

Extended temperature range studies for dry heat microbial reduction

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ABSTRACT

Dry heat microbial reduction is an approved method to reduce the microbial bioburden on space-flight hardware prior to launch to meet flight project planetary protection requirements. Microbial bioburden reduction also occurs if a spacecraft enters a planetary atmosphere (e.g., Mars) and is heated by frictional forces. However, without further studies, administrative credit for this reduction cannot be applied. The killing of *Bacillus subtilis* var. *niger* spores has been examined and lethality data has been collected by placing spores in a vacuum oven or thermal spore exposure vessels (TSEV) in a constant temperature bath. Using this lethality data, a preliminary mathematical model is being developed that can be used to predict spore killing at different temperatures. This paper will present the lethality data that has been collected at this time and the planned future studies. The results show that rapid ramp-up heating times are critical to obtaining valid lethality data at high temperatures because an extensive number of spores are killed before reaching the target temperature. Exploratory experiments have also been performed using a laser to rapidly heat coupons.

INTRODUCTION

Reduction of microbial bioburden prior to launch helps flight projects meet their Planetary Protection requirements. Dry heat is an approved method to reduce the microbial bioburden on flight hardware prior to launch. Flight hardware manufacturing processes occur at a wide range of temperatures. Collection of lethality data at temperatures above 125°C would establish whether any microbial reduction credit can be offered to the flight project for manufacturing processes that occur at temperatures greater than 125°C.

Presently, the dry heat microbial reduction process is the only NASA approved technique available for this application. For general hardware applications, temperatures of 110°C for 47 hours or 125°C for 5 hours, with an absolute humidity constraint are the

requirements. Humidity is controlled during the dry heat microbial reduction process by using either an ambient pressure of 1.15 Torr (which corresponds to 25% relative humidity (RH) at 0°C at 1 atm pressure) or by a flow of dry nitrogen gas with an indirectly measured RH (e.g., 0.1% RH at 125°C) (NPG 8020.12B).

In addition, collecting lethality data under both controlled humidity and uncontrolled humidity conditions provides a baseline to determine at what temperatures the lethality rate of spore killing is not affected by humidity. Often during the manufacturing of composite structures, as well as other types of hardware, conditions are not controlled to the humidity conditions set forth by NASA in NPG 8020.12B. Thus, performing exposures at humidity conditions that are not controlled provides data that can be used to determine how much credit a project can obtain for the flight hardware manufacturing processes used. Likewise, for those projects that deviate from the specified conditions, this lethality data could be used to provide guidelines for any microbial reduction credit that could be offered.

Microbial bioburden reduction also occurs as flight hardware enters the Martian atmosphere. This "in-flight" bioburden reduction occurs at high temperatures (>200°C) that would not be used during the pre-launch dry heat microbial reduction process because it would damage flight hardware. Typically, burn-up and break-up analyses of flight hardware entering planetary atmospheres are conducted and provide temperature data pertinent to bioburden reduction. There is no humidity constraint for "in-flight" sterilization. Lethality data obtained by this task will be useful to flight projects that would like to receive planetary protection credit for the microbial reduction that occurs as the spacecraft enters the Martian atmosphere.

Because temperatures produced during entry of hardware into planetary atmospheres are significantly greater than 125°C, bioburden reduction data for temperatures between 125°C and 500°C will benefit current and future Mars missions. This data will provide

additional dry heat microbial reduction data (e.g., higher temperature for shorter duration) to meet planetary protection requirements, and will provide a basis to evaluate in-flight sterilization of spacecraft parts subjected to these temperatures during Mars atmospheric entry.

This paper describes the preliminary data that has been collected by this in-progress dry heat task. Survivor curves for temperatures from 115°C to 150°C are presented. This data is being used to develop a preliminary mathematical model to predict spore killing at high temperatures. In addition, the results of using a laser to heat a coupon to high temperatures (>200°C) are being assessed.

MATERIALS AND METHODS

GENERATION OF SPORE COUPONS – *Bacillus subtilis* var. *niger* (ATCC#9372; Raven Biological Laboratories, Omaha, NE) was the test organism used in the exposures. Although it has been reclassified as *B. atrophaeus*, for clarity, it will be referred to as *B. subtilis* var. *niger* in this paper.

