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SPACECRAFT STERILIZATION TRAINING MANUAL

Prepared under Contract No. NAS 8-20083 by
Elizabeth A. Quinn

HAYES INTERNATIONAL CORPORATION

For

NASA-GEORGE C. MARSHALL SPACE FLIGHT CENTER
Marshall Space Flight Center, Alabama

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Hayes International Corporation
Huntsville, Alabama

ABSTRACT

Sterilization training programs for shop personnel were initiated at Marshall Space Flight Center in order to train these personnel to make a greater contribution to spacecraft sterilization at the fabrication and assembly levels. This manual covers the material presented in these training courses.

The manual is designed to provide a basic understanding of the microbiological implications in spacecraft sterilization and to present applicable scientific information in layman's terms. A glossary, review questions, and laboratory exercises are included.

APPROVAL

SPACECRAFT STERILIZATION TRAINING MANUAL

By Elizabeth A. Quinn

The information in this report has been reviewed for security classification. Review of any information concerning Department of Defense or Atomic Energy Commission programs has been made by the MSFC Security Classification Officer. This report, in its entirety, has been determined to be unclassified.

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SPACECRAFT
STERILIZATION TRAINING MANUAL

INTRODUCTION

Man is constantly seeking to learn more about himself and the world he lives in. During the last quarter century, science has made tremendous advancements in fields such as medicine, agriculture, and communications. Man's curiosity has led him to explore all parts of Earth's surface, deep beneath the oceans and out into the void of space through which this planet travels.

The origin of life itself is a question for which men have sought an answer down through the ages. Where did it come from? How did life begin? Is life unique to Earth, or does it exist on other planets? Though many theories have been formed, these questions remain unsolved.

The present-day theory of independent evolution of life states that by a combination of certain chemical substances, life might arise. The theory is that this might occur on any world where a suitable physical and chemical environment has existed for a long enough time. If there are other planets where conditions exist which are suitable for life and where life can be found in its very early stages, this theory might be proven true. Conversely, it could be entirely false. The question exists; man is inquisitive; he wants to know. Scientists today look upon traveling to other planets as a means of gaining this knowledge.

If we are to determine whether life does exist on other planets, we must not carry Earth life forms there during explorations. If life is found on another planet, we must be sure that it was there before we arrived, that it was not carried from Earth and placed there by our spacecraft. If a space probe contaminated with Earth organisms lands on another planet, that planet will become likewise contaminated, and the opportunity to biologically assess it will be lost forever.

In order to prevent spreading organisms from our Earth environment until biological studies of other planets can be made, the National Aeronautics and Space Administration has placed contamination constraints on space probes. The Russian government is also concerned. An international planetary quarantine policy has been adopted. This policy requires sterility of planetary landers. This means that any spacecraft intended to land on another planet must not carry any living organism from Earth.

In order to meet this requirement, physical and biological scientists must work together. Those involved in all aspects of the space program must understand the requirements, the problems involved, the techniques necessary, and cooperate to achieve this goal. This includes personnel involved in the vital tasks of spacecraft component fabrication, subassembly, and assembly. Familiarization with the basic phenomena of biological growth and death and principles of decontamination and sterilization is necessary to solve daily problems of the quarantine effort. One must understand what life is, as we know it, and what must be done to eliminate all life in a certain area or on a piece of equipment. One must be aware that hundreds of millions of organisms exist which are so small we cannot see them, yet they are alive, grow, and reproduce. These are the microorganisms (micro meaning small), and found among them are some of the most resistant known life forms. Some microorganisms can withstand severely adverse conditions which quickly kill other living things.

This training manual is designed to provide a basic understanding of the microbiological implications in spacecraft sterilization. The program is arranged to present applicable scientific information in an understandable terminology. Where and why special techniques must be employed are explained and demonstrated. Laboratory exercises are supplied to demonstrate principles and procedures. Charts, illustrations, tables, review questions, and a glossary are furnished to aid the student.

THE MICROSCOPIC WORLD

History

During the last quarter of the seventeenth century, Anton van Leeuwenhoek, a Dutchman whose hobby was making simple microscopes, made a startling

discovery. While looking at a drop of water with one of his self-made lenses, he observed with wonder many "small animalcules and wee beasties" moving about. Leeuwenhoek's microscopes consisted of a single biconvex lens which he ground and mounted in a metal holder. His curiosity led him to focus his lenses on all sorts of substances. He drew and described microorganisms which he saw in rain water, wine vinegar, pepper infusions, scrapings from his teeth, and excreta. He discovered and described blood corpuscles, spermatozoa, protozoa, yeasts, and many bacteria. Leewenhoek's discoveries stimulated others to study microscopic forms of life. Investigations by men such as Louis Pasteur and Robert Koch disclosed the involvement of microorganisms in fermentation and diseases.

Distribution of Microbial Life

Since the discovery of the existence of microorganisms, investigators have proven that they are found practically everywhere. Huge numbers are found in cultivated soils. The richer the soil in organic matter, the more microorganisms it contains. Most of the microbes in soil are beneficial, aiding in fertilization; some disease-causing types, however, are present in soil, especially soil contaminated with human sewage. In moist soils, the microbic population may be as high as a hundred million or more per gram of soil (1 lb equals 454g). Desert soil may contain a few thousand microorganisms per gram.

All water contains microscopic organisms, many of which come from the soil as water runs over or through it. Falling snow collects dust particles which harbor microorganisms. Deep wells and springs usually have fewer numbers, while most lakes have a high population of bacteria, yeasts, molds, algae, and others. Some microorganisms thrive in water which is nearly boiling: hot springs in Yellowstone National Park contain certain varieties which give the water its color. Conversely, some types live best in cold springs or glacial waters.

The surface of the body, the nose, throat, mucous membranes of the mouth, and digestive tracts of human beings and animals are inhabited by numerous species of microorganisms. Most of these are normal flora, but pathogenic (disease-causing) types may be present. Figure 1 shows some types of microorganisms found in the mouth.

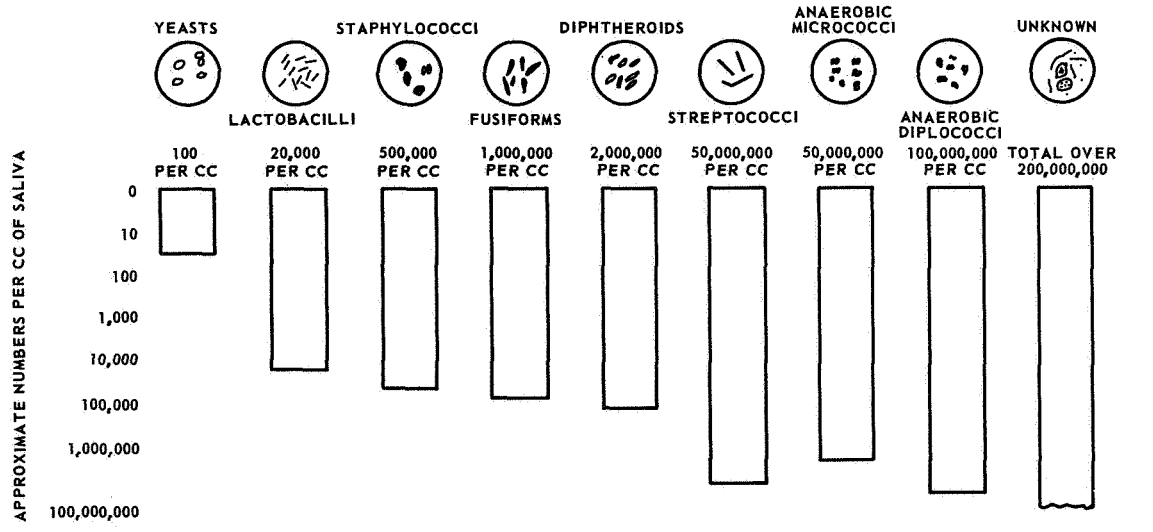


FIGURE 1. COMPLEXITY OF ORAL MICROFLORA

Various insects harbor microscopic organisms on their bodies or within their body cavities and organs. Flies, mosquitoes, ticks, lice, and others often carry pathogenic organisms.

Microorganisms are everywhere: in the air we breath, in the food we eat, on and in our bodies. Although most are so tiny they cannot be seen without the aid of a microscope, they are life forms and share the characteristics of all living things.

Characteristics of Living Things

There are various distinctive ways in which living matter differs from nonliving and which the biologist implements for the description of life itself.

Perhaps the most significant characteristic of living matter is its ability to reproduce itself. At some point in the life cycle, living things give rise to new individuals like themselves or which will become like them through a process of development. This process of reproduction may be sexual or asexual. Sexual reproduction occurs by the combination of two individual organisms of a particular species. Asexual reproduction occurs when the organism alone reproduces itself. Many of the simplest living things reproduce by division of their bodies into two equal parts, each of which continues to live, grow, and reproduce in a similar manner. This is common among the microorganisms.

Another distinguishing characteristic of living things is their ability to carry on metabolism. This process includes all the physical and chemical reactions carried on in order to sustain life. Physical reactions result in the liberation of kinetic energy; chemical changes lead to the formation and elimination of waste materials. To replace materials used and provide a source for more kinetic energy, food is taken into the organism. Thus ingestion, digestion, circulation, assimilation, respiration, and elimination of wastes are all part of the metabolic process.

A third common characteristic of living things is growth, or the orderly increase of all the components of living substance. In all cellular organisms, cell multiplication is a consequence of growth. All living organisms have a natural size limit; as this limit is approached, changes occur which result in cell division. In multicellular organisms, cell multiplication leads to an increase in size of the of the individual; whereas in unicellular (one-cell) organisms, it results in an increase in the number of individuals.

A fundamental trait of all living things is irritability, or the capacity to respond to internal or external stimuli. All living matter is irritable to some extent. Environmental agents such as light, sound, gravity, pressure, temperature, moisture, salinity, acidity, alkalinity, etc., may act as stimuli. Frequently, the response to environmental change is of an adaptive nature; the organism accommodates itself to the change.

Bio-Organization

Living things are biologically classified into three major divisions: the animal kingdom, the plant kingdom, and the Protista. The principle differences between the plants and animals are listed in Table I.

TABLE I. CHARACTERISTICS OF PLANTS AND ANIMALS

Plants
Cells contain chlorophyll
Not actively motile — cannot move at will
Rigid cell walls
Food stored principally as starch
Energy source is photosynthesis
Animals
No chlorophyll
Actively motile — can move at will
Flexible cell membranes
Food stored as glycogen, fat
Energy source is organic matter

Figure 2 illustrates the fundamental classification of living things.

The Protista are made up of a group of organisms standing between the two classical kingdoms in their properties. This group is comprised of a multitude of transitional forms, some showing both

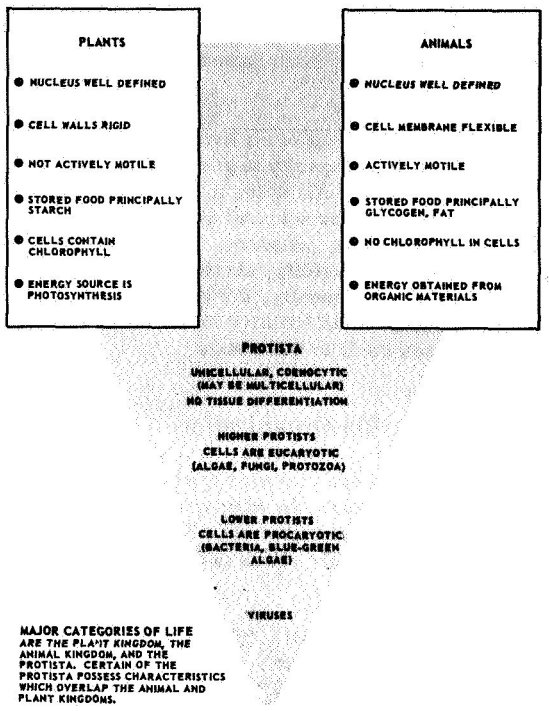


FIGURE 2. BIO-ORGANIZATION

plant-like and animal-like characteristics. Generally, this is the division into which microorganisms are placed. What distinguishes all members of the Protista from higher plants and animals is their relatively simple structure. Many are one-celled, and those that are multicellular lack the differentiation into separate tissues characteristic of the higher animals and plants.

The component groups of the Protista are the algae, the fungi, the protozoa, the bacteria, and the viruses.

The algae are simple plant-like organisms without roots, stems, or leaves. Most algae possess chlorophyll, the green pigment which enables them to produce their own food by photosynthesis. Photosynthesis is the process by which green plants convert carbon dioxide and water, in the presence of sunlight, to carbohydrates, which they use as food. Algae are aquatic and range in size from microscopic unicellular (one-celled) forms to seaweed several feet in length.

The fungi are plant-like forms ranging in size from single-celled, microscopic yeasts to giant multicellular mushrooms and smut balls. The fungi lack chlorophyll, and may be parasites (living on and getting their nourishment from other living organisms) or saprophytes (living on and getting their nourishment from dead organic matter). They reproduce by fission, budding, or by spore formation in spore sacs or stalk-like structures. Fungal spore formation may be asexual or by the combination of two cells, but sexual spores are produced less frequently and in smaller numbers. The structure of some types of fungi and their reproductive structures, or fruiting bodies, is shown in Figure 3. This type of spore formation is for reproductive purposes and should not be confused with bacterial spore formation which is not a means of reproduction.

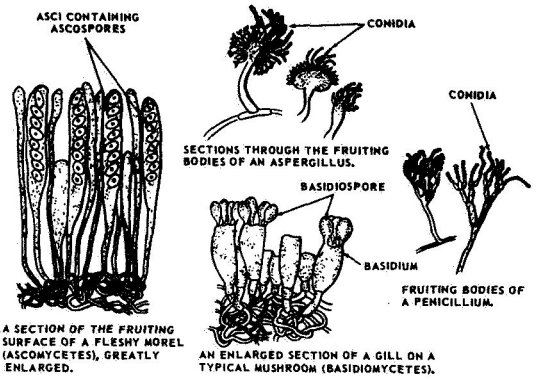


FIGURE 3. STRUCTURE OF SOME TYPES OF FUNGI

Protozoa are simple animal-like organisms. Most lack chlorophyll; some are parasitic, others are free-living and move about capturing and absorbing bits of food. They are predominately microscopic and unicellular.

The bacteria are the smallest, most primitive living microorganisms. They include both plant-like and animal-like species, though most of them are nonphotosynthetic (cannot produce their own food by photosynthesis). Most bacteria are unicellular; many types possess motility, the ability to move about. These are probably the most widely distributed of all living organisms: they are present

in the atmosphere up to four miles above the earth, and have been found in mud three miles beneath the surface of the sea. Some of the most resistant life forms are found among the bacteria. Some cause diseases, such as diphtheria, scarlet fever, pneumonia, tuberculosis, gonorrhea, syphilis, undulant fever, bubonic plague, epidemic meningitis, and tetanus. However, certain types are beneficial in that they aid in soil fertilization, produce useful chemicals by their metabolic activities, and aid in manufacture of dairy products.

The viruses, an extremely interesting and controversial group, are obligate parasites that exhibit characteristics of life only when present inside living cells of a host organism. They multiply only in living tissues or cells, and are very specific in selection of a host. As yet viruses cannot be cultivated in artificial media. The known viruses are disease-producing agents and are so small that they cannot be seen with the best compound microscope. Considerable controversy exists concerning the nature of viruses. One theory is that viruses are living, parasitic, ultramicroscopic cells. The opposing theory states that they are non-living chemical substances having no cell structure. In general, methods which kill or inhibit bacteria, such as heat, ultraviolet light, or certain disinfectants, will have the same effect on viruses. Known viruses are less resistant to adverse conditions than bacterial spores.

Table II summarizes the characteristics of major groups comprising the Protista.

TABLE II. CHARACTERISTICS OF MAJOR GROUPS WHICH COMPRISE PROTISTA

Group	Size Range	Characteristics	Role in Environment
Bacteria	0.5 - 50 microns	Many are motile. All are unicellular. Grow on artificial media. Reproduction is predominantly asexual by binary fission.	Some are agents of disease. Some improve soil fertility and are useful in industrial chemical processes, and others produce antibiotics.
Viruses	10 - 300 millimicrons	Many pass through the finest filters. Grow only in living cells. Are obligate, intracellular parasites.	Cause diseases in man, animals, and plants. Some infect bacteria.
Yeasts	5 - 10 microns	Grow on artificial media. Reproduce by asexual budding or by sexual processes.	A few cause diseases in man. Many are used in industrial fermentation processes for production of alcohols and other organic chemicals.
Molds	5 microns to much larger	Grown on artificial media. Are multicellular. Reproduce by both sexual and asexual means.	Many are used in fermentation industry and in food production. Some cause deterioration of wood, cloth, and other materials. Many are beneficial in the soil. Some cause disease. Others produce valuable antibiotics.
Protozoa	2 - 20 microns	Unicellular animal-like organisms. Many are motile. Some are intracellular parasites. Most protozoa grow on artificial media. Reproduce by both sexual and asexual means.	Many protozoa are active soil forms or are food for aquatic animals. Some cause diseases in man and animals.
Algae	1.0 microns to many feet	Are photosynthetic forms, cells contain chlorophyll. Many are multicellular. Most are aquatic species. Reproduce by sexual means or a variety of asexual processes.	Source of food for aquatic animals

Review Questions

1. When Leeuwenhoek first saw microorganisms with his lenses, what terms did he use to describe them?

2. Could you expect to find any microorganisms in a sample of sand from the Sahara Desert?

3. Why would you expect to find fewer microorganisms in water from a deep well than in water from a shallow lake?

4. What is a pathogenic microorganism?

5. What is a common insect which carries disease-causing microorganisms?

6. What is perhaps the most significant characteristic of living things?

7. Name three additional characteristics of living things.

8. In multicellular organisms, growth leads to an increase in _____.
In one-celled organisms, it results in an increase in _____.

9. What are the three major divisions of living things?

10. Some Protista are animal-like; some are plant-like. What is the major factor that distinguishes all Protista from higher plants and animals?

11. Explain the difference between fungal spore formation and bacterial spores.

12. What group of Protista contains some of the most resistant known life forms?

13. Explain why it is thought that viruses are not living organisms.

It is suggested that at this point the student perform Laboratory Exercises I and II, which are described in detail in Appendix C at the end of this manual.

CHARACTERISTICS OF BACTERIA

We have stated that the most resistant known life forms are found among the bacteria. Certain bacteria survive in water near boiling temperature. Thus, a further understanding of bacterial characteristics is necessary for sterilization considerations.

Bacterial Cells

The smallest unit of matter which exists as a living, individual organism is called a cell. The main structural components of a typical bacterial cell are shown in Figure 4.

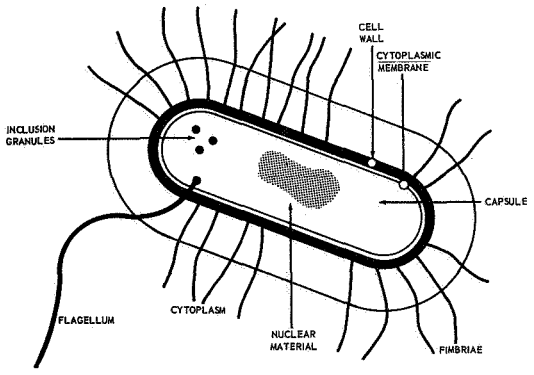


FIGURE 4. DIAGRAM OF A TYPICAL BACTERIAL CELL

A gelatinous, slimy material surrounding some bacteria is called the capsule. Composed of complex chemical compounds, the capsule serves to protect the cell against drying and to hold groups of cells together. It may also serve the cell as a reservoir for stored food or a site of waste disposal. The presence of a capsule on some bacteria increases their infective capacity.

The cell wall is the principle containing structure and gives the cell its shape. The cell wall is

sufficiently rigid to resist distortion and disintegration, and thus provides additional protection to the cell.

Immediately beneath the cell wall is the cytoplasmic membrane. This is a delicate, flexible, semipermeable membrane that controls the passage of nutrients and waste products into and out of the cell. Harsh physical or chemical treatment may result in damage to the cytoplasmic membrane and death of the cell.

Inside the cytoplasmic membrane is the cellular material, or cytoplasm. Colloidal in nature and having a high water content (70 to 85 percent), it contains the cell nucleus and inclusion granules. Concentrated deposits of volutin (ribonucleic acid or polyphosphate), glycogen or iogen (starch-like substances), and fat or sulfur globules are called inclusion granules. These may serve as a source of food or energy for the cell.

The cell nucleus or nuclear material, discernible because of its greater density and unique composition, is the control center of the cell. Regulation of all the life processes of the cell comes from the nucleus. It passes on the genetic information during reproduction. The nuclear material is composed of deoxyribonucleic acid.

Flagella are extremely thin, hair-like appendages that originate in the cytoplasm and protrude through the cell wall. They are proteinaceous and usually several times as long as the cell. These organelles provide the cell with a means of motility; by a waving or whiplike motion of the flagella, cells can move about in a liquid medium. Some very active flagellated bacteria can swim about in very thin films of moisture on the surface of a solid medium. Not all bacteria possess flagella, but those with flagella exhibit a definite pattern and number of appendages. Arrangement of flagella and their number is one criterion for identification of bacterial species. Figure 5 illustrates typical positions of flagella.

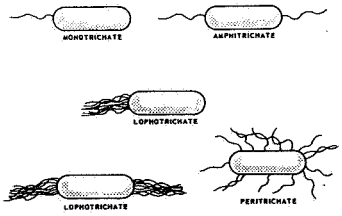


FIGURE 5. SCHEMATIC ILLUSTRATION OF MANNER IN WHICH FLAGELLA MAY BE ATTACHED TO BACTERIAL CELLS

Bacterial Shapes, Sizes, and Forms

The cells of bacteria exist in varied shapes and arrangements. Coccus or spheroidal forms are common and may occur singly or in pairs (diplococci), chains (streptococci), groups of four (tetracocci), clusters or irregular numbers (staphylococci), or cuboidal groupings (sarcinae).

Bacillus or cylindrical, rod-shaped forms do not arrange themselves in groups as do the cocci, but appear as single, unattached cells.

Spirilla, spiral-shaped bacteria, also occur as single unattached cells. These range from wavy, twisted, and curved forms to tightly coiled ones. Comma-shaped bacteria are the vibrios.

Bacterial shapes and patterns of grouping are an important means of identification. Figure 6 shows common bacterial shapes.

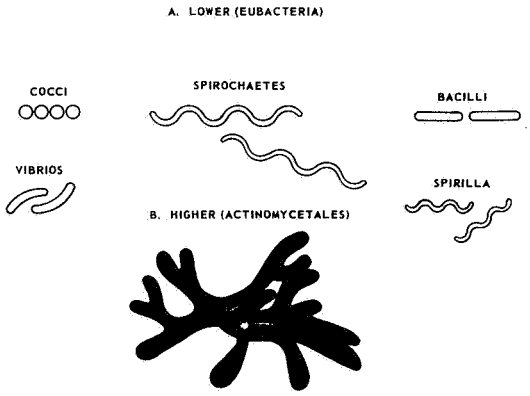


FIGURE 6. PRIMARY CLASSIFICATION OF BACTERIA

Because bacteria are extremely small, their size is expressed in microns (one micron is one thousandth of a millimeter). Their dimensions can be measured by a microscope equipped with a special attachment. Cocci range in size from 0.5 to 1.0 micron. The bacilli average 0.5 micron in diameter and 1.5 microns in length. A volume of one cubic inch could contain approximately nine trillion bacilli. It would take 50 000 cocci of about 0.5 micron in diameter laid side by side to measure one inch.

The weight of a bacterium is very small. An individual bacillus of average size weighs approximately

2×10^{-12} grams, which means that one gram of undried cell substance would contain about 500 000 000 000 cells. Yet one acre of fertile soil one foot deep may contain 300 to 500 pounds of bacterial cells.

Figure 7 shows the relative sizes of matter.

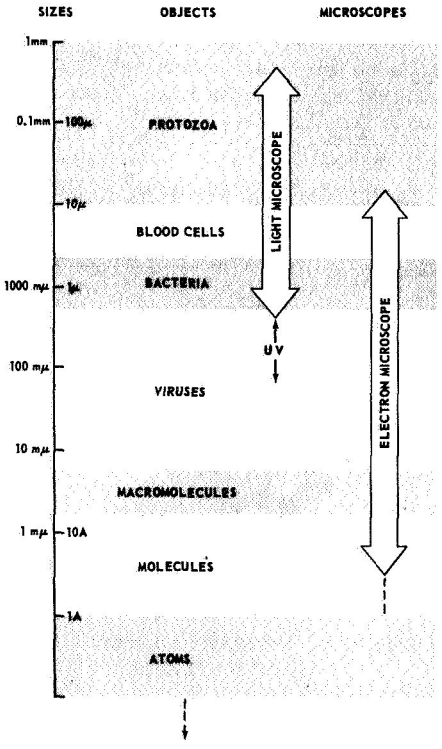


FIGURE 7. RELATIVE SIZES OF MATTER

When bacteria are cultured, they exhibit distinguishing growth characteristics. Masses of cells in colonies, on slants, in stab cultures, or in broth medium present different appearances according to their type. Various cultural traits of bacteria are represented in Figure 8. These morphological features are constant if environmental conditions remain uniform.

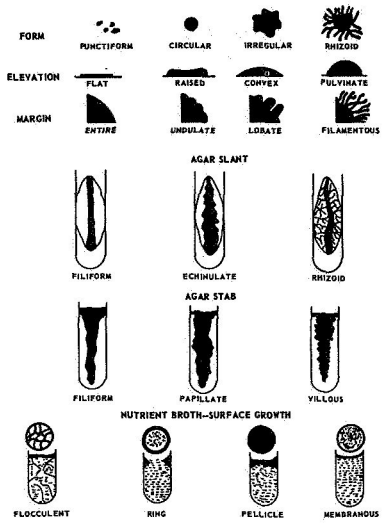


FIGURE 8. CULTURAL CHARACTERISTICS OF BACTERIA

Examination of bacterial colonies usually provides the first data for bacterial identification. A colony may originate from a single cell or from a clump of cells in or on solid culture medium. Colonies imbedded in the medium are called subsurface colonies. Colony form may be punctiform (under one millimeter in size), circular, regular, or rhizoid. They display a variety of elevations (flat, raised, connex, pulvinate), optical properties (colored, opaque, translucent, transparent), and surfaces (smooth, rough, dull, glistening, dry, moist, ringed, ridged). Margin appearance also varies.

Bacterial growth on agar slants and in agar stab cultures shows diverse forms, as does growth in liquid broth media.

Morphology (size, shape, and form) is one of many criteria for classification and identification of bacteria. Figure 9 shows some additional ones.

Reproductive Processes

Bacterial reproduction may be sexual or asexual. Asexual reproduction is by far the more predominant. This asexual process is known as binary fission. Essentially, it consists of the splitting of a single cell into two daughter cells, and it occurs in an orderly sequence of events.

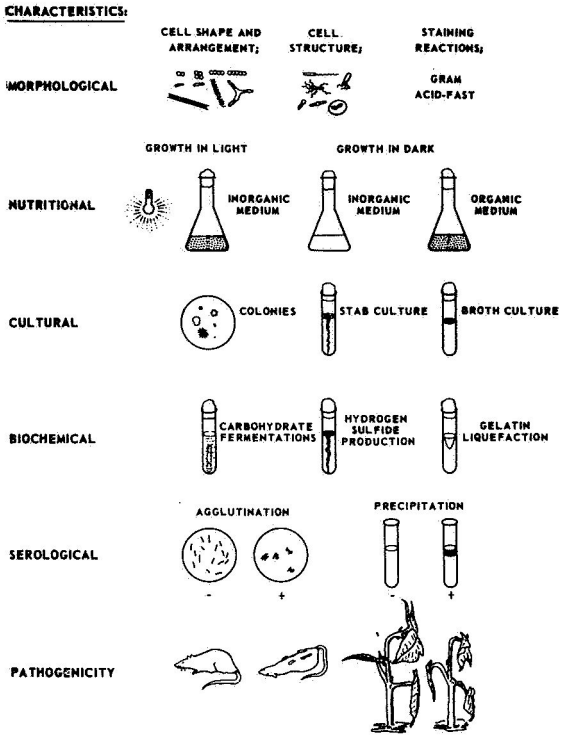


FIGURE 9. SCHEMATIC DIAGRAM ILLUSTRATING GENERAL PLAN TO DETERMINE CHARACTERISTICS OF BACTERIA

Binary fission takes place in two progressive sequels — nuclear division, known as mitosis, and cytoplasmic division. Nuclear division results in an equal distribution of the genetic information contained in the nucleus. Mitosis may be described as a series of stages or phases which are in reality part of a continuous process. Figure 10 shows the mitotic stages.

Figure 10A shows a resting cell with fine, filamentous chromatic material in the nucleus. During the prophase (Fig. 10B and 10C), the nuclear material becomes organized into visible threads, or chromosomes, which bear the genetic specifications for the cell and its descendants. The chromosomes become arranged in the center of the nuclear region and then split longitudinally, thus duplicating themselves. After the chromosomes have duplicated, the metaphase (Fig. 10D) occurs, in which they become

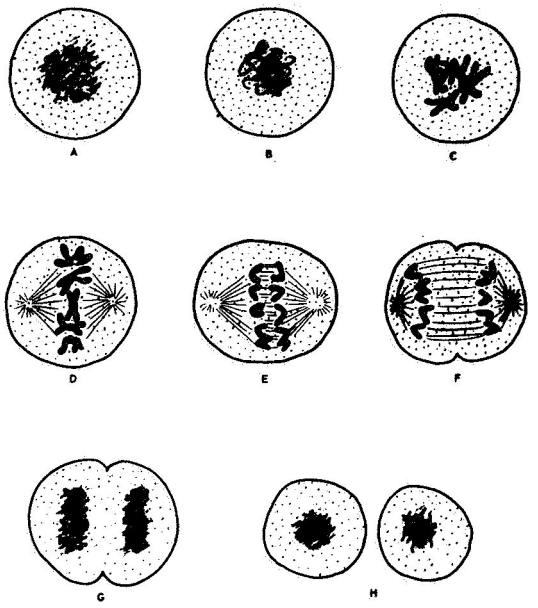


FIGURE 10. STAGES OF NUCLEAR AND CELL DIVISION BY MITOSIS

aligned along the cell's center. At this time, a spindle-shaped structure of protein fibrils develops. This stage is succeeded by the anaphase (Fig. 10E and 10F), during which the duplicate chromosomes gradually separate and move toward the two poles of the spindle. In the final phase or telophase (Fig. 10G), the chromosomes reach the poles, are reorganized into two daughter nuclei, and the spindle disappears. It is in this manner that the two daughter cells become exactly like the parent cell.

Toward the completion of the telophase, the second sequel, cytoplasmic division, begins. A furrow appears between the nuclei and becomes progressively deeper until the two halves become separate (Fig. 10H).

