SUMMARY OF PROGRESS IN THE PROJECT ENVIRONMENTAL MICROBIOLOGY AS RELATED TO PLANETARY QUARANTINE

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SUMMARY

In this reporting period we have been studying several aspects of the behavior of microorganisms associated with soil particles. Details of some of the studies are included in this report. The study to determine the effect of storage time, suspending medium, storage temperature and cleaning procedures on the stability and the dry heat resistance of Bacillus Subtilis var. niger spores is being continued. Heating tests on spores stored for 39 months under the various conditions will be performed in October. We have completed studies on the effect of several levels of water on the survival of Bacillus Subtilis var. niger spores. An abstract of the thesis of Dr. Ronald Jacobson is included in this report and a copy of the thesis will be delivered to Dr. Hall.

We have started growing spore crops of resistant organisms. As soon as spore crops have been produced studies will be carried out to find ways of increasing the kill of these organisms during a sterilization cycle.

PLATE COUNT ANALYSES OF SOIL PARTICLE MICROFLORA AND PARTICLE VIABILITY OF CAPE KENNEDY SOIL FRACTIONS

O. R. Ruschmeyer, I. J. Pflug, R. Gove, and Y. Thun

INTRODUCTION

One major phase of the NASA planetary quarantine program has been especially concerned with the requirements for sterilization of interplanetary space probes to be used for special research missions. The development of procedures for the detection and destruction of microorganisms from various sources responsible for spacecraft contamination has been a prime objective of the program.

During the earlier microbiological investigations, a considerable amount of emphasis was directed toward determining heat effects on survival of certain selected laboratory cultured spore crops of bacterial species. More recently, an interest has also been developed in the indigenous microflora of small soil particles. One source of spacecraft contamination appears to be from these microscopic soil particulates and the attendant microbial populations that are harbored in the soil environment. For this reason investigations to determine the concentrations of the indigenous, aerobic microflora associated with soil particles and studies of dry heat effects on soil particle viability profiles appeared to be desirable. Data obtained from these studies can provide information to aid in assessing the response of the soil contamination problem to dry heat treatment.

The following report deals with results obtained most recently from our laboratory investigations of various particulate fractions of Cape Kennedy soil samples. These studies constitute part of a research effort on the dry heat resistance of microflora and microbial spores which are of direct interest to the NASA interplanetary exploration and quarantine program.

OBJECTIVES

Efforts of this study were directed toward obtaining information relevant to the dry heat resistance of Cape Kennedy soil microflora. The specific

objective of the current project reporting period was to obtain an estimate of the aerobic, mesophilic microbial load associated with selected size ranges of Cape Kennedy soil particles.

MATERIALS AND METHODS

The various phases of experimental work for this project have dealt primarily with the microbial associations as they occurred on soil particles. Laboratory cultured species were not used. Instead, the studies were performed directly with particles that contained aggregates of various microorganisms native to the soil samples. Thus a system was employed that presumably closely approximated a natural soil particle contamination.

Soil Samples

Two samples of Cape Kennedy soil were used in this study. An older soil sample, coded WAJJ series, had been stored in our laboratory following completion of earlier work. This sample was collected in June 1970 by the Spacecraft Bioassay Laboratory. The dry soil was shipped to our laboratory in September 1971 and initially stored at 4°C. In December 1971, the sample was separated into various size fractions and stored at room temperature. A more recently collected Cape soil sample was sent to our laboratory in June 1973. This sample was designated by code as WAKM series and was stored at room temperature in a dry state. The "new" soil has been used extensively in some of the later investigations.

Upon arrival in our laboratory, both soil samples were processed in a Ro-Tap machine to fractionate each sample into particle size ranges using standard sieves (see University of Minnesota, School of Public Health NASA Report #10). Each soil fraction obtained after sieving was stored at ambient laboratory conditions in a clean, covered glass jar until analyzed. Six of the smaller size ranges of particles separated were used in the current project work. The smallest particles analyzed were 44-53 μm and the largest fraction size range was 105-125 μm.

For all the experimental soil particle studies, either a random selected particle series or a dark particle series were used. In the random series, particles were selected without any conscious discrimination for texture,

structure, color, etc. For the dark particle series, deliberate efforts were made to select the more organic or clay-type soil particle and to especially reject quartz-like particles.

Plate Counts of Soil Microflora

Pour plates were used to enumerate microorganisms indigenous to the soil fractions studied. For all plate counts the standard Trypticase Soy Agar media (TSA) was employed.

Plate count analyses of soil microflora associated with individual soil particles were limited to an estimation of the aerobic, mesophilic forms. Because previous studies had shown that higher counts were generally found on dark particles and that lighter colored quartz-like particulates often had very few or no organisms, the current study was limited to only dark soil particles. This was done in order to obtain the best comparison of maximum numbers between soil fractions.

