Challenge Organisms for Inactivation of Viruses by Ultraviolet Treatment

Subject Area: Water Quality
ADDENDUM

Challenge Organisms for Inactivation of Viruses by Ultraviolet Treatment

Water Research Foundation Project #3105


Pending further experimental trials, the two experiments in this report that describe MP-UV inactivation of Ad41 should be disregarded due to potential implications of experimental procedures used. Additional details are provided below.

Medium-pressure (MP) UV inactivation of adenovirus type 41 (Ad41) is described on pages 32-33 of this report. While adenovirus type 2 (Ad2) was found to be more sensitive to MP-UV compared to low-pressure (LP) UV, there appeared to be no difference between MP- and LP-UV for inactivating Ad41 (Figure 3.7, page 32). However, the report states that the Ad41 data should be interpreted with caution due to the low number of experimental replicates and differences in the experimental procedures (pages 33 and 129). Furthermore, the report recommends additional studies to determine the reproducibility of the Ad41 response to MP-UV (pages xxii, 33, 129, and 133).

As stated in the report, exposure of Ad41 to MP-UV was conducted towards the end of the project when secondary cooling of the MP-UV collimated beam apparatus was necessary. This was accomplished using a stand-alone air conditioning unit. However, unpublished data made available after this final report was published indicates the observed response of Ad41 to MP-UV was probably an artifact of this secondary cooling (Linden, unpublished). An MP-UV lamp operated at 70°C produced the expected polychromatic output with emission peaks at approximately 248, 254, 266, 280, 289, and 295 nm. However, when the same lamp was cooled to 27°C, it produced only monochromatic UV at 254 nm (within the range 200 – 300 nm). Therefore, “over-cooled” MP-UV lamps appear to behave similar to monochromatic LP-UV lamps in terms of their output spectrum. Consequently, it is likely that in the experiment in which Ad41 should have been exposed to polychromatic MP-UV, it was actually exposed to monochromatic 254 nm UV. So the reason there was no difference between LP- and MP-UV inactivation of Ad41 was that in both instances the virus was exposed to monochromatic 254 nm UV.

Pending further experimental trials, the two experiments in this report that describe MP-UV inactivation of Ad41 should be disregarded. The issue of over-cooling the MP-UV lamp does not affect any other experiments or data in the report.
Challenge Organisms for Inactivation of Viruses by Ultraviolet Treatment
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Challenge Organisms for Inactivation of Viruses by Ultraviolet Treatment

Prepared by:
Paul A. Rochelle
Metropolitan Water District of Southern California, La Verne, CA 91750
Ernest R. Blatchley III, and Po-Shun Chan
School of Civil Engineering, Purdue University, West Lafayette, IN 47907
O. Karl Scheible, and Chengyue Shen
HydroQual, Inc., Mahwah, NJ 07430

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FOREWORD

The Water Research Foundation (Foundation) is a nonprofit corporation that is dedicated to the implementation of a research effort to help utilities respond to regulatory requirements and traditional high-priority concerns of the industry. The research agenda is developed through a process of consultation with subscribers and drinking water professionals. Under the umbrella of a Strategic Research Plan, the Research Advisory Council prioritizes the suggested projects based upon current and future needs, applicability, and past work; the recommendations are forwarded to the Board of Trustees for final selection. The Foundation also sponsors research projects through the unsolicited proposal process; the Collaborative Research, Research Applications, and Tailored Collaboration programs; and various joint research efforts with organizations such as the U.S. Environmental Protection Agency, the U.S. Bureau of Reclamation, and the Association of California Water Agencies.

This publication is a result of one of these sponsored studies, and it is hoped that its findings will be applied in communities throughout the world. The following report serves not only as a means of communicating the results of the water industry's centralized research program but also as a tool to enlist the further support of the nonmember utilities and individuals. Projects are managed closely from their inception to the final report by the Foundation's staff and large cadre of volunteers who willingly contribute their time and expertise. The Foundation serves a planning and management function and awards contracts to other institutions such as water utilities, universities, and engineering firms. The funding for this research effort comes primarily from the Subscription Program, through which water utilities subscribe to the research program and make an annual payment proportionate to the volume of water they deliver and consultants and manufacturers subscribe based on their annual billings. The program offers a cost-effective and fair method for funding research in the public interest.

A broad spectrum of water supply issues is addressed by the Foundation's research agenda: resources, treatment and operations, distribution and storage, water quality and analysis, toxicology, economics, and management. The ultimate purpose of the coordinated effort is to assist water suppliers to provide the highest possible quality of water economically and reliably. The true benefits are realized when the results are implemented at the utility level. The Foundation's trustees are pleased to offer this publication as a contribution toward that end.

David E. Rager
Chair, Board of Trustees
Water Research Foundation

Robert C. Renner, P.E.
Executive Director
Water Research Foundation
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EXECUTIVE SUMMARY

OBJECTIVES

The overall objective of this project was to identify a non-pathogenic microbe that can be used in challenge tests for ultraviolet (UV) radiation reactor validation at the high doses required for virus inactivation credit. Additionally, dyed microspheres (DMS) were evaluated as UV dosimeters for high dose applications. Specific technical objectives were to: 1) develop UV dose-response curves for adenovirus types 2, 40, and 41 using a consistent cell culture-based quantification assay; 2) assess the level of damage in UV-irradiated adenoviruses; 3) evaluate UV dose-response relationships of a wide variety of potential biological surrogates and DMS, and compare their responses to adenoviruses; 4) conduct demonstration-scale testing of selected biological and non-biological surrogates to identify and resolve scale-up issues; and 5) conduct on-site evaluations with selected surrogates at full-scale UV disinfection facilities.

BACKGROUND

Ultraviolet reactors that are used for drinking water disinfection need to be tested to ensure they are delivering the required doses and achieving the desired level of pathogen inactivation to protect public health. Testing methods can use biological, chemical, or particle surrogates although not all approaches have been approved for reactor validation. The non-pathogenic bacteriophage MS2 and \textit{Bacillus subtilis} spores are currently recognized as the standard challenge organisms for reactor validation because they are more UV resistant than most waterborne pathogens. However, an important group of potentially waterborne viruses, the adenoviruses, is considerably more UV resistant than other pathogens and the standard MS2 and \textit{B. subtilis} biodosimeters. Based on the high UV resistance of adenoviruses, the U.S. Environmental Protection Agency’s (USEPA) Long Term 2 Enhanced Surface Water Treatment Rule (LT2ESWTR) and Ground Water Rule (GWR) require a UV dose of 186 mJ/cm\textsuperscript{2} for 4-log inactivation of viruses. This level of MS2 inactivation is achieved with a dose of approximately 85 mJ/cm\textsuperscript{2}, thereby limiting the utility of MS2 for reactor validation at the high doses required for virus inactivation credit, or other high-dose applications. An alternative challenge organism is required that has similar or higher UV resistance compared to adenoviruses. The ideal challenge organism would be non-pathogenic, easily grown to high concentrations, easily enumerated in a growth or infectivity assay, display minimal shoulder (lag) or tailing of the dose response curve, stable for relatively long periods with minimal die-off, robust under typical testing conditions, and effectively model the reactor’s disinfection efficiency toward the target pathogen.

APPROACH

The response of adenovirus types 2 (Ad2) and 41 (Ad41) and a wide variety of potential challenge microbes to monochromatic low-pressure (LP) UV radiation (254 nm) and polychromatic, medium-pressure (MP) UV was assessed using bench-scale collimated beam instruments. Survival of Ad2 and Ad41 following UV exposure was quantified using a cell culture plaque assay on A549 cells. The challenge organisms comprised standard laboratory strains and native isolates recovered from untreated source waters, and included a variety of
bacteriophages, a virus that infects *Chlorella* spp., bacteria, and bacterial endospores. Dose response data were compared to the benchmark of bacteriophage MS2. The dose responses of three sizes of DMS conjugated to a photo-reactive dye were also evaluated with fluorescence measured by flow cytometry. Action spectra were determined using band-pass filters for selected microbes and DMS to determine the most effective MP wavelengths. Finally, selected challenge microbes and DMS-based Lagrangian actinometry (LA) were tested in two demonstration-scale UV facilities using LP and MP lamps at flow rates of 28 – 2,083 gallons per minute (GPM).

**RESULTS AND CONCLUSIONS**

Low-pressure UV doses of 98 mJ/cm² and 201 mJ/cm² resulted in 3-log inactivation of Ad2 and Ad41, respectively. Medium-pressure UV was more effective than LP-UV for inactivating Ad2 but not Ad41, and action spectra for both viruses indicated that within the range 220 – 280 nm, 220 nm was the most effective wavelength. The formation of thymine dimers in the DNA of UV-exposed Ad2 and their increased accumulation with increasing UV doses demonstrated that there is nothing unique about the DNA or physical structure of adenoviruses that prevents them from being damaged by UV radiation.

None of the bacteriophages were more resistant to UV than MS2 and so were not suitable as alternative challenge organisms for reactor validation. The PBCV-1 virus that infects *Chlorella* spp. more closely resembles adenoviruses compared to bacteriophages but its UV response was within the 90% prediction limits for MS2 provided by the USEPA’s UV Disinfection Guidance Manual (UVDGM), so it offered no advantage over MS2.

The microbes that displayed the highest UV resistance, equal to adenoviruses and in some cases more resistant than adenoviruses, were *Rubrobacter radiotolerans*, *Deinococcus* spp., and endospores of *Bacillus pumilus*. *Deinococcus* spp. and *R. radiotolerans* displayed similar UV resistance as adenoviruses (4-log inactivation at 128 – 186 mJ/cm²) but the shoulder and tailing in their UV dose response curves may limit their utility as practical surrogates. Nevertheless, an indigenous strain of *Deinococcus aquaticus* provided consistent estimates of the doses delivered by a MP 4-lamp demonstration-scale reactor operating at 0.4 MGD. Medium-pressure UV was slightly more effective than LP-UV for inactivating *D. aquaticus*. The action spectrum indicated that 220 and 228 nm filtered UV (UV_{220-228}) was approximately 1.4-fold more effective than UV_{254}, but no more effective than UV_{260}. This may be due to intracellular manganese protecting *Deinococcus* spp. repair proteins from radiation-induced damage. Based on the assumption that bacteriophages that infect *Deinococcus* spp. may be repaired by their host cell and so also display high levels of UV resistance, a variety of strategies were used to isolate *Deinococcus*-specific phage. However, all attempts to isolate them for this project were unsuccessful.

Spores of *B. subtilis*, a standard laboratory strain of *B. pumilus*, and an indigenous isolate of *Bacillus megaterium* all conformed reasonably closely to the UVDGM 90% prediction limits for inactivation of *B. subtilis* spores (3-log inactivation at 51 – 90 mJ/cm²) and so were more sensitive than the LT2ESWTR dose requirements for virus inactivation.

The most promising challenge organisms for UV reactor validation were spores of native strains of *B. pumilus* isolated from low turbidity (1.05 NTU) surface water at the influent of a drinking water treatment plant. The UV dose response of these spores was manipulated by varying the concentration of MnSO₄ in the spore propagation medium. The “tunable” dose response varied from being close to the dose response curve of Ad2 (2-log inactivation at
57 mJ/cm² for *B. pumilus* spores compared to 62 mJ/cm² for Ad2) to being more resistant than adenoviruses (2-log inactivation at 174 – 187 mJ/cm²), depending on the MnSO₄ concentration. Spores of other *Bacillus* spp. could also be made more resistant by culturing the bacteria in media containing MnSO₄. Medium-pressure UV was more effective than LP-UV for inactivating *B. pumilus* spores. A MP-UV dose resulted in 4.8-log inactivation compared to 1.6-log with the same dose of LP-UV. The action spectrum for *B. pumilus* spores was similar to the action spectrum for Ad2, with approximately 8-fold higher inactivation at UV₂20-228 compared to UV₂54. The similarity of action spectra strengthens support for using *B. pumilus* spores in reactor validation.

UV resistant aerobic sporeforming bacteria such as *B. pumilus* are relatively common and easy to isolate from untreated source waters. Suspensions containing 10¹⁰ spores/mL were relatively easy to prepare using standard laboratory procedures and spores could be propagated to sufficiently high enough titers for large-scale reactor testing.

Dyed microspheres with a diameter of 10 µm were more sensitive to UV radiation than 6 µm or 15 µm diameter DMS but all three sizes were most sensitive to UV wavelengths close to 254 nm. All three sizes could be used to characterize UV doses up to 600 mJ/cm². Lagrangian actinometry based on DMS fluorescence measurements characterizes the dose distribution in UV reactors and so shows promise for reactor validation. The relatively strong fluorescence intensity (FI) signals induced by large UV doses are more easily differentiated compared to smaller FI values with lower UV doses, indicating that LA may be well suited for quantification of UV dose distributions in high-dose applications. However, additional work is necessary to fully assess the sources of variability in the method.

Cell culture assays demonstrated that the photosensitive dye used in DMS was not toxic, even at concentrations five orders of magnitude higher than the maximum liquid phase concentration in reactor tests. Substituting a phosphate-based linker for the original silane group that linked the photosensitive dye to the microspheres rendered the DMS unresponsive to MP-UV, even though there was no alteration to the dye itself. Therefore, the phosphate form of DMS cannot be used for MP applications.

Demonstration-scale testing of three UV reactors at two facilities with flow rates of 28 – 2,083 GPM demonstrated that *D. aquaticus* and *B. pumilus* spores could be developed as challenge organisms for reactor validation at the high doses required for LT2ESWTR virus inactivation credit. The reduction equivalent dose (RED) of a 3-lamp low-pressure high-output reactor, based on *B. pumilus* spore biodosimetry at 104 GPM, was 97 mJ/cm² compared to the manufacturer’s nominal dose of 100 mJ/cm² under these conditions. The reactor’s REDs were equivalent to LT2ESWTR virus inactivation credits of 1.2- to 4-log at flow rates of 200 to 25 GPM. There was good agreement between spore inactivation and DMS-predicted inactivation at high flow rates but the correlation was weaker at low flow rates. More work is necessary to increase agreement between DMS and microbial surrogate data since it is unclear whether the poor correlation was due to inaccurate spore or DMS responses.

Microbial-derived RED values for a 4-lamp MP reactor operating at 278 GPM were consistent across three trials and two surrogates (*B. pumilus* spores and *D. aquaticus*). RED values were 26 – 46 mJ/cm² and 101 – 144 mJ/cm² with one and four lamps energized, respectively.

The final objective was to conduct surrogate testing at full-scale UV treatment facilities. However, utility personnel were concerned about the potential public health implications if some of the introduced spores or DMS were not completely removed or inactivated prior to entering
the distribution system, so full-scale testing was not conducted. Although *B. pumilus* is not usually considered as a human pathogen, the extremely rare instances of possible involvement in human infections were enough to cause concern among utility personnel. Consequently, on-site reactor validation with novel challenge organisms may be difficult to implement due to the perception by treatment plant operators and management that the microbes may present a public health threat, even if the challenge organisms are isolated from the influent of drinking water treatment plants.

**RECOMMENDATIONS**

Spores of *B. pumilus* should be considered as alternative challenge microbes for validating UV reactors for virus inactivation credit. Applications include compliance with the LT2ESWTR and GWR, and wastewater treatment for indirect potable reuse. Drinking water, regulatory, and UV industry professionals should decide on the specific UV response that is required of this new tunable surrogate, so that spore propagation conditions can be standardized. This will involve standardizing the type of culture medium, concentration of manganese, and incubation period. Procedures can be standardized on a single isolate, such as *B. pumilus* ASFUVRC isolated in this project, or native *B. pumilus* spores isolated from individual treatment plant influents prior to reactor validation testing at that particular site. Further work is necessary to develop culture conditions that maximize spore yields while retaining the desired level of UV resistance.

Although they have disadvantages such as dose response shoulder and tailing, and difficulty in achieving high concentrations for large-scale testing, *Deinococcus* spp. should also be considered for additional testing and optimization as potential challenge organisms for high dose applications. The focus of this work should be maximizing cell yields in culture.

The advantages of using bacteriophages as challenge organisms include high titers and no concerns about human pathogenicity. Therefore, although none of the tested bacteriophages were more resistant than MS2, studies should continue with selected phages in a variety of hosts that may display a similar repair capacity as the adenovirus/host cell combination.

Additional testing of multiple strains of Ad2, Ad40, and Ad41 is necessary with both LP- and MP-UV systems to determine whether they respond differently to monochromatic and polychromatic UV radiation.

Lagrangian actinometry based on DMS fluorescence measurements should be considered as a practical method for UV reactor validation. However, further work should be conducted to refine the approach and better define the method’s uncertainty. In addition, alternative formulations of DMS that are responsive to both LP- and MP-UV should be investigated.

Utilities, regulators, and public health departments should be engaged in a discussion on the potential public health and public relations issues regarding full-scale reactor validation with challenge organisms and non-biological dosimeters. The outcome should be a process that is protective of public health but allows full-scale testing with surrogates that do not pose a risk to public health, and should include modeling the likelihood of introduced microbes or other dosimeter particles reaching the consumer.

**RESEARCH PARTNER**

U.S. Environmental Protection Agency
CHAPTER 1
INTRODUCTION

REGULATORY BACKGROUND

Ultraviolet (UV) radiation is established as an important primary disinfection process that is effective against a wide range of potentially waterborne pathogens. The application of germicidal UV wavelengths to water inactivates microbes by inducing the formation of dimers in adjacent thymine nucleotides. This destroys the ability of the microbe to reproduce. The photochemistry and biological response of organisms and nucleic acids to UV radiation have been thoroughly researched and well documented (Nickoloff and Hoekstra 1998). UV has been used for many years in relatively small-scale applications to disinfect water intended for pharmaceutical, medical, and food preparation purposes. As part of an overall multi-barrier approach, UV is an effective treatment for control of microbial pathogens in drinking water. Thousands of wastewater treatment plants and hundreds of drinking water treatment plants around the world use UV disinfection as one of their treatment regimes and it has gained acceptance as a disinfectant for drinking water because it is very effective for inactivating many pathogens, including the chlorine resistant protozoan Cryptosporidium spp. (Mofidi et al. 2001; Rochelle et al. 2004). In addition, at the doses typically used to disinfect water, it does not produce disinfection by-products. However, unlike chlorine-based disinfectants it provides no residual disinfection, so chemical disinfectants still need to be used to maintain water quality in the distribution system. The effectiveness of UV disinfection plants is determined by a variety of factors, including the characteristics of the water (suspended solids, color, turbidity), the type and intensity of UV radiation, the configuration of the UV reactor, and the length of time that microbes are exposed to the UV radiation. The world’s largest drinking water UV disinfection plant is currently under construction by the city of New York. The plant will supplement the existing chlorination facility that treats unfiltered Catskill/Delaware supplies. When completed in 2010, the plant will have a capacity of 2.4 billion gallons per day and provide 3-log inactivation of Cryptosporidium, to comply with the Long Term 2 Enhanced Surface Water Treatment Rule (LT2ESWTR; USEPA 2006a).

A report by U.K. Water Industry Research concluded that the energy used by an appropriately designed and operated UV treatment plant would be comparable to, or less than, the energy required by alternative treatment approaches such as ozonation or membrane filtration for Cryptosporidium control (Camm et al. 2008). Consequently, energy usage alone should not be used as justification for discounting UV disinfection as a practical treatment option. Few countries have adopted formal regulations governing the application of UV disinfection to drinking water. In Austria, a dose of 45 ml/cm² is required for municipal water supplies. However, many countries and organizations have issued guidelines and recommendations, with UV doses ranging from 16 to 40 ml/cm² (Masschelein 2002). For many years, the National Sanitation Foundation (NSF) in the United States required a dose of 38 ml/cm² for certification of UV treatment units, which was revised in 2004 to 40 ml/cm² to be consistent with the widely accepted effective dose. This dose provides 3 – 4 log inactivation of most waterborne pathogens.

The intent of the LT2ESWTR is to reduce the incidence of waterborne cryptosporidiosis in the U.S. (USEPA 2006a). The rule includes UV disinfection as a treatment option for those utilities whose source waters are vulnerable to Cryptosporidium contamination. While
Cryptosporidium spp. and Giardia spp.

are readily inactivated by UV radiation, as evidenced by the LT2ESWTR dose requirements, disinfection credits for viruses require much higher doses (186 mJ/cm² for 4-log inactivation; Table 1.1). Based on validation factors described in the USEPA’s UV Disinfection Guidance Manual (UVDGM; USEPA 2006b), the dose for 4-log inactivation of viruses may be as high as 300 mJ/cm² to achieve a validated dose for a particular reactor. UV dose requirements for virus inactivation in the Ground Water Rule (GWR) are the same as those in the LT2ESWTR (USEPA 2006a, 2006c).

**UV Inactivation of Viruses**

Most bacterial and protozoal pathogens of concern to the water industry are easily inactivated by UV radiation, with doses less than 20 mJ/cm² providing at least 3-log inactivation. Although most viruses are typically more resistant to UV, 4-log inactivation can be achieved with UV doses of <40 mJ/cm² (Table 1.2). Based on a review of published low-pressure (LP) UV dose response data, the inactivation rate constants for poliovirus, rotavirus, caliciviruses, hepatitis A virus, and coxsackieviruses were 0.10 – 0.19 cm²/mJ, with maximum inactivation of 4.1 – 5.7 log at UV doses of 28 – 50 mJ/cm² (Hijnen et al. 2006). The high dose requirement for virus inactivation in the LT2ESWTR and GWR was driven by the much higher UV resistance of adenoviruses. With the possible exception of *Acanthamoeba* spp. (Maya et al. 2003), adenoviruses display the highest levels of UV-resistance of any potentially waterborne pathogens so far investigated, with LP-UV doses as high as 150 mJ/cm² necessary to achieve 3-log inactivation, according to some studies (Figure 1.1). This apparent UV resistance is due to host cell-mediated repair of UV damaged adenovirus DNA inside the cells used to quantify virus survival (Day 1974a,b). Therefore, adenoviruses are currently the restrictive organisms in determining future design and dose delivery of UV-based drinking water treatment processes. However, a recent study demonstrated that medium-pressure (MP) UV was much more effective than LP-UV for inactivating adenoviruses (Linden et al. 2007). Approximately 4.3-log inactivation of adenovirus type 2 (Ad2) was achieved with a full-spectrum MP-UV dose of 40 mJ/cm² and 30 – 40 mJ/cm² resulted in 3-log inactivation of adenovirus type 40 (Ad40). These findings may have important consequences for future applications of UV in drinking water treatment.

### Table 1.1

**Regulatory inactivation credits for UV disinfection**

<table>
<thead>
<tr>
<th>Log inactivation credit</th>
<th>UV dose required (mJ/cm²)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Giardia</strong></td>
<td><strong>Cryptosporidium</strong></td>
</tr>
<tr>
<td>1.0</td>
<td>2.1</td>
<td>2.5</td>
</tr>
<tr>
<td>1.5</td>
<td>3.0</td>
<td>3.9</td>
</tr>
<tr>
<td>2.0</td>
<td>5.2</td>
<td>5.8</td>
</tr>
<tr>
<td>2.5</td>
<td>7.7</td>
<td>8.5</td>
</tr>
<tr>
<td>3.0</td>
<td>11</td>
<td>12</td>
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<td>3.5</td>
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<td>15</td>
</tr>
<tr>
<td>4.0</td>
<td>22</td>
<td>22</td>
</tr>
</tbody>
</table>

*Source: USEPA 2006a.*
<table>
<thead>
<tr>
<th>Virus</th>
<th>UV dose (mJ/cm²)</th>
<th>Conditions*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine calicivirus</td>
<td>23</td>
<td>Pre-ozonated treated water</td>
<td>Malley et al. 2004</td>
</tr>
<tr>
<td>Feline calicivirus</td>
<td>29 – 36</td>
<td>Ground water and buffered water</td>
<td>Thurston-Enriquez et al. 2003</td>
</tr>
<tr>
<td>Feline calicivirus</td>
<td>~33</td>
<td>Tap water</td>
<td>De Roda Husman et al. 2004</td>
</tr>
<tr>
<td>Feline calicivirus</td>
<td>~45</td>
<td>UV-B, sterile distilled water</td>
<td>Duizer et al. 2004</td>
</tr>
<tr>
<td>Canine calicivirus</td>
<td>~33</td>
<td>Tap water</td>
<td>De Roda Husman et al. 2004</td>
</tr>
<tr>
<td>Canine calicivirus</td>
<td>~45</td>
<td>UV-B, sterile distilled water</td>
<td>Duizer et al. 2004</td>
</tr>
<tr>
<td>Coxsackievirus B3</td>
<td>33</td>
<td>Phosphate buffered saline</td>
<td>Gerba et al. 2002</td>
</tr>
<tr>
<td>Coxsackievirus B5</td>
<td>36</td>
<td>Phosphate buffered saline</td>
<td>Gerba et al. 2002</td>
</tr>
<tr>
<td>Coxsackievirus B5</td>
<td>29</td>
<td>Phosphate buffered water</td>
<td>Battigelli et al. 1993</td>
</tr>
<tr>
<td>Coxsackievirus B4</td>
<td>~25</td>
<td>Coagulated, filtered surface water</td>
<td>Shin et al. 2005</td>
</tr>
<tr>
<td>Echovirus 1,2</td>
<td>28 – 33</td>
<td>Phosphate buffered saline</td>
<td>Gerba et al. 2002</td>
</tr>
<tr>
<td>Echovirus 1</td>
<td>21</td>
<td>Pre-ozonated treated water</td>
<td>Malley et al. 2004</td>
</tr>
<tr>
<td>Hepatitis A virus</td>
<td>16</td>
<td>Phosphate buffered water</td>
<td>Battigelli et al. 1993</td>
</tr>
<tr>
<td>Murine norovirus</td>
<td>~27</td>
<td>Phosphate buffered saline</td>
<td>Lee et al. 2008</td>
</tr>
<tr>
<td>Poliovirus 1</td>
<td>31</td>
<td>Phosphate buffered saline</td>
<td>Gerba et al. 2002</td>
</tr>
<tr>
<td>Poliovirus</td>
<td>22</td>
<td>Sterile distilled water</td>
<td>Meng and Gerba 1996</td>
</tr>
<tr>
<td>Rotavirus SA-11</td>
<td>42</td>
<td>Phosphate buffered water</td>
<td>Battigelli et al. 1993</td>
</tr>
<tr>
<td>Rotavirus SA-11</td>
<td>61</td>
<td>Pre-ozonated treated water</td>
<td>Malley et al. 2004</td>
</tr>
<tr>
<td>Rotavirus SA-11</td>
<td>39 – 117†</td>
<td>Phosphate buffered saline</td>
<td>Li et al. 2009</td>
</tr>
</tbody>
</table>

* All microorganisms were exposed to low pressure UV irradiation unless indicated otherwise.
† Dose for 4-log inactivation depended on methods used to assay the virus.
Challenge Organisms for Inactivation of Viruses by Ultraviolet Treatment

**Figure 1.1 Inactivation of adenoviruses by UV radiation.** Inactivation data were generated using low-pressure UV (open symbols) or medium-pressure (MP) UV lamps (filled symbols). Data sources: Ballester and Malley 2004; Baxter et al. 2007; Day 1974a and 1974b; Gerba et al. 2002; Jacangelo et al. 2002; Ko et al. 2005; Linden et al. 2007, 2009; Malley et al. 2004; Meng and Gerba 1996; Nwachuku et al. 2005; Thompson et al. 2003; Thurston-Enriquez et al. 2003. The dashed line represents the LT2ESWTR dose requirements for virus inactivation (USEPA 2006a).

There is considerable variation in the published UV response data for some viruses, within individual reports and between studies. For example, Li et al. (2009) reported 3-log inactivation of rotavirus SA-11 with a LP-UV dose of 29 mJ/cm² when virus infectivity was measured using cytopathic effects (CPE) as the cell culture end point assay. However, when cell culture was combined with RT-PCR to quantify surviving viruses, a dose of 87 mJ/cm² was needed for 3-log inactivation. For adenoviruses, there is up to 2-log difference between studies in the amount of inactivation at a particular UV dose for individual viruses, and the LP-UV dose required for 3-log inactivation of adenovirus type 41 (Ad41) varies from 80 to 175 mJ/cm² (Figure 1.1).

There is little consistency between published studies on UV inactivation of adenoviruses (Table 1.3) but the variation in reported UV responses are probably due to a combination of the following factors: normal inter-laboratory variation due to differences in overall laboratory operation; variability inherent in virus-cell culture systems; variation in sensitivity to infection between different cell lines; different assay end-points used to quantify surviving viruses; different post-inoculation incubation periods; different types of UV systems; and difficulties in accurately measuring the delivered UV dose.
### Table 1.3
Assessing UV inactivation of adenoviruses

<table>
<thead>
<tr>
<th>Adenovirus type</th>
<th>Host cells</th>
<th>Incubation period</th>
<th>Assay end-point</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad2</td>
<td>Human fibroblasts</td>
<td>17 days</td>
<td>Plaque</td>
<td>Day 1974</td>
</tr>
<tr>
<td></td>
<td>PLC/PRF/5</td>
<td>24 days</td>
<td>CPE/MPN</td>
<td>Gerba et al. 2002</td>
</tr>
<tr>
<td>Ad2</td>
<td>A549</td>
<td>7 days</td>
<td>RT-PCR</td>
<td>Ko et al. 2003</td>
</tr>
<tr>
<td></td>
<td>A549</td>
<td>21 days</td>
<td>Plaque</td>
<td>Thompson et al. 2003</td>
</tr>
<tr>
<td>Ad2</td>
<td>A549</td>
<td>10 days</td>
<td>CPE/TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Ballester and Malley 2004</td>
</tr>
<tr>
<td>Ad2</td>
<td>A549</td>
<td>14 days</td>
<td>CPE/TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Linden et al. 2007, 2009</td>
</tr>
<tr>
<td>Ad40</td>
<td>PLC/PRF/5</td>
<td>21 days</td>
<td>CPE/TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Meng and Gerba 1996</td>
</tr>
<tr>
<td>Ad40</td>
<td>PLC/PRF/5</td>
<td>24 days</td>
<td>CPE/MPN</td>
<td>Thurston-Enriquez et al. 2003</td>
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<tr>
<td>Ad40</td>
<td>PLC/PRF/5</td>
<td>10 days</td>
<td>CPE/TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Malley et al. 2004</td>
</tr>
<tr>
<td>Ad40</td>
<td>PLC/PRF/5</td>
<td>24 days</td>
<td>CPE/MPN</td>
<td>Linden et al. 2007</td>
</tr>
<tr>
<td>Ad41</td>
<td>G293</td>
<td>7 days</td>
<td>RT-PCR</td>
<td>Ko et al. 2003</td>
</tr>
<tr>
<td>Ad41</td>
<td>PLC/PRF/5</td>
<td>21 days</td>
<td>CPE/MPN</td>
<td>Meng and Gerba 1996</td>
</tr>
<tr>
<td>Ad41</td>
<td>HEK293</td>
<td>7 days</td>
<td>RT-PCR/MPN</td>
<td>Ko et al. 2005</td>
</tr>
</tbody>
</table>

### BIOLOGY, HEALTH EFFECTS, AND OCCURRENCE OF ADENOVIRUSES

Adenoviruses comprise a large group of non-enveloped, icosahedral viruses that are widespread in birds (genus Aviadenovirus) and many mammals (genus Mastadenovirus). Human adenoviruses are classified into six groups or “species” (A – F) comprising 51 serotypes. A recently isolated human adenovirus (Ad52) has been proposed as a seventh species, G (Jones et al. 2007). Adenoviruses are 70 – 110 nm in diameter with a linear double-stranded DNA (dsDNA) genome approximately 35 kb long. The virus capsid contains three types of protein: 12 fiber attachment proteins and their associated penton base proteins, and 240 hexon proteins. They enter cells by receptor-mediated endocytosis, and DNA replication and mRNA synthesis occur in the host cell nucleus.

Adenoviruses cause a broad spectrum of acute and chronic diseases with varying severity in immune-competent and immune-compromised humans with syndromes including non-specific enteric infection (group A), respiratory illness that can be acute and result in pneumonia (groups B, C, and E), conjunctivitis (group D), and gastroenteritis, primarily associated with infants (group F; Foy 1997; Horwitz 1990). Adenovirus infections are spread by aerosolized droplets reaching the upper airways and conjunctiva (eyes), by the fecal-oral route, or nosocomially. Infection is considered endemic in children. The incidence of adenovirus infection peaks in children aged six months to five years but most infections are sub-clinical or result in self-limiting mild gastrointestinal or respiratory illness. However, as with many infectious agents,
they can cause more severe disease in immune-compromised individuals, including acute hemorrhagic cystitis, hepatitis, myocarditis, meningoencephalitis, nephritis, pneumonia, and pertussis-like syndrome. After primary infection the virus may remain latent for prolonged periods in various tissues and fluids. They are increasingly recognized as a cause of significant morbidity and mortality in bone marrow transplant patients, particularly children, due to disseminated infection. While groups B and C are typically associated with disseminated disease, Ad41, which was previously thought to only cause gastroenteritis, has also caused fatal disseminated infection (Slatter et al. 2005). Some adenoviruses in species A and B are oncogenic in selected animal models but have not been associated with cancer in humans. Three human adenoviruses (Ad5, Ad36, and Ad37) are associated with obesity in animals and Ad36 infection may be related to obesity in some humans (Atkinson 2007). In addition, Ad7 has been associated with diseases of the central nervous system.

Outbreaks of adenoviral respiratory disease are often associated with crowded conditions such as military barracks and boarding schools. In the civilian population, the most prevalent adenoviruses causing clinical infections during a two-year survey were Ad3 (35% prevalence), Ad2 (24%), Ad1 (18%), and Ad5 (5%; Gray et al. 2007). Among military trainees, Ad4 (93% prevalence), Ad3 (2.6%), and Ad21 (2.4%) were most prevalent. Out of 764 adenovirus isolates that were sequenced as part of a national surveillance program, only 0.9% were identified as Ad41 and none were identified as Ad40.

Although Ad40 and Ad41 are frequently referred to as the enteric adenoviruses, most human adenoviruses are shed in feces, regardless of the site of infection, so the fecal-oral route may play a role in transmission of all adenoviruses. Other, non-F species adenoviruses also cause gastroenteritis. Ad12 (species A) caused an outbreak of diarrhea with 56% mortality among transplant patients (Jalal et al. 2005) and Ad18 and Ad31 have been isolated from gastroenteritis patients. A recently identified serotype, Ad52, was isolated from a patient with gastroenteritis (Jones et al., 2007). Although sequence analysis of three genes indicated that it was more closely related to species F adenoviruses (Ad40 and Ad41) than other human adenoviruses, the authors proposed a new species (G) containing Ad52 and a simian adenovirus.

