THE POSSIBLE ROLE OF ACTINOMYCETES IN THE BIODEGRADATION OF ENHANCED OIL RECOVERY COMPOUNDS

By

JONELL DOUGLAS

Bachelor of Science

Oklahoma State University

Stillwater, Oklahoma

1980

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
MASTER OF SCIENCE
December, 1983

Thesis
1983
D734p
cop.2

THE POSSIBLE ROLE OF ACTINOMYCETES IN THE

BIODEGRADATION OF ENHANCED OIL

RECOVERY COMPOUNDS

Thesis Approved:

Mary M Grula
Thesis Adviser

E. A. Grula

George V. Odell

Norman N. Durham
Dean of the Graduate College

1170209

ACKNOWLEDGMENTS

My most sincere gratitude and appreciation goes to Dr. Mary M. Grula for her many contributions to my years of study at Oklahoma State University. Her wisdom, encouragement, and patience were respected and will always be remembered.

Appreciation is also extended to Dr. Edward A. Grula and Dr. George V. Odell for their time and effort in serving on my committee.

To my fellow graduate students I wish to express my thanks for their friendship and assistance.

A very special thanks is extended to Dr. Becky Smith for her friendship, encouragement, and her time and effort in helping me to meet my deadlines.

Most of all I thank God for His love and for patiently teaching me that His ways are truly higher than mine.

Finally, I thank my parents and my brother for their love and their many sacrifices throughout the years.

TABLE OF CONTENTS

Chapter | Page

I. INTRODUCTION. 1

II. MATERIALS AND METHODS 10

Microcosm Tests. 10
Isolation and Enumeration of Actinomycetes From the Microcosm Tests. 12
Solid Medium for Selection and Enumeration of Actinomycetes 13
EOR Compounds Tested 13
Preparation of Solutions 15
Isolation and Identification of Microorganisms . . . 16
Degradation of the EOR Compounds by Pure Cultures of Actinomycetes on a Solid Substrate 17
Chemically Defined Medium. 18
Liquid Cultures for Determining Degradation of EOR Compounds. 19
Quantitative Determination of Degradation of Enhanced Oil Recovery Agents Using Chemical or Physical Tests 19
Determination of Extent of Degradation of Polyacrylamides Using Viscosity Measurements. 21
Quantitation of Polyacrylamide Degradation by Screen Factor Measurement 25

III. RESULTS 29

Counts of Actinomycetes in Soil Samples Enriched With Various EOR Compounds 29
Microcosm Tests to Examine the Role of the Acidity of n-DBSA in its Toxicity. 36
Tests With Polyacrylamides 47
Identification of Various Isolates From the Microcosm Tests 60
Tests of Potential Ability of the Isolated Strain to Utilize EOR Chemicals as a Sole Carbon and Energy Source 64
Results of Semiquantitative Analytical Tests 69
Possible Biological Degradation of Polyacrylamides . 73
Possible Biological Degradation of Xanthan Gum . . . 75

IV. DISCUSSION. 76

Chapter Page

LITERATURE CITED. 81

APPENDIX - PROPERTIES OF A *GEOTRICHUM* SPECIES ISOLATED FROM A SOIL ENRICHMENT CULTURE CONTAINING 2-BUTANOL 84

LIST OF TABLES

Table		Page
I.	Actinomycetes and Related Organisms Capable of Biodegrading Hydrocarbons.	8
II.	Sources of Soils Used in Microcosm Tests.	11
III.	Effects of Various Concentrations of Streptomycin in Starch Casein Agar on Growth of Four Eubacteria and Four *Streptomyces*	31
IV.	Effects of Various Concentrations of Cycloheximide in Starch Casein Agar.	32
V.	Microcosm Test with n-Dodecylbenzene Sulfonic Acid. . . .	34
VI.	Microcosm Test With n-DBSA (0.1%)	35
VII.	Actinomycete Counts in Microcosm Tests on Two Soil Samples With n-DBSA, Neutralized n-DBSA, and Soil With HCl	46
VIII.	Microcosm Test Results for Soil C & D With (20% W/W) Polyacrylamides J332-334.	49
IX.	Microcosm Test With Polyacrylamides	50
X.	Microcosm Tests With Marathon (0.1%).	55
XI.	Identification Key for Various Genera of Actinomycetes. .	61
XII.	Microcosm Test Isolates	62
XIII.	Growth of Various Microbial Strains on a Chemically Defined Medium With a Representative EOR Chemical as Sole Carbon-Energy Source.	66
XIV.	Growth of Eight Strains on a Defined Medium With a Petroleum Sulfonate or n-DBSA as Sole Carbon Source . . .	70
XV.	Analysis of n-DBSA Using Methylene Blue Test.	71
XVI.	Analysis of Marathon Using Methylene Blue Test.	72
XVII.	Growth of Eight Strains on a Defined Medium With a Polyacrylamide (J332-334) as Sole Carbon Source	74

Table		Page

XVIII. Growth in Third Serial Cultures Using Various Alcohols as Sole Carbon-Energy Sources. 87

XIX. Growth in Third Serial Cultures Using Various Compounds as Carbon-Energy Sources. 88

XX. Growth in Third Serial Cultures Using Various Compounds as Sole Nitrogen Sources. 89

LIST OF FIGURES

Figure | Page

1. Diagrammatic Representation of the Brookfield Viscometer. . 24

2. Diagrammatic Representation of the Screen Viscometer. . . . 27

3. Actinomycete Counts During a 12 Week Period in the Microcosm Test With n-DBSA Using Soil Sample 38. 38

4. Actinomycete Counts During a 12 Week Period in the Microcosm Test With n-DBSA Using Soil Sample 39. 40

5. Actinomycete Counts During a 12 Week Period in the Microcosm Test With n-DBSA Using Soil Sample D 42

6. Actinomycete Counts During a 12 Week Period in the Microcosm Test With n-DBSA Using Soil Sample 42. 44

7. Actinomycete Counts During a 12 Week Period in the Microcosm Test With Polyacrylamides J332-334 Using Soil Sample 41. 52

8. Actinomycete Counts During a 12 Week Period in the Microcosm Test With Polyacrylamides J332-334 Using Soil Sample 42. 54

9. Actinomycete Counts During a 12 Week Period in the Microcosm Test With Marathon Petroleum Sulfonate Using Soil Sample 38 57

10. Actinomycete Counts During a 12 Week Period in the Microcosm Test With Marathon Petroleum Sulfonate Using Soil Sample 42 59

CHAPTER I

INTRODUCTION

Since the use of chemical agents in enhanced oil recovery (EOR) has increased, it is important to obtain information on the possible environmental effects of these chemicals. Heavy or widespread use of the EOR chemical agents could have a major impact if a spill during transport or leak from the well site occurred. If a spill were to occur and the chemicals were not degraded within a reasonable amount of time, serious environmental deterioration could occur resulting in possible adverse effects for microorganisms, plants, and animals. Therefore, it is important to have information on the biodegradability of the EOR chemicals and the ability of certain soil organisms to bring about biodegradation.

Only about half the crude oil used in the United States is produced in the United States. In fact, only about 50% of the original oil in place is recoverable using primary recovery techniques. The means of recovering more oil after primary recovery involves water flooding of the oil reservoirs (secondary recovery) and chemical flooding (one method of tertiary recovery). Secondary recovery represents about 50% of the U.S. current daily production. After secondary recovery ceases to increase production from the reservoir, a number of other techniques referred to as tertiary recovery methods are employed. Enhanced Oil Recovery consists of both secondary and tertiary recovery techniques.

Chemical flooding of the reservoir is one method utilized in tertiary

recovery. A wide variety of chemicals such as surfactants, petroleum sulfonates, certain primary alcohols, alkali, and certain water-soluble polymers are used in tertiary recovery. It has been estimated that the production of one barrel of oil requires ten pounds of sulfonates, three pounds of alcohol, and one pound of polymers (Foshee, 1976). It is evident that large quantities of EOR compounds must be used to significantly increase oil production from a reservoir. A number of water-soluble polymers are used in micellar-polymer flooding. When solubilized, the polymer soltuions are highly viscous and can serve as mobility control agents in the aqueous phase of the reservoir. The two types of polymers which have been used extensively are 1) polymers of acrylamide, and 2) biological polymers such as xanthan gum, which is an extracellular heteropolysaccharide produced by Xanthomonas campestris strain NRRL B-1459. For a polymer to be useful in Enhanced Oil Recovery, it must maintain certain properties, such as viscosity of its solutions for extended periods of time at relatively high temperatures, various conditions of pressure, pH, ionic strength, and flow rate (McCormick, 1979). In addition to the previously listed ideal properties for enhanced oil recovery, a polymer should be resistant to microbial degradation.

A second group of chemicals used in EOR technologies are the petroleum sulfonates. These materials, produced by sulfonation of crude oil, are an important component of the micellar-polymer flooding process. The petroleum sulfonates are complex mixtures, not pure compounds. The sulfonates produced by sulfonation of crude oil vary in their content of water, free oil, sulfonated hydrocarbons and inorganic salts. They also possess a surfactant quality which allows them to form stable microemulsions in water. This is because of the linkage of a sulfonate group to

aromatic rings, producing a molecule with a hydrophilic portion (aryl sulfonate and a hydrophobic portion (hydrocarbon).

As stated before, it is important to discover whether or not the chemicals used in enhanced oil recovery are susceptible to microbial degradation in the event that a leak from the reservoir or a spill were to occur. The overall goal of this research was to obtain information on the extent to which actinomycetes and other related genera of bacteria contribute to the biodegradation of certain chemicals used in EOR. Also, we were interested in determining the rate and extent of biodegradation of these agents in environments where spills were likely to occur. The project focused on organisms whose natural habitat is the soil, in particular the slower-growing organisms such as the actinomycetes, which would not be selected in ordinary batch, continuous, or semi-continuous enrichment cultures.

The actinomycetes are widely distributed in soil, water, and sediments. In fact, they are second only to eubacteria in abundance (Arai, 1976). They constitute Part 17 of *Bergey's Manual of Determinative Bacteriology* (8th edition), Actinomycetes and Related Organisms. The term actinomycete encompasses two groups of organisms, those known as the coryneform bacteria, and the order *Actinomycetales*.

It should be noted that the Parts in *Bergey's Manual*, 8th edition, are not taxa, or taxomonic groups, but instead were established on a pragmatic basis in which microorganisms were categorized according to a few easily determined criteria so that the *Manual* would be more useful in the identification of microorganisms. Thus Part 17, comprising the organisms with which we are concerned, contains two taxonomically distinct groups of organisms, viz., the Coryneform group of bacteria, and

the Order Actinomycetales.

Actinomycetales are described as "bacteria that tend to form branching filaments which in some families develop into a mycelium. The filaments may be extremely short, as in the Mycobacteriaceae, and Actinomycetaceae, or well-developed as in Streptomycetaceae" (Bergey's, 1974). Although the coryneform group of bacteria do not form branching filaments, the authors of Part 17 point out similarities between human and animal parasites in the genus Corynebacterium, and the genera Mycobacterium (family Mycobacteriaceae) and Nocardia (family Nocardiaceae) in the Order Actinomycetales. The authors believed that these similarities indicated a rather close relationship between the genus Corynebacterium and the mycobacteria and nocardias. Some of the features are: arabinose and galactose as common cell wall sugars, the presence of a common cell wall antigen, and the presence of α-branched, β-hydroxy (mycolic acids) carboxylic acids in the lipids of the organisms. However, based on morphological criteria (at least in part) the nocardias and mycobacteria are placed in a different order from the coryneform bacteria.

One could conclude that the taxonomy of these bacteria was not well understood at the time of publication of the 8th edition of Bergey's Manual (Bergey's, 1974); an Editorial Note (p. 599) stating that "the division of genera between Part 16 and the first section of Part 17 is arbitrary and readers should consult both" tends to reinforce such a conclusion.

Since our approach in doing this study was primarily pragmatic (an attempt to demonstrate slow-growing microorganisms possibly degrading EOR chemicals, in contrast to the more rapidly growing organisms selected for by conventional enrichment methods) the genera we were interested in were

chosen somewhat arbitrarily on the basis of growth rate. This meant excluding most organisms in the coryneform group of bacteria, but including the genus Mycobacterium which is described in the Manual as "slow-growing". The genus is divided into two groups on the basis of growth rate--members of the "rapidly growing group" form visible colonies in about 4 days, whereas members of the slower-growing group require two weeks or longer to form visible colonies. Other genera in the order Actinomycetales are not specifically described as "slow-growing", but they nevertheless grow considerably more slowly on artificial media than most common eubacteria. The three genera that are among the most common in the order, and that would be the most likely to be involved in degradation of chemicals accidentally discharged are Mycobacterium (family Mycobacteriaceae), Nocardia (family Nocardiaceae), and Streptomyces (family Streptomycetaceae).

The Actinomycetales are bacteria, and as such are procaryotic organisms, although some (especially the genus Streptomyces) resemble fungi in the following ways: a) possession of extensively branched hyphae; b) formation of aerial mycelium as well as aerial conidia; and c) growth in liquid culture rarely results in the turbidity associated with bacteria, but rather occurs as clumps or "balls" similar to those associated with fungi. However, the morphology and size of the hyphae, conidia, and individual fragments produced from segmentation of the mycelium are similar in dimensions to bacterial cells.

For purposes of isolation, the following properties of many genera in Actinomycetales may be useful. Many of the genera are slow-growers, requiring anywhere from 48 hrs to 2 weeks for the production of mature colonies at 25°C. They prefer alkaline conditions for growth and usually

will not grow at a pH below 5.0 (El-Nakeeb et al., 1963; Waksman, 1959). The colonies of Streptomyces species on agar media are easily recognizable. Some may produce colonies which develop on the agar surface, have a firm consistency, and adhere tenaciously to the solid substrate. Often, the surface of these colonies may become powdery because of the production of aerial spores (conidia). Pigmentation of aerial mycelium and/or conidia is important in identification. Also the texture of the colonies of some species may be leathery, dry, crusty, or flaky (Waksman, 1963). Most species of Streptomyces are aerobic although a few are facultative anaerobes. The plate count method is the most suitable for enumerating most species of Streptomyces, and other antinomycetes with which we are concerned. Usually the plate counts are not greatly affected by the composition of the medium; this indicates that the organisms can utilize a wide variety of organic substrates (Corke et al., 1956; Crook, 1950; and Kuster, 1964).

The ability to utilize a number of hydrocarbons as sole carbon and energy sources has been reported for many actinomycetes. Abbott (1968), reported a Nocardia species which could utilize a number of alkenes. Also Atlas (1978), Foster (1962), and Kester (1963) have reported that many actinomycetes, especially Streptomyces, are capable of degrading hydrocarbons. Organisms decomposing hydrocarbons are abundantly and widely distributed in soil, water and sediment, especially in areas that have been exposed to hydrocarbons (oil soaked areas, refineries, and oil wells).

Certain genera of soil organisms responsible for a major part of hydrocarbon degradation include Streptomyces, Nocardia, Mycobacterium, Corynebacterium, Brevibacterium, Pseudomonas, Flavobacterium, and a few

filamentous fungi (Klug, 1971; Gilbert, 1978).

Microorganisms can degrade hundreds of hydrocarbon compounds found in petroleum at rates that are dependent in part on molecular structures. However, the range of individual hydrocarbons any particular organism uses for growth is fairly limited (Zobell, 1950). Various investigators have demonstrated the degradation of crude oils, gasolines, lubricating oils, tars, asphalts, illuminating gases, natural and synthetic rubbers, paraffinic waxes, mineral oils, petroleum ethers, natural gases, kerosene, and other mixtures of hydrocarbons (Klug, 1971; Stirling, 1977). However, in many of these complex materials there are hydrocarbon molecules that are very resistant to microbial degradation or that may be bacteriocidal or bacteriostatic. A number of actinomycetes and related organisms have the ability to degrade a variety of hydrocarbons which results in formation of a wide range of metabolic products (Table I).

Since the ability to utilize hydrocarbons is so widespread among the actinomycetes, it might be assumed that these organisms would readily break down EOR chemicals (surfactants, cosurfactants, polyacrylamides, biopolymers) as well. However, very little information is available concerning the ability of various genera of actinomycetes to degrade or utilize these chemicals. A few of the compounds have been considered recalcitrant (Alexander, 1965). However, many actinomycetes have been found to possess a number of oxygenases which can rapidly attack many long-chain hydrocarbons which are considered resistant to microbial attack (Yall, 1979). As stated before the basic purpose of our research in this area is to obtain information on the extent to which actinomycetes and related genera of bacteria may contribute to the biodegradation of chemicals used in EOR. Enrichment methods used previously by our labs at OSU

TABLE I

ACTINOMYCETES AND RELATED ORGANISMS CAPABLE OF BIODEGRADING HYDROCARBONS

Organism	Hydrocarbon
Nocardia salmonicolor	Hexadecane
Nocardia spp.	Diphenylbutane
	Propane
	1-Chloropropane, 1-Chlorobutane
	1-Chloropentane, 1-Chlorohexane
	1-Chlorooctane, 1-Chlorodecane
	Alkylbenzene (Cooxidation)
Nocardia petroleophila	Gaseous cyclohexane
Streptomyces spp.	2,6-dinitro-4-(Trifluoromethyl-)benzanamine
Nocardia spp. and Corynebacterium spp.	C_{19}-C_{30} n-alkanes from crude oil
Mycobacterium rhodochrous	n-Decane
	Gaseous cyclohexane
	Benzene
Mycobacterium smegmatis	Methyl cyclohexane

Products: CO_2, cell substances, organic acids, aldehydes, ketones, alcohols, oxyphenols, pigmented substances (carotenes), methane, butadiene, styrene.

