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Approved for public release; distribution unlimited.

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13. ABSTRACT (Maximum 200 words)
One major barrier to the development of new spore decon technologies is that killing assays take 36-48 hours to perform and require a tedious culture-based assessment of spore viability. Spore sterilization assays can require from seven to twenty one days of incubation in growth medium. We proposed that near-real time optical spore germination assays could be developed into a new type of spore killing assay. The experimental results described in this report were designed to test this hypothesis. Dormant bacterial endospores are highly birefringent due to the anhydrous nature of the spore cytoplasm. Once spores are triggered to germinate by small molecules such as sugars, nucleosides or amino acids, a series of very rapid enzymic reactions occur that leads to the hydrolysis of spore integuments and the rehydration of the spore cytoplasm. The germination process is accompanied by a concomitant 30 - 50% decrease in visible wavelength optical absorbance. Real-time spore germination kinetics can be acquired spectrophotometrically. We report here a comparative analysis of spore killing by several sporicides using established viability assays, live/dead fluorescent microscopy, rapid spectrophotometric and automated scanning microscopic methods.

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(1) List of papers submitted or published under ARO sponsorship **during this reporting period**. List the papers, including journal references, in the following categories:

(a) Manuscripts submitted, but not published

None

(b) Papers published in peer-reviewed journals

Westphal, A., Price, B., Leighton, T. and K. Wheeler. 2003. Kinetics of size changes of individual *Bacillus thuringiensis* spores in response to changes in relative humidity. Proc. Natl. Acad. Sci. USA, 100: 3461.

(c) Papers published in non-peer-reviewed journals or in conference proceedings

None – this is a new project

(d) Papers presented at meetings, but not published in conference proceedings

Leighton, T., Eggum, G and K. Wheeler. 2002. Use of Chlorine Dioxide for Large-area Decontamination. Joint Service Chemical and Biological Decon Conference, San Diego, CA.

Leighton, T. 2001. Microbial decontamination after a Bio/Chemo attack – do we throw away that B-2 bomber? Gordon Research Conference: Illicit Substance Detection: Chemical/Biological Agents, Mount Holyoke College, South Hadley, MA.

(2) **"**Scientific personnel" supported by this project and honors/awards/degrees received

Sky Rashby – B.Sc.

Willian Byrne – B.Sc.

Katie Wheeler – B.Sc.

(3) "Report of inventions" (by title only)

None

(4) "Scientific progress and accomplishments" (Description should include significant theoretical or experimental advances)

Four experimental and technical objectives have been addressed during the project period:

(1) Development of near-real time spectrophotometric assays for sporicidal killing;

(2) Development of live/dead fluorescent microscopy assays for sporicidal bioeffects; and

(3) Correlation of rapid optical sporicidal bioeffects assays with spore viability measurements.

(4) Development of a rapid microscopic method for high-resolution measurements of dimensional changes in individual cells and spores as a function of humidity.

In the following sections we will discuss the progress achieved in these four task areas.

Rapid Optical Assays for Sporicidial Bioeffects

Germination of the bacterial spore is a highly regulated process which is initiated by the presence of a germinant, such as the amino acid L-alanine, sugars, nucleosides etc. which signal the presence of a nutrient environment appropriate for the resumption of vegetative growth. The germination process involves a defined temporal order of events, characterized initially by hydrolysis of the spore coat and cortex, a loss of refractility as the spore rehydrates and the release of Ca^{2+} and DPA. Germinant stimulation along with concomitant spore core hydration leads to the emergence of a rapidly growing vegetative cell.

Numerous studies have shown that sporicidal agents can irreversibly kill spores within one hour. The killing effect can be exerted by any one of several factors, including damage to membranes, receptor or structural proteins, or DNA. While lethal damage could affect early germination processes such as refractility loss, it is also possible that treated spores might be capable of early germination but not resumption of vegetative growth and cell division. We have explored the use of rapid spectrophotometric assays to investigate the effects of sporicidal agents such as bleach (hypochlorite) and hydrogen peroxide on spore germination processes. The goal of this study has been to establish a correlation or set of correlations between viability assays, which are the standard method of assessing killing efficiency, and a much more rapid absorbancy-based method

Experimental Methodology

Spore suspensions were prepared from dry (lyophilized) *Bacillus subtilis* spore stocks. Approximately 3 mg of dried spores were mixed with 1 ml of sterile distilled water, and the resulting suspension was heat-shocked for 30 minutes at 60 °C. If any particulates settled during this time, the supernatant was withdrawn and the particulates discarded. The suspension was then divided for use in plating and spectrophotometric assays

