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Evaluation of Ozonated Water and Ultraviolet Light Water Treatments on *Bacillus subtilis* Spore Efficacy in Spiked Water Samples

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Abstract:

The goal of this water treatment study was to evaluate the effects of ozonated water and ultraviolet light (100–280 nm) on the efficacy of Bacillus subtilis spores. These water treatments are based on the current research for sanitizing bio-contaminated water. However, this study focused on small-scale water treatment units that could be modified or custom-built for a mobile sanitation system used for cleaning field equipment. The factorial study included four factors, resulting in a total of 16 water treatments. The ultraviolet light water treatment (UVC + UVC), with a total exposure time of 60 minutes, resulted in a log10 reduction of 3.51, equivalent to a 99.9% reduction in viable spores. The average spore reduction from UVC treatment increases with exposure time, then reaches an asymptotic plateau between 30 and 60 minutes. The combination of ozonated water with UV radiation yielded promising results in reducing spores. Wastewater technology, based on UVC systems, may be implemented with both mobile power washing systems. Additionally, larger units are required to effectively sterilize bio-contaminated wastewater within farms and storage facilities in the agricultural industry.

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1. INTRODUCTION

Wastewater treatment systems are continually being designed and modified to enhance their effectiveness in sanitizing bio-decontaminated wastewater in the agricultural sector. Numerous commercial wastewater systems are already available for farms and processing facilities that utilize ozonated water and ultraviolet technologies. In addition to these stationary facilities, a subset of mobile or field equipment and transport vehicles in the agricultural sector also requires wastewater sanitation after the initial power washing step. Agricultural field equipment and vehicles would require specialized or custom-built water treatment systems that could be retrofitted onto mobile power washing systems that need to recycle their wastewater. Bio-contaminated wastewater generated from mobile power washers should not be recycled due to the risk of spreading waterborne diseases [1-4].

The US EPA has an active wastewater research program that has investigated the effectiveness of ozonated water and ultraviolet light technology for bio-contaminated water [5]. Ozone or 'activated oxygen', contains three atoms of oxygen, making it a potent sterilization agent to destroy bacteria, viruses, and even odor-causing agents [6-8]. Ozone is highly effective but has a brief life cycle, which is shortened further when it reacts with contaminants through oxidation [6-8]. Ozonated water requires four to five times less exposure time than chlorine during decontamination and can be generated on site due to its short half-life [9]. Over 2,000 facilities globally employ ozone for drinking water treatment, ensuring compliance with EPA regulations and international standards [9]. Ozone has minimal impact on pH and temperature over a broad concentration range [6-8]. Nevertheless, high ozone levels can be hazardous without adequate protection, with OSHA setting exposure limits at 0–10 ppm for an 8-hour workday, five days a week [6-8]. To enhance safety and efficiency in water decontamination, ozone is commonly combined with ultraviolet (UV) light [10-13].

The most energetic segment of UV light, known as UVC, spans wavelengths ranging from 100 to 280 nm. These shorter wavelengths within this range have sufficient energy to be germicidal, provided there is sufficient exposure [13-16]. The overall effectiveness of UVC is very dependent on exposure time, intensity of the lamp(s), and initial general water quality parameters [13-17]. Water treatment systems using UVC light feature a waterproof bulb encased in a glass sleeve, allowing water to flow adjacent to the bulb and exposing any bio-contaminants to the radiation [17]. The United States Department of Health and Human Services established exposure rates in 1966, requiring a minimum dosage of UVC of at least 16,000 μW/cm² on the surface [5].

This study investigated the efficacy of these treatments on *B. subtilis* spores, which are recognized for their resilient spore coats and their use in the most challenging types of water sterilization research. Water samples were spiked with calibrated concentrations of spores. Additional water samples were also spiked with humic acid to simulate turbidity conditions often found in real-world contaminated water. Both ozonated water and UVC light were applied alone or in combination to enumerate the log10 reduction in viable *B. subtilis* spores, employing a factorial experimental design to account for variables such as exposure time, water quality, and treatment combinations.