Bacterial Growth

The term "growth," as applied to microorganisms, means an increase in the number of cells. Increase in population originating from a single cell occurs by geometric progression: $1 \rightarrow 2 \rightarrow 4 \rightarrow 8 \rightarrow 16 \rightarrow 32$, etc. The time interval required for the population to double is called the generation time. Generation

times among the bacteria vary from as little as 12 minutes to as much as several hours, according to the species. The generation time of a particular species may be altered by changes in the amount and kind of nutrients available and changes in the environmental physical conditions.

A growing culture may contain only a few cells at the beginning and many billions of cells at the end. Table III shows the exponential growth of a bacterium with a generation time of 20 minutes.

TABLE III. EXPONENTIAL GROWTH OF A UNICELLULAR ORGANISM WITH GENERATION TIME OF 20 MINUTES

Time (min)	Number of Divisions	Number of organisms		
		Number	Log_2	Log_{10}
0	0	1	0	0.000
20	1	2	1	0.301
40	2	4	2	0.602
60	3	8	3	0.903
80	4	16	4	1.204
100	5	32	5	1.505
120	6	64	6	1.806
140	7	128	7	2.107
160	8	256	8	2.408
180	9	512	9	2.709
200	10	1024	10	3.010

Bacteria inoculated into fresh media and provided with suitable conditions for development pass through a series of growth phases that may be graphically represented as the normal growth curve. Figure 11 shows the typical growth curve of a bacterial culture.

After addition of inoculum to a new medium, there is a period, the lag phase, during which the population remains constant. This is because cells placed in a new environment take time to become accustomed to it before they start to multiply. Length of the lag phase is dependent upon the condition of the inoculum. If the cells are old or are in the spore state, the phase will be comparatively long. An inoculum of young active cells exhibits a shorter lag phase.

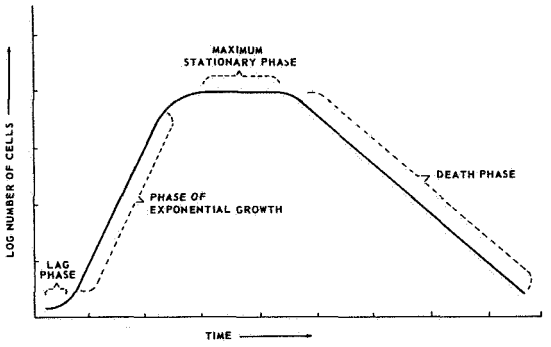


FIGURE 11. GROWTH CURVE OF A BACTERIAL CULTURE

During the logarithmic or exponential phase of growth the cells divide steadily at a rate determined by the generation time of the organism. The rate of growth during this phase is maximal for the organism under the particular conditions of cultivation. All of the cells produced here are viable.

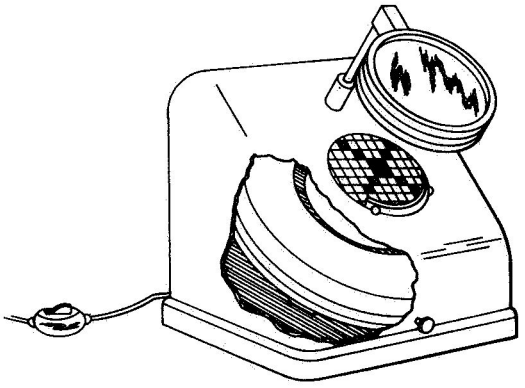
As nutrients become exhausted and toxic metabolic products accumulate, a tapering off takes place. This is termed the stationary phase. Growth rate decreases and eventually a balance between reproduction rate and death rate is reached.

Following the stationary period, the bacteria die at a rate exceeding that of production of new cells. Growth soon ceases and the culture dies. This is the death phase.

The pattern of normal growth for microorganisms may be implemented for predicting the approximate time required for a bacterial species to attain its maximum population under given conditions. For many types of experiments, it is desirable to keep the cells in the exponential phase for prolonged periods, and this can be done by repeatedly transfering the cells to fresh media.

There are several methods for quantitatively measuring bacterial growth, or determining the actual number of cells in a bacterial population. Direct microscopic examination of stained smears, measurement of cell mass by turbidimetric or light scattering techniques, and colony count are common methods. The first two have a disadvantage in that they provide no way to distinguish between viable and nonviable organisms. The colony counting technique is based on the principle that each viable organism

will give rise to one colony. Of course, an aggregate of cells will also give rise to a colony, and resulting counts will be lower than the true number of individuals. Good technique, adequate dilution of the inoculum, and application of clump-breaking procedures such as ultrasonication can be used to obtain separation of the cells. The specimen is usually diluted so that the number of colonies developing on the plate will be within the range of 30 to 300. Within this range the count can be accurate, and the possibility of the growth of one organism interfering with that of another is minimized. Colony counting is facilitated by an instrument combining magnification with an illuminated ruled background (Fig. 12).



PLATES ARE PLACED IN POSITION ABOVE THE RULED PLATFORM AND VIEWED THROUGH THE MAGNIFYING LENS.

FIGURE 12. A BACTERIAL COLONY COUNTER

Bacterial Spores

When environmental conditions become unfavorable for growth, some bacteria undergo transformation to dormant, highly resistant bacterial spores. The bacterial spore is a resting stage of a normal vegetative cell. It has remarkable resistance properties and is capable of remaining viable for extremely long periods. Dried spores have been known to survive for 50 years under adverse conditions. They are highly resistant to physical and chemical agents which kill vegetative cells almost immediately. Heat resistance of bacterial spores is so great that some can survive in boiling water for several hours. No other living cell possesses this degree of thermal resistance. Table IV shows the thermal death times of vegetative cells and spores.

TABLE IV. THERMAL DEATH TIMES OF VEGETATIVE CELLS AND SPORES[a]

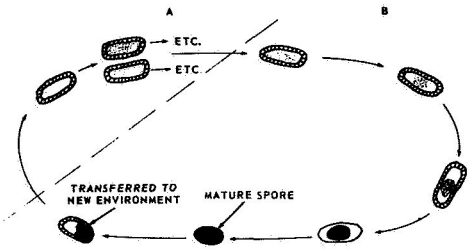
Organism	Vegetative Cells TDT at 53°C (min)	Spores TDT at 99.5°C (min)
Bacillus brevis	4	16
Bacillus cereus	4	6
Bacillus subtilis, S1	4	6
Bacillus subtilis, UT	12	12
Bacillus globigii	6	8
Bacillus mycoides, 420	6	12
Bacillus mycoides, S111	6	8
Bacillus megatherium	6	6
Bacillus fusiformis	6	14
Bacillus mesentericus	22	12

a. Data taken from Williams, O. B. and Zimmerman, C. H., Studies on Heat Resistance III. The Resistance of Vegetative Cells and Spores of the Same Organism. J. Bacteriol. Vol. 61, 1951, pp. 63-65.

The ability to form spores is not possessed by all bacteria. It is extremely rare among the cocci and spirilla. Two genera of the rod-shaped bacteria are characterized by their production of spores: the genus Bacillus, consisting of aerobic organisms, and the genus Clostridium, made up of anaerobic bacteria. These are united in a special family, the Bacillaceae. It is possible that other species now believed to be nonsporeformers may produce spores under conditions yet undetermined.

The mechanism of spore formation is composed of certain definite steps. A typical sporulation cycle is shown in Figure 13.

As vegetative cells mature they begin to exhibit granulation. At the beginning of spore formation, there occurs a gradual localized accumulation of certain cytoplasmic materials. The accumulation becomes progressively more dense and chemically differentiated from the rest of the cytoplasm. This is the preparatory stage.



(A) VEGETATIVE CELLS: REPRODUCTION BY BINARY FISSION. CELLS ARE ACTIVE WITH RESPECT TO GROWTH AND METABOLISM. (B) AT SOME STAGE DURING VEGETATIVE GROWTH, CELLS BEGIN TO SPORULATE. MATURE SPORE IS DORMANT UNTIL CONDITIONS FOR GROWTH ARE SUITABLE, WHEREUPON IT GERMINATES AND ENTERS VEGETATIVE PATTERN OF GROWTH.

FIGURE 13. VEGETATIVE GROWTH AND SPORULATION CYCLE OF A SPORE-FORMING BACTERIUM

Next the accumulation becomes surrounded by an envelope or membrane and is called the forespore. Eventually, this covering changes to a hardened, extremely impermeable (impassable) spore coat. At this time, the spore is mature; the vegetative cytoplasm and cell wall of the original cell disintegrate. The mature spore contains the hereditary units necessary to perpetuate the species.

As soon as environmental conditions again become suitable for growth, spore germination takes place. The spore swells, the spore coat ruptures, and a vegetative cell develops. The empty spore coat is shed as the vegetative cell emerges. Growth and reproduction resume.

Bacterial spores may be seen under the microscope following application of special staining procedures. Spores are difficult to stain with ordinary basic dyes, but once stained they are not readily decolorized. Their resistance to decolorization permits differential staining of spores and vegetative cytoplasm.

The shape, size, and location of spores vary according to the species. Some are spherical, some ellipsoidal, others cylindrical. They may be larger or smaller than the vegetative cell; in some cases, they are large enough to bulge the cell wall and cause a spindle-shaped or drum-stick-like cell. In some species, the spore is located in the center of the cell, whereas in others it may be located in a subterminal or terminal position. Some typical spore positions are shown in Figure 14.

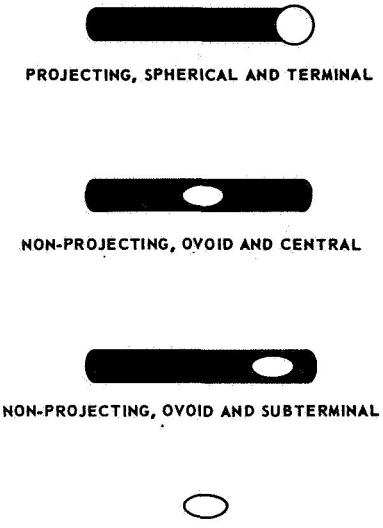


FIGURE 14. SPORE POSITIONS

Bacterial spore formation is not a means of reproduction. Each vegetative cell forms only one spore and on germination the spore gives rise to a single vegetative cell. Bacteria capable of sporulating (forming spores) may grow and reproduce for an indefinite number of generations as ordinary vegetative cells. At a point when nutrients may become scarce or other unfavorable environmental changes occur, these cells enter the spore stage. Thus, the significance of the sporulation process is in perpetuation of the species.

Bacterial spores may remain dormant but viable for years of veritable starvation. They survive disinfectants, antiseptics, extreme desiccation (drying), high termperatures, low temperatures, and ultraviolet light. Vegetative cells are killed by exposure to 60°C for 10 minutes; 100° to 125°C for 10 minutes or more is necessary to kill spores. The relative resistances of bacterial spores and other microorganisms to physical and chemical agents is shown in Table V. It can be seen that bacterial spores are many times more resistant to these agents than are other organisms and vegetative cells.

The unique survival capabilities of bacterial spores have not been fully explained though it has been the subject of much research. The impermeability of the spore coat may explain its resistance to

TABLE V. RELATIVE RESISTANCES OF BACTERIAL SPORES, MOLD SPORES, AND VIRUSES AS COMPARED TO ESCHERICHIA COLI BACTERIA (VEGETATIVE CELLS)[a]

Agent	E. Coli	Bacterial Spores	Mold Spores	Viruses
Phenol	1	100 000 000	1 - 2	30
Formaldehyde	1	250		2
Dry Heat	1	1000	2 - 10	1
Moist Heat	1	3 000 000	2 - 10	1 - 5
Ultraviolet	1	2 - 5	5 - 100	5 - 10

a. Data taken from Rahn, O. "Physical Methods of Sterilization of Microorganisms," Bact. Rev. Vol. 9, 1945, pp. 1 - 47.

toxic chemicals, but heat resistance properties are more puzzling. Heat kills organisms by destruction of essential protein compounds. Certain lipoid materials found in bacterial spores may serve to protect the chemical linkages from destruction. Heat resistance of spores may be attributable to a peculiar molecular arrangement of their constituents; a high concentration of calcium known to exist in spores may have a binding effect on certain compounds in the spore. A major factor in the survival ability of spores is their very low rate of metabolism and negligible respiratory activity. A great proportion of their water content exists as bound water which is so intimately associated with the colloids of the spore that it is essentially removed from the liquid state. This bound water will not participate in freezing or chemical reactions.

Review Questions

1. Explain the purpose of the cell wall. What would you expect to happen to a cell if you shatter its cell wall?
2. What vital function does the cytoplasmic membrane perform?
3. Why couldn't a cell survive without a nucleus?
4. In what way would a bacterial cell be limited if we remove its flagella?
5. What are some characteristics of bacterial colonies which help distinguish one species of bacteria from another?
6. Explain how mitotic division provides daughter cells that are identical to the parent cell.
7. Does the term "growth," applied to bacteria, refer to an increase in size of the organism? Explain.
8. If a certain type of bacterium has a generation time of 10 minutes, how many bacteria would have been produced after 30 minutes? (Assume that there is only one bacterium to begin with and optimum growth conditions exist.)
9. During which phase of the growth curve are the greatest number of viable cells produced?
10. Why is there an eventual death phase in a normal growth curve of a bacterial culture?
11. What is the main advantage of colony counting over other methods of determining the number of organisms?
12. What things can we do to separate bacterial cells so that each colony arises from only one cell?
13. Is bacterial spore formation a method of reproduction? Explain.
14. What can cause bacteria to go into the spore stage?

CULTURING OF BACTERIA

To study microorganisms satisfactorily, we must be able to cultivate them under laboratory conditions. Knowledge of their nutritional and physical requirements is a prerequisite to successful cultivation. The nutritional requirements of bacteria vary widely, and numerous types of culture media are employed to cultivate various species. Bacteria also exhibit radical differences with respect to physical requirements for growth. Provision of proper temperature, oxygen, light, humidity, etc., must be made.

Cultivation Requirements

Nutrition. For growth to occur, all of the necessary nutrients must be furnished to an organism. All microorganisms share a set of basic

nutritional requirements with regard to chemical substances necessary for growth and normal functioning.

Carbon and nitrogen in some form are necessary to all microorganisms, including the bacteria, because these elements are primary components (plus hydrogen and oxygen) of the cell protoplasm. Carbon sources may be carbon dioxide (CO_2) or more complex forms, such as sugars and other carbohydrates. Bacteria, which utilize CO_2 as their sole source of carbon, are termed autotrophs. Those requiring an organic form of carbon are called heterotrophs. Plants utilize nitrogen in the form of inorganic salts, such as potassium nitrate (KNO_3), whereas animals require organic nitrogen compounds, such as the proteins and their degradation products. Bacteria are extremely versatile in this respect. Some use atmospheric nitrogen, others inorganic nitrogen compounds, and some derive nitrogen from proteins or other organic compounds.

All microorganisms require a source of energy for growth. Those capable of employing radiant energy from the sun are designated photographs. Chemotrophs rely upon reactions of chemical compounds to provide their energy. Both types are found among the bacteria.

Bacteria and other living organisms require several metallic elements, such as sodium, potassium, calcium, magnesium, manganese, iron, zinc, copper, phophorus, and cobalt. Bacterial requirements for these elements are only trace amounts. Vitamins and vitamin-like compounds are also necessary to living organisms. Bacteria present a variable pattern in this aspect of nutrition. Some are capable of synthesizing their vitamins from compounds contained in the medium. Others will not grow unless the vitamins are present in the medium.

Water is a necessity for normal growth processes of all living organisms. For bacteria, all nutrients must be in solution before they can enter the cell.

Bacterial types range from those which grow only in the presence of atmospheric oxygen (aerobes) to those which cannot grow in oxygen (anaerobes). Some are obligate in these respects, others may be able to live either in the presence or absence of oxygen. These are the facultative organisms. Some forms, particularly of the parasitic or pathogenic bacteria found in warmblooded animals, require some oxygen but less than that present in the atmosphere. These are termed microaerophilic forms.

The hydrogen ion concentration of a medium, usually expressed in terms of pH, has considerable influence on the rate and extent of bacterial growth. Each type of bacterium has an optimum pH level at which it grows best. Its minimum pH is the most acid medium in which it will grow; its maximum pH is the condition of greatest alkalinity at which growth will occur. Most species grow best at pH level 6.5 to 7.5, or about neutral. Bacteria exist, however, which are extremely acid tolerant and thrive at pH 0.1. Conversely, some types thrive in alkaline environments of pH 9.0. The pH scale is shown in Figure 15.

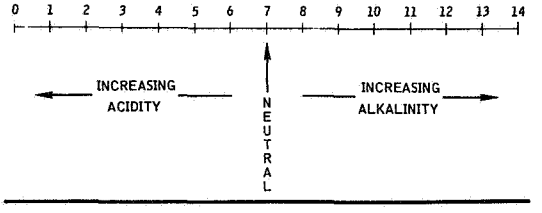


FIGURE 15. THE pH SCALE

No single medium will support growth of all the different types of bacteria. Bacterial growth requirements are many and varied. Therefore, many different types of media are necessary to cultivate them. We can, however, derive a basic medium in which a large number of bacteria can and will grow. This basic medium contains water, a nitrogen compound such as peptone, and a carbon source such as dextrose.

By altering this basic medium through addition of other compounds, selective media can be obtained. Selective media are prepared for only one or just a few types of organisms. If a medium is inoculated with a variety of organisms, only those for which it is suited will grow on it. Other types will be suppressed. If the nutritional requirements of an organism are known, conditions can be provided that will favor development of that organism over that of others.

Examples (Table VI) of a relatively simple liquid and a solid medium which support growth of many common bacteria are nutrient broth (liquid) and nutrient agar (solid).

TABLE VI. COMPOSITION OF NUTRIENT BROTH AND NUTRIENT AGAR

Nutrient Broth		Nutrient Agar	
Beef extract	3 g	Beef extract	3 g
Peptone	5 g	Peptone	5 g
Water	1 l	Agar	15 g
		Water	1 l

Agar is a solidifying agent. Nutrient agar is often called just "agar" by those familiar with its implementation. Media of these types are made up by adding a measured amount of water to a measured amount of dry powdered substance containing all the ingredients. Almost all media are available commercially in powdered form. Solid media are prepared by adding powder to deionized water, heating, and stirring until the powder dissolves. While still hot, the agar is dispensed. It solidifies as it cools. Figure 16 shows students preparing media.

Trypicase soy agar (abbreviated T-soy agar or TSA) or tryptic soy agar are commonly used powdered forms for making solid growth media. Blood serum is commonly added to agar for cultivation of some pathogens.

Nutrient broth is usually made in quantities of 1000 milliliters or less and dispensed into test tubes. Solid agar may be made in petri dishes or test tubes. A prepared agar plate is one into which agar has been dispensed and allowed to solidify. A stab culture tube is one into which agar has been dispensed and allowed to solidify with the tube in a vertical position. Agar slants are tubes into which agar has been dispensed and allowed to solidify with the tubes in a slanted position. The agar solidifies into a slope on which organisms are grown.

Physical Conditions. Just as bacteria vary widely in their nutritional requirements, so do they vary with regard to physical needs.

Temperature is a prime factor. Each species of bacteria will grow at temperatures inside a certain range. On the basis of this, bacteria are divided

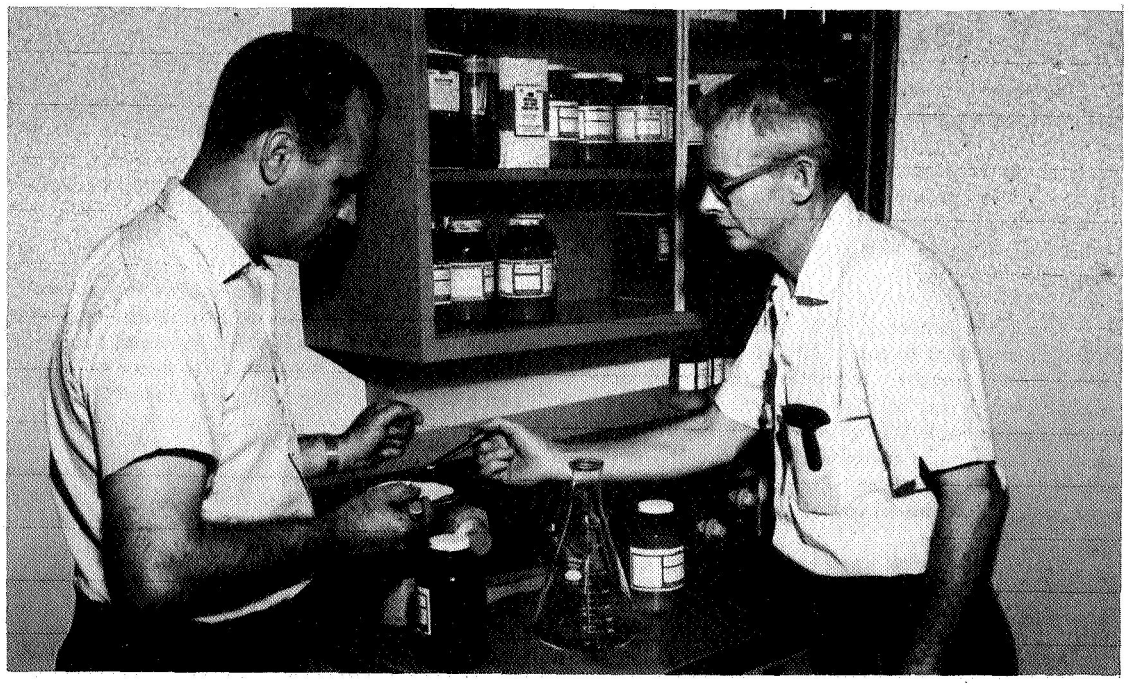


FIGURE 16. STUDENTS PREPARING MEDIA

into certain groups. The phychrophiles grow best at temperatures below 20°C. Mesophiles grow best between 25° and 40°C. Thermophiles prefer temperatures of 45° to 60°C. The optimum growth temperature of an organism is the temperature at which it grows best.

Many bacteria can survive, though they do not grow, at lower or higher temperatures than those at which they grow. Among those which survive much higher temperatures are bacterial spores. Most bacteria survive temperatures far below those optimal for growth. Bacteria can be preserved for long periods of time by rapidly freezing them to about -78°C. The frozen cells survive in a state of "suspended animation" because no metabolism takes place. Freezing is often used in combination with drying to preserve cells for many years.

Another important factor in physical growth conditions is light. Some bacteria grow only in the presence of light.

Pressure affects bacterial growth, although bacteria are fairly adjustable to pressure changes. Several hundred atmospheres would kill bacteria.

Culture Techniques

Bacteria and other organisms grown on a medium in the laboratory are referred to as "cultures." When bacteria are inoculated (placed) into or on a medium and provided with proper growth conditions, crops of cells become visible. These crops may be seen as cloudy suspensions in liquid media, or as localized masses of cells (colonies) on or in a solid medium.

To provide proper growth conditions, equipment called an incubator is used. This is similar in structure to a refrigerator. It provides controlled temperature which may be adjusted to the desired level. A temperature of 37°C, or about 98°F, is commonly used for many types of organisms. Humidity is also provided in the incubator. Figure 17 shows plates being placed into the incubator.

There are several ways to inoculate, or place organisms in, culture media. The method depends largely on the type of medium (liquid or solid). Broth cultures are prepared by placing a small amount of cells into the sterile liquid with a sterile wire loop, swirling to distribute the cells, and incubating the tube.

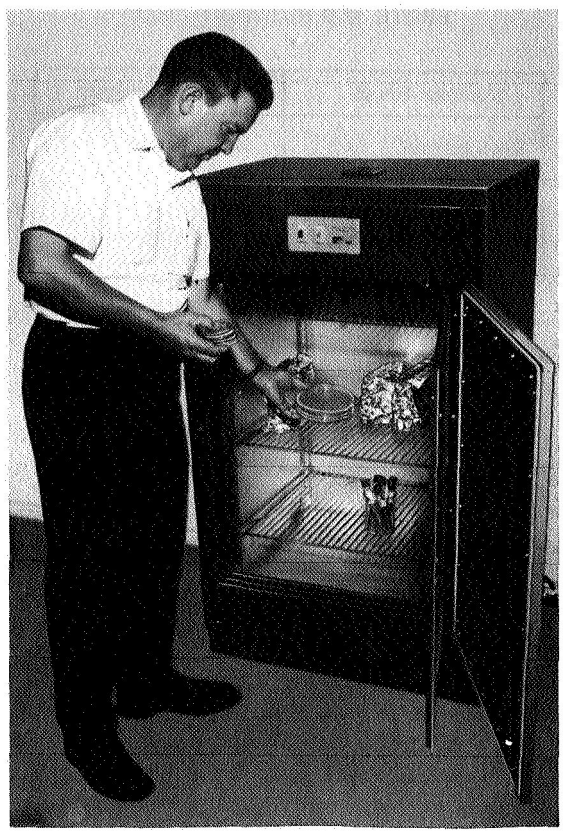


FIGURE 17. STUDENT PLACING PLATES IN INCUBATOR

Stab cultures are made by obtaining a small amount of cells on a sterile wire needle and inserting the wire needle vertically into the sterile agar in a stab culture tube. The needle is removed along the same path. The tube is then incubated.

Agar slants are inoculated with a sterile wire loop. The loop is touched to a colony or crop of cells. A small amount of cells are thus picked up and may be transferred to the surface of the sterile slant and spread over it.

Prepared sterile agar plates may be inoculated in the same way as are agar slants. In both these instances, the growth will be on the surface of the medium.

Poured agar plates are prepared in a somewhat different manner, starting with a suspension of cells in some liquid (alcohol, salt water, etc.). An aliquot (portion) of from 1 ml to 5 ml of this suspension is withdrawn with a sterile pipette. The aliquot is drained from the pipette into a dry, sterile petri dish. Immediately, or before the aliquot dries, about 20 ml of sterile liquid agar is poured or dispensed into the dish. The dish should then be swirled gently to distribute the organisms. The agar, when poured, must of course be warm enough to be in the liquid state. It should not be hot, for it might kill some of the organisms. As soon as the tube or flask agar can be held comfortably in your hand, it will be about the right temperature for pouring or dispensing. Figure 18 shows media being dispensed with a Luer-Lok syringe. Colonies in a poured agar plate may be on the surface or within the agar. Colonies within the agar are called subsurface colonies. These may be smaller than the colonies on the surface because the oxygen supply is less.

Proper culture technique requires sterilizing pipettes, transfer loops and needles, petri dishes, test tubes, dispensing apparatus (syringe), and media. This is accomplished by placing these items in a steam sterilizer, or autoclave, at 121°C for about 30 minutes. Wire loops and needles may be held in the flame of a Bunsen burner or alcohol lamp until they are red hot. Pipettes must be wrapped in paper singly or placed in sealed cans that hold about 25 to 30 pipettes. They must then be autoclaved and must be allowed to remain sterile after removal from the sterilizer. Glass petri dishes may be autoclaved, or plastic, disposable, presterilized ones may be used. Test tubes and flasks of media should be capped loosely for autoclaving. Figure 19 shows materials being placed in the autoclave for sterilizing.

Proper culture technique also requires taking care not to introduce contamination from the air, the table top, your hands, etc., to the culture. Sterile pipettes, syringes, test tube plugs or caps, and transfer loops or needles must not be touched to anything or laid down while they are being used. If you touch the end of a pipette or lay a transfer loop on the table, it may become contaminated with microorganisms other than those you are culturing.

FIGURE 18. STUDENTS DISPENSING MEDIA WITH LUER-LOK APPARATUS

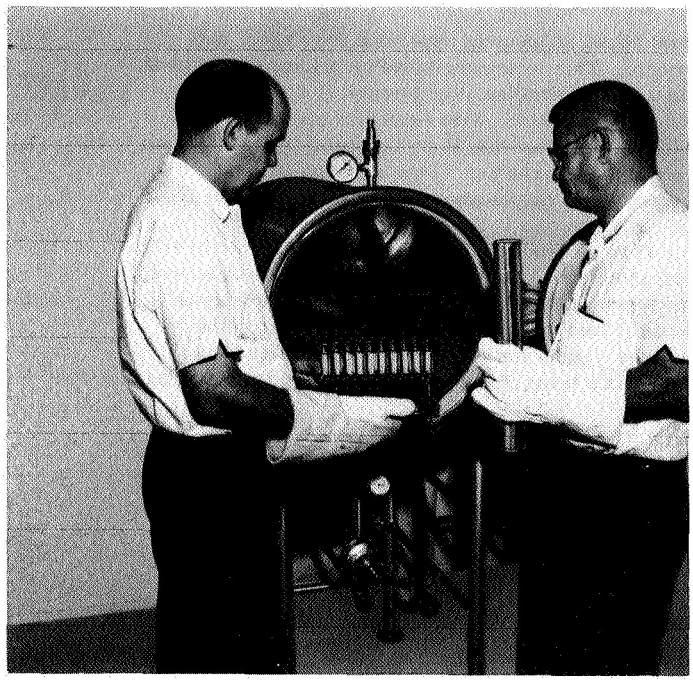


FIGURE 19. STUDENTS USING AUTOCLAVE

Good aseptic technique is, therefore, of prime importance. Test tube plugs or tops should not be put down during transfer operations. Plugs should be held between the fingers and a cap should be held by the little finger while transfer is being made. By curling your little finger around a test tube top and holding it, then turning the tube to remove the top, you can continue to hold the top while transferring the organisms. Figure 20 shows the proper method of holding tubes during culturing.

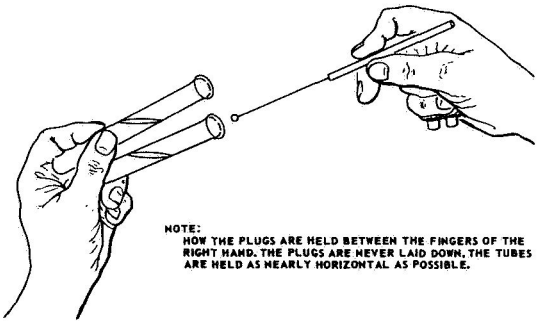


FIGURE 20. METHOD OF HOLDING TUBES WHEN TRANSFERRING CULTURES

In a microbiological laboratory, cultures of various species of microorganisms are maintained as "stock cultures." Thus, a continuous source of various species is at hand. Maintenance of stock cultures is dependent upon regular, periodic transfer to fresh media. Ofter these cultures are kept in a refrigerator. The lowered temperature slows down this metabolic rate and makes transfer to fresh media necessary less often.

Pure Cultures

In order to study microorganisms of different species, it is important to obtain a pure culture of each organism. A pure culture is one in which all of the microorganisms are the same kind. Special techniques are necessary in order to obtain a pure culture and maintain it.

Isolation Techniques. The starting point in obtaining a pure culture is the isolation of a single type of organism. This isolation procedure often must begin with a mixed culture, that is, one in which several types of microorganisms are growing.