Spore viability assays were carried out as follows. At the initial time point, t0, equal volumes of spore suspension and the killing agent were mixed vigorously. A serial dilution was then performed by pipetting 100µl of the treated spore suspension into 9.9 ml of sterilized double distilled water. From this initial dilution a dilution series of $1x10^{-2}$, $1x10^{-4}$, $1x10^{-5}$ and $1x10^{-6}$ was then performed for t0. Four subsequent time points were taken, at t15, t30, t45 and t60 with the same serial dilution performed. Each dilution, at each time point, had aliquots 100µl dispensed onto TBAB agar plates and spread using sterilized glass beads. The plates were incubated at $37^{o}C$ and the colonies enumerated.

For spectrophotometric germination assays, the spore suspension was aliquoted into two polypropylene tubes. Sporicide was added to one of these tubes at a 1:1 ratio to the spore suspension. Immediately after the addition of killing agent, treated and untreated samples were added to a pre-warmed germinant solution in separate plastic cuvettes. In all cases the final germinant concentration after the addition of spore sample was 100 mM L-alanine, 1.65 mM L-asparagine, 2.8 mM D-glucose, 2.8 mM D-fructose, 5 mM potassium chloride, and 25 mM TRIS buffer, pH 8.0. Following a one minute lag period (allowing for onset of germination response), the change in optical density was followed for 10 minutes for both the treated and untreated sample using a Beckman DU-7400 spectrophotometer. These measurements were repeated at 15-minute intervals up to 60 minutes.

Results and Discussion

Plating results are provided in terms of logs of spores neutralized by killing agent. The average count (n_t) of all plates at the most statistically significant dilution (that which provided plate counts in the range of 20-300 colonies/plate) was calculated for each time point. The logs of killing obtained by a given time point (t) was calculated as follows:

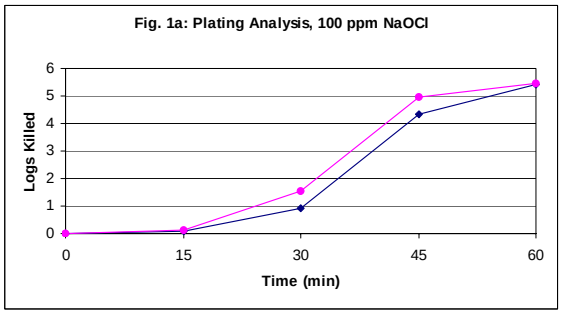
$$\text{logs killed} = \log(n_0 / \max(n_t,1))$$

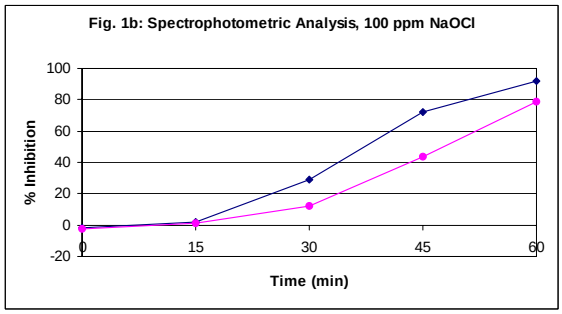
Spectrophotometric results are provided graphically in terms of percent inhibition of optical germination due to treatment with killing agent. The % inhibition was calculated as follows:

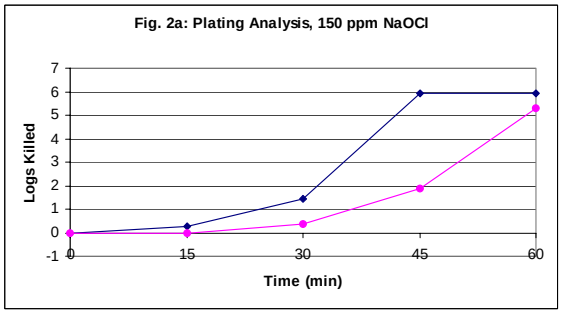
$$\%\ \text{inhibition} = 100*(1-((T_0-T_t)/(C_0-C_t)))$$

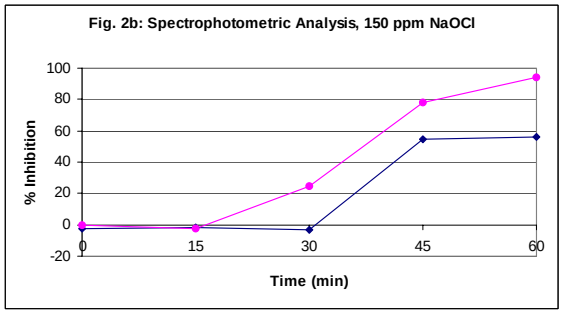
where C_0 and C_t represent the optical density of the control at 0 minutes and at the time point in question respectively, and T_0 and T_t represent the optical density at the same time points for the treated sample.

