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Services Provided in Support of the Planetary Quarantine Requirements of the National Aeronautics and Space Administration under Contract R-137

N68-27647
(ACCESSION NUMBER) (THRU)
15 (PAGES) 1 (CODE)
CR-95555 (NASA CR OR TMX OR AD NUMBER) 04 (CATEGORY)
FACILITY FORM 602

Report No. 22
April - June 1968

Conducted by

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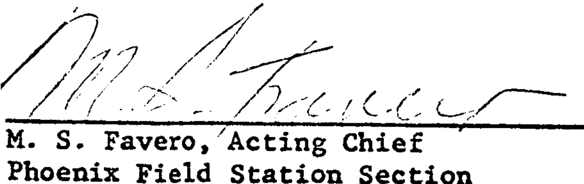
GPO PRICE $ ____
CFSTI PRICE(S) $ ____
Hard copy (HC) 3.00
Microfiche (MF) .65
ff 653 July 65



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July 1968

1. To further evaluate the Sandia Corporation's vacuum probe for sampling surfaces a technique was developed for producing a uniform surface deposition of dust particles contaminated with microorganisms. One gram of sterile aluminum oxide particles having a mean diameter of 10 μ was thoroughly mixed with 1 ml of 95% ethanol containing 1×10^8 spores of Bacillus subtilis var. niger and allowed to dry in a laminar flow clean bench. A 0.1 g portion of the inoculated powder was placed in a plastic 25 x 100 mm centrifuge tube in which three 3/8 in. diameter holes had been drilled through the tube wall 120° apart and 3/4 in. from the tube bottom. The top of the tube was connected to a compressed air supply (70 psi) using a rubber stopper, and the tube was inserted into the top of a 21 x 16 x 11 in. aerosol chamber. A 2 second blast of compressed air dispersed the dust in the chamber, and after a 4 minute calming period stainless steel strips placed on the bottom of the chamber were exposed to the settling dust particles for periods of 1 - 3 minutes. The coefficient of variation observed in the deposition of spores on the exposed strips was consistent from batch to batch and averaged 16% compared to a figure of 60% usually observed in natural microbial deposition studies with naturally occurring airborne microorganisms in this laboratory.

Using procedures described earlier and improved models of the sampling probe provided by Sandia Corporation, recovery of spores from strips was compared with recovery using the standard strip assay procedure. The results of four such experiments are presented in Table 1. Although the probe consistently removed 99% of the spores from the strips, recovery of these spores averaged only 84% of the number recovered using the standard strip assay procedure. The coefficient of variation between strips using the probe was found to be 19% compared to 16% for the standard strip assay procedure.

While no apparent explanation existed for the lower recovery rate observed for the probe it was determined that the probe provided a fairly consistent relative measure of the number of spores on strips contaminated in this manner. Further experiments utilizing aerosols of naturally contaminated dust as well as experiments in actual spacecraft assembly environments at Cape Kennedy are planned.

2. Polyethylene mailing tubes designed for shipment of microscope slides were obtained to determine their suitability for the shipment of inoculated 1 x 2 in. stainless steel strips. Shipment of such strips without alteration of the inocula is necessary for both the conduction of a split-sample program as well as the evaluation of the effectiveness of ultrasonic baths in other laboratories. Because polyethylene does not withstand autoclaving the tubes were sterilized in ethylene oxide (ETO) prior to use. The tubes were uncapped, exposed to ETO (600 mg/L) for 16 hours, recapped and stored in a laminar flow clean bench for 2 weeks. Identical amounts of B. subtilis var. niger spores suspended in 95% ethanol were placed on 40 sterile stainless steel strips and desiccated for 16 hours. Ten strips were retained in petri plates as controls while the remaining 30 strips were placed in 6 mailing tubes, each containing 5 strips. Four tubes were mailed to cooperating laboratories and 2 tubes were retained.

Twelve days after being placed in the petri plates and tubes all strips were removed and assayed. The results were quite instructive and are presented in Table 2. It was found that the mean inoculum of 191 spores (based on 10 controls in petri dishes) was, in most cases, decimated while in the polyethylene mailing tubes. The reduction in the number of spores on the strips in the tubes was attributed to residual off-gassing of ETO from the polyethylene. However, no apparent explanation existed for the significant survival of spores in tubes 3 and 6.

