Pulsed White Light as a Tool for Disinfection

A Major Qualifying Project Report Proposal
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1. Pulsed White Light
2. Bacillus subtilis
3. Ultraviolet Disinfection

Abstract

Pulsed White Light is an effective tool for disinfection when used with photosensitizers. Here the possibility of PWL alone being an effective tool for use against *Bacillus subtilis* spores was explored. Previous work was reviewed, and it was determined that PWL technology is not effective against *B. subtilis* spores without the use of a photosensitizer, refuting previous claims. However, it appears that using pulsed sources of ultraviolet light can be effective at killing over 99% of spores at high concentrations.

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1 Introduction

Previously, work has been done to determine if Pulsed White Light is an effective agent at inactivating *Bacillus subtilis* spores, a model organism for the more pathogenic *Bacillus anthracis*. While it was believed that this was determined, more work needed to be done on the subject to establish the level of effectiveness and the best conditions for such treatment.

Pulsed White Light, or PWL, involves treating an organism with quick, high intensity flashes of white light. In this project, the lamp used was set at the "architectural mode", meaning that over a two-minute irradiation cycle, the organism being studied, in this case *B. subtilis,* was exposed to sixty flashes of light. This light produced 30 joules of energy with a color temperature of 8000K (Different Production, 2005). This light may be 20,000 times more intense than natural light (Kakouros, 2004).

B. anthracis is an organism that can and has been used in bioterrorist attacks. It is easy to grow and prepare. For this reason, a safe and effective way to defend against and clean up after such an attack should it occur needs to be developed. Because bioterrorist attacks may take place under many different conditions, it is necessary to find the most effective way to respond to the attack under these varying circumstances (Atlas, 2001). For this reason, this project explored the effects of time and power of irradiation, cell density, and different light wavelengths on *B. subtilis* survival following PWL treatment.

Recent medical studies have found that PWL, when used in concert with photosensitizers, may not only be effective against bioterrorism, but may also be useful for treating certain infections and cancerous growths. For these reasons, PWL is an important resource that should be further investigated. It is possible that further research

may not only conclude that PWL can be used as a defense against bioterrorist attacks, but also as a treatment of patients who are affected during said attacks. Currently, PWL is used mostly for the disinfection of blood products, in particular against viral contamination, although it has also been used in more clinically based settings as well (Wainwright, 1998). If PWL is capable of treating other infections as has been concluded by Wainwright (1998) and Bertoloni et al. (2000), then it is possible that it could also be used to treat infections such as cutaneous anthrax or, with improved fiber optic technology, inhalation and gastrointestinal anthrax.

Initial research has consisted of attempting to confirm Stavros Kakouros' findings that the lamp used for PWL experiments causes cell death in *B. subtilis* (2004)*,* and expanding upon that by investigating the effects of PWL on higher spore concentrations. His methodology and lamp setup were used and maintained in an attempt to elaborate on his previous research. While doing this work, it was determined that the previous project done using the AF1000 Dataflash Xenon Flash-Lamp did not account for UV light that was produced during cell exposure. Thus, it was determined that while Pulsed White Light when used with photosensitizers may be effective in killing anthrax, treatment with a flash-lamp in the white light spectrum alone does not result in *B. subtilis* death.

To determine if the small percentage of cell growth that was seen after irradiation might be due to an SOS response in response to the UV light instead of statistical chance, a surviving colony was cultured, grown, and washed. These spores were then treated the same as those throughout the experiment to see if they were more resistant to treatment with pulsed UV light. While the spores produced did not seem to express a higher level of resistance to the treatment, more work should be done on more colonies that grow after

pulsed UV treatment to be sure that other bacteria that grew did not do so by the SOS pathway.

2 Background

Before work can be started, one must first understand the materials one is working with. In addition, work that has previously been done must be reviewed both to enhance the current project and to be sure that old work is not simply being duplicated. In the case of this report, *Pulsed White Light as a Tool for Disinfection*, the work of Stavros Kakouros (2004) was to be verified and expanded upon. Basic background information was re-researched, but some not until after data had indicated there were faults with the previous work. Thus this chapter will first review why anthrax must be studied and then briefly discuss why *B. subtilis* is an acceptable model for it. Following this, previous work in the field of pulsed white light will be summarized. Finally, faulty information will be presented followed by corrections and considerations with relation to the AF1000 Dataflash Xenon Flash-Lamp.

2.1 *Bacillus anthracis*

The *Bacillus anthracis* bacterium is an ancient organism that has caused disease in humans for many thousands of years. Humans who work in close contact with infected animals or infected animal products generally contract it. Animals often contract the disease by ingesting dormant spores found on the ground (Inglesby, et al., 2002).

B. anthracis causes three different types of disease in humans by producing three different toxins called *protective antigen*, *lethal factor*, and *edema factor*. Protective antigen allows the virulence factors, lethal toxin and edema toxin, to bind to target cells so they can be transported across the cell membrane. For the bacterium to maintain full virulence, it must also have an antiphagocytic capsule (Inglesby, et al., 2002).

Cutaneous anthrax is the most common infection with about 2,000 cases reported annually worldwide. The infection occurs on the skin and begins with local edema caused by release of toxin. The area then releases serosanguinous fluid that contains bacteria. After this a painless, depressed, black eschar forms along with more edema in the area. After a short period of time, this dries and falls off. With antibiotic treatment, this type of infection is rarely fatal, but without treatment, fatality rates of up to 20% are sometimes seen due to systemic infections that develop (Inglesby, et al., 2002).

