THE MICROBIAL DEGRADATION OF OIL POLLUTANTS

WORKSHOP HELD AT GEORGIA STATE UNIVERSITY, ATLANTA

DECEMBER, 1972

Sponsored by Office of Naval Research, United States Coast Guard and Environmental Protection Agency

EDITORS

D.G. AHEARN AND S.P. MEYERS

CENTER FOR WETLAND RESOURCES
LOUISIANA STATE UNIVERSITY BATON ROUGE, LA.

PUBLICATION NO. LSU-SG-73-01
(1973)

COVER PHOTOGRAPH

Development of the yeast *Candida tropicalis* on oil after 72 hours growth.

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PROCEEDINGS OF A WORKSHOP HELD AT
GEORGIA STATE UNIVERSITY
DECEMBER 4-6, 1972

Sponsored By

The Office of Naval Research
The United States Coast Guard
The Environmental Protection Agency
and
Georgia State University

Workshop Goals

The Workshop objectives were: (1) to determine the present status of knowledge concerning the use of microorganisms in facilitating oil biodegradation, (2) to assess future areas of investigation, (3) to promote cooperative research projects, and (4) to promote communication and exchange of information. To attain these goals, the Workshop was developed for the informal presentation of information. Panel chairmen directed small discussion groups, and members, as well as observers, of the panel were given the opportunity to offer information of interest.

Acknowledgments

Sincere appreciation is extended to Warren L. Cook, who ably served as Program Director, and to Debra Douglas and Vicki Tayloe for their dedication and invaluable assistance in making the Workshop a success. The Public Service Department of Georgia State University, particularly Mr. John Grant, are acknowledged for their excellent and efficient assistance. Mary H. Alston of the Louisiana Water Resources Research Institute was instrumental in final preparation and editing of this report.

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MICROBIAL-FACILITATED DEGRADATION OF OIL: A PROSPECTUS

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Research on microbial utilization of hydrocarbons has been stimulated in recent times, first by a need to develop alternate sources of edible protein and currently, as a result of adverse environmental effects from catastrophic oil pollution. Single-cell protein has been obtained using crude oil or its derivatives as substrates, and is receiving increasing interest as a component of animal and human foods. Although considerable progress has been made in elucidating mechanisms of oil biodegradation, processes for use of microorganisms in treatment of oily pollutants in the environment are only in an early state of development. Elsewhere, practical systems for facilitating the bioconversion of pollutant oils in refinery treatment systems are being tested, but here also the "state of the art" is relatively poor.

Practical applications for a controlled microbiological process of fossil hydrocarbon conversion are numerous, ranging from product formation to removal of harmful materials. Biodegradation of oils is by no means a simple subject since, in nature, hydrocarbon decomposition involves interactions of complex physical, chemical and biological processes, the rates of which are interdependent. Thus, it is understandable that the enzymatic systems and responsible organisms involved in the complete biodegradation of crude oil are ill-defined. Moreover, since crude oils vary in constituency from well to well, the complexity of developing a microbial system for decomposing fossil hydrocarbons becomes even more difficult. Evidence suggests the need to adequately supplement crude oil-enriched waters with proper nutrients (i.e., nitrogen, phosphate) to stimulate and maintain the biodegradation process.

The increasing dependency of industrialized nations on fossil hydrocarbons will not be appreciably altered in the near future. The current "energy crisis," with shortages of oil, will increase requirements for shipping and piping vast amounts of hydrocarbons. Thus, the likelihood of future catastrophic oil pollution will be heightened and chronic oil pollution will continue to be a problem. Practical applications for controlled biodegradation of oil may be developed for transformation of bilge wastes and spent lubricating oils to products not harmful to the environment. Following oil spills, various microbial treatments may be necessary to reduce toxicity and to remove residual oils after mechanical clean-up procedures have been instituted. Presently, defined microbial systems which give a proven increase of oil biodegradation in nature have not been developed. Moreover, neither the immediate or long-term effects of seeding hydrocarbonoclastic microorganisms into the environment have been established. Clearly, further research on the microbial degradation of oil is indicated.

A diversity of research approaches to oil biodegradation is desirable and should continue to be undertaken. However, certain standardizations are required for meaningful evaluation of data. Rates of biodegradation should be obtained with specified hydrocarbon substrates, and microbial culture systems should be characterized as thoroughly as possible. Information on the enzymatic processes for the biodegradation of the recalcitrant fractions of crude oils, i.e., the complex aromatic and bitumen components, is required. In this regard, co-oxidative mechanisms should be given special emphasis. Realistic field evaluations of laboratory data need to be undertaken, with more critical attention given to the sublethal effect of oil and oil products on biological processes of the micro- and macrobiota. Fertilization with nutrients to facilitate natural oil decomposition and fertilization in combination with "microbial seeding" must be further evaluated. Furthermore, broad toxicity studies considering not only metabolites of the seed organisms, but also their potentials for pathogenicity need examination.

This enumeration by no means lists all of the oil biodegradation areas that warrant exploration. Nevertheless it is clear that the practical use of microbial systems to remove pollutant oils will be achieved only through accelerated development of further information on these broad interrelated topics.

MICROBIAL DEGRADATION OF OIL: PRESENT STATUS, PROBLEMS, AND PERSPECTIVES

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My active interest in the microbial degradation of mineral oils dates from 1942 when the American Petroleum Institute started to subsidize such research. Thirty years ago hydrocarbon-oxidizing microorganisms were generally regarded as biological curiosities. With few exceptions, notably methane, most hydrocarbons were believed to be biologically inert or highly refractory to enzymatic attack. Refined mineral oils, petroleum jelly, vaspar, and other hydrocarbon mixtures were commonly used for preserving cultures or for excluding atmospheric oxygen from anaerobes. Most microbiologists viewed with indifferent skepticism the report of Bushnell and Haas (6) on bacterial utilization of gasoline, kerosene, and various mineral oils, although numerous scattered papers on the microbial oxidation of liquid and solid hydrocarbons (41) preceded this classical work.

Papers presented at this Workshop late in 1972 coupled with those listed in major reviews (2,8,10,11,14,15,21,33,37,42) indicate that virtually all kinds of hydrocarbons are susceptible to microbial degradation under favorable conditions. More than 200 species of bacteria, yeasts, and filamentous fungi have been shown to metabolize one or more kinds of hydrocarbons ranging from CH_4 to compounds containing more than 40 carbon atoms. Although there is a wealth of information on the occurrence and kinds of oil-degrading microorganisms in oil-polluted environments, not much is known about their reaction rates or their intermediate degradation products.

Limitations of time and space dictate the minimization of experimental details, data, discussion, and documentation in most sections of the following succinct summation.

1. OCCURRENCE OF HYDROCARBON OXIDIZERS IN NATURE

Microorganisms which oxidize various hydrocarbons are widely distributed in soil and water. They are most numerous and the most varieties occur in places that have been subjected to chronic oil pollution either from natural seeps or by the activities of man. Relatively few hydrocarbon oxidizers, except methane oxidizers, occur in virgin soils remote from oil fields or oil pollution. They are only rarely found in the open ocean except in the vicinity of shipping lanes. Few or none occur in petroleum as it emerges from oil wells or in unpolluted ground waters.

Abundance of Hydrocarbon Oxidizers.--The water in oil sumps and in oil-polluted harbors may contain from 10^3 to 10^6 oil-oxidizing microorganisms per ml. Similarly large populations of such microbes have been found in the topmost layers of bottom deposits beneath chronically oil-polluted water, including fresh, brackish, and saline waters. Between 10 and 10^9 oil oxidizers per ml of bottom ooze were found in 180 out of 192 samples collected from Barataria Bay, Louisiana (46). Quite commonly water and bottom ooze from Gulf Coast and southern California harbors contain 10^2 to 10^6 oil oxidizers per ml while clean beach sand and open ocean water often yield none (44). From month to month at the same station in oil-polluted harbors the population of oil oxidizers sometimes changed by 4 or 5 orders of magnitude.

The abundance and physiological types of hydrocarbon-oxidizing microbes in soil and aquatic environments seem to be influenced by the quantities and kinds of hydrocarbons which have been present. This generalization is based more on laboratory experimentation and prophetic thinking than on field observations.

2. KINDS OF HYDROCARBON OXIDIZERS

One or more species of 70 genera, including 28 bacteria, 30 filamentous fungi, and 12 yeasts, have been shown to oxidize one or more kinds of hydrocarbons. Most of these are listed in various reviews (2,14,21,28).

Enzymes.--Each species generally metabolizes only a narrow spectrum of homologous hydrocarbons. Certain species produce constitutive enzymes, but in a good many species the enzymes which catalyze the oxidation of hydrocarbons are adaptive or inducible. Quite commonly pure cultures of various species have been induced to attack hydrocarbons by cultivating them in appropriate heterotrophic media enriched with one or more hydrocarbons.

Pathogenicity.--Ordinarily oil oxidizers are not infectious for higher organisms. A few species of human pathogens have been induced to metabolize hydrocarbons, but the likelihood of such microorganisms spreading diseases seems rather remote. Machinists whose arms, wrists, and hands are repeatedly exposed to cutting-, cooling-, and quenching-oil emulsions are sometimes afflicted with dermatoses believed by some to be due to bacteria which grow in such emulsions (2). At this Workshop, Dr. Raam Mohan reported that an oil-oxidizing species of *Arthrobacter* acquired pathogenicity for mice.

By depleting dissolved oxygen from oil-polluted water or by producing toxic metabolic products, hydrocarbon-oxidizing microorganisms may injure aquatic animals. Evidence for such injury is largely lacking.

3. KINDS OF HYDROCARBONS OXIDIZED

Virtually all kinds of hydrocarbons appear to be susceptible to microbial degradation under favorable conditions. This generalization is based largely on observations made on several dozen different aliphatic, cycloparaffinic, aromatic, and olefinic hydrocarbons. Although olefins are not found in crude oils, they occur in certain refinery products such as gasoline and

kerosene. Small amounts of olefins are produced by certain organisms. Between 40 and 90% of the molecules in the five major classes of compounds occurring in crude oils (Table 1) have paraffinic side chains, ranging in length from methyl ($-CH_3$) to cetyl ($-C_{16}H_{33}$). It has been conjectured that microbes may degrade certain aromatic and aliphatic compounds by attacking the longer paraffin side chains.

TABLE 1

Average Amounts of Major Classes of Hydrocarbons and Related Compounds Present in Different Petroleums and Gasolines

Component	Percentages in	
	Petroleums	Gasolines
Aliphatic or paraffinic (alkanes)	15-35	25-68
Cycloparaffinic (cycloalkanes; naphthenes)	30-50	5-24
Aromatic (benzene and polynuclear series)	5-20	7-55
Asphaltic (asphaltenes; heterocyclic compounds with oxygen, sulfur, or nitrogen)	2-15	0.1-0.5
Olefinic (alkenes or ethylene series)	nil	0-41

Relative Oxidizability of Various Hydrocarbons.--It is commonly stated that *n*-alkanes are oxidized by pure cultures more readily than *iso*-alkanes, cyclic hydrocarbons, or olefins. This belief may be a built-in artifact resulting from observations on cultures previously enriched in media containing paraffinic components. Certain wild, mixed cultures that developed in media containing cyclic hydrocarbons, notably naphthalenes and polynuclear aromatics, have been found to degrade such compounds more rapidly than *n*-alkanes. Alkane oxidizers are probably more abundant in oil-polluted soil and water owing to the preponderance of alkanes and other kinds of hydrocarbons having paraffin side chains.

Polynuclear Aromatic Hydrocarbons.--Much work has been done on the biosynthesis and biodegradation of polynuclear aromatic hydrocarbons, commonly designated PAH (24,32,45). Several such compounds are carcinogenic, particularly 20-methyl-cholanthrene, a few benz- and dibenz-anthracenes, and 3,4-benzpyrene (also called benz-a-pyrene, or BaP). The carcinogenic 3,4-benzpyrene is not to be confused with the non-carcinogenic 1,2-benzpyrene.

All of these carcinogens and many more non-carcinogenic PAHs have been shown to be degraded by various species of bacteria. Most are also metabolized to some degree by certain invertebrates and higher animals, including mice, rats, dogs, and cats (27,34,40).

The "fingerprints" or chromatograms of PAHs are generally more distinctive than for alkanes or benzene (36). This property has facilitated

the study of their metabolism. Some of the methyl-dibenz-anthracenes are oxidized by specific bacteria about as rapidly as liquid or solid alkanes when dispersed in aqueous media.

3,4-Benzpyrene seems to be the most abundant carcinogenic hydrocarbon in nature. It has been found in the cells, tissues, or organs of many microbes, higher plants, and animals (24,45). It is synthesized by several species of bacteria (22), algae, and higher plants (4,18). However, the major source of 3,4-benzpyrene and other PAHs in air, water, and soil is from the pyrolysis of fossil fuels and the incomplete combustion of other kinds of organic matter as in incinerators, forest fires, etc.

4. FACTORS AFFECTING THE MICROBIAL DEGRADATION OF OIL

The rate at which polluting oils are degraded depends on the chemical composition of the oil, the kinds and numbers of microbes present, and several interrelated environmental parameters.

Oxygen.--For practical purposes, free or dissolved oxygen is essential. From 3 to 4 mg of oxygen is required per mg of hydrocarbon completely oxidized to CO_2 and H_2O. Lesser quantities of oxygen are required when the hydrocarbon is only partly oxidized and when part of the hydrocarbon is converted into microbial biomass. Only a specialized few bacterial species are able to utilize sulfate-oxygen in energy-yielding reactions and not many of these assimilate hydrocarbons. The rate of hydrocarbon oxidation by sulfate-reducing bacteria seems to be very slow. Whether nitrate is used by hydrocarbon oxidizers as a hydrogen acceptor or as a source of nitrogen is controversial.

Dispersion of Oil.--The dispersion of oil in aqueous systems renders it more susceptible to enzymatic attack. Oil can be dispersed in water by emulsification, sonification, blenderization, surface-active agents or detergents, and by adsorption on solid surfaces such as chemically clean silica sand, glass wool, asbestos fibers, etc. An excess of certain detergents may be toxic or the surface tension of the medium may be reduced to a level not tolerated by microbes. A good many microbial species produce surfactants which tend to emulsify oil in water.

Most polluting oils have a tendency to spread rapidly on the surface of water. A gallon of crude oil may spread over an area exceeding one acre in 40 to 100 hr, forming a film about 0.0001 cm thick (3). Such films of oil are more vulnerable to autoxidation and microbial degradation. The spreading rate is influenced by viscosity, density, chemical composition, wind speed, current velocity, temperature, and other factors.

Temperature.--The microbial degradation of oil has been observed at temperatures ranging from the freezing point of seawater (around -2 C) to about 70 C. Most species are most active in the mesothermic range, 20 to 35 C. The rate at which oil is degraded is slower at lower temperatures. Psychrophilic bacteria have been shown to oxidize oil only 5 to 10% as rapidly at -1.1 C as mesothermic species do at 25 C. Most of the oil oxidizers that are active at near zero Celsius fail to grow at 20 C. Certain psychrophilic species are killed within an hour or two at [illegible] 25 C. An

obligate thermophilic bacterium which utilized *n*-tetradecane at 45 to 70 C has been described (20). Its optimum for growth was 55 to 60 C. Neither eurythermic nor euryhaline hydrocarbon oxidizers have been described.

Salinity Requirements.--Although a good many freshwater forms survive for prolonged periods in the sea, only a small percentage of freshwater or terrestrial species are able to reproduce in seawater (salinity around 3.5%). On the other hand, most marine species grow best in media at salinities ranging from 2.5 to 3.5% and poorly or not at all at salinities lower than 1.5 to 2%.

Turbulence.--Shaking or sparging with air tends to mix oil with water and to introduce atmospheric oxygen into cultures, thereby promoting the microbial degradation of oil. Therefore, in reporting quantitative results, one should always state whether conditions of incubation were quiescent or turbulent.

Organic Matter.--Low concentrations of certain organic compounds may promote the growth of hydrocarbon oxidizers by providing accessory growth factors, essential amino acids, or other requirements. Some microbial species have been induced to attack hydrocarbons by cultivating them in nutrient medium enriched with hydrocarbons. High concentrations of utilizable organic matter usually retard or mask the microbial degradation of hydrocarbons. When either oxygen uptake, CO_2 production, microbial growth, or change in pH is employed to indicate hydrocarbon oxidation, all other oxidizable materials must be excluded from the medium.

Concentration of Oil.--The concentration of crude oil in experimental media or in oil-polluted water influences results in numerous ways. When the concentration of oil is relatively low, all fractions are more likely to be attacked. At high concentrations, the components which are most susceptible to microbial degradation will probably be preferentially attacked. Moreover, the toxicity of the oil will be greater at higher concentrations, if it happens to contain water-soluble toxic substances.

Microbial Predators.--Cytophagic protozoans and other invertebrates in oil-polluted environments may limit the microbial population. A good many species of hydrocarbon-oxidizing bacteria and yeasts have been shown to be ingested by a large variety of ciliates and other grazing animals. Such predators may reduce the microbial population from 10^7 or 10^8/ml to only 10^2 or 10^3/ml.

5. CRITERIA FOR THE MICROBIAL DEGRADATION OF OIL

Several different criteria have been employed to demonstrate the microbial degradation of oil or specific hydrocarbons. Most of the methods are faulted for not being sufficiently quantitative and for not showing the degree or completeness of the oxidation. Some of the methods, notably oxygen uptake and microbial growth, are based on the premise that the mineral salts media contain no oxidizable substances except hydrocarbons. The results obtained by any method have little quantitative significance unless the temperature and other essential environmental conditions are specified.

Emulsification.--The emulsification of oil in aqueous media and its decreased adhesion to the walls of glass receptacles is one of the first and most conspicuous signs of microbial activity. However, not all kinds of hydrocarbon-oxidizing microbes emulsify oil and not all kinds of oils undergo such emulsification. The time required for emulsification to become evident tells something about reaction rates.

Disappearance of Oil.--In certain situations the disappearance of oil may be plainly apparent whether due to emulsification or degradation, but visual observations are neither unequivocal nor quantitative. Much more meaningful is the loss in weight of oil determined under carefully controlled conditions. Loss in weight determinations should be supplemented by chromatographic procedures to determine the amounts of various kinds of hydrocarbons which have been degraded. In such experiments one must use appropriate controls and carefully consider the possibilities of the evaporation of certain hydrocarbons, their autoxidation, and adsorption by organisms or other solids in the system.

Autoxidation of crude oil is an on-going process influenced by temperature, sunlight, and inorganic catalysts, especially vanadium (3). Emulsified oil and thin films are more susceptible to autoxidation than large coherent masses. Virtually all constituents in oils may undergo autoxidation (13,30). Hydroperoxides, organic acids, alcohols, esters, ketones, and other oxidation products may be formed. Autoxidation of crude oils in storage reservoirs was found to be from 10 to 50% as rapid as the biochemical oxidation rate (9). Although the rate of autoxidation, like the evaporation of volatile substances decreases with time, both processes continue almost infinitely in crude oil and refined fuel oils exposed to air. Many high boiling-point hydrocarbons slowly sublime or evaporate in open systems at ordinary atmospheric temperatures, e.g., naphthalene. This together with the increased content of oxidation products in "weathered" oils seems to contraindicate the use of "weathered" oils to standardize biodegradation testing procedures, as was proposed during the Workshop.

Oxygen Uptake.--Although beset with technical difficulties and limitations of usefulness, there are reasonably accurate chemical, manometric, and potentiometric methods for determining oxygen uptake by hydrocarbon oxidizers in closed systems. Unless aqueous media are supersaturated with oxygen (which has some disadvantages), the media will contain enough oxygen to oxidize only a few milligrams of hydrocarbons per liter. The rate of oxygen uptake may tell something about how rapidly oil is being oxidized, but it fails to tell how much oil is oxidized unless there is an excess of oxygen. When the oil or hydrocarbon concentration exceeds 3 or 4 mg/liter of aqueous medium, oxygen uptake tells little or nothing about the completeness of the oxidation. For example, 14 molecules (28 atoms) of oxygen could convert 28 hydrocarbon molecules into mono-hydroxy compounds or this same amount of oxygen could oxidize one molecule of nonane to CO_2 and H_2O.

Measuring Oxidation Products.--Determining the quantitites of various kinds of carboxyls, aldehydes, hydroxy compounds, esters, CO_2, and other intermediate or end-products of oxidation is an essential part of the problem. But measuring the amounts of such products may not indicate how much oil or hydrocarbon has been oxidized. Since the microbial oxidation of hydrocarbons

tends to decrease the pH, this property has been used to indicate whether such oxidation has occurred, but it is not a quantitative test for how much oil has been degraded.

Following the course of radioactive carbon in hydrocarbons and their oxidation products has only restricted usefulness. This also applies to the use of oxygen isotopes (O^{18}), which helped to demonstrate the oxidation of nongrowth hydrocarbons (12,23).

Microbial Growth.--Colony counts, increasing turbidity of culture medium, or direct microscopic counts are fairly good indicators of microbial growth. If microbes grow or reproduce in mineral media containing oil as the sole source of energy and building material, they must be assimilating the oil. At their best, though, such methods are only semi-quantitative. There is a wide variance in the percentage (from 20 to 60% or more) of the hydrocarbon that is converted into microbial biomass. Certain cultures continue to oxidize hydrocarbons after growth or reproduction has ceased. Presumably such reactions are catalyzed by pre-formed enzymes.

6. BIODEGRADATION PRODUCTS

Besides CO_2 and H_2O, the principal products resulting from the microbial degradation of hydrocarbons are various hydroperoxides, alcohols, phenols, carbonyls, aldehydes, ketones, and esters. The microbial oxidation of hydrocarbons is usually accompanied by the production of microbial biomass which may be decomposed by autolysis or by predators.

Metabolic Pathways.--Pathways for the metabolism of only a small number of hydrocarbons by relatively few microbial species have been investigated. Most of the literature on the subject has been reviewed elsewhere (1, 7,11,12,14,17,21,26,37,38). The metabolic pathways are so diverse that generalizations are not warranted at this time.

Especially needed is more information on the mechanisms whereby microbes oxidize aromatic hydrocarbons and closely related heterocyclic compounds, some of which contain a little sulfur, oxygen, or nitrogen. Cometabolism is only poorly understood. Also needing more attention is the virtually untouched problem of the ultimate fate and biological effects of intermediate oxidation products.

Fate and Effects of Oxidation Products.--There is a greater tendency for primary and intermediate oxidation products to accumulate in pure cultures than in mixed cultures. This is a commentary on the fact that not many species growing in pure culture convert hydrocarbon quantitatively to CO_2 and microbial biomass. Other things being equal, the more species in the system the greater are the possibilities of there being enzymes capable of catalyzing the oxidation of organic acids, alcohols, ketones, and other hydrocarbon oxidation products.

Whether products resulting from the biodegradation of polluting oil are produced and accumulate in sufficient concentrations to be injurious to aquatic organisms remains to be demonstrated. The probabilities seem remote except in closed systems such as test tubes or aquaria. Also yet to be

determined is whether any of the biodegradation products of carcinogenic hydrocarbons are more potent than the parent compounds under field conditions.

Co-metabolism.--There are instances of hydrocarbons, that do not provide for the growth of a given microbial species, being oxidized by this species that oxidizes another hydrocarbon for growth (12,19,23). Such co-metabolism, co-oxidation, or synergistic action may be of considerable importance in the biodegradation of oil. Nongrowth hydrocarbons may be oxidized to form compounds which may provide for the growth of other organotrophic microorganisms in mixed cultures such as commonly occur in oil-polluted water or soil. Many more studies should be made on such reactions, including observations on more varieties of hydrocarbons and on more microbial species.

Microbial Biomass.--An average of around half of the carbon in growth hydrocarbons metabolized by bacteria and yeasts is converted into cell substance or biomass. The latter consists largely of proteins, nucleic acids, amino acids, purines, pyrimidines, lipids, and polysaccharides. The nutritive value of such microbial biomass is generally high for phagotrophic animals and the decomposition products may nourish other organisms. Although mainly wholesome, there are a few reports of large doses of oil-oxidizing bacteria being harmful to certain phagotrophic animals. The practical question is whether toxic species could reproduce in oil-polluted waters in sufficient quantities to be injurious.

7. RATE OF OIL DEGRADATION

Conspicuous by its absence from the discussions in this Workshop as well as in published papers is meaningful information on the absolute rates of biodegradation of oil pollutants. This hiatus is a reflection of the lack of standard methods and criteria for measuring oil degradation rates. With few exceptions, only relative reaction rates are reported.

Rate Parameters.--The essential units for the expression of absolute rates of hydrocarbon or oil biodegradation must include the amount of substrate oxidized or otherwise degraded, the volume of the system, and the period of time for the observed action to occur. For example, one may report that a given oil was oxidized at an average rate of 0.9 mg/liter/day. So important is temperature in influencing relative reaction rates that this environmental parameter should always be specified.

Unless otherwise specified, it may be taken for granted that the oxidation reaction took place under aerobic conditions. However, it should be specified whether the reported rate occurred in an open (to the atmosphere) or a closed system and whether the latter was filled to capacity with medium. Free or dissolved oxygen may become a rate-limiting factor in a closed system after a few hours or days, depending on the density of the microbial population, temperature, and other conditions.

Along with temperature, one should specify whether conditions were quiescent or what kind of agitation occurred. In open systems particularly, one must employ appropriate uninoculated controls to indicate how much

degradation or loss of hydrocarbons has resulted from evaporation, sublimation, or autoxidation. In pilot plant and field experiments involving large volumes of water, the depth and/or area should be specified since the rate of evaporation, autoxidation, and microbial activity are often maximal at the air-oil-water interface.

Need for Standardization.--Only by the standardization of testing procedures and the standardization of units for expressing rates of microbial degradation of oil pollutants will it be possible to understand and compare the results obtained by various investigators.

8. PROBLEM OF OIL IN FOOD CHAINS

In oil-polluted environments animals may swallow oil droplets along with their normal food, including bacteria, yeasts, phytoplankton, zooplankton, etc., some of which may have adsorbed or ingested polluting oil.

Fate of Ingested Oil.--Part of the oil or its oxidation products may be incorporated in the cells, tissues, or organs of aquatic or soil organisms. Ordinarily most of the ingested oil passes through the digestive tracts of animals unchanged, like the liquid petrolatum used in human and veterinary medicine. (The laxative dose ranges from 50 to 100 ml for adult humans and up to a liter or two for cattle and horses.) Polluting crude and fuel oils may contain various amounts of harmful hydrocarbons. Some of these may be absorbed from the gut and either stored in cells or tissues or degraded in the liver or other organs.

The microbial degradation of hydrocarbons in the gut of certain animals has been reported. More investigations are needed on the extent of such reactions and especially on the toxicity of the degradation products. The metabolism and effects of carcinogenic hydrocarbons in experimental animals have been much more extensively studied than in microorganisms.

Biosynthesis of Hydrocarbons.--In considering the problem of the incorporation of hydrocarbons in the food chain, we must not overlook the biosynthesis of hydrocarbons. Numerous species of bacteria and a great variety of higher plants synthesize liquid and solid hydrocarbons, including carcinogens (4,5,18,22,25).

Earlier literature on the occurrence and biosynthesis of hydrocarbons in bacteria and plants has been reviewed elsewhere (8,16,31,45). Went (39) estimated that millions of tons of terpenes per year are released by terrestrial plants into the atmosphere where they undergo condensation and ultimate precipitation in snow and rain. From 0.1 ppm to > 1% of the carbon content of phytoplankton seems to consist of liquid and solid hydrocarbons. If the average content is only 10 ppm and the annual production of marine phytoplankton is 3×10^{10} metric tons (35), this would provide about 3 million tons of hydrocarbons a year available for incorporation in food chains.

9. ARTIFICIAL SEEDING OF OIL SPILLS

There seemed to be no unanimity in any of the panels on the question of seeding oil spills or artificial inoculation and fertilization. Proponents, including myself, favor further investigations, believing that there may be situations where inoculation might produce beneficial results either by speeding up biodegradation or by providing for the oxidation of a greater variety of hydrocarbons. Opponents argue that if environmental conditions are favorable for the growth of hydrocarbon oxidizers, indigenous and adventitious forms will naturally become established or inducible oxidases will be produced within a few days or weeks.

Possible Harmful Effects.--Opponents of seeding point to the possibilities of upsetting delicate balances among microorganisms in nature by introducing large numbers of alien species (29). Conceivably some of the artificially introduced species could be pathogenic or toxic for man or aquatic organisms. The high BOD of massive amounts of cultures might result in the development of anoxic conditions.

Polyvalent Cultures.--A good many different microbial species would have to be employed to provide for the degradation of the great variety of hydrocarbons in polluting oil. Compatible species would have to be employed. The species should be selected for maximum speed and efficiency in degrading predominant hydrocarbons in oil spills and for minimum toxicity. Psychrophilic varieties should be present for use at low temperatures, say, lower than 8 or 10 C. Mesothermic varieties would have to be prepared for use at higher temperatures. Most likely it would be necessary to have marine species for use in seawater and freshwater varieties for use in freshwater environments.

Shortcomings of Lyophilized Cultures.--Although lyophilization may seem to be a practical way to store and transport cultures, lyophilized preparations have some serious shortcomings. While a few cells among the billions per mg of lyophilized cells may survive for several years, the death rate of the majority is fairly rapid. The short life expectancy of individual cells would make it necessary to use either fairly freshly lyophilized cultures or relatively large amounts. The surviving cells in lyophilized preparations usually have relatively long lag periods for growth when transferred to suitable medium. Other methods for the preparation and transportation of polyvalent cultures should be investigated.

Fertilization of the Milieu.--For maximum growth and biochemical activity of hydrocarbon oxidizers in oil polluted water, it might be necessary to fertilize oil spills with phosphate and/or appropriate forms of nitrogen. Ammonium or urea would probably be best. The amounts to be introduced into the sea would be influenced by the amounts of phosphate and fixed nitrogen already present (nil to 50 µg/liter) and the amounts needed for microbial growth. Depending on oxidation rates and the rates of oxygen replacement by photosynthetic activity and solution from the atmosphere, the depletion of dissolved oxygen could become a limiting factor. The complete oxidation of 1 gram of mineral oil to CO_2 and H_2O would require all of the oxygen normally dissolved in 200 to 300 liters of seawater.

10. FATE OF OIL IN BOTTOM DEPOSITS

High density components of petroleum and hydrocarbons associated with suspended solids, including biomass, tend to sink to the sea floor. Unless dissolved oxygen or essential nutrients become limiting factors, the mud-water interface is usually the scene of greatest microbial activity, especially in shallow water and the intertidal zone. Turbulence in shallow water coupled with the photosynthetic activities of sedentary algae help to replenish the oxygen supply.

