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HARVEY, ILLINOIS

FINAL REPORT
OCTOBER 1965

MICROBIAL UTILIZATION OF HYDROCARBON FUEL FORMULATIONS WITH THE PRODUCTION OF GUMS, SLIMES, SLUDGE AND SURFACE ACTIVE COMPOUNDS

Contract No. DA-19-129-AMC-88(N)

Authors

A. T. Knecht, Jr., Project Leader
J. W. Kools
D. J. Muhlbaier
A. R. Rizzuto

SINCLAIR RESEARCH, INC.
400 E. Sibley Blvd.
Harvey, Illinois

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FOREWORD

This Final Report summarizes work done under Contract DA-19-129-AMC-88(N), "Microbial Utilization of Hydrocarbon Fuel Formulations with the Production of Gums, Slimes, Sludge and Surface Active Compounds", during the period 1 April 1963 to 28 September 1965. This contract was initiated by the Fungicides and Germicides Laboratory, Pioneering Research Division, Quartermaster Research and Engineering Center, Natick, Massachusetts, with Dr. A. M. Kaplan as Project Officer.

Abstract

The role of microorganisms in the deterioration of fuels and in the production of sludges and surfactants was investigated. RP-1 and CITE (Compression Ignition Turbine Engine) fuels supported microbial growth in 10% natural sea water medium. Growth was stimulated by three approved fuel soluble corrosion inhibitors and by the addition of inorganic nitrogen and phosphorus salts to the aqueous medium. However, fuel exposed to microorganisms for 16 weeks still met Military specifications for quality.

The type of hydrocarbon fuel component which supported growth of eight pure and mixed cultures of bacteria and fungi was established. These cultures were then used to produce sludge from fuel and pure hydrocarbons. Methods were outlined for the fractionation and analysis of sludge. Insoluble sludge was found to be primarily microbial cells. Several solvent soluble fractions were characterized as normal cell components and fuel components oxidized by the test organisms. No substantial accumulations of extracellular proteins and carbohydrates were observed.

Comparison of microbial sludge from the laboratory with a field sample indicated both contained essentially the same components. However, microbial sludge was markedly different from normal fuel chemical oxidation products.

Fuel:water emulsions were stabilized by cells and probably microbially oxidized fuel components. Metabolic studies indicated that recognized pathways were followed for the oxidation of n-paraffins and naphthalene.

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MICROBIAL UTILIZATION OF HYDROCARBON FUEL FORMULATIONS WITH THE PRODUCTION OF GUMS, SLIMES, SLUDGE AND SURFACE ACTIVE COMPOUNDS

I. Introduction

Microbiological problems associated with the handling and use of both Military and Commercial jet fuels have received considerable attention in recent years. Information obtained on jet fuel prompted investigations of microbial problems associated with other distillate fuels. Only a paucity of information was available on the role of microorganisms in filter plugging and instability propensities of diesel and heating fuels.

It has been suggested that a number of isolated problems encountered in the past with distillate fuels and attributed to poor fuel quality, may have actually resulted from microbiological contamination[1]. While the potential dangers associated with jet fuel problems are much greater than with other distillate fuels, problems encountered in the latter case can be extremely costly to military and commercial consumers.

The presence of "sludge", "slime", "gum" and "surfactants" in jet fuel bulk storage tanks and in the wing tanks of aircraft contaminated with microorganisms is well documented. While the ability of microorganisms to utilize hydrocarbons and to grow in fuel systems in close association with water has been recognized for some time, their role in the formation of "sludge", "slime", etc. has not been elucidated. The origin, properties, and chemical nature of these materials, and their relationship to microbial activity, also have not been adequately explained.

A project was initiated at Sinclair Research, Inc., under Natick Contract DA-19-129-AMC-88(N), to investigate the mechanisms of microbial attack on fuel and to determine the chemical nature of products formed. Specifically, the objectives as outlined in the original agreement are as follows:

---

(1) A.T. Knecht, Jr. and F.M. Watkins, 1963, "The Influence of Microorganisms on the Analysis of Petroleum Products", Development in Industrial Microbiology, Vol. 4, P. 17-23, AIBS Publication.

1. To establish the ability of various cultures to attack hydrocarbon fuels and to form gums, slimes, sludge and surface active agents.

2. To establish which components of the fuel are attacked in the process of gum, slime, sludge and surfactant formation.

3. To study at least one fuel which is not readily attacked by the cultures and determine the factor(s) for resistance.

4. To determine the chemical nature of the gums, slimes, sludge and surfactants produced by the test organisms. These materials will be compared with similar materials produced by the normal chemical degradation processes which occur in hydrocarbon fuels.

5. To determine how microorganisms utilize hydrocarbon fuel formulations to produce gums, slimes, sludge and surfactants.

6. To determine the probable metabolic pathways involved in the formation of these materials by microorganisms.

II. Summary and Conclusions

1. The ability of RP-1 rocket fuel and CITE (Compression Ignition Turbine Engine) fuel to support microbial growth was demonstrated in laboratory tests. RP-1 fuel was included in the study because of reports that indicated it was resistant to microbial attack. Both fuels still met Military specifications for quality after exposure to microbial activity for 8 and 16 weeks respectively.

2. A blend of additive free CITE fuel was prepared for use in laboratory studies on microbial sludge production. Detailed hydrocarbon type analyses of the fuel were made in addition to the chemical and physical test data outlined in Military specifications for quality.

3. Differences were noted in the extent and type of corrosion which occurred in laboratory tests, depending on whether the system was inoculated or sterile. In metal containers with active water bottoms the corrosion extended an inch or more above the water surface, up the sides of the can. Corrosion was confined to the water covered areas in sterile units. In glass containers, corrosion products from steel wool had a tendency to agglomerate in the active units, and to remain dispersed in the sterile controls.

4. Approved CITE fuel additives, including corrosion inhibitors, anti-oxidants, and a metal deactivator, were evaluated for their effect on microbial growth. DuPont AFA-1 corrosion inhibitor which contains both nitrogen and phosphorus markedly increased microbial growth in 10% sea water solutions exposed to the fuel. Two other corrosion inhibitors, Unicor M (contains nitrogen) and Santolene C (contains phosphorus) also promoted the growth of microorganisms, but to a lesser extent than the DuPont additive. A substantial increase in microbial growth was also noted when nitrogen and phosphorus salts were added to the sea water solution. These studies indicate that some nitrogen and phosphorus containing additives may promote growth in the field and contribute to fuel handling problems.

5. Filterability, stability (existing and potential gums) and water content of CITE fuel exposed to heavy microbial growth were found to be within normal limits. However, fuel containing the corrosion inhibitors, Lubrizol 541 and Tolad 244, after exposure to both active and sterile water bottoms, exhibited poor filterability characteristics.

6. Eight cultures which grew well on CITE fuel were characterized as to the type hydrocarbon fuel component which they utilized. The majority of the cultures utilized paraffinic components of the fuel. This is not unexpected since paraffins constituted approximately 50% of the hydrocarbon types present in the test fuel.

7. Methods were outlined for the fractionation of microbial sludge. Analyses were made primarily on the Infrared Spectrophotometer. IR techniques lend themselves well to this type of investigation. Also, infrared analysis is used throughout the Petroleum Industry and Government for the characterization of materials from petroleum products. Observations on the various fractions are summarized below:

Fraction A - Material insoluble in fuel, water and tri-solvent (benzene-acetone-methyl alcohol mixture). Analyses indicated that this fraction was primarily microbial cells and components thereof. This was established by comparing pure and mixed cultures of bacteria and fungi grown on pure hydrocarbons and on CITE fuel. Comparison of material from the laboratory with microbial sludge from the field indicated that they were basically the same. However, microbial sludge from the laboratory and field differed greatly from a sample of fuel chemical oxidation products.

Fraction B - Material insoluble in fuel and water but soluble in tri-solvent. This fraction was expected to contain normal chemical oxidation products of fuel; however, none were detected because of the excellent stability of the test fuel. Materials extracted by the tri-solvent were primarily cellular lipids. Trace quantities of proteins and carbohydrates, including amino-sugars were also found. Free organic acids were detected in several samples from cultures grown on CITE fuel.

Fraction C - Aqueous phase after distillation. Extracellular proteins and carbohydrates were present in low concentrations in the aqueous media. Small quantities of these materials would, however, support the growth of "scavenger" organisms. A variety of other complex organic structures were detected in several samples. For the most part, these compounds appeared to be microbial pigments. Benzoic and p-toluic acids were extracted from one fermentor run employing a culture which oxidized naphthalenes.

Fraction D - Water soluble materials extracted from the sludge were identified as cellular components. These materials were probably released from the cells due to lysis caused by the distilled water washes.

Fraction E - Hexane soluble materials extracted from the sludge were found to be primarily cellular lipids and residual hydrocarbon fuel components. Free organic acids were observed in extracts of material from fermentor runs with CITE fuel. Emulsions which formed in the fermentor were stabilized by cells, probably because of their lipid content. The free organic acids detected could have contributed to this problem.

8. Metabolic studies with two pure cultures indicated that recognized pathways for the oxidation of n-paraffins and naphthalene were followed. Warburg studies with octane and naphthalene grown cells indicated that octanoic and salicylic acids were intermediate in the oxidation of the respective hydrocarbons. Hexanoic and heptanoic acids were also utilized by the octane cells as were the corresponding hydrocarbons.

9. Pentene-1 and hexene-1 inhibited the oxidation of both n-octane and naphthalene, including the endogenous activity of the paraffin oxidizer. However, the inhibitors had no effect on the endogenous activity of the naphthalene oxidizer

III. Results and Discussion

A. RP-1 Fuel Storage Tests

A sample of RP-1 Rocket Fuel (Natick Voucher No. 3050-63) was supplied by the Natick Laboratories for use in this program. Reportedly, the fuel was resistant to microbial attack. A series of tests were set up to establish this point.

Storage tests were set up with the RP-1 fuel over diluted natural sea water bottoms (ratio of fuel to water, 20:1). The bottoms were inoculated with a composite of contaminated tank bottom samples obtained from a number of fuel oil storage tanks just prior to cleaning. Appendix A describes the procedures employed in setting up the tests and describes the various components used. The variables included in the tests were the presence of iron and the addition of inorganic nitrogen and phosphorus salts to the bottoms. Ferrous metal was included to more closely simulate field storage conditions and to check the effect of iron oxides on microbial growth and fuel quality. Nitrogen and phosphorus salts were added to supplement the sea water which contained only trace quantities of these elements - 0.6 and 0.03 ppm respectively. The RP-1 fuel contained 1.5 ppm nitrogen and 0.4 ppm phosphorus, probably in the form of additives. Most routine culture media contain considerably more nitrogen and phosphorus than the quantities present in this test system.

1. Microbiological Observations

Composite fuel oil water bottoms used to inoculate the test systems contained fungi, yeast, and bacteria, including sulfate-reducers, and appeared to be representative of most natural tank bottom flora. The microbial flora appeared particularly desirable, having been exposed to fuel oil which contains substantial quantities of the same types of paraffins and encompasses the same distillation range as the RP-1 fuel. Also, these two fuels have at least one additive in common.

All units were sampled at bi-weekly intervals over a period of eight weeks. An estimate was made of the number of viable microorganisms present in the water bottoms of series I, II, III and IV (Active units). All sterile control units (V, VI, VII, VIII, IX and X) were checked for contamination in nutrient broth medium. The methods and media employed are presented in Appendices A, B and C.

The results of these analyses are presented in Table I. Maximum numbers were observed in all active units at the end of four weeks. As would be expected, the two series containing added nitrogen and phosphorus had the

TABLE I

Results of RP-1 Fuel Storage Over Sea Water Bottoms-Microbiological Analyses

Test System	pH Initial	pH Final	Estimated Number of Viable Microorganisms/ml. Number of Weeks 0	2	4	6	8
Inoculated Units							
I. Sea Water, steel wool	7.6	5.1	100,000 BF[1](5)[2] T + WF[4]	1,000,000 BF(5)T+WF	10,000,000 BF(5)T+WF	10,000 BYF(3)TF[5]	100,000 BYF(2) TF
II. Sea Water, steel wool	7.2	5.2	"	1,000,000 BF(4) TF	500,000,000 BF(4)T+WF	1,000,000 BYF(3)T+WF	1,000,000 BYF(5) T + WF
III. Sea Water	7.6	7.7	"	5,000,000 B(3)	50,000,000 B(3)	50,000,000 B(4)	10,000,000 B(3)
IV. Sea Water, Nitrogen & Phosphorus	7.2	7.6	"	5,000,000 BF(4) TF	100,000,000 BF(4)T+WF	100,000,000 BF(4)T+WF	50,000,000 BF(4)T+WF
Sterile Control Units							
V. Sea Water, steel wool	7.6	5.3	Sterile				
VI. Sea Water, steel wool, Nitrogen & Phosphorus	7.2	5.3	"				
VII. Sea Water	7.6	8.2	"				
VIII. Sea Water, Nitrogen & Phosphorus	7.2	7.5	"				
IX. Sterile Fuel, Steel Wool (No water phase)			"				
X. Sterile Fuel (No water phase)			"				

1. "BF" stands for Bacteria and Fungi, respectively.
2. Number in parentheses is rough estimate of the number of obviously different colony types present on the TGE Agar plates.
3. "Y" stands for Yeast.
4. "T+WF" = Tan + White Fungi present.
5. "TF" = Tan Fungus present.

highest counts. The source of these elements in the field is apparently of considerable importance in contamination problems. Unfortunately, this point has received little attention.

Photographs (Plates 1, 2 and 3) made initially and after eight weeks storage, clearly demonstrate the build-up of rust in the units containing steel wool. The excessive quantities of rust apparently caused the pH of the water bottoms to drop in series I, II, V and VI. Obviously, microorganisms did not cause the acidic conditions since they also developed in the sterile systems. Yeast forms appeared to be encouraged by the acid environment in series I and II.

A predominance of bacteria was noted in series III which contained only sea water. Perhaps the consistently high pH of the water bottoms favored their growth. Fungus growth at the interface of series I, II and IV is clearly visible in Plates 2 and 3. The tan fungus observed in most of the test series was tentatively identified as a member of the Genus Cladosporium.

Table II compares the results of microbiological analyses obtained on series II units with the results obtained on a duplicate of this series set up with commercial jet fuel (kerosine). Under the conditions of this storage test it appears that the RP-1 fuel supports microbial growth at essentially the same rate as kerosine. Furthermore, on receipt the sample of RP-1 fuel was found to contain viable microorganisms. The organisms found are discussed in Section 4, below.

2. Fuel Quality

Complete fuel quality tests were conducted on composite fuel samples from the three units in each series. The results of these analyses are reported in Table III. Only slight variations were observed, all of which could be explained on the basis of experimental variation. It can therefore be concluded that the quality of the RP-1 fuel as measured by these tests was unaltered by the activities of microorganisms or the presence of other materials in the water bottoms.

When the contents of units from series I, II, III and IV were mixed thoroughly and allowed to stand for a short period of time, the two phases separated rapidly. If surfactants were produced by the microorganisms, their effect was masked by the high salt content of the water bottoms. The clarity of the fuel is demonstrated in the photographs (Plates 2 and 3) by the sharpness of the label attached to the reverse side of the bottle.

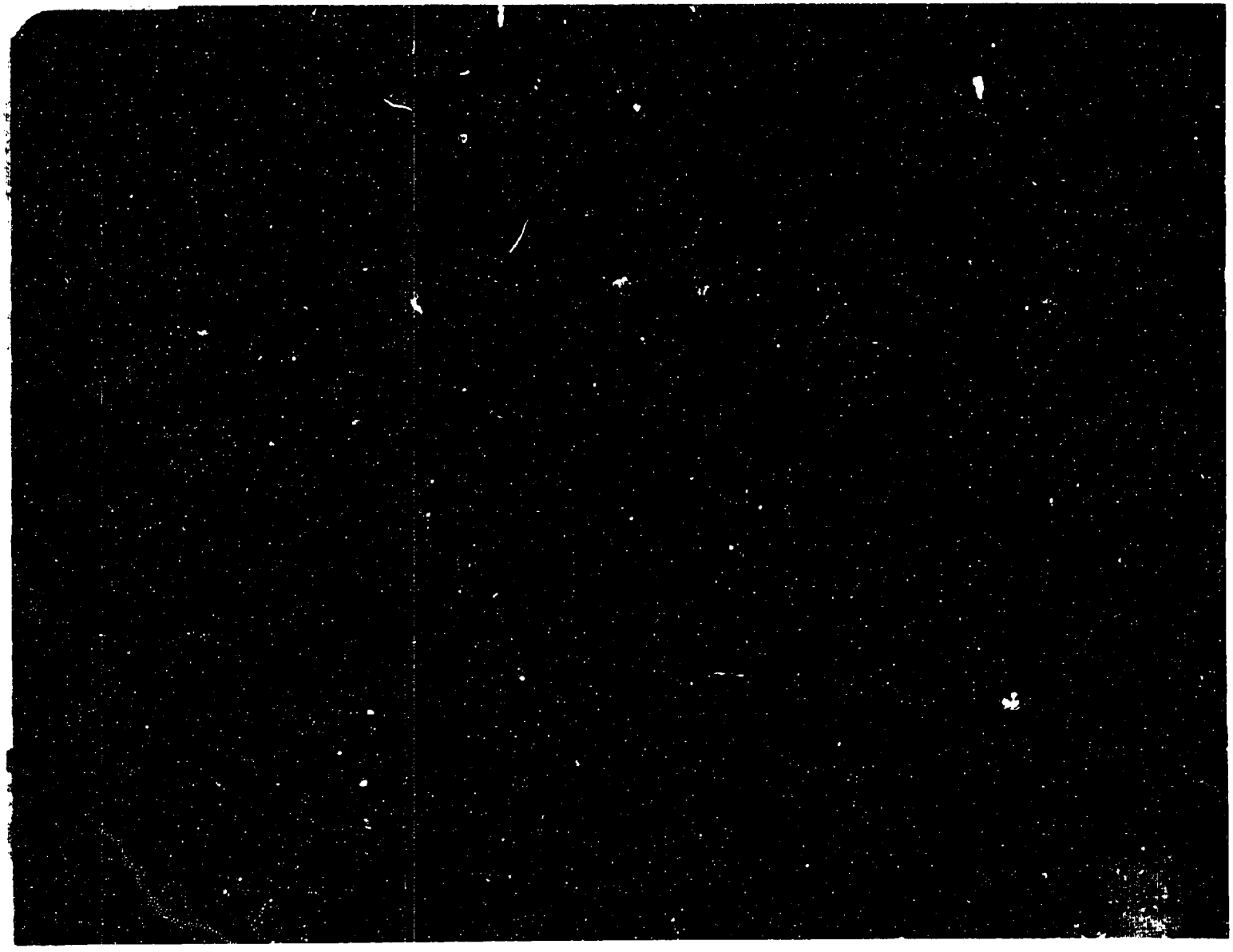

Plate 1. RP-1 Series I, II, III, IV and IX, Initially.

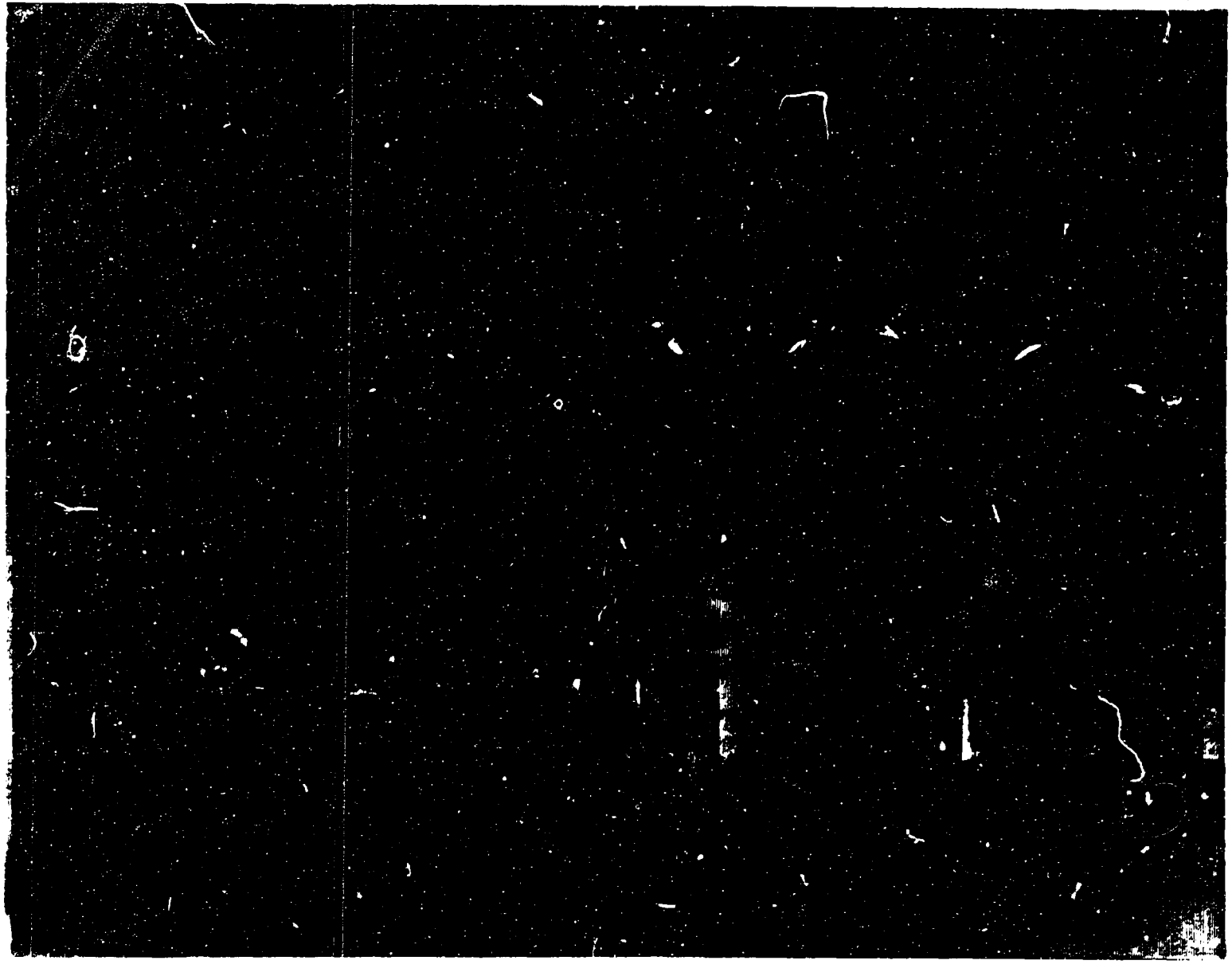

Plate 2. RP-1 Series I, II, V, VI and IX, After 8 Weeks Storage.

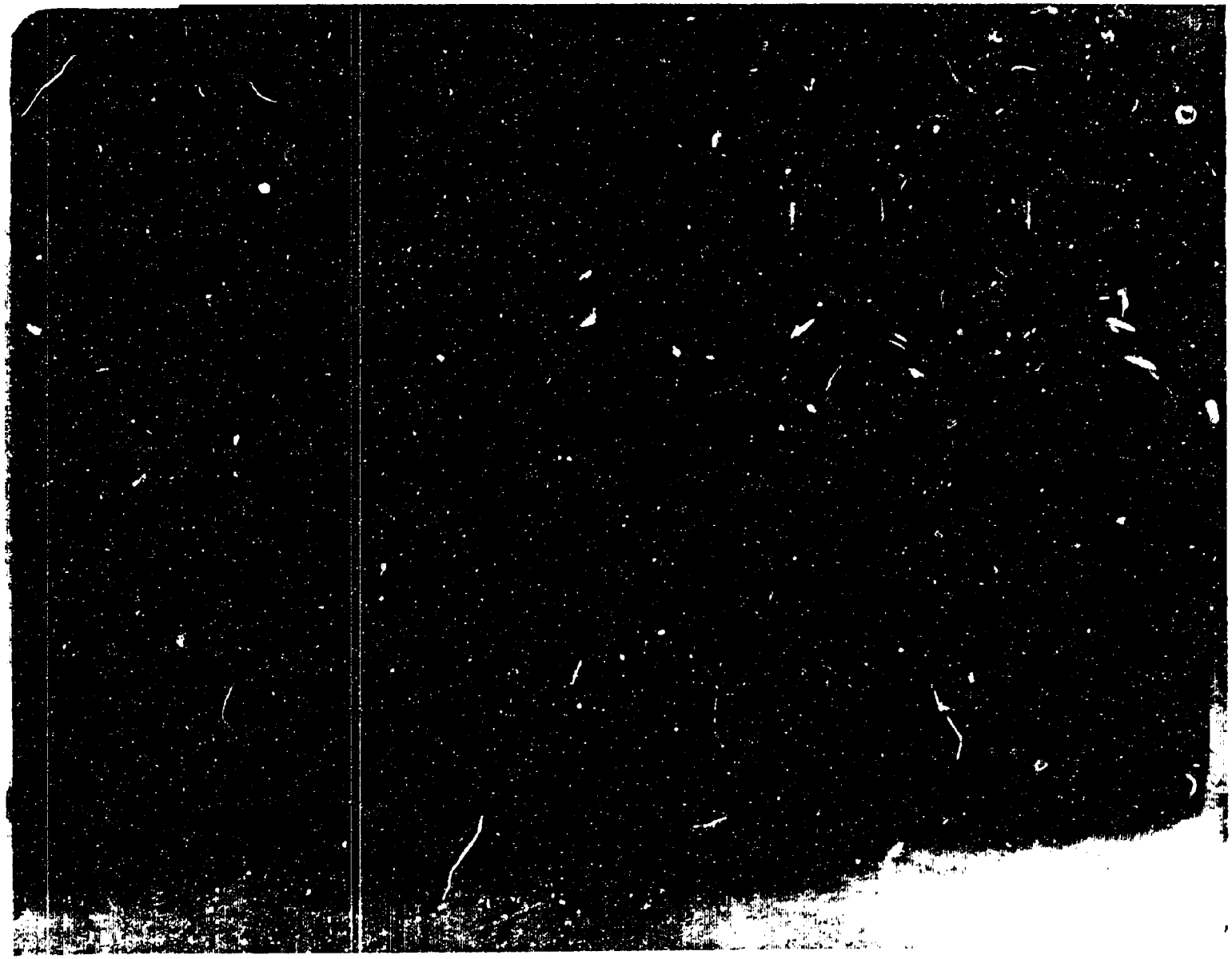

Plate 3. RP-1 Series III, IV, VII, VIII and IX, After 8 Weeks Storage.

TABLE II

RP-1 and Kerosene Fuel Storage Studies - Comparison of Microbiological Data

Test System	Estimated Number of Viable Microorganisms/ml. of Water Bottoms — Number of Weeks: 0	2	4	6	8
II. Sea Water, Steel Wool, Nitrogen & Phosphorus (Composite Inoculum)					
RP-1	100,000 BF[1](5)[2] T + WF[4]	1,000,000 BF(4) TF[5]	500,000,000 BF(4) T + WF	1,000,000 B[3]YF(3)T+WF	1,000,000 BYF(5)T+WF
Kerosene (Commercial Jet Fuel)	"	1,000,000 BF(4) TF	100,000,000 BYF (4) T + WF	500,000 BYF(3)TF	500,000 F TF

1. "BF" stands for Bacteria and Fungi, respectively.
2. Number in parentheses is rough estimate of the number of obviously different colony types present on the TGE agar plates.
3. "Y" stands for Yeast.
4. "T+WF" = Tan and White Fungi present.
5. "TF" = Tan Fungus present.

TABLE III

Results of Quality Tests on RP-1 Fuel After Eight Weeks Storage Over Sea Water

Components of Water Bottoms	ASTM Methods	Mil-R-25576B	Initial Control	Storage Test Systems I	V	II	VI	III	VII	IV	VIII	IX	X
Sea Water				+	+	+	+	+	+	+	+		
Steel Wool				+	+	+	+					+	
N & P Soln.						+	+			+	+		
Inoculum													
Active				+		+		+		+			
Sterile					+		+		+		+		
Quality Tests													
Distillation	D-86												
IBP		TBR(1)	383°F	383°F	384°F	385°F	386°F	385°F	384°F	386°F	384°F	386°F	384°F
5%			399	396	396	396	399	394	396	397	396	398	396
10%		365-410°F	406	407	405	404	404	407	406	403	406	405	404
20%		TBR	415	416	416	414	416	417	416	416	416	416	415
30%		"	423	424	424	422	424	424	424	424	424	424	424
40%		"	429	432	431	430	431	432	430	431	430	431	432
50%		"	437	438	438	437	438	438	439	439	439	439	438
60%		"	445	446	446	444	446	445	446	445	446	446	446
70%		"	453	454	455	453	454	454	454	453	454	454	454
80%		"	464	465	464	463	465	464	464	463	464	464	464
90%		"	478	481	480	478	480	480	480	479	480	480	478
95%		"	491	496	494	495	494	495	494	493	494	494	494
EP		525°F	518	518	520	519	520	518	520	518	520	523	520
Residue (Vol.%)		1.5 Max.	1.0	-	-	-	-	-	-	-	-	-	-
Loss (Vol.%)		1.5 Max.	1.0	-	-	-	-	-	-	-	-	-	-
°API Gravity	D-287	42.0 Min. 45.0 Max.	44.7	44.9	44.8	44.7	44.8	44.8	44.9	44.9	44.9	44.8	44.8
Sulfur, Total (Wt.%)	D-1266	0.05 Max.	0.017										
Existent Gum (mg./100ml)	D-381	7 Max.	0.9	0.5	1.1	0.9	0.8	0.9	0.7	0.9	0.7	0.8	0.7
Potential Gum 16 hr. (mg./100 ml.)	D-873	14 Max.	1.6	1.2	1.0	0.9	0.5	2.0	0.8	1.5	0.8	1.2	1.2
Aromatics, Vol%	D-1319	5.0 Max.	5.7	5.9	5.7	5.7	5.5	6.4	5.5	5.8	5.5	5.8	6.3
Olefins, Vol.%	D-1319	1.0 Max.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Copper Strip Corrosion	D-130	1 Max. 122°F	1A	1A	1A	1A	1A	1A	1A	1A	1A	1A	1A
		212°F (2 hrs)	1B	1B	1B	1B	1B	1B	1B	1B	1B	1B	1B
Flash Point	D-93	110°F Min.	-	-	-	-	-	-	-	-	-	-	-
H_2O Tolerance	FTM(2) VVL791 (3251.7)		1B	1B	1B	1B	1B	1B	1B	1B	1B	1B	1B

(1) TBR = To Be Reported (2) FTM = Federal Test Method

3. Hydrocarbon Type Analyses of RP-1 Fuel

Composite fuel samples from series II (active inoculum, steel wool, and added nitrogen and phosphorus) and series VI (sterile control for series I) were analyzed on the High Temperature Mass Spectrometer, Ultraviolet Spectrophotometer, and Gas Chromatograph. These techniques are routinely used to analyze hydrocarbon fuels. In most cases the distribution of hydrocarbon components in treated and untreated fuels may vary only slightly, therefore requiring extremely sensitive analytical techniques.

The results of studies on RP-1 fuel are reported in Tables IV and V. Analyses by the three analytical techniques failed to demonstrate any significant differences in the hydrocarbon components of the initial fuel sample and the two test samples. It is not too surprising, however, that differences were not observed in these samples, since relatively large volumes of fuel were employed in the storage tests. The amount of hydrocarbon required to support growth of the microorganisms present was probably very small and not limited to any one particular type.

4. RP-1 Fuel Contaminants

The sample of RP-1 rocket fuel provided by the Natick Laboratories was found to contain bacteria and fungi. They were observed in droplets of water on the container bottom after the fuel had been removed. Two cultures were isolated, a fungus and bacteria. The bacterial isolate grew readily on the RP-1 fuel, while the fungus grew very poorly.

An attempt was made to determine which components of the fuel the organisms utilized. Two test series were set up in triplicate with 40 ml of mineral salts solution and 50 ml of sterile RP-1 fuel. One series, number XI, was inoculated while the other, series XII, served as a sterile control. After seven days incubation on a New Brunswick gyrotary shaker at room temperature, the fuel from each series was combined and analyzed. The results are recorded in Tables IV and V.

Mass Spectrometer and Ultraviolet Spectrophotometer analyses failed to demonstrate any differences between the two samples of fuel. Gas Chromatography, on the other hand, revealed slight variations in the distribution of paraffins in the lower molecular weight range (up to C_{12}). Unfortunately, these samples were not run in duplicate and these slight differences may be within the experimental error of the procedure.

It was quite apparent from these studies with RP-1 fuel that direct analysis of exposed fuel would not provide useful information on the hydrocarbon types utilized by microorganisms. An indirect approach employing pure hydrocarbon was chosen. The procedure used and results obtained are presented in Section C-4 of this report.

TABLE IV

HYDROCARBON TYPE ANALYSES OF RP-1 FUEL SAMPLES
(Volume Percent)

Method of Analysis and Hydrocarbon Types	Initial	Storage Tests Eight Weeks		Pure Culture Tests	
		Series II (Active)	Series VI (Sterile)	Series XI (Active)	Series XII (Sterile)
High Temperature Mass Spectrometer (Atlantic Refining Co. Method)					
Paraffins	54.4	53.9	55.1	54.9	53.9
Mono-cycloparaffins and Non-condensed Cycloparaffins	28.2	28.6	27.6	27.5	28.4
Condensed Dicycloparaffins	10.8	10.8	10.7	10.7	11.0
Condensed Tricycloparaffins	1.4	1.6	1.6	1.7	1.6
Alkyl Benzenes	4.1	4.1	4.0	4.1	4.1
Indanes and/or Tetralins	1.1	1.0	1.0	1.1	1.0
Total	100.0	100.0	100.0	100.0	100.0
Ultraviolet Spectrophotometer (ASTM Method D1840-61T)					
Total Naphthalenes	0.12	0.11	0.12	0.12	0.12

TABLE V

CARBON NUMBER DISTRIBUTION OF COMPOUND TYPES
IN SATURATE FRACTIONS[1] OF RP-1 FUEL BY GAS CHROMATOGRAPHY[2]
(Volume Percent)

Carbon Number	Paraffin Type	Fuel Volume Percent in Sample Composites				
		Initial	II	VI	XI	XIII
C_9	Iso- & Cyclo-	0.3	0.6	0.6	0.1	0.4
	Normal	0.4	0.4	0.5	0.3	0.4
C_{10}	Iso- & Cyclo-	1.2	1.5	1.5	1.1	1.6
	Normal	1.9	1.9	1.8	1.8	1.8
C_{11}	Iso- & Cyclo-	4.1	4.4	4.0	4.1	4.0
	Normal	5.1	4.8	4.8	4.8	4.8
C_{12}	Iso- & Cyclo-	10.3	10.0	10.3	10.3	10.3
	Normal	12.0	10.9	11.0	12.4	11.0
C_{13}	Iso- & Cyclo-	14.8	14.6	14.6	15.1	14.6
	Normal	9.8	9.2	9.0	8.9	9.0
C_{14}	Iso- & Cyclo-	16.9	16.3	16.4	16.9	16.4
	Normal	6.8	7.4	6.7	7.1	6.7
C_{15}	Iso- & Cyclo-	9.7	9.4	9.8	9.4	9.8
	Normal	3.1	3.0	3.2	3.2	3.2
C_{16}	Iso- & Cyclo-	3.1	4.7	5.0	3.9	5.0
	Normal	0.4	0.8	0.8	0.5	0.8

(1) All samples were acid treated according to ASTM Method D-1019-62 to remove aromatic and olefin components.

(2) Analyses were made on an F&M Model 500 Temperature Programmed Chromatograph employing a 20' boiling point column containing Sinclair 9150 Bright Stock oil on Chromosorb. The temperature was programmed at a rate of 7.9 °C/min. from 125 to 205°C. Helium was used as a carrier at a flow rate of 25cc/25 sec.

B. Preliminary Studies on Fuel Decomposition Products

In an effort to characterize the products of microbial attack on fuels, for future reference some of the physical properties of sludge and water from storage studies conducted with heating fuels were examined. The water bottoms contai ed fungi, yeast and bacteria, including sulfate-reducers, and closely resembled bottoms observed in highly contaminated storage tanks. A sample of these bottoms was separated into three fractions - fuel, interface sludge and water. The fuel and water fractions were clarified by centrifugations at 25,000 rpm in a Sharples Super Centrifuge. Sediment removed from each fraction had the same appearance as the interfacial sludge. The centrifuged fuel was clear and bright but the water fraction remained slightly hazy and had an orange cast. Solids from the fuel, water and interface were black in color with clumps of gray flocculent material. Microscopic examination of the interface revealed fungus filaments and bacteria and yeast cells in an oil-water emulsion. The emulsion imparted a slimy consistency to the material. The black color was apparently due to iron oxides, sulfides, and small quantities of fuel oil sludge.

The surfactant properties of the sludge were quite apparent as the interfacial material suspended readily in the fuel and water phases after slight shaking. On vigorous shaking a rather stable emulsion was produced. This presented the problem of having a material which can be defined as both sludge and surfactant. It appears that in most systems of this type there are a number of materials which may act as surfactants, including microbial cells, rust, dirt, etc. Also, within a particular sample, several emulsion systems may be present with the continuous phases alternating from water to oil to water. This may explain why some of the interfacial material enters the fuel phase while some suspends readily in the water phase. Microscopic examination of the interface employing fuel soluble and water soluble dyes confirmed the existence of these alternating emulsion systems within the sludge.

Considering the complexity of these materials, it is not surprising that previously accepted definitions of sludge, slime, gum and surfactant are no longer adequate. On the basis of these studies, it was decided that the term "sludge" would be used to describe the interface and all suspended materials or sediment from the fuel and water phases. Solvent extraction procedures will be employed to distinguish between microbiological sludge and normal fuel decomposition products. The tendency of these materials to form emulsions will be noted but the term "surfactant" will be applied only to specific substances or sludge components having surface active properties. The use of the term "slime" will be avoided in the present studies except to describe the consistency of a material. Standard ASTM procedures will be used to establish the presence of existing and potential gums in fuel, thus limiting the use of this term to a specific test.

C. Compression Ignition Turbine Engine Fuel Storage Tests

Compression Ignition Turbine Engine (CITE) fuel was used in all tests throughout the remainder of this program at the request of the Natick Project Officer, Dr. A. M. Kaplan. CITE fuel is being developed by the Army and is intended as a replacement for the several grades of diesel fuels and turbine fuels in the supply system. It is generally a straight-run product having a gravity range from 35° to 55°API and a distillation range of 130 to 575°F. A number of anti-oxidants, corrosion inhibitors and metal deactivators are approved for use in this fuel.

The first series of tests covered in this report were set up with CITE fuel supplied by the Natick Laboratories. All subsequent tests were set up with blends of fuel prepared by Sinclair specifically for this program.

1. Tests on CITE Fuel Supplied by the Natick Laboratories

CITE fuel was stored over sea water and tap water inoculated with a composite of contaminated tank bottom samples from fuel storage tanks. The tests were set up in one-pint bottles (400 ml of fuel and 20 ml of water) and in one-gallon steel containers (1 gal of fuel and 100 ml of water). All units were stored at ambient temperature (75-80°F.) for 16 weeks. Nitrogen and phosphorus were added to half the units in the form of salts dissolved in distilled water. Appendix D describes in detail the components and procedures used in preparing the test units.

The composite storage tank bottoms used to inoculate the tests was similar to the preparation used in the RP-1 fuel study covered in Section A of this report. It was composed of a mixture of bottom samples from fuel oil and kerosine storage tanks. There is a possibility that one or two of the additives used in these two fuels are present in the CITE fuel. Also, the two fuels contain hydrocarbons of the same type that would be expected in the CITE fuel. Thus the microorganisms in the composite bottoms may have been acclimated to some extent to various components of the test fuel. Bacteria, yeast and fungi were present in the composite preparation, including sulfate-reducers.

Fuel stability problems were encountered while setting up the tests, possibly due to a contaminant present in the container in which the fuel was shipped. Fuel of this type can normally be steam sterilized at 15 psi (121°C.) for 20-30 minutes without altering quality. Several days after the fuel was sterilized, a dark brown precipitate was observed in the sample. The material was removed by filtration and was found to be soluble in 1:1 ethanol:benzene mixture. Infrared and Mass Spectrometer analyses gave spectra typical of normal fuel oil sludge.

Sludge formation was observed at room temperature in metal and glass containers, with and without water, and appeared to be accelerated by heating. A sample of the fuel sterilized by filtration also developed the sediment. In addition, the filter plugged after approximately 1000 ml of fuel had been filtered. The accumulation of sludge at the fuel-water interface of steam and filter sterilized fuel is shown in Plate 4.

Storage tests with the CITE fuel were continued and an effort was made to detect any effect the fuel oil sludge may have on the growth of microbial contaminants. Organic nitrogen compounds in the sludge could provide essential nutrients for the microorganisms in the water bottoms.

a. Microbiological Observations

Samples of the water bottoms from each unit were tested bi-weekly for eight weeks and thereafter at four-week intervals over a total period of 16 weeks. The number of viable microorganisms present was estimated on all water samples from inoculated units (I, II, III and IV) and the sterile control units (V, VI, VII and VIII) were checked for contaminants. Appendices B and C present the media and procedures used in the microbiological analyses.

Microbiological data on Group A (Glass units) are presented in Table VI. Very little difference was observed in the numbers of organisms present in the various test systems at any one time interval. All units appeared to reach a maximum viable count between 10 and 50 million microorganisms per ml of water bottoms. Several interesting observations were made on this test group. Bacteria persisted in essentially all units throughout the test period. Also, the pH of all units remained relatively constant. These results are somewhat divergent from those obtained with the RP-1 fuel in which fungi were found consistently in essentially all units and a drop in pH was recorded.

A contributing factor to the persistence of bacteria in the CITE fuel series may have been the relative concentrations of bacteria and fungi in the inoculum used in the two fuel studies. Nitrogen present in the sludge that accumulated in all units and the slightly alkaline pH may have encouraged continued bacterial growth.

Results of microbiological analyses of Group B (Steel units) are presented in Table VII. Some difficulty was encountered in obtaining representative samples from these units because of the heavy accumulations of corrosion products. This influenced the microbial counts, causing some variation. However, the results do suggest that the added nitrogen and phosphorus salts stimulated microbial growth in series II and IV. During the last eight weeks of storage, a gradual decline was noted in the number of viable bacteria present. This was followed by the development of yeast and fungi in the inoculated series (I, II, III and IV). The yeast and fungi were probably encouraged by drop in pH to

Plate 4. CITE Fuel, Steam and Filter Sterilized, over dilute sea water, showing the sludge accumulations after several weeks storage at room temperature (75-78°F.) in the dark.

TABLE VI Results of Microbiological Analyses of Water Bottoms from CIE Fuel Storage Tests, Group A (Glass Units)

Test System (Water Bottoms)	pH			Estimated Number of Viable Microorganisms in Millions per ml.* Time in Weeks						
	Initial	8 Weeks	16 Weeks	0	2	4	6	8	12	16
Inoculated Units										
I. Sea Water	7.1	7.1	7.0	0.50 BF[1] (4)[2]	10.00 B (4)	10.00 B (4)	50.00 B (4)	10.00 B (4)	10.00 B (4)	50.00 B (3)
II. Sea Water, Nitrogen & Phosphorus Salts	7.2	7.0	6.5	0.50 BF (4)	10.00 B (4)	50.00 B (4)	10.00 B (4)	50.00 B (4)	50.00 B (4)	50.00 B (4)
III. Tap Water	7.1	7.3	7.4	0.50 BF (4)	5.00 B (4)	1.00 B (4)	10.00 B (4)	10.00 B (4)	1.00 B (4)	1.00 B (4)
IV. Tap Water, Nitrogen & Phosphorus Salts	7.2	7.0	6.5	0.50 BF (4)	50.00 B (4)	50.00 B (5)	50.00 (4)	50.00 B (4)	100.00 B (4)	50.00 B (5)
Sterile Control Units										
V. Sea Water	7.1	7.0	6.9	Sterile						
VI. Sea Water, Nitrogen & Phosphorus Salts	7.2	6.9	6.9	"						
VII. Tap Water	7.1	7.0	7.4	"						
VIII. Tap Water, Nitrogen & Phosphorus Salts	7.2	7.1	7.0	"						
IX. Fuel, No Water	-	-	-	"						

* - Average of three determinations.
1 - BF stand for Bacteria and Fungi, respectively.
2 - Number in parentheses is rough estimate of the number of obviously different colony types present on TGE agar plates.

TABLE VII Results of Microbiological Analyses of Water Bottoms from CIE Fuel Storage Tests, Group B (Steel Units)

Test System (Water Bottoms)	pH			Estimated Number of Viable Microorganisms in Millions per ml.* Time in Weeks						
	Initial	8 Weeks	16 Weeks	0	2	4	6	8	12	16
Inoculated Units										
I. Sea Water	7.1	6.2	5.0	0.50 BF[1] (4)[2]	0.10 B (4)	0.01 B (3)	1.00 B (3)	0.10 B (4)	0.10 BY (3)	0.10 BY (4)
II. Sea Water, Nitrogen & Phosphorus Salts	7.2	6.8	5.2	0.50 BF (4)	50.00 B (4)	10.00 B (4)	50.00 B (4)	10.00 B (4)	0.01 BY (3)	1.00 BYF (4)
III. Tap Water	7.1	6.9	5.1	0.50 BF (4)	0.10 B (3)	10.00 B (4)	5.00 B (4)	1.00 B (4)	- **	0.10 BYF (4)
IV. Tap Water, Nitrogen & Phosphorus Salts	7.2	7.6	5.4	0.50 BF (4)	50.00 B (4)	10.00 B (4)	50.00 B (4)	100.00 BF (5)	50.00 BF (4)	50.00 BYF (5)
Sterile Control Units										
V. Sea Water	7.1	5.4	5.0	Sterile						
VI. Sea Water, Nitrogen & Phosphorus Salts	7.2	7.3	6.4	*						
VII. Tap Water	7.1	6.5	5.7	*						
VIII. Tap Water, Nitrogen & Phosphorus Salts	7.2	8.5	10.0	*						
IX. Fuel, No Water	-	-	-	*						

* - Average of three determinations.
1 - B-F-Y stand for Bacteria, Fungi and Yeast, respectively.
2 - Number in parentheses is rough estimate of the number of obviously different colony types present on TGE agar plates.
** - Samples were collected but counts were not made.

below 5.5 in all inoculated units. Similar drops in pH were noted in the sterile units without nitrogen, suggesting that chemical reactions were responsible. A sharp rise in pH was noted in sterile control series VIII which contained tap water with added nitrogen and phosphorus.

Despite the difference in environment, there was no evidence of one particular type of bacteria predominating in any of the units. In essentially every case, four or more different types of bacteria were present.

Photographs were made of representative units from each series in Group A (Glass units) and profile samples (Appendix A) from Group B (Steel units) to record changes in appearance (Plates 5-9). The rapid accumulation of sludge in all units was quite apparent at the end of four weeks. Sludge formation appeared to be greater in the units containing sea water (Plate 7). In general, the inoculated units containing nitrogen and phosphorus salts were the most turbid (Plates 7 and 8). Bottom deposits and flakes at the interface in the tubes containing profile samples from the units in Group B are for the most part rust (Plate 9). However, some sludge was observed, as well as turbidity, apparently due to microbial growth. This was particularly discernible in units which contained added nitrogen and phosphorus.

b. Fuel Quality

Fuel quality tests outlined in Military Specification MIL-F-45121A, dated 20 May 1960, and conducted according to the specified ASTM test method, were run on fuel from all units in each group. Composite fuel samples were prepared from the three units in each series. Results of tests on samples from Groups A and B are recorded in Tables VIII and IX. Only slight variations were observed within the two groups, all of which can be explained on the basis of experimental variation. Consistent differences were noted, however, in the initial boiling points (IBP) of samples between the two groups. Samples in Group A (Glass bottles) had consistently lower IBP than samples from Group B (Steel containers). While the IBP is one of the most difficult points to establish, this difference does appear to be real, and is probably due to the sterilization process. The larger volume of fuel in the Group B units (one gallon) required considerably more time to cool than the pint samples in Group A, thus the lower boiling components were more readily lost. This also explains the low IBP of the base fuel sample which was not subjected to sterilization.