Gastrointestinal anthrax is much more rare and is usually contracted by ingesting a large concentration of spores or vegetative bacilli found in undercooked meat of infected animals. In the oral-pharyngeal form of the disease, ulcer in the mouth and esophagus is often followed by edema and sepsis. In the lower gastrointestinal form, lesions are often seen in the cecum and lower ilium. Symptoms rapidly progress from flu-like to bloody diarrhea, abdominal pain, and sepsis (Inglesby, et al., 2002). Diagnosis of this form of the disease is much harder, and because the infection progresses so much more quickly than cutaneous anthrax, it has a much higher fatality rate, estimated to be somewhere between 25% to 60% (The Center for Disease Control, 2004).

Inhalation anthrax occurs when spores are deposited in the alveoli of the lungs and then transported to the mediastinal lymph nodes. After they germinate, the bacteria release toxins that quickly lead to edema, hemorrhage, and tissue death. Initial symptoms are generally flu-like, followed by fever, dyspnea, and shock. If antibiotics are not delivered before the bacteria begin producing toxins, death often occurs. Historical data indicates as many as 89% of those infected with inhalation anthrax die, although with the advent of antibiotics and vaccine treatment, those numbers are believed to have dropped.

It is not yet known how many spores are required to cause this disease. Some estimates say as few as 1 to 3 spores, while some experiments with monkeys indicate a dose of up to 55,000 spores is required to cause disease (Inglesby, et al., 2002).

In more recent times this bacterium has caused great concern because it can easily be used as a weapon in a bioterrorist attack. It is easy to grow in great quantities and is stable in spore form for years. Until the accidental release of anthrax in Sverdlovsk, Russia in 1979 from a bioweapons facility, most of the data we had on anthrax infection came from the few infections in the United States that were contracted under occupational circumstances. The data from Sverdlovsk indicate that up to 250 people might have been infected by the release, with 100 deaths caused by disease. Some of these people did not come down with symptoms for more than 30 days, indicating that anthrax is a severe threat due to its ability to survive in the environment and in the body for extended periods of time, making effective disinfection a vital part of stopping the spread of the disease (Inglesby, et al., 2002).

To date it is thought that at least 13 countries are currently working on developing bioweapons, possibly including *B. anthracis*. The risk of an attack using anthrax finally came to public awareness with the mail attacks that occurred in 2001, although the United States had been studying the threat of weaponization of the organism for years. In this attack there were twenty-two suspected cases of the disease. If an aerosol method of release were used instead of the mail, there would have been many more cases of infection and fatality. This method would go largely undetected until people became ill. One experiment indicated that the aerosolized spores could travel up to 60 miles and still maintain their infectivity. In 1993, it was estimated that if 100kg of

spores were released in an urban area, anywhere between 130,000 and 3 million people would be killed by the attack. For this reason, a quick and effective way to disinfect large areas must be found to stop secondary spread after the spores have settled. The best way to stop primary spread is certainly vaccination, but the FDA has not cleared this treatment for civilian use, and therefore, reactionary procedures must be developed (Inglesby, et al., 2002).

The microbiology of the bacterium is relatively simple and explains its high level of stability. It is an aerobic, spore-forming, nonmotile, gram-positive *Bacillus* that forms a spore about 1μL in length. The spore can survive for years and perhaps centuries in this inactive form before becoming a vegetative bacterium under more favorable conditions. The vegetative bacterium is about 1-8μL long and 1-1.5μL wide. It grows well on standard media at 37˚C; conditions also provided by the human body. If conditions become less favorable for the survival of the bacterium, it can again form a spore and remain dormant for years (Inglesby, et al., 2002).

2.2 *Bacillus subtilis*

Bacillus subtilis is commonly used as a model organism for the pathogen, *Bacillus anthracis*. It is a gram-positive, spore forming, aerobic *Bacillus* bacterium that is a known cause of food spoilage (Prescott, Harley, Klein, p. 966). Because work is often done using *B. subtilis* as a surrogate for *B. anthracis*, a question of how reliable a model it is has come into question. To ensure reliability of results using this model organism, Nicholson and Galeano (2003) compared the inactivation kinetics of both bacteria under 254-nm-wavelength UV. They found that the inactivation kinetics of the two species was

almost identical and concluded that *B. subtilis* could be used as a model organism for *B. anthracis* in UV light experiments.

As with *B. anthracis,* the spore of *B. subtilis* has a different composition than the vegetative cell, thus making it more stable under harsh conditions. In this dormant state, spores have no detectable metabolic activity and have a higher resistance to inactivation by heat, radiation, chemical treatment, and extremely dry conditions. The inside of the spore as well as the spore coat is dehydrated, giving it a higher level of resistance to heat (Driks, 1999). The spore can be broken into three separate structures. The core, or the interior segment, contains the DNA that has been dehydrated and locked in a stable, crystalline state. This is surrounded by the cortex, a thick layer of cross-linked peptidoglycans. Surrounding this is the coat, a shell composed mainly of proteins. This coat is largely responsible for the spore's resistance to unfavorable environments (Driks, 2003).

