QUANTITATIVE ECOLOGY AND DRY-HEAT RESISTANCE OF PSYCHROPHILES

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Luther Winans Jr.

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ABSTRACT

QUANTITATIVE ECOLOGY AND DRY-HEAT RESISTANCE OF PSYCHROPHILES

Microorganisms capable of growth at 7°C were enumerated and isolated from soil samples from the manufacture area (Denver, Colorado) and assembly area (Cape Kennedy, Florida) of the Viking spacecraft. Temperature requirements were determined for these isolates, and those growing at 3°C, but not at 32°C were designated as obligate psychrophiles in this investigation. These were identified to major generic groups, and the population density of obligate psychrophiles from the various groups was determined. It was found that soil samples from the manufacture area contained obligate psychrophiles, none of which were sporeformers, which comprised about 15% of the population isolated at 7°C. The samples from Cape Kennedy contained psychrophilic populations which made up about 16% of the population isolated at 7°C, and 5% of these were aerobic sporeformers.

Dry-heat sensitivity determinations were also made using isolated pure spores and individual, sized soil particles isolated from soil samples obtained from the manufacture and assembly areas associated with the Viki

spacecraft. Dry-heat D-values were determined for those spores that demonstrated growth or survival under a simulated Martian environment.

The organisms used in this investigation were grown on A-K sporulating agar and spores were harvested and washed with sterile phosphate buffer. The final wash and suspension was made using sterile 95% ethyl alcohol. The spores were deposited on stainless steel strips and heated on specially designed hot plates. The experimental conditions used during this investigation were 110°C and 125°C at a relative humidity of 50%.

The results of this limited investigation seem to demonstrate a low resistance to dry-heat by the selected spores. D_{110}-values range from 7.54 minutes to 122.45 minutes, while the D_{125}-values range from less than 1.0 minute to 9.78 minutes. It appears the survival of those spores selected and tested is reduced rapidly by dry-heat at 110°C and even more rapidly by dry-heat at 125°C.

The original soil samples were then sieved into various particle sizes (< 100 > 88, < 88 > 44, < 44 > 22 and < 22μm). Individual soil particles from each group were heated at 110°C and 125°C in a specially designed system at a relative humidity of 50%. The studies were carried out to determine if there was an effect of particle size on the survival of microorganisms associated with these particles.

The results suggested that a less-resistant

population is associated with the small particles or the large particles afford protection for the organisms during the initial heating period.

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This thesis, directed and approved by the candidate's committee, has been accepted by the Graduate Council of Abilene Christian College in partial fulfillment of the requirements for the degree of

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Floyd W Tiemann
Dean of the Graduate School

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Thesis Committee

Chairman

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CHAPTER I

INTRODUCTION

In the exploration of distant planets, it has been recognized that there is a definite need to prevent contamination of the planets under investigation. For this reason, the National Aeronautics and Space Administration (NASA) has, for the past several years, been involved in research in the field of planetary quarantine. This includes research in microbial monitoring of spacecraft, clean-room assembly of spacecraft, determination of microbial growth under simulated planetary environments, the effects of space environments on the survival of microorganisms, sterilization of spacecraft, and numerous other problems associated with preventing contamination of planetary environments with terrestrial microorganisms. All possible factors related to this topic should be investigated to assure that planetary exploration by terrestrial spacecraft does not alter the ecology of the planet under investigation.

Because Mars is the planet recently receiving the most attention in planetary exploration, this present investigation is undertaken to determine if NASA has been excluding an important population of microorganisms from the microbial monitoring systems of the Viking spacecraft

(presently scheduled to land on Mars in 1976), and if so, to determine if these organisms can survive the present sterilization cycle planned for the Viking spacecraft.

The standard incubation temperature now used by the NASA microbial monitoring system is 32-37°C. The objectives of this investigation are (1) the isolation of microorganisms which grow at low temperatures, but not at 32°C, (2) characterization of these isolated organisms, and (3) determination of their sensitivity to dry heat.

The experimental work was done in two parts. First, the isolation and characterization of the microorganisms isolated from the manufacture and assembly areas associated with the Viking spacecraft. This work was done at Hardin Simmons University in Abilene, Texas. Second, heat sensitivity determinations were completed at the University of Minnesota Space Science Center, Division of Environmental Health, School of Public Health. Both parts were supported by National Aeronautics and Space Administration grants.

CHAPTER II

LITERATURE REVIEW

New vistas of space exploration have opened to man in the short span of a decade, and at the beginning of the second decade, it is prudent to briefly examine the history of man's explorations and migrations. In his conquests, man has carried to new lands not only the benefits of his civilization, but also the detriments of contamination, illness, and death. The same problem may also follow him into space and to the most remote galaxies.

Throughout the recorded history of man, innumerable invasions by one species of plant or animal on another has resulted in an epidemic. Early man tried to interpose crude barriers, frequently only distance, between the source of the invading life forms and himself when he was the susceptible host; perhaps the best were the natural barriers of oceans and impenetrable mountain ranges. Periods of quarantine became worldwide practice. Such barriers were reasonably effective in preventing the spread of dangerous organisms from continent to continent and from continent to island. Quarantine measures have been replaced, only in recent time, by diagnostic methods of increased efficiency and by drugs and vaccines to inhibit epidemics.

The huge and presumably hostile space between the planets has served as a natural barrier throughout the ages to prevent the transfer of pathogenic agents--if they exist--between celestial bodies.

As the possibility of planetary exploration becomes less remote quarantine measures must be developed to prevent contamination of the planets under investigation and to prevent contamination of the earth by samples returned from these planets (12,13,34). Investigations in this area, supported by the National Aeronautics and Space Administration (NASA), have brought together biologists, chemists, mathematicians, engineers, and others who have developed a new international discipline known as Planetary Quarantine (35).

This field has expanded rapidly during the past decade and numerous guidelines have been established. Many factors related to spacecraft contamination and sterilization and the growth of organisms in simulated planetary environments have been studied extensively(14,17, 33,37,41,65). These investigations serve as the basis for setting up the guidelines used in the monitoring systems employed in the planetary quarantine effort associated with spacecraft(14,17,24,59). In the undertaking of space travel,consideration must be given to the possibility that undesirable life forms may follow man through space. It is possible that life in our solar system can exist only on Earth because of adverse environmental conditions on

the other planets, but until this is fully determined, planetary quarantine measures are necessary to satisfy two requirements:

1. The instruments carried by, and the automated spacecraft itself, intended to detect life on a planet, must not carry terrestrial life on board, or the instruments will detect it rather than extraterrestrial life.
2. Terrestrial life carried by an automated or manned spacecraft to a planet may reproduce itself and spread on that body, confuse follow-on studies forever, and possibly attack and destroy life indigenous to that planet (35).

In 1957, the National Academy of Sciences (NAS) expressed deep concern that the possibility of contamination caused by space exploration could endanger scientific investigations of the planets. The NAS urged the International Council of Scientific Unions (ICSU) to assist in evaluation of contamination hazards and to encourage development of preventative measures. Subsequently, the ICSU formed an ad hoc committee, Contamination by Extraterrestrial Exploration (CETEX)(12), which recommended adoption of a code of conduct aimed at achieving a compromise between an all-out program of lunar and planetary exploration on one hand, and on the other, providing maximum protection against degradation of future studies.

In 1959 CETEX (13) recommended that the contamination problem was an integral part of the duties of the ICSU Committee on Space Research (COSPAR) established in 1958. COSPAR had been established to provide for continued and expanded cooperation in space science which had been initiated successfully during the International Geophysical Year of 1957-58. As a result, COSPAR assumed responsibility for consideration of the contamination problem and appointed a study group on Standards for Space Probe Sterilization, with members from the United States, U.S.S.R., United Kingdom, Sweden, Belgium, and France (19).

Since this was an area that had not been studied previously, the early work of COSPAR involved the task of developing and clarifying the meaning of spacecraft sterilization, and it was not until the early 1960's that statements began to be developed showing that the problem of planetary quarantine involved significantly more than sterilization of the spacecraft (31). In 1960, Davies and Communtzis(17) recommended a careful inventory of each mission to assess chemical contamination of celestial body in addition to sterile assembly, intrinsic sterilization, and maintenance of the sterile conditions during launch.

In 1964 Sagan and Coleman (65) published a mathematical model for the probability of contamination of Mars, and this serves as a basis for the COSPAR Resolution of 1964(14). This resolution was the first international

agreement on quantitative objectives in terms of probability of events leading to planetary contamination. It offered a basis for sterilization procedures for spacecraft intended for planetary landing or atmospheric penetration.

Although the COSPAR Resolution of 1964 has been modified as knowledge increases, it continues to serve as a framework in the development of present planetary quarantine standards. The essential elements of the framework include a model of the principal contamination parameters and their interrelations, agreements as to which parameters should serve as basic standards, and assignment of quantitative values to the chosen parameters (35). After consideration of these elements, COSPAR (14) recommended a sterilization level such that the probability of a single viable organism on board any vehicle intended for planetary landing would be less than 10^{-4}. Since that time, and after intensive investigations, it was proposed that the probability factor for each mission be altered to state that the probability of a microorganism being deposited on the Martian surface must be less than 10^{-3} (66). In 1966 Craven, McDade, and Light (15) identified at least fourteen mechanisms by which contamination might occur, and later (16) showed the progress that was being made in developing a comprehensive methodology for the analysis of all likely contamination sources.

A spacecraft which is to land on a planet or penetrate the planetary atmosphere presents a variety of problems to the planetary quarantine program from a

microbiological viewpoint. Associated with this type of mission, several possible sources of contamination exist, including (1) failure of the sterilization cycle, (2) recontamination of the sterile capsule, (3) accidental impact of the launch vehicle or its parts, and (4) impact of various ejecta/efflux (52). These problems have required extensive investigations concerning sterilization. Previous knowledge available was associated primarily with the food and pharmaceutical industries. This knowledge could not be readily applied to the unique problems involved in sterilization of a spacecraft such as sterilization of massive surfaces, embedded materials, mated surfaces, and buried contamination.

These factors have led to rapid progressing of sampling techniques involved with spacecraft. In 1966 NASA published Standard Procedures for the Microbiological Examination of Space Hardware (59), and this has been updated as new data are accumulated (22). Of special interest in these standards is the fact that the NASA standard incubation temperature is 32-35°C. At present, all monitoring procedures are excluding the psychrophilic microorganisms.

Since one purpose of the Planetary Quarantine Program is to determine guidelines for the prevention of contamination of Mars with organisms which might grow in the Martian environment, it is essential that all possible groups of microorganisms associated with these planetary

vehicles be studied in this respect. The success of the planetary quarantine measures can only be met with extensive investigation of all factors which might contribute to this problem; therefore, it would seem advisable to determine the presence and concentration of psychrophilic organisms in various areas associated with the Viking spacecraft and to determine their thermal sensitivity.

A great deal of previous research has been conducted on organisms isolated from the manufacture and assembly areas of the Viking spacecraft, but these studies have dealt primarily with the heat resistance of mesophilic sporeformers with some recent studies being conducted with thermophilic spores. Although it is generally accepted that the psychrophiles may not be the most heat resistant of the microorganisms, they should not be excluded from investigations related to planetary quarantine because they may include organisms with the physiological characteristics to grow in the hostile environment of Mars. Also, it is known that some sporeformers, aerobic and anaerobic, possess the ability to grow at low temperatures, and this group includes the more heat resistant microorganisms.

Work done on the ability of organisms to grow in simulated Martian environments has demonstrated that many organisms can grow and survive under these conditions (28,33,32,38,44,47,64,79). However, many factors have not been investigated, and most of these investigators

suggest that there is a definite need for more work in this area. Some of these have also pointed out that because of the conditions on Mars, the most likely organisms to grow on this planet will include those capable of growth at low temperatures (19,33,40,41,51). For these reasons, it appears to be of great value to extend these previous studies to include the isolation and study of psychrophilic organisms from areas directly associated with the Viking spacecraft.

Since the time that interest in prevention of contamination of planets became apparent, the U.S. space agency has also been actively involved in investigations concerning various sterilization procedures for spacecraft and their components (23,31,34,35). Spacecraft sterilization technology has progressed at an ever-increasing pace, from statements of objectives to the design of methodologies for implementing those objectives. The sterilization technology available when the spacecraft sterilization effort began was essentially that which could be borrowed from the pharmaceutical, surgical supply, and canning industries. It has been necessary to greatly expand that technology for application to complex spacecraft designed to travel the great distances of interplanetary space to reach the planets.

The sterilization requirements for a Martian landing capsule, weighing several hundred pounds and including many thousand individual parts--electronic, electrical,

and mechanical--are more severe than those commonly encountered in medical practice. A surgeon's scalpel, for example, must be sterile on the outside, but it is immaterial whether or not its interior is sterile. The chance of the scalpel disintegrating during an operation is extremely remote. A space vehicle intended for landing on Mars, however, must be sterile throughout, at least to the extent of a probability of less than one in a thousand that there will be even a single viable organism anywhere on or within its many component parts. Considerable effort has been devoted, especially in the United States, to the problem of spacecraft sterilization.

The fabrication of a sterile spacecraft involves both biological decontamination, to bring about a substantial decrease in the number of microbes and spores and sterilization, to kill all the organisms present. The procedures proposed for the decontamination and sterilization of space vehicles and their components are based on the use of the following: (1) chemical sterilants; (2) electromagnetic radiation; and, (3) heat.

Sterilization by chemical means includes gaseous, liquid and solid agents. The most common gas used in the sterilization of space-vehicle components at present is ethylene oxide, $(CH_2)_2O$. It is gaseous at room temperature and with relatively small increases in pressure it can be liquified at ordinary room temperature. The gas appears to be effective in killing microorganisms, including

spores, of all kinds. Furthermore, it has essentially no deleterious effects on metals, most plastics, and other materials used in the fabrication of space vehicles and their component parts.

In the pure state, ethylene oxide is readily flammable and the gas can form an explosive mixture with air. Consequently, when employed as a sterilizing agent a nonflammable diluent is usually used. In the United States carbon dioxide or Freon-12 (dichlorodifluoro-methane, CCl_2F_2) is used as a diluent. In the U.S.S.R. methyl bromide is reportedly used (74). Carbon dioxide is used in a mixture of 10% by weight of ethylene oxide and 90% of carbon dioxide. But containment of the mixture in liquid form, under a substantial pressure, requires the use of heavy steel cylinders. When Freon-12 is used, it is mixed in the ratio of 12% by weight of ethylene oxide and 88% of Freon-12. Although the Freon costs more than carbon dioxide, the containers are inexpensive and easy to handle. The ethylene oxide-Freon mixture is maintained as a liquid under moderate pressure at ordinary temperature. Upon opening a valve on the containing cylinder, the gas emerges. Another advantage of this mixture over the one with carbon dioxide is that the Freon diluent also has germicidal properties.

A certain amount of water vapor should be present for ethylene oxide to be effective as a sterilizing material. Bacterial spores which have been partly

desiccated acquire a degree of resistance to sterilization until they reabsorb sufficient moisture. Ethylene oxide mixtures are generally employed at 30 to 50 percent relative humidity and a temperature range of about 20°C to 40°C or more.

One of the outstanding aspects of ethylene oxide as a sterilant is its good penetrating power. Because of this property, components to be sterilized can be wrapped in paper and they can be handled subsequently without risk of recontamination. Although ethylene oxide can readily penetrate into cracks and crevices, it cannot get much below the surface of nonporous solids. Its main use is therefore as a surface sterilant.

Liquids can serve the same purpose as gases for sterilizing the surfaces, but not the interior of solid components. If employed under proper conditions, liquids act more rapidly than gases, mainly because of the higher concentration of the sterilant. As a result of the fairly high surface tension of water, aqueous solutions do not wet surfaces readily, especially if they have a thin film of oil or grease, as is generally the case. Consequently, organic liquids, with lower surface tensions, are usually employed as solvents. A possible alternative is to add a detergent to the aqueous solution to improve its ability to wet surfaces. It should be noted that several so-called liquid sterilizing agents are not active against bacterial spores, but they can, nevertheless, serve to

reduce the total load of microorganisms.

Cleaning, brushing, or immersion in a liquid sterilization solution has been suggested for the decontamination of small electronic parts prior to assembly. Long immersion, to produce sterilization, however, has resulted in deterioration of the components in some instances. One of the best liquid sterilants has been found to be a solution of formaldehyde in methyl alcohol, with or without the addition of water. Brushing with liquid fluorocarbon containing phenol and swabbing with ethyl alcohol have been employed to decontaminate the components of electronic subassemblies.

In the United States, liquid sterilants have found relatively little application, except for the decontamination of small parts. V. I. Vashkov and collaborators in the U.S.S.R., however, reported in 1967 that they had achieved effective destruction of both bacteria and spores by immersion of components for a sufficient time in dilute aqueous solution of hydrogen peroxide to which a detergent had been added to decrease the surface tension(74). Wiping the surfaces of solids with such a solution was also used for decontamination purposes.

Although solid materials are not used for direct sterilization, they can exert the same function in an indirect manner. One of the major problems in the sterilization of space vehicles is the destruction of organisms in the interior of solid components. As seen,

neither gaseous, liquid or solid sterilants are effective in this respect. The problem could be solved, or at least made less severe, if a material with sterilizing properties could be incorporated into the solids from which the component is fabricated.

The second major method of sterilization is through radiation. It is generally accepted that the great majority of microorganisms are unable to survive exposure to ultraviolet rays. Such radiations with wavelength around 2600Å are especially lethal because they are absorbed by and decompose the nucleic acids which are essential to life. Unfortunately microorganisms are often protected from ultraviolet light by small particles to which they are attached or even by surface roughness. Complete sterilization, even of a surface, is thus not always possible. Nevertheless, because ultraviolet radiation can usually destroy exposed microorganisms very quickly, it might be utilized in decontaminating procedures to reduce the microbial load of components of space vehicles.

At best, ultraviolet rays will only kill microorganisms on the surface of a solid, because of the nature of the material being sterilized and because of the low penetrating power, they are unable to penetrate into the interior. The related electromagnetic radiations of shorter wavelength, namely, X-rays and gamma rays, have both considerable penetrating power and sterilizing ability. These two radiations, sometimes referred to as "ionizing

radiations", differ essentially only in their mode of production. X-rays are obtained from conventional X-ray machines by bombarding a target of a heavy element, such as tungsten, with a beam of high-energy electrons, whereas gamma rays are emitted spontaneously by many radioactive substances. Radioactive cobalt-60 sources of gamma rays are readily available. Because of their ability to penetrate some distance into solids, X-rays and gamma rays can destroy organisms in the interior of solid components of space vehicles.

