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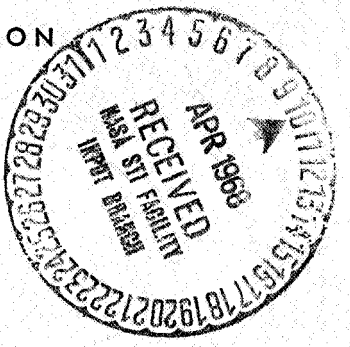

Technical Memorandum 33-331

Growth of Aerobic and Anaerobic Bacteria in Agar Subjected to Freezing and Diurnal Freezing and Thawing

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Prepared Under Contract No. NAS 7-100
National Aeronautics & Space Administration

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Abstract

Surface soils were collected from nine sites on the premises of the Jet Propulsion Laboratory. All samples were mixed and sieved to provide a composite soil sample. The abundance of aerobic and anaerobic bacteria in the sample was determined in trypticase soy agar plates subjected to temperature conditions of (1) room temperature, +25°C, (2) diurnal freezing, −75°C for 16 hr, and thawing, +25°C for 8 hr, and (3) continuous freezing at −75°C. Following two weeks of incubation at the above temperatures, all sets of agar plates were incubated for an additional two weeks at +25°C. No bacteria grew during continuous freezing at −75°C. Aerobes grew during diurnal freezing and thawing at dilutions of 10^{-3} to 10^{-4}. Anaerobes did not grow during diurnal freezing and thawing. All sets of plates showed growth of aerobes and anaerobes by the end of the final two weeks of incubation at +25°C. The abundance of aerobes was greater than anaerobes, but regardless of the first two weeks of temperature conditions, after the second two weeks of incubation at room temperature, survival rates were comparable for sets of aerobic and anaerobic agar plates.

Growth of Aerobic and Anaerobic Bacteria in Agar Subjected to Freezing and Diurnal Freezing and Thawing

I. Introduction

In nature, microorganisms are subjected to freezing and thawing, especially in temperate and polar regions. In both temperate and some polar areas, microflora in soil can winterover, and following the spring thaw, populations are similar in abundance and species composition to that of the soil microbiota during the previous autumn (Ref. 1). In early studies, it was shown that freezing may decrease the number of soil bacterial populations, but at the end of a period of frost, with the thawing of the soil, a large increase in bacterial numbers can occur (Ref. 2). It was also shown that the faster the rate of thawing, the higher the agar plate count. Breaking up of bacterial clumps may contribute to higher counts during thawing, but it is generally assumed that soil bacteria are not eliminated during periods of prolonged freezing. Spring thawing reactivates the soil microflora, with maximum numbers being obtained during the spring and autumn seasons (Ref. 3).

Laboratory studies on the response of microorganisms to cyclic freezing and thawing have been reported in various papers (Refs. 4–9). A variety of substrates were utilized to show survival or growth of microorganisms, and some simulated Martian environmental conditions were utilized in the design of the experiments.

For the purpose of this report, it was desired to obtain information on the abundance of aerobic and anaerobic bacteria in surface soils selected from random sites on the premises of the Jet Propulsion Laboratory. The abundance of these microorganisms was determined in agar utilizing three temperature treatments. No attempt was made to simulate Martian conditions, although the use of a harsh freeze period and a relatively wide temperature range for diurnal freezing and thawing is relevant to survival and/or growth of soil microflora during and following their exposure to harsh environmental conditions.

II. Materials and Methods

A. Soil

Fresh samples of soil were collected from the surface 1 to 3 cm from nine sites at JPL. The exact locations of the sample sites with reference to buildings and facilities are shown in Fig. 1. Soil samples are shown in Figs. 2 through 10.

