Examination of *B. subtilis* var. *niger* Spore Killing by Dry Heat Methods

Michael J. Kempf and Larry E. Kirschner

Biotechnology and Planetary Protection Group, Jet Propulsion Laboratory, California Institute of Technology, Pasadena, CA 91109

Contact: ***Michael.J.Kempf@jpl.nasa.gov***

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Abstract

Dry heat microbial reduction is the only NASA approved sterilization method to reduce the microbial bioburden on space-flight hardware prior to launch. Reduction of the microbial bioburden on spacecraft is necessary to meet planetary protection requirements specific for the mission. Microbial bioburden reduction also occurs if a spacecraft enters a planetary atmosphere (e.g., Mars) and is heated due to frictional forces. Temperatures reached during atmospheric entry events (>200°C) are sufficient to damage or destroy flight hardware and also kill microbial spores that reside on the in-bound spacecraft. The goal of this research is to determine the survival rates of bacterial spores when they are subjected to conditions similar to those the spacecraft would encounter (i.e., temperature, pressure, etc.). *B. subtilis* var. *niger* spore coupons were exposed to a range of temperatures from 125°C to 200°C in a vacuum oven (at <1 Torr). After the exposures, the spores were removed by sonication, dilutions were made, and the spores were plated using the pour plate method with tryptic soy agar. After 3 days incubation at 32°C, the number of colony-forming units was counted. Lethality rate constants and D-values were calculated at each temperature. The calculated D-values were: 27 minutes (at 125°C), 13 minutes (at 135°C), and <0.1 minutes (at 150°C). The 125°C and 135°C survivor curves appeared as concave-downward curves. The 150°C survivor curve appeared as a straight-line. Due to the prolonged ramp-up time to the exposure conditions, spore killing during the ramp-up resulted in insufficient data to draw curves for exposures at 160°C, 175°C, and 200°C. Exploratory experiments using novel techniques, with short ramp times, for performing high temperature exposures were also examined. Several of these techniques, such as vacuum furnaces, thermal spore exposure vessels, and laser heating of the coupons, will be discussed.

Introduction

- NASA Planetary Protection:
  - Responding to international agreement: Committee on Space Research (COSPAR)
- Planetary Protection Goals:
  - To prevent "forward" contamination of extraterrestrial bodies
  - To prevent "back" contamination of sample return missions
- Planetary Protection Functions:
  - Inform, plan, implement, and confirm that the flight project meets the mission specific planetary protection requirements
  - Identify, develop, and test new technologies for use to meet the planetary protection needs of projects

Introduction (cont)

- Dry heat:
  - NASA approved approach for microbial bioburden reduction and terminal sterilization of spacecraft
  - Typical temperature range: 110°C to 125°C with controlled humidity
- *Bacillus* spores:
  - 4 main components (core, cortex, inner spore coat, outer spore coat)
  - Resistant to various chemical and physical agents (i.e., desiccation, heat, oxidizing agents, and UV and γ-radiation)
  - Factors involved in dry heat resistance:
    - DNA protection by small, acid-soluble proteins (SASP)
    - Spore core mineralization

Methods

- **Dry heat exposures with vacuum oven**

  B. subtilis var. *niger* spore coupons were exposed in a vacuum oven (Yamato Scientific America, Inc., Orangeburg, NY) with a vacuum pump (SH-100 dry scroll vacuum pump or TriScroll 300 series dry scroll vacuum pump (Varian Vacuum Technologies, Lexington, MA)). See the next slide for the procedure.

- **Vacuum furnace manufacturers**

  Three vacuum furnace manufacturers were contacted to see if they offered a vacuum furnace to meet the dry heat requirements (small exposure chamber, rapid heat-up to 500°C temperature (maximum), pressure of <1 Torr, possibly down to 10^{-6} Torr, rapid cool down).

- **Thermal spore exposure vessels (TSEV)**

  Stainless steel parts were purchased (AVF Process Controls, San Pedro, CA) and machining modifications were performed at the JPL Machine Shop. A coupon holder was designed and machined to hold 1 spore coupon inside the gland. Two prototype TSEVs were designed; one with a machined gland wall thickness of 0.030" and one with a machined gland wall thickness of 0.010". The prototype TSEVs were tested in a high temperature oven with Xceltherm HT heat transfer fluid (Radco Industries, LaFox, IL).

- **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 sample coupon, without spores, in a non-vacuum environment. 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).

