

BNL 60119
(DE95000159)

EFFECTS OF SELECTED THERMOPHILIC MICROORGANISMS
ON CRUDE OILS AT ELEVATED TEMPERATURES AND PRESSURES

Final Report

By
E. T. Premuzic and M. S. Lin

July 1995

Performed Under Contract No. DE-AC02-76CH00016

Brookhaven National Laboratory
Upton, New York



**Bartlesville Project Office
U. S. DEPARTMENT OF ENERGY
Bartlesville, Oklahoma**

**FOUNDED
1957**

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, expressed or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government.

This report has been reproduced directly from the best available copy.

Available to DOE and DOE contractors from the Office of Scientific and Technical Information, P.O. Box 62, Oak Ridge, TN 37831; prices available from (615) 576-8401.

Available to the public from the National Technical Information Service, U.S. Department of Commerce, 5285 Port Royal Rd., Springfield VA 22161

BNL 60119
Distribution Category UC-122

Effects of Selected Thermophilic Microorganisms
on Crude Oils at Elevated Temperatures and Pressures

Final Report

By
E. T. Premuzic
and
M. S. Lin

July 1995

Work Performed Under Contract No. DE-AC02-76CH00016

Prepared for
U.S. Department of Energy
Assistant Secretary for Fossil Energy

Rhonda Lindsey, Project Manager
Bartlesville Project Office
P.O. Box 1398
Bartlesville, OK 74005

Prepared by
Brookhaven National Laboratory
Department of Applied Science
Upton, NY 11973

Table of Contents

	Page
Abstract.....	vi
1. Objectives.....	1
2. Background.....	1
3. Experimental.....	4
3.1 Bioreactors.....	4
3.2 Temperature/Pressure Units.....	4
3.3 Scaled-up Temperature/Pressure Units.....	7
3.4 Growth and Adaptation of Microorganisms.....	9
3.5 Instrumentation.....	11
(a) Gas Chromatography-Mass Spectrometry.....	11
(b) Metal Complex and Trace Metal Analysis.....	17
(c) Sulfur Analysis.....	17
3.6 Core-Flooding System.....	17
Results and Discussion.....	19
4. Multiple Effects.....	19
4.1 Acidification.....	19
4.2 Variations in the Extent of Emulsification.....	20
4.3 Biochemical Modification of Hydrocarbons.....	31
4.4 Biochemical Modification of Heterocyclic and Organometallic Compounds.....	53
4.5 Microbial Reference Library and its Application.....	65

Table of Contents (cont.)

	Page
5. Analysis of Combined Effects.....	70
5.1 Enhancement.....	70
5.2 Comparison of "Induced" vs. "Indigenous" Bioconversion of Crude Oils.....	83
5.3 Relative Abundance of Major Fractions and their Significance in Biochemical Processes Associated with MEOR..	87
5.4 Duration of Biotreatment and Media Effects.....	96
5.5 Some Chemical Properties of the Emulsified Phase.....	130
5.6 Microscopic Comparison of Reaction Mixtures.....	132
6. Core-Flooding Experiments.....	144
7. Biochemical Mechanisms.....	151
8. International Conference on Microbial Enhanced Oil Recovery.....	161
9. Conclusions.....	164
10. Publications, Reports, and Presentations.....	165
11. Recommendations.....	169
12. References.....	171

Participating Team

and

Acknowledgements

R&D Staff: Eugene T. Premuzic, Mow S. Lin, Bettie Sylvester, Lori Racaniello, Jeffrey Yablon, J.-Z. Jin, Yao Lin, Karlene Hamilton, Guo Kun Ji, Xu Qian Fan, Wei Min Zhou, and Lei Shing (Rina) Wu.

Note: Courtesy of other programs, notably the Educational and Minority programs, contributions of many participants are also acknowledged. These include all the students/minorities/teachers/visiting faculty and joint programs with the Department of Chemical Engineering at Howard University.

We also wish to thank Mitzi McKenna, Corrine Messana, and Sue Walch for the processing, proofing, and editing of this manuscript.

Abstract

During the past several years, a considerable amount of work has been carried out showing that microbially enhanced oil recovery (MEOR) is promising and the resulting biotechnology may be deliverable. At the Brookhaven National Laboratory (BNL), systematic studies have been conducted which dealt with the effects of thermophilic and thermoadapted bacteria on the chemical and physical properties of selected types of crude oils at elevated temperatures and pressures. Particular attention was paid to heavy crude oils from Venezuela, California, Alabama, Arkansas, Wyoming, Alaska, and other oil producing areas. Current studies indicate that during the biotreatment several chemical and physical properties of crude oils are affected. The oils are (1) emulsified; (2) acidified; (3) there is a qualitative and quantitative change in light and heavy fractions of the crudes; (4) there are chemical changes in fractions containing sulfur compounds; (5) there is an apparent reduction in the concentration of trace metals; (6) the qualitative and quantitative changes appear to be microbial species dependent; and (7) there is a distinction between "biodegraded" and "biotreated" oils. The former is more suitable for changes which occur under natural conditions over geological periods of time, and the latter is more applicable to changes brought about by deliberately introduced microorganisms acting over short periods of time. Further, preliminary results indicate that the introduced microorganisms may become the dominant species in the bioconversion of oils. These studies have also generated information which supports the view that the biochemical interactions between crude oils and microorganisms follow distinct trends, characterized by a group of chemical markers. Such markers are useful in the prediction of bioprocessing efficiency prior to core-flooding experiments and

field testing. Core-flooding experiments based on these predictions have shown that compared to commonly used microorganisms, e.g. Clostridium sp., significant additional crude oil recoveries are achievable due to the biochemical action of thermophilic (thermoadapted) microorganisms at elevated temperatures similar to those found in oil reservoirs. In addition, the chemical and biochemical studies conducted at BNL have also shown that the biochemical treatment of crude oils has technological applications in downstream processing of crude oils such as in upgrading of low grade oils and the production of hydrocarbon based detergents.

1. Objectives

The objective of this program is to determine the chemical and physical effects of thermophilic and temperature adapted organisms on a number of geochemically distinct and different crude oils at elevated temperatures and pressures, e.g., 65°C and higher and pressures up to 2500 psi. The information gained in these studies is used to explore the effects of the same set of organisms on samples of oil containing sandstone cores under experimental conditions which match those found in reservoirs, i.e., different temperatures, pressures, salinities, and permeabilities. A number of parameters known to define the best candidate for microbial enhanced oil recovery (MEOR) are known. As defined by laboratory studies generated in this program, the best MEOR candidates will be used for scale-up and technical feasibility studies leading to field applications.

2. Background

Over the past several years active groups at Universities, Government Institutions, and Industry in the U.S.A. and abroad have laid the groundwork for MEOR (King and Stevens, 1987; Grola, 1987; Lazar, 1987; King, 1987; Bryant, 1991). The record indicates that MEOR is a viable technology with a strong scientific and technical basis recently discussed by world experts at the Fourth International Conference on Microbial Enhanced Oil Recovery held at the Brookhaven National Laboratory in September of 1992 (Premuzic and Woodhead, 1993). Although additional studies of the biochemistry, geochemistry, biophysics, geophysics, and microbiology of microorganism-oil interactions are needed, data allow us to suggest several mechanisms and trends of microbial-oil interactions (Donaldson, 1987; Bryant and Douglas, 1987;

Moses, 1991; Jack, 1993; Premuzic et al., 1993). These include: (1) production of gases (CO_2 , H_2 , N_2 , CH_4) which can increase pressure in the reservoir and reduce oil viscosity; (2) microbial production of low molecular weight acids, which cause reservoir rock solubilization; (3) production of biosurfactants which decrease oil surface and interfacial tensions; (4) microbially mediated changes in oil wettability; and (5) production of polymers which facilitate oil mobility. Some of the desirable properties of bacteria to be used in MEOR should include microorganisms capable of a large production of "oil releasing" metabolites (e.g., low molecular weight compounds); they should not require expensive nutrients, they should be anaerobic and/or be capable of growing in the presence of low oxygen concentrations, and be able to withstand relatively high pressures and salinities. Also, such microorganisms should be easily grown in facilities aboveground, remain viable over extended time, and be transportable. Once underground, they should continue to be viable and resume their activity upon refeeding. Further, recent work indicates (Premuzic and Lin, 1991a,b) that biochemical treatment of crude oils may have useful applications in downstream operations.

A number of microorganisms have been found to be present in formation waters and were viable under certain reservoir conditions (Donaldson, 1986). The usefulness of these bacteria appears to be limited because, for these organisms to grow, reservoirs have to be within a given depth, salinity, temperature, and permeability range and also be resistant to increases in the concentration of products generated by their action. Thermophilic organisms live under harsh conditions, such as acidity, alkalinity, and high temperatures (up to 110°C), and some are known to grow under alkaline conditions (Brock, 1978 and 1985). These organisms can use inorganic (e.g., sulfides,

elemental sulfur, ferrous iron) and organic energy sources. Some of these bacteria are also capable of switching from an aerobic to anaerobic metabolism. Further, the natural habitats of these organisms are geothermal brines which means that they are tolerant of high salt concentrations. Thus, the general properties of thermophilic organisms, although not fully explored, possess a number of desirable properties for MEOR.

The MEOR program at BNL has generated data which indicate advantages of using thermophilic organisms (Premuzic and Lin, 1989a-e, 1991a-c; and Premuzic et al., 1993). In addition to properties already mentioned, they are active at temperatures of 75°C, pressures of 2000 psi, tolerate >3% salt (as NaCl) concentrations, bioconvert high molecular weight oil fractions to lighter, and concurrently remove sulfur and trace metals.

Further, in this program, experimental strategies have been developed which allow selected microorganisms to adapt to indigenous crude oil as energy source. Detailed studies have also been conducted on the chemical and biochemical interactions between thermophilic/thermoadapted organisms and crude oils whose chemical and physical properties are known, and to explore the feasibility of MEOR biotechnology based on these interactions. In this report, the progress in studies dealing with the biotreatment of crude oils over a wide range of experimental conditions is discussed. Several topics will be addressed which will include emulsification, qualitative and quantitative changes in the composition of low and high molecular fractions of crude oils, in the composition of fractions containing sulfur and organometallic compounds as well as the trends in the biochemical interactions between microorganisms and crude oils. These trends are characterized by a set of chemical markers which can be used as monitoring and diagnostic tools in the

prediction of the extent of bioconversion of a crude oil. Their mechanistic significance and applicability in core-flooding experiments will also be discussed.

3. Experimental

Interactions between microorganisms and crude oils are complex and involve multiple processes. In order to understand better the chemistry and biochemistry of these processes extensive studies and analyses of several variable parameters are needed. To accomplish some of these requirements we have expanded and/or modified our instrumental capabilities such as Parr reactors, so that many variable parameters can be measured at any given time. Expansion and modification of instrumentation capabilities was carried out as necessary.

3.1 Bioreactors

Commercially available reactors, e.g., Brunswick type model NB-424 (Brunswick, N.J.), and flask type have been used for routine maintenance of microorganisms.

3.2 Temperature/Pressure Units

Pipe-type reactors have been constructed from stainless steel, capable of handling a total volume of 10 ml of fluid, and able to withstand pressures of up to 8000 psi. The pressures are adjustable by means of "external valves and the whole bioreactor unit can be placed in temperature controlled vessels. Under experimental conditions, they were used at 75°C and pressures of 2000-2500 psi. In terms of the biomass and the medium to oil ratio, the oil capacity of such mini-bioreactors is in the order of 0.2-1.0 ml. Typical experimental arrangements are shown in Figures 1 and 2.

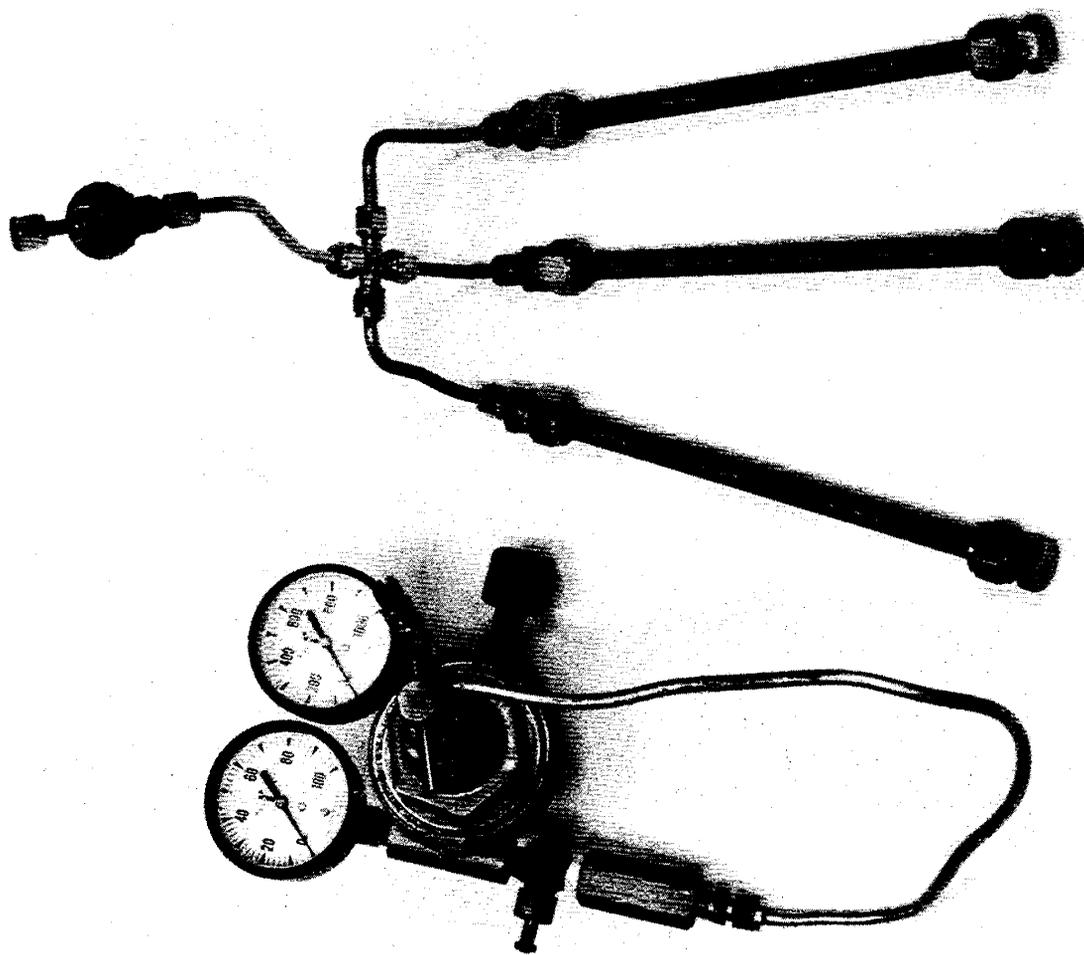


Figure 1. Bioreactor for high temperature and pressure processing.

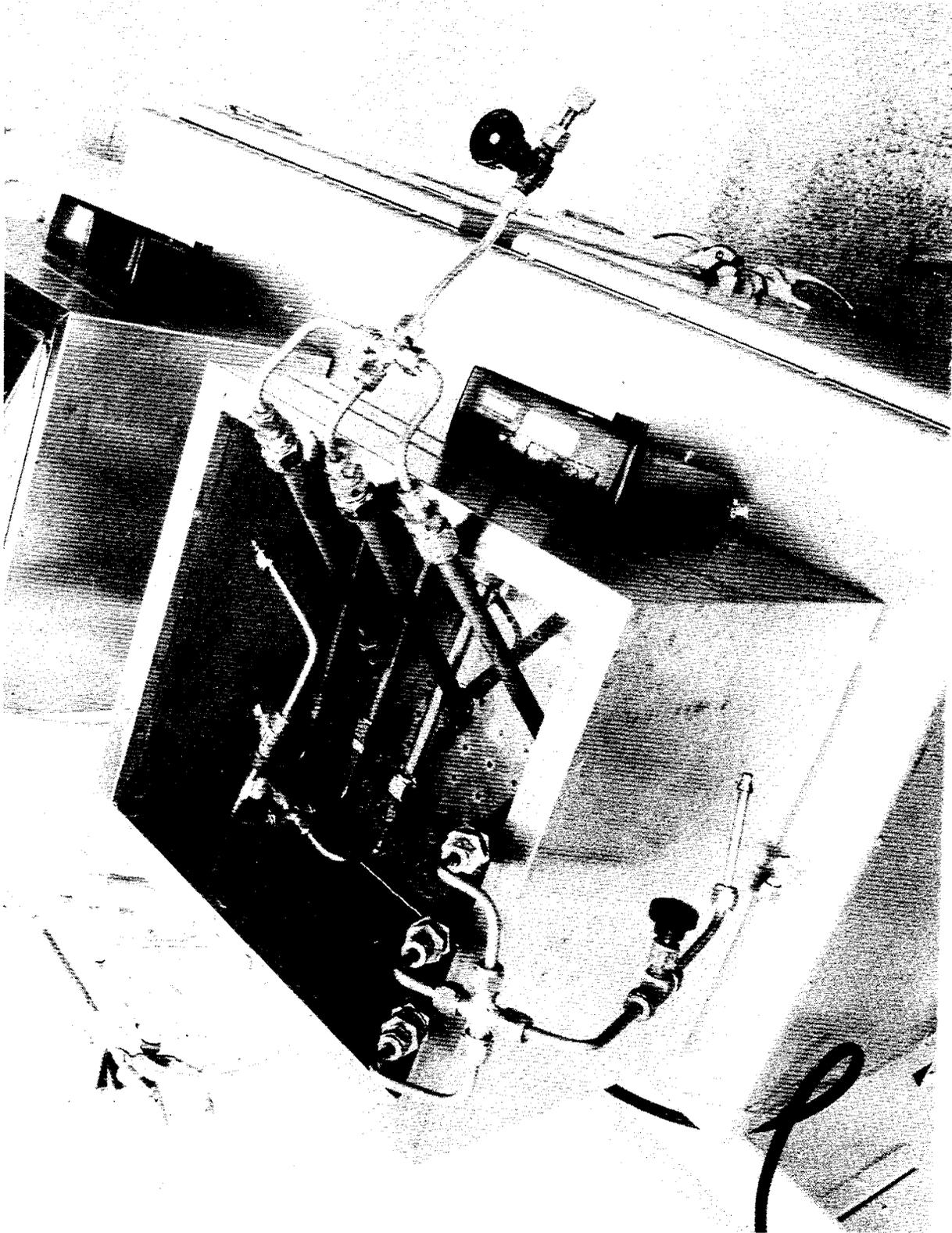


Figure 2. Bioreactors in constant temperature bath.

3.3 Scaled-up Temperature/Pressure Units

For larger scale experimentation (up to 250 ml) a Parr reactor has been modified as shown in Figure 3. This unit is a modified Type-316 stainless steel reactor (5.0" I.D., bolted catalytic reactor, Autoclave Engineers), and is rated to 650°F at 3700 psi pressure and 1000°F at 2000 psi pressure.

The equipment has a removable jacket type three zone furnace. The temperature of the furnace is regulated by a programmable controller (Omega CN-2000 series) consisting of a monitor, relay and thermocouple. This control turns on two 1100 Watt and one 800 Watt element. The control will only heat the vessel to a preset value; if the temperature exceeds that value the controller shuts off the furnace. This controller does not monitor the temperature of the contents of the reaction vessel. Temperature can be regulated to within $\pm 2^\circ\text{F}$.

The reactor contains a 250 ml Teflon liner, which acts as the reaction vessel, encased in a Type-316 stainless steel jacket. The portion of the reaction vessel containing the medium is Teflon lined supporting chemical inertness, mechanical strength, and equivalent thermal expansion. To insure vapor liquid equilibrium throughout the system the gas is recirculated through the system by a magnet drive assembly. The magnet drive assembly consists of an impeller mounted on a shaft driven by an electric motor. The vessel is equipped with appropriate valves and ports to allow for applying gas pressure and gas sampling. The vessel is also equipped with thermowell opening to facilitate other places for thermocouples. The reactor is closed by means of bolted closure. Engineering design for a two-liter capacity temperature/pressure bioreactor has also been completed.

Reactor Design
Growing Thermophilic
Bacteria in Batch Process

Temperature: 65 °C
 Pressure: 1500–2000 psia
 Capacity: 250 ml

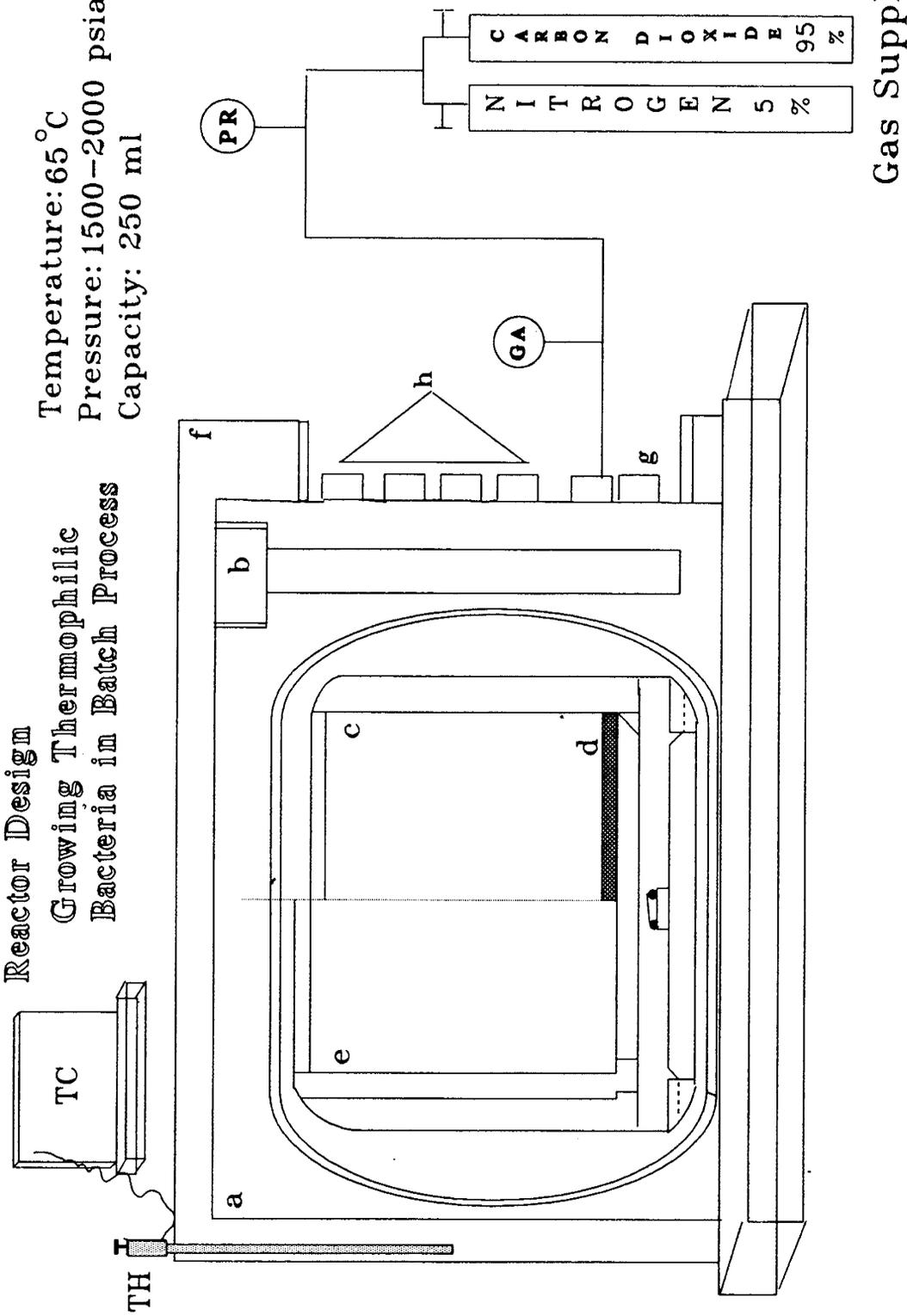


Figure 3. 250 Pressure/Temperature Reactor. Cover (a); 1"-14 Hex. Soc. Cap Screw, 8 (b); stainless steel jacket (c); screen (d); Teflon liner (e); heater jacket (f); heater assembly (GA); thermocouple (TH); pressure regulator (PR); temperature controller (TC); outlet port (g); thermowell openings (h).

3.4 Growth and Adaptation of Microorganisms

Culture media consisted of inorganic salts, e.g., $(\text{NH}_4)_2\text{SO}_4$, MgSO_4 , KH_2PO_4 and crude oil, yeast or peptone extract as a source of carbon. Incubations were carried out at different temperatures (30-80°C) and pressures (200-2500 psi). Through a sequence of different experimental regimes, a methodology has been developed (Premuzic and Lin, Patent Pending) which makes it possible to adapt microorganisms to different temperatures and pressures. In this methodology, modified bioreactors allow for continuous microbial growth in a medium in which a temperature gradient is maintained over a temperature range from 4°C to >85°C. Typical experimental conditions for a series of experiments are shown in Table 1. In all adaptation studies, oil becomes the sole carbon source. In addition to the development of thermoadapted microorganisms, a library, now consisting of about one hundred different thermophilic microorganisms, has been established at BNL. The collection of the thermophilic, acidophilic, and barophilic microorganisms has been accomplished through field trips to extreme environments, such as boiling muds containing natural oil seeps to high saline, geothermal areas, as well as personal contacts. Each microorganism has been coded with a BNL number, e.g., BNL-TH-29, BNL-NZ-3, etc. Routinely, these organisms were grown in the presence of crude oil as a sole source of carbon containing inorganic salts only, referred to as Medium 1. Medium 1 contains the following inorganic salts: K_2HPO_4 , 0.785g; KH_2PO_4 , 0.445g; NaCl , 2.5g; CaCl_2 , 0.25g; $(\text{NH}_4)_2\text{SO}_4$, 0.4g; MgSO_4 , 0.25g. The nutrient is made up of 0.5g of peptone and 0.3g of beef extracts in 1000 ml of medium, referred to as Medium 2. Other media used were: Medium A is medium 1304, supplemented with a non-peptone modified carbon source, B is a nutrient broth containing beef extract supplemented by a non-peptone carbon

Table 1. Examples of Microbial species and treatments.

<u>Microorganism Species</u>	<u>Treatment Conditions</u>			
	Temp. °C range	Medium	Pressure psi	pH
BNL-TH-29 Acidophilic thermophile	60-80	A	up to 2000	1.5-4.5
BNL-TH-31 Acidophilic thermophile	60-80	A	up to 2000	1.5-4.5
BNL-4-21 <u>Acinetobacter</u>	25-75	B	Atm.	6-7.5
BNL-4-22 <u>Arthrobacter</u>	25-75	B	Atm.	6-7.5
BNL-4-23 <u>Achromobacter</u>	25-75	B	Atm.	6-7.5
BNL-4-24 <u>Pseudomonas</u>	25-75	B	Atm.	6-7.5
BNL-4-25 <u>Nocardia</u>	30	C	Atm.	7
BNL-5-32 <u>Methanogenium</u>	55-60	D	Atm.	6-7.5
BNL-TH-1 Acidophilic thermophile	60-80	E	up to 2000	1.5-2.5
BNL-3-25 Acidophilic thermophile	30-60	F	100	1.0-2.5
BNL-4-25 Acidophilic thermophile	30-60	G	100	1.0-2.5

Medium A is medium 1304, supplemented with a non-peptone modified carbon source, B is a nutrient broth containing beef extract supplemented by a non-peptone carbon source. C medium is a yeast extract medium, D and E media are 1442 and 1256, respectively, also supplemented with non-peptone modified carbon source. Medium F is a basal salt solution, and G is a basal salt solution supplemented with iron sulfate.

source. C medium is a yeast extract medium, D and E media are 1442 and 1256, respectively, also supplemented with non-peptone modified carbon source. Medium F is a basal salt solution, and G is a basal salt solution supplemented with iron sulfate. A modified medium, containing 0.08% of added carbon nutrient was used in some experiments. In all cases, the crude oil was present in a large excess (at least six fold). For temperature/pressures studies, each experiment used three bioreactors under identical temperature and pressure conditions. One bioreactor contained culture medium, gases (CO₂, N₂), and the experimental organisms, e.g., BNL-TH-1. The second bioreactor contained oil (0.2-1.0 ml), gases (CO₂, N₂), and culture medium. The third bioreactor contained oil (0.2-1.0 ml), microorganism (BNL TH-1), inorganic salts (same as used in medium), and gases (CO₂, N₂). All were kept for different lengths of time (days-months) at 70°C and 2000 psi. In the course of the experimentation, results suggested modifications in the experimental protocol. These will be addressed and discussed in the body of this report.

3.5 Instrumentation

(a) Gas Chromatography-Mass Spectrometric (GC-MS) Analysis. Initial analyses have been carried out on a Hewlett-Packard 5890 GC-MS system. As needed, a Kratos D390 high resolution mass spectrometer was also made available. Further, a Perkin-Elmer model 8700 gas chromatograph has been purchased and installed as a dedicated instrument for MEOR studies. This instrument is a microprocessor-controlled gas chromatograph with multiramp temperature programming, soft key entry of all temperature and time parameters on color VDU, dual-channel screen graphics, dual-channel integral data handling, and reintegration. Four internal and four external timed events and two RS-232C

communications ports are also available. There is an access to ten methods and Automated Bleed Compensation.

Additional features of the gas chromatograph are:

1. Left channel split/splitless capillary injector.
2. Right channel single packed column injector (1/8 in.).
3. Left channel single Flame Ionization Detector (FID), includes amplifier and combustion gas pneumatics, air, and H₂.
4. Right channel single Flame Photometric Detector (FID), includes amplifier and combustion gas pneumatics, air, and H₂.
5. Left channel pneumatics, capillary pressure control (0-100 psig).
6. Right channel pneumatics, flow controller backed by pressure regulator.
7. The system is also interfaced with a CDS Pyroprobe, Model 120 (Chemical Data Systems, Inc.) pyrolyzer unit.

A Perkin-Elmer Iron Trap Detector (ITD) has also been purchased, installed, and interphased with the Perkin-Elmer model 8700 gas chromatograph (GC). The Iron Trap Detector System has a mass range of 20-650 mass units and has the following features:

1. Heated fused silica transfer line.
 2. Calibrated bleed to introduce calibration compounds.
 3. Air-cooled turbomolecular pump and direct-drive mechanical pump.
 4. Software package for detector control.
 5. Data acquisition; display of chromatograms and spectra, quantitation reports and diagnostics.
 6. Incos library search program and 3000 compound mass spectral library.
- The system also encompasses NBS/EPA Mass Spectral Library containing

over 42,000 mass spectra. The total ion chromatogram is displayed as a function of % peak intensity, y axis, and the chromatographic retention time in minutes or scan numbers expressed in seconds, x-axis. Selected peak identification can be done by comparison of several experimental mass spectral peaks with those of standards on file in the NBS/EPA library. For example, scan number 3702 (retention 62 mins, Figure 5) is entered into the library data base, and the program searches for the closest mass match.

7. Epson Equity III+ computer system contains: PC/AT class performance with a 80286 CPU; 640 kB RAM; 40 mB Winchester disk drive; 1.2 mB, 5.25 inch floppy disk drive.
8. Color CGA monitor.
9. Epson printer/plotter and all required cables and interfaces.

The interphased system has been installed in a separate room specifically converted for computerized analytical work.

A typical example of a GC/MS analysis of a crude oil is shown in Figure 4 (ITD trace). A FID trace for the Recluse, WY, untreated oil is given in Figure 5, and a library search for the Scan Number 3702 is given in Figure 6.

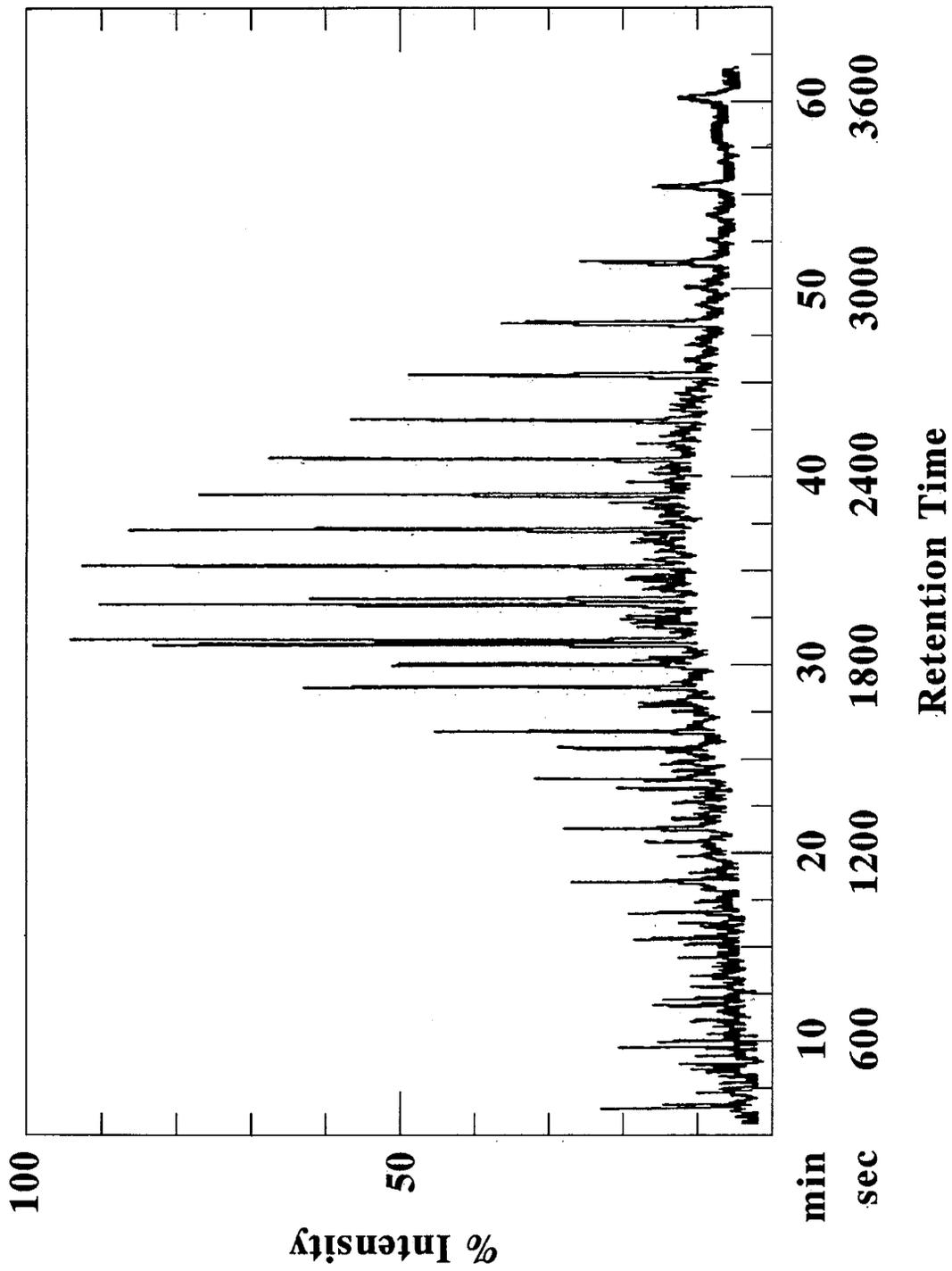


Figure 4. ITD Trace of Recluse, WY, untreated crude.

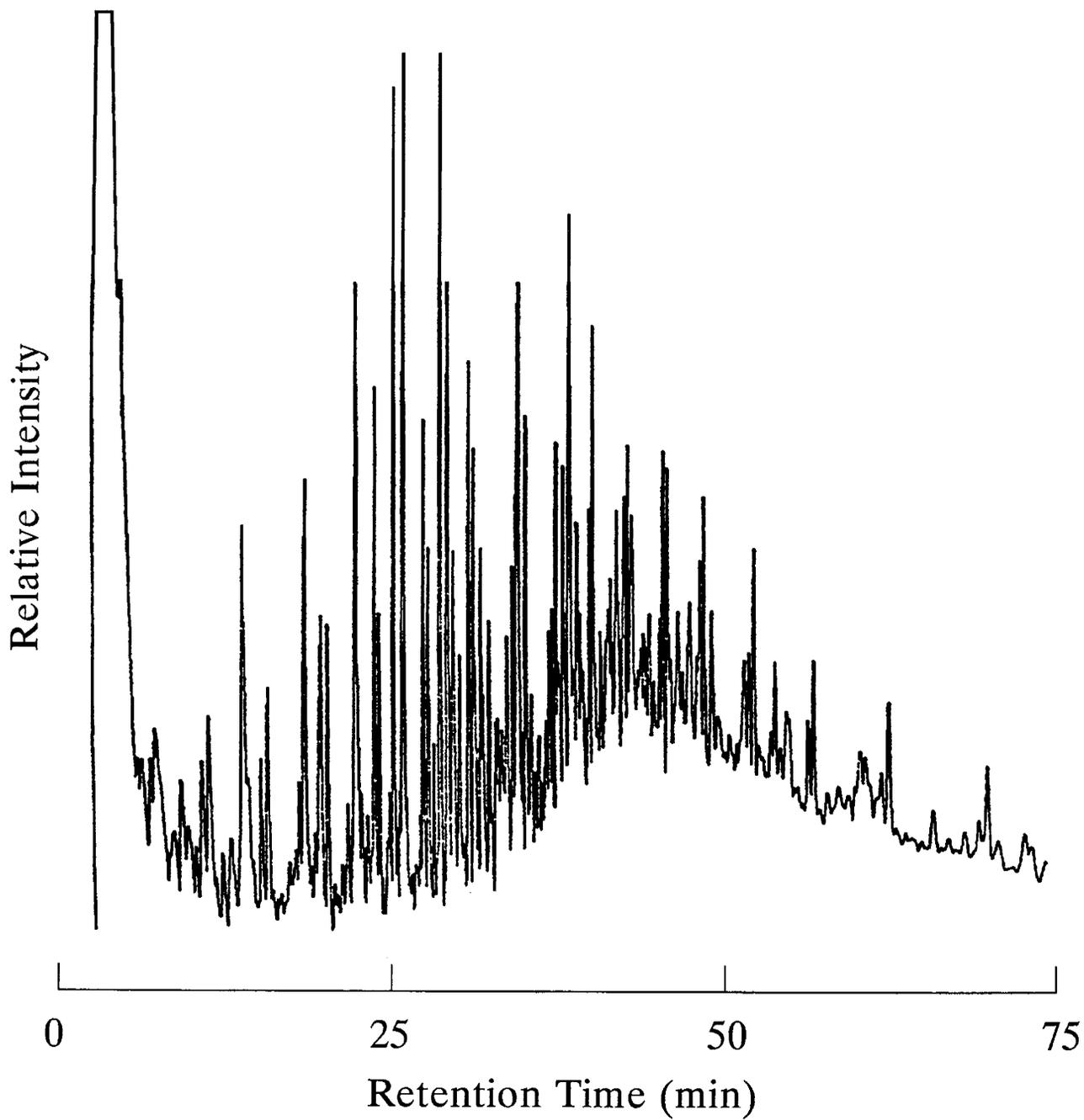


Figure 5. FID trace of Recluse, WY, untreated crude.

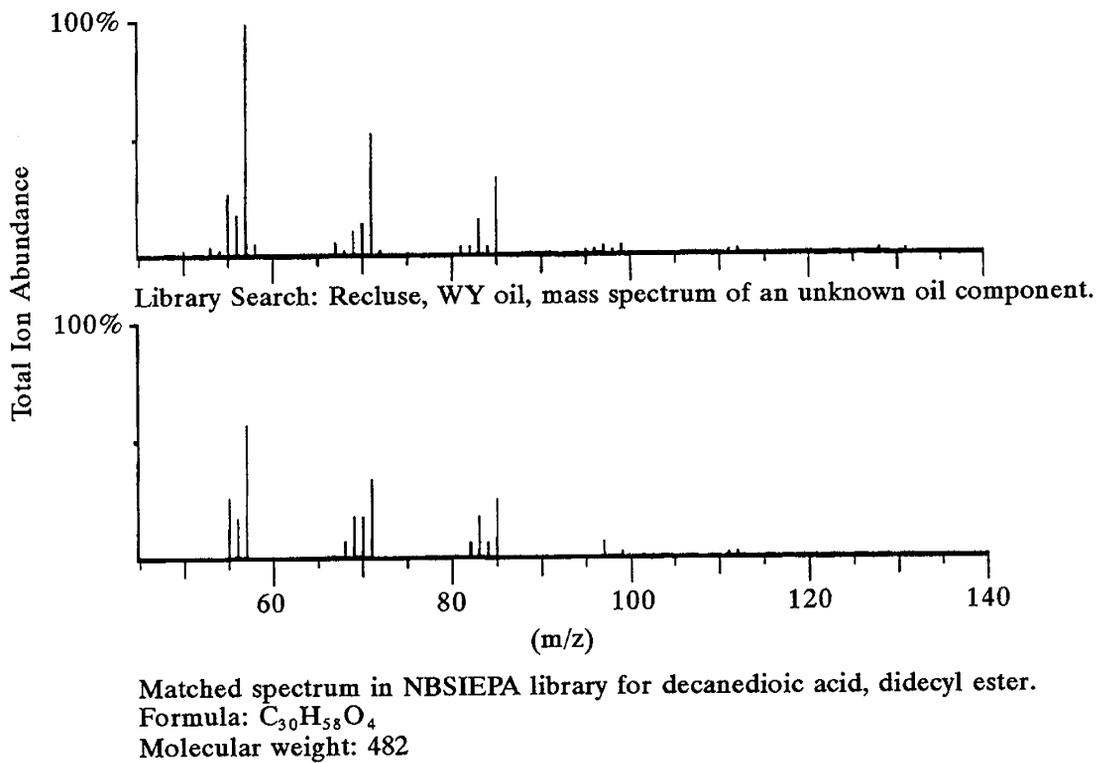


Figure 6. Computerized library search of Scan 3702.

(b) Metal Complexes and Trace Metal Analysis. Hewlett-Packard gas chromatograph model HP 5921A equipped with atomic emission detector was used for the determination of metal complexes. An Induced-Coupled Plasma Mass Spectrometer (ICP-MS), (VG Instruments Plasma Quad PQ 2+) has been used routinely. ICP-MS is capable of detecting and quantifying up to 70 elements in an analysis with a sensitivity of parts per trillion.

(c) Sulfur Analysis. Total sulfur was determined by combustion to SO₂ (Huffman Laboratories, Golden, CO). The organic sulfur compounds were monitored by GC, equipped with FPD, from 40°C-300°C . A J&W, DB-1 column was used throughout. Overall changes in the chemical nature of sulfur compounds were determined by X-ray Absorption Near-Edge Structure spectrometry (XANES), in the National Synchrotron Light Facility at the Brookhaven National Laboratory. This analytical method characterizes changes in groups of sulfur compounds present in oil, e.g., sulfides, thiophenes, and sulfoxides.

3.6 Core-flooding System

The core bioreactor was constructed from 304 stainless 1-1/4" x 12" tubes with a thickness of 0.065 inches, and can be operated up to a pressure of 2600 psi. The tube can be used under controlled temperature conditions and various pressures. The flow diagram for the system is given in Figure 7. Cores made of Berea sandstone cut to appropriate size are introduced into the tube and a tight fit achieved by means of an epoxy treatment of the core.

Flow Diagram for Core-Flooding Bioreactor

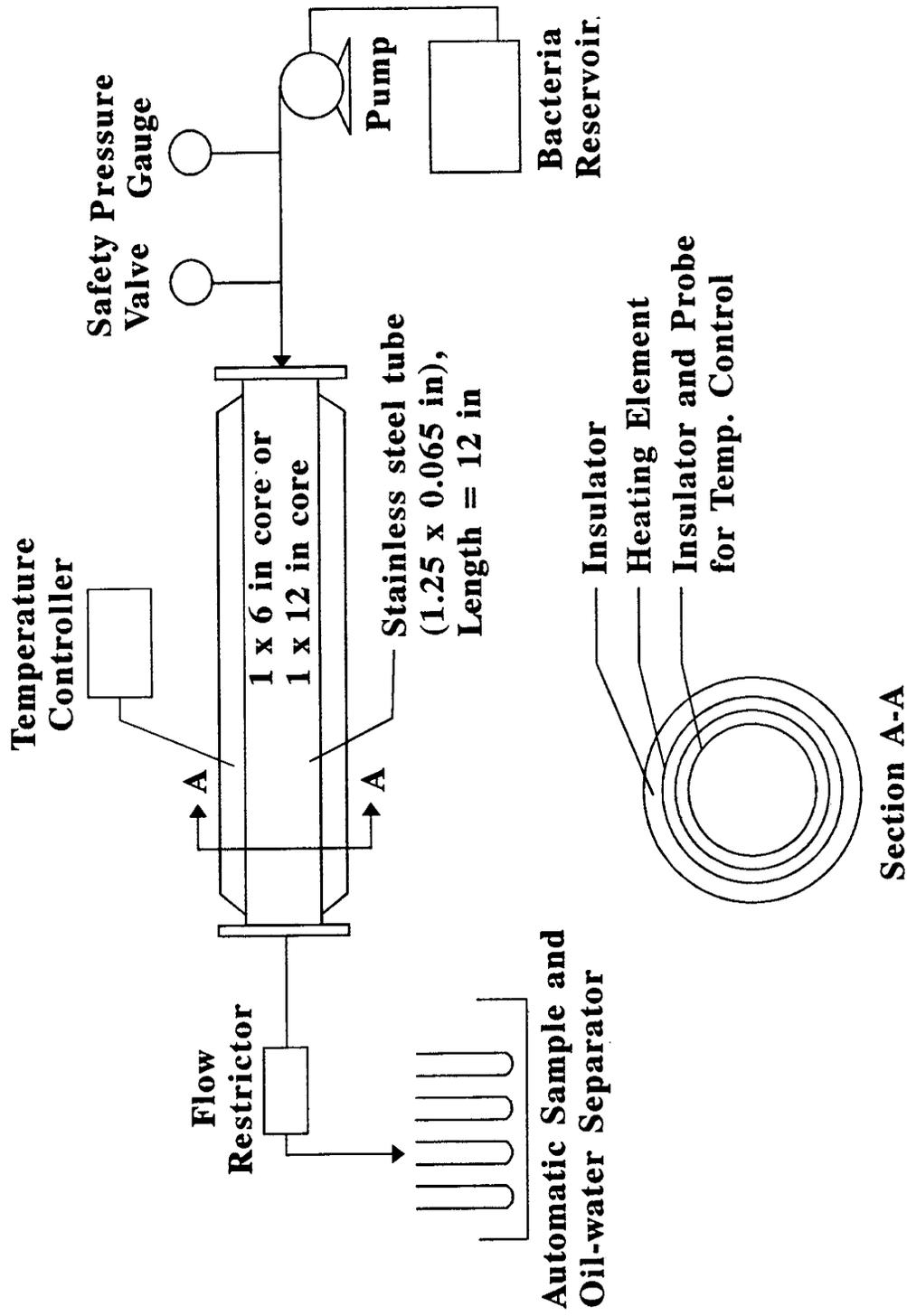


Figure 7. Flow diagram for the core-flooding system.

RESULTS AND DISCUSSION

4. Multiple Effects

Experimental evidence gathered in the early stages of this program, suggested that the interactions between the microbial species and different crude oils are complex multistep processes. Biochemical conversion of the crude oils involved production of acids and emulsifying agents, conversion of higher to lighter molecular weight hydrocarbons as well as reactions with heteroatoms and metal containing chemical constituents present in crude oils. Biochemical reactions involved in the conversion of oils appeared to be also dependent on both the types of microorganisms used and on the chemical composition of the crude. Further, the microbial species considered in these studies, used predominantly indigenous matter as their choice of energy source, which implies considerable intra- and inter-molecular chemical interactions. These complex reactions were followed by multiparameter analyses leading to the formation of a data base allowing for mechanistic and diagnostic evaluation of MEOR processes. Results of these studies will be discussed in the following sections of this report.

4.1 Acidification

Earlier experimental observations have shown that there is a lowering of pH from about pH 5 to as low as pH 1 (See Table 1) of the medium during the acidophilic microbial action on crude oil under our experimental conditions. These results indicated that the aqueous phase should be analyzed for water soluble compounds as possible causes of acidification. For example, a high pressure liquid chromatographic (HPLC) analysis carried out with a sample of PR3 (Teapot Naval Petroleum Reserve #3) crude treated with BNL-4-22 strain

revealed the presence of several organic acids. The result is shown in Figure 8. Assignments for peaks based on authentic standards with retention times in minutes at 8.46, 11.58, 13.84 and 23.87 are lactic, propionic, isobutyric acids and n-butanol, respectively.

Long chain fatty acids may also be produced during the biotreatment of oil. Because such acids are good emulsifying agents, additional studies were directed to characterize such acids. This was accomplished by the use of another strain, BNL-TH-1 and PR-3 crude oil. After the treatment, the culture medium was saponified with sodium hydroxide, and the free acids methylated with methanol/HCl. The produced methyl esters of fatty acids were extracted and analyzed by GC-MS (See Figure 9). The first three fatty acids identified are heptanoic acid (C-7), decanoic acid (C-10), and octadecanoic acid (C-18). The chemical nature of other emulsifying agents is discussed in Section 5.5.

4.2 Variations in the Extent of Emulsification

Concurrent with acidification, an emulsification of the reaction mixture was also observed. Typical results of spectrophotometric analyses of emulsions produced when PR3 crude oil was treated with BNL-TH-29 are shown in Figure 10. Thus, one observes an occurrence of a considerable biochemical reaction when the crude oil is the only source of carbon in the medium as shown by spectrum #2 in Figure 10.

In order to determine the effects of different microorganisms on PR3 and other crudes under the experimental conditions used, a series of experiments in which different types and/or strains of microorganisms were allowed to act upon the same oil have been carried out. The objective of this phase of the experimental program was to develop a data base for efficient "emulsifiers" and "acidifiers" and relate this to experimental conditions and chemical

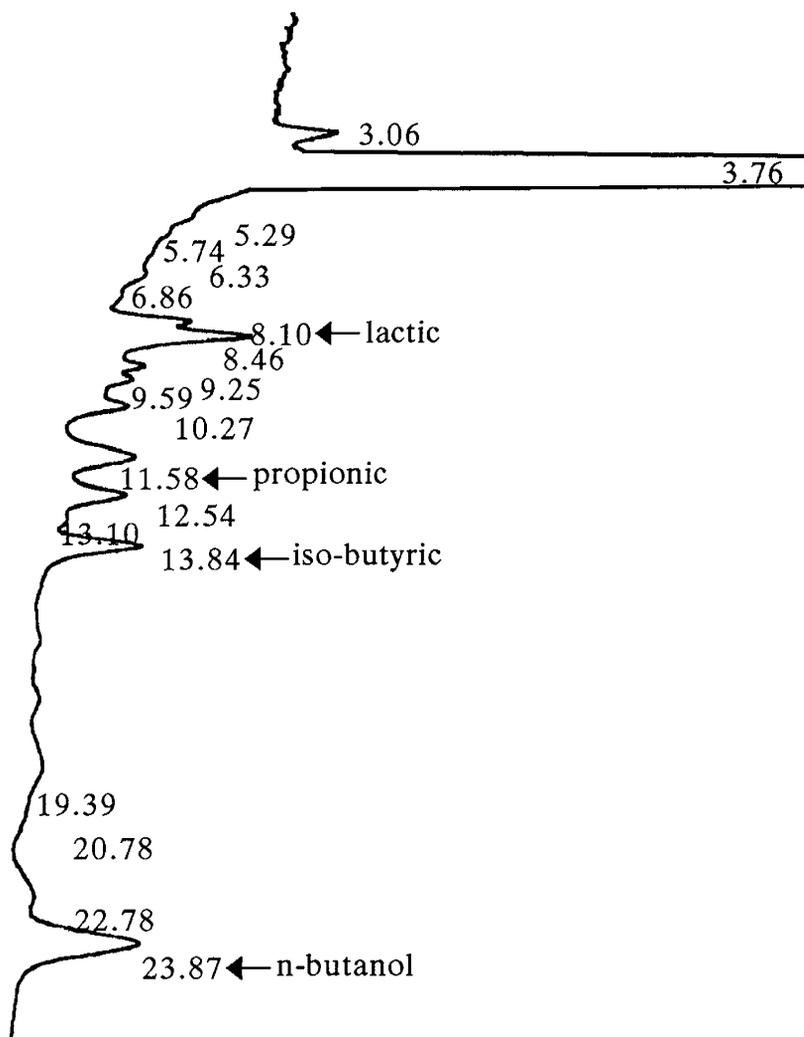


Figure 8. HPLC trace of PR3 + BNL-4-22 + medium.

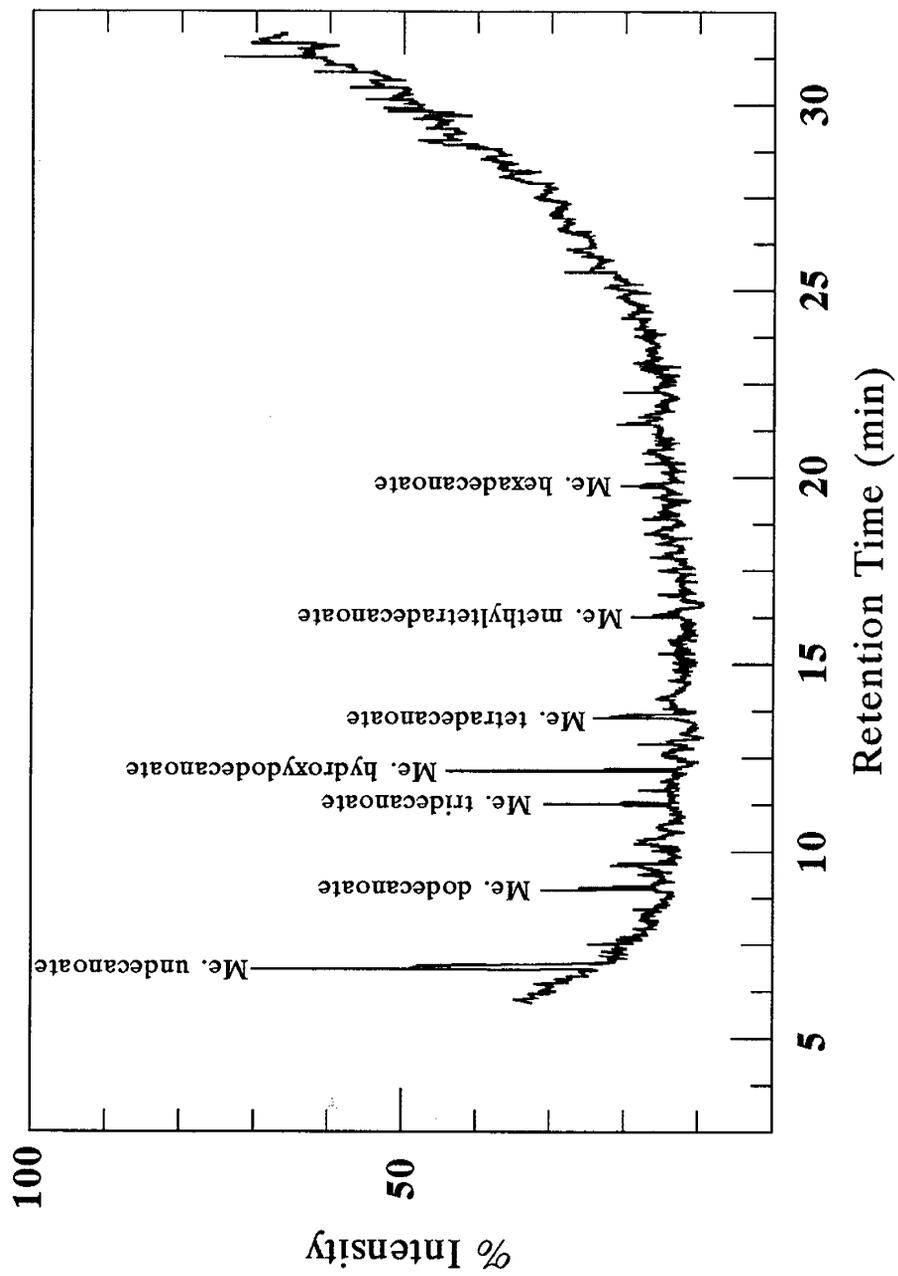


Figure 9. GC-trace of fatty acid methyl esters isolated from the PR3/BNL-TH-1 culture.

