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ENVIRONMENTAL MICROBIOLOGY

AS RELATED TO PLANETARY QUARANTINE

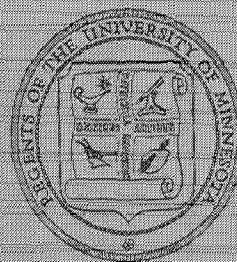
Semiannual Progress Report 4

June 1970

UNIVERSITY OF MINNESOTA

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SCHOOL OF
PUBLIC HEALTH



ENVIRONMENTAL MICROBIOLOGY
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Semiannual Progress Report 4
June 1970

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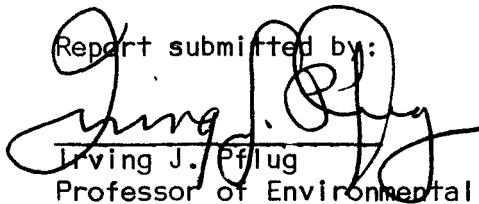
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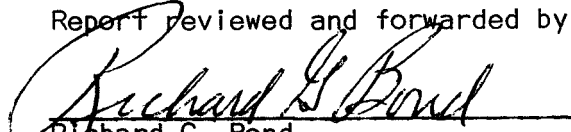
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INTRODUCTION

This report covers research activities during the period December 1, 1969, through May 31, 1970, for the project entitled "Environmental Microbiology as Related to Planetary Quarantine". These studies were conducted by the Division of Environmental Health, School of Public Health, at the University of Minnesota under the auspices of the National Aeronautics and Space Administration--Lawrence B. Hall, Planetary Quarantine Officer.

This is the fourth semiannual report of progress on NASA project NGL 24-005-160. A limited number of earlier reports on this project are available for those who do not have a complete set but who wish to obtain all of our procedures and results in a given area.

In this progress report we have made increased use of an experiment number code for reference purposes. We are using a rather simple seven-unit coding system. The test number code consists of: two letters to identify the project leader, the last digit of the year, three digits to give the day of the year on which the test was conducted or started (1-366) and a letter to designate sub-units of an overall test. In this report we have used test numbers in tables and in text material to make it easier for the reader to grasp differences in experimental procedure and in results of specific tests.

A specific objective in almost every facet of this project is the determination of the effect of water on the survival of spores subjected to a dry heat stress. We know the qualitative effect of water and we now want to determine the quantitative effect of water. These quantitative measurements require a more controlled experimental system validated by adequate statistical analysis. The inter-laboratory study, the study of variables in experiments to determine dry heat D-values on surfaces, the physical measurement of planchet temperatures during heating (Appendix E) and the experimental unit analysis (Appendix B) are all part of our effort to improve control so that we can measure smaller differences.

We are optimistic regarding our closed system studies. We believe we have an experimental system that can be physically defined and that is capable of very close control over a wide range of conditions. We further believe that this system will allow us to quantitatively evaluate the effect of water on closed system D-values. We anticipate that once our closed system has been validated it will be used as the closed system standard dry heat test procedure.

Studies directed toward obtaining dry heat destruction rate data for spores on surfaces as a function of the relative humidity and type of gas in the

heating environment have indicated that there is no difference in the D-value of spores in contact with an air atmosphere compared to those in contact with a nitrogen atmosphere. The effect of humidity in the surrounding atmosphere is significant. The ratios of the D-values obtained in the clean room atmosphere compared to D-values in dry air or nitrogen were 3.2 and 2.8 for Bacillus subtilis at 110 and 125°C respectively and 1.7 for Bacillus firmus. Although we now know the general affect of relative humidity on D-value we must proceed to determine the change in D-value with a change in relative humidity at a range of relative humidities from near zero to about 50% RH.

We have reached a point of transition in our mated surface studies. David Drummond has completed his studies on mated surfaces and has now left the project. An overview of the present status of the destruction rate of microbial spores in mated surface areas has been prepared for the Planetary Quarantine Officer and is included in Appendix C of this report. There is additional work that needs to be done in the mated surface area, however, this work will probably have to await availability of resources.

In the project entitled "Detection of low levels of microbial contamination by chemical methods" our efforts have been directed toward perfecting the technique for thin-layer chromatographic analysis of ATP. During the next project year we will gradually reduce the level of effort on this project and by May, 1971, bring it to an end. The remaining effort in this study will be directed toward calibration of the ATP measurement methods and the preparation of a summary of all of our efforts in this area.

Our overall goal in the dry heat destruction of spores is to fully understand all of the factors that affect spore death. We will then be able to fit spore destruction data from open, mated and closed systems into a single pattern. This end is now visible and we are confident that it will be reached.

I. J. Pflug

SURVIVAL OF MICROBIAL SPORES UNDER SEVERAL TEMPERATURE AND HUMIDITY CONDITIONS

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Division of Environmental Health

Project Contributors: J. Bearman and R. Jacobson
Division of Biometry

INTRODUCTION

In previous progress reports we have reported on experiments to measure survival rates of Bacillus subtilis var. niger spores on surfaces at 22°, 45°, and 60°C as a function of the relative humidity of the surrounding air. Almost 100% survival was observed at 22°C except at >90% relative humidity. However, survival rates were found to decrease significantly at 45° and 60°C when the relative humidity increased to about 50%. Above 50% RH the decrease continued, but was less pronounced.

During the current reporting period the experiment at 45°C has been continued to determine the effect of surface material (glass vs. stainless steel), and extremely dry air (<1% RH) on spore survival. We also attempted to measure box-to-box variation at several temperature-humidity conditions. A study was initiated to obtain survival data at temperatures to 90°C in liquid media.

OBJECTIVE

The objective of this project is to determine the effect of relative humidity and relatively low temperatures (<90°C) on the long-term survival of bacterial spores on surfaces. It is possible that there may be temperature and humidity conditions acceptable to spacecraft engineers which will produce a relatively rapid rate of reduction in the microbial spore population. The effect of the conditions during either space hardware assembly or post-assembly storage could be used to reduce the terminal sterilization bio-load.

Using Bacillus subtilis var. niger spores suspended in distilled water, tests were carried out to obtain D-values under 100% relative humidity conditions. Since some laboratories engaged in dry heat destruction experiments have stored spores in buffered distilled water, tests were carried out using both distilled water and buffered distilled water to determine if the buffer exerts a protective effect.

EXPERIMENTAL PROCEDURES

Tests GS9281A thru H

Procedures for using silica gel and predetermined quantities of water to stabilize relative humidity conditions in sealed plastic containers have been described in Appendix A, Progress Report #3. The technique for obtaining the <1% RH at 45°C was also described in Progress Report #3. This experiment ran for a total of ten weeks and included relative humidities of <2%, ~30%, ~50%, and >90% in the sealed plastic containers as well as a <1% RH in glass bottles prepared in the dry gas chamber.

Tests GS0075A thru D and GS0077A and B

D-values for Bacillus subtilis var. niger (AADA) suspended in distilled water and in buffered distilled water* were evaluated at 60, 75 and 90°C using the successive sampling method. Blood culture bottles, 100 ml capacity were used as the basic container. These bottles have closures designed for sample removal using a hypodermic syringe. The bottles were filled with 70 mls of either distilled or buffered distilled water at room temperature and, using an Eppendorf pipette, 20 μ ls of spore suspension were added to give a concentration of about 10^6 spores per ml.

Heating was carried out in water baths maintained at $\pm 0.5^\circ\text{C}$ of their respective temperatures. For each substrate and heating temperature evaluated three replicate bottles were utilized. The bottles containing the substrate plus spore inoculum were placed in a water bath at time zero. At successive time intervals, each bottle was taken from the bath and shaken by hand. An aliquot was withdrawn using a hypodermic syringe. The quantity of sample withdrawn depended on the expected concentration of surviving organisms. Duplicate samples were taken from each bottle at each sampling time. The bottles were out of the water bath from one to two minutes during sampling resulting in a substrate temperature drop of 2 to 3°C.

The undiluted aliquots were plated immediately in Trypticase Soy agar. The necessary dilutions were made in 50 mls of buffered distilled water and were then plated within five minutes. All samples were incubated for 48 hours at 32°C and

*Butterfield's buffer (Butterfield, C.T. The Selection of Dilution Waters for Bacteriological Examinations. Public Health Reports 48:681, 1933. Reprint 1580.) at pH 7.2 was used in this test. The calculated phosphate content in the substrate was .03125M.

then counted using the guidelines presented in Progress Report #3, except that counts below 20 colonies per plate were utilized in some cases.

RESULTS

Tests GS928IA thru H

The overall results of the 45°C experiment are summarized in Tables 1.1 and 1.2. Data for the ten-week experiment do not indicate any noticeable difference in the survival rate at <1% relative humidity for the dry gas environment compared to the survival rate at <2% RH over silica gel. The <2% RH conditions were measured at 45°C using a Honeywell RH indicator, Model #W611a, range 2%-100%. Since the relative humidity of the boxes containing silica gel was below 2%, which is the lowest limit of the humidity measuring system, we do not have an accurate measurement of the differences in the relative humidities of the two environments. The low survival rate of spores on glass surfaces at the higher humidities noted in Table 1.1 and 1.2 was described in the previous report; this phenomenon has not been investigated further.

TABLE 1.1

Survival of Bacillus subtilis var. niger Spores (AAAA) at 45°C
as a Function of Relative Humidity and Supporting Material

% RH at 45°C	Test No.	Strip Material	Weeks					
			0	2	4	6	8	10
.26-.40*	GS928IA	SS**	2.20E5	9.84E4	9.42E4	6.48E4	7.29E4	6.39E4
< 2	GS928IB	Glass	3.09E5	1.49E5	1.41E5	6.22E4	1.01E5	1.33E4
	GS928IC	Glass	2.74E5	1.72E5	1.10E5	7.59E4	1.15E5	4.14E4
27-32	GS928ID	SS	3.09E5	2.23E5	1.10E5	1.70E4	2.77E4	1.78E2

* Storage humidity calculated at 45°C but measured at 23°C during preparation only.

** Stainless Steel

TABLE 1.2

Survival of *Bacillus subtilis* var. *niger* Spores (AAAA) at 45°C
as a Function of Relative Humidity and Supporting Material

% RH at 45°C	Test No.	Strip Material	Weeks					
			0	1	2	3	4	5
49-55	GS9281E	SS	3.09E5	2.31E5	8.68E4	3.49E3	7.17E2	1.71E2
	GS9281F	Glass	2.74E5	8.38E2	*	--	--	--
90-95	GS9281G	SS	3.09E5	1.33E5	2.84E4	2.25E2	2.5E-1	7.5E-1
	GS9281H	Glass	2.74E5	8.90E1	*	--	--	--

* No colonies observed

Data for duplicate box experiments at various humidity levels using the silica gel method is illustrated in Figures 1.1, 1.2, 1.3, 1.4 and 1.5. The box-to-box agreement at all humidities tested was generally good. In only one instance was there a relatively large difference between the two boxes tested; this is shown in Figure 1.4, 4th week of the 50% RH experiment.

The shape of the survivor curve in experiments where there was a significant die-off is unique. The number of survivors appears to remain nearly constant for a period of time after which the numbers of survivors decrease rapidly. This phenomenon can be observed in Figures 1.3, 1.4, 1.5 and 1.6. A graph of the time when the death rate increases as a function of relative humidity is shown in Figure 1.7. At this point we cannot explain how the relative humidity influences the time at which the death rate increases. The experimental data shown in graphical form in Figure 1.7 indicates that the effect is a linear function of relative humidities between 2 and 50%. It is difficult to understand why the change in the effect of relative humidity should occur at 50% relative humidity.

The objective of the study that was carried out at a relative humidity of <1% was to determine whether the same shape curve of log D versus relative humidity would be obtained at 45°C, for example as was obtained at 100-125°C. Specifically we were trying to prove that at very dry conditions the D-value decreased below a maximum value. In our studies we found no difference between our <1% compared to our <2% relative humidity treatments. Since we are not able to precisely specify the humidity in this <2% or <1% range all we can conclude is that at the dry conditions we evaluated we still obtained maximum rather than minimum D-values.

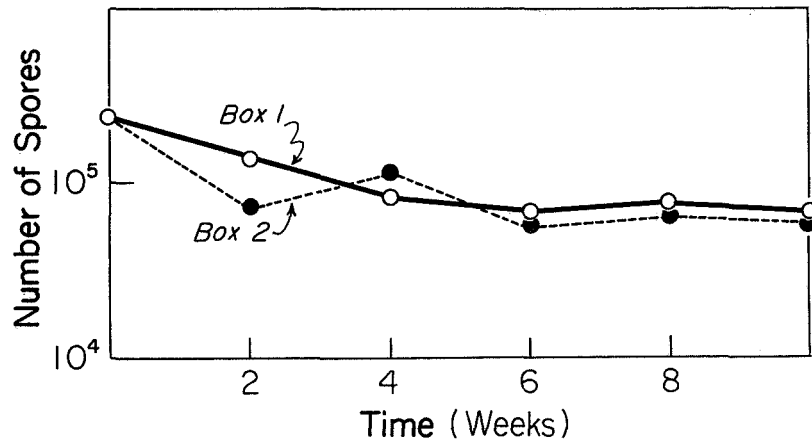


Figure 1.1 - Box-to-Box Variation in the Number of Surviving Spores for Test No. GS9281A (45°C, <1% RH)

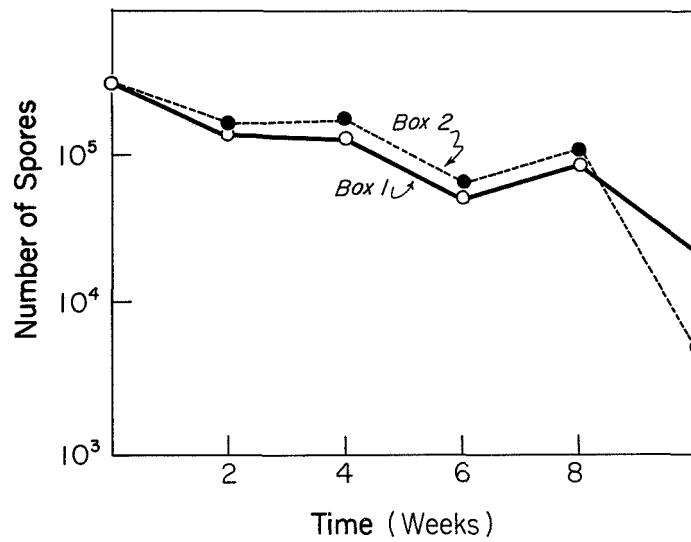


Figure 1.2 - Box-to-Box Variation in the Number of Surviving Spores for Test No. GS9281B (45°C, <2% RH)

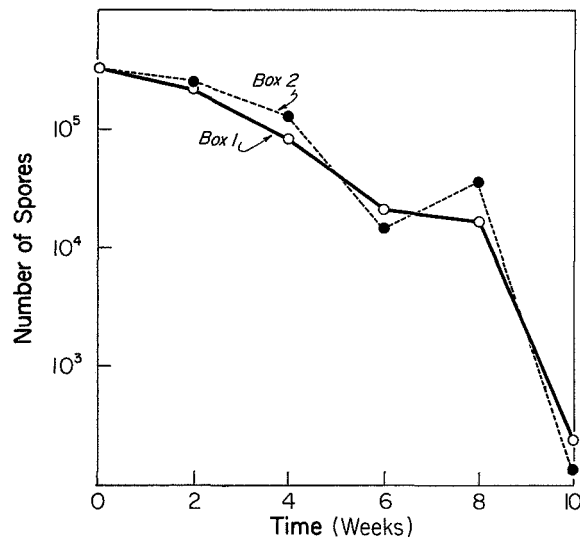


Figure 1.3 - Box-to-Box Variation in the Number of Surviving Spores for Test No. GS9281D (45°C, 30% RH)

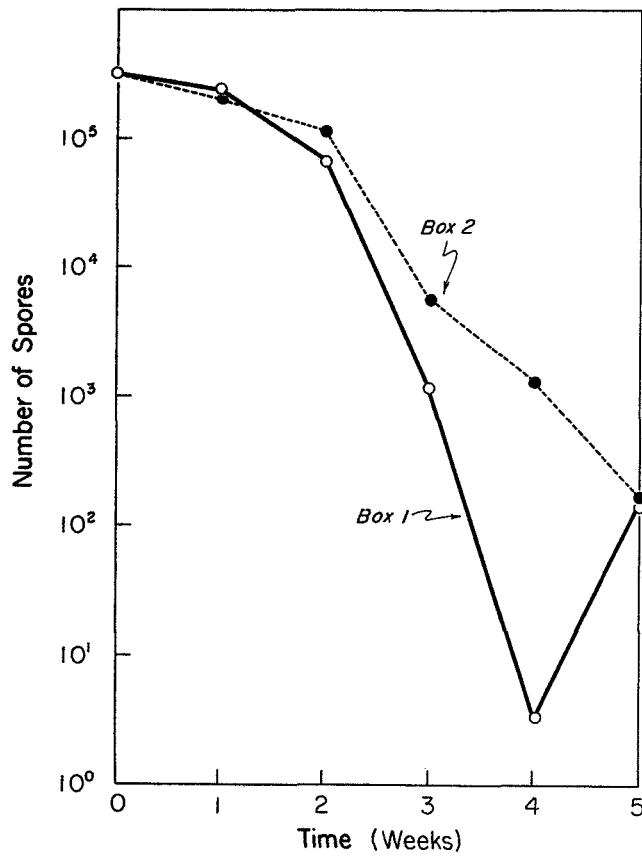


Figure 1.4 - Box-to-Box Variation in the Number of Surviving Spores for Test No. GS9281E (45°C, 50% RH)

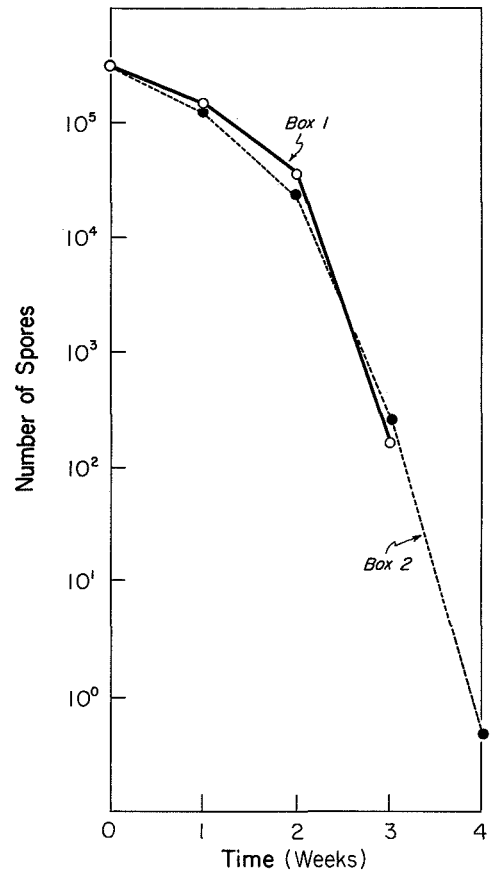


Figure 1.5 - Box-to-Box Variation in the Number of Surviving Spores for Test No. GS9281G (45°C, 90% RH)

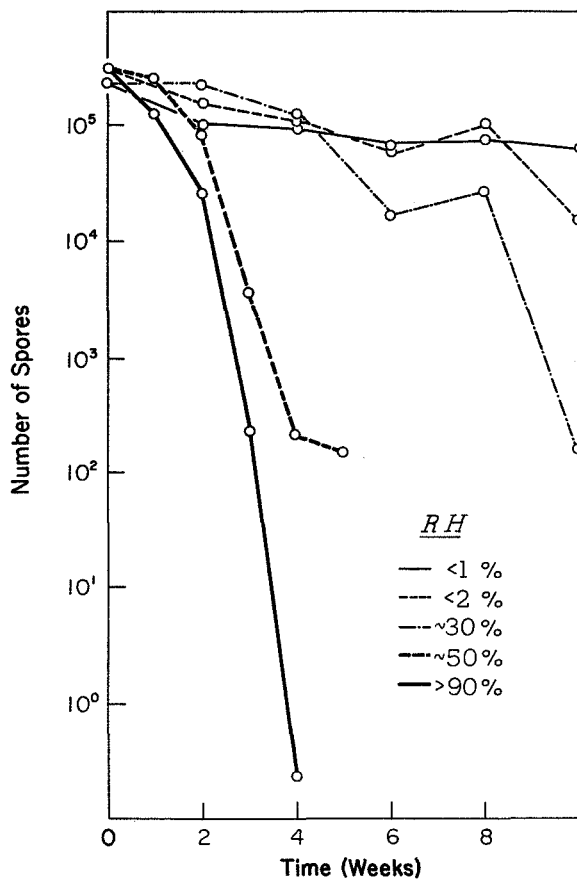


Figure 1.6 - Results of Spore Survival Studies on Stainless Steel at 45°C. Test Nos. GS9281A, B, D, E, and G.

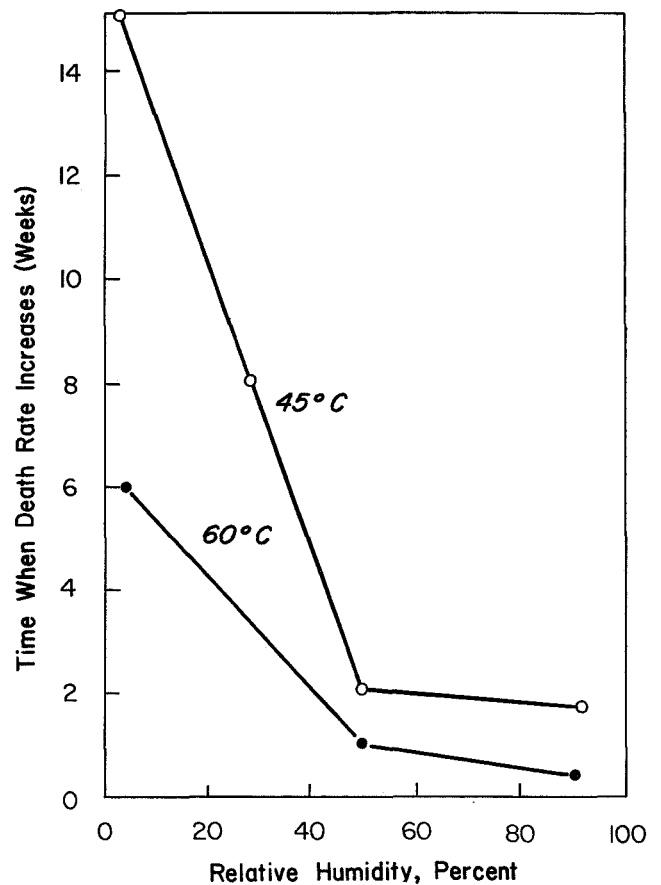


Figure 1.7 - Time at which Death Rate Increases as a Function of Relative Humidity

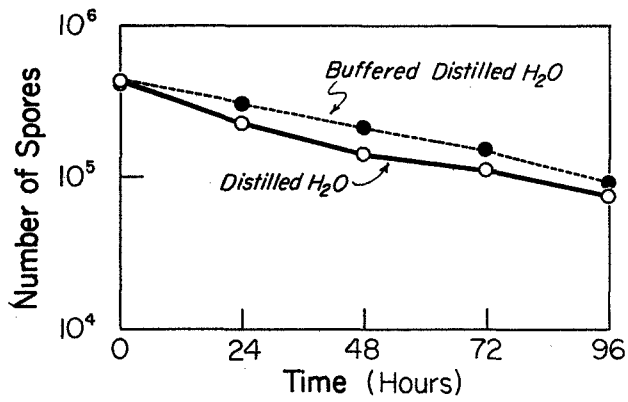


Figure 1.8 - Survivor Curve for Spores in Water at 60°C. Test Nos. GS0075A and B

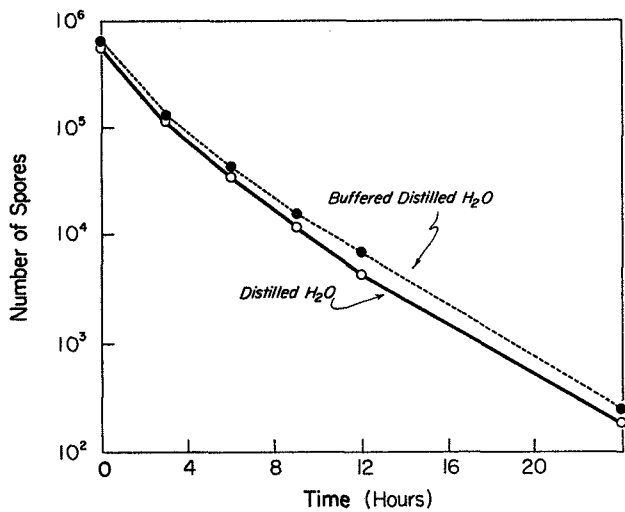


Figure 1.9 - Survivor Curve for Spores in Water at 75°C. Test Nos. GS0075C and D

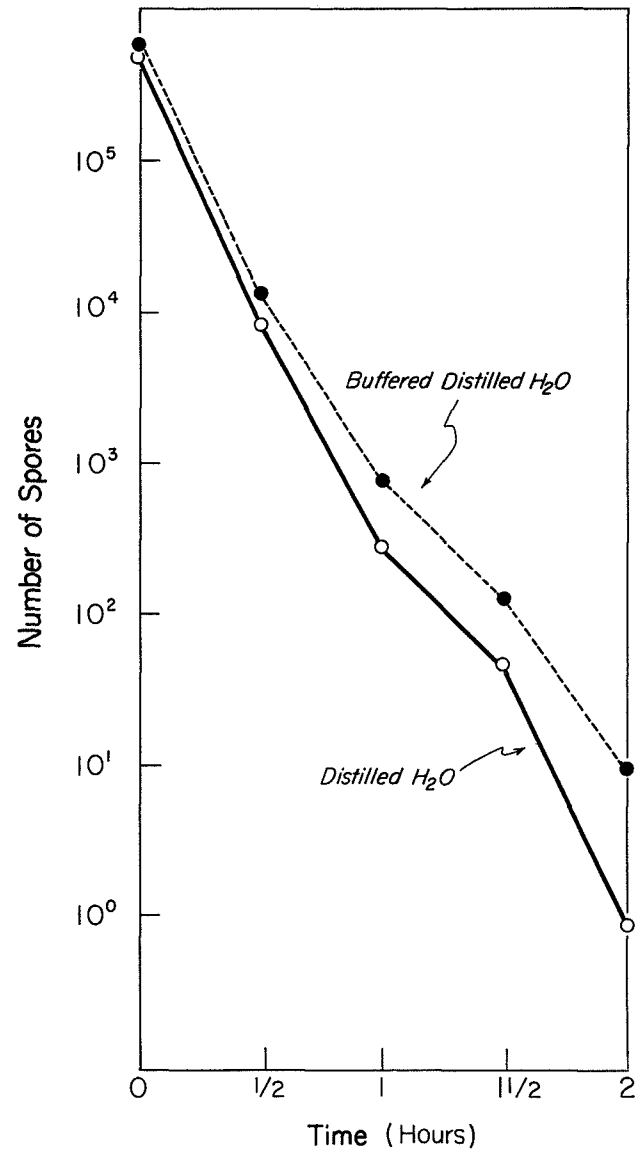


Figure 1.10 - Survivor Curve for Spores in Water at 90°C. Test Nos. GS0077A and B

Tests GS0075A through D and GS0077A and B

The wet heat survivor curves at 60°C, 75°C, and 90°C are summarized in Figures 1.8, 1.9, and 1.10. It can be seen that the buffered distilled water had a consistent, slightly protective effect compared to the non-buffered distilled water at each test temperature.

