



The Validation of Vapor Phase Hydrogen Peroxide Microbial Reduction for Planetary Protection and a Proposed Vacuum Process Specification

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ABSTRACT

The Jet Propulsion Laboratory, in conjunction with the NASA Planetary Protection Officer, has selected the vapor phase hydrogen peroxide sterilization process for continued development as a NASA approved sterilization technique for spacecraft subsystems and systems. The goal is to include this technique, with an appropriate specification, in NPR 8020.12C as a low temperature complementary technique to the dry heat sterilization process.

To meet microbial reduction requirements for all Mars in-situ life detection and sample return missions, various planetary spacecraft subsystems will have to be exposed to a qualified sterilization process. This process could be the elevated temperature dry heat sterilization process (~115°C for 40 hours) which was used to sterilize the Viking lander spacecraft. However, with utilization of such elements as highly sophisticated electronics and sensors in modern spacecraft, this process presents significant materials challenges and is thus an undesirable bioburden reduction method to design engineers. The objective of this work is to introduce vapor hydrogen peroxide (VHP) as an alternative to dry heat microbial reduction to meet planetary protection requirements.

The VHP process is widely used by the medical industry to sterilize surgical instruments and biomedical devices, but high doses of VHP may degrade the performance of flight hardware, or compromise material properties. Our goal for this study was to determine the **minimum** VHP process conditions to achieve microbial reduction levels acceptable for planetary protection.

In order to evaluate the effectiveness of VHP for the inactivation of the standard spore challenge organism, *Geobacillus stearothermophilus*, the STERIS Corporation, under contract to the Jet Propulsion Laboratory (JPL), conducted several series of experiments. The experiments were conducted to determine VHP process parameters that provided significant reductions in spore viability while allowing survival of sufficient spores for statistically significant enumeration. In addition to the obvious process parameters – hydrogen peroxide concentration, number of pulses, and exposure duration – the investigation also considered the possible effect of environmental parameters. Temperature, relative humidity, and material substrate effects on lethality were also studied. Finally, a comparison of assays performed by STERIS and JPL of spore-inoculated coupons exposed to VHP under the same test conditions was included.

Biological indicators were inoculated with more than 1 million *Geobacillus stearothermophilus* (ATCC 7953) spores on stainless steel coupons and packaged in Tyvek/Mylar pouches. For the tests on the effect of material substrates, the same inoculation procedure was employed on the selected material substrates. All exposures were conducted in a STERIS VHP MD2000 Series Sterilization System. The process involves a conditioning phase, injection of liquid hydrogen peroxide, a sterilization phase (in vacuum), and an aeration phase with high-efficiency particulate air filter (HEPA)-filtered air.

The derivation of D-values from the statistically significant (i.e., non-zero) lethality data permitted conservative recommendations for a planetary protection specification.

The outcome of this study provided an optimization of test sterilizer process conditions: VHP concentration, process duration, a process temperature range for which the worst case D-value may be imposed, a process humidity range for which the worst case D-value may be imposed, and robustness to selected spacecraft material substrates.

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BACKGROUND

For interplanetary missions landing on a planet of potential biological interest, United States NASA planetary protection currently requires that the flight system must be assembled, tested, and ultimately launched with the intent of minimizing the bioload taken to and deposited on the planet (Ref. 1 and 2). Future mission requirements will be modified (possibly will be more rigorous) as each successful mission enhances the knowledge base of the planet. To demonstrate compliance with current requirements, there are specific requirements as to the maximum bioload of spore forming organisms allowable upon launch from Earth. There are even more restrictive requirements for surface missions that land in designated locales such as the “special regions” of Mars, or have a goal of either in-situ life detection or an extraterrestrial sample return to Earth.

Historically, compliance has been achieved by sterilizing the flight hardware. In the history of the United States space program, only dry heat (at nominally 125°C) has been approved as a sterilization technique (Ref. 3). This has been and continues to be a valuable and practical technique for many types of hardware. However, recent performance advances in electronics and other thermally sensitive parts make the use of high temperatures unsuitable for some hardware. Another technique utilizing lower temperatures is needed to augment dry heat and provide a viable alternative. Several techniques including ethylene oxide gas, ultraviolet radiation, paraformaldehyde, and chlorine dioxide have been considered (Ref. 4 and 5). These techniques have technical problems such as corrosion of spacecraft materials and issues such as toxicity and carcinogenicity, or organic residue on spacecraft surfaces. An alternative technique, vapor phase hydrogen peroxide (VHP) was selected as a process holding great promise for this specific microbial reduction application.

The utility of VHP as a sterilant has been previously established. Hydrogen peroxide has been very successfully used in the medical industry with no discernable impact on either the component materials or device performance (Ref.6). Advanced Sterilization Products, STERIS Corporation, and Bioquell (and possibly other commercial firms) provide sterilization systems and/or services. However, in the typical medical application, a sufficient (possibly excessive) process to confer sterility and not the minimum (necessary) process, is used. For spaceflight hardware, with potential material and system compatibility issues,¹ knowledge of the minimum adequate process is vital. Further, the sufficient process is usually established by lot sterility testing, that is, by the direct sterility testing of a statistical sample of an item lot. This is often a destructive test not suitable for spaceflight hardware. Also, only one or two spacecraft are typically fabricated, so a statistical sample is not available.

Therefore, this investigation sought a statistically valid parametric characterization of the sterilization process. In order to quantify the lethality, values of the process parameters that would provide significant reductions in spore viability while allowing survival of sufficient spores for statistically significant enumeration were determined. The possible effects of environmental parameters on lethality—i.e., temperature, relative humidity, and

¹ One significant concern has been condensation of water on flight hardware. The condensation of water and hydrogen peroxide can be easily avoided by maintaining proper process conditions (temperature, pressure, and concentration).

material substrate—were also studied. The objective was to permit conservative planetary protection recommendations for a new *in vacuo* process specification to be submitted to the NASA Planetary Protection Officer (PPO) for approval.

In prior years, VHP compatibility with spacecraft materials has been studied at the Jet Propulsion Laboratory (e.g., Ref. 7). Unpublished reports from prior years of the current research activity with STERIS Corporation have provided guidance for the selection of appropriate values of the process parameters. In addition, this overlap provided evidence that the results are repeatable.

EXPERIMENTAL DESIGN

In addition to the obvious process parameters of interest – hydrogen peroxide concentration, number of pulses, and exposure duration – the investigation also considered the possible effect of environmental parameters. Temperature, relative humidity, and material substrate effects on lethality were also studied. Finally a comparison between assays conducted by STERIS and JPL of spore-inoculated coupons exposed to vapor hydrogen peroxide under the same test conditions was performed.

This study consisted of exposing biological indicators, *Geobacillus stearothermophilus* spores dried onto stainless steel coupons, to vaporized hydrogen peroxide sterilant. Five common spacecraft material substrates were also tested. These coupons were inoculated with the same crop of spore suspension as the biological indicators. The coupons, housed in Tyvek/Mylar envelopes, were exposed to VHP in the STERIS MD 2000 sterilizer.

Equipment

The sterilizer used for this study is shown in Figure 1. The STERIS MD 2000 chamber provides vacuum-assisted VHP sterilization. The temperature in the chamber is controlled by a water jacket, and the pressure and duration of the vacuum cycle are programmable. The chamber volume is 784 liters. Evacuation of the air in the chamber enhances diffusion and penetration of the VHP vapor into the item to be sterilized and its packaging within the chamber. The cycle used in this study is as follows:

A constant temperature (settable at a value in the range of 25°C to 45°C) is held by the water jacket. The inoculated coupons are brought to room temperature before testing is initiated. The sterilization cycle passes through the following phases:

A. Leak Test

- 1) The vaporizer and jacket are heated to working temperatures.
- 2) The peroxide reservoir is filled from the cartridge to the weight of peroxide required to complete the programmed cycle.
- 3) The seals on the closed door are filled with compressed air.
- 4) The chamber is evacuated to the required vacuum level.
- 5) The vacuum is held for a stabilizing period and any pressure rise monitored as a leak test.



Figure 1. STERIS MD2000 chamber.

- B. *The Conditioning Phase* consists of a series of pulses with vacuum pulled to a set point of 3 torr (400 N/m^2) and held for the programmed time. HEPA-filtered room air is introduced to replace chamber and load air and to balance the load temperature.
- C. *The Sterilization Phase* consists of one or more pulses (vacuum pulled to 1.0 torr (133 N/m^2); pre-heated VHP drawn into the chamber; held for programmed time of 1 minute). This phase terminates by introduction of filtered room air, followed by a high vacuum.
- D. *Aeration* consists of a series of pulses with the introduction of filtered room air to break the vacuum, followed by re-evacuation. This cycle is repeated for a series of pulses, with a final air break to atmospheric pressure prior to opening of the chamber.

Methods and Materials

Biological Indicator (BI)

Sterile stainless steel coupons (7 x 17 mm) were inoculated with $\geq 1\text{E}6$ *Geobacillus stearothermophilus* ATCC 7953 spores (Lot 1095) at the STERIS Corporation Pinecone facility. The suspension was applied in 10 μL drops and dried on the surface of the coupons and packaged in Tyvek/Mylar envelopes. Figure 2 shows inoculated stainless steel biological indicators without packaging. Figure 3 shows the location of BIs inside the chamber in a typical exposure.



Figure 2. Stainless steel biological indicators shown without Tyvek/Mylar packaging.



Figure 3. Locations of biological indicators inside the chamber in a typical exposure.

Media

The following were used for media preparation:

Tryptic Soy Agar (TSA), lot / expiration date: A050205SK/8-2-05; B050205SK/8-5-05; C050205SK/8-5-05; D050205SK/8-5-05; D042505SK/7-25-05; D051605SK/8-16-05.

Tryptic Soy Broth (TSB), lot/expiration: B060305sk/9-3-05.

Sterile Water (DI H₂O), lot/expiration date: A060905SG/12-9-05

Sterile empty tubes, lot/expiration date: D04250SK/10-25-05; A060905SG/12-9-05.

Sterilant

STERIS' VAPROX 35% hydrogen peroxide, lot/expiration date: PE124D/12-14-05 was used for all the experiments.

VHP monitor

Concentrations of hydrogen peroxide and water vapors were measured with a GUIDED WAVE™ spectrophotometer. This near infrared (IR) sensor has a 0.1mg/L sensitivity for

both VHP as well as water. The peroxide/water probe for the spectrophotometer was modified for vacuum use and mounted within the MD2000 chamber.

Enumeration

Viable spores on unexposed control coupons and on the VHP exposed sample coupons were enumerated by a standard procedure agreed on by STERIS and JPL. Coupons were processed within an hour of exposure to VHP.

Each coupon was removed from the Tyvek/Mylar envelope and placed into a 20 x 150 mm glass test tube containing 10 mL of Tryptic Soy Broth (TSB), which served as a VHP neutralizing medium. This was labeled as the 10^{-1} dilution. These tubes were placed in a Fisher Laboratory Sonication Bath and sonicated for 10 minutes. The coupons were inspected in the tubes to ensure that the dried spores had been visibly removed from each coupon.

After sonication, the TSB was serially diluted 1 in 10 in sterile deionized (DI) water. Exposed coupons were diluted through 10^{-4} . Control coupons were diluted through 10^{-5} . For the exposed coupons, four sets of 2-mL aliquots at each dilution were placed in Petri dishes, and then melted Tryptic Soy Agar (TSA) was added and swirled to mix. For the control coupons, four sets of 2-mL aliquots of the 10^{-4} and 10^{-5} dilutions were placed in Petri dishes, and then melted Tryptic Soy Agar (TSA) was added and swirled to mix. The agar medium was allowed to solidify, and the plates were incubated upside down at 55°C for 48 hours before the colonies were counted.

Experiments

Series 1 – Concentration and Time Effect

The purpose of Series 1 experiments was to determine the VHP concentration, number of pulses, and exposure duration of the biological indicators (BIs) required to provide significant reduction in spore viability while allowing survival of sufficient spores for statistically significant enumeration. Four sets of test conditions were chosen for Series 1 experiments:

- 1) VHP concentration of 0.5 mg/L for one cycle with a one-minute sterilization hold.
- 2) VHP concentration of 0.5 mg/L for two cycles each with a one-minute sterilization hold.
- 3) VHP concentration of 1.1 mg/L for one cycle with a one-minute sterilization hold.
- 4) VHP concentration of 1.1 mg/L for two cycles each with a one-minute sterilization hold.

All Series 1 experiments were performed at a fixed chamber temperature of 35°C . Five replicate runs of five BIs each were exposed under each of the four conditions.

Series 2 – Temperature Effect

The purpose of this series of experiments was to determine the process temperature effect on lethality.

Results from Series 1 experiments at 35°C indicated that a single pulse of nominal one-minute exposure to 1.1 mg/L VHP would provide significant reductions in spore viability while allowing survival of sufficient spores for statistically significant enumeration.

The only test variable was chamber temperature. VHP concentration **C**, exposure duration **t**, and absolute humidity were held constant. During the sterilization phase, the BIs were exposed to a single nominal one-minute pulse at a VHP concentration of 1.1 mg/L with a peroxide concentration of 35 weight%. The nominal hydrogen peroxide concentration and nominal absolute humidity were controlled by the water solution concentration of the injected fluid (1.1 mg/L H₂O₂). Since the injected amount of hydrogen peroxide solution was the same for each run, the absolute humidity was constant for all the runs. The time-integration of the hydrogen peroxide concentration (**Ct**) was calculated after the exposures from the measurements taken by the IR detector. The water vapor concentration (absolute humidity) versus time from the detector was also recorded. Note that despite nomenclature in the task description, relative humidity (RH) was *not* a parameter of these tests because RH varies for the same absolute humidity as the temperature is changed.

Series 2 experiments consisted of exposing $\geq 1E6$ *Geobacillus stearothermophilus* spores, dried onto stainless steel coupons, to VHP. The coupons were placed in Tyvek/Mylar envelopes.

The temperatures were selected to reflect a range appropriate for spacecraft hardware processing, 25°C to 45°C. The lower bound represents room temperature (a highly desirable temperature) and avoids possible condensation of the hydrogen peroxide vapor at the higher concentrations of interest. The upper bound is an arbitrary temperature above which possible undesirable hardware issues (e.g., lifetime degradation due to thermal stress) may arise and where the hydrogen peroxide vapor condensation cannot occur. Higher temperatures would also add complexity to a contemplated spacecraft system-level VHP sterilization procedure. The temperature intervals were selected to limit the run series to five temperatures. The five temperatures were chosen for this series of tests: 25°C; 30°C; 35°C; 40°C; 45°C.

A total of 25 BIs in five sets of five were exposed under each of the five temperatures.

Series 3 – Humidity Effect

The purpose of Series 3 experiments was to determine any humidity effect on lethality of the biological indicators.

Results from Series 1 experiments indicated that a VHP concentration of 1.1 mg/L with one pulse (with a nominal one minute hold time) at a temperature of 35°C would provide sufficient residual viable spores for testing the effect of absolute humidity on lethality.

In this Series *Geobacillus stearothermophilus* spores, dried onto stainless steel coupons, were exposed to VHP at different water vapor concentrations, while holding VHP concentration, exposure duration, and temperature constant.

Eight levels of relative humidity (water vapor concentration at 35°C) ranging from 3% to 50% RH were chosen for this series of tests. Exposure duration, VHP concentration, and temperature were selected based on the results from the Series 1 and 2 experiments.

During each run, a set of five BIs was exposed for a nominal one-minute cycle at a VHP concentration of 1.1 mg/L at 35°C.

A total of 25 BIs in five sets of five were exposed under each of the water vapor concentrations (expressed as relative humidity).

Series 4 – Substrate Effect

The effect of various spacecraft-typical materials (presumably as agents for the decomposition of hydrogen peroxide) exposed to VHP simultaneously with BIs, as a function of their surface area, had been investigated in prior fiscal years. The purpose of the experiments in this series was to determine whether different material substrates on which the *G. stearothermophilus* spores are placed have an impact on VHP sporicidal efficacy. In order to limit the extent of the experimental program, five common spacecraft surfaces were selected, in addition to the standard biological indicator, stainless steel. The material substrates selected for this study were (Figure 4): bare aluminum 6061 (AL), graphite composite (GC), Kapton (K), Aptek 2711 white paint (WP), and Z306 black paint (BP).

The same crop of spore suspension used in Series 1 – 3 was deposited on the five different surfaces, in addition to the stainless steel BIs. There were $\geq 1E6$ *Geobacillus stearothermophilus* spores on each coupon. After drying, the coupons were individually packaged in Tyvek/Mylar envelopes.

The coupons were 13x13mm squares with a small hole in one corner for hanging, if desired. The as-received bare aluminum sheet was 0.81mm thick and coupons were cut to size without further treatment. The graphite/polycyanate composite (M55J/BtCy-1, +45/-45, 20-mil thick) was manufactured using pre-pregs by the Materials Laboratory at JPL. Five-mil thick Kapton sheet was glued down with a film adhesive to a 0.81mm thick aluminum substrate. Both Aptek 2711 and Z306 were coated on aluminum substrate.

