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Effects of Prototype Hydroxylated Water Treatment on Sanitizing Wastewater Spiked with *Bacillus subtilis* Spores

Craig L. Ramsey*

[1] *Retired – US Department of Agriculture, Fort Collins, CO 80526, USA*

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ABSTRACT

A wastewater treatment study evaluated the effects of a prototype hydroxylated water treatment on water spiked with *Bacillus subtilis* spores. The study objectives were to determine the impact of water exposure time and humic acid water treatments on spore inactivation rates. This factorial study included seven water sample collection times and three humic acid concentrations with 21 water treatments. The prototype hydroxylated water treatment system reduced viable *B. subtilis* spore by 2.47 log10 after a 12-minute exposure without any quenching agents in the water. The average *B. subtilis* spore concentration for the control water samples was 4.86 x 105 CFU/ml. Adding humic acid at 0.5% (v/v) as a quenching agent reduced spore inactivation from 3 to 1 log10. Also, adding humic acid as a quenching agent reduced oxidation-reduction potential (ORP) from 600 to about 250 mV. Wastewater treatments based on hydroxylated water technologies can sanitize bio-contaminated water if the water is first filtered to remove most organic contaminants. Alternatively, hydroxylated water treatments should use extended water exposure times, beyond 12 minutes, to minimize the quenching effects of the organic matter in wastewater.

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*Corresponding Author
Email: clramsey37@gmail.com
Tel: +(1) (970) 988-7949

1. Introduction

Decontamination of farm and field equipment, processing facilities, and transport vehicles involves power washing to remove hardened layers of soil, grime, animal/plant tissue, and dried organic matter. Power washing of field equipment or processing facilities results in bio-contaminated wastewater, which must be sanitized before it can be recycled or released into a municipal wastewater disposal system.

This study evaluated the ability of a water treatment system to sanitize wastewater. The prototype wastewater system included a hydroxylated water generator designed by a company that has since gone out of business (former LLC, Prozone Water Products, Huntsville, AL). This efficacy study aimed to determine the ability of hydroxylated water to disinfect tap water spiked with *Bacillus subtilis* spores over several exposure time intervals. The study also included several humic acid treatments to mimic wastewater contaminated with organic matter. This study was designed to determine whether hydroxylated water retained its disinfectant properties over short time intervals. If this test showed any spore efficacy after the hydroxylated water exited the generator, then it may be possible to use hydroxylated water with a power washer system.

Hydroxylated water has the potential for dual functionality in equipment decontamination. Under the right conditions, water containing hydroxyls, or hydroxylated water, can be a surface disinfectant [1-5]. In addition, hydroxylated water can be used to disinfect and sanitize the wastewater produced by power washing to be recycled for repeated power washing [5-8].

The use of hydroxylated water for equipment or facility decontamination presents two separate technical issues. The first potential problem is that hydroxyl radicals (•OH) have a short half-life. Thus, power washing with hydroxylated water may be less effective if the concentration of hydroxyls diminishes below the levels needed to sanitize surfaces during the long transport and spraying times inherent in power washing. The second role of sanitizing the wastewater produced from power washing could be problematic if the wastewater is highly contaminated with organic materials and liquids. Hydroxyls are rapidly quenched by wastewater heavily loaded with organic liquids or solids. This preliminary study partially tested both power washing problems involving hydroxyl concentration levels over time and hydroxyl quenching due to organic loads in the wastewater.

The water treatment system primarily relied on a hydroxylated water generator to sanitize water spiked with *B. subtilis* spores. The hydroxylated water was continuously recycled through the EcoMaster water generator for 12 min. to determine its ability to deactivate *B. subtilis* spores. The design of the EcoMaster allows it to constantly generate ozone, which is not completely consumed during the reactions needed to generate hydroxyls. The excess, non-consumed ozone circulates in the closed-loop system, which extends the hydroxyl generation process. Excess ozone reacts with water to form hydroxyl free radicals [9-12] or with organic solutions in water [13, 14]. This principle of continuous generation of hydroxyls may speak to the problem of the short half-life of hydroxyl radicals. Excess ozone generation may extend the ability of hydroxylated water to sanitize surfaces up to several minutes after leaving the ultraviolet lamb generator and spray nozzle.