The goal of this study was to investigate whether these two water treatments could be adapted for use in a mobile pressure washing system that allows bio-contaminated wastewater to be recycled after power washing field equipment. This is basically a water sterilization study that evaluated the effectiveness of ozonated water and UVC wastewater treatments. The results from this study can also be applied to the design of larger wastewater systems used in farms, storage facilities, and processing plants. Both ozonated water and UVC technologies were evaluated separately and in combination in a two-stage treatment order to determine their ability to inactivate *B. subtilis* spores, a hard-to-kill surrogate spore species. The other study factors included two different exposure times and two water turbidity conditions that assessed any changes in spore efficacy.

2. MATERIALS AND METHODS

2.1. *B. subtilis* Spore Preparation

All *B. subtilis* spore samples were prepared as water suspensions by MicroChem Laboratory (Round Rock, TX). The samples were suspended in water and treated with isopropanol to eliminate vegetative cells, ensuring that only spores remained within the suspensions. Spore suspensions were prepared to achieve an initial spore count of approximately 10^8 spores/ml, while control samples had initial counts of approximately 10^6 spores/ml. Subsequently, the samples were shipped and stored at 4 °C to prevent

spore germination. Control samples were inoculated and analyzed at the MicroChem Laboratory, while a second set of control samples was shipped and stored alongside the treatment samples. The MicroChem lab conducted the culturing and enumeration of viable B. subtilis spores from water suspensions on semi-selective media to determine the colony-forming units (CFU) per treatment.

2.2. Experimental Design

This was a factorial study with four factors, resulting in 16 distinct water treatments. The four factors were: 1) two water treatment exposure times that were 15 and 30 minutes for each stage, 2) two water quality levels that were tap water and tap water + 0.1% humic acid, 3) two types of water treatment that were ozonated water and UVC radiation, and 4) two sequential stages of water treatments. The combination treatments are listed below for the first and second stages of water treatment. All four factors were combined in a fully crossed design, resulting in 16 water treatments (2 exposure times × 2 turbidities × 2 technologies × 2 treatment stages). Humic acid was used to increase turbidity, simulating water with impurities. The first-stage water treatment included ozonated water or UVC radiation, and the second-stage water treatments involved no treatment, ozonated water, or UVC radiation. The first and second stage treatments both included ozonated water and UVC radiation, which are considered two levels within the study factor. The third level was the no treatment in the second stage of water treatment.

When the two exposure times were combined with the two stages of treatments, eight combinations ended at the first stage:

1. ozonated water at 15 and 30 minutes with 0% humic acid
2. ozonated water at 15 and 30 minutes with 0.1% humic acid
3. UVC radiation at 15 and 30 minutes with 0% humic acid
4. UVC radiation at 15 and 30 minutes with 0.1% humic acid

In the second stage, there were also eight combinations:

1. ozonated water at 45 and 60 minutes with 0% humic acid
2. ozonated water at 45 and 60 minutes with 0.1% humic acid
3. UVC radiation at 45 and 60 minutes with 0% humic acid
4. UVC radiation at 45 and 60 minutes with 0.1% humic acid

2.3. Water Treatments

Ozonated water was produced using a custom water generator (Spartan Environmental Technologies, Mentor, OH) with a 140 l insulated tank. Chilled tap water at 2°C was added to the tank before the generator was started. The generator operated until the ozone concentration reached approximately 25 mg/l. Then, two liters of ozonated water were collected, and 40 ml were added to each labeled 100 ml water sample vial. The turbidity treatments involved adding 40 µl of humic acid (Age Old Organics, Plant Natural, Bozeman, MT) to 40 ml of ozonated water.