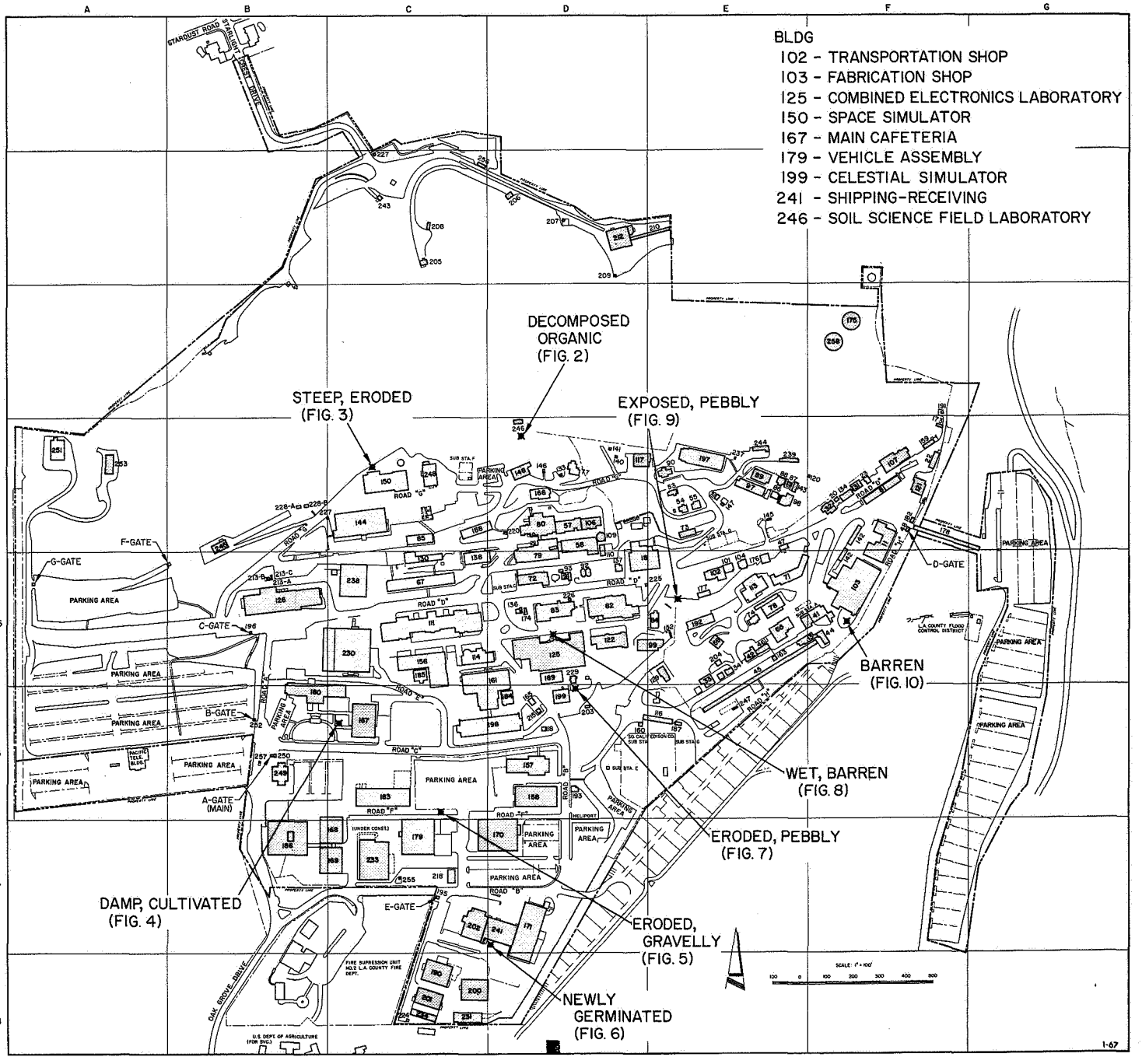

Fig. 1. Soil sample sites

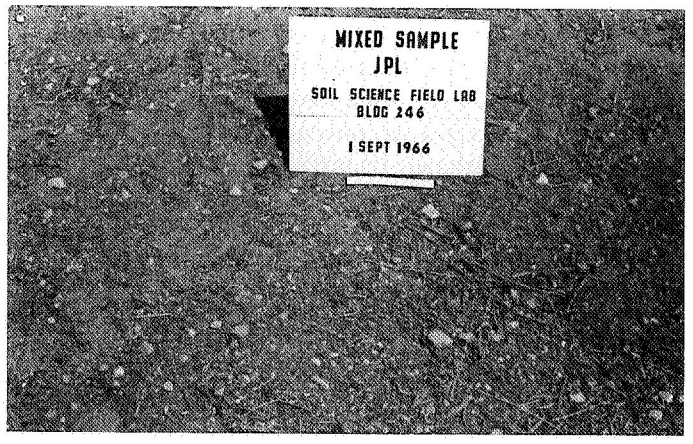

Fig. 2. Layer of partially decomposed organic matter, experimental field plot, in front of Bldg. 246, Soil Science Field Laboratory

