

Note: The arrows on the bars in the graphs indicate that the detection limit was reached at the dose indicated. Thus, the amount of inactivation is equal to or greater than that shown by the bar graph.

Table 2.1. Estimated Dose Required to Achieve 1 to 4 Logs Inactivation of AD40 and MAV-2

UV light	Virus	Log Inactivation			
		1 Log	2 Log	3 Log	4 Log
LP UV	Adenovirus	63	126	189*	252*
	MAV-2	23	45	67*	90*
MP UV	Adenovirus	16	32	48	65*
	MAV-2	16	31*	47*	63*

*The amount of inactivation not actually achieved. These values are extrapolated from the regression line created using observed values.

The results shown in Figure 2.4 and Table 2.1 suggest that the mechanism of inactivation between the mouse and human adenovirus types, or the cell culture infectivity assays used, may differ for LP UV as there are very large differences in the amount of inactivation between these virus types over the range of LP UV doses tested. In contrast, the amount of inactivation by MP UV does not vary significantly, which may indicate that the mechanism for MP UV inactivation is similar for the two viruses tested, or the differences in the cell culture infectivity process was not consequential. MP UV was also found to be more effective at viral inactivation compared to LP UV. Differences in the results between the AD40 and MAV-2 for LP may also be due to the difference in repair efficiency between the cell lines, but this was not specifically investigated.

The observed doses required to inactivate AD40 are higher than previous data; however, these results are based on only one experiment. The highest dose reported in the literature was an extrapolated value of 226 mJ/cm^2 for 4-log reduction of AD40 (Thurston-Enriquez et. al., 2003a). MP UV results for AD40, however, are very similar to previous findings where approximately 60 mJ/cm^2 were required for 4-log AD40 inactivation (Linden et. al., 2007). Recent comprehensive work by Guo et al. (2010) indicated the LP UV dose required for 4-log inactivation of AD40 was approximately 135 mJ/cm^2 for two different cell lines.

These results were repeated but there was continued difficulty in detecting infection with MAV-2. As described later in the report, difficulties propagating and observing cytopathic effect (CPE) and infection in-vivo led to the team investigating the use of another mouse adenovirus—MAV-1. The UV dose–response of MAV-1 was subsequently studied and reported here for comparison to the other adenoviruses UV dose–response.

2.3.3 MAV-1 Cell Culture Infectivity Results from UV Exposures

Because of the difficulties of detecting infection with MAV-2, the team switched to mouse adenovirus type 1 (MAV-1) for mouse infectivity assays. We collaborated with Dr. Kathy Spindler to propagate and run a UV disinfection experiment to determine the UV dose–response of MAV-1.

Virus was received from Dr. Spindler's lab and sent to the University of Colorado-Boulder. An experimental matrix was developed that would establish the UV-dose–response relationship for MAV-1 using cell culture infectivity assays, for LP and MP UV sources. UV exposures were performed over a range of UV doses for both LP and MP UV. A MAV-1 stock titer of approximately 10^8 pfu/mL was spiked into laboratory water to achieve a concentration of approximately 10^6 pfu/mL. The results are presented in Figure 2.5. On the basis of these data, it is apparent that MP is more effective for inactivation of MAV-1 than LP UV. The UV dose required for 4-log inactivation of MAV-1 under MP UV was approximately 30 to 40 mJ/cm^2; whereas the 4-log inactivation dose for LP was 80 mJ/cm^2. On the basis of these results a plan was put into place to use the MAV-1 in both a mouse model and a cell culture model.

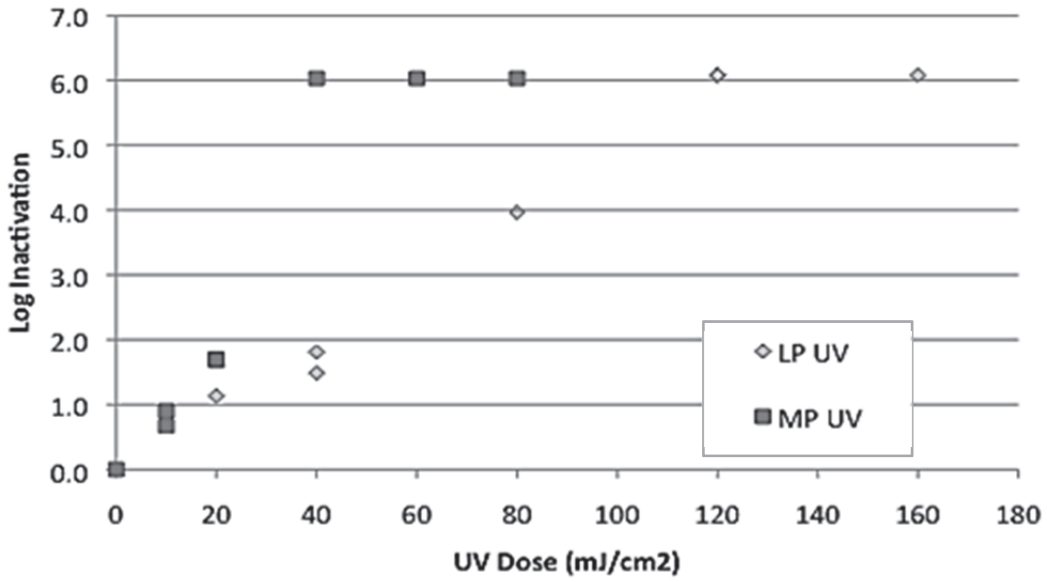

Figure 2.5. UV dose–response for MAV-1 under LP and MP UV irradiation assayed in cell culture.

2.3.4 Comparison of Human Respiratory Adenoviruses' Response to UV Disinfection

A number of experiments were conducted to investigate the UV dose–response of the lower numbered adenoviruses in both laboratory and reuse waters. These adenoviruses are easier to propagate and assay than the enteric adenoviruses, therefore they could be used for more studies. Cell culture infectivity assays for adenovirus type 2 were performed in A549 cells. These included three independent experiments with five doses each of LP and MP UV—0, 10, 25, 50, and 125 mJ/cm^2. All experiments were conducted using PEG-precipitated adenovirus stock in PBS. Data obtained for the AD2 cell culture assays are shown in Figure 2.6. The data are in agreement with data presented in other studies for LP UV (e.g., Meng and Gerba, 1996) and MP UV (Linden et al., 2007).

