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SYSTEMS RESEARCH LABORATORIES

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AEROSPACE MEDICAL RESEARCH LABORATORIES

TECHNICAL REPORT AFAPL-TR-66-91

AUGUST 1966

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**AIR FORCE AERO PROPULSION LABORATORY
RESEARCH AND TECHNOLOGY DIVISION
AIR FORCE SYSTEMS COMMAND
WRIGHT-PATTERSON AIR FORCE BASE, OHIO**

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FOREWORD

This report was prepared by Systems Research Laboratories, Inc., (SRL), Dayton, Ohio, under USAF Contract No. AF33(615)-2692 (Project 3048 "Aviation Fuels," Task No. 304801 "Hydrocarbon Fuels," with the University of Dayton, Research Institute, Dayton, Ohio, Order No. RI 29363, Account No. 93374. The contract was administered by the Air Force Propulsion Laboratory, Research and Technology Division (RTD), Air Force Systems Command, Wright-Patterson Air Force Base, Ohio. The project engineer was Mr. Jack R. Fultz, AFAPL, APFL, Wright-Patterson Air Force Base, Ohio.

This is a final report of the work performed under the experimental program designed by the Biospecialties Branch (MRMBP), 6570 Aerospace Medical Research Laboratories (AMRL) and conducted at AMRL Laboratories during July 1963 through September 1964 with the support of personnel from Systems Research Laboratories, Inc., under Contract No. AF33(657)-11733 (Project 8169, Task No. 816906). This project was administered by the Systems Engineering Group (RTD), Wright-Patterson Air Force Base, Ohio. The project engineer was Mr. E. F. Suhr, SEMSF, Wright-Patterson Air Force Base, Ohio.

The program was initiated to support, extend, and supplement the Air Force Aeronautical System Division programs concerning the microbiological contamination of JP-4 fuels.

The authors wish to acknowledge Mr. Ennio Raimondi of SEFIP, SEG, Wright-Patterson Air Force Base, Ohio, for his assistance with the capacitance measurements during the fuel capacitance probe study.

This technical report has been reviewed and is approved.

ABSTRACT

With the recognition in 1956 that the United States Air Force JP-4 fuels were microbially contaminated, problems concerning malfunctions and corrosion of JP-4 fuel systems were attributed to the presence of microorganisms. The Biospecialties Branch, MRMPB, 6570 Aerospace Medical Research Laboratories, initiated a research effort to support and supplement the Air Force Aeronautical Systems Division program concerned with the relationship of microbial contamination to deleterious changes in JP-4 fuel systems. Results of this program have shown the following: the majority of JP-4 fuel systems contain low levels of viable microorganisms; microorganisms are primarily associated with the water phase and rarely with the fuel phase of a JP-4 fuel/water system; JP-fuels exert a toxic effect on viable microbial cells; and microbial corrosion of aluminum alloys 7075, 2024, and 7178 can occur in the laboratory but does not appear to be the major cause of corrosion in operational systems. 20% ethylene glycol monomethyl ether (EGME) in a laboratory JP-4 fuel/water-bottom acts as an inhibitor to the majority of viable cells present. As a result of engineering design, it is possible that fuel capacitance probes can cease to operate as a result of microbial growth. Micronic filter materials of phenolformaldehyde impregnated paper can support the growth of microorganisms, but growth even after 43 days is insufficient to cause filter clogging, provided the filter is immersed in fuel. In addition, field surveys of JP-4 fuel handling facilities and aircraft have shown that improved housekeeping and the use of EGME have reduced the quantity of microbial contaminants initially found in the jet systems to an insignificant level.

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Section I

INTRODUCTION

With the recognition in 1956 that the United States Air Force JP-4 fuels were microbially contaminated¹, the presence of microorganisms* in gas turbine powered aircraft and related fuel systems and their association with problems of contamination/corrosion have been a matter of controversy. The absence of adequate information on microbiological contamination of aircraft and related equipment initially resulted in erroneous hypothesis of biological and nonbiological problems. Research studies concerned with implicating microorganisms in degradation of fuel cell protective materials, clogging of fuel filters, corrosion of aluminum, and malfunctioning of jet fuel systems have been inconclusive. Difficulty has been experienced in reproducing compatible results in controlled laboratory and field tests. "Stop-gap" control measures have appeared to alleviate suspected problem conditions prior to understanding the basic mechanisms or the environmental contributory factors responsible for the apparent problems.

The USAF became aware of the existence of the microbiological contamination of JP-4 fuels in 1956 when flight operations in some of the USAF tactical organizations were curtailed due to malfunctioning of fuel control systems and refueling equipment. An inspection of the aircraft involved, the B-47 and KC-97, indicated that the malfunction could be the result of an accumulation of sludge. JP-4 bulk storage and hydrant refueling underground tanks appeared to be the source of the sludge. To determine the sludge composition, samples from bulk storage tanks located at Lincoln Air Force Base, Nebraska, Schilling Air Force Base, Arkansas, and Davis-Monthan Air Force Base, Arizona, were collected and sent to the Protective Process Branch, Materials Laboratory, Wright Air Development Center. Microbiological analyses indicated that the sludge was associated with the presence of bacteria and their metabolic by-products^{1,2}.

The finding of sludge in bulk storage tanks was not a new phenomenon, since the Prevention of Deterioration Center, National Research Council, had reported previously (to Bakanauskus in 1958)¹ that sludge accumulation was a common occurrence in tanks used for kerosene-like fuels.

In 1958 the major cause for the crash of a B-52 aircraft³ was attributed to clogging of screens and filters. The clogging appeared to be due to some form of fuel contaminants and ice formation.

*Microorganisms as referred to in this report indicate bacteria, fungi, and yeast.

In 1956 and 1958, the United States Navy and the Royal Austrian Navy became cognizant of the microbiological contamination of jet fuels when analyses of brown sludge found in an aircraft carrier jet fuel storage area indicated the presence of bacteria and fungi. Clogging of filters in the carrier system, fuel and water emulsions, and malfunctioning of aircraft fuel capacitance gages were attributed to the sludge material⁴. Each Navy, after its investigation, concluded that the contamination was obtained from tankers during sea refuelings.

In 1958, following the microbiological analyses of numerous samples of debris obtained from Navy jet fuels (JP-5) and various components of the fuel system, the Microbiological Section of the Organic and Biological Chemistry Branch (OBC) Naval Research Laboratories (NRL), under the Bureau of Naval Weapons, initiated investigations of the microbial contamination of Navy jet fuels. Of the shipped jet fuel samples analyzed at the laboratory, a few indicated that the number and types of microorganisms recovered exceeded that which could be considered as chance contamination⁵.

In addition to analysis of field fuels, laboratory jet fuel contamination studies indicated (1) that mixed microbial cultures altered the filterability of JP-5 fuels, (2) microbial growth in jet fuels was dependent upon the presence of free water, and (3) certain microbial species recovered from jet fuels had a global distribution. Because of the fuel contamination condition, the U.S. Navy in 1957 printed the "Fuel Contamination Information Manual" for distribution to the Naval Aviation Training Divisions⁶.

The first observation of contamination/corrosion associated with JP-4 fuels in wet-wing fuel cell aircraft was reported in 1959. Inspections of the integral fuel tanks of the Electra Aircraft, used by commercial airlines, showed contamination of fuel system gages, pumps and filter screens, and sufficient corrosion of wing panels to require replacement. The conditions observed were attributed to microbiological slime^{4,7}.

The Air Force first became aware of contamination/corrosion of integral wing tanks in 1960, during an inspection of KC-135 Tanker and B-52G Bomber Aircraft assigned to Ramey Air Force Base, Puerto Rico, and Eglin Air Force Base, Florida⁸. The inspection revealed significant wing tank corrosion, the presence in the fuel-cell-sump area of large quantities of gelatinous amorphous material, degradation of sealants and top coatings, and inoperative ground refueling filters. In some areas of the integral wing tanks, sealants were degraded sufficiently to permit fuel leakage⁷. Analysis of the gelatinous material indicated the presence of abnormal amounts of iron oxide, unidentified debris, and microbial contamination. The aircraft in which corrosion was observed required extensive overhauling that resulted in excessive downtime of the aircraft.

Because the preliminary observation suggested an extensive contamination/corrosion condition in aircraft from both Air Force bases, a joint Air Force Department of Defense (DOD) team initiated an inspection of the entire petroleum, oil and lubricants (POL) servicing system of Air Force bases in an attempt to determine the factors contributing to the problem. Undrainable condensate in the sump areas of the wing fuel cell, abnormal amounts of surfactants in the fuel⁹, the use of sea water to purge the pipelines from tanker to bulk storage tanks, and the excess accumulation of water in the bulk storage tanks were found to be contributing factors to the contamination/corrosion condition⁷. Although the problems observed were not directly attributed to microbiological contamination, the Air Force was concerned about the high level of microbial contamination found at Ramey Air Force Base¹⁰. As a result of the AF-DOD findings, the Fuels and Lubricants Division organized a symposium in 1961 to inform the military and industry on fuel contamination⁹.

In 1961 the Strategic Air Command (SAC) requested the assistance of the U.S. Army Biological Laboratories, Fort Detrick, Maryland, in evaluating the SAC contamination/corrosion conditions. The preliminary findings indicated improved housekeeping procedures of bulk storage facilities and associated equipment and higher fuel standards would help control the condition¹¹.

By the end of 1961 approximately 52 governmental and non-governmental agencies were involved in various phases of research on microbiological contamination of fuel¹².

Although the contamination/corrosion conditions were not directly or officially attributed to microbiological contamination, the implication was that microbial contamination could be deleterious to aircraft operation. The Air Force therefore instituted an extensive program to establish control measures and "cause-effective" relationships of microorganisms and contamination/corrosion problems encountered in jet fuel systems. The program was designed to cover the significant areas of the entire problem.

Aeronautical Systems Division (ASD) offices, with the aid of members of the Biospecialties Branch, 6570 Aerospace Medical Research Laboratories (AMRL) assisting as microbiological consultants, issued, in 1962, 11 contracts¹³ to investigate different phases of the microbiological contamination of jet fuels. Overlapping of efforts was incorporated into the programs to insure the development of different approaches to the problem. The studies conducted under the ASD program included: development of microbially resistant coating and sealants for integral fuel cells; development of new fuel specifications to eliminate or control microbial contamination; development of fuel handling procedures for elimination and/or control of microbial contamination from bulk storage tanks to aircraft; development of mechanical

and chemical techniques to detect, kill, and control the microbial contamination of jet fuel; investigation of microbial corrosion of aluminum; determination of the critical level of microbial contamination and environmental conditions necessary for corrosion of aluminum; determination of the ecological factors required for microbial contamination of JP-fuels, formation of sludge, slime, and degradation of fuels and other military products; investigation of the effects of microbial corrosion on mechanical properties of aircraft fuel cell materials; investigation of the effects of microbial growth in JP-4 and avgas on filter/separator components of the Pritchard refueling system (Project BEARS); and preparation of a handbook on petroleum microbiology for use as a standard reference¹³. To further assist the ASD efforts, facilities as well as personnel of the Biospecialties Branch, 6570 AMRL, Wright-Patterson Air Force Base, were made available to extend and supplement the existing programs and evaluate field data.

Contamination/corrosion conditions in JP-4 fuel systems had not changed in the Air Force in 1962 and 1963, since corrosion of wet-wing fuel cells, the presence of sludge, slime, and water in JP-4 fuels, and malfunctioning of fuel control systems were still reported. However, the number of viable microbial cells recovered from JP-fuel samples had significantly decreased. The reduction was attributed to biocidal action of the anti-icing additive (ALA), ethylene glycol monomethyl ether (EGME) and glycerol, which were added to the Air Force JP-4 fuels in April 1962 in final concentrations of 0.1% V/V.^{13,14} The original composition consisted of 90% EGME and 10% glycerine (by volume). The composition of the mixture was changed to 99.6% and 0.4% (designated as MIL-I-27686C) on the basis of corrected solubility data. (The solubility of glycerine in JP-4 fuel, when in the presence of 0.1% EGME, was determined to be approximately 4 PPM.) Depending upon the temperature, rate of water access, and fuel turnover, the EGME builds up to bactericidal concentrations in the fuel water-bottoms. Concentrations of 20% or more in the water-phase are considered biocidal; however, viable microorganisms have been recovered from field and laboratory EGME and glycerol JP-4 fuel/water solutions above the 20% concentrations. A laboratory JP-4 fuel/water system (initiated in November of 1963, and containing 30% EGME and glycerol in the water phase) has indicated, after a two and one-half year shelf life, a viable microbial population of > 100 cells/ml. The population quantity and morphological appearance of the microbial colonies appear to be similar to that observed shortly after a 20% EGME and glycerol solution had been added to the test laboratory system.

During 1963 and 1964 microbiological contamination of USAF JP-4 fuels appeared to be under control, and results of laboratory tests were indicating that the microbiological contamination of JP-4 fuels and associated equipment was not as severe as originally observed. The reduction in the number of microorganisms found in the field JP-4 fuel systems was

attributed to "good housekeeping" procedures, which reduced the amount of water and solid contaminants in the fields, and the use of EGME. However, these procedures are only "stop-gap" measures and are no panacea for the problem if one did exist originally.

The possibility that microorganisms could be associated with jet fuels and cause problems of corrosion/malfunction of aircraft fuel systems and related equipment could not be considered unusual. Microorganisms have been implicated in various problems confronting the petroleum industry. The relationship of microbial contamination to problems observed in the JP-4 fuel system appears to involve both the presence of water in sufficient quantities to permit survival or growth, and the microbes' capability to metabolize hydrocarbon substrates as a source of carbon.

The microbial contamination of jet fuels is world wide and has no geographical boundaries. Over a hundred different types of microorganisms have been recovered from JP-4 fuel samples^{1, 15, 16}. The microbes of the environmental biotic community apparently serve as the inocula with some species having global distribution. However, the number of environmental microbes capable of surviving and/or multiplying in the jet fuels are few⁴. Because microorganisms are ubiquitous and because of the design of the fuel system, it would be impossible to eliminate and/or prevent microbial contamination of jet fuels. However, it is generally assumed that microbial contamination of JP-fuel systems will be more prevalent in tropical areas since the water holding capacity of fuels increases with temperature increase.¹⁷

This report covers the data derived from results of studies that were initiated during the contract to provide information concerning the relevance of the presence of microorganisms in Air Force JP-fuel systems.

Section II

DEVELOPMENT OF LABORATORY TECHNIQUES APPLICABLE FOR FIELD ANALYSIS FOR DETERMINING MICROBIOLOGICAL CONTAMINATION OF JP-FUELS

Of prime importance in the investigations of microbial contamination of JP-fuels was the need for standard laboratory procedures that were simple, specific, and applicable to on-site field analysis. The selection of such a procedure and/or procedures was hampered by the wide variety of microbial types found in the JP-fuel system^{1,10,16,19,20}, and the inability to correlate changes of culture characteristics with those found in standard determinative manuals.

The initial knowledge of the microbiological contamination (numbers and types) associated with JP-fuels was based on data obtained from field samples which were sent to a laboratory for analysis. Because microbial growth is known to be affected by the surrounding environment, the validity of the early findings concerning the types and quantity of microbes found at the sampling site was questionable. From his investigations, Sharpley found that in addition to changes in the number of viable microbial cells present in freshly collected samples and shipped samples, microbial populations of shipped samples change extensively during transportation to the laboratory for analysis.

Because of the numerous standard laboratory procedures that are available for recovery and isolation of specific microorganisms, the procedures initially employed in the determination of JP-4 fuel/water samples was at the discretion of the analyzing laboratory. Of the procedures and media in use, Bakanauskus¹ selected a medium most suitable for heterotrophic bacteria and filamentous fungi. For obligate anaerobic bacteria he used the Brewer plate with thioglycollate; for enumeration and isolation of microbial cells he used the spread plate technique; and to note if the isolate was a hydrocarbon user, he used Bushnell-Haas¹⁸ (B-H) solution overlaid with autoclave sterilized JP-4 fuel. Hazzard⁶ incorporated penicillin into malt agar medium to inhibit the bacteria and permit the growth of fungi, which he considered the primary microbes concerned with microbiological problems of aircraft and related servicing equipment; for laboratory cultivation B-H solution overlaid with sterile kerosene was used. Churchill and Leathen¹⁶ used the spread plate technique and three (3) types of media for enumeration and recovery of bacteria and fungi from the water-bottoms of JP-fuel bulk storage tanks. Swatak²¹ employed potato-dextrose agar for recovery of all microorganisms. Rogers²⁰ employed six (6) media recommended by the "Society of Industrial Microbiology (SIM) Committee

on Microbiological Deterioration of Fuels," and enumeration procedures including the membrane filter technique for fuels and the poured plate technique for water-bottoms. Sharpley^{22,23,24} suggested the use of several types of media for isolation of fuel microbial contaminants. Bagdon²⁵ employed the membrane filter technique with Millipore's TSB medium for recovery; and George²⁶ employed a device to measure the microbial metabolic by-product, carbon dioxide, produced during cellular respiration.

To support the ASD investigation of microbiological contamination of JP-fuels, the Biospecialties Branch (MRMPB, AMRL, Wright-Patterson Air Force Base) initiated as part of the overall program, a study to establish microbiological laboratory procedures and/or procedures which could be applicable for field determinations. Various procedures and media were investigated to determine the best suited for maximum recovery of viable microbes from JP-4 fuel/water samples. Of the analytical procedures employed, the membrane filter technique (for fuels only) afforded a way to sample large volumes of fuel, and the spread plate and poured plate technique yielded a simple and easy way for microbial evaluation.

During the investigation both field and laboratory JP-fuel/water samples were analyzed. The field samples included both shipped samples and those collected on-site.

During evaluation of the spread plate and the membrane filter technique, it was noted (using the spread plate technique) that viable microbial cells were recovered with regularity from a laboratory JP-4 fuel/water system which was subjected to continual, vigorous agitation. A closer observation of the fuel phase showed a suspension of micro-drops of the water-bottom in the fuel phase. To determine if this was the route of microbial contamination of the fuel phase, three additional laboratory JP-4 fuel/water systems were agitated sufficiently to suspend droplets of the water-bottom in the fuel phase. Samples of the fuel were then obtained and subjected to microbial analysis.

The aim of the field microbiological analysis and the laboratory microbial investigation of JP-fuel/water systems was to assist in establishing the presence of microorganisms in JP-fuel/water systems, and, if the microbes were present, were they deleterious to that system. This information would, in turn, assist in determining the measures needed to control and/or eliminate the contaminants.

To facilitate sampling and microbiological analysis of field on-site analysis of JP-fuels, a field kit was constructed. It contained various liquid media, solid media in Millipore plastic disposable petri dishes, sterile hypodermic syringes, cotton swabs, and sterile sampling bottles.

The contents of the kit were altered and changed as the number of field on-site analyses increased ¹³.

METHODS AND MATERIALS

Membrane Filter and Spread Plate Techniques

Initial Determination

The membrane filter and spread plate were the primary techniques tested to determine the method most applicable for JP-fuel/water analysis. The techniques were tested simultaneously to determine the reliability and reproducibility of each procedure. To provide a means for comparison between the two methods, a culture of known cell concentration of Escherichia coli from a human source in Trypticase Soy Broth (TSB) (Baltimore Biological Laboratories) (BBL) was used. A clone of E. coli was obtained by streaking the microorganism on Eosin Methylene Blue (EMB) (Difco) agar plates and incubating at 37° C for 24 hours. A typical E. coli colony was picked and inoculated into a tube containing 10 ml of sterile TSB. The tube was incubated at 37° C for 24 hours. Duplicate 1 ml aliquots of the TSB culture were serially diluted to 10⁻¹⁰ in TSB. One tenth ml aliquots of the 3 highest dilutions were spread over the surface of duplicate plates of TSB and Endo (Difco) agar. Five (5) ml aliquots of each of the three highest dilutions were drawn through duplicate Millipore Field Monitors (No. MH WG 037 HO) with 0.45 micron filters. The monitors were divided into two groups, each group containing one monitor of each of the 3 dilutions. Total count medium (Millipore 0.4 ml ampuls M0 00 000 0T) was added to one group and Endo broth was added to the second group. The spread plates and monitors were incubated at 37° C for 48 hours. The number of viable cells/ml for the spread plates was determined by counting the number of colonies/plate of one dilution, taking the average, and multiplying the number by the dilution of that set of plates. The total number of viable cells/ml on the membrane filter was determined by dividing the number of cells on the filter by the quantity of liquid drawn through the filter membrane.

Membrane Filter Procedure. The membrane filter procedure for the determination of microbiological contamination in JP-fuel/water samples consisted of filtering 10 ml of JP-4 fuel or water through a membrane filter field monitor (0.45 micron filter), followed by filtration of 100 ml of 0.2% Triton X-100 (Rohn and Haas) and an Antifoam B agent (Dow Corning) in sterile distilled water to remove hydrocarbon residues that could prevent capillary migration of the medium from the filter pad through the membrane. The Antifoam B, a silicone substance, was used in a concentration of 10 drops/1000 ml of distilled water. All solutions were drawn through the monitors with a hand syringe (Millipore syringe and valve, No. XX 6200 05). Excess moisture was removed from each

monitor with the aid of the hand syringe. Total count medium (Millipore) was added to each monitor and the monitors were incubated at 35°C for 48 hours. At the termination of the incubation period, the monitors were opened and the filters were stained with malachite green to facilitate the counting of the microbial colonies.

Spread Plate Procedure. The spread plate procedure consisted of serially diluting (10^{-6} or 10^{-8}) an aliquot of a JP-fuel or water in an autoclave sterilized diluent (Searsport JP-4 fuel for fuel samples or TSB for water samples). One-tenth ml aliquots of the 3 highest dilutions were spread over the surface of triplicate plates of TSB agar. The plates were incubated at 30°C for 48 hours. Prior to use the TSB broth and plates were incubated for 48 hours at 30°C to insure the sterility of the plates, (this procedure was used routinely for all laboratory prepared media.) The number of cells/ml was determined as described above.

Membrane Filter Modification Procedure #1-Fuel Quantity

Modification of the membrane filter test procedure was initiated to make the procedure more applicable for JP-fuel analysis and to obtain more reliable results.

Ten ml of JP-4 fuel (10^{-1} dilution) from Laboratory Culture No. 3 were added to sterilized, clear glass milk diluting bottles containing 90 ml of autoclave sterilized JP-4 Searsport fuel (Section III). Serial dilutions of 10^{-2} to 10^{-8} were made from the initial 10^{-1} dilution. Immediately after each dilution (10^0 through 10^{-4}), 0.1 ml aliquots were plated in triplicate to plates of TSB with 1.5% Bacto-agar. The sample was dispersed over the surface of the plate with the aid of a sterile, glass, L-shaped rod. After all dilutions had been made the remaining volume of each dilution (89.9 ml) was drawn through a membrane field monitor. Each monitor was placed in a bomb sampling assembly (Millipore No. XX 64 037 08). The assembly was attached, via a sampling adapter, to a pressure vessel (Millipore Stainless Steel Pressure Vessel No. XX 67 000 05) which was pressurized to 20 psi and contained 1100 ml of 0.2% Triton X-100-Antifoam B in sterile distilled water. The solution had been autoclave sterilized in the vessel for 30 minutes. After cooling of the vessel, the system was pressurized to 20 psi. With the system at pressure, the bomb assembly was flushed with 800 ml of wash solution (Millipore Data Manual ADM-40 pp 17, 1963) as determined by a graduate sampling bottle (Millipore 850 ml polyethylene bottle XX 64 037 10) attached to the bomb assembly for measurement of effluent solutions. With the aid of a hand syringe, excess moisture was removed from each monitor. Plate count medium (Millipore) was added to each monitor. The TSB inoculated plates and the monitors were incubated at 35°C for 48 hours. After incubation, the monitor membranes were stained with malachite green and the total aerobic viable counts of the monitors and the TSB plates were determined as described above.

Membrane Filter Modification #2-Washing Solution.

Microbial contaminated laboratory systems Nos. 3, 4, and 8 were manually agitated for 5 minutes. After agitation, 100 ml aliquots of each fuel were removed and distributed (10/ml bottle) to 10 sterile milk diluting bottles containing 90 ml of autoclave sterilized diluent Searsport JP-4 fuel (10^{-1} dilution). Each group of 10 bottles was divided into two groups (Group A and B, 5 bottles/group). The bottles were agitated and diluted to 10^{-2} (10 ml of the 10^{-1} to 90 ml of diluent fuel). The entire volume of each diluted sample was drawn through a membrane filter field monitor with the aid of a vacuum pump. Monitors from Group A diluting bottles of one laboratory JP-fuel/water system were placed in the bomb sampling assembly and washed with 300 ml of wash solution used as described above. Group B monitors received no washing prior to the addition of the growth medium.

To 9.9 ml of TSB were added 0.1 ml aliquots of fuel from each of the 3 agitated laboratory systems. Aliquots of 0.1 ml of this 10^{-1} dilution were plated to triplicate plates of TSB agar plates and dispersed over the surface of the agar with the aid of an L-rod. Both monitors and TSB agar plates were incubated at 35°C (note change in the incubation temperature) for 48 hours. The total aerobic viable microbial count of the monitors and the plates was determined as discussed above. In addition to the fuel analysis, the water-bottoms of each system were microbially analyzed.

Triton X-100, Antifoam B Effects.

After 5 minutes of agitation, 300 ml of laboratory culture No. 8 were removed and distributed in 10 ml aliquots to 24 sterile milk diluting bottles. The bottles were divided into 4 groups (6 bottles/group). Serial dilutions of 10^{-1} and 10^{-3} were made of each of the 24 bottles. Aliquots of 0.1 ml of each bottle were plated in triplicate on TSB agar. The remainder of each bottle was drawn through a membrane filter field monitor. Monitors of each group contained 6 monitors/dilution. Group A monitors were flushed with a wash solution containing 0.2% Triton X-100; group B were flushed with Antifoam B solution; group C were flushed with a 0.2% Triton X-100 and Antifoam B solution; and group D received no flushing prior to the addition of growth medium. Excess moisture was removed from each monitor with the aid of the hand syringe. Plate count medium (Millipore) was added to each monitor. All monitors and TSB plates were incubated at 35°C for 48 hours. At termination of the incubation period the monitor filters were stained with malachite green and the total counts determined.

MEDIA INVESTIGATION

Various media were investigated in an attempt to find a general medium, one which would serve as a growth medium for the greater number

of microbes found in JP-fuel/water systems and which could be used in the laboratory and for on-site field analysis.

The Biospecialties Laboratory investigated the following as part of the ASD program;

Thioglycollate (Difco).
Brain Heart Infusion Broth (Difco) and the broth with 1.5% Bacto-agar.
Tryptic Soy Broth (Difco) and the broth with 1.5% Bacto-agar.
Trypticase Soy Broth (TSB) (BBL) and the broth with 1.5% Bacto-agar.
TSB with B-H salts and 1.5% Bacto-agar.
TSB with 1% and 3% EGME and glycerol, and 1.5% Bacto-agar.
B-H with 1% and 3% EGME and glycerol, and 1.5% Bacto-agar.
Water from bulk storage tanks and aircraft sumps with 1.5%
Bacto-agar, autoclave and filter sterilized.
B-H with nitrogen.
Sea-Rite solution with 1.5% Bacto-agar.
Water from contaminated laboratory JP-fuel/water systems
with 1.5% Bacto-agar, autoclave and filter sterilized.
1/2 aircraft sump water and distilled water with 1.5% Bacto-agar.
Plain agar (1.5%)
Blood agar.
Sabouraud (Difco) with 1.5% Bacto-agar.
American Petroleum Institute (API) commercially prepared
and laboratory prepared.
Fungi medium ph 5.2 ⁸¹.

Included in the investigation of the media best suited for recovery of the viable microbial cells from JP-fuel/water systems were the use of various diluent liquids. Broths of TSB (BBL), TSB (Difco), Brain Heart Infusion (BHI) (Difco), and autoclaved and filter sterilized Searsport JP-4 fuel were used for diluents.

The initial test to determine the best diluent for JP-fuels consisted of diluting 0.1 ml aliquots of Laboratory Culture No. 8 in 9.9 ml of the above broths. Serial dilutions of 10^{-1} to 10^{-6} were made with each diluent. One-tenth ml aliquots of each of the dilutions of each broth TSB (BBL), TSB (Difco), and BHI were dispersed on the surface of 6 plates of the related solid agars. The diluent JP-4 fuels were plated to 6 plates of each of the 3 solid growth media. The plates of each dilution were divided into 3 groups, A, B and C (2 plates each of each dilution). These were incubated at 35°, 30°, and at room temperature respectively for 48 hours. At the termination of the incubation period, the total viable microbial count was determined as discussed above (see Methods and Materials).

The media listed above were used at various times for a period of one (1) year for the microbial analysis of JP-fuel/water samples.

JP-FUEL/WATER MICROBIAL ANALYSIS

The equipment, method of sampling, and the microbiological contamination determinations for laboratory and/or on-site field analysis of Air Force JP-fuel/water samples which were used by the MRMBP laboratory were as follows:

Collecting containers consisted of sterile 4 or 8 ounce prescription bottles, vacuum serum bottles, gallon jugs, and/or quart mason jars. Other equipment used to obtain samples were aircraft sump drains (quart mason jar lids fitted with a funnel in which was fitted a small-head screw driver), 2 or 10 cc sterile hypodermic syringes fitted with an 18 gage 1 inch needle, Linder sampler¹⁷, thief sampler, and sterile swabs contained in sterile screw capped tubes.

Prior to obtaining any sample from an orifice which was exposed to microbial contamination other than that of the intended sample, the orifice was cleaned with a material (usually cotton) containing 0.1% Roccal[®] solution and the initial effluent discarded. The sample from the source was then obtained in the appropriate sterile containers protected from sunlight, and subjected to analysis within two hours after collection.

The microbiological determination of each sample consisted of total viable aerobic count (cells/ml) (TC), and a non-quantitative determination of anaerobes and/or facultative aerobes, fungi, and sulfate reducers. In addition, a microscopic examination of all samples was done whenever possible. For enumeration of specific microorganisms such as Pseudomonas aeruginosa and iron depositing bacteria, pyocyanin and iron depositing media¹⁸ were used.

For the standard microbiological analysis to determine the quantity of viable aerobic cells in JP-water samples, 0.1 to 1.0 ml of the sample was diluted into 9.9 or 9.0 ml of TSB. The degree of dilution of a sample was determined by its turbidity. Generally, dilutions ranged from 10^{-1} to 10^{-6} . One-tenth ml of each sample of each dilution was plated to duplicate plates of TSB with 1.5% Bacto-agar which had been pre-incubated for 48 hours to insure sterility of the plates. One ml of the samples was added to Thioglycollate broth (Difco) for demonstrating the presence of anaerobes and/or facultative aerobes. The Thioglycollate medium was incubated for 6 days (2 days minimum incubation time) at room temperature. If growth developed, an inoculating loop of the growth was streaked to TSB agar plates to determine if the microorganisms recovered were true anaerobes or facultative aerobes. For determination of the presence of fungi, 0.1 ml aliquots of the water sample were added to duplicate plates of Sabouraud (Difco) (ph 5.8 to 6.2) and 1.5% Bacto-agar. The plates were incubated at room temperature in the dark for a minimum period of 5 days. To isolate the sulfate reducing microbes, 0.1 ml of sample was added to commercially

(Difco) or laboratory prepared API. The vials, or tubes, were incubated at room temperature in the dark for a period of 1 to 6 weeks. The microscopic examination assisted in the determination of the presence of viable and non-viable microbial cells.

The standard microbiological analysis for enumeration of viable aerobic microbial cells in JP-fuels consisted of the same procedure as listed above (except for the sulfate reducing determination). To facilitate field on-site microbial analysis, a metal suitcase was equipped with packages of 1.0 ml sterile pipettes(3/pkg) wrapped in cotton and aluminum foil, sterile L-rods individually wrapped, 2 and 10 cc sterile disposable or glass syringes fitted with 18 gage 1 inch needles, sterile sampling bottles (4 or 8 ounces), sterile sump drains (wrapped in brown-wrapping paper) (the number of each of these items depended upon the number of intended samples to be collected), concentrated Roccal solution (for cleaning sampling spouts and working area), marking implements, cotton or paper towels, portable test tube holder, Linder sampler, and sterile vacuum serum bottles and media, as discussed above.

RESULTS AND DISCUSSION

After the preliminary tests of the membrane filter and spread plate techniques (Table I) indicated comparable recoveries of viable microbes when water was used as a transporting medium, both methods were included in the study to determine the most applicable analysis for detection of microbial contamination of JP-4 fuels. However, as shown in Table II with the change of transporting medium from water to JP-fuels (fuel-to-fuel dilution, Tables II and III(primary test)), erratic results were obtained. In an effort to reduce and/or eliminate the irregularities, the quantity of fuel analyzed was increased. The results of the spread plate technique were inconsistent and higher counts were often obtained from solutions of least concentrations (Table II, Laboratory Culture No. 3). In addition, negative results were obtained with the membrane filter technique (Table III, Test No. 1). To insure that the hydrocarbon residue deposited on the monitor membrane was not a factor in the erratic results obtained, the amount of Triton X-100-Antifcam B wash water was increased from the initial 100 ml to 800 ml (Table III, Test No. 2). In addition to the erratic counts with the increase in volume of wash solutions, microbial-like colonies appeared on the filters after staining with malachite green. Microscopic examinations of the colonial type forms indicated non-biological materials. Microscopic differentiation between true microbial colonies and the colony-like substance was impossible. To determine if the source of the colonial appearing substance was the result of Triton X-100 and Antifoam B, or a combination of both, microbially contaminated JP-4 fuels were diluted with JP-4 fuels and treated with one and/or both of the above materials. This study showed that colony-like material was found on filters obtained from each combination of washed solutions. Thus the post filtration washing procedure

Table 1

Preliminary Determination of Membrane Filter and
Spread Plate Techniques Reproducibility

Membrane Filter Method Cells/ml		Spread Plate Method Cells/ml	
Total Count	Endo Broth	TSB	Endo Agar
2.06×10^9	6.00×10^8	1.67×10^9	1.94×10^9

Test Organism was Escherichia Coli

Table I. Preliminary Determination of Membrane Filter and
Spread Plate Techniques Reproducibility.

Table II

Results of Spread Plate Analysis of Laboratory JP-4 Fuels

Count/ml in Fuel from Undiluted Sample					
Cultures	1 ¹	2	3	4	Count/ml in Water
Laboratory Culture Source	0	0	2.3×10^3	-	$>10^5$
No. 3	TNTC	0	1.4×10^1	<10	3.5×10^8
No. 5*	7.0×10^1	6.6×10^2	9.6×10^1	3.0×10^2	4.7×10^7
Count/ml in Fuel from Fuel Diluted Sample					
Cultures	Dilution	10^{-1}	10^{-3}	10^{-8}	Count/ml in Water
No. 3	a ²	37	2	7	5.5×10^7
	b	68	0	4	
	c	103	0	12	
No. 4*	a	TNTC	2	0	3.0×10^7
	b	176	10	0	
	c	47	12	3	
	Dilution	10^{-0}	10^{-1}	10^{-8}	
No. 3*	a	TNTC	9	0	3.4×10^7
	b	TNTC	6	0	
	c	276	0	0	

*Laboratory System agitated sufficiently to mix the fuel and water.

¹ This experiment was repeated on four separate occasions as indicated by columns 1, 2, 3, and 4.

² Determinations repeated on three separate occasions as indicated by a, b, and c.

Table II. Results of Spread Plate Analysis of Laboratory JP-4 Fuels

Table III

Analytical Procedures for Microbial Determinations of JP-fuel

Laboratory Culture	Membrane Filter Count/ml				Spread Plate Count/ml		Poured Plate Count/ml	
	Monitors				Dilution	Count	Dilution	Count
	Washed		Unwashed					
	Dilution	Count	Dilution	Count				
Primary Test- No. 3 Fuel Only	10 ⁻⁷ - 10 ⁻⁸	0	-	-	10 ⁻⁷ - 10 ⁻⁸	0	-	-
Field Sample Fuel KC135 (100 ml Wash)	10 ⁻¹	0.5	-	-	-	-	-	-
Field Fuel Sample (100 ml Wash)	10 ⁻² - 10 ⁻³	0	-	-	-	-	-	-
#1 Test - Fuel Quantity No. 3 Fuel Only	10 ⁻⁴ - 10 ⁻⁸	0	-	-	10 ⁻⁰ - 10 ⁻⁴	0	-	-
#1 Test - Fuel Quantity No. 3 Fuel Only	10 ⁻¹ - 10 ⁻³	0	-	-	10 ⁻¹ - 10 ⁻³	0	-	-
#2 Test - Increased Wash Solution No. 3 Fuel Only	10 ⁻¹ - 10 ⁻²	0	10 ⁻¹	0	10 ⁻¹ - 10 ⁻³	2.5 x 10 ²	-	-
#2 Test - Increased Wash Solution No. 4 Fuel Only	10 ⁻¹ - 10 ⁻²	0	-	-	10 ⁻¹ - 10 ⁻³	2.7 x 10 ¹	10 ⁻¹ - 10 ⁻³	2.0 x 10 ¹
#2 Test - Increased Wash Solution No. 4 Fuel Only	10 ⁻⁰	TNTC	-	-	-	-	-	-
No. 8 Culture Fuel With Triton X-100 ^a	10 ⁻¹ - 10 ⁻³	0	10 ⁻¹ - 10 ⁻³	0	-	-	-	-
Antifoam B	10 ⁻¹ - 10 ⁻³	0	-	-	-	-	-	-
Triton X-100- Antifoam B	10 ⁻¹ - 10 ⁻³	0	-	-	-	-	-	-
Culture No. 8*	10 ⁻¹	0	-	-	10 ⁻⁰ - 10 ⁻¹	4.6 x 10 ¹	10 ⁻⁰	0
Culture No. 8*	10 ⁻¹	0	-	-	10 ⁻⁰	4.7 x 10 ³	-	-
Culture No. 4*	10 ⁻¹	0	-	-	10 ⁻⁰	4.9 x 10 ²	10 ⁻⁰	0

- Indicates Analysis Omitted.

* Indicates System had been agitated to causing a mixing of fuel and water-bottom

Table III. Analytical Procedures for Microbial Determinations of JP-Fuel.

appears to be responsible for the deposit of this unidentified material. This may be the result of incomplete solubility of the surfactant and Antifoam B agent in wash water. As tabulated in Table II the result obtained from the spread plate procedure indicated microbial contamination of the testing JP-4 fuel, but the results of the test were erratic as previously observed, and dilutions were not indicative of the number of cells actually present in the testing fuel. Dilutions of least concentration indicated as many cells/ml as those of the greatest concentration.

Due to the negative results obtained from the filter technique (Table IV), the weight of the equipment for travel, excessive preparation of materials, limitations as to the number of samples that could be run during a sampling day, the time required for a sample analysis (as compared to the spread plate technique), and the bulkiness of the equipment, the membrane filter technique was discontinued and the spread plate technique was selected as the routine method to ascertain the number of viable aerobic microorganisms present in JP-fuel/water samples. Although erratic results were obtained repeatedly with the membrane filter technique when used in the analysis of JP-fuels, the procedure was used for several on-site field analyses of JP-fuels to determine the presence of low levels of microbes in large volumes of fuel (Table V).

In addition to the membrane filter technique being eliminated as a possible standard technique for JP-fuel microbial analysis, the poured plate method (Table III) was also discontinued as an analytical procedure. Discontinuance was due to (1) significantly lower recovery of viable cells from the JP-fuels, (2) the difficulty of obtaining heating facilities for the medium during on-site field analysis, and (3) the possibility of extrinsic contamination during pouring of the plates under field conditions.

During the program to determine the most applicable analytical method to ascertain the number of microbial cells containing JP-fuels, the mechanism of microbial contamination of the JP-fuels was suggested by the consistent recovery of viable cells from the fuel phase of Laboratory Culture No. 5 (Section III). The system, subjected to a constant agitation sufficient to cause a mixing between fuel and water-bottoms, showed a suspension of water drops in the fuel phase. Thus, in an attempt to assure that testing JP-4 fuels were microbially contaminated, the agitation procedure of Culture No. 5 was repeated on laboratory systems Nos. 3, 5, and 8, sufficiently to cause mixing of the fuel and water-bottom. To correlate the number of cells/ml in the water drops suspended in the fuel phase, the quantitative counts of the water-bottoms of each system were determined. As shown in Tables II and III the results of the spread plate and the membrane filter procedures of agitated fuel systems were similar to previous testing results.

Table IV
 Comparison of Various Wash Solutions Used in the
 Membrane Filter Technique

Dilutions	Triton x-100		Antifoam B		Triton x-100 and Antifoam B			No. Wash				
	10 ⁰	10 ⁻¹	10 ⁻³	10 ⁰	10 ⁻¹	10 ⁻³	10 ⁰	10 ⁻¹	10 ⁻³	10 ⁻⁰	10 ⁻¹	10 ⁻³
Spread plate ¹ (cells/ml)	4.90 x 10 ³	4.75 x 10 ³	4.40 x 10 ³	1.00 x 10 ¹	6.00 x 10 ¹	1.50 x 10 ¹	5.00 x 10 ⁰	0	0	0	5.00 x 10 ²	3.00 x 10 ²
Millipore Monitors 90 ml drawn through each monitor	0	0		0	0	0	0	0	0	0	0	0

¹Counts are averages of 3 plates containing 0.1 ml fuel sample

Table IV. Comparison of Various Wash Solutions Used in the Membrane Filter Technique.

Table V

On-Site Field Filter Membrane and Spread Plate Analysis

Sample Source	Filter Membrane Cells/ml - from Fuel		Spread Plate Cells/ml - from Fuel	
	Sabouraud	Millipore Medium	Sabouraud	TSE
Aircraft Sumps JP-5	2 to 30/52	6 to 34/52	0	0 to $< 1.0 \times 10^1$
Aircraft Sumps JP-5	3 to 9/50	4 to 8/50	0	0 to $< 3.0 \times 10^1$
Aircraft Sumps JP-5	2/50	1/50	0	$< 1.3 \times 10^1$
Aircraft Sumps JP-4	0/110	0/100	0	$< 1.0 \times 10^1$
Ready Tanks JP-5	0/100	0/100	-	$< 2.8 \times 10^1$ to 6.8×10^4
Aircraft Sumps JP-5	19/50	22/50	0	0

Table V. On-Site Field Filter Membrane and Spread Plate Analysis

As indicated on Table VI of the media investigated for quantitative analysis of microbial contaminated JP-fuel/water samples, TSB with 1.5% Bacto-agar, and blood agar plates appeared to give the most dependable and reproducible results. The temperature of 35°C appeared to permit a greater number of cells to grow than did the initial 30°C. The results of the JP-fuel diluent investigation, in addition to the low numbers of microorganisms recovered from field collected JP-4 fuel, indicated that the diluting of JP-fuels was unnecessary. Since approximately 63% of the 125 field fuel samples analyzed during these studies were found to contain no microbial cells, 0.1 ml of sample fuel was considered sufficient to be indicative of the fuel contamination. Of interest from the results of these studies was the decrease in the number of viable cells which were recovered from JP-4 fuels when plating of a sample of the water-drop contaminated JP-fuels and of the diluted fuels (fuel-to-fuel dilution) had been delayed. As a result of these findings and the observations of erratic counts from fuel samples by the spread plate method, investigations described in Sections III and IV were initiated.

CONCLUSION

1. The spread plate procedure was the most dependable for the recovery of the greatest number of cells contaminating the fuel or water phase of a JP-4 fuel/water system.
2. False positive counts of colonies on membrane filters can result from colony-like substances that are hydrophobic and are of a non-biological origin.
3. Mixing of the fuel and water phase in a microbially contaminated JP-4 fuel/water system appears to be necessary to contaminate the fuel phase.
4. Since JP-fuels appear to contain low numbers of microorganisms, and diluting the fuel sample affects the recovery of these microorganisms, the diluting of JP-fuels is unnecessary.

