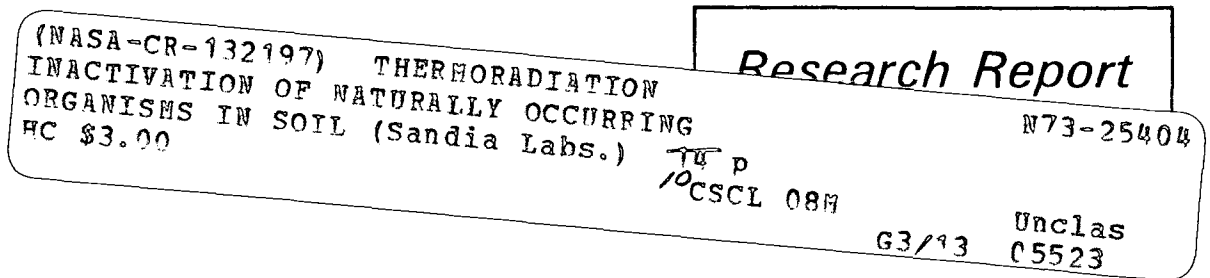


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THERMORADIATION INACTIVATION OF  
NATURALLY OCCURRING ORGANISMS IN SOIL

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### Abstract

Samples of soil collected from Kennedy Space Center near spacecraft assembly facilities were found to contain microorganisms very resistant to conventional sterilization techniques. The inactivation behavior of the naturally occurring spores in soil was investigated using dry heat and ionizing radiation, first separately, then in combination. Dry heat inactivation rates of spores were determined for 105 and 125 °C. Radiation inactivation rates were determined for dose rates of 660 and 76 krad/hr at 25° C. Simultaneous combinations of heat and radiation were then investigated at 105, 110, 115, 120, and 125° C. Combined treatment was found to be highly synergistic requiring greatly reduced radiation doses to accomplish sterilization.

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# THERMORADIATION INACTIVATION OF NATURALLY OCCURRING ORGANISMS IN SOIL

## Introduction

For some time it has been recognized that microbial contamination of spacecraft during assembly at Kennedy Space Center (KSC) might well include naturally occurring organisms. These organisms indigenous to the local soil could be transported via shoes, clothing, tools, etc., into assembly areas and eventually deposited on spacecraft surfaces. To determine what effect these natural organisms might have on spacecraft sterilization rationale, the Phoenix Laboratory, PIIS, began a study using samples of natural soil from around assembly facilities at KSC. They investigated dry heat sterilization of these naturally occurring populations and the resistance of isolates from the hardy sub-population that survived heat treatment. Their findings<sup>1</sup> indicated extraordinarily high resistance to the 125°C dry heat sterilization planned for spacecraft.

This report describes our efforts at Sandia Laboratories to apply the thermoradiation process for more effective sterilization of the naturally occurring organisms.

## Materials and Methods

### Preparation of Samples

Approximately six pounds of soil were collected<sup>2</sup> near Hanger AO at Cape Kennedy and sent to Phoenix on June 17, 1970. The sample consisted of equal amounts from the following three locations: (1) a ditch about 50 yards west of the High Bay doors (a sandy type soil with small sea-shells); (2) a landscaped area at the main (east) entrance of the hanger (a sandy loam soil); and (3) the south end of the hanger near the water cooling tower (a dry sandy-clay soil). The sample was spread in a thin layer on sterile paper, covered, and allowed to dry for 48 hours at room temperature. Dry soil (2771 g) was then dry processed through a stainless steel sieve series (W. S. Tyler Co., Cleveland) down to 0.124 mm screen size to remove rocks, shell particles, and plant particles. A series of rinses in 95% ethanol and then a final sieving with 0.043 mm screen resulted in 4900 ml of soil-spore suspensions in 95% ethanol. Serial dilutions were plated with TSA supplemented with

0.1% soluble starch and 0.2% yeast extract and incubated for 1 week at 35°C.<sup>3</sup> The viable concentration was  $2.7 \times 10^5$  organisms and the approximate weight of soil in the suspension was 0.03 g/ml.