The experiment was repeated using polyethylene mailing tubes sterilized by immersion in 2.0% peracetic acid solution for 20 minutes. These results are presented in Table 3 and indicate the acceptability of the mailing tubes for shipment of inoculated strips when the tubes are properly sterilized in peracetic acid.

3. Attempts were made to develop a model system for studying the probability of release of microorganisms from solids as the result of erosion by airborne abrasives. Methyl methacrylate was selected as the model solid because it (1) simulates the type of non-metalic materials likely to be found on spacecraft (2) can be experimentally contaminated with spores and (3) can be quantitatively assayed for spore concentration by dissolution in acetone. Unfortunately, methyl methacrylate is not easily eroded. An industrial air abrasive unit was found to be ineffective in an attempt to experimentally erode this plastic. Bombardment of the plastic with various types of abrasives indicated that in order to achieve significant erosion in a reasonable period of time, unacceptable temperatures were generated negating the simulation of natural erosion. A search for an acceptable experimental model will be continued.

4. Studies to obtain comparative D_{125C} values of soil suspensions and spore isolates from soil suspensions were continued in order to observe the effects of subculture upon dry heat resistance. As reported earlier (Report Nos. 19, 20, and 21) spore crops derived from soil suspensions appeared to have lost much of their heat resistance upon subculture. No isolate having a D_{125C} value equal to or greater than its parent soil had been observed. It was thought that if this loss in heat resistance by "naturally occurring" spores upon subculture was found to be somewhat constant or predictable, more reliance could be placed on D_{125C} values obtained from sporeformers isolated from space hardware that are subcultured and subsequently sporulated, harvested and cleaned.

   In order to obtain additional data concerning the effects of subculture on the dry heat resistance of naturally occurring spores several soil suspensions exhibiting diphasic dry heat survivor curves were studied. Also included in these dry heat studies were sporeformers isolated from space hardware while in residence at Cape Kennedy. These cultures were maintained on TSA slants until spore crops were produced.

   In the case of soil suspensions, 0.05 ml portions were applied to 1/2 x 1/2 in. stainless steel strips and exposed to dry heat in the manner described previously (Report No. 19). Each soil suspension was exposed to 125 C for 30 minutes on strips inoculated in triplicate. This time interval was selected for these particular suspensions because 30 minutes

was well into the second phase of the diphasic survivor curves. Each strip was placed in 10 ml of buffered distilled water and insonated for 12 minutes in an ultrasonic bath. Varying amounts of rinse fluid were plated with TSA, overlayed, and incubated at 32 C until colonies could be differentiated (usually within 48 hours). Colonies were picked and streaked on TSA for isolation of pure cultures. Cultures were maintained at 4 C on TSA slants and were tested for maximum spore production on six different media incubated at 32 C for 48 hr. The media tested were:

a) Nutrient Agar (Difco) supplemented with 80 ppm calcium chloride and 20 ppm magnesium sulfate,

b) TAM Sporulation Agar (Difco) supplemented with 80 ppm calcium chloride and 20 ppm magnesium sulfate,

c) TAM Sporulation Agar prepared with a boiling water-soil filtrate,

d) A-K Medium #2 (Difco) supplemented with 80 ppm calcium chloride and 20 ppm magnesium sulfate,

e) Tomato Juice Agar (BBL),

f) Charcoal Agar (Difco).

There was much variability in growth and sporulation of each isolate among the six media as well as among the isolates tested on the same media. TAM Sporulation Agar and A-K Medium #2 were the superior media in terms of growth and spore production in the majority of the 47 isolates tested. In many instances, however, good growth of an isolate was not necessarily accompanied by good sporulation. Each isolate usually showed a marked preference for a particular medium with respect to maximum spore production. To date, a total of nine spore crops of soil and spacecraft isolates have been tested for dry heat resistance at 125 C. Table 4 shows D_{125C} values of the spore crops, culture medium used, and comparative D_{125C} values of naturally occurring spores in soil where applicable. Contrary to past observations, four of the five soil isolates had D_{125C} values equal to or greater than those of naturally occurring spores in the parent soils. Two explanations could be given: First, highly heat resistant spores could have been present in the soils in numbers far too low to drastically effect D_{125C} values based on 30 minutes of heating time. Secondly, the spores gained rather than lost heat resistance upon subculture. Even though the former explanation appears to be more attractive than the latter, further investigation into the effects of subculture upon heat resistance of spores is indicated. Nevertheless, even this small sampling clearly shows that spores of *B. subtilis* var. *niger* with a D_{125C} value of approximately 15 minutes is not a "worst case condition". Whether it represents the average or "typical" heat resistance of spores found on spacecraft also is questionable.