Attempts were made to enumerate the special groups of interest among the soil populations capable of growth on TSA media during a one week incubation period at 32°C. Three methods of particle treatment were used for plate counting. These were as follows: (1) Two minute sonication of individual particles in 25 ml phosphate buffer solution followed by appropriate dilutions and plating with TSA media. This procedure provided a standard plate count of the aerobic, mesophilic organisms and included both vegetative types and spore formers. (2) Individual soil particles suspended in 25 ml phosphate buffer were sonicated, heat shocked at 80°C for 20 minutes, cooled in an ice bath and then plated. These plate counts were utilized to provide estimates of the aerobic, mesophilic spore formers. (3) Single soil particles were placed in separate thermal death time cups and heated in aluminum boats at 110°C for one hour. After appropriate cooling, each particle was insonated in 25 ml phosphate buffer and then plated. Data from these plate counts furnished an estimate of the number of aerobic, mesophilic microorganisms resistant to dry heat.

Pour plates from the above procedures were incubated at 32°C for one week. Colonies were enumerated with the aid of a Bactronic Colony Counter. Data were reported as the range and mean count of organisms detected per

particle of soil. For all soil fractions studied, 37 particles were used for each of the three treatment methods employed for microbial analyses. Six soil fractions of WAKM dark particles and one fraction (74-88 μm) of the WAJJ soil series were analyzed in this manner.

RESULTS AND DISCUSSION

For the current project work, the small size of the soil particles (44 to 125 μm) has required the constant use of steroscopic microscopy for particle selection, transfer and detection of microbial growth. This fact, to some extent, limits the experimental study that can be completed in a particular time period. Despite the necessity for meticulous microscopic work, we have processed and analyzed more than 5,000 Cape Kennedy soil particles during past project work and current investigations. These analyses have included determinations of viability profiles and determinations of microbial concentrations associated with particles.

Studies concerned with the viability profiles of soil particles are still in progress and will be reported at a later time. Data presented in this report are the results from analyses to determine concentrations of particle microflora.

The plate count determinations of microbial concentrations associated with soil particles have been completed for six representative small particle soil fractions of the Cape Kennedy WAKM sample series. Only the darker soil particles were used for these plate counts. Earlier work had indicated that the microflora occurred more often and in greater numbers on the dark particulates; therefore, it seemed reasonable that an analysis of the darker particles would yield a better estimate of the maximum concentrations that might be encountered in the soil fractions.

Data obtained from the plate count analyses are presented in Table 1. For each particle size fraction, the mean and range of plate counts are listed. These data are based on the analysis of 37 individual particles for each treatment. In Figures 1 and 2 the plate count values have been plotted on graphs to show the relationship between mean microbial concentrations detected per particle and particle size.

Table I

Microbial Load* Associated with Selected Size Ranges of WAKM Cape Kennedy Dark Soil Particles. Plate Count Estimates for Unheated, Wet Heat and Dry Heat Treated Particles

SOIL CODE	PARTICLE SIZE RANGE (μm)	PLATE COUNTS (ORGANISMS PER PARTICLE)					
		UNHEATED		WET HEAT (20 MIN. AT 80°C)		DRY HEAT (60 MIN. AT 110°C	
		MEAN	RANGE	MEAN	RANGE	MEAN	RANGE
WAKMC	44-53	6.1	1-21	1.8	0-8	1.4	0-5
WAKMD	53-63	9.1	0-32	2.8	0-8	2.0	0-20
WAKME	63-74	12.7	1-35	5.2	0-57	3.5	0-55
WAKMF	74-88	16.2	1-46	5.1	0-35	1.9	0-10
WAKMG	88-105	37.6	1-191	10.8	0-54	11.4	0-54
WAKMH	105-125	55.3	1-356	15.1	1-119	6.5	0-43

*Aerobic, Mesophilic Microorganisms - TSA Media, 32°C, 1 Week Incubation

FIGURE I

MEAN MICROBIAL CONCENTRATIONS DETECTED ON
UNHEATED FRACTIONS OF CAPE KENNEDY DARK SOIL (WAKM)
PARTICLES. (PLATE COUNTS TSA, 32°C, I WEEK).