High-energy (short-wavelength) ionizing radiations are being used for the sterilization of various hospital supplies and also for the preservation of meat and other foodstuffs. The radiation doses required to kill all microorganisms are, however, very large, usually from 2.5 to 5 million rads. Of the available sterilizing agents, only high-energy radiation and heat are capable of killing microorganisms on the surfaces of spacecraft components and also in the interiors. According to L. B. Hall, Planetary Quarantine Officer of the National Aeronautics and Space Administration, "radiation is extremely expensive, hazardous, complex, and damages many materials more than does heat." So the decision has been made in the United States to employ heat as the final terminal sterilization agent (36).

Heat appears to be the most effective and reliable method of killing microbes and spores, both on the surface

and in the interior of solid components. Moist heat has been used for many years for sterilization in hospitals, in bacteriological laboratories, and in the food industry but is not effective for the sterilization of the interior of solid components. While dry heat is less efficient and relatively little has been done in connection with its sterilization properties, it has been chosen as the method of terminal sterilization for the Viking spacecraft because of its ability to sterilize not only the exposed surfaces, but also the interior of all solid components.

In 1961 the National Aeronautics and Space Administration decided that capsules for landing on Mars would be sterilized by heating in a dry atmosphere. At this time systematic investigations were started to determine the effect on microorganisms of temperatures in the range of about 110° to 160°C. The dry-heat sterilization cycles presently employed in industrial and medical fields are too severe to apply to the sterilization of interplanetary spacecraft. Because of the microbial contamination associated with the interiors of certain electronic parts (2,22)the National Aeronautic and Space Administration has decided interplanetary spacecraft shall be sterilized using dry heat, providing that time-temperature combinations can be developed that are compatible with maintaining the functional properties of electronic parts.

In comparison to the accumulated knowledge of the factors that influence microbial resistance to moist heat,

little is known about the factors that affect microbial resistance to dry heat, except the reports of Murrell and Scott (57, 67) on the effects of water activity.

The term "dry heat" obviously implies the application of heat in the absence of water. On closer examination, one becomes aware that some finite value should be established to define the term "dry" or rather its antithesis "wet". A wet or moist-heat sterilization cycle may be defined as one in which the organism is in contact with an environment having an a_w of 1.0 or a water-saturated atmosphere. These conditions are met only when the organism is heated in contact with pure water or saturated steam. This definition of wet heat implies that dry heat is not an equally specific condition, but rather a range of conditions that include such factors as the moisture content of the microorganisms prior to and during heating, the water vapor pressure and flow rate of the gaseous atmosphere in contact with the microorganisms, the chemical and physical composition of the material on or in which the spores are located and the total pressure of the system.

The works of Brannen and Garst and others (8, 9, 11,20) have demonstrated that spores are highly permeable and that a free exchange of water occurs between the spore and its environment. The water activity of spores may be expected, therefore, to change in relation to the water activity of the suspending fluid or with the relative humidity of the atmospheric environment. The ability of

spores to come to water vapor equilibrium with their environment is an important consideration in establishing dry-heat sterilization cycles.

Recent work on the dry-heat resistance of bacterial spores has indicated a critical role for water. Drummond and Pflug(21) showed that the relative humidity of the environment before and during dry-heat exposure may have profound effects on the heat resistance of bacterial spores.

The role of water in the inactivation of intracellular molecules by heat has been a subject for speculation. There has been evidence recently that water has a direct influence on microbial resistance to destruction by dry heat.

The effects of water on the dry-heat destruction rates of spores was first reported by Murrell and Scott (57,67). This initial report has been supported by further studies, dealing directly with water and dry-heat destruction, by Murrell and Scott(57)and Angelatti et al (1). In addition, a considerable number of studies in recent years(1,2,10,11,21,26,46,55,62) report dry-heat destruction rates for specific conditions.

Research on the subject has indicated that the dry-heat destruction rate of microbial spores is a function of the quantity of water in the cell at the time of heating. The quantity of water in the cell at the time of heating is not constant and will depend on certain conditions.

Two physical conditions, open and closed systems, which represent extremes in water movement to or from the cell during heating were defined by Pflug (61). Microorganisms in a closed system are defined as those cells located in such a manner that they are completely surrounded by a solid material that is impervious to the transmission of water vapor. In contrast, microorganisms in an open system are those cells located in a manner so that when they are subjected to a dry-heat sterilization process the cells are in intimate and continuous contact with the surrounding atmosphere. The relative location of the microbial cells, in either a closed system or an open system, is important because of the effect of the physical system on water vapor transfer to and from the cell.

Open and closed systems represent extremes in regard to cell water transfer and a great number of physical conditions lie between the extremes. In the development of sterilization processes for space hardware, the problems of buried microorganisms, surface microorganisms, and microorganisms in mated surface areas must be considered.

Buried microorganisms are completely surrounded by a solid material that is essentially impervious to the transmission of water vapor. In evaluating the physical conditions of microorganisms surrounded by material, the permeability of the material and the dimensions and geometry of the containing object must be considered.

A $D_{125°C}$-value (the D-value is the time it takes at a specific temperature to reduce the population by 90%) of 5 hours is believed to be required for buried microorganisms. Angelotti et al (1) and Paik and Sherry (60) have evaluated the dry heat resistance of Bacillus subtilis var. niger buried in plastic. The results of both groups indicate a maximum $D_{125°C}$-value of 5 hours. On the basis of the data, it is believed that a $D_{125°C}$-value of about 5 hours will be the maximum required value for buried microorganisms on space hardware.

There are at least five factors that may affect the D-value of the sterilization process and must be considered in connection with buried microorganisms. These factors are as follows:

1. The moisture level of the microbial cell at the time of encapsulation
2. The size of the gas volume surrounding the microbial cell
3. The physical and chemical characteristics of the surrounding material, namely, if it binds water tightly, contains absorbed or adsorbed water, or has a high permeability to water
4. Any special pretreatments such as the heat treatment given to some electronic components which could alter the water content of the plastic

5. Any bactericidal properties of the surrounding material (61,63).

Possible locations for buried microorganisms are: the interior of many electronic piece parts, under coatings, and in potting compounds and solid propellants.

Surface microorganisms are located on space hardware in such a manner that the cells are in intimate and continuous contact with the gas atmosphere used to conduct heat to the space vehicle during dry heat sterilization.

This definition thus categorizes microorganisms on surfaces in the open system classification. Therefore, the destruction rate of these organisms will be determined by three factors: (a) initial moisture content of the cell, (b) vapor pressure of the gas surrounding the cell, and (c) the mass transfer of water to or from the cell.

Microorganisms located on the inside surfaces of a hermetic container may be considered a special case if they are in intimate and continuous contact with the internal atmosphere of the container. Each hermetic unit on the spacecraft must be considered as an individual situation.

Mated surface microorganisms are located on the space vehicle in such a way that they cannot be classified either as surface or as buried microorganisms; however, if mated surfaces form a hermetically sealed volume, the microorganisms located therein must be considered buried. The microorganisms in most mated areas, however, appear to

be in a type of open system. Therefore, the same three parameters, mass transfer rate, water content of the cell, and water content of the surrounding gas, will determine the D-value.

The design and establishment of a dry heat sterilization process requires not only the physical condition present, but also an estimate of the numbers of organisms to be killed and the dry-heat destruction characteristics of the microbial population.

The dry-heat destruction of microorganisms is a function of temperature and time. This destruction rate is temperature dependent--increasing the temperature decreases the D-value. In more recent years much of the work on thermal resistance has been expressed in terms of D-values or the time required to reduce a population 90%, assuming approximately logarithmic rates of destruction. Thermal resistance of microorganisms is important in all sterilization processes. For thermal resistance data to be amenable to analytical treatment there must be accepted theories of the death of microorganisms. The logarithmic order of death of microorganisms fills such a need (46,61). In the logarithmic order of death, cells die in a geometric progression where in each equal successive time interval the same fraction of remaining viable cells die.

Knowledge of the logarithmic order is highly important because it permits the microbiologist to compute the death-rate constant K (or its reciprocal,

the D-value). Many early bacteriologists noted the straight line logarithmic order of death under various sterilizing conditions (70,75) and calculated a K value for such reactions. K can be defined by the following equation:

$$K = (1/t)\ (\log N_0 - \log N) \qquad (1)$$

where K is a constant, depending on the organism, temperature, substrate, and the use of logarithms to the base 10; t is the time of exposure, usually in minutes; N_0 is the number of organisms viable at the beginning of the time interval; N is the number of organisms viable at the end of the time interval. Katzin et al (35) pointed cut that a 90% reduction in the population resulted in the following formulation in the equation for K:

$$K = (1/t)\ (\log N_0 - \log 0.1\ N_0) \qquad (2)$$

or $K = 1/t$

or $t = 1/K$

Time t was defined as the decimal reduction time (DRT) which is abbreviated to D. Therefore,

$$D = 1/K$$

The logarithmic model for microbial destruction can now be described by the expression:

$$\log N_t = \frac{-t}{D} + \log N_0 \qquad (3)$$

where N_0 is the initial microbial population; D is the microbial destruction rate, the time to reduce the population by 90% at temperature T; N_t is the population after

t minutes of heating. If the logarithm of the number of survivors is plotted versus time, the resulting curve (commonly referred to as a survivor curve) is a straight line. The thermal destruction model can now be rearranged as:

$$t = D(\log N_0 - \log N_t) \qquad (4)$$

This arrangement is done to emphasize that the term t is the sterilization requirement that must be satisfied by the heat process. It is defined as the "equivalent sterilizing time" or that time at the stablized temperature T sufficient to achieve sterility. This condition holds true not only for heat sterilization but also for chemical sterilization with gases or liquid and for radiation sterilization. Application of this principle is often overlooked in establishing minimum exposure periods or doses for the sterilization of materials or products. If the total load of microorganisms is kept low, and the number of sterilization-resistant types of organisms is kept low, and the exposure periods or doses necessary for sterilization-resistant types of organisms is kept low, then the exposure periods or doses necessary for sterilization can be kept to a minimum. This is one of the chief factors that motivates the use of clean-room conditions not only for the manufacture of spacecraft components but also in the assembly of the spacecraft itself.

From the foregoing, it can be concluded that

microbiological contamination is present in and on spacecraft. To kill all life on spacecraft, two major steps are necessary: (1) The spacecraft and all its components must be assembled under conditions that will keep the level of contamination to a minimum in order that the sterilization cycle will have the least load placed upon it; (2) A final sterilization agent must be applied that will kill all remaining internal and surface contamination. The selection of the method of final sterilization should be based on: (1) The need to achieve both internal and surface sterilization or surface sterilization alone, and (2) the effect of the sterilizing agent on component reliability. There is little to be gained by a spacecraft that is sterile, but inoperative because of sterilization damage. If a final analysis of the risks involved indicates that all components must be internally sterile, only heat or radiation can be used to accomplish this result. Of the alternatives, heat appears to be the best process because of its simplicity and low cost. Heat cannot be used to sterilize spacecraft in the manner commonly used in paramedical practice with high temperatures for short periods of time. Instead, comparatively low temperatures for long periods of time can be used with a minimum of danger to hardware reliability.

In all previous investigations, some aspects have not been considered. Most of the studies were performed on very few species of microorganisms; of those using

soil samples and assaying the survivors, the soils were from areas remote from the spacecraft itself; and isolation and assay procedures used incubation temperatures of 30-32°C. The present investigation has attempted to incorporate many of these changes and specifically to incorporate lower incubation temperatures to restrict the study to psychrophilic microorganisms.

Hawrylewicz has reported that on Mars moisture will likely be available to organisms below 8°C only(38,39) therefore, in order to be a potential contaminant, the organism must possess the ability to grow at lower temperatures and such organisms are not detected by the present microbial monitoring standards of NASA. For these reasons, it appears to be of great value to extend the previous studies to include the isolation and study of psychrophilic organisms from the areas directly associated with the Viking.

It is recognized that there are numerous definitions of psychrophilic organisms including those based on optimum growth range. The latter usually is defined as the formation of microscopic colonies within a certain time period. The definition of psychrophiles has plagued bacteriologists since the term was first used by Schmidt-Nielsen (6,72) in 1902 to describe those bacteria observed by him and by previous workers (25,46,48,50,72) which grew at 0°C. Psychrophiles are a subdivision, along with

mesophiles and thermophiles. Since representatives of the last two groups of microorganisms are defined on the basis of their optimum temperature for growth, it seemed appropriate to the earlier microbiologists that psychrophiles be similarly characterized. Several standard microbiological texts define psychrophiles as microorganisms with an optimum temperature for growth below about 15°C (27,71). One of the main proposals made by Ingraham and Stokes (45) was that psychrophiles should be defined, not on the basis of their optimum temperatures, but on their minimum temperatures for growth. They suggested that psychrophiles be defined as microorganisms which grow well at 0°C within two weeks. They also suggested that good growth be considered as the formation on solid media of colonies visible to the naked eye.

Stokes (72) later suggested that the period of incubation might be shortened to one week at 0°C. Ingraham and Stokes (45) and Stokes (72) deferred to the widespread use of optimum growth temperatures in defining psychrophilic microorganisms by suggesting that these temperatures should continue to be used for subdividing the group into "obligate" psychrophiles, which have an optimum temperature for growth below 20°C and "facultative" psychrophiles which have optimum temperatures above 20°C. This subdivision is useful and appears widely accepted. The vast majority of psychrophiles that have so far been described are facultative psychrophiles, but this may not reflect

the true extent of their distribution in nature (6,73). A whole range of alternative terms for describing psychrophilic microorganisms has been proposed over the years (6,68,78), but none of these has gained wide acceptance.

The overall objective of these studies in Environmental Microbiology as Related to Planetary Quarantine is to gather data that will assist in developing specifications to design, build, and sterilize planetary exploration hardware which will meet the International Committee on Space Research constraints regarding contamination of the planets.

CHAPTER III

MATERIALS AND METHODS

Selection of Samples

In October, 1972, soil samples were obtained from sites at the manufacture area of the Viking spacecraft in Denver, Colorado (M) and from various areas at Cape Kennedy (K) where the spacecraft will be housed in preparation for launching. All samples were taken from around main entrances through which dust contamination might enter (Table 1). All samples were surface samples, no deeper than 5-6 inches, and included grass if it were present at the site. Samples were taken using sterile hand spades and containers.

Isolation of Microorganisms

The samples were returned to the laboratory, and each was thoroughly mixed by shaking in a half-filled sterile container. A ten-gram portion of each was serially diluted in 1.0% peptone to a final dilution of $1:10^6$ prior to plating. A ten-fold dilution scheme was used, and the first bottle (90ml) in each dilution series contained glass beads for better dispersal of soil particles during mixing. Subsequent dilution tubes (ml) were mixed on a Vortex-Genie Mixer (Fisher Scientific, Houston, Texas) to

to assure thorough mixing. One milliliter and one-tenth milliliter aliquots from the first dilution bottle and one-tenth milliliter aliquots from all the dilution tubes were transferred individually to the surface of plates of Trypticase Soy Agar (TSA) and Mycophil Agar (pH 4.0). Media were from Baltimore Biological Laboratory (BBL). Spreading on the agar surface was accomplished with sterile individually wrapped glass spreading rods.

Since one of the major objectives of this investigation was that of isolating microorganisms which grow at low temperatures, isolation was carried out at 7°C. For the purpose of this investigation psychrophiles are defined as those organisms that grow at or below 7°C but not at 32°C. All media used in this investigation were prepared according to manufacturer's directions, allowed to stand at room temperature for 24-36 hours, and then chilled at 7°C for at least 24 hours prior to use. The spreadplate technique was employed to prevent possible damage to psychrophilic organisms by the warm agar required for the pour plate technique. The different media used were prepared from the same "lot" of dehydrated media (BBL), and these "lot" numbers and other pertinent information concerning preparation of media were noted in a log book for control purposes. Differential media and test reagents were tested for reliability with known organisms prior to use in this research, and all manipulations were performed in a laminar flow cabinet (Envirco MiniBench, Model MBC-48,

TABLE 1

SELECTED SAMPLE SITES FROM THE MANUFACTURE AND ASSEMBLY AREAS OF THE VIKING SPACECRAFT

CODE	SOURCE
	From the Manufacture Area in Denver, Colorado
M-1	Outside high bay area on cooling tower side
M-2	West side of high bay area*
M-3	Back of high bay area
	From the Assembly Area at Cape Kennedy
K-1	Bldg.M7-1469 East of low bay door/north side of bldg.
K-2	Bldg.M7-1469 West of low bay door/north side of bldg.
K-3	Bldg.M7-1469 Directly in front on low bay door
K-4	Bldg.M7-1469 East of high bay door/south side bldg.
K-5	Bldg.M7-1469 West of high bay door/south side bldg.
K-6	Bldg.M7-1469 Directly in front of high bay door
K-7	Bldg. AO Directly in front high bay (west) Dark sand
K-8	Bldg. AO Directly in front high bay (west) Light sand
K-9	Bldg. AO From curb directly in front of high bay
K-10	Bldg. AO Main personnel entrance/east side of bldg.
K-11	Bldg. AO From vacuum units inside bldg.
K-12	Bldg. AO Outside main entrance to the clean room

*From roadbed of fill dirt -- not native soil

Albuquerque, New Mexico).

Duplicate plates were prepared for aerobic, anaerobic, and fungal counts. The plates were placed in the 7°C incubator (Freas Model 805) immediately after inoculation, and only the anaerobic plates were allowed to reach room temperature during the manipulations. The anaerobic plates were rechilled after inoculation, placed in Brewer Anaerobic Jars with Gas-Pacs and Anaerobic Indicators (BBL) and placed in the 7°C incubator as soon as anaerobic conditions were achieved as shown by the indicator (approximately 3-4 hours). A freshly inoculated TSA slant of Alcaligenes fecalis (NASA Standard test organism, Center for Disease Control, Phoenix, Arizona) was placed in each anaerobe jar as a biological indicator of anaerobiosis. In no case did this control organism grow in the anaerobe systems. All incubators were monitored with maximum-minimum registering thermometers (Taylor Model No. 5458) which were checked daily. Deviations from 7°C were never more than 1-2°C, and these were usually below 7°C. Slight increases in temperature occurred when samples were being added to or removed from the incubators.

