

An Overview of Surface Heat Microbial Reduction as a Viable Microbial Reduction Modality for Spacecraft Surfaces

T. Brian Shirey[1], Wayne Schubert[2], and James Benardini[3]
Jet Propulsion Laboratory, California Institute of Technology, Pasadena, CA, 91109

In accordance with NASA Planetary Protection (PP) policy requirements, flight project hardware may be required to undergo microbial reduction processes to prevent the forward contamination of target planetary bodies with Earth organisms. Heat microbial reduction (HMR) is the most commonly employed modality used at JPL for reducing the microbial bioburden on flight hardware. In 2013, longstanding HMR specifications were abandoned, and revised specifications were developed which integrated the latest findings on bacterial spore heat resistivity. Revised decimal reduction values (D-values) for time-temperature lethality curves (110 °C to 200 °C) were developed to account for "hardy" bacterial spores that exhibit greater heat resistance than previously understood. Presented here is a comparative analysis of the revised NASA HMR specifications against empirical data compiled from recent JPL studies, and peer-reviewed, published literature. *Bacillus* sp. strain ATCC 29669 displayed high heat resistance, and this strain's 4-log heat lethality curve was comparable to the revised 4-log specification. Spores of *Bacillus atrophaeus* ATCC 9372 displayed less heat resistance, and exhibited D-values which were less than the revised 3-log microbial reduction specifications. Extrapolations indicate that the current 6-log reduction credit applied to 350 °C for 1 hr. and 500 °C for 0.5 sec. is highly conservative. Projections indicate that a 10- to 18-log reduction of both hardy and non-hardy spores may be achievable at bakeouts of 350 °C for 1 hr. The findings reported here indicate the revised NASA HMR specifications from 110 °C to 200 °C are appropriate for achieving 4-log and 6-log reductions with hardy spore populations; however, for non-hardy spore populations, or for temperatures above 200 °C, the specifications are exceedingly conservative.

Nomenclature

JPL	=	Jet Propulsion Laboratory
NASA	=	National Aeronautics and Space Administration
NPR	=	NASA Procedural Requirements
BPPG	=	Biotechnology and Planetary Protection Group
HMR	=	Heat Microbial Reduction
DHMR	=	Dry Heat Microbial Reduction
PP	=	Planetary Protection
D-value	=	Decimal reduction time

I. Introduction

NASA Planetary Protection (PP) policy establishes the microbial reduction requirements for flight project hardware to prevent the forward contamination of planetary bodies with Earth organisms. NASA PP policy is set forth in NASA Procedural Requirement 8020.12D Planetary Protection Provisions for Robotic Extraterrestrial Missions (hereafter referred to as NPR 8020.12D)[1]. NPR 8020.12D sets forth the compliance requirements for the control of terrestrial microbial contamination associated with robotic spacecraft that intend to land, flyby, or otherwise encounter planetary target bodies (forward contamination), as well as the control of contamination of the Earth from material of extraterrestrial origin (backward contamination). Current PP-related activities are monitored by NASA's

[1] Planetary Protection Engineer, Biotechnology and Planetary Protection Group, Jet Propulsion Laboratory, California Institute of Technology, Pasadena, CA.
[2] Research Scientist, Biotechnology and Planetary Protection Group, Jet Propulsion Laboratory, California Institute of Technology, Pasadena, CA.
[3] Planetary Protection Engineer, Biotechnology and Planetary Protection Group, Jet Propulsion Laboratory, California Institute of Technology, Pasadena, CA.

Planetary Protection Officer (PPO), who is responsible for certifying to the NASA Science Mission Directorate Associate Administrator (SMD AA) that all PP requirements have been met prior to launch.

Of the approved microbial reduction processes available for flight project implementation, dry heat microbial reduction (DHMR) is the process most often employed by the Jet Propulsion Laboratory Biotechnology and Planetary Protection Group (JPL BPPG) to ensure compliance with NASA policy on flight hardware biological cleanliness. In 2013; however, NASA revised the longstanding DHMR specifications outlined in NPR 8020.12D in order to integrate the latest findings on heat microbial reduction (HMR) processes. "Dry" (< 25% relative humidity at 0 °C and 1 atmosphere) heat is no longer a strict requirement though current standard practice for flight hardware "bake-outs" employs DHMR. The revised specifications allow for HMR processes that occur under ambient (absolute humidity controlled to 70% RH at 20 °C under 1 atmosphere) humidity conditions. With support from the Mars Program Office, the revisions were based in large part on experimental results generated by the JPL BPPG in conjunction with the European Space Agency, which in turn were used to provide recommendations for the revised NASA specifications[2-4].