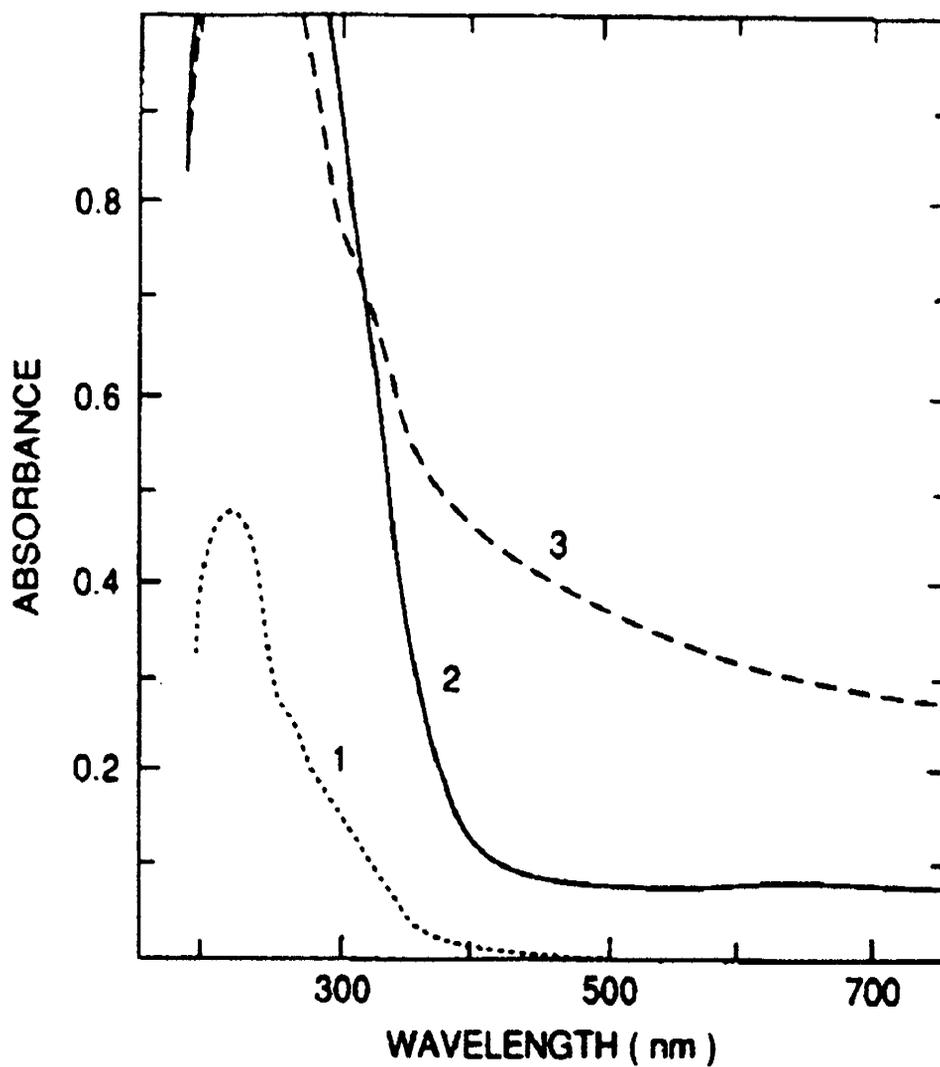


Figure 10. Extent of emulsification due to action of BNL-TH-29 on PR3 at 70°C and 2000 psi.

1. PR3 oil + medium (inorganic salts + yeast extract);
2. PR3 oil + medium (inorganic salts only) + bacteria;
3. PR3 oil + medium (inorganic salts + yeast extract) + bacteria.

changes. In Figure 11, spectra of control (PR3 + culture medium) and the results of treatment with two additional and different strains of microorganisms are shown. Significant differences in the extent of emulsification occur due to the action of different microorganisms. In very small samples of oil, the extent of emulsification is expressed in Klett units (Rosenberg et al., 1979) given by:

$$\frac{1000 \times D}{2} = R \text{ Klett units,}$$

where D is the absorbance determined at 545 nm. In terms of Klett units the difference between the three samples shown in Figure 11 are given in Table 2.

The extent of emulsification during biotreatment also varies with the action of different microorganisms on the same oil. Concurrently, there are changes in the hydrocarbon composition of the crude, which will be discussed in a separate section. However, for the purpose of this discussion, one may postulate that if there are different biochemistries associated with the action of several types of microorganisms on the same oil then it may be reasonable to expect similar microbial species dependent variations in the emulsification effects when a variety of chemically different crudes and their fractions are treated with a number of different microbial species. In order to further clarify these possibilities, a suite of heavy crude oil fractions have been treated by different microorganisms under identical experimental conditions, and the effects compared to those on asphalt. The choice of heavy fractions and asphaltenes have been based on an assumption that under field conditions these fractions represent the "worst case scenario," therefore, the least mobile and recoverable components of crude oils. The results are shown in Table 3 and Figure 12. If biochemical processing is to be effective, then

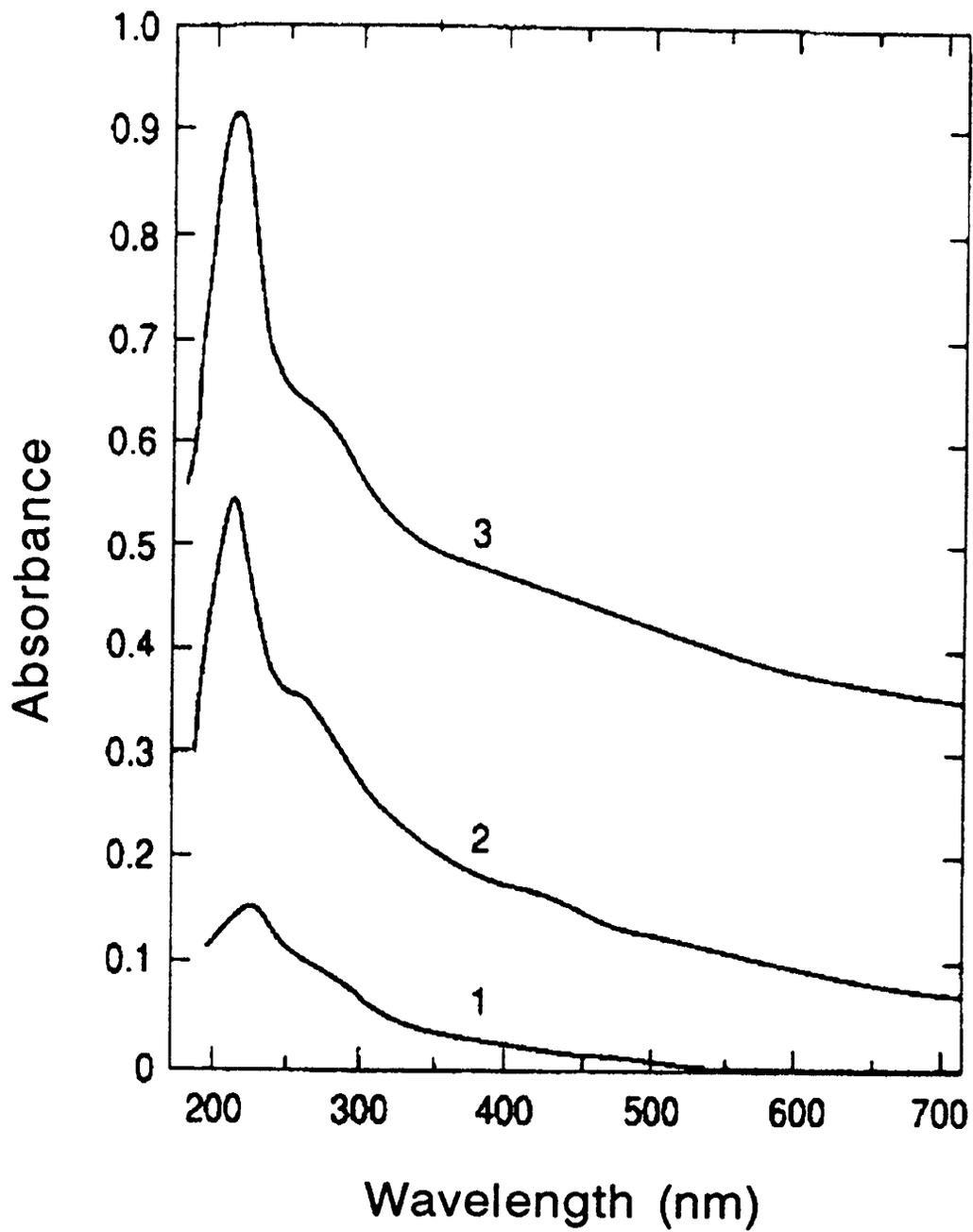


Figure 11. Extent of emulsification due to action of BNL-4-21 and BNL-4-22.
1 = PR3 oil + medium; 2 = PR3 + BNL-4-21; and 3 = PR3 + BNL-4-22.

Table 2. Extent of emulsification of Teapot Naval Reserve, PR3 Crude Oil.

<u>Sample</u>	<u>Klett units</u>
PR3 + culture medium	10
PR3 + BNL-4-21	50
PR3 + BNL-4-22	200

Table 3. Extent of emulsification due to the action of various microorganisms on heavy fractions of crude oils. The results are expressed in Klett units.

Microorganisms	Heavy Oil Fraction				
	Asphalt ¹	Wilmington ² (Calif)	Goch Saran ² (Iran)	Recluse ² (Wyo)	Prudhoe ² (Alaska)
BNL-4-24	35	115	168	250	215
BNL-4-23	250	290	238	225	195
BNL-4-22	275	252	320	175	285
BNL-4-21	475	515	142	600	615

¹Commercially available asphalt, e.g., for highway paving.

²Heavy fractions (>200°C) of crude distillate.

the chemical action should be effective in these fractions. This topic will be further discussed in the section dealing with mechanisms of biochemical conversion of crude oils. Suffice to say at this time that the results shown (Figure 12 and Table 3) strongly support that the extent of emulsification of crudes depends on the microbial species and chemical composition of the oil being treated. These observations were further documented by the following set of experiments.

Several crude oils were incubated with two species of bacteria over a period of two months and the viscosity of the produced emulsions were then measured with a viscometer at 25°C. The results are shown in Table 4.

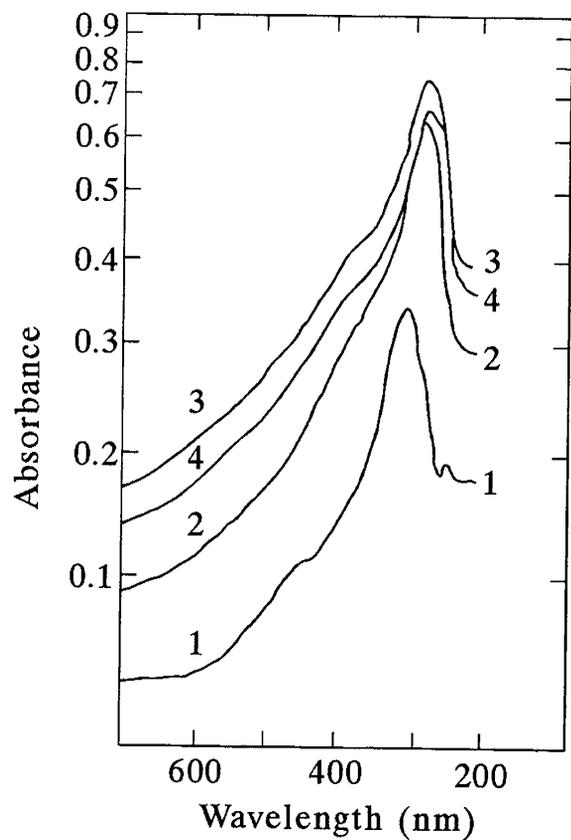


Figure 12. Extent of emulsification (700-200 nm spectral region) of PR3 by BNL-4-24 (1); BNL-4-23 (2); BNL-4-22 (3); BNL-4-21 (4).

Table 4. Variations in viscosity of three crude oils due to the biochemical action of two microorganisms.

Oil	Viscosity before Treatment	Viscosity in Centipoises	
		BNL-4-26	BNL-4-25
Prudhoe Bay (Alaska) Naval Petroleum Reserve	23	3.9	5.2
PR3 (Wyoming)	10	5.3	4.0
Wilmington (California)	400	3.5	3.7

Variations in viscosity are also consistent with the microbial species-oil type dependencies discussed earlier in this section. To further verify this dependence a sample of the Naval Petroleum Reserve (PR3) crude oil was treated with four different types of bacteria under identical experimental conditions. The produced oil emulsions containing the oil were then extracted with methylene chloride and the solvent removed by evaporation. Results shown in Table 5 are both in terms of Klett units and grams of oil per liter of emulsion produced. The corresponding spectrometric analyses is given in Figure 12.

The results summarized in Table 5 show a marked bacterial species dependence on the amount of oil present in the emulsion. Gas Chromatographic-Mass Spectrometric comparison of BNL-4-23 treated PR3 oil in emulsion and control (aqueous phase + nutrients + oil only) shows that C-15 to C-27 hydrocarbons have been emulsified as shown in Figures 13 and 14. The emulsion produced by the biochemical action of microorganisms on crude oil is enriched in C-15, C-18, C-19, C-20, C-21 and C-22 hydrocarbons as shown in the GC trace (Figures 13, 14) of chromatographic analyses conducted under identical

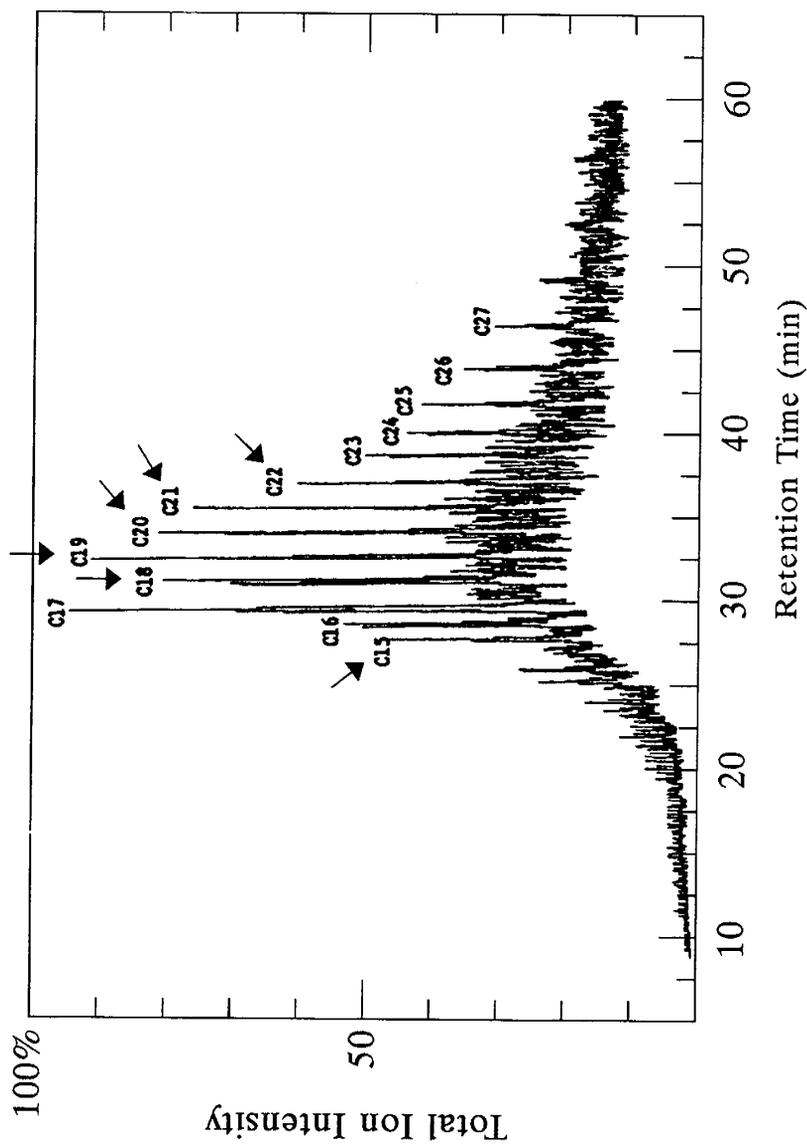


Figure 13. GC trace of biotreated solvent extracted emulsified fraction of the oil.

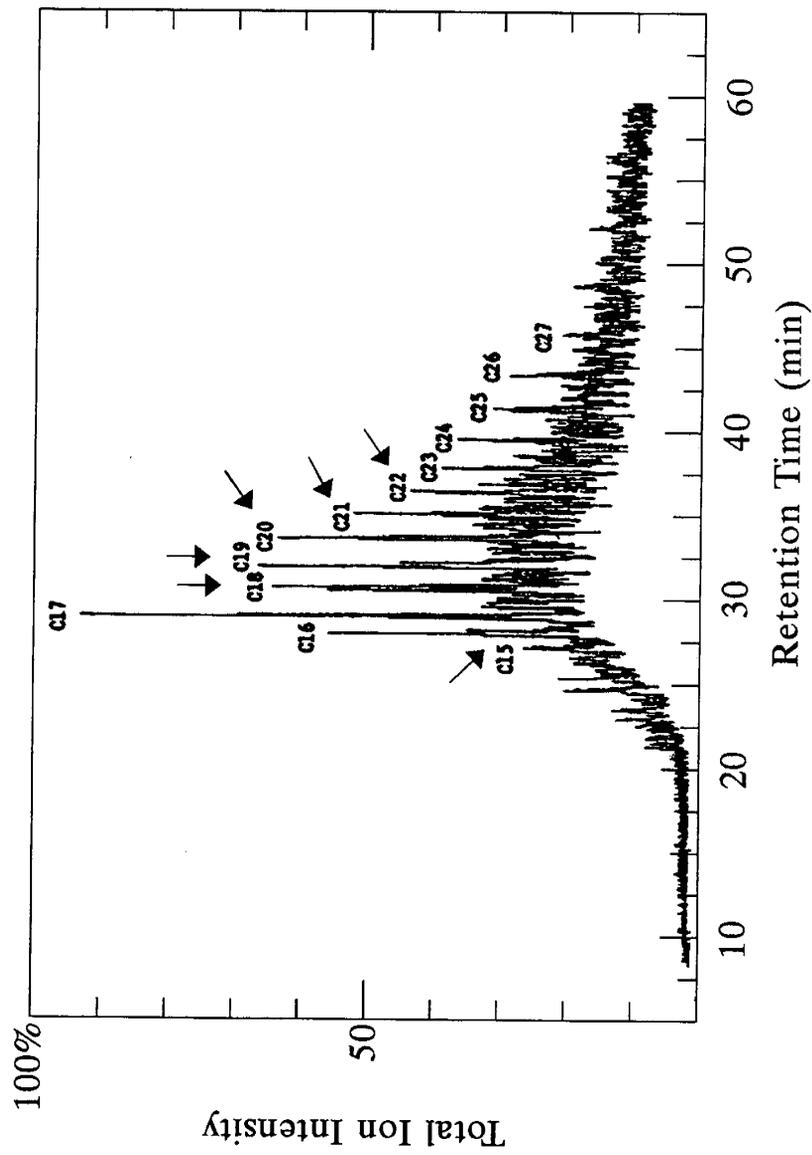


Figure 14. GC trace of control of the non-treated solvent extracted, oil emulsified by nutrient medium only.

Table 5. Variations in the extent of emulsion and the oil content in the emulsion due to the action of four different microorganisms of PR3 crude.

Klett Units	Microbial Strain	Oil Content in Emulsion (g/l)
50	BNL-4-21	3.58
200	BNL-4-22	11.0
700	BNL-4-23	18.0
30	BNL-4-24	3.03

experimental conditions. GC trace in Figure 14 is, therefore, the control for GC-trace in Figure 13. Some loss of lighter hydrocarbons (<C-15) due to the evaporation step in the extraction procedure has also occurred. It is to be noted further, that as shown in Table 5, there is good correlation between the extent of emulsification expressed in Klett units with the amount of emulsified material expressed in grams per liter. These results also indicate that this fast method can be used for a selection of suitable MEOR candidates. The oil contents of the emulsions indicate differences in the relative amounts of oil that have been emulsified. These amounts can be used as another selection parameter. However, it has to be emphasized that such parameters, while practical in terms of rapid scanning, represent only a few of the many parameters affecting the bioconversion and the recovery of crude oils. For example (see Section 3.6), the influence of the inorganic matrix and continuing multiple chemical interactions under reservoir conditions which occur during the bioconversion of the crudes also affect the end products and have to be monitored by different analytical methods.

4.3 Biochemical Modification of Hydrocarbons

Production of organic acids and emulsification of oils during the biotreatment of crudes with microorganisms, a desirable property for MEOR

technology, has also been observed in the biotreatment of oils with mesophilic organisms (e.g., Moses, 1991). However, the significant variations in the effects of microbial species/chemical composition of crudes as observed in the extent of emulsification described in the present studies, have not been noted. The new results imply several biochemical possibilities, particularly intriguing when one considers that the bioconversion of the crudes occurred in media where the oil was either the sole or the predominant source of carbon.

An exhaustive literature search revealed that thermophilic and thermo-adapted microorganisms of the types maintained at the Brookhaven National Laboratory have never been explored as possible agents for MEOR. However, preliminary studies of the action of a thermophilic BNL-TH-1 microorganism on a sample of a Cretaceous, Recluse, Wyoming oil (Thompson et al., 1974) suggested that changes in the chemical composition of hydrocarbons are occurring due to the biochemical action of microorganisms under the experimental conditions used, which involved the pipe bioreactors (see 3.1). The pipe bioreactors are a closed system in which the atmosphere is controlled in the presence of a low concentration of oxygen. The gas composition used was 4% CO₂, 0.2% O₂, and 95.8% N₂ by volume. GC-MS information as indicated by the arrows in Figure 15 shows that there is a redistribution of hydrocarbons in the BNL-TH-1 biotreated Wyoming crude. The analogous detailed study of a biodegraded Monterey, CA, oil showed significant changes due to biotreatment. A GC-MS scan for the organic sulfur hydrocarbon markers M/Z 134 for benzothiophenes, 162 for C2-benzothiophenes and 184 dibenzothiophenes (Figure 16) signals, showed a significant change in the distribution of organosulfur hydrocarbons after the treatment of BNL-NZ-3. The effects of pressure, temperature and medium, (Figure 16 b) are small, while the effects of the

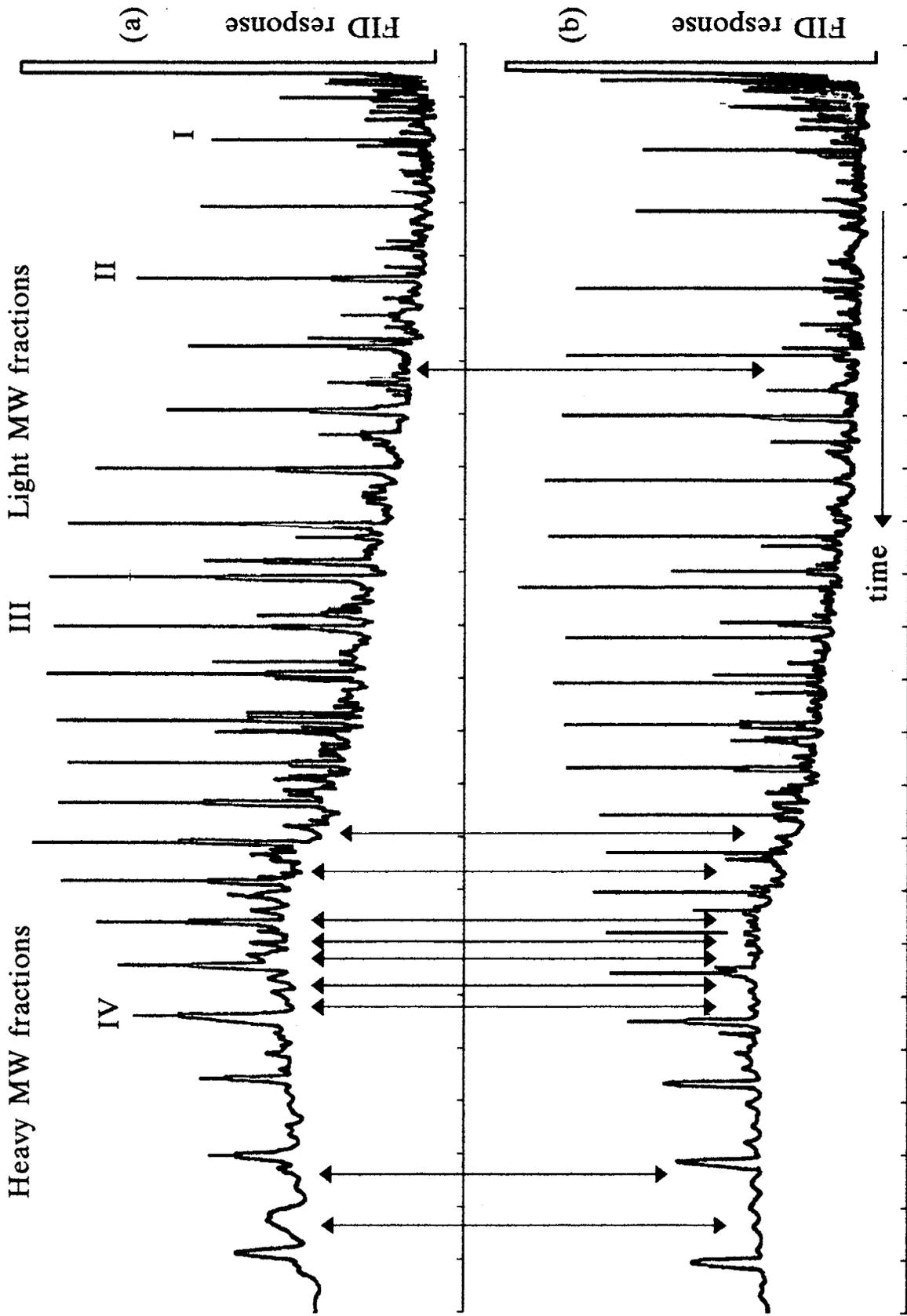


Figure 15. Effect of TH-1 on sample of (Recluse, Wyoming) oil. GC-FID trace of untreated (a) and treated (b) crude.

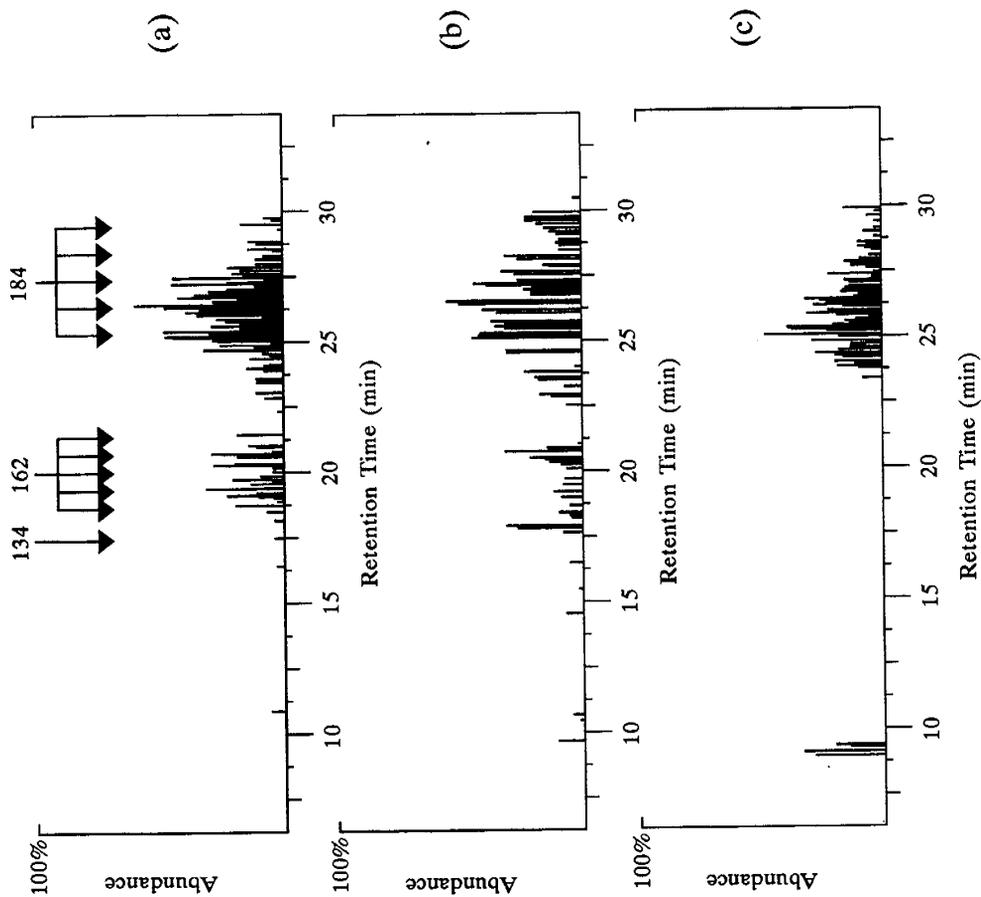
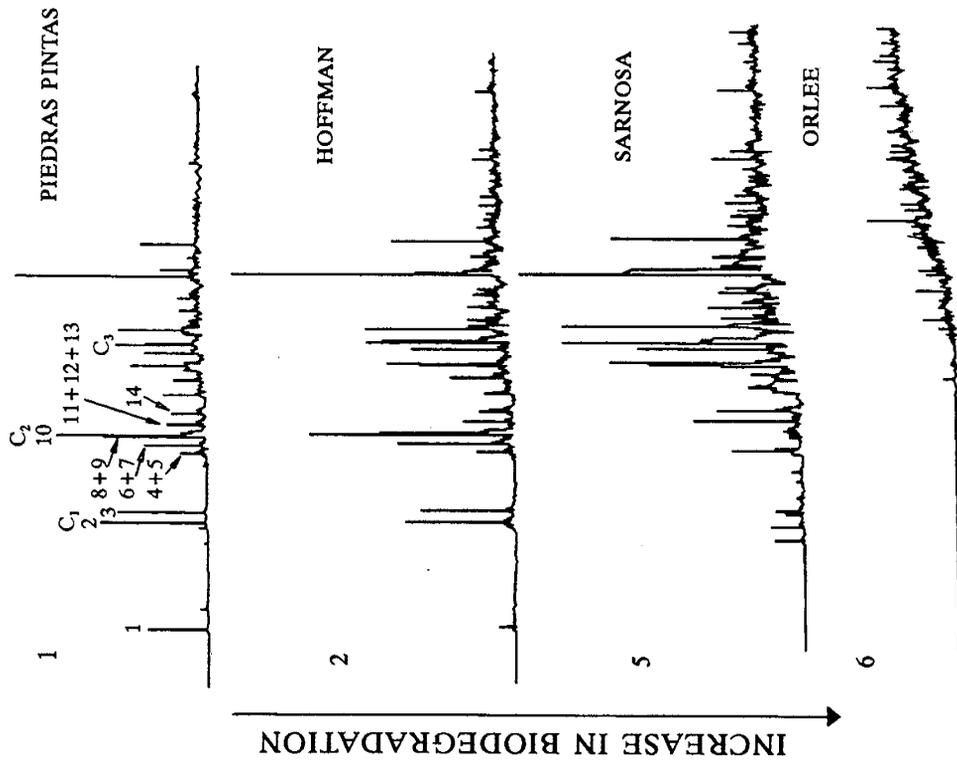


Figure 16. Monterey A851 crude treated at 65°C, under 2000 psi pressure in pipe bioreactors (Fig. 1, Section 3.1) and a defined atmosphere consisting of 4% CO₂, 0.2% O₂, and 95% N₂. Initial crude oil (a); crude oil treated with Medium 1 only (b); crude oil treated with Medium 1 containing BNL-NZ-3. Biomarker standards: M/Z 134, benzothiophenes; M/Z 162, C2-benzothiophenes; M/Z 184, dibenzothiophenes.

BNL-NZ-3 (Figure 16 c) are major. The usefulness of biogeochemical markers in oil exploration, sourcing, migration, and interaction studies has been well documented and recently reviewed by Peters and Moldowan of Chevron (Peters and Moldowan, 1992) and will not be discussed here in detail. Briefly, however, the methodology consists of using a number of organic compounds, ranging from simple aromatics (e.g., naphthalenes, thiophenes, etc.) to complex polycyclic compounds (e.g., steranes, terpanes) isolated from various sedimentary strata, source rocks and crude oils whose chemical properties and geochemical histories correlate well. For example, as shown in Figure 17, a series of substituted naphthalenes has been used to elucidate the natural biodegradation in a suite of South Texas Eocene Oils (Williams et al., 1986).

In the follow-up series of experiments, another sample of a Wyoming crude oil obtained from the Naval Petroleum and Oil Shale Reserve, Casper, WY, (Table 6) was treated with BNL-TH-1 and identical experimental conditions. Preliminary GC-MS analyses of the biotreated oil are shown in Figures 18-21.



PIEDRAS PINTAS

1

2

3

4

5

6

7

8

9

10

11

12

13

14

15

16

17

18

19

20

21

22

23

24

25

26

27

28

29

30

31

32

33

34

35

36

37

38

39

40

41

42

43

44

45

46

47

48

49

50

51

52

53

54

55

56

57

58

59

60

61

62

63

64

65

66

67

68

69

70

71

72

73

74

75

76

77

78

79

80

81

82

83

84

85

86

87

88

89

90

91

92

93

94

95

96

97

98

99

100

101

102

103

104

105

106

107

108

109

110

111

112

113

114

115

116

117

118

119

120

121

122

123

124

125

126

127

128

129

130

131

132

133

134

135

136

137

138

139

140

141

142

143

144

145

146

147

148

149

150

151

152

153

154

155

156

157

158

159

160

161

162

163

164

165

166

167

168

169

170

171

172

173

174

175

176

177

178

179

180

181

182

183

184

185

186

187

188

189

190

191

192

193

194

195

196

197

198

199

200

201

202

203

204

205

206

207

208

209

210

211

212

213

214

215

216

217

218

219

220

221

222

223

224

225

226

227

228

229

230

231

232

233

234

235

236

237

238

239

240

241

242

243

244

245

246

247

248

249

250

251

252

253

254

255

256

257

258

259

260

261

262

263

264

265

266

267

268

269

270

271

272

273

274

275

276

277

278

279

280

281

282

283

284

285

286

287

288

289

290

291

292

293

294

295

296

297

298

299

300

301

302

303

304

305

306

307

308

309

310

311

312

313

314

315

316

317

318

319

320

321

322

323

324

325

326

327

328

329

330

331

332

333

334

335

336

337

338

339

340

341

342

343

344

345

346

347

348

349

350

351

352

353

354

355

356

357

358

359

360

361

362

363

364

365

366

367

368

369

370

371

372

373

374

375

376

377

378

379

380

381

382

383

384

385

386

387

388

389

390

391

392

393

394

395

396

397

398

399

400

401

402

403

404

405

406

407

408

409

410

411

412

413

414

415

416

417

418

419

420

421

422

423

424

425

426

427

428

429

430

431

432

433

434

435

436

437

438

439

440

441

442

443

444

445

446

447

448

449

450

451

452

453

454

455

456

457

458

459

460

461

462

463

464

465

466

467

468

469

470

471

472

473

474

475

476

477

478

479

480

481

482

483

484

485

486

487

488

489

490

491

492

493

494

495

496

497

498

499

500

501

502

503

504

505

506

507

508

509

510

511

512

513

514

515

516

517

518

519

520

521

522

523

524

525

526

527

528

529

530

531

532

533

534

535

536

537

538

539

540

541

542

543

544

545

546

547

548

549

550

551

552

553

554

555

556

557

558

559

560

561

562

563

564

565

566

567

568

569

570

571

572

573

574

575

576

577

578

579

580

581

582

583

584

585

586

587

588

589

590

591

592

593

594

595

596

597

598

599

600

601

602

603

604

605

606

607

608

609

610

611

612

613

614

615

616

617

618

619

620

621

622

623

624

625

626

627

628

629

630

631

632

633

634

635

636

637

638

639

640

641

642

643

644

645

646

647

648

649

650

651

652

653

654

655

656

657

658

659

660

661

662

663

664

665

666

667

668

669

670

671

672

673

674

675

676

677

678

679

680

681

682

683

684

685

686

687

688

689

690

691

692

693

694

695

696

697

698

699

700

701

702

703

704

705

706

707

708

709

710

711

712

713

714

715

716

717

718

719

720

721

722

723

724

725

726

727

728

729

730

731

732

733

734

735

736

737

738

739

740

741

742

743

744

745

746

747

748

749

750

751

752

753

754

755

756

757

758

759

760

761

762

763

764

765

766

767

768

769

770

771

772

773

774

775

776

777

778

779

780

781

782

783

784

785

786

787

788

789

790

791

792

793

794

795

796

797

798

799

800

801

802

803

804

805

806

807

808

809

810

811

812

813

814

815

816

817

818

819

820

821

822

823

824

825

826

827

828

829

830

831

832

833

834

835

836

837

838

839

840

841

842

843

844

845

846

847

848

849

850

851

852

853

854

855

856

857

858

859

860

861

862

863

864

865

866

867

868

869

870

871

872

873

874

875

876

877

878

879

880

881

882

883

884

885

886

887

888

889

890

891

892

893

894

895

896

897

898

899

900

901

902

903

904

905

906

907

908

909

910

911

912

913

914

915

916

917

918

919

920

921

922

923

924

925

926

927

928

929

930

931

932

933

934

935

936

937

938

939

940

941

942

943

944

945

946

947

948

949

950

951

952

953

954

955

956

957

958

959

960

961

962

963

964

965

966

967

968

969

970

971

972

973

974

975

976

977

978

979

980

981

982

983

984

985

986

987

988

989

990

991

992

993

994

995

996

997

998

999

1000

Figure 17. Natural Biodegradation of South Texas Eocene Oils (after Williams et. al., 1986).

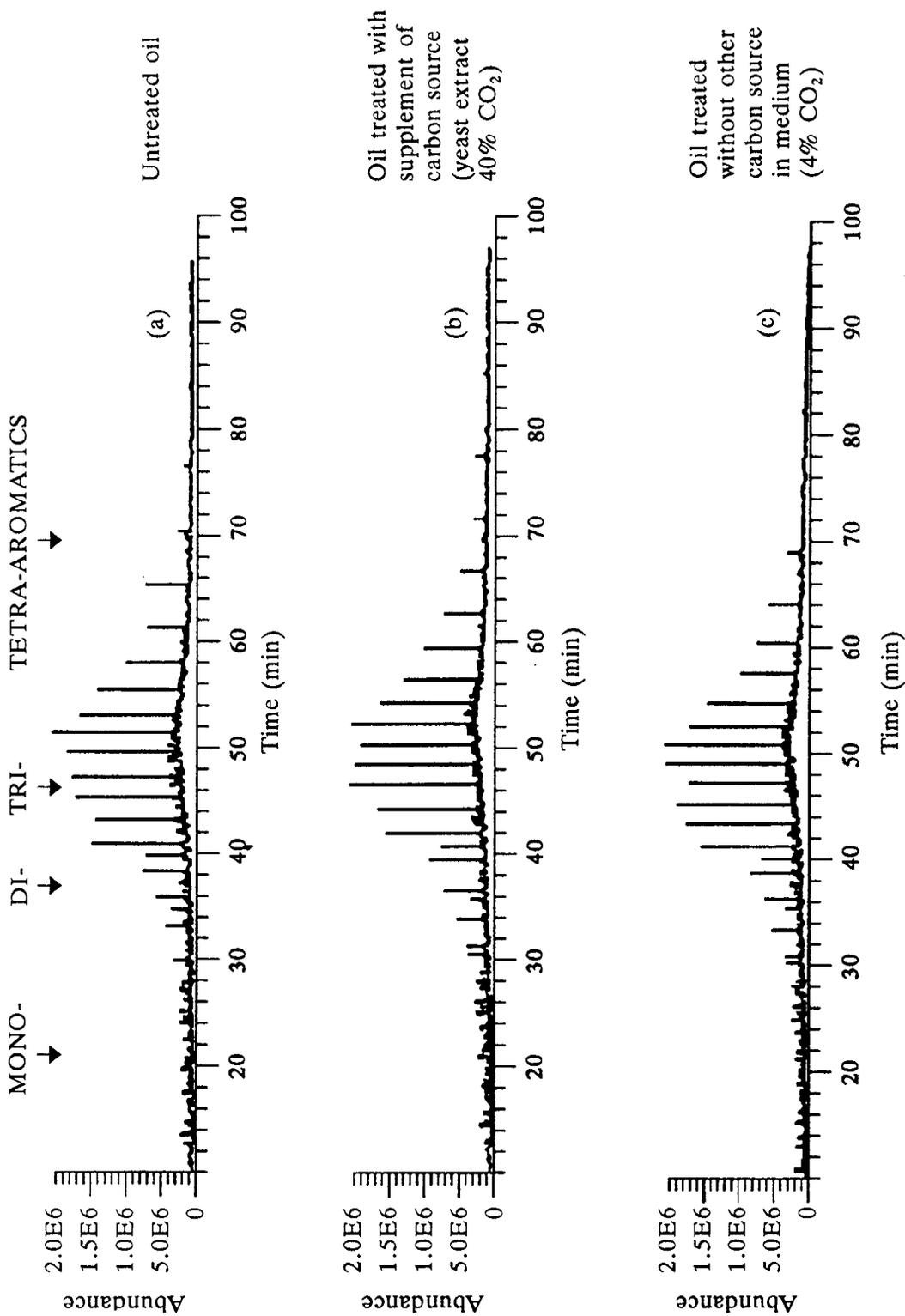


Figure 18. Total Ion Chromatograms of untreated (a) and BNL-TH-1 treated Teapot Naval Petroleum Reserve No. 3, (PR-3) Shannon Formation, WY PR3 crude (b) and (c) in the presence and absence of an added carbon source.

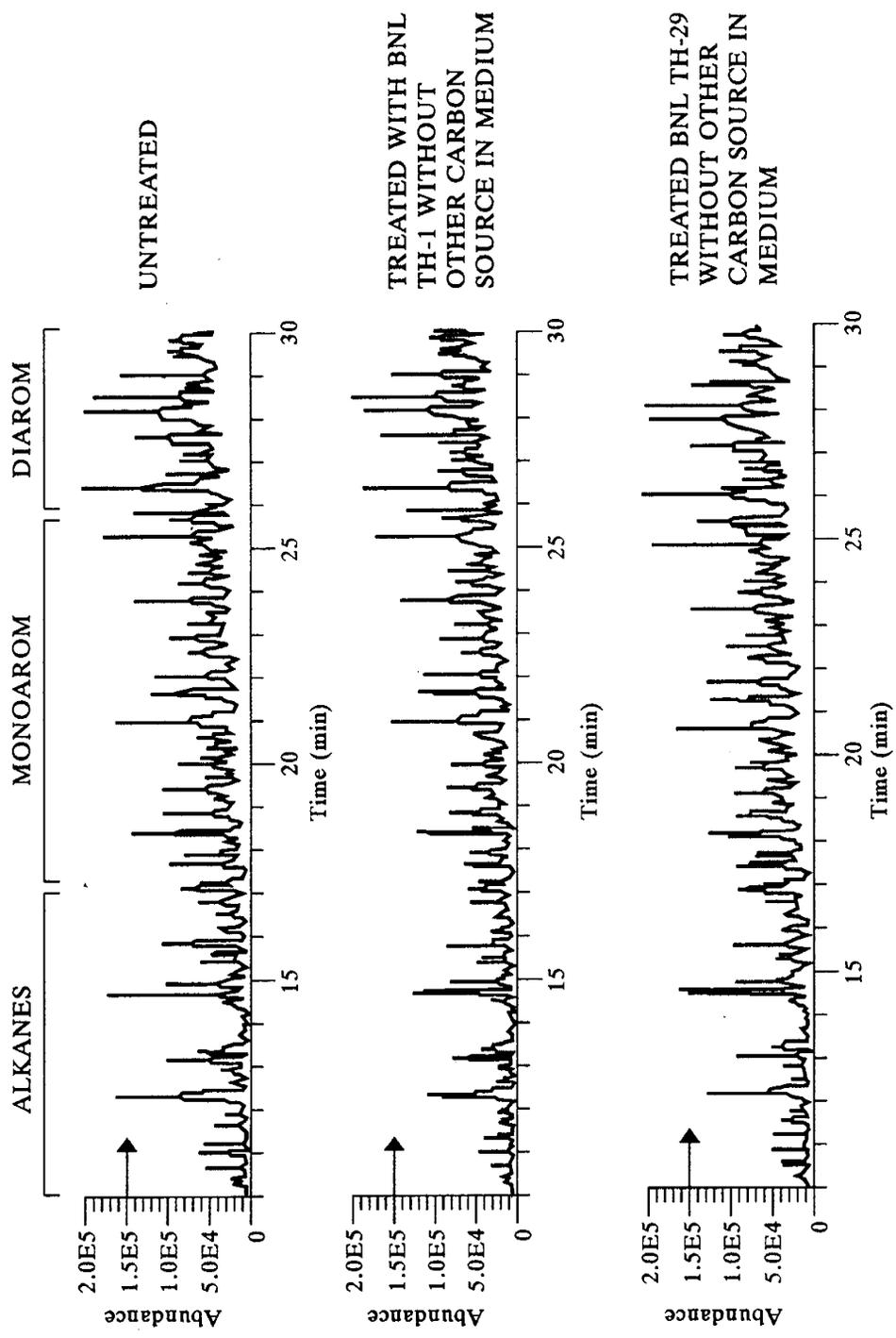


Figure 19. Total Ion Chromatogram (30 Min. Scan) of the PR-3 low molecular weight fractions. Note changes in the peak-heights (arrows) and in the relative distribution of hydrocarbons.

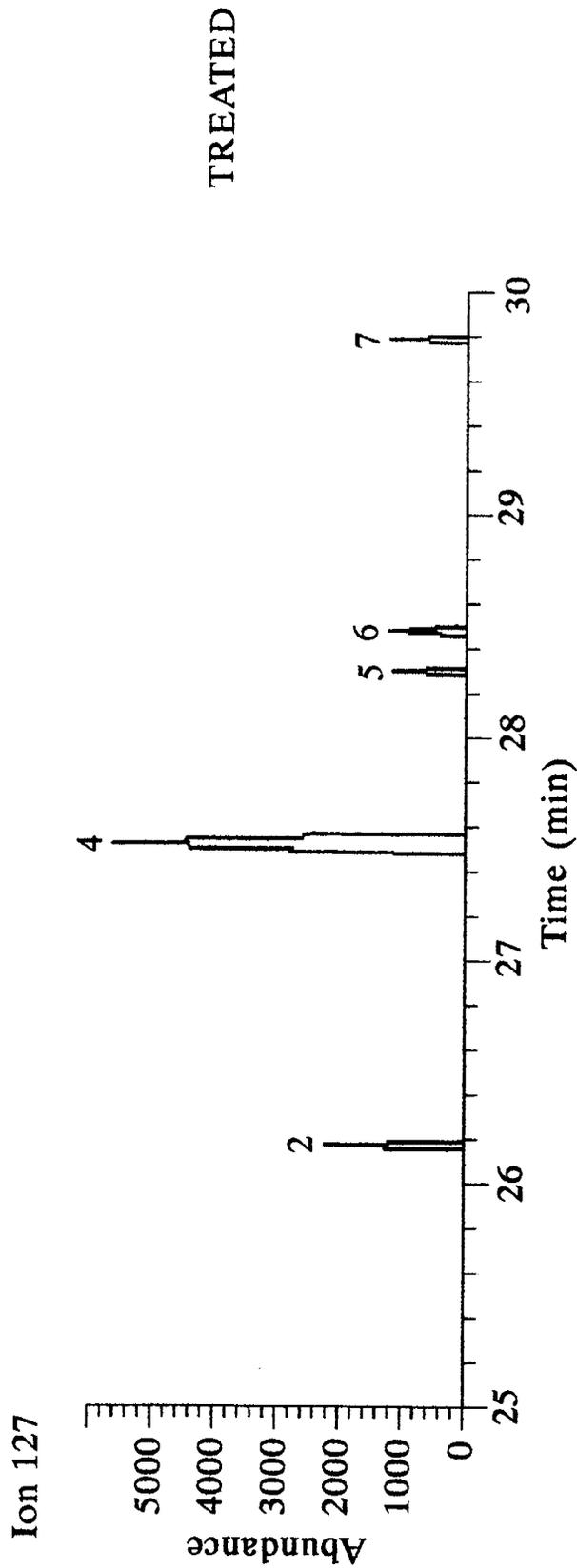
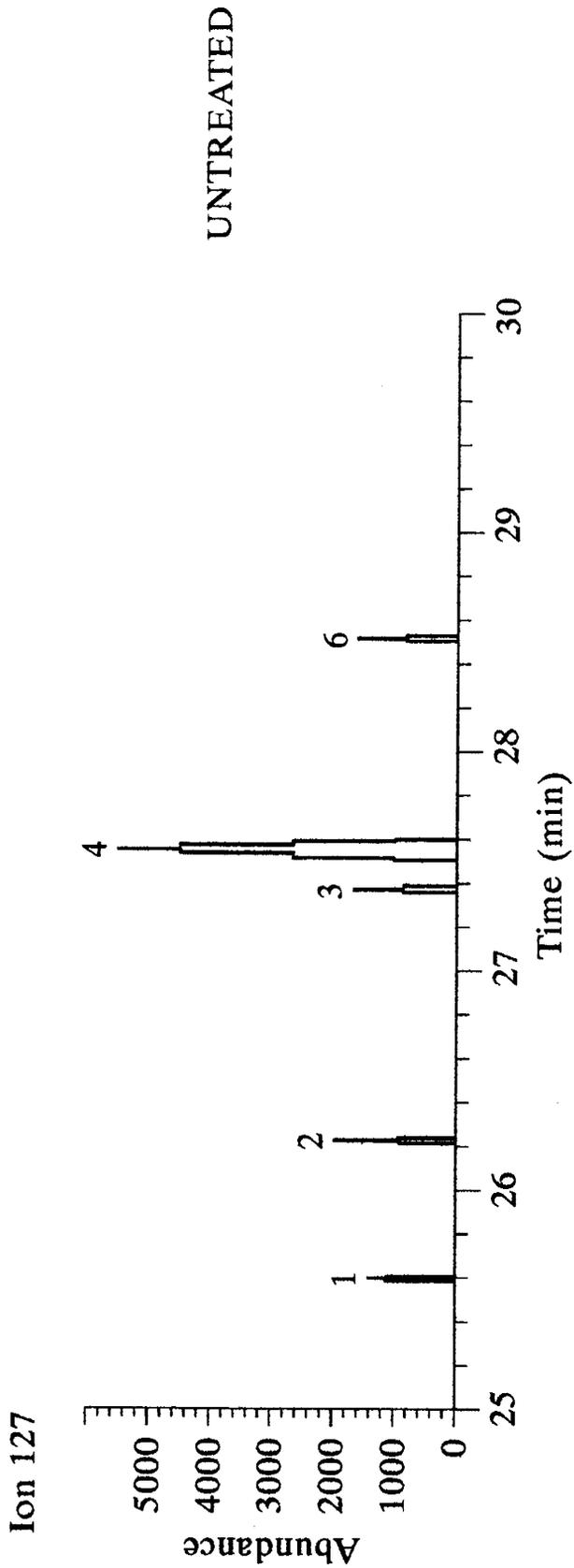


Figure 20. M/Z 127, Mass Fragmentation (Naphthalenes).

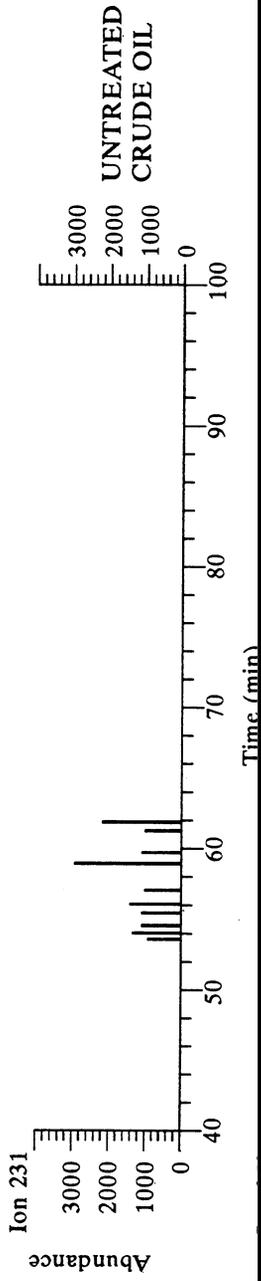


Table 6. Crude Oil Description.*

Company:	Fonix & Scisson	Date:	March 7, 1979
Well No.:	64 SX 10	Lab. No.:	30124-2
Field:	Teapot Naval Petroleum Reserve #3	Location:	Sec. 10-38N-78W
		Formation:	Shannon
County:	Natrona	Depth:	
State:	Wyoming	Analyzed by:	KCM

Sample: Courtesy of E.B. Nuckols, of the DOE, Bartlesville Project Office, Bartlesville, Oklahoma and K.L. Cowan, Naval Petroleum and Oil Shale Reserves, Casper, Wyoming.