In order to test the effects of different culture media upon the heat resistance of a particular isolate, spore crops of *B. subtilis* var. *niger* and isolate XA (Report No. 21) were prepared on four different media employing an active culturing technique. The media used were TAM supplemented with

80 ppm calcium chloride and 20 ppm magnesium sulfate, TAM supplemented with soil filtrate, A-K Medium #2 supplemented with 80 ppm calcium chloride and 20 ppm magnesium sulfate and Charcoal Agar. Cleaned ethanol suspensions of the spore crops were placed in 0.05 ml amounts on 1/2 x 1/2 in. stainless steel strips and assayed for dry heat resistance at 125 C in the manner described earlier (Report No. 19). Results of this study are presently incomplete, but preliminary data suggest no major changes in D_{125C} values from medium to medium. Complete results will be presented in the following Quarterly Report.

In order to assess the degree of heat resistance of naturally occurring anaerobic spores in soil several experiments were performed using soil sample X (Report Nos. 19, 20, and 21). The tests were performed in the same manner described in earlier reports except that all plates were placed in a Brewer anaerobic jar for incubation at 32 C for 72 hours. Survivor curves for naturally occurring anaerobic and aerobic spores in the X-soil sample at 125 C are shown in Figure 1. The slopes of both the first and second phases of the survivor curves are quite similar indicating that the majority of the spores are probably faculative with respect to oxygen requirements. To substantiate this observation, representative colonies of dry heat survivors will be isolated in pure culture and incubated under aerobic and anaerobic conditions. Results will be reported later.

5. A preliminary study was initiated to test survival of viruses in relation to bacterial spores under anaerobic conditions at 25 C and 4 C. One set of 1/2 x 1/2 in. stainless steel strips was inoculated with 0.05 ml of a $TCID_{50} = 10^{4.5}$/ml suspension of ECHO 11 virus in Basal Medium Eagle's (BME) and another set was inoculated with an ethanol suspension of _B. subtilis_ var. _niger_ spores (2.6×10^5/ml). All strips were allowed to air dry in a vertical laminar flow clean bench. Six virus and six spore strips were used for immediate controls: the virus strips were suspended in tubes of 2.5 ml BME and the spore strips in 10 ml of buffered distilled water. The tubes were insonated for 12 minutes in an ultrasonic bath to remove the inocula from the strips and to break up clumps of microorganisms. The spores were diluted appropriately in buffered distilled water and plated in triplicate with TSA while the $TCID_{50}$ of the virus per strip was titered in continuous human embryonic kidney cell culture. To test survival in an anaerobic environment triplicate samples of the spore strips along with the virus strips were placed in Brewer anaerobic jars and stored for periods of 1, 2, 4, and 6 weeks at both 4 and 25 C. Aerobic controls in petri plates were included for the virus strips at both temperatures. At the indicated time intervals, triplicate samples from each exposure condition were taken and assayed in the same manner as the immediate controls. Results showed negligible change in the concentration of spores per strip at both temperatures over the sampling period, while the virus $TCID_{50}$ per strip was lowered 4 logs during the drying procedure. After one week no viruses were detected on strips stored aerobically. Low levels of virus persisted during anaerobic storage throughout the sampling period with only one strip of a triplicate sample, either at 4 or 25 C, being positive ($TCID_{50} \leqslant 10^{0.5}$). Subsequent studies of a similar nature will be conducted employing a more resistant virus as well as bacteriophage.

6. Microbiological studies were initiated on the Apollo 7 Command and Service modules (CSM-101). Microbial contamination isolated from these surfaces by use of the swab-rinse method are shown on Table 5. The exterior surface of the command module was not studied, because it was covered with a metallic-mylar film. Samples will be taken when the film is removed. Due to the configuration of the service module, both vertical and horizontal surfaces were studied. Results confirmed previous studies which indicated that vertical surfaces do not accumulate as high a level of viable microbial particles as horizontal surfaces.

   Table 6 shows the types of aerobic mesophilic microorganisms isolated from the exterior surface of the Apollo 6 command module. A total of 1330 aerobic mesophilic microorganisms were isolated from the command and service modules of the Apollo 6 spacecraft. These will be identified and the results reported at a later date.