References: Fredericsk, 1967, Kester, 1963; Lukins and Foster, 1963; Lusby, 1980; Sielicki et al., 1978; Olivieri et al., 1976; Walker and Colwell, 1976.

have selected for and examined only the faster-growing organisms and thus very little information has been provided on the possible role in biodegradation of EOR compounds by the slower-growing soil microorganisms such as Streptomyces spp. and Nocardia spp.

CHAPTER II

MATERIALS AND METHODS

Microcosm Tests

In order to assess the degradability of the EOR chemicals under conditions that would probably exist in case of a spill, enrichment cultures (or microcosm tests) were prepared. These enrichment cultures were designed to provide long-term enrichment for actinomycetes. These tests represent an attempt to simulate the actual conditions in soil as they would exist if a major spill or leak of the EOR chemicals occurred. The microcosm tests were prepared by adding 15 grams of a soil sample to a sterile petri plate. An appropriate amount of EOR agent was added to obtain a final concentration of 20% (w/w) of the compound. It was assumed that in a situation where a spill or accidental discharge had occurred, that a relatively high concentration of the EOR chemical would be present in surface soil at the site of the spill. Control cultures with no added EOR agent were also prepared for each soil sample. The soil samples used in the microcosm tests were chosen from environments which had probably been exposed to oil products for prolonged periods of time. Also, soils from other environments, such as agricultural lands, were examined (Table II). The soils were collected aseptically from near the soil surface. It was thought that, by obtaining data on actinomycete counts in this way using a number of soil samples from central and eastern Oklahoma, we

TABLE II

SOURCES OF SOILS USED IN MICROCOSM TESTS

Soil	Location	Date Obtained	pH
C	Soil saturated with crankcase oil near Stillwater	12/9/80	6.0
D	Oily soil near a crude oil storage tank North of Bartlesville	12/23/80	6.6
33	Soil from a cattle feeding West of Stillwater	2/6/81	7.5
37	Oil saturated soil from Central Oklahoma	2/6/81	8.0
38	Soil near an oil pump in Eastern Oklahoma	6/1/81	6.0
39	Soil near Highway 51 East of Stillwater	6/1/81	7.4
41	Soil from a flower bed in Stillwater	6/1/81	6.4
42	Soil from a wheat field West of Stillwater	6/1/81	6.4

could get significant information on the role of actinomycetes in biodegradation of EOR chemicals.

The soil samples tested were refrigerated and used for up to two months. Even after six months storage the actinomycete counts remained essentially the same as when the soil was fresh. The microcosm cultures were moistened once a week with sterile distilled water and incubated at room temperature (25°C) for 12 weeks. Actinomycete counts were done on the soil sample itself, on the microcosm mixture initially, and thereafter every two weeks up to 12 weeks.

The initial pH of each soil was determined by using pH paper. Every two weeks in addition to plating for actinomycetes, the pH of the mixture was determined using pH paper.

Counts of the faster-growing bacteria were done by plating on trypticase soy agar (TSA). In some cases we purposely did not control the pH, in others we adjusted the pH of the mixtures or of the compound itself before mixing it with the soil.

Isolation and Enumeration of Actinomycetes From the Microcosm Tests

Initial actinomycete counts were made by diluting and plating out samples from the microcosm tests onto starch casein agar containing streptomycin (to inhibit most fast-growing soil bacteria) and cycloheximide (to inhibit fungi). The starch casein agar (Williams and Davies, 1965) was prepared as follows: starch, 10 g; acid hydrolyzed casein, 10 g; Bacto agar, 17 g; all dissolved in 1 liter of distilled water. The medium was sterilized in an autoclave for 15 min at 15 pounds pressure. Solutions of each antibiotic were prepared and added aseptically to the

sterile, cooled medium. The plates used to make enumerations were incubated at 25°C for 1-2 weeks and then counts were made.

Solid Medium for Selection and Enumeration of Actinomycetes

We needed to develop a medium that would allow the growth of actinomycetes while inhibiting the faster-growing bacteria and fungi. Starch casein agar, buffered to a pH of about 7.5, was chosen to select for and enumerate the genera of interest. The starch casein agar, with antibiotics to inhibit eubacteria and fungi, was suggested by Williams and Davies (1965) for the isolation and enumeration of actinomycetes. We felt it would be desirable to directly determine the effects of concentration of streptomycin and cycloheximide on growth of eubacteria and fungi respectively, and in addition, to determine effects of concentration of such antibiotics on growth of selected species of Streptomyces.

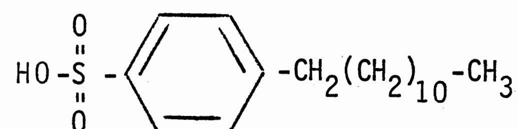
Accordingly, tests were set up using laboratory cultures of four common soil bacteria, and four recently isolated strains of Streptomyces. An analogous test was done using two common soil fungi. Details will be given in the Results section.

EOR Compounds Tested

Two general types of EOR chemicals were used in testing for the possibility of involvement of various genera of actinomycetes in their degradation. The first type was petroleum sulfonates--a type of surfactant produced by treatment of crude oil with sulfuric acid. One representative of this group was a product of Marathon Oil Company, with an approximate composition as follows: Sulfonate, 10%; Mineral Oil, 11%;

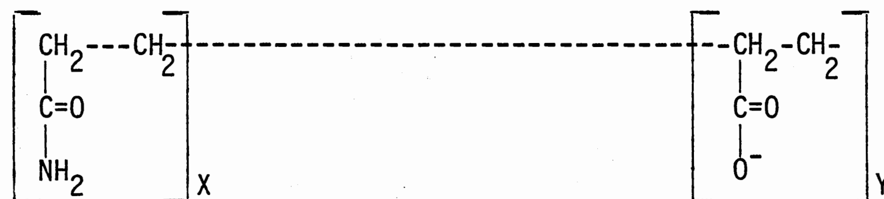
Water, 76%; Inorganic Salts, 3%.

We believed it was advantageous to test, in addition to the crude mixture from Marathon Oil Company, a relatively pure compound to represent this type. For this purpose we chose para-n-dodecylbenzene sulfonic acid, which is easily obtained commercially. The general formula of this compound is:



It is significant that the alkyl side chain is linear; because of this the compound is inherently more biodegradable than if the side chain were branched. Also, the point of attachment of the side chain to the ring, while _para_ to the sulfonic acid group, is not necessarily on the terminal carbon of the dodecyl group. Usually a mixture of isomers with the bond from the C_4 of the ring to any one of several C atoms in the dodecyl group is present.

The second general type of EOR chemical we tested was the polyacrylamides. These are water-soluble copolymers of acrylamide and acrylic acid, of the general formula



The ratio of Y to X is called the degree of hydrolysis of the polymer; the value of this parameter is important in determining the usefulness of a given polymer for enhanced oil recovery purposes. The polyacrylamides most commonly used possess a degree of hydrolysis of 25-35%.

Molecular weights of polyacrylamides usually range from 10^6 to 10^7 daltons. The polyacrylamides we used were obtained as gifts from Dowell Division, Dow Chemical Company, U.S.A., of Tulsa, Oklahoma. They are designated, and have properties as follows:

Designation	Molecular Weight	Degree of Hydrolysis
J_{332}	9-10 x 10^6	25-35%
J_{333}	6-7 x 10^6	25-35%
J_{334}	3-4 x 10^6	25-35%

Preparation of Solutions

Petroleum sulfonates in aqueous solution are acidic; both n-DBSA and the Marathon petroleum sulfonate produced aqueous solutions with a pH in the range of 4.0-5.0.

Both neutralized and non-neutralized n-DBSA were tested. The neutralized n-DBSA was prepared by adding 1 N NaOH to an aqueous solution of n-DBSA. This results in a fairly viscous liquid which was added to the soil samples in the microcosm tests to achieve a 20 percent (v/w) concentration.

Marathon petroleum sulfonate was also tested in both the neutralized and non-neutralized forms, at a concentration of 20 percent.

The polyacrylamides, although they are quite water-soluble on a weight basis, are somewhat difficult to put in solution because even low concentrations produce a highly viscous solution, and the long linear molecules are easily broken by mechanical (shear) stress (Foshee, 1976). Therefore, we carried out the microcosm tests using the dry powders;

appropriate amounts were sprinkled on the soil samples in the petri dishes to achieve a final concentration of 20 percent (w/w). The cultures were then moistured with sterile distilled water.

The polyacrylamides have been considered highly resistant to microbial attack (Foshee et al., 1976). Very little is known of the microbial breakdown of macromolecules consisting of long chains of carbon atoms joined by C-C covalent bonds. A discussion of microbial deterioration of various synthetic rubbers is given in a review by Zyska (Alexander, 1965). Polyacrylamide carbon chains are far longer than the highest molecular weight hydrocarbon subject to microbial attack (40 to 45 carbon atoms). A possible explanation of the resistance of these polymers to microbial attack could lie in the fact that the monomer, acrylic acid, is toxic to microorganisms.

Isolation and Identification of Microorganisms

In this study we planned to isolate and identify a number of species representing various genera which might be characterized as actinomycetes from the microcosm cultures as they incubated. In the event that these strains should prove to be important in the degradation of EOR chemicals in a natural environment, we would expect to find an increase in numbers with time in the microcosm tests, and to find pure cultures that might be capable of using one of the test EOR chemicals as a sole source of carbon-energy.

From the plates prepared for enumeration of actinomycetes in the microcosm cultures, a number of what appeared to be abundant and typical representatives of this group of microorganisms were isolated. The isolates were purified and identified according to their colonial morphology,

Gram reaction, and biochemical tests. The genera tested for their ability to degrade the EOR compounds included Streptomyces spp., Nocardia spp., Corynebacterium spp., and Mycobacterium spp. These cultures were always incubated at room temperature (25°C). The genus Corynebacterium, while not classified in the Order Actinomycetales, is one of the group of coryneform bacteria, and is placed in Part 17, along with the Actinomycetales.

Degradation of the EOR Compounds by Pure Cultures of Actinomycetes on a Solid Substrate

In order to determine if the pure cultures of actinomycetes isolated from the microcosm tests could actually grow on a minimal defined medium with an EOR chemical, the isolates were streaked onto agar plates containing the EOR compound as the sole carbon and energy source. We interpreted growth on this medium as indicating an ability of the organism to degrade the EOR compound and use the EOR compound as a sole carbon and energy source. In tests involving growth on a potential carbon source, it is always essential to do control cultures, handled in the same way except that no carbon source is added. Even with no added carbon source, some growth could well occur on impurities in the medium. The key to obtaining meaningful results is the occurrence of significantly more growth with the carbon source than without it. In any case, a test such as this can be no more than a screening test. The occurrence of good or abundant growth would indicate the need for chemical tests of breakdown of the original molecule and tests for possible end products. Finally, to prove utilization as a carbon source it would be very desirable to show incor-

poration into cell material of a pure radioactively-labeled compound.

Chemically Defined Medium

The following solutions were prepared: 40 g of NH_4Cl was dissolved in 1 ℓ of distilled water; 8.7 g of K_2HPO_4 and 6.8 g of KH_2PO_4 were added to 1 ℓ of distilled water; and 1 g of $MgSO_4 \cdot 7H_2O$ was dissolved in 1 ℓ of distilled water. A trace minerals solution consisting of H_3BO_3, 2.5 μg; $CaCO_3$, 50 μg; $CuSO_4 \cdot 5\ H_2O$, 5 μg; $FeSO_4(NH_4)_2\ SO_4 \cdot 6H_2O$, 250 μg; KI, 10 μg; $MnSO_4 \cdot H_2O$, 10 μg; MoO_3, 5 μg; and $ZnSO_4 \cdot 7H_2O$, 25 μg, all dissolved in 100 ml of distilled water. The basal salts solution for the defined medium was prepared as follows: 60 ml $MgSO_4 \cdot 7H_2O$ solution; 40 ml NH_4Cl solution, 400 ml phosphate solution; 400 ml of trace minerals solution prepared by diluting 2 ml of the trace metals in 498 ml of distilled water; and adding 1100 ml of distilled water. The medium was solidified by adding 18 g of Bacto-agar. The agar was melted and sterilized by autoclaving at 15 psi for 15 min., poured into sterile petri plates, and a sterile solution of the EOR compound was added to the plates, which would serve as the sole carbon and energy source. Final concentrations of 0.1% and 0.2% EOR compounds were tested. The isolates were streaked onto the plates and incubated at 25°C for 1-2 weeks. Control cultures containing no EOR compound were also prepared. After incubation, the amount of growth for each organism was estimated visually and rated on a scale of 0 to 4+ (4+ being excellent growth; 0 = no growth).

In addition to the controls with no added EOR compound, we prepared and streaked plates with 0.1 or 0.2 percent glucose. Glucose is utilizable as a carbon-energy source by most actinomycetes; it should support abundant growth.

Liquid Cultures for Determining Degradation of EOR Compounds

Liquid cultures were also prepared in order to corroborate and possibly extend the results obtained using isolated pure cultures on solid media. Physiochemical tests for possible degradation of EOR compounds were done using these media.

A sterile solution of the EOR chemical was added to the sterile basal medium to achieve a final concentration of 0.1% or 0.05% (v/v). Inoculum was 0.3 ml of lyophilized cells suspended in sterile buffered saline. These cultures were incubated on a rotary shaker at 25°C for 1-2 weeks. After incubation, the cultures were scored for the amount of growth. Controls containing only the EOR compound (no cells), cells in basal medium with no carbon-energy source, and 0.1% or 0.05% glucose were also prepared.

Quantitative Determination of Degradation of Enhanced Oil Recovery Agents Using Chemical or Physical Tests

Tests based on growth of microorganisms must be supplemented with physiochemical tests whenever possible in order to confirm degradation. Surface active components of petroleum sulfonates, i.e., alkylbenzene sulfonates can be estimated by the use of the Methylene Blue Active Substances (MBAS) test. This test is based on the property of certain aryl sulfonates of reacting with methylene blue to form blue compounds which are soluble in chloroform. A decrease in the chain length of the alkyl group below six carbon atoms will result in a marked decrease in MBAS activity. In the case of n-dodecylbenzene sulfonic acid, the MBAS test,

by use of a standard curve, could be applied as a quantitative measure of the degradation of the alkyl side chain of the molecule to a number of carbon atoms below six. It is believed that in natural ecosystems (e.g., bodies of water), and in sewage treatment plants, initial breakdown of linear alkylbenzene sulfonate molecules occurs via a β-oxidation process operating on the side chain after oxidation of the terminal methyl group to a carboxyl. Loss of MBAS activity is referred to in the detergent industry as "primary biodegradation."

In the case of the Marathon petroleum sulfonate, loss of MBAS activity would indicate that one or more components with surfactant activity (ability to reduce surface tension of aqueous solutions) was undergoing primary biodegradation.

Pure cultures of actinomycetes were tested for ability to bring about primary degradation of ABS surfactant molecules in the following general way: Either n-dodecylbenzene sulfonic acid (n-DBSA), or Marathon petroleum sulfonate was added to basal medium in a final concentration of 0.1 percent or 0.05 percent. An inoculum of 0.3 ml of rehydrated lyophilized cells was added, and cultures were incubated at $25^{o}C$ on a rotary shaker (180 rpm) for one week, after which the MBAS test was performed. Uninoculated controls were also incubated.

The MBAS test itself is carried out as follows: One ml of methylene blue solution (0.12 g methylene blue chloride + 200 g anhydrous Na_2SO_4 + 26 ml concentrated H_2SO_4, all in 4ℓ of deionized water) is added to 9 ml of water in a large test tube. Five ml of chloroform and 1 ml of liquid culture is added and the tube is vortexed for one minute. The chloroform layer (bottom layer) is withdrawn with a Pasteur pipette, placed in a spectronic 20 cuvette, and the absorbance measured at 650 nm. Known con-

centrations of both n-DBSA and Marathon petroleum sulfonate were run as standards and standard curves were prepared.

Determination of Extent of Degradation of Polyacrylamides Using Viscosity Measurements

One way to estimate the concentration of polyacrylamide present is to determine the absolute viscosity in centipoises of a solution of the polymer. A decrease in viscosity of the polymer solution would indicate degradation (lowering of average molecular weight; however, in some cases breaking of cross-linking could reduce viscosity to a certain extent) of the polymer had occurred.

When working with polyacrylamide solutions, care must be taken to prevent breaking of the large molecules by mechanical shearing. For instance, solutions of the polyacrylamides should not be shaken or agitated. Also, the polymers are sensitive to UV light (Foshee et al., 1976) and high concentrations of ferrous or ferric iron in the medium which cause spontaneous degradation of the molecule. Also, oxygen can break down the polymer very rapidly in the presence of a reducing agent (Foshee et al., 1976).

