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Ahmet Aydogan & Mirat D. Gurol

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Application of Gaseous Ozone for Inactivation of *Bacillus subtilis* Spores

Ahmet Aydogan and Mirat D. Gurol
Civil and Environmental Engineering Department, San Diego State University, San Diego, CA

ABSTRACT

The effectiveness of gaseous ozone (O_3) as a disinfectant was tested on *Bacillus subtilis* spores, which share the same physiological characteristics as *Bacillus anthracis* spores that cause the anthrax disease. Spores dried on surfaces of different carrier material were exposed to O_3 gas in the range of 500–5000 ppm and at relative humidity (RH) of 70–95%. Gaseous O_3 was found to be very effective against the *B. subtilis* spores, and at O_3 concentrations as low as 3 mg/L (1500 ppm), ~3-log inactivation was obtained within 4 hr of exposure. The inactivation curves consisted of a short lag phase followed by an exponential decrease in the number of surviving spores. Prehydration of the bacterial spores has eliminated the initial lag phase. The inactivation rate increased with increasing O_3 concentration but not >3 mg/L. The inactivation rate also increased with increase in RH. Different survival curves were obtained for various surfaces used to carry spores. Inactivation rates of spores on glass, a vinyl floor tile, and office paper were nearly the same. Whereas cut pile carpet and hardwood flooring surfaces resulted in much lower inactivation rates, another type of carpet (loop pile) showed significant enhancement in the inactivation of the spores.

INTRODUCTION

In response to a possible terrorist attack involving chemical and/or biological warfare agents, tools and strategies are being developed. These include early detection sensors, crisis management planning, forensics, and decontamination strategies. Anthrax is considered as the most current and immediate public health threat that can be created by a warfare agent. Anthrax is caused by the *Bacillus anthracis* spore, a bioorganism that adapts well to extreme environmental conditions. When properly prepared, the anthrax spores can be aerosolized and become available in lethal dosages for inhalation or body contact. The spores may be introduced into the ambient air by opened contaminated packages or envelopes, or through the ventilation systems of buildings. Because of their size, the spores after being introduced into the air primarily settle onto surfaces, such as desks, furniture, clothing, walls, rugs, floors, and so forth.

Although chlorine dioxide has been used for the clean-up of federal buildings after the recent anthrax attack, ozone (O_3) gas is expected to be a more appropriate and effective disinfecting agent for decontamination of air and the surfaces in rooms, dwellings, offices, buildings, and so forth, that may have been exposed to anthrax spores. O_3 is known to be an effective oxidant both in gas phase and water, in addition to being a strong disinfectant. In water, it can destroy chemicals, such as cyanide and chlorinated aromatic and aliphatic compounds. One of the classic applications of O_3 in the gas phase has been its use as a deodorizing agent in uninhabited homes, office buildings, and cars in which fires have occurred, leaving smoke odors entrapped behind walls, ceilings, under floors, and on furniture.[1] In such cases, the proven remediation procedure is to seal the affected areas, station an appropriately sized O_3 generator, and turn it on. After O_3 oxidizes the organic residues, such as smoke and polycyclic aromatic hydrocarbons, the generator is turned off, the enclosure is opened, and fresh air is allowed to enter. A similar approach can be envisioned to decontaminate enclosures exposed to biological or chemical warfare agents, including buildings and subway systems, as well as the surfaces in these spaces. As opposed to nongaseous disinfectants, gaseous O_3 can diffuse to completely fill the entire space, including the air ducts, cracks, ventilation systems, and porous material to inactivate bacteria, viruses, spores, and cysts and to degrade biological and chemical toxins. As opposed to chlorine dioxide, the decontaminated space is expected to require no post-treatment cleanup, because O_3 naturally decomposes into oxygen in a matter of hours. Directing O_3 through catalytic destruction units, if necessary, can additionally accelerate decomposition of O_3.

O_3 is generated in gas form from pure oxygen or air, and, because of its instability (O_3 reverts to oxygen), it must be generated and applied on-site, at its point of use.[2] It can be used directly in the gas phase to inactivate airborne microorganisms or dissolved in water to inactivate microorganisms in water. Earlier studies showed the effectiveness of O_3 in inactivating spore-forming bacteria in water, including bacillus species, such as *Bacillus subtilis* and

IMPLICATIONS

This paper reports the results obtained from inactivation of Bacillus spores with gaseous O_3 under different environmental and operational conditions. These results can be used for remediation of enclosed spaces by O_3 in case of a terrorist attack involving anthrax spores. O_3 was found to be very effective against the spores in gas phase, and the extent of inactivation of spores varied with the contact time, O_3 gas concentration, the RH of air, prehydration level of spores, and the type of material the spores rest on. No material damage was observed during these studies.