After inoculation of the plates for counting purposes, 1.0 ml aliquots were transferred from the 1:10 dilution to triplicate tubes of synthetic media designed for isolation of blue-green algae, green algae, green sulfur bacteria, purple sulfur bacteria, purple non-sulfur bacteria, Nitrobacter, Nitrosomonas, Ferrobacillus and

Thiobacillus. These media were prepared according to formulations given by Stanier, Doudoroff, and Adelberg (54) and Frobisher (17). The inoculated tubes were incubated at 7°C for 30-60 days and examined both macroscopically and microscopically (phase-contrast and staining). Media inoculated for the cultivation of phototrophic organisms were placed in a lighted incubator. Cultures showing positive results were subcultured into fresh media, incubated for another 30 days and again examined. No attempts were made to count these organisms, only to demonstrate their ability to grow at 7°C.

Growth Temperature Studies

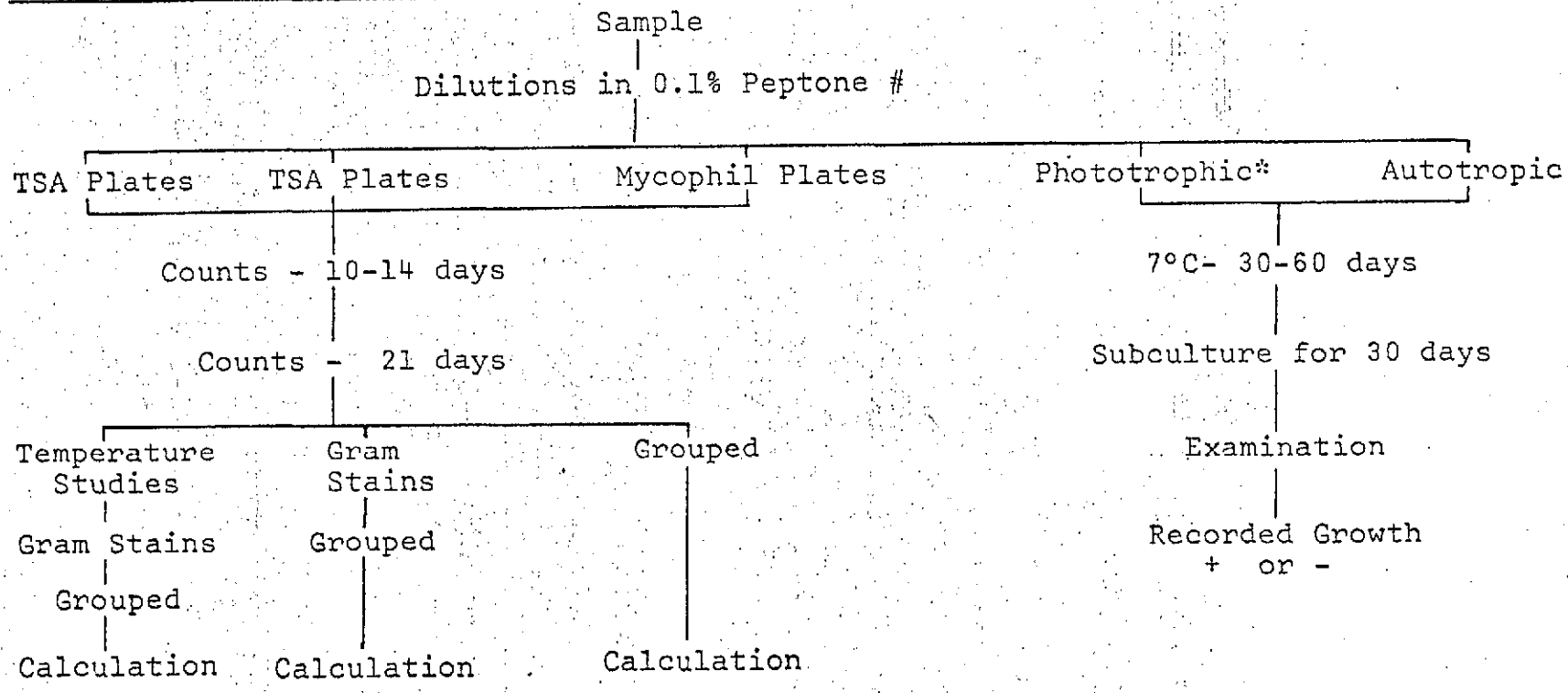
Plates showing countable (30-300 colonies) results after 14 days incubation were selected, and all colonies from these were transferred to each of three TSA slants for incubation at 3°C (10-14 days), 24°C (3-5 days), and 32°C (48 hours). Plates with higher counts were examined for low populations of organisms which did not appear on the countable plates. After growth had occurred, the results were recorded and organisms showing growth at 3°C, but not at 32°C, were classified as psychrophilic, according to the definition used in this investigation.

Identification of Isolates

All isolates from the manufacture area (M) were examined individually by staining and biochemical testing. From these results, the temperature studies, and colonial

FIGURE 1

SAMPLE TREATMENT



#All media stored at 7°C for at least 24 hours prior to use.

*Lighted incubator at 7°C

characteristics, the organisms were identified to major generic groups. From these detailed procedures it became apparent that the organisms could be grouped on the basis of temperature studies and colonial characteristics at the different temperatures. For this reason, it was decided to alter the procedures for the samples from Cape Kennedy. All colonies from countable plates of these samples were transferred to TSA slants for determination of temperature requirements as described previously. The isolates from individual samples were then grouped on the basis of these temperature studies and careful examination of colonial characteristics at all temperatures at which they grew. The mean number of isolates from each sample was 104. Differences in these characteristics caused the organism to be placed into different groups. After these were grouped, random tubes were selected from each group for staining and biochemical testing. Selection of tubes was performed by using standard random tables, and the number of tubes chosen depended upon the size of the group. In all cases at least one-half of the isolates within a group, or a mean number of at least 52 isolates per sample, were selected for staining and biochemical testing. The results from these procedures allowed the generic grouping of organisms for the determination of distributions of the different types.

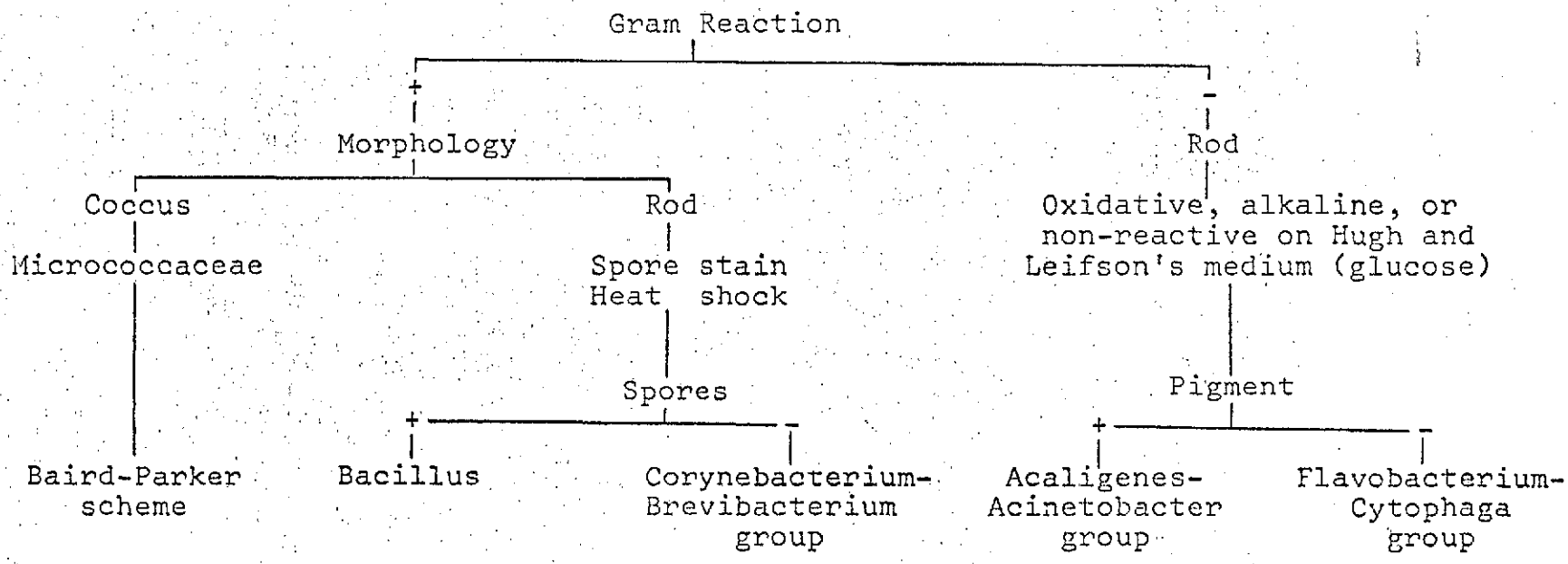
The isolates were gram-stained, tested for motility using a wet mount and phase-contrast microscopy, and

subjected to selected biochemical tests. The biochemical tests used were Hugh-Leifson's test (glucose), Kovac's oxidase test, nitrate reduction, citrate utilization, starch hydrolysis, gelatin liquefaction, casein hydrolysis, acetoin production, methyl red utilization, phenylalanine decarboxilation, litmus milk reactions, catalase production, indole production, urea hydrolysis, Seller's, H_2S production, and fermentation of lactose, sucrose, mannitol, and glucose. Although all of these tests are not included in the identification scheme, they were useful in differentiating isolates within the various generic groups. Organisms thought to be sporeformers were grown on AK-2 Sporulating Agar (BBL) at either 7°C (10-14 days) or 24°C (3-5 days). These were then spore-stained by the cold method of Bartholomew and Mittwer (18) to demonstrate production of spores.

From these results, the organisms were placed into major groups according to the scheme presented in Fig. 2 (p. 38). Members of the Micrococcaceae were identified according to the method of Baird-Parker (3). No streptococci were isolated. The Corynebacterium-Brevibacterium group may also include members of the genera Arthrobacter, Microbacterium, and Cellulomonas (29,68,69). but attempts were not made to differentiate these. The Alcaligenes-Acinetobacter group may also contain members of other closely-related genera such as Achromobacter, but according to J. M. Shewan (Personal communication,

FIGURE 2

Diagnostic key for grouping of isolates from soil samples associated with the Viking spacecraft



January 31, 1973) this genus is being absorbed into the Alcaligenes. The Acinetobacter genus is included in this group because of inconsistencies in the results of the oxidase and motility tests. As a general rule, organisms of this group are oxidase negative and non-motile. The taxonomic relationship of the Flavobacterium-Cytophaga group and related organisms is still under debate (69); therefore, they have been placed into a single group in this scheme.

The fungi were identified to genus according to the methods of Barnett and Hunter (4) and Barron (5), and the yeasts were identified following the methods of Lodder (53). This was performed with the assistance of Dr. John Brandsberg, Center for Disease Control, Kansas City, Kansas.

All aerobic isolates were subjected to anaerobic conditions to determine which ones were facultative anaerobes. Since evidence indicates that only a trace amount of oxygen is present in the Martian atmosphere, the most likely organisms to grow in this environment will be anaerobes or facultative anaerobes. The anaerobic isolates were subjected to aerobic conditions to determine if they were obligate or facultative anaerobes.

Since two mechanisms of contamination of the spacecraft are inadequate terminal sterilization and recontamination after sterilization, it is likely that the contaminating flora, if it is present, will consist primarily of

sporeforming organisms. For this reason, all isolates were cultured on AK-2 Sporulating Agar (BBL) and spore-stained in an attempt to demonstrate spore formation. Because some isolates did not readily demonstrate spores, they were then washed and suspended in phosphate buffer (pH 7.0), heat-shocked at 80°C for 15 minutes (22), and plated on TSA plates to show survival.

Representatives of all isolates were lyophilized for future reference by washing a 48 hour TSA culture with 2 ml of 10% skim milk, placing in a Virtis Vac Vial, quick-freezing in a dry ice-acetone mixture, and drying on a Virtis Unitrap lyophilizer (Virtis Co., Inc., Gardiner, New York) for 24 hours.

Preparation of Spore Suspension

Spore suspensions were made using fifteen pure cultures, isolated from the original soil samples, which survived exposure to a simulated Martian environment (NASA Research, Hardin Simmons University, Abilene, Texas; unpublished data).

The organisms used in this investigation were grown on AK-2 Sporulating Agar (BBL). The medium was prepared according to the directions on the bottle. Prior to pouring the medium into the plates and after the medium had cooled it was supplemented with 0.8 milliliter of a sterile 10% calcium chloride solution.

Pure cultures of the desired organisms were washed with sterile phosphate buffer (pH 7.0) then heat shocked

at 80°C for fifteen minutes. Using the heat shocked suspension, the surface of a supplemented AK plate was inoculated using a sterile cotton swab. Plates were incubated at 7°C for 3-5 days or until sporulation occurred. A heavy suspension of spores was then prepared from the AK plates in sterile deionized water. The suspension was heat shocked at 80°C for fifteen minutes. Ten fresh plates of AK agar were inoculated with the spore suspension using a cotton swab. The plates were incubated at 7°C for 3-5 days. Each plate was checked for sporulation by preparing stained smears and examining microscopically. Any plate that did not have a least 90% sporulation after 5 days was discarded.

The growth of each plate was collected using cold sterile deionized distilled water. Using a Bronson Sonogen A Series ultrasonic bath the suspension was insonated in cold 0.3% Tween 80 for thirty minutes at 25 Khz/sec. to break up vegetative cells. The temperature of the bath was monitored and maintained at 5-10°C by the addition of ice. The spore suspension was then centrifuged at 2400 rpm in a refrigerated centrifuge (International Model B-20) kept at 4°C. The spores were washed six times in sterile phosphate buffer by centrifugation and then rinsed twice in ethanol by centrifugation. The final suspension was stored in sterile 95% ethanol. The final spore suspension was checked microscopically by making a smear and staining by the Schaeffer and Fulton Modification of the Virtz Method of Staining Bacterial Spores. The spore suspensions were then

titered using a ten-fold dilution scheme and plated out on TSA plates. Incubation was carried out at 7°C for 3-5 days. All spore suspensions were adjusted to approximately 1×10^8 spores per milliliter by the addition or removal of alcohol.

D-Value Determination

The objective of this experiment was to compare the survival characteristics of different psychrophilic spores under identical conditions. All operations, prior to incubation of the plates, were carried out in a down-flow laminar flow clean room. The clean room was maintained at 21°C and at a relative humidity of 50%.

The spores were supported on 1/2 inch square stainless steel strips (planchets), type 302, 0.015 inch thick. The strips were prepared by washing in non-ionic detergent, rinsing five times in tap water and then twice in distilled water. The planchets were dipped in 99% isopropyl alcohol and then ethyl ether and allowed to dry. They were placed into petri dishes and sterilized in a hot air oven at 180-200°C for two hours.

Every heating trial utilized 24 permanently numbered rectangular copper boats, each containing two 1/2 inch square planchets which laid flat in a shallow groove running the length of each. The copper boats were cleaned with sterile 95% ethyl alcohol and arranged in numerical order in four rows of six boats each, on a sterile stainless steel sheet. The sterile planchets were then aseptically

transferred to each boat, two per boat, and deposited with the desired spore suspension. An Eppendorf microliter pipette was used for depositing 0.01 milliliters of the ethanol suspension on each planchet. The order of deposition was made in a snake-like pattern. The deposits began with the first row of planchets on the first row of boats and proceeded from left to right. Next the first row of planchets on the second row of boats was inoculated from right to left. After one planchet had been inoculated on each boat, the process was repeated for the second row of planchets and continued until deposits had been made on all planchets (Fig. 3). In order to maintain even distribution, the spore suspension was remixed (after every five deposits) using a Vortex-Genie mixer. The boats containing inoculated planchets were transferred to another sterile tray in the order they were to be placed later on a hot plate. Hot plate position was determined by the use of a table of random numbers.

The deposited spores were allowed to condition for 20-24 hours in the laminar downflow clean room. The boats and planchets were shielded from direct airflow by a sterile stainless steel tray placed over them, although the system was left open on all sides.

Using a table of random numbers twenty-one of the boats were assigned treatment times (heating times) and the other three boats were used as controls (non-heated boats). Each treatment time (T_1 through T_7) plus the

Figure 3: Example of Boat Arrangement for Spore Deposits (From Ex.p LW4015B)

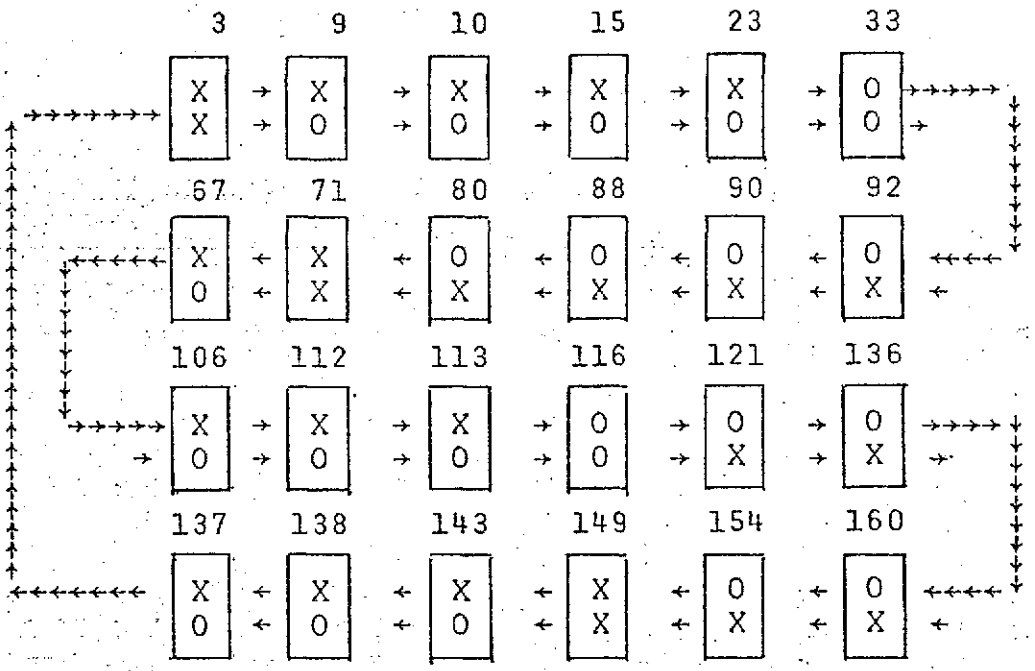


Figure 4: Example of Hot Plate Boat Sequence (From Exp. LW4015B)

	Thermocouple Boat			Thermocouple Boat			Thermocouple Boat	
1	80	T_1	8	106	T_7	15	3	T_4
2	160	T_3	9	10	T_7	16	88	T_4
3	138	T_5	10	149	T_1	17	137	T_5
4	33	T_6	11	116	T_7	18	23	T_2
5	121	T_6	12	15	T_1	19	67	T_6
6	136	T_4	13	90	T_2	20	92	T_3
7	71	T_5	14	113	T_3	21	143	T_2

controls (T_0 or N_0) consisted of three boats. Heating schedules (Tables 1 & 2) and hot plate placement diagrams (Fig. 4) were made for each experiment. The copper boats were placed on the hot plate and heated for the designated times. Upon the completion of each heating interval, the designated boat was removed immediately from the hot plate and placed on a cooling plate for a minimum of three minutes. A special holding tool was used to handle all boats.