Spore coupon exposures in vacuum oven

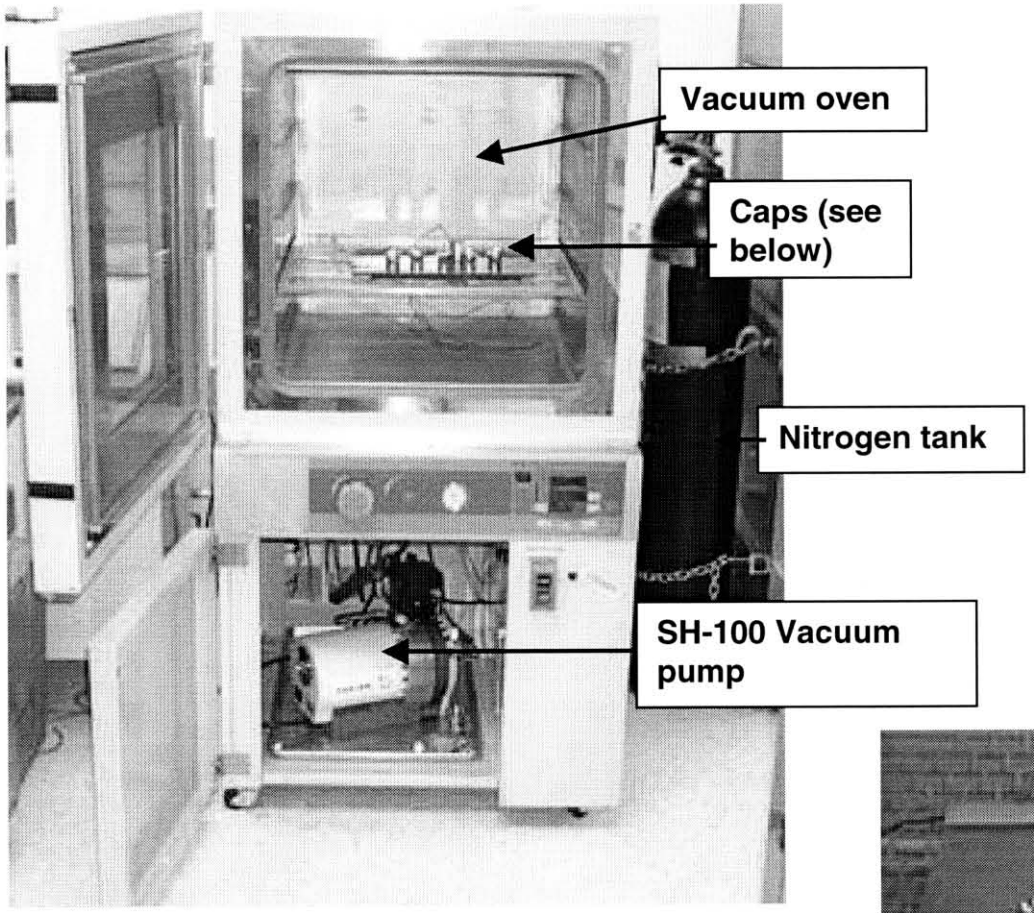
- Pre-exposure: *B. subtilis* var. *niger* (ATCC 9372) spore coupons (10^8 spores/coupon) were placed into 28 mm diameter stainless steel caps, covered with aluminum foil, placed into the preheated oven, and the vacuum pump started. Typical ramp times were 10 to 15 minutes to reach the exposure temperature and pressure.

- Exposure: Timing for the exposure was started when the oven thermocouples were within 1°C of the exposure temperature and the vacuum pressure was <1 Torr. 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 3 minutes to backfill the oven with nitrogen.

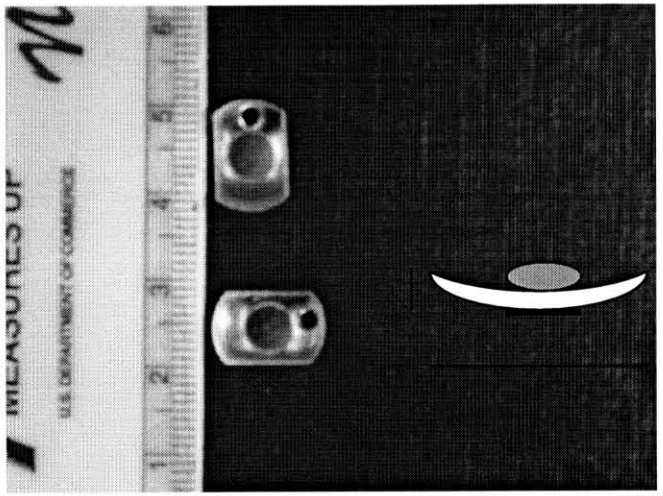
- Post-exposure processing: The coupons were aseptically transferred to sterile screw-cap tubes containing 10 ml of sterile water. The tubes and coupons were sonicated for 2 minutes at 25 kHz to remove the spores. Appropriate dilutions of the spore suspensions were plated in Difco tryptic soy agar (TSA) using the pour plate method. Five (5) spore coupons were heated at each time point. Three (3) spore coupons were used as the unheated controls at each time point. The agar plates were counted after 3 days incubation at 32°C.

- Plate counting and analysis: 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 the heated and unheated coupons.