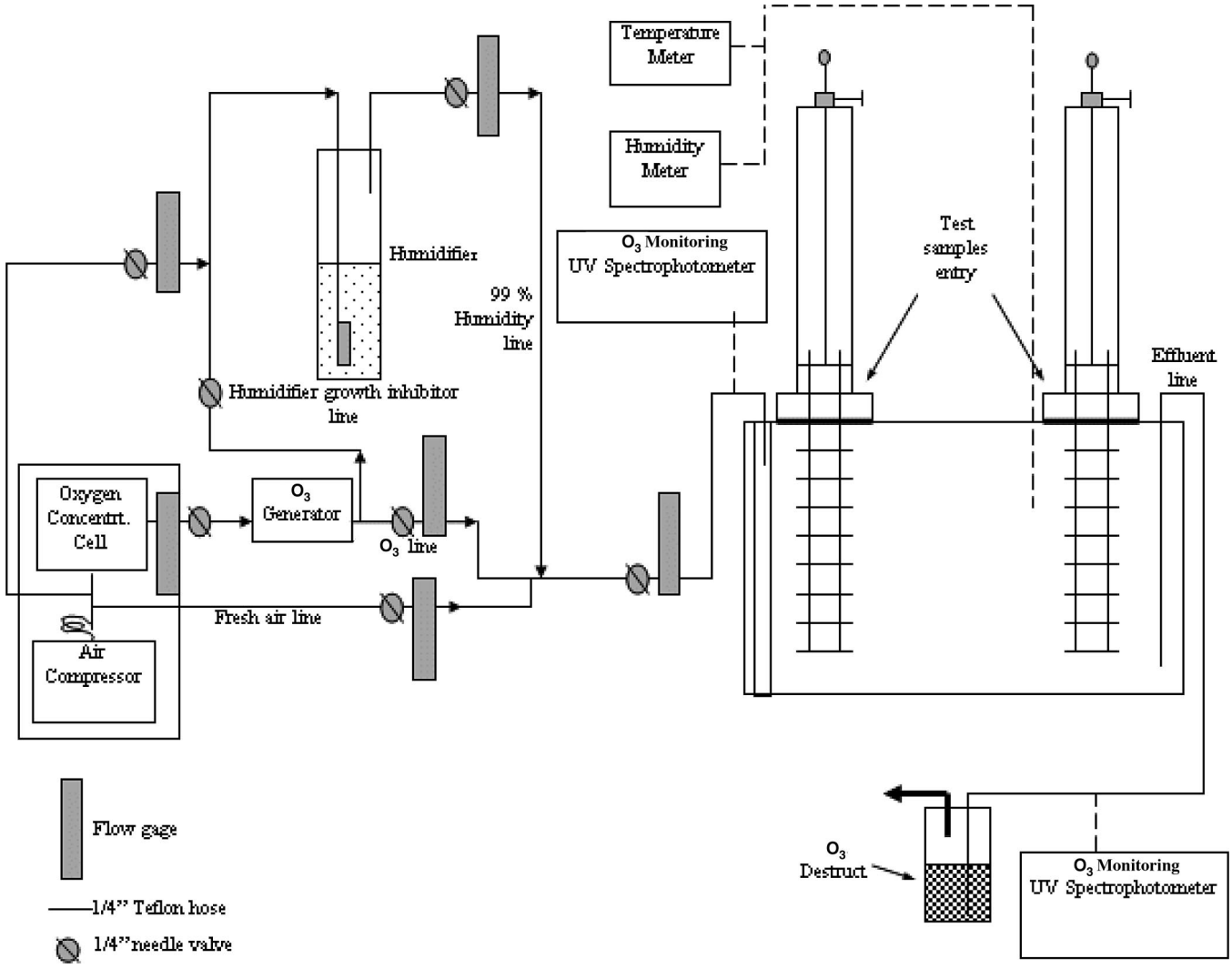

Figure 1. Schematic diagram of ozonation test chamber.

Bacillus globigii, which belong to the same genus as *B. anthracis*, that causes anthrax yet are not pathogens. The effectiveness of O_3 on *B. anthracis,* as well as on other potential biowarfare agents, including toxin of *Clostridium botulinum* and influenza virus in ozonated water, was demonstrated through a study conducted for the Chemical Corps of Fort Detrick, MD, as early as 1956.[3] This study has determined that O_3 was equally effective on *B. anthracis* and *B. subtilis*.