In addition to evaluating the quality of the fuel, the water bottoms in each group were checked for emulsifying tendencies. This was accomplished with the units in Group A by vigorously shaking the bottles and allowing them to stand for a short period of time. The speed at which the two phases separated was noted. Only the inoculated units which contained tap water had a tendency to emulsify. The units in Group A were tested only at the end of the 16 weeks storage period to avoid introducing mixing as a variable in the study.

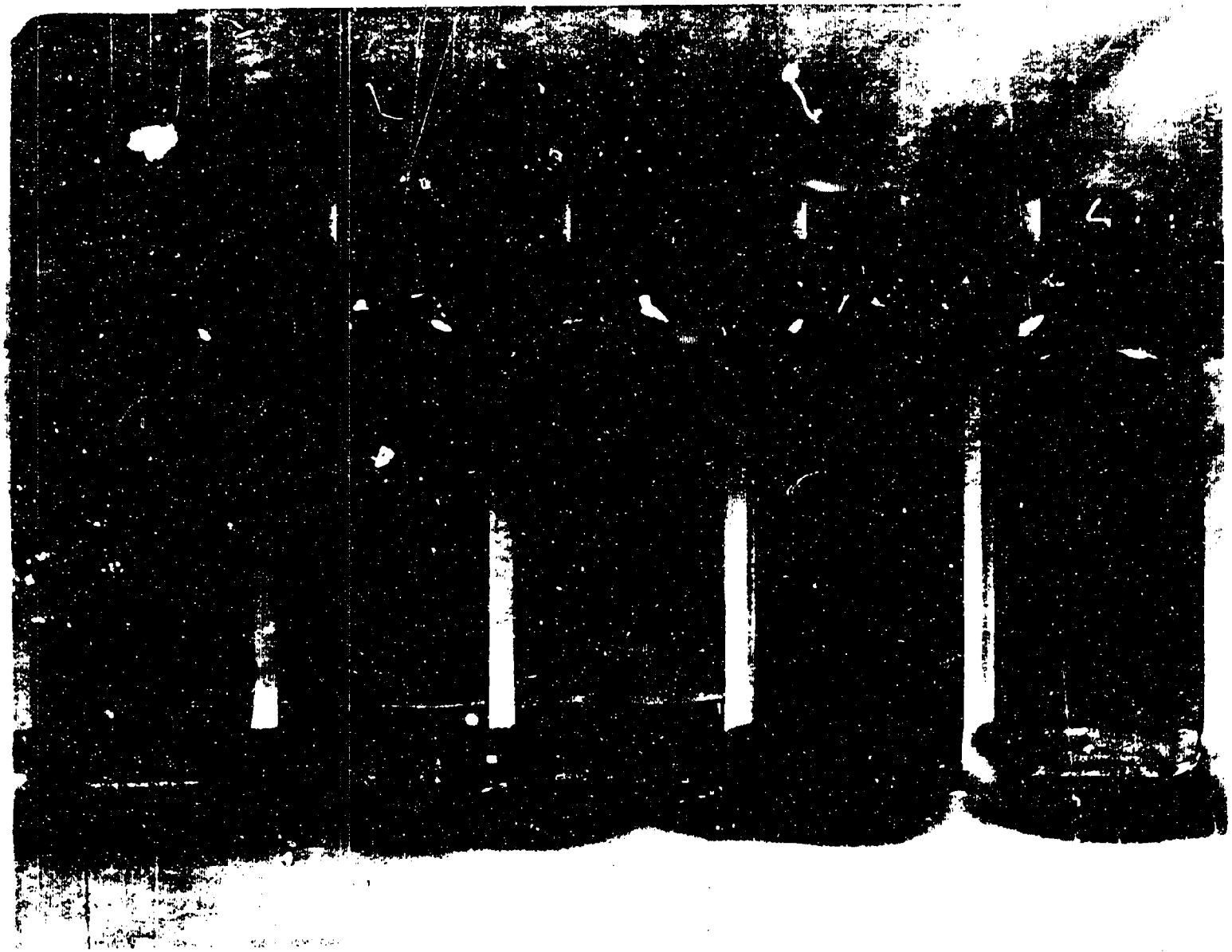

Plate 5. CITE Fuel Group A, Series I, II, III, IV and IX, Initially.

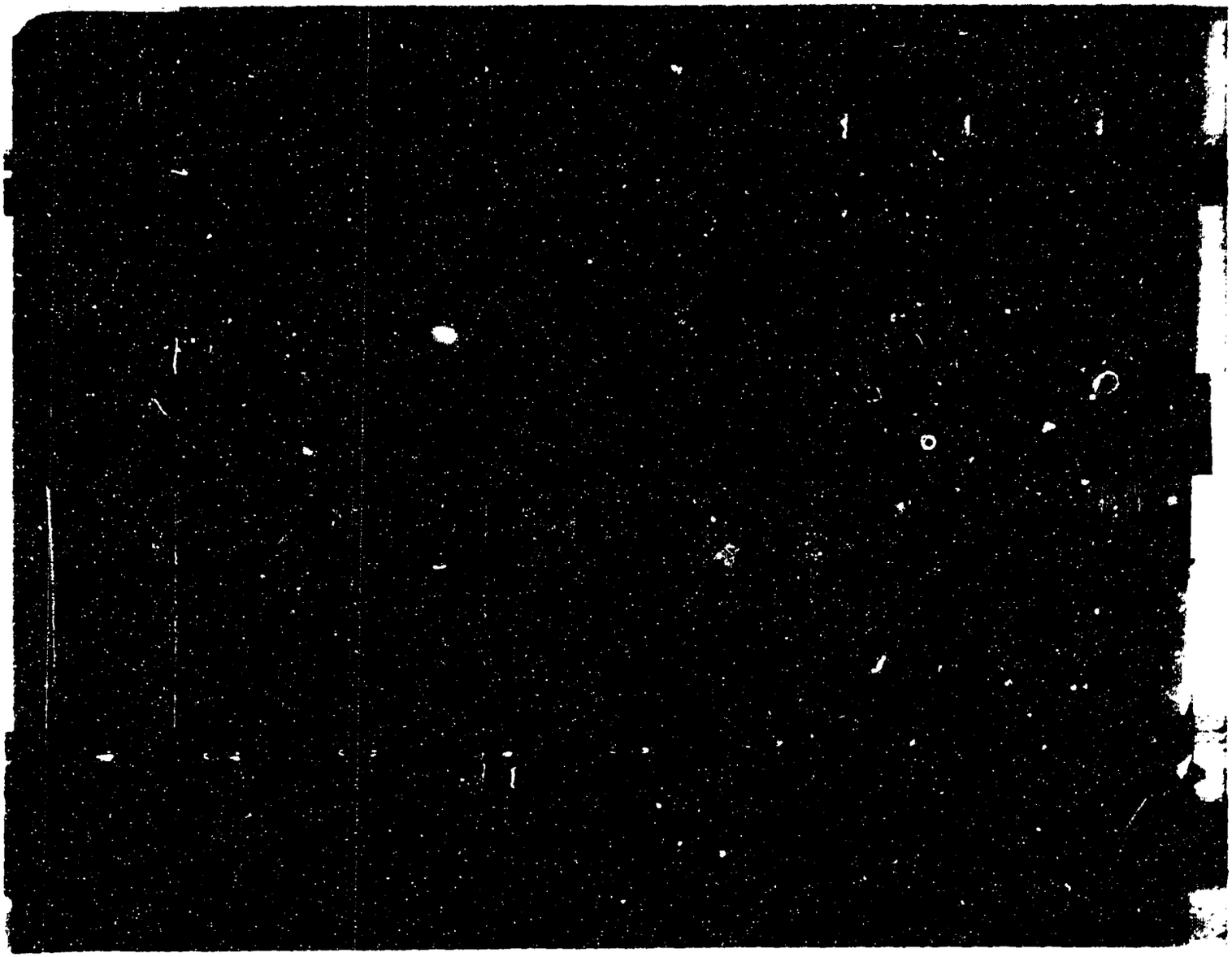

Plate 6. CITE Fuel Group B, Series I through IX, Initially.

Plate 7. CITE Fuel Group A, Series I, II, V, VI and IX, After 16 Weeks Storage.

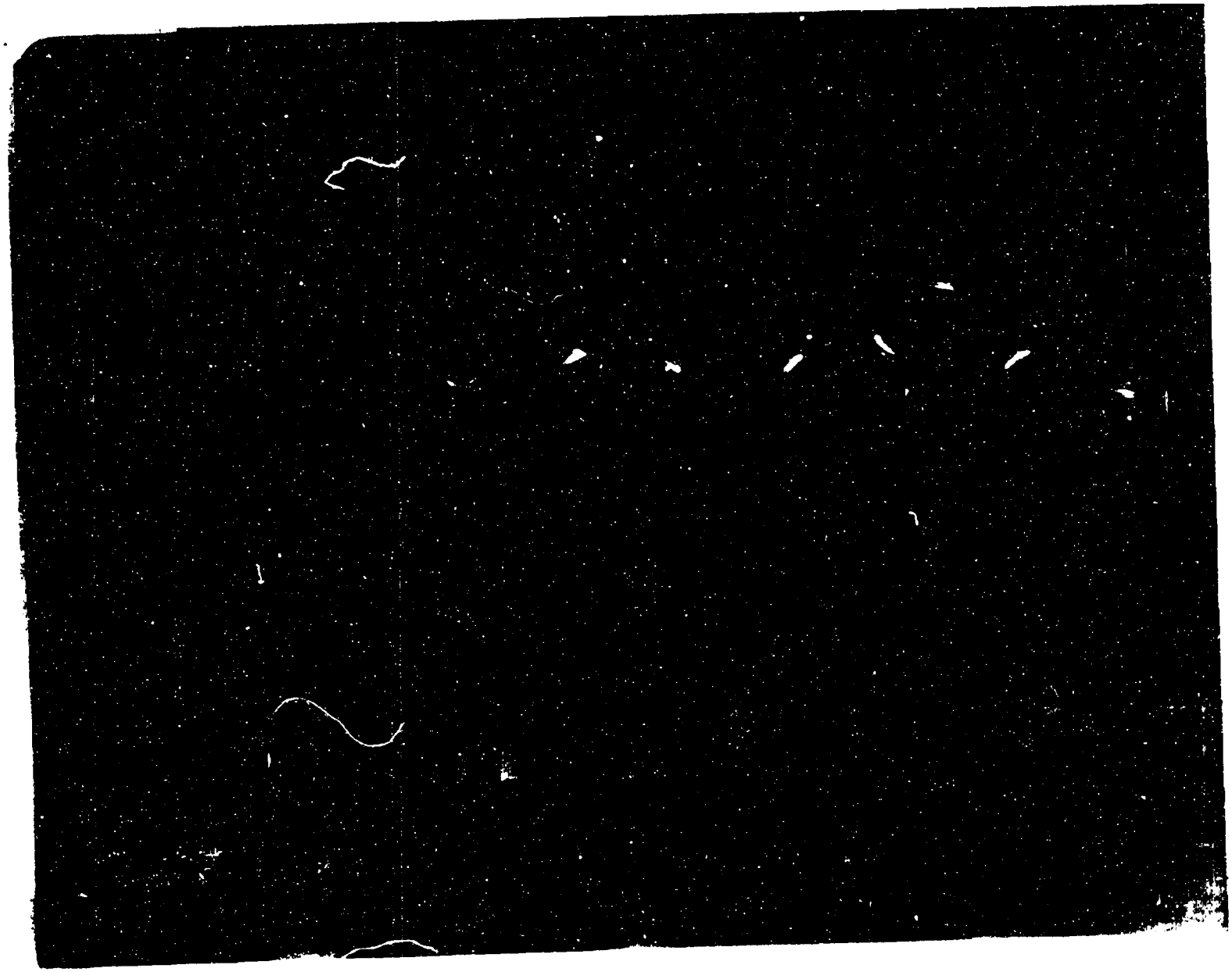

Plate 8. CITE Fuel Group A, Series III, IV, VII, VIII and IX, After 16 Weeks Storage.

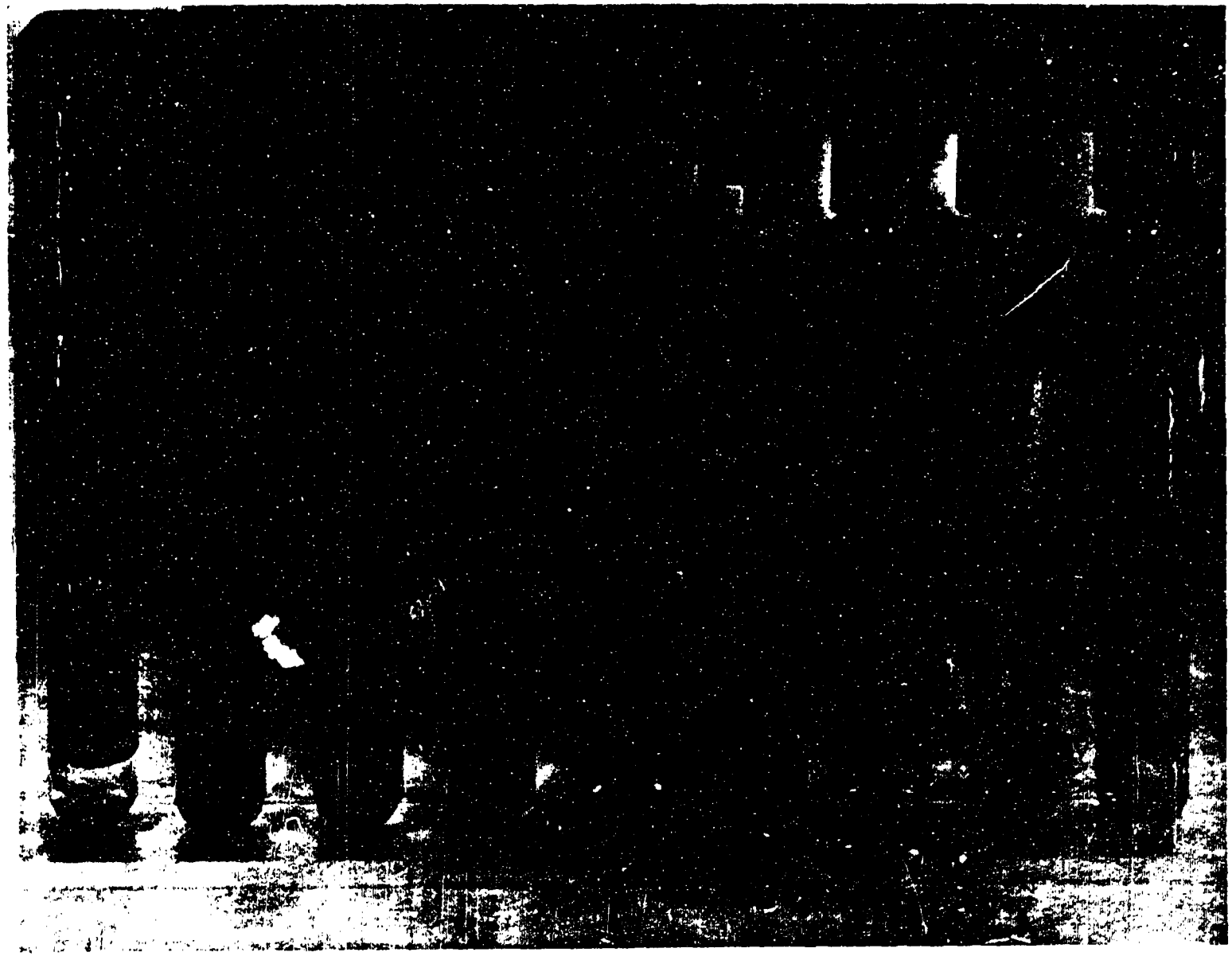

Plate 9. CITE Fuel Group B, Series I through IX, After 16 Weeks Storage.

TABLE VIII Results of Quality Tests on CIE Fuel After 16 Weeks Storage Over Sea Water and Tap Water In Glass

Components of Test System	ASTM Method	Initial Control Fuel	Storage Test Systems(1) I	II	III	IV	V	VI	VII	VIII	IX
Sea Water			x	x			x	x			No Water Bottoms
Tap Water					x	x			x	x	
Nitrogen & Phosphorus Salts				x		x		x		x	
Inoculum			Active				Sterile				
Quality Tests											
Distillation	D-86										
IBP		115°F	138°F	136°F	138°F	135°F	146°F	142°F	136°F	146°F	156°F
5%		176	194	196	204	197	204	209	196	207	216
10		206	218	224	229	222	229	232	220	231	236
20		247	253	258	263	256	262	260	254	260	262
30		278	276	283	286	282	286	286	279	284	286
40		304	306	314	314	314	317	317	312	316	320
50		340	346	352	347	352	352	352	350	353	358
60		370	372	378	375	376	376	381	376	380	385
70		402	398	404	404	404	406	407	404	405	407
80		427	425	430	429	428	430	430	428	430	430
90		454	449	455	456	458	458	456	455	457	457
95		476	465	478	478	482	481	478	480	482	481
EP		507	507	509	508	508	506	508	504	510	504
Residue (Vol.%)		1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.5
Loss (Vol.%)		1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Gravity, °API	D-287	49.2	47.7	48.0	48.2	48.5	47.9	47.7	47.9	47.7	47.1
Existent Gum (mg/100ml)	D-381	2.0	3.1	3.5	3.1	2.4	3.2	3.5	3.0	1.9	3.0
Potential Gum, 16 hrs. (mg/100ml)	D-873	4.9	5.5	4.4	4.6	5.1	6.4	4.6	5.2	4.4	6.2
Aromatics (Vol.%)	D-1319	21.1	22.5	23.5	23.5	22.5	23.0	22.0	23.0	23.5	23.5
Olefins (Vol.%)	D-1319	0.4	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Copper Strip Corrosion	D-130										
122°F.		1A	1A	1A	1A	1A	1A	1A	1A	1A	1A
212°F.		1A	1A	1A	1A	1A	1A	1A	1A	1A	1A
Total Nitrogen (Kjeldahl), ppm		20	21		19		22		22		

(1) Samples analyzed were composites of fuel from the three units in each series.

TABLE IX **Results of Quality Tests on CIE Fuel After 16 Weeks Storage Over Sea Water and Tap Water In Steel**

Components of Test System	ASTM Method	Initial Control Fuel	Storage Test System (1) I	II	III	IV	V	VI	VII	VIII	IX
Sea Water			x	x			x	x			No Water Bottoms
Tap Water					x	x			x	x	
Nitrogen & Phosphorus Salts				x		x		x		x	
Inoculum			Active				Sterile				
Quality Tests											
Distillation	D-86										
IBP		115°F	162°F	152°F	158°F	161°F	167°F	162°F	150°F	156°F	161°F
5%		176	220	223	221	217	229	224	216	216	222
10		206	234	242	244	239	250	244	236	236	242
20		247	269	272	270	268	276	275	263	262	269
30		278	300	298	296	294	302	300	288	286	293
40		304	336	328	325	324	334	328	320	320	326
50		340	365	360	359	355	362	360	356	358	358
60		370	394	390	376	384	376	384	384	385	386
70		402	417	412	412	408	410	408	408	407	408
80		427	441	436	435	431	433	432	432	430	430
90		454	470	464	462	457	459	458	457	457	458
95		476	500	489	484	478	482	482	483	481	482
EP		507	516	514	511	505	512	510	508	504	505
Residue (Vol.%)		1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.5	1.5	2.0
Loss (Vol.%)		1.0	2.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.5
Gravity, °API	D-287	49.2	46.8	47.2	47.1	47.1	46.7	47.0	47.1	47.1	46.8
Existent Gum (mg/100ml)	D-381	2.0	2.6	2.5	2.5	2.6	2.4	2.2	2.8	2.6	3.1
Potential Gum, 16 hrs. (mg/100ml)	D-873	4.9	4.7	4.6	4.5	5.0	5.0	4.6	4.6	5.2	5.2
Aromatics (Vol.%)	D-1319	21.1	24.0	23.0	23.5	24.5	24.0	23.5	23.0	24.0	24.5
Olefins (Vol.%)	D-1319	0.4	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.0	0.0
Copper Strip Corrosion	D-130										
122°F.		1A	1A	1A	1A	1A	1A	1A	1A	1A	1A
212°F.		1A	1A	1A	1A	1A	1A	1A	1A	1A	1A
Total Nitrogen (Kjeldahl), ppm		20	21		20		20		21		

(1) Samples analyzed were composites of fuel from the three units in each series.

All profile samples (Appendix D) collected from the units in Group B were checked for emulsifying tendencies. However, only the units in series IV, tap water with nitrogen and phosphorus, showed any sign of emulsification. This was first observed in the 4-week samples.

c. Analysis of Sludge

Considerable quantities of sludge accumulated at the interface of both active and sterile units in Group A (Glass units). This material was apparently insoluble in both the water and fuel phases of the system and its formation appeared to be influenced by steam sterilization, as pointed out earlier (Plate 4). The amount of sludge present in each unit was determined gravimetrically at the end of the 16-weeks test period by the methods described in Appendix E.

Three fractions were obtained and their weight determined:

A) Insoluble Material - Insoluble in fuel, water and benzene-acetone-methyl alcohol solvent mixture. This fraction will be referred to later as microbial sludge.

B) Fuel Sludge - Material insoluble in fuel and water but soluble in benzene-acetone-methyl alcohol solvent mixture.

C) Non-volatile, water soluble materials and fine particles which may have passed through the filter pads.

The results of these determinations are recorded in Table X. The quantities of sludge formed in the presence of sea water and tap water were greater than the quantity formed in series IX which contained only fuel. There is some indication that sea water bottoms may enhance formation of this insoluble material more than tap water.

In the active test series without added nitrogen and phosphorus (I and III), substantially less insoluble sludge (fraction A) was found than in the sterile control units (V and VII). This suggests that the bacteria utilized or in some manner altered the material. In active units II and IV which contained added nitrogen and phosphorus this trend is not as distinct compared to the controls, units VI and VIII. Nitrogen and phosphorus would appear to be important factors in this phenomenon.

It is difficult to explain this observation without further analytical work. However, several possible explanations may be offered. If the solubility of this insoluble material was altered by biological action it may have re-dissolved in the fuel phase or in the water phase as a volatile component. Another possibility is that the organic components of this material were utilized by the bacteria and then lost by evolution of CO_2.

TABLE X

Results of Gravimetric Determinations of Sludge

(Average Weight in mgs per Test Unit)[1]

	Active Series				Sterile Series				
	I Sea Water	II Sea Water N & P	III Tap Water	IV Tap Water N & P	V Sea Water	VI Sea Water N & P	VII Tap Water	VIII Tap Water N & P	IX Fuel Only
A[2]	10.3	37.2	9.7	18.9	29.0	48.0	33.0	12.3	2.3
B[3]	8.0	8.9	10.1	7.1	10.3	9.5	7.3	5.6	6.5
C[4]	663.0	659.0	31.0	89.0	680.0	683.0	40.0	88.0	-

(1) Two units from each series were checked and the average weight of the materials found is recorded in the table.

(2) Insoluble Material - Insoluble in fuel, water and benzene-acetone-methyl alcohol solvent. Agreement between duplicate determinations was very good, varying in most cases by only a few mgs.

(3) Fuel Sludge - Material insoluble in fuel and water but soluble in benzene-acetone-methyl alcohol solvent.

(4) Non-volatile, water solubles and fine particles which may have passed through the filter pads.

Infrared spectra of fractions A and B from series V are presented in Figures 1 and 2. Similar patterns were obtained on samples from active series. These spectra indicate that fraction A may contain an inorganic sulfate or phosphate while fraction B appears to be typical fuel oil sludge. The spectrum of fraction B indicates the presence of hydroxy and carbonyl structures characteristic of such sludges.

An observation of interest was the fact that the water bottoms from the inoculated units filtered very slowly compared to the sterile controls. This is of particular significance since, in some cases, they contained less sludge than the sterile units. Microscopic examination of sludge from the inoculated series revealed the presence of bacterial cells and debris.

It is quite apparent that the CITE fuel used in these tests was atypical and was probably contaminated. While the observations on sludge formation are most interesting, they do not appear to be typical materials that would be expected from good CITE fuel. Therefore, we discontinued our analysis of the sludges formed in tests with this particular fuel.

d. Observations on Corrosion

During the course of this investigation we have considered a number of factors which might contribute to problems in the field. One of the factors considered was the presence of iron. Although we have not been able to clearly establish the role of iron in fuel problems associated with microorganisms, we have observed some interesting effects of microorganisms on corrosion. While examining the contents of several one-gallon steel containers used in CITE fuel storage tests, we noted differences in the extent of corrosion above the fuel:water interface. Plate 10 shows sections of two of these containers. Corrosion in containers with active water bottoms (both sea water and tap water) extended an inch or more up the sides of the can. In the sterile controls, corrosion was limited to surfaces in contact with water. The water depth in these containers did not exceed 1/4".

Differences in corrosion were also noted in the additive evaluation study. Corrosion products from the steel wool in Series C and D (Active and Sterile) appeared different (see Section 2). In the active units, the corrosion products had a tendency to agglomerate, while in the sterile units they remained dispersed.

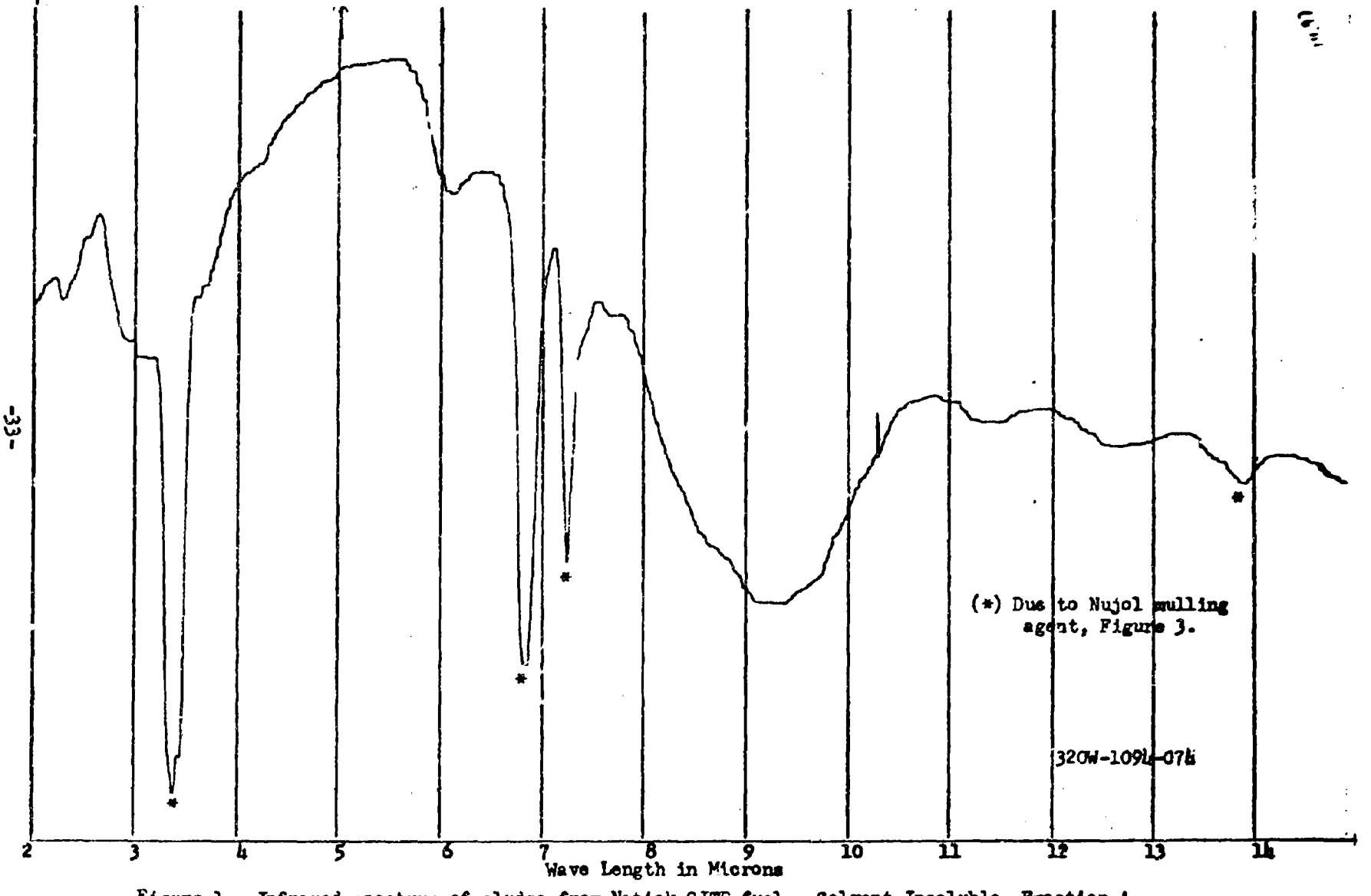

Figure 1. Infrared spectrum of sludge from Natick CITE fuel - Solvent Insoluble, Fraction A.

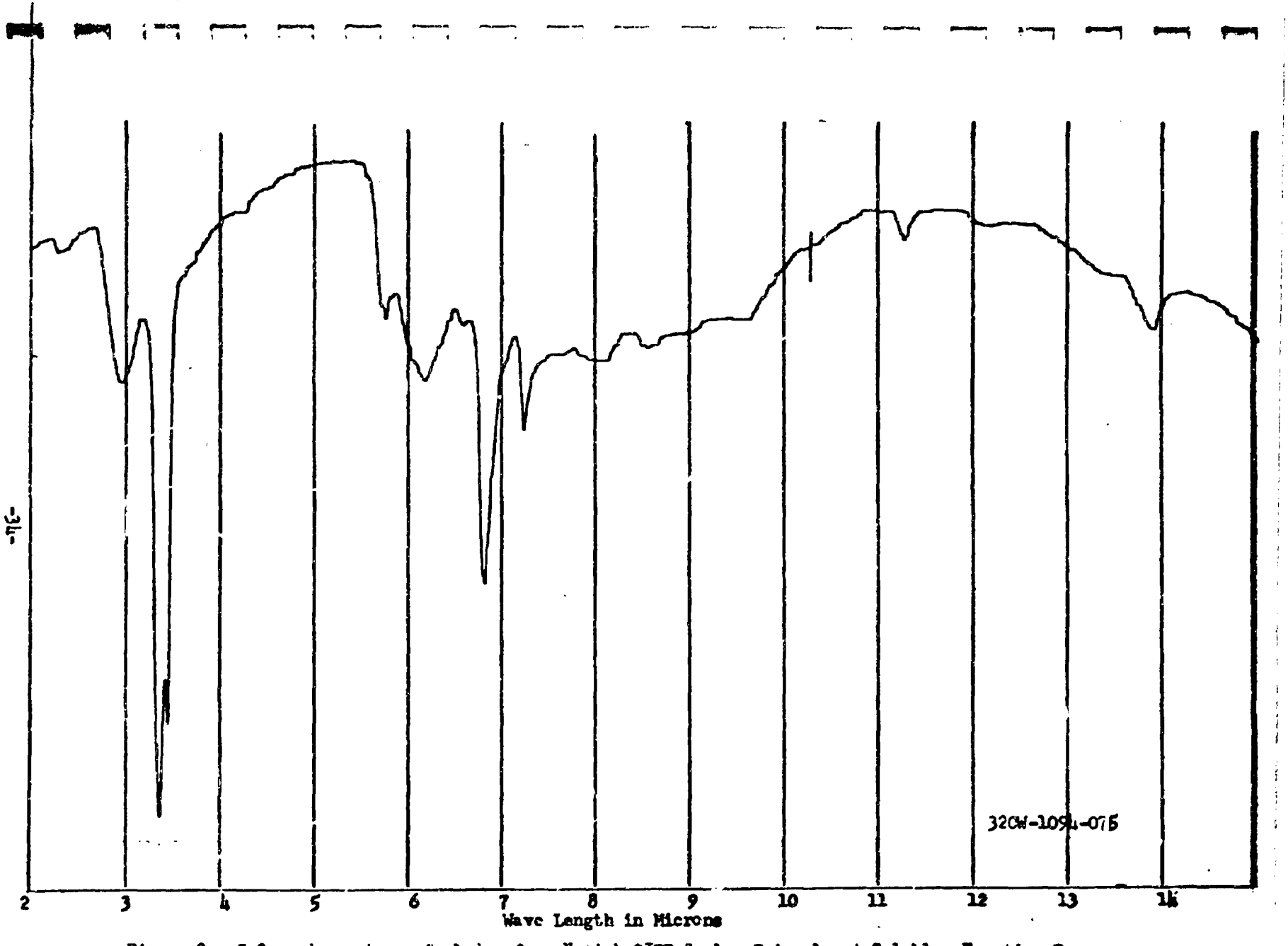

Figure 2. Infrared spectrum of sludge from Natick CITE fuel - Tri-solvent Soluble, Fraction B.

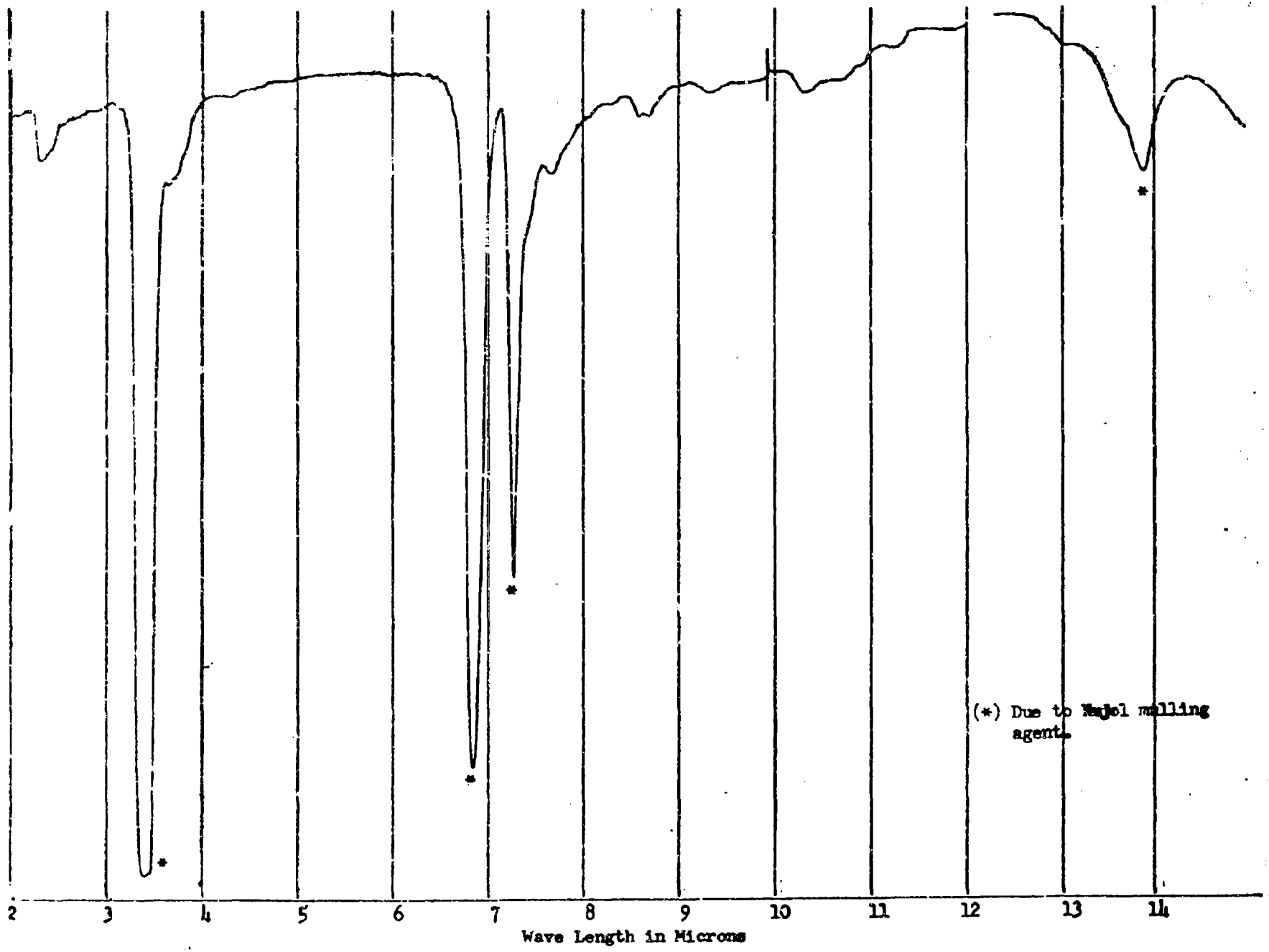

Figure 3. Infrared spectrum of Nujol milling agent.

Plate 10. Photograph showing corrosion of steel containers used in CMTE Fuel Storage Tests.

e. Observations on Contaminated CITE Fuel Samples from Natick

The CITE fuel employed in the initial storage tests was supplied by Natick. The unusual amount of sludge which formed in the test systems suggested that the fuel may have been inadvertently contaminated. Samples of two fuel shipments received at Natick were forwarded to us for analysis and comparison with the apparently contaminated shipment. One-pint samples of the fuel were steam sterilized at 15 psi for 20 minutes, allowed to cool, and then stored for six weeks in the dark at 30°C.

Distillation data and other observations on the fuel samples are presented in Table XI. Fuel shipped under Voucher No. A 23080-3087-33441 appeared to be relatively stable and contained no free water. The distillation results indicated that the fuel employed in our initial storage test was from this shipment. Sludge formation was much greater in fuel from the other shipment (Voucher No. A 23080-3046-9005010-29870). It also had a noticeably darker color. Five of the seven samples from drums shipped under the second voucher number contained water bottoms. Analyses of these bottoms indicated that microorganisms were present.

f. Experimental CITE Fuel Blends

Two blends of CITE fuel were prepared to meet Military Specification MIL-F-46005A(MR), dated 15 May 1963. One blend was prepared with straight run components to produce a highly stable fuel. The other blend was prepared with straight run and catalytic cracked components to produce a potentially unstable fuel. We were of the opinion that these fuels represented the extremes which could be supplied under the above specification. Because of the expected instability of the latter fuel blend without the necessary stabilizing additives, only enough fuel was prepared to meet the requirements of our tests. A relatively large volume of the stable fuel was prepared to avoid variations in composition which may occur between batches.

Blend 1 is part of a batch of additive-free CITE fuel supplied to Dr. A. M. Kaplan under Order No. 19129-F4-1544(N). This fuel was used in all subsequent work presented in this report. Routine fuel specification data are shown in Table XII. In addition, detailed hydrocarbon type analyses were made on the fuel blend.

Low (room) temperature mass spectrometer analysis was made on the fuel fraction boiling below 400°F. (Table XIII). This type of analysis provided a breakdown of the major hydrocarbon types present, including paraffins, cycloparaffins (mono-, di and tri-), alkylbenzenes, indans and/or tetralins, and naphthalenes. A further breakdown of the alkylbenzenes and paraffins was also obtained. The carbon number distribution of the paraffins must be considered semi-quantitative, since the isoparaffins also contribute to the peaks obtained.

TABLE XI

Analyses of CITE Fuel Samples from Natick

Voucher No. A23080-3087-33441

	Drum Number 1	2	3	4	Fuel Used in Initial Storage Tests
IBP(1)	120	117	116	116	115
50%	341	340	342	340	340
EP	507	509	508	508	507
Color	C&B(3)	C&B	C&B	C&B	C&B
Sludge Formation(2)	Trace	+	Trace	+	+
H_2O Bottoms	-	-	-	-	-
Microbial Contamination	-	-	-	-	-

Voucher No. A23080-3046-9005010-29870

	Drum Number 5	6	7	8	9	10	12
IBP	147	138	144	139	137	149	144
50%	362	360	361	356	356	358	362
EP	498	492	498	498	498	494	496
Color	C&B	C&B	C&B	C&B	C&B	C&B	C&B
Sludge Formation	++	++	++	++	++	++	++
H_2O Bottoms	+	-	+	+	+	-	Trace
Microbial Contamination	+	-	+	+	+	-	+
pH of Water Bottoms	6.5	-	7.3	8.1	7.9	-	-

(1) ASTM Test Method D-86.

(2) After stream sterilization in soft glass followed by several days storage. Sludge formation greater in 29870 shipment than in 33441.

(3) C&B indicate Clear and Bright

TABLE XII

Chemical and Physical Test Data
On Additive Free Compression Ignition Engine Fuel Blend 1

Characteristic	Results	ASTM Test Method
Distillation		D86
Initial	134	
5	182	
10	200	
20	229	
30	256	
40	284	
50	319	
60	360	
70	399	
80	426	
90	454	
95	478	
End Point	494	
Residue (Vol. %)	1.5	
Distillation Loss (Vol. %)	0.5	
°API Gravity at 60°F.	51.9	
Existent Gum, mg/100 ml	0.2	D381
Total Potential Residue, 16 hr. Aging, mg/100 ml.	0.4	D873
Sulfur, total - per cent by weight	.043	D1266
Reid Vapor Pressure, psi	2.4	D323
Freezing Point, °F.	-70	D1477
Cetane Number	41.7	D613
Viscosity, kinematic		
at 100°F., centistokes	0.9045	D445
at minus 30°F., centistokes	3.132	D445
Aromatics, Vol. %	8.0	D1319
Olefins, Vol. %	0.0	D1319
Copper Strip Corrosion Classification Bomb, 2 hours, 212°F.	1A	D130
Thermal Stability		(1)
Change in pressure drop in 5 hrs., inches mercury	0.2	
Preheater deposit rating	0	
Water Reaction	1	FTM3251

(1) Conducted in a CFR fuel coker using a preheater temperature of 300°F., a filter temperature of 400°F., and a fuel flow rate of 6 pounds per hour over the 5 hour test period.

TABLE XIII

Hydrocarbon Type Analysis of CITE Fuel Blend 1

Fraction: IBP-400°F. (70% of Total Sample)

Low Temperature Mass Spectrometer(1)

	Volume %
Paraffins	46.3
Mono-cycloparaffins	41.7
Dicycloparaffins	3.4
Tricycloparaffins	0.3
Alkyl-benzenes	8.0
Indans and/or Tetralins	0.2
Naphthalenes	0.1
	100.0

Paraffins(2)

	C_6	7.4
	C_7	11.7
Avg. Carbon # 7.83	C_8	11.2
Avg. Mol Wt. 110.6	C_9	8.8
	C_{10}	4.3
	C_{11}	2.9
	C_{12}	-
		46.3

Alkyl-benzenes(3)

	Benzene	0.4
Avg. Carbon # 8.15	Toluene	1.3
Avg. Mol. Wt. 108.1	Molecular Weight 106	2.6
	" " 120	2.1
	" " 134	1.1
	" " 148	0.5
	" " 162	-
		8.0

(1) ASTM method for hydrocarbon types in low olefinic gasoline by Mass Spectrometry. (Published for information only by ASTM Committee D-2).

(2) These data were derived in conjunction with the above method, but the procedure employed is not actually a part of the method. The procedure employs data used to calculate the average number of carbon atoms in the paraffin fraction of the sample. These data are semiquantitative in that the iso-paraffins may contribute to the peaks obtained.

(3) Sinclair method and calibration data.

The 400°F. plus fraction was analyzed with the high temperature mass spectrometer (Table XIV). Two analyses were made, one on the total sample and the other on an aromatic and olefin free preparation. This enabled us to obtain a better separation of the various hydrocarbon types present. The distribution of n-paraffins reported for this fraction is also semi-quantitative.

The carbon number distribution of the paraffins in the total sample was determined by means of gas chromatography (Table XV). The results are reported in volume per cent, assuming that the peaks obtained reflect only the paraffin components of the fuel.

TABLE XIV

Hydrocarbon Type Analysis of CLTE Fuel Blend 1

Fraction: 400°F. plus (30% of total sample)

High Temperature Mass Spectrometer

Method I (Aromatics + Olefins Removed)

	Volume %
Paraffins	58.7%
Aromatics	0.5
Mono-cycloparaffins	26.65
Dicycloparaffins	12.59
Tricycloparaffins	1.99
	100.00

Method II (Aromatics + Olefins Removed)

n-paraffins	
C_{10}	0.2
C_{11}	0.5
C_{12}	18.1
C_{13}	22.1
C_{14}	35.6
C_{15}	12.1
C_{16}	6.4
C_{17}	2.8
C_{18}	2.2
	100.0

Method III (Aromatics + Olefins Present)

Paraffins	47.5
Cycloparaffins	33.9
Alkyl Benzenes	7.9
Indanes, etc.	4.3
Indenes, etc.	0.4
Naphthalenes	3.0
Biphenyls	1.9
Fluorenes	0.5
Tricyclic aromatics	0.6
	100.0

METHOD I Shell Method and Calibration Data
Presented to ASTM Committee E-14 at Meeting in New Orleans, La., June 3-8, 1963.

Paper #3. Mass Spectrometric Analysis of Middle Distillate Saturate Hydrocarbons. A. Hood, P. R. Mommessin, and B. K. Fritts.

Sample was acid treated according to ASTM Method D1019-62 to remove aromatic and olefin components.

METHOD II Sinclair Method and Calibration Data
Aromatic and olefin components removed as in Method I. Data are semiquantitative in that iso-paraffins may contribute to the peaks.

METHOD III Atlantic Refining Company Method for fractions containing aromatic components.

TABLE XV

Carbon Number Distribution of Paraffins in CITE Fuel Blend 1 by Gas Chromatography* (Total Sample)

Carbon Number	Paraffin Type	Volume %**
C_6	Iso- + Cyclo-	0.2
	Normal	0.4
C_7	Iso- + Cyclo-	2.4
	Normal	3.6
C_8	Iso- + Cyclo-	6.7
	Normal	2.8
C_9	Iso- + Cyclo-	9.1
	Normal	3.4
C_{10}	Iso- + Cyclo-	8.1
	Normal	4.1
C_{11}	Iso- + Cyclo-	6.6
	Normal	4.3
C_{12}	Iso- + Cyclo-	6.2
	Normal	5.5
C_{13}	Iso- + Cyclo-	8.2
	Normal	4.8
C_{14}	Iso- + Cyclo-	7.2
	Normal	3.4
C_{15}	Iso- + Cyclo-	8.0
	Normal	1.6
C_{16}	Iso- + Cyclo-	3.0
	Normal	0.4
		100.0

* Analyses were made on an F & M Model 500 Temperature Programmed Chromatograph employing a 20' boiling point column containing Sinclair 9150 Bright Stock oil on Chromosorb. The temperature was programmed at a rate of 7.9°C/min. from 125 to 205°C. Helium was used as a carrier at a flow rate of 25 cc/25 sec.

** Based on the assumption that the total sample was composed of iso-, cyclo-, and normal paraffins.

2. CITE Fuel Additive Evaluation

Preliminary storage tests with RP-1 and CITE fuels indicated that nitrogen and phosphorus were limiting factors in the development of microbial sludge in fuel-water systems. A possible source of these elements in the field would be fuel components, including additives. Therefore, storage tests were set up employing the additive-free CITE fuel blend to evaluate the influence of qualified corrosion inhibitors, anti-oxidants, and a metal deactivator on microbial growth and sludge formation.

The tests were set up in one-pint glass sample bottles with 400 ml of sterile fuel and 20 ml of 10% sea water, with and without iron (steel wool) (Appendix F). The inoculum employed was a composite of contaminated tank bottom waters from kerosine, gasoline, JP-4, diesel and heating fuel storage tanks, and contained bacteria, yeast and fungi, including sulfate-reducers. Pertinent data on the six corrosion inhibitors tested, including nitrogen (N) and phosphorus (P) determinations, are presented in Table XVI. Table XVII contains similar data on the five anti-oxidants and the metal deactivator tested.

a. Microbiological Observations

Estimates of the number of viable microorganisms present in the water bottoms at various time intervals during the 16-week storage period are presented in Tables XVIII and XIX. Only three of the twelve additives tested significantly affected microbial growth in the adjacent water bottoms. Additive I, DuPont AFA-1 corrosion inhibitor, had the most pronounced effect. The number of microorganisms present in the water bottoms reached 500 million per ml at the end of eight weeks (Table XVIII). This stimulatory effect was apparently due to nitrogen and phosphorus supplied by the DuPont additive (contained 2.41% N and 5.28% P).