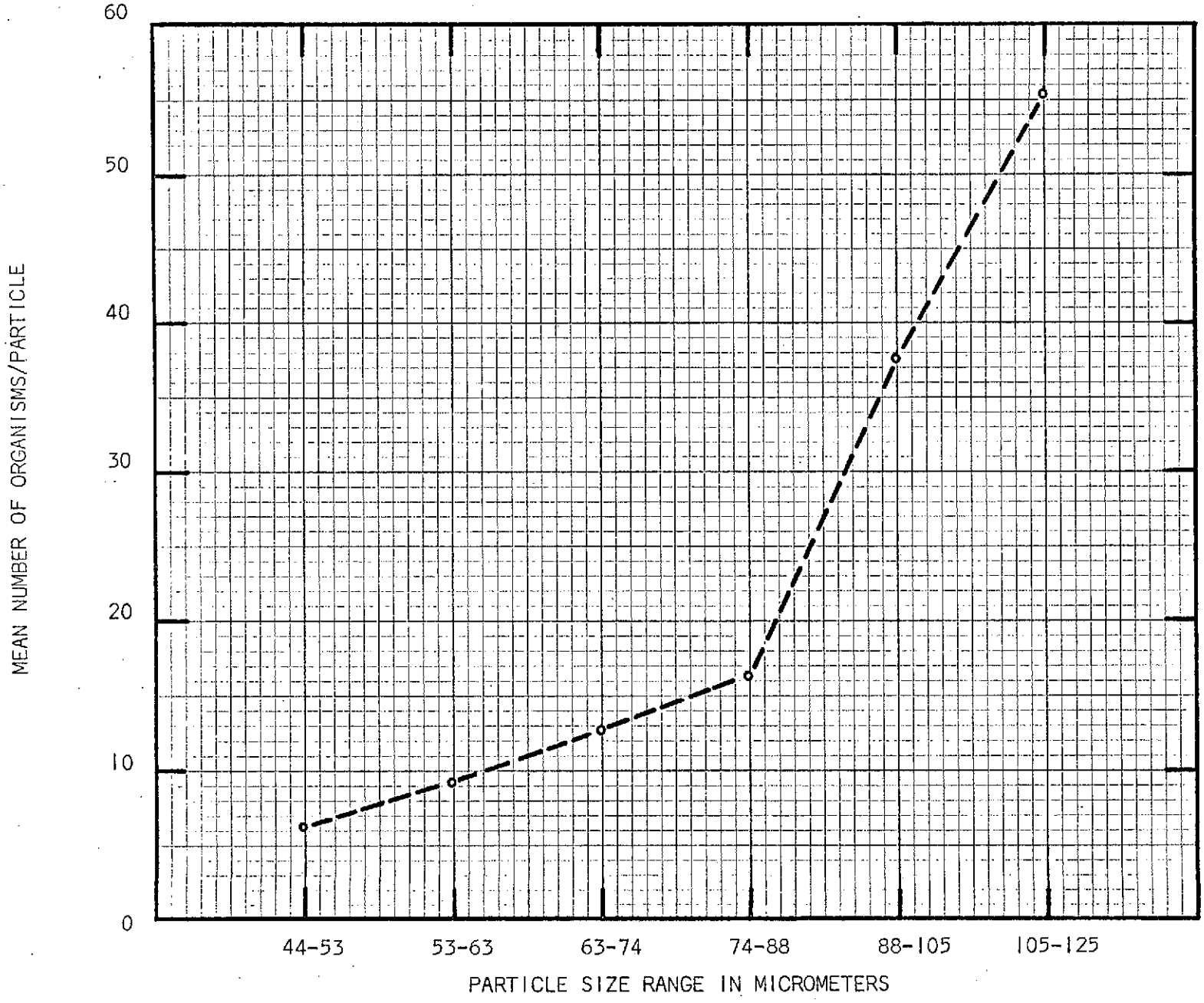

FIGURE 2

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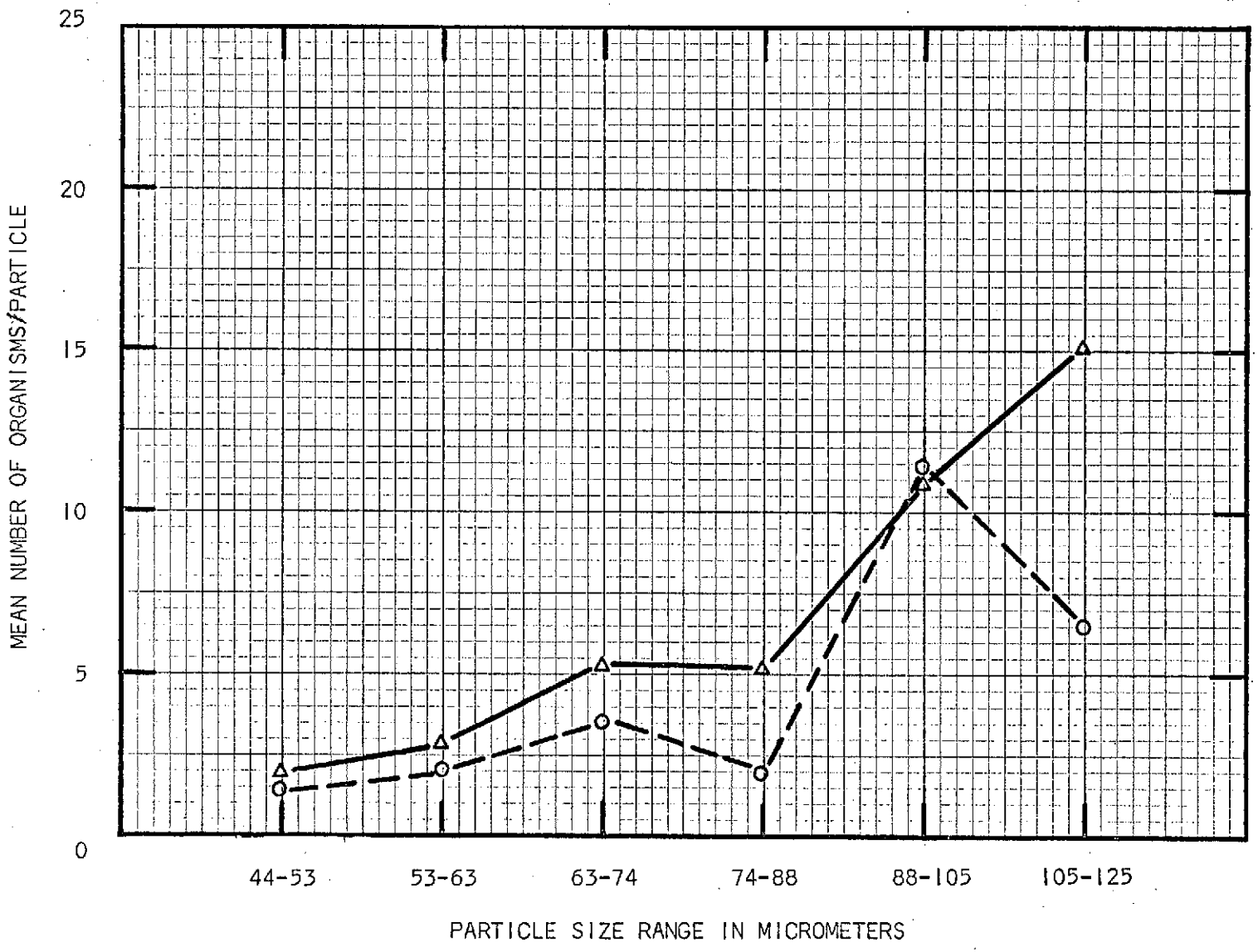

Examination of the plate count data indicates a considerable range in the aerobic, mesophilic microbial concentrations associated with individual particles. It is also evident that as the particle size increased, there was a readily detectable increase in the range of the counts. For example, in unheated particles of the 105-125 µm soil fraction, the number of organisms per particle ranged from one to over 300. The graphed data for the unheated soil (Figure 1) indicates a definite rise in the mean microbial concentration as the particle size increases. The numbers of organisms per particle increase moderately as the particle size increases up to the 74-78 µm range. Beyond that size a more marked rise in microflora numbers per particle was observed and for the 105-125 µm fraction a mean count of about 55 organisms per particle was detected in the unheated soil particles. This value is approximately nine times higher than the mean concentration of six organisms per particle found on unheated particles in the 44-53 µm range.

The data in Figure 2 show the results from analyses of heated soil particles. The microflora detected after wet heat treatment of particles at 80°C for 20 min. are most likely representative of the aerobic, mesophilic spore formers. The trend of these data also shows the continued rise in number of organisms with an increased particle size. Maximum counts of 15 organisms per particle were found for the 105-125 µm soil fraction. This is an average concentration which is over seven times that of the smallest (44-53 µm) particles studied.

The trend of the graph showing the relationship for particles treated with dry heat at 110°C for one hour is not as clearly defined. However, these data also suggest a general increased microbial count per particle for dry heat resistant forms as the particle size range increased.