*Tillman and Martinsen, 1984.

In the 100 min. scan (Figure 18), there is a notable difference in the hydrocarbon envelope, regardless whether a supplemental carbon source has been added or not. Further, the expansion of the 30 min. scan (Figure 19) shows changes in the total ion chromatogram of alkanes and monoaromatics as well as some fine structural differences between the action of the two organisms. A GC-MS single ion scan for hydrocarbon marker M/Z 127 is indicative of the naphthalene group of compounds (Figure 20). Note that after treatment, peaks 1 and 3 are removed, while peaks 5 and 7 appear as a consequence of a breakdown in the higher molecular weight fraction. The results of naphthalene biconversion mimic those of natural biodegradation (e.g. South Texas oil), but on a much shorter time scale (days vs. millions of years). Similarly, scans for hydrocarbon markers the M/Z 231 and M/Z 253, characteristic of the tri- and mono-aromatized steranes show significant changes in the fragmentation patterns of the treated and untreated crude (Figure 21).

Experimental results discussed in this section indicate that GC-MS specific ion scans are representative of the more detailed mass fragmentation scans for hydrocarbon markers. Similarly, extent of emulsification expressed in Klett units (KU) is also a good indicator of the biotreatment effects. Therefore, for routine analyses and comparative monitoring of multiple samples by GC-MS (specific ion) spectrometry and emulsification measurements (Klett units) represent good "experimental markers" to follow biochemical conversion of crude oils by deliberately introduced microorganisms. Treatment of PR3 crude with BNL-4-21 and BNL-4-22 resulted in emulsified products measuring 50 and 200 Klett units (KU), respectively. GE-MS analyses of these samples shown in Figures 22 and 23, indicate that in the C6-C13 region, there is a major change in the composition of PR3 after treatment with either of the microorganisms used. Thus, in the C13-C28 region, i.e., in the area of higher molecular weight components of the crude, there are significant qualitative changes. In both cases, the duration of the biotreatment was three weeks. It is to be understood that these experiments have not been carried out under optimum conditions, but are a part of a series of experimental studies aimed at a better understanding of processing conditions, and process effects based on the "marker" analyses. In a peak-by-peak comparison of relative intensities, BNL-4-22 appears to cause a larger alteration in the heavy end of the PR3 crude, a result consistent with the earlier observation expressed in Klett units, see, for example, Table 2. In fact, in terms of MEOR and other biochemical processing of oils (e.g., bioremediation) it is reasonable to assume that no single microbial and/or biochemical system may satisfy all the requirements of an efficient process. It is quite possible that mixed microbial cultures, combined with other recovery technologies may ultimately

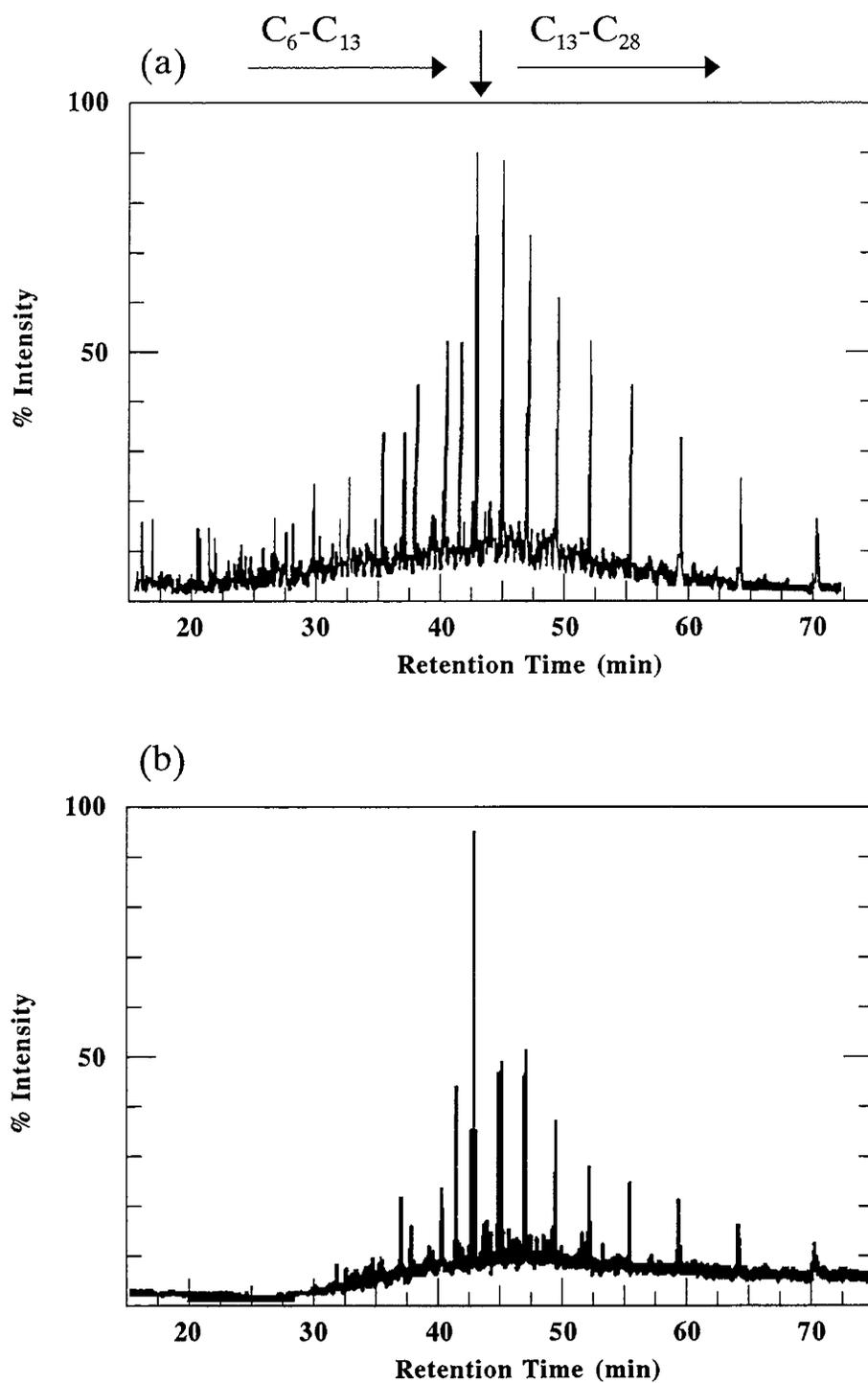


Figure 22. GC-MS scan of (a) PR3 untreated; (b) PR3 treated with BNL-4-21.

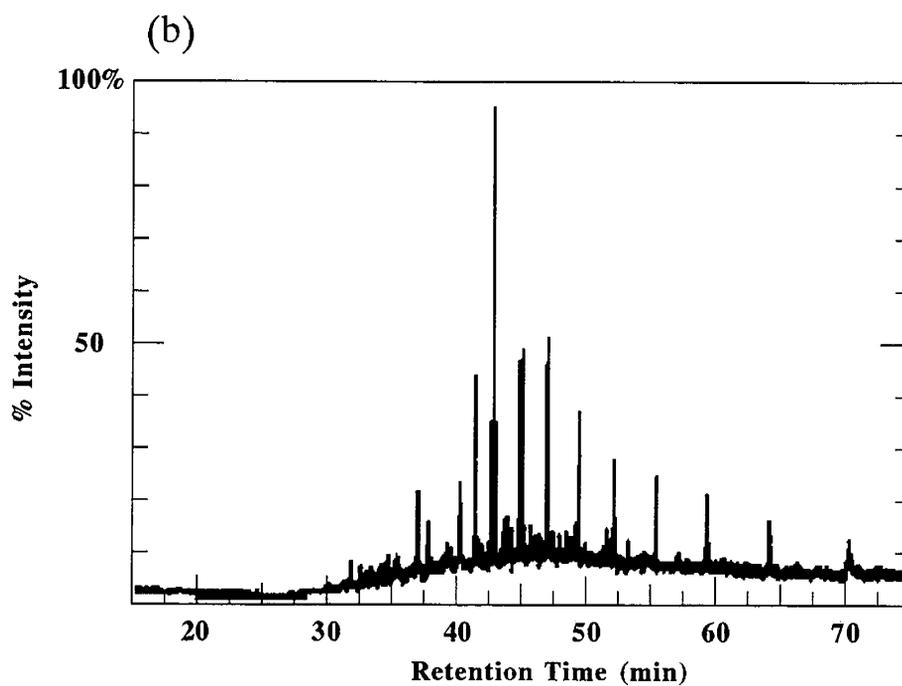
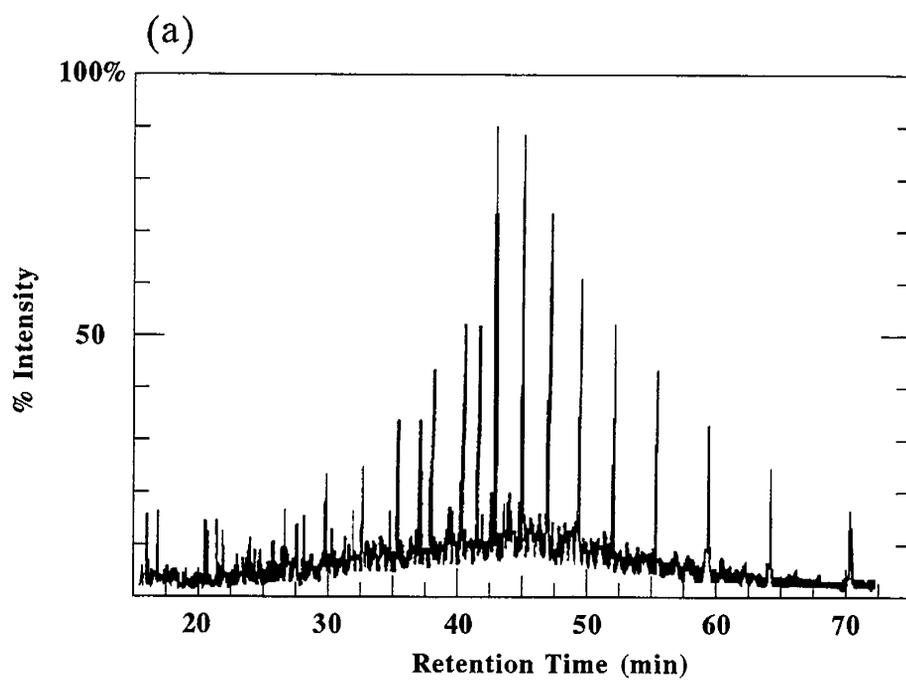


Figure 23. GC-MS scan of (a) PR3 untreated; (b) treated with BNL-4-22.

Table 7. Comparison of emulsification activity of selected BNL strains to those isolated from the formation waters (FW) taken from a shallow Texas oil well. Extent of emulsification expressed in Klett units.

<u>Microbial Strain</u>	<u>Crude Oil</u>		
	PR3	Wilmington, CA	Asphalt*
LB1	164	164	79
LB3	168	110	112
LB4	143	90	100
FW	144	84	120
BNL-4-23	300	290	250
BNL-4-22	200	252	275
BNL-4-21	325	515	475

* Commercial road paving grade.

prove to be the most efficient processing strategies. Further, there may be differences in the action of indigenous microorganisms, such as those found in formation waters and those intentionally introduced into the system. A series of experiments which allowed to explore this possibility was a result of an opportunity which made it possible to compare the effects of microbial isolates obtained from formation waters of a Texas oil well to those produced by microorganisms from the BNL collection. Samples of oil/formation waters (Texas source proprietary) were obtained with the prior knowledge that they came from a source containing light oil, rich in paraffinic compounds. The formation water (FW) yielded three microbial isolates LB1, LB3, and LB4 whose emulsification activity was compared to that of several BNL strains under experimental conditions described earlier. The results of these experiments are shown in Table 7.

In terms of the extent of emulsification, the effect of the LB1, LB3, and LB4 isolates on Wilmington, CA, crude is significantly smaller than the affect of the BNL-4-23, 22, and 21, i.e., microorganisms deliberately introduced into

the bioprocess. It is this series of experiments that, for the first time, also suggested that the effects due to different microbial species used and deliberately introduced into the system may act differently to the indigenous ones. This possibility, together with variations in the chemical composition of the crude oils before and after biotreatment, may play a significant role both in MEOR as well as in bioremediation processes, and/or the downstream biochemical conversion of oils. With this in mind, exploratory work at BNL has been further extended to comparative studies between the chemical and physical effects caused by BNL thermal and oil adapted "stock" strains and the effects caused by organisms isolated from other sources, such as, for example, oil seeps.

It has been mentioned earlier (vide infra) that in the mass spectrometric analysis of mixtures containing organic compounds, such as crude oils, it is customary to use characteristic mass signals produced during fragmentation of organic molecules in the course of mass spectrometric analyses. Typical examples are C_4H_9 (M/Z 57) for alkanes, C_7H_7 (M/Z 91) for substituted aromatics, etc., all of which are characteristic molecular markers (Peters and Moldowan, 1992). In the Brookhaven studies, such markers are used routinely as diagnostic tools in systematic studies of oil-microbial interactions. For example, biotreatment of the Teapot Naval Petroleum Reserve #3 (PR3) with BNL strain BNL-4-24 at 65°C under 2000 psi of nitrogen and 80 psi of carbon dioxide for two weeks yielded the following results: The single ion chromatogram monitored for mass 57 (Figure 24) shows that the lighter alkanes (up to C16) are degraded over that period of time, while hydrocarbons larger than C16 and up to C30, based on peak comparison were about 80% degraded. Similarly, Figure 25 shows the effect of the biotreatment on alkylarenes (M/Z 91).

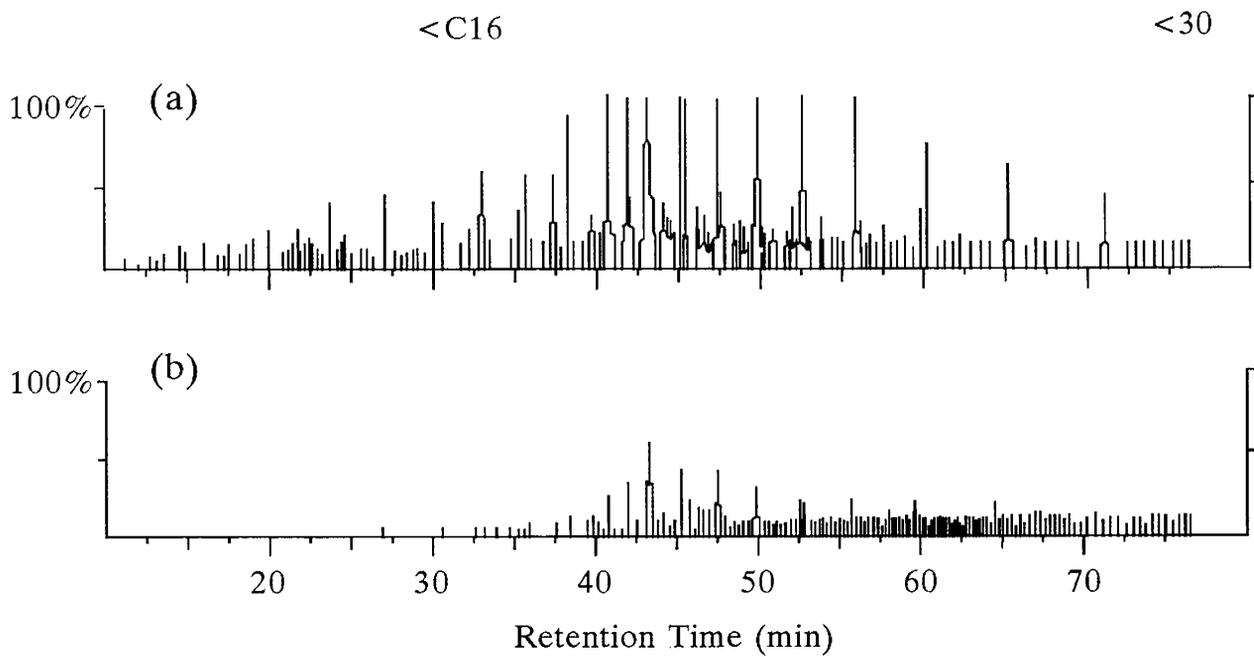


Figure 24. Degradation of hydrocarbons, (a) before treatment and (b) after treatment, of M/Z 57 ion trace of PR3 crude treated with BNL-4-24 at 65°C under 2000 psi of N₂ and 80 psi CO₂.

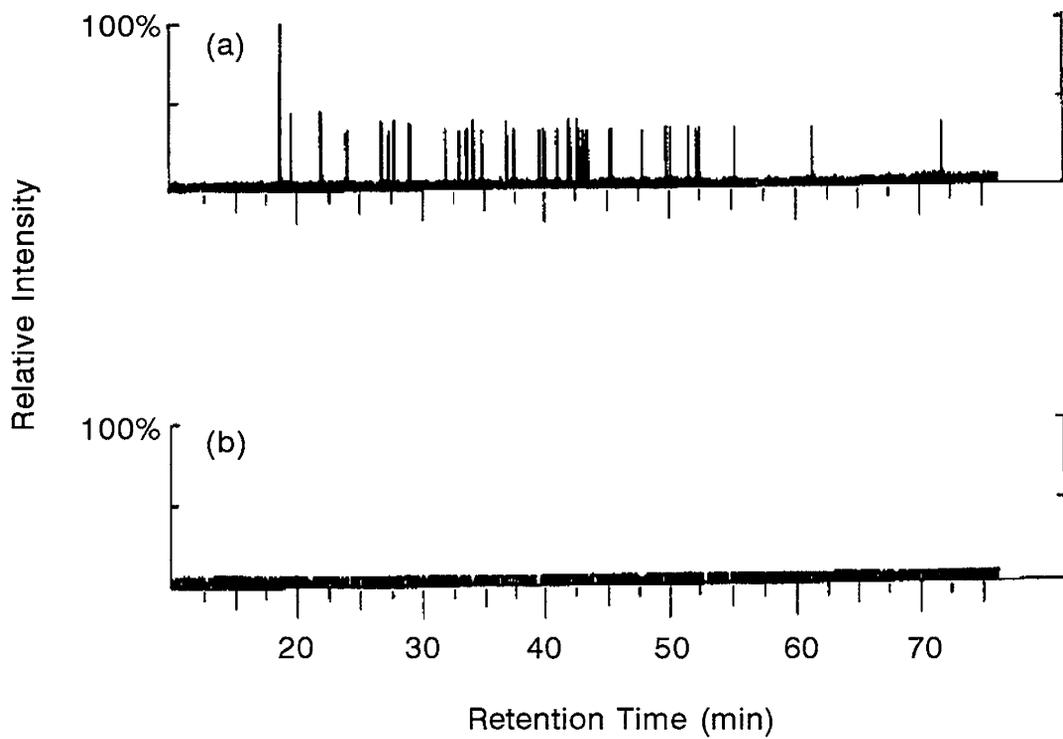


Figure 25. Degradation of alkylanes, (a) before treatment and (b) after treatment, M/Z 91 ion trace of PR3 crude treated with BNL-4-24.

Biodegradation of cyclic saturated hydrocarbons, e.g., bicyclic sesquiterpanes(M/Z 123) also occurs, as shown in Figure 26. In these studies, the ion-scans and other diagnostic parameters (such as monitoring of sulfur markers, discussed later), are used in the development of a data base which ultimately determines trends and variations in the composition of crude oils due to biotreatment by different strains of microorganisms under experimental conditions used.

It has been mentioned earlier that the use of heavy oil fractions (>200°C/5 mmHg) and asphalt may be indicative of possible mechanisms of microbial-oil interactions, particularly those involving heavy crude oils. To further explore this possibility, several heavy, high sulfur, crudes have been studied systematically. Thus, biotreatment of Boscan heavy crude oil from Venezuela with different microorganisms from the Brookhaven collection leads to significant chemical changes in the composition of the crude. For example, biotreatment of Boscan crude oil with BNL-4-22 microorganism causes the following changes in the chemical composition of crude oil as shown in Figures 27 and 28:

- (i) Decrease in the C20 to C30 alkanes.
- (ii) Increase in the <C20 type alkanes.
- (iii) There is an overall formation of lighter hydrocarbons.

Experimental data discussed in this section allow to conclude that chemical changes in the hydrocarbon composition of crude oils brought about by the action of microorganisms can be followed by monitoring key marker compounds.

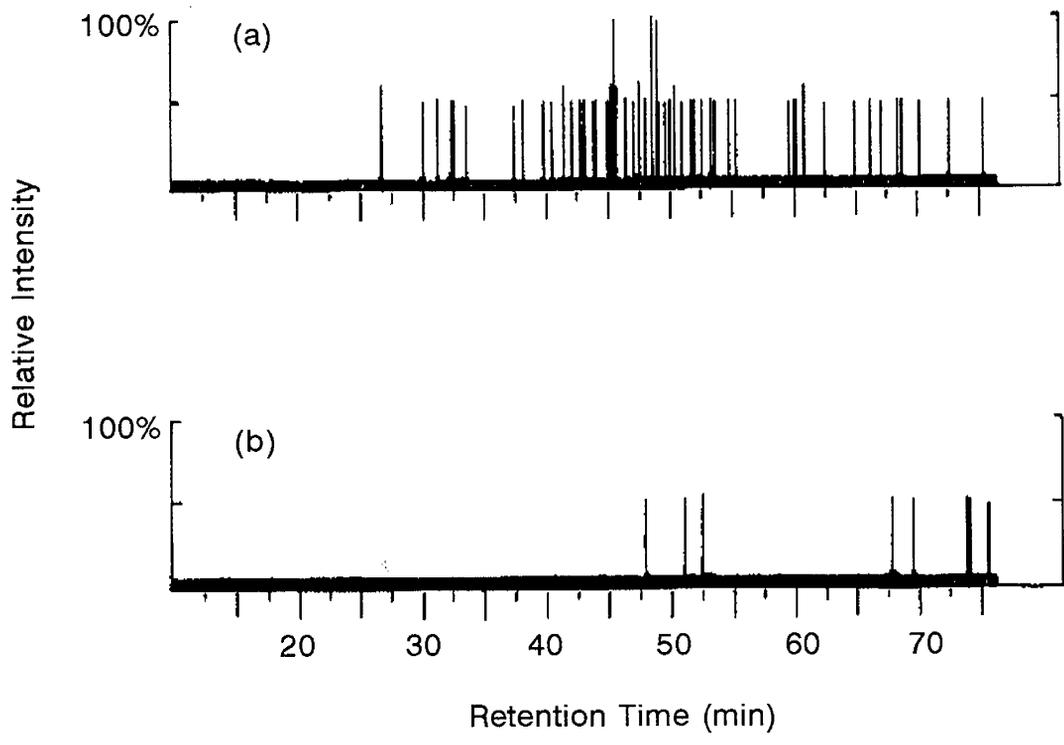


Figure 26. Degradation of cyclic hydrocarbons, (a) before treatment and (b) after treatment, M/Z 123 ion trace of PR3 crude treated with BNL-4-24.

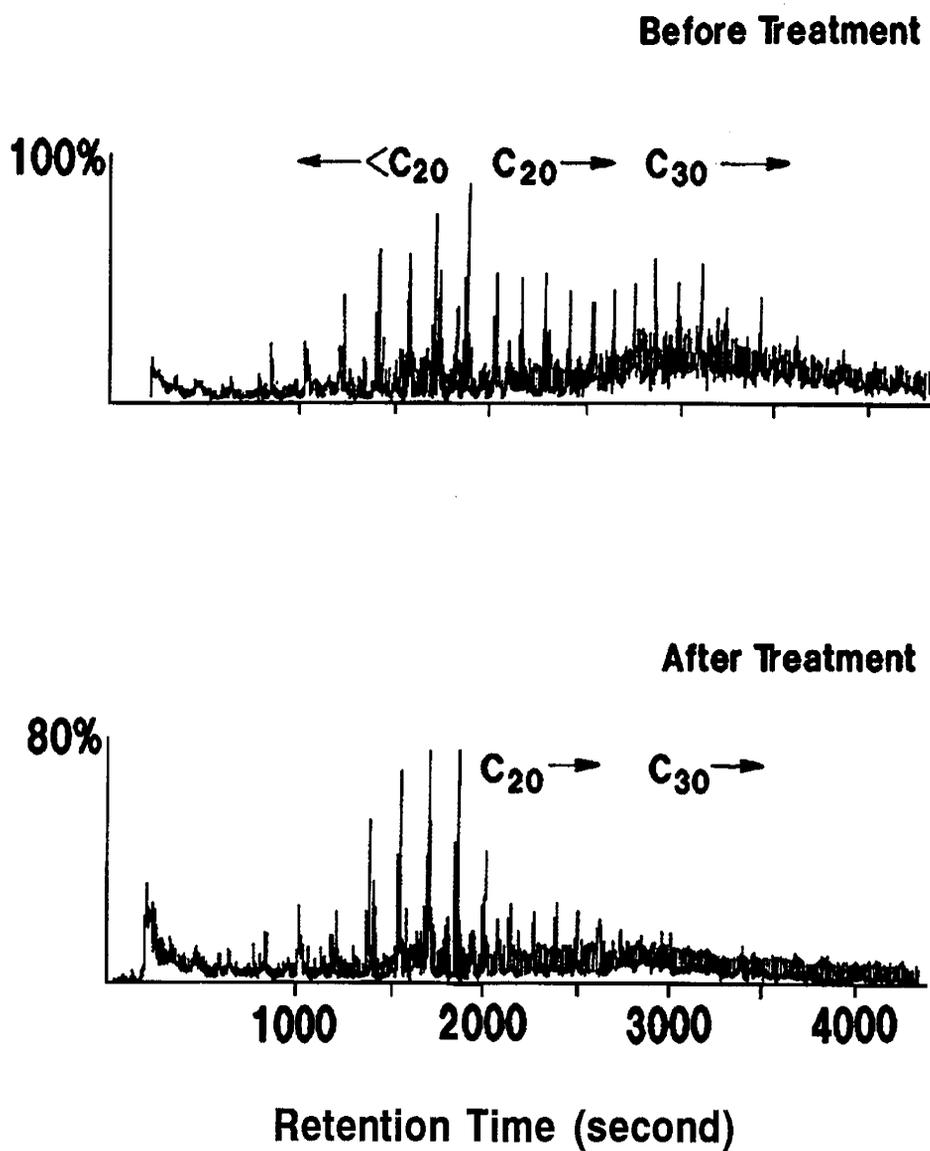


Figure 27. Aliphatic fraction of Boscan Crude M/Z 57 scan: (a) before treatment and (b) after the treatment with BNL-4-22.

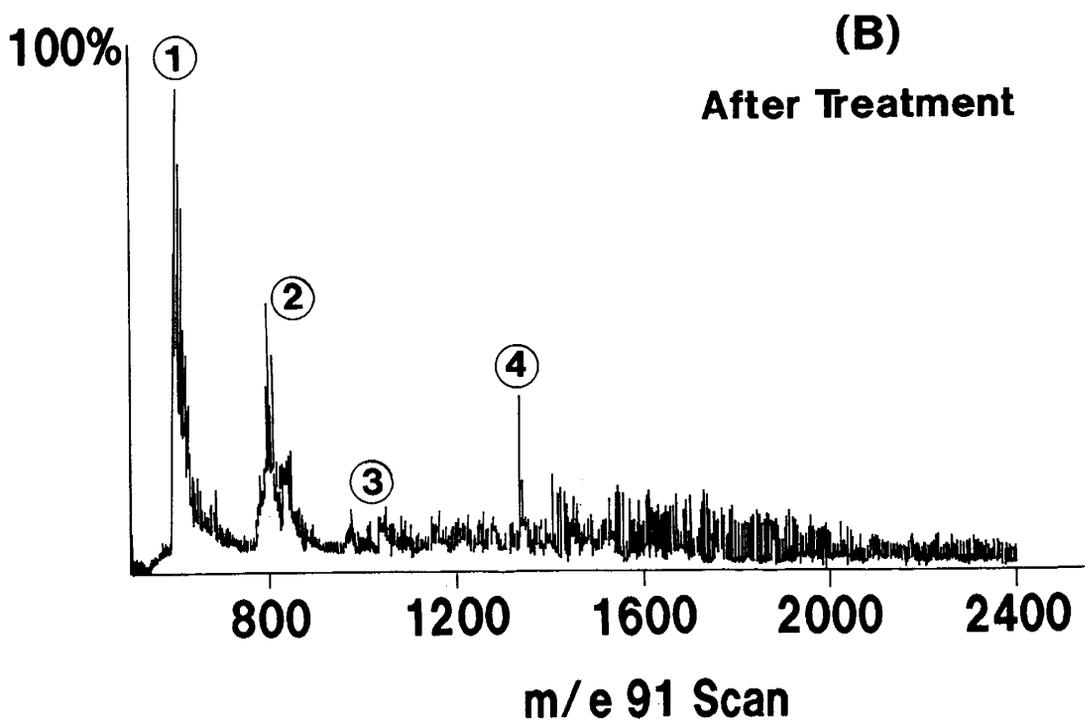
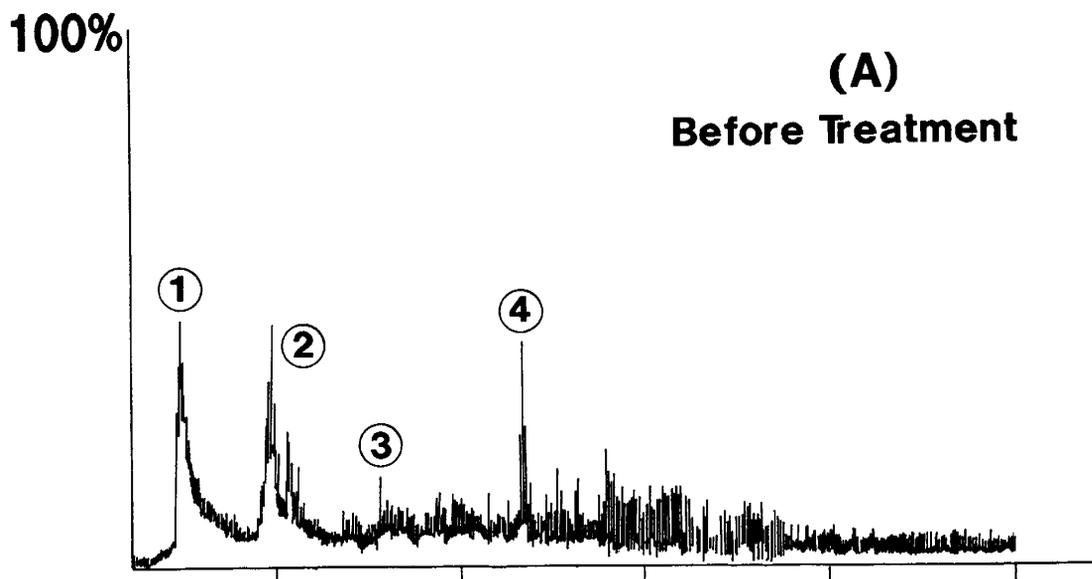


Figure 28. Aromatic fraction of Boscan crude m/e 91 scan: (a) before treatment and (b) after the treatment with BNL-4-22. Legend: 1 Toluene; 2 C2-Benzene; 3 C3-Benzene, and 4 Naphthalene.

4.4. Biochemical Modification of Heterocyclic and Organometallic Compounds

Microbial treatment of Recluse, Wy, crude oil with BNL-TH-1, a thermophilic microorganism, at 70°C and 2000 psi, yielded a product different from the starting crude oil. In order to study this observation further, a flame photometric detector (FPD) has been installed and calibrated. FPD works as a flame spectrophotometer and for certain elements, e.g., sulfur, phosphorous, and chlorine can be used as a specific, highly sensitive detector. In this program, the FPD detector has been used for the detection of sulfur compounds, including those which may be trapped in the gas phase present in bioreactors. An understanding of the changes brought about by biochemical action on the sulfur compounds determine whether sulfur compounds are modified or degraded biochemically in a manner which does not produce volatile products such as H₂S gas.

A combination of GC equipped with an FPD and mass-spectrometry also determines the chemical-structural changes which may occur in organic sulfur constituents of crude oils under experimental conditions used. Application of the FPD detector for the identification of small molecular weight volatile sulfur containing compounds (including H₂S) is also possible. Therefore, the Perkin-Elmer 8700/ITD system used in these studies, has been equipped with a splitter, which allows one portion of the sample to be analyzed by GC/FPD, specifically for sulfur and the other is simultaneously analyzed by mass spectrometry.

Treatment of Teapot Naval Petroleum Reserve #3 crude (PR3) with a temperature and pressure adapted organism BNL-4-24 at 65-70°C, and 1400 psi, resulted in considerable changes in the composition of sulfur compounds.

Table 8. Total sulfur content of Boscan and Cerro Negro Venezuelan oils before and after biotreatment.

Oil	Total % Sulfur	% Loss
Untreated Boscan	5.49	--
Boscan treated with BNL-4-22	4.14	-25
Boscan treated with BNL-4-23	4.84	-11
Boscan treated with BNL-4-24	4.92	-10
Untreated Cerro Negro	4.37	--
Cerro Negro treated with BNL-4-24	3.10	-29
Cerro Negro treated with BNL-4-23	3.74	-25
Cerro Negro treated with BNL-4-22	3.21	-27

Data shown in Figure 29 include identified molecular markers for organic sulfur compounds. Qualitative and quantitative changes in the composition of PR3 sulfur compounds are clearly evident. Studies, analogous to those conducted with PR3, have been extended to heavy Venezuelan oils known to contain high concentrations of sulfur and are, therefore, particularly suitable for these studies. Through the courtesy of W.D. Peters and P.W. Woodward of BPO/NIPER, two samples of high sulfur content Venezuelan oils (Boscan and Cerro Negro) have been obtained. These oils were then subjected to biotreatment. Particular attention was paid to the changes in the sulfur components. Changes in the total sulfur contents of the Venezuelan crudes brought about by treatment of the oils by three microorganisms from the BNL collections are given in Table 8.

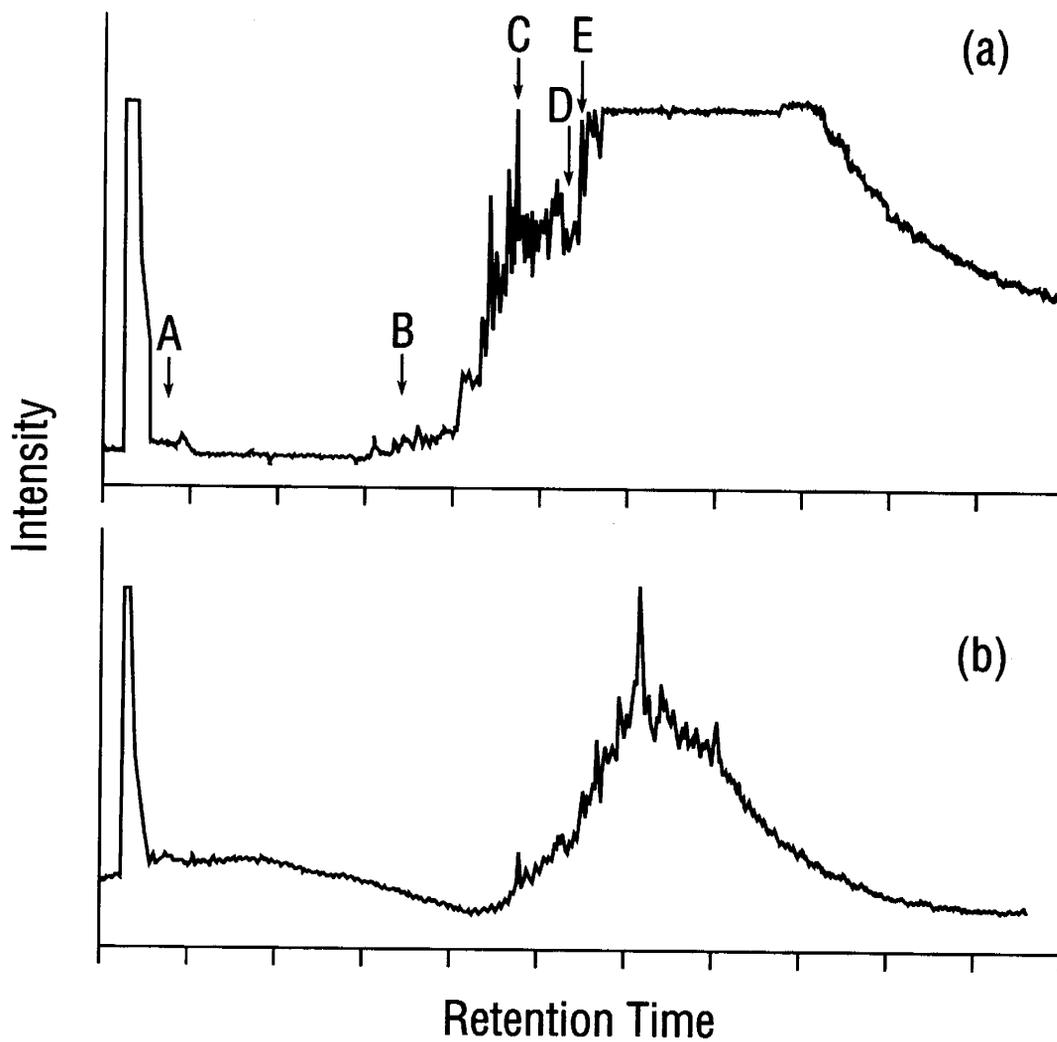


Figure 29. Gas Chromatography-Flame Photoemission Detector (GCFPD) trace of untreated Wilmington, CA, crude (sulfur specific trace) (a) and treated with BNL-4-24 (b). Molecular markers A: Thiophene; B: Benzothiophene; C: Phenyl Sulfide; D: Dibenzothiophene; E: C-1 Dibenzothiophene.

A substantial decrease in sulfur content is evident. In Figure 30, GC/FPD scans for untreated and BNL-4-24 treated Cerro Negro oil are shown. In all cases, identical experimental conditions have been used. Treatment of Cerro Negro crude with BNL-4-24 results in an almost 50% decrease in sulfur-containing compounds. In order to compare the effects of different microorganisms on the same oil, Cerro Negro crude was treated under identical experimental conditions with several different strains of microorganisms from the BNL collection. Results of the gas chromatographic analysis showed that while there is an overall similarity in terms of the concentrations of organic sulfur compounds, there is also fine structural differentiation depending on which particular microbial types have been used as shown in Figure 31. These results represent the first comparative analysis of biochemical modification and/or degradation of organic sulfur-containing compounds characterized by molecular markers ranging from thiophene to dibenzothiophene.

Cerro Negro and Boscan are very viscous oils with high contents of metals and sulfur (NIPER 1988, 1989). For such oils, conventional direct injection into GC-MS has to be occasionally modified because of solubility problems as well as retention of residues on the chromatographic column. An alternative technique is the application of pyrolysis combined with gas chromatography and mass spectrometry as used, for example, in the study of Monterey, CA, crudes, to be discussed in a different section.

Biotreatment of Boscan crude oil from Venezuela containing an average of 5% sulfur with BNL-4-22, BNL-4-23, and BNL-4-24 for seven days resulted in an overall lowering of organic sulfur content by 10-25%. Although kinetic studies under optimum conditions have as yet to be carried out, BNL-4-22

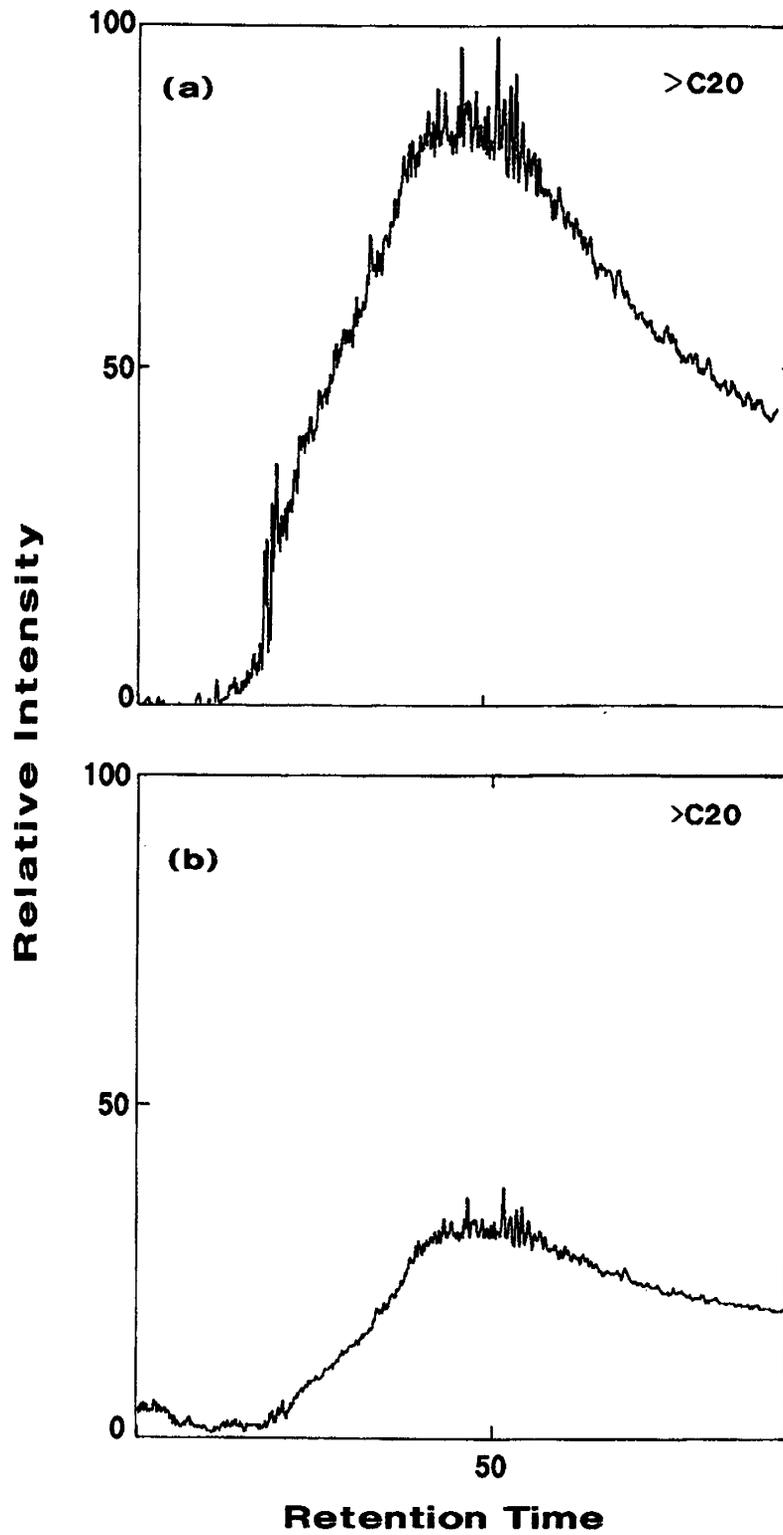


Fig. 30. FPD trace of untreated Cerro Negro (a), and treated with BNL-4-24 (b).

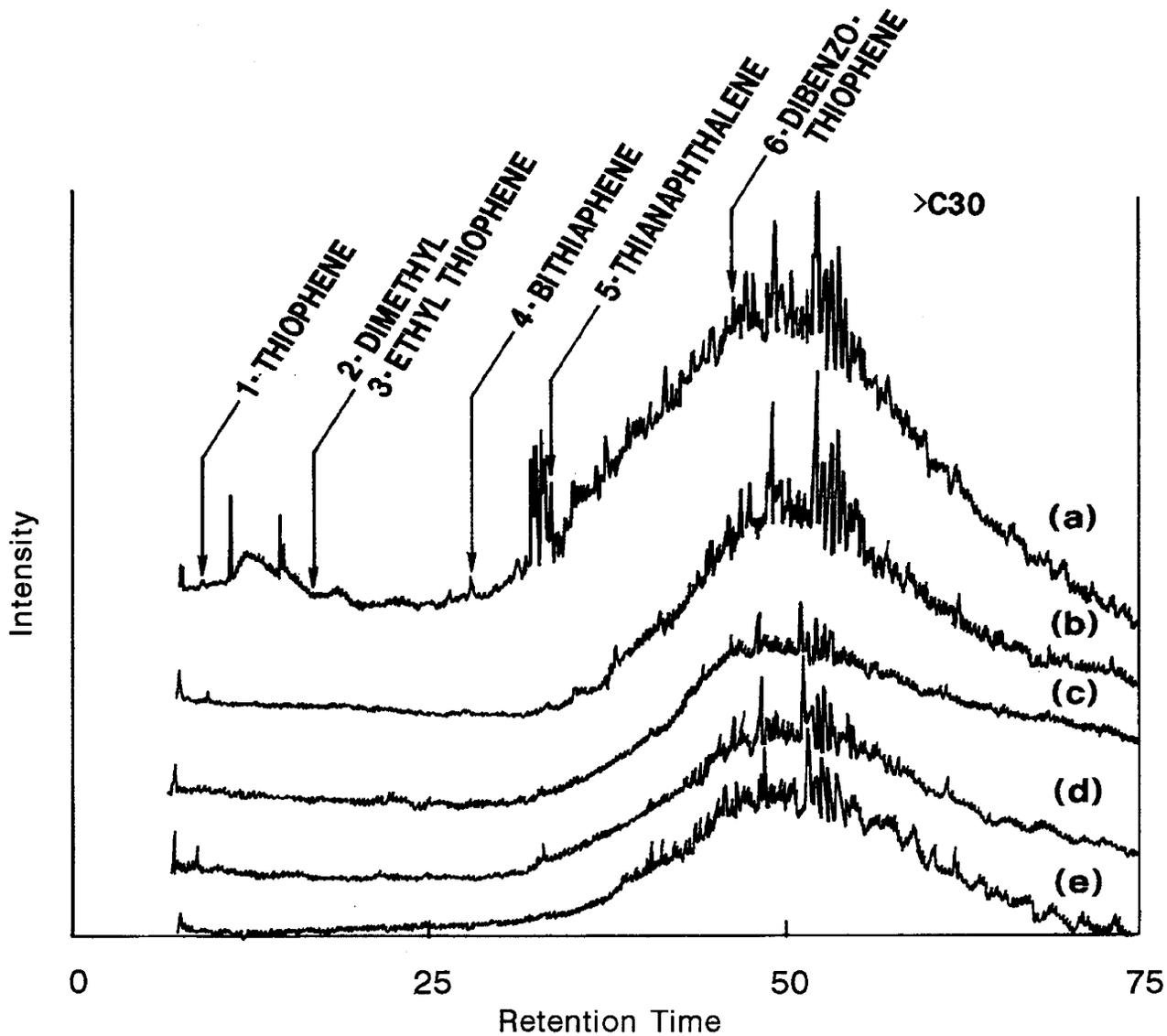


Figure 31. FPD traces of Cerro Negro crude treated and processed under identical conditions. The data are for <C30 range of compounds. (a) untreated; treated with: BNL-4-21 (b), BNL-4-24 (c), BNL-4-23 (d); BNL-4-22 (e).

removed 25% of original sulfur in seven days. As a follow up of the Cerro Negro studies, a detailed GC-MS analysis of untreated and treated Boscan crude has also been carried out. A GC trace using a flame photometric detector is shown in Figure 32. The results are consistent and show an overall decrease in the content of organic sulfur, with significant changes, both qualitatively and quantitatively in the content of thianaphthalenes. By and large, there is an overall decrease in the content of various substituted thianaphthalenes with the exception of thianaphthalene itself (peak 2, Figure 32). This result is being further explored in order to determine whether this particular trend is characteristic of BNL-4-22 interaction with Boscan crude oil and differs from other microbial interactions studied in this program. Complementary to the GC/FPD/MS analysis, which reflects detailed changes in chemical composition of crude oils, XANES (X-ray Absorption Near-Edge Structure) spectrometry reflects total changes in the chemical nature of sulfur compounds (Waldo et al., 1991). Results of comparative analyses by XANES of Boscan and Cerro Negro crude oils are given in Table 9. These results show that the biotreatment decreases the sulfide and thiophene contents of the crudes and increases the sulfoxide contents. Since volatile products from biotreatment containing sulfur have not been detected to date, while there is a significant decrease in the total organo-sulfur content, a possible implication of these results is that the products are soluble in the water phase which is always present in the culture medium. Detailed experimental protocol has been developed to investigate the chemical nature of the "sulfoxides" and other products which may be present in the aqueous phase. A possible outcome of this could be that some of the products may be water soluble sulfones contributing to the concurrent emulsification of the oil.

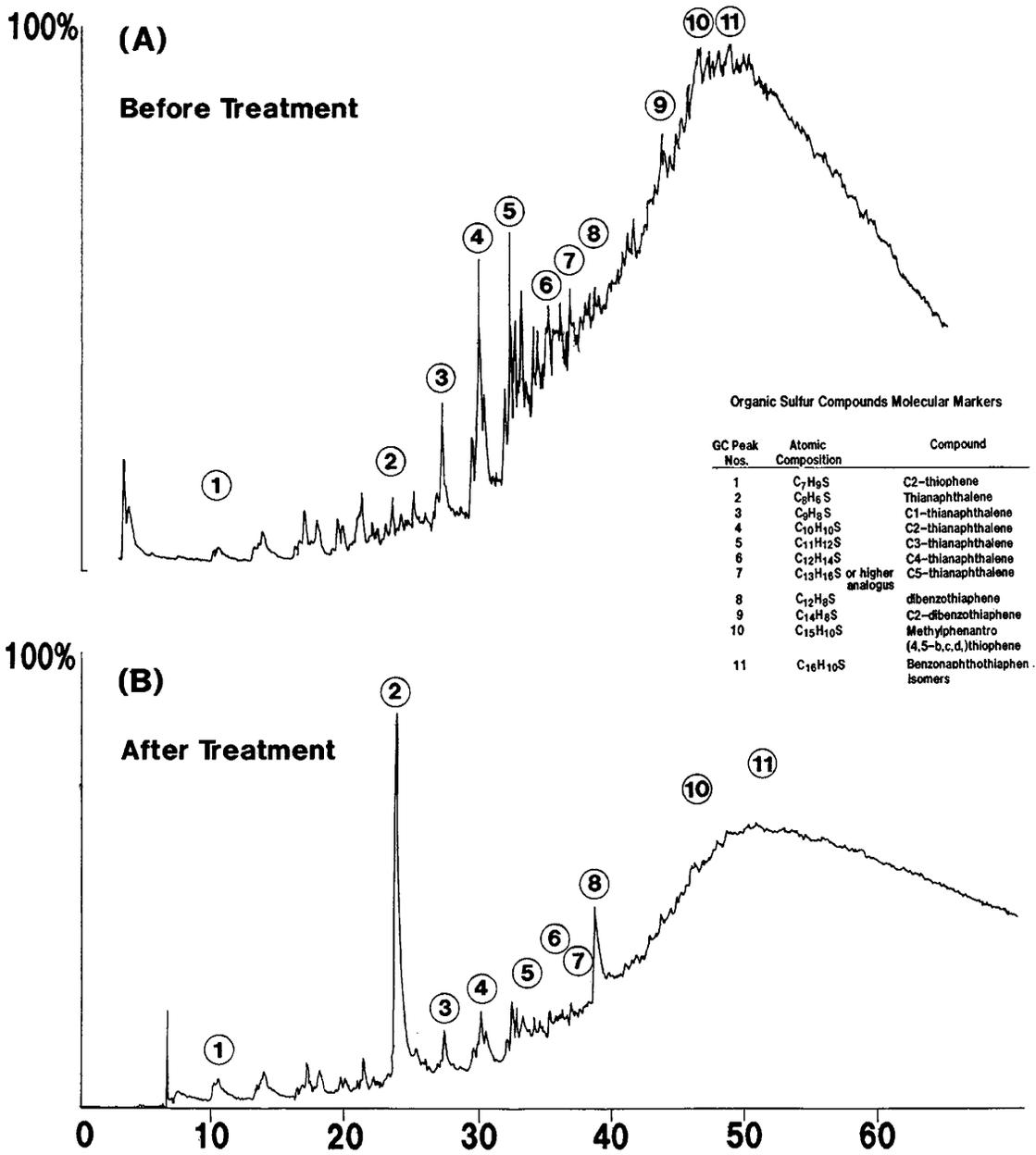


Figure 32. Trace of Boscan crude oil: (A) before and (B) after the treatment with BNL-4-22.

Table 9. XANES analysis of sulfides, thiophenes and sulfoxide contents of untreated and treated crude oils.

			Relative Content		
			$\begin{array}{c} \text{R} \\ \diagdown \\ \text{S} \\ \diagup \\ \text{R}' \end{array}$		$\begin{array}{c} \text{O} \\ \\ \text{R} - \text{S} - \text{R}' \end{array}$
Crude Oil	Microorganisms	Treatment	Sulfide	Thiophene	Sulfoxide
Boscan	0	untreated	0.198	0.738	0.064
	BNL-4-22	treatment	0.159	0.655	0.186
	BNL-4-23	treatment	0.121	0.743	0.135
Cerro Negro	0	untreated	0.147	0.781	0.072
	BNL-4-22	treatment	0.179	0.683	0.138
	BNL-4-23	treatment	0.103	0.713	0.184

Trace metals present in crude oils exist as complexes and organometallic compounds and are an integral part of the crude oil composition. During the biotreatment of crude oils, both acidification and emulsification occurs as well as solubilization in the aqueous phase of some hydrocarbon components of the crudes. Because such chemical changes may influence trace metal composition of crude oils, their chemical properties have also been studied in biotreated and untreated oils. There are several experimental techniques available for this purpose. In the first phase of these studies, a gas chromatograph (GC) equipped with an atomic emission detector (AED) has been used. This analytical technique separates the metal complexes by GC and metals are then detected selectively by means of their specific emission wavelengths in microwave induced plasma. With appropriate calibration metal species can be easily identified. For example, biotreatment of Wilmington, CA, crude with BNL-4-22 resulted in a considerable reduction of the nickel porphyrin complex as shown in Figure 33. In this analysis, the GC system was

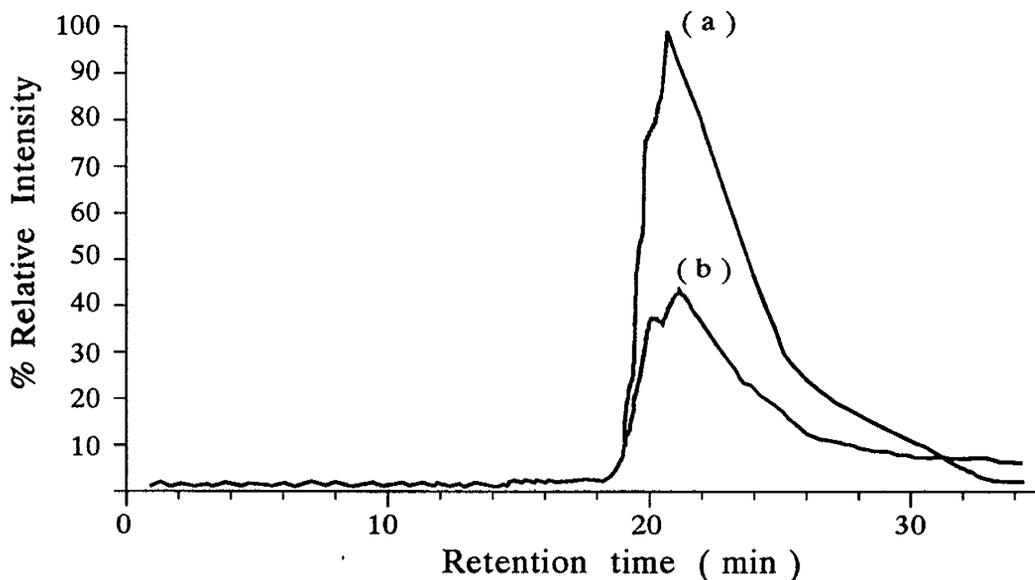


Figure 33. Reduction of nickel porphyrin content in Wilmington, CA, crude (a) before treatment and (b) after treatment with BNL-4-22.

calibrated with nickel octaethyl porphyrin eluting at 18.5 mins and cobalt-octaethyl porphyrin eluting at 19.1 mins. This is the first clear indication of removal of trace metals from a crude oil by means of biotreatment under our experimental conditions.

