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Hydrazine Inactivates *Bacillus* Spores

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Abstract

Hydrazine (N_2H_4) is a liquid rocket engine propellant. It is similar to ammonia in physical properties but more reactive.

Planetary Protection places requirements on the maximum number of viable bacterial spores that may be delivered by a spacecraft to another solar system body. Therefore, for such space missions, the spores that may be found in hydrazine are of concern. A proposed change in processing procedures that eliminated a 0.2 µm filtration step prompted this study to ensure that no microbial contamination issue existed, especially since no information was found in the literature to substantiate bacterial spore inactivation by hydrazine.

A set of experiments was carried out to determine if hydrazine is a self-sterilizing solution, capable of inactivating bacterial spores. *Bacillus atrophaeus (B.subtilis)* ATCC 9372 is a standard biological indicator for sterilization procedures.

Dry spores were exposed to hydrazine. Spores were filtered and washed with water. Resulting spore populations were enumerated by pour plates.

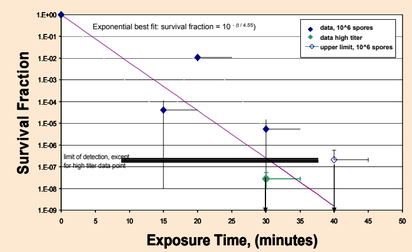
Significant reductions in spore populations were observed for all exposures. Exposures of 60 minutes or longer yielded no growth (survival fraction less than 10^{-6}). Since bacterial spores were inactivated in liquid hydrazine, the substantial quantities of the fluid held in spacecraft tanks stored for months or longer can be considered sterile.

Experimental Matrix

Sample or Control Description	Spores Present	Hydrazine Present	Time of Exposure	Filtration & Wash Step	Petri-Dish Plating Step
Spore Challenge	✓	✓	15, 20, 30, 40, 60, 120 minutes; 24 hours	✓	✓
Positive Control	✓	No Hydrazine used	15, 20, 30, 40, 60, 120 minutes; 24 hours	✓	✓
Negative Control	No spores Present	✓	20, 40, 120 minutes	✓	✓
Residual Hydrazine Control	None – Spores added to filter after wash step	✓	20, 40, 120 minutes	✓	✓
Residual Hydrazine Control, Matched Population Control	None – Spores added to clean test tube	No Hydrazine used	No Hydrazine exposure	No Filtration	✓
Diluted Hydrazine Control	✓	✓	~5 minutes to dilute Hydrazine	✓	✓
Hydrazine Evaporation Control	✓	✓	6 to 24 hours	No Filtration	✓
Media Control to ensure prepared agar is sterile	No Spores present	No Hydrazine used	No Hydrazine exposure	No Filtration	✓

✓ = conditions met (step was included)

Spore Inactivation by Hydrazine



Hydrazine Evaporation Control Experiment

Hydrazine was evaporated under a stream of dry nitrogen gas.

- Hydrazine or H_2O control added to 10^9 dry *Bacillus* spores.
- Results: Triplicate vials resulted in a 10^{-9} population reduction in about 6 hours, compared to H_2O.

Resulting Populations	
Water Evaporation	Hydrazine Evaporation
$6.4 \times 10^9 \pm 4.3 \times 10^8$	0

Introduction

- A Mars orbiting spacecraft currently being built, will not be able to filter the bulk liquid rocket propellant hydrazine, because the filtration rate will be impractically slow.
- A specified spore-bioburden accounting estimate must be applied to the unfiltered hydrazine volume, making the total spacecraft bioburden unacceptably high.
- This estimate could be lowered if new data supports a lower estimate.

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Method for Hydrazine Assay

- Commercially prepared *Bacillus atrophaeus* (*B. subtilis* var. niger) spores ATCC 9372 placed in serum vials (~10^6 spores) and desiccated overnight.
- Hydrazine added to serum vial and allowed to contact spores for specified lengths of time.
- Reaction "stopped" or slowed by addition of water.
- Spores collected onto a Teflon filter and extensively washed with water.
- Spores on filters were resuspended in H_2O, serially diluted and pour-plated in TSA.
- Plate counts used to assess spore survival.