It was noted in these counts that actinomycetes associated with the soil particles were often able to survive the one hour dry heat treatment at 110°C. However, these organisms apparently were unable to survive the 80°C, 20 minute wet heat since no actinomycete colonies were observed on plates from wet heat treated particles. It appears that soil actinomycetes are somewhat more resistant to dry heat than wet heat.

It should be recognized that these plate count data represent only the aerobic, mesophilic soil microorganisms capable of growth on TSA media at 32°C within one week. Since many aerobic spore formers would be included in this group, the data provide information that may be helpful in establishing sterilization requirements. At the present time enumeration of all the microbes indigenous to a particular soil would be an enormous task that would likely be beyond the capability of most, if not all, general laboratories.

The data in Table II provide a comparison of plate count estimates for the longer stored (WAJJF) and the more recently collected (WAKMF) dark soil particles. These data indicate that the proportion of WAJJF particles retaining viable microorganisms was similar to results for the WAKMF soil. Also of interest was the fact that the older stored soil (WAJJF) yielded a somewhat higher mean count of organisms per particle for all categories of microflora counted. For example, the average count per particle of aerobic, mesophilic microflora for the WAJJF soil was 28 as compared to 16 for the WAKMF particles. For the longer stored WAJJF soil, the mean count per particle after wet heating at 80°C for 20 minutes was approximately 13 organisms. This count is about two times higher than that obtained for WAKMF soil which received the same heat treatment. A similar pattern is evident for the counts of dry heat resistant forms. These results suggest that storage of soils may not necessarily reduce the number of heat resistant microbial forms which are indigenous to a particular soil.