Consistent with other observations, discussed above, it might be reasonable to expect that different strains of microorganisms may vary in the extent of their action on organometallic compounds present in crude oils. To test for this possibility, the California (Wilmington) crude was treated with two different strains of microorganisms and the result is shown in Figure 34. These results show that under experimental conditions used, the biotreatment caused variations in the concentration and distribution of metal complexes, including a complete removal of the trace metal. Until further studies have been conducted, it is reasonable to assume that the metal complexes have been converted to species soluble in the aqueous phase. The resolved peaks within

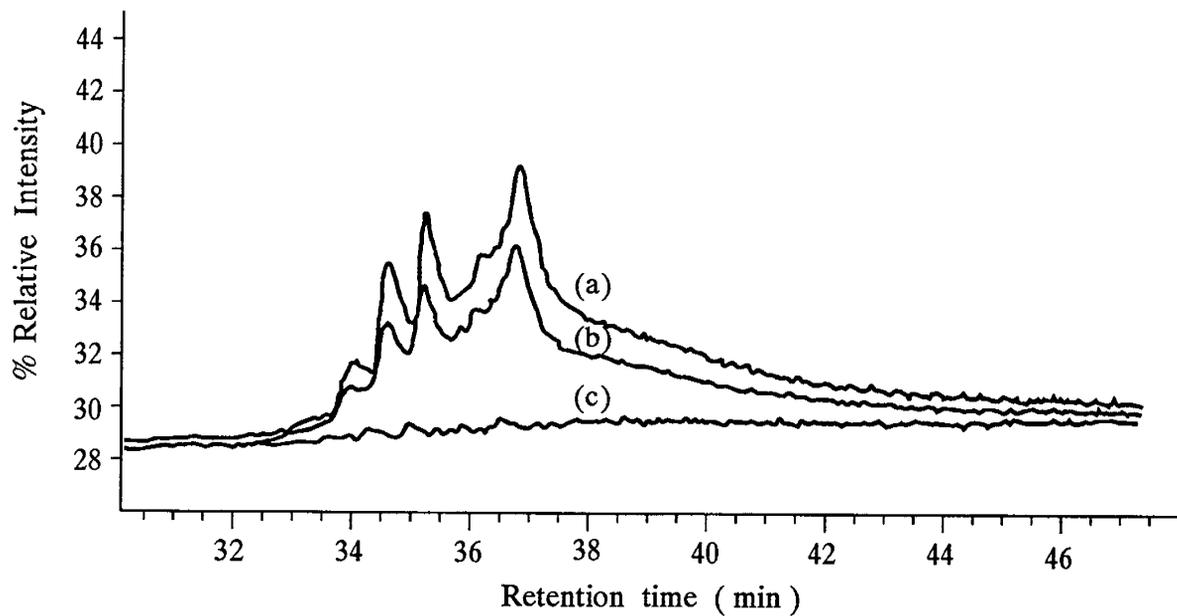


Figure 34. GC-AED analyses of Ni porphyrin contents of California crude: untreated (a); treated with BNL-3-25 (b); and BNL-4-25 (c).

Table 10. Changes in the selected trace metals content of Cerro Negro oil treated with BNL-4-23.

Metal	Metal Content $\mu\text{g/ml}$	
	Untreated	Treated
V	3330	2290
Ni	926	639
Mg	78	6.8
Sr	9.6	1.7
Mn	15.8	1.8

the 33 to 38 min resolution time, are nickel porphyrin in complexes. Areas under the curve are proportional to quantities of the complexes. The area under curve (b) and (c) for the biotreated samples, are lower than the untreated sample.

Another powerful analytical tool for simultaneous multi-element analysis is the Inductively Coupled Plasma-Mass Spectrometry (ICP-MS, Fison, VG PQ2 Plus). In the ICP-MS technique (Chicarelli et al., 1990), the total metal concentrations are determined. A multi-element analysis of Cerro Negro untreated and treated crude, Table 10, showed that several trace metals are being concurrently "removed" from the crude.

Since the effect of different microorganisms on the same oil varies (and vice versa), the metal studies have been extended to the comparison of the effects of several different strains on the total nickel and vanadium contents of Cerro Negro crude. The results are shown in Table 11.

Consistent with previous results, biochemical processes involving reactions with organometallic compounds in crude oils appear to be also microbial species dependent. It is to be noted that such reactions will also depend on the chemical nature of organometallic compounds and the chemical

Table 11. Variations in the total content of nickel and vanadium in Cerro Negro biotreated by different strains of microorganisms.

Microorganism	Metal	Treated ppm	Untreated ppm	% Metal Removed
BNL-4-24	Ni	186	246	25
	V	276	493	38
BNL-4-22	Ni	12	246	95
	V	6	493	99
BNL-4-23	Ni	160	246	35
	V	204	493	58
BNL-TH-29	Ni	168	246	32
+				
BNL-TH-31	V	212	493	57
BNL-2-45	Ni	122	246	51
+				
BNL-3-26	V	157	493	68

composition of the crude oils, particularly in terms of saturates, aromatics, and polar fractions. Until further studies have been conducted, it is reasonable to assume that the metals removed from the organic oil phase, were converted into water soluble species. However, even at this stage of the studies, these results already suggest important industrial applications in petroleum refining processes.

4.5 Microbial Reference Library and Its Application

The BNL reference library BNL contains at the present time a collection of ~100 aerobic and anaerobic thermophilic and thermoadapted microorganisms. About one half of these are strictly thermophilic, growing at >65°C. In addition to temperature regimes, most are capable of growth in inorganic salt media with oil either as the sole source of carbon or in the presence of an 0.08% additional organic nutrient. For the thermoadapted microorganisms, a

prototype continuous temperature gradient incubator has been constructed and tested. This highly efficient incubator can culture microbial strains in a continuous mode over a temperature range of 4°C to 100°C. This is a unique tool (Patent Pending) for the development of thermo-adapted bacteria capable of interaction with crude oils under different temperature regimes, including low and high extremes, and high salt concentrations (brines). In the temperature-pressure adaptation studies, particular attention has been given to microorganisms which are not initially strictly thermophilic and barophilic. The adapted organisms currently on hand, represent a substantial addition to the consortium of microorganisms available for biochemical studies. In terms of applications, several different microbial treatments in combination with other EOR methods may be useful. For example, in some cases, under field conditions, combined MEOR and, say, water and/or steam flooding may be the technology of choice. In such circumstances, an advanced knowledge of process conditions is advantageous and possibly critical. In Figures 35, 36, and 37, an untreated PR3 and PR3 treated with an aerobic microorganism, BNL-4-25, and a methanogenic thermophilic microorganism, BNL-5-32, are compared. As expected (compare with Figure 27), there are significant changes in the 1200 to 2500 scanning range as indicated by the lack of lower molecular weight species.

In the >C20 region, differences in the peak intensities and lack of some high molecular weight peaks are also evident. Although extensive kinetic and process optimization studies are as yet to be conducted, the available data indicate that BNL-4-25 may be a more efficient microorganism than BNL-5-32. However, information of this kind together with other chemical and biomarker information, if made available prior to core flooding experiments and

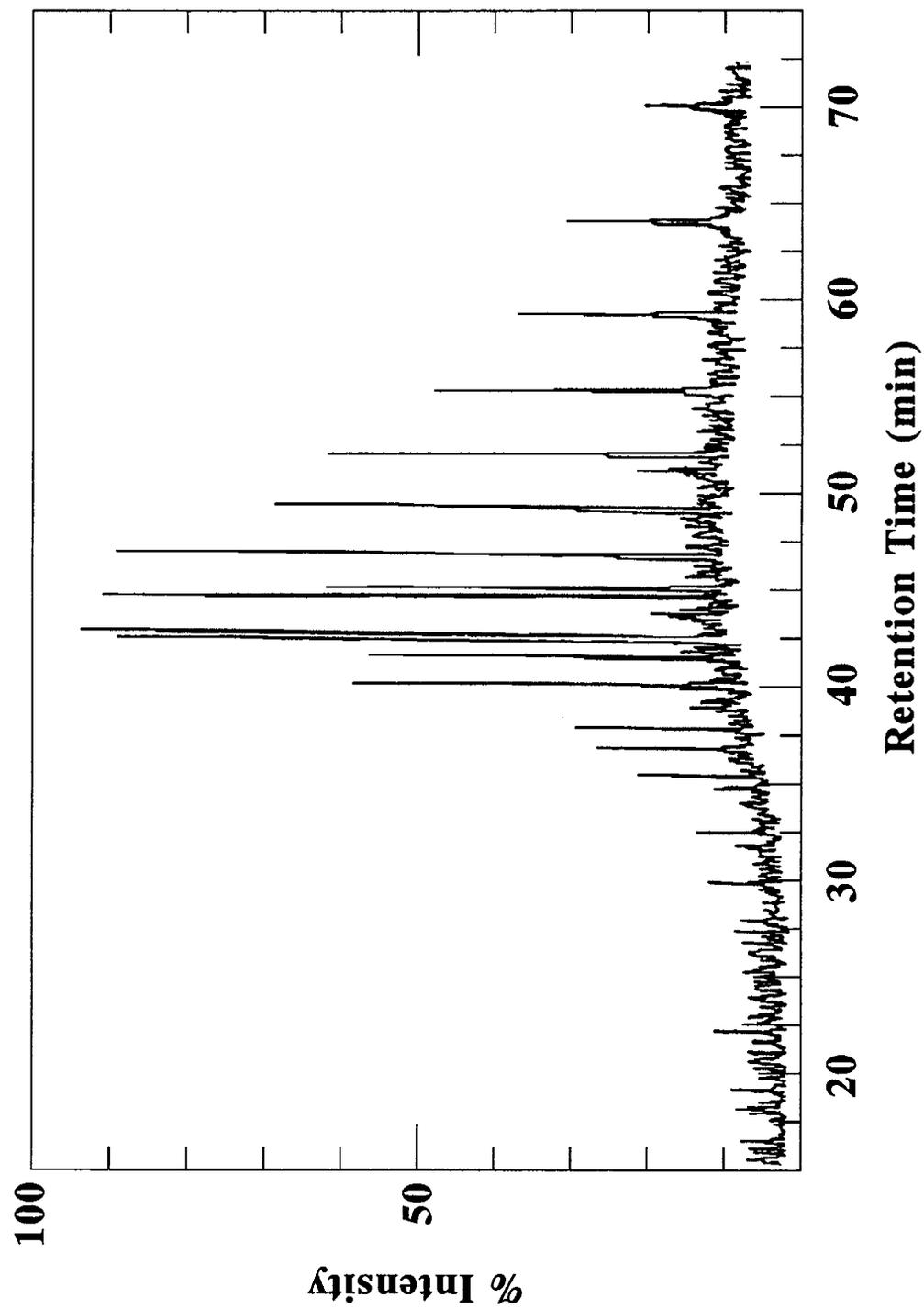


Figure 35. Untreated PR3 control.

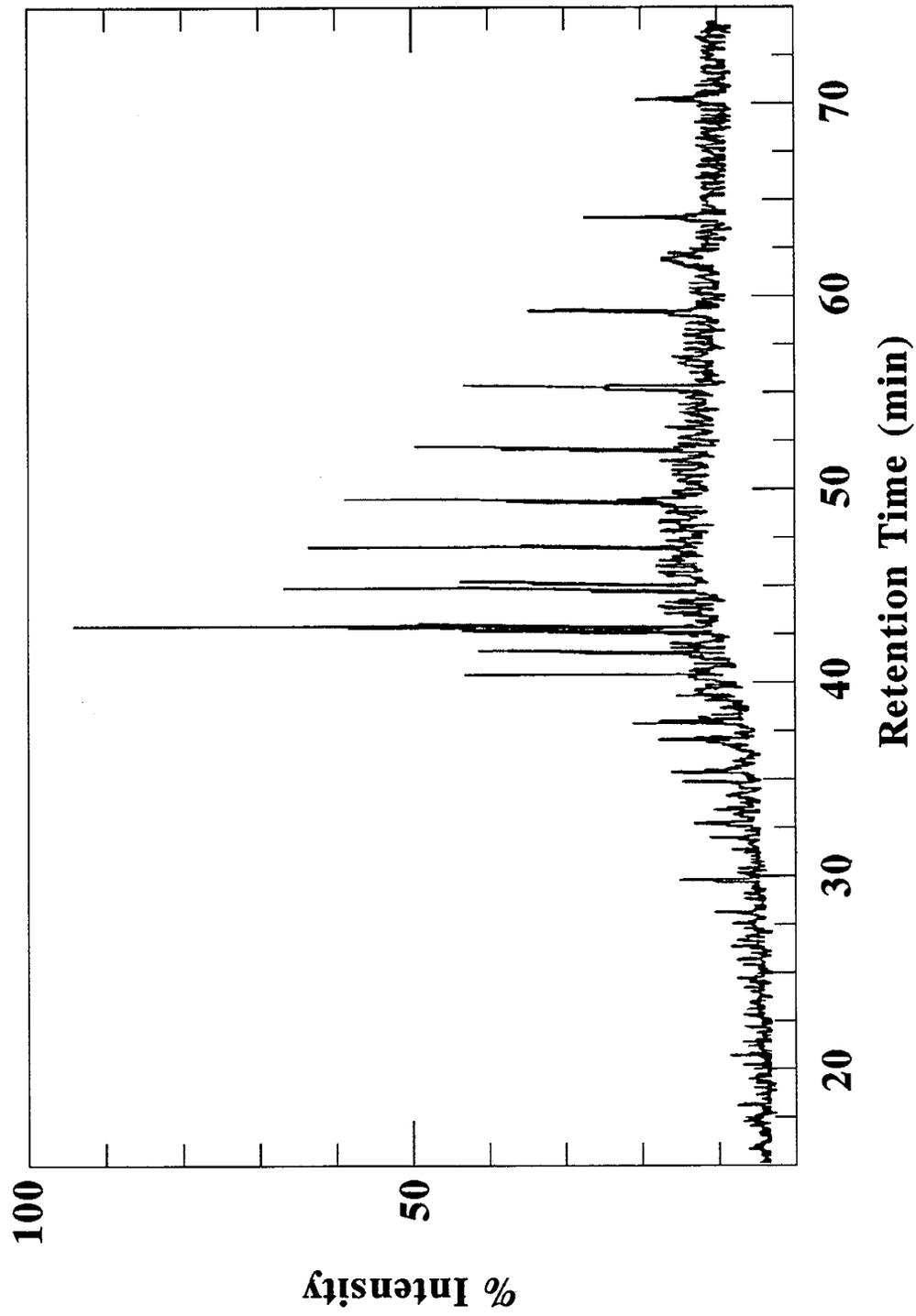


Figure 36. PR3 treated with aerobic BNL-4-25 strain.

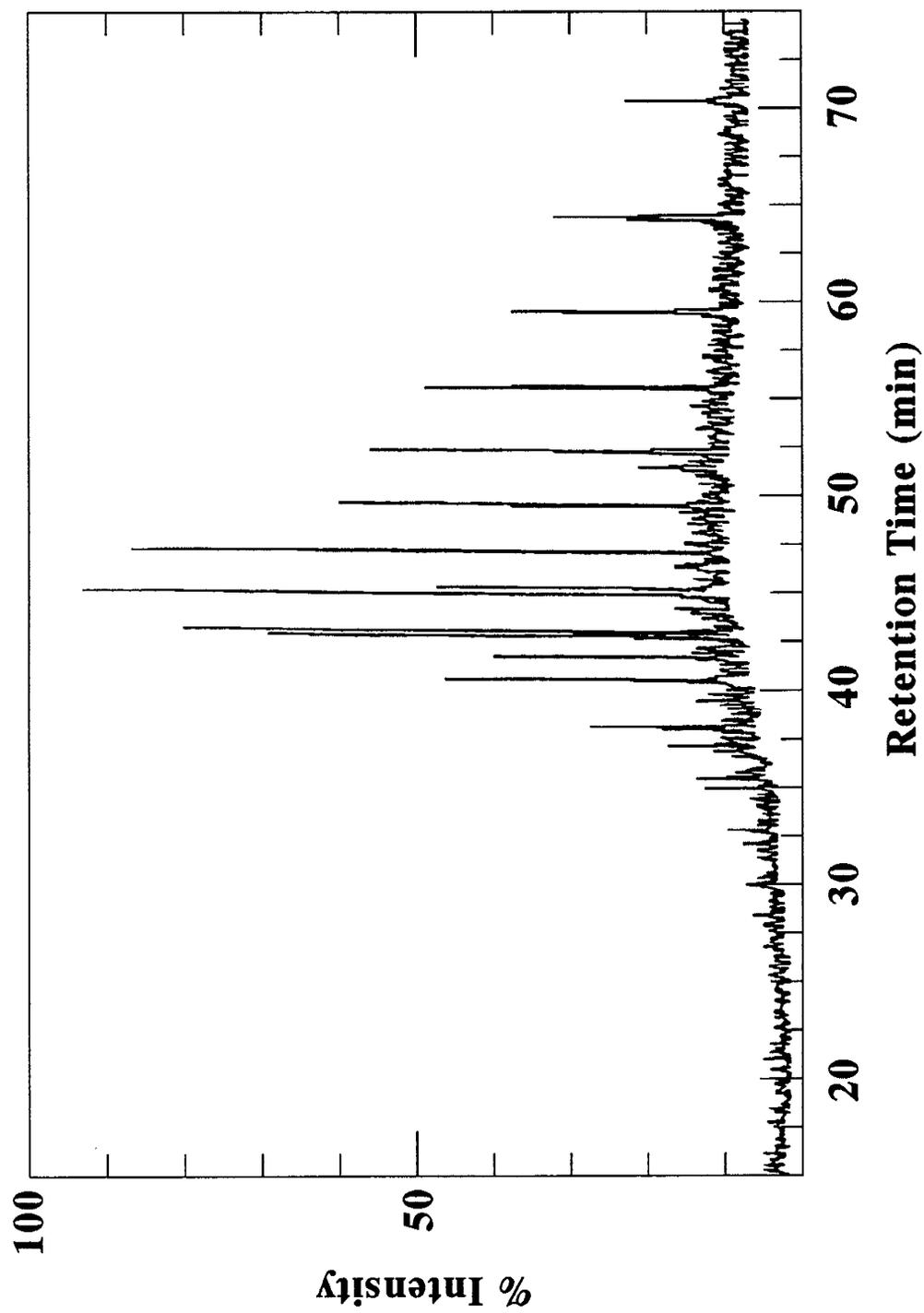


Figure 37. PR3 treated with an anaerobic, methanogenic thermophilic BNL-5-32 strain.

ultimately field trials, may allow predicting the effects and the efficiency of biochemical treatment of oils. This, in turn, will allow better planning of cost-efficient large scale operations.

Both pyrolysis mass spectrometry (PY-MS) (Boon et al., 1981; Donnison et al., 1986) and pyrolysis chromatography (Py-GC) (Needelman and Stuchberg, 1977) have been used to characterize and identify bacterial strains with good accuracy. In this program, bacteria useful to MEOR have been characterized similarly by PY-GC with ion trap mass spectrometer (Py-GC-MS). In the routine procedure, bacterial cultures were centrifuged in an ultracentrifuge and the pellets freeze-dried for pyrolysis experiments.

Pyrolysis was carried out by using 1 mg samples spiked with polybutylstyrene as internal standard in a Chemical Data Systems Model 190 pyroprobe. Typical GC-MS fingerprints of novel microbial species are shown in Figures 38-42. GC-MS fingerprinting techniques, together with routine morphological characterization assures continuity in culture maintenance.

5. Part II. Analysis of Combined Effects

5.1 Enhancement

Experimental evidence discussed in previous sections, has identified trends in biochemical interactions between microorganisms and crude oils. These trends indicate that different strains of microorganisms acting on the same oil interact differently. The reverse is also true, namely that the interaction of a single microbial strain with different oils is likewise variable. Thus, treatment of Wilmington, CA, crude oil with several microorganisms (Table 12) has shown that BNL-4-24, although not the best emulsion producer, is highly efficient in terms of conversion of organic sulfur

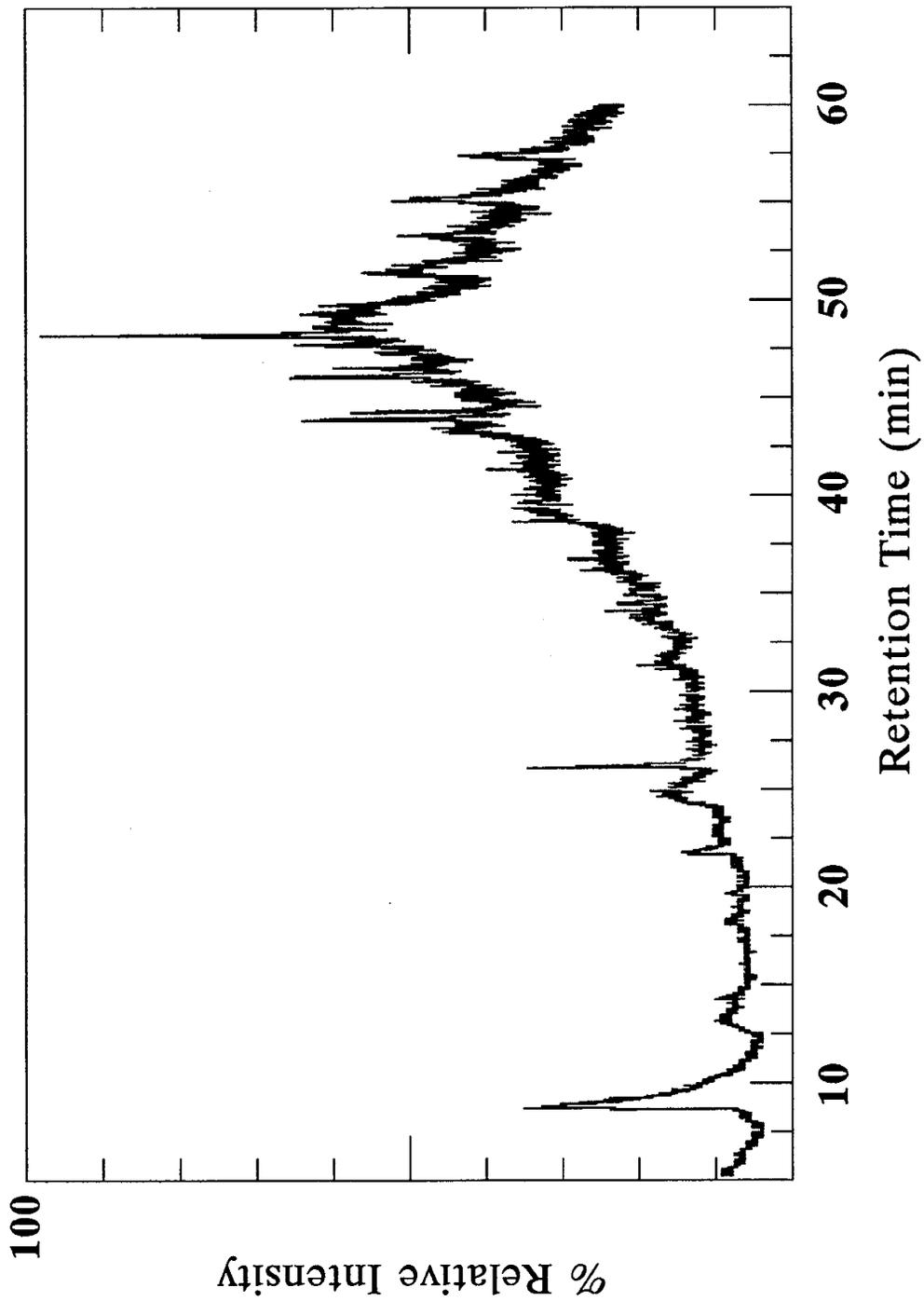


Figure 38. Pyrolysis Gas Chromatography Mass Spectrometry (total ion chromatogram) fingerprint of thermophilic microorganism BNL-4-21.

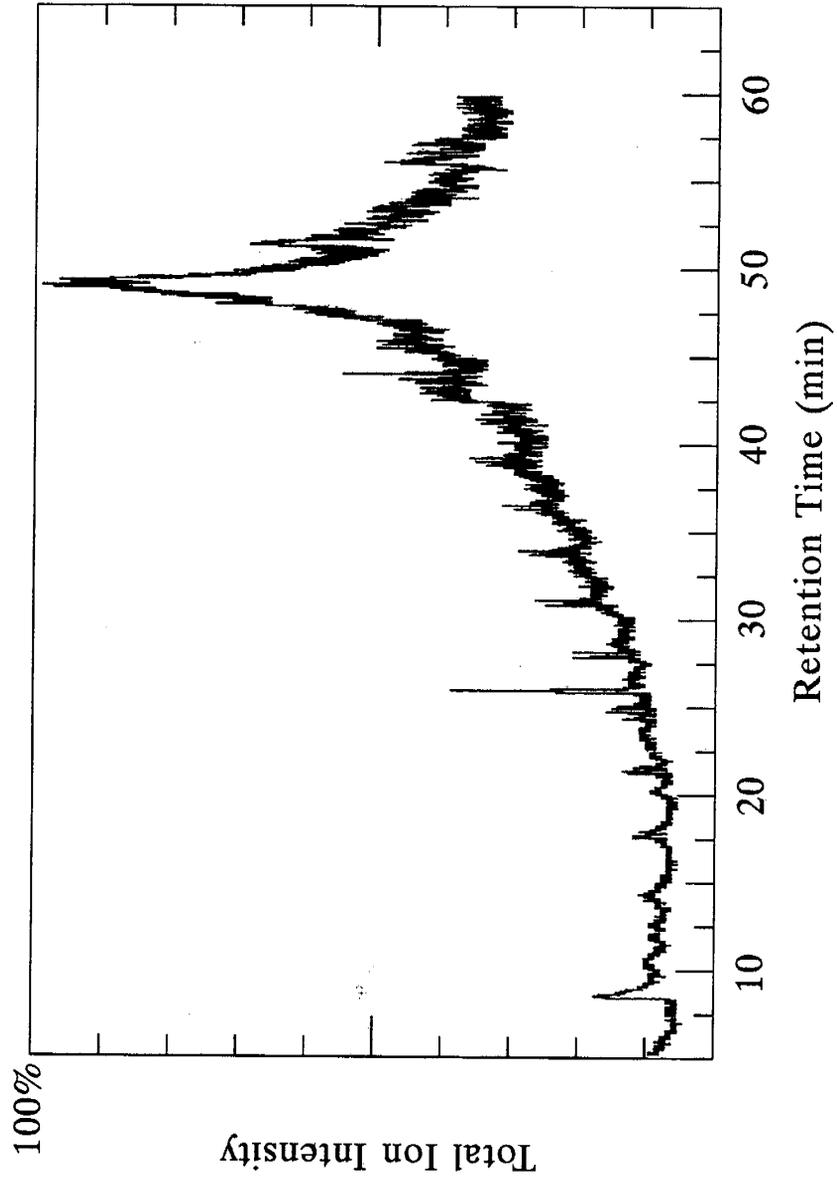


Figure 39. Pyrolysis Gas Chromatography Mass Spectrometry (total ion chromatogram) fingerprint of thermophilic microorganism BNL-4-22.

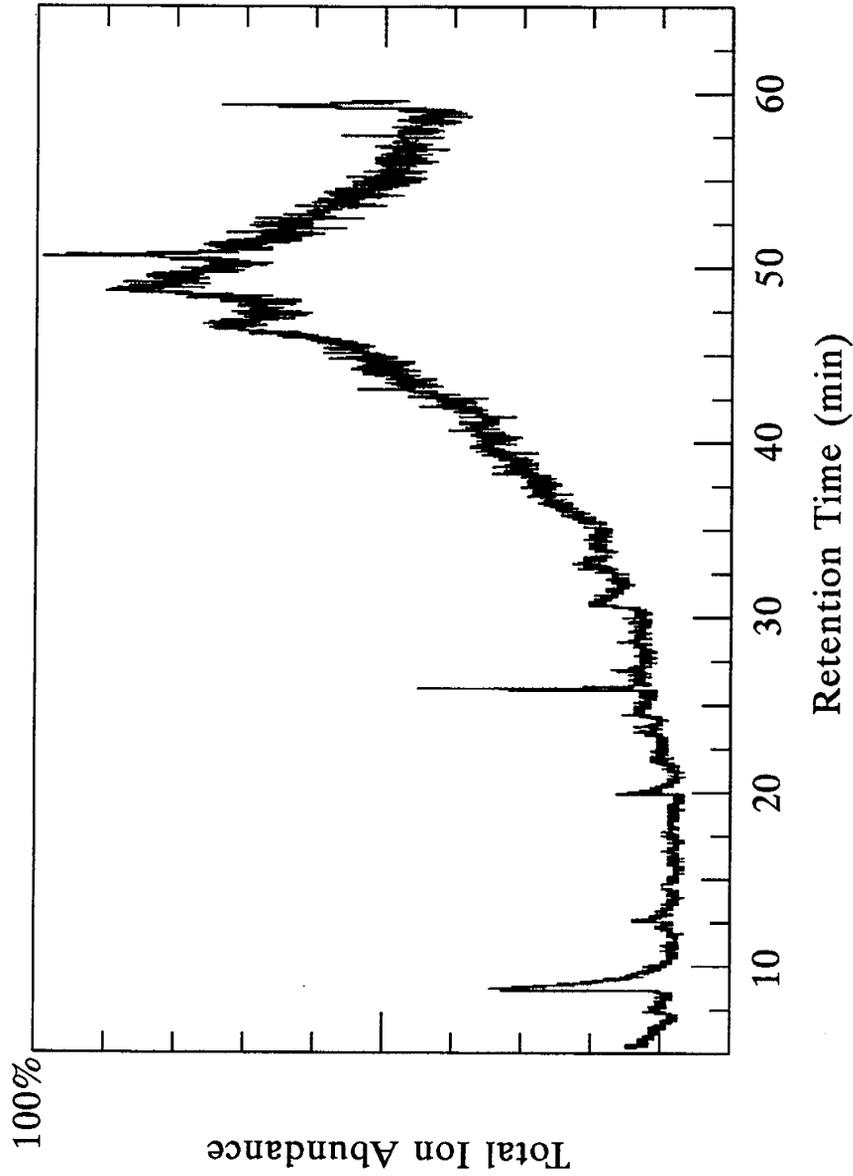


Figure 40. Pyrolysis Gas Chromatography Mass Spectrometry (total ion chromatogram) fingerprint of thermophilic microorganism BNL-4-23.

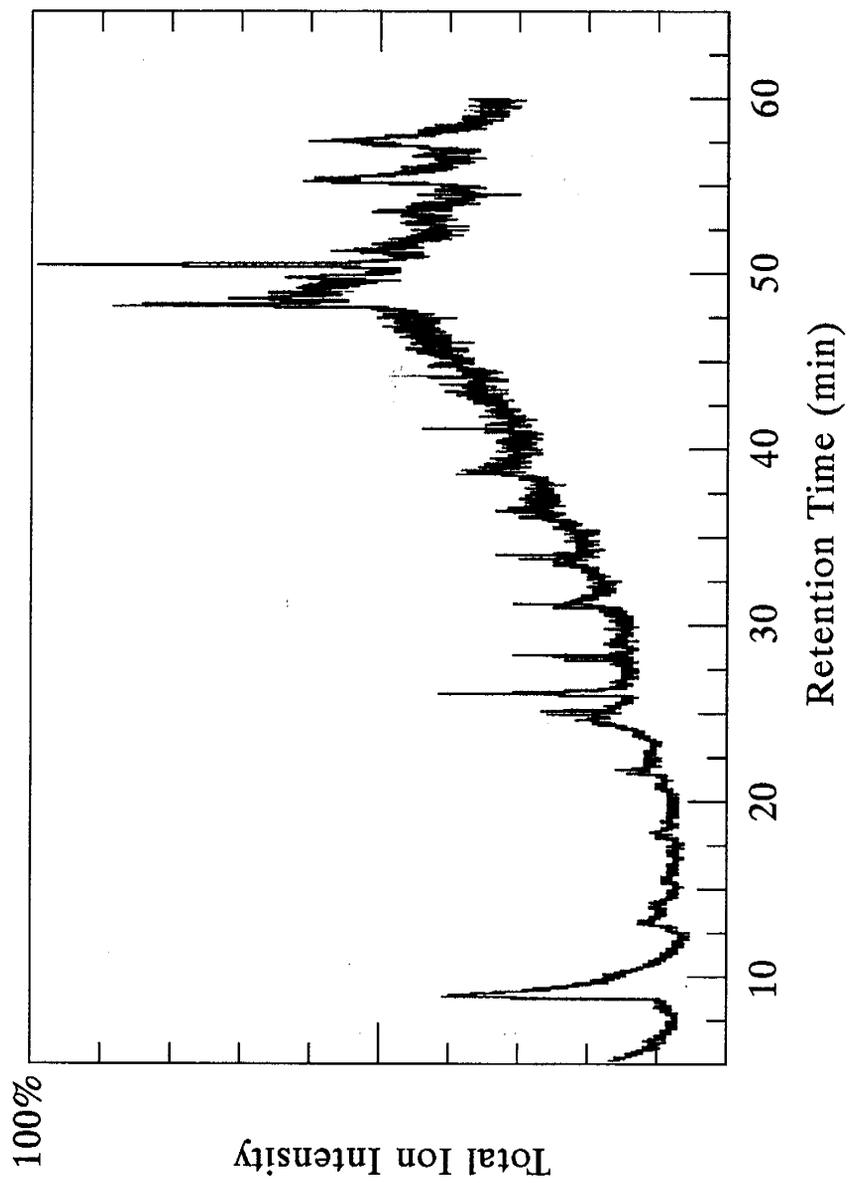


Figure 41. Pyrolysis Gas Chromatography Mass Spectrometry (total ion chromatogram) fingerprint of thermophilic microorganism BNL-4-24.

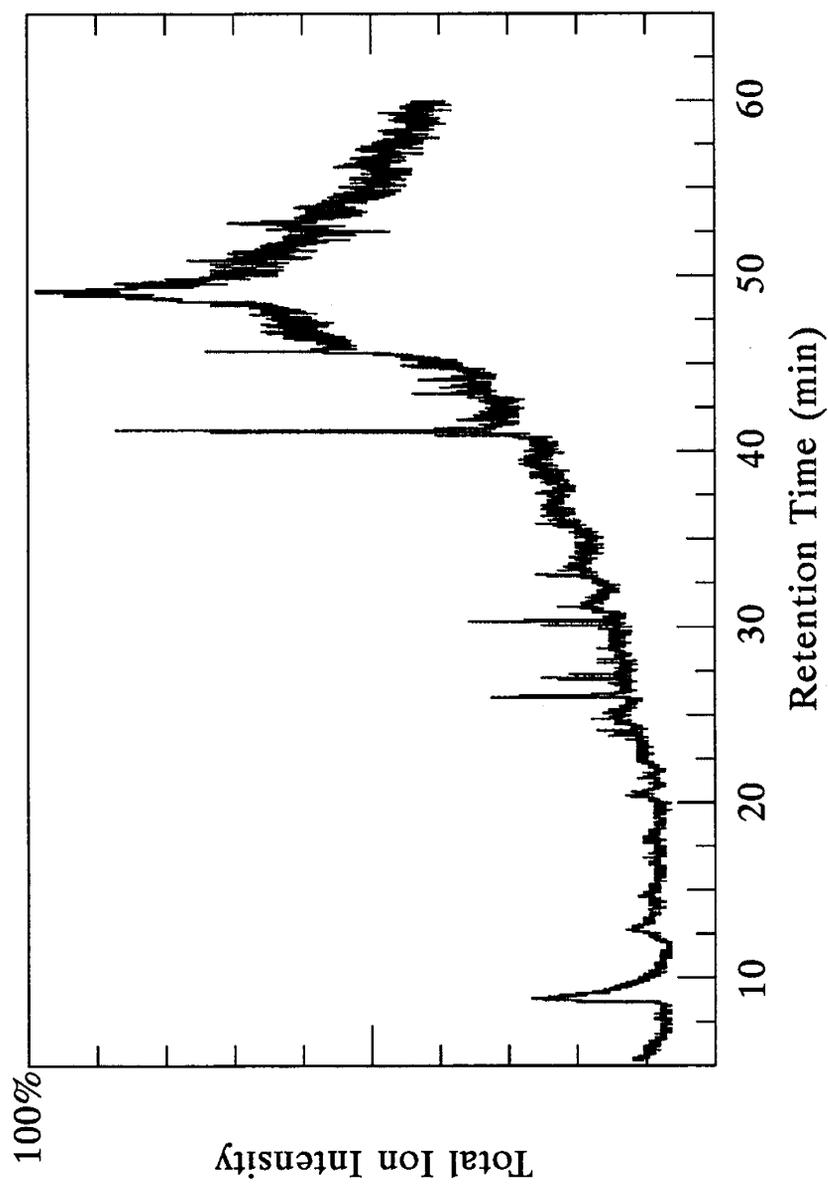


Figure 42. Pyrolysis Gas Chromatography Mass Spectrometry (total ion chromatogram) fingerprint of thermophilic microorganism BNL-NZ-3.

Table 12. Microbial Treatment of Wilmington Crude.

Microorganism	Days incubated	Viscosity at the end of biotreatment in centipoise (cps)	Emulsion in Klett units
BNL-4-21	20	3.6	180
BNL-4-22	20	3.0	400
BNL-4-23	23	3.0	180
BNL-4-24	30	3.6	55
Control	55	3.3	7.5

compounds as shown by examples given in Figures 43, 44, and 45, previously discussed in the section dealing with heteroatoms and organometallic compounds. All gas chromatographic traces are on the same scale and run under identical experimental conditions. It is interesting to note that the same organism used in this experiment, when acting on heavy fractions (200°C fraction) of Wilmington oil, produced a larger extent of emulsification than when acting on the whole crude oil (see p. 25, Sec. 4.2, Table 3).

In some instances, thermophilic microorganisms react with crude oils in a manner which causes the crude to be more efficiently emulsified. This information implies that chemically different emulsifying agents may be produced and the yields of naturally produced emulsifying agents, may also vary as a function of microbial species and the chemical composition of oils. If this is so, then some microbial species may be more suitable as producers of surface active agents while others may be better biochemical converters of crudes. Consortium of such organisms may enhance the overall effect. While additional R&D will further clarify this concept, preliminary experiments have been conducted in which the "enhancement effect" was tested. Thus, a sample of the Wilmington crude oil was emulsified with Tergitol (a commercial

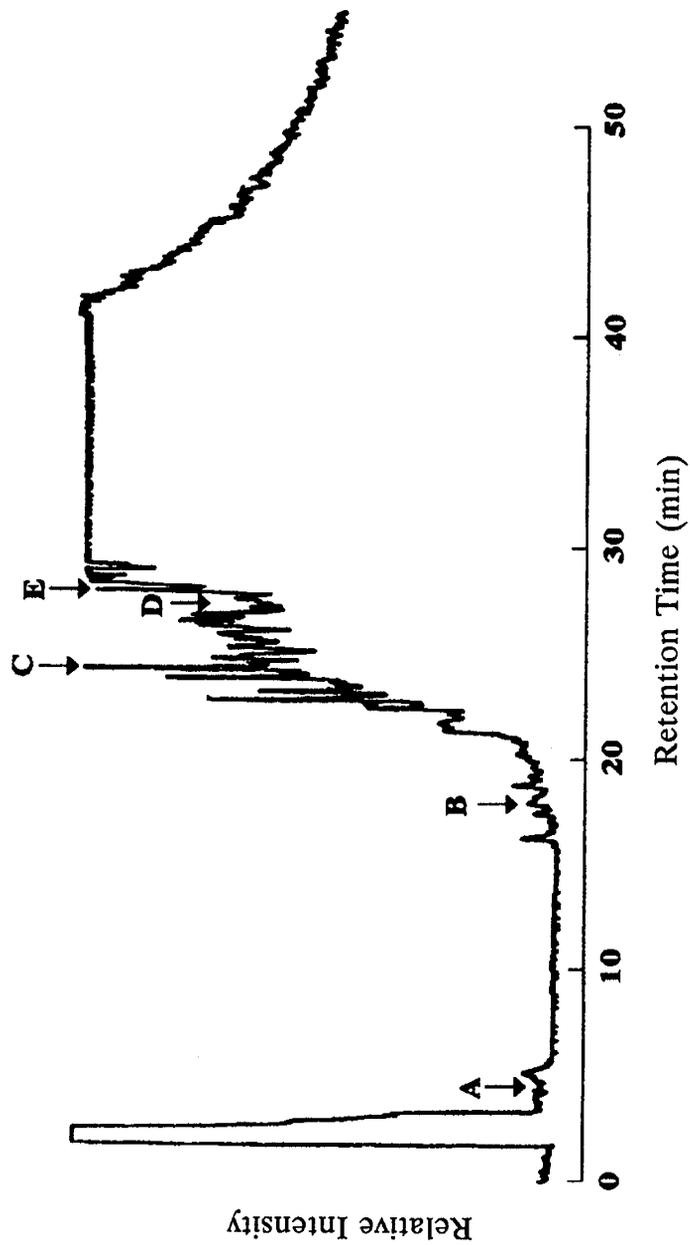


Figure 43. Gas Chromatography-Flame Photoemission Detector (GCFPD) trace of untreated Wilmington, CA, crude (sulfur specific trace). Molecular Markers A: Thiophene; B: Benzothiophene; C: Phenyl Sulfide; D: Dibenzothiophene; E: C-1 Dibenzothiophene.

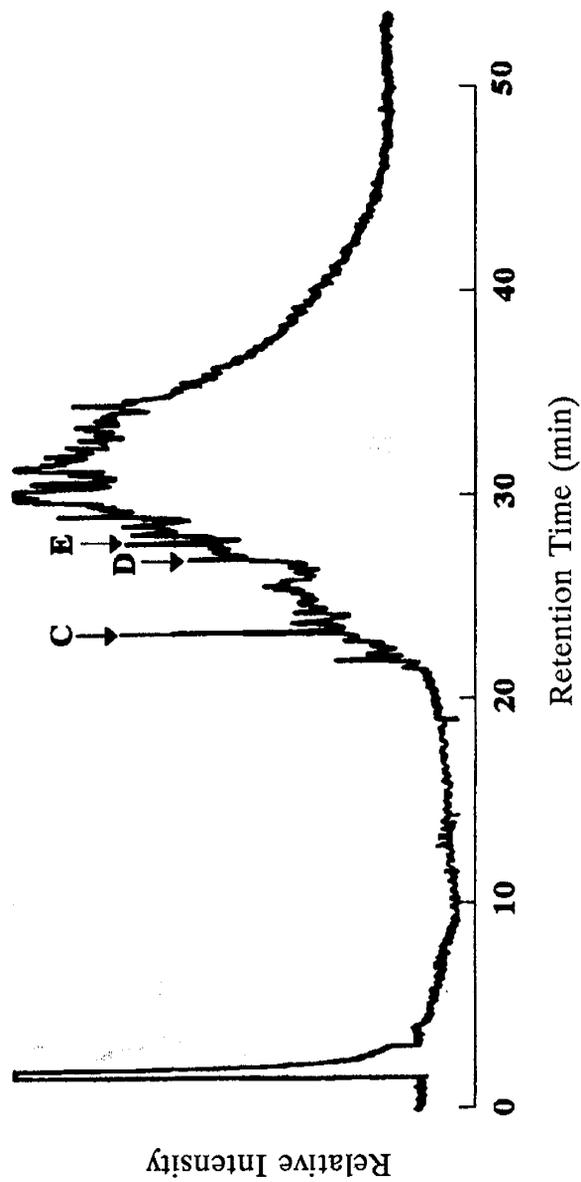


Figure 44. Gas Chromatography-Flame Photometric Detector (GCFPD) trace (sulfur emission intensity) of Wilmington, CA, crude biotreated with BNL-4-21. C, D, E, as in Figure 42.

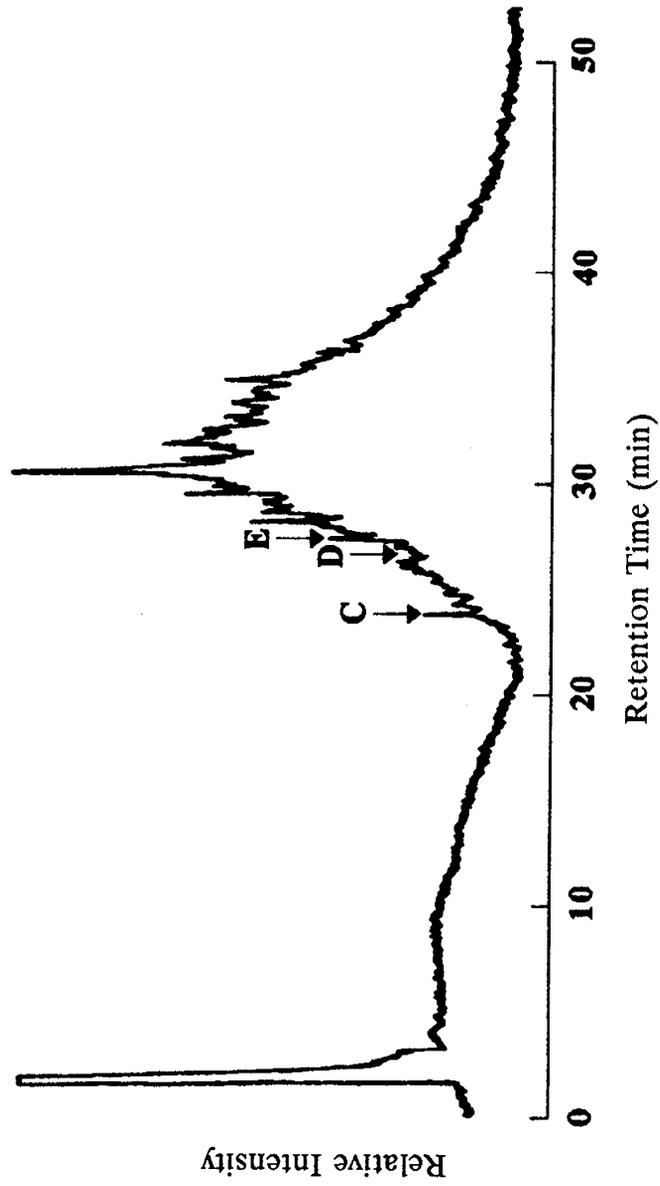


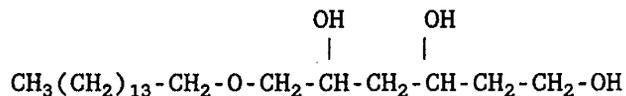
Figure 45. Gas Chromatography-Flame Photometric Detector (GCFPD) trace (sulfur emission intensity) of Wilmington, CA, crude biotreated with BNL-4-24. C, D, E, as in Figure 43.

Table 13. BNL-4-24 Treatment of Pre-emulsified Wilmington Crude Oil.

	<u>Days Incubated</u>	<u>Viscosity at the end of biotreatment in cps</u>	<u>Emulsion in Klett units</u>
Untreated Oil	0	400	
BNL-4-24 + 0.1% oil + 1% Tergitol	7	2.79	7400
BNL-4-24 + 0.1% oil + 0.5% Tergitol	7	3.83	8000

emulsifying agent) and then treated with BNL-4-24. The results are shown in Table 13. Compared to Figures 42 and 44, a considerable enhancement is achievable in terms of extent of emulsification and in terms of removal of organic sulfur compounds as shown in Figures 46 and 47, all in a much shorter period of time (seven days, compare Table 13). Further, comparable effects are observed with higher and lower concentrations of Tergitol which in large-scale operations represents a significant cost advantage. Similar experiments have been carried out with Boscan and Cerro Negro oils. The results are consistent and are shown in Table 14.

Tergitol is a hydrocarbon based emulsifying agent, whose chemical composition is shown below.



The use of tergitol does not imply that it is the most appropriate emulsifying agent. It is, however, a C-21 hydrocarbon and in that sense resembles some of the hydrocarbons identified in the emulsified phases of the crude oils used in these studies (see Section 5.5).

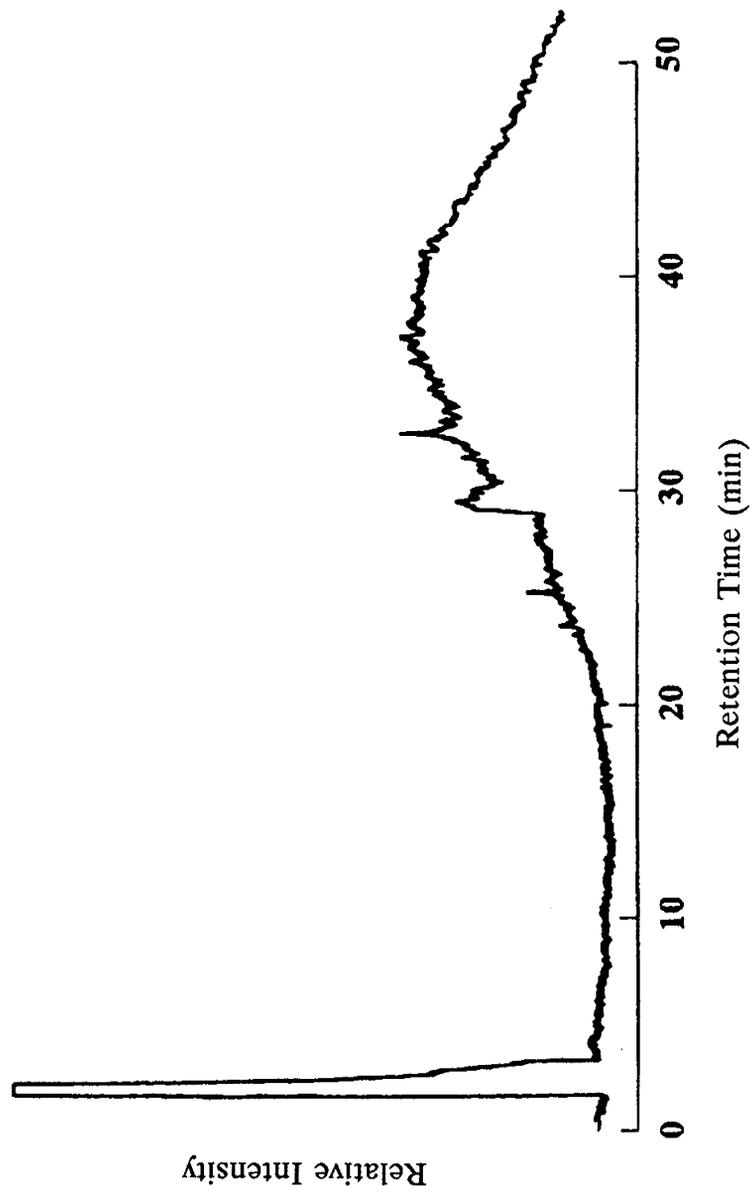


Figure 46. Gas Chromatography-Flame Photoemission Detector (GCFPD) trace of Wilmington, CA, crude pre-emulsified with 1% Tergitol and biotreated with BNL-4-24.

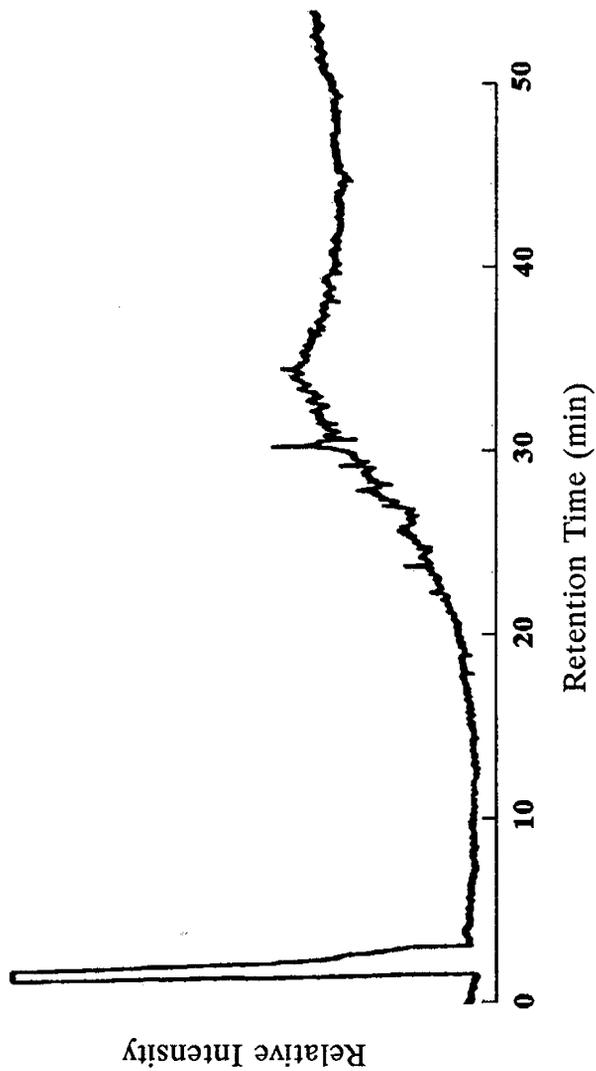


Figure 47. Gas Chromatography-Flame Photoemission Detector (GCFPD) trace of Wilmington, CA, crude pre-emulsified with 0.5% Tergitol and biotreated with BNL-4-24.

Table 14. Biotreatment of Pre-emulsified Crude Oils.