All heating times were completed before processing the planchets. The copper boats were picked up by means of the special holder and transferred to a sterile-gloved hand. Each of the two planchets was transferred spore-side down into a dry, sterile, pre-labeled 125 ml Erlenmeyer Flask. To begin processing, 25 or 50 ml of sterile phosphate buffer was added to each flask. The amount of buffer was based on the estimated number of survivors. See Table 2 for the dilution scheme. Each flask, containing the planchets and buffer solution, was suspended individually in the ultrasonic tank using a rubber-coated, Stoddard-type test tube holder attached to the wall of the bath. (The flask was suspended vertically in the tank and was positioned so that the level of tank solution [0.3% v/v Tween 80,aqueous] was level with the buffer solution in the flask). Each flask was insonated at 25 Khz per second for two minutes. After insonation the flask was removed and one milliliter of suspension

TABLE 2

TIME SCHEDULE FOR HEATING BOATS
(From Exp. LW4015B)

BOAT #	TIME ON	TIME OFF	MINUTES TIME	T_X
80	1:00:00	1:02:00	2	1
160	1:00:15	1:16:15	6	3
138	1:00:30	1:15:30	15	5
33	1:00:45	1:15:45	15	5
121	1:01:00	1:31:00	30	6
136	1:01:15	1:31:15	30	6
71	1:01:30	1:11:30	10	4
106	1:01:45	2:01:45	60	7
10	1:02:15	2:02:15	60	7
149	1:02:30	1:04:30	2	1
116	1:02:45	2:02:45	60	7
15	1:03:00	1:05:00	2	1
90	1:03:15	1:07:15	4	2
113	1:03:30	1:09:30	6	3
3	1:03:45	1:13:45	10	4
88	1:04:00	1:14:00	10	4
137	1:04:15	1:19:15	15	5
23	1:04:45	1:08:45	4	2
67	1:05:00	1:35:00	30	6
92	1:05:15	1:11:15	6	3
143	1:05:45	1:09:45	4	2
9	control	--	0	T_0
112	control	--	0	T_0
154	control	--	0	T_0

TABLE 3

EXAMPLE OF SCHEDULED TIME OF BOAT REMOVAL & DILUTION SERIES
(From Exp. LW 4015B)

Time Off		Hot Plate Position			Total	Blanks		Amounted Plated		
Scheduled	Actual	Location	Boat#	T_x	Time Minutes	1st	2nd	0.1	1.0	10
Control	--	--	9	0	0	50	50	25500	2550	255
Control	--	--	112	0	0	50	50	25500	2550	255
Control	--	--	154	0	0	50	50	25500	2550	255
1:02:00	1:22:00	1	80	1	2	25	25	6250	625	62.5
1:04:30	1:04:30	10	149	1	2	25	25	6250	625	62.5
1:05:00	1:05:00	12	15	1	2	25	25	6250	625	62.5
1:06:15	1:06:15	2	160	3	6	50	--	500	50	5
1:07:15	1:07:15	13	90	2	4	25	25	6250	625	62.5
1:08.45	1:08:45	18	18	2	4	25	25	6250	625	62.5
1:09:30	1:09:30	14	113	3	6	50	--	500	50	5
1:09:45	1:09:45	21	143	2	4	25	25	6250	625	62.5
1:11:15	1:11:15	20	92	3	6	50	--	500	50	5
1:11:30	1:11:30	7	71	4	10	50	--	500	50	5
1:13:45	1:13:45	15	3	3	4	50	--	500	50	5
1:14:00	1:14:00	16	88	4	10	50	--	500	50	5
1:15:30	1:15:30	3	138	5	15	25	--	250	25	2.5
1:15:45	1:15:45	4	33	5	15	25	--	250	25	2.5
1:19:15	1:19:15	17	137	5	15	25	--	250	25	2.5
1:31:00	1:31:00	5	121	6	30	25	--	250	25	2.5
1:31:15	1:31:15	6	136	6	30	25	--	250	25	2.5
1:35:00	1:35:00	19	67	6	30	25	--	250	25	2.5
2:01:45	2:01:45	8	106	7	60	25	--	250	25	2.5
2:02:15	2:05:15	9	10	7	60	25	--	250	25	2.5
2:02:45	2:07:00	11	116	7	64.15	25	--	250	25	2.5

transferred to another 25 or 50 ml blank. The second blank was mixed by shaking and aliquots of 0.1 ml, 1.0 ml, and 10.0 ml were each plated in duplicate, using 15 X 100mm disposable petri plates and disposable pipettes. Planchets from later heating times were processed using only a single 25 or 50 ml blank. The ten milliliter aliquots were plated with 15 ml of one-and-one-half strength TSA; all other volumes were plated with 20 ml single-strength TSA. The expected D-value was used as the basis for selecting the heating time and the volumes to be plated so that countable plates would be obtained at each time interval. The last heating time was calculated to yield approximately 1×10^2 spores per planchet.

The plates were inverted and incubated for 3-5 days at 7°C and counted with the aid of a Bactronic Counter (New Brunswick Scientific, New Brunswick, New Jersey). Preliminary D-values and y-intercepts were determined by plotting on semilogarithmic paper, the number of survivors (logarithmic scale) versus the heating time in minutes (linear scale) and drawing a "best fit" line, excluding N_0. The estimated number of surviving spores at each heating time was recorded on special computer data sheets and forwarded to the biomedical computer center for computer analysis. The computer program utilized a least squares regression analysis (ignoring N_0) to estimate a D-value and y-intercept (Y_0) for each time-temperature treatment combination.

In all experiments the spores were heated at 110°C and 125°C on a specially designed hot plate. The temperature was accurately determined with thermocouples attached to two special copper boats. The thermocouples were connected to a temperature recording potentiometer.

Expected sources of variation were the physical characteristics of the boats, the position of the boats on the hot plate, the order of deposition, and the order of treatment. The experimental design included a randomization scheme designed to reduce the chance of systematic effects due to these sources.

Separation of Soil Into Different Particle Sizes

To study the dry heat resistance of spores associated with soil particles of a specific size range, the original soil samples from the manufacture (M) and assembly (K) were sorted into four groups, <100 >88, <88 >44, <44 >22, <22μm. The separation was done with sterile mechanical sieves. After each use, the sieves were washed in a non-ionic detergent, then rinsed three times in tap water and three times in deionized distilled water. They were then rinsed twice with 95% isopropyl alcohol, dried and wrapped in foil. They were sterilized in a hot air oven at 180°C for 2 hours.

The sieves were assembled under laminar flow and raw soil was placed in the top unit. The assembled sieve unit was placed on a shaker assembly and allowed to operate

for one hour. At the end of the hour, the assembly was returned to the laminar flow area and separated soil particles were collected into sterile, pre-labeled screw top jars.

Effect of Particle Size on the Dry Heat Destruction Rate

An experimental program was designed to establish whether soil particle size has an effect on the survival of microbial flora associated with these particles. D-values for various particles were determined using a fraction-negative type system.

Special stainless steel boats were employed, each of which contained 100 wells. They were cleaned and washed as described earlier for the stainless steel planchets.

Individual particles of a specific size, were isolated using a stereoscope (75X) under laminar flow. The particles were sorted and collected on a sterile glass plate under the stereoscope. One particle was aseptically transferred to each of the hundred wells on each of twenty boats. The boats were placed in sterile glass petri plates and allowed to condition in the clean room for 16-20 hours. Using a table of random numbers each boat was assigned a heating time and position on the hot plate. This experiment was set-up using four boats for each heating time interval and four heating times. Four boats were used as unheated controls and represented the original population

(N_0). Schedules were prepared similar to those mentioned earlier with the boat-planchet system.

At the end of the conditioning period the boats were heated as scheduled. Immediately upon the completion of each heating time, the designated boat was removed from the hot plate and placed on a cooling plate for at least five minutes. All heating times were completed before processing was begun.

For processing, each well containing a soil particle was filled with melted TSA maintained at 45°C. After all wells were filled, the boats were placed back into sterile pre-labeled glass petri plates and were incubated at 7°C for 2-3 weeks.

They were examined daily, after the third day, for growth in the wells. The total number of positive wells in each boat was determined. The average number of positive wells per boat at each time interval was plotted against the heating time in minutes.

Heating temperatures for this experiment were 110°C and 125°C. Temperature monitoring was done as described for the boat-planchet system.

CHAPTER IV

RESULTS

Population Studies

The population distribution of organisms capable of growth at 7°C from areas associated with the Viking spacecraft is one of the primary objectives of this study. To determine the population distribution, soil samples were obtained from the manufacture area in Denver, Colorado (M) and from the assembly area in Cape Kennedy, Florida (K). These organisms were isolated aerobically and anaerobically on TSA and Mycophil Agar with the results of these counts presented in Table 4 (p.53). These are based on counts from duplicate plates. No organisms were isolated anaerobically on Mycophil Agar. The recorded temperature during the incubation period was 7 ± 2°C. During periods when the incubator was not opened, the temperature stabilized at 6°C. As can be seen, the total counts from the manufacture area (3.9×10^5 - 9.1×10^6) appeared to be approximately two logs higher than those from the assembly area (5.6×10^3 - 1.5×10^5). A comparison of the distribution of major types of isolated organisms (aerobic, anaerobic, and fungi) also appears in Table 4 (p.53). In all but one sample (K-4), aerobic counts were the higher, ranging from 18.6% in K-4 to 97.7% in M-1. Sample K-4 contained

TABLE 4

TOTAL COUNTS AND COUNTS OF FUNGI AND AEROBIC AND AEROBIC BACTERIA ISOLATED FROM SOIL SAMPLES ASSOCIATED WITH THE VIKING SPACECRAFT*

SAMPLE	TOTAL	AEROBIC	ANAEROBIC	FUNGI
M-1	1.74×10^6	1.7×10^6 (97.7)#	2.7×10^4 (1.6)	1.3×10^4 (0.7)
M-2	3.94×10^5	3.5×10^5 (88.8)	4.4×10^4 (11.1)	4.5×10^2 (0.1)
M-3	9.10×10^6	7.7×10^6 (84.6)	3.0×10^3 (0.03)	1.4×10^6 (15.4)
K-1	6.04×10^4	4.8×10^4 (79.5)	9.6×10^3 (15.9)	2.8×10^3 (4.6)
K-2	8.18×10^4	5.1×10^4 (62.3)	2.8×10^4 (34.2)	2.8×10^3 (3.5)
K-3	1.60×10^4	1.3×10^4 (78.1)	2.1×10^3 (13.1)	1.4×10^3 (8.8)
K-4	3.60×10^4	6.7×10^3 (18.6)	2.4×10^4 (66.7)	5.3×10^3 (14.7)
K-5	2.73×10^4	2.3×10^4 (84.2)	3.9×10^3 (14.3)	4.1×10^2 (1.5)
K-6	1.02×10^5	9.4×10^4 (92.2)	4.1×10^3 (4.1)	3.7×10^3 (3.7)
K-7	1.89×10^4	1.3×10^4 (69.3)	1.7×10^3 (9.0)	4.1×10^3 (21.7)
K-8	7.14×10^3	6.5×10^3 (91.0)	2.5×10^2 (3.5)	3.9×10^2 (5.5)
K-9	5.64×10^3	4.2×10^3 (74.0)	4.5×10^2 (8.0)	9.9×10^2 (18.0)
K-10	2.45×10^4	1.9×10^4 (77.6)	2.2×10^3 (9.0)	3.3×10^3 (13.4)
K-11	8.38×10^3	6.1×10^3 (73.0)	1.9×10^3 (23.0)	3.8×10^2 (4.0)
K-12	1.47×10^5	1.4×10^5 (95.1)	6.9×10^5 (4.7)	2.3×10^2 (0.2)

*Cells/gm of soil
% of total

a higher percentage of anaerobes (66.7%). The anaerobic counts were higher than the fungal counts in ten of the fifteen samples. The anaerobes comprised from less than 1% in M-3 to 66.7% in K-4, and the fungi, from 0.1% in M-2 to 2.17% in K-4.

Attempts to isolate phototrophic and autotrophic organisms resulted in only a few blue-green algae, euglenoids, diatoms, and green algae. These were isolated from only five of the samples and are shown in Table 5 (p.55). Tentative identification for most of the isolates are given. On primary isolation, several samples appeared to exhibit growth of various types of bacteria in small populations in the chemically defined media for isolation of autotrophic bacteria. Upon subsequent attempts at subculturing these in fresh media, none were able to grow. Perhaps they had been obtaining nutrients and energy from the small amounts of soil added to the initial tubes upon inoculation and could not grow on subculture. Because these organisms comprised such a small portion of the total population, they are the least likely to grow in the Martian environment (23) and least likely to survive sterilizing temperatures, it was decided not to include these in the remaining investigation; therefore, only tentative identification is given for them.

Temperature Studies

It is recognized that there are various definitions of psychrophilic organisms (25,45,68,69,78). It is not

TABLE 5

PHOTOTROPHIC ORGANISMS ISOLATED FROM SOIL SAMPLES ASSOCIATED WITH THE VIKING SPACECRAFT (Tentative identification)

TYPE OF MEDIUM	SAMPLE				
	M-1	K-1	K-2	K-6	K-7
Blue-green algal medium	Unicellular (Chroococcus)	Euglenoid + Unicellular (Chroococcus)	--	Biflagellate, Unicellular with large chloroplast (Chlamydomonas)	--
Green algal medium	Masses of long chains of irregular cells - branched filaments	Euglenoid + Unicellular (Chlorella) + diatoms	Euglenoid + Unicellular (Chlorella)	Long, filamentous alga (Ulothrix)	Long, filamentous alga (Ulothrix)

No autotrophic organisms showed growth on subculture
Phototroph were not isolated from samples which have been omitted
Photosynthetic bacteria were not isolated

the purpose of this investigation, however, to answer this question. It is important to describe the conditions used for such a definition as set forth in this investigation. In order to include as many potential psychrophiles as possible, primary isolation was performed at 7°C. Subsequent temperature studies included growth at 3°C, 24°C, and 32°C. Organisms showing growth at 3°C in 10-14 days, but not at 32°C are defined in this project as obligate psychrophiles. Many of these did show growth at 24°C, and are shown in Table 6(p.57). Because some investigators prefer a more rigid definition (78), results also show the percent of organisms which grow at 3°C, but not at the other two temperatures. The results presented in Table 6 (p.57)are given as the percent of the aerobic counts from Table 4 (p.53). As can be seen in the second column, if a more rigid definition of psychrophilic organisms is preferred, the percentages range from 0.0% in K-3, K-11, and K-12 to 16% in K-2. According to the definition of psychrophiles as used here (growth at 3°C, but not at 32°C), the results range from 2.3% in K-4 to 56.4% in K-8, as shown in the final column. Even though the total population is higher in the samples from Denver, the percent of obligate psychrophiles appears to be higher in the samples from Cape Kennedy. This is especially true in the group which includes organisms growing at 3°C, but not at 24°C and 32°C. The results given for percent of obligate psychrophiles from these soil samples may appear high, but

TABLE 6

PERCENT OF ORGANISMS ISOLATED AEROBICALLY FROM THE ORIGINAL SOIL SAMPLES WHICH GREW AT 3°C BUT NOT AT 32°C

Sample	3 C+* 24 C- 32 C-	3 C+ 24 C+ 32 C-	Total Obligate Psychrophiles
M-1	2.0**	24.0	26.0
M-2	2.0	6.0	8.0
M-3	3.7	9.1	12.8
K-1	10.0	26.0	36.0
K-2	16.0	12.0	28.0
K-3	--	9.5	9.5
K-4	2.3	--	2.3
K-5	8.3	--	8.3
K-6	6.8	11.4	18.2
K-7	7.1	12:2	19.3
K-8	9.9	46.5	56.4
K-9	2.1	25.0	27.1
K-10	10.0	13.7	23.7
K-11	--	27.6	27.6
K-12	--	2.1	2.1

* +=Growth
-=No growth
** Given in % of aerobic count in Table 4

it must be remembered they are based upon counts in which the organisms were originally isolated at 7°C.

Because only comparatively low populations of fungi were present except for a few samples, and because they were demonstrated to be of little problem in areas related to planetary quarantine (35), they were not included in the remainder of this investigation. Temperature studies were not performed on them, although they have been identified to genus.

Identification

The fungi isolated were identified to genus only with the results presented in Table 7 (p.59). The molds were not identified to species, but it was observed that most genera showed only one or two different species, with the exception of Penicillium. The yeasts from the manufacture area were all determined to be Cryptococcus albidus. Those from Cape Kennedy were identified as C. albidus, Cryptococcus laurentii, Rhodotorula rubra, or Rhodotorula minuta.

Organisms isolated anaerobically from the manufacture (M) and assembly (K) areas were stained and found to consist predominantly of sporeformers. Those isolated from the manufacture area consisted of approximately 70-80% sporeformers and 20-30% gram-positive non-sporeforming rods. Samples from the assembly area (Cape Kennedy) yielded almost 100% sporeformers. During further testing,

Table 7. PERCENT OF DIFFERENT FUNGI WHICH WERE ISOLATED FROM THE ORIGINAL SOIL SAMPLES AT 7°C ON MYCOPHIL AGAR

Manufacture Area

	Alt*	Cep	Chr	Cla	Cry	Fus	Pen	Rho	Rhi	Ulo
M-1	4.6	-	16.9	4.6	36.9	9.2	-	4.6	3.1	20.0
M-2	-	0.9	39.8	-	0.9	-	40.7	-	-	17.6
M-3	5.0	-	12.5	-	60.0	5.0	7.5	-	-	10.0

Cape Kennedy Area

	Alt	Asp	Chr	Cla	Cry	Gen	Pen	Rho	Tub	Ulo
K-1	-	-	-	10.0	-	-	90.0	-	-	-
K-2	8.1	-	-	31.6	2.9	44.9	8.8	3.7	-	-
K-3	8.7	-	42.8	-	2.2	12.3	29.0	5.1	-	-
K-4	2.8	-	0.9	-	11.0	-	4.6	-	-	80.7
K-5	-	7.4	1.1	-	4.6	-	7.5	3.5	-	75.9
K-6	41.6	1.4	1.4	-	27.8	2.8	15.3	6.9	-	2.8
K-7	-	-	-	-	-	-	97.6	-	-	2.4
K-8	5.2	-	-	2.6	-	-	-	-	5.1	87.1
K-9	17.4	5.0	7.2	-	31.1	-	2.9	18.8	-	-
K-10	-	18.2	18.2	-	3.0	18.2	36.4	-	-	6.1
K-11	6.6	27.9	8.2	-	6.6	-	26.2	21.3	-	3.3
K-12	-	-	27.0	-	5.4	-	28.4	5.4	-	33.8

*Alt = Alternaria
Asp = Aspergillus
Cep = Cephalosporium
Chr = Chrysosporium
Cla = Cladosporium
Cry = Cryptococcus
Gen = Geniculosporium
Fus = Fusarium
Pen = Penicillium
Rho = Rhodotorula
Rhi = Rhizoctonia
Tub = Tubercularia
Ulo = Ulocladium

it was determined that nearly all of these were facultative aerobes belonging to the genus Bacillus. It was also determined that many of the aerobic isolates were facultative. From these results it was shown that representatives of the organisms isolated anaerobically would be selected from those isolated aerobically. It was then decided to exclude the anaerobes from further investigation since obligate anaerobes were not demonstrated.