It is not known whether the differences observed in the "new" and stored soil samples are primarily due to differences in microbial populations at the time soils were collected. No information is available on the particle microbial counts at the time of collection at the Cape Kennedy site. However, the plate count data of Table II indicate that particles will retain substantial numbers of viable organisms even under storage conditions and that these numbers are comparable to those found on particles of more recently collected soils.

Table II

Comparison of Old, Stored Cape Kennedy Soil (WAJJF) With More Recent Sample (WAKMF). Plate Count Estimates of Microbial Load and Heat Treatment Effect on Viability of 74-88 μm Dark Particles

TREATMENT	WAJJF SOIL		WAKMF SOIL	
	PROPORTION POSITIVE*	ORG/PARTICLE MEAN AND RANGE	PROPORTION POSITIVE	ORG/PARTICLE MEAN AND RANGE
None	33/37 (0.892)	28.2 (0-194)	37/37 (1.00)	16.2 (1-46)
Wet Heat (80°C, 20 Min)	31/37 (0.838)	13.4 (0-182)	34/37 (0.919)	5.1 (0-35)
Dry Heat	30/37 (0.810)	5.5 (0-35)	27/37 (0.729)	1.9 (0-10)

* Refers to proportion of particles with viable microorganisms.

WAJJF soil was stored in laboratory since September 1971. WAKMF was obtained in June 1973.

CONCLUSIONS

The laboratory studies completed recently with Cape Kennedy soil particles have provided additional data to supplement previous information on microbial concentrations and heat resistance. Results from these microbiological analyses of soil particles allow for the following conclusions:

1. There is a considerable range in the values of aerobic, mesophilic microbial counts associated with different size soil fractions. The range becomes more extended with increased particle size.

2. As the soil particle size increases, there is an increase in the mean microbial concentration per particle.

3. Plate counts of aerobic, mesophilic organisms in unheated soils yielded a mean concentration of about six organisms per particle for the smallest (44-53 μm) soil fraction. The counts increased consistently with particle size to a mean value of approximately 55 organisms per particle for the largest (105-125 μm) soil fraction investigated.

4. Aerobic, mesophilic counts for sonicated particles heated at 80°C for 20 minutes yielded mean values of about two organisms per particle for the smallest particles. The mean concentration for the 105-125 μm particles was approximately 15 organisms per particle. A somewhat similar relationship was noted for counts of organisms resistant to 110°C dry heat for one hour.

5. Some actinomycetes associated with the soil fractions could survive dry heat treatment at 110°C for one hour.

6. Soil particles stored under ambient laboratory conditions for 2.5 years yielded aerobic, mesophilic plate counts which were comparable or slightly greater than the counts for more recently collected soil.

FUTURE WORK

1. Determination of soil particle viability profiles for selected treatments at 110°C.

2. Reproducibility of the viability profiles.

3. Effect of the number of particles on the viability profile. (i.e. -- effect of particle load).

4. Effect of particle type and size on particle viability.

ABSTRACT

The Application of Biometrical Principles in the Study of Dry Heat Destruction of Bacterial Spores

Ronald Leslie Jacobson

Dry heat destruction tests were conducted in an open system using Bacillus subtilis var. niger spores on stainless steel planchets. Survival characteristics were associated with the test conditions of surface temperature and water content in the nitrogen gas environment. Test temperatures were 90 C, 110 C and 125 C while water levels were about 10, 100 and 1000 parts per million (ppm). Results of tests conducted in this system were combined with results obtained using similar test equipment in a laminar down flow clean room containing air with a water content of 13,000 ppm.

Straight line survivor curves were generally obtained for each test when logarithms of the estimated number of survivors were plotted opposite heating times. The logarithms of D-values increased linearly when plotted opposite the logarithms of ppm of water. $D_{110\ C}$-values ranged from 21 minutes at 5.5 ppm of water to 265 minutes at 13,000 ppm. Intercept ratios (logarithm of the ratio of the estimated number of spores in the innoculum obtained by extrapolating the regression line survivor curve to zero heating time and the directly estimated number in the deposit) generally increased as water level was increased in the nitrogen environment from a low of -1.5 at 5.5 ppm to a high of 0.2 at 870 ppm. Results varied at 13,000 ppm. Estimating regression equations for D-values and intercept ratios were obtained by the method of weighted least squares and combined to form a survivor curve prediction model.