Coupons containing *B. subtilis* var. *niger* spores on their surface were prepared by spotting 10 µl of a 10¹⁰ spores/ml suspension onto thin stainless steel discs. The coupons were allowed to dry for two days at room temperature inside a glass petri dish placed in a biosafety hood. The spore coupons were stored inside these petri dishes at room temperature until they were used.

SPORE COUPON EXPOSURES IN A VACUUM OVEN – A vacuum oven (Yamato Scientific America, Inc., Orangeburg, NY) and pump (TriScroll 300 series dry scroll vacuum pump (Varian Vacuum Technologies, Lexington, MA)) were used in these studies (Figure 1, top). Three thermocouples were installed to monitor the internal oven temperature. This vacuum oven was used for both the controlled humidity and the uncontrolled humidity exposures.

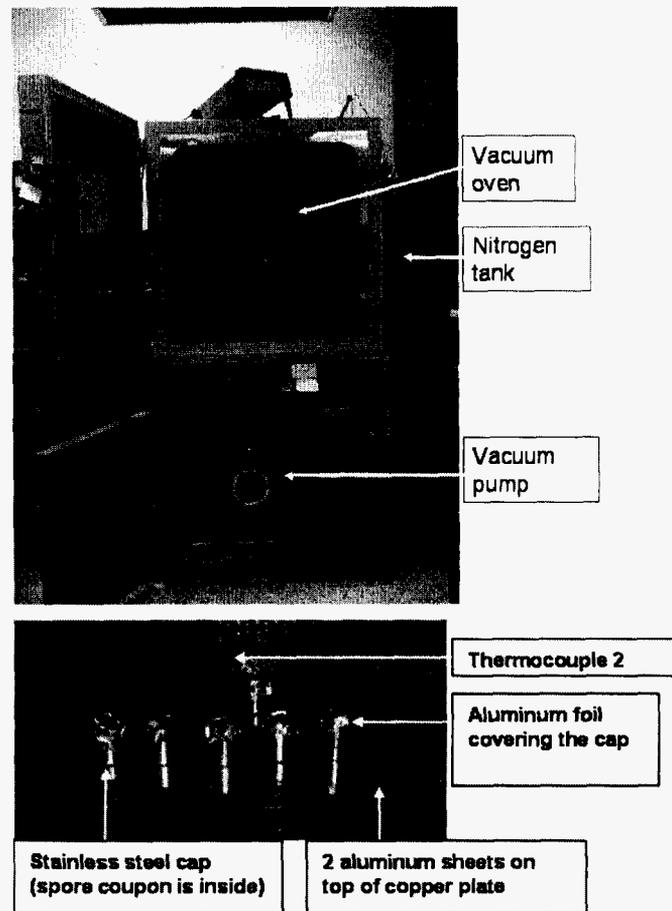


Figure 1. Vacuum oven (top) and stainless steel caps (bottom) used in the controlled and uncontrolled humidity spore coupon exposures.

For controlled humidity exposures, the vacuum oven was set at constant temperatures varying from 115°C to 200°C for any given run (with a vacuum of <1 Torr). Five (5) spore coupons were heated to the designated exposure temperature. Three (3) additional spore coupons were used as the unheated controls in each run. Spore coupons were exposed to the designated temperature by placing the coupons in stainless steel caps (28 mm test tube caps). The caps were covered with aluminum foil and placed on top of aluminum sheets in the preheated oven (at the exposure temperature) (Figure 1, bottom). The exposure time was started when the pressure was <1 Torr and the temperature of two of the three thermocouples in the oven were within 1°C of the exposure temperature. On average, it took 12.5 minutes for the vacuum oven to evacuate and the spore coupons to reach the exposure temperature. To stop the exposure, the vacuum pump was stopped and the vacuum released by filling the oven with dry nitrogen gas. In general, it took about 3 minutes to attain atmospheric pressure and be able to open the door. At the end of the exposure, the caps with the spore coupons were removed from the oven and the spore coupons were processed as described below.