Compared to vegetative cells, spores are several orders of magnitude more resistant to 254nm UV treatment. This is in part due to the change that occurs in DNA in the spore coat. Upon irradiation, thymine dimers are formed. These are repaired during germination. Another special quality of the spore coat is that it contains a large amount of dipicolinic acid (DPA). This chemical increases spore resistance to wet heat and dry full spectrum light treatment. DPA also keeps the spore from spontaneously germinating at inappropriate times (Slieman and Nicholson, 2000). However, *B. subtilis* spores are not completely unaffected by UV treatment. Previous work on the topic showed that treatment of 10^7 to 10^9 spores in suspension by high intensity UV light exposure (1-4 pulses) led to spore inactivation (Sonenshein, 2001).

2.3 Previous Work Using Pulsed White Light

According to previous research done by the Pennsylvania State University's Graduate School of Architectural Engineering (2002), the light produced by a xenon lamp such as the AF1000 Dataflash produces a spectrum very similar to that of natural light, but with 20,000 times more intensity. This light has a peak at about 350nm, followed by a gentle sloping off into the infrared. Moreover, compared to typical UV treatment, a dose of light treatment an order of magnitude lower is required to get the same effect. However, the lamp also releases a large amount of light in the UV range (Pulsed Light and PEF).

PWL is currently used by the pharmaceutical packaging industry. In previous experiments, only two to three pulses of light were used to completely eradicate bacteria and fungal spores. *B. subtilis* spores at a concentration of 10^8 were inactivated with 1-3 pulses of light at $0.75J/cm^2$. This effect was attributed to UV damage to the nucleic acid and lysis of the cell wall caused by the heat produced by the light. However, the disintegration effect observed was also accomplished in the absence of UV light. Therefore it seems that Pulsed White Light is an effective and economical way to kill various microorganisms. But further attention must be paid to the type of light released during these and previous PWL experiments (Pulsed Light and PEF).

In work done by Xue and Nicholson, *B. subtilis* spores were exposed to light from the UV-C range of 254nm through the UV-B range of 290nm-320nm, up through visible light. It was found that with the wavelength increase, the resistance of the spores to the deleterious effects of the light decreased from 33-fold in the UV-C range to 6-fold in the UV-A sunlight only range. This seems to indicate that UV light is less effective at killing

B. subtilis spores then was previously thought and that visible light plays a larger role in the inactivation of bacterial spores. The authors claim that that this higher light wavelength causes damage to the DNA or other photosensitive molecules that the cells cannot repair at this level, while at lower light wavelengths the spores have developed mechanisms to address such damage (Xue and Nicholson, 1996).

Other work on the topic has come to similar conclusions, in particular that if a spore does manage to survive the initial irradiation, repair by a spore photoproduct may occur during germination, allowing the bacteria to thrive. It has been shown that the wavelength of light to which these spores are so resistant exists in the UV-C range of 254nm (Setlow, 2001). However, other data seem to be in direct opposition to this fact and so it seems that more work still needs to be done on the topic to clarify the effectiveness of different UV treatments.

Medical trials have also been done using visible and UV light to kill pathogens of a wide variety including bacteria, viruses, and various other parasites including drug-resistant strains of the pathogens. While photodynamic antimicrobial chemotherapy (PACT) is currently mostly used to sterilize blood products, it is also being developed for clinical uses. Tumors and infections are treated with low power systems that produce wavelengths ranging anywhere from 400-1000nm. Something as simple as a halogen light that produces light in the 600nm range can be used. This technique requires the surface being exposed to be treated with a photosensitizer before being exposed to the light of a given intensity for a given amount of time. It is hoped that with increased fiber optic technology this treatment can be improved to treat internal tumors and infections in addition to those that are currently successfully treated on the surface of the body. This

treatment may be useful in cases of drug resistant infections, or infections that are not responding to other forms of treatment. It is possible that this technology may even be useful against anthrax infections. What is important to remember about this technology is that in these procedures a chemical photosensitizer such as porphyrin derivatives and phthalocyanines are also used in addition to the Pulsed White Light and reliable, repeatable results have been obtained (Wainwright, 1998).

2.4 The AF1000 Dataflash Xenon Flash-Lamp

"As a light source for this study the AF-1000 lamp of High End systems was used. This is one of the most powerful strobes that are currently on the market. The AF-1000 is a xenon flash-lamp [used primarily for theatrical purposes]. The AF-1000 has the ability to produce white light pulses up to 120 [per] second. The color temperature of this lamp is 8000K compared to natural sunlight color temperature, which is about 5600K. The actual lamp that the fixture was operated with is made from Pyrex glass. This serves as a UV radiation shield allowing only near UVA and visible light to hit the lighted object. The Pyrex glass transmits light from 380nm and above" (Kakouros, 2004).

While the above information appeared to be reliable, the experiments run over the period of this project indicated that there was something faulty with the facts presented above. At this point, the properties of the lamp were further explored, during which time it was learned that the envelope of the lamp was in fact composed of quartz, and not Pyrex glass (Different Production, 2005). It was further determined that a color temperature of 8000K produces light with a peak at 362nm (IMSA, 2002). This means

that when the lamp was activated, the spores were also being exposed to UV light and that it was likely that it was this part of the spectrum that was actually killing the bacteria. In addition, the Pyrex glass assayed in the spectrophotometer for this project allowed transmittance of UV light with wavelengths of 280nm and up. It is interesting to note that the wavelength of UV produced by the flash-lamp likely reaches as low as at least 254nm, the wavelength used by Nicholson and Galeano in their inactivation experiments on *B. subtilis* and *B. anthracis*. In addition, Pyrex allows a substantial amount of UV-B light through, indicating that even if bacterial kill was achieved with a Pyrex light, UV-B light was quite possibly responsible for spore death and not the white light.