CONCLUSIONS

Conclusions drawn in the study of survival as a function of temperature and humidity conditions indicate that there is a significant change in the rate of survival with time. The time at which this change in the survival rate occurs appears to be a function of relative humidity. In studies to determine the reproducibility of our data we found that box-to-box variation was relatively small.

In our studies of the D-value of spores of Bacillus subtilis in water we have obtained consistent straight-line survivor curves. There were small differences between distilled water and the buffered distilled water, however, statistical analysis has not been carried out to determine whether or not these differences are significant.

At 60°C it is possible to compare D-values for Bacillus subtilis var. niger at 2%, 50%, and 90% over silica gel and at 100% in distilled and buffered distilled water. The data collected thus far indicates maximum D-values at 2% and in water (100%) with minimum D-values at 50 and 90% relative humidity. This is just the reverse of results that were obtained at 110 and 125°C where the maximum D-values were found at intermediate, 35 to 50%, relative humidities and minimum D-values were obtained at very low and at 100% relative humidity. Needless to say these data are very disturbing since they do not fit the same pattern. We hope in the future to explain these seemingly conflicting results.

FUTURE WORK

Experiments at 75 and 90°C for a range of relative humidity conditions are our priority items in this project. However, establishing and verifying these conditions is a reasonably difficult laboratory problem and to date it has not been solved. These experiments thus must await the completion of the apparatus which will prove the subsequent testing program to be reliable. These experiments can then get under way. We plan to continue using Bacillus subtilis var. niger (AAAA or AADA). In subsequent studies we plan to use

Bacillus firmus (VDLB) and another variety of Bacillus subtilis.

A group of experiments which will determine the behavior of spores at several water activity levels are being planned. Sucrose and glycerol systems will be used to give water activities of .85, .90 and .99 and to evaluate these conditions at 45, 60, 75 and 90°C. Along with this study we plan to evaluate acetone at 45°C and ethyl and isopropyl alcohol at 45 and 60°C. Both acetone and alcohol are used as storage substrates for spores and therefore their bactericidal characteristics should be known.

THE EFFECT OF HUMIDITY, LOCATION, SURFACE FINISH AND
SEPARATOR THICKNESS ON THE DRY HEAT DESTRUCTION OF
BACILLUS SUBTILIS VAR. NIGER SPORES LOCATED BETWEEN MATED SURFACES

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Division of Environmental Health
Project Contributors: J. Bearman and R. Jacobson
Division of Biometry

INTRODUCTION

In Progress Reports 1, 2, and 3, the mated surface experimental system was described and the experimental results to date were summarized. In this report the data processing system will be described. This system is capable of handling data from several of our projects. It reduces considerably the time required for certain analyses to be performed.

OBJECTIVE

The objective of this project is to measure the thermal death parameters of Bacillus subtilis var. niger spores in a mated surface system and to determine the effect of certain varieties of the environment and surfaces on thermal destruction. The work reported here has the additional objective of providing a new data processing tool for use within the planetary quarantine effort.

DISCUSSION

To assist in the processing of data from the mated surface system, as well as the data from other experimental systems used in these laboratories, a data processing system has been developed and is centered around a Control Data Corporation 3300 digital computer. Experimental data is recorded by the experimenter or by another person on special forms, from which punch-card decks are prepared for input into the computer. The output comes from a high-speed printer.

The use of the computer has been necessitated by increased volumes of data and by a need for more complex routine analyses of the data. As experimental systems became more streamlined and as laboratory personnel became accustomed to them, they were able to generate larger volumes of data. To maximize the utilization of the information provided by each experiment,

statistical analysis and other manipulation of the data must be carried out as soon as the raw data are available. A desk calculator could be used to perform most of the analyses, however, the computer does it in less time and also provides the added advantages of storage of the data and of a more detailed printer output format. More specifically, we believe that the D-value (i.e. the time to kill 90% of the cells in a sample) is inadequate by itself to describe the behavior of the cells in the experimental system. We believe that in addition to the D-value, confidence intervals and the y-intercept of the regression line are needed. Analyses of variance and information about the shape of the survivor curve are also needed. To carry out these analyses frequently requires a transformation of the data to obtain constant variance. For example, the mated surface system requires a logarithmic transformation of the data.

The computer also provides other more general benefits. Since a standard format for experimental data has been established the data accumulated over a period of months or years can easily be pulled together. Computer output can be prepared quickly and sent to groups who are carrying out cooperative experiments. For this purpose, and when preparing tables for reports, a considerable saving in proofreading is achieved. The punch-card deck is proofread when it is produced and if the program is accurate, the correct output will be produced each time.

The choice of punch-card input and printer output was made in the interest of flexibility during the developmental stages of the program. The system can be adapted for other types of input and output (e.g. teletype) if that later becomes desirable. When a sufficient amount of experimental data is accumulated, mass storage of data on tape or discs will be considered.

In developing the data analysis system many arbitrary decisions had to be made. One of the first and most important decisions that had to be made was which bit of experimental data should be used as the starting point in the data analysis. In our spore destruction rate test system, the spores were inoculated onto plates and heated. The plates were then dropped into flasks to which buffer solution was added. The flasks containing the plates and buffer solution were then sonicated for two minutes. The buffer solution containing the spores from the plates was then plated at several dilution levels. The plates were incubated and counted using the guidelines in Appendix E, Progress Report #3. The data from one dilution were selected for calculating the final number of survivors per strip. These data consisted of the count on plate 1, the count on plate 2 and the dilution factor. At this point we have the alternative of develop-

ing a system in which we put the data from plate count 1, plate count 2 and the dilution factor into the computer or we allow the researcher to manipulate D-values and we only put the final estimated number of spores on each strip into the computer. In our spores in mated surface areas study we chose to utilize the estimated number of spores on each strip for the following reasons:

1. Recovery methods might change. For example, most probable number techniques might be used, but nearly every microbiological procedure at some point gives an estimate of the number of spores in the original sample.
2. Different microbiologists have different ways of expressing dilution factors which could have led to confusion if plate counts and dilution factors were used.

After we had gotten this system operating it became apparent that there were certain advantages to utilizing the plate counts and the dilution factor in our computer input. This procedure has been used in some of our other projects.

Another problem which arose was that of adjusting the computer system to make it acceptable to the laboratory personnel and to make it provide all the information which was needed. In order to input the information to the data handling system, forms (Figures 2.1, 2.2, and 2.3) which were understandable to both laboratory personnel and the lab service personnel had to be designed. Although it is hoped that eventually the lab personnel will record their own data on the coding forms, in the initial use of the system another person was utilized to collect and record data. This was necessary to minimize the initial stages in the use of the system. A set of short instructions, given on pages 15-18, were prepared to assist in the use of the data forms.

Sample outputs from the three programs which have been completed are shown in Tables 2.1, 2.2, and 2.3 on pages 20 and 21. (The three programs are available on request.) PROGRAM DATA 2 carries out linear regressions on the data then prints out data and results; a typical print out is shown in Table 2.1. PROGRAM RAW DATA prints a table of the data with information about the experiment and with a summary of experimental results; a typical print out is shown in Table 2.2.

Our programming was carried out so that we were quite flexible in analysis. To allow for experimental differences, the D-value, y-intercept and their confidence limits were computed in three different ways:

1. Each observation was taken to be an experimental unit. This was appropriate when samples were treated independently of one another.
2. The mean for each treatment time was taken to be an experimental unit. This was appropriate, for example, when all samples for a given treatment were placed in a single sample carrier.
3. The D-value was calculated separately for each column of the data table and the results were averaged. This was appropriate when each column of data represented the behavior of a different position on a test surface.

It should be noted that each of these approaches produced the same D-value but considerably different confidence limits. When predicting the reliability of a sterilization process it is extremely important that the proper experimental unit is identified and used in the analysis.

In one of our results tables we show a negative D-value in the confidence limits. A negative D-value will result if the confidence limit of the slope includes zero. In theory, a negative D-value would be interpreted as resulting from multiplication of the organisms. In this program a negative D-value indicates that the upper limit D-value is approaching infinity; the survivor curve is horizontal meaning no decrease in the microbial population.

PROGRAM P00LER A combined the results of up to twenty tests and then carried out the same calculations as were performed by PROGRAM DATA 2. A typical print out is shown in Table 2.3.

During the development of these programs an attempt was made to fit the computer to the existing laboratory situation and to preserve the roles of the personnel. Over a period of time, statisticians, programmers, and microbiologists begin to consult about these programs and about functions to be performed by future programs. This is necessary to foster the development of improved data-handling systems and the development of experiments using the rapid data-handling capabilities of the computer which were not previously possible.

A computer program presently under development by the Biometry Division uses the data decks and input sub-routine of this system to print summary tables of the data. The statisticians in the Biometry Division have indicated that these tables, which include raw data values as well as means, will be of considerable utility to people who wish to carry out other specialized statistical tests and analyses. The program has been written and is now in the revision and debugging stage. In addition, programs to assist with the analysis of variance and to analyze the shape of the survivor curve are in the flow-chart stage. Programs to carry out complete analyses of variance will not be written since the analysis of variance should be

tailored to each experimental system, and to each set of experiments performed. Instead the program will calculate sums of squares according to a simple set of instructions coded on a special form. The analysis will then be completed on a desk calculator. The shape of the survivor curve will be analyzed by fitting higher-order polynomials to the survivor curve and comparing these with the fit provided by a linear regression. Such a procedure will not solve the problem of interpreting the shape of survivor curves but it will indicate whether curvatures discovered in particular experiments are statistically significant, i.e., are more than can reasonably be attributed to chance.

Instructions for Data Forms 1 and 2

Information Card

col	Experiment Code
1-2	Initials of the experimenter
3-6	In a one-day experiment this should be the same as col 9-12 (year and day treatment began). In a multi-day experiment use the coded date for the first day of the experiment.
7	Sequentially assigned letter A-Z to differentiate sets which otherwise would have the same experiment number.
9	Last digit of the year (e.g. 0 for 1970)
10-12	Sequential number of the day (e.g. for January 9, would be written 009; October 20 would be written 293 except in leap year.) This number can be found on most desk calendars.
13	Not used at this time
15-19	Spore code. If your present spore code has only 4 letters, leave col 19 blank.
20-24	Not used at this time but the experimenter may wish to record the data for future reference.
25-27	Apparatus code. Col 25 must be Ø, M, or C for open, mated or closed system respectively. Col 26 and 27 refer to specific treatment systems. Currently the legal codes are: Ø (open) 01 Oven with hanging strips 02 Hot plate in clean room 03 Hot plate in glove box 04 Strips in plastic boxes 05 Strips in glass jars M (mated) 01 Heat block C (closed) 01 Bolted blocks

Data processing codes. (Only cols. 29-33 are currently used)

29	Option number. If 0 is recorded, column D-values will not be calculated. If 1 is recorded, column D-values will be calculated. N.B: If 1 is recorded here the same number of columns must be filled for each data card having a treatment time greater than 0.
30-31	Sequential number for sets of an experiment having more than one data set.
32-33	Total number of sets in the experiment.
41-44	Conditioning temperature in degrees Centigrade. The computer places a decimal point between cols. 43 and 44.
45-49	Conditioning RH in percent. Unless decimal point is filled in, computer will place it after col. 49.
50-53	Conditioning time in hours. Unless a decimal point is filled in, the computer will place it after col. 53.
54-60	Not used at present.
65-68	Treatment temperature in degrees Centigrade. The computer places a decimal point between cols. 67 and 68.
69-73	Treatment RH in percent. This should be the actual RH at the treatment temperature. Unless a decimal point is filled in, the computer will place it after col. 73.
74-74	Number of treatment times. Not used at present.

Comment Cards

Comment cards may be omitted; the maximum number is 6 per data set. These may contain any information desired. The information is reproduced exactly as recorded.

Data Cards (minimum = 1, maximum = 15)

col.

1-7	The experiment code must be exactly the same as on the information card.
9-15	Treatment time in minutes. The treatment time may be recorded in 3 ways. In all cases the number should be right justified. 1. As a number without decimal point. In this case the computer will insert a decimal point after col. 15. 222. As a number with decimal point. 3. In exponential format e.g. 52,500,000 may be recorded as 5.25E08; the decimal point should be included in the left half of the number.
	Except for very long treatment times, method 1 or 2 is recommended as least confusing. If there is a card with treatment time = 0 it must come before other data cards.
21-80	Data. The data must be recorded in exponential form. Examples: This number could be recorded as

5.67×10^8

567E8

90. ($=9.00 \times 10^1$)	900E1
6.5 ($=6.50 \times 10^0$)	650E0
0.0	000E0
2735. ($=2.74 \times 10^3$)	274E3

If option 1 has been chosen (col. 29 of information card) each data card with treatment time greater than zero must have the same number of columns of data.

Results Card

This card provides a quick summary of results. It may be omitted. Normally the results card would be prepared after the data set has been run once to provide the needed information.

col.

1-7	Experiment code. Must be same as on information card.
9-15	Log of N_0 . Decimal point must be filled in.
16-22	S.E. of Log of N_0 . Decimal point must be filled in.
23-25	Number of observations used to determine N_0 . The computer will insert a decimal point after col. 25.
28-34	Log of y-intercept. Decimal point must be filled in.
35-41	S.E. of Log of y_0 . Decimal point must be filled in.
42-44	Number of observations used to determine y_0 . The computer will insert a decimal point after col. 44.
47-53	Slope of regression line. Minus sign (if negative slope) and decimal point must be included.
54-60	S.E. of slope of regression line. Decimal point must be filled in.
61-63	Number of observations used to determine the slope. The computer will insert a decimal point after col. 63.

Title Card

The title card is used to title and, optionally, to number the columns of the data table. It may be omitted.

1-7	Experiment code must be the same as that on the information card.
9-33	Title for the columns of the data table.
	Recommended titles:
	Position number --if the column number is significant
	Strip number(unordered)--if the column number is not significant
34-57	Optional numbers for the columns. Cols. 34-35 may have the number for data col. 1; cols. 36-37 may have the number for data col. 2, etc.
	If col. 34 and col. 35 are blank, the data columns will be numbered 1-12 automatically.

Program Deck for RAW DATA or DATA 2

After the \$LGØ card, place all the data sets to be run in the order desired. After the last deck, place a blank card followed by an end-of-the-file card. N.B: A blank card at any other point will abort the program.

Instructions for Form 3 for Program PØØLERA

This form is used in conjunction with Program PØØLERA to pool a number of data sets. The output format is similar to that of Program DATA 2.

The following maxima apply:

1. 20 data sets per pool request
2. 10 pool requests per sheet
3. 100 different data sets requested per sheet

Each pool request consists of 1 or 2 cards. If a request involves pooling of 1 to 10 sets only one card is needed and col. 2 of the card must be left blank. If a pooling of 11 to 20 sets is requested the first card must be labeled A in col. 2 and the second must be labeled B in col. 2.

Fill in the requests from left to right.

The selection of option 0 or 1 (see instructions for Form 1 col. 29) will be controlled by the last data set in each pool request. If the output from option 1 is to be meaningful, all data sets in the pool request must have the same number of columns for each data card with a treatment time greater than zero.

In all cases, data cards with treatment time equal to 0 are excluded from processing.

Output Controls

If a 1 is placed in col. 3, data tables will be printed for each set.

If a 1 is placed in col. 4, the pooled table of logs will be suppressed.

Program Deck

After the \$LGØ card, place the punched deck from Form 3. This must end in a STØP card. Then insert the data sets followed by a blank card and the end-of-file card. The data sets must, of course, include all those requested; in addition extraneous sets may be included as long as the total does not exceed 100.

TABLE 2.1

Printout from PROGRAM DATA 2

THIS IS A POOLING OF THE FOLLOWING DATA SETS
 DDB320I DDB320J DDB320K DDB320L

TABLE OF LOGS OF (DATA + 1.0)

TIME	COUNTS					
22.50	3.3224	3.0216	3.7102	3.9948	3.7560	3.1764
45.00	2.6665	2.9886	3.1339	3.3524	3.3804	3.4234
67.50	0	0	1.3222	2.0645	2.2455	2.0755
22.50	2.6542	3.1402	3.6485	3.6436	3.7227	3.6465
45.00	1.8195	1.2041	2.4216	2.6010	1.9708	2.3784
67.50	1.5250	1.7284	2.5786	2.6010	2.8122	2.8998
22.50	3.2044	3.3224	3.6485	3.7521	3.7521	4.0171
45.00	2.3032	1.4914	2.7701	2.9299	2.8954	3.0535
67.50	2.0453	2.5729	1.3222	2.3729	2.9595	3.3858
22.50	3.0973	3.1764	3.5564	3.4067	3.4773	3.6795
45.00	2.0828	2.0934	3.1075	3.3581	3.5741	2.9818
67.50	0	1.1303	1.9469	2.0374	2.2068	1.8195

TABLE OF MEANS FOR EACH TREATMENT TIME

TIME	MEANLOG	SELOG	MEAN	.95 C.I.		NO. OF OBS.
22.50	3.4803	.3307	3.02E 03	6.26E 02	1.46E 04	24
45.00	2.6659	.6401	4.63E 02	2.20E 01	9.74E 03	24
67.50	1.9022	.9177	7.98E 01	1.01E 00	6.29E 03	24

DVALUE CALCULATION USING EACH INPUT DATUM AS AN EXPERIMENTAL UNIT, NZERO EXCLUDED

BIOMED COMP

NUMBER OF THIS RUN IS POOLED DATA

X = TIME IN MINUTES

Y = LOG OF NO. SPORES IN ORIGINAL SAMPLE

N = 72 XBAR = 45.00000

YBAR = 2.68280 10**YBAR = 4.82E 02

STD DEV X = 18.50010 STD DEV Y = .92846

STD DEV Y/Y = .66889 RHO = -0.69877

Y-INTERCEPT = 4.26091 SLOPE = -0.03507

SE Y-INT = .20856 SE SLOPE = .00429

TABLE OF DVALUE, ANTILOG INTERCEPT, AND .95 CONF IVALS

ESTIMATE LOWER C.L. UPPER C.L.

D VALUE(MINUTES) 28.52 22.93 37.71

DVALUE CALCULATION USING THE GEOMETRIC MEAN FOR EACH TREATMENT TIME AS THE EXPERIMENTAL UNIT, NZERO EXCLUDED

BIOMED COMP

NUMBER OF THIS RUN IS POOLED DATA

X = TIME IN MINUTES

Y = LOG OF NO. SPORES IN ORIGINAL SAMPLE

N = 12 XBAR = 45.00000

YBAR = 2.68280 10**YBAR = 4.82E 02

STD DEV X = 19.18806 STD DEV Y = .77974

STD DEV Y/Y = .41317 RHO = -0.86299

Y-INTERCEPT = 4.26091 SLOPE = -0.03507

SE Y-INT = .31556 SE SLOPE = .00649

TABLE OF DVALUE, ANTILOG INTERCEPT, AND .95 CONF IVALS

ESTIMATE LOWER C.L. UPPER C.L.

D VALUE(MINUTES) 28.52 20.19 48.55

INTERCEPT 1.82E 04 3.61E 03 9.21E 04

TABLE OF COLUMN BY COLUMN VALUES. NUMBER OF OBSERVATIONS IN EACH COLUMN VALUE IS 12

NO.	LOG ICPT	SE ICPT	SLOPE	SE SLOPE	XBAR	10**YBAR	INTERCEPT	.95 CI INTERCEPT	D-VALUE	.95 CI D-VALUE	NO.
1	4.23703	.44981	-0.04838	.01008	45.00	1.15E 02	1.73E 04	1.40E 03 2.13E 05	20.67	14.12 38.59	1
2	3.96306	.57608	-0.04016	.01185	45.00	1.43E 02	9.18E 03	4.77E 02 1.77E 05	24.90	15.02 72.77	2
3	4.61227	.29444	-0.04108	.00606	45.00	5.81E 02	4.10E 04	9.03E 03 1.86E 05	24.35	18.32 36.27	3
4	4.43988	.21774	-0.03179	.00448	45.00	1.02E 03	2.75E 04	9.01E 03 8.42E 04	31.46	23.94 45.67	4
5	4.18373	.35055	-0.02491	.00721	45.00	1.16E 03	1.53E 04	2.53E 03 9.23E 04	40.14	24.40 113.19	5
6	4.12947	.38522	-0.02410	.00793	45.00	1.11E 03	1.35E 04	1.87E 03 9.73E 04	41.49	23.94 155.33	6

COLUMN D-VALUES

MEAN POOLED SAMPLE SE .95 CONF IVAL FOR THE MEAN
 28.52 .00339 22.84 37.95

TABLE 2.2
Printout from PROGRAM RAW DATA

DATA TABLE FOR EXPERIMENT NUMBER DD9141A
EXPERIMENT STARTED ON DAY-141, YEAR-1969. SPORES-AAAC .
APPARATUS TYPE-MATED , NAME-HEATBLOCK
SAMPLES CONDITIONED AT 23.0 C, 90 PCT. R.H. FOR 18 HOURS.
SAMPLES TREATED AT 125.0 C, .34 PCT. R.H. FOR TIMES LISTED.
COMMENTS-INVESTIGATION OF A SLOW-KILL TREATMENT OVER A 6 HOUR PERIOD: HANDLING
AND ENVIRONMENTS ARE IDENTICAL TO THE CONDITIONED-HIGH,TREATED LOW
PORTION OF EXPERIMENT DD9066.
TIME(MIN.) POSITION NUMBER

	1	2	3	4	5	6	7	8	9	10	11	12
0	1.72E 05	1.63E 05	1.72E 05	2.33E 05	1.84E 05	1.85E 05	2.24E 05	2.21E 05	2.17E 05	1.47E 05	1.78E 05	2.11E 05
60.00	2.98E 04	2.80E 04	6.65E 04	7.70E 04	7.00E 04	6.40E 04						
210.00	8.88E 02	1.01E 03	2.50E 03	3.53E 03	2.23E 03	2.28E 03						
360.00	0	0	1.00E 01	1.30E 01	0	0						

SUMMARY OF RESULTS FROM POOLED DATA
NZERO NOT INCLUDED IN D-VALUE OR INTERCEPT CALCULATIONS

	ESTIMATE	NO REPS	.95 CONFIDENCE LIMITS	
D-VALUE	68.97	18	60.25	80.63
Y-INTERCEPT	6.70E 05	18	2.08E 05	2.16E 06
NZERO	1.90E 05	12	1.38E 05	2.62E 05
LOG ICPT/LOG NZERO	1.10			

TABLE 2.3
Printout from PROGRAM POOLER A

DATA TABLE FOR EXPERIMENT NUMBER DU9141A
EXPERIMENT STARTED ON DAY-141, YEAR-1969. SPORES-AAAC .
APPARATUS TYPE-MATED , NAME-HEATBLOCK
SAMPLES CONDITIONED AT 23.0 C, 90 PCT. R.H. FOR 18 HOURS.
SAMPLES TREATED AT 125.0 C, .34 PCT. R.H. FOR TIMES LISTED.
COMMENTS-INVESTIGATION OF A SLOW-KILL TREATMENT OVER A 6 HOUR PERIOD: HANDLING
AND ENVIRONMENTS ARE IDENTICAL TO THE CONDITIONED-HIGH,TREATED LOW
PORTION OF EXPERIMENT DD9066.
TIME(MIN.) POSITION NUMBER

	1	2	3	4	5	6	7	8	9	10	11	12
0	1.72E 05	1.63E 05	1.72E 05	2.33E 05	1.84E 05	1.85E 05	2.24E 05	2.21E 05	2.17E 05	1.47E 05	1.78E 05	2.11E 05
60.00	2.98E 04	2.80E 04	6.65E 04	7.70E 04	7.00E 04	6.40E 04						
210.00	8.88E 02	1.01E 03	2.50E 03	3.53E 03	2.23E 03	2.28E 03						
360.00	0	0	1.00E 01	1.30E 01	0	0						

SUMMARY OF RESULTS FROM POOLED DATA
NZERO NOT INCLUDED IN D-VALUE OR INTERCEPT CALCULATIONS

	ESTIMATE	NO.OBS.	.95 CONFIDENCE LIMITS	
D-VALUE	68.97	18	60.25	80.63
Y-INTERCEPT	6.70E 05	18	2.08E 05	2.16E 06
NZERO	1.90E 05	12	1.38E 05	2.62E 05
LOG ICPT/LOG NZERO	1.10			