All samples were then spiked with 400 µl of *B. subtilis* spore suspension, and a timer was set for either 15 or 30 minutes. At the end of each exposure time, the samples undergoing a second treatment were immediately re-exposed to ozonated water or UVC radiation. If samples were designated for the second treatment or had no further treatment, they were neutralized with 6.4 ml of 2.5% sodium thiosulfate (Fisher Scientific, Pittsburgh, PA).

The UVC treatments were exposed to a home aquarium unit that had two 9-watt UVC lamps (Green Killing Machine, AA Aquarium, Pure Essence Health). The fully enclosed units had UVC lamps approximately 11.4 cm long, with an estimated surface area of about 220–230 cm². The two lamps emit short-wave ultraviolet light with an average wavelength around 254 nanometers. For UVC water samples, 40 µl of tap water was collected into labeled samples; depending on the treatment, these samples were either left untreated or spiked with 0.1% humic acid. All samples received 400 µl of *B. subtilis* spore suspension. The spiked water samples were then exposed to UVC light for the first and second stages of water treatment. The samples were then neutralized with 6.4 mL of 2.5% sodium thiosulfate.

The oxidative reduction potential (ORP) and pH data were collected from all water samples using an Orion 3 Star pH/ORP multi-meter (ThermoScientific, Waltham, MA). Both water properties were collected at 5-minute

intervals up to 75 minutes after the initial collection time began. The ORP values were then analyzed over time to assess the degradation rate of ozone resulting from increasing water temperatures.

2.4. Dosage Estimates for the UVC Lamps and Exposure Times

The dosage for the two 9-watt UVC lamps was estimated using standard measurements for similar bulbs. These estimates assume a UV wavelength of 254 nm and a total lamp surface area of about 224 cm². The literature suggests that the dosage rate for a 90% kill, 1-log reduction is approximately 11,600 µW·s/cm² (11.6 mJ/cm²) for most bio-contaminants [18]. The dosage rate for a 99% kill, or 2-log reduction, is approximately 22,000 µW·s/cm² (22 mJ/cm²), and about 33 mJ/cm² for a 99.9%, or 3-log reduction.

The two 9-watt lamps had an estimated power of 18 J/s (2 × 9 W = 18 W). The following assumptions and equations were used to estimate the UVC intensity at the glass surface of the lamps, based on published properties of comparable UVC lamps used for fish aquariums.

UVC efficiency ≈ 35% (a reasonable mid-range assumption for low-pressure mercury lamps).

UVC radiant power = 18 J/s × 0.35 ≈ 6.3 J/s = 6.3 W of germicidal light.

Estimated quartz surface area = 224 cm² (for both lamps combined).

Irradiance (power per unit area) = 6.3 W/224 cm^2 = 0.028 W/cm^2

or 28 mW/cm², right at the glass surface.

Estimated in-water irradiance ≈ 28 mW/cm² × 0.65 ≈ 18 mW/cm².

Exposure times = 15 min. x 60 s = 900 s

30 min. x 60 s = 1,800 s

UVC dosage = intensity per unit area x exposure time

15 min. = 18 mW/cm2 x 900 s = 16,200 mJ/cm2

30 min. = 18 mW/cm2 x 1,800 s = 32,400 mJ/cm2

45 min. = 18 mW/cm2 x 2,700 s = 48,600 mJ/cm2

60 min. = 18 mW/cm2 x 3,600 s = 64,800 mJ/cm2

2.5. Data Analysis

This study employed the SAS JMP software (SAS Institute Inc., Clary, NC) for designing experiments (DOE) to minimize the number of samples needed. Each experiment was structured as a fractional factorial design to exclude all higher-order interaction terms beyond two-way interactions, as well as to facilitate statistical analysis and significance testing (α = 0.05). The viable spores per sample [CFU/sample] were analyzed using two approaches: estimating the probability of viable spores remaining after treatment and calculating log10 reduction. The probability of recovering viable spores from treated samples was determined by transforming the raw data. Specifically, it was calculated as:

$$\text{Probably Viable Spores} = \frac{\text{Treated CFU per sample}}{\text{Control CFU per sample}}$$