The soil suspension described above was used as the inoculum for all the experiments. The suspension was maintained at 4°C during storage. Prior to use, the soil-spore suspension was inoculated for two minutes to break up clumps within the ethanol suspension and was continuously agitated during inoculation to prevent settling. The samples were prepared by pipeting 0.1 ml of the suspension onto the surface of aluminum foil discs. The discs were 1.25-inch diameter cut from biological grade aluminum foil 0.0015-inch thick. The samples were then allowed to air dry until all of the ethanol had evaporated. When dry, the inoculated discs were assembled on aluminum strips, 1.50 inches wide by 0.020 inch thick. Four sample discs were placed on each strip, a single clean foil disc was placed over each sample, and then another aluminum strip placed on top and held in place with wire clamps. This assembly of the discs clamped between two strips permitted considerable handling and suspending of the assembly in a vertical position without loss or damage to the sample discs. The assembled sample strips were then placed in a desiccator over Drierite in a vacuum for 15 hours prior to exposure to the sterilization environment. All of the inoculation and assembly operations were performed in a Class 100 laminar airflow clean room.

The use of covered foil as a substrate for the inoculum was evaluated and the inactivation rates using this substrate was compared to rates using open coupons. This was done in order to relate this study to previous work by PHS Phoenix Laboratory. A comparative experiment was performed using the covered foils, 1-inch square by 0.020-inch thick coupons of aluminum, and stainless steel. The coupons were attached to 1.5-inch wide aluminum strips with small wire hooks, leaving the inoculated surface exposed. This type of assembly required extreme care in handling to prevent sample loss or damage. With all other experimental procedures being identical, no differences in the resulting inactivation rates was detected between the covered foil discs or the open coupons of either aluminum or stainless steel.

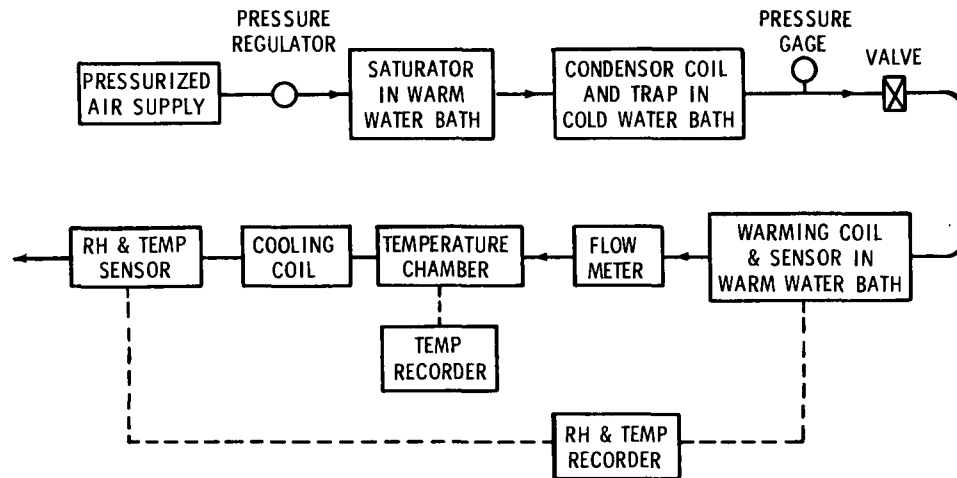


Figure 1. Equipment Setup for Thermoradiation Experiment with Controlled Humidity

### Exposure Methods

The thermal environment was provided by a recirculating air temperature chamber having a volume of 0.578 ft<sup>3</sup> with a rail arrangement in the door to hold the aluminum strips. The temperature was controlled and recorded to an accuracy of  $\pm 0.2^\circ\text{C}$ . The radiation environment was provided by the Sandia Gamma Irradiation Facility (GIF). This facility contained remote handling equipment to introduce and remove the source and included visual, physical, and electrical access with necessary safety controls. The cobalt-60 source was introduced in a corner of the cell, which is 7 by 8 by 8.5 feet. The dose rates ranged from  $1 \times 10^6$  rad/hr to  $4 \times 10^3$  rad/hr depending on the location of the sample within the cell.