NZERO DATA SUMMARY

LOG (DATA+1.0)	5.2355	5.2122	5.2355	5.3674	5.2648	5.2672	5.3502	5.3444	5.3365	5.1073	5.2504	5.3243
LOG NZERO	=	5.2796		NZERO	=	1.90E 05						
SE LOG NZERO	=	.0635		.95 CONF	INTERVAL=	1.38E 05		2.63E 05				
NO. REPS	=	12										

TABLE OF LOGS OF (DATA + 1.0)

TIME	COUNTS					
60.00	4.4742	4.4472	4.8228	4.8865	4.8451	4.8062
210.00	2.9489	3.0048	3.3981	3.5479	3.3485	3.3541
360.00	0	0	1.0414	1.1461	0	0

TABLE OF MEANS

TIME	MEANLOG	SELOG	MEAN	.95 C.I.		NO. OF OBS.
60.00	4.7137	.1980	5.17E 04	1.60E 04	1.67E 05	6
210.00	3.2677	.2371	1.85E 03	4.55E 02	7.54E 03	6
360.00	.3646	.5658	2.32E 00	8.13E-02	6.60E 01	.6

Table 2.3 continued

DVALUE CALCULATION USING EACH INPUT DATUM AS AN EXPERIMENTAL UNIT, NZERO EXCLUDED

BIOMED COMP

NUMBER OF THIS RUN IS 009141A

X = TIME IN MINUTES

Y = LOG OF NO. SPORES IN ORIGINAL SAMPLE

N	=	18	XBAR	=	210.00000
YBAR	=	2.78199	10**YBAR	=	6.05E 02
STD DEV X	=	126.02521	STD DEV Y	=	1.89340
STD DEV Y/X	=	.51240	RHO	=	-0.96492
Y-INTERCEPT	=	5.82635	SLOPE	=	-0.01450
SE Y-INT	=	.23973	SE SLOPE	=	.00099

TABLE OF DVALUE, ANTILOG INTERCEPT, AND .95 CONF IVALS

	ESTIMATE	LOWER C.L.	UPPER C.L.
D VALUE(MINUTES)	68.98	60.29	80.60
INTERCEPT	6.70E 05	2.08E 05	2.16E 06

DVALUE CALCULATION USING THE GEOMETRIC MEAN FOR EACH TREATMENT TIME AS THE EXPERIMENTAL UNIT, NZERO EXCLUDED

BIOMED COMP

NUMBER OF THIS RUN IS 009141A

X = TIME IN MINUTES

Y = LOG OF NO. SPORES IN ORIGINAL SAMPLE

N	=	3	XBAR	=	210.00000
YBAR	=	2.78199	10**YBAR	=	6.05E 02
STD DEV X	=	150.00000	STD DEV Y	=	2.21485
STD DEV Y/X	=	.59489	RHO	=	-0.98180
Y-INTERCEPT	=	5.82635	SLOPE	=	-0.01450
SE Y-INT	=	.68175	SE SLOPE	=	.00280

TABLE OF DVALUE, ANTILOG INTERCEPT, AND .95 CONF IVALS

	ESTIMATE	LOWER C.L.	UPPER C.L.
D VALUE(MINUTES)	68.98	19.95	~47.31
INTERCEPT	6.70E 05	1.46E-03	3.09E 14

TABLE OF COLUMN BY COLUMN VALUES. NUMBER OF OBSERVATIONS IN EACH COLUMN VALUE IS 3

NO.	LOG ICPT	SE ICPT	SLOPE	SE SLOPE	XBAR	10**YBAR	INTERCEPT	.95 CI INTERCEPT	D-VALUE	.95 CI D-VALUE	NO.		
1	5.60634	.66603	-0.01491	.00274	210.00	2.98E 02	4.04E 05	1.39E-03	1.17E 14	67.05	20.11	-50.25	1
2	5.59700	.73094	-0.01482	.00301	210.00	3.05E 02	3.95E 05	2.04E-04	7.68E 14	67.46	18.86	-42.77	2
3	5.73445	.43604	-0.01260	.00179	210.00	1.22E 03	5.43E 05	1.56E 00	1.89E 11	79.33	28.25	-98.16	3
4	5.81177	.49741	-0.01247	.00205	210.00	1.56E 03	6.48E 05	3.10E-01	1.36E 12	80.21	26.00	-73.90	4
5	6.12277	.86642	-0.01615	.00356	210.00	5.39E 02	1.33E 06	1.30E-05	1.36E 17	61.92	16.28	-34.32	5
6	6.08577	.89363	-0.01602	.00368	210.00	5.27E 02	1.22E 06	5.37E-06	2.76E 17	62.42	15.94	-32.58	6

COLUMN D-VALUES

MEAN	68.98	PUOLED SAMPLE SE	.00118	.95 CONF IVAL FOR THE MEAN	57.04	87.25
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DETECTION OF LOW LEVELS OF MICROBIAL CONTAMINATION ON SURFACES BY CHEMICAL APPROACHES

Project Personnel: V. Goppers and I. J. Pflug
Division of Environmental Health

INTRODUCTION

Studies during the past several years have been directed toward developing a chemical method that can be used for quickly enumerating surface microbial contamination. Our program has been based on an analysis of ATP (adenosine triphosphate). We have chosen to follow an ATP analysis utilizing thin-layer chromatography plus a sensitive photometer system.

OBJECTIVE

The immediate objective of this project is to determine the minimum concentration of ATP that can be reproducibly analyzed by the thin-layer chromatography-microscope photometer system and then to determine the effect of interfering substances on this determination. Activities during this reporting period have all been directed toward improving different phases of the analysis technique.

For ultimate sensitivity the microscope photometer requires a high quality, thin-layer chromatographic plate with special surface characteristics and a high degree of purity of the thin-layer material. In order to fulfill those requirements, time has been devoted to the following phases of technique development:

1. Purification of the thin-layer material
2. Improving the surface quality of the plate
3. Preparing chromatographic plates with uniform layer thickness
4. Improving the method of sample application
5. Developing aids that will make it possible to rapidly prepare several thin-layer plates at the same time under uniform conditions.
6. Studies of factors that affect the development of the chromatogram so that interfering conditions such as humidity in the surrounding laboratory atmosphere can be minimized.

Experiments carried out

1. The material used for the thin-layer was cellulose powder MN-300

obtained from Machery, Nagel and Co., in Germany. This material has been prepared especially for chromatographic use, however, for our applications both a preliminary and a final purification are required to separate out trace amounts of interfering substances.

Two different methods were developed and used for the preliminary purification of the cellulose powder. Initially the impurities were extracted from the cellulose powder using n-butanol in a Soxhlet unit. In the second method about 50 grams of the MN-300 cellulose powder were suspended in a glass-stoppered Erlenmeyer flask containing 500 ml of the same solvent system that will be used in the chromatographic procedure. The flask was shaken for 30 minutes in an automatic shaker and then filtered. The solid residue was rinsed with n-butanol and dried under vacuum until the solvent was removed. This dried powder was used for the preparation of the micro thin-layer plates.

The final purification (to remove air fall out contamination) of the prepared micro thin-layer plates was obtained by chromatographic action of the solvent system that will be used in the final separation procedure. The solvent system was allowed to travel the whole length of the plate in ascending action. Following chromatographic cleaning, the plates were removed from the solvent system and dried. At this point the plates were clean and ready for use, however, they had to be protected from outside contamination. When inspected in ultraviolet light, the chromatographically cleaned thin-layer plates showed a bright fluorescent band at 75% distance from the starting line. The area used for the separation of the adenosine compounds is below this fluorescent line.

2. Studies were carried out to improve the surface quality of the thin-layer plates. To eliminate the reflected light from the surface of the thin-layer plates during microscope photometer measurement, the plates must be prepared from cellulose material consisting of relatively small, even-size particles. The final dried surface must be smooth and even. To improve the surface of the plate, small amounts of several inert materials were added to the cellulose powder. Improvements were noticed after the addition of 0.5 to 2% hydrated calcium sulfate (plaster of Paris). This additive caused the plates to set faster and the surface was more even; unfortunately the additive interfered with the separating action and no separation occurred.

The liquid used for the preparation of the basic cellulose slurry was evaluated. Acetone was used instead of water. The plates prepared with acetone had entirely different physical properties than those prepared with water; the layer was not as compact and the developing time was much shorter. Inspection

of the developed chromatograms in the microscope photometer revealed that although the plates were excellent for qualitative use they were not usable for quantitative evaluation since the outlines of the separated compounds were blurred due to the porosity of the layer.

It was decided that the cellulose powder must be mixed with water and improvements made in the preparation of the slurry. Different mixing methods were tried with the best results being obtained by homogenizing the cellulose with water in a Waring blender for 30 to 45 seconds. Time is critical! This method produced plates with even surfaces.

3. Different techniques were tried to obtain uniform thicknesses on the micro thin-layer plates. First, the plates were prepared by a dipping method. Another way involved spraying the suspension on large groups of plates. Both methods failed to produce the required quality of the chromatographic layer. It turned out that the best way to prepare the plates was by rapidly delivering a measured quantity of the suspension on the plate and distributing it evenly by gentle tilting. The coated plates were allowed to dry at room temperature, and were then activated for 15 minutes at 110°C and stored in a desiccator.

4. To prepare groups of micro thin-layer chromatograms for evaluation in the microscope photometer, improvements were needed in the method of sample application. In regular thin-layer chromatography the prepared standard solution or the solution of analysis is spotted on a pre-determined place on a thin-layer plate, dried and then developed. To evaluate the chromatographic plates using the microscope photometer system the whole procedure must be more mechanized in order to utilize the higher precision of the measuring system. All chromatograms must be prepared under similar conditions.

Spotting the sample at a certain geometrical location on each plate appeared to be the solution. An applicator assembly was constructed consisting of a laboratory jack with an attached Lucite frame with millimeter scales in two directions. A ten microliter (smallest division 1 μ l) Hamilton syringe was located above the Lucite frame. The micro thin-layer plates were lined up on a glass plate and moved from right to left on the Lucite platform. Using this set-up, the middle point of the micro plate and the desired distance from the end could be precisely located. When the plate was in the proper position under the syringe, the platform was raised until the needle of the syringe touched the thin-layer plate. The desired volume was delivered, the platform lowered, the next micro slide moved into position, and the procedure repeated.

In this manner the spots were very precisely located and the delivered quantities were accurate.

5. It was impossible to handle a group of plates by hand as single units and to prepare them under identical conditions of time, temperature, humidity, etc. To simultaneously place all the micro plates in the chromatography chamber and remove them all at the same time, special glass holding racks were designed. Glass rods (3 mm diameter) were bent so as to form a rack that would hold thirteen micro slides. This unit was made completely of glass because it would come in contact with the solvent system during the developing procedure. Using this holder it was possible to place all of the plates in the solvent system and remove them all at the same time. This system also served to reduce contamination by less handling.

6. The quantity of interfering material must be reduced as the sensitivity of an analysis is increased. In measuring quantities of less than 0.1 μg , one of the major problems is reducing interference.

The primary agents which interfere with the direct scanning of fluorescent compounds on thin-layer plates are: humidity, temperature, and time. Beside these three factors there can be chemical changes (e.g. oxidation or loss of fluorescence if the material is exposed too long to ultraviolet light).

One of the most troublesome interfering factors is humidity. The dry thin-layer plates removed from the desiccator absorb water from the air and reach a high water level equilibrium in a few minutes. In fluorescent analysis the moisture in the thin-layer suppresses the appearance of the fluorescence.

The influence of water content in the thin-layer in fluorescent analysis was observed many years ago in connection with the separation of DANS(1-Dimethyl-amino-naphthol - 5 sulfonic acid amide) derivatives and quantitative estimation of amines and amino acids in the range 10^{-8} to 10^{-12} M. Seiler and Wiechmann (1963¹, 1966²) described the influence of water on these derivatives and suggested spraying the chromatograms with triethanolamine, isopropanol (20:80). Following a long drying period, the chromatograms were scanned. The interfering humidity effect on the thin-layer under direct fluorescent analysis of the DANS derivatives was also reported by G. Pataki and E. Strasky (1966)³.

¹N. Seiler, G. Werner, and M. Wiechmann. *Naturwissenschaften* 50, 643(1963).

²N. Seiler and M. Wiechmann. *S. Analyt. Chemie* 220, 109 (1966).

³G. Pataki and E. Strasky. *Chimia (Aarau)* 20, 361 (1966).

Moisture content in the thin-layer was studied by D. Jänchen(1968)⁴. This author used the ideas of Seiler and Wiechmann and published data obtained by comparing chromatograms sprayed with a triethanolamine reagent to those that had not been sprayed. He also introduced the importance of the time element.

A group of experiments, using both chemical approaches and variations in drying, was carried out to improve the quality of ATP fluorescence by reducing or eliminating the effect of water.

The use of chemical approaches to eliminate or reduce the influence of water

Developed and partially dried plates were sprayed with triethanolamine/isopropanol (1:4) to eliminate the influence of humidity on the fluorescence of ATP. In tests carried out thus far, this procedure has not eliminated the problem since after spraying and drying the plates for two hours, we still were not able to obtain the desired clear fluorescent pattern. Additional investigations of this method are still in progress.

We are presently investigating the sequence of events in the development and drying of the plates that leads to fluorescence. By trying to learn the sequence of events, we will perhaps be able to see where the water interferes and what steps can be taken to either eliminate or modify the process so that the desired results are obtained. There is also a possibility that the negative results that we have obtained by this method could have resulted from diffusion of compounds on the plates. If the quantities of separated material are below 1 μg and the area of the spot is less than 1 mm in diameter, then a small amount of diffusion to spread out the fluorescent molecules can have a major effect on the appearance of the final chromatogram.

Another chemical approach that was used to eliminate the influence of humidity was the treatment of the developed chromatograms with ammonia vapors to change the pH of the chromatographic layer by neutralizing the acetate ions. Only acetate ions remain after all solvents except water have evaporated. This procedure did not solve the problem either.

A third approach to solving the water problem was through the use of a different solvent system in which the quantities of water present, hopefully, will not interfere with the fluorescence of the ATP compounds. Using a solvent system containing isopropanol/ammonia/water(60:35:5) for the separation of nucleic compounds in quantities from 1 μg to 0.01 μg the results were very good in that

⁴D. Jänchen and G. Pataki. J. Chromatogr. 33, 391 (1968)

the developing time was short and the separation of the standard compounds was excellent. The method was tried with ATP from E. coli. While this solvent system was excellent for separating pure substances it lacked eluting and resolving power in the presence of interfering substances. It was possible to see the fluorescent compounds after a five minute development time but they were overlapped 80% by another substance. It is possible that by applying the multiple developing method the system can be used but then the developing time alone will be two or three hours.

Variations in air drying

An experiment was carried out in which the dry air from a 52 cm long x 11 cm in diameter silica gel column was simply directed over a group of chromatograms which had been just removed from the chromatographic developing chambers. After five minutes the chromatogram located 5 cm below the air outlet was in perfect condition for evaluation using the microscope photometer but the rest of the plates were not of the required quality.

Another group of the developed chromatograms was placed on glass plates and put into a desiccator cabinet. Air from the drying column was blown into the bottom part of the desiccator and the excess air was released at the top corner. The temperature was 22°C and the humidity of the incoming air was below 2%. The chromatograms were divided into five groups and were removed at different times for examination in ultraviolet light at 360 nm. Three groups were taken out of the desiccator after 35 minutes. This experiment was repeated with the chromatograms placed on filter paper instead of glass plates because condensation of moisture was observed in the first experiment. The fourth group was examined after ten minutes and the fifth after 35 minutes. This condensation appeared to have been caused by the fast evaporation of the volatile solvents in the thin-layer. In both cases the fluorescence of ATP in ultraviolet light started to appear after five minutes in the drying unit. However, the development of the fluorescence did not improve with increased length of time in the desiccator. After five minutes of drying time the chromatograms were not of sufficient quality to make the readings in the microscope photometer.

An experimental apparatus equipped with a heater, humidity sensor and air flow meter and containing a reduced-area outlet section to give a high velocity was constructed for studying the drying of thin-layer plates. The air feed to this unit was from the silica gel column described above. The air discharge end of the apparatus was constructed so that a 20 x 20 cm plate could be placed in the air stream as support for twelve micro thin-layer plates.

Groups of chromatograms were prepared with standard ATP according to the procedures outlined previously. They were dried in the unit under different velocity, temperature, and time conditions. When the plates were removed from the drying unit the chromatograms were examined in ultraviolet light at 360 nm and rated according to the appearance of the ATP fluorescence using the following scale: very good-1, good-2, fair-3, and unsatisfactory-4.

In the testing program, the micro thin-layer plates were all numbered and the position of the plate on the 20 x 20 cm glass plate was identified so that its chromatogram quality could be related to position. However, in tests thus far we have found no position effect.

Test 1 was carried out with the relative humidity less than 2%, air temperature of 22°C, air flow rate 0.9 scfm and time in the unit 5, 10, 15, and 25 minutes.

The results of this test were negative in that all chromatograms at all times evaluated rated four. It was observed that after five minutes in the air flow apparatus, the chromatograms were floating in water. At this point we are concluding that this phenomena resulted from water condensation on the cold micro plates. The cold plate condition was due to the evaporation of the highly volatile compounds in the solvent system.

Test 2 was carried out with a relative humidity of less than 2% at 22°C, an air flow rate of 0.9 scfm and times of 5, 10, 15, and 25 minutes. This experiment was similar to Test 1 except that filter paper was placed between the micro thin-layer plates and the large support plates. The results of this test were still negative for all positions at all times.

In Test 3 the humidity was less than 2% at 22°C however, the air flow was increased to 1.25 scfm at time periods of 5, 10, 15 and 25 minutes. There was no noticeable difference between the results from Tests 1 and 2.

In Test 4 the relative humidity was less than 2% at 22°C, the velocity across the plates was between 750 and 800 feet per minute and the time periods were 5, 10, 15, and 25 minutes. After 5 and 10 minutes the chromatograms were rated 3; whereas, after 15 and 25 minutes they were rated 4.

In Test 5 the air temperature was increased to 27°C, the relative humidity was 2%, and the velocity across the plates was 527 feet per minute. Drying times of 5, 15, and 25 minutes were evaluated and all chromatograms were rated 4.

Test 5 was repeated except the temperature was increased to 29°C and all of the chromatograms were rated 4.

Summary of Tests 1 through 6

Test 4 is the only one that has shown any promise. Since this test had the highest velocity used, the effect of velocity will be studied further. Additional experiments will be carried out to try to pin point the conditions necessary for optimum development of the chromatograms.

FUTURE WORK

In this project we shall continue to direct our effort toward improving the several phases of the analytical techniques that are still not satisfactory.

DRY HEAT DESTRUCTION RATES OF
BACILLUS SUBTILIS VAR. NIGER IN A CLOSED SYSTEM

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INTRODUCTION

This study is a continuation of the study first reported in Progress Report #3, December, 1969. A number of experiments have been carried out using the same heat-block system described in Progress Report #3. Most of the experiments reported at this time are concerned with evaluating and controlling system variation as well as approaching the objectives stated in the third report.

OBJECTIVE

The objective of this project is to determine the dry heat D-values of microorganisms in closed systems. Specifically it is to determine (1) the relationship of spore D-value to spore water content, (2) the effect of the atmospheric volume per spore on the D-value of spores with different initial water contents (3) the effect of pressure on D-value, (4) the effect of water absorption and water vapor transfer characteristics of plastic materials on the D-values of microorganisms adjacent to or imbedded in these materials, and metals versus spores encapsulated in plastics.

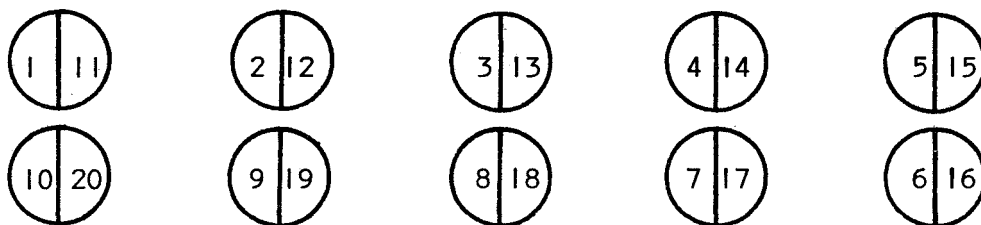
EXPERIMENTAL PROCEDURE

Test Procedure

The Bacillus subtilis var. niger spore crop AAOE (see spore code details, Appendix B, Progress Report #3) was used in all experiments. The stock spore suspension was resuspended in distilled water so that an 0.02 ml droplet would contain 1×10^6 spores. The spores were deposited on the discs using an 0.02 ml Eppendorf push-button pipette.

Stainless steel discs were punched out of type 302 full-hard 0.01" shim stock. These discs were thoroughly cleaned according to NASA standard procedures and dry heat sterilized for 2 hours at 180°C. The discs were placed on a sterile stainless steel sheet for inoculation in a clean room. Generally, two 0.02 ml spore suspension deposits were placed on each disc. Before starting the pipetting

operation the spore suspension was mixed a few seconds using a vibrating automatic vortex mixer and was re-mixed after each ten pipettings. The inoculating scheme order typical for the tests described below was as follows:



The discs were randomly assigned to heating times. Both the discs, blocks and o-rings or gaskets were equilibrated in an ambient temperature clean room (35 to 50% RH at 22-24°C) or in plastic isolators at controlled relative humidity and temperature (i.e. <1%, 25%, 35% or 50% at 22°C).

The blocks were assembled, heated, disassembled and the spore discs processed as described in Progress Report #3.

Six sets of experiments were carried out. Both a description of the experiment and the progress are listed below.

EXPERIMENTS CONDUCTED

Heating before conditioning

In an attempt to control undue variation it was decided to investigate the effects of preheating the blocks and Viton o-rings just prior to the conditioning period. One set of blocks and o-rings was heated after cleaning and prior to conditioning. A second set of blocks and o-rings was similarly cleaned but was not heated prior to conditioning. Both sets of blocks and o-rings were conditioned for 18 hours at 37% relative humidity at 22°C in the clean room. The discs with spores used in both sets were prepared as usual and conditioned along with the blocks. The block units were then assembled, heated for a given time period (4 hours), disassembled, and the discs assayed for survivors. It appeared that there was less block-to-block variation in spore survival in the preheated blocks than in the non-preheated blocks.

Atmospheric volume

In another set of experiments a preliminary attempt was made to determine if there was an effect of atmospheric volume on the survival of spores. In the first experiment the number of survivors from blocks with 0.25" recessed (1.185" diameter) tops was compared to the number of survivors from blocks with flat tops.

The spores, blocks and o-rings were conditioned at about 47% RH at 22°C for 18 hours. Fewer spores survived in the blocks with the 0.25" recessed top than in the flat-top blocks.

A second experiment was carried out except this time 0.50" recessed tops were used in place of the 0.25" recessed tops. No obvious differences in spore survival were observed between flat and recessed tops. Much further study needs to be and will be done in this area.

Location of spores in blocks

In each of these three systems, flat-top, 1/4" recess top, and 1/2" recess top, inoculated discs were placed both directly facing the tops and with a cover disc between the top and inoculated disc. Generally, in all of the systems tested, more spores were found to have survived with the cover disc on than without it.

Effect of o-ring material

The effect of Viton and Teflon o-rings at two different relative humidities on the survival of spores was also briefly investigated. Flat-top blocks were sealed with both Viton and Teflon o-rings. One-half of the Teflon and Viton o-rings were preheated and conditioned at <1% in jars dessicated with silica gel. The other half of the o-rings, the spores and the blocks were conditioned in a glove box at 28% RH at 22°C for 18 hours. The blocks were then assembled in the glove box, torqued, and heated for 90 minutes at 125°C.

At <1% relative humidity the spores sealed with Teflon o-rings had a longer D-value than did the spores sealed with the Viton o-rings. At 28% RH the opposite was true. Also the spores sealed with the Teflon o-rings appeared to show no change in D-value from the one conditioning relative humidity to the other. However, the spores tested with the Viton o-rings at 28% RH showed a distinctively higher D-value than did the spores in blocks with o-rings equilibrated at <1% RH.

Effect of water in spores

An attempt was made here to determine whether or not the water in the spore deposit contributed significantly to the total water in the system. To carry out this test we compared the results of four spore deposits per disc

with two spore deposits per disc and all other conditions held constant. The results of this test showed no obvious difference in the number of survivors between units with two spore deposits compared with units with four spore deposits on the same disc.

CONCLUSIONS

1. Heating of the blocks and their component parts prior to conditioning is important in reducing block-to-block variability.
2. Apparently more spores survive when covered with a cover disc than when uncovered in the block system. This may be related to diffusion of water to or from the spores as in a mated system (per D. Drummond).
3. Not enough data has been acquired to make any firm conclusions regarding the effect of atmospheric volume on spore survival.

FUTURE WORK

In the next reporting period we hope to complete studies that will generate data to establish the reliability of our system. We plan to then proceed to evaluate the effect of different amounts of atmospheric volume and the equilibration of the spores and blocks at different relative humidities.

DRY HEAT DESTRUCTION RATES OF MICROORGANISMS ON SURFACES AS A FUNCTION OF RELATIVE HUMIDITY: DEVELOPING DRY HEAT D-VALUES

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INTRODUCTION

The plan to use a dry nitrogen environment for terminal sterilization in the Viking '75 mission has created an immediate need for information concerning surface spore-destruction rates under dry nitrogen conditions. Considerable data are available regarding the dry heat destruction rates of spores which have been equilibrated and heated in ambient clean-room environments. Since the Viking '75 lander will be sterilized in a dry nitrogen atmosphere it is imperative to obtain comparable data for that environment.

In Progress Report #3 (December, 1969) equipment and methods were described for obtaining surface dry heat destruction data as a function of relative humidity, i.e., for a dry gas environment (air or nitrogen at <1% RH at 22°C) and an ambient environment (22°C and 35% RH). Some data were also presented for Bacillus subtilis var. niger (spore code AAOE) tested at 110°C.

During this reporting period experiments at 110 and 125°C using the same apparatus and spore suspension have been completed. In addition, data have been collected for a second spore suspension, Bacillus firmus (spore code VDLB). This spore was used because it was a dry heat resistance organism isolated from the Mariner '69 spacecraft.