Microbial Populations.--In chronically oil-polluted regions, one or two orders of magnitude more oil oxidizers are often found per unit volume of oozes at the mud-water interface than in the overlying water. Aerobic hydrocarbon-oxidizing bacteria predominate. The abundance of such bacteria decreases rapidly with depth in bottom deposits. In situations where hydrocarbons are deposited on the sea floor more rapidly than they are degraded, they tend to be buried in the sediments. Anaerobic degradation by sulfate-reducing bacteria appears to take place at rates which are so slow that it is only of academic or geological interest.

The foregoing generalizations are based largely on the demonstration of various kinds of oil oxidizers in bottom sediments (46). Field studies should be conducted to determine the kinds and quantities of hydrocarbons and their degradation products in bottom deposits in chronically polluted regions.

Tarballs.--Tarballs constitute a special case of persistent oil pollutants on beaches and the sea floor. They consist of varying proportions of tars, waxes, asphaltenes, greases, oxidation products, etc., intermixed with silt, sand, shell fragments, skeletal remains, and other detritus. They are believed to be built around small chunks of bituminous material which accumulate concentric layers of material as they are rolled about by the surf on the sea floor. Chromatographic analyses of certain tarballs demonstrate the presence of specific components which indicate the source of the polluting oil. Others are highly nondescript. Some of the tarballs are beached by water movements and many more are transported to deeper water.

The relatively low surface area exposed to water, free oxygen, and microbial enzymes tends to protect tarballs from autoxidation or biodegradation. Most of the ether-soluble components of tarballs undergo microbial degradation when dispersed in seawater media. This can be demonstrated by dissolving about 100 mg of tarball material in 100 ml of spectrographic grade solvent such as ether or hexane. Ten ml of this solution is then introduced into each of several 100-200 ml glass bottles or flasks containing a layer of ignited silica sand, ground glass, glass wool, or asbestos fibers. After thorough mixing, the solvent is evaporated, leaving about 10 mg of tarball material dispersed on inert solid surfaces. Now appropriately inoculated seawater medium is introduced. After a few days incubation various tests can be made to demonstrate microbial growth and/or oxidation of the tarball material.

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A THRENODY CONCERNING THE BIODEGRADATION OF OIL IN NATURAL WATERS

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INTRODUCTION AND DISCUSSION

Crude oil, and many of its derivatives, is an extremely complex mixture of many chemical species. In one reference petroleum, Mair (15) found 234 hydrocarbons alone where individual concentration by volume varied between 2.3% and 0.0004%. Moreover, the mixture differs from oil field to oil field, and even from well to well. In addition, the sea receives many non-fossil hydrocarbons from the decay of its biota. Oil also appears in an array of physical forms, i.e., monomolecular films, films several centimeters thick, films on rock, sediment or suspended matter, or as water in oil emulsions or oil in water emulsions or as tar balls.

The sea considered as a medium for microbial growth varies in concentration of nutrients from place to place and from time to time in any one locality. Temperatures vary from Arctic cold to tropical heat. The microorganisms inhabiting the medium comprise many hundreds of species of bacteria, fungi and protozoa whose biology in the natural environment is largely unexplored. The term "microbial degradation of oil in the marine environment" means therefore the degradation of a complex and variable mixture of hundreds of substrates by unknown mixed populations of microorganisms, in an erratically changing medium. No wonder workers in this field sometimes complain of its difficulty.

The confusion is compounded by the fact that biodegradation is not the only means whereby the oil is changed chemically. Other processes, such as evaporation, solubilization, photo-oxidation and possibly other abiological mechanisms, operate at the same time. The task of untangling the skein of events is formidable and bold generalizations are clearly ill advised.

Some of the inconsistencies in the literature due to various workers approaching this problem in different ways have been pointed out already (6, 7). An attempt will be made here to examine the limitations of some of the methods in use to study this problem, and the difficulties of interpreting the data once they have been obtained.

PROBLEMS ARISING FROM THE DESIGN OF EXPERIMENTS

Unlike his counterpart in macrobiology, the microbial ecologist is limited in the field observations he can make. Even basic ecological parameters such as the number of organisms involved, or their biomass or their

taxonomic position, present many difficult problems. The micro-ecologist must perforce devise experiments which mimic the natural environment as closely as possible, and then he must cautiously extrapolate the results to the natural environment. These difficulties are particularly acute when it is desirable to obtain kinetic information. Paradoxically, a great deal of field work is now necessary in order to monitor his laboratory data and theories.

Unfortunately, there is a strong tendency in considerable oil degradation work to treat what is essentially an ecological problem as if it were solely a biochemical one. This can be illustrated by the manner in which the batch culture technique has been used. It is a characteristic of this technique that all substrates are in excess concentrations. Obviously, this is a desirable feature when growing bacteria for enzyme production, etc. At sea, even in the nutrient rich coastal waters, nutrients (especially nitrogen) establish the limits to bacterial growth. The problem is not solved by simply reducing the experimental nitrogen level down to sea water values, because the organisms rapidly take the concentration down to the limiting value. In contrast, at sea, the nitrogen is replenished so that the rate of change of concentration is much slower and depends upon a diversity of hydrographic and biological factors unrelated to oil degradation. In experiments some attempt must be made to replenish the nitrogen at a rate and in a chemical form that is found in nature.

Another useful feature of the batch culture method in biochemistry is the accumulation of intermediate and end products. Additions are sometimes made to the medium to enhance this effect. However, by changing the composition of the medium, accumulating intermediates may influence the rate of oil dissimulation by, for example, pH change or the increase in concentration of inhibitors or stimulants. This accumulation would not usually be expected to occur in the natural environment.

Again, batch culture methods are usually operated with pure or single (axenic) cultures, but sometimes a mixed culture, either a fortuitous natural mixture as from soil or water or an artificial mixture of several known pure strains, is preferred. Which of these alternatives represents situations in the marine environment most closely? Meadows and Anderson (16) examined marine sand grains and showed that while the whole grain could be considered as supporting a mixed culture, various facets and small areas were inhabited by colonies of single cultures. If an oil globule has the same population structure, then the degradation rate would be the resultant of the rates for each of the several pure cultures on its surface. In contrast, when a classical batch culture is seeded with a natural mixture of organisms a selection gradient is set up which has a number of the following consequences:

(a) Firstly, the mixture is unstable and rapidly changes so that one or two organisms become predominant. These will be species which grow most rapidly under the culture conditions used and are best able to use the carbon source provided. The "enrichment" of the desired bacteria has of course been a powerful tool in microbiological research and has been extensively used in selection of hydrocarbonoclastic bacteria (8,25). It is important to realize, however, that the whole environment, not the carbon source alone, is acting selectively. Incubation temperature, oxygen diffusion rates, etc., also play a part. Further complications are added in that in oil experiments a large number of possible substrates are added simultaneously so that a multiple

enrichment is occurring with organisms in competition for nutrients and growth factors, possibly forming antibiotics or acting synergistically. This "whole environment" pressure may be demonstrated by a pertinent illustration used by Van Niel (24) in a paper which should be read by anyone concerned with microbial ecological problems. Van Niel points out, "Söhngen found that on mineral agar plates, inoculated with soil suspensions and exposed to hydrocarbon vapours, colonies of saprophytic mycobacteria appear in abundance. . . . But in liquid elective cultures, inoculated with the same soil samples, one generally finds pseudomonads as hydrocarbon oxidizers."

(b) Secondly, there will be a succession of dominant organisms. Van Niel (12,24) again gives several illustrations from different environments. This succession is due initially to the removal of the most easily degraded substrates, and secondly to formation of byproducts of degradation within the medium. These byproducts may in turn be further degraded by other bacterial species. Many such intermediates are known from hydrocarbon digestion (1). Recently in this department it was noted that an old batch culture containing sea water with fuel oil recovered from a slick had turned bright green. Microscopic and other examinations showed that the color was due to a thriving culture of green sulphur bacteria. The latter are photosynthetic anaerobes requiring hydrogen sulphide. Presumably conditions in the culture had become anaerobic, hydrogen sulphide had been formed from the sulphur-containing substrates in the oil, or from sulphates in the sea water and the culture had been left in the light. On the basis of this evidence alone, it would be clearly misleading to classify green sulphur bacteria as "oil decomposers."

Since the greater part of the oil does not go into solution, but remains as discrete globules or films of various dimensions, experiments with oil do not conform to the usual pattern of batch cultures, which makes it even more difficult to interpret such experiments in terms of the natural environment. Well-established growth constants, i.e., the saturation constant, the yield factor and specific growth rate, that are used to describe the batch culture kinetics mathematically, all have reference to dissolved substrate and homogeneous media, which is clearly not the situation in oil experiments. Probably all that can be safely deduced from these batch culture experiments is that many microorganisms from the aquatic environment can, under laboratory conditions, degrade certain substances present in or derived from crude oil.

In view of these difficulties, microbial ecologists have examined the possibility of using continuous culture techniques (12). These have several clear advantages over batch culture. It is possible to control many factors such as nutrient concentration, pH and oxygen tension. There are, however, a number of disadvantages which may account for the fact that continuous culture has not been used extensively in oil degradation studies. The standard reaction vessel of a continuous culture does not have the best shape for this purpose, since the volume of water out of contact with the oil is much greater than that in contact with it. Because changes taking place in oil experiments are slow, lasting at least several days if not weeks, the dilution rate must be equally slow in order to monitor changes in nutrient concentration or oxygen tension in the sea water. Nevertheless, changes in oxygen tension between water entering and leaving an open system have been observed by Gibbs (personal communication) using a considerably modified apparatus.

Again, the biphasic nature of the system poses theoretical problems in the application of the kinetic constants based on substrate in solution, and practical difficulties in retaining the oil in the reaction vessel. The oil can be retained as a captive slick by running the medium into and out of the system, underneath the oil. This has some attraction as presumably this is what happens in a calm sea assuming there is some movement of the oil over the water.

Alternatively, the oil could be entrapped in an inert material. In both cases it is desirable to make provision for sampling the oil, and the bacteria both in the oil and at the water-oil interface. Continuous culture experiments would be adversely affected if the bacteria began to produce natural surfactants to emulsify the oil, thereby changing the surface area available to bacterial attack and possibly even allowing some of the substrate to be washed out. Continuous culture also exerts a selective pressure on a mixed culture (23); however, the selection made by a continuous culture is different from that induced by batch enrichment cultures (13).

Very few experiments have been devised which mimic the situation when oil is deposited on a beach. Johnson (14) obtained some valuable results using sand columns and natural sea water. Work in progress with similar objectives in this department has mostly served to demonstrate how little is known about the natural variations in chemistry and microbiology of a sea beach. After two years' study, it may now be possible to construct model beaches whose characteristics will be sufficiently close to those of a natural beach in order to make meaningful extrapolations.

As an alternative to laboratory studies various workers have successfully exploited a situation where accidentally spilt oil is trapped in sediment or retained within an enclosed area (e.g., 5,11). In open water, however, oil slicks are difficult to follow for more than a few hours and tend to disappear by wave action, so that the oil needs to be "tethered" by some means. A raft in Langstone Harbour, Portsmouth, England, has been adapted for this purpose, and floating oil has been retained under natural conditions for about one and a half years. Ideally, a number of such experiments operating under different hydrographical regimes are required.

INTERPRETATION OF THE DATA

Having decided on what methodological approach to employ, the next problem is how to measure the changes in the system and to decide what significance, if any, should be given to the changes. Basically three possible types of measurements are available: a) a change in bacterial or fungal numbers or biomass, an increase being interpreted as an acceleration in the rate of oil degradation; b) changes in a metabolite in the system which are interpreted in terms of changes in the oil; and c) measurement of changes in the oil itself. Of the aforementioned, (a) is likely to be most inexact and the most difficult to interpret.

(a) The difficulties encountered in enumerating microorganisms by well-established methods, such as the viable plate and M.P.N. techniques, are too well known to be reiterated here (17,19,20). Again the two-phase nature of oil experiments causes added complications since the bacteria may be found in both the aqueous phase, in the oil and at the interface. Extracting the

bacteria from the oil is difficult. Chemical biomass determinations, as for example, using adenosine triphosphate (10) are in an early stage of development and need further evaluation. Fungi (non-yeast forms), because they are present as mycelia rather than single cells, are difficult to estimate.

Even if these difficulties were removed, some problems would remain. It is generally agreed among microbial ecologists that it is "impossible to evaluate from numbers alone, the ecological significance of an organism in a given habitat" (4). The activity considered in terms of molecules of substrate "turned over" in unit time is not directly proportional to the change in cell numbers or in biomass. Numbers alone provide some indication of the relative turnover rates in two environments providing it is reasonable to assume that the two populations have the same structure and are equally active. Moreover, as Mitchell (18) has noted, a large bloom of microorganisms sets in motion intermicrobial predator mechanisms which operate to reduce microbial concentrations. Andrews (personal communication) has observed protozoa ingesting bacteria growing in a batch culture with Kuwait crude oil as carbon source.

(b) Alternatively, the rate of change in a metabolite in the system may be used and its variation taken to indicate a change in the oil. Oxygen is the most common metabolite involved because of the ease and comparative accuracy of the Winkler titration or oxygen sensitive electrodes. Because the oil is such a complex mixture, the estimations should be maintained for a long period; furthermore, it is undesirable to extrapolate because of increasing resistance to degradation of the successive residues. For the same reason, calculations based on an overall C.O.D. have their limitations. Another difficulty can arise when natural sea water is used. Batches of sea water taken from one place from time to time, and aged under identical conditions, were found to have different residual oxygen uptake even after four months storage. This can be partly overcome by collecting the water in large quantities at a sufficient distance offshore. The main disadvantage of oxygen uptake measurements is to interpret their significance in terms of oil biodegradation, for oxygen uptake can be due to abiotic as well as biological activity. In addition, there is no indication as to which part of the substrate mixture is being oxidized, or whether the components are being mineralized to carbon dioxide and water, or only as far as an intermediate.

The degree of mineralization can be measured by carbon dioxide evolution. This, however, is liable to error from several sources. Some 2% to 3% of the organic carbon in the cell biomass is accounted for by carbon dioxide fixation (21). In sea water the carbonate buffer system also introduces some sources of error. As with oxygen determinations, no information is obtained as to which substrate is attacked and whether intermediates are formed.

(c) Since the basic problem is to discover how the oil changes in time periodic chemical analysis of the oil itself is very desirable. But again there are difficulties, two of which arise in obtaining the sample. Securing a "representative" sample is always a problem in ecological work, and can only be solved by repeated sampling to demonstrate either the homogeneity or the heterogeneity of the oil. Secondly, the removal of salt water and other extraneous matter can be a nuisance, particularly with weathered oil or with emulsions. Very low levels of oil such as monomolecular layers can be difficult to deal with in this respect.

Having obtained a good sample it is first necessary to distinguish between fossil hydrocarbons and those of recent origin. Fortunately, this is not as difficult as would first appear (3). Beyond that, the method used depends upon the desired result, the sophistication of the analytical apparatus and the amount of time and money available. It is "fashionable" at the moment to utilize gas chromatographic techniques showing the removal of alkanes. This is very valuable, but it must also be remembered that the alkanes constitute only a fraction of the total hydrocarbons, even in the most alkane-rich oils. Of the 234 hydrocarbons listed by Mair (15), thirty-three were *n*-alkanes, accounting for approximately 24% of the oil by volume. About 16% by volume was made up of alkanes with boiling points below 235 C and would evaporate in a day or so in temperate and tropical waters. The remaining 7% would be expected to be biodegraded. Thus, after evaporation (presumably to be reversed by being washed down in rain elsewhere) and biodegradation, 75% of the oil still remains!

Weighing of residual oil has the attraction of simplicity. Unfortunately the net weight is not solely due to oil loss but to gains in weight due to oxidation. Nevertheless, if details of the changes in specific components are not acquired, it is probably the most useful when many samples have to be processed.

If the circumstances permit, it is advisable to monitor the oil changes in several ways as several recent authors have done (e.g., 2).

CONCLUSIONS

This essay has been entitled a *threnody*, that is, a "song of lamentation." It is not by any means a song of despair! Rather, it is largely a plea for a more ecological approach to the problem of oil degradation--an insistence that when oil is spilled into water, the physics, chemistry and biology of the water must be taken into account. New ideas and new apparatus are required such as Gibbs (9) has used in his semi-open system as a first approach to the problem, and its later developments (personal communication). Other possible ways of measuring heterotrophic activity, as yet unexplored in oil degradation, were suggested by Strickland (22). Also implied in the present paper is that research on this topic requires the cooperation of microbiologists, oil chemists and marine chemists. It is also a plea for recognition of our ignorance, and of the complexity of the problem. Broad generalizations, whether optimistic or pessimistic, are out of place. Our knowledge of what part microorganisms play in the ecology of estuaries, coastal waters and the deep oceans is at best sketchy. A great deal of hard, but exciting work lies ahead.

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HYDROCARBON UTILIZATION BY *CLADOSPORIUM RESINAE*

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The filamentous fungi include a large number of diverse species and strains which can grow at the expense of hydrocarbons. Nevertheless, in comparison to knowledge of hydrocarbon-using bacteria and yeasts, relatively little is known of hydrocarbon-using filamentous fungi. Considerable fundamental information must be obtained in order to establish the role of fungi in hydrocarbon-containing ecosystems and to assess their potential in the treatment of oil-polluted environs.

We have concentrated on a single organism, *Cladosporium (Amorphotheca) resinae*, which is one of the most prominent hydrocarbon-using fungi. *C. resinae* is considered part of the normal soil microflora (17), and can be isolated from marine and estuarine waters (1), from fresh water (22) and from sewage sludge (3). It has been called the "creosote fungus" because of its frequent isolation from wood impregnated with creosote. The term is probably a misnomer, since the organism does not use creosote. However, while other organisms are inhibited or killed by creosote, *C. resinae* survives. The fungus is also known as the "kerosene fungus" because it is a principal contaminant of jet fuels. The role of *C. resinae* in contaminated jet fuel and its biology were reviewed by Parbery (18).

This paper summarizes some of the work from our laboratory designed to answer basic questions about *C. resinae*.

METHODS AND RESULTS

Organisms.--The two strains of *C. resinae* used were isolated from contaminated jet fuel systems. They have been deposited with the A.T.C.C. and have been assigned accession nos. 22711 and 22712, respectively. Media and culture conditions have been described (4,5,6).

Substrates Utilized.--Each strain was tested for ability to grow on 55 individual hydrocarbons as sole source of organic carbon with NH_4NO_3 as the nitrogen source. One strain grew on 31 of the compounds and the other on 33 (4). Some representative data are presented in Table 1. As with many hydrocarbon-using organisms, growth was best on *n*-alkanes of intermediate chain length. Compounds which supported growth include alkanoic alcohols

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and acids, *n*-alkenes, and some cyclic and aromatic compounds. Growth was slower on hydrocarbons than on carbohydrates or amino acids. Hexadecanoic acid was the best substrate tested, supporting four to five times as much total growth as glucose, glutamate or casein hydrolysate. However, growth was slower, i.e., requiring 20 days to reach a stationary phase in shaken flasks versus 4 to 6 days on glucose or amino acids.

TABLE 1

Growth of *C. resinae* on Glucose, on Amino Acids and on Hydrocarbons

Carbon source	Quiescent cultures		Shaken flasks	
	Growth (mg dried cells per 100 ml)	pH at harvest[a]	Growth (mg dried cells per 100 ml)	pH at harvest[a]
Glucose	150	5.3	370	3.2
Glutamic acid			400	6.2
Casein hydrolysate			380	7.3
Dodecane	105	3.0	28	5.4
Hexadecane	57	3.4	47	5.5
1-Dodecanol	8	5.0	47	5.5
1-Hexadecanol			102	5.0
Dodecanoic acid	51	4.6	40	4.6
Hexadecanoic acid			1540	3.7
Dodecene-1	8	5.5		
Cyclohexane	3	5.4		
Benzene	4	5.4		
o-Xylene	5	5.5		

[a]Glucose and amino acid cultures were harvested after 4-6 days incubation, hexadecanoic acid cultures were harvested after 20 days, and other hydrocarbons after 32-36 days. In all cultures the pH was adjusted to 6.0 after addition of carbon source.

Growth on glucose or hydrocarbon was accompanied by decreased pH in the medium. Agitation during incubation increased growth and acid accumulation in some instances and decreased them in others (Table 1). Similar apparently anomalous results have been reported in yeast fermentations (20). Of six pesticides examined, none served as sole carbon source (4). Aldrin and malathion enhanced growth on hexadecane; and aldrin, dieldrin, DDT, and malathion enhanced growth on glucose, suggesting that high concentrations of pesticides which can occur in oil slicks (21) would not inhibit oil degradation by *C. resinae*.

Cells did not grow on alkane aldehydes, but polarographic determination of oxygen consumption indicated that the aldehydes were oxidized by glucose-grown cells (24). Hexane supported only limited growth; it was not oxidized by whole cells and it repressed endogenous oxygen consumption.

Moreover, hexane interfered with hexadecane oxidation and phase contrast micrographs suggest that it may exert a solvent effect on cell membranes such as indicated for bacteria (12). Hexane, hexanol, and hexanal were oxidized by cell-free preparations, however, supporting the conclusion that short chain compounds exert a toxic effect on cell membranes (24).

In preliminary screening of substrates for cooxidation, four oxidizable substrates were examined in combination with each of 12 potential co-substrates. Each oxidizable substrate was screened for increased oxygen consumption in the presence of the potential co-substrate (23). Seven combinations showed enhanced oxygen uptake (Table 2). Cells and culture fluids from growing cells, from resting cells and from cell-free preparations were extracted with solvents and subjected to gas chromatography. No cooxidation products were observed. Two of the potential co-substrates were examined to establish the basis for their stimulation of oxygen consumption. Toluene and *p*-xylene were added individually to cells oxidizing C^{14}-hexadecane. In each test, $C^{14}O_2$ production was increased 12-30% by the addition, and the amount of radioactivity assimilated into cell material increased 12-25% (23). Thus, certain non-metabolizable hydrocarbons appear to stimulate oxidation and assimilation of other hydrocarbons, probably by increasing permeability.

TABLE 2

Combinations of Substrates which Yielded Increased Respiration

Substrate	Potential co-substrate	$Q^a_{O_2}$
n-Hexadecane	none	4.3
n-Hecadecane	cyclohexane	11.7
n-Hexadecane	*p*-xylene	21.2
n-Hexadecane	phenol	6.3
n-Hexadecane	cyclohexane	4.5
n-Hexadecane	toluene	23.4
Glutamate	none	4.0
Glutamate	anthracene	4.2
Glutamate	naphthalene	4.2
Glutamate	phenanthrene	4.2

$^aQ_{O_2}$ = µl oxygen consumed per milligram cell dry weight per hour. Values are corrected for endogenous oxygen consumption.

Alkane Utilization.--Additional aspects of alkane utilization have been examined. The enzymes for *n*-alkane oxidation are constitutive because: i) cells culture on glucose consumed oxygen without a lag when transferred to hydrocarbons or their oxygenated derivatives, ii) cycloheximide did not inhibit oxygen uptake when cells were transferred from glucose to hydrocarbons, iii) hydrocarbon-grown cells did not have higher Q_{O_2} values on hydro-

carbons than did glucose-grown cells which were transferred to hydrocarbons, iv) isocitrate lyase levels were not higher in hydrocarbon-grown cells than in glucose- or glutamic acid-grown cells (24).

The major pathway for initial oxidation of alkanes involves

$$RCH_2CH_3 \xrightarrow{NAD(P)H} RCH_2CH_2OH \xrightarrow{NAD(P)} RCH_2\overset{O}{\overset{\|}{C}}H \xrightarrow{NAD(P)} RCH_2COOH.$$

This conclusion is based on studies with hexadecane wherein we observed: i) oxidation of the compounds by whole cells, ii) stimulation of oxygen uptake by the appropriate co-enzymes in cell-free preparations, iii) accumulation of the appropriate reduced co-enzymes in the presence of cyanide or azide in cell-free preparations, iv) recovery of 65-85% of the radioactivity supplied as C^{14}-hexadecane in the presence of trapping amounts of any one of the proposed intermediates (25).

Cellular Lipids.--Cells were cultured on glucose or individual *n*-alkanes and the total cellular fatty acid composition was determined. As with other fungi, the predominant fatty acids were 16:0, 18:1 and 18:2 (6). Cells grown on 10-, 11-, or 12-carbon *n*-alkanes showed little or no correlation between growth substrate and homologous or related fatty acids (Table 3). Cells grown on *n*-tridecane or *n*-tetradecane showed slight (*ca.* 6%) correlation between growth substrate and cellular fatty acids. Thus, fatty acids produced from *n*-alkanes appear to be metabolized via β-oxidation and cellular fatty acids are synthesized *de novo*.

TABLE 3

Effect of Carbon Source on Homologous Cellular Fatty Acids

Fatty acid methyl ester	Percent of total fatty acids in cells grown on					
	Glucose	Decane	Undecane	Dodecane	Tridecane	Tetradecane
10:0[a]	-	-	-	-	-	-
11:0	tr	tr	tr	-	-	-
12:0	tr[b]	-	tr	tr	-	-
13:0	tr	-	tr	-	1.2	-
14:0	tr	tr	tr	tr	0.9	6.5
15:0	0.7	tr	0.5	tr	2.2	tr
Total odd-carbon fatty acids	2.2	1.8	3.2	1.9	8.4	tr

[a]Carbon number: number of double bonds.

[b]Less than 0.5% of total peak area was considered trace (tr).

Cellular phospholipids were characterized by thin-layer chromatography and by identification of water-soluble esters resulting from mild alkaline hydrolysis. Major phospholipids of glucose- or *n*-alkane-grown cells were phosphatidyl ethanolamine, phosphatidyl choline, phosphatidyl serine, and cardiolipin or a cardiolipin-like compound (14)

The total cell lipid and cellular hydrocarbon content were 2-3 times greater in hydrocarbon-grown *C. resinae* than in cells cultured on glucose or on glutamic acid (14,26). Extracellular hydrocarbons were removed and cellular hydrocarbons were extracted and analyzed by gas chromatography. No unsaturated hydrocarbons were detected. In cells grown on glucose, *n*-hexadecane (*n*-16) and a branched 17-carbon compound (br-17) comprised 98% of the cellular hydrocarbons. In glutamate-grown cells, *n*-13, *n*-14, *n*-16 and br-17 constituted 76% of the hydrocarbons. Cells cultured on *n*-decane or *n*-undecane showed no correlation between cellular hydrocarbon and hydrocarbon growth substrate, but cells cultured on 12-, 13-, 14-, 15-, or 16-carbon *n*-alkane contained that alkane as their principal cellular hydrocarbon (Table 4). The total of cellular hydrocarbons with an odd number of carbons was significantly higher in cells grown on odd-carbon hydrocarbons (C-11, C-13, C-15) than in cells cultured on even-carbon hydrocarbons (C-10, C-12, C-14, C-16).

TABLE 4

Relation Between Hydrocarbon Growth Substrate and Homologous Cellular Hydrocarbons

Cellular hydrocarbon	Percent of total hydrocarbons after growth on						
	Decane	Undecane	Dodecane	Tri-decane	Tetra-decane	Penta-decane	Hexa-decane
n-10	0.4						
n-11		0.1					
n-12			77				
n-13				78			
n-14					65		
n-15						92	
n-16							61
Total odd-C hydrocarbons	2.4	49	11	83	27	93	33

DISCUSSION

C. resinae resembles a number of hydrocarbon-using bacteria and yeasts in that it grows slowly on hydrocarbons. Moreover, limited growth is obtained on cyclic and aromatic compounds. However, the two strains examined do grow on a variety of substrates. Combinations of hydrocarbons and other nitrogen sources may enhance activity and are under examination. Moreover, somatic growth alone may not be an adequate criterion. In one experiment

using C^{14}-hexadecane, 93% of the hydrocarbon metabolized was oxidized to CO_2 and only 7% was assimilated. If similar data are obtained with other substrates, the organism may be a useful "seeding" organism, since considerable hydrocarbon could be mineralized without accumulation of a large biomass.

Other questions should also be posed. An organism which can metabolize a compound in pure culture when that compound is the sole carbon source available, and when nitrogen is not limiting, may not metabolize the compound in mixed culture with a number of alternate substrates present and when nitrogen is limiting. Contrariwise, symbiotic relations may exist between organisms, cometabolism may occur, and certain compounds may enhance oxidation of other compounds, as with toluene and *p*-xylene stimulation of hexadecane metabolism in the present study. The constitutive nature of *n*-alkane pxidation suggests that a prior period of adaptation is not necessary, and that *C. resinae* would have a competetive advantage over a number of other hydrocarbon users in which hydrocarbon-oxidizing systems must be induced.

Comparison of cells grown on glucose with cells grown on *n*-alkanes indicates that cellular lipids are affected by growth substrate; *n*-alkane-grown cells contain more total lipids. Some bacteria and yeasts show a correlation between alkane growth substrate and cellular fatty acids (7-9,15,16) while others do not (2,10,13,19). To our knowledge, *C. resinae* is the only filamentous fungus examined in this regard; we reported that growth on *n*-alkanes does not greatly affect total cellular fatty acids (6). The major phospholipids are the same in glucose- or *n*-alkane-grown cells, but fatty acids varied from one phospholipid to another and were influenced by growth substrate. These variations will be the subject of a future report.

Growth substrate had a marked effect on cellular hydrocarbons. Most fungi contain a range of hydrocarbons in which C_{18-22} and C_{27-31} predominate. Cells grown on glucose or glutamate do not fit this pattern. Cells grown on C_{11} to C_{15} *n*-alkanes yielded no cellular hydrocarbon longer than C_{28} (26). The strong correlation between cellular hydrocarbons and *n*-alkane growth substrates of C_{12} or longer are consistent with the view that *C. resinae* transports *n*-alkanes into the cell and that the initial oxidation is intracellular, as demonstrated for other microorganisms elsewhere in this volume (11).

Cladosporium resinae accumulates hydrocarbons, assimilating and oxidizing them by mechanisms that have been demonstrated for a number of bacteria and yeasts. Results summarized here may provide a beginning for evaluation of the role of *C. resinae* in aquatic and terrestrial environs, and for determining its potential as a seeding organism for oil slicks. Of equal importance with surface slicks, however, is the need to deal with accumulations of hydrocarbons in sediments. The most refractory hydrocarbons are deposited where they can be toxic to benthic organisms and thus provide a reservoir from which they may enter the food chain. Such bottom accumulations are particularly important in shallow water systems such as lakes, streams, marshes and estuaries.