This study's goal was to evaluate the ability of a prototype hydroxyl water generator to deactivate *B. subtilis* spores suspended in five gallons of water. The study's central hypothesis was that extending the water treatment time would increase the log10 reduction of the spore counts. A second hypothesis was that an organic challenge, such as humic acid mixed into the five-gallon water tank, would quench the hydroxyl free radicals, lower the UV transmission in the water tube, and thereby reduce the log10 reduction of the *B. subtilis* spores.

2. Materials and Methods

2.1. Study Design

This study includes two separate studies using similar prototype hydroxylated water systems. The first study at the Prozone facilities consists of a factorial design with two study factors fully crossed. The crossed study factors were seven water sample collection times and three humic acid concentrations, totaling 21 water treatments. These water treatments were replicated only twice, resulting in a study total of 42 water samples. The seven water sample collection times are 0, 2, 4, 6, 8, 10, and 12 minutes after starting the water pump. The three humic acid concentrations were 0, 0.025, and 0.05% v/v (Age Old Organic humic acid, Mendota, IL). The second study was conducted at a private facility with a slightly different prototype of a hydroxylated water generator. The second study was considered a preliminary study; therefore, this article will not present the methods and data analysis from this study. Neither study was fully replicated due to insufficient funding to initiate and complete standardized *B. subtilis* spore assays for both prototype hydroxylated water systems. Both studies were designed to generate enough data to justify further funding for more detailed and fully replicated studies for both prototype systems.

2.2. *B. subtilis* Spore Preparation

All *B. subtilis* spore samples were prepared by MicroChem Laboratory (Round Rock, TX) using EPA-certified protocols. The spore samples were prepared as water suspensions. This lab also assayed and quantified the viable *B. subtilis* spores for all the control and treated water samples for the Prozone study. Samples were suspended in water and treated with isopropanol to kill any vegetative cells so that only *B. subtilis* spores would remain within the suspensions. The initial spore counts from the culture plates were approximately 10^8 Colony Forming Units per ml (CFU/ml), while control samples had initial counts of 10^6 CFU/ml. All *B. subtilis* spore samples were shipped in insulated boxes with ice packs and stored at 4ºC until the day of the study. Baseline control samples were inoculated and assayed at the MicroChem lab, while the transport and storage control samples were shipped and stored along with the treatment samples. The samples were stored in the field in portable coolers with ice packs during the study period. Upon completion of the experiment, treated samples were returned to 4ºC until they were returned to MicroChem in insulated boxes with ice packs. Samples were assayed by culturing viable *B. subtilis* spores from the water suspensions on a semi-selective media to enumerate the viable spore count per treatment (CFU/ml) and log10 reduction for each water sample.

2.3. EcoMaster Hydroxylated Water Generator

The prototype water treatment system included a hydroxylated water generator (EcoMaster PZ-784, formerly made by Prozone Water Products, Huntsville, AL) (Fig. **1**). The EcoMaster unit contained a hybrid arc tube that generates hydroxyl free radicals from water and ozone molecules using an Advanced Oxidation Processing (AOP). This EcoMaster model was designed to clean and sanitize large swimming pools with water volumes up to 151,416 l. The EcoMaster water generator contains one water tube and two ultra-violet lamps with two wavelengths that generate 1) germicidal ultraviolet (UV) radiation (254 nm wavelength) and 2) ultra-violet light (185 nm wavelength) to convert air and water droplets into ozone. The dual wavelength lamps generate ozone injected into water, which is then converted into hydroxyl free radicals by the 254 nm UV wavelength.