Additives II and III, Unicor M (0.88% N and 0.001% P) and Santolene C (0.002% N and 0.326% P) also stimulated microbial growth. The fact that phosphorus is limiting in the sea water bottoms (0.03 ppm P), apparently explains the slightly higher counts obtained with the Santolene C than obtained with Unicor M. The remaining corrosion inhibitors, antioxidants and the metal deactivator had very little noticeable effect on microbial growth in the test system (Tables XVIII and XIX).

Only slight variations in pH were observed in the water bottoms of active and sterile test units. Iron had a slight tendency to make the water bottoms more acidic. However, only one series with iron had a pH below 6 at the end of the first 8-week storage period.

TABLE XVI

Qualified Corrosion Inhibitors

(Qualified under Specification MIL-I-25017B)

Number	Additive & Source	Analysis(1) % Nitrogen	Analysis(1) % Phosphorus	Concentrations Tested(2) mg/L
I	AFA-1 E. I. duPont	2.41	5.28	46.1
II	Unicor-M Universal Oil Products	0.88	<0.001	57.6
III	Santolene C Monsanto Chemical Co.	<0.001	0.326	46.1
IV	TRI-182 Texaco, Inc.	<0.001	<0.001	57.6
V	Lubrizol 541 The Lubrizol Corp.	<0.001	<0.001	57.6
VI	Tolad 244 Petrolite Corp.	1.00	<0.001	57.6

(1) Some of the analyses were repeated employing more sensitive techniques, and therefore the figures are slightly different from those reported in Table I, Quarterly Report No. 3.

(2) Maximum concentration authorized under Specification MIL-I-25017B.

TABLE XVII

Qualified Antioxidants and Metal Deactivator

(Authorized by Specification MIL-F-46005A(MR) dated 15 May 1963)

Number	Additive	Analysis % Nitrogen	Analysis % Phosphorus	Concentration Tested mg/L Active Ingredient(1)
Antioxidants				
VII	2,6-Ditertiary-butyl-4-methylphenol	<0.001	0.010	24
VIII	N,N'-Disecondary-butyl paraphenylene diamine (12.72% Theoretical Nitrogen)	12.51	0.008	24
IX	2,4-Dimethyl-6-tertiary-butyl phenol	0.003	0.012	24
X	2,6-Ditertiary-butyl phenol	<0.001	0.010	24
XI	Mixed tertiary-butyl phenols 75% 2,6-Ditertiary-butyl phenol 10-15% 2,4,6 Tertiary-butyl phenol 10-15% Ortho-tertiary-butyl phenol	<0.001	0.014	24
Metal Deactivator				
XII	N,N'Disalicylindene-1,2-propane-diamine (10% Theoretical Nitrogen)	17.4	<0.001	5.8

(1) Maximum concentration authorized under Specification MIL-F-46005A(MR).

TABLE XVIII

Estimated Number of Viable Microorganisms in Water Bottoms from CIME Fuel Additive Evaluation Tests - Corrosion Inhibitors

Additive Number		Estimated Number in Millions per ml* Time in Weeks 0	2	4	6	8	12	16	Final pH Active/Sterile
I	No Iron	0.5BF(4)**	50.0B(5)	50.0B(5)	100.0B(5)	500.0B(5)	100.0B(5)	100.0B(4)	6.0/6.7***
	Iron	"	50.0B(5)	50.0B(4)	100.0B(5)	100.0B(5)	50.0B(5)	50.0B(5)	5.9/6.7
II	No Iron	"	10.0B(5)	50.0B(4)	1.0B(4)	1.0B(5)	10.0B(4)	1.0B(4)	6.3/6.6
	Iron	"	10.0B(4)	10.0B(4)	1.0B(4)	10.0B(5)	1.0B(3)	1.0B(3)	6.7/6.6
III	No Iron	"	50.0B(5)	50.0B(5)	50.0B(5)	50.0B(5)	50.0B(5)	50.0B(5)	6.7/6.6
	Iron	"	50.0B(5)	50.0B(4)	1.0B(4)	10.0B(4)	50.0B(5)	10.0B(5)	6.6/6.3
IV	No Iron	"	10.0B(4)	1.0B(4)	1.0B(3)	1.0B(4)	1.0B(4)	1.0B(4)	6.7/6.7
	Iron	"	10.0B(4)	1.0B(3)	1.0B(3)	1.0B(5)	1.0B(4)	1.0B(3)	6.5/6.6
V	No Iron	"	1.0B(4)	10.0B(4)	1.0B(4)	0.1B(4)	10.0B(4)	1.0B(4)	6.5/6.2
	Iron	"	10.0B(4)	1.0B(4)	1.0B(4)	1.0B(3)	1.0B(4)	1.0B(4)	6.4/6.4
VI	No Iron	"	10.0B(4)	1.0B(4)	1.0B(3)	1.0B(4)	1.0B(4)	1.0B(4)	6.6/6.5
	Iron	"	50.0B(5)	10.0B(4)	1.0B(3)	0.1B(4)	1.0B(3)	1.0B(2)	6.6/6.5
Control - No Additive									
XIII	No Iron	"	1.0B(3)	1.0B(4)	1.0B(3)	1.0B(4)	1.0B(4)	1.0B(3)	6.8/6.8
	Iron	"	1.0B(4)	0.01B(2)	0.1B(4)	0.1B(4)	1.0B(3)	1.0B(3)	6.5/6.7

* Average of three determinations.

** BF stands for Bacteria and Fungi, respectively. The number in parentheses is a rough estimate of the number of obviously different colony types present on TGE agar plates.

*** The initial pH of all water bottoms was adjusted to 7.0.

TABLE XIX

Estimated Number of Viable Microorganisms in Water Bottoms
from CFTE Fuel Additive Evaluation Tests - Antioxidants and Metal Deactivator

Additive Number		Estimated Number in Millions per ml.* Time in Weeks							Final pH Active/Sterile
Antioxidants		0	2	4	6	8	12	16	
VII	No Iron	0.5BF(4)**	10.0B(4)	0.1B(3)	1.0B(4)	1.0B(5)	1.0B(4)	1.0B(3)	6.8/6.8***
	Iron	"	10.0B(4)	0.01B(3)	0.1B(3)	1.0B(4)	1.0B(3)	1.0B(3)	6.8/6.7
VIII	No Iron	"	50.0B(4)	1.0B(4)	1.0B(3)	1.0B(3)	1.0B(3)	0.1B(3)	6.8/6.6
	Iron	"	1.0B(4)	0.1B(3)	1.0B(3)	1.0B(3)	1.0B(3)	0.1B(3)	6.7/6.5
IX	No Iron	"	10.0B(5)	1.0B(3)	1.0B(4)	1.0B(4)	1.0B(4)	0.1B(3)	6.8/6.8
	Iron	"	10.0B(4)	0.1B(3)	0.1B(3)	1.0B(3)	10.0B(4)	1.0B(4)	6.7/6.7
X	No Iron	"	1.0B(4)	10.0B(3)	1.0B(4)	10.0B(4)	10.0B(5)	1.0B(3)	6.8/6.8
	Iron	"	1.0B(4)	1.0B(3)	1.0B(3)	1.0B(4)	1.0B(4)	1.0B(4)	6.4/6.7
XI	No Iron	"	10.0B(4)	1.0B(4)	1.0B(3)	0.1B(3)	1.0B(3)	1.0B(4)	6.8/6.7
	Iron	"	10.0B(4)	0.1B(3)	0.1B(4)	1.0B(4)	1.0B(4)	10.0B(4)	6.8/6.7
Metal Deactivator									
XII	No Iron	"	1.0B(4)	1.0B(4)	1.0B(3)	10.0B(4)	1.0B(4)	10.0B(4)	6.8/6.9
	Iron	"	10.0B(5)	0.1B(4)	0.1B(3)	1.0B(3)	1.0B(4)	1.0B(3)	6.7/6.6
Control - No Additive									
XIII	No Iron	"	1.0B(3)	1.0B(4)	1.0B(3)	1.0B(4)	1.0B(4)	1.0B(3)	6.8/6.8
	Iron	"	1.0B(4)	0.01F(2)	0.1B(4)	0.1B(4)	1.0B(3)	1.0B(3)	6.5/6.7

* Average of three determinations.

** BF stands for Bacteria and Fungi, respectively. The number in parentheses is a rough estimate of the number of obviously different colony types present on TGE agar plates.

*** The initial pH of all water bottoms was adjusted to 7.0.

Earlier studies with CITE fuel indicated that the 10% sea water bottoms used in the test system were deficient in both nitrogen and phosphorus (0.06 ppm N and 0.003 ppm P). To further substantiate this observation and to provide a control for the additive series, the following four control series were set up with water soluble nitrogen and phosphorus salts:

Control Group XIII		Salts Added to 10% Sea Water Bottoms	mg/20 ml
Series A-1	No Iron	NH_4NO_3	40
and		$NaH_2PO_4 \cdot H_2O$	20
Series C-1	Iron	K_2HPO_4	40
Series A-2	No Iron	NH_4NO_3	40
Series A-3	No Iron	$NaH_2PO_4 \cdot H_2O$	20
		K_2HPO_4	40

The results of these tests are reported in Table XX. Heavy microbial growth was noted in the water bottoms of all four test series. However, the growth present in Series A-1 was noticeably heavier than in the other series. It should be pointed out that the estimated number of microorganisms reported in Table XX for the series containing fungus growth is probably low. It was essentially impossible to obtain a representative sample of water bottoms from these units.

An observation of interest was the fact that fungi developed in Series A-1, A-3 and C-1 but not in A-2, which contained only nitrogen. This may possibly explain the predominance of bacteria in some field samples, and heavy fungus growth in others.

The increased microbial growth and sludge formed in units containing Additives I, II and III, and those units with added nitrogen and phosphorus, Group XIII Series A-1,2,3 and C-1, are clearly visible in Plates 12, 13, 14 and 25 (16 weeks). This is particularly true when they are compared with the initial photograph (Plate 11) or with photographs of the appropriate sterile control units. Heavy fungus growth in the nitrogen and phosphorus series is quite obvious in Plate 25. Photographs showing the appearance of the other test units after 16 weeks are also included.

TABLE XX

Estimated Number of Viable Microorganisms in Water Bottoms Covered with CIIE Fuel - Effect of Nitrogen and Phosphorus Salts

Control Group XIII Series	10% Sea Water plus		Estimated Number in Millions per ml.* Time in Weeks 0	2	4	6	8	16	Final pH
A-1	Nitrogen & Phosphorus	No Iron	0.5BF(4)**	50.0B(4)	500.0B(5)	500.0BF(5)	100.0BF(5)	100.0BF(5)	7.0***
C-1	Nitrogen & Phosphorus	Iron	"	100.0B(4)	500.0BF(6)	500.0BF(5)	100.0BF(5)	50.0BF(5)	7.9
A-2	Nitrogen	No Iron	"	10.0B(4)	10.0B(4)	10.0B(4)	1.0B(4)	10.0B(4)	6.1
A-3	Phosphorus	No Iron	"	10.0B(4)	50.0B(4)	50.0B(5)	50.0B(5)	100.0BF(5)	7.4
A	-	No Iron	"	1.0B(3)	1.0B(4)	1.0B(3)	1.0B(4)	1.0B(3)	6.8
C	-	Iron	"	1.0B(4)	0.01B(2)	0.1B(4)	0.1B(4)	1.0B(3)	6.5

* Average of three determinations.

** BF stands for Bacteria and Fungi, respectively. The number in parentheses is a rough estimate of the number of obviously different colony types present on TGE agar plates.

*** The initial pH of all water bottoms was adjusted to 7.0.

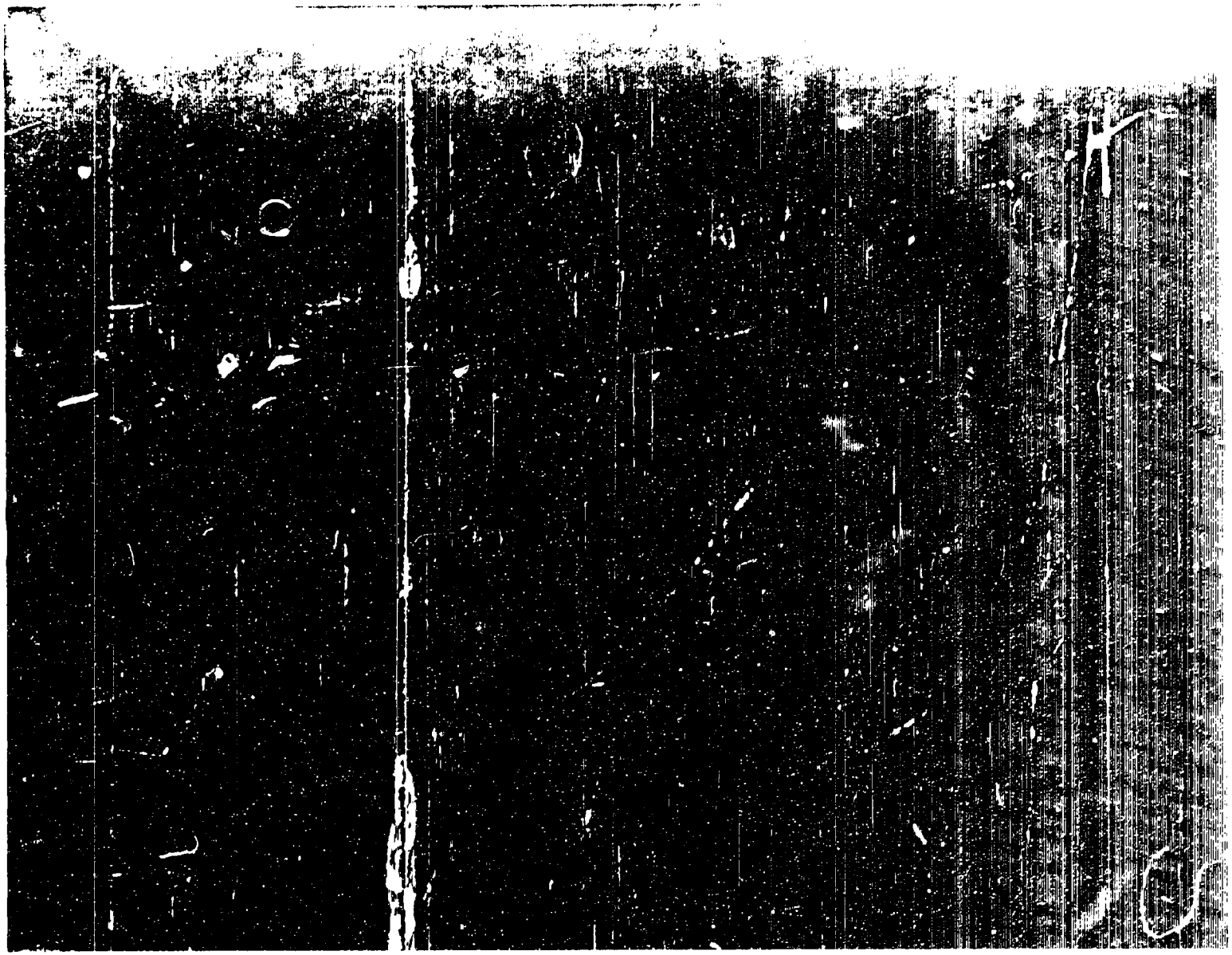

Plate 11. CITE Fuel Additive Group XIII, Control Without Additives at Start of Test.

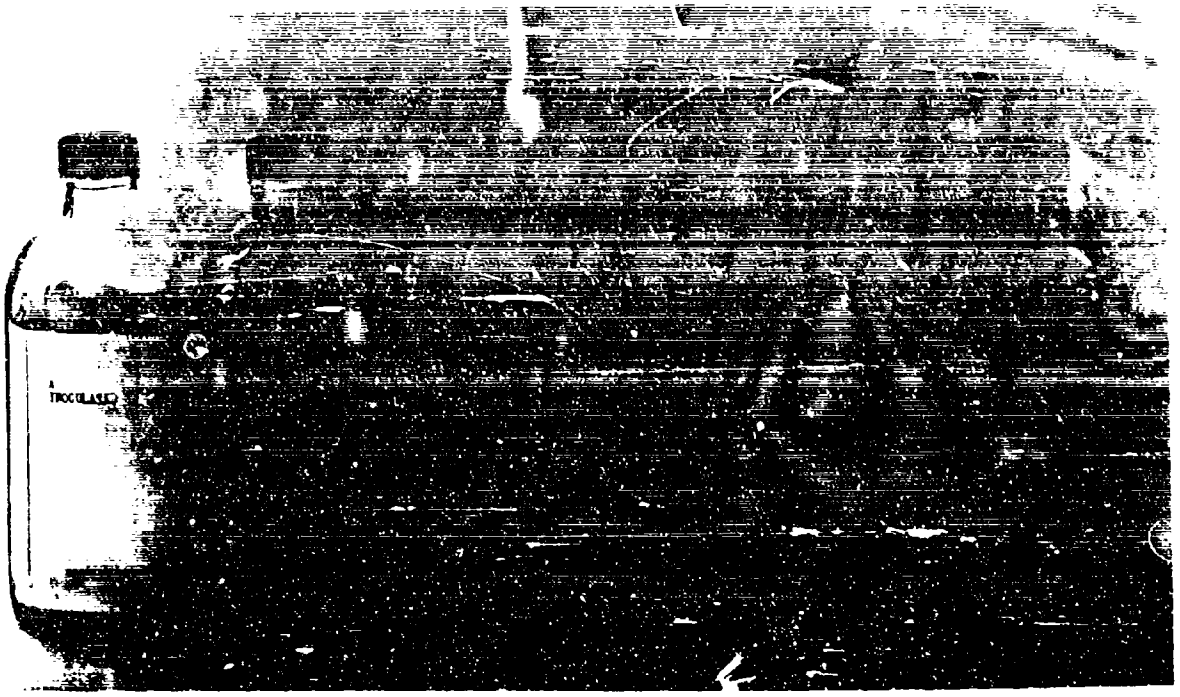

Plate 12. CITE Fuel Additive Group I, DuPont AFA-1 Corrosion Inhibitor - 16 Weeks

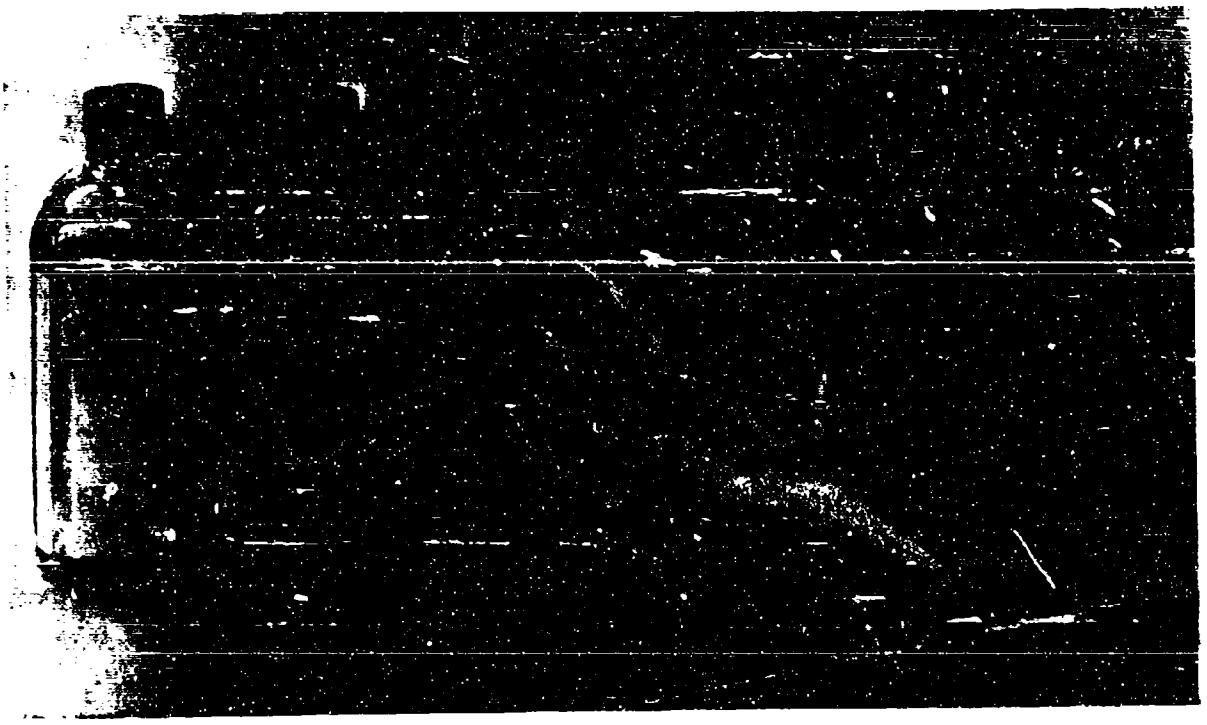

Plate 13. CITE Fuel Additive Group II, Unicor-M Corrosion Inhibitor - 16 Weeks.

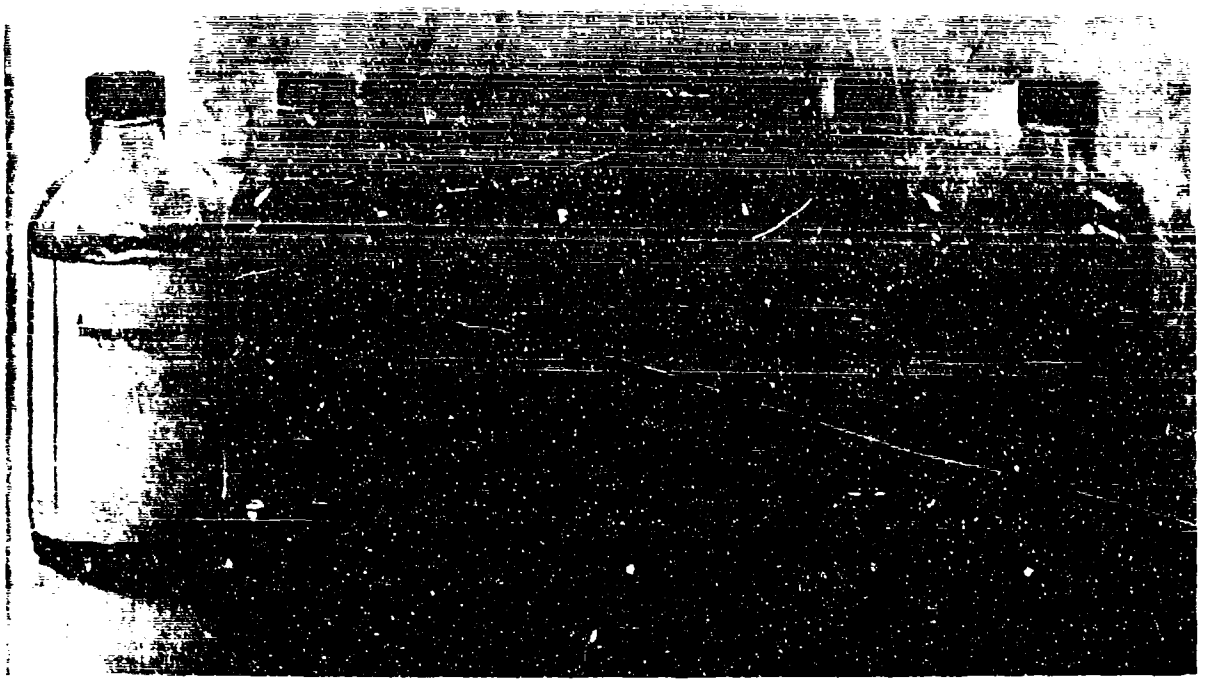

Plate 14. CITE Fuel Additive Group III, Santolene C Corrosion Inhibitor - 16 Weeks.

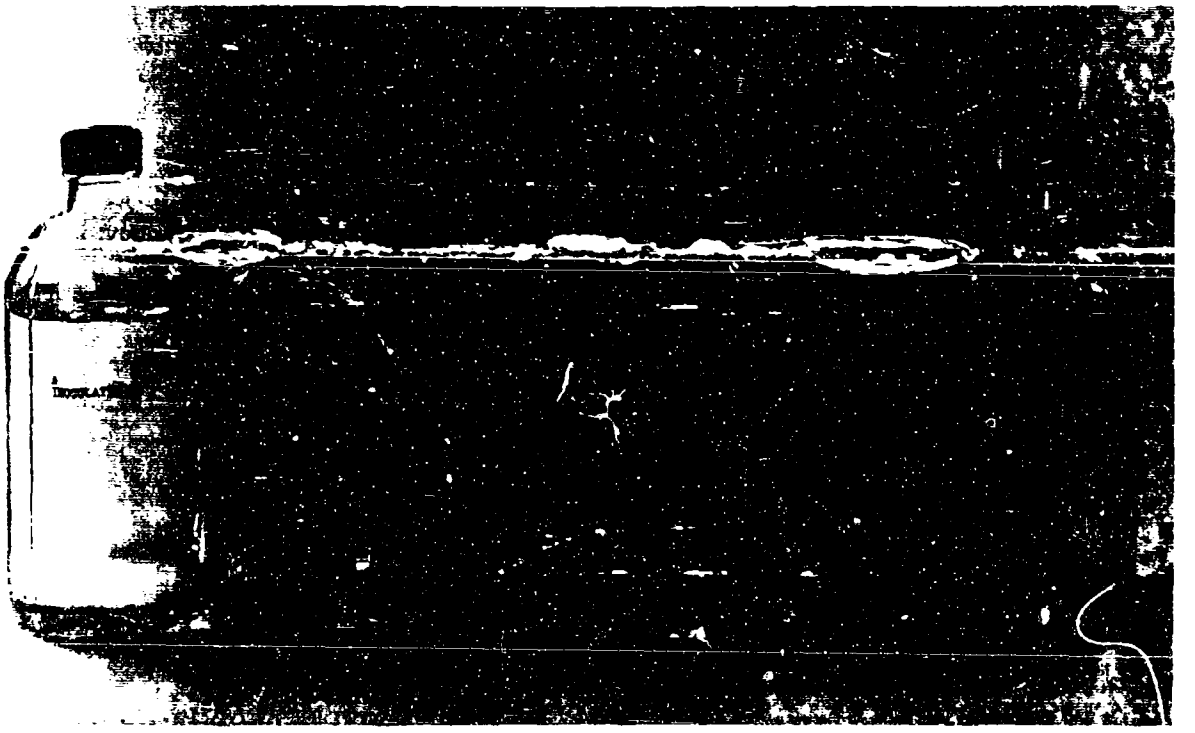

Plate 15. CITE Fuel Additive Group IV, TRI-182 Corrosion Inhibitor - 16 Weeks.

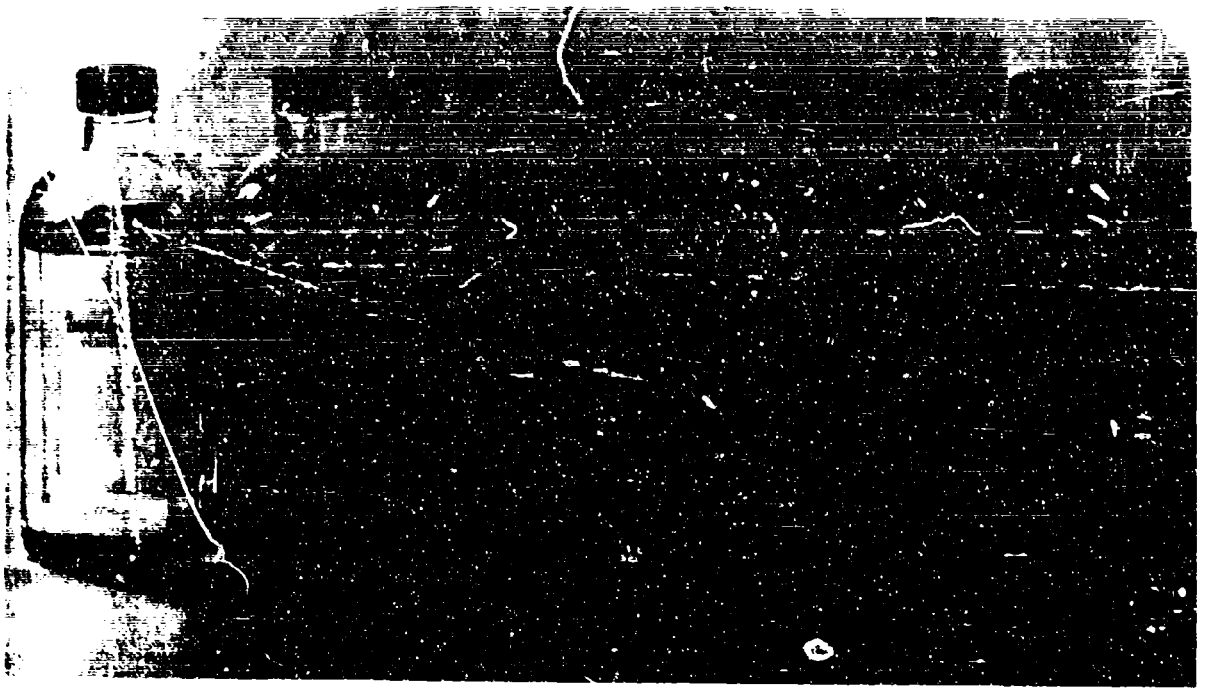

Plate 16. CITE Fuel Additive Group V, Lubrizol 541 Corrosion Inhibitor - 16 Weeks.

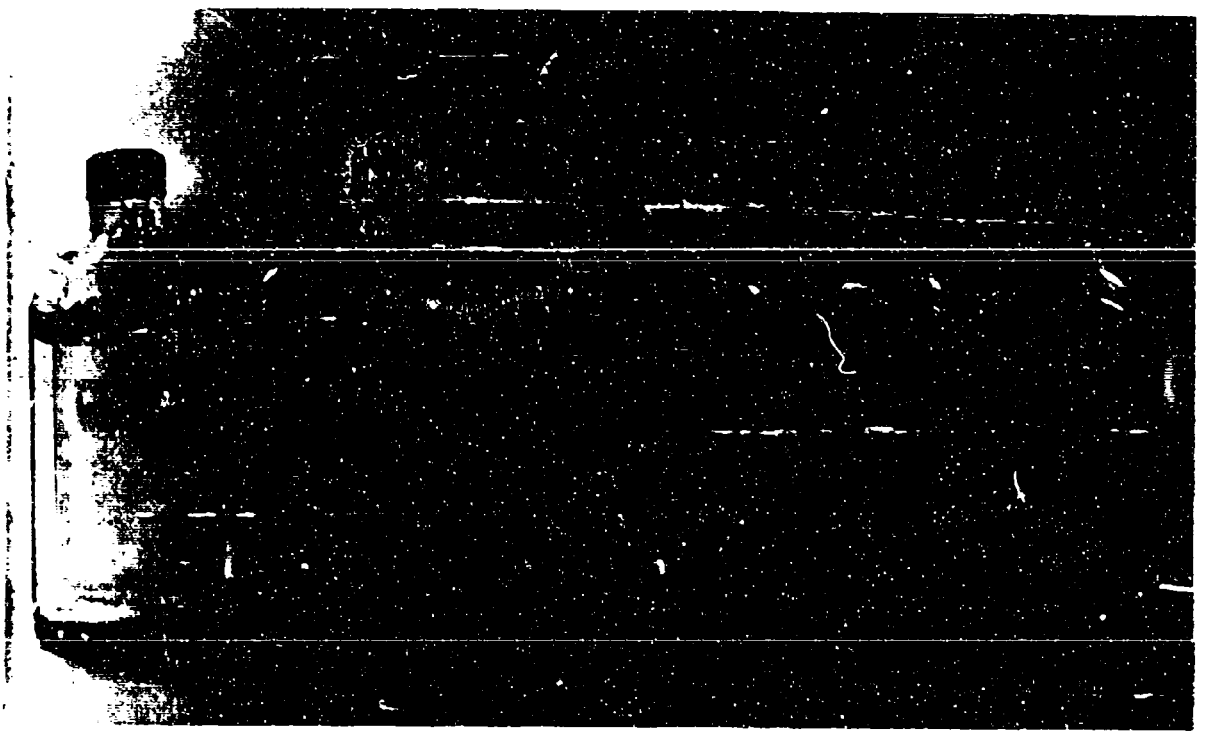

Plate 17. CITE Fuel Additive Group VI, Tolad 244 Corrosion Inhibitor - 16 Weeks.

Plate 18. CITE Fuel Additive Group VII, 2,6-Ditertiary-butyl-4-methylphenol Antioxidant - 16 Weeks.

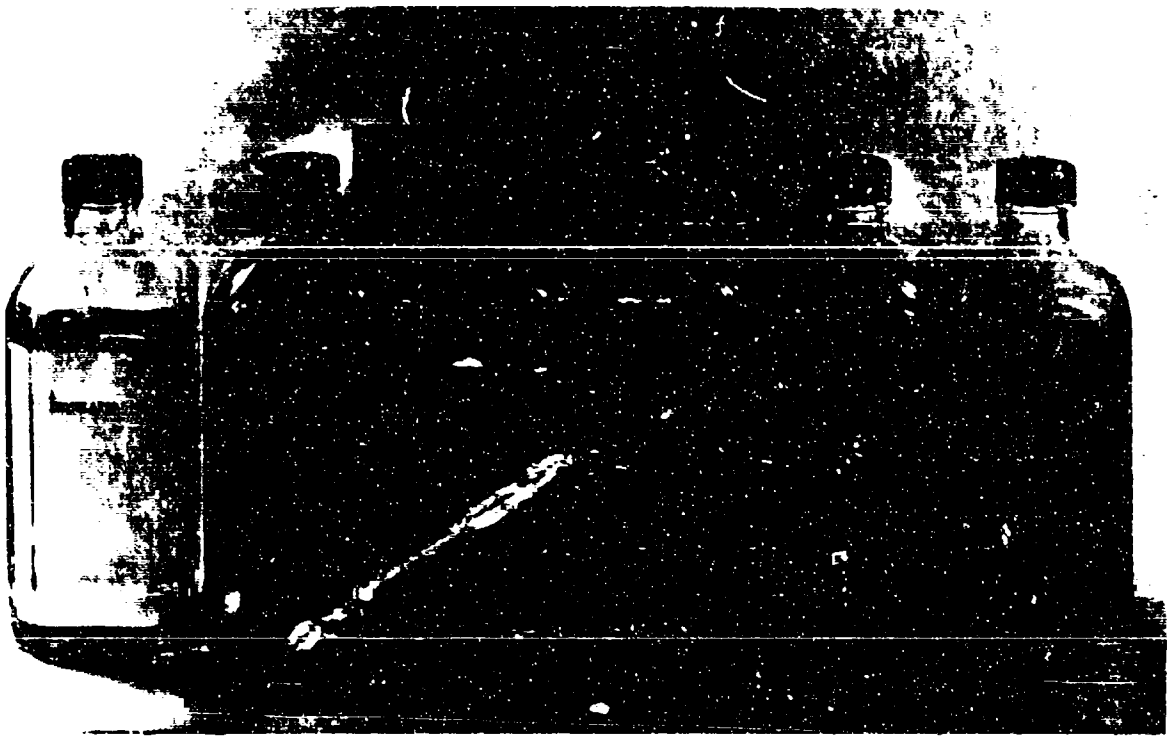

Plate 19. CITE Fuel Additive Group VIII, N,N-Disecondary-butyl-paraphenylene-diamine Antioxidant - 16 Weeks.

Plate 20. CITE Fuel Additive Group IX, 2,4-Dimethyl-6-tertiary-butyl-phenol Antioxidant - 16 Weeks.

Plate 21. CITE Fuel Additive Group X, 2,6-Ditertiary-butyl-phenol Antioxidant - 16 Weeks.

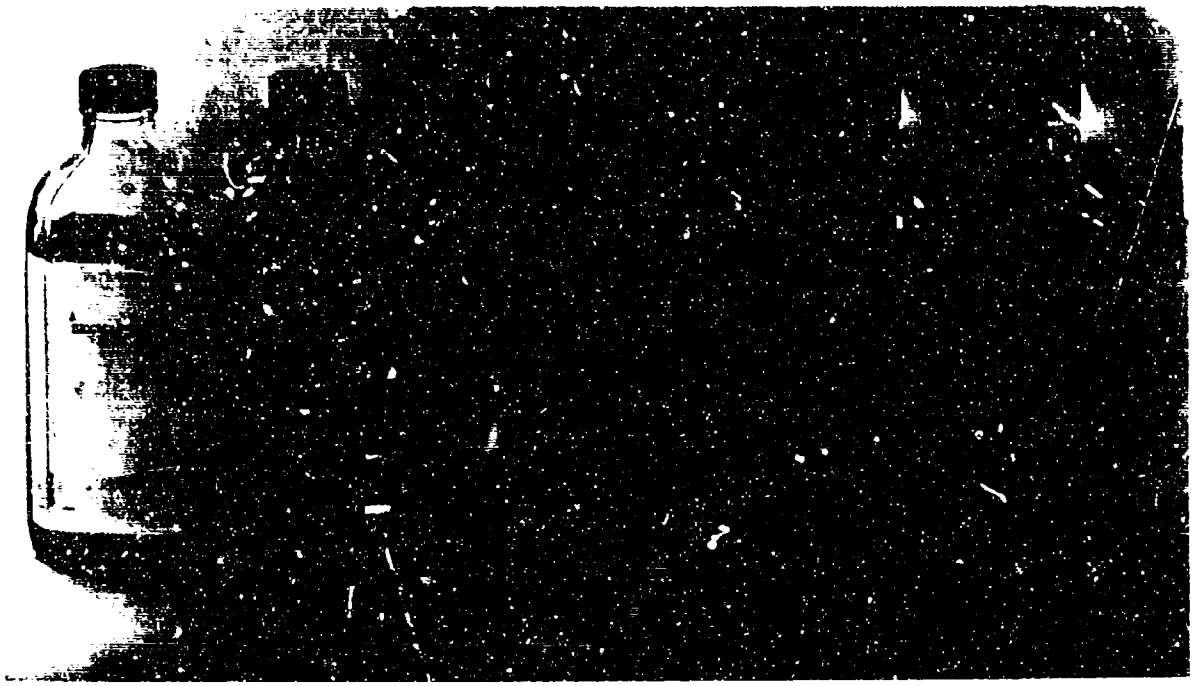

Plate 22. CITE Fuel Additive Group XI, Mixed Tertiary-butyl-phenol Antioxidant - 16 Weeks.

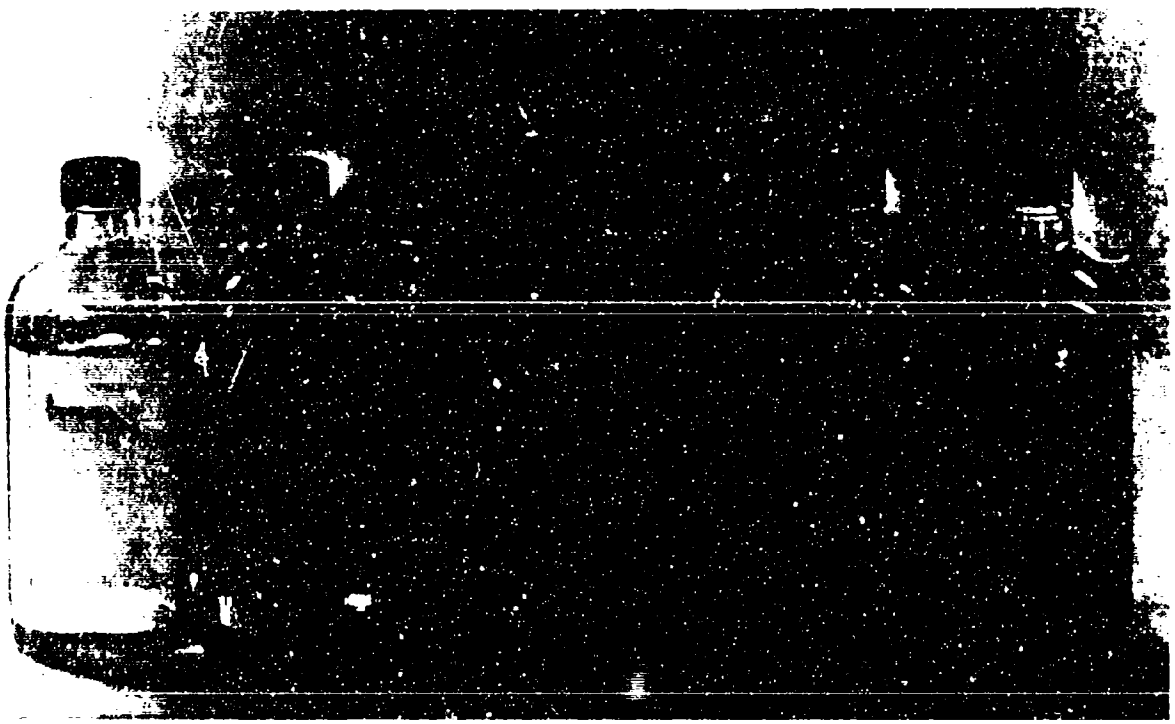

Plate 23. CITE Fuel Additive Group XII, N,N'-Disalicylidene-1,2-propane-diamine Antioxidant - 16 Weeks.

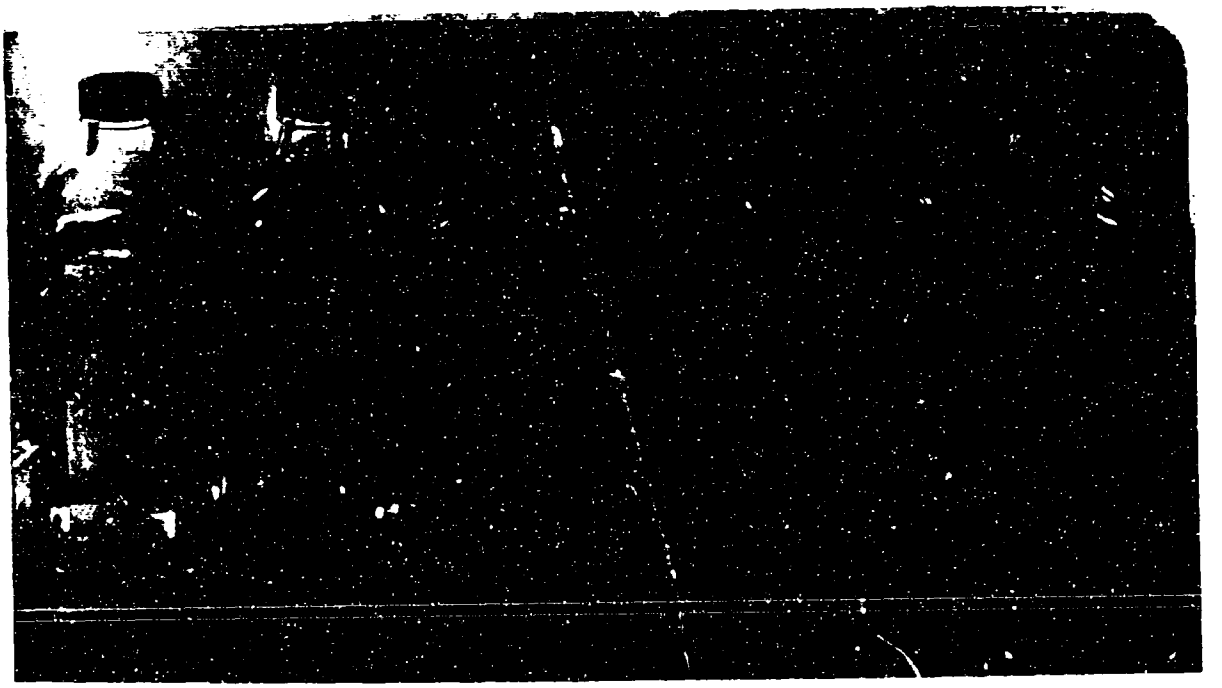

Plate 24. CITE Fuel Additive Group XIII, Control Without Additives - 16 Weeks.

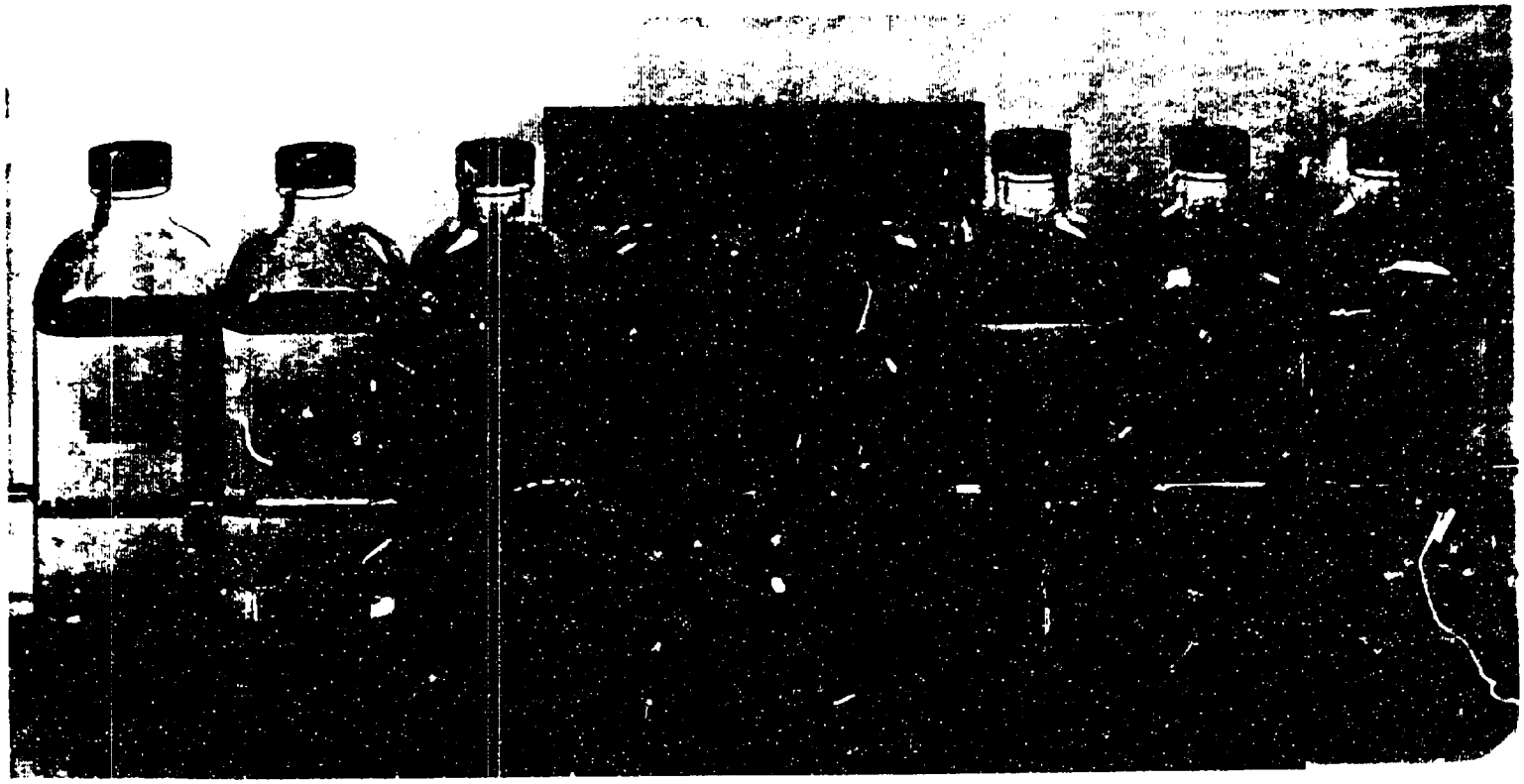

Plate 25. CITE Fuel Additive Group XIII, Control With Water Soluble Nitrogen and Phosphorus - 16 Weeks.

b. Fuel Quality

An effort was made to determine if fuel characteristics, such as stability, filterability and water content, would be affected by microbial growth in adjacent water bottoms. Earlier studies with CITE and RP-1 fuels indicated that they would still meet Military specifications after exposure to microbial growth. While the tests employed are apparently adequate for quality control, they may not be sensitive enough to detect slight changes produced by microorganisms.