Another series of experiments has also been carried out to determine the effect that vapor pressure changes in the ambient environment had on the $D_{125^{\circ}\text{C}}$ value of spores on surfaces.

OBJECTIVE

The specific objectives of the experiments carried out during the current reporting period were as follows:

1. To develop D-values for two spore suspensions (AAOE and VDLB) at 110 and 125°C as functions of relative humidity (<1% vs. ~35% at 22°C) using both air and nitrogen as the surrounding gas.

2. To determine the effect of a range of ambient room temperatures and water vapor pressures on $D_{125^{\circ}\text{C}}$ -values using Bacillus subtilis var. niger (AAOE) as the test spore suspension.

EXPERIMENTAL PROCEDURE

The apparatus and experimental procedures utilized for the dry gas versus ambient environment experiments were described in detail in Progress Report #3.

In previous experiments using Bacillus subtilis var. niger, the conditioning relative humidity (<1% vs. ~35% at 22°C) and the dry gas used (air or nitrogen) did not significantly affect the D-value. For this reason and to reduce time and effort it was decided to condition Bacillus firmus spores (VDLB) only at ~35% RH and 22°C and to use only nitrogen at 110°C as the dry gas. We believe that these are the conditions that will be most applicable to the sterilization of the Viking '75 lander.

Another variation was introduced in the MH0061 tests in order to obtain information on the shape of the survivor curve when the survival level was less than 10^2 spores per deposit. To gather this information, four additional copper boats with five planchets per boat were heated in both the dry gas and the ambient environment. Using data from the first experiments, an upper and lower limit of the heating time it required to reduce the survivor level to approximately 10^{-1} spores per deposit was estimated. The upper and lower time values were 50 and 60 minutes for the dry gas and 120 and 140 minutes for the 35% RH and 22°C environment. Two boats were removed from the hot plate at each of the test times. Each planchet was then placed directly into a tube of nutrient broth (TSA) which was then incubated for one week and observed for growth. Using the fraction-negative data result, an MPN value was then calculated for each time interval using the formula of Halvorson and Ziegler.* $N_u = \ln(r/q) - 2.303 \log_{10}(r/q)$ where r = number of units per replication and q = number negative.

A series of experiments was carried out to ascertain whether relative humidity or water vapor pressure controlled the D-value of spores on surfaces. These experiments were carried out using Bacillus subtilis var. niger spores (AAOE). For each test condition a $D_{125^{\circ}\text{C}}$ -experiment was carried out in the manner described in Progress Report #3. On each day one temperature and relative humidity condition

*Halvorson, H. O. and N. R. Ziegler (1933). Applications of statistics in bacteriology. I. A means of determining bacterial population by the dilution method. J. Bact. 25:101-121.

was established in the morning and another in the afternoon with a D-value experiment carried out for each condition. The experimental program of any given day was conducted in one of three phases: (1) constant vapor pressure with varied temperature, (2) constant temperature and varied vapor pressure, and (3) constant temperature and vapor pressure. The temperature and vapor pressure were set utilizing the thermostat and humidistat when possible, but hand operation of the steam control valve was necessary for some conditions. The experimental program was carried out in the spring of the year making it very difficult to achieve a low vapor-pressure condition. Therefore most of the variation had to be achieved by raising the vapor pressure above the average level. Although the data have been gathered, they have not been analyzed as of the date of this report. Rather than simply listing raw data in this report and the analysis in the next report we decided to wait and present both the raw data and the analysis at the same time.

RESULTS

In Table 5.1 are listed the experimental conditions for all tests and the resulting D-values. In this table the tests are listed in chronological order.

TABLE 5.1
Experimental Conditions and D-value
Results for All Tests Carried Out

Test No.	Heating temp. °C	Spores	Conditioning* environment	Heating* environment	D-value (mins.)
MH9308A	110	AAOE	DA	DA	43.1
" "B	110	AAOE	DA	AA	140.7
" "C	110	AAOE	AA	DA	40.4
" "D	110	AAOE	AA	AA	163.4
MH9322A	110	AAOE	DA	DA	42.3
" "B	110	AAOE	DA	AA	142.0
" "C	110	AAOE	AA	DA	42.5
" "D	110	AAOE	AA	AA	166.6
MH9328A	110	AAOE	DN	DN	38.6
" "B	110	AAOE	DN	AA	177.6
" "C	110	AAOE	AA	DN	43.3
" "D	110	AAOE	AA	AA	139.6
MH9336A	110	AAOE	DN	DN	49.1
" "B	110	AAOE	DN	AA	147.7
" "C	110	AAOE	AA	DN	37.7
" "D	110	AAOE	AA	AA	161.2

Table 5.1 cont.

TABLE 5.1 Continued

Test No.	Heating temp. °C	Spores	Conditioning* environment	Heating* environment	D-value (mins.)
MH0040A	125	AAOE	DN	DN	7.4
" "B	125	AAOE	DN	AA	29.4
" "C	125	AAOE	AA	DN	7.4
" "D	125	AAOE	AA	AA	32.3
MH0047A	125	AAOE	DA	DA	9.3
" "B	125	AAOE	DA	AA	15.9
" "C	125	AAOE	AA	DA	6.9
" "D	125	AAOE	AA	AA	18.8
MH0055A	125	AAOE	DN	DN	7.7
" "B	125	AAOE	DN	AA	16.9
" "C	125	AAOE	AA	DN	8.5
" "D	125	AAOE	AA	AA	17.9
" "E	125	VDLB	AA	DN	23.7
" "F	125	VDLB	AA	AA	38.9
MH0061A	125	AAOE	DA	DA	6.7
" "B	125	AAOE	DA	AA	20.5
" "C	125	AAOE	AA	DA	6.2
" "D	125	AAOE	AA	AA	16.8
" "E	125	VDLB	AA	DA	22.1
" "F	125	VDLB	AA	AA	37.5
MH0068A	110	AAOE	AA	DN	69.0
" "B	110	AAOE	AA	AA	158.7
" "C	110	VDLB	AA	DN	128.2
" "D	110	VDLB	AA	AA	200.0
MH0078A	110	AAOE	AA	DN	69.4
" "B	110	AAOE	AA	AA	137.0
" "C	110	VDLB	AA	DN	104.2
" "D	110	VDLB	AA	DN	200.0

*DA = Dry Air

DN = Dry Nitrogen

In Table 5.2 the D-value data obtained at 110°C in the dry gas and ambient environments for the Bacillus subtilis var. niger spores (AAOE) are summarized. The D-value data at 125°C are summarized in Table 5.3. Table 5.4 includes the $D_{110^{\circ}\text{C}}$ and the $D_{125^{\circ}\text{C}}$ -data obtained for Bacillus firmus (VDLB). Typical survivor curves for both spore suspensions at the two temperatures studied are illustrated in Figures 5.1-5.4.

TABLE 5.2

$D_{110^{\circ}\text{C}}$ -values(mins.) for Bacillus subtilis
var. niger (AAOE) as a Function of
Conditioning and Heating Environment

Conditioning Environment	Heating Environment		
	Clean Room	Dry Air	Dry Nitrogen
Clean Room	163.4, 166.6 139.6, 161.2 158.7, 137.0	40.4, 42.5	43.3, 37.7 69.0, 69.4
Dry Air	140.7, 142.0	43.1, 42.3	---
Dry Nitrogen	177.6, 147.7	---	38.6, 49.1

TABLE 5.3

$D_{125^{\circ}\text{C}}$ -values(mins.) for Bacillus subtilis
var. niger (AAOE) as a Function of
Conditioning and Heating Environment

Conditioning Environment	Heating Environment		
	Clean Room	Dry Air	Dry Nitrogen
Clean Room	18.8, 16.8 32.3, 17.9	6.9, 6.2	7.4, 8.5
Dry Air	15.9, 20.5	9.3, 6.7	---
Dry Nitrogen	29.4, 16.9	---	7.4, 7.7

TABLE 5.4

D-values(mins.) for Bacillus firmus (VDLB)
Conditioned in the Clean Room as a Function
of Heating Temperatures and Environment

Temperature	Heating Environment		
	Clean Room	Dry Nitrogen	Dry Air
110°C	200.0 200.0	128.2 104.2	
125°C	38.9 37.5	23.7	22.1

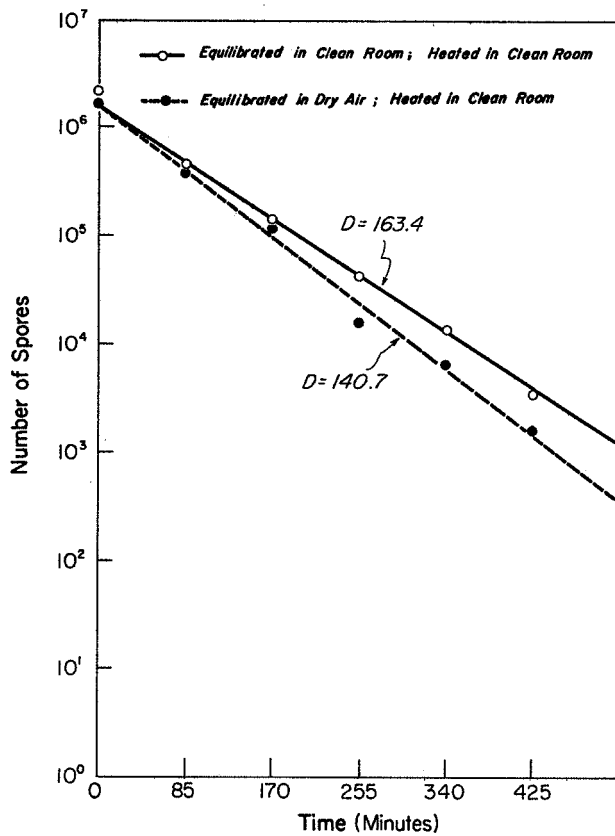


Figure 5.1 - Survivor curves for *Bacillus subtilis* var. *niger* (AAOE) spores equilibrated in dry air and in the clean room and heated on a hot plate at 110°C in the clean room.

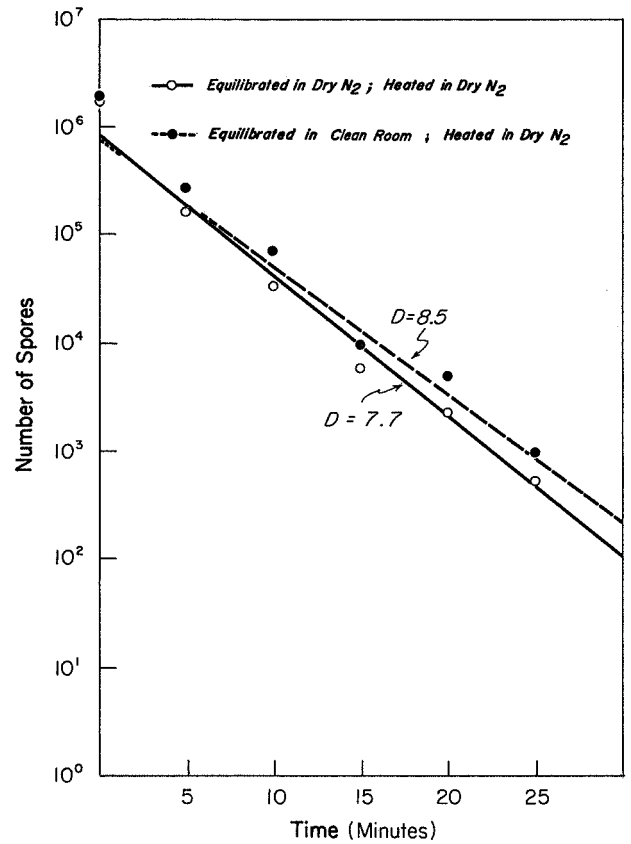


Figure 5.2 - Survivor curves for *Bacillus subtilis* var. *niger* (AAOE) spores equilibrated in dry nitrogen and in the clean room and heated on a hot plate at 125°C in dry nitrogen.

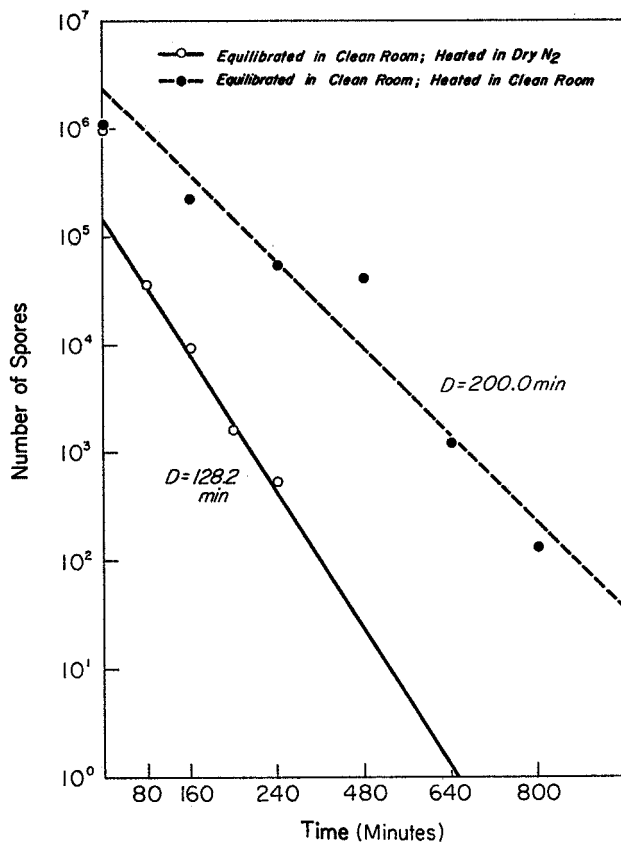


Figure 5.3 - Survivor curves for *Bacillus firmus* (VDLB) spores equilibrated in the clean room and heated on a hot plate at 110°C in both the clean room and dry nitrogen

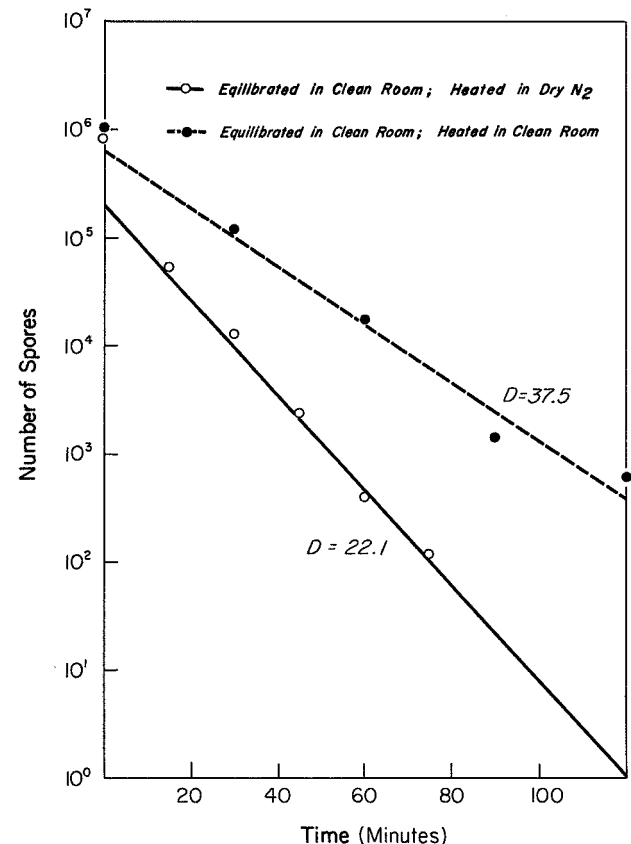


Figure 5.4 - Survivor curves for *Bacillus firmus* (VDLB) spores equilibrated in the clean room and heated on a hot plate at 125°C in both the clean room and dry nitrogen.

The most obvious discovery that was made through examination of these data was the existence of major differences between the D-values of the spores heated in the dry gas environment and the D-values of spores heated in the 35% RH-22°C environment. It was also apparent from these results that the affect of the relative humidity of the conditioning environment on the D-value was very much smaller than the affect of relative humidity of the heating environment. This suggests that either spores equilibrate very quickly to the moisture conditions of the heating environment or that in the first few seconds of heating the moisture content of the spores is reduced to a new equilibrium level that is not substantially influenced by the initial water content.

We observed no consistent differences between the D-value of spores heated in a dry air atmosphere and those heated in a dry nitrogen atmosphere. This is in agreement with the ideas put forth by Angelotti (1967)* who concluded after evaluating the existing data on the affect of different gases that the affect of the gas atmosphere itself was very small and that the water content of the gas atmosphere was the critical variable.

The D-values for the Bacillus firmus (VDLB) were longer than the D-values for Bacillus subtilis (AAOE) at all conditions tested. The ratio of $\frac{D_{B. firmus}}{D_{B. subtilis}}$ was not constant; changing the heating atmosphere affected the ratio more than changing the temperature. The ratios were 2.45 and 3.05 in the dry gas and 1.3 and 1.8 in the clean room for temperatures of 110 and 125°C respectively. These results suggest that the effect of humidity during heating is a function of spore species. However, since these spore crops were grown on different media this difference may be a growth medium manifestation. Future tests should answer this question.

The data at 110 and 125°C are summarized in Table 5.5. The mean D-values for dry gas include the data from both the dry nitrogen and the dry air. To facilitate a comparison of the dry gas and clean room environment, ratios of $\frac{D_{clean room}}{D_{dry gas}}$ were calculated. The ratio for Bacillus subtilis (AAOE) was 3.2 at 110°C and 2.8 at 125°C. The ratio for Bacillus firmus (VDLB) was 1.7 for both temperatures.

*Angelotti, R. (1967). Protective Mechanisms Affecting Dry-Heat Sterilization. COSPAR Technique Manual Series, Manual #4. Sterilization Techniques for Instruments and Material as Applied to Space Research.

TABLE 5.5

Summary Table of Mean D-values and Ratio of $\frac{D_{\text{clean room}}}{D_{\text{Dry gas}}}$

Temperature °C	Spores	Mean D-values(min.)		Ratio
		Dry gas	Clean room	
110	<u>B. subtilis</u>	47.5	153.5	3.2
	<u>B. firmus</u>	116.2	200.0	1.7
125	<u>B. subtilis</u>	7.5	21.1	2.8
	<u>B. firmus</u>	22.9	38.2	1.7

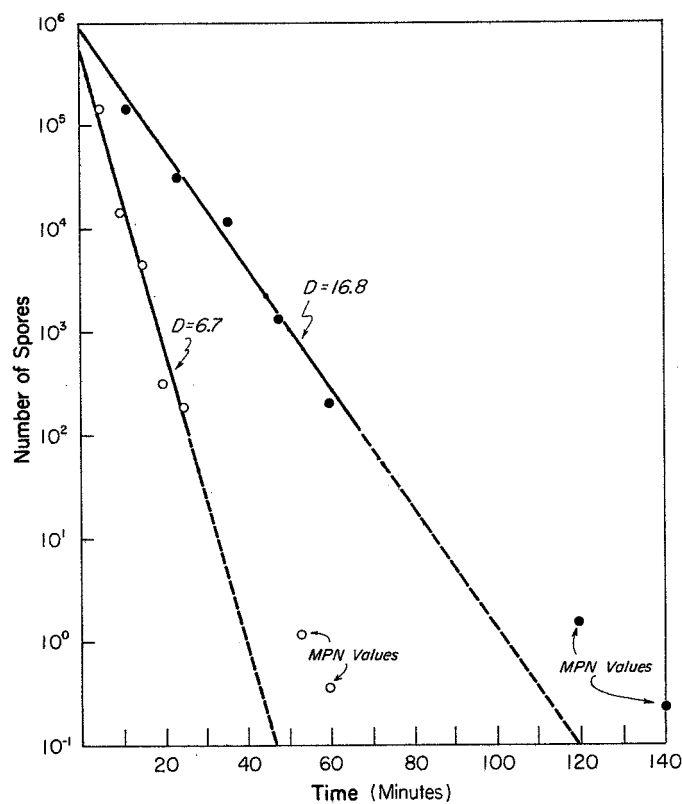


Figure 5.6 - Survivor curves for *Bacillus subtilis* var. *niger* (AAOE) spores equilibrated and heated in the clean room at 125°C (D=16.8 min.) and equilibrated and heated in dry air at 125°C (D=6.7 min.) showing the two data points for each curve in the survivor range N_0 to 10^{-1} determined by MPN methods.

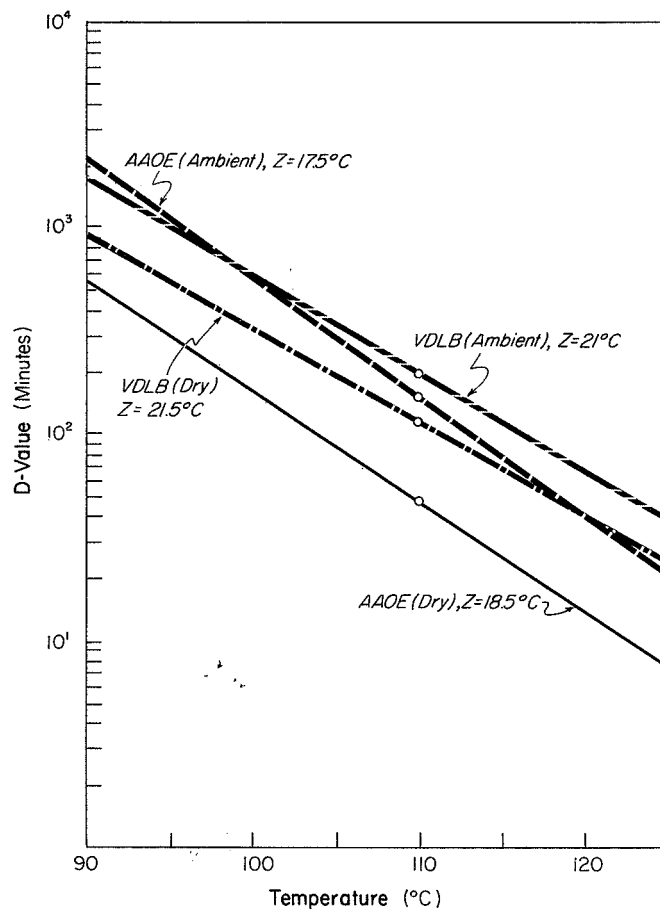


Figure 5.5 - Thermoresistance curves for *Bacillus subtilis* var. *niger* (AAOE) and *Bacillus firmus* (VDLB) spores for both dry and ambient conditions using the data at two temperature points (110 and 125°C) extrapolated to 90°C .

On the basis of the data obtained at 110°C and 125°C for the two spore suspensions, projected z-values were estimated. Figure 5.5 illustrates the z-value curves constructed from the available data. It is interesting to note that slightly higher z-values were noted for Bacillus firmus (VDLB) than for Bacillus subtilis var. niger (AAOE). As a result it is projected that at about 110°C the two spore species will have similar D-values under ambient heating conditions, while at temperatures below 100°C AAOE spores will have the larger D-value. Conversely at temperatures above 125°C the D-value of VDLB would appear to be increasingly larger than the D-value of AAOE.

Figure 5.6 illustrates the result of the experiment utilizing the MPN approach to establish the direction of the survivor curve below a survival level of 10^2 spores per deposit. The results of this experiment indicate a longer survival time in this 10^0 to 10^{-1} range than that obtained by extrapolating the survivor curve obtained by plate counting methods. In future experiments we plan to carry out fraction-negative MPN tests as part of all studies in which we are establishing survivor curves using plate counting techniques.

In Table 5.6 are shown the ranges of relative humidity values (calculated at test temperature) of the gas surrounding the spores for the data in Tables 5.1, 5.2, and 5.3.

TABLE 5.6
Relative Humidity Ranges Calculated at Test
Temperatures for Gas Surrounding Spores

Temp.	Clean Room	Dry Gas Box
110°C	0.57-0.75%	0.002-0.022%
125°C	0.33-0.42%	0.001-0.006%

In the clean room the relative humidity was measured before and after each test using a sling psychrometer. The relative humidity and temperature of the clean room during a test was recorded using a hydrothermograph. In the clean room the relative humidity and temperature ranged from 31%-21°C to 38%-23°C which calculates to a range of 0.57-0.75% relative humidity at 110°C and 0.33-0.42% relative humidity at 125°C. The relative humidity of the gas in the glove box was measured using a Bell and Howell moisture analyzer and gas sampling system (see Appendix F). This water measuring system had a low level accuracy limit of 20 µg water. The calculated relative humidities at 110 and 125°C

corresponding to 20 mg per liter are 0.002% and 0.001% respectively. The water level in the gas was often below the instrument measurement level however, we are listing the low level measurement limit in Table 5.6 as the lower level relative humidity in the glove box. Statistical analysis of the data in Tables 5.2, 5.3, and 5.4 will be included in the next progress report.

CONCLUSIONS

On the basis of completed experiments we note that dry gas (air or nitrogen at <1% RH at 22°C) significantly reduces D-values at 110°C and 125°C for both spore species tested compared with heating the same spores under ambient conditions (~35% RH at 22°C). The relative humidities of the conditioning environment and type of dry gas do not appear to be significant factors, but the two spore species differ in both the dry heat resistance level and the relative effect of the environment during heating.

FUTURE WORK

Priority will be given to the following items during the next reporting period:

1. D-values at 100°C and 90°C for spores AAOE and VDLB.
2. Statistical analysis of the data at 90, 100, 110 and 125°C.
3. $D_{125^{\circ}\text{C}}$ -values for AAOE and VDLB at relative humidities intermediate between 0.001 and 0.3%.

DRY HEAT DESTRUCTION RATES OF MICROORGANISMS ON SURFACES:
STUDIES TO EVALUATE POSSIBLE SOURCES OF VARIATION
IN THE EXPERIMENTAL SYSTEM

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INTRODUCTION

In experiments to determine dry heat D-values, we have been troubled by unexplained variability in experiment-to-experiment results under supposedly identical conditions. While careful experimental design and randomization of uncontrollable factors have helped to reduce bias, it is felt that the continuous tightening up of experimental procedures may help to reduce the magnitude of variation between D-values.