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MICROBIAL DEGRADATION OF AROMATIC HYDROCARBONS

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Bacteria capable of growth on benzene, toluene, ethylbenzene, naphthalene and biphenyl were isolated from soil. Mutagenesis led to the isolation of different bacterial strains that will accumulate the initial products of hydrocarbon oxidation. The results obtained suggest that *cis*-hydroxylation is a common reaction in the microbial oxidation of aromatic hydrocarbons.

Aromatic hydrocarbons are derived almost exclusively from crude petroleum. The toxicity of benzene and its alkyl-substituted derivatives is well known (3). In addition, certain polycyclic compounds are potent carcinogens. Aromatic hydrocarbons are used extensively in fuels, as industrial solvents, and in the synthesis of dyes, polymers, explosives, pesticides and many other products of everyday use. Consequently, it is not surprising that these aromatics find their way into the environment where they are regarded as environmental pollutants. Sources of such pollution include automobile exhaust fumes, oil deposition (accidental, natural, or otherwise) and emissions from chemical plants. It has been stated that the key to the biodegradation of oil lies in the isolation of microorganisms that will preferentially attack aromatic hydrocarbons (2).

Mammals oxidize aromatic hydrocarbons to arene oxides which can add water to form *trans*-dihydrodiols or glutathione to form premercapturic acids. Arene oxides can also isomerize to phenolic products by a reaction mechanism called the NIH shift (12). It is significant to note that arene oxides of carcinogenic hydrocarbons are more carcinogenic than the parent compounds (1).

Certain bacteria are capable of utilizing aromatic hydrocarbons as the sole source of carbon and energy for growth. *Pseudomonas putida* can utilize benzene, toluene and ethylbenzene as growth substrates. The enzyme that catalyzes the initial oxidation of the aromatic nucleus has been partially purified from toluene-grown cells (8). Subsequent studies have shown that the enzyme is extremely sensitive to air. The enzyme was prepared in 0.05 M KH_2PO_4 buffer, pH 7.2, containing 10% (v/v) ethanol. Immediately after preparation the extract was stored under nitrogen at 4 C. Extracts prepared in this manner lost activity over a period of seven days. The enzyme has a broad substrate specificity indicated by its ability to oxidize a variety of different hydrocarbons (Table 1). The effect of different halogen substituents on toluene oxidation is shown in Table 2. When *P. putida* was grown on glucose no enzyme activity could be detected.

TABLE 1

Oxidation of Aromatic Hydrocarbons
by Toluene Dioxygenase[a]

Substrate	Relative Activity (%)
Toluene	100
Benzene	46
Ethylbenzene	82
Propylbenzene	73
Butylbenzene	63
Isopropylbenzene	63
Isobutylbenzene	53
Sec-butylbenzene	49
Tert-butylbenzene	47

[a]Reaction mixtures contained in a final volume of 2.0 ml: KH_2PO_4 buffer, pH 7.2, 75 µmoles; $FeSO_4 \cdot 7H_2O$, 0.2 µmole; NAD^+, 0.8 µmole; ammonium sulfate extract, 16 mg of protein and substrate 0.2 µmole in 10 µl of 95% ethanol. Oxygen consumption was measured polarographically with a Clark oxygen electrode. Results are corrected for endogenous absorption in the absence of substrate.

TABLE 2

Oxidation of Substituted Toluenes
by Toluene Dioxygenase[a]

Substrate	Relative Activity (%)
Toluene	100
p-Fluorotoluene	48
p-Chlorotoluene	34
p-Bromotoluene	24
p-Iodotoluene	21
o-Xylene	23
m-Xylene	24
p-Xylene	36
p-Ethyltoluene	18
p-Cymene	5

[a]Conditions as described in Table 1.

To gain some insight into the metabolic intermediates involved in degradation of the above hydrocarbons, *P. putida* was subjected to mutagenesis with nitrosoguanidine. This treatment led to isolation of a strain, *P. putida* 39/D, that could no longer utilize benzene, toluene, or ethylbenzene as growth substrates. When *P. putida* 39/D was grown with glucose in the presence of benzene, *cis*-3,5-cyclohexadien-1,2-diol (*cis*-benzenediol) accumulated in the culture medium. The *cis*-benzenediol was isolated and shown to be identical to a synthetic sample. An enzyme present in the parent strain of *P. putida* oxidized *cis*-benzenediol to catechol. *P. putida* 39/D was incubated with benzene in the presence of $^{18}O_2$. The *cis*-benzenediol formed was isolated and shown by mass spectroscopy to contain two atoms of isotopic oxygen (5). Thus, the enzyme catalyzing the initial oxidation of benzene is a dioxygenase. Toluene and its para-substituted halogenated derivatives and ethylbenzene were oxidized by *P. putida* 39/D to *cis*-dihydroxycyclohexadiene derivatives (Table 3). The products from each substrate were isolated and characterized by conventional organic techniques (6,7,10). The absolute stereochemistry of the product formed from toluene was shown to be (+)-*cis*-3-methyl-3,5-cyclohexadien-1(S),2(R)-diol by X-ray analysis.

TABLE 3

Products Formed from Substituted Aromatic Hydrocarbons by *P. putida* 39/D

Substrate	Product
Toluene	*cis*-3-methyl-3,5-cyclohexadien-1,2-diol
Ethylbenzene	*cis*-3-ethyl-3,5-cyclohexadien-1,2-diol
p-Fluorotoluene	*cis*-3-methyl-6-fluoro-3,5-cyclohexadien-1,2-diol
p-Chlorotoluene	*cis*-3-methyl-6-chloro-3,5-cyclohexadien-1,2-diol
p-Bromotoluene	*cis*-3-methyl-6-bromo-3,5-cyclohexadien-1,2-diol

Naphthalene was utilized as a growth substrate by an unidentified *Pseudomonas* sp. Mutagenesis with nitrosoguanidine led to the isolation of a strain that oxidized naphthalene to 1,2-dihydro-1,2-dihydroxynaphthalene. Previous literature reports identified *trans*-1,2-dihydro-1,2-dihydroxynaphthalene as an intermediate in naphthalene degradation (13). However, the product formed by the *Pseudomonas* sp. was firmly identified as (+)-*cis*-1(R),3(S)-dihydroxy-1,2-dihydronaphthalene (11). The structure was assigned on the basis of proton magnetic resonance spectroscopy, formation of an isopropylidine derivative and reduction to (-)-2(S)-hydroxy-1,2,3,4-tetrahydro-

naphthalene. Four different microorganisms oxidized naphthalene through *cis*-1,2-dihydroxy-1,2-dihydronaphthalene. One of these bacteria, *Pseudomonas* NCIB 9816, was previously reported to oxidize naphthalene through *trans*-1,2-dihydroxy-1,2-dihydronaphthalene. An enzyme from the *Pseudomonas* sp that catalyzes the oxidation of (+)-*cis*-naphthalenediol to 1,2-dihydroxynaphthalene has been purified to homogeneity. The enzyme is specific for the (+)-isomer and catalyzed a 93% resolution of a synthetic sample of (±)-*cis*-1,2-dihydroxy-1,2-dihydronaphthalene.

Biphenyl is utilized as a growth substrate by an organism that has been tentatively identified as a *Beijerinckia* species. The bacterium will grow at pH 3.5 under nitrogen-fixing conditions. Mutagenesis with nitrosoguanidine resulted in the isolation of a strain, *Beijerinckia* B8/36, tbat accumulated a hydroxylated cyclohexadiene derivative from biphenyl during growth on succinate. The product was isolated and identified as *cis*-3-phenyl-3,5-cyclohexadien-1,2-diol. The ability of this product to give an isopropylidine derivative with 2,2'-dimethoxypropane provides strong evidence for a *cis*-configuration of the hydroxyl groups in the isolated product (9).

It is apparent that *cis*-hydroxylation of aromatic hydrocarbons by bacteria is a common phenomenon (4). In Figure 1 are shown *cis*-diols that have been isolated and identified as metabolic intermediates in the bacterial oxidation of different aromatic hydrocarbons.

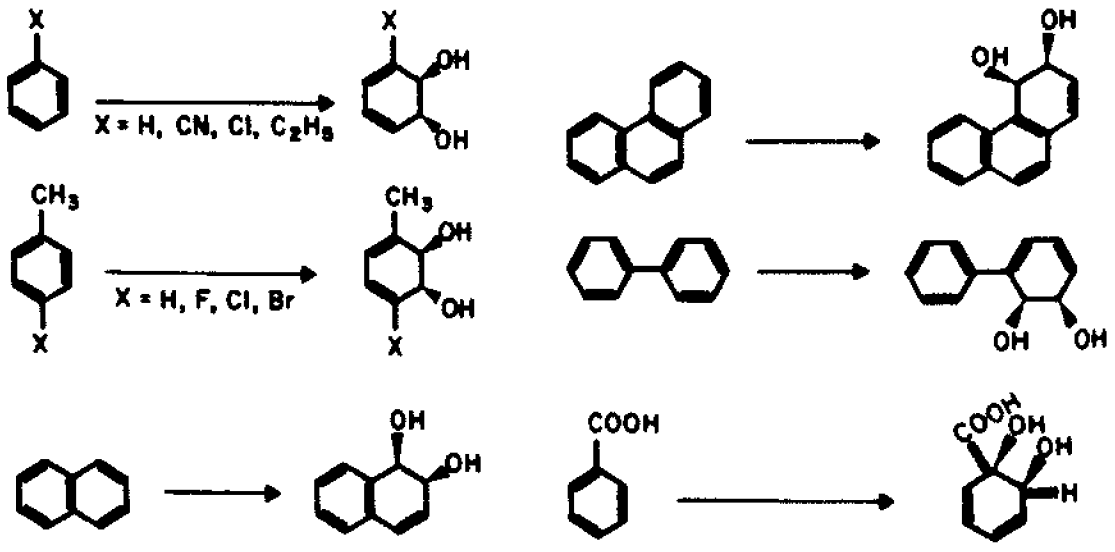

Figure 1. *cis*-Dihydrodiols Produced by Bacteria

Differences in mechanisms utilized by bacteria and mammals to oxidize aromatic hydrocarbons are illustrated in Figure 2. Mammals incorporate one atom of molecular oxygen into aromatic hydrocarbons with the formation of arene oxides. Subsequent enzymatic addition of water leads to the formation of *trans*-dihydrodiols. The latter compounds can undergo $NADP^+$-dependent oxidation to catechols. Bacteria utilize both atoms of molecular oxygen to form *cis*-dihydrodiols. The cyclic peroxide (dioxetane) intermediate is hypothetical. Oxidation of *cis*-dihydrodiols is NAD^+-dependent and, as with the mammalian system, also leads to formation of catechols. The mechanisms of enzymatic oxygen fixation remain unsolved, and probably will not be resolved until purified enzyme systems are available.

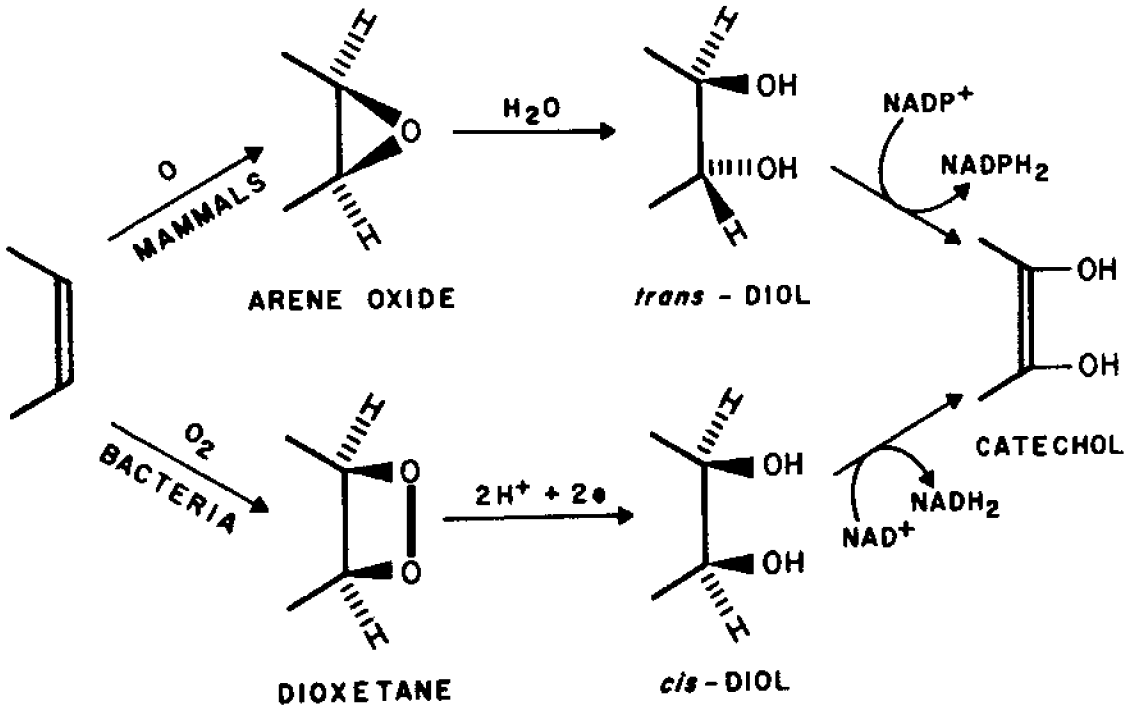

Figure 2. Initial Reactions Utilized by Mammals and Bacteria to Oxidize Aromatic Hydrocarbons

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MICROBIAL DEGRADATION OF OIL AND HYDROCARBONS IN CONTINUOUS CULTURE

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Preliminary results are presented which support the proposed use of continuous culture techniques to study oil degradation. Experiments with both pure and mixed bacterial populations have shown that slow but significant degradation rates can be obtained with an undisturbed, two-phase (water-hydrocarbon) system.

INTRODUCTION

The rate of degradation of oil slicks in aquatic ecosystems depends on a large number of environmental factors, including proper oxygen concentration, availability of inorganic nutrients, degree of dispersal and emulsification, removal rate of toxic end products, and optimal conditions of temperature and pH (7). The study of microbial oil degradation under laboratory conditions has classically involved use of batch culture techniques. These techniques, though very useful experimentally, are greatly limited in their application to natural environmental situations because many of the above factors exert a time-dependent influence on degradation rates and are difficult to monitor and control in batch culture (6). The closed nature of the batch culture also artificially affects the succession of microorganisms involved in oil degradation. Significantly, these successions, and the competitive factors which control them, are a function of the batch culture technique itself and do not reflect the inherent limitations present in the natural environment (3).

Study of the degradation of oil and petroleum hydrocarbons is further beset by problems related to the water insolubility of such compounds. Thus, in order to increase availability to the hydrocarbonoclastic microorganism, it is necessary to mechanically disperse or emulsify the substrates. In this regard, batch culture techniques work very well and, as a result, force oil degradation studies to be carried out in closed systems.

The use of continuous culture techniques has only recently been applied to microbial ecology and the study of various degradative processes (4). This is due, in part, to the application of continuous culture theory to pure culture-single substrate systems and has therefore found minimal use in study of mixed populations and their degradation of complex organic materials. It

is important, however, to emphasize that continuous culture offers several advantages over batch culture in study of natural systems (3,6).

First, since one is dealing with an open system, bacterial population densities can be regulated. The regulation of this biomass is based on growth conditions which are not time-dependent as they are in batch culture. Thus, size, composition, and change within a microbial population are functions of: a) continuous culture conditions (1), b) the nature of the substrate and its availability to the organisms, and c) the interaction of members of the population with themselves and their surroundings (2).

Secondly, toxic products and metabolic wastes will not accumulate in a continuous culture system and their effect on the degradation process will not increase with time. This situation is typical of a natural aquatic environment where a continual removal or dilution of these products is expected.

Thirdly, bacterial succession involved in the degradation of oil can be more easily studied and monitored (5). As Jannasch has shown (3), the chemostat will enrich for specific metabolic types depending on the particular conditions employed. With a mixed population and a complex organic substrate such as oil, several enrichments will occur simultaneously. These can be followed by examining the population and/or observing chemical changes in the substrate itself.

The study of the degradation of petroleum hydrocarbons in a continuous culture system presents a number of complicated problems, the most obvious of which is the water insolubility of the substrate. The purpose of this paper is to describe a continuous culture system, developed in our laboratory, which eliminates many of these problems and allows examination of oil degradation in an open versus a closed system.

MATERIALS AND METHODS

The design of a continuous culture system to study insoluble substrates, such as hydrocarbons, was based on maintaining a stable two-phase system in which hydrocarbons float on the surface of a water column. The water, or nutrient solution, is then continuously passed underneath the hydrocarbon layer. To obtain basal rates of degradation, emulsification of the hydrocarbon was kept to a minimum. To supply sufficient oxygen to the water phase, constant aliquots were continually cycled out of the growth vessel, aerated, and then recycled back into the growth vessel. The system employed is described in Figure 1.

For initial tests of the workability of the system, a single hydrocarbon, octane, was used as the sole carbon and energy source. Inflowing medium contained NH_4Cl (100 mg/l), phosphate buffer (100 mg/l, pH 7.2), $MgSO_4$ (10 mg/l) and distilled water. To monitor disappearance of the octane layer, a fat soluble dye (Sudan III) was added to the octane at a concentration of 50 µg/ml. Rates were measured as a function of increasing dye concentration in the octane layer.

Cell numbers were determined by bacterial enumeration using plate count agar (Difco) and/or minimal salts agar in an octane atmosphere.

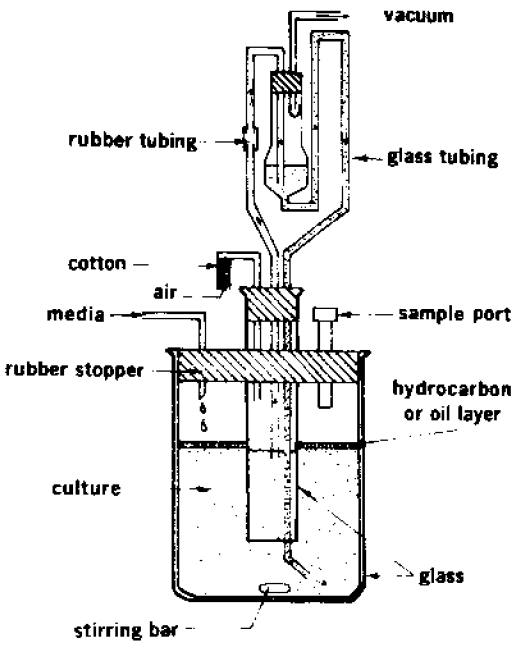

Figure 1. Continuous Culture System

RESULTS

Evidence demonstrating that hydrocarbons can be degraded in an undisturbed two-phase continuous culture system has been derived from both pure culture and mixed culture experiments. Using isolate J-10 in pure culture (obtained from batch culture enrichments on octane), degradation rates of 16.2 µg of octane consumed per hour were obtained. This rate was determined by following the decrease in percent transmittance (550 µm) of the Sudan III dye in the octane layer. The results are shown in Table 1.

As can be seen from the control system, the removal of octane was not due to solubilization or evaporation. This observed rate appears to be a function of the ability of the bacterium to degrade the octane at the hydrocarbon-water interface *without* the aid of emulsification or physical dispersion. It therefore represented a minimal rate.

Evidence for actual degradation of the octane was derived from various observations. First, within 18-24 hours after inoculation, pH decreased rapidly to values of 4.5 and lower. As a result, phosphate buffer was needed to prevent cessation of growth. The pH drop presumably resulted from the oxidation of the octane to fatty acid end products. Secondly, a steady state population (7×10^8 cell/ml) could be maintained. When the octane was completely consumed, the bacterial population was washed out of the continuous culture system. Third, if ammonium chloride was eliminated from the inflowing medium, wash-out occurred, and octane removal ceased. Fourth, the presence of fatty acids could be detected in the effluents from the continuous culture system indicating that actual oxidation of the octane had occurred.

TABLE I

Rate of Sudan III Dye Concentration in Octane Layer in Continuous Culture System with Dilution Rate = 0.05 hr^{-1}

Time (hrs)	Control[a] (%T)	Experimental (%T)
0	82.1	81.5
48	82.4	79.8
96	81.9	78.0
168	82.0	77.2
192	81.8	76.5
240	82.2	73.0
288	82.2	71.0
385	81.1	67.0
505	81.2	63.0

[a]System was not inoculated.

Mixed bacterial populations, obtained from fresh samples of Lake Ontario water, were also tested for their ability to degrade octane. Degradation rates of the mixed culture proved to be faster than rates obtained with the batch culture isolate. As noted in Table 2, degradation commenced about the tenth day (240 hrs) and continued at a rate of 57.1 µg of octane consumed per hour using a dilution rate = 0.03^{-1}.

Examination of the bacterial population during this experiment showed that the population density increased slowly, eventually reaching a steady state at the 10-12 day period (Table 3).

It was expected with the mixed population system that a selection of the fastest growing octane utilizer would occur after 10-15 retention volumes (3). Examination of colonial morphologies on agar plates indicated that this selection process did occur. A particular colony, designated M_2, predominated after 8 retention volumes (6 days) and eventually after 14 retention volumes, constituted >90% of the population in the continuous culture system. As a pure culture M_2 grew well in an octane and salts medium. It has also been cultivated in a chemostat ($D = 0.03\ hr^{-1}$) using octane as the sole carbon and energy source. The rate of degradation of octane obtained was very similar to that seen with the mixed population (see Table 2). Thus, it appeared that the degradation of octane in the mixed culture system commenced at the same time M_2 reached 90% of the population. The degradation rate of the mixed population was, therefore, a reflection of the activities of a single bacterial species.

When the above enrichment experiment was repeated using the same water sample but a different dilution rate ($0.06\ hr^{-1}$), a second, completely different, species (M_1) was selected and eventually predominated. Again, it appeared that degradation of the octane did not begin until M_1 was predominant. It should be noted that the degradation rate (16.5 µg/hr) with M_1 was about three times faster than M_2.

TABLE 2

Rate of Sudan III Dye Concentration in Octane Layer with Mixed Bacterial Populations (Dilution rate = 0.03 hr^{-1})

Time (hrs)	Control[a] (%T)	Experimental (%T)
0	88.2	87.5
48	88.0	87.5
96	88.0	87.8
120	88.0	89.9
168	87.5	89.1
216	89.5	87.9
240	89.9	88.0
264	90.0	85.0
336	85.5	84.3
360	85.6	82.0
448	89.0	80.0
472	89.0	77.1
505	88.0	73.0
529	87.0	70.0
553	88.2	64.1
600	88.2	60.0

[a]System was not inoculated.

TABLE 3

Change in Bacterial Cell Numbers during Octane Degradation

Time (hrs)	$\log_{10}$ cell numbers
0	5.7
96	8.0
168	9.1
288	9.5
360	9.9
472	9.8
529	9.6
600	9.8

Experiments to test the effect of flow rate, nutrient levels, pH, dispersion, surface area, and other parameters on these enrichments and degradation rates are presently being conducted.

Preliminary evidence has also suggested that our isolates may produce an emulsifying agent or surfactant under certain conditions. This has been observed in experiments where the removal rate of octane from the hydrocarbon layer suddenly increased approximately eight-fold or higher and persisted until the hydrocarbon was completely consumed. Work is now in progress to determine the conditions which stimulated this rate increase and to establish how it was carried out by the bacteria.

Continuous culture systems have also been set up to monitor the degradation of diesel oil by mixed bacterial populations. Due to the light brown color of the oil, the Sudan III dye method was also used to monitor rates. Preliminary results indicated fluctuations in %T of the dye but a steady decrease in the %T was observed overall. The rate was slower than the mixed culture octane system. Some degree of enrichment has also been detected. In some instances, as few as two bacterial types predominated at one time despite the complexity of the substrate, but their enrichments were always transient. The fluctuation in predominant species appeared to be characteristic of the degradation pattern. Work is in progress to study this system further including gas chromatographic analysis of the diesel oil.

DISCUSSION

It would appear that bacterial degradation of hydrocarbons and crude oil products can occur without mechanically dispersing or emulsifying the hydrocarbon in the aqueous phase. Although this is probably atypical for the degradation of oil slicks in aquatic environments, it does allow a study to be carried out in a continuous culture system. As such, the number of individual factors affecting the degradation rate which can be tested is tremendous. Of these, dispersion and emulsification are two very important aspects to consider.

The use of the dye method for monitoring rates also appears very reliable and simple; however, its usefulness may be limited to purified hydrocarbons and their mixtures (artificial oils). The development of gas chromatographic techniques will greatly aid and complement the dye method.

The preliminary results presented here illustrate the importance of comparing axenic versus mixed population studies, and simple versus complex substrates, as a means of obtaining practical information to be used successfully in predicting the fate of oil in aquatic environments.

ACKNOWLEDGEMENT

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UTILIZATION OF CRUDE OIL HYDROCARBONS BY MIXED CULTURES OF MARINE BACTERIA

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Marine bacteria degraded hydrocarbons in crude oils in an enriched seawater medium. Normal and branched paraffins were preferentially utilized. Utilization rates were inversely related to chain length.

During the past 50 years increasing quantities of crude oils and derivative hydrocarbons have been released into the world ocean without regard for ultimate diagenesis or its potentially adverse effects toward marine organisms. Currently, annual maritime oil spillage is estimated to account for the loss of a minimum of 10^6 metric tons/year (1). The volume of hydrocarbons derived from terrestrial sources such as runoff of spent lubricants, industrial effluents and the erosion of asphalt surfaced roads may be of similar magnitude.

Biogeologically, hydrocarbons have been continuously introduced into the world ocean through a) natural crude oil seepages, b) the addition of some fraction of the 200×10^6 metric tons of volatile plant hydrocarbons annually released into the atmosphere (7), and c) through the annual production of 10^5-10^7 metric tons of hydrocarbons derived from marine photosynthesis. Despite the natural occurrence of these recent hydrocarbons at very low levels in marine waters, localized large spillages of fossil crude oils or their distillation products have produced acute biological and aesthetic damages. While the acute biological effects are highly visible, the ecological subtleties of sublethal exposure to petroleums are virtually unknown. Catastrophic oil spills, the ubiquity of "tar lumps" on the ocean surface, and a rising public awareness of environmental issues have provided incentives toward understanding the response of the environment to hydrocarbon pollutants.

Microorganisms, especially bacteria, are essential for diagenesis of hydrocarbons in the marine environment (6). While it is known that bacteria can degrade many types of pure hydrocarbons (2), bacterial utilization of the hydrocarbons contained in a substrate as complex as crude oil has not been well described. Indeed, utilization of mixed substrates by bacteria is a facet of microbiology which is only now achieving recognition. Inasmuch as approaches to petroleum microbiology have employed "classical" concepts of bacteriology, i.e., pure cultures obtained from soils grown on pure hydrocarbons, the purpose of this study was to isolate naturally-occurring mixed populations of marine bacteria on crude oils and to quantitatively determine *in vitro* their degradation capabilities.

MATERIALS AND METHODS

Bacterial Cultures and Inoculation Procedures.--Mixed cultures of petroleum-utilizing marine bacteria were isolated from a wide variety of oil-contaminated marine sediments and waters (Table 1). The cultures were isolated on previously described (5) enriched seawater medium (ESW) plus sterile crude oil. These cultures were maintained routinely on ESW agar slants overlain with crude oil. Crude oils were sterilized through 0.45 micron membrane filters resistant to hydrocarbons. Crude oils used consisted of Louisiana crude oil (a light paraffin crude oil, Lafitte Field, La.), Kuwait (Gulf Oil Kuwait crude #LS-3355), and Venezuelan crude (Lagotraca-Bachequero Field).

TABLE 1

Mixed Culture Nomenclature

Name	Isolation Location
C-63	California coastline near working oil rig
BHMO	Oily sediment in Boston Harbor
SOCLA	Refinery drainage ditch in Pennsylvania, leading to bay
TPLA	Harbor at Turkey Point Marine Laboratory, Florida State University
NOLA	Oil slick on Mississippi River near New Orleans
OILRIG	Vicinity of working oil rig near New Orleans in the Gulf of Mexico

An oil-free cell suspension for degradation experiments was prepared by streaking an ESW agar plate with bacterial cells from a 24 hour-ESW slant and then inverting the plate bottom over the plate cover. The latter was filled with 0.5 ml of the crude oil studied. After 24 hours incubation at 25 C, vapor-grown cells were carefully scraped from the agar surface and suspended in sterile ESW to an optical density of 0.1. This oil-free suspension was used to inoculate 500 ml flasks containing 200 ml of ESW. Fifty microliters of a selected crude oil were dispensed into the flasks with a sterilized microliter syringe. Flasks were sealed with foil, covered with screw caps, and incubated on a rotary shaker at 160 rpm. Control flasks containing only ESW plus sterile crude oil were provided. Calculations indicated that the oxygen content within the sealed flask was adequate over the experimental time periods selected.

Solvent Extraction and Analytical Procedures.--Growth flasks were removed from the rotary shaker at selected intervals. The water phase of each flask was then acidified to a pH of 2.0 and exhaustively extracted twice with a freshly-distilled solvent mixture consisting of 25 ml of benzene:ethyl ether (2:1, v/v). Each extract was then filtered through washed anhydrous sodium sulfate in Whatman No. 50 paper. The solvent mixture was removed through aspiration using a heated water bath (60 C).

The residual crude oils were analyzed with gas chromatography or

silica gel column chromatography. A Varian-Aerograph 1740 gas chromatograph equipped with dual columns operating in a differential mode was used. Linear temperature programming allowed for a constant rate of temperature increase over the range 40 C to 300 C at 6 C/min. Chromatographic columns (5' x 1/8" S.S.) packed with 3% SE-30 on 100/120 Varaport were used.

Unknown *n*-paraffins and other selected hydrocarbons were identified by comparison with retention times for standard pure compounds on polar and unpolar (FFAP) stationary phases. Changes in *n*-paraffin peak concentrations were calculated using the naturally-occurring (in crude oils) isoprenoid hydrocarbons pristane and phytane as true internal standards. The technique of standard addition was used to determine the absolute amounts of pristane, phytane and normal paraffins for initial calibrations.

Specific utilization of major classes of hydrocarbons contained in crude oils was determined by chromatographic comparison of degraded crude oils with uninoculated crude oil controls. Degraded and control samples were dissolved in a small volume of heptane and transferred to a chromatographic column (9 mm I.D.) containing 18 gm of heat-activated silica gel (28-200 mesh heated for 24 hours at 260 C). The samples were then chromatographed sequentially with distilled solvents of increasing polarity in order to elute the following compound classes: (a) saturated paraffins (heptane); (b) aromatics and naphthenics (CCl_4 and benzene); (c) polar compounds (methanol) (4). Eluted fractions were collected and weighed in tared aluminum dishes (to nearest 0.1 mg) after solvent removal.