Log10 spore reductions were calculated where A is the number of viable spores recovered from control samples, and B is the number of variable spores recovered from the treated samples using the equation:

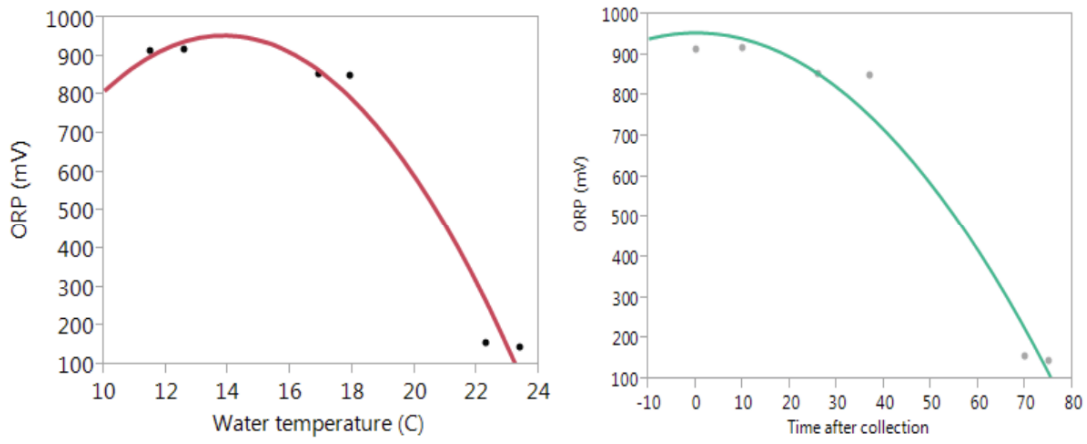
$$\text{Log10 Reduction} = \text{Log}(A / B)$$

These are two different methods for transforming viable spore counts, both of which include control counts in ratios. This study evaluated whether either spore transformation influenced the results. A Generalized Linear Model (GLM) was used to analyze the statistics within each factorial study, and the maximum likelihood procedure included all terms, study factors, and two-way interaction terms. Each GLM included all significant factors and interaction terms in order to predict the log10 reduction values. Hidden replications were used to limit the interaction model terms to only two-way interactions. This interaction restriction avoids three and four-way deciphering dilemmas and increases the overall strength of the final model.

3. RESULTS

The pH values of ozonated water samples averaged 7.3 across all four water samples. Injecting ozone into tap water did not alter the pH values of the samples, even at high levels of ozone gas. The ozonated water ORP values decreased non-linearly when regressed over water temperature and exposure time over the 75-minute measuring period (Figure **1**).

The first stage of water treatment involved either ozonated water or UVC light, followed by no treatment,



A B

Figure 1: The quadratic relationship between ORP and water temperature (**A**) and exposure time (**B**).

Table 1: The Generalized Linear Model (GLM) Results with Fixed Effects for all Three of the Study Factors

Source	DF	ChiSquare	Prob>ChiSq
First Stage Water Trt	3	560.15	<0.0001
Exposure Time	1	11.65	0.0006
Second Stage Water Trt	2	58.98	<0.0001

ozonated water, or UVC light. Exposure time and the second water treatment were significant factors in the model. There were no two-way interaction terms in the final GLM, meaning that none of the three key study factors influenced each other; instead, each had an independent effect on the viability of B. subtilis spores. Three out of four study factors were statistically significant (Table **1**). The addition of humic acid, used to simulate water turbidity, was not statistically significant. Thus, the only terms in the final model were the type of water treatment and exposure time, which affected the log10 reduction of spores.