Ozone (O_3) was produced from two ozone generators built by Prozone (PZ2-4V model). The O_3 output at 6.89 and 20.68 kPa pressures is 2.832×10^4 and 8.495×10^4 cm^3/hr. The compressed ozone was injected into the water lines before the water entered the EcoMaster unit. The ozonated water passed into a quartz tube within the EcoMaster, exposing the ozonated water to ultraviolet light (254 nm). Combining the compressed ozone generator with the hydroxylated water generator generates excess O_3 that is not consumed within the EcoMaster unit and transported in the water lines. The unconsumed O_3 flowing in the water lines continuously generates additional hydroxyl free radicals in the water lines. The UV light converts the ozone into hydroxyl free radicals that react with all organic compounds and deactivate all microbes in the wastewater. The free radicals' ability to inactivate *B. subtilis* spores is a function of ozone concentration, UV lamp properties, and length of water treatment time.

The Prozone prototype system consisted of a closed-loop water treatment system (Fig. **1**). This system included two compressor ozone generators (PZ2-4V model), which are the two vertical, gray cylinders in the center of the board in Fig. (**1**). The silver unit on the center left of the board contained the EcoMaster hydroxyl generator with ultra-violet lamps. A five-gallon water tank is on the right side of the board, and the water pump is on the lower right of the board. Three water sample collection ports are built into the water lines to allow water sample collection during each treatment. The ports were located on the back of the panel for easy access. The prototype system included several in-line sensors that monitored pH and Oxidation Reduction Potential (ORP) when the water pumps were turned on. The ORP for each water sample was recorded at the two-minute sampling time.

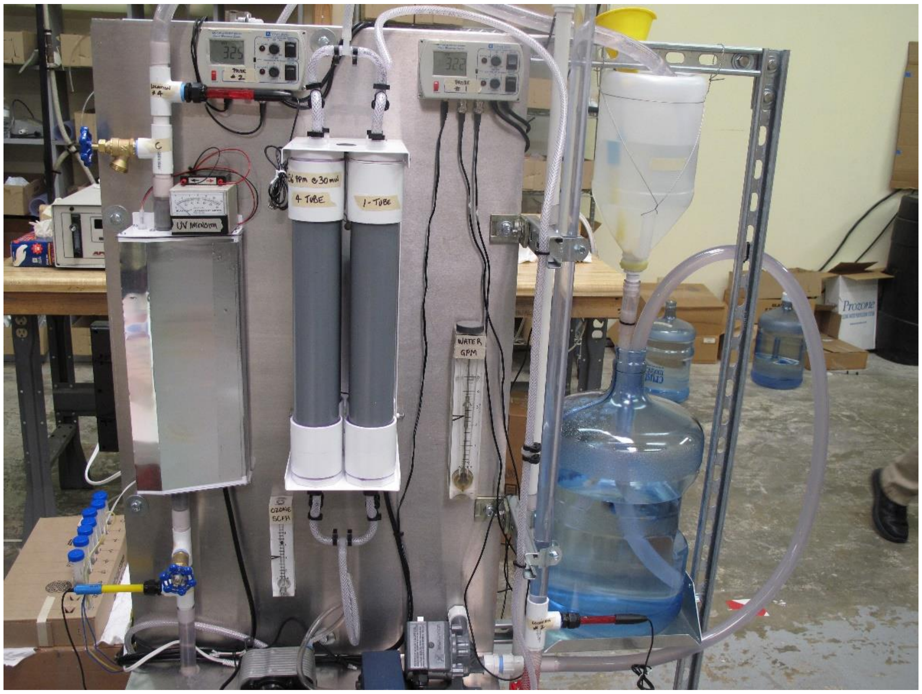

Figure 1: Photo of prototype hydroxylated water generator for wastewater sanitation study.

Most commercial water treatment systems use patented methods of generating ozone and ultraviolet (UV) light to create hydroxyls inside a chamber to sanitize bio-contaminated water. Larger advanced oxidation process (AOP) systems use ozone generators based on corona discharge technology and oxygen concentrators, generating nitrogen gas compounds that pollute air. The EcoMaster generator uses UV lamps to convert O_2 to O_3 without generating toxic nitrogen-based gases.