OBJECTIVE

The objective of this project was to evaluate possible sources of variation in D-value determinations. Four different experiments have been completed during the current reporting period directed toward this end; they are (1) boat to-boat variation, (2) hot plate with and without shield, (3) effect of boat location on hot plate and (4) effect of the boat position with regard to the direction of air flow. The experiments and their results are outlined below.

Boat-to-boat variation: Tests GS0041, GS0042, and GS0043

An experiment was carried out to examine boat-to-boat differences as a possible source of experimental variation in our planchet-boat-hot plate system.

Bacillus subtilis var. niger (AAAA) spores were used in this experiment. The inoculation, spore recovery, and counting techniques described in Progress Report #3 were used in this study. The initial inoculum was approximately 10^6 spores per stainless steel planchet. The spores were heated on the hot plate at 125°C in the clean room. A plastic shield was in place over the hot plate in all tests.

In each test two copper boats each containing five planchets were subjected

to each of five heating time periods. Uniform heating time intervals were used. The heating times were selected so that at the fifth heating time approximately 10^2 spores per planchet remained viable.

The test was carried out one time per day on three consecutive days.

Results and discussion

The results of the three experiments GS0041, GS0042, and GS0043 are summarized in Table 6.1. The data from the analysis of variance is presented in Table 6.2. The observed D-values were approximately 14 minutes.

TABLE 6.1
Results of Tests GS0041, GS0042, and GS0043

Test	$\hat{\beta}$	$SD(\hat{\beta})$	D(min)	95%CI
GS0041	-0.0788	0.0075	12.69	10.41-16.26
GS0042	-0.0699	0.0059	14.31	11.98-17.76
GS0043	-0.0671	0.0048	14.90	12.79-17.86

TABLE 6.2
Analysis of Variance of Experiments
GS0041, GS0042 and GS0043

Source	df	SS	MS	F
Day	2	5.004	2.502	6.9*
Time	4	189.073	47.268	130.6*
Day x time	8	2.504	0.313	0.9
Among boats within treatments	15	5.437	0.362	13.4*
Among strips within boats	60	1.626	0.027	6.8*
Among plates within strips	90	.369	0.004	
Total	179	204.012		

*Significant at $\alpha = .01$

In order to investigate the boat-to-boat sources of variability, the variable $y = \log_{10}$ (estimated number of survivors) based upon a single plate count was

considered. Estimates of the components of variance were obtained and are presented in Table 6.3.

TABLE 6.3.
Components of Variance.

Source	Expected Mean Square	Estimates
Among boats within treatments	$\sigma_{\rho}^2 + 2\sigma^2 + (2)(3)\sigma_{\beta}^2$	$\hat{\sigma}_{\beta}^2 = 0.0559$
Among strips within boats	$\sigma_{\rho}^2 + 2\sigma^2$	$\hat{\sigma}^2 = 0.0115$
Among plates within strips	σ_{ρ}^2	$\hat{\sigma}_{\rho}^2 = 0.0041$

Using these estimates as truth and assuming the validity of the model, various combinations of the numbers of boats, strips per boat, and plates per strip were considered. The objective was to minimize the experimental error (mean square within treatments). It was concluded that experiments in this series which involved five heating times, two boats per heating time, three strips per boat, and two plates per strip did appear to optimize the use of the equipment within the constraints of size of hot plate, number of desired heating intervals, and the total number of plates to be processed.

Hot plate with and without shield: Tests MH0133A and B and MH0152A and B

An experiments was carried out to evaluate the effect of the hot plate air shield on the D-values of spores heated on the hot plate. The usual practice has been to shield the hot plate located in the horizontal laminar flow clean room. The shield was initially installed to reduce the air velocity across the hot plate.

Bacillus subtilis var. niger (AAOE) spores were used. Heating was carried out at 125°C. The inoculation, spore recovery and counting techniques described in Progress Report #3 were used in this study. Four heating times were used with two boats at each heating time and three planchets were centrally located on each boat.

The experiment was carried out on two different days. On day #1 the shield was in place for the morning experiments (MH0133A) but was removed for the afternoon experiment (MH0133B). On day #2 the shield was removed in the morning (MH0152A) and in place for the afternoon experiment (MH0152B).

The hot plate record charts were monitored and the planchets in all tests were heated at $125^{\circ}\text{C} \pm 1^{\circ}\text{C}$. For tests MH0133A and B the relative humidity in the clean room was $45 \pm 2\%$ and the temperature was $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$. For tests MH0152A and B the relative humidity in the clean room was 35.5% and the temperature 23°C .

Results and discussion

The results of tests MH0133A and B and MH0152A and B are summarized in Tables 6.4 and 6.5. The results suggest that the D-value is lower with the shield in place.

Table 6.4

Comparison of Survivor Data for Tests MH0133A and B and MH0152A and B at 125°C in the Clean Room With and Without Hot Plate Air Shield

Treatment	Test No.	With shield		Without shield	
		Mean	95%CI *	Mean	95%CI
0(N _o)	1	2.09E6**	---	1.81E6	---
	2	2.34E6	---	2.59E6	---
15 min.	1	3.88E5	3.80E5, 3.95E5	2.70E5	2.03E5, 3.59E5
	2	7.11E5	6.31E5, 8.00E5	4.02E5	1.88E5, 8.57E5
30 min.	1	8.13E4	3.80E3, 1.74E6	9.53E4	1.22E4, 7.44E5
	2	2.33E5	2.29E5, 2.36E5	9.39E4	5.95E4, 1.48E5
45 min.	1	1.88E4	8.54E2, 4.12E5	1.72E4	3.49E3, 8.49E4
	2	1.97E4	7.43E3, 5.20E4	3.14E4	2.05E4, 4.79E4
60 min.	1	3.56E3	2.76E3, 4.58E3	5.10E3	1.09E2, 2.39E5
	2	2.80E3	1.37E1, 5.74E5	4.71E3	2.88E3, 7.68E3

*Error degrees of freedom at each treatment where confidence limits are given is one

**2.09E6 = $(2.09) \times 10^6$

Test 1 with shield = MH0133A

Test 1 without shield = MH0133B

Test 2 without shield = MH0152A

Test 2 with shield = MH0152B

TABLE 6.5

D_{125°C}-values as a Function of Presence or
Absence of a Plexiglass Hot Plate Shield

Experimental code	Statistic	Shield present	Shield absent
MH0133A,B	D-value 95%CI IR*	22.2 20.18,24.74 0.992	25.4 22.06,29.86 0.970
MH0152A,B	D-value 95%CI IR*	18.1 15.47,21.83 1.069	23.9 21.57,26.84 0.975

$$*IR = \frac{\log y_o}{\log N_o}$$

where y_o = estimated initial count from estimated survivor curve (data obtained at times not equal to zero)

N_o = estimated initial count from data obtained at time = 0

If we assume that the temperature of the spores was 125°C in all cases then the shield would appear to have an effect on the rate of water loss from the spores.

The air temperature three inches above the hot plate was measured; with the shield in place it was about 40°C, however, without the shield it was near the room temperature of 22°C.

If we assume that the vapor pressure is constant throughout the clean room then the effect of the shield is to reduce the relative humidity gradient as we move away from the planchet. If a lower RH at small distances from the planchet would produce a lower water level around the spores then we would expect a decrease in the D-value.

Effect of boat location on hot plate: Tests MH0112A and B

An experiment was carried out to determine whether there were measurable differences in the quantity of heat energy received by spores on different planchets as a function of the boat location on the hot plate. The spores themselves were used as biological indicators. Differences in the number of spores per planchet would be indicative of differences in the integrated time-temperature effect at the planchet.

Spores of Bacillus subtilis var. niger (AAOE) were used in this experiment. The inoculation, spore recovery, and counting techniques described in Progress Report #3 were used in this study. All boats were heated on the hot plate at 125°C for 45 minutes. There were three planchets centrally located on each boat. A zero time control of one boat with three planchets was used in all tests. All tests were conducted with the plastic shield in place. Two tests were carried out with two boats located at each corner of the hot plate, and two boats with thermocouples to monitor and control temperature. There were ten boats on the hot plate in each test with the test boats number 1-8.

Figure 6.1 (see page 53) shows the relative locations of the ten boats and the partitioning of the hot plate into four positions.

Results and discussion

The results of tests MH0112A and B are shown in Table 6.6.

TABLE 6.6

Mean Survivor Counts per Boat after 45 Minutes at 125°C
as a Function of Position on Hot Plate and Time of Test

Test No.	Position				All Posits.
	1	2	3	4	
MH0112A(am)	1.15E4	1.82E4	1.07E4	1.15E4	1.26E4
MH0112B(pm)	8.69E3	1.19E4	1.04E4	7.34E3	9.44E3
Average	1.00E4	1.47E4	1.05E4	8.00E3	1.09E4

This experiment was designed to evaluate boat position on the hot plate as a source of variability in the estimated number of survivors. One estimate was associated with each boat. On the average, the estimated number of survivors was larger in the morning than in the afternoon.

The two boats in each position were assumed to be representative of the respective position effect. An analysis of variance of the $\log_{10}$ (estimated number of survivors) is given in Table 6.7.

TABLE 6.7

Analysis of Variance Table for Experiment to Calculate
Positional Effects on Clean Room Hot Plate

Contrast	Source	df	MS	F
1	a.m. vs. p.m.	1	0.065223	8.78*
2	positions 1,2 vs. 3,4	1	0.032762	4.41
3	positions 1,3 vs. 2,4	1	0.011447	1.54
4	positions 1,4 vs. 2,3	1	0.051943	6.99**
5	contrast (1) x (2)	1	0.002598	0.35
6	contrast (1) x (3)	1	0.015030	2.02
7	contrast (1) x (4)	1	0.003692	0.50
	error	8	0.007432	

* 0.01 $P(F_{1,8} 8.78) 0.025$

** 0.025 $P(F_{1,8} 6.99) 0.05$

The treatment sum of squares was partitioned into seven orthogonal contrasts. Significant differences appeared in contrast (1), morning versus afternoon results, and in contrast (4), positions 1 and 4 versus 2 and 3.

Based upon the results of this experiment it would appear that a 45-minute heating period of spore type AAOE would result in a non-uniform treatment of the spores located at the various positions of the hot plate. Previous experiments have taken account of this possible phenomenon by randomly assigning boats to positions on the hot plate. However, further investigation of position effects might shed some light on the cause of such effects and enable the experimenter to reduce some of the experimental noise.

Effect of the boat position with regard to the direction of air flow:

Tests MH0131A and B

An experiment was carried out to determine whether there were measurable differences in the quantities of heat energy received by the spores on boat planchets which were placed differently in relation to the direction of air flow. Differences in the number of spores surviving per planchet would be indicative of differences in the integrated time-temperature effect at the

planchet. The spores themselves were used as biological indicators.

Spores of Bacillus subtilis var. niger (AAOE) were used in this experiment. Inoculation, spore recovery and counting techniques described in Progress Report #3 were used in this study. All boats were heated on the hot plate for 45 minutes at 125°C. There were three planchets individually located on each boat. All tests were conducted with the plastic shield in place and a zero time control consisting of one boat with three planchets.

The temperature recorder monitoring the temperature of the hot plate during tests MH0131A and B indicated that the planchet temperature should be 121°C ±1°C. The temperature in the clean room was 22°C ±1°C and the relative humidity was 43% ±2%.

Two tests were carried out with eight boats grouped together near the center of the hot plate. One test was run in the morning and one in the afternoon. In the morning test (MH0131A) the eight boats were placed so that the long axis of the boat was parallel to the direction of the air flow as shown in Figure 6.2. In the afternoon test (MH0131B) the eight boats were oriented so that the long axis of the boat was perpendicular to the direction of air flow as shown in Figure 6.3.

Results and discussion

The results of these two tests to determine the effect of boat position with regard to the direction of air flow are shown in Table 6.8. An analysis of variance of boat means is shown in Table 6.9. Four areas were designated on the hot plate: they were upper left, upper right, lower left and lower right. In the analysis the eight boats (each with three planchets) were paired according to this partition. It was assumed that each pair provided an estimate of experimental error with one degree of freedom.

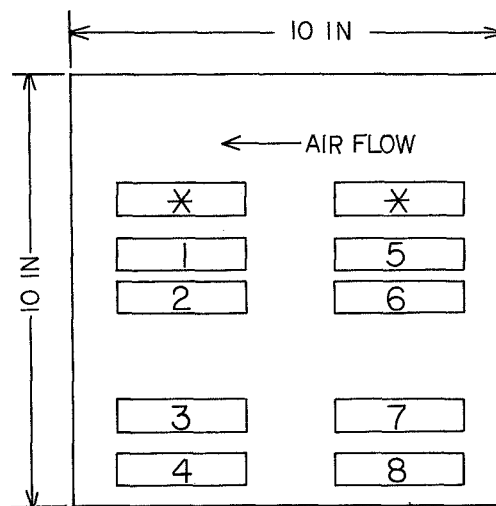


Figure 6.1 - Boat position on hot plate in experiments MH0112A and B to determine if there are boat temperature differences on the hot plate.

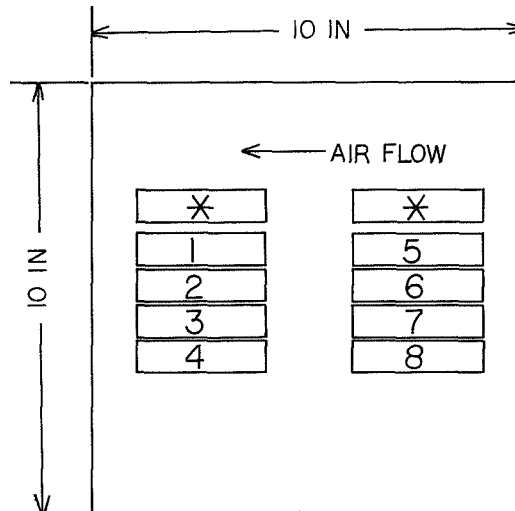


Figure 6.2 - Boat position on hot plate in experiment MH0131A to determine if there are temperature differences in boats. Air flow parallel to long axis of boats.

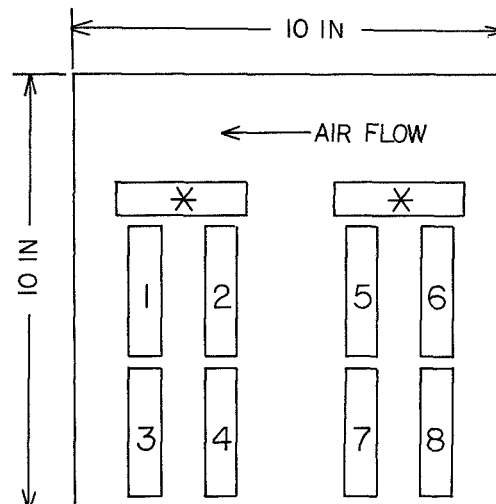


Figure 6.3 - Boat position on hot plate in experiment MH0131B to determine if there are temperature differences in boats. Air flow perpendicular to long axis of boats.

* Temperature reference boats

TABLE 6.8

Mean Survivors per Boat for Tests MH0131A and B
as a Function of Boat Number and Position

Location on hot plate	Boat number	MH0131A boat parallel to air flow	MH0131B boat perpendicular to air flow	Means
Left side	1	2.42E4	2.63E4	2.94E4
	2	2.84E4	3.82E4	
	3	2.32E4	5.78E4	
	4	2.09E4	2.87E4	
	mean	2.40E4	3.59E4	
Right side	5	1.66E4	2.17E4	1.84E4
	6	1.42E4	1.55E4	
	7	3.29E4	2.06E4	
	8	1.72E4	1.43E4	
	mean	1.91E4	1.77E4	
	means	2.14E4	2.52E4	2.33E4

TABLE 6.9

Analysis of Variance Table for Experiment to Evaluate
Position Effects and Orientation Relative to
Airflow for Boats on Clean Room Hot Plate

Contrast	Source	df	MS
1	Horizontal vs. vertical (am vs. pm)	1	0.012435
2	left vs. right	1	0.0141773 6.55*
3	upper vs. lower	1	0.004407
4	(2) x (3)	1	0.008717
5	(1) x (2)	1	0.030611
6	(1) x (3)	1	0.002425
7	(1) x (4)	1	0.029150
	error	8	0.021659

* $0.025 < P(F_{1,8} > 6.55) < 0.050$ (significant at $\alpha = .05$)

It appeared that there was a difference between those values obtained on the left side and those obtained on the right side of the hot plate. This difference was consistent for the a.m. and p.m. runs.

It should be noted that the differences between the left and right sides of the hot plate were greater in the afternoon than in the morning. This suggests an effect due to orientation of the boat on the hot plate. It should be recognized that orientation as a source of variability was confounded with time of day (runs). The large difference observed was thought to be due more so to orientation than runs because previous experiments had produced small differences between successive runs conducted on the same day. A horizontal position of the boat (as opposed to vertical position) appeared to reduce the variability between boats. The reasons for this were not clear.

Another run of the same experiment without air flow may provide more interesting data to assist in further examination of the observed effects.

INTER-LABORATORY D-VALUE EXPERIMENTS

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INTRODUCTION

During the last few years a number of geographically diverse laboratories have been carrying out research on dry heat D-value determination in accord with the requirements of the NASA Planetary Quarantine Program. The wide variety of dry heat systems, spore preparation protocols and viable spore recovery techniques in use has resulted in extremely variable D-value results being reported for supposedly identical spore species. In an effort to sort out the effects of the known variables and to determine the extent of reproducibility of data within and among various laboratories, the Planetary Quarantine Office requested that information be obtained on Inter-laboratory experiments. In response to this request, the University of Minnesota School of Public Health and the N.C.D.C. Phoenix Field Station laboratories initiated such a series of experiments in January of 1970.

EXPERIMENTAL PROCEDURE

The series of experiments was carried out in two parts. In the first segment, a team of two persons traveled from Minneapolis to Phoenix to work side-by-side with personnel from the Phoenix laboratory. Each team carried out $D_{125^{\circ}\text{C}}$ -value determinations in duplicate, on two consecutive days, on a spore crop from the Minneapolis laboratory (AAAA) and on a spore crop from the Phoenix laboratory. The spore deposition, conditioning, heating, processing, and counting were all carried out using the Phoenix laboratory techniques. Several weeks later, the Phoenix team traveled to Minneapolis and the entire experiment was repeated, this time utilizing the techniques of the Minneapolis laboratory. In each segment, the visiting personnel spent one day acclimating themselves to the methods of the host laboratory and practicing their techniques.

The following factors were standardized between the two laboratories:

1. Bacillus subtilis var. niger was the spore species utilized. However, Minnesota spores had been cultured on supplemented TAM medium while Phoenix

spores had been cultured on SSM-10 medium.

2. Initial deposits of approximately 10^6 spores were made on 1/2" x 1/2" stainless steel planchets.
3. Plating medium (TSA), incubation time (48 hours) and incubation temperature (32°C) were also standardized.
4. All data were processed by the University of Minnesota group. A straight line was fitted to the transformed data (ignoring N_0) by the method of least squares.

Salient features of the techniques utilized in each laboratory were as follows:

A. Phoenix laboratory

1. Spores. An ethanol suspension of Bacillus subtilis var. niger was deposited with a 0.025 ml dropper pipette from a large test tube which was insonated for twelve minutes each time spores were removed. (The Minnesota spores were in separate vials for each trial, and thus were insonated only once before use.)
2. Conditioning. After the planchets were inoculated, (between 8:00 and 9:00 a.m.) they were kept in closed petri plates in a laminar flow room until 4:00 p.m. They were taken to the laboratory and placed in a dessicator over dry silica gel. A vacuum was pulled and sodium hydroxide-dried air was flushed through the dessicator five times. The spores were then left in the dessicator in a vacuum overnight (~16 hours).
3. Heating system. A dry-heat oven was used for spore heating. Planchets were held with alligator clips attached to hangers. The hangers were placed in the oven at 5-minute intervals for varying lengths of time (longest heating time first). One and one-half minutes were added to each heating time for heat-up correction. Neither the location of the planchet on the hanger nor the hanger-location in the oven was randomized. The spore strips which were processed by the Minnesota team were in position #1 on the hanger and those from the Phoenix team were in position #2. (The two spore crops were heated separately.) Six heating intervals were utilized ending with approximately 10^2 spores per deposit. After heating, the strips were placed in a laminar-flow hood to cool for at least two minutes.
4. Processing. Following cooling the strips were placed in cotton-plugged test tubes containing 10 ml of cold buffer and three glass beads. These tubes were collectively insonated for twelve minutes in a test tube

rack which was placed on the bottom of the tank. Before plating, the contents of each tube were mixed on a vortex mixer. Glass plates and pipettes were used and 99 ml of buffered distilled water were used for dilution.

B. Minnesota Laboratory

1. Spores. An ethanol suspension of Bacillus subtilis var. niger was used. A separate 5 ml vial was used for each trial. Deposits were made from a $f \times 10^7$ spores-per-ml suspension with a 20 μ l Eppendorf pipette. The contents of each vial were mixed on a vortex mixer following every nine deposits. Deposits were made on the stainless steel planchets and were then placed on rectangular copper boats (5 planchets/boat). This entire process was carried out in a laminar-flow room.
2. Conditioning. After deposition, spores were protected from direct air flow by a metal deflector. When they were visibly dry, they were conditioned in the laminar-flow room for 16-18 hours.
3. Heating system. Five heating intervals were used. The copper boats containing the stainless steel planchets were placed on an aluminum hot plate in the clean room. At the pre-selected time intervals, the randomly allocated boats were removed for processing and were cooled on a cold plate for at least three minutes following removal from the heat.
4. Processing. The planchets were transferred individually (contaminated side down) to 125 ml Erlenmeyer flasks. Four planchets from each boat were processed, while the fifth was kept as a spare. Fifty ml of phosphate buffer were added to each flask and the flasks were insonated individually for two minutes and suspended in the bath so that the buffer was level with the tank solution. Duplicate aliquots of three dilutions were plated for each planchet at each heating treatment. Plastic plates and pipettes were used and 50 ml of buffered distilled water were used for dilutions.

RESULTS

Differences in counters were observed when the experiments were conducted at Phoenix. (Similar data was not available from the Minnesota portion of the series of experiments.) On the first day of the Phoenix series both teams

counted all plates. The results are summarized in Table 7.1.

TABLE 7.1
Summary of Counter Differences

Run	Spore	Plating Team	N	r	p	Average Difference
1	P	P	20	19	0.95	1.8
2	P	M	24	24	1.00	2.7
3	M	P	24	19	0.79	10.0
4	M	M	24	15	0.62	7.1
Total			92	77	0.84	

P = Phoenix M = Minnesota

N = number of plates counted

r = number of plates for which the Minnesota spore count was higher than the Phoenix spore count

$p = r/N$

When the Minnesota spores were counted the average difference between the counts of the two teams was smaller than that observed when the Phoenix spores were counted. It appears that this difference had little effect on the D-value.

$D_{125^{\circ}\text{C}}$ -values for the series of experiments are presented in Table 7.2. It will be noted that separate D-values are reported for counting teams on day #1 in the Phoenix experiments. Those plates were counted independently by each team. On day #2 in Phoenix each team counted only those plates which it had processed. In Minneapolis each team counted an aliquot from each dilution for each planchet and the D-values were based on the combined count. The reason that separate counts were abandoned after the first day was that they were logistically very difficult and subsequent analysis indicated that the D-values were very similar regardless of counting team. Consistent differences between the main effects of location, day and spore are observed in Table 7.2. The Phoenix spores had larger D-values than the Minnesota spores. D-values were larger for the respective spores in Phoenix than they were at Minnesota. The first day D-values for the respective spores in either location were larger than the D-values obtained on the second day.

TABLE 7.2

 $D_{125^{\circ}\text{C}}$ -values for Inter-laboratory Experiments

Location	Day	Spore	Plating Team	Counting Team*	
				Phoenix(P)	Minn.(M)
P *** (31.01)	first: 1-13-70 (34.71)	P (45.52)	P (45.74)	42.59**	48.89
			M (45.30)	45.58	45.01
		M (23.91)	P (25.12)	25.17	25.07
			M (22.70)	22.63	22.76
	second: 1-4-70 (27.31)	P (34.77)	P (34.17)	34.17	-----
			M (35.37)	35.37	-----
		M (19.85)	P (19.68)	-----	19.68
			M (20.01)	-----	20.01
M (20.83)	first: 1-27-70 (21.98)	P (28.96)	P (27.91)	27.95	27.86
			M (30.02)	29.38	30.65
		M (15.01)	P (15.14)	15.08	15.19
			M (14.88)	15.00	14.76
	second: 1-28-70 (19.68)	P (25.79)	P (25.47)	24.83	26.10
			M (26.12)	25.90	26.33
		M (13.57)	P (14.29)	13.98	14.59
			M (12.85)	12.80	12.89

* D-values calculated for data obtained at Phoenix are based upon counts for two plates per planchet. D-values calculated for data

obtained at Minnesota are based upon counts for one plate per planchet.

** Data from 40-minute heating period was omitted when calculating this D-value.

*** Bracketed values are group means of estimated D-values. For example:

1) Phoenix location--

$$31.01 = \frac{45.74 + 45.30 + \dots + 20.01}{8}$$

2) First day, Phoenix location--

$$34.71 = \frac{45.74 + 45.30 + \dots + 22.70}{4}$$

CONCLUSIONS

The belief that the difference in spores and the different heating methods used would yield significantly different D-values was confirmed. The consistent decrease in D-value from day #1 to day #2 was certainly not expected and is unexplainable at this time. Day-to-day variation in the results of dry heat studies is a problem. These variations may be due to yet unknown factors that are now uncontrolled and that affect the dry heat destruction rate of microorganisms.

FUTURE WORK

Clarification of the effects of spore suspension differences and heating and processing effects was very beneficial to both laboratories involved. It would appear that additional inter-laboratory experiments involving other laboratories engaged in dry heat D-value determination may prove quite useful to the Planetary Quarantine program.

APPENDIX A: COLLECTION AND ANALYSIS OF DATA

The following is a brief description of the protocol that was followed in the collection and analysis of spore survival data. This discussion is limited to the type of experiment that involves the use of planchets and boats.