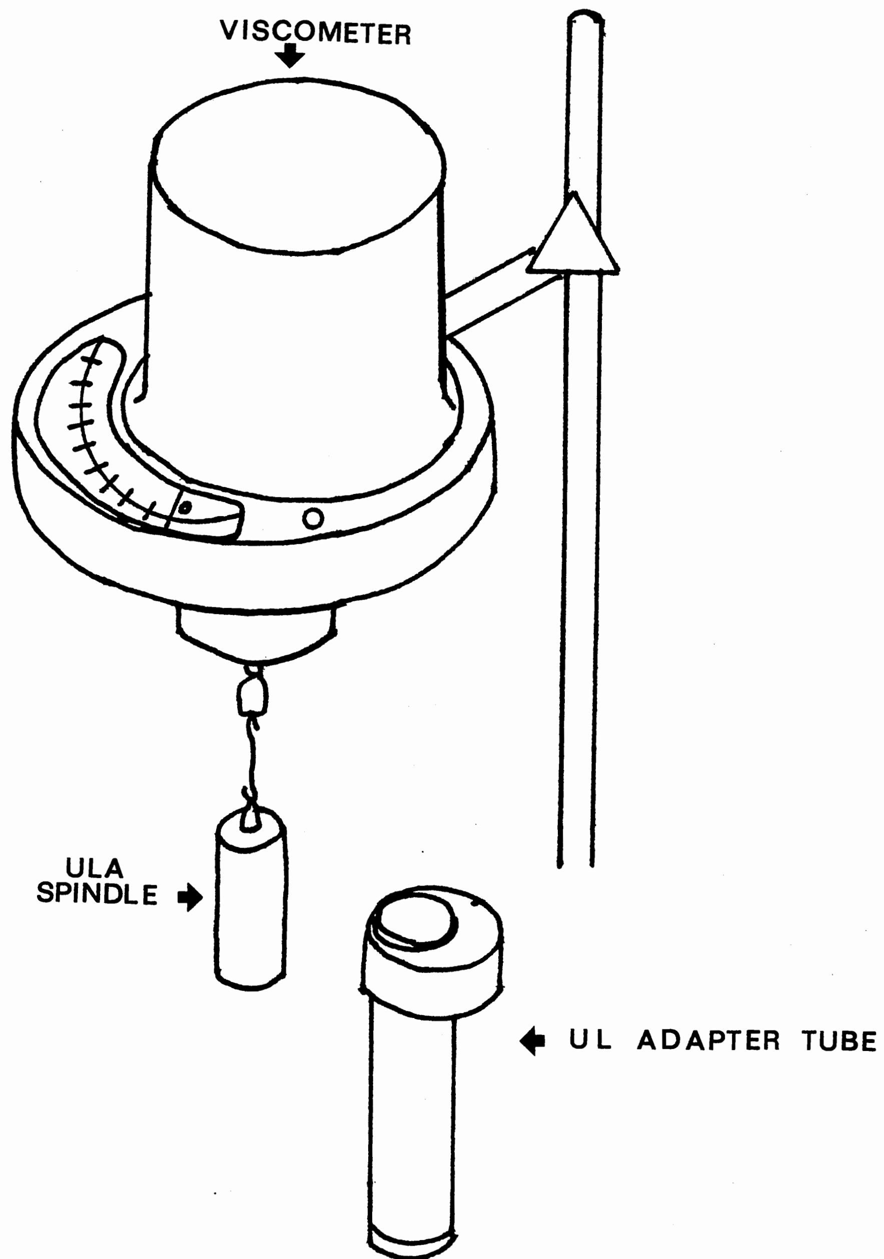
To prevent contamination in the liquid cultures, it was necessary to autoclave solutions of the polymers (10 min at 15 psi). Autoclaving in the presence of molecular oxygen may also contribute to polymer dedegradation (G. Sewell and M. Grula, unpublished data).

Although there have been no reports in the literature of microorganisms being able to degrade polyacrylamide through utilization of the polymer as a carbon-energy source, it is widely believed in the oil indus-

try that they stimulate the growth of bacteria, and that they are harmed (degraded in some way) by the presence and/or growth of the microorganisms. On the basis of the structure of the polymer, it does not appear that they could efficiently serve as a source of carbon/energy (M. Grula, personal communication). However, laboratory data indicate that at least some bacteria, when grown in the presence of the polymer, actually bring about degradation as shown by loss of viscosity. This effect could be direct or indirect; the mechanism is not understood yet.

To determine if possible degradative ability in isolated actinomycetes existed, cultures were prepared as they were for the MBAS tests. The polymers used were J332, J333, and J334, at concentrations of 0.1 or 0.05 percent. Liquid cultures were prepared as follows: 50 ml of basal salts medium in a 125 ml Erlenmeyer flask; final concentration of 0.1% or 0.05% polyacrylamide; and 0.3 ml rehydrated, lyophilized cells. Control cultures with only polyacrylamide (no cells) were also prepared. The cultures were incubated at 25°C for 1-2 weeks with no shaking. After incubation, the solutions were passed through a 500 mesh screen to remove the cells and the viscosity of the cultures was determined using the Brookfield Viscometer, Model LVT with a UL adapter (Figure 1). The viscosity was read after 10 min at room temperature (25°C). Viscosity readings were taken at 3 speeds; 6 rpm, 12 rpm, and 30 rpm whenever possible. As the speed increases, the amount of shearing or shear rate also increases. The viscosity of the sample cultures were compared to the viscosity of controls of known polymer concentrations to determine whether there was a decrease in viscosity resulting from the presence of the organisms. The increase in growth in the cultures was also monitored and rated on a scale of 0 to 4+ (4+ being excellent growth; 0 = no growth).

Figure 1. Diagrammatic Representation of the Brookfield Viscometer

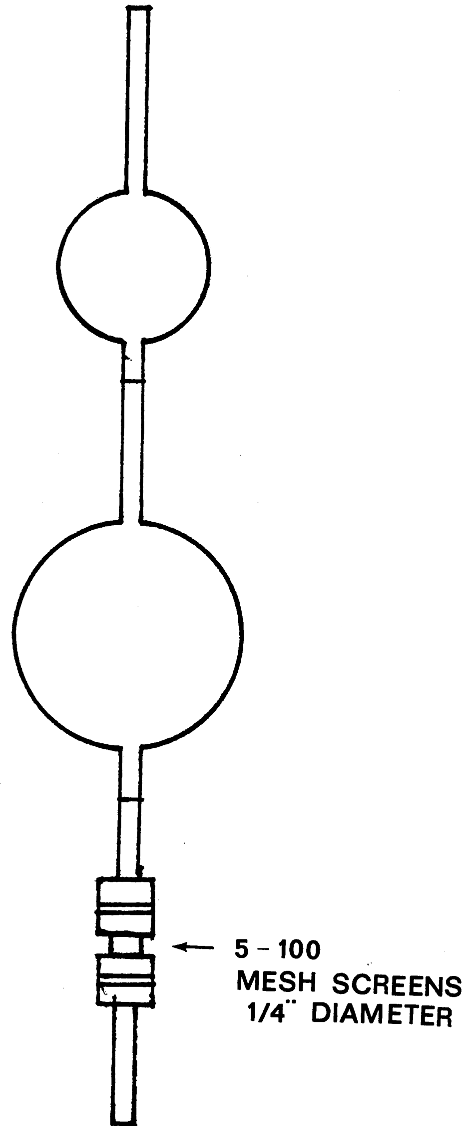

VISCOMETER
ULA
SPINDLE
UL ADAPTER TUBE

Quantitation of Polyacrylamide Degradation by Screen Factor Measurement

Another method used to measure undegraded polyacrylamide concentration is the use of screen factor. Screen factor has been of considerable value as an easily measured quantity reflecting the activity of the polyacrylamide solutions more reliably than absolute viscosity measurements (Foshee et al., 1976). The standard screen factor or screen viscometer can be seen in Figure 2. Two sources of error in measuring screen factors may occur; 1) because of plugging the screens with insoluble material, or 2) physical degradation of the polymer solution by pulling it through the screens too rapidly during the loading of the instrument. Screen factor is expressed as a ratio of the time required for a certain volume of polymer solution to pass through the instrument to the time required for the same volume of pure water.

Liquid cultures for screen factor analysis were prepared as follows: 60 ml sterile basal medium in a 125 ml Erlenmeyer flask; 0.1% or 0.05% polyacrylamide; and 0.3 ml of cells. The cultures were incubated at $25^{o}C$ for 1-2 weeks without shaking. After incubation the cultures were filtered to remove the biomass through a 200 mesh screen using a vacuum. The samples were loaded into the instrument by applying a vacuum of about one foot of water. The samples were run at room temperature. Between runs the screen viscometer was rinsed with 0.8% buffered saline, 3% hypochlorite bleach, and distilled water. To determine screen factors (F_s) the flow time of the polymer solution is divided by the flow time of the basal medium ($F_s = t_p/t_s$). Basal medium was used instead of water because the cultures were prepared in basal medium. The screen factor is very sensitive to temperature fluctuations so care must be taken to main-

Figure 2. Diagrammatic Representation of the Screen Viscometer


5 - 100
MESH SCREENS
1/4" DIAMETER

tain a constant temperature during runs. The amount of degradation of each polymer was determined by comparing the screen factor values of the actinomycete cultures with controls of known polymer concentration.

CHAPTER III

RESULTS

Counts of Actinomycetes in Soil Samples Enriched With Various EOR Compounds

Microcosm tests or long-term enrichment cultures were prepared to obtain information on the types of actinomycetes present in various soil samples enriched with an EOR compound and how their numbers changed with time. Most experiments were conducted for 12 weeks; an increase in counts of these organisms, as compared with the initial counts, would indicate that the EOR chemical was being attacked or degraded by the actinomycetes. Such an increase in number should not occur in a control culture, containing no EOR chemical, being run concurrently with the experimental cultures. We have somewhat arbitrarily adopted a 10-fold increase in count over the control as being indicative of stimulation of growth of the actinomycete population by the EOR agent. This stimulation might occur because the chemical (or a component thereof) was actually being utilized, probably as a carbon-energy source, by the organisms. No more definitive conclusions than these can be drawn from the microcosm cultures. A decrease in the count with time of actinomycetes, because of the complexity of the conditions (large numbers of other organisms will be present, as well as a variety of nutrients from the soil which did not occur in the control), would indicate that the EOR compound was

somewhat toxic to these soil organisms.

Ten soil samples were tested for the presence of actinomycetes which were able to degrade the EOR chemicals. As each soil sample was collected (aseptically), the source, date of collection, and pH were recorded for each soil (Table II).

Tests were set up simply by adding 20 percent by weight of an EOR chemical to a portion of soil, mixing, and incubating at ambient temperature. Counts (plate) of actinomycetes were done initially, and at two week intervals thereafter. It was hoped in this way to closely simulate conditions that might exist in soils close to the surface if a large scale spill occurred.

Since most soils contain a large and varied population of bacteria and fungi, it was necessary to selectively inhibit the growth of these organisms when doing actinomycete counts.

The effects of various concentrations of cycloheximide (an antifungal antibiotic) and streptomycin were tested using eight normal soil organisms, including four species of *Streptomyces*, four representative species of soil bacteria, and two genera of fungi. The test cultures were obtained from the stock culture collection at Oklahoma State University. The *Streptomyces* species were isolated from various soil samples. The bacteria were streaked onto starch casein agar (pH 7.5) containing various concentrations of streptomycin ranging from 30 to 60 μg/ml. The streptomycin was added after autoclaving the agar. Concentrations of cycloheximide ranging from 40 to 250 μg/ml were tested with *Penicillium* and *Aspergillus* spp. and *Streptomyces*. The results are given in Tables III and IV. The bacteria tested were inhibited at 60 μg/ml of streptomycin and growth of none of the *Streptomyces* spp. (except for #2) was

TABLE III

EFFECTS OF VARIOUS CONCENTRATIONS OF STREPTOMYCIN IN STARCH CASEIN AGAR ON GROWTH OF FOUR EUBACTERIA AND FOUR *STREPTOMYCES*

Organism	Streptomycin Concentration (µg/ml) 30	40	50	60
Bacillus subtilis	+	+	+	-
Pseudomonas aeruginosa	+	+	-	-
Micrococcus luteus	-	-	-	-
Enterobacter aerogenes	+	+	+	-
Streptomyces 1	+	+	+	+
Streptomyces 2	+	-	-	-
Streptomyces 3	+	+	+	+
Streptomyces 4	+	+	+	+

+ = Visible growth; - = no visible growth.

Plates were observed after 3 days incubation at 25°C.

Streptomycin Sulfate obtained from Sigma Chemical Company.

TABLE IV

EFFECTS OF VARIOUS CONCENTRATIONS OF CYCLOHEXIMIDE IN STARCH CASEIN AGAR

Cycloheximide Concentration (μg/ml)	*Penicillium*	*Aspergillus*	*Streptomyces* 1	*Streptomyces* 2
40	-	+	+	+
60	-	+	+	+
100	-	+	+	+
150	-	+	+	+
200	-	+	+	+
250	-	-	+	+

+ = Visible growth; - = no visible growth.

Plates were observed after 3 days incubation at 25°C.

Cycloheximide was obtained from Sigma Chemical Company.

inhibited at 60 μg/ml. Growth of Penicillium and Aspergillus was inhibited at 250 μg/ml and the Streptomyces spp. tested showed no inhibition of growth in the range of 40 to 250 μg/ml cycloheximide. From these results it was concluded that a concentration of 250 μg/ml cycloheximide and 60 μg/ml streptomycin wouldinhibit the fungi and faster-growing bacteria, and thus allow for the growth of the actinomycetes.

The first chemical we tested was n-dodecylbenzene sulfonic acid (n-DBSA), a surface-active, water-soluble compound which is probably found in many crude petroleum sulfonates. Preliminary tests with n-DBSA showed that the majority of soil samples became sterile within 3 to 4 weeks. In these tests we had purposely not adjusted the pH because we felt it would more nearly simulate actual conditions in the soil if a spill occurred, than if we had adjusted the pH. However, to get information on the role of the acidity of the molecule in lowering microbial counts, we set up a test in which neutralized n-DBSA was compared with the non-neutralized material. The results are shown in Tables V and VI. Table V shows the results for soil samples 38, 39 and D; whereas Table VI gives the results for soil sample 42.

Again, the non-neutralized n-DBSA destroyed all microorganisms growing on starch casein in soil 38 and D within 4-6 weeks. Soil 39, however, maintained a significant population up to 10 weeks incubation at 25°C. This soil was more highly buffered (as shown by the pH values) than the other soils tested, and may have had a more resistant population. At 10 and 12 weeks, however, the population of organisms growing on starch casein agar had fallen off to essentially zero, even though the pH had fallen no lower than 6.0. Even at 10 weeks incubation, the pH was still 6.4, as high as it was with Soil D and neutralized n-DBSA.

TABLE V

MICROCOSM TEST WITH n-DODECYLBENZENE SULFONIC ACID*

	APC (cfu/g) pH								
	Control			n-DBSA			Neutralized n-DBSA		
Time	38	39	D	38	39	D	38	39	D
0 time	9.7×10^5	5.3×10^6	5.8×10^4						
pH	6.2	7.4	6.6						
2 wks	4.5×10^5	3.9×10^6	4.8×10^6	30	6.7×10^6	30	4.6×10^5	1.6×10^8	1.9×10^8
	6.2	7.4	6.6	5.0	6.4	5.5	5.5	6.8	6.4
4 wks	2.0×10^5	5.7×10^7	6.0×10^6	0	9.6×10^6	10	9.0×10^6	1.5×10^8	2.2×10^8
	6.4	6.6	6.4	4.7	5.0	4.0	5.7	6.6	6.0
6 wks	1.6×10^5	4.5×10^6	9.7×10^5	0	3.0×10^5	55	6.7×10^4	1.0×10^8	8.7×10^7
	6.4	6.8	6.4	5.0	6.2	4.0	6.0		6.4
8 wks	1.9×10^5	5.2×10^6	1.0×10^6	10	3.8×10^5	0	4.5×10^5	8.3×10^7	2.9×10^8
	6.4	7.2	6.4	5.0	6.4	4.0	6.4	6.8	6.2
10 wks	2.9×10^5	1.2×10^6	3.3×10^4	0	10	0	8.0×10^5	9.5×10^3	4.2×10^6
	6.4	7.0	6.4	5.0	6.4	4.0	6.6	7.0	6.4
12 wks	4.7×10^5	6.0×10^5	3.4×10^5	0	0	0	1.0×10^7	1.9×10^6	5.5×10^7
	6.4	6.8	6.4	4.0	6.0	5.0	6.6	6.9	6.4

APC = Aerobic plate counts; cfu = colony forming units. *n-DBSA tested at 20% v/v in the microcosm tests.
APC done on Starch Casein Agar with streptomycin and cycloheximide.

TABLE VI

MICROCOSM TEST WITH n-DBSA (0.1%)*

Time			Soil Sample 42 cfu/g
Zero		pH	1.0×10^5 6.4
2 Weeks	Control	6.4	2.5×10^5
	n-DBSA	6.8	1.0×10^6
4 Weeks	Control	6.4	3.5×10^5
	n-DBSA	6.8	2.7×10^6
6 Weeks	Control	6.4	5.1×10^6
	n-DBSA	6.8	4.5×10^6
8 Weeks	Control	6.4	3.0×10^6
	n-DBSA	7.0	6.1×10^5
10 Weeks	Control	6.4	3.4×10^6
	n-DBSA	7.0	3.6×10^6
12 Weeks	Control	6.4	5.1×10^6
	n-DBSA	7.0	1.2×10^6

*Buffered to pH 7.0.