RESULTS AND DISCUSSION

Rapidly growing oil-degrading cultures consistently dispersed and metabolized thin layers of Louisiana, Kuwait and Venezuelan crude oils in ESW within 24 hours. Substrate dispersal and cell growth produced large increases in optical density. Plate counts indicated that cell populations usually increased from initial inoculum values of 10^5 cells/ml (i.e., petroleum-utilizing cells) to final average cell populations of 10^7 cells/ml. Visible oil emulsification did not occur without nutrient salts enrichment.

The most active cultures were overwhelmingly composed of pleomorphic rods ranging in length from 0.5 to 8.0 microns. Cocci were also observed in many of the cultures. Colonies growing on ESW agar plates were typically white or cream colored, translucent, entire or undulate. The most active cultures contained colonies which tended to spread. Several distinct colony types appeared in each mixed culture. All cultures grew well on kerosene, mineral oil, glucose in seawater, 2216 E heterotroph medium and liquefied gelatin.

Typical changes in the *n*-paraffin composition of a degraded Louisiana crude oil are illustrated in Figures 1-3. This sequence of chromatograms reveals utilization of *n*-paraffins from C-16 to C-30 with a selective preference for straight chain, unbranched hydrocarbons. Pristane and phytane, examples of branched alkanes, were utilized only after the removal of normal paraffins and therefore illustrated a sequential pattern of substrate utilization. These data reveal why pristane or phytane could be used as internal standards for quantification of *n*-paraffin utilization.

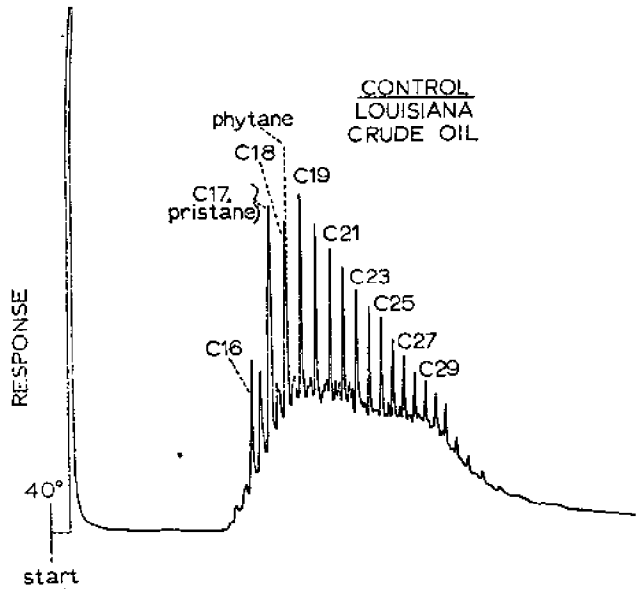

Figure 1. Gas Chromatogram of the Louisiana Crude Oil Control

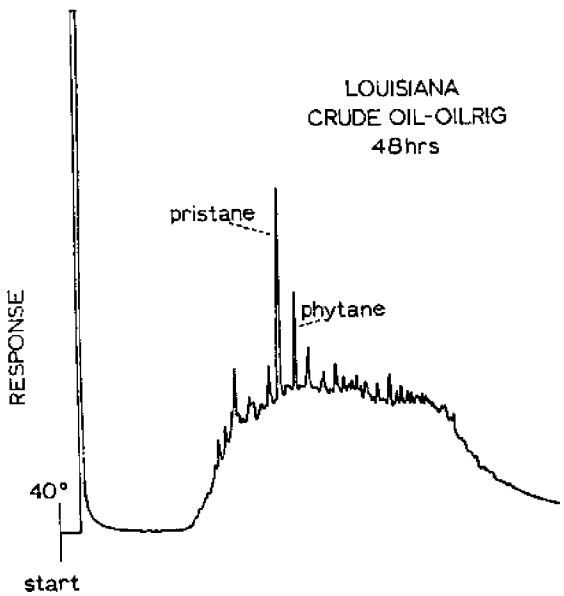

Figure 2. Gas Chromatogram of Louisiana Crude Oil Degraded by OILRIG for 48 hours

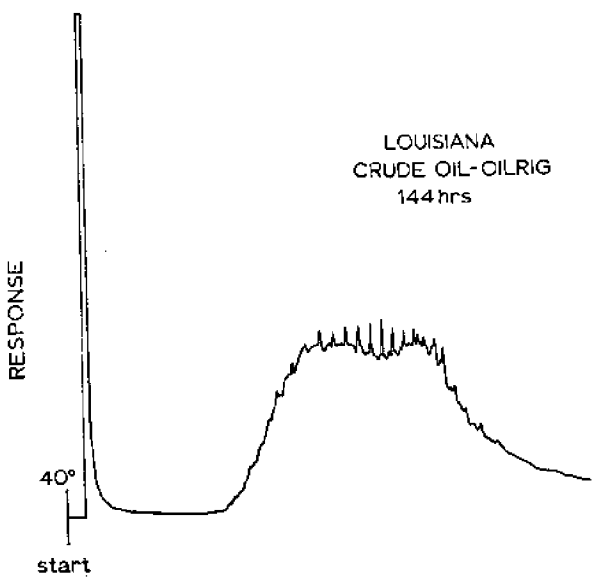

Figure 3. Gas Chromatogram of Louisiana Crude Oil Degraded by OILRIG for 144 hours

The results of a series of experiments designed to quantitate *n*-paraffin utilization in different crude oils by mixed cultures of marine bacteria are summarized with a series of composite chromatograms which indicate how *n*-paraffin composition varied during incubation.

The data in Figures 4 through 8 indicated that *n*-paraffins in Louisiana crude oil from C-10 to C-26 were utilized. Within this range, a carbon chain length preference (i.e., odd versus even) was not evident. During the incubation period the ratio of pristane to phytane was constant. The average daily rate of total *n*-paraffin utilization, based on the summation of the absolute amounts of *n*-paraffins C-10 to C-30 (Table 2), was 846.5 ± 289.9 micrograms/day at 30 C.

Changes in *n*-paraffin composition and summation data for Kuwait crude oil are given in Figures 9 to 11 and Table 3. Phytane was used as the internal standard since pristane was not well resolved from *n*C-17. The data reveal utilization of *n*-paraffins C-14 to C-30. The average daily rate of total *n*-paraffin utilization was 258.5 ± 32.4 micrograms/day. The peak remaining at the *n*C-17 position after 48 hours incubation (Figures 10 and 11) possessed the same retention time as pristane. Pristane disappeared after 96 hours incubation with the OILRIG culture.

Data from gas chromatographic analysis of degraded Venezuelan crude oil are shown in Figures 12 and 13 and in Table 4. Phytane was again utilized as the internal standard. Extensive degradation of *n*-paraffins occurred. Summation values clearly showed complete removal to baseline of *n*-paraffins C-14 to C-30 within 96 hours incubation. Both phytane and pristane were utilized during the final 48 hours of incubation. The average daily rate of total *n*-paraffin utilization was 186.1 ± 7.4 micrograms/day. Gas chromatograms of the saturated paraffin fraction of this oil indicated that branched paraffins other than pristane and phytane were degraded after 96 hours.

In summary, *n*-paraffin degradation patterns were similar for different major crude oils. However, the rates of total *n*-paraffin utilization were significantly larger with the Louisiana crude oil. This oil contains roughly twice the amount of normal paraffins found in Kuwait and Venezuelan crude oils.

To determine if there was a relationship between the rate of utilization/oxidation and molecular size, the amounts of selected *n*-paraffins in the growth flasks were plotted as a function of time. The results (Figures 14 to 16, Louisiana crude oil; Figure 17, Kuwait crude oil; Figure 18, Venezuelan crude oil) revealed that the rates of utilization were not linear with respect to time. For this reason rate constants were not calculated and the average daily rates (or rates calculated for the time required to exhaust the substrate) are shown for each selected hydrocarbon. The data summarized in Table 5 for Louisiana crude oil reveal a relationship between the rate of utilization and *n*-paraffin chain length; *n*C-10 and *n*C-15 paraffins were utilized at higher average rates than *n*C-20 or *n*C-25 paraffins. In many instances, the rate curves exhibit a hierarchy of slopes during the incubation period, i.e., the slopes being inversely related to chain length. Similar observations were made with Kuwait crude oil (Table 6). In Venezuelan crude oil, however, the *n*C-20 paraffin appeared to be utilized at a faster rate than the *n*C-15 (Table 7).

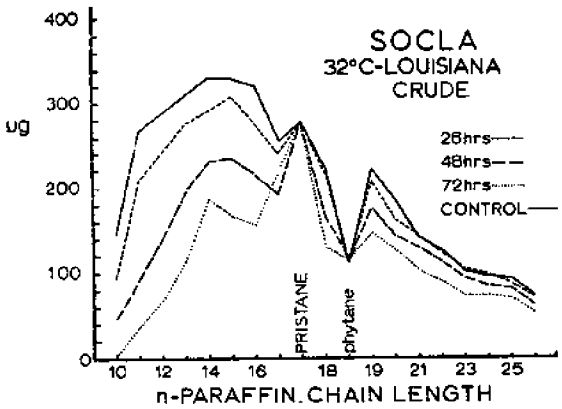

Figure 4. Quantitative changes in *n*-paraffin composition in a Louisiana crude oil incubated with the mixed marine bacterial culture SOCLA in ESW for the indicated period. *n*-Paraffin content (µg) is based on 50 µl of crude oil.

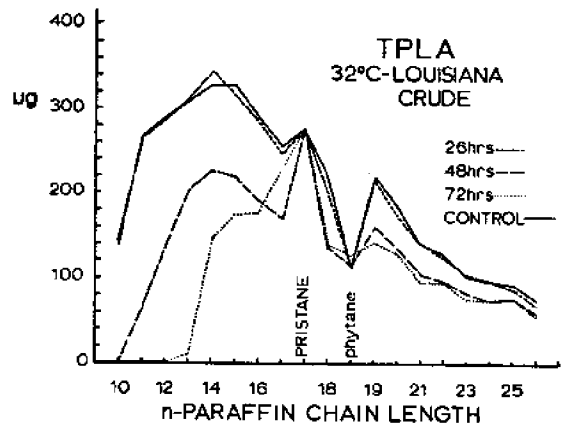

Figure 5. Quantitative changes in *n*-paraffin composition in a Louisiana crude oil incubated with the mixed marine bacterial culture TPLA in ESW for the indicated period. *n*-Paraffin content (µg) is based on 50 µl of crude oil.

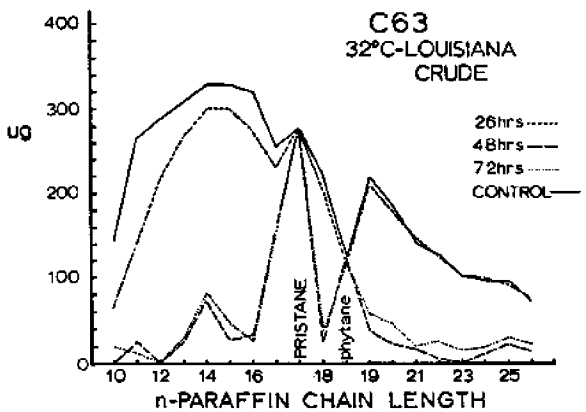

Figure 6. Quantitative changes in *n*-paraffin composition in a Louisiana crude oil incubated with the mixed marine bacterial culture C-63 in ESW for the indicated period. *n*-Paraffin content (µg) is based on 50 µl of crude oil.

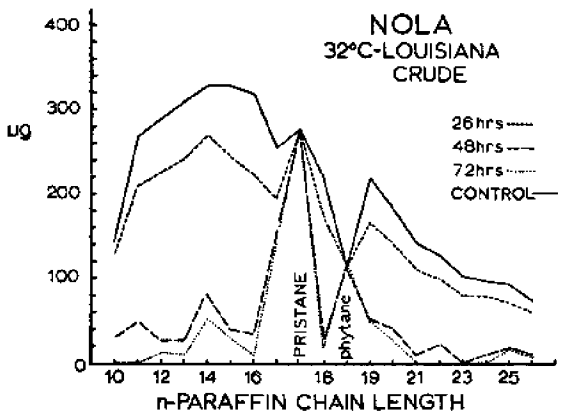

Figure 7. Quantitative changes in *n*-paraffin composition in a Louisiana crude oil incubated with the mixed marine bacterial culture NOLA in ESW for the indicated period. *n*-Paraffin content (µg) is based on 50 µl of crude oil.

TABLE 2

Summation of Peak Concentrations (µg) of the *n*-Paraffins C-10[a] to C-26 in Louisiana Crude Oil Following Incubation at 32 C in ESW (160 rpm) with Selected Mixed Marine Bacterial Cultures

Time (hrs)	Cultures							
	OILRIG	SOCLA	NOLA	C-63	BHMO	TPLA	CONTROL	
26	3067.7	3511.4	067.7	3355.4	3757.9	3752.4		Summation
	26	8	20	12	2	2		% Utilized
48	720.4	2771.9	750.5	731.4	3127.9	2500.8		Summation
	81	27	80	81	22	34		% Utilized
72	581.3	1983.0	632.8	879.3	2043.3	1788.7	3815.5	Summation
	84	48	83	80	46	53		% Utilized
	1152.7	610.9	1060.9	978.7	590.8	677.1		Average Rate of Utilization (µg/day)[b]
	±57.6	±30.5	±53.0	±48.9	±29.5	±33.9		

[a]Normal and saturated paraffins with boiling points below C-10 had evaporated within 48 hours.

[b]Precision = 5%.

TABLE 3

Summation of Peak Concentrations (µg) of the *n*-Paraffins C-14 to C-30 in Kuwait Crude Oil Following Degradation with Mixed Cultures of Marine Bacteria in ESW at 20 C (160 rpm)

Time (hrs)	Cultures				
	C-63	OILRIG	SOCLA	CONTROL	
24	1247.4	1212.4	1368.9		Summation
	8	11	0		% Utilized
48	438.5	234.7	1114.1		Summation
	68	83	18		% Utilized
96	326.6	>234.7	422.5	1361.7	Summation
	76	>83	69	0	% Utilized
	258.8	>281.8	234.8		Average Rate of Utilization (µg/day)[a]
	±12.9	±14.1	±11.7		

[a]Precision = 5%.

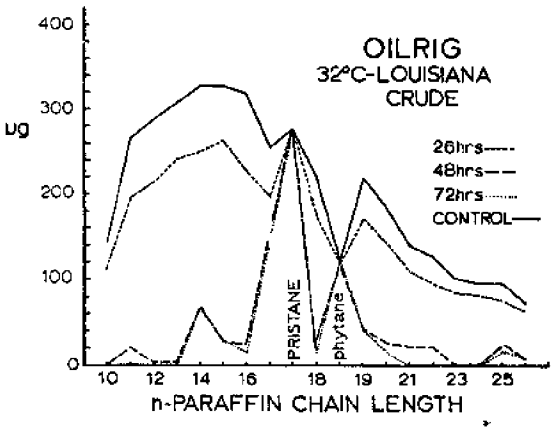

Figure 8. Quantitative changes in *n*-paraffin composition in a Louisiana crude oil incubated with the mixed marine bacterial culture OILRIG in ESW for the indicated period. *n*-Paraffin content (µg) is based on 50 µl of crude oil.

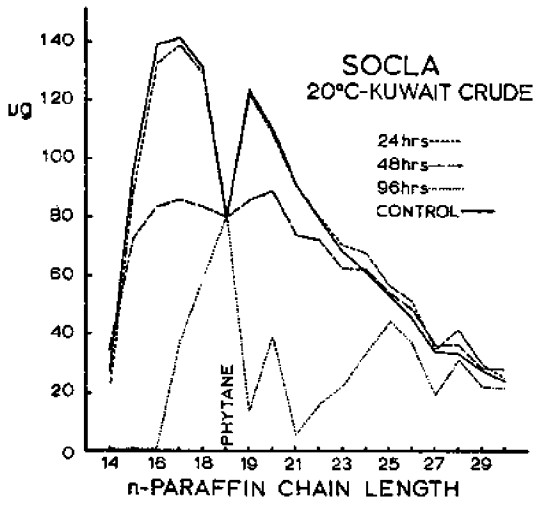

Figure 9. Quantitative changes in *n*-paraffin composition in a Kuwait crude oil incubated with the mixed marine bacterial culture SOCLA in ESW for the indicated period. *n*-Paraffin content (µg) is based on 50 µl of crude oil.

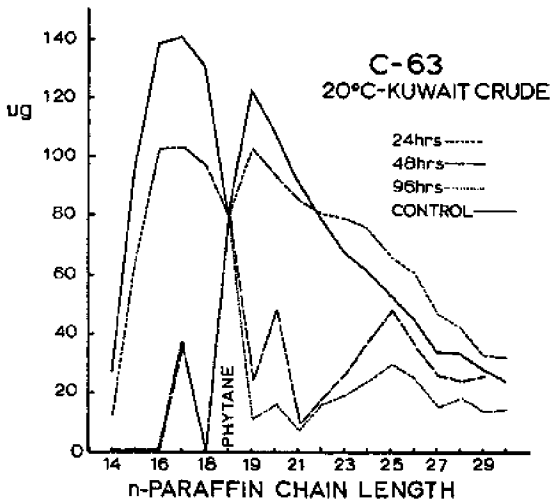

Figure 10. Quantitative changes in *n*-paraffin composition in a Kuwait crude oil incubated with the mixed marine bacterial culture C-63 in ESW for the indicated period. *n*-Paraffin content (µg) is based on 50 µl of crude oil.

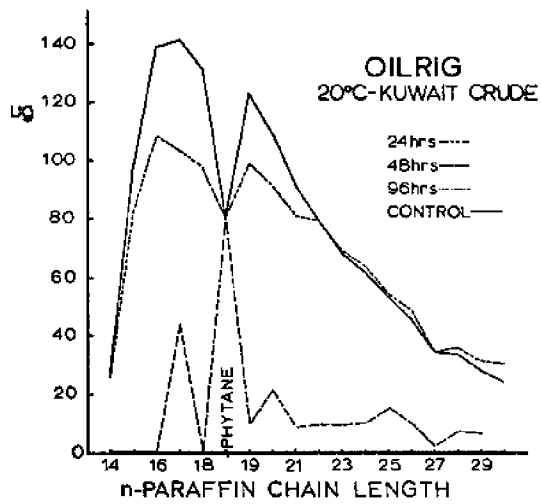

Figure 11. Quantitative changes in *n*-paraffin composition in a Kuwait crude oil incubated with the mixed marine bacterial culture OILRIG in ESW for the indicated period. *n*-Paraffin content (µg) is based on 50 µl of crude oil.

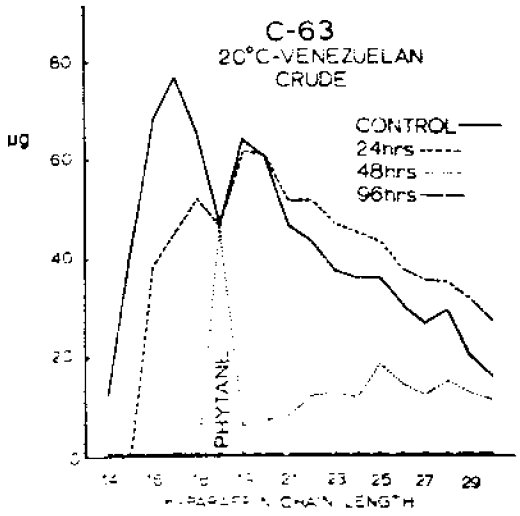

Figure 12. Quantitative changes in *n*-paraffin composition in a Venezuelan crude oil incubated with the mixed marine bacterial culture C-63 in ESW for the indicated period. *n*-Paraffin content (µg) is based on 50 µl of crude oil.

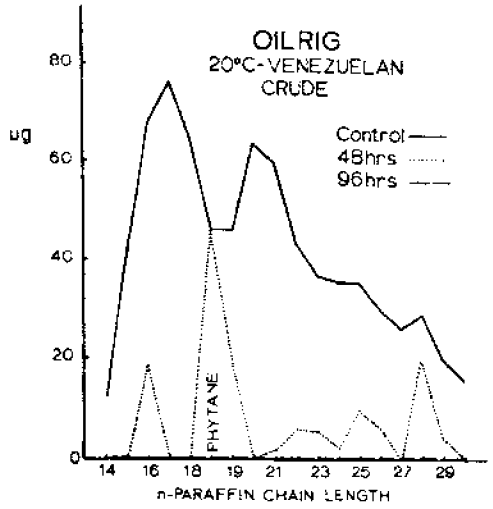

Figure 13. Quantitative changes in *n*-paraffin composition in a Venezuelan crude oil incubated with the mixed marine bacterial culture OILRIG in ESW for the indicated period. *n*-Paraffin content (µg) is based of 50 µl of crude oil.

TABLE 4

Summation of Peak Concentrations (µg) of the *n*-Paraffins C-14 to C-30 in Venezuelan Crude Oil Following Incubation with Selected Mixed Cultures of Marine Bacteria in ESW at 20 C (160 rpm)

Time (hrs)	Cultures OILRIG	Cultures C-63	CONTROL	
24	n.a.	740.7		Summation
		0.7		% Utilized
48	197.7	243.2		Summation
	63	67		% Utilized
96	0.0	0.0	744.3	Summation
	100	100		% Utilized
	186.1 ±9.3	186.1 ±9.3		Average Rate of Utilization (µg/day)[a]

[a]Precision = 5%.

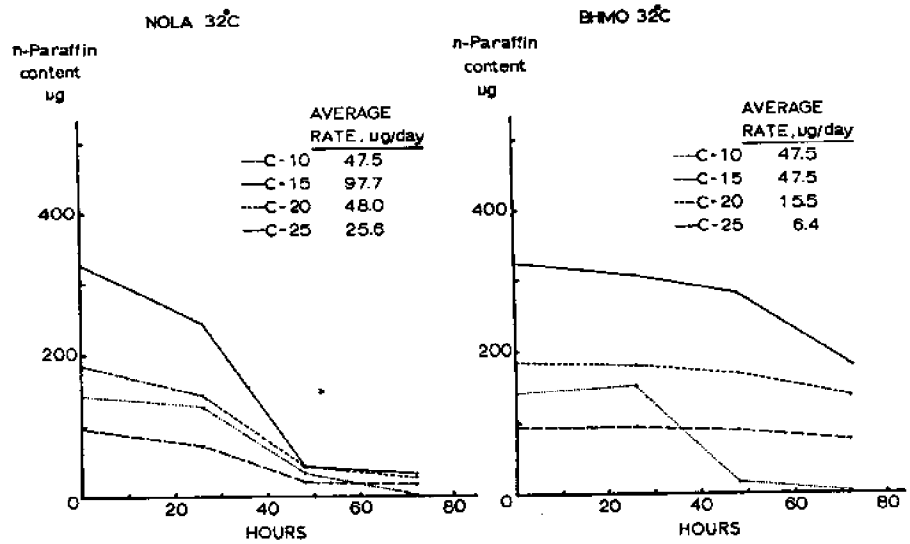

Figure 14. Change in the content of specific *n*-paraffins in a Louisiana crude oil during incubation with the mixed marine bacterial cultures NOLA and BHMO in ESW at 32 C. Average rates of utilization for the entire incubation period are indicated.

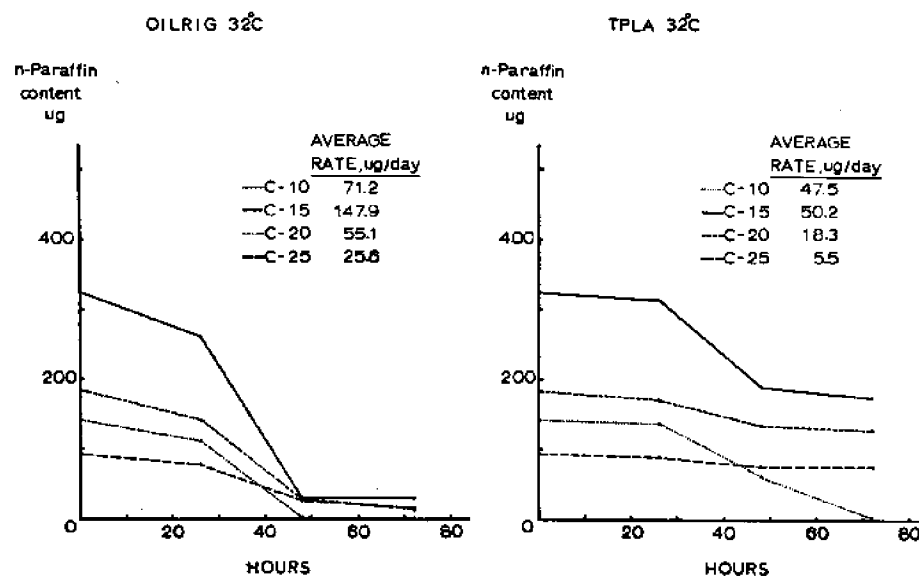

Figure 15. Change in the content of specific *n*-paraffins in a Louisiana crude oil during incubation with the mixed marine bacterial cultures TPLA and OILRIG in ESW at 32 C. Average rates of utilization for the entire incubation period are indicated.

TABLE 5

Rates of Utilization (µg/day) of *n*-Paraffins of Selected Chain Lengths by a Series of Mixed Cultures of Marine Crude Oil Degrading Bacteria Growing in ESW on a Louisiana Crude Oil at 32 C (160 rpm)

Culture	*n*C-10	*n*C-15	*n*C-20	*n*C-25
TPLA	47.5	50.2	18.3	5.5
SOCLA	46.6	52.9	18.3	7.3
NOLA	47.5	97.7	48.0	25.6
OILRIG	71.2	147.9	55.1	25.6
BHMO	47.5	47.5	15.5	6.4
C-63	41.1	143.1	51.5	13.0
Mean Rate µg/day	50.5 ±13.1	90.2 ±51.4	28.9 ±21.2	21.4 ±13.6

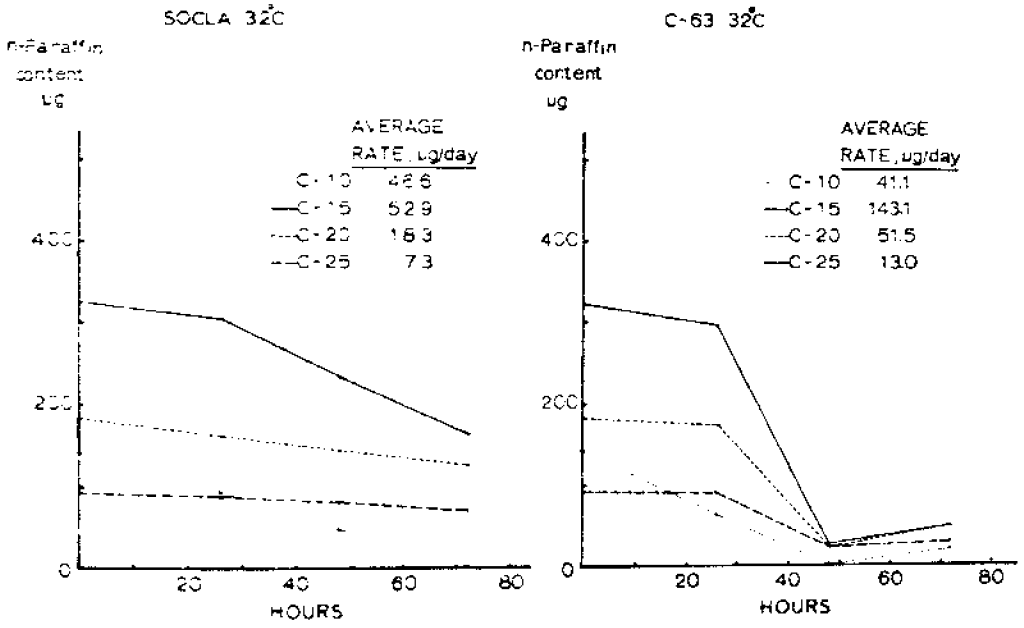

Figure 16. Change in the content of specific *n*-paraffins in a Louisiana crude oil during incubation with the mixed marine bacterial cultures C-63 and SOCLA in ESW at 32 C. Average rates of utilization for the entire incubation period are indicated.

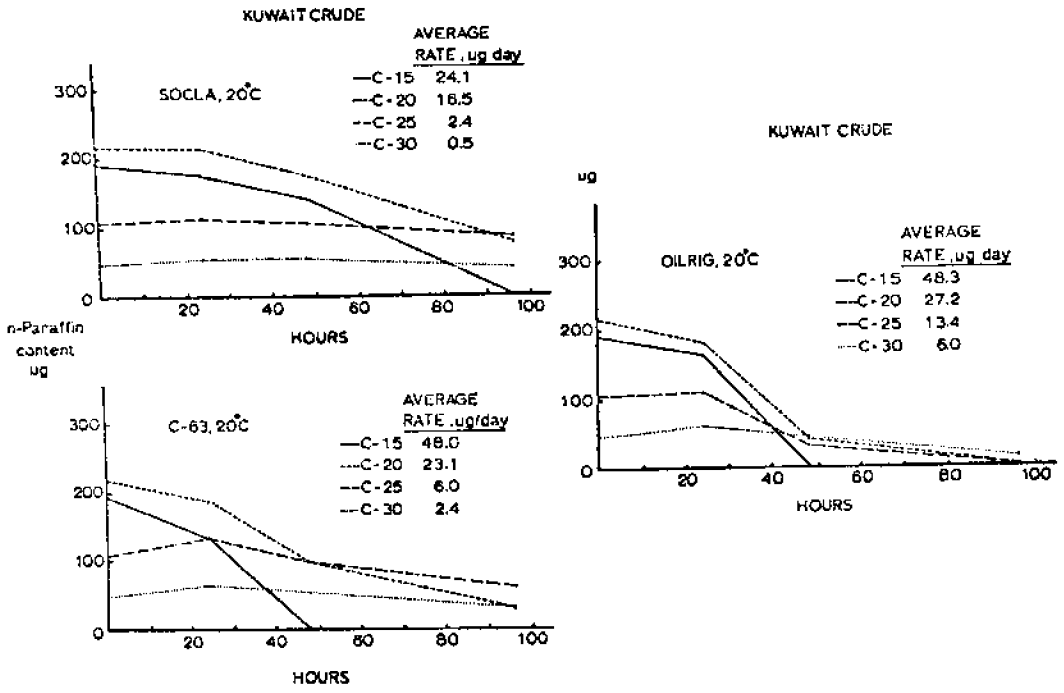

Figure 17. Change in the content of specific *n*-paraffins in a Kuwait crude oil during incubation with the mixed marine bacterial cultures SOCLA, C-63, and OILRIG in ESW at 20 C. Average rates of utilization for the entire incubation period are indicated.