Prior to revision, standard NASA practice for DHMR was based on processes validated in the NASA Viking mission era (circa 1960's). The Viking era specification was subsequently implemented forward into the current Mars Science Laboratory-era (MSL) era. Among other restrictions, those parameter specifications provided a narrow temperature range (104 °C to 125 °C) for the DHMR of flight hardware. The time-temperature decimal reduction values (D-values; the time required to reduce the size of the microbial population by one log [90%]) provided in the pre-revised specifications were based on heat-lethality data applied to the most appropriate biological indicator species known at that time. Since that time however, there has been a notable increase in the DHMR knowledge-space, and includes a more in-depth understanding of bacterial spore resistance to heat. Recent research has confirmed that the heat-hardy bacterial spore population was more resistant than previously known, which led to the recommendation that a revision of NASA's DHMR specifications should be considered.

Presented here is a comparative analysis of the newly-revised NASA HMR specifications against empirically-derived laboratory data compiled from reviews of historical, published literature, as well as data synthesized from more recent investigations conducted by the JPL BPPG. The primary objective of this effort was to elucidate the current margin between NASA specifications and empirically-derived data, thus providing information on the degree of conservatism within the revised NASA HMR specifications. This spore lethality data will help determine whether the degree of conservatism in the revised specifications for HMR are justified and appropriate based on the current understanding.

II. Dry Heat Microbial Reduction Historical Background

To provide historical context to the current HMR approach, and to form a foundation for comparisons, a thorough literature review of relevant, peer-reviewed, heat microbial reduction studies was performed. This review also served to highlight the progress toward developing a more complete understanding of bacterial spore resistance to heat. The metabolically dormant bacterial spore is extremely resilient to environmental perturbations, characterized by an increased resistance to high temperatures and other damaging environmental effects[5]. For example, in the first half of the 20th century, spore heat-resistance was most often attributed to novel physiological traits and unusual extracellular secretions, whereas it is now understood that spore killing by dry heat occurs largely, if not completely through DNA damage[6], and this process is dissimilar to that of wet-heat spore inactivation process. With continued progress within the field of DHMR process biology, advancements in the application of dry heat to ensure spacecraft cleanliness have also emerged. For instance, although wet-heat is much more effective at spore destruction than dry heat[6,7], spacecraft surface sensitivity and compatibility with wet-heat make it an impractical microbial reduction modality. Thus, the current depth of knowledge regarding the efficacy of DHMR for NASA flight hardware has led to it becoming the gold-standard in spacecraft surface decontamination. It is clear that the cornerstone of this knowledge base was set in place by numerous foundational studies, and has been continually reinforced through the better part of a century of research into heat microbial reduction.

In the 1920's, investigators had already begun probing the science behind spore heat lethality[8,9]. The findings from much of that early work have since been expounded upon with our current understanding of heat microbial reduction. Early work on thermal death time curves by Esty and Bigelow in the 1920's set the groundwork for spore heat lethality studies, and led to a resurgence of investigations in this field as evidenced by the efforts of Pflug and colleagues in the 1950's, 1960's and decades beyond[10-22]. Whereas some of that early work concluded that spore heat resistance varied considerably between species and depended on several key, yet unknown factors, we are now equipped with a much broader understanding of bacterial inactivation kinetics which allows us to throw light upon many of those unknown factors.