Table VI

Effect of Media on Microbial Recovery

Studies	Culture Source	Media				
		TSB	B-H	B-H 3%	Fungi pH 5.2	Sabouraud
a				Cells/ml	*	
	5 ¹	$2.0 \times 10^{4.6}$	0	1.0×10^4	0	0
	7 ¹	1.2×10^6	7.0×10^5	1.2×10^5	4.7×10^5	9.7×10^5 (bacteria)
b		TSB	TSB 3%	B-H	B-H 1%	B-H 3%
	Column	2.4×10^3	1.3×10^2	1.0×10^1	4.5×10^1	6.5×10^1
	8 ¹	TWTC	TWTC	2.7×10^3	-	2.9×10^3
	3 ¹	2.9×10^6	2.1×10^6	1.8×10^6	-	2.2×10^4
c		TSB	TSB B-H	TSB 1%	TSB 3%	TSB 30%
	Column ¹	1.3×10^3	1.0×10^2	-	1.0×10^2	-
	Column ¹	1.1×10^3	-	1.1×10^1	9.0×10^1	0
d		TSB	Fungi pH 5.2	B-H with Dextrose		
	Glass ¹ Column with 30% AIA	1.4×10^2	-	4.3×10^1		
	Field ¹ Sample	2.0×10^1	-	0		
	Culture ² 7% AIA	0	0	0		
e		TSB	Blood Agar			
	4 ¹	4.6×10^6	3.8×10^6			
	3 ¹	5.5×10^6	7.3×10^7			
	7 ¹	2.3×10^8	3.8×10^8			
	8 ¹	2.8×10^8	2.9×10^8			

Table VI. Effect of Media on Microbial Recovery.

Table VI
Effect of Media on Microbial

Studies	Culture Source	Media							
		TSB	TSB & B-H	TSB 1%	TSB 3%	B-H 1%	B-H 3%	Field Water	
								F	A
f	Column ¹	1.7×10^2	9.2×10^1	1.4×10^2	1.1×10^2	2.5×10^1	4.0×10^1	0	0
	Column ¹	1.4×10^2	1.5×10^2	1.1×10^2	1.1×10^2	6.0×10^1	2.3×10^1	0	0
	Column ¹	1.4×10^2	9.3×10^1	1.4×10^2	9.3×10^1	2.3×10^1	0	0	0
	7 ¹	1.7×10^6	-	-	-	1.2×10^6	4.0×10^5	-	-
	3 ¹	-	-	-	-	6.5×10^5	7.1×10^5	-	-
		TSB	TSB 3% AIA	Sump Water Agar	Fungi pH 5.2	B-H			
						Alone	3% AIA		
g	Field ¹ Sample	1.2×10^2	6.0×10^1	0	4.0×10^1	0	0		
	Column ¹ with 30% AIA	1.6×10^2	8.0×10^1	-	-	3.0×10^1	1.5×10^2		
	Column ¹ with 3% AIA	1.8×10^2	1.5×10^1	-	-	1.9×10^1	1.3×10^2		
	Column ¹ with 3% AIA	2.2×10^2	1.4×10^2	-	-	-	8.3×10^1		

Table VI. Effect of Media

VI

Microbial Recovery

Media								
Field Water		B-H without any Nitrogen	Laboratory Water		Sea Rite Agar	Sump Water	Fungi PH 5.2	B-H
F	A		A	F				
0	0	0	0	0	0	-	-	-
0	0	4.7×10^1	0	0	0	-	-	-
0	0	1.3×10^1	0	0	0	-	-	-
-	-	-	-	-	-	0	1.4×10^6	1.6×10^6
-	-	-	-	-	-	-	(Bacteria) 2.0×10^6	9.7×10^5

AIA

0

$\times 10^2$

$\times 10^2$

$\times 10^1$

Tables 6a through 6g represent individual studies of the effect of media on microbial recovery.

* Numbers in table refer to average number of cells/ml of 3 replicates

- Indicates that the analysis was omitted.

1 Indicates water analyzed.

2 Indicates fuel analyzed.

A Autoclaved

F Filtered

t of Media on Microbial Recovery.

Section III

DISTRIBUTION OF MICROORGANISMS IN JP-4 FUEL/WATER SYSTEMS

Early reports of investigations concerning microbial contamination of JP-4 fuel/water systems implied that microbial growth was most abundant at the fuel/water interface,^{9, 16, 17, 20} and that the microbial cells stratified in systems containing both JP-4 fuel and free water. Therefore, the depth at which a fuel sample was obtained in a JP-4 fuel/water system was considered as a major cause for the variation in results obtained concerning the number and kinds of microbial cells recovered. Efforts to establish causes for variation in microbial population in laboratory JP-4 fuel/water systems and the relationship of microbial population in water-bottoms to those in fuels were facilitated by the use of laboratory systems of various JP-4 fuels and waters microbially contaminated. These efforts led to additional investigations concerning the effect on the microbial population of different metals in a JP-4 fuel/water system as well as the effect of climatic changes.

During the efforts concerned with selecting and establishing optimal analytical methods for laboratory and field analysis, one approach used the application of column devices which permitted sampling at several points above and below the fuel/water interface without disturbing the interface of the system. This facilitated the collecting of data on stratification of microorganisms and the effect of depth on the number of microorganisms at various levels in fuel/water systems. The systems were established to simulate environment conditions in the field, or to force the microorganisms to use the JP-4 fuel as a source of carbon, thus causing migration of the microbial cells from the water phase into the fuel phase.

METHODS AND MATERIALS

Microbial Analysis

The total number of viable aerobic microorganisms present in contaminated JP-fuel/water systems was determined by the spread plate technique. Serial dilutions of a 1:10 to a 1:10,000,000 were made of the water in TSB. Aliquots of 0.1 to 1.0 ml of the 3 highest dilutions were plated in triplicate on TSB with 1.5% Bacto-agar. The average number of colonies present on plates on one dilution, multiplied by the dilution plated, indicated the number of viable aerobic microbial cells/ml present in the system at time of sampling. The presence of anaerobes was not determined since quantitation of anaerobes is time consuming, and analysis of only aerobes was sufficient to indicate microbial distribution as required for this study.

Initially, fuels were diluted in Searsport JP-4 fuel; however, when it became evident that diluent JP-4 fuels, autoclave or filter sterilized, exerted a toxic effect on viable microbial cells, 0.1 ml aliquots of fuel were plated in triplicate directly on TSB with 1.5% Bacto-agar. All inoculated plates were incubated at 35°C for 48 hours.

Laboratory Systems

Laboratory Supply Culture. Samples obtained in the field of JP-4 fuel/water and their resident microbial contaminants were used to establish a standard laboratory culture. The system was composed of a 5 gallon clear glass carboy and a composite of contaminated JP-4 fuel/water samples collected from the field 6 to 12 months prior to the establishment of the laboratory culture. The system was replenished with additional microbial cells, water, and JP-4 fuels as such samples were received from the field. Total aerobic microbial counts were determined periodically.

Additional Laboratory Systems. Nine laboratory cultures were established during this study. These cultures will be described in greater detail in various sections of this report. However, the general information pertaining to these cultures is presented in Table VIII.

Culture No. 1. Laboratory Culture No. 1 consisted of a 5 gallon clear glass carboy with a 1 L beaker serving as a cover to prevent air contaminants from entering the system. Prior to use the carboy was washed with a laboratory detergent, rinsed with 0.1% Roccal (Sterwin Chemicals, Inc.) solution and 3 rinses of sterile distilled water. The system was composed of 4 L of non-sterile Searsport JP-4 fuel with 0.1% EGME and glycerol; 4 L of a B-H-1 (see section on Laboratory Systems 2a and 2b below) solution heated to 212°F, cooled for 12 hours and filtered through a No. 5 grade filter paper to eliminate all sediment and a 3 ml microbial inoculum of 10^6 cells/ml from the water-bottom of the laboratory supply culture. The container was placed in a wooden support rack and positioned over a Magnastir (Harshaw Scientific Company, Number H 600 60). The liquids were agitated for 48 hours which was sufficient to cause a slight vortex of the fuel and water-bottom. At the end of 48 hours the system was maintained in a quiescent state. Samples of the fuel and water were obtained with sterile pipettes. The culture was analyzed periodically. Water-bottom samples were serially diluted in TSB with 1.5% Bacto-agar. Aliquots of 0.1 ml of fuels were plated directly on triplicate plates of TSB with 1.5% Bacto-agar. All inoculated plates were incubated at 35°C for 48 hours.

Laboratory Systems 2a and 2b - Static Drum Storage Tests. Cultures 2a and 2b, the static drum storage facility (Figure 1), consisted of two 208 L (55 gallon) drums, one aluminum and one steel, equipped with end sampling valves located at the fuel/water interface to facilitate the sampling of fuel and water, and end viewing panels of glass for viewing the system. The



Figure 1. Laboratory Systems 2a and 2b Facility.

drums were installed on their sides in a drum rack located outside the building as shown in Figure 1. The drums, which had been cleaned by the manufacturer after construction, were used as received. The manufacturer's method of cleaning consisted of a sodium hydroxide washing, water rinses, and a 15 minute drying period at 300° F.

The fuel/water-bottom and microbial inoculum of systems 2a and 2b were as follows: to each drum was added 113 L (30 gal) of non-sterile Searsport JP-4 fuel with 0.1% EGME and glycerol obtained from Wright-Patterson Air Force Base, Ohio (delivered 1 August 1963); and 45.5 L (12 gal) of rain water that had been collected (1) in a rusty 208 L (55 gal) steel drum for use in the steel test drum; and (2) in a 208 L (55 gal) galvanized garbage can for use in the aluminum test drum. Two weeks after the system had been established 1 L of Bushnell-Haas-1 (composed of 0.02 gm $MgSO_4$, 1.00 gm KH_2PO_4 , 1.00 gm K_2HPO_4 , 1.00 gm NH_4NO_3 , 0.02 gm $CaCl_2$ and 0.05 gm $FeCl_3$ per 1000 ml distilled water) was added to each drum. The microbial inoculum was a 225 ml composite of 10^8 cells/ml of microorganisms obtained from the water-bottom of the laboratory supply culture. The drums were kept in a quiescent state for 7 months at which time it became necessary to rotate the drums to facilitate sampling of the water phase. Each drum was observed for a period of 9 months.

Prior to obtaining fuel and water samples in 8 ounce sterile clear glass prescription bottles with screw caps, the outer portion of the sampling valve was cleaned with 0.1% Roccal solution and the inner surface flushed with the fluid to be analyzed.

Stratification Systems. The stratification studies consisted of three parts (A, B, and C). Systems of Studies A and B simulate field conditions, and the system of Study C was constructed to force the microbes to use the JP-4 fuel of the system as the sole source of carbon.

The column devices consisted of two tall columns; one was of aluminum (606 3T6 - - 3003), height 190 cm and an inner diameter of 10.2 cm; the second was of clear pyrex glass, height 112 cm and an inner diameter of 15.2 cm. Each column was equipped with a removable lid with an atmospheric vent for elimination of fuel vapors and Buna-N plugged sampling ports along one side. The sampling ports were held in place by metal bands. The columns were installed as shown in Figures 2 and 3.

A non-sterile Searsport JP-4 fuel without EGME and glycerol was obtained from ATFL Section, Wright-Patterson Air Force Base, Ohio, 26 July 1963.

The water-bottoms of the columns were composed of 20% Sea-Rite salt solution (Study A), diluted sea water, (40% distilled water, 40% boiled



Figure 2. Aluminum Stratification Column

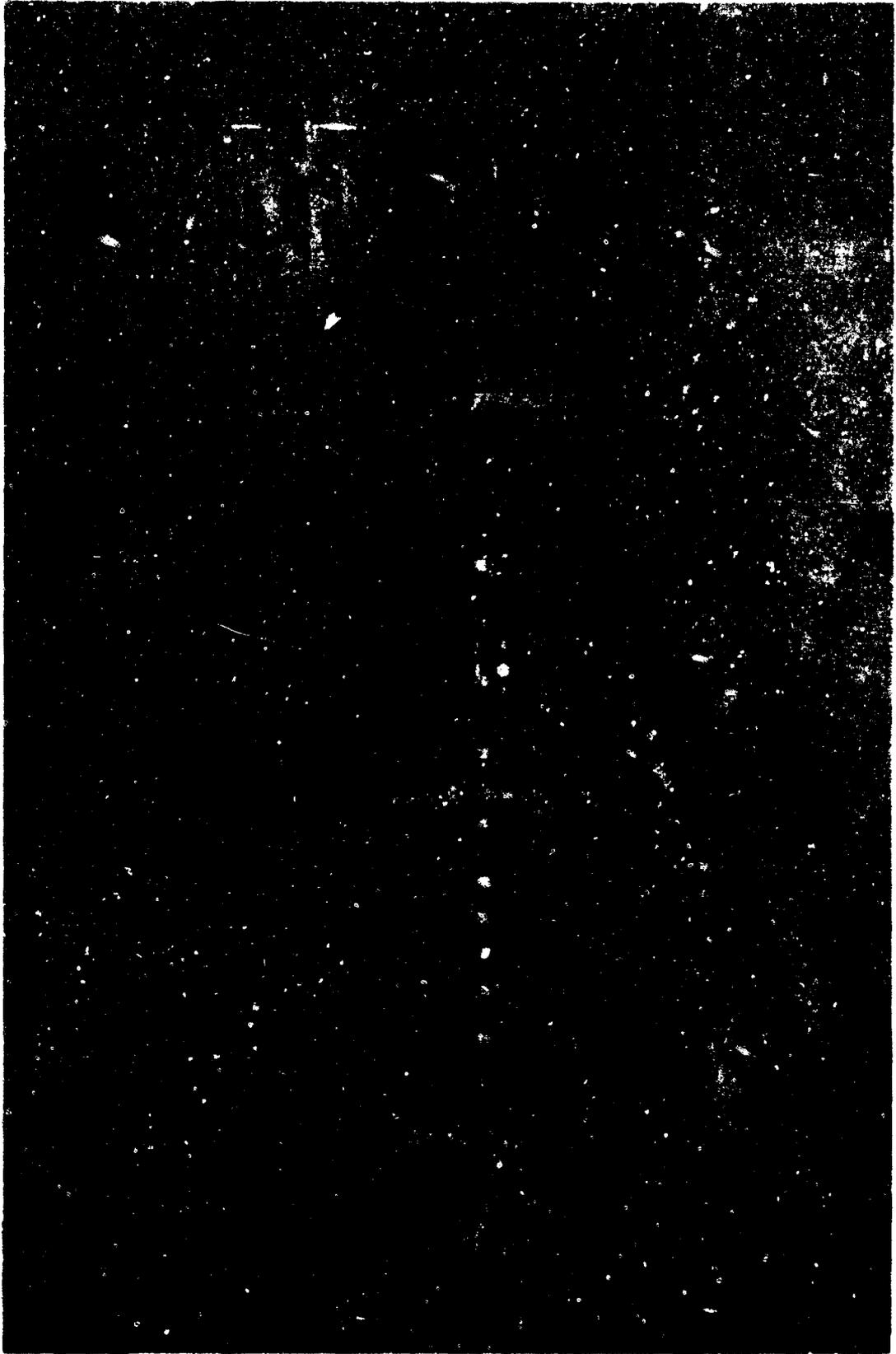


Figure 3. Glass Stratification Column

tap water, and 20% aged natural sea water) (Study B) , and the B-H-1 mineral salt solution (Study C).

To eliminate extraneous dirt and debris the columns were cleaned prior to use. However, complete sterilization of the columns was considered impractical due to the construction and size of the columns. The aluminum column was flushed, via the top opening, several times with demineralized water (Bantam Demineralizer, Model BD-1, 10 gph). The glass column was flushed, via the top opening, with 0.01% Roccal; rinsed 3 times with sterile distilled water, and air dried for 4 days prior to use.

The initial filling of the aluminum column (Study A) contained 2.5 L of Sea-Rite salt solution, 4.5 L of JP-4 fuel, and 2.5 ml of 10^7 cells/ml obtained from the water-bottom of Laboratory Culture No. 3 (Table VIII), and an unknown number of an inherent microbial population derived from the ambient air or as a result of incomplete sterilization of the column. Due to adding the fluids via the top opening, a mixing of the fuel and the water occurred. Study A was discontinued after 48 hours of observation due to the lack of survival rate data of the microbial population in the Sea-Rite salt solution. For Study B the aluminum column was emptied, flushed several times with demineralized water, and filled as follows: 2.5 L of diluted, aged natural sea water, 2.5 ml of the water-bottom of Laboratory Culture No. 3, and 4.5 L of the JP-4 fuel used in Study A. Study B was terminated after 82 days of observation.

The glass column (Study C) was filled with 4 L of JP-4 fuel, 2 L of B-H-1 mineral salt solution, and a microbial inoculum consisting of 4.0 ml of a composite of 10^7 cells/ml of 4 laboratory cultures, 1.0 ml of 10^2 cells/ml of a microbially contaminated field JP-4 fuel/water sample containing 21% EGME and glycerol, and 10^3 cells/ml of the inherent microbial population. To prevent the mixing of the fuel and water, the fuel was added to the glass column via the top opening 3 days prior to the addition of the water-bottom. The water-bottom was then added via a polyethylene tube attached to a bottom spout of the column (Figure 4). After 3 days and prior to the addition of the inoculum via the bottom sampling port, the water and the fuel were sampled and analyzed for the presence of microbial contamination. Study C was terminated on the 58th day of observation.

Aliquots of fuel and water were withdrawn from the columns through the sampling ports with a sterile syringe fitted with 18 to 21 gage needles (Figure 4). Aseptic techniques were followed during sampling to prevent exogenous microbial contamination of the column. Prior to the removal of a fuel or water sample, the sampling ports were cleaned with 0.1% Roccal solution and 70% alcohol. During the observation period of Studies A and B (aluminum column), samples were obtained and analyzed from all ports. However, due to the results obtained from Studies A and B, all fuel ports



Figure 4. Sampling Glass Stratification Column

of the glass column in Study C were not sampled or analyzed each time a water sample was obtained and analyzed.

RESULTS AND DISCUSSION

The results of microbial determinations (cells/ml) of laboratory systems indicated that growth rates of cells in water-bottoms of laboratory initiated JP fuel/water systems did not reach the magnitude observed when cells are inoculated into a laboratory medium such as TSB. The highest count observed in any system was 10^9 cells/ml. This occurred in Culture No. 1 during the development of a fuel/water interface matting. As indicated in Table VII the maximum count obtained from the laboratory culture source composed of only field materials was 10^8 cells/ml with an average of 10^7 cells/ml for the entire observation period. The count of 10^8 cells/ml appeared to be the average magnitude reached by most of the laboratory cultures.

Of importance but not indicated in Table VIII was the inability to recover viable microbial cells from the fuel phase unless the system was sufficiently agitated to cause a mixing of the fuel/water. However, this was not always true since viable microbial cells were infrequently recovered from laboratory JP-fuel/water systems. After scrutinizing sampling procedures the apparent cause was found to be the method of sampling the system. During sampling of the water-bottom, water would be drawn up through the fuel phase and, without permitting sufficient time for settling-out of the water droplets from the fuel, samples of the fuel would be obtained for microbial determinations. Of interest during the program was the color change of the fuel and water of the Laboratory Source Culture and of Laboratory Cultures numbered 3 and 4. Originally the fuel and water were colorless, but with time the colors changed to an amber and a bile green respectively.

Although the early literature indicates that viable microbial cells stratified in fuel/water systems with the major concentration occurring at the fuel/water interface, the phenomenon was not evident in the JP-4 fuel/water cultures reported in this study. Other than the matting of microorganisms which occurred at the fuel/water interface of Laboratory Culture No. 1 and Study C Stratification Column Culture, the results of the investigation indicated that viable microbial cells are reasonably uniformly distributed throughout the water phase and not present at all in quiescent JP-4 fuels.

The pellicle or mat of microorganisms noted at the fuel/water interface of Laboratory Culture No. 1 developed within a 72-hour period after agitation of the system had been stopped; by the 11th day the matting was fairly thick and cohesive and the viable aerobic microbial count of the water-bottom had increased from 10^3 to 10^9 cells/ml (Table IX). The pellicle gradually settled to the bottom, only to be replaced by one of equal thickness.

Table VII

Viable Microbial Count of Laboratory Supply Culture

Time of Sampling in Days After Initiation of System	Counts/ml from Water Phase	Counts/ml Fuel Phase
0	not determined	
2	3.4×10^6	1×10^1
13	5.4×10^8	0
25	1.0×10^7	-
46	2.0×10^6	-
65	2.6×10^6	-
80	1.7×10^4	-
83	1.0×10^2	-
90	3.3×10^2	-
108	1.9×10^3	-
127	1.0×10^3	-
133	1.6×10^7	-
184	4.8×10^7	0
200	1.4×10^7	-
221	2.4×10^6	-
288	2.1×10^8	-
339	4.2×10^7	-
354	1.9×10^6	0
360	2.6×10^7	-
374	1.1×10^7	-
376	1.3×10^7 Terminated	0

- indicates analysis omitted

Table VII. Viable Microbial Count of Laboratory Supply Culture.

Viable Microbial Count of Laboratory JP-4 Fuel/Water Cultures 3 through 9

Culture	Agitation	Total Observation Time in Months	Fuel JP-4 Source	Water Used	¹ High Count	¹ Low Count	24 hrs	1 week	1 month	2 months	5 months	Terminal Count
No. 3	+	11	Searsport	Sea H ₂ O	$\frac{10^7}{1W}$	$\frac{10^5}{4H}$	1.0×10^5	2.2×10^7	3.5×10^7	4.7×10^7	3.2×10^6	5.5×10^6
No. 4	+	8	Maytag	Sea H ₂ O	$\frac{10^7}{4th H}$	$\frac{10^2}{7th H}$	1.7×10^7	-	3.0×10^7	1.0×10^5	1.4×10^9	1.4×10^9
No. 5	+ Vigorously	4	Searsport	Sea H ₂ O	$\frac{10^7}{1W}$	$\frac{10^6}{6W}$	-	7.5×10^7	1×10^7	-	-	2.3×10^6
No. 6	+	4	None	Sea H ₂ O	$\frac{10^8}{1W}$	$\frac{10^5}{3W}$	-	8.9×10^8	1.8×10^5	-	-	3.4×10^5
No. 7	-	8	Searsport (NH ₄) ₂ SO ₄	B-H mod	$\frac{10^7}{5H}$	$\frac{10^5}{8H}$	9.6×10^6	4.6×10^6	2.2×10^6	1.1×10^6	4.8×10^7	1.7×10^6
No. 8	-	8	Searsport	B-H-I	$\frac{10^8}{24 hrs}$	$\frac{10^3}{8H}$	4.4×10^8	2.3×10^8	6.0×10^7	1.4×10^8	1.2×10^8	1.4×10^5
No. 9	-	10	Searsport	B-H-I	$\frac{10^7}{5H}$	10^4	7.9×10^3	4.1×10^4	4.8×10^4	3.5×10^5	5.3×10^7	3.3×10^5

¹ Date shows count/ml over time of analysis in weeks.

Table VIII. Viable Microbial Count of Laboratory JP-4 Fuel/Water Cultures 3 through 9.

Table IX

Viable Microbial Count of Laboratory Culture 1

Date of Sampling	Fuel Phase Cells/ml	Water Phase Cells/ml
10-3-63	6.67×10^0	$>2.00 \times 10^3$
10-4-63	0	-
	+ Stopped agitation +	
10-7-63	0	6.43×10^8
10-8-63	0	7.65×10^8
10-9-63	0	1.85×10^9
	+ Pellicle formation +	
10-14-63	0	3.07×10^8
10-16-63	0	1.46×10^9
	+ Thick Pellicle +	
10-21-63	0	1.20×10^9
10-28-63	0	4.57×10^8
11-5-63	0	2.60×10^8
11-15-63	0	2.40×10^8
12-4-63	-	1.63×10^8
12-23-63	0	1.33×10^7
12-27-63	-	$>1.00 \times 10^7$
1-31-64	-	1.80×10^8
2-10-64	-	1.25×10^7
2-13-64	-	2.50×10^7
3-5-64	0	4.85×10^8
3-26-64	-	3.00×10^8
6-7-64	-	4.90×10^7
7-9-64	-	4.30×10^7 Terminated

- indicates analysis omitted

Table IX. Viable Microbial Count of Laboratory Culture No. 1

This reaction occurred several times. Eventually only small islands of the surface pellicle were formed. The island matting displayed the same cohesiveness as did the over-all matting. Microscopic examinations of the matting of Culture No. 1 showed rods of varying sizes with a large rod as the predominating type. The matting formed in the glass column was different from that of Culture No. 1 in that it was not as thick nor as cohesive, and was easily dispersed. The matting of microbial cells at the fuel/water interface was not considered a result of microbial cell stratification but was considered as a normal characteristic of growth for these cells. It is suggested that the growth of particular species of microbes would occur at the interface regardless of the overlaying medium (air included). Therefore, it is possible that these types of microorganisms may have been responsible for the interface surface matting described in early literature.

The results obtained from Laboratory Cultures 2a and 2b, concerning the effects of internal and external environmental factors on microbial growth rates, are shown in Table X. The counts obtained from the steel drum water-bottom remained approximately at 10^6 cells/ml up to the 22nd week of sampling, at which time the ambient temperature changed to below freezing. The counts of the aluminum drum during the same interim of time were intermittent, fluctuating from 10^5 to 10^7 and finally less than 10 cells/ml. Thus, as indicated by the data, the environment of the steel drum water-bottom composed of water collected in a rusty container was more favorable to the survivability of microbial cells than was the aluminum drum water-bottom environment. The differences of growth rate noted may be attributed to growth promoting or maintaining material present in water containing rust particles. Conversely, it is doubtful that microbial growth inhibitory substances were present in the water-bottom of the aluminum drum. It is also possible that the differences in cell counts between the two drums may be the result of the difference in heat adsorption of the drum material. As the ambient temperature decreased, the number of viable cells present in both systems diminished, until finally, at the 43rd week of observation, cell counts of both drums dropped to zero and remained there until the termination of the experiment. Thus, the observed correlation of microbial concentration with ambient temperatures is in agreement with the observations of higher microbial activity in JP-4 fuel systems located in warmer climates.

The significance of this investigation was the lack of recovery of microbial cells in the fuel phase either prior to or after the freezing of the water-bottoms.

The data from the studies concerning stratification of microbial cells in JP-fuel/water systems and optimal depths at which JP-4 fuel/water samples can be drawn for maximum recovery of viable microbial cells indicated (1) that stratification of viable microbial cells did not occur in the water-phase or in the quiescent fuel-phase; (2) that fuels used in the study did not support or harbor the viable microbial cells that were inoculated into the system;

Table X

Viable Microbial Count of Laboratory Cultures 2a and 2b

Sampling time 1963-64	Steel Drum		Aluminum Drum	
	Fuel- Cells/ml	Water- Cells/ml	Fuel-Cells/ml	Water -Cells/ml
Aug 6	0	3.0×10^5	$>1.0 \times 10^1$	2.5×10^7
Aug 12	0	1.5×10^5	0	1.9×10^6
Aug 16	B-H-I added			
Sept 9	0	3.2×10^6	0	1.5×10^5
Oct 4	0	7.7×10^5	0	6.5×10^7
Oct 11	0	3.8×10^6	0	4.6×10^6
Oct 18	0	1.5×10^6	0	1.0×10^5
Oct 25	0	5.8×10^6	0	2.0×10^5
Nov 1	0	2.4×10^6	0	6.2×10^4
Nov 8	0	2.1×10^6	0	0
Nov 19	0	1.6×10^6	0	0
Dec 4	0	3.5×10^6	0	$<3.0 \times 10^0$
*				
Jan 20	0	$<1.0 \times 10^1$	0	$<1.0 \times 10^1$
Feb 2	-	$<1.0 \times 10^1$	-	$<1.0 \times 10^1$
Feb 12	-	$<1.0 \times 10^1$	-	$<1.0 \times 10^1$
April 6	-	0	-	0
April 24	-	0	-	0
April 29	-	0	-	0
May 8	-	0	-	0
May 15	-	0	-	0
May 20	-	0	-	0
May 21	0	0	0	0
**				
May 21	0	0	0	0

- indicates analysis was omitted

* change in climatic environment

** after agitation and termination of study

Table X. Viable Microbial Count of Laboratory Cultures 2a and 2b.

and (3) that under the test procedures used, microbial cells did not migrate from the water phase into the fuel phase. Thus, because of the absence of microbial cell stratification in the water phase, the sampling of the water phase at any depth should be adequate to indicate the degree of microbiological contamination present in the JP-4 fuel/water system.

As is indicated in Table XI viable microbial cells were recovered from the fuel phase of Study B for a period of 2 weeks. However, the presence of the cells was not as a result of microbial growth supportability of the fuel. If the fuel was able to support the growth of viable microorganisms, cell counts would have been obtained at later dates from the system as well as from the fuel of Study C. Possible explanations for the counts are as follows: During the construction of the system, the water-bottom was added via the top vent; thus the area to be occupied by fuel was subjected to microbially contaminated water. Since the inner surface of the container was not smooth and the Buna-N port coverings protruded into the inner diameter of the container, sites were available to which small amounts of water could adhere. Although the water-bottom counts in Study B (Table XI) in the 3rd and 4th week (Port No. 3) and of Study C (Table XI) on the 1st and 2nd week (Ports No. 3 and 4, located at the fuel/water interface) appear to be a form of cell stratification, it is not considered as such due to the short duration (compared to the total period of observation) at which it is observed.

Of importance from each part of the distribution study was the finding that microbial activity was absent in the JP-4 fuel phase of all laboratory JP-4 fuel/water systems unless the system had been agitated or handled in such a manner to contaminate the fuel with water containing viable microbial cells. Thus, the results from the previous studies and this study suggested that the recovery of viable microbial cells from the fuel phase in field systems resulted from a sample obtained from an agitated system. As was indicated by the results, recovery of viable microorganisms from fuel was dependent upon the presence of water. The presence of the water in turn was dependent upon the handling of the fuel prior to sampling. Water is dispersed into the fuel phase by agitation or by the vortex action created during filling or removal of JP-fuel from its storage facility. Sufficient settling time of JP fuels is necessary for valid microbial determinations.

The results of these studies are in agreement with the findings obtained from on-site fuel analysis of field samples as well as routine laboratory analysis of fuel. The implications of these studies is discussed in detail in Section IV.

CONCLUSIONS

1. Viable microorganisms do not stratify in fuel or water of a JP-4 fuel/water system with the exception of pellicle formers.

Table XI
 Counts of Viable Microbial Cells/ml of Stratification Columns

System	Sampling Port No.	Initial Water-Bottom Population	Time						
			24 hrs	1 week	2 weeks	3 weeks	4 weeks	2 months	3 months
Study B Aluminum Column	8		0	0	$<1.0 \times 10^1$	0	0	0	0
	7		$<0.3 \times 10^1$	$<1.0 \times 10^1$	$<0.3 \times 10^1$	0	0	0	0
	6		0	$<0.7 \times 10^1$	6.7×10^2	0	0	0	0
	5		0	0	6.7×10^1	0	0	0	0
	Fuel †	4		0	0	2.3×10^1	0	0	0
Water †	3	} 6.5×10^5	7.7×10^3	2.0×10^6	2.3×10^5	6.8×10^4	1.7×10^5	8.6×10^4	4.6×10^4
	2		9.0×10^2	2.1×10^6	2.7×10^5	1.5×10^5	1.9×10^5	1.1×10^5	4.1×10^4
	1		1.3×10^3	1.0×10^6	4.7×10^5	1.8×10^5	1.9×10^5	1.3×10^5	5.7×10^4
Study C Glass Column	11		-	-	0	-	-	0	EGME-Glycerol Study Initiated
	10		-	-	-	-	-	0	
	9		-	-	0	-	-	-	
	8		-	-	-	-	-	-	
	7		-	0	0	0	-	-	
	6		-	0	-	0	-	0	
	5		-	0	0	0	-	0	
Fuel †	4		0	0 *	1.1×10^7	3.0×10^6	-	4.1×10^3	
Water †	3	} 2.6×10^5	5.3×10^5	1.3×10^7	3.4×10^6	2.8×10^6	-	3.7×10^6	
	2		4.1×10^5	6.7×10^6	2.9×10^6	4.2×10^6	-	2.3×10^6	
	1		4.7×10^5	5.2×10^6	1.7×10^6	3.9×10^6	-	4.3×10^6	

Study A not included due to lack of comparative data.

- indicates analysis omitted

Time in days and months are approximate

* additional B-H-1 added, raising interface between parts 4 and 5.

Table XI. Counts of Viable Microbial Cells/ml of Stratification Columns.

2. Water is needed for microbial growth in JP-4 fuel/water systems.

3. The use of simulated field water-bottoms does not give maximum yield of viable microbial cells.

4. Viable microbial cells do not migrate (as described by the conditions of this study) measurable distances from the water-phase to the fuel phase.

Section IV

TOXICITY OF JP-4 FUELS TO VIABLE MICROBIAL CELLS

The data presented in the previous sections indicated that viable microorganisms were rarely associated with the fuel phase in JP-4 fuel/water systems, but were generally confined to the water-bottom; however, some instances of recovery of viable microbial cells from fuels have been reported in literature. DeGray and Killian¹⁵ discussed recovery of microbial cells from diesel fuels; an interim report by Sohio²⁹ discussed recovery of viable microbial cells from jet fuels collected 6 months prior to analysis; and Swatek, Omieczvnski, and Digman³¹ reported finding viable cells in shipped turbine fuels.

The sporadic occurrence of low levels of viable cells from laboratory and field JP-4 fuels reported herein suggested the possibility of (1) inadvertent contamination due to sampling procedures, (2) plating-out of the cells on the walls of the analyzing containers, (3) suspension in the fuels of microdrops of contaminated water, or (4) an inherent toxicity of the fuel constituents to viable microbes. Since JP-4 fuel is a mixture of gasoline and kerosene fractions which have solvent characteristics, it would be expected that there would be some effect on biological material suspended in them, particularly if the material (in this case viable microbial cells) were not protected by sufficient quantities of water. Therefore, to evaluate the proposed hypothesis, laboratory studies were designed to determine the following: (1) The effect of agitation of a microbially contaminated JP-4 fuel/water system on the occurrence of viable microorganisms in the fuel phase; (2) Survivability of cells in (a) different grades of JP-fuels, (b) JP-4 fuels obtained from different refineries, and (c) JP-4 fuels from microbially contaminated fuel/water systems; (3) the effect of sterilization on the JP-4 fuels used as diluents for microbial analysis; (4) if the microbes from fuels contaminated with water were attributable to plating-out of the cells on the walls of the test containers.

To ascertain if the recovery of microbes from JP-4 fuel was due to the presence of droplets of water suspended in the fuel, the period of time of the dispersion and/or suspension of microbially contaminated droplets of water in a fuel phase was measured.

Various methods were used to attempt to suspend microbial cells in JP-4 fuel. The methods utilized included: (1) the suspension of microbial cells in water droplets in JP-fuels; (2) seeding of JP-4 fuel with fungal material followed by a bacterial inoculum; (3) the addition to water-free JP-4 fuel of (a) intact microbial colonies recovered from JP-4 fuel systems,

and (b) the sediment collected by centrifugation of a microbially contaminated JP-fuel/water-bottom; and (4) the suspension of cells in JP-4 fuels by vigorous agitation of microbial contaminants in minimal quantities of TSB, buffered water (ph 7.2), B-H-1 solution, or aircraft sump water.

To determine if fuel additives contributed to the suspension of viable microbial cells in the fuel phase, the addition of surfactants (Tween-80 and Triton X-100) to the JP-4 fuel was also investigated.

METHODS AND PROCEDURES

Microbiological Analyses

The total number of viable aerobic microorganisms present in the contaminated JP-fuels was determined as described in the previous section. Fuels were analyzed undiluted and in 10-fold dilutions (up to 1:10,000) in JP-fuels of the same composition. Aliquots of 0.1 or 1.0 ml of all dilutions were plated in triplicate on TSB with 1.5% Bacto-agar. The fuel was dispersed over the growth medium with the aid of an L-rod or by gingerly rotating the plate from side to side. The plates were incubated at 35°C for 48 hours. The number of viable microbial cells per/ml present in the system at the time of sampling was determined by multiplying the average number of colonies present on the plates exhibiting between 30 and 300 colonies by the appropriate dilution factor.

The number of viable aerobic microbial cells/ml in the water-bottoms of systems employed in this program was determined by serially diluting the water-bottoms into TSB and applying the same procedure as discussed above.

Agitation Studies

Culture No. 5. Laboratory Culture No. 5 system consisted of a non-sterile glass tissue culture jar (Figure 5) containing 400 ml of non-sterile Searsport JP-4 fuel, 160 ml of boiled tap water, 160 ml of distilled water, 80 ml of organic free (cartridge filtration, Bantam Demineraliser Model BD-1, 10 gph) sea water, 8 ml of a composite of microorganisms from the Laboratory Culture (Section III), and 1 ml of API (Difco) medium containing sulfate-reducing microorganisms.

The fuel/water system was agitated by a propeller stirrer that was activated by Mag-Mix magnastir, causing a vortex at the fuel/water interface and a vigorous mixing of the JP-4 fuel and water-bottom.

Laboratory Culture No. 3. Laboratory Culture No. 3 system had been initiated 2-1/2 months prior to the study. The system consisted of a 5-gallon clear glass carboy (Figure 6) with a Buna-N gasket neck seal and a cork plug in the sampling port, and contained 5 L of unsterilized Searsport JP-4 fuel, 2 L of

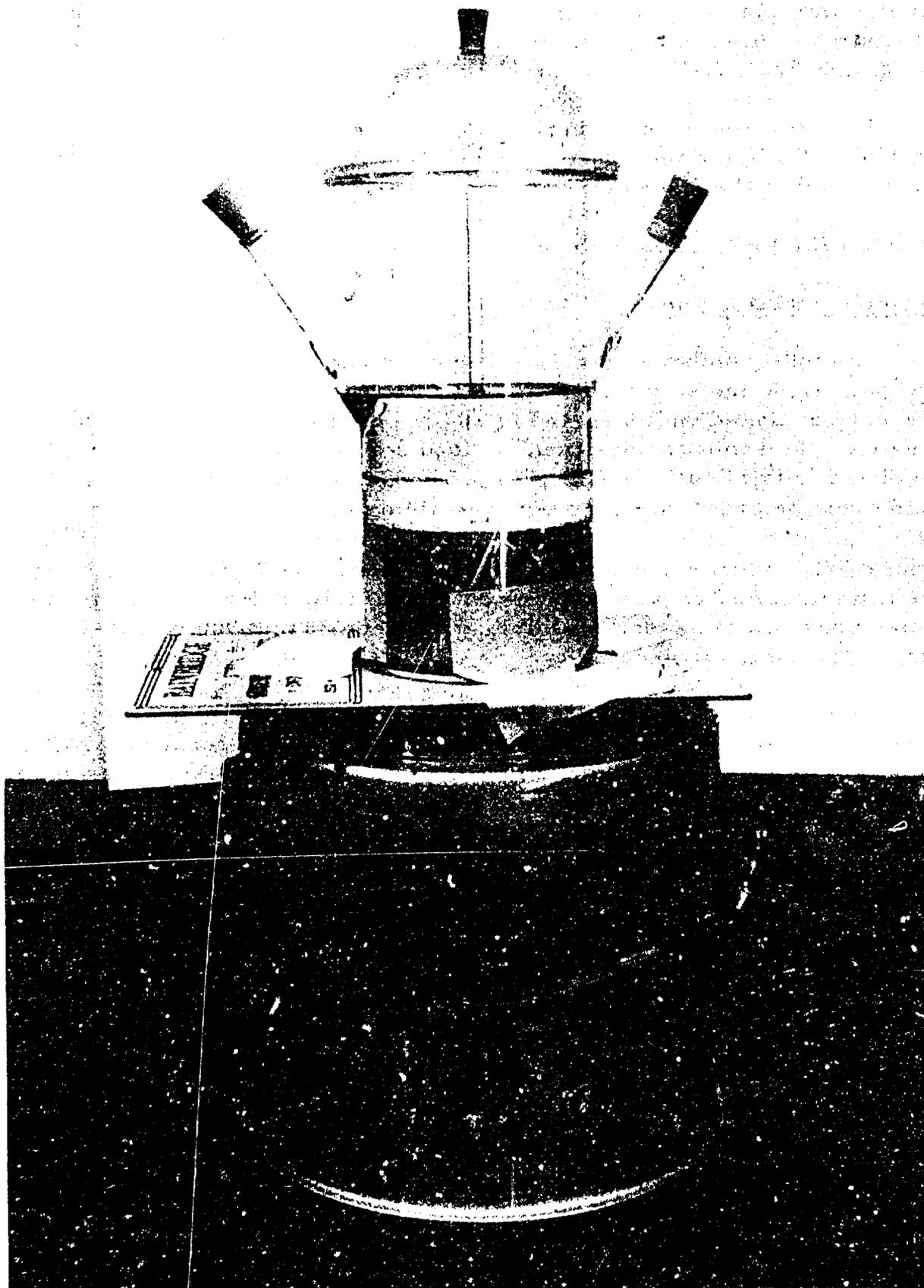


Figure 5. Laboratory Culture No. 5

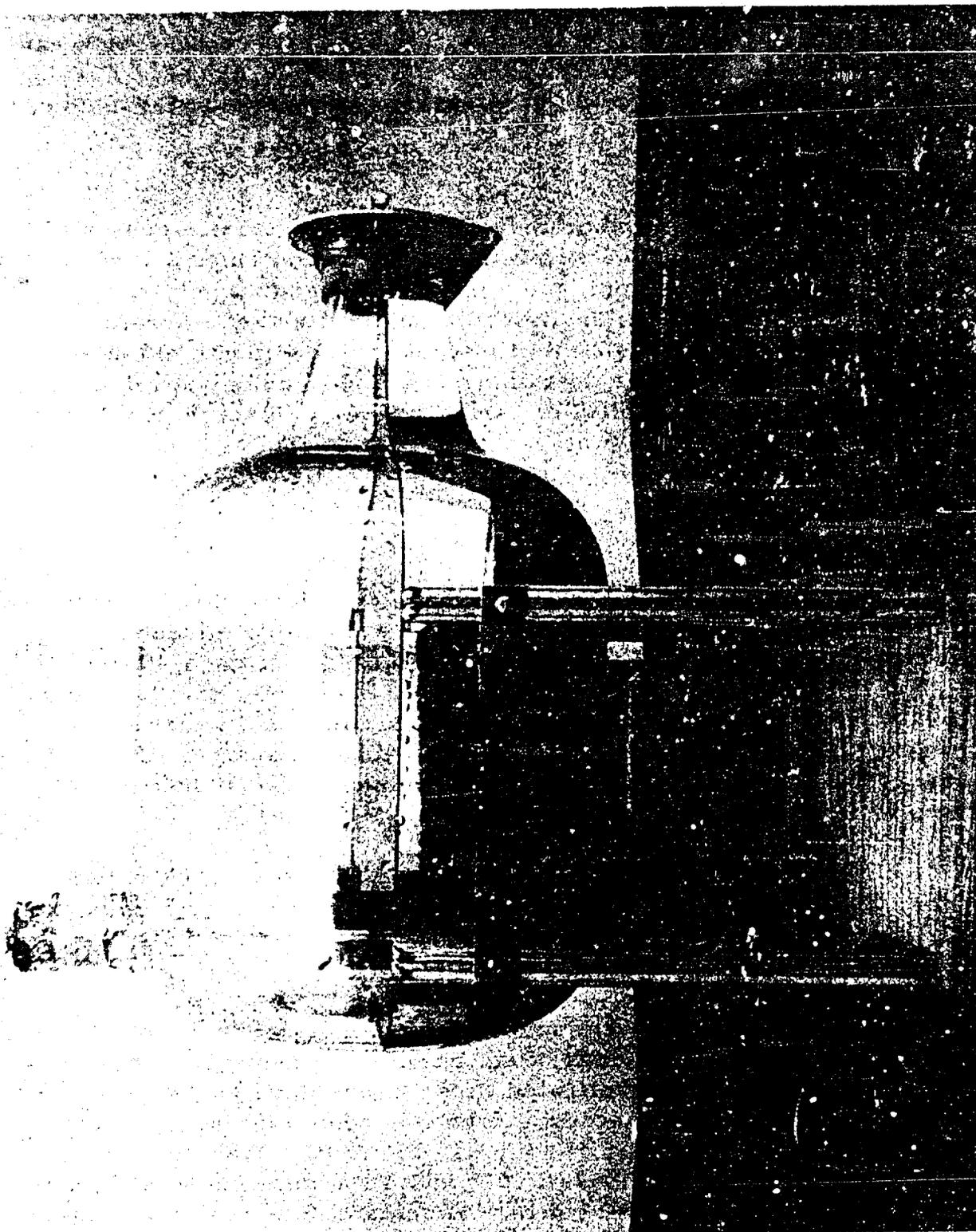


Figure 6. Laboratory Culture No. 3

boiled tap water, 2 L of distilled water, 1 L of autoclaved sea water, 100 ml of water-bottom from the Laboratory Supply Culture (Section III), and 9.5 ml of API medium inoculated with sulfate-reducing microorganisms. All fluids were added via the port, therefore excessive mixing of the fuel, microbial inoculum, and water occurred. The water-bottom was circulated by means of a Magnastir (Harshaw magnastir, Harshaw Scientific Company, Catalog #H60060) sufficiently to cause a slight movement of only the water-bottom.

