METHODOLOGY OF MEASURING INTERNAL CONTAMINATION IN SPACECRAFT HARDWARE

V. W. Greene, Ph.D.

Bailus Walker, Jr., M.P.H.

Orin A. Anderson, B.A.

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I Background Information

A. Quantitative Microbiology and Sterilization Guidelines

Accurate and reproducible techniques for measuring the viable microbial contamination of spacecraft hardware are essential to the success of the planetary quarantine program as it is presently envisioned. In order to prescribe any realistic sterilization treatment, it is necessary to know, among other things, both the total contamination load and the destruction kinetics (i.e., "D" values) for the most resistant organisms under the given environmental conditions. The validity of either of these estimates (i.e., total load and "D" values) can be no more reliable than the accuracy of the microbiological assay techniques employed in their determination. Techniques which consistently underestimate the true count might lead to unwarranted confidence in a given sterilization treatment; techniques which consistently overestimate the true count might lead to the imposition of an unnecessarily severe sterilization treatment (leading to unnecessary constraints on material selection and design); techniques which are accurate for certain contamination levels but inaccurate for others will lead to false "D" values; and techniques which are basically inconsistent lead to questions about the validity of the whole program.

B. Drawbacks of Classical Methodology

The consequent need for accurate and reproducible methods in quantitative microbiology is faily well recognized and understood. However, the inadequacy of existing assay methods is not as well appreciated. Historically, quantitative microbiology as it concerns "sterility" evaluation has been the concern of hospital laboratories, food microbiologists, and environmental hygienists.

During the past fifty years, workers in these disciplines have developed for their specific needs a number of quantitative assay methods, which, for the most part, are either technically inappropriate or insufficiently precise for NASA's purposes. To start with, nearly all good bacteriological enumeration methods are designed for assaying inert liquids or smooth surfaces. Some work, of course, has been done with air and powders, but most microbiologists admit that the accuracy and reliability of enumeration techniques for systems other than liquids and surfaces leave much to be desired. Secondly, most existing techniques are designed for homogeneous materials, or materials which can be made homogeneous (i.e., by shaking, blending, or grinding). The desire to assay dozens of different materials independently (such as metals, adhesives, coatings, lubricants, fuel and encapsulants) with equally reliable techniques and then adding the counts to get a valid estimate of total load is beyond both the technology and statistics of current microbiology. Finally, microbiological assay techniques have never really been needed for the interior of any solid harder than cheese or muscle tissue; thus, any questions NASA workers might have about interior contamination of spacecraft hardware could not be properly answered with existing methodology.

C. NASA Standard Procedures Manual

Despite these difficulties, considerable progress has been made toward the adaptation of several classical enumeration techniques for NASA's purposes. Indeed, a standard procedures manual[1] has been prepared which outlines a number of useful and reliable techniques for air sampling, surface sampling and assessment of interior contamination of small piece parts.

This manual is being regularly upgraded, and has gone far to standardize methodology, particularly in the area of surface and air contamination. There is still some question, however, whether the techniques described in the manual are adequate for interior contamination assay.

D. Importance of Internal Contamination

Admittedly, surface contamination is the greatest concern to NASA, since these organisms have the greatest chance of being liberated to contaminate a planet. However, the planetary quarantine program is also concerned with "interior" contamination which can be liberated by solids fracturing during a hard impact or by abrasive erosion of components after landing. This concern is demonstrated by the current policy choice of dry-heat sterilization (a treatment which penetrates to the interior of the smallest and most remote component) in distinction to some less drastic sterilization treatment (e.g., ethylene oxide or autoclaving) which is essentially nonpenetrating.

Consequently, if total microbial load need be known and if "D" values must be determined for interior contaminants, assay methods for interior contamination should be as accurate as those for the more accessible surface contaminants. Conversely, if the interior assay methods are not as accurate, some idea should be gained about the extent of their inaccuracy, or at least about inherent reproducibility. It is in this area that a great gap in our knowledge exists.

E. Interior Contamination Assay Problems

The limitations of interior contamination assay procedures exist only in part because of historical unprecedence. The subject is quite complex and is permeated with microbiological and technological difficulties. For

example, the very diversity of materials which must be assayed presents significant problems. The surfaces of such materials as Lucite, epoxy, and silicone rubber can be sampled by one single and reliable method. However, the need to "break up" each of these materials (in order to release any embedded microorganism) before culturing might involve as many solid reduction techniques or solvents as materials being assayed. A further drawback is introduced by the potential bactericidal action of the solid reduction process, such as heat liberated during pulverization, or by toxic solvents. Still further, the problem of culturing a few organisms in a milieu of potentially bacteriostatic polymers continually leads to suspicion of negative or "sterile" results.

Some of these considerations are well covered in the standard NASA procedures for piece part interior contamination. However, another complexity should also be recognized. All too frequently, the term "interior contamination" is used to imply a single subject entity. Actually, it is possible to identify at least three different types of "interior contamination" categories, each related to each other, but each presenting its own unique challenge to microbiological assay:

a. Interior Contamination of Piece Parts

   This is the subject dealt with in the NASA standard procedures manual. Piece parts are the primary units of hardware such as transistors, capacitors, resistors, diodes, etc. Those which are not subjected to a sporicidal stress during or after manufacture might very well be internally contaminated.

b. Occluded Contamination

   During assembly of components or modules, any number of coatings, encapsulants and adhesives are used which may themselves be con-

taminated or which may cover surface contaminants. When the coatings harden, the contaminants become "internal" (i.e., not amenable to surface assay and surface sterilization techniques). It is quite possible to assemble a circuit board using internally sterile piece parts, (and to assume that the only contamination is "surfaceborne") only to find that the hitherto "surface" contaminants are now embedded in potting compound and have thus become occluded or internal.

c. Inaccessible Contamination

This type of contamination is also originally surfaceborne, but becomes inaccessible to surface assay or surface sterilization treatments when two surfaces become intimately pressure mated, (e.g., microorganisms on the threads of a bolt screwed tightly into its receptacle, or two flat contaminated surfaces securely clamped together). Inaccessible contaminants are never truly "embedded" (thus differentiating them from "a" and "b"), but cannot be considered surface contaminants from the viewpoint of assay or nonpenetrating lethal treatment.

(These categories are not only pertinent to problems of microbiological assay and sterilization but obviously also pertain to probability of contamination release and perhaps to types and numbers of contaminants present. However, for the purpose of this report, they will be considered only in the light of microbial quantitation.)

The above-mentioned illustrations, dealing with categories of internal contaminants, the diversity of materials potentially contaminated, and the problems of viable recovery and culturing, emphasize the need for further

work in the field of internal contamination assay methodology in order to determine realistically the total loading and true "D" values of microorganisms associated with space hardware.

F. Literature Review

The available literature indicates that four groups of researchers have been prominent in the field of internal contamination methodology in recent years. The Ft. Detrick workers[2,3,4,5] assayed electron tubes, condensers, resistors, diodes, coils, capacitors, thermistors, connectors, cores and chokes by pulverizing the piece parts into sterile broth under aseptic conditions. The solid reduction techniques involved hammers, saws, files, and mortar and pestle. This work was not really a methodology study as much as a survey of shelf items; thus, it created an awareness seven years ago of the potential problem, but did not systematically investigate methodology.

The JPL workers and their subcontractors in the Dynamic Science Corporation[6,7] defined both the microbiological and engineering problems of interior contamination assay. They used ball mills, blenders, drills, mortar and pestle, microtomes, abrasive devices and saws to liberate embedded spores from paraplast, parlidion, plaster of paris, and a number of polymer coatings and encapsulants. They concluded that in the absence of a suitable, nontoxic solvent, sawing is one of the best solid reduction techniques and that great care must be taken to neutralize the toxic residues of epoxies during culturing.

The CDC-Phoenix workers[8,9,10,11] studied the lethal effects of pulverization (by mortar and pestle, and blender mill) of B. subtilis spores embedded in plaster of paris, in dental inlay material and in paraplast. Good recoveries (70 - 95%) were observed in fresh samples, suggesting no lethal pulverization effects; however, die away of organisms occurred in all solids after storage.

A number of solvents were tested for sporicidal effect. It was found that acetone, dimethylformamide, ethanol, methanol, isopropyl alcohol and pyridine exerted no adverse effect on viability after three weeks' storage. On the other hand, benzene, chloroform, carbon tetrachloride, diethyl ether, freon, toluene, tetrahydroduran and xylene could be used only for brief exposure periods. Ethylene diamine was rapidly toxic. Further studies on artificially contaminated methyl methacrylate (Lucite) showed a considerable decrease in viable count during polymerization and a "slower but still significant" die away during subsequent storage, depending on time and temperature. Contrary to their experience with plaster of paris, pulverization of Lucite in a blender mill for one minute destroyed 85% of the inoculated spores. Furthermore, at least five minutes of grinding was needed to release all of the spores from the Lucite matrix, and this treatment was more lethal than the one minute grinding.

The Robert A. Taft Sanitary Engineering Center investigated a number of methodological problems during their study of destruction kinetics of spores embedded in different solids.[12,13,14,15,16] They were able to recover good yields of inoculated spores from balsa wood ($\sim$100%) after maceration in a Waring Blendor, and from Lucite (40-100%) after dissolution in acetone, in marked contrast to the experience of the Phoenix group. A method was developed to recover embedded spores from epoxy resin by grinding in a modified Waring Blendor with a silicone carbide grinding disc, and this technique permitted them to ascertain that the "D" value of spores embedded in different solids might vary as much as 100%. As a sidelight to their work, they established a standard technique for measuring toxicity of different plastics and showed that certain of these polymers exert a far greater effect on viable recovery than any of the recovery methods per se.