Perhaps the most widely used method of isolation is the streak plate method. A suitable sterile agar medium is poured or dispensed into a sterile petri dish and allowed to solidify. With a sterile wire loop or needle, a small amount of growth from a broth culture, a bacterial suspension, or a mixed colony is picked up. This is streaked (Fig. 21) across the

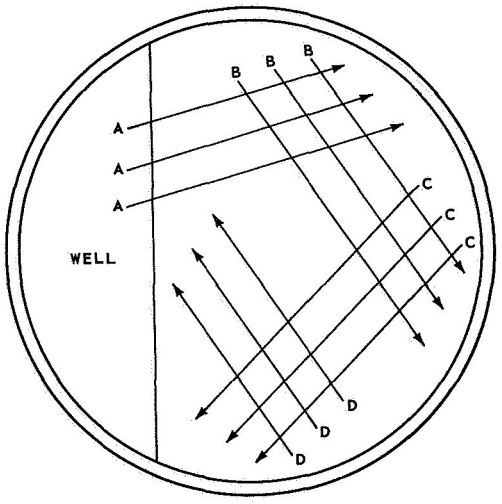


FIGURE 21. STREAK PLATE METHOD FOR PURE CULTURE ISOLATION

surface of the agar on one side of the plate. The loop or needle is then flamed (held in the flame of a Bunsen burner or alcohol lamp until it is red hot) and more streaks are made. This second set of streaks is made by pulling the loop or needle across the first streaks. This serves to drag bacteria out in a long line from the initial streaks, and thin the bacteria out. Following this series of streaks, the loop or needle is again flamed. More streaks are made, pulling the bacteria out from the second set of streaks. The plate is then incubated for 24 hours or longer and examined. Isolated colonies should appear along the second or third streakings. Organisms from these isolated colonies may then be transferred with a sterile wire loop to sterile nutrient agar or broth for subsequent use.

The purpose of the streak plate, and the purpose of other pure culture procedures, is to thin or separate the microorganisms so that individual cells are isolated. These isolated cells will then reproduce on a medium and result in colonies. All the organisms in a colony that originated from one organism will be of the same type, and hence, a pure culture.

When several different organisms are present in a medium with one type predominating, the loop dilution technique may be applied to obtain a pure culture. Figure 22 illustrates this procedure. The mixture of bacteria is suspended in a suitable diluting fluid, such as one percent saline (salt water). A sterile transfer loop is dipped into this suspension and a loopful of the fluid transferred to a second tube (Fig. 22A). The second tube contains a warm, liquid, sterile agar medium and must be swirled gently to distribute the organisms. A similar transfer with a sterile loop is made from this tube to

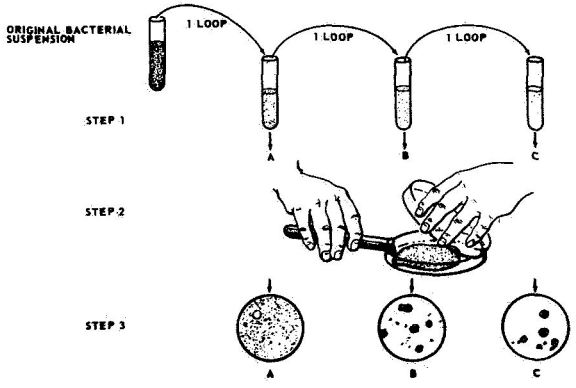


FIGURE 22. LOOP DILUTION TECHNIQUE FOR PURE CULTURE ISOLATION OF BACTERIA

another tube (B), which also contains a warm, liquid, sterile agar medium. This tube is similarly swirled and a loopful of its contents transferred to tube (C) of sterile medium. The contents of each tube are poured into separate sterile petri dishes, allowed to solidify, and incubated. After incubation for about 24 hours (incubation time is dependent upon the growth rate of the particular type of organism), the plates are examined for the one which contains isolated colonies. Portions of the growth from these colonies may then be transferred to fresh sterile media.

If we need to isolate a particular organism from a mixed culture, we may use special media. Usually these media are agar types. A special medium that favors the growth of a particular species is often used. This type of approach is used if one is looking for or expects to find a particular species. The mixed culture is inoculated into or on the specific, sterile medium and incubated under conditions (temperature, humidity) optimum for the desired organism. If the organism is there, it can then be expected to grow and form colonies. These selective media are extremely valuable in diagnosis of diseases.

The most ideal and most difficult method of securing a pure culture involves the isolation of a single cell by mechanical means. Special equipment called a micromanipulator is used in conjunction with a microscope to pick a single organism from a drop of liquid. A tiny glass tube, called a micropipette, is used to pick up the cell. The cell is then placed on sterile media for incubation. This technique is reserved for use in highly specialized studies and requires a skilled operator.

Maintenance Techniques. After a pure culture is obtained, one is faced with the problem of maintaining it for various time periods. This involves several special techniques. The American Type Culture Collection in Washington, D.C., is devoted entirely to the preservation of pure cultures of all types of microorganisms. Some of the ways pure cultures are maintained are periodic transfer to fresh media, refrigeration, and lyophilization. The time intervals at which transfers to fresh media are made depend upon the species of organisms. Also, the proper medium must be employed and the proper storage temperature maintained for each type of organism. Proper identification and careful labeling are of vital importance.

Some species of microorganisms can be successfully preserved for weeks or months by keeping them

in a refrigerator, but transfer to fresh media is eventually necessary.

Lyophilization is the most effective procedure for preservation of pure cultures. This is rapid freezing in combination with drying. Afterward, if kept in sealed tubes and stored at very low temperatures, many organisms remain viable (living) for up to 20 years. This is one of the main procedures used at the American Type Culture Collection for maintaining its tremendous numbers of pure cultures.

Operations involving pure cultures are often performed in an isolator or glove box that provides a sterile working environment.

Review Questions

1. In a microbiological laboratory, why is it necessary to have many different types of media?

2. How are bacteria classified on the basis of their need for oxygen?

3. What is the difference between nutrient broth and nutrient agar?

4. How are media made in the laboratory?

5. What is a prepared agar plate?

6. How are bacteria grouped according to temperature requirements?

7. What is a culture?

8. What is an incubator? What two important growth conditions does it provide?

9. _____ a medium means to place organisms in or on the medium.

10. For the poured agar plate method of culturing bacteria, the liquid agar should not be extremely hot. Explain. What is a simple test to tell if the medium is about the correct temperature?

11. Explain how laboratory materials are sterilized.

12. What does "flame the loop" mean?

13. Assume you are going to transfer part of a suspension of bacteria from a tube to a sterile petri dish, using a sterile pipette. As you remove the pipette from its wrapping, you brush it against your hand. You must then lay this pipette aside and get another sterile pipette. Explain.

14. Why are stock cultures kept in a refrigerator?

15. If you wish to obtain a pure culture, what four methods could you use?

16. What is lyophilization? Why may it be called "suspended animation?"
What is its advantage over other methods of pure culture maintenance?

It is suggested that at this point the student perform Laboratory Exercises III, IV, and VII, which are described in detail in Appendix C at the end of this manual.

MICROBIOLOGICAL TECHNIQUES

The Microscope

Because microorganisms are so small that most of them cannot be seen with the unaided eye, the microscope is one of the most important tools of the microbiologist.

Today, there are two kinds of microscopes used extensively in research. These are the light microscope and the electron microscope. Light microscopes magnify by a series of optical lenses. Electron microscopes illuminate an object with a beam of electrons instead of a beam of light and employ magnets instead of lenses. Images can be magnified to a much greater extent with an electron microscope. The usual light microscope magnifies up to 100 times. An electron microscope magnifies objects 10 000 to 80 000 times.

The kind of microscope used most commonly in microbiological laboratories is a type of light microscope called the compound microscope. It uses double magnification and can magnify objects up to 10 000 times without appreciable loss of clarity. The mechanics of using this microscope are not complicated but require practice. The student should

handle this instrument very carefully because it is a precision instrument and can be easily damaged by carelessness.

A typical laboratory compound microscope is shown in Figure 23. Light is reflected by the mirror (A) into the substage condenser (B), where light is focused onto the stage (D) above. The object to be examined is placed on the stage and held in place with the stage clip (E). The light passes through an opening in the stage and is focused on the object. Attached to the revolving nosepiece (G) are the objective lenses (F). The nosepiece can be rotated so that the desired lens can be used. There are usually three, sometim ur, objective lenses. The first magnifies 10 es, the second about 30 times, the third about 40 times, the fourth (oil immersion lens) about 100 times.

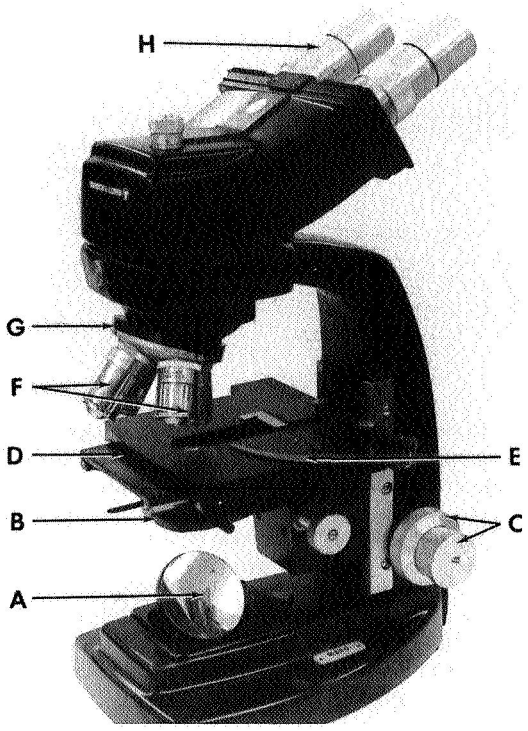


FIGURE 23. TYPICAL LABORATORY MICROSCOPE

The light passes through the objective lens to the ocular or eyepiece (H). The ocular magnifies 10 times. Thus, as shown in Table VII, double magnification is obtained. To focus the entire lens system, there are adjustment knobs (C). The coarse adjustment brings the object into approximate focus, and the fine adjustment is used for final, precise focusing.

TABLE VII. MAGNIFICATIONS OBTAINED WITH VARIOUS OBJECTIVES ON MICROSCOPE

Objective Lens		Ocular		Magnification
10	×	10	=	100
30	×	10	=	300
40	×	10	=	400
100	×	10	=	1000

The objective lens which magnifies 100 times is an oil immersion lens. It is the lens most frequently used in microbiological work and requires great care in operation. It focuses extremely close to the specimen or object. The procedure for its use is as follows:

1. Place a microscope light in front of the mirror and adjust the angle of the mirror so that maximum light is focused on the stage.

2. With the coarse adjustment knob, raise the objectives well above the stage. Rotate the nosepiece so that the oil immersion lens is centered above the condenser.

3. Place the prepared glass slide on the stage and lock in place with the stage clip. Add a drop of special immersion oil directly on the smear to be examined.

4. With the coarse adjustment knob, slowly lower the oil immersion lens until it touches the oil. Watch from the side of the microscope as this is done. When the lens touches the oil it will be very close to the slide, but should not touch the slide. The lens could be damaged if you allow it to touch the slide.

5. Looking through the eyepiece, very carefully adjust the lens with the coarse adjustment knob until the specimen comes into view. Only a very small adjustment is usually required.

6. Use the fine adjustment knob to obtain sharp focus. At this time you may need to further adjust the light to obtain the best possible view.

7. When the examination is completed, gently wipe the oil from the lens with lens paper.

Essentially this same procedure is used to examine microorganisms with the other objective lenses. The main difference is that oil is not used. The lens in use should be brought close to the slide while the examiner watches from the side to be sure the lens doesn't touch the slide. With the coarse adjustment knob, the image is carefully brought into focus. Finer focus is obtained with the fine adjustment knob.

A standard microscope slide, a piece of glass about 1- by 2-inches, is used for mounting organisms to be studied under the microscope. It is possible to study living cells in what is called a "wet mount." A drop of fluid containing the organisms is placed on the slide and covered with a cover slip, which is a small square of very thin glass. The organisms in the liquid can then be examined microscopically. This procedure can be used to study living microorganisms to determine whether they are motile (capable of moving) or to observe cell division.

Staining Procedures

To study shape, size, form, internal structures, flagella, and spores of bacteria under the microscope, the microbiologist employs staining procedures. This involves coloring the cells or parts of the cells with a chemical dye. Living organisms cannot usually be examined in this way. Most applications of staining kill the organisms, but the procedures have definite advantages. The cells are much more clearly visible after staining, and differences between cells can be seen by appropriate staining (differential staining).

The essential steps in staining procedures are preparation of a smear, fixing the smear, and application of one or more stains. A smear is prepared by placing a drop of sterile water on a slide and, with a sterile transfer loop, mixing into the drop a small amount of organisms from a culture. The mixture is then spread gently with the loop to make a thin smear. The smear is fixed on the slide by slowly passing the slide through the flame of a Bunsen burner or alcohol lamp. The heat dries the smear and causes the organisms to stick to the slide. The slide is then allowed to cool. The procedure of making and fixing a smear can be seen in Figure 24.

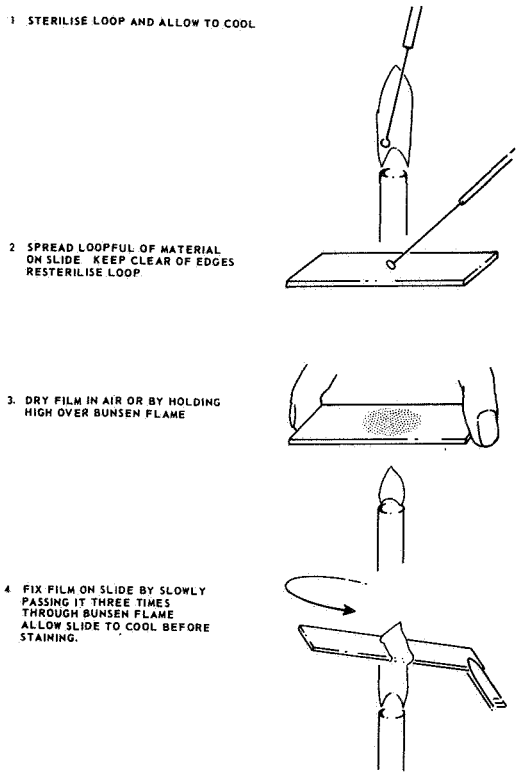


FIGURE 24. PROCEDURES FOR SLIDE PREPARATION

The coloration of bacteria by application of a single stain is called "simple staining." Staining which differentiates between bacterial cells or parts of bacterial cells is called "differential staining." This is slightly more elaborate because the cells are exposed to more than one dye.

One of the most important differential staining procedures is the gram stain. This procedure is widely used in microbiology. Figure 25 shows the steps of this procedure.

Bacteria stained by the gram method fall into two groups: (1) gram-positive bacteria that retain the methyl violet and are colored purple, and (2) gram-negative bacteria that lose the methyl violet and, stained by the basic fuchsin, are colored red. The bacteria take up different colors because

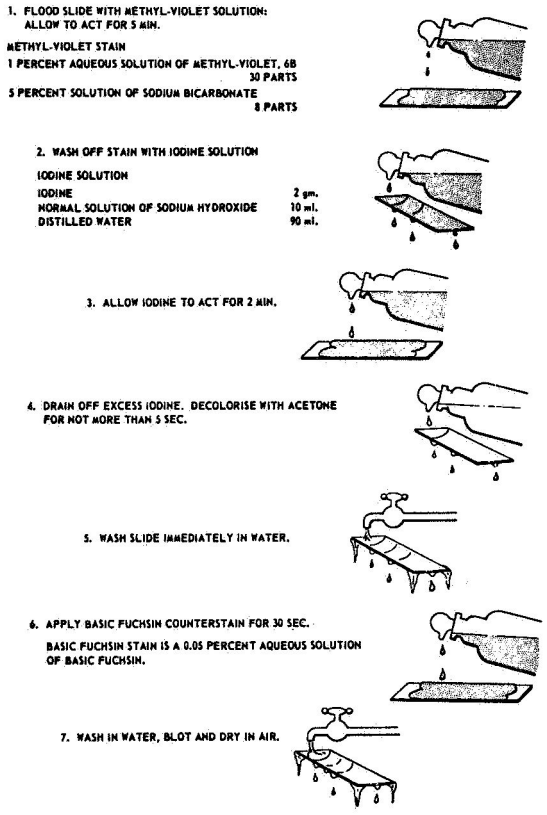


FIGURE 25. GRAM STAINING PROCEDURE

of differences in the cell surface chemical structure. These fundamental differences are used as a major basis for classifying bacteria.

Differential staining may be used to demonstrate certain structures such as flagella, capsules, nuclei, etc. An important staining procedure of this type is the spore stain. The steps in this procedure are shown in Figure 26.

The bacterial spores retain the Malachite green dye and are colored green. Vegetative cells take up the safranin (red) stain. This method, called the Wirtz-Conklin method, is one of several spore staining procedures employing various dyes.

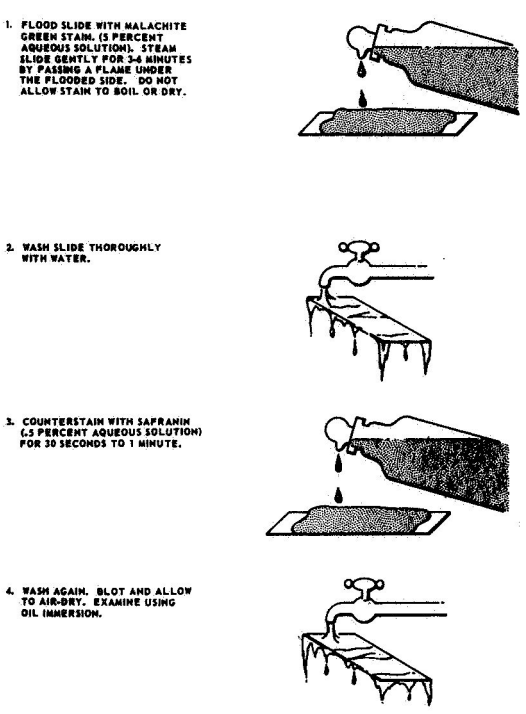


FIGURE 26. SPORE STAINING TECHNIQUE

Controlled Contamination

Purpose. To check sterilization — to test whether something is actually sterile after autoclaving (or some other sterilizing method) — the microbiologist uses a biological control. A biological control is made by placing a certain number of specific organisms on or into an object. This contaminated object is then subjected to sterilization procedures (for example, autoclaving) for a measured time. The object can then be removed and checked, through assay procedures, to determine whether all the organisms were killed. If the microbiologist finds that all the organisms were killed, then he has shown that the sterilizing method was effective. If, however, some of the organisms remain alive, then the method did not sterilize the object. In this way sterilization can be checked or controlled.

This process of using living organisms to check sterilization methods is called "controlled contamination." It is controlled because a certain known number of a certain known type of organism are used.

For controlled contamination, a pure culture of the desired organism must be obtained. Then special procedures must be employed to enumerate viable organisms and to obtain a certain number of them for controlled contamination.

Serial Dilution. A special procedure that is widely used for controlled contamination is serial dilution. It provides a means of enumerating viable organisms and obtaining a suspension of a known number of cells for contamination purposes. The serial dilution procedure is shown in Figure 27.

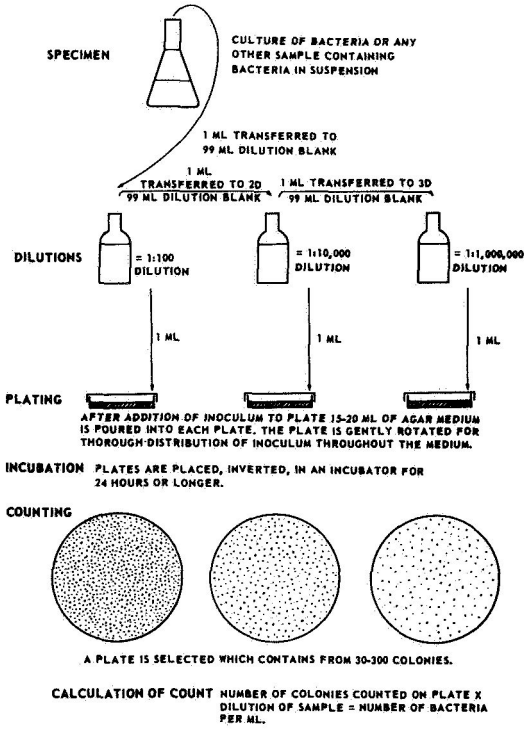


FIGURE 27. SERIAL DILUTION PROCEDURE

Beginning with a heavy suspension of bacterial cells in a liquid (for example, one percent saline), a series of dilutions are made until the number of bacteria is reduced to the desired number. This is accomplished through a series of dilution blanks, which are tubes or bottles of sterile liquid (for example, one percent saline). The bottles or tubes contain equal amounts of sterile liquid — either 9 ml or 99 ml, depending upon the amount of contaminating suspension needed. One milliliter of the original bacterial suspension is transferred with a sterile pipette to the first dilution blank. The blank is swirled to distribute the organisms. One milliliter of this dilution is pipetted (with another sterile pipette) into a sterile petri dish. Twenty milliliters of sterile liquid agar are poured or dispensed into the dish; the dish is swirled and allowed to solidify. Starting with the first diultion, one milliliter is pipetted into another dilution blank and the foregoing procedure repeated. The dilution series is continued in this manner through six or seven blanks. Each time, the number of bacteria is reduced to one-tenth that of the preceding dilution. After incubation the colonies on the plates are counted to confirm the number of bacteria in each dilution.

One disadvantage of this procedure, when it is used with vegetative cells in saline, is that controlled contamination of an object must be effected as soon as the suspension is prepared. The vegetative cells will not survive long enough for preconfirmation of the number of organisms in the contaminating suspension. Therefore, the microbiologist must choose what he judges to be the correct dilution for controlled contamination purposes, use it, and then wait for the plates to be incubated before he can confirm the number of organisms.

Alcohol Sporulation. As stated previously, vegetative cells used for controlled contamination will not remain viable in suspension long enough for preconfirmation of their number. The diluted suspension must be immediately used to contaminate the desired objects, then 24 to 48 hours later the number of bacteria can be accurately determined. To overcome this disadvantage, the microbiologist can use a suspension of bacterial spores. Because of their survival capabilities, bacterial spores remain viable long enough for enumeration before the suspension is actually used for controlled contamination.

A crop of spores of the nonpathogenic aerobic bacteria Bacillus subtilis variety niger may be produced by special techniques. The spores of this organism are ideal for use in biological controls because they are extremely resistant and are not pathogenic. They can, therefore, be used safely in the laboratory without introducing health hazards. Also, this species is aerobic, which simplifies incubation requirements.

After the crop of spores is washed and rid of vegetative debris, the spores can be suspended in 100 percent ethyl alcohol without loss of viability. A dilution series, employing blanks of 100 percent ethyl alcohol, may be prepared. The actual number of organisms in each dilution can then be checked. While the enumeration procedures are being carried out, the dilutions can be stored in a refrigerator without loss of viability. After the number of bacteria in each dilution is confirmed, the correct dilution may be chosen for controlled contamination.

For controlled contamination with either vegetative cells or spores, a dilution containing approximately 1000 organisms or 100 organisms per milliliter is frequently used. An aliquot (for example, one milliliter) of this dilution is withdrawn with a sterile pipette and drained onto the object to contaminate it. The liquid, i.e. saline or alcohol, is then allowed to evaporate, leaving the cells deposited on the object.

Biological indicators are available commercially. One type of these indicators is the spore strip. This is a strip of filter paper containing a certain number of dried bacterial spores. Spore strips may be included in an autoclave load with materials to be sterilized, subsequently transferred to a medium, and incubated to check for sterilization.

Aerosol Contamination. Another method of introducing controlled contamination is by aerosol dissemination of organisms. This can be accomplished by suspending the organisms in a liquid and forcing the liquid through a nozzle which reduces it to fine droplets producing a spray or cloud. The organisms can, in effect, be sprayed onto an object, but this is actually quite complicated and very difficult to perform effectively. Parameters, such as number of organisms, density of the liquid, size of the nozzle orifice, die-off of the organisms, and physical decay of the cloud, must be carefully measured and controlled. This technique is therefore reserved for highly trained microbiologists.

Review Questions

1. What is one limitation of light microscopes, as compared to electron microscopes?

2. Explain the functions of (a) the microscope mirror, (b) the stage, (c) stage clip, (d) coarse adjustment knob, (e) fine adjustment knob, (f) objective lenses, and (g) revolving nosepiece.

3. If the oil immersion lens itself magnifies 100 times, explain how a magnification of 1000 times is achieved when this lens is used.

4. Why is great care required when one is using the oil immersion lens?

5. If one wanted to determine whether a certain organism were motile, would he set up (a) a wet mount or (b) a stained smear? Which procedure would be used if one wanted to study bacterial spores?

6. How is a smear prepared for microscopic examination? What does fixing a smear mean?

7. What staining technique is commonly used as a basis for classifying bacteria?

8. If a bacterial culture containing spores is subjected to a Wirtz-Conklin spore stain procedure, what color will the spores appear?

9. Explain the purpose of controlled contamination.

10. What is a disadvantage of using vegetative cells in a serial dilution procedure for controlled contamination?

11. What spore-forming bacteria are ideal for use as a biological control? Why?

12. What is the process called by which organisms are sprayed onto an object?

It is suggested that at this point the student perform Laboratory Exercises V, VI, and XII, which are described in detail in Appendix C at the end of this manual.

ENVIRONMENTAL SAMPLING

To sterilize an object or area, it is first necessary to assess the microbial contamination present. The amount and type of microbial contamination determine the sterilizing techniques required and the duration of their application. Contamination on surfaces, in the air, in liquids, and inside solid materials must be assessed. Microbiological assay of these areas after their exposure to the sterilizing method is required to verify the method's effectiveness.

Several general sampling techniques exist; research is being conducted to improve these and to

develop new, more efficient methods. Appendix B, "NASA Standard Procedures for the Microbiological Examination of Space Hardware," is an official document that describes the NASA approved microbiological sampling procedures. These procedures were standardized for sampling all space hardware and space hardware assembly, test, and launch facilities required to conform to standards of the NASA Planetary Quarantine Office. Both sampling procedures sanctioned in the NASA standard procedures and proposed methods of sampling will be discussed in this section.

Surface Sampling Methods

Stainless Steel Strips. The NASA standard procedure for sampling microbial contamination accumulating on surfaces is the stainless steel strip method (Appendix B). The heat shock treatment — placement in an 80°C water bath for 20 minutes — serves to kill any vegetative cells that have settled on the stainless steel strip. Any bacterial spores that might be present are separated in this manner from the vegetative cells. The heat shock technique is thus employed to obtain the number of vegetative cells and the number of spores in the total assay.

The NASA standard procedures specify that all manipulations of sterile items be performed in a laminar-flow clean bench meeting class 100 air cleanliness requirements. Figure 28 shows a clean bench of this type. The laminar-flow clean bench of the class 100 type employs circulation of filtered air to provide a work area having fewer than 100 particles greater than 0.5 micron in size per cubic foot of air. High efficiency filters are used to achieve this level of cleanliness. Frequent monitoring of the operational efficiency of clean benches is necessary to maintain quality assurance.

The laminar flow concept is used for bioclean rooms as well as for bioclean work stations (clean benches). Two different laminar flow concepts are shown in Figure 29: the air down-flow type and the air cross-flow type.

In the stainless steel strip sampling procedure, an ultrasonic bath removes collected organisms from the strips. The ultrasonic bath is simply a tank containing some liquid — usually water — into which the bottles containing the strips are placed. Then high frequency sound waves are transmitted into the bath. This sets up a scrubbing action that removes the organisms from the surface of the strip, freeing them into the recovery solution (one percent peptone water). Figure 30 shows use of an ultrasonic bath.

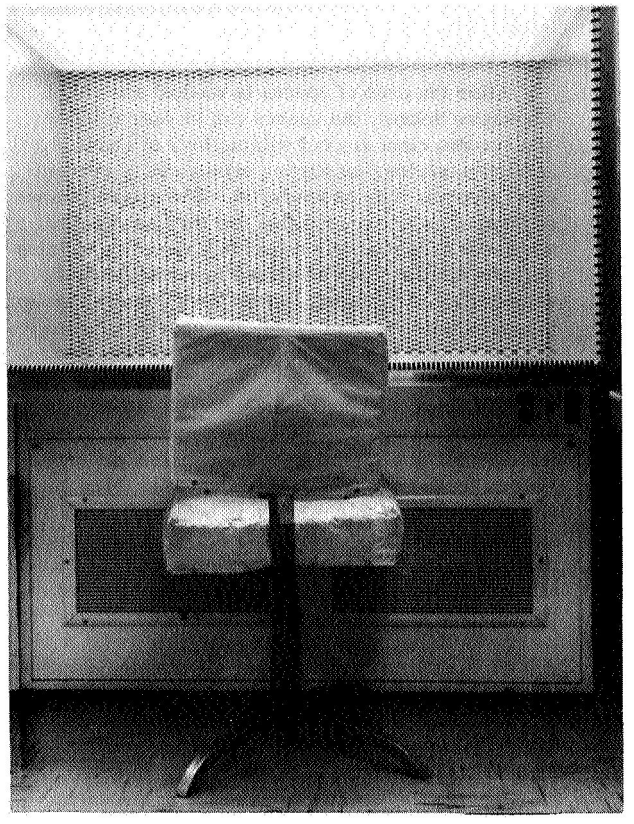


FIGURE 28. LAMINAR FLOW CLEAN BENCH

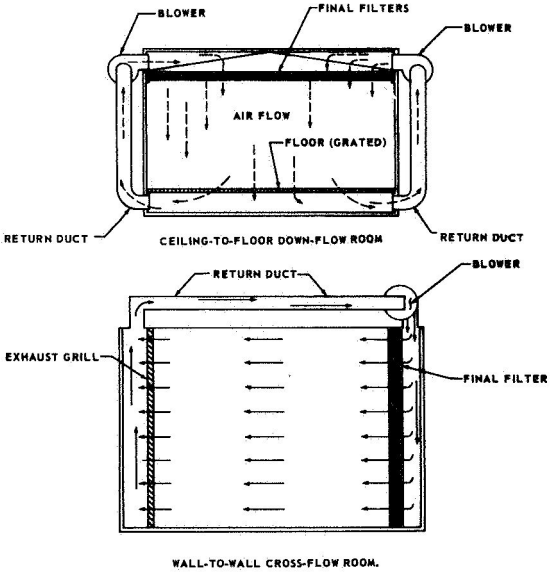


FIGURE 29. LAMINAR FLOW CONCEPTS

FIGURE 30. STUDENTS OPERATING ULTRASONIC BATH

The stainless steel strip assay also provides for enumeration of anaerobic, as well as aerobic, organisms. Anaerobic incubation — incubation in the absence of oxygen — is accomplished with an anaerobic jar. This is an airtight cylindrical jar which holds the plates that are to be incubated. The air is then pumped out of the jar or the oxygen is removed by chemical reaction. (Chemical removal is done by placing into the jar an envelope containing chemicals which react to produce hydrogen. The jar is then sealed, and, as the hydrogen is produced inside, a reaction takes place with the oxygen in the jar until all the oxygen is depleted.) After mechanical or chemical removal of oxygen, the jar is placed into the incubator at the desired temperature.