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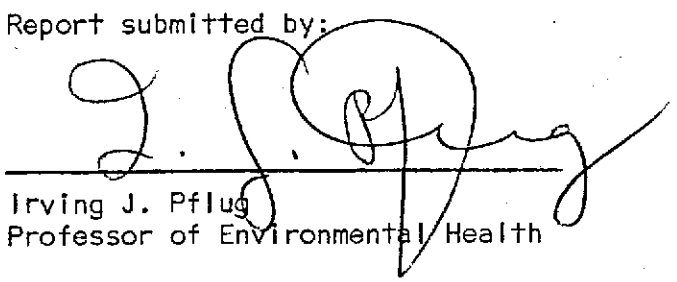

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*Aerobic, Mesophilic Microorganisms - TSA Media, 32°C, 1 Week Incubation

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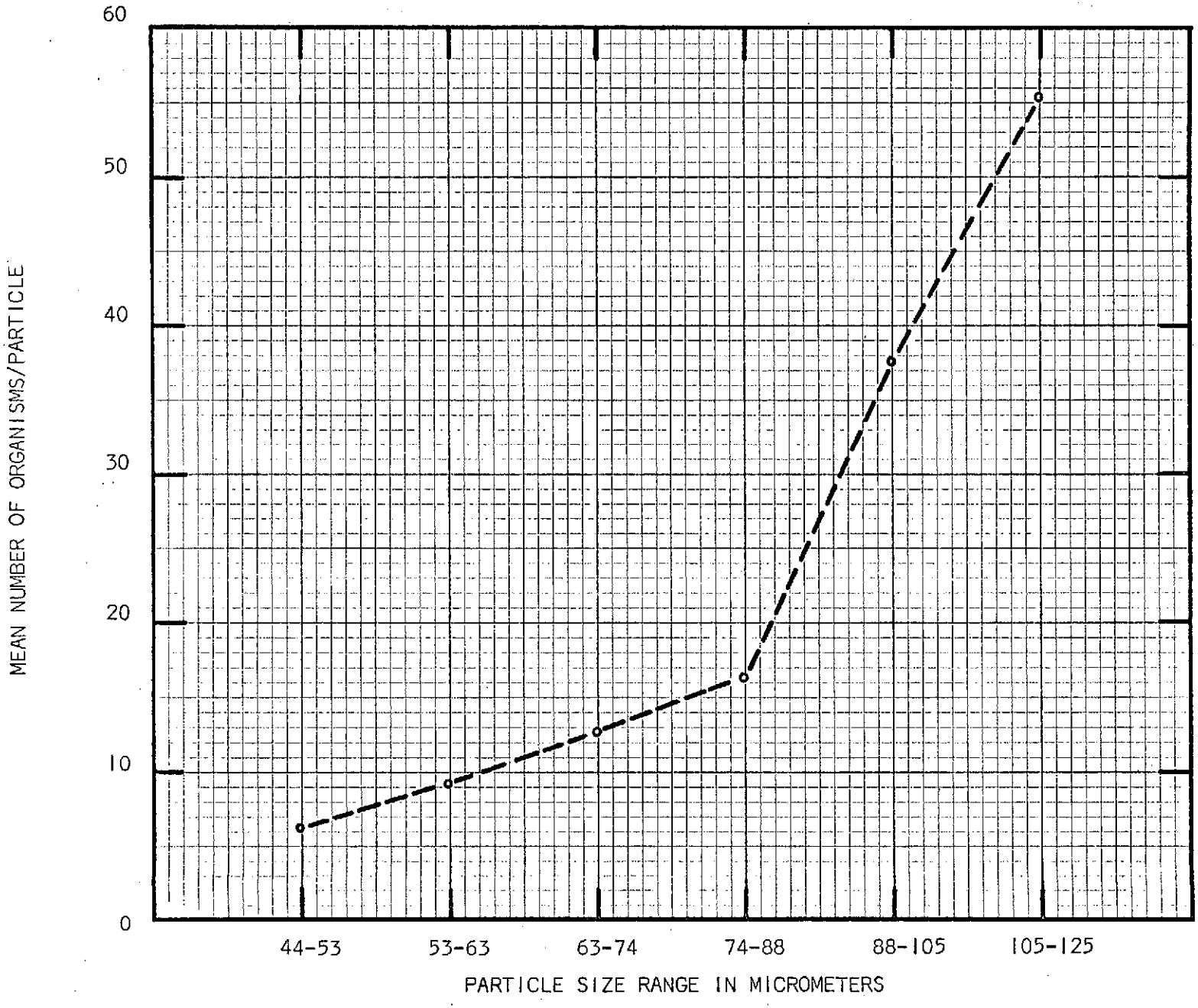