G. Statement of the Problem

In the preceding discussion, the complexity and importance of interior contamination assay methodology was pointed out:

a. Interior contaminants contribute to the total viable load of the spacecraft.

b. The destruction kinetics of interior contaminants might be different from surface contaminants.

c. Although the risk of interior contamination release is not known, their numbers and "D" values must be known before a sterilization treatment can be certified as valid (unless the probability of release $\longrightarrow$ 0).

d. The accuracy of estimating interior contamination load and "D" value is a function of the accuracy of the assay method.

e. Considering the diversity of materials which might contain embedded microorganisms and spores, it is difficult to visualize a single assay method that will be universally applicable. Rather, a number of methods might have to be employed for interior assay.

f. Unless perfectly reliable methods are available to assay each type of material that can harbor internal contaminants, the reliability or precision of the best method employed for each must be determined.

g. The most recent NASA standard procedures for microbiological assay provide no guidelines for interior contamination methodology beyond the piece part level. Since contamination can feasibly be introduced during further assembly steps, methods must be available to cope with this occluded and inaccessible contamination.

Consequently, a research program was undertaken in the School of Public Health of the University of Minnesota to attempt to answer some of these questions. Specifically, the following problems were attacked:

1. What are the most appropriate methods for recovering inoculated spores from a number of different solids? What kind of reproducibility and precision can be obtained with these methods?
2. What are the effects of surface sterilization treatments on occluded contamination?
3. How long do occluded contaminants survive under different storage conditions?

Concomitantly, a number of other side issues were approached, such as the probability of release of embedded spores during impact, and the optimal methods of artifically inoculating and culturing certain solid materials. Some of this work duplicates and overlaps with the efforts of the Phoenix and the Taft Center workers, since it was conducted simultaneously. Nonetheless, in this area of very little knowledge, corroboration and reinforcement might be welcome.

The research project, summarized in this report, was initiated in February 1966 and was terminated in April 1967. It represents 20 man months of effort.

II Experimental Work

A. Materials Studied

During the course of this program, six different polymer materials were employed as model systems:

1. Paraffin - a solid paraffin wax (MP 50-55 C), insoluble in water and alcohol, but soluble in benzene and ether.
2. Paraplast - a histological embedding compound (MP 61-62 C), obtained from Fisher Scientific Company.
3. Rigidax (w.s.) - a water soluble polymer of unknown formulation, obtainable from M. Argueso Company (Mamaroneck, New York). Dry Rigidax flakes were melted at 70 C, poured into glass molds and allowed to solidify at room temperature for 24 hours, to yield a uniformly hard and strong rigid plastic.
4. Silicone rubber (RTV11) - The liquid silicone rubber (General Electric Company) was mixed with catalyst (Thermolite-12) and allowed to polymerize at room temperature for 24-48 hours into a resilient, flexible plastic.
5. Epoxy C-7 - The liquid resin and activator "E" (both obtained from Armstrong Products) were mixed and poured at room temperature. Within four days a hard transparent epoxy was available.
6. Lucite - Methyl methacrylate strips were dissolved in acetone; the solution was poured into the appropriate mold and allowed to dry in a laminar flow hood. Complete polymerization required 18 days at room temperature and yielded clear plastic models free of bubbles.

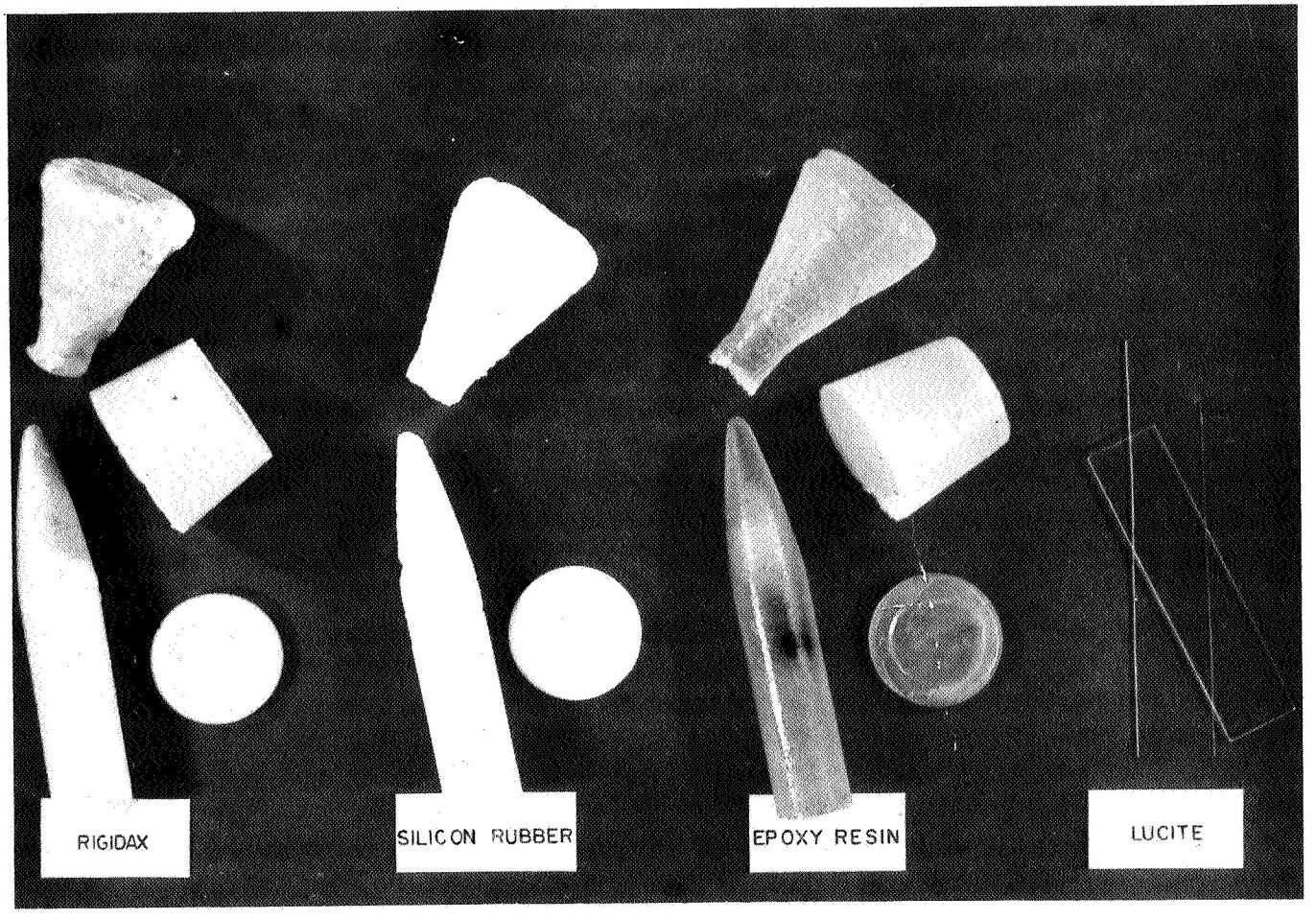


Figure 1: Artificially Inoculated Model Systems Employed In Methodology Studies

In each case, the molten liquid or dissolved plastics were inoculated with known concentrations of spores of Bacillus subtilis var. niger (B. globigii) and were thoroughly mixed before being allowed to polymerize. After hardening, they were subjected to the various solid reduction techniques, sterilization treatments, and storage experiments discussed below. Some of the materials studied (i.e., epoxy, silicone rubber, and Lucite) were chosen because they represent solids which are actually encountered in spacecraft hardware and which may harbor occluded contaminants. The other materials studied (paraffin, paraplast, and Rigidax) are quite unrelated to practical spacecraft solids, and were chosen because of their ability to answer general technical questions such as the lethal effect of pulverization, efficacy of culturing techniques, known positive controls, etc.

The inoculated plastics were allowed to polymerize in various types of molds in order to provide a variety of test modules with different configurations and size. Several of these modules are shown in Figure 1. Since the prime objective of this study was an evaluation of methods for recovery of spores from plastic interiors, it was originally hoped that experimental bias could be avoided by using a variety of plastics, a variety of geometric configurations, and a variety of known contamination levels in each plastic-configuration combination. It was subsequently found that module configuration had no influence on recovery experience, and most of the data in this report are based on the disc modules, prepared by pouring the inoculated liquids into aluminum planchets.

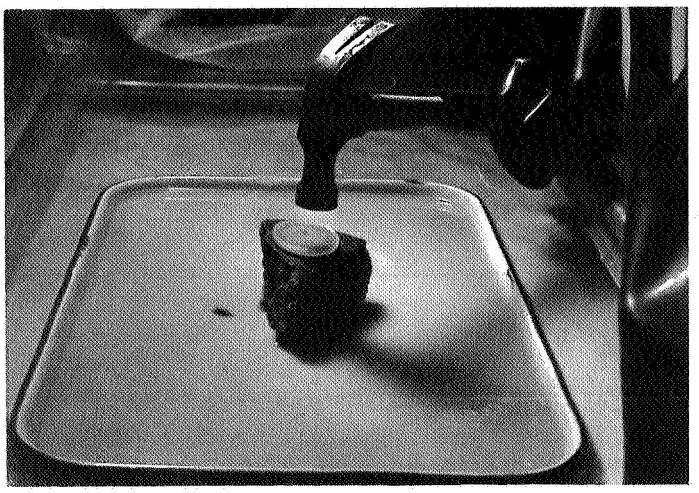
B. Release of Internal Contaminants by Impact Shattering

The probability that an embedded or occluded spore will be released to contaminate a planet can be calculated only after considering such variables as force of impact or erosion, friability of material, location of spore in the solid matrix, degree of contamination, and many others. It will be a difficult equation to solve, and yet it is of great interest in planetary quarantine. Similarly, it is difficult to design a satisfactory experiment to simulate the problem empirically. A meaningful test would require a simulated Martian surface and atmosphere, a scale model (or models!) of internally contaminated landing craft, and a variety of impacting conditions.