Moisture content of the air in the temperature chamber was controlled by a system<sup>4</sup> of flowing pressurized air through a saturator in a warm water bath and then through a condenser coil and water trap in a cold water bath. The air was allowed to expand into a coil in the warm water bath and then introduced into the temperature chamber. Adjustments of the air pressure and the temperature of the cold water bath provided the relative humidity (RH) control desired. The relative humidity for these experiments was controlled at 30% RH at  $25^\circ\text{C}$ . Relative humidity measurements of the air were made at the input to the temperature chamber with a dewpoint indicator. In addition, continuous measurements were provided by lithium chloride sensors and a strip chart recorder. The RH value was controlled within  $\pm 1\%$  RH during the experiments. The

rate of air flow into the temperature chamber was controlled to 0.5 cfm and the air pressure in the chamber was regulated at about 0.8 in. w. g. by adjustment of a bleeder valve on the chamber.

For each experiment, the temperature chamber was placed in the GIF cell at the appropriate distance from the cobalt-60 source for the desired dose rate. The chamber was positioned so the sample strips assembled with the foil discs were vertical and the face of the strips was perpendicular to the direction of the gamma rays. The temperature chamber controller and temperature recorder were located outside the cell with necessary cable connections passed through the cell wall. The humidity control system was also located outside the cell with the input air to the temperature chamber penetrating the cell wall. A block diagram of the equipment setup is shown in Figure 1. Silver phosphate or cobalt glass dosimeters, depending on the dose range, were placed on selected sample strips to verify the computed dose rates.

### Recovery Methods

Each sample strip when removed from the temperature chamber was wrapped in sterile aluminum foil and returned to the clean room facilities for recovery operations. From 20 to 30 minutes were required to transport the samples from the remote reactor area where the GIF is located to the Class 100 clean room facilities.

Each sample strip containing four replicate samples represents a single data point. Each of the samples from the strips was placed in a separate 50 ml beaker containing 10 ml of sterile, 0.1 percent Tween 80 water. The samples were then insonated for 2 minutes to remove the organisms from the foil discs. Care was exercised in placing the foil discs in the beakers to assure separation of the inoculated disc and cover disc, and complete wetting and submersion of both discs in the water. During the ultrasonic treatment, occasional agitation of the beakers kept the discs separated and prevented cold welding together of the two discs. This protocol assured good recovery of the organisms from the foil discs. The insonation is accomplished with the beakers immersed in the ultrasonic water bath to a level just above the water level in the beaker.

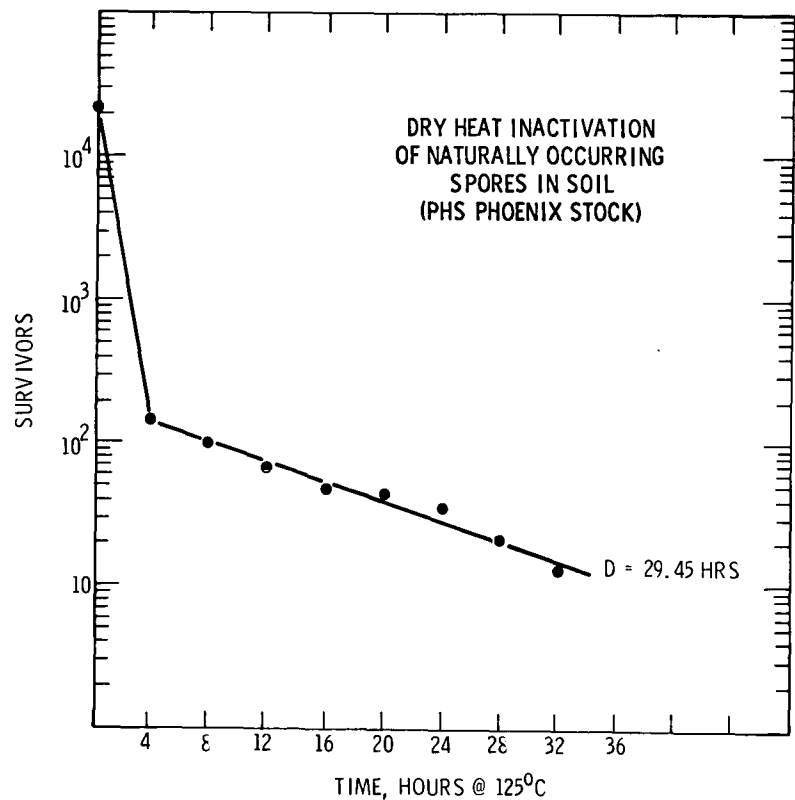
Additional ten-fold serial dilutions were made as required. Dilutions were plated out in duplicate on Trypticase Soy Agar supplemented with 0.1% soluble starch and 0.2% yeast extract underlay, overlaid with the same type of media and then placed in an incubator maintained at 35° C. Plates were counted after 1 week in the incubator.