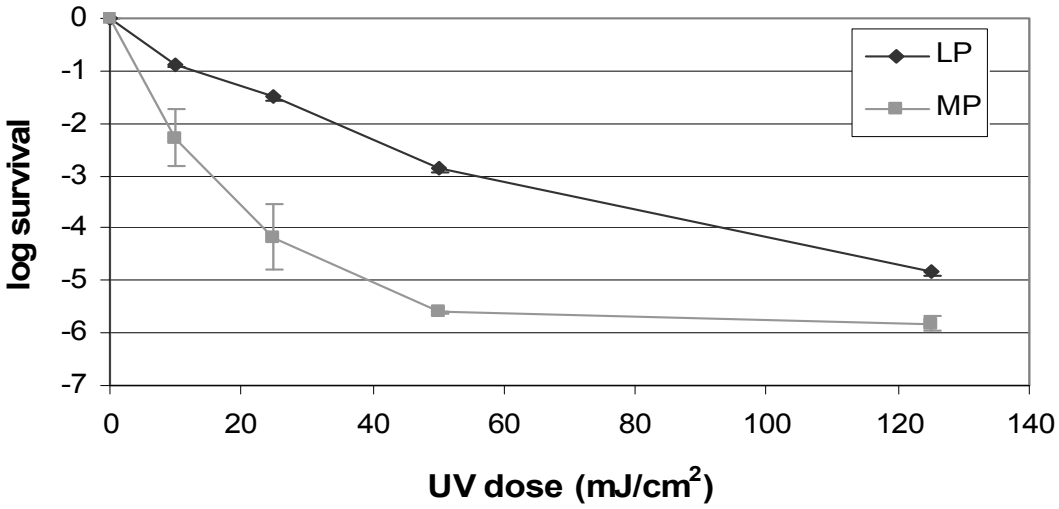

Figure 2.6. UV dose–response for adenovirus type 2 for LP and MP UV.

Bench scale LP UV and MP UV experiments were also performed comparing three different adenovirus types (2, 4, and 5). These experiments were carried out in three different waters from water reuse facilities; however, only two waters provided results that were valid.

Unfortunately, titer levels of AD2 were not high enough, so 4-log reduction was not achieved. However, titers of AD5 were high enough to achieve 4-log reduction. As was shown previously, MP UV is more effective at reducing adenoviruses compared to LP UV. These results are presented in Figures 2.7 and 2.8.

As far as the sensitivity to the two types of UV light, AD5 appears to be the most sensitive. It is difficult to determine if there are differences in UV effectiveness between the different water types because of the low level of adenoviruses and the similarity between the inactivation curves. However, AD4 in Pinellas County water appears to have increased resistance to MP UV inactivation (and possibly LP UV) compared to the other viruses tested. Note, however, that the two reuse waters compared were similar in having low absorbance levels below 250 nm, and the bench scale collimated beam testing accounts for differences in absorbance in the dose calculations.

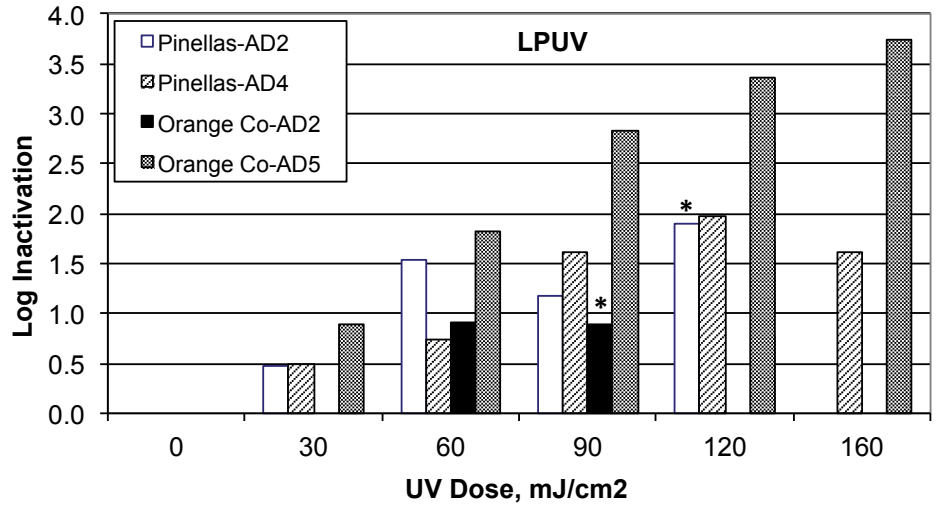

Figure 2.7. LP UV inactivation of three types of adenoviruses in two different reuse waters.

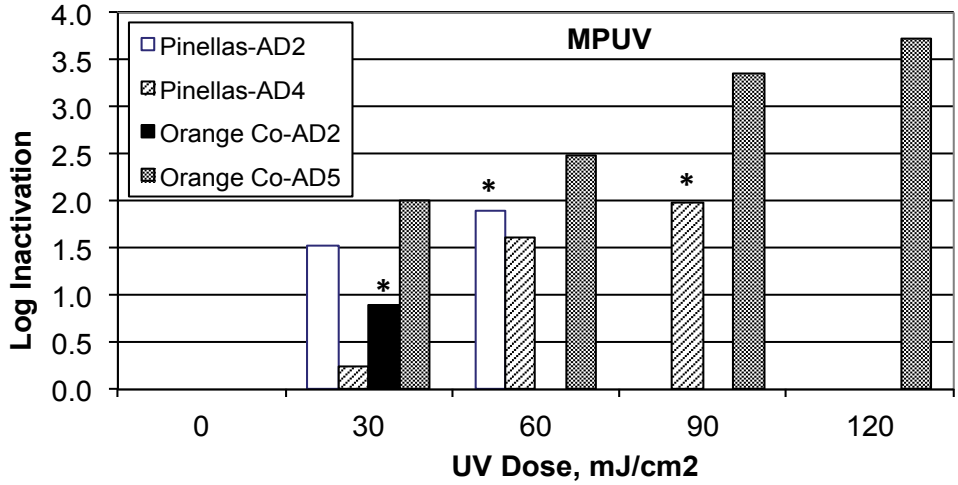

Figure 2.8. MP UV inactivation of three types of adenoviruses in two different reuse waters.

2.4 Conclusions

UV disinfection experiments were performed on a number of different adenovirus types spanning lower numbered respiratory adenoviruses, enteric adenoviruses, and murine adenoviruses. All viruses were assayed using cell culture infectivity. All adenoviruses, exhibited more resistance to UV irradiation from LP UV lamp sources (monochromatic UV at 254 nm) and higher susceptibility to MP UV (polychromatic UV across the 200 to 300 nm bandwidth). The murine adenoviruses were more susceptible to LP UV than the human adenoviruses. When assayed in waters of different quality, there was no detectable difference in the dose–response that was due to water quality, in the waters tested.

Chapter 3

An Animal Model for Assessing the Inactivation of Adenovirus by UV Light

3.1 Objective

The objective was to evaluate mouse adenovirus as a potential model for estimating the infectiousness of human adenoviruses after UV inactivation.