The closed-loop water system has a pump capacity of 2.5 gallons per minute; thus, every two minutes, all five gallons of water are circulated through the system. The total water treatment time of 12 minutes used in this study allows the five gallons to be treated 4.8 times. After starting the water pump, seven water samples were collected at two-minute intervals (0, 2, 4, 6, 8, 10, and 12 min.) for the duration of each water treatment. Before each water treatment, five gallons of tap water were spiked using a concentrated solution of *B. subtilis* spores at a 1 ml spore concentrate ratio to one liter of water. The average diluted spore count was 10^6 CFU/ml. After each water treatment, the entire water tubing in the closed loop system will be purged of all water, and a new tank of untreated water will replace the old tank of water.

Figure 2: Image of *B. subtilis* spore concentrate prepared by Microchem laboratory.

Figure 3: Image of humic acid used as an organic challenge by adding it to the five-gallon water tank at 0.025 or 0.05% v/v.

2.4. Supplemental Study

A second study used a similar water treatment system to evaluate whether slight differences in prototype systems could affect *B. subtilis* spore efficacy. Both prototype systems combined ozone and ultraviolet components to generate hydroxyl radicals in a closed-loop water system to inactivate the spores. However, the supplemental study only had two replicates and didn't include any physicochemical water property measurements. Also, the supplemental study was conducted by a private company that used its own non-standardized methods for determining viable spore counts. Independent microbiology laboratories such as Microchem use assay protocols for spore viability that are based on EPA-standardized protocols. Therefore, the second study's spore efficacy results will only be presented as unofficial and preliminary evidence to verify whether both studies had similar results.

2.5. Data Analysis

Microchem assayed the water samples from the main study and supplied the final viable spore count (CFU/ml) for the control and water treatment samples. The viable spore count was converted into log 10 reduction values to estimate *B. subtilis* spore deactivation rates for each water treatment. Log10 spore reductions were calculated from the following equation:

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

A = number of viable spores recovered from control samples

B = number of variable spores recovered from the treated samples

A generalized linear model (GLM) used the maximum likelihood procedure to analyze the data and predict log10 reduction values for each water treatment. A multivariate test correlated log10 reduction with the study variables. Regression tests were conducted to determine the relationships between the study factors. All the results were considered significant at $\alpha = 0.05$.

3. Results

Data analysis shows that both study factors and their two-way interaction were highly significant (Table **1**). The interaction term in the GLM model requires that the log 10 estimates be predicted for all 21 water treatments (Table **2**).

Table 1: Least Squares model results for the factorial study factors and their interaction.

Term	Prob>|t|
Intercept	<.0001
Time at water sample collection (min after starting pump)	<.0001
Humic acid organic challenge (%)	<.0001
Time at water sample collection*Humic acid organic challenge	<.0001

Table 2: Predicted log10 reduction for 21 treatments based on the water sample collection time (first column) and humic acid concentration (first row).

Time at Water Sample Collection (min)	Predicted log10 Reduction (0% ha)	Predicted log10 Reduction (0.025% ha)	Predicted log10 Reduction (0.05% ha)
0	0	0	0
2	0.38	0.23	0.07
4	0.79	0.48	0.17
6	1.21	0.73	0.26
8	1.63	0.99	0.35
10	2.05	1.24	0.44
12	2.47	1.5	0.53

The water treatment with the optimal log10 reduction (2.47) was a sample collection time of 12 min. and 0% humic acid. Average *B. subtilis* spore concentration for the control water samples was 4.86 x 10^5 CFU/ml. The analysis shows that water sample collection time increased the deactivation of B. subtilis spores, i.e., higher log10 reduction values. However, humic acid decreased the deactivation of *B. subtilis* spores with lower log 10 values. The addition of humic acid appears to either quench the ozone or hydroxyl free radicals and/or decrease UV transmission into the water tube.

There was a linear increase in log10 reduction as the water sample time increased at 0% humic acid (Fig. **4**). The linear increase in log10 reduction shows that ozonated water combined with a short exposure to hydroxylated within the EcoMaster unit results in a 0.3 to 0.4 log10 reduction in spores for every time the five gallons of water cycles through the water treatment loop (Table **2**). This linear relationship may eventually reach an asymptote where extending the exposure time will have a decreasing effect on log10 reduction.