The stainless steel strip procedure may be adapted for use in conjunction with controlled contamination to check sterilization methods. One proposed adaptation utilizes alcohol sporulation procedures. Bacillus subtilis variety niger spores in 100 percent ETOH (ethyl alcohol) may be used for controlled contamination of stainless steel strips. The strips may then be exposed to decontaminating or sterilizing methods. Subsequent assay requires only aerobic incubation because Bacillus subtilis is an aerobic organism. Heat shock is also eliminated because the procedure employs spores only. This proposed procedure is outlined in Figure 31. It is emphasized that this procedure is not an environment sampling procedure, but a controlled contamination procedure, and is not sanctioned by the NASA

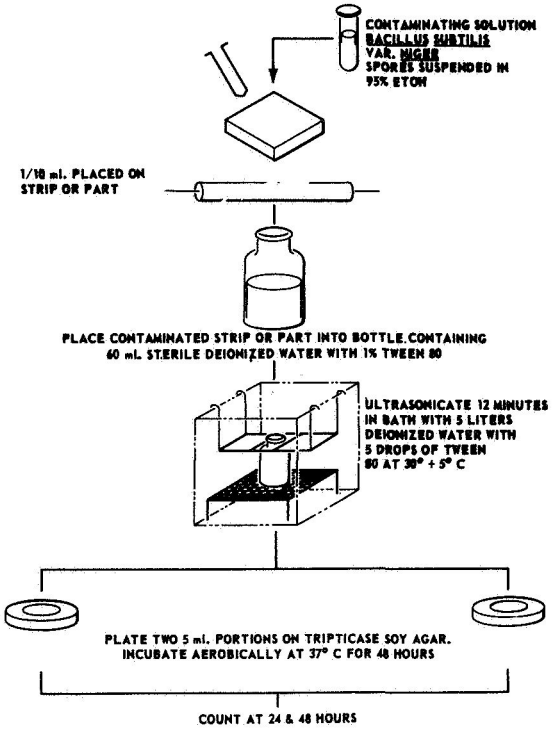


FIGURE 31. SCHEMATIC OUTLINE OF PIECE-PART AND STAINLESS STEEL STRIP ANALYSIS USING ALCOHOL SPORULATION METHOD

standard procedures. It is, however, a workable, time-saving adaptation of the standard stainless steel strip method, and its inclusion in the standard procedures has been proposed. The alcohol sporulation procedure of controlled contamination provides the additional advantage of dealing only with bacterial spores, thus providing the most stringent known parameter for evaluating sterilization methods.

Another adaptation of the stainless steel strip method for sampling microbial contamination accumulating on surfaces has been proposed. This consists of the use of flexible sampling strips instead of stainless steel strips. Flexible strips are more easily attached or fitted to curved or irregular surfaces, and can be bent around angles or corners. Research is being conducted to compare flexible film strips with stainless steel strips and to determine whether, in fact, the flexible film is more effective. Figure 32 shows one type of flexible film strips in comparison with the stainless steel type.

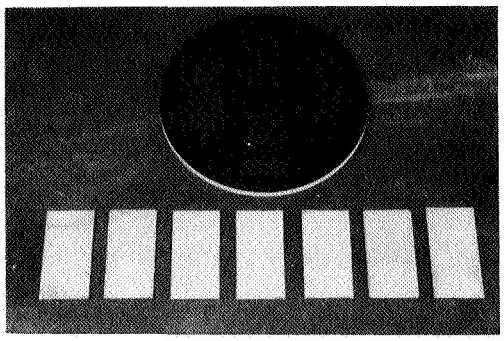


FIGURE 32. STAINLESS STEEL STRIPS AND FLEXIBLE FILM STRIPS

Swab. The NASA standard procedure for microbial sampling of curved and irregularly shaped surfaces is the swab procedure (Appendix B). Again, all manipulations of sterile items are to be performed in a class 100 laminar flow clean bench. Ultrasonication is again used to dislodge the collected organisms from the swab, and both aerobic and anaerobic organisms are enumerated.

Rodac. For microbiological sampling of flat surfaces, the NASA standard procedures (Appendix B) specify the Rodac plate procedure. The Rodac plate is a prepared sterile agar plate with an agar surface of approximately four square inches. The Rodac plate is specially prepared so that the agar rises slightly above the edge of the dish. To apply the Rodac, the dish lid is removed and the plate inverted over the surface to be sampled. One edge of the agar is first touched to the test surface, then through a progressive rolling motion the entire agar surface is touched to the test surface. The lid is replaced and the plate is incubated. The Rodac may be used to sample microbial contamination on any flat surface including the skin, worktables, floors, walls, ceilings, or clothing.

The Rodac plate method gives no differentiation between vegetative cells and spores from the test surface. However, enumeration of both aerobic and anaerobic organisms is possible with the Rodac.

Vacuum Probe. The vacuum probe is a recently proposed method for sampling large surfaces with

light microbial loads. Essentially, this device employs suction and filtration for sampling surfaces. Negative pressure produced by a vacuum source (for example, a vacuum pump) produces a suction air flow. The probe tip is passed over the test surface, thus sucking into the device any microorganisms present. As the air passes through the device, it is filtered by a high efficiency membrane filter. Microorganisms that have been picked up are thus caught on the filter. After sampling of the test surface, the contaminated filter is removed and placed on a prepared agar plate, covered with molten agar, and incubated. Organisms that have been collected on the filter will then grow and produce countable colonies.

The vacuum probe has been reported to effect a high percentage removal of microorganisms from smooth surfaces. Another aspect of this device that may be advantageous is that it leaves no residue on the test surface, as the swab and Rodac often do. The vacuum probe method is a suggested one, and is not included in the current NASA standard procedures.

Air Sampling Methods

Volumetric. Volumetric sampling methods are used for quantitative sampling of biocontamination in the air. The NASA standard procedures (Appendix B) specify three types of volumetric samplers: long-term slit sampler, short-term slit sampler, and cascaded-sieve sampler.

Reyniers Sampler. Representative of the long-term slit samplers is the Reyniers, which samples the air at the rate of 1.0 ft^3/min for a maximum of 60 minutes. The Reyniers slit sampler is approved by the NASA standard procedures (Appendix B). The sampler is connected to a vacuum pump, and air is drawn through a narrow slit. The slit is positioned above the surface of a prepared agar plate which slowly rotates. The plate completes one revolution in one hour. As the air is drawn through the slit, particles in the air are impacted upon the agar surface. After incubation, the colonies on the plate are counted to determine the amount of viable contamination that was present in the air. From the rate of air flow and sampling time, the number of viable particles per unit volume of air sampled may be calculated.

Anderson Sampler. The Anderson sampler is a short-term type that separates airborne viable particles into discrete sizes. Termed a cascaded sieve sampler, the Anderson can be calibrated to sample 1.0 ft^3 of air per minute. The Anderson is NASA-approved (Appendix B), and the sampling time specified for its use is 15 minutes. The NASA standard procedures state that a maximum of 15.0 ft^3 of air shall be sampled on a given set of plates with this type of sampler.

The Anderson sampler consists of six stages that separate particles into six different sizes and impact the particles on prepared agar plates. The stages are numbered one through six, starting at the top of the sampler and going down. Each stage has small jets, or holes, drilled in it, through which the air passes. Figure 33 illustrates the Anderson sampler, and how the air flows through it. The size of the jets grows progressively smaller from stage 1 down. Thus, the larger particles are collected near the top of the sampler, while smaller ones are collected near the bottom. The approximate size range of particles collected on each stage may be seen in Table VIII.

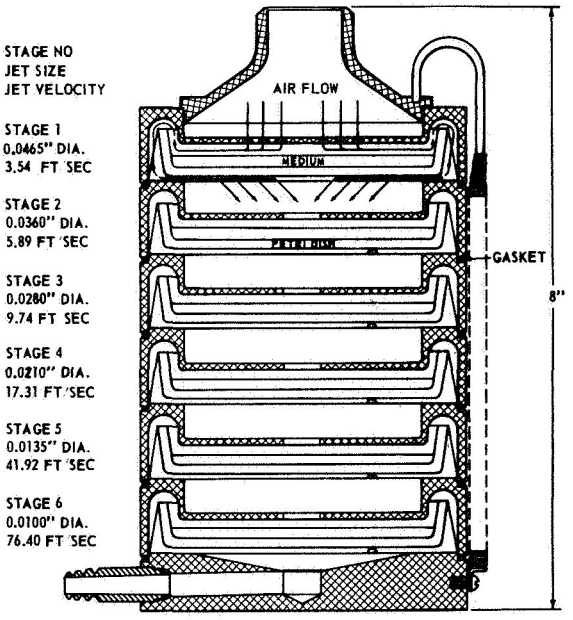


FIGURE 33. THE ANDERSON PARTICLE-SIZING AIR SAMPLER

Petri dishes specially designed for uniform dimension and breakage resistance are recommended for use with the Anderson sampler. About 27

TABLE VIII. PARTICLE SIZE DISTRIBUTION IN ANDERSON SAMPLER

Stage Number	Particle Size
1	9.2 microns and larger
2	5.5 to 9.2 microns
3	3.3 to 5.5 microns
4	2.0 to 3.3 microns
5	1.0 to 2.0 microns
6	1.0 micron and smaller

milliliters of sterile nutrient agar are dispensed into each Petri dish and allowed to solidify. After sampling, the agar plates are incubated and the colonies counted.

Impingement Samplers. The types of volumetric air samplers previously discussed were impactors — they impact or deposite particles directly onto solid surfaces. Another type of volumetric sampler is the impinger that traps organisms in a liquid medium. Impingers direct air through a jet and against the surface of a liquid. Suspended particles in the air are collected in the liquid. Some impingers, called the air washing type, bubble the air through a liquid to collect the suspended particles.

Impingement samplers may possess an advantage in that they may more effectively break up clumps of organisms. In order to accurately determine the number of organisms in any type of sample, aggregates or clumps of the organisms must be broken apart or separated. A clump of several organisms, if placed on a culture medium, will develop into a single colony. To be able to enumerate individual organisms by the colony-counting technique, clumps must be disintegrated so that each organism will develop a discrete colony. Because of air velocity or bubbling action, fragmentation of clumps of organisms occurs in impingement samplers.

The impingement type of sampler is not included in the NASA standard procedures.

Gravimetric. Approved in the NASA standard procedures (Appendix B), the gravimetric method provides a simple means of sampling airborne contamination. Particles suspended in the air are allowed to settle on prepared agar plates. This sedimentation process however, has several drawbacks, one of which is that it does not yield true quantitative counts. This is because large particles may settle readily; whereas smaller ones may not. Also, air movement may prevent gravimetric settling of airborne particles. The gravimetric method requires a longer sampling time than volumetric methods. It does, however, furnish a simple way of obtaining a qualitative evaluation of contamination of the air — determination of what types of viable contamination are present.

Piece-Part Interiors

Many materials and piece-parts may contain interior contamination, or viable microorganisms trapped inside them. Because of the manufacturing and processing conditions for some piece-parts, i.e. high heat or toxic chemicals, microorganisms inside them are killed. But many other parts do not undergo manufacturing conditions harsh enough to kill microorganisms. Therefore, materials (such as rubber, epoxy, lubricants, lucite, etc.) may carry viable microorganisms embedded in them. If these contaminated materials are used in a spacecraft sent to another planet, the interior contamination could be released if the materials should break apart on impact. The planet would then be contaminated with Earth organisms. Consequently, not only the surfaces but also the interiors of piece-parts must be sterile.

To check sterilization, methods of sampling piece-part interiors must be devised. We must be able to determine how many viable contaminants are actually present, and to verify their destruction by the sterilizing method applied.

Sampling interiors of piece-parts presents many problems. One is that, in order to release embedded microorganisms, a material must be ground or pulverized to dust-size (about 1.0 micron or less) particles. The grinding or pulverizing processes generate heat, which might kill the organisms. Embedded organisms might also be crushed by these harsh processes. However, grinding or pulverizing seem, at present, to be the most feasible methods. The possibility of dissolving contaminated materials in some liquid solvent, then filtering to remove the microorganisms, has been considered. But the solvents necessary to dissolve most materials would kill microorganisms. Research is being conducted to develop techniques to assay internal contamination more efficiently.

The NASA standard procedures approve the Pica Blender Mill for assay of piece-part interiors (Appendix B). This device pulverizes materials by impacting and milling actions. Materials are pulverized in a recovery fluid (one percent peptone water). Portions of the fluid and pulverized particles are plated out, incubated, and colonies are counted.

Review Questions

1. To sterilize an object or area, why is it first necessary to assess the microbial contamination present?

2. What is the NASA approved method for sampling microbial contamination accumulating on surfaces?

3. What is the purpose of heat shock as applied in the stainless steel strip assay?

4. What is the purpose of ultrasonication as applied in the stainless steel strip assay?

5. Explain how anaerobic incubation is achieved.

6. What adaptation of the stainless steel strip method may be used for controlled contamination?

7. Why is it best to use bacterial spores for controlled contamination to check sterilization methods?

8. What is a possible advantage of flexible film strips over the stainless steel type?

9. What is the NASA standard procedure for sampling curved or irregular surfaces?

10. What is the NASA standard procedure for sampling flat surfaces?

11. The Radac sampling procedure provides no differentiation between vegetative organisms and bacterial spores. Explain.

12. What is the NASA specified sampling time for Reyniers sampler?

13. Name a NASA-approved volumetric air sampler that separates airborne particles into discrete sizes?

14. Name three drawbacks of gravimetric air sampling.

15. Of the two NASA-approved air sampling methods, which one is best for quantitative determinations?

16. For spacecraft intended to land on another planet, why must piece-part interiors be free of viable contamination?

It is suggested that at this point the student perform Laboratory Exercises VIII, IX, X, and XI, which are described in detail in Appendix C at the end of this manual.

DECONTAMINATION AND STERILIZATION

In the previous sections, we have become familiar with microorganisms, seen their general cell structures, and learned that microscopic life is so widely distributed that microorganisms are found almost everywhere. We have seen that certain types of microorganisms are much more resistant to adverse conditions than are others and can survive under extremely harsh circumstances.

Also discussed in a previous section were the characteristics of living things, such as metabolism, reproduction, growth, and irritability. The biologist uses these characteristics to define life as we know it, to distinguish living matter from nonliving.

It has been established that, in order to determine whether life exists on other planets, we must not contaminate these planets with Earth organisms. This requires that any spacecraft sent to another planet must be sterile, at least until that planet has been biologically assessed. It is perhaps necessary to clarify and concretely define sterility.

Concepts and Requirements

Sterile is an absolute term meaning the absence of life. Something is either sterile or it is not sterile; it cannot be partly sterile or 50 percent or 99.99 percent sterile. To sterilize an object or area, then, means to destroy all living matter on or in that object or area.

On the other hand, if some part — but not all — of the living matter is removed from an object or

area, it is said to be decontaminated. Thus, a decontaminating agent is one which can only be relied upon to destroy part of the living matter. This is usually because decontaminating agents are not strong enough or harsh enough to kill highly resistant forms of life such as bacterial spores.

An obvious prerequisite to verification of sterility is the definition of life itself. Before we can say that sterility has been achieved, we must establish a definite difference between living matter and nonliving. Essentially, we must have some criteria according to which we can state that life exists or has been destroyed. The characteristics mentioned before — metabolism, reproduction, growth, and irritability — are such criteria. Perhaps the most important of these is the ability to reproduce because this criterion is most often used as the final, most decisive one. Metabolism, growth, and irritability are often not as obvious or as easily proven as is the ability to reproduce. For example, bacterial spores demonstrate a very low rate of metabolism — one that is almost impossible to detect. Although a bacterial spore is viable (living), no growth occurs in the spore state. But a viable spore will reproduce itself if provided with the right environment, as will any viable organism presently known. Therefore, we rely on the ability to reproduce as the foremost criterion of life.

The achievement of sterility may be by several methods. The method used depends largely on the type of contamination and the amount of contamination present. As discussed in the previous section, the type and amount of contamination may be determined by various sampling techniques. Tables IX, X, XI, and XII show comparisons of contamination levels in the air, on surfaces, induced by handling, and resulting from fallout, as determined by various sampling techniques. These tables have been included here to emphasize the distribution of microbial life. It is interesting to note that, according to these tables, even hospital operating rooms have a relatively high level of contamination.

TABLE IX. FALLOUT CONTAMINATION ON STAINLESS STEEL STRIPS

Location	Colonies/Sq Ft
Manufacturing area	5×10^4
Hospital operating room	5×10^4
Conventional clean room	1×10^3
Laminar downflow room	<1

TABLE X. CONTAMINATION LEVELS ON VARIOUS SURFACES

Location	Colonies/Rodac Plate	
	After Activity	After Cleaning
Hospital patient rooms		
Walls	<10	
Overbed tables	110	50
Floors	230	75
Operating room and floors	50	
Conventional clean room		
Bench tops	3-5	
Laminar flow room		
Cross flow		
Floors	1-5	<1
Walls	<1	<1
Downflow		
Walls	<1	<1

TABLE XI. LEVELS OF AIRBORNE MICROORGANISMS IN SEVERAL LOCATIONS

Location	Approx. No. Colonies/ft^3 of Air
Industrial plant	10-25
Home	30-50
Hatchery	>100 000
Hospital	
Patient room	20
Operating room	10
Clean Rooms	
Conventional	0.1-1.0
Laminar flow	
Vertical	0.001
Horizontal (upstream)	0.001
Horizontal (downstream)	0.02-0.10

TABLE XII. MICROBIAL CONTAMINATION FROM HUMAN HANDLING

Protection Used while Handling Nuts and Bolts	Average Number of Colonies on One Nut and Bolt Assembly	
	Open Lab	Downflow Clean Room
None	122	4
Ivory soap wash	13	40
Hexachlorophene scrub		
2 min	8	
5 min		0.4
Sterile gloves	0.4	0.3

Methods

There are several existing methods for controlling microbial life, or effecting decontamination or sterilization. Figure 34 shows some of these, the degree of their effectiveness, the required temperature for their use, and the length of exposure time.

From Figure 34, it can be seen that detergents, boiling water, or chemicals such as formaldehyde or alcohol are not sterilizing agents. Because we are concerned primarily with sterilization, we shall discuss some of the physical and chemical methods which may be used to achieve it.

Physical. The metabolism of an organism is dependent upon chemical reactions. These chemical reactions are influenced by temperature. For all organisms, there is a maximum temperature above

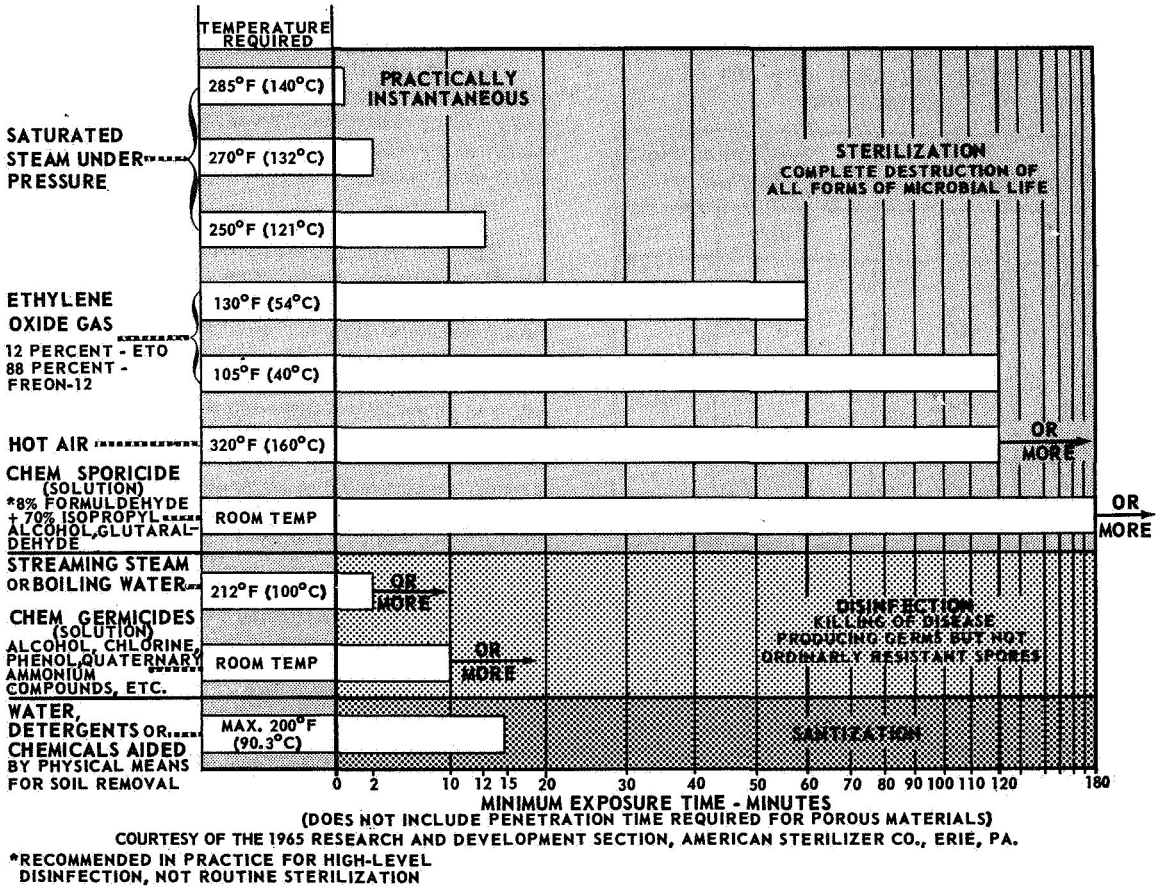


FIGURE 34. METHODS FOR CONTROLLING MICROBIAL LIFE

which they cannot survive. Death of the organism results because the high temperatures alter chemical compounds inside the organism. The application of heat is one of the most effective, economic, and reliable sterilizing methods.

Sterilization by heat may be accomplished in two ways: dry heat or wet heat. Of the two, wet heat in the form of saturated steam under pressure is the most dependable method for destruction of all forms of microbial life. Microorganisms are destroyed at lower temperatures when moisture is present, probably because the chemical alterations are enhanced by the presence of water. Also, the exposure time necessary to effect sterilization with moist heat is less. Table XIII shows a comparison of exposure times for moist heat and dry heat sterilization at various temperatures. The times shown in this table are recommended for materials which have been decontaminated and carry a relatively low level of microbial contamination.

TABLE XIII. EXPOSURE TIME-TEMPERATURE COMPARISONS FOR MOIST AND DRY HEAT STERILIZATION

Temperature		Sterilization Time in Minutes	
(°F)	(°C)	Moist	Dry
240	115	30	
250	121	12	
270	132	2	
285	140		180
320	160		120
356	180		<60

Among all living organisms, bacterial spores are the most resistant in their capacity to withstand destructive methods. As with other microorganisms, bacterial spores are more susceptible to moist heat than to dry heat. Even with the application of moist heat, a temperature of 120°C for 15 minutes or longer is required to kill bacterial spores. Bacterial vegetative cells are destroyed in about five to ten minutes at temperatures of 55° to 65°C (moist heat). The differences in effectiveness of moist heat and dry heat with bacterial spores is illustrated by Tables XIV and XV.

Factors which affect the heat resistance of microorganisms are the nature of the carrier, the cleanliness of the carrier, the type of microorganisms, and the number of microorganisms. For example, more stringent conditions would be required to kill 1 000 000 microorganisms on a given area than would be necessary to kill 1000 microorganisms on that same area. This is due in part to a protection phenomenon: if many organisms are clumped together, those near the center of the clump are protected by the surrounding ones. The nature of the carrier, or contaminated material, also affects the necessary exposure time to achieve sterilization. Soil contaminated with bacterial spores is more difficult to sterilize than are glass or paper strips contaminated with bacterial spores. Also, the "cleanliness" of the carrier is an important factor. It has been found that if micoorganisms are occluded in (surrounded by) dust, their resistance is increased. The presence of organic debris has a similar affect, as does occlusion of microorganisms in salt crystals such as sodium chloride or calcium chloride.

Although sterilization by moist heat is possible at lower temperatures and with shorter exposure times, many materials are damaged by wet heat and cannot be sterilized in this way. Materials deteriorated by wet heat are often sterilized by dry heat.

The type of sterilizer which employs steam under regulated pressure is called an autoclave. Steam under pressure is used only to reach higher temperatures; pressure itself has no other contributing effect in the sterilizing process. Table XVI shows how pressure affects the temperature of steam. The autoclave is essentially a double-jacketed steam chamber equipped with devices which permit the chamber to be filled with saturated steam and maintained at a certain temperature and pressure for any period of time. The autoclave is a vital unit in any microbiological laboratory and is used widely in hospitals. For general purposes, the autoclave is operated at about 15 pounds of pressure (121°C). Sterilization time may be varied according to the materials, types of containers, and volume of the load.

The equipment used for dry heat sterilization may be an electric or gas oven. Maintenance of uniform temperature throughout the oven may be aided by a fan installed in the oven to create air circulation.

TABLE XIV. TYPICAL DESTRUCTION TIME (IN MINUTES) OF BACTERIAL SPORES SUBJECTED TO MOIST HEAT[a]

Organism	212°F (100°C)	221°F (105°C)	230°F (110°C)	239°F (115°C)	248-250°F (120-121°C)	257°F (125°C)	266°F (130°C)	267°F (135°C)	Investigator
B. anthracis	2								Schneiter, Kolb
B. anthracis	5-15								Stein, Rogers
B. anthracis	5-10	5-10							Murray
B. cereus	6								Schneiter, Kolb
B. stearo-thermophilus					12				Perkins
B. subtilis	6-17								Schneiter, Kolb
B. subtilis	10								Ecker
Cl. botulinum	330	100	32	10	4				Esty, Meyer
Cl. botulinum			30	10	4				Hoyt, Chaney, Cavell
Cl. botulinum	300	120	90	40	10				Tanner, McCrea
Cl. botulinum	300	40			6				Weiss
Cl. oedematiens			10	4	1				Hoyt, Chaney, Cavell
Cl. oedematiens			15						Ecker
Cl. septicum			5						Ecker
Cl. tetani	5-15	5-10							Murray, Headlee
Cl. welchii	5-10								Headlee
Cl. welchii		5							Ecker
Putrefactive anaerobe 3679	780	170	41.6	15.6	5.6				McCulloch
Thermophiles	834	405	100	40	11-12	3.9-4.6	1.7-2.2	0.7-0.9	Bigelow
Soil bacilli	660			15					Ecker
Soil bacilli	1020	420	120	15	6				Konrich

a. J. J Perkins in Reddish, G. F. (ed.), Antiseptics, Disinfectants, Fungicides, and Chemical and Physical Sterilization, Lea and Febiger, Philadelphia, 1957.

TABLE XV. DESTRUCTION TIME (IN MINUTES) OF BACTERIAL SPORES BY DRY HEAT AT DIFFERENT TEMPERATURES[a]

Organism (dry spores)	248°F (120°C)	266°F (130°C)	284°F (140°C)	302°F (150°C)	320°F (160°C)	338°F (170°C)	356°F (180°C)	Investigator
B. anthracis	45	20						Murray
B. anthracis			180					Koch et al
B. anthracis				60				Stein, Rogers
B. anthracis			180					Park, Williams
B. anthracis	60				9			Oag
B. subtilis				60				Perkins, Underwood
Cl. botulinum	120	60	60	25	25	15	10	Tanner, Dack
Cl. septicum						7		Oag
Cl. tetani		35	15					Murray, Headlee
Cl. welchii	50	15	5					Headlee
Cl. welchii						7		Oag
Garden soil					30	15		Eacker, Smith

a. J. J. Perkins (ed.), Manual of Sterilization and Disinfection, American Sterilizer Company, Erie, Pennsylvania.

TABLE XVI. TEMPERATURES OF STEAM UNDER DIFFERENT PRESSURES (AT SEA LEVEL)

Steam Pressure (lb/sq in.)	Temperature (°C)	Temperature (°F)
0	100.0°	212.0°
5	109.0°	228.0°
10	115.0°	239.0°
15	121.5°	250.7°
20	126.5°	259.7°

Chemical. Many chemical agents exist which destroy microorganisms. A great number of these possess only decontaminating capabilities. Among those which effect sterilization — or destruction of all known microbial life — is ethylene oxide.

Ethylene oxide is a powerful sterilant, effective even against bacterial spores. Since liquid ethylene oxide is extremely damaging to most materials, the chemical is usually employed in the gaseous state. Gaseous ethylene oxide has the advantages of superior penetration and relatively little damaging effects on materials. Because of these properties, it can be used to sterilize many objects or materials otherwise unsterilizable. It has been used to sterilize books, leather, wool, spices, cotton, rayon, silk, nylon, cardboard, paper, plastics, food, straw, rubber, pottery, semiprecious stones, linoleum, cellophane, oil paintings. It is widely used in the medical fields to sterilize garments, sheets, delicate instruments, electronic equipment, and surgical supplies.

Liquid ethylene oxide will cause burns if it comes in contact with the skin. Gaseous ethylene oxide is moderately toxic if inhaled; irritates the eyes, nose, and throat; and will numb the sense of smell if exposure is continuous. Exposure to the gaseous form may cause nausea and vomiting. The gas has an easily detected odor which serves as a warning signal if exposure occurs. Its odor is similar to that of acetic acid.

Pure ethylene oxide is extremely flammable and explosive in concentration of as little as 3 percent in air. These hazards are eliminated when

ethylene oxide is mixed with an inert gas such as Freon or carbon dioxide. A commonly used mixture consists of 88 percent Freon 12 and 12 percent ethylene oxide. This mixture is nonflammable and nonexplosive. The mixture may, however, separate if released into the air, because of molecular weight differences of the two compounds. Therefore, care should be taken to prevent leaks from containers of the gas mixture, piping, or from the exposure chamber.

Sterilization with ethylene oxide may be most efficiently carried out in a gas sterilizer similar to an autoclave. Automatic gas sterilizers are available which provide controlled humidity, gas concentration, and temperature — parameters upon which the sterilizing efficiency of the gas depends. Figure 35 shows a typical ethylene oxide-Freon 12 cycle for a chamber of this type.

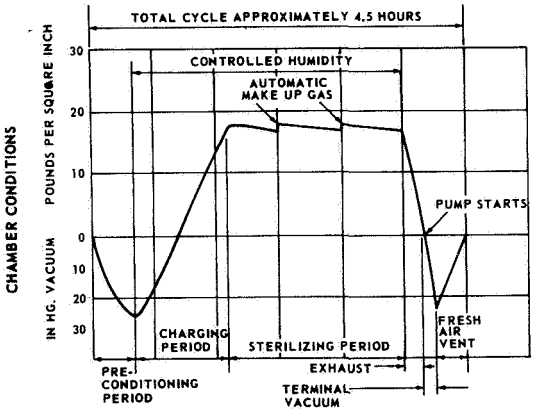


FIGURE 35. TYPICAL ETHYLENE OXIDE-FREON 12 EXPOSURE

The extent of "wetness" of the organisms themselves and the relative humidity of the atmosphere inside the chamber play a vital part in ethylene oxide's sterilization capabilities. Organisms can be preconditioned, or prewetted, to aid sterilization. It has been found that organisms which are desiccated (dry) are not as susceptible to sterilization by ethylene oxide. These facts are illustrated in Figure 36. Both graphs show sterilization of Bacillus subtilis spores was most quickly achieved at 33 percent relative humidity after the spores were conditioned at the same relative humidity. Efficiency drops off as the relative humidity goes below 33 percent and as it goes above 33 percent. Thus, when organisms are preconditioned (prewetted) there is an optimum humidity level for their destruction by ethylene oxide.