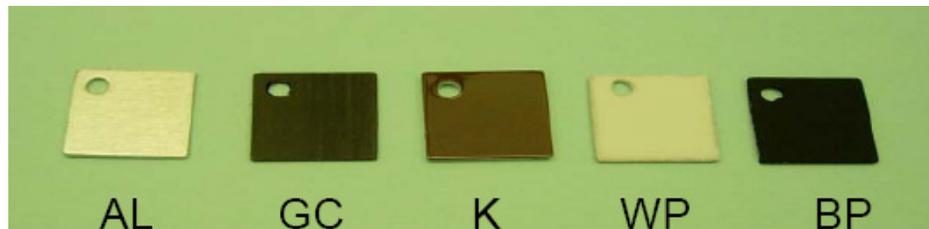


Figure 4. Photo of five common spacecraft material substrates.

All the coupons were subjected to a standard Freon vapor degreasing cleaning. The coupons were then sterilized in an Ultra Violet Products CL-1000 Cross-Linker chamber. The coupons were placed on a 15 cm x 15 cm quartz plate. The plate was suspended at the corners, inside the UV sterilizer. The quartz plate permitted ultraviolet light to pass through and be reflected from the chamber walls to sterilize the bottom side of the coupon. The coupons were UV sterilized for 15 minutes under full power. After UV treatment, the sterilized coupons were aseptically packaged into clean anti-static bags for shipment. The white and black paint coupons were subdivided into individual compartments by heat sealing the anti-static bags. This packaging scheme protected the sensitive surfaces during shipment.

Ten microliters of the same crop of spore suspension used for biological indicators in Series 1 – 3 was used to inoculate these sterile substrate coupons. Each coupon had $\geq 1E6$ *Geobacillus stearothermophilus* spores. After drying, the coupons were packaged in Tyvek/Mylar envelopes, with the inoculated surface facing Tyvek (Figure 5).

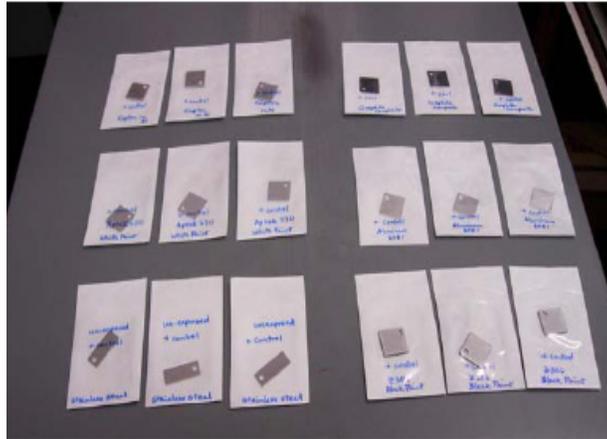


Figure 5. Coupons in Tyvek/Mylar envelopes.

Time and concentration parameters of the cycles were chosen based on the results of Series 1 – 3 experiments. After the determination that 35°C was the least effective temperature over the range studied in run Series 2 and 50% RH at 35°C was the least effective humidity over the range 10 to 50% in run Series 3, we selected 50%RH, 35°C, 1.1 mg/L H₂O₂, nominal 1-min duration as the test parameters for these Series 4 experiments.

During each run, five substrate coupons of one material were exposed to a nominal one-minute cycle at a VHP concentration of 1.1 mg/L at 35°C in 50% relative humidity, along with one stainless steel BI as control. Five replicate runs were performed for each of the five materials.

Series 5 – Validation

Run series 5 consisted of an inter-comparison of assays of biological indicators and inoculated material coupons (*Geobacillus stearothermophilus*) exposed to hydrogen peroxide under selected conditions representative of the prior tests of 2005 conducted solely by STERIS. In these tests, the biological indicators and material coupons had been prepared by Pinecone and were from the same lot employed in all of the 2005 tests. The selected conditions were: stainless steel BIs in Tyvek at 5.2%RH, 35°C, 1.1 mg/L H₂O₂, 1-min duration in MD2000 in vacuum during exposure, the same at 5.2% RH and 25°C, the same at 50% RH and 35°C, and black paint (Z306) coupons in Tyvek at 50%RH, 35°C, 1.1 mg/L H₂O₂, 1-min duration. On the basis of the prior tests, the survival fractions were expected to be in the range 10⁻² and 10⁻³, which would provide statistically useful absolute survivor numbers (for a 10⁶ nominal initial number) for the inter-comparison. Five replicate runs at each condition, with ten samples from each run to be split between STERIS and JPL were planned. Unfortunately, for unknown reasons, there were not enough of the previously prepared BIs to fulfill this plan. Therefore, in the runs with BIs, only in the first two replicate runs at 5.2% RH and 35°C did STERIS assay a

full complement of five exposed BIs. In the other three replicate runs and in all the other conditions with BIs, STERIS received only three exposed BIs. This deviation from the plan necessitated a slightly more complicated form of the statistical analysis. In addition to the comparison of the STERIS data to the JPL data, the results of these exposures by STERIS were statistically compared to prior STERIS tests. The statistical methods used are shown in the Appendix, Statistical Analysis Method Details.

RESULTS

Run Series 1 Concentration and Time Effect

The survival fraction N/N_0 (colony forming units, CFU, of the exposed sample divided by the mean CFU of the unexposed controls) for each of five runs averaged across position for the VHP-exposed coupons are presented in Table 1. The N/N_0 for each of five positions averaged across the runs for the VHP-exposed coupons are presented in Table 2.

The results of run series 1 confirmed that the data from the previous (unpublished) report could be repeated. The exposures in run 1 were selected in the mid-range of the expected survival fractions from the previous tests to obtain statistically significant data, for the runs to follow. Zero survival is not useful because only an upper limit on the survival is obtained, and statistics cannot be performed. Both the concentration C (the ratio of

Table 1. Concentration and time effect on lethality, survival fraction, of *G. stearothermophilus* at 35°C for each replicate run, averaged over positions (no correction for Ct variation between runs).

Run / Run Conditions	A1	B1	C1	D1	E1
0.5 mg/L, 1 pulse x 1 minute	2.76E-02 ±3.88E-02	5.02E-02 ±6.59E-02	1.04E-01 ±1.18E-01	7.88E-03 ±9.45E-03	1.67E-02 ±2.05E-02
0.5 mg/L, 2 pulses x 1 minute	8.54E-06 ±1.11E-05	3.20E-04 ±3.65E-04	5.59E-04 ±9.37E-04	5.32E-05 ±8.36E-05	1.22E-04 ±2.45E-04
1.1 mg/L, 1 pulse x 1 minute	9.59E-05 ±2.17E-04	1.62E-04 ±3.80E-04	9.12E-07 ±1.17E-06	1.69E-06 ±2.74E-06	1.22E-03 ±1.38E-03
1.1 mg/L, 2 pulses x 1 minute	4.92E-07 ±7.15E-07	2.46E-07 ±3.28E-07	3.71E-06 ±4.37E-06	1.07E-06 ±1.45E-06	2.00E-06 ±2.15E-06

Table 2. Concentration and time effect on lethality, survival fraction, of *G. stearothermophilus* at 35°C for each BI and its position, averaged over runs (no correction for Ct variation between runs).

Position / Run Conditions	1	2	3	4	5
0.5 mg/L, 1 pulse x 1 minute	4.04E-02 ±6.24E-02	9.22E-02 ±1.16E-01	4.87E-02 ±5.61E-02	1.35E-02 ±1.94E-02	1.18E-02 ±1.82E-02
0.5 mg/L, 2 pulses x 1 minute	2.01E-04 ±2.83E-04	1.10E-04 ±1.49E-04	5.48E-04 ±9.36E-04	6.36E-05 ±1.48E-04	1.41E-04 ± 3.28E-04
1.1 mg/L, 1 pulse x 1 minute	3.71E-04 ±4.76E-04	1.00E-04 ±2.35E-04	3.19E-04 ±7.61E-04	5.38E-04 ±1.28E-03	1.48E-04 ±3.41E-04
1.1 mg/L, 2 pulses x 1 minute	1.70E-06 ±2.32E-06	5.93E-07 ±7.39E-07	1.87E-06 ±2.61E-06	8.54E-07 ±1.18E-06	2.10E-06 ±3.47E-06

injected mass of hydrogen peroxide to the chamber volume) and the exposure duration t were varied to demonstrate that Ct is the proper exposure parameter. In Figure 6, note that the two N/N_0 data points from 0.5 mg/L for 120 s and 1.1 mg/L for 60 s ($Ct = 60$ (mg/L)s and $Ct = 66$, respectively) agree very well. This approach was based on a model of the lethality of hydrogen peroxide where the inactivation rate is taken to be proportional to the hydrogen peroxide concentration C and first order kinetics are assumed (a typical assumption). Then the survival fraction, N/N_0 is given by

$$\frac{N}{N_0} = 10^{-Ct/D}$$

Here t is the time and D is the D-value (exposure Ct required to reduce the population by a factor of ten).

Also shown in Figure 6 are the data (X_s) from the previous run 11 in the MD2000 conducted at 45°C. Although the same hydrogen peroxide solution concentration, 35% by weight, was used in the previous runs in the MD2000, the previous runs' temperature was 45°C. This temperature change was due to a misunderstanding and due to a desire for a lower temperature process. A small correction based on the temperature dependence of run series 2 has been applied.² The agreement of N/N_0 was fair.³

Note that in all of the graphs of survival fraction, the lower error bars displayed are based on the standard deviation of the logarithm of the mean N/N_0 . This measure was taken to allow Excel to plot "representative" error bars when the standard deviation was larger than the mean (in these semi-log plots). However, for statistical analysis, the standard deviations of the mean N/N_0 were used. They reflect the experimental values of the standard deviations of both N and N_0 .

When the survival fraction was plotted against the actual time-integrated value of Ct , the agreement improved (Figure 7).⁴ The integrated value of Ct was obtained from real-time concentration data from the Guided Wave™ IR detector. The use of the integrated value of Ct is extremely important because it represents the actual exposure, which is typically much larger than the nominal value. The nominal value of Ct neglects the part of the exposure when the concentration is less than the nominal value, especially late in the exposure (e.g., after the concentration plateau is over and the concentration is decreasing). This effect is about a factor of two in the MD2000 runs (both the series reported here and the previous runs in the prior fiscal year. Therefore, for comparison purposes in Figure 7, the values of Ct for the runs in prior years in the MD2000 were doubled to approximate the effect. (No digital IR detector data was available in prior years.) Finally, the previous comparable data (run 11) from the biological indicator evaluator resistometer (BIER) vessel (also obtained at 45°C and also with a small correction to 35°C) are also shown in Figure 7 (triangles). The BIER vessel is a small (28.3 L) experimental chamber specially fabricated by STERIS for the contract with JPL.

² The appearance in Figure 6 of the previous data with and without the correction is barely visible.

³ See later discussion, next figure (Figure 7) and especially next footnote.

⁴ The improvement is relative to the comparison shown in Figure 6. In the final analysis, the D-value for run 2 was 35.5 ± 4.5 (mg/L) s and for the non-zero survivor data points of the previous work (run 11) after all corrections were applied, 38.7 (mg/L) s. The separate results are statistically the same.

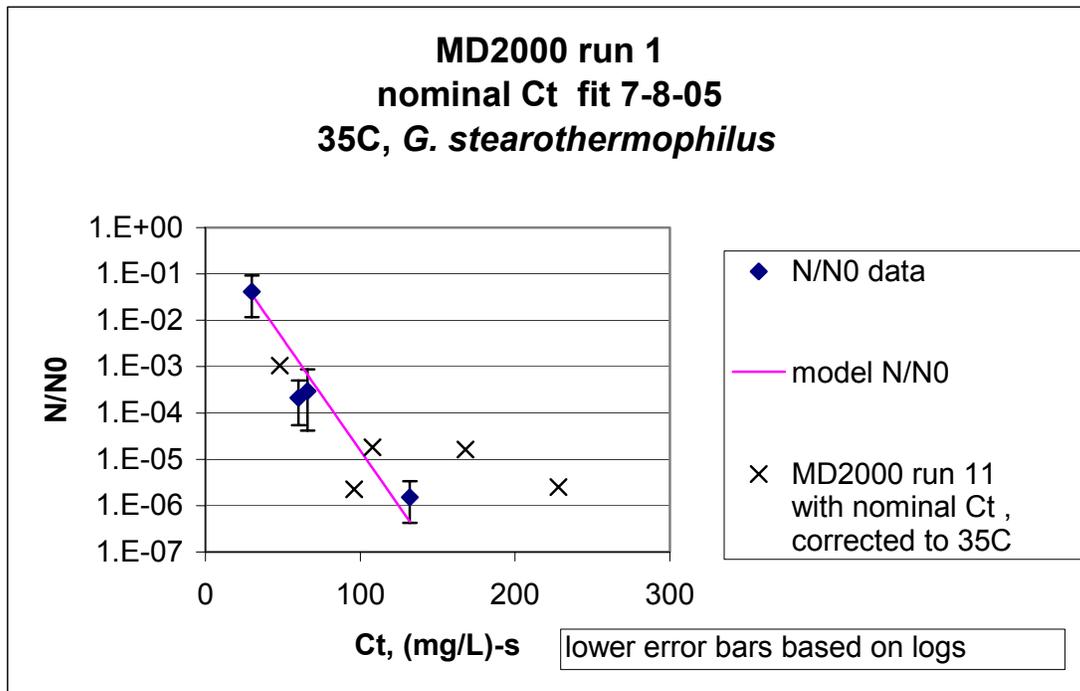


Figure 6. Comparison of survival fractions versus nominal Ct between previous MD2000 run 11 and those for the 2005 runs. Note previous MD2000 run 11 was conducted at 45°C, corrected to 35°C.

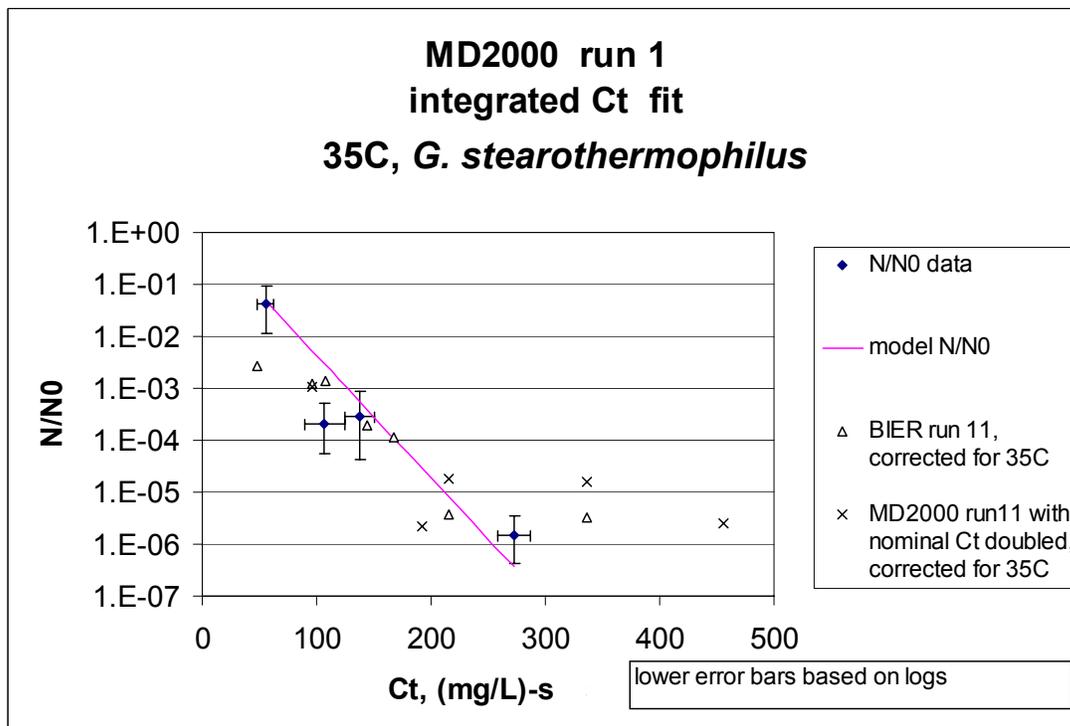


Figure 7. Comparison of survival fractions versus integrated Ct between previous MD2000 run 11 and those for the 2005 runs. Note previous BIER and MD2000 run 11 were conducted at 45°C, corrected to 35°C.

It features very accurate control of the pre-programmed exposure and a hydrogen peroxide (and water vapor) sensor, which calculates and records the time-dependent VHP and water vapor concentrations. The MD2000 is a sterilizer, with no sensor and many interlocks that make short exposures at relatively low VHP concentrations difficult. The previous run 11 results implied that something had changed in going from a small chamber to a larger one.

The survival fractions of the BIER vessel runs are shown in terms of their nominal Ct values, again with a small correction from 45°C to 35°C. The concentration tailing effect was not observed in the BIER runs. Both the rise and fall of VHP concentration were also much more rapid than in the larger MD2000. Note that the integrated Ct values for the MD2000 data are necessary to bring the previous BIER data into agreement with the previous MD2000 data.

Although the hydrogen peroxide concentration versus time in the MD2000 was not a “square wave,” the data from the IR detector were reproducible. This reproducibility was essential for the statistical treatment of the replicate runs at a given condition; the survival fraction variance otherwise could have been dominated by the variance in Ct . A typical data set from the IR detector for the five replicate runs for nominal 1.1 mg/L, 60 second injection is shown in Figure 8.