## Experimental Results

Experimentation with the naturally occurring spores in soil was directed primarily toward the response of natural organisms to dry heat treatment, radiation treatment, then the combination of dry heat and gamma radiation.

The results of the first baseline experiment to determine dry heat resistance of the organisms in soil is shown in Figure 2. The viable sample population, beginning at  $2 \times 10^4$  organisms (zero heat treatment), undergoes a rapid reduction to about  $10^2$  organisms during the first treatment period. This initial drop, which was found to be characteristic of organisms in soil, is followed by a second logarithmic phase of destruction. The second phase represents a very heat resistant sub-population of approximately 1.0% of the original sample. Excepting radiation inactivation, this bi-phasic order of death was noted in all dry heat and thermoradiation experimentation. The second phase, on the logarithmic part of the survivor curve,

Figure 2. Dry Heat Inactivation at 125° C



was used as a basis of comparison for various treatments. Using the method of least squares, the data was fit with a straight line in order that slopes or D-values\* might be compared. In Figure 2, then,

The third curve on Figure 3 is the results of simultaneously combining dry heat and radiation (thermoradiation). The thermoradiation D-value was found to be approximately one hour as com-

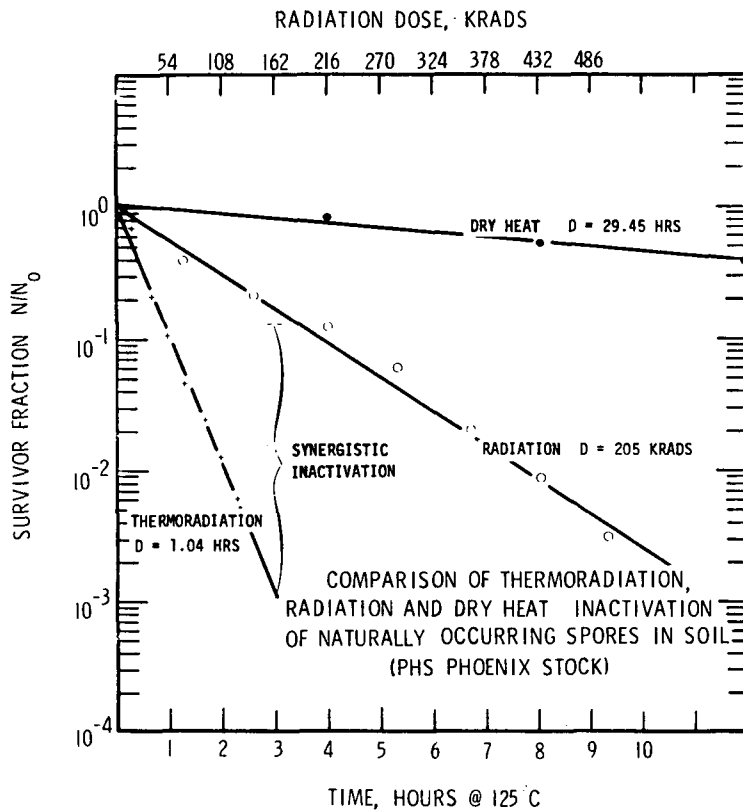


Figure 3. Comparison of Thermoradiation, Radiation, and Dry Heat Inactivation

the D-value determined for the resistant sub-population is 29.45 hours. This value is roughly 50 to 100 times the D-value for heat resistant *Bacillus subtilis* var. *niger* spores.