TABLE 6

Rates of Utilization (µg/day) of Selected *n*-Paraffins in a Kuwait Crude Oil Degraded by Mixed Cultures of Marine Hydrocarbon-Utilizing Bacteria in ESW (160 rpm) at 20 C

Culture	*n*C-15	*n*C-20	*n*C-25	*n*C-30
SOCLA	24.1	16.5	2.4	0.5
C-63	48.0	23.1	6.0	2.4
OILRIG	48.3	27.2	13.4	6.0
Mean Rate (µg/day)	40.1 ±15.9	22.3 ±6.5	7.3 ±6.0	3.0 ±3.0

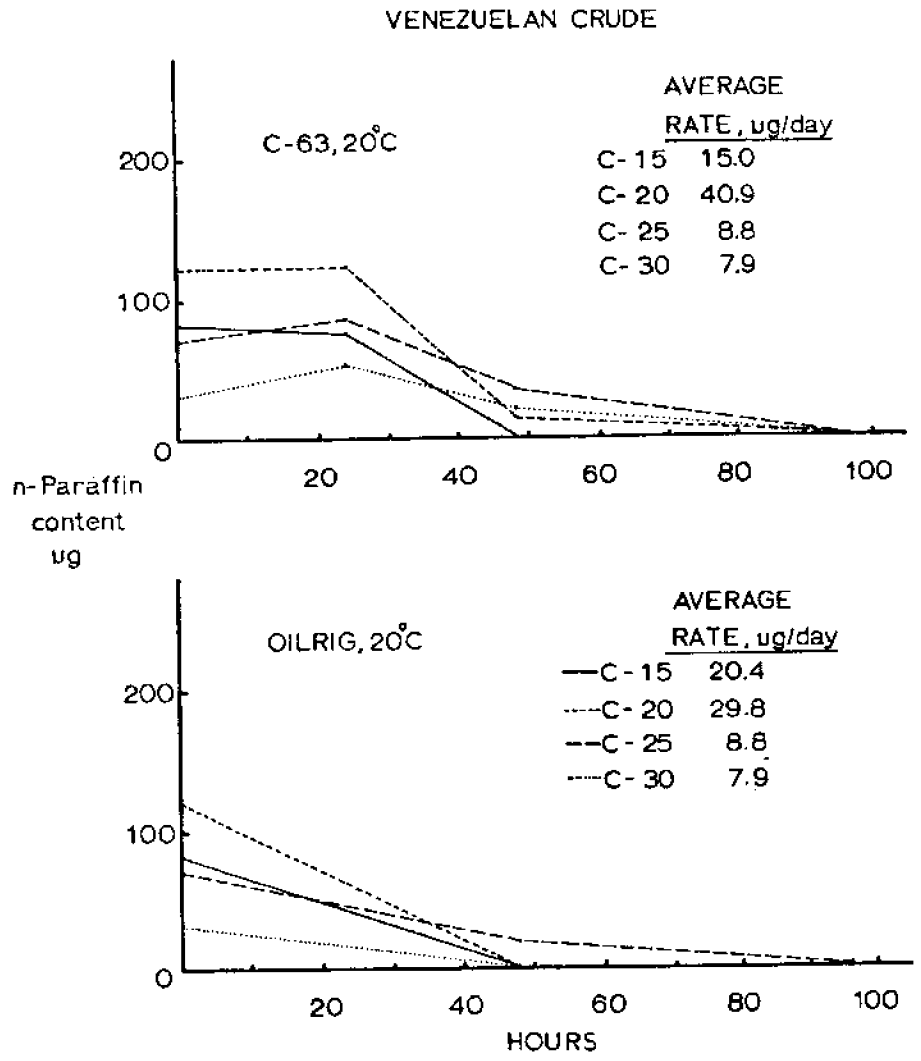

Figure 18. Change in the content of specific *n*-paraffins in a Venezuelan crude oil during incubation with the mixed marine bacterial cultures C-63 and OILRIG in ESW at 20 C. Average rates of utilization for the entire incubation period are indicated.

TABLE 7

Rates of Utilization (µg/day) of Selected *n*-Paraffins in a Venezuelan Crude Oil Degraded by Mixed Cultures of Marine Hydrocarbon-Utilizing Bacteria in ESW (160 rpm) at 20 C

Culture	*n*C-15	*n*C-20	*n*C-25	*n*C-30
OILRIG	20.4	29.8	8.8	7.9
C-63	15.0	40.9	8.8	7.9
Mean Rate (µg/day)	17.7 ±4.6	35.4 ±9.7	8.8 ±0.4	7.9 ±0.4

In a similar type of study using mixed cultures and mixed carbohydrate substrates, Mateles and Chian (3) measured different rates of utilization for sugars in a mixture. This illustrated a situation analogous to diauxie or the sequential use of one substrate before another. Further experimentation is required to determine if the observed *n*-paraffin rate hierarchies are a function of aqueous hydrocarbon solubilities or are perhaps related to a membrane transport mechanism. The latter hypothesis is appealing since although complete dispersion of the crude oil generally occurred within 24 hours of incubation, the rate hierarchy was continued.

Activated silica gel column chromatography permitted reproducible fractionation of degraded crude oils into major chemical groups. The data shown in Tables 8, 9 and 10 show typical weight changes in these chromatographic fractions for representative crude oils degraded by marine bacteria. The significant changes in oil composition were the utilization of saturated paraffins (heptane fraction). Within the precision limits of this technique, degradation of the aromatic-naphthenic fractions (CCl_4 and benzene fractions) in whole crude oils was not significant.

To determine if branched alkanes would support adequate growth in the absence of *n*-paraffins, a Louisiana crude oil was rendered *n*-paraffin-free by treatment with Linde 5A molecular sieve. The results of an experiment in which a selected mixed culture was incubated with such an oil are shown in Table 11. These data revealed that a relatively long lag period (96 hours) occurred before significant degradation of the sieve-treated fraction commenced.

It is evident from these studies that mixed populations of marine bacteria isolated on selected crude oils have a clear preference for normal and then branched paraffins. Inasmuch as hydrocarbon degradation in crude oils is sequential, further research utilizing steady-state conditions (without loss of oil) is required to evaluate the range and rates of hydrocarbons utilized or oxidized in crude oils.

TABLE 8

Weight[a] Changes in Various Chromatographic Fractions in a Louisiana Crude Oil Following Degradation by Selected Mixed Cultures of Marine Bacteria at 20 C (160 rpm)

Fraction	Control (96 hrs)	24 hrs	48 hrs	96 hrs	Total Change	% Loss of Control	Average Daily Rate of Saturated Paraffin Uptake
	OILRIG						
Heptane	34.9±2.1	34.9±2.1	35.2±2.1	25.0±1.5	-9.9±4.2	28±10.1	2.5±0.9 mg/day
CCl_4	2.8±0.8	2.9±0.8	3.4±0.9	2.2±0.6	-0.6±1.4		
Benzene	8.4±0.4	8.1±0.4	7.6±0.4	9.3±0.5	+1.2±0.9		
Methanol	8.3±0.9	6.9±0.8	6.8±0.7	9.1±1.0	+0.8±1.9		
	SOCLA						
Heptane	34.9±2.1	35.9±2.2	32.1±2.0	30.7±1.9	-4.2±4.0	12±11.4	1.0±1.0 mg/day
CCl_4	2.8±0.8	2.8±0.8	2.5±0.7	3.2±0.9	+0.4±1.7		
Benzene	8.4±0.4	8.3±0.4	9.1±0.5	9.4±0.5	+1.0±0.9		
Methanol	8.3±0.9	7.5±0.8	8.6±0.9	8.6±0.9	+0.3±1.8		

[a]Weight changes in mg, technique precision values indicated.

TABLE 9

Weight[a] Changes in Various Chromatographic Fractions in a Kuwait Crude Oil Following Degradation by Mixed Cultures of Marine Bacteria in ESW at 20 C (160 rpm)

Fraction	Control (96 hrs)	24 hrs	48 hrs	96 hrs	Total Change	% Loss of Control	Average Daily Rate of Utilization
			SOCLA				
Heptane	10.6±0.9	10.8±0.9	11.1±1.0	5.8±0.5	-4.8±1.4	45±13	1.2±0.4 mg/day
CCl_4	2.7±0.4	2.4±0.3	2.5±0.4	2.2±0.3	-0.5±0.7		
Benzene	8.9±1.1	9.7±1.2	8.4±1.1	6.8±0.9	-2.1±2.0		
Methanol	10.8±1.5	11.7±1.6	11.4±1.6	11.3±1.5	+1.5±3.0		
			C-63				
Heptane	10.6±0.9	9.6±0.8	6.1±0.5	6.9±0.6	-3.7±1.5	35±14	0.9±0.4 mg/day
CCl_4	2.7±0.4	2.8±0.4	2.5±0.4	2.1±0.2	-0.6±0.7		
Benzene	8.9±1.1	8.7±1.1	7.6±1.0	7.6±1.0	-1.3±2.1		
Methanol	10.8±1.5	11.0±1.5	12.4±1.7	11.1±1.5	+0.3±3.0		
			OILRIG				
Heptane	10.6±0.9	10.4±0.9	8.1±0.7	5.3±0.5	-5.3±1.4	50±13	1.3±0.4 mg/day
CCl_4	2.7±0.4	2.8±0.4	2.0±0.3	2.1±0.3	-0.6±0.7		
Benzene	8.9±1.1	9.3±1.2	7.1±0.9	7.3±0.9	-1.6±2.0		
Methanol	10.8±1.5	10.7±1.5	10.7±1.5	12.6±1.7	+1.8±3.2		

[a]Weight changes in mg, technique precision values indicated.

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TABLE 10

Weight[a] Changes in Various Chromatographic Fractions in a Venezuelan Crude Oil Following Degradation by Mixed Cultures of Marine Bacteria in ESW at 20 C (160 rpm)

Fraction	Control (96 hrs)	24 hrs	48 hrs	96 hrs	Total Change	% Loss of Control	Average Daily Rate of Utilization
	C-63						
Heptane	12.4±1.1	12.4±1.1	9.7±0.8	7.6±0.7	-4.8±1.8	39±14.0	1.2±0.5 mg/day
CCl_4	1.2±0.2	1.8±0.3	1.3±0.2	0.7±0.1	-0.5±0.3	42±25.2	
Benzene	7.1±0.9	7.5±0.9	7.5±0.9	6.4±0.8	-0.7±1.7		
Methanol	8.9±1.2	10.9±1.5	10.8±1.5	10.6±1.5	+1.7±2.1		
	OILRIG						
Heptane	12.4±1.1	12.7±1.1	9.0±0.8	7.9±0.7	-4.5±1.8	36±14.0	1.1±0.5 mg/day
CCl_4	1.2±0.2	1.4±0.2	1.8±0.3	1.0±0.1	-0.2±0.3		
Benzene	7.1±0.9	7.6±1.0	6.7±0.8	6.5±0,8	-0.6±1.7		
Methanol	8.9±1.2	11.7±1.6	9.4±1.3	9.8±1.3	+0.9±3.0		

[a]Weight changes in mg, technique precision values indicated.

TABLE 11

Weight[a] Changes in Various Chromatographic Fractions in a Louisiana Crude Oil Treated with Molecular Sieve (5A) Following Degradation with the Mixed Marine Bacterial Culture OILRIG at 20 C in ESW (160 rpm)

Fraction	Control (96 hrs)	24 hrs	48 hrs	96 hrs	Total Change	% Loss of Control	Average Daily Rate of Utilization
			1				
Heptane	16.1±1.0	--	15.5±0.9	15.8±1.0	-0.3±2.0		0.0 mg/day
CCl_4	1.6±0.4	1.9±0.5	1.8±0.5	0.9±0.2	-0.7±0.6		
Benzene	3.9±0.2	4.6±0.2	5.2±0.3	4.2±0.2	+0.3±0.4		
Methanol	3.1±0.3	3.0±0.3	2.5±0.3	3.3±0.4	+0.2±0.7		
			2				
Heptane	20.1±1.2	23.4±1.4	20.7±1.3	13.8±0.8	-6.3±2.0	31±10.0	1.1±0.3 mg/day
CCl_4	1.3±0.4	2.2±0.6	2.1±0.6	1.8±0.5	+0.5±0.9		
Benzene	5.9±0.3	4.8±0.2	6.5±0.3	4.4±0.2	-1.5±0.5	25±8.1	
Methanol	2.1±0.2	2.2±0.2	2.2±0.2	5.1±0.6	+3.0±0.8		

[a]Weight changes in mg, technique precision values indicated.

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MICROBIAL DECOMPOSITION PATTERNS USING CRUDE OIL

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A mixed culture of microorganisms, acclimated to decomposition of crude oil, was used in a series of incubation experiments to determine the sequence of microbial degradation in a Santa Barbara, California, crude oil. The molecular weight distribution of the crude oil was: C_1 to C_{10}, 29%; C_{10} to C_{30}, 52%; and greater than C_{30}, 19%. At least 15%, by weight, of this oil was composed of *n*-alkanes, or straight chain paraffins. Gas chromatograms of the stock crude oil were characterized by (in order of prominence), an envelope of *n*-paraffins, six highly resolved isoprenoid hydrocarbons, and more than a hundred smaller peaks that were partially resolved above the base envelope. Microbial degradation of the crude oil is initially characterized by a rapid disappearance of the *n*-paraffin envelope. This degradation starts with the low molecular weight components and progresses toward the higher molecular weight compounds. The isoprenoids are also progressively reduced simultaneously with the reduction of the paraffins. In addition, the base envelope and fine fingerprint region subsequently undergo degradation and the base envelope becomes progressively skewed toward the higher molecular weight end of the chromatogram. Biodegradation of the Santa Barbara crude oil by a mixed microbial population was initiated simultaneously on all components, but the sequential patterns of decomposition were affected by rate differences. Chromatograms of naturally weathered oils collected from southern California beaches are remarkably similar to chromatograms of oil samples which have undergone extensive microbial decomposition in the laboratory.

INTRODUCTION

Oil spilled on the ocean's surface undergoes a series of chemical and physical changes, generally referred to as weathering. This process begins almost immediately after the occurrence of a spill and progresses at a rate which is controlled by external environmental conditions (20,23).

Processes such as evaporation, dissolution, chemical oxidation and microbial degradation all serve to "weather" or modify a spilled oil with resultant changes in its distinguishing physical and chemical characteristics. Thus, the initial oil is altered to weathered oil and is found in the form of slicks, beach tars and tar balls. It is also sorbed in sediments, dispersed in the water column, or found in any of several other forms that depend on the environmental conditions and the length of exposure.

In the first few hours after a spill, the chemical and physical

changes taking place on the water's surface are dominant in determining the subsequent character of the exposed oil (20). However, as the residence time of the oil on the water increases, biological processes begin to operate and rapidly gain in significance. It has been suggested that, ultimately, it is the activity of bacteria which stabilizes and mitigates the effects of an oil spill (6,11,15,16,22,23).

Selective degradation of crude petroleums by bacteria has been demonstrated using both pure and mixed cultures (1,10,19). The *n*-alkanes have been regarded as being the most readily-utilized fraction of petroleum, while other hydrocarbons were more resistant (1,10,15,23). The use of pure cultures of microorganisms has been instrumental in increasing understanding of enzyme mechanisms involved in petroleum decomposition (7,14,18,21). However, the limited metabolic activity of pure cultures has led to some confusion concerning substrate preference, accumulation of intermediates, and the oxidation sequence which occurs when an oil is subjected to bacterial attack.

In this study, gas chromatographic techniques allowed for the separation of a sequence of changes which occurred as an oil was degraded by a mixed bacterial culture. A parallel relationship is expected to occur in the natural weathering of an oil.

MATERIALS AND METHODS

Chemistry.--Oil samples were analyzed without prior preparation using a Hewlett Packard Model 700 Gas Chromatograph equipped with a linear temperature programmer. Conditions similar to recommendations of the Western Oil and Gas Association (12) were used: 10 ft x 1/8 inch O.D.stainless steel columns, 80/100 mesh Chromosorb W (AW-DMCS) coated with 10% OV-101; dual flame ionization detectors; helium flow of 40 ml per minute; chart speed of 1/4 inch per minute; and disc integration. Samples, usually dissolved in chloroform, were injected onto the column at an initial oven temperature of 80 C. After 4 minutes, during which period the solvent had eluted from the column, the oven temperature was programmed to 320 C with an incremental temperature rise of 5 C per minute. Prior to each injection, the column resolution (splitting between n-C_{17} and pristane) and detector sensitivity (calibrated with n-C_{16}) were carefully monitored to insure reproducibility. The columns were balanced for operation in a dual mode to eliminate baseline drift, and periodically conditioned with a silylating agent (Silyl 8, Pierce Chemical Co.). Duplicate analyses were performed and the solvents were checked for interfering impurities.

The positions of the straight-chain (normal) paraffins on the chromatograms were assigned on the basis of their separation with 3 Å molecular sieves (5). The branched isoprenoid hydrocarbons forming a homologous series C_{14}, C_{15} (farnesane), C_{16}, C_{18} (norpristane), C_{19} (pristane) and C_{20} (phytane) were assigned from similarity to published chromatograms (9). The amount of heavy end components which did not elute from the column was estimated by subtracting a weight calculated from the integration of the area C_1 to C_{30} from a gravimetric weight of injected oil. The integration was corrected for the solvent-obscured region by comparison with a sample of the same oil injected without solvent.

Microbiology.--A mixed culture of microorganisms acclimated to decomposition of crude oil was used as the inoculum for the microbiological studies. This culture was obtained by means of an enrichment procedure using waste sludge from a secondary sewage treatment plant as the starting source. The microorganisms of the sludge, which grow as "flocs" or zoogleal masses distributed in the culture liquid, were allowed to settle for 30 minutes and the overlying liquid was decanted and discarded. The settled culture was restored to its original volume with primary sewage effluent which also served as the sole source of added nutrients. Crude oil (1 ml per liter) was added to the mixture which was then incubated for 48 hours at room temperature. Compressed air was used for aeration. This procedure was repeated every 48 hours until six transfers of the culture biomass into fresh sewage-petroleum medium had been accomplished. This method had the advantage that a large mixed population of microorganisms capable of rapidly degrading a crude oil was maintained in an active state.

For quantitative determinations, a 100 ml aliquot of the freshly resuspended enrichment culture (0.2 gram dry weight per liter of medium) was transferred, prior to the daily feeding with oil, to a 6 liter bottle. Crude oil (0.1 ml per 100 ml) was added directly to the culture and another 2 drops was added to a glass wool pad in the cap of the vessel. The latter was used to maintain a vapor phase of the more volatile fractions in the bottle. The bottle, which contained enough air to oxidize all of the oil, was tightly sealed and incubated at room temperature (23 C) with vigorous stirring by a magnetic stirrer.

Sample Preparation.--Direct solvent extraction of degraded oil from the bacterial culture preparatory to gas chromatography resulted in the formation of persistent oil and water emulsions. In order to overcome this problem, the oil was quantitatively recovered by centrifuging and employing sequential extractions of the water phase and of the separated bacterial sediment. The water phase was extracted twice with 5 ml of chloroform, which previously had been used to rinse the culture bottle. The chloroform extracts were added to the bacterial sediment and subjected to ultrasonic treatment for 30 seconds (Biosonik, 20,000 cps, 100 watts). After centrifugation, the chloroform was decanted from the resulting bacterial sediment and the extraction of the sediment repeated a second time. Subsequently, the solvent extracts were combined and the total volume measured. Aliquots ranging between 10 to 20 microliters of the chloroform extracts were used for gas-chromatographic analyses. These relatively large volumes of solvent injected caused only a tail on the solvent peak and did not affect the resolution of components with molecular weights greater than C_{11}.

Since chloroform elution obscured the chromatogram in the region from C_1 to C_8, evaporation of the light molecular weight fractions of the crude oil was not detectable in the experiments. Slight losses of the components from C_9 to C_{12} occurred during prolonged handling of the chloroform extracts. Therefore, comparisons of the C_{10} to C_{12} regions were based upon oils freshly extracted from the bacterial cultures. By adjusting the volume of extract injected into the gas chromatograph, relative to the total volume of the extract, the various chromatograms were made comparable.

RESULTS

Characterization of the Oil Substrate.--The crude oil used in this study was obtained directly from a producing well in the Santa Barbara Channel. The gas chromatogram of this oil in chloroform (Fig. 1, top) showed in order of prominence: a) an envelope of *n*-paraffins, *n*-C_9 to *n*-C_{30}; b) six highly resolved isoprenoid hydrocarbons; and c) over a hundred smaller peaks partially resolved from a base envelope. The non-resolved base envelope was nearly the same height from C_{10} to C_{27} above the zero baseline. The following molecular weight distributions were observed in this crude oil: C_1 to C_{10}, 29%; C_{10} to C_{30}, 52%; and the heavy ends greater than C_{30}, 19%. The *n*-alkanes, or straight chain paraffins, were estimated to compose at least 15% by weight of this oil.

Microbial Degradation.--To determine the sequence of events and the extent of degradation when a crude oil was exposed to an acclimated population of microorganisms, a series of incubation experiments was set up. The large stock of a mixed microbial population allowed the selection of incubation times to be varied.

The most rapid and noticeable changes occurred in the *n*-paraffins after 4 hours of incubation. For comparison with the original crude oil, the chromatograms are presented in Figure 1, top and middle. A simplified outline of the *n*-paraffin envelope is presented for clarity in Figure 2, top, to illustrate the changes in shape that occurred after 4 hours of incubation. In the lower half of Figure 2, the percentage decrease in each *n*-paraffin is calculated on the basis of its resolved peak height. A definite molecular weight preference is noticeable for the lower molecular weight homologs. An extraction with carbon disulfide revealed that the lower molecular weight *n*-paraffins, *n*-C_5 to *n*-C_8, were less degraded in a reverse order from the maximum of degradation observed in the *n*-C_8 to *n*-C_{10} region. The complete decrease of the *n*-paraffins to amounts below the resolution limits required less than 10 hours under the conditions of this experiment.

The outline of the isoprenoid peaks and the changes in the shape of its envelope with time is shown in Figure 3, top. In the degradation of the isoprenoid hydrocarbons, C_{14} to C_{20}, a preference in terms of molecular weight was observed for the lower homologs (Fig. 3, bottom). Approximately 20 hours were required for the isoprenoid hydrocarbons to be decreased to below their resolution limits.

Further changes were observed in the shape of the base envelope, which represents the bulk of the components in the oil. The initial base envelope became progressively skewed during the incubation of the oil (Fig. 4). Again, a preference for the lower molecular weight components was indicated from the gas chromatograms. The higher molecular weight components of the base envelope, C_{25} and above, appeared to be the most resistant to microbial attack and decreased only slightly after 26 hours of incubation. The percentages of degradation, which were calculated from integrations, are summarized in Table 1.

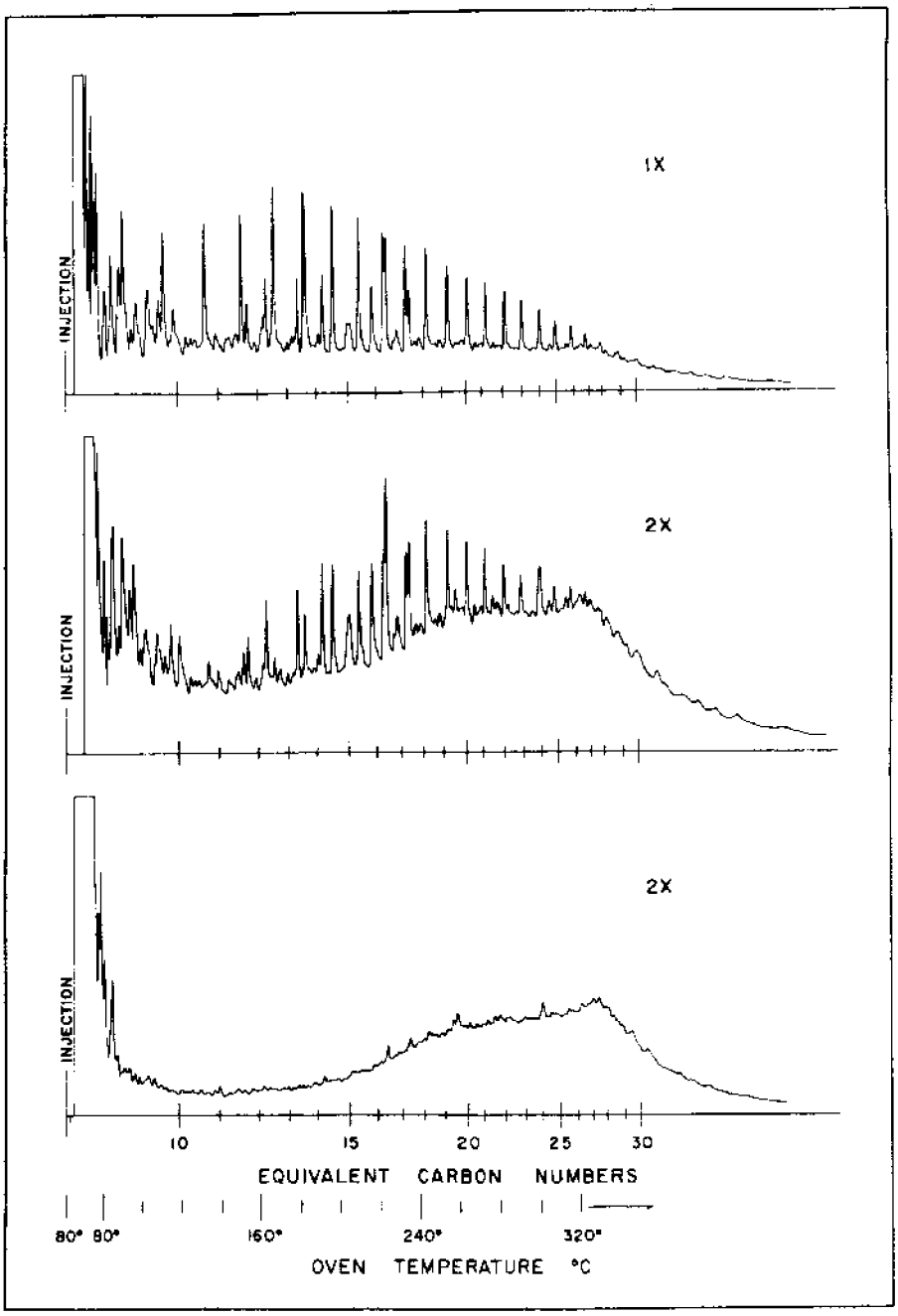

Figure 1. Gas Chromatograms of Crude Oil at 0-time (top) and after 6 hours (center) and 26 hours (bottom) of incubation with a mixed population of microorganisms. Note change in attenuation in top chromatogram.

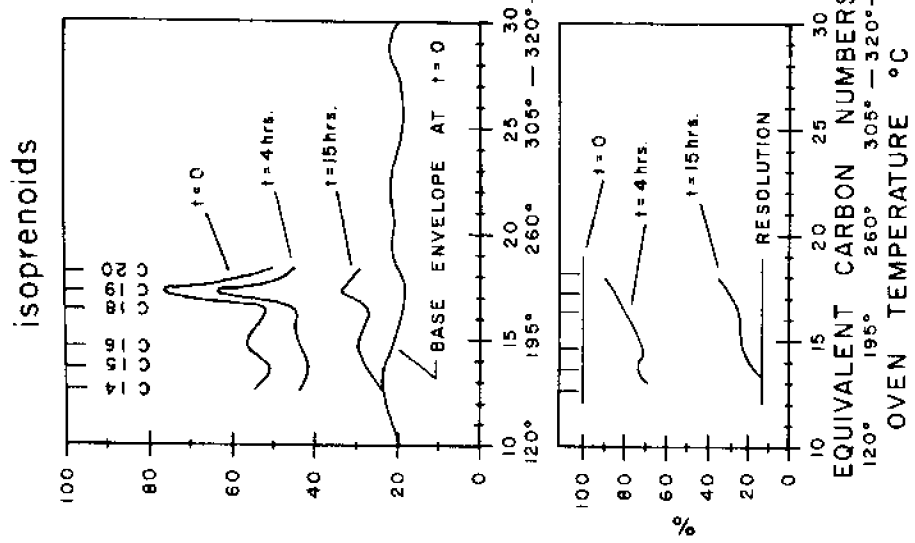

Figure 3. Changes in shape and amount of the enclosing envelopes of the six principal isoprenoid peaks. Top = envelopes formed by connecting maximum peak heights. Bottom = percentage decrease in peak heights as compared to resolved heights above the baseline.

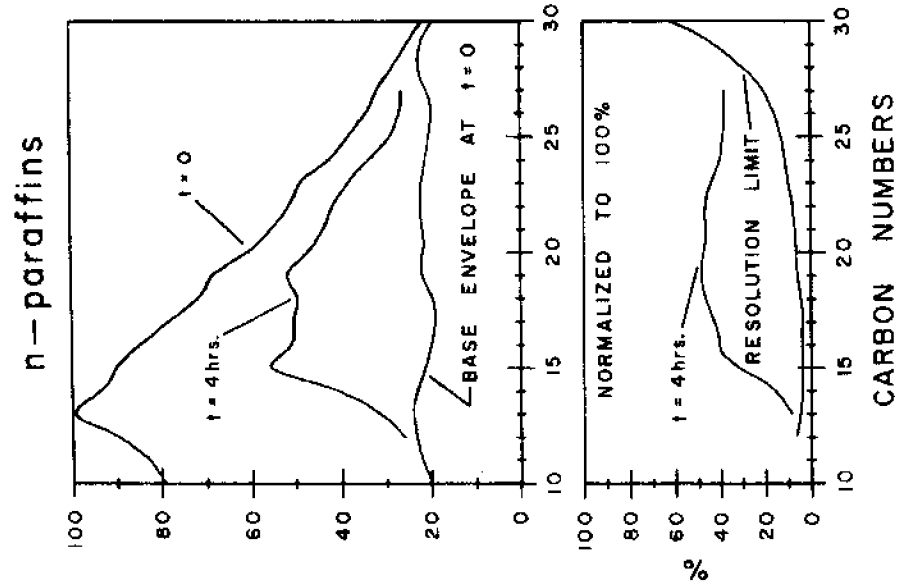

Figure 2. Changes in shape and amount of the enclosing envelopes of *n*-paraffin peaks with increasing incubation time. Top = envelopes formed by connecting maximum peak heights. Bottom = percentage decrease in peak heights as compared to resolved heights above the baseline.