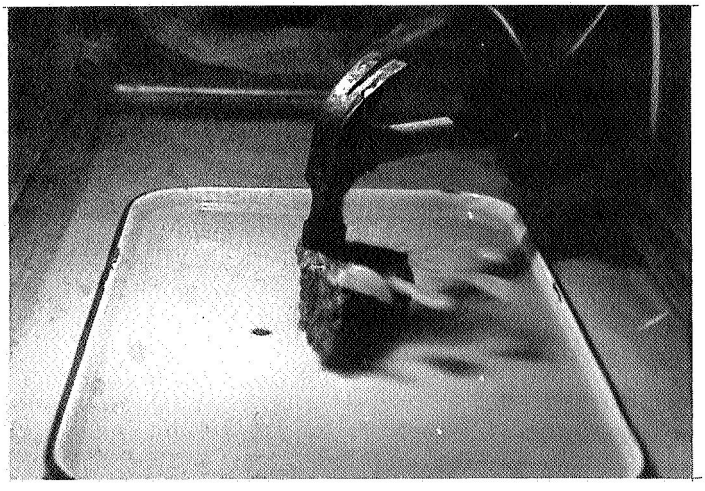
However, several attempts were made during this study to gain an idea about the approximate quantitative relationship between original contamination load and the contamination released from plastic interiors by crude impact shattering. Inoculated epoxy and paraffin discs were surface sterilized in peracetic acid and were aseptically introduced into a sterile glove box which contained a pan of sterile agar, a block of sterile granite, a sterile hammer, and several loaded Andersen Air Samplers. In certain trials, the discs were presoaked in liquid nitrogen before testing. In each case, the disc was placed on the granite block and shattered with a single blow of the hammer. Air samples were taken to measure the viable aerosol thus produced, and the pan of agar was allowed to incubate undisturbed in the glove box for several days to indicate the extent of surface contamination which occurs in the immediate vicinity of "impact."

The experiment is illustrated in Figure 2 and the data of six trials are summarized in Table 1. Although admittedly crude, these data are nevertheless interesting because they suggest a fairly consistent ratio of $1:10^{-4}$

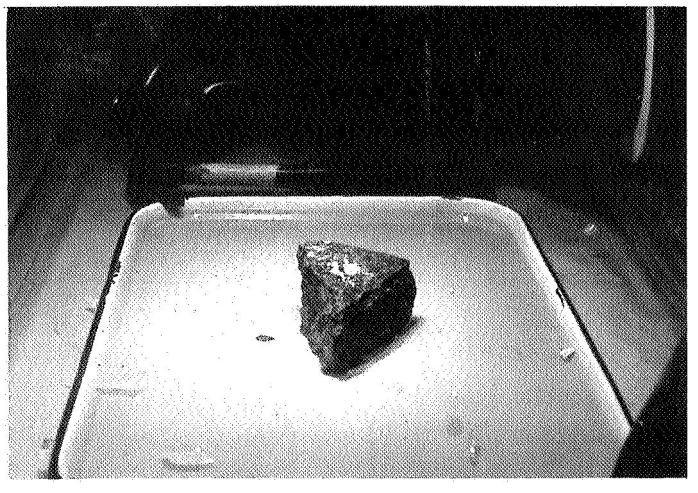
1. Positioning of internally contaminated disc on sterile granite anvil. Pan contained sterile nutrient agar. Complete operation was conducted aseptically in the glove box.



2. Preparing to shatter epoxy disc with sterile hammer.



3. Moment of impact. Note formation of both large fragments and fine powder.



4. After shattering, material was allowed to incubate undisturbed for several days at room temperature.

Figure 2: Release of Internal Contamination by Impact Shattering

Table 1: Release of Internal Contamination by Impact Shattering

Trial	Material	Internal Contamination Load (spores/cast)	Surface Contaminants Released By Shattering (colonies/tray)	Airborne Contaminants Released By Shattering ($colonies/ft^3$)
1	Paraffin (chilled in liquid N_2)	$\sim 10^9$	$\sim 10^5$	$\sim 10^3$
2	Paraffin (chilled in liquid N_2)	$\sim 10^6$	322	25
3	Paraffin (chilled in liquid N_2)	$\sim 10^5$	16	-
4	Epoxy (C-7) (unchilled)	$\sim 10^8$	$\sim 10^4$	-
5	Epoxy (C-7) (chilled in liquid N_2)	$\sim 10^8$	$\sim 10^4$	$\sim 10^3$

between the original count per cast and the resulting colonies on the tray of agar. The illustrations reveal that only a small fraction of the solid matrix was finely pulverized, and that most of the plastic mass (and concomitantly, the majority of the embedded spores) was retained in relatively large fragments. Thus the recurring ratio of 10^{-4} should not be considered as a constant that can be extrapolated safely to other plastics or other impact stresses. Rather it should be considered primarily as a verification of the assumption that the amount of contamination released will be closely and consistently related to the contamination load.

This experiment also revealed some interesting sidelights about airborne contaminants released during shattering. As expected, the viable count per cubic foot of air in the glove box depended on the original contamination load of the plastic. However, the size classification of the aerosol, determined by the cascade impactor Andersen device, showed that at least fifty per cent of the viable organisms were associated with particles $<5u$ in diameter. Particles in this range are considered truly airborne under terrestrial conditions and can travel great distances on wind currents without gravitational sedimentation.

Any implications from these trials should be treated with caution, considering the superficial and oversimplified experimental approach. Nevertheless, a few generalizations might still be valid. On the one hand, these data may be used to minimize the hazard of internal contamination. If a $1:10^{-4}$ ratio between load and release has any meaning, the constraints on interior sterilization might be relaxed without compromising the planetary quarantine obligation. On the other hand, the demonstration that interior contaminants were consistently detectable on surfaces and in air

after simply hitting a piece of epoxy with a hammer emphasizes again the importance of plastic interiors as a potential source of viable contaminants for other planets. The resolution of this paradox can only come after further effort and more sophisticated experimental attacks on this problem.

C. Preliminary Bacteriological Culturing Studies

During the course of this project it was necessary to conduct a number of unrelated but critical experiments to standardize bacteriological enumeration techniques. Since a major objective of this program was to measure the precision and reliability of spore recovery techniques from solid interiors, it was essential to know the precision and reliability of the culturing techniques per se.

Table 2 illustrates one of these trials in which standard pour plates were compared to membrane filters. Two spore suspensions were used, one in water and the other in acetone. The aqueous suspension was handled in routine fashion: Triplicate decimal dilutions were made in distilled water; aliquots from each dilution were delivered to petri dishes and poured with Trypticase Soy Agar (TSA); aliquots from the same dilution blanks were then passed through membrane filters (Millipore HA, 0.45u) which were incubated on previously poured and hardened TSA plates. The acetone suspension was diluted in filter-sterilized acetone dilution blanks; aliquots were plated directly as above, and replicate aliquots were filtered through solvent resistant membranes (Gelman Alpha 8, 0.2u) which were incubated as above. The data suggest that there was no significant difference between the standard pour plate and the HA filter technique. However, there was a

Table 2: Influence of Culturing Techniques on Apparent Spore Recovery

Sample	Replicate		Standard Pour Plate Count	Membrane Filter Count
Aqueous Spore Suspension	1		3.09×10^8	2.83×10^8
	2		3.04×10^8	2.75×10^8
	3		2.80×10^8	2.76×10^8
		Av	2.98×10^8	2.78×10^8
Acetone Spore Suspension	1		1.93×10^5	1.14×10^5
	2		2.10×10^5	1.02×10^5
	3		1.58×10^5	1.49×10^5
	4		1.49×10^5	1.13×10^5
		Av	1.77×10^5	1.19×10^5

slight but consistent diminution in count on the solvent resistant filters when compared to the standard pour plate. From a practical point of view, the counts were not sufficiently disparate to preclude the use of the membrane filters as labor saving devices. The difference should be remembered, however, during the subsequent discussion of spore recoveries from Lucite.

The precision that could be expected from the culturing techniques is again illustrated in Table 3. These experiments dealt with a suggestion by the CDC-Phoenix workers[8] regarding the use of freshly poured "molten" agar as a substrate for membrane filter incubation. Five replicate Rigidax modules (containing the same spore loads) were dissolved and diluted in water. Four replicate epoxy discs were similarly suspended and diluted after pulverization. Six aliquots from each of the terminal dilution blanks were filtered through HA membranes. Three of these filters were incubated on previously poured and hardened TSA plates, while the other three were incubated on freshly poured and partially solidified TSA. The counts from the fifty-four plates indicated no practical advantage that would be gained by using "molten" agar, particularly in light of the inconvenience involved. More important, however, was the demonstration of reproducibility which could be attained by this culturing method. The data are presented here in detail rather than in summary to provide an insight into the close agreement between tests. It is evident that the agreement between replicate Rigidax modules would be more than welcome in any quantitative microbiology work. Similarly, the agreement between replicate plates in the epoxy series was very encouraging. Even the rather marked differences between the different epoxy modules did not

Table 3: Influence of Physical State of Agar on Apparent Spore Recovery by Membrane Filters

Sample	Trial		Count/Plate "Molten Agar"			Count/Plate "Solid Agar"		
Dissolved Rigidax	1		31	23	30	39	21	33
	2		35	47	34	26	37	31
	3		27	28	29	26	20	27
	4		36	34	35	29	27	26
	5		30	39	39	33	28	33
		Av		33			28	
Pulverized Epoxy C-7 (mortar and pestle)	1		114	113	115	101	102	103
	2		101	107	106	91	97	94
		Av		109			98	
Pulverized Epoxy C-7 (pica blender)	1		201	203	206	221	220	228
	2		147	141	147	229	226	225
		Av		174			225	

prove to be any serious disadvantage. On the one hand, the differences in pulverization technique (which will be discussed in detail in a subsequent section of this report) could be clearly demonstrated. On the other hand, the maximum variation encountered (174 $\pm$ 33) was well within the tolerated experimental error experienced in food and dairy bacteriology.