The radiation resistance of the naturally occurring populations were next investigated. Samples were exposed at room temperature (25°C) to gamma radiation from a cobalt-60 source. Temperature and moisture-conditioned air was supplied to the sample chambers. The rate of flow provided one air change per minute. The results of irradiation are shown in Figure 3. In this figure, the data from Figure 2 dry heat inactivation is repeated in order to simplify comparisons of the inactivation rates with various treatments. The second curve is the results of radiation at room temperature with 54 krad/hr. The D-value was found to be 205 krad (3.8 hours) for the entire population which is a very high resistance indeed.

pared to 3.8 hours for radiation alone or 29.4 hours for heat alone. For an example of the synergism obtained, the singular effects of heat and radiation when added would reduce the population by 1 log after 3 hours of treatment. Thermoradiation, for the same time and total dose, reduced the population by 3 logs. If we examine the slope or D-values of these examples, the singular effects of heat and radiation, if considered in an additive sense, would result in a D-value of 3.37 hours. The thermoradiation D-value, 1.04 hours, is less than 1/3 that of the additive effects. This level of synergism means that sterilization can be accomplished in 1/3 of the time at temperature and with 1/3 the normal dose.

Additional experiments were performed to determine the characteristics of thermoradiation treatment at temperatures below 125°C. For this series a constant dose rate, sampling time, and moisture environment were maintained throughout with only temperatures as a variable. The D-values based on the resistant sub-populations were found to be 57 minutes at 125°C, 60 minutes at 120°C,

\*D-value is the time at temperature or the radiation dose required to reduce the viable population by 90%.

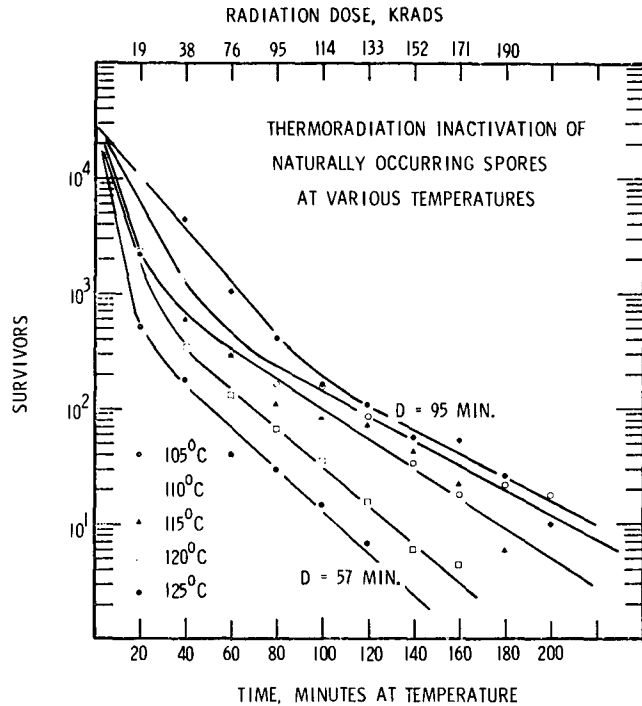
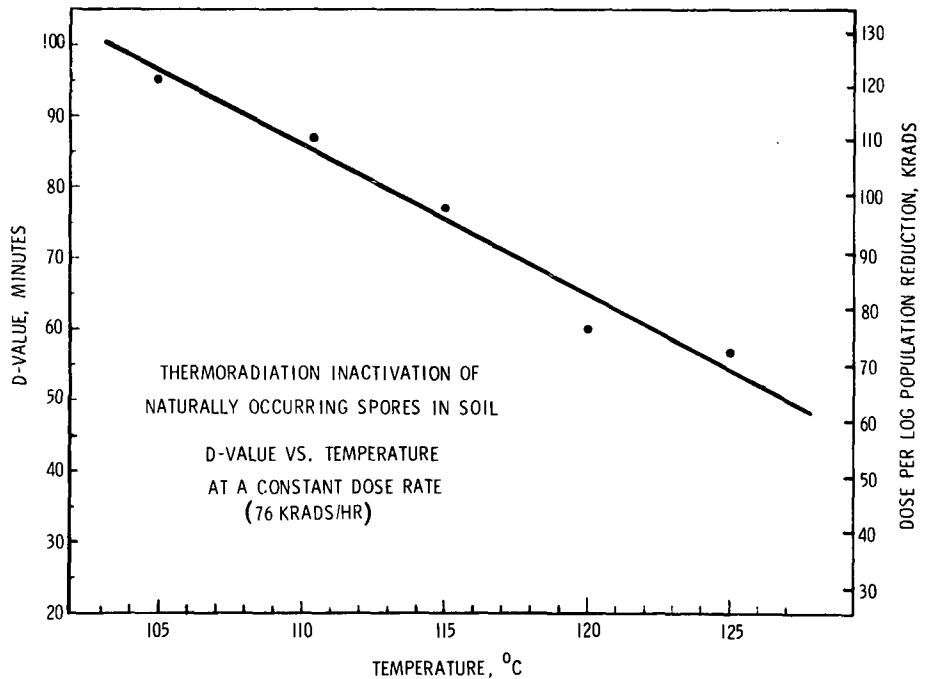