For uncontrolled humidity exposures, the oven was set at a range of temperatures to give exposures of 115°C, 125°C, and 135°C. No vacuum was used during the exposures. Again, five (5) spore coupons were heated to the exposure temperature for each run and three (3) spore coupons were used as the unheated controls. Spore coupons were exposed as described above. In addition, during the uncontrolled humidity exposures, a thermocouple from a digital thermometer (model # Fluke 52 K/J thermometer, John Fluke Mfg. Co., Inc, Everett, WA) was attached to the concave surface of an uninoculated surrogate stainless steel coupon. The thermocouple/coupon set-up was then taped so that the coupon rested on the interior bottom of the cap. The cap was covered with aluminum foil and the surrogate was placed into the oven at the same time as the spore coupons. The temperature of the surrogate coupon/thermocouple set-up was monitored and recorded during the exposure. At the end of the exposure, the caps with the spore coupons were removed from the oven and the spore coupons were processed as described below. The temperature and humidity of the lab where the exposures were performed was measured by a hygrometer (Dickson, model # TM121; Addison, IL).

PROCESSING OF SPORE COUPONS - After each exposure, the spore coupons were processed as follows. The coupons were aseptically transferred to sterile screw-cap tubes containing 10 ml of sterile deionized water. The tubes and coupons were sonicated (25 kHz) for 2 minutes to remove the spores (NHB 5340.1B). Appropriate dilutions of the spore suspensions were made in sterile deionized water and plated in tryptic soy agar (TSA; Difco) using the pour plate method. The colonies on the agar plates were counted after 3 days incubation at 32°C. The number of colony forming units per ml (CFU/ml) was determined for each of the 8 spore coupons and the average CFU/ml was determined for both the heated and unheated coupons.

CONSTRUCTION OF THERMAL SPORE EXPOSURE VESSEL (TSEV) – The TSEV consists of a metal gland (VCR, socket weld, 0.5" internal diameter, stainless steel (item code: H-VGS-8S-B-31L)) that was machined to a wall thickness of 0.010" and a butyl rubber tube stopper (Figure 2). The gland was obtained from and machined by AVF Process Controls (San Pedro, CA).

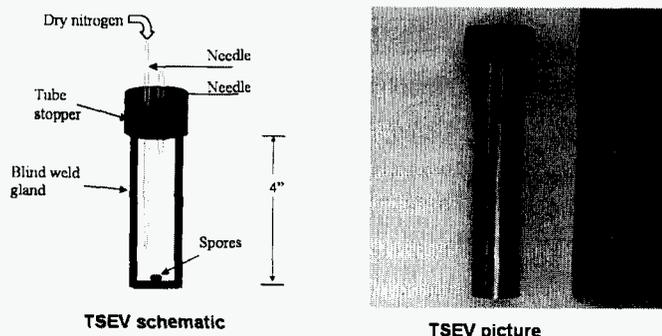


Figure 2. A schematic (left) and picture (right) of the thermal spore exposure vessel (TSEV) used in the uncontrolled humidity TSEV exposures.

EXPOSURES WITH TSEV – A constant high temperature bath (model 6330; Hart Scientific, American Fork, UT) was used for the high temperature exposures with the TSEVs. A bath lid was modified to accommodate the TSEV-3 glands. Silicone oil (Type 200.50) was used in the bath.

The TSEVs were inoculated with *B. subtilis* var. *niger* spores (10^8 or 10^9 spores per TSEV) and allowed to dry at least 2 days at room temperature in a biosafety hood. Butyl rubber tube stoppers were placed into each TSEV. Five (5) inoculated TSEVs were heated in the oil bath while three (3) inoculated TSEVs were used as unheated controls. The inoculated TSEVs and a surrogate TSEV containing a thermocouple were placed into the lid which served as a sample holder. The lid with the TSEVs was placed into the preheated silicone oil bath and the temperature of the surrogate TSEV was recorded with an Agilent 34970 data logger. The temperature profile was displayed on the LabView software program. The TSEVs were exposed for a set period of time. A timer was started when the vessels went into the oil bath. When the timer stopped, the lid with the vessels was removed from the oil bath and placed into an ice bath to rapidly cool the TSEVs. After the vessels had cooled to room temperature, the TSEVs were processed to recover the viable spores.

RECOVERY AND PROCESSING OF SPORES FROM TSEV EXPOSURES - The spores were recovered from the TSEV by adding 2 ml of sterile deionized water and sonicating for 2 minutes (25 kHz; minimum of 0.35 W/cm²). After the sonication, the 2 ml volume was aseptically removed and transferred to a screw-cap glass test tube. The 2 ml water wash, 2 minute sonication, and transfer to the test tube were performed a total of 4 times to collect as many spores as possible. The final volume collected in the test tube was 8 ml. The spore suspension in the test tube was brought to a final volume of 10 ml with sterile deionized water. Appropriate dilutions of the spore suspensions were made in sterile deionized water and plated in tryptic soy agar (TSA) using the pour plate method. The colonies on the agar plates were counted after 3 days of incubation at 32°C. The number of colony forming units per ml (CFU/ml) was determined for each of the 8 exposure vessels and the average CFU/ml was calculated for both the heated and unheated vessels.