   Sites for microbiological sampling on the surface of the Lunar Module-3, ascent (interior and exterior) and descent stages were selected. The results obtained from this study will be reported in the next quarter. Microbiological studies of the SIV-4 and Instrument Unit (IU) surfaces will be performed as soon as authorization is obtained from the respective contractors.

   Levels of microbial contamination in the intramural environments of the Manned Spacecraft Operations Building and Back Pack Life Support Clean Room and the Spacesuit Clean Room were continued. Figure 2 shows the results obtained in the Back Pack Life Support and Spacesuit Clean Rooms. Results obtained from studying the Lunar Module (LM) descent and ascent stage work stands and the Command Module (C/M) and Service Module (S/M) work stands are shown in Figure 3. The increased activities in the C/M and S/M work stands could account for the high level of microbial contamination observed in these areas. Levels of microbial contamination accumulating on stainless steel strips exposed in the Integrated Test Stand #2 are shown in Figure 4.

   Modification of the rooms in the Vehicle Assembly Building (VAB) which are being converted into a microbiological assay laboratory and modification of the Trailer Laboratory are progressing and should be completed by the next quarter.

7. All construction and modification of the bioclean room has been completed. The room has undergone a final cleaning and decontamination, has been furnished and is now in operation. Measurements of air and surface microbial contamination in the unoccupied room are presently being made.