Diluent Studies

Laboratory Culture No. 8. Laboratory Culture No. 8 system consisted of a 1-gallon clear glass jug with a screw-cap lid, and contained 2 L of JP-4 fuel, 2.5 L of B-H water-bottom, and a microbial inoculum consisting of (1) 2 ml of water-bottom containing microorganisms of the Laboratory Supply Culture (Section III), (2) 2 ml of microbes of a 4 month old shipped JP-4 fuel/water-bottom sample containing 21% EGME and glycerol and having a cell count of 5.0×10^2 cells/ml, and (3) several intact colonial forms of the 4 month shipped JP-fuel/water-bottom sample isolated on TSB agar plates.

Test Procedures

Although the counts/ml of the fuel phase of Culture No. 5 during agitation were erratic, a base line of the number of viable microbial cells in the fuel phase was established prior to the initiation of the agitation study. The stirrer was stopped for a period of 144 hours, and 0.1 aliquots of the fuel were plated at designated times on triplicate TSB agar plates to determine the presence of viable cells. With each fuel analysis the microbial count of the water-bottom was also determined. Two additional 144 hour tests were made of Culture No. 5.

To observe similar results in another laboratory culture system, Laboratory Culture No. 3 was manually shaken for a period of 5 minutes, 0.1 ml aliquots of the fuel were plated on triplicate plates of TSB agar, and all inoculated plates incubated for 48 hours at 35°C.

Fuel to Fuel Dilution. Ten ml aliquots of Searsport JP-4 fuel from Laboratory Culture No. 8 (after 5 minutes of agitation of the system) containing a suspension of microbially contaminated water-drops were added to 160 ml clear glass milk dilution (MD) screw cap bottles containing 90 ml of autoclaved and/or filtered Searsport JP-4 fuel. The bottles were vigorously shaken and two additional dilutions of 10^{-2} and 10^{-3} were made (1.0 ml to 99 ml of diluent fuel) of each MD bottle. Aliquots of 0.1 ml of the diluted JP-4 fuel were taken immediately from each dilution bottle and plated in triplicate on glass Petri plates containing 20 ml of TSB agar. Prior to incubation of the plates at 35°C for 48 hours, all inoculated plates remained

at room temperature for approximately 1 hour to allow for evaporation of JP-fuel vapors. After incubation the viable count was determined as discussed previously.

Grades of JP-fuel. Aliquots of 10 ml of Searsport JP-4 fuel (after 5 minutes of agitation of the system) from Laboratory Culture No. 8 with a suspension of microbially contaminated water-drops were added to sterile MD bottles containing 90 ml of autoclaved or filtered JP-6, XJP-4, and Searsport JP-4 fuel (control). The MD bottles were vigorously shaken for 1 minute every 15 minutes for 1 hour. After each shaking, 1.0 ml aliquot samples were plated to triplicate glass Petri dishes containing 20 ml of TSB (BBL) agar. The plates remained at room temperature for approximately 1 hour and were then incubated at 35°C for 48 hours.

Time and Sterilization Fuel Studies. Ten ml aliquots of the water-bottom of Laboratory Culture No. 8 were added to 18 (3 each/fuel/method of fuel sterilization) sterile MD bottles containing 90 ml of autoclaved and/or filtered JP-6, XJP-4 and Searsport JP-4 fuel. A control MD bottle of 90 ml of the overlaying JP-4 fuel of Culture No. 8 was siphoned into a sterile MD bottle prior to the initiation of the study. The sterility of the siphoned control fuel and the quantity of micro-contamination of the water-bottom of Culture No. 8 was determined. Each MD bottle was vigorously shaken for 1 minute every 15 minutes for 1 hour. Immediately after each shaking, 1.0 and 0.1 ml aliquots of fuel of each bottle were plated in triplicate on TSB agar. One ml fuel aliquots were plated in glass Petri dishes and 0.1 ml fuel aliquots were plated in plastic Petri dishes.

Plating-Out Study. At the end of 1 hour after agitation and analysis, the JP-fuel of the 1st set of MD bottles in the test above was decanted to a second set of sterile MD bottles. In 15 minutes the second set of MD bottles was shaken and the fuel was analyzed and decanted into a third set of MD bottles. This procedure was followed every 15 minutes for 1 hour. To the second MD bottle and each decanted MD bottle thereafter, 10 ml of TSB were added. The bottles were rotated to facilitate the rinsing of any microbial cells from the walls of the MD containers. The MD bottles were incubated at 35° C for 48 hours.

The same procedure as listed in the paragraph above was repeated at a later date with the exception that TSB with 2.0% Bacto-agar was solidified on the sides and bottom of the second, third, etc., MD bottles.

Cell Suspension Tests

The contamination of JP-fuels by the suspension of droplets of microbially contaminated water has been discussed as pertained to the previous tests, as was the primary procedure utilized to contaminate all JP-fuels for microbial contamination determinations.

To a sterile 8 ounce prescription bottle containing 50 ml of non-sterile Searsport JP-4 fuel were added several loops of surface growth of fungal material from Sabouraud agar medium. (Caution was taken to include only surface growth.) Following a storage period of 10 days in the dark of the test container, several loops of surface growth of bacterial colonies from TSB agar plates were added. The viability of both the fungal and bacterial inoculum was determined at time of inoculation by streaking the inoculant material to Sabouraud and TSB agar plates. The test container was vigorously shaken to disperse the bacterial material and observed for visible growth for a period of 60 days. The fuel was analyzed periodically for microbial growth.

To an 8 ounce sterile prescription bottle containing 50 ml of non-sterile Searsport JP-4 fuel showing no visible free water was added intact microbial colonies recovered from contaminated field JP-fuel samples on TSB agar plates. The test container was stored in the dark and observed for a period of 60 days for visible growth. The fuel was analyzed periodically for bacterial activity.

To a duplicate set (A and B) of triplicate 50 ml screw capped tubes containing 30 ml of filter sterilized Searsport JP-4 fuel was added a sediment composite of microorganisms. The sedimented cells for set A were obtained by centrifuging 30 ml (3 tubes of 10 ml/each centrifuge tube) of Laboratory Culture No. 1 and discarding the supernate. Set B cells were obtained as were set A cells with the exception of re-suspending the cells in a phosphate buffered water and recentrifuging. After the sediment of cells were added to the 50 ml screw cap tubes, the tubes were agitated vigorously for three minutes. The test containers were placed in the dark and observed for a period of 30 days. The fuels were periodically analyzed for viable microbial cells with minimal handling to prevent any agitation.

The same procedure as described in the paragraph above was followed to suspend, in JP-4 fuels, viable microbial cells that were contained in TSB, phosphate buffered water (pH 7.2), B-H-1, and aircraft sump water. Non-sterilized Searsport JP-4 fuel (25 ml) containing varying quantities of either Tween 80, Triton X-100, Tween 80 and Triton X-100, or Triton X-100 and Antifoam B agent was dispensed in 50 ml sterile screw capped glass test tubes. Compositated, centrifuged microbial cells from Laboratory Culture No. 1 were then added to the 50 ml test tubes. The quantity of cell composite was sufficient to cover the bottom of the test tube. The tubes were vigorously shaken to disperse the cells in the fuels. The procedure was then reversed, i. e., the cells were added first, followed by the addition of the surfactant and then the JP-4 fuel. The test containers were placed in the dark at room temperature for 24 hours. The presence of viable microbial cells in the fuel and bottom material was determined by the procedure described previously.

RESULTS AND DISCUSSION

As a result of (1) the discrepancies in laboratory data of static and agitated laboratory JP-4 fuel/water systems concerning recovery of viable microbial cells from the fuel phase (Table XII); (2) the unsuccessful attempts in establishing a microbial population in a JP-4 fuel environment by any method other than the suspension of microbially contaminated water-drops through fuel/water agitation; and (3) the consistency in recovery of viable cells from the fuel phase and the appearance of water-drops in the fuel (caused by continuous agitation of the system) in Culture No. 5 (Table XII); the hypothesis was formed that JP-4 fuels were microbially contaminated by the suspension or dispersion of microbiologically contaminated water-drops in the fuel phase. In an attempt to substantiate this hypothesis, the agitation of Culture No. 5 was stopped and the number of viable microbial cells/ml versus time was determined. As shown in Table XIII, the count/ml decreased irregularly with time. Additional agitation and static time studies of Cultures No. 5 and 3 (Table XIII and Figures 7 and 8) indicated similar results.

Initially the decrease and erratic cell counts were attributed to the size of the water-drops obtained with the sampled fuel. However, discrepancies between fuel phase and water-bottom count/ml indicated possible plating-out of the cells on the walls of the diluting containers or a toxic effect of the JP-4 fuel to viable microbial cells. The absence of microbial growth in TSB medium obtained from washings of empty fuel to fuel dilution bottles, and the lack of microbial colonies on thin-coatings of TSB agar affixed to the walls of diluting bottles, indicated that the decrease of cell counts from a fuel phase resulted from some factor other than plating-out of the cells.

Due to the inconsistency in the number of viable cells/ml recovered in fuel-to-fuel dilutions of one dilution (Table XIV) or in a dilution series (Table XV and Figure 9), it was suspected that JP-4 fuel was exerting a biocidal effect on the microbial cells. In an attempt to verify these results, the method of sterilization of the fuel was investigated. Dilution studies were conducted with both filtered and autoclaved fuel samples. As shown in Tables XV and XVI (Figures 9, 10, and 11) the results of these tests indicated that filtered fuel was somewhat less inhibitory to the survivability of microbes than was autoclaved fuel. Sample platings of a dilution series of each fuel are shown in Figures 13, 14, 15 and 16. Further testing was conducted utilizing different grades of JP-fuels, JP-4 fuels from different refineries, and JP-4 fuel from a microbially contaminated fuel/water system. As indicated in Tables XV and XVI and Figures 9, 11 and 12, of the fuels tested, JP-6 was least toxic to the microorganisms and Searsport JP-4 fuel was most toxic. However the results in general indicated that all fuels were toxic. As shown in Table XVI and Figures 10, 11, 16, and 18,

Table XII

Routine Counts of Laboratory JP-4 Fuel/Water
Systems Used in Toxicity Studies

Laboratory System	Time After Initiation of the System in Days	Viable Microbial Count/ml in Fuel	Viable Microbial Count/ml in Water
No. 3 Static System	1	0	$>1.0 \times 10^5$
	7	$>5.0 \times 10^0$	2.2×10^7
	33	$>1.0 \times 10^1$	3.5×10^7
	34	5.2×10^1	5.5×10^7
	35	1.3×10^2	-
	36	1.3×10	-
	37	0	-
	38	$>1.0 \times 10^5$	-
	40	3.3×10^0	-
	41	0	4.1×10^7
	46	1.3×10^1	-
	47	6.7×10^0	-
	50	6.3×10^1	4.7×10^7
	71	0	8.7×10^6
	80	0	2.7×10^7
86	0	5.5×10^6	
99	0	5.1×10^5	
114	0	4.0×10^6	
11 months	-	5.5×10^6	
No. 5 Agitated System	10	7.0×10^1	$>7.5 \times 10^7$
	40	-	$>1.0 \times 10^7$
	53	6.5×10^2	-
	54	9.6×10^1	-
	72	3.1×10^2	-
	74	1.5×10^1	8.9×10^6
	94	7.0×10^1	4.7×10^7
	107	2.0×10^1	6.3×10^6
143	-	2.3×10^6	
No. 8 Static System	1	0	-
	5	0	4.4×10^8
	11	0	2.6×10^8
	24	0	1.2×10^8
	52	0	6.0×10^7
	53	0	3.3×10^7
	68	0	1.1×10^8
	74	0	1.4×10^8
	106	-	3.1×10^7
	121	-	1.4×10^8
8 months	-	1.4×10^5	

indicates analysis omitted

Table XII. Routine Counts of Laboratory JP-4 Fuel/Water Systems
Used in Toxicity Studies

Table XIII

Recovery of Viable Microbial Cells/ml from JP-4 Fuel/Water Systems No. 3 and 5

System	Fuel Count Cells/ml Before	Water Count Cells/ml Before	Viable Cells/ml in Fuel After Agitation														
			0	15 min	30 min	50 min	120 min	150 min	24 hrs	48 hrs	144 hrs						
No. 3 1st test	6.3×10^1	4.7×10^7	3.4×10^2	5.7×10^1	-	2.0×10^1	-	-	7.3×10^1	-	-	7.3×10^1	-	-	-	-	
			3.0×10^2	2.8×10^1	1.0×10^1	1.0×10^1	7.3×10^1	-	-	7.3×10^1	-	-	-	-	-	-	-
No. 5 1st test motor agitation	9.7×10^1	-	-	6.7×10^1	-	4.7×10^1	-	0	-	-	7.3×10^1	-	-	-	-	-	
			-	7.0×10^2	-	9.7×10^1	6.3×10^1	2.7×10^1	6.7	-	-	-	-	-	-	-	-
			-	3.3×10^1	1.0×10^1	0	0	-	-	-	-	-	-	-	-	-	-
2nd test manual agitation	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
3rd test	-	-	4.3×10^1	-	-	-	-	-	-	-	-	-	-	-	-	-	

- indicates analysis omitted

Table XIII. Recovery of Viable Microbial Cells/ml from JP-4 Fuel/Water Systems No. 3 and No. 5.

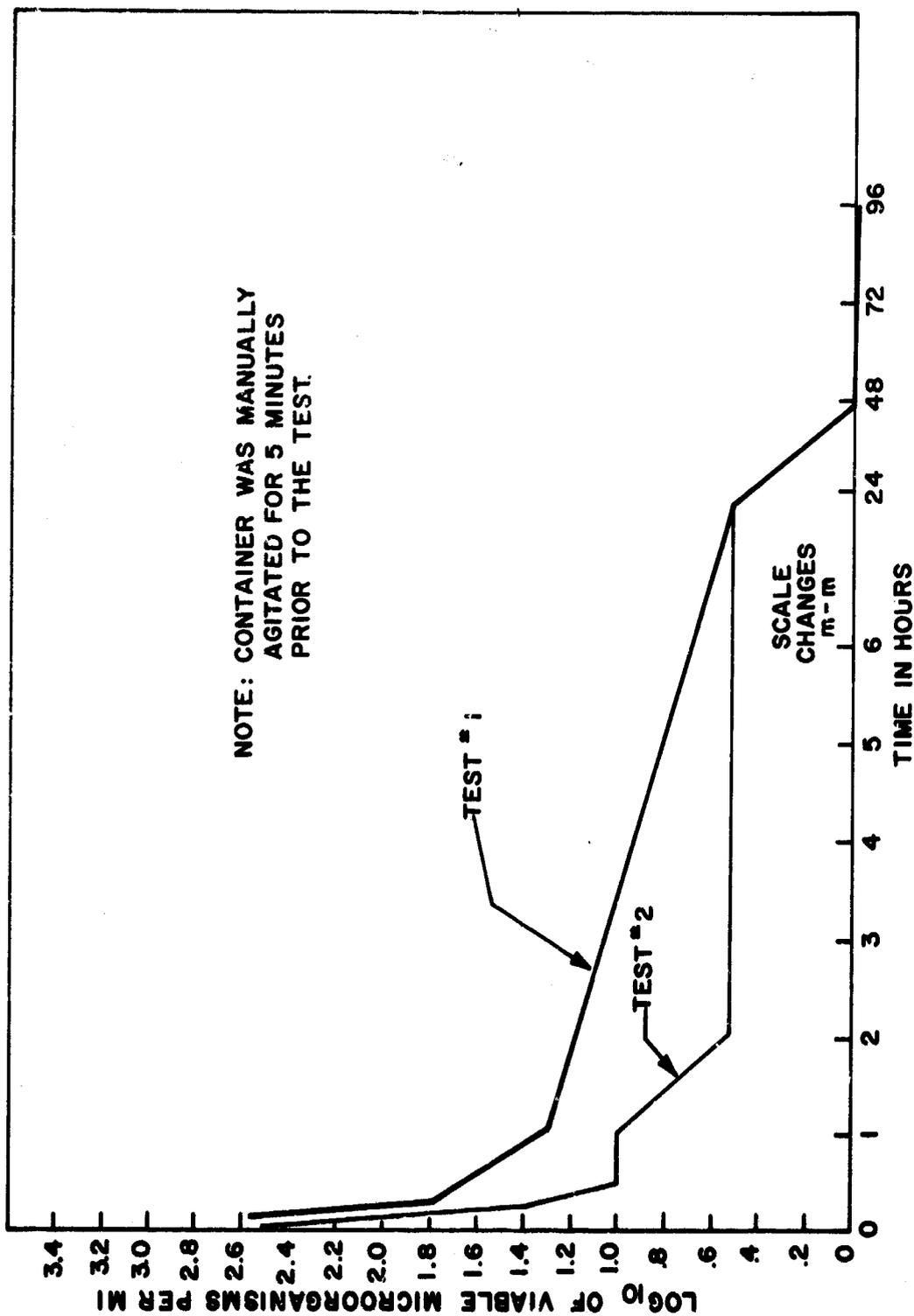


Figure 7. Survival Rate of Laboratory Culture No. 3 Microbial Cells Suspended in Water Drops in JP-4 Fuel.

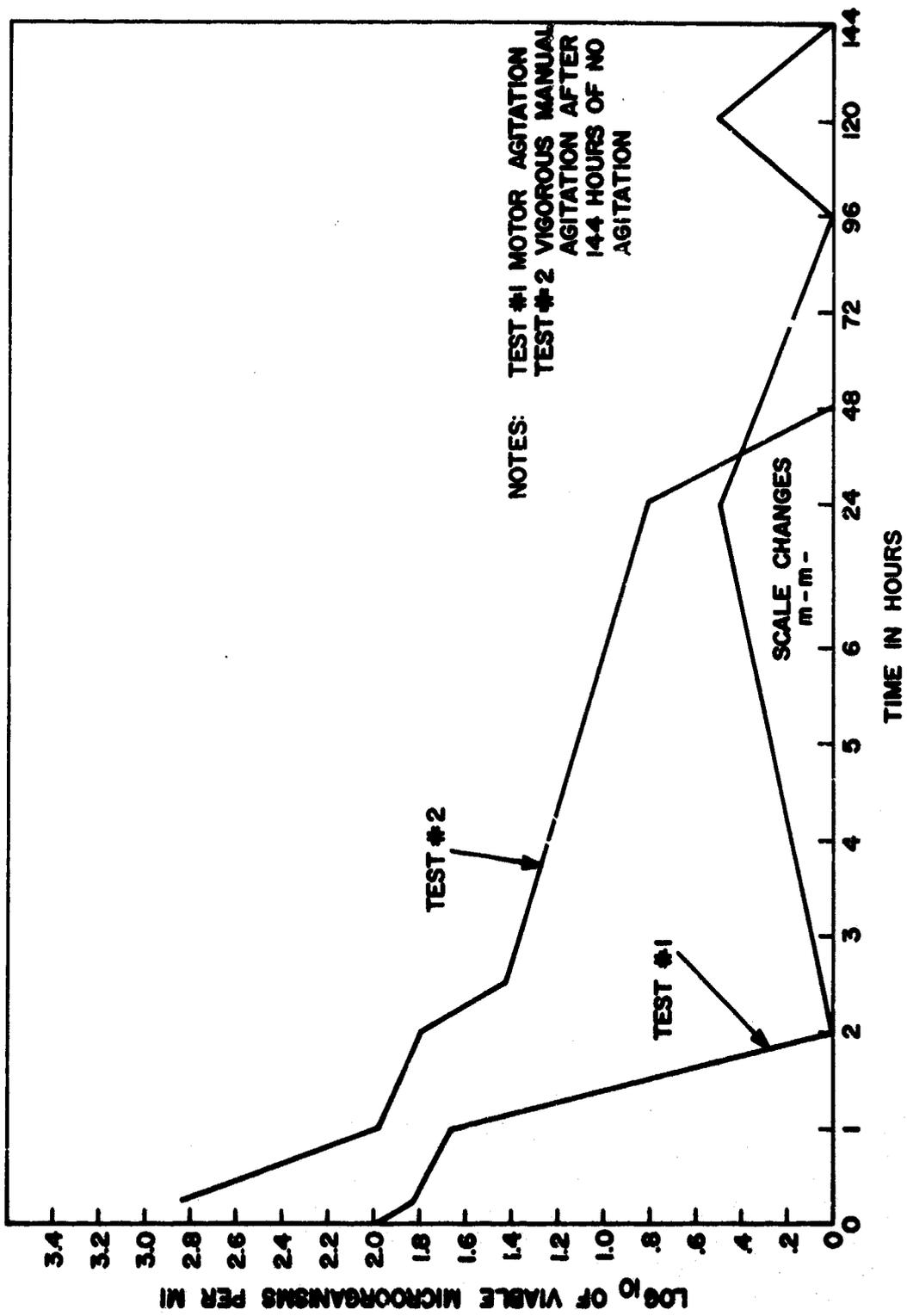


Figure 8. Survival Rate of Laboratory Culture No. 5 Microbial Cells Suspended in Water Drops in JP-4 Fuel.

Table XIV

Toxicity of JP-Fuels to Viable Microbial Cells

Laboratory Fuels	Fuel Autoclaved		Fuel Filtered		
	Time Minutes	1st Test Viable cells/ml	2nd Test Viable cells/ml	1st Test Viable cells/ml	2nd Test Viable cells/ml
XJP-4	0	TNTC	TNTC	TNTC	TNTC
	15	1.50×10^3	2.9×10^2	1.60×10^3	1.20×10^2
	30	3.00×10^2	1.7×10^2	2.80×10^2	4.50×10^1
	45	9.00×10^1	3.5×10^1	3.80×10^2	5.00×10^0
	60	-	2.0×10^1	-	3.50×10^1
Searsport JP-4	0	TNTC	2.90×10^3	TNTC	$>3.5 \times 10^3$
	15	1.60×10^3	4.00×10^1	3.33×10^3	5.00×10^0
	30	3.00×10^1	0	2.10×10^3	0
	45	5.00×10^0	5.00×10^0	9.10×10^2	1.00×10^0
	60	-	0	-	5.00×10^0
JP-6	0	TNTC	TNTC	TNTC	TNTC
	15	2.40×10^2	$>3.5 \times 10^3$	1.00×10^1	3.50×10^3
	30	5.00×10^1	1.80×10^3	0	1.10×10^3
	45	5.00×10^0	4.50×10^2	0	1.60×10^2
	60	-	5.00×10		0

Table XIV. Toxicity of JP-Fuels to Viable Microbial Cells

Table XV

Toxicity of Autoclave vs Filter Sterilized Diluent JP-Fuels to Viable Microbial Cells

Laboratory Fuels		Fuel		Autoclaved		Fuel		Filtered	
		10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	10^{-2}	10^{-3}
XJP-4	1st Test	TNTC	9.40×10^3	7.50×10^3	TNTC	TNTC	TNTC	1.34×10^4	1.15×10^4
	2nd Test	TNTC	6.65×10^3	1.50×10^3	TNTC	TNTC	TNTC	3.15×10^3	5.00×10^2
Seasport JP-4	1st Test	TNTC	1.12×10^4	1.50×10^3	TNTC	TNTC	TNTC	1.34×10^4	8.00×10^3
	2nd Test	2.93×10^2	4.00×10^0	0	TNTC	TNTC	3.50×10^2	7.50×10^0	0
JP-6	1st Test	TNTC	TNTC	2.61×10^5	TNTC	TNTC	TNTC	TNTC	2.14×10^5
	2nd Test	TNTC	1.39×10^4	9.00×10^3	TNTC	TNTC	TNTC	1.19×10^4	3.00×10^3

Table XV. Toxicity of Autoclave vs Filter Sterilized Diluent JP-Fuels to Viable Microbial Cells.

Table XVI

Toxicity of JP-Fuels to Viable Microbial Cells:
Sampling Aliquots/ Time Study

	Time Minutes	Fuel			
		1.0 ml	0.1 ml	1.0 ml	0.1 ml
Ramey JP-4	0	TNTC	8.17×10^2		
	15	1.46×10^2	4.10×10^2		
	30	1.50×10^2	3.50×10^2		
	45	1.87×10^2	3.30×10^2		
	60	1.29×10^2	4.03×10^2		
	75	9.30×10^1	1.90×10^2		
	95	1.35×10^1	4.33×10^1		
	105	1.13×10^1	2.67×10^1		
	200	5.00×10^0	3.67×10^1		
		Fuel Autoclaved		Fuel Filtered	
		1.0 ml	0.1 ml	1.0 ml	0.1 ml
X JP-4	0	TNTC	1.25×10^3	TNTC	9.77×10^3
	15	2.51×10^2	4.57×10^2	2.00×10^2	4.30×10^2
	30	1.33×10^2	2.13×10^2	1.36×10^2	2.50×10^2
	45	1.28×10^2	1.87×10^2	1.16×10^2	1.57×10^2
	60	1.23×10^2	2.33×10^2	9.00×10^1	1.10×10^2
	75	5.00×10^1	9.67×10^1	3.13×10^1	8.33×10^1
	95	4.16×10^1	7.33×10^1	2.80×10^1	6.33×10^1
	105	3.83×10^1	4.67×10^1	2.20×10^1	5.00×10^1
	200	3.30×10^1	6.00×10^1	2.40×10^1	4.33×10^1
JP-6	0	TNTC	2.00×10^3	TNTC	1.43×10^2
	15	4.00×10^2	1.34×10^3	TNTC	1.21×10^3
	30	4.00×10^2	9.30×10^2	TNTC	1.14×10^3
	45	4.00×10^2	7.35×10^2	TNTC	1.15×10^3
	60	3.44×10^2	5.60×10^2	TNTC	1.10×10^3
	75	6.23×10^1	1.30×10^2	3.00×10^2	7.20×10^2
	95	1.37×10^1	1.25×10^2	4.03×10^1	1.33×10^2
	105	9.00×10^0	6.50×10^1	1.13×10^1	7.67×10^1
	200	8.33×10^0	7.67×10^1	1.20×10^1	4.00×10^1
Searsport JP-4	0	TNTC	9.80×10^2	TNTC	7.83×10^2
	15	1.53×10^2	3.73×10^2	1.45×10^2	2.93×10^2
	30	1.47×10^2	3.20×10^2	1.50×10^2	1.33×10^2
	45	1.61×10^2	2.60×10^2	5.40×10^1	1.30×10^2
	60	2.97×10^1	1.90×10^2	8.70×10^1	1.17×10^2
	75	2.00×10^1	1.13×10^2	4.00×10^0	7.67×10^1
	95	3.00×10^1	9.00×10^1	1.00×10^0	4.00×10^1
	105	0.57×10^0	9.00×10^1	1.00×10^0	4.00×10^1
	200	5.00×10^0	8.00×10^1	1.00×10^0	3.33×10^1

Numbers in table refer to average number of cells/ml of 3 replicates.

Table XVI. Toxicity of JP-Fuels to Viable Microbial Cells:
Sampling Aliquots/Time Studies

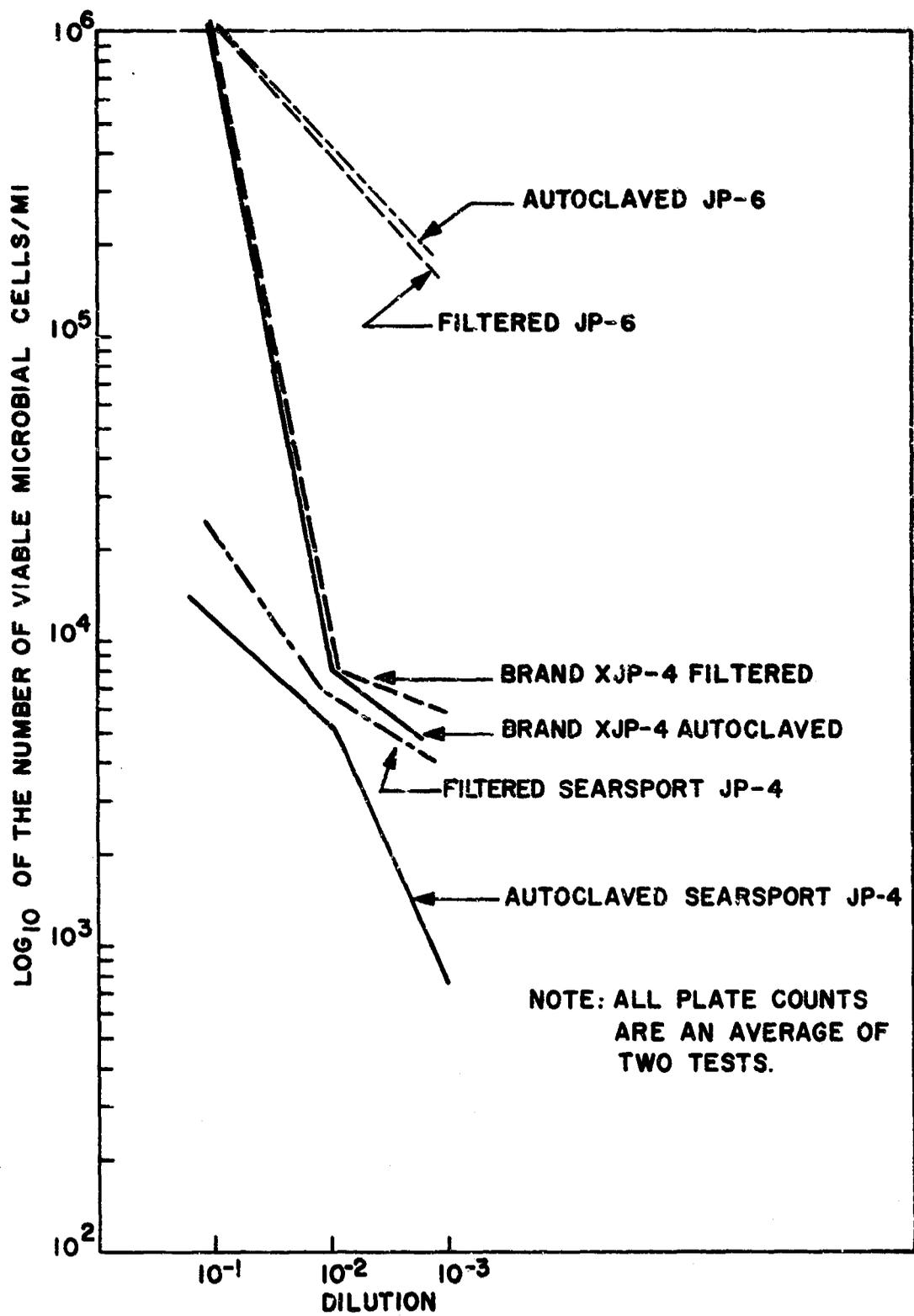


Figure 9. Dilution Study: Toxicity of JP-Fuels to Viable Microbial Cells.

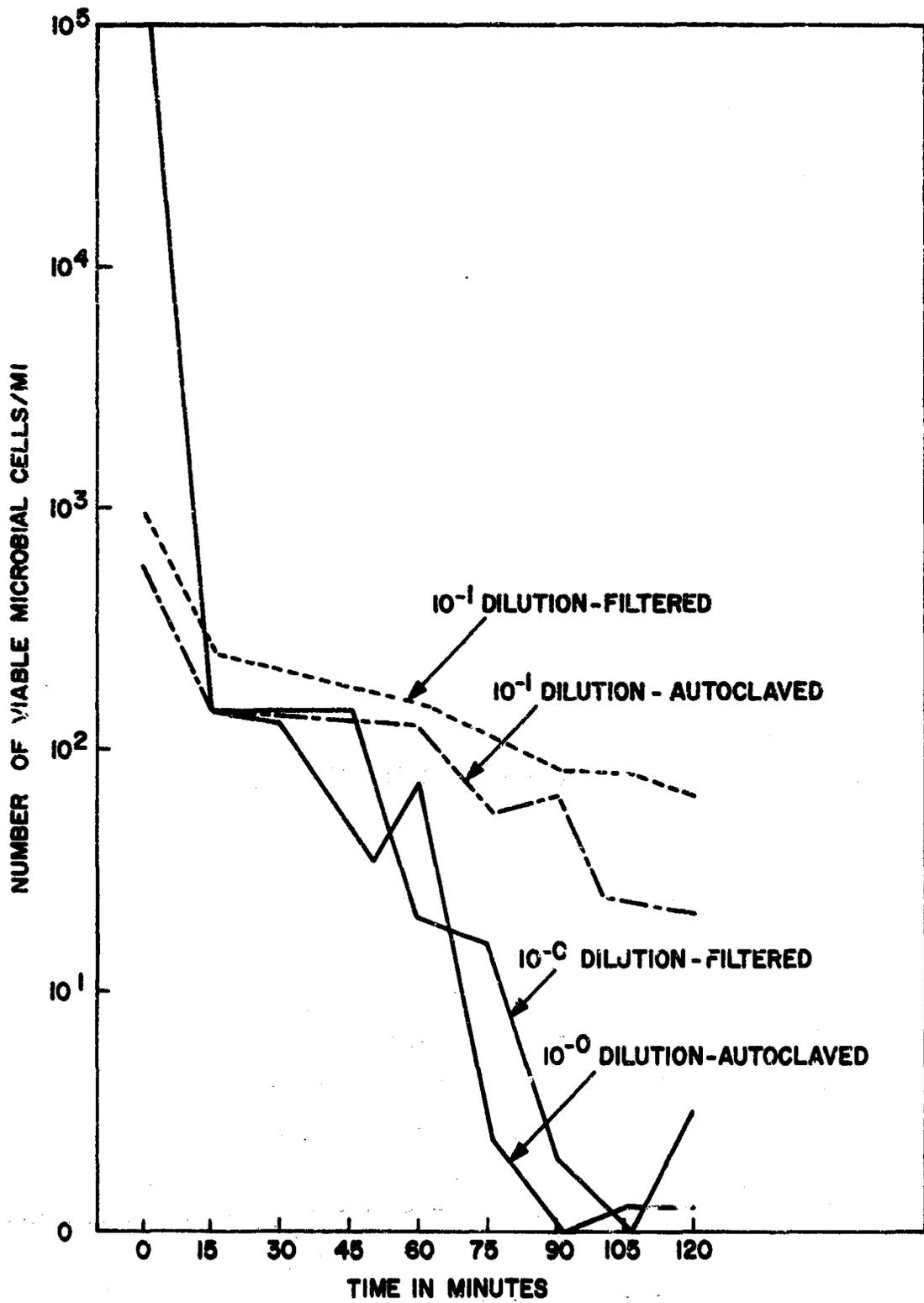


Figure 10. Toxicity of Searsport JP-4 Fuel to Viable Microbial Cells: Autoclave versus Filter Sterilization and Quantity of Fuel Plated.

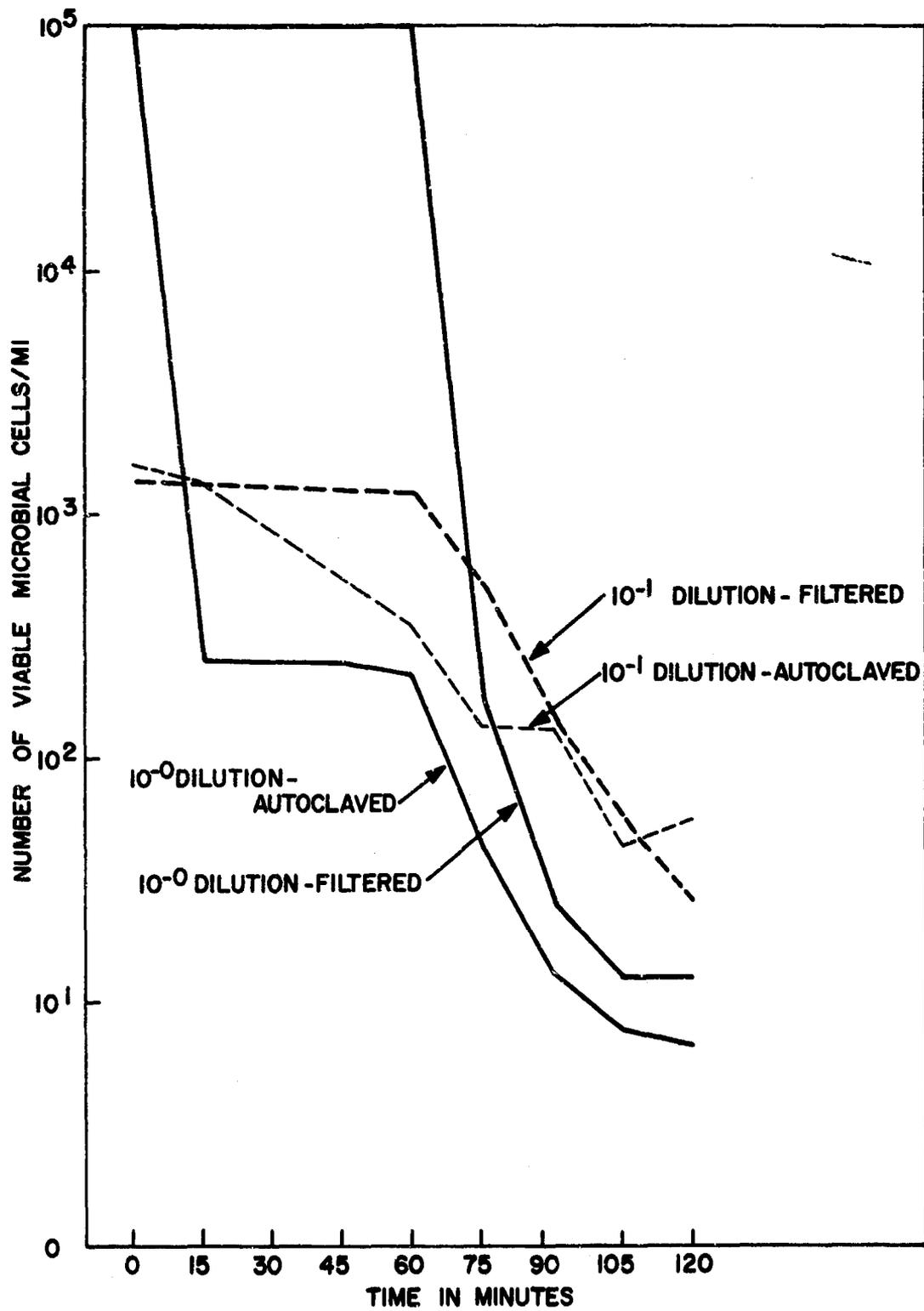


Figure 11. Toxicity of JP-6 Fuel to Viable Microbial Cells: Autoclave Versus Filter Sterilization and Quantity of Fuel Plated.

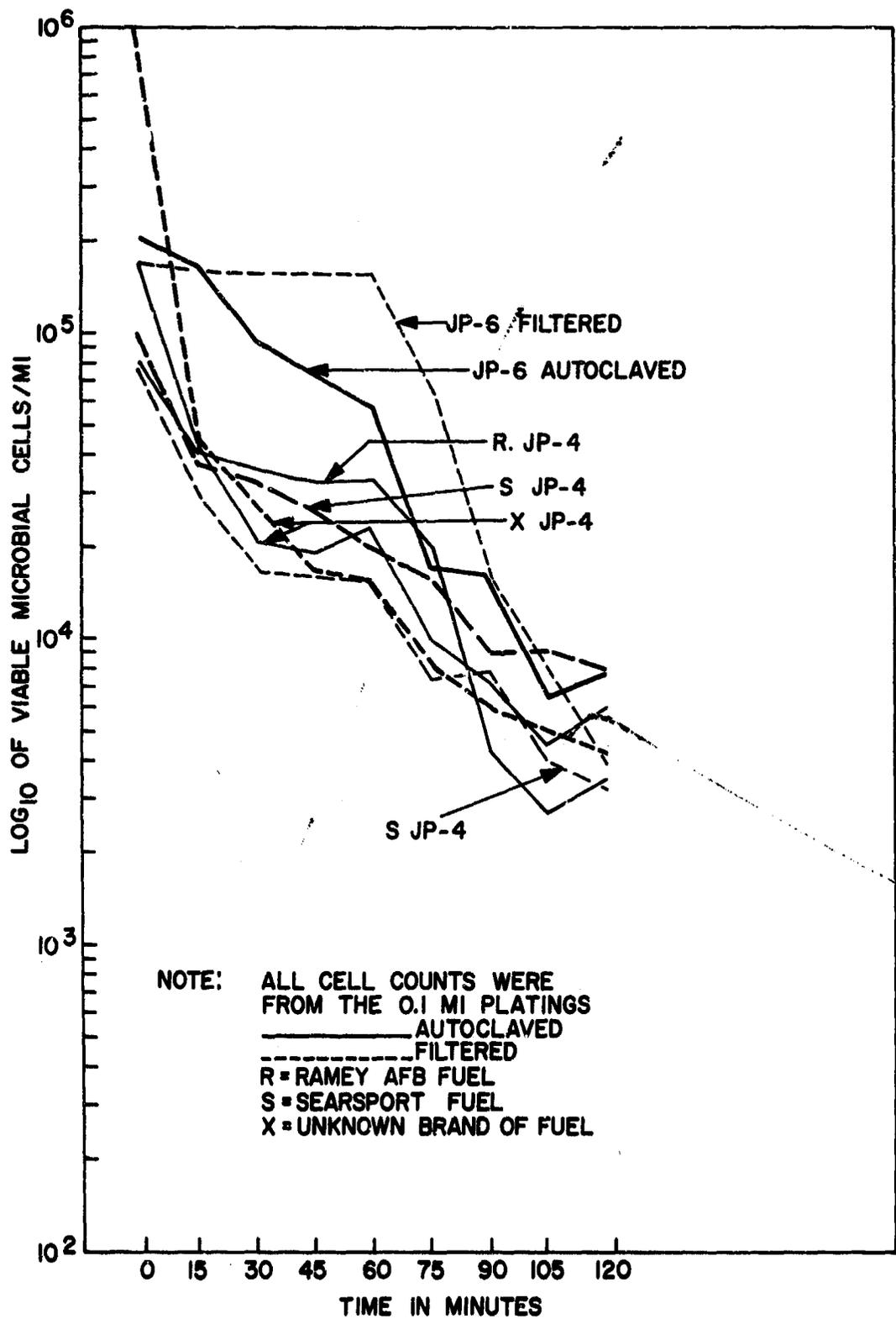


Figure 12. JP-Fuels Toxicity to Viable Microbial Cells Suspended in Microdrops of Water.

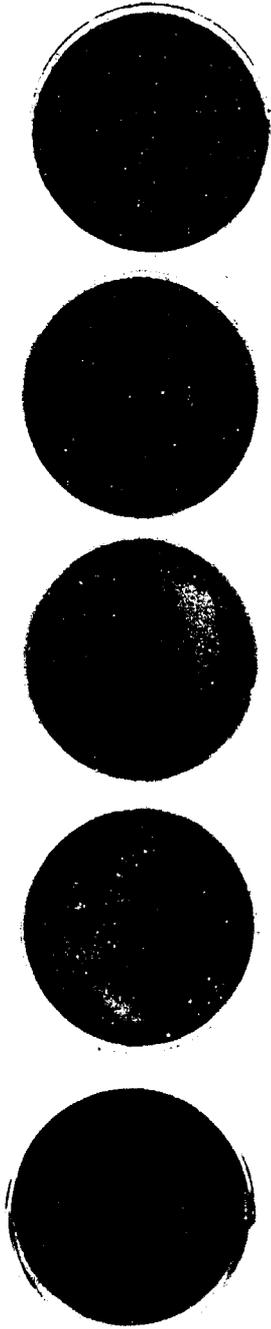
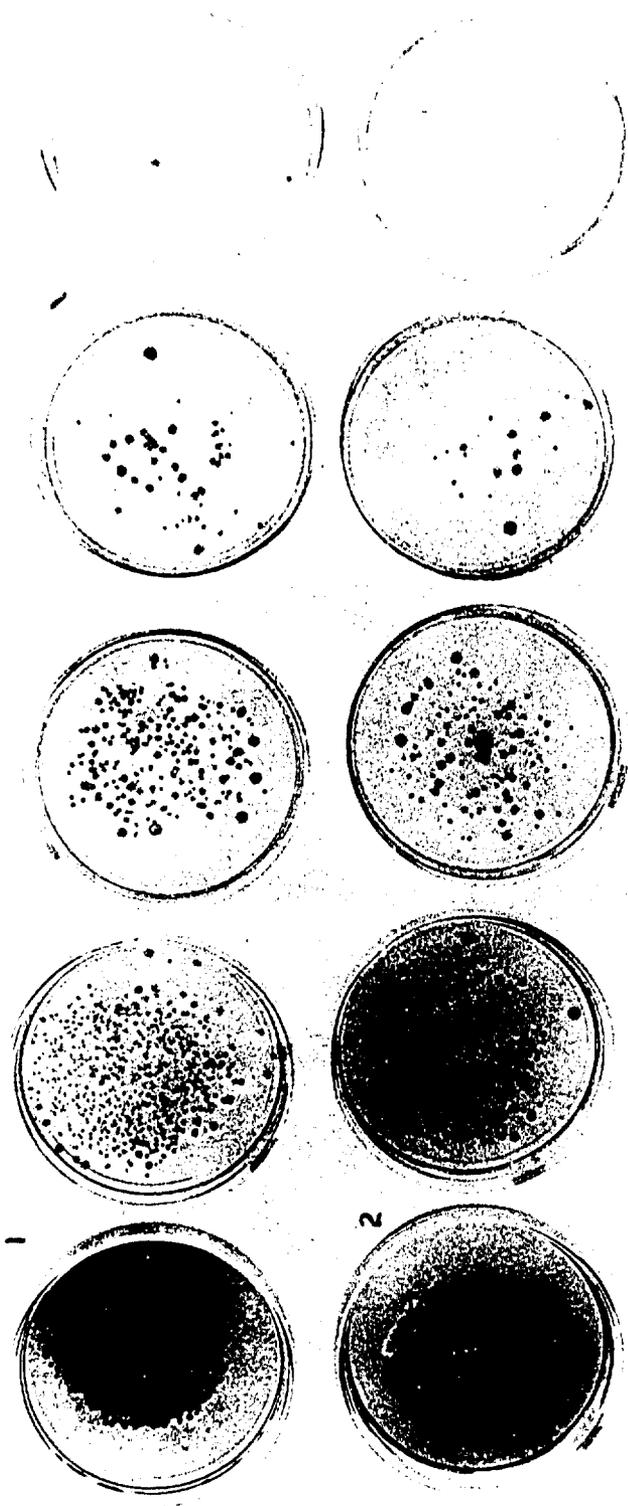


Figure 13

Figure 13. Recovery of Viable Cells from Washings of JP-4 Fuel with TSB.