2.5 SOS Response Induction in *E. coli*

Miller et al. worked with *E. coli* bacteria during an experiment involving induction of the SOS response to antibiotic treatment. Their findings indicated that the signal transduction system in the bacteria stopped cell division during antibiotic treatment, stopping damage from occurring to the cells, thus allowing them to survive and proliferate. While this particular mechanism occurred in the cell wall and was mediated by genes responsible for cell wall proteins, another mechanism, the DNA damage-induced mediated path also exists. The group showed that mutation of specific genes was able to affect the ability of the bacteria to survive antibiotic treatment, thus proving that SOS signaling has a genetic basis that may be passed on vertically in bacteria and is not simply luck or chance on the part of the bacteria (Miller, et al., 2004). It is therefore thought that a similar mechanism may be involved in the survival of some bacteria during treatment with the pulsed light in the DNA damage induced pathway.

This hypothesis is due to the fact that UV light treatment damages the cellular DNA involved in this second pathway.

3 Materials and Methods

Research was conducted using *Bacillus subtilis* as a model for *Bacillus anthracis*. All experiments were run in triplicate to ensure reliability and accuracy of data. Filters were always dried before being irradiated to ensure repeatability of procedure. After irradiation, filters were plated and incubated at 37ºC for an average of 20-24 hours.

3.1 Preparation of Spores

Stock solutions of the *B. subtilis* spores used in this project had previously been prepared from a single colony of bacteria. This colony was then used to inoculate 20ml of 2xSG media. This media was incubated for between 6 and 8 hours at 37 ºC in a shaker, before being transferred to duplicate flasks of 100mL of 2xSG media. This was then incubated for an additional 24-48 hours at 37ºC in a shaker. After this time, spores were placed in the refrigerator and allowed to settle before the purification process to remove any vegetative cells began (Kakouros, 2004).

To purify the spores, each sample was centrifuged for an hour, after which time the supernatant was poured out. Then the spores were resuspended in 10mL dH_2O and transferred to 15mL conical tubes. These tubes were centrifuged for 40 minutes at 3000rpm. Again the supernatant was poured out and the process of washing and centrifuging was repeated 40 times. Then the remaining spores were combined (Kakouros, 2004) and saved at a stock concentration of 3.56×10^9.

3.2 Preparation of the AF1000 Dataflash Xenon Flash-Lamp

The research and work on the lamp had already been done by Stavros Kakouros. For this reason, his setup and procedure as described in his MQP can be found in the section below with corrections where necessary.

"The lamp fixture was positioned firmly, approximately 1 meter above the lab bench. The plastic dome of the fixture was removed and during irradiation the lamp was used without it. However, a cardboard shade was fashioned to protect student's eyes from the light. A clamp and stand were used to position the lighted sample exactly 10cm from the light source. Figure 1 shows the experimental setup for irradiation of a sample with the Dataflash AF1000. All samples were treated by supplying power to the lamp until the desired numbers of flashes were obtained. The settings of the lamp for intensity, duration, and flash rate were set by manipulation of the dip switches at the base of the lamp shown in Figure 2" (Kakouros, 2004). For all experiments run during this project, the "architectural mode" was used, and the corresponding dip switch settings can be seen in Figure 3. This mode of use produces 60 pulses of light over 2 minutes. The light produces 30 joules of energy with a color temperature of 8000K (Different Production, 2005).

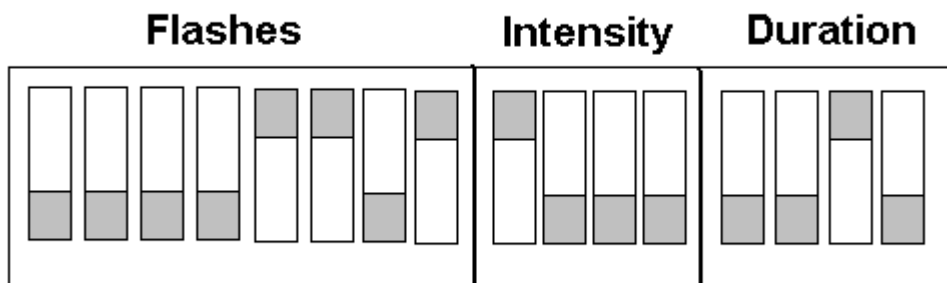**Figure 1: The Experimental Setup for Irradiation of Samples**

(Kakouros, 2004)

Figure 2: Dip Switches

(Kakouros, 2004)

Figure 3: Architectural Mode Dip Switch Settings

3.3 Most Effective Number of Irradiation Cycles

After the spores were grown and isolated, serial dilutions of samples were performed to get the desired spore concentration for a given experiment. To verify Kakouros' findings, 1×10^4 spores were prepared by taking 28μL of 3.56×10^8 spores and performing serial dilutions. These spores were then added to 5mL dH_2O to get a consistent sample across the entire filter and were then filtered using sterilized membranes from Gelman Sciences. Filters were then placed in sterile petri dishes and allowed to dry in the 37ºC incubator. Using sterile forceps, filters were then placed on a screen and flashed for 1, 2, or 3 cycles and were then moved to Millipore plates containing LB agar, where they were incubated at 37ºC for approximately 21 hours. In addition to making three plates containing 1×10^4 spores for each time of irradiation, one plate was not irradiated to test the viability of the spores, and another went through the entire procedure without adding any spores to verify sterility. These two controls were used for every experiment run to maintain that procedures were being done correctly.