The aerobic bacteria were identified to major generic groups including Micrococcus, Bacillus, Coryne-bacterium-Brevibacterium (C-B), Alcaligenes-Acinetobacter (A-A) and Flavobacterium-Cytophaga (F-C). Different isolates within each major group were recognized, and representatives of these have been lyophilized. The results of these determinations are presented in Table 8.

Samples from the manufacture area appear to contain primarily members of the C-B group (36% in M-1 to 7.6% in M-3) and members of the genus Micrococcus, especially subgroup 8 (6% in M-2 to 22% in M-1). Of interest is that these samples appear to contain only low percentages of aerobic sporeformers (2.0% in M-2 and 1.8% in M-3). In contrast to this, samples from Cape Kennedy seem to contain primarily members of the genus Bacillus (8% in K-9 to 75% in K-10) and the C-B group (5% in K-5 to 8.6% in K-7), with the exceptions of K-5 which contains a high percentage of the A-A group (70%) and K-9 which contains a high percentage of Micrococcus subgroup 7 (50%).

Table 8. NUMBER AND PERCENT OF THE MAJOR GROUPS OF ORGANISMS ISOLATED AT 7°C FROM SOIL SAMPLES ASSOCIATED WITH THE VIKING SPACECRAFT*

Sample	Bac. #	C-B	A-A	F-C	Mic. 1	Mic. 7	Mic. 8	Yeasts &Molds	No growth on subculture
M-1	- -	6.1×10^5 (36.0)	2.4×10^5 (14.0)	6.8×10^4 (4.0)	2.7×10^5 (16.0)	6.8×10^4 (4.0)	3.7×10^5 (22.0)	- -	6.8×10^4 (4.0)
M-2	7.0×10^3 (2.0)	2.3×10^5 (66.0)	3.5×10^4 (10.0)	- -	3.5×10^4 (10.0)	7.0×10^3 (2.0)	2.1×10^4 (6.0)	- -	1.4×10^4 (4.0)
M-3	1.4×10^5 (1.8)	5.4×10^6 (69.8)	- -	4.2×10^5 (5.5)	- -	6.9×10^4 (0.9)	1.6×10^6 (20.2)	1.4×10^5 (1.8)	- -
K-1	1.9×10^4 (40.0)	1.3×10^4 (28.0)	9.6×10^2 (2.0)	1.9×10^3 (4.0)	- -	1.9×10^3 (4.0)	- -	8.6×10^3 (18.0)	1.9×10^3 (4.0)
K-2	1.9×10^4 (38.0)	1.3×10^4 (48.0)	3.1×10^3 (6.0)	2.0×10^3 (4.0)	- -	- -	1.0×10^3 (2.0)	1.0×10^3 (2.0)	- -
K-3	6.5×10^3 (50.1)	4.6×10^3 (35.1)	9.1×10^1 (0.7)	1.2×10^3 (8.9)	- -	3.9×10^2 (3.0)	- -	- -	2.9×10^2 (2.2)
K-4	2.6×10^3 (39.1)	2.7×10^3 (43.5)	3.6×10^2 (5.3)	2.6×10^2 (3.8)	- -	1.0×10^2 (1.5)	- -	2.6×10^2 (3.8)	2.0×10^2 (3.0)
K-5	3.5×10^3 (15.0)	1.2×10^3 (5.0)	1.6×10^4 (70.0)	- -	- -	2.3×10^3 (10.0)	- -	- -	- -

(continued)

Table 8. (continued)

Sample	Bac. #	C-B	A-A	F-C	Mic. 1	Mic. 7	Mic. 8	Yeasts &Molds	No growth on subculture
K-6	$1.2x10^4$	$3.8x10^4$	-	$2.2x10^3$	$3.1x10^4$	$1.1x10^4$	-	-	-
	(12.5)	(40.9)	-	(2.3)	(33.0)	(11.3)	-	-	-
K-7	$1.4x10^3$	$1.1x10^4$	$9.1x10^1$	-	-	$3.8x10^2$	-	-	-
	(10.7)	(85.7)	(0.7)	-	-	(2.9)	-	-	-
K-8	$1.3x10^3$	$2.9x10^3$	$5.5x10^2$	$9.1x10^1$	-	-	-	-	-
	(19.6)	(45.1)	(25.5)	(8.4)	-	-	-	-	-
K-9	$3.5x10^2$	$1.1x10^3$	-	$3.5x10^2$	-	$2.1x10^3$	-	$8.8x10^1$	$1.8x10^2$
	(8.4)	(27.0)	-	(8.3)	-	(50.0)	-	(2.1)	(4.2)
K-10	$1.4x10^4$	$4.1x10^3$	$9.5x10^1$	$9.5x10^1$	-	$2.1x10^2$	$2.1x10^2$	$9.5x10^1$	-
	(74.8)	(21.6)	(0.5)	(0.5)	-	(1.1)	(1.1)	(0.5)	-
K-11	$3.1x10^3$	$1.7x10^3$	$1.9x10^2$	$3.8x10^2$	-	$4.7x10^2$	$9.8x10^1$	$1.9x10^2$	-
	(50.7)	(27.6)	(3.1)	(6.2)	-	(7.7)	(1.6)	(3.1)	-
K-12	$2.8x10^4$	$1.2x10^5$	$9.8x10^2$	$5.6x10^3$	-	-	-	-	-
	(12.0)	(83.3)	(0.7)	(4.0)	-	-	-	-	-

*Cells/gm soil
#Bac. = Bacillus
C-B = Corynebacterium-Brevibacterium
A-A = Alcaligenes-Acinetobacter
F-C = Flavobacterium-Cytophaga
Mic. = Micrococcus

Table 8(p.61) also includes the number of organisms in each group per gram of soil from the various samples. One interesting result seen in this table is that the actual number of bacilli per gram of soil is similar when the Denver samples are compared to the Kennedy samples. Even though the percentage of bacilli in the aerobic population of the Denver samples is quite low, M-3 contains the largest population of aerobic sporeformers (1.4×10^5 cells/gm soil).

Distribution of Obligate Psychrophiles

Based upon the temperature studies and distribution, the percent and number of obligate psychrophiles (growth at 3°C, but not at 32°C) within each group were determined and are presented in Table 9(p.64). The majority of psychrophiles from the manufacture area belong primarily to the C-B group (6% in M-2 to 10% in M-1) or to Micrococcus subgroup 8 (2% in M-2 to 8% in M-1). Of the organisms isolated from this area, only 2.0% proved to be gram-negative rods. Samples from Cape Kennedy contained a more diverse population, but the majority of obligate psychrophiles from these samples were also either gram-positive rods or gram-positive cocci. Sample K-1 contained a large percent (18%) of psychrophilic fungi in its population isolated aerobically on TSA, and samples K-8 and K-4 had a fairly high population of gram-negative rods (16.9% and 8.3%, respectively). No obligate psychrophilic bacilli were isolated from the Denver samples, but 10 of

Table 9. NUMBER AND PERCENT OF OBLIGATE PSYCHROPHILES IN THE MAJOR GROUPS OF ORGANISMS ISOLATED AT 7°C FROM SOIL SAMPLES ASSOCIATED WITH THE VIKING SPACECRAFT

Sample	Bac.+	C-B	A-A	F-C	Mic. 1	Mic. 7	Mic. 8	Yeasts &Molds	Total
M-1	-#	1.7×10^{5}* (10.0)	0	3.4×10^{4} (2.0)	1.0×10^{5} (6.0)	0	1.4×10^{5} (8.0)	-	4.4×10^{5} (26.0)
M-2	0@	2.1×10^{4} (6.0)	0	-	0	0	7.0×10^{5} (2.0)	-	2.8×10^{4} (8.0)
M-3	0	6.3×10^{5} (8.2)	-	0	-	0	3.5×10^{5} (4.6)	0	9.8×10^{5} (12.8)
K-1	2.9×10^{3} (6.0)	1.9×10^{3} (4.0)	9.6×10^{2} (2.0)	9.6×10^{2} (2.0)	-	1.9×10^{3} (4.0)	-	8.6×10^{3} (18.0)	1.7×10^{4} (36.0)
K-2	6.1×10^{3} (12.0)	4.1×10^{3} (8.0)	2.0×10^{3} (4.0)	2.0×10^{3} (4.0)	-	-	0	0	1.4×10^{4} (28.0)
K-3	9.6×10^{2} (7.4)	1.8×10^{2} (1.4)	0	0	-	9.1×10^{1} (0.7)	-	-	1.2×10^{3} (9.5)
K-4	1.5×10^{2} (2.3)	0	0	0	-	0	-	0	1.5×10^{2} (2.3)
K-5	0	0	1.9×10^{3} (8.3)	-	-	0	-	-	1.9×10^{3} (8.3)

(continued)

Table 9. (continued)

Sample	Bac.+	C-B	A-A	F-C	Mic. 1	Mic. 7	Mic. 8	Yeasts &Molds	Total
K-6	3.2×10^{3} (3.4)	9.6×10^{3} (10.2)	-	0	2.2×10^{3} (2.3)	2.2×10^{3} (2.3)	-	-	1.7×10^{4} (18.2)
K-7	3.7×10^{2} (2.9)	2.1×10^{3} (16.4)	0	-	-	0	-	-	2.5×10^{3} (19.3)
K-8	5.5×10^{2} (8.5)	1.7×10^{3} (26.8)	1.1×10^{3} (16.9)	2.7×10^{2} (4.2)	0	-	-	-	3.6×10^{3} (56.4)
K-9	0	3.5×10^{2} (8.3)	-	8.8×10^{1} (2.1)	-	6.1×10^{2} (14.6)	-	8.8×10^{1} (2.1)	1.1×10^{3} (27.1)
K-10	4.2×10^{3} (22.1)	2.1×10^{2} (1.1)	0	0	-	0	0	9.5×10^{1} (0.5)	4.5×10^{3} (23.7)
K-11	7.0×10^{2} (11.5)	4.2×10^{2} (6.9)	0	2.3×10^{2} (3.8)	-	2.3×10^{2} (3.8)	9.8×10^{1} (1.6)	0	1.7×10^{3} (27.6)
K-12	9.8×10^{2} (0.7)	9.8×10^{2} (0.7)	9.8×10^{2} (0.7)	0	-	-	-	-	2.9×10^{2} (2.1)

*Cells/gm of soil
#This type was not isolated originally.
@This type was isolated originally, but none was psychrophilic.
+Bac. = Bacillus
C-B = Corynebacterium-Brevibacterium
A-A = Alcaligenes-Acinetobacter
F-C = Flavobacterium-Cytophaga
Mic. = Micrococcus

TABLE 10

MEAN NUMBER AND PERCENT OF THE MAJOR GROUPS OF ORGANISMS AND OBLIGATE PSYCHROPHILES FOR ALL SAMPLES FROM THE MANUFACTURE AND ASSEMBLY AREAS OF THE VIKING SPACECRAFT

	Mean No.* (Mean %)	Mean No.Psy. (Mean% Psy.)	Mean No. (Mean %)	Mean No. Psy. (Mean % Psy.)
Bac.@	4.9×10^4 (1.5)	- -	9.3×10^3 (26.1)	1.7×10^3 (4.7)
C-B	2.1×10^6 (63.9)	2.7×10^5 (8.4)	1.8×10^4 (50.0)	1.8×10^3 (5.1)
A-A	9.1×10^4 (2.8)	- -	2.0×10^3 (0.3)	5.7×10^2 (1.6)
F-C	1.6×10^5 (5.0)	1.1×10^4 (0.3)	1.2×10^3 (3.4)	2.8×10^2 (0.8)
Mic.1	1.0×10^5 (3.1)	3.4×10^4 (1.0)	2.6×10^3 (7.3)	1.8×10^2 (0.5)
Mic.7	4.8×10^4 (1.5)	- -	1.5×10^3 (4.3)	4.2×10^2 (1.2)
Mic.8	6.5×10^5 (20.0)	1.7×10^5 (5.1)	1.1×10^2 (0.3)	- -
Fungi	4.6×10^4 (1.4)	- -	8.2×10^2 (2.4)	7.1×10^2 (2.0)
No growth on sub-culture	2.7×10^4 (0.8)	- -	2.1×10^2 (0.6)	- -
TOTALS	3.3×10^6 (100.0)	4.9×10^5 (14.8)	3.5×10^4 (100.0)	5.6×10^3 (15.9)

*Cells per gm of soil
@Bac. = Bacillus
C-B = Corynebacterium-Brevibacterium
A-A = Alcaligenes-Acinetobacter
F-C = Flavobacterium-Cytophaga
Mic. = Micrococcus

the 12 samples from Cape Kennedy contained members of this genus which grew at 3°C, but not at 32°C (0% in K-5 and K-11 to 22% in K-10). The total psychrophilic counts for the Kennedy samples were quite low, ranging from 150 cells/gm of soil (K-4) to 6,100 cells/gm of soil (K-2).

Means for Sample Areas

The means for the three sample sites of the manufacture area and the twelve sites from the assembly area are presented in Table 10(p.66). This table provides a representation of the aerobic population distribution for the two major areas associated with the Viking spacecraft. It indicates that approximately 14.8% (4.9 X 10^5 cells/ gm soil) of the aerobic population originally isolated at 7°C from the manufacture area consists of obligately psychrophilic organisms. Of this population, 14.5% consists of gram-positive rods or cocci, none of which were sporeformers. It also indicates that approximately 15.9% (5.9 X 10^3 cells/gm soil) are aerobic sporeformers. The remaining populations were gram-negative rods.

Dry Heat Studies of Psychrophilic Spores

It has been demonstrated that psychrophilic sporeformers are present in the Cape Kennedy soils from areas which will be used for assembly of the Viking spacecraft. Many of these isolates failed to grow at 32°C on primary

isolation, and it was felt that these may have been largely excluded from the present microbial monitoring systems employed by NASA. For this reason, it was felt that dry-heat studies should be performed on representative psychrophilic sporeformers isolated during these investigations. The organisms selected for this investigation possessed characteristics which would cause them to be of interest to planetary quarantine research. They are sporeformers, they are capable of growth at low temperatures, they are facultative aerobes, and they have demonstrated the ability to grow in a simulated Martian environment.

The experimental runs of this present experiment have primarily been to explore the scope of the problem of survival of psychrophilic spores. The objective of this study was to make a preliminary survey of the survival of those psychrophilic organisms isolated from the soil samples obtained from the assembly areas of the Viking spacecraft. The experimental conditions used during this investigation were 110°C and 125°C at a relative humidity of 50%.

The results of this limited investigation seem to demonstrate a fairly low resistance to dry heat by the selected spores. D_{110}-values range from 7.54 minutes (K-3-27) to 122.45 minutes (K-12-33) while the D_{125}-values range from less than 1 minute (K-3-27 and K-11-123) to 9.78 minutes (K-3-110). It appears the survival of those spores selected and tested are affected rapidly by

dry-heat at 110°C and even more rapidly by dry-heat at 125°C. The results are summarized in Table 21 (p.83). The D-values and y-intercepts of the regression lines, the 95% confidence interval for each, and the intercept ratio (IR) where IR equals log Y_0/logN_0, are recorded in the data table. The IR is a gross measure of the shape of the survivor curve during the initial portion of the heat treatment. If the IR value is greater than 1.0 the curve will be concave downwards (the curve may be thought to have a shoulder). If the IR value is less than 1.0 the initial portion of the curve is concave upwards. The IR value is used to help characterize the survivor curve and to test if the line is linear. An IR value of 1.0 designates a linear line.

Results and comparison of the D_{110}-values of the fifteen spore suspensions are summarized in Table 21, while typical survivor curves are shown in Figures 5(p.73), 7(p.75), and 13(p.81). It is obvious that regardless of which suspension was used, the dry-heat exposure resulted in a significantly lower (about a four-fold reduction) number of survivors. The survivor curve for Spore K-2-38 (Fig. 5) and K-12-33 (Fig. 7) both show a fairly rapid population drop followed by a longer period of slower population decline. Each shows IR values of less than 1.0, which would follow from the definition of the Intercept Ratio. As can be seen by Table 21, seven of the fifteen samples show IR values of less than 1.0. Of these,

spore K-3-27 shows a much lower IR value denoting a fairly pronounced population decrease at the beginning of the heating period followed by a longer slower population decrease. The D_{110}-value of spore K-3-27 is 11.30 minutes. The survivor curve shown in Figure 13 for spore K-3-27 does show this deviation from linearity as predicted by the IR value. The other spores when heated at 110°C showed IR values of greater than 1.0, or had a longer initial population decrease followed by a more rapid population decrease. As seen by the IR values in Table 21, none of these survivor curves have very pronounced shoulders. The IR values range from a low of 1.022 to a high of 1.042, denoting a fairly linear response by the remaining seven spore suspensions. Table 11(p.73) 13 (p.75) and 19 (p.81)are typical data arrays used for analysis and construction of survivor curves. As can be seen, the Y-mean, the standard deviation (S.D.), the upper confidence interval (U-C.I.), and the lower confidence interval (L-C.I) are given for each time interval. For each survivor curve, point to point D-values are also calculated. This is done to help analyze the curves and the total reaction of the spores to the heat system. The point to point D-values can also give an indication of the shape of the survivor curve. In Table 13 (p.75)the point to point D-values indicated that the initial heating period for Spore K-12-33 produced a D_{110}-value of 54.08 minutes with the next three heating periods producing larger and increasing D_{110}-values,

from 113.15 minutes to 146.87 minutes, indicating a decrease in the slope of the line or a slowing down of the spore death rate, followed again by a decrease in the D_{110}-values. The point to point D-values can also show any "tailing" effect, as demonstrated by spore K-12-33 (Fig. 7). The last time interval for spore K-12-33 has a D_{110}-value of 165.15 minutes. This value is much greater than the earlier values, which indicates a lingering spore population. It would appear from this that in establishing a D-value for a particular spore, care must be taken to include this resistant, lingering population to overcome any "tailing" effects that may be present.