Stability characteristics of fuel exposed to microbial attack were determined by ASTM test methods D-381 and D-873 (existent and potential gums). The results of these tests on fuel from additive groups I, II, III and XIII, are presented in Table XXI. Only slight differences were observed, and there was no apparent correlation with microbial activity or presence of water in the test systems.

Filterability tests were run on CITE fuel from all groups in the additive test, including the series with added nitrogen and phosphorus salts. The test method employed was developed by Continental Oil Company for the evaluation of No. 2 fuel oils and diesel fuels (Conoco Method 207-58). Results of these tests are presented in Table XXII. There was no apparent effect on filterability due to microbial activity in the water bottoms. However, two of the corrosion inhibitors, Lubrizol 541 and Tolad 244, adversely affected fuel filterability when stored over active or sterile water bottoms. In both cases, a white sediment accumulated at the fuel water interface. This material suspended quite readily in the fuel on gentle mixing. Since all fuel samples were collected after mixing, this material may have been responsible for the poor filterability characteristics of the fuel.

The water content of fuel from Groups I and XIII was determined by the Karl Fischer method. Results of these analyses are presented in Table XXIII. Only slight differences were noted in the water content of fuels from the two groups, and in fuel from different test series within the respective groups.

TABLE XXI

Existent and Potential Gum* Content of CTE Fuel from Additive Evaluation Studies after 16 Weeks Storage

Series	10% Sea Water Bottoms	Type Gum	Additive Group, mg gum/100 ml Fuel I	II	III	XIII
A	Active - No Iron	Existent	0.4	1.5	0.6	0.3
		Potential	0.5	1.9	0.7	0.4
B	Sterile - No Iron	Existent	0.4	1.6	0.5	0.4
		Potential	0.4	1.9	1.1	0.5
C	Active - Iron	Existent	0.5	2.2	0.4	0.3
		Potential	0.5	2.3	0.9	0.8
D	Sterile - Iron	Existent	0.5	1.7	0.5	0.4
		Potential	0.7	1.9	0.4	0.3
E	Sterile Fuel Only - No Iron	Existent	1.0	1.2	0.5	0.4
		Potential	0.6	1.3	0.5	0.7
F	Sterile Fuel only - Iron	Existent	1.1	1.2	0.4	0.4
		Potential	1.1	1.1	0.5	0.5
A-1	Active plus N + P - No Iron	Existent	-**	-	-	0.4
		Potential	-	-	-	0.7
A-2	Active plus N - No Iron	Existent	-	-	-	0.3
		Potential	-	-	-	0.4
A-3	Active plus P - No Iron	Existent	-	-	-	0.4
		Potential	-	-	-	0.6
C-1	Active plus N + P - Iron	Existent	-	-	-	0.4
		Potential	-	-	-	0.4

* Existent Gum - ASTM Test Method D 381; Potential Gum - ASTM Test Method D 873.

** (-) denotes that sample was not tested or that series was not included in group.

TABLE XXII

Filterability Test* Data on CITE Fuel from Additive Evaluation Study after 16 Weeks Storage

Series	10% Sea Water Bottoms	Additive Groups - Time in Seconds Required to Filter 100 ml												
		I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
A	Active - No Iron	70	61	69	62	514** (50 ml)	335 (95 ml)	59	59	58	57	56	58	62
B	Sterile - No Iron	97	58	67	59	509 (50 ml)	295	54	64	56	52	60	58	63
C	Active - Iron	87	58	65	72	525 (50 ml)	408	-***	-	-	-	-	-	-
D	Sterile - Iron	62	56	63	58	552 (72 ml)	455	-	-	-	-	-	-	-
E	Sterile Fuel only, No Iron	60	58	60	57	64	70	58	66	61	56	58	58	56
F	Sterile Fuel Only, Iron	57	64	57	63	55	61	-	-	-	-	-	-	-
A-1	Active plus N + P, No Iron	-	-	-	-	-	-	-	-	-	-	-	-	55
A-2	Active plus N, No Iron	-	-	-	-	-	-	-	-	-	-	-	-	65
A-3	Active plus P, No Iron	-	-	-	-	-	-	-	-	-	-	-	-	62
C-1	Active plus N + P, Iron	-	-	-	-	-	-	-	-	-	-	-	-	53

* Filterability Test Method developed by Continental Oil Co. (Method 207-58) employing No. 1 Whatman filter paper and a constant head pressure of 14 inches of water on the fuel during the filtration.

** Filters were considered plugged when a fuel sample required more than 500 seconds to filter. The volume of fuel filtered when plugging occurred is reported (in parentheses).

*** (-) denotes that sample was not filtered or that particular series was not included in test group.

TABLE XXIII

Karl Fischer Water Analysis on CITE Fuel
from Additive Evaluation Study after 16 Weeks Storage

		Additive Group - ppm H_2O	
Series	10% Sea Water Bottoms	I	XIII
A	Active, No Iron	43	60
B	Sterile, No Iron	47	60
C	Active, Iron	42	34
D	Sterile, Iron	43	68
E	Sterile Fuel Only, No Iron	30	34
F	Sterile Fuel Only, Iron	34	35
A-1	Active (N + P), No Iron	-	40
C-1	Active (N + P), Iron	-	40

c. Analysis of Sludge

"Sludge" accumulations in the additive evaluation studies (water and fuel insoluble materials other than corrosion products) were observed only in units with increased microbial activity. Corrosion products precluded the study of sludge in units containing steel wool (Series C, D and F). Quantitative gravimetric determinations were made on four fractions of the sludge:

Fraction A - Insoluble Material (Insoluble in fuel, water, and benzene-acetone-methyl alcohol solvent).

Fraction B - Fuel Sludge (Material insoluble in fuel and water but soluble in benzene-acetone-methyl alcohol solvent).

Fraction C - Non-volatile, water solubles (including NaCl) and fine particles which may have passed through the Millipore filter disc.

Fraction D - Material from the water bottoms collected on 0.45 micron Millipore filter disc after Fraction A has been collected.

Details of the procedures employed are presented in Appendix E. The results of analyses on units from Groups I, II, III and XIII are reported in Table XXIV. Those units exhibiting heaviest microbial growth contained the most sludge, particularly the nitrogen and phosphorus unit (Group XIII, Series A-1). Only a trace of benzene-acetone-methyl alcohol solvent soluble material (Fraction B) was found in the bottoms examined. Material collected on the Millipore filter disc (Fraction D) was similar to that collected on the glass filter pad (Fraction A). Microscopic examination of the Millipore disc revealed the presence of bacterial cells and fungus mycelium.

Several discrepancies were noted in the quantities of water soluble materials (Fraction C) present in the Control Group XIII, particularly in Series A-1. The water bottoms in Series A-1, 2 and 3 contained nitrogen and phosphorus salts in addition to the salts present in the sea water. The results suggest that the salts were incorporated into the microbial sludge which formed.

To further characterize the sludge in these systems, Fractions A and B from Group XIII, Series A-1, were analyzed in the infrared spectrometer. The absorption spectrum obtained on Fraction A was similar to but not the same as the one shown in Figure 1. Subsequent studies reported in Section D-1-a, and shown in Figures 4 and 5 therewith, indicated that the spectrum constituted the infrared absorption characteristics of the bacterial cells and fungus mycelium present. Infrared analysis of Fraction B indicated that it was primarily hydrocarbon, probably fuel components.

TABLE XXIV

Results of Gravimetric Determination of Sludge from Additive Evaluation Units after 16 Weeks Storage

Additive Group	10% Sea Water Bottoms, Series Without Iron	Fraction, Weight in mgs/test unit			
		A(1)	B(2)	C(3)	D(4)
I	A Active	10.1	5.2	76.8	18.3
	B Sterile	0.7	1.6	87.1	0.0
	E Sterile fuel only	0.5	-	-	-
II	A Active	1.3	2.5	78.7	0.6
	B Sterile	1.3	2.4	75.8	0.0
	E Sterile fuel only	0.8	-	-	-
III	A Active	3.3	0.3	80.1	2.3
	B Sterile	3.1	0.1	78.7	0.2
	E Sterile fuel only	0.1	-	-	-
XIII	A Active	0.7	0.7	86.8	0.1
	B Sterile	0.3	0.0	76.6	0.0
	C Sterile fuel only	0.0	-	-	-
	A-1 Active (N + P)	48.4	8.7	88.8	31.6
	A-2 Active (N)	1.5	0.8	97.7	0.4
	A-3 Active (P)	25.1	1.3	106.6	4.2

(1) Insoluble Material - Insoluble in fuel, water and benzene-acetone-methyl alcohol solvent.

(2) Fuel Sludge - Material insoluble in fuel and water but soluble in benzene-acetone-methyl alcohol solvent.

(3) Non-volatile, water solubles (including NaCl) and fine particles which may have passed through the filter pads. Reported as mg/20 ml of water bottoms.

(4) Material not removed by initial filtration through glass filter pad and having a particle size greater than 0.45 microns. The material was collected on a 0.45 micron Millipore filter disk.

3. CITE Fuel Storage Tests - Effects of Fuel Stability and Mixing

Two other variables which may influence the growth of microorganisms and sludge formation in fuel-water systems were investigated. The effect of fuel stability and occasional mixing of the water and fuel phase were considered. The two additive-free fuel blends were employed in this study: stable straight-run fuel, Blend 1 (used in Additive Evaluation), and potentially unstable fuel containing straight-run and cracked stock, Blend 2.

Tests were set up in 8-oz. square bottles containing 200 ml of fuel and 10 ml of 10% sea water, with and without iron. The various series were set up in the same manner as the Additive test (Appendix F) with the above exceptions. Duplicate groups were set up with each fuel for comparison of static incubation with occasional mixing.

Blend 2, the less stable fuel, was expected to form typical fuel sludge. Unfortunately, the fuel was more stable than anticipated. After 24 weeks, there was no noticeable change in the appearance of the fuel or in the microbial growth in the water bottoms (Table XXV). Also, intermittent shaking had very little effect on growth. It should be pointed out that these tests were set up with additive-free fuel, and with 10% sea water bottoms, deficient in nitrogen and phosphorus.

The tests were discontinued after six months when it was obvious that no change in the fuel had occurred. Intermittent shaking also failed to significantly influence the microorganisms in the water bottoms. This was apparently due to the lack of nitrogen and phosphorus in the water bottoms. No other potentially unstable fuel blends were prepared because of the difficulties of meeting the Military specifications. Since our efforts under this program were confined to CITE fuel, we did not pursue this area any further.

TABLE XXV

Estimated Number of Viable Microorganisms in Water Bottom
- The Effect of Fuel Stability and Mixing on Microbial Growth

Test System	Estimated Number in Million per ml* Time In Weeks 0	8	16	24
Static				
Blend 1 (Stable)				
No Iron	0.5 BF(5)**	1.0 B(4)	1.5 B(3)Y***	1.0 B(2)Y
Iron		1.0 B(2)	1.0 B(3)	0.5 B(2)Y
Blend 2 (Unstable)				
No Iron		1.0 B(4)	1.0 B(4)Y	0.5 B(1)Y
Iron		1.0 B(3)	1.0 B(2)	0.5 B(2)Y
Mixing				
Blend 1 (Stable)				
No Iron		1.0 B(4)	1.5 B(4)Y	1.0 B(3)Y
Iron		1.0 B(3)	1.0 B(3)Y	0.5 B(2)Y
Blend 2 (Unstable)				
No Iron		10.0 B(3)	1.5 B(2)Y	1.0 B(2)Y
Iron		1.0 B(3)	1.0 B(3)Y	0.5 B(2)Y

* Average of three determinations.

** BF stands for Bacteria and Fungi, respectively. The number in parentheses is a rough estimate of the number of obviously different colony types present on TGE agar plates.

*** The initial pH of all water bottoms was adjusted to 7.0.

***(Y) An organism which formed a yellow colony on TGE agar predominated in these units. When transferred to sea water: fuel medium, the organism did not grow. It appears to be a scavenger type.

4. Studies on Hydrocarbon Fuel Components Utilized by Microorganisms

In preliminary studies with RP-1 rocket fuel we attempted to establish which hydrocarbon components of the fuel were utilized by the microbial flora present. Hydrocarbon type analysis of exposed fuel failed to reveal any significant changes in fuel composition (Tables IV and V). Apparently, the organisms utilized such small quantities of the fuel that it was impossible to detect any change. Also, when mixed cultures were employed, a variety of hydrocarbon types were probably attacked, further complicating the analysis. In an effort to more directly establish which hydrocarbon types were being utilized, studies were initiated with pure compounds. A number of hydrocarbon types which might be found in a typical CITE fuel blend were selected.

The cultures used in these studies were selected from Sinclair's stock culture collection. Some twenty pure and mixed cultures isolated from fuel-water systems were checked for their ability to grow on additive-free CITE fuel (Blend 1). The tests were set up in two basal media, mineral salts solution and 10% sea water containing added nitrogen and phosphorus salts. Twelve of the twenty cultures checked grew profusely on the CITE fuel in both test media. Growth was somewhat slower under static conditions than when incubated on a shaker. However, the amount of growth present in the systems at the end of one week apparently varied only slightly. No quantitative measurements were employed, only visual comparisons were made.

The twelve cultures which grew well on the CITE fuel were transferred to mineral salts solution overlaid with a blend of pure n-paraffins (Appendix G). All but two cultures grew well on the hydrocarbon blend. The two cultures which did not appear to utilize the n-paraffins were isolated from RP-1 fuel. They apparently utilized aromatic components of the fuel.

Additional studies were made using the composite inoculum employed in the storage tests. Enrichments were made and several pure cultures isolated.

a. Mixed Culture Studies

Seventeen purified hydrocarbons and a synthetic mixture were exposed to the composite tank bottom water sample used to inoculate earlier storage tests. Included were n-heptane, n-octane, n-tridecane, n-tetradecane, isooctane, 2-methylheptane, 2-methylhexane, benzene, toluene, meta-xylene, 1,2,4-trimethylbenzene, n-propylbenzene, isopropylbenzene, naphthalene, biphenyl, 1,3-dimethylcyclohexane, and a mixture of cycloparaffins (including cyclopentane, methylcyclopentane, cyclohexane and methylcyclohexane). All purified hydrocarbons used were either Phillips Research Grade (99+ Mol %), American Petroleum Institute or National Bureau of Standards Reference Compounds. Because of volatility problems, particularly with the lighter hydrocarbons, growth experiments were carried out in a Warburg respirometer. Details of the procedures are presented in Appendix H. The studies

were conducted in 10% sea water, containing nitrogen and phosphorus salts (Appendix B). Carbon dioxide was continually absorbed from the system by KOH in the center well of each Warburg flask.

Growth, as reflected by oxygen uptake, was observed within a week as summarized in Table XXVI under Inoculum I. These results were subsequently duplicated using tightly capped 4-ounce bottles containing 20 ml of 10% sea water solution. Every 24 hours, the caps were loosened to allow air exchange. Incubation took place under shake conditions at 30°C.

Bacteria predominated in units containing the isopropylbenzene, biphenyl and naphthalene, while mixtures of bacteria and fungi were observed growing on the normal paraffins. Continued enrichment of the mixed cultures on the individual hydrocarbons resulted in accelerated growth and selection of one or two organisms. Attempts were then made to isolate pure cultures for further study.

b. Characterization of Pure and Mixed Cultures

Cultures from the above study were serially enriched on n-heptane, n-octane, n-tridecane, n-tetradecane, isopropylbenzene, naphthalene and biphenyl. After four transfers, the n-paraffin cultures were streaked on 10% sea water-hydrocarbon agar plates (Appendix B). A mixture of n-paraffins (Appendix G) was employed as a carbon source in this medium. Isolated bacterial colonies were transferred to 10% sea water solution and the hydrocarbon on which the culture was enriched. Fungi were isolated by placing a piece of the fungal growth from initial enrichment units on the surface of the solidified medium. After several days the fungi grew away from the initial streak, permitting their isolation from the peripheral area without bacterial contamination. Organisms which utilized naphthalene, biphenyl and isopropylbenzene were also isolated. The biphenyl and isopropylbenzene isolates grew extremely slow and were very difficult to maintain. Furthermore, the CITE fuel used was deficient in these two hydrocarbon types and would not support growth. These two isolates, therefore, were not subjected to further study.

The naphthalene culture was checked for its ability to grow on the following substituted naphthalenes:

1-Methylnaphthalene
2-Methylnaphthalene
1,2-Dimethylnaphthalene
1,3- "
1,5- "
1,6- "
1,7- "
2,3- "
2,6- "

TABLE XXVI

Comparison of Growth of Pure and Mixed Cultures on Various Pure Hydrocarbons

Hydrocarbon	Inoculum I	Inoculum II	Inoculum III	Inoculum VII	Inoculum VIII
n-Heptane	+	+*	-**	-	-
n-Octane	+	+	-	-	-
n-Tridecane	+	+ (weak)	-	+	+
n-Tetradecane	+	+ (weak)	-	+	+
iso-Octane	-	-	-	-	-
2-Methylheptane	-	-	-	-	-
2-Methylhexane	-	-	-	-	-
Benzene	-	-	-	-	-
Toluene	-	-	-	-	-
o-Xylene	-	-	-	-	-
m-Xylene	-	-	-	-	-
1,2,4-Trimethylbenzene	-	-	-	-	-
n-Propylbenzene	-	-	-	-	-
iso-Propylbenzene	+	-	-	-	-
Naphthalene	+	-	+	-	-
Biphenyl	+	-	-	-	-
Cycloparaffin Mixture	***-	-	-	-	-
Phenol	-	-	-	-	-
CITE Fuel	+	+	+	+	+

*(+) Indicates hydrocarbon supported grow of culture on several transfers.

**(-) Indicates hydrocarbon failed to support growth of culture.

*** Cycloparaffin Mixture contained equimolar concentrations of the following:

Cyclopentane
Methylcyclopentane
Cyclohexane
Methylcyclohexane

Only slight growth was noted on 2-methylnaphthalene, while heavy growth was noted on 1,2-methylnaphthalene. The growth on the latter compared quite closely to that obtained on naphthalene itself. Growth was generally observed in less than 24 hours on these two hydrocarbons.

Cultures which utilized n-paraffins were transferred to 10% sea water solution containing individual n-paraffins from C_5 to C_{14} and C_{16} (C_{15} was not available in purified form). Growth was observed visually and the results are recorded in Table XXVII. The n-heptane and octane cultures appeared to be the same organism, utilizing the same hydrocarbons. For this reason, only the n-octane culture (Inoculum VI) was included in the table. The culture enriched on n-tridecane grew best on n-paraffins in the C_7 to C_{10} range. This was in contrast to the n-tetradecane enrichment which grew best on C_{10} to C_{16} n-paraffins.

In addition to the composite tank bottom (Inoculum I) and the enrichment culture reported above, four other cultures were examined for their ability to grow on CITE fuel and to utilize various hydrocarbon types. They were identified as follows:

Inoculum II - Pseudomonas sp. (Isolated from JP-4).

Inoculum III - Pure culture of bacteria isolated from RP-1, Rocket fuel sample supplied by Natick.

Inoculum VII - Cladosporium resinae (Isolated from JP-4).

Inoculum VIII - Mixed culture of bacteria and fungi obtained from Sohio.

The results of these studies are reported in Table XXVI. Inocula II, VII and VIII grew primarily on the n-paraffins, while Inoculum III grew only on naphthalene. Apparently Inocula VII and VIII prefer the higher molecular weight normal paraffins in contrast to Inoculum II which in this study grew on C_7, C_8, C_{13} and C_{14} paraffins. A further check on Inoculum II (a pure Pseudomonas sp. isolated from JP-4) indicated that the organism was capable of utilizing n-paraffins from C_6 to C_{16}. Inoculum III (a pure unidentified bacterial culture isolated from RP-1 fuel), on the other hand, utilized 2-methyl-, 1,2-dimethyl-, and 2,3-dimethyl- substituted naphthalene in addition to naphthalene, but failed to grow on the other methyl derivatives. These two pure cultures were isolated from two different fuels, which differ primarily in paraffin content rather than in the amount of aromatics present. The RP-1 fuel is a narrow boiling fraction with a distillation range of 365-410 compared with 137-476 for JP-4.

TABLE XXVII

Comparison of Growth of Purified Cultures Enriched On n-Octane, n-Tridecane And n-Tetradecane on Eleven n-Paraffins

n-Paraffin	Organisms Enriched On		
	n-Octane Inoculum VI	n-Tridecane Inoculum V	n-Tetradecane Inoculum IV
Pentane	-	-	-
Hexane	+++	-	-
Heptane	++++	++++	-
Octane	+++	++++	++
Nonane	++++	+++	-
Decane	++	++++	++
Undecane	+	++	+
Dodecane	±	+	++
Tridecane	-	±	+
Tetradecane	-	-	+
Hexadecane	-	-	++

* Growth was observed visually and rated as follows:

- \- No visible growth
- ± Questionable growth
- \+ Slight growth
- ++ Moderate growth
- +++ Moderate to heavy growth
- ++++ Heavy growth

The preference of the tank bottom isolates for n-paraffins is quite obvious from the results reported in this section. This is not surprising since paraffins are generally the predominate hydrocarbon type in test fuel employed. In the following sections we employed eight cultures which we considered representative of typical tank bottom flora. Also included were mixed natural mixtures of organisms obtained from the field. All test cultures were characterized in this section as to the hydrocarbon types which would support their growth or the growth of at least one member of the mixture. In addition to the four identified above, the following were included in this group:

Inoculum I	-	Tank bottom composite. Same preparation used in setting up the additive evaluation.
Inoculum IV	-	Pure fungus culture isolated from the tank bottom composite (Inoculum I) on n-tetradecane.
Inoculum V	-	Mixed culture containing bacteria and fungi. Obtained by enriching the tank bottom composite (Inoculum I) on n-tridecane.
Inoculum VI	-	Same as V, except enrichment carried out on n-octane.

D. Production and Analysis of Microbial Sludge

Earlier studies with CITE fuel demonstrated the ability of microorganisms to grow in water bottoms at the expense of hydrocarbon components of the fuel. Also demonstrated was the need for suitable nitrogen and phosphorus sources for the production of microbial sludge in these systems. Based on this and other information obtained in these studies, three systems were employed for the controlled production of microbial sludge. In the first system the sludge was produced under static conditions in one-gallon glass bottles. The second system employed conventional laboratory fermentation techniques for the production of large quantities of sludge. CITE fuel was used in the static while both CITE fuel and pure hydrocarbons were used in the fermentor.

A third series was set up under static conditions employing n-hexadecane as a substrate and two fungus cultures, Inocula IV and VII. This series was set up under conditions which favor fungus growth in an effort to obtain large quantities of sludge for comparison with bacterial sludge produced in the fermentor.

1. Static Test System

Preliminary tests were set up in gallon bottles containing 160 ml of 10% sea water solution (Appendix B) and approximately one gallon of filter sterilized CITE fuel (Additive-free, Blend 1). The units were inoculated with various mixed and pure cultures of both bacteria and fungi characterized in the above section and identified as follows:

Inoculum I	-	Tank bottom composite. Same preparation used in setting up the additive evaluation.
Inoculum II	-	*Pseudomonas sp.* (Isolated from JP-4).
Inoculum III	-	Pure culture of bacteria isolated from RP-1 fuel sample supplied by Natick.
Inoculum IV	-	Pure fungus culture isolated from the tank bottom composite (Inoculum I) on n-tetradecane.
Inoculum V	-	Mixed culture containing bacteria and fungi. Obtained by enriching the tank bottom composite (Inoculum I) on n-tridecane.
Inoculum VI	-	Same as V, except enrichment carri i out on n-octane.
Inoculum VII	-	*Cladosporium resinae* (Isolated from JP-4).
Inoculum VIII	-	Mixed culture of bacteria and fungi obtained from SOHIO.

After six weeks storage under static conditions at 30°C., the contents of each unit were separated into three fractions according to the procedure outlined in Appendix E.

A. Insoluble Material - Insoluble in fuel, water, and a benzene-acetone-methyl alcohol solvent mixture. (Triple solvent.)

B. Fuel Sludge - Material insoluble in fuel and water but soluble in a benzene-acetone-methyl alcohol solvent mixture.

C. Non-volatile, water soluble materials and fine particles which may have passed through the filters employed.

Gravimetric determinations of Fractions A and B are reported in Table XXVIII. The results indicate that Inocula I, IV and VIII produced the most microbial sludge under the test conditions employed. In general, there does not appear to be any correlation between the quantity of microbial sludge produced (Fraction A) and the amount of fuel sludge (Fraction B) observed. Variations in the quantity of fuel sludge may be due to the presence of materials extracted from Fraction A. Such compounds as lipids and high molecular weight alcohols, and possibly some polysaccharides and proteins, may be extracted by the solvent mixture employed.

a. Observations on Fraction A

The water, fuel and triple solvent insoluble material, Fraction A, from each of the eight test systems was examined by infrared spectroscopy. Eight inocula were employed in this study in an effort to establish if there were any significant differences in the IR absorption characteristics of pure cultures and natural mixtures grown in a fuel:water system. Careful examination of the spectra indicated that each sample possessed slightly different absorption properties. However, all absorbed strongly in the 9-10 micron range. Differences in spectra are apparently due to inherent differences in the microorganisms present. Characteristic of all spectra was the absorption band at 5.95-6.1 microns which is normally duo to amides. In these samples the band could represent the peptide bonds of proteins. Representative IR spectra are presented in Figures 4 and 5.

TABLE XXVIII

Gravimetric Determinations on Fractions A and B

(Weight in gms/1000 ml water bottoms)

Fraction	Inoculum							
	I	II	III	IV	V	VI	VII	VIII
A(1)	1.228	0.682	0.831	1.029	0.800	0.744	0.827	1.295
B(2)	0.142	0.035	0.079	0.059	0.106	0.283	0.248	0.238

(1) A - Insoluble Material - Insoluble in fuel, water and benzene-acetone-methyl alcohol solvent mixture.

(2) B - Fuel Sludge - Material insoluble in fuel and water but soluble in benzene-acetone-methyl alcohol solvent mixture.

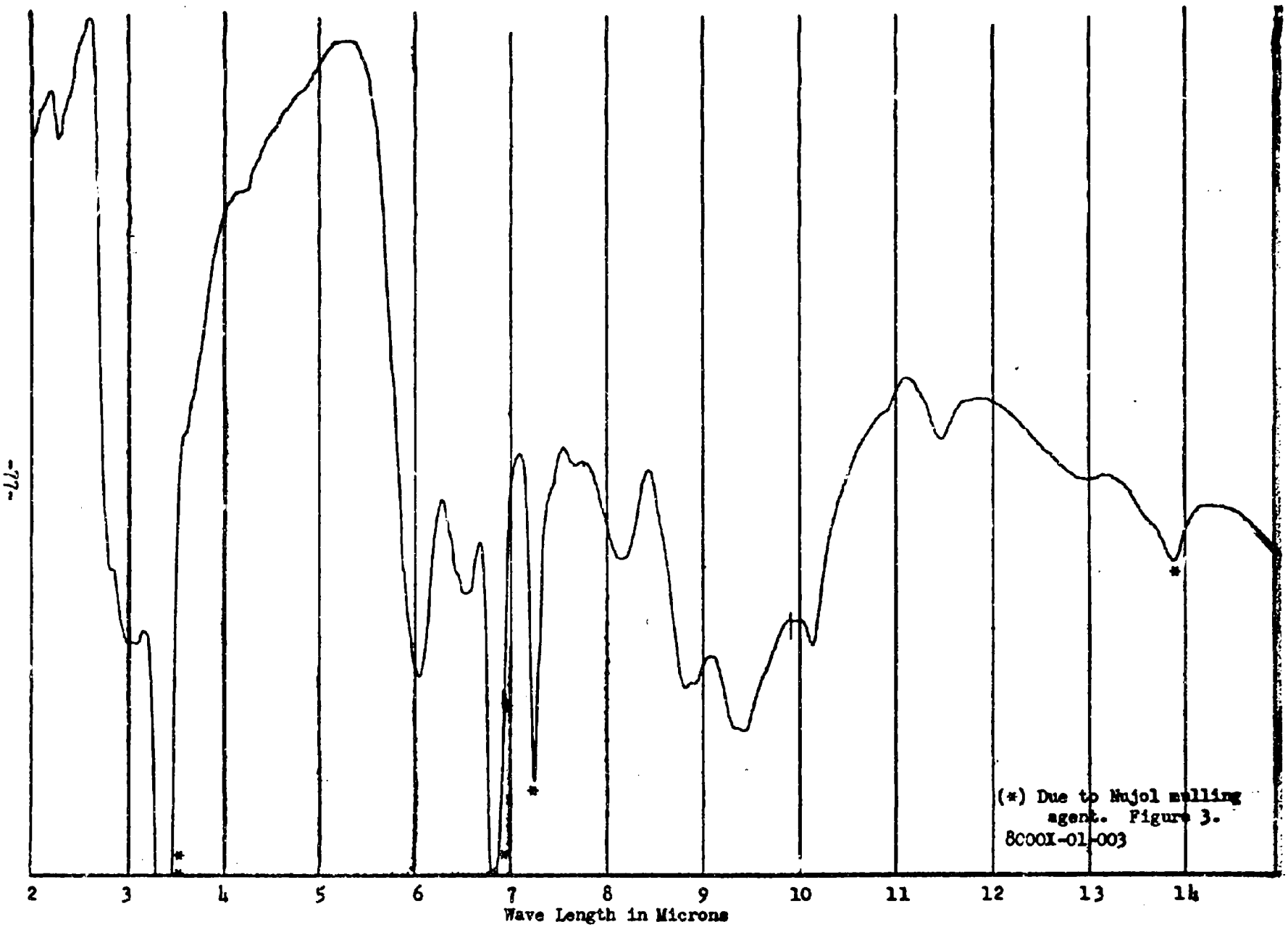

Figure 4. Infrared spectrum of Fraction A, Inoculum II.

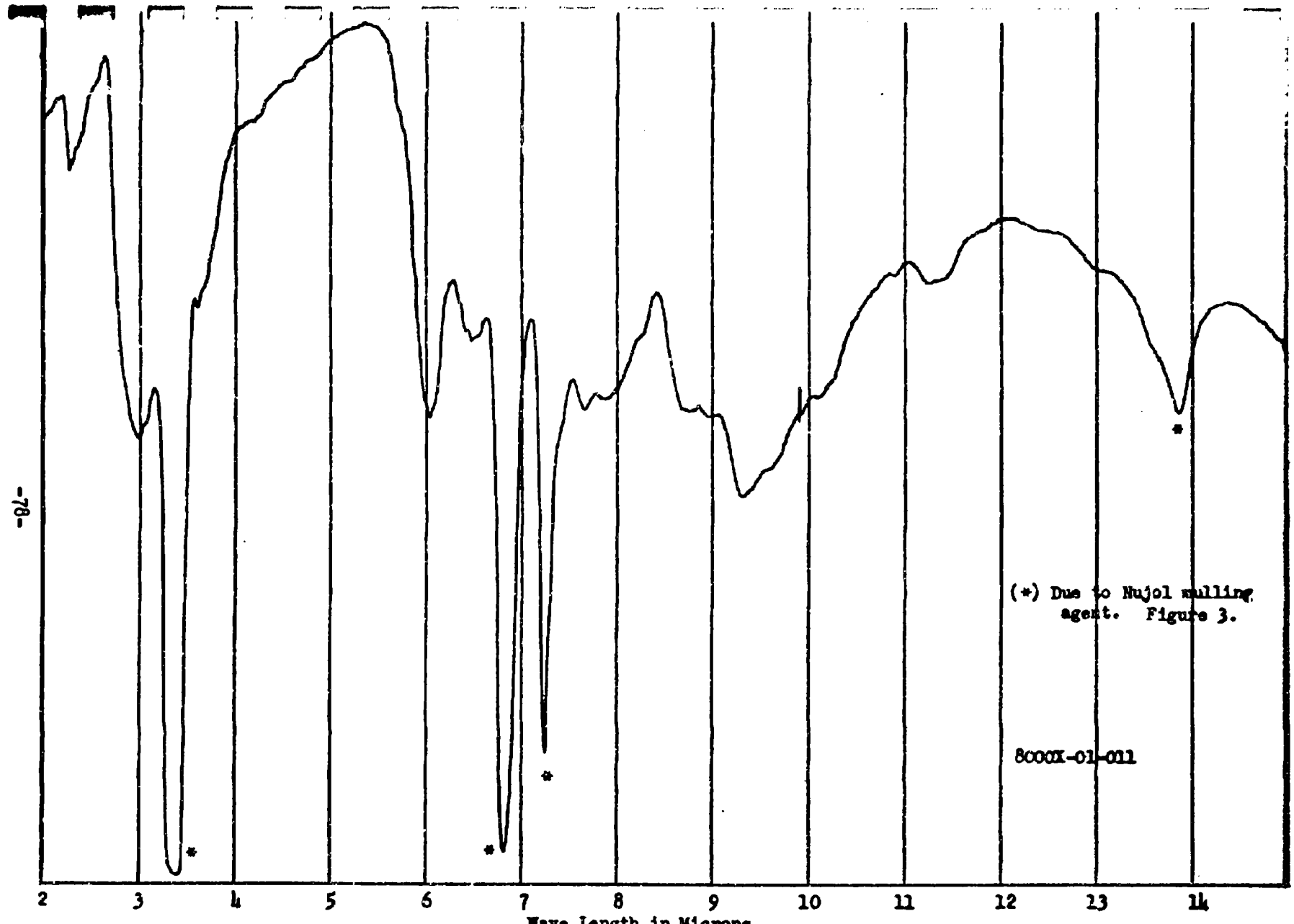

Figure 5. Infrared specturm of Fraction A, Inoculum VIII.

Reports in the literature on the IR absorption characteristics of microorganisms attribute the 9.5 band to cellular carbohydrates[1,2]. The IR spectra of starch and dextrin show strong absorption bands between 8.7 and 10.0 microns. While these bands are in the right range, they are considerably broader and do not appear to be the same as the microbial sludge. However, spectra of some mono- and di-saccharides show strong absorption bands at 9-10 microns[3], suggesting that the cellular material is of lower molecular weight than the starch and dextrin.

b. Observations on Fraction B

Analysis of the triple solvent soluble material, Fraction B, from the eight test systems was also confined to infrared spectroscopy. Representative spectra obtained on these samples are presented in Figures 6-10. It should be pointed out that some of the samples were run as Nujol mulls. Absorption bands due to hydrocarbons are quite apparent in the spectra of samples run without the mulling agent. The presence of absorption bands between 5.6 and 5.9 microns on spectrograms of all samples indicates oxidized compounds, either fuel components or residual solvent. However, absorption in this range was not observed in any of the insoluble sludge samples (Fraction A) which were solvent extracted, somewhat ruling out the solvent. This would also tend to indicate that the solvent extraction of Fraction A removed essentially all of the material which absorbs at 5.6 to 5.9.

Examination of the IR spectra obtained on Fraction B revealed two groups of inocula having similar spectra (Figure 7). One group consisted of Inocula I, VI and VII (Figure 6), and the other included II and V. The remaining spectra appeared to be distinctly different from those in the two groups. It is not surprising that Inocula I, VI and VII have similar spectra since VI and VII are enrichments of I. However, Inoculum V should also be in this group for the same reason, but it has a distinctly different spectrum, possibly reflecting a predominate microbial type not present in the other inocula.

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(1) Greenstreet, J.E.S. and Norris, K.P., "The Existence of Differences Between the Infrared Absorption Spectra of Bacteria", Spectrochimica Acta 9, 177-198 (1957).

(2) Norris, K.P., "Infrared Spectra of Microorganisms", Advances in Spectroscopy, Volume II, pp. 293-330 (1961), edited by H.W. Thompson, Interscience Publishers, Inc., N.Y.

(3) Katlafsky, B. and Keller, R.E., "Attenuated Total Reflectance Infrared Analysis of Aqueous Solutions", Analytical Chemistry 35, (11) 1665-1670, (1963).

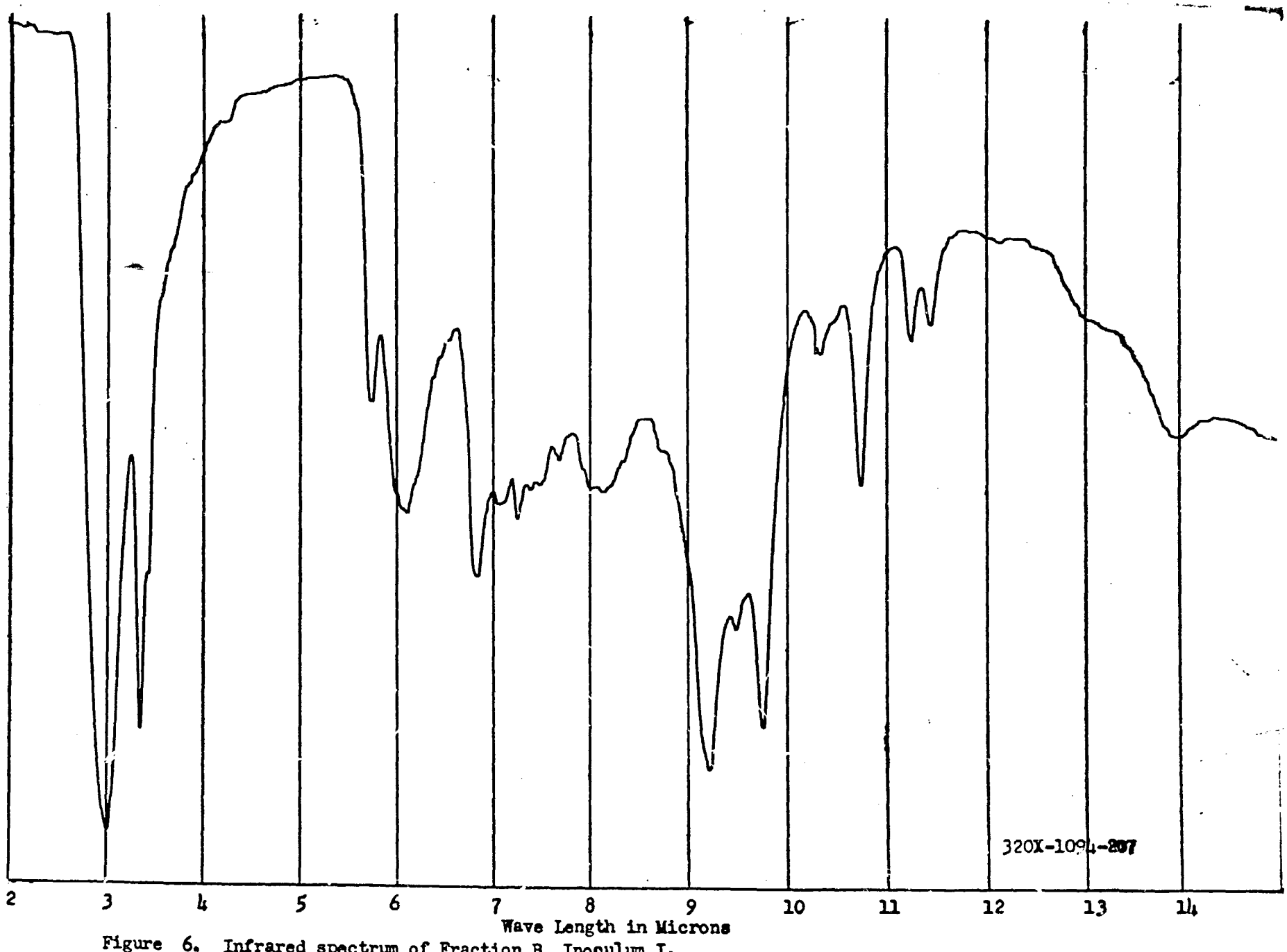

Figure 6. Infrared spectrum of Fraction B, Inoculum I.

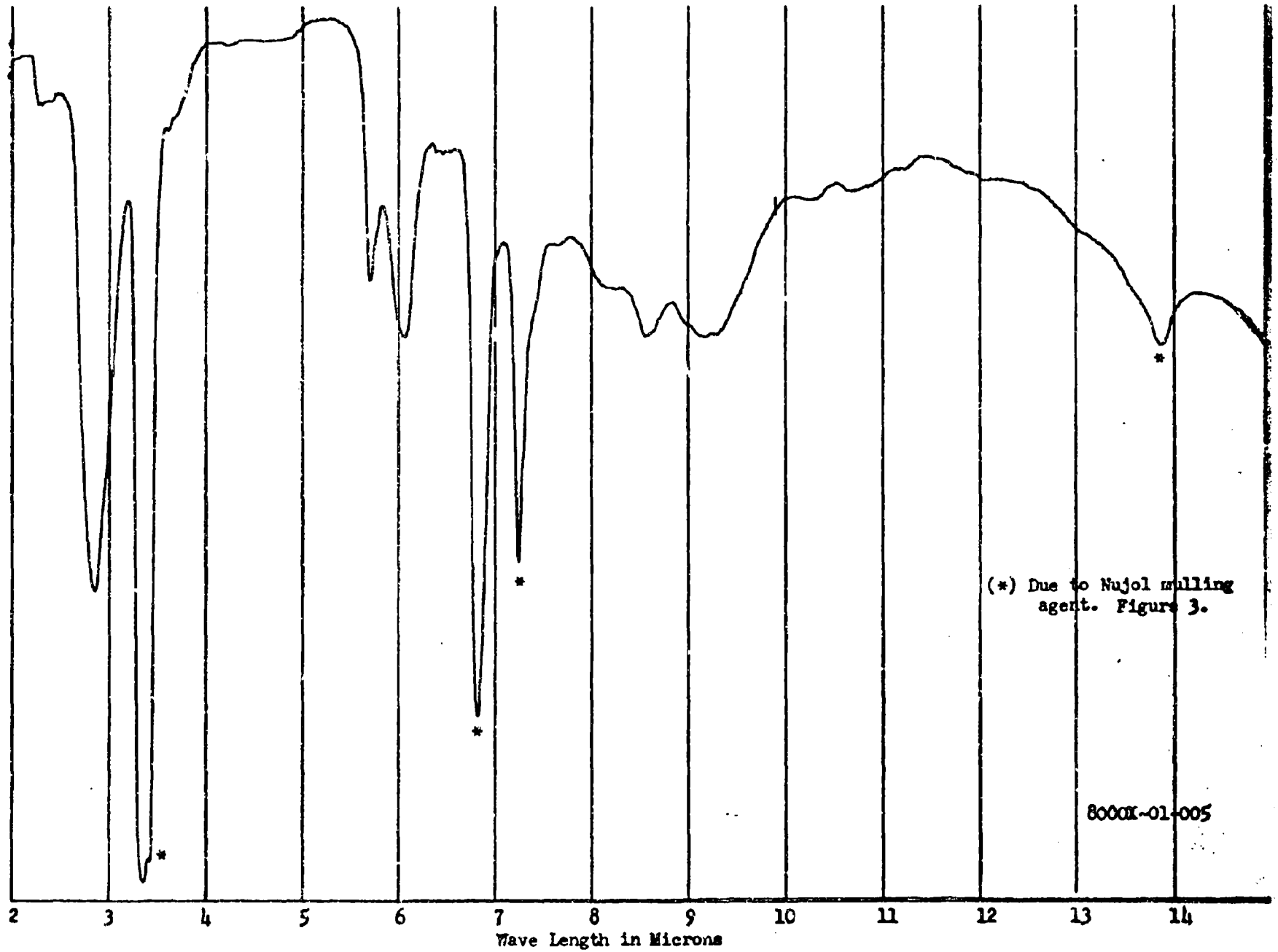

Figure 7. Infrared spectrum of Fraction B, Inoculum II.

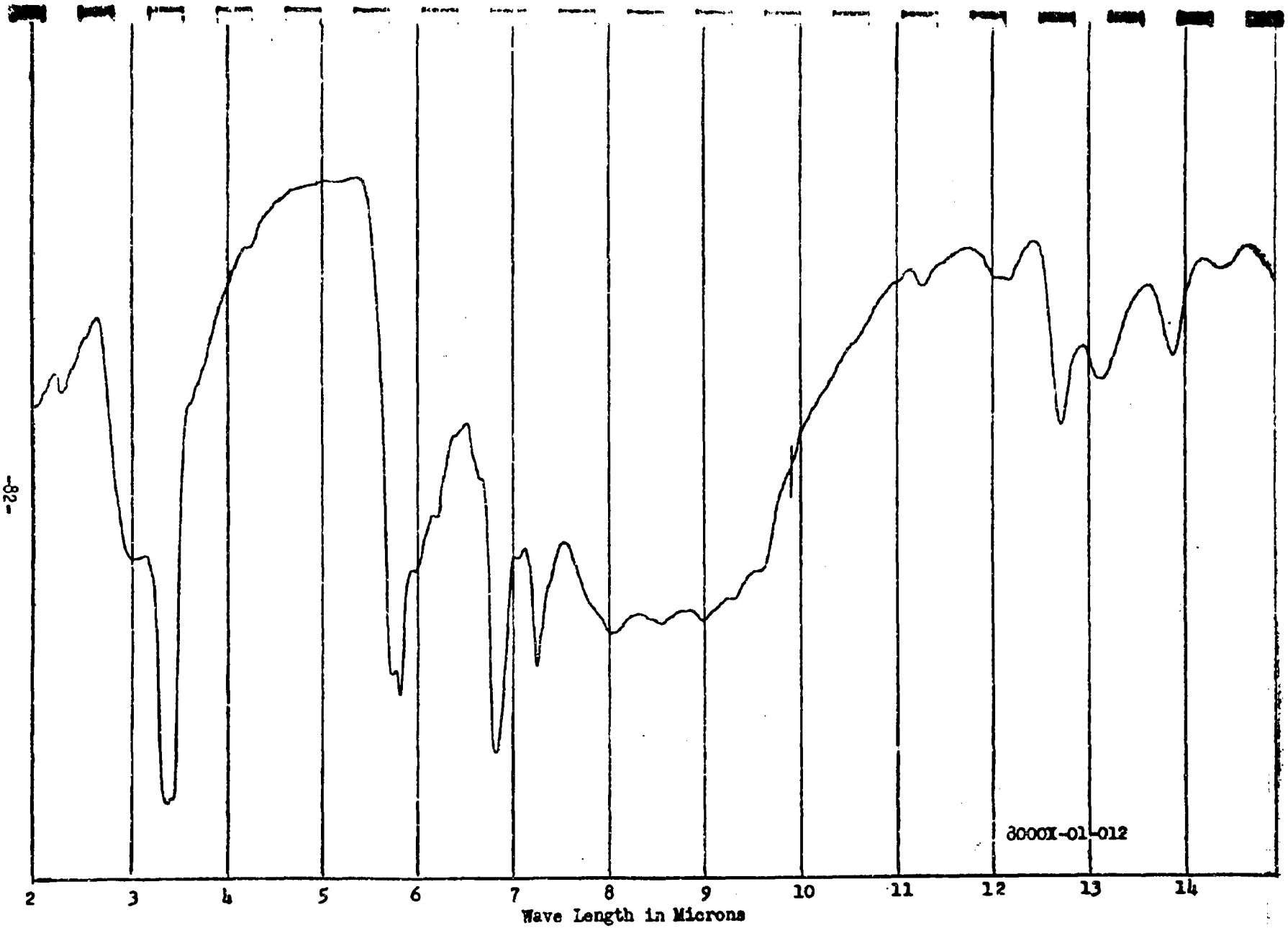

Figure 8. Infrared spectrum of Fraction B, Inoculum III.