The recently abandoned NASA DHMR specifications were rooted in decades old science, and recent work indicated that at least a portion of that science was no longer relevant for current missions. Moreover, the Viking-era specifications were ill-equipped to face the increasingly stringent requirements of NASA PP policy. The previous Viking-era NASA specifications offered a narrow range at which DHMR could be performed on flight hardware. The limitation was, in part, due to the dearth of relevant information on spore inactivation at temperatures beyond 100 °C. Beginning in the late 1960's and early 1970's a number of investigators set out to better understand spore inactivation at higher temperatures[23-25]. In 1967, Busta[23] presented temperature survivor curves for *Bacillus* spores for temperatures above 100 °C and suggested they have concave, Weilbullian-like distributions; a finding which was verified and confirmed much later[26]. In 1968, Angelotti et al.[25] were among the first to perform exhaustive tests on the dry-heat resistance of *B. subtilis* spores deposited on spacecraft-relevant materials. That study identified several factors which affect microbial resistance to dry heat, and generated data that later proved useful in the development of the first spacecraft sterilization cycles. These mid-late century studies, set the framework for our current understanding of DHMR and how that can be utilized in a flight hardware setting.

Recent studies on DHMR have advanced our understanding of spore inactivation kinetics and spore heat-lethality response. There have been numerous linearity spore survival curves presented over the entirety of heat microbial reduction research, and the results of that work have led to a variety of alternative spore survivability models[27]. Much of that work led to the development of novel formulas for calculating D-values and z-values for microbial heat lethality curves, but has also led to some highly conservative assumptions. In 2003, Conesa reported that survival curves of *B. subtilis* spores obtained under isothermal conditions followed first order kinetics; however, under non-isothermal conditions the spores displayed convex survival curves which were generally flat during initial ramp up, then displayed a rapid decrease in survival when temperatures reached approximately 100 °C[27]. In contrast, later work concluded that the survival curves of most spores were not log linear, and that microbial inactivation does not typically follow first-order kinetics, implying that the probability of a lethal event at a given temperature is constant and does not depend on the duration of heat exposure[26]. The authors of that study indicated that non-linear spore survival curves were due to a mixed spore population, and proof of first-order kinetic spore inactivation required confirmation by isolating the subpopulations and independently determining their rate constants. It has become clearer in recent years, that the underlying science behind spore lethality from dry heat is a muti-faceted, complex subject which requires the continuous update of prior assumptions as the knowledge in this field develops.

III. NASA Dry Heat Microbial Reduction Specifications

In accordance with NPR 8020.12D, microbial reduction for planetary spacecraft shall be accomplished only by an approved microbial reduction process. These processes must be based on rigorous examination of supplied data, and must demonstrate conclusively the biological effectiveness and reproducibility of the microbial reduction method under consideration. NPR 8020.12D defines the time-temperature dependence parameter D-value of 0.5 hours (at 104 °C to 125 °C) for surface DHMR, 1 hour for mated surfaces, and 5 hours for encapsulated (spores embedded within non-metallic spacecraft material) materials. The parameter further specifies the heat-lethality parameter definition as the time required to destroy 90% of the non-hardy microbial spore population subjected to dry heat at 125 °C. The conditions to achieve absolute sterility as defined in NPR 8020.12D are 500 °C for ≥ 0.5 seconds. The specifications outlined by the Viking-era requirements were appropriate for calculating lethality up to the level of the hardy (heat resistant) fraction, but could not be used to predict lethality greater than a 3-log reduction for microbial spore populations on spacecraft surfaces. The constraints of the Viking-era specifications are less appropriate for integration into current missions, and were not derived from the most recent research findings. Thus, the search began for providing NASA with recommendations on an updated and current approach for the implementation of DHMR processes.

The HMR specifications shown in Table 1 and Table 2 are the revised NASA HMR specifications which are now implemented for NASA Mars missions with HMR requirements. Where the Viking-era requirement allowed for microbial reduction credit for temperatures ≤125 °C, the revision extends that range to 200 °C. In addition, the revised specification allows for a microbial log reduction of up to 6-log for HMR processes above 125 °C.