3.2 Approach Method—Evaluating an Appropriate Adenovirus Surrogate

3.2.1 Mouse Adenovirus Type 2

Because of economic reasons, a small animal model was needed to carry out our objectives. Mouse adenoviruses, MAV-1 and MAV-2, have been used in previous studies and methods for their detection using both mice and cell culture have been reported (Kring et al., 1995; Moore et al., 2003). Mouse adenoviruses are approximately the same capsid makeup and size as human adenoviruses. Mouse adenoviruses are approximately 70 nm and human adenoviruses are approximately 80 nm in diameter. Both have icosohedral capsid architecture with fibers projecting from the capsid's vertices.

Murine adenovirus type 2 (MAV-2), a fecal pathogen of mice, was originally selected for this study. MAV-2 is an enteric virus that infects the gastrointestinal tract and is excreted in feces for up to 6 months. This virus also causes wasting disease and pathology to the mucosal epithelium of ileum and cecum of athymic (nude) mice. MAV-2 was considered to be an appropriate surrogate to human enteric adenoviruses because (a) the mode of transmission (fecal-oral) and disease (enteric infection) are similar in nature; (b) both are double stranded DNA viruses (previously reported to be extremely resistant to UV treatment based on mammalian cell culture methods); and (c) both belong to the Mastadenovirus genus.

3.2.1.1 MAV-2 Propagation and Assay Development Methods

The CMT-93 cell line, derived from a 19-month male mouse having rectal carcinoma was grown and passed according to recommendations from the American Type Culture Collection (ATCC; http://www.atcc.org). Briefly, cells were subcultured every five to seven days by removing medium (ATCC formulated Dulbecco's Modified Eagles's Medium [MEM] with 10% fetal bovine serum) and rinsing with Tris (pH 7); 1 to 2 ml of a 0.25% trypsin and 0.03% EDTA solution was added and incubated at room temperature until the cells detached. New 162 cm^2 cell culture flasks (non-vent) were inoculated with cells and fresh medium (subcultivation ratio between 1:4 and 1:10) and incubated at 37 ^oC.

MAV-2, strain K87, was generously provided by Dr. Susan Compton of Yale University. This virus was propagated in CMT-93 cells. After decanting 10% MEM and rinsing with TRIS (pH 7), full monolayers (>85% confluent) of CMT-93 cells in 162 cm^2 flasks were inoculated with approximately 10,000 MPN/ml MAV-2 and incubated at room temperature for 30 min. After incubation, 0% MEM was replaced in the flask and the flask was incubated

at 37 ^{o}C until >90% of the monolayer showed CPE. Flasks were then frozen (-20 ^{o}C) until concentration and purification steps were performed.

Human adenovirus type 2 was initially purified and concentrated using two different methods. The first method used polyethylene glycol (PEG) precipitation followed by chloroform extraction, which resulted in a viral stock suspended in phosphate buffered saline (PBS). The second method used direct chloroform extraction of the viral supernatant from cell culture and resulted in a viral stock suspended in cell culture medium. Some of each type of stock was stored for long-term use at -80 °C. The second method required less researcher time and fewer materials, and it resulted in viral stock of a higher titer according to cell culture infectivity assays, which would lend itself better to dilution.

3.2.1.2 Assessment of Methods for the Enumeration of Viruses in Cell Culture

Using the previously described methods, we achieved viral stock concentrations ranging from 1000 to 50,000 MPN/ml. These are very low titers, similar to what we get for human enteric adenoviruses. For the purposes of achieving 4 or higher log reduction in our water disinfection studies, we decided to determine if we could significantly increase our sensitivity for MAV-2 detection by assessing different viral titer methods.

We took two approaches to determine if we could increase our sensitivity for detecting MAV-2. We assessed whether or not inoculating virus-containing samples onto monolayers was more sensitive than simultaneous inoculation of virus and freshly passed cells ("cells in suspension"). We also assessed whether multiple cell culture passages would result in higher virus titers. Descriptions of these studies and their findings are described in sections 3.2.1.3 and 3.2.1.4.

3.2.1.3 Monolayer Versus "Cells in Suspension"

Traditional methods for the enumeration of viruses in cells include inoculation of the virus onto cell monolayers and observation of CPE in a series of wells in order to determine the viral concentration ($TCID_{50}$ or MPN). In our previous work with human adenoviruses (Thurston-Enriquez et al., 2003a, 2003b), we inoculated virus-containing samples into freshly passed cells in 24-well cell culture dishes followed by incubation at 37 ^{o}C. We wanted to determine if this method or monolayer inoculation resulted in higher sensitivity of detection. Two experiments were carried out to see if there was a significant difference between the sensitivity of these methods.

In the first study, we determined the concentration of AD40 using both the monolayer and cell suspension methods (Figure 3.1). Five replicate 24-well trays were observed for CPE for both methods and carried out for at least 15 days post inoculation (21 days post inoculation for cell suspension method). Incubation of 21 days was not possible for the monolayer method because of significant cell monolayer deterioration (unable to determine CPE). Using the cell suspension method and 21 days incubation, the concentration of AD40 in our sample was significantly higher using the cell suspension method compared to the monolayer method. However, no difference was observed between the two methods at 12 and 15 days post inoculation. For days 2 through 7 post inoculation, the observed viral titer was higher for monolayer inoculated samples. This is not surprising because the monolayers in the cells in suspension assays would not have completely formed, making CPE difficult to visualize.

The second experiment was conducted with CMT-93 cells and MAV-2. This experiment was conducted similarly to the AD40 &PLC/PRF/5 experiment just described. For this study, the monolayer method performed very poorly for the observance of CPE by MAV-2. We observed a buildup of acid that quickly deteriorated the cell monolayer making it very difficult to identify virus infection (Figure 3.2). Because of the difficulties of acid production we decided to try multiple passages to determine if we could achieve higher sensitivity using the monolayer method.