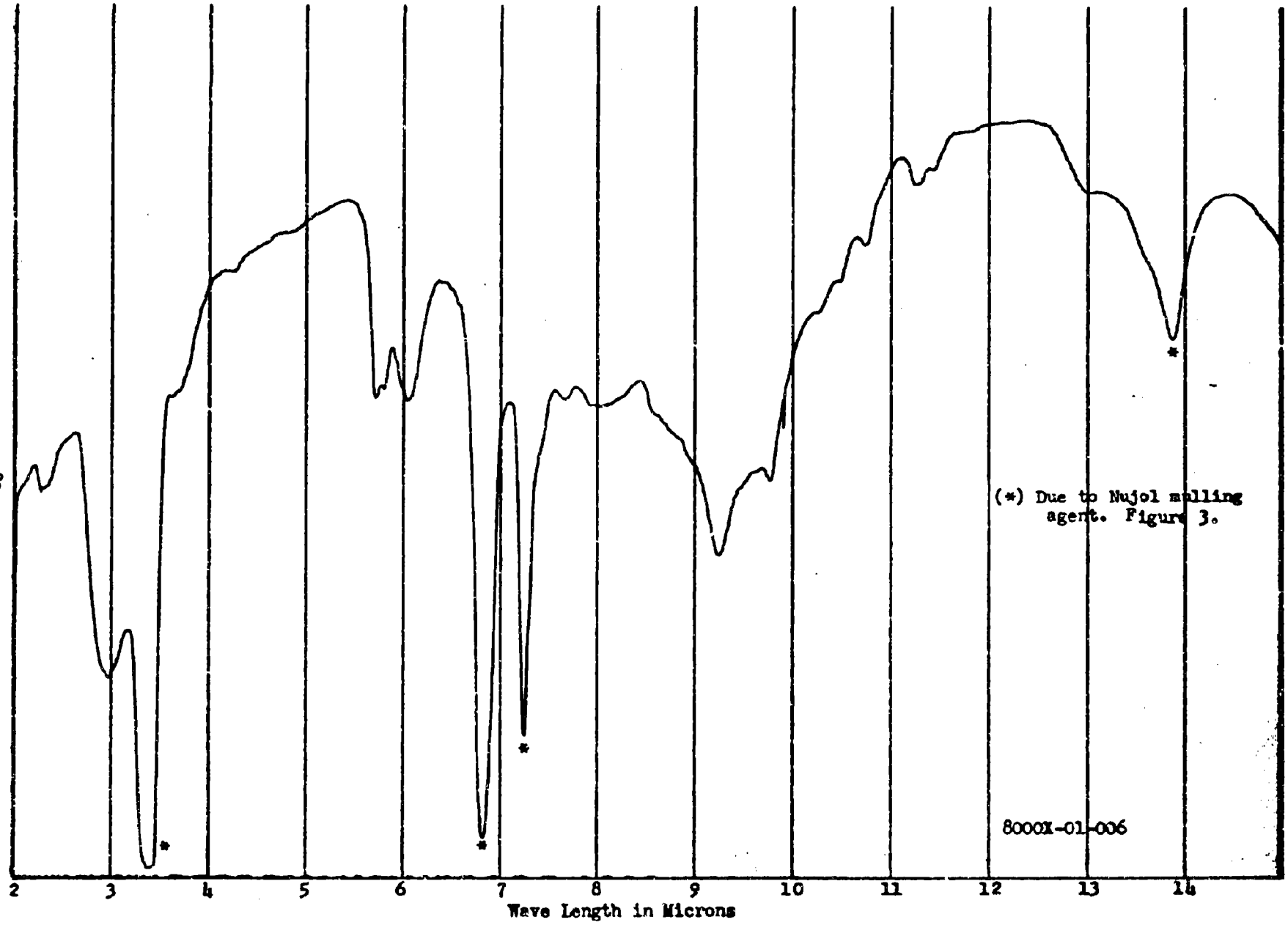

Figure 9. Infrared spectrum of Fraction B, Inoculum IV.

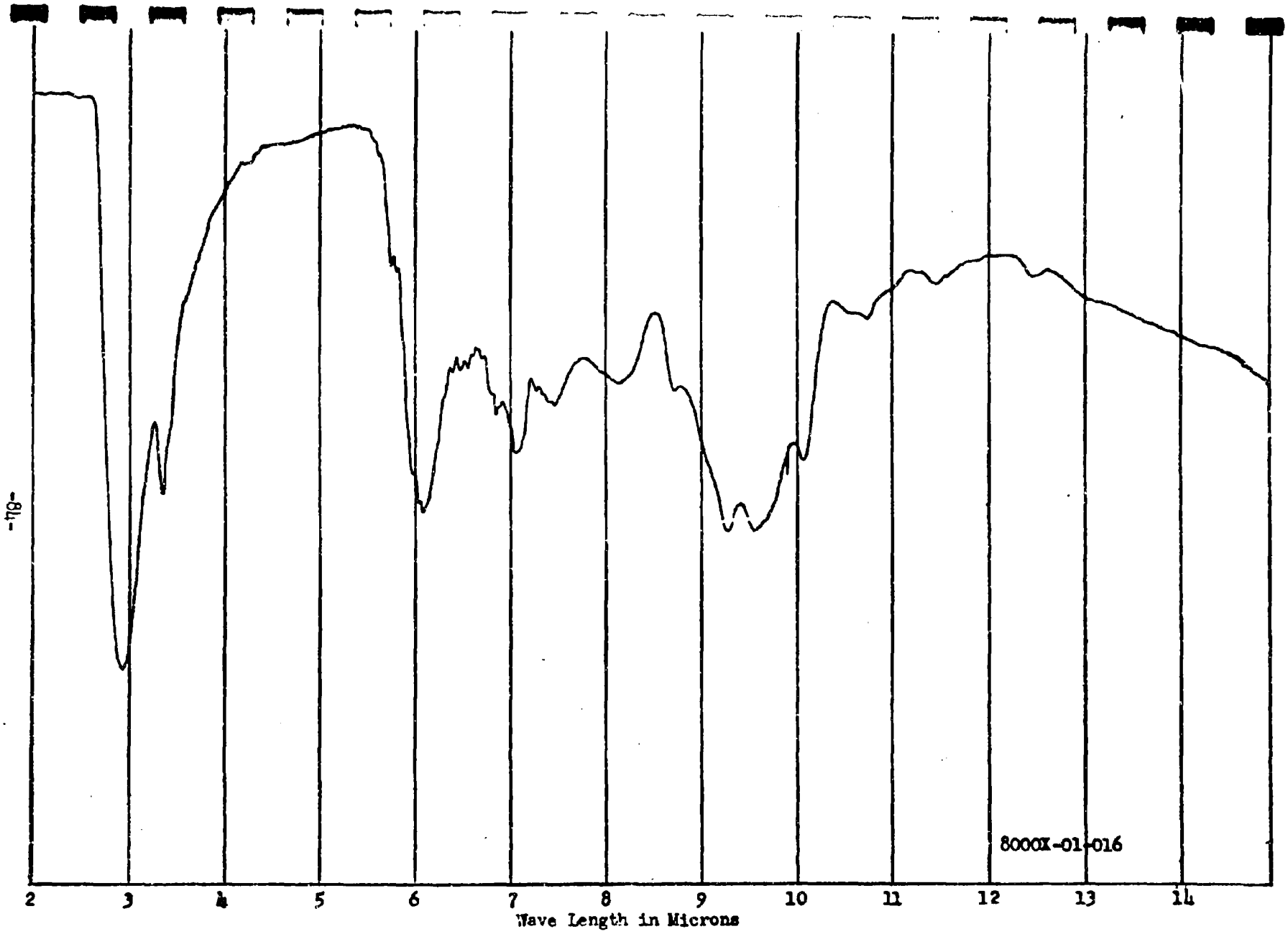

Figure 10. Infrared spectrum of Fraction B, Inoculum VIII.

An interesting observation was the presence of absorption bands between 9 and 10 microns in the triple solvent soluble fraction similar to the bands noted in the Fraction A spectra. The bands were particularly sharp in Figures 6 and 10, but somewhat obscure in Figure 8 (Inoculum III). Absorption in this range suggests the presence of triple solvent soluble carbohydrate-like materials. Some cellular polysaccharides, for example, are alcohol soluble.

Absorption bands were also observed between 5.95 and 6.1 microns in all samples of Fraction B with the possible exception of Inoculum III (Figure 8). Bands in this range are usually associated with amides, suggesting the presence of triple solvent soluble proteins, polypeptides, or amino sugars.

The atypical spectrogram obtained on the solvent soluble fraction of Inoculum III (Figure 8) is of particular interest since the organism involved is the only one tested which utilizes aromatic hydrocarbons (naphthalene and several substituted naphthalenes). Absorption bands usually assigned to substituted aromatics were observed in the 12.25 to 13.5 micron range. The presence of aromatics is further evidenced by the absorption bands at approximately 6.2 microns. Other absorption bands of interest were observed in the 5.6-5.9 and 5.95-6 ranges, and at 8 microns. In general, these bands are associated with oxidized organic compounds such as esters and organic acids. These observations confirm that hydrocarbon fuel components other than paraffins are susceptible to microbial attack.

c. Observations on Fraction C

Detailed analyses of Fraction C (water bottoms free of microbial sludge), were carried out in several steps as outlined in Appendices I and J. The results of these analyses are discussed below in three parts, each dealing with a particular type of analysis.

1) Proteins

The presence of proteins in water bottoms containing active microorganisms would normally be expected as a result of the elaboration of extracellular enzymes and cell lysis. Several methods of quantitating the protein present in the cell-free water bottoms (Fraction C) were considered. Two techniques were selected because of their sensitivity and specificity. Since most tests for proteins have inherent limitations, the two procedures were employed in an effort to minimize the errors involved. To eliminate interfering substances which might be present in the water bottoms, an aliquot of each water bottom was dialysed against running tap water. Determinations were made before and after dialysis.

Results of the quantitative measurement of proteins in the water bottoms are presented in Table XXIX. The presence of interfering substances is indicated by the lower readings obtained after dialysis. Since both methods are dependent on the presence of tyrosine in the protein molecule, any other phenolic compound

TABLE XXIX

Quantitative Protein Determinations on Water Bottoms, Fraction C

(mg/l)

Procedure	Inoculum I	II	III	IV	V	VI	VII	VIII
Before dialysis[1]								
Lowry Method[2]	250	1006	819	463	538	469	638	656
280/260 Method[3]	2025	513	450	-(4)	1513	-	1581	1563
After dialysis[5]								
Lowry Method	63	275 (219)	181	63	144	38	119	156
280/260 Method	250	444 (294)	338	150	425	88	369	425

(1) Analysis made on water bottoms before dialysis against running tap water.

(2) Lowry, O. H., Rosenbrough, N. J., Fan, A. L., and Randall, R. J., 1951, J. Biol. Chem., 193, 265.

(3) Warburg, O., and Christian, W., 1942, Biochem. Z., 310, 384.

(4) Readings were made but they were off the scale.

(5) Analysis made on water bottoms after dialysis against running tap water.

present will also be detected. Analysis of water bottoms from sterile control units and of CITE fuel revealed the presence of phenolic materials. The fuel contained 76 ppm of phenols. This is within the normal range for a straight-run product having a distillation range comparable with CITE fuel. Therefore, the values obtained after dialysis are more realistic. Even these figures should be considered slightly high because it is difficult to completely eliminate the interfering substances. We were not able to adjust the figures obtained with the various protein tests because the organisms present probably utilized some of the phenolics. Nevertheless, variations in the protein content were noted between the water bottoms containing different inocula. Inoculum II, a Pseudomonas sp. apparently produced the most extracellular protein.

If these data are considered qualitative rather than quantitative, we can conclude that protein is present in the water bottoms in all of the test systems. Also, we can speculate that it may contribute to the stability of the emulsion which is normally observed in microbial sludge at the fuel:water interface in the field as well as in the laboratory.

In reviewing our techniques for determining proteins, Dr. M. J. Kronman of Natick pointed out several inadequacies in our procedures. The Lowry and 280/260 methods for determining protein are dependent on the tyrosine content of the protein involved. In comparing mixtures of proteins, differences in composition may be reflected as differences in concentration. We appreciate the significance of this point but, after reviewing available procedures, decided that perhaps the error involved would be minimized rather than enhanced by the fact that we were dealing with mixtures of proteins. The error would be great only if we were dealing with very unusual proteins. Furthermore, because of the apparent low protein content of our samples we were required to use these two methods. The conventional biuret procedure requires considerably more material and in addition does not work in the presence of ammonium salts. Dialysis could have been employed to eliminate the salt but we could not compensate for the low protein content. An unsuccessful attempt was made to precipitate the protein with trichloroacetic acid.

At the time we were conducting these analyses, we were unaware of the presence of phenolic compounds in the water phase. Undoubtedly, these materials were interfering with both procedures. Dialysis probably eliminated much of this material but some of the higher molecular weight compounds probably remained in the sample.

Further attempts were made to determine the protein content of water bottoms produced in Fermentor Studies and are covered in Section D-2 of this report.

2) Carbohydrates

Efforts to quantitate the amount of carbohydrate material present in the water bottoms first involved a review of available procedures. Two techniques which reportedly determine total carbohydrate were selected, the Anthrone and the Indole methods. However, only the Anthrone procedure (Appendix I) proved of any value in testing the water bottoms. The NH_4NO_3 in the 10% sea water medium interfered with the Indole test.

The results obtained with the Anthrone technique are presented in Table XXX. In all cases, the carbohydrate content of the water bottoms was relatively low. Readings obtained on the samples were at the lower limits of the test's sensitivity. For this reason, the per cent error, which is normally greater in this range, was magnified when corrections were made for dilution and the results adjusted to mg per liter.

Qualitatively this test procedure confirmed the presence of carbohydrate in the water bottoms. It is possible that this material, like the protein, may also help stabilize the emulsion present in microbial sludge samples before extraction.

Dr. F. W. Parrish of Natick commented on our techniques for determining total carbohydrates. He recommended the use of the phenol method of Smith, F., et al, (Analytical Chemistry 28, 350, 1956), because it has distinct advantages over the Anthrone method which we employed. This method was evaluated and interference encountered from nitrates present in the system. With respect to the Anthrone procedure, the results obtained (Table XXX) were definitely at the lower limits of the test's sensitivity. The data presented were based on the original volume of water bottoms which varied from 160 to 320 ml and were adjusted to mg/liter for convenience. However, in the process of fractionating the samples, the water phase was diluted considerably before the Anthrone determinations were made.

Dr. Parrish also pointed out that, despite the apparent low concentration of carbohydrates, we should be able to obtain ample material for structural determinations from a few liters of solution. Unfortunately, it is impractical to obtain this volume of solution with our present test system.

The following tests were employed and the resulting interferences noted:

Test Procedure	Interfering Agents
Anthrone	NO_3 (ppt) Phenol (color)
Indole	NO_3 (ppt) Phenol (color)
Phenol-H_2SO_4	NO_3 (Color forms. A material having a broad absorption band is also produced. Since the quantity of NO_3 varies, adequate controls are essentially impossible.)

TABLE XXX

Total Carbohydrate Content of Water Bottoms, Fraction C

(mg/l)

Procedure	Inoculum							
	I	II	III	IV	V	VI	VII	VIII
Anthrone[1]	64	75	156	172	25	66 (45)	76	127

(1) Ashwall, G., 1957, Colorimetric Analysis of Sugars, in Methods in Enzymology II, 84, Edited by Colowick, S. P. and Kaplan, N. O., Academic Press, Inc., N. Y.

3) Non-Protein and Non-Carbohydrate Components

A series of distillations and extractions were conducted on an aliquot from each water bottom sample (Fraction C), following closely the procedures generally employed in fermentation analyses (Appendix J). Because of the questionable sensitivity of the analytical procedures employed, the results are presented in a qualitative form. It appears that most of the classes of compounds detected in the analyses were present in low concentrations, if present at all.

Results of these tests are summarized in Table XXXI. Of the eight samples analyzed, only the water bottoms containing Inoculum III (RP-1 pure bacterial culture) gave positive test results. The identity of the compounds present in the two distillates was not established. Further tests to determine the nature of the materials present were not made because of the relatively small quantity of samples available. Larger quantities of material were obtained and analyzed in the Fermentor Studies reported in Section D-2 of this report.

TABLE XXXI

Summarization of Analyses for Non-Protein and Non-Carbohydrate Components of Water Bottoms (Fraction C) Static Test

Analytical Procedure	Inoculum							
	I	II	III	IV	V	VI	VII	VIII
Volatile Neutrals - Analysis of distillate								
Dichromate Test	-(1)	-	+(2)	-	-	-	-	-
NMR (CCl_4 Extract)	-	-	±	-	-	-	-	-
Volatile Acids - Analysis of steam distillate								
Titrable Acidity	-	-	+	-	-	-	-	-
CCl_4 Extractable, non-volatile neutrals								
NMR	-	-	-	-	-	-	-	-
CCl_4 Extractable, non-volatile acids								
NMR	-	-	-	-	-	-	-	-

(1) - = Negative test, no compounds observed.

(2) + = Positive test, a small amount of material detected.

2. Fermentor Studies

The data obtained with the static system reported in Section D-1 yielded useful information on the growth of microorganisms in fuel systems but failed to provide adequate material for detailed analyses of sludge and water bottoms. To conveniently produce sufficient quantities of these materials, a single unit New Brunswick MicroFerm fermentor was used. Details of the system are presented in Appendix K. Because of the relatively large volumes of material involved, some modification of earlier fractionation procedures was required (Appendix L). The interfacial emulsion which formed in the runs with CITE fuel was handled separately.

Five fermentor runs were made employing two pure cultures and a composite of contaminated tank bottom water samples. The two pure cultures were grown on the pure hydrocarbons which they utilized most readily and on CITE fuel. Details of the cultures used are presented in Section C-4 of this report. The following fermentor runs were made:

Run 1 - Inoculum II (*Pseudomonas sp.*) on n-octane.
Run 2 - Inoculum III (RP-1 culture, pure) on naphthalene.
Run 3 - Inoculum I (Composite tank bottom sample) on CITE fuel.
Run 4 - Inoculum II on CITE fuel.
Run 5 - Inoculum III on CITE fuel.

Fermentor run 3 consisted of a two-stage process involving incubation under dynamic conditions in the fermentor followed by static conditions under a large volume of fuel (Appendix K). Following the fermentor run which produced heavy bacterial growth, the contents of the fermentor were divided in half. The two portions were used to inoculate two 40-liter glass carboys containing sterile CITE fuel. We anticipated heavy fungus growth to develop under the static conditions. The pH of the water phase was approximately 5 when the fermentor run was completed. To insure fungus growth, the carboys were re-inoculated with an aliquot of Inoculum I. This two-stage process more closely approaches field conditions where both bacteria and fungi are present. The fermentor simulated the turbulence of pumps which tend to produce emulsions, and the carboys simulated the relatively quiescent tank bottoms.

Fermentor run 2 had to be repeated because of excessive foaming and the use of a large amount of silicone antifoam agent. The silicone made analysis of the sludge and water bottoms impossible. Fermentor run 5 was also repeated, but only because of difficulties in separating the emulsion which formed. The water phase, including the cells, was evaporated to dryness at 105°C. The run was repeated in order to obtain an aqueous fraction for analysis.

Each fermentor run was handled separately and fractionated in the manner described in Appendix L. The fractions obtained were analyzed in an effort to characterize the materials present. In previous studies we reported only three fractions:

Fraction A - Insoluble Residue - Insoluble in fuel, water, and benzene-acetone-methyl alcohol solvent mixture (triple solvent).

Fraction B - Fuel Sludge - Material insoluble in fuel and water but soluble in benzene-acetone-methyl alcohol solvent mixture (triple solvent).

Fraction C - Water Bottoms - Water soluble, non-volatile materials.

In an effort to obtain better separation of materials the procedures were extended to include the following fractions (Appendix B):

Fraction D - Water Soluble Extract - Materials extracted from the sludge with distilled water.

Fraction E - Hexane Solubles - Materials extracted from the sludge with n-hexane. In the past this fraction has been considered as part of the fuel phase and not reported.

The weights of the various fractions were obtained and are presented in Table XXXII. Very little difference was noted in the yield of sludge from runs 1, 3 and 4. Run 2 yielded very little sludge, probably because of the short duration of the test. The high yield reported for run 5 was due to the inclusion of salts present in the water bottom.

Only trace quantities of hexane and triple solvent soluble material were present in the sludge obtained from runs 1 and 2 (pure hydrocarbons). Small but significant quantities of these two fractions were obtained when CITE fuel was employed (runs 3, 4 and 5). Relatively large quantities of water soluble materials were obtained in runs 1, 2 and 4. A substantial portion of this material is probably due to cell lysis. Also, it was difficult to completely eliminate inorganic salts from this fraction. The sludge from run 5 contained a large quantity of inorganics since the entire water phase had to be evaporated to dryness to facilitate handling.

Analytical data obtained on each fraction are discussed below. Results from the various fermentor runs are compared.

TABLE XXXII

Results of Gravimetric Determinations on Microbial Sludge from Fermentor Studies

Run Number	Test System	Sludge Yield gm/L Water (Dry Wt.)	Weight (Dry) Per Cent of Fraction: Water Soluble Materials (Fraction D)	Tri-Solvent Soluble Materials (Fraction B)	Hexane Soluble Materials (Fraction E)	Residue(1) (Fraction A)
1	Inoculum II on n-octane	1.35	16.4	Trace(2)	Trace	83.6
2	Inoculum III on naphthalene	0.30	16.0	Trace	Trace	84.0
3	Inoculum I on CITE Fuel (2 Stage)(3)					
	Sludge	1.23	-	2.0	0.3	97.7
	Interface Residue	0.20	-	4.7	1.0	94.3
4	Inoculum II on CITE fuel					
	Sludge	1.25	7.5	0.3(3)	4.8(3)	87.4
	Interface Residue	0.12	Trace	Trace	Trace	99+
5	Inoculum III on CITE fuel	7.82(4)	61.5	9.3	2.2	27.0

(1) Residue (Fraction A) - Microbial Sludge, insoluble in fuel, water, n-hexane, and benzene-acetone-methyl alcohol solvent mixture (Tri-solvent).

(2) Less than 0.1% present.

(3) These two fractions were combined. The small amount of material reported as tri-solvent soluble, apparently is cellular material which was suspended in the extract. Essentially all of the tri-solvent soluble material was also soluble in n-hexane.

(4) The entire water phase, including cells, was evaporated to dryness.

a. Observations on Fraction A

Elemental analyses of Fraction A from the various fermentor runs are presented in Tables XXXIII and XXXIV. Considerable variations in the relative concentrations of C, H, O, N and P in microbial cells can be expected since many factors, including the genetic make-up of the organisms, influence cell content. Emission Spectrograph analysis (Table XXXIV) indicated that phosphorus was a major metal in all sludge samples. Calcium was also a major metal in the sludge from runs 2, 4 and 5. The variations noted probably reflect differences in growth conditions or in preparation of the sludge, as well as factors intrinsic to the organisms involved. Data from run 5 was not included because we were not able to account for the inorganic salts present.

The following empirical formulae were derived from the data in Table XXXIII:

Run 1 - $C_{44}H_{80}O_{15}N_{10}P$
Run 2 - $C_{39}H_{65}O_{11}N_{9}P$
Run 3 - $C_{62}H_{110}O_{23}N_{13}P$
Run 4 - $C_{29}H_{62}O_{12}N_{8}P$

Variations in element content are again reflected by these formulae. The effect of substrate and conditions is demonstrated in the formulae obtained on runs 2 and 4 which employed the same organism. These empirical formulae appear relatively reduced when compared with $C_{55}H_{77}O_{22}N_{11}P$ obtained by Porges, et al* for activated sludge produced in a simulated waste disposal system. In Porges' system not only were the conditions oxidative but the substrate was more oxidized.

IR spectra obtained on samples of Fraction A (water, fuel and triple solvent insoluble material) from the five fermentor runs were essentially the same. Figures 11 and 12 are representative of the spectra obtained on these fractions. The spectrum of Fraction A from run 5 was altered somewhat by the presence of inorganic salts. Also, there were no apparent changes in the sludge spectra before and after extractions. Interfacial sludge from runs 4 and 5 had spectra similar to those shown in Figures 11 and 12.

All spectra possessed strong absorption bands in the 5.5 to 6.5 micron range, indicating higher relative concentrations of protein than observed with the same cultures under static conditions (Figures 4 and 5). This was probably due to more favorable growth conditions in the fermentor. A dynamic system provides a better supply of oxygen and substrate, thus encouraging rapid growth. Under these conditions there is probably less tendency for carbohydrate to accumulate in the cells and a greater tendency for protein synthesis.

(*) Porges, N. et al, 1956, "Principles of Biological Oxidation", Biological Treatment of Sewage and Industrial Wastes, Edited by McCabe, B.J. and Eckenfelder, W.W., Vol. 1, pages 35-48, Reinhold Publishing Corp.

TABLE XXXIII

Elemental Analysis of Microbial Sludge - Fraction A

Element	Percent			
	Run 1 Inoculum II (n-Octane)	Run 2 Inoculum III (Naphthalene)	Run 3 Inoculum I (CITE Fuel)	Run 4 Inoculum II (CITE Fuel)
Carbon	42.0	45.9	47.72	38.7
Hydrogen	7.0	6.3	7.11	6.8
Oxygen	19.6	17.6	24.30	20.7
Nitrogen	13.0	12.5	11.40	11.6
Phosphorus	2.7	3.0	1.95	3.4
Sulfur	0.8	0.3	0.70	0.6
Sodium	0.3	0.3	0.06	0.5
Potassium	1.0	0.4	0.16	1.2
Ash	2.0	11.9	7.33	11.8
% Recovery	88.4	98.2	100.73	95.3

TABLE XXXIV

Emission Spectrograph* Metal Analysis of Microbial Sludge Ash

Relative Concentration	Run 1 Inoculum II (n-Octane)	Run 2 Inoculum III (Naphthalene)	Run 3 Inoculum I (CITE)	Run 4 Inoculum II (CITE Fuel)
Major	P	P Ca	P	P Ca Na
Minor	Si Fe Ca Mg	Zn Ni	Ca Fe	
Trace	Al Ag Cu Cr Pb Sn	Fe Al Cu Cr Pb Ti	Si Mn Cu Zn	Fe Pb Cu Cr Si Al

* 1.5 Meter Grating Emission Spectrograph, Applied Research Laboratories instrument.

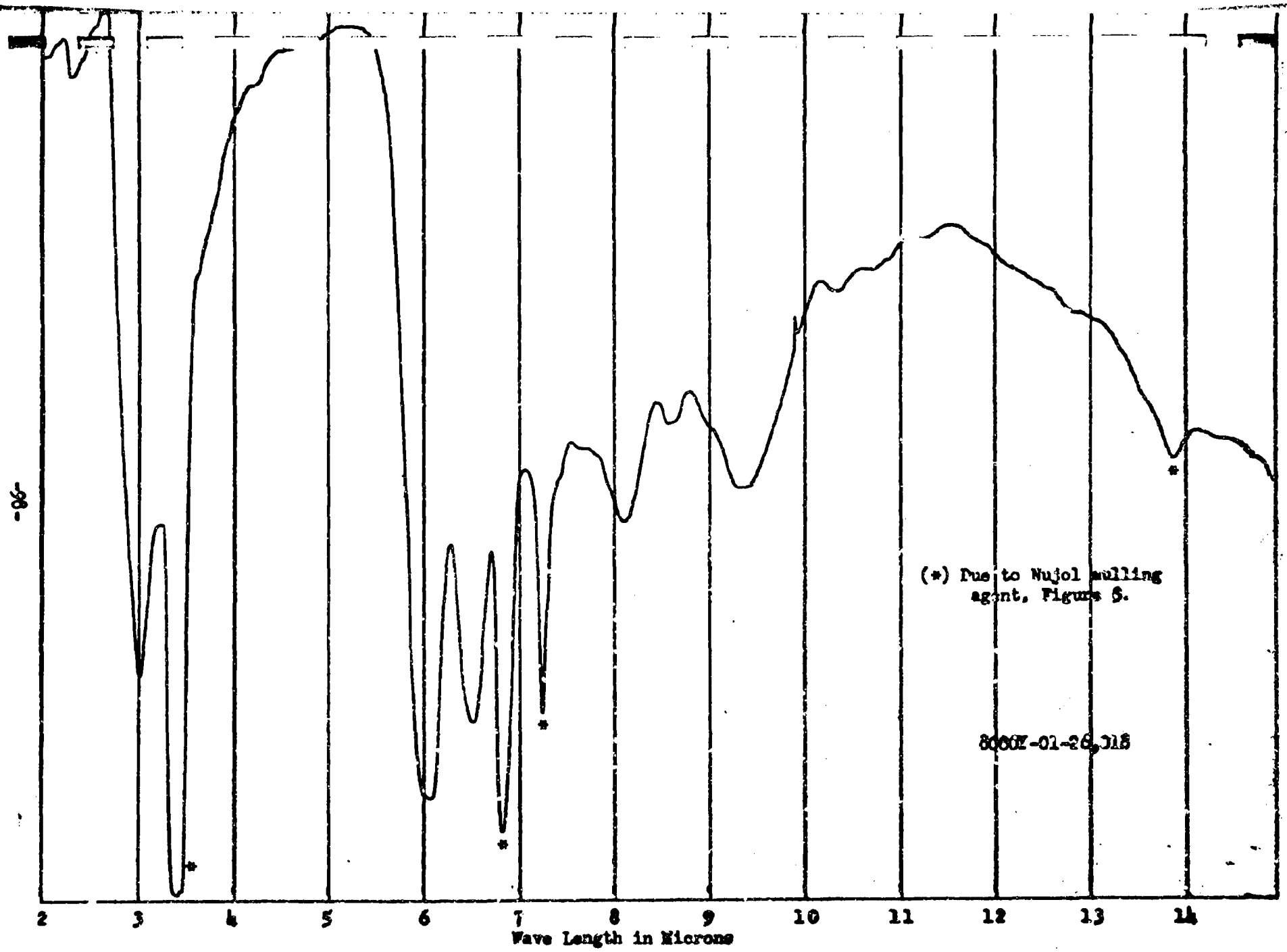

Figure 11. Infrared spectrum of Fraction A, fermentor Run 1.

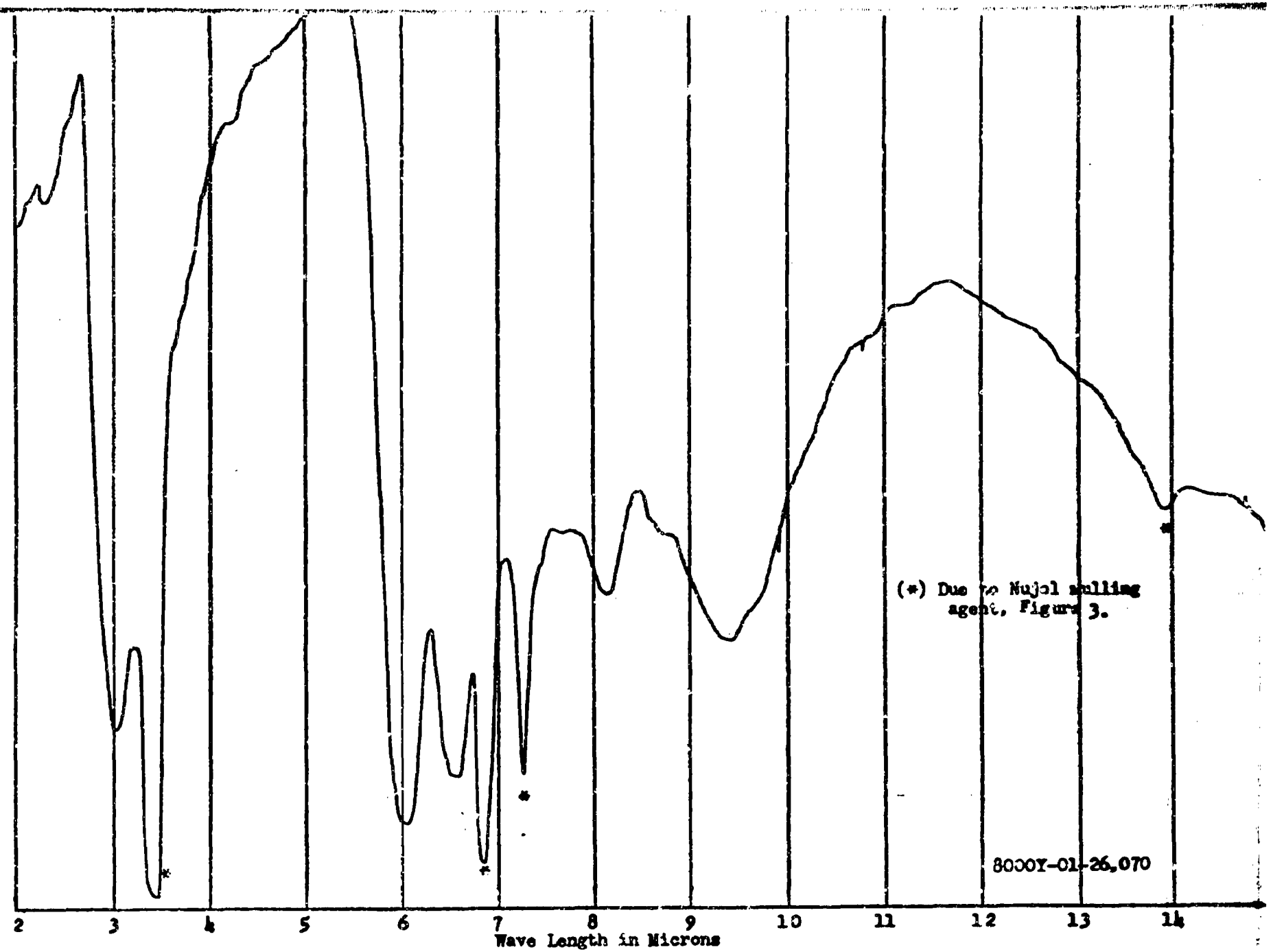

Figure 12. Infrared spectrum of Fraction A from Fermentor Run 2.

Absorption bands are also present at 3.0 and 6.54 microns which were not normally present or only vaguely discernible in other sludge spectra. These bands are usually assigned to secondary amines and amine or metal soaps respectively. The lack of bands in these regions in the other sludge spectra was probably due to the relative concentrations present or masking by other components. Bands at these two wave lengths and in the 5.9 to 6.05 range are characteristic of many proteins, and tend to confirm the high protein content of the cells.

In addition to the bands in the 5.5 to 6.5 micron range, all spectra showed strong absorption bands in the 9-10 micron region. According to Morris(1) absorption in this region is due primarily to cellular polysaccharides. Normally, bands in this region, more specifically at 9.1 to 9.5, are assigned to "-C-O-" and "-S=O" stretching and "P-O-C" vibrations. However, when these structures are present, another band is observed at 10.6 microns. In the IR spectra of sludge samples (Fraction A) this band is generally not observed, or is only vaguely discernible.

A more recent article by Linker and Jones(2) reported studies on polysaccharides produced by a Pseudomonas sp. IR spectra of their polysaccharide preparation possessed strong absorption bands between 9 and 10 microns and at 10.6 microns. In an effort to establish the presence or absence of a 10.6 micron band, crude separations of the sludge (Fraction A) from run 1 were made employing TCA (trichloroacetic acid) precipitations (Appendix M). The IR spectrum of the sludge, Fraction A, before TCA treatment, is presented in Figure 11 and shows the typical absorption band at 9.5 microns and a questionable band at 10.6 microns. These two bands were quite pronounced in the supernatant solution after TCA precipitation of the protein (Figure 13). Apparently, protein or other TCA precipitable sludge components masked the 10.6 micron band.

Carbohydrates, glycols, and some organophosphates all possess absorption bands in these two regions. The class most likely present in the sludge would be the carbohydrates and probably their phosphoric acid esters. This would tend to confirm Harris' observation.

It should be noted that the amide band in the 5.9 to 6.01 micron range is still present in the purified preparation. This is probably due to the presence of non-TCA precipitable proteins, polypeptides, amino acids, or possibly nitrogen containing carbohydrates.

- -

(1) Morris, K.P., 1961, Infrared Spectra of Microorganisms, pp 293-330, Advances in Spectroscopy, Volume II, edited by Thompson, H.W., Interscience Publishers, Inc., N.Y.

(2) Linker, S.A. and Jones, R.S., 1964, A Polysaccharide Resembling Alginic Acid from a Pseudomonas Microorganism. Nature 204, (4954) 187.

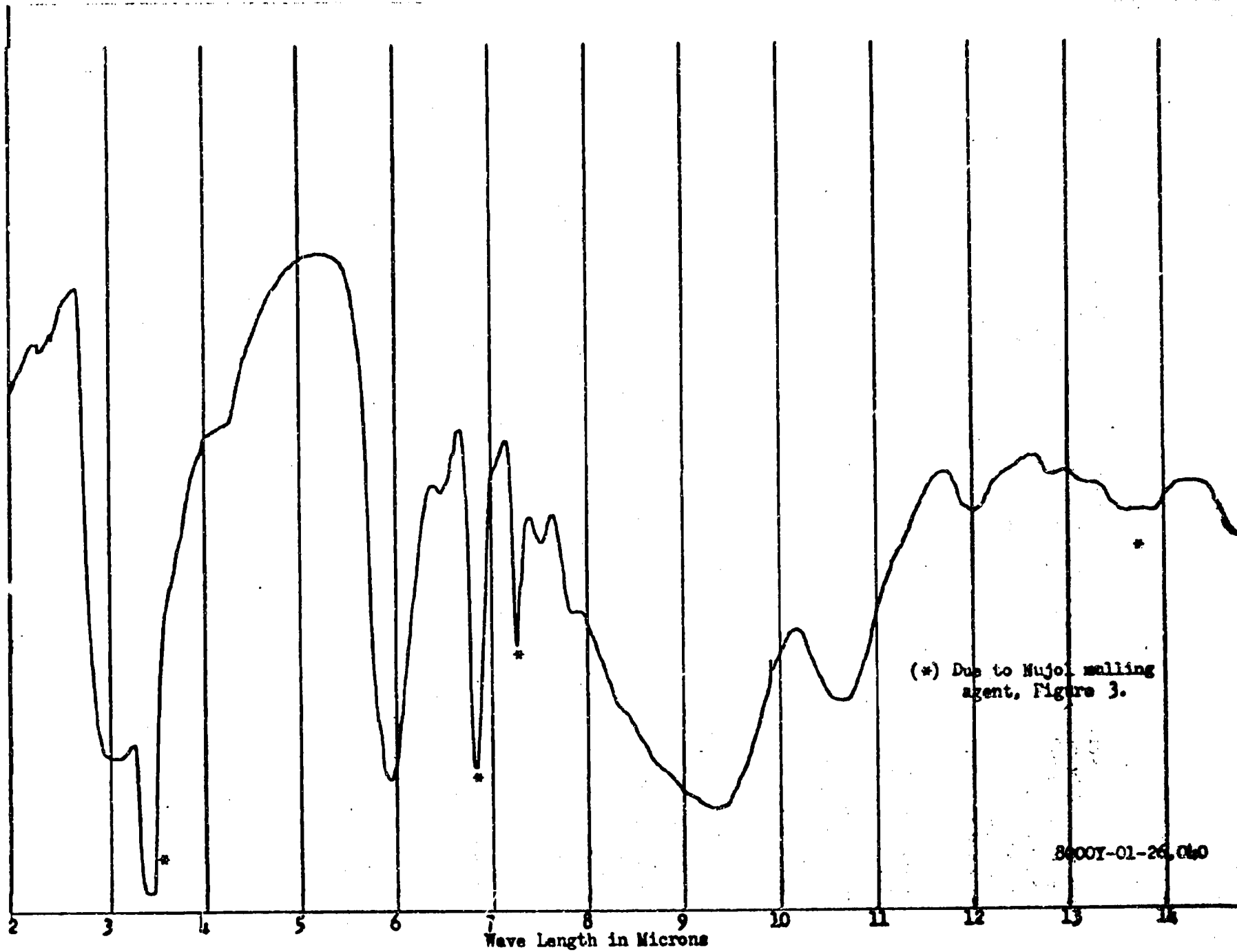

Figure 13. Infrared spectrum of purified sludge fraction (Fermentor Run 1) showing strong absorption band at 9.5 microns.

b. Observations on Fraction B

The triple solvent soluble fractions from the fermentor runs were analyzed by infrared spectroscopy. A representative spectrum is shown in Figure 14. In general, the spectra obtained resemble the one shown in Figure 7 (Fraction B, Static Test). However, the absorption bands are clearer in Figure 14. Of particular interest are the ester bands at 5.72 and 8 to 9 microns. These bands apparently reflect the presence of lipid materials extracted from the cells. Also present are bands generally assigned to proteins and carbohydrates. The triple solvent evidently extracted these cell components or perhaps caused lysis which released these materials into the solvent. Similar spectra were obtained on triple solvent soluble components of interfacial sludge collected from fermentor runs 4 and 5. The ester components of the sludge which are clearly visible in these spectra are apparently lipid components of the cells. These materials appear to have acted as surfactants and stabilized the emulsions which formed in the fermentor runs employing fuel.

Further solvent separation of the triple solvent soluble fraction from run 4 produced a material which was also soluble in n-hexane. The spectrum of this fraction is shown in Figure 15. Ester bands at 7.2 and 8-9 microns are very clear as is the band between 9-10 microns usually assigned to carbohydrate components. The hydrocarbon peaks (3.5, 6.8, 7.3 and 13.8) are probably due to residual hexane and fuel components.

A similar separation was made on the triple solvent soluble material from run 3. The hexane soluble fraction had a spectrum similar to Figure 15. IR analysis of the hexane insolubles indicated that the materials which absorb in the 7 to 10 micron range were absent (Figure 16). Peaks associated with acid carbonyls, amides and metal soaps were present. In addition, bands at 6.2, 6.6 and in the 11 to 13 micron range were present, suggesting aromatic structures. These peaks probably represent trace quantities of microbial pigments. The spectrum of an ethanol extract of the tri-solvent soluble material (Fraction B) is presented in Figure 17. Because of the variety of structures present, distinct bands were not obtained. However, some of the absorption bands do warrant comment. A strong secondary amine band is present at 3.0 microns and a strong soap band at 6.54 microns. The broad band between 5.5 and 6.2 microns apparently represents three and possibly four peaks; ester carbonyl at 5.75; acid carbonyl at 5.82; amide carbonyl at 5.9-6.01; and possibly an aromatic ring band at 6.25. Some evidence of aromatic substitution is also present. This is indicated by the bands above 10 microns.

This ethanol extract was also examined on the High Mass Spectrophotometer. While the results are not conclusive, the following compounds or similar materials may have been present in the sample. The spectrum indicated the presence of a mixture of aromatic acids, acetates and possibly ketones having molecular weights ranging through mass 322. Not all of the sample volatilized, since there was a small amount of residue. The predominate component peaks in the order of greatest peak height were as follows:

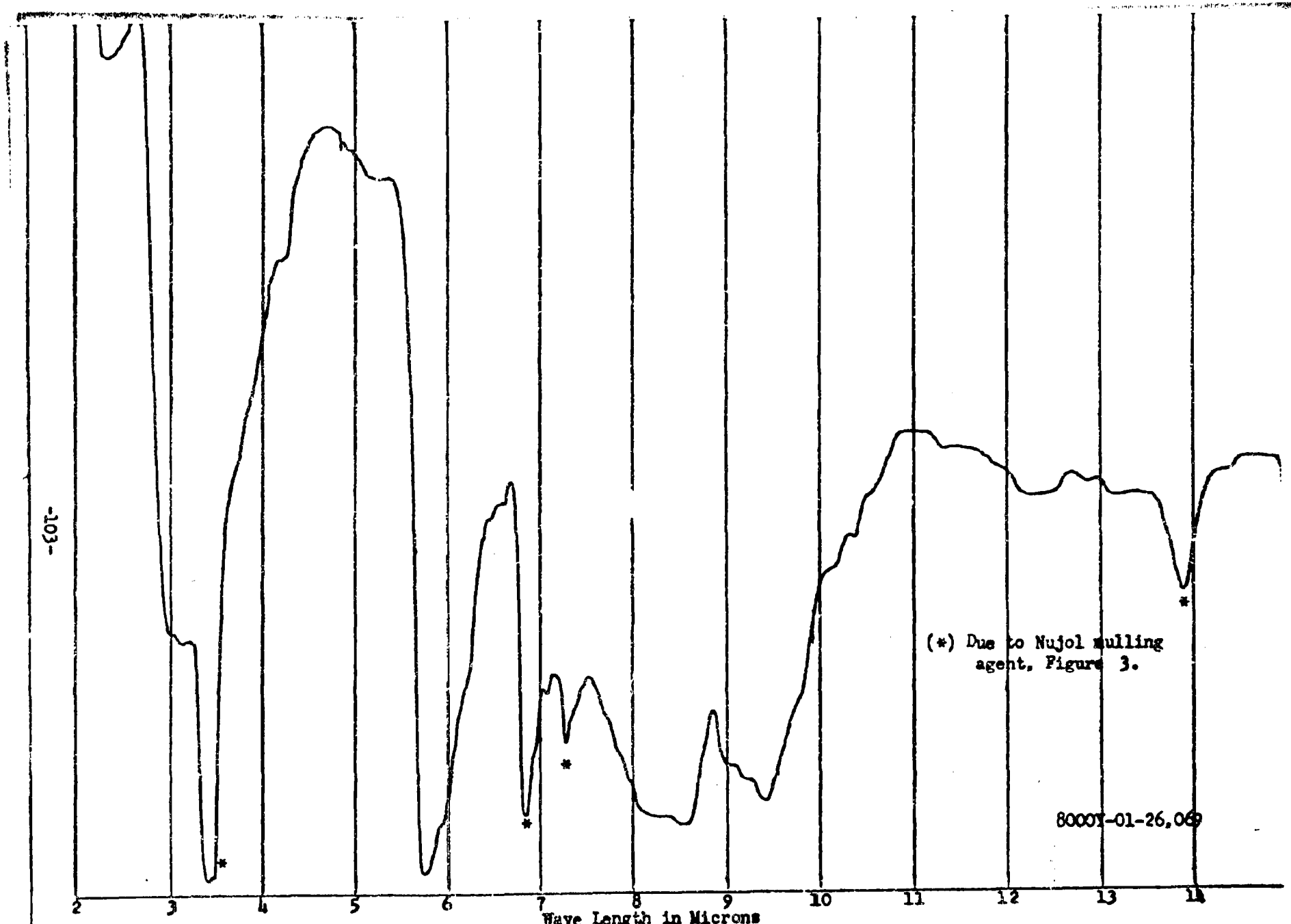

Figure 14. Infrared spectrum of tri-solvent soluble (Fraction B) materials from Fermentor Run 2.

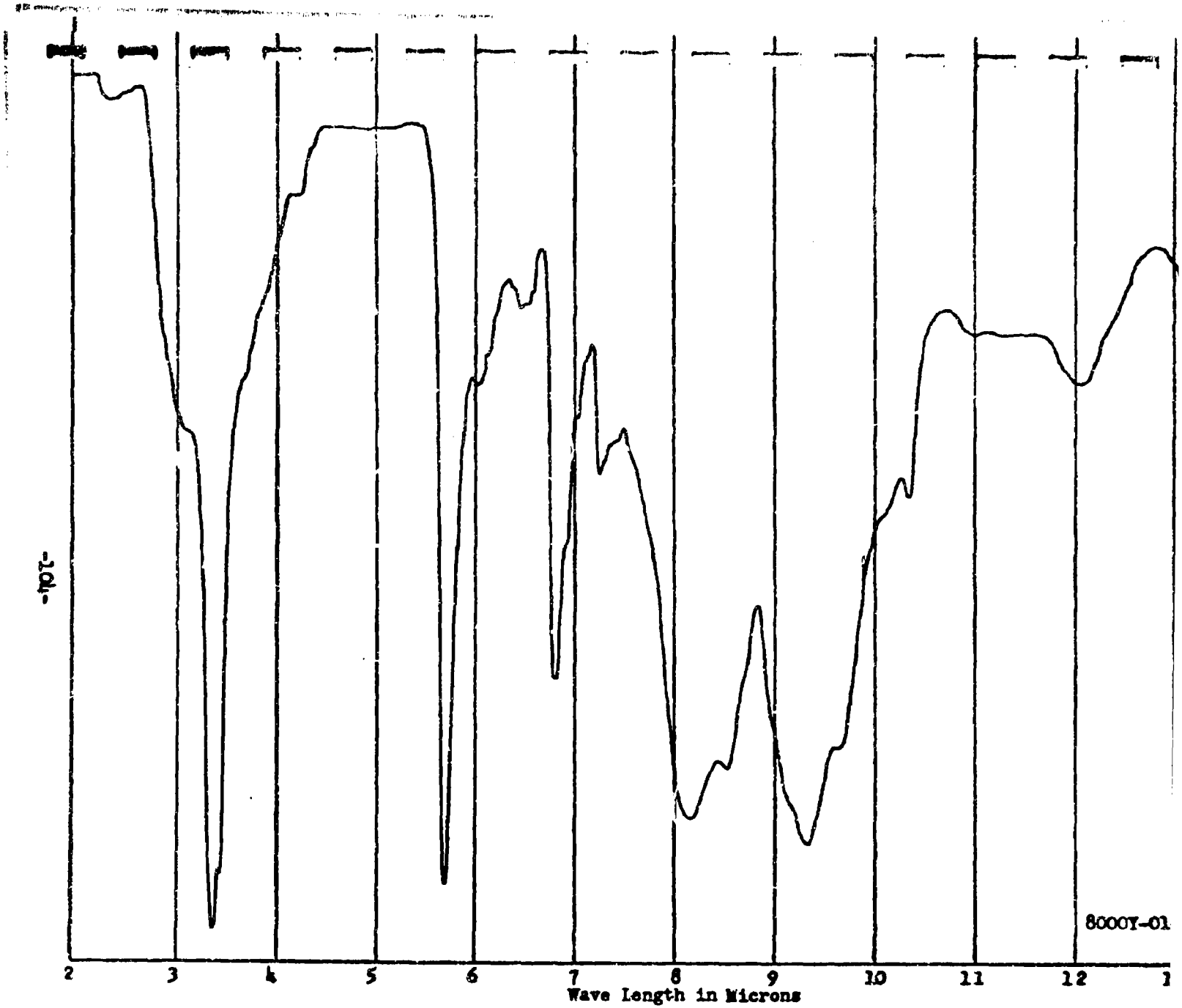

Figure 15. Infrared spectrum of tri-solvent and n-hexane soluble material extracted from slud
Fermentor Run 4.