Table 1 indicates the revised D-values required to achieve a 2 or 3-log microbial reduction for surfaces and encapsulated materials. To calculate the time required for a 2 or 3-log microbial reduction, the D-values in Table 1 must be calculated by multiplying the D-value by factor of 2 or 3 respectively. To achieve a 4 to 6-log reduction, the D-values in Table 2 must be used. A 4, 5, or 6-log reduction can be achieved by multiplying the respective D-values in Table 2 by a factor of 1, 2, or 3, respectively; however, a maximum of 4-log reduction can be credited for temperatures < 125 °C. In addition, temperature dependent D-values for mated surfaces shall be calculated by multiplying the D-values in Table 2 by a factor of 2, and for encapsulated bioburden by a factor of 10. For example, to calculate the time required to achieve a 6-log reduction for a mated surface at 140 °C, the equation for T > 130 °C in Table 2 must be used, and multiplied by a factor of 3 for a 6-log reduction, and also by a factor of 2 to account for mated surfaces (i.e. $3 * 2 * 10^{(-19.1595 + 8320.082 / (T + 273))}$). The current specification does not allow for a log microbial reduction that is less than 2 or greater than 6 for any configuration or condition.

Table 1. Heat microbial reduction D-values for reducing spore population by 2 to 3 orders of magnitude.

Configuration	D-Value, hours	Humidity	Temperature, T, °C
Surfaces	$0.5 * 10^{((125 - T) / 21)}$	Dry	$110 \leq T \leq 140$
Surfaces	$0.0965 * 10^{((140 - T) / (23 * T / 140))}$	Dry	$T > 140$
Surfaces	$0.0965 * 10^{((140 - T) / 18))}$	Ambient	$110 \leq T \leq 140$
Surfaces	$0.0965 * 10^{((140 - T) / (23 * T / 140))}$	Ambient	$T > 140$
Surfaces	$10 * 0.5 * 10^{((125 - T) / 21)}$	Uncontrolled	$110 \leq T \leq 140$
Surfaces	$10 * 0.0965 * 10^{((140 - T) / (23 * T / 140))}$	Uncontrolled	$T > 140$
Encapsulated	$5 * 0.5 * 10^{((125 - T) / 15)}$	Uncontrolled	$116 \leq T \leq 125$
Encapsulated	$5 * 0.5 * 10^{((125 - T) / 21)}$	Uncontrolled	$125 \leq T \leq 140$
Encapsulated	$5 * 0.0965 * 10^{((140 - T) / (23 * T / 140))}$	Uncontrolled	$T > 140$

Table 2. Heat microbial reduction D-values for reducing spore population by 4 to 6 orders of magnitude.

Configuration	D-Value, hours	Temperature, °C
Surfaces, mated, and encapsulated	$10^{(-3.5991 + 2049.0923 / (T + 273))}$	$110 \leq T \leq 130$
Surfaces, mated, and encapsulated	$10^{(-19.1595 + 8320.082 / (T + 273))}$	$T > 130$

IV. Laboratory studies on Dry Heat Microbial Reduction

The JPL-directed laboratory studies presented here, were conducted to provide empirical inputs and recommendations for the revision of the Viking-era NASA HMR specifications[2,4]. As noted in Dry Heat Exposures of Surface Exposed and Embedded *Bacillus atrophaeus* Spores[2], there were a number of claims regarding dry heat lethality rates for temperatures between 104 °C to 125 °C; however, many of the observations were never validated experimentally. To provide relevant inputs into the revised specifications, the JPL studies required the development of novel techniques which would be complementary to prior studies, sufficient enough to allow for meaningful statistical analyses, rigorous enough to pass peer-review, and to measure the disparity in lethality rate constants applicable to spacecraft surfaces. For additional comparative context, a review of historical data regarding heat microbial reduction was also compiled and compared against heat-lethality curves calculated from the revised NASA specifications.

V. Dry Heat Microbial Reduction Analysis and Findings

A. Bacterial Spore Indicator strains and reference data

Reference data for comparisons were compiled from a review of published literature, and data derived from laboratory-derived experiments conducted at JPL. The historical data reviewed includes heat microbial reduction studies on spores of various *Bacillus* species and includes strains of *Clostridium* sp. The data compiled from studies

performed at JPL include the bacterial spore reference strains *B. atrophaeus* ATCC 9372, and *Bacillus* sp. ATCC 29669. All of the data presented were derived from bacterial spores and do not reflect heat lethality of bacterial vegetative cells.

B. atrophaeus ATCC 9372 strain spores (hereafter referred to as *B. atrophaeus*) represents the non-heat hardy historical indicator for DHMR processing, and is often used as the industry standard biological indicator species for heat lethality. This species of bacteria is among the most heat resistant of the common lab *Bacillus* spp. laboratory strains, and was used to develop the Viking-era DHMR specifications[3]. *B. atrophaeus* is also a NASA cleanroom-associated species.