Vacuum oven, pump, and spore coupons



Spore coupons for exposures



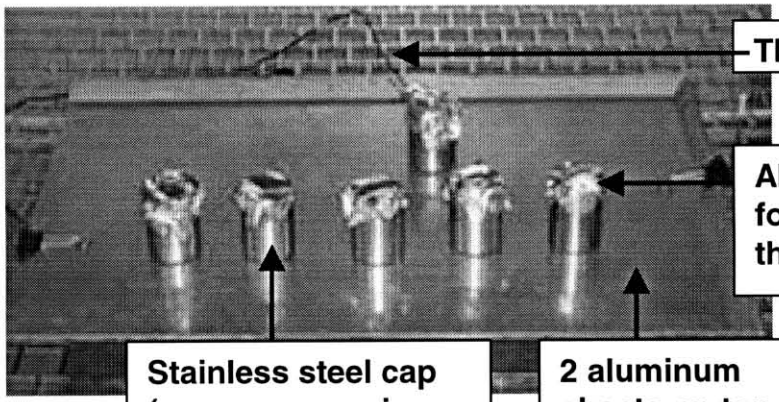
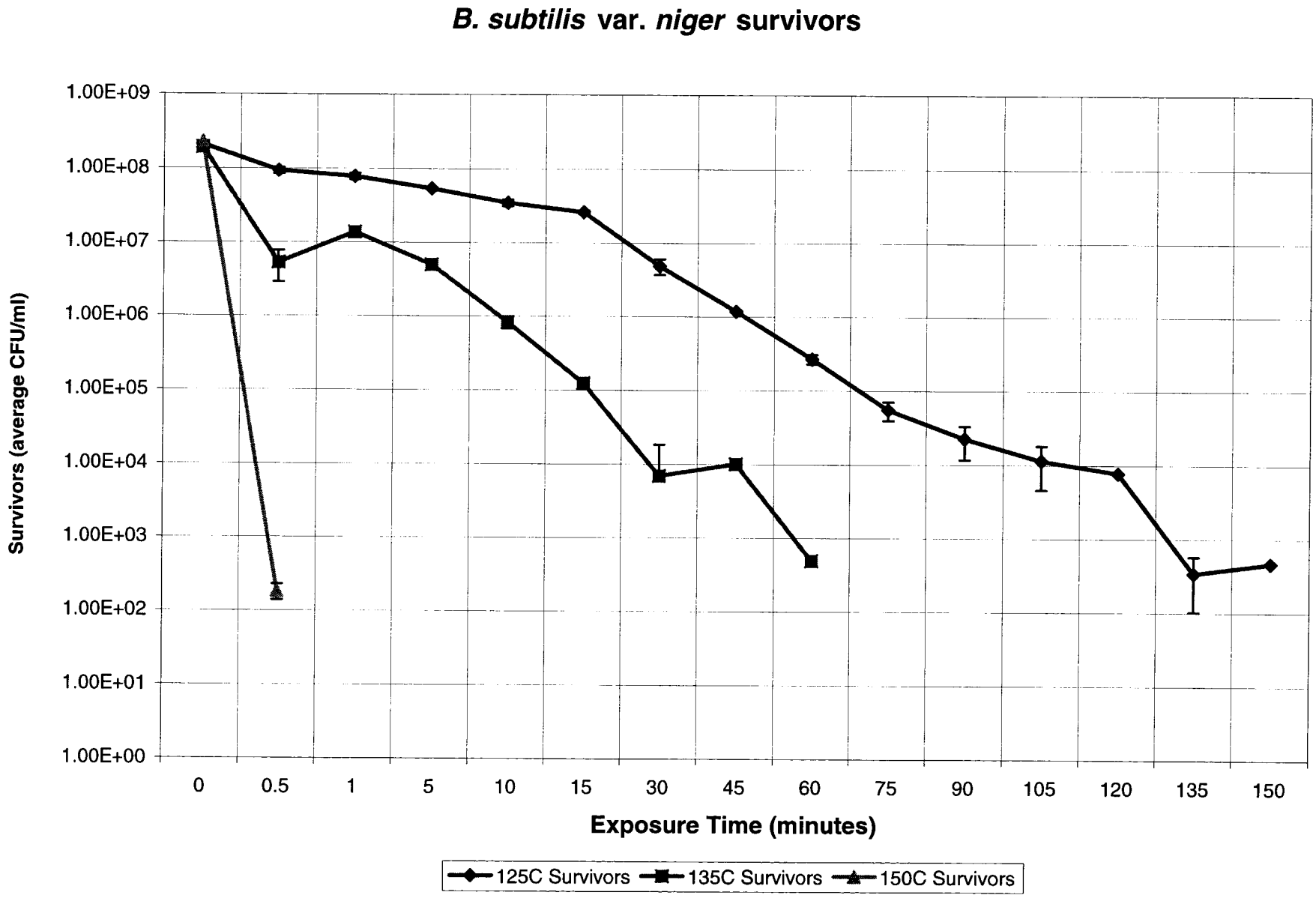