Generally, increasing the exposure time of the treatment resulted in higher spore reduction values, with a few exceptions. Ozone as the sole treatment showed inferior results with a probability of 98% and 91% viable spores remaining after 15 and 30 minutes, respectively (Table **2**). The UVC + UVC water treatment had a probability of 0.63% and 0.03% viable spores remaining after 15 and 30 minutes of exposure time, respectively. Ozone performed better when UVC was added as the second stage of water treatment.

The best overall treatment was the UVC + UVC water treatment with the 60-minute exposure time. Increasing the exposure time from 15 to 30 minutes resulted in a 0.6% increase in the number of remaining viable spores (Table **2**). The UVC + UVC water treatment had a viable spore probability of only 0.03%, which parallels

Table 2: The Log10 Reduction Means Based on the Least Squares Means Model. The Means are Listed by Five Water Treatments and by Two Exposure Times. The Probability of Viable Spores is also Listed in the Last Two Columns for the Five Water Treatments and Two Exposure Times

Water Treatment	Spore log10 reduction		Probability of viable spores	
	15 minutes	30 minutes	15 minutes	30 minutes
Ozone + No Second Stage	0.0046	0.038	0.9894	0.9153
UVC + No Second Stage	1.73	2.89	0.0185	0.0013
Second stage treatments	**30 minutes**	**45 minutes**	**30 minutes**	**45 minutes**
Ozone + UVC	1.54	2.63	0.0289	0.0024
UVC+ Ozone	1.41	2.44	0.0396	0.0036
UVC+UVC	2.2	3.51	0.0063	0.0003

the spore reduction results. The UVC + UVC treatment resulted in a log10 reduction of 3.51, corresponding to a 99.9% total reduction in viable spores. The average spore reduction counts for UVC treatments at 15, 30, 45, and 60 minutes of exposure (Figure **2**) increase with time but begin to level off between 30 and 60 minutes. These results indicate an asymptotic response in log10 reduction to increasing exposure time.

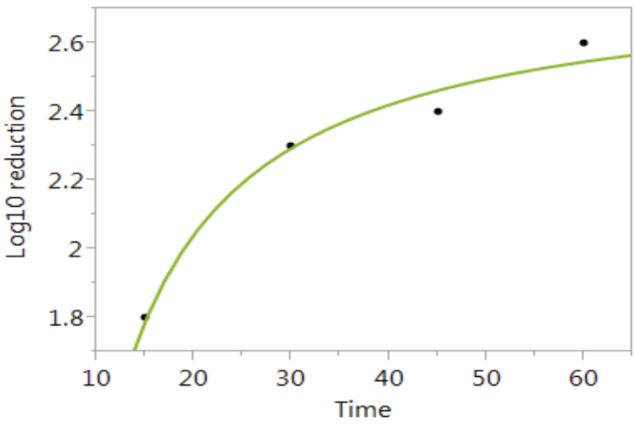


Figure 2: Average log10 reduction for UVC + UVC water treatment over 60 minutes.

4. DISCUSSION

Ozone naturally decomposes into oxygen, especially in warmer water, under light, or in the presence of organic matter [18]. The ozone monitoring results show a rapid degradation back into oxygen due to increasing water temperature during the 75-minute measurement period (Figure **1**). Ozonated water treatments were most effective when collected directly from the generator tank within 20 minutes. Unlike temperature, which impacted ozonated water, pH did not show an effect. The water used in the experiment was near neutral, and even after ozonation, the average pH stayed close to 7.3 across all samples. Adding *B. subtilis* spores did not alter the pH, as the low concentration of humic acid added to the samples did not significantly impact the pH. With increased humic acid, the pH may rise, potentially causing ORP values to become more negative, thereby improving the water's physico-chemical disinfection properties [19-20]. The ORP of the ozonated water peaked near 900 mV, when the water temperature ranged from 14 to 16 °C (Figure **1**).

