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Hydrazine vapor inactivates *Bacillus* spores

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Abstract

NASA policy restricts the total number of bacterial spores that can remain on a spacecraft traveling to any planetary body which might harbor life or have evidence of past life. Hydrazine, N_2H_4, is commonly used as a propellant on spacecraft. Hydrazine as a liquid is known to inactivate bacterial spores. We have now verified that hydrazine vapor also inactivates bacterial spores. After *Bacillus atrophaeus* ATCC 9372 spores deposited on stainless steel coupons were exposed to saturated hydrazine vapor in closed containers, the spores were recovered from the coupons, serially diluted, pour plated and the surviving bacterial colonies were counted. The exposure times required to reduce the spore population by a factor of ten, known as the *D*-value, were 4.70 ± 0.50 h at 25 °C and 2.85 ± 0.13 h at 35 °C. These inactivation rates are short enough to ensure that the bioburden of the surfaces and volumes would be negligible after prolonged exposure to hydrazine vapor. Thus, all the propellant tubing and internal tank surfaces exposed to hydrazine vapor do not contribute to the total spore count.

Keywords: Hydrazine; Planetary Protection; Spores; Lethality rate; *Bacillus*; *D*-value

1. Introduction

NASA policy restricts the total number of bacterial spores on any spacecraft encountering solar system bodies where extinct or extant life could exist. A total spore budget of 5×10^5 spores at launch was specified in Mars Science Laboratory (MSL) Project Planetary Protection Implementation Plan and summarized by Benardini et al. (2014b). Hydrazine, N_2H_4, is commonly used as a propellant on spacecraft. An investigative study was carried out to establish the survival rate of bacterial spores exposed to the vapor of the liquid rocket fuel hydrazine. The purpose of this work was to provide experimentally established bacterial spore inactivation rate data used in calculating the bioburden associated with the propulsion system for the Mars Science Laboratory Mission.

The MSL propellant tanks could not undergo dry heat microbial reduction (DHMR) because the internal liner called a diaphragm, or bladder, was made from a rubber material designated AF-E-332 (Weiss and Guernsey, 2013). There were engineering concerns that this material could have been potentially damaged by heating. It was suspected that the material could become brittle or less flexible if subjected to standard dry heat microbial reduction DHMR temperatures of 125 °C for 50 or more hours. The diaphragm or bladder is an integral component of the propellant tank system and could not be handled independently. The DHMR treatment was not considered to be a viable engineering approach due to uncertainties of material compatibility with elevated temperature.

If this research had not been carried out, it would have been necessary to assign the large spore density of 30 spores/cm^{3}for the volume and 10^5spores/m^2 for the surfaces of the bladder and tank (a value assigned for uncontrolled manufacturing), as specified in NASA Procedural

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Requirements, NPR 8020.12 (NASA, 2011). The MSL descent stage propulsion system had three propellant tanks, each carrying approximately 133 kg of high purity hydrazine (Weiss and Guernsey, 2013). The spore bioburden that would have been assigned to the hydrazine alone would have exceeded the total allowable spore bioburden for the entire spacecraft. This study was complimentary to efforts invoked to reduce the bioburden from all spacecraft components (Benardini et al., 2014a, b).

This work describes the research study performed to understand and account for the additional bioburden associated with the hydrazine propellant system. Previous studies in our laboratory revealed that the rocket propellant hydrazine (N_2H_4) inactivated bacterial spores by contact in liquid form. Experiments with liquid hydrazine carried out to determine the resistance of bacterial spores showed unequivocally that the spores were completely inactivated within 60 min (Schubert et al., 2008). In an earlier study *Bacillus subtilis* spores were exposed in two different liquid hydrazine propellants (Godding and Lynch, 1965). Monomethyl hydrazine (MMH) rapidly inactivated spores within two hours. Spore inactivation by 1, 1-dimethyl hydrazine (unsymmetrical dimethyl hydrazine, UDMH) was not as rapid and required more than 24 h to completely inactivate all spores. Thus, spores can be inactivated by these three forms of hydrazine often used as propellants.