Table of *B. subtilis* var. *niger* survivors after dry heat

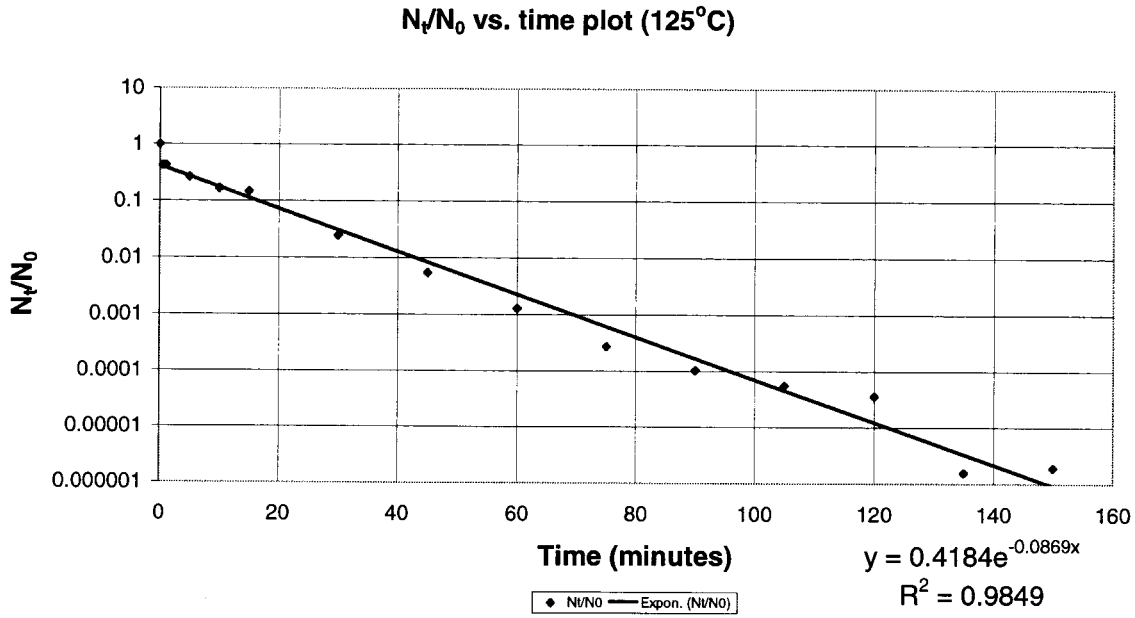
	Average Survivors (N_t) (CFU/ml)							
Time (min)	125°C	135°C	150°C	160°C	175°C	200°C		
0	2.11E+08	1.94E+08	2.31E+08	2.39E+08	2.18E+08	2.17E+08		
0.5	9.43E+07	5.34E+06	1.84E+02	Too Few	Not Tested	Too Few		
1	7.88E+07	1.38E+07	Too Few	Too Few	Too Few			
5	5.41E+07	5.01E+06						
10	3.50E+07	8.33E+05						
15	2.62E+07	1.25E+05						
30	4.88E+06	6.96E+03						
45	1.18E+06	1.01E+04						
60	2.68E+05	4.91E+02						
75	5.54E+04	Too Few						
90	2.26E+04	Too Few						
105	1.14E+04							
120	7.80E+03							
135	3.39E+02							
150	4.62E+02							
165	Too Few							
182	Too Few							
Calculated lethality rate constant (k)	0.0869	0.1799	28.252					
Calculated D-value (minutes)	26.5	12.8	0.08					
[a] = unheated CFU/ml is based on the average unheated CFU/ml from all exposures at that temp.								
Too few = growth, but not enough growth to be statistically relevant (i.e., less than 30 colonies (average))								

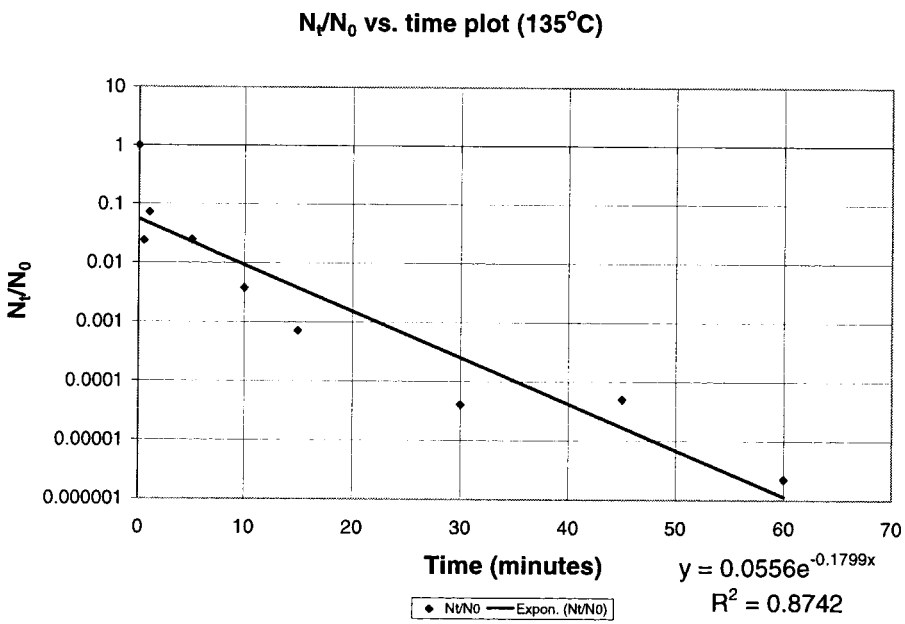
$$k = \pm 2.303 \frac{1}{D}$$

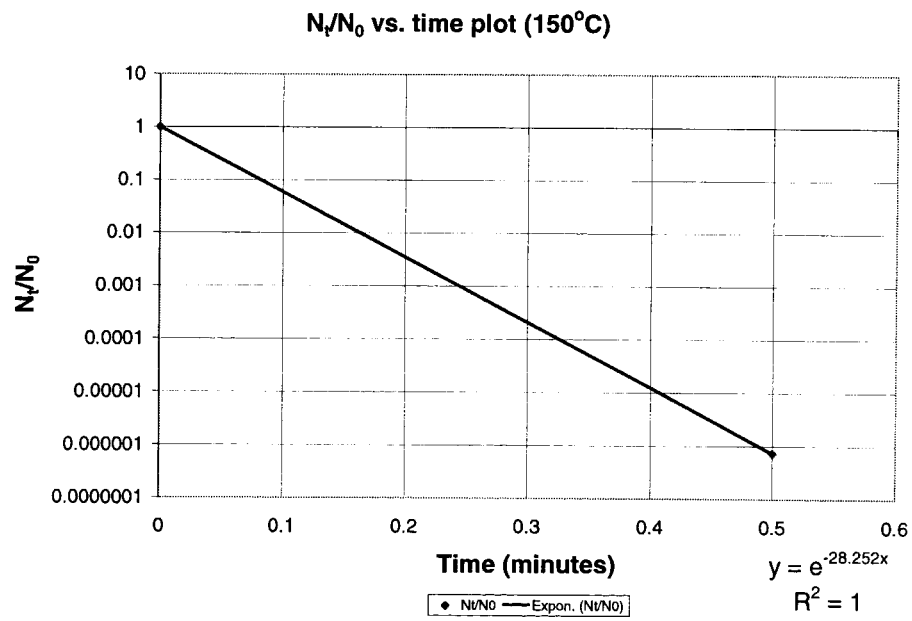
Graph of *B. subtilis* var. *niger* survivors after dry heat