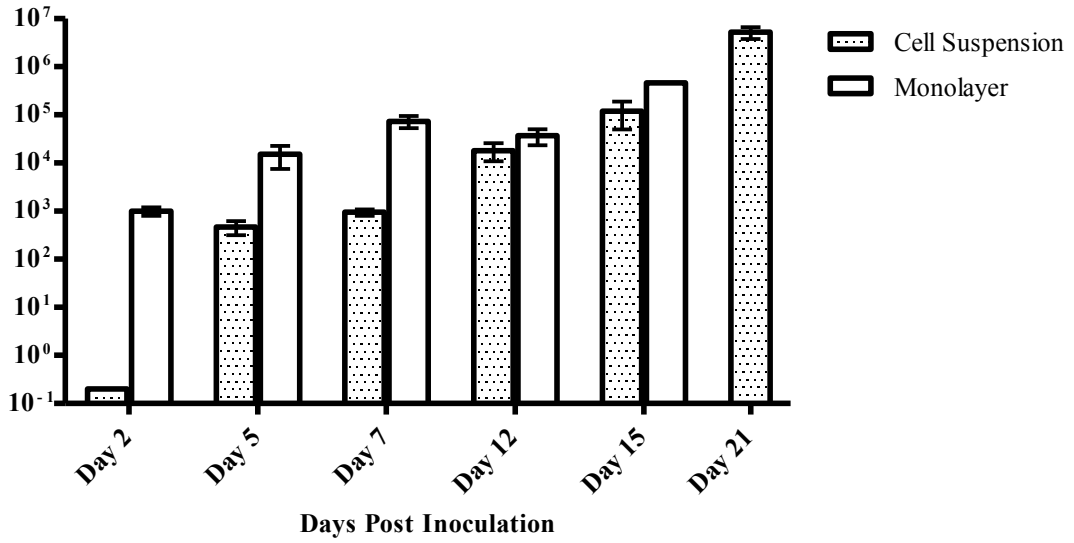

Figure 3.1. Comparison of time taken for the observance of adenovirus type 40 to cause CPE in PLC/PRF/5 cells using two methods for determining viral titer: inoculation onto full monolayers or into freshly passed cells ("cell suspension").

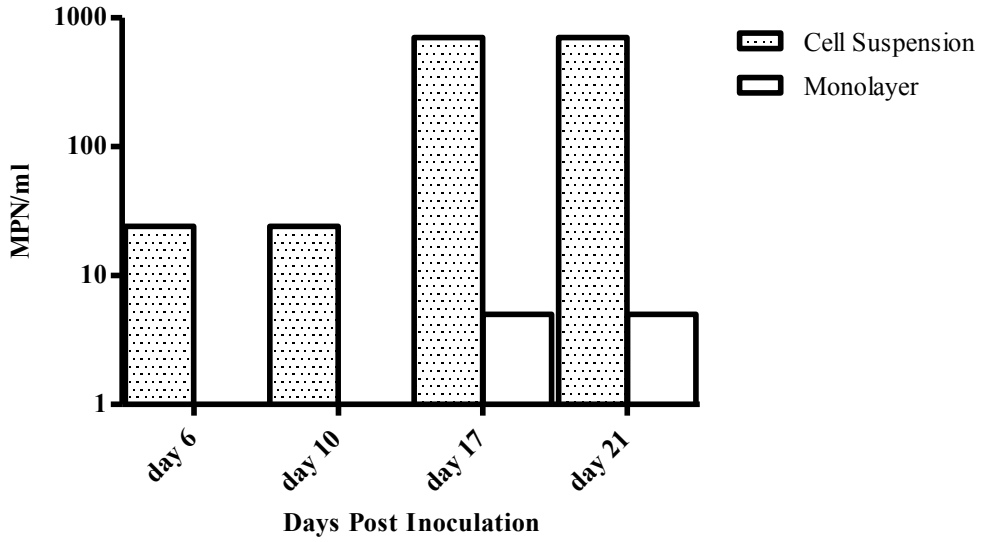

Figure 3.2. Comparison of time taken for the observance of MAV-2 to cause CPE in CMT-93 cells using two methods for determining viral titer: inoculation onto full monolayers or into freshly passed cells ("cell suspension").

3.2.1.4 Assessment of Multiple Cell Culture Passages

Multiple passages were performed on samples containing MAV-2 in order to determine if we could increase the sensitivity of detection. We found a significant increase in observed titer after three passages but no difference with the fourth passage (Figure 3.3). Although we could achieve higher titers with three passages, the titer observed was not significantly different than the titer observed using the cells in suspension method with an incubation time of 21 days. Because three passages were necessary, this did not decrease the time taken to observe CPE. On the basis of these tests we decided to conduct our assays using cells in suspension and carry out incubation for 21 days. Additional passages were performed if significant deterioration of cells occurred.

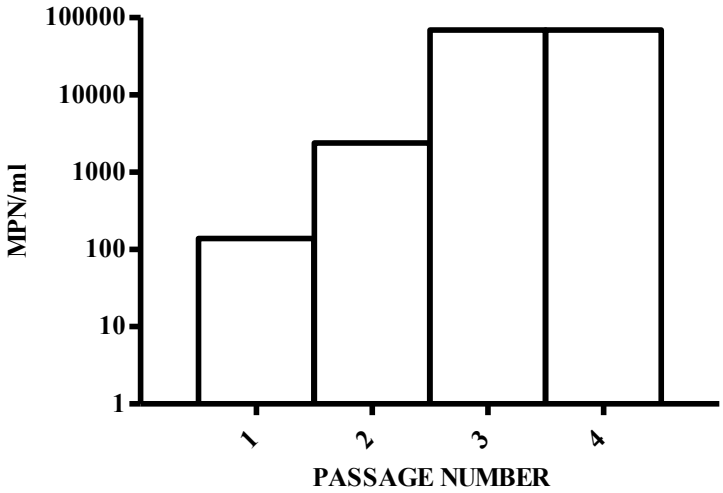

Figure 3.3. Comparison of the titer observed after multiple passages. No difference between multiple passages and determining viral titer with "cells in suspension" over a 21-day period.

3.2.1.5 Determination of Cross-Reactivity Between Adenovirus Cell Lines

In order to determine if the cell line used for detection of the human adenoviruses (PLC/PRF5) was susceptible to MAV-2 infection, we inoculated PLC/PRF5 monolayers with MAV-2. We did the same for CMT-93 cells and human adenovirus types 2, 4 and 40. We did not observe any CPE for any of these assays.

3.2.1.6 Development of PCR and Quantitative Real-Time PCR for Adenoviruses

A traditional PCR method (Table 3.1) was first developed for the detection of MAV-2. The target of this PCR method is a portion of the hexon gene resulting in a 151 base pair product. This method was to be used as a verification of MAV-2 in cell culture samples because of the low cost and simplicity of this technique.

Although traditional PCR is useful for detection purposes, it gives no information on the concentration and infectivity of the target organism. Thus, we felt it important to develop a method that combines quantitative real-time PCR (QPCR) and cell culture. First, the QPCR method was developed and optimized using viral copy standards. We determined that our QPCR protocol does not react with human adenoviruses types 2 and 40. We did have cross-reactivity with traditional PCR. Thus, our probe is specific to MAV-2 and was needed for specificity of detection.

Table 3.1. PCR and QPC Primers, Probe, and Reaction Protocol

MAV-2 Left Primer	GGCGACCGATTCGTACTTTA
MAV-2 Right Primer	AAACACACGGTGCGAATACA
MAV-2 Probe	AACAAGTTCCGTAACCCCAC
PCR Reaction Protocol	95 ^{o}C 10 min (1x) 95 ^{o}C 3 s, 56 ^{o}C 10 s, 65 ^{o}C 1 min (45x)

3.2.1.7 MAV-2 Infectivity

Because there is currently no information concerning the minimum infectious dose or the appropriate time of incubation for observation of intestinal changes, two different batches of mice were inoculated with MAV-2 at varying concentrations and were necropsied at various times. These studies were necessary for the development and optimization of a molecular detection method for MAV-2 in feces. Observation of inclusion bodies in intestinal sections (microscopic detection in infected tissue) was used to confirm infection.