Experimental work was begun under the assumption that in nominal spacecraft operating conditions, the diaphragms would be permeable to vapor hydrazine and that the total volumes and the surface on the opposite side from the liquid hydrazine would become saturated with hydrazine vapor. The diaphragm material AF-E-332 has relatively low but measurable permeability to hydrazine (Ballinger et al., 1995; Martin, 1973). Typical liquid propulsion system configurations and the diaphragm configurations shown by Ballinger et al. (1995) indicated that there were significantly large surface areas and volumes to be of concern for any associated bioburden. These included the internal volume of the rubber diaphragm material itself and the inner surfaces of the tanks opposite the liquid hydrazine. It was conjectured that if the hydrazine vapor also proved sporicidal, the inner surfaces of the tanks, the remaining tank volumes, the propellant lines and the diaphragm material could all be credited with a reduced bioburden. With this objective in mind, a series of experiments were carried out to assess the survival of bacterial spores to saturated hydrazine vapor.

2. Materials and methods

The biological indicator *Bacillus atrophaeus*, also known as *Bacillus subtilis var. niger* (ATCC 9372), was used. These spores show substantial resistance to dry heat (Kempf et al., 2008) as well as harsh chemicals, radiation, and desiccation (Block, 2001). They have been used in a previous liquid hydrazine study (Schubert et al., 2008). *B. atrophaeus* spores have been extensively studied as models for sterilization (Setlow 2006). Spore coupons were supplied by Raven Labs (a division of Mesa Laboratories Inc.), Omaha, NE. The coupons, or industrial-use Bio-Indicators (BIs), were made of stainless steel with a hole to aid in the suspension of the coupon over the liquid hydrazine. Each coupon contained 10^6 *Bacillus atrophaeus* spores dried on the flat surface of the dish-shaped carrier.

Several variations of a prototype exposure system were built and tested. The design criteria were to provide a saturated vapor environment for the spores that could be set up safely and easily, as well as being able to rapidly terminate the exposure. The final simple design is depicted in Fig. 1.

Bent glass rods were used to facilitate handling a group of coupons as a set. The coupons were suspended from the rod without touching one another or contacting any other surface. Then, the rods with coupons were placed onto polypropylene beakers, notched at the top rim to support the glass rod and coupons and prevent them from falling. This suspended the coupons directly above a small glass vial into which hydrazine could be transferred. In turn, the polypropylene beaker was placed inside of a 400 mL wide mouth jar with screw cap and Teflon liner.

The experiment was initiated by placing the coupons and glass rod in the jar, pipetting 1 mL of liquid hydrazine into the glass reservoir and then closing the cap. Initial tests with litmus paper confirmed by a strong alkaline reaction that hydrazine vapor filled the chamber within 5 s. This was expected given the temperature, the small volume of the test apparatus, and the vapor pressure of hydrazine (between 1 and 2 kPa at experimental temperatures). Because the actual flight configurations called for the tanks to be kept at either room temperature or heated to 35–36 °C (Krylo et al., 2008), the exposure temperatures were set at 25 °C and 35 °C.

The exposures were stopped at predetermined times by opening the lid of the jar and removing the supporting glass rod with all 5 coupons. Coupons were then allowed to outgas for 60 min before further handling.

The survivor fraction was estimated by growth in nutrient medium. Samples were processed by the NASA standard assay procedure (NASA, 2010). Coupons were transferred to 10.0 mL of sterile water. Spores were removed from the coupons by vortex mixing followed by ultrasonic vibration. Serial dilutions were made for the spore suspension from each test coupon and aliquots were distributed to petri dishes with Trypic Soy Agar. Positive and negative controls were included. Colonies resulting from the growth of individual spores were observed at 24, 48 and 72 h, and under the conditions tested with the spores used, showed equivalent colony formation at 48 and 72 h. The plates were counted after incubating for 48 h and the results were reported as colony forming units (CFUs).