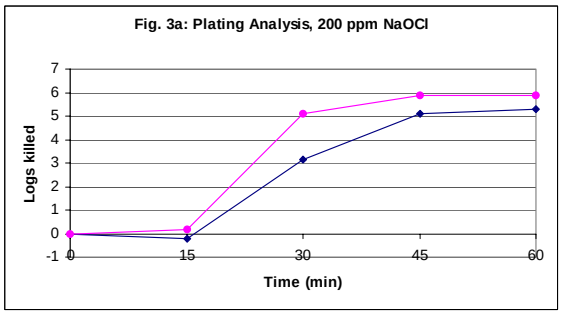
Figures 1-3: NaClO Sterilization Results (◆, trial 1; ●, trial 2).

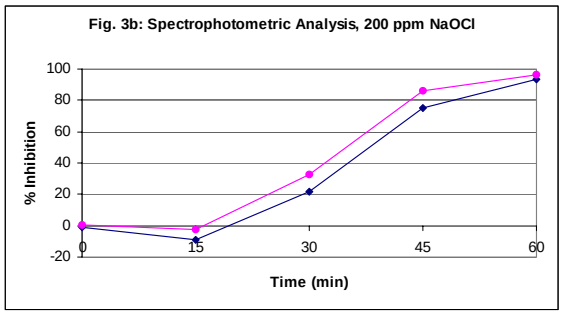






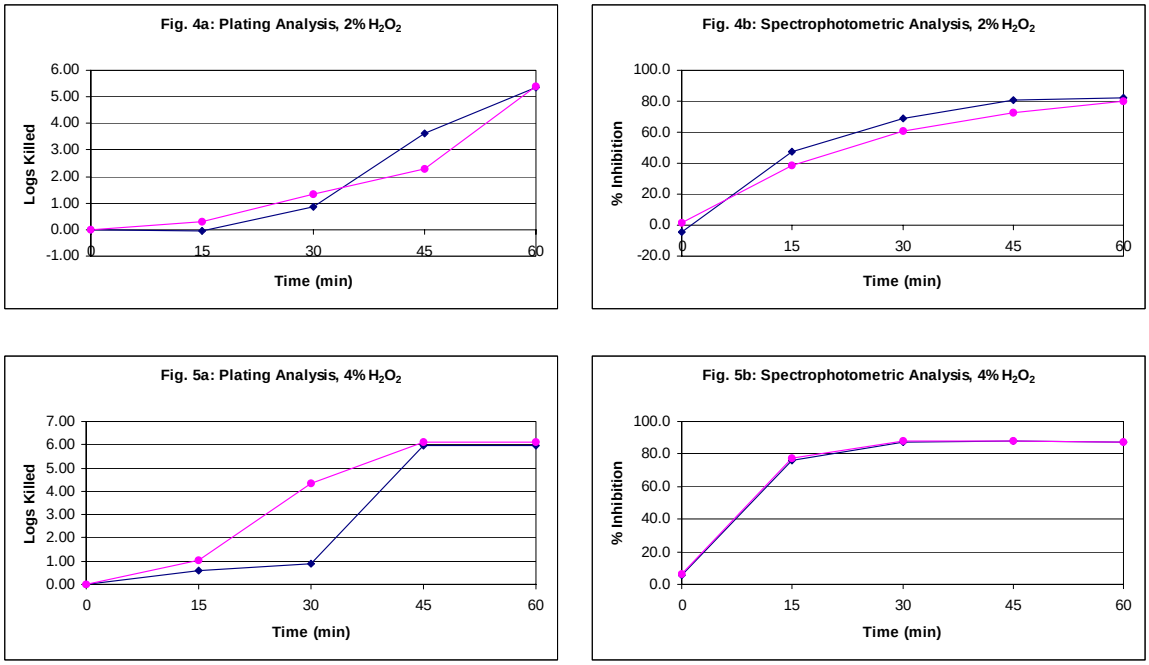






Analysis of sodium hypochlorite results: Levels of killing derived from viability assays for the three concentrations examined here, 100 ppm, 150 ppm and 200 ppm, (figures 1a, 2a, 3a respectively) showed killing of between 5 and 6 logs. In all cases the first treated time point, t15, showed little change in logs killed at all concentrations. Not until the next time point, t30, was there any appreciable level of spore killing. There is thus evidence for a lag in the killing effect of hypochlorite, perhaps due to the presence of a general damage threshold necessary to prevent germination. These results are consistent with previous studies demonstrating that sodium hypochlorite spore killing is not exponential.

Figures 4,5: H_2O_2 Sterilization Results (◆, trial 1; ●, trial 2).



Analysis of hydrogen peroxide results: A notable feature of treatment with this sporicide is that, as the contact time increased, the individual colony size produced by exposed spores notably decreased. This points to a damaging effect by this agent either on the primary germination response pathways, leading to drastically reduced germination kinetics, or on genetic information necessary for resumption of optimal vegetative growth.

The results for 2% and 4% hydrogen peroxide treatment (figures 4a and 5a respectively) showed some killing at t_{15}, more evident than with the hypochlorite results, particularly at the 4% concentration. However it is at the next time point, t_{30}, that the killing effect became particularly evident. The 2% treated spores showed killing of approximately one log for both trials. The 4% treated spores showed a wider range of killing, one and four logs for trial #1 and trial #2 respectively. However at the next time point, t_{45}, the data for the 4% treated spores showed a similar level of killing in both cases, at approximately six logs. The final time point, t_{60}, showed logs of killing of 5.34 for trial #1 and 5.39 for log #2 with the 2% treatment and 5.99 and 6.11 logs for trials#1 and #2 with the 4% treatment. It should be noted that, as would be expected, the rate of increase in killing, especially in the t_{30} to t_{45} time period, was much greater with the higher concentration.

Analysis of factors unique to the spectrophotometric assay: The spectrophotometric results represent the change in optical germination response over time during exposure to a killing agent and display different characteristic patterns for each killing agent. Nonetheless, these patterns seem to be consistent when considering killing agents separately. Peroxide seems to inhibit germination very rapidly following initial exposure, with inhibition asymptotically approaching a maximum level (usually between 75% and 98% inhibition). The pattern for