Oil	Microorganism	Media*	Treatment**	Emulsion in Klett units
Boscan	BNL-4-22	Nutrient added	0	210
	BNL-4-23	Nutrient added	0	140
	0	control	0	5
Cerro Negro	BNL-4-22	Nutrient added	0	120
	BNL-4-23	Nutrient added	0	145
	0	control	0	24
Boscan	BNL-4-22	no Nutrient	Emulsified	6000
	BNL-4-23	no Nutrient	Emulsified	5500
	0	control (Oil only)	Emulsified	2800
	BNL-4-22,23	control (Bacteria)	Emulsified	2600
Cerro Negro	BNL-4-22	no Nutrient	Emulsified	3800
	BNL-4-23	no Nutrient	Emulsified	4800
	0	control (Oil Only)	Emulsified	2800
	BNL-4-22,23	control (Bacteria)	Emulsified	2600

*Nutrient added means an addition of 100 ml of nutrient made up of 0.5g of peptone plus 0.3g of beef extract in 1000 ml, i.e. 0.08% of nutrient added.

** "0" means that the organisms were grown under conditions in which the organism generated the emulsification process in the medium. "Emulsified" means that the oil was pre-emulsified and then biotreated. The resulting emulsion is the cumulative result of pre-emulsification and emulsification generated by the microorganism.

5.2 Comparison of "Induced" vs. "Indigenous" Bioconversion of Crude Oils

In the course of the discussion dealing with the combined effects of microbial treatment of crudes, the possible role of the indigenous microorganisms which may be present in the crude oil has not been discussed. In this and several following sections, this topic will be addressed. Indigenous microorganisms (see, for example, Hunt, 1979; Tissot and Welte, 1984) are microbes found in formation waters and reservoirs which over geological periods of time alter crude oils, usually leading to the formation of heavy crudes. In the experimental studies under discussion, the "indigenous"

Table 15. Reaction Mixtures.

Reaction Mixture, RM₁

- (a) oil + medium 1
- (b) oil + medium 2
- (c) oil + medium 1 + emulsifier
- (d) oil + medium 2 + emulsifier

Reaction Mixture, RM₂

- (a) medium 1 + bacteria*
- (b) medium 2 + bacteria
- (c) medium 1 + bacteria + emulsifier
- (d) medium 2 + bacteria + emulsifier
- (e) medium 1 + bacteria + oil
- (f) medium 2 + bacteria + oil
- (g) medium 1 + bacteria + oil + emulsifier
- (h) medium 2 + bacteria + oil + emulsifier

*deliberately introduced bacteria, e.g., BNL strains

microorganisms are considered as those either present in the original crude or introduced during shipping and handling, or a combination of both possibilities, all viable under the experimental conditions used. In this laboratory, two types of media are used routinely. The first, called Medium 1, contains no organic carbon, and the second, called Medium 2, contains Medium 1 plus 0.08% nutrient added (see Section 3.4). In addition, some experiments have also been carried out with pre-emulsified oils followed by biotreatment. Tergitol (0.0075%) was used routinely for pre-emulsification in all of the experiments discussed in this and the following sections.

A series of experiments have been conducted in which several combinations as defined in Table 15 have been tested. The results, shown in Tables 16 and 17, clearly indicate that after a 40- or a 20-day treatment, significant differences in the end effect can be observed. In these experiments, the differences were measured by the extent of the resulting emulsions. Three

Table 16. Bioconversion of BOSCAN Crude Oil.

Microorganism + Media	Total Volume (mls)	%Oil	Incubation (days)	Viscosity	Emulsion (OD545nm)	Klett Units (OD545nm x 500)
BNL-4-22	203	0.57	40	2.6	0.1	50
BNL-4-23	203	0.54	40	2.4	0.02	10
BNL-4-24	203	0.52	40	2.4	0.01	5
BNL-NZ-2	203	0.51	40	2.5	0.045	22.5
BNL-NZ-3	203	0.54	40	2.4	0.001	0.5
BNL-NZ-5	203	0.51	40	2.4	0.02	10
Oil	203	0.5	40	2.4	0.02	10
BNL-4-21	203	0.47	40	2.5	0.138	69
BNL-4-22	203	0.48	40	2.5	0.049	24.5
BNL-4-23	203	0.47	40	2.8	0.14	70
BNL-NZ-2	203	0.49	40	2.6	0.032	16
BNL-NZ-3	203	0.51	40	2.6	0.1	50
BNL-NZ-5	203	0.51	40	2.8	0.12	60
Oil	203	0.5	40	2.6	0.178	89

Table 17. Bioconversion of pre-emulsified BOSCAN Crude Oil.

Microorganism + Media	Total Volume (mls)	%Oil	Incubation (days)	Viscosity	Emulsion (OD545nm)	Klett Units (OD545nm x 500)
BNL-4-22	203	0.51	40	2.2	3.15	1575
BNL-4-23	203	0.51	40	2.2	5.43	2715
BNL-4-24	203	0.52	40	2.6	1.5	750
BNL-NZ-2	203	0.49	40	2.4	2.05	1025
BNL-NZ-3	203	0.52	40	2.4	2.5	1250
BNL-NZ-5	203	0.5	40	2.5	5.65	2825
Oil	203	0.51	40	2.7	4	2000
BNL-4-22	203	0.649	20	2.4	4.59	2295
BNL-4-23	203	0.567	20	2.7	9.4	4700
BNL-4-24	203	0.519	20	2.4	6.82	3410
BNL-NZ-2	203	0.49	40	2.6	0.032	16
BNL-NZ-3	203	0.51	40	2.6	0.1	50
BNL-NZ-5	203	0.51	40	2.8	0.12	60
Oil	203	0.5	40	2.6	0.178	89

important consequences due to the "RM type" and duration of the treatment were also evident. One is that biotreatment with Medium 1 and without pretreatment does not encourage growth of microorganisms in the absence of oil. The second observation is that treatment with Medium 2 of crude oil alone produces an emulsified product larger than that produced by Medium 1. This effect may be due to the "indigenous" microorganisms as defined above. The presence of oil has an obvious effect, because the extent of emulsions is smaller in samples with microbes only. Thirdly, as shown in Table 17, in which the crude oil has been emulsified prior to biotreatment, a sizable enhancement in the end product is observed. Further, shortening the duration of biotreatment from 40 to 20 days, appears in some cases to be more efficient, e.g., BNL-4-23, BNL-NZ-2. The results generated by these experiments, indicate even stronger the versatility as well as the complexity of biochemical interactions between crude oils and microorganisms under the experimental conditions used.

5.3 Relative Abundance of Major Fractions and Their Significance in Biochemical Processes Associated with MEOR

Exploratory studies have been conducted which in addition to sulfur and trace metal chemistry, also address the biochemical effects on heavy fractions of crude oils, e.g., asphaltenes, maltenes, and saturates. This phase of the project was carried out in collaboration with the Chevron Oil Research Company of Richmond, California. The fractionation treatment involves dissolution in toluene, precipitation of asphaltenes with n-pentane, and the fractionation of the n-pentane soluble fraction, i.e., maltenes by high pressure liquid chromatography to yield saturate, aromatic, and polar fractions. Each fraction is then subjected to biotreatment and analysis. Typical results for Cerro Negro are given in Table 18.

Table 18. Cerro Negro Crude Oil; Relative Abundance of Major Fractions.

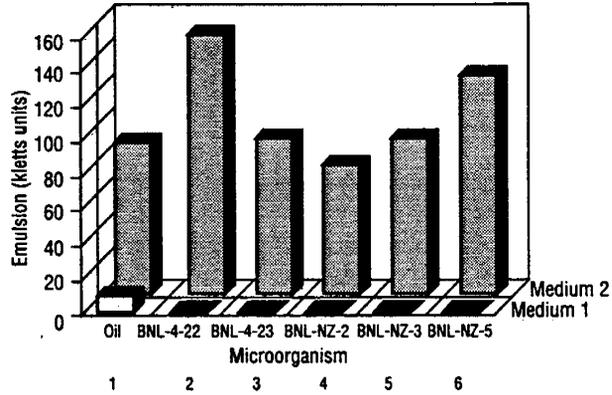
	Wt. % Asphaltenes	Wt. % Maltenes	Saturates (% of Maltenes)
Before Biotreatment	19.7	80.3	15.1
After Biotreatment with BNL-4-22	9.2	90.8	10.8

Extension of this work to other oils will be discussed later. However, it has to be emphasized that Boscan oil is heavy due to immaturity and Cerro Negro is heavy due to biodegradation by indigenous microorganisms over geological periods of time. Both oils are rich in heavy fractions (i.e., asphaltenes, maltenes), however, derive their chemical composition by different processes. The importance of such processes is reflected in several ways. As discussed earlier, there is a species specificity both in terms of microorganisms and types of oils used. Further, the biochemical processes which occur during the biotreatment of crude oils appear to be also influenced by other factors such as (i) the oil is the sole source of carbon; (ii) the carbon source other than oil has been added to the medium; and (iii) the presence of microbially produced or added detergents. Microbial treatment of crude oils, such as in MEOR, is routinely referred to as "biodegradation." This term may be misleading, particularly in view of actual end-products observed. Under the experimental conditions in which selected organisms interact with crude oils, various fractions of the crudes are affected in different ways. Heavy fractions may be biochemically split into smaller molecular weight fractions by means of chemical reactions involving intra- and inter-molecular rearrangements, hydroxylation, and changes of sulfur,

nitrogen, and oxygen bridges. Electron transfer may occur via active group transfer and change in organometallic complexes, followed by rearrangements yielding a variety of natural products with chemical properties significantly different from the starting complex organic matrix. These processes may occur during several phases of biotreatment with a net result of chemically altered crude oil. Therefore, the term "biotreatment" may be a more appropriate term, encompassing a multiplicity of biochemical and chemical reactions occurring concurrently as a direct consequence of the microbial intervention. Typical results such as shown in Figures 48 and 49 will be discussed in these terms. Medium 1 and 2 (RM_1 , RM_2) have been described (in Section 5.2). For the purpose of this discussion it is to be remembered that RM_1 has no carbon and RM_2 contains 0.08% of added nutrient. In Figure 49, the biotreatment experiment was repeated with RM_1 in the presence of a detergent over a period of 20 and 40 days. Thus, in Medium 1 (see Figure 48), indigenous microbes cause a negligible amount of emulsification, e.g., oil plus Medium 1 or Medium 2 (1 in Figure 48), while the BNL-strains do not grow at all. However, as a consequence of the interaction between oil, Medium 1 and BNL-4-22 (compare Figures 48,2a with 48,1) there is a significant increase in emulsification. Further, the extent of emulsification varies with different microorganisms (e.g., Figures 48,2a-6a) consistent with previous observations (Premuzic and Lin, 1991a,b). In this experiment the emulsions produced are due to the action of the microorganisms on oil only, because the oil is the sole source of carbon. In Medium 2, which contains a carbon nutrient, on addition of oil and microorganisms, a modified effect is observed (Figures 48,2a-6a). The observed effect is due to the action of microorganisms on the oil and the nutrient. The added nutrient represents 0.08% of added carbon while the concentration of

Biotreatment Controls

40 Days Medium 1 vs. Medium 2

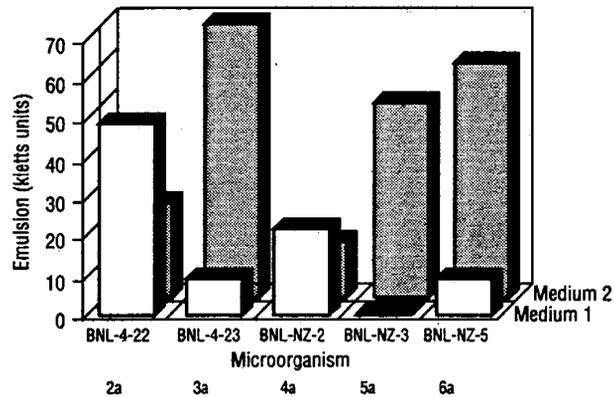


Legend: 1 = Boscan oil alone + RM₁ or RM₂; 2-6, BNL microorganism + RM₁ or RM₂.

Shows the effect of indigenous organisms.

Boscan Biotreatment

40 Days Medium 1 vs. Medium 2

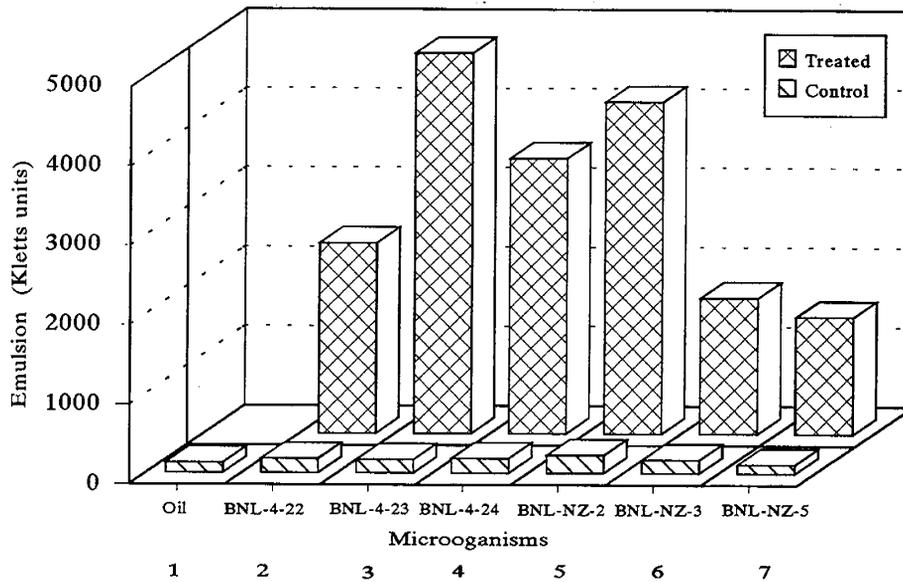


Legend: 2a-6a = Boscan oil + BNL microorganisms + RM₁ or RM₂.

Figure 48. Bioconversion of Venezuelan Boscan crude oil.

Boscan Biotreatment in Medium 1

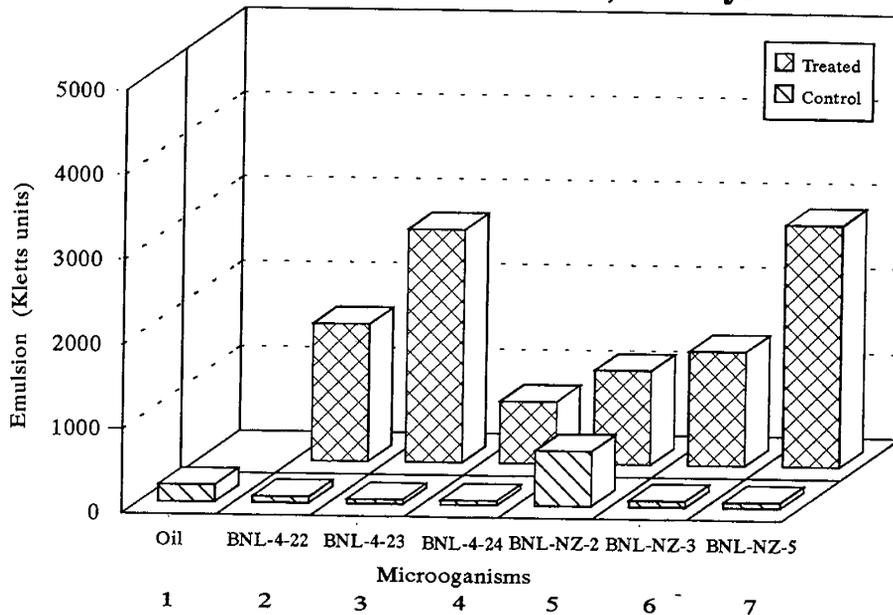
Plus Emulsifier, 20 Days



Legend: 1 = oil + detergent + RM₁; 2-7 = BNL microorganism + detergent + RM₁
20 day treatment.

Boscan Biotreatment in Medium 1

Plus Emulsifier, 40 Days



Legend: 1 = oil + detergent + RM₁; 2-7 = BNL microorganism + detergent + RM₁
40 day treatment.

Figure 49. Bioconversion of Venezuelan Boscan Crude Oil in Presence of a Detergent.

total oil added averages at 0.5%. For the purposes of this discussion, it is reasonable to assume that, although not directly comparable, the oil carbon contribution to the biochemical conversion of the oil exceeds that due to the non-oil nutrient added. However, the observed gross effect is, nevertheless, a consequence of microbial action on both sources of carbon, which may well influence the biochemical pathways involved, and may be the cause of the observed overall differences which appear to exist between biotreatment in Medium 1 and Medium 2. Other than media, these experiments have been carried out under identical experimental conditions. In a concurrent experiment, 0.0075% of detergent Tergitol has been added to the oil and the emulsified oil then treated with microorganisms under identical experimental conditions over a period of twenty- and forty-days. The results for experiments carried out in Medium 1 are given in Figure 49. Under these experimental conditions, shorter periods of treatment appear to be effective suggesting that optimization of the variables in the biochemical processing of oils may lead to short treatment intervals. The corresponding changes in the hydrocarbon, organosulfur, and organometallic chemical markers in biotreated Boscan and Cero Negro oils have already been discussed in Sections 4.3 and 4.4.

Analogous biochemical treatment of several domestic, high sulfur content, heavy crude oils also with high contents of the asphaltene/maltene fractions yielded comparable results. Thus, for two immature offshore Monterey oils, A836, 3.2% S and A837, 4.0% S are given in Tables 19 and 20, and Figure 50 follow similar trends in the biochemical processes associated with microbial treatment of oils. The data also indicate that in some cases, addition of detergent does not enhance the overall bioprocess. For example, compare the effects of BNL-4-23 on A836 and A837. In the absence of the detergent, there

Table 19. Monterey Oil A836 Biotreatment.

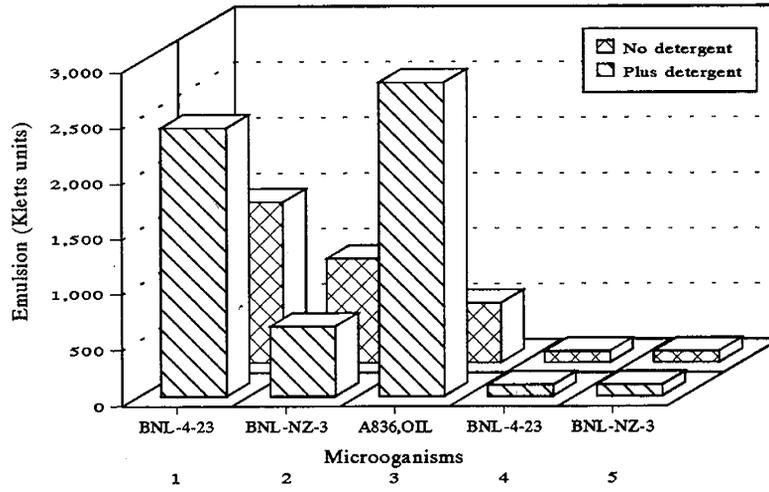
Microorganism	Media	Volume (ml)	%Oil	Incubation (Days)	Optical Density (545nm)	Klett Units (ODx500)
NO DETERGENT EXPT I						
BNL-4-23	Media 2	201	0.496	20	4.800	2400
BNL-NZ-3	Media 2	201	0.491	20	1.240	620
A836 Control	Media 2	200	0.503	20	5.600	2800
BNL-4-23 Control	Media 2	200	0.000	20	0.158	79
BNL-NZ-3 Control	Media 2	200	0.000	20	0.121	60.5
DETERGENT EXPT I 0.0075% TERGITOL						
BNL-4-23	Media 2	201	0.445	20	1.000	500
BNL-NZ-3	Media 2	201	0.546	20	2.160	1080
A836 Control	Media 2	200	0.548	20	1.040	520
BNL-4-23 Control	Media 2	200	0.000	20	0.190	95
BNL-NZ-3 Control	Media 2	200	0.000	20	0.134	67

Table 20. Monterey Oil A837 Biotreatment.

Microorganism	Media	Volume (ml)	%Oil	Incubation (Days)	Optical Density (545nm)	Klett Units (ODx500)
NO DETERGENT EXPT I						
BNL-4-23	Media 2	201	0.503	20	0.760	380
BNL-NZ-3	Media 2	201	0.543	20	3.400	1700
A837 Control Oil	Media 2	200	0.502	20	4.000	2000
BNL-4-23 Control	Media 2	200	0	20	0.158	79
BNL-NZ-3 Control	Media 2	200	0	20	0.121	60.5
DETERGENT EXPT I 0.0075% TERGITOL						
BNL-4-23	Media 2	201	0.479	20	4.000	2000
BNL-NZ-3	Media 2	201	0.496	20	3.760	1880
A837 Control Oil	Media 2	200	0.487	20	0.510	255
BNL-4-23 Control	Media 2	200	0	20	0.190	95
BNL-NZ-3 Control	Media 2	200	0	20	0.134	67

Monterey Oil A836 Biotreatment

20 Days Detergent vs. No Detergent



Monterey Oil A837 Biotreatment

20 Days Detergent vs. No Detergent

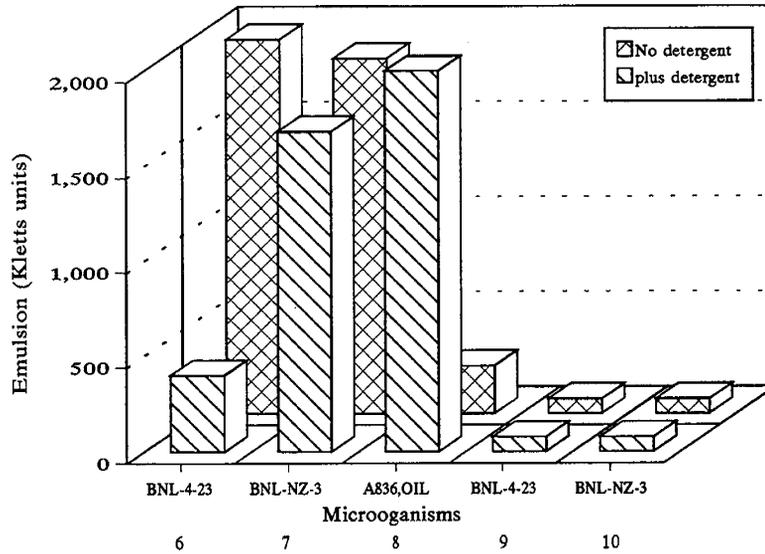


Figure 50. Bioconversion of Monterey Oils: 20 Day Biotreatment in Medium 2.

Controls:
 3 & 8 = Oil + M2
 4 & 9 = BNL-4-23 + M2
 5 & 10 = BNL-NZ-3 + M2

is a significant effect on A837 compared to the effect on A836. The net effect is reversed in the presence of the detergent. In both cases the controls are comparable (3 and 8, in Figure 50). BNL-NZ-3 is significantly more active in A837 than in A836. NZ group of organisms represents isolates containing closely related thermophilic microorganisms. Because complete characterization requires a considerable expenditure of time and funds, unavailable under the programmatic terms of the present project, biochemical conversion of crudes by these organisms should be considered as being due to the group. Arbitrary labelling simply allows for possible refinements on further characterization in the future. The effect of "indigenous microorganisms" to be discussed later (Section 5.6) is also noticeable. Medium-2 contains 0.08% of added nutrient, therefore, the emulsification observed in experimental controls 3 and 8, must be due to the growth of organisms present in the oil whose growth was promoted by the addition of the nutrient.

5.4 Duration of Biotreatment and Media Effects

The biochemical processes discussed in previous sections which occur during the biotreatment of crude oils are influenced not only by the type of microorganisms used, but also by the composition of the reaction mixture and duration of the treatment. In order to further refine these observations, Monterey Crude Oil A836, an immature, off-shore Monterey California crude, was treated for seven days and for twenty days in the presence and absence of Tergitol in Medium 3. The medium is a modification of Medium 1, where the sulfate was replaced by a chloride. The choice of Tergitol, a C-21 carbon emulsifying agent containing ethylene oxide and hydroxy functional groups, was based on other data discussed in Section 5.5.

The biotreatment results for A836 are given in Table 21. The corresponding Gas Chromatography-Mass Spectrometry (GC-MS) analyses of A836 treated with BNL-4-22 and BNL-4-23 in Medium 3 and absence of Tergitol are given in

Figure 51. This means that no external source of a terminal electron acceptor, such as nitrate or sulfate, has been added to the reaction mixture. Therefore, biochemical reactions resulting in the bioconversion of the oil must be due to either internal electron transfer reactions involving the N, S, O, or residual oxygen in the medium. All GC-MS analyses have been carried out under identical experimental conditions, concentration, and instrument settings, allowing for direct comparison of spectra. As expected, significant changes occur after a twenty-day treatment in the presence and/or absence of the detergent (0.0075% Tergitol). The BNL-4-23 case, where an exceptionally high extent of emulsification relative to other samples was measured, has to be re-examined by additional experimental work.

Results given in Table 21 show that a seven day biotreatment is useful and further studies with other oils should indicate whether the shorter, i.e., less than twenty day treatments could be considered for routine applications. GC-MS analyses shown in Figure 51, clearly indicate a decrease in the higher molecular weight fractions (retention time 30 minutes and more). They also indicate fine changes in the shorter retention time region. Since the Monterey A836 crude oil is high in sulfur (4%) a GC analyses using a sulfur specific detector (FPD) has been carried out with untreated and biotreated samples. The results are shown in Figure 52. The chromatographic analyses for organic sulfur compounds and the results have been recorded under identical experimental conditions (injected amount, retention time, and attenuation) allowing for direct comparison of the chromatographic scans. Within the experimental limits these results show a 50% or better conversion and/or removal of organic sulfur compounds from the oil phase.

Table 21

Microorganism	Detergent	Media	Volume (ml)	Monterey Oil A836			Biotreatment			Klett Units (OD x 500)	pH
				%Oil	Incubation (days)	Viscosity (Centipoise)	Optical Density (545nm)	Viscosity (Centipoise)			
BNL-4-22	No	Media 3	125	0.484	7	2.5	0.0060	2.5	3.000	3.50	
BNL-4-23	No	Media 3	125	0.434	7	2.5	0.0050	2.5	2.500	3.50	
Control Oil	No	Media 3	125	0.518	7	2.6	0.0010	2.6	0.500	3.50	
BNL-4-22 Control	No	Media 3	125	0.000	7	2.4	0.0100	2.4	5.000	3.50	
BNL-4-23 Control	No	Media 3	125	0.000	7	2.5	0.0010	2.5	0.500	3.50	
Control/Control*	No	Media 3	125	0.000	7	2.5	0.0000	2.5	0.000	3.50	
BNL-4-22	No	Media 3	125	0.491	20	NA	0.0570	NA	28.490	4.00	
BNL-4-23	No	Media 3	125	0.510	20	NA	0.1935	NA	96.725	4.25	
Control Oil	No	Media 3	125	0.518	20	NA	0.0540	NA	27.010	4.25	
BNL-4-22 Control	No	Media 3	125	0.000	20	NA	0.0292	NA	14.595	4.25	
BNL-4-23 Control	No	Media 3	125	0.000	20	NA	0.0107	NA	5.365	4.00	
Control/Control*	No	Media 3	125	0.000	20	NA	0.0194	NA	9.710	4.00	

*Medium Control (medium only)

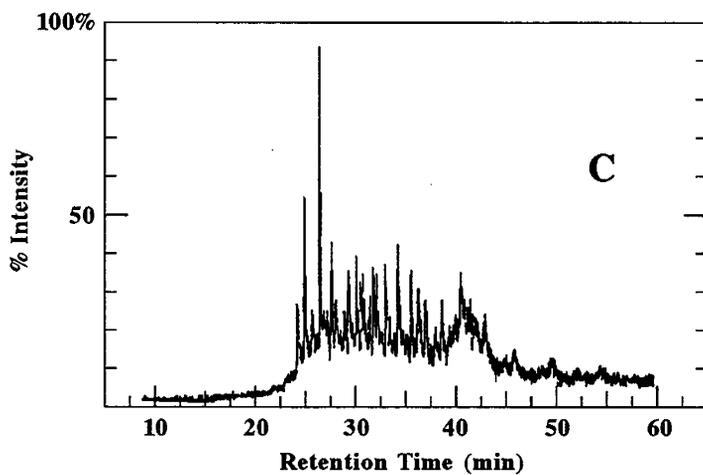
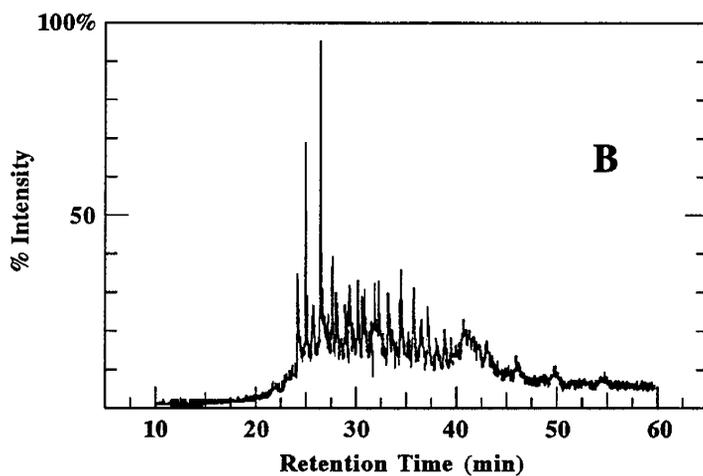
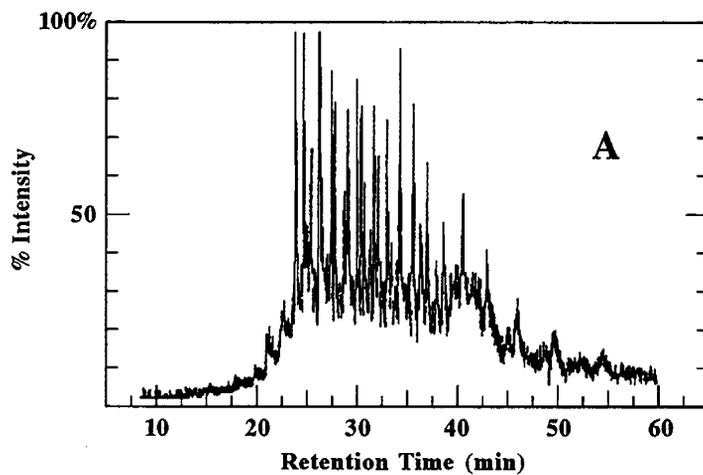


Figure 51. Gas Chromatography-Mass Spectrometry (GC-MS) scan of Monterey crude A836: (A) Control; (B) treated with BNL-4-22; (C) treated with BNL-4-23. (Medium 3, no Tergitol.)

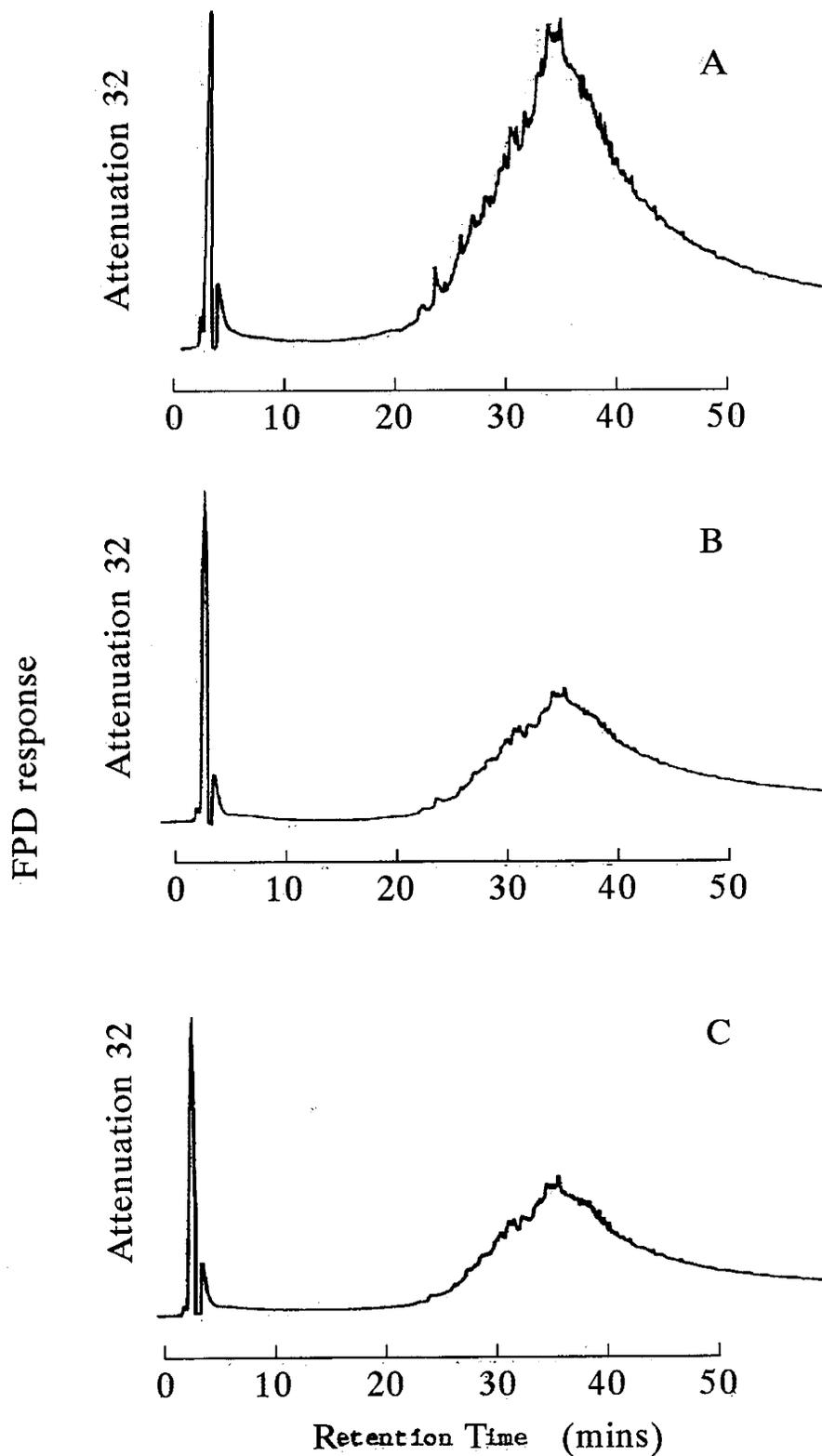


Figure 52. Gas Chromatographic Analyses of Monterey A836 Crude. Flame Photometric Detector (FPD) trace of: (A) Control A836; (B) treated with BNL-4-22; (C) treated with BNL-4-23.

Analogous experimental strategy was used in the study of another immature off-shore Monterey crude oil (A837) and an onshore biodegraded heavy crude (A851). The results of these studies were recently discussed at the Fourth International Microbial Enhanced Oil Recovery Conference (Premuzic and Woodhead, 1993). Medium 2 and 3 were used throughout. The experiments were carried out under identical experimental conditions in the presence and absence of a detergent (e.g., Tergitol) over a period of twenty days and a period of seven days. The results summarized in Tables 22-26 consistently show that the biochemical interaction between selected microorganisms and oils follows distinct patterns. Treatment of Monterey A837 with BNL-4-22, BNL-4-23, and BNL-NZ-3 leads to the conversion of heavier hydrocarbon fractions of crude oil to lighter. Furthermore, there are differences in the effects due to the presence or absence of Tergitol. For all cases in this series of experiments, a seven-day treatment appeared to be sufficient to bring about the biochemical conversion. A working hypothesis discussed in Section 7, is based on the premise that the biochemical conversion starts by an attack on the complex structure, possibly on the N,S,O rich polar components of the maltene fraction derived from the asphaltene component of the crude. In the three crude oils considered, A836, A837, and A851, the highest concentration of this fraction is in A851. The overall redistribution of the relative amounts of heavy and light hydrocarbons are shown in Figures 51, 53, and 58. The hydrocarbon profiles determined by GC-MS analyses also show that BNL-4-22 and BNL-4-23 are not very effective in the biotreatment of A851 (see also Figure 58). BNL-NZ-3 on the other hand, is remarkably effective.

Table 22. Seven and Twenty Day Biotreatment of Monterey Oil A837 in Medium 3 and in the Absence of an Emulsifying Agent.

Microorganisms	Medium	%Oil	Incubation (Days)	Extent of Emulsification in Klett Units
BNL-4-22	Medium 3	0.489	7	16.875
BNL-4-23	Medium 3	0.534	7	3.710
BNL-NZ-3	Medium 3	0.482	7	7.265
Control, Oil	Medium 3	0.486	7	1.205
BNL-4-22 Control*	Medium 3	0.000	7	3.550
BNL-4-23 Control*	Medium 3	0.000	7	1.825
BNL-NZ-3 Control*	Medium 3	0.000	7	2.105
Medium/Control**	Medium 3	0.000	7	0.080
BNL-4-22	Medium 3	0.476	20	40.885
BNL-4-23	Medium 3	0.476	20	7.225
BNL-NZ-3	Medium 3	0.476	20	5.675
Control, Oil	Medium 3	0.480	20	5.465
BNL-4-22 Control*	Medium 3	0.000	20	9.080
BNL-4-23 Control*	Medium 3	0.000	20	3.670
BNL-NZ-3 Control*	Medium 3	0.000	20	5.105
Medium/Control**	Medium 3	0.000	20	3.160

* Medium plus organism only.

** Medium only.

Table 23. Seven and Twenty Day Monterey Oil A837 Biotreatment in Medium 2 and 3 in the Presence of an Emulsifying Agent.

Microorganisms	Medium	%Oil	Incubation (Days)	Extent of Emulsification in Klett Units
BNL-4-23	Medium 2	0.479	21	2000.000
BNL-NZ-3	Medium 2	0.496	20	1880.000
A837 Control, Oil	Medium 2	0.487	23	255.000
BNL-4-23 Control	Medium 2	0.000	20	95.000
BNL-NZ-3 Control	Medium 2	0.000	20	67.000
BNL-4-22	Medium 3	0.567	20	13.000
BNL-4-23 Control, Oil	Medium 3	0.510	20	9.000
BNL-4-22 Control	Medium 3	0.500	20	7.000
BNL-4-23 Control	Medium 3	0.000	20	26.000
Medium/Control (65C)	Medium 3	0.000	20	15.000
	Medium 3	0.000	20	19.500
BNL-4-22	Medium 3	0.490	7	18.785
BNL-4-23 Control, Oil	Medium 3	0.521	7	10.010
BNL-4-22 Control	Medium 3	0.530	7	6.035
BNL-4-23 Control	Medium 3	0.000	7	5.150
Control/Control	Medium 3	0.000	7	5.125
	Medium 3	0.000	7	3.465

Table 24. Seven and Twenty Day Biotreatment of Monterey Oil A851 in Medium 2 in the Absence of an Emulsifying Agent.

Microorganisms	Medium	%Oil	Incubation (Days)	Extent of Emulsification in Klett Units
BNL-4-23	Medium 2	0.537	20	2000.000
BNL-NZ-3	Medium 2	0.490	20	2000.000
A851 Control, Oil	Medium 2	0.516	20	1280.000
BNL-4-23 Control	Medium 2	0.000	20	79.000
BNL-NZ-3 Control	Medium 2	0.000	20	60.500
BNL-4-22	Medium 2	0.482	7	282.310
BNL-4-23	Medium 2	0.480	7	155.935
BNL-NZ-3	Medium 2	0.479	7	130.510
A836 Control	Medium 2	0.494	7	5.900
BNL-4-22	Medium 2	0.478	20	407.525
BNL-4-23	Medium 2	0.476	20	116.25
BNL-NZ-3	Medium 2	0.490	20	261.07
A836 Control	Medium 2	0.484	20	60.98
Medium/Control	Medium 2	0.000	20	4.58

Table 25. Seven and Twenty Days of Biotreatment of Monterey Oil A851 in Medium 3 in the Absence of an Emulsifying Agent.

Microorganisms	Medium	%oil	Incubation (Days)	Extent of Emulsification in Klett Units
BNL-4-21	Medium 3	0.628	7	3.355
BNL-4-22	Medium 3	0.628	7	4.220
BNL-4-23	Medium 3	0.625	7	3.435
BNL-NZ-3	Medium 3	0.629	7	4.020
Control, Oil	Medium 3	0.626	7	3.615
BNL-4-21 Control	Medium 3	0.628	7	1.160
BNL-4-22 Control	Medium 3	0.000	7	3.760
BNL-4-23 Control	Medium 3	0.000	7	1.970
BNL-NZ-3 Control	Medium 3	0.000	7	3.275
Medium/Control	Medium 3	0.000	7	2.915
BNL-4-22	Medium 3	0.484	20	12.605
BNL-4-23	Medium 3	0.484	20	14.205
BNL-NZ-3	Medium 3	0.476	20	6.720
Control, Oil	Medium 3	0.480	20	1.070
BNL-4-22 Control*	Medium 3	0.000	20	9.080
BNL-4-23 Control*	Medium 3	0.000	20	3.670
BNL-NZ-3 Control*	Medium 3	0.000	20	5.105
Medium/Control**	Medium 3	0.000	20	3.160

Table 26. Biotreatment of Monterey Oil A851 in Medium 2 and 3 in the Presence of an Emulsifying Agent.

Microorganisms	Medium	%Oil	Incubation (Days)	Extent of Emulsifi- cation in Klett Units
BNL-4-23	Medium 2	0.490	20	940.000
BNL-NZ-3	Medium 2	0.511	20	1360.000
A851 Control	Medium 2	0.494	23	1380.000
BNL-4-23 Control	Medium 2	0.000	20	95.000
BNL-NZ-3 Control	Medium 2	0.000	20	67.000
Medium/Control	Medium 2	0.000	20	4.6

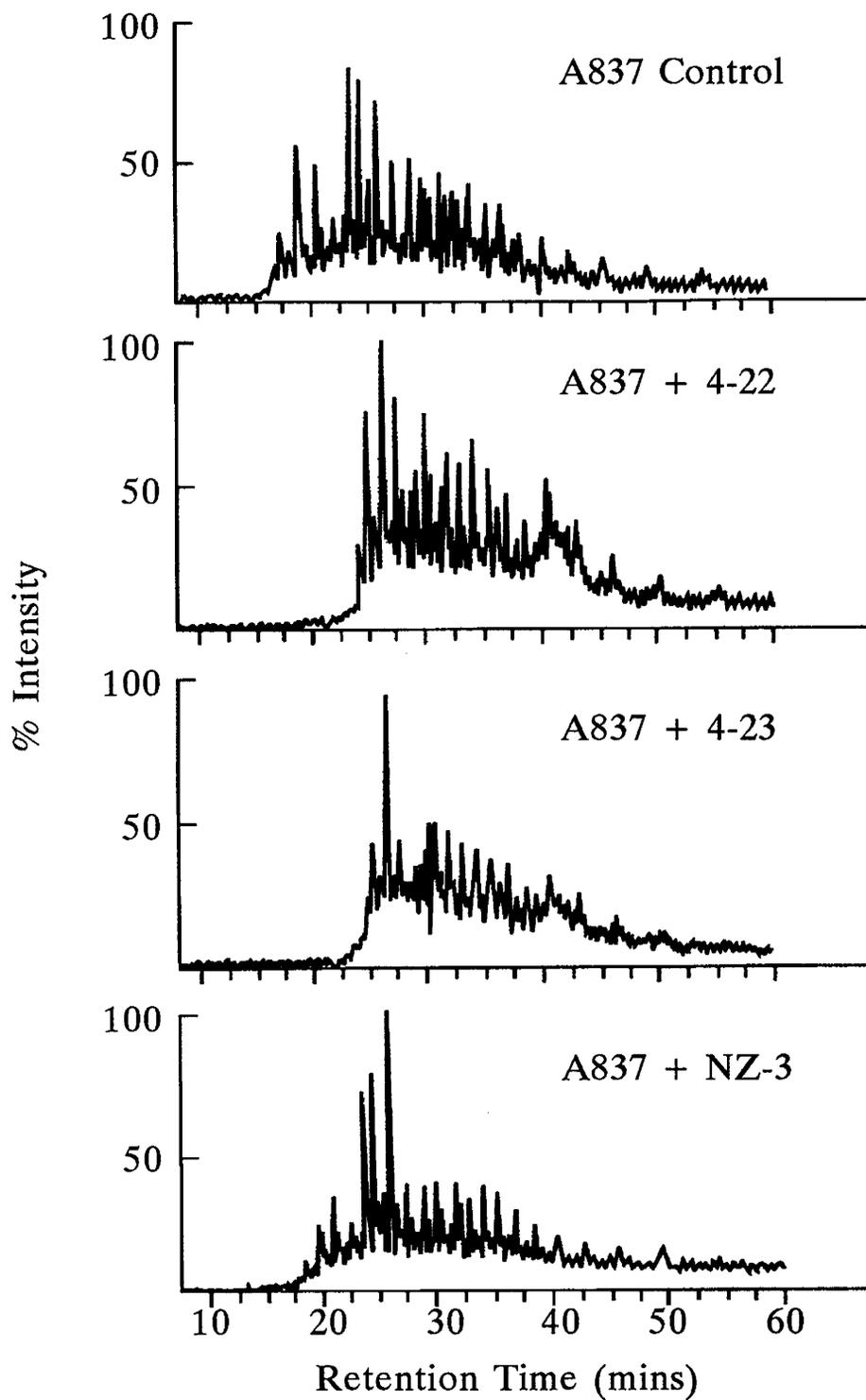


Figure 53. GC-MS Fragmentograms, m/z 57, Monterey, CA, crude A837 treated with BNL-4-22, BNL-4-23, and BNL-NZ-3.

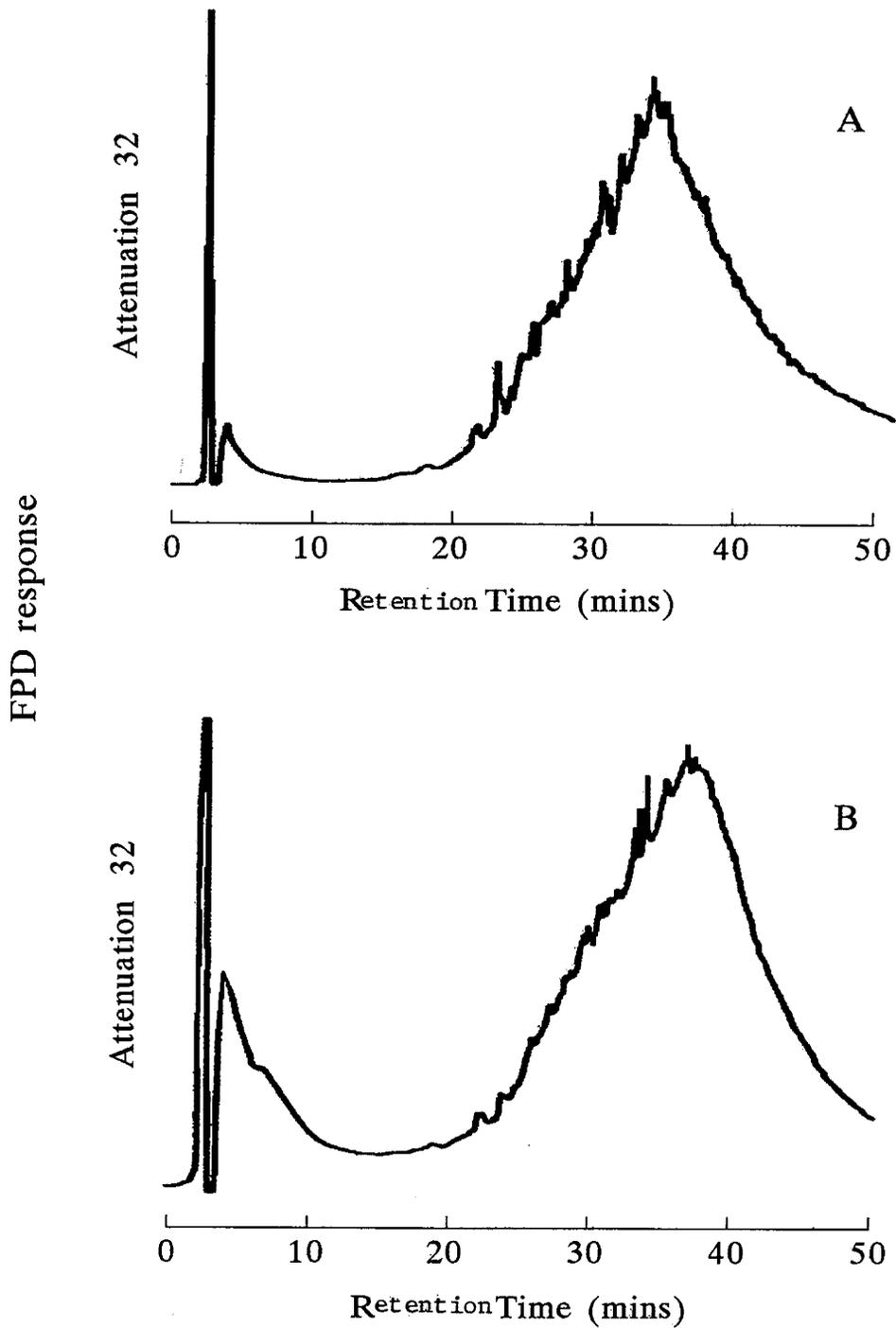


Figure 54. Gas Chromatographic trace with Flame Photoemission Detector (FPD) of, A: Monterey A836 control; B: Monterey A836 treated with BNL-NZ-3. Medium 3, seven days, no Tergitol.

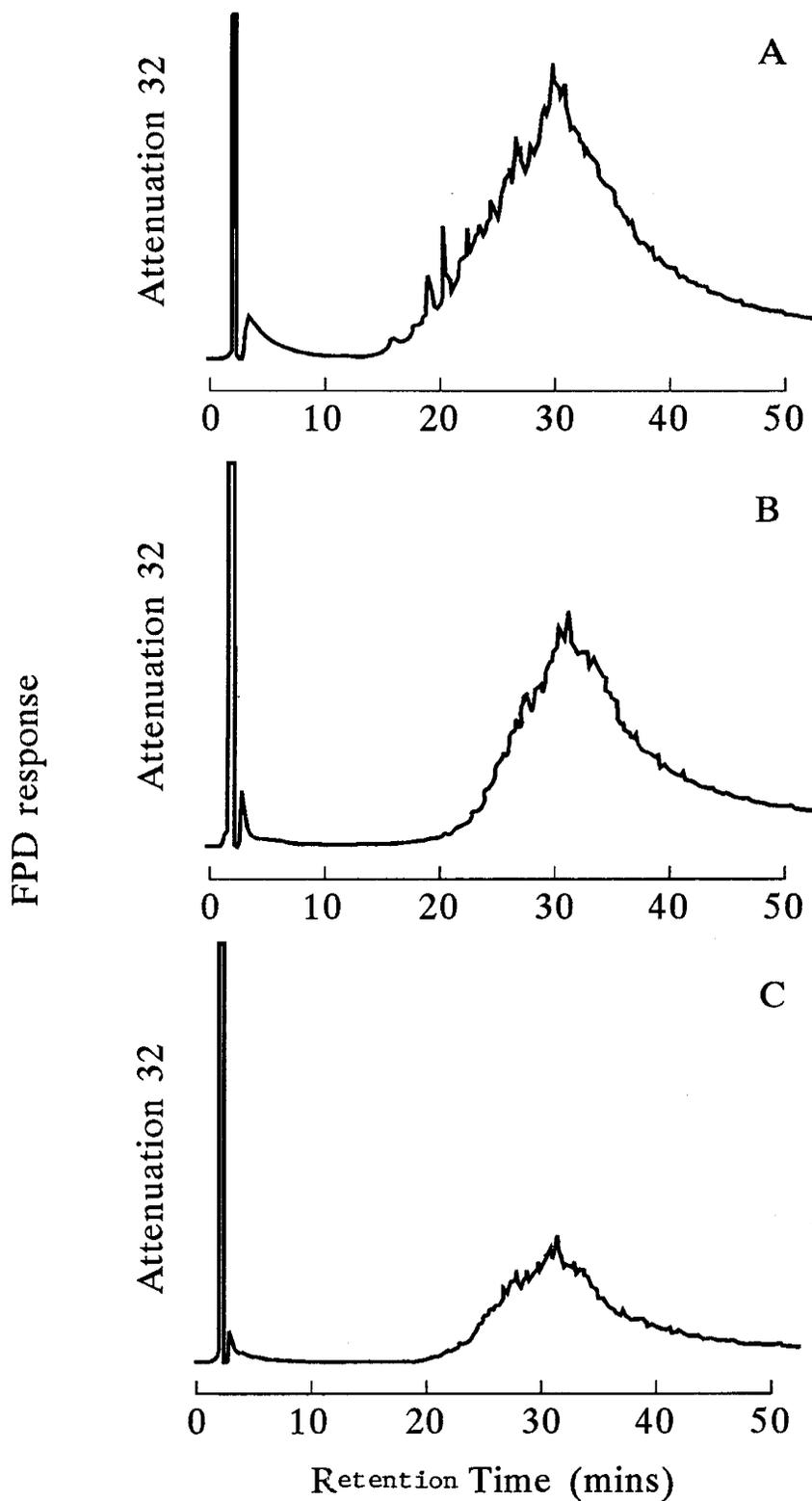


Figure 55. Gas Chromatographic trace with Flame Photoemission Detector (FPD) of, A: Monterey A837 control; B: Monterey A837 treated with BNL-4-22; C: Monterey A337 treated with BNL-NZ-3. In Medium 3, for seven days, in the absence of Tergitol.