Results and comparison of the D_{125}-values are also shown in Table 21 (p.83). Typical survivor curves at 125°C are shown in Figures 9 (p.64) and 11(p.73). D_{125}-values range from less than 1 minute, for spores K-3-27 and K-11-123, to 9.78 minutes, for spore K-3-110. D-values and other data for spores K-3-27 and K-11-123 are not given (Table 21) due to the difficulty of running D-values of less than 1 minute and because it was felt that due to the extremely low resistance of the spores further reruns and tests were not necessary. The IR values range from 0.833 to 1.102. In Fig. 9 the survivor curve for spore K-3-110 appears to be concave upwards and should have an IR value of less than 1.0. From Table 21 it is seen that spore K-3-110 does have an IR value of less than 1.0 (0.926). Figure 11, the survivor curve for spore K-2-38,

shows a survivor curve which is concave downward and should have an IR value greater than 1.0. In Table 21, an IR value of 1.041 is shown for spore K-2-38. Tables 15 (p.77) and 17 (p.79) give the y-mean, standard deviation, upper and lower confidence intervals, and point to point D-values for spores K-3-110 and K-2-38.

After the survivor curves were obtained and analyzed, a regression line was calculated. Figures 6 (p.74), 8 (p.76), 10 (p.78), 12 (p.80), and 14 (p.82) shows typical regression lines at 110°C and 125°C. The final regression lines are computed and drawn by the Biomedical computer. The regression line is calculated using all time intervals except T_0 or N_0. From the regression line, the D-values (the negative inverse of the slope) and the y-intercept (Y_0) can now be obtained. Tables 12 (p.74), 14 (p.76), 16 (p. 78), 18 (p. 80), and 20 (p.82) show summary statistics that can be obtained from the regression line. The final D-value for a specific spore at a specific temperature is taken from the regression line.

Tables 15, 16, 17, 18, and Figures 9, 10, 11, and 12 are examples of typical data obtained from experimental runs at 125°C while Tables 11, 12, 13, 14, 19, 20, and Figures 5, 6, 7, 8, 13, and 14 are examples of typical data obtained from experimental runs at 110°C.

Effect of Particle Size On the Dry-Heat Destruction Rate

The results of these experiments showed a trend

Table 11: Survivor curve data with D-values calculated from adjacent periods for Spore K-2-38 when heated at 110°C

Time(Min.)	Y-mean	S.D.	U-C.I.	L-C.I.	D-value
0	4.492	0.019	4.571	4.412	12.51
2	4.331	0.034	4.477	4.186	15.53
5	4.138	0.034	4.285	3.992	9.22
10	3.596	0.013	3.653	3.539	7.58
15	2.937	0.030	3.067	2.807	14.90
30	1.930	0.056	2.188	1.672	

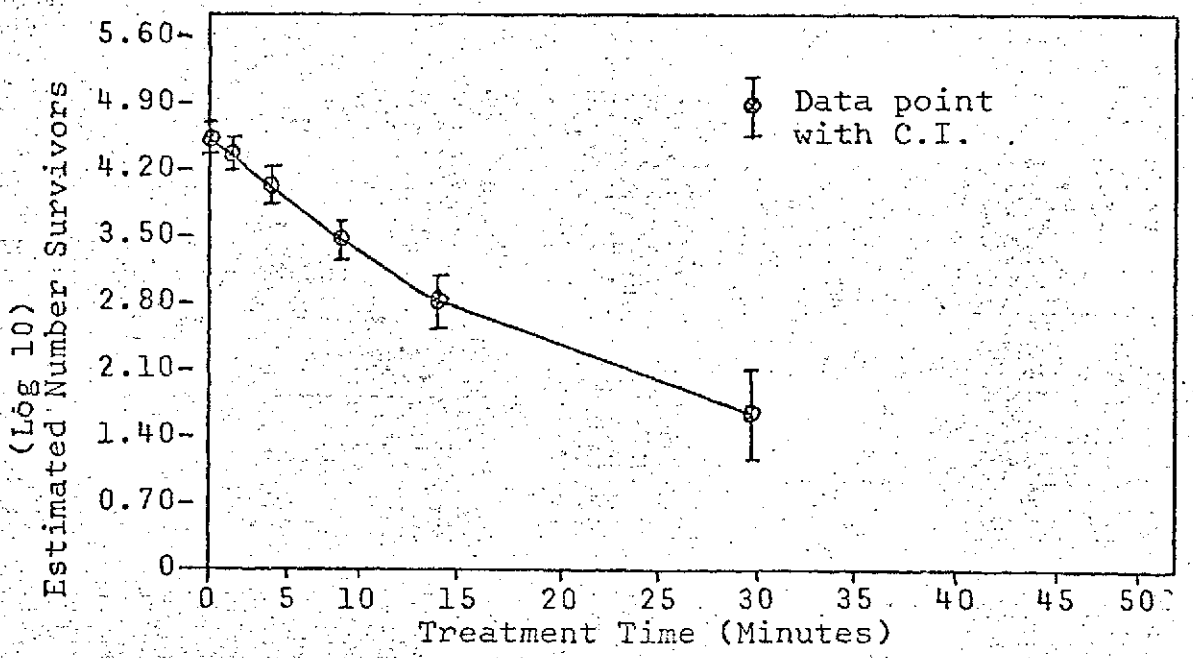


Figure 5: Survivor Curve for Spore K-2-38 when heated at 110°C

Table 12: Summary Statistic for Regression Line Obtained from K-2-38 when heated at 110°C

Summary Statistics *	Value	95% Confidence Limits Lower	Upper
D-value	11.3624	10.4370	12.4678
Slope	-0.0880	-0.0958	-0.0802
Intercept	4.4780	4.3544	4.6015

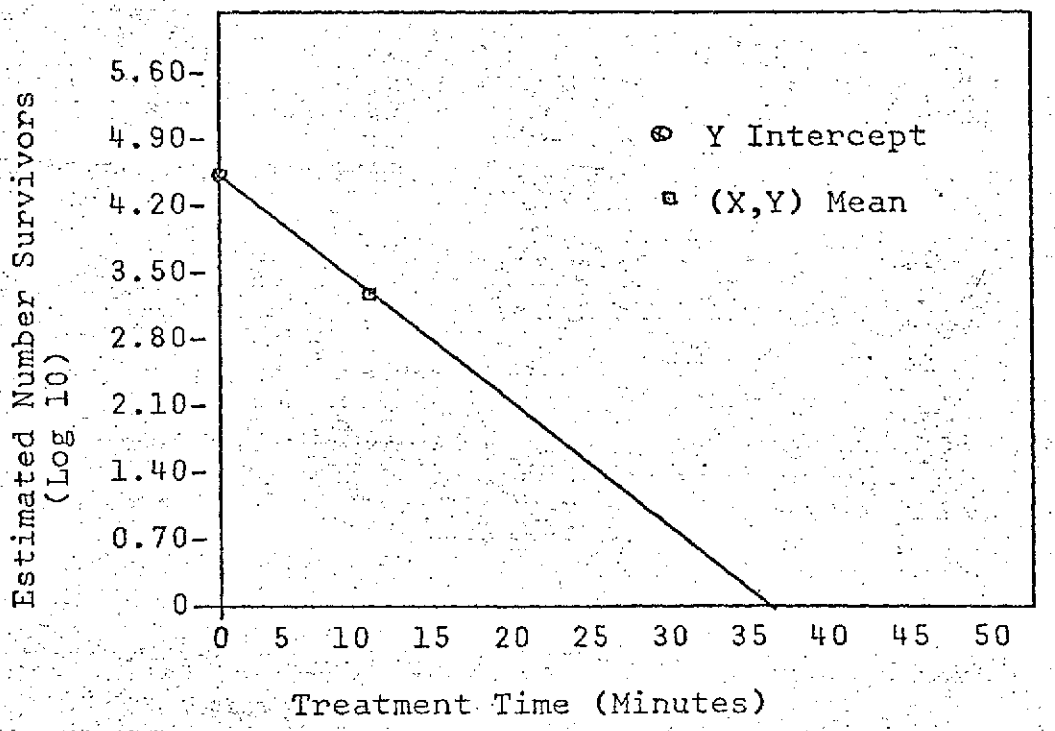


Figure 6: Regression Line for Spore K-2-38 when heated at 110°C

Table 13: Survivor curve data with D-values calculated from adjacent periods for spore K-12-33 when heated at 110°C

Time(Min.)	Y-mean	S.D.(Y-mean)	U-C.I.	L-C.I.	D-value
0	4.906	0.022	5.002	4.810	54.08
30	4.351	0.085	4.717	3.985	113.15
60	4.086	0.028	4.206	3.967	122.33
90	3.841	0.035	3.992	3.689	146.87
120	3.637	0.010	3.681	3.593	136.93
150	3.417	0.036	3.571	3.264	76.15
180	3.024	0.018	3.102	2.945	164.15
240	2.659	0.016	2.728	2.590	

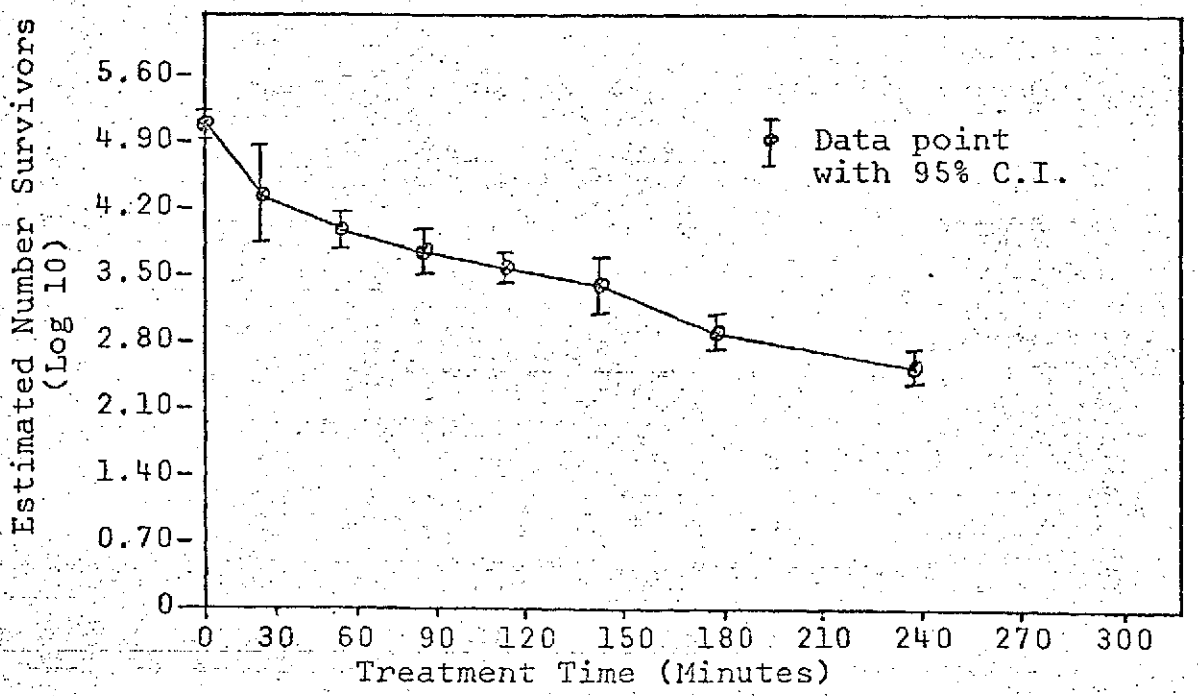


Figure 7: Survivor Curve for Spore K-12-33 when heated at 110°C

Table 14: Summary Statistics for Regression Line Obtained from Spore K-12-33 when heated at 110°C

Summary Statistics *	Value	95% Confidence Limits Lower	Upper
D-value	122.4500	115.1603	130.7329
Slope	-0.0082	-0.0087	-0.0076
Intercept	4.5890	4.5155	4.6615

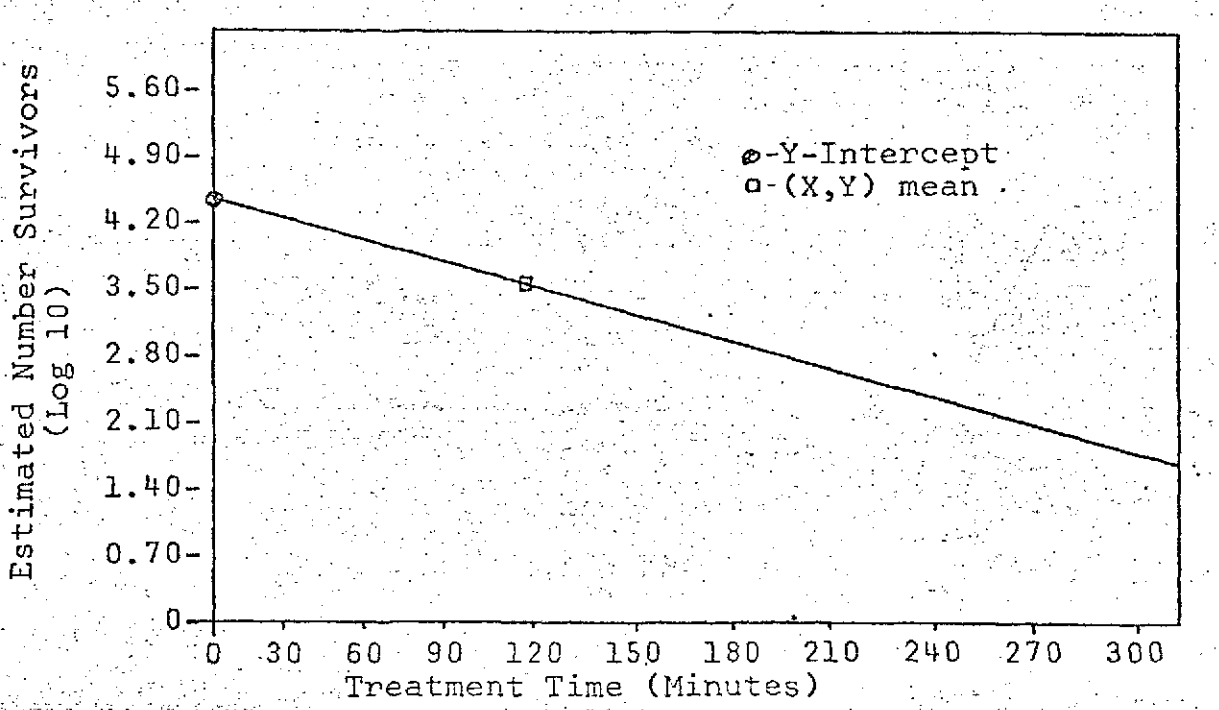


Figure 8: Regression Line for spore K-12-33 when heated at 110°C

Table 15: Survivor curve data with D-values calculated from adjacent periods for spore K-3-110 when heated at 125°C

Time(Min.)	Y-Mean	S.D.(Y-Mean)	U-C.I.	L-C.I.	D-value
0	5.890	0.0159	5.958	5.822	
					6.32
5	5.099	0.0471	5.301	4.896	
					7.02
10	4.387	0.0033	4.401	4.373	
					8.69
15	3.812	0.0034	3.826	3.797	
					12.23
20	3.403	0.0600	3.661	3.145	
					7.35
25	2.722	0.0349	2.873	2.572	
					21.80
30	2.493	0.0424	2.676	2.311	
					9.28
35	1.954	0.0512	2.175	1.734	

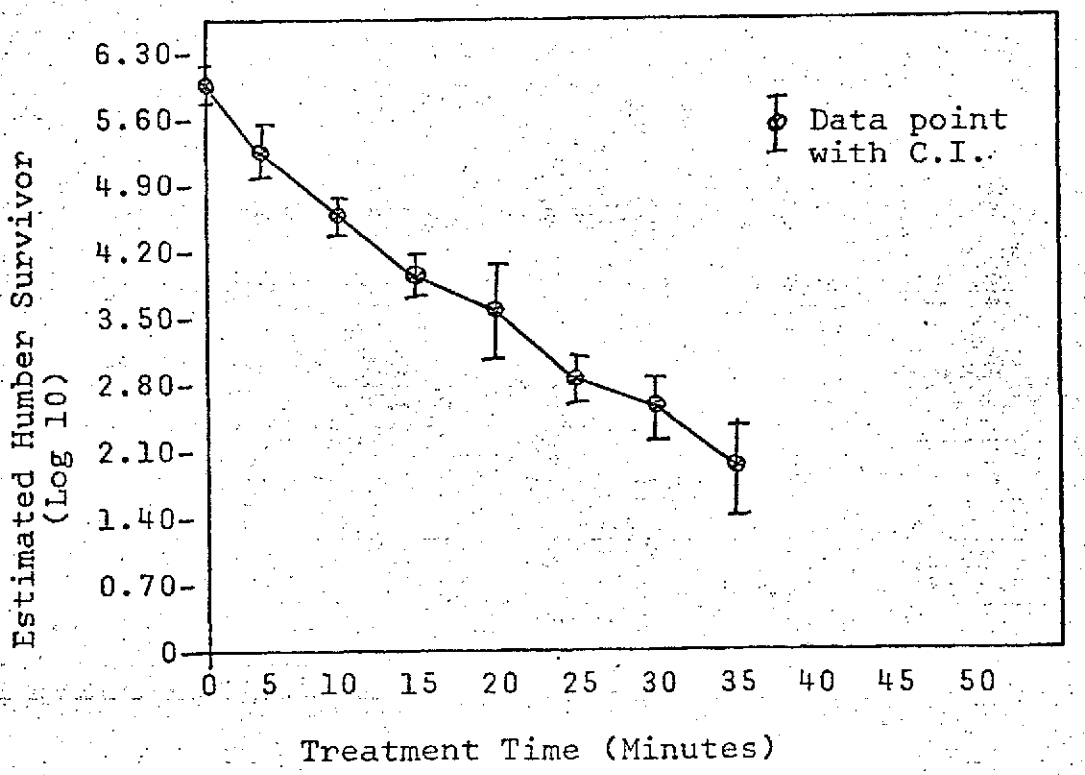


Figure 9: Survivor Curve for Spore K-3-110 when heated at 125°C

Table 16: Summary Statistics for Regression Line obtained from Spore K-3-110 when heated at 125°C