Two earlier publications have shown that O_3 is also effective on bacterial spores in gas-phase applications. Ishizaki et al.[4] examined the sporicidal activity of O_3 on different Bacillus spores for different O_3 concentrations (250–1500 ppm). At high relative humidity (RH), the difference between the inactivation rates of different strains was relatively small, and ~5-log reduction was obtained in <2 hr. Currier et al.[5] presented experimental results on *Bacillus globigii* spores at an O_3 concentration of 9000 ppm. O_3 has been shown to be very effective against bacillus spores at this high concentration by leaving undetectable numbers (>5-log reduction) in <1 hr. Both Ishizaki et al.[4] and Currier et al.[5] have observed O_3 to be less effective at humidity levels <60%. These studies demonstrate the feasibility of spore inactivation by gaseous O_3. However, additional information is needed on the rate of inactivation of the spores at various O_3 concentrations, humidity, and the type of material surface that the spores rest on, as well as the physical effect of O_3 on the material.

This research is focused on investigation of the proper operational conditions for O_3 gas to effectively inactivate *B. subtilis*, a spore forming nonpathogenic bacteria that belongs to the genus Bacillus and shares the same physiological characteristics as *B. anthracis*. The variables that were tested in the O_3 test chamber included O_3 concentration in air, the RH of the air in the test chamber during ozonation, and the degree of hydration of the spores before exposure to O_3. In addition, the effect of carrier material on inactivation rates was tested by placing the spores on strips of different surface materials, such as glass, carpet, paper, hardwood, and vinyl floor tile, and the material was observed for any physical damages after exposure to O_3.

EXPERIMENTAL WORK

Ozonation Test Chamber

Figure 1 shows a diagram of the ozonation test system. The test chamber has been constructed of plexyglass to

allow transparency for observation. The chamber is 76 cm long, 56 cm deep, and 30 cm wide (volume 128 L), and it is sealed perfectly. The system has been constructed and assembled specifically for this study by Pure-O-Tech Inc. The system has its own air compressor, a Sequal oxygen concentrator and a Pure-O-Tech O_3 generator, that is capable of producing 4% O_3 in gas. The test samples are placed into the chamber through two sample entry windows at the top of the chamber and are completely sealed. Three gas streams are directed to the chamber to create the desired conditions in the chamber in terms of O_3 concentration, humidity level, and the total gas flow through the chamber: the O_3 gas stream from the O_3 generator, the airstream from the air compressor, and the humidified airstream, which is produced by bubbling in sterile water. The O_3 concentration is monitored by a UV spectrophotometer by measuring the absorbance at 254 nm. The RH and temperature are measured by humidity/temperature sensor, which was placed inside the chamber. The experiments were conducted at room temperature, 21–22 °C. The RH has been controlled by changing the proportion of the two inlet air streams.

Preparation of Spores

B. subtilis sp. ATCC 6633 was obtained from the Microbiology Laboratories of the Biology Department of San Diego State University. The strain was grown in tryptic soy broth with Schaeffer sporulation media [K_2HPO_4, 10.5 g/L; KH_2PO_4, 3.5 g/L; $MgSO_4 \cdot 7\ Hr_2O$, 0.05 g/L; $CaCl_2$, 0.05 g/L; $MnCl_2 \cdot 4H_2O$, 0.005g/L; and $Fe_2(SO_4)_3 \cdot 7\ Hr_2O$, 0.005 g/L],[6] and 1 week was allowed for growth and sporulation at 30 °C and moderate aeration. The culture in the form of vegetative cells and spores was harvested and heat shocked at 65 °C for 15 min to kill the vegetative cells. Afterwards, the suspension was centrifuged, washed, and resuspended in distilled water. Temperature was brought to 37 °C, and the suspension was additionally treated with 0.5% sodium dodecylsulfate and lysozyme (1 mg/mL) for 30 min to completely eliminate the vegetative cells. The suspension was filtered through 2-μm glass fiber filter paper to remove the debris. The remaining cells (spores) were centrifuged, washed, and resuspended in distilled water five times, and the final stock spore solution was stored in a refrigerator at 3 °C. Necessary dilutions were applied to bring stock spore solution concentration to $\sim 10^8$ colony forming units/mL.

Preparation of Test Samples

Spores were exposed to O_3 on strips of material in common use in offices and residences, for example, glass, carpet, paper, vinyl floor material, and hardwood. Microscope slides were used as glass carrier. Two different carpet samples were tested; the first one was a new loop pile carpet, and the second one was part of an old used cut pile carpet. Parts from an old office desk were used as finished hardwood samples. Two different types of new vinyl floor tiles were obtained from a hardware store. Regular office print paper was used as the paper sample.