A. Estimating survivor curves

1. Experimental methods

- a. A suspension of spores is prepared.
- b. Planchets are placed on the boats.
- c. Equal volumes of the suspension are deposited on the planchets according to protocol (see Appendices C and D, Progress Report #3).
- d. Several boats are prepared and randomly assigned treatment times, $t_0, t_1, \dots, t_k$.
- e. Treatment t_i is given to the i^{th} boat.
- f. Planchets are processed; two or three dilutions are made for each planchet and two plates are prepared from each dilution.
- g. Plate counts are made for all dilutions.

2. Statistical methods

- a. Two plate counts are selected for each planchet according to the guidelines given in part B.
- b. The plate count (v), is multiplied by the appropriate dilution factor (f), to yield an estimate of the number of survivors on the planchet (w). Thus, $w = v \times f$.
- c. The variable of interest is the log (base 10) of w denoted by y ; $y = \log_{10} w$.
- d. The arithmetic mean of $\{y\}$, the set consisting of the logs of the estimated number of survivors for one boat, is calculated for each boat. This is $\bar{y}_b = \frac{1}{2p} \sum_{i=1}^{2p} y_{bi}$, where p equals the number of planchets per boat.
- e. The $\bar{y}_b$'s are assumed to be random variables which could reasonably be assumed to be normally distributed had $\bar{y}_b$ been based on a relatively large number of observations. Due to the relatively small number of observations at each treatment time, the assumption of the normality of $\bar{y}$ is questionable. Another assumption (which appears to be somewhat tenuous) is that $\bar{y}_b$ can be satisfactorily

described by the model

$$\bar{y}_b = \alpha + \beta t_b + \epsilon$$

where: α = intercept

β = slope

t_b = treatment time for boat b

ϵ = stochastic component with mean equal to zero.

- f. Estimates are obtained for α and β by the method of least squares ($\bar{y}_b$, where $t_b = 0$, are excluded from the analysis).
- g. The variance of ϵ is estimated.
- h. Confidence intervals are constructed for α and β as $(\hat{\alpha}_1; \hat{\alpha}_2)$ and $(\hat{\beta}_1; \hat{\beta}_2)$.
- i. D-value equals $-1/\beta$. An estimate of the D-value is $D = -1/\hat{\beta}$.
- j. A confidence interval is constructed for D as $(\hat{D}_1; \hat{D}_2)$ where

$$\hat{D}_1 = -1/\hat{\beta}_1$$

$$\hat{D}_2 = -1/\hat{\beta}_2$$

and $\hat{\beta}_j < 0$, otherwise $\hat{D}_j = \infty$

- k. The index ratio (IR) is calculated

$$IR = \frac{\hat{\alpha}}{\bar{y}_b} \text{ where } t_b = 0.$$

B. Guidelines for selecting plate counts

1. Two or three dilutions are made for each planchet.
2. Two aliquots are plated for each dilution.
3. The set of plates from the same dilution for all planchets on one boat which yield acceptable counts are denoted as the experimental results.
4. The selected plates are determined according to the following guidelines taken from Appendix E, Progress Report #3:
 - a. Count colonies on all plates; >600 colonies = TNTC.
 - b. Any TNTC or zero count disqualifies that dilution.
 - c. Calculate mean colony count for each dilution. Use dilution yielding mean of 30-300 colonies per plate. Use highest mean if two dilutions qualify.
 - d. If no dilution qualifies, mean of >20 or <500 colonies per plate is used. Again use highest mean if two dilutions qualify.
 - e. If no dilution qualifies as above, data time point is considered lost.

R. Jacobson

APPENDIX B: EXPERIMENTAL DESIGN, LABORATORY CONTROL AND STATISTICAL ANALYSIS

In the investigation of reproducibility of inter-laboratory data (discussed in detail in the section entitled "Inter-laboratory D-value Experiments), experimental results showed what we thought were certain day-to-day differences. These differences were reported at the February, 1970, meeting of the Planetary Quarantine Advisory Committee. In trying to pin point the sources of these apparent day-to-day differences we came upon some results that we think may illuminate some important points in experimental design as well as in data analysis.

These inter-laboratory experiments were performed with one boat per heating time. Each boat contained five inoculated planchets of which only four were evaluated; one planchet was carried as a reserve. In re-examining the results of this experiment we realized that, rather than treating the the planchet as the experimental unit as we had done, the true experimental unit was the boat. Furthermore, since only one boat was used per heating time, we had no proper estimate of the true experimental error. It is possible that the day-to-day significant difference may have been significant only because of using the improper estimate of experimental error. In sterilization experiments, as everywhere, the appropriate experimental error must be determined.

To measure the day-to-day variability with the proper experimental error, we designed a set of experiments (GS0041, GS0042, and GS0043) where two boats were subjected to each of five heating times, three planchets per boat. The heating temperature was 125°C.

When we analyzed the data from experiment GS0042 considering the planchet as the experimental unit, we obtained the results shown in Figure 1; note the tight confidence belts. In this erroneous way of looking at the data we have six experimental units (six planchets) per heating time.

The correct way of handling the data from experiment GS0042 is to consider the boat as the experimental unit. An estimate of the number of survivors on each boat was calculated by averaging the estimates obtained from each of the planchets. The results when these same raw data were analyzed using the boat as the experimental unit are shown in Figure 2; note the wider confidence belts.

The relative differences in the widths of the confidence belts can be attributed to two facts that both involve the number of experimental units: (1) two different estimates of experimental error and (2) two different

t-distributions. When planchets were considered to be the experimental units, an assumption of no effect assignable to boats was implied. Thus, the expected mean squares(EMS) for boats, analogous to Table 6.3 of this report, were assumed to be equal to the EMS for planchets. The two estimates of the "experimental error" were pooled and associated with the respective pooled degrees of freedom. The difference in the weighting of the two sets of sum of squares(when pooling) contributed to the resulting small estimate of experimental error. The "degrees of freedom" associated with the estimates of experimental error was larger when planchets were erroneously used as the experimental units than when boats were so designated. This implied the use of two different t-distributions when constructing the two sets of confidence belts.

The effect of looking at this experiment incorrectly was to claim a more precise estimate of experimental error than justified; we thought that we had more discrimination power with which to judge the day effects than that which we actually possessed. What we had previously thought could be day effects based upon the experimental results could no longer be justified.

To emphasize the above observed effect we have combined the data from Figures 1 and 2 and present the results in Figure 3. The estimated line is the same in both cases, however, the differences in confidence belts are apparent. Both sets of confidence belts are claiming to bracket the true regression line with 95% confidence. Table I presents the ten boat estimates of the $\log_{10}$ (number of survivors) at specified times. If all combinations of one estimate at each of the five treatment times are obtained, each combination can be used to obtain a least squares line.

TABLE I
Data from Test GS0042 Used in the Analysis

Heating time(mins.)	Boat I.D.	$\log_{10}$ --estimated No. of survivors
10	10A	5.1063
10	10B	5.0474
20	20A	4.2278
20	20B	4.1108
30	30A	3.2262
30	30B	3.8017
40	40A	2.5317
40	40B	2.9777
50	50A	2.2734
50	50B	2.3007

Table 2 shows all 32 possible combinations and the respective D-values.

TABLE 2
D-values Calculated for all Possible Combinations
of the Ten Values Observed

Line No.	Boat I.D.										D-values (mins.)
	10A	10B	20A	20B	30A	30B	40A	40B	50A	50B	
1	*		*		*		*		*		13.58
2	*		*		*		*			*	13.68
3	*		*		*			*	*		14.46
4	*		*		*			*		*	14.57
5	*		*			*	*		*		13.58
6	*		*			*	*			*	13.68
7	*		*			*		*	*		14.46
8	*		*			*		*		*	14.57
9	*			*	*		*		*		13.80
10	*			*	*		*			*	13.91
11	*			*	*			*	*		14.71
12	*			*	*			*		*	14.83
13	*			*		*	*		*		13.80
14	*			*		*	*			*	13.91
15	*			*		*		*	*		14.71
16	*			*		*		*		*	14.83
17		*	*		*		*		*		13.80
18		*	*		*		*			*	13.91
19		*	*		*			*	*		14.71
20		*	*		*			*		*	14.83
21		*	*			*	*		*		13.80
22		*	*			*	*			*	13.91
23		*	*			*		*	*		14.71
24		*	*			*		*		*	14.83
25		*		*	*		*		*		14.03
26		*		*	*		*			*	14.14
27		*		*	*			*	*		14.97
28		*		*	*			*		*	15.09
29		*		*		*	*		*		14.03
30		*		*		*	*			*	14.14
31		*		*		*		*	*		14.97
32		*		*		*		*		*	15.09

* Boat data value used in calculation

The two lines with minimum and maximum slope (maximum and minimum D-values) are plotted in Figure 3. One argument might be to think of these lines as a third set of confidence belts based upon the original data. Where do they lie in relation to the other two sets? It is interesting to note that their position is in part between the two other sets.

Another interesting point was brought to light by this experiment. There are only 16 distinct D-values among the 32 given in Table 2. If the heating times are equally spaced with an odd number of time points employed and the same number of boats are at each point, the data values obtained at the central time point provide no information for estimating the slope of the survivor curve. This means that by carefully selecting an even number of equally spaced heating times, all of the data obtained will be used when estimating the D-value. By using more than two distinct heating times (e.g. four), analysis of the non-linear aspect of the survivor curve is possible.

Experimental design has a significant effect on laboratory efficiency and the quality of the results. When we looked at our experimental design we found that without increasing the number of man-hours expended we could increase the precision of our experiments by reducing the number of plachets per boat and increasing the number of boats. (This aspect is discussed in detail in section 6 of this report.) We also observed that when $\log D$ is a linear function of time, by decreasing the number of heating times from four to two (one short and one long) an equal number of experiments can be carried out using 50% of the man-power effort with less than a 50% reduction in precision. (Note: this applies only when $\log D$ is a linear function of time.)

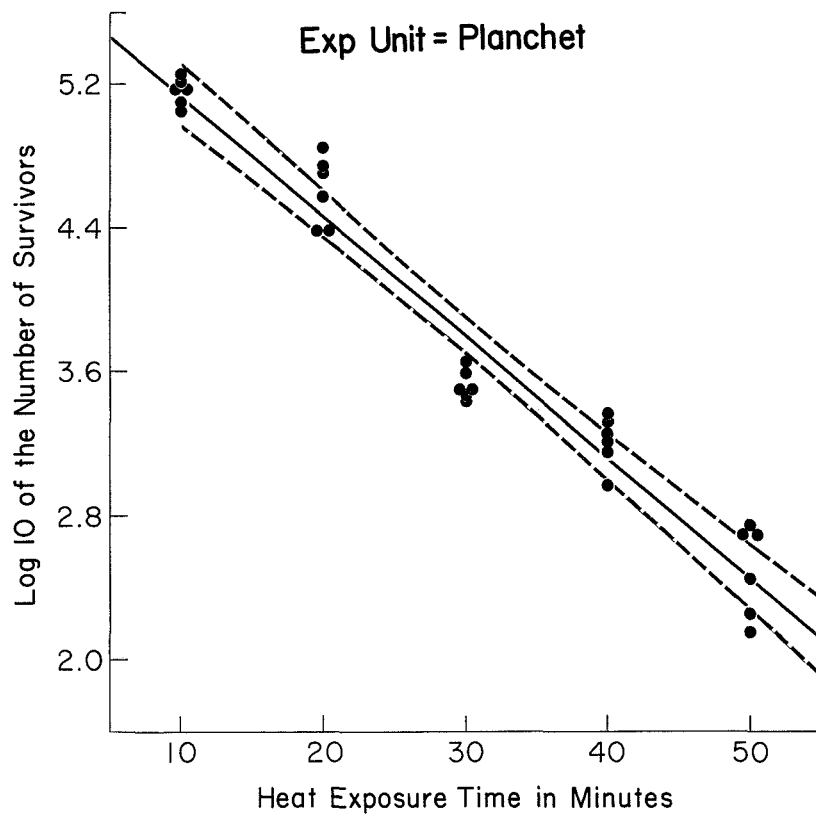


Figure 1 - Least-squares line and confidence belts when the planchet was used as the experimental unit

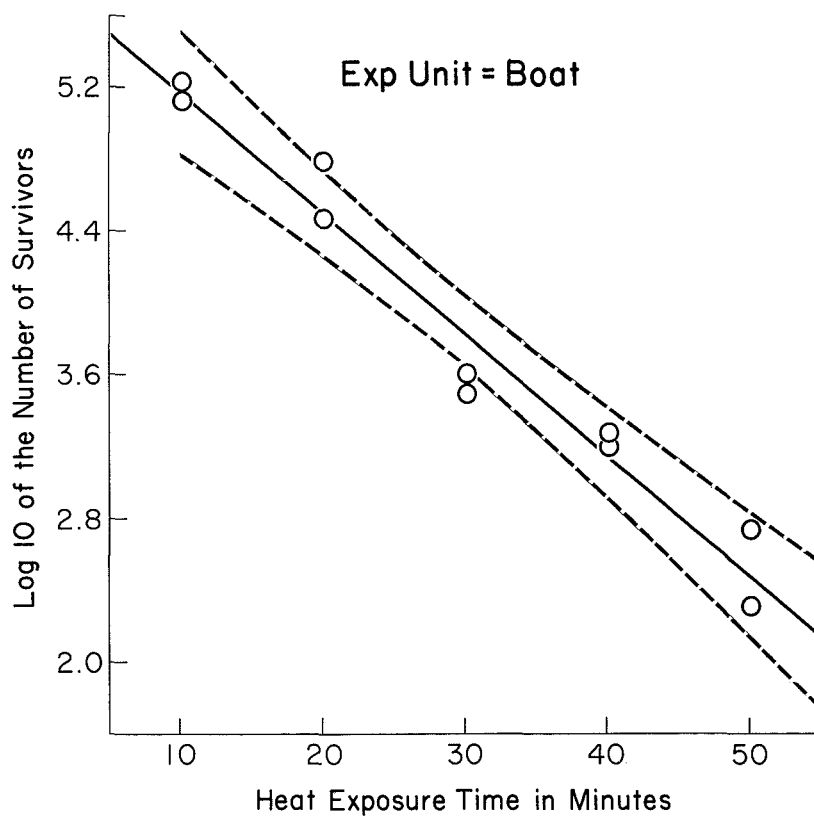


Figure 2 - Least-squares line and confidence belts when the boat was used as the experimental unit

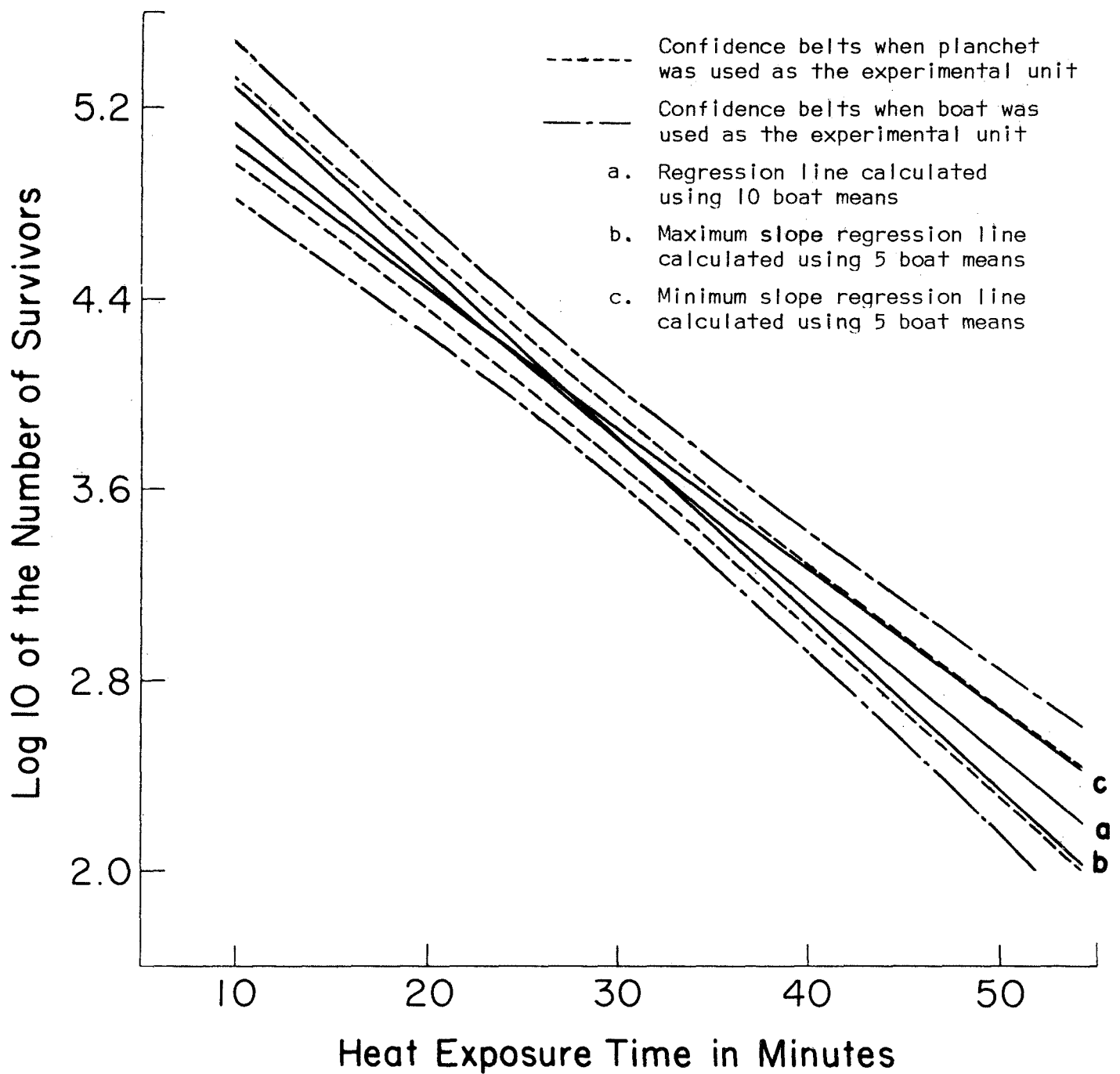


Figure 3 - Effects of using the inappropriate experimental error term

APPENDIX C: DISCUSSION OF D-VALUES IN MATED SURFACE AREAS

Introduction

Since it was recognized by NASA that microorganisms on surfaces, in mated surface areas and encapsulated die at different rates, several laboratories have carried out experiments directed toward developing D-values for microorganisms in mated surface areas. These studies were carried out at different times and were directed toward answering different questions pertaining to dry heat death rates of microorganisms. At this point in time it seemed desirable to review the research findings of these several laboratories concerning D-values of microorganisms in mated surface areas.

D-values of spores in mated surface areas

Studies to determine the D-values of spores in mated surface areas have been conducted by the Public Health Services Laboratory, Cincinnati, Ohio, the Jet Propulsion Laboratory, Pasadena, California, and the University of Minnesota, Minneapolis, Minnesota. In this report I will try to put these several pieces of data together to give an overall view of the status of microbial destruction in mated surface areas.

The Jet Propulsion Laboratory has carried out several studies to determine D-values as a function of mated surface pressure. In these studies the spores were placed on small planchets and equilibrated at relatively low humidities and then heated at pressures from near zero to 200,000 psi in the JPL "thermal conductance apparatus". They found that at pressures as high as 200,000 psi, the $D_{125^{\circ}\text{C}}$ -value was only two times the zero pressure D-value.

At the University of Minnesota, Drummond studied the dry heat destruction rate of microbial spores in a simulated mated surface apparatus where the spores were deposited at nine different locations on a 6" x 8" stainless steel sheet. The first stainless steel sheet was covered with a second; the two sheets were then placed in a package and the package (with one 6-inch edge open) was inserted between two blocks. Drummond equilibrated his spores at very low (less than 2% RH at 23°C) and very high (about 90% RH at 23°C) humidity levels. When he equilibrated his spores at low humidities the mated surface D-values were only slightly higher than open system D-values for similar spores. In spores equilibrated at the high humidity levels he found that the D-values were greatly increased. At high humidities there was also a tendency for the spores in the center of the

6" x 8" sheets to show a significantly higher D-value than the spores near the open edge. On the other hand, when he tested the spores equilibrated at low humidities, he found the D-value of the spores near the edge was similar to the D-value of the spores in the center of the 6" x 8" sheets.

Angelotti et al. carried out two series of experiments where they evaluated the D-value of spores in a mated surface configuration. They produced the mated surface area by depositing the spores on a washer face and then forcing this washer face against a second stainless steel facing. The two washer facings were held together by a nut and bolt which was tightened to a prescribed torque level. Each mated surface unit was sealed using an oxy-gas flame in a sterile, thermal-death-time tube. Duplicate samples were exposed in a silicon oil bath for various times at test temperature. At the end of the heat treatment the tubes were cooled, washed, surface disinfected and then opened. Both discs were aseptically placed in a test tube and the number of surviving microorganisms determined.

In Angelotti's 9th progress report, $D_{125^{\circ}\text{C}}$ -values of 47.5 and 44.5 were reported for spores in the mated surface apparatus in this system. In the 10th report a z-value of 32°C was reported for these data.

As reported in their 12th progress report, they repeated the tests with the mated joint sealed with epoxy resin and reported $D_{125^{\circ}\text{C}}$ -values of 165.0 minutes. The mated surface unit with the epoxy resin was also placed in a glass TDT tube and flame sealed before heating.

To be able to evaluate the mated surface affect found by Angelotti, a comparable open surface D-value is needed. Angelotti reported D-values of 8.3 and 8.9 minutes for spores on stainless steel surfaces heated in TDT tubes where the spores and air in the tube were dried over silica gel prior to being sealed in the TDT tube. If we assume that the D-value of spores at relative humidities (RH) of 30 to 40% is twice the D-value of spores at near zero percent RH (based on the results of the University of Minnesota studies which were reported in December, 1969) then the D-values of Angelotti's spores on surfaces at 30 to 40% RH would have been 16 to 18 minutes.

If we now compare this calculated surface D-value of 16 to 18 minutes with the $D_{125^{\circ}\text{C}}$ -value results of 47.5 and 44.5 of Angelotti's first experiment we observe that these mated surface values are about 2.5 times greater than the comparable calculated open surface values. It is probable that these D-values of 47.5 and 44.5 minutes are larger than a mated surface exposed to a circulating atmosphere because of the effect of the sealed tube coupled with the large surface area of the mated surface apparatus. The large z-value of 32°C suggests a

a different type of destruction system. (The behavior of this system is discussed at length on page 3 of the 12th progress report of Angelotti.) I believe that we can conclude that the glass TDT tube was responsible for the D_{125} -values being as large as 47.5 and 44.5.

In the second set of experiments of Angelotti et al., the D-value of 165.0 minutes which was obtained was probably due to a higher water content again due to the system. When the mated surface joint is covered with epoxy and the test unit is sealed in a glass tube again we have a mated surface system plus a major modification. In this type of encapsulated system we may have a moderately high water content depending on the pre-sealing treatment, the properties of the epoxy, and the area of exposed metal. Perhaps this system corresponds more closely to the conditions reported by Drummond when he equilibrated his spores at high humidities prior to testing in his mated surface system. In the light of present knowledge, these data are for a specific experimental system different than a spacecraft mated surface area and therefore are not applicable to the sterilization of a mated surface on a spacecraft which will de-gas to a low water level.

I believe that the above discussion establishes the fact that some of the data reported for experimental mated surface systems is in reality not applicable to the present-day mated surface problem on spacecraft. In the overall Planetary Quarantine program these data have served as guidelines for other research, and may also be valuable in showing what happens if we go from a mated to a hermetic surface and include polymeric materials that may adsorb or contain water.

Discussion of water transfer in and out of mated surfaces

Let us now think about the physical phenomena that are going on in our open and mated surface systems. Let us assume from the start that the D-value is a function of the water conditions surrounding the spore during the heating period and that over the range of humidity conditions that we are studying and at the test temperature the D-value will increase with increasing water content. In other words, we are working on the left side of a parabolic type of curve with an approximate equation of $x^2 - x = -0.125y$ where $y = \log D_T$ and x is the relative humidity as a ratio.

The primary difference between an open surface, a mated surface and an encapsulated surface is the degree to which water vapor can escape during heating. We assume that when the water molecules on an open surface move into

the vapor state, especially when they clear the boundary layer, they are essentially lost from the surface. In the mated and closed systems it is more difficult for water molecules to escape. If the atmospheric volume surrounding the spore is large compared to the volume of the spore and if the spores and surfaces have been equilibrated at a low humidity, it appears that when heated to about 125°C, the spores will lose sufficient water so that their D-value will approach the D-value for spores on open surfaces heated in a moderate humidity condition.

The initial water present in the system is critical because only an extremely small quantity of water must be lost from the spores during the heating period to change the resistance of the microorganisms from a high $D_{125^{\circ}\text{C}}$ -value of one to three hours to the normal open surface D-value of fifteen minutes. Apparently the pressure forcing the system together is not critical when we are dealing with rather rough and incompressible surfaces.

The movement of water out of mated surface areas is through a diffusion process which means that the rate of water movement will be a direct function of the size and length of the channel compared to the size of a water molecule. In the JPL studies in general, one-half-inch square planchets on which spores were deposited in a droplet were used. This suggests that the maximum distance from the center of the planchet to a free atmosphere surface was 0.25 inch. The relative diffusion distances for the studies done at JPL and the University of Minnesota are 0.25 inches and 1-4 inches respectively. In other words, the diffusion distance in the University of Minnesota studies was from 4 to 16 times greater than that for the JPL studies.

In the JPL studies the planchets were subjected to compressive stresses of up to 200,000 psi. In the University of Minnesota studies, the pressures were relatively low, being only 10.4 psi. Since in both cases relatively hard metal surfaces were being dealt with, it is probable that the size of the diffusion channel did not vary by more than a factor of two or three from the relatively low pressures used by the University of Minnesota to the very high pressures used in certain tests at JPL.