FIGURE 2

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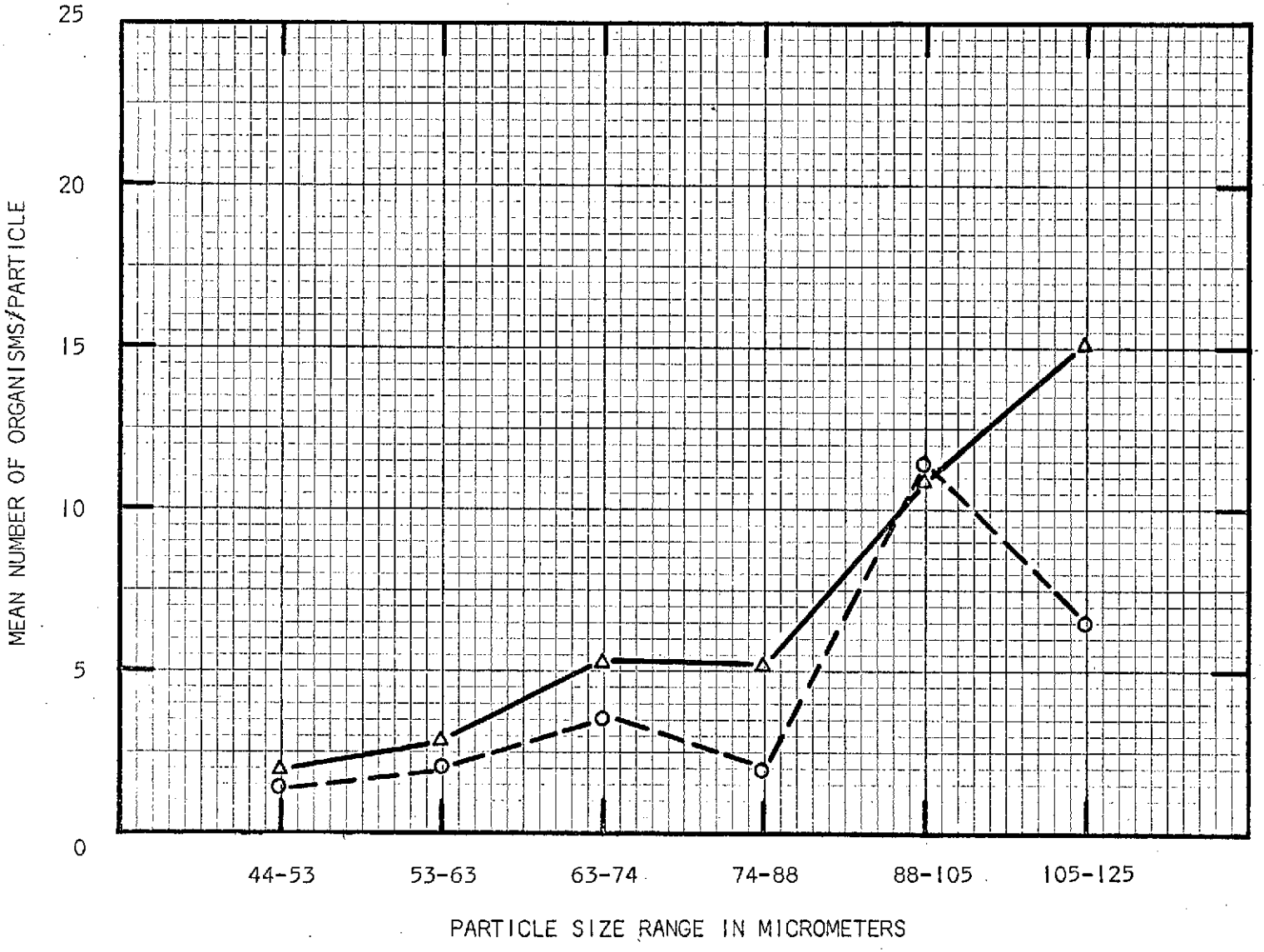

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The data in Figure 2 show the results from analyses of heated soil particles. The microflora detected after wet heat treatment of particles at 80°C for 20 min. are most likely representative of the aerobic, mesophilic spore formers. The trend of these data also shows the continued rise in number of organisms with an increased particle size. Maximum counts of 15 organisms per particle were found for the 105-125 μm soil fraction. This is an average concentration which is over seven times that of the smallest (44-53 μm) particles studied.

The trend of the graph showing the relationship for particles treated with dry heat at 110°C for one hour is not as clearly defined. However, these data also suggest a general increased microbial count per particle for dry heat resistant forms as the particle size range increased.

It was noted in these counts that actinomycetes associated with the soil particles were often able to survive the one hour dry heat treatment at 110°C. However, these organisms apparently were unable to survive the 80°C, 20 minute wet heat since no actinomycete colonies were observed on plates from wet heat treated particles. It appears that soil actinomycetes are somewhat more resistant to dry heat than wet heat.

It should be recognized that these plate count data represent only the aerobic, mesophilic soil microorganisms capable of growth on TSA media at 32°C within one week. Since many aerobic spore formers would be included in this group, the data provide information that may be helpful in establishing sterilization requirements. At the present time enumeration of all the microbes indigenous to a particular soil would be an enormous task that would likely be beyond the capability of most, if not all, general laboratories.