The first batch of mice were inoculated with a dose of approximately $10^{3.5}$ MPN/ml based on CPE in CMT-93 cells. Fecal samples were collected before inoculation and 2, 9, and 14 days after inoculation. The mice were necropsied 14 days postinfection and all sections appeared healthy (i.e., uninfected). Three of 11 inoculated mice were positive by PCR by day 9 (feces). However, the intestinal sections did not have any inclusion bodies. The lack of observed infection may be due to missing the "window of infection" and the initial virus concentration may have been too low.

On the basis of the first mouse study, 15 additional mice were infected with a higher titer ($\sim 10^5$ MPN/ml) of virus and necropsied earlier (in hopes of catching the window of infection). Of the mice inoculated, only two of seven mice were positive for the virus in their feces. As in the first study, none of the mice had characteristic viral inclusion bodies in their small intestine or duodenum. These mice were necropsied seven days postinfection. Immunohistochemistry on intestinal and duodenum sections of the two mice that appeared to be excreting MAV-2 in their feces was conducted as an additional method to confirm infection. The sections were stained with a fluorescent antibody for MAV-2 but were not found to be positive.

3.2.1.8 MAV-2 Results and Discussion

Despite a great deal of effort in the development of assays to detect MAV-2 by cell culture and PCR for mouse infectivity studies, we were unsuccessful in detecting active infection in mice. None of the mice had virus in the intestinal sections despite high inoculants containing 10^5 MPN/ml. To our knowledge a sensitive assay was not available at the time of these experiments and the time and funds required to develop a more sensitive assay was not possible under the duration and funding of this project.

3.2.2 Mouse Adenovirus Type 1

Difficulties concerning the observance infection of mice with MAV-2 prompted the exploration of respiratory mouse adenovirus type 1 (MAV-1) as a workable surrogate for evaluating the in vivo response of UV inactivated adenoviruses.

MAV-1 causes obvious CPE in 3T6 cells and can be easily propagated to high doses (up to 10^8 pfu/ml). Viral replication can also be easily detected in multiple organs, but especially in the brain and spleen (Moore et al., 2003). In addition, a plaque assay method useful for the quantification of virus in solutions is available. Collaboration was established with Dr. Spindler who has worked with this virus for more than 20 years. Furthermore, her research team has developed and has had extensive experience with the detection of MAV-1 using plaque and ELISA assays.

3.2.2.1 MAV-1 Propagation and Assay Development Methods

Published methods for the viral propagation and assay of MAV-1 were followed (Cauthen and Spindler, 1999).The cell line, mouse 3T6, and the MAV-1 virus were maintained by our collaborator, Dr. Spindler, at the University of Michigan.

3.2.2.2 Mouse Infectivity

Three separate mouse experiments were conducted to determine the infectious dose of MAV-1 in female NIH Swiss outbred mice as used for MAV-1 infection studies previously (Kring et al., 1995). MAV-1 stock titers varying from 10^4 to 10^6 pfu/ml were diluted in phosphate buffered saline (pH 7) to achieve a range in concentration from 0.1 pfu/ml to 100,000 pfu/ml. After inoculation, the actual dose of virus per mouse varied from 0.03 to 30,000 pfu/ml. The number of mice inoculated for each virus dose was between five and ten. At least two mice were inoculated with PBS and served as a negative control.

Mice were inoculated intraperitoneally. Specifically, 0.3 ml inoculations of the diluted stock virus solutions were made in the lower left quadrant of the body cavity (Figure 3.4). The mice were euthanized when they exhibited disease symptoms, and the remaining mice were euthanized 12 days postinoculation. The time between inoculating and euthanizing mice was chosen on the basis of previous MAV-1 research (Moore et al., 2003). Eight days was reported to be the appropriate time for acute infection to occur when a MAV-1 dose of 1000 to 10,000 pfu was inoculated into susceptible mice. Because our UV inactivation studies may result in lower inoculation doses, we extended the infection period by four days.

The morbidity ratio (MR) was calculated using the following equation:

MR = # of mice that died during study period* / total number of mice studied*

*For a given condition. For example if 10 mice were inoculated with 3 pfu of MAV-1 and 7 died, the MR = 0.7.

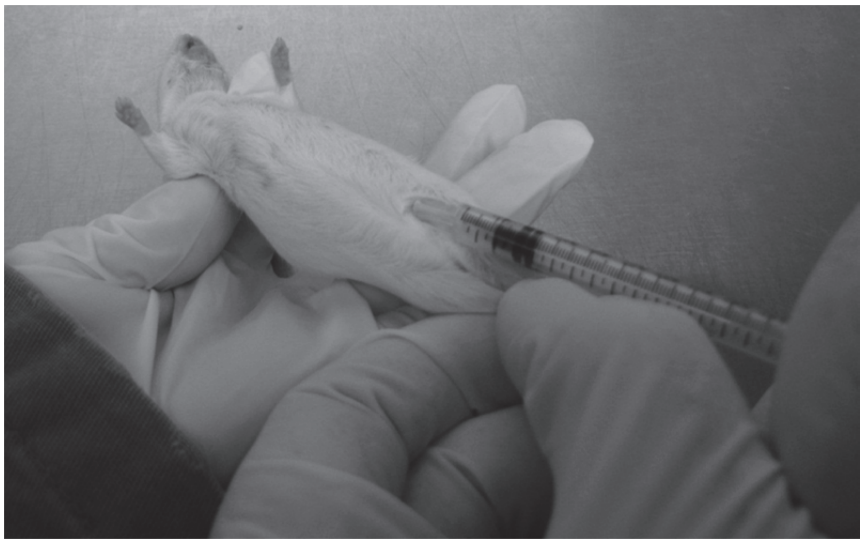

Figure 3.4. Inoculation of MAV-1 intraperitoneally into NIH Swiss bred mice.