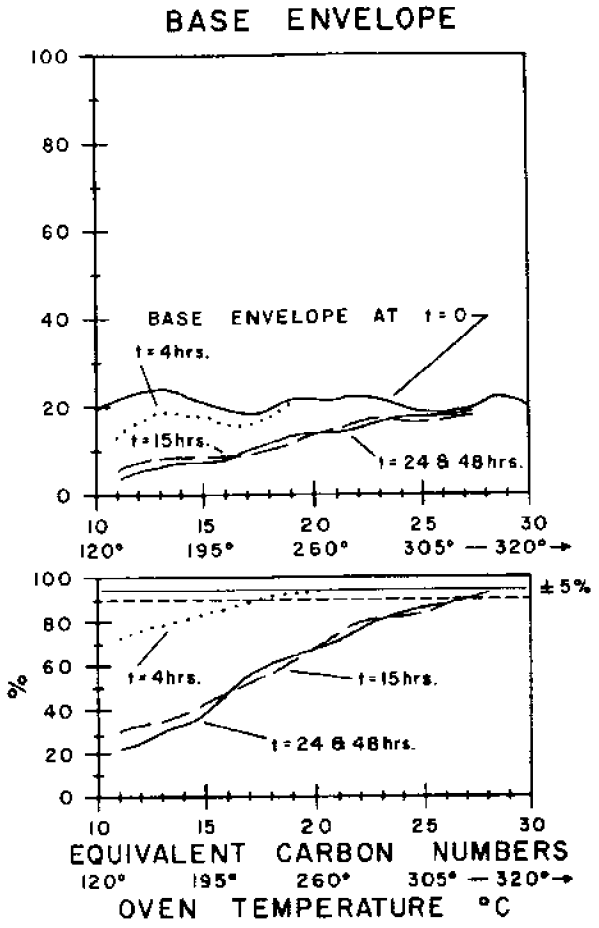

Figure 4. Changes in the amount and shape of the unresolved base envelope with increasing incubation time. Top = maximum envelope formed by connecting the tops of the fine peaks projecting above baseline. Bottom = percentage change in the envelope outline as compared to the baseline of the chromatogram.

Experimental Controls.--Several controls were included in these experiments to assure that the observed changes were biological in nature, and not due to physical or chemical effects. As indicated in the methods section, the stock microbial culture was supplied with crude oil every 48 hours. This addition of oil to the stock culture led to the accumulation of a non-degraded residual material in the cell suspension. The amount varied according to the particular addition sequence of oil and primary sewage effluent as well as the amount of cell mass. Prior to each incubation run, 100 ml of the acclimated stock culture was extracted to obtain a residue blank. The gas chromatogram of this material had the same appearance as that of the highly degraded oil observed after 26 hours of incubation (Fig. 1, bottom). A correction was made, during analysis of the chromatograms, by subtracting this small amount of non-degraded material.

TABLE 1

Changes in Crude Oil Incubated with an Acclimated Bacterial Culture

Time of incubation	Reduction in portion of the oil between C_{10} and C_{36}	Reduction of total oil from C_{10}[a] (less volatiles)
Hours	%	%
0	0	0
4	21	15
15	54	39
26	60	44
48	64	48
18 autoclaved	8	6

[a]Relative composition of total oil less volatiles:

	%
n-alkanes	16 ± 3
isoprenoids	2.0 ± 0.5
Base envelope	82 ± 3

A sample of the culture was heated to boiling in order to kill or inactivate the bacterial cells prior to the addition of the crude oil and incubated, while undergoing stirring, for 18 hours. Less than a 10% decrease in any of the fractions was visible after extraction and gas chromatographic analysis, indicating that living organisms are required to degrade the oil.

An additional experiment was carried out using an unacclimated culture of sewage-oxidizing microorganisms. This experiment also resulted in less than a 10% change in the chromatogram after 24 hours.

Integration of the peak areas of the gas chromatograms was the basis for determining the amount of decomposition of the oil during these experiments. The validity of the correction factors and the accuracy of this method were checked by quantitatively recovering the oil from the cultures and gravimetrically determining the amount present before and after incubation.

Gravimetric weights of the extracts from which the chloroform had been evaporated were compared with the weight calculated from the integration of the peak areas of the gas chromatograms. Table 2 lists the results of a series of these calculations involving: a) the conversions of the integrations of C_1 to C_{30} to weights, b) correction for the culture blank, and c) addition of weights corresponding to materials above C_{30}. These were then compared to the actual weights of the oil recovered from the experimental cultures, which were corrected for the gravimetric weight of residual oil in the culture blanks.

TABLE 2

Recovery of Oil from Bacterial Cultures: Comparison of Amounts Predicted by Integration of the Gas Chromatogram and the Amounts Actually Extracted from the Cultures

Sample	Calculated weight (integration and residue estimates)	Actual weight mg	Difference
0.1 ml	calibration	80	--
oil minus volatiles	57	62	-8%
composite T = 0	73.5	70	-5%
18 hr. sterile	66	63	+5%
4 hr.	75.5	79	-4%
15 hr.	63	71	-10%
24 hr.	52	58	-10%
48 hr.	49.5	52	-5%

DISCUSSION

The intent of the biological approach used in this study was to define the microbial potential for and the course of degradation in a crude oil, as well as to gain insight into the rates and conditions in the natural environment.

The validity of the experimental approach is based on at least two broad assumptions. It is assumed that only rarely will an oil spill be acted upon by a pure culture of microorganisms. It is also assumed that under natural conditions, the most significant changes in petroleum composition and quantity, from an ecological standpoint, will occur after the bulk of an acclimated population has been developed (3). It was thus decided that if one wants to determine the sequence of changes occurring during weathering, a fairly large actively metabolizing microbial population should be used (8,23). In these experiments, it was desirable to permit the fullest expression of metabolic potential of the microbes.

An important aspect of this study is that the enrichment culture technique was not limited to merely selection of organisms that can grow on oil, but rather a selection for organisms that can *degrade* oil. The value of this additional capability is emphasized by the phenomenon of cooxidation described by Leadbetter and Foster (13). In this process, hydrocarbons that cannot be utilized as growth substrates by certain bacteria may be oxidized by the same organisms during their growth on other organic substrates. As a consequence of the method of enrichment employed, a large stable inoculum was available.

It is possible that in specific instances some reactions may be different in sea water, but in the main, it is assumed that analogous metabolic

processes occur in the sea as in fresh water (3). The fact that sea water was not used for these experiments is therefore not an overriding consideration.

This study demonstrates that the biological modification of a crude oil follows a definite and predictable sequence of changes. The first changes are purely physical, characterized by the disproportionate disappearance of the more volatile low molecular weight hydrocarbons. The interpretation of the loss of the low molecular weight normal paraffins is complicated, however, by the finding that these compounds, n-C_8 and above, undergo bacterial decomposition when contained in closed systems designed to avoid evaporation losses. Once microbial decomposition is initiated, the amounts of the long chain paraffins start to decrease and the shape of their enclosing envelope is altered. The lighter n-alkanes are degraded at the most rapid rate, but there is a simultaneous decrease in all of the peak heights, progressing from the light to the heavy molecular weight components. The decomposition of the paraffins occurs rather quickly; by the end of 10 hours in the experiments presented here, the entire n-paraffin envelope has disappeared. The order of decrease is governed by molecular weight with no odd or even carbon number preference being apparent.

Simultaneously with the attack on the n-paraffins, there occurs a progressive reduction in the amounts of the branched isoprenoid hydrocarbons. Significant changes in the isoprenoid content as a function of molecular weight were observed after only 4 hours. The decrease in the isoprenoid hydrocarbons indicates that these compounds are not as resistant to biological degradation as once believed. The fact that the peaks of these compounds start to decay almost immediately, and at differential rates, cautions against using them as internal standards when working with mixed microbial populations (1,2,15).

The base envelope containing the unresolved cycloalkanes and aromatics also undergoes some degradation. The attack is also more pronounced in the light end of the molecular weight distributions, and progresses at a decreasing rate towards the heavier end. After the first 26 hours, the decay rate slows down rather markedly with only a slight (5%) decrease being observed in the next 24 hours. The observed rate change reflects the relative biological stability of the high molecular weight fraction. This fraction becomes the predominant material as decomposition continues and there is a progressive skewing of the chromatogram toward the heavier ends. This apparent increase in the high-molecular fraction of weathered oil has often been observed (4) but the significance of biological changes to account for it has been recognized by few investigators (17). The increase is relative and becomes apparent only after extensive biological decomposition has occurred.

Slight changes in the high molecular weight fraction, on the order of 5% per day, are almost impossible to detect due to the limited resolution of the C_{30} and above fractions by the gas chromatographic conditions used.

It is important to note when examining the results of this investigation that by using a well-acclimated mixed population of bacteria, the attack on all components of the crude oil began simultaneously. There was no evidence of a diauxie effect (preferential utilization of substrate) as has been observed with relatively pure cultures of microorganisms. The sequential pattern of decomposition observed in the studies reported here is due

primarily to rate differences, and probably reflects the activity of different segments of the microbial population.

The progressive changes that a given crude oil undergoes when exposed to a mixed microbial population may vary in rate and degree of attack according to many environmental factors. However, it is anticipated that the sequence of events will occur in the order indicated by these studies. Thus, given enough time, some crude oils will eventually degrade in an aerobic environment to a level similar to the 26-hour chromatograms (Fig. 1, bottom).

A chromatogram of a sample of heavily weathered oil collected from a California beach is presented in Figure 5. There is a striking similarity between this chromatogram and the chromatogram produced from the 26 hours of incubation with the mixed bacterial population. Since the original reservoir suspected for this weathered sample is known to contain both *n*-paraffin and isoprenoid hydrocarbons, it is inferred that this sample had undergone extensive microbial decomposition during the removal of the *n*-paraffins and isoprenoid hydrocarbons.

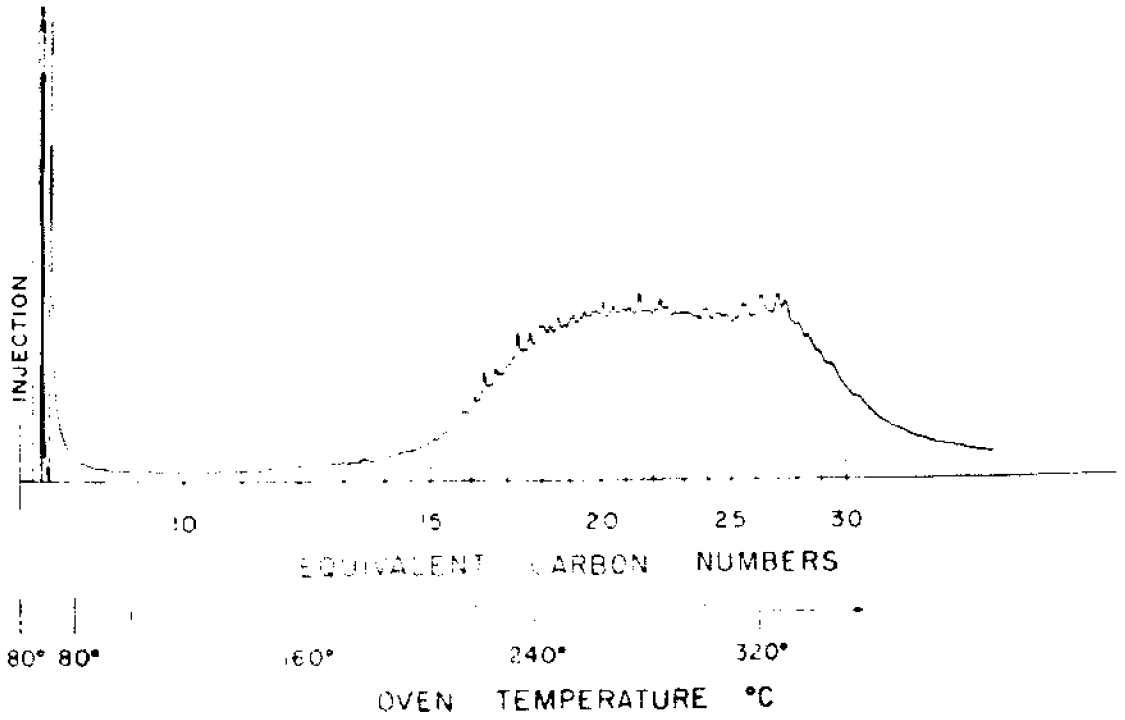

Figure 5. Chromatogram of a Weathered Sample of Crude Oil Collected from a Southern California Beach. The parent source of this oil is known to contain the missing paraffin and isoprenoid peaks.

Since the apparently biologically-resistant high molecular weight fraction of petroleum contains potentially toxic and/or carcinogenic compounds (23,24), it is important to determine its ultimate fate in the environment. In order to follow the changes that fraction undergoes, and to investigate its potential entry into the food chain, information on component identification and rates of breakdown is essential. Further progress in the area of microbial decomposition of crude oil awaits the development and application of appropriate analytical techniques to permit the resolution of these chemical species.

ACKNOWLEDGMENTS

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CONSIDERATIONS IN APPLICATION OF MICROORGANISMS TO THE ENVIRONMENT FOR DEGRADATION OF PETROLEUM PRODUCTS

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Considerations in the use of microbial preparations for the degradation of oil pollutants are presented. Besides obvious effects of nutrients on the environment, factors associated with emulsification of the oil pollutant, i.e., increased solubility and toxicity of the residue, and factors in application of the preparation (aerosolization) are discussed.

INTRODUCTION

During the past few years, there has been extensive thought and research (coupled with some well publicized demonstrations) given to possible application of microorganisms to degrade petroleum products inadvertently released into the environment (1,15). In theory, microbial preparations would be applied to the spilled petroleum and, through metabolic activities of the microorganisms, the petroleum would be degraded in time. This technique would be particularly useful in inaccessible areas or where normal cleanup operations would be extremely difficult, i.e., marshes, etc. Certainly, microbial preparations could provide a supplementary tool to current methods of oil removal. However, use of such a technique presents areas of concern perhaps even more important than the question of effectiveness of the material. These concerns should be considered and examined further before widespread implementation of the technique.

BACKGROUND

The Microbial Preparation.--A single microorganism does not usually have the enzymatic capability to metabolize all of the many varieties of compounds that would be present in a spilled oil (24,21). Thus, a mixture of microorganisms including fungi (19), yeasts (5), and bacteria (14), and representing a multitude of enzymatic capabilities would be used.

These microorganisms may be from pure culture stocks with known characteristics and enzymatic capabilities and combined to form a suitable preparation. Alternatively and probably more successfully, mixtures of organisms may be obtained from natural sources, i.e., soils, sediments or waters, using enrichment techniques without further culture purification. In any event,

the preparation will include a number of organisms, possibly from a variety of sources. Some additives may be included to facilitate dispersal, maintain culture viability, or to minimize losses into the water base.

Method of Application.--The microbial preparation may be applied in any of three basic forms: a wet slurry, a dry powder, or as granules.

The wet slurry may be released in large volumes from a boat, with the turbulence from the screw providing the mixing. Alternatively, the slurry may be sprayed from either a boat or airplane which would offer more economical coverage of the spilled petroleum.

The dry powder could be prepared as an air-slurry and handled in a similar manner as an aerosolized dust, again from a ship or airplane. If desired, the powder could be combined with, or attached to, small nutrient oleophilic or hydrophobic particles and aerosolized.

The microbial preparation could be pelletized or capsulated with nutrients to form larger particles. These large particles or granules would be prepared to dissolve in either the oil or water phase soon after contact (18).

Aerosols.--In applying the microbial preparation by wet or dry spray (aerosol), a large volume dispersal in large droplets would most probably be used. The predominant particle sizes from these conventional short-range sprayers range from 40-100 microns to large "rain" drops and the aerosols usually contain from 10^3 to 10^5 particles per liter. Despite this relatively large size, their production is inevitably accompanied by the generation of much smaller particles (<10μ) and by even larger numbers (10^6-10^{12}) of particles, some smaller than 1.0μ (12).

The aerosol in the immediate area of its production can be measured in particles per cubic meter. Downwind of this point or line source the concentration of particles decreases rapidly, in fact almost exponentially in relation to the distance, wind velocity, lapse rate and original particle size. The ability of single microbial cells, particularly pathogens, to initiate reactions means that tolerable levels are usually in the "parts per hundred billion" region (or picograms per cubic meter), as contrasted to the "parts per million" levels frequently accepted for toxic chemicals.

DISCUSSION

The deposition of aerosols in the lungs of man during breathing constitutes an important mechanism for the entry of toxins or pathogenic organisms into the body. The upper part of the respiratory tract is protected by an efficient ciliary and mucous blanket mechanism for the rapid removal of particles deposited in the upper airways. Particles reaching the deeper bronchiole and alveolar regions which comprise the "tennis court" area of the lungs are removed more slowly (17).

Particles in the 1 to 3μ range and in sizes below 0.3μ deposit in these deep pulmonary spaces where a large fraction is retained (10,13). Davies et al. (8) and Davies (9) estimated that 30 to 60% of such small particles entering the respiratory system penetrate the deepest parts of the respiratory system and are deposited firmly on tissue surfaces. These may be

removed by the mucous blanket or phagocytes (or alveolar macrophages), move into the lymph system, be sequestered by a tissue reaction; or, if even moderately soluble, these can be dissolved and transferred into the blood stream. Some years ago Dautrebande (7) observed that the rate of transport of a given volume of soluble and submicron size material into the blood stream was ten to twelve-fold greater from alveolar tissue than from the gastrointestinal tract.

The presence of potential human or animal pathogens in the microbial preparations used for seeding oil spills can create hazards either during the initial aerosolization or from the secondary aerosolization of spume or spray from the water surface. We are concerned not only for overtly pathogenic microorganisms, but also for those opportunistic pathogenic organisms able to produce infection only under certain conditions of stress, or lowered resistance of the host. Microbial preparations proposed for oil degradation should be grown under scrupulously controlled conditions and screened to assure that these types of organisms are not included. This is particularly important for microbial preparations derived from enrichment processes without further purification. This screening is not a simple process and in the latter case, if done properly, can require extensive testing using animal species as hosts. Adequate consideration must be given to possible bacterial or even viral contaminants which may directly, or through the products of metabolism, contain teratogenic or oncogenic materials. Mixed microbial cultures of high concentration and with unclearly defined biological characteristics are not easily described, nor should they be treated lightly.

In regard to the non-pathogenic organisms: if man or animal is exposed to even low doses of such aerosol, the foreign proteins included can cause allergic reactions or sensitization with dramatic results. "Allergies" in the generic sense, and particularly from aerosols of fungi, pollens, spores, dusts, and powders, are well known. However, it is of interest that there have been reported cases of adverse reactions to many of the proteins and materials most likely to be found in oil control mixtures. Bohm (3) has reported a response against peptone, a common component in nutrient broths. Flind (11) has reported similar results from enzymes produced by *B. subtilis*, an almost ubiquitous spore-forming organism. Salvaggio et al. (20) observed reactions from a polysaccharide, while Bernstein and Safferman (2) showed allergic responses to green algae and speculated that these could be human respiratory allergens. A complete listing is beyond the scope of this brief review, but suffice it to say that spraying any proteinaceous material in a manner permitting respiratory reception by man or animal should not be done without serious consideration of those possible effects. In particular, the problem of possible infection, allergic reaction or sensitization is of especially serious concern to those people directly associated with the dispersal of any microbial preparation, particularly if this is done on a repetitive basis which tends to aggravate sensitivity problems. For example, if a penicillin-producing organism is a likely constituent, sensitivity to some of these strains could deny the use of the drug penicillin to the individual concerned.

Regardless of the method of application, another problem is the possible effect of the increased density of microorganisms in the environment. This includes the possible pathogenicity of the microorganisms to marine

organisms, the toxicity of the metabolic products derived from petroleum degradation (21), providing a food base for various plankters and subsequent effects (5).

The application of the microbial preparation, with nitrogen and phosphorus nutrients, to an oil spill provides the potential for emulsification of the petroleum, as well as an obvious potential for additional nutrient pollution. Emulsification of the petroleum results in an increase in the surface area of the pollutant leading to its accelerated biodegradation. However, this action can be a "mixed blessing" since increasing the surface area of the petroleum decreases the diffusion time required for the more water-soluble components (both aliphatic and aromatic) to leach into the water column. The aliphatic components are metabolized more rapidly than the aromatic and naphthenic fractions (14,23) thereby leaving more of the latter fractions in the water. The toxicity of benzene and alkyl substituted derivatives is well known (4). The increased toxicity of a Mississippi crude to the common guppy, *Lesbiates reticulatus*, upon addition of yeasts and subsequent emulsification (5) may be a result of this process described. Granted, ultimately the aliphatic, aromatic and naphthenic fractions would leach from the oil slick to the same degree under normal weathering processes; however, the more soluble compounds are leached more rapidly when the oil slick is emulsified, resulting in their higher concentration in the water column.

It is anticipated that the emulsified oil residue will be *more* toxic as a result of the biodegradation process. With further biodegradation of the less soluble oil fractions remaining in the residue, the aliphatic fraction will be degraded more rapidly than the aromatic and naphthenic fractions (15,23). Thus, the more toxic and slower biodegraded aromatic and naphthenic fractions will tend to be concentrated in the residue.

Biodegradation of an oil slick results in the formation of extracellular metabolic products, the effects of which should be considered. Aerobic biodegradation of the hydrocarbon molecule commences with the incorporation of molecular oxygen into the hydrocarbon (22). The resulting metabolic products may be more toxic and often have a greater solubility than the precursor compound. Biodegradation of an oil slick, emulsified oil, and the soluble fraction could conceivably result in a greatly increased toxic organic load in the water column due to increased solubility differences of the metabolic products. As an example, the conversion of butylbenzene to 3-phenylpropionic acid by a *Pseudomonas* species (6) results in a solubility increase of greater than 100 times to a level of 6 gm/liter of water.

The increased levels of both the more soluble, light weight, hydrocarbons and their metabolic derivatives, as a result of the above factors associated with biodegradation and concurrent emulsion, could have an adverse impact on the chemoreception of various marine invertebrates (16).

We would not preclude the use of microbial preparations, but would advise that careful consideration should be given to possible side effects before adopting their widespread use.

Figure 1 summarizes the areas of concern in applying microbial preparations to oil to hasten its removal. The major concern resulting from emulsification (though these same processes occur during the normal weathering of the slick), is that the time frame is greatly shortened; thus, the factors of

evaporation and dilution of the soluble and metabolic products by the water column do not play as large a role in keeping the toxic compounds at lower levels.

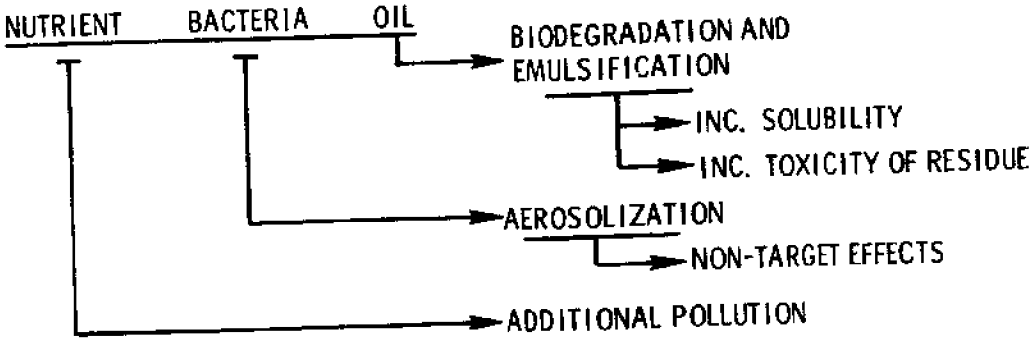

Figure 1. General Areas of Concern in Applying Microbial Preparations to Oil

The problems and potential dangers in aerosolization of the microbial preparation should be considered in regard to meteorological conditions, location to land (human and animal inhabitants), non-target effects in the marine environment, types of organisms in the preparation, and quality control of the preparation.

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STUDIES ON THE DEGRADATION OF PETROLEUM BY FILAMENTOUS FUNGI

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The initial report in 1895 of microbial growth on hydrocarbon substrates was that of Miyoshi (16) with the observation that *Botrytis cinera*, a Deuteromycete, would attack the paraffin used in embedding cellulose membranes. In 1906, Rahn reported on fungi that extensively utilized hydrocarbon substrates. The same year Söhngen published his classical work on methane-utilizing bacteria (22) and later demonstrated that microbes would degrade benzene, petroleum and paraffinic hydrocarbons (23). The utilization of various hydrocarbon substrates by yeasts and filamentous fungi has received extensive study and has been considered in a recent review by Klug and Markovetz (15). These authors compiled a list including 27 genera of filamentous fungi and 10 genera of yeasts that utilize aliphatic hydrocarbons as source of carbon and energy.

Studies were initiated in this laboratory on the degradation of petroleum by microorganisms maintained in stock culture and originally selected for their ability to grow on hydrocarbon substrates. The results suggested that many of the constituent parts of complex petroleum could be utilized by these organisms but none could mineralize a significant part of the oil. It was evident, however, that the fungi available were superior to bacteria in the amount of growth attained on various crude oils (5). Tests with microbes isolated by enrichment with crude oil as substrate confirmed that fungi were more efficient in our system in mineralizing petroleum hydrocarbons (6,7). This report is concerned with several aspects of these hydrocarbon degradation studies.

MATERIALS AND METHODS

Enrichment.--Mud and water samples were collected from coastal areas of North Carolina. Enrichment with crude oil as substrate (19) resulted in the isolation of several hundred bacterial cultures and a number of yeasts and filamentous fungi. Similar experiments were conducted with soil and water obtained from Bermuda. The basal medium for isolation was sea water supplemented with NH_4Cl (0.25 mg/ml) and Na_2HPO_4/KH_2PO_4(0.03 mg/ml). All organisms could utilize one or more hydrocarbons as sole source of carbon and energy. Isolation of microorganisms from primary enrichment was accomplished by the well plate method (7).

Substrate Specificity.--All substrate specificity experiments were carried out with sea water (10 ml media/50 ml flask) supplemented with

phosphate and nitrogen (see above). All liquid and solid substrates were added at 0.2% and gaseous substrates were added as previously described (18). Inoculum consisted of a drop of a 72 h culture obtained by growth on a suitable substrate. Fungal cultures were homogenized in a sterile blendor cup prior to addition to test substrates. An inoculated control without substrate was added for each organism. All tests were incubated at 26 C. The bacteria were placed on a rotary shaker and the fungi were run in both shake and stationary culture.

Utilization of Crude Oil.--Crude oil was placed on the surface of a sea water medium supplemented with a source of N and P. The amount of mycelium produced was determined by dry weight as previously described (7). In some instances the residual crude oil present after growth was determined by gravimetric analysis (7).

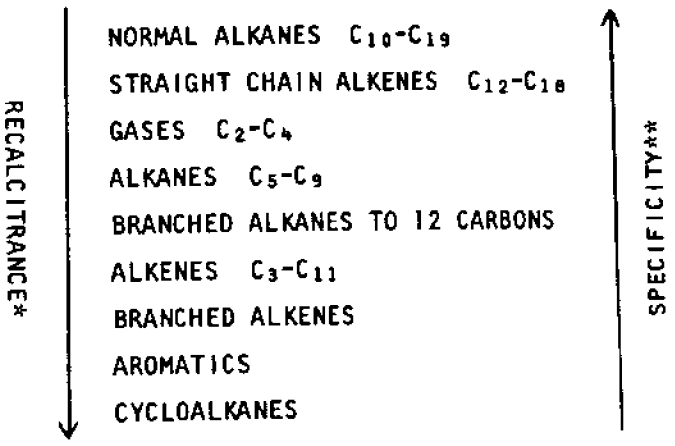
Crude Oils Used in These Studies.--Crude oil and petroleum samples were obtained from a number of oil companies and were labeled as follows: Gulf Oil Company--South Louisiana Crude, Mesa Crude, Kiwait Crude, Nigerian Crude, Leona Crude, Merey Crude and No. 6 Fuel Oil; Pennsylvania Refining Co.--Pennsylvania Crude Oil; Shell Oil Co.--Light Off-Shore Crude and Heavy Off-Shore Crude Oil; Texaco--South Louisiana Regular Crude and West Texas/New Mexico Sour Crude Oil; Phillips--Mixed base/mid Continent Crude Oil; Esso--Bunker 6 and Brega Crude Oil; Atlantic Richfield--San Andreas (West Texas), North Russel Devonian (Texas) Crude, Prudhoe Bay/Sadlerochit (Alaska), Swanson River Field (Alaska), Markham Zone (Springer, Okla.), Off Shore Louisiana Crude, San Joaquin (Venezuela) and Lagunillas (Venezuela) Crude Oil; Cosden Oil and Chemical Co.--West Texas Sour Crude and West Texas Sweet Crude Oil.

RESULTS

Enrichment culture with alkenes, xylenes, etc., as substrate, and estuarine water and mud collected from littoral areas of North Carolina as inoculum, yielded a considerable number of hydrocarbon-utilizing bacteria, yeasts, actinomycetes and fungi. These organisms were isolated in pure culture and tested for substrate specificity. Results are presented in Table I. The number of organisms in soil and water samples that could utilize *n*-alkanes was determined by an end point dilution technique. The soil or water sample was diluted in physiological saline and one milliliter of the selected dilution was placed in a mineral salts medium containing *n*-tetradecane as substrate. There were 10^2 to 10^4 organisms per gram of original soil or water sample that could utilize the alkane as carbon and energy source. In contrast, we have not been able to isolate an organism in pure culture that would grow on cyclohexane (H. W. Beam and J. J. Perry, MS in preparation). None of the organisms isolated in this study required vitamins or other growth factors. On isolation, the bacteria from sea water or marine habitats grew better in the N- and P-supplemented sea water medium than in mineral salts. However, the requirement for sea water was lost after repeated transfer after which they grew well in the L-salts medium routinely used in this laboratory (20). Filamentous fungi were unaffected by the presence or absence of sea water and equivalent cell mass was attained in either a mineral salts medium or N- and P-supplemented sea water with hydrocarbons as growth substrate.

TABLE 1

Relative Biodegradability of Various Hydrocarbon Substrates



*The number of microorganisms isolated that would grow on the substrates listed decreased from top to bottom.