The D-values were obtained from an Excel unweighted linear regression (including the “origin,” $N/N_0 = 1$ for $Ct=0$) of the logarithm (base 10) of the survival fraction versus Ct data. The D-value is the inverse of the slope obtained by this method.

The integrated Ct exposure leads to a significant correction in the D-value, as the graphs for run series 1 with the nominal and integrated values of Ct show (Figure 9 and Figure 10, respectively). The D-values are 20.8 and 42.4 (mg/L)s, respectively. Obviously the more conservative (larger) D-value, based on the entire exposure, is the conservative value needed to predict the survival fraction or to ensure sterility in the treatment of flight hardware. Of course, if this conservative D-value were used in the treatment of flight hardware, but the nominal Ct were also applied (e.g., no hydrogen peroxide detector was available), the results would be extra conservative.

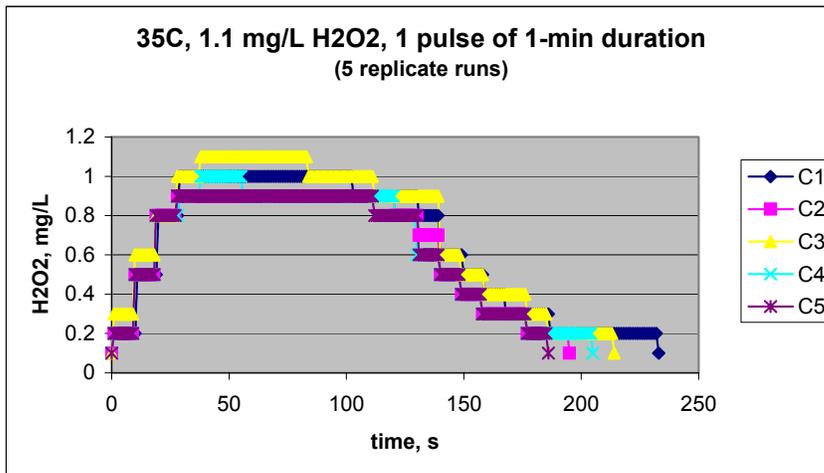


Figure 8. Hydrogen peroxide concentration vs. time for indicated 5 runs.

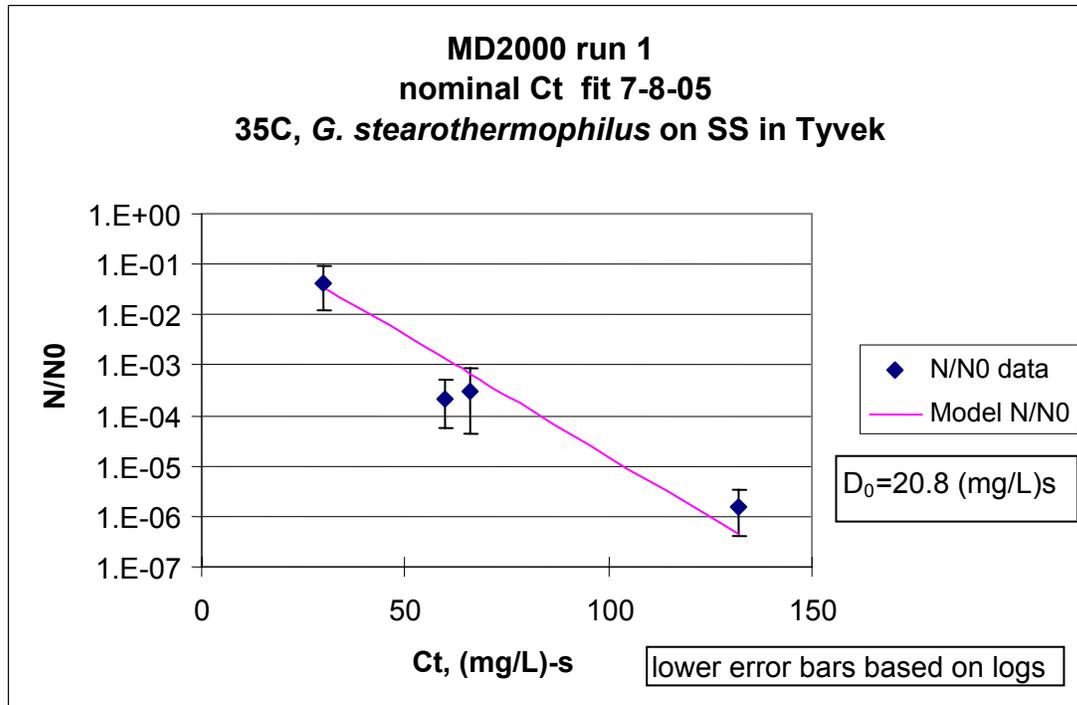


Figure 9. Survival fraction as a function of nominal Ct, with a linear regression fit to $\log(N/N_0)$.

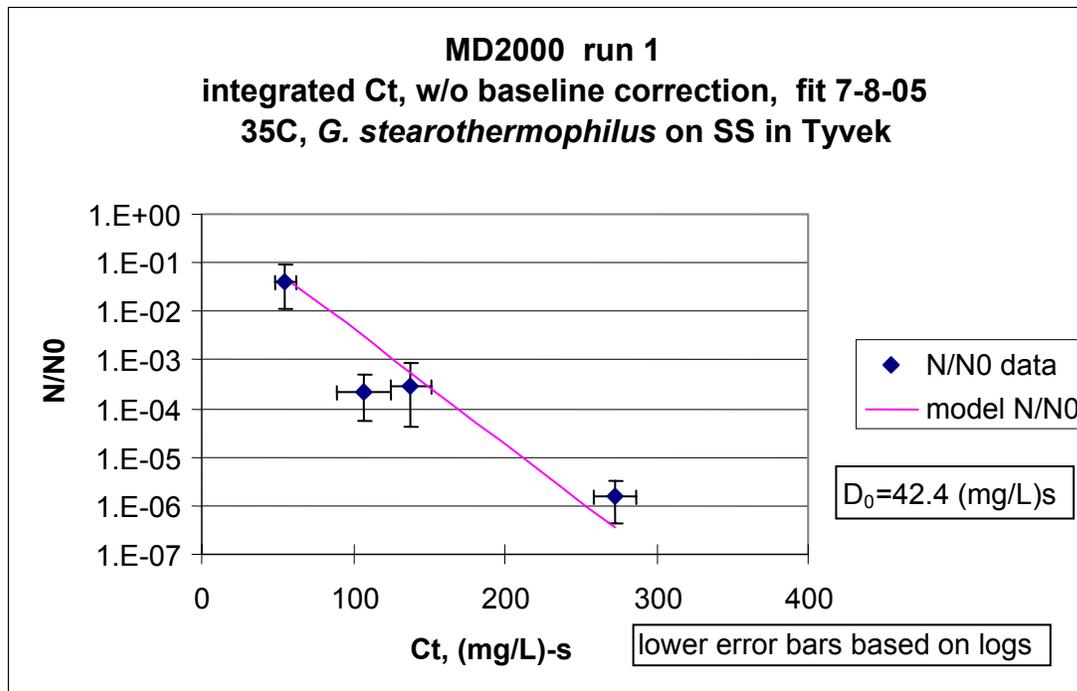


Figure 10. Survival fraction as a function of integrated Ct (uncorrected for baseline offset), with a linear regression fit to $\log(N/N_0)$. Error bars for the integrated Ct values (experimental standard deviation) are also shown.

Finally a baseline correction (for the detector offset output at zero concentration) was applied. The results are displayed in Figure 11. This is a small correction to eliminate the zero concentration offset of the IR detector. The offset is available from the detector data record before the hydrogen peroxide is injected. (See for example Figure 8.) The time integration was manually terminated when the readings returned to that value. Each concentration reading was then manually corrected for this offset value by subtraction. A typical value of the offset was 0.1 mg/L. So for a nominal 1.1 mg/L injection concentration, it is about a 10% correction. This correction renders the D-value slightly less conservative (down from 42.4 (mg/L)s to 35.5 (mg/L)s). This correction ought to be applied to the data from the treatment of flight hardware. However, since the effect of integrated Ct is much larger and in the opposite sense, it would be acceptable to use the conservative D-value in the treatment of flight hardware with the nominal Ct value, without any baseline correction (as would be necessary if no hydrogen peroxide detector were available).

Although all of these runs were conducted at 35°C, two different injection amounts of hydrogen peroxide were employed, 0.5 and 1.1 mg/L H₂O₂ mass per chamber volume (and thus also different water content). Therefore the humidity (and proportionately the RH) were not controlled. Specifically for the 35% (by weight) hydrogen peroxide employed, the two injection amounts correspond to 0.9 and 2.0 mg/L of water. As we shall see, in run series 3 the effect of humidity was investigated. After series 3 was completed, the results of that series were applied to these data. The small correction is shown in Figure 12. The triangles represent the results with the humidity correction and the diamonds the values without the correction.

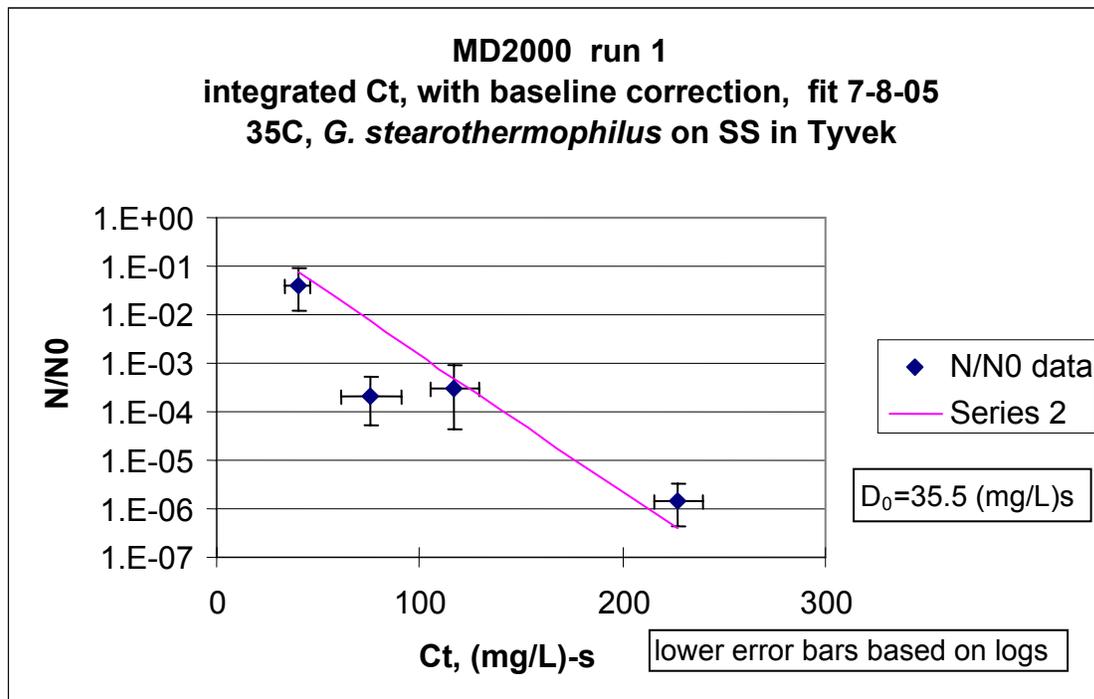


Figure 11. Survival fraction as a function of integrated Ct (corrected for baseline offset), with a linear regression fit to $\log(N/N_0)$. Error bars for the integrated Ct values (experimental standard deviation) are also shown. Same as Figure 10, except baseline correction has been applied.

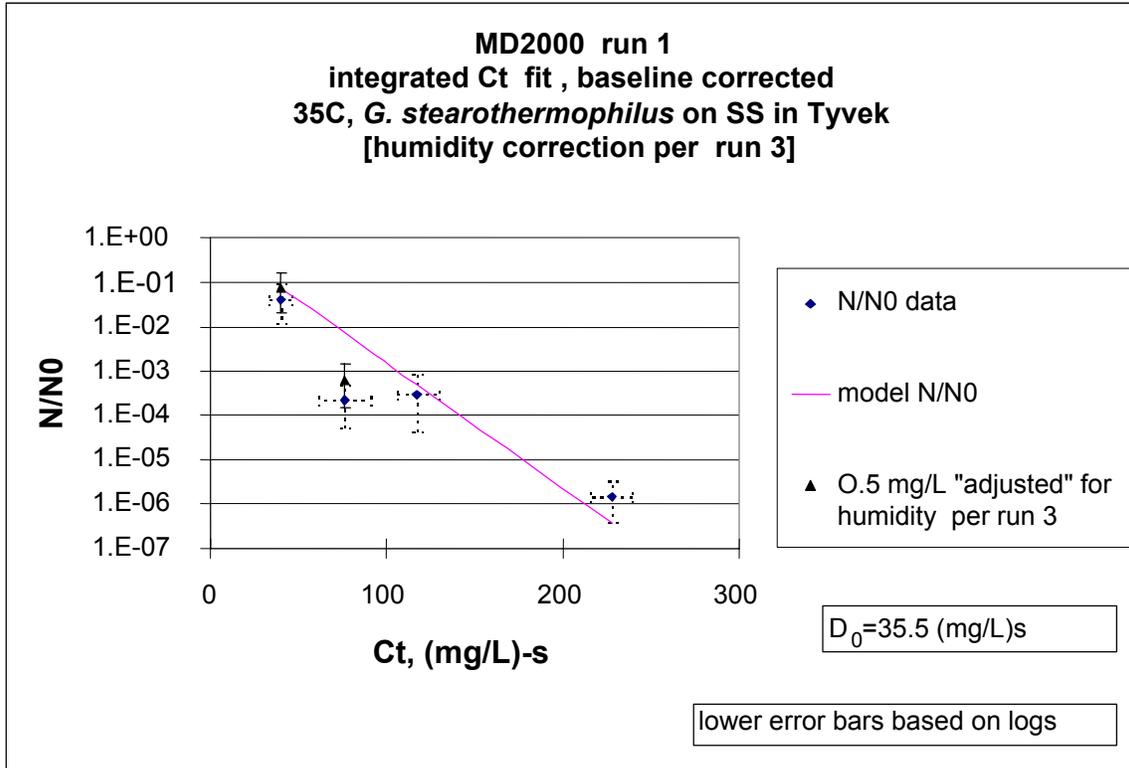


Figure 12. The survival fractions at the two lowest Ct values (from 0.5 mg/L injections) adjusted for humidity dependence per run series 3. The data presented in Figure 11 is repeated to indicate the small size of the effect.

Run Series 2 Temperature Effect

The N/N_0 for each of five runs averaged across position for the VHP-exposed coupons are presented in Table 3. The N/N_0 for each of five positions averaged across the runs for the VHP-exposed coupons are presented in Table 4.

Table 3. Temperature effect on lethality, survival fraction, (at 1.1 mg/L H_2O_2 , 1-min duration and indicated temperature), averaged over positions (no correction for Ct variation between runs).

Run/ Run Condition	A2	B2	C2	D2	E2
25°C	2.05E-05 ±2.28E-05	7.55E-06 ±1.63E-05	9.26E-07 ±2.08E-06	2.45E-06 ±4.90E-06	0.00E+00 ±na
30°C	0.00E+00 ±na	3.32E-04 ±5.31E-04	1.17E-04 ±2.22E-04	5.14E-04 ±1.09E-03	8.14E-05 ±1.02E-04
35°C	3.85E-04 ±3.62E-04	1.23E-04 ±8.37E-05	1.41E-04 ±1.40E-04	1.22E-03 ±2.70E-03	2.69E-05 ±5.30E-05
40°C	3.00E-05 ±1.90E-05	1.62E-05 ±1.69E-05	1.68E-05 ±2.52E-05	7.27E-05 ±1.18E-04	1.21E-05 ±1.59E-05
45°C	4.72E-07 ±7.74E-07	1.67E-06 ±3.75E-06	0.00E+00 ±na	3.73E-06 ±5.83E-06	2.37E-06 ±2.70E-06

Table 4. Temperature effect on lethality, survival fraction, (at 1.1 mg/L H₂O₂, 1-min duration and indicated temperature), averaged over runs (no correction for Ct variation between runs).

Position/ Run Condition	1	2	3	4	5
25°C	1.75E-05 ±2.49E-05	1.28E-06 ±2.05E-06	2.69E-06 ±4.86E-06	2.36E-06 ±4.70E-06	7.64E-06 ±1.66E-05
30°C	5.45E-04 ±1.08E-03	2.28E-05 ±3.96E-05	2.69E-04 ±5.53E-04	1.96E-05 ±2.13E-05	1.87E-04 ±2.16E-04
35°C	1.45E-03 ±2.59E-03	2.10E-04 ±3.09E-04	7.87E-05 ±1.10E-04	1.03E-04 ±2.28E-04	5.56E-05 ±8.18E-05
40°C	5.10E-05 ±2.23E-05	1.31E-05 ±1.94E-05	1.06E-05 ±1.30E-05	8.84E-06 ±1.22E-05	6.43E-05 ±1.18E-04
45°C	3.3-E-06 ±3.37E-06	3.89E-06 ±6.01E-06	9.35E-07 ±1.79E-06	1.20E-07 ±2.71E-07	0.00E+00 ±na

The overall mean values of the survival fractions versus temperature are shown in Figure 13. Here a small temperature effect was observed in the hydrogen peroxide concentration time profile. Therefore the survival fractions were corrected for the different mean Ct at each temperature. The Ct values were obtained and treated as previously discussed. The effective D-values of the mean survival fractions are plotted in Figure 14. The effective D-value for a single set of repeated exposures is defined as

$$D_{\text{eff}} = -Ct / \log (N/N_0)$$

Ordinarily, a true D-value is related to a linear regression of N/N₀ versus Ct (as the data has been modeled). This procedure was used to normalize the data at each temperature (for unequal mean values of Ct) so that the lethality in terms of the temperature dependence of the D-values could be compared. (See Appendix, Statistical Analysis Method Details, for a derivation of the experimental deviation of D_{eff}.)