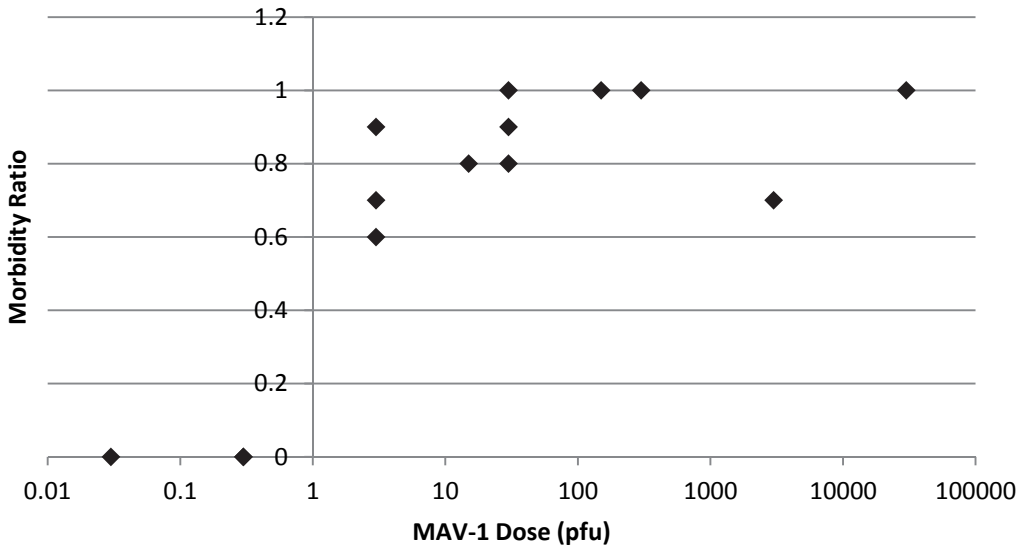

Figure 3.5. MAV-1 dose inoculated into mice and corresponding morbidity ratio after 12 days post inoculation.

3.2.2.3 MAV-1 Results and Discussion

Three experiments were conducted to identify minimum MAV-1 concentrations that resulted in morbidity. Knowing the minimum MAV-1 concentrations that produce infection was critical in the adequate design of our disinfection studies.

Our results show that very low doses result in high morbidity (Figure 3.5). Less than 5.0 pfu, for example, causes approximately 80% morbidity in inoculated mice (average of three experiments). However, the data from these experiments show variability in higher doses. This variability in the concentrations that result in a MR=1.0 may be due to a low sensitivity of this mouse infectivity method. Identification of morbidity was defined as death of the mouse or by the observation of viral inclusion bodies in necropsied sections of the brain. None of the necropsied samples were observed to have changes consistent with viral infection; thus, the morbidity ratio for these experiments was based solely on mouse death.

The variability in the MR at higher doses is likely due to the fact that viral infection was not detected. A more sensitive method that identifies virus in infected tissues may be more appropriate in evening out the variability in MR. Another factor in the variability between experiments and for MR at higher doses, may be due to the fact that experiments were carried out for 12 days. Longer experimental times may eliminate the variability between experiments and doses.

3.3 Results and Discussion

3.3.1 MAV-2 and MAV-1 Assay Development

We were unable to develop a mouse infectivity assay for MAV-2 and moved ahead with developing and analyzing MAV-1 as a potential surrogate for human adenovirus. Like previous MAV-1 infectivity studies, our work shows that MAV-1 infects mice at low doses, less than 5.0 pfu causes 80% morbidity. In order to use the MAV-1 mouse model for

determining the in vivo infectivity of UV-irradiated adenoviruses, a more sensitive and stable method to detect 4-logs inactivation was needed.

3.3.2 Comparison of Mouse Infectivity and ELISA Methods for the Determination of MAV-1 Infection

An ELISA method for the detection of MAV-1 in the brain of inoculated mice was assessed, not only to provide a confirmation of infectivity, but also to compare mouse infectivity data. This comparison turned out to be important in developing a more sensitive and less variable infectivity detection method.

For all mice under study, sections of brain tissue were removed immediately after mice were euthanized. These sections were fixed and sent, on ice, overnight to the Spindler laboratory where they were prepared and analyzed according to previously published methods (Welton and Spindler, 2007).

Figure 3.6 shows the curves of infectivity experiments where (a) mouse morbidity observation (morbidity ratio) and (b) ELISA was used to identify infection. ELISA detected infection in more mice than observation of morbidity alone. Variability between replicate experiments was less in ELISA versus morbidity observation alone. Figure 3.7 shows only the morbidity curve from ELISA results. This curve shows the steep slope at low doses and achieving a MR=1.0, or 100 % infectivity, by a dose of 30 pfu.

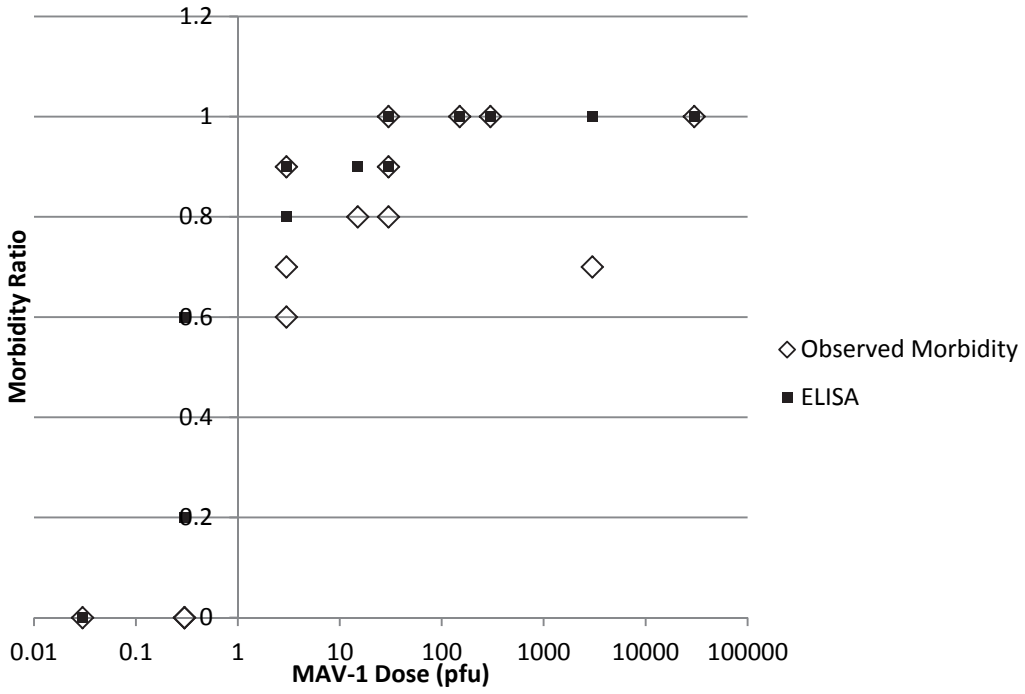

Figure 3.6. Comparison of morbidity curves for mouse morbidity and ELISA methods.