Some studies indicate relatively high levels of adenovirus occurrence in environmental waters although results are often based on PCR detection and not the production of cytopathic effects in cell culture. They are common in raw sewage and are relatively stable in aquatic environments (Enriquez 2002). Human adenoviruses were detected in 81.5% of raw sewage samples (Komninou et al. 2004) but although they are frequently detected in untreated wastewater, they are less prevalent and present at much lower concentrations than reoviruses or enteroviruses (Sedmak et al. 2005). Adenoviruses belonging to species A, C, D, and F were detected by cell culture coupled with RT-PCR in 75% of surface waters impacted by domestic and industrial wastewaters (Lee et al. 2004). Direct PCR without cell culture detected adenovirus DNA in 16 – 45% of river water samples (Choi and Jiang 2005; Fong et al. 2005; Haramoto et al. 2005). Direct nested PCR on concentrated water samples detected adenovirus DNA in 22% and 5% of raw and treated water samples, respectively (Van Heerden et al. 2005). Based on sequences of amplified hexon gene fragments, the majority of the human adenovirus detected in treated drinking water were types 2, 40 and 41. Cell culture coupled with PCR detected human adenoviruses in 13% of raw water and 4.4% of treated water samples (van Heerden et al. 2003). Infectious Ad40/41 were detected by integrated cell culture RT-PCR on BGMK cells in 38% of untreated surface water samples (Chapron et al. 2000). Quantitative real-time PCR detected human adenovirus DNA in all tested river water samples and in all GAC-filtered samples from a
drinking water treatment plant using chlorine and ozone disinfection, although the concentration was approximately 100-fold lower than in untreated water (Albinana-Gimenez et al. 2006). Although, human adenoviruses were detected by real-time PCR in 16% of urban river samples, no infectious adenoviruses were detected using a virus plaque assay on HEK-293A and A549 cells (Choi and Jiang 2005), suggesting that the majority of adenoviruses in raw waters are non-infectious, the real-time PCR assay had a high proportion of false-positives, or the human adenoviruses present in the samples were unable to form plaques in the particular assay used.

A few outbreaks of adenovirus infection have been associated with recreational water and a waterborne outbreak with 595 cases of conjunctivitis and pharyngitis was linked to a contaminated lake (Craun et al. 2003). Adenovirus types 3 and 4 have also been associated with outbreaks linked to recreational use of water (D’Angelo et al. 1979; Martone et al. 1980) and enteric adenoviruses were identified as one of the agents causing acute gastroenteritis in a waterborne outbreak (Kukkula et al. 1997). Because adenoviruses are detected in water and can cause adverse health effects, they have been listed on all of the USEPA Candidate Contaminant Lists (CCL), including the recent CCL3 (USEPA, 2008). However, although they have been associated with waterborne outbreaks of gastroenteritis, adenoviruses are usually recovered from the implicated water along with a host of other pathogens, such as norovirus, rotaviruses, and bacterial pathogens. Consequently, definitively linking adenoviruses with waterborne disease outbreaks is difficult.

Although highly resistant to UV disinfection, adenoviruses are readily controlled by most conventional chemical disinfectants such as chlorine, chlorine dioxide, and ozone (Table 1.4). Chloramines are less effective. Some authors have suggested sequential disinfection as the most appropriate option for controlling adenoviruses in drinking water. For example, a UV dose of 40 mJ/cm² followed by a chloramine CT of 27 mg-min/L resulted in 4-log inactivation of Ad2 (Ballester and Malley 2004).
Table 1.4
Chemical disinfection of adenoviruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Disinfectant, CT (mg-min/L)</th>
<th>Conditions</th>
<th>Inactivation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad2</td>
<td>chlorine, 0.27</td>
<td>pH 8, 5°C</td>
<td>4-log</td>
<td>Cromeans et al. 2010</td>
</tr>
<tr>
<td>Ad40</td>
<td>chlorine, &lt;0.04</td>
<td></td>
<td>4-log</td>
<td></td>
</tr>
<tr>
<td>Ad41</td>
<td>chlorine, &lt;0.03</td>
<td></td>
<td>4-log</td>
<td></td>
</tr>
<tr>
<td>Ad2</td>
<td>monochloramine, 990</td>
<td></td>
<td>2-log</td>
<td></td>
</tr>
<tr>
<td>Ad40</td>
<td>monochloramine, 360</td>
<td>pH 8, 5°C</td>
<td>2-log</td>
<td></td>
</tr>
<tr>
<td>Ad41</td>
<td>monochloramine, 190</td>
<td>pH 8, 5°C</td>
<td>2-log</td>
<td></td>
</tr>
<tr>
<td>Ad5, Ad41</td>
<td>chlorine, 0.22</td>
<td>pH 8.5, 5°C</td>
<td>4-log</td>
<td>Baxter et al. 2007</td>
</tr>
<tr>
<td>Ad5, Ad41</td>
<td>chloramine, 350</td>
<td>pH 8.5, 5°C</td>
<td>2.5-log</td>
<td></td>
</tr>
<tr>
<td>Ad2</td>
<td>chloramine, 100</td>
<td>pH 8, 25°C</td>
<td>4-log</td>
<td></td>
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<tr>
<td>Ad2</td>
<td>chlorine, 1.22</td>
<td>pH 8, 5°C</td>
<td>3.7-log</td>
<td>Ballester and Malley 2004</td>
</tr>
<tr>
<td>Ad2</td>
<td>chloramine, 265</td>
<td>pH 8, 5°C</td>
<td>1.2-log</td>
<td></td>
</tr>
<tr>
<td>Ad40</td>
<td>chlorine, 0.24</td>
<td>pH 8, 5°C</td>
<td>4-log</td>
<td>Thurston-Enriquez et al. 2003</td>
</tr>
<tr>
<td>Ad40</td>
<td>chloramine dioxide, &lt;0.12 – 1.59</td>
<td>pH 6 – 8, 5 – 23°C</td>
<td>4-log</td>
<td>Thurston-Enriquez et al. 2005a</td>
</tr>
<tr>
<td>Ad40</td>
<td>ozone, 0.07 – 0.6</td>
<td>pH 7, 5°C</td>
<td>4-log</td>
<td>Thurston-Enriquez et al. 2005b</td>
</tr>
<tr>
<td>Ad40</td>
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</tr>
<tr>
<td>Ad40</td>
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<td>pH 7, 5°C</td>
<td>2-log</td>
<td></td>
</tr>
<tr>
<td>Ad40</td>
<td>chloramines, 60 – 120 [3:1]</td>
<td>pH 7, 19°C</td>
<td>2-log</td>
<td></td>
</tr>
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<td>Ad40</td>
<td>chloramines, 20 [7:1]</td>
<td>pH 7, 19°C</td>
<td>2-log</td>
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</tr>
<tr>
<td>Ad40</td>
<td>ozone, &lt;0.5</td>
<td>pH 7, 5°C</td>
<td>2-log</td>
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</tbody>
</table>

*Biodosimetry*

Regular system maintenance and correct dose monitoring are critical for continuous and effective performance of UV disinfection systems. Systems should be installed, operated, and validated using standardized procedures, within their intended normal operating ranges, to achieve adequate UV dose with a safety margin. The delivered UV dose distribution depends on the UV intensity, the water flow rate, and the UV transmittance of the water. One of the challenges of UV for drinking water treatment is the difficulty in measuring the UV dose distribution delivered by a reactor or its actual inactivation efficacy against pathogens during full-scale operation. Consequently, methods are needed to validate drinking water treatment plants that employ UV disinfection to ensure they are delivering UV doses that are protective of public health. Since pathogens cannot easily be used for full-scale validation, these methods typically involve inactivation of non-pathogenic challenge organisms or non-biological...
approaches such as chemical or particle actinometry. The single-stranded RNA bacteriophage MS2 is frequently used as a biodosimetry challenge organism for reactor validation because it is non-pathogenic, considerably more UV resistant than many bacterial, viral, or protozoan pathogens, and can be grown to high titers. Biodosimetry is used to calculate the dose delivered by a UV reactor based on inactivation of the challenge organism in the reactor compared to the response of the microbe in a bench-scale collimated beam test. However, UV reactors produce dose distributions rather than a single UV dose. Due to these dose distributions, the UV dose measured by a challenge microbe may be different from the UV dose that would be measured with the target pathogen. This source of uncertainty is addressed in the UVDGM by incorporating a safety factor termed the reduction equivalent dose (RED) bias (USEPA 2006b). The UVDGM includes testing protocols for using surrogates for validating reactors, and defines the 90% prediction limits for the UV response in collimated beam tests for bacteriophage MS2 and *Bacillus subtilis* spores. However, MS2 and *B. subtilis* spores are more sensitive to UV radiation than adenoviruses, thereby limiting their utility for reactor validation at the high doses required for virus inactivation credit. A low-pressure UV dose of 60 mJ/cm² results in approximately 3-log inactivation of both organisms compared to at least 100 mJ/cm² to achieve 3-log inactivation of adenoviruses. There is currently no testing protocol or suitably resistant surrogate for validating reactors at the high doses (186 mJ/cm²) required for 4-log virus inactivation.

A single UV inactivation surrogate is not feasible due to the widely varying responses of microorganisms to UV radiation. Therefore, to ensure adequate protection of public health where UV is applied for disinfection of viruses, it is necessary to select surrogates whose response to UV allows characterization of reactor-applied doses needed for inactivation of the most resistant viral pathogens. Inactivation of human viruses and bacteriophages by UV irradiation cannot be predicted by the type and size of the virus or the type of the genome (Shin et al. 2005). In addition, analysis of regions of the *Deinococcus radiodurans* genome suggests that a high GC content is not a prerequisite for radiation resistance (Lio and Vannucci 2000). Therefore, surrogate microbes can only be selected based on empirical testing and comparison with the target pathogen. The ideal characteristics of a challenge organism for UV reactor validation include:

- Non-pathogenic
- Growth to high concentrations
- Easily enumerated in a growth or infectivity assay
- Effectively model the reactor’s disinfection efficiency toward the target pathogen
- Minimal shoulder (lag) or tailing of the dose response curve
- Stable for relatively long periods of time with minimal die off
- Robust under typical testing conditions

There are a surprising variety of bacteria that display high tolerance to ionizing radiation at doses 100 – 1,000 times higher than can be tolerated by most microorganisms. These include *Deinococcus* spp., *Rubrobacter radiotolerans*, *Thermococcus gammatolerance*, and the cyanobacterium *Chroococcidiopsis* (Billi et al. 2000; Ferreira et al. 1999; Jolivet et al. 2003). Ionizing radiation causes double-stranded DNA breaks, which are efficiently repaired by these
bacteria. Since they have efficient repair systems, most bacteria that are resistant to ionizing radiation are also resistant to high doses of UV radiation.

NON-BIOLOGICAL DOSIMETERS

Non-biological approaches to reactor validation include chemical and Lagrangian Actinometry (LA). Chemical actinometers undergo a radiation-induced reaction at certain wavelengths, for which the quantum yield is known. The photochemical conversion, which can be degradation of substrate or production of a derivative, is directly related to the number of photons absorbed. Measuring the reaction rate allows calculation of the absorbed photon flux. Examples of chemical actinometers include the nucleoside uridine with an absorbance peak at 262 nm and potassium iodide/iodate, which absorbs all wavelengths similarly in the 200 – 280 nm region. Potassium iodide/iodate measures incident fluence whereas uridine measures germicidal fluence (Jin et al. 2006).

A potential limitation of biodosimeters is that they measure an average UV dose. However, due to the range of fluid travel paths within a reactor vessel and variations in UV intensity, continuous flow UV reactors produce a distribution of UV doses rather than an average dose. Consequently, methods have been developed to measure dose distributions to allow improved characterization of reactor performance. For the LA method, dyed microspheres (DMS) that are responsive to UV radiation have been proposed as suitable validation surrogates. All successful applications of LA to date have involved a type of DMS that comprises a photosensitive dye attached to synthetic microspheres. Upon exposure to germicidal UV radiation, the non-fluorescent parent compound (dye) undergoes a permanent photochemical change to yield a brightly fluorescent photoproduct. The fluorescence intensity of an exposed particle can then be related to the UV dose it has been subjected to. Injection of a large population of these DMS into a UV reactor allows measurement of the dose distribution delivered by the reactor (Blatchley et al. 2006, 2008; Shen et al. 2007). LA-derived dose distribution data can be used to develop quantitative predictions of reactor behavior. Knowledge of the UV dose distribution allows for accurate prediction of the performance of a UV reactor relative to any photochemical or photobiological endpoint for which accurate photochemical kinetics (or UV dose-response behavior) are known. Consequently, LA has been proposed as a method for reducing uncertainty in prediction of reactor performance with the ability to predict reactor behavior relative to many photochemical endpoints.

Methods using computational fluid dynamics (CFD) have also been developed for predicting UV doses delivered by UV reactors (Mofidi et al. 2004). These methods use coupled models describing turbulent flow, microbial transport, UV intensity, and microbial inactivation.

MECHANISMS OF UV INACTIVATION AND REPAIR

The heterocyclic ring structures of nucleic acids absorb radiation with a maximum near 260 nm and so DNA damage is usually considered the most important effect of an organism’s exposure to UV radiation. Depending on the amount of UV exposure and various properties of the organism, this damage may be repaired with no long term consequences for the organism, silent or deleterious mutations may be formed, or the organism may die as a result of UV exposure. The primary type of UV-induced DNA damage is the generation of photoproducts between adjacent bases. The major photoproducts are cyclobutane pyrimidine dimers (CPDs)
between adjacent thymine residues (thymine dimers) and a pyrimidine-pyrimidone product (6-4 photoproduct; Rothschild 1999). Both types of DNA damage are mutagenic and highly deleterious because they prevent passage of both DNA replication and transcriptional machinery. Therefore, a cell or virus with a UV-induced damage cannot express the genetic information in the region of the genome affected, nor can it accurately copy its DNA and divide. Such a cell or virus may die or lose infectivity, or if it survives, become altered and begin to accumulate genetic changes. Additional UV-induced damage includes: hydroxylation of cytosine and uracil, cytosine-thymine dimers, cross-linking between DNA and proteins, inter-strand cross-linking of DNA, and chain breakage or denaturation of DNA (Rothschild 1999). However, many prokaryotic and eukaryotic organisms have DNA repair mechanisms that reduce or eliminate UV-induced damage. These repair mechanisms include: nucleotide excision repair (NER) mediated by a complex of 15 – 20 proteins; photoreactivation mediated by light activated photolyase enzymes; UV-specific endonucleases; and base excision repair mediated by CPD-specific glycosylases. In addition, some organisms also have the ability to delay cellular processes once DNA damage is detected. These cell cycle delays are mediated by checkpoint genes and they increase a cell’s chances of surviving DNA damage by allowing time for the repair proteins to operate prior to DNA replication (Weinert and Lydall 1998). With some exceptions such as bacteriophage T4 and Mimivirus (Kemper 1998; Raoult et al. 2004), viruses do not encode DNA repair functions. Instead, they are dependent on their host cells to provide DNA repair mechanisms, such that, even if UV-induced damage in virus DNA is reparable, if the virus is plated on repair-deficient cells, the virus will not be able to replicate or cause cytopathic effects in these cells.
CHAPTER 2
MATERIALS AND METHODS FOR MICROBIAL ANALYSES

MICROBIAL CULTURE AND ENUMERATION TECHNIQUES

All of the organisms used in this study, along with their respective sources, types of genome, and basic culturing conditions are listed in Table 2.1.

Bacteriophages

Bacteriophages MS2 and PP7 are structurally and genetically similar single stranded RNA (ssRNA) phages with different hosts. Phages PRD1, Bam35, and PBCV-1 are structurally similar to adenoviruses in overall architecture, vertex recognition spikes, and possessing double stranded DNA (dsDNA) genomes with inverted terminal repeats and terminal proteins. The PRD1 major coat protein is similar to the major hexon coat protein of human adenoviruses (Benson et al. 1999). Although their genome sequences are distinct these dsDNA viruses may share a common evolutionary ancestor (Ravantti et al. 2003) and adenoviruses may have evolved from a virus very similar to PRD1. PRD1 has a relatively high GC content and its UV response has been studied previously but the two reports provide very different results, one indicating marked sensitivity while the second demonstrated increased resistance (Meng and Gerba 1996; Shin et al. 2005). Structurally similar, Bam35 infects a Gram-positive host and PBCV-1 infects a eukaryotic host, possibly making it more analogous to adenoviruses. PM2 is also structurally similar to PRD1, using different mechanisms for host infection. The DNA of bacteriophage Phi29 is linked to a terminal protein which circularizes it and initiates replication using a mechanism of strand displacement and starting simultaneously from either end of the DNA, as in adenoviruses (Salas et al. 1995). The response of phage T7 to UV radiation has been investigated previously and demonstrated extreme sensitivity, similar to Cryptosporidium (Fallon et al. 2007). However, in this earlier study it was plated on a strain of E. coli (ATCC 11303) derived from E. coli strain B that is UV sensitive. This strain has a mutation (lon-) that causes cells to elongate and inhibits cell division in response to recognition of UV-induced damage. Consequently, once the host’s DNA repair proteins recognize UV damaged DNA, the cells elongate and die. Thus the UV sensitivity of T7 may have been a reflection of the UV sensitivity of the host E. coli. Also, T7 displays host cell reactivation when assayed on repair proficient cells (Chiang and Harm 1976). Bacteriophage T4 has a low GC content (35%) and so may be more susceptible to UV-induced damage. However, it is unique among the coliphages because it encodes a DNA repair endonuclease (T4 endonuclease V) that can repair damaged DNA in both itself and its host cells (Kemper 1998).

Bacteriophage MS2 was propagated in E. coli host strain ATCC 15597. For initial collimated beam tests, 1 mL of a 4 – 6 h tryptone-glucose-yeast extract (TGY) broth culture of E.coli 15597 and 1 mL of an MS2 stock suspension containing $10^8$ plaque forming units (pfu) were added to 3 mL of melted TGY agar in a 10 mL glass test tube, mixed by vigorously rolling the tube, and poured onto the surface of a TGY agar plate. Plates were incubated for 18 h at 37°C. MS2 particles were harvested by flooding the plates with 5 mL of saline-calcium buffer and incubating at 21°C for 30 min. The resulting suspension from multiple plates was aspirated into 30 mL centrifuge tubes and centrifuged at 10,000×g for 17 min at 4°C. Disposable syringe
filters were pre-treated by passing 10 mL of TGY broth through them and then used to filter the supernatant containing MS2 particles. The filtrates from approximately 15 agar plates were combined and stored at 4°C. For large-scale experiments, two milliliters of an overnight culture of *E. coli* 15597 were inoculated into 500 mL of pre-warmed TGY broth in a 2 L flask and incubated for 4 – 6 h at 37°C in a shaking incubator at 125 rpm. This culture was inoculated with 1 mL of a 10^4 dilution of MS2 stock suspension and incubated for 18 h at 37°C without shaking. MS2 particles were harvested by adding 3.4 mL of chloroform to 170 mL of the broth culture, inverting the tube 10 times to mix and lyse the host cells, and centrifuging at 10,000×g for 17 min at 4°C. The supernatants containing MS2 particles from multiple broth cultures were transferred into a sterile glass bottle and stored at 4°C.

Other bacteriophages were propagated in their respective host organisms (Table 2.1) grown in nutrient-rich media and incubated at 28°C (PM2) or 37°C (all other bacteriophages). Bacteriophage stocks were prepared by inoculating approximately 1 × 10^7 pfu of purified phage particles into 500 mL of a 6 h culture of host bacteria and incubating at 28 – 37°C for 18 h. Bacteriophage particles were harvested by addition of chloroform (2% final concentration), mixing by inversion, and centrifugation for 15 min at 10,000×g and 4°C. Phage titers were typically 0.01 – 1 × 10^12 pfu/mL. When stored at 4°C, MS2 and most of the other bacteriophages were stable for at least a month. However, Bam35 was used within two weeks of propagation because it is unstable and rapidly loses viability at 4°C (Ackermann et al. 2004). For assaying phages by a quantitative agar overlay plaque method, UV-irradiated and non-exposed samples were diluted in saline-calcium buffer to a total volume of 4 mL, mixed with 1 mL of a 4 h broth culture of host bacteria and melted TGY top agar, and poured onto triplicate TGY agar plates. Plaques were enumerated following 18 h incubation at 37°C.

**Eukaryotic Viruses**

*Paramecium bursaria* Chlorella virus 1 (PBCV-1; genus *Chlorovirus*, family *Phycodnaviridae*) is a double-stranded DNA virus that infects certain types of unicellular chlorella-like green algae. It is a large, icosahedral virus that forms plaques in lawns of host algae. It has a linear 330 kb genome and carries an endonuclease gene that repairs UV-damaged DNA (Furuta et al. 1997). The algal host for PBCV-1 is Chlorella NC64A, which is an endosymbiont of the protozoan *Paramecium bursaria*. The host strain Chlorella spp. NC64A and the virus PBCV-1 were provided by Dr. James van Etten (University of Nebraska, Lincoln). Virus stocks were prepared by culturing in Chlorella sp. NC64A maintained in modified Bold’s basal medium (MBBM) containing 10 µg/mL of tetracycline at 25°C in an illuminated incubator. After 72 h incubation cultures were centrifuged at 5,000 rpm, the pellet was discarded and Triton X-100 was added to the supernatant (final concentration of 1%) to solubilize the green pigment. The suspension was centrifuged at 18,000 rpm for 50 min at 18°C and after discarding the supernatant the virus pellet was resuspended in 50 mM Tris-HCl, pH 7.8. PBCV-1 suspensions were quantified before and after UV irradiation using an agar overlay method with MBBM. Plaques were enumerated in triplicate after 72 h incubation at 25°C in a continuously illuminated light box.
### Table 2.1
Organisms used in this study

<table>
<thead>
<tr>
<th>Organism</th>
<th>Genome*</th>
<th>Host</th>
<th>Source‡</th>
<th>Culture conditions‡</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteriophages</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS2</td>
<td>SS RNA</td>
<td>Escherichia coli</td>
<td>ATCC(^\text{§}) 15597-B1 (ATCC 15597)</td>
<td>TGY, 37°C</td>
</tr>
<tr>
<td>PP7</td>
<td>SS RNA</td>
<td>Pseudomonas aeruginosa</td>
<td>ATCC 15692-B2 (ATCC 15692)</td>
<td>NB+0.5% NaCl, 37°C</td>
</tr>
<tr>
<td>PRD1</td>
<td>DS DNA</td>
<td>Escherichia coli</td>
<td>ATCC BAA769-B1 (ATCC BAA769)</td>
<td>TSA, 37°C</td>
</tr>
<tr>
<td>Bam35</td>
<td>DS DNA</td>
<td>Bacillus thuringiensis</td>
<td>FHRCBV(^\text{†}) 410 (FHRCBV 1410)</td>
<td>LB, 37°C</td>
</tr>
<tr>
<td>Phi29</td>
<td>DS DNA</td>
<td>Bacillus subtilis</td>
<td>FHRCBV 243 (FHRCBV 1243)</td>
<td>TSA, 37°C</td>
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<tr>
<td>PM2</td>
<td>DS DNA</td>
<td>Pseudoalteromonas espejana</td>
<td>ATCC 27025-B1 (ATCC 27025)</td>
<td>AMS, 28°C</td>
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<td>T7</td>
<td>DS DNA</td>
<td>Escherichia coli</td>
<td>ATCC11303-B38 (ATCC 11303)</td>
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</tr>
<tr>
<td>T4</td>
<td>DS DNA</td>
<td>Escherichia coli</td>
<td>ATCC 11303-B4 (ATCC 11303)</td>
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<td>6631-B1 (236)</td>
<td>DS DNA</td>
<td>Bacillus pumilus</td>
<td>ATCC 6631-B1 (ATCC 6631)</td>
<td>TSA, 37°C</td>
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<tr>
<td><strong>Bacteria</strong></td>
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<td>Bacillus subtilis</td>
<td>DS DNA</td>
<td>NA</td>
<td>ATCC 6633</td>
<td>TSA, 37°C</td>
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<td>Bacillus megaterium</td>
<td>DS DNA</td>
<td>NA</td>
<td>Environmental isolate BHPCC</td>
<td>NB, 37°C</td>
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<tr>
<td>Bacillus pumilus</td>
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<td>ATCC 7061, ATCC 6631, ATCC 27142, Environmental isolates ASFUVR, ASFUVR</td>
<td>NB and TSA, 37°C</td>
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<td>Deinococcus radiodurans R1</td>
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<td>ATCC 13939</td>
<td>NB+1% glucose, 28°C</td>
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<td>Deinococcus radiopugnans</td>
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<td>NA</td>
<td>ATCC 19172</td>
<td>NB+1% glucose, 28°C</td>
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<td>Deinococcus aquaticus</td>
<td>DS DNA</td>
<td>NA</td>
<td>Environmental isolate DPHPCD</td>
<td>NB+1% glucose, 28°C</td>
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<td>Rubrobacter radiotolerans</td>
<td>DS DNA</td>
<td>NA</td>
<td>ATCC 51242</td>
<td>NB, 37°C</td>
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<tr>
<td>Lactobacillus plantarum</td>
<td>DS DNA</td>
<td>NA</td>
<td>ATCC 14917</td>
<td>Lactobacilli MRS, 37°C</td>
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<tr>
<td>Methyllobacterium spp.</td>
<td>DS DNA</td>
<td>NA</td>
<td>Environmental isolate G1</td>
<td>R2A, 28°C</td>
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<td><strong>Viruses of eukaryotes</strong></td>
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</tr>
<tr>
<td>PBCV-1</td>
<td>DS DNA</td>
<td>Chlorella spp, NC64A</td>
<td>J. Van Etten, University of Nebraska</td>
<td>MBBBM, 22°C</td>
</tr>
<tr>
<td>Adenovirus 2</td>
<td>DS DNA</td>
<td>A549 human lung carcinoma cells(^{††})</td>
<td>ATCC VR846</td>
<td>Ham’s F-12, 37°C</td>
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<tr>
<td>Adenovirus 40</td>
<td>DS DNA</td>
<td>A549 human lung carcinoma cells(^{††})</td>
<td>T. Cromeans, Centers for Disease Control</td>
<td>DMEM, 37°C</td>
</tr>
<tr>
<td>Dugan (clone 6A)</td>
<td>DS DNA</td>
<td>A549 human lung carcinoma cells(^{††})</td>
<td>T. Cromeans, Centers for Disease Control</td>
<td>DMEM, 37°C</td>
</tr>
<tr>
<td>Adenovirus 41</td>
<td>DS DNA</td>
<td>A549 human lung carcinoma cells(^{††})</td>
<td>T. Cromeans, Centers for Disease Control</td>
<td>DMEM, 37°C</td>
</tr>
</tbody>
</table>

*DS DNA, Double stranded DNA; SS RNA, Single stranded RNA. †Source of bacterial host in parentheses. ‡TGY, Tryptone-Glucose-Yeast extract medium; NB, Nutrient broth; TSA, Tryptic soy medium; LB, Luria broth; AMS, Ammonium mineral salts medium; MRS, Man-Rogosa-Sharpe medium; R2A, R2 agar; MBBM, Modified Bold Basal Medium; DMEM, Dulbecco’s Modified Eagle’s Medium. §American Type Culture Collection. ††Felix d’Herelle Reference Center for Bacterial Viruses. ††Source for host cells: ATCC CCL-185. NA, Not applicable.
Bacteria

Bacteria obtained from the ATCC were reconstituted and cultured following the supplier’s instructions, using the culture conditions described in Table 2.1. *Bacillus megaterium* (BPHPCC) and *Deinococcus aquaticus* (DPHPCD) were isolated from raw treatment plant influent on R2A agar plates following exposure of the sample to 40 mJ/cm² of low-pressure UV radiation. They were identified by fatty acid analysis (Microbial ID, Newark, DE) and sequencing a PCR-amplified fragment of the 16S ribosomal RNA (rRNA) gene. *Methylobacterium* spp. G1 was a naturally occurring isolate recovered from laboratory tap water and identified by PCR-sequencing of the 16S rRNA gene. For identification of bacteria by sequence analysis, approximately 90% of the 16S rRNA gene was amplified using 0.25 μM of universal primers (Lane 1991) and standard amplification conditions with a high-fidelity PCR kit (Easy-A; Stratagene, La Jolla, CA) and primer annealing at 60°C.

Forward primer: 27F, 5’- AGA GTT TGA TCC TGG CTC AG-3’
Reverse primer: 1392R, 5’- ACG GGC GGT GTG TA/G C-3’

The resulting 1.36 kb amplification product was gel-purified (QIAquick Gel Extraction Kit, Qiagen, Inc., Valencia, CA), TA-cloned into the vector pCR4-TOPO, and electroporated into *E. coli* TOP10 electrocompetent cells (Invitrogen, Carlsbad, CA). Clones were grown on LB agar plates containing 50 μg/mL kanamycin sulfate and incubated at 37°C overnight. Plasmids containing cloned 16S rRNA genes were purified using spin columns (QIAprep Spin Miniprep Kit, Qiagen) and inserts were sequenced using T3 and T7 universal primers (Seqwright, Houston, TX). Details of the phylogenetic analysis are provided in the legend for Figure 2.1.

Naturally occurring UV resistant isolates of spore-forming bacteria were recovered from raw surface water at a treatment plant influent (1.05 NTU, pH 7.02, 134 mg CaCO₃/L). Water samples were heated at 80°C for 15 min, cooled on ice, and then concentrated by centrifuging at 5,000 × g at 4°C for 15 min. The resulting pellet was resuspended in 15 mL of sterile deionized water and then exposed to LP-UV irradiation at doses up to 320 mJ/cm² of. Surviving colonies were streaked for isolation, cultured overnight in nutrient broth, and fresh spore suspensions prepared by heating at 80°C for 15 min to kill vegetative cells. Two isolates (ASFUVRA and ASFUVRC) that displayed high UV resistance in subsequent collimated beam experiments were identified as *Bacillus pumilus* by fatty acid analysis (Microbial ID) and sequencing a PCR-amplified fragment of the 16S rRNA gene (Figure 2.1). A strain of *B. pumilus* (SAFR032) was also provided by Dr. K. Venkateswaran at the California Institute of Technology Jet Propulsion Laboratory (Pasadena, CA).

For UV experiments, bacteria were grown in broth culture and cells harvested by centrifuging at 4,000×g at 4°C for 15 min. Cell pellets were washed by repeated (∗×2) resuspension in phosphate buffered water (PBW; 0.3 mM KH₂PO₄, 2 mM MgCl₂, pH 7.2) and centrifugation. The final cell pellet was resuspended in 10 mL of PBW prior to UV exposure.

Bacteria were enumerated by 10-fold serial dilution and triplicate plating on nutrient agar or tryptic soy agar incubated at 28°C (*Deinococcus* spp. and *Methylobacterium* spp. G1) or 37°C (all other bacteria).
Figure 2.1 Evolutionary relationships of UV resistant bacteria isolated for this project (boxed), based on 16S ribosomal RNA gene sequences. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor 1969). All positions containing gaps and missing data were eliminated from the dataset. There were a total of 714 aligned positions in the final dataset spanning positions 28 – 821 in the *Escherichia coli* 16S rRNA gene (Lane 1991). Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007). The scale bar indicates the number of base substitutions per nucleotide site. The tree was rooted with the *Aquifex pyrophilus* sequence. Sequences with boxed names were generated by this project and GenBank accession numbers are provided for all reference sequences.
A variety of media were used for propagating spores of *B. pumilus*:

Columbia broth (Difco, Becton Dickinson, Franklin Lakes, NJ):

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount (per L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic Digest of Casein</td>
<td>10 g</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>5 g</td>
</tr>
<tr>
<td>Proteose Peptone No. 3</td>
<td>5 g</td>
</tr>
<tr>
<td>Tryptic Digest of Beef Heart</td>
<td>3 g</td>
</tr>
<tr>
<td>L-Cysteine HCl</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>5 g</td>
</tr>
<tr>
<td>Magnesium Sulfate (anhydrous)</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Ferrous Sulfate</td>
<td>0.02 g</td>
</tr>
<tr>
<td>Sodium Carbonate</td>
<td>0.6 g</td>
</tr>
<tr>
<td>Tris (Hydroxymethyl) Aminomethane</td>
<td>0.83 g</td>
</tr>
<tr>
<td>Tris (Hydroxymethyl) Aminomethane HCl</td>
<td>2.86 g</td>
</tr>
</tbody>
</table>

Supplemented with 0.9 mM MnSO₄·4H₂O

Modified sporulation medium (UV Disinfection Guidance Manual; USEPA 2006b):

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount (per L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO₄·H₂O</td>
<td>280 mg</td>
</tr>
<tr>
<td>KCl</td>
<td>1.11 g</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>3.1 mg</td>
</tr>
<tr>
<td>Nutrient broth</td>
<td>8.9 g</td>
</tr>
</tbody>
</table>

Supplemented with 1 mM MnSO₄·4H₂O

AK Agar #2 (BBL; Becton Dickinson):

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount (per L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic digest of gelatin</td>
<td>6 g</td>
</tr>
<tr>
<td>Pancreatic digest of casein</td>
<td>4 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>3 g</td>
</tr>
<tr>
<td>Beef extract</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>1 g</td>
</tr>
<tr>
<td>Manganous sulfate</td>
<td>0.3 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15 g (omit for broth)</td>
</tr>
</tbody>
</table>
For collimated beam tests, cells were cultured in 100 mL of medium (primarily sporulation medium supplemented with 1 mM MnSO₄) and incubated at 37°C for 5 days on a rotating platform at 125 rpm. For larger, demonstration-scale testing, cells were cultured in multiple flasks containing 1 L of medium. Spores were harvested by centrifugation at 4,000×g for 15 min at 4°C and the resulting pellet was washed by repeated resuspension in PBW and centrifugation. Final pellets were resuspended in 10 mL of PBW for collimated beam tests or 100 mL of PBW for demonstration-scale tests. These suspensions were then heated at 80°C for 20 min to kill vegetative cells. Spore suspensions were stored at 4°C. For some experiments, spore suspensions were sonicated for 10 min at room temperature in a 40 kHz ultrasonic bath (Branson 1200, Branson Ultrasonics Corp., Danbury CT).

For large-scale testing, spores of *B. pumilus* ASFUVRC were also propagated using AK agar plates. One milliliter aliquots of an overnight TSB broth culture were spread onto ten 150 mm diameter AK Agar plates that were incubated at 37°C for 5 days. The resulting lawn of bacterial growth was resuspended in 5 mL of sterile deionized water using a cell scraper and the individual suspensions were combined in a 200 mL centrifuge tube. Following centrifuging at 4,000 × g for 30 min at 4°C, the pelleted material was washed by repeated resuspension in sterile deionized water and centrifugation. The final pellet was resuspended in 100 mL of PBW and heated at 80°C for 20 min. This procedure typically generated 100 mL of a spore suspension containing an average of 7 ×10⁹ spores (CFU)/mL.