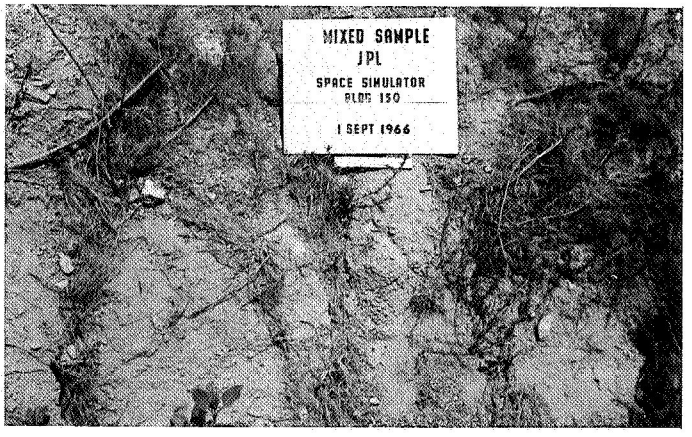

Fig. 3. Steep, eroded, weedy bank in back of northwest corner of Bldg. 150, Space Simulator

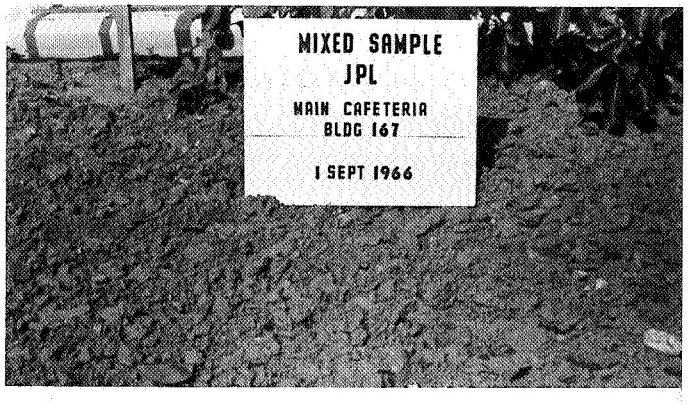

Fig. 4. Damp, cultivated shrub bed, containing several cigarette butts, west end of patio, Bldg. 167, Main Cafeteria

Fig. 5. Eroded, gravelly, and weedy bank next to driveway, northeast corner of Bldg. 179, Vehicle Assembly

Fig. 6. Damp shrub bed, adjacent to steps, containing newly germinated weeds, scattered cigarette butts and other small artifacts, south entrance, Bldg. 241, Shipping–Receiving

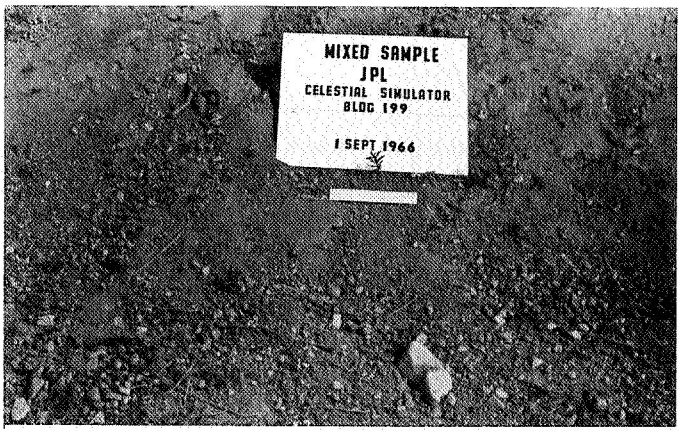

Fig. 7. Eroded, pebbly band of dumped soil, northeast end of Bldg. 199, Celestial Simulator