The results of the studies carried out by the two laboratories are in general in line with these observations of the relative diffusion distance and the size of the diffusion channels, where we see that going from essentially zero to 200,000 psi caused a change in D-value by only a factor of 2. In comparing the JPL and University of Minnesota results the affect of the length of the channel comes into play only when there is a large amount of water that must diffuse out of the system as in those experiments where the plates and spores

were equilibrated at high humidities.

I believe that one of the important variables that we are working with is the diffusion gradient; this is the difference between the water concentration conditions surrounding the mated surface and the water condition at the spore site. The initial water condition at the spore site is determined by the total water available and consists not only of the water in the spore but the water adsorbed by the steel surface itself.

The present plan is for space hardware to be sterilized in a dry nitrogen atmosphere. Let us now look at the respective water conditions in the mated surface area compared to the water conditions in the external gas atmosphere and the specific conditions inside the mated surface area.

In the worst case situation there will probably be the deposition of organic material, microorganisms, and water on a surface before the surface becomes mated due to the touch, a finger, a tool or other dirty object. All of the area constituting the mated surface will have been in equilibrium with an atmosphere containing water prior to the mating, therefore there will be some water absorbed on the surfaces. As the spacecraft hardware containing the mated surface is heated, water will be lost from the surface and any microorganisms on the surface. The concentration of water molecules in the mated surface area at the beginning of heating will certainly be much greater than that of water molecules in the nitrogen gas. Therefore, water molecules will diffuse from the mated surface area into the surrounding gas and be carried away. The movement of water by diffusion is a function of the relative size of the pathway compared to the size of the molecule and, of course, varies inversely with the length of the path.

The time required for the water concentration to drop to a significant low level will be a function of the quantity of water in the mated surface area. I interpret the results of Drummond, where he obtained high D-values with high equilibrium humidity conditions, as representing the condition of a large reservoir of water which requires a long period of time to diffuse out of the area. When there are only small quantities of water present, the time for this water to diffuse out is sufficiently short that the water condition surrounding the spores approaches those of a spore on an open surface.

There are some other interesting details that must be considered, however, I do not know at this instant where they fit into the overall picture. For instance, if we have only a few microorganisms in a mated surface area, undoubtedly the water contributed by the surface will be far in excess of the

water contributed by the spores; surface finish and surface treatment will probably both have an effect on the water absorption capability of a surface and a degassing treatment where a vacuum is pulled prior to the final sterilization cycle will undoubtedly remove the water from both surfaces and spores. However, if it removes a significant amount of water from the mated surface area then there will be that much less water in the total surface area during heating with the same result as if it had been originally removed from the spores. Lastly, we must know the relationship between the quantity of water in the spore during heating and the D-value.

Conclusions

My conclusions on the mated surface problem at this time are itemized as follows:

1. Water is the agent that is producing the variability or change in D-values.
2. If we observe the results of Dave Drummond's research we see that the quantity of water in the spore in the mated surface area, having an affect on the D-value equilibration of spores at relative humidity levels of about 90%, increased the D-values. Equilibration of spores at relatively low humidities resulted in D-values that were not too different from the D-values he obtained for similar spores in an open system.
3. When Drummond equilibrated his spores at low humidities, the D-value of spores near the edge compared with the D-value of spores in the center of the 6" x 8" sheets was not significantly different.
4. When JPL studied the D-values of spores on planchets the area of the JPL planchets was small. In a thermal conductance apparatus they found that increasing the pressure from essentially 0 to 200,000 psi only increased the D-value by a factor of 2. (The spores were equilibrated at a relatively low humidity.)
5. JPL used materials that were treated so as to be identical to materials on a spacecraft.
6. In studies to date at both JPL and the University of Minnesota, when the spores were equilibrated under conditions considered to be dry, or even under clean room conditions of 35% RH, the D-values did not differ from the D-value of microorganisms on an open surface by more than a factor of 2.
7. The two exceptions to D-values of spores in mated surface areas being of

the same order as D-values of spores on surfaces were:

- a. The results obtained from Angelotti's studies when mated surface units were sealed with epoxy and in turn were sealed inside glass tubes during heating and
 - b. the results when Dave Drummond in his mated surface experiments equilibrated the spores at about 90% relative humidity.
8. The high humidity, high D-value data of Drummond is not applicable to our spacecraft sterilization problem because the spacecraft will:
 - a. be assembled in a much lower humidity controlled environmental area and
 - b. be subject to a vacuum de-gassing treatment
 9. The high D-value results of Angelotti also are not applicable to mated surfaces on spacecraft because the mated surface was inside of a sealed glass tube.
 10. Mated surfaces which contain plastics or unusual oxide surfaces should be tested to assure that they do not increase the D-value of spores in these mated surface areas.
 11. Subject to the above qualifications the present mated surface D-value of one hour (or twice the open system D-value) is reasonable and should continue to be used.

I. J. Pflug

APPENDIX D: VARIABILITY IN MICROBIAL SPORE RECOVERY TECHNIQUES¹

Introduction

In experimental research studies there is a certain amount of variation in data that is attributable to procedural differences. If variation in technique can be limited, the statistical variation in the results will be smaller, allowing the researcher to reach more definitive conclusions regarding his results.

A research study reported in Progress Report #2 in which we attempted to measure the effect of air velocity on the survival of microbial spores deposited on stainless steel strips produced some interesting and unpredictable results. The unpredictability of these results may have been due to laboratory techniques or some phenomenon that at this time is still unexplainable.

The experimental procedure involved exposing inoculated stainless steel strips (1" x 2" x .024") in an air duct and then assaying the strips for microbial survival. The liquid suspension on the strip was allowed to dry over night in a vertical downflow clean room and the spot that was left was then assumed to consist of individual spores. The strips were then subjected to a treatment process or used as controls. The treatment process consisted of exposing the strips in the air duct to a controlled temperature and velocity air stream for periods of one, two, and three weeks. The strips were then individually removed from the air duct and placed in 125 ml flasks to which 50 mls of buffer were added. The flask, buffer and strip were sonicated to remove the remaining viable spores and aliquots were pipetted into petri dishes. Liquid media was added and the plates were counted after 48 hours incubation.

Experimental Variables

In the techniques described above there were several sources of human and mechanical errors that may have introduced unwanted variability. Some of the most obvious sources were the following:

1. Pipetting spores from the spore suspension onto the strips.
2. Strips contaminated during transfer to treatment apparatus.

¹ The material in this report was taken, in part, from a Plan B paper which was presented by the senior author to the School of Public Health, University of Minnesota.

3. Strips contaminated during transfer from apparatus to flask after treatment.
4. Differences in volume of buffer in dilution blank bottle that was added to the flask.
5. Pipetting diluted suspension from flask to petri dish.
6. Differences in plate counting procedures.

In the initial deposit of the spore suspension a Misco λ -pipetter made by Micro Chemical Specialities was used. This pipette utilizes an adjustable piston pump that meters and delivers a measured volume from an attached reservoir. The pumping mechanism in this pipette clogged several times, causing the tip to deliver unequal amounts of suspension. Some of the variability in results may have been caused by this factor. A new device, the Eppendorf Microliter pipette, was then purchased for use in metering these depositions. The Eppendorf device contains a barrel piston to which a disposable tip is attached; each time a deposition is made the tip will refill from the stock suspension. In this system the spore suspension never reaches the pumping mechanism. The Eppendorf pipette system worked out very well in this experiment and is currently being used in this laboratory.

The possible sources and the prevention of contamination in microbial recovery techniques have always been somewhat difficult to evaluate. This subject has been treated in detail by Runkle and Phillips (1969). In this laboratory, the sources of contamination were either manipulative sources or environmental sources. The first category included such things as transferring the strips to flasks and plating the suspension, while the second source pertained primarily to the air and other physical sources in the recovery room.

The recovery procedure was carried out in a laminar downflow clean room. In this room (which is approximately 10' x 10') the air is supplied to plenums above the entire ceiling. The air is pre-filtered and conditioned and then passed through absolute filters (99.97% efficient on 0.3 micron DOP) and into the room. The air discharged from the filters then flows downward towards the floor at a velocity of 90 fpm. The floor is completely perforated and provides the exhaust for the down flowing air. When this room is functioning properly there should be no contamination from the air.

The possibility of contamination due to manipulative sources may occur during handling of the spore strips either before or after the treatment process. In our work, the strips were transferred with sterile forceps and every precaution was taken to maintain an aseptic condition.

Although we had no positive way of detecting the presence of contamination from these two sources we obtained an indication of it by observing the colonies on the plates. This was possible because a specific microbial spore was used to measure the effectiveness of a given treatment. This spore produced a microbiologically specific, and easily identifiable colony; if any other colony appeared on the media plates, the plates were assumed to be contaminated. The presence of foreign colonies was not observed during any experimental tests and therefore no sources of contamination affected the experimental results.

Another part of the recovery procedure, the handling of the strips after the treatment process, took place in the clean room. The strips were placed in sterilized 125 ml beakers to which buffer was added from a dilution blank bottle that had been prepared in advance. After a two-minute sonication a small volume of suspension was pipetted into plastic petri dishes. The standard volumes pipetted were 0.1, 1.0, and 10.0 mls. However, in this experimental procedure only the 0.1 ml of suspension was required because the treatment process was such that there were high levels of survival. The pipetting was done with a 1.1 ml disposable pipette of the type used in the dairy industry. After several experiments using this type of pipette, it appeared that this operation could introduce experimental errors. Eppendorf microliter pipettes were then obtained and adapted for use in transferring 0.1 ml of the suspension to the petri dishes. However, there was no observed reduction in experimental variability.

By the process of elimination and a qualitative examination of the bottles it seemed appropriate to look at the variability introduced by the dilution blank bottles. This visual examination revealed that there was some variation in the level of liquid in the bottles. A study was then undertaken to examine this variable more closely.

Dilution blank variability

As a result of the analysis presented above and the qualitative examination of the liquid level in several dilution blank bottles blanks associated with a strip for one experimental run were recorded. The dilution blank bottles were weighed prior to their use and again after the contents had been poured into the flask. The weight difference was the volume of buffer present. In Table 1 the weight of the buffer is compared with the subsequent plate counts.

and the parameters that are used in a statistical analysis of these data are shown.

TABLE I
Weight of Buffer and Subsequent Plate Counts*

Dilution blank #	Weight of buffer (x)	Plate counts (y)
1	41.7	363
2	45.6	262
3	46.6	247
4	47.6	274
5	47.3	231
6	47.1	364
7	47.2	372
8	46.5	306
9	46.7	337
10	47.3	338
11	41.1	369
12	47.6	345

Statistical Parameters

$$\begin{aligned}\Sigma x &= 552.3 & \Sigma y &= 3809 \\ \Sigma x^2 &= 25,474.51 & \Sigma xy &= 174,849.9 & \Sigma y^2 &= 1,238,101 \\ (\Sigma x)^2 &= 25,419.61 & \frac{\Sigma x \Sigma y}{n} &= 175,309.2 & (\Sigma y)^2 &= 1,209,040\end{aligned}$$

$$\bar{x} = \frac{\Sigma x}{n} = 46.0 = \text{Mean weight of buffer}$$

$$\bar{y} = \frac{\Sigma y}{n} = 317 = \text{Mean plate count}$$

A linear regression-statistical analysis (Bartsch 1968) was then performed on the data. The analysis was of a Model II type, i.e., both of the variables being random. This type of analysis is centered around determining the constants α and β in the linear algebraic equation $y = \alpha + \beta x$.

The squared vertical deviations from the mean values were then determined.

$$\Sigma(x-\bar{x})^2 = \Sigma x^2 - \frac{(\Sigma x)^2}{n} = 54.9$$

$$\Sigma(y-\bar{y})^2 = \Sigma y^2 - \frac{(\Sigma y)^2}{n} = 29,061$$

$$\Sigma(x-\bar{x})(y-\bar{y}) = \Sigma xy - \frac{\Sigma x \Sigma y}{n} = -459.3$$

Constants α and β were calculated with these values. The value of β was given by $\beta = \frac{\Sigma(x-\bar{x})(y-\bar{y})}{\Sigma(x-\bar{x})} = \frac{-459.3}{54.9} = -8.36$

The slope of the line was therefore a negative 8.36.

The value of α (y-intercept) was determined by using the mean values of x and y in the linear equation $y = \alpha + \beta x$. The values of $\bar{x}$ and $\bar{y}$ were taken from Table I and produced the following:

$$\begin{aligned}\bar{y} &= \alpha + \beta \bar{x} \\ 317 &= \alpha + (-8.36)(46.0) \\ \alpha &= 317 + 384.6 \\ \alpha &= 701.6\end{aligned}$$

The linear regression equation for this data was then given by

$$y = 701.6 - 8.36x.$$

This equation may be used to predict values of y (plate counts) given various values of x (buffer weight). Confidence intervals may be constructed for $\bar{y}$ given specific values of x . However, since the x values (buffer weight) do occur in a random nature these statistics are not of much benefit and another statistic that is much more relevant is the correlation coefficient. The correlation coefficient (Bartsch 1968), usually designated as ρ , is a ratio of the cross product variation to the square root of the individual variations. A statistical estimate of this coefficient is given by

$$\hat{\rho} = r = \frac{\sum (x - \bar{x})(y - \bar{y})}{\sqrt{\sum (x - \bar{x})^2 \sum (y - \bar{y})^2}}$$

Applying the parameters previously calculated the value for this data was

$$\begin{aligned}\hat{\rho} = r &= \frac{-459.3}{\sqrt{54.9 \times 29,061}} &= \frac{-459.3}{1263.1} = -.36\end{aligned}$$

In interpreting the meaning of this figure it was necessary to test the hypothesis that the correlation coefficient was zero ($\rho=0$). If this hypothesis ($H_0: \rho=0$) was accepted there was insufficient evidence of an association at the level tested. The test statistic

$$t(n-2) = \frac{r\sqrt{n-2}}{\sqrt{1-r^2}}$$

was distributed as student's t with $n-2$ degrees of freedom (Bartsch 1968). Substituting the value of r and n into the above equation resulted in

$$\begin{aligned}t(n-2) &= \frac{r\sqrt{n-2}}{\sqrt{1-r^2}} = \frac{-.36\sqrt{12-2}}{\sqrt{1-(-.36)^2}} \\ &= \frac{.36 \times 3.16}{.87} = 1.226\end{aligned}$$

A significant t with 10 degrees of freedom is 1.093, 1.372, and 1.812 for the 30%, 20%, and 10% level respectively (Geigy 1962). The calculated test value (1.226) was then significant at about the 20% level. This was interpreted as meaning there was a relationship between the buffer weight and the plate counts at the 20% confidence level. It also seemed reasonable that this level could be raised if the other factors in the technique were improved thereby putting more of the experimental error into this factor. Another point worthy of note is the fact that the correlation coefficient was negative. A negative correlation coefficient indicated a decrease in y (plate count) with an increase in x (buffer weight). This was expected in a situation involving dilution blanks i.e., more diluent results in fewer organisms per ml, thereby reducing plate counts.

This analysis established the fact that considerable experimental error was introduced into the experiments by variation in the weight of buffer in the dilution blank bottles. Therefore the dilution blank bottle preparation and techniques that would reduce this variability were looked at more closely.

Blank preparation

The dilution blank bottles were prepared by first mixing 34.0 gms of potassium dihydrogen phosphate (KH_2PO_4) with 500 ml of distilled water (NASA Standard Procedures 1968). The pH was adjusted to $7.2 \pm .1$ with 1 normal NaOH and then diluted to one liter with distilled water. This solution was pipetted into smaller bottles with a syringe type automatic pipettor. Plastic lids were loosely attached and the bottles were autoclaved. After autoclaving the lids were tightened and the bottles were stored until used.

When this preparation procedure was reviewed with the laboratory personnel it was apparent that there were three possible times when error could be introduced into this operation: when putting the buffer in the bottle, during autoclaving, and when taking the buffer out of the bottle.

The syringe was fixed at one end and the plunger of the automatic pipettor was attached to a rotating adjustable eccentric (Baltimore Biological Laboratory Model 40). Check valves were located in the inlet and outlet hose connections. The inlet tube of the syringe was placed in the large flask containing the buffer and the outlet tube was used to fill the individual bottles. The strokes per minute of the syringe plunger were adjusted so that the bottles could be filled one by one without turning the machine off. The filling procedure is quite routine but requires a certain amount of manual dexterity. About the only

ways to affect the amount of buffer in the dilution blank bottles is by spilling and pipettor malfunction.

After the bottles were filled the lids were loosely attached and the dilution blanks were autoclaved. The liquid probably escaped during the cooling portion of the autoclave cycle. If the liquid is cooled too rapidly it boils and vapor is released. Also if there is uneven cooling of the bottles differing amounts of liquid will be lost. It is fairly obvious that the vapor is going to escape much easier when the lids are loose than when they are tight. However, when the idea of tightening the caps during autoclaving was suggested considerable opposition to this idea was expressed. The basic reason given was that the dilution blank bottles might break. However, if the autoclave is operated and vented properly before the door is opened this should not occur.

The third factor that may be a source of error is careless removal of the buffer solution from the dilution blank bottles. When the blank was used it was merely poured into the flask until the liquid stopped running out. This method of removal was quite crude but appeared to be adequate.

Before any experimenting had been done it seemed that the greatest loss of solution might be due to loose bottle lids during the autoclaving cycle. Evaluation of this procedure could best be made by tightening the lids on one set of dilution blanks and comparing them to another set. The possibility of a different container was also suggested. The medium-size baby food jar was chosen because they were fairly inexpensive, had a gasketed lid, could withstand autoclaving, and were easy to obtain.

Experimental design

An experiment was designed using 18 standard dilution blank bottles and nine baby food jars. All the containers were washed, dried, weighed, assigned a number, and then filled with buffer solution using the normal filling procedure. The containers were then weighed again and the weight and jar number recorded.

The 18 standard bottles were randomly divided into two groups. The two groups of standard blanks and one group of baby food jars made up the three groups of bottles evaluated. The lids were hand tightened on the baby food jars and nine of the standard dilution blank bottles. On the remaining nine dilution blank bottles the lids were left loose. The bottles were all autoclaved at the same time and after cooling, the weight of each bottle was again

determined. The last operation was to pour the liquid solution out of the bottles and weigh each of the bottles again. This pouring operation was done just as it was done under normal operating conditions.

The weights as recorded are shown in Tables 2, 3, and 4. The weighings were made on a triple beam balance and recorded to the nearest tenth of a gram. Four weighings were made of the following:

1. The empty and dry bottle
2. Bottle filled with buffer solution
3. Bottle and solution after autoclaving
4. Bottle after pouring out buffer solution

TABLE 2
Weights(in grams) of Bottles with Loose Caps
at Various Stages in the Experiment

Bottle No.	Empty & dry	After filling	After autoclaving	After empty but not dry
1	128.3	177.7	174.8	128.2
2	128.1	177.1	174.4	128.2
3	129.7	179.4	176.5	130.3
4	128.4	177.7	175.5	129.3
5	131.1	180.5	177.1	131.9
6	129.6	178.7	175.8	130.2
7	130.0	178.7	176.0	130.6
8	131.0	180.3	177.6	131.6
9	128.4	177.8	174.8	129.1

TABLE 3
Weights(in grams) of Bottles with Tight Caps
at Various Stages in the Experiment

Bottle No.	Empty & dry	After filling	After autoclaving	After empty but not dry
1	140.6	190.0	188.5	141.3
2	131.2	180.5	179.8	131.7
3	131.3	180.5	179.5	131.9
4	133.6	182.9	182.6	134.2
5	129.8	179.1	178.7	130.4
6	131.9	181.1	180.4	132.2
7	128.2	176.3	174.3	128.8
8	128.1	177.4	176.1	128.8
9	132.4	181.2	180.3	133.1

TABLE 4
Weights(in grams) of Baby Food Jars
at Various Stages in the Experiment

Bottle No.	Empty & dry	After filling	After autoclaving	After empty but not dry
1	95.6	143.3	143.3	96.2
2	94.2	143.0	143.0	95.1
3	91.2	140.4	140.4	92.2
4	92.8	141.4	141.1	93.8
5	92.4	141.4	141.4	93.5
6	94.1	139.5	139.4	94.8
7	93.6	142.3	142.2	94.6
8	93.5	142.2	142.1	94.2
9	94.2	143.0	143.0	95.0

Data analysis

The raw data in Tables 2, 3, and 4 were used to develop the data in Tables 5, 6, and 7 prior to statistically examining the three sources of variation. The weight data was reduced to differences in the following manner:

Table 5

50 - weight after filling - weight empty and dry

Table 6

weight after filling - weight after autoclaving

Table 7

weight after emptying - weight empty and dry

An analysis of variance table was prepared for each set of data. The sources of variation were calculated using the following formula:

$$\begin{aligned}
 1. \text{ Total S.S.} &= \sum \sum x_{ij}^2 - \frac{(\sum \sum x_{ij})^2}{rt} \\
 2. \text{ Replicates S.S.} &= \sum x_{.j}^2 - \frac{(\sum \sum x_{ij})^2}{rt} \\
 3. \text{ Bottle Type S.S.} &= \sum \frac{x_{i.}^2}{r} - \frac{(\sum \sum x_{ij})^2}{rt} \\
 4. \text{ Error S.S.} &= \sum \sum x_{ij}^2 - \frac{\sum x_{.j}^2}{t} - \frac{\sum x_{i.}^2}{r} + \frac{(\sum \sum x_{ij})^2}{rt}
 \end{aligned}$$

r = number of replicates = 9

t = number of bottle types = 3

The critical difference between means was then calculated using Tukey's

(Bartsch 1968) scheme. This critical difference was given by:

$$c.d. = \frac{1}{2} \sum |c_i| q_{1-\alpha} [t, t(r-1)] \left[\frac{M.S. \text{ error}}{r} \right]^{1/2}$$

TABLE 5

Differences in Weights(in grams) of
Buffer after Pipetting

Bottle No.	Std. bottles loose caps	Std. bottles tight caps	Baby food jars
1	0.6	0.6	2.3
2	1.0	0.7	1.2
3	0.3	0.8	1.2
4	0.7	0.7	1.4
5	0.6	0.7	1.0
6	0.9	0.8	4.6
7	1.4	0.9	1.3
8	0.7	0.7	1.4
9	0.6	1.2	1.2
Σx	6.8	7.1	15.6
$\bar{x}$	.75	.78	1.73

Analysis of Variance Table

Source	S.S.	d.f.	M.S.
Bottles	5.55	2	2.78
Error	11.37	24	.47
Total	16.92	26	

$$\text{Critical Difference} = q(3, 24) \sqrt{\frac{M.S. \text{ within}}{r}} = 3.49 \sqrt{\frac{.47}{9}} =$$

$$3.49 \times \sqrt{.052} = 3.49 \times .23 = 0.80$$

TABLE 6

Differences in Weight(in grams) of
Buffer after Autoclaving

Bottle No.	Std. bottles loose caps	Std. bottles tight caps	Baby food jars
1	2.9	1.5	0.0
2	2.7	0.7	0.0
3	2.9	0.5	0.0
4	2.6	0.3	0.3
5	2.8	0.4	0.0
6	2.9	0.7	0.1
7	2.7	2.0	0.1
8	2.7	1.3	0.1

cont.

Bottle No.	Std. bottles loose caps	Std. bottles tight caps	Baby food jars
9	<u>3.0</u>	<u>0.9</u>	<u>0.0</u>
Σx	25.2	8.3	0.6
$\bar{x}$	2.8	.92	.06

Analysis of Variance Table

Source	S.S.	d.f.	M.S.
Bottles	35.18	2	17.59
Error	<u>2.80</u>	<u>24</u>	<u>.11</u>
Total	37.98	26	

$$\text{Critical Difference} = q(3, 24) \sqrt{\frac{\text{M.S. within}}{r}} = 3.49 \sqrt{\frac{.12}{9}} =$$

$$3.49 \times \sqrt{.012} = 3.49 \times .11 = .38$$

TABLE 7

Differences in the Weight(in grams)
of Liquid Removed from Jars

Bottle No.	Std. bottles loose caps	Std. bottles tight caps	Baby food jars
1	0.9	0.3	0.4
2	0.0	0.5	0.9
3	0.6	0.6	0.6
4	0.9	0.6	1.0
5	0.8	0.6	1.1
6	0.6	0.3	0.7
7	0.5	0.6	1.0
8	0.6	0.7	0.6
9	<u>0.7</u>	<u>0.7</u>	<u>0.8</u>
Σx	5.7	4.9	7.1
$\bar{x}$	.63	.54	.79

Analysis of Variance Table

Source	S.S.	d.f.	M.S.
Bottles	.28	2	.14
Error	<u>1.09</u>	<u>24</u>	<u>.04</u>
Totals	1.37	26	

$$\text{Critical Difference} = q(3, 24) \sqrt{\frac{\text{M.S. within}}{r}} = 3.49 \times \sqrt{\frac{.04}{9}} = 3.49 \times \sqrt{.004} = 3.49 \times .07 = .24$$

In this case with the significance level of 5%, the c.d. was reduced to:

$$\text{c.d.} = q_{.05}[t, t(r-1)] \left(\frac{\text{M.S. error}}{r} \right)^{1/2}$$

The tabled values of $q_{1-\alpha}$ with (3,24) degrees of freedom was 3.49. The critical difference in all of the three experiments was then given by:

$$\text{c.d.} = 3.49 \left(\frac{\text{M.S. error}}{r} \right)^{1/2}$$

Discussion of results

The results in Table 8 indicate that in pipetting there was a significant difference between the means of the baby food jars and either of the standard bottles. This difference was not expected because the pipetting operation did not change from bottle to bottle and variation due to pipetting should have been equal in all three cases. However, our laboratory technician reported that when filling the baby food jars it was difficult to prevent the liquid from splashing out. This factor resulted in a significant critical difference.

The results of the autoclaving operation indicate that baby food jars were superior to either of the other two types of containers. This was shown not only by the critical difference but also by the analysis of variance. Table 6 shows the bottle mean square equal to 17.59 which is very large compared to the mean square of the error (0.11). This indicates that variance in the experiment is due to the bottles and not to random error.

The liquid removal operation did not appear to be different for either type although the difference related to volume variation in the two types of jars was significant.