For microscopic observation, spore suspensions were stained with malachite green. This involved air-drying 100 µL of the suspension onto a glass microscope slide followed by heat fixation. The slide was flooded with 5% aqueous malachite green, heated to the point of steaming for 5 min, rinsed with water and then counterstained with safranin. Spores were observed and photographed using a Zeiss AX10 microscope at 1000× magnification.

### Deinococcus Phage Isolation from Sewage

Various attempts were made to isolate bacteriophage that infect *Deinococcus* spp. from sewage. Primary clarified sewage was obtained from a wastewater treatment plant and 10 mL samples were mixed with 525 µL of chloroform and held at 4°C overnight. After centrifuging at 4,000 × g at 4°C for 30 min, the supernatant was passed through a 0.22 µm porosity membrane filter. One milliliter portions of the filtrate were mixed with 1 mL cultures of *D. radiodurans*, *D. radiopugnans*, and *D. aquaticus* (cultured in nutrient broth + 1% glucose) as potential host bacteria and 4 mL of 0.5% nutrient agar, and plated as agar overlays. Plates were incubated at 28°C for five days. Sewage samples were also filtered through 5 µm porosity filters, centrifuged at 10,000 × g at 4°C for 15 min, followed by ultracentrifugation of the supernatant at 45,500 × g for 3 h. The resulting pellet was resuspended in saline calcium buffer and then plated using the agar overlay method and three strains of *Deinococcus* spp. listed above. All plates were checked daily for plaque formation.

Samples of raw sewage were irradiated with 100 mJ/cm² of LP-UV radiation using a Calgon collimated beam apparatus. One milliliter portions of these samples were mixed with three *Deinococcus* spp. isolates and plated as agar overlays as described above, with and without prior treatment with chloroform.

Primary clarified sewage samples were also exposed to LP-UV doses of 50 – 200 mJ/cm² and 30 surviving *Deinococcus*-like colonies (based on colony morphology and color) were streaked for isolation and then cultured at 28°C for 3 d in nutrient broth. The isolates were plated
as lawns (100 µL volumes) on nutrient agar and incubated at 28°C for five days. Plates were checked daily for plaque formation.

**ADENOVIRUS PROPAGATION AND ENUMERATION**

Adenovirus type 2 strain VR846 (Ad2) was obtained from the American Type Culture Collection (ATCC). Adenovirus type 40 (Ad40; Dugan strain, clone 6A) and adenovirus type 41 (Ad41; strain v1930, clone 11A) were obtained from T. Cromeans (Centers for Disease Control and Prevention). These clones of Ad40 and Ad41 were obtained by repeated plaque selection and sub-passaging on A549 cell monolayers to obtain viruses that plaqued efficiently on these cells (Cromeans et al. 2008). All adenoviruses were propagated in A549 human lung carcinoma cells and enumerated using a quantitative plaque assay (QPA). For propagation, Ad2 was inoculated at a multiplicity of infection of 0.1 into 80 – 90% confluent A549 cells in 150 cm² flasks. A549 cells were cultured in Ham’s F12 medium with Glutamax supplemented with 2% fetal bovine serum (FBS), 1.2 mM L-glutamine, 15 mM HEPES buffer, 45 mg/mL sodium bicarbonate, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 µg/mL amphotericin B. Flasks were incubated in a humidified incubator at 37°C in 5% CO₂ for 4 – 5 days (80 – 90% cytopathic effects, CPE). Virus particles were recovered by three cycles of freezing at -80°C and thawing at room temperature followed by centrifugation at 900×g for 10 min at 4°C to remove cell debris, concentrated by ultracentrifugation at 45,000×g, and resuspended in PBS. The titers of virus particles prepared in this way were typically 0.1 – 1 × 10⁷ pfu/mL. Viruses were stored at -80°C.

Ad41 was propagated in a similar manner except that A549 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) and flasks were incubated for 14 – 17 days prior to harvesting the virus once 70 – 100% CPE was obtained. Fresh cell culture medium was added after 7 days incubation. For Ad41, virus infected cell suspensions were transferred to a centrifuge tube, frozen at -80°C for 30 min, thawed at room temperature for 1 h, and then centrifuged at 900×g for 10 min at 4°C. The resulting supernatant containing virus particles was diluted with PBS and used for UV inactivation experiments.

For enumeration of Ad2 by QPA, 10-fold dilutions of the virus were inoculated in triplicate onto A549 monolayers grown in 6-well plates, overlaid with 0.3% Bacto agar in Ham’s F-12, 1% FBS and incubated for 7 d. The agar overlay was removed by inverting the 6-well plate and firmly slapping the underside. This dislodged the overlay but did not damage the underlying monolayer. Following removal of the agar overlay, the monolayers were stained with 20% Gram’s crystal violet at room temperature for 10 min, and plaques were enumerated once the stain was removed. A similar method was used for enumeration of Ad41 by QPA but with recently described modifications (Cromeans et al. 2008). Ten-fold dilutions of Ad41 were inoculated in triplicate onto A549 monolayers grown in 6-well plates in DMEM containing 4% FBS, overlaid with 0.5% SeaKem agarose and incubated for 7 d. If no plaques were observed, a second agarose overlay was applied and the plates incubated for a further 7 d prior to staining with Crystal Violet. Adenovirus type 2 plaques were 1.5 ± 0.75 mm in diameter (mean ± standard deviation, N = 100) compared to 0.82 ± 0.75 mm for Ad41 (N = 50).

**Confirmation of Infection by Electron Microscopy**

The presence of infectious viruses in cell culture plaques generated by Ad2 following UV irradiation was confirmed by transmission electron microscopy (TEM). To generate viruses for
morphological analysis by TEM, 1 mL of an Ad2 stock suspension that was propagated from a plaque was inoculated into a monolayer of A549 cells grown in Ham’s F-12 medium in a 150 cm² flask and incubated at 37°C for 7 days. Viruses were recovered by three cycles of freezing at -80°C and thawing at 21°C followed by centrifugation at 900×g for 10 min at 4°C, and centrifugation of the resulting supernatant at 45,000×g for 60 min. The virus pellet was resuspended in 1 mL of PBS, and purified by Freon extraction and exclusion chromatography using Sephadex G-50 columns. Viruses were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 3 min, transferred to a 3 mm copper grid and then background stained by floating the grid on 3% uranyl acetate for 3 min. Viruses were visualized using a FEI-Philips CM300 transmission electron microscope at the Central Facility for Advanced Microscopy and Microanalysis at the University of California, Riverside.

UV EQUIPMENT AND COLLIMATED BEAM EXPERIMENTS

Microorganisms were exposed to monochromatic UV radiation using a collimated beam apparatus (Calgon Carbon Corp.) with a low-pressure (LP) lamp mounted above a 25 cm long, 6.5 cm inside diameter collimating tube, and an air-actuated shutter. Since such devices do not produce a beam with truly parallel rays, a more appropriate term is quasi-parallel beam apparatus. Radiation was collected from the end of the lamp. The lamp was allowed to warm up for 1 h prior to experimental use. The petri factor was determined three times over a two year period during the course of the project using a fiber optic cable connected to an IL1700 radiometer (International Light, Inc., Newburyport, MA), and a servo-controlled X-Y platform. Beam uniformity was determined by moving the fiber optic detector in two perpendicular transects across the beam and recording radiometer readings at 5 mm intervals. The petri factor was calculated by averaging the radiometer readings at each of 121 positions relative to the center of the beam as described previously (Bolton and Linden 2003; Mofidi et al. 2001). The average petri factor was 0.74. The LP lamp emitted monochromatic radiation with peak output at 254 nm. This equipment was also used for medium-pressure (MP) UV experiments with a polychromatic 1-kW UV bulb located centrally over the collimating tube. The MP lamp had emission peaks at 248, 266, 280, 289, and 295 nm. The petri factor in MP configuration was 0.95. This equipment was over 10 years old at the start of the project and the onboard fan was unable to adequately cool the MP bulb during operation. Therefore, a stand-alone air-conditioning unit was used to pre-cool the air intake for the lamp housing. Experiments with bacteriophage MS2 demonstrated that this configuration did not affect performance of the MP lamp.

A second collimated beam system, built by co-investigator E. R. Blatchley III, was used in the later stages of the project. This equipment used a 45 cm long Trojan LP lamp (UV705/Adv5), was air-cooled, with a manually operated shutter. It produced 60 mm and 70 mm diameter collimated beams through a 32 cm collimating tube positioned below the center of the UV lamp. The output characteristics of the collimated beam were determined by measuring beam intensity as a function of time after ignition of the lamp and intensity of the beam as a function of location in the plane of irradiation. All measurements of beam intensity were performed using a fiber optic radiometer detector (International Light Model P2) and an IL1700 radiometer. The fiber optic detector tip was attached to a micro-positioning device. All measurements were performed with the active surface of the detector located 2 cm below the bottom of the collimator box and the detector located in the center of the beam. The results indicated that the system should be allowed to “warm up” for 10 minutes before initiating experiments (Figure 2.2). After
allowing the output of the system to stabilize, the uniformity of the beam was measured by moving the fiber optic detector across the beam in 1 mm increments using the micro-positioning device. For each aperture configuration, two perpendicular transects were performed. The petri factor for this LP-UV unit was 0.95.

Test organisms were suspended at concentrations of approximately $10^6$ plaque or colony forming units per mL in 10 mL of dechlorinated treatment plant filter effluent. Plant effluent samples were dechlorinated by adding sodium thiosulfate to a final concentration of 0.002% followed by analyzing for residual chlorine using DPD reagent (Hach, Loveland, CO). Typical annual averages for this water are 0.07 NTU (range 0.05 – 0.08), pH 8.13 (range 8.07 – 8.21), and 18.5°C (range 12.3 – 25.3°C). The UV absorbance spectrum of plant effluent (Figure 2.3) was measured over the range 200 – 400 nm with a UV1201S spectrophotometer (Shimadzu, Columbia, MD). Sample depth was 0.45 cm and samples were continuously mixed using a stir bar and magnetic stirrers. Samples were exposed for variable lengths of time at a constant distance from a constant intensity UV lamp to achieve different UV dosages. UV irradiance was measured with calibrated International Light IL1700 and IL1400A radiometers equipped with SED240 and SEL240 detectors, respectively, before and after each exposure of microorganisms to UV light. Radiometers at all project institutions (MWD, Purdue, HydroQual) were calibrated annually by International Light. An example calibration certificate is provided in Appendix A.

The average UV fluence and exposure time measured to the nearest 0.1 sec were used to calculate the applied dose in collimated beam experiments using the method described in the UV Disinfection Guidance Manual, Appendix C (USEPA 2006b) and a dose calculation spreadsheet (Bolton and Linden, 2003). Following UV exposure, 10-fold serial dilutions of test samples were plated in triplicate. The amount of inactivation was calculated as the surviving fraction of the test microbe relative to the zero dose control (Equation 2.1), which was a suspension of the microbe placed under the collimating tube with no UV exposure, for the period required to achieve the highest UV dose in that particular experiment.

$$Inactivation = -\log_{10}\left(\frac{N_d}{N_0}\right)$$
(2.1)

where: $N_d$ = concentration of microbes surviving at each UV dose
$N_0$ = concentration of microbes in zero UV dose control

UV intensity for selected collimated beam experiments at MWD was measured using an IL1700 radiometer and an IL1400A as a secondary QC check. The mean difference in readings between the two radiometers was 0.86% ($N = 31$) with a range of 0 – 8.3%. Only a single comparison had a difference greater than 5% (8.3%) while the remaining 30 comparisons were all less than 5%. The correlation coefficients between the two radiometers were 0.89 and 0.95 for the entire dataset and excluding the one outlier (8.3%), respectively.

For all bench-scale experiments, UV intensity of the collimated beam lamp was measured prior to organism exposure and afterwards; the average of the two values was used to calculate the UV dose. The average difference between the before and after exposure radiometer readings was 1.4% ($N = 238$, excluding 14 values above 5% that were not included in analyses).

A series of UV bandpass filters (FS10-50; Andover Corp., Salem, NH) was utilized to determine the wavelength-specific response of selected microbial surrogates. The half-peak bandwidths for the filters were 9–11 nm. Transmittance scans of all of the filters were performed using a UV-VIS spectrophotometer over the wavelength range of 200 – 300 nm (Figure 2.4).
Figure 2.2 Measured output intensity of LP-UV collimated beam apparatus built for this project, with 60 mm aperture installed.

Figure 2.3 Representative UV absorbance spectra of drinking water treatment plant effluent samples over a 12 month period. Lines remained flat beyond 300 nm.
Figure 2.4 Transmittance spectra of optical band-pass filters. The transmitted wavelength is indicated above each peak and the half-peak bandwidths were 9 – 11 nm.

ASSESSING UV DAMAGE IN MICROBES

The extent of UV-induced DNA damage was assessed through the use of monoclonal antibodies that bound to cyclobutane thymine dimers (mouse IgG1 clone H3; Sigma) and 6-4 photoproducts (mouse IgG1; Kamiya Biomedical Company). Control experiments demonstrated that these antibodies did not bind to DNA that had not been exposed to UV. Following exposure of a variety of microbes to increasing doses of UV radiation, genomic DNA was extracted using a commercial extraction kit (MOBIO, Carlsbad, CA), digested with the enzyme HindIII (37°C, 3 h), separated by agarose gel electrophoresis, and blotted to a positively charged nylon membrane using standard techniques (Sambrook et al. 2001), without staining or photographing to avoid exposure of the extracted DNA to UV radiation from the transilluminator. The membrane was air-dried at room temperature, washed for 30 min in 0.1 M maleic acid, 0.15 M NaCl, pH 7.5 containing 4% blocking reagent (Roche), and then incubated at 37°C for 30 min in 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 2% blocking reagent containing 2 µg/mL of unlabeled anti-thymine dimer or anti-6-4 photoproduct antibody. This was followed by 30 min incubation at 37°C in 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 2% blocking reagent containing alkaline phosphatase-labeled goat anti-mouse IgG1 (SouthernBiotech, Birmingham, AL). Following three 15 min washes in 0.1 M maleic acid, 0.15 M NaCl, pH 7.5, containing 0.3% Tween-20, and incubation in a chemiluminescent substrate (CPD Star, Roche) diluted 1 in 100 in 0.1 M Tris, 0.1 M NaCl, 50 mM MgCl₂, pH 9.5 at room temperature for 15 min, the chemiluminescent signal was captured digitally using a FluorChem 8900 instrument (Alpha InnoTech). The same basic detection procedure was also used for adenovirus DNA that was extracted and applied to nylon membranes without prior digestion and for adenovirus that was applied directly to membranes and lysed by the addition of 0.4 M NaOH.
CHAPTER 3
UV DISINFECTION OF ADENOVIRUSES

Enteric adenoviruses (Ad40 and Ad41) have been referred to as “fastidious” because they are typically more difficult to culture than other adenoviruses; they require lengthy incubation periods, often produce low titers, and may produce limited or no cytopathic effects (CPE). Previous studies on UV inactivation of adenoviruses used most probable number, CPE/TCID$_{50}$, RT-PCR, and plaque assays on human hepatoma (PLC/PRF/5), lung adenocarcinoma (A549), embryonic kidney (G293), and skin fibroblast cell lines to enumerate surviving viruses (see Table 1.3). However, it is important to use the same assay methods when comparing disinfection kinetics of different organisms to ensure that differences in response are not due to differences between the assays. Although Ad2 could be enumerated in a plaque assay on the A549 cell line and the G293 cell line is typically used to propagate Ad40 and Ad41, at the start of this project there was no routine plaque assay for the enteric adenoviruses. A goal of the project was to use the same quantitative plaque assay (QPA) on a single cell line for Ad2 and Ad41/Ad40 so that their UV responses could be compared directly. Therefore, considerable effort was expended in implementing and refining an Ad41 plaque assay in A549 cells based on a recently published method (Cromeans et al. 2008), so that the response to UV could be assessed using the same cell line. The strain of Ad41 used in the current study (strain v1930, clone 11A) was obtained by repeated plaque selection and sub-passaging on A549 cells to obtain viruses that plaqued efficiently (Cromeans et al. 2008). Ad2 and Ad41 both produced consistent patterns of roughly circular plaques on A549 monolayers (Figure 3.1) with diameters of $1.5 \pm 0.75$ mm (mean $\pm$ standard deviation, $N = 100$) for Ad2 and $0.82 \pm 0.75$ mm ($N = 50$) for Ad41. No UV inactivation data were generated for Ad40 because, although it could also be enumerated using the A549 QPA, titers of propagated Ad40 were lower than for Ad41, it produced smaller plaques than Ad41, and did not plaque as consistently or efficiently. Confirmation that plaques were formed by infectious adenoviruses was provided by transmission electron microscopy on viruses that were propagated from a plaque produced by a UV irradiated suspension of Ad2. Although the virus fibers cannot be discerned in the images, these viruses displayed typical adenovirus morphology with triangular faces of the icosahedral structure clearly visible (Figure 3.2).

Low-pressure UV inactivation of Ad2 and Ad41 is displayed in Figure 3.3. Aggregate data from 11 experiments demonstrated that a LP-UV dose of 150 mJ/cm$^2$ resulted in 4-log inactivation of Ad2, based on the best fit polynomial regression. Aggregate data from three experiments demonstrated 2.2-log inactivation of Ad41 at the same UV dose based on a linear regression. If inactivation data for UV doses above 200 mJ/cm$^2$ are omitted and a linear regression used for both virus dose response curves, a dose of 150 mJ/cm$^2$ resulted in 4.2-log and 2.2-log inactivation of Ad2 and Ad41, respectively (Figure 3.3B).

As reviewed by Yates et al. (2006), there is considerable variability within the published UV responses of various types of adenoviruses, possibly due to one or a combination of the following: different cell lines and quantification assays, a variety of virus propagation methods, different methods for assaying infection in cell cultures, and different types of UV equipment and UV dose measuring methods. Nevertheless, all of the studies generally support the conclusion that adenoviruses are much more resistant to UV disinfection than any other potentially waterborne pathogens so far investigated. Based on published data, the USEPA’s
LT2ESWTR established a required UV dose of 186 mJ/cm² (not including safety factor) for 4-log inactivation of viruses (USEPA 2006a).

Figure 3.1 Adenovirus 2 (A) and adenovirus 41 (B) plaque assays on A549 cells. Triplicate wells were inoculated with 10-fold dilutions of the virus and stained with Crystal Violet following incubation and removal of agar overlays. Three wells on the left of panel B have cleared completely due to overwhelming infection.
Figure 3.2 Transmission electron micrographs of adenovirus 2 particles propagated on A549 cells. Released viruses were fixed in glutaraldehyde and background stained with uranyl acetate. The scale bars in the lower left and upper right panels are 20 nm.
Figure 3.3 Low-pressure UV inactivation of adenovirus types 2 (solid line) and 41 (dashed line). Surviving viruses were enumerated by a plaque assay on A549 cell monolayers. A) All data. The UV dose requirement for virus inactivation credit under the LT2ESWTR is included (dashed line). B) Doses above 200 mJ/cm² omitted.
The Ad2 and Ad41 inactivation results from the current project are in general agreement with published data for these viruses (Figure 3.4; Ballester and Malley 2004; Baxter et al. 2007; Gerba et al. 2002; Jacangelo et al. 2002; Ko et al. 2005; Linden et al. 2007, 2009; Thurston-Enriquez et al. 2003) although the Ad41 response was slightly more resistant than that reported in other studies.

For some experiments, the infectivity of Ad2 surviving UV irradiation was confirmed by a second passage on A549 cells. Following exposure to 30 – 125 mJ/cm² of low-pressure UV, Ad2 viruses were inoculated onto monolayers and incubated for 7 d following the standard plaque assay conditions. Without staining the monolayer, plaques were recovered by stabbing a pipet tip through the agar overlay into a plaque and removing the agar plug. Following homogenization, the suspension was serially diluted and inoculated onto fresh monolayers in six-well plates. Following incubation, removal of the agar overlay, and staining with crystal violet, plaques were enumerated. Dilution of the original plaque suspension to 10⁻⁵ and 10⁻⁶ generated average plaque densities of 19 and 3 pfu per monolayer, respectively. This demonstrated that the original plaques were formed by infectious adenovirus particles that survived UV irradiation and were not due to cytotoxicity of inactive virus components.

![Figure 3.4](LT2ESWTR- Virus)

**Figure 3.4 Comparison of low-pressure UV inactivation data from this report (Ad2, solid line; Ad41, dotted line) with published data for Ad2, Ad40, and Ad41 and dose requirements in the LT2ESWTR. Literature references are provided in the legend for Figure 1.1.**

Experiments were conducted to better define the variability in the Ad2 cell culture assay. It was not possible to expose replicates to exactly the same UV dose because of the variability introduced by actual radiometer measurements of UV intensity and measuring exposure time in 0.1 second increments. The results demonstrated the degree of variability within immediate duplicates compared to inter-day replicates (Figure 3.5). The data presented for each replicate data point are
based on the mean number of virus plaques in triplicate monolayers. The mean inter-day variability of inactivation was 0.5-log compared to a mean intra-day variability of 0.18-log. The response of adenovirus particles suspended in phosphate buffered water during LP-UV exposure was the same as when the virus was suspended in treatment plant filter effluent (Table 3.1).

Infectivity of UV-irradiated Ad2 was also assessed following various holding periods prior to inoculation of cell monolayers. Following exposure of Ad2 suspensions to LP-UV, the virus was either immediately inoculated onto A549 monolayers or held at 4°C for up to 48 h prior to inoculating cells. The aggregate results from two experiments demonstrated no statistical difference in the amount of inactivation for holding periods up to 48 h (Figure 3.6). These experiments were conducted over two ranges of UV doses (41.5 – 47.2 mJ/cm² and 108.5 – 123.6 mJ/cm²) since the delivered dose was dependent on individual radiometer readings and exposure time measured to the nearest 0.1 seconds. Therefore, to allow statistical analysis, inactivation data were normalized as UV dose per log inactivation. One-way analysis of variance demonstrated no significant difference between the various holding periods ($P = 0.87, N = 4$ at each holding period). The apparent resistance of adenoviruses to UV radiation is due largely to repair mechanisms within their host cell, which repair UV-induced DNA damage (Day 1974a, 1974b; Rainbow 1977). Therefore, delaying inoculation of UV irradiated adenoviruses into host cells should not affect the amount of inactivation since the host’s repair mechanisms are not affected by the storage conditions of the virus. UV-induced lesions involve chemical modification of DNA, producing thymine dimers and (6-4) photoproducts. Once formed, these products are stable unless removed by enzymatic cellular processes. They do not cause further degradation of the DNA molecule upon storage. Therefore, the efficiency of host cell repair mechanisms that are responsible for the apparent UV resistance of adenoviruses is not reduced when introduction of UV-irradiated viruses into host cells is delayed.

Table 3.1

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Average (N = 2) log inactivation at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>40 mJ/cm²</td>
</tr>
<tr>
<td>Treatment plant filter effluent, pH 7.8</td>
<td>1.44</td>
</tr>
<tr>
<td>Phosphate buffered water, pH 7.4</td>
<td>1.39</td>
</tr>
</tbody>
</table>

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Figure 3.5 Intra- and inter-day variability in UV response of adenovirus 2. Duplicate experiments (same symbol) were conducted on two separate days and compared to the aggregate UV response for Ad2 (-----, N = 9 experiments, 60 datapoints).

Figure 3.6 Effect of delayed inoculation of cell monolayers following UV irradiation on the response of UV irradiated adenovirus 2. The error bar represents the maximum inter-day variability in the amount of Ad2 inactivation (1.2 log from Figure 3.5). The line was plotted through the mean UV dose and corresponding mean log inactivation.
Full-spectrum medium-pressure (MP) UV collimated beam experiments were conducted for Ad2 and Ad41. The number of individual experiments for each virus and lamp type was: LP-Ad2, N = 11; MP-Ad2, N = 2; LP-Ad41, N = 2; and MP-Ad41, N = 2. Consequently, the relatively low number of MP-UV datapoints should be considered when comparing with LP-UV data. Medium-pressure, polychromatic UV was more effective than monochromatic LP-UV for inactivating Ad2 (Figure 3.7). A LP-UV dose of 150 mJ/cm² resulted in 4-log inactivation of Ad2 but the same amount of inactivation was achieved with only 86 mJ/cm² of MP-UV. However, there was no difference between LP- and MP-UV for inactivating Ad41 (Figure 3.7). Low and medium pressure doses of 150 mJ/cm² achieved 2.16-log and 2.15-log inactivation, respectively, of Ad41.

Figure 3.7 Comparison between low-pressure (solid lines) and medium-pressure (dotted lines) UV lamps for inactivation of adenoviruses.

The Ad2 data agrees with a recent study that reported higher inactivation of Ad2 and Ad40 by MP-UV compared to LP-UV (Linden et al. 2007). Using an A549 cell culture assay and quantifying surviving viruses by 50% tissue culture infective dose, Linden et al. (2007) reported approximately 2.3-log and 5.3-log inactivation of Ad2 at 60 mJ/cm² of LP-UV and MP-UV, respectively. The ratio of MP to LP inactivation of Ad2 at 60 mJ/cm² for this project was 1.7 compared to 2.3 in the Linden et al. study. These authors also reported approximately 1-log and >4-log inactivation of Ad40 at 60 mJ/cm² of LP-UV and MP-UV, respectively. The increased efficacy of MP-UV compared to LP-UV for Ad2 inactivation was also demonstrated at full-scale at flow rates of 200 – 900 GPM (Linden et al. 2009). The authors suggested that the LT2ESWTR LP-based UV dose requirements are inappropriate for achieving cost-effective virus inactivation because MP-UV is so much more effective than LP-UV for Ad2 inactivation.

The reason for the difference in relative germicidal effectiveness of LP- and MP-UV between Ad41 in the current study and published Ad40 data (Linden et al. 2007) is not clear. It may be due to a difference between Ad40 and Ad41 in their response to MP-UV or different
experimental procedures. Ad41 plaqued less efficiently on A549 cell monolayers compared to Ad2 but inefficient plaque formation would probably not result in the observed behavior of Ad41. Inefficient plaque formation would lead to an apparent increase in sensitivity. Because the same cell culture and virus quantification methods were used for both viruses, differences should not be due to experimental variation, so Ad41 appears to be more resistant to UV than Ad2. Since the high level of resistance is actually a measure of the repair capacity of the host cells and both viruses were assayed in the same cells, this suggest that Ad41 may be repaired more efficiently than Ad2, or it may interact with the host cell in a different way that allows more efficient repair.

However, the MP-UV inactivation data for Ad41 generated by the current project should be interpreted with caution due to the low number of experiments that were conducted and differences in the way the two viruses were exposed to UV. Due to the relatively low titers of Ad41 obtained by propagation in A549 cells, virus particles were not suspended in treatment plant effluent water for UV exposure as they were for Ad2 (and all other microbes). Instead Ad41 was suspended in diluted cell culture medium, which may have impacted its response to UV due to absorbance by organic components in the medium. The absorbance spectrum of the suspension matrix was measured for all collimated beam experiments and factored into the UV dose calculation. Although the UV absorbance of diluted cell culture medium was higher than either treatment plant effluent or PBS, this increased absorbance was accounted by increasing the period of UV exposure to achieve target UV doses. For Ad2 suspended in treatment plant effluent, the average exposure period under the collimated UV beam (normalized relative to calculated dose) was 2.8 seconds per unit dose of UV (mJ/cm²). For Ad41 suspended in diluted cell culture medium, the average exposure period was 6.2 seconds per unit dose of UV. In addition, there was no difference in the amount of inactivation with either LP- or MP-UV when comparing undiluted cell culture medium and PBS as the suspension matrix for exposure of Ad41 to UV (Table 3.2). So if undiluted cell culture medium did not affect the response of Ad41 to MP-UV, the diluted medium that was used in the dose response experiments would not have affected its response.

Table 3.2

<table>
<thead>
<tr>
<th>Suspension matrix*</th>
<th>Type of UV</th>
<th>Dose (mJ/cm²)</th>
<th>Log inactivation</th>
<th>Inactivation/mJ</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM</td>
<td>Low-pressure</td>
<td>85.9</td>
<td>1.4</td>
<td>0.016</td>
</tr>
<tr>
<td>PBS</td>
<td>Low-pressure</td>
<td>90.4</td>
<td>1.5</td>
<td>0.017</td>
</tr>
<tr>
<td>DMEM</td>
<td>Medium-pressure</td>
<td>80.1</td>
<td>1.7</td>
<td>0.021</td>
</tr>
<tr>
<td>PBS</td>
<td>Medium-pressure</td>
<td>79.3</td>
<td>1.3</td>
<td>0.016</td>
</tr>
</tbody>
</table>

* DMEM, Dulbecco’s Modified Eagle’s Medium; PBS, Phosphate buffered saline

These findings indicate that the difference in MP-UV response between the strains of Ad2 and Ad41 used in this study was not due to differences in experimental handling of the viruses. Nevertheless, further studies are necessary to determine whether there is a genuine and reproducible difference in MP-UV responses between Ad2 and Ad41 and whether all strains of Ad41 display the same responses to LP- and MP-UV.
Five band-pass filters with peak transmitted wavelengths of 220, 239, 254, 260, and 280 nm and peak band widths of 9 – 11 nm were used to develop action spectra for Ad2 and Ad41 to determine the most effective inactivation wavelengths (Figure 3.8). For Ad2, the most effective wavelength was 220 nm with approximately 10-fold higher inactivation than at 254 nm. This agrees with the action spectrum previously reported for Ad2 (Linden et al. 2007; Malley et al. 2004). However, although the action spectrum for Ad41 displayed the same overall shape as Ad2 and the most effective wavelength was again 220 nm, the relative differences between wavelengths were not as pronounced for Ad41. Inactivation of Ad41 at 220 nm was only 1.7-fold higher than inactivation at 254 nm. Action spectra are compared and discussed in more detail in Chapter 4.

Viruses and bacteriophages are intracellular organisms that are entirely dependent on their host organisms (animal, in vitro cell culture, or bacterium) for replication and propagation. In general, they have small genomes that encode their essential structural proteins and may also encode genes that direct some aspects of host cell operation. However, they are dependent on host cell functions for nucleic acid replication, transcription, and translation. With a few exceptions such as bacteriophage T4 and Mimiviruses (Kemper 1998; Raoult et al. 2004), viruses do not carry genes for DNA repair proteins and so are also dependent on their host cell to repair nucleic acid damage. Therefore, viruses cannot be considered as discrete entities when investigating factors that affect their survival. Instead, the virus-host complex should be considered.

Several studies have reported that UV irradiated adenoviruses are repaired by the nucleotide excision repair (NER) mechanisms of their host cell (reviewed by Yates et al. 2006), and Ad2 displayed the same susceptibility to UV radiation as most other organisms when surviving viruses were enumerated using a plaque assay in repair-deficient cells (Figure 3.9, data adapted from Day 1974a). The normal cells in Figure 3.9 were human skin fibroblasts and Ad2.
assayed on these cells displayed a UV response close to the LT2ESWTR dose requirements for virus inactivation. XPC cells do not have a functional NER pathway that operates on bulk DNA but they do have an active transcription coupled repair (TCR) mechanism that operates during DNA transcription (Day 1974a). The UV response of Ad2 assayed on these partially repair-deficient cells was similar to the response of MS2. The XP-1 cell line is deficient in both NER and TCR pathways so Ad2 assayed on these cells was extremely sensitive to LP-UV with approximately 4-log inactivation at 12 mJ/cm².

Figure 3.9 Effect of host cell repair status on the sensitivity of adenovirus type 2 (Ad2) to UV radiation. Following irradiation, Ad2 was assayed on normal cells and repair-deficient mutants (XPA and XPC). Adenovirus 2 data for XP-1, XPC, and normal cells were adapted from Day (1974a). MS2 (-----) and additional Ad2 data (......) are from this project.