Because O_3 demand of glass is expected to be minimal, and microorganisms on impervious surfaces may be hard to inactivate according to Gilbert et al.[7] and Ishizaki et al.,[4] glass strips were used as the base test material. Spore solution in the amount of 50 μL was placed and distributed on each strip surface. The strips were then dried at room temperature in a safety cabinet for 3 hr and conditioned in desiccators for ≥3 days. The RH in desiccators was maintained at <1% by silica gel. When preconditioning of samples was required at different humidity levels, constant humidity levels were maintained by the following saturated salt solutions: $BaCl_2$, 90%; and NaCl, 75%.

Hydrodynamic Characterization of the Test Chamber

Flow distribution inside the ozonation chamber was analyzed by tracer tests using O_3 gas itself as the tracer. O_3 was introduced into the chamber at a constant flow rate and concentration, and the inflow and effluent O_3 concentrations were monitored by an UV spectrometer. O_3 breakthrough curves were obtained at various residence times (RT) values, where RT is defined as the volume of the chamber divided by the flow rate of gas mixture through the chamber. Numerical data analysis has showed that the flow regime was very close to complete mix conditions, because normalized effluent concentration curves overlapped with ideal complete mix curves. The effluent from the chamber stabilized after a period equivalent to four RT. These tests provided enough evidence that complete mixing conditions were maintained in the chamber, that is, all of the test strips placed in the chamber were exposed to the same levels of O_3, and, furthermore, influent, chamber, and effluent O_3 levels were the same after four RTs.

Experiment and Recovery of Spores

The O_3 generator was turned on, and the test strips were placed in the chamber in duplicates through the two entry points after O_3 concentration and RH were stabilized at the desired values beyond four RT. The strips were exposed to O_3 for a period of ≤4 hr. Duplicate samples of test strips were removed at various sampling times.

The strips were then transferred into 50 mL of physiological phosphate buffer solution, and ultrasonication was applied for recovery of the spores. The remaining number of spores on each strip was determined together with the control, which provided the initial number of spores. The physiological phosphate buffer solution contained 10 mM K_2HPO_4, 1 g/L $MgSO_4$, 0.05% Tween-20, and 1 mM sodium thiosulfate (to quench O_3). It is highly unlikely that mild sonication could affect the activity of the spores, yet control experiments were performed to determine whether there would be any inactivation because of sonication with a Branson B-220 sonication bath. Similar experiments were conducted to determine the sonication time required to recover >90% of spores from all of the carrier material strips.

A spore solution was sonicated for 2 hr and viability of spores where determined at several time intervals. These control experiments indicated that 2 hr of sonication did not cause a significant change in the viability of the spores. The control experiments for determination of the recovery time were conducted in a similar manner. The test strips with the known amount of spores were put in the buffer solution, and sonication was applied. The number of spores in the solution was determined at several time intervals. Within 90 min of sonication, >90% of

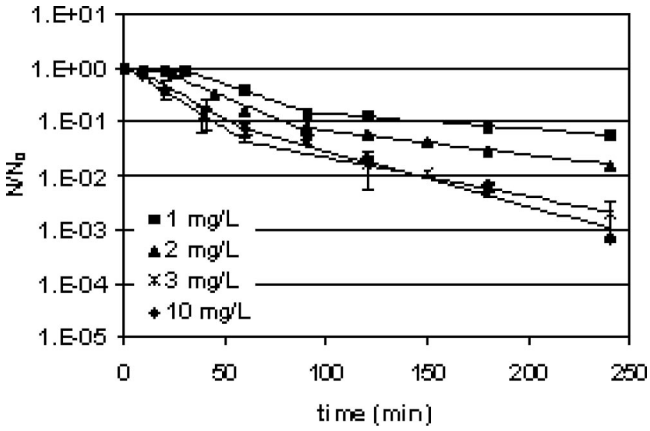

Figure 2. Effect of O_3 concentration on inactivation of *B. subtilis sp.* spores at 90% RH and 22 °C.

the spores were recovered from the glass, vinyl, and the hardwood strips. The recovery from the carpet and paper strips did not exceed 50%, but the recovery ratio was consistent throughout the experiments.

Determination of Number of Spores

The spread plate viable counting method[8] was used to determine the number of the spores recovered by sonication in the buffer solutions. Required serial dilutions were applied to the sample solutions using sterile phosphate buffer solution as the diluent. Inoculum samples of 0.1 mL from each dilution tube were spread on tryptic soy agar plates. Agar plates were incubated at 30 °C for 16–24 hr, and colonies formed on agar surface were counted. If the number of spores in the sample was very low (<100/mL), the pour plate viable counting method[8] was used instead.