DEVELOPMENT OF MODEL – A preliminary mathematical model was developed using MATHCAD software (Mathsoft, Inc., Cambridge, MA). The model predicts microbial reduction as a function of temperature and exposure time. The model assumes that the microbial reduction follows standard first order kinetics and the Arrhenius equation for the time dependence of

the survival rate constants. The model takes into consideration the microbial reduction that occurs during the temperature ramp-up and cool down by using the Arrhenius parameters. It can be used to analyze experimental data, predict microbial reduction at high temperatures not accessible experimentally, and predict total microbial reduction when temperature varies.

LASER HEATING OF SPORE COUPONS – An Argon laser system (Sabre DBW15, Coherent, Inc., Santa Clara, CA) with power outputs of 7.5W, 10W, 12.5W, and 15W was used to heat a stainless steel coupon in a non-vacuum environment. The coupon did not contain any spores. Heating was on the side of the coupon that would be opposite the spores. The experiment was performed using an all lines mirror (a mixture of 488 nm and 514 nm wavelength light). Data (temperature and time) were recorded and a graph of the data was plotted.

RESULTS AND DISCUSSION

SPORE COUPON EXPOSURES IN A VACUUM OVEN (CONTROLLED HUMIDITY) – Figure 3 shows a plot of the survivors (N_t) for the controlled humidity spore coupon exposures in a vacuum oven. Concave-downward curves are seen in the 115°C, 125°C, and 135°C exposures. There appears to be some tailing occurring in the 125°C survivor curve. The 150°C survivor curve is based on only two points: the initial unheated point (N_0) and one exposure data point (0.5 minute exposure).

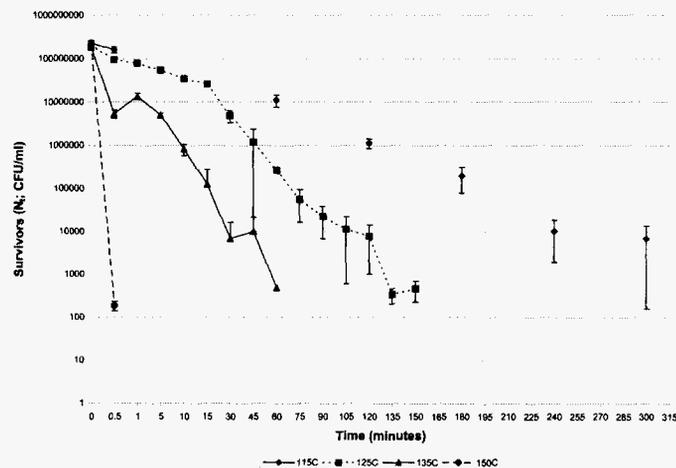


Figure 3. Semi-log plot of the survivors versus time from the controlled humidity spore coupon exposures in the vacuum oven.

Concave-downward survivor curves have a low but increasing rate of destruction during the initial stress period and then exhibit logarithmic destruction (constant rate) at longer times. Concave-downward survivor curves are often described as having a “shoulder”; “multihit” inactivation processes have been proposed as a cause (Moats, 1971). In addition, extensive clumping of cells can result in a delay before the start of logarithmic

decline, resulting in a shoulder (Pflug, et al., 2001). Concave-downward curves are seen in 20 to 25% of spore survivor curves (Pflug, et al., 2001).

“Tailing” is seen in the 125°C controlled humidity exposure. “Tailing” is caused by numerous factors including: faulty experimental testing equipment or procedures (Pflug, 1999); the presence of small numbers of large clumps of cells in the population; and heterogeneity or cell-to-cell differences in heat resistance within the population (Cerf, 1977; Sharpe and Bektash, 1977).