For a wide variety of cell lines with differing repair capacities, the UV dose required for 2-log inactivation of Ad2 ranged from 4 mJ/cm² to 114 mJ/cm² (Table 3.3), which is almost a 30-fold difference between the extremes. The 254 nm dose required for the same level of inactivation in other viruses (Herpes simplex, SV40) was also much lower when the surviving virus was assayed on repair-deficient cell lines compared to repair competent cells (reviewed by Day 1974b). These data confirm the importance of the host cell in determining the response of viruses to UV irradiation. Consequently, the responses of adenoviruses to UV radiation reported in this and other studies are a measure of the repair capacity of the cell line used to assay and quantify the virus, rather than intrinsic “resistance” of the virus. Furthermore, the level of UV “resistance” can be increased in adenoviruses by low level exposure of the host cells to UV (up to 1.5 mJ/cm²) prior to assaying the UV-irradiated virus in a process termed UV-enhanced host cell reactivation (Francis and Rainbow 1999). The use of different cell lines (presumably with differing repair capacities) to assay adenoviruses may explain some of the discrepancies in published inactivation data for some adenoviruses.
Table 3.3

UV inactivation of adenovirus type 2 when assayed in different cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Repair capacity*</th>
<th>Assay type</th>
<th>Dose (mJ/cm²) for 2-log inactivation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1119</td>
<td>+</td>
<td></td>
<td>94</td>
<td>Day 1974a</td>
</tr>
<tr>
<td>1161C</td>
<td>+/-</td>
<td></td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>1166C</td>
<td>+/-</td>
<td>Plaque</td>
<td>33</td>
<td>Day 1974a</td>
</tr>
<tr>
<td>1201A</td>
<td>-</td>
<td></td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>XP-1A</td>
<td>-</td>
<td></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>10 normal cell lines</td>
<td>+</td>
<td></td>
<td>92 – 105</td>
<td></td>
</tr>
<tr>
<td>1165</td>
<td>+</td>
<td></td>
<td>114</td>
<td></td>
</tr>
<tr>
<td>1254</td>
<td>+</td>
<td></td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>XW</td>
<td>+/-</td>
<td>Plaque</td>
<td>71 – 75</td>
<td>Day 1974b</td>
</tr>
<tr>
<td>1199B</td>
<td>+/-</td>
<td></td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>1223A</td>
<td>-</td>
<td></td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>1157D</td>
<td>-</td>
<td></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>A549</td>
<td>?†</td>
<td>Plaque</td>
<td>63</td>
<td>This study</td>
</tr>
<tr>
<td>A549</td>
<td>?†</td>
<td>CPE/TCID₅₀</td>
<td>55</td>
<td>Ballester and Malley 2004</td>
</tr>
<tr>
<td>PLC/PRF/5</td>
<td>?†</td>
<td>CPE/MPN</td>
<td>78</td>
<td>Gerba et al. 2002</td>
</tr>
</tbody>
</table>

* Cell lines were fully repair-competent (+), deficient in one or more repair pathways (+/-), or completely repair-deficient (-).
† DNA repair status unknown

The increased effectiveness of MP-UV compared to LP-UV is due to the shorter MP wavelengths (220 – 230 nm) that are absorbed by proteins, damaging their structure and properties. LP-UV at 254 nm is absorbed minimally or not at all by proteins. Adenovirus DNA that is damaged by UV₂₅₄ can be efficiently repaired by host cell repair mechanisms so long as the virus can penetrate the cell and the viral DNA can migrate to the host cell nucleus. However, repair cannot occur if the virus cannot penetrate the host cell. The shorter wavelengths in MP-UV may damage the coat proteins that Ad2 uses to attach to host cell receptors. If the virus cannot attach to the host cells, they cannot be engulfed by the cell’s endocytosis process and therefore cannot be repaired. However, if the Ad41 MP-UV data are confirmed, the full spectrum and spectral sensitivity data suggest that its proteins may be protected from UV₂₂₀–₂₃₀.
Detecting UV-Induced DNA Damage in Adenoviruses

An antibody-based assay was used to demonstrate that UV irradiated adenoviruses accumulate DNA damage similar to other organisms. Increasing doses of UV irradiation resulted in increased thymine dimer accumulation in *Escherichia coli*, *Deinococcus radiodurans*, and *Bacillus pumilus* spores (Figure 3.10A). Although direct quantitative comparisons could not be made between organisms, the results clearly demonstrated that the amount of DNA damage in Ad2 increased as the virus was exposed to increasing doses of UV radiation (Figure 3.10B and 3.10C). Both thymine dimers and (6-4) photoproducts were detected. UV irradiation of adenoviruses results in a variety of photolesions, including thymine dimers, cross-linking, and alkaline-labile damage; irradiation of Ad2 induced 30 thymine dimers per lethal hit (Rainbow 1977). It was also reported that UV-irradiation caused a delay in DNA synthesis in adenoviruses (Mak and Mak 1974). Host-mediated repair of UV-irradiated adenoviruses has also been demonstrated (Day 1974a, 1974b; Rainbow 1977). Generally, 1% of an organism’s thymine nucleotides are converted to pyrimidine dimers when the organism is exposed to 50 mJ/cm² of UV radiation (Earl et al. 2002), which equates to 162 and 168 dimers in Ad2 and Ad40, respectively, based on their nucleotide composition (45% and 49% AT) and genome size (35,937 and 34,214 bases). According to Day (1974a), cyclobutane pyrimidine dimers are produced at a rate of 1.5 dimers per genome per mJ/cm² in UV exposed Ad2. Based solely on genome size and a higher AT content, it is expected that Ad40 and Ad41 (49% AT) would accumulate more thymine dimers than Ad2 (45%), so the increased resistance of Ad41 compared to Ad2 demonstrated in this and other studies cannot be attributed to less UV-induced DNA damage throughout the genome. In addition, a PCR-based assay demonstrated that increasing UV doses induce lesions in adenovirus DNA at a rate consistent with other organisms (Eischeid et al. 2009). These results demonstrated that there is nothing unique about the properties of adenovirus DNA or physical structure that prevents UV-induced DNA damage.
Figure 3.10 Detecting UV-induced DNA damage with fluorescently labeled antibodies. A) DNA was extracted from bacterial suspensions exposed to various levels of LP-UV, digested with the restriction enzyme EcoRI, separated by electrophoresis on an agarose gels, and transferred to a positively-charged nylon membrane, followed by application of anti-thymine dimer antibody. B) Adenovirus 2 particles were concentrated by ultracentrifugation, deposited directly on the membrane and lysed with sodium hydroxide prior to application of antibody. C) DNA was extracted from Ad2 and applied to a membrane with a dot blot manifold prior to application of the antibodies. Positive control was E. coli exposed to 80 – 100 mJ/cm² of LP-UV.
CHAPTER 4
UV DISINFECTION OF MICROBIAL SURROGATES

A wide variety of microorganisms were evaluated during this project, some of which had been previously investigated and others that had not. The UV response of these organisms could be generally categorized into three groups: 1) organisms that were extremely sensitive to UV and are therefore not suitable as surrogates for UV inactivation of adenoviruses, although they may be suitable as surrogates for other, less resistant pathogens such as Cryptosporidium spp.; 2) organisms that were moderately resistant but provided no benefit over the current MS2 biodosimeter; and 3) microbes that displayed similar or higher levels of UV resistance than adenoviruses and may therefore be suitable surrogates for reactor validation at the higher doses required for virus inactivation credit.

BACTERIOPHAGE MS2

The UV response of bacteriophage MS2 has been well characterized so it was used as a benchmark throughout this project to ensure that UV equipment was operating within expected bounds, and to confirm consistency across different pieces of equipment and when changes were made to equipment location or configuration. The response of MS2 to low-pressure UV (LP-UV) was consistent with expectations based on the 90% prediction limits provided by the UVDGM (USEPA 2006b), suggesting that appropriate methods were used for operation of the UV equipment, microbial exposure, UV irradiance measurements, and dose calculations (Figure 4.1A). Two LP-UV collimated beam instruments were used during the project: a commercial Calgon unit with radiation collimated from one end of the UV lamp (petri factor = 0.76) and a unit built by E. R. Blatchley specifically for this project with radiation collimated from the center of the UV lamp (petri factor = 0.95). With very few exceptions, all of the MS2 inactivation data with both pieces of equipment were within the UVDGM 90% prediction interval (Figure 4.1A) and agreed with much of the published data (Batch et al. 2004; Fallon et al. 2007; Hargy et al. 2005; Malley et al. 2004; Shin et al. 2005). The Calgon instrument was relocated twice during the project, including placement inside a hood for pathogen containment, and was also equipped with an ancillary air-conditioning unit to prevent overheating when it was used with a medium pressure bulb during the later stages of the project. In all cases, MS2 test results demonstrated that performance of the unit was not adversely affected by these changes.

Although most of the MS2 UV data generated by this project was within the UVDGM 90% prediction limits, much of it was outside an alternative set of boundaries provided in the National Water Research Institute UV Disinfection Guidelines (Figure 4.1B; NWRI 2003). The two instruments used to measure UV irradiance for bench-scale collimated beam experiments were calibrated three times during the course of the project. The instruments were an IL1700 radiometer with SED240 detector and IL1400 hand-held radiometer with SEL240 detector. Example calibration certificates for each instrument are provided in Appendix A. Therefore, the difference between MS2 dose response data produced by this project and the NWRI guidelines were not due to inaccurate radiometer measurements. Since the same calibrated radiometers were used for all microbes throughout the project, and the objective of the project was to compare relative responses, the overall conclusions regarding the response of surrogates compared to adenoviruses are not affected by the difference between MS2 responses generated by this project.
and the NWRI guidelines. In fact, it has been suggested that the NWRI guidelines should be revised since they are not in agreement with much of the published data and do not consider tailing in the MS2 dose-response (Hargy et al. 2005; Malley et al. 2004).

A series of experiments was conducted to better understand the variability and factors affecting UV inactivation of MS2. Variability in LP-UV inactivation of MS2 was evaluated by conducting duplicate collimated beam experiments at four UV doses on three separate days (Figure 4.2A). The inter-day variability was no greater than the intra-day variability. Maximum variability within a replicate dataset was 0.34-log at 59 mJ/cm². For most of the collimated beam experiments in this study, microbes were suspended in treatment plant effluent. However, many reports of UV inactivation are for microbes suspended in phosphate buffers of various types. There was no difference in the effectiveness of UV when MS2 was suspended in phosphate buffered water (pH 7.4) compared to treatment plant effluent (pH 6.85, 0.06 NTU; Figure 4.2B).

Bacteriophages are intracellular microbes that are dependent on their host bacteria for successful replication and propagation. They are also dependent, to varying degrees, on their host for repair of damaged nucleic acids. Therefore, the role of the host bacterium in UV inactivation of bacteriophages should be considered. Repair of UV-induced damage is inducible in many organisms by exposure to low doses of UV radiation. However, the LP-UV response of MS2 assayed in *E. coli* that had first been exposed to 11 mJ/cm² of UV was the same as MS2 assayed in non-exposed *E. coli* (Figure 4.2C). Although as a bacterial virus, MS2 is dependent on its host bacterium for survival, the UV response of the phage and host were remarkably different (Figure 4.2C). A UV dose of 11 mJ/cm² achieved 4-log inactivation of the *E. coli* host compared to 70 mJ/cm² for inactivation of MS2 to the same level. Consequently, the UV response of MS2 is an inherent property of the virus or of the virus/host association and not just a function of host sensitivity or repair capacity. The role of host versus virus was also investigated by assaying two very different bacteriophages (T7 and MS2) on the same *E. coli* host (15597). Phage T7 assayed on *E. coli* 15597 and the host bacterium itself displayed identical responses to UV and were far more sensitive than MS2 (Figure 4.2D). This provides further evidence that the UV-response of MS2 is an inherent property of the virus or of the virus/host association and not just a function of host sensitivity or repair capacity.

Some reports have indicated that MS2 may be more sensitive to MP-UV compared to LP-UV (Batch et al. 2004; Linden and Mofidi 2003; Malley et al. 2004). However, the authors all stated that the apparent increased sensitivity may have been due to differences in UV dose measurement and calculation rather than genuine difference in response of the virus. This is important since current protocols for UV reactor validation are based on low-pressure collimated beam dose response curves for MS2 regardless of whether the reactor being validated uses low-pressure monochromatic or medium-pressure polychromatic UV lamps. In the current study, as for the LP-UV data, most of the MP-UV inactivation data for MS2 were within the UVDGM 90% prediction limits for LP-UV disinfection of MS2 but outside of the NWRI boundaries (Figure 4.3). There was no difference between MP-UV and LP-UV dose response curves for MS2 inactivation.
Figure 4.1 Low-pressure UV inactivation of bacteriophage MS2 using two collimated beam instruments (Calgon CB and Blatchley CB). Dashed lines represent the UVDGM 90% prediction interval for MS2 inactivation (A; USEPA 2006b) and boundaries for acceptable performance described in the NWRI guidelines (B; NWRI 2003).
Figure 4.2 Investigating variability in the LP-UV response of MS2. A) Duplicate experiments performed on three separate days; B) Assessing MS suspension matrices; C) The effect of UV-irradiating host cells (11 mJ/cm²) on MS2 inactivation; D) The role of the host in determining bacteriophage response to UV radiation. For all graphs, the UVDGM 90% prediction limits for MS2 are indicated by dashed lines.
Figure 4.3 Comparison between medium-pressure and low-pressure UV inactivation of bacteriophage MS2. Dotted lines represent the UVDGM 90% prediction interval for MS2 inactivation (A; USEPA 2006b) and boundaries for acceptable performance described in the NWRI guidelines (B; NWRI 2003).
ALTERNATIVE BACTERIOPHAGES AND VIRUSES

The LP-UV dose responses of a variety of bacteriophages, with different types of genome and widely differing bacterial hosts, were evaluated and compared to MS2 (Figure 4.4). The responses of bacteriophages PP7 and PM2 overlapped the MS2 dose response curve within the range of doses investigated with 3-log inactivation at approximately 50 mJ/cm². Like MS2, PP7 is a single-stranded RNA levivirus but it infects Pseudomonas aeruginosa rather than Escherichia coli. PM2 is a double-stranded DNA virus that infects Pseudoalteromonas espejiana. Bacteriophages PRD1, Bam35, Phi29, T4, and T7 were more sensitive with 4-log inactivation at 35.5, 29.8, 16.3, 13.7, and 8.3 mJ/cm², respectively. Two published reports on UV inactivation of phage PRD1 presented conflicting data. Meng and Gerba (1996) reported 3-log inactivation with a low-pressure UV dose of 23 mJ/cm² making PRD1 more sensitive than MS2. According to Shin et al. (2005), this dose would only achieve 1.1-log inactivation so that PRD1 was more resistant than MS2 and potentially a good surrogate for adenovirus disinfection. Both studies used low-pressure UV and the same host strain to enumerate the phage (Salmonella typhimurium LT2) so the reasons for the discrepancy are not clear. Results from this project are in agreement with Meng and Gerba (1996) with 3-log inactivation at 26.7 mJ/cm². The results demonstrated that none of the bacteriophages tested were any better than MS2 as virus inactivation surrogates.

![Figure 4.4 Low-pressure UV inactivation of a variety of bacteriophages. The UVDGM 90% prediction limits for MS2 are indicated by dotted lines.](image)

Of all the bacteriophages tested, T4, T7 and φ29 (all double-stranded DNA viruses) were the most sensitive to UV irradiation. Although not an objective of this project, there is also a
need to develop a more representative challenge organism for UV disinfection of Cryptosporidium. All three of these bacteriophages may be worth considering for reactor validation at doses required for Cryptosporidium inactivation credit. A previous study also indicated that T7 might be a suitable Cryptosporidium surrogate (Fallon et al. 2007).

Although phages T4 and T7 were more sensitive than MS2 and not suitable as virus inactivation surrogates, their responses to UV were evaluated when they were assayed on a variety of host cells with varying genetic backgrounds and repair capacities to further investigate the role of the host in the observed sensitivity or resistance to UV radiation. The biggest impact on UV sensitivity for bacteriophage T4 was from a mutation in the viral genome that disabled its endonuclease V gene (endV) that is involved in repair of DNA damage (Figure 4.5). The UV dose required for 4-log inactivation of the wild type phage was 13.6 mJ/cm² compared to 3 mJ/cm² for the endV mutant when both viruses were assayed on the same *E. coli* host strain (11303). However, assaying the wild type phage on one of the alternate hosts (BAA1025) also increased its sensitivity by reducing the dose required for 4 log inactivation to 6 mJ/cm². Thus, the host has some role in the UV-sensitivity/resistance of bacterial viruses. The response of bacteriophage T7 was similar in all hosts (Figure 4.6).

Adenoviruses are double-stranded DNA viruses that infect complex eukaryotic cells whereas bacteriophages infect relatively simple bacterial cells, and MS2 is a single stranded RNA virus. Therefore, a double-stranded DNA virus that infects eukaryotic cells was evaluated as a potential challenge organism. The virus PBCV-1 is a double-stranded DNA virus that infects a *Chlorella* sp. endosymbiont of the protozoan *Paramecium bursaria*. It is structurally similar to adenoviruses and carries genes for DNA repair. However, the LP-UV dose response curve for PBCV-1 was similar to bacteriophage MS2, with most of the data falling within the UVDGM 90% prediction limits for MS2 (Figure 4.7). Therefore, PBCV-1 is no better than MS2 as a surrogate for UV inactivation of adenoviruses. It is possible that a more resistant UV response could be obtained for PBCV-1 by manipulating the host’s growth conditions to maximize DNA repair capabilities or using alternative hosts.
Figure 4.5  Low-pressure UV inactivation of bacteriophage T4 in a variety of host *Escherichia coli* strains. The dashed line corresponds to the LP-UV response of MS2.

Figure 4.6  Low-pressure UV inactivation of bacteriophage T7 assayed in a variety of host *Escherichia coli* strains. The dashed line corresponds to the LP-UV response of MS2.
Figure 4.7 Inactivation of *Chlorella* sp. virus PBCV-1 by LP-UV. The dose response of PBCV-1 was compared to MS2, the UVDGM 90% prediction limits for MS2 (dashed lines), and the LT2ESWTR dose requirements for virus inactivation credit. Insert: Plaques of PBCV-1 in a lawn of *Chlorella* sp. NC64A.

**UV INACTIVATION OF BACTERIA**

To obtain naturally occurring UV-resistant bacteria, untreated influent water from one of MWD’s treatment plants was exposed to low-pressure UV radiation and the surviving bacteria plated on R2A agar (routinely used to enumerate heterotrophic bacteria, HPC). This native population of heterotrophic bacteria was very sensitive to UV; approximately 3-log inactivation was achieved with a LP-UV dose of 9.3 mJ/cm² (Figure 4.8). Although there was some scatter within the UV dose response of mixed native HPC populations, the average response was almost identical with previous work conducted at MWD that demonstrated 1-, 2-, and 3-log inactivation of native HPC bacteria with low-pressure UV doses of 4.9, 9.7, and 14.5 mJ/cm², respectively (Mofidi and Linden 2004). Colonies of HPC bacteria that survived the highest dose were subcultured and exposed to UV again. Three of these isolates displayed increased UV-resistance compared to the mixed native HPC population (Figure 4.8). Isolates were identified by fatty acid methyl ester analysis and PCR amplification and sequencing of most of the 16S rRNA gene. Two isolates were identified as *Bacillus megaterium* and a third as a strain most closely related to *Deinococcus aquaticus* (99% similarity of 16S rRNA gene sequences; Figure 4.9).
Challenge Organisms for Inactivation of Viruses by Ultraviolet Treatment

The responses of the two native *B. megaterium* isolates were similar and the average LP-UV dose required for 3-log inactivation of the two strains was 67 mJ/cm² (Figure 4.8). *Deinococcus aquaticus* DPHPCD was more resistant with a dose of 95 mJ/cm² required for 3-log inactivation. The naturally occurring *D. aquaticus* isolate (DPHPCD) was selected for additional evaluation along with two other strains of *Deinococcus* spp. Based on aggregate data from multiple experiments, 3-log inactivation of *D. radiodurans* R1, *D. radiopugnans*, and *D. aquaticus* DPHPCD was achieved with LP-UV doses of 101, 122, and 107 mJ/cm², respectively (Figure 4.9). Of the three *Deinococcus* spp., *D. radiopugnans* was the most resistant with very little inactivation below 50 mJ/cm².

Bacteria that are resistant to ionizing radiation typically also have elevated resistance to UV radiation. Some species of methylobacteria are moderately resistant to ionizing radiation (Rainey et al. 2005) so a pink-pigmented bacterium previously isolated from MWD’s system and identified as *Methylobacterium* sp. (strain G1) was tested. However, exposure of this isolate to 30 mJ/cm² of LP-UV resulted in approximately 4-log inactivation (data not shown), making it more resistant than many bacteria but not suitable as a virus surrogate.

A high intracellular level of manganese is thought to play a role in resistance to ionizing radiation and has been suggested as a possible mechanism for elevated radiation resistance in *Deinococcus* spp. (Daly et al. 2004, 2007), so bacteria that accumulate high concentrations of Mn²⁺ may display UV resistance. This was the rationale for testing the UV response of *Lactobacillus plantarum*, a Gram-positive bacterium in which high intracellular Mn²⁺ protects...
against oxidative stress (Nierop Groot et al. 2005). However, the strain of *L. plantarum* tested for this project was very sensitive to LP-UV with >5-log inactivation at 16 mJ/cm² (data not shown).

*Deinococcus* spp. are non-pathogenic, non-sporulating, non-motile obligate aerobic bacteria that grow readily in undefined rich media at mesophilic growth temperatures (28 – 37°C). They typically grow as diplococci (two cell clusters) in the early stages of the growth cycle and as tetracocci (four cell clusters) in the later stages. The most studied species, *D. radiodurans*, is extremely resistant to ionizing radiation, UV, desiccation, and chemical and oxidative-induced damage. It has an extremely high GC content and contains a full complement of DNA repair genes. Rather than just survive ionizing radiation, it can actively grow when exposed to continuous gamma radiation (Venkateswaran et al. 2000). *Deinococcus* spp. are commonly found in dry environments such as desert soils and were the most common type of bacteria isolated from desert soils exposed to high doses of ionizing radiation (Rainey et al. 2005). They are also often found in sewage and they constitute a component of the heterotrophic population in surface waters. For example, various *Deinococcus* spp. have been isolated from MWD’s untreated source waters that pass through the Colorado Desert.

The mechanisms and evolution of extreme stress resistance are not fully understood but these resistance phenotypes appear to have emerged in the *Deinococcus* lineage by progressive amassing of cell-cleaning systems from different sources, but not by acquisition of novel DNA repair systems (Makarova et al. 2007). Most of the research on mechanisms for resistance in *Deinococcus* spp. has focused on ionizing radiation rather than UV but repair pathways may be the same for both types of radiation. Hypotheses for the extreme radiation resistance in *Deinococcus* spp. include: 1) chromosome alignment, morphology, and/or repeated sequences that facilitate genome reassembly; 2) uncharacterized genes encoding functions that enhance the efficiency of DNA repair; and 3) manganese complexes that protect proteins, but not DNA, from oxidation during irradiation, so that conventional enzyme systems involved in recovery and repair survive and function with far greater efficiency.
Figure 4.9  Response to low-pressure UV of *Deinococcus* spp. compared to the LT2ESWTR virus inactivation requirements (dashed line).
The survival of *D. radiodurans* under extreme conditions is linked to its highly efficient ability to reassemble its genome without errors even after being exposed to high levels of ionizing radiation that “shatter” its chromosomes (Lovett 2006). However, the proteins that repair damaged nucleic acids are also damaged by exposure to ionizing radiation, so DNA repair alone cannot explain the extreme resistance of these bacteria. Recent studies have demonstrated that high levels of intracellular manganese, and specifically a high manganese to iron ratio, play a role in protecting *D. radiodurans* proteins from ionizing radiation and desiccation (Daly et al. 2004, 2007) so that they can still function to repair radiation-induced DNA damage.

Potential disadvantages of *Deinococcus* spp. as challenge organisms for UV reactor testing and validation include growing them to a sufficiently high titer to allow large-scale testing, the pronounced shoulder and tail in their UV response, and their growth habit as diplococcic and tetrads. When cultured in non-supplemented nutrient broth, the concentration of *D. radiodurans* cells was typically $1–2 \times 10^6$ cfu/mL. Therefore, the growth of *D. radiodurans* R1 was investigated in a variety of media with additional growth factors and additional carbon sources. Maximum growth yields were obtained in nutrient broth supplemented with 1% glucose, various minerals (0.5 mg/L EDTA, 3 mg/L MgSO₄·7H₂O, 0.5 mg/L MnSO₄·H₂O, 1 mg/L NaCl and salts of iron, cobalt, calcium, zinc, and copper at 0.1 mg/L) and a mixture of vitamins (Table 4.1). The growth yield in this medium was approximately 1,000-fold higher ($1.1 \times 10^9$ cfu/mL) than in non-supplemented nutrient broth. This titer was high enough for demonstration-scale testing at 0.5 MGD but would not be sufficient for large, full-scale testing. However, *D. radiodurans* cells grown under these conditions displayed approximately 1.5-log increased inactivation compared to cells grown in just nutrient broth, making them less suitable as surrogates for the LT2ESWTR UV dose requirements for virus inactivation. In contrast to the results with *D. radiodurans*, *Deinococcus radiopugnans* displayed approximately 1.5-log greater resistance when grown in the vitamin, mineral, and glucose supplemented broth but with a pronounced S-shaped dose response curve due to a substantial response lag and tail.

The UVDGM provides guidelines for the use of challenge organisms that demonstrate a dose response shoulder (lag) and tailing (flattening of the curve at higher doses) in collimated beam tests (USEPA 2006b). UV sensitivity is defined as sensitivity over the linear-log region of the dose response curve that occurs between any inactivation shoulder and the onset of tailing. Furthermore, a challenge microbe should only be used to demonstrate reduction equivalent doses (RED) values greater than or equal to twice the point at which the shoulder ends. Tailing should not begin until at least 1-log inactivation beyond the measured reactor inactivation range. Following these guidelines, the use of *D. radiodurans* as a challenge microbe would be limited to a reactor testing range of 60 – 120 mJ/cm², *D. aquaticus* DPHPCD to 20 – 120 mJ/cm², and *D. radiopugnans* to doses >100 mJ/cm².

*Deinococcus* species typically grow in liquid culture as diplococci and tetrad clusters that are approximately 5 µm in diameter. Consequently, obtaining monodispersed suspensions of individual cells may not be possible. If three cells in a tetrad are inactivated by UV radiation but not the fourth, the 75% inactivated tetrad will still produce a colony; for organisms that grow as tetrads, a colony forming unit may contain 1 – 4 cells. Consequently, survival frequencies for a single-celled population cannot be determined experimentally. To address the possibility of tetrads or clumps of tetrads affecting the UV dose response, *D. radiodurans* R1 cell suspensions were passed through 5 µm porosity membrane filters either before exposure to UV or following UV exposure, prior to plating. The response of these filtered cells was the same as the control unfiltered cell suspension (Figure 4.10). A UV dose of 60 mJ/cm² resulted in 4.1, 4.0, and 3.9-
log inactivation for cells that were unfiltered, filtered before UV exposure, and filtered after UV exposure, respectively. For this experiment, *D. radiodurans* was grown in nutrient broth supplemented with 1% glucose and minerals, which produced cells that were more sensitive to UV compared to cells cultured in non-amended nutrient broth.

Since there was considerable variability within the response of *Deinococcus* spp. to similar UV doses (see Figure 4.9), duplicate experiments were conducted on separate days to determine the amount of inherent variability. Although intra-day replicates were generally closer together than inter-day replicates for *D. radiodurans*, the maximum variability values were similar (Figure 4.11). Maximum inactivation variability between intra-day duplicates was 0.6-log and 0.9-log for inter-day variability.

### Table 4.1

Growth yields of *Deinococcus radiodurans* R1 in various media formulations

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>Mean ± s.d. (cfu/mL)</th>
<th>N*</th>
<th>Yield †</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone-Glucose-Yeast extract + methionine</td>
<td>9.9 ± 6.7 × 10⁴</td>
<td>3</td>
<td>0.07</td>
</tr>
<tr>
<td>Tryptone-Yeast extract + 0.4% glycerol</td>
<td>6.3 ± 1.4 × 10⁵</td>
<td>3</td>
<td>48.5</td>
</tr>
<tr>
<td>Tryptone-Yeast extract + 20 mM glucose + 10 mM MgSO₄</td>
<td>1.4 ± 0.1 × 10⁸</td>
<td>3</td>
<td>107.7</td>
</tr>
<tr>
<td>Tryptic soy broth</td>
<td>2.1 ± 0.3 × 10⁸</td>
<td>3</td>
<td>161.5</td>
</tr>
<tr>
<td>Nutrient broth</td>
<td>1.3 ± 0.6 × 10⁶</td>
<td>3</td>
<td>1.0</td>
</tr>
<tr>
<td>Nutrient broth + lactate</td>
<td>3.5 ± 0.2 × 10⁵</td>
<td>3</td>
<td>0.3</td>
</tr>
<tr>
<td>Nutrient broth + 1% glucose</td>
<td>3.0 ± 0.9 × 10⁵</td>
<td>3</td>
<td>23.1</td>
</tr>
<tr>
<td>Nutrient broth + 2% glucose</td>
<td>1.9 ± 0.3 × 10⁷</td>
<td>3</td>
<td>14.6</td>
</tr>
<tr>
<td>Nutrient broth + 1% glucose + acetate</td>
<td>2.7 ± 0.2 × 10⁷</td>
<td>3</td>
<td>20.8</td>
</tr>
<tr>
<td>Nutrient broth + 1% glucose + vitamins</td>
<td>2.9 ± 0.2 × 10⁷</td>
<td>3</td>
<td>22.3</td>
</tr>
<tr>
<td>Nutrient broth + 1% glycerol</td>
<td>4.1 ± 0.3 × 10⁷</td>
<td>3</td>
<td>31.5</td>
</tr>
<tr>
<td>Nutrient broth + 1% glucose + yeast extract</td>
<td>1.5 ± 0.2 × 10⁸</td>
<td>3</td>
<td>115.4</td>
</tr>
<tr>
<td>Nutrient broth + 1% glucose + minerals ‡</td>
<td>8.0 ± 2.9 × 10⁹</td>
<td>6</td>
<td>615.4</td>
</tr>
<tr>
<td>Nutrient broth + 1% glucose + vitamins § + minerals</td>
<td>1.1 ± 0.05 × 10⁹</td>
<td>3</td>
<td>846.2</td>
</tr>
</tbody>
</table>

* Number of replicates. For each replicate, bacterial colonies were enumerated in triplicate.
† Growth yield relative to non-amended nutrient broth. Nutrient broth contains beef extract and peptone.
‡ The primary components of the trace mineral supplement (ATCC) were EDTA (0.5 mg/L), MgSO₄·7H₂O (3 mg/L), MnSO₄·H₂O (0.5 mg/L), NaCl (1 mg/L) and salts of iron, cobalt, calcium, zinc, and copper at 0.1 mg/L.
§ The vitamin supplement contained pyridoxine hydrochloride (10 mg/L), and riboflavin, thiamine, nicotinic acid, calcium pantothenate, para-aminobenzoic acid, and thioctic acid, all at 5 mg/L.
Figure 4.10 The effect of filtering suspensions of *Deinococcus radiodurans* R1 on the organism’s response to LP-UV radiation. Cells were cultured in nutrient broth supplemented with 1% glucose and minerals.

\[
y = -0.0012x^2 + 0.2277x - 5.2572 \\
R^2 = 0.9864
\]

Figure 4.11 Variability in low-pressure UV inactivation of *Deinococcus radiodurans* R1. Duplicate experiments were performed on two separate days (○ and ◊) and bacterial colonies were enumerated in triplicate. Cells were cultured in nutrient broth supplemented with 1% glucose and minerals.
Rubrobacter radiotolerans is a red pigmented aerobic bacterium that is related to the high G+C content Gram-positive bacteria (actinobacteria; Kausar et al. 1997). Similar to Deinococcus spp., it is highly resistant to gamma irradiation (Yoshinaka et al. 1973). It displayed a similar LP-UV response as Deinococcus spp. with similar shoulder and tail, requiring 146 mJ/cm² for 3-log inactivation (Figure 4.12) and a good correlation with the LT2ESWTR dose requirements for UV inactivation of viruses. However, the organism grows slowly, requiring 9 – 12 day incubation periods, and does not grow to high titers, which limits its utility as a practical challenge microbe for large-scale UV testing.

Although MP-UV appeared to be slightly more effective against D. aquaticus DPHPCD (Figure 4.13), considering the variability demonstrated in Figure 4.11, the MP-UV dose response was essentially the same as the LP-UV response. A direct comparison between MP- and LP-UV using the same bacterial suspension supported this conclusion (Figure 4.13, inset). Based on a polynomial regression analysis of aggregate data, 3-log inactivation was achieved with MP- and LP-UV doses of 85 and 94 mJ/cm², respectively.

![Figure 4.12 Low-pressure UV inactivation of Rubrobacter radiotolerans.](image-url)
A microorganism that is used as a pathogen surrogate for reactor validation should be sensitive to conventional chlorine disinfection so that it can be inactivated following the reactor test, even though it is not pathogenic. In batch-reactor bench tests, a chlorine CT of 180 mg-min/L (3 mg/L for 60 min), which is the standard decontamination condition for test water held in the clearwell of MWD’s demonstration-scale treatment plant, achieved >5-log inactivation of *D. radiodurans* and *D. radiopugnans*. Challenge microbes should also be relatively stable in storage so that fresh stocks do not need to be produced for each experiment or validation trial. Cell suspensions of *D. radiodurans*, *D. radiopugnans*, and *D. aquaticus DPHPCD* were stable at 4°C with no significant change in cell concentration for up to three months.

**DEINOCOCCUS SPP. PHAGE**

Since strains of *Deinococcus* spp. are highly resistant to UV radiation, it may be reasonable to speculate that phages of *Deinococcus* spp. would also display high levels of resistance to UV radiation, because they may be protected and repaired by the same mechanisms that the host cell uses. However, no phages have been isolated from *Deinococcus* spp., although this may be due to the relatively few attempts to isolate them rather than their actual absence.
The genome of *D. radiodurans* R1 contains a mu-like prophage integrated into the bacterial chromosome (Morgan et al. 2002) but the conditions under which its lytic cycle can be induced are not known. Isolating native bacteriophage from environmental samples that infect Gram-negative and Gram-positive bacteria is relatively straightforward and phages that infect *Thermus* spp. have been isolated (Yu et al. 2006). *Thermus* is a genus of bacteria that is related to *Deinococcus* and forms part of the broader *Thermus-Deinococcus* eubacterial phylum. Over 100 diverse phages were isolated from alkaline hot springs and were identified as *Myoviridae*, *Siphoviridae*, *Tectiviridae*, and *Inoviridae*, but most of them were narrowly host-specific.

For this project, all attempts to isolate bacteriophages that formed plaques on strains of *Deinococcus* spp. were unsuccessful. This included agar overlays of chloroform-treated primary clarified sewage using the ATCC strains of *D. radiodurans* R1 and *D. radiopugnans*, and the natural isolate *D. aquaticus* as potential host bacteria, as well as agar overlays of LP-UV irradiated sewage samples on the same hosts. In addition, 30 naturally occurring pigmented *Deinococcus*-like isolates recovered from sewage following exposure to 50 – 200 mJ/cm² of UV were plated as agar overlays to determine whether any of them were hosts to indigenous plaque-forming phage. Plaques did not form on any of these isolates. While not exhaustive, this study confirmed that plaque-forming bacteriophages specific to *Deinococcus* spp. are not common. *Deinococcus* phages may exist but they may not form plaques on the three host strains used, they may not form plaques at all on any host strains, they may be present in concentrations too low to be detected by the methods used, or their isolation may require conditions that were not provided in this project. Further attempts to isolate bacteriophages of *Deinococcus* spp. were beyond scope of this project.

**INACTIVATION OF BACTERIAL SPORES**

Bacterial spores are typically more resistant to physical and chemical stresses than vegetative cells. *Bacillus subtilis* spores are used as biosimeters for heat and ethylene oxide sterilization, and UV disinfection; the UVDGM provides protocols for UV reactor validation based on inactivation of *B. subtilis* spores. *Bacillus subtilis* 6633 was demonstrated to be a suitable disinfection surrogate for *Bacillus anthracis* (Nicholson and Galeano 2003) and is more UV resistant than most other strains of *B. subtilis*. In this study, LP-UV inactivation data for spores of *B. subtilis* 6633 were within the UVDGM 90% prediction limits for spores with 3-log inactivation at 59 mJ/cm² (Figure 4.14), similar to a previously reported LP-UV dose of approximately 50 mJ/cm² for 3-log inactivation of two strains of *B. subtilis* and *B. anthracis* (Nicholson and Galeano 2003). Spores of a standard laboratory strain of *Bacillus pumilus* (ATCC 7061) had a similar doses response while spores of native *Bacillus megaterium* isolate BPHPCC were slightly more resistant (3-log inactivation at 86 mJ/cm²; Figure 4.14).