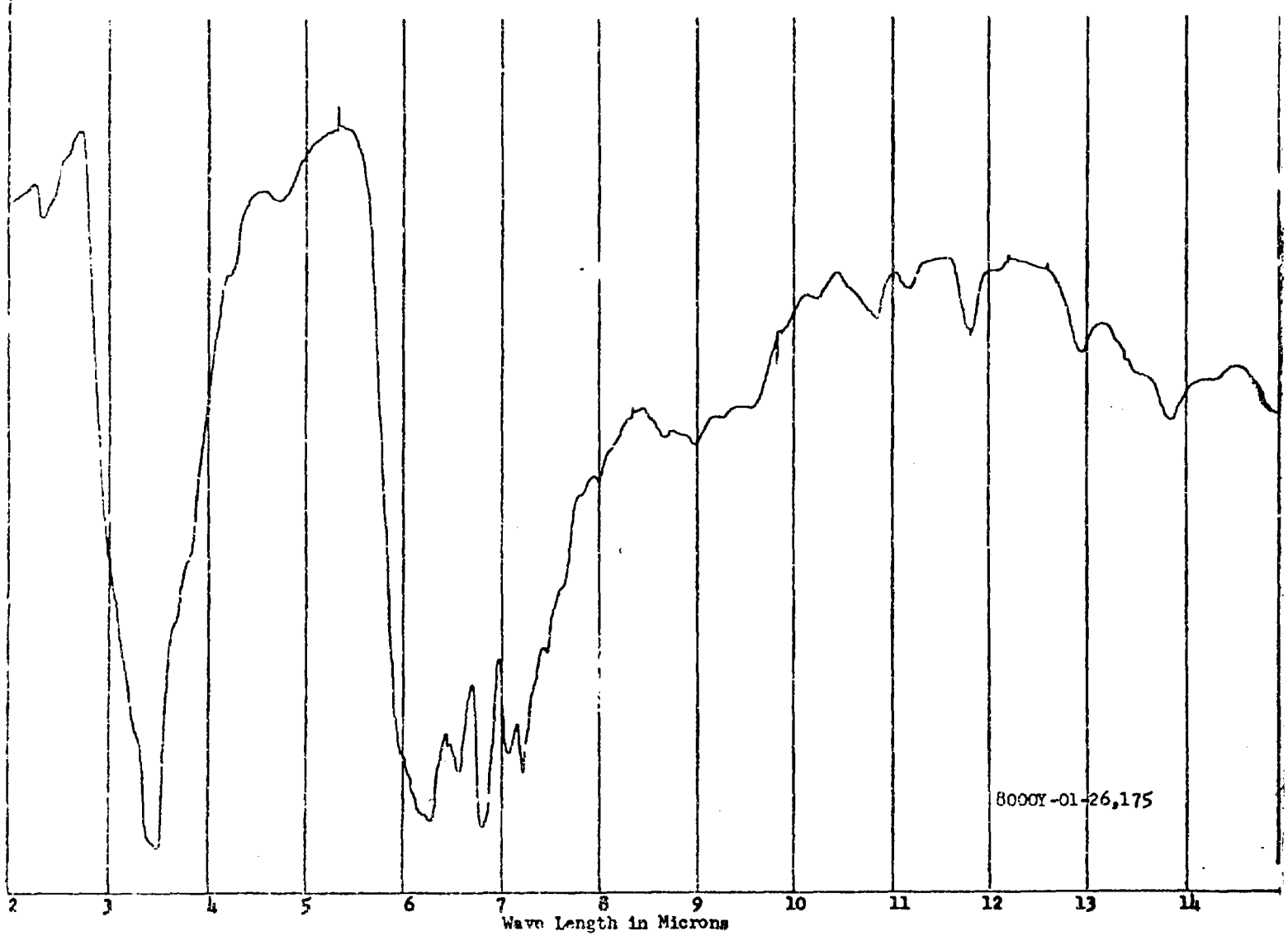

Figure 16. Infrared spectrum of tri-solvent soluble, n-hexane insoluble material extracted from the sludge obtained from Fermentor Run 3.

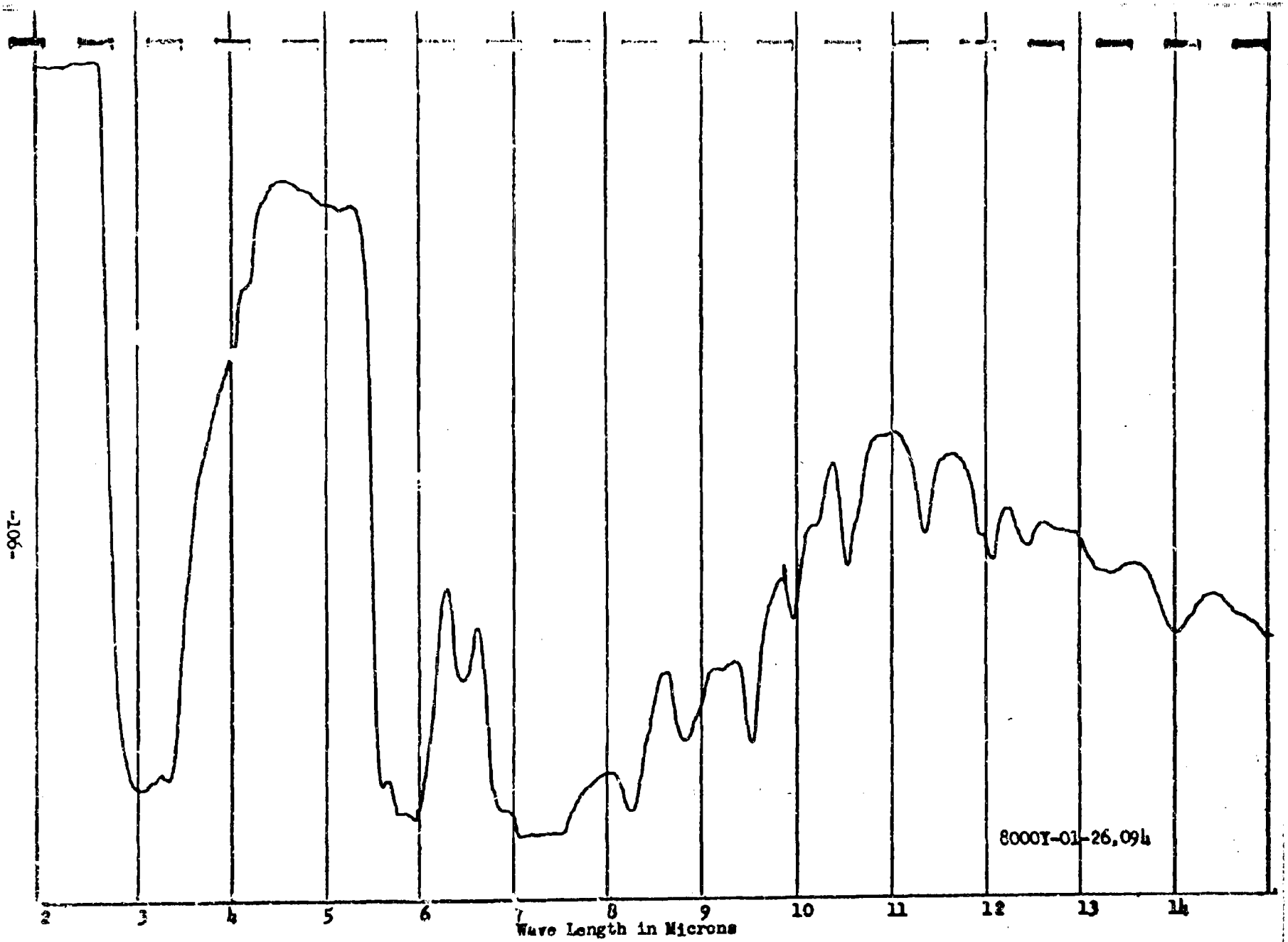

Figure 17. Infrared Spectrum of tri-solvent soluble, ethanol insoluble, material extracted from the sludge obtained from Fermentor Run 5. Ethanol solubles were inorganic.

Mass Number	Possible Compounds
136	m-Toluic acid
122	Benzoic acid
138	Salicylic acid
152	Methyl salicylate
126	C_9 Olefin
112	C_8 Olefin
110	Catechol
166	Ethyl salicylate
150	Methyl-o-toluate or ethyl benzoic acid
134	Methyl acetophenone or C_{10} alkyl benzene
120	C_9 Alky benzene
124	Dihydroxy toluene

Peak heights of the above components were too small to make positive identification possible. Additional peaks were observed between 166 and 322 mass but they were too weak to permit even tentative identification. However, the data tend to substantiate the infrared observations.

They also correspond to likely metabolic intermediates which might be produced since Inoculum III, used in this run, is quite specific for naphthalene and substituted naphthalenes.

c. Observations on Fraction C

This fraction, water soluble materials, was analyzed for proteins, carbohydrates and other organic components. The results of these analyses are discussed below.

1) Proteins

Results of protein determinations on the aqueous phase, Fraction C, from the various fermentor runs are presented in Table XXXV. A modified biuret test was employed. Protein was not detected in the samples from runs 1 and 2 which utilized pure hydrocarbons as substrates. However, when fuel was employed, varying amounts of protein were detected in the samples. This may have resulted from cell lysis due to the excessive washings with distilled water and n-hexane required to separate the emulsions which formed.

The pre[illegible] even small quantities of protein in the water bottoms supports the co[illegible] hydrocarbon utilizers may supply nutrients for non-hydrocarbon utili[illegible] [illegible]vengers) in tank bottoms.

TABLE XXXV

Results of Protein Determinations on the Aqueous Phase from Fermentor Runs

Run Number	Protein Determination[1] mg/85 ml
1	-
2	-
3	8.73
4	3.80
5	2.44

(1) Modified biuret procedure reported by Beisenherz, et al., (Zeitschrift fur Naturforschung 8b, 576-577, 1953)

2) Carbohydrates

Our efforts to detect carbohydrates in the aqueous phase were frustrated by the presence of sodium nitrate in the medium. The problems encountered are discussed in detail in Section D-1-c of this report. Despite the availability of relatively large samples of water bottoms from the fermentor studies, we were unable to eliminate the sodium nitrate interference. No quantitative measurements of carbohydrate content were therefore made. It would appear, however, from earlier tests that only trace quantities of carbohydrate are present in the water phase employing the tests qualitatively. Also, there was no apparent change in the viscosity of the water bottoms which might be expected if excessive accumulations of polymeric substances such as polysaccharides were present.

3) Non-Protein and Non-Carbohydrate Components

The cell free aqueous phase obtained from the fermentor runs was concentrated in vacuo and subjected to a series of distillation and extraction procedures outlined in Appendix J. Results of analyses of the various fractions are presented in qualitative form in Table XXXVI. They indicate, with the possible exception of runs 4 and 5, that no substantial quantities of volatile neutrals or acids accumulated. In runs 4 and 5, trace quantities of volatile neutrals were detected but further analysis indicated that the materials were hydrocarbon components of the fuel.

Following the distillations, the water bottoms were extracted with ether rather than carbon tetrachloride to facilitate handling. All water bottoms contained ether extractables, both acidic and neutral. An attempt was made to characterize these fractions by infrared absorption techniques.

Materials extractable under both acidic and basic conditions were obtained from run 1. The IR spectrum (Figure 18) indicates the presence of very complex and diverse organic structures, probably microbial pigments. No one component was present in a significant quantity to permit complete or even partial identification. Similar observations were made on spectra obtained on acidic and basic ether extracts of run 2 water bottoms. However, in these spectra additional bands were present which indicated the presence of aromatic hydrocarbons, probably residual naphthalene. There was also some evidence of aromatic acids. This is not surprising since the organism employed utilizes naphthalene and the pathway includes a number of oxidized aromatic intermediates.

Fermentor runs 3, 4 and 5 were set up with CITE fuel. IR spectra of the various ether extracts revealed the presence of trace quantities of phenolics in essentially every sample. This observation was anticipated since the fuel used contained approximately 70 ppm of these materials. In addition to the phenolics, the complexity of the spectra indicated that a variety of other compounds were present in the extracts but only a few structures were clearly

TABLE XXXVI

Summarization of Analyses for Non-Protein and Non-Carbohydrate Components of Water Bottoms (Fraction C) - Fermentor Studies -

Analytical Procedure	Fermentor Run Number 1	2	3	4	5
Volatile Neutrals Analysis of distillate Dichromate Test	-(1)	-	-	Trace	Trace
Volatile Acids Analysis of Steam Distillate Titrable Acidity	-	-	-	-	-
Ether Extractables, non-Volatile neutrals. (IR Analysis)	+(2)	+	+	+	+
Ether Extractables, non-Volatile acids (IR Analysis)	+	+	+	+	+

(1) - = Negative test, no volatile or extractable organic compounds detected.

(2) + = Positive test for volatile or extractable organic compounds.

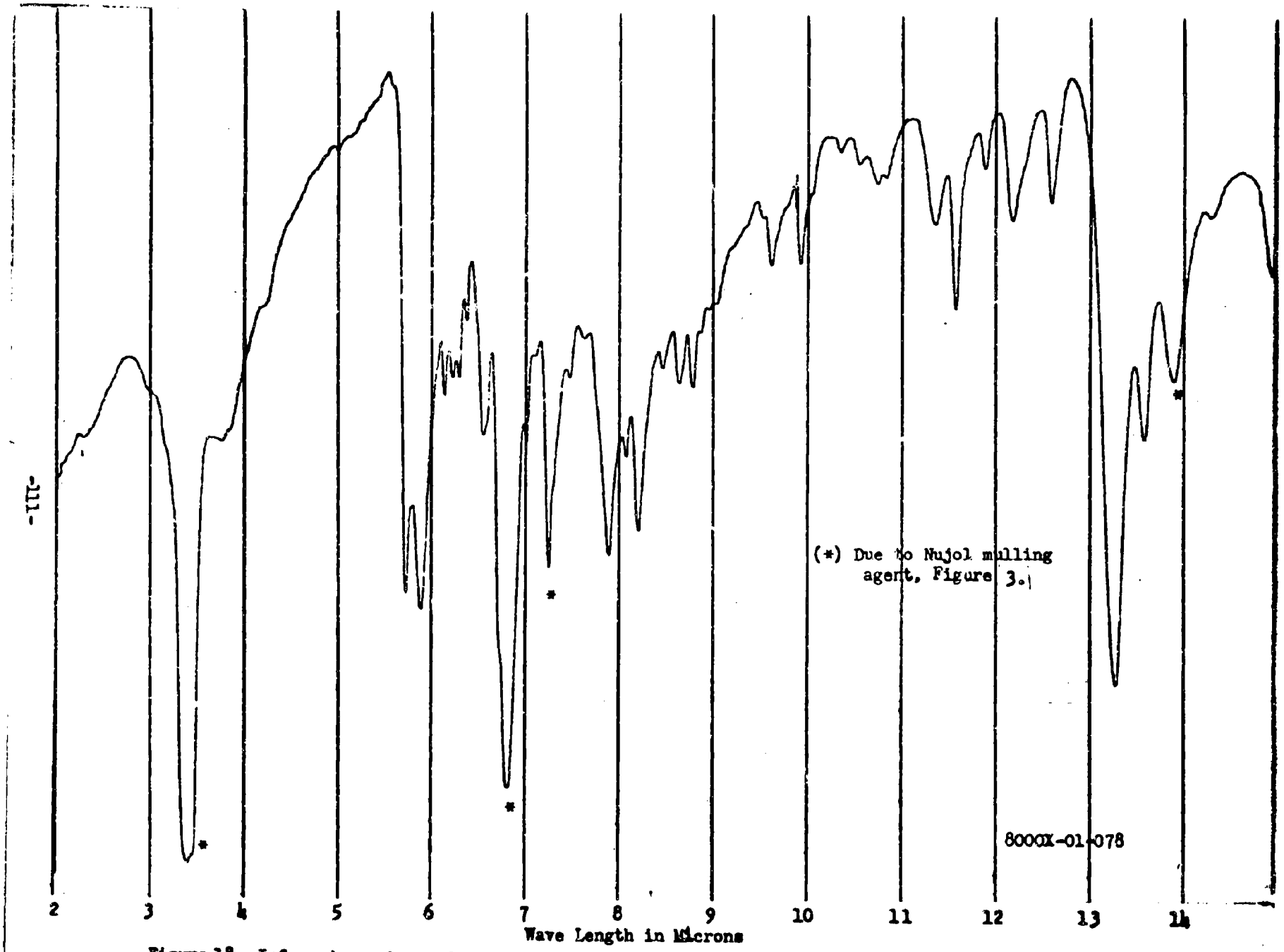

Figure 18. Infrared spectrum of ether extract of water phase from fermentor Run 1, after distillations.

discernible. Characterization of the components was again handicapped by the small quantities of samples. Aromatic and aliphatic acids and nitrogen containing compounds were indicated by spectra of ether extracts from runs 3 and 4. The multiplicity of compounds indicated by the spectra is not surprising since the substrate, fuel, contains a variety of hydrocarbon types. It is unlikely that any one fuel component is present in any significant quantity. The results also suggest, particularly in run 4, that some co-oxidation occurred. The culture employed in run 4 utilized only n-paraffins in earlier culture studies (Section C-4-b). The presence of aromatic acids in the water bottoms suggests either co-oxidation of methyl groups or oxidation of short chain (C_2-C_{10}) alkyl benzenes.

The neutral extract from run 5 was similar to the extracts obtained with runs 3 and 4. However, the acid extract yielded substantial quantities of organic acids. The IR spectrum presented in Figure 19 was obtained on a sample of this fraction and indicated the presence of benzoic and p-toluic acids. Compounds of this type might be anticipated since the test culture utilizes naphthalene and methyl naphthalenes. This supports the observations reported in Section D-2-b with extracts of evaporated water bottoms from an earlier run. In this earlier sample a series of oxidized aromatic compounds including m-toluic and benzoic acids were identified by IR and the Mass Spectrophotometer.

d. Observations on Fraction D

During separation of the various fractions from fermentor runs it was necessary to wash the sludge (bacterial cells) repeatedly with distilled water. In the static series reported in Section D-1, the water washings were combined with the water bottoms. Since there appeared to be substantial quantities of material in the water washings obtained with the fermentor series, they were examined separately as Fraction D. The samples were evaporated to dryness at 105°F. and analyzed in the infrared spectrophotometer.

Comparisons of IR spectra on fractions from the various fermentor runs indicated that they contained essentially the same types of material. A typical spectrum is presented in Figure 20. It is obvious that the water soluble materials are cellular components as they have essentially the same absorption bands as Fraction A (Figures 11 and 12). The components were probably released from the cells as a result of lysis. As pointed out in the first part of this section, measurable quantities of H_2O soluble materials were obtained from runs 1 and 2 (Table XXXII). In both cases the cells were washed repeatedly with distilled water.

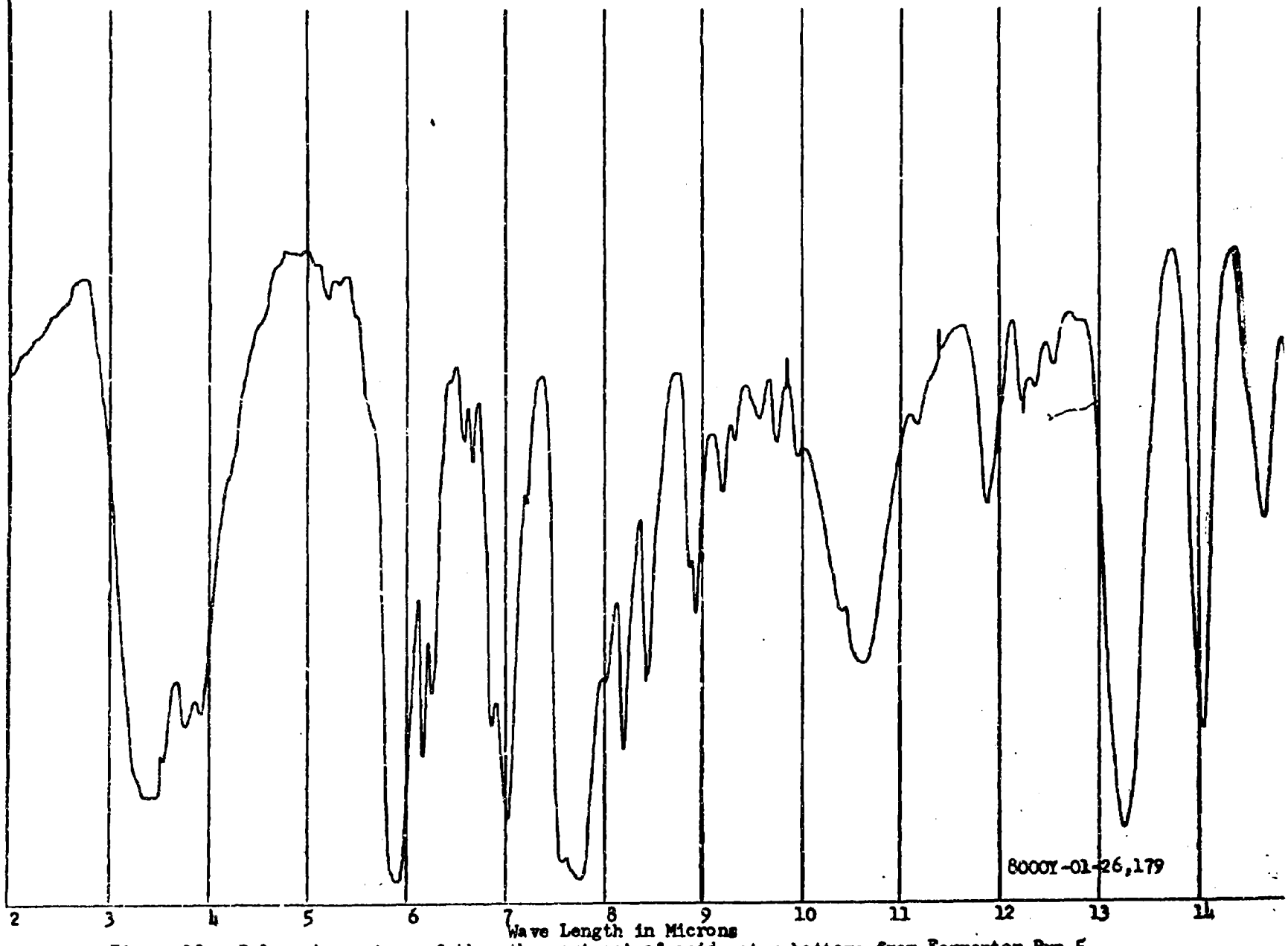

Figure 19. Infrared spectrum of the ether extract of acid water bottoms from Fermentor Run 5.

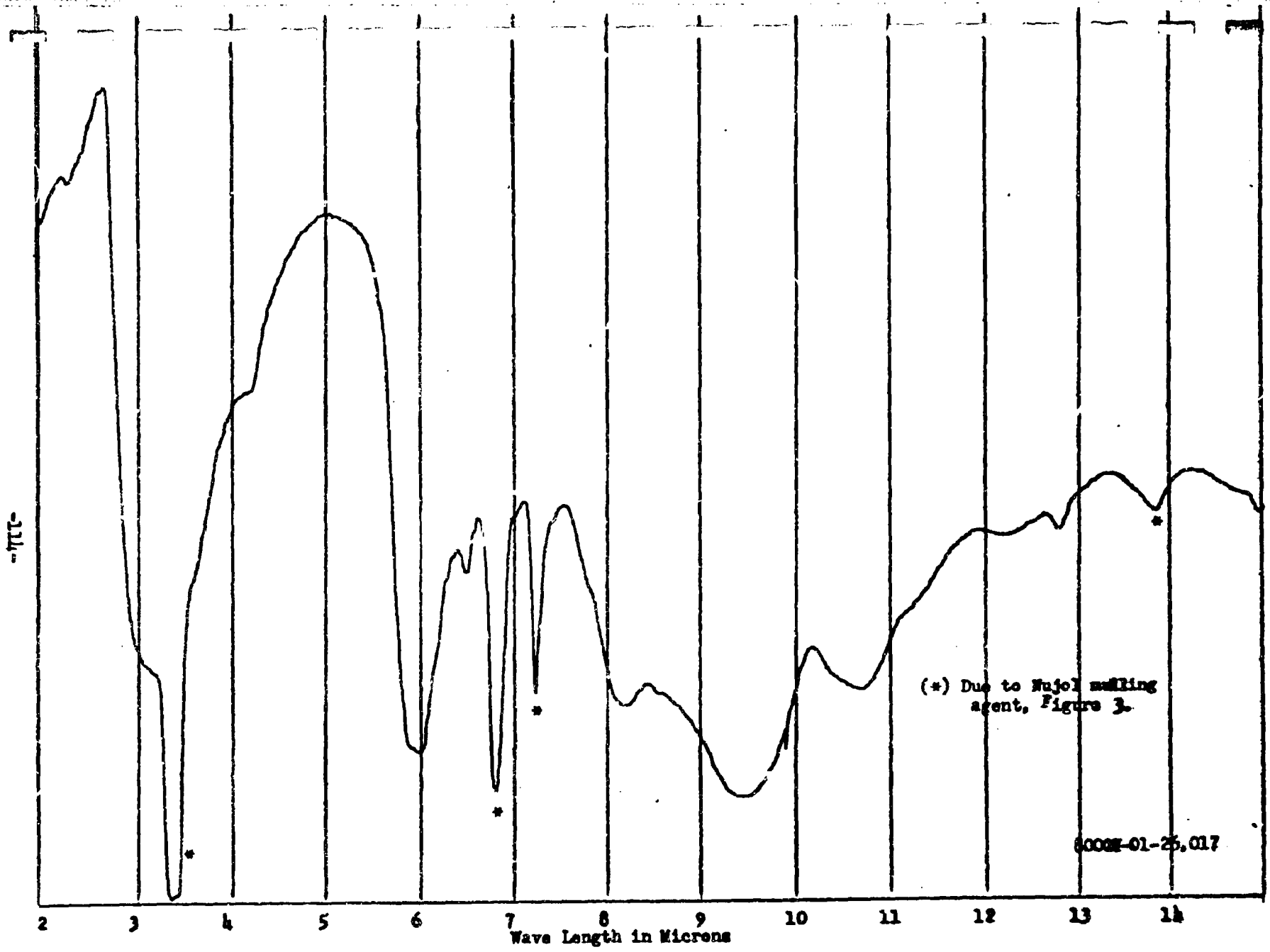

Figure 20. Infrared spectrum of water soluble extract (Fraction D) of microbial sludge, Fermenter Run 1.

e. Observations on Fraction E

Sludge components soluble in n-hexane were analyzed in the infrared spectrophotometer. Representative spectra are presented in Figures 21 and 22. The first spectrum (Figure 21) is representative of the hexane soluble obtained when pure hydrocarbons were employed as the substrate. Bands assigned to esters are present at 2.8, 5.72, and 8 to 9 microns. This material is probably cellular lipids extracted by the n-hexane.

The spectrum in Figure 22 is representative of fractions, including those from interface sludge, obtained from systems employing CITE fuel. In contrast to the first samples from runs 1 and 2, the hexane soluble materials obtained from runs 3, 4 and 5 were primarily free organic acids. There are some faint ester bands present, indicating at least trace quantities of these materials. Bands assigned to aliphatic acids are present at 5.8 and 10.75 microns. In addition, bands assigned to aromatic structures, probably phenolics from the fuel, are present at 6.25 and 7.75 to 7.8 microns. Widening of the band at 3 to 4 microns also suggests phenolics.

Differences noted in the chemical nature of n-hexane solubles from cells grown on pure hydrocarbons and from cells grown on CITE fuel are difficult to explain. With the pure hydrocarbons it is likely that the conditions favored the production of cells, CO_2 and H_2O, without the accumulation of intermediate oxidation products. The substrate was probably limiting in these runs. However, in the fuel runs there was an excess of substrate. Also there was a chance for co-oxidation which did not exist in the pure hydrocarbon systems. Thus it would appear that the major difference between the two systems was the accumulation of products. This observation is substantiated by the fact that only the water bottoms (Fraction C) from CITE fuel runs contained organic acids and other oxidized products.

The presence of organic acids in association with the cells is somewhat unusual. It would appear that the acids are present on the surface of the cells since they were readily extracted by the hexane. Undoubtedly, the acids would be involved along with the cells in stabilizing the emulsions which form in fermentor runs with fuel. There is also the possibility that the acids had poor solubility in the water washes and were centrifuged out with the cells. This point was not resolved.

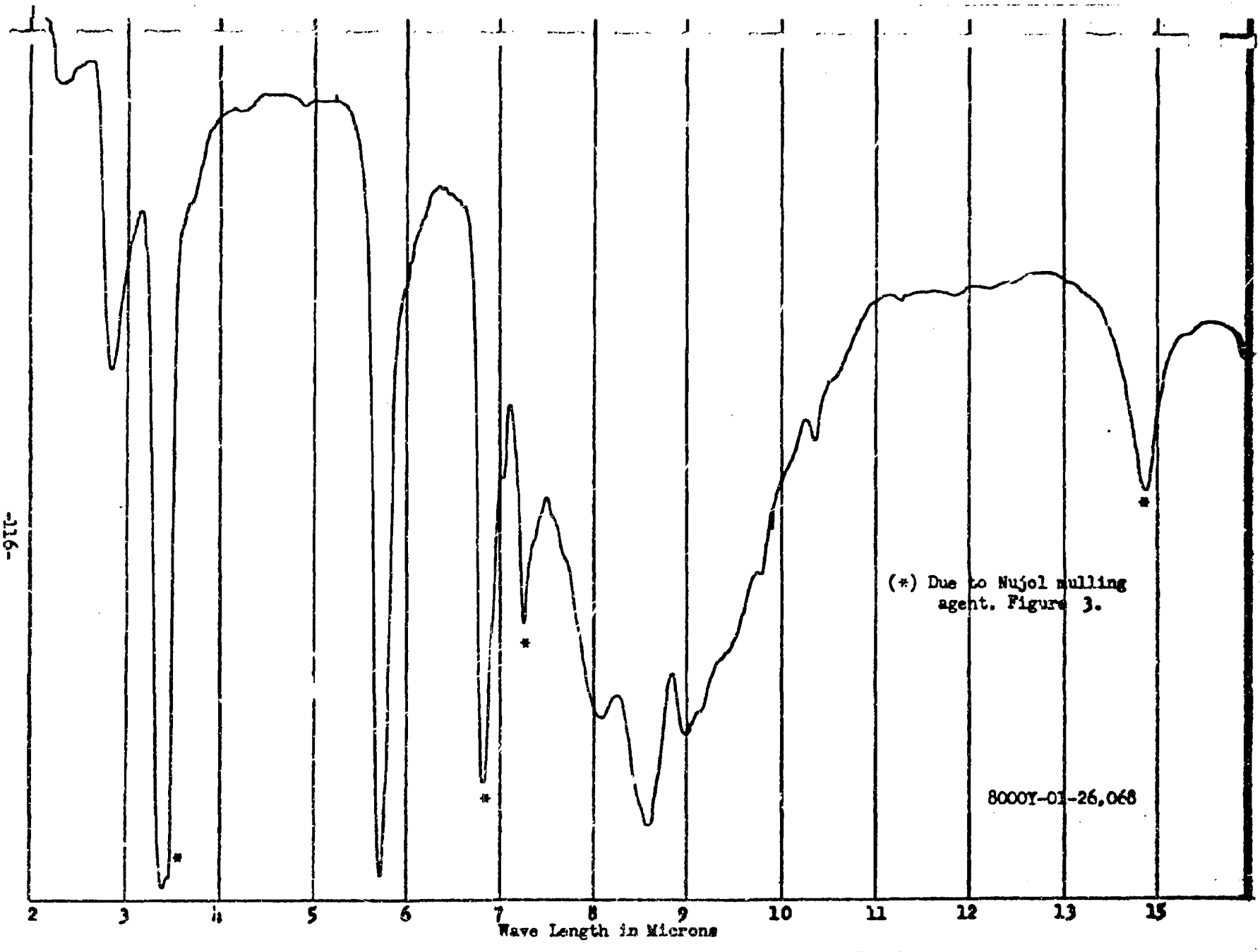

Figure 21. Infrared spectrum of n-hexane extract of sludge from Fermentor Run 2.

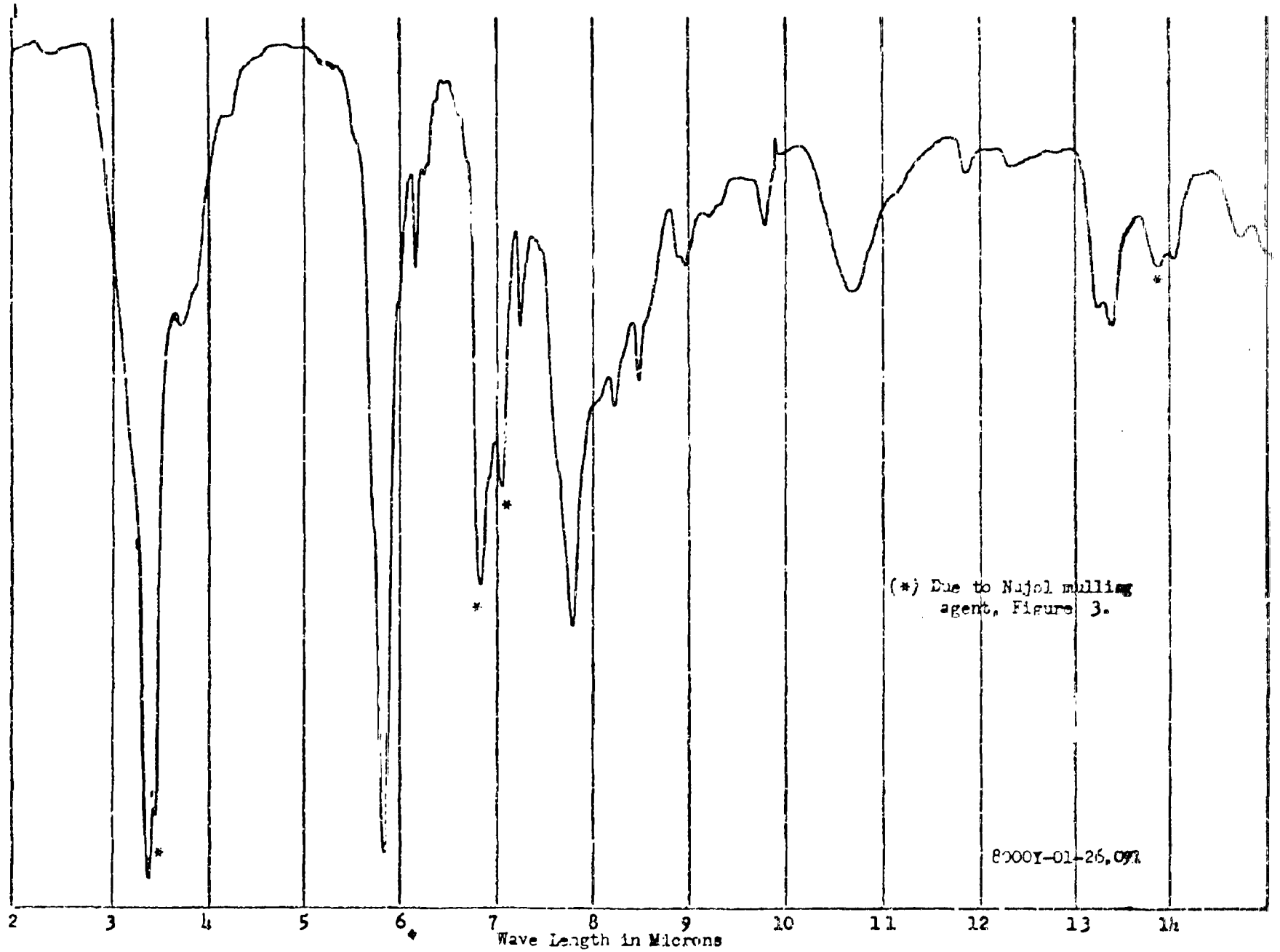

Figure 22. Infrared Spectrum of n-hexane soluble material (Fraction E) extracted from sludge, Fermentor Run 5.

3. n-Tetradecane Series

A static test series was set up in gallon bottles with two fungal cultures, Inocula IV and VII, employing n-tetradecane as the substrate. This series was set up to produce a large quantity of fungal mycelium or sludge for analysis and comparison with bacterial sludge from the fermentor runs. Details of the test series are presented in Appendix N. Contents of the test units were collected and fractionated according to the procedures outlined in Appendix E.

Fractions obtained and their weight percentages are presented in Table XXXVII. Sludge yields from the two series were essentially the same. The most significant difference between the two cultures was the large percentage of tri-solvent and hexane soluble material extracted from Inoculum IV sludge. Only trace quantities of materials soluble only in n-hexane (Fraction E) were obtained.

Elemental analysis of the insoluble sludge, Fraction A, are presented in Tables XXXVIII and XXXIX. The following empirical formulae based on these data indicate that the sludges were not appreciably different from other laboratory samples and the field sample reported in Section E-1.

Inoculum IV - $C_{50}H_{88}O_{20}N_8P$

Inoculum VII - $C_{59}H_{89}O_{35}N_5P$

In general, there does not appear to be any significant differences in the elemental analysis of bacterial cells (see Section D-2-a) and fungal mycelium.

Infrared spectra obtained on the insoluble residue (Fraction A) from Inocula IV and VII units were essentially the same. The spectrum of material from Inoculum IV is shown in Figure 23. In general, this spectrum is similar to many obtained on samples of sludge from the laboratory and field during the course of this investigation.

Analysis of the tri-solvent soluble materials (Fraction B) indicated that the main components of the fraction obtained from Inoculum VII were inorganic. Fraction B from the Inoculum IV series, however, contained cellular components (Figure 24). Bands associated w cellular proteins and carbohydrates are present at 3, 6, and 9-10 microns. Hydrocarbon bands are also present probably due to residual n-tetradecane.

Qualitative analytical data obtained on the water bottoms (Fraction C) are presented in Table XL . No volatile components were obtained with the distillation procedures. However, materials were obtained from the water bottoms, after distillation, by ether extraction under both acidic and basic conditions. The fractions were analyzed by IR absorption techniques. Examination of the

TABLE XXXVII

Results of Gravimetric Determinations on Microbial Sludge
n-Tetradecane Series

Test Culture	Sludge Yield gm/L Water (Dry Wt.)	Weight (Dry) Percent of Fraction			
		Tri-Solvent Soluble Material (Fraction B)	Hexane Soluble Material (Fraction E)	Tri-Solvent & Hexane Soluble Material (1)	Residue[2] (Fraction A)
Inoculum IV	1.93	2.5	0.3	8.5	88.7
Inoculum VII	1.82	4.6	0.2	-	95.2

(1) This fraction represents material which is soluble in both tri-solvent and n-hexane. Since some material was present which was soluble only in tri-solvent and in n-hexane, this additional fraction was handled separately.

(2) Residue (Fraction A) - Microbial sludge, insoluble in fuel, water, n-hexane, and benzene-acetone-methyl alcohol (Tri-Solvent).

TABLE XXXVIII

Elemental Analysis of Microbial Sludge (Fraction A)
n-Tetradecane Series

Element	Percent	
	Inoculum IV	Inoculum VII
Carbon	43.63	43.19
Hydrogen	6 52	5.42
Oxygen	35.00	34.00
Nitrogen	7.89	4.06
Phosphorus	2.27	1.89
Sulfur	0.60	0.54
Sodium	0.25	1.16
Potassium	1.40	1.01
Ash	12.20	10.95
% Recovery	109.76	102.22

TABLE XXXIX

Emission Spectrograph[1] Metal Analysis
of Microbial Sludge Ash
n-Tetradecane Series

Relative Concentration	Inoculum IV	Inoculum VII
Major	Na Ca P	Ca P
Minor	Si Fe	Na
Trace	Cu Al Mg Pb	Si Al Fe Cu

(1) 1.5 Meter Grating Emission Quantograph, Applied Research Laboratories Instrument.

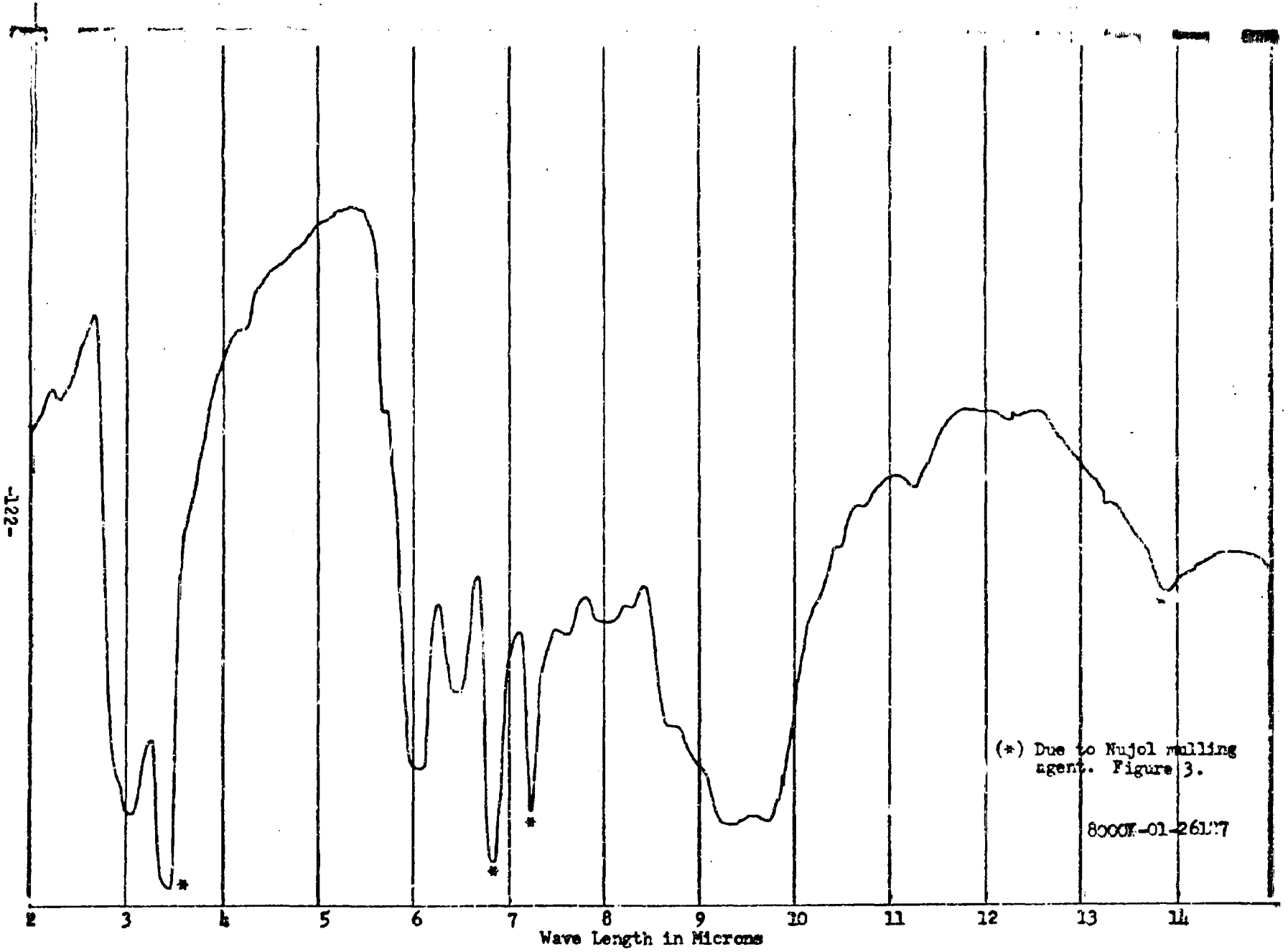

Figure 23. Infrared spectrum of microbial sludge (Fraction A) produced by Inoculum IV grown under static conditions.

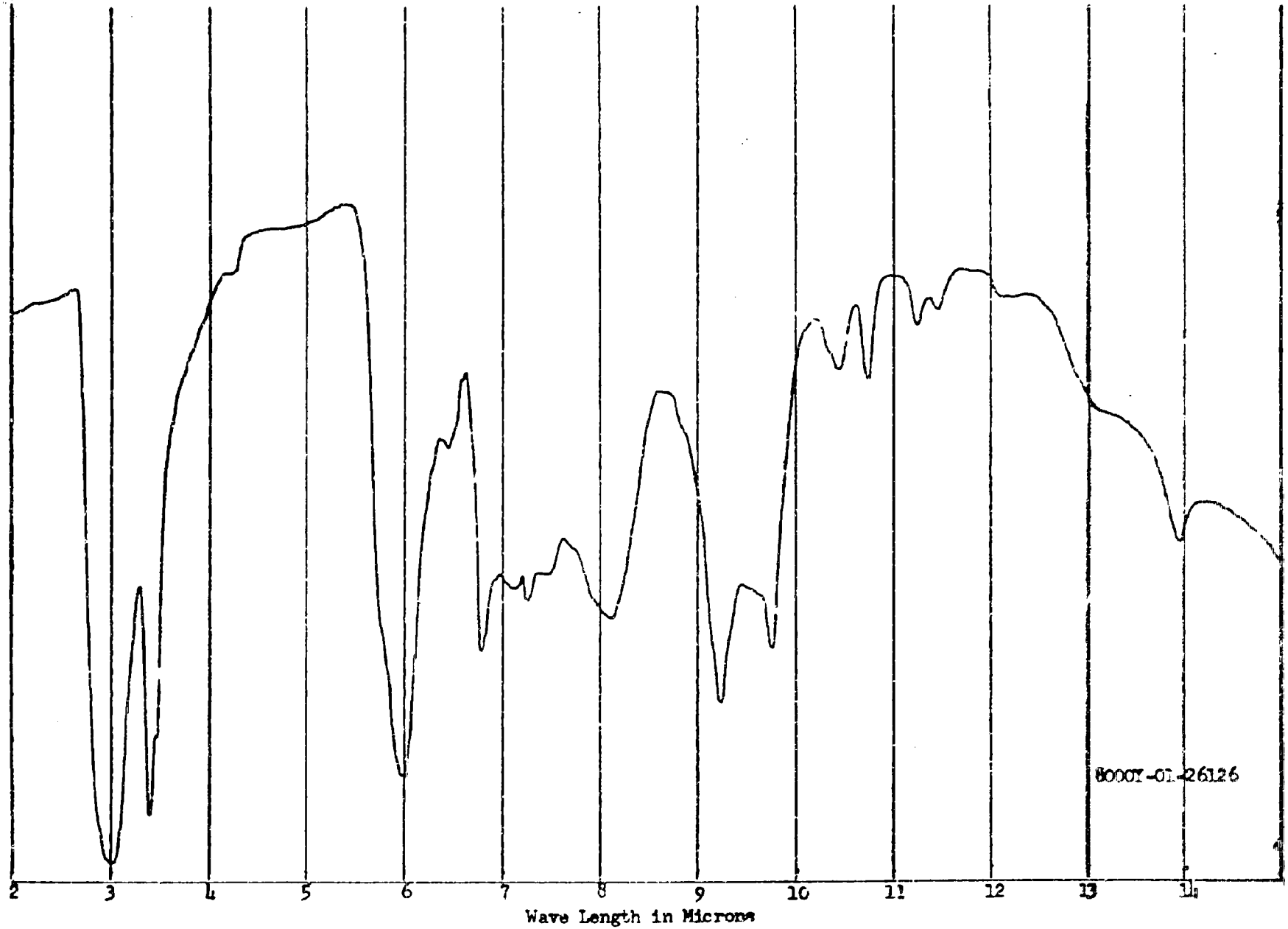

Figure 24. Infrared spectrum of the tri-solvent soluble material (Fraction B) produced by Inoculum IV grown under static conditions.