**Any organism isolated on the compounds further down the list would generally grow on those above.

The results of our studies suggested that, by our isolation procedures, the major group of organisms in the environment that grew on paraffinic hydrocarbons comprised the mycobacteria and related organisms. Fewer fungi were isolated but these were more effective in mineralizing hydrocarbons and crude oil. Among the filamentous fungi isolated, the most effective were strains of *Cunninghamella elegans* (7) and *Penicillium zonatum* (11). Other isolates that grew well on hydrocarbons were strains of *Aspergillus versicolor*, *Cephalosporium acremonium* and *Penicillium ochro-chlorens*.

The amount of crude oil utilized by the fungi was determined by recovery of residual substrate after growth, as previously described (7). The maximum amount utilized during growth of *C. elegans* and *P. zonatum* was 85-92% of paraffin base crude oil. These organisms utilized 30 to 65% of asphalt base crude oil. The growth rate was affected by the temperature of incubation. The optimum temperature for *C. elegans* was 30 C and for *P. zonatum* was 37 C.

The amount of N and P essential for maximum utilization of crude oil was determined. Growth was better on NH_4Cl than on $NaNO_3$, and 0.25 mg/ml N was sufficient for mineralization of crude oil.

DISCUSSION

The utilization of crude oil by microbes present in marine environments has been followed in a number of laboratories (1,2,3,10,13,14,21,24,25). The bacteria have been studied in greater detail and these organisms have been found to utilize the paraffinic fraction of crude oil more effectively than

the asphaltic fractions. The utilization of various hydrocarbons by yeasts and filamentous fungi has received considerable attention (9,15) and some studies on fungal degradation of petroleum have appeared (5,6,7). Most of the organisms described to date are more effective in degrading intermediate length and gaseous alkanes. Some can degrade aromatic compounds but little is known of the biodegradation of the naphthenes. Since paraffinic hydrocarbons are of widespread occurrence in living cells, it would follow that microbes capable of growth on these substrates would be more prevalent than those organisms that degrade the asphaltic fractions.

Despite the widespread occurrence of hydrocarbon-degrading organisms (20), the more recalcitrant fractions of petroleum are prevalent in all environments in which spillage of oil occurs (12,17). Blumer and Sass (3) followed the persistence of oil at the site of a massive fuel oil spill at Buzzard's Bay off West Falmouth, Massachusetts. These workers found the readily biodegradable alkanes remained in bottom deposits in the area after two years. These results and others (12,17) indicate that the level of hydrocarbon pollution in the biosphere will increase in proportion to the amount of oil spilled into the environment. The ever-rising need for oil as an energy source indicates that oil pollution will continue to be a problem.

The effect of petroleum on the total environment has not been fully explored nor do we know what effects these products will have on marine food chains. Reports on the accumulation of hydrocarbon pollutants in shellfish (4,8) have appeared. It is evident that persistent pollution by petroleum is deleterious and means must be found to limit spillage and also to remove contaminating oil from the environment.

Our results suggest that filamentous fungi might have greater potential than bacteria in cleansing the environment of spilled petroleum. The fungi degrade greater quantities of oil during growth, in part perhaps due to their development as a mat on the surface of the oil. Bacteria and yeasts grow in oil droplets where oxygen and mineral nutrients might be limiting. Another advantage is the ability of fungi to form spores that retain viability over extended periods of time without refrigeration. Spores could be accumulated for use as "seed" inocula when and where they might be needed.

Experiments with *C. elegans* and *P. zonatum* suggest that a means of adding sources of P and N must be found if large amounts of oil are to be degraded. Isolation of filamentous fungi that can grow on the more recalcitrant asphaltic fractions of crude oil are now underway.

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MICROBIAL DEGRADATION OF CRUDE OIL AND THE VARIOUS HYDROCARBON DERIVATIVES

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The utilization of various petroleum products by a crude oil-grown bacterial culture has been studied using manometric and gas chromatographic techniques. A comparison of the gas chromatogram before and after growth showed that the *n*-alkane fraction had been preferentially utilized. Manometric techniques demonstrated that the crude oil-grown cells could also rapidly degrade *n*-alkanes, hydrocarbon alcohols, methyl ester of fatty acids and alkylbenzenes.

INTRODUCTION

Crude and refined petroleum products which enter natural waters as wastes and spills are susceptible to microbial decomposition (4). Treatment of refinery wastewater with aeration pond actually involves the destruction of these oily materials by microorganisms prior to discharge. Microbial degradation of pure hydrocarbon compounds has received extensive attention (5,7), but until recently there has been little work on the microbial degradation of crude oil. This can be attributed to the technical difficulties encountered in the analytical complexities (1). The purpose of this paper is to present additional information about the biodegradation of crude oil and the various hydrocarbon derivatives.

MATERIALS AND METHODS

Microorganism and Growth Medium.--The crude oil-degrading culture, CMOl, was isolated from a local refinery soil using a technique developed in this laboratory (6). The organism is a short gram negative rod, the complete identification of which has not been completed. The basal mineral salt medium consisted of the following (in grams per liter): K_2HPO_4 anhydrous, 0.66; KH_2PO_4, 0.41; $MgCl_2 \cdot 6H_2O$, 0.10; $FeCl_2 \cdot 4H_2O$, 0.05; $MnCl_2 \cdot 4H_2O$, 0.002; and $(NH_4)_2SO_4$, 1.0. The final pH of the medium was 6.9, and it was sterilized at 15 lb for 15 min. Crude oil was sterilized separately under the same condition and was added aseptically to the medium at a concentration of 5 ml/liter.

Dry Weight Determination and Manometric Study.--Fifty ml of culture suspension was filtered through the loosely packed glass wool and 30 ml of the

filtrate was centrifuged at 20,000 x g for 20 min followed by two washes in distilled water. The cell pellet was taken up in 5 ml of distilled water and dried for 20 hr at 105 C.

Oxygen uptake was followed at 20 C with conventional methodology using the Gilson Differential Respirometer. After preincubation for 5 min, the substrate was tipped into the main chamber. The CO_2 was absorbed in 0.2 ml of 20% KOH placed in the center well. The total fluid volume of each Warburg flask was 3.2 ml.

Gas Chromatographic Analysis of Crude Oil.--Crude oil was analyzed with a Beckman GC-65 analytical gas chromatograph equipped with a dual flame ionization detector. The dual 1.8 m x 0.63 cm O.D. stainless steel column contained 2% OV-1 liquid phase on 80-100 mesh chromosorb G-AW-DMCH. The oven temperature was programmed from 60 C to 300 C at a rate of 5 C/min and was kept at 300 C isothermally for another 20 min. The injection port temperature was 250 C and helium was used as the carrier gas at a flow rate of 60 ml per min.

For analysis of biodegraded crude oil, 100 ml of culture suspension was centrifuged at 20,000 x g for 20 min at 35 C and the supernatant was extracted twice with 5 ml of *n*-hexane. The emulsion was broken by centrifugation at 5000 x g for 10 min at 4 C and the top clear solvent layer containing crude oil could be easily transferred to a 10 ml volumetric flask with a Pasteur pipet. This extract is suitable for immediate gas chromatographic analysis.

RESULTS AND DISCUSSION

The bacterial culture, CM01, was selected for this study from 19 isolates on the basis of its rapid growth on crude oil as a sole carbon and energy source. Figure 1 shows the effect of crude oil concentration on the cell yield after 2 days growth at 20 C. In this experiment various amounts of crude oil were added to 250 ml Erlenmeyer flasks, containing 100 ml of basal medium, on a rotary shaker at 250 strokes per min. It is obvious that the cell yield is a function of the crude oil concentration in the growth medium up to 6 ml per liter. Further increase in the oil concentration did not result in significant increase in cell yield.

The gas chromatogram pattern of Canadian crude oil is shown in Figure 2. The major hydrocarbon compounds were identified by their retention time when compared with the authentic compounds. It seems that *n*-alkanes are the major components of the Canadian crude oil. The ability of culture CM01 to attack the Canadian crude oil is demonstrated in Figure 3. The gas chromatogram indicates that all *n*-alkanes except *n*-decane, *n*-undecane, *n*-heptadecane and *n*-octadecane had been degraded extensively by this culture. However, no attempt was made to quantitate the individual *n*-alkanes degraded in the crude oil by integrating the corresponding peak area, because all methods available for routine measurement of hydrocarbon biodegradation are subject to various positive and negative errors and give only relative values (2).

Manometric technique has been extensively used in this paper to study the degradation of various hydrocarbon compounds. It has been accepted that the amount of oxidizable materials and the rate of oxidation characterize

either the degradability of the material tested or the number of bacteria responsible for the degradation (3). Since the amount of added bacterial cells in each Warburg flask was constant, the rate of oxygen consumption would reflect the degradability of the hydrocarbon compounds tested.

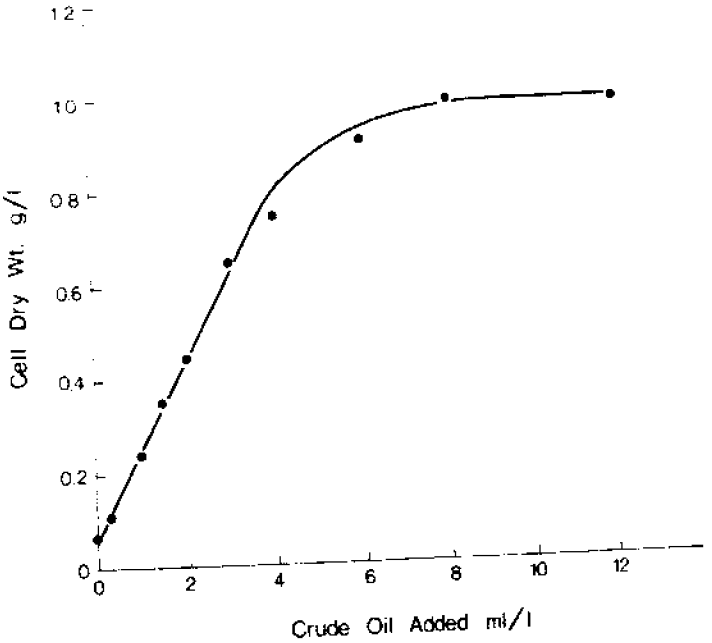

Figure 1. Effect of Canadian Crude Oil on the Cell Yield of Culture CM01. Each Erlenmeyer flask contained 100 ml of basal medium, 1 ml of cell inoculum and various amounts of Canadian crude oil. The experiments were carried out at 20 C on a rotary shaker at 250 strokes/min for 2 days.

In all the manometric experiments, the culture CM01 was grown on crude oil mineral salt medium in a 14-liter New Brunswick fermenter for 24 hr at 20 C, harvested, washed twice in mineral salt medium and resuspended in 0.05 M phosphate buffer (pH 7.0). Details of the individual manometric experiment are listed in the legend under each figure. As expected, hydrocarbon bacteria will attack petroleum products at different rates due to the variation of the components in the products. This phenomenon is clearly shown in Figure 4 in which the crude oil-grown cell degraded the crude oil (QO_2 = 38) faster than the Bunker C fuel oil (QO_2 = 17) and the kerosene (QO_2 = 5.3). Therefore, experiments were conducted to test the capability of culture CM01 to attack various gas chromatographic pure *n*-alkanes from $C_6 - C_{38}$ (Figs. 5, 6). Since *n*-octadecane and the above *n*-alkanes are solid at room temperature, it is of interest to note that the culture CM01 could rapidly attack these solid hydrocarbons. Before any substrate is oxidized, it has to be carried across the

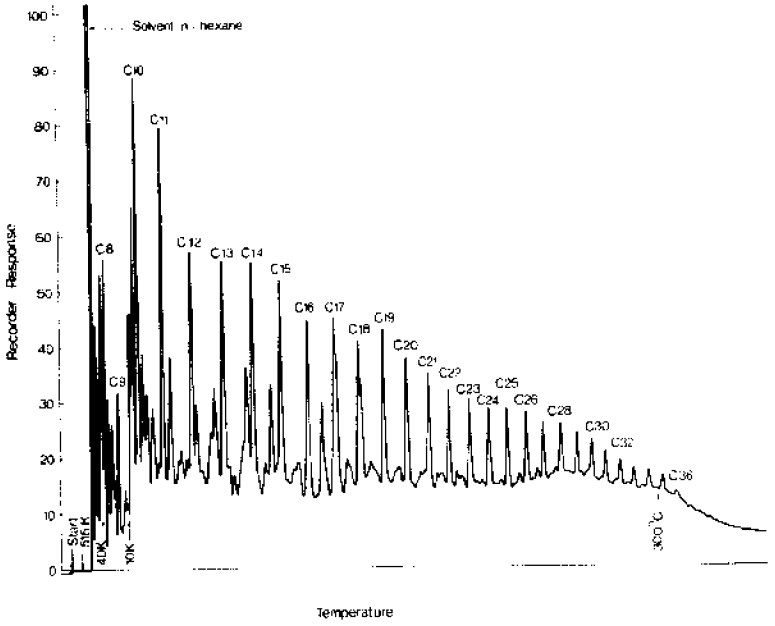

Figure 2. Gas Chromatogram of Canadian Crude Oil

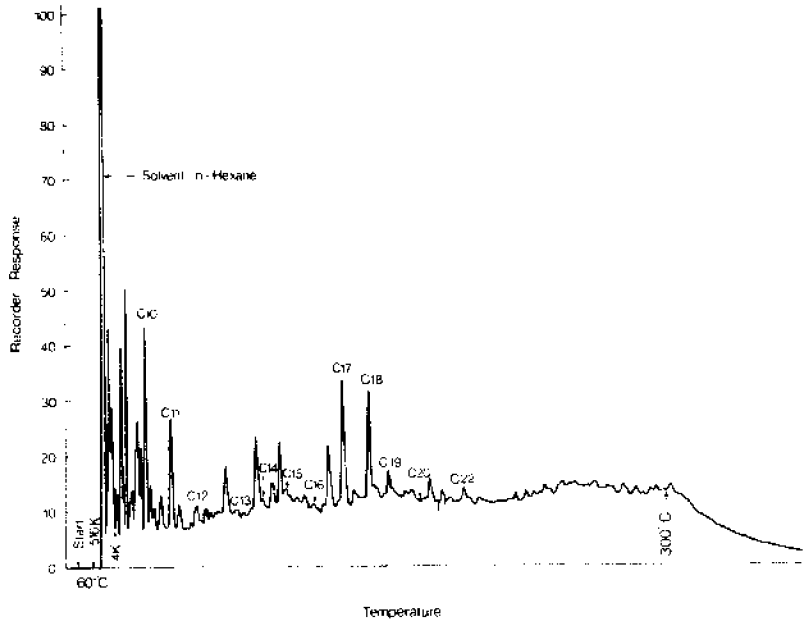

Figure 3. Gas Chromatogram of Canadian Crude Oil after the Growth of Culture CM01 for 2 Days

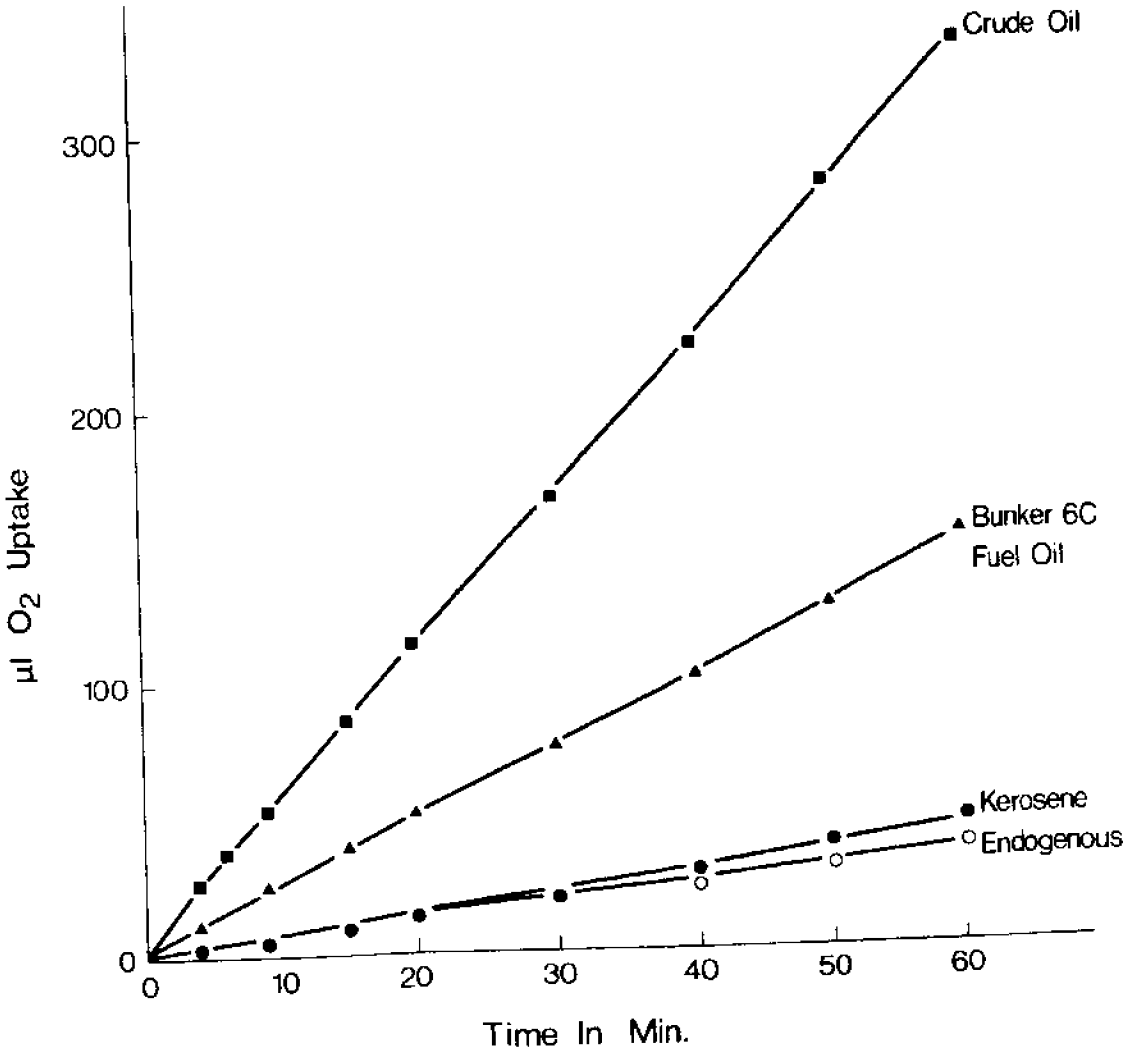

Figure 4. The Oxidation of Various Petroleum Products by Culture CM01. Each Warburg flask contained 1 ml of 0.05 M phosphate buffer (pH 7.0), 1 ml of cell suspension (8.8 mg dry wt.) and 100 µl of the substrate. The total fluid volume of each Warburg flask was 3.2 ml.

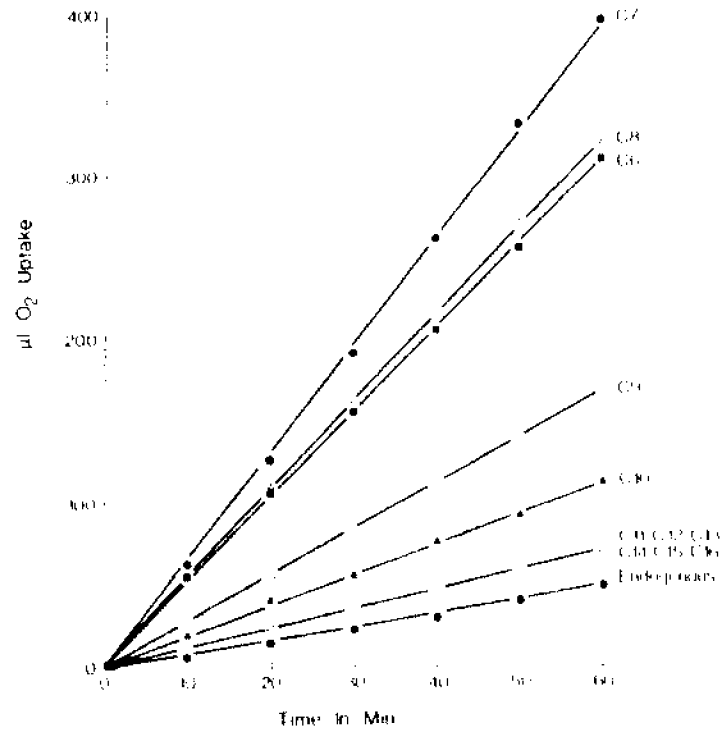

Figure 5. Effect of Various *n*-Alkanes on the Rate of Oxygen Consumption by Culture CM01. Each Warburg flask contained 1 ml of 0.05 M phosphate buffer (pH 7.0), 1 ml of cell suspension (6.7 mg dry wt.) and 100 µl or 50 mg of gas chromatographic pure *n*-alkanes.

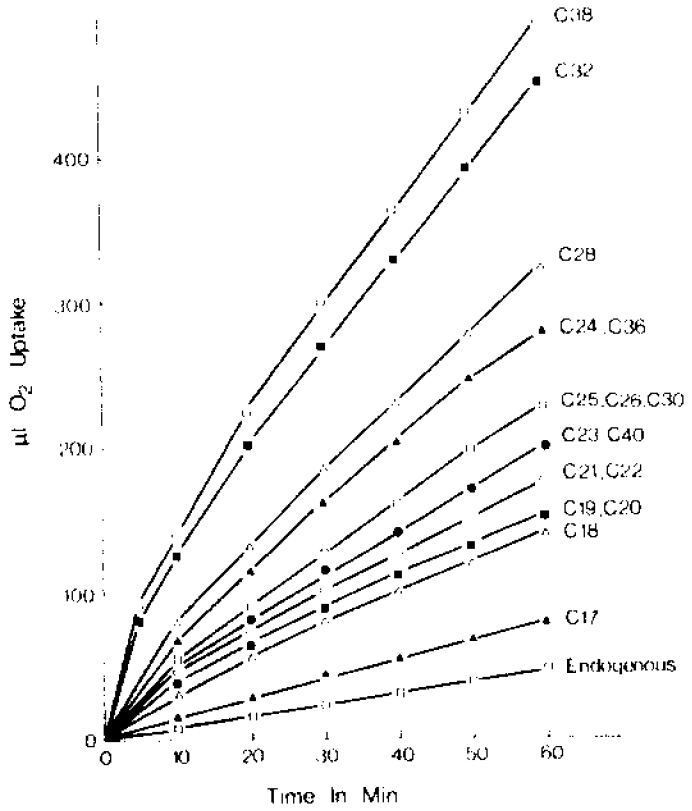

Figure 6. Effect of Various *n*-Alkanes on the Rate of Oxygen Consumption by Culture CM01. The experimental conditions were the same as in Fig. 5 except that each flask contained 7.2 mg cells.

cell membrane. How could these solid hydrocarbons be transported across the cell membrane? The bacterium must possess a unique transport system, because the oxygen consumption was immediate after tipping the solid hydrocarbon substrate into the main chamber of the Warburg flask. Another conclusion which can be drawn from this experiment is that there appears to be no preference for odd or even carbon substrate by the culture CM01.

Hydrocarbon alcohols have also been tested for their biodegradability by culture CM01. This may have some practical application in the future as it is known that the oil herders used in oil pollution control are long chain, high molecular weight alcohols. Therefore, it would seem advisable to insure that these oil herders did not remain in the environment after the oil clean up. It can be seen that most of the hydrocarbon alcohols tested were susceptible to degradation by the hydrocarbon bacterium (Fig. 7). The methyl ester of various fatty acids was also subject to degradation by culture CM01 indicating that the bacterium could hydrolyze the ester linkage of the fatty acid ester (Fig. 8). Several commercial oil emulsifiers are actually derived from the fatty acid ester. Recent study in this laboratory has indicated that the degradation of the oil emulsifier starts from the splitting of the ester linkage.

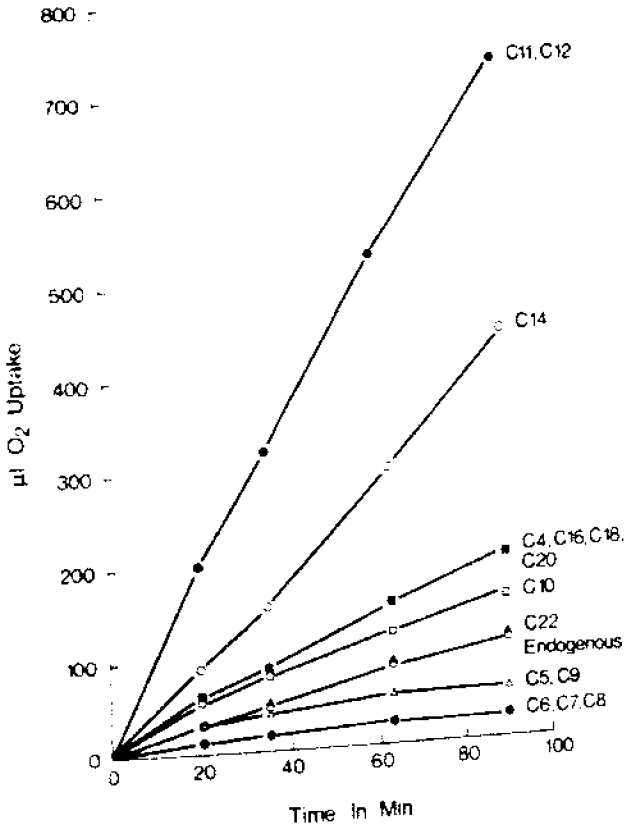

Figure 7. Oxidation of Various Hydrocarbon Alcohols by Culture CM01. Each Warburg flask contained 1 ml of 0.05 M phosphate buffer (pH 7.0), 1 ml of cell suspension (6.5 mg dry wt.) and 100 µl or 50 mg substrate.

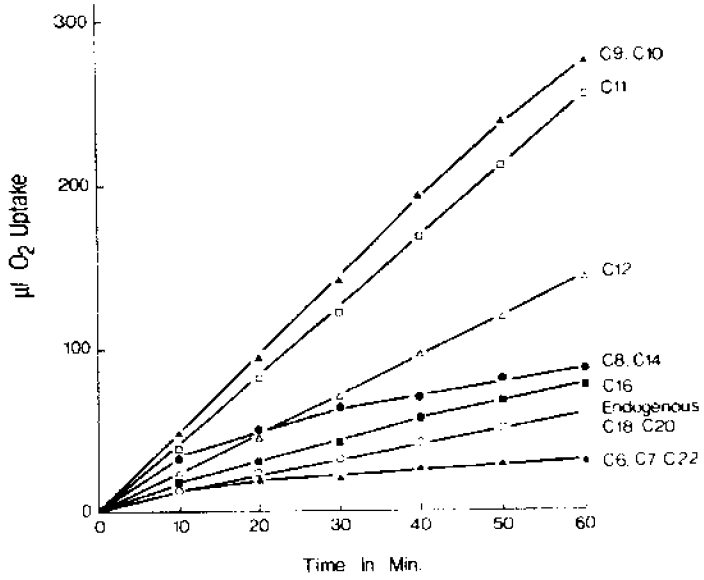

Figure 8. Oxidation of Methyl Ester of Fatty Acids by Culture CM01. Each Warburg flask contained 1 ml of 0.05 M phosphate buffer (pH 7.0), 1 ml of cell suspension (5.6 mg dry wt.) and 100 µl or 50 mg of substrate.

The effect of the side chain length of alkylbenzene on the rate of oxygen consumption by culture CM01 has also been studied. In this experiment 100 µl of gas chromatographic pure alkylbenzene was used as substrate to test their biodegradability (Fig. 9). As noted in Figure 10, benzene, toluene and ethyl benzene were inhibitory to the bacterium. Figure 11 summarizes the effect of carbon number of substrate, including *n*-alkanes, hydrocarbon alcohols and methyl ester of fatty acids, on the rate of oxygen consumption by culture CM01. Apparently, there is no definite pattern in the bacterial oxidation of these hydrocarbon substrates. The obvious conclusion which can be drawn from this study is that hydrocarbon bacteria, represented by culture CM01, possess a ready ability to degrade various petroleum products.

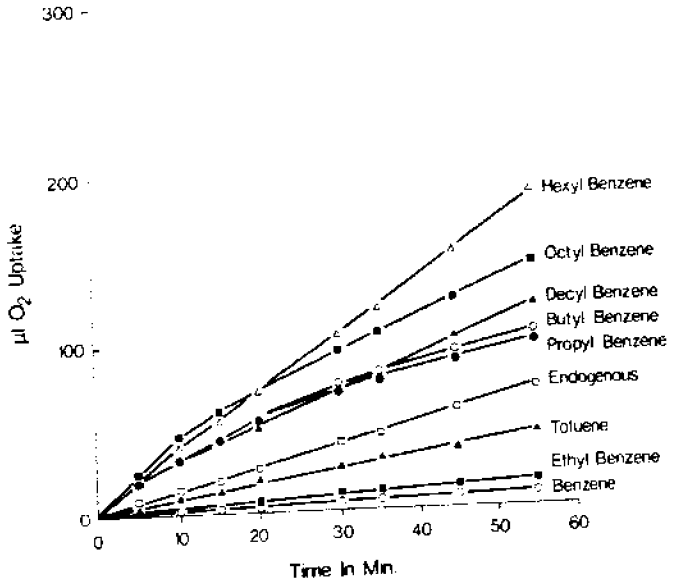

Figure 9. Oxidation of Various Alkylbenzene by Culture CM01. Each Warburg flask contained 1 ml of 0.05 M phosphate buffer (pH 7.0), 1 ml of cell suspension (7.3 mg dry wt.) and 100 µl of substrate.

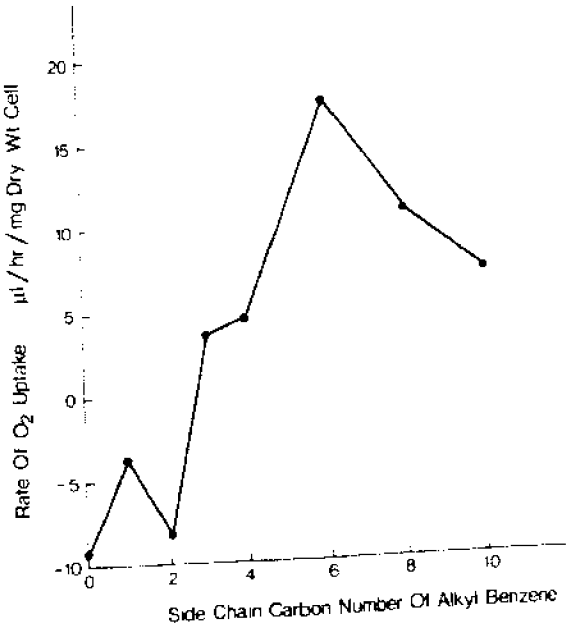

Figure 10. Effect of Side Chain Carbon Number of Alkylbenzene on the Rate of Oxygen Consumption by Culture CM01. The experimental conditions were exactly the same as in Fig. 9.