bleach is similar for later time points, but up to the 15-minute time point there seems to be a lag in inhibition. It is possible that this corresponds to the time required to accumulate a certain level of randomly distributed damage (a normal distribution, which would translate into a sigmoid curve over time similar to the one observed). However, a treated sample that displayed the same germination kinetics as the control at time zero often displayed a negative inhibition (or, in other words, better germination kinetics than the control) after 15 minutes of exposure to bleach. This would correspond with the hypothesis that exposure to bleach disrupts the spore coat mildly at first, thereby in fact facilitating the initial stages of germination. This process might even be analogous to heat shock; a process designed to artificially “age” recently generated spores to improve germination kinetics. If this were the case it would account for the difference in curve form between bleach and peroxide. Low levels of peroxide seemed to have a similar effect, although only for time zero. These studies suggest that optical interrogation of spore germination could form the basis for a near-real time assay of spore killing effects that has the potential to revolutionize the analysis of sporicidal processes, kinetics and mechanisms. This type of assay could provide a platform technology for high-throughput screening of chemical libraries for new chemical entities that interfere with spore germination processes.

Rapid Fluorescent Assays for Sporicidial Bioeffects

Fluorescent stains have proven useful for the rapid assessment of bacterial viability. Commercially available "live/dead" stains, used in combination with fluorescent microscopy, are frequently employed in evaluating the membrane integrity of vegetative bacterial cells. This study attempts to extend the use of the popular BacLight™ stain kit to the analysis of bacterial spore integrity. Results obtained by treating dormant spores with various killing agents indicate that differential fluorescent staining can reveal to a significant extent the integrity of the various barriers between the spore core and the environment.

Live/dead staining relies on the application of two fluorescent nucleic acid stains to a population of vegetative cells. The first stain (green), which can pass through membranes in the presence of DMSO, enters all cells in the population unimpeded, thereby labeling the population and making every cell visible through the fluorescent microscope. The second stain (red) is incapable of passing through intact membranes and stains only cells with damaged membrane integrity. Stained cells then emit light at an average wavelength between the emission ranges of the two stains. In this assay live bacteria stain green and dead bacteria strain red. In a population of vegetative cells, membrane damage that allows both stains to enter the cell generally indicates a lack of viability.