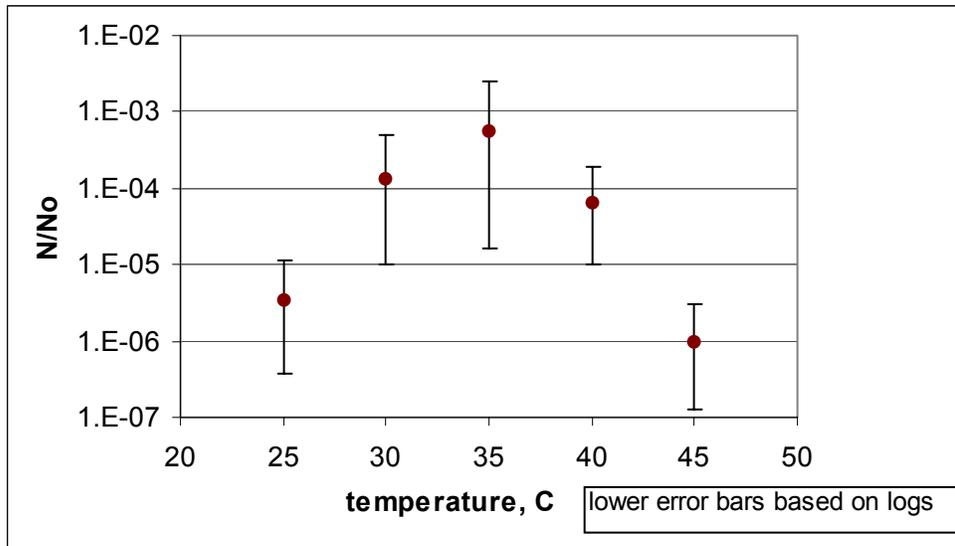


Figure 13. *G. stearotherophilus* survival fraction for fixed 1.1 mg/L 60-s injection of H₂O₂ as a function of temperature, corrected for integrated Ct.

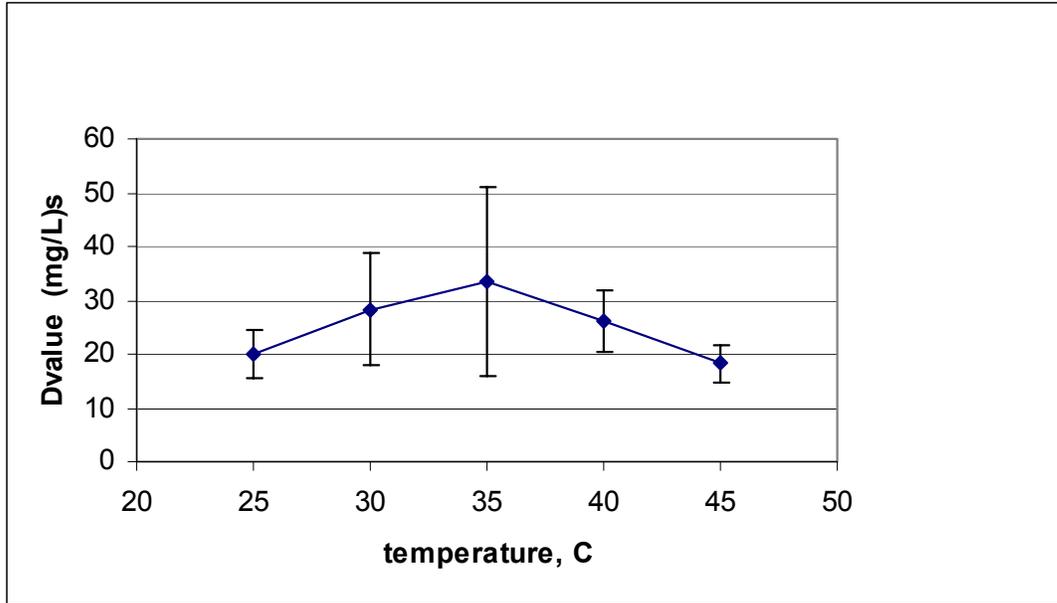


Figure 14. *G. stearothermophilus* D-values for fixed 1.1 mg/L 60-s injection of H₂O₂ as a function of temperature, corrected for integrated Ct.

As mentioned above, it is possible to interpret the data as a RH effect (i.e., with the absolute humidity constant, assume that the only temperature effect is by way of the RH). This interpretation of the data is shown in Figure 15. A separate line of runs would be required to discover and determine other combined temperature and RH dependences. However, this additional work is not necessary for the purposes of this task.

The temperature dependence of the survival fraction was statistically analyzed. An analysis of variance (ANOVA) test (Excel version) was performed on all (25) of the values for N/N_0 in the five replicate runs with the (assumed) five equivalent positions, for each of the five temperatures. The overall mean of N_0 was used. If these values were not corrected for the variation in Ct, the ANOVA indicated that the temperature dependence was real (the effect of at least one temperature was different than the others) with a probability of 0.90.⁵ With an approximate correction for the small variation in Ct, this value was 0.915. The minor change reflected the small variation in Ct (between runs at different temperatures). These statistical tests cannot reveal which specific temperatures contribute to the significant effect observed. For that purpose, a pair-wise t-test was more useful.

In addition, the assumptions that the replicate runs were statistically equivalent and that the positions of the exposed BIs were equivalent were tested. A t-test of the minimum and maximum (for the set of replicate runs) observed mean (averaged over position) N/N_0 and standard deviations, for each temperature, showed no significant difference at 0.90. Also, a t-test of the minimum and maximum (for the set of positions) observed mean (averaged over replicate runs) N/N_0 and standard deviations, for each temperature, showed no significant difference at 0.90.

⁵ The proper statistical statement is that the conclusion that there is a significant temperature effect may be incorrect with a probability of 0.10. Values shown are 1-P.

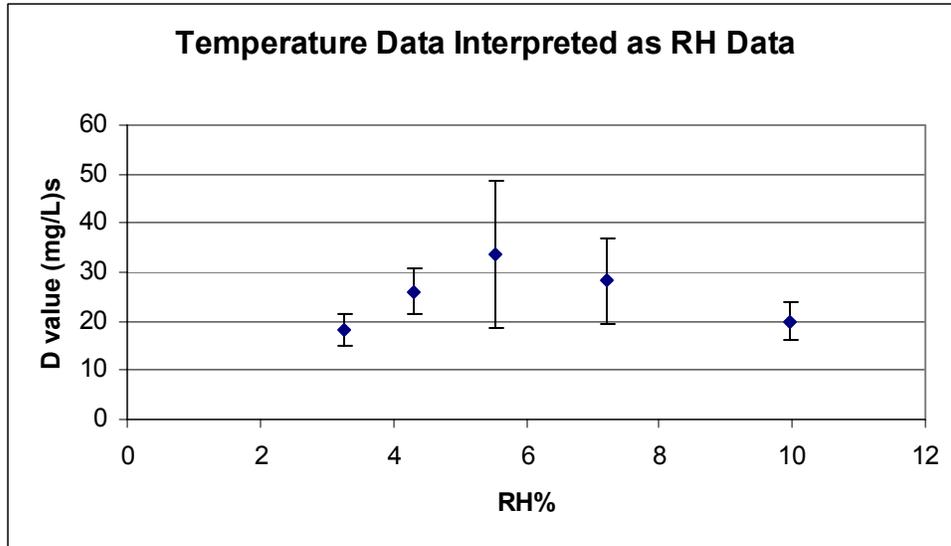


Figure 15. D-values shown in Figure 14, with the temperature at constant humidity interpreted as relative humidity (RH).

Student's *t*-test was used to determine whether pairs of means of N/N_0 at adjacent temperatures were significantly different. Although the integrated *Ct* values were reasonably controlled, an approximate correction was again applied. (See Appendix, Statistical Analysis Method Details.)

Table 5. Results for pair-wise *t*-test, using temperature data means of survival fraction.

Temperature compared, °C	N/N_0 different at 0.9?	Probability different
25, 30	no	0.80
30, 35	no	0.40
35, 40	no	0.55
40, 45	yes	0.97

Another approach that fits the design of this series is Dunnett's test,⁶ where one of the values of temperature is considered the control (reference) and all the other temperature values are treatments. The test determines whether the result from each temperature is significantly different than the result from the reference value. This test was applied to the survival fractions N/N_0 (corrected for *Ct*), with 40°C chosen as the reference (since 40 to 45°C produced the only possible significant difference). The significance implied in Table 5 decreased only to slightly less than 0.95. This outcome apparently is a real effect. In Dunnett's test, the statistical test includes the pooled mean standard error of all of the

⁶ C. W. Dunnett, "New Tables for Multiple Comparison with a Control," *Biometrics*, Vol. 20, No. 3, 1964, and C. W. Dunnett, "A Multiple Comparison Procedure for Comparing Several Treatments with a Control," *Journal of the American Statistical Association*, Vol. 50, 1955.

treatment (i.e., temperatures) measurements as indicative of the variation.⁷ In a pair-wise comparison, only the standard deviations of the data for the two temperatures being compared are considered. However, pair-wise comparisons always indicate more significant differences than actually exist.

Nevertheless, to err on the side of conservatism, one must accept the possibility that the effectiveness of hydrogen peroxide on *G. stearothermophilus* may be minimal at 35°C (equivalently D-value is maximal), on the range of 25 to 45°C.

Run Series 3 Humidity Effect

The N/N₀ values for each of five runs averaged across position for the VHP-exposed coupons are presented in Table 6. The N/N₀ values for each of five positions averaged across the runs for the VHP-exposed coupons are presented in Table 7.

Table 6. Relative humidity effect on lethality, survival fraction, (at 35°C, 1.1 mg/L H₂O₂, 1-min duration), averaged over positions (no correction for Ct variation between runs).

Run/ Run Condition	A3	B3	C3	D3	E3
3% RH	5.09E-05 ±8.28E-05	6.94E-05 ±1.30E-04	1.48E-04 ±1.29E-04	2.36E-04 ±4.41E-04	9.25E-05 ±2.00E-04
5.2% RH	3.85E-04 ±3.54E-04	1.23E-04 ±8.00E-05	1.40E-04 ±1.37E-04	1.22E-03 ±2.69E-03	2.69E-05 ±5.27E-05
8.1% RH	1.49E-05 ±1.98E-05	3.47E-06 ±4.30E-06	1.92E-05 ±2.49E-05	3.02E-06 ±2.04E-06	3.23E-06 ±6.95E-06
13.2% RH	9.37E-06 ±1.82E-05	5.83E-07 ±1.31E-06	9.39E-06 ±1.46E-05	na ⁸	na
20% RH	3.26E-05 ±4.26E-05	1.16E-07 ±2.59E-07	4.19E-06 ±4.94E-06	4.47E-06 ±6.23E-06	1.73E-06 ±3.57E-06
30% RH	5.78E-07 ±1.30E-06	5.12E-05 ±7.91E-05	5.78E-07 ±1.30E-06	1.04E-06 ±1.45E-06	1.62E-06 ±3.63E-06
40% RH	3.79E-06 ±7.28E-06	8.42E-06 ±1.51E-05	1.20E-07 ±2.70E-07	1.76E-06 ±3.94E-06	1.22E-05 ±1.62E-05
50% RH	1.05E-05 ±6.85E-06	0.00E+00 ±na	4.17E-06 ±6.42E-06	3.51E-07 ±5.23E-07	2.31E-05 ±3.05E-05

The mean values of the survival fractions versus RH are shown in Figure 16. Only the two lowest RH values (3 and 5.2%) appeared to yield different results.

A large variation in integrated Ct between runs at different RH values was observed. Therefore the correction for Ct (by way of the effective D-value) was essential to analyze the data. The result of this correction is shown in Figure 17. Now a large RH dependence is apparent. Equivalently, the D-value versus RH is plotted in Figure 18.

⁷ Also for a planned Dunnett's analysis, the number of replicates of the control treatment should be a factor of at least the square root of one less than the number of treatments, more than the number of replicates.

⁸ The original five runs at 13.2%RH were accidentally run at 1.3 mg/L VHP concentration. Due to constraints, only three correct runs at 13.2% were then conducted. The runs at 1.3 mg/L were analyzed and corrected for the larger Ct values corresponding to the higher concentration. As may be seen in Figure 18, these two runs also line up with the other data very well.

Table 7. Relative humidity effect on lethality, survival fraction, (at 35°C, 1.1 mg/L H₂O₂, 1-min duration), averaged over runs (no correction for Ct variation between runs).

Position/ Run Condition	1	2	3	4	5
3% RH	1.65E-04 ±1.29E-04	1.12E-05 ±1.85E-05	5.15E-05 ±5.74E-05	2.13E-04 ±4.51E-04	1.56E-04 ±2.06E-04
5.2% RH	1.45E-03 ±2.57E-03	2.09E-04 ±3.06E-04	7.86E-05 ±1.09E-04	1.03E-04 ±2.27E-04	5.55E-05 ±8.10E-05
8.1% RH	2.18E-05 ±2.10E-05	2.76E-06 ±4.46E-06	1.03E-06 ±1.45E-06	1.39E-06 ±1.68E-06	1.69E-05 ±2.08E-05
13.2% RH	2.33E-06 ±1.56E-06	3.55E-06 ±3.20E-06	1.43E-05 ±2.38E-05	1.17E-05 ±2.04E-05	3.85E-07 ±6.70E-07
20% RH	2.59E-05 ±4.54E-05	7.59E-06 ±1.16E-05	2.31E-07 ±3.19E-07	5.79E-06 ±5.55E-06	3.59E-06 ±8.04E-06
30% RH	1.66E-05 ±3.43E-05	3.57E-05 ±8.00E-05	0.00E+00 ±na	0.00E+00 ±na	2.77E-06 ±3.33E-06
40% RH	6.01E-06 ±1.35E-05	1.60E-06 ±3.26E-06	2.22E-06 ±3.82E-06	1.05E-05 ±1.53E-05	5.92E-06 ±1.33E-05
50% RH	2.00E-05 ±2.87E-05	1.52E-06 ±2.75E-06	1.01E-05 ±1.66E-05	5.09E-06 ±7.74E-06	1.51E-06 ±3.38E-06

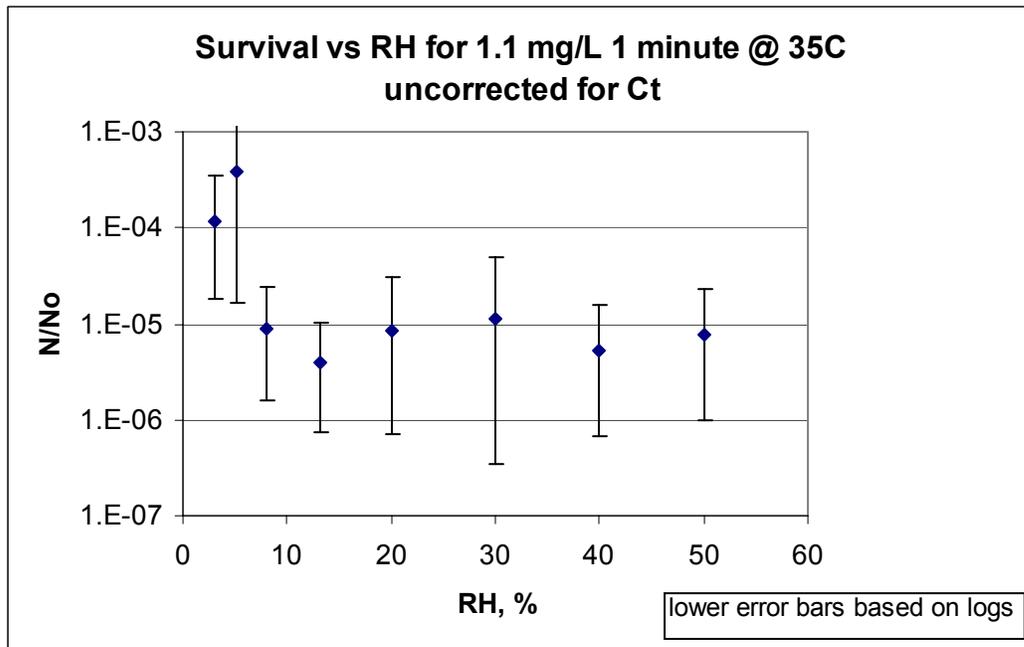


Figure 16. Survival fraction versus RH(%), uncorrected for Ct variation between runs at different RH.