Conversely, when organisms have not been prewetted, ethylene oxide effectiveness increases as the relative humidity increases. These relations between humidity and ethylene oxide effectiveness are caused by rather complicated processes of dynamic exchange of water. The complex principles behind these dynamic exchanges of water will not be discussed here; it is sufficient to say that relative humidity is an important parameter for ethylene oxide sterilization.

Temperature of exposure and concentration of ethylene oxide are also important parameters for ethylene oxide sterilization. Temperature affects penetration and pressure of the gas. It also aids the chemical reactions by which ethylene oxide kills microorganisms. The sterilization efficiency of the gas is also dependent upon the gas concentration, within certain limits. In general, effectiveness of the gas increases as the gas concentration increases, provided humidity and temperature are controlled. Beyond certain limits of temperature and humidity, the effect of high concentrations is negligible.

It is emphasized that the use of ethylene oxide for sterilization purposes involves relatively small fire or health hazards as long as the gas is used in an inflammable mixture (such as 88 percent Freon-12 percent ETO). The gas mixture should be used in an exposure chamber specially constructed for sterilization with ethylene oxide so that concentration, temperature, and humidity can be controlled. The major safety precautions which should be taken are prevention of leakage and detection of leaks if they occur. Adequate ventilation should be provided in the area where the chamber or gas containers are located. The easily-detected odor of ethylene oxide provides quick warning to the operator if a leak occurs. Pressure gages in the chamber and on the gas containers provide a simple method of leak detection — a sudden drop in pressure is good indication of leakage. Special ETO indicators are also available.

The area where ethylene oxide is located should be strictly a "no smoking" area. In case of leakage, all ignition sources (gas burners, electric motors, etc.) should be shut down immediately and the area evacuated by all personnel.

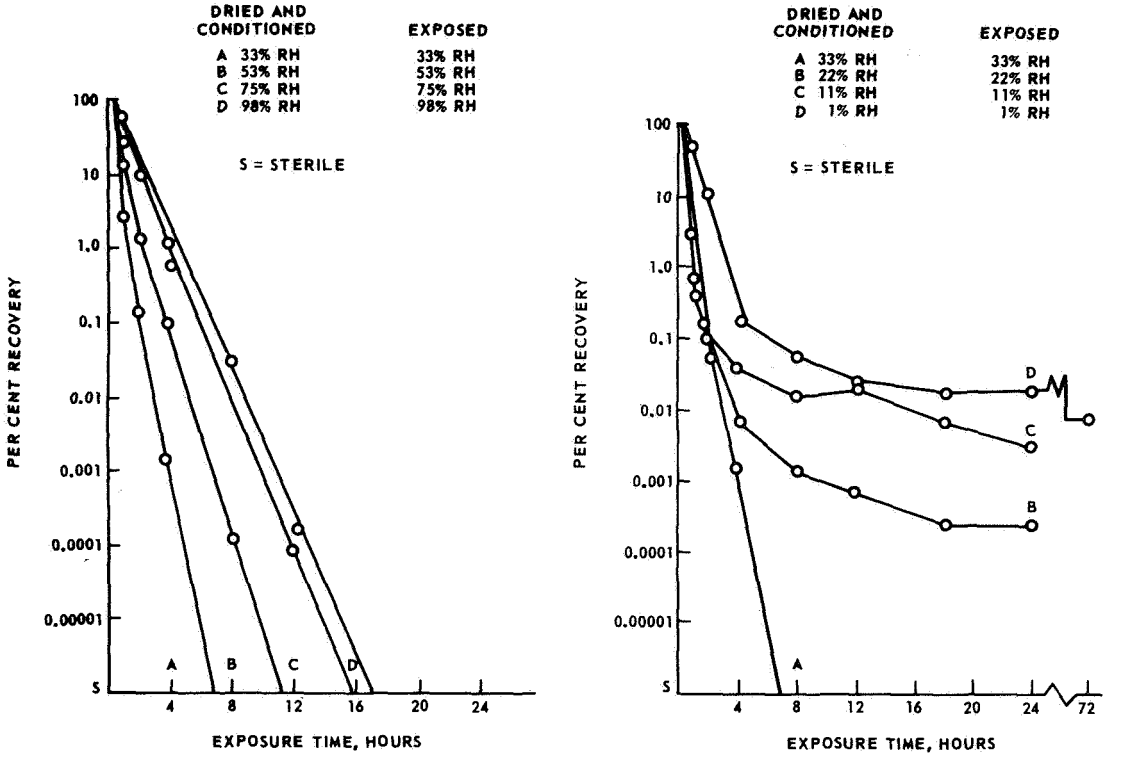


FIGURE 36. EFFECT OF RELATIVE HUMIDITY ON ETHYLENE OXIDE STERILIZATION

Review Questions

1. What is the meaning of "sterile"?
2. Explain the difference between decontaminating agents and sterilizing agents.
3. What is the major criterion used to distinguish living matter from nonliving?
4. Is boiling water a sterilant?
5. How does the application of heat kill microorganisms?
6. Of wet heat and dry heat, which is more efficient? Why?
7. What are four factors which affect the heat resistance of microorganisms?
8. Why are more stringent conditions required to kill 1 000 000 microorganisms on a given area than would be required to kill 1000 microorganisms on that same area?
9. Though wet heat effects sterilization in a much shorter time and at lower temperatures, dry heat must often be used instead. Explain.

10. Explain the effect of pressure as it is used in the steam autoclave.

11. What operating pressure is commonly used in a laboratory autoclave?

12. What is the equipment used for dry heat sterilization?

13. What are two advantages of gaseous ethylene oxide as a sterilizing agent?

14. What property of ethylene oxide provides a warning of exposure to the gas?

15. The 12 percent ethylene oxide-88 percent Freon 12 gas mixture is nonflammable and nonexplosive. Safety hazards may, however, develop if the mixture is released into the air. Why?

16. What are three parameters upon which the sterilizing efficiency of ethylene oxide depends?

17. What are four safety precautions which should be taken for the use of ethylene oxide?

18. In case of ethylene oxide gas leakage, what action should be taken?

It is suggested that at this point the student perform Laboratory Exercises XIII and XIV, which are described in detail in Appendix C at the end of this manual.

SPACECRAFT STERILIZATION

Requirements

NASA has set up certain sterility requirements for unmanned spacecraft intended to land on or penetrate the atmosphere of another planet. These requirements have been established in cooperation with other countries of the world. The Planetary Quarantine Policy states that no act will be performed which may exclude a planet as a base for the search for extraterrestrial life. Current Planetary Quarantine constraints include the following:

PROBABILITY OF CONTAMINATION FOR THE PERIOD OF BIOLOGICAL EXPLORATION OF A PLANET WILL BE 10^{-3}

(This means that the lander will be sterilized and that only once in every 1000 missions would a lander have any viable microorganisms.)

PROBABILITY OF CONTAMINATION OF THE PLANET BY THE FLIGHT SPACECRAFT WILL BE 10^{-3}

(This means that only once in every 1000 missions could a planet be contaminated with microorganisms from an orbiter.)

These requirements to prevent transporting Earth forms of living organisms to other planets are quite severe. Meeting these stringent requirements presents many problems.

Problems

One of the first considerations in achievement of spacecraft sterility is the wide distribution of microbial life in the Earth environment. Table XVII shows some common parts of our environment and their average microbial population.

TABLE XVII. MICROBIAL DISTRIBUTION IN SOME PARTS OF THE EARTH ENVIRONMENT

Location	Number of Microorganisms
Soils	10^4 to 10^8/g
Water	Up to 10^4/cc
Ocean Sediment	10^5 to 10^7/g
"Clean" Air	1 to 10/ft^3
"Dirty" Air	10^3 to 10^5/ft^3
Stratosphere	Up to 10^3/ft^3
Human Skin	1 to 10^4/$in.^2$
Plant Surfaces	10^3 to 10^6/$in.^2$
Sewage	10^6 to 10^7/cc
Floors	Up to 10^3/$in.^2$
Raw Milk	10^3 to 10^5/cc

Human beings are one of the major sources of microbial contamination. People carry many types of microorganisms on and in their bodies, and one of the greatest problems in the program is to prevent humans from contaminating a sterile spacecraft. A person taking mild exercise is exhaling over 10^6 (100 000) particles per minute, and many of these are viable microorganisms. Our hands, mouth, nose, hair, and skin constantly shed living microorganisms.

Keeping in mind that microbial life is found almost everywhere in tremendous numbers and that some microorganisms are very difficult to destroy, we can see that spacecraft sterilization presents a multitude of problems in design considerations alone. Material and component surfaces and interiors must be sterilized. Sterility, once achieved, must be maintained throughout component or system repairs, should such repairs be necessary. Sterility must be maintained throughout vehicle launch operations and as the craft passes through the contaminated Earth atmosphere on its way into space. The accomplishment of sterility itself is a problem of great magnitude because many materials are degraded or destroyed by the extreme conditions necessary to effect sterilization. Performance reliability of many components may be greatly reduced or destroyed by exposure to sterilization methods. Therefore, materials must be used and components designed to withstand sterilization.

To satisfy contamination constraints, problems of achieving spacecraft sterility and of maintaining that sterility must be overcome. There are numerous potential sources of contamination before launch, during flight, and after the craft reaches its destination. All of these sources must be anticipated and precautions taken for each. Figure 37 shows some potential sources of contamination.

Procedures

Many studies have been performed to determine the most effective and feasible procedures for spacecraft sterilization. Among the methods suggested are sterilization by chemical means, by heat, and by the conditions of outer space. The latter, automatic sterilization by the passage of the vehicle through interplanetary space, has been evaluated and judged inadequate. Experiments have shown that microorganisms can survive the extremely high vacuum of outer space. The penetrating radiations

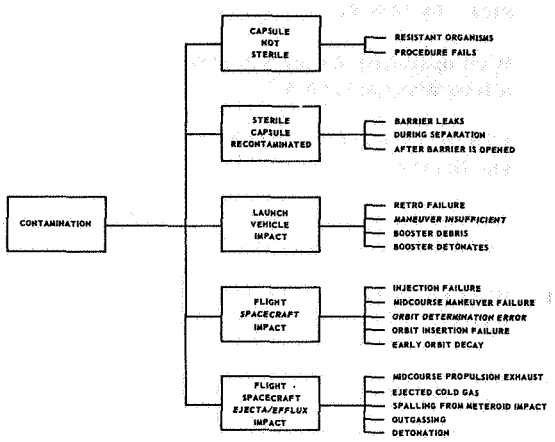


FIGURE 37. POTENTIAL CONTAMINATION SOURCES

of outer space are not sufficiently intense to assure sterility, and because internal temperature of a space vehicle is controlled to about room temperature, sterility would not be effected by heat during space flight. Therefore, space vehicles must be sterilized before they leave Earth.

Dry heat is the only approved technique for spacecraft sterilization at the present time. A temperature of 125°C (257°F) for 64 to 92 hours constitutes the presently proposed dry heat exposure for spacecraft. Present plans call for sterilization of the lander and decontamination of the orbiter. Any parts of the orbiter which touch the lander must, of course, be sterile. The spacecraft will be assembled in clean rooms to reduce the initial number of microorganisms. The sterile capsule will be enclosed in a shroud, or barrier, to protect it from contamination until it leaves Earth atmosphere.

Ethylene oxide is presently approved for decontamination purposes only. There is a possibility that the ETO-Freon 12 gas mixture will be authorized for limited sterilization at some future time. The proposed ethylene oxide-Freon 12 exposures for compatibility testing are shown in Table XVIII. The proposed time, temperature, gas concentration, and relative humidity conditions for three different levels are shown: parts and materials, TAT (type approval or prototype items), and FAT (flight acceptance of flight quality items).

TABLE XVIII. PROPOSED ETHYLENE OXIDE TEST ENVIRONMENTS

Test Condition	Test Level		
	Parts and Materials	TAT	FAT
Time at temp, hr	28	26	24
Temp, °C	50 ±2	50 ±2	40 ±2
Ethylene oxide gas conc, mg/l	600 ±50 (6 psig)	600 ±50 (6 psig)	500 ±50 (3 psig)
Relative humidity, percent	50 ±5	50 ±5	40 ±5
Number of cycles	6	6	1

Figure 38 shows the gas pressures necessary to effect the above ETO concentration in an ETO-Freon 12 gaseous environment.

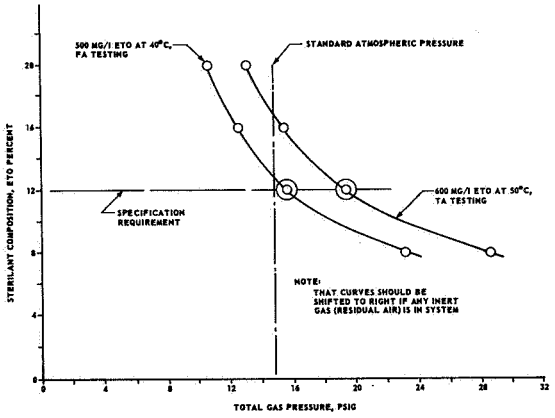


FIGURE 38. GAS PRESSURE-ETO CONCENTRATION RELATIONSHIP

The approved ethylene oxide decontamination method consists of exposure to 12 percent ETO-88 percent Freon 12 (at 40° to 50°C and 35 to 55 percent relative humidity) for 24 to 28 hours.

It is possible that the time of exposure for terminal heat sterilization can be reduced if the presterilization bioload can be sufficiently reduced. Thus, problems such as deterioration of materials and reduced reliability of components could be cut down. Prolonged heating is very damaging to some materials. Effective bioload reduction requires clean assembly in addition to application of decontamination techniques. Therefore, it is important for personnel involved in all mission phases, including fabrication and assembly, to have an understanding of the requirements and microbiological implications for spacecraft sterilization.

Review Questions

1. What is the Planetary Quarantine Policy?
2. What numerical constraints does the Planetary Quarantine Policy include?
3. What is the average number of microorganisms present on the human skin?
4. Contamination from human beings is one of the greatest problems in the spacecraft sterilization program. Explain.
5. Will the passage of the spacecraft through outer space be adequate to sterilize it? Why?
6. What is the presently approved sterilization method for spacecraft?
7. What chemical has been approved for decontamination of the spacecraft?
8. Why will the craft be assembled in clean rooms?
9. How will the sterile capsule be protected from contamination as it passes through Earth atmosphere?
10. What is the value of reducing the bioload before terminal sterilization?

APPENDIX A

GLOSSARY

aerobe — oxygen-requiring organism

agar — microbiological media containing dried polysaccharide extract of red algae (Rhodophyceae) as a solidifying agent

aliquot — a part or portion of the whole; usually a measured amount of fluid

amino acid — an organic compound containing both amino (NH_2) and carboxyl (COOH) groups

amphitrichous — having a single flagellum at each end of a cell

anaerobe — an organism which lives in the absence of oxygen

antiseptic — an agent which prevents or arrests the growth of organisms

aseptic — free of microbial contamination

asexual — a type of reproduction in which only one organism reproduces itself

assimilation — the synthesis by which living organisms incorporate environmental materials into their own substances

atom — the smallest particle of an element

autoclave — an apparatus using steam under pressure for sterilization

autotrophs — microorganisms which use only inorganic materials as a source of nutrients; CO_2 is their sole source of carbon

bacilli — rod-shaped bacteria

bacterial spore — resistant stage of a bacterial cell formed by members of the Clostridium and Bacillus genera

bactericidal — capable of killing bacteria

bacteriostatic — preventing the growth of bacteria without killing them

binary fission — an asexual process by which some microorganisms reproduce

biology — the science of life, living organisms, and life processes

capsule — a gelatinous slime layer surrounding the cell wall of some microorganisms

carbohydrate — organic compounds composed of carbon, hydrogen, and oxygen; examples: sugars and starches

cell — the fundamental structural unit of living matter

centrifuge — an apparatus used to separate or remove particulate matter suspended in a liquid by centrifugal force

chemotroph — an organism which relies upon reactions of chemical compounds to provide its energy

chlorophyll — a green light-trapping pigment essential for photosynthesis

chromosome — a gene-containing filamentous structure in a cell nucleus; the number per cell nucleus is constant for each species

circulation — the movement of food throughout the living body for use in various parts; may take place within the cell, by osmosis from cell to cell, or by means of a circulatory system such as the blood

class 100 clean station — no more than 100 particles over 0.5 micron in size per cubic foot

cocci — spheroidal bacteria

colloid — finely divided matter dispersed through continuous medium; may be solid in liquid (starch in water) or liquid in immiscible liquid (mayonnaise)

colony — a microscopically visible growth of microorganisms on a solid culture medium; may originate from only one organism

compound — a substance formed by the chemical union of two or more elements

conidia — asexual spores of a mold

culture — a mass of cells grown in or on a culture medium

cytology — study of the structure and function of cells

cytoplasm — the living matter of a cell inside the cytoplasmic membrane; contains the nucleus and inclusion granules

decontamination — reduction of living matter but not sterilization

deoxyribonucleic acid (DNA) — a type of nucleic acid occurring in the nuclei of living cells; contains phosphoric acid, D-2-deoxyribose, adenine, guanine, cytosine, and thymine

desiccation — removal of water; drying

digestion — enzymatic processes by which insoluble foodstuffs from the environment are rendered soluble in body fluids

dilution — the process of increasing the proportion of diluent to particulate matter of viable cells

dilution, serial — successive dilution of a specimen

diplococci — cocci occurring in pairs

disinfectant — an agent that frees from infection by killing the vegetative cells of microorganisms

element — the simplest type of matter; elements cannot be chemically broken down into simpler substances; everything we know is made up of elements, either by themselves or in combination with each other; oxygen is an element, as are hydrogen, iron, carbon, and nitrogen

enzyme — an organic catalyst produced within an organism

eucaryotic cell — a type of cell possessing a well-defined nuclear membrane

exoenzyme — an enzyme excreted by a microorganism into the environment; also called extracellular enzyme

facultative organism — one that can either utilize free oxygen or grow without it

fastidious organism — one that is difficult to isolate or cultivate on ordinary culture media; requires special growth factors, vitamins

fermentation — anaerobic oxidation of carbohydrate and carbohydrate-like compounds by enzyme action of microorganisms; gaseous oxygen is not involved in this energy-yielding process

flagellum — a flexible, whip-like appendage which some bacterial cells possess and which is used as an organ of locomotion

flora — microorganisms present in a given situation; examples: intestinal flora, soil flora, mouth flora

fungal spore — a primitive unicellular reproductive body

fungi — plantlike members of the Protista which lack chlorophyll and are filamentous in structure; may be parasitic or saprophytic

gene — a segment of a chromosome: repository of a unit of genetic information

generation time — time required for the doubling of a bacterial population or cell division

genus — a taxonomic division embracing related species; plural: genera

germ — a pathogenic microorganism

glucose — a monosaccharide carbohydrate called dextrose or grape sugar, used as an energy source by many microorganisms

gram stain — a differential stain by which bacteria are classified as gram-positive or gram-negative, depending on whether they retain or lose the primary stain (crystal violet) when subjected to a decolorizing agent

growth — increase in number of microorganisms

growth curve — graphic representation of the growth of bacteria in a culture medium

halophile — a microorganism whose growth is accelerated by or dependent on high salt concentration

heterotroph — a microorganism which is unable to use CO_2 as its sole source of carbon and which requires one or more organic compounds

host — an organism harboring another as a parasite or infectious agent

hydrolysis — the process by which a substrate is split to form two end products by the intervention of a water molecule

inclusion granules — concentrated deposits of volutin (ribonucleic acid or polyphosphate), glycogen or iogen (starch-like substances), fat globules, or sulphur globules present in the cytoplasm

incubation period — the time period during which microorganisms inoculated into a medium are allowed to grow

ingestion — the taking in of food

inhibition — refers to prevention of growth of microorganisms

inoculation — the artificial introduction of microorganisms into a culture medium

inoculum — the material containing microorganisms and used for inoculation

intracellular — within a cell

lipid — a fat or fat-like substance

liter — metric unit of capacity equal to the volume of one kilogram of water at 4° C and at standard atmospheric pressure

macro — prefix meaning large

macroscopic — visible without the aid of a microscope

media — substances used to provide nutrients for the growth and multiplication of microorganisms

mesophile — a bacterium which grows best at the moderate temperature range (25° to 40° C)

metabolism — the process which includes all the physical and chemical reactions carried on in order to sustain life

metabolite — any chemical used in metabolic processes

micro — prefix meaning small

microaerophilic — microorganisms which require some oxygen but less than that present in the atmosphere

micron — a unit of measurement that is one thousandth of a millimeter, abbreviated μ

millimicron — one thousandth of a micron, abbreviated $m\mu$

milliliter — one thousandth of a liter, abbreviated ml

mitosis — that part of binary fission consisting of nuclear division

molecule — the smallest unit of a compound that retains the chemical identity of that compound

mold — a fungus characterized by a filamentous structure

monotrichate — having a single flagellum

morphology — study of the size, shape, structure, and arrangement of living organisms

nucleic acids — a class of molecules composed of joined nucleotide complexes; the principle types are deoxyribonucleic acid (DNA) and ribonucleic acid (RNA)

nucleus — the structure of the cell composed of DNA and containing the chromosomes; plural: nuclei

obligate aerobe — an organism which grows only in the presence of free oxygen

obligate anaerobe — an organism which grows only in the absence of free oxygen

organic compound — a compound of carbon, differentiated from a noncarbon, or inorganic compound

parasite — an organism which derives its nourishment from a living host; does not necessarily cause disease

pathogenic — capable of producing disease

peptone — partially hydrolized protein

petri dish — a double glass or plastic dish used for cultivating microorganisms

pH — a number scale used to express the acidity or alkalinity of a substance; the scale ranges from pH 0 (very acid) to pH 14 (very alkaline); a pH of 7 is neutral

photosynthesis — the synthesis of carbohydrate material from carbon dioxide in the presence of chlorophyll, water, and light

phototroph — an organism capable of employing radiant energy for growth

polysaccharide — a carbohydrate formed by the combination of many molecules of monosaccharide; examples: starch, cellulose, glycogen

procaryotic cell — a type of cell in which the nuclear substance is not enclosed within a membrane

proteins — complex organic nitrogenous compounds composed of an extremely large number of amino acids joined through peptide linkages

protoplasm — living substance, living matter, or living material of a cell

protozoa — unicellular animal-like organisms

psychrophile — a microorganism which grows best at temperature below 20°C

pure culture — a culture containing only one species of organism

respiration — the process which includes all the gaseous interchanges of of a living organism

saprophyte — an organism living on dead organic matter

sarcinae — cubiodal groupings of cocci

serology — a branch of science which treats of serum

serum — the fluid portion of blood after coagulation; red and white cells and fibrin are removed

sexual — a type of reproduction requiring combination of two organisms of a particular species

smear — a thin film of material spread on a glass slide for microscopic examination

solution — a chemical state in which some substance (gas, liquid, solid) is mixed with a liquid

species — a taxonomic unit including only one kind of organism; a subdivision of a genus

spirilla — spiral-shaped bacteria

sporicidal — capable of killing bacterial spores

sporulation — production of spores

stain — a dye used to color microorganisms as an aid to visual inspection

staphylococci — cocci occurring in clusters of irregular numbers

sterile — free of all living organisms

stimuli — physical or chemical changes in the internal or external environment of a living organism

streptococci — cocci occurring in chains

substrate — a substance acted upon, as by an enzyme

synthesis — the putting together of simpler compounds or elements to form a more complex product

taxonomy — the system of naming and classifying organisms

tetracocci — cocci occurring in groups of four

thermal death time — time necessary to kill a particular species of microorganism at a given temperature

thermophile — an organism that grows best at temperatures of 50°C or higher

tissue — an aggregation of cells devoted to the performance of a particular function

TSA — abbreviation for Tryticase Soy Agar, a type of media on which microorganisms are grown

ultrasonic — sound waves of high intensity; used in microbiological assay procedures

unicellular — made up of one cell

vegetative cell — a bacterial cell in the stage of active growth as opposed to the spore stage

viable — living

vibrio — comma-shaped bacteria

virus — an intracellular obligate parasitic microorganism much smaller than bacteria

yeast — a unicellular type of fungus

APPENDIX B

STANDARD PROCEDURES FOR THE MICROBIOLOGICAL EXAMINATION OF SPACE HARDWARE

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APPENDIX B

STANDARD PROCEDURES FOR THE MICROBIOLOGICAL EXAMINATION OF SPACE HARDWARE

FOREWORD

This manual presents standard procedures for the microbiological examination of space hardware that have been developed in response to the requirements of the National Aeronautics and Space Administration (NASA) Planetary Quarantine Program. The NASA desires that these standard procedures be evaluated under all possible in-use situations. Criticisms, both positive and negative, comments, and remarks are solicited and should be sent to the NASA central microbiological monitoring laboratory:

Communicable Disease Center
U.S. Public Health Service
4402 North Seventh Street
Phoenix, Arizona 85014

The NASA Task Force that developed this manual included Joseph J. McDade (chairman), Carl W. Bruch, Martin S. Favero, Donald Vesley, and James B. Ingles (technical editor).

The manual was reviewed by a committee consisting of Robert Angelotti, Richard G. Bond, John Brewer, Charles Craven, Frank Farmer, Jack Fooks, Lloyd Herman, Robert K. Hoffman, John J. Iandlo, Alexander S. Irons, Jack D. Johnson, T. W. Kethley, I. J. Pflug, G. Briggs Phillips, John Ulrich, and William Walter.

The work of the Task Force and the many individuals who reviewed this manual is acknowledged and appreciated. The manual is approved by Lawrence B. Hall, Planetary Quarantine Office, Bioscience Programs Office, NASA, and Orr E. Reynolds, director, Bioscience Programs Office, NASA.

INTRODUCTION

Objective

The objective of this manual is to provide the standard procedures that shall be used to:

1. Assess the degree of microbial contamination of the intramural environment of space hardware assembly, test, and launch facilities.

2. Assess the microbial contamination on and within space hardware.

3. Assess the microbial contamination deposited on space hardware handled by technical personnel.

Scope

The standard procedures contained in this document apply to all space hardware and space hardware assembly, test, and launch facilities that are required to meet the standards established by the NASA Planetary Quarantine Office.

Organization

This section describes the objective, scope, and organization of the document, and outlines the required method for processing deviations from the procedures described in detail in the succeeding section. The three procedures contained in the succeeding section provide detailed instructions for implementing the test objectives. The last section describes the data recording and processing requirements.

Three appendixes supplement the main body of the manual and provide a complete listing of all references (Appendix B-a), requirements for the preparation and sterilization of equipment (Appendix B-b), and preparation and sterilization methods for the various required culture media (Appendix B-c).

Deviations

Any change from the details of these procedures shall be considered a deviation. Requests for deviation (with justification) shall be submitted in writing to the Planetary Quarantine Officer, Code SB; Bioscience Programs Office, National Aeronautics and Space Administration; Washington, D.C. 20546. Deviation from a given procedure shall not be permitted until written approval is granted to the requestor by the NASA Planetary Quarantine Officer.

PROCEDURES

All of the operations described in these procedures shall be performed by competent personnel in NASA-approved laboratories. The professional and technical qualifications of all associated personnel shall be as specified by NASA management.

General Notes

The following general notes apply to all operations described in these procedures:

1. Any deviation from the operations or equipment specified in these procedures (or appendixes) shall require the written approval of the NASA Planetary Quarantine Officer, as stated in the subsection, Deviations, of the INTRODUCTION to this report.

2. All operations involving the manipulation of sterile items and sample processing shall be performed in a laminar-flow clean bench meeting the Class 100 air-cleanliness requirements of Federal Standard No. 209 (Appendix B-a:1).

PROCEDURE NO. 1

ASSESSMENT OF MICROBIAL CONTAMINATION OF INTRAMURAL ENVIRONMENT OF SPACE HARDWARE ASSEMBLY, TEST, AND LAUNCH FACILITIES

Sampling of Microbial Contamination Accumulating on Surfaces

Method. Use the sampling strip method (Fig. B-1) to assess the degree of microbial contamination accumulating on surfaces exposed to intramural air. Prepare sterile, stainless-steel strips (16 gage, 1 in. × 2 in.) per Appendix B-b. Place flat trays containing sterile, stainless-steel strips in the areas to be sampled. The number and location of sampling sites shall be as prescribed by NASA management. When in place at each sampling site, carefully remove the aluminum foil covering each tray. Using sterile forceps, rearrange the sterile strips to form a monolayer. The duration of strip exposure and the sampling intervals shall be specified by NASA management. Collect six stainless-steel strips per site at each sampling interval. Wearing disposable, sterile surgical gloves, and using separate sterile forceps, asceptically place each strip to be assayed into a separate, dry, sterile, screw-capped bottle (Appendix B-b). Cover the screw cap of each bottle with aluminum foil to minimize the possibility of contaminating the lip of the bottle. Store the strips contained in the bottles in an insulated container maintained at 25° to 35°C. Assay the strips contained in the bottles within two hours after collection.

In a laminar-flow clean bench[1], aseptically add 50 milliliters of sterile, one percent peptone water (Appendix B-c) maintained at 25° ±5°C to each bottle containing a stainless-steel strip. Unless fewer samples are collected, process a minimum of 15 exposed strips with a period of 60 minutes.

Place the bottles containing one percent peptone water and the stainless-steel strips to be assayed in an ultrasonic bath (Appendix B-b) and ultrasonicate for 12 minutes. After ultrasonication, plate the contents of a given bottle as follows:

1. All operations involving the manipulation of sterile items (e.g., sample processing, filling Petri or Rodac plates with sterile trypticase soy agar) shall be performed in a laminar-flow clean bench meeting class 100 air-cleanliness requirements, as specified in Appendix B-a:1.