Lethality rate constant curves







Arrhenius plot

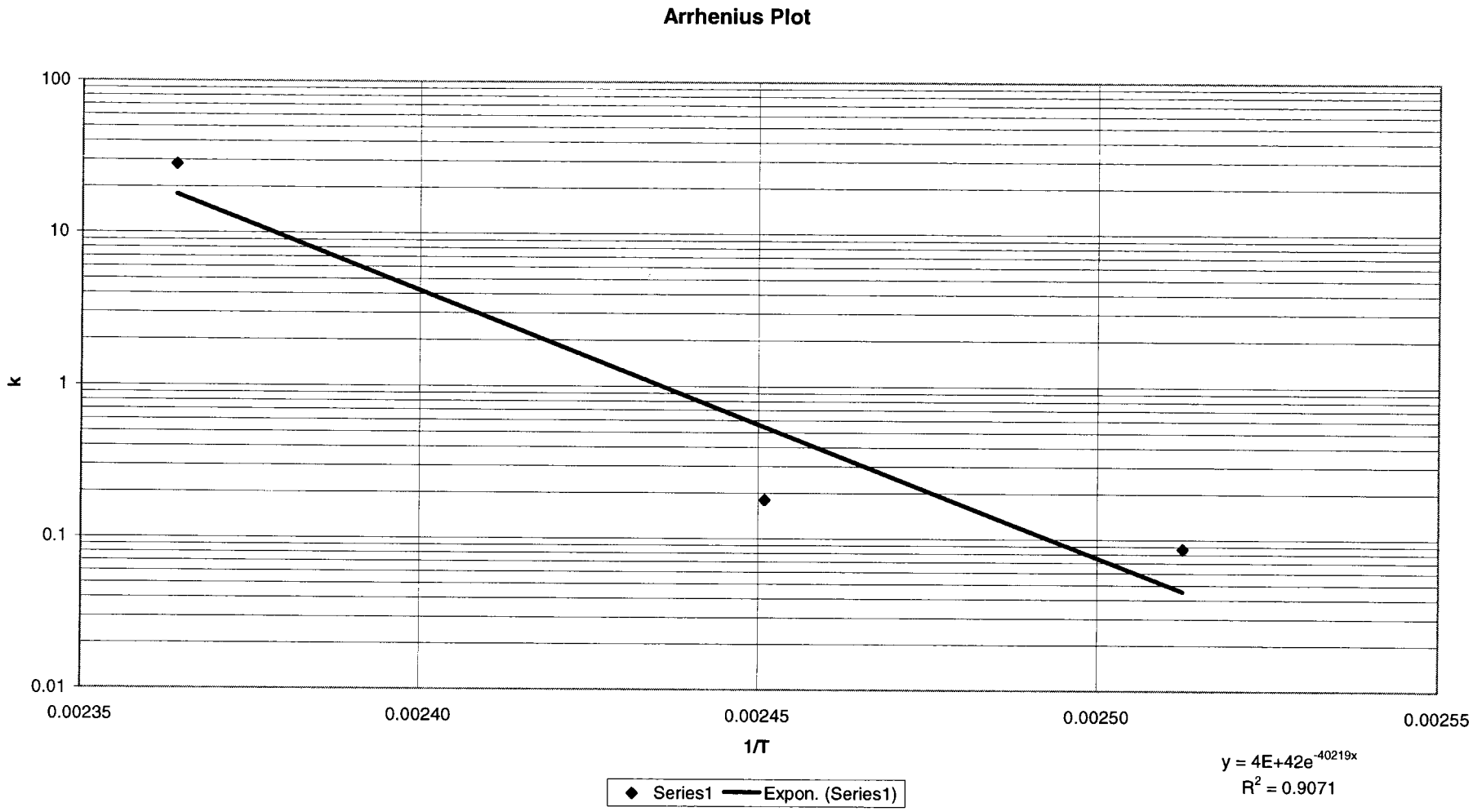


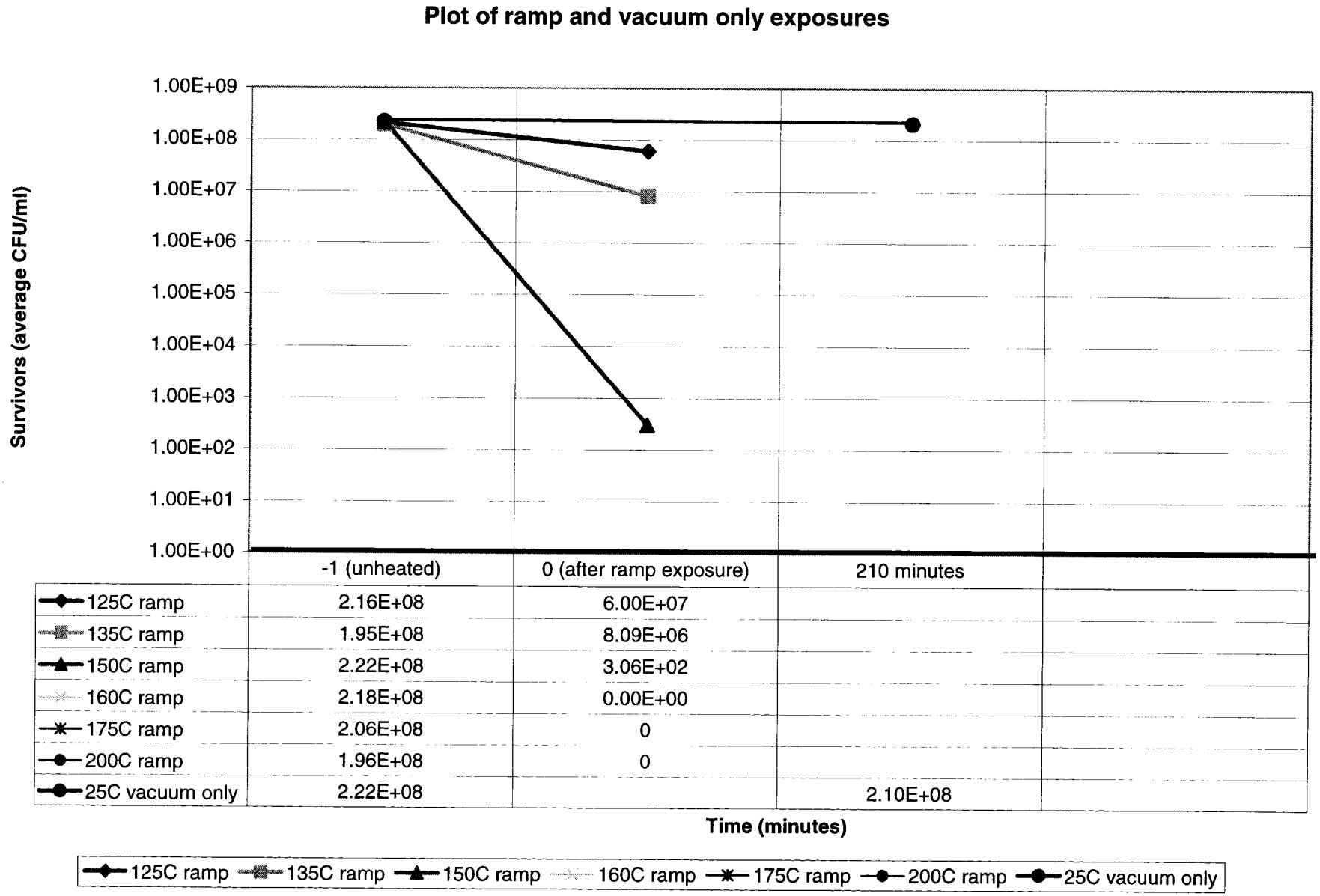
Table for Arrhenius plot				
Exposure Temperture (°C)	Absolute Temperature (T) (in K)	1/Absolute Temperature (T)	Lethality rate constant (k)	Correlation Coefficient (R^2)
125	398	0.00251	0.0869	0.9849
135	408	0.00245	0.1799	0.8742
150	423	0.00236	28.252	1

Lethality rate constants and D-values for *B. subtilis* var. *niger* spores exposed to dry heat

Temperature (°C)	Lethality rate constant (k) (from N_t/N_0 vs. time plots)	Calculated D-value (minutes)
125	0.0869	26.5
135	0.1799	12.8
150	28.252	0.08

$$k = \pm 2.303 \frac{1}{D}$$

Ramp and vacuum only exposures



	-1 (unheated)	0 (after ramp exposure)	210 minutes	
125C ramp	2.16E+08	6.00E+07		
135C ramp	1.95E+08	8.09E+06		
150C ramp	2.22E+08	3.06E+02		
160C ramp	2.18E+08	0.00E+00		
175C ramp	2.06E+08	0		
200C ramp	1.96E+08	0		
25C vacuum only	2.22E+08		2.10E+08	