Because the volume of the vacuum oven was large (0.22m³), the temperature ramp-up time was long and a significant fraction of the spores were killed before reaching the target temperature. The amount of spore killing that occurred during the ramp-up varied for the exposures. The approximate spore killing was: 0.5 to 1 log reduction for the 115°C exposures, 0.5 to 1 log reduction for the 125°C exposures, 1 to 2 log reduction for the 135°C exposures, and 6 log reduction for the 150°C exposures. Significant spore killing (7 or 8 log reduction) during the ramp-up to the conditions resulted in insufficient data to draw curves for the 160°C, 175°C, and 200°C exposures.

Lethality rate constants and D-values were determined for each of the controlled humidity exposures at 115°C, 125°C, 135°C, and 150°C obtained by using the vacuum oven. The lethality rate constant was determined from the slope of the line from the N_t/N_0 versus time plot for each exposure (data not shown). The lethality rate constant at varying temperatures is required to generate an Arrhenius plot. The lethality rate constants were used to determine D-values for the exposure temperatures. The lethality rate constant, k , is the reciprocal of the D , (D -value), but there is also a conversion factor to go from natural logs to logs to the base ten. The D -value at each temperature was calculated from the equation:

$$D = 2.303/k$$

where “ k ” is the lethality rate constant and “ D ” is the D -value.

Table 1 shows the preliminary lethality rate constants and D -values for the controlled humidity exposures using the vacuum oven.

Exposure conditions	Temperature (°C)	Lethality rate constant (k)	Calculated D -value
Vacuum oven, controlled humidity	115	0.0359	64.2 minutes
	125	0.0869	26.5 minutes
	135	0.1799	12.8 minutes

	150	28.252	0.08 minutes
Oven, humidity not controlled	115	0.0171	134.7 minutes
	125	0.0654	35.2 minutes
	135	0.2463	9.4 minutes
TSEV, humidity not controlled	125	0.0655	35.2 minutes
	150	1.8493	1.3 minutes

Table 1. Lethality rate constants and D-values that were determined from the exposure data.

SPORE COUPON EXPOSURES IN A VACUUM OVEN (UNCONTROLLED HUMIDITY) – In order to determine if humidity has an effect on the kill rates, both controlled (with vacuum) and uncontrolled (without vacuum) humidity exposures were performed. Uncontrolled humidity exposures were performed at 115°C, 125°C, and 135°C using spore coupons in the vacuum oven which was not evacuated during the exposures. In addition, the temperature of a surrogate coupon was also monitored during the uncontrolled humidity exposures. The temperature and relative humidity of the room were also recorded during the exposures.

Figure 4 shows a plot of the survivors (N_t) for the uncontrolled humidity spore coupon exposures in a vacuum oven. Again, a concave-downward survivor curve is seen in the 115°C exposures, and a sigmoidal survivor curve is seen in the 125°C exposures. After an initial shoulder, a straight-line survivor curve is seen in the 135°C exposures. There appears to be some “tailing” occurring in the 115°C and 125°C survivor curves.

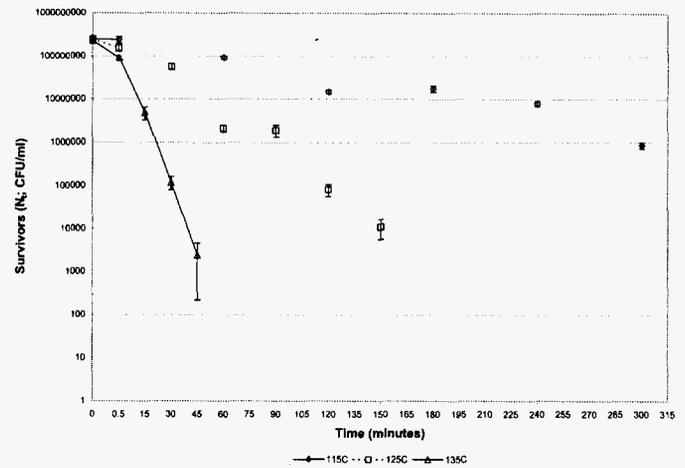


Figure 4. Semi-log plot of the survivors versus time from the uncontrolled humidity spore coupon exposures in the vacuum oven. No vacuum was used during the exposures.

Sigmoidal survivor curves occur infrequently and are usually due to unique conditions (Pflug, et al., 2001). Combinations of the factors that produce concave-upward and concave-downward curves result in sigmoidal survivor curves. Concave-upward curves can result when the test suspension contains subpopulations with different levels of resistance (Pflug, et al., 2001).