Prolonged Hydrazine Exposures*	
Starting Population = $1.6 \times 10^6 \pm 5 \times 10^5$	
Exposure Time	Resulting Population
1 hour	0
2 hours	0
24 hours	0

* Triplicate determinations for each time point.

Negative Controls	
	CFU
Reagent Hydrazine, filtered and washed*	0

*Three replicate experiments

Physical Changes Imply Damage

Resuspended in Water, vortex mixed

Hydrazine Treated Control (Water Treated)

Resuspended in Water, vortex mixed and sonicated

Hydrazine Treated Control (Water Treated)

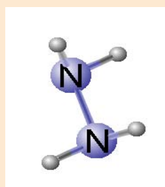
- Physical changes of spores were noted after hydrazine treatment
- Clumping and reduced turbidity observed upon resuspension in water
- Implies biochemical changes and cellular damage

A large volume of Hydrazine

- The volume of hydrazine planned for a current mission is 1.2×10^6 cm^3 (mass = 1187 kg = 2617 lbs).
- Without filtration the default specification of 30 spores per cm^3 for non-metallic volumes would have to be applied.
- This would result in a spore burden estimate of 3×10^7, a number that is unacceptably high.

Properties of Hydrazine

- N_2H_4
- Strong reducing agent.
- Infinitely soluble in water.
- Alkaline pH.
- Similar to ammonia.
- Modifies DNA bases; Tags C & T residues in Maxim-Gilbert sequencing.
- Removes N-acetyl groups from N-acetylated amino sugars.



Residual Hydrazine Control

This control was done to insure that any residual hydrazine on the filter is not contributing to failure of spores to grow.

Method

- Spores were added to hydrazine treated filter and paired untreated filter
- Similar wash steps as normal protocol

Resulting Populations	
Unexposed Filter	Hydrazine Treated Filter
$3.1 \times 10^5 \pm 2.3 \times 10^4$	$2.4 \times 10^5 \pm 3.3 \times 10^4$
$2.6 \times 10^5 \pm 3.6 \times 10^4$	$2.4 \times 10^5 \pm 1.9 \times 10^4$
$2.7 \times 10^5 \pm 2.0 \times 10^4$	$3.2 \times 10^5 \pm 5.1 \times 10^4$

Residual Hydrazine Control Conclusion

- Similar populations in both treated and untreated samples
- Residual hydrazine on the filter is not interfering with the main experimental set

Pre-Diluted Hydrazine Control

Question: Is there spore inactivation due to contact with a dilute solution of hydrazine? During the filtration step spores are exposed to a 17% solution of hydrazine.

Method

- Premix 2.0 ml hydrazine (17%) plus the 10 ml sterile water
- Add solution to dried spores in vial
- Filter after 5 minutes (typical timing)
- Dilute and do plate count

Resulting Population	
Starting Population	After Treatment
3.2×10^6	$9.0 \times 10^3 \pm 5.4 \times 10^3$

Pre-Diluted Hydrazine Control Conclusion

- 2.4 log reduction indicates there is a significant amount of spore inactivation at the hydrazine dilution step

Conclusions

- Hydrazine rapidly inactivated *Bacillus atrophaeus* spores.
- Water solutions of hydrazine also inactivated *B. atrophaeus* spores.
- D-value (time in minutes required to reduce a population by 1 log) was roughly 5 minutes. This result is comparable to the inactivation rates for ethylene oxide (4.6 minutes) and dry heat at 160°C (2.6 minutes) for the same crop of spores*.
- Spores, typically chosen as the most difficult sterilization challenge, were readily inactivated, giving confidence for applying a greatly reduced bioburden estimate in bulk hydrazine.
- Hydrazine was found to be a self-sterilizing solution.

* data provided by commercial spore supplier

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