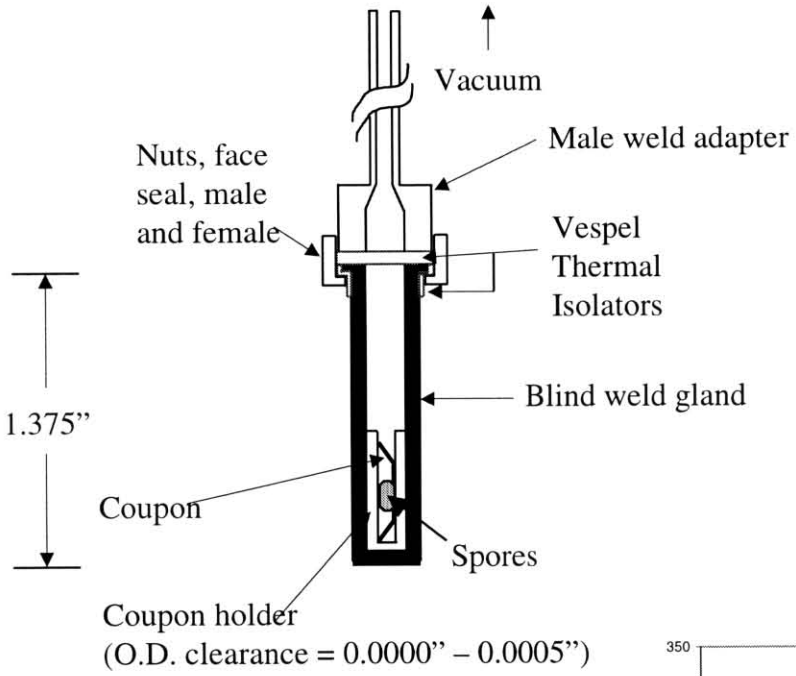
Vacuum oven conclusions

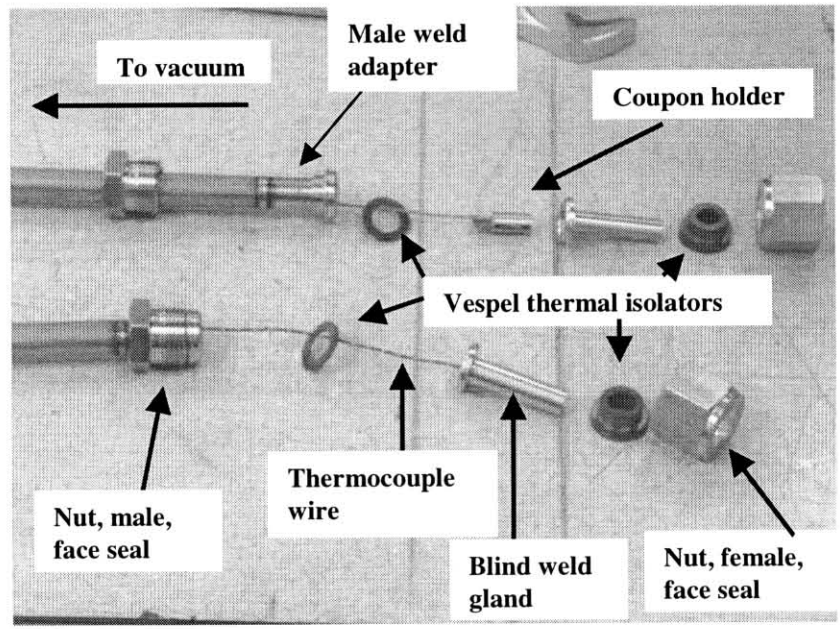
- Vacuum oven results
  - Lethality rate constants and D-values were calculated at 125°C (0.0869; 26.5 minutes), 135°C (0.1799; 12.8 minutes), and 150°C (28.252; 0.08 minutes) for *B. subtilis* var. *niger* spores on coupons.
  - 125°C and 135°C survivor curves appeared as concave-downward curves
    - Possible reasons: clumping of spores on coupons; variability of resistance in spore population
  - Exposures at 150°C and above were not possible, due to the extended ramp times to temperature and pressure, resulting in spore killing prior to reaching the exposure conditions.
  - Vacuum (0.1 Torr) alone did not result in spore killing at 25°C.
- Future work
  - Exposures with different *Bacillus* species (environmental isolates from spacecraft assembly facilities and flight hardware)
  - Exposures with spores in different surface configurations (mated and encapsulated)

High temperature exposures

- Goals
  - To perform exposures and collect data above 200°C to determine lethality rate constants and D-values
- Significance
  - Provides a foundation to evaluate sterilization of spacecraft parts as they enter the Mars atmosphere
  - Has potential to reduce the planetary protection implementation cost to a flight project
- Needs
  - Rapid ramp up to temperature and ramp down cooling
    - Small exposure chamber
  - Controlled environment
  - Temperature range: 200°C up to 500°C (maximum)
    - Temperatures <200°C also beneficial
  - Rapid data acquisition methods

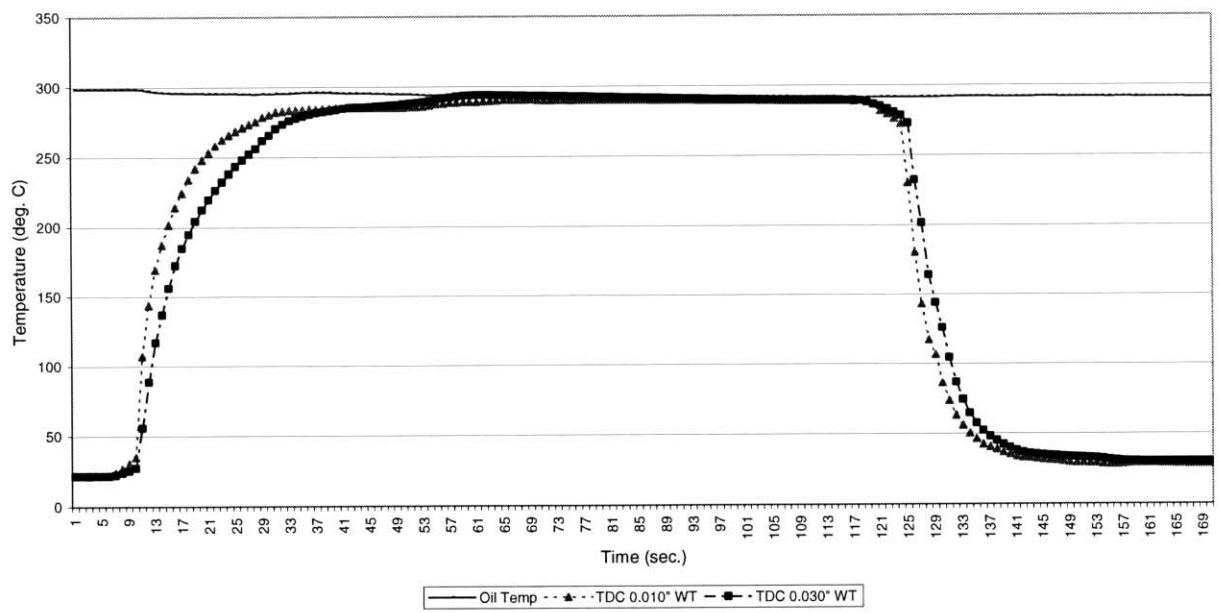
Thermal Spore Exposure Vessel (TSEV)