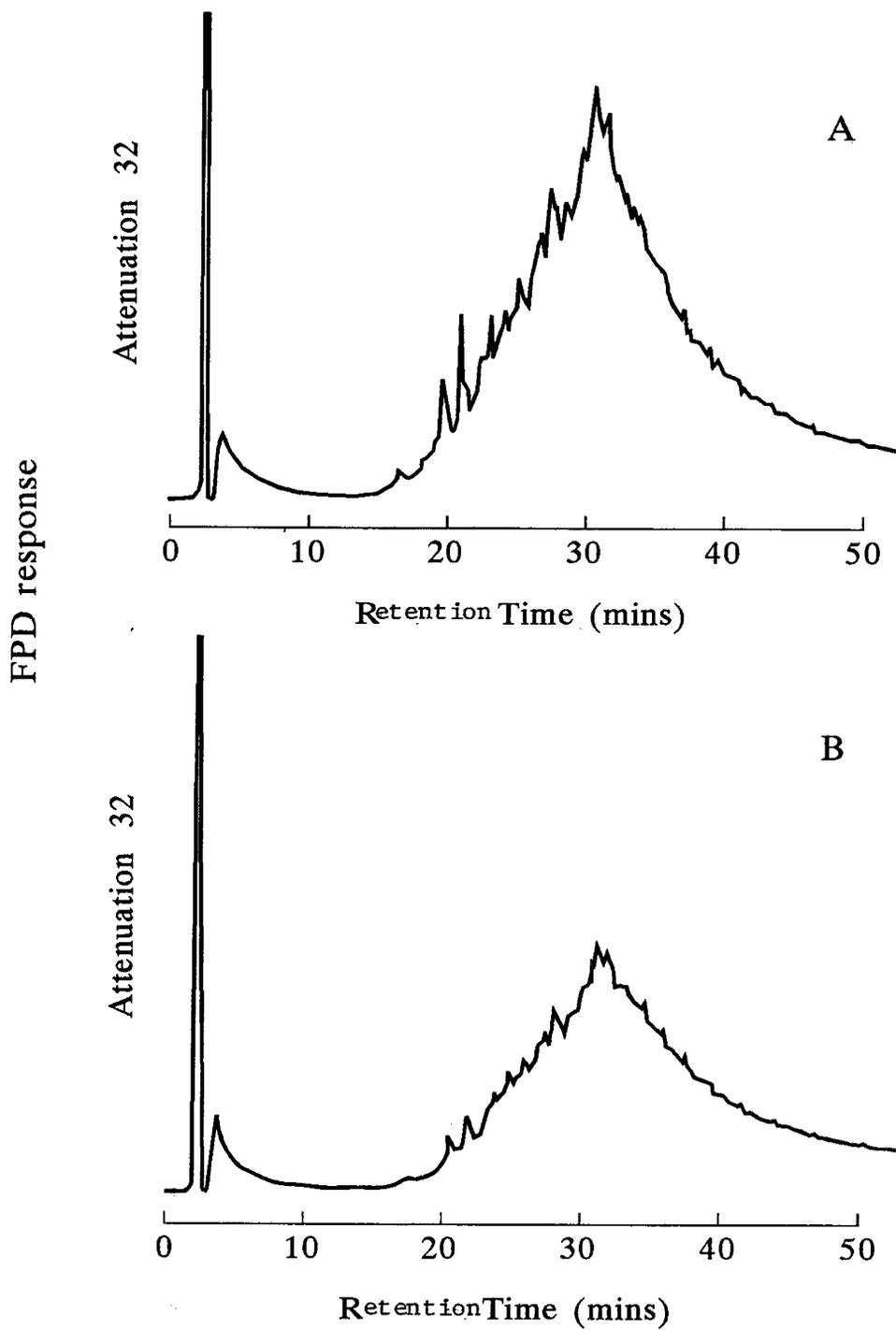


Figure 56. Gas Chromatographic trace with Flame Photoemission Detector (FPD) of, A: Monterey A837 control; B: Monterey A837 treated with BNL-NZ-3. Medium 3, seven days, no Tergitol.

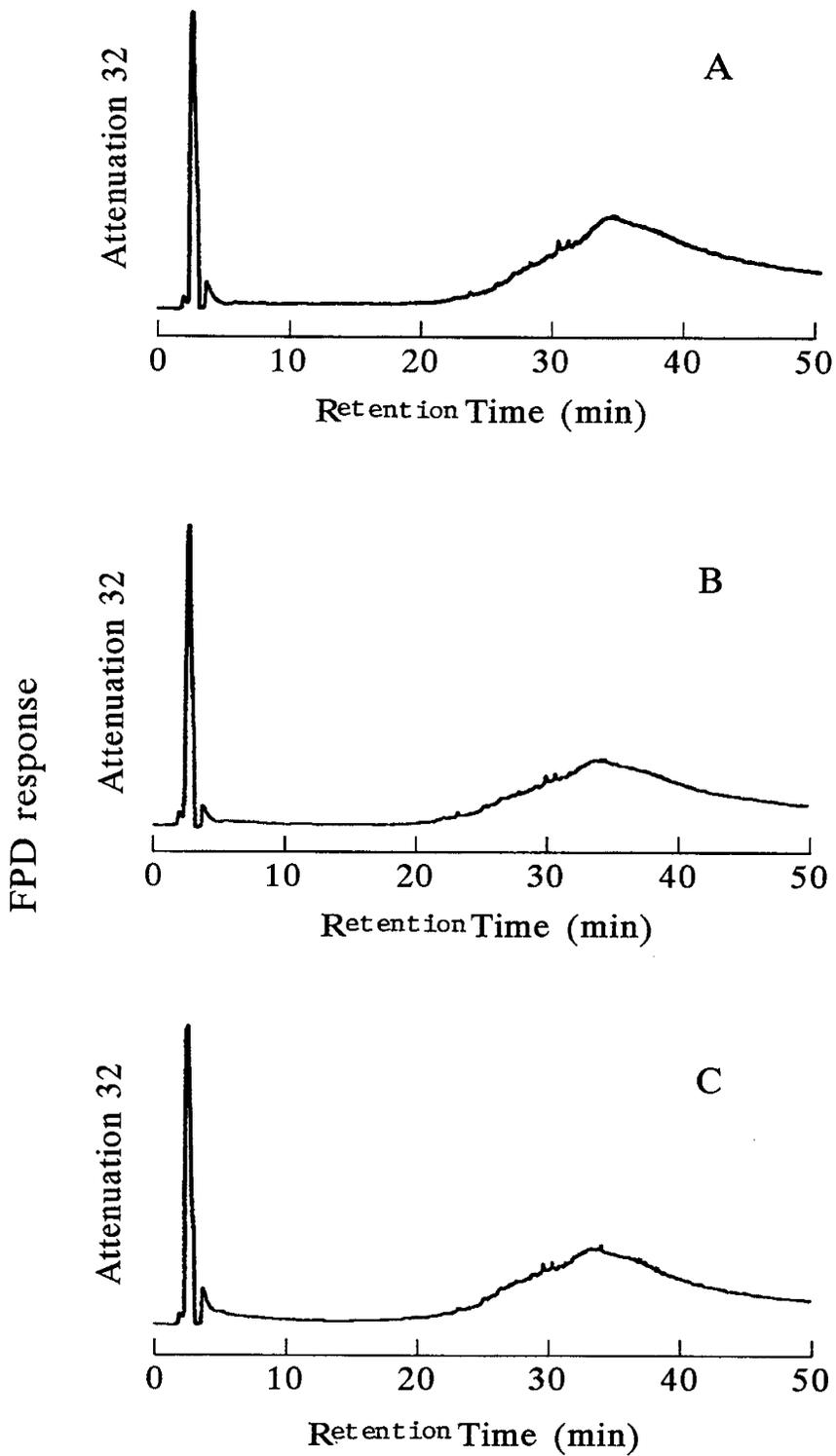


Figure 57. Gas Chromatographic trace with Flame Photoemission Detector (FPD) of, A: Control; B: Monterey A851 + BNL-4-22; C: Monterey A851 + BNL-4-23. Medium 3, seven days, no Tergitol.

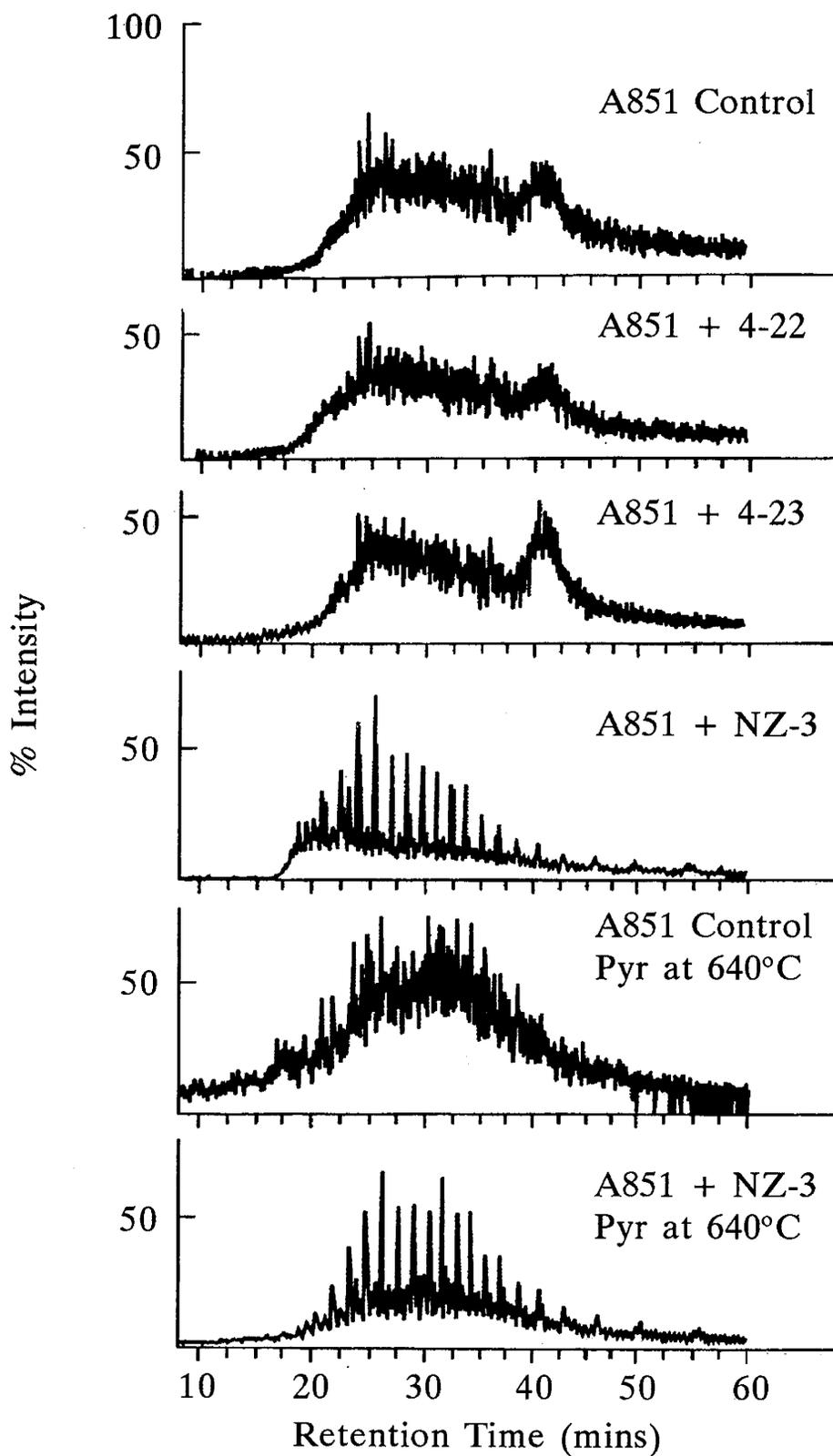


Figure 58. GC-MS Fragmentograms, m/z 57, Monterey, CA, crude A851 (1) treated with BNL-4-22, BNL-4-23, and BNL-NZ-3 and (2) Control and Pyrolyzed Control and BNL-NZ-3 at 640°C.

Further biochemical evidence which supports a mechanism or mechanisms by which microorganisms may interact with crude oils by converting the complex N, S, O structures is offered by a comparative chromatographic analysis of organosulfur compounds present in Monterey A836, A837, and A851 before and after biotreatment. Biotreatment of Monterey A836 with BNL-4-22 and BNL-4-23 results in changes of organosulfur composition, which differ to those due to the biotreatment of A836 with BNL-NZ-3 as shown in Figure 54. Relative to control, there appear to be fine structural compositional changes without an overall decrease in organic sulfur content. However, biotreatment of Monterey A837 with BNL-4-22, BNL-4-23, and BNL-NZ-3 under identical experimental conditions results in significant overall changes in hydrocarbon distribution and decrease in organic sulfur content as shown in Figures 55 and 56 with a small effect in A851 as shown in Figure 57. In this series of experiments, the Monterey A851, an onshore highly biodegraded California crude oil, was considered a test case in terms of differentiation of "biotreated" and "biodegraded" crude oil. Biotreatment of this crude oil further indicated the importance of "diagnostic" evaluation of the biotreatment, i.e., an initial small-scale evaluation of the microorganisms potential for the biochemical conversion of the oil. All the experiments referred to in this discussion were run under identical experimental conditions (e.g. sample treatment, concentration, and chromatographic conditions). Thus, treatment of A851 with BNL-4-22 and BNL-4-23 had no effect as shown in Figure 58. However, treatment of A851 with BNL-NZ-3 resulted in major changes in the composition of A851 also shown (Figure 58). This thermophilic isolate breaks down A851 into a much more volatile hydrocarbon mixture with a significant increase in the lower molecular weight $<C_{30}$ hydrocarbons. The corresponding FPD gas

chromatographic trace shown in Figure 59 is consistent and offers additional support for the proposed mechanism(s) that the initial biochemical reactions may occur in the heavy N, S, O portions of asphaltenes. A qualitative test for the bioconversion of the crude in terms of the sulfur heteroatom is offered by a comparison of the two FPD scans in Figure 59. In order to plot the FPD traces in a manner consistent with previous analyses, the sensitivity of the chromatograph (i.e., attenuation) was set at 32. At that setting, the response was off-scale (Figure 59(A)) and the attenuation had to be reduced to 64 or by 50% (Figure 59(B)) to get a full response on that scale.

Pyrolysis of A851 at 640°C (Figure 58) produced a volatile fraction with some enrichment in the lighter ends of the crude, however, not very effective when compared to biotreatment. Pyrolysis at 640°C of the BNL-NZ-3 treated oil produced a hydrocarbon product whose GC-MS fragmentogram compares to the non-pyrolyzed one (Figure 58), however, with a loss of very light fractions in the C₁₀ - C₁₈ scan region, consistent with the GC-MS results.

Similar trends in the biochemical effects due to microbial treatment of crudes have been observed with other types of oils, two of which will be discussed in the next few paragraphs.

An Arkansas (°API=20, S=4.2%) and an Alabama crude (°API=19, S=4.6%) were treated in an analogous manner to those used for the California oils. The results of tests with the Arkansas crude are given in Tables 27, 28 and 29 and in Figures 60 and 61. In the presence of Tergitol, BNL-4-23 appears to be an efficient bioconverter over a period of seven days. The controls are oil + medium + detergent (oil-C), oil + medium (control-C), and organisms + medium + Tergitol (BNL-#-C). In the absence of Tergitol, BNL-4-23 appears to be a weaker emulsifier and requires a longer period of time to be effective.

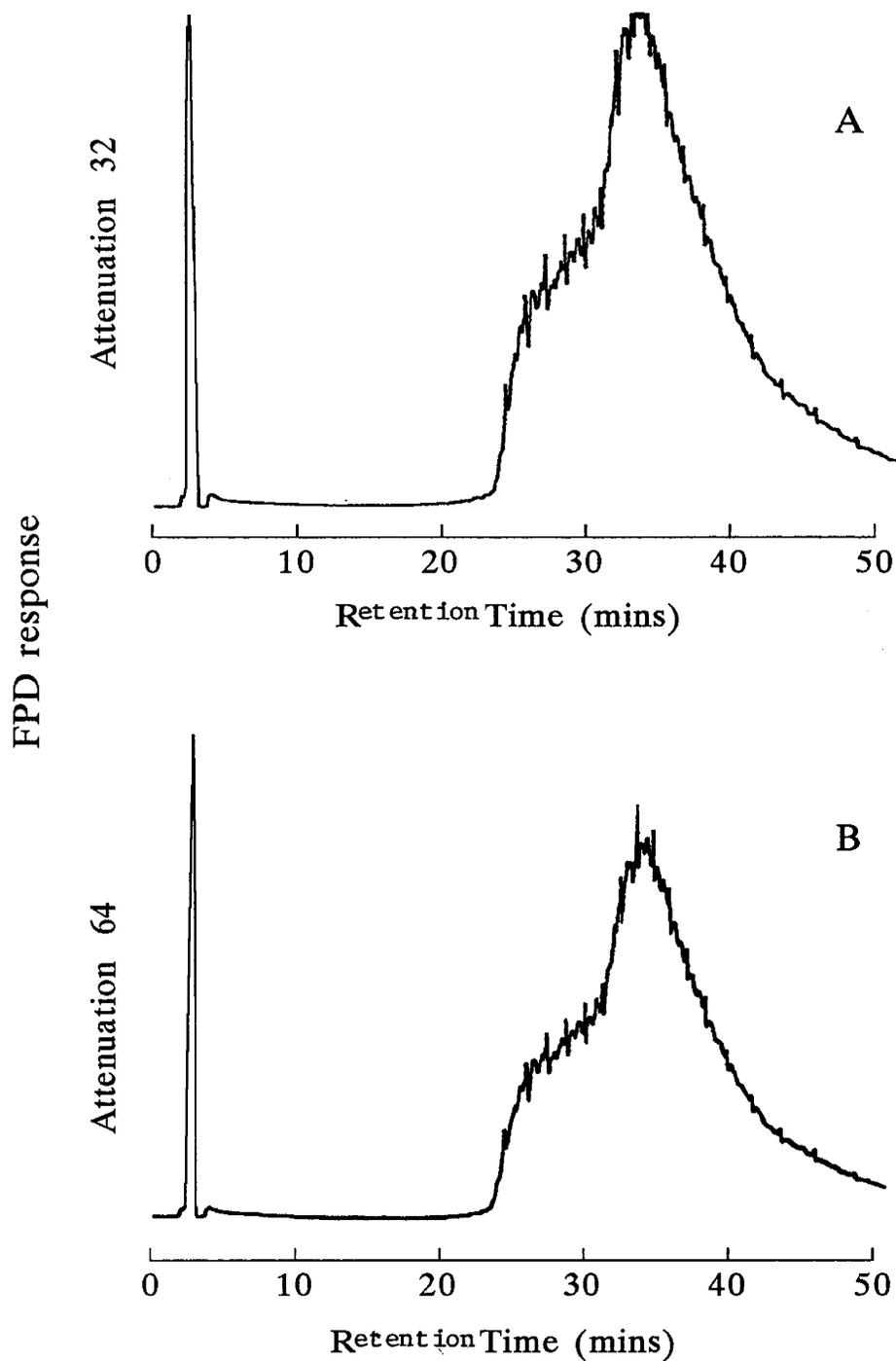


Figure 59. Gas Chromatographic trace with Flame Photoemission Detector (FPD) of Monterey A851 crude treated with BNL-NZ-3. Medium 3, seven days, no Tergitol. Figure 59(A) shows a scan at attenuation of 32 and 59(B) at attenuation 64 (see text for discussion).

Table 27. Biotreatment of Arkansas B70116 Crude in Medium 3 in the Presence of a Detergent (Tergitol) Over a Period of Seven and Twenty Days.

Microorganisms	Medium	%Oil	Incubation (Days)	Klett Units (OD x 500)	pH
BNL-4-22	Medium 3	0.497	7	17.300	4.00
BNL-4-22-Control	Medium 3	0.000	7	51.800	4.25
BNL-4-23	Medium 3	0.513	7	142.000	4.00
BNL-4-23-Control	Medium 3	0.000	7	61.950	4.00
Control, Oil	Medium 3	0.494	7	27.850	4.00
Medium/Control	Medium 3	0.000	7	10.500	4.25
BNL-4-22	Medium 3	0.516	20	43.000	3.50
BNL-4-22 Control	Medium 3	0.000	20	26.000	3.50
BNL-4-23	Medium 3	0.518	20	61.500	3.50
BNL-4-23 Control	Medium 3	0.000	20	15.000	3.50
Control, Oil	Medium 3	0.510	20	15.500	3.50
Medium/Control	Medium 3	0.000	20	19.500	3.50

Table 28. Biotreatment of Arkansas B70116 Crude in Medium 3 in the Absence of a Detergent Over a Period of Seven and Twenty Days.

Microorganisms	Medium	%Oil	Incubation (Days)	Klett Units (OD x 500)	pH
BNL-4-22	Medium 3	0.519	7	5.000	3.50
BNL-4-23	Medium 3	0.588	7	0.500	3.50
Control, Oil	Medium 3	0.500	7	0.000	3.50
BNL-4-22 Control	Medium 3	0.000	7	5.000	3.50
BNL-4-23 Control	Medium 3	0.000	7	0.500	3.50
Medium/Control	Medium 3	0.000	7	0.000	3.50
BNL-4-22	Medium 3	0.489	20	38.750	4.25
BNL-4-23	Medium 3	0.479	20	54.595	4.25
Control, Oil	Medium 3	0.526	20	13.625	4.25
BNL-4-22 Control	Medium 3	0.000	20	14.595	4.25
BNL-4-23 Control	Medium 3	0.000	20	5.365	4.00
Medium/Control	Medium 3	0.000	20	9.710	4.00

Table 29. Arkansas B70116 Crude Biotreated with BNL-NZ-3.

Microorganisms	Detergent	Medium	%Oil	Incubation (Days)	Klett Units (OD x 500)	pH
BNL-NZ-3	No	Medium 3	0.532	7	3.16	4.5

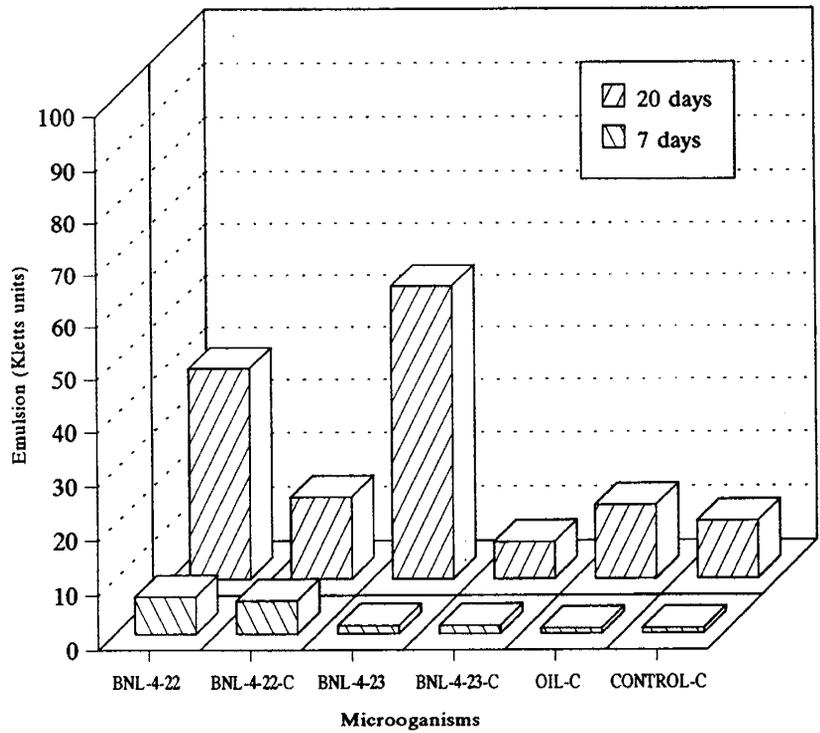


Figure 60. Arkansas Oil B70116 Biotreatment
7 vs 20 Days; Media 3 - Tergitol

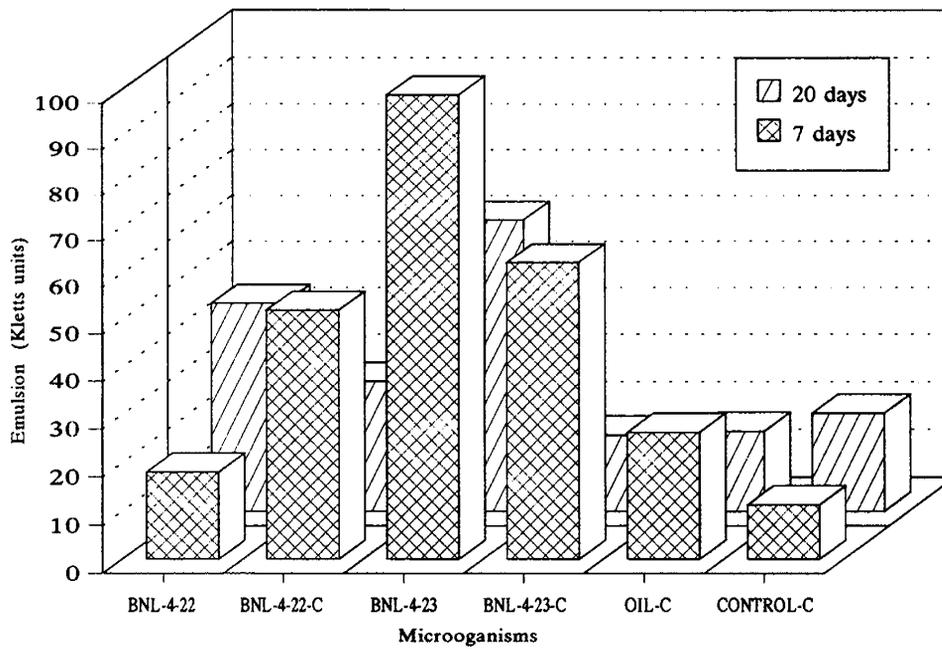


Figure 61. Arkansas Oil B70116 Biotreatment
7 vs 20 Days; Media 3 + Tergitol

However, Gas Chromatography-Mass Spectrometry (GC-MS) analyses and the corresponding gas chromatography analyses for organosulfur compounds indicate little or no change due to the biotreatment as shown in Figures 62 and 63, although relative to the control there is some decrease in the overall content of organosulfur components of the crude. These results may be interpreted in the following manner. Biotreatment of Arkansas crude with BNL-4-22 and BNL-4-23 produces emulsification and some small changes in the sulfur content representative of an overall redistribution of hydrocarbons within the major fractions. To explore further the feasibility of the occurrence of such processes, a sample of Arkansas crude was treated with BNL-NZ-3, an efficient bioconverter of Monterey crude, particularly A851. The results are presented in Table 29 and Figures 64 and 65. Thus, GC-MS analysis shows small changes in the hydrocarbon envelope, particularly evident in the expanded (Figure 65) region, and even more evident in the FPD chromatograms shown in Figure 66. Analogous experiments with Alabama crude support the view that biotreatment may cause in some oils significant changes in the hydrocarbon matter as well as decreases in the heterocyclic contents of the organic phase, or it may cause smaller changes due to inter- and intra-molecular rearrangements leading to re-distribution of the crude oil components such as those observed in the bioconversion of the Alabama and Arkansas crudes.

Thus, the results of emulsification studies using Alabama B69112 crude oil given in Table 30 and Figure 67 show effects of BNL-4-22, BNL-4-23, and BNL-NZ-3 to be lower than on other crudes tested so far, the overall trends are consistent: emulsification is achieved with the crude oil being the sole carbon source. Similarly, changes in organic sulfur content and sulfur speciation are also evident as shown in Figures 68 and 69, and Table 31. The small chemical changes that occur in biochemical conversion of the Alabama

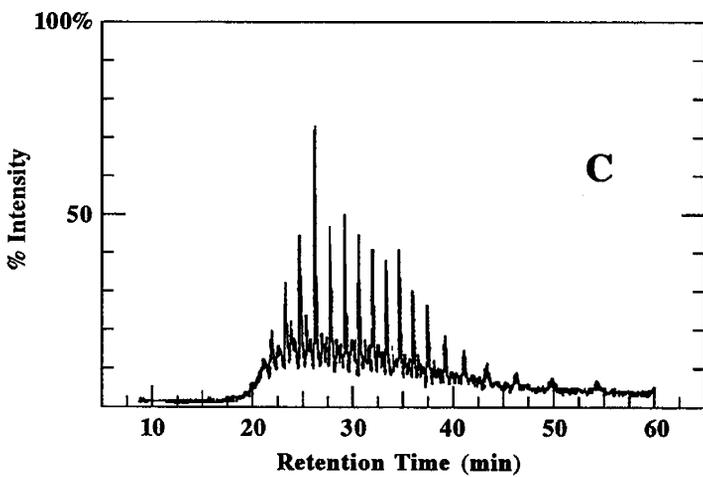
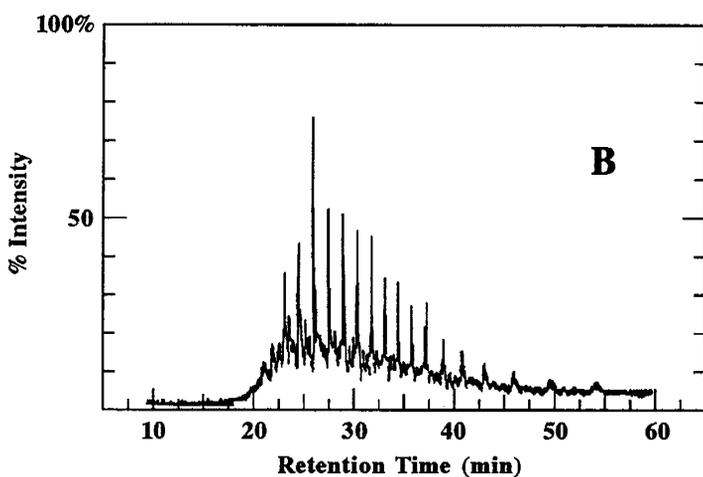
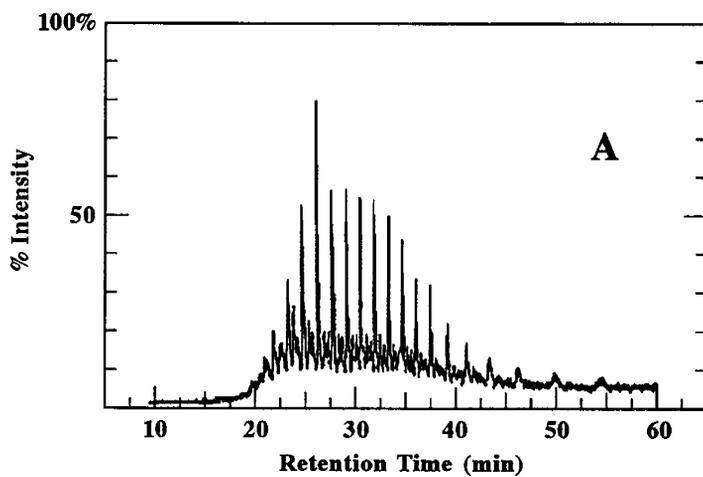


Figure 62. GC-MS analyses of Arkansas B70116, A: Control; B: treated with BNL-4-22; C: treated with BNL-4-23.

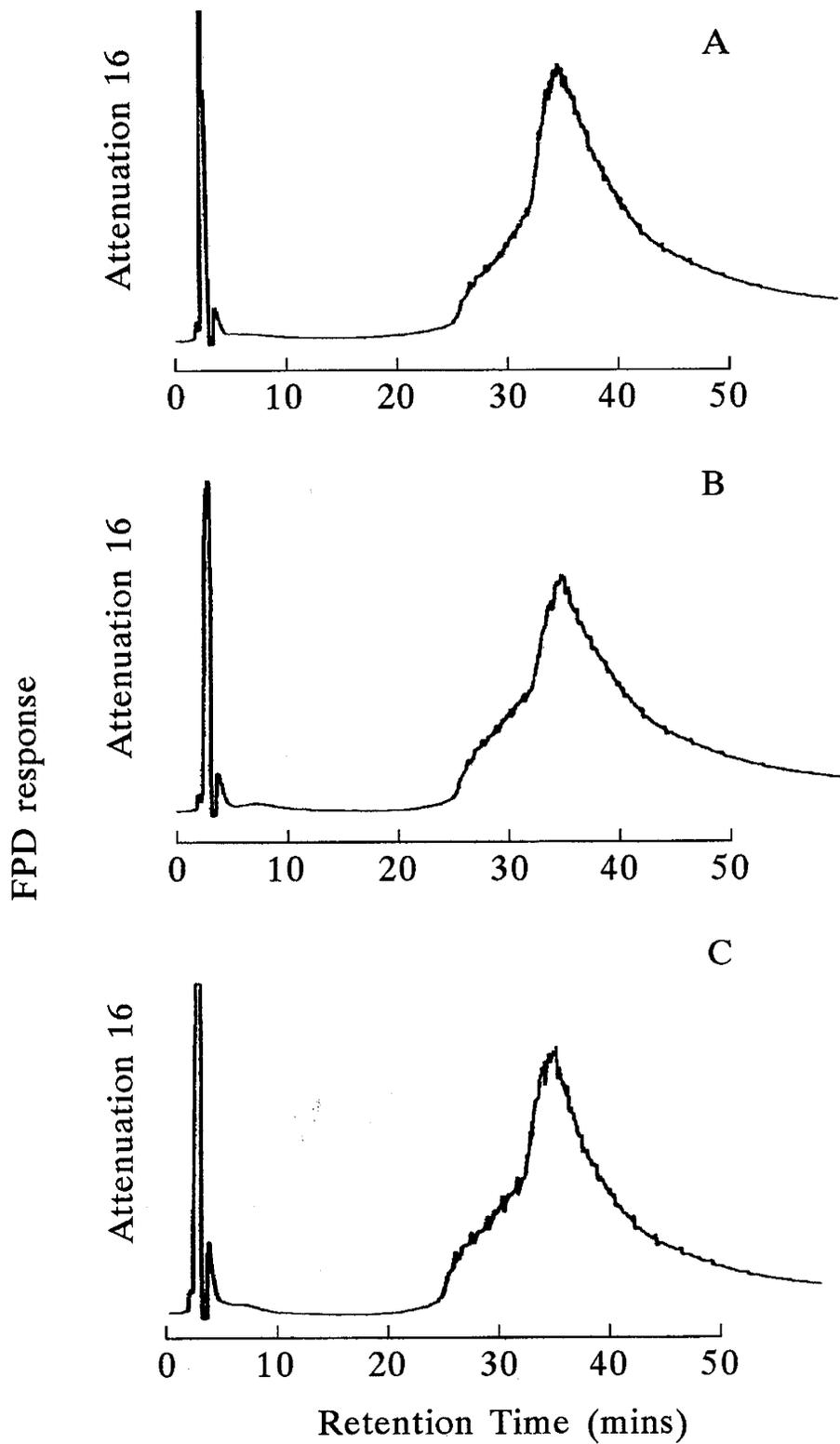


Figure 63. Chromatograms, Flame Photoemission Detector (FPD) trace of Arkansas B70116, A: Control; B: treated with BNL-4-22; C: treated with BNL-4-23.

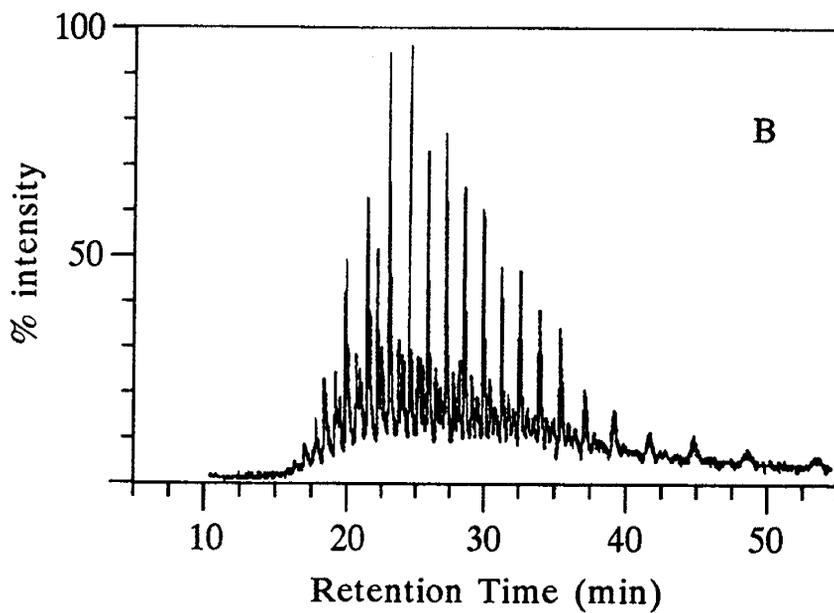
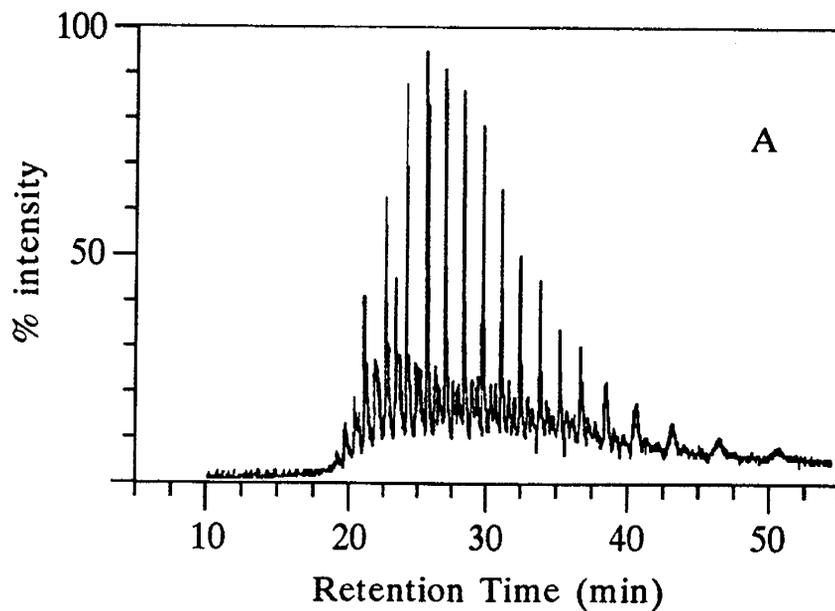


Figure 64. Arkansas B70116 Gas Chromatographic-Mass Spectrometry (GC-MS) fragmentograms, A: Control; B: treated with BNL-NZ-3.

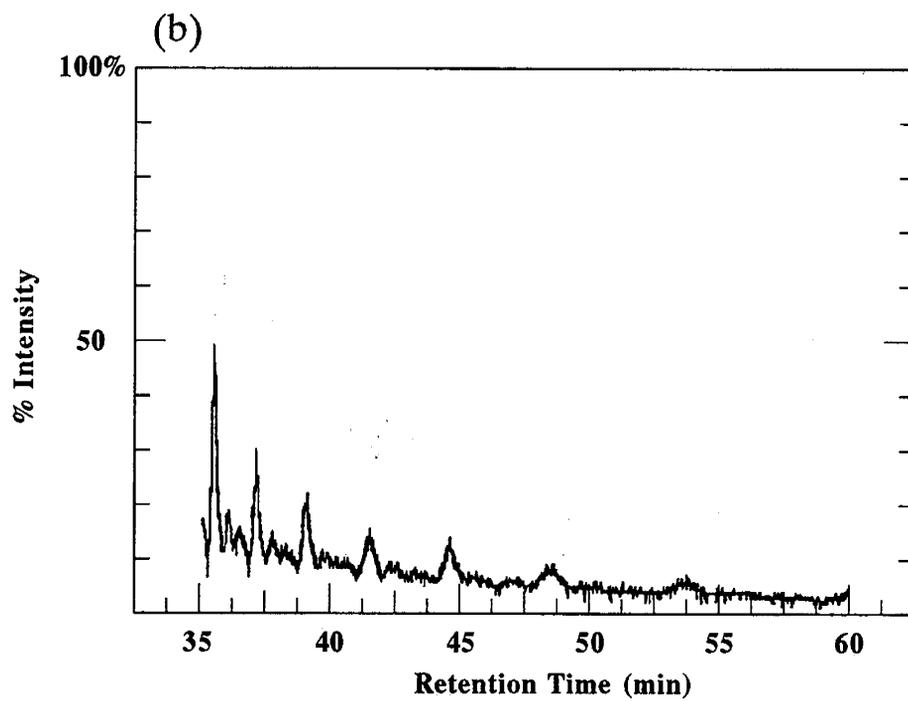
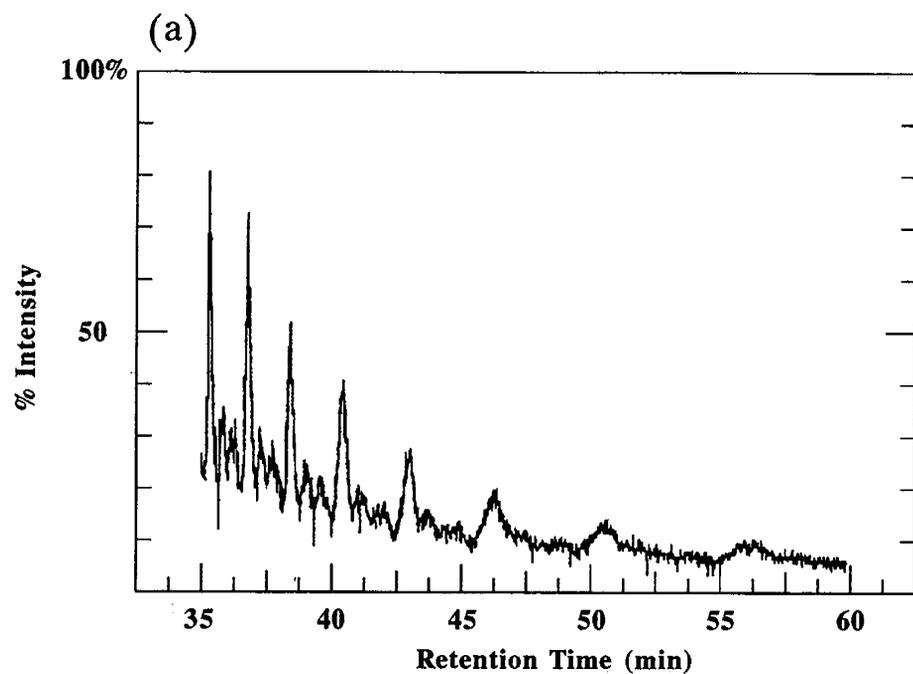


Figure 65. Arkansas B70116 Gas Chromatographic-Mass Spectrometry (GC-MS) fragmentograms expanded scans (2100-3600). A: Control; B: treated with BNL-NZ-3.

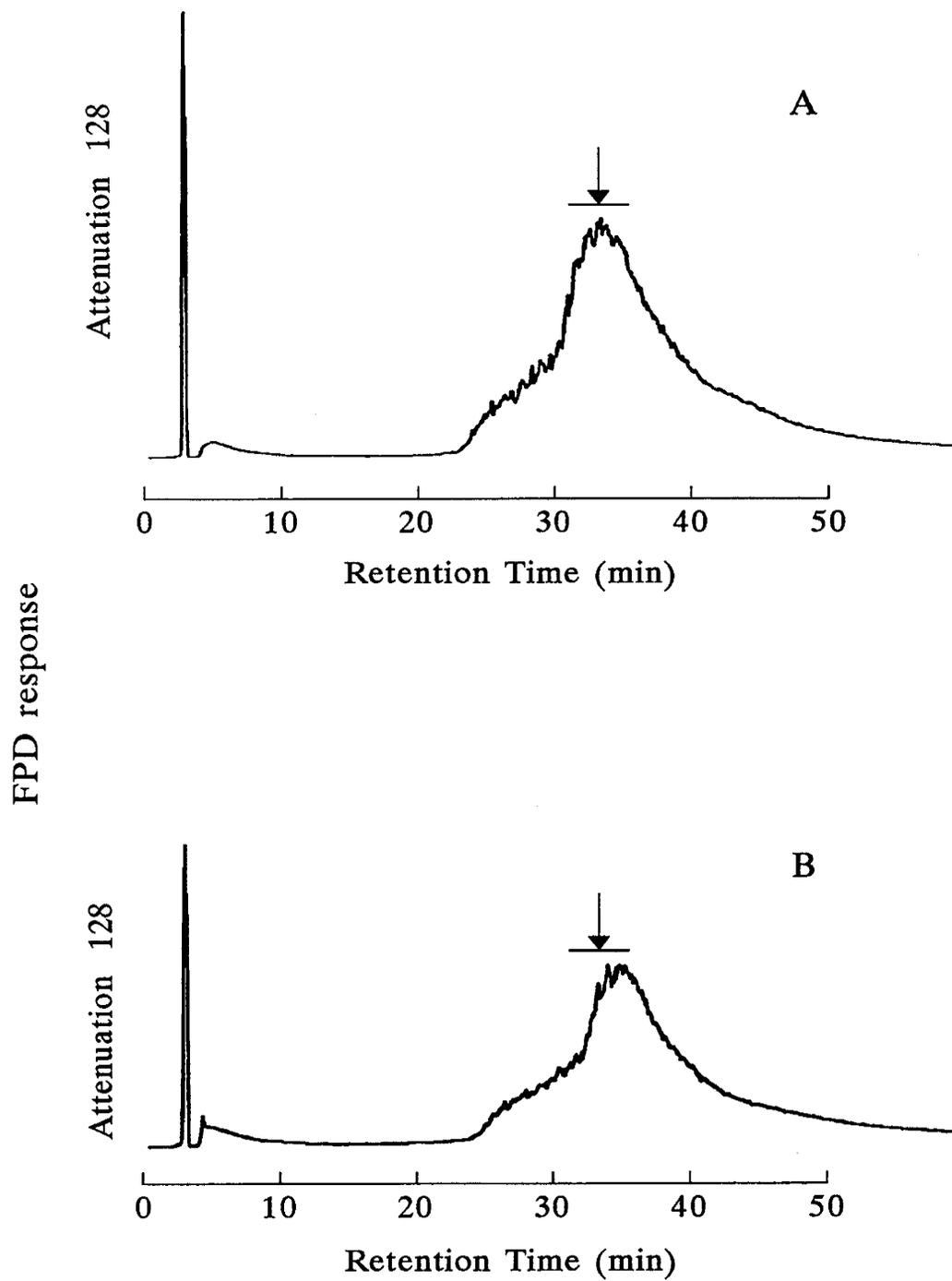


Figure 66. Chromatograms, Flame Photoemission Detector (FPD) trace of Arkansas B70116, A: Control; B: treated with BNL-NZ-3; seven days, medium 3, no Tergitol.

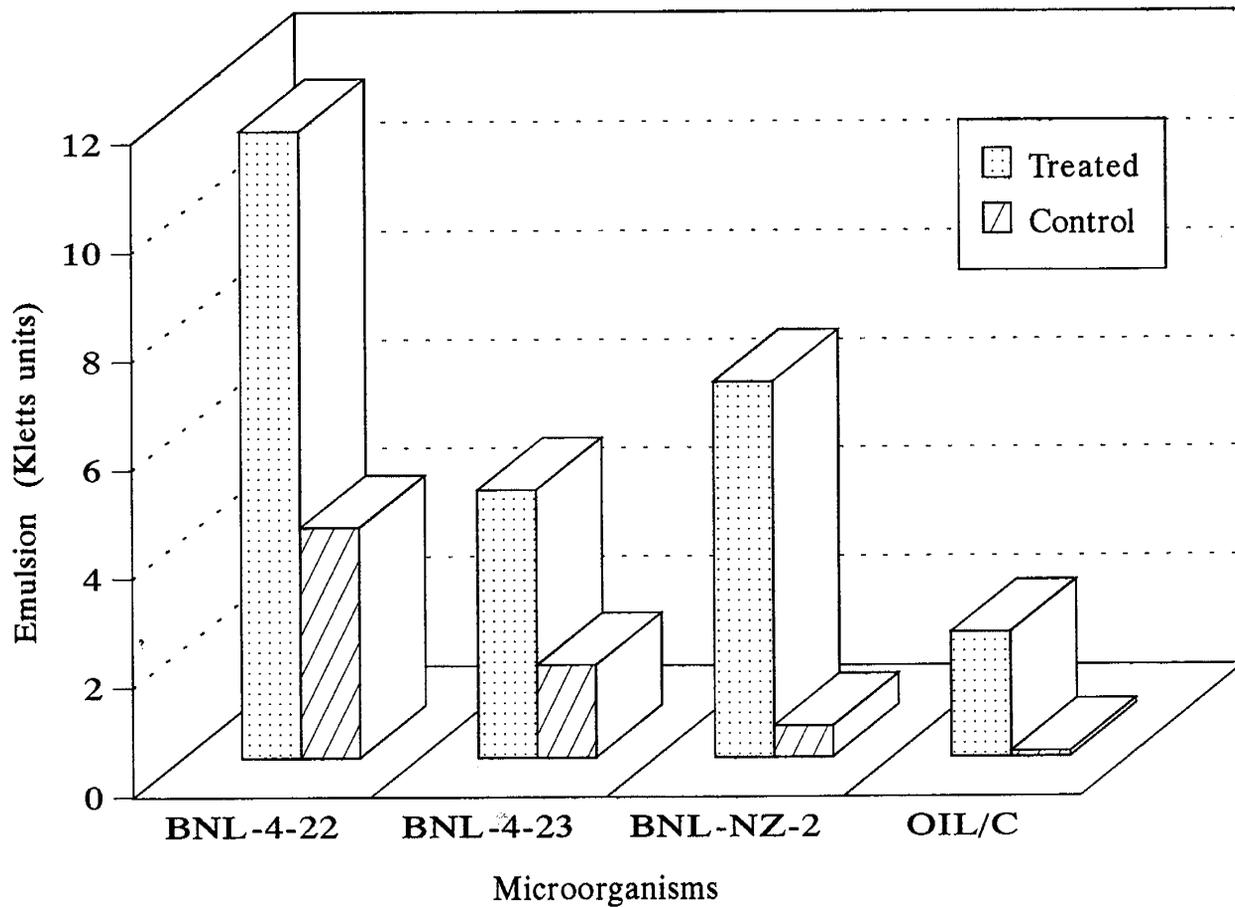


Figure 67. Alabama oil B69112 biotreatment; 7 days; Media 3.

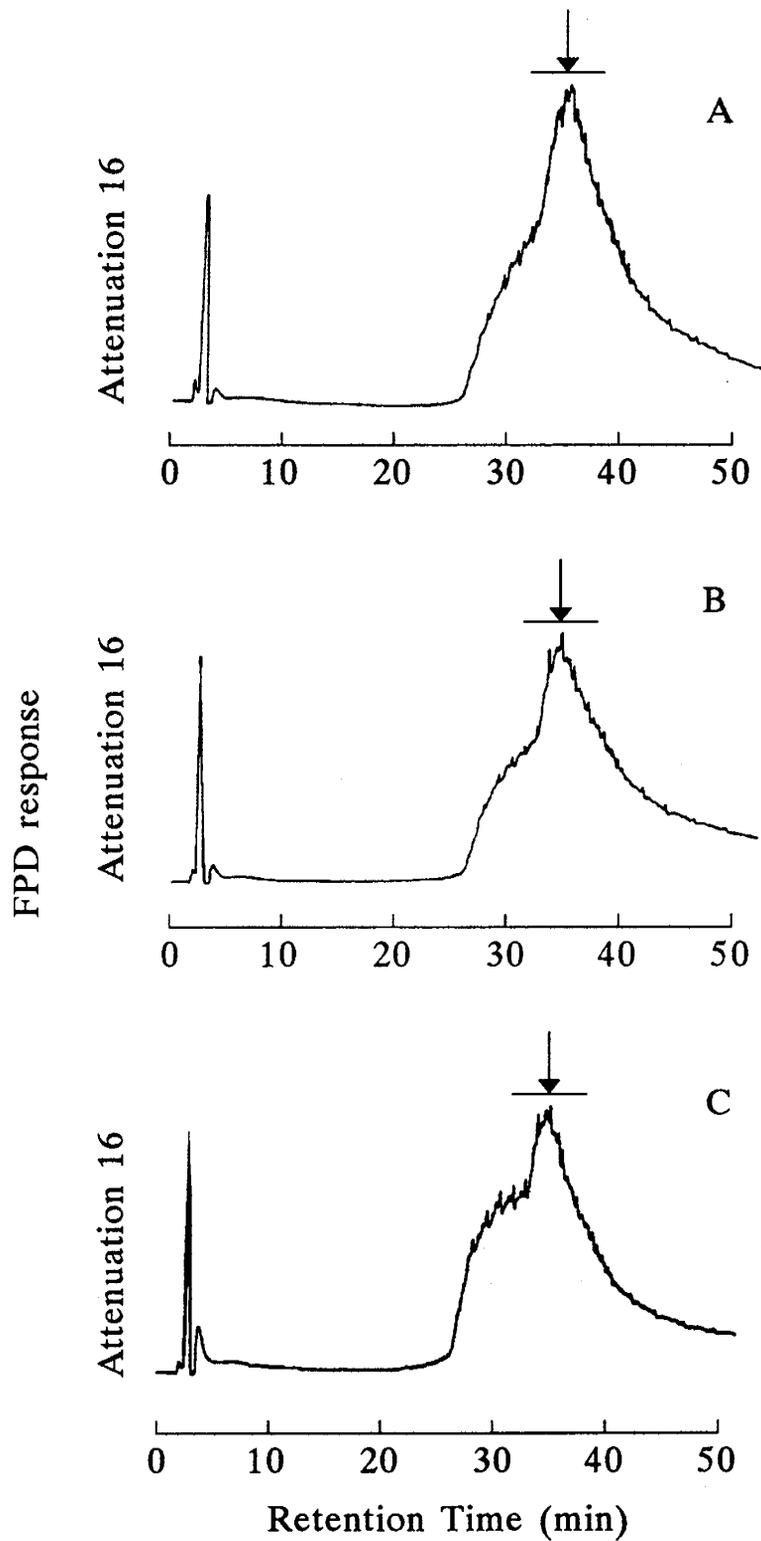


Figure 68. Flame Photoemission Detector (FPD) traces of Alabama B69112 crude oil. A: Control; B: treated with BNL-4-22; C: treated with BNL-4-23.