These studies attempt to apply the same principles, but to different ends. Rather than inferring viability, the goal was to determine whether dead spores had been affected by disruption of permeability barriers or by other means. By applying the same stains to a population of bacterial spores it should be possible to tell whether the inner core (the sole location of DNA within the spore) is protected from or exposed to the killing environment. Normally the spore core should be protected, but exposure to certain chemical agents is believed to disrupt the spore's natural permeability barriers. The use of differential fluorescent staining is a promising technique to characterize sporicidal agents based on their effects on spore integrity.

Experimental Methodology

Dry, non-heat-shocked spore stocks of *Bacillus cereus* 6A1 were exposed to the following sterilizing treatments:

- 30 minutes exposure to 1500 ppm NaOCl
- 30 minutes exposure to 15% H2O2
- 1 hour exposure to 2000 ppm formaldehyde
- 30 minutes exposure to sterilizing pressure/temperature (in a standard laboratory autoclave)

Spores were exposed to sporicides by mixing a liquid killing agent with a homogenized aqueous spore suspension, with the exception of autoclaving, which was applied to a dry spore sample placed in a screw-top flask. A control sample was also prepared consisting of spores exposed to sterile distilled water for 1 hour. Finally, ClO2 sterilization was accomplished by placing either several cotton spore strips containing approximately 10^8 *Bacillus subtilis* variant niger spores or glass cover slips coated with dry *Bacillus cereus* 6A1 spores in a gas-tight chamber along with the chemical reagents necessary to produce and maintain 1000 ppm gas-phase ClO2 concentrations for the duration of the 2 hour exposure time.

Spores were then recovered from each sample. Recovery from spore strips was accomplished by shearing the strips in a coffee grinder and then placing the fragments in sterile distilled water and vortexing. The aqueous samples were exposed to propidium iodide and Syto® 9 for 20 minutes at concentrations of 40 μM and 6.68 μM, respectively. The stains were then washed away through successive cycles of centrifugation and resuspension in sterile distilled water. The final resuspensions were passed through 0.2-micron black Poretics™ polycarbonate filters (Osmonics®), which were mounted on charged slides using Polymount™ mounting medium. Glass coverslips were applied and sealed onto the slide using commercially available nail polish.

Samples were visualized using a Zeiss® Axiophot™ fluorescent microscope equipped with a fluorescence filter set. A video link and image capture software were used to record computer image files.

Results and Discussion

Video capture results for each sterilization technique described above are shown in the following Figures. The selected images provided accurately characterize of all the images obtained throughour these experiments, and image results did not vary significantly within any given sterilization regime.

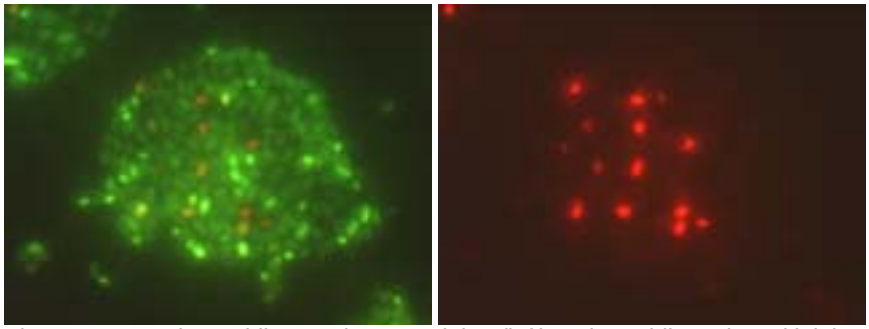


Figure 1: Control; propidium and Syto staining (left) and propidium alone (right).