The regression analysis also shows that the humic acid challenge reduced spore efficacy (Fig. **4**). This relationship shows that high rates of humic acid are a significant organic challenge for ozone and hydroxyl generation/activity. High rates of humic acid appear to quench the ozone and hydroxyl free radical activity, thereby reducing log10 reduction. Also, high rates of humic acid significantly lower the transmission of ultraviolet light in water, reducing hydroxyl generation.

The log10 reduction data was also correlated with the oxidation-reduction potential (ORP) for the three humic acid treatments (Fig. **5**). A smoother graph shows a sigmoidal relationship between ORP and water sample time for humic acid added at 0% (v/v). However, when humic acid was added at 0.025 or 0.5%, there was a linear relationship between ORP and water sample time (Fig. **5**). The reduction in ORP from close to 600 mV to about 250 mV with the addition of humic acid shows that humic acid is a strong quenching agent. The organic matter in humic acid readily accepts electrons and is reduced by the positively charged ozonated water.

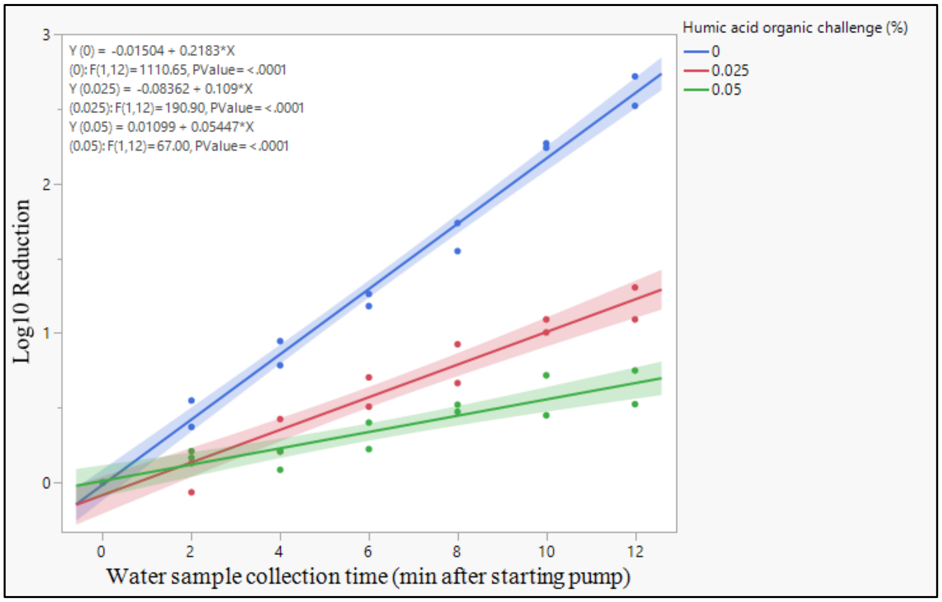

Figure 4: Regression model of log10 reduction over water sample collection time for three humic acid levels (legend shows percent humic acid).