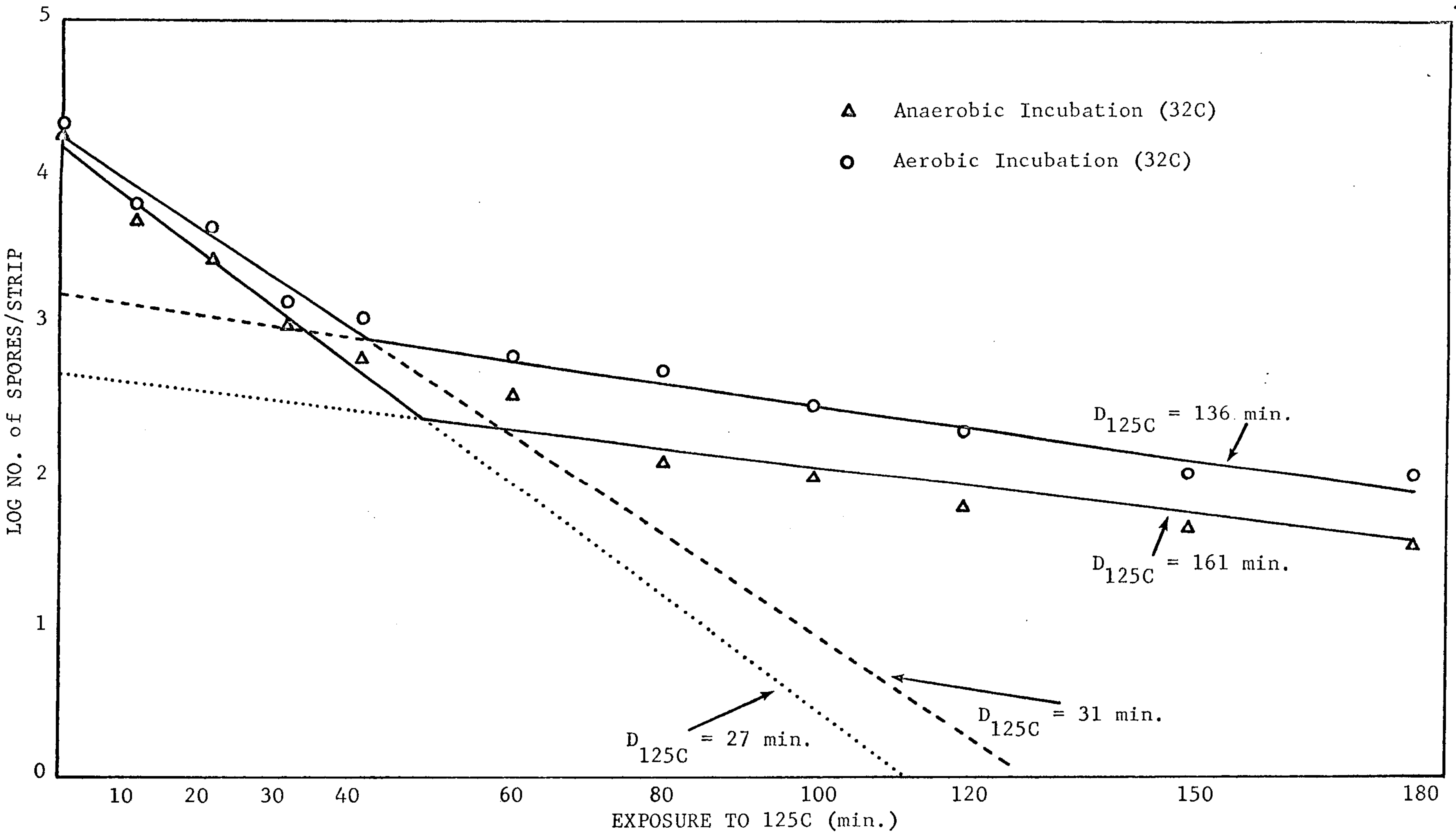

Figure 1. DRY HEAT INACTIVATION OF SPORES IN X-SOIL AT 125C CULTURED AEROBICALLY AND ANAEROBICALLY

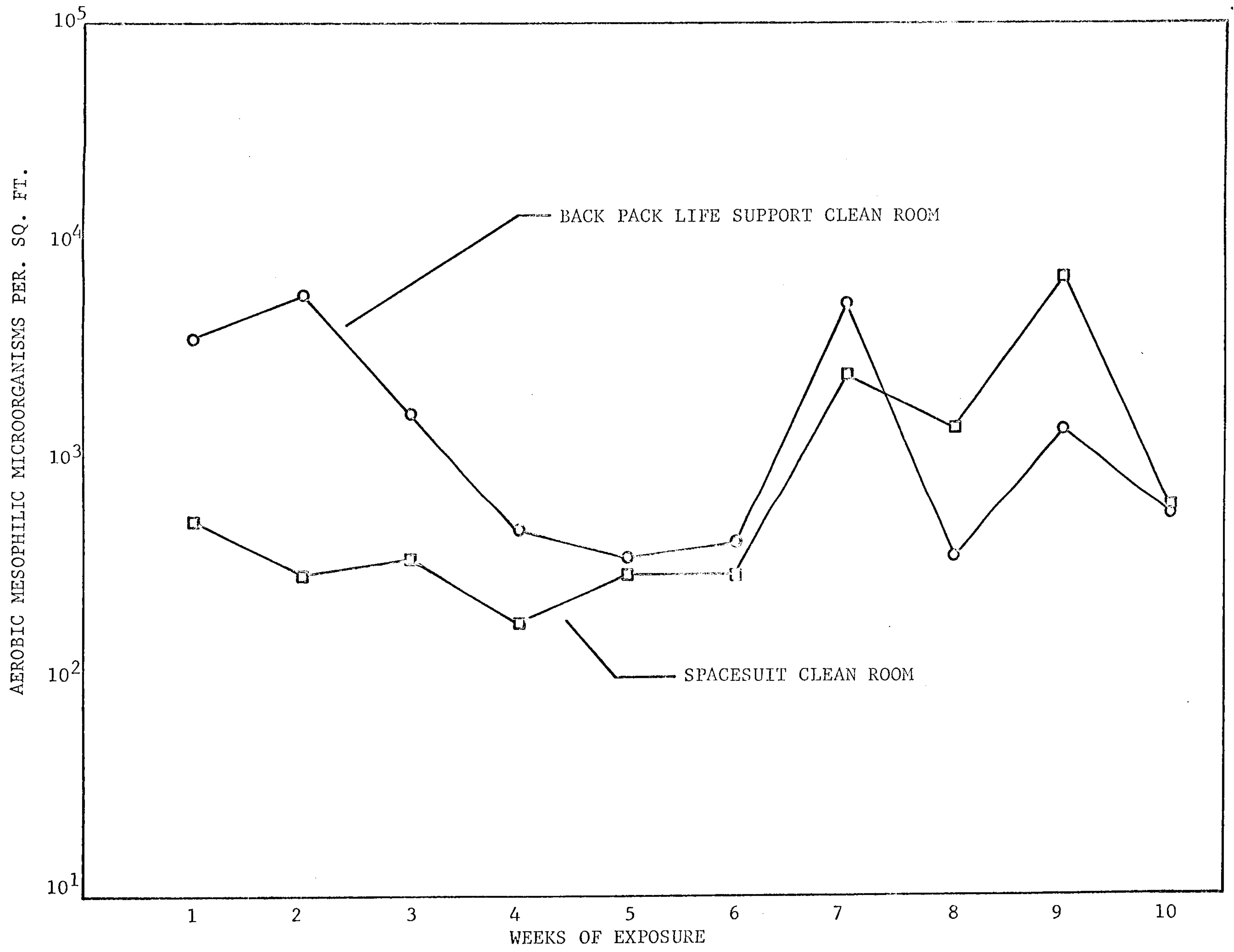

Figure 2. FALLOUT CONTAMINATION IN THE