With the exception of several minor experiments, which employed Serratia marcescens, all of the recovery trials dealt with spores of B. globigii. Consequently, there was no need to conduct elaborate trials using a variety of media and/or incubation conditions (which might be the case in a search for the different naturally occurring contaminants within space hardware). Trypticase Soy Agar, incubated aerobically at 35 C for 48 hours, was adequate for the comparative studies conducted. However, any work with spores does involve the problem of spore germination, a process usually aided by heat shocking. Suggestions by the CDC-Phoenix group based on work by Busta et.al.[17] indicated that the heat shocking step could be avoided if calcium dipicolinate was added to the medium. The advantage of this procedure was verifed by a series of experiments with pulverized epoxy discs, summarized in Table 4. Although the supplemented TSA yielded consistently higher counts, the magnitude of difference was not great enough to warrant adaption of this medium for routine culturing purposes.

Some further preliminary investigations dealt with the viability of the standard B. globigii spores (used as inocula) after exposure to different organic solvents. It is axiomatic in any internal contamination recovery work that matrix dissolution is superior to any other method for releasing of embedded spores. However, the potential toxicity of the

Table 4: Influence of Calcium Dipicolinate Supplementation on Apparent Spore Recovery from Pulverized Epoxy Resin

	Unsupplemented TSA Agar		TSA Agar + Ca Dipicolinate*	
Trial 1				
(10 replicates)	$\bar{x}$	1.8×10^6	$\bar{x}$	2.3×10^6
	σ	0.19	σ	0.16
Trial 2				
(10 replicates)	$\bar{x}$	1.7×10^6	$\bar{x}$	2.2×10^6
	σ	0.20	σ	.35

* 50 mMoles $CaCl_2$ + 40 mMoles sodium dipicolinate

solvent (assuming that a suitable solvent is available) might outweigh any advantage gained by this method of spore release. Consequently, solvent toxicity evaluations become extremely important in this type of work from both the sporicidal and the germination inhibition points of view. A series of experiments, summarized in Table 5, compared the gross effects of solvent exposure. Duplicate spore suspensions were inoculated into distilled water and into the solvents listed. At various time intervals aliquots were taken from each and cultured. The data show that the common organic solvents, acetone, benzene, and ether had no deleterious effects on either germination or viability even after several days. On the other hand, the two epoxy strippers tested exerted a lethal and/or inhibitory effect after a relatively short exposure. It should be noted that these experiments were not exhaustive and that unpublished communications from several sources suggest that nontoxic epoxy solvents do exist. No doubt, some of these solvents will be of great value for internal contamination recovery. However, during the course of this project no suitable solvents for either epoxies or silicone rubber were encountered.

D. Preliminary Recovery Experiments with Soluble Polymers

During the initial stages of this project, considerable attention was paid to the inoculation and recovery of spores from Rigidax, paraffin, and paraplast. As mentioned earlier, these materials are quite unrelated to "spacecraft plastics" and could be considered as completely artificial systems. They did have some definite experimental advantages, however, because of their solubility properties. For example, paraffin and paraplast are insoluble in water but soluble in ether and benzene, respectively.

Table 5: Survival of Spores in Organic Solvents

Solvent	Inoculum Size	Exposure Period	Survival % $\left(\frac{\text{Spores Recovered}}{\text{Spores Inoculated}} \times 100\right)$
Acetone	10^7	30 days	100%
Armstrong Epoxy Stripper	10^7	1 hour	7%
Methyl Dichloride and Formic Acid	10^8	15 minutes	0.1%
Benzene	10^8	3 days	100%
Ether	10^8	3 days	100%

Thus aqueous slurries of these polymers could simulate other water insoluble plastics for culturing purposes, while organic solvents would provide "total recovery" controls of the same material. Similarly, during investigations of embedded spore survival, where loss of viability is often confounded by unknown spore liberation efficiencies, the availability of soluble plastic simulants from which all of the embedded spores could be theoretically recovered was of great value.

To a large extent during the first few months of this work, most of the experiments dealing with these artificial systems were "learning experiments," designed mainly to improve and standardize those inoculation and recovery methods which would later be employed with the other, more realistic, but insoluble polymers. Table 6 summarizes several dozen preliminary experiments that were performed. The data represent the best viable recovery values consistently observed after repeated trials with different levels of internal contamination (10^3 to 10^7/gm), different inoculation methods, and different recovery approaches. They are expressed as percentages of the maximum theoretical count obtainable (i.e., $\frac{\text{count in plastic}}{\text{count in inoculum}} \times 100$).

Rigidax, a dense (1.6 gms/cc), hard (Rockwell hardness "L" = 78) plastic, which is completely soluble in water, consistently yielded total or near total recoveries of the embedded spores regardless of the inoculum used. This polymer was used extensively in subsequent experiments as a "total recovery" control in studies of pulverization effects, heating, ethylene oxide treatments, and storage.

The recovery efficiences from paraffin and paraplast were disappointing. The maximum yields, after dissolving in suitable organic solvents, were 80% and 36%, respectively. It should be pointed out, however, that recoveries of

Table 6: Recovery of Inoculated Spores from Soluble Model Systems

Material	Inoculation Technique	Recovery Technique	Recovery Experience
Rigidax	Acetone spore suspension	Aqueous solution	100%
	Aqueous spore suspension	Aqueous solution	100%
	Lyophilized spore powder	Aqueous solution	97%
	Lyophilized Serratia marcescens	Aqueous solution	15%
Paraffin	Acetone spore suspension	Ether solution	80%
	Acetone spore suspension	Waring Blendor-plated as aqueous slurry	60%
	Lyophilized spore powder	Waring Blendor-plated as aqueous slurry	20%
Paraplast	Acetone spore suspension	Benzene solution	36%
	Acetone spore suspension	Waring Blendor-plated as aqueous slurry	12%
	Lyophilized spore powder	Waring Blendor-plated as aqueous slurry	11%

less than 100% did not preclude the further use of these materials. Since the recovery experience was quite reproducible, simple paired experiments (i.e., treated vs. untreated modules) with paraffin and paraplast yielded some useful information in subsequent trials.

The data in Table 6 suggest an apparent interaction between the material used, the inoculation technique and the recovery technique. Thus the same method of disintegration (Waring Blendor) recovered 60% of the spore inoculum from paraffin when an acetone inoculum was used and only 20% when a lyophilized powder was added directly to the melted material. On the other hand, comparable trials with paraplast showed a different discrepancy. In the latter case, a significant difference was observed only when the recovery technique was changed. It seems that such factors as spore clumping, particle wettability, and particle size reduction all play a role in recovery efficiencies.

For the purpose of this work, the inoculation-recovery combinations chosen were based on the optimal yields for each material. Rigidax inoculation was made with lyophilized spore powder added directly to the melt; recovery was accomplished by aqueous dissolution. Paraffin was inoculated with acetone spore suspension; recovery was accomplished by ether dissolution. Paraplast was also inoculated with acetone spore suspensions; recovery was accomplished by benzene dissolution. It should be noted that some work was also done with the inoculation-recovery combinations not shown in this table (i.e., lyophilized spore inoculum-organic solvent recovery), but not enough data were gathered to report these values with confidence.

This table also presents some of the preliminary information obtained with nonsporeforming inocula, in this case, lyophilized Serratia marcescens. Despite repeated efforts, this inoculum could not be recovered from

polymerized plastics in any appreciable quantities. The recoveries were often inconsistent, and further efforts in this direction were discontinued.

E. Pulverization Methodology

In the introduction to this report a number of problems related to spore recovery from solids were enumerated. It was indicated that only some of the problems, such as culturing, were bacteriological in nature, whereas the real critical challenges involved the liberation of the spore from its embedding matrix. Only two alternatives were presented: 1. Dissolving the solid in a nontoxic solvent (if available), and 2. Pulverizing or disintegrating the solid to a particle size range approximating the spore (i.e., $\sim$ 1-2u).

During the course of this study, considerable attention was paid to the physical and bacteriological effects of different pulverization methods. In one series of experiments the particle size distribution of pulverized epoxy was studied. One group of epoxy modules was disintegrated by mortar and pestle, another group by Pica Blender-Mill*, and the third group by both of these methods, sequentially. The resulting powders were examined microscopically, and representative samples were size classified. The data from these trials, summarized in Table 7, provide at least three interesting inferences:

1. Although disintegration with mortar and pestle or Pica mill yields powders that are visibly similar, there is a considerable difference in their particle size distribution. Of the two methods, the Pica mill is a much more efficient pulverizer. Furthermore, the combination of both treatments, namely preliminary disintegration by mortar and pestle followed by Pica mill

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* Pitchford Manufacturing Corporation, Pittsburgh, Pennsylvania

Table 7: Particle Size Distribution of Epoxy after Pulverization

Pulverization Method	Numbers of Particles (%) * 0-10u	11-40u	41-200u	200u
Mortar and pestle	28.9	43.3	26.4	0.4
Pica Blender	31.3	64.2	4.2	0.3
Mortar and pestle followed by Pica Blender	71.1	23.3	5.4	0.2

* Size classified microscopically

treatment, is more efficient that either treatment alone. These results demonstrate the need for both pulverization steps, and the sequence of manual pregrinding, followed by Pica mill blending, became the standard disintegration process for nonsoluble plastics.