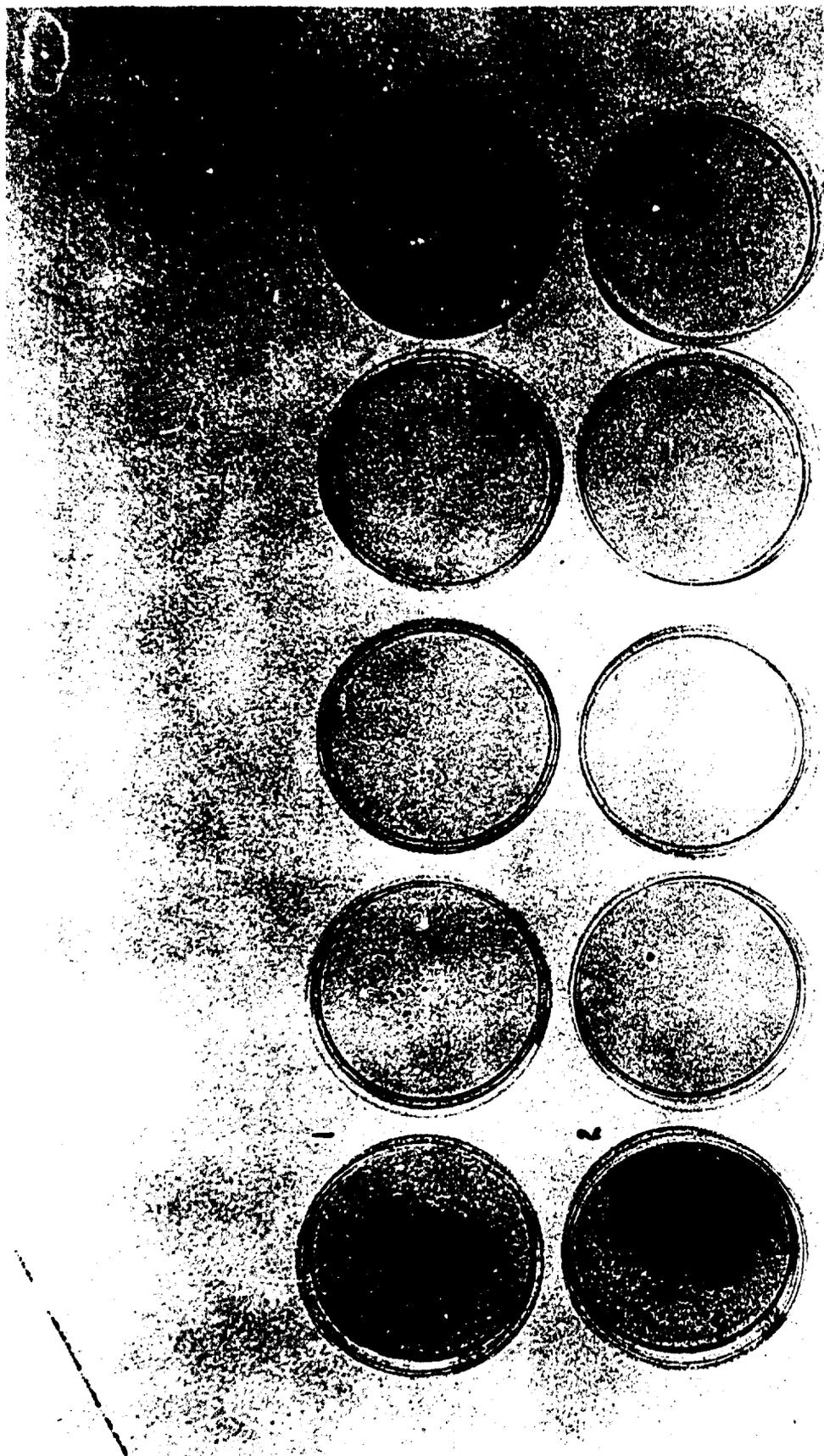


JP-6

Figure 14. Recovery of Viable Cells/ml from Contaminated JP-6 Fuel.



Figure 15. Recovery of Viable Cells/ml from Contaminated XJP-4 Fuel.



SF
JP-4

Figure 16. Recovery of Viable Cells/ml from Contaminated Searsport JP-4 Fuel.

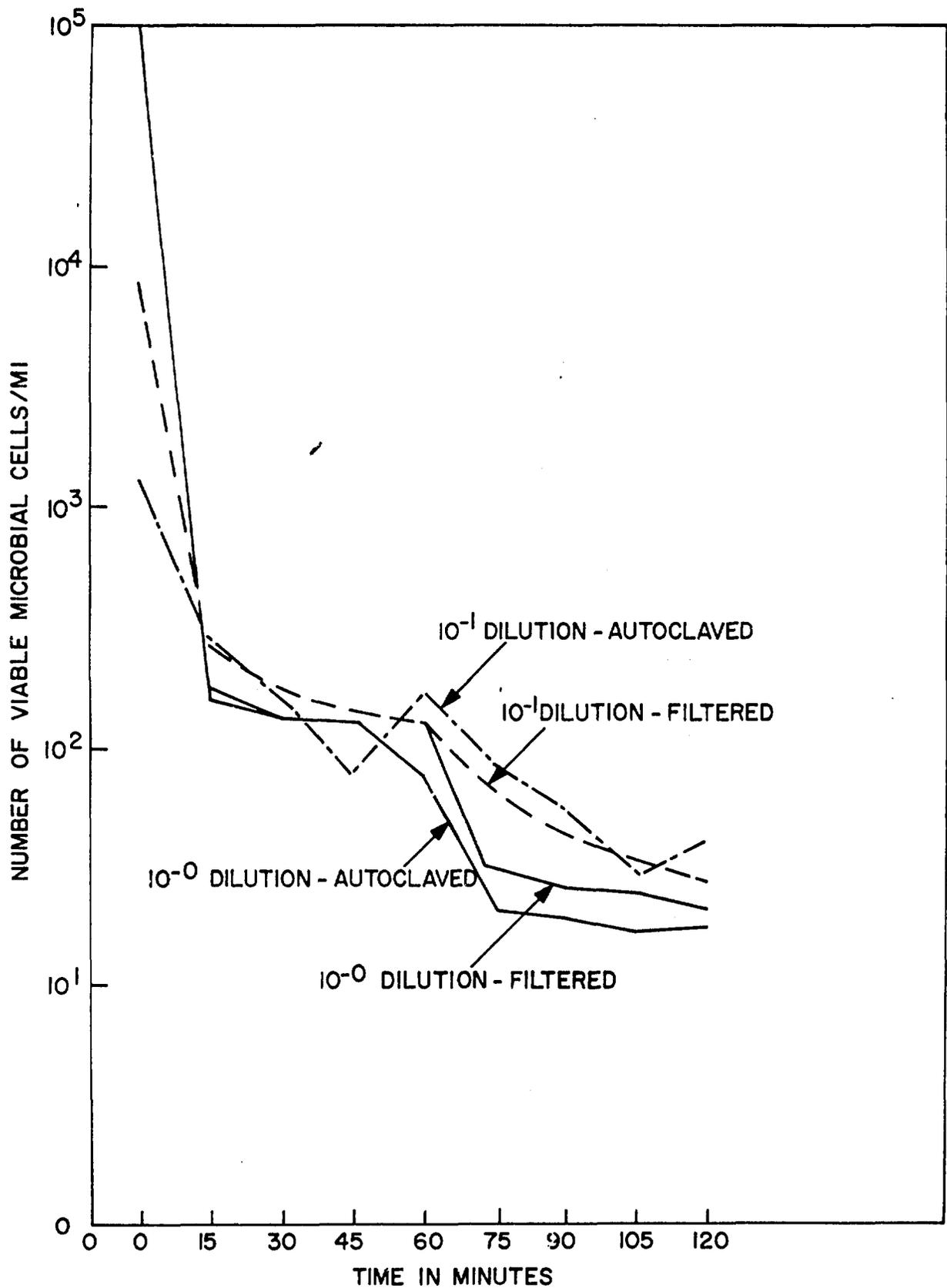


Figure 17. Toxicity of XJP-4 Fuel to Viable Microbial Cells: Autoclave versus Filter Sterilization and Quantity of Fuel Plated.

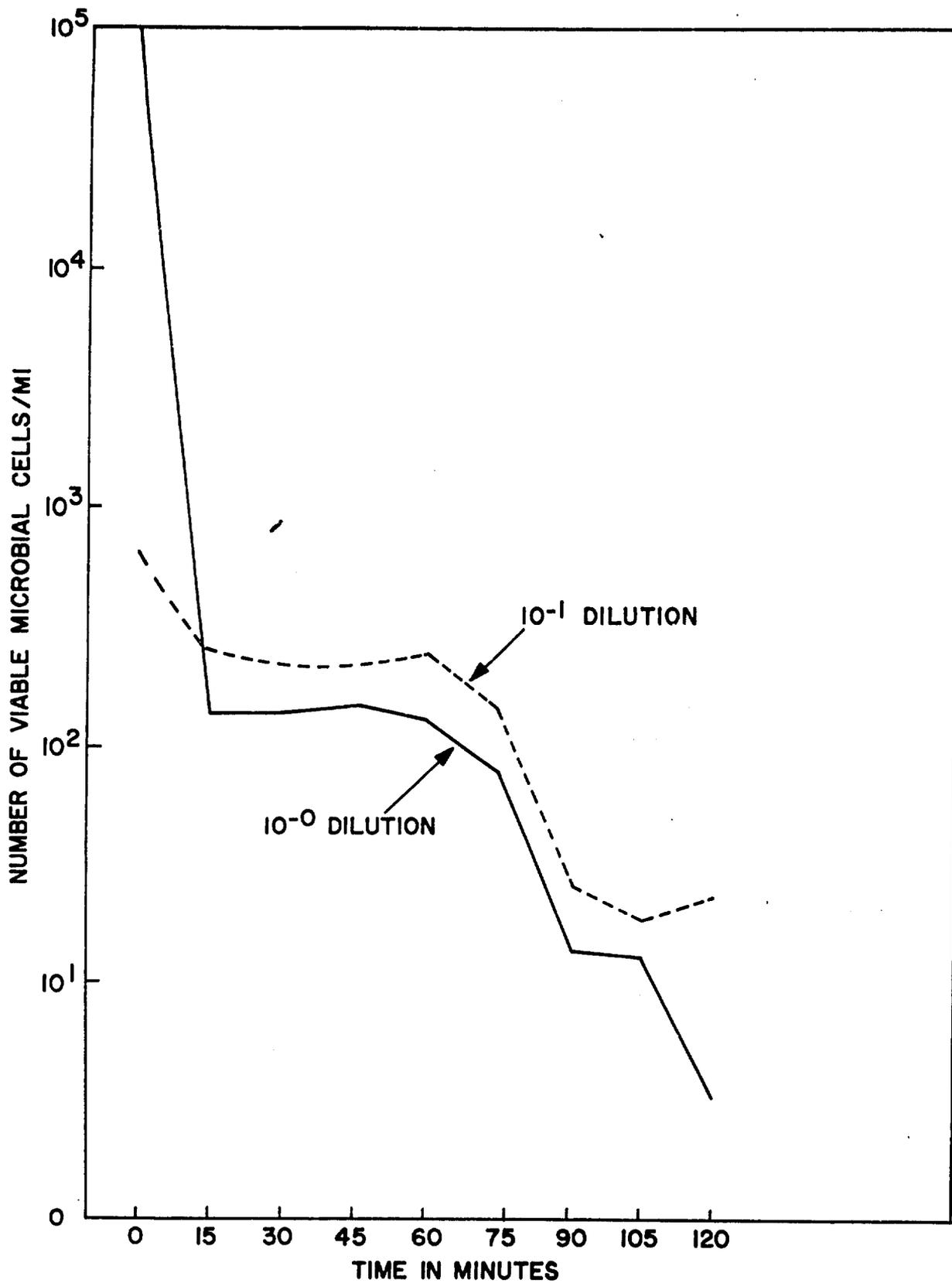


Figure 18. Viability of Microbial Cells in JP-4 Fuel to Quantity of Fuel Plated

the toxicity of fuel samples was directly proportional to the size of the sample plated. The consistently lower counts obtained with the 1.0 ml aliquot of fuel was a further indication of a toxic or mechanical effect on the suspended cells. The lower counts obtained with the larger volume were probably due to the inhibitory hydrocarbon residues that remained on the agar surface. The number of cells/ml obtained from platings of 1.0 or 0.1 ml were sufficiently different to warrant the use of smaller quantities of fuel for the analysis of JP-4 fuel samples. Thus, in all subsequent analysis, 0.1 ml volumes of fuel were utilized.

CONCLUSIONS

1. JP-4 fuels are contaminated by dispersing microbiologically contaminated droplets of water in the fuels, either by the method of sampling or by the handling procedure prior to sampling.
2. JP-4 fuels exert a toxic effect on viable microorganisms and this toxicity is dependent upon the time the microbes are in contact with the fuel, the sterilization method of the diluent fuels, and the grade of the JP-fuel.
3. Sterilization of diluent fuels by filtration is least inhibitory to the recovery of viable microbial cells.
4. Erratic counts of microbial cells/ml were not the result of plating out of the cells on the walls of the diluting MD containers. Erratic counts are, however, a result of the conditions listed in 2 above.
5. The difference in number of cells recovered from a fuel sample warrant the use of 0.1 ml for routine quantitative analysis.

Section V

THE EFFECT OF EGME AND GLYCEROL ON VIABLE MICROBIAL CELLS

During the Air Force investigations to develop fuel-water soluble additives for control of microbiological contamination of JP-fuels, laboratories concerned with the use of ethylene glycol monomethyl ether (EGME) and glycerol as an anti-icing additive for JP-4 fuels indicated that EGME-glycerol was a potential bacteriostatic agent.* In 1962, after the Air Force initiated the use of EGME-glycerol in JP-4 fuels as an anti-icing additive, field data of microbial contamination of JP-4 fuel/water samples from aircraft and ground servicing equipment confirmed the laboratory findings. The material designated MIL-I-27686C was added to the fuel in concentrations of 0.1% v/v and is currently composed of 99.4% volume EGME-glycerol and 0.6% volume of glycerol.

The bacteriostatic property of EGME-glycerol results from its partitioning to the water phase of a JP-fuel system resulting in concentrations of bacteriostatic levels. Initially a concentration of EGME and glycerol of less than 10% was considered biostatic. However, during the past three years viable bacterial cells have been recovered from field JP-4 fuel/water-bottoms containing concentrations of EGME-glycerol in excess of 20% (20% concentrations are currently considered bacteriostatic). Although results of tests performed during the initial use of EGME-glycerol to adapt microorganisms to high concentrations of the material¹¹ were negative, the increase in concentration required for lethal effect does suggest a tolerance development by the microbial flora of JP-4 fuel/water systems.

Because of a possible development of resistant microbial strains to EGME-glycerol in the field, laboratory tests were designed to determine the effects of increasing concentrations of EGME-glycerol on growth of microorganisms and the bacteriocidal level of EGME-glycerol (in water) for microbial cells which had either no exposure or prior exposure to EGME-glycerol. In addition, to determine the effect of other biocides on the growth of microbial cells surviving in a 25% EGME-glycerol environment, Biobor JF[®] (U.S. Borax Chemical Corp.), and beta-nitrostyrene were investigated.

Of interest during the study was the recovery of microorganisms from JP-4 fuel/water-bottoms containing high levels of EGME that displayed

* The effect of EGME was never proven as bacteriostatic or bacteriocidal.

alpha and beta hemolysis when streaked on blood agar plates (see Figures 19 and 20. Figure 21 shows the colonial morphology of the microbes on TSB plates.) Biochemical analyses identified three of the hemolytic colonies as Bacillus pumilus, B. subtilis, and B. cereus.³⁰

METHODS AND MATERIALS

Microbial Analysis

The microbial analysis of fuel and water was performed as described in previous sections: aliquots of 0.1 ml were plated in triplicate on TSB containing 1.5% Bacto-agar. After incubation at 35°C for 48 hours the cell count/ml was determined.

EGME-Glycerol Studies

Materials. The test containers for the studies were (1) a tall glass column containing Searsport JP-4 fuel and a microbially contaminated B-H water-bottom (Section II, Figure 3) initiated 58 days prior to the study; (2) sterile 500 ml flasks, beaker capped; and (3) 50 ml sterile screw cap tubes.

The EGME-glycerol was composed of 99.4% ethylene glycol monomethyl ether and 0.6% glycerol. It was combined and stored in the dark at room temperature. The quantity of EGME-glycerol required by each testing container to give a specific percent was dependent upon the volume of the water-bottom of that test container.

The Biobor JF was used as delivered to the laboratory. It was added to the fuel of the testing container, prior to adding the microbially contaminated water, in quantities sufficient to partition to the water to the desired concentrations. The beta-nitrostyrene was obtained from the POL Section of Wright-Patterson Air Force Base and was used as received in the laboratory. It was added to the fuel as was Biobor JF.

The glass column contained 9 L of non-sterile EGME-glycerol-free Searsport JP-4 fuel, 2 L of B-H-1 salt solution, and a composite of microbial cells of 1 ml each of Laboratory Cultures No. 6, 7, 8, and 9, and 1 ml of a contaminated field sample of JP-4 fuel/water-bottom containing 21% EGME and glycerol.

A second experiment utilized twelve 500 ml sterile flasks containing 200 ml of a filter sterilized (0.45 micron Millipore filter) Searsport JP-4 fuel and 200 ml of sterile B-H-1 solution. A 4 ml water-bottom composite obtained from 4 sampling ports of the glass column (prior to the addition of the EGME-glycerol to the column) was the initial microbial inoculum (10^{-7} cell/ml).



Figure 19. Beta Hemolysis of 18 Hour Cultures selected from Colonies Shown in Figure 21.

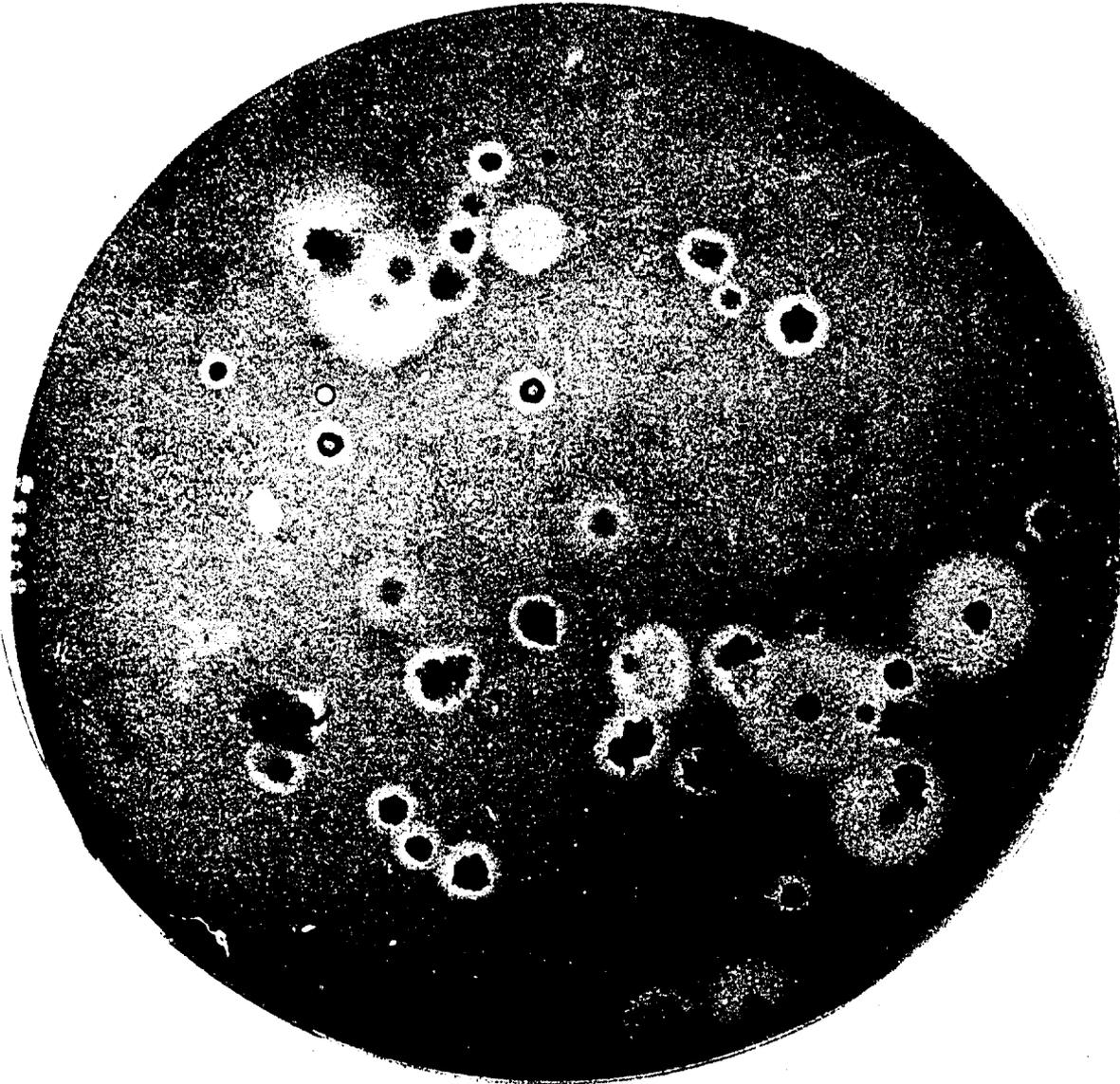


Figure 20.

Beta Hemolysis of Colonies Plated Directly to Blood Agar
from a JP-4 Fuel/Water-Bottom Sample.

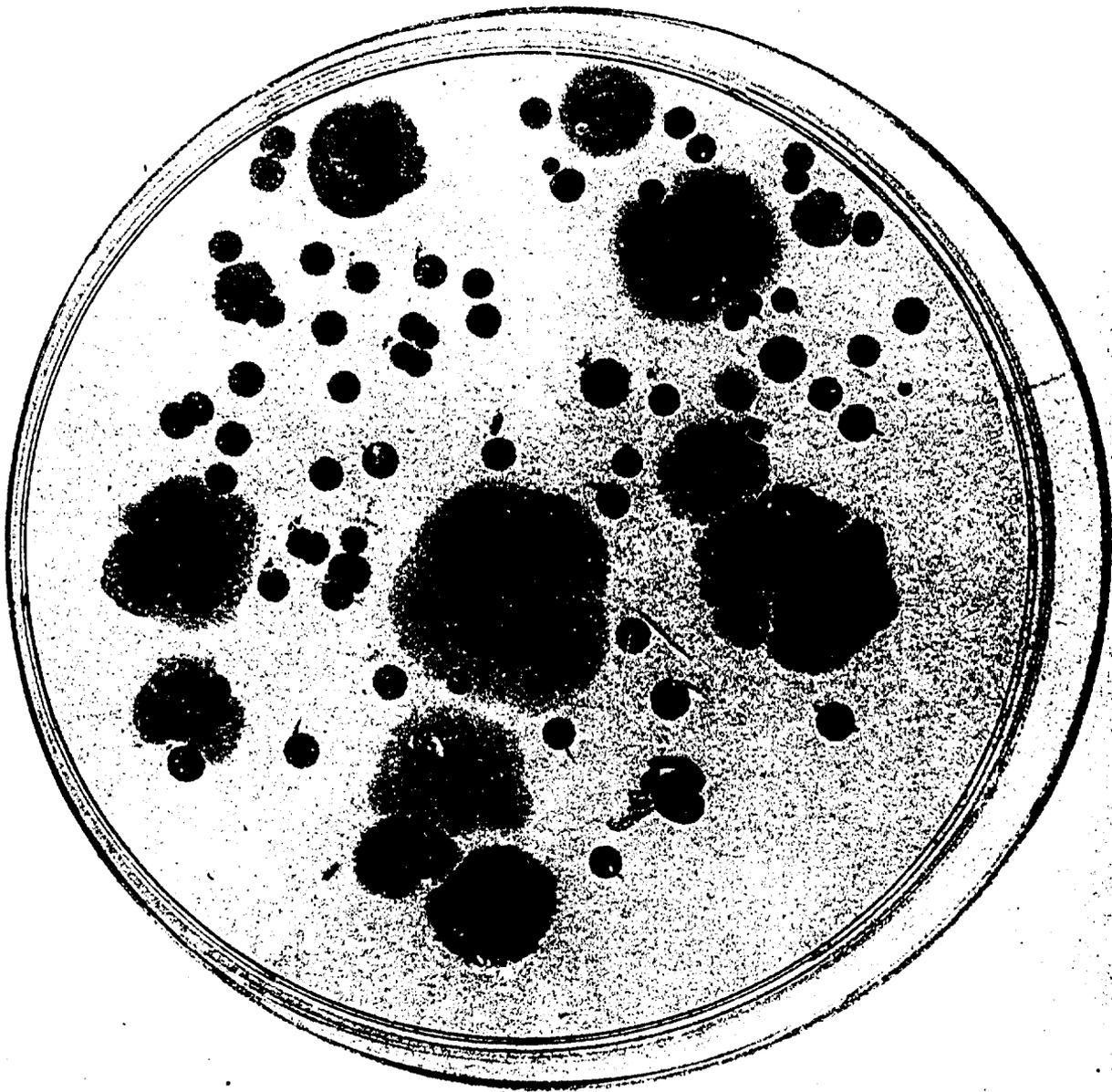


Figure 21.

Colony Morphology of Bacteria from A Field JP-4
Fuel/Water Sample Containing 21% EGME and Glycerol.

To each of 20, 50 ml sterile screw-capped tubes were added 20 ml of sterile JP-4 fuel and 10 ml of B-H solution. The microbial inoculum was a composite of cells obtained from the glass column water-bottom during the time it contained 25% EGME-glycerol. Aliquots of 0.1 ml of the inoculum were added to each test container.

Test Procedures. After 58 days of observation of the glass column system for stratification of microbial cells in the fuel and/or in the water phase, 2% v/v of EGME-glycerol (40 ml) was added to the water-bottom via the bottom sampling port. The entire system was agitated to disperse the EGME-glycerol solution. Microbial analysis for total number of viable aerobic microbial cells in the water-bottom was obtained prior to and at designated times after the addition of the EGME-glycerol. The column was kept at room temperature in the dark. On the eighth day a concentration of EGME-glycerol sufficient to make a 5% v/v in the water-bottom was added via the bottom sampling port. The system was agitated to disperse the EGME-glycerol solution. The percent of EGME-glycerol was gradually increased over a period of 49 days until a 30% v/v in the water-bottom was obtained. The increases were in 5% increments. Samples for microbial analysis were obtained from the system via the sampling port (Figure 4, Section III) with 2 cc sterile hypodermic syringes fitted with 18 gage needles.

To two of the twelve 500 ml flasks containing sterile JP-4 fuel and B-H-1 solution was added 2 ml of a microbial cell composite obtained from the glass column water-bottom prior to the addition of EGME-glycerol to the column. The flasks were designated as A (test flask) and A₁ (hold flask, used as the source for EGME-glycerol-free microbial cells). Subsequent flask inocula were obtained from the A₁ hold culture. After an initial 18 hour incubation period at 35°C, flask A₁ was stored in the dark at room temperature, and to the water-bottom in flask A was added sufficient EGME-glycerol (4.0 ml) to obtain a 2.0% v/v solution. After flask A was agitated to disperse the EGME-glycerol solution it was placed in the dark at room temperature. The total number of viable microorganisms of flask A water-bottom was determined prior to and at specific times after the addition of the EGME.

Eight days following the initiation of flasks A and A₁, 4.0 ml of flask A₁ were added to flasks designated B (test flask) and B₁ (hold flask). Flasks B and B₁ were incubated at 35°C for 18 hours. After incubation, flask B₁ was set aside in the dark at room temperature. To flask B water-bottom was added sufficient EGME-glycerol to make a 5% solution. Microbial analysis, agitation, and storage facilities for flask B was as flask A. A quantity of EGME-glycerol sufficient to make a 5% v/v solution was added to flask A. Prior to and at designated times after the second addition of EGME-glycerol, the total number of viable microbial cells present

in flask A was determined. Seven days following the initiation of flask B and B₁, 4.0 ml of the water-bottom from flask B₁ were added to flasks designated C (test flask) and C₁ (hold flask). After flasks C and C₁ were incubated for 18 hours at 35°C, flask C₁ was stored in the dark at room temperature, and to the water-bottom of flask C was added sufficient EGME-glycerol to make a 10% solution. The flask was agitated to disperse the EGME-glycerol and was then placed in the dark at room temperature. The total number of microbes of the water-bottom of flask C was determined prior to and at prescribed times after the addition of EGME-glycerol. To flasks B and A water-bottoms was added an additional quantity of EGME-glycerol to obtain a 10% solution of EGME-glycerol (a 5% increase). Flasks A and B were agitated, microbially analyzed, and stored as with previous additions of EGME-glycerol. Except for the addition of EGME-glycerol in 5% increments (15, 20, 25, ...) the same procedure as discussed above for C was followed for all subsequent test flasks (D, E, F, ...).

To obtain a uniform 5% increment increase of EGME-glycerol in the test flasks (A, B, C, ...) the water-bottom of the test flasks initiated prior to the 7 day test interval were treated with additional EGME-glycerol. The percent of EGME-glycerol was added and/or increased in the water-bottoms of the flasks until 40% v/v was obtained. When less than 10/ml cells were recovered from any one flask to which EGME-glycerol had been added, the observation of that test flask was discontinued. Thus, this experimental design permitted the exposure of cells to both initially different EGME-glycerol concentrations and uniformly increasing concentrations.

To determine EGME-glycerol microbial growth dependencies, a modification of the gradient plate method³¹ was used. Plates were composed of two solidified agar layers of TSB with (bottom layer) and without 30% EGME-glycerol. The EGME-glycerol medium was prepared by adding 4.5 ml of filter sterilized EGME-glycerol to 15 ml of sterile TSB with 2.0 percent Bacto-agar and solidified at a 20 degree angle. Over this medium was added TSB with 1.5% Bacto-agar. Aliquots of 1 ml of a microbially contaminated water-bottom containing EGME-glycerol were spread over the surface of the plates. Control plates of two layer TSB with 1.5% Bacto-agar were also used. The inoculated plates were incubated at 35°C for 48 hours. The location of the colonies on the medium surface would indicate if the microorganisms were dependent upon the EGME-glycerol.

Two procedures were utilized for the addition of the microbes to the prepared test plates. (1) Microorganisms from the glass column containing 25% EGME-glycerol were plated on TSB plates. A colonial type of the most predominant type on the plate was selected after a 48 hour incubation period at a temperature of 35°C and streaked to the surface of the test plate.

(2) microorganisms as in 1 above were diluted to 10^{-6} in TSB. An aliquot of 1.0 ml of the 10^{-6} dilution was added to 9 ml of melted TSB agar (as for the poured plate techniques). Control plates of two layered TSB with 1.5% Bacto-agar were also used. The inoculated plates were incubated at 35°C for 48 hours. The location of the colonies on the medium surface would indicate if the microorganisms were dependent upon EGME-glycerol.

Biobor and Beta-Nitrostyrene Test

After the glass column water-bottom contained 25% v/v of EGME-glycerol, 0.1 ml aliquots of the water-bottom were added to sterile screw capped test tubes containing 50 ml of sterile B-H-1 solution and JP-4 fuel containing Biobor or beta-nitrostyrene (in concentrations considered biocidal). The number of cells/ml of the B-H-1 bottom inoculum was determined prior to and at designated times after the addition of the inoculum to the test containers.

Hemolytic Cell Recovery

Samples of JP-4 fuel/water-bottoms were plated on blood agar plates (BA). The plates were incubated at 35°C for 24 hours or less.

RESULTS AND DISCUSSION

Results of the study indicated that some microorganisms can survive in a water environment of a JP-4 fuel/water system containing EGME-glycerol. From the compiled data shown in Table XVII, a decrease in viable cells in the column occurred within 24 hours with the 10% increase of EGME-glycerol. However, this decrease, a factor of 10, was not considered significant. A second factor of a log decrease in cells was noted with the increase to 20% EGME-glycerol. A third and significant decrease (a factor of 100, 1000 from the initial count) occurred at some time between the second and the sixth day. This number of viable cells remained fairly constant over a period of 26 months. Microbiological determination of the system after transfer to a 1 gallon dark glass container indicated, after an additional 12 month period, a viable microbial count of greater than 100 cells/ml. Of interest was the change in the predominating colonial forms that occurred with the increase of EGME-glycerol to 20%. The change in colonial morphology was characteristic of colonies recovered (Figures 19 and 20) from a water-bottom of a contaminated JP-4 fuel/water sample obtained from Ramey Air Force Base, Puerto-Rico. (As indicated in procedures above, an aliquot of the Puerto-Rican microbial inoculum had been added to the column as part of the inoculum for contaminating the system).

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Table XVII

Effects of EGME on Viable Microbial Growth

Total Time in Days	Time ¹	EGME Total %	Column ²	A 2%	A ^{**} 1%	B 5%	C 10%	D 15%
0	Prior		3.60×10^6	7.00×10^6	7.13×10^7			
1	24 hrs	2%	6.10×10^6	1.68×10^6	2.28×10^7			
8	Prior		3.60×10^6	3.10×10^2	6.15×10^6	1.11×10^7		
9	24 hrs	5%	1.48×10^6	$<1.00 \times 10^1$	9.67×10^6	1.38×10^7		
10	48 hrs		7.88×10^6	$<1.00 \times 10^1$	-	2.53×10^8		
13	5 days		8.92×10^5	$<1.00 \times 10^1$	-	1.66×10^7		
15	Prior		2.74×10^6	T	-	1.87×10^8	2.37×10^8	
16	24 hrs	10%	1.90×10^5		8.00×10^5	2.14×10^6	2.26×10^6	
25	Prior		1.71×10^5		3.67×10^3	7.00×10^7	4.15×10^7	6.15×10^7
26	24 hrs	15%	3.85×10^5		<10	2.50×10^4	9.67×10^6	7.87×10^6
33	Prior		-		T	-	-	-
34	24 hrs	20%	3.33×10^5			2.50×10^4	$<1.00 \times 10^5$	3.00×10^4
35	46 hrs		8.62×10^4			$<1.00 \times 10^3$	2.13×10^4	1.36×10^4
39	6 days		1.95×10^2			$<1.00 \times 10^1$	2.95×10^3	2.95×10^3
40	Prior		2.07×10^2			$<1.00 \times 10^1$	4.30×10^2	1.97×10^2
43	72 hrs	25%	3.54×10^2			$<1.00 \times 10^0$	$<1.00 \times 10^1$	2.70×10^1
47	Prior		2.15×10^2			T	$<1.00 \times 10^0$	5.00×10^0
49	48 hrs	30%	5.90×10^2				$<1.67 \times 10^0$	$<1.00 \times 10^0$
55	8 days		2.70×10^2				0	0
60	21 days		2.30×10^2				T	T
123	76 days		1.60×10^2					
333	26 wths		1.70×10^2					

¹ Time refers to sampling time of each EGME addition.

² The microbial count/ml of the water-bottom is an average of the 4 sampling water-bottom ports, in turn each of the 4 were an average of triplicate plates. The column was initiated 58 days prior to EGME-glycerol study. During the twenty days prior to the EGME-glycerol study an average count of 4.00×10^6 cells/ml was obtained.

Table XVII. Effects of EGME on Viable Micro

Table XVII

of EGME on Viable Microbial Growth

*	Flasks*							
	B 5%	C 10%	D 15%	E 20%	F 25%	G 30%	H 35%	I 40%
10 ⁷								
10 ⁷								
10 ⁶	1.11 x 10 ⁷							
10 ⁶	1.38 x 10 ⁷							
	2.53 x 10 ⁸							
	1.66 x 10 ⁷							
	1.87 x 10 ⁹	2.37 x 10 ⁸						
10 ⁵	2.14 x 10 ⁶	2.26 x 10 ⁶						
10 ³	7.00 x 10 ⁷	4.15 x 10 ⁷	6.15 x 10 ⁷					
	2.50 x 10 ⁴	9.67 x 10 ⁶	7.87 x 10 ⁶					
	-	-	-	-				
	2.50 x 10 ⁴	<1.00 x 10 ⁵	3.00 x 10 ⁶	6.00 x 10 ⁶				
	<1.00 x 10 ³	2.13 x 10 ⁴	1.36 x 10 ⁵	3.00 x 10 ⁵				
	<1.00 x 10 ¹	2.95 x 10 ³	2.95 x 10 ⁵	1.35 x 10 ⁵				
	<1.00 x 10 ¹	4.30 x 10 ²	1.97 x 10 ⁵	3.21 x 10 ⁵	1.60 x 10 ⁸			
	<1.00 x 10 ⁰	<1.00 x 10 ¹	2.70 x 10 ³	1.17 x 10 ³	1.00 x 10 ⁸			
	T	<1.00 x 10 ⁰	5.00 x 10 ²	2.06 x 10 ²	2.50 x 10 ³	1.95 x 10 ⁸	9.00 x 10 ⁷	7.00 x 10 ⁷
		<1.67 x 10 ⁰	<1.00 x 10 ⁰	<1.00 x 10 ⁰	3.00 x 10 ¹	3.90 x 10 ³	0 2 ⁴ hrs	0
		0	0		<1.00 x 10 ⁰	0	T	T
		T	T	T	T	T		

- analysis omitted

T Flask culture terminated

* With the exception of the columns cells/ml the numbers in table refer to average number of cells/ml of 3 replicates.

** -Flask A₁ at 15 days inoculated with 2% EGME-glycerol.

mg
plates.
10⁶

Effects of EGME on Viable Microbial Growth.

2

As shown in Table XVII, the results of the studies in the test flasks indicated that shock doses of greater than 30% of EGME-glycerol concentrations were required for "fast-kill" (within 24 hours). The biocidal effect of the 30% concentration required a period of 8 days. As indicated from the results the microbes of flask A and A₁ (EGME-glycerol added after 15 days) appeared to be more susceptible to the effects of EGME-glycerol than were the microbes initially treated with 5, 10, or 15% EGME-glycerol. In addition, the growth cycle of the microbes of flask B, C, and D appeared to be similar, a decrease to less than 10 cells/ml occurring in 3 weeks after the addition of the initial EGME-glycerol concentration (Figure 18).

The bacteriostatic concentration of EGME-glycerol effective for the microbes utilized in these tests appeared to be approximately 20%. Table XVIII shows the cells/ml in each test flask during the initial concentration of EGME-glycerol. Of the cells/ml recovered, a significant reduction is not apparent until the shock dose of 25% at 2 hours. Contrary to this are field samples which indicate microorganisms in concentrations greater than 20%. This may be attributable to adaptation occurring over a period of time greater than of these studies, or the presence of partially protective substances in the field water-bottoms. Since the greater majority of field samples analyzed did not contain viable cells and the composition of aircraft sump water varies considerably¹⁷, the former possibility appears most probable.

The results shown in Table XIX indicate that older cultures were more resistant to the EGME-glycerol biostatic effects than were the 18 hour cultures (Total count determined 24 hours after the addition of EGME-glycerol showed a greater number of the cells from older cultures survived than did in the 18 hour cultures.)

The results of the gradient plate method indicated that the microbial cells recovered from the water-bottom of the glass column containing a high concentration of EGME-glycerol were not mutants nor were they EGME-glycerol dependent. The colonial growth was restricted to the area of least EGME concentration, thus suggesting an inhibitory action of the EGME-glycerol substance.

Of those JP-4 fuel/water samples plated to blood-agar plates, the cells isolated from high level EGME-glycerol environments gave clearer and faster hemolysis than did cultures from lesser concentrations of EGME-glycerol. Fourteen different morphological colonies were recovered from the shipped JP-4 fuel/water-bottom sample containing 21% EGME-glycerol (Table XX). Each colony displayed some degree of alpha or beta hemolysis. Three of the colonies displaying beta-hemolysis (prior to shipment) were identified by biochemical analysis. They were identified as Bacillus pumilus.

Table XVIII

Growth of Flask Cultures A through I with Initial Concentrations of EGME-Glycerol^a

Flask No.	A	B	C	D	E	F	G	H	I
9 EGME-glycerol	2%	5%	10%	15%	20%	25%	30%	35%	40%
Time									
Prior to EGME	7.08×10^6	1.11×10^7	8.33×10^7	6.15×10^7	-	1.40×10^8	1.95×10^8	3.00×10^7	7.00×10^7
0 + 20 min	6.15×10^6	-	-	2.00×10^7	1.00×10^6	4.00×10^7	2.50×10^5	1.00×10^4	3.00×10^3
0 + 60 min	6.15×10^6	1.30×10^7	1.06×10^8	1.30×10^7	6.0×10^5	9.75×10^6	4.50×10^5	1.00×10^4	1.50×10^3
0 + 90 min	1.84×10^6	-	-	-	-	-	-	-	5.00×10^1
0 + 120 min	8.26×10^5	6.90×10^6	6.27×10^7	-	3.45×10^6	4.25×10^6	1.5×10^5	-	1.50×10^1
0 + 150 min	1.52×10^6	-	-	1.50×10^7	-	-	1.0×10^5	-	9.67×10^0
0 + 180 min	1.56×10^6	-	-	-	-	-	-	-	8.00×10^0
1 day	1.68×10^6	1.30×10^7	2.26×10^8	7.87×10^6	6.00×10^6	-	-	0	0
2 days	-	2.53×10^8	-	-	3.00×10^7	-	3.90×10^3	-	-
3 days	-	-	-	-	-	1.00×10^5	-	-	-
4 days	-	-	-	-	-	-	-	-	-
5 days	-	1.66×10^7	6.60×10^7	-	-	-	-	-	-
6 days	-	-	-	-	1.35×10^5	-	-	-	-
7 days	-	1.87×10^9	4.15×10^7	-	3.21×10^5	2.80×10^5	2.5×10^0	0	0

- Indicates analysis omitted

Numbers in table refer to average number of cells/ml of 3 replicates.

Table XVIII. Growth of Flask Cultures A through I with Initial of EGME-Glycerol.