The ozonated water treatment alone had the lowest spore efficacy results. This suggests that ozonated water alone is not highly effective and should be combined with UVC radiation to produce hydroxylated water, a promising wastewater technology [21-24]. A hydroxylated water study by [21] evaluated the combined effects of ozonated water exposed to UVC radiation on the efficacy of *B. subtilis* spores. The hydroxylated water treatments resulted in a 2.5 log10 reduction in *B. subtilis* spores after a 12-minute exposure in tap water samples.

The UVC water treatment was effective when applied during both stages, with a total treatment time of 60 minutes. The average reduction in spore counts for UVC water treatments at 15, 30, 45, and 60 minutes of exposure increased over time, reaching an asymptotic plateau between 30 and 60 minutes of exposure. If the estimated UVC irradiance was approximately 18 mJ/cm², based on the light efficiency calculation above, and the exposure times were 900 s (15 min) and 1,800 s (30 min), then the dosage could be estimated. The irradiance dosage for the two 9-watt UVC lamps, at 900 and 1,800 s (15 and 30 minutes), is 16,200 and 32,400 mJ/cm², resulting in a 1.7- and 2.9-log10 reduction in spore viability. The 60-minute dosage for the UVC + UVC water treatment was 64,800 mJ/cm², resulting in a 99.9% or 3.51 $\log_{10}$ reduction in *B. subtilis* spores. These results align well with wastewater sanitation research, which published an average efficacy of *B. subtilis* spores at 99%, corresponding to a 2 log10 reduction, when exposed to a dosage of 22,000 uWs/cm² [25-27].

The UVC lamps in this study had a very low power rating of 9 W. Also, the water samples spiked with B. subtilis spores were not recirculated in the UVC unit. Therefore, the study design was modified to include longer exposure times of 15, 30, 45, and 60 minutes to simulate extended recycling times for wastewater in closed power washing systems. Most wastewater treatment systems with UVC technology utilize lamps with significantly more power, ranging from 40 to 200 W for low-flow systems to 200 to 1,000 W for large municipal systems. The dosage equation multiplies the UVC lamp's " water irradiance power" by the estimated exposure time as the wastewater flows past the lamps. This equation can be readily modified by substituting the expected water flow and exposure times from this study back into the results to predict the UVC lamp wattage required to maintain a target log10 reduction. For example, suppose the exposure time was reduced to 10 s for a longer lamp. In that case, the UVC lamp wattage should be powerful enough to generate 3,240 mWs/cm2 at the lamp surface to achieve a 2.9 log10 reduction in *B. subtilis* viable spores (see UVC lamp dosage equations).

Although the addition of humic acid did not significantly influence the spore efficacy of either water treatment, it is recognized that water turbidity can negatively affect UVC transmission within water [15]. Water turbidity is a significant concern when recycling wastewater from power washing vehicles or field equipment that frequently contains mud or excess road grime. Many pressure washing systems lack filtration mechanisms to eliminate substantial levels of organic materials prior to water reuse; therefore, testing higher turbidity levels with humic acid or other approaches could be highly relevant in simulating real-world conditions.

Enhancing wastewater systems by using higher wattage UVC lamps would reduce exposure times. Properly designed wastewater systems enable highly bio-contaminated water to be sanitized in the recycled water tanks of mobile decontamination units, thus allowing for safe reuse with minimal concerns. Further research is needed to determine the optimal UVC light intensity and exposure time for mobile pressure washers, particularly in contexts with higher turbidity, which can present additional challenges.

The goal of this study was to evaluate the effects of various combinations of ozonated water and UVC treatments on water samples spiked with B. subtilis spores, with the aim of utilizing these treatments as a potential wastewater sanitation system for power washing systems. The results from this study could also be used to assist in the design of larger wastewater sanitation systems for agricultural producers, processing facilities, and truck transportation companies. The agricultural sector has numerous needs for sanitizing or decontaminating mobile field equipment, tractors, and trucks, as well as farms, storage systems, and processing facilities.

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