2. Even the most efficient pulverization process (i.e., the combination of manual and machine treatment referred to above) could in no way be considered ideal. In order for a spore to germinate it must be in intimate contact with moisture and nutrients. An impervious and insoluble barrier of any dimension will effectively inhibit growth (and consequent detection) of a viable spore. The fact that nearly one third of the powder particles were greater than 10u suggests that many spores, even in a fine powder, were still "embedded" and would not be detected by culturing techniques.

3. In addition to those plastic particles between 11 and 40u, (23% of the total number), the particles > 40u (5.6% of the total number) would also contribute significantly to nonrecovery. Actually, the expression of these data as per cent of total numbers can be quite misleading. The 0.2% of the particles > 200u contained nearly 23% of the total polymer mass, and the 5.4% of the particles between 40 and 200u contained more than 71% of total mass.* Thus, nearly 94% of the plastic matrix, even after disintegration, were really particles of considerable size. If a similar proportion of spores were associated with these particles, then recovery would pose a major problem. This matter will be reconsidered in a subsequent section of the report dealing with actual spore recovery experience.

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* Mass = (numbers of particles in a given range) x (mean diameter)^3
Total mass = Σ masses of particles in each size group

Pulverization processes should not only reduce the particle size of embedding matrices, but should also exert no deleterious effect on spore viability. A number of experiments were performed to evaluate this parameter, using different embedding materials and different pulverization methods. Essentially, these were all "paired" trials, in which replicate modules of paraplast, paraffin, and Rigidax were used. Half of the replicates served as untreated controls, while the other half were pulverized, either in a Pica mill or by mortar and pestle. In certain trials the polymer modules were presoaked in liquid nitrogen before pulverization to render them more friable: in the remaining cases the pulverization was carried out at room temperature. As a further check on the potential sporicidal effects of Pica Blender-mill treatment, spore powder and peptone water spore suspensions were added directly to the blending vial without prior encapsulation in plastic. The results of this work, representing the mean recovery values from at least four test modules per treatment are presented in Table 8. The data show that the pulverization treatments employed exert little if any influence on spore viability. Furthermore, the work with Rigidax, which measured viable recoveries in each of four particle size categories (obtained by sieving the pulverized powder), demonstrated that the yield after pulverization was quite independent of the degree of disintegration effected, and the original inoculum size. If pulverization were to injure the spores, either by mechanical erosion or by heat, this effect would be most noticeable in the finest powder fraction. The evidence gathered essentially eliminates this concern.

Further experiments in pulverization methodology dealt with the optimal time required for maximum spore recovery. Insufficient treatment might not release the spores; excessive treatment might be injurious. The results from several of these trials are shown in Table 9. In the first experiment,

Table 8: Effect of Pulverization on Spore Viability

Material	Pulverization Technique	Spore Recovery Technique	Viable Spores/Gram Untreated Controls	Viable Spores/Gram Pulverized Samples
Dry mixture of spores and talc	Pica Blender - one minute	Standard plating	2.2×10^8	7.2×10^7
Wet suspension of spores in peptone water	Pica Blender - one minute	Standard plating	2.2×10^8	8.0×10^7
Paraplast	Mortar and pestle (cryogenic)	Dissolved in benzene	6.5×10^6	6.1×10^6
	Mortar and pestle	Dissolved in ether	3.8×10^5	4.6×10^5
Paraffin	Mortar and pestle (cryogenic)	Dissolved in ether	4.3×10^5	3.6×10^5
Rigidax	Mortar and pestle (room temp.)	Dissolved in water	3.1×10^7	3.2×10^7
	Mortar and pestle (cryogenic)	Dissolved in water	1.5×10^6	2.3×10^6
	Pica Blender (cryogenic)	Dissolved in water	2.8×10^2	3.1×10^2
	Mortar and pestle (room temp.)			
	< 44u	Dissolved in water	$.8 \times 10^4$	7.7×10^4
	44 - 62u	Dissolved in water	8×10^4	6.5×10^4
	62 - 125u	Dissolved in water	8×10^4	6.8×10^4
	> 125u	Dissolved in water	8×10^4	7.1×10^4

Table 9: Influence of Pulverization Time on Recovery of Inoculated Spores from Solid Interiors

Material	Pulverization Technique	Pulverization Time (minutes)	Recovery (% of original inoculum)
Paraffin	Waring Blendor	5	16.1
		15	20.4
		30	40.0
		60	65.0
Epoxy	Pica Blender-"dry"	0.5	0.3 - 1.0
		1.0	3.0 - 10.0
		2.0	51.0 - 55.0
		3.0	1.0 - 4.0
		4.0	0.3 - 2.0
		5.0	2.0 - 3.0
	Pica Blender-"wet" (peptone water)	0.5	0.3 - 1.0
		1.0	13.0 - 17.0
		2.0	63.0 - 65.0
		3.0	8.0 - 12.0
		4.0	3.5 - 4.0
		5.0	6.0 - 8.0

several discs of paraffin containing approximately 10^7 spores/gm were first disintegrated under liquid nitrogen with mortar and pestle. The powder was then added to a 0.5% triton solution (10 gms powder to 190 ml aqueous detergent) in sterilized Waring Blendor cups. After the time periods shown in the table, aliquots were removed and cultured by membrane filtration. The data, expressed as per cent of the original inoculum recovery, show that spore release improved consistently with continued pulverization in the Waring Blendor.

In the epoxy experiments a number of discs containing approximately 3×10^6 spores/gm were also disintegrated cryogenically with mortar and pestle. One gram samples of the powder were then further pulverized in the Pica mill (with or without peptone water) for different time periods and plated. These data show that the optimal treatment time in the Pica Blender was two minutes, and this exposure was subsequently adopted as the standard pulverization method.

In addition to the pulverization techniques mentioned above (Waring Blendor, mortar and pestle, and Pica mill, individually and in combination), the possibilities of using a "cryogenic sanding" approach was also investigated. The latter method employed an electrically driven sanding device and a stainless steel beaker fitted with a small vice. The material to be disintegrated was clamped in the vice, the beaker was filled with liquid nitrogen, and the sander was applied to erode the surface. Since the complete system (beaker, liquid nitrogen, sanding disc and module surface) was sterile, it was possible to elute the abraded polymer with sterile water and to consider any spores recovered as being liberated from the plastic's interior. The data obtained with this approach will be presented in the subsequent discussion on epoxy and silicone rubber research.

F. Accuracy and Reliability of Spore Recovery Methods

1. Polymerized Epoxy

After the preliminary work on pulverization and culturing methodology and the preliminary experiments with artificial soluble polymer systems had been completed, the focus of attention was shifted toward the major objective of this project, namely the determination of the accuracy and precision of viable spore recovery methods for epoxies, silicone rubber, and Lucite.

Table 10 summarizes the recovery values from inoculated epoxy modules. These data represent the combined results of several hundred trials with different module configurations, different initial inoculum sizes, and different solid reduction techniques. Even though no attempt was made in this table to define the individual experimental parameters precisely, it is readily apparent from the general summary that only the Pica Blender treatment yielded adequate recoveries. Neither mortar disintegration, nor the Waring Blendor, nor cryogenic sanding (individually and in combination with each other) ever recovered more than 20% of the theoretical contamination, and indeed, most of the recoveries with these methods were less than 10%. On the other hand, the Pica mill yields were significantly and consistently higher.

These data agree in some measure with what might be expected from the particle size data previously shown in Table 7. There it was shown that most of the particles in powders from the Pica mill were significantly smaller than those from manually pulverized powders and would thus be more amenable for subsequent culturing of viable spores. In addition, it now appears that grinding in the Pica mill probably creates cracks and fissures in the epoxy particles as well as being responsible for size reduction.

Table 10: Recovery of Inoculated Spores from Polymerized Epoxy by Various Solid Reduction Techniques

Solid Reduction Technique	Inoculum Size Tested (spores/gm)	% Recovery
Mortar and pestle (room temperature)	10^6 - 10^7	< 1
Mortar and pestle (cryogenic)	10^3 - 10^7	1 - 7
Mortar and pestle + Waring Blendor (room temperature)	10^6 - 10^7	1 - 10
Mortar and pestle + Waring Blendor (cryogenic)	10^6 - 10^7	1 - 20
Sanding (cryogenic)	10^5 - 10^8	1 - 6
Sanding + Waring Blendor (cryogenic)	10^4 - 10^8	1 - 8
Mortar and pestle + "dry" Pica mill (cryogenic)	10^1 - 10^6	40 - 62
Mortar and pestle + "wet" Pica mill (with peptone water) (cryogenic)	10^4 - 10^6	63 - 98
Mortar and pestle + "wet" Pica mill (with peptone water)	10^2	40 - 50

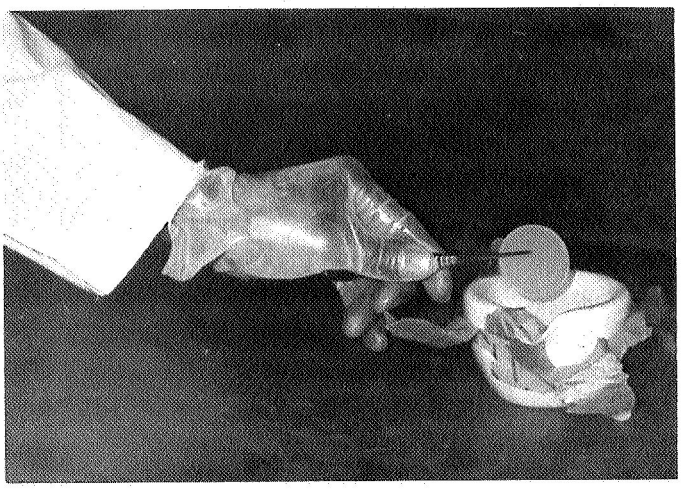
Otherwise it would be difficult to explain viable recoveries ranging from 40% to 98% in powders where 94% of the mass is associated with particles >40u.