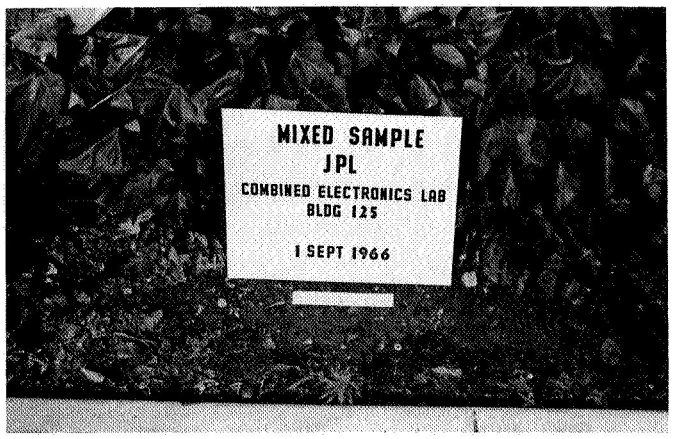

Fig. 8. Wet, barren patch of soil with miscellaneous small artifacts adjacent to sidewalk, north main entrance, Bldg. 125, Combined Electronics Laboratory

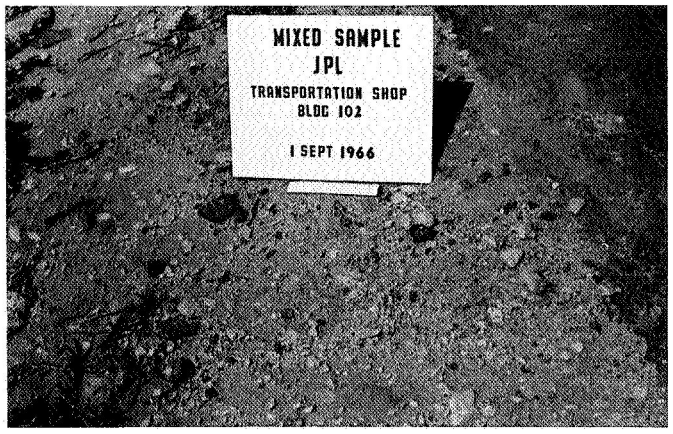

Fig. 9. Exposed, pebbly patch of barren soil between encroaching ice plant and asphalt pavement, south edge of parking lot, Bldg. 102, Transportation Shop

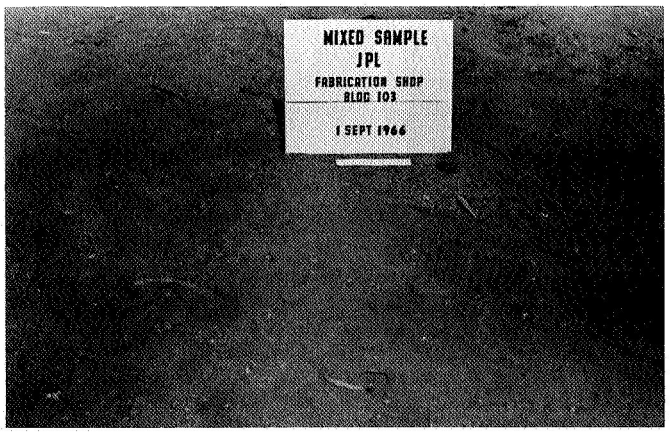

Fig. 10. Barren soil, disturbed by foot traffic, southeast corner of Bldg. 103, Fabrication Shop

No attempt was made to use aseptic procedures in the collection of samples because most of the soils were taken from obviously contaminated areas, e.g., areas adjacent to walkways or pavement and showing footprints and miscellaneous discarded items such as buttons, thread, cigarette butts, etc. (Figs. 2–10). Approximately 2 kg of surface soil was taken at each site and stored in sterilized, plastic-lined soil-sample sacks.

All nine samples were thoroughly mixed in the sample sacks, and an aliquot of the resulting homogeneous soil was taken for a soil moisture determination. The total sample was then passed through a sieve with 2-mm openings, and all material $\geq$2 mm (gravel, pieces of organic matter, and various artifacts) was discarded. The soil materials $\leq$2 mm were then spread evenly on aluminum foil and allowed to air-dry for four days before repackaging into sample sacks. Aliquots of the composite sample were later used for physical, physico-chemical, and chemical analyses for the microbiological experiment.