This could indicate that the fraction of n-DBSA molecules which is un-ionized at pH 6.4 is toxic for the organisms in soil 39. Since initial neutralization of the n-DBSA resulted in continued high populations of actinomycetes in Soils 38 and D over a 12 week period, it appears that pH is a major factor in the toxicity of n-DBSA in the soil microcosm tests, but is is not the only factor as shown by a major decline in counts in Soil 39 while the pH remained at a non-toxic level (Table V).

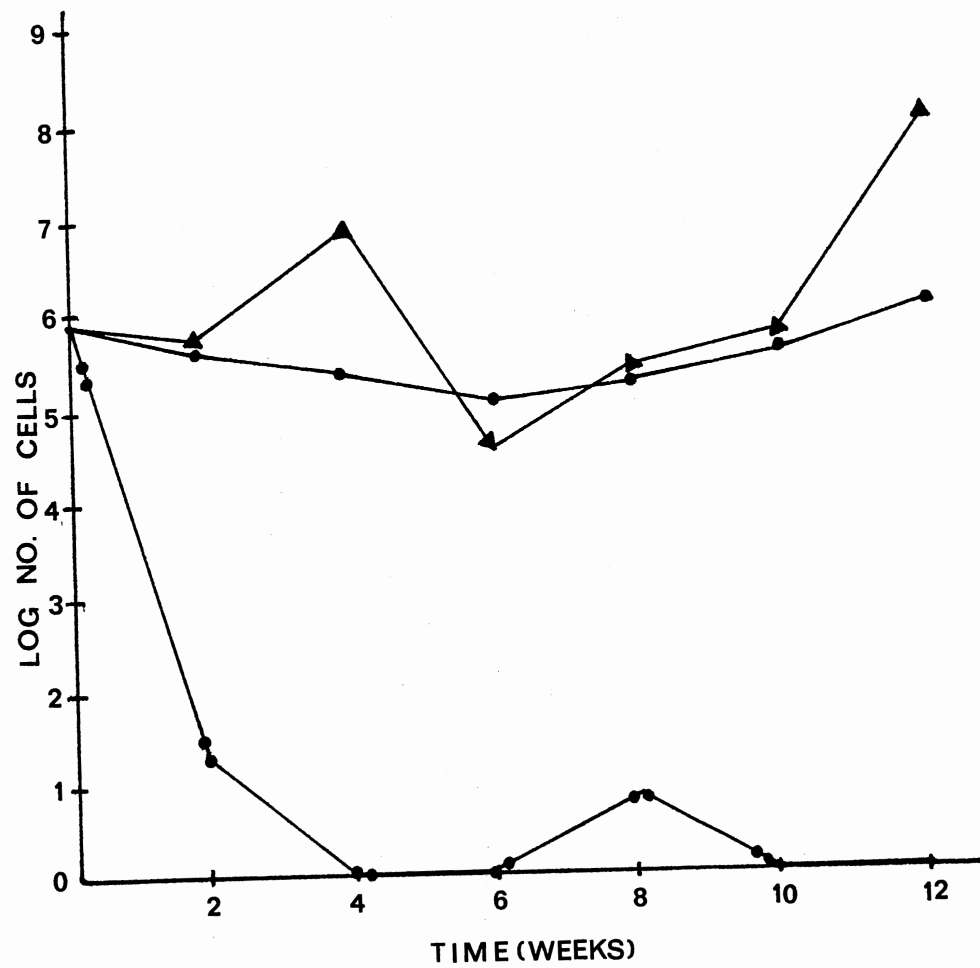
The counts with neutralized n-DBSA added to soil, in all three cases, are higher throughout most of the time than the controls; sometimes by a factor of 10 or more. This simulation by n-DBSA indicates that the organism may be utilizing the substance.

Soil sample #42 was also used in microcosm cultures with 0.1% n-DBSA (buffered to pH 7.0). With this soil sample there were no significant differences in numbers of actinomycetes between the control and the soil with n-DBSA. Lack of toxicity at pH 7, a pH value at which n-DBSA is undoubtedly highly ionized, is compatible with the possibility that toxicity fails to occur because the molecule is not getting into the cell. It is possible that toxicity of n-DBSA at low pH values (5 or less) occurs, at least in part, because the unionized molecule is able to more readily enter the cell. It would be interesting to do control studies in which the pH was adjusted to 4.0 - 4.5 with some other acid, and without n-DBSA. The data from the microcosm tests using n-DBSA are also shown graphically in Figures 3 through 6.

Microcosm Tests to Examine the Role of the Acidity of n-DBSA in its Toxicity

In the earlier microcosm tests using n-DBSA, it was observed that

Figure 3. Actinomycete Counts During a 12 Week Period in the Microcosm Test With n-DBSA Using Soil Sample 38



9
8
7
6
5
4
3
2
1
0
LOG NO. OF CELLS
2
4
6
8
10
12
TIME (WEEKS)
CONTROL
n-DBSA
NEUTRAL n-DBSA

Figure 4. Actinomycete Counts During a 12 Week Period in the Microcosm Test With n-DBSA Using Soil Sample 39

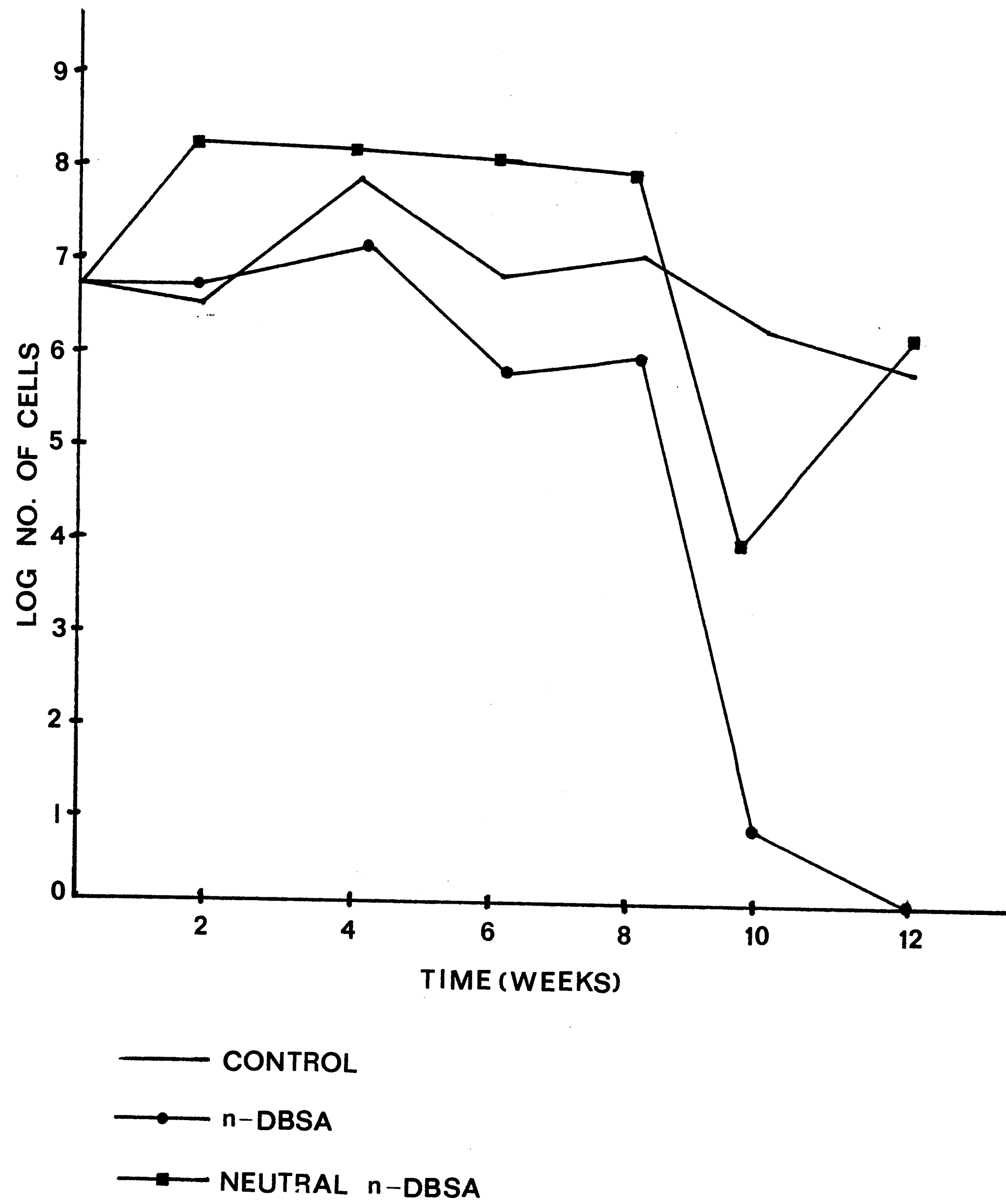

LOG NO. OF CELLS
0
1
2
3
4
5
6
7
8
9
2
4
6
8
10
12
TIME (WEEKS)
CONTROL
n-DBSA
NEUTRAL n-DBSA

Figure 5. Actinomycete Counts During a 12 Week Period in the Microcosm Test With n-DBSA Using Soil Sample D

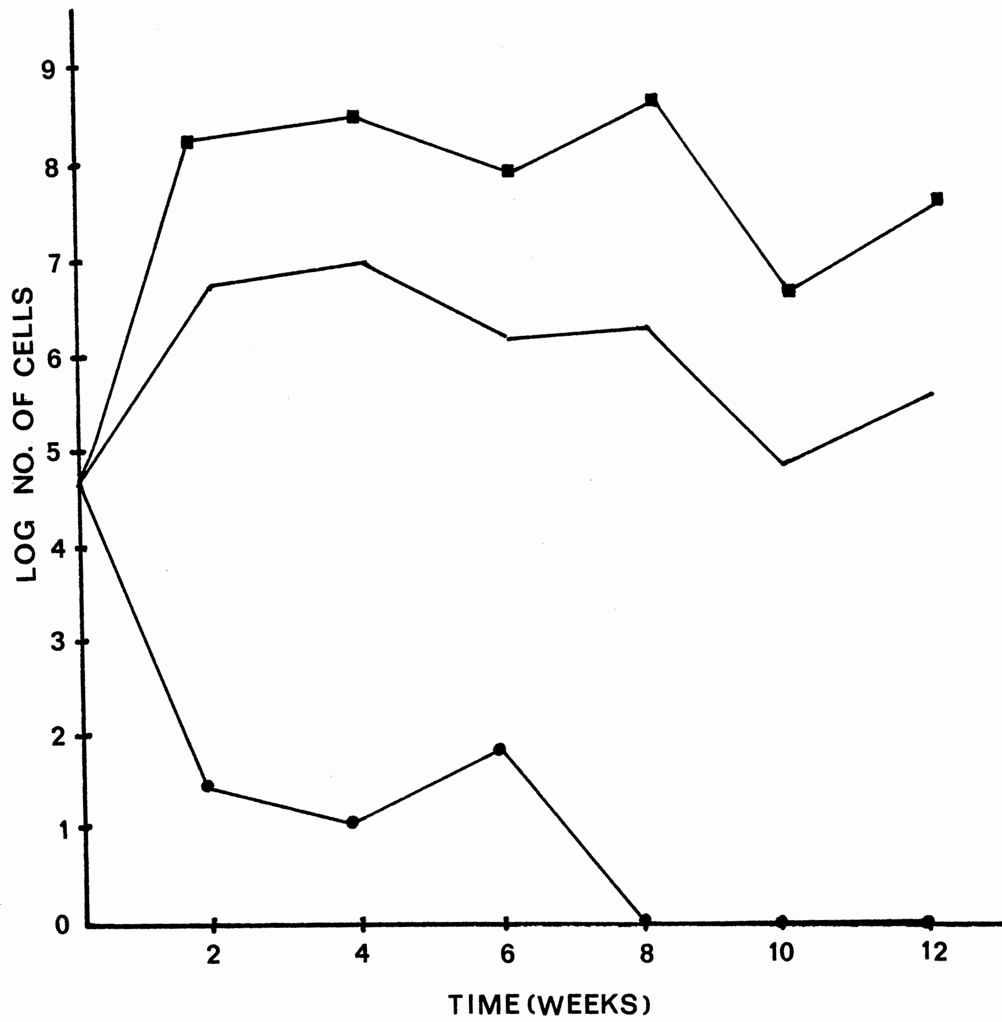
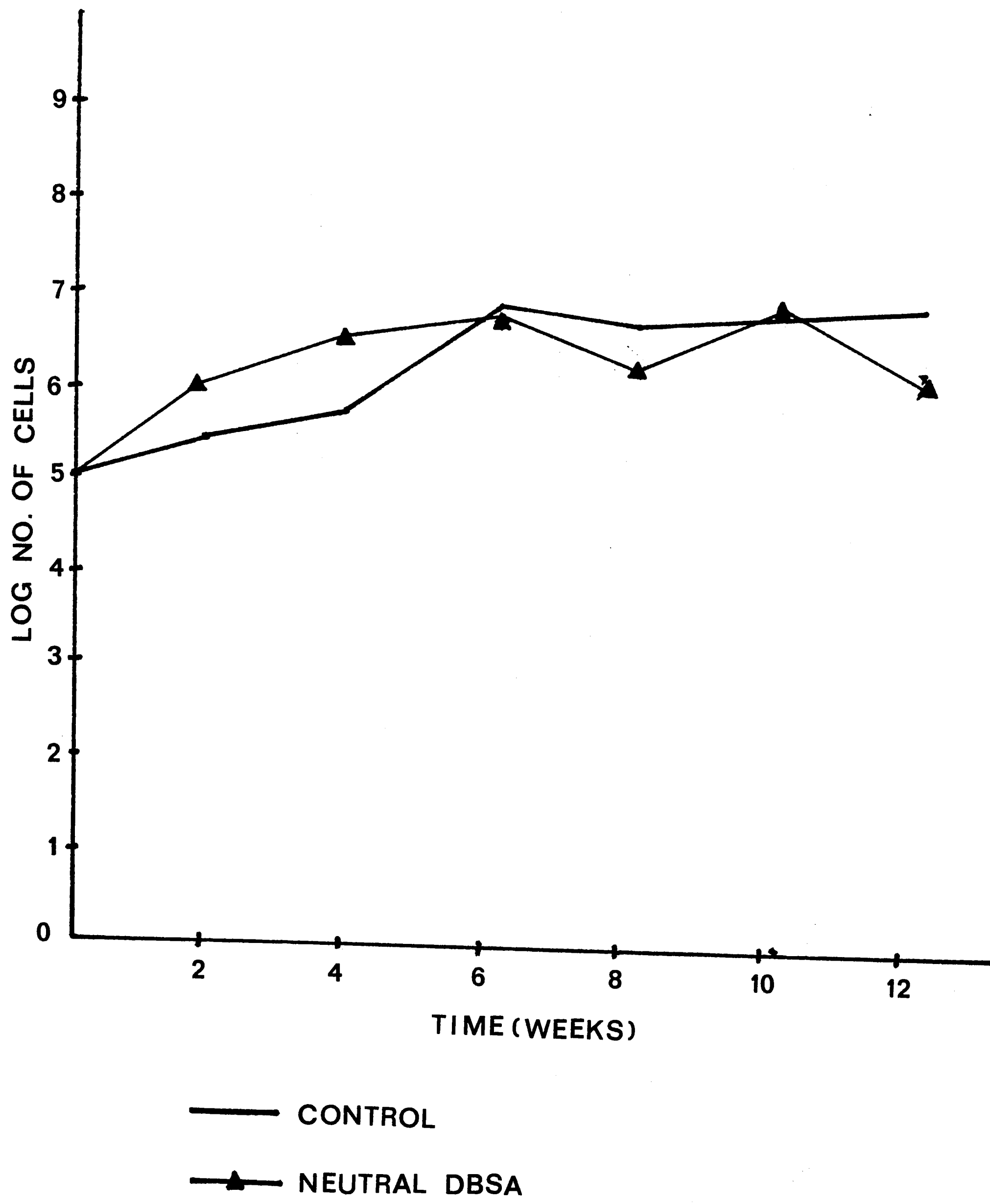

LOG NO. OF CELLS
0
1
2
3
4
5
6
7
8
9
2
4
6
8
10
12
TIME (WEEKS)
CONTROL
n-DBSA
NEUTRAL DBSA

Figure 6. Actinomycete Counts During a 12 Week Period in the Microcosm Test With n-DBSA Using Soil Sample 42


9
8
7
6
5
4
3
2
1
0
LOG NO. OF CELLS
2
4
6
8
10
12
TIME (WEEKS)
CONTROL
NEUTRAL DBSA

with the unneutralized n-DBSA, actinomycete and total plate counts fell off to nearly zero in poorly buffered soil. In a soil sample with a higher buffer capacity this did not happen. Neutralizing the n-DBSA with NaOH removed some toxicity for soil microorganisms, and with Soils 39 and D, at two, four, and six weeks, resulted in a greatly enhanced count in the presence of n-DBSA. A toxicity of non-neutralized n-DBSA which may have been due to a factor other than pH, occurred with Soil sample 39. The pH of the mixture at 6 weeks was 6.2, whereas the control with 6.8. Actinomycete count with the non-neutralized n-DBSA was just 1-50 to 1-100 of the control value, and the pH was not low enough itself to produce this kind of toxicity.

These observations are consistent with the hypothesis that the n-DBSA molecule, unionized, is inherently toxic in addition to the toxicity resulting from a low pH. It is known that in the case of other organic acids and other species of microorganisms, that the unionized molecule is more toxic than the anion, as indicated by toxicity as a function of pH (low toxicity at a high pH where the molecule is nearly 100 percent ionized; the charged molecule does not easily penetrate the cell envelope, whereas an uncharged molecule does).