TABLE 8
Summary of Means and Differences

	Std. bottles loose caps	Std. bottles tight caps	Baby food jars	Critical difference
Pipetting mean	.75	.78	1.73	.80
Autoclaving mean	2.80	.92	.07	.38
Liquid removal mean	.63	.54	.79	.24

Conclusions and recommendations

Although all six of the factors mentioned previously may have contributed to the overall experimental variability, most of this variation came from the dilution blank bottles. More specifically, the differences in the amount of solution in the bottles was due to the autoclaving process.

It is therefore recommended that baby food jars employed in a pilot study to determine if it is feasible to replace the present process. This pilot study will require a slightly different nozzle on the automatic pipettor but this can be developed. After its feasibility has been established, the new process can be adopted and a quality control program instituted to insure a consistent amount of buffer in each dilution bottle.

The quality control program should be initiated by taking a sample of prepared bottles and calculating a mean and standard deviation. A tolerable limit should then be established based on the sample data. After subsequent batches of bottles are prepared, a random sample should be taken and the sample compared with the established mean and standard deviation.

Another factor that should be considered in the quality control program is the chemical contamination of the buffer bottles. This contamination may be associated with the bottles now in use and changing to the new bottles will eliminate the problem. However, it may be worthwhile in the future to institute some type of chemical quality control program as well as a solution quality control program.

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APPENDIX E: STUDY OF THE PLANCHET AND BOAT TEMPERATURE IN RELATION TO THE HOT PLATE TEMPERATURE

In the planchet-boat-hot plate system used to expose microbial spores to dry heat in atmospheres of different relative humidity, it is of vital importance to know the relative temperature of the planchet, boat and hot plate. In the planchet-boat-hot plate system the temperature of the hot plate is controlled but the spores are on the planchet and the temperature of the spores is the critical temperature! Hot plate temperature is controlled manually through the manipulation of a variable transformer. This control is based on temperature measurements of either the boats fitted with thermocouples or the thermocouples directly connected to the hot plate.

A study was carried out to determine the relative hot plate, boat and planchet temperature under several possible conditions of use.

Apparatus

In studies to determine the effect of temperature and relative humidity on the destruction rate of microbial spores on surfaces two identical hot plates were used--one located in the glove box and one in the clean room. In these calibration studies the same two hot plates were used. The hot plates were 10" wide, 10" long and 2" thick and the top of the plate was milled flat. Each plate was fitted with two 150-watt heaters (Chromalox type C-206). A cartridge type unit containing a thermistor and a copper-constantan thermocouple was also located in the plate.

Four new copper boats were constructed having the same dimensions as the other boats being used in this project. A 5/64 inch diameter hole was drilled through the center of the long side of the boat about 0.025 inches from the bottom. The copper and constantan thermocouple wires (30 gauge) were prepared in such a way that approximately 1/8 inch at the end of each wire was thoroughly cleaned of insulation and lacquer; the rest of the wire remained lacquered and insulated. Both the copper and constantan thermocouple wires were inserted into the hole in the boat until the ends were in a position half-way through the boat. The thermocouple was then formed by allowing 50:50 tin-lead solder to flow into the hole in the boat from the end opposite the end in which the thermocouple wires had been inserted. The thermocouple junction was prepared in this manner to insure that there would be thermal contact between the thermocouple junction and the boat material and that there would be an isothermal area of

wire in the boat adjacent to the thermocouple junction. In the process of soldering the thermocouple into the boats, the boat surface became highly oxidized. To remove this oxide layer, the boats were cleaned in hydrochloric acid and then polished with #600 emery paper; this was the same procedure that was originally used to finish the boats.

The planchets were $1/2" \times 1/2"$ squares cut from 0.015" thick stainless steel shimstock type 302. Burns from the cutting operation were removed. A copper-constantan thermocouple made from 0.002 inch diameter wire was soldered to each planchet using 50:50 tin-lead solder. To effect this soldering operation the planchet was first treated with a drop of stainless steel soldering flux placed in the center of the planchet. The thermocouple junction was then immersed in this droplet of flux. The area was then heated and a very small quantity of solder was added. The quantity of solder added in this operation was kept to a minimum so that the change in heat capacity of the planchet would be minimal. Soldering the thermocouple to the planchet produced a relatively small amount of oxidation on the side of the planchet opposite the thermocouple junction. It was judged that the heat conduction properties of the planchet would not be greatly affected by this oxidation. Removing this oxidation would have been extremely difficult because of the fragility of the thermocouple wires.

The four boats with thermocouples and the four planchets with thermocouples were assembled into a unit with a thermocouple support board. A piece of masonite board 2" wide, 8" long, and $1/4"$ thick was used as a support for the 30 gauge copper-constantan lead wires. The leads on the thermocouples on the planchets were trimmed to about five inches and these 0.002 inch diameter wires were soldered to respective 30 gauge copper and constantan lead wires. The boat thermocouple wires were also trimmed and connected to 30 gauge lead wires. The board, lead wires, and junctions are all shown in Figure 1.

A Honeywell Multipoint temperature recording potentiometer was used to measure the temperatures of the thermocouples. The temperature recorder was a 12-channel unit; the range was 0 to 150°C with a one-minute print cycle. Temperature values could be read from the chart to $\pm 0.25^{\circ}\text{C}$.

Testing the thermocouples

The planchets were placed on their respective boats directly above the boat thermocouple. The boats were then placed on a small aluminum block. The block with the boats and the planchets was placed in a constant temperature oven and the thermocouple leads were connected to the recorder. The oven, block,

boats and planchets were allowed to reach thermal equilibrium and then the temperatures of the planchets and boats were measured over a one-hour period. These evaluation tests were carried out at 110, 125, and 140°C. The boat temperatures were all very close and were well within the 0.25 readability of the chart. The temperatures of the planchets were generally within 1/4°C of the temperature of their respective boats. It was noted that the recorder had a tendency to oscillate or hunt before printing each of the planchet temperatures. We believe this was caused by the high resistance of the 0.002 inch diameter thermocouple wire used for the planchet thermocouples. Very small thermocouple lead wire to the planchet was used because a large wire (30 gauge) would not let the planchet lay flat on the boat and would thus impede the conduction heat transfer from the boat to the planchet.

Temperatures of planchets, boats and hot plate

The temperature measurement tests were carried out in a horizontal laminar flow clean room. The hot plate was located on a table in the clean room; air flowed horizontally across the top of the hot plate. In Figure 2 is shown the hot plate equipped with a plastic shield that was thought to be necessary to maintain a uniform hot plate-boat-planchet temperature.

Three tests were performed. The first was carried out with the plexiglass shield in place, the second with the plexiglass shield removed, and the third with the shield in place but with all of its vent holes blocked. In all tests the boats were located in the center of the hot plate as shown in Figure 3.

The temperature measurements were made in a similar manner in all tests. The Multipoint recorder was connected so that sequentially the temperature of the first boat (thermocouple #2) was recorded, then the temperature of the planchet on that boat (thermocouple #3) then the temperature of the next boat (thermocouple #4), and its planchet (thermocouple #5) and so on for the remaining boat-planchet pairs. The temperature measurement cycle was repeated once each minute.

In each of the three series of tests, the planchet temperatures were evaluated in positions A, B, C, D, and E (see Figure 4). At the beginning of the test the planchet with a thermocouple was placed in position A at the extreme upwind end of the boat. Four planchets without thermocouples were located at B, C, D, and E. This procedure was repeated for each of the four boats. After the temperature readings of the planchets, boats, and hot plate had been taken with the planchet in position A, the thermocouple planchet was

moved to position B. After it had equilibrated, measurements were taken again over a fifteen minute period. This procedure was repeated until the relative temperatures of the planchet, boat, and hot plate had been evaluated at points A, B, D, C, and E.

After the temperature data had been gathered they were tabulated as follows: For each boat, the temperature difference between the boat and the planchet at position A was determined. For example, the average temperature of planchet thermocouple #3, was subtracted from the average temperature of boat thermocouple #2, and similarly for planchet #5 and boat #4, planchet #9 and boat #8 and planchet #11 and boat #10 as shown for the sample of data in Table 1.

TABLE 1

Average temperature reading for each thermocouple at position A for hood in place
(Temperatures estimated to nearest $1/8^{\circ}\text{C}$)

Thermocouple number	Temperature $^{\circ}\text{C}$	Temperature Difference $\Delta^{\circ}\text{C}$
2	126	2 $3/4$
3	123 $1/4$	
4	126	2
5	124	
8	126	1 $1/4$
9	124 $3/4$	
10	126	1 $1/2$
11	124 $1/2$	

This procedure was repeated for the temperatures obtained with the planchets in positions B, C, D, and E. Temperatures were estimated to the nearest $1/8^{\circ}\text{C}$.

For each of the five planchet positions, the four computed temperature differences for the four planchet-boat combinations were averaged to give an average temperature difference at each position.

The entire process was repeated for the three hood positions producing the overall results shown in Table 2.

Discussion of results

The results are shown graphically in Figure 5. The difference (in degrees C) between the planchet and the boat is shown as a function of the planchet position. In all three tests there was a tendency for the temperature of the planchet in position E (which was downwind) to be higher than the other planchets. This

effect was minimal when the hood was used with holes blocked.

In the majority of the thermal destruction tests either two or three planchets located in the center of the boat were used. When the hood was removed the temperature difference for the three center locations was smaller than for the other two conditions.

Conclusions

Based on these tests, the data suggest that the best results will be obtained by using the hot plate with the hood removed and with three planchets located at the three center planchet positions. For this condition the three planchets showed an average temperature difference of less than $1/8^{\circ}\text{C}$. In terms of temperature control, the hot plate would be operated so that the average boat temperature is 2°C above the desired design temperature to compensate for the temperature drop from the boat to the planchet. In other words, if we were testing at 125°C then we would adjust the system to maintain a boat temperature of 127°C , to assure a planchet temperature of 125°C .

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John Steiner

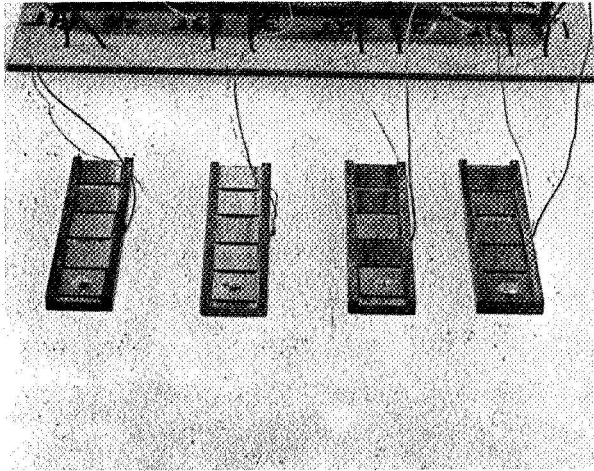


Figure 1 - Boats and planchets with thermocouples

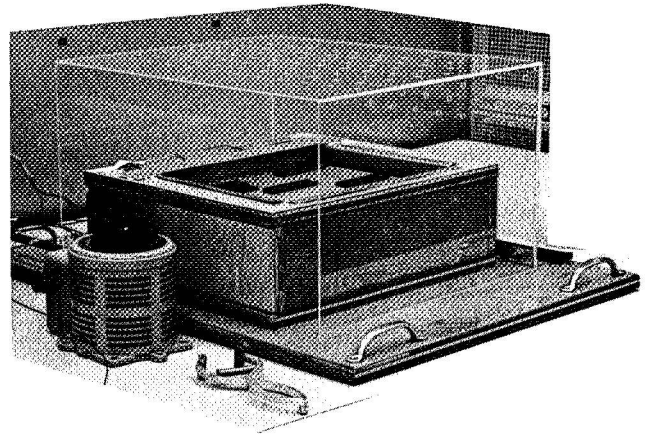


Figure 2 - Hot plate with plastic shield in place

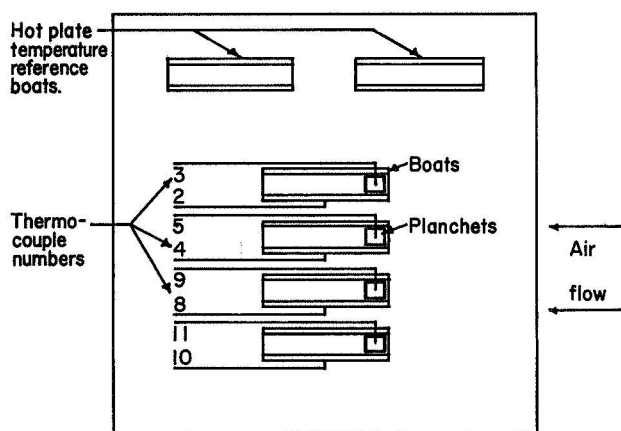


Figure 3 - Diagram of hot plate with the four boats in place and the thermocouple planchets in position A.

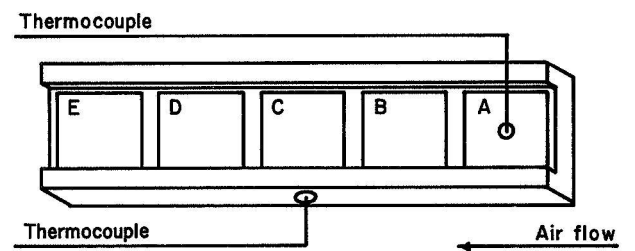


Figure 4 - Planchet positions on boat. Thermocouple planchet in position A.

TABLE 2

Boat-planchet temperature differences as a function of planchet position and presence or absence of hood
(Temperatures estimated to nearest 1/8°C)

Temperature differences at positions ($\Delta^{\circ}\text{C}$)

	A	B	C	D	E
Hood in place	1 7/8	1 5/8	1 5/8	2	2 7/8
Hood in place with holes blocked	1 5/8	2	2 1/4	2 1/4	2 3/8
Hood removed	2 1/8	2	2 1/8	2 1/8	2 3/4

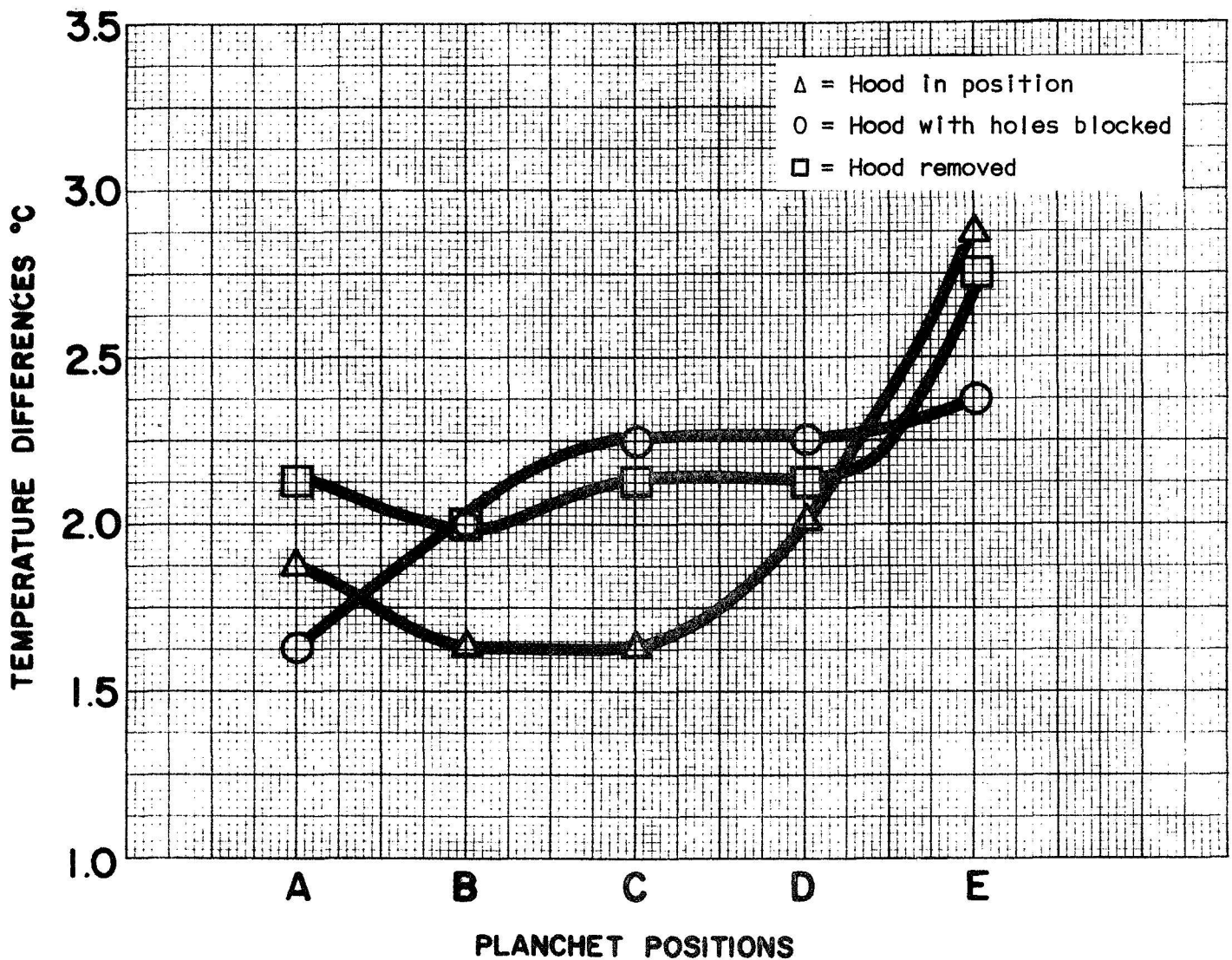


Figure 5 - Temperature differences between planchets and boats as a function of planchet position

APPENDIX F: MEASUREMENT OF THE WATER CONTENT OF THE GAS USED IN THE GLOVE BOXES IN THE PROJECT "DRY HEAT DESTRUCTION RATES OF MICROORGANISMS ON SURFACES"

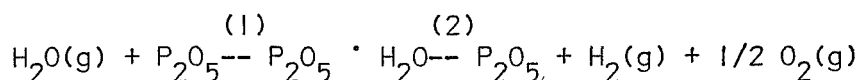
Introduction

In the dry heat destruction of microbial spores on surfaces, the water content of the gas surrounding the spores has an effect on the destruction rate. In the study of "Dry heat destruction rates of microorganisms on surfaces" we determined the D-value for spores heated in the clean room (35% RH at 22°C) and in glove boxes provided with a dry compressed nitrogen or air atmosphere. We monitored the temperature and humidity in the clean room using a hydrothermograph which was adjusted daily using relative humidity values calculated from sling psychrometer measurements. This system could not be used to measure the relative humidity of the low water-content gas atmosphere in the glove boxes; therefore, the measuring system described below was developed and used.

The system for determining the maximum water content of the atmosphere in the glove boxes was developed around a Bell and Howell CEC type 26-321A moisture analyzer. The operation of the moisture analyzer, description of the sampling system, operation procedure of the system, calculation of data and calibration of the equipment are described below.

Principles of operation of the moisture analyzer

The moisture analyzer operation is based on a coulometric principle utilizing an electrolytic cell which has the unique property of responding only to water. When electrolyzing water, the cell consumes current proportional to the amount of water. Therefore, a measure of the electrolysis current corresponds to a measure of the amount of water present. In our procedure, we are measuring the total moisture present in a measured volume of the sample gas. The carrier gas sweeps the moisture into the cell through a port in the Teflon end-piece and through a length of coiled glass tubing inside the cell. Two parallel, helically wound platinum wires, affixed to the inside of the glass tubing serve as electrodes. A thin layer of phosphorus pentoxide (P_2O_5) is deposited on the electrodes. The entire assembly is encased in epoxy resin for protection. Water is first absorbed onto the P_2O_5 film and then converted by electrolysis into molecular hydrogen and oxygen. The chemical and electrochemical reactions are as follows:



In these reactions, (1) represents a simple chemical absorption of gaseous water

by P_2O_5 which renders the film electrically conductive and (2) represents the electrolysis into the hydrogen and oxygen component elements which is accompanied by a transfer of electric charge from one electrode to the other. The electrolysis current drives the integrator, and because the cell current is integrated as a function of time a measurement of the total moisture present is made; this is independent of the carrier gas flow rate.

The CEC 26-321A moisture analyzer is equipped with zeroing circuitry to cancel out the measurement of any residual water in the carrier gas being supplied to the instrument. However, there is a residual count rate equivalent to 0.3 micrograms/minute that must be subtracted from the final reading. In our operation the moisture analyzer was zeroed each time it was used.

Description of the gas sampling system

The Bell and Howell CEC 26-321A moisture analyzer was designed to measure the water in a sample of material, for example, a quantity of spores. To use this instrument to measure the water in the atmosphere of the glove boxes it was necessary to construct a sampling system. The sampling system used is shown diagrammatically in Figure 1.

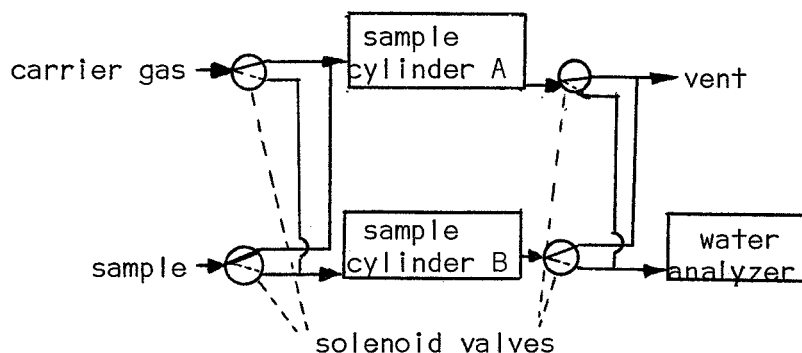


Figure 1 - Diagram of gas sampling system. The solenoid valves are controlled by a two-position manually operated switch. In position 1 the carrier gas is directed through sample cylinder A to the water analyzer while the gas being sampled flows through sample cylinder B and is vented. When the switch is moved to position 2 the carrier gas moves the sample of gas in sample cylinder B into the water analyzer while a new sample is collected in sample cylinder A.

In the first sampling system the two sample cylinders were made out of brass and were connected by copper and brass pipe and valves. The large cylinder had a volume of 892 ml and the small cylinder had a volume of 166 ml. To reduce water absorption both cylinders were covered with heating tape to maintain a high cylinder wall temperature. After a few months of operation the system was rebuilt using two 153 ml Teflon cylinders, Teflon pipe and stainless steel valves.

The double glove box experimental chamber which was described in Progress Report No. 3 was equipped with sampling ports so that the sampling system could be connected to either the conditioning chamber or the heating chamber. Samples from the smaller volume cylinder required approximately 3-5 minutes to run through the analyzer while samples from the larger volume cylinder required about 12-15 minutes.

Calculation of relative humidity

The water analyzer indicates the μg of water in the sample. To calculate relative humidity:

1. The indicated water value was corrected for the background count (0.3 $\mu\text{g}/\text{min}$ was subtracted)
2. The size of the gas sample was incorporated to yield water content in mg/liter which through the aid of psychometric tables was converted into vapor pressure.
3. Relative humidity was calculated using the equation

$$\text{Relative humidity (\%)} = \frac{\text{vapor pressure}}{\text{saturated vapor pressure}} \times 100$$

The saturated vapor pressure of water for the temperature of interest was obtained from psychometric tables.

Calibration of the water measuring system

Each time the water analyzer was used the zero of the instrument was adjusted using dry nitrogen as the carrier gas. Two tanks of reference gas (nitrogen plus a specified quantity of water vapor) were used to check the calibration of the system at water levels of about 600 ppm.

The attempts to calibrate the gas sampling and analysis system using the reference gas were not completely successful. On each day each successive analysis yielded a higher reading than the previous analysis. For example, the experiment carried out on April 4 yielded much higher water readings than

the one carried out on February 18. The reason for this phenomenon is not known. It is possible that the measuring system was not adequately pre-dried for the April 4 experiment or that the gas from the reference tanks was not homogeneous (i.e. water content of the gas increased as the pressure in the tank decreased). However, in all instances the moisture analyzer indicated more water than the reference gas analysis furnished by the supplier.

All calibration measurements to date indicate that if there is an error the indicated water content is higher than the actual water present. Recently, after the dry gas chambers had been resealed and after the Teflon sampling cylinders had been installed, water measurements were less than the 20 μg accuracy limit of the analyzer. Thus, every indication is that the actual moisture content of the air surrounding the spores during heating is often less than the level which can accurately be measured by the current system.

Errors in water measurement

A major problem in the measurement of low levels of water is the absorption of water by surfaces. In our sampling system the absorption of any water by the surfaces of the sampling cylinders will result in an error in the indicated quantity of water in the gas since we will be measuring not only the water in the gas but the difference between the quantity of water absorbed by surfaces during the sampling and at the end of the measurement period. When we were using the brass sampling cylinders we consistently obtained higher water readings for samples from the small cylinder than from the larger cylinder. Comparative tests of the 892 ml brass cylinder, 166 ml brass cylinder and the two 153 ml Teflon cylinders using the same reference gas indicated similar μg water/ml for the two Teflon and large brass cylinders but a greater number of μg water/ml for the small brass cylinder. It seems probable that the larger value for the small brass cylinder compared to the large brass cylinder was due to the larger surface to volume ratio of the small brass cylinder. The similar values for the large brass compared to the Teflon cylinders reflects the lower water absorption of Teflon compared to brass.

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PUBLICATIONS AND PRESENTATIONS

Publications in the Open Literature

Pflug, I. J. "Dry Heat Destruction Rates for Microorganisms on Open Surfaces, in Mated Surface Areas and Encapsulated in Solids of Spacecraft Hardware". Life Sciences and Space Research VII. North-Holland Publishing Co. Amsterdam, The Netherlands. 1970.

Presented at Meetings

Drummond, David W. "Statistical Processing of Microbiological Data with the Digital Computer", presented at the meeting of the American Association for Contamination Control in Anaheim, California, April 20, 1970.

Vesley, Donald. "Dry Heat Destruction of Microbial Spores as a Function of Relative Humidity", a technical paper presented at the annual meeting of the American Society for Microbiology, Boston, Massachusetts. April, 1970.