Bacillus sp. ATCC 29669 is an atypical species of *Bacillus*, and also associated with NASA flight assembly cleanrooms. While this species is one of the most heat-resistant spore-forming organisms known, its presence in NASA cleanroom is a rare occurrence. The heat-resistance of ATCC 29669 represents the heat-hardy fraction within the NASA heat lethality curves, carrying a D-value for DHMR that is approximately 20 to 35 times that of *B. atrophaeus*[4]. Using ATCC 29669 allowed for a conservative heat-resistant reference strain which justified the recommendation of increasing the upper practical DHMR limit to 200 °C[3]. Per NPR 8020.12D, the heat-hardy organisms represent only a fraction (the heat-hardy fraction) of the total spore population, which has been confirmed by more recent studies.

B. Current NASA Heat Lethality Specifications Versus Historical Data

Bacterial spore heat-lethality data (n = 67) were compiled[28] into Figure 1 in order to present a comparison to the revised 3-log, 4-log, and 6-log heat-lethality curves derived from the revised D-values for spacecraft surfaces (Table 1; ambient). The data is presented as a representative snap-shot on historical laboratory studies on spore heat microbial reduction, and forms the foundation for comparison to current NASA specifications. Comparisons indicate that the majority of empirically-derived historical data for heat microbial reduction would have D-values that are lower than the NASA D-values for a 3-log reduction for surfaces under ambient conditions.

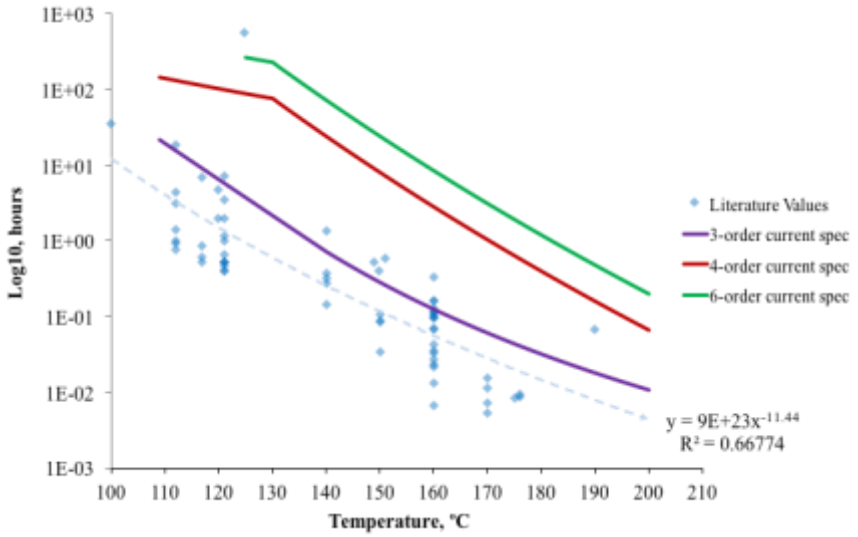

Figure 1. Empirical results from published literature compared to the revised NASA specification heat-lethality curves for 3-log, 4-log, and 6-log heat microbial reductions.

C. Current NASA Heat Lethality Specifications Versus *Bacillus atrophaeus* Reference Strain

For the JPL study on *B. atrophaeus* heat lethality, the spores were exposed to temperatures ranging from 115 °C to 170 °C. *B. atrophaeus* data presented in Figure 2 represents the summation of those results.

Results from JPL laboratory studies on *B. atrophaeus* heat lethality[2] closely resemble that of the historical data shown in Figure 1. This finding is not surprising as *B. atropheaus* is considered the industry standard biological indicator for spore heat lethality studies, thus should fall in line with the majority of historical data. The JPL investigators determined that the data produced from laboratory experiments using *B. atrophaeus* could be used to revise NASA's longstanding approach on heat microbial reduction, and were in fact used to revise NASA's D-values and are currently implemented in NASA missions.