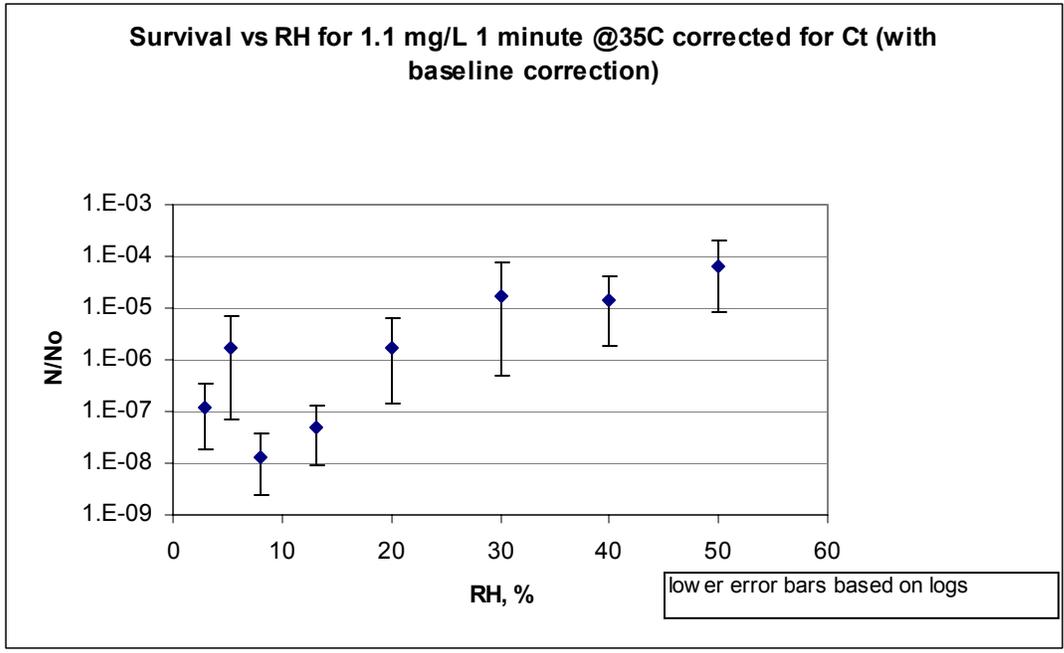


Figure 17. Survival fraction versus RH(%), corrected for Ct variation between runs at different RH.

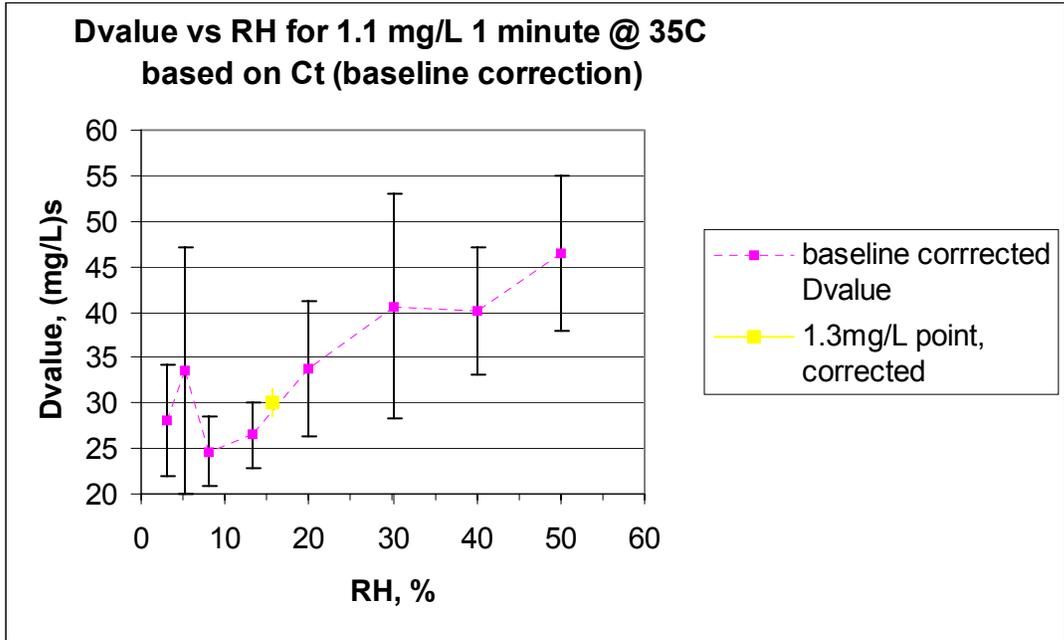


Figure 18. D-value versus RH(%), corrected for Ct variation between runs at different RH.

Also shown in Figure 18 is the D-value for a series of exposures at 13.2% RH that was inadvertently conducted with 1.3 mg/L injections instead of 1.1 mg/L. The D-value was corrected for the Ct difference. (This result shows the power of the Ct model to represent the data.)

Finally the data of run series 2 on temperature may be interpreted as RH runs, as previously discussed. This comparison is shown in Figure 19.

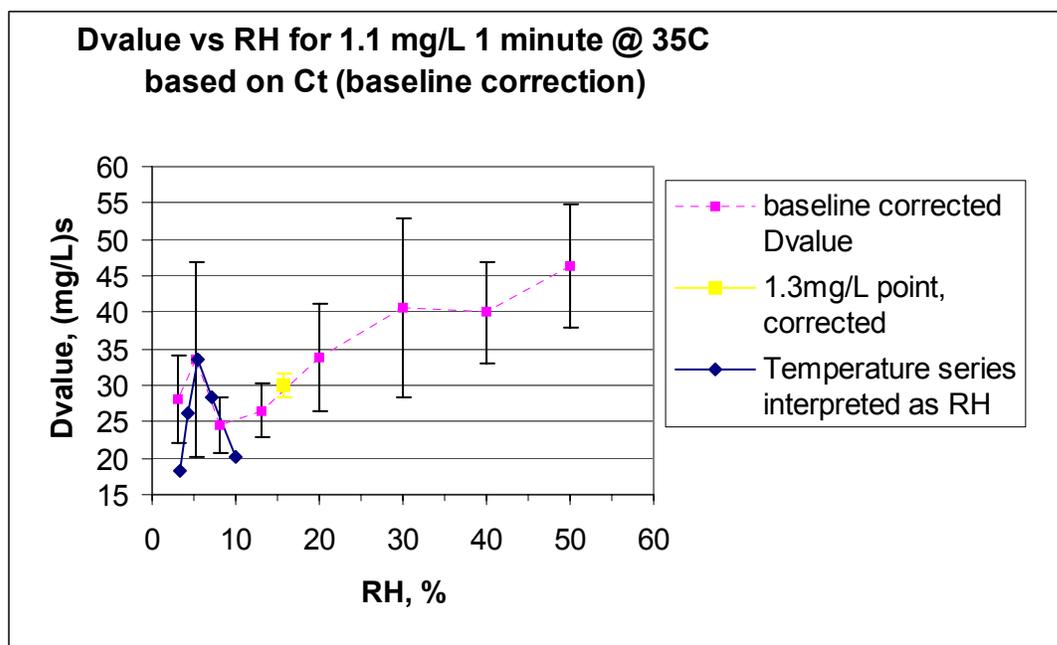


Figure 19. D-value versus RH(%), corrected for Ct variation between runs at different RH. Data of Figure 18 with data of run series 2 on temperature added for comparison.

The effect of absolute humidity on the survival fraction (expressed as RH at 35°C) was statistically analyzed. An ANOVA test was performed on all (25) of the values for N/N_0 in the five replicate runs with the (assumed) five equivalent positions for each of the five values of humidity. The overall mean of N_0 was used. If these values were not corrected for the variation in Ct , the result was that the humidity dependence was real (the effect of at least one humidity level was different than the others) with a probability of 0.975⁹ (see Table 8). With a correction for the large variation in Ct , this value was 0.9999. The major changes reflected the large variation in Ct (between runs at different humidity values).

The ANOVA test was also applied excluding some of the humidity values, namely 3% and 5.2% and 3%, 5.5%, 8.1% and 13.2%, in order to distinguish which mean values were significantly different than the others. As soon as the 3% and 5.2% RH mean values were eliminated, the means uncorrected for Ct variation were no longer significantly different (1-P much less than 90%). (This result is as expected by the data shown in Figure 17.) However, in the ANOVA of the values of N/N_0 corrected for Ct variations, the mean values continued to be significantly different even after these exclusions. These results are summarized in Table 8.

In addition, the assumptions that the replicate runs were statistically equivalent and that the positions of the exposed BIs were equivalent were tested. A t-test of the minimum and maximum (for the set of replicate runs) observed mean (averaged over position) N/N_0 and standard deviations, for each humidity, showed no significant difference at 0.90. Also, a t-test of the minimum and maximum (for the set of positions) observed

⁹ The proper statistical statement is that the conclusion that there is a significant temperature effect may be incorrect with a probability of 0.025.

mean (averaged over replicate runs) N/N_0 and standard deviations, for each humidity, showed no significant difference at 0.90.

Table 8. ANOVA test results for humidity data, one minus probability of coincidental differences in means (1-P).

RH Data Included	1-P for survival fraction uncorrected for Ct	1-P for survival fraction corrected for Ct
ALL	0.975	0.9999
8.1 to 50%	0.05	0.999
20 to 50%	0.14	0.98

To reveal which specific humidity values contribute to the significant effect observed, a pair-wise *t*-test was more useful than an ANOVA. Student's *t*-test was used to determine whether pairs of means of N/N_0 at adjacent humidity values were significantly different (see Table 9). The approximate correction for the variation in **Ct** was again applied.

Table 9. Results for pair-wise *t*-test, humidity data means.

RH compared, %	N/N_0 different at 0.9?	Probability different
8.1 , 13.2	yes	0.95
13.2 , 20	yes	>0.99
20 , 30	yes	0.98
30 , 40	no	0.11
40 , 50	yes	>0.99

Once again, Dunnett's test was applied, where one of the values of humidity is considered the control (reference) and all the other humidity values are treatments. The test determines whether the result from each humidity is significantly different than the result for the reference value. This test was applied to the survival fractions N/N_0 (corrected for **Ct**), with 30% RH (arbitrarily) chosen as the reference. The probabilities implied in Table 9 decreased to less than 0.95, with the exception of the 50% RH value differing from the 30% RH value at the 0.95 level (but not at 0.99). This outcome apparently is a real effect. In Dunnett's test, the statistical test includes the pooled mean standard error of all of the treatment (i.e., humidity values) measurements. In a pair-wise comparison, only the standard deviations of the two humidity values being compared are considered. Pair-wise comparisons always indicate more significant differences than actually exist.

These results indicated that humidity does have an effect on VHP sporicidal efficacy. It appears that increasing humidity will increase the survival of spores exposed to the same concentration of VHP for the same duration. Again to err on the side of conservatism, one must accept that the effectiveness of hydrogen peroxide on *G. stearothermophilus* may be minimal at 50% RH at 35°C (equivalently D-value is maximal), on the range of 13.2 to 50% RH.

Run Series 4 Substrate Effect

The N/N_0 values for each of five runs averaged across position for the VHP-exposed material substrate coupons are presented in Table 10. The N/N_0 values for each of five positions averaged across the runs for the VHP-exposed material substrate coupons are presented in Table 11.

Table 10. Substrate effect on lethality, survival fraction, (at 50% RH, 35°C, 1.1 mg/L H₂O₂, 1-min duration), averaged over positions (no correction for Ct variation between runs).

Run/ Run Condition	A3	B3	C3	D3	E3
black paint	5.0E-04 ±5.8E-04	9.8E-04 ±7.8E-04	4.4E-04 ±3.9E-04	5.7E-04 ±6.2E-04	4.7E-04 ±5.5E-04
white paint	8.2E-02 ±5.6E-02	1.8E-01 ±6.6E-02	3.0E-01 ±1.7E-01	2.7E-01 ±1.3E-01	2.3E-01 ±5.6E-02
aluminum	5.0E-07 ±5.3E-07	6.4E-06 ±9.2E-06	8.9E-06 ±1.1E-05	2.5E-05 ±2.5E-05	9.7E-06 ±2.1E-05
Kapton	0.0E+00 ±na	4.8E-06 ±9.7E-06	6.3E-07 ±1.4E-06	0.0E+00 ±na	0.0E+00 ±na
graphite composite	0.0E+00 ±na	2.5E-07 ±3.5E-07	0.0E+00 ±na	3.8E-07 ±8.5E-07	0.0E+00 ±na

Table 11. Substrate effect on lethality, survival fraction, (at 50% RH, 35°C, 1.1 mg/L H₂O₂, 1-min duration), averaged over runs (no correction for Ct variation between runs).

Position/ Run Condition	1	2	3	4	5
black paint	3.8E-04 ±1.2E-04	6.8E-04 ±7.7E-04	3.0E-04 ±2.1E-04	7.0E-04 ±7.1E-04	9.0E-04 ±7.6E-04
white paint	1.5E-01 ±9.5E-02	2.8E-01 ±1.9E-01	1.9E-01 ±7.7E-02	1.7E-01 ±6.5E-02	2.9E-01 ±1.4E-01
aluminum	2.0E-05 ±2.8E-05	3.5E-06 ±3.5E-06	2.8E-06 ±3.4E-06	7.3E-06 ±1.5E-05	1.8E-05 ±1.9E-05
Kapton	6.3E-07 ±1.4E-06	4.4E-06 ±9.9E-06	2.5E-07 ±5.6E-07	1.3E-07 ±2.8E-07	0.0E+00 ±na
graphite composite	3.8E-07 ±8.5E-07	1.3E-07 ±2.8E-07	0.0E+00 ±na	0.0E+00 ±na	1.3E-07 ±2.8E-07

The survival fractions obtained from run series 4 are plotted in Figure 20. The calculated D-values are shown in Figure 21, Figure 22, and Figure 23. Because the D-value for the white paint substrate is so much larger than the others, a semi-log plot is shown (Figure 21). However, a linear plot (Figure 22) better reflects how little apparent effect all of the other substrates had on the D-value. Finally, a linear plot with the white paint omitted (Figure 23) guides the eye toward other possible dependences (e.g., black paint).

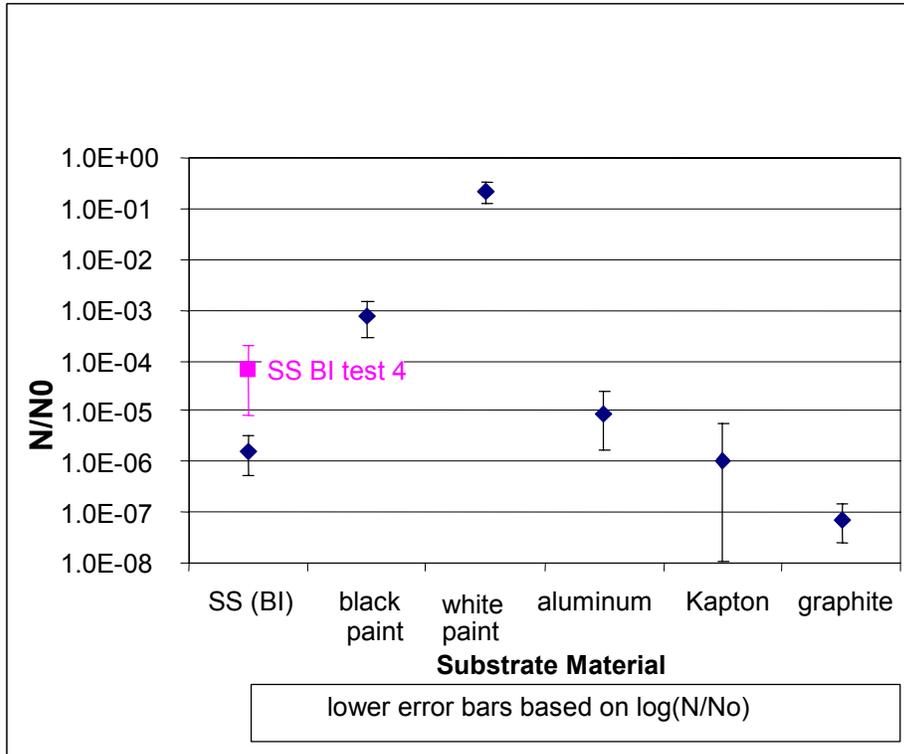


Figure 20. Survival fraction versus substrate for 1.1 mg/L (of chamber), 60 s nominal injection at 35°C and 50% RH. Survival fractions corrected for Ct variability and baseline. Also shown is the appropriate mean stainless steel BI result from run series 3, for comparison.