Hence, we did an experiment to indirectly test this hypothesis. A microcosm test, using two soil samples was set up (as previously) with n-DBSA, neutralized n-DBSA, and with soil acidified to pH 4.5 with HCl. Twenty percent by weight of non-neutralized n-DBSA lowers the pH of most poorly buffered soil samples to approximately 5.0. Data obtained in this experiment are given in Table VII.

On the basis of the results available after six weeks incubation, we could conclude that: a) at two weeks, neutralized n-DBSA produced a

TABLE VII

ACTINOMYCETE COUNTS IN MICROCOSM TESTS ON TWO SOIL SAMPLES WITH n-DBSA, NEUTRALIZED n-DBSA, AND SOIL WITH HCl

Time		Soil Samples			
			43 (cfu/g)		E (cfu/g)
0			2.6×10^5		4.0×10^4
		pH	6.4	pH	6.4
2 Wks	Control	6.4	2.5×10^5	6.5	4.2×10^4
	n-DBSA	5.0	7.3×10^4	5.0	6.2×10^4
	Neut. n-DBSA	6.8	2.6×10^5	6.8	1.8×10^6
	Acidic (HCl)	4.5	7.8×10^4	4.5	3.2×10^4
4 Wks	Control	6.4	1.2×10^5	6.4	4.5×10^4
	n-DBSA	5.0	3.0×10^3	4.8	3.4×10^3
	Neut. n-DBSA	6.9	1.7×10^5	6.8	3.2×10^5
	Acidic	4.5	0	4.5	0
6 Wks	Control	6.4	5.4×10^4	6.4	3.9×10^4
	n-DBSA	4.5	8.4×10^2	4.8	3.0×10^2
	Neut. DBSA	6.8	1.4×10^5	6.8	1.6×10^5
	Acidic	4.5	0	4.5	0

Cultures were plated onto Starch Casein Agar containing streptomycin and cycloheximide.

large increase in population of actinomycetes in soil sample E (soil from near a pipeline), but not in Soil sample 43 (from near a wheat field). At six weeks there is a slight increase in the populations of actinomycetes in the neutralized cultures, as compared to the control cultures, with both soil samples. The soil samples acidified to pH 4.5 with HCl provided a more toxic environment for actinomycetes than soils brought to pH 4.5 with n-DBSA. It is of interest that the toxicity required more than two weeks to manifest itself.

At four weeks, a decrease in plate counts of actinomycetes in the n-DBSA was noted; this decline in population was quite pronounced at six weeks. This did not happen with neutralized n-DBSA; in fact, the populations here were somewhat higher than those of the controls. It does appear that the low pH plays a role in the toxicity of n-DBSA; however, it has not been ruled out that the unionized form of n-DBSA may also be a factor in its toxicity. More organisms survived at a pH in the range 4.5 - 5.0 in the presence of n-DBSA than when acidification was accomplished with HCl; this could be interpreted as a protective effect of n-DBSA. In order to get an answer to the question of relative toxicities of low pH and unionized n-DBSA, one probably should go to a simpler system than soil (e.g., a pure culture in a chemically defined medium).

Tests With Polyacrylamides

Another group of EOR chemicals tested were the polyacrylamide polymers. A preliminary microcosm test with the three polyacrylamides (J332, J333, J334) showed counts on starch casein at 7, 9, 10, 11 and 12 weeks significantly higher (10 to 100 fold) with the polymers than in the control cultures (no polymer). The pH values were also elevated

from 1.2 to 1.6 units in the cultures with polymer as compared to the control (Table VIII).

Since the reason for the increase in pH and its relationship (if any) to the increase in the actinomycete population occurring in the presence of the polymer was unknown, the following experiment was set up: microcosm cultures using two new soil samples (Soil 41 and 43) and each of the three polymers were prepared along with two controls: 1) no polymer and pH not adjusted; and 2) no polymer, pH adjusted to 8.0. The actinomycete counts on starch casein agar were done at two week intervals. The results are given in Table IX and Figures 7 and 8.

In all of the microcosm tests it is evident that different soil samples behave differently. In Sample 41, for example, there is no significant difference between the control soils and those mixed with polymers. With Sample 42, however, polymers J333 and J334 showed counts significantly higher (20 to 40 fold) than either control at six weeks. This increased population was not maintained in subsequent counts. Further tests must be done to get the final answer. The contrast between this and the previous experiment in which stimulation by polymer was observed may have resulted from use of a different basal medium.

One other EOR compound that was tested was Marathon petroleum sulfonate. The results are seen in Table X and Figures 9 and 10. The pH values of the soil microcosm tests for soils 38 and 42 remained fairly stable. There was never more than a 3 to 4 fold increase in the counts of actinomycetes seen in the cultures with Marathon petroleum sulfonate (0.1%) as compared with the control cultures which contained no Marathon. In fact, after eight weeks incubation at 25°C, a slight decrease in the numbers of actinomycetes was seen. However, the Marathon did not appear

TABLE VIII

MICROCOSM TEST RESULTS FOR SOIL C & D WITH (20% W/W) POLYACRYLAMIDES J332-334

Time		Soil Samples				
		Soil C (cfu/g)			Soil D (cfu/g)	
0			1.2×10^7			1.0×10^7
		pH	6.2		pH	6.8
2 Weeks	Control	6.0	1.1×10^7	Control	6.6	1.1×10^7
	J332	6.8	2.2×10^7	J332	7.0	1.5×10^7
	J333	7.0	TNTC	J333	7.0	TNTC
	J334	7.0	TNTC	J334	7.2	TNTC
5 Weeks	Control	6.0	4.9×10^7	Control	6.6	3.0×10^7
	J332	6.8	3.9×10^7	J332	7.0	3.8×10^7
	J333	7.0	3.3×10^7	J333	7.0	3.0×10^8
	J334	7.2	2.9×10^8	J334	7.0	2.6×10^8
7 Weeks	Control	6.0	4.7×10^4	Control	6.6	1.1×10^5
	J332	7.4	2.4×10^7	J332	7.0	9.8×10^5
	J333	7.2	1.2×10^7	J333	7.0	6.0×10^6
	J334	7.4	1.1×10^7	J334	7.2	7.9×10^6
9 Weeks	Control	6.0	8.9×10^4	Control	6.4	9.0×10^4
	J332	6.8	1.9×10^7	J332	6.8	9.0×10^5
	J333	7.0	1.3×10^7	J333	7.0	8.8×10^5
	J334	7.2	3.4×10^7	J334	7.2	6.8×10^5
10 Weeks	Control	6.2	1.2×10^6	Control	6.6	9.1×10^5
	J332	6.8	3.1×10^7	J332	6.8	1.1×10^6
	J333	7.0	1.5×10^7	J333	7.2	1.6×10^5
	J334	7.0	7.1×10^8	J334	7.2	6.7×10^6
11 Weeks	Control	6.2	6.4×10^5	Control	6.4	3.7×10^5
	J332	7.4	5.4×10^7	J332	7.6	9.5×10^5
	J333	7.6	8.6×10^6	J333	7.2	6.7×10^6
	J334	8.0	1.1×10^8	J334	7.8	1.5×10^6
12 Weeks	Control	6.4	1.4×10^5	Control	6.2	1.1×10^5
	J332	7.6	1.9×10^8	J332	7.2	5.3×10^5
	J333	7.8	1.2×10^7	J333	7.6	7.5×10^4
	J334	8.0	7.8×10^6	J334	7.8	8.9×10^5

(cfu/g) = Colony forming units/gram.

TNTC = Too numerous to count; greater than 6,500 x highest dilution plated.

TABLE IX

MICROCOSM TEST WITH POLYACRYLAMIDES

Time			Soil Samples 41 (cfu/g)			Soil Samples 42 (cfu/g)
0			3.2×10^6			7.4×10^6
		pH	6.4		pH	6.4
2 wks	Control	6.4	1.0×10^7	Control	6.2	5.7×10^6
	Control	8.0	1.0×10^6	Control	7.6	1.6×10^8
	J332	7.4	5.4×10^5	J332	8.0	1.0×10^8
	J333	8.0	2.5×10^6	J333	7.2	3.2×10^7
	J334	7.4	3.4×10^5	J334	7.2	4.8×10^7
4 wks	Control	6.4	3.8×10^5	Control	6.4	1.1×10^6
	Control	8.0	1.2×10^5	Control	8.0	3.8×10^6
	J332	7.6	1.2×10^5	J332	7.2	3.3×10^6
	J333	8.0	2.4×10^5	J333	7.0	2.9×10^5
	J334	7.6	2.7×10^5	J334	7.6	1.4×10^6
6 wks	Control	6.4	2.0×10^6	Control	6.2	1.3×10^6
	Control	8.0	1.2×10^7	Control	7.8	3.8×10^6
	J332	8.0	5.5×10^6	J332	7.4	2.2×10^6
	J333	8.5	1.2×10^7	J333	8.0	3.4×10^7
	J334	7.8	2.2×10^6	J334	8.0	7.2×10^7
8 wks	Control	6.4	3.1×10^6	Control	6.2	4.8×10^6
	Control	8.0	5.7×10^6	Control	7.8	1.2×10^6
	J332	8.0	2.4×10^6	J332	7.4	3.1×10^6
	J333	8.5	2.7×10^6	J333	8.0	2.4×10^5
	J334	7,8	2.8×10^5	J334	8.0	3.8×10^5
10 wks	Control	6.4	1.4×10^6	Control	6.4	6.0×10^5
	Control	8.0	5.0×10^6	Control	8.0	2.5×10^7
	J332	8.0	3.4×10^5	J332	7.4	9.2×10^6
	J333	8.0	1.4×10^6	J333	7.8	4.8×10^5
	J334	7.8	3.3×10^6	J334	7.6	8.4×10^6
12 wks	Control	6.4	1.1×10^6	Control	6.4	5.3×10^5
	Control	8.0	9.5×10^6	Control	8.0	7.2×10^6
	J332	8.0	2.9×10^6	J332	7.4	5.0×10^6
	J333	8.0	2.3×10^6	J333	7.8	2.0×10^6
	J334	7.8	2.3×10^6	J334	7.6	2.0×10^6

cfu = Colony forming units.

Figure 7. Actinomycete Counts During a 12 Week Period in the Microcosm Test With Polyacrylamides J332-334 Using Soil Sample 41

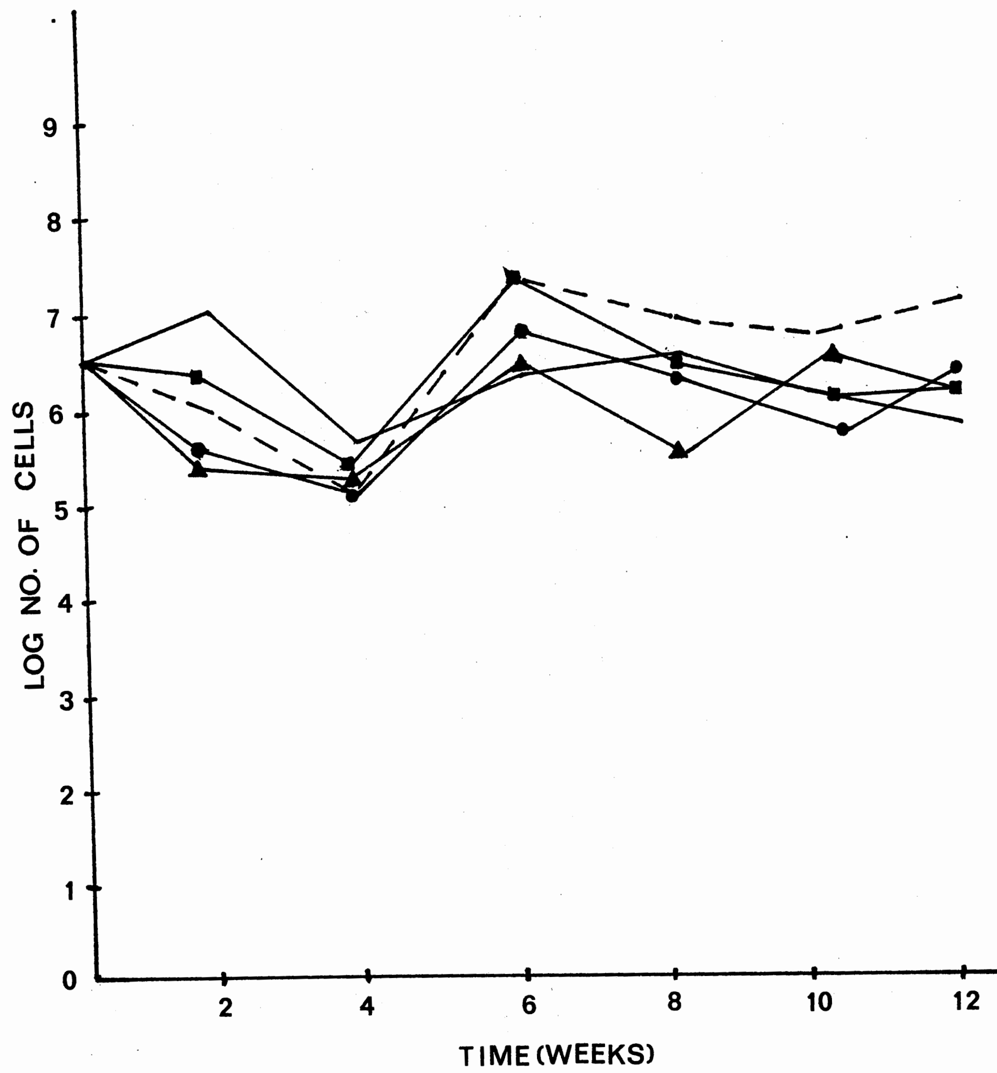

LOG NO. OF CELLS
0
1
2
3
4
5
6
7
8
9
2
4
6
8
10
12
TIME (WEEKS)
CONTROL— pH 6.4
CONTROL— pH 8
J332
J333
J334

Figure 8. Actinomycete Counts During a 12 Week Period in the Microcosm Test With Polyacrylamides J332-334 Using Soil Sample 42