D. Current NASA Heat Lethality Specifications Versus *Bacillus* spp. ATCC 29669 Reference Strain

In addition to studies on the non-heat hardy B. atrophaeus spores, JPL research scientists also investigated the heat lethality effects on the high heat resistant *Bacillus* sp. ATCC 29669 strain[4]. Researchers within the JPL Planetary Protection and Biotechnology Group incorporated this cleanroom-associated biological indicator into their recommendation for the newly revised NASA D-values as its title of "hardy" organism remains in place. The data generated from JPL's recent study further supported the expansion of the process specification to temperatures beyond 125 °C, along with loosening the dry requirement for HMR. With the results provided on ATCC 29669, NASA's revised HMR specifications is extended to account for one of the most heat-resistant spore forming strains that has ever been characterized from a NASA spacecraft assembly environment.

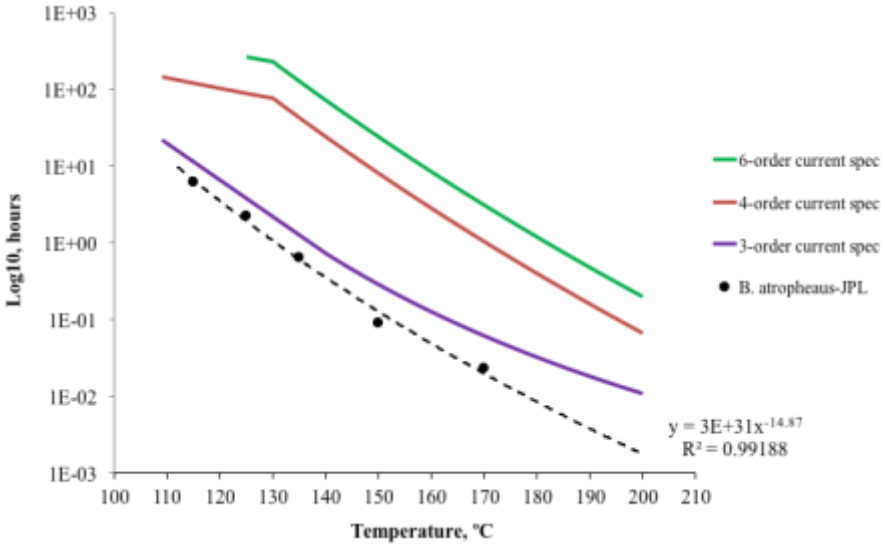

Figure 2. A 4-log heat-lethality curve of *B. atrophaeus* compared to revised NASA heat lethality curves for 3-log, 4-log, and 6-log heat microbial reductions.

Although the adoption of expanded process specifications has provided more options for planning and implementation of microbial bioburden reduction for compliance with PP requirements, the incorporation of data derived from studies on ATCC 29669 has led to much higher conservatism in the NASA HMR specifications. Figure 3 shows the comparison between ATCC 29669 heat lethality curve for a 4-log reduction against the revised NASA specifications for 3-log, 4-log, and 6-log microbial reductions.

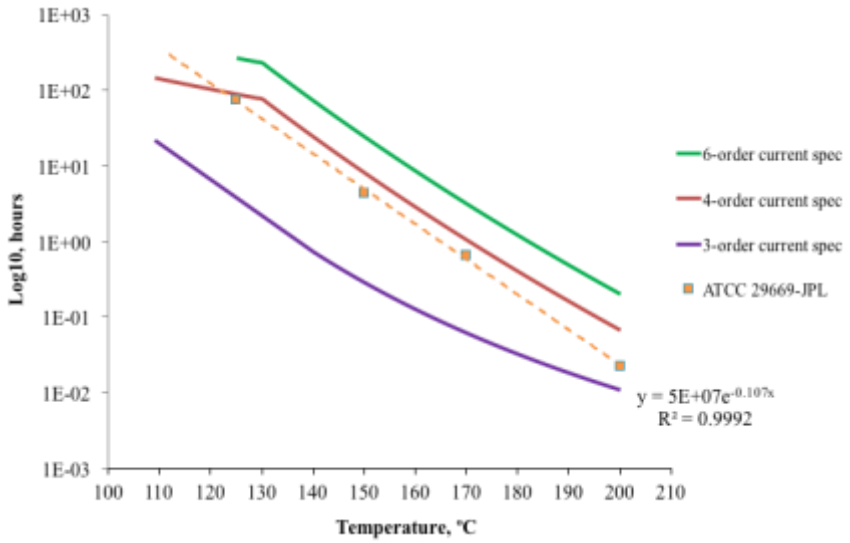

Figure 3. A 4-log heat-lethality curve of *Bacillus* sp. ATCC 29669 compared to revised NASA heat lethality curves for 3-log, 4-log, and 6-log heat microbial reductions.