Table XIX

Comparison of Microbial Growth of 18 Hour and X-day cultures
in an Environment of EGME-Glycerol

Time	EGME Added	Flasks*						
		A ₁	A	B ₁	B	D ₁	D	
Proce	2%	7.13 x 10 ⁷	7.00 x 10 ⁶					
1/2 hr		1.18 x 10 ⁷	4.15 x 10 ⁶					
1 hr		8.60 x 10 ⁶	4.15 x 10 ⁶					
2 hrs		1.10 x 10 ⁷	8.05 x 10 ⁵					
1 day		2.28 x 10 ⁶	1.68 x 10 ⁶					
2 days		-	-					
3 days		-	-					
5 days		1.06 x 10 ⁸	-					
7 days		6.15 x 10 ⁷	2.00 x 10 ⁴		1.11 x 10 ⁷			
14 days		-	3.10 x 10 ²	3.46 x 10 ⁷	-			
1/2 hr		5%	7.00 x 10 ⁷	-	7.45 x 10 ⁷	1.38 x 10 ⁷		
1 hr			4.55 x 10 ⁷	<1.00 x 10 ¹	4.55 x 10 ⁷	6.90 x 10 ⁶		
2 hrs			9.95 x 10 ⁷	<1.00 x 10 ¹	4.00 x 10 ⁶	1.38 x 10 ⁷		
6 hrs			6.45 x 10 ⁶	<5.00 x 10 ⁰	-	2.53 x 10 ⁸		
1 day	9.67 x 10 ⁶		<1.00 x 10 ¹	4.23 x 10 ⁷	-			
7 days	-		T	-	1.66 x 10 ⁷			
14 days	-		-	-	1.87 x 10 ⁹			
1/2 hr	10%		2.23 x 10 ⁶	-	1.12 x 10 ⁸	2.90 x 10 ⁸	3.8 x 10 ⁸	6.15 x 10 ⁷
1 hr			2.87 x 10 ⁶	-	1.05 x 10 ⁸	8.63 x 10 ⁷	5.02 x 10 ⁸	2.00 x 10 ⁷
2 hrs			1.43 x 10 ⁶	-	3.90 x 10 ⁷	1.79 x 10 ²	4.90 x 10 ⁶	1.30 x 10 ⁷
6 hrs			-	-	-	-	4.30 x 10 ⁷	1.50 x 10 ⁷

Table XIX. Comparison of Microbial Growth of 18 Hour and X-day cultures in an Environment of EGME-Glycerol

Microbial Growth of 18 Hour and X-day Cultures
in Environment of EGME-Glycerol.

10%	1/2 hr					1.12 x 10 ⁸	2.90 x 10 ⁸	3.8 x 10 ⁸	6.15 x 10 ⁷
	1 hr	2.23 x 10 ⁶	-	1.05 x 10 ⁸	8.63 x 10 ⁷	5.02 x 10 ⁸	2.00 x 10 ⁷		
	2 hrs	2.87 x 10 ⁶	-	3.90 x 10 ⁷	1.79 x 10 ²	4.90 x 10 ⁶	1.30 x 10 ⁷		
	6 hrs	1.43 x 10 ⁶	-	-	-	4.30 x 10 ⁷	1.50 x 10 ⁷		
	1 day	-	-	6.80 x 10 ⁷	2.14 x 10 ⁶	-	-		
	2 days	8.00 x 10 ⁵	-	-	-	-	-		
	6 days	5.67 x 10 ⁵	-	-	-	-	-		
15%	7 days	4.50 x 10 ⁴	-	1.70 x 10 ⁷	7.00 x 10 ⁷	3.07 x 10 ⁷	7.87 x 10 ⁶		
	1/2 hr	3.67 x 10 ³							
	1 hr			1.53 x 10 ⁶	3.75 x 10 ⁶	1.00 x 10 ⁶	1.30 x 10 ⁶		
	2 hrs			8.00 x 10 ⁵	3.95 x 10 ⁶	4.00 x 10 ⁶	2.77 x 10 ⁶		
	3 hrs			3.15 x 10 ⁵	1.30 x 10 ⁶	2.10 x 10 ⁷	1.57 x 10 ⁶		
	1 day			-	7.00 x 10 ⁵	-	-		
	7 days			3.67 x 10 ³	2.50 x 10 ⁴	6.67 x 10 ⁵	3.00 x 10 ⁶		
20%	1/2 hr								
	1 hr			4.23 x 10 ¹	2.50 x 10 ⁴	9.67 x 10 ³	1.97 x 10 ⁵		
	2 hrs								
	1 day								
	7 days								

A₁, B₁, C₁ are original hold cultures

A, B, C are test 18 hour cultures

- indicates analysis omitted

T indicates the test culture was discontinued

* numbers in table refer to average number of cells/ml of 3 replicates.

2

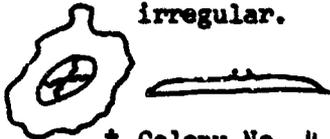
* Colony No. 1, round, raised, white-cream, irregular edge, mucoid, under raised area.



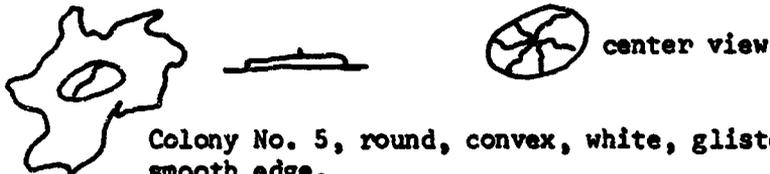
Colony No. 2, round, convex, white, glistening, mucoid, irregular edge.



* Colony No. 3, large rough, white center, crinkled, edge irregular.



* Colony No. 4, round umbonate, white, irregular edge, rough, radiating ridges.



Colony No. 5, round, convex, white, glistening, mucoid, fairly smooth edge.



Colony No. 6, round umbonate, white cream, irregular edge, rough radiating ridges.



Colony No. 7, round, white, irregular edge, shows up frequently in laboratory cultures contaminated with these microbial cells.



Colony No. 8, small, round, convex, white, glistening, mucoid, irregular edge.

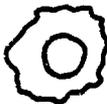
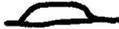


Table XX.

Colonial Morphology of 21% EGME-Glycerol
Surviving Field Sample Microorganisms.



Colony No. 9, white, mucoid, convex, glistening, similar to No. 2 except for edge.



Colony No. 10, white (stark white) similar to No. 3 except this colony comes off of medium easily.



Colony No. 11, cream, rough, irregular edge.



Colony No. 12, white, very rough, small, irregular colony, popcorn appearing colony.



Colony No. 13, very large, white, spreading, flat, rough,



Colony No. 14, cream, small to minute, round, entire, radiating ridges.

† Cultures recovered sent to U.S. Army Natick Laboratories for biochemical identification.

Table XX. Colonial Morphology of 21% EGME-Glycerol Surviving Field Sample Microorganisms.
(Continued)

B. subtilis, and B. cereus.³⁰ It was found that long storage of these cultures on solid media under refrigeration or repeated transfers of the cultures to laboratory media drastically reduced the hemolytic capabilities of the cells.

Although the following discussion is not directly related to the study, it is included in this section because of the potential implication it has to the development of EGME-glycerol resistant strains by transduction. Transduction is the transfer of genetic material from one cell to another via temperate bacteriophages, resulting in the formation of a hybrid clone. Thus, microbes experiencing this process may possess unusual biochemical capabilities.

During the program to contaminate JP-4 fuels, the appearance of phage-like plaque was frequently observed on 2 morphologically different colonial forms. These microbes were recovered from the water-bottom of a system four months old which had been plated on TSB agar plates after a minimum incubation period of 72 hours (48 hours at 35°C followed by 24 hours at room temperature). Both colonies produced an extra cellular, water soluble, diffusible pigment, one which was fluorescent under ultraviolet light. The plaques occurred at room temperatures and with a color change of the colony. The plaque phenomenon would continue until only small islands of the original microbial colony remained. The colors changed from a green-yellow to a brown-yellow for one colony type and from a green to a darker shade of green for the other colony form. Results of biochemical determinations³² tentatively identified the two colonial forms as Pseudomonas fluorescens and Pseudomonas aeruginosa. Ferry, Fisher and Fisher³³ and Burke³⁴ suggested that this type of plaque formation was not a phage activity but autolyses. Results of their studies indicated that P. aeruginosa, after several months of sub-culturing on laboratory mediums, spontaneously lysed, producing plaque areas resembling those of phage lyses.

The possibility exists that transduction might occur in JP-fuel environments and result in the formation of microbial strains possessing the capability to induce degradation in JP-fuel systems.

CONCLUSIONS

1. Viable microbial cells may persist in water-bottoms containing various levels of EGME-glycerol.
2. Concentrations greater than 20% but less than 30% appear to reduce microbial concentrations in laboratory systems over a period of three weeks.
3. An initial concentration of 30% or more appears to exert a biocidal effect in laboratory systems within one week.

4. The presence of low levels of viable cells in field samples containing greater than 20% EGME-glycerol, or laboratory samples initially containing low levels of EGME-glycerol, suggest a slow adaptation or the presence of protective substance in aircraft sump water.

5. The possibility of transduction occurring in JP-4 systems is indicated. This may have significance with respect to the appearance of microbes possessing increased capability for fuel system degradation.

Section VI

MICROBIOLOGICAL CORROSION OF ALUMINUM ALLOYS AND THE USE OF THE CORROSOMETER PROBE AS A MEANS OF DETECTING MICROBIAL CORROSION OF ALUMINUM ALLOYS

Direct evidence implicating microorganisms or their by-products with corrosion of aluminum alloys of integral fuel tanks under field conditions and under controlled laboratory studies has been inconclusive or has yielded inconsistent results. This is due to a lack of knowledge of the relationship between the environmental factors and microbial metabolism in JP-fuel systems. Of the problems of aircraft and associated equipment attributable to microbial contamination, that of corrosion appears to be the most controversial. Digman³⁶, Cockey, Hodge, Iverson, and Wilkes³⁸, Swatek³⁷, Hawks and Edington³⁸, Hedrick, Miller, Halkias, and Hildebrand³⁹, Baumgartner⁴⁰, Hill⁴¹, and Blanchard⁴² presented data that indicate the corrosion of aluminum alloys can be induced by the presence of microorganisms or their metabolites. However, Powelson³⁸ and Calvelli⁴³ suggest that non-biological materials are of major importance in the corrosion problem of jet fuel systems.

To determine the involvement of microorganisms (viable and non-viable) in the processes of aluminum alloy corrosion, laboratory studies were initiated to induce microbial corrosion of aluminum alloys 7075, 7178, and 2024.

Although evidence that unequivocally attributed corrosion of integral fuel tanks to microbial activity was lacking during the early investigations of microbial contamination of JP-fuels, microbial contamination of fuel cells was considered as one of the primary factors involved with the corrosion of integral wing fuel tanks. Thus, a means to detect microbial growth would assist in correlating microbial activity with corrosion of the integral wing fuel cells. The Corrosometer probe (Magna Products, Inc., Santa Fe Springs, California, (Figure 22) originally designed for use in iron corrosion studies was evaluated in 1962 by Dooley and West⁴⁴ as a means to detect microbiological contamination in JP-4 fuels. These authors suggested that the Corrosometer probe, if fabricated from aircraft-type metals and equipped with appropriate electrodes, could be used to detect microbial growth in a fuel/water system. To evaluate these findings, Corrosometer probes of aluminum alloys 7075 and 2024 were constructed, and a study was initiated to determine if the Corrosometer probe could be successfully employed as a technique for the detection of corrosion by microorganisms in a JP-4 fuel/water system in the field.

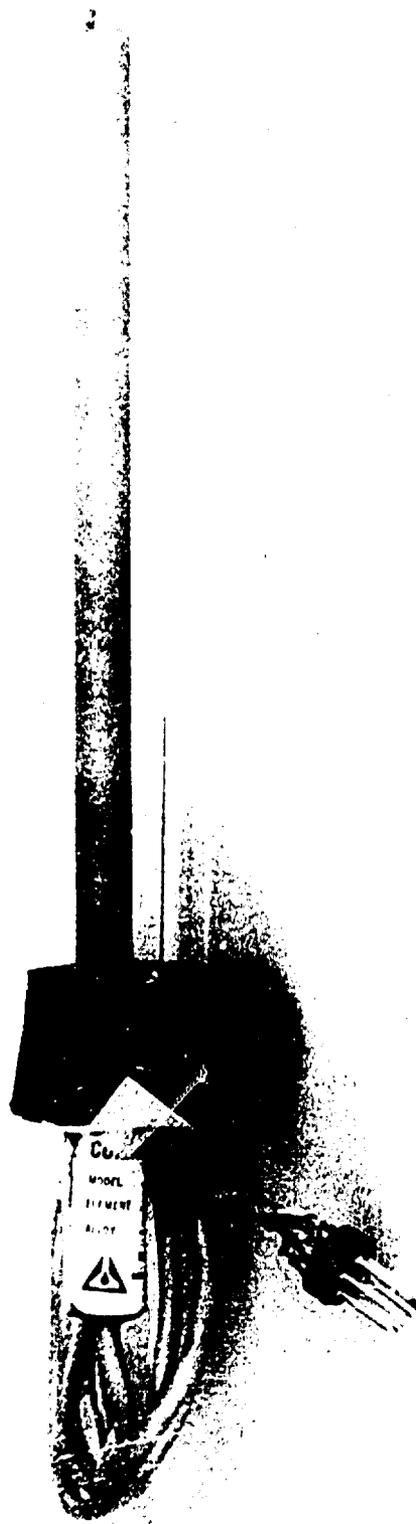


Figure 22. Aluminum Strip Corrosometer Probe

METHODS AND MATERIALS

Microbial Analysis

The sterility of a system and the total number of viable aerobic microorganisms of a test system was determined by the spread plate technique. Serial dilutions of the water-bottoms were made in TSB, and aliquots of 0.1 ml of the three highest dilutions plated in triplicate on TSB with 1.5% Bacto-agar. Fuel analysis consisted of direct plating on the TSB solid growth medium. All systems were subjected to microbiological analysis throughout the experimental period.

Corrosion Study

Materials. To study the effects of microbiological corrosion of 7075, 7178 and 2024 aluminum alloys, coupons 7.62 x 1.27 x 0.16 cm were machine cut from sheets of the metal. In addition, coupons of aluminum alloy 2024 were machine cut from various depths of a solid bar of the alloy to determine if quenching, and therefore granular structure, influenced corrosion of aluminum alloy 2024. All specimens were coded, emery-buffed on one side, washed with a detergent, rinsed twice with distilled water, rinsed with 70% and 100% alcohol for 1 hour, rinsed 3 times with sterile distilled water, and placed in a 4-ounce sterile, wide mouth glass bottle. (See Figures 23 and 24.)

When the JP-4 fuel and the water were added to the test container, the coupon was positioned so that it would be exposed to fuel, water, and a vapor phase.

The JP-4 fuels used in the investigation were obtained from two different sources, Maytag and Searsport. The fuels were used as delivered to the laboratory, or filter sterilized through a 0.45 micron Millipore filter, depending upon the experimental design.

The various water-bottoms employed were: diluted sea water (2 parts boiled tap water, 2 parts distilled water, and 1 part sea water); de-ionized water (Bantam Demineralizer, Model BD-1); distilled water; TSB; and B-H mineral salts solution 1 through 6. (The chemical composition of B-H-2 through 6 is the same as B-H-1 (Section III) with the following exceptions: B-H-2 replaced 1 g $(\text{NH}_4) \text{NO}_3$ with 1 g $(\text{NH}_4)_2\text{SO}_4$; B-H-3 lacked $(\text{NH}_4)_2\text{SO}_4$; B-H-4 contained 0.2g $(\text{NH}_4)_2\text{SO}_4$; B-H-5 contained 0.06 g $(\text{NH}_4)_2\text{SO}_3$; and B-H-6 contained 0.01 g $(\text{NH}_4)_2\text{SO}_4$.

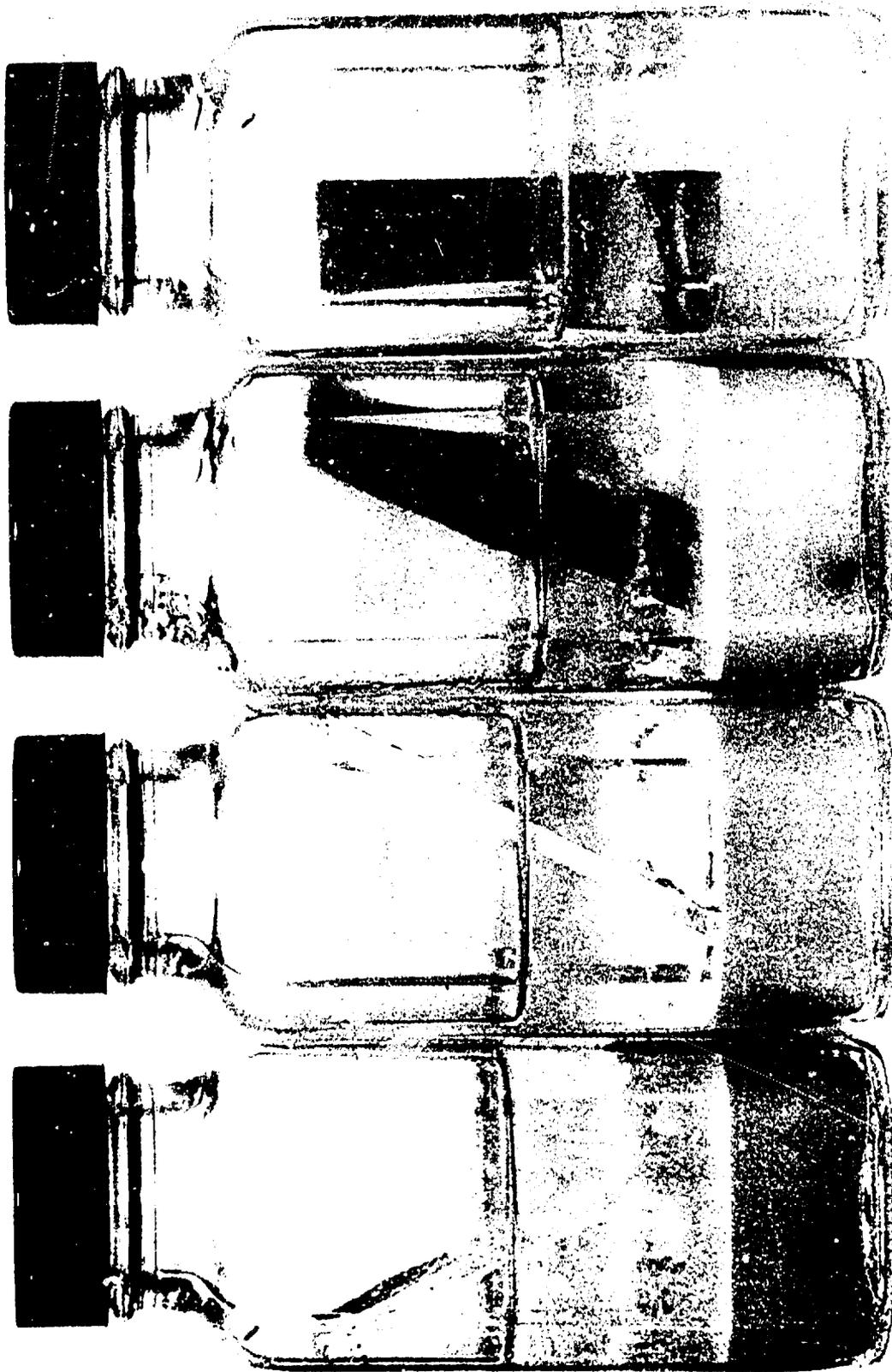


Figure 23. Test System for Corrosion Studies.

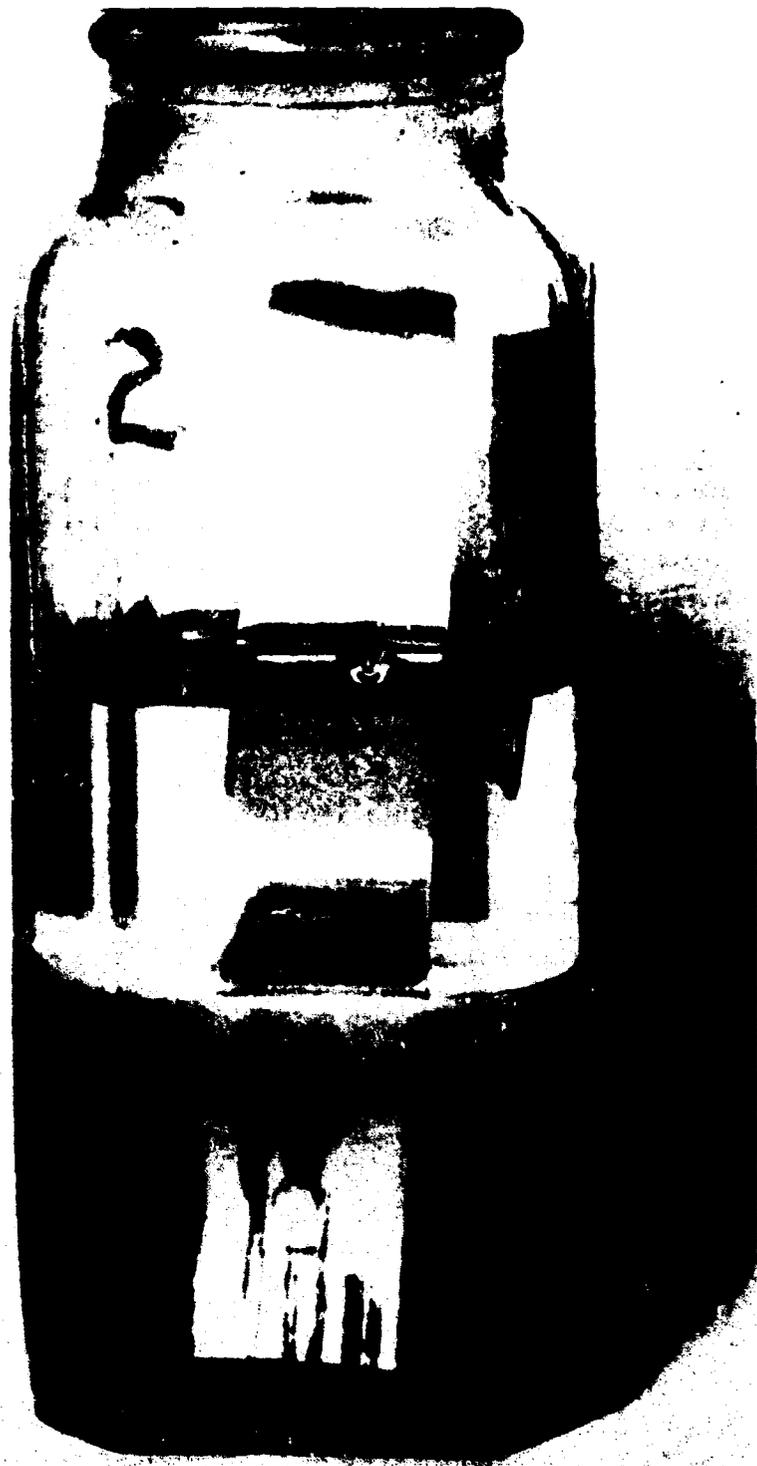


Figure 24.
Test Container for Corrosion Studies Showing
Intergranular Corrosion of Aluminum Coupons

Procedures

Sea Water vs B-H-1. One ml of 10^8 microbial cells/ml and scrapings of corrosion from aluminum alloy 7075 containing viable microorganisms were added to 2 of four 500 ml flasks containing 100 ml diluted sea water or B-H-1 solution overlaid with 100 ml of Searsport JP-4 fuel and 1 coupon each of 2024 and 7075. Inocula were not added to the other 2 flasks, designated as controls. The flasks were incubated at room temperature in the dark. Each coupon was observed daily for visual indication of corrosion. The flasks containing the sea water were terminated after a 30-day observation period. The coupons of the B-H-1 system were removed and examined after a 90-day observation period.

Aircraft Sump Water. Samples of aircraft sump water were obtained from the Propulsion Fluid System Branch (SEJIF), Wright-Patterson Air Force Base. The samples were divided into two parts; one part was used as delivered, the other was autoclave sterilized. Coupons of alcohol-sterilized aluminum alloys 7178 and 2024 were placed in each water portion contained in sterile 3.78 L (1 gal) clear glass jugs. The coupons were suspended in each system by a surgical gut suture. All systems were incubated at room temperature in the dark. Visual observations were made daily to note the development of corrosion. The coupons were removed and examined after 60-day observation periods. Sump samples as received in the laboratory were subjected to microbial and microscopic analysis.

Diluted Sea Water, Distilled Water, Deionized Water, B-H-1 through 6, and Viable vs Non-Viable Microbial Cells. Two separate studies were initiated under this program. Studies A and B were designed to determine the participation of viable and non-viable microbial cells in the corrosion process of aluminum alloys and the effect of water-bottoms of various compositions on microbial growth and corrosion of aluminum alloys 7075, 7178, and 2024. Study B also included the study of the influence of the quenching process on corrosion of aluminum alloy 2024.

The viable inoculum for Study A was obtained from the water-bottoms of Laboratory Cultures Numbers 3 and 4. An aliquot of 50 ml (10^6 cells/ml) was centrifuged, the supernate was decanted, and the sediment washed with 50 ml of buffered water pH 7.2, centrifuged 3 times, and re-suspended in 20 ml of each of the following sterile water-bottoms: diluted sea water, distilled water, deionized water, and B-H-1 solution. The non-viable inoculum of Study A was a composite of 5-day old microbially contaminated broth cultures of TSB obtained from field JP-4 fuel/water samples. An aliquot of 50 ml of the broth was autoclaved for 15 minutes at 121°C , 15 psi, and centrifuged (Automatic Servall Superspeed Centrifuge #KSB-3(4)A) for 15 minutes at 1.0×10^4 rpm. The supernate

was decanted and the sediment washed 3 times with 50 ml of buffered water pH 7.2, centrifuged after each of the 3 washings, and re-suspended in 20 ml of the same water-bottoms as those used for the re-suspension of viable cells.

The viable and non-viable microbial inoculum of Study B was obtained from the water-bottom of Laboratory Culture No. 8 (10^8 cells/ml). With the exception that both the viable and non-viable cells were re-suspended in 60 ml of sterile water-bottoms (diluted sea water, distilled water, de-ionized water, B-H-1, and TSB) the inocula were treated as for Study A.

To 4-ounce, sterile, clear glass square test bottles containing 30 ml of sterile Searsport JP-4 fuel overlaying 30 ml of water-bottom were added 2 ml of viable or 6 ml of non-viable microbial suspension. Each of the bottles were tightly capped and placed on a reciprocating laboratory shaker with a horizontal excursion of 4 cm at 76 oscillations/minute (Figure 25). The shaker remained at room temperature and was kept in the dark as much as possible. Each coupon was observed daily for visual corrosion. After a thirteen day exposure period the coupons were removed, rinsed in 70% dehydrated alcohol, air dried under aseptic conditions, and examined.

Nitrate vs Nitrite. This study was designed to determine the effect of microbially produced nitrite on the corrosion of aluminum alloy 7075, 7178, and 2024. The viable and non-viable inocula were obtained from the water-bottoms of Laboratory Cultures Numbers 3 and 8 (10^6 cells/ml and 10^8 cells/ml). Aliquots of 50 ml of each water-bottom were autoclaved to obtain the non-viable inoculum. Both the autoclaved and non-autoclaved water-bottoms were centrifuged, the supernate decanted, and the sediment washed and centrifuged three times in B-H solution without nitrogen (B-H-3) and re-suspended in 200 ml of B-H-3. To test containers, as used in Studies A and B, containing 30 ml of sterile Searsport JP-4 fuel and 30 ml of B-H solution containing various concentrations of nitrogen (see page 89) were added 2 ml of viable or 6 ml of non-viable microbial inoculum. Each water-bottom system was replicated 5 times for each of the three different alloys used. The bottles were tightly capped and placed on the shaking mechanism. The shaker and the bottles were left at room temperature and kept in the dark as much as possible. Each coupon was observed weekly for visual corrosion. In addition, each bottle inoculated with viable cells was checked for nitrite (sulphanilic acid reagent), nitrate (diphenylamine reagent), and ammonia (Boch and Benedict modification of Nessler's reagent). After a 90-day exposure period, the coupons were removed and rinsed in 70% dehydrated alcohol, air dried under aseptic conditions, and examined.

At the termination of the study, viable cells from the deionized water-bottom were centrifuged and washed three times with B-H-3, re-suspended in B-H-3, and inoculated into B-H-3 broth and agar (Special Agar-Noble,

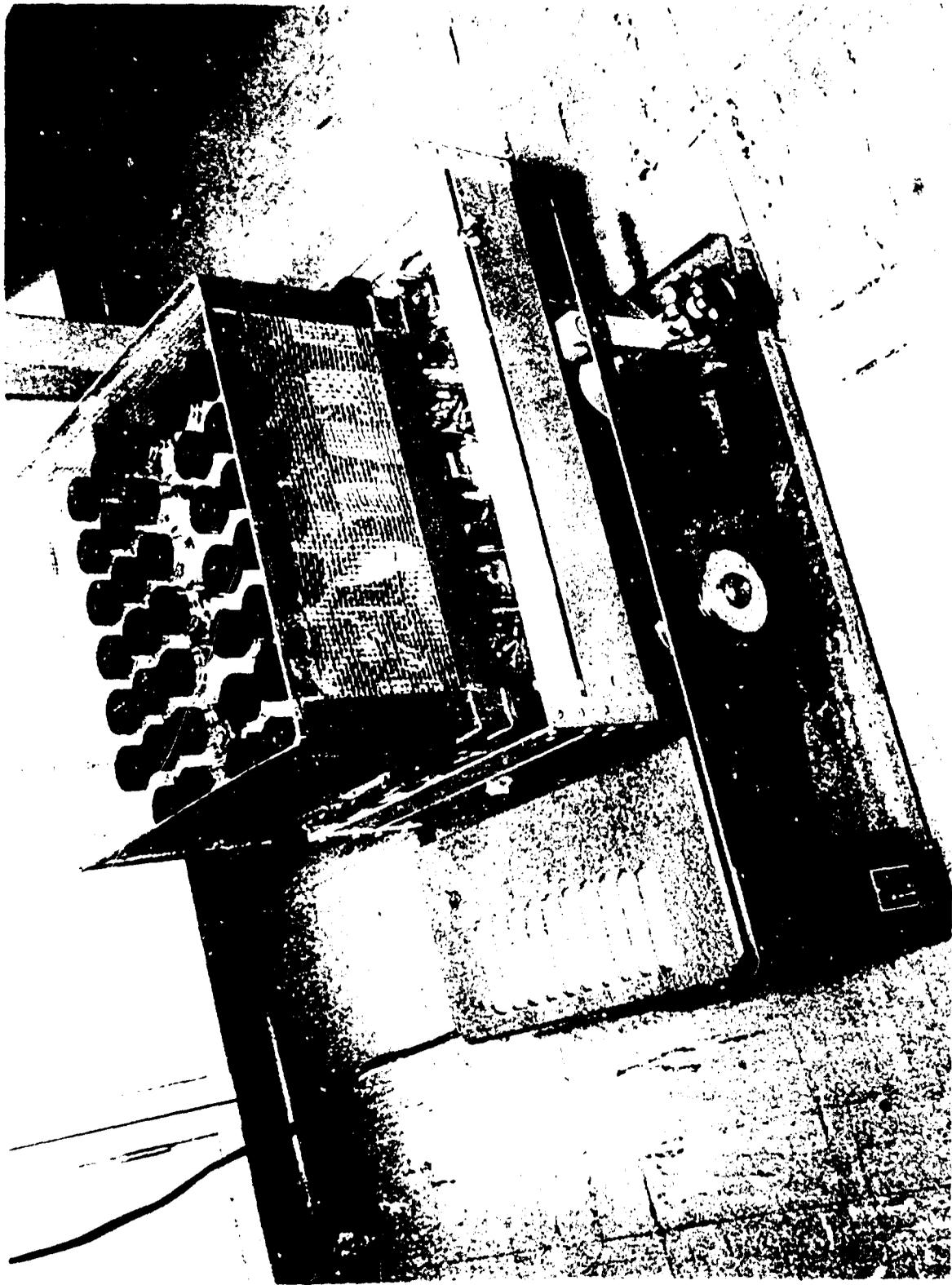


Figure 25. Corrosion Study Agitation System.

Difco). An aliquot of 0.1 ml of the microorganisms were plated in triplicate. The inoculated media were divided into three groups. A and B groups were placed in anaerobic jars and flushed with nitrogen gas or carbon dioxide. The jars were placed in a 30°C incubator for 72 hours. Group C was placed directly into the 30°C incubator for 72 hours. After the incubation time the tubes and plates showing growth from the anaerobic jars were inoculated on additional nitrogen free solid medium and incubated at 30°C for 48 hours.

CORROSOMETER STUDY

Material

Ten strip corrosometer probes of 3 types of aluminum, model 1036 (Figure 25), were employed in the study. The probes used were 4 each of aluminum alloy 2024 and 7075, and 2 of steel 8001. The testing containers consisted of sterile 1000 ml flasks fitted with Buna-N stoppers to seal the flasks and to hold the probes securely in place (Figure 26). Each probe, prior to insertion in a test container, was cleaned by soaking that portion of the probe to be inserted inside the test container in a 0.1% Roccal solution for 3 hours and rinsing 2 times with sterile distilled water. The corrosion of each probe was measured with a Corrosometer (Model CK-2 Magna Products Inc., Santa Fe Springs, California). The corrosion in mils/yr can be determined by the following formula:

$$\text{mils/yr} = \frac{\text{Change in dial divisions}}{\text{Change in time in days}} \times (0.365) (8) \text{ or } (4)$$

(8) factor for steel probe

(4) factor for aluminum probe

For the purposes of this study, the data were recorded as change in resistance (dial reading) with time.

The microbial inoculum of viable cells was a composite of Laboratory Culture Numbers 8 and 2 (10^6 cells/ml) and fungal scrapings from a Sabouraud agar plate of a field JP-4 fuel/water sample. The composite was diluted so that the starting inoculum was 10^3 cells/ml.

Procedures

Each probe was secured firmly in sterile 1000 ml flasks containing 100 ml of sterile B-H-1 solution and 250 ml of filter sterilized JP-4 Searsport fuel overlay. The strips were placed in the containers so that each was exposed to fuel and the water-bottom of B-H-1. Prior to adding the microbial inoculum, an initial reading of each probe was obtained. The

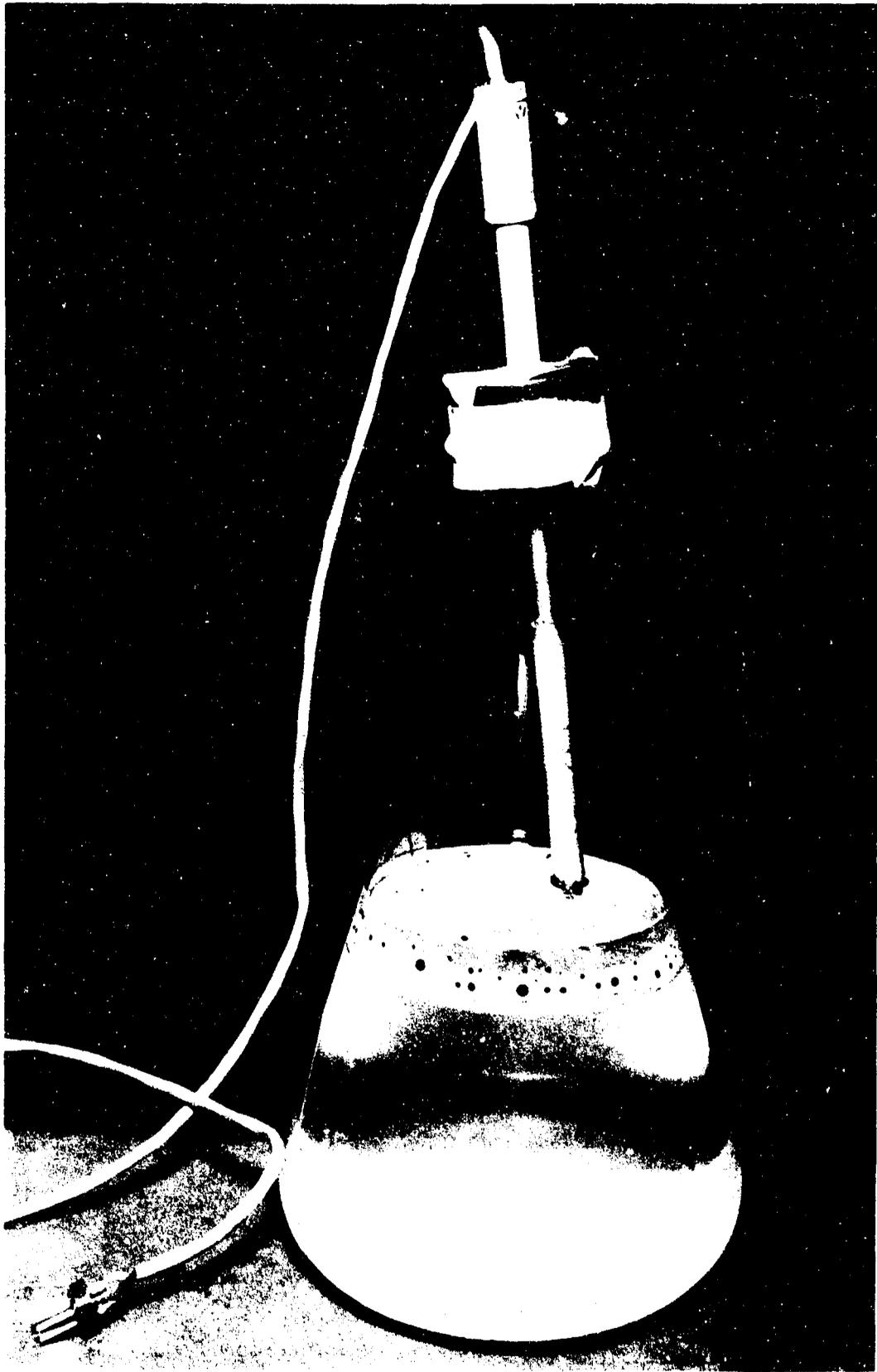


Figure 26. Test System for Corrosion Study.

microbial inoculum of 0.1 ml was added to flasks designated 2, 5, 6, 9, and 10. The microbial population of each inoculated flask was determined daily until a stationary phase of the culture had been reached and maintained for a period of three days. All test containers were incubated at room temperature and in the dark, and were handled only for microbial and corrosion determination. The change of resistance (dial readings) of each strip was determined periodically. After a 46 day observation period the probes were removed and the strips examined for visual corrosion.

RESULTS AND DISCUSSION

Due to the heavy incrustment of corrosion of aluminum alloys 7075 and 2024 in the first phase of this study, all test flasks containing sea water were discarded after 32 days of observation. The corrosion noted was of a pit-type for alloy 2024 and intergranular for alloy 7075. Of the coupons in B-H-1, no corrosion other than blackening occurred during the 60 days of observation (Table XXI, Figure 27, Group C). The terminal microbiological count was 10^8 cells/ml in the sea water, 10^7 cells/ml in B-H-1 and 0 cells/ml in the control (uninoculated) flasks. Since corrosion differed significantly in the sea water and the B-H-1 coupons and the microbial counts in the water-bottoms were similar, a correlation between the presence of viable microorganisms and aluminum corrosion was not evident. The possibility does exist that the environment provided by the sea water permitted the microbes to carry out metabolic processes that manifested in corrosion. However, the corrosiveness of sea water is well documented. It was also found that aircraft sump water samples were always corrosive to 7178, 7075, and 2024 (Table XXII, Figure 27, Group A). However, the degree of corrosion would vary from one sample to the next. Of special interest were the microscopic examinations of the sump samples. Although the viable counts ranged from $0 < 10^3$ cells/ml, the microscopic analysis usually indicated that the system contained a greater microbial contamination than was shown on the growth plates for total counts.

Tables XXIII and XXIV show the cell counts of the water-bottoms of diluted sea water, distilled water, deionized water, and B-H-1 utilized in Studies A and B. As can be noted, terminal counts varied between the various water-bottoms. However, the count appeared to have had no relationship to the corrosion process. Of the three alloys tested, 2024 appeared the most resistant to corrosion. Figures 28 through 33 show the aluminum coupons at the termination of Studies A and B. (Note the pit-type corrosion of alloy 2024 in Figure 28.)

In the microbially contaminated test bottles containing TSB, a gelatinous covering of coupons occurred. Microscopic examination indicated the presence of bacterial cells. The covering did not appear to inhibit corrosion and/or increase or decrease the rate of corrosion.

Stable emulsions as seen in Figure 34 at the fuel/water interface were a common occurrence in JP-4 fuel/water systems during the early

Table XXI

Sea Water vs B-H-1 Corrosion Study of Aluminum Alloys 7178 and 2024

Observation time in Days	Aluminum Alloy 7178					Aluminum Alloy 2024					
	2	3	15	32	60	2	3	15	32	60	
Culture Source											
Sea H ₂ O uninoculated	bl	ab ab	+ bl;b	+ +	Terminated	0 0	ab ab	ab ab	+ +	Terminated	
Sea H ₂ O inoculated	bl	ab ab	+ bl;b	+ +	Terminated	0 0	ab ab	ab ab	+ +	Terminated	
B-H-1 uninoculated	0 0	0 0	0 0	0 0	0 0	Terminated	0 0	0 0	0 0	0 0	Terminated
B-H-1 inoculated	0 0	0 0	0 0	0 0	bl bl	Terminated	0 0	0 0	0 0	bl bl	Terminated

Bacterial Count

Initial Count Cells/ml	Culture Source	Terminal Count/ml	pH
3.33×10^4	Sea H ₂ O uninoculated	0	-
	Sea H ₂ O inoculated	1.30×10^8	-
	B-H-1 uninoculated	0	-
	B-H-1 inoculated	1.40×10^7	5.0 ±

Note:  each triangle represents one side of the coupon

- bl = blackening
- ab = air bubbles
- b = bubbles
- 0 = no corrosion
- + = corrosion found
- / = two sides of aluminum strips

Table XXI. Sea Water vs B-H-I Corrosion Study of Aluminum Alloys 7178 and 2024.

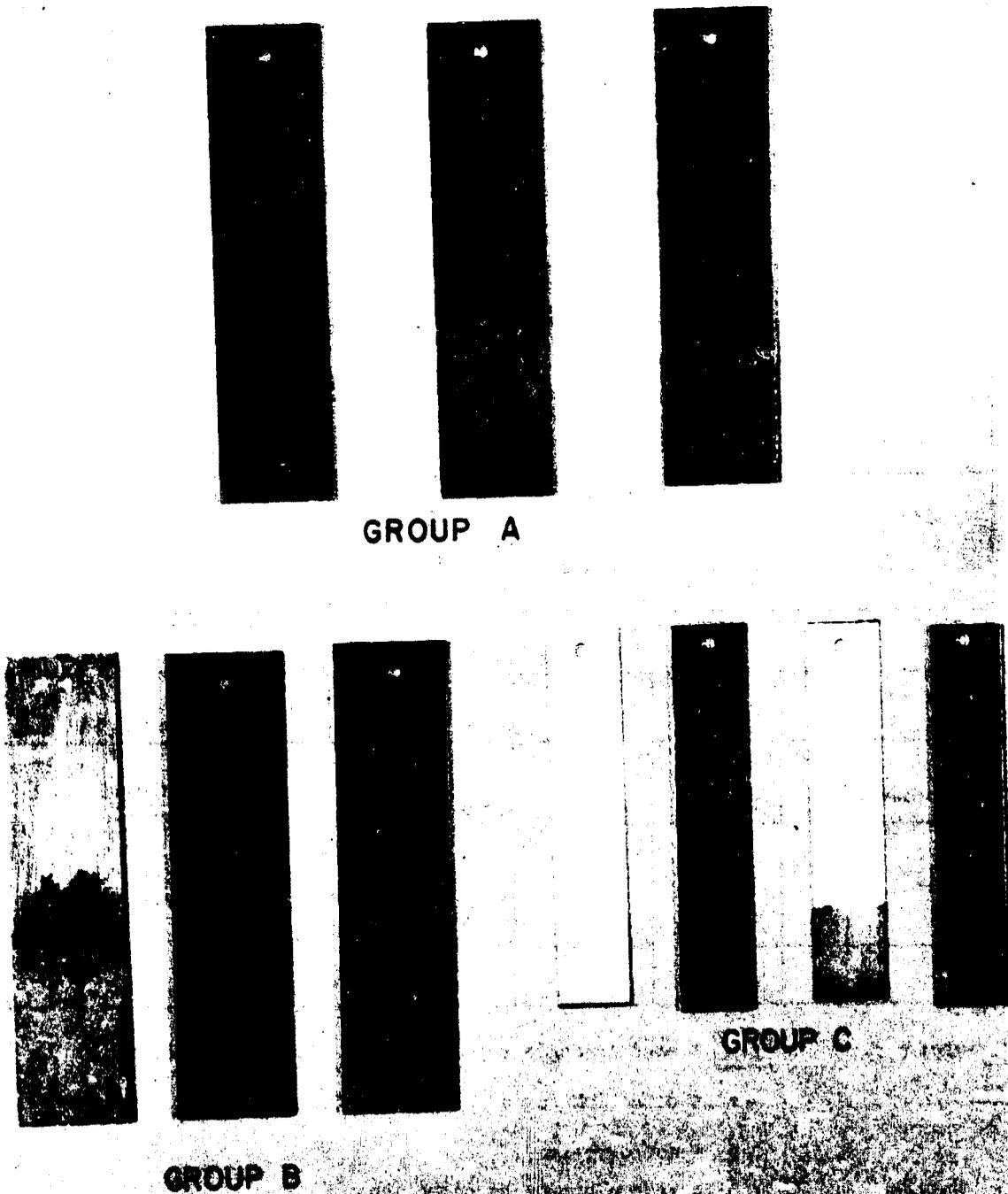


Figure 27.
Aluminum Corrosion of Coupons in Aircraft Sump Water,
B-H-1 Solution and Laboratory Culture No. 3 System.