Figure 4. Thermoradiation Inactivation at Various Temperatures

77 minutes at 115°C, 89 minutes at 110°C, and 95 minutes at 105°C (Figure 4). The interesting aspect of this series is the manner in which the shape of the inactivation curve changes. As the temperatures are lowered, the sharp initial drop usually experienced at 125°C softens, requiring a longer exposure time to reach the second phase.

As the temperature is further reduced, the thermoradiation curve approaches a straight line which is, in fact, the case for radiation of naturally occurring spores at room temperatures (Figure 3). The D-values presented in Figure 4 are plotted as a function of temperature in Figure 5 to illustrate the

Figure 5. D-Value Versus Temperature at a Dose Rate of 76 krad/hr





temperature/dose relationship. For example, at 105°C with a 95 minute D-value, the radiation dose per log population reduction would be 121 krad/log. As the temperature is elevated (constant dose rate), the D-value drops to 57 minutes with a total dose of 72 krad per log population reduction or roughly 60% of the radiation required at the lower temperature.

## Conclusions

Naturally occurring organisms present a very difficult but realistic contaminant for spacecraft sterilization. Although organisms are frequently either heat resistant or radiation resistant, the naturally occurring spores are both. As such, these organisms have been a desirable adjunct to our previous studies that were based primarily on the use of B. subtilis var. niger as a working organism. However, the synergistic behavior to certain combined treatments was in good agreement with observations of the inactivation of other bacterial spores, bacteriophage, proteins, viruses, and yeast.<sup>5-8</sup> Although the soil organisms were found to be highly resistant to both heat and radiation, the degree of synergism appears higher than for B. subtilis. We experienced thermoradiation inactivation rates of 3.25 times the additive effects of heat and radiation, whereas for B. subtilis thermoradiation rates of about 2.6 times the singular additive rates were found to be the upper limit.<sup>9</sup> We are presently completing the mathematical characterization of the response of naturally occurring spores to treatment for all temperatures and dose rates of interest. This has been done for B. subtilis in order that dose rate - temperature combinations could be selected to yield the highest level of synergism. The utility of this approach to determine treatment parameters is that thermal and radiation exposure can be minimized.

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