BACK PACK LIFE SUPPORT AND SPACESUIT CLEAN ROOMS

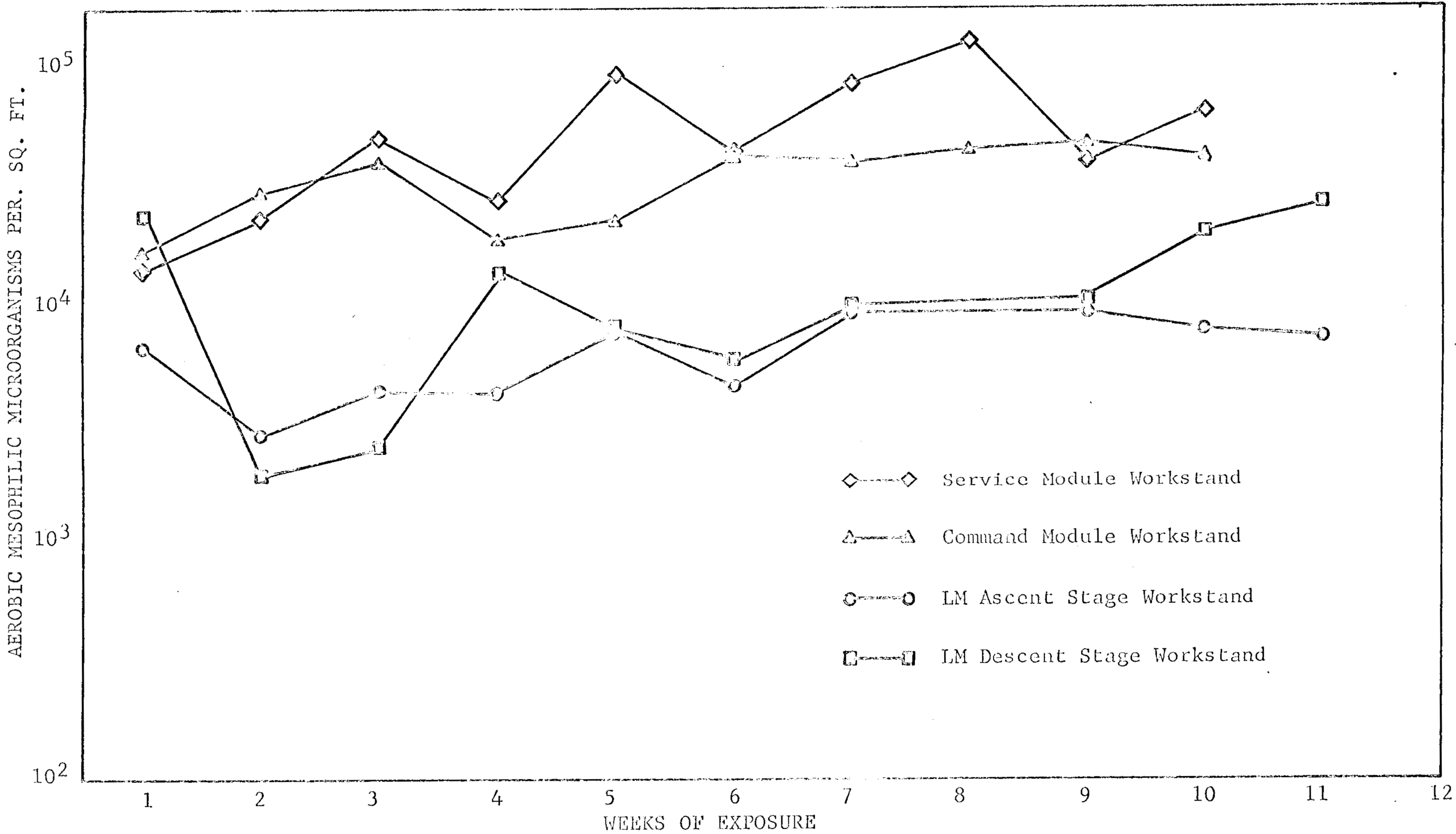

Figure 3. FALLOUT CONTAMINATION IN FOUR APOLLO AREAS

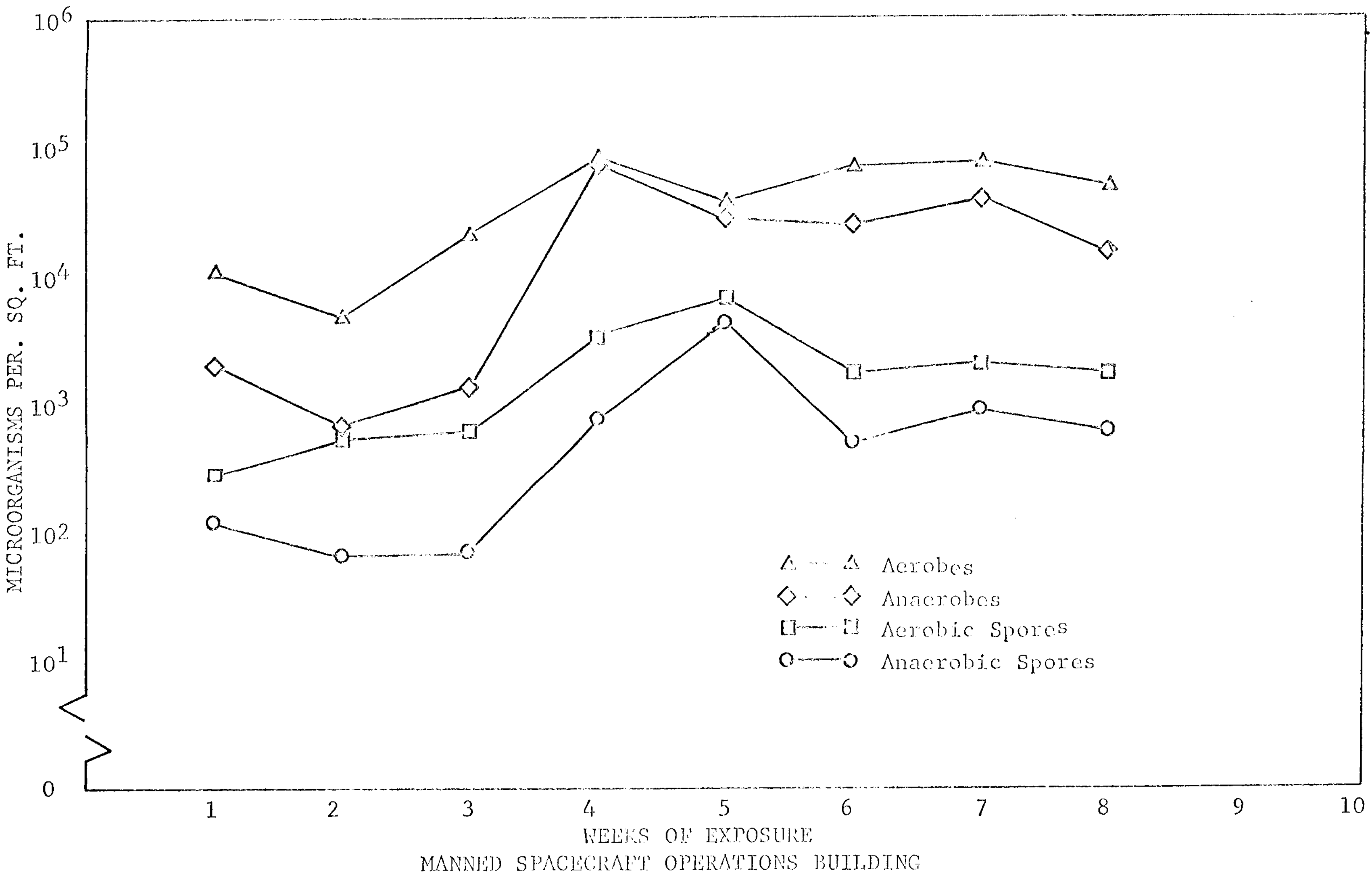

Figure 4. FALLOUT CONTAMINATION IN THE AREA OF THE INTEGRATED TEST STAND #2

TABLE 1. COMPARISON OF THE STANDARD STRIP ASSAY PROCEDURE WITH THE VACUUM PROBE IN RECOVERY OF *B. SUBTILIS* VAR. *NIGER* SPORES FROM SURFACES OF STAINLESS STEEL FALLOUT STRIPS