One other point should be made regarding this table. There appeared to be a significant difference between recovery experience with epoxy containing 10^4 - 10^6 spores/gm and epoxy containing 10^2 spores/gm. Not enough work was done with lower counts to determine whether recovery efficiency would continue to decrease with still lower inoculum levels.

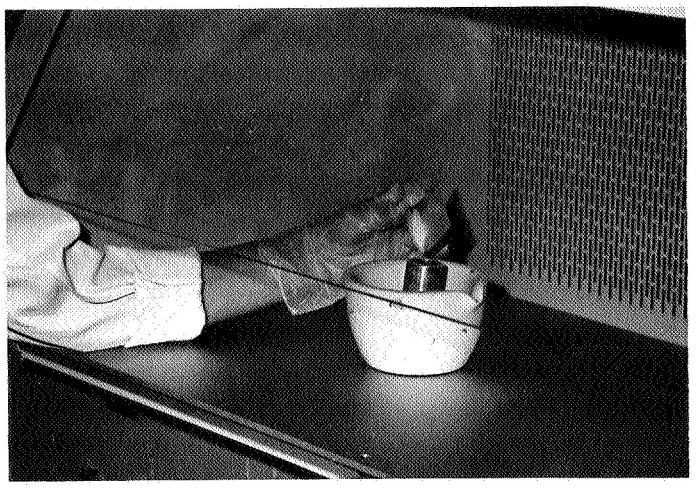
On the basis of these data, it was evident that Pica blending was the best recovery method available for epoxy. Further work was then initiated to ascertain the degree of precision and reproducibility that could be achieved with this technique. Tables 11 and 12 provide some insight into this matter, and Figure 3 illustrates the salient aspects of the methodology.

The data in Table 11 are the crude figures reproduced from a typical experiment. Ten replicate epoxy discs were pulverized in the manner shown in Figure 3 and were plated in triplicate. The extent of the variation observed within the triplicate plates ranged from $\pm$16% of an individual sample mean to $\pm$1%. The variation between the means of the ten replicate discs was less than $\pm$33%. When considered in the light of usual bacteriological enumeration experience, these data illustrate the high degree of reproducibility attainable by epoxy pulverization in a Pica mill.

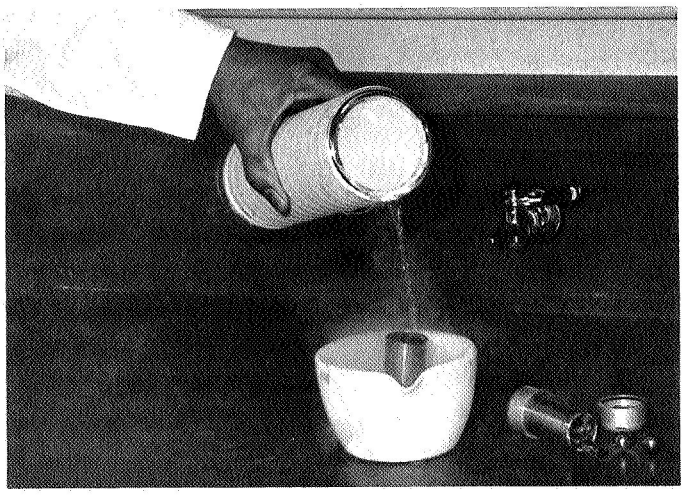
Table 12 demonstrates both reproducibility and the degree of accuracy it was possible to achieve with epoxy. These are the results of work with twenty replicate epoxy discs made from a batch of resin inoculated with 3×10^6 spores/gm. Each disc was disintegrated by mortar and pestle and then further pulverized for two minutes in the Pica mill. In the "dry" experiments, the powder from the mortar and pestle was added directly to



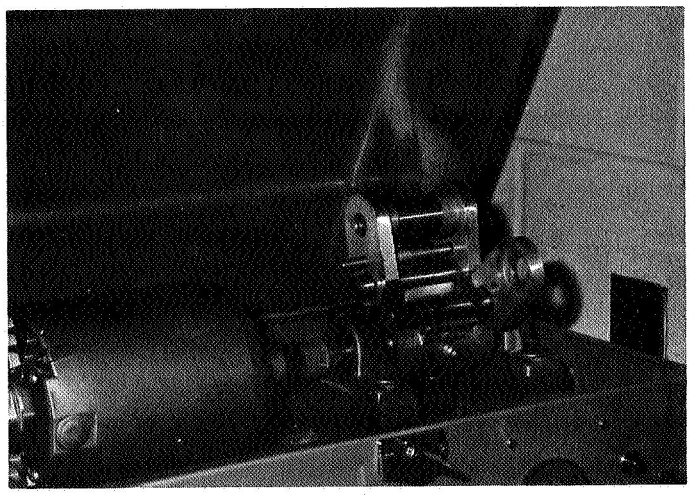
1. Chilling epoxy cast prior to preliminary break up with mortar and pestle.



2. Adding partly reduced epoxy powder to pica blender vial.



3. Chilling blender vial and contents prior to final pulverization.



4. Pica blender mill with vial locked into place.

Figure 3: Cryogenic Solid Reduction Technique Used for Recovering Viable Spores from Epoxy

Table 11: Reproducibility of Viable Counts from Replicate Epoxy Discs

Disc Number	Counts on Individual Plates (Dilution 10^{-3}) A	B	C	Disc Mean Count/gram
1	179	179	164	1.7×10^5
2	164	228	184	1.9×10^5
3	157	182	214	1.8×10^5
4	138	144	170	1.5×10^5
5	261	200	221	2.4×10^5
6	208	216	210	2.1×10^5
7	210	208	206	2.1×10^5
8	261	249	270	2.6×10^5
9	280	276	274	2.8×10^5
10	277	261	275	2.7×10^5

Table 12: Recovery of Inoculated Spores from Polymerized Epoxy by Cryogenic Pulverization in Pica Mill

Sample Replicate	% Recovery* "Dry" Pulverization Standard Plating	Membrane Filter	Sample Replicate	% Recovery* "Wet" Pulverization Standard Plating	Membrane Filter
1	49	57	11	77	67
2	55	42	12	67	89
3	47	59	13	76	89
4	61	52	14	87	79
5	62	43	15	77	77
6	48	50	16	63	78
7	56	46	17	74	87
8	54	50	18	89	74
9	55	58	19	83	96
10	46	56	20	98	76
mean	53	52		79	81

* Inoculum = 3×10^6 spores/gm; % recovery is based on viable count/disc compared to maximum theoretical yield/disc. (Means of three plates/sample-plating combination)

the blender vial without weighing; after Pica mill treatment, the powder was weighed out and diluted; triplicate aliquots were plated directly and another set of triplicate aliquots were membrane filtered. In the "wet" experiments, the dry powder from the mortar was weighed out and an appropriate amount (usually one gram) was added to ten mls of peptone water in a chilled Pica vial; after two minutes of Pica mill treatment, an aliquot of the peptone water-pulverized epoxy slurry was diluted and plated and membrane filtered in triplicate. It was evident that the culturing procedure was not important. It was also evident that the agreement between replicate discs was quite satisfactory. Most encouraging, however, was the observation that "wet" Pica mill pulverization consistently yielded recoveries that clustered around 80% of the theoretical maximum. (As mentioned earlier, with lower original counts, a lower recovery percentage was observed - but not enough work was done with contamination levels below 100/gm to measure reliability with confidence.)

2. Silicone rubber

Silicone rubber modules provided the least satisfactory spore recovery experience. The polymer is resilient and non-friable and thus resists classical pulverization methods. Similarly, its resistance to extreme cold limited the use of any cryogenic approaches which might render it disintegratable. After many failures in trying to degrade the matrix by mortar and pestle, Pica mill, and Waring Blendor, the only recourse which yielded any consistent recoveries was a combination of cold sanding followed by cryogenic Pica mill treatment. Even this "best" approach did not recover much more than 10% of the theoretical maximum. The data summary of work with silicone rubber is given in Table 13. These values represent three separate trials with ten modules per trial.

Table 13: Recovery of Inoculated Spores from Silicone Rubber by "Cold Sanding" and Pulverization

Trial	Inoculum Size (spores/gram)	Culturing Technique	% Recovery (minimum of three replicates/trial)
1	1×10^5	membrane filtration	6.5 (4 - 8)
2	3.3×10^6	membrane filtration	7 (6 - 8)
3	3.3×10^6	membrane filtration	10 (8 - 14)

3. Lucite

Polymerized methyl methacrylate, or Lucite, is an excellent model system for spacecraft plastics. It is easy to inoculate; it hardens into a clear, hard plastic; it can be cut, machined, disintegrated, and moulded; and it dissolves in a nontoxic solvent. Many experiments were performed with this material, mainly in support of the Taft Center workers who were using it to measure "D" values of embedded spores. The results of nine trials, using a minimum of five replicate modules per trial are shown in Table 14.

It appears that the viable recovery experience with this system is not too different from that with the epoxy. Recoveries ranged from 46% to 98% with a mean of 74%, and within the inoculum sizes tested (3×10^3 to 2.6×10^7 spores/gm) there was no significant correlation between concentration and recovery percentage. There is some indication that recoveries by membrane filtration were lower than recoveries by direct pour plates. This result is most likely caused by the inherent characteristics of the culturing technique (cf. Table 2) rather than by the spore recovery methodology.