Nonmicrobiological properties for the composite sample are shown in the following listing[1]:

(1) Physical properties

In situ H_2O = 5.8%

Air dry H_2O = 1.6%

Mechanical analysis

sand = 49.3%

silt = 27.7%

clay = 23.0%

Texture = sandy clay loam

(2) Physico-chemical properties

pH, saturated paste = 7.1

Eh, saturated paste = +413 mv

EC, saturated paste = 4000 μmhos at 25°C

pH, 1:5, soil: H_2O extract = 7.7

Eh, 1:5, soil: H_2O extract = +483 mv

EC, 1:5, soil: H_2O extract = 1800 μmhos at 25°C

Cation exchange capacity = 5.1 meq/100 gm soil

[1]C, N, H, P, and S analyses by Elek Microanalytical Laboratories, Torrance, California. Elemental abundance, except C, N, H, P, and S, by Pacific Spectrochemical Laboratory, Inc., Los Angeles, California, O_2 calculated by difference. Cation exchange capacity determined by Lois Taylor, JPL Bioscience Section. Percent by weight of soil, unless otherwise indicated.

(3) "Available" ions, 1:5, soil: H_2O extract, ppm

P = 2.5	Fe = <<2
K = <5	NH_4 = 2
Ca = <20	NO_2 = 3
Mg = 5	NO_3 = 50
Al = <2	SO_4 = <<150
Mn = <<0.5	Cl = <20

(4) Carbon, nitrogen, hydrogen values

Total H = 0.43%

Total C = 1.42%

Organic C = 1.36%

Carbonate C = 0.06%

Total N = 0.08%

Organic N = 0.08%

Total C: total N = 17.8

Organic C: organic N = 17.0

Total C: total H = 3.3

Total H: total N = 5.4

(5) Elemental abundance, %

O = 44.0	N = 0.08
Si = 25.5	Ba = 0.063
Al = 9.7	Mn = 0.057
K = 7.1	Sr = 0.042
Na = 3.3	Zr = 0.041
Fe = 3.25	Pb = 0.033
Ca = 2.45	Cr = 0.0145
C = 1.42	Ga = 0.0051
Mg = 1.10	V = 0.0051
Ti = 0.53	Co = 0.0028
H = 0.43	Ni = 0.0026
P = 0.10	

Since the sample was not a representative of any one kind of soil, and included both natural and added materials which did not result from *in situ* processes of soil formation and development, it could not be classified and can only be considered as "nondescript soil." However, a comparison of properties of the sample with those of typical southwestern U.S. desert soils does show a number of close similarities in properties (Refs. 10 and 11). The main differences between the composite JPL sample and typical desert soils are the higher contents of organic carbon, organic nitrogen, and nitrate nitrogen in the JPL sample. Most of the carbon and nitrogen was in the organic form. Sulfur was <0.000%. *In situ* moisture content was higher for the composite JPL sample than for desert soils because some of the JPL sample sites had recently been irrigated.

B. Experimental Methods

Ten-gram aliquots of homogeneous, sieved, air-dry soil were placed in milk dilution bottles containing 90 cc of sterile, distilled water. All bottles were then shaken vigorously at full speed for 20 min on a wrist-action shaker. Subsequent to dispersal of the samples, 1-cc aliquots of the supernatant suspension were serially transferred to 9 cc of distilled water in dilution tubes. Portions of 1-cc per each dilution of 10^{-1} to 10^{-6} were transferred to duplicate sets of plastic petri plates. Duplicate control plates containing 1 cc of sterile H_2O were also prepared. Warm agar (BBL trypticase soy agar at 45°C) was poured into each plate and the plates swirled to disperse the samples throughout the agar medium. Approximately 20 to 25 cc of agar was used for each plate.

Duplicate sets of plates including controls were subjected to the following temperature conditions for a two-week period of incubation:

(1) Room temperature, +24 to +26°C.

(2) Diurnal freezing and thawing, −75°C in dry ice for 16 hr and +25°C for 8 hr at room temperature – including time to completely freeze and completely thaw (see Table 1).

(3) Continuous freezing, −75°C in dry ice.

Table 1. Equilibration times for agar plates subjected to freezing and thawing

O_2 tension	Temperature, °C	Time, hr
Anaerobes	freeze, −75	≃4
Anaerobes	thaw, +25	≃2
Aerobes	freeze, −75	≃3.5
Aerobes	thaw, +25	≃3

After two weeks of incubation under the above temperature conditions, all plates were incubated at room temperature for an additional two weeks.