Table XXII

Aircraft Sump Water Corrosion Study of Aluminum Alloys 7075 and 2024

Aluminum Alloy 7075					
Observation time in days	2	15	30	60	Terminated
Autoclaved	+ 0	+ +	+ +	+ +	Extensive granular corrosion
As is	+ 0	granular + +	+ +	+ +	
pH				5.0±	
Aluminum Alloy 2024					
Observation time in days	2	15	30	60	Terminated
Autoclaved	0 0	+ 0	* *	* *	Small amount of pit corrosion
As is	0 0	pit + +	+ +	+ +	
pH				5.0±	

Note:  = each triangle represents one side of the coupon
 + = corrosion found
 0 = no corrosion
 * = minute amount of corrosion

Table XXII. Aircraft Sump Water Corrosion Study of Aluminum Alloys 7075 and 2024.

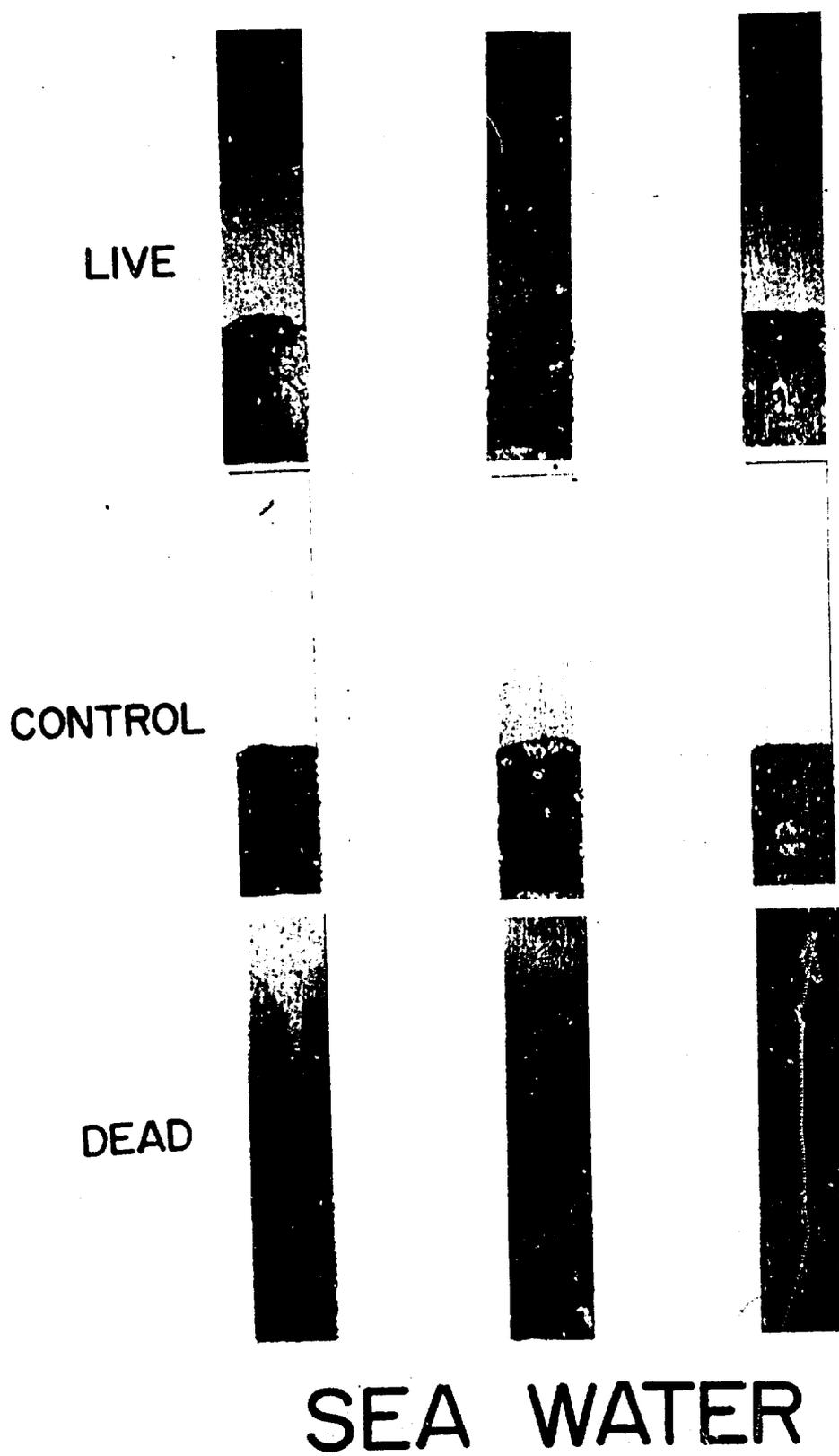


Figure 28.
Study A, Corrosion of Aluminum Alloys 7178,
2024 and 7075 (left to right) in Sea Water.

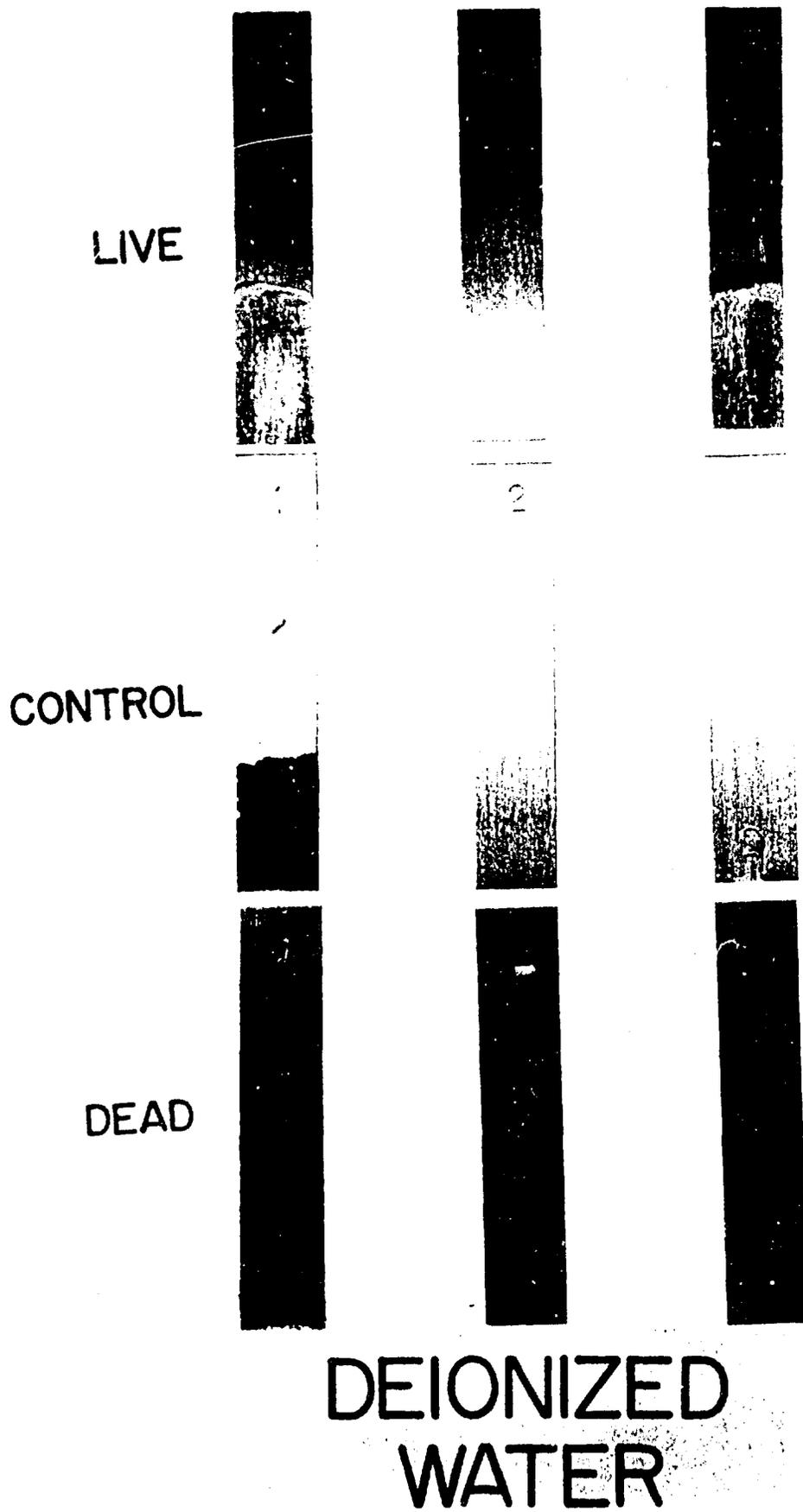


Figure 29.
Study A, Corrosion of Aluminum Alloys 7178, 2024
and 7075 (left to right) in Deionized Water.

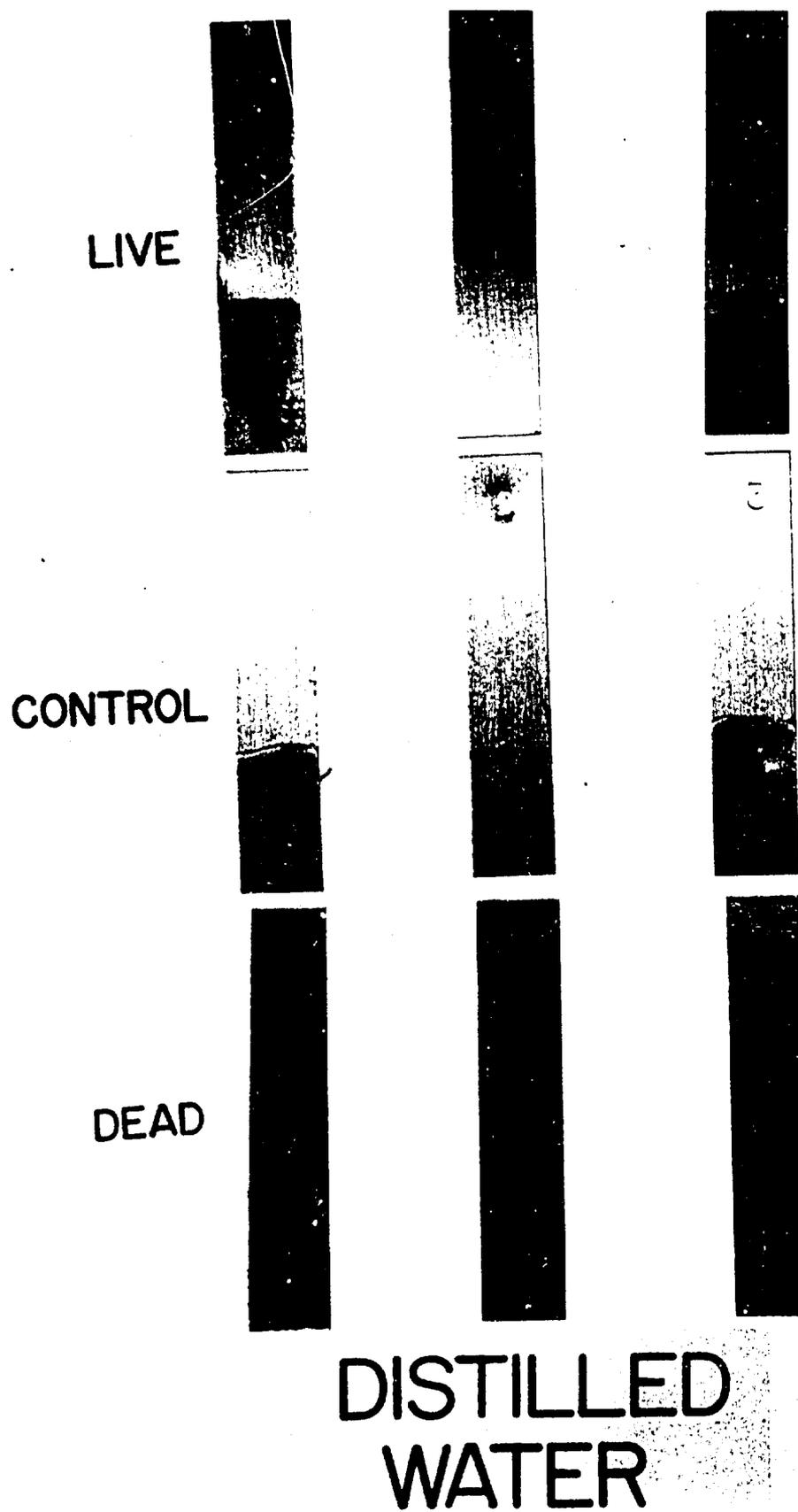


Figure 30.
Study A, Corrosion of Aluminum Alloys 7178, 2024,
and 7075 (left to right) in Distilled Water.

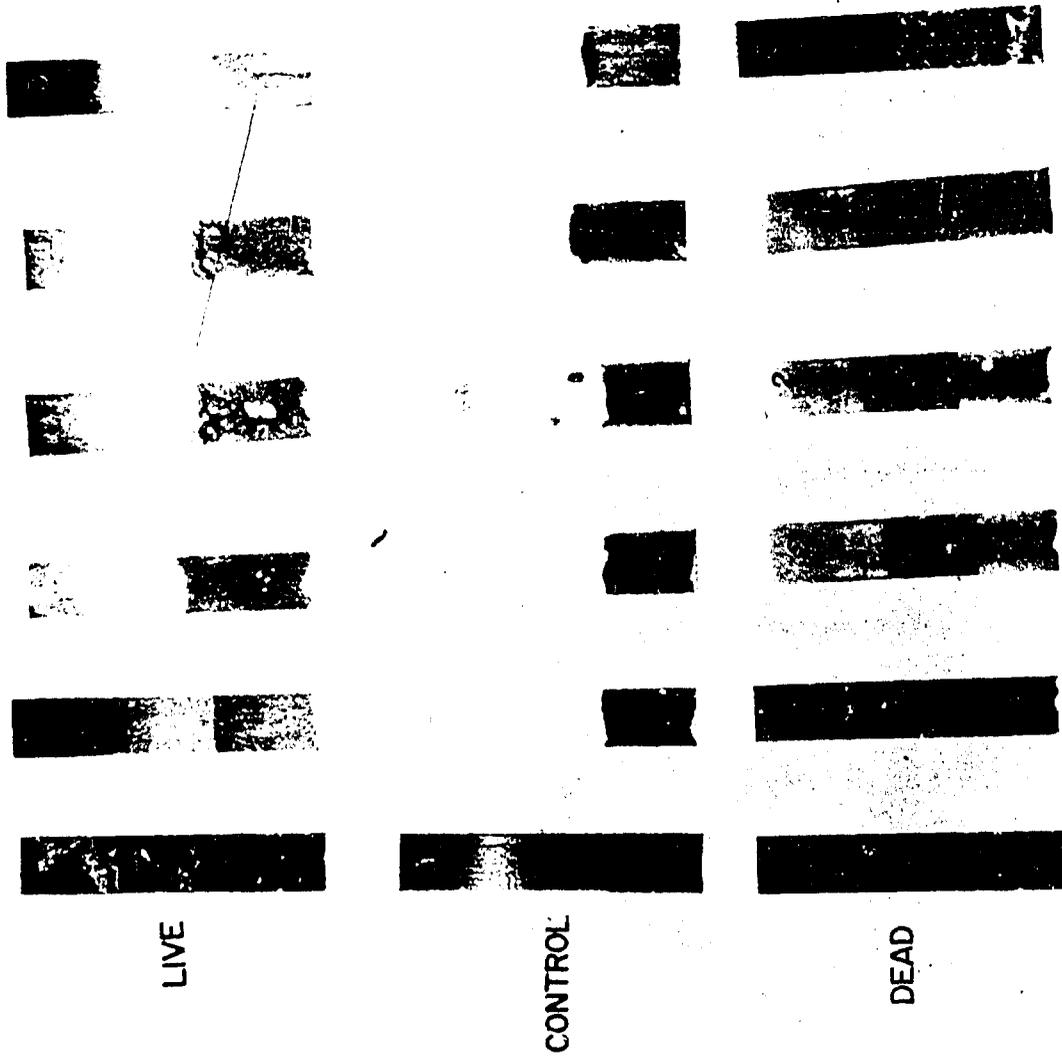


Figure 31. Study B, Corrosion of Aluminum Alloys 7178, 2024 (inner cuts of 2024) and 7075 (left to right) in Sea Water.

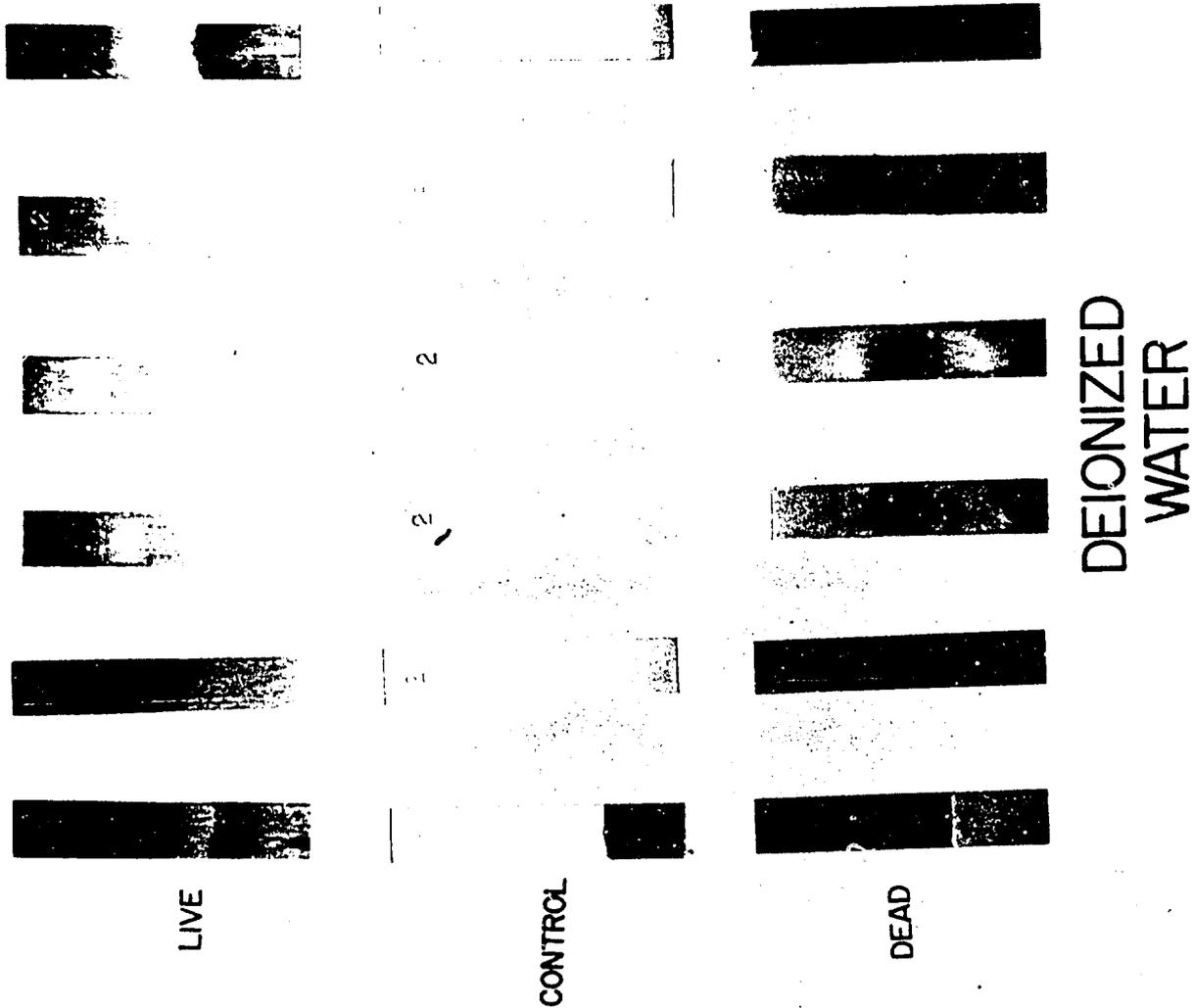


Figure 32. Study B, Corrosion of Aluminum Alloys 7178, 2024 (inner cuts of 2024) and 7075 (left to right) in Deionized Water

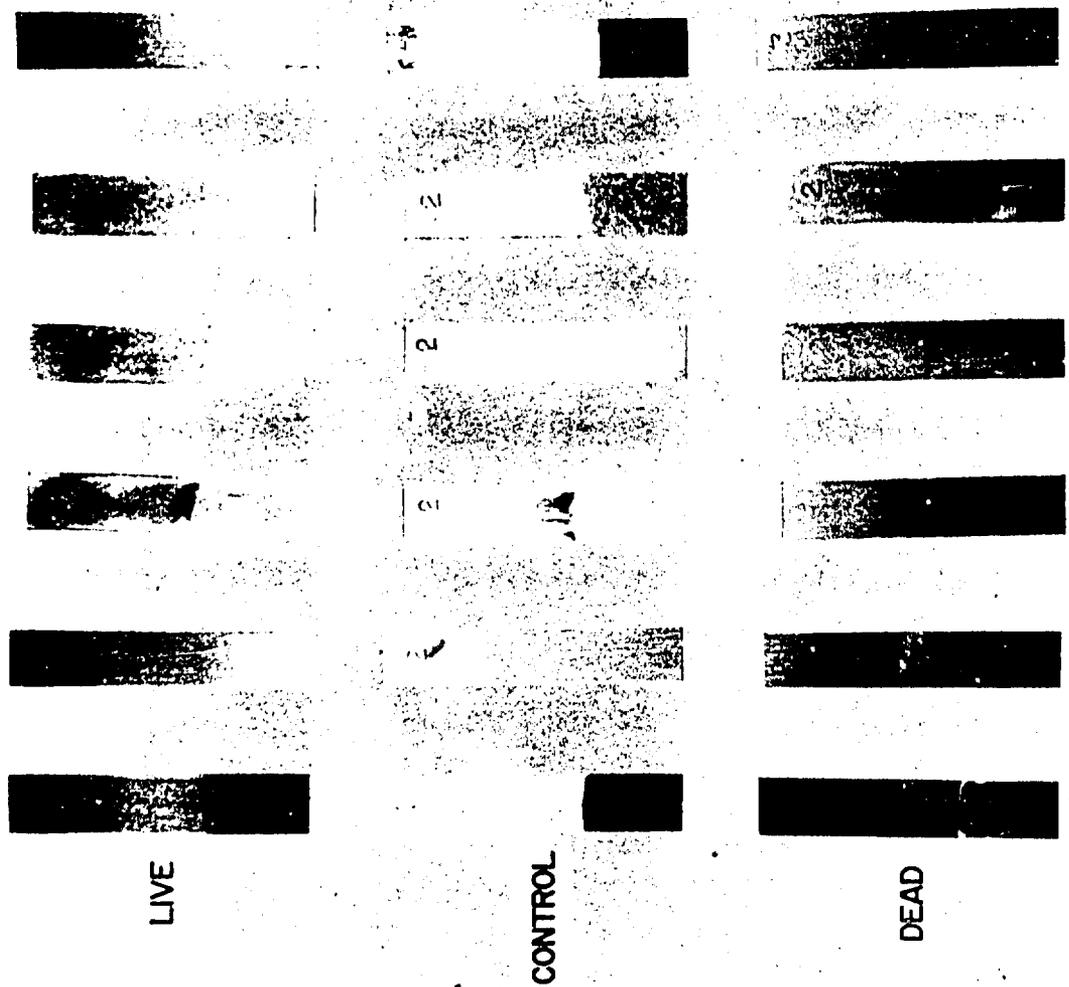


Figure 33. Study B, Corrosion of Aluminum Alloys 7178, 2024 (inner cuts of 2024) and 7075 (left to right) in Distilled Water

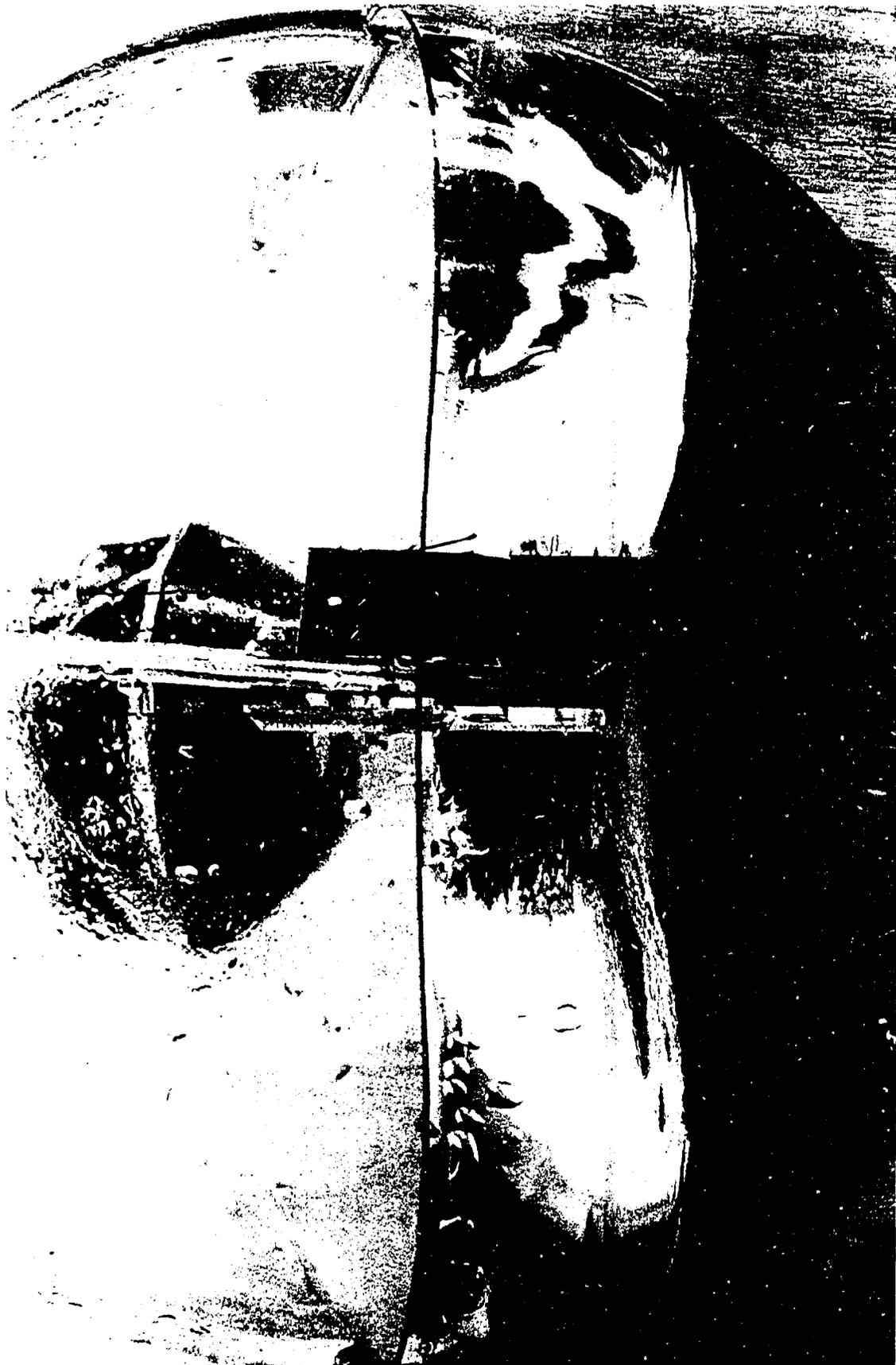


Figure 34. Culture No. 3 Showing Stable Emulsion of Fuel/Water Interface.

Table XXIII

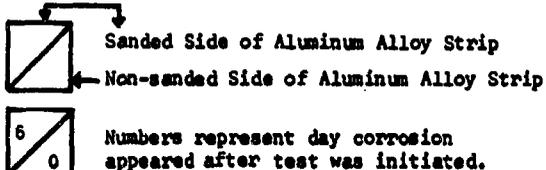
Corrosion Study A

A. Time Corrosion Appeared in Days

Water-Bottoms	Control			Live Microbial Cells			Dead Microbial Cells		
	7178	2024	7075	7178	2024	7075	7178	2024	7075
Diluted Sea Water	1 6	6 0	1 5	1 5	6 0	1 5	1 1	6 0	1 5
Deionized Water	5 13	0 0	5 0	2 6	0 0	2 5	8 13	0 0	2 0
Distilled Water	6 7	0 0	3 6	2 5	0 0	2 8	6 8	0 0	6 0
B-H-I	5 0	0 0	2 0	8 13	0 0	6 13			
TSB	6 0	0 0	2 0	13 0	0 0	6 0			

B. Viable Cell Count/ml of Inoculum, Initial and Terminal

Water-Bottoms	Initial Count ¹	Aluminum Alloys		
		7178	2024	7075
Diluted Sea Water	8.30×10^4	$< 1.00 \times 10^5$	1.50×10^7	$< 1.00 \times 10^5$
Deionized Water	1.46×10^5	$< 1.00 \times 10^5$	1.80×10^6	1.30×10^5
Distilled Water	1.58×10^5	4.30×10^5	3.67×10^5	$< 1.00 \times 10^5$
B-H-I	1.50×10^5	7.80×10^6	2.36×10^6	9.60×10^6
TSB	1.37×10^5	2.57×10^7	2.80×10^6	1.41×10^7



¹ Number of Cells/ml determined theoretically after cells had been washed, centrifuged, and resuspended in 20 ml of designated water-bottom.

Table XXIII.

Corrosion Study A: A-Time Corrosion Appeared in Days.
B-Viable Cell Count/ml of Inoculum, Initial and Terminal.

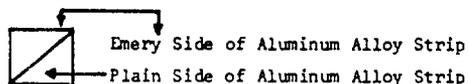
Table XXIV
Corrosion Study B

A. Time Corrosion Appeared in Days

Water Bottoms	Control						Live Microbial Cells						Dead Microbial Cells					
	7178	2024	2024			7075	7178	2024	2024			7075	7178	2024	2024			7075
			a	b	c				a	b	c				a	b	c	
Diluted Sea Water	<1 4	5 13	4 4	5 5	4 4	<1 4	<1 4	12 12	4 4	4 4	4 4	<1 4	<1 4	4 12	4 5	4 4	4 4	<1 5
Deionized Water	12 12	0 0	0 0	0 0	0 0	0 0	12 12	0 0	0 0	0 0	0 0	0 0	4 13	0 0	0 0	0 0	0 02	<1 12
Distilled Water	4 12	0 0	0 0	0 0	12 12	4 12	4 12	0 0	13+ 13+	0 0	0 0	<1 12	13 13	0 0	12 12	0 0	0 0	0 0
B-H-I	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	13 0
TSB	4 0	0 0	0 13+	13+ 0	13+ 13+	<4 0	<1 0	0 0	0 12	13+ 0	0 0	<1 13	<1 0	0 0	0 12+	13+ 13+	0 0	5 12

B. Viable Count/ml of Inoculum, Initially and Terminal

Water Bottoms	Initial Count	Aluminum Alloys					
		7178	2024	2024a	2024b	2024c	7075
		Terminal Count/ml					
Diluted Sea Water	1.19×10^6	$<1.00 \times 10^5$	5.10×10^6	7.67×10^6	2.85×10^8	4.73×10^6	5.97×10^6
Deionized Water	5.37×10^5	1.67×10^5	2.00×10^5	$<1.00 \times 10^5$	$<1.00 \times 10^5$	1.00×10^5	$<1.00 \times 10^5$
Distilled Water	7.68×10^5	$<1.00 \times 10^5$	$<1.00 \times 10^5$	$<1.00 \times 10^5$	3.30×10^4	$<1.00 \times 10^5$	$<1.00 \times 10^5$
B-H-I	6.62×10^5	2.50×10^5	4.53×10^6	1.30×10^7	1.30×10^8	1.57×10^7	2.80×10^6
TSB	8.12×10^5	1.00×10^5	2.60×10^8	1.60×10^8	2.70×10^8	1.48×10^7	$<1.00 \times 10^5$



Numbers represent day corrosion appeared after test was initiated on 12/11/63.

† = possible corrosion

Number of Cells/ml determined theoretically after the cells had been washed, centrifuged and resuspended in 60 ml of designated water-bottom.

Table XXIV.

Corrosion Study B: A-Time Corrosion Appeared in Days.
B-Viable Count/ml of Inoculum, Initial and Terminal.

investigation of microbial contamination of jet fuels. At the present time they are very rarely seen. However, in the nitrate vs nitrite study using varying amounts of nitrogen in the growth medium, those test containers microbially contaminated and having the greatest concentration of $(\text{NH}_4)_2\text{SO}_4$ developed a stable emulsion within 24 hours after initiation. No correlation was evident between the emulsion and occurrence of corrosion of the aluminum alloys.

Due to the lack of information concerning the factors pertaining to the growth and metabolic activity of the microbial cells when in an environment of JP-fuel, attempts to correlate corrosion and microbial activity of aluminum have been unsuccessful. Reproducibility of corrosion exclusively associated with microbial activity on aluminum alloys, even under stringent laboratory conditions, is difficult to obtain and is unpredictable. The "cause-effect" relationship between microbial cells and aluminum alloy corrosion could not be affirmed by the results of these studies. The study concerned with microbial metabolism (nitrate vs nitrite), initiated as the result of Blanchard's studies,^{4,4} did not appear to implicate microorganisms in aluminum alloy corrosion (Table XXV and XXVI). Thus, contrary to Blanchard's^{4,2} suggestion, microbial cells did not appear to have influenced the corrosiveness of the environment.

Although corrosion of coupons was more apparent in some of the test bottles containing viable cells than in other test containers containing identical systems, the final observation showed corrosion of some coupons in all systems. Table XXV shows that non-viable and controlled test containers had more coupons which corroded than did the viable inoculated systems. This cannot be explained based upon the present studies. Also, the number of viable cells present appears to be unrelated to the corrosion of the alloys (Table XXV).

Although field reports suggested that corrosion of aluminum alloys was associated with microbial contamination of JP-fuel systems, the results of each of the laboratory corrosion studies of aluminum alloys 2024, 7178, and 7075, under the conditions of the testing environment, did not give significant evidence to unequivocally implicate microorganisms with the corrosion problem.

Results of the studies concerning the ability of microorganisms obtained from JP-fuel to fix nitrogen (from the nitrate-nitrite studies) were negative. Since growth occurred in the absence of oxygen (Azotobacter species requires oxygen for nitrogen fixation while Clostridium pasteurianum is anerobic) and in apparently nitrogen free media, this growth is attributable to the presence of nitrogen in the deionized water and/or substituents of the media used. Of interest from the study was the development, after 6 days, of a gelatinous material in the nitrogen-free broth

Table XXV

Study C - Corrosion of Three Aluminum Alloys
in Various Water-Bottoms, Effect of NO₃ or NO₂
(Terminal Determinations)

B-H Water Bottoms	Corrosion			Side Corrosion			Ammonium			Nitrate			Nit
	V	D	C	V	D	C	V	D	C	V	D	C	V
Alloy 7178													
2	2/5	2/5	2/5	5/5	4/5	5/5	3/5	5/5	4/5	0/5	1/5	0/5	0/5
3	2/5	0/5	4/5	5/5	4/5	3/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
1	1/5	0/5	0/5	0/5	0/5	0/5	3/5	5/5	5/5	4/5	5/5	2/5	2/5
4	0/5	-	0/5	1/5	-	0/5	5/5	-	5/5	4/5	-	3/5	3/5
5	0/5	-	0/5	5/5	-	4/5	0/5	-	0/5	0/5	-	0/5	0/5
6	0/4	-	0/5	4/4	-	5/5	0/5	-	0/5	0/5	-	0/5	0/5
Alloy 2024													
2	1/5	5/5	3/5 1/5	1/5			5/5	5/5	5/5	0/5	0/5	0/5	0/5
3	4/5 3/5	3/5	5/5 2/5	0/5			0/5	0/5	0/5	0/5	0/5	0/5	0/5
1	0/5	0/5	0/5	0/5			1/5	5/5	5/5	1/5	5/5	5/5	0/5
4	2/5	-	0/5	0/5			1/5	-	5/5	1/5	-	5/5	0/5
5	0/5	-	0/5	0/5			0/5	-	0/5	0/5	-	0/5	0/5
6	0/5	-	4/5 3/5	0/5			0/5	-	1/5	0/5	-	0/5	0/5
Alloy 7075													
2	1/5	1/5	1/5	5/5	5/5	5/5	3/5	4/5	5/5	1/5	4/5	2/5	0/5
3	1/5	1/5	0/5	5/5	3/5	5/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
1	0/5	0/5	0/5		0/5	0/5	4/5	5/5	5/5	3/5	5/5	5/5	1/5
4	0/5	-	0/5		-	1/5	2/5	-	5/5	1/5	-	3/5	0/5
5	0/4	-	2/5	3/4	-	0/5	0/4	-	3/5	0/4	-	2/5	0/5
6	0/5	-	0/5	4/5	-	5/5	0/5	-	0/5	0/5	-	0/5	0/5

V = Inoculated with viable microorganisms
D = Inoculated with dead microorganisms
C = Control

2 = The number of bottles positive for the specification determination.
The number of bottles in the set.
5 = All sets contain 5 except for 7178 F inoculated and 7075 E inoculated.

Table XXV. Study C - Corrosion of Three Aluminum Alloys in Various Water-Bottoms, Effect of NO₃ or NO₂ (Terminal Determinations).

Table XXV

Study C - Corrosion of Three Aluminum Alloys
in Various Water-Bottoms, Effect of NO₃ or NO₂
(Terminal Determinations)

Corrosion	Ammonium			Nitrate			Nitrite			pH Range			Initial pH	Emulsion in Inoculated Bottles	
	C	V	D	C	V	D	C	V	D	C	V	D			C
5	5/5	3/5	5/5	4/5	0/5	1/5	0/5	0/5	0/5	0/5	4.0-4.5	6.8	6.8-7.0	7.0±	0/5
5	3/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	6.4-7.0	7.0	7/8	7.0±	0/5
5	0/5	3/5	5/5	5/5	4/5	5/5	2/5	2/5	0/5	0/5	3.5-6.0	6.4-6.6	6.0-6.8	6.5±	1/5
-	0/5	5/5	-	5/5	4/5	-	3/5	3/5	-	0/5	3.5-6.6	-	6.4-6.6	6.5±	1/5
-	4/5	0/5	-	0/5	0/5	-	0/5	0/5	-	0/5	4.0-6.8	-	6.8	7.0±	0/5
-	5/5	0/5	-	0/5	0/5	-	0/5	0/5	-	0/5	6.8-7.0	-	6.8	7.0±	0/5
		5/5	5/5	5/5	0/5	0/5	0/5	0/5	0/5	0/5	4.0	6.0-7.0	6.8	7.0±	0/5
		0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	6.8	6.0-6.8	6.0-6.8	7.0±	0/5
		1/5	5/5	5/5	1/5	5/5	5/5	0/5	0/5	0/5	4.5-6.4	6.8	6.0-6.8	6.5±	2/5
		1/5	-	5/5	1/5	-	5/5	0/5	-	0/5	3.5-6.5	-	6.4	6.5±	3/5
		0/5	-	0/5	0/5	-	0/5	0/5	-	0/5	5.0-6.8	-	6.8	7.0±	0/5
		0/5	-	1/5	0/5	-	0/5	0/5	-	0/5	4.5-6.4	-	6.8	7.0±	0/5
5	5/5	3/5	4/5	5/5	1/5	4/5	2/5	0/5	0/5	0/5	3.5-4.0	6.0-7.0	6.0-7.0	7.0±	0/5
5	5/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	6.8	6.8-7.0	6.0-7.0	7.0±	0/5
5	0/5	4/5	5/5	5/5	3/5	5/5	5/5	1/5	0/5	0/5	3.5-6.8	6.0-6.8	6.0	6.5±	0/5
-	1/5	2/5	-	5/5	1/5	-	3/5	0/5	-	0/5	4.0-6.8	-	6.0	6.5±	2/5
-	0/5	0/4	-	3/5	0/4	-	2/5	0/5	-	0/5	4.0-6.8	-	6.0-7.0	7.0±	0/5
-	5/5	0/5	-	0/5	0/5	-	0/5	0/5	-	0/5	4.0-6.8	-	6.0-7.0	7.0±	0/5

2 = The number of bottles positive for the specification determination.

0/5 / 3/5 = indicates 2 sets within that group.

5 = The number of bottles in the set.
All sets contain 5 except for 7178 F inoculated and 7075 E inoculated.

- Corrosion of Three Aluminum Alloys in Various Water-Bottoms, Effect of NO₃ or NO₂ (Terminal Determinations).

2

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Table XXVI

Study C - Viable Microbial Count/ml for Each Test Bottle for 1st 3rd 11th weeks and at 13th week.

Viable Microbial Cells in Test Containers with alloys	B-d Wt% Bottoms	Bottles of one series*					
		a	b	c	d	e	
7178	2	8.50 x 10 ⁷	1.15 x 10 ⁸	5.00 x 10 ⁷	4.50 x 10 ⁶	1.45 x 10 ⁸	
	3	1.00 x 10 ⁶	1.00 x 10 ⁸	1.00 x 10 ⁶	1.00 x 10 ⁶	1.00 x 10 ⁸	
	1	2.85 x 10 ⁸	1.90 x 10 ⁸	8.50 x 10 ⁷	1.70 x 10 ⁷	1.60 x 10 ⁸	
	4	4.45 x 10 ⁸	2.00 x 10 ⁸	7.00 x 10 ⁸	8.00 x 10 ⁸	8.50 x 10 ⁸	
	5	4.40 x 10 ⁶	3.35 x 10 ⁶	4.35 x 10 ⁶	4.30 x 10 ⁶	4.00 x 10 ⁸	
	6	1.00 x 10 ⁶	-				
2024	2	2.55 x 10 ⁸	7.50 x 10 ⁷	5.00 x 10 ⁶	2.85 x 10 ⁶	8.00 x 10 ⁶	
	3	1.00 x 10 ⁶					
	1	1.02 x 10 ⁹	1.01 x 10 ⁹	6.70 x 10 ⁹	9.00 x 10 ⁷	5.75 x 10 ⁹	
	4	1.12 x 10 ⁶	8.00 x 10 ⁸	1.00 x 10 ⁸	1.55 x 10 ⁸	1.98 x 10 ⁸	
	3	1.00 x 10 ⁶	5.30 x 10 ⁸	4.55 x 10 ⁸	4.15 x 10 ⁶	3.25 x 10 ⁶	
	6	1.00 x 10 ⁶					
7075	2	7.50 x 10 ⁷	6.55 x 10 ⁶	3.50 x 10 ⁶	5.90 x 10 ⁶	1.05 x 10 ⁸	
	3	1.00 x 10 ⁸	1.00 x 10 ⁸	1.00 x 10 ⁸	1.00 x 10 ⁹	1.00 x 10 ⁸	
	1	9.45 x 10 ⁹	2.10 x 10 ⁹	1.20 x 10 ⁸	1.07 x 10 ⁷	9.00 x 10 ⁸	
	4	2.06 x 10 ⁸	3.27 x 10 ⁸	5.40 x 10 ⁸	3.50 x 10 ⁸	8.35 x 10 ⁸	
	5	7.00 x 10 ⁸	6.35 x 10 ⁶	3.55 x 10 ⁶	3.90 x 10 ⁸	-	
	6	1.00 x 10 ⁶					
7178	2	1.23 x 10 ⁸	1.00 x 10 ⁷	1.00 x 10 ⁷	3.33 x 10 ⁷	1.33 x 10 ⁸	
	3	1.27 x 10 ⁷	5.67 x 10 ⁶	6.00 x 10 ⁷	1.00 x 10 ⁶	1.03 x 10 ⁷	
	1	4.00 x 10 ⁶	6.70 x 10 ⁷	2.10 x 10 ⁷	6.67 x 10 ⁶	1.33 x 10 ⁷	
	4	6.67 x 10 ⁸	1.00 x 10 ⁸	1.00 x 10 ⁸	1.33 x 10 ⁸	4.27 x 10 ⁸	
	5	2.33 x 10 ⁸	2.40 x 10 ⁸	4.30 x 10 ⁷	3.67 x 10 ⁸	3.13 x 10 ⁸	
	6	1.33 x 10 ⁸	1.27 x 10 ⁸	8.33 x 10 ⁷	1.67 x 10 ⁸	-	
2024	2	3.00 x 10 ⁷	1.00 x 10 ⁷	1.00 x 10 ⁷	1.00 x 10 ⁷	1.67 x 10 ⁷	
	3	2.17 x 10 ⁸	3.33 x 10 ⁷	4.67 x 10 ⁷	1.33 x 10 ⁷	4.33 x 10 ⁷	
	1	5.27 x 10 ⁸	6.33 x 10 ⁶	1.00 x 10 ⁷	1.07 x 10 ⁷	1.00 x 10 ⁸	
	4	6.47 x 10 ⁷	6.67 x 10 ⁶	1.00 x 10 ⁶	2.00 x 10 ⁶	3.80 x 10 ⁸	
	5	1.40 x 10 ⁸	3.40 x 10 ⁸	2.00 x 10 ⁸	3.97 x 10 ⁸	3.33 x 10 ⁸	
	6	1.03 x 10 ⁸	1.63 x 10 ⁸	1.10 x 10 ⁸	1.07 x 10 ⁸	1.03 x 10 ⁸	
7075	2	1.33 x 10 ⁷	3.33 x 10 ⁷	1.33 x 10 ⁷	3.33 x 10 ⁷	1.00 x 10 ⁷	
	3	1.03 x 10 ⁷	1.20 x 10 ⁷	8.00 x 10 ⁷	8.33 x 10 ⁷	7.00 x 10 ⁸	
	1	1.40 x 10 ⁸	1.00 x 10 ⁷	1.67 x 10 ⁸	1.00 x 10 ⁶	2.00 x 10 ⁶	
	4	4.33 x 10 ⁸	1.00 x 10 ⁸	7.06 x 10 ⁸	6.67 x 10 ⁶	6.67 x 10 ⁶	
	5	3.17 x 10 ⁸	3.87 x 10 ⁸	2.33 x 10 ⁸	2.80 x 10 ⁸	-	
	6	1.00 x 10 ⁸	1.23 x 10 ⁸	1.20 x 10 ⁷	2.13 x 10 ⁸	8.33 x 10 ⁷	
7178	2	1.00 x 10 ⁶	5.33 x 10 ⁶	11 weeks	2.38 x 10 ⁷	3.33 x 10 ⁷	
	3	1.00 x 10 ⁶	2.42 x 10 ⁷	11 weeks	3.12 x 10 ⁷	8.33 x 10 ⁶	
	1	1.13 x 10 ⁸	1.23 x 10 ⁷	11 weeks	1.30 x 10 ⁶	8.33 x 10 ⁵	

Table XXVI. Study C - Viable Microbial Count/ for 1st, 3rd, 11

medium inoculated with the suspected nitrogen fixer. A repeat of the procedure resulted in similar results. Microscopic analysis of the gelatinous material indicated long chains of bacterial cells.