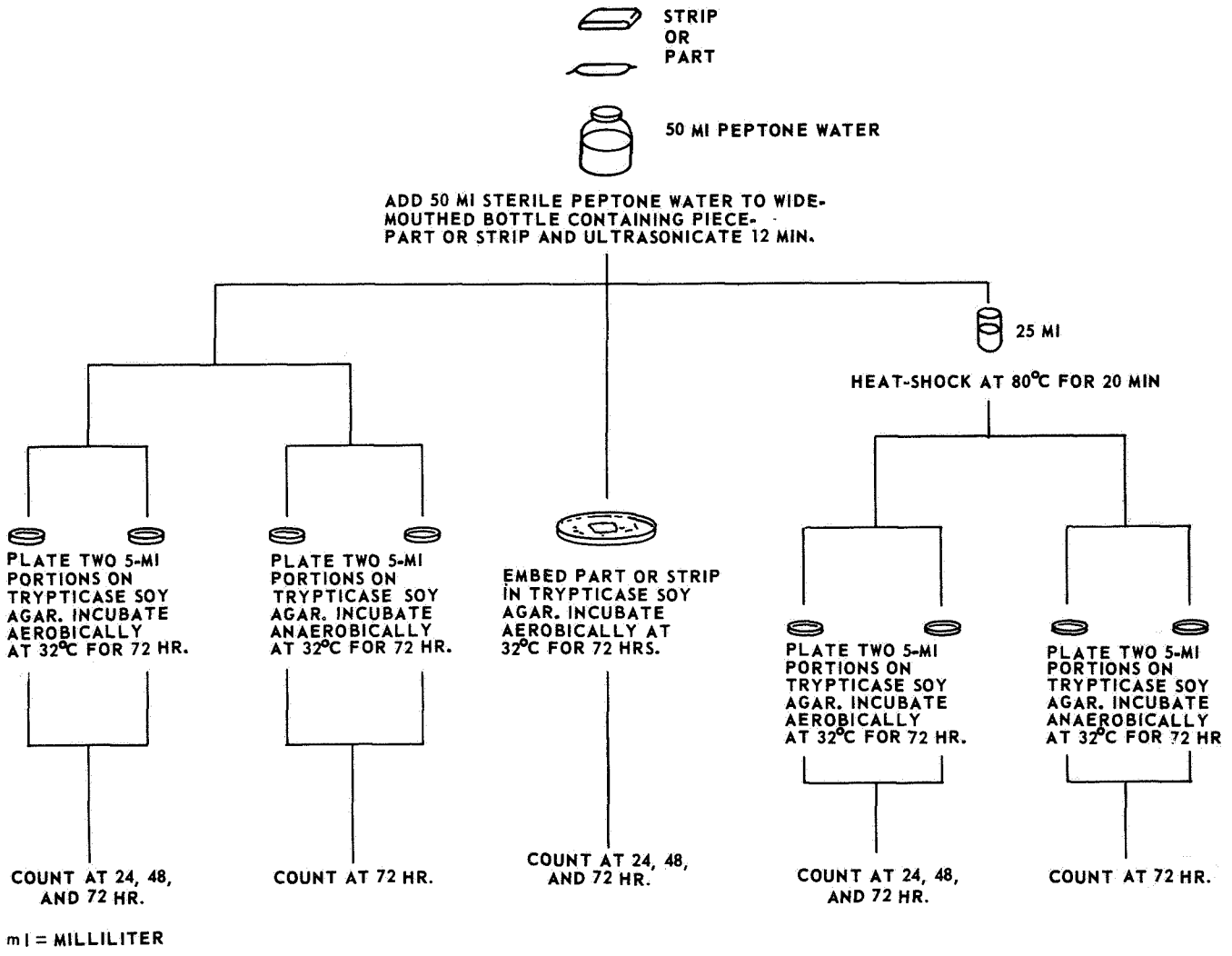

FIGURE B-1. SCHEMATIC OUTLINE OF PIECE-PART AND STAINLESS-STEEL STRIP ANALYSIS

Nonheat-Shocked Portions. In a laminar-flow clean bench, aseptically pipette 5.0-milliliter portions of the liquid into four 100-millimeter-diameter Petri plates (Appendix B-b). Add 20 milliliters of sterile, molten (50°C) trypticase soy agar (Appendix B-c) to each plate and mix the contents by gentle swirling. Allow the mixture to solidify. Using sterile forceps, aseptically remove the stainless-steel strip from the bottle and place the strip on a 9 ±2 milliliter layer of solidified, sterile trypticase soy agar (Appendix B-b) contained in a 100-millimeter-diameter Petri plate. Add 20 milliliters of sterile molten (50°C) trypticase soy agar to cover the stainless-steel strip completely.

Heat-Shocked Portions. Use the liquid remaining in each bottle for spore assays. In a laminar-flow clean bench, aseptically pipette a 25.0-milliliter portion of the rinse fluid remaining in each bottle into the bottom of a separate, sterile test tube (1-in. dia ×8-in.) taking care not to contaminate the sides of the tube. Heat-shock the rinse fluid contained in each test tube in an 80°C water bath for 20 minutes. Make certain the water bath level is at least one inch above the level of the liquid contents of each test tube being heat-shocked. Aseptically pipette 5.0-milliliter portions of the heat-shocked liquid from a given tube into four 100-millimeter-diameter Petri plates. Add 20 milliliters of sterile, molten (50°C) trypticase soy agar to each plate and mix the contents by gentle swirling. Allow the mixture to solidify.

Incubation. Aerobically incubate two nonheat-shocked and two heat-shocked samples. Anaerobically incubate (Appendix B-b) the remaining four samples from each series. Aerobically incubate the plated stainless-steel strip. Incubate all samples at 32°C for 72 hours. Perform colony counts of the aerobically incubated samples after 24, 48, and 72 hours. Do not remove the Petri plate covers until the final count after 72 hours. Perform colony counts of the anaerobically incubated samples after 72 hours. In the event of spreading colonies or subsequent laboratory accidents, the highest colony count obtained previously shall constitute the value to be recorded.

Calculation of Results. The average colony count from each of the two plates in each series (nonheat-shocked aerobic and anaerobic portions, heat-shocked aerobic and anaerobic portions) multiplied by ten equals the number of microorganisms per stainless-steel strip. Add the colony count from the plated stainless-steel strip (nonheat-shocked, aerobically incubated) to the previously calculated number of nonheat-shocked, aerobic microorganisms. The average values for the six stainless-steel strips exposed during a sampling interval equals the result for each site. Also record the range of values for the six stainless-steel strips.

Controls. Process a minimum of six sterile, unexposed, stainless-steel strips as sterility control check items on the entire assay procedure. Processing shall be during one of the assays conducted each day as prescribed by NASA management.

Microbiological Sampling of Environmental Surfaces[1]

Flat Surfaces. Use the Rodac plate method (Appendix B-a:4) to sample all flat surfaces, including rigid surfaces (worktables, floors) and nonrigid surfaces (clothing, packaging materials).

Prepare the Rodac plates (or equivalent) per Appendix B-b. Use trypticase soy agar for routine sampling. If germicidal solutions have been applied to a surface to be sampled, use trypticase soy agar with neutralizers (Appendix B-c).

Remove the cover of the Rodac plate. Impress the agar surface in the bottom half of the Rodac plate onto the surface to be sampled. Replace the cover on the Rodac plate. Collect a minimum of four samples from nonrigid surfaces such as clothing and packaging materials. Collect a minimum of 12 samples from each worktable top and a minimum of 12 samples from floor surfaces in the vicinity of each work area. After sampling, place the Rodac plates in an insulated container maintained at 25° to 35°C. Incubate all Rodac plates within two hours after sampling.

Incubation. Aerobically incubate 75 percent of the samples from each sampling site. Anaerobically incubate (Appendix B-b) the remaining 25 percent of the samples. Incubate all samples at 32°C for 72 hours. Perform colony counts of the aerobically incubated samples after 24 and 72 hours. Do not remove the Rodac plate covers until the final count is made after 72 hours. Perform colony counts of the anaerobically incubated samples after 72 hours. In the event of spreading colonies or subsequent laboratory accidents, the highest colony count obtained previously shall constitute the value to be recorded.

Calculation of Results. Express the results as the number of colonies per Rodac plate.

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1. Appendix B-a:1 and 2 describe techniques and problems associated with the microbiological sampling of surfaces.

Curved and Irregularly Shaped Surfaces. Use the cotton swab method (Appendix B-a:3) to sample curved and irregularly-shaped surfaces. Aseptically remove a sterile cotton swab from its container (Appendix B-b) and moisten the head of the swab in five milliliters of sterile, one percent peptone water against the interior wall of the tube.

Hold the swab handle to make about a 30-degree-angle contact with the surface to be sampled. Rub the head of the swab slowly and thoroughly over approximately four square inches of surface. Rub the swab over the four-square-inch surface three times, reversing direction between successive strokes. Return the head of the swab to the original tube of one percent peptone water. Break off the head of the swab below any portion of the handle that was touched by the sampler. Allow the swab head to drop into the liquid and replace the screw cap.

Place the tubes in an ultrasonic bath (Appendix B-b) and ultrasonicate for 12 minutes. After ultrasonication, process the liquid contents of each tube as follows:

Nonheat-Shocked Portions. In a laminar-flow clean bench, aseptically pipette 1.0-milliliter portions into two 100-millimeter-diameter Petri plates. Add 20 milliliters of sterile, molten (50°C) trypticase soy agar to each plate and mix the contents by gentle swirling. Allow the mixture to solidify.

Heat-Shocked Portions. Heat-shock the remaining one percent peptone water in each tube by placing the tube with the liquid and swab-head in an 80°C water bath for a total of 20 minutes. Make certain the water bath level is at least one inch above the level of the liquid contents of each test tube being heat-shocked. In a laminar-flow clean bench, aseptically pipette 1.0-milliliter portions of the heat-shocked fluid into two 100-millimeter-diameter Petri plates. Add 20 milliliters of sterile, molten trypticase soy agar to each plate and mix the contents by gentle swirling. Allow the mixture to solidify.

Dilutions. If deemed necessary, make serial, 10-fold dilutions of the one percent peptone water after the ultrasonication process is completed. Plate portions of each dilution as described above.

Incubation. Aerobically incubate one nonheat-shocked sample and one heat-shocked sample from each swab assay. Anaerobically incubate (Appendix B-b) the remaining nonheat-shocked and heat-shocked samples from that swab. Incubate all samples at 32°C for 72 hours. Perform colony counts of the aerobically incubated portions after 24, 48, and 72 hours. Do not remove the Petri

plate covers until the final count is made after 72 hours. Perform colony counts of the anaerobically incubated samples after 72 hours. In the event of spreading colonies or subsequent laboratory accidents, the highest colony count obtained previously shall constitute the value to be recorded.

Calculation of Results. Express the results as the microbial contamination per four square inches of surface. The resultant colony count for a given portion multiplied by five (multiplied by the dilution factor, if decimal dilutions are made) equals the number of microorganisms per four square inches of surface.

Controls. Process a minimum of six sterile swabs as sterility control items. Processing shall be during one of the assays conducted each day as prescribed by NASA management.

Microbiological Sampling of Intramural Air[1]

Volumetric — Sequential. For hourly sequential sampling of intramural air, use a long-term slit sampler (Reyniers or equivalent, Appendix B-b). Each sampler shall be equipped with a clock motor that will rotate the sample plate one revolution per hour and be calibrated to collect air at the rate of one cubic foot per minute. The volume of air sampled with each plate (Appendix B-b) shall not exceed 60 cubic feet.

Volumetric — Intermittent. For the intermittent sampling of intramural air, use a short-term slit sampler (Elliott or equivalent, Appendix B-b). If a requirement exists to characterize the airborne viable particles into discrete sizes, use a cascaded-sieve sampler (Andersen or equivalent, Appendix B-b). Each short-term slit sampler and each cascaded-sieve sampler shall be calibrated to collect air at a rate of one cubic foot per minute. The volume of air sampled with a given plate (Appendix B-b) shall not exceed 15 cubic feet and the sampling period shall not exceed 15 minutes. Incubate and assay each set of six plates in a sample collected with the cascaded-sieve sampler as a unit.

The sampling frequency and the sampling locations at which air samples shall be collected shall be as prescribed by NASA management. Unless specified otherwise, use trypticase soy agar as the medium to collect and culture all air samples.

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1. Appendix B-a:5 presents a comprehensive review of air sampling devices.

Volumetric — Incubation. Aerobically incubate all hourly, sequential air samples collected on 150-millimeter-diameter Petri plates (plates of this size are too large to be placed in a conventional Brewer anaerobic jar). Aerobically incubate 75 percent of the intermittent air samples from a given location. Anaerobically incubate the remaining 25 percent of the intermittent samples. Incubate all samples at 32° C for 72 hours. Perform colony counts of the aerobically incubated samples after 24, 48, and 72 hours of incubation. Do not remove the Petri plate covers until the final count is made after 72 hours. Perform colony counts of all anaerobically incubated samples after 72 hours. In the event of spreading colonies or subsequent laboratory accidents, the highest colony count obtained previously shall constitute the value to be recorded.

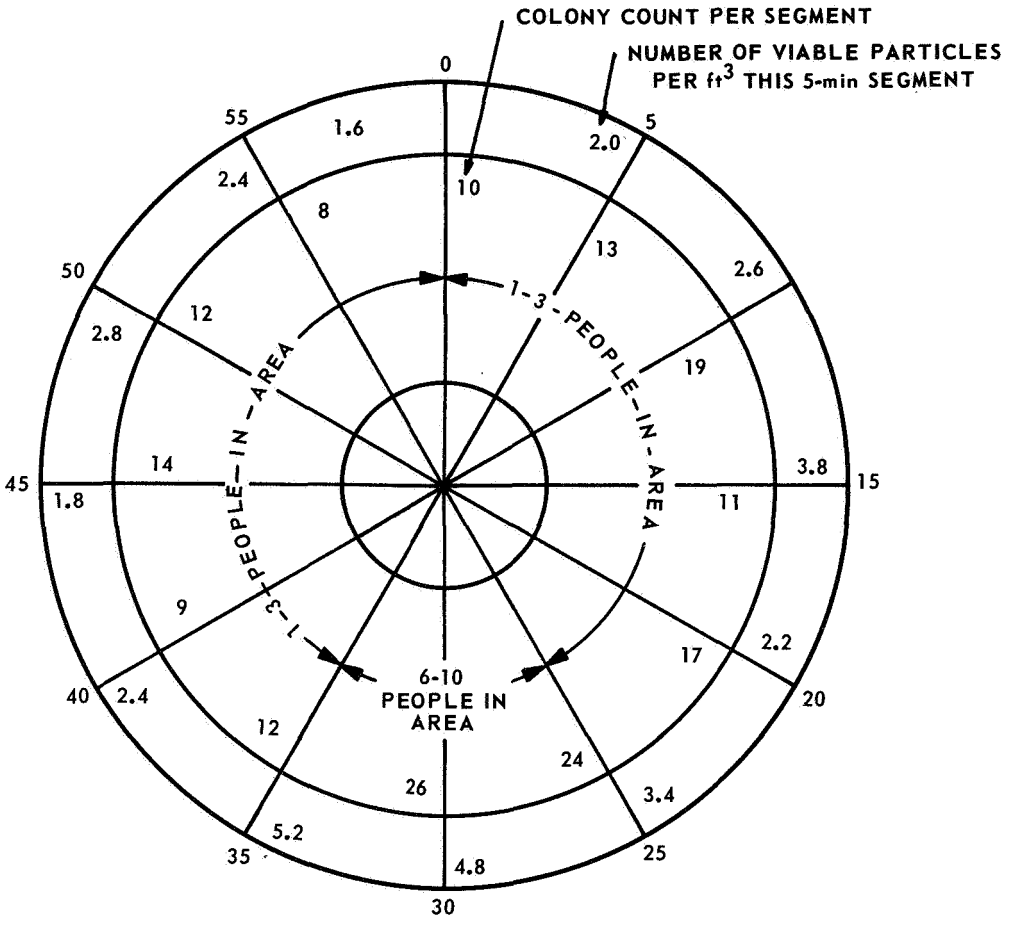
Volumetric — Calculation of Results. Long-Term Split Sampler. The next section contains a data sheet (Fig. B-2) used for a Reyniers slit sampler. Each segment (1/12) represents a 5-minute sample, thereby giving the number of airborne viable particles per five cubic feet of air. This value, divided by five, converts the count per segment to viable particles per cubic foot of air.

Volumetric — Calculation of Results. Short-Term Slit Sampler. For the short-term slit sampler, use a system similar to that used for the long-term slit sampler.

Volumetric — Calculation of Results. Cascaded-Sieve Sampler. The duration of the sampling time in minutes divided into the sum of the corrected colony count for all of the plates (Table B-I) gives the number of viable particles per cubic foot of air. The following calculations are typical for a 15-cubic-foot sample:

Stage	Observed count	Corrected count (Table B-I)	Viable particles per ft^3 air
1	48	51	3.40
2	33	34	2.26
3	16	16	1.06
4	8	8	0.53
5	2	2	0.13
6	1	1	0.07
		112	

$$\frac{112 \text{ corrected count}}{15 \text{ ft}^3 \text{ air}} = 7.5 \text{ viable particles per ft}^3 \text{ air}$$



DATE: 15 APR 66 TECHNICIAN: M. CHRISTENSEN

(1) SAMPLER LOCATION: JPL SAF BUILDING - MARINER PTM AREA

(2) TIME OF OPERATION: ON: 0800 OFF: 0900 TOTAL TIME: 60 MIN

(3) BACTERIOLOGICAL MEDIUM: TRYPTICASE SOY AGAR

(4) INCUBATION OF SAMPLE: AEROBIC TEMPERATURE: 32 C TOTAL TIME: 72 HR

REMARKS: SAMPLER LOCATED 5.0 FT FROM FLOOR AND APPROXIMATELY 5.0 FT FROM MARINER PTM. PERSONNEL ACTIVITY MODERATE.

FIGURE B-2. SAMPLE SHEET FOR REPORTING RESULTS OBTAINED WITH LONG-TERM SLIT SAMPLER

TABLE B-I. CONVERSION TABLE FOR USE WITH ANDERSEN CASCADED-SIENE SAMPLER (REF. 6)

r[a]	P[b]	r	P	r	P	r	P	r	P	r	P	r	P	r	P	r	P
1	1	41	43	81	91	121	144	161	206	201	279	241	369	281	485	321	649	361	931
2	2	42	44	82	92	122	146	162	208	202	281	242	372	282	488	322	654	362	942
3	3	43	45	83	93	123	147	163	209	203	283	243	374	283	492	323	659	363	952
4	4	44	47	84	94	124	148	164	211	204	285	244	377	284	495	324	664	364	963
5	5	45	48	85	96	125	150	165	213	205	287	245	379	285	499	325	670	365	974
6	6	46	49	86	97	126	151	166	214	206	289	246	382	286	502	326	675	366	986
7	7	47	50	87	98	127	153	167	216	207	292	247	384	287	506	327	680	367	998
8	8	48	51	88	99	128	154	168	218	208	294	248	387	288	509	328	686	368	1010
9	9	49	52	89	101	129	156	169	220	209	296	249	390	289	513	329	692	369	1023
10	10	50	53	90	102	130	157	170	221	210	298	250	392	290	516	330	697	370	1036
11	11	51	55	91	103	131	159	171	223	211	300	251	395	291	520	331	703	371	1050
12	12	52	56	92	105	132	160	172	225	212	302	252	398	292	524	332	709	372	1064
13	13	53	57	93	106	133	162	173	227	213	304	253	400	293	527	333	715	373	1078
14	14	54	58	94	107	134	163	174	228	214	306	254	403	294	531	334	721	374	1093
15	15	55	59	95	108	135	165	175	230	215	308	255	406	295	535	335	727	375	1109
16	16	56	60	96	110	136	166	176	232	216	311	256	409	296	539	336	733	376	1125
17	17	57	61	97	111	137	168	177	234	217	313	257	411	297	543	337	739	377	1142
18	18	58	63	98	112	138	169	178	236	218	315	258	414	298	547	338	746	378	1160
19	19	59	64	99	114	139	171	179	237	219	317	259	417	299	551	339	752	379	1179
20	21	60	65	100	115	140	172	180	239	220	319	260	420	300	555	340	759	380	1198
21	22	61	66	101	116	141	174	181	241	221	322	261	423	301	559	341	766	381	1219
22	23	62	67	102	118	142	175	182	243	222	324	262	426	302	563	342	772	382	1241
23	24	63	69	103	119	143	177	183	245	223	326	263	429	303	567	343	779	383	1263
24	25	64	70	104	120	144	179	184	246	224	328	264	432	304	571	344	786	384	1288
25	26	65	71	105	122	145	180	185	248	225	331	265	434	305	575	345	794	385	1314
26	27	66	72	106	123	146	182	186	250	226	333	266	437	306	579	346	801	386	1341
27	28	67	73	107	125	147	183	187	252	227	335	267	440	307	584	347	808	387	1371
28	29	68	75	108	126	148	185	188	254	228	338	268	443	308	588	348	816	388	1403
29	30	69	76	109	127	149	186	189	256	229	340	269	447	309	592	349	824	389	1438
30	31	70	77	110	129	150	188	190	258	230	342	270	450	310	597	350	832	390	1476
31	32	71	78	111	130	151	190	191	260	231	345	271	453	311	601	351	840	391	1518
32	33	72	79	112	131	152	191	192	262	232	347	272	456	312	606	352	848	392	1565
33	34	73	81	113	133	153	193	193	263	233	349	273	459	313	610	353	857	393	1619
34	36	74	82	114	134	154	194	194	265	234	352	274	462	314	615	354	865	394	1681
35	37	75	83	115	136	155	196	195	267	235	354	275	465	315	620	355	874	395	1754
36	38	76	84	116	137	156	198	196	269	236	357	276	468	316	624	356	883	396	1844
37	39	77	86	117	138	157	199	197	271	237	359	277	472	317	629	357	892	397	1961
38	40	78	87	118	140	158	201	198	273	238	362	278	475	318	634	358	902	398	2127
39	41	79	88	119	141	159	203	199	275	239	364	279	478	319	639	359	911	399	2427
40	42	80	89	120	143	160	204	200	277	240	367	280	482	320	644	360	921	400	[c]

[a]Observed counts [b]Corrected particle counts [c]Quantitative limit of stage (approx 2628 particles) is exceeded

Volumetric — Calculation of Results. Gravimetric. Use agar settling plates containing trypticase soy agar for the gravimetric sampling of air. A detailed evaluation of the procedure for use of agar settling plates in laminar-airflow clean benches is being conducted. Upon completion of this evaluation, a procedure will be prepared.

PROCEDURE NO. 2

ASSESSMENT OF MICROBIAL CONTAMINATION ON AND WITHIN SPACE HARDWARE

Piece-Part Exteriors

Method. In a laminar-flow clean bench[1], use sterile forceps to aseptically add the piece-part to be assayed to 50 milliliters of sterile, one percent peptone water (Appendix B-b) contained in a screw-capped bottle (Appendix B-b).

Ultrasonicate the bottles containing one percent peptone water and the piece-part to be assayed for 12 minutes. After ultrasonication, plate the liquid contents of a given bottle as follows:

Nonheat-Shocked Portions. In a laminar-flow clean bench, aseptically pipette 5.0-milliliter portions into four 100-millimeter-diameter Petri plates. Add 20 milliliters of sterile, molten (50°C) trypticase soy agar to each plate and mix the contents by gentle swirling. Allow the mixture to solidify.

Aseptically remove the piece-part from the bottle with sterile forceps and place the item on a 9 ±2 milliliter layer of solidified trypticase soy agar contained in a 150-millimeter-diameter Petri plate (Appendix B-b). Add sufficient sterile, molten (50°C) trypticase soy agar to completely cover the piece-part. (Obviously, only those piece-parts small enough to fit inside a 150-millimeter-diameter Petri plate can be plated directly.)

Heat-Shocked Portions. Use the liquid remaining in each bottle for spore assays. In a laminar-flow clean bench, aseptically pipette 25-milliliter

1. All operations involving the manipulation of sterile items (sample processing, filling Petri or Rodac plates with sterile trypticase soy agar) shall be performed in a laminar-flow clean bench meeting class 100 air-cleanliness requirements, as specified in Appendix B-a:1.

portion of the rinse fluid remaining in each bottle into a separate, sterile (1-in. dia × 8-in. long) plugged with cotton. Take care not to contaminate the sides of the test tube with the liquid being added. The liquid contained in each tube shall be heat-shocked in an 80 °C water bath for 20 minutes. Make certain the water bath level is at least one-inch above the level of the liquid contents of each test tube being heat-shocked. Then, aseptically pipette 5.0-milliliter portions of the heat-shocked liquid from a given bottle into four 100-millimeter-diameter Petri plates. Add 20 milliliters of sterile, molten (50 °C) trypticase soy agar to each dish and mix the contents by gentle swirling. Allow the mixture to solidify.

Incubation. Aerobically incubate two nonheat-shocked portions and two heat-shocked portions. Anaerobically incubate (Appendix B-b) the remaining four portions from each series. Aerobically incubate the plated piece-part. Incubate all portions at 32 °C for 72 hours. Perform colony counts of the aerobically incubated portions after 24, 48, and 72 hours. Do not remove the Petri plate covers until the final count after 72 hours. Perform colony counts of the anaerobically incubated portions after 72 hours. In the event of spreading colonies or subsequent laboratory accidents, the highest colony count obtained previously shall constitute the value to be recorded.

Calculation of Results. Express results as microbial contamination per piece-part surface. The (average) value from the two nonheat-shocked anaerobic, the two heat-shocked anaerobic, or the two heat-shocked aerobic portions from a given piece-part, multiplied by ten equals the number of microorganisms on the surface of that piece-part. The (average) value from the aerobic portions (multiplied by five), plus the colony count per plated piece-part, equals the total number of aerobic mesophilic microorganisms.

Test for Bacteriostatic and Bactericidal Substances. After incubation and counting of all portions, inoculate the agar surface of each Petri plate showing no evidence of microbial growth with 0.1 milliliter of an aqueous suspension containing 10^2 spores of the Fort Detrick strain of Bacillus subtilis var. niger[1]. Spread the inoculum over the entire agar surface with a sterile glass rod bent at a 75 degree angle (Appendix B-b). Aerobically incubate the Petri plates inoculated with spore suspension at 32 °C for a minimum of seven days. Growth of this spore inoculum indicates that the hardware item was not bacteriostatic or bactericidal. If bacteriostasis does occur, the assay of piece-parts composed of bacteriostatic material shall be repeated as described above, using trypticase soy agar with neutralizers (Appendix B-b).

1. May be obtained from the Communicable Disease Center, U.S. Public Health Service, 4402 North Seventh Street, Phoenix, Arizona 85014.

Controls. Process six sterile, stainless-steel strips daily as sterility control check items during at least one series of piece-part assays. The time and frequency of sterility control assays shall be as prescribed by NASA management.

Piece-Part Interiors

Method. Surface Decontamination. Using peracetic acid (Appendix B-b), surface decontaminate duplicates of each piece-part to be assayed.

Pulverization. Pulverize solids with a Pica Blender-Mill[1] (or equivalent). The blender employs a hand-pestle type of motion that achieves particle-size reduction by a combination of impact and milling action. The mortar is a tubular, stainless-steel vial approximately 2 1/2 inches long (30 milliliter capacity) × 1-inch ID[2]. The tube is fitted with removable end caps that are held in place by rubber O-rings. Stainless-steel washers are installed between the tube and the end caps. Each tube contains one 3/8-inch-diameter stainless-steel rod pestle and three 5/8-inch stainless-steel balls.

To prepare the Pica Blender-Mill for use, insert the rod and stainless-steel balls into the tubular mortar, wrap in Kraft paper, and autoclave at 121°C for 15 minutes. Individually wrap the O-rings and end caps in Kraft paper and autoclave at 121°C for 15 minutes. In a laminar-flow clean bench, assemble one end of the mortar, using a sterile 3 inch × 3 inch piece of 4-mil plastic file (e.g., polyethylene or Mylar) between the washer and end cap to ensure a liquid-tight seal.

Aseptically pipette 10.0 milliliters of sterile, 1.0 percent peptone water into the open end of the vial. Aseptically place the sample to be pulverized into the open end of the vial, and seal the vial as previously described. Attach the assembled vial to the blender-mill. (Process three vials simultaneously.)

For operations of more than one minute in duration, cool the vial containing the sample and broth to 4°C before processing. After the pulverization process, aseptically open one end of the vial and aseptically remove the contents. Aseptically pipette two 2.0-milliliter portions of the vial contents into two

1. Pitchford Manufacturing Corp., 1901 Painter Run Road, Pittsburgh, Pa., Catalog No. 3800.

2. Pitchford Manufacturing Corp., Catalog No. 201.

separate 150-millimeter-diameter Petri plates. Add 100 milliliter of sterile, molten (50°C) trypticase soy agar. Plate two additional 2.0-milliliter portions as above, except that 100-millimeter-diameter Petri plates and 20-milliliter portions of sterile, molten (50°C) trypticase soy agar shall be used.

Incubation. Aerobically incubate the 150-millimeter-diameter Petri plates. Anaerobically incubate (Appendix B-b) the 100-millimeter-diameter Petri plates. Incubate all samples at 32° C for seven days. Perform colony counts of the aerobically incubated samples after 24, 48, and 72 hours and after seven days. Do not remove Petri plate covers until after the final count is made after seven days. Perform colony counts of the anaerobically incubated samples after seven days. In the event of spreading colonies or subsequent laboratory accidents, the highest colony count obtained previously shall constitute the value to be recorded.

Calculation of Results. Express results as colonies per unit volume of plated sample.

Controls. Include a control for each sample to determine if material in the sample exhibits inhibitory properties. A spot plate technique shall be used. Spot 0.01 milliliter of the processed sample suspension on a previously seed and dried agar plate. Aerobically incubate the cultures at 32°C and observe for zones of inhibition at 8, 24, 48, and 72 hours. Use standard sporeforming microorganisms for such control, including Bacillus subtilis var. niger and Clostridium sporogenes. In addition, any organism isolated from the interior of a sample shall be retained and also used for subsequent controls. At least five such isolates shall be employed. If inhibition is observed, add appropriate neutralizers to the culture medium.

Subsystems, Systems, and Landing Capsules

The following definitions apply to the space hardware specified in this category:

1. Subsystems — combinations of modules and components

2. System — assembly of subsystems capable of operating as a total unit

3. Landing capsule — that part of the space hardware that impacts the extraterrestrial surface

Exterior Surfaces. Method. Hardware design shall provide for the inclusion of detachable stainless-steel strips (16 gage, 1 in. × 2 in.) on the surface of each flight item. The time of attachment, the location, and the number of these strips shall be specified by NASA management. Before terminal heat sterilization, aseptically remove these stainless-steel strips using sterile forceps and individually assay each strip according to the procedure schematically outlined in Figure B-1 and described in detail in the first section of PROCEDURE NO. 1.

Interiors. Presently, there is no microbiological requirement to sample the interiors of hardware other than piece-parts.

PROCEDURE NO. 3

ASSESSMENT OF MICROBIAL CONTAMINATION DEPOSITED ON SPACE HARDWARE HANDLED BY TECHNICAL PERSONNEL

Method

Using sterile forceps, place the test item in the handler's right palm. Require the handler to close his right hand into a fist, then open his right hand and transfer the test object into his left palm. Require the handler to close his left hand into a fist, the open his left hand and insert the test item into a dry, sterile, assay bottle, taking care to avoid touching the rim of the bottle as he inserts the test item.

Require each handler to manipulate a minimum of six test items. Assay each test item as outlined in the first section of PROCEDURE NO. 1.

Incubation

Perform incubation as described in the first section of PROCEDURE NO. 1.