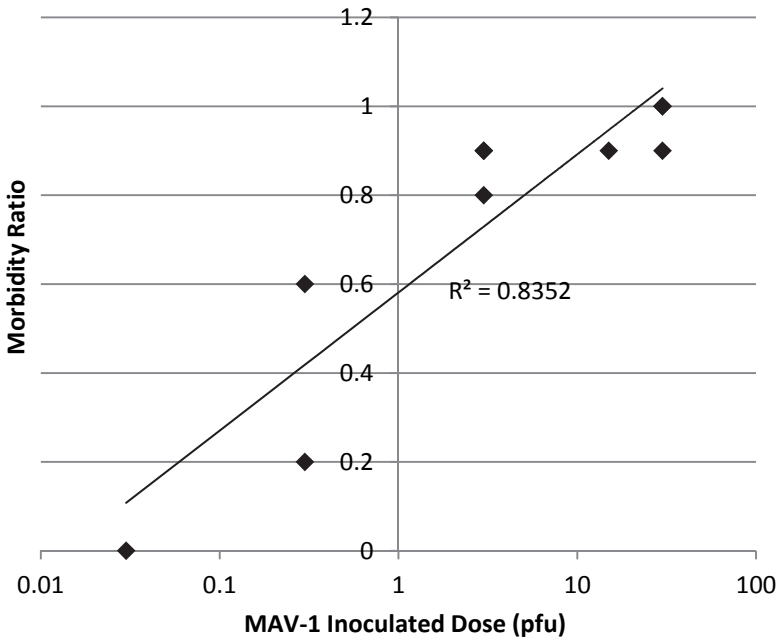

Figure 3.7. Morbidity curve based on ELISA results for MAV-1 infection in mice.

Because of the wide variability in determining infectivity only on the basis of observed infection and death in mice, an established ELISA method was evaluated. The ELISA method was initially included in this study as a confirmation of infection. Not only did it serve that purpose, but the ELISA method also increased the sensitivity of the mouse infectivity assay. The mouse infectivity method (observation of morbidity and mortality) combined with analysis of all brain sections by ELISA proved to be a more sensitive method for determining infectivity as well as evening out the variability in results that were observed with the mouse infectivity method alone. On the basis of these results, we analyzed all UV disinfection experiments using the combined method.

Understanding the dose where all mice are infected was important in the design of UV inactivation experiments where we sought to achieve 4-logs inactivation. Through these ELISA experiments, we observed 100% infection at viral doses as low as 30 pfu.

Chapter 4

UV Inactivation Studies Using Mouse Infectivity Method Compared to Plaque Assay

4.1 Objective

The objective was to use a mouse infectivity method to assess the inactivation of adenoviruses under UV disinfection and evaluate the mouse model in comparison to cell culture infectivity.

4.2 Approach Methods—Mouse Infectivity

Virus preparation, mouse inoculation, mouse morbidity, and ELISA methods were the same as described earlier.

The concentration of virus in samples was calculated using the MPN method that has been described previously for determining the UV inactivation of Giardia in water using a gerbil infectivity method (Linden et al., 2002). Briefly, the number of mice that were positive (a positive ELISA reaction) or negative for MAV-1 infection out of the total number of mice inoculated for a given set of experiments were used to estimate the MPN using the Thomas equation. Thus, the MPN unit is the number of infectious virus (viral detection in brain tissue by ELISA) divided by the total number of viruses.

4.3 Results and Discussion—UV Light Inactivation of MAV-1 and Infectivity in Mice

Figure 4.1 shows the UV inactivation curves developed from mouse infectivity data. A linear inactivation curve, similar to what has been shown for other adenoviruses, was observed for LP UV data. Unlike LP UV, the inactivation curve for MP UV experiments did not have as good a linear fit ($R^2 = 0.63$) but fit well to a logarithmic curve ($R^2 = 0.98$). The apparent tailing off of the MP UV data may be an indication of the need for "multiple hits" of MP UV for virus inactivation at higher log inactivation levels. Estimated required doses to achieve 1 through 4-logs inactivation are shown in Table 4.3. Estimated (Table 4.3) and actual doses (Table **4.1**and Table **4.2**) observed for 1 to 4 logs inactivation of MAV-1 are also given.

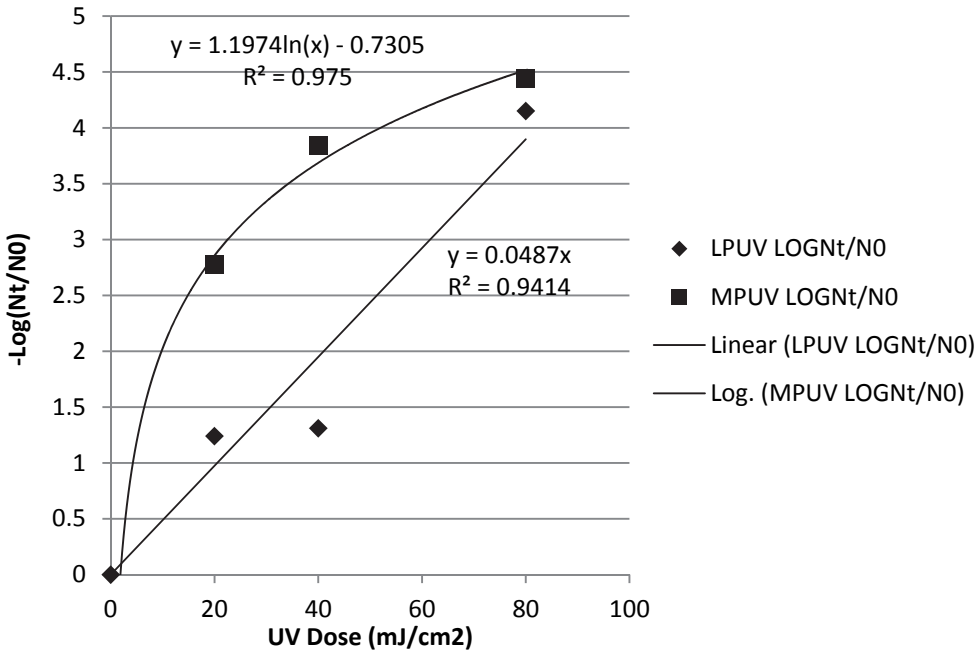

Figure 4.1. UV light inactivation of MAV-1 as determined by mouse infectivity.

Table 4.1. Comparison of Mouse Model and Plaque Assay Log Reduction of MAV-1 by LP UV in PBS

LP UV Experiment	Mouse Model LOG Reduction and Average Log Reduction	Plaque Assay LOG Reduction and Average Log Reduction
LP UV dose: 20	1) 1.2*	1.1
LP UV dose: 40	1) 1.3*	1.7
LP UV Dose: 80	1) 3.9 2) 4.4 Average: 4.2	4.0

*One experiment conducted.