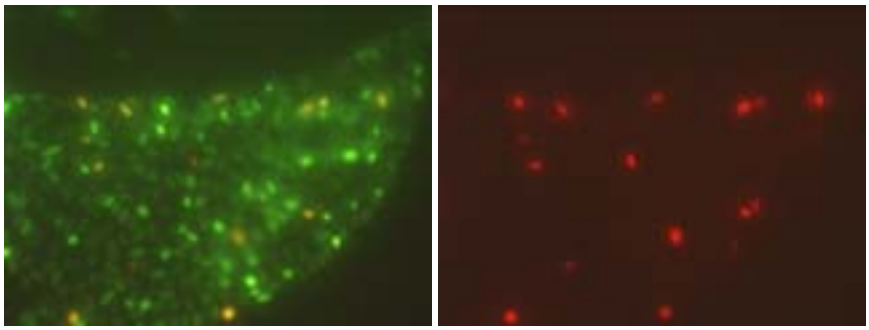


Figure 2: Peroxide; propidium and Syto staining (left) and propidium alone (right).

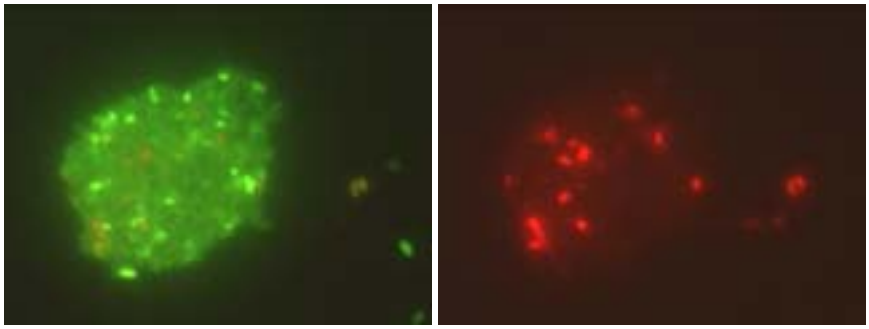


Figure 3: Formaldehyde; propidium and Syto staining (left) and propidium alone (right).

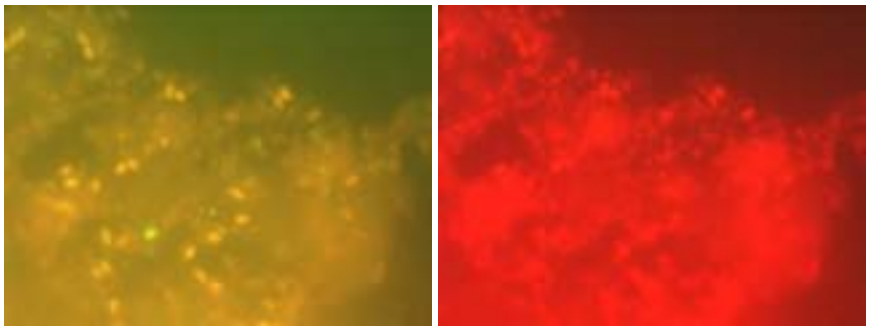


Figure 4: Bleach; propidium and Syto staining (left) and propidium alone (right).

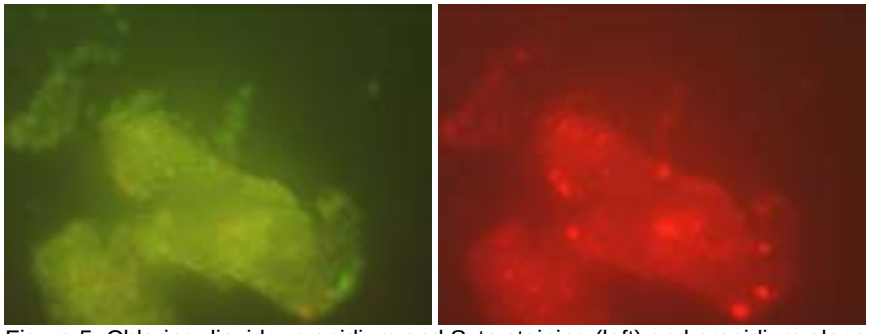


Figure 5: Chlorine dioxide; propidium and Syto staining (left) and propidium alone (right).

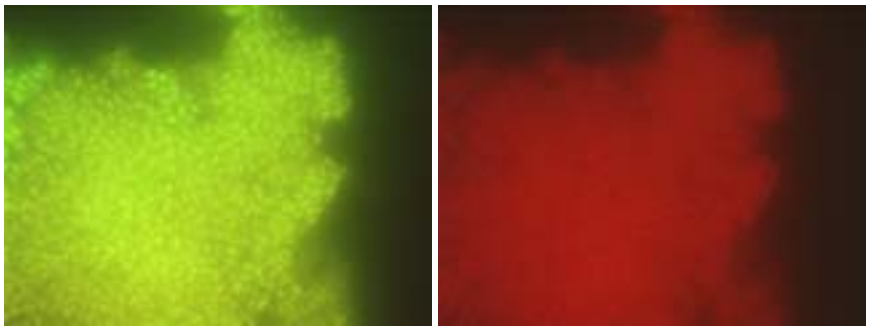


Figure 6: Autoclave, propidium and Syto staining (left) and propidium alone (right).