One set of each plate subjected to the experimental temperature conditions was incubated aerobically. For these plates, protection was provided from subliming dry ice by placing the plates in a sealed, plastic, disposable glove bag. During room-temperature incubations, the bags containing aerobic plates were opened.

A second set of plates subjected to the above experimental temperature conditions was incubated anaerobically. Anaerobic plates were also placed in a sealed, plastic, disposable glove bag. All bags containing anaerobic plates were evacuated three times to 30-mm Hg and flushed with 100% CO_2. After the final evacuation, the bags were filled with CO_2. However, each evening, before the bags containing anaerobic plates were placed in the dry ice, they were partially deflated to allow their inclusion in the dry ice chamber. The following morning, when the bags were removed, they were again filled with CO_2.

Before the bags of aerobic and anaerobic plates were placed in the dry ice chamber, they were stacked upright in wire holders. Plates were not inverted because thawing of the agar increased the sol state, and the agar no longer adhered to the petri plate. All plates were removed from the wire holders during the thaw period and spread in a single layer on the bottom of the plastic glove bag.

All plates were examined macroscopically for growth during the experiment. Evident growth was checked daily, and appropriate plate counts were made. When discrete colonies could not be determined, or spreading growth was evident, the entire dilution plate was indicated as positive for growth. Whenever possible, colonies were counted with a Quebec Colony Counter.

At the termination of the experiment, bacteria from typical colonies or spreading growth were examined microscopically after gram staining.

III. Results and Discussion

The results of incubation of agar plates for the first two weeks of incubation at various temperatures are shown in Table 2. The results of incubation of the same sets of all agar plates for an additional two weeks at room temperature are shown in Table 3. For agar plates where it was not possible to count discrete colonies, the plates were counted as positive for growth at the highest dilution.

Table 2. Growth of bacteria in agar plates subjected to various incubation temperatures[a, b]

O_2 tension	Temperature conditions, °C	Bacteria per gm soil
Anaerobic	Room temperature, +25	800,000
Anaerobic	Cycled, −75 to +25	0
Anaerobic	−75	0
Anaerobic	Controls (all sets)	0
Aerobic	Room temperature, +25	5,400,000
Aerobic	Cycled, −75 to +25	1,000 to 10,000[a]
Aerobic	−75	0
Aerobic	Controls (all sets)	0

[a] Spreading or confluent bacterial growth; discrete colonies not possible to count. Positive growth recorded for highest dilution.

[b] Initial two-week incubation period.

Table 3. Growth of bacteria in agar plates subjected to final room temperature incubation[a]

O_2 tension	Previous temperature conditions, °C	Bacteria per gm soil
Anaerobic	Room temperature, +25	800,000
Anaerobic	Cycled, −75 to +25	200,000
Anaerobic	−75	100,000[b]
Anaerobic	Controls (all sets)	0
Aerobic	Room temperature, +25	5,400,000
Aerobic	Cycled, −75 to +25	1,000,000[b]
Aerobic	−75	1,000,000[b]
Aerobic	Controls (all sets)	0

[a] Results of incubation for additional two weeks of same sets of plates as recorded for Table 2.

[b] Spreading or confluent bacterial growth; discrete colonies not possible to count. Positive growth recorded for highest dilution. For example, 100,000 indicates growth at 10^{-5} dilution, but not at 10^{-6}.

It is evident from a comparison of Tables 2 and 3 that the abundance of aerobic bacteria is higher than the abundance of anaerobes in the mixed JPL soil, regardless of incubation conditions. The abundance and ratios of both of these groups of microorganisms are slightly higher

than for plate counts obtained for eight southwestern U.S. California desert surface soils in the Colorado, Yuma, Mohave, and Great Basin Deserts (Refs. 10 and 12). The values compare favorably with abundance of aerobes and anaerobes obtained for a partially grass-covered alpine soil plot at 12,400 ft in the White Mountain Range of California (Ref. 13). For regions such as the Valley of 10,000 Smokes in Alaska (Ref. 14), the Atacama Desert in Chile (Ref. 15), the eastern Sahara (unreported data), and the Kau Desert of Hawaii (unreported data), abundance of aerobes is considerably higher, and the abundance of anaerobes can be negligible if growth is attempted using trypticase soy agar in CO_2. A more favorable climatic regime, more organic matter, and application of soil management factors (fertilizer increments, cultivation, induced contaminants, irrigation, etc.) undoubtedly have a favorable effect on the abundance of bacteria in the composite JPL soil sample.