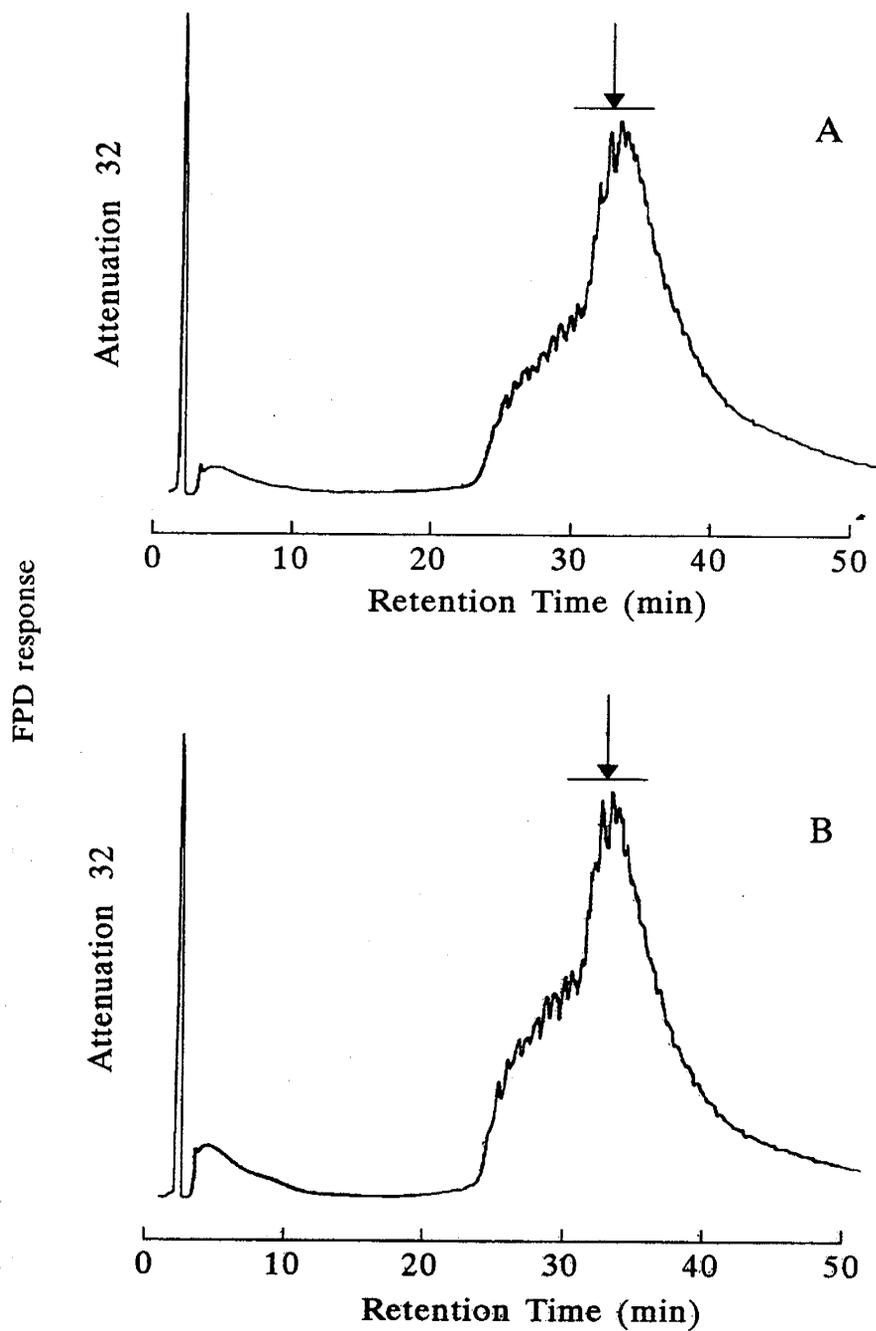


Figure 69. Gas Chromatograms, Flame Photoemission Detector (FPD) trace of Arkansas B69112 crude oil. A: Control; B: Treated with BNL-NZ-3.

Table 30. Alabama Oil B69112 Biotreatment*

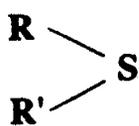
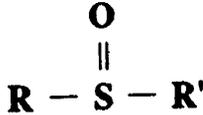
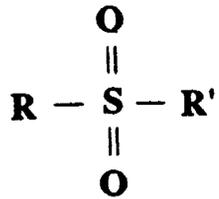
MICROORGANISM	DETERGENT	MEDIA	VOLUME	XOIL	INCUBATION	OPTICAL DENSITY	KLETT UNITS	pH
BNL-4-22	NO	Media 3	125	0.500	7	0.0229	11,473	4.5
BNL-4-23	NO	Media 3	125	0.517	7	0.0097	4,828	4.5
BNL-NZ-3	NO	Media 3	125	0.476	7	0.0137	6,835	5.25
Control, Oil	NO	Media 3	125	0.494	7	0.0043	2.16	4.38
BNL-4-22 Control	NO	Media 3	125	0.000	7	0.009	4.25	4.5
BNL-4-23 Control	NO	Media 3	125	0.000	7	0.003	1.658	4.5
BNL-NZ-3 Control	NO	Media 3	125	0.000	7	0.001	0.505	5.5
Medium/Control	NO	Media 3	125	0.000	7	3.2E-05	0.016	4.38
BNL-4-22	NO	Media 3	125	0.543	20	0.2084	104	3.75
BNL-4-23	NO	Media 3	125	0.487	20	0.0202	10	4.00
CONTROL, OIL	NO	Media 3	125	0.490	20	0.0109	5	4.00
BNL-4-22 Control	NO	Media 3	125	0.000	20	0.0292	15	4.25
BNL-4-23 Control	NO	Media 3	125	0.000	20	0.0107	5	4.00
Medium/Control	NO	Media 3	125	0.000	20	0.0194	10	4.00

*Averages of triplicate experiments.
Control, Oil = oil and medium only.
Medium/Control = oil + medium.

crude when treated with BNL-4-22, BNL-4-23, and BNL-NZ-3 are also reflected in their gas-chromatographic-mass spectromic (GC-MS) characteristics as well as their XANES analysis as shown in Table 31.

A redistribution of organosulfur species and a decrease of heavier fractions is also consistent with the GC-FPD traces in the region of the chromatograms indicated by the arrows in Figures 66, 68, and 69. The Alabama oils are derived from reservoirs in the upper part of the Smackover Formation (Jurassic) and are known to have been subject to transformations (Claypool and Mancini, 1989), which may have led to chemically distinct types of oils differing from all the others tested at BNL. Therefore, it is possible that the three species of microorganisms under the experimental conditions used, may not be the best choices and/or optimum conditions for these particular types of oil. This possibility should be further explored. However, the overall experimental observations discussed in this section allow us to conclude that trends in the biochemistries of interaction between microorganisms and crude oils are not random and follow distinct paths. Further, some

Table 31. XANES (X-ray Absorption Near Edge Structure) analysis of organic sulfur species in biochemically converted Alabama B69112 crude.

Chemical types of organosulfur structures				
				
	Sulfide	Thiophene	Sulfoxide	Sulfone
crude, control	0.308	0.535	0.130	0.028
crude+BNL-4-22	0.351	0.476	0.132	0.039
crude+BNL-4-23	0.322	0.523	0.129	0.026
crude+BNL-NZ-3	0.301	0.536	0.129	0.033

biosystems are more effective than others and some microbial species are better inducers of some processes than others, such as for example "emulsification" vs. "biocracking" or breakdown of heavy ends of crudes. At this stage of the MEOR mechanistic R&D effort, these phenomenon might best be summarized by a comparison of a detailed study of the emulsification effect of BNL-4-23 on the Monterey A837 California crude. The results shown in Figure 70 are consistent and clearly indicate that in addition to microbial species specificity vs. chemical composition of the crude, there also exists a need to predetermine medium, pretreatment, and duration parameters. Medium 1 and 3 are inorganic media, where in Medium 3, the sulfate has been replaced by a chloride salt. Medium 2 contains 0.08% of added carbon. The experimental scenarios described in Figure 70 suggest that in terms of emulsification for Monterey A837, the best experimental conditions are in the order of E > D > F. Clearly similar comparisons of chemical markers, microorganisms, and geochemistry of fields should lead to a data base suitable for the prediction of the efficiency of biochemical processing of oils in reservoirs.

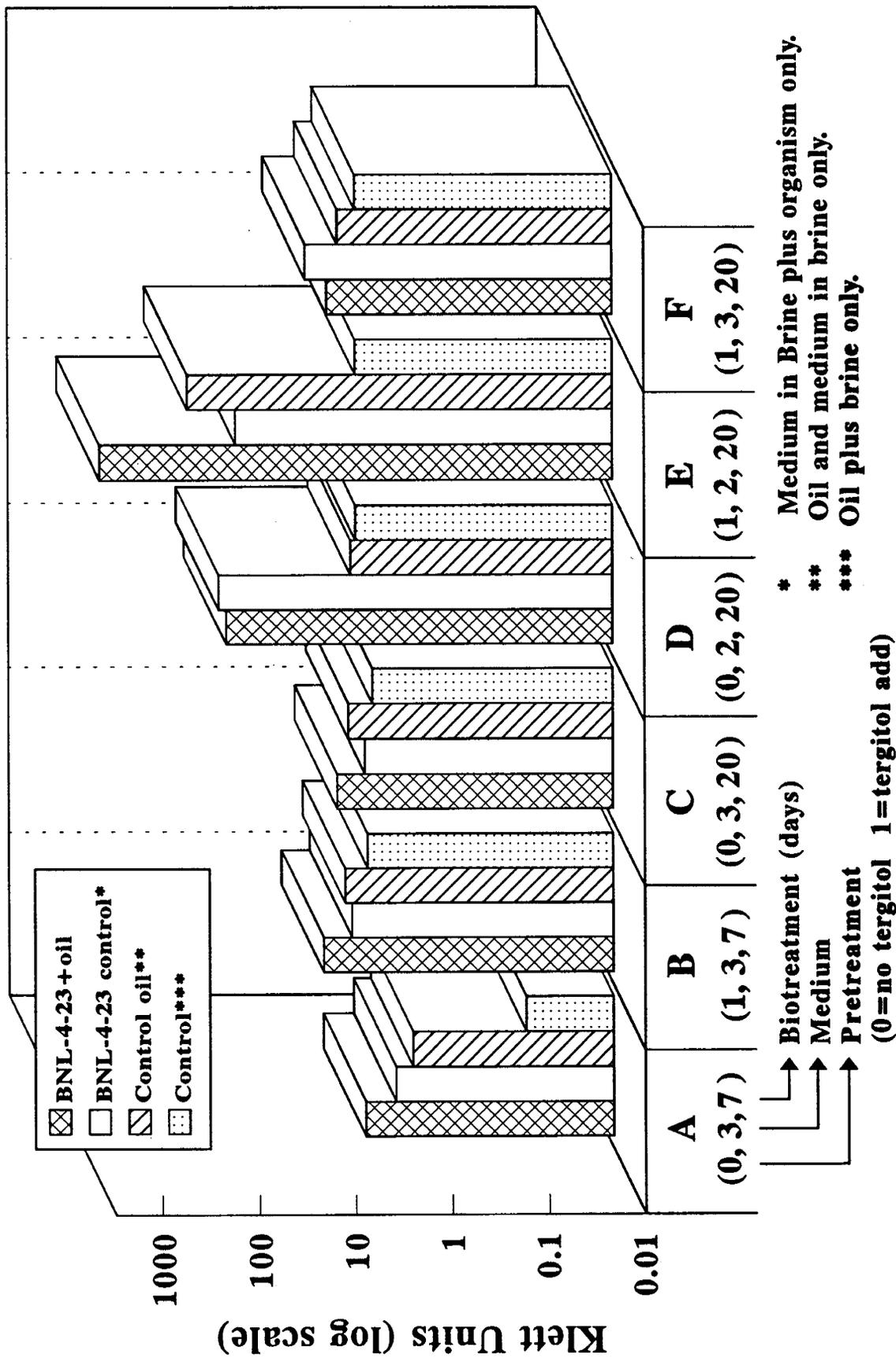


Figure 70. Emulsification effects of BNL-4-23 (includes microorganism + oil + medium in brine) on Monterey A837 crude.

5.5 Some Chemical Properties of the Emulsified Phase

In Section 4.1, several fatty acids present in the aqueous/emulsified phase have been described. These included heptanoic, decanoic, and octadecanoic acids. Organic acids such as lactic, propionic, and isobutyric acids have also been identified. Further exploratory work has identified other hydrocarbons present in the emulsified phase.

Thus, a heavy fraction of the Prudhoe Bay crude treated with BNL-4-24 yielded an aqueous emulsion measuring 215 units on the Klett scale. The emulsion was extracted with chloroform and analyzed by GC/MS. The result shown in Figure 71 indicates that hydrocarbons in the range of C13 to C25 are higher and present in the emulsion. While detailed chemical analyses of the aqueous and the emulsified phases contents after biotreatment have as yet to be carried out, production of long chain aliphatic hydrocarbons with different functional groups from crude oils is a reasonable assumption (Zajic and Mahomed, 1984). It is to be understood that this does not imply that the choice of Tergitol, a hydrocarbon type surface active agent, used routinely in the studies described in this report, is the best one, nor does it imply that only a single surface active agent is produced during the biotreatment of crude oils. Indeed, if the microbial production of detergents from crude oils is consistent with other observations, all of which indicate that after biotreatment the chemical composition of the end product depends on a number of biochemical factors, several chemically distinct classes of detergents which can be biosynthesized from crude oils might be anticipated. This possibility is further supported by the following experiments. Treatment of Wilmington crude with BNL-TAQ-1 yielded an aqueous phase which was chemically processed for derivatization of surfactants and metabolites leading to

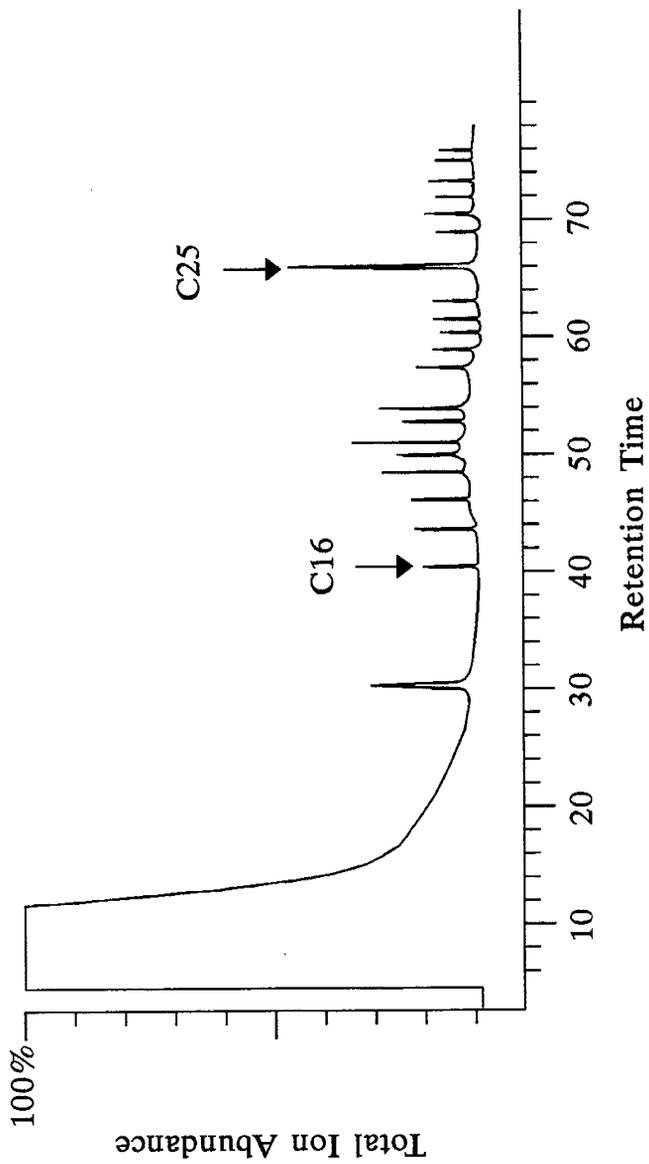


Figure 71. Heavy fraction from Prudhoe Bay crude treated with BNL-4-24, GC/MS trace of the chloroform extracted emulsion.

trimethyl-silyl (TMS) derivatives. The silyl derivatives were then analyzed by gas chromatography/mass spectrometry and marker compounds identified by matching with library data. In addition to already mentioned derivatives (e.g., propionic acid) several aromatic acids, for example, 1,2-phenyldicarboxylic and phenylpropionic acids have been identified. It is worth noting that this is the first time that such compounds have been identified in the aqueous phase under the experimental conditions used in these studies. Typical examples are given in Figures 72 and 73. Further work will identify the chemical nature, as well as the efficiency of other components and surface active agents present in the aqueous phase.

5.6 Microscopic Comparison of Reaction Mixtures

In order to extend our capabilities for gross product characterization and gain a better handle on the possible presence and the effects of indigenous microorganisms, a microscopic examination of several "reaction mixtures" (RM) has been carried out. The results are shown in Figures 74 to 78. Thus, comparison of Figure 74 with Figures 75 and 78 shows that after forty days of biotreatment of Boscan crude in the presence of an emulsifier, the predominant strain is the introduced one. Differences in the effects due to variations in microbial species deliberately introduced and oils used are also apparent in microscopic comparisons as shown in Figures 79 through 83. In view of these preliminary results, concurrent with chemical analysis, a systematic microscopic analysis of RMs was initiated. Preliminary results from these analyses obtained for two Monterey and an Arkansas crude oil are given in Figures 84 through 90. Note, in all of the experiments, Medium 2 was used (i.e., a medium which contains 0.08% of added organic nutrient other than the crude oil) in the presence and the absence of the detergent. Medium 2 was chosen

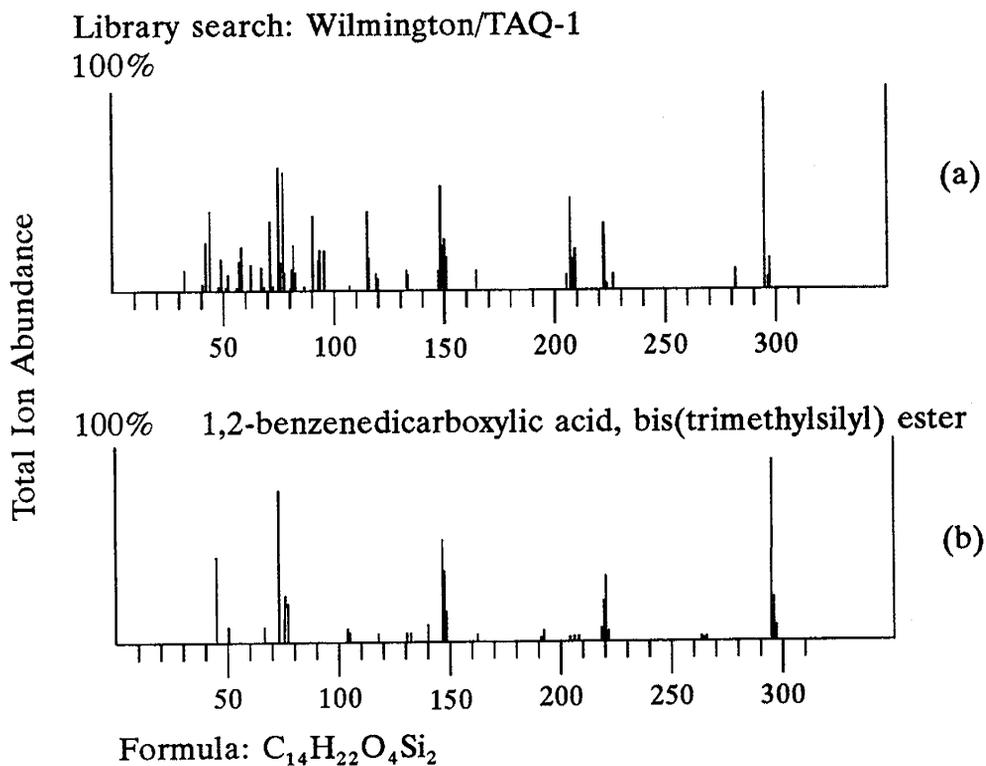


Figure 72. Mass fragmentogram of Wilmington crude BNL-TAQ-1 system TMS derivativized sample (a) and the corresponding library match compound, 1,2-benzenedicarboxylic acid, bis-trimethyl-silyl ester.

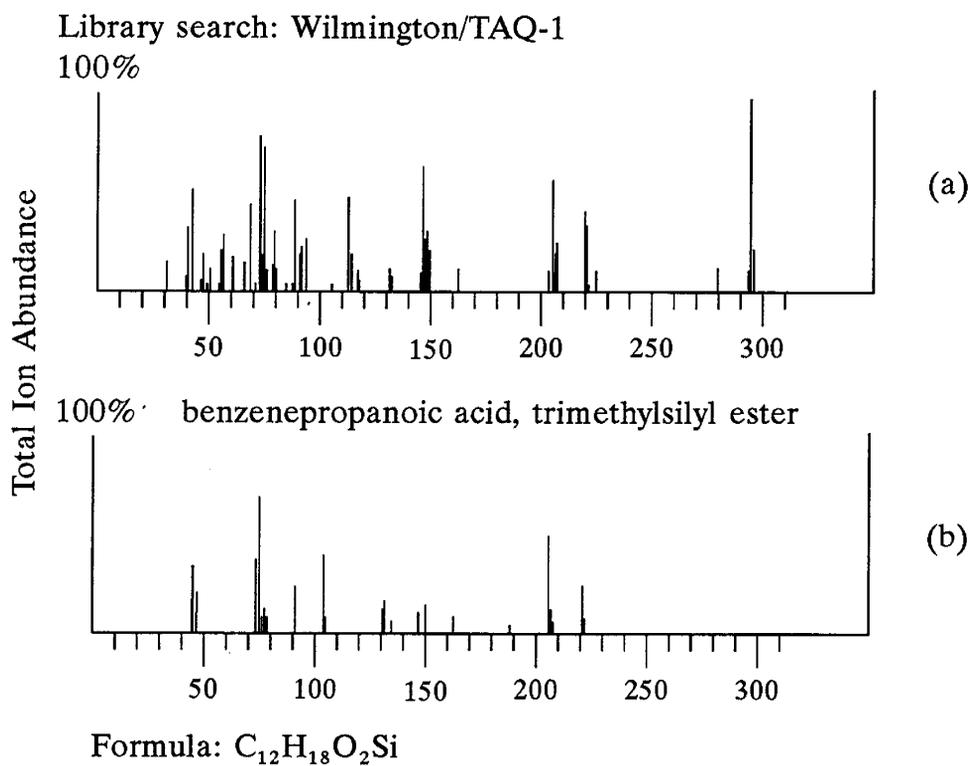


Figure 73. Mass fragmentogram of Wilmington crude BNL-TAQ-1 system TMS derivativized sample (a) and the corresponding library match compound benzene propanoic acid, trimethyl-silyl ester.

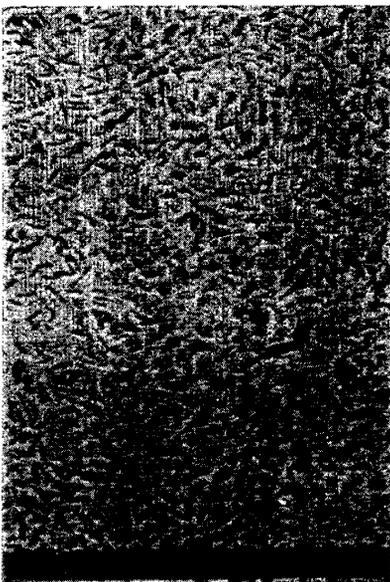


Figure 74. Boscan + emulsifier + Media. 40 day treatment.

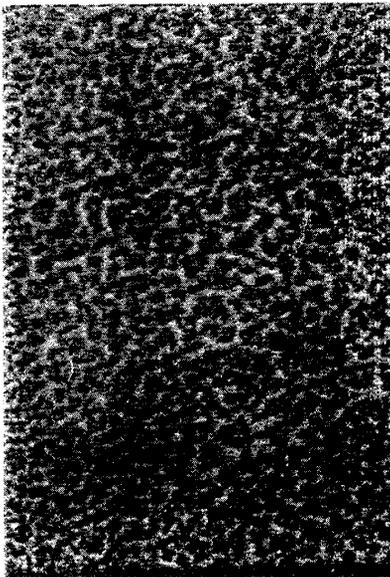


Figure 75. Media + emulsifier + BNL-4-21. 40 day treatment.

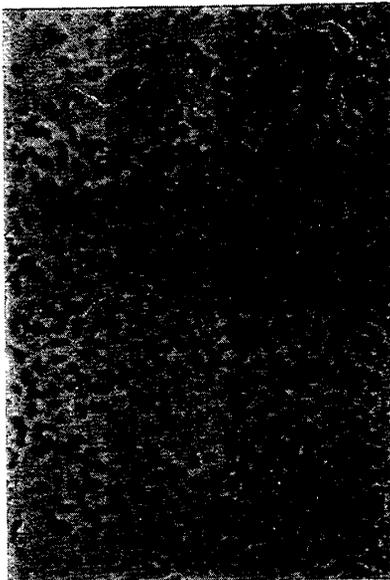


Figure 76. Media + emulsifier + BNL-4-22. 40 treatment.



Figure 77. Media + emulsifier + Boscan + BNL-4-21. 40 day treatment.



Figure 78. Media 2 + emulsifier + Boscan + BNL-4-22. 40 day treatment.

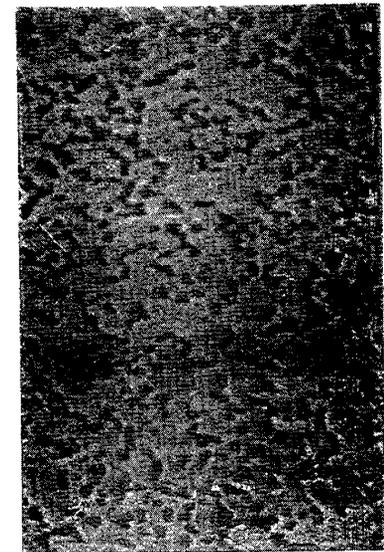


Figure 79. Boscan + Media 2.
40 day treatment.

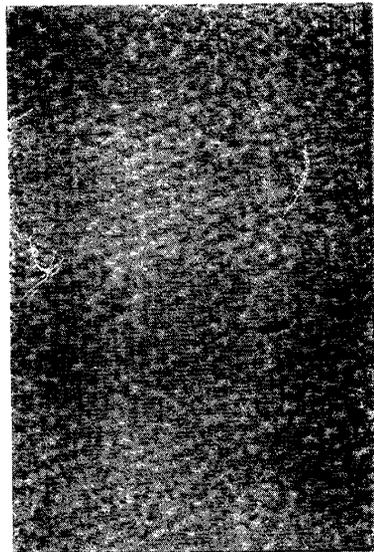


Figure 80. Media 2 + BNL-4-23.
40 day treatment.

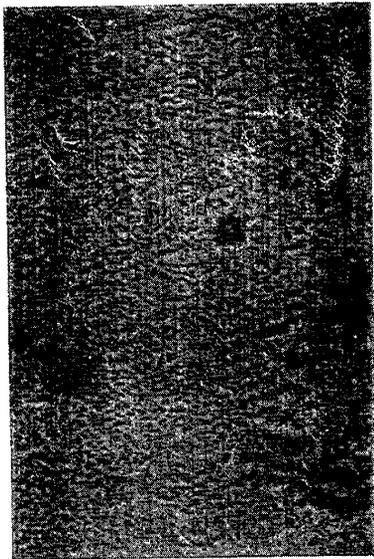


Figure 81. Cerro Negro + Media
+ BNL-4-23. 40day treatment.

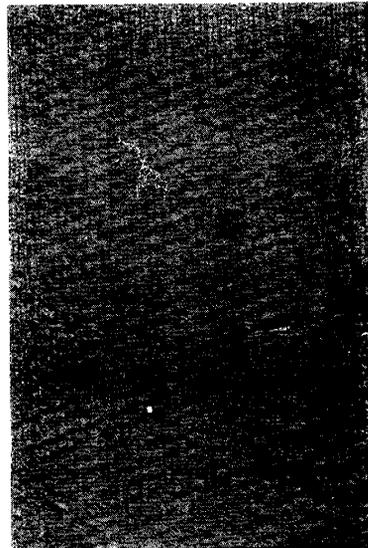


Figure 82. Boscan + Media 2
+ BNL-4-23. 40 day treatment.

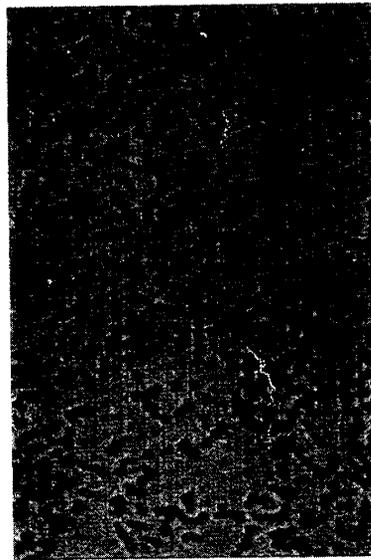
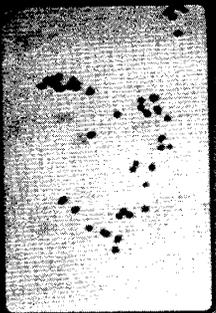


Figure 83. Cerro Negro + Media 2
+ BNL-4-22. 40 day treatment.

4



4. Monterey Oil A836
Control
Minus Detergent

5



5. Monterey Oil A836
+ BNL-NZ-3
Minus Detergent

6



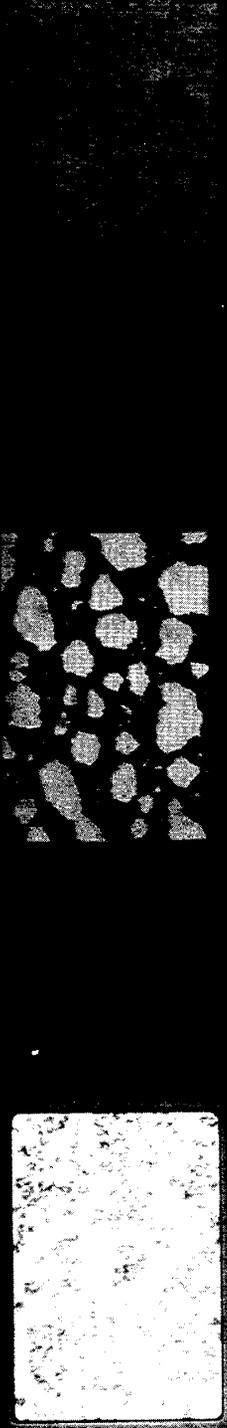
6. Monterey Oil A836
+ BNL-4-23
Minus Detergent

Figure 84. Microscopic images of control and biotreated Monterey A836 crude oil. Medium 2, no Tergitol, seven days.

3

2

1



- 1. Monterey Oil A836
Control
Plus Detergent

- 2. Monterey Oil A836
+ BNL-NZ-3
Plus Detergent

- 3. Monterey Oil A836
+ BNL-4-23
Plus Detergent

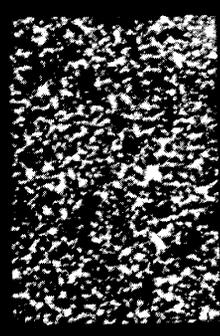
Figure 85. Microscopic images of control and biotreated Monterey A836 crude oil. Medium 2, plus Tergitol, seven days.

12



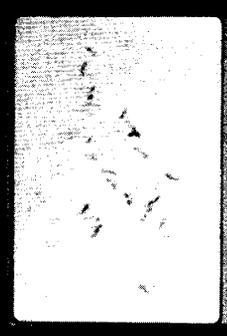
12. Monterey Oil A837
+ BNL-4-23
Minus Detergent

11



11. Monterey Oil A837
+ BNL-NZ-3
Minus Detergent

10



10. Monterey Oil A837
Control
Minus Detergent

Figure 86. Microscopic images of control and biotreated Monterey A837 crude oil. Medium 2, no Tergitol, seven days.

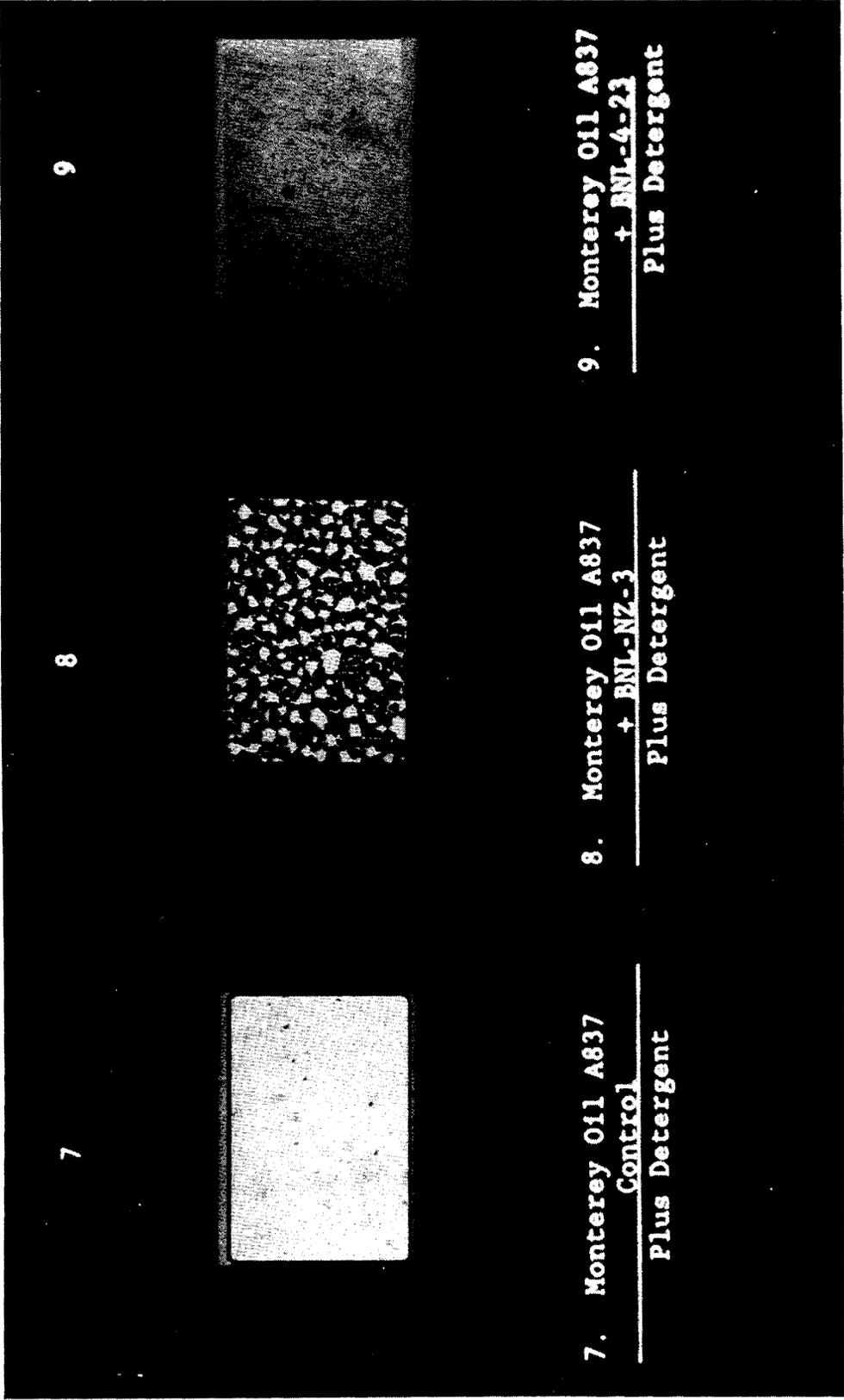


Figure 87. Microscopic images of control and biotreated Monterey A837 crude oil. Medium 2, plus Tergitol, seven days.

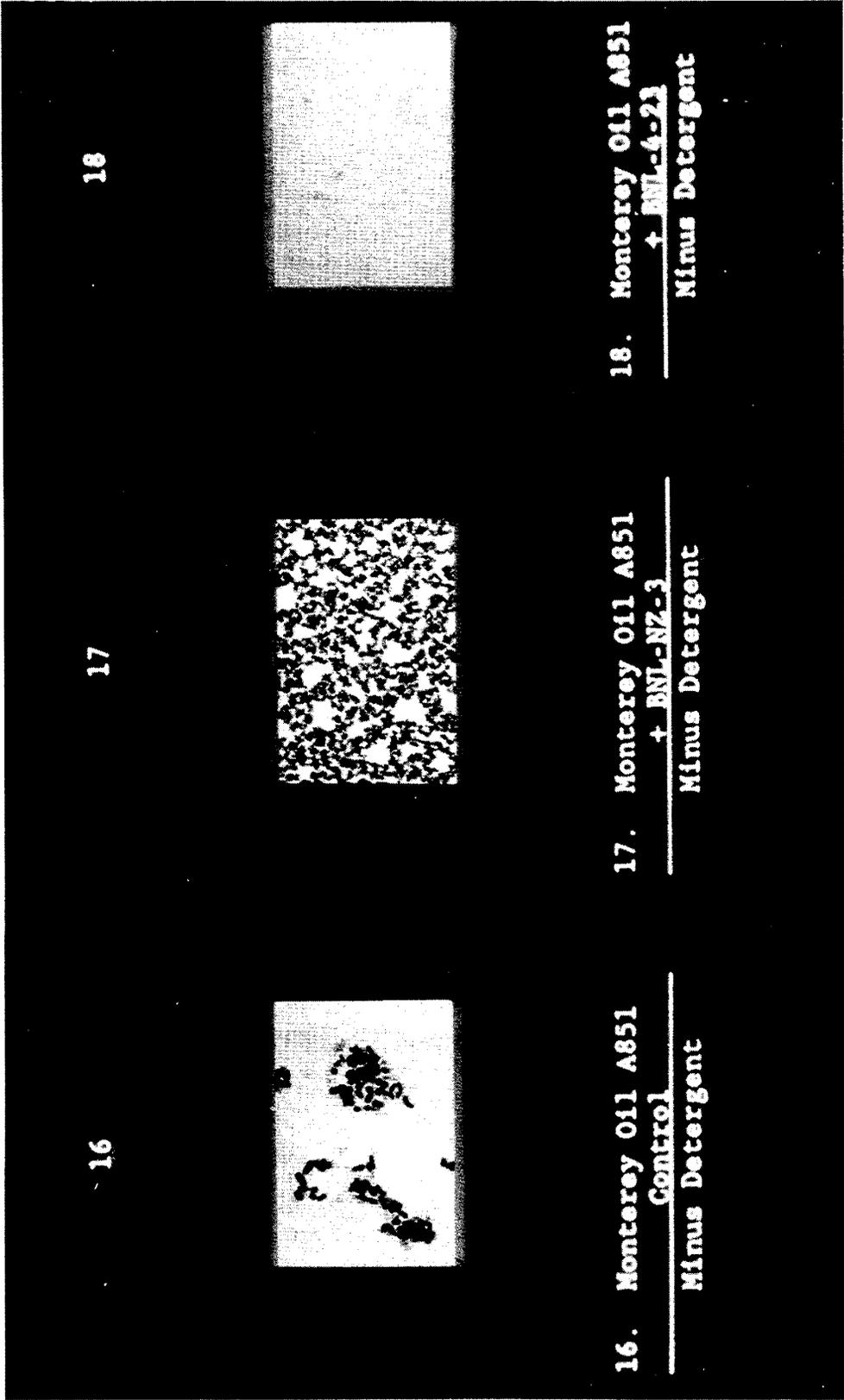
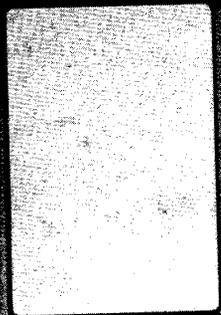


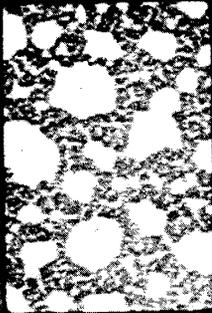
Figure 88. Microscopic images of control and biotreated Monterey A851 crude oil. Medium 2, no Tergitol, seven days.

13



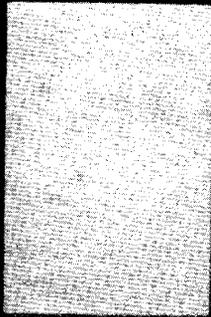
13. Monterey Oil A851
Control
Plus Detergent

14



14. Monterey Oil A851
+ BNL-NZ-3
Plus Detergent

15



15. Monterey Oil A851
+ BNL-4-23
Plus Detergent

Figure 89. Microscopic images of control and biotreated Monterey A851 crude oil. Medium 2, plus Tergitol, seven days.

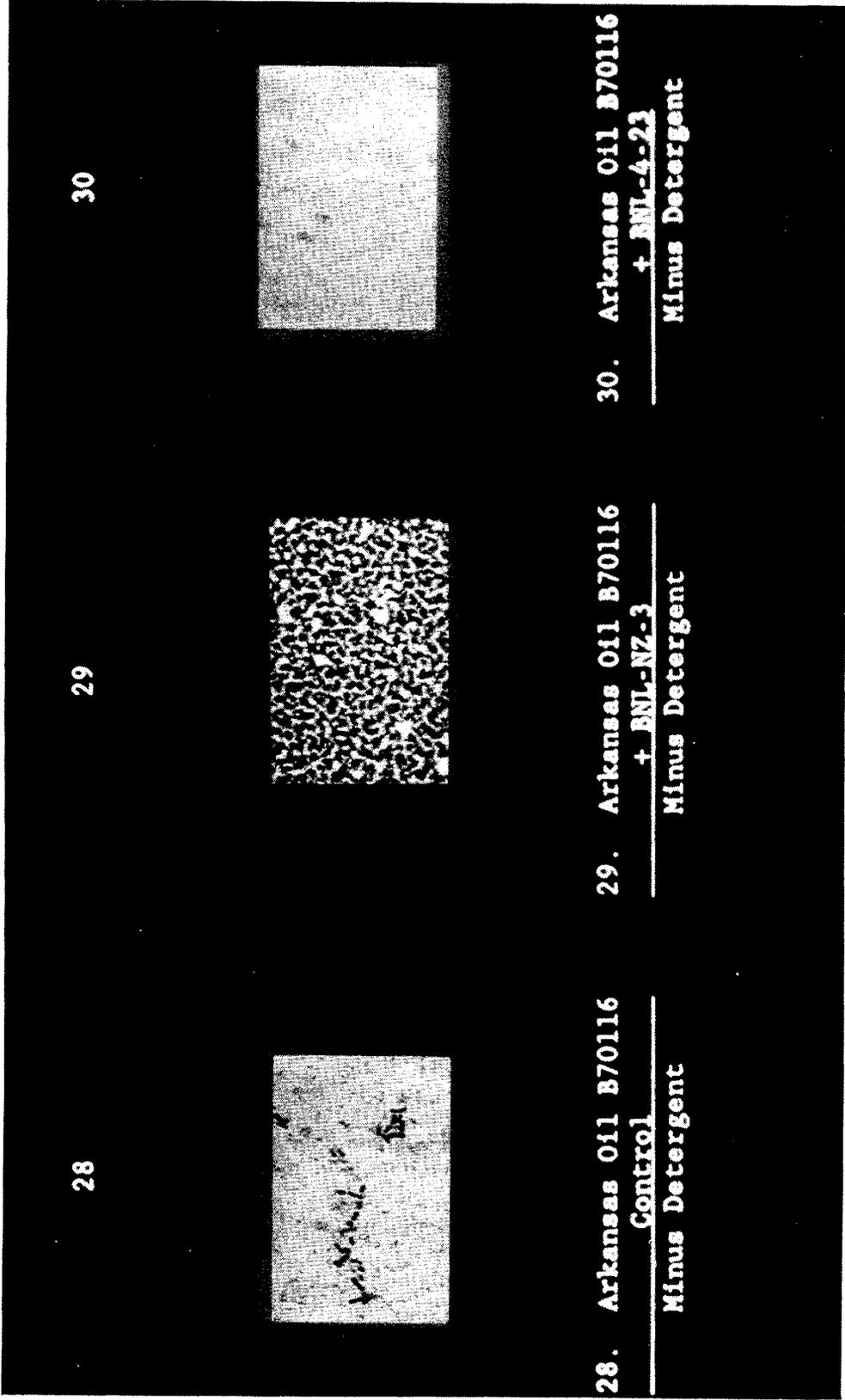


Figure 90. Microscopic images of control and biotreated Arkansas crude oil. Medium 2, no Tergitol, seven days.

deliberately to encourage the growth of indigenous microorganisms. In all cases, the microscopic characteristics change significantly upon the introduction of the test organisms. Also note that shorter treatment periods have been applied.

Addition of a detergent did not apparently interfere with the action of introduced microbial species. Another important aspect of these results should also be emphasized. A851 crude is an onshore biodegraded crude oil, as compared to A836, an offshore crude oil which is heavy because of immaturity, discussed in Section 5.3, means that the chemical history of the two oils, also differs. These preliminary observations therefore indicate that in the case of A851, the action of "indigenous" microorganisms which, over the geological periods of time, led to the formation of the heavy A851 oil, via different biochemical routes affecting the lighter fractions. Hence, the introduced microbial species convert further an already "biodegraded" oil by a series of biochemical reactions affecting the heavy ends of crudes. A considerable research effort is needed in this important area in order to further verify these observations. If indeed, the introduced organisms are the species which drive the biochemical conversion of oils, then some of the negative aspects associated with the action of "indigenous" microorganisms may be understood better.

6. Core-flooding Experiments

The core-flooding apparatus system described in Section 3.6, has been used routinely in all of the experiments discussed in this Section. For the sake of clarity, the schematics of the core-flooding apparatus are reproduced in Figure 91.

Flow Diagram for Core-Flooding Bioreactor

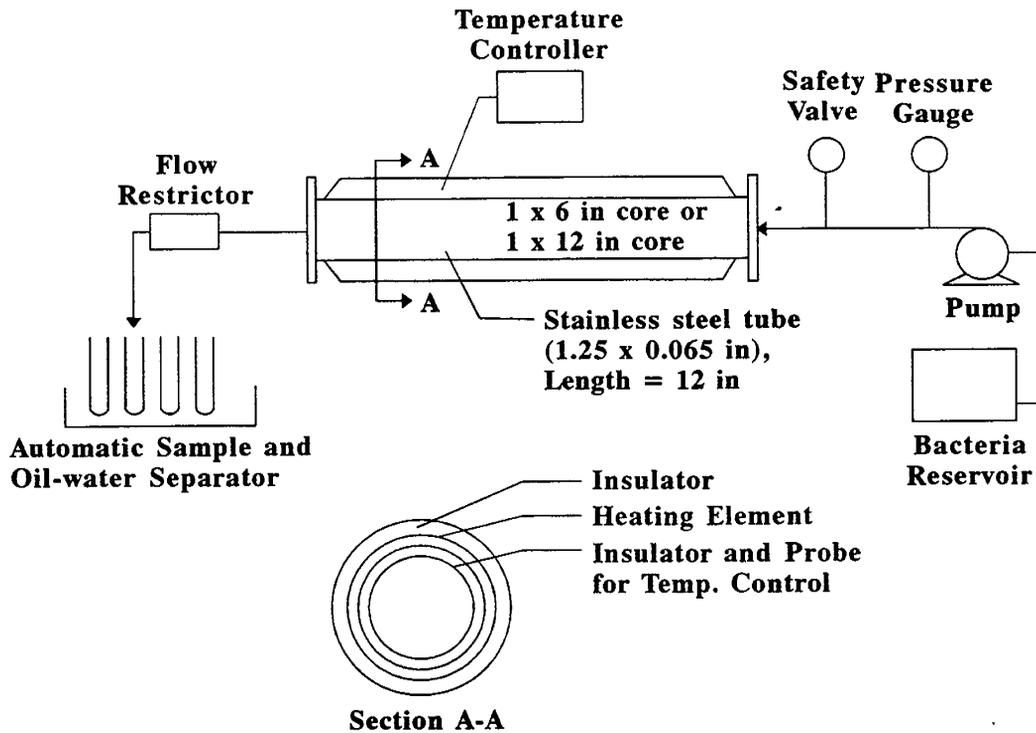


Figure 91. Flow Diagram for Core-flooding Bioreactor.

Berea sandstone (200 Milli-darcys) has been cut and fitted into the stainless steel "bioreactors." A sample of Wilmington, CA, crude oil (Table 32) was used in several experiments in which BNL organisms known to bioconvert this crude oil have been tried out in the core-flooding experiments.

The results of the first series of core-flooding experiments with four microorganisms from the BNL collection given in Table 33 show significant additional oil recovery under the experimental conditions used. Each column in the table represents an average of three separate core-flooding experiments. All experiments were carried out under identical experimental conditions as follows.

Table 32. Data from routine analyses of Wilmington, CA, crude oil.
(Bartlesville sample 71052, Dooley et al., 1994)

gravity, °API, 19.4
gravity specific, 0.938
sulfur percent 1.59

The core was saturated with a known amount of the crude oil and then flooded with brine at 65° C, which displaced about 30% of the original oil. Brine treatment was then followed by the introduction of microbial culture in brine under pressure at 65°C. The cell count was determined in the original culture brine medium which was then allowed to develop in the core over a period of six days, after which the additional oil recovered was collected and the cell count repeated in the brine exudate.

These highly promising core-flooding experiments have been extended to other oils and microorganisms from the BNL collection. For example, treatment of a Prudhoe Bay, Alaska, crude oil with two thermophilic organisms, TAQ-1 and TAQ-2, yielded results shown in Table 34. The experiments have been carried out under identical experimental conditions as those used for Wilmington crude.

The original TAQ-1 culture contained 1.7×10^5 cells/ml. The cell count in brine exudate dropped by an order of magnitude to 5.71×10^4 after biotreatment of the crude oil with TAQ-1 compared to no change in the TAQ-2 cell count under identical experimental conditions. In both cases, the pH became alkaline, i.e., pH 7.760 and pH 6.583 before biotreatment, and pH 8.44 and 7.98, respectively, after the biotreatment with TAQ-1 and TAQ-2.

Table 33. Recovery of Wilmington Crude from Brea Sandstone cores at 65°C.

Core absorbed oil sample, wt. (g)	16.3	16.7	16.7	16.7
Pressure (psi)	44	44	30	30
Microorganisms	BNL-NZ-3	-4-22	TAQ-2	TAQ-1
Surface tension of culture (dynes/cm)	70.7	70.0	70.7	62.7
Oil recovered by brine (g)	5.2	8.9	5.7	7.8
Oil recovered by growing microorganisms (g)	5.7	2.8	3.2	4.1
Oil recovery by brine (%)*	31	53	34	47
Additional oil recovery by growing microorganisms (%)**	50	36	29	34

Table 34. Recovery of Prudhoe Bay Crude from Berea Sandstone Cores at 65°C.

Core absorbed oil sample, wt. (g)	16.7	16.7
Pressure (psi)	40	40
Microorganisms	TAQ-1	TAQ-2
Surface tension of Culture (dynes/cm)	72.036	70.702
Oil recovered by brine (g)	5.5	5.0
Oil recovered by growing microorganisms (g)	1.7	4.5
Oil recovery by brine (%)*	33	29.9
Additional oil recovery by growing microorganisms (%)**	15.2	38.5

Samples of crude oils obtained through courtesy of the U.S. DOE, Bartlesville Operations Office.

$$* \quad \% \text{ Oil recovery by brine} = \frac{\text{oil recovered by brine (g)}}{\text{oil sample wt. (g)}} \times 100$$

$$** \quad \% \text{ Additional oil recovered by growing microorganisms} = \frac{\text{oil recovered by microorganisms (g)}}{\text{oil sample wt. (g) -- oil recovered by brine (g)}} \times 100$$

The differences in the effects due to TAQ-1 and TAQ-2 might be due to several reasons. A simple explanation might be a difference in rates between growth TAQ-1 and TAQ-2 under the experimental conditions used. Or, it may be due to the specificity of biochemical action of microorganisms discussed in previous sections. A preferential adsorption of TAQ-1 to the core may have also caused a lower count of TAQ-1 population in the exudate. Regardless, this is another example which illustrates a practical need to examine the mechanisms of oil displacement by different microbial cultures in terms of biochemical, chemical, and physical interactions between microorganisms, oils, their metabolic products, and the inorganic matrices of the core material.

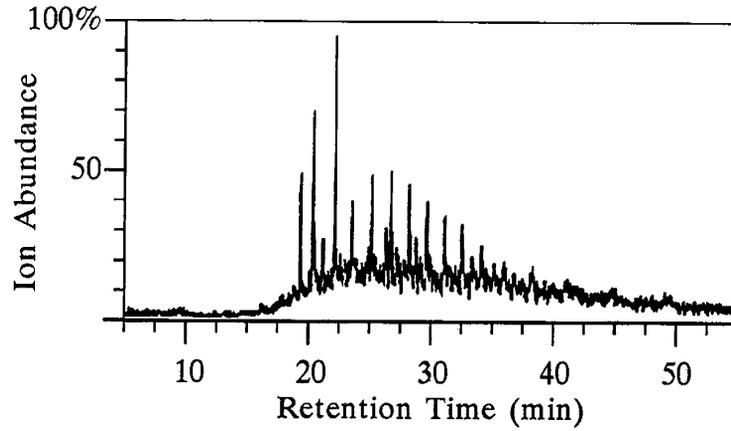
The next series of core-flooding experiments further supports this view. A sample of Wilmington, CA, crude oil was treated with mesophilic and thermophilic microorganisms and the results are shown in Figure 92. These results show two important aspects of the thermophilic process(es): (1) there is a significant additional recovery of crude oil with thermophilic microorganisms and (2) variations due to different species also occur under the experimental conditions used in core-flooding experiments. Further, as shown in Figure 93, relative to recovery of crude by saline drive (Figure 93 (b)), additional lighter crude is being recovered by treatment of Wilmington with BNL-NZ-3. The results of the above experiments further support the diagnostic significance of chemical markers in predicting efficiency of biochemical treatment. Analysis of the M/Z 57 chemical markers scans reveals "new peaks" appearing at lower retention time, an area of the chromatogram which corresponds to the lighter C-20 hydrocarbons. The saline drive recovery is, therefore, indicative of the chromatographic effect of the sandstone, i.e., retention of the

**OIL RECOVERY AS % OF RESIDUAL OIL IN CORE AT 65°C
(WILMINGTON, CA, CRUDE)--6 DAY TREATMENT**

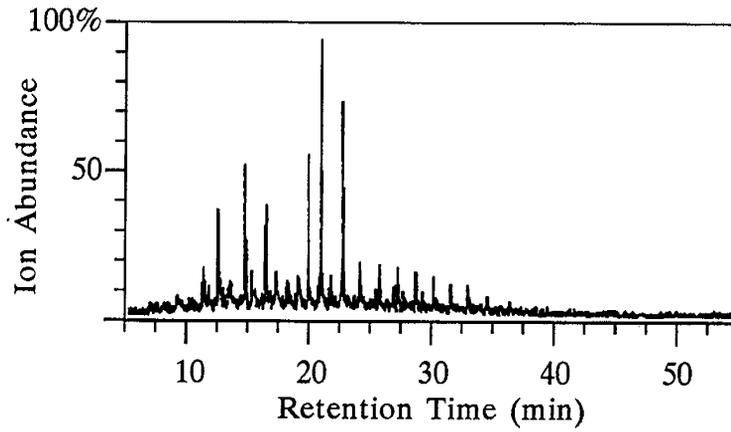
	Recovered by brine only	Flooding core temperature	Recovered by biotreatment (additional oil)
<i>Clostridium acetobutylicum</i> *	26	65°	25
<i>Clostridium collagenovorans</i> *	33	65°	24
Thermophilic BNL-TAQ1	36	65°	34
Thermophilic BNL-TAQ2	34	65°	29
Thermophilic BNL-NZ-3	28	65°	46
Thermoadapted BNL-4-23	38	65°	28
Thermoadapted BNL-4-22	41	65°	31

* Fully grown outside core at 28°C, then applied to the core at 65°C.