RESULTS AND DISCUSSION

Effect of O_3 Concentration on Inactivation

Spores of *B. subtilis sp.* dried on glass strips and preconditioned at <1% RH were exposed to different O_3 concentrations at 90% RH and 22 °C ambient temperature. The experimental results are presented in Figure 2, which depicts the ratio of number of live spores remaining to the number of initial live spores in log units over exposure time. Duplicate samples were drawn at each time, and each experiment was conducted in duplicate. Each data point represents the average of the readings, and the error bars shown on graphs correspond to one standard deviation.

The results show a short lag phase followed by an exponential decrease in the number of surviving spores at low O_3 concentrations of 1 and 2 mg/L. The lag phase shortens considerably at higher O_3 concentrations. When the O_3 concentration is high, penetration/destruction through the spore coat is expected to be faster, thus resulting in a decrease in lag time. The exponential decrease seems to be composed of two different rates, a steep decrease followed by tailing off. A similar behavior in the survival curves of bacteria, viruses, or spores in water is frequently reported in the literature. Several theories regarding this behavior have been proposed.[9] Some researchers consider this observation an experimental error, whereas others attribute it to nonhomogeneity of the bacterial population or clumping of organisms. It was also suggested that the number of organisms is getting too small after extended exposure times where the measurement becomes a discrete rather than a statistical event.[7]

Table 1. *D* values obtained with different O_3 concentrations at 90% RH for inactivation of *B. subtilis sp.* spores on glass strips at 22 °C.

O_3 Concentration (mg/L)	RH (%)	First-Stage *D* Value (min)	Second-Stage *D* Value (min)
1	90	79	360
2	90	65	226
3	90	43	136
10	90	47	98

The results also show that increasing the O_3 concentration from 1 to 3 mg/L increased the rate of inactivation of spores. Yet, beyond 3 mg/L, no additional rate increase was obvious. This observation indicates that inactivation of spores could be limited by the rate of mass transfer of O_3 beyond this O_3 concentration.

Yokoya and York[10] proposed use of the term "*D* value" to express the death rate or the apparent resistance of the organisms. It was defined as the time, in minutes, needed to reduce the population by 90% or 1-log, at a constant dosage. The *D* values are obtained from the slopes of the inactivation curves where $D = 1/\text{slope}$. The D values calculated for both stages of inactivation curves were listed in Table 1. As O_3 concentration was increased from 1 to 3 mg/L, the *D* values for both stages decreased significantly, whereas there was no significant difference between the *D* values for 3 and 10 mg/L.

The *D* values obtained in two earlier studies were considerably lower than the values obtained in this study. Ishizaki et al.[4] determined the *D* values as 12–13 min for different strains of *B. subtilis* spores at 90% RH and 3 mg/L O_3 concentrations. However, they observed much longer lag phases, 50–65 min, under the same conditions. Currier et al.[5] did not present any *D* values for their study in which 9000 ppm (18 mg/L) of O_3 was applied for deactivation of *B. globigii* spores. Their results showed either no lag phase or very short ones and >4-log reduction in the number of spores in 30 min, thus resulting in <10 min for the *D* values. In our study, a larger *D* value of >40 min and considerably short lag phase of <10 min were obtained for inactivation at 90% RH. Although the general inactivation trend was similar in these three studies, the inactivation rates were not close to each other. A similar situation was observed in water disinfection with O_3 studies, as several researchers have obtained different survival rates for various species of *B. subtilis* spores.

An explanation for the differences in inactivation kinetics might be the physiological differences among the spores, which may change with the change in the sporulation medium. The physiological differences include the thickness, composition, and/or density of the spore coat, cortex, and inner membrane, which have been identified as possible barriers presented by spores against disinfectants. For example, Bloomfield and Arthur[11] showed that the resistance of *B. subtilis* spores to chlorine-releasing agents depends on both spore coat and the cortex.

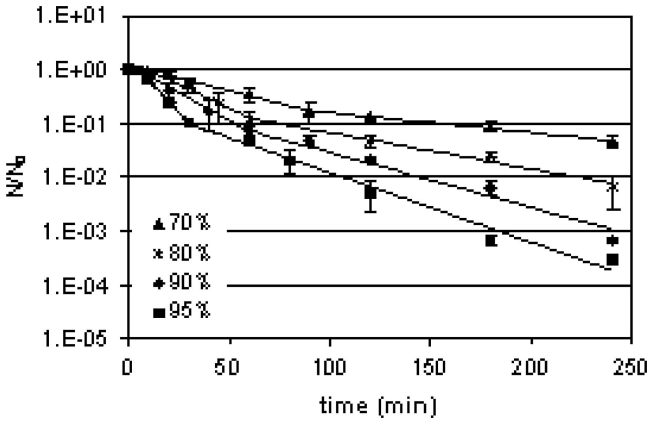

Figure 3. Effect of RH on inactivation of *B. subtilis sp.* spores at 10 mg/L O_3 concentration and 22 °C.