Peroxide (Fig. 2) and formaldehyde treatment (Fig. 3) produce staining results that appear identical to the control (Fig. 1), with spores emitting faint to strong green ("live") fluorescence, indicating DNA staining with Syto but not with propidium. Spores treated with NaOCl (Fig. 4), ClO2 (Fig.5), and autoclaving (Fig. 6) all stain with both Syto and propidium, giving them a yellow to orange appearance when viewed with a long pass filter. These treatments also seem to encourage the formation of spore "clumps" (perhaps resulting from the accumulation of debris from damaged spores or alterations in the spore coat surface), which complicates visualization.

The images obtained for sterilizing treatments of hydrogen peroxide and formaldehyde make a convincing case for the hypothesis that these agents are able to kill bacterial spores without causing significant disruption to spore permeability barriers. Spores treated with these agents displayed staining patterns identical to those of untreated spores. This would tend to favor explanations for the killing effect based on damage to germinant receptors or other spore integument targets rather than damage to the spore core membrane.

Conversely, the images pertaining to ClO_2, NaOCl, and autoclave treatments would seem to indicate that these sterilization techniques rely on (or at least result in) damage to spore permeability barriers. However, while the results for formaldehyde and peroxide treatments seem to rule out the possibility that damage to permeability barriers was involved in their sterilizing effects, the results for the other agents were not sufficient to prove that such damage is the cause of sterilization rather than a side effect. It should be possible to further investigate the level of association between these observations and the killing effect by repeating experiments at the threshold of sterilization, perhaps at a 50% effective treatment level. If such a treatment failed to result in any propidium staining, it would seem likely that permeability barrier disruption was a secondary effect observed only following strong treatments above the threshold necessary to achieve a minimal level of killing through other means.

Another issue that bears further consideration is the form in which spores are exposed to the treatments. While liquid spore suspensions were used in most cases, autoclaved and ClO_2-treated spores were exposed in dry form, either adhering to a cotton fiber matrix (ClO_2) or as a powder on a glass surface (ClO_2 and autoclaving). In both cases, uneven distribution/exposure could introduce artifacts into the analysis. Furthermore, if spores damaged by sterilization had a different level of adherence to the cotton strips than undamaged spores, they would be either over- or underrepresented in slides prepared from spores that were successfully removed by soaking. While the cover slip method circumvents these problems, the distribution of spores on the slip was visibly uneven, and the spores were not truly monodispersed. In future trials spores ocujld be exposed to sterilizing heat/pressure in an aqueous suspension for comparison. Aqueous exposure may also be possible with chlorine dioxide Repeated four- to six-hour exposures of spore suspensions to 50 - 500 ppm ClO_2 would be expected to produce observable killing effects, which could then be correlated to fluorescent staining results.

These results suggest that live/dead staining provides a very useful optical/chemical system to probe the effects of sporicides on spore structure. Live/dead staining provides a rapid optical method to confirm the sporicidal effects of ClO_2, NaOCl, and autoclave treatments. This capability may be of particular value in the case of bleach, which is the most widely used DoD sporicide. As documented by other workers and confirmed here, bleach sporicidal effects are exerted after an unpredictable lag period. The availability of a rapid live/dead fluorescent assay to confirm bleach efficacy would be of considerable value in validating the performance of hypochlorite sterilization procedures.

Rapid Microscopic Assays for Spore Shape Changes

It has not been technically feasible to make direct, nondestructive, high-resolution measurements of dimensional changes in individual spores as a function of humidity. We report such measurements, show that the size of individual spores is an increasing function of humidity, and show that there appear to be two distinct time constants in the swelling curve. These results alter the traditional view that, when not germinating, spores are entirely static. The implications of our results for the location of the water in the spore and for inactivation of spores by gases such as chlorine dioxide, formaldehyde, and ethylene oxide will also be discussed.