3.4 Highest Cell Concentration Reliably Killed During Irradiation

From this point, it was determined that allowing a filter containing 1×10^4 spores to go through 3 cycles of irradiation was effective at killing over 99% of the *B. subtilis* spores. To find the highest concentration of spores that could be effectively irradiated, the procedure above was followed, but this time using spores at concentrations of 1×10^6, 1×10^5, and 1×10^4. All the filters were irradiated for 3 cycles, were plated, and the kill factor was again determined. Even at concentrations of 1×10^6, over 99% of the spores were killed.

3.5 Exploration of Light Wavelengths Involved in Cell Death

After determining that the limits of the lamp's ability to kill high concentrations of cells is greater than can reliably be deduced, the most effective light wavelength for killing *B. subtilis* spores was explored. IR Reflecting, IR Absorbing, Green, Yellow, Blue, UV Blocking, Pyrex, and the plastic dome that had been removed from the light, were sterilized and placed over filters containing preparations of 1×10^5 spores to act as light filters. The different preparations were irradiated for three cycles each before being plated and allowed to grow overnight.

To determine which wavelengths of light all the filters were blocking, they were placed in a spectrophotometer and their percent transmittance from 200nm to 750nm was measured and graphed. Points where all the filters blocked various light wavelengths were noted and are reported below.

3.6 Exploration of SOS Response in *B. subtilis* Survival

A single colony of *B. subtilis* was sterilely removed from a plate that had been treated with a concentration of 10^6 spores and then irradiated for 3 cycles with a loop. This plate had approximately 147 colonies growing on it. The colony was placed in a sterile 20mL beaker containing 75mL 2xSG media and 75μL 100x metals. This was then incubated at 30˚C for five days. After this time the culture was separated into two centrifuge tubes, which were spun at 3000rpm for 2 hours. The supernatant was poured off and 10mL of sterile water was added to each tube. The spores were agitated and the tubes were again spun for 2 hours before supernatant was poured off. This procedure was followed five more times to ensure any vegetative cells were removed. Then the spore concentration was determined using the spectrophotometer and a standard curve for enumeration of *B. subtilis*. One tube was found to have a concentration of 3.02 x 10^9 spores. The other tube gave widely varying concentrations and therefore was not used. The spores were diluted to a stock concentration of 1 x 10^8. From here serial dilutions were done and filters were exposed to 1 x 10^5 spores. These were irradiated for 3 cycles and plated along with growth and sterility controls.

4 Results

Data from the course of the entire project can be found below, presented in linear fashion for ease of comprehension and viewing. All experiments were performed in triplicate to ensure accuracy in reporting. These data are summed up and reported, with careful discussion of these findings found in the following section.

4.1 Determination of the Most Effective Number of Irradiation Cycles

The following table shows the results of trials run during which time 1,000 spores were irradiated for one, two, or three cycles.

Table 1: Percent Kill versus Number of Irradiation Cycles

Number of Cycles	Number of Colonies	Percent Kill
1	0	N/A
0	~1300	N/A
1	190	81%
1	342	65.80%
1	260	74%
2	24	97.60%
2	8	99.20%
2	0	100%
3	2	99.80%
3	2	99.80%
3	0	100%

The average percent kill for spores that underwent one cycle of irradiation was 73.6%. For spores that underwent two cycles, the average percent kill was 98.93%. For spores that underwent three cycles, 99.87% of the spores were killed.

4.2 Determination of the Highest Cell Concentration Reliably Killed

The following table shows the results of trials run to determine the highest number of spores reliably killed by three cycles of irradiation.

Table 2: Percent Kill versus Number of Spores Irradiated

Number of Spores	Number of Cycles	Number of Colonies	Percent Kill
0	1	0	N/A
1,000	0	774	N/A
10,000	3	32	99.68%
10,000	3	4	99.96%
10,000	3	85*	99.15%
100,000	3	140*	99.86%
100,000	3	87	99.91%
100,000	3	281*	99.72%
1,000,000	3	476	99.95%
1,000,000	3	147	99.99%
1,000,000	3	158	99.98%

The * next to several of the numbers in the "Number of Colonies" column indicates a large clustering of colonies along one edge of the filter, accounting for the majority of the colonies seen on the plate.

The average percent kill for 1×10^4 spores was 99.6%. For 1×10^5 spores the percent kill rose to 99.83%, and for 1×10^6 spores the percent kill was 99.97%. Higher spore concentrations with 99% kill proved difficult to accurately count (data not shown).

4.3 Determination of Light Wavelengths Involved in Cell Death

The following table shows the results of the trials run to determine if specific light wavelengths were responsible for cell death. Spore concentrations of 1×10^5 were exposed to three cycles of irradiation except in the case of the controls. For the sterility control, there was zero growth during both runs, and for the viability control, approximately 1,200 colonies grew each time.