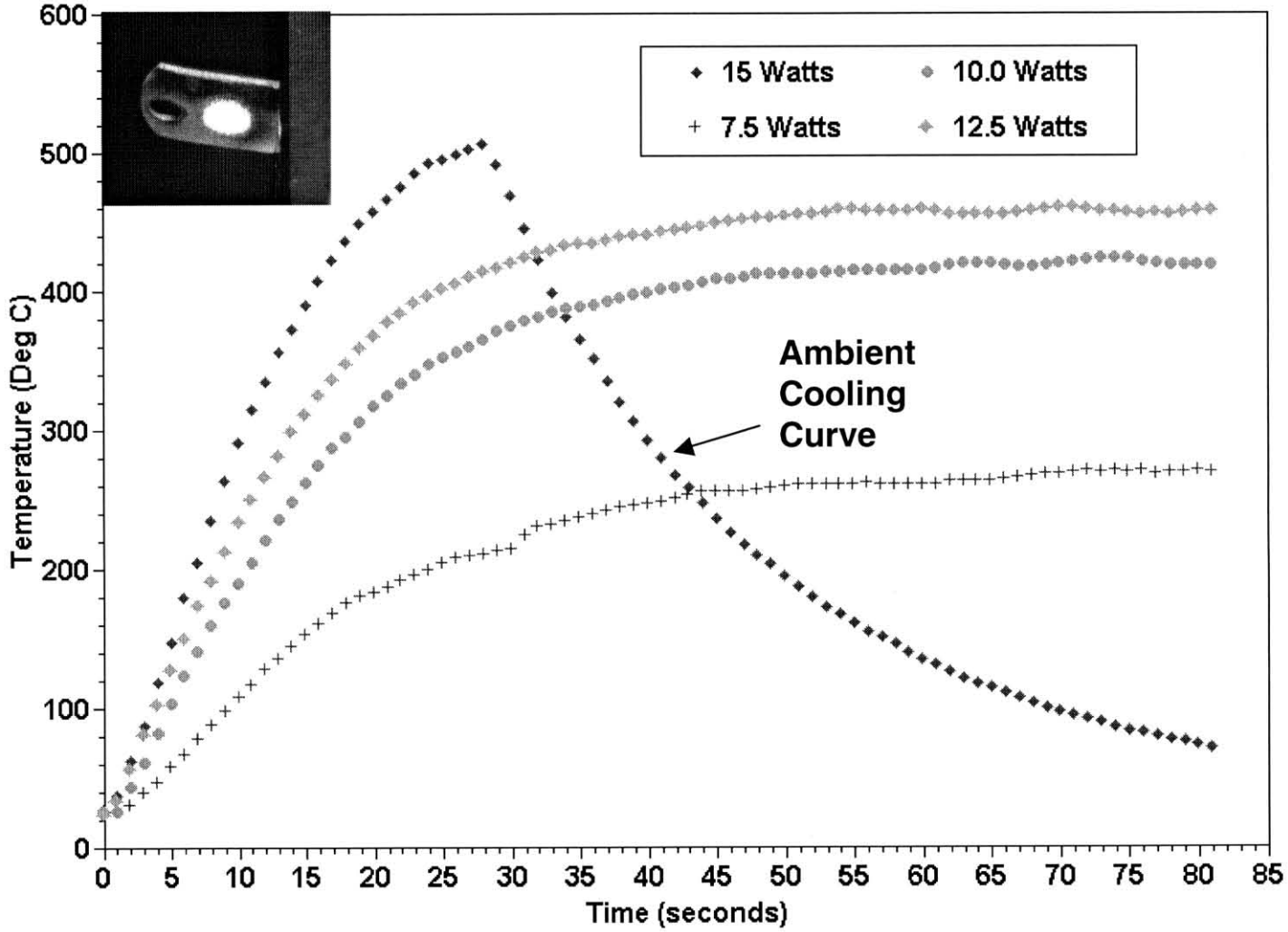


Thermal Spore Exposure Vessel, w/o coupon & holder, ~300 deg. C

-All parts are stainless steel; insulator is Vespel.
-Two thicknesses were designed: 0.03" and 0.01".
-Length of gland: 1.375"
-Mass of entire TSEV: 12.6g (0.01"); 6.7g (0.01" w/o coupon holder)



Rapid heating of stainless steel coupons with an Argon laser



High temperature conclusions

- Vacuum furnace
  - Based on cost and necessary modifications to furnaces to meet specific requirements, vacuum furnaces were not chosen for the high temperature work
- TSEV
  - 0.010" thickness TSEV resulted in faster ramp times to the exposure temperature than the 0.030" thickness TSEV
  - Additional testing and modifications to the TSEV are necessary to collect high temperature data
- Laser heating
  - Fast ramp times and 500°C exposures are possible
  - Additional testing and system development are necessary before this method can be used to collect high temperature data

Acknowledgements

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- Personnel
  - Robert S. Leland, Virgil Miriles, and Jose Rodriguez (thermal engineers) and Andy Lamborn (manufacturing engineer) for assistance in designing and testing the thermal spore exposure vessels
  - James Lambert for performing the laser heating of spore coupons
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