VI. Degree of Conservatism in the Revised NASA Heat Microbial Reduction Specifications

The current approach for implementation of NASA's revised HMR specifications includes an incorporation of some degree of conservatism. When the revised specifications are compared to empirically-derived data, the degree

of conservatism within the revised specification is most notable for *B. atrophaeus* which represents the more common spore strain encountered in NASA assembly facilities (Table 3). This conservatism in the revised specification can exceed 2 orders of magnitude for a 6-log reduction for *B. atrophaeus*. The degree of conservatism within the revised NASA specifications when compared to the heat-hardy ATCC 29669 strain are less pronounced however. Only at 125 °C and for a 4-log microbial reduction does the revised NASA specification enter into conservatism with respect to ATCC 29669, and at no point from 100 °C to 200 °C does the degree of conservatism in the revised NASA specification exceed 1-log.

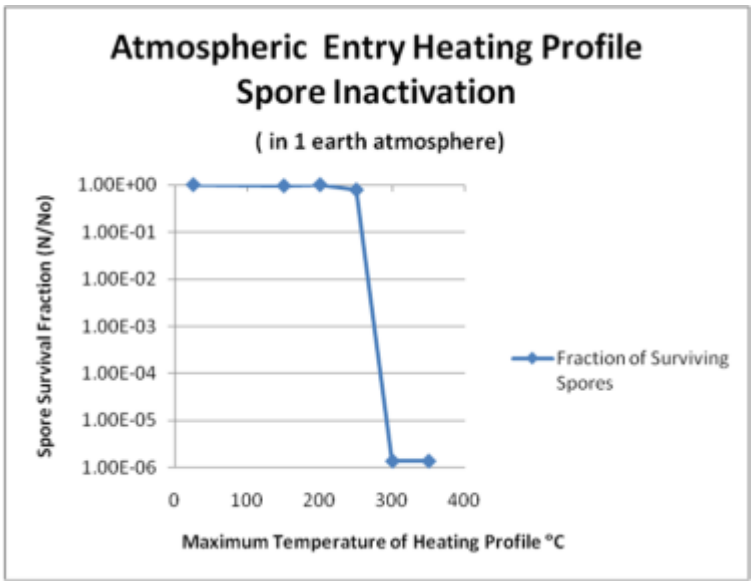

Figure 4. Rapid heating profile of *B. atrophaeus* spores with target temperature reached within 90 seconds from initiation of heating.

While there are some gaps in our understanding of the spore inactivation rates at temperatures beyond the range of NASA specifications, extrapolations from the laboratory data presented here indicate that the maximum allowable 6-log reduction credit is highly conservative at temperatures beyond 200 °C. Using a custom designed rapid heating system, temperatures between 125 °C and 350 °C have been preliminarily tested by the JPL BPPG. Figure 4 displays the temperature profile for a rapid temperature increase, where the target temperature was reached in less than 90 seconds. These findings indicate that the spores will be inactivated at temperatures considerably less than the current specification value of 500 °C for 0.5 seconds. Although this preliminary data represents a single data set, the temperature inactivation curve closely parallels that of other recent studies on high temperature-short time spore inactivation studies[29-31].

Table 3. Heat microbial reduction log-margins in hours for empirical data derived from literature and JPL studies for a) 3-log, b) 4-log, and c) 6-log microbial reductions.

a)

3-log	$\triangle \log_{10}(\text{time}) = \log_{10} \frac{Time\ Ref}{Time\ Spec}$				
	112 ºC	**125 ºC**	**150 ºC**	**170 ºC**	**200 ºC**
Literature	-0.83	-0.68	-0.26	-0.49	-0.81
***B. atropheaus*-JPL**	-0.36	-0.35	-0.44	-0.55	-0.95
ATCC 29669-JPL	+1.20	+1.18	+1.25	+0.90	+0.20

b)