Experiment	No. of Strips	Mean No. Microorganisms Recovered		% Recovery
		Std. Assay	Probe Assay	
1	20	426	372	87
2	18	135	110	82
3	27	212	169	80
4	25	459	383	83
Overall	90	313	262	84

TABLE 2. EXPOSURE OF INOCULATED STAINLESS STEEL STRIPS IN POLYETHYLENE TUBES STERILIZED IN ETO

Strip No.	Number of Microorganisms Recovered from Strips						
	Petri Plate Controls	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6
1	232	2	0	42	1	0	108
2	218	3	0	34	2	0	128
3	186	0	0	31	1	0	102
4	157	0	0	44	1	0	95
5	198	0	0	26	0	0	136
6	211						
7	166						
8	194						
9	168						
10	184						
Average	191	1	0	35	1	0	114

TABLE 3. EXPOSURE OF INOCULATED STAINLESS STEEL STRIPS IN POLYETHYLENE TUBES STERILIZED IN 2% PERACETIC ACID SOLUTION

Strip No.	Number of Microorganisms Recovered from Strips						
	Petri Plate Controls	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6
1	361	329	337	339	308	312	328
2	366	421	334	362	349	305	312
3	309	326	344	311	364	360	322
4	321	287	330	315	302	361	316
5	336	357	366	323	334	316	332
6	360						
7	344						
8	325						
9	324						
10	385						
Average	343	344	342	330	331	331	322

TABLE 4. DEATH RATE OF SPORE ISOLATES FROM SOIL AND SPACECRAFT AT 125 C

Isolate	Source	Sporulation Medium[1]	D_{125C} (min)	D_{125C} of 2nd Phase, Parent Soil Suspension (min)[2]
G-2	G-soil (McDonnell, St. Louis)	A-K #2	109	38.
J-1	J-soil (Langley R.C., Hampton, Va.)	A-K #2	49	36.
J-2	J-soil (Langley R.C., Hampton, Va.)	A-K #2	36	36.
M-1	M-soil (Marshall S.F.C., Huntsville, Ala.)	A-K #2	73	42.
XA	X-soil (Phoenix)	TAM	56	126.
CK-2	Surveyor #6 (Cape Kennedy)	TAM	15	--
CK-4	Surveyor #6 (Cape Kennedy)	TAM	46	--
CK-6	Surveyor #6 (Cape Kennedy)	A-K #2	8	--
CK-10	Surveyor #6 (Cape Kennedy)	A-K #2	10	--

[1]Both media were supplemented with 80.0 ppm calcium chloride and 20 ppm magnesium sulfate.

[2]The D_{125C} value given is the largest value exhibited by the diphasic soil. See Report No. 20.

TABLE 5. LEVELS OF MICROBIAL CONTAMINATION DETECTED ON THE APOLLO COMMAND AND SERVICE MODULES (CSM-101)

Source	Date	Area Sampled (sq. in.)	Microorganisms per Square Inch			
			Aerobes	Anaerobes	Aerobic Spores	Anaerobic Spores
Command[1] Module (Interior)	6-6-68	120	85.50	40.88	0.20	0.17
Service[1] Module (Exterior)	6-3-68	80[2]	17.38	3.06	0.94	0.50
		60[3]	3.50	2.67	0.67	0.00

[1] Samples taken while Command and Service Modules were located in the Manned Spacecraft Operations Building.

[2] Horizontal surface.

[3] Vertical surface.

TABLE 6. TYPES OF AEROBIC MESOPHILIC MICROORGANISMS ISOLATED FROM THE EXTERIOR SURFACE OF THE APOLLO 6 COMMAND MODULE

Types of Microorganisms	Number	Percent
Staphylococcus epidermidis	67	35.1
Staphylococcus aureus	3	1.6
Micrococcus spp.	33	17.3
Bacillus spp. (sporeformers)	6	3.1
Brevibacterium-Corynebacterium group	13	6.8
Gram negative bacilli	10	5.2
Molds	48	25.1
Yeasts	5	2.6
Actinomycetes	2	1.0
Miscellaneous	4	
Total	191	