Native aerobic spore-forming (ASF) bacteria were isolated by exposing untreated surface water to LP-UV. The characteristics of this water were turbidity of 1.05 NTU, pH 7.02, and hardness of 134 mg/L measured as CaCO₃. The average concentration of culturable spores from ASF bacteria in surface water was $4.2 \times 10^4$ cfu/L (N = 5). This concentration was reduced to $1.8 \times 10^4$ cfu/L (N=6) following exposure to LP-UV doses of $13 - 56$ mJ/cm², $2 \times 10^2$ cfu/L (N=3) after 220 mJ/cm², and $1.3 \times 10^2$ cfu/L (N=3) at 320 mJ/cm², equivalent to 1-log inactivation of uncultured indigenous spores at approximately 180 mJ/cm² (Figure 4.15A).
Two colonies that survived 320 mJ/cm² were subcultured and identified by fatty acid methyl ester analysis and 16S rDNA amplification and sequencing. Two of these isolates, ASFUVRA and ASFUVRC were identified as *Bacillus pumilus* (see Figure 2.1). Spores of these native isolates were more resistant to LP-UV than two ATCC strains (7061 and 27142). A dose of 160 mJ/cm² resulted in approximately 2-log inactivation, which is considerably more resistant than Ad2 and the dose required for virus inactivation in the LT2ESWTR (Figure 4.15B). The dose response of the native *B. pumilus* spores was similar to the response of Ad41.

The UV response and inactivation of bacterial spores is species and strain specific and depends on the media and procedures used to prepare spore suspensions. In this project the presence of manganese sulfate (MnSO₄) in the media used for *Bacillus pumilus* spore propagation strongly influenced the level of UV resistance. ASFUVRA and ASFUVRC spores were most sensitive to UV when grown in sporulation medium with no MnSO₄ and were most resistant when the spores were propagated by culturing in sporulation medium containing 1 mM MnSO₄ (Figures 4.16 and 4.17). There was a direct correlation between the concentration of MnSO₄ in the spore propagation medium and the level of UV resistance, expressed as the UV dose required for 3-log inactivation (Figure 4.17B). Manganese is needed for efficient sporulation in *Bacillus* spp. so culturing cells in medium containing no or very little manganese (0 and 0.01 mM MnSO₄) may cause inefficient or incomplete sporulation that produces spores with reduced resistance to UV.
Figure 4.15 Low-pressure UV inactivation of bacterial spores. A) Indigenous aerobic spores in raw surface water were exposed to LP-UV following heat inactivation of vegetative cells but without culturing. B) Inactivation of *Bacillus pumilus* spores from cultured isolates.
Figure 4.16 Low-pressure UV dose response of *Bacillus pumilus* isolate ASFUVRA. Spores were propagated by culturing the isolate in the presence and absence of MnSO₄.

An additional strain of *B. pumilus* (SAFR032) was provided by Dr. K. Venkateswaran at the California Institute of Technology Jet Propulsion Laboratory (Pasadena, CA). Spores of this strain have previously been shown to be resistant to various environmental stresses (Newcombe et al. 2005). Spores of this strain were also highly resistant to LP-UV and, as with the two native sporeformers recovered from MWD source water, the dose response could be manipulated by varying the concentration of MnSO₄ in the culture medium used for spore propagation (Figure 4.18). The LP-UV dose required for 3-log inactivation of spores of the three strains of *B. pumilus* in the absence of manganese were 59, 77, and 76 mJ/cm² for ASFUVRA, ASFUVRC, and SAFR032, respectively. For spores propagated in medium containing 1 mM MnSO₄, the doses required for 3-log inactivation were greater than 150 mJ/cm² for all three strains. A dose of 250 mJ/cm² resulted in only 2-log inactivation of *B. pumilus* SAFR032. These results indicate that the UV response of *B. pumilus* spores can be manipulated to more closely correlate with the response of adenoviruses or other UV resistant pathogens. The dose response of Ad2 was within the region of inactivation covered by *B. pumilus* spores propagated with 0 – 0.03 mM MnSO₄.
Figure 4.17 The effect of MnSO$_4$ in spore propagation culture medium on UV resistance of *B. pumilus* ASFUVRC. A) Dose responses of spores propagated in culture medium containing various concentrations of MnSO$_4$ compared to dose response curves for Ad2 and LT2ESWTR-Virus dose requirements. B) Correlation between MnSO$_4$ concentration in spore propagation medium and the LP-UV dose required for 3-log inactivation.
Figure 4.18 Effect of MnSO₄ in spore propagation culture medium on UV resistance of B. pumilus isolate SAFR032.

Since manganese in the spore propagation medium had such a pronounced effect on the UV response of native B. pumilus spores, a limited number of experiments were also conducted with B. subtilis spores (ATCC 6633) and spores of a standard laboratory strain of B. pumilus (ATCC 7061). Spores of B. subtilis 6633 propagated in liquid medium containing 1 mM MnSO₄ displayed the same high level LP-UV resistance as B. pumilus ASFUVRC spores (Figure 4.19). However, B. pumilus 7061 spores propagated in the presence of manganese were just as sensitive as spores propagated without manganese (Figure 4.19). These results demonstrate that the affect of manganese on the response of Bacillus spp. spores is not universal for all species and strains of spore-forming bacilli.

Other studies have also reported high UV resistance in Bacillus spp. spores. A dose of approximately 175 mJ/cm² was necessary for 3-log₁₀ inactivation of indigenous B. pumilus spores isolated from a source water reservoir (Collins and Malley 2006), and 2.6- and 5.4-log inactivation was reported at 150 and 250 mJ/cm², respectively, with no evidence of tailing up to 250 mJ/cm² (Malley et al. 2007). Spores of another B. pumilus isolate, recovered from equipment surfaces exposed to ultra-cleaning procedures, required a dose of 350 mJ/cm² for 4.5-log₁₀ inactivation (Link et al. 2004), and only 1-log inactivation was reported for Bacillus atrophaeus spores exposed to 133 mJ/cm² (Yung and Ponce 2008).

There are also a few reports of high UV resistance in other microbes. Spores of Aspergillus niger required a LP-UV doses of 450 mJ/cm² for 2.5-log inactivation (Petri and Odegaard 2008), Acanthamoeba spp. cysts required 145 mJ/cm² for 4-log inactivation (Maya et al. 2003), and a dose of approximately 100 mJ/cm² resulted in 2-log inactivation of spores of environmental isolates of sulfite-reducing Clostridium perfringens (Hijnen et al. 2004).
Challenge Organisms for Inactivation of Viruses by Ultraviolet Treatment

Figure 4.19 Effect of 1 mM MnSO₄ in spore propagation medium on LP-UV responses of spores of various strains of Bacillus spp.

The growth conditions and medium composition used for propagating Bacillus spp. spores influence the phenotypic properties of the spores. For example, increasing the incubation period prior to harvesting spores increased resistance of B. subtilis spores to various chemical agents and heat (Gilbert et al. 1995). In this study, spores of B. pumilus ASFUVRC propagated by culturing in Columbia broth were more resistant to LP-UV than spores from bacteria cultured in sporulation medium in the presence and absence of manganese (Figure 4.20). Incubation of B. pumilus ASFUVRC in sporulation medium plus 1 mM MnSO₄ for 15 days prior to harvesting spores produced spores with higher UV resistance compared to spores harvested from a five day old culture (Figure 4.21). The LP-UV dose for 1-log inactivation of spores from 15 day old cultures (172 mJ/cm²) was approximately 2.5-fold higher than for spores from 5 day old cultures (74 mJ/cm²). This is possibly due to increased accumulation of intracellular manganese in spores generated by cells that are cultured for longer periods. However, once a spore suspension is prepared, the UV response of spores does not change with storage.

The Bacillus subtilis spore production method detailed in the UVDGM includes multiple rounds of sonication. Therefore, the effect of sonication on the UV dose response of B. pumilus ASFUVRC was assessed using spores propagated in sporulation medium containing 1 mM MnSO₄ and in AK broth. Following heating at 80°C for 20 min to kill vegetative cells, cold spore suspensions were sonicated at 40 kHz for 10 min. There was no significant difference in the dose response curves between sonicated and non-sonicated spore preparations prepared in either medium, indicating that sonication is not necessary for this B. pumilus strain (Figure 4.22).
Figure 4.20 The effect of culture medium and manganese on UV resistance of Bacillus pumilus ASFUVRC spores.

Figure 4.21 Increased UV resistance in Bacillus pumilus ASFUVRC spores following prolonged incubation in sporulation medium containing 1 mM MnSO$_4$ prior to harvesting.
Figure 4.22 The effect of sonication on UV resistance of spores. Spores of *Bacillus pumilus* ASFUVRC were propagated in sporulation medium containing 1 mM MnSO₄ (A) or AK Broth (B).
One of the requirements of a practical surrogate for large-scale reactor validation is the ability to generate sufficiently high titers of the surrogate microbe. Propagating *B. pumilus* spores in sporulation medium, Columbia broth, and nutrient broth resulted in spore concentrations of approximately $10^8$/mL, which is sufficient for bench-scale and low-flow demonstration-scale experiments. However, higher spore concentrations are necessary for high-flow demonstration-scale and full-scale applications. Therefore, a commercially prepared medium, AK Agar #2, which was developed for production of *Bacillus subtilis* spores, was evaluated. The procedure involves growing the organism on solid agar plates for 5 days at 35°C, washing off the resulting growth in PBS, concentrating cells by centrifugation, and then heating at 70°C for 30 min to kill vegetative cells. This medium and procedure generated concentrations of approximately $10^{10}$ spores/mL. However, spores propagated on AK Agar displayed a hybrid UV response, similar to spores produced using MnSO$_4$-containing broth at UV doses $\leq 50$ mJ/cm$^2$ but appearing to be more sensitive at higher doses, as with spores produced without MnSO$_4$ (Figure 4.23). AK Agar #2 contains 3 g/L of MnSO$_4$ (1.8 mM) but since cells grow on the agar surface they may not be able to accumulate as much intracellular MnSO$_4$ as broth-grown cultures. Sommer and Cabaj (1993) reported that *B. subtilis* spores sporulated on the surface of a solid medium were more sensitive to LP-UV compared to spores that were cultured in liquid medium. Therefore, *B. pumilus* ASFUVRC spores were produced by culturing in AK broth, based on the manufacturer’s formulation but omitting the agar. These spores were highly resistant to LP-UV irradiation with 2.1-log inactivation at 400 mJ/cm$^2$. This same pattern of resistance was also observed with the SAFR032 isolate of *B. pumilus* (results not shown). Table 4.2 provides a summary of the dose requirements for inactivation of *B. pumilus* spores propagated in various media, based on best fit regressions of their LP-UV dose response curves.

Ideally, spores should be prepared fresh for each experiment or validation trial. However, this is not always practical so it may be necessary to use older spore preparations. Therefore, spores of *B. pumilus* ASFUVRC were stored at 4°C for up to 12 months. There was a general trend of decreased viability as the age of spore suspensions increased with an average loss in viability (assessed by enumeration of spores on agar plates) of 0.07-log per month of storage. The viable count was reduced by 0.93-log after 12 months storage at 4°C but a second preparation of ASFUVRC spores and spores of *B. pumilus* SAFR032 were only reduced by 0.08-log after 5 months storage. These data indicate that *B. pumilus* spores are relatively stable in storage at 4°C and will be suitable for assessing UV reactor performance for at least three months after preparing the suspension.

An additional requirement for UV reactor challenge organisms is that they should be prepared as monodispersed suspensions without clumping. Clumped cells are likely to be protected to some extent in a UV irradiated suspension thus increasing the apparent resistance of the organism. Microscopic observation of malachite green stained suspensions of spores prepared by each of the methods of spore propagation used in this project demonstrated that the spores were relatively evenly distributed with no substantial clumping (Figure 4.24). A few dead vegetative cells were observed (stained pink in original images) but the majority of the cells were spores (stained green in original).

Medium pressure UV was considerably more effective than LP-UV for inactivating *B. pumilus* ASFUVRC spores (Figure 4.25). An MP-UV dose of 120 mJ/cm$^2$ resulted in 4.8 log inactivation compared to 1.6 log inactivation with the same dose of LP-UV. The data in Figure 4.25 were combined from multiple experiments with both LP- and MP-UV but spores
for all experiments were propagated in the same manner, culturing bacteria in sporulation medium containing 1 mM MnSO₄. In a direct comparison using the same spore stock, 2-log inactivation was achieved with MP- and LP-UV doses of 43 and 148 mJ/cm², respectively.

Table 4.2
Dose requirements for inactivation of *B. pumilus* spores and viruses

<table>
<thead>
<tr>
<th></th>
<th>Low-pressure UV dose required for inactivation:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-log</td>
</tr>
<tr>
<td>LT2ESWTR</td>
<td>58</td>
</tr>
<tr>
<td>Adenovirus 2</td>
<td>31</td>
</tr>
<tr>
<td>Adenovirus 41</td>
<td>81</td>
</tr>
</tbody>
</table>

*Bacillus pumilus* spores

<table>
<thead>
<tr>
<th></th>
<th>1-log</th>
<th>2-log</th>
<th>3-log</th>
<th>4-log</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASFUVRA, 1 mM MnSO₄</td>
<td>99</td>
<td>187</td>
<td>276</td>
<td>*</td>
</tr>
<tr>
<td>ASFUVRA, 0 mM MnSO₄</td>
<td>*</td>
<td>22</td>
<td>52</td>
<td>122</td>
</tr>
<tr>
<td>ASFUVRC, 1 mM MnSO₄</td>
<td>82</td>
<td>174</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>ASFUVRC, 0.1 mM MnSO₄</td>
<td>53</td>
<td>102</td>
<td>151</td>
<td>*</td>
</tr>
<tr>
<td>ASFUVRC, 0.03 mM MnSO₄</td>
<td>47</td>
<td>73</td>
<td>103</td>
<td>137</td>
</tr>
<tr>
<td>ASFUVRC, 0.01 mM MnSO₄</td>
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<td>61</td>
<td>90</td>
<td>122</td>
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<tr>
<td>ASFUVRC, 0 mM MnSO₄</td>
<td>36</td>
<td>56</td>
<td>77</td>
<td>97</td>
</tr>
<tr>
<td>ASFUVRC, 1 mM MnSO₄⁺</td>
<td>172</td>
<td>345</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>ASFUVRC, AK Agar (1.8 mM MnSO₄)</td>
<td>37</td>
<td>53</td>
<td>77</td>
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<tr>
<td>ASFUVRC, AK Broth (1.8 mM MnSO₄)</td>
<td>197</td>
<td>353</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>SAFR032, 1 mM MnSO₄</td>
<td>149</td>
<td>276</td>
<td>*</td>
<td>*</td>
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<tr>
<td>SAFR032, 0 mM MnSO₄</td>
<td>26</td>
<td>52</td>
<td>77</td>
<td>103</td>
</tr>
</tbody>
</table>

* Required extrapolation beyond available data.
⁺ Spores were propagated by incubating cells for 15 day prior to harvesting the spores, instead of the usual 5 days.
Figure 4.23 LP-UV responses of *Bacillus pumilus* ASFUVRC spores propagated in different culture media.

Figure 4.24 Malachite green stained spores (darker, smaller cells →) and dead vegetative cells (lighter, larger cells △) of *Bacillus pumilus* ASFUVRC.
**Figure 4.25 Comparison of low- and medium-pressure UV for inactivating *Bacillus pumilus* ASFUVRC spores.**

*Bacillus pumilus* is a ubiquitous Gram-positive, aerobic, rod-shaped endospore-forming bacterium that is often isolated from a wide variety of soils, water, plants, environmental surfaces, and the interior of Sonoran desert basalt (Benardini et al. 2003). *Bacillus pumilus* isolates were also recently recovered aboard the International Space Station from hardware surfaces (Newcombe et al. 2005). The endospores of many strains of *B. pumilus* typically demonstrate elevated resistance to unfavorable conditions such as low or no nutrient availability, extreme desiccation, H₂O₂, UV and gamma radiation, and chemical disinfection, when compared to the spores of many other *Bacillus* species. Humans and animals are commonly exposed to *B. pumilus* due to its ubiquitous occurrence in the environment. In addition, *B. pumilus* is included in many probiotic supplements for both human and animal consumption (Duc et al. 2004) and some strains are used as biological pesticides for agricultural applications (USEPA 2004). *Bacillus pumilus* is generally regarded as non-pathogenic for the general population, including children and infants. Of the non-anthrax *Bacillus* species, *B. cereus*, *B. licheniformis*, and *B. pumilus* may be more pathogenic in severely immunosuppressed hosts than other common *Bacillus* species such as *B. subtilis* or *B. megaterium*. However, *B. pumilus* has rarely been reported as a human pathogen, and even then it was under unusual surgical and clinical conditions (Bentur et al. 2007). Some strains produce a putative emetic toxin and extracellular proteases that may contribute to tissue damage in periodontitis (From et al. 2005; Johnson et al. 2008).

*Bacillus pumilus* is a common environmental bacterium and two naturally occurring strains that produced UV resistant spores were isolated from raw water for this project (ASFUVRA and ASFUVRC). Raw water samples from a potential participating utility were also assessed for the presence of UV resistant ASF. The results confirmed that *B. pumilus* is relatively common in untreated source waters. Average ASF concentrations in samples from two locations were 17 and 180 cfu/L. Selected colonies were identified by fatty acid methyl ester (FAME) analysis as *Bacillus cereus* and *Bacillus mycoides*. ASF isolates were also recovered following
exposure of source water samples to 100 mJ/cm² of LP-UV. These isolates were all identified as *Bacillus* species by FAME: *B. pumilus*, *B. mycoides*, *B. cereus*, and *B. megaterium*.

Bacterial spores are typically 10 – 50 times more resistant to UV₂₅₄ radiation than actively growing cells due to a difference between spores and vegetative cells in the UV photochemistry of DNA, and relatively error-free repair of UV-induced DNA damage in spores (Nicholson et al. 2000). The major UV photoproduct in the DNA of growing bacterial cells is a cyclobutane thymine-thymine dimer (thymine dimer) with bonding between the fifth and sixth position carbon atoms on adjacent thymine residues. However, the primary DNA damage in bacterial spores exposed to 254 nm UV radiation is 5-thyminyl-5,6-dihydrothymine, which is called Spore Photoproduct (SP). Endospores of *Bacillus* spp. contain large amounts of small acid-soluble proteins (SASP) bound to their DNA. These proteins are synthesized during the later stages of the sporulation process and upon binding to DNA shift its conformation from the usual B form to the A form. This structurally changed DNA complexed with SASP results in the formation of SP instead of thymine dimers or (6-4) photoproducts upon exposure to UV radiation. In *B. subtilis* spores, SP damage is repaired by the spore enzyme SP lyase during germination and the SP lyase gene (*splAB* operon) is also present in the genome of *B. pumilus* SAFR032, along with genes for many different types of DNA repair mechanisms (Gioia et al. 2007). Repair of SP by SP lyase is a very efficient process and accounts for the generally higher UV resistance of spores compared to vegetative cells. NER also has a role in repairing spore DNA upon germination but to a lesser extent than SP lyase.

**BACTERIAL ACTION SPECTRA**

The medium pressure action spectrum for *D. aquaticus* DPHPCD was examined using eight bandpass filters with wavelengths spanning 220 – 289 nm and peak band widths of 9 – 11 nm. The most effective wavelengths were 220 – 228 nm and 260 – 270 nm (Figure 4.26A). The action spectrum was similar to that reported for spores of *Bacillus subtilis* 6633, which were most sensitive to 265 nm UV (Mamane-Gravetz et al. 2005). The lack of a pronounced inactivation peak at UV₂₂₀₋₂₂₈ in the *D. aquaticus* action spectrum is consistent with the similarity of the LP-UV and MP-UV dose response curves for this organism and the suggestion that *Deinococcus* spp. proteins are protected from UV damage by intracellular manganese (Daly et al. 2007). The action spectra for two other species of *Deinococcus* displayed the same overall shape as *D. aquaticus* DPHPCD but with more pronounced inactivation at UV₂₂₀₋₂₂₈ (Figure 4.26B).

The action spectrum for *B. pumilus* ASFUVRC spores is displayed in Figure 4.27. Of the wavelengths tested, highest inactivation occurred at 220 – 228 nm (approximately 8-fold higher inactivation than at 254 nm). This is different from the action spectrum for *B. subtilis* spores, which were reported to be most sensitive to UV₂₆₅ (Mamane-Gravetz et al. 2005) but displayed no significantly increased sensitivity at any wavelength relative to UV₂₅₄. The ASFUVRC action spectrum was similar to the Ad2 spectrum. The absorbance spectrum for a spore homogenate (prepared by repeated freezing and thawing of a spore suspension) was displayed the same overall shape as the inactivation action spectrum. A comparison of action spectra for a variety of microorganisms, including Ad2, demonstrated the same general pattern: highest inactivation at 220 – 230 nm, a steep decline in effectiveness to a minimum at 240 – 250 nm, a relatively broad peak at 260 – 280 nm, followed by a decline (Figure 4.28). The primary difference between organisms was the amount of inactivation at UV₂₂₀₋₂₂₈ relative to UV₂₅₄. This ratio varied from almost 12-fold for Ad2 to less than 2-fold for Ad41 and *D. aquaticus* DPHPCD. The *B. pumilus*
action spectrum was essentially the same as the Ad2 action spectrum from this project and an earlier study (Malley et al. 2004). The only difference was the relative increased effectiveness at the lower wavelengths. This pattern of relative inactivation efficiency is to be expected based simply on the UV absorbance properties of biological molecules. Nucleic acids and proteins have absorbance maxima at 260 nm and 220 – 230 nm, respectively, so most damage to DNA occurs at wavelengths around 260 nm whereas proteins are damaged at the shorter wavelengths. The absorbance peak for thymine nucleotides is 260 – 270 nm. UV-induced DNA damage can be repaired to varying extents but UV-damaged proteins are irreparable. Adenovirus proteins are involved in attachment of the virus to host cells so if these proteins are damaged by UV radiation, the virus will not be able to attach and subsequently infect cells and replicate, regardless of whether or not their DNA can be repaired. Proteins complexed with DNA in the virus core are also involved in the infection process. In addition, Ad2 DNA accumulated the same number of lesions regardless of whether the virus was exposed to LP- or MP-UV (Eischeid et al. 2009). Therefore, the increased efficacy of UV220-228 relative to UV254 and of MP-UV relative to LP-UV can be explained by damage to virus proteins. Considering the intimate role of SASP in protecting spore DNA from the lethal effects of thymine dimer formation, it is likely that the increased effectiveness of UV220-228 against B. pumilus ASFUVRC is due to damage to SASP proteins.

For microbes that demonstrated a marked difference between LP and MP inactivation (e.g., Ad2 and B. pumilus ASFUVRC spores), the action spectra displayed a dramatically higher inactivation at 220 nm relative to 254 nm. However, for both organisms that displayed similar responses to LP-UV and MP-UV (Ad41 and D. aquaticus, data plotted but no lines in Figure 4.28) there was only slightly increased inactivation at 220 nm relative to 254 nm. This demonstrates a consistency between two very different organisms in their response to MP-UV and indicates that the similarity in the LP-UV and MP-UV dose responses of Ad41 are genuine and not an artifact of experimental procedures. The action spectra for Ad41 and D. aquaticus DPHPCD were almost identical to the spectral sensitivity of B. subtilis 6633 spores (Mamane-Gravetz et al. 2005).

However, UV action spectra should be interpreted with caution, particularly at shorter wavelengths such as 220 – 228 nm. Band-pass filters are not ideal for assessing the relative effectiveness of different wavelengths because radiation transmittance, and hence the fluence rate, is reduced by the filters (Linden et al. 2007). Therefore, longer exposure periods are required to achieve equivalent doses. For example, to achieve a UV dose of approximately 200 mJ/cm² for B. pumilus spores in this project, exposure times for the 220, 228, 239, 254, 260, 270, 280, and 289 nm filters were 1105, 653, 490, 349, 302, 531, 641, and 495 seconds, respectively, compared to 18 seconds for unfiltered radiation. Time-fluence reciprocity has been demonstrated for MS2 and B. subtilis spores (Sommer et al. 1998). Therefore, sensitivity of B. pumilus spores at 220 nm and 228 nm may be artificially elevated due to time-fluence reciprocity, even though calculated doses based on radiation transmittance of the filter and exposure times were the same.
Figure 4.26 Medium pressure action spectra for *Deinococcus* spp. Duplicate experiments were conducted for *D. aquaticus* DPHPCD (A) and single experiments were performed for *D. radiodurans* and *D. radiopugnans* (B). Best-fit polynomial regressions were used to generate all of the curves.
Figure 4.27 Medium pressure UV action spectrum for *Bacillus pumilus* ASFUVRC (solid line) compared to absorbance of spore homogenate (dashed line).

Figure 4.28 Comparison of UV action spectra for a variety of microbes.
A compounding factor may be that inactivation levels at 254 nm filtered MP may be artificially low due to the physical properties of the filter. A filtered MP<sub>254</sub> UV dose of 55 mJ/cm<sup>2</sup> resulted in 0.34-log inactivation of Ad2 compared to 1.5 – 2-log with the same dose of LP<sub>254</sub>. Similarly, MP<sub>254</sub> doses of 68 and 199 mJ/cm<sup>2</sup> resulted in 0.24- and 0.42-log inactivation of <i>B. pumilus</i> ASFUVRC spores whereas 1-log and 2 – 3-log inactivation, respectively, would be expected based on the LP<sub>254</sub> dose response.

**INACTIVATION OF <i>BACILLUS PUMILUS</i> PHAGE**

In contrast to UV resistant <i>B. pumilus</i> ASFUVRA and ASFUVRC spores, a bacteriophage that infects <i>B. pumilus</i> was very sensitive to LP-UV (Figure 4.29). Bacteriophage 6631-B is a double-stranded DNA virus and its UV response was similar to that of the T-phages described earlier, with 6-log inactivation at approximately 20 mJ/cm<sup>2</sup>. The primary UV damage repair mechanism in <i>Bacillus</i> spp. spores, SP lyase, operates during spore germination. However, phage 6631-B was assayed on actively growing vegetative cells of <i>B. pumilus</i> following UV irradiation. Consequently, SASP and SP lyase would not have been available to change the conformation of phage 6631-B DNA and repair UV-induced DNA damage in the phage genome, assuming that these proteins could even interact with phage DNA.

![Figure 4.29 Low pressure UV inactivation of bacteriophage 6631-B assayed on three host strains of Bacillus pumilus.](image-url)
Lagrangian actinometry (LA) is a method for measuring the dose distribution delivered by a UV reactor (Blatchley et al. 2006a). In LA, microspheres are conjugated with a photochemically-active dye that is sensitive to germicidal UV radiation. The product of the photochemical reaction is a brightly fluorescent compound. The fluorescence intensity of individual microspheres can be correlated with the dose of UV radiation received by the microsphere. Injecting a large population of microspheres into a reactor and exposing them to UV radiation followed by collection downstream of the irradiated zone and measuring the fluorescence intensity of each microsphere by flow cytometry, allows the dose distribution delivered by the reactor to be estimated. Flow cytometry allows measurement of the fluorescence intensity of a large number of microspheres in a relatively short period of time; typically, at least 10,000 microspheres are analyzed per sample.

**DESCRIPTION OF DYED MICROSPHERES**

The dyed microspheres (DMS) developed at Purdue University are synthetic microspheres modified by attachment of a photochemically active dye to their surface. The dye is a nucleoside analog, \((E)-5\)-[2-(methoxycarbonyl)-ethenyl]cytidine (hereafter referred to as S). When subjected to germicidal UV radiation, S undergoes a permanent photochemical change to yield 3-β-D-ribofuranosyl-2,7-dioxopyrido[2,3-d]pyrimidine (hereafter referred to as P; Figure 5.1A). In the absence of UV radiation, S and P are extremely stable, with shelf lives on the order of decades or longer. Characteristics of S and P have been described in detail in a series of publications (Bergstrom et al. 1982; Fang et al. 2003; Shen et al. 2005). Optical behavior of S and P are crucial to their application in UV disinfection systems. Specifically, S is a strong absorber of radiation across the entire germicidal spectrum, and the quantum yield for conversion from S to P is high for all germicidal wavelengths. S is a non-fluorescent compound, while P is brightly fluorescent, with an excitation maximum at 330 nm and an emission maximum at 385 nm. Moreover, P is photochemically stable. Therefore, photochemical conversion of S → P yields a permanent optical change that can be measured easily and rapidly by conventional instrumentation. In turn, these characteristics allow quantification of UV dose delivery via a fluorescence measurement. The dye is conjugated to microspheres through a series of chemical linkages (Fang et al. 2008; Figure 5.1B).

DMS response was quantified as a change in fluorescence intensity (FI) distribution for a population of microspheres. The magnitude of the FI change is related to the dose of radiation received by the particle. Fluorescence intensities for individual microspheres within a sample were measured by flow cytometry; typically sample analysis would involve optical measurements for at least 10,000 DMS per sample. Analysis of the FI distribution among a population of DMS provides a method whereby the dose distribution can be quantified. As such, incorporating DMS as a non-biological surrogate represents a method for detailed characterization of UV reactors. When applied in conjunction with biodosimetry and numerical simulations, this method provides extremely detailed information about the fundamental behavior of UV systems.

The useable UV dose range of the DMS may be modified by controlling the surface
loading of the dye on the microsphere. Because the dye is attached to the microsphere through an avidin-biotin linkage, the surface loading of the biotinylated dye (the form we use in these experiments) is controlled by the surface loading of the avidin group per microsphere. The manufacturer of the microspheres used in this work (Bangs Laboratories, Fishers, IN) indicated that they are able to accomplish the tightest control of avidin loading by application of monolayer coverage of the avidin group to the microsphere surface. Therefore, the avidin loading, and hence dye loading per microsphere was varied by working with different sizes of microspheres that were produced with a monolayer of streptavidin (Table 5.1).

Table 5.1
Characteristics of dyed microspheres

<table>
<thead>
<tr>
<th>Mean diameter</th>
<th>Coating</th>
<th>Density (g/cm³)</th>
<th>Binding capacity (biotin µg/µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal</td>
<td>Actual</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 µm</td>
<td>5.60 µm</td>
<td>Streptavidin</td>
<td>1.10</td>
</tr>
<tr>
<td>10 µm</td>
<td>9.95 µm</td>
<td>SuperAvidin</td>
<td>1.06</td>
</tr>
<tr>
<td>15 µm</td>
<td>15.68 µm</td>
<td>SuperAvidin</td>
<td>1.06</td>
</tr>
</tbody>
</table>

Figure 5.1 Chemistry of dyed microspheres. A) Basic UV photochemistry of \((E)-5-[2-(methoxycarbonyl)-ethenyl]cytidine\) \((S)\) in aqueous solution (adapted from Bergstrom et al. 1982). \(S\) first undergoes a photoisomerization reaction to yield the intermediate indicated in brackets, which in turn spontaneously reacts to yield \(P\) as a stable, fluorescent product. B) Schematic representation of the chemical groups incorporated into dyed microspheres.
DMS ANALYSIS METHODS

There are five steps to characterize a LP-UV reactor’s disinfection capabilities using DMS:

1. Conduct dose-response experiments for DMS under a collimated beam UV device.
2. Conduct flow-through dose distribution experiments for DMS with the UV reactor.
3. Calculate dose distribution based on the results from the previous two steps.
4. Obtain dose-response behavior of a target microorganism by conducting collimated beam experiments or reference to published data.
5. Calculate disinfection of the microorganism based on the results from the third and fourth steps using a segregated flow model.

The FI distribution of samples from the collimated beam experiment (step 1) varies in a regular manner such that interpolation methods can be applied to accurately estimate FI distributions for doses between the dose values that are included in step 1. As such, this interpolation function defines the dose-response behavior of the DMS over the range of UV doses applied with the collimated beam.

The fluorescence response of DMS measured from a flow-through sample (step 2) is a linear combination of many fluorescence responses of DMS measured from dose-response samples (from step 1). The linear combination of interest is the combination of doses delivered by the reactor (i.e., dose distribution). A deconvolution algorithm is applied to determine the “best” linear combination of doses that accounts for the fluorescence distribution of the DMS sample collected from the reactor, subject to an appropriate optimization constraint. Therefore, the interpolation matrix developed from the data collected in step 1 can be applied to describe the data from step 2. The combination of UV doses that provides the best fit to the data is the dose distribution delivered by the reactor. Once the dose distribution is known, the performance of the reactor can be accurately calculated using a segregated-flow model approach. The segregated flow model requires information about the dose distribution and the kinetics of the reaction(s) of interest.

Aqueous suspensions of DMS were exposed to a range of UV doses using a flat plate collimated beam apparatus (Blatchley 1997) with a water-cooled, medium-pressure lamp (Aquionics, Inc., Erlanger, KY). Irradiance was measured using a calibrated IL1700 radiometer (International Light). The wavelength-dependent dose-response behavior of the DMS was evaluated by passing full-spectrum MP-UV radiation through the same optical band-pass filters used to assess microbial action spectra (FS10-50, Andover Corp.; see Figure 2.4 for transmittance spectra of filters). DMS suspensions were exposed to filtered MP-UV in a shallow, well-mixed batch reactor placed under the collimated beam for a range of pre-defined exposure periods. Dose delivery from the collimated beam to the aqueous DMS suspension was calculated using Equations 5.1 and 5.2.

For demonstration-scale testing, DMS were recovered from large volume samples (typically 10 L) of process water downstream of the UV reactor by filtration through 3 µm (nominal porosity) nitrocellulose membrane filters. Membrane filtration was performed on-site immediately following sample collection. The filters were then placed in centrifuge tubes, wrapped in aluminum foil, packed on ice, and shipped by overnight courier to Purdue University. Filter-captured DMS were resuspended in a small volume of deionized water followed by
agitation on a vortex mixer and sonication for 3 min. Following removal of the filter, the DMS suspension was sonicated for an additional 3 min to ensure separation of DMS from ambient particles in the concentrated water sample. Samples were centrifuged at 1,750 x g for 10 min and the supernatant was reduced to 1 mL. These suspensions were analyzed by flow cytometry.

\[
I_{\lambda,\text{avg}} = \frac{I_{\lambda,0}}{\alpha_{\lambda} H} \left[ 1 - \frac{n_{\lambda,\text{air}} - n_{\lambda,\text{water}}}{n_{\lambda,\text{air}} + n_{\lambda,\text{water}}} \right] (1 - e^{-\alpha_{\lambda} H}) \quad (5.1)
\]

\[
D_{\lambda,\text{avg}} = I_{\lambda,\text{avg}} \times t \quad (5.2)
\]

where 

- \( I_{\lambda,\text{avg}} \) = average UV intensity in aqueous suspension at wavelength \( \lambda \) (mW/cm²)
- \( I_{\lambda,0} \) = incident intensity at free surface of aqueous suspension (mW/cm²)
- \( \alpha_{\lambda} \) = absorbance coefficient at \( \lambda \) nm (cm⁻¹)
- \( n_{\lambda,\text{air}} \) = refractive index for air at wavelength \( \lambda \)
- \( n_{\lambda,\text{water}} \) = refractive index for water at wavelength \( \lambda \)
- \( H \) = depth of aqueous solution
- \( t \) = exposure time (sec)
- \( D \) = average UV dose delivered to aqueous suspension at wavelength \( \lambda \) (mJ/cm²).