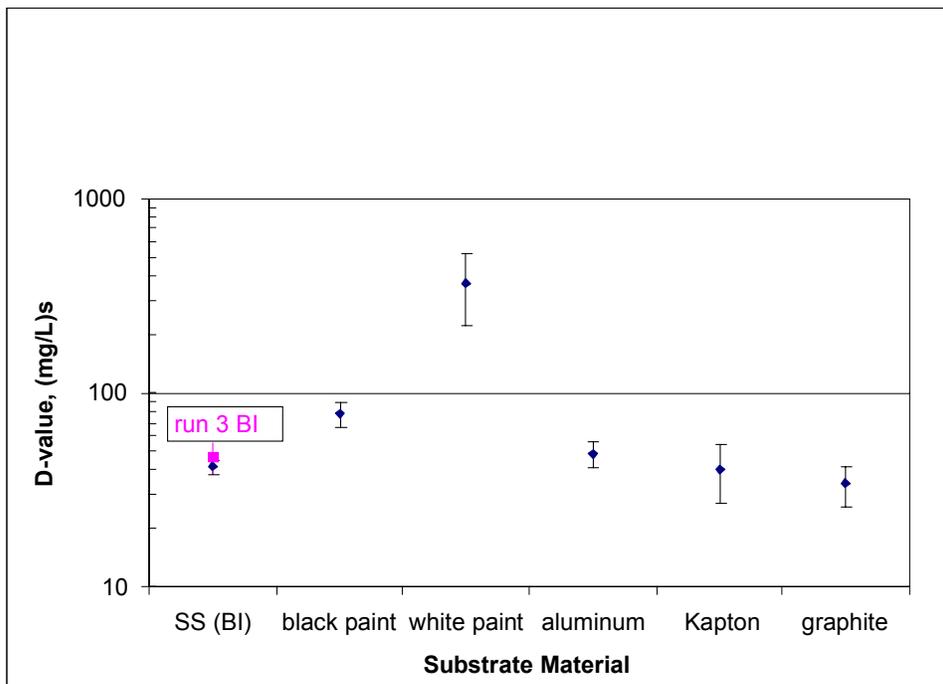


Figure 21. D-value versus substrate for 1.1 mg/L (of chamber), 60 s nominal injection at 35°C and 50% RH. Also shown is the appropriate mean stainless steel BI result from run series 3, for comparison. Note that this is a semi-log plot.

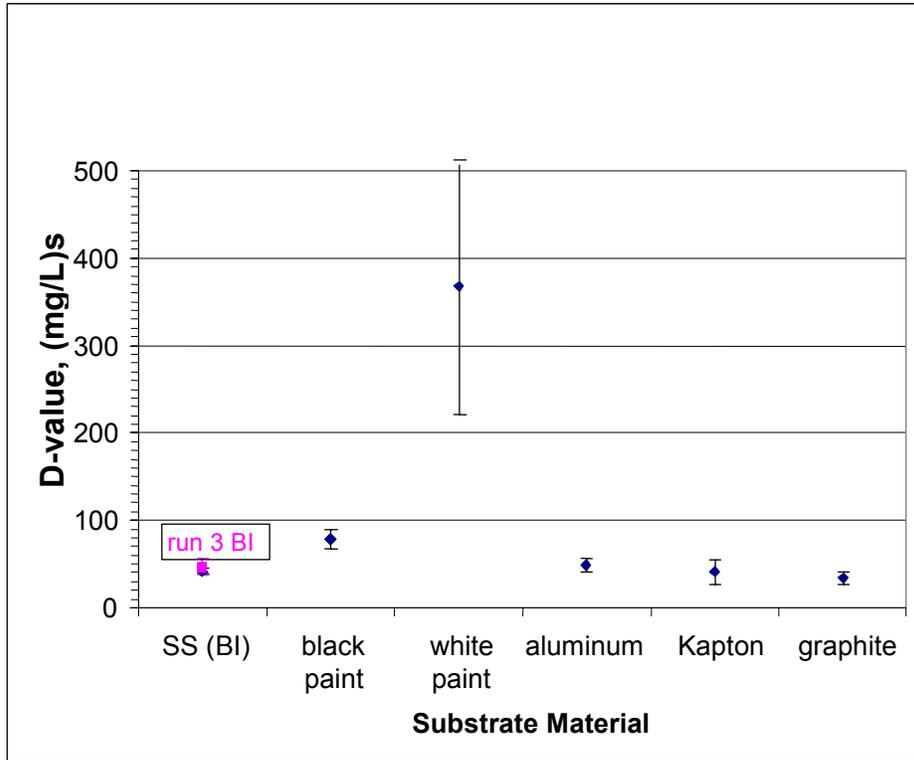


Figure 22. D-value versus substrate for 1.1 mg/L (of chamber), 60 s nominal injection at 35°C and 50% RH. Also shown is the appropriate mean stainless steel BI result from run series 3, for comparison. This is a linear version of Figure 21.

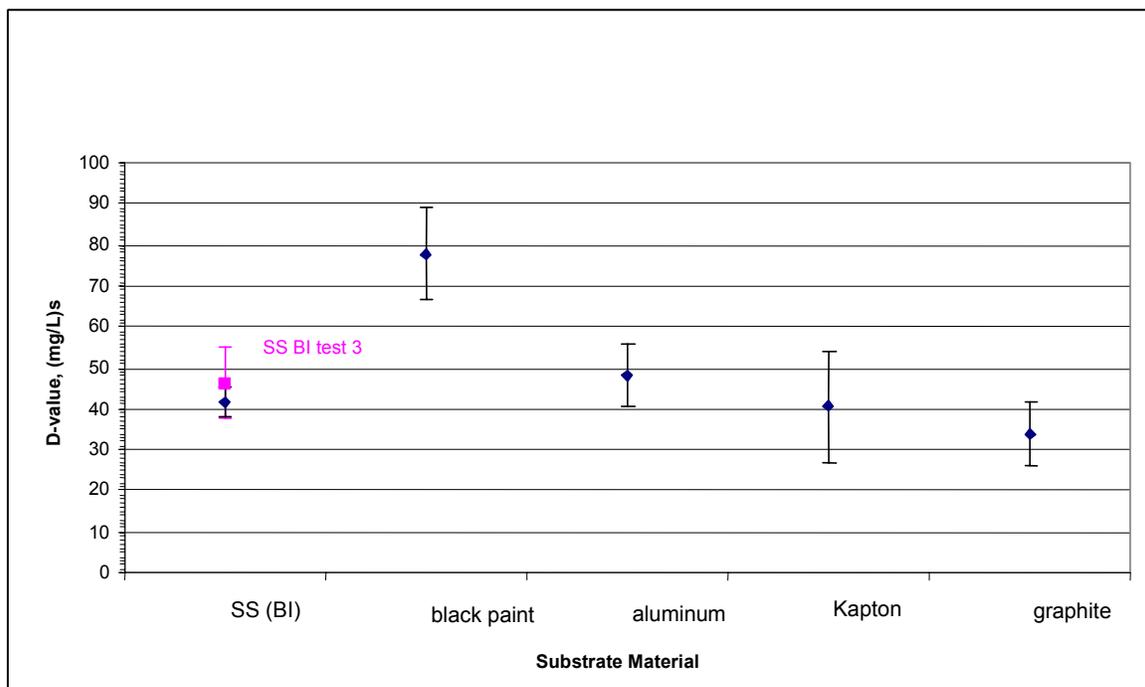


Figure 23. D-value versus substrate for 1.1 mg/L (of chamber), 60 s nominal injection at 35°C and 50% RH. Also shown is the appropriate mean stainless steel BI result from run series 3, for comparison. Same as Figure 22 with the white paint result omitted for clarity and rescaled.

As before, the results of run series 4 were statistically analyzed. Unsurprisingly, the ANOVA showed a substrate dependence with 0.98 significance. However, this statistical test cannot reveal which specific substrates contributed to the significant effect observed (although the white paint was obvious). For a pair-wise t-test on N/N_0 , there is an obvious natural pairing. The actual pairing of interest is each substrate versus the stainless steel (SS) of the standard BI. These analyses have been conducted with the N/N_0 corrected for Ct variability. (These corrections were very small because the values of integrated Ct in the five replicate runs were very well reproduced.) The results are given in Table 12.

Table 12. Results for pair-wise (to SS) t-test, substrate data means.

Substrate compared to SS	N/N_0 different at 0.9?	Probability different
Black paint	yes	0.9999
White paint	yes	0.999999
Aluminum	yes	0.98
Kapton	no	0.35
Graphite composite	yes	0.9999

This series is made to order for Dunnett's test, where the stainless steel BIs were considered the control and all the other substrates were treatments.¹⁰ The test determined whether the result for each substrate was significantly different than the result for stainless steel. This test was applied to the survival fractions N/N_0 (both corrected and uncorrected for Ct). As expected for the small corrections, the results in this test were exactly the same. However, only the white paint substrate was significantly different than the stainless steel control surface (0.99). This outcome apparently was a real effect. In Dunnett's test, the statistical test includes the pooled mean standard error of all of the treatment (i.e., substrate) measurements. In a pair-wise comparison, only the standard deviations of the data for the two substrates being compared are considered. Pair-wise comparisons always indicate more significant differences than actually exist.

Some effort was made to understand the extremely large survival on the white painted surface. Figure 24 is a photograph of the six pairs of substrate coupons with unexposed controls on the left and VHP-exposed on the right. A scanning electron microscope (SEM) was used to further investigate the substrates, both unexposed and exposed to VHP (not the same samples). These figures are Figure 25 through Figure 30.

¹⁰ However, as pointed out previously: For a planned Dunnett's analysis, the number of replicates of the control treatment (i. e., the SS substrate) should be a factor of at least the square root of one less than the number of treatments more than the number of replicates.

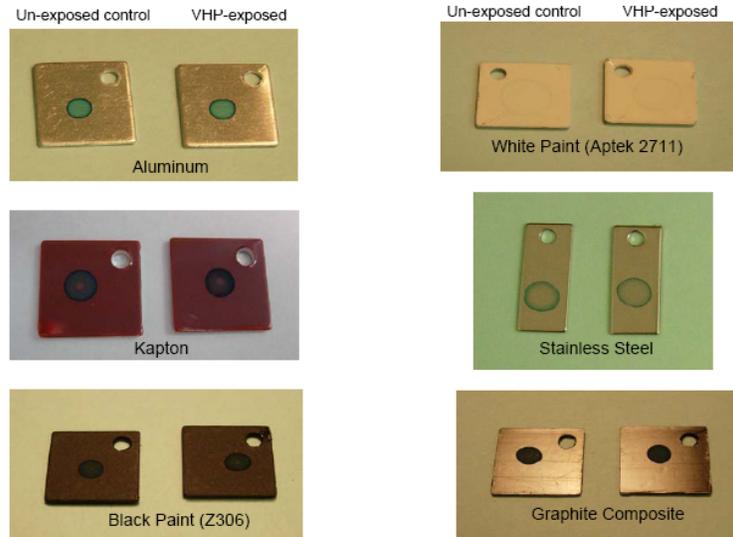


Figure 24. Pairs of coupons with unexposed controls on the left and VHP-exposed on the right.

Stainless Steel

Typical scanning electron micrographs of *G. stearotherophilus* spores on stainless steel coupons are shown in Figure 25 for unexposed control and VHP-exposed coupons, on the left and right, respectively.

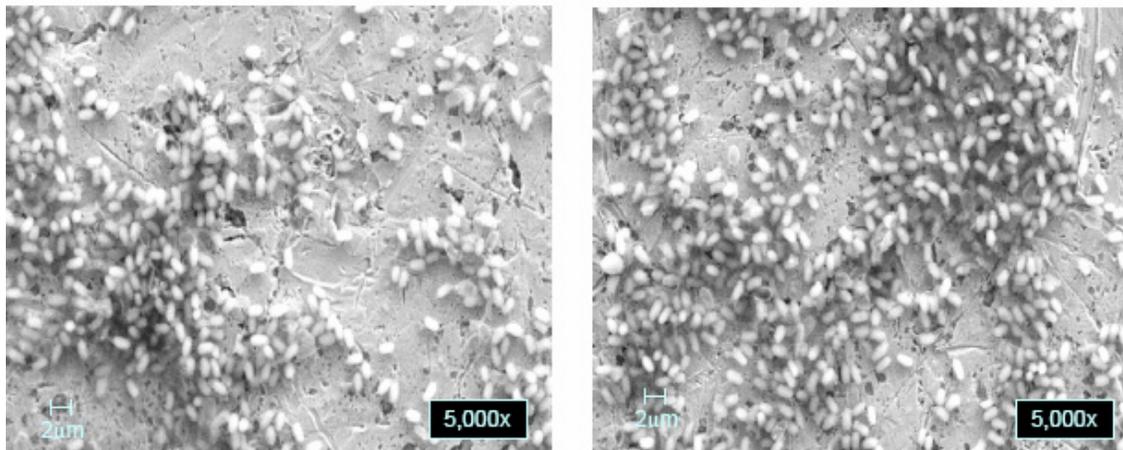


Figure 25. SEM photos of unexposed control (left) and VHP-exposed (right) stainless steel coupons.

Z306 – Black Paint

A three-log reduction was observed on Z306-coated aluminum substrate. The chemical composition of the paint may have increased the degradation of the VHP and thereby provided localized protection from the sporicidal effect. Figure 26 is SEM photos of an unexposed control and a VHP-exposed black paint, Z306 coupon.

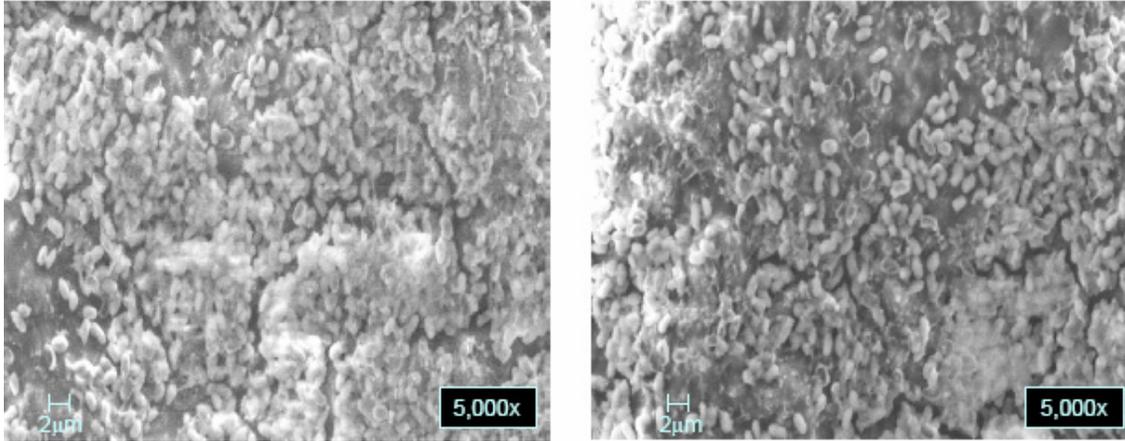


Figure 26. SEM photos of unexposed control (left) and VHP-exposed (right) Black Paint, Z306 coupons.

Aluminum 6061

Results showed a five-log reduction in lethality, with no difference in surface morphology or appearance of spores between the unexposed control and VHP-exposed coupons. The magnification used was not intended to reveal individual cell morphological changes, however. Figure 27 is SEM photos of an unexposed control and a VHP-exposed aluminum coupon.

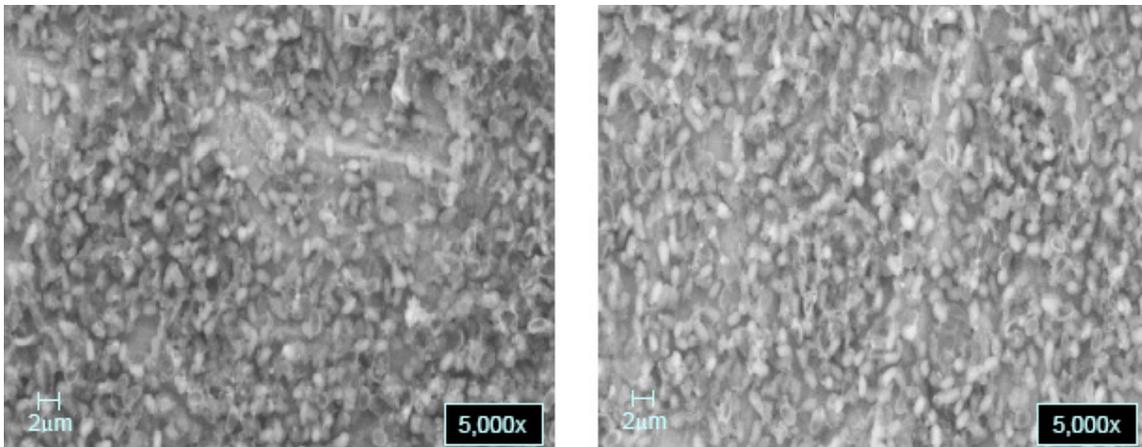


Figure 27. SEM photos of unexposed control (left) and VHP-exposed (right) aluminum coupons.

Graphite Composite

The results were a six-log reduction after VHP exposure, with no differences in surface morphology or spore appearance. Figure 28 is SEM photos of an unexposed control and a VHP-exposed graphite composite coupon.