Table 3: Percent Kill for Different Light Blocking Filters

Filter Type	Number of Colonies	Percent Kill
IR Reflecting	TNTC	100%
IR Reflecting	TNTC	100%
IR Reflecting	135	99.98%
IR Absorbing	TNTC	100%
IR Absorbing	TNTC	100%
IR Absorbing	33	99.99%
Blue	TNTC	100%
Blue	TNTC	100%
Blue	TNTC	100%
Yellow	TNTC	100%
Yellow	TNTC	100%
Yellow	TNTC	100%
Green	TNTC	100%
Green	TNTC	100%
Green	TNTC	100%
Plastic Casing	TNTC*	See Below
Plastic Casing	TNTC*	See Below
Plastic Casing	TNTC*	See Below
UV Blocking	TNTC	100%
UV Blocking	TNTC	100%
UV Blocking	TNTC	100%
Pyrex	80	99.92%
Pyrex	22	99.98%
Pyrex	36	99.96%
None	6	99.99%
None	0	100%
None	17	99.98%

TNTC = Too Numerous To Count

The * is next to the TNTC from the Plastic Casing because it had a small hole in it. In the area where the bacteria were exposed directly to the light, no cell growth was observed (Figure 4).

Figure 4: Growth of Bacteria Irradiated under Plastic Casing
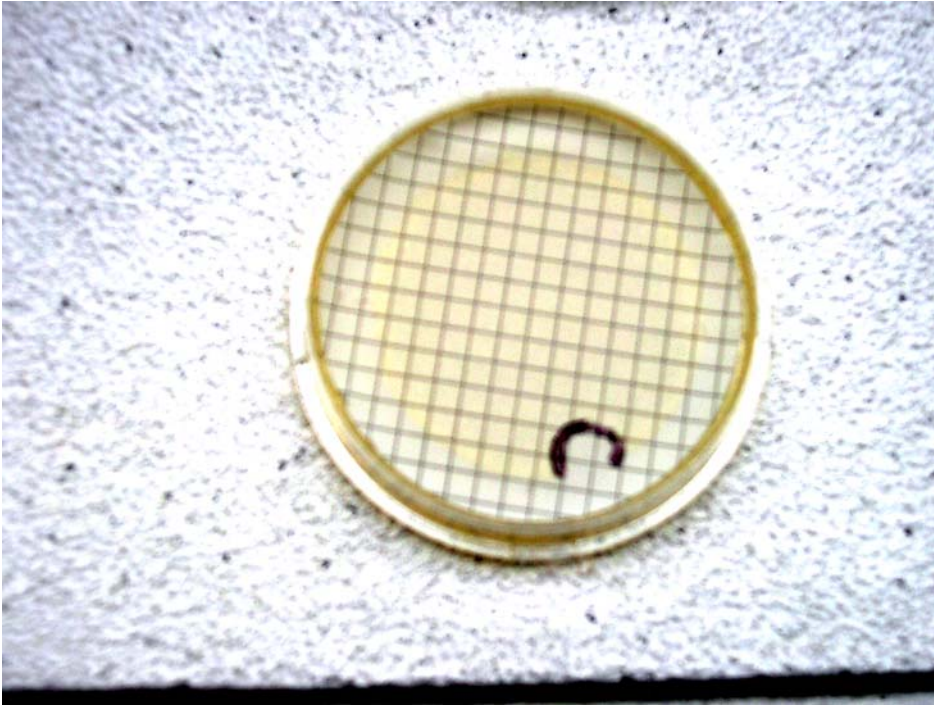
The area where no bacterial growth is observed is outlined in black for ease of viewing.

To determine which light wavelength all the filters were blocking, each filter was placed in the spectrophotometer and percent transmittance was found from 200nm to 750nm. In the graph below, the percent transmittance of each filter is charted from 200nm to 550nm.

Figure 5: Percent Transmittance for All Filters from 200nm to 550nm
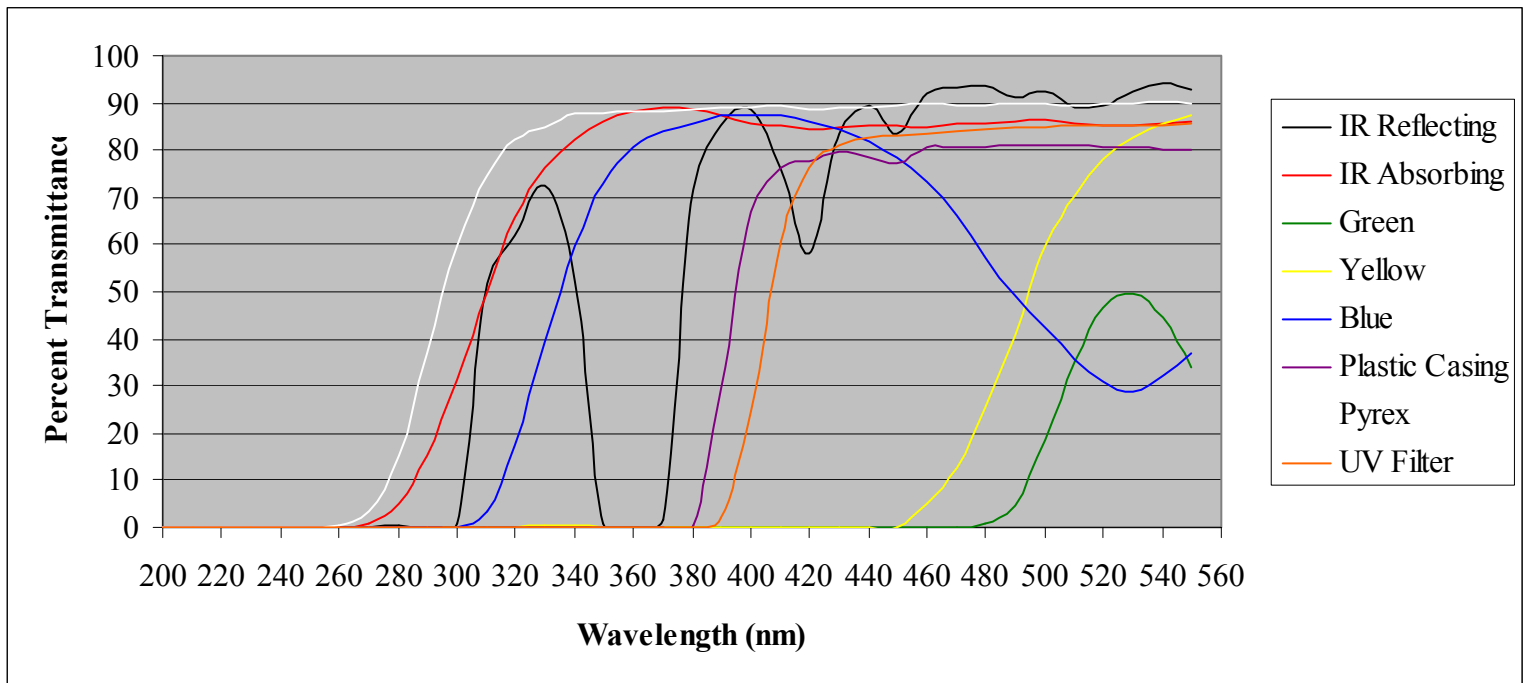
4.4 Exploration of SOS Response in *B. subtilis* Survival