The data in Table II provide a comparison of plate count estimates for the longer stored (WAJJF) and the more recently collected (WAKMF) dark soil particles. These data indicate that the proportion of WAJJF particles retaining viable microorganisms was similar to results for the WAKMF soil. Also of interest was the fact that the older stored soil (WAJJF) yielded a somewhat higher mean count of organisms per particle for all categories of microflora counted. For example, the average count per particle of aerobic, mesophilic microflora for the WAJJF soil was 28 as compared to 16 for the WAKMF particles. For the longer stored WAJJF soil, the mean count per particle after wet heating at 80°C for 20 minutes was approximately 13 organisms. This count is about two times higher than that obtained for WAKMF soil which received the same heat treatment. A similar pattern is evident for the counts of dry heat resistant forms. These results suggest that storage of soils may not necessarily reduce the number of heat resistant microbial forms which are indigenous to a particular soil.

It is not known whether the differences observed in the "new" and stored soil samples are primarily due to differences in microbial populations at the time soils were collected. No information is available on the particle microbial counts at the time of collection at the Cape Kennedy site. However, the plate count data of Table II indicate that particles will retain substantial numbers of viable organisms even under storage conditions and that these numbers are comparable to those found on particles of more recently collected soils.

Table II

Comparison of Old, Stored Cape Kennedy Soil (WAJJF) With More Recent Sample (WAKMF). Plate Count Estimates of Microbial Load and Heat Treatment Effect on Viability of 74-88 μm Dark Particles

TREATMENT	WAJJF SOIL		WAKMF SOIL	
	PROPORTION POSITIVE*	ORG/PARTICLE MEAN AND RANGE	PROPORTION POSITIVE	ORG/PARTICLE MEAN AND RANGE
None	33/37 (0.892)	28.2 (0-194)	37/37 (1.00)	16.2 (1-46)
Wet Heat (80°C, 20 Min)	31/37 (0.838)	13.4 (0-182)	34/37 (0.919)	5.1 (0-35)
Dry Heat	30/37 (0.810)	5.5 (0-35)	27/37 (0.729)	1.9 (0-10)

* Refers to proportion of particles with viable microorganisms.

WAJJF soil was stored in laboratory since September 1971. WAKMF was obtained in June 1973.

CONCLUSIONS

The laboratory studies completed recently with Cape Kennedy soil particles have provided additional data to supplement previous information on microbial concentrations and heat resistance. Results from these microbiological analyses of soil particles allow for the following conclusions:

1. There is a considerable range in the values of aerobic, mesophilic microbial counts associated with different size soil fractions. The range becomes more extended with increased particle size.

2. As the soil particle size increases, there is an increase in the mean microbial concentration per particle.

3. Plate counts of aerobic, mesophilic organisms in unheated soils yielded a mean concentration of about six organisms per particle for the smallest (44-53 μm) soil fraction. The counts increased consistently with particle size to a mean value of approximately 55 organisms per particle for the largest (105-125 μm) soil fraction investigated.

4. Aerobic, mesophilic counts for sonicated particles heated at 80°C for 20 minutes yielded mean values of about two organisms per particle for the smallest particles. The mean concentration for the 105-125 μm particles was approximately 15 organisms per particle. A somewhat similar relationship was noted for counts of organisms resistant to 110°C dry heat for one hour.

5. Some actinomycetes associated with the soil fractions could survive dry heat treatment at 110°C for one hour.

6. Soil particles stored under ambient laboratory conditions for 2.5 years yielded aerobic, mesophilic plate counts which were comparable or slightly greater than the counts for more recently collected soil.

FUTURE WORK

1. Determination of soil particle viability profiles for selected treatments at 110°C.

2. Reproducibility of the viability profiles.

3. Effect of the number of particles on the viability profile. (i.e. -- effect of particle load).

4. Effect of particle type and size on particle viability.

ABSTRACT

The Application of Biometrical Principles in the Study of Dry Heat Destruction of Bacterial Spores

Ronald Leslie Jacobson

Dry heat destruction tests were conducted in an open system using Bacillus subtilis var. niger spores on stainless steel planchets. Survival characteristics were associated with the test conditions of surface temperature and water content in the nitrogen gas environment. Test temperatures were 90 C, 110 C and 125 C while water levels were about 10, 100 and 1000 parts per million (ppm). Results of tests conducted in this system were combined with results obtained using similar test equipment in a laminar down flow clean room containing air with a water content of 13,000 ppm.

Straight line survivor curves were generally obtained for each test when logarithms of the estimated number of survivors were plotted opposite heating times. The logarithms of D-values increased linearly when plotted opposite the logarithms of ppm of water. $D_{110\ C}$-values ranged from 21 minutes at 5.5 ppm of water to 265 minutes at 13,000 ppm. Intercept ratios (logarithm of the ratio of the estimated number of spores in the innoculum obtained by extrapolating the regression line survivor curve to zero heating time and the directly estimated number in the deposit) generally increased as water level was increased in the nitrogen environment from a low of -1.5 at 5.5 ppm to a high of 0.2 at 870 ppm. Results varied at 13,000 ppm. Estimating regression equations for D-values and intercept ratios were obtained by the method of weighted least squares and combined to form a survivor curve prediction model.