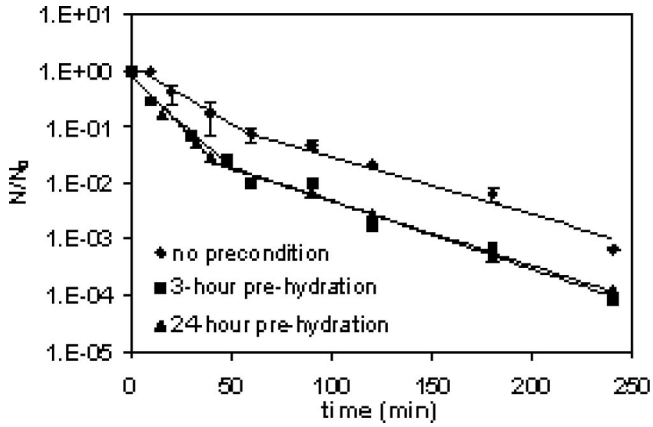

Figure 4. Effect of prehydration at 90% RH on inactivation of *B. subtilis sp.* spores by 10 mg/L O_3 concentration at 90% RH and 22 °C.

Gould[12] has postulated that cortex peptidoglycan is responsible for both mechanical protection of the *Bacillus cereus T* spores and maintaining dehydration of the protoplast. His studies suggest that dipicolinic acid in the spore protoplast plays a role in the dormancy and resistance of spores. Yokoya and York[10] showed that different sporulation mediums resulted in different resistance of *Bacillus* spores to heat. The primary lethal effect of sporicidal agents is on the spore protoplast or protoplasmic membrane, but their activity is largely determined by their ability to produce degradation of not only coat but also cortex permeability barriers.[11–13]

Effect of RH

B. subtilis sp. spores conditioned at <1% RH have been exposed to 10 mg/L O_3 concentration at different RH values. The results are presented in Figure 3. The positive effect of RH on inactivation efficiency is very evident. The inactivation rate has increased as RH in air was increased. All of the survival curves showed two-stage behavior and *D* values increased with a decrease in RH (Table 2). The duration of the initial lag phase has decreased by an increase in humidity. The *D* values obtained at 95% RH were almost four times smaller than the values obtained at 70% RH. It is common to explain the lag phase as a necessity to reach a minimal damage in the spore coat before inactivation occurs.[9,10,14,15]

Ishizaki et al.[4] observed a similar relation between the inactivation rate and RH. They tested a RH range of 50–95%, and the inactivation rate has increased proportionally with the increase in RH. The presence of certain amounts of moisture in the spore surface seems to be essential for rapid penetration of O_3 and/or reaction of O_3 with the surface structure of spores.

On the contrary, Currier et al.[5] obtained significantly less kill at RH >80–85% when spores of *B. globigii* were exposed to 18 mg/L O_3 at ambient temperature. They used clumped spores during their experiments, and large variations between experimental runs were observed at high humidity levels. Currier et al.[5] suggested that capillary condensation can occur between the spores at higher humidity levels as they observed a sharp increase in water adsorption >80% RH They stated that trapped water would provide a significant mass transport resistance for gas trying to penetrate, thus reducing the global kill rate. However, we believe that if the spores are not in clumped form, the space between spores may be large enough to prevent capillary condensation. Therefore, additional increase in RH may not affect mass transport significantly. Thus, an increase in humidification of the spores surface should result in acceleration of inactivation rate, as observed in this research.

Table 2. *D* values obtained with 10 mg/L of O_3 concentration and different RHs for inactivation of *B. subtilis sp.* spores on glass strips at 22 °C.

O_3 Concentration (mg/L)	RH (%)	First-Stage *D* Value (min)	Second-Stage *D* Value (min)
10	70	107	274
10	80	56	149
10	90	47	98
10	95	26	77

Effect of Prehydration

To investigate the effect of prehydration of spores on the inactivation rate by O_3, spores of *B. subtilis sp.* that were dried on glass strips were subjected to prehydration at 90% RH for periods of 3 and 24 hr. These strips were then subjected to inactivation in the chamber at 10 mg/L of O_3 concentration and 90% humidity. The results were compared with the results obtained without prehydration in Figure 4. Prehydration has eliminated the initial lag phase and resulted in higher initial inactivation rate (first stage) only.

The *D* values are presented in Table 3. Apparently, 3-hr prehydration was sufficient to eliminate the lag phase and increase the rate of inactivation, indicating no additional advantage with longer prehydration.