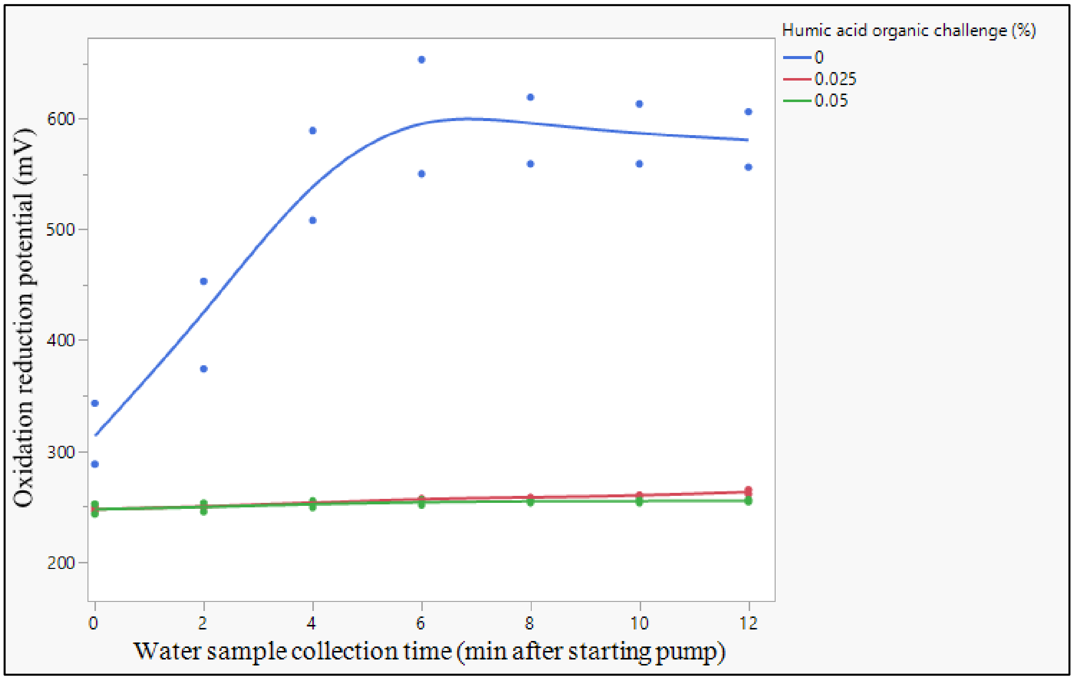

Figure 5: Smoother graph for oxidation-reduction potential over water sample collection time for three humic acid treatments (legend).

A linear relationship exists between ORP and *B. subtilis* spore inactivation rates (Fig. **6**). This is a strong, direct linear relationship similar to the relationship between water sample time and log10 reduction of *B. subtilis* spores (Fig. **4**).

The multivariate test shows that water sample time and ORP were positively correlated with log10 reduction (Table **3**). The strength of the correlation between log10 reduction and water sample time and ORP was 0.99 and 0.74, respectively, and both correlations were highly significant (Table **3**). However, increasing water sample time had a more substantial effect on log10 reduction than increasing ORP rates.

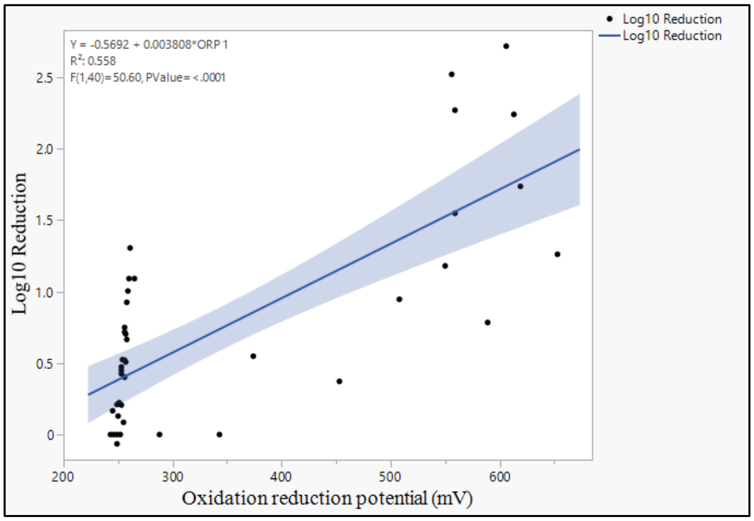

Figure 6: Regression model for log 10 reduction over oxidation-reduction potential.

Table 3: Multivariate correlations between log10 reduction of *B. subtilis* spores and water sample time and ORP.

Variable	by Variable	Correlation	Signif. Prob.
Log10 Reduction	Water sample time	0.9946	<.0001
ORP	Water sample time	0.7597	0.0016
ORP	Log10 Reduction	0.7379	0.0026

The *B. subtilis* spore efficacy of the prototype system in the second study was comparable to that of the prototype system designed by Prozone. Analysis of the data for the second study showed a quadratic relationship between log10 reduction and water sample collection time (*p*-value = <0.0001 and r^2 = 0.9583) (Fig. **7**). The quadratic relationship for log10 reduction results shows an approximate 2.5 – 2.7 reduction after a 12 min. exposure time using the second prototype water system (Fig. **7**).