The results of the study concerned with the use of the corrosometer probe as a device to indicate microbial induced corrosion in JP-fuel systems are shown in Table XXVII and Figures 35 to 40. The change in corrosometer dial readings shown in Figure 40 was selected over corrosion in mils/year in order to show more clearly the changes of the probe in microbially inoculated and non-inoculated JP-4 fuel/water systems. Table XXVII presents a tabulation of the dial readings obtained with the corrosometer probes, the calculated change in dial readings, temperature variation during the study, and the microbial concentration. Figure 40 is a graphic presentation of these data. It can be seen in Table XXVII and Figure 40 that the corrosometer reading of the steel probe showed a definite relationship between the JP-fuel systems microbially inoculated and non-inoculated. The inoculated system exhibited a linear rate of corrosion throughout the experimental period. Some change in the resistance in the uninoculated system was also noted. Of particular interest was the continuation of the corrosion process of the probe while the bacterial count reached a stationary phase about the twelfth day and remained there for approximately 28 days. Since the dial readings (resistance) did not alter to any great degree in the non-inoculated JP-systems, these data indicate that products of microbial metabolism were responsible for the corrosion and that these products were either present in excess at the termination of the log phase of growth (1st through the 12th day) or were continually produced by the microbes during the stationary phase of growth. Thus, the corrosion of steel appeared to be directly influenced by the activity of the viable microorganisms in this experimental system. This is in agreement with Dooley and West⁴⁸ and with literature concerning iron corrosion.

The results with the aluminum probes indicate that aluminum alloys as used in aircraft are not susceptible to microbially induced corrosion under the conditions of this experiment. Figures 36 and 38 show that the alloy probes 2024 and 7075 were corroded to a slight degree in the microbially inoculated JP-fuel system. The degree of resistance, however, was not sufficient to warrant the use of the probes as corrosion indicators in JP-fuel systems. The observation in the corrosion study (Section VI) of the resistance of 2024 alloy to visible corrosion and the finding that the 2024 corrosometer probe is a somewhat more sensitive indicator of corrosion cannot be explained on the basis of the available data. Figures 35 through 39 indicate the visible corrosions of various corrosometer probes used in this study.

The evaluation of the use of the aluminum alloy strip corrosometer probes as a means of detecting microbially contaminated aircraft

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Table XXVII

Record of Corrosionmeter Probes in Microbially Inoculated and Uninoculated JP-4 Fuel/Water Systems

Total time in Days	Temperature °C	Iron Probe 8001				Aluminum Probe 2024			
		Microbial Count/ml		Change in Dial Readings		Microbial Count/ml		Change in Dial Readings	
		Inoculated	Uninoculated	Inoculated	Uninoculated	Inoculated	Uninoculated	Inoculated	Uninoculated
0	25.0	-	0	-	-	-	-	-	-
1	25.5	-	0	11.0	12.4	2.50×10^3	0	2.0	2.30
2	25.0	2.09×10^5	0	26.0	21.7	2.10×10^5	0	8.5	5.30
5	26.0	-	-	38.5	28.7	-	-	12.5	0.30
6	25.5	1.04×10^7	-	42.5	33.7	8.2×10^7	0	16.5	1.6
7	25.5	6.30×10^7	-	47.5	35.7	1.6×10^8	0	22.0	1.85
8	26.0	-	-	52.5	35.7	-	-	25.0	0.85
11	32.0	$<1.00 \times 10^9$	-	74.5	40.7	3.60×10^8	0	27.5	-2.35
12	27.5	-	↑	86.5	46.7	-	-	34.5	3.35
13	26.5	-	↑	91.0	47.2	-	-	35.0	3.1
14	26.5	-	↑	95.0	46.2	-	-	35.0	1.85
15	26.0	-	↑	101.5	49.2	-	-	37.0	3.85
18	26.0	1.18×10^9	↑	115.0	49.2	8.80×10^8	-	36.5	3.1
19	26.0	-	↑	119.5	50.2	-	-	36.5	2.35
20	26.0	-	↑	124.5	51.7	-	-	37.0	4.1
21	26.5	-	↑	130.5	53.2	-	-	37.0	3.6
22	26.0	-	↑	132.0	52.7	-	-	37.0	3.85
25	26.75	-	↑	149.5	51.7	-	-	39.5	0.35
26	28.0	-	↑	158.5	55.7	-	-	33.5	1.35
27	27.5	-	↑	172.5	61.7	-	-	37.5	5.1
28	28.0	1.02×10^9	↑	180.0	62.2	1.40×10^9	-	39.5	8.35
29	28.0	-	↑	186.5	61.7	-	-	38.5	7.1
32	28.5	-	↑	201.5	59.7	-	-	33.5	3.6
33	28.0	1.76×10^9	↑	210.5	61.7	3.16×10^9	-	35.5	3.6
34	27.0	-	↑	217.5	62.7	-	-	37.5	4.85
35	28.5	-	↑	226.5	65.7	-	-	36.7	6.6
36	-	-	↑	238.5	66.2	-	-	39.5	7.1
39	28.0	-	↑	258.5	69.7	-	-	40.5	7.85
40	28.0	-	↑	261.5	67.7	-	-	39.5	7.85
41	28.0	1.65×10^9	↑	271.5	70.2	2.15×10^9	-	40.5	9.1
42	28.0	-	↑	288.5	69.2	-	-	40.5	8.85
43	29.0	-	↑	298.0	70.2	-	-	41.5	8.85
46	29.0	-	↑	328.5	76.7	-	-	43.5	8.6
47	29.0	-	↑	342.5	72.7	-	-	44.0	9.5
48	29.75	7.33×10^7	↓	348.5	68.7	1.19×10^9	0	41.5	6.85

* an average of 2 probe readings

- indicates analysis omitted

Table XXVII. Record of Corrosionmeter Probes in Microbially Inoculated and Uninoculated JP-4 Fuel/Water Systems.

Table XXVII

Record of Corrosometer Probes in Microbially Inoculated and Uninoculated JP-4 Fuel/Water Systems

		Aluminum Probe 2024				Aluminum Probe 7075			
Change in Dial Readings		Microbial Count/ml		Change in Dial Readings		Microbial Count/ml		Change in Dial Readings	
Inoculated	Uninoculated	Inoculated	Uninoculated	Inoculated	Uninoculated *	Inoculated	Uninoculated	Inoculated	Uninoculated
-	-	-	-	-	-	-	-	-	-
12.4	-	2.50×10^3	0	2.0	2.35	1.0×10^3	0	0.9	1.85
21.7	-	2.10×10^5	0	8.5	5.35	1.89×10^5	0	4.9	6.1
28.7	-	-	-	12.5	0.35	-	0	-2.6	-0.9
33.7	-	8.2×10^7	0	16.5	1.6	1.54×10^8	-	-0.1	1.1
35.7	-	1.6×10^8	0	22.0	1.85	8.83×10^8	-	0.15	1.35
35.7	-	-	-	25.0	0.85	-	-	-1.1	1.1
40.7	-	3.60×10^8	0	27.5	-2.35	2.89×10^8	-	-4.6	-2.4
46.7	-	-	-	34.5	3.35	-	-	1.65	4.35
47.2	-	-	-	35.0	3.1	-	-	1.65	4.35
46.2	-	-	-	35.0	1.85	-	-	-0.1	2.6
49.2	-	-	-	37.0	3.85	-	-	2.15	5.35
49.2	-	8.80×10^8	-	36.5	3.1	4.63×10^8	-	1.15	4.6
50.2	-	-	-	36.5	2.85	-	-	1.15	4.1
51.7	-	-	-	37.0	4.1	-	-	1.65	5.1
53.2	-	-	-	37.0	3.6	-	-	1.4	5.35
52.7	-	-	-	37.0	3.85	-	-	0.65	4.85
51.7	-	-	-	33.5	0.35	-	-	-4.1	-0.15
55.7	-	-	-	33.5	1.35	-	-	-2.85	1.1
61.7	-	-	-	37.5	5.1	-	-	2.15	5.85
62.2	-	1.44×10^9	-	39.5	8.35	2.5×10^8	-	5.15	8.85
61.7	-	-	-	38.5	7.1	-	-	3.15	7.1
59.7	-	-	-	35.5	3.6	-	-	-1.35	2.6
61.7	-	3.16×10^9	-	35.5	3.6	1.68×10^9	-	-0.1	2.85
62.7	-	-	-	37.5	4.85	-	-	0.65	3.85
65.7	-	-	-	39.	6.6	-	-	4.65	7.35
66.2	-	-	-	39.3	7.1	-	-	4.65	6.35
69.7	-	-	-	40.5	7.85	-	-	6.65	7.35
67.7	-	-	-	39.5	7.85	-	-	6.65	7.35
70.2	-	2.25×10^9	-	40.5	9.1	1.95×10^9	-	8.15	8.35
69.2	-	-	-	40.5	8.85	-	-	7.4	7.6
70.2	-	-	-	41.5	8.85	-	-	9.9	9.1
70.7	-	-	-	43.5	8.6	-	-	9.65	9.85
72.7	-	-	-	44.0	9.6	-	-	10.65	9.6
68.7	-	1.53×10^8	0	41.5	6.85	2.2×10^8	0	7.65	4.85

↑
Analysis omitted
↓

Record of Corrosometer Probes in Microbially Inoculated and Uninoculated JP-4 Fuel/Water Systems.



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Figure 35.
Corrosion of Iron Corrosometer Probes
(No. 1 from Uninoculated Test System,
No. 2 from Microbially Contaminated Test System).

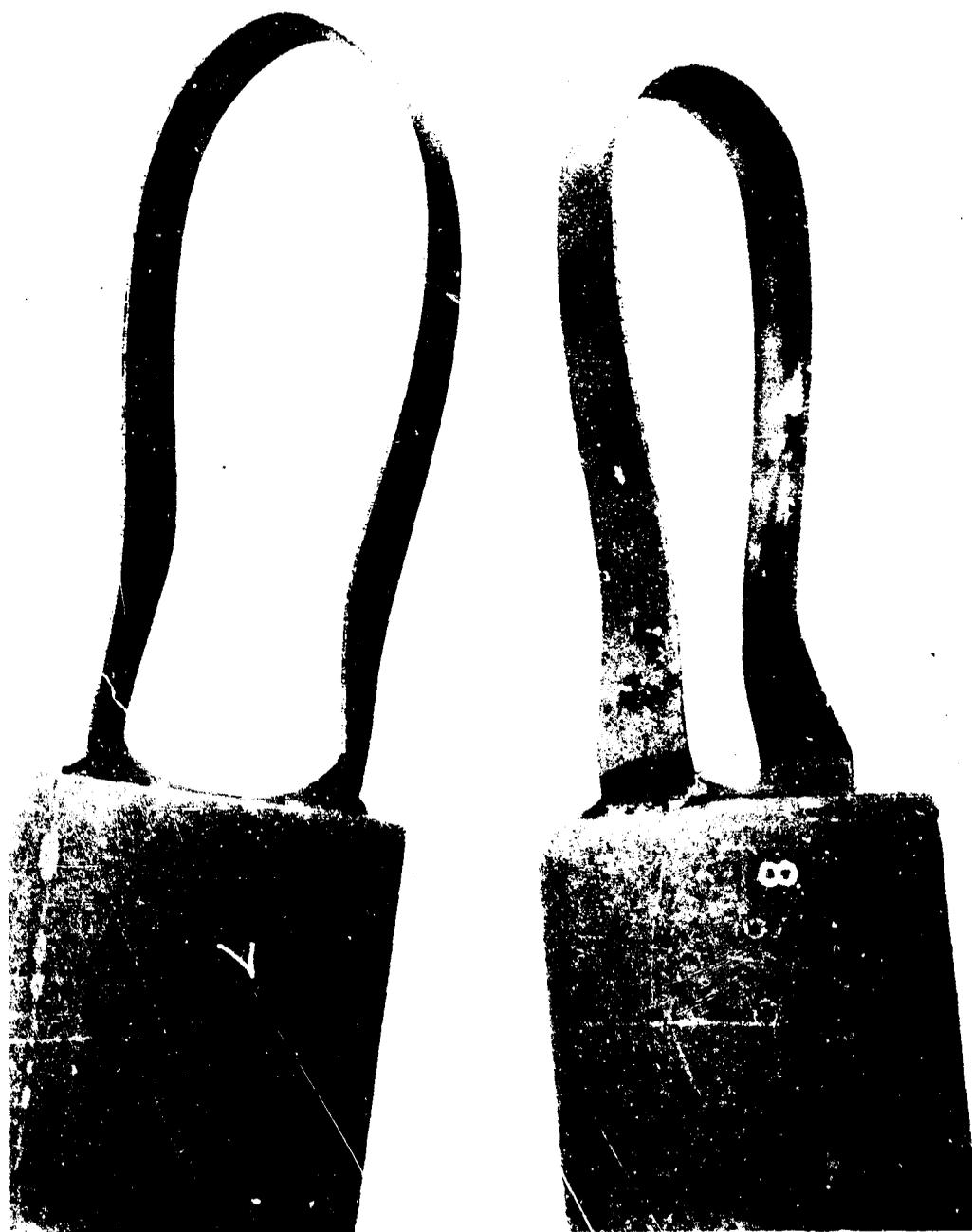


Figure 36.
Corrosion of Aluminum Corrosometer Probes 2024
from Microbially Contaminated Test System.

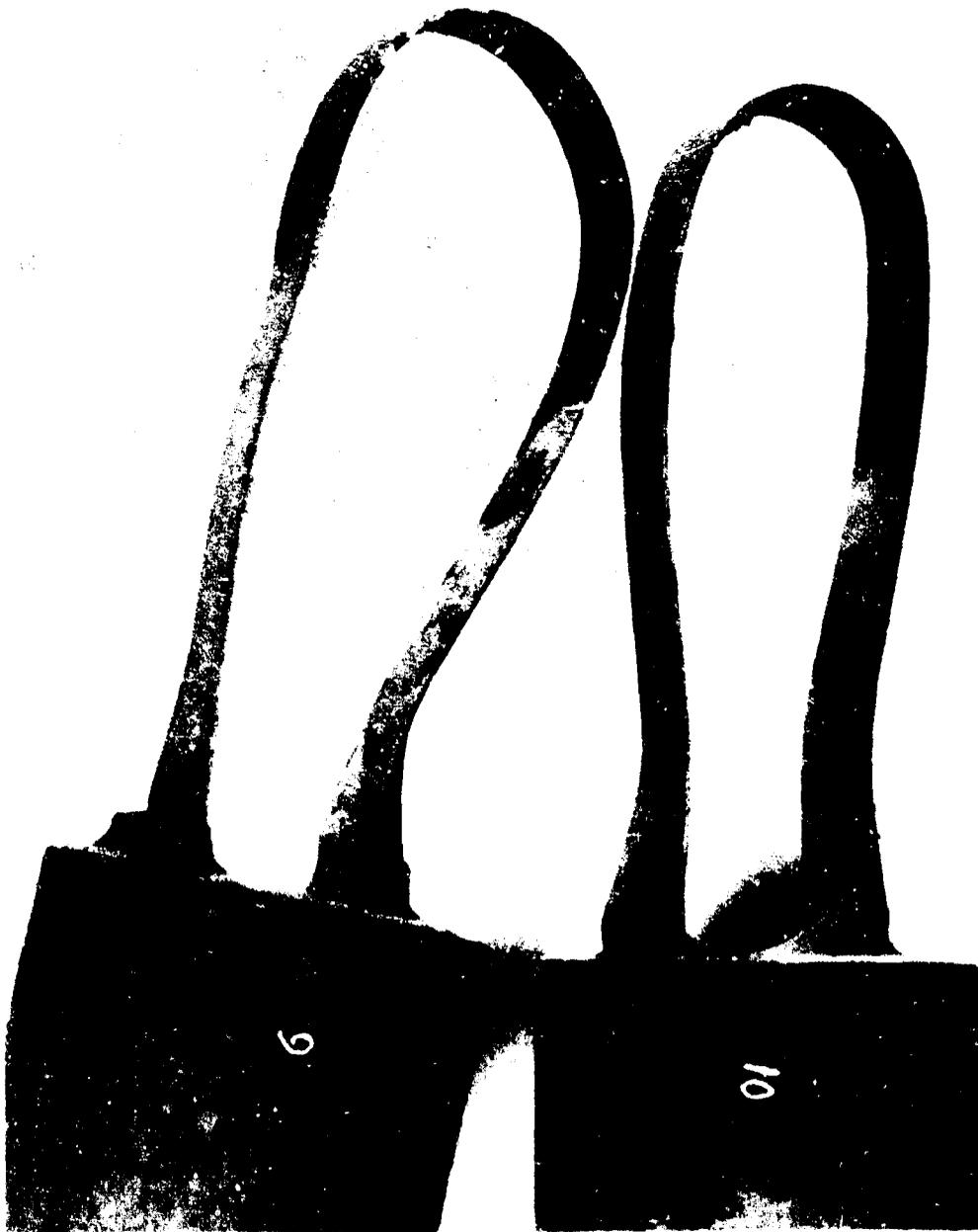


Figure 37.
Corrosion of Aluminum Corrosometer Probes
2024 from Uninoculated Test System.

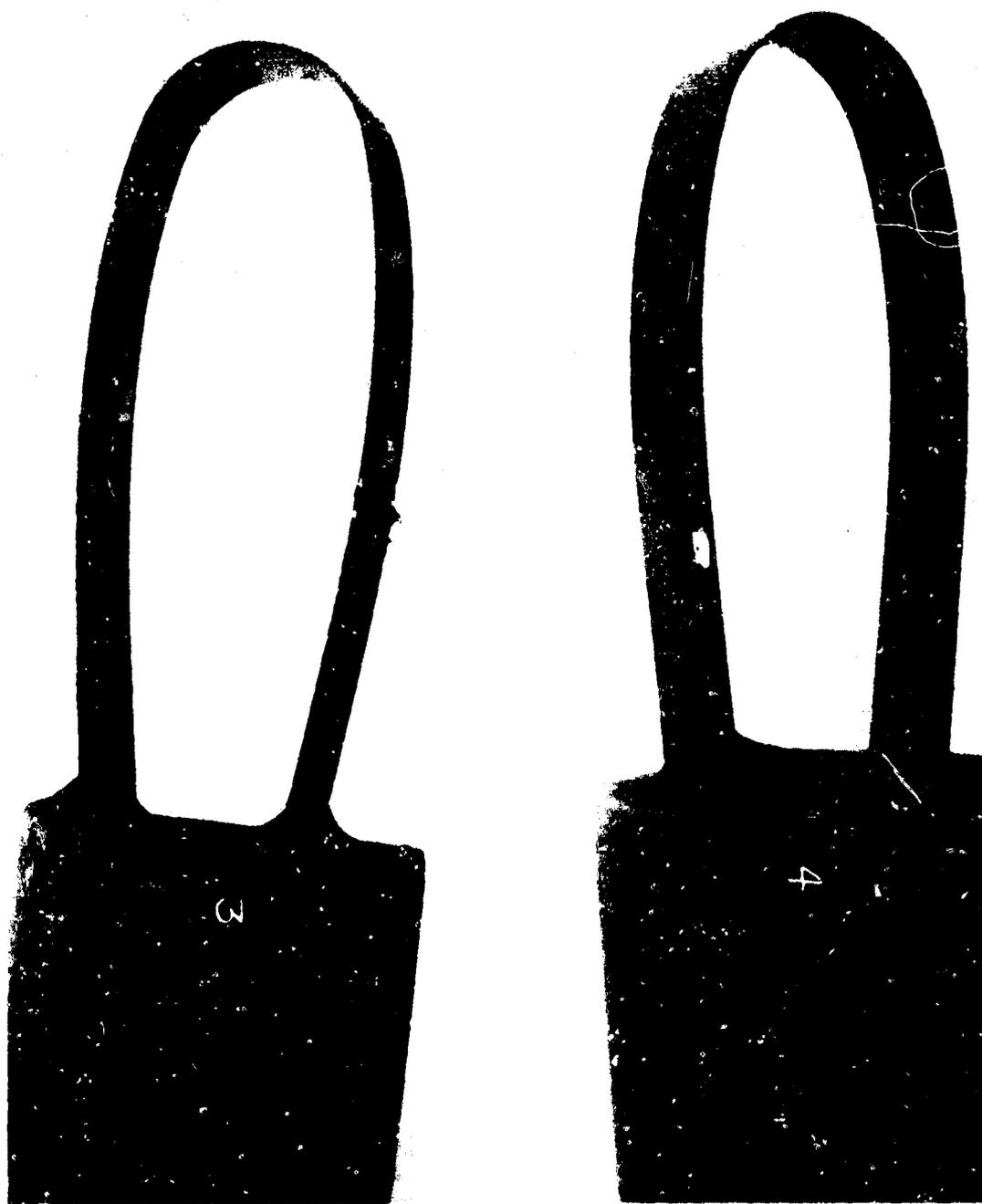


Figure 38.
Corrosion of Aluminum Corrosometer Probes
7075 from Microbially Contaminated Test System.



Figure 33.
Corrosion of Aluminum Corrosometer Probes 7075
from Uninocular Test System.

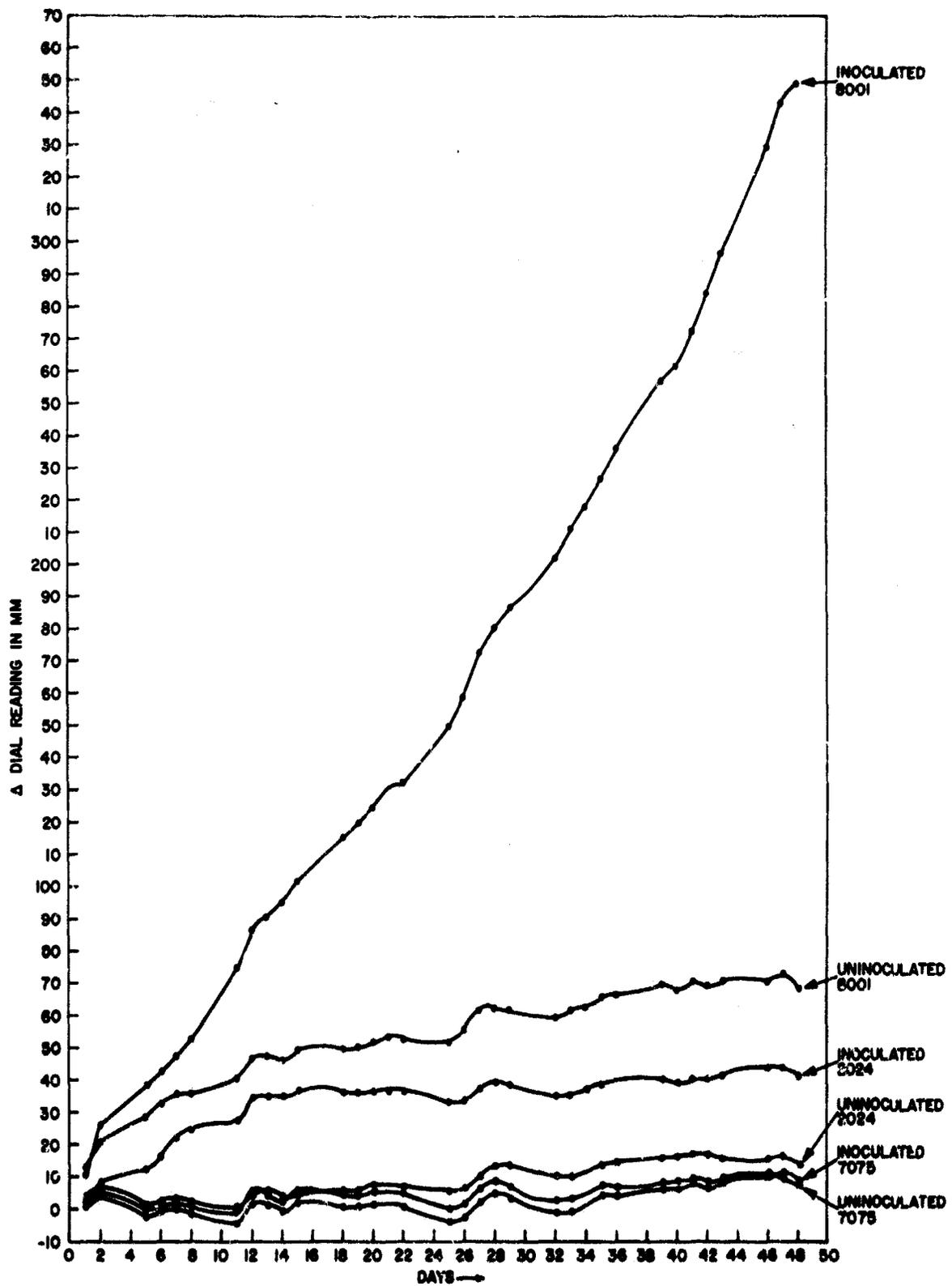


Figure 40.
 Change in Corrosometer Dial Readings of Probes Iron 6001, and Aluminum Alloys 2024 and 7075 in Microbially Inoculated and Non-inoculated JP-4 Fuel/Water Systems.

JP-fuel systems was a preliminary study. However, with the attention now given to fuel handling procedures and the use of EGME-glycerol in JP-fuels, a device to detect microbial corrosion in aircraft is no longer in demand. Microbial corrosion of aluminum of the integral fuel tanks is no longer considered a major factor in the overall corrosion problem still in existence. In fact, microorganisms in JP-fuel systems may never have been a problem, or at least not a problem of the magnitude initially believed.

The implication that microbial corrosion is not considered a problem at this time is not to be interpreted as meaning that microbial corrosion is non-existent or will not become a problem in the future. Mutant or adaptable strains from the present population of hydrocarbon microbes contaminating the JP-fuels may increase to sufficient quantities to create microbial corrosion problems or perhaps other types of fuel system degradation.

CONCLUSIONS

1. The tests performed did not unequivocally implicate microorganisms with the corrosion of aluminum alloys 7178, 2024, and 7075.
2. Sea water, even in diluted form, with or without microbial contaminants, was corrosive to aluminum alloys 7178, 2024, and 7075.
3. Aircraft sump water appeared to be as corrosive to aluminum alloys 7178, 2024, and 7075 as was the sea water.
4. Of the aluminum alloys included in the coupon corrosion studies, alloy 2024 was the most resistant. In the event of corrosion, 2024 exhibited a pit-type corrosion and 7075 exhibited an intergranular type.
5. Of the microorganisms surviving in a JP-4 fuel and deionized water system and tested for a nitrogen fixation, nitrogen fixation was not evident.
6. The studies concerning the use of aircraft structural aluminum in corrosometer probes as a detection device for microbial corrosion of integral fuel cells was a preliminary study with evidence indicating that the rate of corrosion was insufficient to show microbially induced corrosion.

Section VII

EFFECT OF MICROBIOLOGICAL CONTAMINATION ON FUEL GAGE CAPACITANCE PROBES

The presence of microorganisms and microbially produced material has been implicated in the production of slime and sludge in JP-4 fuel/water systems.^{1, 46} The slime and sludge have, in turn, been suspected as a cause for the malfunctioning of fuel gage capacitance probes^{9, 47, 48, 49} of aircraft. However, direct evidence of microbial involvement in fuel gage malfunctioning was not available in the literature reviewed.

To determine the relationship of microorganisms and the microbial slime or sludge to the malfunctioning of aircraft fuel gage capacitance probes, probes of four USAF aircraft were installed in simulated field JP-4 fuel/water systems, contaminated and non-contaminated, and observed for a period of time. During the period of observation the probes were attached to a capacitance bridge to determine the stability of the fuel measurements. To correlate probe malfunctioning to microbial population, the water-bottom of each test system was sampled periodically and the number of viable aerobic microbial cells were calculated. Since microbial population and probe malfunctioning were to be correlated, each probe was disassembled and examined at the termination of the study.

METHOD AND MATERIALS

Microbiological Analysis

Viable microbial cells in the water-bottoms of each test system were determined by the spread plate technique (Section II).

Probe Study

Materials. The test containers consisting of two 208 L (55 gal.) aluminum drums and two 20.3 x 20.3 x 20.3 cm Buna-N coated simulated aircraft fuel cells with removable lids, were fitted with probe sleeves to rigidly hold each probe in place during the test period. Sleeves of aluminum for three of four probes and a vent for the escape of fuel vapors were installed into holes cut along one side of each drum. One Buna-N plugged sampling port, secured in place by a metal band approximately 180° from the probes, facilitated the sampling of the water-bottoms of each drum for microbial enumeration. End gages attached to each drum enabled the observation of the fuel-water interface (Figure 41). Sleeves of Buna-N were affixed in the lids of the model fuel cell tanks. The water-bottoms of each model tank were sampled via a

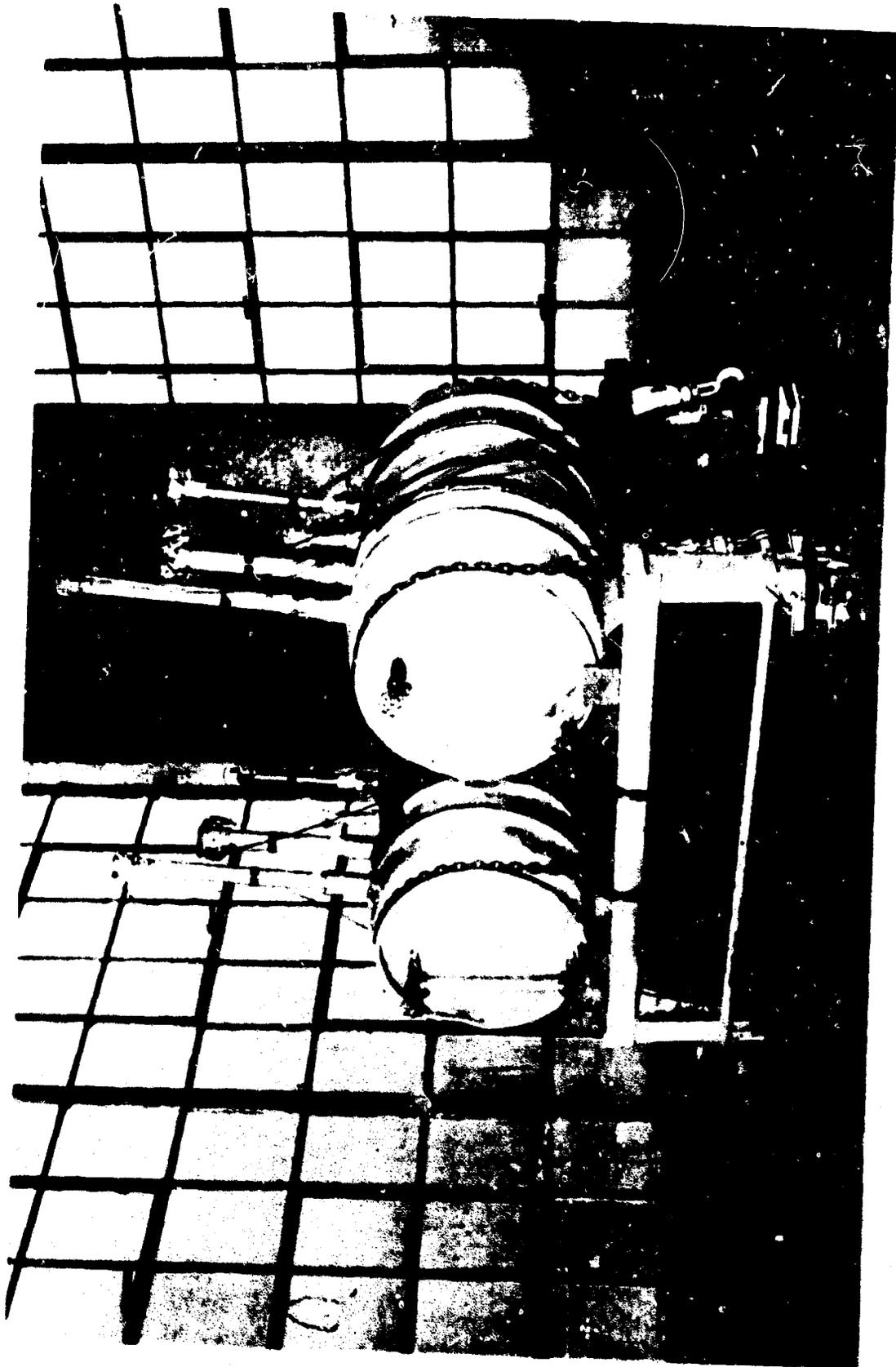


Figure 41. Test System for Fuel Gage Capacitance Probe Study.

Buna-N plugged sampling port which was located at the base of the tanks (Figure 42). The ports were secured in place by a metal band.

The test JP-4 fuel was an anti-icing free fuel obtained from a Wright-Patterson Air Force Base storage facility.

The water-bottoms were composed of an autoclave sterilized B-H-1 salt solution. The solution was initially prepared in double strength, sterilized, cooled, and diluted to the proper concentration. The solution was autoclaved in an 18.9 L (5 gal.) clear glass carboy for a period of 30 minutes. After sterilization the water was cooled for 96 hours at room temperature.

The microbial inoculum consisted of a composite of 10^4 cells/ml of air contaminants (determined after the concentration of B-H-1 solution had been diluted) and 1.4 ml of Laboratory Cultures 7, 8, and 9.

The gage probes of the study were:

Liquidometer Pin used in the F-105

Part No. Ea772 GCB1662
Stock No. 6680 827-4626
Order No. AF36 600 9128
Serial No. A96339-23
Cap. 73. 74 and 73.65 μMF

Minneapolis Honeywell Pin used in the KC-97

Part No. JG7013 A58
Stock No. 15-19423-74
Spec. No. 372442-701
Serial No. 10383 and 10419

Avien Pin used in the C-124

Part No. 155-022-222
Stock No. 6680-090-1852
Control No. AF36-600-13465
Serial No. A406 and A393

Simmons Pin used in the F-102

Part No. 381056-A0652
Stock No. 6680-561-4732
Order No. AF36 600-10834
Serial No. 4185H

Attached to each probe was a central capacitance bridge (precision type P582, General Radio Co., Serial No. 103, Cambridge, Mass.) to indicate the operational status of each probe.



Figure 42. Sampling Fuel Gage Capacitance Test Containers.

To determine if the environmental temperature could be correlated with erroneous readings of fuel capacitance probes, the environmental temperature was recorded (Honeywell-Brown Electric roll type recorder).

All containers were placed on a large tilt-table platform that tilted 15° 11 times/min (Figure 43a and 43b) to facilitate agitation of each test system.

Procedures. With probe sleeves welded in place, the drums were washed with hot tap water containing a laboratory detergent, rinsed once with 0.1% Roccal solution and steam sterilized for 2 hours. Immediately after steaming all orifices were capped with sterile cotton and aluminum foil coverings.

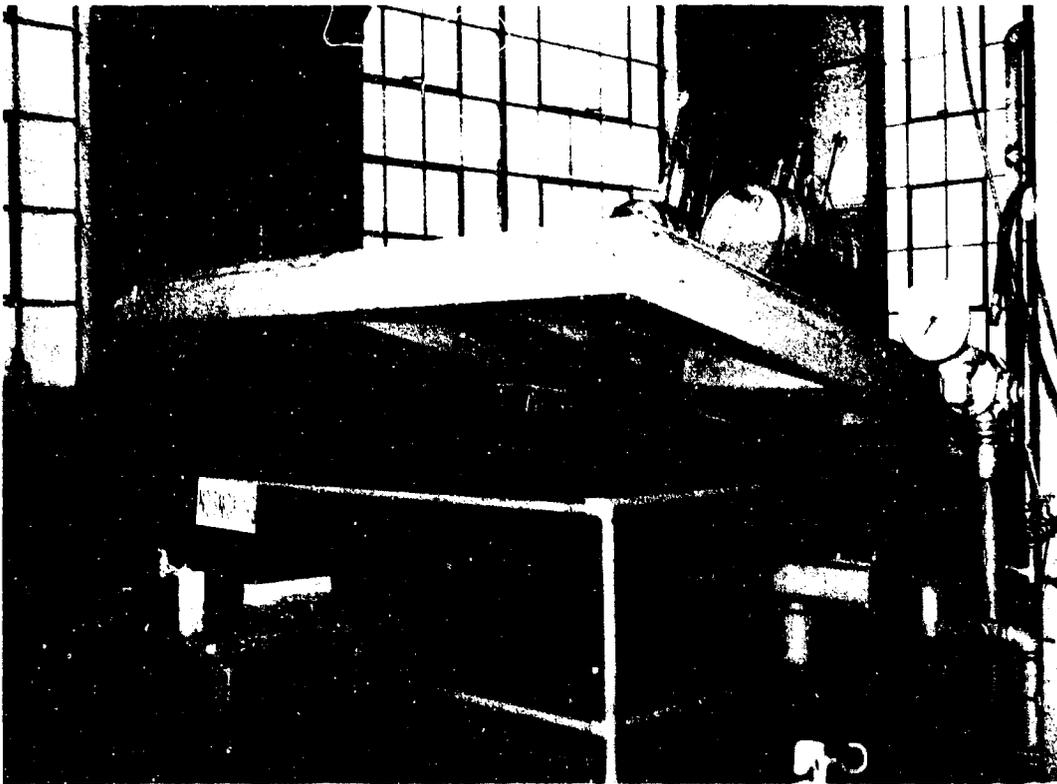
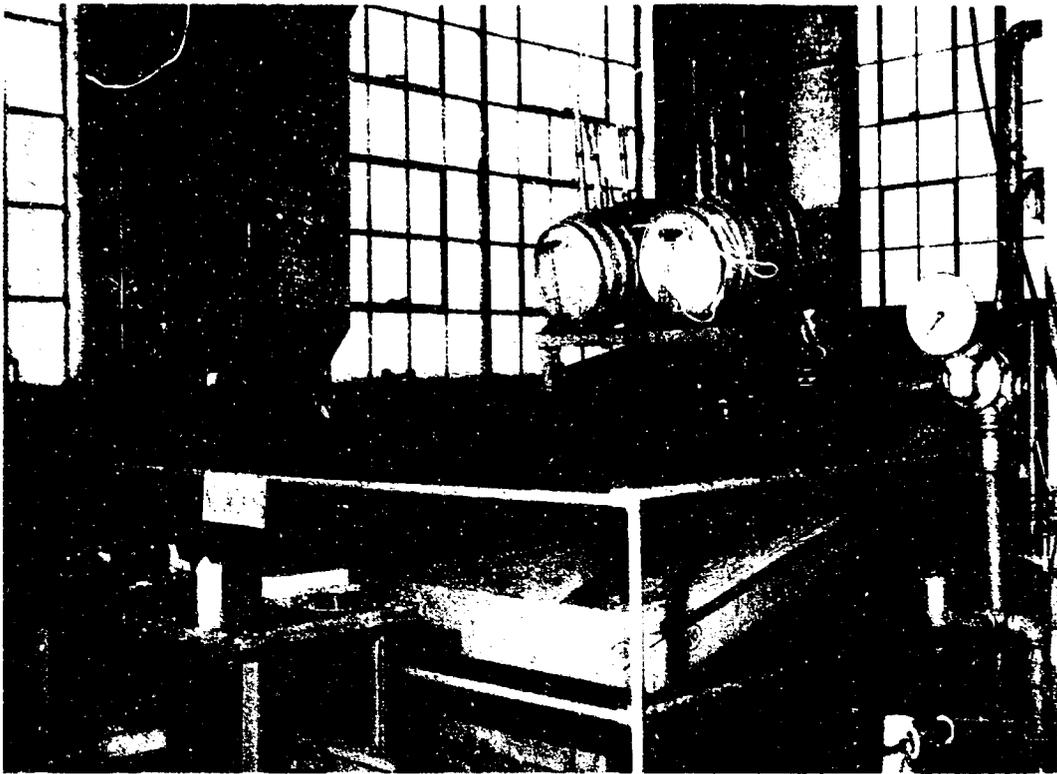
The two model fuel cells were cleaned with hot tap water containing a detergent, rinsed once with distilled water, rinsed once with a 0.1% Roccal solution, and rinsed twice with sterile distilled water. The cleaned removable lids were immediately set in place and securely fastened. The Buna-N sleeve gasket was immediately covered as were the drum probe sleeves.

Each probe was soaked, up to the connecting capacitance connectors, in 0.1% Roccal solution for 3 to 5 hours, rinsed with 3 L of sterile distilled water, packaged in sterile brown wrapping paper while wet, and dried in a hot air oven at approximately 35°C overnight.

The growth support characteristic of the test JP-4 fuel to microbial cells was determined by observing, for a period of 2 weeks prior to the test period, the viable microbial cell count of a system consisting of 300 ml each of JP-4 fuel and B-H-1 solution.

Prior to inserting the probes in each test container, or positioning the test containers on the tilt-table platform, 96.5 L (26 gal.) of JP-4 fuel were filter sterilized through a 0.45 micron Millipore membrane filter (laboratory sterilized filter holder No. YY30-142-00) directly into each drum via the fume exhaust sleeve. To each model wing tank 200 ml of similarly sterilized fuel were added.

The addition of contaminated or non-contaminated B-H-1 solution to each drum and model fuel tank followed the positioning and securing of each test container to the tilt table platform. To minimize the amount of water in each testing container, but to assure probe contact with a suspension of water-bottom material during the testing agitation period 15 L or 10.2 cm of water-bottom (at the lowest point of the drum) were added via the fume exhaust sleeve to each drum, and 50 ml of B-H-1 solution were added to each model wing tank. The microbiological contamination level (or sterility) of each water-bottom was determined within 3 hours after the addition of the B-H-1 to the fuel. Prior to the addition of the B-H-1 solution, the sterility



Figures 43a and 43b. Test System for Fuel Gage Capacitance Probe Study showing the tilt of the Agitation Table.

of all fuel was determined by obtaining 0.1 ml aliquots of fuel via the bottom sampling ports, and plating to TSB with 1.5% Bacto-agar plates.