Neither continuous freezing at −75°C nor diurnal freezing and thawing at −75 to +25°C had an appreciable effect on the final, survivable numbers of aerobes or anaerobes as shown by the final two weeks of incubation of all plates at room temperatures (Table 3). However, no growth of anaerobes was evident, and growth of aerobes was delayed for plates subjected to the first two weeks of freeze–thaw cycling as is shown by a comparison of results in Tables 2 and 3. Growth of aerobes in the 10^{-1} dilution plates was apparent as agar became discolored by bacterial growth after 9 days of freeze–thaw cycling.

At room temperature, anaerobic incubation may take up to one week longer than aerobic incubation before growth of all observable colonies can be obtained. It took approximately one week before anaerobic plates subjected to freeze–thaw cycling showed growth at room temperature.

Some fungi and algae were present in the composite soil sample. Some *fungi imperfecti* species survived and grew on the aerobic plates subjected to diurnal freezing and thawing after the plates were incubated at room temperature. Fungi have the ability to grow slowly during exposure to short periods of thawing after a number of days of diurnal freezing and thawing (Ref. 16). No algae were evident, although it was not expected that they would show growth on trypticase soy agar.

Table 4 gives a brief description of some of the bacterial colonies and the morphology of the bacteria from colonies in the plates. Both cocci and rods and, especially, gram-negative forms were present. Soil diptheroids were also present. Effects of freezing or diurnal freezing and thawing were not apparent according to observed forms of survivable bacteria.

IV. Conclusions

As indicated in experiments by previous investigators, a variety of microorganisms can grow under simulated Martian conditions, including diurnal freezing and thawing (Refs. 4–9). In most cases, the microorganisms were

Table 4. Colony and bacteria appearance after four weeks of incubation

O_2 tension	Temperature conditions, °C	Dilution plate	Colony appearance	Microscopic appearance
Anaerobic	Cycled, −75 to +25	10^{-5}	Slightly spreading grayish white	Large gram-negative rods, some club-shaped, i.e., containing gram-positive granules (diptheroids)
Anaerobic	Cycled, −75 to +25	10^{-3}	Spreading yellowish and grayish white	Same as above
Anaerobic	−75	10^{-4}	Thin, spreading golden yellow	Long, slender, and very short gram-negative rods
Anaerobic	−75	10^{-5}	Spreading, grayish white	Gram-negative rods
Anaerobic	+25	10^{-5}	Small, thin, translucent	Gram-negative diplococci
Aerobic	+25	10^{-6}	Spreading, thin, yellowish orange	Gram-negative very short rods and gram-positive to gram-variable long rods
Aerobic	+25	10^{-6}	Raised, puttery, yellowish	Gram-negative diplococci
Aerobic	+25	10^{-6}	Raised, creamy, grayish white	Gram-negative cocci
Aerobic	+25	10^{-6}	Raised, cavitated, dark orange centered	Gram-negative and gram-positive rods containing gram-positive granules (diptheroids)

contained in liquid substrates or soil. Our results show that both aerobes and anaerobes obtained from soil can survive after subjection to diurnal freezing and thawing for fourteen consecutive days in agar plates. Aerobic bacteria demonstrated the ability to grow during the final days of thaw. Anaerobic agar plates were more subject to effects of freezing and thawing than were aerobic agar plates as shown by the formation of larger ice crystals and greater disintegration of the agar (Fig. 11). All aerobic and anaerobic agar plate counts differed by less than two orders of magnitude after the final two weeks of incubation, regardless of the previous two weeks' treat-