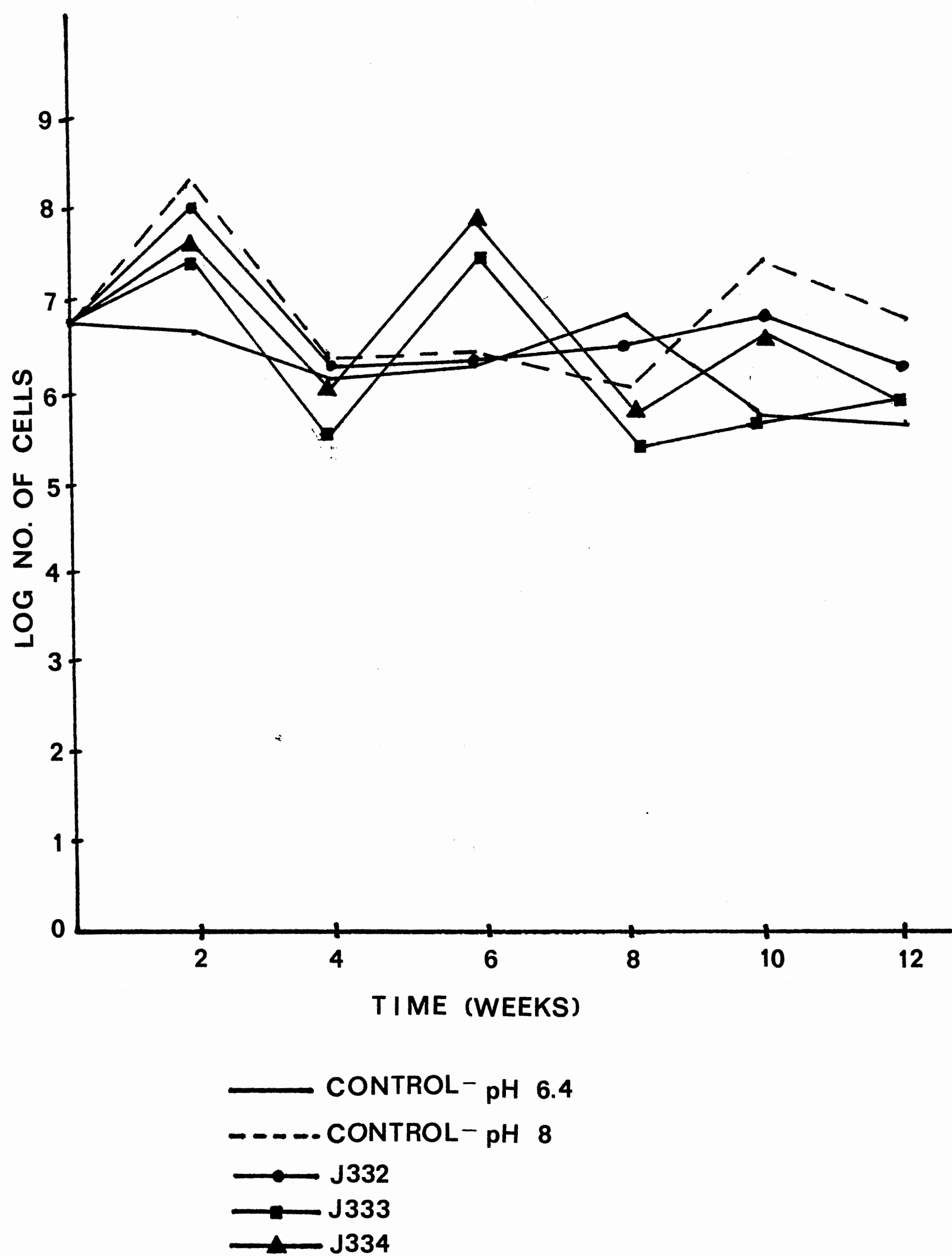
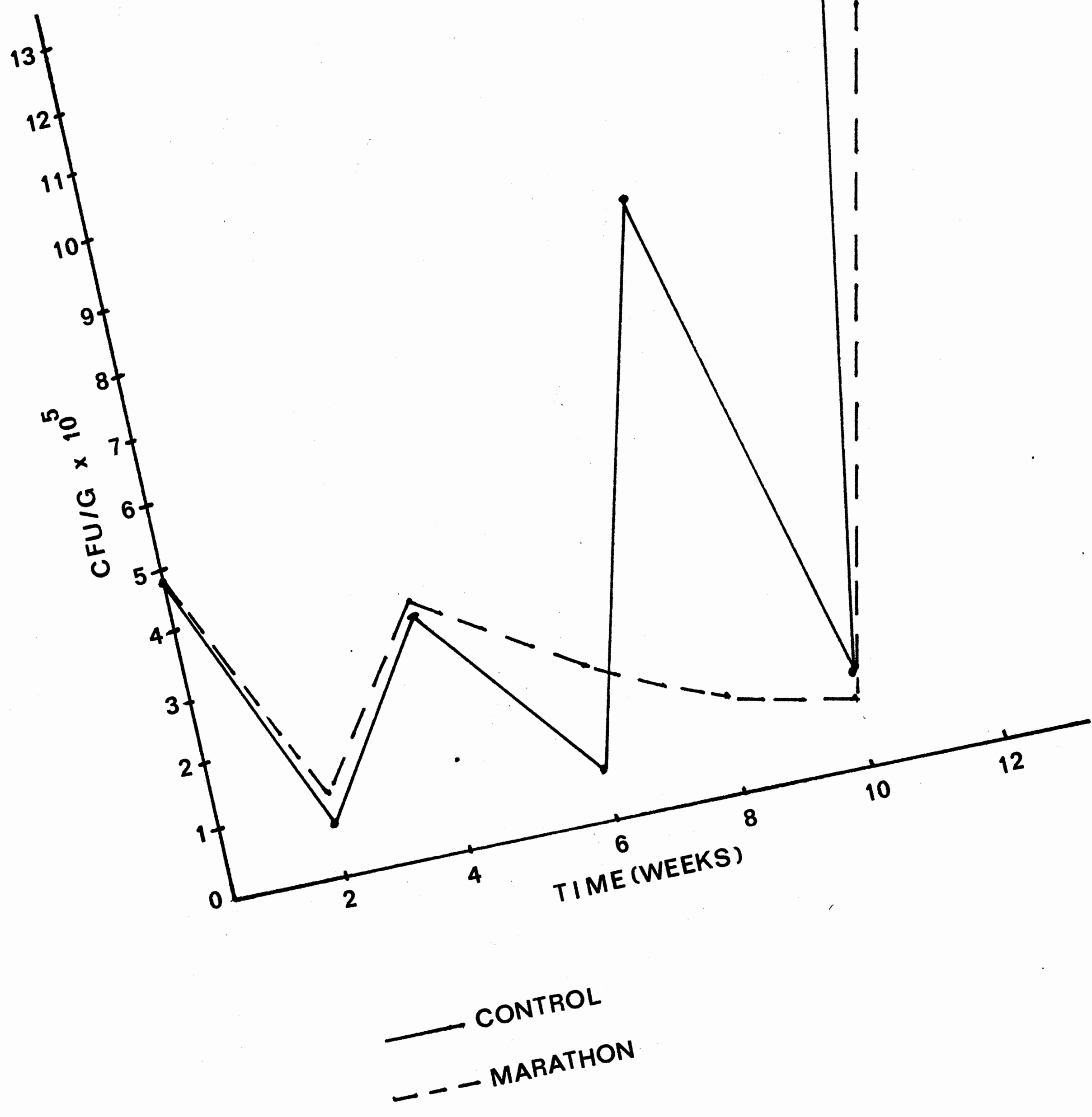

9
8
7
6
5
4
3
2
1
0
LOG NO. OF CELLS
2
4
6
8
10
12
TIME (WEEKS)
CONTROL- pH 6.4
CONTROL- pH 8
J332
J333
J334

TABLE X

MICROCOSM TESTS WITH MARATHON (0.1%)

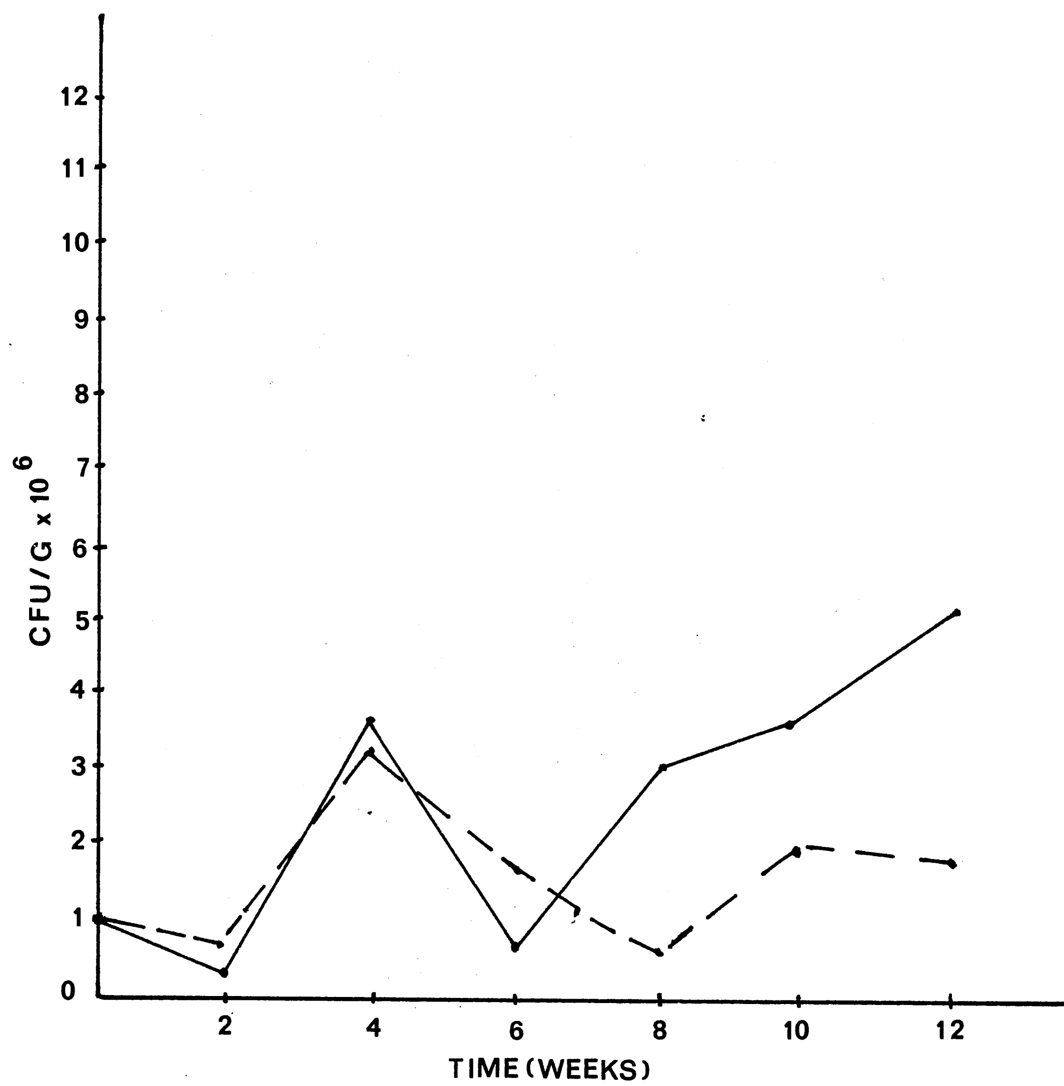
Time	Soil #38		Soil #42	
	Control	Marathon	Control	Marathon
Zero pH	4.8×10^5 6.4		1.0×10^6 6.4	
2 Weeks	7.2×10^4 6.4	1.1×10^5 6.4	2.5×10^5 6.4	4.5×10^5 6.4
4 Weeks	3.5×10^5 6.4	3.7×10^5 6.6	3.5×10^6 6.4	2.9×10^6 6.6
6 Weeks	6.7×10^4 6.4	2.1×10^5 6.6	5.1×10^5 6.4	1.7×10^6 6.6
8 Weeks	8.9×10^5 6.4	1.5×10^5 6.4	3.0×10^6 6.4	5.4×10^5 6.4
10 Weeks	1.5×10^5 6.4	9.8×10^4 6.6	3.4×10^6 6.4	2.0×10^6 6.6
12 Weeks	2.2×10^6 6.4	1.1×10^6 6.6	5.2×10^6 6.4	1.9×10^6 6.6

Figure 9. Actinomycete Counts During a 12 Week Period in the Microcosm Test With Marathon Petroleum Sulfonate Using Soil Sample 38



13
12
11
10
9
8
7
6
5
4
3
2
1
0
CFU/G x 10^5
2
4
6
8
10
12
TIME (WEEKS)
CONTROL
MARATHON

Figure 10. Actinomycete Counts During a 12 Week Period in the Microcosm Test With Marathon Petroleum Sulfonate Using Soil Sample 42



12
11
10
9
8
7
6
5
4
3
2
1
0
CFU/G x 10^6
2
4
6
8
10
12
TIME (WEEKS)
CONTROL
MARATHON

to be toxic to the actinomycetes since the counts remained close to those seen in the control cultures for both soils 38 and 42. In all the microcosm tests, the majority of colonies present on the plates of starch casein agar appeared to be Streptomyces species. A few Mycobacterium, Nocardia, and Corynebacterium species were also seen on the plates of starch casein.

Identification of Various Isolates From the Microcosm Tests

A number of colonies of Streptomyces and other genera of interest were isolated from the various microcosm tests using TSA agar slants. These isolates were purified by streaking four times serially onto TSA plates. A number of biochemical tests were done to identify these organisms, and the colonial and cellular morphology were also used in identification. The descriptions in Bergey's Manual (8th Edition) were used as a key to identify the isolates according to their physical and biochemical properties. Table XI gives some key features used to identify each genus. These characteristics were also used to key the isolates to species. A summary of the various biochemical tests results and the final identifications of the strains are shown in Table XII. The isolates were identified on the basis of how closely similar their properties were to those shown in Table XI and the description given in Bergey's Manual. These eight isolates represent the most abundant types of microorganisms in terms of numbers of colonies present on the plates from the microcosm tests. Colonies that appeared to be Nocardia, Streptomyces, Mycobacterium, and Corynebacterium were purposely chosen from the plates. All the isolates were Gram-positive and catalase positive.

TABLE XI

IDENTIFICATION KEY FOR VARIOUS GENERA OF ACTINOMYCETES

Streptomyces:

Colonial Morphology: small, leathery, may produce aerial mycelium
Microscopic Appearance: slender, branched mycelium
Gram Reaction: Gram-positive
Oxygen Requirements: Aerobic
Optimum Growth Temperature: 25-35°C
Production of dark brown melanoid pigments
Spore Chain Morphology: straight or coiled
Growth in Glucose
Growth slow-usually 48 hours to 7 days
Produce a variety of pigments

Mycobacterium:

Colonial Morphology: small, circular, moist, smooth
Microscopic Appearance: rods, may be filamentous
Pigment: yellow or orange colonies
Oxygen Requirements: Aerobic
Slow growth-usually within 48 hours; Acid-fast

Nocardia:

Colonial Morphology: leathery, dry, crusty
Microscopic Appearance: mycelium which fragments to rods or coccoid rods
Aerial hyphae sometimes formed
Obligate Aerobes: some species are Acid-fast
Growth Slow: Optimum temperature is 25°C

Corynebacterium:

Colonial Morphology: straight to slightly curved rods; may see snapping divisions or pallisades
Gram Reaction: Gram-positive
Pigments: Yellow, Orange
Oxygen Requirements: Aerobic or facultative anaerobes
Non-acid fast; Gorwth sometimes slow

TABLE XII

MICROCOSM TEST ISOLATES

Organism and Source	Microscopic Appearance	Colonial Morphology	Spore Chain Morph.	C Source	Pigment	Melanoid Pigment and Acid-Fast Reaction
Streptomyces #39 (n-DBSA)	Slender hyphae	Leathery, circular	Spiral	GLU GAL	White mycelium	+, dark brown non-Acid fast
Streptomyces albo-flavus #41-(PAA)	Slender branched hyphae	Circular, rough	Straight	GLU	Yellow-white	- non-Acid fast
Mycobacterium flavescens #38-(n-DBSA)	Rods	Circular, moist small	-	GLU	Yellow	- Acid fast
Corynebacterium #42 (PAA)	(Pallisades) slightly curved	Circular, moist small	-	GLU GAL	Yellow	- Non-Acid fast
Nocardia rugosa #39-(n-DBSA)	Fragments branched hyphae,	Circular, pasty wrinkled	-	GLU GAL	Cream	+, dark brown with age Non-Acid fast
Nocardia sp. #38-(PAA)	Fragments branched hyphae	Circular, dry, flaky	-	GLU	Pink	- Acid fast
Mycobacterium phlei #42-(PAA)	Coccoid rods	Small, circular mucoid	-	GLU	Yellow	- Acid-fast

TABLE XII (Continued)

Organism and Source	Microscopic Appearance	Colonial Morphology	Spore Chain Morph.	C Source	Pigment	Melanoid Pigment and Acid-Fast Reaction
Streptomyces griseus #38-MAR	Slender branched hyphae	Leathery, small grey	Straight	GLU GAL	Grey-white	+, dark brown Non-Acid fast

GLU = growth inglucose broth.

GAL = growth in galactose broth.

All the strains grew at 25°C within 48 to 72 hours. The Mycobacterium species were also acid-fast which is a major identifying characteristic of this genus.

Tests of Potential Ability of the Isolated Strain to Utilize EOR Chemicals as a Sole Carbon and Energy Source

Each strain was tested for its ability to utilize the EOR compounds under study as a sole carbon and energy source. We assumed that growth of the microorganism on a chemically defined medium containing the EOR compound (0.2% or 0.1%) indicated the possibility that the organism in question could degrade and utilize the EOR chemical as a carbon and energy source. This would be true only if the chemicals used in the medium were extremely pure, and the strains did not utilize agar. Control cultures, containing no carbon-energy source, are essential in tests of this kind. Only if significantly greater growth were to occur in the presence of the carbon-energy source could one conclude that the EOR compound might support growth as a carbon-energy source. Also, control cultures with glucose were inoculated. The amount of growth on the plates containing an EOR compound was compared to growth on the controls and rated subjectively. The plates with no EOR agent showed no growth of any of the strains which indicated that impurities in the medium, or agar itself, were not able to support growth of any of the strains. Therefore, any growth seen on the test plates should be because of the EOR agent. This test alone does not tell much about the mechanism by which the EOR chemical supports growth, or to what extent the EOR agent is broken down. The plates were inoculated with washed cells suspended

in sterile buffered saline. The cultures used in preparing the cell suspensions had been incubated at 25°C for one week. The plates were incubated at 25°C and checked for growth every two days. After one week the amount of growth was compared to the control plates and rated subjectively. The results of these tests are given in Table XIII.

The fact that we observed no growth of any strain on the control (no carbon-energy source), and fair to good growth of all strains with glucose in the medium allows certain conclusions. First, since there were very few differences between 0.1 percent and 0.2 percent, we had, in effect, duplicate cultures, thus enhancing validity of results overall. n-Dodecylbenzene sulfonic acid supported growth poorly--no growth in three cases, and poor growth in four cases. The n-DBSA was neutralized to pH 7 prior to addition to the medium. Despite the neutralization of the DBSA, very little growth was seen on the plates.

Marathon petroleum sulfonate also supported growth poorly, but a little better (with strains 1, 3 and 7) than n-DBSA. This may have occurred because of the greater content of unreacted hydrocarbon in the Marathon preparation.

The polyacrylamides supported a surprising amount of growth; fair growth with all strains except one in the case of J332 and J333, and with all strains except two in the case of J334. Every strain except one (Pseudomonas aeruginosa) grew on at least two of these polymers, and five grew on all three polymers. This is fairly good evidence these polymers are somehow providing a utilizable carbon-energy source for the organisms. This does not mean that the polymer itself is being degraded and utilized; for example, J332 has an average molecular weight of 9-10 x 10^6 daltons, and there is no enzyme system known which would attack a

TABLE XIII

GROWTH OF VARIOUS MICROBIAL STRAINS ON A CHEMICALLY DEFINED MEDIUM WITH A REPRESENTATIVE EOR CHEMICAL AS SOLE CARBON-ENERGY SOURCE

Compound	1	2	3	4	5	6	7
0.1% GLU	++	++	++	++	++	+++	+++
0.2% GLU	++	++	++	++	++	+++	+++
0.1% n-DBSA*	0	+	0	+	+	0	+
0.2% n-DBSA	0	+	0	0	+	0	+
0.1% MARA.	++	+	+	0	+	0	++
0.2% MARA.	++	0	+	0	+	0	++
0.1% J322	++	++	++	+	++	++	++
0.2% J322	++	++	++	0	++	++	++
0.1% J333	++	++	++	++	++	0	++
0.2% J333	++	++	++	++	++	0	++
0.1% J334	++	++	++	+	++	0	++
0.2% J334	++	++	++	0	++	0	++
Control	0	0	0	0	0	0	0

1 - *Streptomyces* sp.
2 - *Streptomyces alboflavus*
3 - *Mycobacterium flavescens*
4 - *Corynebacterium* sp.
5 - *Nocardia rugosa*
6 - *Pseudomonas aeruginosa*
7 - *Bacillus subtilis*

Scale for Growth Rating: 0 = no growth; + = poor; ++ = fair; +++ = good; ++++ = excellent.