Calculation of Results

The average colony count from each of the two plates in each series (nonheat-shocked aerobic and anaerobic portions, heat-shocked aerobic, and

anaerobic portions multiplied by ten equals the number of microorganisms per test item. Add the colony count from the plated test item (nonheat-shocked, aerobically incubated) to the previously calculated number of nonheat-shocked aerobic microorganisms. The average values for the six test items exposed during a sampling interval equals the result for each site. Also, record the range of values for the six test items.

DATA RECORDING AND PROCESSING

Primary emphasis has been placed on asporogenous and sporogenous, aerobic and anaerobic, heterotrophic mesophiles. Data record forms (Fig. B-2 is typical) shall include untreated data as well as calculated results per unit volume or surface area for the above listed microorganisms. Also, include the microbial contamination per plated item, such as stainless-steel strip or piece-part. The data record sheet format and all processing and reduction of data shall be as specified by NASA management.

APPENDIX B-a

REFERENCES

1. Federal Standard No. 209. Clean Room and Clean Work Station Requirements, Controlled Environments. 16 December 1963.

2. Greene, V. W. and Herman, L. G. Problems Associated with Surface Sampling Techniques and Apparatus in the Institutional Environment. Jour. Milk & Food Technology, vol. 24. 1961. pp. 262-265.

3. Vesley, D. Surface Sampling Techniques for the Institutional Environment. Present Status. Proceedings National Conference on Institutionally Acquired Infections. 1963. pp. 101-103.

4. Hall, L. B. and Hartnett, M. J. Measurement of the Bacterial Contamination on Surfaces in Hospitals. Public Health Reports. vol. 79. 1964. pp. 1021-1024.

5. Wolf, H. W., et al. Sampling Microbiological Aerosols, Public Health Mongraph No. 60, 1959.

6. Andersen, A. A. New Sampler for the Collection, Sizing and Enumeration of Viable Airborne Particles. Jour. Bact., vol. 76. 1958. pp. 471-484.

APPENDIX B-b

PREPARATION AND STERILIZATION OF EQUIPMENT[1]

Stainless-Steel Surfaces

Cleaning. Wash in hot tap water containing a nonionic detergent (7×, Haemo-Sol, or equivalent.) Rinse three times with hot distilled water. Rinse with isopropyl alcohol. Rinse with ethyl ether. Drain dry.

Preparation for Sterilization. Strips for Space Hardware. Requirements for strips to be attached to flight hardware shall be resolved between the cognizant project office and NASA management. The preparation and sterilization of the strips to be attached shall be resolved during such negotiations.

Preparation for Sterilization. Microbial Fallout Sampling Strips. Using forceps, place clean, dry stainless-steel strips (50 per tray) on flat noncorrosive metal trays (11 in. × 15 in. × about 0.006 in.). The metal trays shall not have raised edges.

Arrange the stainless-steel strips in a monolayer on each tray. Wrap each tray with aluminum foil. Make certain that the entire tray is covered and that the foil is not punctured in the process.

Sterilization. Sterilize the wrapped trays with heat at (175°C for 2 hr). After sterilization, select one tray as a sterility control for each ten trays processed. Select ten stainless-steel strips at random from each sterility control tray and assay these strips as described in the first section of PROCEDURE NO. 1.

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1. All operations involving the manipulation of sterile items (e.g., filling Petri or Rodac plates with sterile trypticase soy agar) shall be performed in a laminar-flow clean bench meeting class 100 air-cleanliness requirements as specified in Appendix B-a:1.

Forceps

Clean stainless-steel forceps (at least 6 in. long) as described in the first section of PROCEDURE NO. 1 and individually package in aluminum foil or in capped test tubes. Sterilize with dry heat at 175° C for two hours.

Screw-Capped Bottles

Type. Use wide-mouth, screw-capped, square, four-ounce bottles (Bussey Products Company, 2750 West 35th Street, Chicago 32, Illinois; Catalog No. 1463, or equivalent).

Cleaning. Wash bottles and screw caps in hot tap water containing detergent (7×, Haemo-Sol, or equivalent). Rinse five times with tap water. Rinse five times with distilled water. Drain dry.

Sterilization. Cover each bottle with a layer of aluminum foil to a level one inch below the mouth of the bottle. Sterilize the aluminum-foil covered bottle with dry heat at 175° C for two hours. Sterilize screw caps in an autocalve at 121° C for 15 minutes. In a laminar-flow clean bench, aseptically place sterile screw caps on the sterile bottles. Replace the aluminum foil over the capped bottles. Following sterilization, aseptically add 25.0 milliliters of sterile trypticase soy broth (Appendix B-c) to six bottles selected at random as sterility controls from any lot of 50 processed bottles. Incubate the sterility controls at 32° C for a minimum of 72 hours.

Ultrasonic Bath

The ultrasonic bath (Branson Instruments: generator, A-300; tank, LT-80 power control, PC-30; or equivalent) shall conform to the following specification:

1. Frequency shall be 25 kc/sec.

2. Power output in relation to bottom surface area of tank shall be at least 2.3 W/sq inch (0.35 W/cm^2).

3. If the ultrasonic bath is not automatically tuned, tuning shall be performed according to the manufacturer's directions.

4. Inside surfaces of the bath shall be stainless steel.

5. Glass bottles containing piece-parts or stainless-steel strips shall be supported on the bottom of the tank.

6. Tank fluid shall be an aqueous solution of 0.3 percent by volume polyoxyethylene sorbitan mono-oleate (Tween 80).

7. Temperature of bath fluid shall be at least 25° C and shall not exceed 37° C. Bath fluid shall be changed periodically to prevent heat-up.

8. Bath liquid shall be at least one inch above the level of the liquid in the bottles being ultrasonicated.

Petri Plates

Type. Commercially available, disposable, sterile 100-millimeter-diameter and 150-millimeter-diameter Petri plates or Pyrex glass Petri plates of similar diameter are equally acceptable.

Sterile, disposable Petri plates have the advantage of being ready for immediate use. Pyrex plates are reusable and should be washed, according to the procedure outlined under subsection Screw-Capped Bottles. Cleaning. in this appendix. Place clean, dry glass Petri plates into metal Petri plate containers. Sterilize packaged Petri plates with dry heat at 175°C for two hours.

Rodac Plates

Add 15.5 milliliters of sterile trypticase soy agar to each Rodac plate. Wrap prepared batches of Rodac plates securely in Parafilm M (or equivalent). Incubate prepared and wrapped plates 72 hours at 32° C. Unwrap and discard contaminated plates. Rewrap and refrigerate plates at 4° to 6° C until 12 to 18 hours before use. Again, unwrap plates and leave at room temperature until used. Use sterile Rodac plates within 18 hours after unwrapping and within seven days of initial preparation.

Cotton Swabs

Use commercially available cotton swabs (nonabsorbent cotton), firmly twisted to 3/16 inch by 3/4 inch over one end of a six-inch wooden applicator stick. The swabs shall be packed individually in a protective container, cotton plugged test tubes, covered test tubes with swab heads away from the closure. Sterilize swabs by autoclaving at 121°C for 15 minutes.

Volumetric Air Samplers

Calibration and Preparation. All volumetric air samplers (Reyniers, Model FD-100 A; Andersen cascaded-sieve Model 0203; or Elliott, Model No. B; or equivalents) shall be equipped with a calibrated inline flowmeter (Fisher and Porter or equivalent). A minimum vacuum source of 1.5 cubic feet per minute free air shall be required for each sampler. Before initial operation and at monthly intervals thereafter, calibrate all samplers to draw air at a velocity of one cubic foot per minute through the samplers.

Preparation of Volumetric Air Sampler Plates. Andersen Sampler. Use plates designed for use with the Andersen sampler. Prepare each plate to contain 27 ±0 milliliters of sterile trypticase soy agar. Wrap prepared batches of plates securely in Parafilm M or equivalent. Incubate prepared and wrapped plates at 32°C for 72 hours. Unwrap and discard contaminated plated. Rewrap and refrigerate plates at 4° to 6°C until 12 to 18 hours before use. Again, unwrap plates and leave at room temperature until used. Use sterile plates within 18 hours after unwrapping and within seven days of initial preparation.

Preparation of Volumetric Air Sampler Plates. Elliot Sampler. Use sterile, 100-millimeter-diameter Petri plates. Prepare each plate to contain 27.0 ±2.0 milliliters sterile trypticase soy agar. Wrap prepared batches of plates securely in Parafilm M or equivalent. Incubate prepared and wrapped plates at 32°C for 72 hours. Unwrap and discard contaminated plates. Rewrap and refrigerate plates at 4° to 6°C until 12 to 18 hours before use. Again unwrap plates and leave at room temperature until used. Use sterile plates within 18 hours after unwrapping and within seven days of initial preparation.

Preparation of Volumetric Air Sampler Plates. Reyniers Sampler. Use sterile, 150-millimeter-diameter Petri plates. Prepare each plate to contain 85.0 ±2.0 milliliters of sterile trypticase soy agar. Wrap prepared

batches of plates securely in Parafilm M or equivalent. Incubate prepared and wrapped plates at 32°C for 72 hours. Unwrap and discard contaminated plates. Rewrap and refrigerate plates at 4°C to 6°C until 12 to 18 hours before use. Again, unwrap plates and leave at room temperature until used. Use sterile plates within 18 hours after unwrapping and within seven days of initial preparation.

Data Recording. Figure B-2 shows a sample sheet for reporting results obtained with the long-term slit sampler. A conversion table [B-2:6] for correcting particle counts with the Andersen sampler is shown in Table B-I.

Peracetic Acid Decontamination

Use a freshly prepared aqueous solution of 2.0 percent peracetic acid and 0.1 percent nonionic detergent to surface decontaminate piece-parts to be assayed. Perform the decontamination procedure in a laminar-flow clean bench. Using sterile forceps, completely immerse the piece-part to be assayed in the freshly prepared peracetic acid solution for 20 minutes. Remove the piece-part and allow it to drain into the peracetic acid solution for 30 seconds. Place the decontaminated piece-part in a sterile, 150-millimeter-diameter Petri plate and cover it with the Petri plate cover.

Place the covered Petri plate/piece-part combination in a laminar-flow clean bench. Remove the Petri plate cover and allow the class 100 filtered air to pass over the piece-part for 20 minutes. After air drying, the piece-part is ready for assay.

Ethylene Oxide Decontamination

Expose items to be decontaminated to a pre-ETO moisturization at 40 ±10 percent relative humidity for 60 minutes. Expose items to be decontaminated to a gaseous mixture of ETO-F12 at a temperature of 130° ±5°F and 40 ±10 percent relative humidity. Heat-labile materials, including certain plastics, require 500 ±50 milligrams ethylene oxide per liter of chamber volume for six hours. Other items require 800 ±50 milligrams of ethylene oxide per liter of chamber volume for three hours.

Preparation of Anaerobic Conditions

Establish and maintain anaerobic conditions in Brewer jars or equivalent. Use gaseous hydrogen and a catalyst to remove residual oxygen. Place a desiccant, such anhydrous $CaCl_2$, in the bottom of each Brewer jar. Include the aerobic indicator organism Alcaligenes faecalis and the anaerobic indicator organism Clostridium sporogenes in each jar[1].

APPENDIX B-c

PREPARATION AND STERILIZATION OF CULTURE MEDIA[2]

One-Percent Peptone Water

Suspend 10 grams Bacto peptone (or equivalent) in one liter of distilled water. Adjust to pH 7 with HCl or NaOH. Dispense as required. Sterilize by autoclaving at 121°C for 15 minutes.

Trypticase Soy Agar

Suspend 40 grams dehydrated trypticase soy agar (Baltimore Biological Laboratory, Baltimore, Md.; or equivalent) in one liter of distilled water. Heat gently with frequent agitation and boil for one minute. Dispense as required. Sterilize by autoclaving at 121°C for 15 minutes.

Trypticase Soy Agar With Neutralizers

Prepare and sterilize the required amount of sterile, molten (50°C) trypticase soy agar. In a laminar-flow clean bench, aseptically add sufficient

1. Cultures of both indicator organisms may be obtained from the Communicable Disease Center, U.S. Public Health Service, 4402 North Seventh Street, Phoenix, Arizona 85014.

2. All operations involving the manipulation of sterile items (e.g., filling Petri or Rodac plates with sterile trypticase soy agar) shall be performed in a laminar-flow clean bench meeting class 100 air-cleanliness requirements as specified in Appendix B-a:1.

sterile (autoclaved individually and cooled at 60°C) polyoxyethylene sorbitan mono-oleate (Tween 80) and liquid lecithin to make concentrations of 0.5 percent (Tween 80) and 0.07 percent (lecithin) by volume. Mix thoroughly and use within 30 minutes.

Trypticase Soy Broth

Suspend 30 grams of dehydrated trypticase soy broth (Baltimore Biological Laboratory or equivalent) in one liter of distilled water. Mix thoroughly and warm gently until solution is complete. Dispense as required. Sterilize by autoclaving at 121°C for 15 minutes.

Trypticase Soy Broth With Neutralizers

Prepare and sterilize the required amount of sterile trypticase soy both. In a laminar-flow clean bench, aseptically add sufficient sterile (autoclaved individually and cooled at 60°C) polyoxyethylene sorbitan mono-oleate (Tween 80) and liquid lecithin to make concentrations of 0.5 percent (Tween 80) and 0.07 percent (lecithin) by volume. Mix thoroughly and use within 30 minutes.

APPENDIX C

LABORATORY EXERCISES

Apparatus frequently used in these exercises are shown in Figures C-1 and C-2.

Laboratory Exercise I
Bacterial Distribution

1. Purpose — To demonstrate the presence of bacteria on the human body and objects in our environment.

2. Materials Needed (per student)

   a. One sterile cotton swab

   b. One pair sterile gloves

   c. One sterile prepared plate of blood agar

   d. Five prepared sterile plates T-soy agar

3. Procedure

   a. touch your lips firmly to surface of blood agar plate

   b. Touch your fingers to surface of one T-soy agar plate

   c. Swab you throat and roll swab over surface of one T-soy agar plate

   d. Cough directly onto surface of one T-soy agar plate

   e. Carefully remove the sterile glove from its envelope and aseptically put it on; touch gloved fingers to surface of one T-soy agar plate

   f. Rub your gloved fingers over desk or table top and touch them to surface of another T-soy agar plate

   g. Label each plate clearly; place plates in incubator for 24 hours at 37° C and then count colonies; record results on data sheet shown in Figure C-3.

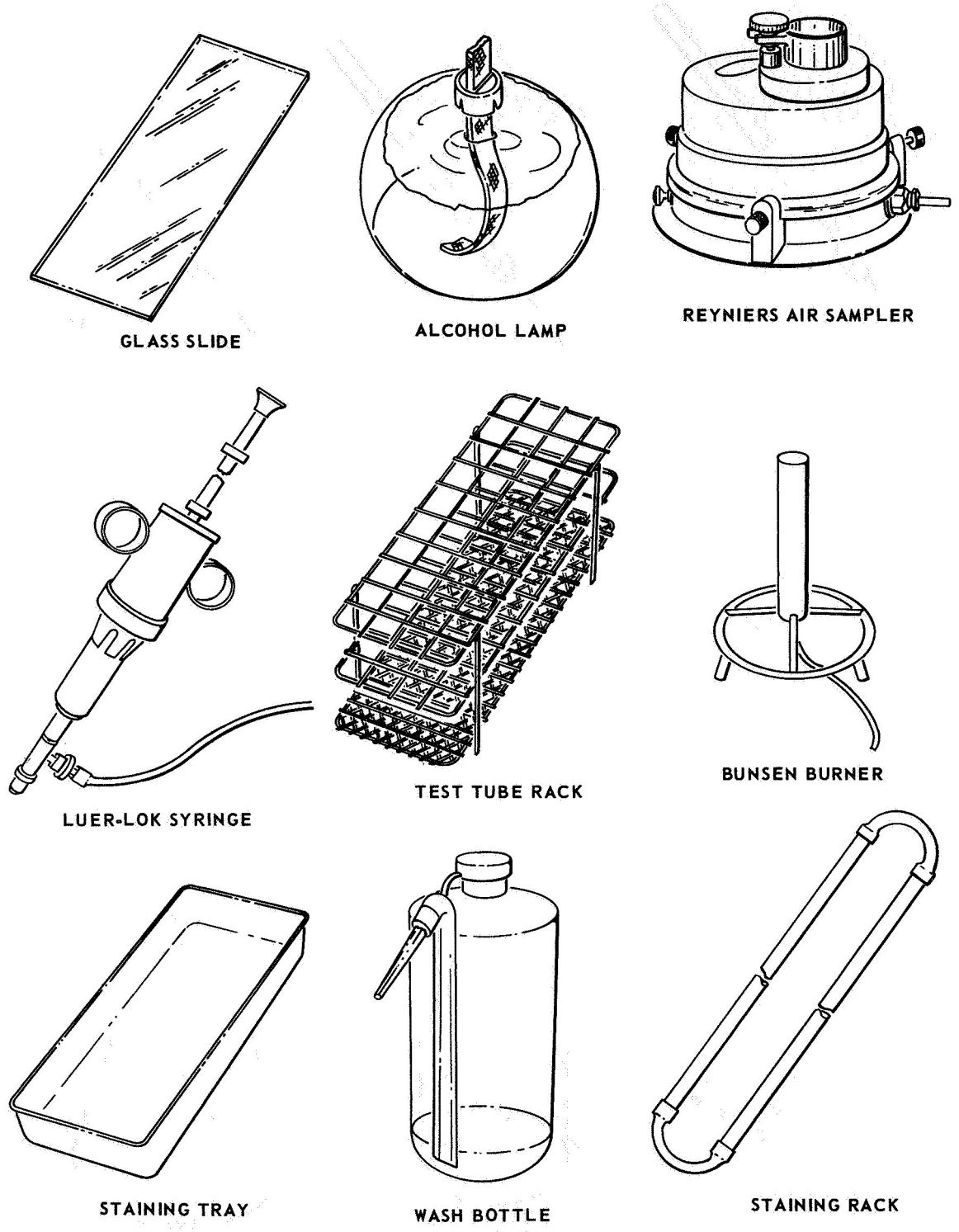

FIGURE C-1. LABORATORY APPARATUS

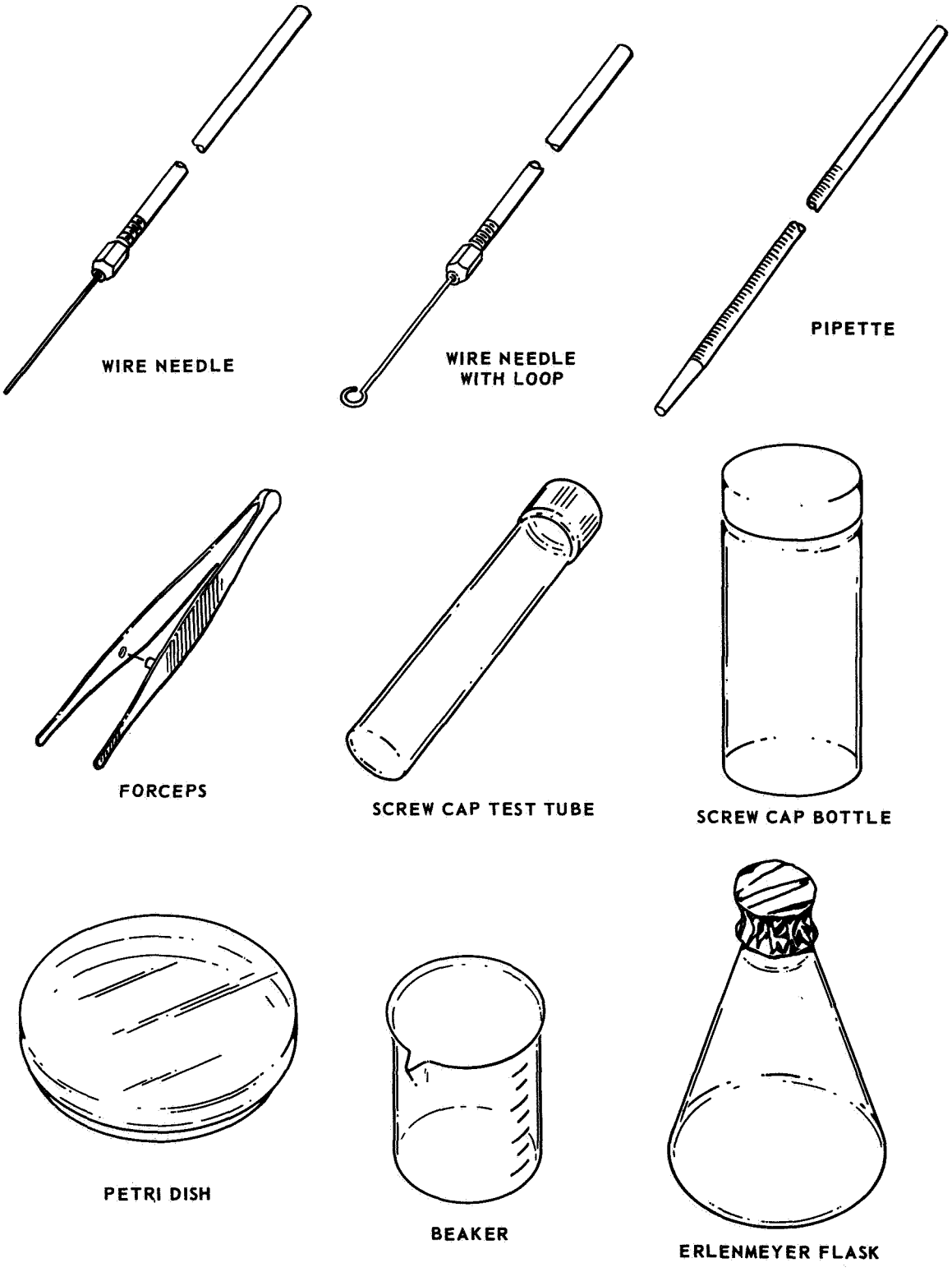

FIGURE C-2. ADDIDIONAL LABORATORY APPARATUS

Plate	No. of Colonies
Kiss	
Fingers	
Sterile glove	
Contaminated glove	
Throat swab	
Cough	

FIGURE C-3. BACTERIAL DISTRIBUTION DATA SHEET

Laboratory Exercise II

Contamination From Human Handling

1. Purpose — To demonstrate that handling a part will introduce increased biological contamination.

2. Materials Needed (per student)

a. Two sterile test items

b. Two sterile 4-ounce screw cap bottles

c. Two sterile 4-ounce bottles with 60 ml sterile peptone each

d. Two sterile test tubes

e. Sixteen sterile empty Petri dishes

f. Two sterile T-soy agar plates

g. Two pairs sterile forceps

h. Six sterile 10 ml pipettes

i. Twenty tubes with 20 ml liquid sterile T-soy agar each

3. Procedure

a. Place one of test items in your with hand and close your hand into a fist.

b. Transfer object to left hand and close hand over it.

c. Insert object into one of the sterile screw cap bottles.

d. Place sterile test item, using sterile forceps, into the other sterile screw cap bottle.

e. Aseptically pour 50 ml of sterile peptone water into each bottle.

f. Place the bottles in the ultrasonic bath for 12 minutes.

Item	Non-Heat Shock		Heat Shock
	Aerobic	Item	Aerobic
Handled			
(A)			
(B)			
Control			
(A)			
(B)			

FIGURE C-4. HUMAN HANDLING, AEROBIC PLATES: 24-HOUR COUNTS

Item	Non-Heat Shock		Heat Shock
	Aerobic	Item	Aerobic
Handled			
(A)			
(B)			
Control			
(A)			
(B)			

FIGURE C-5. HUMAN HANDLING, AEROBIC PLATES: 48-HOUR COUNTS

Item	Non-Heat Shock		Heat Shock
	Aerobic	Item	Aerobic
Handled			
(A)			
(B)			
Control			
(A)			
(B)			

FIGURE C-6. HUMAN HANDLING, AEROBIC PLATES: 72-HOUR COUNTS

Item	Non-Heat Shock	Heat Shock
Handled		
(A)		
(B)		
Control		
(A)		
(B)		

FIGURE C-7. HUMAN HANDLING, ANAEROBIC PLATES: 72-HOUR COUNTS

g. Using a sterile 10 ml pipette for each bottle, transfer four 5 ml aliquot from each bottle into four sterile Petri dishes. Label the dishes NHS (non-heat shock).

h. Add 20 ml sterile T-soy agar to each dish and swirl contents gently.

i. Using sterile forceps, aseptically remove each item and place in a prepared T-soy agar plate. Cover each with 20 ml T-soy agar.

j. Using two sterile 10 ml pipettes, place into a sterile test tube 25 ml of the remaining fluid in each jar. Place the tubes in the 80° C water bath for 20 minutes.

k. Using two more sterile pipettes, repeat step g.

l. Repeat step h.

m. Incubate half of each set of plates aerobically, the other half anaerobically. Label clearly.

n. Record colony counts at 24, 48, and 72 hours for aerobically incubated plates (Figs. C-4 through C-6); count and record the colonies at 72 hours for anaerobic plates (Fig. C-7).

Laboratory Exercise III
Inoculation of Culture Media

1. Purpose — To demonstrate various means of inoculating culture media

2. Materials Needed (per student)

   a. Wire loop

   b. Small beaker of 100 percent alcohol

   c. Bunsen burner or alcohol lamp

   d. Two sterile T-soy agar slants

   e. Two sterile T-soy agar stab culture tubes

   f. Two sterile tubes of nutrient broth

g. Two sterile prepared plates of T-soy agar

h. One pair sterile forceps

i. Serratia marcescens stock culture

3. Procedure

NOTE: In this exercise you will be handling capped test tubes frequently. You will have to remove and replace caps. Good aseptic technique requires that you not touch these caps to anything or lay them down so as to contaminate the tubes when the caps are replaced. You may remove a cap by curling your little finger around it to hold it and turning the tube. You may continue to hold it in this manner with your little finger during transfer operations.

a. Broth Culture

(1) Flame wire loop to red heat. (This means to hold the wire in the flame of an alcohol lamp or Bunsen burner until it is red hot.) Allow to cool. Do not touch the loop to anything or lay it down after it has been flamed.

(2) After the loop is cool (You may test this by touching it to the edge of the agar in the culture tube; if it sizzles, it is too hot.) transfer a small amount of Serratia marcescens from the stock culture tube to a tube containing sterile nutrient broth. (Care should be taken not to touch the loop to the edges of the tubes or to anything else while transferring the organisms.) Shake the wire loop gently in the broth to disperse the organisms in the fluid.

(3) Flame the wire loop again.

(4) Repeat step (2) for the second tube of nutrient broth. Flame the loop after transfer.

(5) Place the broth tubes in the incubator for 24 hours at 37°C.

(6) Observe the tubes by holding them up to a light and gently tapping them or swirling them. Growth of the organisms is evidenced by the increased cloudiness of the broth. (These may be incubated for an additional 24 hours and observed again if so desired.)

b. Agar Slant Culture

(1) Flame the wire loop.

(2) After the loop is cool, transfer a small amount of Serratia marcescens from the stock culture tube to the surface of a sterile agar slant tube. Be careful not to touch the loop to the edges of the tubes or to anything else during the transfer.

(3) Gently spread the organisms over the surface of the agar slant with the wire loop. Replace the cap.

(4) Flame the wire loop.

(5) Repeat steps (2) and (3) for the second agar slant. Flame the loop after transfer.

(6) Place the slants in the incubator for 24 hours at 37°C. Observe. (These may be incubated for an additional 24 hours and observed again to illustrate increased growth.)

c. Dish Culture

(1) Flame the wire loop.

(2) After the loop has cooled, transfer a small amount of Serratia marcescens from the stock culture tube to the surface of a prepared sterile agar plate. Take care not to touch the loop to the edge of the tube or plate or anything else during the transfer.

(3) Gently spread the organisms over the surface of the agar in the plate. Replace the lid.

(4) Flame the loop.

(5) Repeat steps (2) and (3) for the second prepared sterile agar plate. Flame the loop after transfer.

(6) Place these plates in the incubator for 24 hours at 37°C. Observe. (These may be incubated for an additional 24 hours and observed again if so desired.)

d. Stab Culture

(1) With forceps, carefully straighten the loop to a wire needle.

(2) Flame the wire needle.

(3) With the wire needle, pick up a small amount of Serratia marcescens from the stock culture tube. Be careful not to touch the needle to the tube edge or to anything else.

(4) Holding the wire needle as steady as possible, plunge it straight down into the middle of the agar in a sterile stab culture tube. Push the needle down about one inch below the agar surface. Carefully draw out the needle, holding it steady so you do not break the agar. Recap the tube.

(5) Flame the needle.

(6) Repeat steps (3) and (4) for the second sterile agar stab culture tube. Flame the needle after transfer.

(7) Place the stab culture tubes in the incubator at 37° C for 24 hours. Observe. Incubate for an additional 24 hours and observe again.

Laboratory Exercise IV

Pure Culture Isolation

1. Purpose — To demonstrate the streak plate method for obtaining the isolation of a single type of organism.

2. Materials Needed (per student)

a. Two sterile prepared T-soy agar plates

b. Wire loop

c. Serratia marcescens stock culture

d. Bunsen burner or alcohol lamp

e. Escherchia coli stock culture

3. Procedure

a. Flame the wire loop and allow to cool. Do not touch the loop to anything or lay it down after flaming it.

b. With the wire loop, pick up a small amount of Serratia marcescens from the stock culture tube. Gently make three parallel streaks across one side of the surface of a sterile T-soy agar plate. Make the streaks about one-fourth-inch apart. (Turn to Fig. 20 in the main body of the text for an illustration.)

c. Flame the loop. Allow to cool.

d. Make three more parallel streaks across a fresh portion of the agar surface. Start these streaks by crossing the previous three streaks. (See Fig. 20.)

e. Flame the loop. Allow to cool.

f. Repeat step d. (See Fig. 20.)

g. Flame the loop.

h. Replace the lid on the streaked plate and place it in the incubator at 37° C for 24 hours. Observe

i. Repeat steps a-h, using the Escherchia coli stock culture.

Laboratory Exercise V

Gram Staining Procedure

1. Purpose — To demonstrate the technique applied and the results obtained in a gram staining procedure.

2. Materials Needed (per student)

a. Glass slide

b. Wire loop

c. Bunsen burner or alcohol lamp

d. Bacillus subtilis culture

e. Escherchia coli culture

f. Methyl violet staining solution

g. Iodine solution

h. Ethyl alcohol

i. Wash bottle (water)

j. Basic fuchsin staining solution

k. Blotting towel

l. Staining tray and slide rack

3. Procedure

a. Place two drops of water on the glass slide, about one inch apart. Flame the wire loop and allow it to cool. Transfer a small amount of Bacillus subtilis from the culture to one drop of water and make a thin smear.

b. Flame the loop and repeat step a, applying the Escherchia coli organisms to the second drop of water.

c. Fix the smears on the slide. (See Fig. 23 in main body of text.)

d. Flood the slide with methyl violet and allow to act for five minutes.

e. Wash off stain with iodine solution. Allow the iodine to act for two minutes.

f. Drain slide and flush with alcohol for five seconds.

g. Wash slide with water.

h. Flood slide with basic fuchsin and allow to act for 30 seconds.

i. Wash slide again with water, blot, and allow to dry.

j. Examine both smears under the microscope, using the oil immersion lense. Bacillus subtilis organisms will appear purple and rod-shaped. These are called gram positive. Escherichia coli organisms are gram negative and will be stained red.