**FLOW CYTOMETRY ANALYSIS**

Flow cytometry was performed on an Epics Altra cell sorter (Beckman-Coulter, Miami, FL) using the 351 nm line of an Argon 5-watt Enterprise laser (Coherent, Inc., Santa Clara, CA) with power set at 60 mW for the UV line. Individual microspheres were excited with the UV line and scatter and fluorescence signals were collected simultaneously: fluorescence was collected in linear mode for wavelengths between 380 to 408 nm with PMT2; forward angle light scatter (FS) was collected using the 351 nm laser excitation; and side scatter (SS) was collected in the orthogonal position for wavelengths shorter than 380 nm with PMT1 (Figure 5.2). FS is proportional to the size of particles and SS is a representation of particle granularity.

![Figure 5.2 Schematic illustration of flow cytometry setup.](image-url)
Flow cytometry analyses allowed measurement of several optical properties, including particle size, granularity, and FI. Particle population data for each sample were refined by “gating” of the size, granularity, and FI signals. Since the size, granularity, and FI of the DMS were known from analysis of microsphere stock suspensions, this gating procedure was used to eliminate measurements from ambient particles and non-conforming measurements. An example of gating for three different sizes of DMS through a low-pressure high-output (LPHO) 3-lamp reactor (Trojan Technologies, London, ON) at 25 GPM is presented in Figure 5.3. On the basis of size and granularity alone, DMS signals show considerable overlap with background particles. However, gating based on FI and granularity makes it possible to distinguish DMS signals from those of ambient particles.

Figure 5.3. Flow cytometry data of 25 GPM effluent samples for three different sizes of DMS. For particles in each sample, three optical parameters were measured: size (forward scatter), granularity (side scatter) and fluorescence intensity (FI). Size versus granularity (panels a – c) and FI versus granularity (panels d – f) for three different sizes of DMS are shown. Gating boundaries, as indicated by the white and grey ovals, allow differentiation of DMS from ambient particles. The 10 µm and 15 µm DMS are higher in all three parameters and allow better discrimination against background particles than the 6 µm DMS.

The variability inherent in FI measurement by flow cytometry represents a complicating feature in FI quantification for these microspheres. Therefore, a modified analysis method involving an internal standard was developed to account for the background variability of flow cytometry. The internal standard was developed using a different size of DMS than the target
microsphere. The internal standard was subjected to a pre-defined, single-valued dose under a collimated beam. For example, the two internal standards used for 15 μm DMS samples were 10 μm DMS with UV$_{254}$ doses of 20 and 400 mJ/cm$^2$. The reason for using a different size of DMS as the internal standard is that the data of samples and internal standards could be easily separated in cytometry by the size-dependent parameter, forward light scatter or the granularity-dependent parameter, side light scatter. The reason for using multiple internal standards of different doses in a sample is to generate a calibration curve in each sample. The flow cytometer generates a fluorescence intensity distribution of DMS with an arbitrary unit (au1). The method involves calibrating this arbitrary unit to another arbitrary unit (au2) based on the same instrument and scale, but with reduced inherent uncertainty in the cytometer. The au2 for the internal standard with UV$_{254}$ dose of 20 mJ/cm$^2$ was assigned a value of 100 and the au2 for the internal standard with UV$_{254}$ dose of 200 mJ/cm$^2$ was assigned a value of 750. Therefore, the values of au1 and au2 could vary between samples; in turn the variation in these signals could be used to account for (and eliminate the effects of) drift in the cytometer signal.

Factors that lead to drift in the FI signal from a cytometer include: variation of laser power, fluid pressure, temperature, voltage of PMT, or optical alignment. In applying this approach, it was assumed that cytometer variations were expressed in a similar manner among the test and internal standard microspheres, and therefore, the variations in FI signals for the internal standard could be used to adjust measured FI responses for the test microspheres.

The dose-response results of 15 μm DMS for 6 different wavelengths are presented in Tables 5.2 – 5.7 (wavelengths = 220, 228, 239, 254, 260, and 270 nm). All of these data were developed based on the use of multiple internal standards. The FI with au1 of the sample and two internal standards were measured by flow cytometry at the same time and are listed in columns B, C, and D of Tables 5.2 – 5.7. A value of 100 was selected as au2 for the internal standard M20 (10 μm DMS with UV$_{254}$ dose of 20 mJ/cm$^2$); a value of 750 was selected as au2 for the internal standard M400 (10 μm DMS with UV$_{254}$ dose of 400 mJ/cm$^2$). The adjusted FI with au2 is listed in column E ($E$, Equation 5.3).

\[
E = 100 + \frac{[(B-C) \times 750 - 100]}{(D-C)}
\]  

(5.3)

where:
- $E$ = adjusted fluorescence intensity
- $B$ = sample fluorescence intensity
- $C$ = M20 internal standard fluorescence intensity
- $D$ = M400 internal standard fluorescence intensity

By following this approach, it was possible to quantify the dose-response behavior of the microspheres more precisely than with previously applied methods, such that DMS UV dose response was more consistent between experiments.

More detailed information about LA, including additional details of flow cytometry methods as applied to LA, can be found in previous publications on this topic (Blatchley et al. 2006, 2007, 2008; Shen et al. 2007). A comprehensive summary of the method and detailed protocol for its application is presented in the Water Research Foundation report for Project #4112 "Develop and Demonstrate UV Reactor Validation Protocol Using Dyed-Microspheres Actinometry" (co-sponsored by NYSEDRA).
### Table 5.2
Mean fluorescence intensity of 15 μm DMS with two internal standards, M20 and M400 for ten different UV220 doses.

<table>
<thead>
<tr>
<th>UV220 dose (mJ/cm²)</th>
<th>Sample</th>
<th>Internal standard M20</th>
<th>Internal standard M400</th>
<th>Adjusted FI (au2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>115.3</td>
<td>74.6</td>
<td>764.0</td>
<td>138</td>
</tr>
<tr>
<td>50</td>
<td>125.3</td>
<td>74.5</td>
<td>758.0</td>
<td>148</td>
</tr>
<tr>
<td>100</td>
<td>134.2</td>
<td>74.5</td>
<td>753.8</td>
<td>157</td>
</tr>
<tr>
<td>150</td>
<td>144.1</td>
<td>74.8</td>
<td>745.2</td>
<td>167</td>
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<td>153.0</td>
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<td>174</td>
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<td>753.8</td>
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<td>76.3</td>
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<td>177.6</td>
<td>75.1</td>
<td>752.4</td>
<td>198</td>
</tr>
<tr>
<td>450</td>
<td>181.6</td>
<td>74.7</td>
<td>749.1</td>
<td>203</td>
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</table>

### Table 5.3
Mean fluorescence intensity of 15 μm DMS with two internal standards, M20 and M400 for ten different UV228 doses.

<table>
<thead>
<tr>
<th>UV228 dose (mJ/cm²)</th>
<th>Sample</th>
<th>Internal standard M20</th>
<th>Internal standard M400</th>
<th>Adjusted FI (au2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>116.2</td>
<td>75.1</td>
<td>772.3</td>
<td>138</td>
</tr>
<tr>
<td>50</td>
<td>131.7</td>
<td>75.4</td>
<td>768.8</td>
<td>153</td>
</tr>
<tr>
<td>100</td>
<td>149.5</td>
<td>75.2</td>
<td>769.3</td>
<td>170</td>
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<tr>
<td>150</td>
<td>168.5</td>
<td>75.2</td>
<td>769.9</td>
<td>187</td>
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<td>200</td>
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<td>75.0</td>
<td>767.0</td>
<td>206</td>
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<td>250</td>
<td>205.5</td>
<td>75.5</td>
<td>767.3</td>
<td>222</td>
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<td>300</td>
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<td>76.9</td>
<td>766.0</td>
<td>255</td>
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<td>400</td>
<td>257.1</td>
<td>75.7</td>
<td>763.8</td>
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<td>284</td>
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Table 5.4
Mean fluorescence intensity of 15 μm DMS with two internal standards, M20 and M400 for ten different UV239 doses.

<table>
<thead>
<tr>
<th>UV239 dose (mJ/cm²)</th>
<th>Sample</th>
<th>Internal standard M20</th>
<th>Internal standard M400</th>
<th>Adjusted FI (au2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>780.8</td>
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<td>145.2</td>
<td>76.7</td>
<td>777.0</td>
<td>164</td>
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<td>100</td>
<td>183.2</td>
<td>76.3</td>
<td>775.6</td>
<td>199</td>
</tr>
<tr>
<td>150</td>
<td>223.4</td>
<td>76.7</td>
<td>776.3</td>
<td>236</td>
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<td>76.1</td>
<td>772.6</td>
<td>275</td>
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<td>250</td>
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<td>775.4</td>
<td>310</td>
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<tr>
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<td>428</td>
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</table>

Table 5.5
Mean fluorescence intensity of 15 μm DMS with two internal standards, M20 and M400 for ten different UV254 doses.

<table>
<thead>
<tr>
<th>UV254 dose (mJ/cm²)</th>
<th>Sample</th>
<th>Internal standard M20</th>
<th>Internal standard M400</th>
<th>Adjusted FI (au2)</th>
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</thead>
<tbody>
<tr>
<td>0</td>
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<td>96.9</td>
<td>750.0</td>
<td>154</td>
</tr>
<tr>
<td>50</td>
<td>178.6</td>
<td>97.6</td>
<td>747.8</td>
<td>181</td>
</tr>
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<td>385.5</td>
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<td>736.2</td>
<td>394</td>
</tr>
<tr>
<td>450</td>
<td>413.6</td>
<td>96.9</td>
<td>737.3</td>
<td>421</td>
</tr>
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</table>
Table 5.6
Mean fluorescence intensity of 15 μm DMS with two internal standards, M20 and M400 for ten different UV$_{260}$ doses.

<table>
<thead>
<tr>
<th>UV$_{260}$ dose (mJ/cm$^2$)</th>
<th>Sample</th>
<th>M20*</th>
<th>Internal standard M400†</th>
<th>Adjusted FI (au2)</th>
</tr>
</thead>
<tbody>
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<td>0</td>
<td>150.9</td>
<td>97.1</td>
<td>742.4</td>
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<td>150</td>
<td>250.5</td>
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<td>746.5</td>
<td>253</td>
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<td>200</td>
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<td>98.8</td>
<td>746.7</td>
<td>291</td>
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<td>250</td>
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<td>753.2</td>
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<td>101.3</td>
<td>757.0</td>
<td>355</td>
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<td>390.0</td>
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<td>387</td>
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<td>400</td>
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<td>450</td>
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<td>102.6</td>
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</table>

Table 5.7
Mean fluorescence intensity of 15 μm DMS with two internal standards, M20 and M400 for ten different UV$_{270}$ doses.

<table>
<thead>
<tr>
<th>UV$_{270}$ dose (mJ/cm$^2$)</th>
<th>Sample</th>
<th>M20*</th>
<th>Internal standard M400†</th>
<th>Adjusted FI (au2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>150.6</td>
<td>97.4</td>
<td>751.8</td>
<td>153</td>
</tr>
<tr>
<td>50</td>
<td>184.7</td>
<td>98.0</td>
<td>749.7</td>
<td>186</td>
</tr>
<tr>
<td>100</td>
<td>227.5</td>
<td>97.8</td>
<td>751.0</td>
<td>229</td>
</tr>
<tr>
<td>150</td>
<td>270.6</td>
<td>97.5</td>
<td>747.8</td>
<td>273</td>
</tr>
<tr>
<td>200</td>
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<tr>
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<td>98.6</td>
<td>749.3</td>
<td>377</td>
</tr>
<tr>
<td>300</td>
<td>400.0</td>
<td>98.2</td>
<td>752.4</td>
<td>400</td>
</tr>
<tr>
<td>350</td>
<td>440.0</td>
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<td>470.9</td>
<td>98.0</td>
<td>747.4</td>
<td>473</td>
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<tr>
<td>450</td>
<td>508.3</td>
<td>97.8</td>
<td>748.5</td>
<td>510</td>
</tr>
</tbody>
</table>

Footnotes for Tables 5.2 – 5.7:
* M20: 10 μm DMS with UV$_{254}$ dose of 20 mJ/cm$^2$, a value of 100 of au2 was selected for it.
† M400: 10 μm DMS with UV$_{254}$ dose of 400 mJ/cm$^2$, a value of 750 of au2 was selected for it.
‡ Use (C,100) and (D,750) to generate linear interpolation equation (Equation 5.3)
WAVELENGTH-DEPENDENT DOSE-RESPONSE BEHAVIOR OF DMS

The wavelength-dependent dose-response behavior of DMS was represented by the measurement of particle-specific fluorescence intensity (FI) for large populations of microspheres using flow cytometry (target populations for each sample were >10,000 microspheres). The mean FI responses of the 6, 10 and 15 μm DMS for each dose-wavelength combination are illustrated in Figures 5.4 – 5.6.

As the UV dose increased, the mean FI value increased in a roughly linear manner, and the population of FI responses shifted to higher mean values with greater spread of the population of responses. For all three microsphere sizes, the DMS were most sensitive to UV radiation in the vicinity of 254 nm; the dose-response behavior of the microspheres became less steep as the wavelength was shifted to values above or below this value. The 10 μm DMS were the most sensitive to UV radiation across the spectrum of wavelengths.

An example of the wavelength-dependent dose-response results of the three different sizes of DMS with ten different UV\textsubscript{254} doses is shown in Figure 5.7. The 10 μm DMS displayed the greatest sensitivity to UV doses. Although there were some inconsistent conditions (e.g., doses, pre-irradiation, calibration) between the datasets for the three sizes of DMS, the 10 μm DMS clearly had the largest fluorescence intensity change with corresponding UV\textsubscript{254} dose increments.

The results of collimated beam tests demonstrated that although the 10 μm DMS were the most sensitive to UV radiation, all three sizes of DMS could be used to quantify UV doses in the range 0 – 600 mJ/cm\textsuperscript{2}. Unfortunately, part way through the project, the manufacturer of the biotinylated dye stopped producing the original formulation of the dye due to the cost, scale, and complexity of the synthesis. Producing the dye involves non-routine synthesis that is not widely available through commercial laboratories. The original chemistry used to conjugate the fluorophore S to streptavidin-coated microspheres involved a silyl acetal linkage (silane group in Figure 5.1B; Fang et al. 2008). As an alternative, the manufacturer substituted phosphate for the silane group. These new phosphate-linked DMS generated the expected response to LP-UV radiation (254 nm). However, they generated little or no response with full-spectrum MP-UV, even though S was converted to P. We assume the lack of response to MP-UV was attributable to photobleaching of P resulting from replacement of the silane group by phosphate. Consequently, the new DMS formulation cannot be used for medium-pressure applications. However, they were used for demonstration-scale testing of LP-UV reactors. The results of demonstration-scale testing of DMS are described in Chapter 6.
Figure 5.4 Mean fluorescence intensity (FI) response of 6 µm dyed microspheres for each UV dose-wavelength combination. For all wavelengths, mean FI increased with UV dose over a broad range of UV doses (0 – 450 mJ/cm²). The most sensitive range of wavelengths was between 239-280 nm. The highest mean FI increase was approximately 50 FI units with UV260 dose = 450 mJ/cm².
Figure 5.5  Mean fluorescence intensity (FI) response of 10 µm dyed microspheres for each UV dose-wavelength combination. For all wavelengths, mean FI increased with UV dose over a broad range of UV doses (0 – 450 mJ/cm²). The most sensitive range of wavelengths was between 239-280 nm. The highest mean FI increase was approximately 700 FI units with UV_{260} dose = 450 mJ/cm².
Figure 5.6 Mean fluorescence intensity (FI) response of 15 µm dyed microspheres for each UV dose-wavelength combination. For all wavelengths, mean FI increases with UV dose over a broad range of UV doses (0 – 450 mJ/cm²). The most sensitive range of wavelengths was between 260-280 nm. The highest mean FI increase was approximately 400 FI units with UV₂₆₀ dose = 450 mJ/cm².
Figure 5.7  Wavelength-dependent dose-response results of three different sizes of dyed microspheres in the UV$_{254}$ dose range 0 – 600 mJ/cm$^2$. The response was measured as fluorescence intensity (FI) and UV dose increases from left to right across the graph.
DOSE DISTRIBUTION DECONVOLUTION

Lagrangian actinometry is the only available method to measure the UV dose distribution delivered by a UV reactor. It is this attribute of the LA method that defines its greatest strength relative to other methods of reactor analysis and validation. Dose distribution measurement by LA is based on a combination of physical measurements and numerical analysis of the resulting data. The following text provides an abridged description of UV dose distribution measurement by LA. Additional details of this method are available in previous publications (Blatchley et al. 2006, 2007, 2008; Shen et al. 2007). In addition to these existing references on the topic, a detailed description of the application of LA for reactor validation is expected to be available in early 2010 as a report for Water Research Foundation Project 4112 (which was conducted as a partnership with NYSERDA) entitled: "Develop and Demonstrate UV Reactor Validation Protocol Using Dyed-Microspheres Actinometry". This report is being developed as a protocol to describe the application of LA for UV reactor validation. The protocol has been developed in a manner and structure that are intentionally parallel to those used to develop the UVDGM, to provide users with a comprehensive description of LA for validation of UV reactors.

UV dose-response experiments are conducted by subjecting aqueous suspensions of dyed microspheres to a range of controlled UV doses using a well-mixed, shallow batch reactor and a collimated UV source. The changes in FI with UV dose from these experiments have been observed to be quite regular, as shown in Figure 5.7. Therefore, it is possible to use curve fitting to interpolate the UV dose-response behavior for doses that lie between those used in the actual dose-response experiment. Previous experience has demonstrated that the 4-parameter Weibull distribution works well for this curve-fitting process.

Non-linear regression is used to allow a fit of the 4-parameter Weibull distribution to the dose-specific DMS FI responses for each dose in a dose-response experiment. Mathematically, the 4-parameter Weibull distribution is defined by Equation 5.4. An example of the 4-parameter Weibull distribution fitted to FI data is illustrated in Figure 5.8.

\[
y = a \times \left( \frac{(c-1)}{c} \right)^{\frac{(c-1)}{c}} \left( \frac{x-X_0}{b} + \frac{(c-1)}{c} \right)^{c-1} \exp \left( - \left( \frac{x-X_0}{b} + \frac{(c-1)}{c} \right)^{c} + \frac{c-1}{c} \right) \quad (5.4)
\]

for \( c - 1 \geq 0 \) and \( \frac{x-X_0}{b} + \frac{(c-1)}{c} \geq 0 \)

for \( c - 1 < 0 \) and \( \frac{x-X_0}{b} + \frac{(c-1)}{c} < 0 \)

where

- \( y \) = density function
- \( x \) = fluorescent intensity
- \( a \) = scale parameter
- \( b \) = width parameter
- \( c \) = shape parameter
- \( X_0 \) = center parameter

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Figure 5.8  Experimental data and a Weibull-fitted curve of fluorescence intensity distribution of 10 µm DMS at a UV dose of 200 mJ/cm².

For each FI histogram of a dose-response experiment that is fitted by the method described above, a dose-specific value of each model parameter is defined. These parameters vary in a regular, predictable manner, thereby allowing interpolation (Figure 5.9). Each parameter becomes a function of dose. Therefore, for a certain dose value, these four parameters can be predicted and substituted in the Weibull distribution. Then the fluorescent distribution of DMS at that dose can be predicted. Non-linear regression allows good fitting to the observed behavior of the parameters a, b, and x₀. However, the fit of a smooth function to parameter c is often poor. On the other hand, the ability of the Weibull function to fit observed data is not heavily dependent on parameter c. Based on these interpolation schemes, it is then possible to describe the dose-response behavior of the DMS as a matrix (Equation 5.5):

\[
A = \begin{bmatrix}
  a_{11} & a_{12} & \cdots & a_{1m} \\
  a_{21} & a_{22} & \cdots & a_{2m} \\
  \vdots & \vdots & \ddots & \vdots \\
  a_{n1} & a_{n2} & \cdots & a_{nm}
\end{bmatrix}
\]

(5.5)

where

- \( n \) = number of intervals of FI distribution
- \( m \) = number of intervals of dose distribution
- \( a_{11}, a_{21}, \ldots, a_{n1} \) = the FI histogram of dose #1
- \( a_{12}, a_{22}, \ldots, a_{n2} \) = the FI histogram of dose #2
- \( \vdots \)
- \( a_{1m}, a_{2m}, \ldots, a_{nm} \) = the FI histogram of dose #m
Figure 5.10 provides a graphical illustration of a typical DMS dose-response matrix. These results may be interpreted as the analog of a UV disinfection kinetic model fitted to data from a UV dose-response experiment for a microorganism.

![Graphs showing dose-response data for parameters a, b, c, and x0](image)

Figure 5.9 Interpolations of the four parameters for Weibull distribution.
Challenge Organisms for Inactivation of Viruses by Ultraviolet Treatment

Figure 5.10  Fluorescence intensity distribution predicted by Weibull density function for the 10 µm DMS. Sixty one fluorescence intensity histograms are shown corresponding to UV doses ranging from 0 to 600 mJ/cm² with an interval of 10 mJ/cm². Each curve can be written as a column vector. Collectively, 61 curves can be written in 61 column vectors, which form a matrix.

Following this logic, the FI distribution of the DMS through a continuous-flow UV reactor (where a UV dose distribution is delivered) may be described as a linear combination of the matrix A (DMS dose-response behavior) and the dose distribution, as shown in Equation 5.6:

\[ Ax = b; \]

\[
\begin{bmatrix}
  a_{11} & a_{12} & \cdots & a_{1m} \\
  a_{21} & a_{22} & \cdots & a_{2m} \\
  \vdots & \vdots & \ddots & \vdots \\
  a_{n1} & a_{n2} & \cdots & a_{nm}
\end{bmatrix}
\begin{bmatrix}
  x_1 \\
  x_2 \\
  \vdots \\
  x_m
\end{bmatrix}
= 
\begin{bmatrix}
  b_1 \\
  b_2 \\
  \vdots \\
  b_n
\end{bmatrix}
\]

(5.6)

where  \( x = \) dose distribution vector with \( m \) intervals

\( b = \) FI distribution vector of a flow-through DMS sample with \( n \) intervals

The solution to Equation 5.6 is the vector \( x \), which represents the dose distribution. However, there are several sources of error associated with matrix A and vector b. These include errors associated with instrumental measurements (e.g., flow cytometry, radiometry), and errors associated with manufacturing of the microspheres. Because of these errors, it is extremely unlikely that an exact solution to Equation 5.6 will be found; that is to say, \( b \) does not belong to the range of A. Therefore, the approach used in developing an estimate of the vector \( x \) (i.e., the UV dose distribution) is to identify a vector, \( x' \) such that \( Ax' \) is as close as possible to \( b \). The
Euclidean norm is selected to measure the difference between $Ax'$ and $b$. In other words, we are trying to minimize the sum of residual squared errors (as shown in Equation 5.7).

$$\left( \sum_{i=1}^{n} (Ax'_i - b_i)^2 \right)^{1/2}$$

(5.7)

This approach is mathematically analogous to the application of conventional regression analysis. In searching for this solution, it is also necessary to apply two constraints, so as to ensure that the solution is consistent with the known behavior of DMS. Specifically, these constraints are that each element in $x'$ cannot be negative, and that the sum of elements in $x'$ equals one. An executable program has been written in MATLAB to search for this solution.

**INACTIVATION PREDICTION**

Accurate predictions of microbial inactivation in a continuous-flow reactor can be made by integration of the UV dose distribution with measured dose-response behavior for the challenge organism using a mathematical analog of the segregated-flow model (Equation 5.8; Chiu et al. 1999; Blatchley et al. 2006, 2008):

$$\frac{N}{N_0} = \int_0^\infty \left( \frac{N}{N_0} \right)_{\text{batch}} E(D) d(D) \approx \sum \left( \frac{N}{N_0} \right)_{\text{batch}} E(D_i) \Delta D_i$$

(5.8)

where:
- $N$ = concentration of the viable target organisms in the effluent (cfu/mL)
- $N_0$ = concentration of the viable target organisms in the influent (cfu/mL)
- $(N/N_0)_{\text{batch}}$ = fraction of target organisms that retain viability after batch irradiation using a collimated UV source
- $E(D)$ = dose distribution (mJ/cm$^2$)

The description of UV dose-response behavior for the target microorganism will be in the form of a mathematical expression. Many such models are available in the literature. Examples of appropriate microbial UV dose-response models include the series-event model (Severin et al. 1983), which includes the single-event (or “Chick-Watson” model as a special case); and the Phenotypic Persistence and External Shielding (PPES) model (Pennell et al. 2008). The term $E(D) d(D)$, or $E(D_i) \Delta D_i$, in Equation 5.8 represents the fraction of particles receiving a given UV dose, which is available once the dose distribution is known.

The segregated flow model is based on the hypothesis that no “exchange” of material occurs among reactive components of the system. In the application of the segregated flow model to photochemical reactors, the reactive components of interest are the photochemical targets; in the case of disinfection applications, these “targets” are waterborne microbes. Therefore, the basic assumption of the segregated flow model is satisfied rigorously in UV disinfection applications. The segregated-flow model has been demonstrated to provide accurate descriptions of the behavior of photochemical reactors, when accurate estimates of the dose distribution and dose-response behavior are available (Blatchley et al. 2006, 2008).
POTENTIAL FOR APPLICATION TO HIGH DOSE SYSTEMS

The ability of DMS to provide a distinct, measurable signal over the entire range of UV doses that will characterize a reactor’s dose distribution is vital to the application of LA. If DMS are available that have this attribute, it is then possible to estimate the UV dose distribution delivered by a reactor by the combination of interpolation of the dose-response behavior from a collimated beam experiment, and the deconvolution method described above.

For application of LA to high-dose applications, the relatively strong fluorescence intensity signals that result from large UV doses is beneficial to LA, in that these large fluorescence values are more easily differentiated from smaller FI values that correspond with smaller UV doses. By extension, this suggests that LA may be even better suited for quantification of UV dose distributions in the high dose range, than those that are more conventionally applied in water disinfection systems.

ASSESSING THE TOXICITY OF (E)-5-[2-(METHOXYCARBONYL)-ETHENYL]CYTIDINE (S)

Dyed microspheres have previously been used in full-scale treatment plants but precautions were taken to minimize the risk of microspheres entering the distribution system. In one study using Fluorabrite YG microspheres at an ozone plant, effluent from the test treatment trains was discharged to a storm drain and filters were backwashed prior to returning the plant to normal service (Marinas et al. 1999). The 5.6 μm DMS used in the current project have been applied in large-scale low-pressure UV tests (20 – 60 MGD) but the effluent from the reactors was disposed by slowly feeding it to a full-scale wastewater treatment facility (Blatchley et al. 2008).

The UV-sensitive compound S attached to the microspheres is a nucleoside analogue and some of these compounds have anti-viral activity and may therefore present a toxicity hazard to water consumers downstream of a reactor that has been tested with DMS. A range of toxicological properties have been reported for various anti-retroviral nucleoside analogues, including peripheral neuropathy, myopathy, and pancreatitis (Moyle 2000). However, other nucleoside analogues with broad microbicidal activity did not demonstrate any adverse effects in toxicological studies (D’Cruz 2003).

Due to the concerns of introducing a fluorescent cytidine analogue into demonstration-scale and full-scale treatment processes, the toxicity of S was assessed using a cell culture and 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay by Mi Ran Kim, under the supervision of Dr. Sulma Muhammed at the Purdue University Cancer Center. In considering the fate of S in drinking water systems, it is anticipated that human exposure would be through ingestion of water. As such, three types of target cell were selected for the toxicity test: kidney cells, liver cells, and pancreatic cancer cells. The MTT assay is a quantitative colorimetric method to determine cell proliferation and cytotoxicity of potentially toxic compounds (Niks and Otto 1990; Vellonen et al. 2004). It utilizes MTT, a yellow tetrazolium salt, that is reduced to formazan (purple color) by the succinate-tetrazolium reductase system in cells. This system is part of the respiratory chain of mitochondria and is active only in metabolically-active cells. Addition of an appropriate solvent or detergent results in the liberation of the crystals, which are solubilized. The number (concentration) of surviving cells is assumed to be directly proportional to the concentration of the formazan product created. Formazan concentration is measured...
colorimetrically at 500 – 600 nm using a spectrophotometer. The MTT assay is an indirect measure of metabolic activity in cells. Other viability tests sometimes give different results, as many different conditions can increase or decrease metabolic activity. Therefore, it is possible for environmental factors other than the target compound to influence the results of the MTT assay.

Materials and Methods

Liver epithelial cells (THLE-2), kidney epithelial cells (293T/17), pancreatic cancer cells (Paca2) were obtained from ATCC. Dulbecco’s Modified Eagle’s Medium (DMEM) was from Sigma and Bronchial Epithelial Growth Medium (BEGM) bullet kit was from Lonza Inc. (Walkersville, MD). RPMI 1640 media was from Mediatech, Inc. (Manassas, VA), fetal bovine serum was from Atlas Biologicals, Inc. (Fort Collins, CO), L-glutamine was from Sigma, and dimethyl sulfoxide (DMSO) was from Acros Organics (Morris Plains, NJ).

All cell manipulations were conducted in a sterile environment. THLE-2, 293T/17, and Paca2 cells were grown in accordance with ATCC-recommended procedures. Growing cells were trypsinized and seeded at approximately 4,000 cells/well into individual wells of a 96-well plate. Cells were maintained overnight in a humidified, 5% CO2 atmosphere in an incubator at 37°C. The following day, fresh RPMI 1640 media (supplemented with 10% fetal bovine serum and 1% L-glutamine) was added to wells in the 96-well plate with the test compound at concentration of 0.01 pM – 20 μM. Each exposure was replicated 4 – 6 times and incubated for 72 hours. Cells treated only with media served as a negative control, while Doxorubicin (a drug widely used cancer chemotherapy) was also tested as positive toxicity indicator. To each well, 20 µL of a 0.5% MTT solution was added and the plates were incubated for an additional 4 h at 37°C. All media was removed from the wells and 200 µL DMSO was added to dissolve the insoluble purple formazan product into a colored solution. Absorbance of the solution in each well was measured at 570 nm using a Molecular Device microplate reader (Union City, CA) equipped with SoftMax Pro Software. This absorbance reading was used as an indirect measure of the number of viable cells in each well.

For each experiment, an optical density (OD) measurement was replicated for the growth medium alone. The average of these OD measurements was subtracted from each measurement of OD for the control samples (i.e., wells in the 96-well tray containing growth medium and cells) and the exposed samples (i.e., wells in the 96-well tray containing growth medium, cells, and a known, non-zero-concentration of S). The OD measurements for which contributions of the growth medium had been subtracted was used as a measure of absorbance attributable to viable cells. These measurements were normalized against the same measurement for the control sample, in which the S concentration was zero. The results of the tests are presented graphically in the form of normalized OD as a function on dose (concentration) of the agent of interest.

Results of Toxicity Tests

All cells treated with Doxorubicin displayed evidence of toxicity at concentrations of 100 nM and 1 μM. This response is typical of the cell lines used in this study, and as such, these data provide an indication that the specific cell cultures used in this test were representative of other healthy cultures of these same cells and that toxicity could be detected. The responses of each of the cell lines to exposure to S are summarized in Figures 5.11 – 5.13. For each cell line, two
rounds of testing were conducted, the first with four replicates of each combination of cell line and \( S \) concentration, and the second round with six replicates of each \( S \) concentration. Toxicity would result in a significant decrease in OD. For all three cell lines, there was no evidence of toxicity for the range of \( S \) concentrations used in this study.

From the perspective of DMS application to on-site testing of UV reactors, it is important to consider the liquid-phase concentration of \( S \) that could result from application of DMS in testing of a UV system at a drinking water facility. Based on applying approximately 30,000 DMS/L in a reactor test, a streptavidin surface loading of 2.3 \( \mu \)g/mL for the 6 \( \mu \)m microspheres, and a 1:1 molar ratio of streptavidin (MW = 633) and the biotinylated form of compound \( S \) (phosphate form, MW = 771) giving a streptavidin:\( S \) mass ratio of 1.218, the maximum liquid phase concentration of \( S \) in reactor tests under normal operating conditions will be \( 1.1 \times 10^{-5} \) \( \mu \)M, if a worst case scenario of all the biotinylated \( S \) remaining in solution is assumed (i.e., no conjugation to the microspheres). Since there was no measured toxicity at 20 \( \mu \)M of \( S \), which is five orders of magnitude higher than the maximum liquid-phase concentration in reactor tests, it can be concluded that the introduction of these DMS into a drinking water treatment train does not pose a significant toxicity hazard. In addition, the dye is conjugated to the microsphere surface and therefore is not likely to be biologically available.
Figure 5.11  Response of THLE-2 (liver epithelial) cells to (E)-5-[2-(Methoxycarbonyl)-ethenyl]cytidine (S) exposure. The symbols in each figure represent the mean of replicate measurements, while the error bars indicate the standard deviation. For the 23 June tests, n=4; for the 27 June tests, n=6.
Figure 5.12  Response of 293T/17 (kidney epithelial) cells to \((E)-5-[2-(\text{Methoxycarboxyl})-\text{ethenyl}]\text{cytidine (S) exposure. The symbols in each figure represent the mean of replicate measurements, while the error bars indicate the standard deviation. For the 23 June tests, } n=4; \text{ for the 27 June tests, } n=6.\)
Figure 5.13 Response of Paca2 (pancreatic cancer) cells to \((E)-5-[2-(Methoxycarbonyl)ethenyl]cytidine (S)\) exposure. The symbols in each figure represent the mean of replicate measurements, while the error bars indicate the standard deviation. For the 23 June tests, \(n=4\); for the 27 June tests, \(n=6\).
CHAPTER 6
DEMONSTRATION-SCALE EXPERIMENTS

Demonstration-scale tests were conducted at two sites: MWD’s F. E. Weymouth Water Treatment Plant in La Verne, CA and the UV Validation and Research Center of New York, which is located at the Gloversville-Johnstown Joint Wastewater Treatment Facility, Johnstown, NY and operated by HydroQual. The purpose of these experiments was to determine whether potential surrogates that appeared promising in collimated beam bench tests could be used on a larger scale. They were not intended to validate the UV reactors nor account for all possible variables in UV reactor testing.