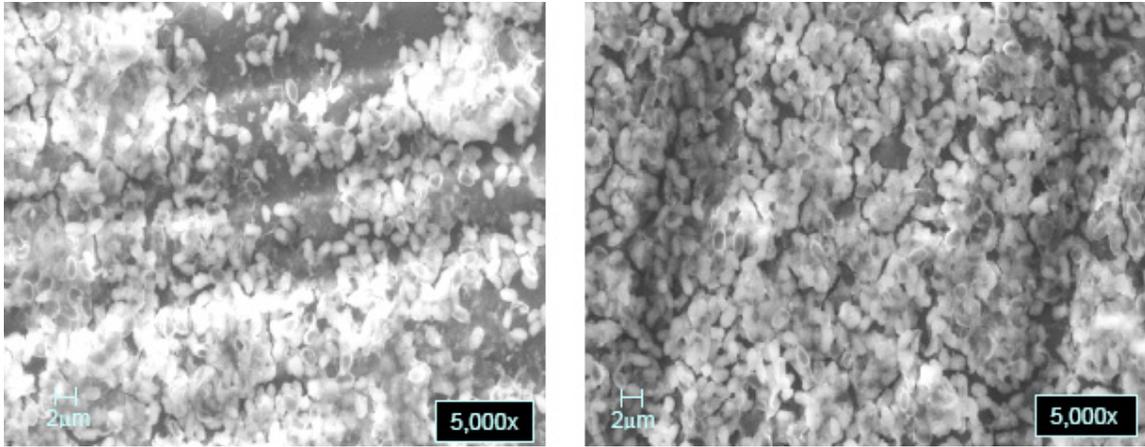


Figure 28. SEM photos of unexposed control (left) and VHP-exposed (right) graphite composite coupons.

Kapton

The results were a six-log reduction after VHP exposure. There were no differences in surface morphology or spore appearance between unexposed and VHP-exposed coupons. The magnification used was not intended to reveal individual cell morphological changes. Figure 29 is SEM photos of an unexposed control and a VHP-exposed Kapton coupon.

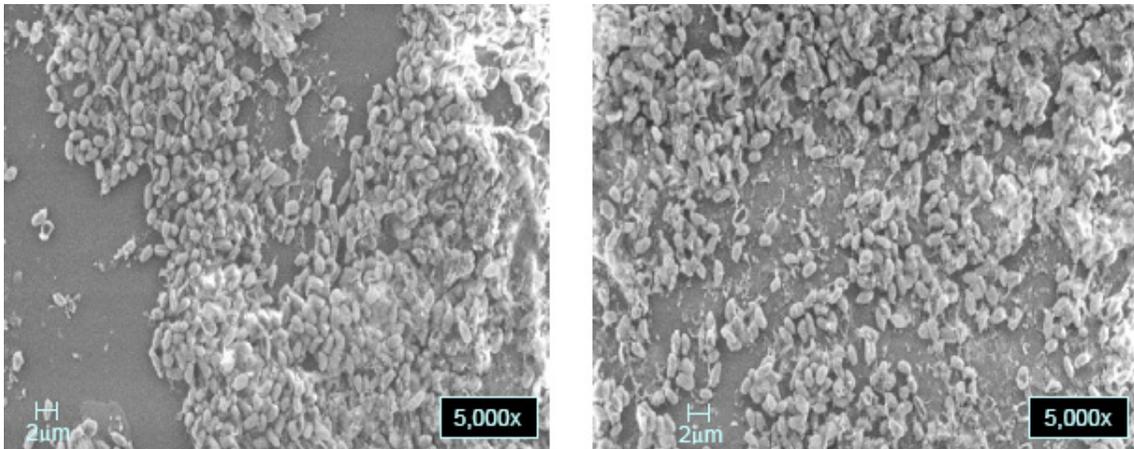


Figure 29. SEM photos of unexposed control (left) and VHP-exposed (right) Kapton coupons.

Aptek 2711 – White Paint

A one-log reduction was observed with Aptek 2711-coated aluminum substrate. When the spore suspension was applied to these paint-coated coupons, it was noted that the suspension immediately spread edge to edge; and during assaying, some of the White Paint came off in the medium. It is probable that the paint was lifted during inoculation and some spores were occluded by paint particles. The shielding of these spores from exposure to VHP could explain the high spore survival seen with these coupons.

Microscopy of the white paint revealed that the spores were piled up on top of one another and located down in the surface pores, the scale of which exceeded the size of the

spores (Figure 30). It is hypothesized that this arrangement caused the high survival. Further, this condition was caused by the inoculation of the substrates with $\sim 10^6$ spores onto a small area (a few cm^2). This surface density is not representative of natural fallout. This interpretation was also based on the apparent large effect of spore surface density on stainless steel (relatively without spore pockets) observed and resolved in the task in the previous year. Black paint also showed less lethality than the other substrates, but the difference was not statistically significant.

Nevertheless, for the purposes of this task, one must accept the possibility that the effectiveness of hydrogen peroxide may strongly depend on substrate material and surface characteristics.

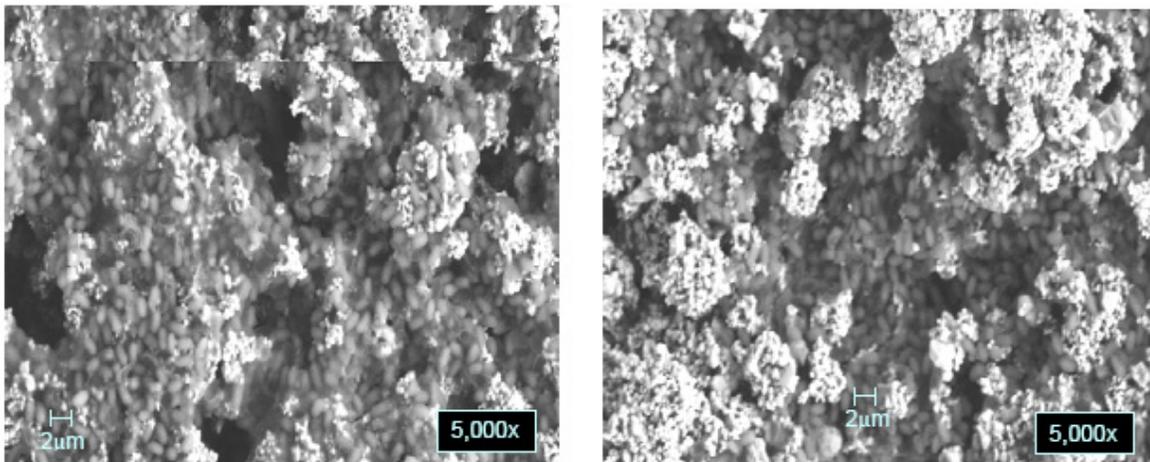


Figure 30. SEM photos of unexposed control (left) and VHP-exposed (right) White Paint, Aptek 2711 coupons.

Run Series 5 Validation

Data Results

The purpose of this series was to compare the STERIS assay procedures to the JPL procedures on coupons that had been identically prepared and exposed. The results, mean N/N_0 and standard deviations, for the exposed BIs and samples are summarized in Table 13. In addition, the pooled results of the assays of the unexposed BIs (controls) are provided.

The analysis of these data was difficult because two of the four conditions (stainless steel BIs in Tyvek at 5.2%RH, 35°C, 1.1 mg/L H_2O_2 , 1-min duration and stainless steel BIs in Tyvek at 50%RH, 35°C, 1.1 mg/L H_2O_2 , 1-min duration) produced only zeroes (each BI in each run!). This result precluded a statistical inter-comparison. However, both sets of data were all zeroes and in agreement. Further, because there was only one black paint unexposed control for STERIS, the value shown is not a mean, and the standard deviation could not be calculated. No statistical comparison for these runs was possible. However, in the prior test series on materials, there was an additional data point for STERIS' assay of an unexposed black paint sample. This value for N_0 , 2.03×10^6 appears to be consistent with the present value, 1.76×10^6 (no statistical test possible).

Table 13. Summary of experimental mean survival fractions, unexposed control CFUs and standard deviations.

Run Conditions	STERIS		JPL	
	N/N ₀	s	N/N ₀	s
Stainless steel, 5.2%RH, 35°C, 1.1 mg/L H ₂ O ₂ , 1-min duration	3.77E-05	5.03E-05	7.12E-05	9.95E-05
Stainless steel, 5.2%RH, 25°C, 1.1 mg/L H ₂ O ₂ , 1-min duration	0	n/a	0	n/a
Stainless steel, 50%RH, 35°C, 1.1 mg/L H ₂ O ₂ , 1-min duration	0	n/a	0	n/a
Black paint (Z306), 50%RH, 35°C, 1.1 mg/L H ₂ O ₂ , 1-min duration	8.10E-05	1.03E-04	2.27E-04	4.74E-04
Controls				
Stainless steel unexposed control, N ₀ cfu	2.73E+06	3.43E+05	2.98E+06	2.01E+05
Black paint (Z306) unexposed , N ₀ cfu	1.76E+06	n/a	3.08E+06	3.69E+05
Unexposed (pooled), N ₀ cfu	2.49E+06	5.59E+05	3.0E+06	2.37E+05

Finally, the experiment was designed and conducted such that the results of Table 13 merely compared the results of the STERIS-processed assays of BIs, simultaneously exposed to VHP under various conditions, to those processed by JPL personnel. One would expect that the differences in performance would reflect variances in operators and in the specific assay protocols. The same comparison could have been obtained from BIs and inoculated black paint samples with various inoculation values (blind experiment), without any hydrogen peroxide exposure.

STATISTICAL ANALYSIS RESULTS

Unexposed Controls

The comparison of the STERIS controls vs. JPL controls provided information on samples of high spore numbers (N₀), without any effects from hydrogen peroxide. Both the F test on the standard deviations (s) and the t-test on the means (m) of the value of N₀ were conducted. A priori, one must assume that both statistics may be different because different personnel performed the assay and the procedures may be different in minor details. For example, different sonicators with different frequency and power were used. Unfortunately, because normalization was not possible, the statistics may also differ due to variation in the actual N₀ for the samples. One may attempt to control this variation by performing the F test on the fractional standard deviations (s/N₀). Actually, this involves some circular logic and leads to no changes in the results. The experimental values used are shown in Table 13. In addition, the fractional standard deviations are given below in Table 14.

Table 14. Experimental fractional standard deviations for unexposed controls.

Controls	STERIS	JPL
	s/N ₀	s/N ₀
Stainless steel unexposed control, N ₀	0.13	0.07
Black paint (Z306) unexposed , N ₀	n/a	0.12
Unexposed (pooled)	0.22	0.08

STERIS Controls vs. JPL Controls (unexposed BIs only)

The comparison of the STERIS controls vs. JPL controls, unexposed BIs only, provided information on samples on a relatively smooth substrate. The statistical tests indicated that the standard deviations of N_0 were equal; i.e., the null hypothesis could not be rejected at 90%. (However, they are different at 85%.) Similarly the means of N_0 are the same (could not be shown different at the 90% confidence level), by the means test for equal variances ($t = 1.6$; $t_c(0.1) = 1.8$). (However, they are different at 83%.)

STERIS Controls vs. JPL Controls (unexposed black paint only)

The comparison of the STERIS controls vs. JPL controls, unexposed black paint only, provided information on samples on a “rough” substrate. Because there was only one unexposed black paint sample assayed by STERIS, no mean or standard deviation was available. If one assumes that the population standard deviations are equal, one can still test the means (although the black paint sample assayed by STERIS was merely a single value). In this case, one cannot reject the null hypothesis at 90% ($t = 2.4$; $t_c(0.1) = 2.9$). No difference was demonstrated.

STERIS Controls vs. JPL Controls (pooled unexposed controls)

Finally, it is possible to pool all of the unexposed controls (BIs and black paint) and to neglect possible substrate effects (after all, there is no hydrogen peroxide exposure). The interesting result was obtained that the standard deviations of N_0 were different; the null hypothesis was rejected at 95%. This may reflect a true difference, unobtainable from the black paint unexposed controls. Nevertheless, the pooled means of N_0 are equal (can't be shown different at the 90% confidence level, ($t = 1.8$; $t_c(0.1) = 2.4$)).

Exposed BIs and Samples

This part of the data addressed the main objective: for BIs and samples exposed to H_2O_2 , do STERIS and JPL obtain the same results when processing the coupons? Are the mean survival fractions statistically the same? Note that the BIs and samples were inoculated and exposed at the same time under identical conditions.

Therefore, mean N/N_0 is the appropriate statistic. Since N and N_0 are uncorrelated, mean N/N_0 is simply mean N divided by mean N_0 (for the STERIS data and the JPL data separately). The experimental standard deviations of the means were calculated by the usual method. (See Appendix, Statistical Analysis Method Details.) All of the values that were statistically tested may be found in Table 13.

Exposed BIs

For the stainless steel BIs exposed at 5.2%RH, 35°C, and 1.1 mg/L H_2O_2 , for a 1-min duration, the null hypothesis that the STERIS values and JPL values of the variances are the same was rejected by the F test at the 95% confidence level. Therefore, they were assumed unequal for the purpose of the statistical test on the means of the survival fraction (N/N_0). Use of Student's t -test for unequal variances indicated that the mean values were the same. The null hypothesis could not be rejected at 90% ($t = 1.0$; $t_c(0.1) = 1.7$). The 95% confidence interval for the difference in the population means was:

$$-2.82 \times 10^{-5} < \mu_1 - \mu_2 < 8.67 \times 10^{-5}$$

These extrema are of the order of magnitude of the means themselves. (See Table 13) Therefore, for factor-of-two type conclusions, there was no difference between STERIS and JPL results.

As noted above, no statistical comparison was possible for the two sets (STERIS, JPL) of runs: stainless steel, 5.2%RH, 25°C, 1.1 mg/L H₂O₂, 1-min duration, and stainless steel, 50%RH, 35°C, 1.1 mg/L H₂O₂, 1-min duration. However, the null survivor results were consistent with each other.

Exposed Black Paint Samples

For the black paint samples, exposed at 50%RH, 35°C and 1.1 mg/L H₂O₂, for a 1-min duration, the null hypothesis that the variances are the same was rejected by the F test on the variances at 95% confidence. Therefore, they were assumed unequal for the purpose of the statistical test on the means of the survival fraction. Use of Student's t-test for unequal variances indicated that the mean values were the same. The null hypothesis could not be rejected at 90% ($t = 1.0$; $t_c(0.1) = 1.7$). The 95% confidence interval for the difference in the population means was:

$$-1.05 \times 10^{-4} < \mu_1 - \mu_2 < 2.93 \times 10^{-4}$$

These extrema are of the order of magnitude of the means themselves. Therefore, for factor-of-two type conclusions, there was no difference between STERIS and JPL results.

Comparison with Previous Experiments

Although data comparison between tests was not part of the experimental plan, the data of this validation test may be statistically compared to the data of prior test series. The pooled (STERIS and JPL) data of the validation test were compared to previous STERIS data obtained in the task; the present STERIS data were also compared to analogous previous data. Specific comparisons included the stainless steel BIs exposed at 5.2%RH, 35°C, and 1.1 mg/L H₂O₂, for a 1-min duration, and the black paint samples at 50%RH, 35°C, 1.1 mg/L H₂O₂, for a 1-min duration.

However, mean survival fraction (N/N_0) is not an appropriate statistic for these comparisons because the H₂O₂ exposures were not well controlled (even though the nominal concentration and duration are fixed). Therefore, the effective D-values, D_{eff} , were calculated, which correct for the variations in Ct.

For the stainless steel BIs in Table 15, the statistical treatment of the pooled STERIS and JPL series (column 1) compared to the prior results by STERIS (column 3) showed that the mean values of D_{eff} were significantly different. The null hypothesis was rejected at 90% ($t = 5.9$; $t_c(0.1) = 1.67$). In fact, the level of confidence is greater than 99.999%, based on 44 and 25 samples, respectively. The 95% confidence interval for the difference in the population means was:

$$9.2 < \mu_1 - \mu_2 < 16.6$$

Table 15. Summary of experimental mean D-values and standard deviations.