Table 3. Effect of prehydration on the *D* values at 10 mg/L of O_3 concentration on glass strips at 22 °C.

Prehydration	RH (%)	First-Stage *D* Value (min)	Second-Stage *D* Value (min)
None	90	47	98
3 hr at 90% RH	90	31	83
24 hr at 90% RH	90	27	87

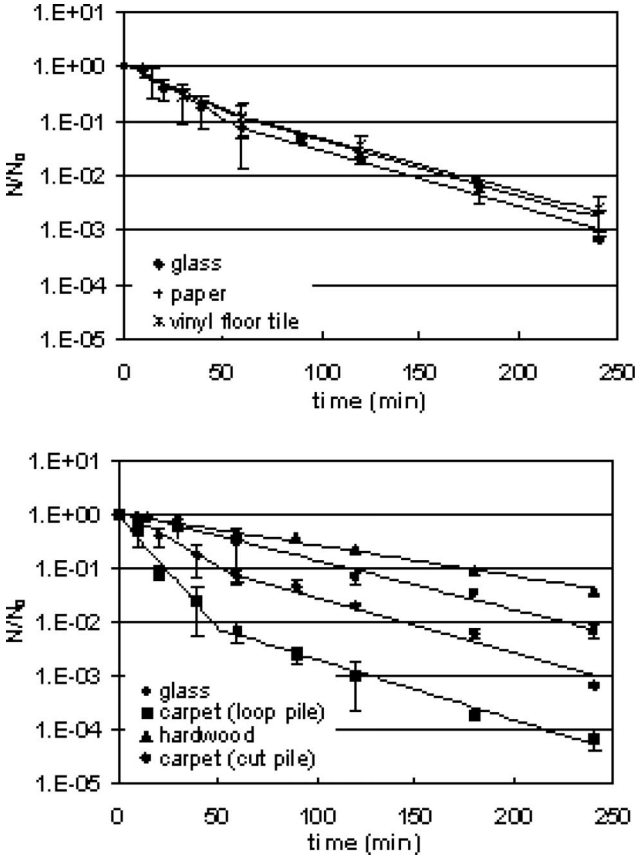

Figure 5. Effect of carrier material on inactivation of *B. subtilis sp.* spores at 10 mg/L O_3 concentration, 90% RH, and 22 °C.

Two possible explanations can be provided for the effect of humidity on inactivation rates: (1) when spores sense the presence of high humidity, they might think the "stress" conditions are over; they might become more perceptive to outside stimulus, and, as a result, O_3 might penetrate and destruct spores more easily; and (2) O_3 is a more powerful biocide in water than in air; under high RH conditions, there might be microcondensations through spore surfaces, and O_3 dissolution in these will result in faster inactivation. Both phenomena may be effective simultaneously, because the increased humidity also enhances inactivation of spores with other gaseous disinfectants, such as chlorine dioxide and ethylene dioxide.[7]

Effect of Carrier Materials Surface

Spores placed on the surface of different test materials were exposed to 10 mg/L O_3 at 90% RH. The survival curves and the *D* values obtained for these experiments are presented in Figure 5 and Table 4.

Inactivation of spores on glass, vinyl floor tiles, and paper were not significantly different from each other. The results on the two vinyl carriers were similar; thus, they are shown as one sample. Thus, it can be concluded that the vinyl and paper surfaces used in this study did not enhance or delay the inactivation of spores in comparison with glass.

Two different carpet types tested in the study produced completely different results from each other. The new loop pile carpet sample has resulted in much faster spore inactivation than the glass surface. The used cut pile carpet sample, on the other hand, resulted in slower inactivation than glass. Compared with the glass surface, slower inactivation was observed on the finished hardwood sample as well. Over the duration of the experiment, the inactivation curves obtained on these two surfaces, the cut pile carpet and the hardwood, did not exhibit the two stages observed for other surfaces, probably because the extremely slow rate of inactivation required a longer experimentation period to reach the second stages. Overall, the results confirm the expectations that spores resting on different surfaces would inactivate at different rates by gaseous disinfectants like O_3. In fact, at the end of 4 hr of ozonation, the inactivation among different surfaces varied by a factor of >500.

Table 4. *D* values obtained with different carrier materials for inactivation of *B. subtilis sp.* spores with 10 mg/L O_3 concentration at 90% RH at 22 °C.