The following table shows the results of trials run to determine if an SOS response may have caused the cultured colony to survive previous irradiation.

Table 4: Percent Kill for a Previously Surviving Colony

Number of Spores	Number of Cycles	Number of Colonies	Percent Kill
0	1	0	N/A
1,000	0	820	N/A
10,000	3	0	100.00%
10,000	3	2	99.98%
10,000	3	1	99.99%

The average percent kill for 1×10^{6} spores was 99.99%

5 Discussion

This project required many steps to produce reliable and accurate numbers as well as to come to reliable conclusions about what was actually leading to spore death. With careful sterile technique, it was determined that three cycles of irradiation led to a percent kill of over 99%. Table 1 illustrates the stepwise way in which more cycles of irradiation led to greater percentages of cell death. From these data it was determined that three cycles of irradiation was the most effective at killing the highest percentage of spores. Therefore, in later procedures spores were exposed to three cycles.

In Table 2, greater numbers of spores were irradiated to determine where there was a plateau in effectiveness of treatment with the light. At a concentration of 10^6, 99.9% of cells were being killed. However, because of the high concentration this meant that there were still up to several hundred colonies growing on the media. Therefore it was determined that higher concentrations, while still being effected by the light, were unrealitic to work with and try to quantitate.

It was also noted on Table 2 that there was some clustering near the edges of some of the filters. At first there was concern that there might have been contamination. However, it was noted that the part of the filter that had more cell growth was always on the edge and the bacteria showed the same morphology as those on viability control plates. It was then noticed that the clustering of bacterial colonies only occurred when the filter was off to the side and was not directly under the light. Instead the plastic casing was blocking some of the light from the lamp. After further research was conducted, this was explained by the fact that the casing was blocking the UV waves that were leading to the spore death.

In Table 3 it can be seen that all the filters except for Pyrex were capable of blocking some wavelength of light that allowed the cells to survive. Except for the Pyrex glass and two other cases, only the controls with no filters over them produced a countable number of colonies, with complete cell death in one of those cases. In the case of the IR Reflecting and IR Absorbing filters that corresponded to cell death, a simple explanation exists. In both of those runs, the filters fell directly onto the samples being irradiated, likely leading to cell death due to the heat. In the other two runs using those filters, they were held off the cells using sterilized toothpicks to keep the filters from causing this cell death due to extreme heat produced by the light. This precaution appears to have been effective.

Another interesting point that arose when using the filters involved a piece of casing from the lamp itself. This circle of plastic was removed before the current project even began, presumably for ease of use when irradiating samples. However, when questions arose due to the clustering of bacteria on some of the filters (Table 2), it was decided that this would also be used in the light filtering experiment. When the samples were allowed to grow, there were too many colonies to count *except* where a hole in the casing existed. In this area where the entire spectrum of light was able to reach the spores, no cell growth occurred (Figure 4).

When it was initially found that all the filters used blocked some wavelength of light that when blocked allowed the bacteria to grow, they were placed in a spectrophotometer and readings of percent transmittance from 290nm to 750nm were taken (data not shown). It was noted that the only point where the percent transmittance for all the filters coincided or was virtually zero or was in the area of 290nm. Therefore,

the percent transmittance down to 200nm was determined and all percents transmittance were graphed. To be sure the light killing the bacteria really was in the UV range, Pyrex glass and a UV blocking filter were also used for irradiation runs (Table 3), placed in the spectrophotometer, and graphed (Figure 5).

In the case of the Pyrex glass, spectrophotometry showed that it allowed a lower wavelength of light through than the other filters, which led to cell death. At 280nm, percent transmittance by the glass was 15.3%. The IR Absorbing filter with 5% transmittance, provided the next nearest percent transmittance at that wavelength. At 290nm, a wavelength associated with UV-B light; there was 36.8% transmittance from the Pyrex glass while the IR Absorbing filter allowed only a 15.1% transmittance, less than half as much light. Cell death was noted on the Pyrex exposed cultures with over 99% of the spores killed, while on the IR Absorbing plates, there was too much cell growth to count. This indicates that UV light in the range below 300nm was causing cell death, not white light.