Experimental Methodology

The scanning microscopic system consisted of a Leitz microscope, a computer-controlled stage, a CCD camera, and a Linux workstation with an image acquisition card. The system can rapidly scan a surface, such as a microscope slide, automatically measuring and recording the size of every object on the surface. In a typical application, the image of each object is fitted to an ellipse, so five parameters (x and y position, semiminor and semimajor axis, and orientation) are recorded for each detected object. Each measurement is derived from many pixels, so the absolute precision of a single measurement is better than 50 nm. Furthermore, by measuring the same object multiple times, the precision can be improved dramatically. By averaging over 100 measurements, we have achieved a precision in size measurement of better than 5nm.

Results and Discussion

We determined the time history of the measurement of the semimajor axis of 80 spores and three small glass calibration spheres. The semiminor axis data are similar. For these runs, the slide was first exposed to dry air (measured RH = 3.0%). We then switched to a humid line (RH = 95%, noncondensing).The humidity as measured in a small chamber just upstream of the sample chamber increased to >70% RH within 30 s of the transition and was above 80% within 1 min. Subsequently the flow was switched back to dry air. We determined the amplitude of swelling for the particles by comparing the average semimajor or semiminor axes in the 10 measurements before changing humidity to those between 10 and 20 measurements afterward. We found the semimajor swelling amplitude to be 51 - 54 nm. For the semiminor axes, the amplitudes were 37.7 - 41.4 nm. Calibration particles exhibited very small but significant changes in size.

To investigate the time constant for swelling, we used an ensemble-average of the 80 spores that showed the lowest noise. The time scale for the rapid swelling observed just after the RH is increased is not well resolved even in a high size-resolution dataset. The swelling values are approximately equal to the time constant for the observed change in humidity of the apparatus. We conclude that we have not resolved the intrinsic time scale for spore swelling even in these high-resolution data, and can only place an upper limit on this time scale at ~50 s.

This rapid swelling component does not, however, appear to be adequate to describe the data for longer time scale responses. We found that an additional increase in size, of the same form as the first but with a smaller amplitude and longer time scale, fit the data well. Independent maximum likelihood fits to the data gave amplitudes for this increase of 13 nm (semimajor axis) and 11 nm (semiminor axis), and a time constant of ~8 min for both semimajor and semiminor axes.

There are several implications of our results:

- First and foremost, the view that dormant spores are entirely static while waiting for nutrient germination cues must be reconsidered. The dynamic response of the dormant spore to a change in one environmental parameter (humidity) may play a role in preparing the spore for a change in another parameter (nutrient cueing of germination). Further study of spore swelling may increase our understanding of the mechanism of spore dormancy and emergence from the dormant state.
- At a more fundamental level, it may be possible in future work to dissect the functions of various core and cortex proteins in dehydration and rehydration during germination. One could, for example, examine whether mutants of *B. subtilis* in which cortex cross-linking is altered have swelling that is altered.
- Along these same lines, it may be possible to use our automated microscopic technique to search for distinct phases of swelling during spore germination by comparing the swelling of wild type spores and cortex-related mutants.
- The fast and slow time constants of for spore swelling may represent, respectively, time for diffusion of water into the spore coats and cortex and time for diffusion of water into the spore core.
- Various authors have studied the effect of RH on inactivation of bacterial spores by gas-phase ethylene oxide, formaldehyde, and chlorine dioxide. All of them reported that spore killing efficacy increased with RH, approaching 100% for RH greater than ~70%, but opinions differed as to the role of humidity. Our discovery of spore swelling in response to a large increase in RH provides a natural explanation for these observations. In geometric terms, swelling of a spore increases the diameter of channels through which inactivating gases can pass. A small increase in pore size could significantly increase the equilibrium concentration of the gases inside the spore.

(5) “Technology transfer” (any specific interactions or developments which would constitute technology transfer of the research results). Examples include patents, initiation of a start-up company based on research results, interactions with industry/Army R&D Laboratories or transfer of information which might impact the development of products.

This project provides a mechanistic understanding of sporicidal mode of action and high-throughput screening technologies required to develop the next-generation of spore decon agents. These are crucial capabilities for advancing DoD asset and personal protection research. These data will allow the development of spore decon technologies with improved performance, safety and efficacy.

Professor Leighton is an advisor and program reviewer for DARPA, MRMC and DDRE in the areas of medical countermeasures and decon. Technologies developed from the proposed research will be brought to the attention of these agencies for possible inclusion in their development and acquisition plans.

c. “Copies of technical reports,” which have not been previously submitted to the ARO, must be forwarded with the Interim Progress Report.

None

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