A description of the best recovery methods employed for viable spores in plastic interiors and a general comparison of each method's accuracy and reliability is presented in Table 15. Essentially, these data are a digest of the results already presented in the preceding discussion and are tabulated here as a summary of the methodology research. It should be emphasized that although these data are based on hundreds of trials and are quite reproducible, they may not be valid if extrapolated to other polymers, to other organisms, or to concentrations of organisms untested in these trials.

Table 14: Recovery of Inoculated Spores from Lucite by Dissolving in Acetone

Trial	Inoculum Size (spores/gram)	Culturing Technique	% Recovery (minimum of five replicates/trial)
1	3×10^3	pour plate	74 (70 - 78)
2	2.1×10^5	pour plate	75 (59 - 83)
3	2.2×10^5	pour plate	81 (72 - 95)
4	3.0×10^5	pour plate	88 (72 - 98)
5	2.3×10^5	pour plate	85 (68 - 98)
6	2.6×10^7	pour plate	77 (60 - 86)
7	3×10^3	solvent resistant membrane filter	73 (67 - 85)
8	2.2×10^5	solvent resistant membrane filter	64 (46 - 80)
9	2.2×10^5	solvent resistant membrane filter	54 (52 - 68)

Table 15: Recovery of Inoculated Spores from Solid Interiors: Summary of Experience with Optimal Techniques

Material		Recovery Experience (based on theoretical maximum)
Epoxy resin	Presoak in liquid N_2 → pulverize with pestle in mortar → add known weight of powder to prechilled Pica mill vial → add known volume of peptone water → grind for two minutes → plate aliquots on TSA agar containing Ca dipicolinate	80% * (range: 40 - 98%)
Silicone rubber	Presoak in liquid N_2 → abrade with electrically driven sanding disc → add abraded particles to Pica mill vial → add liquid N_2 → grind dry for two minutes → weigh out known quantity of powder and dilute in peptone water → plate aliquots on TSA agar containing Ca dipicolinate	7% ** (range: 4 - 14%)
Lucite	Dissolve in filter sterilized acetone → make dilutions in acetone → plate aliquots directly on TSA agar containing Ca dipicolinate	74% *** (range: 46 - 98%)
Rigidax	Dissolve in sterile water → dilute and plate or concentrate by membrane filtration and plate	99% **** (range: 97 - 100%)

* See Tables 10 and 12

** See Table 13

*** See Table 14

**** See Table 6

G. Survival of Embedded Spores

After completing the methodology experiments, it was decided to employ the technical experience acquired in several studies of embedded spore survival and inactivation. A number of disc modules of epoxy and Rigidax containing approximately 10^6 spores/gm were prepared and randomly allocated to storage at 3, 6, 20, and 32 C, respectively. At regular intervals during a six month period, four discs of each plastic were selected from each storage condition and were assayed by the standard procedures described in Table 15. The data from these trials are plotted in Figures 4 and 5, each data point representing the mean count of four replicate discs.

These charts suggest that storage temperatures exert a marked effect on embedded spore survival. At near freezing conditions, there was considerably less diminution in viability than at elevated temperatures. It might be argued that this observation is an artifact and that it represents mechanical recovery problems rather than true viability effects. In other words, it is possible that the plastic continues to polymerize gradually at warmer temperatures and increasingly interferes with spore liberation, while the low temperature storage inhibits polymerization and permits easy fragmentation. (Low recovery counts would result from both lowered viability and poorer liberation.) Although the latter possibility should not be discounted completely, the evidence tends to support an actual viability effect. In the first place, the diminution in count at elevated temperatures was evident not only in epoxy, but even more dramatically in Rigidax, which is completely soluble and presents no liberation problems. Secondly, the effect of temperature was not consistent with time (as would be expected if polymerization was responsible), but appeared to be diphasic, with an initial diminution

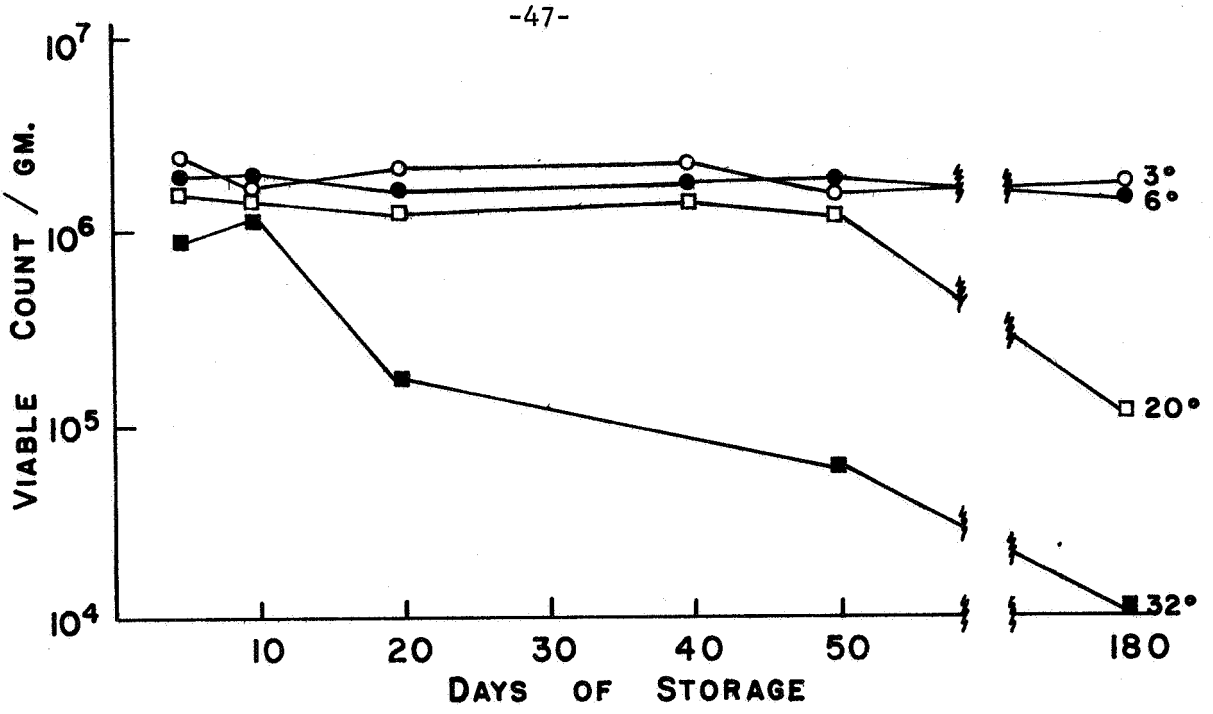


Figure 4: Survival of Spores Embedded in Rigidax

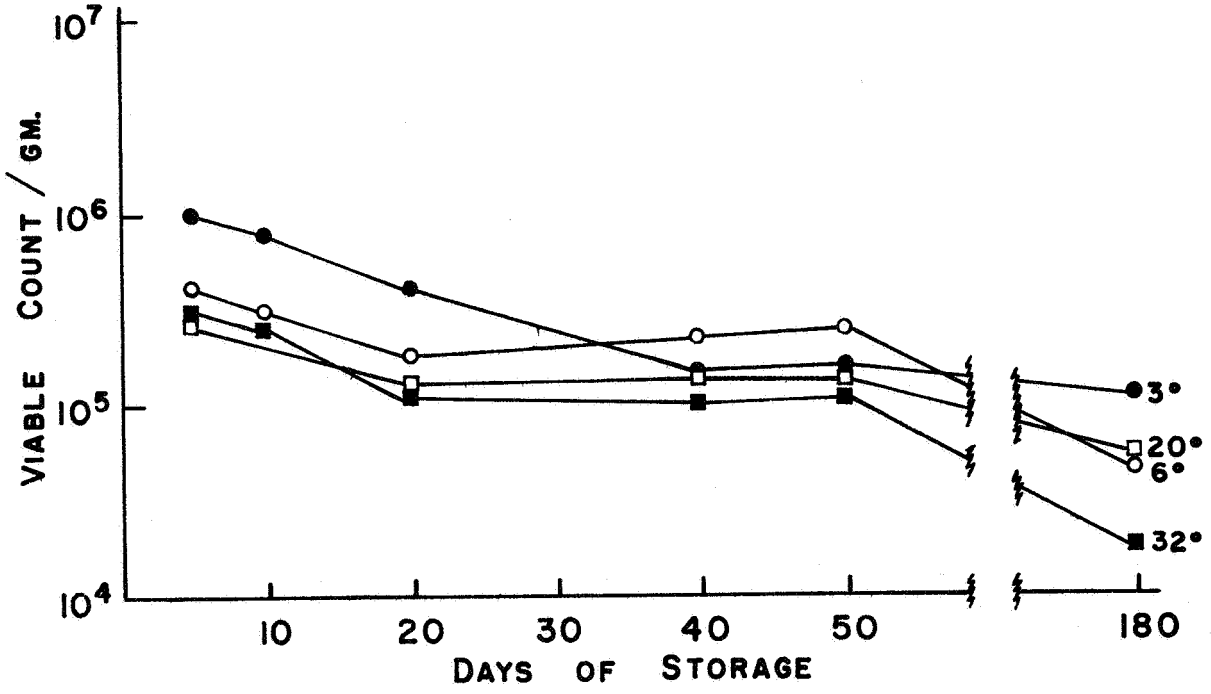


Figure 5: Survival of Spores Embedded in Epoxy

during the first three weeks, followed by a stable period of a month, and ending with a slow decrease during the last few months.

There appeared to be some type of temperature-plastic interaction. In Rigidax the difference between refrigerator and elevated temperatures was very marked, whereas in epoxy, only the high and low extremes differed significantly from each other, and even these differences were dampened. This effect might be due to a combination of both effects mentioned above, namely the additive influences of true viability loss and diminished recoverability, each contributing a different and unknown loss at each temperature.