When the possibility of an SOS response leading to cell survival was explored, it was found that a second treatment of the cultured colony with pulsed UV light did not indicate any genetic predisposition towards this response. In fact, less cell survival was noted than is normal, indicating that it is likely the spore that originally survived to produce a colony was lucky, one which may in fact have been initially damaged by the light as indicated by the inability of later clones to resist light treatment. However, it is possible that other colonies that grew during this project did survive via SOS responses and therefore, more work on the topic should be done. Perhaps in a later project every colony from a plate could go through the cloning and irradiation process, allowing us to

see if any surviving cells showed a genetic predisposition to resistance to the light treatment.

As can be seen from the data above, previous Major Qualifying Projects are not always reliable and therefore, data and facts from them must be rechecked before solid assertions are made. Going back to the sources of information on the lamp, it was discovered that the light was in fact made of quartz, not Pyrex as had been previously stated and assumed throughout the current project. This meant that the light being emitted contained wavelengths in the UV spectrum. Upon further inspection, it was found that the color temperature produced by the light is 8000K, which has a peak at about 362nm. This indicates that the flash-lamp releases light that is well in the UV range. Viewing Figure 5, one can see that it was this light and not the white light that was causing the cell death.

While it is disappointing to discover that the pulsed white light alone was not causing cell death, it is interesting to note that 30-Joule pulses of ultraviolet light are highly effective at killing high concentrations of *B. subtilis* spores. It is possible that future experiments may be done to determine exactly what wavelengths are responsible and whether a greater percentage of cell death can be achieved with a larger range of UV light. Also, it should be noted that PWL as a technology should not be given up on but should still be studied as a tool for of disinfection, especially when used with a photosensitizer.

6 References

AF 1000. Different Production. Online. Accessed 27 January 2005.
http://differentproduction.com/highendaf1000.html

Anthrax: Technical Information. The Center for Disease Control. Online. Accessed 27 January 2005. http://www.cdc.gov/ncidod/dbmd/diseaseinfo/anthrax_t.htm

Atlas, Ronald. "Bioterrorism Before and After September 11". Critical Reviews in Microbiology. 27 (2001): 355-379.

Bertoloni, Giulio, et al. "Photosensitizing activity of hematoporphyrin on *Staphylococcus areus* cells." Biochemica et Biophysica Acta 1475 (2000): 169-174.

Driks, Adam. "*Bacillus subtilis* Spore Coat". Microbiology and Molecular Biology Review. 63 (1999). 1-20.

Driks, Adam. "The dynamic spore". Proceedings of the National Academey of Sciences of the Unites States of America. 100 (2003): 3007-3009.

Horrell, Mark. "Energy Balance of the Earth: Theoretical Blackbody Curve for 8000K". Online. Accessed 27 January 2005. http://staff.imsa.edu/science/geophysics/atmosphere/energy/8000k.html

Inglesby, Thomas., et al. "Anthrax as a Biological Weapon, 2002." Journal of the American Medical Association. 287 (2002): 2236-2252.

Kakouros, Stavros. "Pulsed White Light as a Defence Against Bioterrorism." Major Qualifying Project. Worcester Polytechnic Institute. Worcester, MA. 14 January 2004.

Miller, Christine, et al. "SOS Response Induction by β-Lactams and Bacterial Defense Against Antibiotic Lethality". Science. 305 (2004): 1629-1631.

Nicholson, W., Galeano, B. "UV resistance of Bacillus anthracis spores revisited: validation of Bacillus subtilis spores as UV surrogates for spores of *B. Anthracis* Sterne". Applied and Environmental Microbiology. 69 (2003): 1327-1330.

Prescott, L., Harley, J., Klein, D. Microbiology *Fifth Edition*. McGraw Hill, New York. 2002.

"Pulsed Light and PEF". Penn State Department of Architectural Engineering. Online. Accessed 2 March 2005. http://www.engr.psu.edu/ae/iec/abe/control/pulsed_light.asp

Setlow, P. " Resistance of spores of Bacillus species to ultraviolet light". <u>Environmental and Molecular Mutagenesis</u>. 38 (2001): 97-104

Slieman, T., Nicholson, W. "Role of Dipicolinic Acid in Survival of *Bacillus subtilis* Spores Exposed to Artificial and Solar UV Radiation". <u>Applied and Environmental Microbiology</u>. 67 (2001): 1274-1279.

Sonenshein, Abraham. "Killing of Bacillus Spores by High-Intensity Ultraviolet Light". <u>Xenon Corporation</u>. 28 November 2001. Online. Accessed 25 February 2005. <u>http://www.xenoncorp.com/products/images/Final_Report.pdf</u>

Wainwright, Mark. "Photodynamic antimicrobial chemotherapy (PACT)." <u>Journal of Antimicrobial Chemotherapy</u> 42 (1998): 13-28.

Xue Y., Nicholson WL. "The two major spore DNA repair pathways, nucleotide excision repair and spore photoproduct lyase, are sufficient for the resistance of Bacillus subtilis spores to artificial UV-C and UV-B but not to solar radiation". <u>Applied Environmental Microbiology</u>. 62 (1996): 2221–2227.