After an initial shoulder, which is most likely due to the spore killing that occurs on the ramp-up to the conditions, the 135°C uncontrolled humidity survivor curve is a classic log-linear survivor curve. A straight line through the initial population (N_0) represents a classic log-linear survivor curve. Surprisingly, classic log-linear survivor curves are seen in approximately only 40% of spore survivor curves (Pflug, et al., 2001).

Lethality rate constants and D-values were determined for each of the uncontrolled humidity exposures at 115°C, 125°C, and 135°C in the runs using the vacuum oven without vacuum. The lethality rate constants and D-values were determined as described above. Table 1 shows the lethality rate constants and D-values for the uncontrolled humidity exposures using the vacuum oven (without vacuum).

UNCONTROLLED HUMIDITY EXPOSURES WITH TSEV – Figure 5 shows a plot of the survivors (N_t) for the uncontrolled humidity spore coupon exposures in the thermal spore exposure vessels (TSEV). A sigmoidal survivor curve is seen in the 125°C exposures. After an initial shoulder, a straight-line survivor curve with tailing is seen in the 150°C exposures. Conditions that can result in sigmoidal survivor curves and tailing have been described earlier.

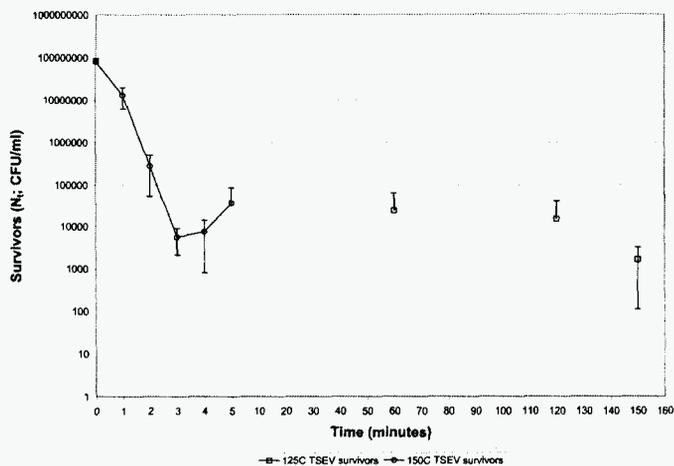


Figure 5. Semi-log plot of the survivors versus time from the uncontrolled humidity spore exposures using the TSEV in the high temperature silicone oil bath.

Lethality rate constants and D-values were determined for each of the uncontrolled humidity exposures using the TSEV. The lethality rate constants and D-values were determined as described earlier. Table 1 shows the lethality rate constants and D-values for the uncontrolled humidity exposures using the TSEV. The lethality rate constant and corresponding D-value for the 150°C TSEV exposures are based on all of the data points. Interestingly, if the data from the 4 minute and 5 minute exposures are not included in the calculations, the lethality rate constant is 3.2734 with a corresponding D-value of 0.7 minutes (or 42 seconds). The tailing that is seen which results in the 4 and 5 minute exposure results may be outlying data points. Repeating some of the 150°C exposures in the TSEV without controlled humidity may provide results that fall closer to the straight-line curve that is observed prior to tailing. Alternatively, repeating the exposures and obtaining similar results would suggest that there is a subpopulation of resistant spores that requires a longer heating exposure to be killed.

LASER HEATING OF COUPON – An Argon laser system with power outputs of 7.5W, 10W, 12.5W, and 15W was used to directly heat a sample coupon in ambient conditions (i.e., no vacuum or controlled humidity). The coupon did not contain any spores. The laser was directed at the side of the coupon that would be opposite the spores. Figure 6 shows the results of the laser heating of a coupon.

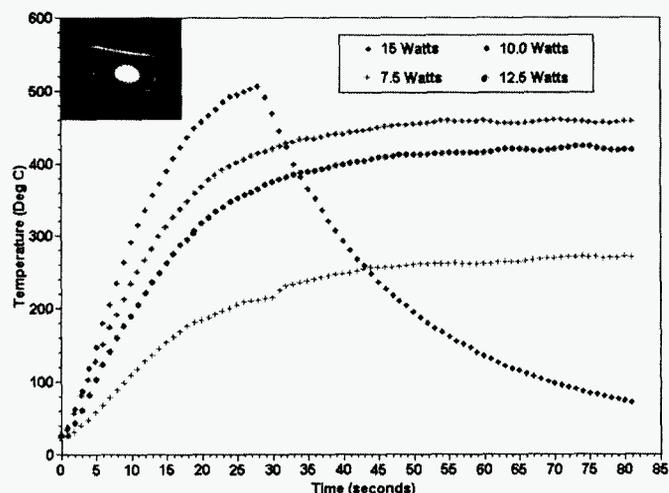


Figure 6. Heating profiles from using an Argon laser to heat a stainless steel coupon.