DESCRIPTION OF FACILITIES

The 3-MGD (maximum flow) demonstration-scale UV research platform at MWD is a Calgon Sentinel enclosed pipe reactor equipped with four 4-kW medium-pressure lamps mounted perpendicular to the flow of water. It is installed within a 5-MGD demonstration-scale treatment plant (Figure 6.1) with 24 inch inlet and outlet pipes. New lamps were installed in the reactor for this project. Disinfection experiments were started 2 – 4 hours after a filter backwash to ensure steady state operation of the filter process. Surrogate microbe suspensions (2 L volumes) were injected through a 1 inch diameter perforated stainless steel pipe that spanned the full width of the UV reactor inlet pipe. Previous MS2 biodosimetry, numerical simulations, and computational fluid dynamic and irradiance (CFD-i) modeling indicated that this method of injection accomplished complete cross-sectional mixing within the reactor (Mofidi et al. 2004). Samples were withdrawn immediately downstream of the reactor through a second perforated stainless steel pipe spanning the diameter of the effluent pipe. Water flow through the reactor was controlled by an 18-inch butterfly valve to provide flow rates of 0.4 – 3 MGD (63 – 473 m³/h). Treated water was captured in a 100,000 gallon clearwell, which was empty at the beginning of the test. At the end of the test, the UV reactor was shut down and the filter effluent valve immediately upstream of the UV reactor was shut, causing the filter to overflow and reject settled water to waste. The clearwell water was held with at least 3 mg/L residual chlorine for at least two hours. Following chlorine contact, this water was pumped to the filter building sump containment basin of MWD’s full-scale water treatment plant. This sump water was then processed by the full-scale facility’s wash water reclamation plant.

The demonstration-scale facility operated by HydroQual has a modular design with four functionally similar test streams: 4-inch test stream, 1 MGD maximum flow; 12-inch test stream, 7.5 MGD; 24-inch test stream, 7.5 – 40 MGD; 36-inch test stream, 40 – 60 MGD. A schematic of the validation facility configuration is shown in Figure 6.2. The functional elements of the 24-inch test stream are, in progressive downstream order:

- A manifold to combine the flow of up to eight pumps.
- An injection point for water modifiers and microorganisms, which is connected to the injection premix system in the control trailer.
- A 24-inch High Efficiency Vortex (HEV) in-line, static mixer to assure homogeneous mixing of the test organism and the UVT absorber in the challenge water stream.
- A straight length of pipe that allows fully developed flow prior to the flow meter.
• A 24-inch electromagnetic flow meter with a 4-20 mA output signal that can be used for data logging and for input to the test unit PLC.
• An influent sampling point.
• The UV disinfection test unit with necessary piping to create the proper hydraulic simulation.
• A second 24-inch in-line HEV mixer to assure homogenous mixing of the dosed effluent stream exiting the test unit.
• An effluent sampling point.
• Valves to accommodate recirculation to the source tank or dumping to a waste tank.

The facility uses several large concrete tanks that are used to prepare source water for challenge testing, or to accept testing effluent. The clean source water is staged in a clean-water tank with an active volume (useable for testing, after accounting for the minimum depth required for pumping) of 0.75 Mgal constructed from two existing tanks with a breached common wall, and another clean-water tank with an active volume of 1.3 Mgal. Modified and seeded water is disposed to an effluent tank with a volume of 1.3 Mgal, which can overflow into a tank with a volume of 0.8 Mgal. The accumulated effluent is slowly pumped (up to 1000 gpm) into the treatment stream of the wastewater plant for treatment and final disposal. Because of this disposal procedure to an active wastewater treatment plant, the injection of most microbiological surrogates and/or microspheres is acceptable and does not require any special permitting or handling.

Filtered, high-quality potable water from a surface water supply (93 to 97% UVT at 254 nm) is delivered by the Johnstown Water Company Water Treatment Plant, Johnstown, NY via a local hydrant at rates up to 1,200 gpm. The water is dechlorinated with sodium bisulfite at the point of discharge into the source water tanks.

Three samples were collected at the influent and effluent ports provided with each test stand. The first of the three biodosimetry samples were collected from a sample tap that had been freely flowing for at least 30 seconds, then the tap was allowed to flow throughout the sample collection sequence. All biodosimetry samples were collected in individually packaged pre-sterilized containers and shipped on ice to MWD by overnight courier. DMS surrogate testing involved collection of larger samples, typically 5 to 20 liters, in opaque containers. This volume was adjusted based on the targeted number of particles to be captured for analysis. The collected sample was filtered on-site through a 0.45 µm membrane filter, the filter was removed and stored under covered and chilled conditions, and shipped to the Purdue University laboratories for analysis.
Figure 6.1 Schematic of MWD’s demonstration-scale 3-MGD UV facility showing tie-in with the full-scale treatment plant.
EVALUATION OF SPORES AND DMS IN LOW-PRESSURE REACTORS

A Trojan Technologies three-lamp, low-pressure high-output (LPHO) reactor at the Gloversville - Johnstown Joint Wastewater Treatment Facilities was tested with the phosphate-form of DMS and Bacillus pumilus ASFUVRC spores. Three different sizes of DMS (6, 10 and 15 μm) were used in the test. The reactor was operated under conditions that were anticipated to yield relatively high doses of UV₂₅⁴ radiation. Tests involved simultaneous application of LA and biodosimetry. A computational fluid dynamics (CFD) simulation was not available for this reactor but based on average intensity and residence time calculations, a nominal dose of 100 mJ/cm² was predicted for UV₂₅⁴ water transmittance of 95% at a 107 GPM flow rate, or UV₂₅⁴ water transmittance of 90% and 86 GPM flow rate. Assuming the average dose delivered by the reactor is inversely proportional to the flow rate, a flow range of 25 – 200 GPM should achieve a nominal dose range of 400 – 50 mJ/cm² with water UV₂₅⁴ transmittance of 95%. Four operating conditions of the reactor were tested, as summarized in Table 6.1.

The UV reactor was installed in a 4” test line in an upflow configuration. The water source was dechlorinated Johnstown tap water. A submersible pump was installed to draw water
to the reactor. The reactor was plumbed with an influent valve and an effluent valve to maintain desired pressure and flow rate through the reactor. An injection side-loop premix system was installed at the influent pipe in order to inject DMS and the challenge microorganism. Plumbing to the reactor included $90^\circ$ bends immediately before and after the reactor.

*B. pumilus* ASFUVRC spores from two separate preparations, cultured in sporulation broth containing 1 mM MnSO$_4$, were combined and shipped to the HydroQual facility by overnight courier. The spores and DMS were injected into the reactor feed and samples of reactor influent and effluent water were collected for enumeration. Spores samples were returned to MWD by overnight courier for enumeration. DMS and *B. pumilus* ASFUVRC spores were injected via a pre-mix system upstream of the UV reactor. Spores were injected simultaneously with the 10 µm DMS but spores were not included with the 6 µm or 15 µm DMS tests. Triplicate samples were analyzed for each test condition; DMS were analyzed by flow cytometry and surviving spores were enumerated on agar plates. The sampling interval was at least one residence time between the inlet and outlet sampling points. For DMS analysis, 10 L of the fluid was collected for each sample. For biodosimetry analysis, 0.4 L of the fluid was collected for each sample. Pre-sterilized containers were used for collection of samples used in biodosimetry. The *B. pumilus* spores were injected together with the 10 µm DMS, but the 10 L and 0.4 L samples for DMS and biodosimetry analyses, respectively, were collected separately. Although they were collected in the adjacent period of time, they should not be considered as the same batch of a sample because of differences in sample volume, sample collection time, and time required to collect each sample.

### Table 6.1

<table>
<thead>
<tr>
<th>Flow rate (GPM)</th>
<th>Flow rate (L/s)</th>
<th>UV$_{254}$ transmittance (%)</th>
<th>Nominal average dose$^*$ (mJ/cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>1.58</td>
<td>95</td>
<td>400</td>
</tr>
<tr>
<td>50</td>
<td>3.15</td>
<td>95</td>
<td>200</td>
</tr>
<tr>
<td>100</td>
<td>6.31</td>
<td>95</td>
<td>100</td>
</tr>
<tr>
<td>200</td>
<td>12.62</td>
<td>95</td>
<td>50</td>
</tr>
</tbody>
</table>

$^*$Estimated as the product of average intensity and residence time.

DMS Dose Responses, Flow-Through Responses, and Dose Distributions in a 3-Lamp LPHO reactor

Dose-response experiments at 0, 10, 20, 50, 100, 200, 400, and 600 mJ/cm$^2$ (300 mJ/cm$^2$ for 6 µm DMS only) were conducted for each size of DMS. Dose-response results for the 6, 10 and 15 µm phosphate-form DMS are shown in Figure 6.3. The 10 µm DMS had the greatest sensitivity to UV irradiation. The settings of the flow cytometer for each size of DMS were adjusted to ensure the entire response matrix for each size of DMS was in the linear range of the instrument. The settings of the flow cytometer were fixed for each size of DMS to ensure dose-response and flow-through samples were measured under the same condition.

Flow-through FI results for the 6, 10 and 15 µm DMS are shown in Figure 6.4. Triplicate samples collected at the lower flow rates were generally characterized by increased variability (*i.e.*, poorer reproducibility). This may have been due to instability of the reactor at lower flow rates. One of the triplicate samples of 6 µm DMS at 50 gpm had very low FI response; most of
the FI response was within the same range as the control (dose 0) samples. This sample was double checked by running it again in the flow cytometer after several other samples; the results of this repeated analysis were essentially identical to those of the first sample. This indicates the measurement should be correct. It is unclear why the microspheres in this sample were apparently not exposed to germicidal UV radiation; the cause of this behavior was not identified and constraints of the project made it impossible to repeat the experiment.

Each dose-response curve was fitted with a four-parameter Weibull equation and the dose-response matrix was generated by interpolating the four parameters against dose within the range and increment of interest. Dose distributions of the DMS, deconvolved from the dose-response matrix and flow-through FI distributions, demonstrated that the reactor delivered a wide range of doses, especially at low flow rates (Figure 6.5). A wide dose distribution indicated low efficiency of inactivation. Factors that could influence the width of a delivered dose distribution include water transmittance, reactor geometry, configuration of lamp installation, and fluid dynamics within the reactor. Operation of this reactor at the two lowest flow rates required partially closing the reactor’s effluent valve, which may have caused recirculation and instability within the reactor.
Figure 6.3  UV$_{254}$ dose-response of 6 μm (A), 10 μm (B), and (C) 15 μm DMS between 0 and 600 mJ/cm$^2$. UV doses increase from left to right across the graphs.
Figure 6.4. Fluorescence intensity (FI) responses of flow-through triplicate samples of 6 μm (A) and 10 μm (B) dyed microspheres at four different flow rates in a 3-lamp LPHO reactor.
Figure 6.4 (continued) Fluorescence intensity (FI) response of flow-through triplicate samples of 15 μm (C) dyed microspheres at four different flow rates in a 3-lamp LPHO reactor.
Figure 6.5 Cumulative dose distribution of 6 μm (A) and 10 μm DMS (B) in a 3-lamp LPHO reactor. Reactor flow rates increase from left to right across the graphs.
Figure 6.5 (continued) Cumulative dose distribution of 15 μm DMS (C) in a 3-lamp LPHO reactor. Reactor flow rates increase from left to right across the graphs.

**Biodosimetry Results for a 3-Lamp LPHO Reactor**

The LP-UV dose response for *B. pumilus* ASFUVRC spores was determined in a collimated beam bench-scale test at MWD (Figure 6.6) conducted in parallel with the demonstration testing of the LPHO reactor. The UV dose-response was essentially first-order (*i.e.*, log-linear) over the entire dose range used in this experiment and 3-log10 inactivation required a UV254 dose of 264 mJ/cm². Biodosimetry involved simultaneous collection of reactor influent and effluent samples. The log10 inactivation and corresponding reduction equivalent doses (RED) of triplicate samples at each flow rate are summarized in Table 6.2 and Figure 6.7. At the lowest flow rate of 25 GPM, 2.4- to 3.1-log10 inactivation was achieved. The average RED delivered by the Trojan reactor were 65, 105, 154, and 244 mJ/cm² at flow rates of 200, 100, 50, and 25 GPM, respectively (Table 6.2). Regression analysis of these data (R² = 0.998) showed that the calculated RED at 107 GPM was 97 mJ/cm², which is close to the manufacturer’s nominal dose of 100 mJ/cm² at 107 GPM and 95% transmittance. Based on the relationship between UV dose requirement and log inactivation provided in the LT2ESWTR, these RED values equate to virus inactivation credits of 1.2-log at 200 GPM to 5.3-log at 25 GPM (although 4-log is maximum inactivation credit provided by the LT2ESWTR).
Figure 6.6 LP-UV dose-response of *Bacillus pumilus* ASFUVRC spores in a single collimated beam test (●). Spores were propagated by growing the bacterium in sporulation medium containing 1 mM MnSO₄. Additional collimated beam inactivation data for these spores from other experiments are included for comparison (◊).
Table 6.2
Inactivation of *Bacillus pumilus* ASFUVRC spores in a LPHO UV reactor*

<table>
<thead>
<tr>
<th>Flow rate (GPM)</th>
<th>Log inactivation</th>
<th>Reduction equivalent dose, RED (mJ/cm²)</th>
<th>Equivalent virus inactivation credit†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Individual</td>
<td>Average</td>
</tr>
<tr>
<td>25</td>
<td>2.78</td>
<td>245</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>2.37</td>
<td>211</td>
<td>244</td>
</tr>
<tr>
<td>25</td>
<td>3.13</td>
<td>275</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1.55</td>
<td>141</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1.90</td>
<td>171</td>
<td>154</td>
</tr>
<tr>
<td>50</td>
<td>1.65</td>
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<td></td>
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<td>100</td>
<td>0.92</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>1.36</td>
<td>125</td>
<td>105</td>
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<tr>
<td>100</td>
<td>1.09</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>0.64</td>
<td>64</td>
<td></td>
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<tr>
<td>200</td>
<td>0.72</td>
<td>71</td>
<td>65</td>
</tr>
<tr>
<td>200</td>
<td>0.58</td>
<td>59</td>
<td></td>
</tr>
</tbody>
</table>

* Triplicate samples were analyzed for each flow rate and *B. pumilus* spores were enumerated in triplicate for each sample.
† Based on virus inactivation credits in LT2ESWTR, not adjusted by validation factors.
‡ Calculated as 5.3-log but 4-log is maximum inactivation credit.
Challenge Organisms for Inactivation of Viruses by Ultraviolet Treatment

Figure 6.7  Reduction equivalent dose (RED) in a 3-lamp LPHO reactor, estimated by Bacillus pumilus ASFUVRC spore biodosimetry (♦, solid line). Error bars are one standard deviation (N = 3). The equivalent LT2ESWTR virus inactivation credits for each flow condition are also plotted (○, dashed line).

Prediction of Inactivation in a 3-Lamp LPHO Reactor by DMS

Spore inactivation was predicted by DMS data for each sample collected and each operating condition by integration of the dose-response behavior and the dose distribution corresponding to each sample based on the segregated-flow model, as described previously (see Chapter 5 and previous references). Higher variability in both the DMS and spore data is reflected in the weak correlation between DMS predicted inactivation and measured spore inactivation at low flow rates (Figure 6.8). However, there was good agreement between biodosimetry with B. pumilus ASFUVRC spores and all three sizes of DMS at high flow rates. The most variable data set was for 6 µm DMS (Table 6.3) but this was heavily biased by a single value at 50 GPM that did not conform to the rest of the data. Despite the variability in the levels of inactivation measured by spore biodosimetry (average coefficient of variation, CV = 13.7%) and predicted by DMS (average CV = 10.1 – 31.5%), there was strong agreement when spore biodosimetry inactivation data was compared with the predicted inactivation based on all three sizes of DMS combined (Figure 6.8B; \( r = 0.998 \)). Although spores were only injected along with 10 µm DMS, the correlation between biodosimetry and DMS predicted inactivation were similar for all three sizes of DMS; correlation coefficients were 0.703, 0.847, and 0.934 for 6 µm, 10 µm, and 15 µm DMS, respectively, compared to ASFUVRC spores.
Table 6.3
Variability in inactivation data generated by biodosimetry and DMS predictions for a 3-lamp LPHO reactor

<table>
<thead>
<tr>
<th>Flow rate (GPM)</th>
<th>Coefficient of variation for (\log_{10}) inactivation, based on triplicate samples:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spores</td>
</tr>
<tr>
<td>25</td>
<td>13.69</td>
</tr>
<tr>
<td>50</td>
<td>10.69</td>
</tr>
<tr>
<td>100</td>
<td>19.70</td>
</tr>
<tr>
<td>200</td>
<td>10.85</td>
</tr>
<tr>
<td>Mean</td>
<td>13.7</td>
</tr>
</tbody>
</table>

Figure 6.8 Correlation between dyed microspheres (DMS) and *Bacillus pumilus* ASFUVRC spore biodosimetry for measuring UV inactivation in a 3-lamp LPHO reactor. The test was conducted at four flow conditions (25 – 200 GPM) and \(\text{UV}_{254}\) transmittance of 95%. Biodosimetry spores were simultaneously injected with only the 10 µm DMS. The dashed line indicates the ideal 1:1 correlation. Data were plotted for individual triplicate samples (A) and averaged data (B).

Evaluation of Spores and DMS in an 18-lamp LPHO Reactor

A second test was conducted at the HydroQual facility using an 18-lamp, LPHO reactor (18AL40; Trojan Technologies). This was an L-shaped reactor with a 16-inch reactor chamber diameter and lamps parallel to water flow. Water entered the reactor horizontally and exited...
Vertically through 10-inch inlet and outlet flanges. Spores of *B. pumilus* ASFUVRC were propagated to a high concentration using AK Agar plates. An 800 mL suspension was prepared containing $9.5 \times 10^9$ spores/mL giving a total spore titer of $7.6 \times 10^{12}$. The spore suspension was prepared at MWD and shipped via overnight courier to the HydroQual testing facility. Upon completion of the test, reactor influent and effluent samples were returned to MWD by overnight courier for processing. The period between sample collection and inoculation of spore enumeration agar plates was <24 h. The phosphate form of the 6 µm DMS were also used for this test. The test was conducted at four flow conditions (85 – 1,800 GPM) with the lamps operating at maximum power and water with UV transmittance of 95%.

Dose distributions of the DMS, deconvolved from the dose-response matrix and flow-through fluorescence distributions, again demonstrated that the reactor delivered a wide range of doses, especially at lower flow rates (Figure 6.9). Variability in DMS data was also greatest at the lower flow rates.

The collimated beam dose response was generated at MWD using the same spore preparation that was used for demonstration-scale testing. A dose of 250 mJ/cm² resulted in 4-log₁₀ inactivation of spores (Figure 6.10A). Although spore propagation on AK Agar yielded high spore concentrations, the dose response of these spores was not as resistant as spores propagated in liquid medium containing 1 mM MnSO₄ and tended towards tailing at high UV doses (as discussed in Chapter 4).

Based on *B. pumilus* ASFUVRC spore biodosimetry, the 18-lamp LPHO reactor achieved 1.28-, 1.97-, 3.08-, and 4.0-log inactivation at flow rates of 1800, 670, 240, and 85 GPM, respectively (Figure 6.10B). This equated to RED values of 43.2, 67.4, 138, and 250 mJ/cm² at flow rates of 1800, 670, 240, and 85 GPM, respectively. The corresponding LT2ESWTR virus inactivation credits for these RED values are 0.7-, 1.2-, 2.9-, and 4.0-log (calculated as 5.5-log but 4-log is maximum credit). Predicted spore inactivation based on DMS data averaged 2.73-, 3.97-, 4.66-, and 4.71-log at 1800, 670, 240, and 85 GPM, respectively, which did not correlate well with biodosimetry data. DMS-predicted inactivation using the spore dose response curve from the previous large-scale experiment (Figure 6.6) displayed good agreement with inactivation measured by spore biodosimetry at high flow rates but they diverged as the reactor flow rate decreased (Figure 6.10B). The reasons for the poor correlation between *B. pumilus* spores and DMS in this test are not clear but may have been due, in part, to the non-linear LP-UV dose response behavior of the spores in the collimated beam test (Figure 6.10A), although the same spore suspension was used for the collimated beam and large-scale tests.
Figure 6.9 Cumulative dose distribution deconvolution for 6 µm dyed microspheres in an 18-lamp LPHO reactor at the indicated flow rates. Deconvolution was accomplished using the Linear least squares (Lsqlin; A) and Find minimum of constrained nonlinear multivariable (Fmincon; B) functions of the MATLAB package.
Challenge Organisms for Inactivation of Viruses by Ultraviolet Treatment

Figure 6.10 Measuring inactivation in an 18-lamp LPHO reactor. A) Dose response of *Bacillus pumilus* ASFUVRC spores in a LP-UV collimated beam test. The dashed lines correspond to the level of inactivation measured in the LPHO reactor test at the indicated water flow rates and indicate the reduction equivalent doses achieved at each flow rate. B) Comparison between spore biodosimetry measured inactivation and DMS predicted inactivation. DMS predictions were based on a previous spore dose response (DMS predicted, A; see Figure 6.6 for dose response) or the dose response generated for this experiment (DMS predicted, B; panel A in this figure).

**EVALUATION OF *BACILLUS PUMILUS* SPORES AND *DEINOCOCCUS AQUATICUS* IN A MEDIUM-PRESSURE REACTOR**

The phosphate form of DMS could not be used in MWD’s demonstration-scale reactor because of photo-bleaching of the dye caused by MP-UV (see Chapter 5). Therefore, MWD’s demonstration-scale 4-lamp reactor was tested with *D. aquaticus* DPHPCD and *B. pumilus* ASFUVRC spores. Both of these microbes were originally isolated from Metropolitan’s untreated source water. In previous experiments, the germicidal weighted dose distribution for MWD’s MP-UV reactor, based on CFD-i modeling and operational parameters of 3 MGD flow, 97% light transmittance, water temperature of 56°F, and turbidity of 0.06 NTU, had a minimum and maximum of 33 and 1,053 mJ/cm², respectively, with an average of 154 mJ/cm² (Mofidi et al. 2004). Compensating for germicidal effectiveness, the resulting equivalent dose of 75 mJ/cm² corresponded to 3.4 log inactivation of MS2.
Both organisms were injected simultaneously upstream of the reactor using a chemical metering pump through a perforated pipe spanning the diameter of the reactor’s inlet pipe. Treated water samples were collected downstream of the reactor from a port connected to another perforated pipe spanning the diameter of the effluent pipe. The reactor was operated at four dose conditions (1, 2, 3, or 4 lamps energized) as well as a zero dose (no lamps energized). The influent water flow rate was 0.4 ± 0.1 MGD, controlled by an 18” butterfly valve.

The LP-UV dose response curves from collimated beam tests are displayed in Figure 6.11A. Since there was no difference in the LP-UV and MP-UV responses of *D. aquaticus* DPHPCD, the LP-UV dose response generated in the concurrent collimated beam test was used to calculate RED values for the MP-UV demonstration-scale reactor. However, *B. pumilus* ASFUVRC spores were more sensitive to MP-UV than LP-UV, so RED values were calculated using both MP- and LP-UV dose response data. Up to 4.9-log and 4.5-log inactivation was achieved for *B. pumilus* ASFUVRC spores and *D. aquaticus*, respectively, when all four UV lamps in the reactor were energized. Using the low-pressure collimated beam dose response for each organism, the calculated RED values based on each microbe’s data were similar (Table 6.4). The RED values based on the MP-UV spore data were substantially lower. For this experiment, spores were propagated by culturing *B. pumilus* ASFUVRC on AK Agar plates, to ensure a high enough titer for the test. However, as described in Chapter 4, spores propagated on AK Agar do not display the high level UV resistance of spores produced in sporulation broth containing supplementary MnSO$_4$, even though AK Agar contains a high concentration of MnSO$_4$.

The calculated RED values for this reactor based on *B. pumilus* spores and *D. aquaticus* were 33 – 75% higher than those previously determined in 2002 using MS2 as the biodosimeter (Figure 6.11B). This may have been due to the previous MS2 tests being conducted at 0.5 MGD whereas the tests for this project were at 0.4 MGD. Both of these flow rates are somewhat uncertain because of the difficulty in precisely controlling water flow at the demonstration-scale facility using an 18” butterfly valve. The plant operators estimated that the uncertainty in reactor flow rate was ± 0.1 MGD. Consequently, the previous MS2 test may have been conducted at a flow rate closer to 0.6 MGD while the tests for this project may have used a flow rate as low as 0.3 MGD.
Figure 6.11 Demonstration-scale testing of selected surrogate microbes in a 4-lamp Calgon Sentinel medium-pressure UV reactor at a flow rate of 0.4 MGD. A) LP-UV dose response curves were generated for *Bacillus pumilus* spores (isolate ASFUVRC) and *Deinococcus aquaticus* (isolate DPHPCD) using bench-scale collimated beam equipment. The dotted line corresponds to the MP-UV response of ASFUVRC spores. B) Inactivation in the reactor was compared to MS2 data obtained previously at MWD using this reactor at a flow rate of 0.5 MGD (▲).
This experiment was repeated but *Bacillus pumilus* ASFUVRC spores were propagated in sporulation medium + 1 mM MnSO$_4$ rather than on AK agar plates. The difference in spore propagation medium is reflected in the more resistant spore dose response while the *D. aquaticus* DPHPCD dose responses were similar in both experiments (Figure 6.12). The RED values based on the *D. aquaticus* dose response (26 – 130 mJ/cm$^2$; Table 6.5) were similar to the first test of this MP reactor (46 – 141 mJ/cm$^2$; Table 6.4). However, the spore derived RED values (based on the LP-UV dose response), averaged across all four lamp conditions, were 1.9 times higher than the *D. aquaticus* derived REDs. The RED values based on the MP-UV spore data were again substantially lower.

### Table 6.4
**Inactivation of microbial surrogates in a 4-lamp medium-pressure UV reactor operating at a flow rate of 0.4 MGD- Test 1**

<table>
<thead>
<tr>
<th>Number of UV lamps energized</th>
<th><em>Bacillus pumilus</em> ASFUVRC spores*</th>
<th><em>Deinococcus aquaticus</em> DPHPCD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log inactivation</td>
<td>RED† (mJ/cm$^2$)</td>
</tr>
<tr>
<td></td>
<td>LP‡</td>
<td>MP§</td>
</tr>
<tr>
<td>1</td>
<td>0.62</td>
<td>37.4</td>
</tr>
<tr>
<td>2</td>
<td>2.17</td>
<td>76.4</td>
</tr>
<tr>
<td>3</td>
<td>4.11</td>
<td>125.1</td>
</tr>
<tr>
<td>4</td>
<td>4.86</td>
<td>144.0</td>
</tr>
</tbody>
</table>

* Spores propagated on AK Agar. † Reduction equivalent dose. ‡ Based on the dose response in low-pressure collimated beam tests (Figure 6.11A). § Based on aggregate dose response in medium-pressure collimated beam tests (see Chapter 4).

### Table 6.5
**Inactivation of microbial surrogates in a 4-lamp medium-pressure UV reactor at a flow rate of 0.4 MGD- Test 2**

<table>
<thead>
<tr>
<th>Number of UV lamps</th>
<th><em>Bacillus pumilus</em> ASFUVRC spores*</th>
<th><em>Deinococcus aquaticus</em> DPHPCD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log inactivation</td>
<td>RED† (mJ/cm$^2$)</td>
</tr>
<tr>
<td></td>
<td>LP‡</td>
<td>MP§</td>
</tr>
<tr>
<td>1</td>
<td>0.45</td>
<td>62.3</td>
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<td>2</td>
<td>1.04</td>
<td>127.1</td>
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<tr>
<td>3</td>
<td>1.55</td>
<td>183.2</td>
</tr>
<tr>
<td>4</td>
<td>1.89</td>
<td>220.5</td>
</tr>
</tbody>
</table>

* Spores were propagated in sporulation medium containing 1 mM MnSO$_4$. † Reduction equivalent dose. ‡ Based on dose response in low-pressure collimated beam tests (Figure 6.12). § Based on aggregate dose response in medium-pressure collimated beam tests (see Chapter 4).
A third trial of the 4-lamp demonstration-scale MP-UV reactor was conducted at a flow rate of 0.4 MGD. Spores were propagated in sporulation broth containing 1 mM MnSO₄ to maximize their UV resistance and both LP- and MP-UV dose response curves were generated for both microbes using the collimated beam equipment (Figure 6.13) but the spore LP-UV data were not used to calculate RED values. The \emph{D. aquaticus} LP- and MP-UV, and spore MP-UV derived RED values were all similar for all four lamp conditions (Table 6.6). Comparing the three 0.4 MGD trials of this 4-lamp MP-UV reactor demonstrated variability in the calculated RED values, particularly with 3- and 4-lamps energized (Table 6.7). The ASFUVRC-derived RED values in the second trial were approximately 1.7-fold higher than the average of the first and third trials. Nevertheless, the calculated RED values from all three trials, using both microbes, were reasonably consistent.

The final trial of MWD’s 4-lamp reactor was conducted at the maximum flow rate of 3 MGD and used just \emph{B. pumilus} ASFUVRC spores as the challenge organism with a MP-UV collimated beam dose response curve. Inactivation levels ranged from 0.68-log to 5.83-log. RED values of 17.8 \text{mJ/cm}^2 with one lamp energized to >80 \text{mJ/cm}^2 with all four lamps energized were consistent with earlier data for this reactor at the same flow rate using MS2 as the biodosimeter (Table 6.8).

These demonstration-scale trials conducted at both MWD and the HydroQual facility with flow rates ranging from 0.04 – 3 MGD (28 – 2,083 GPM) demonstrated that two microbes,
*D. aquaticus* and *B. pumilus* spores, with similar or higher UV resistance compared to adenoviruses, could be developed as practical challenge organisms.

![Graph](image)

**Figure 6.13** UV dose responses of *Bacillus pumilus* ASFUVRC spores and *Deinococcus aquaticus* DPHPCD in collimated beam tests.

**Table 6.6**

Inactivation of microbial surrogates in a 4-lamp medium-pressure UV reactor at a flow rate of 0.4 MGD- Test 3

<table>
<thead>
<tr>
<th>Number of UV lamps</th>
<th><em>B. pumilus</em> ASFUVRC*</th>
<th><em>D. aquaticus</em> DPHPCD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log inactivation RED-MP</td>
<td>Log inactivation RED-LP</td>
</tr>
<tr>
<td>1</td>
<td>1.08</td>
<td>31.2</td>
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<tr>
<td>2</td>
<td>2.43</td>
<td>63.2</td>
</tr>
<tr>
<td>3</td>
<td>3.26</td>
<td>80.0</td>
</tr>
<tr>
<td>4</td>
<td>4.72</td>
<td>130</td>
</tr>
</tbody>
</table>

* Spores were propagated in sporulation medium + 1 mM MnSO₄.
**Table 6.7**
Consistency of biodosimetry-derived RED values across all 0.4 MGD demonstration-scale tests

<table>
<thead>
<tr>
<th>Number of UV lamps energized</th>
<th>Reduction equivalent dose (mJ/cm²)</th>
<th>Bacillus pumilus ASFUVRC</th>
<th>Deinococcus aquaticus DPHPCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial 1</td>
<td>Trial 2</td>
<td>Trial 3</td>
<td>Trial 1</td>
</tr>
<tr>
<td>1</td>
<td>37.4</td>
<td>62.3</td>
<td>31.2</td>
</tr>
<tr>
<td>2</td>
<td>76.4</td>
<td>127.1</td>
<td>63.2</td>
</tr>
<tr>
<td>3</td>
<td>125.1</td>
<td>183.2</td>
<td>80.0</td>
</tr>
<tr>
<td>4</td>
<td>144.0</td>
<td>220.5</td>
<td>130</td>
</tr>
</tbody>
</table>

* Average of LP- and MP-derived RED values.

**Table 6.8**
Inactivation of Bacillus pumilus spores in a 4-lamp medium-pressure UV reactor at a flow rate of 3 MGD

<table>
<thead>
<tr>
<th>Number of UV lamps</th>
<th>B. pumilus ASFUVRC*</th>
<th>Log inactivation</th>
<th>RED (mJ/cm²)</th>
<th>MS2 RED (mJ/cm²)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.68</td>
<td>17.8</td>
<td>18.2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.99</td>
<td>29.6</td>
<td>31.9</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4.12</td>
<td>57.9</td>
<td>43.6</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5.83</td>
<td>&gt;80</td>
<td>52.5</td>
<td></td>
</tr>
</tbody>
</table>

* Spores were propagated on AK agar plates.
† Based on MWD’s 2002 data for 3 MGD.
CHAPTER 7
DISCUSSION AND CONCLUSIONS

The primary objective of this project was to identify a non-pathogenic challenge organism that effectively models a UV reactor’s disinfection efficiency towards virus inactivation, as described by the LT2ESWTR inactivation tables. The surrogate(s) should be applicable to large-scale UV reactor validation for virus inactivation credit. The project identified a variety of highly UV resistant microbes (Table 7.1). These were various species of Deinococcus, Rubrobacter radiotolerans, and Bacillus pumilus spores, including native strains of D. aquaticus and B. pumilus isolated from untreated source water. The microbes that were most resistant and most suitable as new challenge organisms were spores of native isolates of B. pumilus (isolates ASFUVRA and ASFUVRC). The response of these spores to UV radiation could be manipulated by varying the concentration of manganese (in the form of MnSO₄) in the culture medium used to propagate the spores (Chapter 4). This “tunable” dose response could be varied from being close to the dose response curve of Ad2 to being more resistant than adenoviruses or the LT2ESWTR dose requirements for virus inactivation. Therefore, B. pumilus spores should be developed as alternative challenge microbes for validating UV reactors for virus inactivation credit. The availability of this highly UV resistant biodosimeter could assist utilities that are considering UV treatment to comply with the virus inactivation credits of the LT2ESWTR and GWR. Applications to recycled waste water for indirect potable reuse, fabrication, and general industrial use are also likely.

A second project objective was to examine the potential for dyed microspheres (DMS) to function as non-biological surrogates for validating UV reactors that are intended for inactivation of UV-recalcitrant microorganisms such as adenoviruses (Chapter 5). Three different sizes of DMS (nominally 6 μm, 10 μm, and 15 μm) were examined to determine their UV₂₅₄ dose-response behavior. The 10 μm DMS provided the greatest response to germicidal UV₂₅₄ exposure. In addition, the wavelength dependence of germicidal UV dose-response behavior was characterized for each of the three DMS sizes. However, application of DMS to polychromatic reactors depends on the availability of a dye molecule that is photoreactive to a broad spectrum of germicidal UV radiation. Two forms of DMS were used in the project, one with a silane linkage between the fluorophore S and the microspheres, and a second that used a phosphate linkage. While the silane-form of the dye produced a measurable response to LP- and MP-UV, the phosphate form only responded to LP-UV. The phosphate-form generated no response when exposed to full-spectrum MP-UV, possibly due to photobleaching of the fluorescent product P. Therefore, for the dye formulation that was used in this project, the silane-form of the dye is required for application to polychromatic reactors.