4-log*	$\triangle \log_{10}(\text{time}) = \log_{10} \frac{Time\ Ref}{Time\ Spec}$				
	112 ºC	**125 ºC**	**150 ºC**	**170 ºC**	**200 ºC**
Literature	-1.64	-1.93	-1.55	-1.60	-1.48
***B. atropheaus*-JPL**	-1.12	-1.59	-1.72	-1.66	-1.57
ATCC 29669-JPL	+0.37	-0.07	-0.03	-0.21	-0.47

c)

6-log	$\triangle \log_{10}(\text{time}) = \log_{10} \frac{Time\ Ref}{Time\ Spec}$				
	112 ºC	**125 ºC**	**150 ºC**	**170 ºC**	**200 ºC**
Literature	-	-	-2.08	-1.90	-1.78
***B. atropheaus*-JPL**	-	-	-2.25	-1.96	-1.92
ATCC 29669-JPL	-	-	-0.56	-0.51	-0.77

Figure 5 shows an extrapolation of ATCC 29669 heat microbial reduction data to 500 °C. This data indicates that the current NASA specifications of 6-log reduction for 500 °C for 0.5 seconds is highly conservative. The projection was generated from the 12-log extrapolation of the most conservative estimate derived from laboratory data. This data indicates the time required for a 12-log reduction of ATCC 29669 under DHMR at temperatures from 200 °C to 500 °C ranges from 6 minutes to 62 microseconds, respectively.

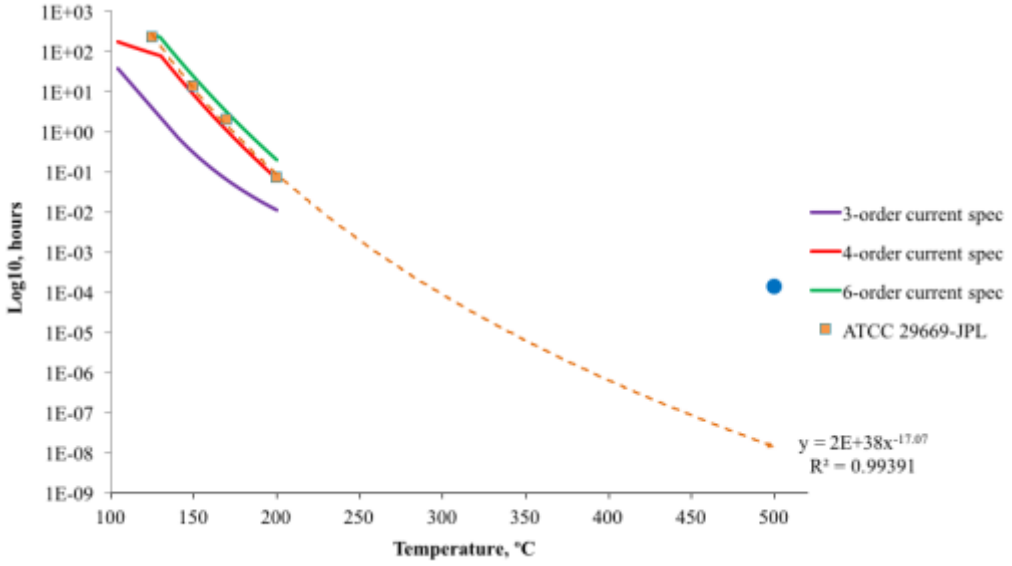

Figure 5. Laboratory derived results and projected curve for 12-log reduction of *Bacillus* spp. ATCC 29669 compared to NASA revised specifications for dry heat microbial reduction.

VII. Conclusion

Recent studies on DHMR have advanced our understanding of spore inactivation kinetics and spore heat-lethality response. The findings reported here indicate the revised NASA HMR specifications from 110 °C to 200 °C are appropriate for achieving 4-log and 6-log reductions with hardy spore populations; however, for non-hardy spore populations, or for temperatures above 200 °C, the specifications are exceedingly conservative. For more detailed information regarding HMR at temperatures above 200 °C, additional studies are necessary. This provides an initial assessment on the degree of conservatism within the newly-revised NASA HMR specifications, and serves as a starting point for discussions on future work which may further elucidate this unique field of research.

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