TABLE XL

Summarization of Analyses for Non-Protein and Non-Carbohydrate Components of Water Bottoms (Fraction C) n-Tetradecane Series

Analytical Procedure	Inoculum IV	Inoculum VII
Volatile Neutrals Analysis of distillate Dichromate Test	-(1)	-
Volatile Acids Analysis of Steam distillate Titrable Acidity	-	-
Ether Extractables, Non-Volatile Neutrals (IR Analysis)	+(2)	+
Ether Extractables, Non-Volatile Acids (IR Analysis)	+	+

(1) - = Negative test, no volatile or extractable organic compounds detected.

(2) + = Positive test for volatile or extractable organic compounds.

spectra revealed absorption bands assigned to a variety of structures including major bands assigned to amides, acid carbonyl and hydrocarbons. Spectra obtained on the ether extractables from the Inoculum IV series are shown in Figures 25 and 26. Spectra on the Inocula VII samples were similar and therefore were not included. It would appear from the variety of structures present that the ether solubles are pigments or cellular components. While absorption bands assigned to organic acids are present, it does not appear that they are aliphatic as might be expected. The strong band at 10.75 microns which is usually associated with aliphatic acids is not present. Aliphatic acids would be expected if degradation of the tetradecane was incomplete. However, the presence of bands assigned to nitrogen containing structures tends to support the conclusion that these materials are pigments or cellular components.

Hexane soluble sludge components (Fraction E) from Inocula IV and VII appear to be cellular lipids. The material from Inoculum IV that was soluble in tri-solvent and n-hexane was identical to Fraction E. Figure 27 is the IR spectrum obtained on this fraction. Ester bands are present at 5.72 and 8 to 9 microns. Hydrocarbon bands, probably representing residual n-tetradecane, are also present.

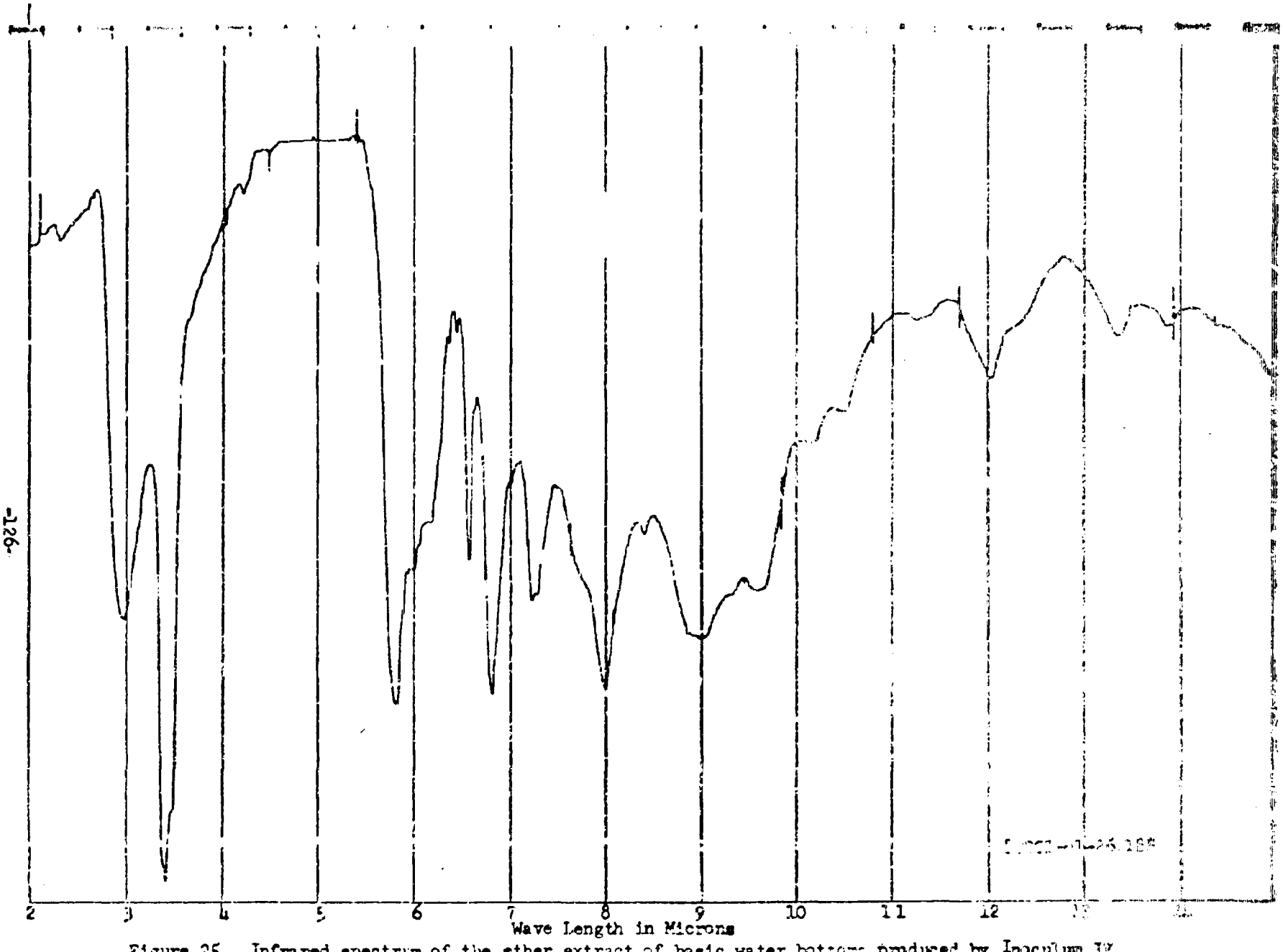

Figure 25 Infrared spectrum of the ether extract of basic water bottom produced by Inoculum IV grown under static conditions.

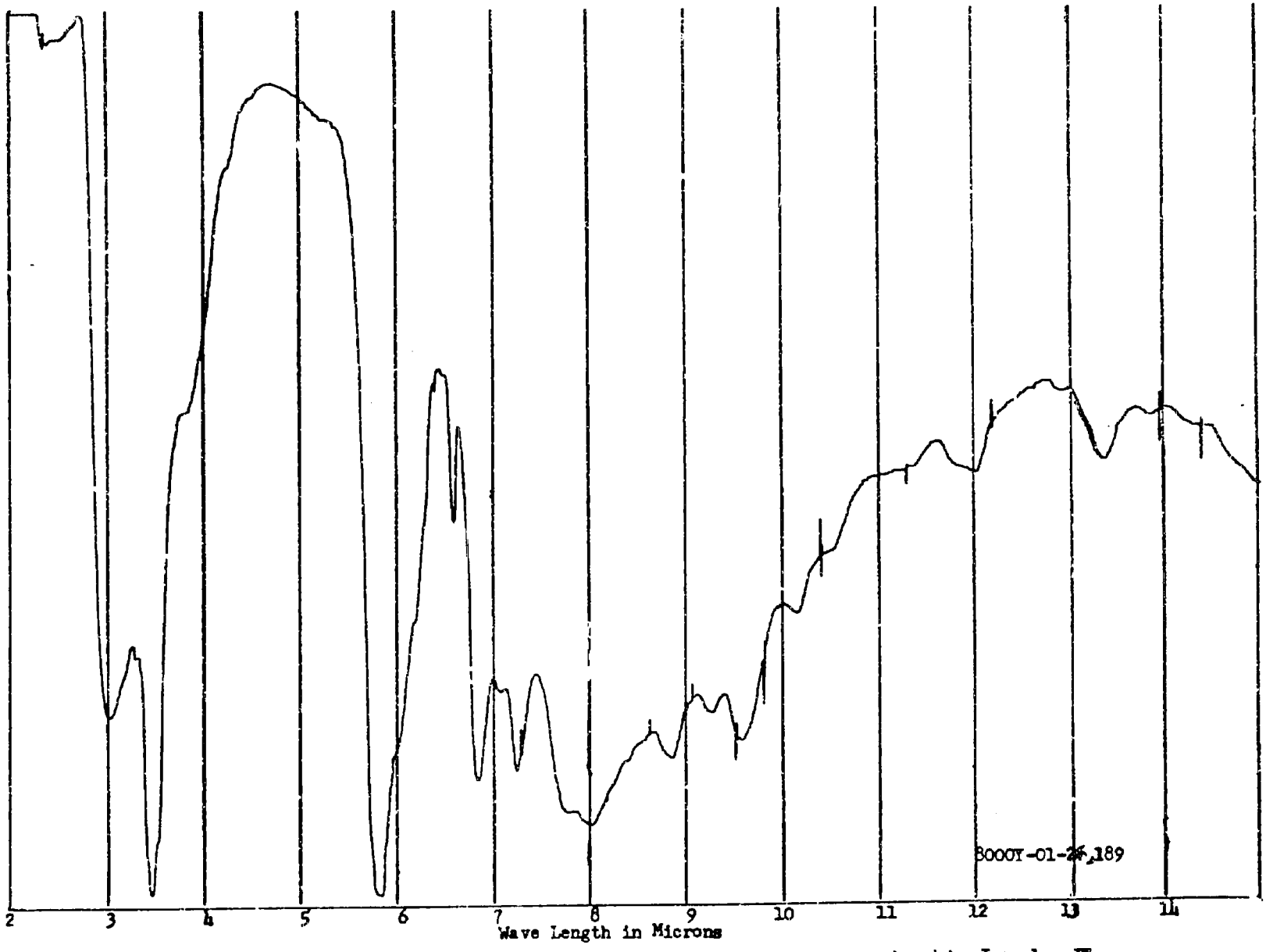

Figure 26. Infrared spectrum of the ether extract of acid water bottoms produced by Inoculum IV grown under static conditions.

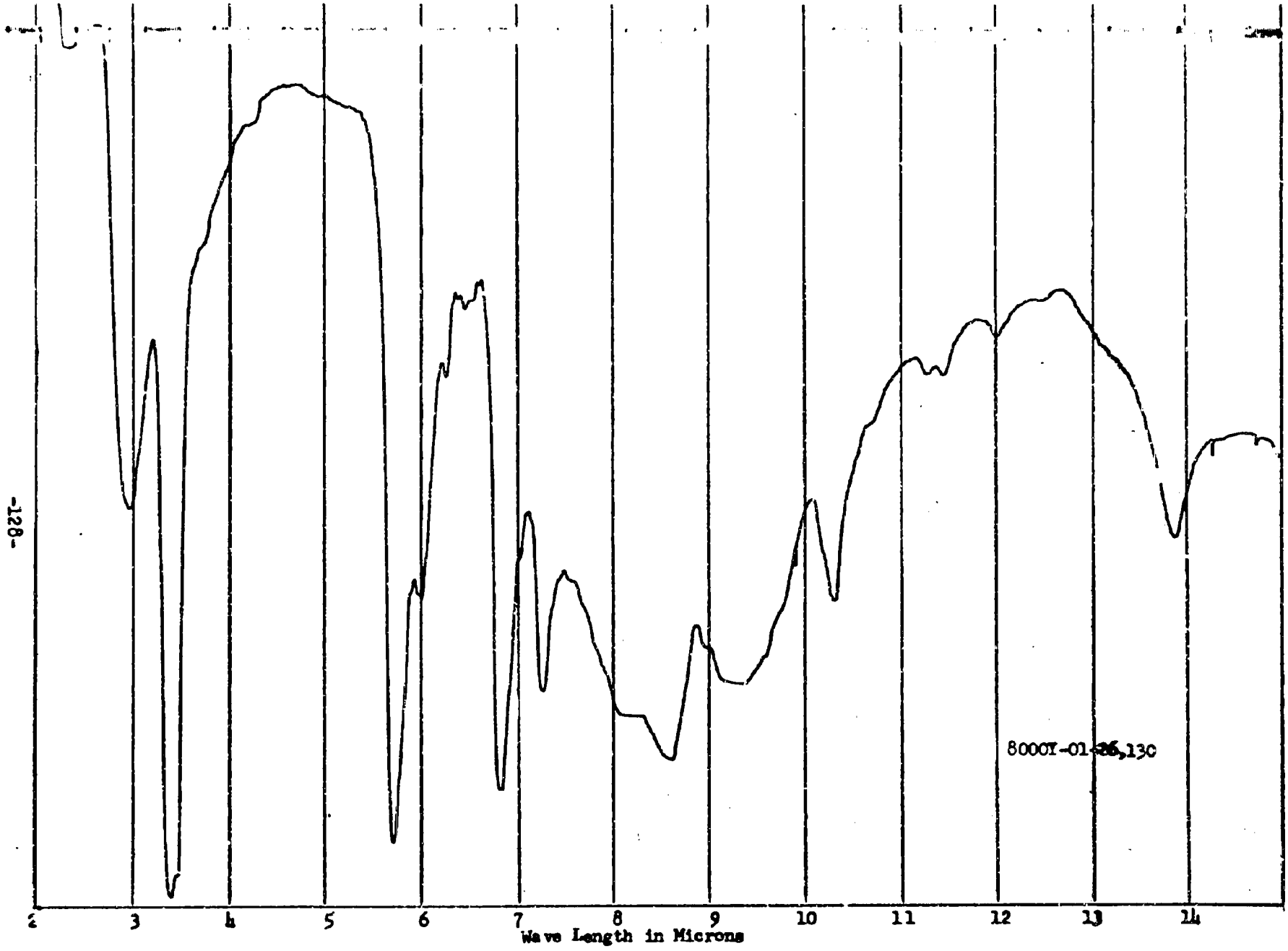

Figure 27. Infrared spectrum of n-hexane soluble material (Fraction 1) produced by Inoculum IV grown under static conditions.

E. Analysis of Sludge Samples from the Field

To complete our investigation of microbial sludge it appeared desirable to have samples of material from the field to compare with laboratory preparations. Since such information is not generally available in the literature, analysis of sludge samples from the field were undertaken. Two samples were selected for detailed study. One sample (111X-01-32,115) was obtained from a small diesel fuel storage tank that had been out of service for several years. The other sample (8000Y-01-26,160) was a diesel fuel filter which appeared to be partially plugged with normal fuel oxidation products. Each sample was fractionated in the same manner as sludge from laboratory test systems.

A sample of water bottoms from a CITE fuel storage tank was provided by the Natick Laboratories (Army Shipping Document, Voucher No. 4212, 6 August 1964) for analysis. Preliminary examination indicated that the sample was atypical, being highly contaminated with rust, dirt and other unidentified materials of non-biological origin. Therefore, detailed analyses were not undertaken.

1. Analyses of Sample No. 111X-01-32,115

Microscopic examination of this sample revealed the presence of typical fungus mycelium and bacterial cells. Culture studies indicated that only a few of the microorganisms present were still viable. Detailed analyses of the sample were made and the results are presented and discussed below.

Weight percentages of the various fractions obtained are presented in Table XLI . The major liquid portion of the sample was fuel. Only a trace of free water was present. This was due to the fact that the sludge was collected on a filter during tank cleaning operations. A substantial quantity of water soluble materials (Fraction D) was obtained from the sludge while only a small amount of tri-solvent soluble materials (Fraction B) was extracted. Since this is a field sample one would normally expect to find more fuel oxidation products.

Results of elemental analysis of the insoluble residue, Fraction A, are presented in Tables XLII and XLIII. An empirical formula of $C_{51}H_{84}O_{20}N_5P$ was obtained. A comparison of the element content of the various sludge samples will be presented later in this section. It should be noted that this sludge sample contained a substantial quantity of iron oxide. This is typical of samples from the field and is the reason iron was included in earlier storage tests.

Infrared analysis of Fraction A (Figure 28) indicated that it was essentially the same as materials obtained from laboratory studies, particularly in the static series with Inoculum I. Some similarity was also noted in the IR spectrum of Fraction B (Figure 29) from this field sample and the spectrum of the same fraction from the static series with Inoculum I. These observations confirm that the sludge produced in the laboratory systems can be expected to form in the field. We have also obtained spectra similar to the one in Figure 28 on other samples from the field which were subsequently found to contain microorganisms.

TABLE XLI

Gravimetric Determinations on Diesel Fuel Sludge
Sample No. 111X-01-32,115

Fraction	Weight in Grams
Total Sample	162.4
Oil Fraction (including hexane soluble material)	126.8
A - Insoluble Residue	15.6
B - Triple Solvent Soluble	0.3
D - Water Soluble Material	10.8

% Recovery - 94.5

TABLE XLII

Elemental Analysis of Fraction A Obtained from Field Samples

Element	Percent Present Sample Number 111X-01-32,115	 8000Y-01-26,160
Carbon	37.06	63.80
Hydrogen	6.20	7.30
Oxygen	27.40	13.70
Nitrogen	5.70	2.60
Phosphorus	2.25	0.04
Sulfur	0.30	1.90
Sodium	0.13	0.52
Potassium	0.20	0.06
Ash	15.47 (5.6% Iron)	5.90
		95.82

TABLE XLIII

Emission Spectrograph* Metal Analysis of Fraction A Ash
- Field Samples -

Relative Concentration	Sample Number 111X-01-32,115		Sample Number 8000Y-01-26,160
Major	Fe		Zn Fe
Minor	B Zn K Cr Cu	Si Mg	
Trace	Pb Mn Al Sn Ni Ca		Si Mn Cu Mg Ni Al

* 1.5 Meter Grating Emission Quantograph, Applied Research Laboratories Instrument.

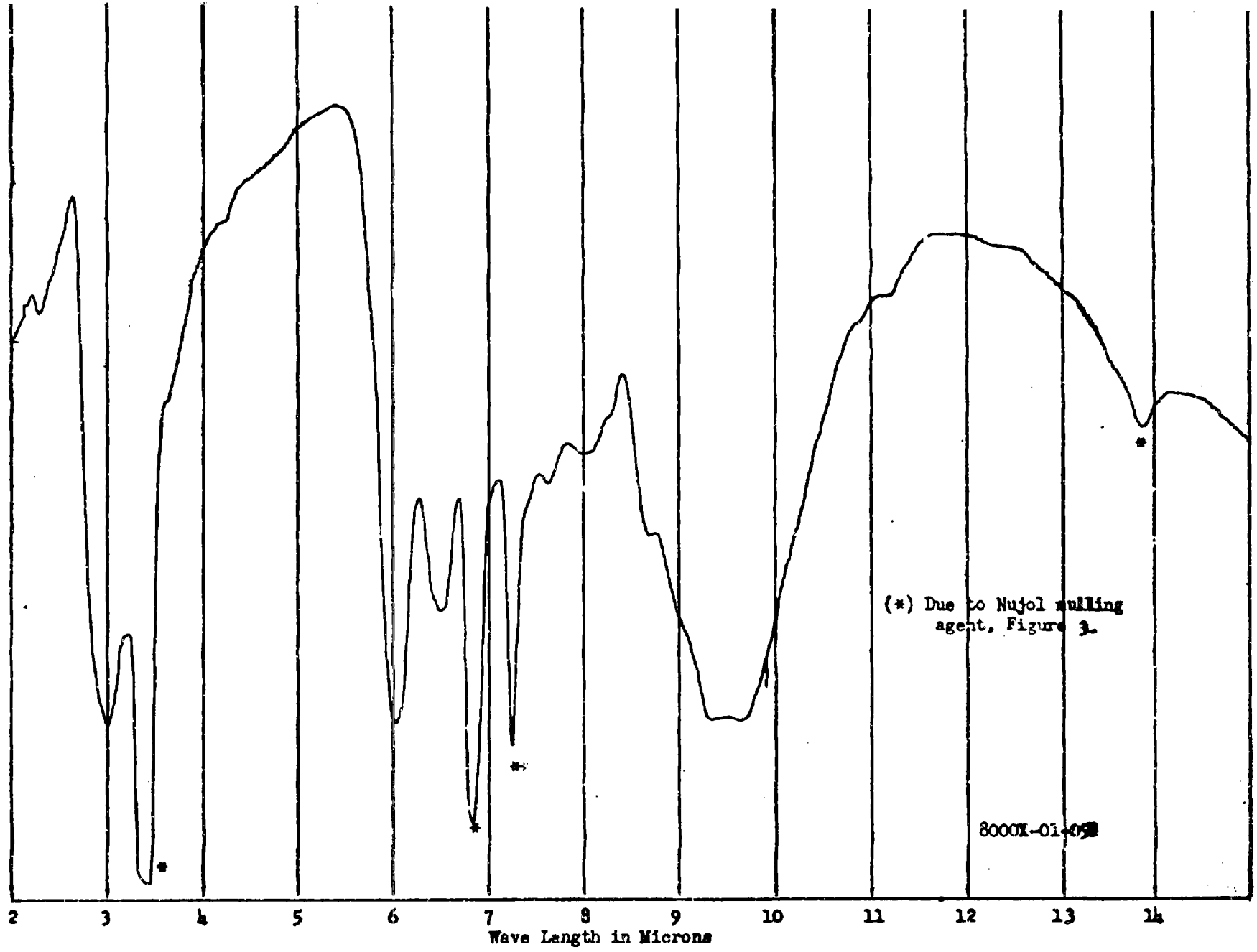

Figure 28. Infrared spectrum of Fraction A obtained from a diesel fuel sludge sample.

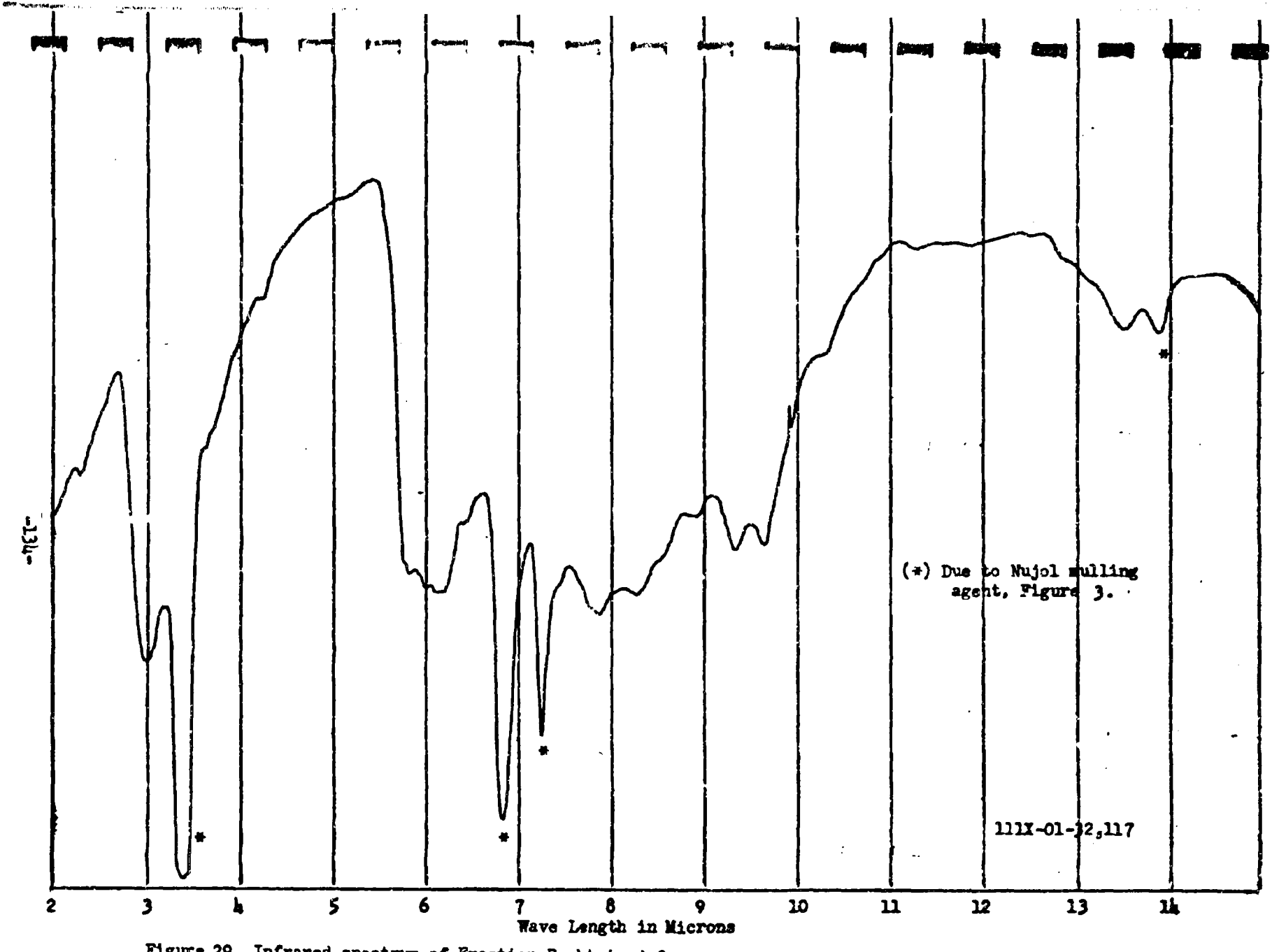

Figure 29. Infrared spectrum of Fraction B obtained from diesel fuel sludge sample.

2. Analyses of Sample No. 8000Y-01-26,160

Sludge produced in laboratory systems and sludge from the field (Sample No. 111X-01-32,115) have been analyzed and the various components characterized. Unfortunately, in the laboratory systems normal chemical oxidation products (fuel sludge) did not form. This was apparently due to the excellent stability of the fuel used. Since one objective of this program was to compare microbial sludge with fuel sludge, a filter which was partially plugged with diesel fuel oxidation products was obtained and analyzed.

The filter element was cut in small pieces, extracted with n-hexane to remove fuel, dried, and extracted with tri-solvent for three days in a Soxhlet Extractor. The tri-solvent was evaporated off and the residue obtained was washed with n-hexane and water. Only the material insoluble in hexane and water, and soluble in the tri-solvent, was analyzed. However, there appeared to be a trace of water insoluble inorganic material present in the tri-solvent soluble fraction despite efforts to eliminate it.

Infrared analysis of the tri-solvent soluble fraction, fuel sludge, indicated that the material is significantly different from microbial sludge. The spectrograph obtained is presented in Figure 30. In general, the spectrum resembles the one obtained on the CITE fuel sludge (Figure 2). Careful examination reveals that the amide band (5.9 microns) which is characteristic of microbial matter is missing. Also, a C=N band (6.25 microns), a hydroxyl band (3.0 microns) and an acid carbonyl band (5.8 microns) are present in the fuel sludge spectrum but are not normally found in spectra of microbial sludge. The sharp band between 9 to 10 microns which is characteristic of microbial sludge is present in the fuel sludge spectrum and is apparently due to inorganic sulfates and phosphates. Bands at 6.3 microns and in the 13.5 micron range indicate that the fuel sludge contains some aromatic components.

In general, the spectrum obtained on the sludge is typical of the normal oxidation products usually found in fuel systems. However, it should be pointed out that chemical oxidation products vary depending on the crude oil, refining and blending procedures, and the additives used.

Elemental analyses of the sludge are presented in Tables XLII and XLIII. These analyses indicate that the fuel sludge is considerably different chemically than the microbial sludge. The most significant differences in the two materials are their relative phosphorus, nitrogen, and oxygen contents. There is a 100-fold difference in the carbon to phosphorus ratios in the two materials:

Fuel Sludge (8000Y-01-26,160)	1600:1 C:P
Microbial Sludge (111X-01-32,115)	16:1 C:P

An empirical formula of $C_{28}H_{38}O_5N$ was obtained on the fuel sludge compared to $C_8H_{15}O_4N$ on the microbial sludge sample from the field (111X-01-32,115). The oxygen and nitrogen content of the fuel sludge is lower than that of the microbial material. These same differences are apparent when the C, H, O, N and P content of fuel sludge is compared with that of microbial sludge from laboratory systems.

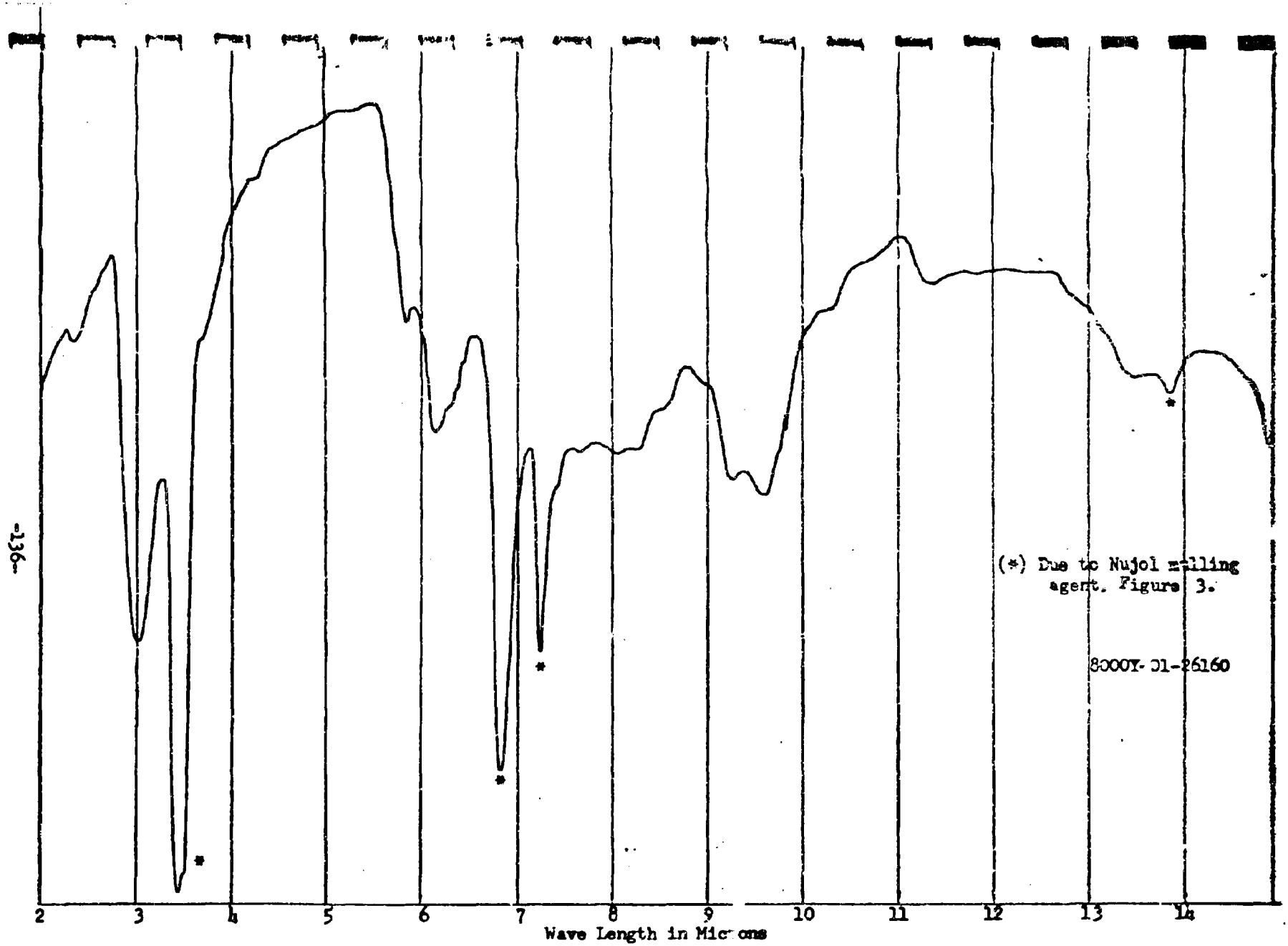

Figure 30. Infrared spectrum of normal fuel oxidation products.

F. Metabolic Intermediates in Hydrocarbon Oxidations

To elucidate the metabolic pathways involved in the microbial utilization of hydrocarbon fuels under aerobic conditions, pure cultures and purified hydrocarbons were employed. Because of the complex nature of fuels, possible interreactions and synergistic effects, studies with mixed cultures and fuels were not undertaken. We are of the opinion that results from such a study would be difficult to interpret and would therefore be of little help. Also appreciated is the fact that data obtained with defined systems must be considered with some reservation.

The approach employed to establish intermediates in the oxidation of various hydrocarbons was based on Stanier's theory of "Simultaneous Adaptation".(*) Cells grown on a particular hydrocarbon were tested in the Warburg respirometer for their ability to oxidize a suspected intermediate. According to Stanier's theory, a compound can be considered an intermediate if it logically fits into the proposed pathway and is readily oxidized by the organisms. Any delay in oxidation of a compound is considered indicative of an adaptive system, and the compound is therefore excluded from the pathway. However, discretion must also be used here since solubility, permeability, and toxicity problems may be responsible for the delay. Solubility and toxicity problems may be overcome by the use of several different substrate concentrations. The problem of permeability has not been completely solved; however, the use of dried cells has been suggested as a solution.

1. Inoculum II on n-Octane

Preliminary studies with octane grown cells (Inoculum II) indicated that octanoic acid is an intermediate product. Results of a typical Warburg run are shown in Figure 31. The octanoic acid was actually utilized at a more rapid rate than the n-octane. This is probably due to several factors; namely, 1) octanoic acid is more water soluble than octane, 2) octanoic acid may be transported across the cell membrane more readily than the hydrocarbon, 3) the initial oxidation of octane is probably limiting, and 4) the concentration of n-octane used may be inhibitory.

To test the concept that the hydrocarbon was inhibitory, various concentrations of n-octane were checked in the Warburg. Figure 32 presents the results of this study and indicates that there is no apparent inhibition at the concentrations tested. It appears that n-octane oxidation proceeds at a slow rate due to some other factor or factors.

(*) Stanier, R. Y. (1947) Simultaneous Adaptation: A New Technique for the Study of Metabolic Pathways. J. Bacteriology 54, 339-348.

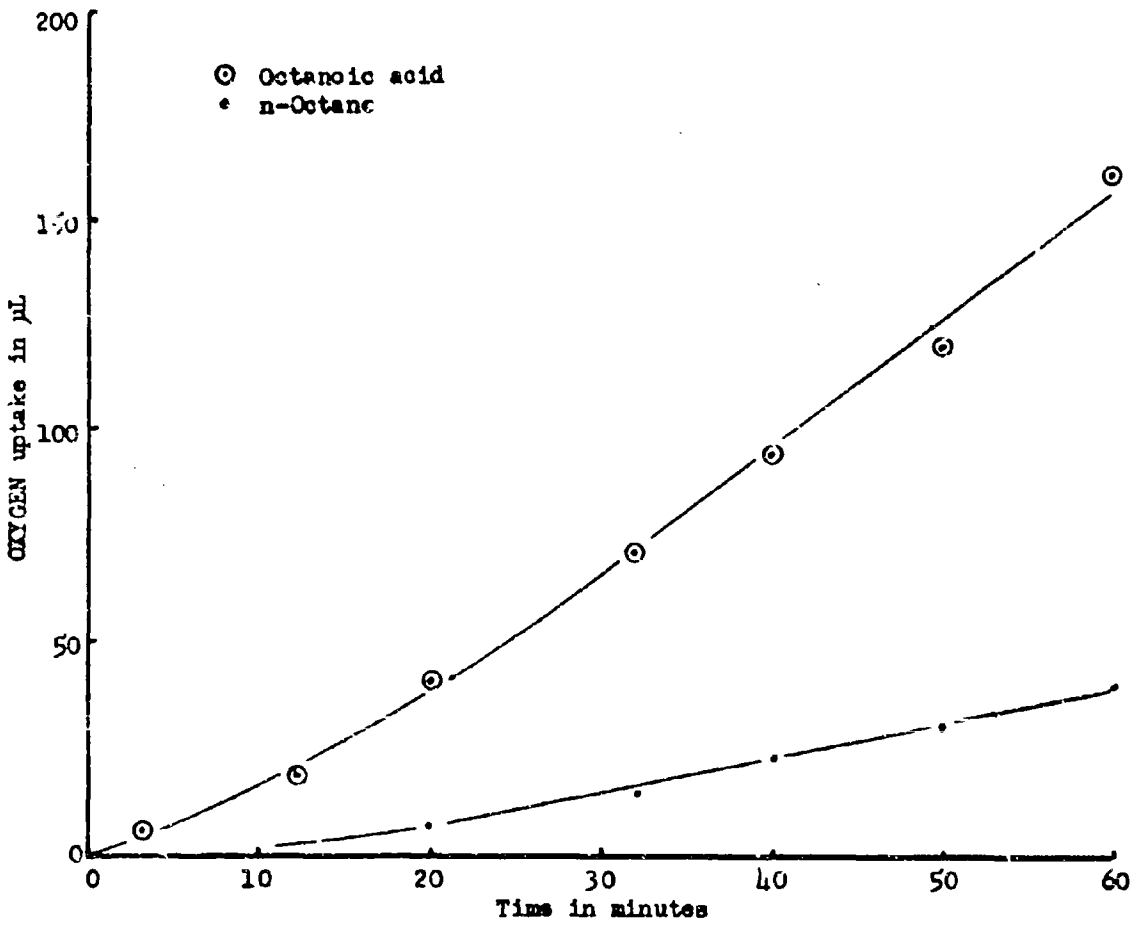

Figure 31. Oxidation of octanoic acid and n-octane by cells grown on n-octane.

Each flask contained 2.7 ml of cell suspension in dilute sea water solution and 0.2 ml of 16 per cent KOH in the center well. Three tenths of a ml of n-octane or 10 mg of the sodium salt of octanoic acid were added to the cups. Air atmosphere.

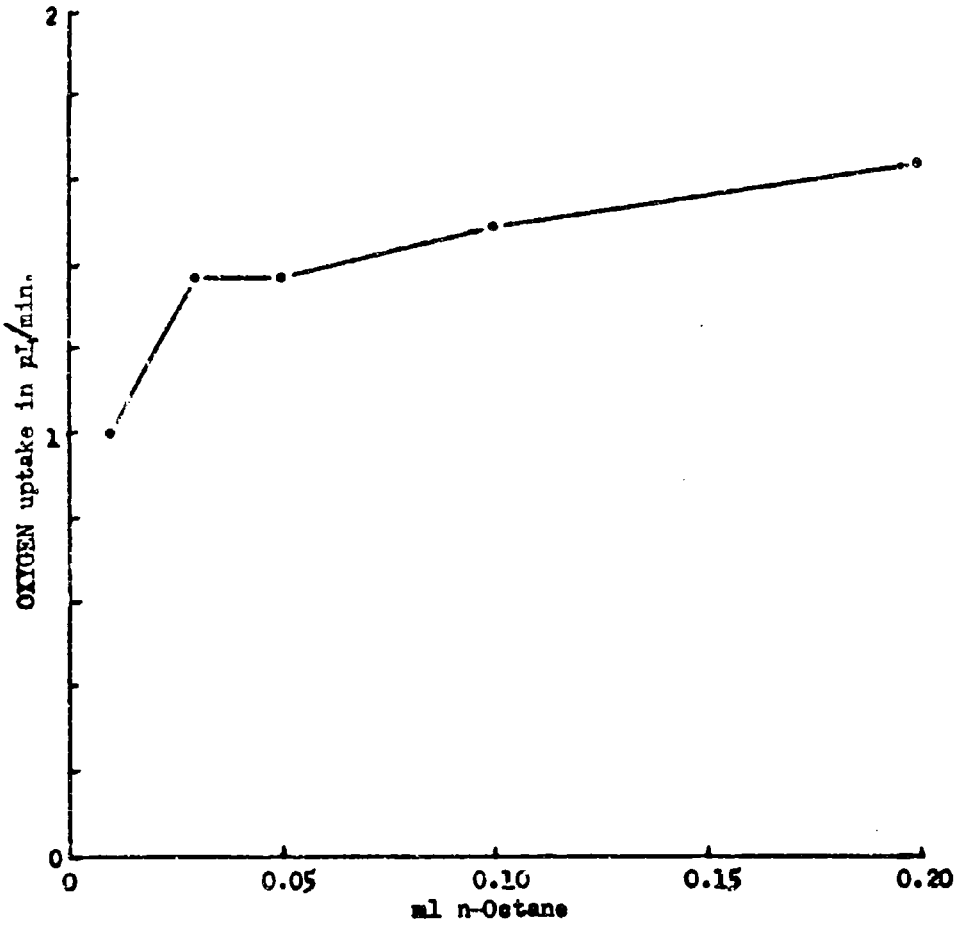

Figure 32. The effect of substrate concentration on the oxidation of n-octane.

Each flask contained 3 ml of a 48 hour cell suspension of Inoculum II (4.61 mg dry wt. per ml).in 0.02 M phosphate buffer, pH 7.0. The center well contained 0.2 ml of 20 % KOH. Octane was added from the side arm. Temperature was maintained at 30 °C and the atmosphere was air.

A second study was made on the effect of various concentrations of octanoic acid on oxidation rates. Heptanoic and hexanoic acids were also included in this study. From the results recorded in Table XLIV, it can be seen that hexanoic acid was not inhibitory at the highest concentration used, 150 μM, but that this concentration of both heptanoic and octanoic acids completely inhibited oxidation. Lower concentrations of these two acids were oxidized at reasonable rates.

2. Inoculum III on Naphthalene

One of the organisms (Inoculum III) isolated from the mixed tank bottom sample (Inoculum I) utilized naphthalene, 1,2-dimethylnaphthalene, and 2-methylnaphthalene (Section C-4). In this study an attempt was made to confirm the degradative pathway(1) of these compounds by observing the oxidation of possible intermediates in the Warburg respirometer. Unfortunately, only salicylic acid, of the commonly accepted metabolic intermediates, is readily available. Coumarin and 1,2-naphthoquinone used in this study are not considered in the direct pathway of naphthalene oxidation. Furthermore, the latter compound is reported to be inhibitory(2). 1-Naphthol and 2-naphthol have been implicated in the pathway but seem not to be generally accepted(3). The rate of oxidation of these compounds by cells grown on naphthalene is shown in Table XLV.

It should be noted that only two compounds, naphthalene and salicylic acid, are oxidized immediately; all the others were oxidized after approximately a 30-minute lag. Based on the "Simultaneous Adaptation" concept, only salicylic acid should be considered in the pathway. However, it is of interest that 1,2-naphthoquinone exhibits its known inhibition only after 30 minutes. This, therefore, suggests the possibility that solubility and permeability problems may cause the lag and that some of the other compounds may be intermediates.

Also, the cells used in this study had a rather high endogenous rate. Although they were washed 3 times in buffer, it might be hypothesized that it took 30 minutes for the naphthalene adsorbed on or contained in the cells to be utilized before the other compounds were oxidized. This might infer that the site of salicylic acid oxidation is different than that of the other chemicals.

While these data do not establish the exact metabolic pathway for naphthalene oxidation, they nevertheless indicate that salicylic acid is an intermediate.

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(1) Davies, J.I. and Evans, W.C., 1964. Oxidative Metabolism of Naphthalene by Soil Pseudomonads. Biochem. J. 91, 251-261.

(2) Murphy, J.F., 1953. The Bacterial Dissimilation of Naphthalene. Ph.D. Thesis, Pennsylvania State College.

(3) Klausmeier, R.E., 1958. Microbial Oxidation of Naphthalene. Ph.D. Thesis, Louisiana State University.

TABLE XLIV

The Effect of Substrate Concentration on the Rate of Oxidation of Hexanoic, Heptanoic and Octanoic Acids by n-Octane Grown Cells

	Oxidation Rate (µL O_2 Uptake/min.)		
µM of Acid*	Hexanoic	Heptanoic	Octanoic
10	1.44	1.40	1.36
50	1.64	1.34	1.64
100	1.71	0.96	1.22
150	1.74	0	0

Each flask contained 1 ml of a 48 hour cell suspension of Inoculum II grown on n-octane (4.61 mg dry wt/ml) in 0.02 M, pH 7.0 phosphate buffer. The center well contained 0.2 ml of 20% KOH. The acid salts were added from the side arm. The temperature was maintained at 30°C. Air Atmosphere.

* Sodium salts of the various acids were employed to prevent drastic pH changes and to minimize solubility problems.

TABLE XLV

Rates of Oxidation of Various Compounds
Associated with Naphthalene Dissimilation

	µL O_2 Consumed/Min.
Naphthalene (6 µM)	.65
1,2-Dimethylnaphthalene (6 µM)	.47 *
2-Methylnaphthalene (6 µM)	.50 *
1-Naphthol (3.5 µM)	.85 *
2-Naphthol (3.5 µM)	.81 *
Salicylic Acid (6 µM)	1.74
Coumarin (6 µM)	.29 *
1,2-Naphthoquinone (3 µM)	-.82 *

Each Warburg flask contained 3 ml of a 24-hour cell suspension of Inoculum III grown on naphthalene (5.89 mg dry wt./ml) in 0.02 M phosphate buffer, pH 7.0. The temperature was maintained at 30°C. The center well contained 0.2 ml of 20% KOH. Air atmosphere.

(*) Exhibited a 30 minute lag.

3. Metabolic Inhibitors

Evidence was presented by Blanchard and Goucher(*) which indicated that certain olefins are inhibitory to the oxidative processes of some fuel organisms. They did not mention the possibility that this inhibition might provide useful information on mechanisms of microbial oxidation of hydrocarbon fuel components and the degree of susceptibility of various fuels to microorganisms. These studies were apparently made with organisms which preferentially utilized paraffin components of jet fuel. For these reasons we initiated a study employing cultures which oxidize different hydrocarbon types (Inoculum II, n-paraffins, and Inoculum III, naphthalene and methyl naphthalenes). Pentene-1 and hexene-1 were employed as inhibitors.

Because the inhibitors are extremely volatile, it was necessary to dissolve them in CITE fuel. This reduced the amount of volatilization and facilitated measurement of pressure changes in the Warburg flasks. Also, since CITE fuel contains relatively large quantities of octane but essentially no naphthalene, it was necessary to add naphthalene to fuel used with Inoculum III.

Results of the Warburg studies are shown in Tables XLVI and XLVII. It should be noted that in both cases the olefins inhibited oxygen uptake. However, they completely inhibited oxidation by the octane grown cells, including the endogenous (Table XLVI), while only oxidation of the naphthalene by Inoculum III cells was inhibited (Table XLVII). Two possible explanations for this effect are 1) the differential permeability of the cells for the inhibitor, and 2) differences in the sites of oxidation of the hydrocarbons or intermediates.

It might also be noted that the rate of oxidation when both CITE fuel and naphthalene were present was less than that of naphthalene alone, but more than that of CITE fuel. The reason for the latter observation can be explained by the fact, as mentioned before, that the amount of naphthalene is limiting in CITE fuel. However, the reason for the observation that naphthalene alone caused an increased oxygen consumption relative to that of CITE fuel with added naphthalene (5.0 to 3.1 μL O_2 consumed per minute) may be due to an inhibitor present in the CITE fuel or to a difference in the availability of the naphthalene when it is dissolved in fuel. Subsequent studies did not resolve these points.

(*) Mechanism of Microbiological Contamination of Jet Fuel and Development of Techniques for Detection of Microbiological Contamination. Quarterly Progress Reports Nos. 4 and 5 prepared under Contract No. AF 33(657)-9186 for the Air Force Aero Propulsion Laboratory Research and Technology Division, Wright-Patterson Air Force Base, Ohio, by Melpar, Inc., Falls Church, Va. Authors G.C. Blanchard and C.R. Goucher.