Carrier Material	First-Stage *D* Value (min)	Second-Stage *D* Value (min)
Glass	47	98
Vinyl floor material	65	103
Office paper	64	98
Carpet–loop pile	24	89
Carpet–cut pile	109	109
Hardwood	174	174

The slower inactivation observed for the hardwood and the cut pile carpet samples could perhaps be explained by emission of O_3-reactive substances (volatile organic compounds) from these surfaces. Such substances might consume O_3 gas within the boundary layer over the surface before O_3 reaches the spores on the material surface. In other words, these materials may exert an "O_3 demand." For example, Molhave et al.[16] reported that several types of latile organic compounds, mainly in the form of terpenes, are emitted from indoor wood surfaces. For the cut pile carpet, additional mass transfer limitation in terms of slower diffusion of O_3 through an irregular boundary layer may also play a role in slowing the inactivation rate. However, this will not be the case for the hardwood surface, which was observed under the microscope to be as smooth as the glass surface.

For the loop pile carpet, the enhancement in the inactivation rate compared with the glass surface might be the result of fumigative effect of chemicals produced from the reaction of O_3 with the carpet surface. For example, Weschler[17] and Morrison and Nazaroff,[18] who studied the interactions between O_3 and various carpet samples at typical indoor O_3 concentrations of 30–400 ppb (0.06–0.8 μg/L) reported the formation of a variety of volatile organic carbons, generally aldehydes, including formaldehyde. Formaldehyde is frequently used as a gaseous disinfectant, and is also shown to be effective against *B. subtilis sp.* spores.[19] Reactions between O_3 and unsaturated hydrocarbons, many found in wood products, are known to produce a variety of products, ranging from short-lived, highly reactive free radicals to stable, highly oxidized species, such as hydrogen peroxide.[20] Hydrogen peroxide and various free radicals are also effective disinfectants.

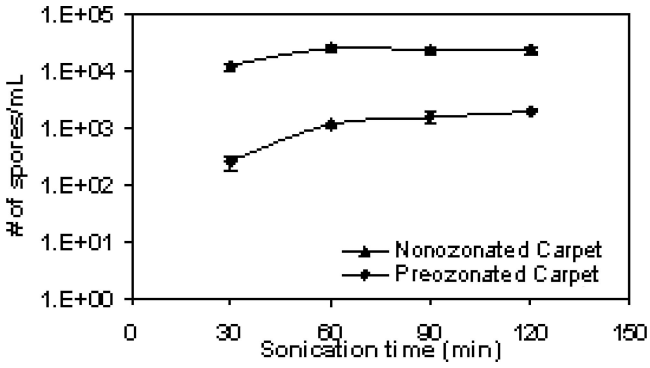

Figure 6. Recovery of spores from preozonated and nonozonated loop-pile carpet strips.

We performed additional experiments to test the hypothesis that reaction byproducts of O_3 with the loop pile carpet can, in fact, function as a disinfectant and inactivate the spores. Samples of loop pile carpet strips were ozonated for 4 hr and kept in desiccators for 24 hr. Then, spores were placed on both ozonated and nonozonated carpet strips and dried in desiccators for 3 days. The recovery of spores from these carpet strips as a function of sonication time is presented in Figure 6. At the end of 2-hr of sonication, >90% less live spores were recovered from the preozonated carpet strips compared with the nonozonated ones. This is equivalent to the inactivation of spores by 1-log on preozonated carpet strips. This 1-log reduction may be attributable to inactivation caused by fumigative secondary emissions resulting from the reaction of O_3 with the carpet surface. In fact, the difference between the remaining spores on the loop pile carpet and the glass strips at the end of 4 hr of ozonation is also 1-log (Figure 5), with the implication that the enhanced inactivation observed on the loop pile carpet samples is because of secondary emissions resulting from the reactions between O_3 and the carpet surface.

Observations on Physical Effect of O_3 Exposure on Carrier Material

At the conclusion of the ozonation experiments after 4 hr, carrier material was inspected visually and by touch. No damage as a result of ozonation or no difference between before and after ozonation was apparent.

CONCLUSIONS

The results presented here demonstrate that gaseous O_3 can be very effective against *B. subtilis* sp. spores. O_3 at 3 mg/L (1500 ppm) produced ~3-log reduction within 4 hr at 90% RH and 22 °C on glass surface. No additional benefit was observed in terms of increased inactivation rate at higher O_3 concentrations. Higher humidity levels during ozonation, as well as prehydration of the spores, additionally increased the rate of inactivation. The type of surface on which the spores rest may decrease or increase the inactivation rate of the spores significantly.

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About the Authors

Ahmet Aydogan is a Ph.D student and Mirat D. Gurol is the Blasker Chair Professor of Environmental Engineering in Civil and Environmental Engineering Department of San Diego State University. Address correspondence to: Mirat D. Gurol, Civil and Environmental Engineering Department, San Diego State University, San Diego, CA; phone: +1-619-594-0391; fax: +1-619-594-8078; e-mail: mgurol@mail.sdsu.edu.