As aseptically as possible the probes were unwrapped and each inserted into its designated sleeve. The probes were affixed just above the fuel-water interface. Aluminum foil caps were placed over all probes to prevent any additional air contaminants from entering the testing systems.

Initial readings of each probe were made upon connection to the capacitance bridge apparatus. The 208 L drums and both model wing tank systems were agitated 3 times per week for 1 hour over a period of 3 months. The observation period of the control drum was extended 3 weeks. Capacitance readings and microbial population determinations were scheduled for all systems prior to and directly after each agitation. All samples of the systems were obtained via the base sampling ports with a sterile hypodermic syringe fitted with an 18 gage needle (Figure 42).

At the termination of the observation period, all probes were removed and disassembled. Swab samples of all adhering slime material were streaked to solid growth medium to determine the presence of viable microorganisms. In addition, slime-like material was examined microscopically.

Upon completion of the study, all test containers were emptied and inspected for any deterioration. Swab samples of areas indicating deterioration were subjected to microbial analysis.

RESULTS AND DISCUSSION

Of the four types of probes tested, one probe (Liquidometer Pin of the F-105 aircraft) ceased to give capacitance readings one month after exposure to microbiologically contaminated JP-4 fuel/water (Figure 44). When the malfunctioning probe was disassembled, a bridge of slime was observed between the inner surface of the inner capacitance plate and the support rods (Figure 45) which stabilize the plate. Each of the other probes showed similar gelatinous adhering material on the inner portions of probe plates. However, a bridge of slime was not noted in any of the other probes between the probe plates and support pins. Except for the support pins of the probe that malfunctioned, which were at the base of the probe (Figure 44), all other support pins or structures were located close to or above the middle of the probe plates, or were situated so that a bridge of slime could not be formed between supporters and capacitance plates.

On the 80th day of observation the water-bottom of the control drum was found to be contaminated (Table XXVIII). To determine if the Liquidometer Pin of the F-105 aircraft would become inoperative as did the similar probe in the microbially contaminated drum, the control drum was observed for an additional 3 weeks. Approximately 1 month after the

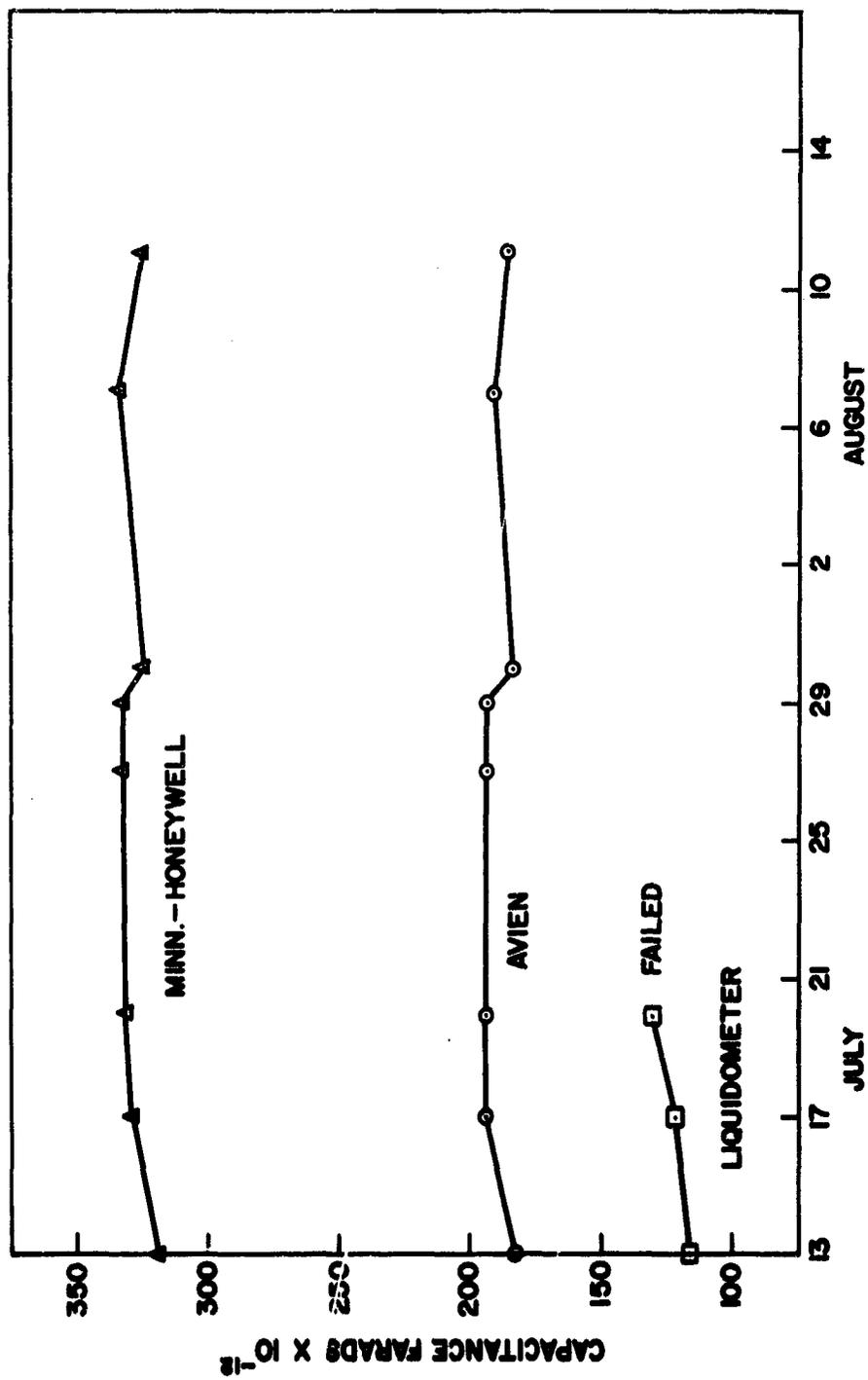


Figure 44. Capacitance Readings of Fuel Capacitance Probes Contained in Control Drum After Microbial Contamination Disassembled.

Table XXVIII

Control Drum Contamination, Cells/ml

Sampling Time in Days	Cells/ml	
	Prior to Agitation	After Agitation
80	TNTC	TNTC
89	TNTC	Not Run
92	1.45×10^7	Not Run
101	1.05×10^6	5.50×10^6
108	4.67×10^6	1.17×10^7
114	7.00×10^6	AF3600-9128 malfunctioned 7.00×10^6
120	no agitation	1.87×10^6

Table XXVIII. Control Drum Contamination, Cells/ml.

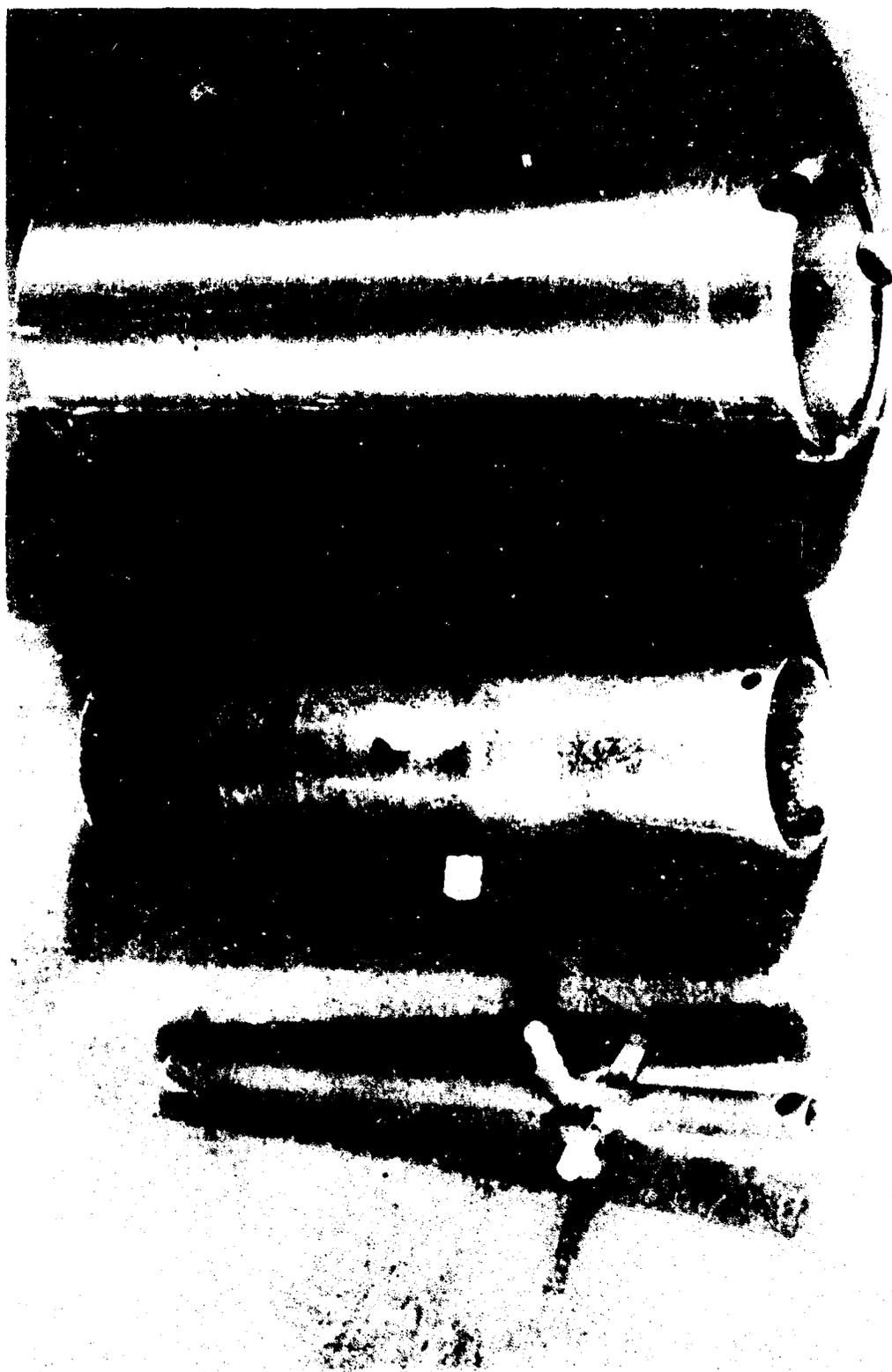


Figure 45. Liquidmeter Pin eatt2gd b 1662 used on F-105 Aircraft.

water-bottom of the control drum had become microbially contaminated, the Liquidometer pin ceased to give a capacitance reading (Figure 44). To assure that the pin had malfunctioned, capacitance readings were continued for an additional 2 weeks. At that time observation of the system was terminated, and the liquidometer probe was disassembled. The bridge of slime between support rods and capacitance plates was again observed. Microbiological analysis indicated that the slime was of biological origin or at least associated with microbial activity. Under the conditions of this test the results indicate microorganisms can cause the malfunctioning of fuel gage capacitance probes.

Although fluctuations were noted in capacitance readings of all probes (Figures 46, 47, 48, 49), no correlation between microbial population (Table XXIX) and capacitance readings could be made, for the number of microbial cell/ml reached its peak (10^7 for the drums and 10^8 cells/ml for the wing tanks) within 10 days and maintained approximately this level throughout the observation period. A decrease to 10^6 cells/ml was noted 2 weeks prior to termination of the study in the simulated wing tanks, (Table XXIX). These fluctuations may have been due to temperature changes.

Small areas of deterioration were noted in the 208 L drum. Swab samples of each of the areas indicated the presence of viable microbial cells. No deterioration was noted of the coating material of the simulated fuel cell tanks.

CONCLUSIONS

Under the conditions of this study it is concluded that viable microbial cells can cause the malfunctioning of fuel gage capacitance probes if the engineering design is such that it will permit the microbes to form a gelatinous bridge between capacitance plates.

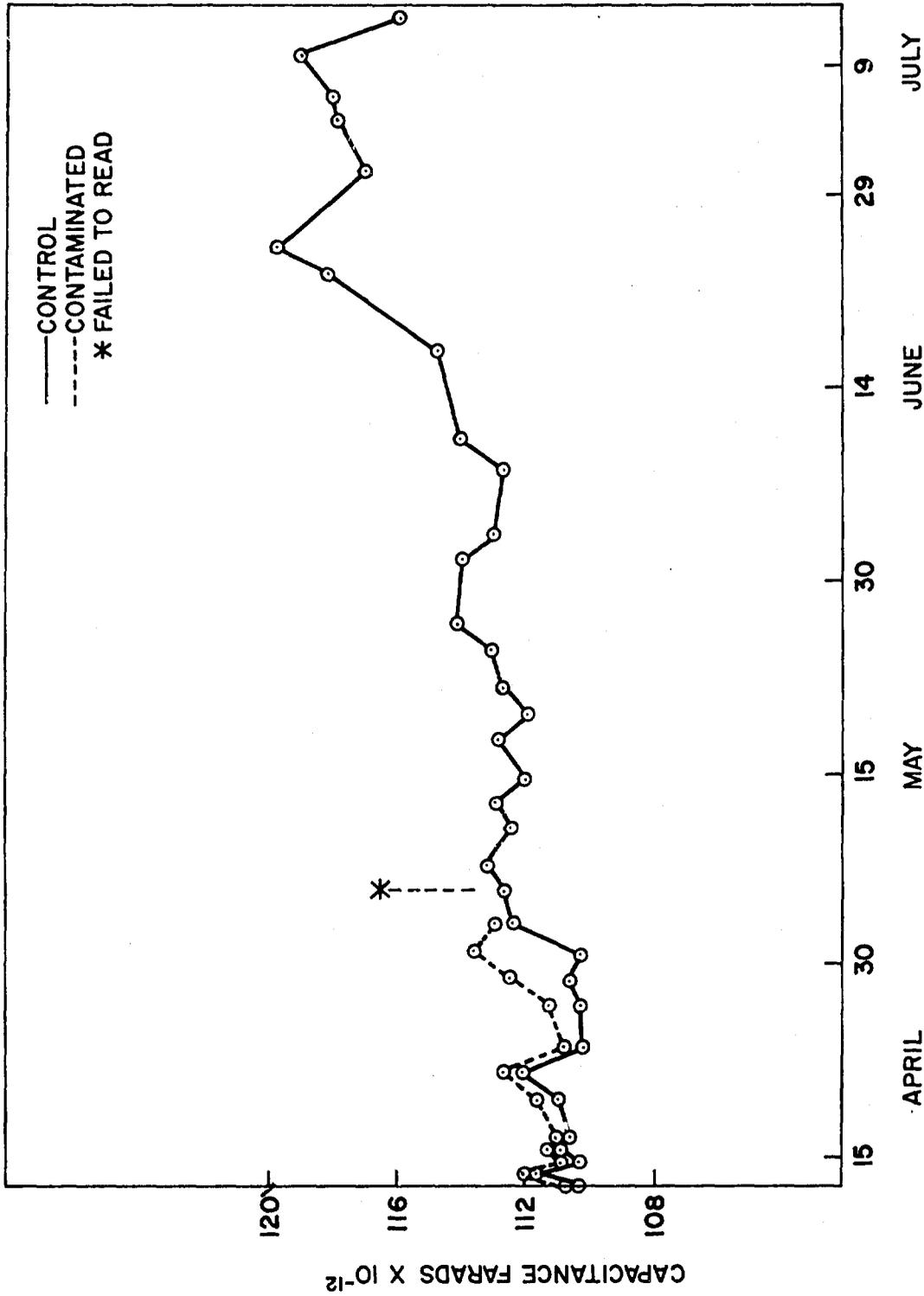


Figure 46. Capacitance Readings of Fuel Capacitance Probe, Liquidometer Pin Used on F-105.

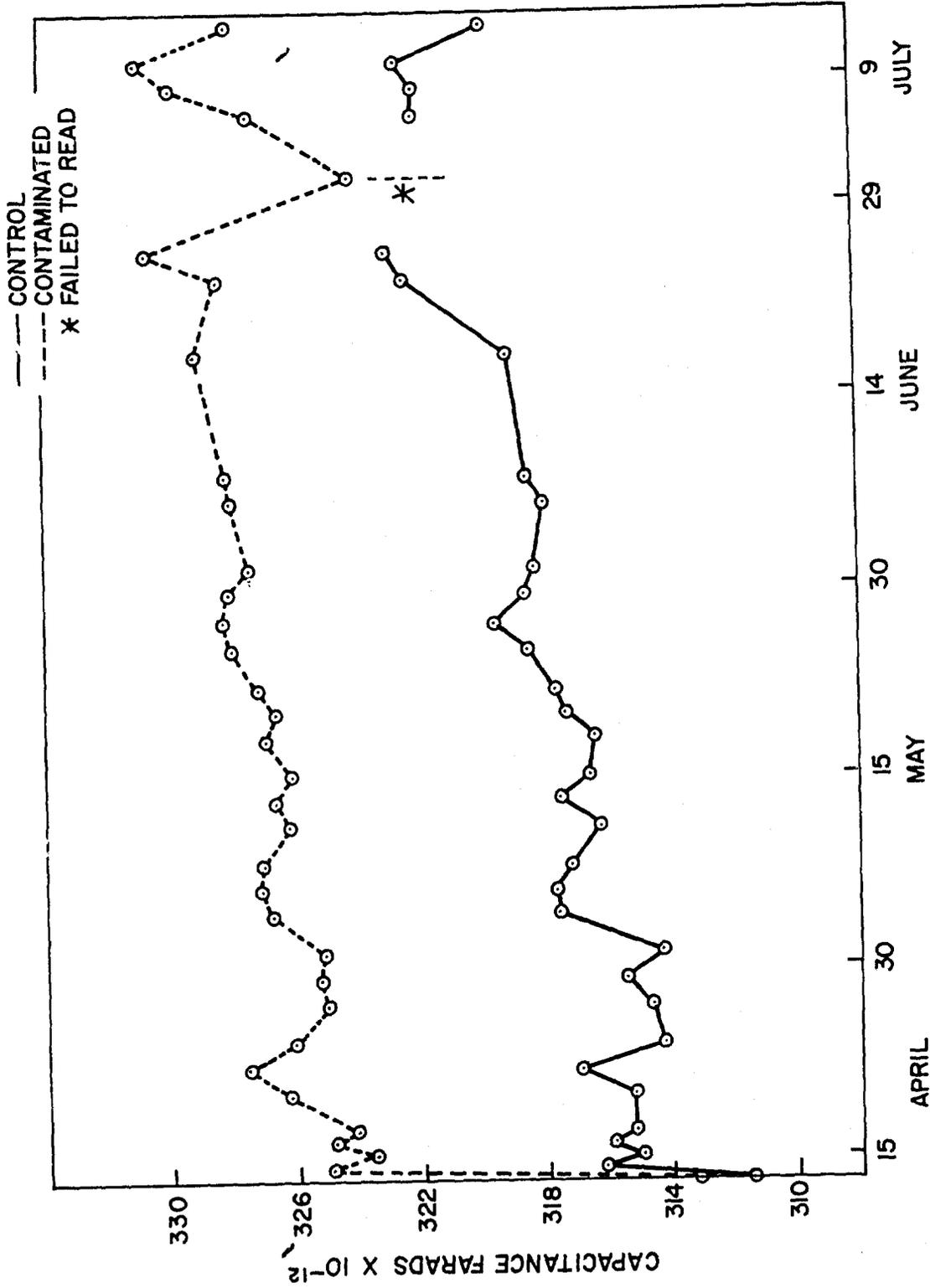


Figure 47. Capacitance Readings of Fuel Capacitance Probe, Minn Honeywell Pin Used on KC-97

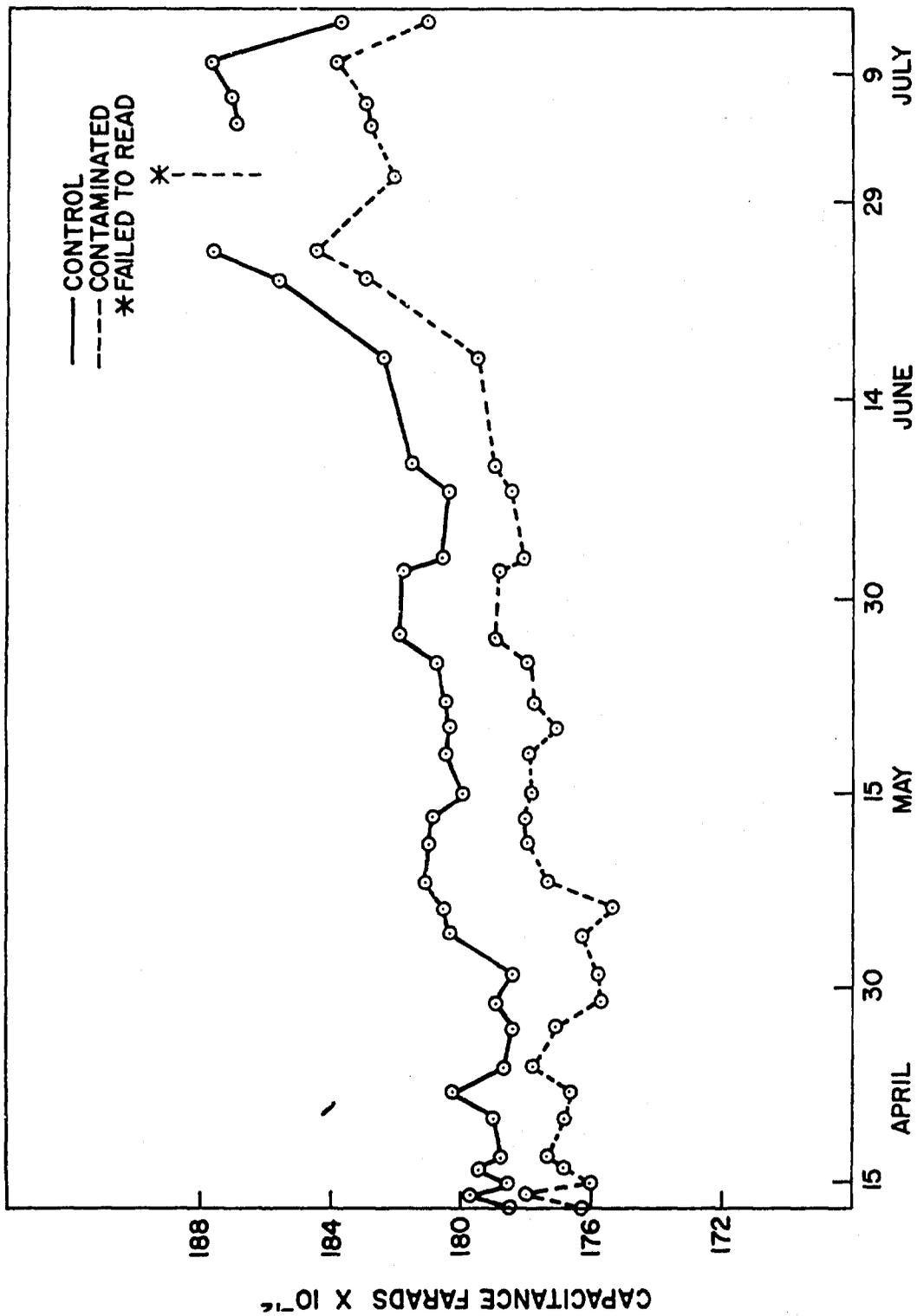


Figure 48. Capacitance Readings of Fuel Capacitance Probe, Avien Pin Used on C-124.

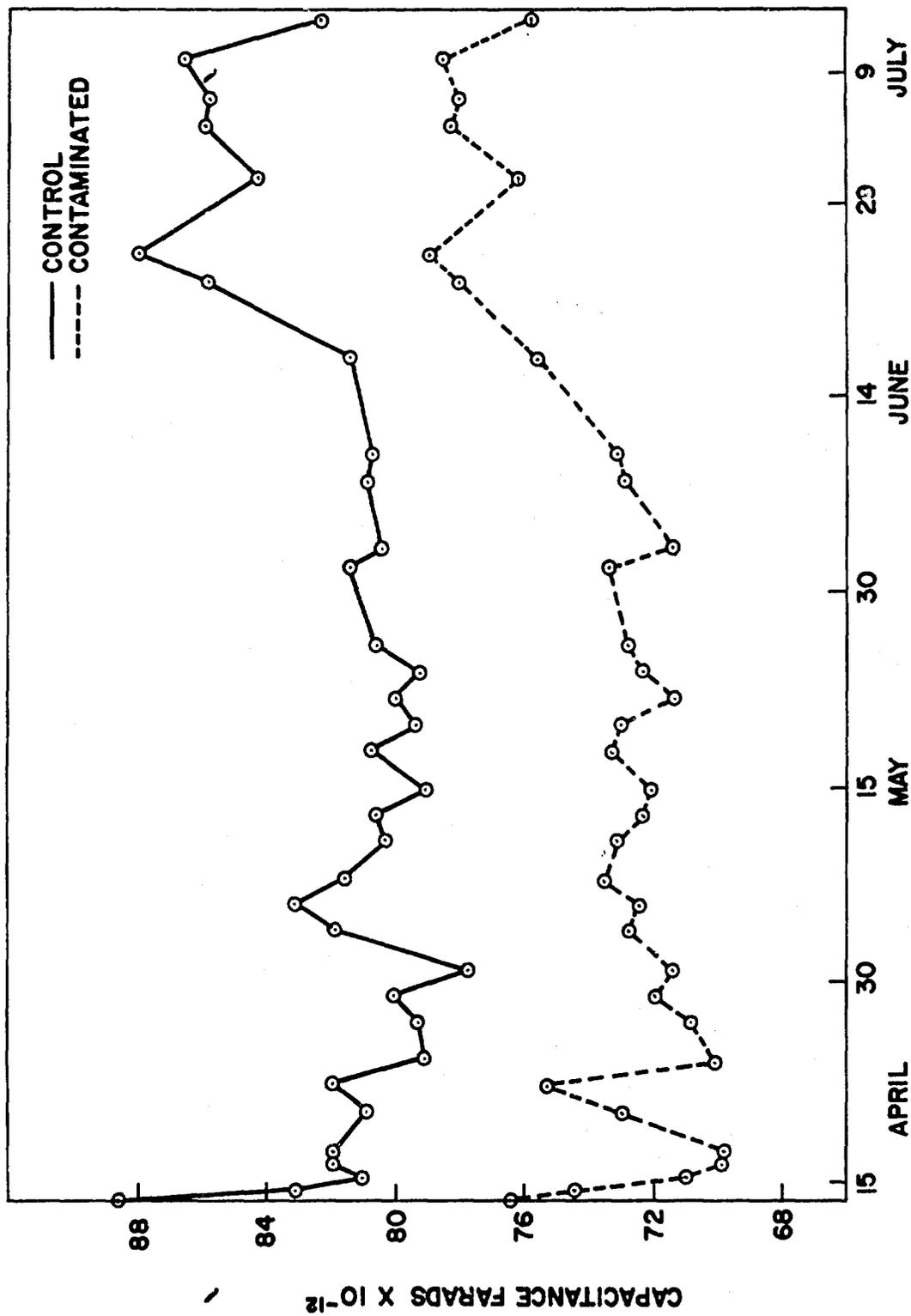


Figure 49. Capacitance Readings of Fuel Capacitance Probe, Sinmonds Pin Used on F-102.

Table XXIX

Viable Cell Count/ml for Microbially Contaminated Containers
for Fuel Capacitance Study

Date of Sampling in Days	Large Drum		Wing Tank		
	Prior to agitation	After agitation	Prior to agitation	After agitation	
	Cells per ml		Cells per ml		
	1.08×10^8 ← original inoculum → 1.08×10^8				
3	(no agitation)	2.00×10^8	(no agitation)	1.64×10^8	
5	3.93×10^7	2.67×10^7	1.40×10^8	6.00×10^7	
7	6.33×10^7	8.37×10^7	7.87×10^7	1.05×10^8	
10	4.27×10^7	8.00×10^8	1.03×10^8	1.40×10^8	
12	1.08×10^7	2.13×10^6	2.33×10^8	4.30×10^8	
14	5.03×10^6	8.63×10^6	5.97×10^8	4.33×10^8	
17	4.63×10^6	1.06×10^7	2.50×10^8	3.87×10^8	
19	5.30×10^6	2.10×10^7	3.30×10^8	4.39×10^8	
21	9.67×10^5	2.77×10^7	1.43×10^8	1.33×10^8	
22	8.00×10^5	1.35×10^7	2.05×10^9	4.17×10^8	
25	-	2.77×10^7	3.40×10^8	1.31×10^7	
27	6.30×10^5	7.30×10^6	2.47×10^8	1.19×10^7	
30	1.83×10^6	-	2.30×10^8	-	AF3600-9128 malfunctioned
32	3.30×10^6	3.93×10^6	2.23×10^8	2.20×10^8	
34	5.47×10^6	1.32×10^7	2.23×10^8	7.63×10^8	
37	7.67×10^5	1.09×10^7	3.67×10^7	4.30×10^7	
39	1.67×10^7	3.33×10^7	1.10×10^7	1.12×10^8	
41	9.00×10^6	2.67×10^7	2.07×10^7	3.00×10^7	
44	2.10×10^7	3.53×10^7	3.83×10^7	3.23×10^7	
46	1.27×10^7	3.67×10^7	3.09×10^8	1.04×10^8	
54	1.30×10^6	3.67×10^7	1.13×10^9	9.00×10^8	
61	2.63×10^6	1.50×10^7	2.28×10^9	7.03×10^8	
68	-	3.91×10^7	-	1.10×10^8	
75	1.03×10^7	1.61×10^8	3.67×10^6	3.70×10^8	
80	6.90×10^6	3.80×10^6	2.00×10^6	2.67×10^6	
89	1.80×10^7	-	1.23×10^6	-	
92	-	4.05×10^7	-	8.50×10^6	

- indicates analysis omitted

Table XXIX. Viable Cell Count/ml for Microbially Contaminated Containers for Fuel Capacitance Study.

Section VIII

MICROBIOLOGICAL GROWTH SUPPORTABILITY OF MICRONIC FUEL FILTER/SEPARATOR MATERIAL

The probability that microorganisms were involved in the clogging of filter elements used in refueling equipment for JP-fuels was implied in earlier studies of microbial contamination of USAF JP-fuels⁴⁹. However, an on-site field study⁵⁰ of a refueling system grossly microbiologically contaminated indicated that microbes had little to no effect on the clogging of filter/separators of that system. The field program, designated "Bacteriological Effects on Aircraft Refueling Systems" (BEARS), was initiated in an idle Pritchard refueling system located at Kindly AFB. Operational tests were conducted on two underground storage tanks, one designated as a control, and the other inoculated with microorganisms recovered from other microbially contaminated fuel system water-bottoms. The results of the study showed no degradation of the JP-fuel, and no significant changes in the performance of the filter/separators. In addition, of the filters checked, the number of viable microbial cells recovered were insufficient to cause filter clogging.

Although the results of the BEARS study had tentatively concluded that microbes were not of concern in filter/separator clogging, the possibility that microbes were involved was suggested when a microscopic examination of material adhering to the surface of a micronic filter (Figure 50) obtained from an operational field refueling system indicated a gross microbial contamination. The microscopic analysis showed the material to be an amorphous, brown, greasy substance and a mass of bacteria and fungal hyphae. However, in direct opposition to the microscopic analysis, the plate counts to determine the total number of viable microbial cells of the adhering material showed a low level of microbial contamination. To determine the effects of the microbial contamination on filter element material, various sections of the element were removed and analyzed (microscopically and microbiologically). The results of the tests indicated that of the layers checked, no degradation of the filter material was visually evident, although viable microbial cells were present throughout the element.

As a result of the micronic filter analysis, a study program was initiated to determine the growth supportability of the micronic fuel filter/separator material.



Figure 50. Micronic Filter Recovered from Lockborn A.F.B., Ohio

METHODS AND MATERIALS

Materials

The test containers consisted of sterile, 50 ml, screw-capped clear glass test tubes and 5 cm glass petri dishes. To obtain a high humidity environment, a chamber was fabricated as follows. A 25.4 x 25.4 cm museum jar was chambered on a piece of glass. A 15.2 cm petri dish bottom with sterile distilled water to provide the moisture was placed inside the museum jar. A dessicator plate was placed over the petri dish to serve as a platform for the test containers. The system was made airtight by sealing the museum jar to the glass plate with Plasticine®.

The testing fuel was a JP-4 fuel, filter sterilized through an 0.45 micron Millipore filter.

A new, intact micronic filter as received at the POL Section at Lockbourne AFB, Ohio, was used as the testing material in the study. The material was a compressed fiber, phenol formaldehyde impregnated paper (percent of chemicals not known). Strips of 1.3 x 3.8 cm of the filter were cut under aseptic conditions and placed in 4 sterile screw-capped jars. Two of the jars containing the strips were autoclave sterilized for 15 minutes and oven dried for 2 days. The other 2 jars containing filter strips were stored at room temperature until used.

Nutritional media of TSB with 1.5% Bacto-agar and Sabouraud with 1.5% Bacto-agar (final pH 6.8) were used for recovery of bacteria and fungi. Non-nutritional media of plain 1.5% Bacto-agar and 1.0% Special (Difco) agar were used as moisture pads for the micronic filter cut section analysis.

Procedures

The program consisted of four tests.

Test 1. To 18 plates (6 plates each of partially jelled TSB, Sabouraud, and Bacto-agar) were added 2 sterile or non-sterile filter strips (3 plates each). The areas at each end of the strips were designated as either F (fungi) or B (bacteria). To each end of the strips in the area designated as "F" was added a 0.1 ml aliquot of a composite of viable fungal growth suspended in 3 ml of B-H-1 solution. The fungi were obtained from JP-4 fuel water samples from Lockbourne AFB and Laboratory Culture No. 1 plated on Sabouraud agar plates. A 0.05 ml composite of bacterial cells was added to each end of the strips marked "B". The bacterial composite was composed of 1.0 ml of sterile B-H-1 solution, 1 ml of 10^2 cells/ml of a shipped JP-4 fuel water sample with 21% EGME, and 1 ml of 10^7 cells/ml of a

modified B-H-1 solution containing $(\text{NH}_4)_2\text{SO}_4$ showing a stable microbial emulsion. After the inoculum liquid was absorbed by the filter material, the 18 plates were placed at room temperature in the dark for 8 days. The plates were checked daily for visible growth. On the 8th day, the inoculated area of each filter strip was teased apart and microscopically examined.

Test 2. To 20 plates (10 each of partially jelled Sabouraud and Special agar) were added sterile and/or non-sterile filter strips. To the end of each strip, (as in Test 1) 0.1 ml aliquots of fungal composite was added. The fungal inoculum was composed of 3 ml of sterile B-H-1 solution containing viable fungi obtained from JP-4 fuel water samples from Schilling AFB and Laboratory Culture No. 1 inoculated on plates of Sabouraud agar. The plates of each medium with the filter strips were divided into 2 groups, A and B. Group A plates were treated as in Study 1. To the surface of Group B plates, after the liquid inoculum had been absorbed by the filter material, was added 1.0 ml of sterile JP-4 fuel. By rotating the plate, the fuel was dispersed over the surface of the agar. The plates were placed in the dark at room temperature for 15 days. Each plate was checked daily for growth. At the termination of the test, the inoculated section of each strip or an area with fungal growth was scraped clean of surface growth and then teased apart and microscopically examined for penetration of the inoculated fungi into the filter fibers.

Test 3. Sixteen sterile (autoclaved) filter strips and sixteen unsterile filter strips were added to a microbial suspension composed of (1) 15 ml of sterile B-H-1 solution, (2) fungal growth (13 days old) from Sabouraud agar plates recovered from JP-4 fuel water samples from Ramey Air Force Base storage tanks No. 24 and 41, and (3) 1 ml of Thioglycollate medium containing bacteria from Ramey Air Force Base storage tank No. 41. The strips were soaked for 1 hour in the microbial composite suspension, then removed and stacked to permit drainage of excess inoculum. Thirty-two, 50 ml, sterile screw-capped tubes were divided into 4 groups. Groups A and B tubes contained 25 ml of sterile EGME-free JP-4 fuel. Group C and D tubes contained 25 ml of sterile 0.1% EGME JP-4 fuel. To group A and C were added the filter strips which had been presterilized before impregnating with the microbial composite discussed above. To group B and D were added the unsterile, microbially impregnated filter strips. Each strip was completely submerged in the fuel. The tubes were placed in the dark at room temperature for 43 days. The tubes were checked daily for visible growth. Seven days after the study had been initiated, one tube of each group was removed from storage. The filter strip from each tube was removed and cut into 3 sections. Section 1 was pressed on a TSB agar plate, removed, and placed on a Sabouraud agar plate. Section 2 was placed on a Bacto-agar plate. Section 3 was teased apart and microscopically examined. The Sabouraud and Bacto-agar plates were placed in the dark at room temperature for a minimum of 5 days. The TSB plates were

incubated at 35°C for 48 hours. The same procedure was followed on the 14th, 27th, 34th and 43rd day of observation of the tubes. Visible growth on some filter strips on the 7th day of observation.

Test 4. To the inoculum of test 3 were added 24 filter strips, 12 each sterilized and non-sterilized. The strips were soaked for one hour. Three each of the sterilized and non-sterilized filter strips were drained of excess fluid. The remaining 18 strips (9 each of the sterilized and non-sterilized) were dried at approximately 34-36°C for 36 hours in a drying oven. To empty, uncovered petri dishes were added one of the following types of filter strips: controls (uninoculated), oven dried, and moist filter strips. The petri plates were placed in the fabricated humidity chamber. The chamber was sealed and placed in an incubator at 30°C for 33 days. Without breaking the seal, the strips were checked daily for microbial growth.

The remaining oven dried, sterilized and non-sterilized filter strips were added to 50 ml screw capped tubes containing 25 ml of JP-4 fuel with and without 0.1% EGME as described in Test 3 above. The tubes were placed in the dark at room temperature for 33 days. The tubes were checked daily for microbial growth.

Upon termination of the study, all strips were removed from their test containers and aseptically cut into 3 parts. Each section was subjected to analysis as described above in Test 3.

RESULTS AND DISCUSSION

The result of Test 1, which was initiated to determine if the micronic filter material could support microbial growth, and upon which all the following tests were contingent, indicated that the micronic filter material would support the growth of microorganisms if sufficient moisture were available. In addition, the microscopic analysis indicated that mycelium of the fungi had invaded the inner portion of the filtering material.

The results of Test 2, which was initiated to determine the growth supportability of the filter material when saturated with JP-4 fuel, confirmed the finding of Test 1 and indicated fungi could grow and invade the inner portion of the filtering material providing moisture were available.

The results of Test 3, which was initiated to determine if filter strips suspended in JP-4 fuel with and without 0.1% EGME and glycerol would maintain the viability of fungal material with a minimal amount of moisture, indicated that viable fungi were isolated from the filter matrix and hence could survive the environmental conditions established in this test.

The results as shown on Table XXX and XXXI of Test 4, which was initiated to determine if microbial cells dried on the filtering material would

Table XXX

Results of Test 4 Micronic Filter Study

Results from High Humidity Chamber

Treatment of Filter Strips	Sterilized Filter						Non-sterilized Filter																	
	TSB			Sab			1.5% Agar			TSB			Sab			1.5% Agar								
Control	0			0			0			0			0			0								
Drained Dry	+B			+BF			+F			+B			+F			+F								
	+B			+BF			+F			+B			+BF			+F								
	+BF			+F			+F			+BF			+BF			+F								
Dried Hot Air Oven	+B			+F			0			+B			+BF			omitted								
	+B			+BF			0			+BF			+F			+F								
	+F			+F			+F			+F			+F			+F								
Spray	+B			+B			+F			+BF			+B			0								
	+B			+BF			+F			+B			+BF			+F								
	+BF			+BF			+F			+BF			+BF			+F								
	JP-4 fuel						JP-4 fuel with AIA						JP-4 fuel						JP-4 fuel with AIA					
	TSB		Sab		Agar		TSB		Sab		Agar		TSB		Sab		Agar		TSB		Sab		Agar	
Dried Hot Air Oven	+		+		0		+		+		+		+		+		+		+		+		+	

Key to table

+ indicates growth observed on plate

† indicates some growth

0 indicates no growth

F indicates fungi

B indicates bacteria

Sab Sabouraud agar

Agar 1.0% non-nutrient solid growth agar

Table XXX. Results of Test 4 - Micronic Filter Study.

Table XXXI

Results of Microscopic of Micronic Filters from Test 4

Treatment of Filter Strips	Sterilized Filter	Non-Sterilized Filter
Control	negative	negative
Drained dry	Small amount of Fungal growth Small amount of Fungal growth Small amount of Fungal growth	Fungal growth - Hyphae, and Spores Fungal growth - Hyphae, and Spores Fungal growth - Hyphae, and Spores
Dried Hot Air Oven	Heavy Fungal growth many spores Small amount of Fungal growth few spores Very heavy growth, many spores	Fungal growth with spores possible bacterial rods Fungal growth with few spores. Fungal growth with few spores bacterial rods present
Spray Contaminated	Small amount of Fungal growth Small amount of Fungal growth Small amount of Fungal growth	Very small amount of Fungal growth Moderate amount of Fungal growth Fungal growth few spores
Dried Hot Air Oven in Fuel With AIA	negative growth negative growth small amount of Fungal growth	Small amount of Fungal growth negative growth negative growth
Dried Hot Air Oven in Fuel Without AIA	Fungal growth few spores negative growth Fungal growth	negative growth Fungal growth Fungal growth

Table XXXI. Results of the Microscopic Analysis of Micronic Filters from Test 4.

grow when the combination was subjected to (1) JP-4 fuel with and without 0.1% EGME and glycerol, and (2) a high humidity environment, confirmed the findings of the 3 previous tests and indicated that dried filtering material supported the viability of microbial cells.

Of interest was the similarity of microbial growth on the sterile and non-sterile filtering material and in JP-4 fuels with and without 0.1% EGME. However, the microbial growth observed after 43 days, would not have been sufficient to have caused the filter to become clogged.

CONCLUSIONS

Although the micronic filter material supported microbial growth, the degree of growth was insufficient to cause filter clogging.

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DOCUMENT CONTROL DATA - R&D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author) Systems Research Laboratories, Inc., 500 Woods Drive, Dayton, Ohio 45432		2a. REPORT SECURITY CLASSIFICATION Unclassified	
		2b. GROUP	
3. REPORT TITLE Microbial Contamination of USAF JP-4 Fuels			
4. DESCRIPTIVE NOTES (Type of report and inclusive dates) Final August 1965 through June 1966			
5. AUTHOR(S) (Last name, first name, initial) Finefrock, Viola H. London, Sheldon A.			
6. REPORT DATE August 1966		7a. TOTAL NO. OF PAGES 184	7b. NO. OF REFS 49
8a. CONTRACT OR GRANT NO. AF33(615)-2692		9a. ORIGINATOR'S REPORT NUMBER(S) 773	
b. PROJECT NO. 8169			
c. Task No. 816906		9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report) AFAPL-TR-66-91	
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11. SUPPLEMENTARY NOTES		12. SPONSORING MILITARY ACTIVITY Air Force Aero Propulsion Laboratory RTD, AFSC Wright-Patterson Air Force Base, Ohio	
13. ABSTRACT With the recognition in 1956 that the United States Air Force JP-4 fuels were microbially contaminated, problems concerning malfunctions and corrosion of JP-4 fuel systems were attributed to the presence of microorganisms. The Biospecialties Branch, MRMPB, 6570 Aerospace Medical Research Laboratories, initiated a research effort to support and supplement the Air Force Aeronautical Systems Division program concerned with the relationship of microbial contamination to deleterious changes in JP-4 fuel systems. Results of this program have shown the following: the majority of JP-4 fuel systems contain low levels of viable microorganisms; microorganisms are primarily associated with the water phase and rarely with the fuel phase of a JP-4 fuel/water system; JP-fuels exert a toxic effect on viable microbial cells; and microbial corrosion of aluminum alloys 7075 2024, and 7178 can occur in the laboratory but does not appear to be the major cause of corrosion in operational systems. Twenty percent ethylene glycol monomethyl ether (EGME) in a laboratory JP-4 fuel/water-bottom acts as an inhibitor to the majority of viable cells present. As a result of engineering design, it is possible that fuel capacitance probes can cease to operate as a result of microbial growth. Micronic filter materials of phenolformaldehyde impregnated paper can support the growth of microorganisms, but growth even after 43 days is insufficient to cause filter clogging, provided the filter is immersed in fuel. In addition, field surveys of JP-4 fuel handling facilities and aircraft have shown that improved housekeeping and the use of EGME have reduced the quantity of microbial contaminants initially found in the jet systems to an insignificant level.			

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