*n-DBSA was neutralized to pH 7 prior to addition to the medium.

$--\left[\begin{array}{c} CH_2\text{-}CH_2\text{-} \\ | \\ C{=}O \\ | \\ NH_2 \end{array} \right]_n$ chain of this magnitude. The agent supporting growth may be some impurity in the polymer entirely unrelated to polyacrylamide. Whatever it is, it appears to be present to an equal degree, and to support fairly good growth of all organisms used except *Pseudomonas aeruginosa* (a control--not isolated from EOR enrichment cultures) and *Corynebacterium* sp. The extent of growth of *Bacillus subtilis* (another control) is surprising. The stimulation of growth of eubacteria (fast growing bacteria) by polyacrylamides is in accord with observations reported earlier by this laboratory. We have observed here a stimulation of growth of two *Streptomyces* species, a *Mycobacterium* species, and a *Nocardia* species. The mechanism of stimulation is unknown, but it apparently occurs with a wide variety of actinomycetes.

Growth with n-DBSA and the Marathon petroleum sulfonate in most cases was considerably less than with the polyacrylamides. The occurrence of growth here is not in accord with the results of the microcosm test. We cannot explain this discrepancy at the present time. Indeed, n-DBSA did not permit growth of *Streptomyces* sp., of *Mycobacterium flavescens*, or of *Pseudomonas aeruginosa*. The Marathon product did not permit growth of the *Corynebacterium* strain nor of *Pseudomonas aeruginosa*. In two cases, 0.2 percent appeared toxic, whereas minimal growth occurred with 0.1 percent of the agent.

Our data from the pure cultures using a solid medium seem to indicate that a number of the isolated cultures were able to utilize either polyacrylamides or petroleum sulfonates. Since growth alone is not

enough to draw final conclusions as to degradation by a given species of the compound in question, it was essential to determine if growth of the organism in liquid culture medium in the presence of the substance (EOR chemical) was accompanied by a decrease in the concentration of the EOR compound. If the amount of growth could be correlated with the extent of degradation of the polymer, it would indicate that the organism was indeed able to degrade the EOR agent and utilize the compound as a carbon-energy source.

To detect a possible decrease in concentration, we did semiquantitative tests, which could be run on a large number of samples, and would be sufficient to show a correlation between amount of growth and extent of degradation of the substance. To test for polyacrylamide degradation, we decided to determine viscosity of the polyacrylamide solution. Aqueous solutions of acrylamide polymers are highly viscous which is why these polymers are useful in enhanced oil recovery. Shear stress, or almost any kind of mechanical stress, will break the carbon-carbon covalent bonds in the backbone of the molecule. This degradation of the molecule results in a loss of absolute viscosity (centipoises) and a loss of screen factor.

In order to determine if the EOR chemical was being degraded by any of our microbial strains, we grew each culture in the liquid basal defined medium containing either Marathon petroleum sulfonate, n-DBSA, or one of the polymers J332-J334 as a sole carbon-energy source. Each agent was tested at two concentrations, 0.05 percent and 0.1 percent. Media were made up in 25 ml volumes in Erlenmeyer flasks. The cultures were incubated at 25°C for one week.

Results on growth obtained with the petroleum sulfonate cultures are

given in Table XIV. In comparing results in Table XIV with those given in Table XIII (four of the same strains, *Streptomyces* sp., *Streptomyces alboflavus*, *Mycobacterium flavescens*, and *Nocardi rugosa* were used), it is apparent that there are some inconsistencies in the extent of growth observed. This is particularly noticeable with *Mycobacterium flavescens*, designated number 3 in both cases. In the first experiment (Table XIII), *M. flavescens* showed no growth in 0.1 percent n-DBSA, and poor growth in Marathon 0.1 percent; whereas in the second experiment the isolate (*M. flavescens*) showed good growth both in 0.1 percent Marathon and 0.1 percent n-DBSA.

In the second experiment (Table XIV), eight actinomycete-related isolates were used, and no control cultures of eubacteria. Four out of the eight showed good growth on 0.1 percent Marathon, and seven of eight showed either good or fair growth on 0.1 percent n-DBSA. This is a considerable improvement over the growth reported in Table XIII.

Results of Semiquantitative Analytical Tests

The Methylene Blue Active Substances (MBAS) test results are given for n-DBSA and for the Marathon preparation in Tables XV and XVI respectively. The results of the MBAS test were compared with the results of the amount of growth (Table XIV) to determine if an increase in growth could be correlated with a high MBAS activity. Control cultures (uninoculated controls) were also prepared as a check to see if either evaporation or uncatalyzed breakdown of substrate was occurring. Neither of these occurrences were seen and no degradation of the n-DBSA or of the Marathon petroleum sulfonate occurred in the uninoculated controls.

There appears to be some degradation brought about by some of the

TABLE XIV

GROWTH OF EIGHT STRAINS ON A DEFINED MEDIUM WITH A PETROLEUM SULFONATE OR n-DBSA AS SOLE CARBON SOURCE

Isolate	0.1% Mar	0.05% Mar	0.1% n-DBSA	0.05% n-DBSA	0.1% Glucose
1	++	++	++	+	++++
2	+++	++	++	+	+++
3	+++	++	+++	+	+++
4	+++	++	++	+	++++
5	++	++	+	+	++++
6	++	++	++	++	++++
7	+++	+++	++	++	++
8	++	++	++	++	++++

1 - *Streptomyces* sp.
2 - *Streptomyces alboflavus*
3 - *Mycobacterium flavescens*
4 - *Corynebacterium* sp.
5 - *Nocardia rugosa*
6 - *Mycobacterium phlei*
7 - *Nocardia* sp.
8 - *Streptomyces griseus*

Scale for Growth Rating: 0 = no growth; + = poor; ++ = fair; +++ = good; ++++ = excellent.

Controls: Control with no EOR agent (only cells) was prepared for each isolate; growth in controls was used to rate growth in cultures containing an EOR agent.

Control with only EOR agent was also run; no growth appeared in any of the controls with EOR agent only.

Amount of growth in all controls (1-8) was Poor (+).

TABLE XV

ANALYSIS OF n-DBSA USING METHYLENE BLUE TEST

Organism	μg DBSA Added	μg DBSA Remaining *	%n-DBSA Degraded
M. phlei	20	15.7	21%
	10	7.5	25%
S. griseus	20	15.0	25%
	10	7.0	30%
M. flavescens	20	15.0	25%
	10	9.0	10%
Corynebacterium	10	8.5	15%
Streptomyces alboflavus	10	3.0	70%
Streptomyces	10	7.5	25%
Nocardia	10	7.5	25%
Control (only DBSA)	20	20	0%

Controls containing only cells were done for each isolate--a fair amount of growth was seen in the cultures.

*the MBAS activity remaining may be due to not only intact DBSA remaining, but also benzene sulfonates with six or less carbon chains

TABLE XVI

ANALYSIS OF MARATHON USING METHYLENE BLUE TEST

Organism	% Volume Added	% Volume Remaining *	% Degraded
M. phlei	0.2	0.17	14%
S. griseus	0.2	0.14	28%
M. flavescens	0.2	0.17	16%
Corynebacterium	0.1	.065	35%
Streptomyces alboflavus	0.1	.07	30%
Streptomyces	0.1	.076	24%
Nocardia	0.1	.078	22%
Control (only Mar.)	0.2	0.2	0%

Controls containing only cells were done for each isolate--a fair amount of growth was seen in each culture.

*the MBAS activity remaining may be due to not only intact DBSA, but also benzene sulfonates with six or less carbon chains

isolates, particularly Streptomyces alboflavus on n-DBSA, and Corynebacterium sp., Streptomyces griseus, and Streptomyces alboflavus on Marathon. All these isolates demonstrated fair to good growth, as compared to control cultures (no EOR agent), in the liquid cultures (Table XIV).

Possible Biological Degradation of Polyacrylamides

Polyacrylamide polymers--those which are linear, as ours were, are extremely susceptible to nonbiological degradation arising from two sources: a) Mechanical, or shear degradation. Any violent (as in a Waring blender) agitation, or even moderate agitation (gentle shaking, or ordinary stirring) will readily break some of the carbon-carbon covalent bonds of the long thin molecule, reducing average molecular weight, and hence the viscosity (both absolute and screen factor) of the solution; b) chemical degradation, resulting from the presence of molecular oxygen. The extent of breakdown is greater if a reducing agent such as Fe^{++} ion is present. This loss of intact polymer molecule is thought to result from a free radical induced depolymerization. Very few definite conclusions could be drawn from the viscosity results and screen factor tests. Preliminary experiments with the polyacrylamides solutions indicated that at least some of the strains may bring about a loss in screen factor or viscosity of the solutions.

Table XVII gives results obtained in defined medium growth experiments using one of the polyacrylamides as a sole carbon-energy source. Growth of all eight of the strains, at the time of observation, appeared to be relatively heavier than growth in the previous experiment in which polyacrylamides were tested for ability to support growth on a solid

TABLE XVII

GROWTH OF EIGHT STRAINS ON A DEFINED MEDIUM WITH A POLYACRYLAMIDE (J332-334) AS SOLE CARBON SOURCE

Isolate	0.1% J332	0.05% J332	0.1% J333	0.05% J333	0.1% J334	0.05% J334	0.1% Glucose
1	++	+	++	+	++	++	++
2	+	+	+	+	++	+	++
3	+	+	++	+	++	++	+++
4	++	++	+++	+++	++	++	+++
5	++	+	++	++	++	+	++
6	+++	+++	++	++	+++	+++	+++
7	++	++	++	++	++	++	++
8	+++	++	+++	++	+++	++	+++

1 - Streptomyces sp.
2 - Streptomyces alboflavus
3 - Mycobacterium flavescens
4 - Corynebacterium sp.
5 - Nocardia rugosa
6 - Mycobacterium phlei
7 - Nocardi sp.
8 - Streptomyces griseus

Scale for Growth Rating: 0 = no growth; + = poor; ++ = fair; +++ = good; ++++ = excellent.

Controls: Control (only cells) was prepared for each isolate; growth in controls was used to rate growth in cultures containing an EOR agent.

Controls with only EOR agent were also run; no growth appeared in any of the controls with only EOR agent.

Amount of growth in all controls (1-8) was Poor (+).

medium. There were no significant differences between the three polymers, nor between the two concentrations of one polymer.

Possible Biological Degradation of Xanthan Gum

The basic biodegradability of Xanthan Gum under aerobic conditions is not in doubt, but very little is known of the microbiology of the process, or the biochemical mechanism of breakdown. According to Cadmus et al. (1982), Xanthan degrading organisms are not abundant in nature. However, such organisms do exist; Cadmus et al. (1982) have described an extracellular enzyme system (probably a mixture of carbohydrases) that degrades Xanthan gum, which they referred to as Xanthanase.

In order to gain information on the possibility of the existence of a Xanthanase produced by actinomycetes, and a possible role of this group of organisms in Xanthan gum degradation, we set up microcosm tests using the soil samples E and 43. When first set up (20% w/w Sigma Xanthan gum--a relatively pure preparation), heavy mold growth over the entire plates occurred in a few days. These plates were discarded, and a second set was prepared in which 250 μg/g of cycloheximide was mixed thoroughly with the soils. Again, the molds overgrew the cultures. However, when plated out on starch casein medium with streptomycin and cycloheximide added, a number of actinomycete colonies were seen. It was not possible to get a definite plate count due to the mold contaminants; however, it does appear that some actinomycetes possess the ability to utilize the Xanthan gum.

CHAPTER IV

DISCUSSION

It appears that a few of the isolated strains can degrade at least some of the EOR compounds in pure culture. In particular, several species (*Mycobacterium flavescens*, *Nocardia rugosa*, and *Streptomyces alboflavus*) grow on n-DBSA as sole carbon source and concomitantly reduce the MBAS activity.

Relatively heavy growth of *Streptomyces alboflavus*, *Mycobacterium flavescens*, *Corynebacterium* sp., and *Nocardia* sp. occurred in 0.1 percent Marathon petroleum sulfonate. Other isolates grew moderately. This is not surprising in view of the probable complexity of that mixture. All isolates grew moderately to well on 0.1 percent n-DBSA.

Reduction of MBAS activity represents loss of surfactant capability, and (as indicated previously) is termed "primary biodegradation." In a preliminary test of ability to reduce the methylene blue activity (MBAS) of our isolates, we observed, using seven strains, that the MBAS was apparently reduced significantly by all strains, with both n-DBSA and the Marathon preparation (Tables XV and XVI). Extent of reduction varied from ten percent to seventy percent (the latter figure was obtained with *Streptomyces alboflavus* in n-DBSA). To confirm the earlier results, tests were set up for each substrate, using *Streptomyces alboflavus* and *Mycobacterium phlei* with n-DBSA, and *Streptomyces griseus* and *Nocardia* sp. with Marathon petroleum sulfonate. All these species had previously

shown a relatively high degree of reduction of MBAS. The confirmatory test results were very close to the first results seen in Tables XV and XVI. This indicates that these organisms possess the ability to readily degrade the petroleum sulfonates tested. The relatively large number of strains of actinomycetes capable of primary biodegradation of n-DBSA observed in this study contrasts with the difficulty of isolating eubacteria (ordinary fast-growing strains) from Oklahoma soils which have this capability (Grula, 1981). Numerous attempts to isolate such organisms from soil samples failed. Only in using activated sludge from the Perry (Oklahoma) wastewater treatment plant was success finally achieved in getting a mixed culture capable of producing primary biodegradation of n-DBSA (Pennington and Grula, unpublished data). None of the components of this mixed culture could, individually, grow on n-DBSA as a sole carbon source, nor reduce the MBAS activity.

If the actinomycetes as a group should prove, upon further study, to be as active (as pure cultures) against n-DBSA, and other linear alkyl benzene sulfonates, they would certainly be a major factor in the biodegradation of linear alkyl benzene sulfonates in nature. As far as we know, no one has yet reported degradation of linear alkyl sulfonates by an actinomycete. This remains a fertile field for investigation.

Regarding possible polyacrylamide biodeterioration by actinomycetes, the situation is quite different. The term "biodeterioration" is used instead of "biodegradation" because of the improbability that these polymers can be biodegraded in the usual sense. "Biodeterioration" has been used to mean a change in properties, or a lowering of quality, or damage to a substance or structure resulting indirectly from the activities of microorganisms (Rose, 1981). Most materials which undergo biodeteriora-

tion are normally considered to be relatively resistant to microbial attack (stone, wood, paper, plastics, paint films). Polyacrylamides fit in this category very well. The evidence indicates that they undergo biodeterioration, but the mechanism is unknown.

Although the first microcosm cultures done with actinomycetes showed populations in the presence of the polymer to be up to several hundred fold higher than in its absence, it is not certain that these high counts consisted exclusively of actinomycetes. Aerobic eubacteria are markedly stimulated in this type of culture by polyacrylamides (Rose, 1981). In subsequent experiments the level of streptomycin was raised from 10 to 60 μg/ml, a level which very effectively inhibited eubacteria. In the latter experiments, the marked stimulation of the microbial population by polyacrylamide seen in the first trial did not occur.

The fact that the polymers, when added as a sole carbon source, allowed growth of pure cultures of actinomycetes in a chemically defined medium requires an explanation. Since no growth occurred in the controls, to which no carbon source had been added, it appeared as though the polymer was being attacked. One possibility that should be considered is that small amounts of monomers (acrylic acid or acrylamide) contaminating the polymer could support growth of the microorganisms. Other impurities present in the polymer might also have growth-promoting activity. Tests of growth-promoting activity of these polyacrylamides should be repeated using polymer solutions that have been dialyzed, or have undergone further purification procedures.

Tests of reduction in screen factor, or in absolute viscosity by growth of these microorganisms unfortunately are difficult to interpret, because of the great variability of the controls. Culture #6 (Mycobac-

terium phlei), on J333, produced what appeared to be a very significant reduction in screen factor; however, the same organism had no effect on polymer J334. It is hard to distinguish a trend in the results. However, in looking at overall apparent reductions in viscosity and screen factor, it is possible that the intermediate and lower molecular weight polymers such as J333 (MW 5-6 x 10^6) and J334 (MW 3-4 x 10^6) were degraded (as shown by apparent loss of viscosity or screen factor) to a greater extent than the high molecular weight polymer J332 (MW 9-10 x 10^6). It is possible that the high molecular weight polymer could be more resistant to biodeterioration than the polymers of lower molecular weight. If loss of viscosity were related, even indirectly, to growth and metabolic activities of the organism, then we should see a positive correlation between amount of growth of a given strain on a polymer, and degree of loss of viscosity of that polymer solution. A comparison of the results on growth (Table XVII) with loss of viscosity or screen factor could be used to demonstrate this possible correlation. In the preliminary viscosity tests and screen factor analysis, no such positive correlation was seen. Perhaps a more quantitative experiment, with more reliable measurements of viscosity and screen factor would show a correlation.