Table 4.2. Comparison of Mouse Model and Plaque Assay Log Reduction for MAV-1 by MP UV in PBS

MP UV Experiment	Mouse Model LOG Reduction and Average Log Reduction	Plaque Assay LOG Reduction and Average Log Reduction
MP UV dose: 20	1) 2.8*	1.8
MP UV dose: 40	1) 3.2	
	2) 4.4	>6
	Average: 3.8	
MP UV Dose: 80	1) 4.8	
	2) 4.0	>6
	Average: 4.4	

*One experiment conducted.

The inactivation of adenoviruses by UV light illustrated in Tables 4.1 and 4.2 provides an important insight into the comparison of the use of cell culture infectivity and animal infectivity for assessing the effectiveness of UV for disinfection of viruses. For LP UV light at 254 nm, which mainly causes damage to the viral genome, there was almost no difference in the UV dose–response of adenoviruses when assayed in cell culture or an animal model as reported in Table 4.3. Both models also proved again that MP UV light was much more effective than LP UV light for inactivation of adenoviruses.

What was not evident from these data is that the use of an animal model provided a different outcome or interpretation of the UV inactivation of adenoviruses compared to cell culture. The data may suggest differences in inactivation mechanisms between LP UV and MP UV when comparing animal and cell culture infectivity results. At higher MP UV doses, it appears that there is a population of adenoviruses that is capable of infecting animals although this was not observed in the cell culture assays. Over 6 logs of inactivation was observed at MP UV doses of 40 and 80 in cell culture, whereas a maximum of 4 logs was observed in mouse studies. These differences cannot be teased out from our studies; however, it may suggest that viral damage is at a level that still allows infection in vivo but not in vitro. Although this study was not comprehensive enough to prove specifically that animal models were similar to cell culture models for assessing infectivity of viruses, as was shown for *Cryptosporidium* (Rochelle et al., 2002), the evidence presented herein certainly points to this likelihood and provides more confidence in cell culture results as being representative of what would be expected in an animal infectivity case for LP UV studies. Interesting, in all cases, the doses for both LP UV and MP UV inactivation were lower than those required in the US EPA Long Term 2 Enhanced Surface Water Treatment Rule (US EPA, 2006). The US EPA virus requirements were developed using an extensive statistical evaluation of all data available for LP UV inactivation of adenovirus and the 80% credible interval was selected to be conservative.

Table 4.3. Comparison of US EPA UV Dose Requirements (mJ/cm^2) and Estimated Doses Required to Achieve 1 through 4 Logs Inactivation of MAV-1 for Mouse Infectivity and Plaque Assays

Log Inactivation	Mouse Infectivity Assay		Plaque Assay		US EPA UV Dose Requirements*
	LP UV	MP UV	LP UV	MP UV	
1	21	4.3	20	8	58
2	41	10	40	16	100
3	62	23	60	20	143
4	82	52	80	25	186

*US EPA, 2006

Chapter 5

Conclusions and Recommendations for Future Work

5.1 Conclusions

All previous research on adenovirus UV inactivation was performed using monochromatic UV 254 nm light. The data indicated that adenovirus are extremely resistant to UV irradiation. However, the data developed in this research strongly suggests that polychromatic UV light from common MP UV sources is two to ten times more effective than monochromatic UV 254 nm. This enhancement is likely due to the fact that low UV wavelengths (<240 nm) have up to 6 times greater effectiveness than 254 nm, on the basis of some action spectra work done by Linden et al. (2007). These data were developed in both clean water and reclaimed water matrices.

Working with scientists at the United States Department of Agriculture (USDA), we used animal adenoviruses that are closely related to human adenoviruses and developed a link between cell culture and animal infectivity data for the first time. The inactivation of adenoviruses by UV light provided an important insight into the comparison of the use of cell culture infectivity and animal infectivity for assessing the effectiveness of UV for disinfection of viruses. For LP UV light at 254 nm, which mainly causes damage to the viral genome, there was almost no difference in the UV dose–response of adenoviruses when assayed in cell culture or an animal model. Both models also proved that MP UV light was much more effective than LP UV light for inactivation of adenoviruses.

What was not evident from these data is whether the use of an animal model provides a different outcome or interpretation of the UV inactivation of adenoviruses compared to cell culture. It was initially hypothesized that comparing animal and cell culture models may unveil some of the clues as to why adenovirus is seemingly so much more resistant than other viruses to UV disinfection, but this was not the case. Although this study was not comprehensive enough to prove specifically that animal models were similar to cell culture models for assessing infectivity of viruses, the evidence presented certainly points to this likelihood and provides more confidence in cell culture results as being representative of what would be expected in an animal infectivity case.

5.2 Potential Relevance and Future Applications

Clearly, virus inactivation is essential in water reuse applications. If any pathogens are of concern when membranes are used and the formation of free chlorine is challenging, the primary pathogen risk relates to viruses. Currently, the US EPA has set a dose level of 186 mJ/cm^2 for 4-log reduction of all viruses on the basis of adenovirus data in drinking water. If these doses are carried over to reclaimed water, the size of the required UV system will be much larger than needed for most other viruses, as well as bacteria and protozoan parasites.

The results indicating that MP is much more effective than LP UV, which supports a growing body of evidence stating the same, can have some significant impact on the required UV

doses to use for achieving inactivation of adenoviruses. System design for log inactivation of viruses using UV processes should seriously consider the use of MP UV and work early on with local regulators to evaluate the use of a lower dose than is specified in the US EPA regulations, based on LP UV studies. This may also save reclaimed water utilities a lot of money in disinfection costs, operating at a lower dose. Facilities considering a reduction in MP UV dose below that required by regulations should be careful to evaluate the effects of water absorbance, water layer, and UV lamp emission in the low wavelength region and consider monitoring of the low wavelengths responsible for enhanced virus inactivation. Some reclaimed waters may have high absorbance in the wavelength region of greatest impact (<240 nm) for virus inactivation so the benefit of MP UV may be limited. Furthermore, any UV system validation testing and control algorithms for dose delivery need to account for low wavelength effects when considering enhancements for virus inactivation.

The work on animal infectivity compared to cell culture for virus assessment is some of the first work of its kind. Although much was revealed from our study, these results should be repeated with other viruses, including adenoviruses, and animal models. Of interest to future research is the use of a virus–animal model that utilizes an oral route for ingestion of the viruses, which was not possible using the MAV-1 model.

This research should also spur some more interest in better understanding why the polychromatic UV sources are so much more effective than LP monochromatic UV light for adenoviruses. Insights based on molecular biology investigation into the role of UV damage of viral proteins and types of DNA damage based on different UV wavelengths is recommended on the basis of our findings. A study to define the role of DNA repair both in vitro and in vivo on the survival of UV-disinfected adenoviruses is also recommended. These studies could lead to further insights into disinfection behavior of viral pathogens.

Finally, this work provided a positive cooperative link between the USDA and the WateReuse Research Foundation, which hopefully will lead to future collaborations in the area of animal–water interactions.

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