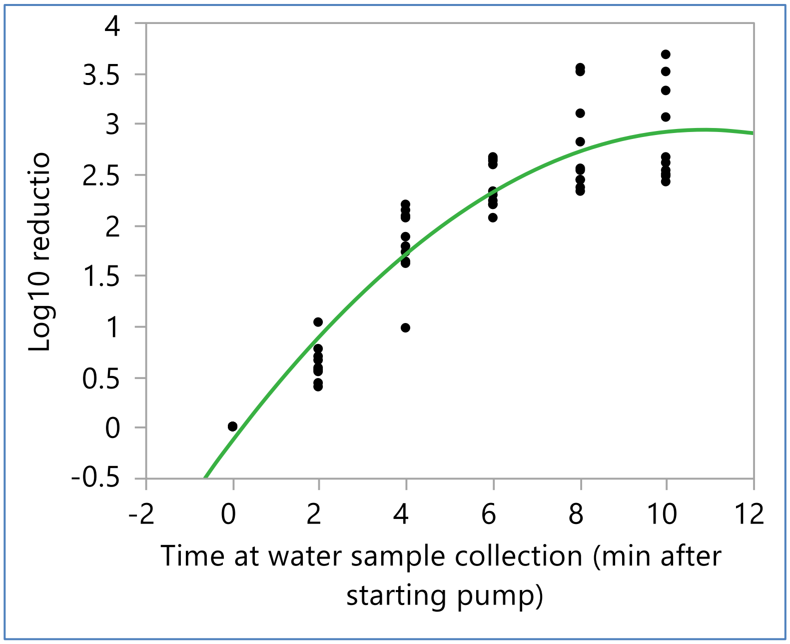

Figure 7: Log10 reduction of *B. subtilis* spores in water samples using the prototype hydroxylated water system in the second study (unpublished data, Ramsey).

4. Discussion

A wastewater study by Matin *et al*. [15] evaluated the efficacy of a combination of hydrogen peroxide, ferrate, and UV treatments on *B. subtilis* spores. Their optimal treatment included all three study factors combined together, which resulted in a log10 reduction of 2.5 in viable spores. The combination of oxidants and UV radiation treatments in the Matin study generates hydroxyl radicals similar to the protocol systems described in this study. Both study treatments produced similar spore efficacy results that approximated 2.5 – 2.7 log10 reduction of *B. subtilis* spores.

By manipulating three factors, it is possible to increase log10 reduction using a hydroxylated water generator. The first factor is increasing the water treatment time in a closed-loop system. The second factor is to increase the concentration of hydroxylated water by adding larger or more hydroxyl and ozone generators in the water system. Finally, a third factor would be increasing water quality by adding filters before water enters the hydroxylated water system. All these methods could be combined or considered individually for their cost effectiveness for increasing log10 reduction. Extension of water treatment time is a low-cost method for increasing log10 reduction if there are no time constraints, such as with cleaning swimming pools or spas. A two-stage water treatment that includes a water filter stage and a hydroxylated water treatment stage should be cost-effective for most wastewater treatments involving food processing facilities. Water treatment for high water volume conditions, such as food processing facilities or municipal water treatment plants, would probably require high-capacity, hydroxylated water generators to minimize the water treatment time so that water can be recycled quickly for reuse.

5. Conclusion

The potential of hydroxylated water treatment for reducing B. subtilis spores in wastewater. However, organic matter, like humic acid, can hinder its effectiveness. Simple adjustments—such as increasing exposure time, enhancing hydroxylated water concentration, or pre-filtering contaminants—can improve results. A practical approach for industries is a two-stage system that first filters water before applying hydroxylated treatment, ensuring efficient and cost-effective wastewater purification.

Conflict of Interest

The author declares that there is no conflict of interest.

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