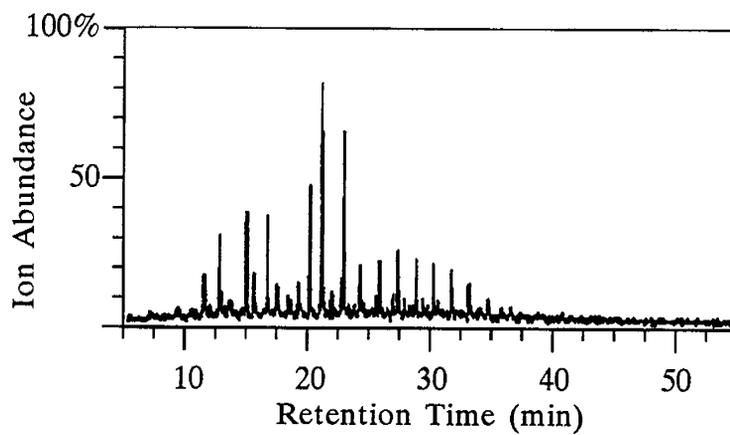
Figure 92. Oil recovery as % of residual oil in core at 65°C (Wilmington, CA, crude), six-day treatment.



(a) Untreated Wilmington crude oil.



(b) Wilmington crude recovered by saline drive.



(c) Wilmington crude recovered by BNL NZ-3.

Figure 93. Core-flooding experiments. Wilmington crude M/Z 57 chemical marker scans.

heavy fractions, and chromatographic redistribution with simultaneous concentration and fractionation of the mobile phase. Analogous experimental observations, shown in the chromatogram of the BNL-NZ-3 core-flooding experiment shown in Figure 93 (c), therefore, must be due to a bioconverted heavy fraction of the oil also enriched in lighter hydrocarbons and then chromatographed as above.

The results discussed in this section allow us to conclude that an intense R&D effort in the area dealing with the complex chemical and biochemical interactions between oils-biomass-inorganic matrix under different regimes of temperature, pressure, and salinity, is needed. The use of chemical markers will clearly lead to the development of diagnostic tools characteristic to the bioconversion of crudes, which will lead to better technologies and field applications.

7. Biochemical Mechanisms

The data discussed in the previous sections show that the bioconversion of oils is subject to many variables. Multiple chemical and biochemical processes occurring in a complex medium involve many parallel and sequential reactions, which experimentally may be difficult, if not impossible to individually isolate and monitor. However, a practical solution to monitor such processes is to identify sets of chemical compounds which can serve as signals that characterize an overall sequence of chemical events. These chemical markers are characteristic of a particular set of chemical conversions and transformations peculiar to a complex matrix, which may be organic, inorganic, or a combination of both. Such "chemical markers" are used successfully in oil exploration studies and are known as biomarkers. In fact,

they are molecular fossils derived from living organisms and can be found in both oils and source rock bitumines. These compounds can be identified and measured in complex mixtures by a variety of analytical techniques varying from Gas Chromatographic Mass Spectrometric (GC-MS) analyses, to isotope ratios and X-ray Absorption Near Edge Structure (XANES) analyses. The chemical markers are used in the characterization of source rocks, depositional environments, maturity, migration, and the age of source rock. Similarly, within an oil reservoir they can indicate the extent of conversion, for example, due to biodegradation caused by indigenous micro-organisms over geological periods of time. R&D effort at BNL has shown that biotreatment of heavy crudes with thermophilic and thermoadapted microorganisms, involves interactions which fall into identifiable categories and follow recognizable patterns, are not random and can be monitored by characteristic chemical markers in a manner similar to that used in oil exploration studies mentioned above. These may involve the use of specific mass fragmentation patterns of chemical markers characterized by typical GS-MS scans which, for example, may show the extent of bioconversion of heavier C-30 fractions of crudes to lighter, containing <C-20 hydrocarbons, Similarly, measurement of the distribution of organo-sulfur compounds by GC, using a sulfur specific detector indicates that biochemical conversion treatment of heavy crudes decreases the concentration of organo-sulfur compounds in the oil. Such analytical studies have been extended to a suite of heavy crude oils (see previous sections and cited references), yielding data which consistently indicated that biochemical conversion of crudes leads to changes in hydrocarbon distribution favoring lighter fractions, a decrease in the concentration of organo-sulfur compounds and changes in the organometallic composition. These studies have also shown

that the action of a single species of microorganisms on a series of different oils, varies. The reverse also holds. Some are good metal removers, or good emulsifiers, or good hydrocarbon and sulfur converters, and others are not. Further, deliberately introduced microorganisms seem to act differently from "indigenous ones." This is further supported by experiments in which different types of heavy crudes have been treated under identical experimental conditions. Specifically, the Venezuelan (Boscan and Cerro Negro) and the California (Monterey) oils belonging to two types of heavy crudes. One set is heavy because it is immature (Boscan and Monterey A836, A837). The other is heavy because of biodegradation during geological time (Cerro Negro, Monterey A851) due to indigenous microorganisms. As mentioned above, there are changes in organosulfur constituents. There is a formation of sulfoxides and sulfones as indicated by XANES analysis. Biochemical synthesis from crude oils of compounds containing such functional groups could be a plausible explanation as the reason for the lowering of the organic sulfur content observed by sulfur specific gas chromatographic analysis. Since sulfoxides and sulfones are soluble in aqueous phase, these forms of sulfur formed by the bioconversion of the initial oil become soluble in the aqueous phase, resulting in the observed overall lowering of the organic sulfur content in the organic (i.e. oil) phase. Changes in the chemistry of the organometallic and trace metal species as indicated by GC-AED and ICP-MS analyses indicate decreases in the concentration of metals and metal complexes in the organic phase.

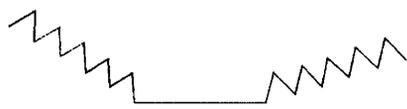
Based on the above discussion the experimental evidence currently in hand allows to suggest the following mechanisms: The biochemical conversion of crude oils may involve reactions with the heavy ends of crudes and proceed via a depolymerization process, which involves inter- and intra-molecular

reactions at active sites containing heteroatoms (S,N,O) and metals. The structure of asphaltenes and resins representative of the heavy ends of crude oils are shown in Figure 94 and of typical metal complexes in Figure 95. The association, aggregation, and coalescence of asphaltene and resin units into micelles and multilamellar vesicles occurs through bonding via sulfur, nitrogen, oxygen, and metal bridges. This leads to the formation of a complex, three dimensional structure, as shown in Figure 96. Biochemical reactions at the heteroatoms and bridges would convert such a structure into a less rigid and viscous, lighter hydrocarbon enriched material.

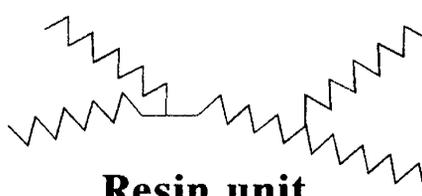
If this mechanistic hypothesis is valid, then based on the chemical similarity between asphaltenes and bituminous coals whose typical chemical structure is shown in Figure 97, organisms which act on heavy crude oils should act in a similar manner on low grade coals. Some of the experimental results supporting this hypothesis follow (for details see Crawford, 1993; Premuzic et al., 1993b,c and 1994; Lin et al., 1993; and Lin et al., 1991). Thus changes in the trace metal contents after the biotreatment of Kentucky No. 8 coal are shown in Table 35, and the effects on the sulfur content in Table 36. The results show an overall lowering in organic sulfur contents.

Further, XANES analyses (Table 37) analogous to that carried out with heavy crudes are consistent and show variations in the chemical sulfur speciation. Similarly, hydrocarbon analysis, also suggest that biotreatment causes structural conversion and redistribution of hydrocarbons as shown in Figure 98. The GC-MS scan of the mass 57 chemical marker shows qualitative and quantitative changes in the spectra following an enrichment in the lighter, less rigid organic fractions of bituminous coal.

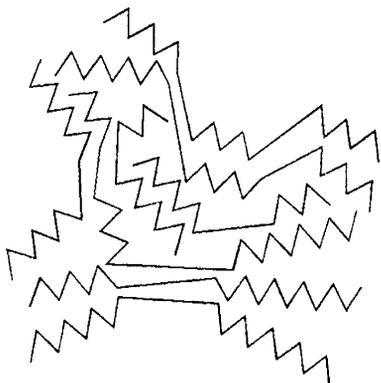
While additional studies are needed to verify the mechanisms involved in biochemical treatment of oils, the data discussed in this section support the proposed mechanisms involving heavy ends of crudes.



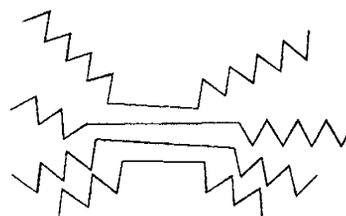
Asphaltene unit



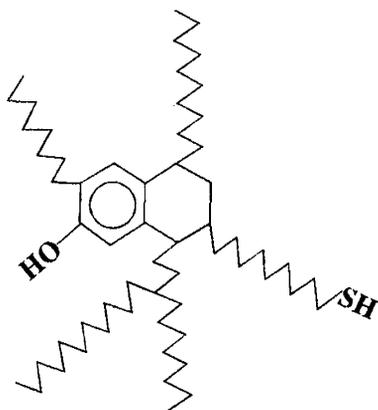
Resin unit
 NW ~800 to 3,000



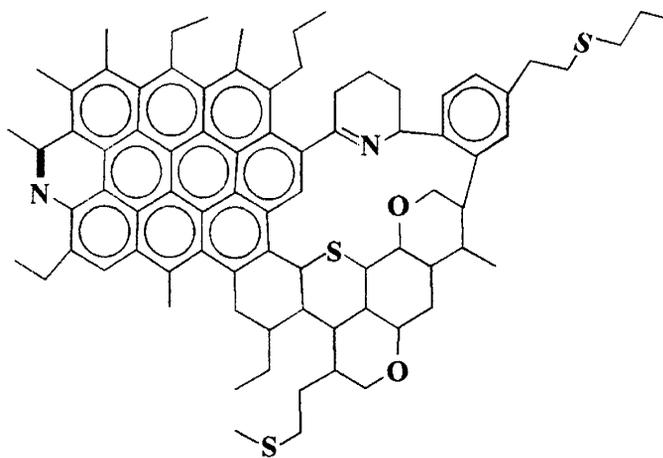
Asphaltene micelle
 MW > 30,000



Asphaltene particle
 MW ~3,000 to 10,000



Schematic resin molecule



Schematic asphaltene molecule

Figure 94. Structure of asphaltenes and resins (after Hunt, 1979).

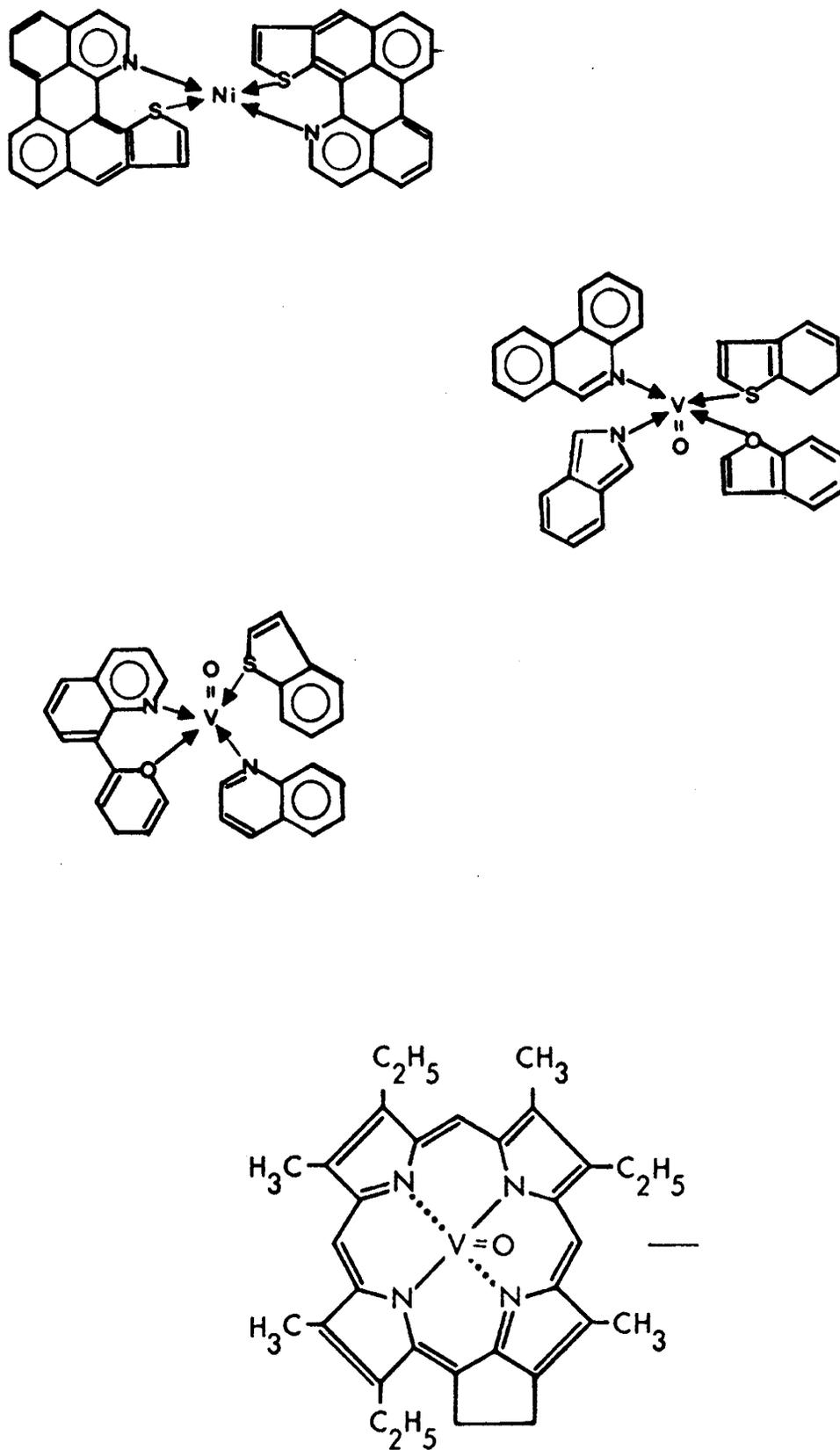


Figure 95. Organo-Metallic compounds found in crude oils (after Yen, 1975).

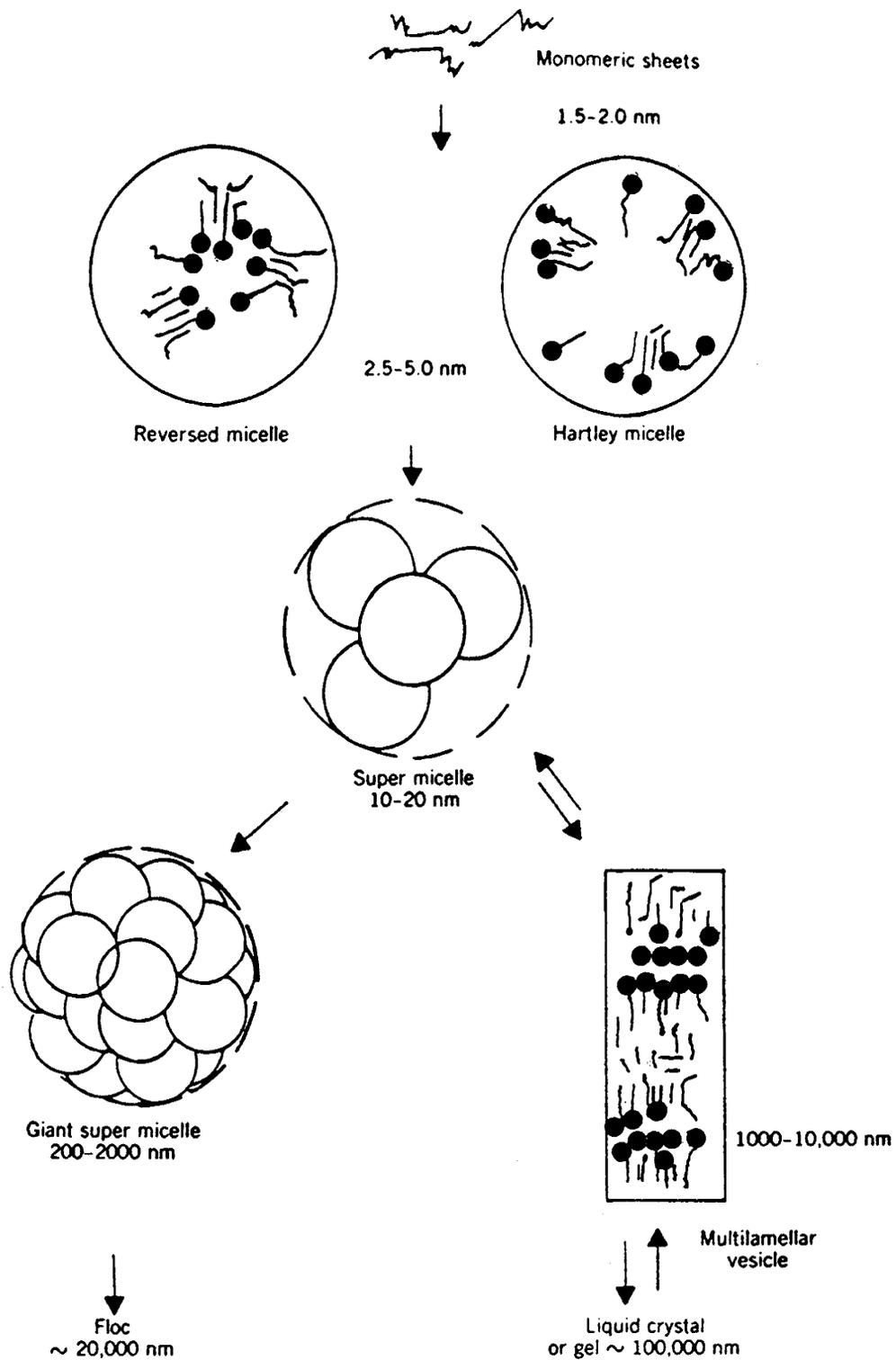


Figure 96. Association, aggregation, and coalescence of asphaltic materials to form micelles. ● denotes functional group, e.g., S, N, and O (after Yen, 1990).

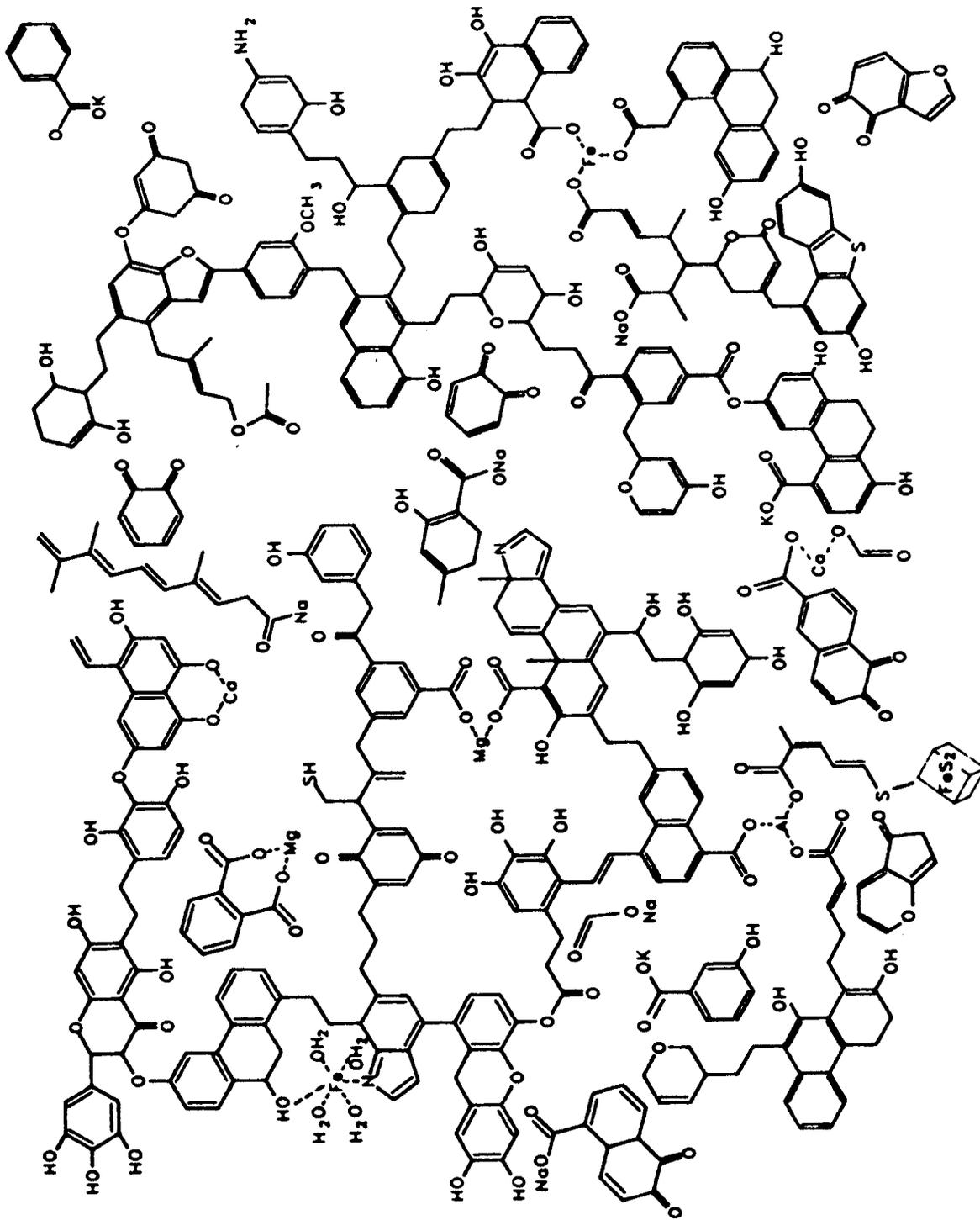


Figure 97. Organic components of low rank coals (after Faison, 1993).

Table 35. Trace Metals Contents ($\mu\text{g/g}$).

Trace Metals	Untreated Kentucky No. 8	Biotreated Kentucky No. 8*
V	157	99
Mn	168	41
Cu	143	0
Sr	1400	1040
Y	80	57
Zr	148	88
La	83	53
Ce	148	140
Pb	76	1
Th	34	23
U	19	0

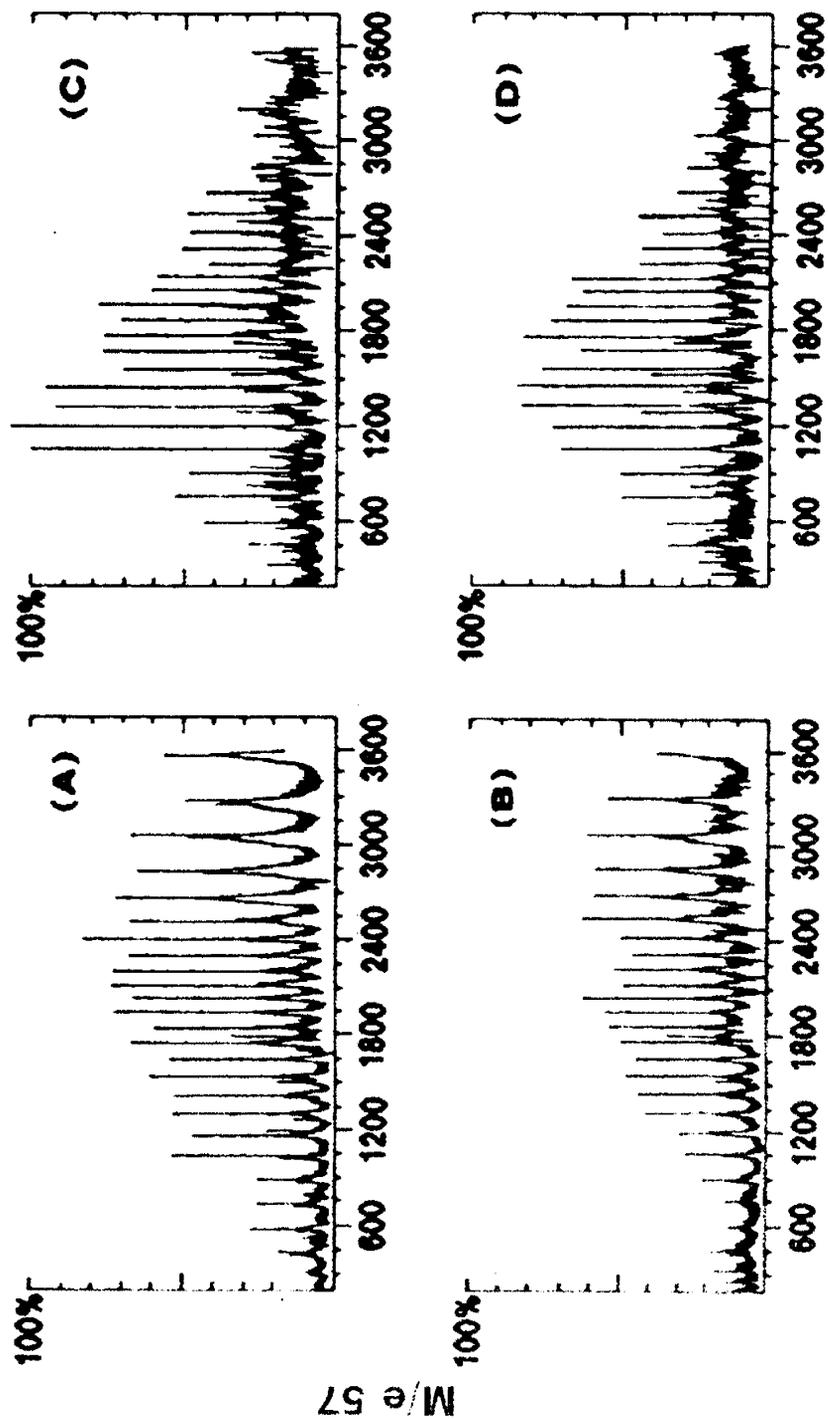
Table 36. Organic Sulfur Removal in Acid Washed Coals.

Biotreatment	Kentucky No. 8 Bituminous
Control	2
Mesophilic BNL-4-23	26
BNL-4-24	6
Control	2
Thermophilic BNL-TH-31	31

Table 37. XANES analysis of biotreated Kentucky No. 8 bituminous coal.*

	Sulfides	Thiophenes	Sulfoxides	Sulfones	Sulfates
Untreated	0.262	0.397	0.133	0.074	0.234
BNL-NZ Control	0.240	0.441	0.121	0.045	0.153
BNL-NZ-3 Treated	0.234	0.408	0.086	0.047	0.194
BNL-NZ-5 Treated	0.208	0.296	0.089	0.053	0.179
BNL-TH-29 Control	0.184	0.303	0.109	0.055	0.239
BNL-TH-29	0.198	0.198	0.177	0.045	0.173

*Measured in mole fractions.



Retention Time (min)

Figure 98. Mass 57 hydrocarbon scan of untreated (A, C) and treated (B, D) samples of Kentucky No. 8 and lignite coals.

8. International Conference on Microbial Enhanced Oil Recovery

The highly successful Fourth International Conference on Microbial Enhanced Oil Recovery (MEOR) was held at Brookhaven National Laboratory, Upton, New York, U.S.A., in September of 1992. There were approximately fifty four Investigators/Presenters in attendance, representing twelve countries, namely, Australia, Canada, China, Japan, Norway, Romania, Russia, Saudi Arabia, Trinidad (West Indies), United Kingdom, United States of America, and Venezuela. The Proceedings of the Conference were published by Elsevier, Amsterdam, in 1993. The following is a complete list of the topics covered at the MEOR Conference, as well as the names of the authors and/or presenters.

Contents: Proceedings of the 1992 International Conference "Microbial Enhancement of Oil Recovery--Recent Advances." Edited by E.T. Premuzic and A. Woodhead, Developments in Petroleum Science Volume 39, 446 pages, 1993.

Preface. Introduction to the Fourth International MEOR Conference (F. Burtch). Plenary Address: "M.O.R.E." to M.E.O.R.: An Overview of Microbially Enhanced Oil Recovery (T.R. Jack). **Selection and Characterization of Microbial Systems.** Use of Natural Microflora, Electron Acceptors and Energy Sources for Enhanced Oil Recovery (G.T. Sperl, P.L. Sperl, D.O. Hitzman). Bug Rock: Bacteriogenic Material Precipitation Systems for Oil Patch Use (T.R. Jack et al.). Chemical Markers of Induced Microbial Transformations in Crude Oils (E.T. Premuzic et al.). Characterization of Xanthan Gum Degrading Enzymes from a Heat-stable, Salt-tolerant Bacterial Consortium (J.A. Ahlgren). Subsurface Application of *Alcaligenes eutrophus* for Plugging of Porous Media (Y. Li et al.). Halotolerant and Extremely Halophilic Oil-oxidizing Bacteria in Oil Fields (S.S. Belyaev et al.).

The Use of Slime-forming Bacteria to Enhance the Strength of the Soil Matrix (I.C.Y. Yang et al.). Parameters Affecting Microbial Oil Mobilization in Porous Media (A.K. Stepp et al.). Behavior of Microbial Culture Product (PARA-BAC^R) Isolates in Anaerobic Environments (D.R. Schneider). Aqueous Microbial Biosurfactant Solutions Exhibiting Ultra-low Tension at Oil-water Interfaces (T. Ban, T. Sato). The Compatibility of Biosurfactants on Degassed Oil and the Displacement Efficiency of Biosurfactant/Sulphonate-Alkaline-Polymer System (S.T. Gao, T.L. Qin). Comparative Analysis of Microbially Mediated Oil Recovery by Surfactants Produced by *Bacillus licheniformis* and *Bacillus subtilis* (S.L. Fox et al.) Noninvasive Methodology to Study the Kinetics of Microbial Growth and Metabolism in Subsurface Porous Materials (M.J. McInerney et al.). Adhesion of Microbial Cells to Porous Hydrophilic and Hydrophobic Solid Substrata (T. Ban, S. Yamamoto). Modeling of MEOR. A Mathematical Model for Microbially Enhanced Oil Recovery Process (X. Zhang, R.M. Knapp, M.J. McInerney). Effect of Hydrophobicity of the Solid Substratum on Oil Displacement in the Hele-show Model (T. Ban, H. Kamo). Field Applications. Microbially Enhanced Oil Recovery Field Pilot, Payne County, Oklahoma (J.D. Coates et al.). Microbial Hydraulic Acid Fracturing (V. Moses et al.). A Pilot Test of EOR by In-Situ Microorganism Fermentation in the Daqing Oilfield (C.Y. Zhang, J.C. Zhang). The Application of Microbial Enhanced Oil Recovery to Trinidadian Oil Wells (U. Maharaj, M. May, M.P. Imbert). MEOR, Recent Field Trials in Romania: Reservoir Selection, Type of Inoculum, Protocol for Well Treatment and Line Monitoring (I. Lazar et al.). Microbial-Enhanced Waterflooding Field Pilots (R.S. Bryant et al.). Microbial Characteristics and Metabolic Activity of Bacteria from Venezuelan Oil Wells (H. Bastardo, L. Vierma, A. Estevez). A Nutrient Control Process for Microbially Enhanced Oil Recovery Applications (G.E. Jenneman, J.B. Clark,

P.D. Moffitt). Characteristics of Enriched Cultures and their Application to MEOR Field Tests (S.-Y. Wang, Y.-F. Xue, S.-H. Xie). On-site Bioaugmentation Treatment of Petroleum Tank Bottom Wastes: A Case Study (F.K. Hiebert et al.). Six Years of Paraffin Control and Enhanced Oil Recovery with the Microbial Product, Para-Bac™ (L. Nelson, D.R. Schneider). Causes and Control of Microbially Induced Souring (M.J. McInerney et al.). Additional Oil Production During Field Trials in Russia (M.V. Ivanov et al.). Isolation of Thermophilic Bacteria from a Venezuelan Oil Field (G. Sanchez, A. Marin, L. Vierma). Potential of MEOR. The Potential for MEOR from Carbonate Reservoirs: Literature Review and Recent Research (R.S. Tanner et al.). Using Bacteria to Improve Oil Recovery from Arabian Fields (M.H. Sayyoub, M.S. Al-Blehed). On Towards the Real World (V. Moses). Abstracts. Comparison of the Properties of Commercial Xantham Gum with a Xantham Gum Produced by *Xanthomonas campestris*™ Using Lactose as Sole Source of Carbon (F. Paz, G. Trebbau, L. Vierma). A Mathematical Model to Optimize Fermentation in *Xanthomonas campestris* (E. Rodriguez). Thermophilic Bacteria from Petroleum Reservoirs (G. Grassia, A.J. Sheehy). Index.

9. Conclusions

- Current experimental evidence shows that biochemical interactions between microorganisms and crude oils follow distinct trends.
- Biotreatment causes a decrease in the total content of organic sulfur compounds, apparently by mechanisms which convert the compounds into water soluble species.
- Biotreatment removes trace metals from the oil phase.
- With some microbial species, there is an increase in <C20 compounds with an overall formation of lighter hydrocarbons.
- Evidence has been gathered which implies that some microbial species biochemically prefer to convert higher molecular weight compounds to smaller, while others favor formation of better emulsions.
- Efficient emulsifying agents may be generated by some microorganisms in medium containing crude oil as the sole source of carbon.
- Spin-offs from this work may have useful downstream application, for example, biochemical processing of residuum.
- In comparison to the conventional experiments with Clostridium sp., a series of core-flooding experiments at elevated temperatures and pressures have led to substantial (>30%) additional oil recoveries.
- Current experimental evidence suggests a biochemical mechanism involving depolymerization process(es) in the heavy ends of crudes.
- Chemical markers show distinct promise as diagnostic tools in the evaluation of extent and the nature of biochemical conversion of crude oils, in either in situ or downstream applications.
- A successful International Conference on Microbial Enhanced Oil Recovery was organized and held at BNL in September of 1992, and the Proceedings published by Elsevier.

10. Publications, Reports, and Presentations

- Lin, M. S., Sylvester B. J., and Premuzic, E. T. Biochemical Desulfurization and Demineralization of Coals with Mixed Microbial Cultures, in "Proceedings: Second International Symposium on the Biological Processing of Coal," EPRI GS-7482, pp. 79-85, 1991.
- Lin, M. S. and Premuzic, E. T. Applications of bacteriorhodopsin in membrane mimetic chemistry. In Advances in Membrane-Mimetic Chemistry and its Applications T. F. Yen, Editor, Plenum Press, N.Y, 1993. (BNL 49026).
- Lin, M. S., Premuzic, E. T., Manowitz, B., Jeon, Y. J. and Racaniello, L. Biodegradation of coals. Fuel, 27, 1667-1672, 1993.
- Premuzic, E. T. and Lin, M. S. Effects of selected microorganisms on crude oil at elevated temperatures and pressures. Annual Report BNL 42048, 1988.
- Premuzic, E. T. and Lin, M. S. Interactions between thermophilic microorganisms and crude oils. In Bioprocessing of Fossil Fuels Workshop, Vienna, VA, August 8-10, 1989, P. E. Bayer, Ed., Conf. 890884, 1989.
- Premuzic, E. T. and Lin, M. S. Effects of selected thermophilic microorganisms on crude oils at elevated temperatures and pressures, Enhanced Oil Recovery 55, 79, DOE/BC-88/3 (DE89000730), 1989a.
- Premuzic, E. T. and Lin, M. S. Effects of selected thermophilic microorganisms on crude oils at elevated temperatures and pressures, Enhanced Oil Recovery 56, 106-107, DOE/BC-88/4 (DE89000742), 1989b.
- Premuzic, E. T. and Lin, M. S. Effects of selected thermophilic microorganisms on crude oils at elevated temperatures and pressures, Enhanced Oil Recovery, 57, 71, DOE/BC-89/1 (DE89000755), 1989c.
- Premuzic, E. T. and Lin, M. S. Effects of selected microorganisms on crude oil at elevated temperatures and pressures. Informal Report BNL 43185, 1989d.
- Premuzic, E. T. and Lin, M. S. Prospects for thermophilic microorganisms in microbial enhanced oil recovery (MEOR). Presented at the 1990 International Conference on Microbial Enhanced Oil Recovery, Norman, OK, May-June 1, 1990. (BNL 43789).
- Premuzic, E. T. and M. S. Lin. Interaction between thermophilic microorganisms and crude oils: Recent developments. Resources, Conservation, and Recycling, 5, 177-284, 1991.
- Premuzic, E. T. and Lin, M. S. Prospects for thermophilic microorganisms in microbial enhanced oil recovery (MEOR). Proc. Microbial Enhanced Oil Recovery, Norman, OK, May-June 1, 1990. E. Donaldson, Editor, Chapter R-18, pp. 277-96, Elsevier Press, NY, 1991.

- Premuzic, E. T. and Lin, M. S. Prospects for thermophilic microorganisms in microbial oil recovery, SPE 21015, pp. 143-148, 1991.
- Premuzic, E. T. and Lin, M. S. Effects of selected thermophilic microorganisms on crude oils at elevated temperatures and pressures. Quarterly Report (10/1/90-12/31/90) BNL 45787, Jan. 1991.
- Premuzic, E. T. and Lin, M. S. Effects of selected thermophilic microorganisms on crude oils at elevated temperatures and pressures. Quarterly Report (01/01/91-03/31/91) BNL 46055, Apr. 1991.
- Premuzic, E. T. and Lin, M. S. Effects of selected thermophilic microorganisms on crude oils at elevated temperatures and pressures. Quarterly Report (04/01/91-06/30/91) BNL 46403, July 1991.
- Premuzic, E. T., and Lin, M. S. Effects of selected thermophilic microorganisms on crude oils at elevated temperatures and pressures. Quarterly Report (07/01/91-09/30/91) BNL 46872, Nov. 1991.
- Premuzic, E. T., and Lin, M. S. Effects of selected thermophilic microorganisms on crude oils at elevated temperatures and pressures. Annual Summary Report FY 1991, BNL 47046, Dec. 1991.
- Premuzic E. T., and Lin, M. S. Effects of selected thermophilic microorganisms on crude oils at elevated temperatures and pressures. Annual Report FY 1991, BNL 47046, Dec. 1991.
- Premuzic, E. T., and Lin, M. S. Effects of selected thermophilic microorganisms on crude oils at elevated temperatures and pressures. Quarterly Report (09/01/91-12/30/91) BNL 47046, Feb. 1992.
- Premuzic, E. T., and Lin, M. S. Effects of selected thermophilic microorganisms on crude oils at elevated temperatures and pressures. Quarterly Report (01/01/92-03/30/92) BNL 47555, May 1992.
- Premuzic, E. T. and Lin, M. S. Effects of selected thermophilic microorganisms on crude oils at elevated temperatures and pressures. Quarterly Report (04/01/92-06/30/92) BNL 47741, July 1992.
- Premuzic, E. T. and Lin, M. S. Effects of selected thermophilic microorganisms on crude oils at elevated temperatures and pressures. Quarterly Report (07/01/92-09/30/92) BNL 48381, October 1992.
- Premuzic, E. T. and Lin, M. S. Effects of selected thermophilic microorganisms on crude oils at elevated temperatures and pressures. Quarterly Report (10/01/92-12/31/92) BNL 48560, January 1992.
- Premuzic, E. T. and Lin, M. S. Patent pending, 1993.
- Premuzic, E. T. and Woodhead, A., Eds. Microbial Enhanced Oil Recovery - Recent Advances. Proc. of the 1992 International Conference on Microbial Enhanced Oil Recovery, Developments in Petroleum Science, Vol. 39, Elsevier, Amsterdam, 1993.

- Premuzic, E. T., Lin, M. S., and Manowitz, B. Comparison of biochemical microbial effects in enhanced oil recovery. Proc. Bioremediation and Bioprocessing, American Chemical Society, Denver, March 1993.
- Premuzic, E. T., Lin, M. S., and Manowitz, B. Parallel trends in the biotreatment of fossil fuels (abstract). Presented at the American Chemical Society Conference, Denver, March 28-April 2, 1993.
- Premuzic, E. T., Lin, M. S., and Jin, J.-Z. Developments in geothermal waste treatment biotechnology. Geothermics, 21(5,6), 891-899 (1993). (BNL 47156).
- Premuzic, E. T., Lin, M. S., Racaniello, L. K., and Manowitz, B. Chemical markers of induced microbial transformations in crude oils. In Microbial Enhancement of Oil Recovery--Recent Advances, E. T. Premuzic and A. Woodhead, Eds., Vol. 39, pp. 37-54, Elsevier Science Publishers, Amsterdam, 1993. (BNL 48992).
- Premuzic, E. T., Lin, M. S., Jin, J.-Z., Manowitz, B., and Racaniello, L. Biochemical alteration of crude oils in microbial enhanced oil recovery. Proc. Biohydrometallurgical Technologies, Jackson Hole, Aug. 1993, A. E. Torma, M. L. Apel, and C. L. Brierley, Editors, pp. 401-13, Minerals, Metals, and Materials Society, 1993. (BNL 48049).
- Premuzic, E. T., Lin, M. S., and Jin, J. -Z. Recent developments in geothermal waste treatment biotechnology (abstract). Presented at the Ninth International Conference in the Environment, Toronto, Sept. 12-17, 1993. (BNL 49271).
- Premuzic, E. T., Lin, M. S., and Jin, J. -Z. Geothermal Waste Treatment Biotechnology. Proc. Geothermal Program Review XI: "Geothermal Energy--The Environmentally Responsible Energy Technology for the Nineties, Berkeley, Apr. 1993, pp. 216-219, US DOE, CONF 930484.
- Premuzic, E. T., Lin, M. S., and Manowitz, B. The significance of chemical markers in bioprocessing of fossil fuels. Alghero, Italy, 1993 Fourth International Symposium on Bioprocessing of Fossil Fuels. In "Fuel Processing Technology," Special Edition, 1994 (in press).
- Premuzic, E. T. and Lin, M. S. Effects of selected thermophilic microorganisms on crude oils at elevated temperatures and pressures - Annual Report 1992. Informal Report BNL 48572, Apr. 1993.
- Premuzic, E. T. and Lin, M. S. Effects of selected thermophilic microorganisms on crude oils at elevated temperatures and pressures. Quarterly Report (10/01/92-12/31/92) BNL 485602, Apr. 1993.
- Premuzic, E. T. and Lin, M. S. Effects of selected thermophilic microorganisms on crude oils at elevated temperatures and pressures-- Quarterly Report (01/01/93-03/31/93) BNL 49196, June 1993.
- Premuzic, E. T. and Lin, M. S. Effects of selected thermophilic microorganisms on crude oils at elevated temperatures and pressures-- Quarterly Report (04/01/93-06/30/93) BNL 49602, Sep. 1993.

Patents

AUI has title to the inventions developed under this research program, has filed a U. S. Patent application on these inventions which are pending before the U. S. Patent Trade Office, and has initiated an act of licensing programs aimed at commercializing these technologies.

Premuzic, E. T. and Lin, M. S. Biochemically Enhanced Oil Recovery and Oil Treatment. U.S. Patent No. 5,297,625 (1994).

Premuzic, E. T. and Lin, M. S. Biochemical Solubilization of Metals from Residual Geothermal Brines and Sludges. U.S. Patent No. 5,366,891 (1994).

11. Recommendations

1. Expand the studies to other immature or biodegraded and heavy crude oils, particularly those representing large domestic oil reserves.
2. Continue exploratory work dealing with chemical biomarkers under varying conditions, biomass to oil ratios, and different strains of microorganisms already on hand.
3. Expand core-flooding experiments and focus on the "diagnostic" utility of precore-flooding experiments in which the efficiency of the process is determined by the analyses of chemical biomarkers. Focus on the chemical and biochemical interactions between the biomass, crude oil, and the inorganic matrix. Identify common chemical markers.
4. Expand the GC/MS data base and complement with other chemical information and use it as a tool in optimization studies. Include GC-MS characterization of thermophilic and thermoadapted microbial species tested.
5. Determine the differences between the application of pregrown biomass vs. biomass grown in situ.
6. Explore the chemical nature of the biochemically produced emulsifying agents in cultures containing crude oil as the only source of carbon.
7. Determine trends in the nature and extent of emulsification vs. pre-emulsification.
8. Expand collaborative efforts particularly in the areas which involve detailed studies of the chemical nature of crude oil fractions containing key molecular markers. These should include saturated fractions, aromatic, polar fractions, metals, and heteroatoms.
9. Expand studies in the area of scaling up and pilot plant requirements for the emerging biotechnology.

10. Expand XANES studies of changes of organic sulfur compounds due to biotreatment of crude oils.
11. Develop collaborative efforts with industry in this country and abroad in areas of in situ and downstream processing of crude oils.
12. Explore the utility of oil bioconversion in the development of remediation.

12. References

- Boon, J. J., DeBoer, W. R., Kruyssen, F. J., and Wonters, J. T. M. Pyrolysis mass spectrometry of whole cells, cell walls and isolated cell wall polymers of Bacillus subtilis var. niger WM. J. of Microbiology 122, 119-127 (1981).
- Brock, T. D. Thermophilic Microorganisms and Life at High Temperatures, pp. 465, Springer Verlag, New York, 1978.
- Brock, T. D. Life at high temperature. Science 20, 132-38 (1985).
- Bryant, R. S. Microbial enhanced oil recovery and state of the art review. In Research Needs to Maximize Economic Productivity of the Domestic Oil Resource, NIPER-527 (DE9200101), Ch. 5, pp. 63-80, 1991.
- Bryant, R. S. and J. Douglas. IITRI/NIPER, pp. 449-56, SPE 16284, Society of Petroleum Engineers, 1987.
- Chicarelli, M. I., Eckardt, C. B., Owen, C. R., Maxwell, J. R., Eglinton, G., Hutton, R. C., and Eaton, A. N. Application of inductively coupled plasma-mass spectrometry in the detection of organometallic compounds in chromatographic fractions from organic rich shales. Org. Geochem., 15(3), 267-274, 1990.
- Claypool, G. C. and Mancini, E. A. Geochemical relationships of petroleum in Mesozoic reservoirs to carbonate source rocks of Jurassic smackover formation, Southwestern Alabama. The Am. Assoc. of Pet. Geologists Bull., 73(7), 906-924, 1989.
- Crawford, D. L., Editors. "Microbial Transformations of Low Rank Coals," CRC Press, Boca Raton, 1993.
- Donaldson, E. C. Pro. of the First International MEOR Workshop, April 1-3, 1986, pp. 247-56, DOE/BC/10852-1, 1987.
- Donnison, A. M., Gutteridge, C. S., Norris, J. R., Morgan, H. W., and Daniel, R. M. A preliminary grouping of New Zealand Thermus strains by pyrolysis-mass spectrometry. J. of Analytical and Appl. Pyrolysis 9, 281-295 (1986).
- Faison, B. D. The chemistry of low rank coal and its relationship to the biochemical mechanisms of coal biotransformation, in "Microbial Transformations of Low Rank Coals," Ed. D. L. Crawford, CRC Press, Boca Raton, 1993.
- Grola, M. M. Proc. of the First International MEOR Workshop, April 1-3, 1986, pp. 152-213, DOE/BC/10852-1, 1987.
- Hunt, J. M. Petroleum geochemistry and geology, W. H. Freedman and Co., San Francisco, 1979.

- Jack, T. R. "M.O.R.E." to M.E.O.R.: An overview of microbially enhanced recovery, in "Microbial Enhancement of Oil Recovery--Recent Advances," Proceedings of the 1992 International Conference on Microbial Enhanced Oil Recovery, Eds. E. T. Premuzic and A. Woodhead, Elsevier, Amsterdam (1993) 7-16.
- King, J. W. and Stevens, D. A., Editors. Proc. of the First International MEOR Workshop, April 1-3, 1986, DOE/BC/10852-1, 1987.
- King, J. W. MEOR technical status and assessment of needs. DOE/BC/10852-2, DE7-001227, 1987.
- Lazar, I. Proc. of the First International MEOR Workshop, April 1-3, 1986, pp. 124-51, DOE/BC/10852-1, 1987.
- Lin, M. S., Sylvester B. J., and Premuzic, E. T. Biochemical Desulfurization and Demineralization of Coals with Mixed Microbial Cultures, in "Proceedings: Second International Symposium on the Biological Processing of Coal," EPRI GS-7482, pp. 79-85, 1991.
- Lin, M. S., Premuzic, E. T., Manowitz, B., Jeon, Y. J. and Racaniello, L. Biodegradation of coals. Fuel Journal, submitted. (BNL 47367).
- Moses, V. MEOR in the field: Why so little? In Microbial Enhancement of Oil Recovery - Recent Advances, E. C. Donaldson, Editor, Ch. 1-3, pp 21-28, Elsevier, New York, 1991.
- Needleman, M. and Stuchberg, P. The identification of microorganisms by pyrolysis gas liquid chromatography. In Analytical Pyrolysis, C. E. Jones and C. A. Cramers, Elsevier Scientific Publishing Co., New York, pp. 77-88, 1977.
- NIPER reports: NIPER 160 (1988), NIPER 161 (1988), NIPER 324 (1988), NIPER 289 (1989), NIPER 322 (1989), NIPER 323 (1989).
- Peters, K. E. and Moldowan, J. M. The biomarker guide, Prentice Hall, New Jersey, 1992.
- Premuzic, E. T. and Lin, M. S. Effects of selected microorganisms on crude oil at elevated temperatures and pressures. Annual Report BNL 42048, 1988.
- Premuzic, E. T. and Lin, M. S. Interactions between thermophilic microorganisms and crude oils. In Bioprocessing of Fossil Fuels Workshop, Vienna, VA, August 8-10, 1989, P. E. Bayer, Ed., Conf. 890884, 1989e.
- Premuzic, E. T. and Lin, M. S. Effects of selected thermophilic microorganisms on crude oils at elevated temperatures and pressures, Enhanced Oil Recovery 55, 79, DOE/BC-88/3 (DE89000730), 1989a.

- Premuzic, E. T. and Lin, M. S. Effects of selected thermophilic microorganisms on crude oils at elevated temperatures and pressures, Enhanced Oil Recovery 56, 106-107, DOE/BC-88/4 (DE89000742), 1989b.
- Premuzic, E. T. and Lin, M. S. Effects of selected thermophilic microorganisms on crude oils at elevated temperatures and pressures, Enhanced Oil Recovery, 57, 71, DOE/BC-89/1 (DE89000755), 1989c.
- Premuzic, E. T. and Lin, M. S. Effects of selected microorganisms on crude oil at elevated temperatures and pressures. Informal Report BNL 43185, 1989d.
- Premuzic, E. T. and M. S. Lin. Interaction between thermophilic microorganisms and crude oils: Recent developments. Resources, Conservation, and Recycling, 5, 177-284, 1991a.
- Premuzic, E. T. and Lin, M. S. Prospects for thermophilic microorganisms in microbial enhanced oil recovery (MEOR). Proc. Microbial Enhanced Oil Recovery, Norman, OK, May-June 1, 1990. E. Donaldson, Editor, Chapter R-18, pp. 277-96, Elsevier Press, NY, 1991b.
- Premuzic, E. T. and Lin, M. S. Prospects for thermophilic microorganisms in microbial oil recovery, SPE 21015, pp. 143-148, 1991c.
- Premuzic, E. T. and Lin, M. S. Patent pending, 1993.
- Premuzic, E. T. and Woodhead, A., Eds. Microbial Enhanced Oil Recovery - Recent Advances. Proc. of the 1992 International Conference on Microbial Enhanced Oil Recovery, Developments in Petroleum Science, Vol. 39, Elsevier, Amsterdam, 1993.
- Premuzic, E. T., Lin, M. S., and Jin, J.-Z. Developments in geothermal waste treatment biotechnology. Geothermics, 21(5,6), 891-899 (1993b). (BNL 47156).
- Premuzic, E. T., Lin, M. S., Racaniello, L. K., and Manowitz, B. Chemical markers of induced microbial transformations in crude oils. In Microbial Enhancement of Oil Recovery--Recent Advances, E. T. Premuzic and A. Woodhead, Eds., Vol. 39, pp. 37-54, Elsevier Science Publishers, Amsterdam, 1993a. (BNL 48992).
- Premuzic, E. T., Lin, M. S., Jin, J.-Z., Manowitz, B., and Racaniello, L. Biochemical alteration of crude oils in microbial enhanced oil recovery. Proc. Biohydrometallurgical Technologies, Jackson Hole, Aug. 1993, A. E. Torma, M. L. Apel, and C. L. Brierley, Editors, pp. 401-13, Minerals, Metals, and Materials Society, 1993c. (BNL 48049).
- Premuzic, E. T., Lin, M. S., and Manowitz, B. The significance of chemical markers in bioprocessing of fossil fuels. Italy, 1993 Fourth International Symposium on Bioprocessing of Fossil Fuels. In "Fuel Processing Technology," Special Edition, 1994 (in press).

- Rosenberg, E., Zuckerberg, A., Rubinowitz, C., and Gutnick, D. L. Emulsifier of Arthrobacter RAG-1: Isolation and emulsifying properties. Appl. Environ. Microbiol. 37(3), 402-408 (1979).
- Thompson, C. J., Dooley, J. E., Vogh, J. W., and Hirsch, D. E. Analyzing heavy ends of crude. Hydrocarbon Processing, 93-98 (1974).
- Tillman, R. W. and Martinsen, R. S. 1984. The Shannon shelf-ridge sandstone complex Salt Creek anticline area, Powder Basin, Wyoming. In Silicastic Shelf Sediments, R. W. Tillman and C. T. Siemers, Editors, pp. 85-142, Soc. of Economic Paleontologists and Mineralogists, Sp. Pub. 34.
- Tissot, B. P. and Welte, D. H. Petroleum formation and occurrence. Springer Verlag, New York, p. 699, 1984.
- Waldo, G. S., Carlson, R. M. K., Moldowan, J. M., Peters, K. E., and Penner-Hahn, J. E. Sulfur speciation in heavy petroleums. Information from x-ray absorption near-edge structure. Geochimica and Cosmochimica 55, 801-14, 1991.
- Williams, T. A., Bjørøy, M., Dolcator, D. L., and Winters, J. C. Biodegradation in South Texas Eocene oils - Effects on aromatics and biomarkers. Organic Geochem. 10, 451-461, 1986.
- Yen, T. F. "The role of trace metals in petroleum," Ann Arbor Science Publ., 1975.
- Yen, T. F. and Chen J. Transport of microorganisms to enhance soil and groundwater bioremediation. In T. Burszyski (Ed.), Proceedings, Hazmacon, ABAB, Anaheim, CA, Vol. II, 1990.
- Zajic, J. E. and Mahomed, A. Y. Biosynthesis of surface active agent. In R. M. Atlas, Petroleum Microbiology, McMillan Pub. Co., NY, Ph. 6, pp. 221-97, (1984).