Summary Statistics *	Value	95% Confidence Limits Lower	Upper
D-value	9.7830	9.2410	10.3900
Slope	-0.1022	-0.1082	-0.0962
Intercept	5.4540	5.3200	5.5890

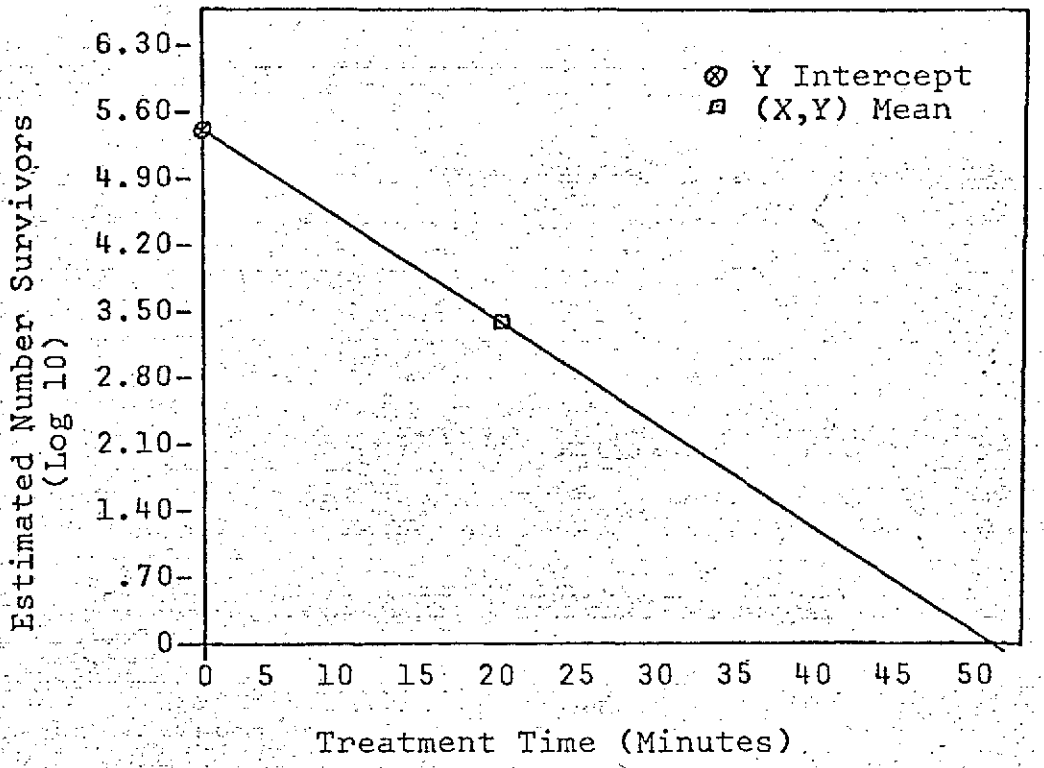


Figure 10: Regression Line for Spore K-3-110 when Heated at 125°C

Table 17: Survivor curve data with D-values calculated from adjacent periods for spore K-2-38 when heated at 125°C

Time(Min)	Y-Mean	S.D.(Y-mean)	U-C.I.	L-C.I.	D-value
0	4.4879	0.01859	4.5679	4.4079	
2	3.5771	0.01736	3.6518	3.5024	2.20
5	1.9353	0.05323	2.1643	1.7062	1.83

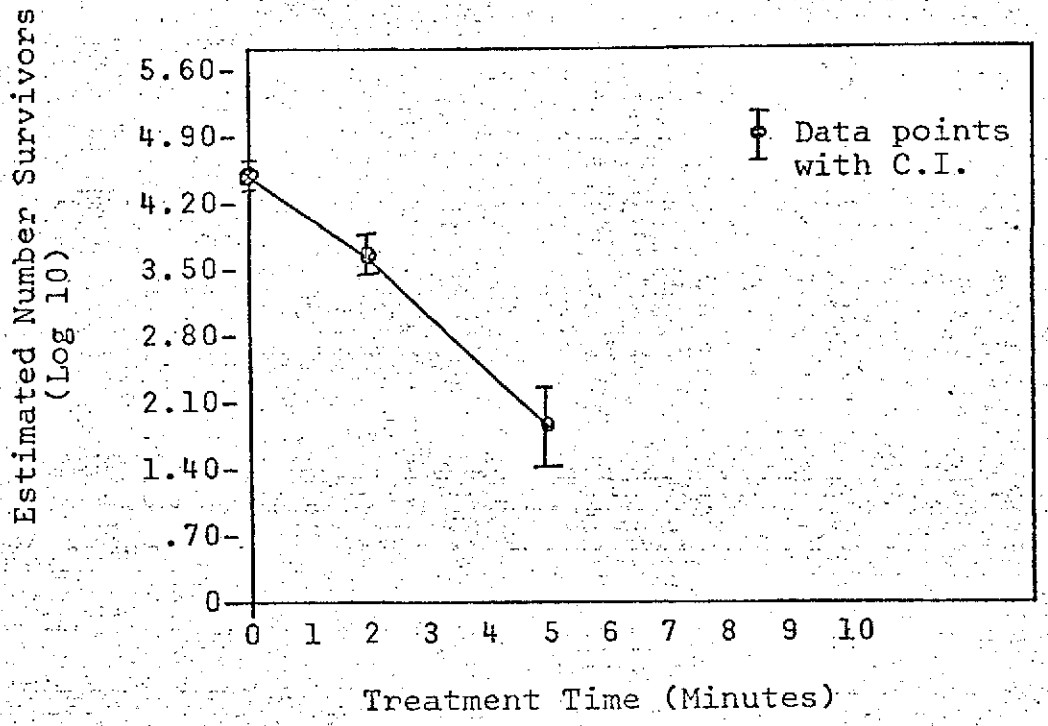


Figure 11: Survivor Curve for Spore K-2-38 when heated at 125°C

Table 18: Summary Statistics for Regression Line Obtained from Spore K-2-38 when heated at 125°C

Summary *	Statistics Value	95% Confidence Limits Lower	Upper
D-value	1.8270	1.6690	2.0180
Slope	-0.5473	-0.5991	-0.4955
Intercept	4.6720	4.4740	4.8690

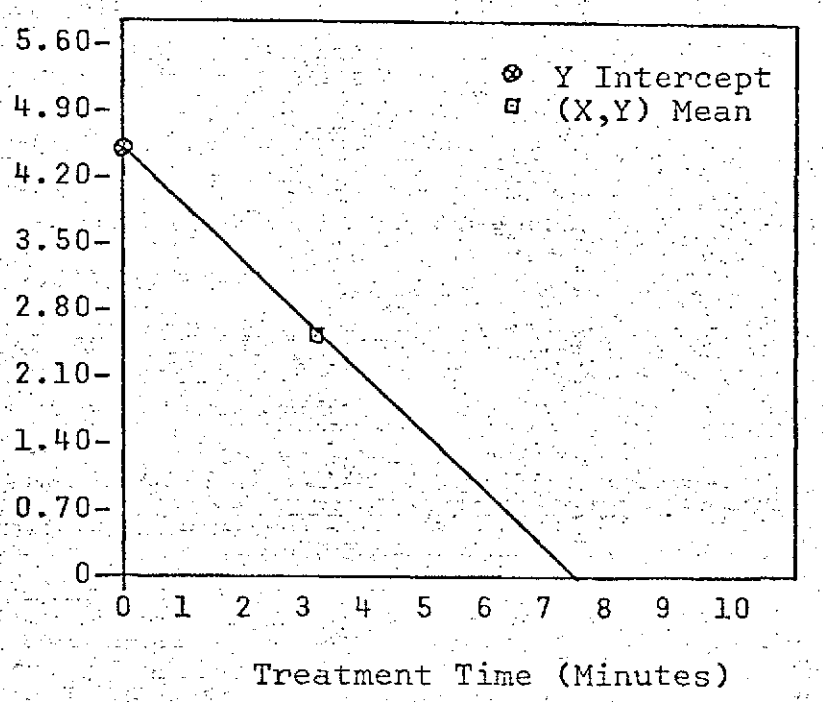


Figure 12: Regression Line for Spore K-2-38 when Heated at 125°C

Table 19: Survivor Curve Data with D-values Calculated from Adjacent Periods for Spore K-3-27 when Heated at 110°C

Time(Min.)	Y-Mean	S.D.(y-mean)	U-C.I.	L-C.I.	D-value
0	6.593	0.0181	6.671	6.515	
					3.73
5	5.253	0.0239	5.356	5.150	
					5.36
15	3.388	0.0403	3.561	3.215	
					9.89
30	1.872	0.0587	2.124	1.619	

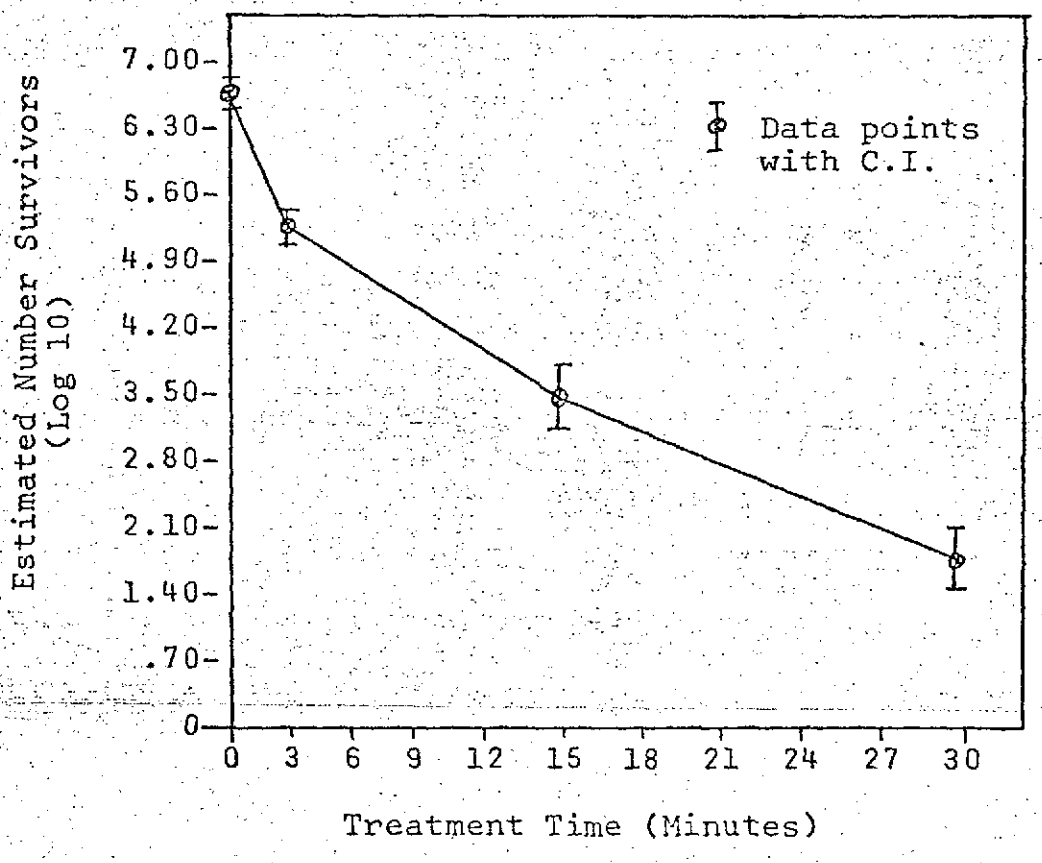


Figure 13 : Survivor Curve for Spore K-3-37 when Heated at 110°C

Table 20: Summary Statistics for Regression Line Obtained from Spore K-3-27 when heated at 110°C

Summary Statistics *	Value	95% Confidence Limits Lower	Upper
D-value	7.5430	6.4880	9.0080
Slope	-0.1326	-0.1541	-0.1110
Intercept	5.7140	5.2920	6.1360

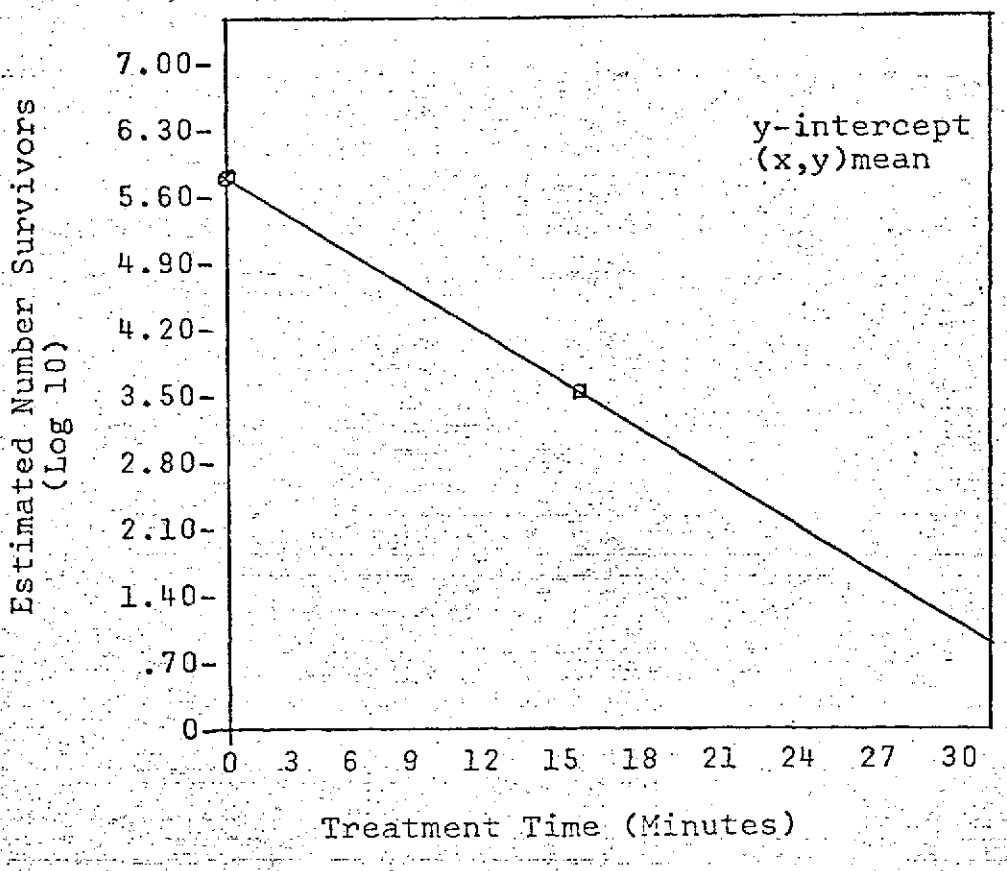


Figure 14: Regression Line for Spore K-3-27 when Heated at 110°C

Table 21: Summary of D-value Data for Pure Culture Psychrophilic Spores Isolated from Cape Kennedy

Sample (Spore)	Temperature °C	Condition %R.H.	D-value Minutes	95% Confidence Limits Lower	Higher	Regression Line N_0	Y_0	I.R.
K-1-6	110°	50%	30.10	28.89	31.42	6.64	6.58	0.991
	125°	50%	4.01	3.55	4.62	6.63	7.11	1.070
K-1-8	110°	50%	21.28	20.15	22.53	6.67	6.82	1.022
	125°	50%	2.99	2.81	3.21	6.68	7.35	1.100
K-2-38	110°	50%	11.30	10.44	12.47	4.49	4.48	0.997
	125°	50%	1.83	1.009	2.018	4.49	4.67	1.041
K-3-27	110°	50%	7.54	6.49	9.01	6.59	5.71	0.867
	125°	50%	--*	--	--	--	--	--
K-3-89	110°	50%	8.10	7.59	8.68	6.20	6.29	1.015
	125°	50%	3.03	2.390	4.118	6.162	5.934	0.9629
K-3-110	110°	50%	43.37	33.55	49.57	6.21	5.95	0.959
	125°	50%	9.78	9.24	10.39	5.89	5.45	0.926
K-4-118	110°	50%	15.74	14.24	17.60	6.96	6.34	0.911
	125°	50%	2.67	2.24	3.29	6.96	5.79	0.833
K-7-140	110°	50%	17.91	16.99	18.91	6.99	7.29	1.042
	125°	50%	3.82	3.52	4.17	6.97	7.69	1.102
K-8-42	110°	50%	34.16	32.39	36.14	7.00	7.28	1.031
	125°	50%	5.21	4.912	5.547	7.05	7.57	1.074
K-10-99	110°	50%	29.81	27.49	32.57	6.90	7.08	1.026
	125°	50%	4.73	4.35	5.18	6.90	7.18	1.041

Table 21 continued.

Sample (Spore)	Temperature °C	Condition %R.H.	D-value Minutes	95% Confidence Limits Lower	Higher	Regression Line N_0	Y_0	I.R.
K-11-38	110°	50%	30.27	27.68	33.40	6.12	6.22	1.016
	125°	50%	3.63	3.33	4.04	6.14	6.35	1.082
K-10-113	110°	50%	33.09	31.67	34.65	6.70	6.18	0.921
	125°	50%	4.70	4.48	4.93	6.89	7.09	1.029
K-11-123	110°	50%	12.175	10.80	13.86	6.53	5.91	0.906
	125°	50%	--*	--	--	--	--	--
K-7-137	110°	50%	22.74	20.32	25.04	6.51	6.74	1.035
	125°	50%	3.70	3.27	4.26	6.44	6.89	1.070
K-12-33	110°	50%	122.45	115.16	130.73	4.906	4.590	0.9352
	125°	50%	6.94	6.56	7.36	6.76	7.31	1.080

* D-values were less than 1.0, therefore no other data could be computed.

for the organisms associated with large particles to be more resistant to dry-heat than those organisms associated with smaller particles. Duplicate dry-heat tests at 110°C and 125°C were conducted on each separation. Each data point is the average of eight boats (800 particles) or of two heating runs. The survivor curves of the particle size experiments are shown in Figures 15(p.87) and 16(p.88). Figure 15 shows the survivor curve of each separation when heated at 110°C while Figure 16 shows the survivor curves when heated at 125°C.