Laboratory Exercise VI

Spore Staining Procedure

1. Purpose — To demonstrate the technique applied and the results obtained in a Wirtz-Conklin spore staining procedure.

2. Materials Needed (per student)

   a. Glass slide

   b. Wire loop

   c. Bunsen burner or alcohol lamp

   d. Bacillus subtilis culture

   e. Malachite green staining solution

   f. Safranin staining solution

   g. Wash bottle (water)

   h. Blotting towel

   i. Staining tray and slide rack

3. Procedure

   a. Place a small drop of water on the glass slide. Flame the wire loop until it is red hot and then allow it to cool.

   b. With the wire loop, transfer a small amount of Bacillus subtilis from the culture to the drop of water and make a thin smear.

   c. Fix the smear on the slide (See Fig. 23 in main body of text.)

d. Flood the slide with Malachite green staining solution and steam the slide for three to six minutes by passing it repeatedly over a flame. Do not allow the stain to boil or dry.

e. Rinse the slide with water for about 10 seconds.

f. Flood the slide with safranin staining solution and allow it to act for 30 seconds to one minute.

g. Wash with water, gently blot, and allow to dry.

h. Examine the stained smear under the microscope, using the oil immersion lense. The bacterial spores appear green, while the vegetative cells are pink or red.

Laboratory Exercise VII

Preparation for a Microbiological Assay

1. Purpose — To familiarize the student with certain preparation and sterilization procedures required to conduct a microbiological assay.

2. Materials Needed (per student)

a. Test tubes and caps

b. Test tube rack

c. Luer-lok syringe

d. Ten ml pipettes

e. One ml pipettes

f. Wrapping paper

g. Autoclave tape

h. Two 1500 ml Erlenmeyer flasks

i. T-soy agar (dehydrated)

j. Peptone (dehydrated)

k. Screw cap bottles

l. Petri dishes

m. Cotton

n. Forceps

o. Stainless steel strips

p. Autoclave bag

q. Rodac plates

3. Procedure

a. Preparation of Glassware, Forceps, Strips, and Media:

(1) Plug pipettes with cotton and wrap with paper. (See Fig. C-8.) Label according to size.

(2) Wash, dry, and rack test tubes.

(3) Wrap syringe in a soft paper towel and place in autoclave bag. Label according to size.

(4) Prepare one liter peptone water and one liter T-soy agar. To prepare peptone water, weigh out 10 grams of powdered peptone and add to 1000 ml of deionized water in a 1000 ml Erlenmeyer flask. Adjust the pH to 7 by adding a few drops of sodium hydroxide. Use pH indicator paper to tell the pH level, stirring the flask contents constantly. To prepare T-soy agar, weigh out 40 grams of powdered agar and add to 1000 ml deionized water in a 1000 ml Erlenmeyer flask. Heat gently on the hot plate while stirring constantly until the powder dissolves.

(5) Wrap strips and forceps in paper. (See Fig. C-9.)

(6) Wash, dry, and cap flask and bottles.

(7) Sterilize all glassware, instruments, strips, and media in the autoclave.

NOTE: Proper method of wrapping pipettes and strips to facilitate aseptic removal from wrapping paper may be seen in Figures C-8 and C-9.

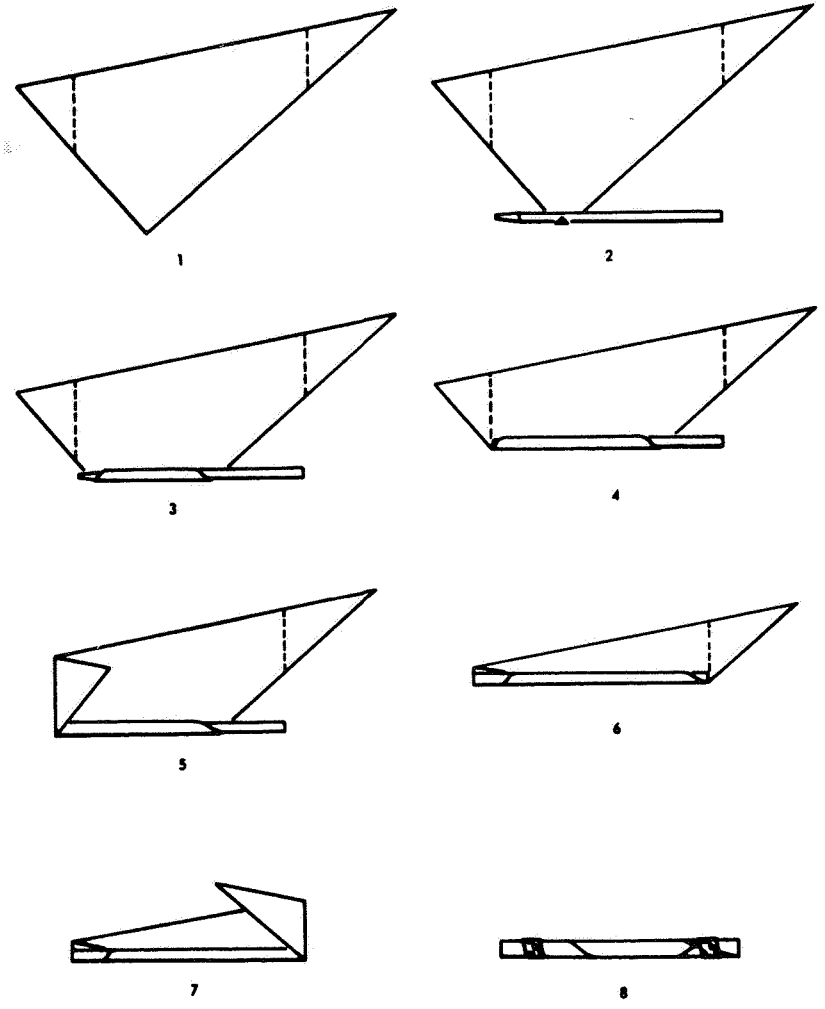

FIGURE C-8. PIPETTE WRAPPING PROCEDURE

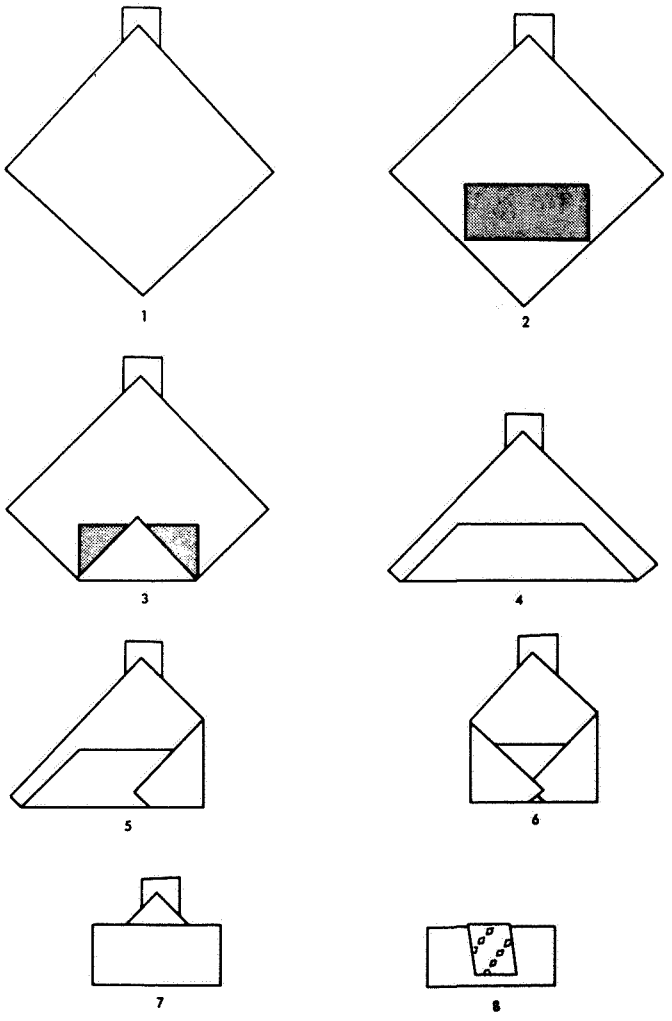

FIGURE C-9. STRIP WRAPPING PROCEDURE

b. Operation of Autoclave

(1) Close vent valve.

(2) Open chamber trap shut-off valve.

(3) Set steam control valve.

(4) Open steam supply valve.

(5) Allow the pressure to rise to 15 pounds. Maintain for 15 minutes.

(6) After correct dwell time has elapsed, close steam supply valve.

(7) Open vent valve.

(8) When chamber pressure reaches zero, autoclave may be opened.

c. Dispensing Media

(1) Aseptically dispense with Luer-lok syringe 5 ml sterile peptone water into each test tube.

(2) Aseptically dispense 50 ml sterile peptone water into each screw cap bottle.

(3) Aseptically dispense with Luer-lok syringe 20 ml sterile T-soy agar into each Petri dish.

(4) Aseptically dispense approximately 15.5 ml sterile T-soy agar into each Rodac plate.

(5) Immediately after dispensing agar, flush syringe with hot water, wrap, and label for sterilization.

d. Preincubation

(1) After the agar has solidified, wrap plates in aluminum foil and place in the incubator 24 hours to check sterility.

Laboratory Exercise VIII

Stainless Steel Strip Assay

1. Purpose — To familiarize the student with the stainless steel strip method which is used in sampling microbial contamination accumulating on surfaces.

2. Material Needed (for each student)

a. Two stainless steel strips, one previously exposed to the air for 24 hours and one sterile strip

b. Two empty 4 ounce screw cap bottles

c. Two 4 ounce screw cap bottles with 60 ml of sterile peptone in each

d. Two pairs sterile forceps

e. Two sterile test tubes

f. Sixteen sterile empty Petri dishes

g. Two sterile T-soy agar plates

h. Marking pencil

i. Six sterile 10 ml pipettes

j. Twenty tubes with 20 ml liquid sterile T-soy agar each

3. Procedure

a. Using sterile forceps, remove strip from tray and place in empty four-ounce bottle with exposed side face down.

b. Aseptically pour 60 ml of peptone water into the bottle containing the strip.

c. Place bottle in ultrasonic bath and ultrasonicate for 12 minutes.

d. Using a sterile 10 ml pipette, transfer 5 ml aliquots into 4 Petri dishes, add 20 ml of TSA to each plate, and gently swirl contents. Label these plates NHS (Non-Heat Shock).

e. Using sterile forceps, aseptically remove the strip and place in the prepared TSA plate and cover with another 20 ml of TSA and allow to solidify.

f. Using a sterile 10 ml pipette, place 25 ml of the remaining fluid in a sterile test tube, and place tube in the 80°C water bath for 20 minutes.

g. Using another sterile pipette, repeat step d.

h. Place the plates in the incubator at 37°C for both aerobic and anaerobic conditions with plates inverted.

i. Record colony counts for aerobically incubated plates at 24, 48, and 72 hours (Figs. C-10 through C-12). For anaerobic incubated plates, record colony counts at 72 hours (Fig. C-13).

j. Repeat steps a-i using the control strip.

Laboratory Exercise IX

Cotton Swab Technique

1. Purpose — To familiarize the student with the cotton swab technique for sampling surfaces.

2. Materials Needed (per student)

a. Two sterile cotton swabs

b. Two test tubes each containing 5 ml of sterile peptone water

c. Sixteen sterile Petri dishes (empty)

d. Marking pencil

e. Six sterile 1 ml pipettes

f. Eighteen tubes with 20 ml sterile liquid T-soy agar each

g. Data sheets are shown on Figures C-14 through C-17.

Strip	Non-Heat Shock		Heat Shock
	Aerobic	Strip	Aerobic
Exposed			
(A)			
(B)			
Control			
(A)			
(B)			

FIGURE C-10. STAINLESS STEEL STRIP, AEROBIC PLATES: 24-HOUR COUNTS

Strip	Non-Heat Shock		Heat Shock
	Aerobic	Strip	Aerobic
Exposed			
(A)			
(B)			
Control			
(A)			
(B)			

FIGURE C-11. STAINLESS STEEL STRIP, AEROBIC PLATES: 48-HOUR COUNTS

Strip	Non-Heat Shock		Heat Shock
	Aerobic	Strip	Aerobic
Exposed			
(A)			
(B)			
Control			
(A)			
(B)			

FIGURE C-12. STAINLESS STEEL STRIP, AEROBIC PLATES: 72-HOUR COUNTS

Strip	Non-Heat Shock	Heat Shock
Exposed		
(A)		
(B)		
Control		
(A)		
(B)		

FIGURE C-13. STAINLESS STEEL STRIP, ANAEROBIC PLATES: 72-HOUR COUNTS

Swab	Non-Heat Shock Aerobic	Heat Shock Aerobic
Exposed		
(A)		
(B)		
Control		
(A)		
(B)		

FIGURE C-14. COTTON SWAB, AEROBIC PLATES: 24-HOUR COUNTS

Swab	Non-Heat Shock Aerobic	Heat Shock Aerobic
Exposed		
(A)		
(B)		
Control		
(A)		
(B)		

FIGURE C-15. COTTON SWAB, AEROBIC PLATES: 48-HOUR COUNTS

Swab	Non-Heat Shock Aerobic	Heat Shock Aerobic
Exposed		
(A)		
(B)		
Control		
(A)		
(B)		

FIGURE C-16. COTTON SWAB, AEROBIC PLATES: 72-HOUR COUNTS

Swab	Non-Heat Shock	Heat Shock
Exposed		
(A)		
(B)		
Control		
(A)		
(B)		

FIGURE C-17. COTTON SWAB, ANAEROBIC PLATES: 72-HOUR COUNTS

3. Procedure

a. Select an area approximately four square inches in size.

b. Aseptically remove a sterile cotton swab from its container and moisten the head of the swab in peptone water.

c. Hold swab handle to make about a 30-degree angle contact with surface to be sampled.

d. Rub head slowly and thoroughly over surface three times, reversing direction between successive strokes.

e. Return the head of swab to original tube of one percent peptone water.

f. Break off the head of swab below any portion of the handle that you touched.

g. Allow swab head to drop into liquid and replace screw cap.

h. Place the other sterile swab directly into tube of peptone water and break off below that portion which was touched. This will be the sterility control check. Do not touch the swab head to any surface.

i. Place both tubes in an ultrasonic bath and ultrasonicate for 12 minutes.

j. Assay according to Experiment VIII, steps d-i with two exceptions: pipette only 1 ml aliquots into each Petri dish and do not plate out the swabs.

Laboratory Exercise X

Rodac Plate Technique

1. Purpose — To familiarize student with Rodac plate method for sampling surfaces.

2. Materials Needed (per student)

a. Five prepared sterile Rodac plates

b. Marking pencils

3. Procedure

a. Remove cover from Rodac plate.

b. Impress agar surface in bottom half of Rodac plate onto surface to be sampled.

c. Replace cover on Rodac plate. Label accordingly.

d. Sample the following areas.

(1) Floor

(2) Wall

(3) Clothing

(4) Skin

(5) Desk

e. Place the plates in the incubator at 37°C aerobically for 24 hours. Count the colonies (Fig. C-18).

Rodac Plate Sample Number	Number of Colonies	Number of Colonies Per Sq In.
1		
2		
3		
4		
5		

FIGURE C-18. RODAC PLATE DATA SHEET

Laboratory Exercise XI

Air Sampling Techniques

1. Purpose — To acquaint the student with both volumetric and gravimetric air sampling.

2. Materials Needed (work in groups of three)

   a. Andersen sampler

   b. Six prepared sterile TSA Andersen plates

   c. Three prepared sterile TSA plates

   d. Vacuum pump

   e. Reyniers sampler

   f. One prepared sterile Reyniers TSA plate

3. Procedure

   a. Volumetric Air Sampling — Andersen Sampler

   (1) Unfasten spring apparatus by placing one hand on top of sampler.

   (2) Starting with bottom stage, place an Andersen plate with lid removed on that stage. Repeat for the 5, 4, 3, 2, 1 stages. Be sure the plates are labeled for the appropriate stage.

   (3) Remove cover on top of sampler and turn vacuum pump on.

   (4) Sample the air for a period of 15 minutes.

   (5) Incubate plates aerobically at 37°C and count the colonies at 24, 48, and 72 hours.

   b. Gravimetric

   (1) Remove lids from two of remaining TSA plates and expose to air for 15 minutes and one hour.

(2) The third plate will be a control. Do not remove lid.

(3) Incubate the plates aerobically at 37°C and count the colonies at 24, 48, and 72 hours (Fig. C-19).

NOTE: a and b may be repeated, using anaerobic incubation.

c. Volumetric Air Sampling — Reyniers Sampler

(1) Turn the three knurled knobs on the sides of the sampler counterclockwise to remove the top of the sampler.

(2) Wind the timer.

(3) Place the sterile Reyniers agar plate in the sampler and remove the lid of the plate. Mark the dish to indicate its position under the slit when sampling starts.

(4) Replace sampler top. Make sure the notch in the top is in line with the projection on the base.

(5) Adjust the height of the slit to 2 mm above the agar surface. Lift the height indicator and turn it until it makes a seal with the gasket. This prevents leakage.

(6) Adjust vacuum to 1 ft^3 per minute and start the timer.

(7) Sample the air for one hour. Take the plate out and place in the incubator for 24 hours at 37°C. Observe.

(8) Marking on the bottom of the plate, divide it into 12 equal pie-shaped segments. Each of these segments will represent 5 minutes sampling time. By counting the colonies in each of these segments, totaling the counts, and dividing by the number of segments, obtain the average number of organisms for a 5 minute sampling period (Fig. C-20).

If the rate of air flow was 1 ft^3/min for 5 minutes, calculate the number of viable organisms per ft^3 of air:

$$\frac{\text{Average No. Organisms per Segment}}{5}$$

Stage No.	Colony Count			Total Ft^3 Sampled	Particles per $Feet^3$
	24 Hr	48 Hr	72 Hr		
				Total Viable Particles/ft^3 =	

Date of sample _____

Time of sample _____

Site of sample _____

Duration of sampling _____

Rate of air flow _____

Incubation Anaerobic _____ Aerobic _____

FIGURE C-19. ANDERSEN DATA SHEET

Sampling Plates	No. of Colonies
1. Control 2. 15 Minutes 3. 1 Hour	

FIGURE C-20. GRAVIMETRIC AIR SAMPLING DATA SHEET

Laboratory Exercise XII

Controlled Contamination With A Known Organism

1. Purpose — To demonstrate the procedure utilized to contaminate a certain object with a known level of a specific type of bacteria.

2. Materials Needed (per student)

   a. Seven sterile stainless steel strips

   b. One slant of Bacillus subtilis

   c. One rack of sterile tubes with 9 ml sterile saline each

   d. One can of 1 ml sterile pipettes

   e. Two sterile 10 ml pipettes

   f. Seven sterile standard size Petri dishes

   g. Sterile forceps

   h. Beaker of 100 percent ethyl alcohol

   i. Sterile test tube

   j. Bunsen burner or alcohol lamp

3. Procedure

   a. Wash the slant with 5 ml of sterile saline and place the suspension into a sterile test tube. Add an additional 5 ml of saline to the suspension using another 5 or 10 ml pipette.

   b. Set up a series of eight test tubes with this original suspension as the first tube in the series, followed by seven saline blanks (tubes of sterile saline). Label these tubes in the following manner: 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2.

c. Starting with the original suspension, transfer 1 ml of the suspension into the saline blank labeled 10^9. After mixing this dilution, transfer 1 ml of it into the next saline blank. Continue this procedure until the dilution series has been completed. Be sure to use a different pipette between each dilution.

d. Use the dilution blank labeled 10^2 to contaminate the stainless steel strips. Perform the contamination process in the laminar flow clean bench.

e. Contamination process: transfer 1 ml of the 10^2 suspension onto the strip which has been placed in a sterile Petri dish. Partially remove the lid to allow the suspension to air dry. After the strips have dried, the lids should be replaced on the Petri dishes.

Laboratory Exercise XIII

Decontamination Agents

1. Purpose — To demonstrate the degree of decontamination effected by a 70 percent alcohol bath. For this exercise, 70 percent alcohol will be used as representative of the group of agents which decontaminate but do not sterilize.

2. Materials Needed (per student)

a. Three stainless steel strips previously contaminated from a Bacillus subtilis culture

b. Three bottles, each containing 60 ml sterile peptone

c. Four pairs sterile forceps

d. Two 70 percent ethyl alcohol baths

e. Twelve sterile Petri dishes

f. Three prepared sterile T-soy agar plates

g. Three sterile test tubes

h. Nine sterile 10 ml pipettes

i. Fifteen tubes with 20 ml sterile liquid T-soy agar each

3. Procedure

a. One of the contaminated strips will be assayed as a control to give the number of organisms present before decontamination procedure.

b. Place one of the contaminated strips into an alcohol bath for five minutes.

c. Remove the strip with sterile forceps and place into a bottle of peptone water.

d. Place bottle in ultrasonic bath for 12 minutes.

e. Using a sterile 10 ml pipette, transfer 5 ml aliquots into two Petri dishes. Add 20 ml T-soy agar to each dish and swirl gently. Label these NHS (Non-Heat Shock) plates.

f. Using forceps again, remove the strip and place in a prepared plate of T-soy agar. Cover with 20 ml sterile liquid T-soy agar and allow to solidify.

g. With a sterile 10 ml pipette, place 25 ml of the remaining fluid into a sterile test tube and place the tube into the 80° C water bath for 20 minutes.

h. Using another sterile pipette, repeat step e. Label these plates HS (Heat Shock).

i. Place the plates in the incubator at 37° C. Because Bacillus subtilis is an aerobic organism, eliminate anaerobic incubation.

j. Record colony counts at 24, 48, and 72 hours.

k. Repeat steps b-j with the third strip, placing it in the alcohol bath for 20 minutes.

NOTE: Use data sheets shown on Figures C-21 through C-23. All three strips may be processed simultaneously to save time if so desired.

Strip	Non-Heat Shock		Heat Shock
	Aerobic	Strip	Aerobic
Alcohol bath			
5 min			
20 min			
Control			

FIGURE C-21. DECONTAMINATION AGENTS, ALCOHOL BATH, AEROBIC PLATES: 24-HOUR COUNTS

Strip	Non-Heat Shock		Heat Shock
	Aerobic	Strip	Aerobic
Alcohol bath			
5 min			
20 min			
Control			

FIGURE C-22. DECONTAMINATION AGENTS, ALCOHOL BATH, AEROBIC PLATES: 48-HOUR COUNTS

Strip	Non-Heat Shock		Heat Shock
	Aerobic	Strip	Aerobic
Alcohol bath			
5 min			
20 min			
Control			

FIGURE C-23. DECONTAMINATION AGENTS, ALCOHOL BATH, AEROBIC PLATES: 72-HOUR COUNTS

Laboratory Exercise XIV

Wet and Dry Heat for Decontamination and Sterilization

1. Purpose — To demonstrate the effectiveness of dry heat and wet heat for decontamination and sterilization.

2. Materials Needed (per student)

a. Four stainless steel strips, previously contaminated from a Bacillus subtilis culture

b. Four sterile glass Petri dishes

c. Four bottles, each containing 60 ml sterile peptone

d. Four pairs sterile forceps

e. Sixteen sterile disposable Petri dishes

f. Four prepared sterile T-soy agar plates

g. Four sterile test tubes

h. Twelve sterile 10 ml pipettes

i. Sixteen tubes with 20 ml sterile liquid T-soy agar each

3. Procedure

a. Place each of the contaminated strips into a sterile glass Petri dish.

b. Autoclave one strip for 15 minutes at 121° C. Autoclave one strip for 30 minutes at 121° C.

c. Place the other two strips in the dry heat oven at 125° C. Remove one strip after 5 hours; remove the other after 24 hours.

d. Assay each strip as directed in steps c-j, Laboratory XIII. Use data sheets shown on Figures C-24 through C-26.

Strip	Non-Heat Shock		Heat Shock
	Aerobic	Strip	Aerobic
Autoclave			
15 min			
30 min			
Dry heat			
5 hr			
24 hr			

FIGURE C-24. AEROBIC PLATES: 24-HOUR COUNTS

Strip	Non-Heat Shock		Heat Shock
	Aerobic	Strip	Aerobic
Autoclave			
15 min			
30 min			
Dry heat			
5 hr			
24 hr			

FIGURE C-25. AEROBIC PLATES: 48-HOUR COUNTS

Strip	Non-Heat Shock		Heat Shock
	Aerobic	Strip	Aerobic
Autoclave			
15 min			
30 min			
Dry heat			
5 hr			
24 hr			

FIGURE C-26. AEROBIC PLATES: 72-HOUR COUNTS

REFERENCES

1. Pelczar, Michael, Jr., and Reid, Roger D.: Microbiology. McGraw-Hill Book Company, Inc., New York, New York, 1958.

2. Stanier, Roger Y., Doudoroff, Michael and Adelberg, Edward A.: The Microbial World. Prentice-Hall, Inc., Englewood, New Jersey, 1957.

3. Galigher, Albert E., and Kozloff, Eugene N.: Essentials of Practical Microtechnique. Lea and Febiger, Philadelphia, Pennsylvania, 1964.

4. Wolcott, Robert H.: Animal Biology. McGraw-Hill Book Company, Inc., New York, New York, 1940.

5. Sarles, William, Frazier, William, Wilson, Joe, and Knight, Stanley: Microbiology. Harper and Brothers, New York, New York, 1956.

6. Gelhardt, Louis P., and Anderson, Dean: Microbiology. The C. V. Mosby Company, St. Louis, Missouri, 1954.

7. Guyer, Michael F., and Lane, Charles, E.: Animal Biology. Harper and Row, New York, New York, 1964.

8. Gillies, R. R., and Dodds, T. C.: Bacteriology Illustrated. The Williams and Wilkins Company, Baltimore, Maryland, 1965.

9. Bryan, Arthur H., Bryan, Charles A., and Bryan, Charles G.: Bacteriology Principles and Practices. Barnes and Noble, Inc., New York, New York, 1962.

10. Garrod, Lawrence P.: The Becton, Dickinson Lectures on Sterilization. Some Difficulties in the Sterilization of Surgical Equipment. Presented at Seton Hall College of Medicine and Dentistry, Jersey City, New Jersey, February 25, 1958.

11. Perkins, John J.: The Becton, Dickinson Lectures on Sterilization. Sterilization by Heat. Presented at Seton Hall College of Medicine and Dentistry, Jersey City, New Jersey, November 19, 1957.

REFERENCES (Continued)

12. Phillips, Charles R.: The Becton, Dickinson Lectures on Sterilization. Gaseous Sterilization. Presented at Seton Hall College of Medicine and Dentistry, Jersey City, New Jersey, February 25, 1968.

13. Spaulding, Earle H.: The Becton, Dickinson Lectures on Sterilization. Chemical Disinfection. Presented at Seton Hall College of Medicine and Dentistry. Jersey City, New Jersey, April 1, 1958.

14. Brewer, John H.: The Becton, Dickinson Lectures on Sterilization. The Control of Sterilization Procedures. Presented at Seton Hall College of Medicine and Dentistry. Jersey City, New Jersey, April 10, 1959.

15. Public Health Monograph No. 60: Sampling Microbiological Aerosols. United States Department of Health, Education, and Welfare, Public Health Service, Washington, D.C., 1965.

16. Quinn, Elizabeth A.: Ethylene Oxide — A Literature Survey. MEL Technical Report MD-197-67, Hayes International Corp., November 20, 1967.

17. Snow, E. B.: Sterilization of Unmanned Planetary Spacecraft — A State of the Art Report. MEL Technical Report MD-111-66, Hayes International Corp., August 17, 1966.

18. Austin, Philip R., and Timmerman, Stewart W.: Design and Operation of Clean Rooms. Business News Publishing Company, Detroit, Michigan, 1965.

19. Terry, John E.: Sterilization of Unmanned Planetary Spacecraft. United States Army Missile Command, Redstone Arsenal, Alabama, November 15, 1965.

20. Anderson Air Samplers and Accessory Equipment. Anderson Samplers and Consulting Service, Provo, Utah.

21. James, Ann N.: Evaluation of Alcohol Sporulation Method. MEL Technical Report MD-209-67, Hayes International Corp., December 27, 1967.

REFERENCES (Continued)

22. Greene, V. W., Walker, Bailus, and Anderson, Orin A.: Methodology of Measuring Internal Contamination in Spacecraft Hardware. Final Report on Research Project no. MGR-24-005-063, University of Minnesota, Minneapolis, Minnesota, June 1967.

23. Dugan, V. L.: Principles of Operation of the Vacuum Probe Microbiological Sampler. Sandia Laboratories, Albuquerque, New Mexico, August 1967.

24. Thimann, Kenneth V.: The Life of Bacteria. The Macmillan Company, New York, New York, 1963.

25. The Society of American Bacteriologists, Committee on Bacteriological Technic: Manual of Microbiological Methods. McGraw-Hill Book Company, Inc., New York, New York, 1957.

26. Harris, Albert H., and Coleman, Marion B., Editors: Diagnostic Procedures and Reagents. American Public Health Association, Inc., New York, New York, 1963.

27. Procedures Manual for Planetary Spacecraft to be Sterilized by Heating. Volume III. Biological Handbook for Engineers. Prepared for NASA by General Electric Company, 1967.

28. University of Minnesota School of Public Health: Short Course on Basic Environmental Microbiology. June 1967.

29. Standard Procedures for the Microbiological Examination of Space Hardware. National Aeronautics and Space Administration, Washington, D.C., June 1966.

30. Jet Propulsion Laboratory: Environmental Specification Voyager Capsule Flight Equipment, Type Approval and Flight Acceptance Procedures for the Heat Sterilization and Ethylene Oxide Decontamination Environments. JPL volume 50503-ETS, January 1966.

31. Phillips, Charles R., and Hoffman, Robert K.: Sterilization of Interplanetary Vehicles. Science. Vol. 132, no. 3433. October 14, 1960, pp. 991-995.

REFERENCES (Concluded)

32. Hurgeton, John C.: The Sterilization Training Program Presented to MEL Valve Clinic and Tube Cleaning Personnel. MEL Technical Report MD-192-67, Hayes International Corp., September 1967

33. Davies, Richard W., and Comuntzis, Marcus G.: The Sterilization of Space Vehicles to Prevent Extraterrestrial Biological Contamination. Jet Propulsion Laboratory, Pasadena, California, August 1959.

34. Ernst, Robert R., and Kretz, Albert P., Jr.: Compatibility of Sterilization and Contamination Control with Application to Spacecraft Assembly. Wilmot Castle Company Research Laboratory, Rochester, New York.

35. Nowistsky, A. M.: Spacecraft Sterilization Techniques and Equipment. Johnson Publishing Co., Boulder, Colorado, 1965.

36. Claghorn, Allen: Sterilization With Ethylene Oxide Gas Mixtures. Reprinted from Inhalation Therapy, vol. 11, no. 5. October 1966.