The final objective of the project was to conduct testing of selected surrogates at full-scale UV treatment facilities. Following successful demonstration-scale testing up to 3 MGD, the intent was to test B. pumilus spores and DMS at full-scale. Three utilities originally agreed to participate for this phase of the project. However, following detailed discussions with laboratory and management personnel from the utilities, they were not prepared to allow testing of proposed surrogates. Utility personnel were concerned about the potential public health implications, real or perceived, if some of the introduced spores or DMS were not completely removed or inactivated prior to entering the distribution system.
Bacillus pumilus is not considered a human pathogen but it has been recovered from human infections (Bentur et al. 2007), although its role as the primary cause of the infection was not demonstrated. Nevertheless, the inability to guarantee zero probability of infection caused concern among participating utility personnel so they were reluctant to allow testing. Naturally occurring ASF and UV resistant B. pumilus spores were isolated from the source water of one of the utilities (up to 180 spores per liter, Chapter 4) but the concentration of indigenous ASF was at least two orders of magnitude lower than the planned spike concentration. So, although the same types of bacteria are naturally present in untreated plant influents, the utilities were not prepared to spike influent waters with spore concentrations at least 100-fold higher than background.

Different types of DMS have been used previously in full-scale treatment plants but precautions were taken to minimize the risk of microspheres entering the distribution system. In one study using Fluorabrite YG microspheres at an ozone plant, effluent from the test treatment trains was discharged to a storm drain and filters were backwashed prior to returning the plant to normal service (Marinas et al. 1999). The 6 μm DMS used in the current project were previously applied in large-scale LP-UV tests (20 – 60 MGD) but the effluent from the reactors was disposed by slowly feeding it to a full-scale wastewater treatment facility (Blatchley et al. 2008). The utilities for the current project were concerned about the potential health hazards of introducing a fluorescent photoreactive compound into a drinking water treatment plant. Two of the utilities were located in the western United States in regions suffering from a multi-year drought and none of them were prepared to discharge large volumes of water to waste. Information on the non-toxicity of the photosensitive dye in the DMS (Chapter 5) was not available at the time that full-scale testing was being discussed.

The understandable difficulty in obtaining acceptance for full-scale, on-site testing of alternative dosimeters suggests that such testing may be restricted to dedicated large-scale facilities such as the research platform operated by HydroQual. An advantage of such facilities is that in addition to surrogate microbes or other dosimeters, pathogens can be tested directly, so long as the waste stream is adequately treated to completely inactivate the pathogen. For example, a recent report described large-scale tests at HydroQual’s Johnstown facility in which Ad2 disinfection was studied using a MP-UV reactor at flow rates of 200 – 900 GPM (Linden et al. 2009). Over 4-log inactivation of Ad2 was achieved with an MS2 RED of 100 – 160 mJ/cm². The authors suggested that the low-pressure UV-based dose requirements for virus inactivation credit included in the LT2ESWTR (see Table 1.1 in Chapter 1) are too high to ensure cost-effective virus inactivation. Furthermore, they concluded that MP-UV is a best available technology for 4-log virus disinfection.

Similar to B. pumilus ASFUVRC in this project, a strain of B. pumilus isolated from raw water was cultured to concentrations up to 10¹⁰ cfu/mL (Collins and Malley 2006). Doses of 93 and 210 mJ/cm² resulted in 1-log and 4-log inactivation, respectively, and there was no evidence of tailing up to 250 mJ/cm². The authors suggested that indigenous biodosimeters have several advantages over standard laboratory strains of microorganisms. Obtaining regulatory permits for on-site validation could be easier with native isolates and there may be less public concern regarding the use of native organisms in drinking water treatment plants, compared to non-native organisms. In addition, native biodosimeters could also be used in treatment plants that might impact sensitive ecosystems, which would preclude the use of non-native strains.

However, the experience of this project and discussions with various utilities suggest that novel challenge microbes and non-biological dosimeters may not be acceptable for on-site
reactor validation testing unless unequivocal proof can be provided that they present no risk to public health, even if the microbes are indigenous to the source water feeding a particular treatment plant. There may need to be a process similar to the current NSF certification program for materials that come into contact with drinking water.

Spores of the fungus *A. niger* are highly resistant to UV light with a dose of approximately 450 mJ/cm² being required for 2.5-log inactivation (Petri and Odegaard 2008). Spores were cultured to a high density and used to measure REDs up to 410 mJ/cm² in demonstration-scale testing of a UV reactor at flow rates of 18 GPM (0.025 MGD) and 555 GPM (0.78 MGD). However, *A. niger* is a human pathogen causing ear and lung infections (aspergillosis) and is therefore not suitable as a challenge organism for on-site reactor validation.

One of the requirements of suitable challenge organisms for large-scale reactor validation is that they can be grown to sufficiently high titers. From a logistics standpoint, the primary obstacle to full-scale testing of *B. pumilus* spores is generating a sufficient number of spores. For this project, an 800 mL suspension containing $9.5 \times 10^9$ spores/mL (total = $7.6 \times 10^{12}$) was achieved by scaling up the spore propagation procedure. Forty agar plates and two weeks were needed to produce this number of spores but most of the time was multi-day incubation periods. Although it will not be possible to achieve MS2-like yields of spores from a single culture, spore yields 10- or even 100-fold higher could be achieved with a dedicated effort in a relatively short period of time. Demonstration-scale testing at 3 MGD was accomplished with $2.3 \times 10^{12}$ spores, which were easily generated using standard laboratory culture procedures. The highest spore concentrations were achieved with *B. pumilus* cultured on AK agar plates. However, the dose response of these spores is not as resistant as spores grown in liquid medium containing 1 mM MnSO₄ and not linear. Therefore, more work is necessary to increase spore concentrations obtained in liquid medium or to manipulate conditions and media components so that spores propagated on solid media have higher UV resistance.

High concentrations of bacteria used for industrial, biotechnology, and pharmaceutical purposes are typically produced by fermentation in continuous cultures using chemostats with regulated nutrients, pH, and temperature. However, the discontinuous sporulation process in *Bacillus* spp. does not lend itself to continuous culture. Consequently, medium- to large-scale batch processes are typically used. A 10 L sequential batch process produced $2.3 \times 10^{10}$ *B. subtilis* spores per mL in 22 hours for a total of $2.3 \times 10^{14}$ spores (Evans and Harris-Smith 1971), and a fed-batch process generated a 2 L suspension containing $7.4 \times 10^9$ *B. subtilis* spores per mL (Monteiro et al. 2005). These studies, along with the spore concentrations achieved in this project, demonstrate that achieving high spore concentrations is not an obstacle to adopting *B. pumilus* spores as a challenge organism for validating UV reactors at the high doses required for LT2ESWTR virus inactivation credit.

In this project and the Collins and Malley (2006) study discussed above, native *B. pumilus* strains isolated from source waters were cultured in the laboratory to produce high concentrations of spores for reactor testing. Other authors have suggested the use of indigenous aerobic spores for reactor validation without prior culturing (Mamane-Gravetz and Linden 2004). The concentration of aerobic spores in six natural waters was $1 – 100$ spores per mL and 1-log₁₀ inactivation of these indigenous spores was achieved with UV doses of 47 – 77 ml/cm². However, the response of indigenous spores is likely to be site and season specific due to the diversity of naturally occurring ASF. The concentration of ASF bacteria in source waters tested for this project was <0.02 – 42 spores/mL. Therefore, naturally occurring spores that have not been propagated to high concentrations by laboratory culture cannot be used to validate reactors at the high levels of inactivation needed for viruses.
### Table 7.1
Low-pressure UV dose requirements for microbial inactivation

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Dose required for inactivation (mJ/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-log$_{10}$</td>
</tr>
<tr>
<td>Adenovirus 2</td>
<td>31</td>
</tr>
<tr>
<td>Adenovirus 41</td>
<td>1</td>
</tr>
<tr>
<td>Bacillus pumilus ASFUVRA*</td>
<td>99</td>
</tr>
<tr>
<td>Bacillus pumilus ASFUVRC*</td>
<td>82</td>
</tr>
<tr>
<td>Bacillus pumilus ASFUVRC†</td>
<td>32</td>
</tr>
<tr>
<td>Bacillus pumilus SAFR032*</td>
<td>149</td>
</tr>
<tr>
<td>Rubrobacter radiotolerans</td>
<td>66</td>
</tr>
<tr>
<td>Deinococcus radiopugnans</td>
<td>65</td>
</tr>
<tr>
<td>Deinococcus aquaticus DPHPCD</td>
<td>52</td>
</tr>
<tr>
<td>Deinococcus radiodurans R1</td>
<td>47</td>
</tr>
<tr>
<td>Bacillus megaterium</td>
<td>35</td>
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<tr>
<td>Bacillus pumilus 7061</td>
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<td>Bacillus pumilus 27421</td>
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</tr>
<tr>
<td>Chlorella virus PBCV-1</td>
<td>24</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>21</td>
</tr>
<tr>
<td>Bacteriophage MS2</td>
<td>19</td>
</tr>
<tr>
<td>Bacteriophage PP7</td>
<td>14</td>
</tr>
<tr>
<td>Bacteriophage PM2</td>
<td>14</td>
</tr>
<tr>
<td>Methylobacterium sp.</td>
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<tr>
<td>Bacteriophage PRD1</td>
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<tr>
<td>Bacteriophage Bam35</td>
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</tr>
<tr>
<td>Bacteriophage Phi29</td>
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</tr>
<tr>
<td>Lactobacillus plantarum</td>
<td>-†</td>
</tr>
<tr>
<td>Bacteriophage T4</td>
<td>-†</td>
</tr>
<tr>
<td>Bacteriophage 6631-B1</td>
<td>-†</td>
</tr>
<tr>
<td>Bacteriophage T7</td>
<td>-†</td>
</tr>
</tbody>
</table>

* Spores propagated in 1 mM MnSO$_4$.
† Spores propagated in 0.01 mM MnSO$_4$.
‡ Requires extrapolation beyond the measured data range.
CONCLUSIONS

Adenovirus types 2 and 41 were enumerated using the same quantitative plaque assay on the easily maintained A549 cell line. Low-pressure UV doses of 98 mJ/cm² and 201 mJ/cm² resulted in 3-log inactivation of Ad2 and Ad41, respectively. The Ad2 data were in agreement with published reports for this virus (Ballester and Malley 2004; Gerba et al. 2002; Linden et al. 2009) while the Ad41 response was slightly more resistant than most published data for the “enteric” adenoviruses (Baxter et al. 2007; Jacangelo et al. 2002; Ko et al. 2005; Linden et al. 2007; Thurston-Enriquez et al. 2003). Infectivity of Ad2 surviving UV irradiation was confirmed by plucking plaques and secondary passage in cell culture, and by electron microscopy. Medium-pressure UV was more effective than LP-UV for inactivating Ad2 but not Ad41, and the action spectrum for Ad2 indicated that within the range 220 – 280 nm, 220 nm was the most effective wavelength (approximately 10-fold higher inactivation compared to 254 nm). Although 220 nm was also the most effective wavelength for inactivating Ad41, the differences between wavelengths were not as pronounced as they were for Ad2. The Ad41 MP-UV data should be interpreted with caution since Ad41 and Ad2 were not assayed using exactly the same procedures due to difficulties in obtaining sufficiently high titers of Ad41. Adenovirus 2 was suspended in treatment plant effluent water for all collimated beam experiments whereas Ad41 was suspended in diluted cell culture medium. However, control experiments demonstrated that the failure of MP-UV to increase inactivation of Ad41 relative to LP-UV was not an artifact of experimental procedures.

Based on the LP-UV data for Ad2 generated by this project, the LT2ESWTR and GWR dose requirements for virus inactivation credit appear to be appropriate. However, the dose requirements for Ad2 inactivation can be reduced substantially for MP-UV systems because Ad2 is more sensitive to MP-UV compared to LP-UV.

Linden et al. (2007, 2009) demonstrated that MP-UV is more effective than LP-UV for inactivating Ad2 and Ad40. Results of the current project for Ad2 support this conclusion. Based on demonstrations of the increased effectiveness of MP-UV in bench-scale collimated beam and full-scale field trials, Linden et al. (2009) concluded that the UV dose requirements for virus inactivation credit in the LT2ESWTR are not appropriate for ensuring cost-effective virus inactivation. Further work is necessary to determine whether this holds true for Ad41. The results of the current study indicated that Ad41 displays the same resistance to MP-UV as it does to LP-UV. However, this finding needs to be independently corroborated (or refuted) because of the limited amount of Ad41 MP-UV data that was generated and the different suspension matrices that were used for Ad41 and Ad2 collimated beam experiments. Additional replicates of the Ad41 MP-UV response experiments could not be performed because of project time constraints and failure of the medium-pressure collimated beam apparatus.

Ad2 is easier to work with than Ad40/41 and can be cultivated to higher titers. Therefore, there may be a preference to use Ad2 rather than Ad40/41 for testing UV systems. However, if the MP-UV responses of Ad41 and Ad2 are significantly different, using Ad2 as a UV inactivation model for all adenoviruses may not be appropriate. An action spectrum correction factor would need to be applied to the RED and it would be necessary to prove that the correction factor was sufficient to protect public health. In addition, although Ad2 targets the respiratory system, it is still a human pathogen and so, from a public health perspective, on-site reactor validation with Ad2 is no less problematic than using the enteric adenoviruses (Ad40 and Ad41).
The formation of thymine dimers in the DNA of UV exposed Ad2 and their increased accumulation with increasing UV doses (Chapter 3), along with published data showing that the apparent resistance of adenoviruses to UV is due to host cell mediated DNA repair (Day, 1974b; Rainbow, 1977), demonstrated that there is nothing unique about the DNA or physical structure of adenoviruses that prevents them from being damaged by UV radiation.

A wide variety of bacteriophages were tested but none of them were more resistant to UV than MS2 and so were not suitable as alternative challenge organisms for virus inactivation (Chapter 4). However, the advantages of using bacteriophages as challenge organisms include high titers and the lack of concern regarding human pathogenicity. Therefore, studies should continue with selected phages in a variety of hosts that may display a similar repair capacity as the adenovirus/host cell combinations. The response to UV of MS2 was shown to be, in part, an inherent property of the virus and not driven solely by the sensitivity or resistance to UV of the host bacterium used to assay the bacteriophage following UV irradiation.

The PBCV-1 virus that infects *Chlorella* spp. was considered to be more closely akin to adenoviruses because, like adenoviruses, it infects eukaryotic cells rather than bacteria and has a double stranded DNA genome. Although its UV dose response was slightly more resistant than the average MS2 data for this project, its response was within the 90% prediction limits for MS2 inactivation provided by the UV Disinfection Guidance Manual, so it offered no advantage over MS2 as a challenge organism.

*Deinococcus* spp. and *Rubrobacter radiotolerans* displayed similar UV resistance as adenoviruses (4-log inactivation at 128 – 186 mJ/cm²) but the shoulder and tailing in their UV dose response curves may limit their utility as practical surrogates. Nevertheless, an indigenous strain of *Deinococcus aquaticus* provided consistent estimates of the doses delivered by a 4-lamp demonstration-scale reactor operating at 0.4 MGD (Chapter 6).

Medium-pressure UV was only slightly more effective than low pressure UV for inactivating *D. aquaticus* and the action spectrum indicated that although 220 and 228 nm filtered UV (UV<sub>220-228</sub>) was approximately 1.4-fold more effective than UV<sub>254</sub>, it was no more effective than UV<sub>260</sub>. This may be due to intracellular manganese protecting *Deinococcus* spp. proteins (including DNA repair proteins) from radiation-induced damage (Daly et al. 2004, 2007) so that they are not as sensitive to UV exposure as proteins in other bacteria.

Based on the assumption that bacteriophages that infect *Deinococcus* spp. may be repaired by their host cell and so also display high levels of UV resistance, a variety of strategies were used to isolate *Deinococcus*-specific phage. However, there are no reports of such phage and all attempts to isolate them for this project were unsuccessful.

The most promising candidates as suitable challenge organisms for UV reactor validation were spores of native strains of *Bacillus pumilus* (ASFUVRA and ASFUVRC) isolated from the influent of a drinking water treatment plant. The UV dose response of these spores could be manipulated by varying the concentration of manganese (in the form of MnSO₄) in the spore propagation medium. The dose response curve could be varied from being close to the dose response curve of Ad2 (2-log inactivation at 57 mJ/cm² for *B. pumilus* spores compared to 62 mJ/cm² for Ad2) to being more resistant than the LT2ESWTR dose requirements for virus inactivation (2-log inactivation at 174 – 187 mJ/cm²) or more resistant than Ad41. Consequently, *B. pumilus* spores are suitable as a UV inactivation surrogate for all adenoviruses.

Spores of *Bacillus subtilis*, a standard laboratory strain of *Bacillus pumilus*, and an indigenous isolate of *Bacillus megaterium* all conformed reasonably closely to the UVDGM 90% prediction limits for inactivation of *B. subtilis* spores (3-log inactivation at 51 – 90 mJ/cm²) and
so were less resistant than the LT2ESWTR dose requirements for virus inactivation. However, the dose response of some of these strains could also be manipulated by propagating the spores in media containing manganese to increase their resistance.

Medium-pressure UV was more effective than low pressure UV for inactivating \textit{B. pumilus} spores. A MP-UV dose of 120 mJ/cm$^2$ resulted in 4.8-log inactivation compared to 1.6-log inactivation with the same dose of LP-UV. The action spectrum for \textit{B. pumilus} spores was almost the same as the action spectrum for Ad2, with approximately 8-fold higher inactivation at UV$_{220-228}$ than at UV$_{254}$. The similarity of action spectra strengthens support for \textit{B. pumilus} spores as suitable challenge organisms for reactor validation.

A summary of inactivation data demonstrates that MP-UV was more effective than LP-UV for inactivating some, but not all, microorganisms (Table 7.2). Of the five microbes for which the two types of UV radiation were compared, the LP/MP dose ratios for 2-log inactivation ranged from 1.1 for Ad41 (no or little difference between LP- and MP-UV) to 3.4 for \textit{B. pumilus} ASFUVRC spores (MP-UV far more effective than LP-UV). Some of these results concur with published data (MS2 and Ad2) while others appear to disagree. In the current project MP-UV was no more effective than LP-UV for inactivating the species F adenovirus, Ad41. However, Linden et al. (2007) reported increased effectiveness of MP-UV for inactivating the similar species F adenovirus, Ad40. Therefore, follow-up studies are needed to confirm or refute the reported responses of adenoviruses to MP-UV.

UV resistant aerobic sporeforming bacteria such as \textit{B. pumilus} are relatively common and easy to isolate from untreated source waters. Suspensions containing $10^{10}$ spores/mL were relatively easy to prepare using standard laboratory procedures and spores could be propagated to sufficiently high enough titers for large-scale reactor testing and validation. Based on microscopic observation, spores were evenly distributed with minimal clumping.

\begin{table}[h]
\centering
\begin{tabular}{lcc}
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\textbf{Microbe} & \textbf{Dose for 2-log inactivation} & \textbf{LP/MP ratio} \\
 & \textbf{LP-UV} & \textbf{MP-UV} \\
\hline
Bacteriophage MS2 & 34 & 29 & 1.2 \\
\textit{Deinococcus aquaticus} DPHPCD & 63 & 52 & 1.2 \\
\textit{Bacillus pumilus} ASFUVRC spores & 148 & 43 & 3.4 \\
Adenovirus type 2 & 62 & 30 & 2.1 \\
Adenovirus type 41 & 141 & 134 & 1.1 \\
\hline
\end{tabular}
\caption{Summary of low-pressure vs. medium-pressure UV inactivation of microbes}
\end{table}

Dyed microspheres with a diameter of 10 µm were more sensitive to UV than 6 µm or 15 µm diameter DMS but all three sizes were most sensitive to UV radiation close to 254 nm. Additionally, all three sizes could be used to characterize dose distributions in the range 0 – 600 mJ/cm$^2$ in collimated beam experiments and flowing reactors. Lagrangian actinometry based on DMS fluorescence measurements characterizes the dose distribution in UV reactors and so shows promise for reactor validation. The relatively strong fluorescence intensity (FI) signals induced by large UV doses are more easily differentiated compared to smaller FI values with
lower UV doses, indicating that LA may be well suited for quantification of UV dose distributions in high-dose applications. However, additional work is necessary to fully assess the variability of the method.

Cell culture based assays demonstrated that the photosensitive dye used in the DMS was not toxic, even at concentrations five orders of magnitude higher than the maximum liquid phase concentration expected in reactor tests.

Changes to the chemistry involved in linking the photosensitive dye \(E-5-[2-(\text{methoxycarbonyl})-\text{ethenyl}]\text{cytidine}\) to the microspheres altered the response of DMS to UV radiation, even though there was no alteration to the dye itself. Specifically, substituting a phosphate-based linker for the original silane group rendered the DMS unresponsive to MP-UV radiation and thus unsuitable for MP applications.

Demonstration-scale testing of three UV reactors at two facilities with flow rates ranging from 28 to 2,083 GPM demonstrated that \(D. \text{aquaticus}\) and \(B. \text{pumilus}\) spores could be developed as practical challenge organisms for reactor validation at the high doses required for virus inactivation (Chapter 6). The RED of a 3-lamp LPHO reactor based on \(B. \text{pumilus}\) spore biodosimetry at a flow rate of 104 GPM was 97 mJ/cm\(^2\) compared to the manufacturer’s nominal dose of 100 mJ/cm\(^2\) under these conditions. In this experiment, the reactor’s REDs were equivalent to LT2ESWTR virus inactivation credits (not adjusted for validation factors) of 1.2-log to 4-log (maximum allowable inactivation credit) at flow rates of 200 to 25 GPM.

Based on \(B. \text{pumilus}\) ASFUVRC spore biodosimetry, an 18-lamp LPHO reactor achieved RED values of 43 to 250 mJ/cm\(^2\) at flow rates of 1800 to 85 GPM. These values equate to unadjusted LT2ESWTR virus inactivation credits of 0.7-log to 4-log.

There was good agreement between measured spore inactivation and DMS-predicted inactivation at high flow rates in LPHO reactors but the correlation was weaker at low flow rates. More work is necessary to improve the correlation between DMS and microbial surrogates; it is not clear whether the poor correlation in some experiments was due to inaccurate spore or DMS responses in the reactor.

Microbi ally-derived RED values for a 4-lamp MP-UV reactor operating at 278 GPM were reasonably consistent across three trials and two surrogates (\(B. \text{pumilus}\) spores and \(D. \text{aquaticus}\)). RED values were 26 – 46 mJ/cm\(^2\) with one lamp energized and 101 – 144 mJ/cm\(^2\) with all four lamps turned on.

Average \(B. \text{pumilus}\) ASFUVRC spore-derived RED values for the same 4-lamp MP-UV reactor operating at 2,083 GPM were within 7% of previously derived RED values for the same reactor based on MS2 biodosimetry. These data provide further support for the suitability of \(B. \text{pumilus}\) spores as a challenge organism for reactor validation.

On-site validation is important in confirming that a reactor performs as intended. However, on-site reactor validation with novel challenge organisms may be difficult to implement due to the perception by treatment plant operators and management that the microbes may present a public health threat, even if the challenge organisms are isolated from the influent of drinking water treatment plants.

**RECOMMENDATIONS**

Spores of \(B. \text{pumilus}\) should be considered as an alternative challenge microbe for validating UV reactors for virus inactivation. Drinking water, regulatory, and UV industry professionals should decide on the specific UV response that is required of this new tunable
challenge organism so that the spore propagation conditions can be standardized. This will involve standardizing the type of culture medium, concentration of manganese, and incubation period during spore propagation, because all of these affect the response of the spores to UV radiation. Procedures could be standardized on a single isolate, such as the strain of *B. pumilus* isolated in this project (ASFUVRC), or native *B. pumilus* spores could be isolated and cultured from individual treatment plant influents prior to reactor validation testing at that particular site.

Further work is necessary to develop culture conditions that maximize spore yields while retaining the desired level of UV resistance. In addition, more large-scale (full-scale) testing of the spore biodosimeter is necessary using a wider variety of reactor types and testing conditions. An inter-laboratory round robin study should be conducted to determine the reproducibility and variability of all aspects of the spore methodology.

Although they have some disadvantages such as dose response shoulder and tailing and difficulty in achieving sufficiently high concentrations for large-scale testing, *Deinococcus* spp. should also be considered for additional testing and optimization as potential challenge organisms. The focus of this work should be maximizing cell yields in culture.

Bacteriophages offer various advantages over vegetative bacteria and spores as challenge organisms, including the ability to culture them to high titers and no concerns about human pathogenicity. Therefore, although none of the tested bacteriophages were more resistant than MS2, studies should continue with selected phages in a variety of hosts that may display a similar repair capacity as the adenovirus/host cell combination.

Additional testing of multiple strains of Ad2, Ad40, and Ad41 should be conducted with both LP-UV and MP-UV systems to assess whether the viruses respond differently to monochromatic and polychromatic UV radiation.

Lagrangian actinometry also demonstrated promise as a method for validating UV reactors that are intended for inactivation of UV-recalcitrant microorganisms, or for other high-dose applications. The primary benefit of LA relative to other validation methods (i.e., biodosimetry) is the ability to measure a dose distribution, which in turn allows for reductions in uncertainty and bias in reactor validation. However, additional work is needed to better define the uncertainty associated with applying this approach to reactor validation. In addition, alternative formulations of DMS that are responsive to both LP-UV and MP-UV should be investigated.

The demonstration-scale experiments involving LA generated poorer sample reproducibility than has been observed in other applications of LA to date. This behavior was observed in samples that were collected from a reactor that was being operated at a flow rate well below its recommended flow rate range. It was hypothesized that the relatively poor reproducibility observed in these replicate samples was attributable to instability of the reactor under these operating conditions. If this is the case, then it suggests another potential advantage of LA, in the sense that it represents a method that is sufficiently sensitive to allow identification of the effects of flow instability. However, additional experiments are needed to characterize the validity of this hypothesis, and more generally to identify the ability of LA to provide accurate measurements of the UV dose distribution delivered by a UV reactor under conditions designed to deliver high UV doses. As an extension of this logic, it appears that LA may also represent an appropriate method for characterization of UV photoreactors that are used to bring about purely photochemical changes in fluid composition. In these applications, which often involve reactors that are designed to deliver large UV doses, predictions of reactor behavior would be based on the segregated flow model, but would include the dose distribution delivered by the reactor and...
an appropriate description of the kinetics of the photochemical reaction of interest. Future experimentation should be directed to this issue.

Finally, there is a need to engage utilities, regulatory agencies, and public health departments in a discussion on the potential public health and public relations issues relating to the use of challenge organisms and non-biological dosimeters for full-scale on-site reactor validation. This may involve modeling the likelihood of introduced microbes or other dosimeter particles reaching the consumer, based on input concentrations, flow and volume of the treatment plant, the amount of UV-induced inactivation, sensitivity to post-UV chemical disinfection, holding periods and volume of water storage facilities downstream of the UV reactor, and residence time in the distribution system.
APPENDIX A
EXAMPLE RADIOMETER CALIBRATION CERTIFICATES

ELECTRICAL INSTRUMENTATION CALIBRATION REPORT
DUE DATE: 18-Jan-07

This document that the instrument described below meets or exceeds all manufacturer specifications and has been compared to standards which are directly traceable to the National Institute of Standards and Technology.

Date: 18-Jan-06 Certificate #: 601189705E SO#: 117972
Temp: 21 degrees C Humidity: 26 % Procedure: 146300-REVD

Rendered To: Metropolitan Water District
Instrument/Model-S/N: IL1700 #3470
Calibration/Repair Remarks:

Parts (If Needed):

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<th>As Found Permissible Error</th>
<th>Applied Current</th>
<th>Adjusted Readings</th>
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Tolerance after repair and/or calibration: Out In X
COMBINED NIST AND KEITHLEY TRANSFER UNCERTAINTY IS AS FOLLOWS: 1mA=+/0.025%, 100μA=+/0.025%, 1uA=+/0.025%, 100nA=+/0.035%, 10nA=+/0.065%, 1nA=+/0.065%, 100pA=+/0.25%

The above instrument was compared to the Keithley Current Calibrator/Source Model 263 SN 0621350 calibrated on 01APR 05, which is traceable to NIST.

Calibrated By: [Signature] Checked By: [Signature]

*For authorized copies of this certificate please contact International Light, Inc.*
International Light certifies that the instrument described below has been compared with the laboratory working standards whose calibrations are traceable to the U.S. National Institute of Standards and Technology and whose procedures are in accordance with the requirements of ANSI/NCSL Z540-1-1994, ISO 10012-1:1992(E) and ISO/IEC Guide 25:1990(E).

Rendered to: METROPOLITAN WATER DISTRICT

Detector: SED240 #5213

Input Optic: W #912W

Filter: G #18915

Misc.: N/A

Spectral Response (half power points):

(PIR) PEAK IRRADIANCE RESPONSE SENSITIVITY FACTOR AS CALIBRATED ON: 22-Mar-2006

3.78E+4 (A)(cm2)(W-1) assuming monochromatic irradiance at 254nm

-2.83% *Change In Sensitivity From Previous Calibration Date: 27-Dec-2004

Tolerance As Found: In √ Out

Tolerance As Left: In √ Out

Unit will read directly in watts per square centimeter when used with an IL1700 and the Sensitivity Factor Above. Calibrated with IL1700 #3470.

CALIBRATED WITH IL1700 +5V BIAS: ON  √ OFF

REFERENCE PLANE: Groove ONE formed by filter or diffuser elements and next element, counted from front surface of assembly.

*Difference includes intrinsic detector change, NIST recertification updates, lab experimental error or modifications to the hardware adjustments.

PRIMARY STANDARD: U.S. National Institute of Standards and Technology Detector Response

U1023 - January 1997 - NIST Test No. 844/257423-96/2  D204 - January 1997 - NIST Test No. 844/257423-96/1

N.I.S.T. Uncertainty: 200-250nm = 5% 250-400nm = 10% 400-900nm = 0.31% 900-1000nm = 0.38% 1000-1100nm = 2.93%

INTERNATIONAL LIGHT PRIMARY TRANSFER STANDARDS:

IL-J01, #02, #275, #139, #1490, #522, #627  JAN 1998 - JAN 2008

IL Transfer Uncertainty to Customer = +/- 6.5% plus NIST uncertainty above.

LIGHT SOURCE: 6a Low Pressure Mercury

LAMP OUTPUT: 8.40E-5 W/cm2

INSTRUMENTATION: #1029 Radiometer

PROCEDURE: PIR6a

TEMPERATURE: 21 degrees C

HUMIDITY: 14

CALIBRATED BY: unchecked

CHECKED BY: R. S. Cooper

Calibration Technician

QA Manager, Calibrations

FOR AUTHORIZED COPIES OF THIS CERTIFICATE OR OTHER INFORMATION PLEASE REFER TO THESE NUMBERS. THIS CERTIFICATE SHALL NOT BE REPRODUCED EXCEPT IN FULL WITHOUT THE WRITTEN APPROVAL OF INTERNATIONAL LIGHT, INC.

Calibration Date: 22-Mar-06  Certificate No: 660225512  FO/Sales Order #: 117972

IL RECOMMENDS AN ANNUAL CALIBRATION CONFIRMATION INTERVAL. INTERVALS OF CONFIRMATION MAY NEED TO BE ADJUSTED DEPENDING ON RESULTS OF PRECEDING CALIBRATIONS.
REFERENCES


References


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ABBREVIATIONS

µL  Microliter
µm  Micrometer

Ad2  Adenovirus type 2
Ad40  Adenovirus type 40
Ad41  Adenovirus type 41
ASF  Aerobic spore formers
AT  Adenine/thymine
ATCC  American Type Culture Collection
au  Arbitrary units

CFD  Computational fluid dynamics
cfu  Colony forming units
CPD  Cyclobutane pyrimidine dimers
CPE  Cytopathic effects
CT  Disinfectant concentration × exposure time
CV  Coefficient of variation

DMEM  Dulbecco’s Modified Eagle’s Medium
DMS  Dyed microspheres
DMSO  Dimethyl sulfoxide
DNA  Deoxyribonucleic acid
dsDNA  Double stranded deoxyribonucleic acid

FAME  Fatty acid methyl ester
FI  Fluorescence intensity

GC  Guanine/cytosine
GPM  Gallons per minute
GWR  Ground Water Rule

HEV  High efficiency vortex

kW  Kilowatts

L  Liter
LA  Lagrangian actinometry
LP  Low pressure
LPHO  Low-pressure high-output
LT2ESWTR  Long Term 2 Enhanced Surface Water Treatment Rule
MBBM  Modified Bold’s Basal Medium
Mgal  Million gallons
MGD  Million gallons per day
min  Minutes
mJ/cm²  Millijoules per square centimeter
mL  Milliliters
mM  Millimolar
MP  Medium pressure
MPN  Most probable number
mRNA  Messenger ribonucleic acid
MTT  Dimethylthiazol tetrazolium
MW  Molecular weight
MWD  Metropolitan Water District of Southern California
ng  Nanograms
N  Number
NB  Nutrient broth
NER  Nucleotide excision repair
nm  Nanometers
NTU  Nephelometric turbidity units
OD  Optical density

\( P \)  3-β-D-ribofuranosyl-2,7-dioxopyrido[2,3-d]pyrimidine

PBCV-1  *Paramecium bursaria Chlorella* virus
PBW  Phosphate buffered water
PCR  Polymerase chain reaction
PEG  Polyethylene glycol
pfu  Plaque forming units
PMT  Photomultiplier tube

QPA  Quantitative plaque assay
RED  Reduction equivalent dose
rRNA  Ribosomal ribonucleic acid
RT-PCR  Reverse transcriptase polymerase chain reaction

\( S \)  (E)-5-[2-(methoxycarbonyl)-ethenyl]cytidine
SASP  Small acid soluble proteins
SP  Spore photoproduct
ssRNA  Single stranded ribonucleic acid

TCID\(_{50}\)  50% tissue culture infective dose
TCR  Transcription coupled repair
TGY  Trytone glucose yeast extract
<table>
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<tr>
<td>TSA</td>
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<tr>
<td>USEPA</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>UV_{254}</td>
<td>Ultraviolet radiation filtered to the indicated wavelength</td>
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<tr>
<td>UVDGM</td>
<td>Ultraviolet Disinfection Guidance Manual</td>
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