In the analysis of the survivor curve test results it can be observed that there was a consistently larger number of surviving organisms associated with the large particles than with the medium size particles. This same type of relationship existed between the medium and small size particles; there was a larger number of surviving organisms associated with the medium size particles than with the smaller sized particles. The difference appeared at the shortest heating time of 5 minutes and reached a more or less stable condition after 20 minutes. Following heating times of from 20 to 140 minutes for 110°C and 30 to 120 minutes for 125°C a stable pattern developed in which the relative number of survivors was highest for the larger particles and lowest for the smallest particles. There may be some variation but, considering all of the manipulations required, the consistency of this data would indicate that the differences in survival associated with

particle size are real.

In the comparison of the results at 110°C and at 125°C, it can be seen that the survival at 125°C is much less than at 110°C. The initial survivor drop appears greater at 125°C, while both 110°C and 125°C produce survivor curves that appear to level off some after about 15 to 20 minutes. At 110°C particle sizes of <100 >88μm <88 >44μm and <44 >22 showed survivors after 140 minutes, while there were no survivors in the time periods at 125°C. Those particles less that 22μm showed survivors at 110 minutes when heated at 110°C and at only 50 minutes when heated at 125°C. A summary of the particle size data is given in Table 22 (p.89). As can be seen, about 76% of the viable population associated with the large particles survived 60 minutes of heating whereas, approximately 7.8% of the viable organisms associated with the smallest particles survived 60 minutes of heating at 110°C. At 125°C about 52% of the viable population associated with the larger particles survived 60 minutes of heating, while 0% of the viable population associated with the smallest particles survived 60 minutes of heating. D-values were not calculated due to the non-linear response observed from the survivor curves at both 110°C and 125°C. These results seem to demonstrate again a fairly low resistance to dry-heat by psychrophilic organisms.

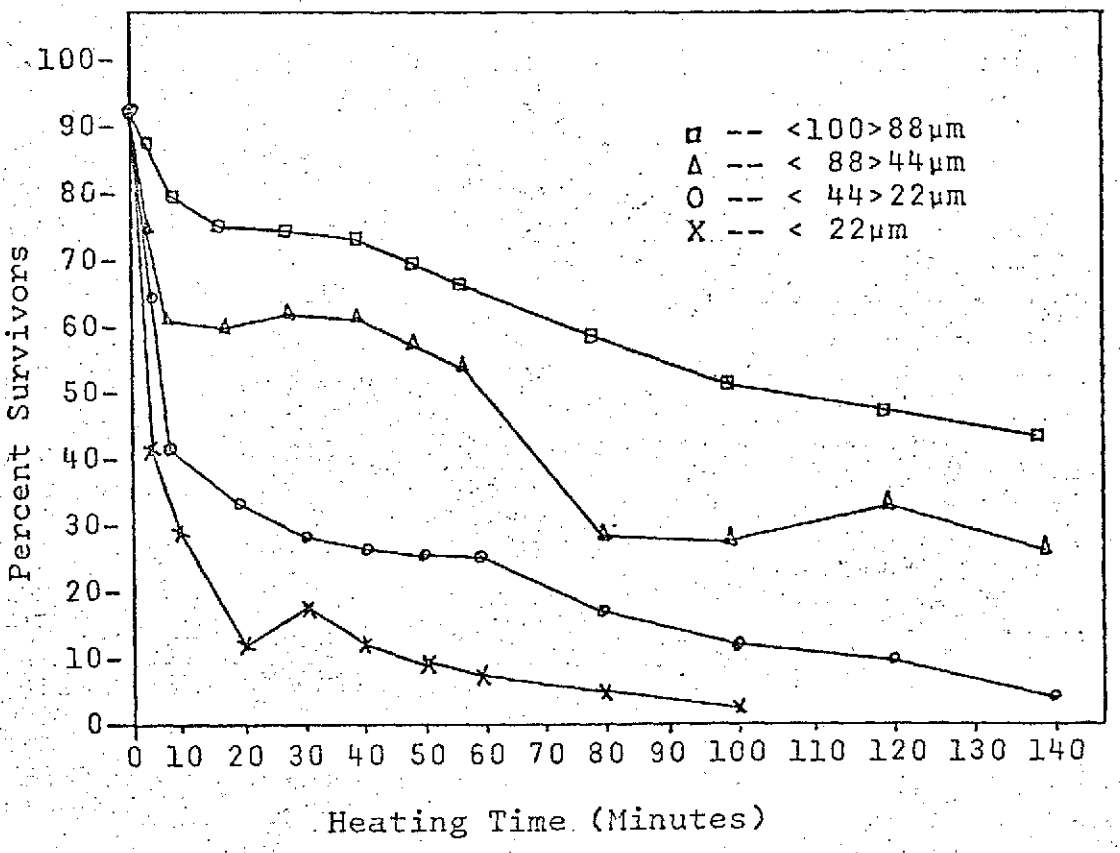


Figure 15: Effect of Particle Size on the Dry-Heat Destruction Rate when Heated at 110°C

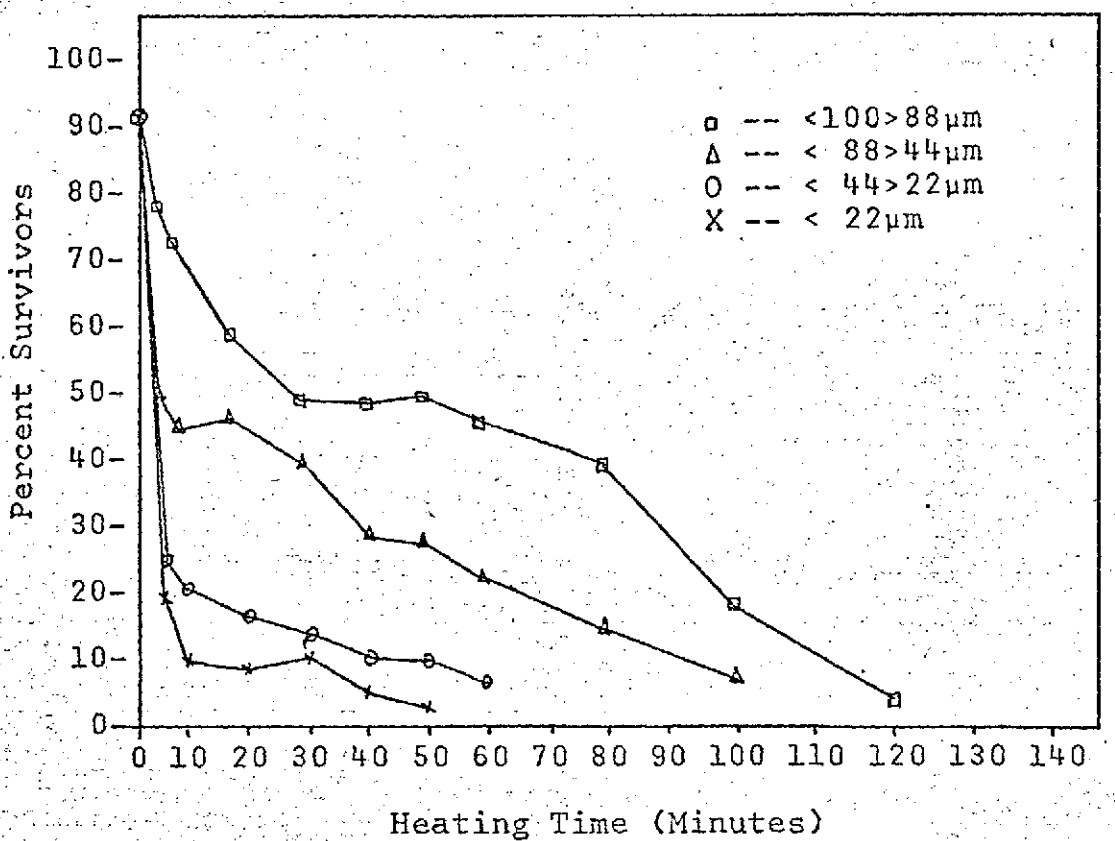


Figure 16: Effect of Particle Size on the Dry-Heat Destruction Rate when Heated at 125°C

TABLE 22

PERCENT SURVIVORS FOR PARTICLES SIZED FOR DIFFERENT EXPOSURE TIMES IN MINUTES AT 110°C AND 125°C

Particle Size	Temp.	30	60	90	120
<100 >88μm	110°C	87	76	63	53
	125°C	55	52	33	43
< 88 >44μm	110°C	71	61	32	40
	125°C	42	24	13	0
< 44 >22μm	110°C	31	29	18	12
	125°C	17	7.8	0	0
< 22μm	110°C	20	7.8	4.4	0
	125°C	10	0	0	0

CHAPTER V

DISCUSSION

The primary objective of planetary quarantine activities associated with the investigation of Mars is prevention of contamination of the spacecraft. Attempts are made to meet this objective to assure that the Martian surface is not contaminated with organisms which might alter the pristine state of the planet and to assure that contaminating organisms on the spacecraft do not interfere with life detection experiments. Because these objectives are so vital to the exploration of Mars, the National Aeronautics and Space Administration has developed detailed standards for the microbial monitoring of the Viking spacecraft which is scheduled to land on Mars in 1976 (77). Intensive investigations have also been performed to determine a terminal sterilization procedure which will accomplish its purpose without interfering with the engineering designs of the spacecraft. Dry heat has been selected as the method of sterilization. (31, 61).

Included in the NASA standards (59) for monitoring the spacecraft is incubation of samples at 32°C. It is known that numerous organisms cannot grow at this temperature, and one objective of this investigation was to demonstrate that these organisms are possibly being

overlooked in the microbial monitoring of the spacecraft. For this reason, all investigations were performed at temperatures below 32°C, and initial isolation was performed at 7°C. From the results of this primary isolation, as presented in Table 4 (p.53), it can be seen that the soil samples from the manufacture area contain high populations of organisms possessing the ability to grow at low temperatures. The samples from Cape Kennedy show populations averaging about two logs less than those from Denver. In all but one sample, it is seen that the aerobic population is the highest. Further investigations, however, show most organisms incubated aerobically and anaerobically were facultative.

Even though these organisms were isolated at 7°C, it was necessary to determine if they could grow at 32°C; therefore, the second objective of this investigation was to determine the percentage of obligate psychrophiles in the samples. According to some definitions, mesophilic organisms usually do not grow below 10°C (78); therefore, it would be expected that all of these isolates should be psychrophiles. On the other hand, Ingraham (45) defines psychrophiles as organisms possessing the ability to grow at 0°C in 10 days. Because this investigation used incubation temperatures of 3°C, 24°C and 32°C, it was necessary to define the term as used in this project. For purposes of this investigation, organisms growing at 3°C in 10-14 days, but not at 32°C are designated as

psychrophiles. As can be seen in Table 6 (p.57), 9 of the original 15 samples contained obligate psychrophiles among their populations, ranging from 15% to 55% of the total isolates per sample. The highest percentages are found in the soil samples from Cape Kennedy. In order to better characterize these samples, the results have been presented in such a way that those who prefer a more rigid definition of psychrophiles can see those results in the second column of Table 6 (p.57).

In the studies related to temperature requirements every colony was transferred from the original, countable plates to TSA slants for incubation at the different temperatures. These investigations included isolates ranging from 50 organisms per sample to 190 organisms per sample, the mean number of isolates per sample being 97. Because isolations were made at 7°C and because several samples showed reasonably high concentrations of psychrophilic organisms, the results presented in Table 6 (p.57) demonstrate clearly that many organisms are not being included by current monitoring techniques.

The temperature of isolation was used to selectively isolate organisms not capable of growth at 32°C. Some investigators (41,42) feel sporeforming bacteria will not grow at this low isolation temperature while others (45) have demonstrated that these bacteria can grow at such a low temperature. This question is of major interest to planetary quarantine because the sporeformers include

organisms which are most resistant to terminal dry-heat sterilization. Therefore, a third objective of this investigation was to determine the distribution of different types of organisms isolated at 7°C from areas associated with the Viking spacecraft and to determine the percentage of these that were obligate psychrophiles. The results presented in Table 8 (p.61), reveal that the majority of organisms making up the populations in several samples are non-sporeformers. However, populations sampled from six of the twelve collections from Cape Kennedy each contain more than 35 percent sporeformers. Although the percentages from the manufacture area are lower, the actual numbers of sporeformers per gram of soil for samples M-2 and M-3 are as great as or greater than those from the Cape Kennedy samples. These results indicate that both types of samples do contain sporeforming populations capable of growth at 7°C.

Even though these isolates did grow at 7°C, it was necessary to determine if any were really psychrophiles according to our definition. From Table 9 (p.64), it can be seen that all of the sporeformers from the Denver samples grow at 32°C. Psychrophilic populations other than sporeformers are demonstrated, but these are less important due to their susceptibility to the terminal dry-heat sterilization cycle. These non-sporeformers cannot be overlooked completely, however, because of the possibility of these organisms being associated with small soil particles which

could afford protection from the dry heat cycle. The samples from Cape Kennedy do contain sporeformers incapable of growth at 32°C and hence, not detected by NASA monitoring standards.

These results can be more clearly seen as composite results in Table 10 (p. 66). This table shows that approximately 15% of the soil populations at the manufacture area are not being selected by current monitoring procedures, but apparently not including sporeformers. Approximately 16% of the soil populations from Cape Kennedy are not assayed by current techniques, and this total includes about 5% which are psychrophilic sporeformers. In other words, it appears that current monitoring procedures may be excluding as many as 1.7×10^3 sporeformers per gram of soil due to the use of the single incubation temperature of 32°C. It is felt that these numbers are probably low because of the single isolation temperature of 7°C. If other isolation temperatures were used, other populations might be demonstrated which are not capable of growth at 32°C. These results are of importance, however, because they demonstrate a large population, including sporeformers, which may not be included in microbial monitoring procedures associated with the spacecraft. Since investigations, such as dry-heat studies, related to planetary quarantine constraints have been performed only on organisms isolated at 32°C, it appears that there are many organisms in the vicinity of the spacecraft which have not been investigated.

Organisms growing below 8°C may possess the characteristics favorable to growth in the Martian environment(40). Therefore, not only may the organisms isolated in this investigation be excluded by current monitoring procedures, but they may also include the organisms best adapted to grow on and contaminate Mars.

Previous investigations on the effects of dry-heat used organisms which were isolated from areas remote from the spacecraft and were either mesophiles or thermophiles. The third objective of this investigation was to make a preliminary survey of the dry-heat resistance of psychrophilic microorganisms isolated directly from the two main areas associated with the Viking spacecraft. The results of this study, as shown in Table 19 (p.81), indicate that pure isolates of psychrophilic spores are fairly sensitive to dry heat at 110°C and even more sensitive at 125°C. D_{110}-values in this investigation ranged from 7.54 minutes to 122.45 minutes while D_{125}-values range from less than 1 minute to 9.78 minutes. I. J. Pflug(21) has reported D_{110}-values working with Bacillus subtilis var niger, of 136 minutes to 167 minutes and D_{125}-values of 16.6 minutes to 32.3 minutes. As can be seen in Table 19 (p.81), only spore K-12-33 approaches these D_{110}-values. Spore K-12-33 has a D_{110}-value of 122.454 minutes while B. subtilis var niger, has a D_{110}-value of approximately 151 minutes. At 125°C, none of the psychrophilic isolates showed D-values approaching those of B. subtilis var niger.

The highest psychrophilic D_{125}-value among our isolates is 9.783 minutes (K-3-110). Bacillus subtilis var niger is now used as one of the standard dry-heat indicator organisms, due to its fairly high resistance to dry-heat. Comparing survivor curves, given in Tables 9-17, and the IR values from Table 19, it can be seen that the heating response appears to be nearly linear. Any deviation that occurs in the survivor curve from a linear response can be related to many factors. The simple logarithmic model requires that the survivor curve be a straight line where $Y_0=N_0$. Failure of an experimentally determined survivor curve to meet this criterion indicates that the data does not completely fit the model. However, the model itself imposes several restrictions on the experimental program;

1. The spore suspension being evaluated must be genetically, chemically, and physically uniform.
2. The condition of the heat destruction test must be constant and identical on a test-to-test basis.
3. The overall handling procedures including the media, incubation temperature, and recovery method must be identical and constant (61).

It is very difficult to completely control all these variables to a degree where the variation will not affect the shape of the survivor curve. When a non-linear survivor curve is obtained the question must be raised whether variations in the experimental protocol are

responsible for the unique shape of the survivor curve or it is due to other factors.

Recent work on the dry-heat resistance of bacterial spores has indicated a critical role for water. Drummond and Pflug(21) showed that the relative humidity of the environment before and during dry-heat exposure may have profound effects on the heat resistance of bacterial spores. For this reason the relative humidity throughout this investigation was controlled and kept at 50%±2. However, no attempt was made to study the effect of relative humidity on the dry-heat resistance of psychrophilic spores. Further investigations should be conducted to establish the psychrophilic response to various dry-heat cycles at different relative humidities.

It has been established experimentally that the dry-heat resistance of psychrophilic spores isolated in the pure state is low. These same psychrophilic organisms when heated in their natural mixed populations with soil particles of various sizes appear to have a high survival ability. The studies of the effect of soil particle size on the survival of microorganisms associated with them suggest that there is a direct relationship between particle size and survival time of microorganisms. Figures 13 (p.81) and 14 (p.82) show the survivor curves at 110° and 125° for organisms mixed with various particle sizes. These survivor curves are non-linear and any real comparison with the D-value curves would be very difficult. Figures 13,

14 and Table 20 (p.82) show a population with lower resistance associated with smaller particles and a more resistant fraction associated with the larger particles. This could mean that the larger particles are giving more protection against heat than are the smaller particles. Other explanations for the suggested increase in survival associated with the larger particles could be that more organisms are associated with the larger particles than with the smaller ones; or there may be differences in chemical composition between the larger and smaller particles. As the population with a specific particle size increases or decreases so will the time needed to obtain sterilization since the total time needed to obtain sterility is not only dependent on temperature but also on the total microbial population.

In summary, this investigation has proved the existence of psychrophilic populations in the soil samples associated with the Viking spacecraft. It has also demonstrated obligate psychrophiles in the samples, including sporeformers, which cannot grow at the current incubation temperature used in microbial monitoring of the spacecraft. However, these psychrophilic organisms demonstrate low dry-heat resistance at 110°C and 125°C. To say that all psychrophiles are sensitive to dry-heat should not be extrapolated from these results, but it would appear that under the present conditions of this investigation, those isolates from the manufacture and assembly

areas of the Viking spacecraft would not survive the presently planned sterilization cycle of a temperature between 110°C and 125°C for 30 hours. From the results of this investigation, it appears that organisms associated with large soil particles are more resistant than those associated with smaller particles.

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