TABLE XLVI

The Effect of Pentene-1 and Hexene-1 on the Rate of Oxidation of CITE Fuel by n-Octane Grown Cells

	$\mu L\ O_2$ Consumed/Min.
Endogenous	2.6 *
CITE Fuel (1 ml)	2.5 *
CITE Fuel (1 ml) + 0.5 ml Pentene-1	0
CITE Fuel (1 ml) + 0.5 ml Hexene-1	0

Each Warburg flask contained 1.5 ml of a 48-hour cell suspension of Inoculum II grown on n-octane (3.02 mg dry wt./ml) in 0.02 M phosphate buffer, pH 7.0. The center well contained 0.2 ml of 20% KOH. Temperature was maintained at 30°C. The results were corrected for pentene-1 and hexene-1 volatility but not for endogenous. Air atmosphere.

(*) These rate figures were obtained from the linear portion of the curves. In the case of the endogenous, the rate dropped substantially after the first 30 minutes, while the CITE fuel units continued at essentially the same rate until the run was discontinued.

TABLE XLVII

The Effect of Pentene-1 and Hexene-1 on the Rate of Oxidation of CITE Fuel and Naphthalene by Naphthalene Grown Cells

	µL O_2 Consumed/Min.
Endogenous	1.4
CITE Fuel (1 ml)	2.4
Naphthalene (100 mg)	5.0
Naphthalene (100 mg) + CITE Fuel (1 ml)	3.1
Naphthalene (100 mg) + CITE Fuel (1 ml) + 0.5 ml Pentene-1	1.5
Naphthalene (100 mg) + CITE Fuel (1 ml) + 0.5 ml Hexene-1	1.7

Each Warburg flask contained 1.5 ml of a 24-hour cell suspension of Inoculum III grown on naphthalene (5.89 mg dry wt./ml) in 0.02 M phosphate buffer, pH 7.0. The center well contained 0.2 ml of 20% KOH. Temperature was maintained at 30°C. The results were corrected for pentene-1 and hexene-1 volatility but not for endogenous. Air atmosphere.

IV. Recommendations for Future Studies

1. The effect of microorganisms on the quality of other more commonly used fuels should be investigated.

2. A more detailed investigation should be made on the mechanisms involved in the microbial utilization of hydrocarbon fuel components. Emphasis should be placed on the determination of factors, including structural characteristics, which influence the rate and degree of attack.

3. The relative importance of microorganisms in the corrosion of ferrous metals should be investigated. An effort should also be made to determine the association of corrosion products with fuel contamination and microbial sludge problems.

4. A more practical investigation should be made to evaluate various means of controlling microbial growth in fuel systems, including the use of biocides, filter devices, etc.

* * * * * * * * *

A P P E N D I C E S

APPENDIX A

RP-1 Storage Tests

Container: One pint, soft glass, French square sample bottles sterilized in a dry oven at 170°C for four hours. Bottles were capped with aluminum foil during the sterilization process. Plastic screw caps with aluminum liners were sterilized separately by autoclaving at 15 psi for 20 minutes (121°C).

Fuel: RP-1 fuel supplied by U. S. Army Natick Laboratories (Voucher No. 3050-63) was sterilized by filtering through a 0.45 micron Millipore filter. Approximately 400 ml. of the sterilized fuel was added aseptically to each test unit after the water bottoms had been added.

Water bottoms: Sea water, tap water, salt solution and inocula were added to the bottles to give a total of 20 ml. water bottoms. The units were set up in triplicate according to the following table.

Test Series	Sea Water[1]	Tap Water[2]	Active Inoculum[3]	Sterile Inoculum[4]	Salt Solution[5]	Steel Wool[6]	RP-1 Fuel
I	10 ml.	9 ml.	1 ml.			+	400 ml.
II	"	8 ml.	1 ml.		1 ml.	+	"
III	"	9 ml.	1 ml.				"
IV	"	8 ml.	1 ml.		1 ml.		"
V	"	9 ml.		1 ml.		+	"
VI	"	8 ml.		1 ml.	1 ml.	+	"
VII	"	9 ml.		1 ml.			"
VIII	"	8 ml.		1 ml.	1 ml.		"
IX	--No Water-----					+	"
X	--No Water-----						"

Footnotes

1. Natural sea water from the Atlantic Ocean was employed in these tests. The water was aged several months before use. It was sterilized in a pyrex container by autoclaving at 15 psi for 20 minutes(121°C). The sea water contained 0.6 ppm nitrogen, 0.03 ppm phosphorus, and 20,200 ppm chloride ion.

2. Tap water used in the tests was sterilized in a pyrex container by autoclaving at 15 psi for 20 minutes (121°C). This water is purified Lake Michigan water having a total hardness of approximately 125.

3. The active inoculum consisted of a composite of contaminated water bottom samples collected from a number of bulk fuel oil storage tanks just prior to cleaning. The composite was diluted with an equal volume of distilled water before use. An estimated 1,000,000 bacteria, yeast, and fungi were present in the diluted bottoms. Sulfate-reducers were also present.

4. A portion of the above mentioned tank bottom composite was sterilized in a pyrex container by autoclaving at 15 psi for 20 minutes (121°C.).

5. Salt solution used to supplement the nitrogen and phosphorus in the water bottoms contained 40 mg. NH_4NO_3, 20 mg. $NaH_2PO_4 \cdot H_2O$, and 40 mg. K_2HPO_4 per ml. of distilled water, or 14 mg. of nitrogen and 11.62 mg. of phosphorus. The final levels of nitrogen and phosphorus in the 20 ml. of water bottoms were 700 and 580 ppm respectively.

6. A small ball of steel wool was added to the respective units. The steel wool was rinsed with benzene and then with acetone to remove rust inhibitors and residual oil. It was sterilized in a dry air oven at 170°C for four hours.

For comparative purposes, three units were set up as in Series II with commercial jet fuel (Kerosine) substituted for the RP-1 fuel.

Storage Conditions: All units were stored at 30°C in the dark with caps loose to permit air exchange.

Sampling: All units were sampled at 2 week intervals over a period of 8 weeks. Since all tests were set up at the same time, only one inoculated series was sampled initially. The pH of the water bottoms was checked initially and at the end of the 8 week period.

Microbiological Analyses: One tenth ml. of the water bottoms from units in series I through IV was added to 9.9 ml. of sterile distilled water to give an initial dilution of 1:100. TGE and Sabouraud agar plates were streaked as described in Appendix C. The remaining series were checked for sterility by adding 0.1 ml. from each replication in a series to 20 ml. of nutrient broth.

APPENDIX B

Microbiological Media Employed

Sabouraud Maltose Agar (Difco)

Neopeptone	10 g.
Maltose	40 g.
Bacto-Agar	20 g.*
Distilled water	1000 ml.

Plate Count Agar (TGE)(Difco)

Bacto-Tryptone	5.0 g.
Bacto-Yeast Extract	2.5 g.
Bacto-Dextrose	1.0 g.
Bacto-Agar	20.0 g.*
Distilled water	1000 ml.

Nutrient Broth (Difco)(Modified)

Nutrient Broth Difco	8.0 g.
Dextrose	1.0 g.
Yeast Extract	2.5 g.
Distilled water	1000 ml.

Twenty ml. aliquots dispensed in 4 oz. bottles prior to sterilizing at 15 psi for 20 minutes. pH 7.0 after sterilization.

* The usual Agar content is 15 g. per liter. More (20 g) was used in these tests to stiffen the culture media and facilitate streaking.

Mineral Salts Solution

$NaH_2PO_4 \cdot H_2O$	1.00 g.
K_2HPO_4	2.00 g.
NH_4NO_3	2.00 g.
$MgSO_4 \cdot 7H_2O$	0.30 g.
$MnCl_2 \cdot 4\ H_2O$	0.01 g.
$CaCO_3$	0.01 g.
$FeSO_4 \cdot 7\ H_2O$	0.01 g.
Distilled water	1000 ml.

Twenty ml. aliquots in 4 oz. bottles pH adjusted to 7.4 before dispensing and sterilizing at 15 psi for 20 minutes (121°C).

Ten Per Cent Sea Water Solution

Natural Sea Water(1)	100 ml.
Tap Water (Chicago)	900 ml.
NH_4NO_3	2 gm.
$NaH_2PO_4 \cdot H_2O$	1 gm.
K_2HPO_4	2 gm.

The solution was adjusted to pH 7.4 prior to sterilization. Aliquots were dispensed as desired and the solution steam sterilized at 15 psi for 20 minutes.

Fuel or hydrocarbon substrates were added to the solution after sterilization. The individual fuels and hydrocarbons were sterilized separately by filtration through a 0.45 micron Millipore filter.

Agar plates containing a n-paraffin mixture (Appendix G) were prepared by adding 2% Difco agar to the above solutions prior to sterilization. After sterilization, and while still hot, 2% hydrocarbon was added and the medium was passed through a hand homogenizer (E. H. Sargent, No. S-61615) directly into sterile Petri dishes.

(1) Natural sea water obtained from the Atlantic Ocean. The water was aged several months before use. It contained 0.6 ppm nitrogen, 0.03 ppm phosphorus, and 20,200 ppm chloride ion.

APPENDIX C

Dilution Streak Technique

A standard platinum loop is used to deliver and spread 0.01 ml. of sample or appropriate initial dilution evenly over the upper half of the agar plate (Figure 1, A). The loop is then flamed (heated until glowing), cooled, and a diagonal streak approximately 5 cm. long is made with the flat of the loop through the inoculated half of the plate. The material picked up is spread evenly over the lower right quadrant of the plate (Figure 1, B). The loop is again flamed, cooled, and a streak made across the lower quadrant into the remaining quadrant. The material so collected is spread evenly over the last quadrant (Figure 1, C). The plate is then incubated for 24 to 48 hours at ambient temperature or at 30 to 35°C. depending on available facilities or the type of organisms involved. Longer incubation periods may be employed for the detection of fungi.

It is necessary to cool the loop in sterile water between streaking steps because it is made of heavy gauge wire and cools slowly. Four ounce French square wide mouth bottles are excellent containers for the sterile water.

After incubation, plates are read quantitatively by a scale such as that shown in Figure 2 which is based on an undiluted sample. The scale will obviously vary with the initial dilution employed.

The number of organisms present may only be estimated to the nearest whole or half $\log_{10}$. Examples of dilution streak plates employing undiluted samples are shown in Figure 3. The last quadrant of each plate, in this case will determine the estimated number of organisms per ml. Plate A has less than 10 colonies in this quadrant and ~100 in the opposite section; therefore, the count is estimated at 10^6 or 1,000,000 per ml. In Plate B the last quadrant has 10-100 colonies and more than 100 in the opposite section; therefore, the count is estimated at $10^{6.5}$ or 5,000,000 per ml. The use of exponents is quite handy but may result in some confusion as a result of using "half logs." "Half logs" in this case indicates a value half that of the next whole log as the count estimated for Plate B.

Care must be taken to avoid overlapping the various sections of the plate while streaking. Also, particular care must be taken to avoid contaminating the sterile water used to cool the loop. As in all bacterial counting procedures, the technique is only as good as the inoculum employed. The sample should therefore be shaken vigorously so that a representative inoculum may be obtained.

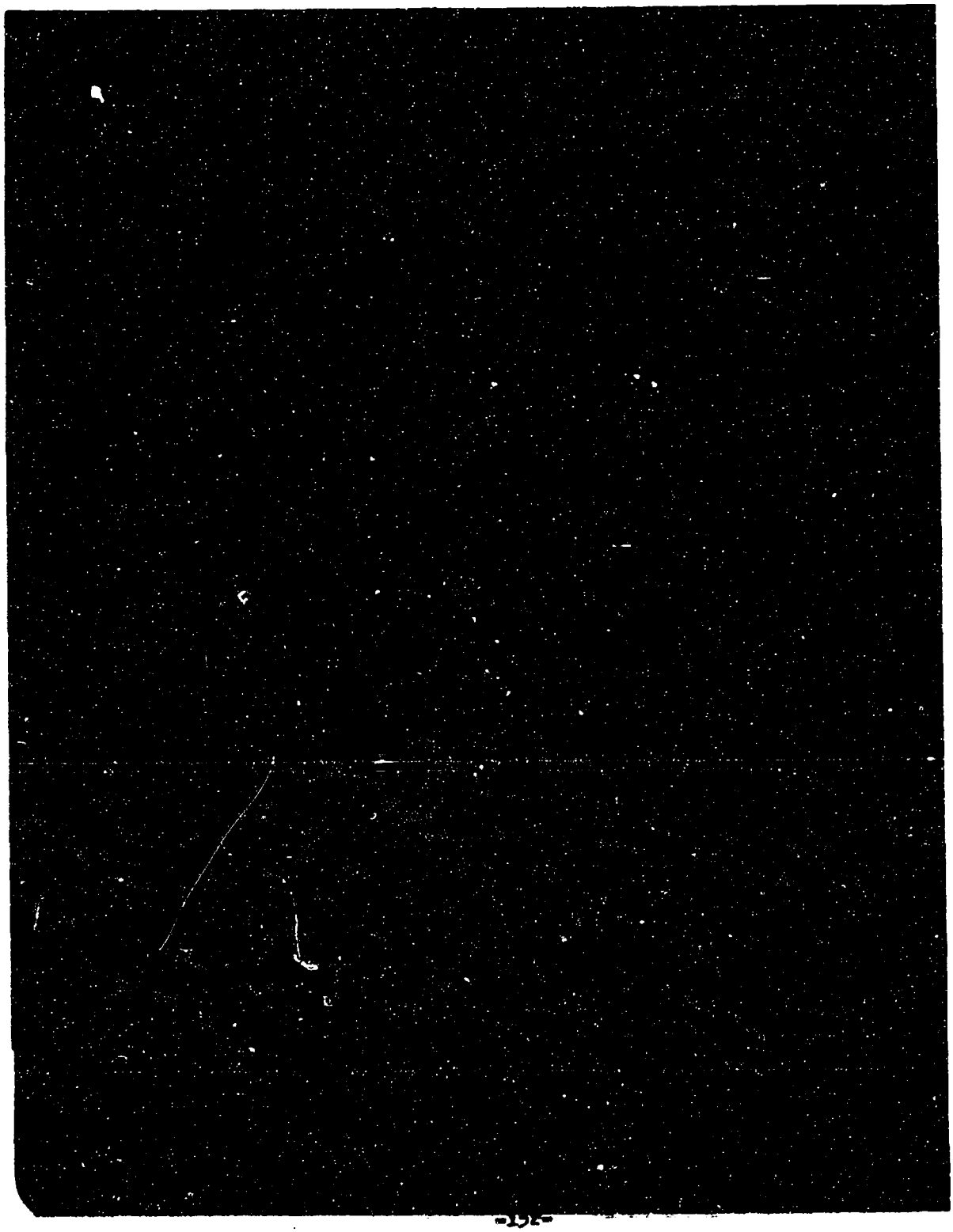

Figure 1. Successive steps in serial dilution streaking of a single plate for estimating the number of microorganisms per ml of sample.

Number of Colonies Observed in Section		Estimated Number of Organisms per ml of Sample
1-10	=	100 or 10^2
10-100	=	1000 or 10^3
100-1000	=	10,000 or 10^4

~100 in Section to Right, Plus:

Colonies		Estimated Number/ml
1-10	=	1,000,000 or 10^6

>100 in Section to Right, Plus:

Colonies		Number/ml
10-100	=	5,000,000 or $10^{6.5}$

~1000 in Section Above, Plus:

Colonies		Estimated Number/ml
1-10	=	100,000 or 10^5
10-100	=	500,000 or $10^{5.5}$

Figure 2

Scale for Estimating Number of Organisms per ml on Serial Dilution Streaked Plates

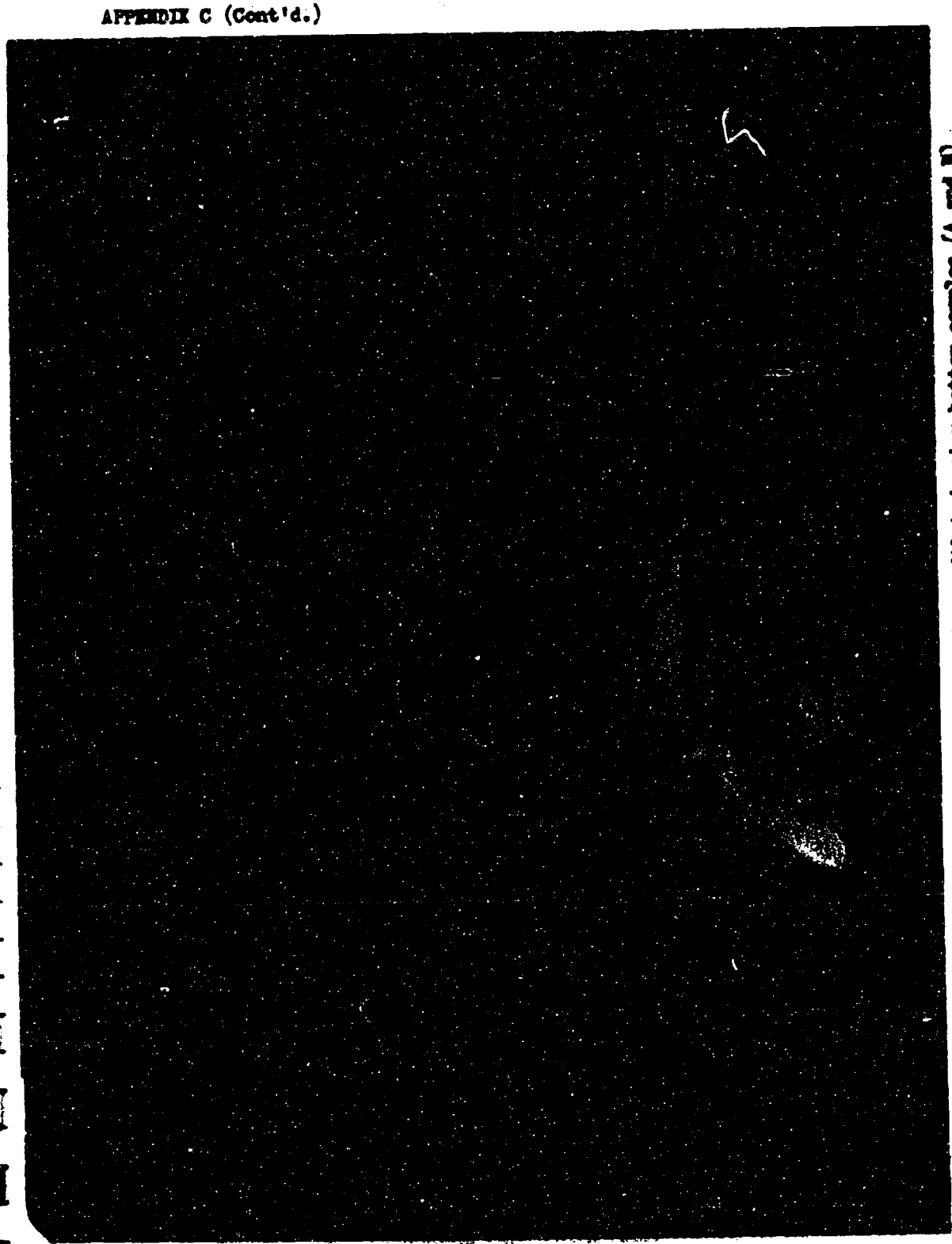

Figure 3. Examples of dilution streak plates. Two undiluted water bottom samples (A and B) inoculated with a pure culture were used for this demonstration.

APPENDIX D

CITE Fuel Storage Tests

Containers: Group A - One pint, soft glass, French square bottles containing 400 ml of CITE fuel were steam sterilized at 15 psi for 20 minutes (248°F). Twenty ml of water bottoms were added after the units had cooled to room temperature.

Group B - One gallon lug type, uncoated, steel containers, 6½ inches in diameter and 7-1/8 inches high (see Figure 1), were filled with 1 gallon of CITE fuel and steam sterilized at 15 psi for 20 minutes (248°F). After the units had cooled to room temperature, 100 ml of water bottoms was added.

Water Bottoms: Sea water, tap water, salt solution and inocula were added to the bottles and steel containers according to the following table. All units were set up in triplicate.

Group A (Glass Bottles)
(Contents in ml.)

Series	Sea Water[1]	Distilled Water[2]	Tap Water[3]	Inoculum[4] Active	Inoculum[4] Sterile	Salt Solution[5]	CITE Fuel[6]
I	10	9		1			400
II	10	8		1		1	"
III			19	1			"
IV			18	1		1	"
V	10	9			1		"
VI	10	8			1	1	"
VII			19		1		"
VIII			18		1	1	"
IX							"

Group B (Steel Containers)
(Contents in ml.)

I	50	45		5			1 gallon
II	50	40		5		5	"
III		95		5			"
IV		90		5		5	"
V	50	45			5		"
VI	50	40			5	5	"
VII		95			5		"
VIII		90			5	5	"
IX							"

(See following page for footnote references)

APPENDIX D (Cont'd.)

Footnotes:

(1) Natural sea water from the Atlantic Ocean was employed in these tests. The water was aged several months before use. It was sterilized in a pyrex container by autoclaving at 15 psi for 20 minutes. The sea water contained 0.6 ppm nitrogen, 0.03 ppm phosphorus, and 20,200 ppm chloride ions.

(2) Distilled water was sterilized in pyrex as described above.

(3) Tap water used in the tests was sterilized in a pyrex container as described above. This water is purified Lake Michigan water having a total hardness of approximately 125.

(4) The inoculum employed was a composite of contaminated water bottom samples collected from a number of bulk fuel oil and kerosine storage tanks. The composite was diluted with an equal volume of distilled water before use. An estimated 1,000,000 bacteria, yeast and fungi were present in the diluted composite. Sulfate-reducers were also present. A portion of this material was sterilized as described above for the sterile control units.

(5) Salt solution used to supplement the nitrogen and phosphorus in the water bottoms contained 40 mg. NH_4NO_3, 20 mg. $NaH_2PO_4 \cdot H_2O$, and 40 mg. K_2HPO_4 per ml. of distilled water, or 14 mg. of nitrogen and 11.62 mg. of phosphorus. The final levels of nitrogen and phosphorus in the water bottoms were 700 and 580 ppm respectively.

(6) CITE fuel was supplied by U. S. Army Natick Laboratories, (Voucher No. 3050-63) and was reportedly prepared according to Military Specification MIL-F-45121A dated 20 May 1960.

Storage Conditions: All units were stored at room temperature (75-80°F.) with caps loose to permit air exchange. Glass units were stored in the dark to prevent changes in the fuel catalyzed by light.

Sampling: All units were sampled at 2-week intervals over a period of 8 weeks. Two additional samples were collected at 4-week intervals. The overall storage time was 16 weeks. Since all tests were set up at the same time and in a similar manner, only one inoculated series was sampled initially. The pH of the water bottoms was checked initially, at 8 weeks and at the end of 16 weeks. Profile samples were collected from the steel containers to permit visual observations of the fuel and water bottoms. This was accomplished by employing sterile pyrex glass tubes approximately 12 inches in length and 10 mm ID. The tubes were used like pipettes and the large diameter tubing provided a relatively undisturbed profile sample. The tubes were carefully discharged into sterile screw cap test tubes for examination and photographing. Vigorous shaking of the tubes followed by a period of settling was used to observe emulsification tendencies.

APPENDIX D (Cont'd.)

Microbiological Analyses: One tenth ml. of the water bottoms from units in series I through IV, both Test A and B, was added to 9.9 ml. of sterile distilled water to give an initial dilution of 1:100. Difco Plate Count Agar (TGE) and Sabouraud Maltose Agar were streaked as described in Appendix C, to estimate the numbers of viable microorganisms present. The remaining units in both Test A and B were checked for sterility by adding 0.1 ml. from each replication in a series to 20 ml. of Nutrient Broth. Media employed are presented in Appendix B.

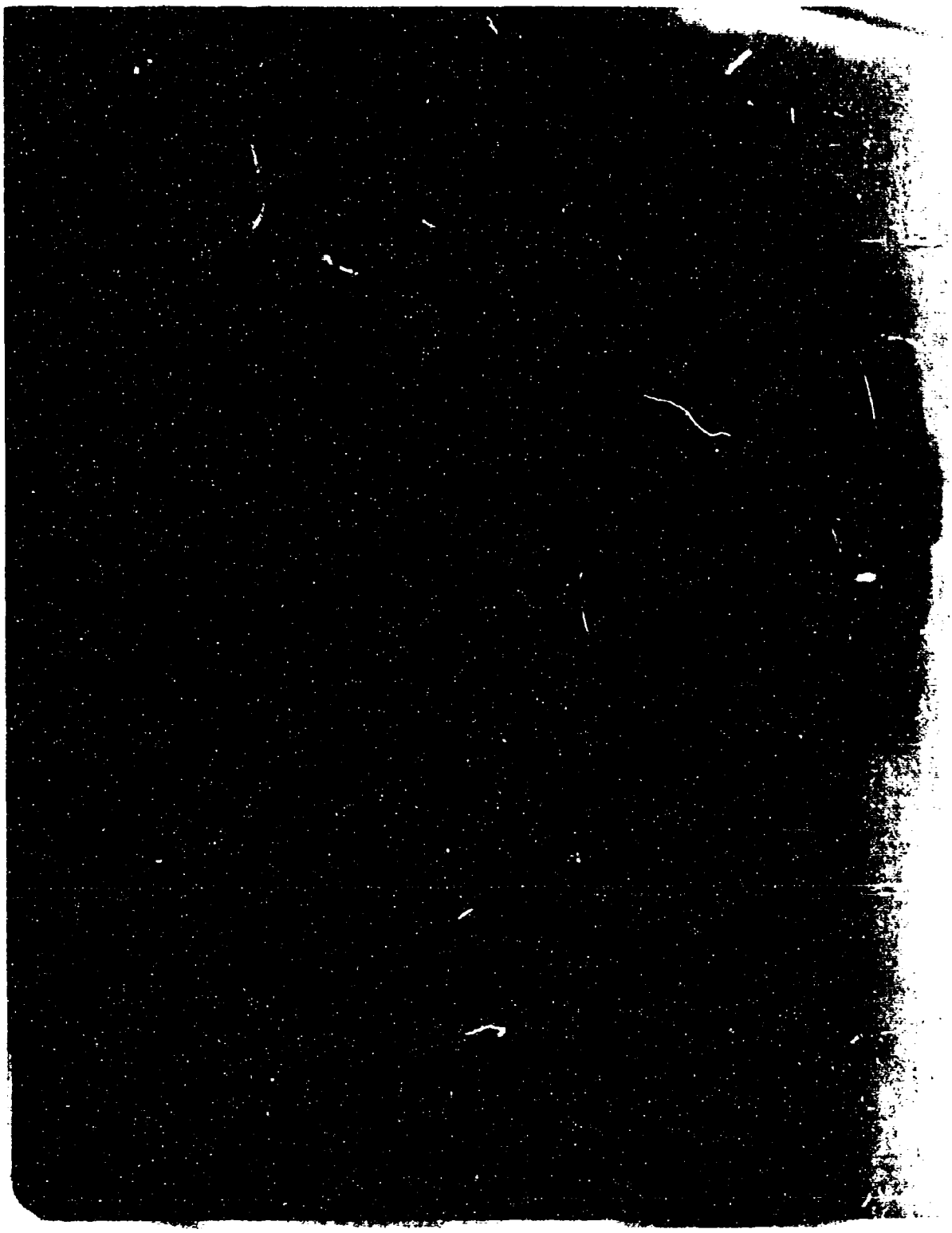

Figure 1. One-Gallon Lug Type, Uncoated, Steel Container Used in CIFS Fuel Storage Tests, Group 4.

APPENDIX E

Gravimetric Determination of Sludge

The amount of sludge formed in glass units without added iron was determined gravimetrically. Approximately 300 ml. of fuel was decanted from each unit to prepare composite samples of fuel for Quality Tests. The remaining 100 ml. of fuel and the water bottoms were filtered, and the amount of sludge present determined. An outline of the equipment and procedures employed follows:

Equipment: Filter unit comprised of a Coors Porcelain Gooch Crucible, F-7, with Glass fiber filter paper, No. 934-AH (R. Reeve Angel and Co., Inc.)

Filtering flasks with side tubes, pyrex.

Glass filter funnels, pyrex.

Solvents: n-Hexane (Commercial Grade)

Fuel Sludge Solvent: Equal portions of benzene, acetone and methyl alcohol. (ASTM Standards of Petroleum Products, Vol. I, 1961. Appendix XVI, pp. 1204-1207).

Procedure: 1. The remaining 100 ml. of fuel in each unit was carefully decanted and filtered through a tared filter unit. Hexane was used to remove residual fuel from the pad after the initial filtration. The filter unit was then washed with Fuel Sludge Solvent and the extract was transferred to a tared evaporating dish. The filter unit was then dried to constant weight.

2. The water bottoms and interface material were filtered through another tared filter unit. Several washings with distilled water were necessary to completely remove all sediment from the bottles. After the filtration, the residue was washed thoroughly with hexane, dried and washed with Fuel Sludge Solvent. The final extract was added to the material obtained in the first step. The filter unit was then dried to constant weight.

3. The evaporating dish containing Fuel Sludge Solvent extracts from steps 1 and 2 was placed in an oven to evaporate the solvent and dried to constant weight.

4. The filtered water phase was adjusted to a volume of 100 ml. and a 10 ml. aliquot transferred to a tared crucible. Water was evaporated and the units dried to constant weight.

APPENDIX E (Cont'd.)

The following materials were obtained employing this procedure on two units from each series:

A. Insoluble Material - Insoluble in fuel, water, and benzene-acetone-methyl alcohol solvent mixture.

B. Fuel Sludge - Material insoluble in fuel and water but soluble in benzene-acetone-methyl alcohol solvent mixture.

C. Non-volatile, water soluble materials and fine particles which may have passed through the filter pads.

APPENDIX F

CITE Fuel Additive Evaluation

Containers: The tests were set up in one-pint, soft glass, French square sample bottles with screw caps. The bottles were capped with aluminum foil and sterilized in a dry air oven at 170°C. for 3 hours. The screw caps were sterilized separately in an autoclave.

CITE Fuel: Additive-free CITE fuel, Blend 1, was sterilized in bulk in a sealed stainless steel cylinder by heating at 200°F. for 8 hours. After cooling to room temperature, 400 ml of the fuel were dispensed aseptically into sterile bottles.

Water Bottoms: Ten percent natural aged sea water, diluted with tap water, was employed. The active water bottom was prepared by making a 5% solution of the inoculum in sterile 10% sea water. In a similar manner, a 5% solution of inoculum in 10% sea water was sterilized by autoclaving at 15 psi for 20 minutes to be used as sterile water bottoms. The pH of both solutions was adjusted to 7.0 before they were dispensed in the bottles.

Inoculum: The inoculum employed was a composite of contaminated water bottom samples collected from a number of bulk kerosine, gasoline, JP-4, diesel, and heating fuel storage tanks. The composite was first diluted with an equal volume of distilled water and then mixed thoroughly in a Waring blender for 2 minutes. A final 1:5 dilution was made with distilled water before adding the inoculum to the 10% sea water as described above. The final 1:5 dilution of the inoculum contained an estimated 5,000,000 microorganisms, including bacteria, yeast and fungi. Sulfate-reducers were also present.

Additives: An appropriate dilution of each additive was prepared in CITE fuel, and steam sterilized at 15 psi for 20 minutes. The additive solution was then added to the 400 ml of fuel in each bottle to give the desired concentration (Tables XVI and XVII). After adding the appropriate dilution to the fuel, the contents of each bottle were thoroughly mixed.

Storage Conditions: All units were stored in the dark at 30°C. with caps loose to permit air exchange.

Sampling: All units were sampled bi-weekly over a period of eight weeks. The pH of the water bottoms in each unit was checked at the end of eight weeks.

Microbiological Analyses: One tenth ml of the water bottoms from active units was added to 9.9 ml of sterile distilled water to give an initial dilution of 1:100. Difco Plate Count Agar (TGE) and Sabouraud Maltose Agar were streaked as described in Appendix C, to estimate the number of viable microorganisms present. The sterile control units were checked at four and eight weeks by adding 0.1 ml from each replication in a series of 20 ml of Nutrient Broth. Media employed are presented in Appendix B.

Details of Test Series:

Series Designation	ml of Sterile CITE Fuel	ml of 10% Sea Water		Iron*
		Active	Sterile	
A	400	20		
B	400		20	
C	400	20		+
D	400		20	+
E	400			
F	400			+

(*) + Indicates the addition of a small piece of sterile steel wool. The steel wool was washed with benzene and acetone to remove residual oil and corrosion inhibitor. It was then sterilized in a dry air oven at 170°C. for 3 hours.

APPENDIX G

Preparation of Pure Hydrocarbon Blend

The following blend of pure hydrocarbons was prepared on a weight basis to give equimolar concentrations of the various components. The blend was sterilized by filtering through a 0.45 micron Millipore filter, and dispensed aseptically as needed.

Constituents of Pure Hydrocarbon Blend - n-Paraffins

Hydrocarbon*	Grams in Mixture**
n-Heptane	44
n-Octane	50
n-Nonane	56
n-Undecane	67
n-Dodecane	76
n-Tetradecane	88
n-Hexadecane	100

(*) All hydrocarbons were Phillips grade pure or 99+ Mol. %.

(**) Weights of each hydrocarbon were calculated to give equimolar concentrations in the final blend.

APPENDIX H

Warburg Manometric Procedures

The apparatus used in this study was a constant temperature Warburg Respirometer (American Instrument Co., Inc.) equipped with 125 ml BOD flasks with center well. The contents of the flasks were as follows:

Flask			
	10% Sea Water Solution (Appendix B)		29.0 ml
	Hydrocarbon	Liquid	1.0 ml
		or	or
		Solid (Powder)	0.5 gm
	Inoculum		1.0 ml
Center Well			
	10% KOH		1.0 ml.

All studies were conducted at 30°C. with agitation (72 cycles per minute with amplitude of 4 cm) in an atmosphere of air.

The following purified hydrocarbons (Phillips Research Grade, 99+ Mol % American Petroleum Institute or National Bureau of Standards compounds) were tested in this system:

n-Paraffins	Heptane
	Octane
	Tridecane
	Tetradecane
Iso-Paraffins	Isooctane
	2-Methylheptane
	2-Methylhexane
Aromatics	Benzene
	Toluene
	meta-Xylene
	1,2,4-Trimethylbenzene
	n-Propylbenzene
	Isopropylbenzene
	Naphthalene
	Biphenyl
Cycloparaffins	1,3-Dimethylcyclohexane
	A mixture of: Cyclopentane
	Methylcyclopentane
	Cyclohexane
	Methylcyclohexane

APPENDIX I

Procedures for the Determination of Proteins and Carbohydrates

Proteins

1. Lowry method with Folin Phenol Reagent (Lowry, O. H., et al., 1951, J. Biol. Chem., 193, 265)

   Reagents:

   Folin-Ciocalteau Phenol Reagent (E. H. Sargent & Co.) diluted 1:2 in distilled water before use.

   Protein Reagent: prepared by adding 1.0 ml of 2% $CuSO_4 \cdot 5H_2O$ and 1.0 ml 4% sodium tartrate solution to 100 ml of 4% Na_2CO_3 solution. (Prepared as needed.)

   Protein Standard: Bovine serum albumin.

   Procedure:

   1. Add 0.5 ml of sample to a calibrated colorimeter tube.
   2. Add 5.0 ml of Protein reagent and allow to stand approximately 10 minutes in a 45°C. water bath.
   3. Add 0.5 ml Folin Reagent, mix thoroughly, and let stand 15 minutes at room temperature.
   4. Read in Spectronic 20 colorimeter at 660 millimicrons.

2. Spectrophotometric Method (Warburg, O., and Christian, W., 1942 Biochem. Z., 310, 384)

   Protein was determined spectrophotometrically by measuring light absorption at 280 and 260 millimicrons in a Cary Recording Spectrophotometer, Model 11 MS. The data given by Warburg and Christian for known mixtures of crystalline enolase and yeast nucleic acid were used to correct for nucleic acid content. A 5 ml silica cell (d=1.0 cm) was employed.

3. Modified Biuret (G. Beisenherz, et al. 1953 Zeitschrift fur Naturforschung 8b, 576-577).

   Reagents:

   Biuret Reagent: Nine grams of $KNaC_4H_4O_6 \cdot 4H_2O$ are dissolved in 400 ml of 0.2 M NaOH. Three grams of finely powdered $CuSO_4 \cdot 5H_2O$

and 5 grams of KI are then added. Each salt was dissolved completely before adding the next. The solution was then adjusted to 1000 ml with 0.2 M NaOH.

Protein Standard: Bovine serum albumin

Procedure:

1. Cell free water bottoms were prepared by centrifugation at 5000 rpm for 30 minutes.

2. Fifteen ml of 3 M trichloroacetic acid (TCA) were added with agitation, to 85 ml of the cell free water sample or a dilution thereof.

3. The solution was allowed to stand for 1 to 3 minutes and was then centrifuged at 5000 rpm for 30 minutes. The supernate was decanted and the precipitate was washed 1 to 3 times with 0.5 M TCA. The number of washings depended on the amount of excess salts present.

4. Five ml of Biuret reagent were added with stirring to the precipitate. The volume was then adjusted to 10 ml with distilled water.

5. After allowing 30 minutes for color development, the optical density of the solution was determined on a Baush and Lomb Spectronic 20 colorimeter at 546 mu. A blank containing 5 ml of Biuret reagent and 5 ml of distilled water was employed.

6. The quantity of protein present was determined from a bovine serum albumin standard curve.

Dialysis

To remove water soluble materials which interfered with the analysis, aliquots representing 10% of the water bottoms were dialyzed against running tap water for a minimum of 16 hours. Protein determinations were made before and after dialysis.

APPENDIX I (Cont'd.)

Carbohydrates

Anthrone Method (Ashwell, G., 1957, Colorimetric Analysis of Sugars, in Methods in Enzymology II, 84, Edited by Colowick, S. P. and Kaplan, N. O., Academic Press, Inc., N. Y.

Reagent: Two grams of anthrone is dissolved in a liter of concentrated Sulfuric Acid.

Procedure:

1. 10 ml of Anthrone Reagent was added to a test tube and chilled to 15-20°C.

2. 5 ml of sample or standard was carefully layered over the reagent and allowed to chill.

3. The tubes were then shaken vigorously while still immersed in the bath.

4. The tubes were then brought to room temperature and placed in a 90°C. bath for 16 minutes.

5. Upon removal from the bath, they were cooled to room temperature and read at 625 mu in a Spectronic 20 Colorimeter.

APPENDIX J

Methods Employed for the Determination of Non-Protein and Non-Carbohydrate Compounds

The presence of neutral and acidic compounds determined by employing the procedures outlined by A. C. Neish (Analytical Methods for Bacterial Fermentations, Report No. 46-8-3, 1952, National Research Council of Canada).

Step 1. Distillation of volatile neutrals. A 50 ml. aliquot of each water bottom was adjusted to pH 8.0 with NaOH and distilled. Approximately 25 ml of distillate were collected.

Step 2. Steam distillation of volatile acids. The residue from Step 1 (25 ml) was adjusted to pH 4 with phosphoric acid, and steam distilled. Approximately 250 ml of distillate were collected.

Step 3. Extraction of non-volatile neutrals. The residue from Step 2 (25 ml) was adjusted to pH 8.0 with NaOH. A 10 ml aliquot was then extracted with 1.0 ml of redistilled carbon tetrachloride.

Step 4. Extraction of non-volatile acids. The portion of unextracted residue from Step 3 (15 ml) was then adjusted to pH 4.0 with phosphoric acid. A 10 ml aliquot was then extracted with 1.0 ml of redistilled carbon tetrachloride.

The presence of neutral compounds in the distillate from Step 1 was determined by the Acid Dichromate procedure described by Neish. In addition, a carbon tetrachloride extract of the distillate was prepared and analyzed in a Varian Model A-60 NMR. The carbon tetrachloride extracts prepared in Steps 3 and 4 were also analyzed by the NMR.

Titration with 0.01 N NaOH was used to establish the presence of volatile organic acids in the steam distillate from Step 2.

APPENDIX K

Data on Fermentor Operations

A single unit MicroFerm Laboratory Fermentor, manufactured by New Brunswick Scientific Co., Inc., New Brunswick, N.J., was employed in these studies. Sea water medium as described in Appendix B, Quarterly Progress Report No. 4, was used in this system.

Run No. 1 - Inoculum II on n-Octane

The fermentor was set up with 10 liters of sea water solution (pH 7.4) containing 50 ml of n-octane (Phillips Pure Grade, 99+ Mol. %). A 5% inoculum of 24-hour cells was employed.

Air Flow	5 liters per minute
Mixing Rate	400 rpm

Octane was added continually at a rate of 3 ml per hour during the entire run because of hydrocarbon loss due to volatility. Silicone anti-foam (Dow Corning Anti-foam A) was added sparingly while the fermentor was operating. Approximately 1 ml of anti-foam was added. The fermentor was shut down after 119 hours of continuous operation. Final pH of the medium was 4.8.

Run No. 2 - Inoculum III on Naphthalene

The fermentor was set up with 10 liters of sea water solution (pH 7.4) and 25 grams of naphthalene (USP Grade). A 5% inoculum of 24-hour cells was employed.

Air Flow	3 liters per minute
Mixing Rate	600 rpm

The fermentor was operated continuously for a period of 26 hours before being shut down because of foaming. Final pH of the medium was 6.9.

Run No. 3 - Inoculum I on CITE Fuel

The fermentor was set up with 5 liters of sea water solution and 5 liters of CITE fuel. A 5% inoculum (based on aqueous phase) was added to the system.

Air Flow	5 liters per minute
Mixing Rate	400 rpm

After 36 hours operation, the fuel phase had been reduced by 1.5 liters. This volume was replaced by an aliquot 0-30% cut of the fuel. Another aliquot was added on the third day. The pH was unchanged up to this time. On the fourth day heavy growth was observed along with emulsification. Anti-foam was not added to the system. The pH had dropped to 5.0. At this time the unit was shut down and allowed to stand for 3 days. The contents of the fermentor were divided in half and used to inoculate two 40-liter carboys containing CITE fuel.

Run No. 4 - Inoculum II on CITE fuel

The fermentor was set up with 5 liters of sea water solution (pH 7.4) and 5 liters of CITE fuel, Blend 1 (QPR 2).

Air Flow	5 liters per minute
Mixing Rate	600 rpm

The fermentor was shut down after 78 hours of continuous operation. A heavy stable emulsion formed on the third day of operation. The final pH of the water bottoms was 6.2.

Run No. 5 - Inoculum III on CITE fuel

The fermentor was set up as described above for Run 4. After 140 hours of continuous operation the fermentor was shut down. During the first 72 hours, no significant amount of growth was apparent in the system. At that time 10 grams of naphthalene (USP Grade) was added. Forty-eight hours after the addition of naphthalene, only slight microbial growth had occurred and the pH had dropped to 6.7. During the next 20 hours, heavy growth developed and a thick emulsion formed. Final pH of the water bottoms was 5.0.

APPENDIX L

Procedures Employed to Separate Phases Obtained in the Fermentor Runs

Fractionation of sludge and water bottom samples was carried out as outlined in Appendix E. In addition, two other fractions, a water extract and a n-hexane extract, which had previously been combined with the water bottoms and fuel phases respectively, were handled separately. They were given the following designations.

Fraction D - Water soluble extr[illegible]: Materials extracted from the sludge with dis[illegible]ed water. The water bottoms, Fraction C, were handled separately.

Fraction E - Hexane solubles: Materials extracted from the sludge with n-hexane.

Separations employed on the fermentor contents after each run are presented below. Three phases were obtained from each fuel run. Fractionations were made on the sludge fraction and on the residue obtained from the emulsions. No attempt was made to analyze the fuel phases at this time.

Fermentor Runs 1 and 2 - Inoculum II on n-Octane and Inoculum III on Naphthalene

Microbial sludge (cells) were readily collected by centrifugation at 5000 rpm.

Fermentor Runs 3 and 4 - Inoculum I on CITE (2 stage) and Inoculum II on CITE fuel

1) An initial separation of the fuel, interface and water phases was made in a separatory funnel. In Run 3 the bulk of the fuel was decanted before the volume could be handled in a separatory funnel.

2) The interfacial emulsion was washed extensively with distilled water. Cells were removed from the water washings by centrifugation at 5000 rpm. Following the water treatment, the emulsion was washed extensively with n-hexane in an effort to remove the fuel phase. The remaining emulsion was evaporated to dryness at 105°C.

3) The fuel phase was relatively clear but was filtered through No. 1 Whatman paper to remove traces of water.

4) Cells were removed from the water bottoms by centrifugation at 5000 rpm.

Fermentor Run 5 - Inoculum III on CITE fuel

An attempt was made to separate the three phases as described above for Run 5. The fuel was separated easily along with the emulsion phase. However, centrifugation at speeds up to 25,000 rpm (Sharples Supercentrifuge) would not remove the cells from the water phase.

The interfacial emulsion was treated as described above and then collected on No. 1 Whatman filter paper in a Buchner funnel. Hydrocarbon and water were forced through the filter paper. The paper was then dried at 105°C and the residue collected.

To facilitate analysis of the water bottoms, the entire layer, including cells, was evaporated to dryness at 105°C. The dried residue was subjected to the routine extractions and analyses.

This run was repeated and the water bottoms collected. Cells were removed by filtration and centrifugation to obtain water bottoms for analysis.

APPENDIX M

Fractionation of Microbial Sludge

The following procedure was employed in an effort to obtain a relatively pure carbohydrate fraction for infrared analysis.

1. Microbial sludge from Fermentor Run 1 was autoclaved and dried at 105°C overnight.

2. Ten ml of 10% TCA (trichloroacetic acid) were added to one gram of dried sludge. The mixture was stirred for 1.5 hours at 0°C.

3. The precipitate was then collected by centrifugation at 5000 rpm at 0°C., and washed three times with 10% TCA solution.

4. The supernate and all TCA washings were combined and evaporated to dryness.

5. The dried acid soluble material, which included carbohydrates, was washed three times with 100 ml aliquots of ether. This was followed by three washes with absolute ethyl alcohol to remove the TCA.

6. The final preparation was dried and an IR spectrum prepared.

APPENDIX N

n-Tetradecane Series

One gallon square milk bottles were employed as culture containers for two fungus cultures, Inocula IV and VII. The bottles were set up as follows.

400 ml	Sea Water Solution, pH 7.4 (QPR 4)
4 ml	n-Tetradecane (Filter sterilized)
1 ml	Inoculum (Including clump of fungus mycelium)

The bottles were incubated on their sides to increase the surface area available for fungus growth.

APPENDIX O

Analytical Procedures

1. Carbon and hydrogen	Macrocombustion Apparatus Model 1237A Hallikainen Instruments Berkley, Calif.
2. Ash (%)	ASTM Procedure D-482-63 ASTM Standards 1964, Part 17, pp 218-19
3. Oxygen	Sinclair method involving chromatographic determination of carbon monoxide after reduction pyrolysis. Gas Chromatographic Determination of Total Oxygen in Organic Materials, Boys, F. L. and Dworak, D. D., to be published in "Developments in Applied Spectroscopy" vol. 4, Plenum Press
4. Sulfur	Peroxide Bomb Combustion Technique Parr Manual No. 121, p. 28 Parr Instrument Co. 211 53rd St. Moline, Ill.
5. Nitrogen	Modified Coleman Nitrogen Analyzer, Model 29, Unit equipped with gas chromatograph.
6. Phosphorus	ASTM Procedure D-1091-58T ASTM Standards 1964, Part 17, pp. 387-396
7. Sodium and potassium	Beckman DU with Flame Photometer attachment
8. Metals (Qualitative)	1.5 Meter Grating Emission Spectrograph Applied Research Laboratories Instrument

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Sinclair Research, Inc., Harvey, Illinois

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by A. T. Knecht, Jr., J. W. Koole, D. J. Muhlbaier, A. R. Rizzuto

Final Report, 15 November 1965, 175 pp -illus -tables
(Contract DA-19-129-AMC-86(N)) Unclassified Report

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Fuel-water emulsions were stabilized by cells and probably microbially oxidized fuel components. Metabolic studies indicated that recognized pathways were followed for the oxidation of n-paraffins and naphthalene.

UNCLASSIFIED

1. Fuel Contamination
2. Diesel Fuels
3. Molds (Organisms)
4. Fungi
5. Microorganisms
6. Bacteria
7. Storage Tanks

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Gums	3					
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