It should be noted that diminution of spore viability is not an uncommon phenomenon in the bacteriology laboratory, and that decreased spore counts during storage have also been observed in surface contamination studies. (In the latter case, the effect has often been attributed to "blow off.") However, the work reported herein should lead to a re-examination of the theories advanced to account for spore loss. Obviously, an embedded spore cannot be blown away. Furthermore, an embedded spore should theoretically be isolated from the common "toxic" stresses of the environment, such as humidity and oxygen.

Several experiments were also performed with embedded spores exposed to heat. Although insufficient data were gathered to establish valid "D" values, the results of these trials, summarized in Table 16, yield some interesting insights into spore inactivation.

Again, there was a difference between the rates of kill in Rigidax and in epoxy, with the latter providing some measure of protection (cf. Figures 4 and 5). Furthermore, the inactivation rates in both cases were considerably slower than those observed for surfaceborne suspensions of the same organism. If a straight line were interpolated between the initial counts and the

Table 16: Viability of Embedded Spores after Exposure to Heat

Material	Exposure	Viable Count/Gram (mean of five replicates) Unheated Control	Heated Sample
Rigidax	135 C x 1 hour (dry)	3.1×10^6	4.2×10^5
	x 3 hours		1.8×10^5
	x 5 hours		2.0×10^5
	x 7 hours		7.0×10^4
	x 24 hours		4.4×10^2
	x 48 hours		$< 1 \times 10^2$
Polymerized Epoxy	135 C x 1 hour (dry)	3.3×10^7	3.5×10^7
	x 3 hours		5.1×10^7
	x 5 hours		4.7×10^7
	x 7 hours		1.0×10^7
	x 24 hours		3.5×10^4
	x 48 hours		7.0×10^1
Polymerized Epoxy	120 C x 30 minutes (autoclave)	3.3×10^6	2.2×10^6
Unpolymerized Epoxy	120 C x 30 minutes (autoclave)	3.3×10^6	2.6×10^3

counts after 24 hours, "D" values in the range of 6 to 8 hours could be estimated. These values, though crude, are well beyond any other dry heat constants reported for B. globigii. Of interest also are the data shown for autoclaving exposures. Spores in completely polymerized epoxy (i.e., after four days) were essentially untouched in the autoclave. On the other hand, freshly poured epoxy apparently permitted moisture penetration since a simple thirty minute exposure in the autoclave destroyed nearly 99.9% of the spores contained therein. These data emphasize once again the futility of considering interior contamination as a simple, single entity. Both recoverability and thermal sensitivity of embedded microorganisms depend to a large extent on the type of material in which they are encapsulated.

Toward the end of the program, some work was also done with ethylene oxide treatment of embedded spores. The results of this work were remarkably inconsistent. The data shown in Table 17 are typical of these variable results. During different trials with three different plastics exposed to similar ETO exposures, inactivation values ranged from more than four orders of magnitude to no apparent destruction. These values might reflect differences of penetration of sterilant and moisture into different polymers or batches of the same polymer. On the other hand, they might refer to the artifact mentioned above, where the sterilizing gas influences polymerization and consequent liberation of spores. In any event, the data support the contention that interior contamination cannot be effectively (or consistently) inactivated by surface sterilization treatments.

As a corollary to the ethylene oxide experiments, an attempt was made to measure indirectly the degree of sterilant penetration into Rigidax. Several modules of the polymer were prepared with an inoculum of $\sim 10^9$/gm. These were

Table 17: Viability of Embedded Spores after Exposure to Ethylene Oxide

Material	Trial	Viable Count/Gram Control	Viable Count/Gram Exposed to ETO *
Paraplast	1	1.0×10^5	2.0×10^3
	2	1.8×10^6	2.0×10^2
	3	1.5×10^7	2.3×10^6
	4	7.8×10^7	3.7×10^7
Rigidax	1	5.0×10^8	1.0×10^6
	2	1.4×10^8	1.0×10^4
	3	1.7×10^8	4.1×10^7
	4	8.0×10^8	1.8×10^4
Epoxy	1	7.6×10^4	5.0×10^4
	2	6.4×10^3	7.8×10^3

* Standard four hour cycle in commercial ethylene oxide autoclave

subjected to a four hour exposure in a commercial gas autoclave and were then dissolved gradually in sterile water. At intervals during the dissolving process, the modules were removed for measuring, and simultaneously, an aliquot of the solution was plated. The relationship between the viable count and the degree of dissolution is shown in Table 18. The control data show a gradual increase in count as the module dissolved. It may be assumed that the inoculum was uniformly distributed in the plastic, and that the increase resulted simply from an incremental contribution to the solvent. On the other hand, similar increments of the gas sterilized modules yielded considerably lower spore counts. Since even the final increment was only slightly more contaminated than the two or three preceding ones, it may be assumed that the gas penetrated to the interior of the module. However, the penetration was not uniform. The spores in the outer layers suffered a kill of over six logarithms. The spores in the intermediate layers were less affected, and those in the center of the module survived best.

These data are not unexpected. They merely point out that surface sterilizing treatments may have some kind of influence on interior contaminants (although they cannot be relied upon for sterilization). They also show how artificial polymer systems may be used to study interior contamination and decontamination phenomena.

Table 18: Effect of Ethylene Oxide on Spores Embedded at Different Depths in Rigidax

Distance from Surface (mm)	Viable Count/ml of Solution Control	Viable Count/ml of Solution Exposed to ETO *
0.5	4.9×10^7	$< 10^1$
1.5	8.2×10^7	4×10^3
2.5	1.4×10^8	5×10^3
4.0	3.8×10^8	4×10^3
7.0	8.0×10^8	1.8×10^4

* Standard four hour cycle in commercial ethylene oxide autoclave

III Summary and Conclusion

A number of general statements can be made about the assessment of viable microbial contamination found in the interior of spacecraft hardware:

1. Estimates of sterilization effectiveness will depend on knowledge of the total contamination load and the destruction rates of embedded organisms. This knowledge, in turn, will depend on the accuracy and realiability of the assay technique employed.
2. The accuracy of assay techniques for interior contamination are functions of a) the efficacy of the method used to liberate embedded organisms, and b) the efficacy of the culturing method used. Since these two functions are usually confounded, the best approach toward measuring the efficacy of any given technique is an empirical one, using controlled artificial inocula and model solid systems.
3. No simple assay method is suitable for measuring the interior contamination of all potentially contaminated materials. Where possible, dissolving in a nontoxic solvent is the preferred technique for liberating embedded organisms. Failing this, some methods of nonlethal fine grinding or pulverization must be employed.
4. Interior contaminants may be subdivided into three general categories, each endowed with its own problems of assay, sterilization and potential hazard to planetary quarantine:
   a. Internal contamination of piece parts.
   b. Occluded contamination in potting compounds, adhesives, and encapsulating plastics.
   c. Inaccessible contamination on intimately mated surfaces.

The research program upon which this report is based concerned itself almost exclusively with assay problems of the second category listed above: Occluded contaminants in polymerizable plastics. The following general and specific conclusions were reached on the basis of the experimental data presented in this report:

1. The accuracy and reliability of any estimate of viable contamination in the plastics studied is a function of:
   a. The specific material in which the organisms are embedded.
   b. The method used to liberate the viable spores.
   c. The nature of the inoculum used, and in certain cases, the actual concentration of viable spores in the inoculum.
   d. The elapsed time between contamination and assay, and the temperature at which the material was stored.
   e. Interactions of the above.
2. Simple shattering of contaminated plastics containing $> 10^4$ spores/gm consistently yielded detectable viable spores in the air and on surfaces in the immediate vicinity.
3. Culturing of liberated spores did not present any real difficulties. Trypticase Soy Agar supplemented with Calcium dipicolinate yielded suitable recoveries. There was no real advantage gained by the use of membrane filters (compared to standard pour plates) or freshly poured "molten" agar (compared to prepoured hardened agar). The precision of the culturing data, using spores liberated from plastics, was as good as that experienced in classical bacteriological quantitation.
4. Different organic solvents exert different toxic effects on spores. Acetone, benzene, and ether were not deleterious.

5. Soluble polymer systems, such as paraffin, paraplast, and Rigidax, are useful models whereby to study recovery and pulverization methodology. Water soluble Rigidax was an excellent simulant for hard polymers and permitted recovery of $\sim$ 100% of the original inoculum.
6. Pulverization in a Pica Blender mill for up to two minutes did not exert any deleterious effect on spore viability. Longer exposures were sporicidal. Briefer exposures were inadequate for epoxy size reduction.
7. Polymerized epoxy, containing 10^4 - 10^6 spores/gm, consistently yielded approximately 80% of its theoretical spore load after a preliminary cryogenic disintegration with mortar and pestle followed by two minutes of pulverization with a Pica Blender. Lower yields (40 - 50%) were obtained when the original inoculum was $\sim 10^2$/gm.
8. Silicone rubber, containing 10^5 - 10^6 spores/gm, yielded approximately 7% of its theoretical spore load after cryogenic sanding and pulverization.
9. Lucite, containing 10^3 - 10^7 spores/gm yielded approximately 74% of its theoretical spore load after dissolving in acetone.
10. The best methods developed and studied for spore recovery from epoxy, silicone rubber, and Lucite provided consistent and reproducible results.
11. Embedded spores survived best under near freezing conditions and demonstrated significant and consistent diminution of viability (and/or recovery) at warmer temperatures.

12. Dry heat at 135 C required more than seven hours to lower the embedded spore concentration in epoxy by one logarithmic cycle.
13. Ethylene oxide did not penetrate beyond the surface of hardened epoxy. However, it did penetrate into the interior of other plastics and exerted some sporicidal effects in Rigidax and paraplast, depending on density and degree of polymerization.

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