Xanthan gum, unlike the polyacrylamides, enhanced considerably the actinomycete counts in microcosm cultures. It is interesting that the soil sample obtained from near a pipeline showed a much greater increase in the actinomycete population in the presence of Xanthan gum than did the soil sample obtained from a wheat field. Many more samples would have to be run before any definite conclusions could be drawn.

If a spill should occur (in many environments in Oklahoma) the

chances are very good that fast-growing eubacteria--probably predominantly pseudomonads--would rapidly increase in numbers in areas where the concentration of EOR agent was not extremely high. In the long term, however, certain actinomycetes would survive, increase in numbers, and possibly bring about a biodegradation of the agent.

While we have evidence that actinomycetes could be involved in deterioration of polyacrylamides and degradation of components of petroleum sulfonates, we have no information on how these actions might take place in terms of biochemical mechanisms. As indicated in the Introduction and Literature Review section, polyacrylamides, being of non-biological origin (Xenobiotic), and possessing very long chains of CH_2-groups linked by carbon-carbon double bonds, are in all probability non-biodegradable in the usual sense.

The results obtained from this study are significant from a strictly empirical standpoint. This study was an attempt to obtain information on the possible role that actinomycetes might play in the biodegradation of EOR chemicals. The most significant finding may well be the relatively large number of pure cultures of actinomycetes which are able to degrade (at least through primary biodegradation) linear alkyl benzene sulfonates, as exemplified by n-DBSA.

LITERATURE CITED

Abbott, B. J., and L. E. Casida, Jr. 1968. Oxidation of alkenes by a Nocardia. J. Bacteriol. 96(2): 925-930.

Alexander, M. 1965. Biodegradation: problems of molecular recalcitrance and microbial fallibility. Adv. Appl. Microbiol. 7:35-80.

Arai, T. 1976. Actinomycetes: the boundary microorganisms. Toppan Co., Tokyo, Japan.

Atlas, R. M. 1978. Microorganisms and petroleum pollutants. Bioscience 28(6): 387-391.

Bergey's Manual of Determinative Bacteriology (8th Edition). 1974. R. E. Buchanan and N. E. Gibbons, eds., p. 599-855. Williams and Wilkins Company, Baltimore, Maryland.

Cadmus, M. C., L. K. Jackson, K. A. Burton, R. D. Plattner, and M. E. Slodki. 1982. Biodegradation of Xanthan Gum by Bacillus sp. Appl. Environ. Microbiol. 44:5-11.

Corke, C. T., and F. E. Chase. 1956. The selective enumeration of actinomycetes in the presence of large numbers of fungi. Canad. J. Microbiol. 2:12-16.

Crook, P., C. C. Carpenter, and P. F. Klens. 1950. The use of sodium propionate in isolating actinomycetes from soils. Science 112:656.

Dulaney, E. L., A. H. Larson, and E. O. Stapley. 1955. A note on the isolation of microorganisms from natural sources. Mycologia 47:420-422.

El-Nakeeb, M. A., and H. A. Lechevalier. 1963. Selective isolation of aerobic actinomycetes. Appl. Microbiol. 11:75-78.

Foshee, W. C., R. R. Jennings, and T. J. West. 1976. "Preparation and testing of partially hydrolyzed polyacrylamide solutions". (Paper presented at the 51st Annual Fall Technical Conference and Exhibition of the Society of Petroleum Engineers of AIME, New Orleans, Louisiana, October, 1976). Dow Chemical, USA. 1976.

Foster, J. W. 1962. Hydrocarbons as substrates for microorganisms. Antonie van Leeuwenhoek 28:241-274.

Fredericks, K. M. 1967. Products of the oxidation of n-decane by Pseudomonas aeruginosa and Mycobacterium rhodochrous. Antonie van Leeuwenhoek 33:41-48.

Gilbert, P. D. 1978. Microbial degradation of crude mineral oils at sea. J. Gen. Microbiol. 108(1):63-70.

Grula, M. M., and E. A. Grula. 1979. "Biodegradation of materials used in enhanced oil recovery". Report DOE/BC30201-13, Contract DE-AT19-78BC-30201, Oklahoma State University, Stillwater, Oklahoma.

Hestrin, S. 1949. The reaction of acetylcholine and other carboxylic acid derivatives (esters, lactones) with hydroxylamine and its analytical application. J. Biol. Chem. 180:249-261.

Kester, A. S., and J. W. Foster. 1963. Diterminal oxidation of long-chain alkanes by bacteria. J. Bacteriol. 85:859-869.

Klug, M. J., and A. J. Markovetz. 1971. Utilization of aliphatic hydrocarbons by microorganisms. Adv. Microbial Physiol. 5:1-39.

Kuster, E., and S. T. Williams. 1964. Selection of media for the isolation of streptomycetes. Nature 202:928-929.

Lukins, H. B., and J. W. Foster. 1963. Methyl ketone metabolism in hydrocarbon-utilizing mycobacteria. J. Bacteriol. 85:1074-1087.

Lusby, W. R., and J. Oliver. 1980. Metabolism of 2,6-dinitro-4-(trifluoromethyl) benzenamine by a Streptomyces isolated from soil. J. Agric. Food Chem. 28(3):641-644.

McCormick, C. L., H. H. Neidlinger, R. D. Hester, and G. C. Wildman. 1979. "Improved polymers for enhanced oil recovery - synthesis and rheology." First Annual Report, Contract #EF-77-S-05-5603, Department of Energy.

Olivieri, R., P. Bacchin, A. Robertiello, N. Oddo, L. Degen, and A. Tonolo. 1976. Microbial degradation of oil spills enhanced by a slow-release fertilizer. Appl. Environ. Microbiol. 31(5):629-634.

Ottow, J. C. G. 1972. Rose bengal as a selective aid in the isolation of fungi and actinomycetes from natural sources. Mycologia 64:304-315.

Patel, R. N., C. T. Hou, A. I. Laskin, P. Derelanko, and A. Felix. 1979. Oxidation of secondary alcohols to methyl ketones by yeasts. Appl. Environ. Microbiol. 38(2);219-223.

Porter, J. N. 1971. Prevalence and distribution of antibiotic producing actinomycetes. Adv. Appl. Microbiol. 14:73-92.

Rose, A. H., Ed. 1981. Economic Microbiology, Vol. 6, Microbial Biodeterioration. Chapter 1. Academic Press, New York.

Sielicki, M., D. D. Focht, and J. P. Martin. 1978. Microbial degradation of polystyrene and 1,3-diphenylbutane. Can. J. Microbiol. 24(7):798-803.

Stirling, L. A., and R. J. Watkinson. 1977. Microbial metabolism of alicyclic hydrocarbons: Isolation and properties of a cyclohexane degrading bacterium. J. Gen. Microbiol. 99(1):119-126.

Strassman, M., J. B. Shatton, and S. Weinhouse. 1960. Conversion of α-acetolactic acid to the valine precursor, α,β-dihydroxyisovaleric acid. J. Biol. Chem. 235:700-705.

Waksman, S. A. 1959. The Actinomycetes: their nature, occurrence, activities, and importance. Williams and Wilkins, Baltimore, Maryland.

Waksman, S. A. 1963. The Actinomycetes: the classification, identification, and description of genera and species. Williams and Wilkins, Baltimore, Maryland.

Walker, J. D., and R. R. Colwell. 1976. Long chain n-alkanes occurring during microbial degradation of petroleum. Can. J. Microbiol. 22(6):886-891.

Westlake, D. W. S., A. M. Jobson, and F. D. Cook. 1978. In situ degradation of soil in a soil of the boreal region of the Northwest Territory. Can. J. Microbiol. 24(3):254-260.

Williams, S. T., and F. L. Davies. 1965. Use of antibiotics for selective isolation and enumeration of actinomycetes from soil. J. Gen. Microbiol. 38:251-261.

Yall, I. 1979. Petroleum-eating bacteria. Natural History 88:42-46.

ZoBell, C. E. 1950. Assimilation of hydrocarbons by microbes. Adv. Enzymol. 10:443-486.

APPENDIX

PROPERTIES OF A GEOTRICHUM SPECIES ISOLATED FROM A SOIL ENRICHMENT CULTURE CONTAINING 2-BUTANOL

In our studies of the compunds used in Enhanced Oil Recovery, we have isolated a *Geotrichum* species which can utilize secondary butanol as a sole carbon-energy source. Secondary butanol is used in EOR as a cosurfactant in micellar-polymer flooding. The *Geotrichum* was isolated from a soil enrichment culture with 1 percent secondary butanol (2-butanol) as the sole carbon and energy source.

Most of the soil samples tested in enrichment experiments with 2-butanol were negative for growth. In one of the cultures, however, growth was heavy and the concentration of 2-butanol in the culture supernatant as determined by gas chromatography decreased markedly. The culture supernatant was plated onto TSA (tryptic soy agar) and SDA (Sabouraud's dextrose agar) and incubated at 25°C for 48 hours. The organism isolated from the culture supernatant was identified as *Geotrichum* sp. This identification was based on the cellular and colonial morphology of the culture.

Geotrichum is a common soil inhabitant and a member of the subdivision Deuteromycotina or "Fungi Imperfecti." It is obligately aerobic, rarely pathogenic, and is characterized by the formation of arthrospores. Arthrospores are produced by the fungi during resting stages. The

arthrospores of *Geotrichum* resemble large bacillary rods. The production of arthrospores by our isolate was one of the key characteristics used in its identification.

The culture we isolated produced a white, short, cottony colony on SDA plates incubated at 25°C. The colonies were relatively small. Wet mounts stained with Lactophenol Cotton Blue were done to observe the cellular morphology. Long, branching hyphae approximately 3-5 μm in diameter were seen. Also, an abundance of arthrospores were observed. Based on these characteristics, our isolate was identified as a *Geotrichum* species. Identification of the *Geotrichum* to species was not attempted.

The *Geotrichum* isolate was of interest to us due to its ability to readily utilize 2-butanol when very few other soil organisms seem to do so. Experiments were set up to determine what other compounds the *Geotrichum* could use as a carbon-energy source. Liquid cultures were prepared by adding the carbon compound (1 percent) to basal salts medium in a 125 ml Erlenmeyer flask, and 0.5 ml of a *Geotrichum* cell suspension was added to each flask. The suspension of cells was prepared by adding washed cells to sterile buffered saline. The cultures were incubated on a shaker at 25°C for 48 hours. Two serial transfers were made, each after 48 hours. The purpose of the transfers was to eliminate the possibility of significant growth resulting from nutrients adhering to cells, etc. After incubation, the amount of growth in the culture was compared to a control containing only cells and rated on a scale of 0 to 4+ (4+ being excellent growth). The growth of the *Geotrichum* in liquid culture was flocculant and not well dispersed. The amount of growth, as given by this scale, is certainly related to the readiness with which the

organism is able to utilize a certain compound. Our objective at this time was to carry out screening tests on a large number of compounds in order to see which ones could serve as a sole carbon-energy source. Some of the metabolic capabilities of an organism can be learned from this approach. The results are seen in Tables XVIII and XIX. It will be noted that very clear cut differences among the compounds occurred. Except for ethanol, the primary alcohols were not utilized. It is surprising that 2-pentanol and 2-hexanol supported no growth. Three hexoses, glucose, fructose, and mannose, were readily utilized but sucrose, a disaccharide, supported no growth. Failure to grow on methanol, formate, or methylamine strongly indicates that the organism cannot utilize one-carbon compounds and therefore would not be considered a methylotroph. The moderate growth on acetate indicates the possible presence of a glyoxalate cycle. The good growth seen on methyl ethyl ketone suggests that this may be one of the initial intermediates in the metabolism of 2-butanol by the Geotrichum.

Also, serial transfer cultures using various compounds as sole nitrogen sources were prepared. The cultures were prepared using the basal salts medium as before with the exception that the NH_4Cl was omitted. The cultures contained a 1 percent concentration of a nitrogen compound. The results are seen in Table XX.

From the results it can be seen that the organism cannot utilize NH_4Cl as a sole nitrogen source in a chemically defined medium. This Geotrichum requires one or more amino acids for growth, and quite possibly (but not necessarily) one or more B-vitamins. Given the essential amino acid(s), ammonia is readily assimilated, as seen by the growth stimulation by NH_4Cl in the presence of peptone or casein hydrolyzate,

TABLE XVIII

GROWTH IN THIRD SERIAL CULTURES USING VARIOUS ALCOHOLS AS SOLE CARBON-ENERGY SOURCES*

Alcohol	Growth
Methanol	0
Ethanol	4+
n-Propanol	0
n-Butanol	0
2-Propanol	0
2-Butanol	4+
2-Pentanol	0
2-Hexanol	0
Mannitol	2+
Control (only cells)	0

*0.5% concentration

TABLE XIX

GROWTH IN THIRD SERIAL CULTURES USING VARIOUS COMPOUNDS AS CARBON-ENERGY SOURCES*

Compound	Growth
Control (only cells)	0
Sugars:	
Fructose	4+
Glucose	4+
Mannose	4+
Sucrose	0
Salts of Organic Acids:	
Formate	0
K fumarate	3+
Na acetate	2+
Na malate	4+
Na lactate	4+
Na propionate	3+
Na pyruvate	4+
Li acetoacetate	1+
Misc.:	
2-Butanol	3+
Methyl ethyl ketone	3+
Methylamine	0
L-alanine	3+

*0.5% concentration

TABLE XX

GROWTH IN THIRD SERIAL CULTURES USING VARIOUS COMPOUNDS AS SOLE NITROGEN SOURCES*

Compound	Growth
Control	0
NH_4Cl	0
Peptone	2+
Casein Hydrolysate*	2+
Yeast Extract	2+
Peptone + NH_4Cl	4+
Casein Hydrolysate + NH_4Cl	3+
Yeast Extract + NH_4Cl	3+

*Casein hydrolysate used was acid hydrolyzed; obtained from Sigma Chemical Company.

or yeast extract.

The supernatant of a liquid culture containing 1 percent 2-butanol and 0.5 ml of Geotrichum cells suspended in buffered saline and incubated at $25^{o}C$ for 48 hours, was examined by flame ionization gas chromatography to determine the products of oxidation of 2-butanol by the Geotrichum. The oxidation products were identified by the comparison of the retention times with a standard. Samples from the cultures were aseptically removed at various times starting at 3 hours, and continuing for every three hours for a period of 60 hours. The samples were centrifuged and refrigerated until time of analysis for solvent and acid production. A number of solvents such methy ethyl ketone at 24 hours, acetone at 18 hours, propanol and isopropanol at 36 hours, and methanol or formaldehyde at 6 hours were detected. The column that was used was unable to distinguish between methanol and formaldehyde. Probably the most significant observation, and the most interpretable, is the presence of methyl ethyl ketone, the immediate oxidation product of secondary butanol. It is harder to account for acetone, n-propanol, isopropanol, and methanol-formaldehyde in terms of any known metabolic reaction.

In addition to the gas chromatography analysis, attempts were made to detect carboxyl-containing compounds by the use of a paper chromatographic method for detecting the phenylhydrazone derivatives of such compounds (Strassman, et al., 1960). Supernatants from the Geotrichum cultures containing secondary butanol were analyzed by this method. The derivatives of phenylhydrazone were spotted onto Whatman number one paper and developed in n-butanol--0.5 N NH_3--and ethanol (70:20:10) for four to five hours. No conclusive results were obtained from these experiments; however, some type of carbonyl compound seems to be present in the

supernatants.

The Hestrin Test which detects ester or lactone linkages was done (Hestrin, 1949). Also, the Carbonyl test which tests for keto acids and other carbonyl compounds was done using the Geotrichum culture supernatants. The results of these tests also confirm the presence of one or more compounds containing a carbonyl group in the metabolic product(s) of secondary butanol by the Geotrichum.

There has been a recent report by Patel et al. on the oxidation of secondary alcohols to methyl ketones by yeast (Patel et al., 1979). They found that certain yeasts oxidized 2-butanol to 2-butanone. Our preliminary data also seem to indicate that 2-butanone is the first intermediate produced in the oxidation of 2-butanol.

Further experiments need to be done to determine the mode of attack of 2-butanol by the Geotrichum. The elucidation of this mechanism could prove very significant since relatively few soil organisms seem to possess the ability to oxidize secondary butanol.

VITA 2

Jonell Douglas

Candidate for the Degree of

Master of Science

Thesis: THE POSSIBLE ROLE OF ACTINOMYCETES IN THE BIODEGRADATION OF ENHANCED OIL RECOVERY COMPOUNDS

Major Field: Microbiology

Biographical:

Personal Data: Born in Seoul, Korea, January 11, 1958, the daughter of Jack and Dorothy M. Douglas.

Education: Graduated from Sooner High School, Bartlesville, Oklahoma, in May 1976; received the Bachelor of Science degree in Microbiology from Oklahoma State University in May 1980; completed requirements for the Master of Science degree at Oklahoma State University in December, 1983.

Professional Experience: Graduate teaching assistant, Oklahoma State University, 1981-1983; research assistant, Oklahoma State University, 1980-1981.