Run Conditions	Pooled STERIS and JPL, Validation Series		STERIS Validation Series		STERIS Prior Series	
	D _{eff}	s	D _{eff}	s	D _{eff}	s
Stainless steel, 5.2%RH, 35°C, 1.1 mg/L H ₂ O ₂ , 1-min duration	24.8	4.5	24.7	3.8	39.5	17.6
Black Paint (Z306), 50%RH, 35°C, 1.1 mg/L H ₂ O ₂ , 1-min duration	70.6	19.2	35.5	4.6	77.8	11.4

A comparison of the STERIS data in this study (column 2) to previous STERIS data (column 3) yielded similar results. The mean values of D_{eff} were significantly different. The null hypothesis was rejected at 90% (t = 3.8; t_c(.1) = 1.8). In fact, the level of confidence is greater than 99.95%, based on 19 and 25 samples, respectively. The 95% confidence interval for the difference in the population means was:

$$7.2 < \mu_1 - \mu_2 < 23.8$$

Similarly, for the black paint samples, the statistical treatment of the pooled JPL and STERIS data (this series) compared to the prior results by STERIS showed that the mean values of D_{eff} were significantly different. The null hypothesis was rejected at 90% (t = 1.73; t_c(0.1) = 1.67). However, the level of confidence is only 91.3%, based on 50 and 25 samples, respectively. The 95% confidence interval for the difference in the population means was:

$$-1.1 < \mu_1 - \mu_2 < 10.1$$

Because the mean exposure values for the black paint samples are virtually identical, the survival fractions N/N₀ may be compared directly, without the complication of D_{eff}. Here the means of N/N₀ were found to be significantly different. This difference probably arose from the increased experimental standard deviations (or variances) from the logarithm dependence of D_{eff} on N/N₀. The null hypothesis was rejected at 90% (t = 4.2; t_c(0.1) = 1.67). In fact, the level of confidence is 99.99%, based on 50 and 25 samples, respectively. The 95% confidence interval for the difference in the population means was:

$$1.96 \times 10^{-4} < \mu_1 - \mu_2 < 9.43 \times 10^{-4}$$

A comparison of the STERIS data in this validation series to previous STERIS data for the black paint samples yielded similar results. The mean values of D_{eff} were significantly different. The null hypothesis was rejected at 90% (t = 2.65; t_c(.1) = 1.68). The level of confidence is greater than 98.9%, based on 25 and 25 samples, respectively. The 95% confidence interval for the difference in the population means was:

$$3.1 < \mu_1 - \mu_2 < 22.5$$

Again, because the Ct values of the exposures were similar, a direct comparison of the means of N/N₀ was possible. This raised the level of confidence, by modest amount, to 99.98% (t = 4.1; t_c(0.1) = 1.68). The 95% confidence interval was:

$$3.2 \times 10^{-4} < \mu_1 - \mu_2 < 9.3 \times 10^{-4}$$

Statistical Treatment of the Zero Survival Results

Although the zero survival fraction results for stainless steel BIs at 5.2%RH, 25°C, 1.1 mg/L H₂O₂, 1-min duration and stainless steel BIs at 50%RH, 35°C, 1.1 mg/L H₂O₂, 1-min duration could not be analyzed, it was possible to analyze for statistical anomalies in comparison with the prior results by STERIS. A large kill at 5.2%RH and 25°C is not completely unexpected. In the previous (temperature) series of tests by STERIS, the survival fraction at 25°C under identical conditions was $3.6 \times 10^{-6} \pm 8 \times 10^{-6}$, while at 35°C it was $1 \times 10^{-3} \pm 7 \times 10^{-3}$. In this series, the 35°C result (4.1×10^{-5}) was significantly smaller than in the prior series. So an N/N₀ of zero at 25°C should be expected. A t-test analysis of the previous data set indicates that the probability (for the average of another series, like the present one) of the result N/N₀= 1×10^{-7} (equivalent to zero for N₀ < 10⁷ spores) is 0.33. In fact fifteen of the twenty-five previous data points were zeroes. No correction is needed here because the Ct values were virtually identical, 104 and 105 (mg/L)s (previous exposure and current, respectively).

The previous survival fraction at 50%RH and 35°C under identical conditions on stainless steel (BIs) was $1.5 \times 10^{-6} \pm 1.7 \times 10^{-6}$ (substrate material series). Here thirteen of the twenty-five previous data points were zeroes. However, a correction was needed to compare the results because the Ct values were quite different, 237 and 271 (mg/L)s (previous exposure and current, respectively). With the correction, the expected survival fraction in the present series would be $3.3 \times 10^{-7} \pm 3.6 \times 10^{-7}$. A t-test analysis of the data set (corrected for Ct) indicated that the probability (for the average of another series, like the present one) of the result N/N₀= 1×10^{-7} (equivalent to zero for N₀ < 10⁷ spores) is 0.26.

CONCLUSIONS

The lethality dependence on VHP concentration and duration of exposure compared very well with that of prior years. The variations of survival fractions for replicate run and biological or substrate samples exhibited the factor of two behavior typical of microbial lethality testing. D-values have been extracted from the current data. The comparison with previous results also demonstrated that different chambers could produce similar results under the same conditions.

For VHP concentrations C as small as 0.5 mg/L and as large as 1.1 mg/L, the lethality was characterized by the exposure Ct for durations in the range 40 to 200 seconds. The dependence on temperature was statistically significant over the range 25 to 45°C. Hydrogen peroxide was least effective at 35°C; the D-value was maximal. The D-value varied by a factor of about 1.5. Hence the D-value for 35°C may be used to bound the problem, with some amount of conservatism. However, the absolute humidity (not the relative humidity) was held constant in the series for temperature effects (series 2).

The effect of absolute humidity was statistically significant over the range corresponding to RH at 35°C of 5 to 50%. The D-value varied by a factor of about 1.8, increasing monotonically (but not linearly) with humidity. Hydrogen peroxide was least effective at an absolute humidity corresponding to 50% at 35°C; the D-value was maximal. Hence the D-value for 50% RH at 35°C may be used to bound the problem, with some amount of conservatism. The temperature was held constant at 35°C in run series 3. It is not appropriate to state the results in terms of RH for series 2 because run series 2 did not have the RH constant. Further, the entire experimental program was not designed to investigate effects of simultaneous changes to multiple factors, for example, temperature and relative humidity, independently.

Different substrates were also found to produce statistically significant results. Aptek 2711 white paint caused the least effective exposures or maximum D-value. The results were far out of line with the others, which did not vary significantly. Further study to determine whether the result is an artifact of an unrealistic loading of the surface porosity is indicated.¹¹ Nevertheless, Z306 black paint, which was not significantly different, exhibited the same trend. The establishment of a larger D-value for a proposed process could bound the result for the black paint. However, the white paint result remains an issue. As a matter of conservatism, either the use of white paints with a low ratio of solar absorptance to infrared emittance must be avoided, the paint samples must be tested in VHP, or the specification taken must be high enough to bound such white paints.

The validation series of experiments has shown that the STERIS assay process and the JPL assay process for stainless steel BIs and for inoculated black paint substrates yielded statistically similar results. For these simultaneous exposures, the values of the mean survival fraction are sufficient for comparison. Although the variances of mean survival fractions for stainless steel BIs in Tyvek at 5.2%RH, 35°C, 1.1 mg/L H₂O₂, 1-min duration in MD2000 in vacuum during exposure were different ($P=0.95$), the means were statistically the same (only $P=0.69$ of being different). For the black paint (Z306)

¹¹ An appropriate study might employ deposition of the *Geobacillus stearothermophilus* spores onto white paint samples by a nebulizer system (e.g., the existing one at JPL), followed by VHP exposures per the present work.

coupons in Tyvek at 50%RH, 35°C, 1.1 mg/L H₂O₂, 1-min duration, again the variances were different (P=0.95), but the means were statistically the same (only P=0.66 of being different). The possible differences in the means will not create an undue degree of margin in a planetary protection setting, where a factor-of-two precision is typically an acceptable result.

For the unexposed controls, the comparison was also favorable. The stainless steel BIs mean N₀ values were statistically the same at the 90% confidence level. The variances were the same (at P=0.90). The two mean values of N₀, for each pooled set (JPL and STERIS) of unexposed controls, were also statistically the same at the 90% confidence level. However, here the variances were different (P=0.95).

For the two cases (stainless steel BIs in Tyvek at 5.2%RH, 25°C, 1.1 mg/L H₂O₂, 1-min duration exposure and the same at 50% RH and 35°C) where no survival was observed, the JPL and STERIS data agreed with no statistical test possible.

A serious issue arose from the comparison of the STERIS data with previous series of experiments by STERIS earlier in 2005. Here the effective D-values must be used to account for different exposure histories. However, this indicated that the two sets of data differ significantly. Nevertheless, although the variances were not significantly different, the mean effective D-values for stainless steel BIs at 5.2%RH, 25°C, 1.1 mg/L H₂O₂, 1-min duration exposures were very significantly different (P=0.99). For the black paint (Z306) coupons at 50%RH, 35°C, 1.1 mg/L H₂O₂, 1-min duration, the mean effective D-values were also statistically different (P=0.99). Since the records of the IR sensor from the previous runs are unavailable, this finding either arose from the known issue of inhomogeneous BIs (at marginal sterilization) or personnel variations. However, the possible differences in the means will not create an undue degree of margin in a planetary protection setting, where factor-of-two precision is typically an acceptable result.

Finally, the statistical likelihood of the zero survivors in the other two exposure series was considered, with the use of the prior STERIS data. For this purpose, a mean of zero was interpreted to mean seven orders of magnitude reduction in these test series (appropriate for N₀ < 10⁷). Corrections for C_t via D_{eff} were required because the exposures were not equal to the prior series. Although there were no standard deviations available, it was still possible to conclude that the stainless steel BIs at 5.2%RH and 25°C would be 33% likely to be all zeroes. This result came from N/N₀ because the exposures in the present work were almost exactly equal. The stainless steel BIs at 50%RH and 35°C would be 26% likely to be all zeroes (result from D_{eff} comparison). Thus, this result should have been foreseen. However, the inability to compare zero results was only a minor loss for the purposes of run series 5, for the planetary protection program.

Possible PP Specification Based on Present Results

In vacuo, the D-value for hydrogen peroxide reduction of surface spore burden is 100 (mg/L)s (i.e., the time-integral C_t of the mass concentration in the volume of the chamber C required to reduce the population by a factor of ten). This value bounds the results for the environmental parameters specified below and for all substrates, except the white paint.

The exposure shall be measured in real time for the vapor concentration of hydrogen peroxide. The hydrogen peroxide vapor concentration shall be no lower than 0.5 mg/L.

The temperature shall be in the range 25 to 45°C. The absolute humidity shall be in the range 2 mg/L to 20 mg/L (corresponding to a RH of 5 to 50% at 35°C).

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APPENDIX – STATISTICAL ANALYSIS METHOD DETAILS

The experimental standard deviation of a set of measured survival fractions was calculated in the usual manner for “error propagation,” i.e.,

$$s(N/N_0) = (N/N_0) \sqrt{[s(N)/N]^2 + [s(N_0)/N_0]^2}$$

where N is the mean number of survivors assayed after exposure, N_0 is the mean pre-exposure number, and $s()$ denotes the experimental standard deviation of the quantity inside the parentheses.

The experimental deviation of the effective D-value is slightly more difficult, but was derived from the generalized rule for “error propagation,” i.e.,

$$s^2(y) = \left[\frac{\partial y}{\partial a} \right]^2 s^2(a) + \left[\frac{\partial y}{\partial b} \right]^2 s^2(b) + \dots$$

where y is a function of (a, b, \dots) , the measured parameters and $\partial y / \partial a$ denote the partial derivative of y with respect to a (and so on) with the other parameters held constant. Since our definition of an effective D-value is $(Ct) / \log(NN_0)$, where Ct is the “exposure” (time integral of H_2O_2 concentration),

$$s^2(D_{\text{eff}}) = \frac{s_{Ct}^2}{\log^2(N/N_0)} + \left\{ \frac{s_{(N/N_0)}^2 [Ct \ln(10)]}{-[(N/N_0) \ln^2(N/N_0)]} \right\}^2 = D_{\text{eff}}^2 \left\{ \frac{s_{Ct}^2}{(Ct)^2} + \frac{s_{(N/N_0)}^2}{[(N/N_0) \ln(N/N_0)]^2} \right\}$$

Note that this result differs substantially from the usual form when $y = a^j b^k$.

The general approach to a statistical comparison of the STERIS data sets with the JPL data sets was Student’s t -test. This test requires the calculation of the statistic t for statistically independent measurement sets. With the assumption (supported by the nature of the data or verified by an F test of the experimental variances) that the population standard deviations are equal, one first calculates the pooled estimate of the common sample (experimental) standard deviation:

$$S_p = \sqrt{(s_1^2 + s_2^2) / 2}$$

and then

$$t_c = |(\bar{y}_1 - \bar{y}_2) / [S_p \sqrt{2/n}]|$$

where s_1 and s_2 are the sample standard deviations, and \bar{y}_1 and \bar{y}_2 are the sample means of each data set. The degrees of freedom are $2(n-1)$.

The statistical comparison by Student’s t -test of two sets of data with unequal numbers of

data points, n_1 and n_2 (samples), requires a slightly more complicated version of the formulas:

$$S_p = \sqrt{[(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2] / (n_1 + n_2 - 2)}$$

$$t_c = |(\bar{y}_1 - \bar{y}_2) / [S_p \sqrt{(1/n_1 + 1/n_2)}]|$$

Here the degrees of freedom are $n_1 + n_2 - 2$.

The confidence interval for the hypothetical difference in the population means $\mu_1 - \mu_2$ is given by

$$(\bar{y}_1 - \bar{y}_2) - t_{\alpha/2} SE \leq \mu_1 - \mu_2 \leq (\bar{y}_1 - \bar{y}_2) + t_{\alpha/2} SE$$

$$SE = S_p \sqrt{(1/n_1 + 1/n_2)}$$

where $1 - \alpha$ is the degree of confidence. The value of t for a given α may be looked up in a table or found as a worksheet function in Excel. Note that the Excel function TINV provides a double-tailed value of t for a given value of α and degrees of freedom. The table lookup requires one to look for the single-tailed value corresponding to $\alpha/2$ (and therefore corresponding to the double-tailed value for α).

The usual null hypothesis test is performed by a determination of whether $|t_c| > t_{\alpha/2}$. If so, the null hypothesis (that $\mu_1 - \mu_2 = 0$) is rejected with a significance (confidence) level $1 - \alpha$; otherwise $\mu_1 = \mu_2$ cannot be rejected. Excel also has a function TDIST, which can be used to determine $1 - \alpha_c$, the probability (corresponding to t) that the population means are different.

If the population standard deviations/variances are shown (see F test, below) or known to be statistically different (as for the intercomparison of JPL and STERIS data), then a different statistical form is required, which approximately follows the t distribution.

$$t_c = |(\bar{y}_1 - \bar{y}_2) / \sqrt{(w_1 + w_2)}|$$

and the degrees of freedom v is

$$v = \frac{(w_1 + w_2)^2}{w_1^2 / (n_1 - 1) + w_2^2 / (n_2 - 1)}$$

where $w_i = s_i^2 / n_i$.

The $1 - \alpha$ confidence interval is unchanged

$$(\bar{y}_1 - \bar{y}_2) - t_{\alpha/2} SE \leq \mu_1 - \mu_2 \leq (\bar{y}_1 - \bar{y}_2) + t_{\alpha/2} SE$$

except in this case

$$SE = \sqrt{(w_1^2 + w_2^2)}.$$

The same null hypothesis tests apply.

Dunnett's test is used to test each sample mean of **a-1** treatments \bar{y}_i against the sample mean \bar{y}_a of one more treatment that is the control. It is a modification of the t-test. The null hypotheses are $\mu_i = \mu_a$, where μ_i is the population mean for the i^{th} treatment and μ_a is the population mean of the control treatment. They are rejected at significance $1 - \alpha$ if

$$|\bar{y}_i - \bar{y}_a| > d_\alpha(a-1, f) \sqrt{(SE / (\mathbf{an} - \mathbf{a})) (1/n_a + 1/n_i)}$$

where n_i are the total number of runs for the i^{th} treatment, n_a are the total number of runs for the control treatment,

$$SE = \sqrt{\sum_{i=1}^a \sum_{j=1}^{n_i} (y_{ij} - \bar{y}_i)^2}$$

$f = an - a$, and $d_\alpha(a-1, f)$ may be found in tabular form (C. W. Dunnett, "New Tables for Multiple Comparison with a Control," Biometrics, Vol. 20, No. 3, 1964). It is important to note that this test is more useful if the experimental design has n_a at least as large as $\sqrt{a-1}$.

The standard deviation/variance null hypothesis test with the **F** statistic for the experimental standard deviations (variances) is performed as described here.

$$F_c = \frac{s_1^2}{s_2^2}$$

The null hypothesis that the ratio of the population standard deviations (or variances) is unity is rejected if $F_c < F_{1-\alpha/2}$ or $F_c > F_{\alpha/2}$, with a significance (confidence) level $1 - \alpha$. If the null hypothesis is rejected, then to that significance level the population standard deviations (or variances) are presumed to be different, to that confidence level. The degrees of freedom are $n_1 - 1$ and $n_2 - 1$.

The two-sided level of confidence interval for the ratio of the population standard deviations (variances) is given by:

$$F_c / F_{\alpha/2} < \frac{\sigma_1^2}{\sigma_2^2} < F_c / F_{1-\alpha/2}$$

Again, Excel provides a function FINV that gives the value of **F** for a given value of α and degrees of freedom.

A very important use of the F statistic test is analysis of variance (ANOVA). The usual application is for a case with n measurements of each of **a** treatments. This test will indicate whether one or more of the treatments has a population mean significantly different than the others. The complete variance is decomposed into two parts, MSEE and MStr, where MSEE is the experimental variance within treatments and MStr is the experimental variance between treatments.

$$MSSE = \frac{1}{(an - a)} \sum_{i=1}^a [\langle \bar{y} \rangle - \bar{y}_i]^2 \quad \langle \bar{y} \rangle = \left(\frac{1}{a} \right) \sum_{i=1}^a \bar{y}_i \quad \text{and} \quad \bar{y}_i = \left(\frac{1}{n} \right) \sum_{j=1}^n y_{ij}$$

$$MSStr = \frac{1}{(a - 1)} \sum_{i=1}^a \sum_{j=1}^n [\bar{y}_i - y_{ij}]^2$$

and $F_C = MSStr/MSSE$. Excel has an ANOVA program to perform this analysis on the data set y_{ij} . However, if $SSE = \sum_{i=1}^a [\langle \bar{y} \rangle - \bar{y}_i]^2$, which is (a-1) times the sample variance of

the set of means for the treatment, and $SST = \sum_{j=1}^n [\langle \bar{y} \rangle - y_{ij}]^2$, which is (an-1) times the sample variance of all the data set y_{ij} , have already been calculated, it may be easier to perform the analysis manually. In that case, note that

$$SST = SSE + SStr$$

so that

$$MSStr = \frac{1}{(a - 1)} [SST - SSE].$$