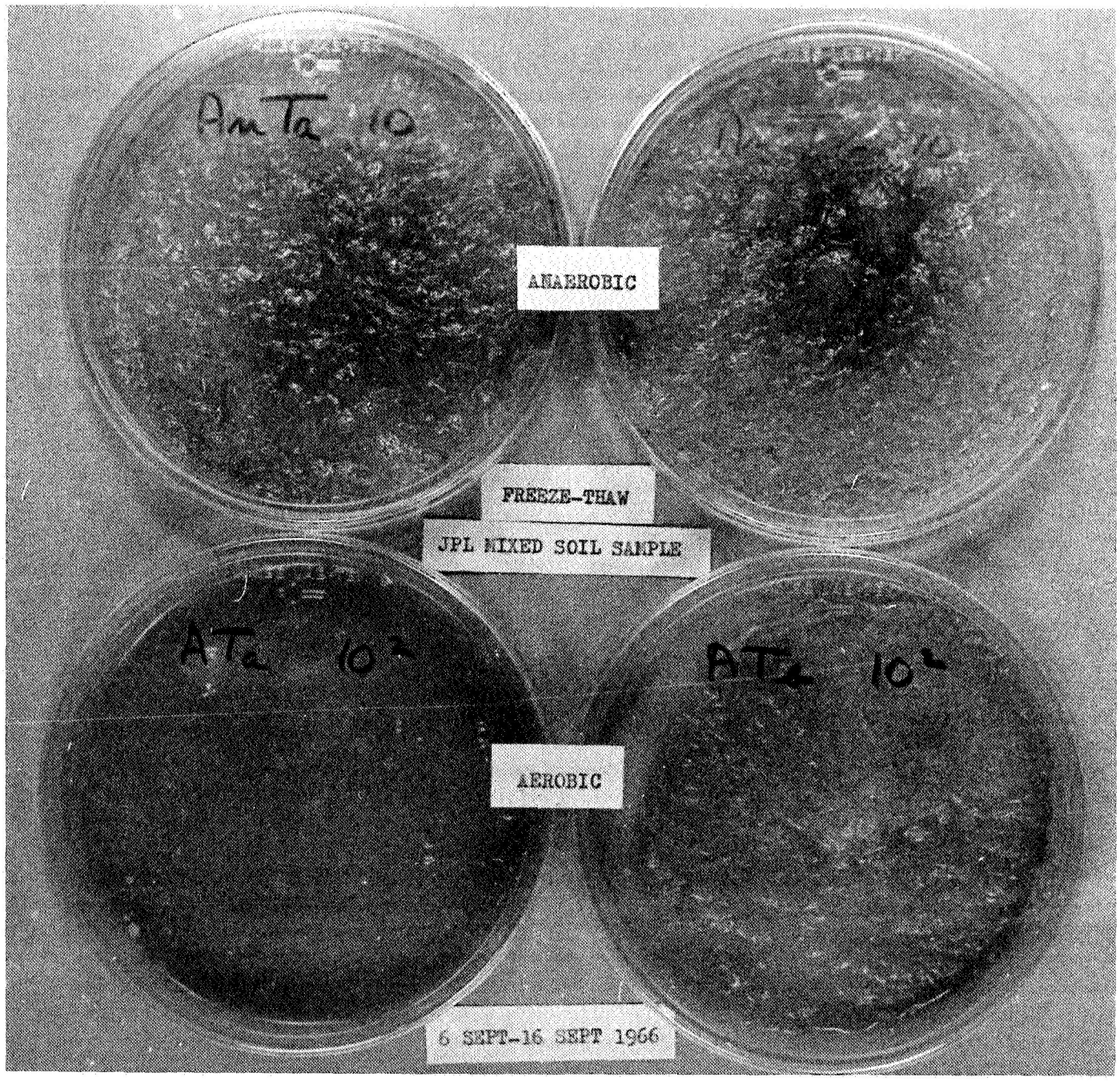

Fig. 11. Anaerobic and aerobic trypticase soil agar plates showing condition of agar following freezing and thawing (anaerobic agar plates show greater degree of disintegration)

ment of continual freezing at −75°C or diurnal freezing and thawing at −75 to +25°C.

Environmental factors, which are of obvious importance in incubating aerobes and anaerobes, include various soil properties such as structure, texture, aggregation state, and porosity. Soil quality and quantity are also pertinent as are physical subdivisions of organics and salts, pH, Eh, and various moisture suctions and gases. Those factors which are relevant to extended periods of freezing and diurnal freezing and thawing of microflora populations of *in situ* soils are especially important.

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