Based on the preliminary experiment, rapid heating of the spore coupons is possible with an Argon laser. Table 2 shows the time it took to reach high temperature with different laser power settings.

Laser power (W)	Coupon Temperature (°C)	Time to reach temperature (seconds)
7.5	200	24
10	300	18
12.5	400	25
15	500	26

Table 2. Ramp-up heating times it took for the laser heated coupon to reach the target temperature.

At 200°C, the coupon took approximately 17 seconds to cool down under ambient conditions (i.e., there was no cooling apparatus used).

Additional research and development of the laser heating system is necessary before this method can be used to collect experimental data. All of the exposures were transient exposures, i.e., the laser was used to get the coupon to the temperature as quickly as possible, not to hold the temperature. Additional research and development are necessary to control the laser power to reach and maintain the exposure temperature. A method to simultaneously heat several (i.e. 5) spore coupons, while also measuring the temperature of the coupons is needed. Technology development is also necessary to design a vessel/carrier that the spore coupons can be

placed in to meet the exposure conditions. In addition, the costs of supplies, maintenance, and operation of a laser system have not been extensively studied.

FUTURE WORK – Exposures at 175°C in the TSEV without controlled humidity will be performed. After the uncontrolled humidity exposures are completed, controlled humidity exposures in the TSEV will be performed over the same range of temperatures. The results of the controlled and uncontrolled humidity exposures in the TSEV will be compared to the vacuum oven results. Exposures with different *Bacillus* spore species will also be performed and compared to the *B. subtilis* var. *niger* results. Candidate spore species must be identified, obtained, grown and sporulated before lethality data can be collected.

The exposures described in this paper have been performed with surface exposed spores. It will be necessary to perform exposures by using different configurations of the spores, such as mated surface spores and encapsulated spores. *B. subtilis* var. *niger* spores and other identified *Bacillus* spore species will need to be tested in the different spore configurations. The mathematical model will need to be developed further and validated with experimental data. If necessary, the model will be modified. Finally, an experimental approach needs to be identified to perform the high temperature (>200°C) exposures. The high temperature experimental approach may require the modification of a current system or may require custom-design and construction of a system specific for this application.

CONCLUSIONS

Using two experimental approaches (a vacuum oven and thermal spore exposure vessels), exposures have been performed on *B. subtilis* var. *niger* spores to determine the spore killing that occurs at different temperatures under different humidity conditions. This data has been used to develop a preliminary mathematical model that can be used to predict spore killing at different temperatures. This data and mathematical model will be used to predict spore killing at higher temperatures and to develop guidelines to determine the amount of microbial reduction credit a flight project may receive for processes over a wide range of temperatures.

High temperature lethality data is needed to provide a baseline to determine the amount of spore killing that occurs during atmospheric entry. This is especially important for flight projects whose flight hardware components do not reach 500°C for 0.5 seconds (thus, sterility) during atmospheric entry. The experimental approach to perform high temperature (>200°C) exposures will be challenging. A rapid, if not instantaneous, heating ramp-up to the exposure temperature will be necessary to minimize spore killing that occurs on the ramp-up to the high temperatures. It may be possible to modify an existing heating system to be able to meet the requirements necessary for

performing high temperature exposures. Alternatively, a custom-built system, specific for performing high temperature exposure, may be necessary to collect the data. Additional research and development is necessary to get a system that is able to collect the high temperature data necessary to evaluate spore killing during simulated atmospheric entry.

ACKNOWLEDGMENTS

The research described in this paper was carried out by the Jet Propulsion Laboratory, California Institute of Technology, under a contract with NASA.

Funding for this work was provided by JPL's Mars Program Office. We thank current and former Mars Program Planetary Protection Managers Karen Buxbaum, Jim Campbell, and Dave Beatty for their support of this task.

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DEFINITIONS, ACRONYMS, ABBREVIATIONS

D-value: the time required to reduce the bacterial population 10-fold (or 90%) at a specified temperature.