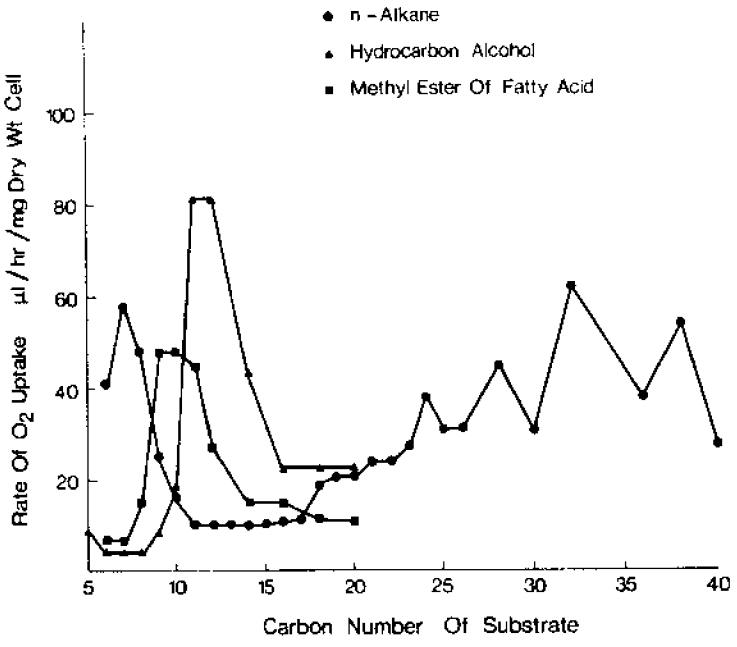

Figure 11. Effect of the Carbon Number of *n*-Alkanes, Hydrocarbon Alcohols and Methyl Ester of Fatty Acids on the Rate of Oxygen Consumption by Culture CM01

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MICROBES AND PETROLEUM: PERSPECTIVES AND IMPLICATIONS

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An ultra-structure study of *Acinetobacter* sp grown on paraffinic and olefinic hydrocarbons demonstrated cytoplasmic sequestering of hydrocarbons. Induced membrane synthesis was additionally demonstrated as a result of hydrocarbon metabolism. Increased cellular and extracellular lipid synthesis was qualitatively and quantitatively documented during hydrocarbon metabolism. These studies serve to emphasize our lack of detailed knowledge concerning the consequences which may arise from this relationship.

Hydrocarbon microbiology has persisted for the past half century as an ephemeral scientific curiosity with the exception of those applied aspects related to petroleum technology and commercial exploitation. Detailed and exhaustive studies concerning the fate and effect of diverse and complex fractions of petroleum in and on the environment are difficult if not impossible to find. The presence of petroleum in the environment, either accidentally or naturally, has created a myriad of crash programs attempting to evaluate and analyze the environmental impact of petroleum.

Our composite knowledge of mechanisms by which diverse microorganisms grow at the expense of hydrocarbons has been periodically reiterated (1,7,13, 23,30). These reviews serve to emphasize the paucity of knowledge concerning detailed mechanisms operative in microbial hydrocarbon oxidation processes. A great number of studies have been directed toward a compilation of hydrocarbon-utilizing microorganisms with very few investigators undertaking studies into the detailed mechanisms of hydrocarbon assimilation and utilization. The exact nature of biochemical mechanisms which regulate hydrocarbon oxidations by microorganisms are poorly understood at best.

This laboratory has concentrated its efforts on a hydrocarbon-utilizing microorganism in pure culture growing on defined and chemically pure hydrocarbons. Our studies are aimed at defining the cultural, physiological, biochemical, and enzymological parameters of microbial growth on hydrocarbons.

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The microorganism used in these studies has been described as an *Acinetobacter* sp (2,10) with the former epithet *Micrococcus cerificans* (4). This bacterium is capable of prolific growth on a variety of alkanes and alk-*1*-enes greater than 10 carbons in length. The structure of many compounds arising from the growth of this microorganism on specific hydrocarbons has been reported (5,11,27,28,29) as well as has the biosynthesis of characteristic wax esters (6,11). Detailed biochemical analyses with this microorganism have defined specific aspects of hydrocarbon metabolism (16,17,18,19,20,21). The present report extends these studies with observations concerning the effect and fate of hydrocarbon on a microorganism in pure culture.

MATERIALS AND METHODS

Organism and Growth Conditions.--All studies were carried out with the HOI-N strain of *Micrococcus cerificans* (*Acinetobacter* sp) as described by Finnerty et al. (4). The organism was grown on a mineral medium consisting of (in grams per liter): $(NH_4)_2SO_4$, 2; KH_2PO_4, 4; Na_2HPO_4, 6; $MgSO_4 \cdot 7H_2O$, 0.02; $CaCl_2 \cdot 2H_2O$, 0.001; $FeSO_4 \cdot 7H_2O$, 0.001; pH 7.5.

n-Alkanes and *n*-*1*-alkenes (Humphrey Chemical Co., New Haven, Conn.) were added to a final concentration of 1%.

Organisms were also grown on nutrient broth (0.8%)-yeast extract (0.5%), acetate (2%), and ribose (2%) for comparative studies. All hydrocarbon and non-hydrocarbon growth substrates were added to the defined mineral medium.

Cultures were grown on a gyratory shaker at room temperature (25 C) to the late exponential growth phase and harvested by centrifugation at room temperature. Cell pellets were washed two times with the mineral medium to remove the external non-metabolized hydrocarbon.

Fixation.--Two fixation procedures were used. Method 1 followed the techniques described by Kellenberger et al. (12). In method 2, the procedure of Glauert and Thornley was used (9). A cell suspension was mixed with equal parts of 5% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.3) containing 2 mg of $CaCl_2$ per ml.

Dehydration and Embedding.--The fixed cells were dehydrated by one of two procedures. Method 1 involved processing the samples through a graded series of water-ethanol mixtures followed by propylene oxide. These samples were infiltrated with Epon according to Luft (15) or with Maraglas as described by Freeman et al. (8). In method 2, the samples were dehydrated by processing through a graded series of Durcupan as described by Staubli (26) and embedded in Araldite. Ultrathin sections were cut on a Reichert OMU-2 ultramicrotome and mounted on uncoated 300 mesh copper grids. The sections were stained with lead citrate (25) followed by uranyl acetate and examined in a Phillips-200 electron microscope operating at 80 Kv.

Extraction of Cells.--Cell pellets obtained from the growth of cells on specified substrates were washed 5 times with distilled water by repeated centrifugation. Each cell pellet was processed through a graded series of water-ethanol mixtures as used in the dehydration for EM (50:50, 30:70, 5:95,

5:95, 0:100, 0:100, v/v). The alcohol extracts were pooled and reduced to dryness. All alcohol extracts reduced to dryness except those obtained from hexadecane and hexadec-1-ene grown cultures. The alcohol extracts were dissolved in chloroform, dried with anhydrous sodium sulfate, and dissolved in hexane for analysis by gas chromatography.

Gas Liquid Chromatography (GLC).--A Packard gas chromatograph, series 7500, consisting of a dual-column oven with coiled-glass columns (4 mm inside diameter, 1.83 m long) was used for the analysis of alcohol extracts. The detection system was an argon ionization detector with column support systems consisting of liquid phases of 10% Apiezon L and 20% diethylene glycol succinate (DEGS) on a support of 70-80 Anakrom A. Operating conditions were: column temperature, 120 C; detector temperature, 190 C; injection temperature, 180 C; outlet temperature, 205 C; argon flow rate, 60 ml/min; chart speed, 2.5 min/inch.

X-Ray Diffraction Analysis.--Cultures grown on specified hydrocarbon and non-hydrocarbon substrates were washed extensively and lyophilized. These cell preparations were encapsulated in a Mylar film and cooled to -10 C with a cold air stream. The cooled specimens were exposed to a 1 mm diameter beam of Ni-filtered X-rays from a Cu anode and a flat film was set perpendicular to the beam approximately 5.3 cm behind the specimen (flat-plate forward-reflection technique). The exposed film was quantitatively analyzed by microphotometry along a diametric line.

Scanning Electron Microscopy.--Cell preparations for scanning electron microscopy were either fixed in glutaraldehyde or dried unfixed onto specimen stubs, coated with gold-palladium (40:60) in a vacuum evaporator and viewed in a Cambridge Stereoscan Scanning Electron Microscope.

RESULTS

Early studies with *Acinetobacter* spp HO-1-N actively growing at the expense of hexadecane indicated the hydrocarbon was dispersed as an oil in water emulsion in the culture medium. Interestingly, hexadecane did not disperse into stable emulsions when shaken with a) uninoculated medium, b) bacteria (*Escherichia coli, Aerobacter aerogenes, Bacillus subtilis*) unable to metabolize hydrocarbons, or c) bacteria not preinduced to hydrocarbon growth. Bacteria preinduced to growth on hydrocarbons were essential for the formation of apparent stable hexadecane in water emulsions. A physical relationship between preinduced bacteria and microdroplets of hexadecane was noted by light microscopy in that the bacteria adhered to the surface of the hydrocarbon droplet.

This phenomenon was studied in further detail by scanning electron microscopy. *Acinetobacter* spp was grown on hexadecane, sampled during exponential growth, and prepared for scanning electron microscopy. Figures 1 and 2 reveal the surface of hydrocarbon microdroplets to be uniformly covered with bacteria.

A detailed study was initiated into the ultrastructure of *Acinetobacter* spp grown on hydrocarbon and non-hydrocarbon substrates. Ultra-thin sections prepared from cells grown on acetate, ribose, or nutrient broth-yeast extract (NBYE) were identical with respect to their fine-structure detail.

Figure 1. Scanning Electron Micrograph of *Acinetobacter* sp growing on hexadecane (HC) and sampled during the mid exponential growth phase. 875 X.

Figure 2. Scanning Electron Micrograph of *Acinetobacter* sp cells coating the surface of the hydrocarbon-hexadecane. The surface of the hydrocarbon is irregular with the bacteria randomly positioned on all surfaces. D indicates individual bacteria. 1825 X.

Figure 3 is a representative thin-section of NBYE-grown cells. In contrast, cells obtained from growth on hydrocarbons exhibited unique characteristics that served to distinguish them from non-hydrocarbon-grown bacteria. Figure 4 is a thin-section prepared from hexadecane-grown bacteria. Multiple inclusion bodies are readily apparent and served to characterize an ultra-structure feature associated with only hydrocarbon-grown bacteria. This fine-structure inclusion body was a characteristic feature associated with growth of *Acinetobacter* spp on a homologous set of alkanes varying in chain length from 12-20 carbon atoms. One piece of experimental evidence used to establish the specificity of the inclusions is shown in Figure 5. The cells were obtained from colonies of *Acinetobacter* spp growing on the surface of a mineral salts-agar medium in the presence of hexadecane vapors. The bacterial colonies were never in direct physical contact with liquid hexadecane. This experiment demonstrates the presence of multiple inclusions as a characteristic fine-structure detail.

Further evidence to establish the physical integrity of the inclusions was obtained by freeze-etch procedures. Figures 6 and 7 are micrographs of freeze-etch studies of *Acinetobacter* spp grown on hexadecane. Structural elements identical to those observed in thin-sections are present indicating the physical integrity of these inclusion bodies in hydrocarbon-grown bacteria.

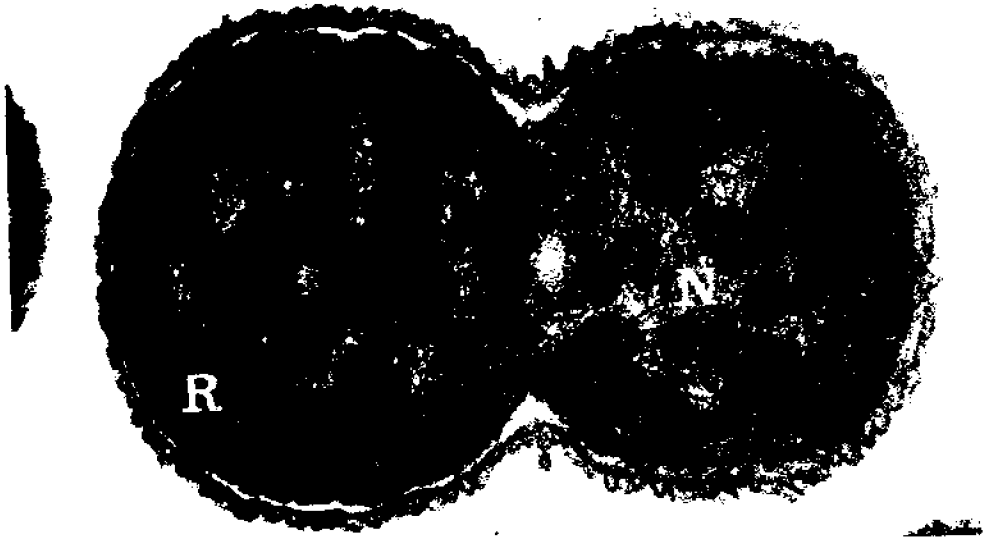

Figure 3. Electron micrograph of *Acinetobacter* sp grown on nutrient broth-yeast extract. Ribosomes (R) and nuclear material (N). 71,500 X.

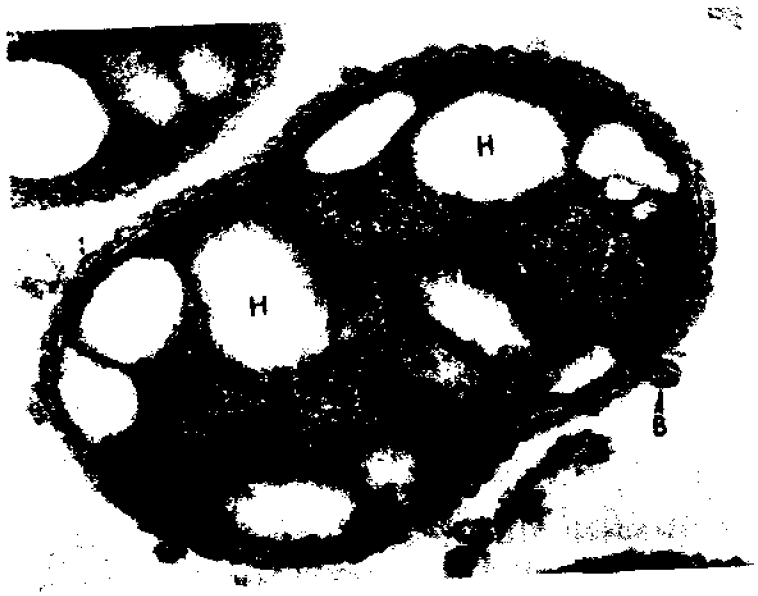

Figure 4. Ultra-thin sections of *Acinetobacter* sp grown on hexadecane medium showing the hydrocarbon inclusion bodies (H). Multiple inclusions are characteristic of alkane-grown bacteria. 82,000 X.

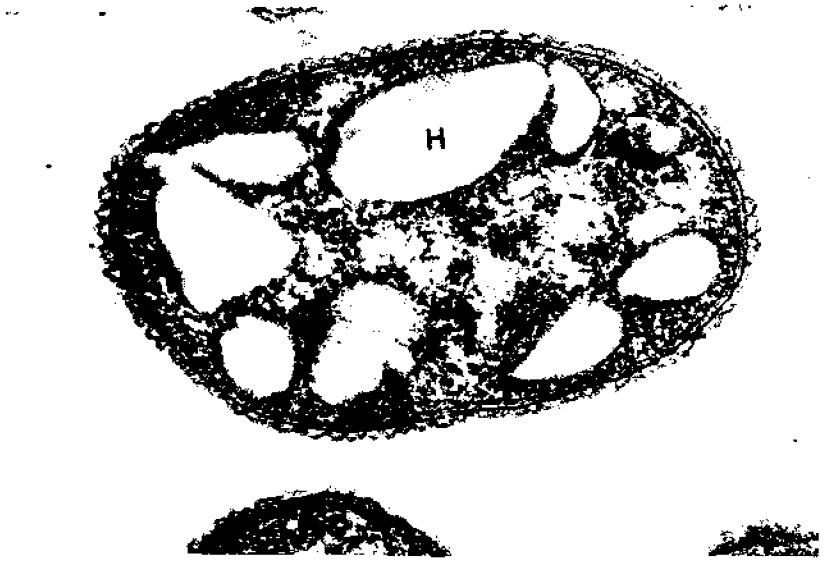

Figure 5. Ultra-thin section of *Acinetobacter* sp grown on hexadecane vapors. 76,000 X.

Alk-1-enes as sole carbon sources exhibited a different but still characteristic fine-structure pattern. Figure 8 shows a thin-section of hexadec-1-ene grown cells with two osmiophilic inclusion bodies. These inclusion bodies are characteristic of alkene-grown bacteria and appear as strongly osmiophilic bodies only when water-soluble dehydration and embedding procedures are used. The use of conventional procedures which require organic solvents removes the osmiophilic inclusions. The latter can also be extracted from thin-sections by floating the section on organic solvents (e.g., chloroform, hexane, ether).

The chemical identification of these inclusion bodies was established by gas chromatography and X-ray diffraction. Positive identification of pentadecane, hexadecane, hexadec-1-ene, heptadecane, and octadecane were confirmed in alcohol extracts of cells grown on these respective hydrocarbons by gas chromatography. Analyses by low temperature X-ray diffraction of lyophilized cells established the identity of the inclusion bodies. Figure 9 is a densitometric tracing of the X-ray diffraction pattern of heptadecane, while Figure 10 is the densitometric tracing obtained from cells which had been grown on heptadecane. The X-ray diffraction pattern allowed a positive identification of heptadecane in the bacteria. Positive identifications for the hydrocarbons specified above have also been obtained confirming the gas chromatographic evidence. Figure 11 represents a control where NBYE cells were analyzed by X-ray diffraction. It was not possible to correlate this pattern to a hydrocarbon structure providing conclusive evidence as to the chemical identity of the inclusion bodies.

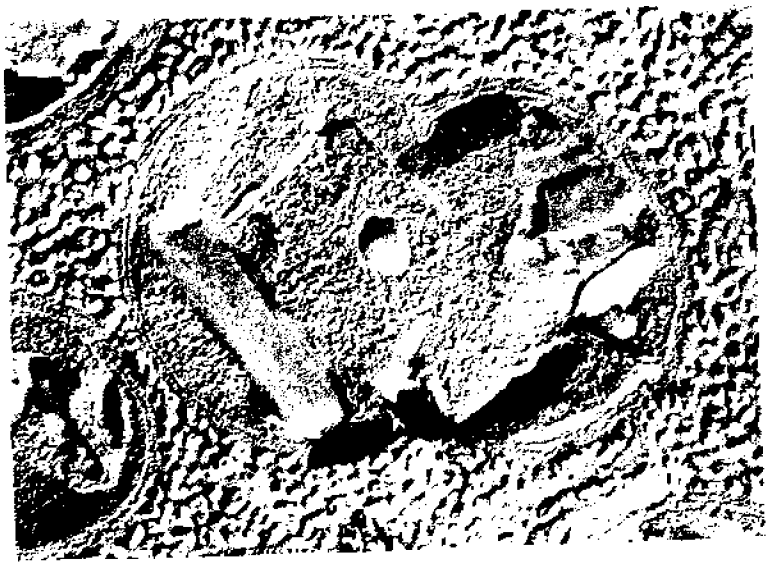

Figure 6. Freeze-etch of *Acinetobacter* sp grown on hexadecane. 22,000 X.

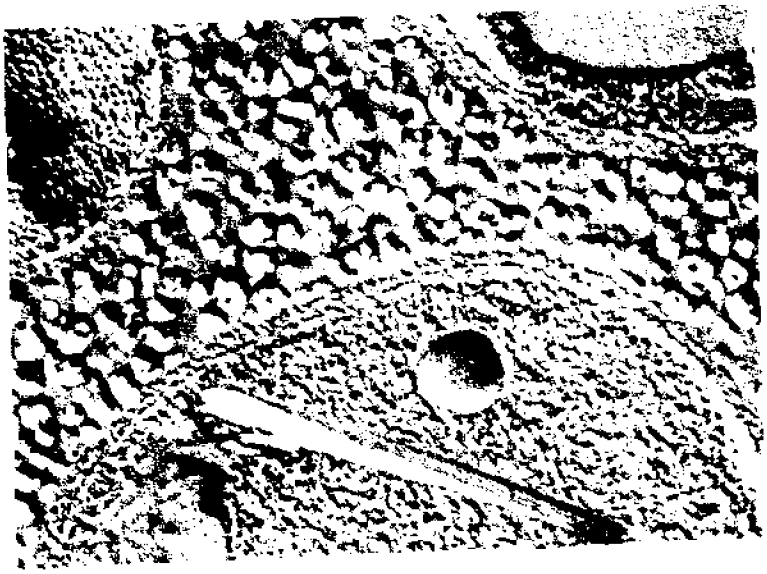

Figure 7. Freeze-etch of *Acinetobacter* sp grown on hexadecane. 83,000 X.

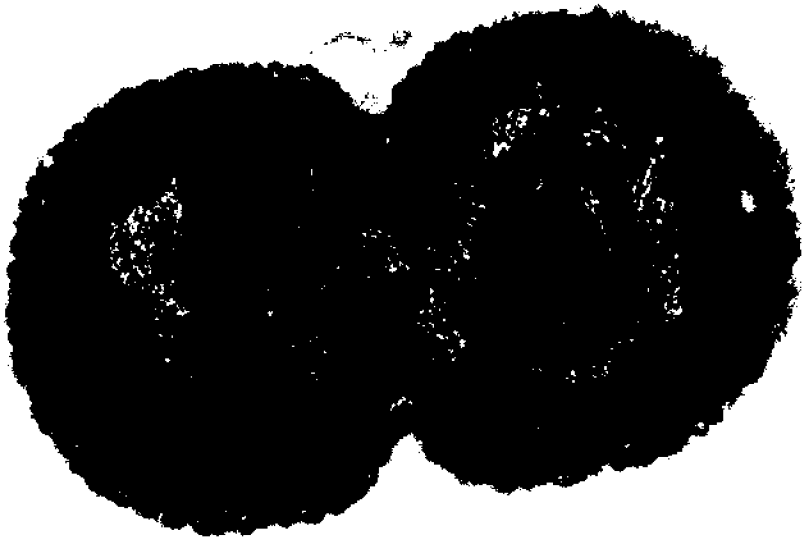

Figure 8. Ultra-thin section of *Acinetobacter* sp grown on hexadec-1-ene. Cells processed with durcupan so that the unsaturated, osmiophilic hydrocarbon appears as electron dense inclusions. 105,000 X.

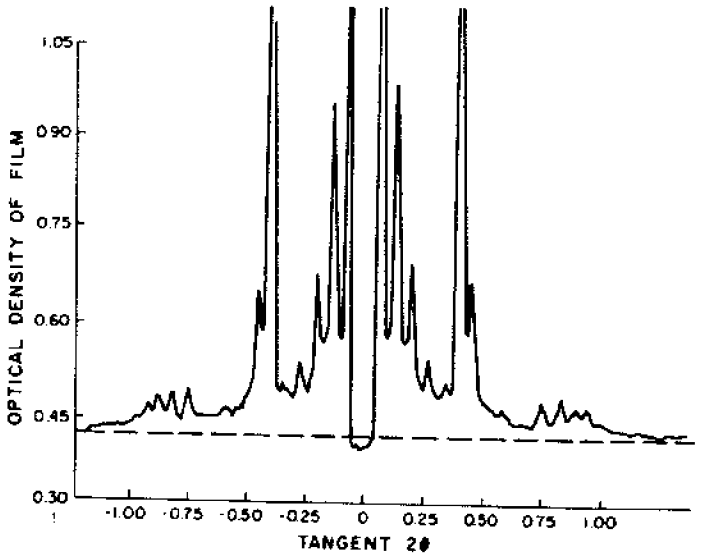

Figure 9. Densitometric tracing of X-ray diffraction pattern of *n*-heptadecane.

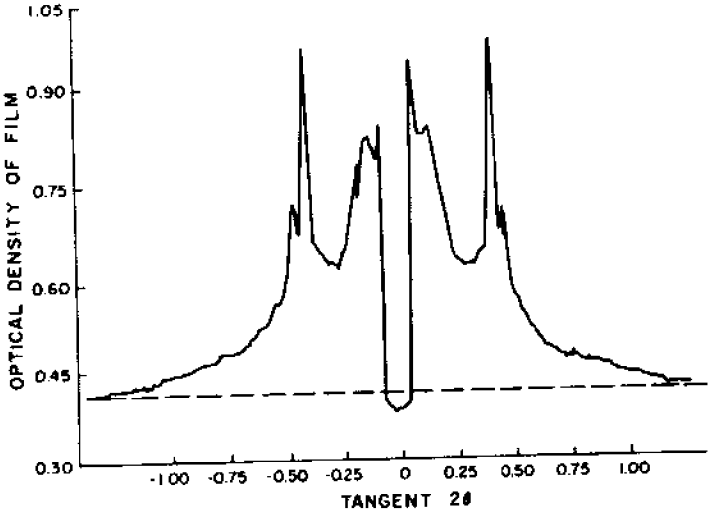

Figure 10. Densitometric tracing of X-ray diffraction pattern of *Acinetobacter* sp grown on heptadecane.

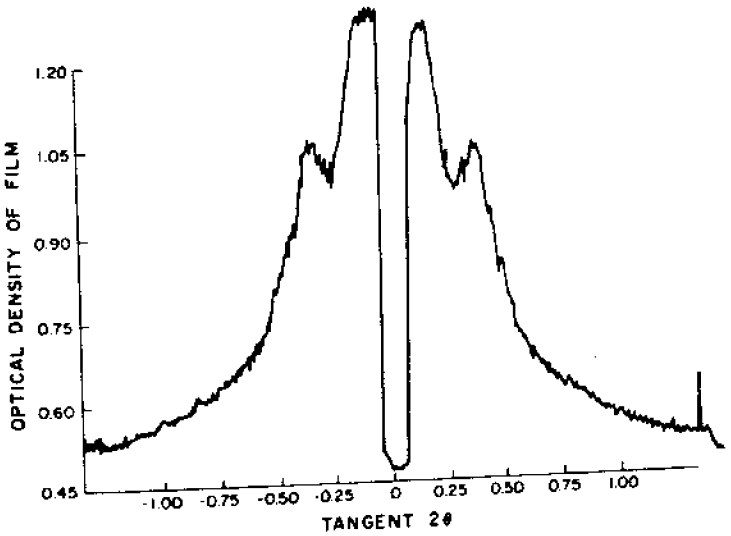

Figure 11. Densitometric tracing of X-ray diffraction pattern of *Acinetobacter* sp grown on nutrient broth-yeast extract.

An assessment of the generality of hydrocarbon cytoplasmic sequestering by microorganisms in the natural environment was initiated. Crude oil was placed with a sample of pond water. Figure 12 is a thin section prepared from microorganisms grown under these conditions. Hydrocarbon inclusions are observed as fine-structure inclusions. Further studies were carried out with a tar ball collected from a large petroleum spill off Whalebone Bay, Nova Scotia, in March, 1972. Portions of this tar ball (5 g) were placed in a mineral salts medium and incubated with shaking at room temperature. Excellent microbial growth occurred within 4 days. Figure 13 is a scanning electron micrograph prepared directly from this tar ball culture flask. A complex amorphous structural array is apparent. Increased magnification shows the presence of bacteria (Figure 14). Thin-sections were prepared from microorganisms which grew on components of this tar ball. Figure 15 shows identical cytoplasmic inclusions as noted with *Acinetobacter* growing on hexadecane. Bacteria, algae, and protozoa were observed in these thin-sections. These tar ball cultures have yielded approximately 15 presumptively different species of microorganisms.

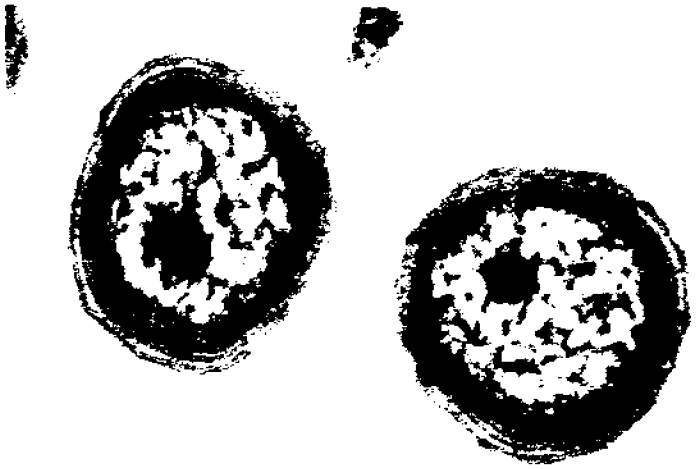

Figure 12. Electron micrograph of fresh pond water bacteria developing on crude oil. 93,000 X.

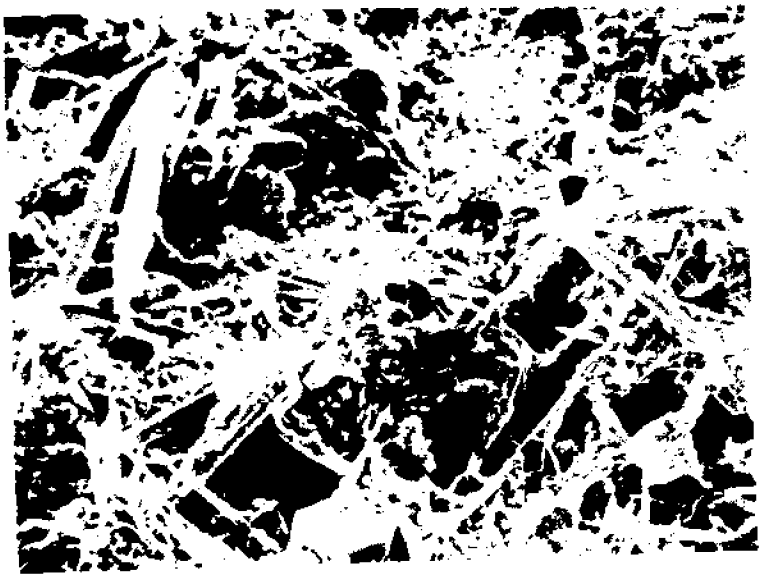

Figure 13. Scanning electron micrograph of microbial growth on a tar ball. 1500 X

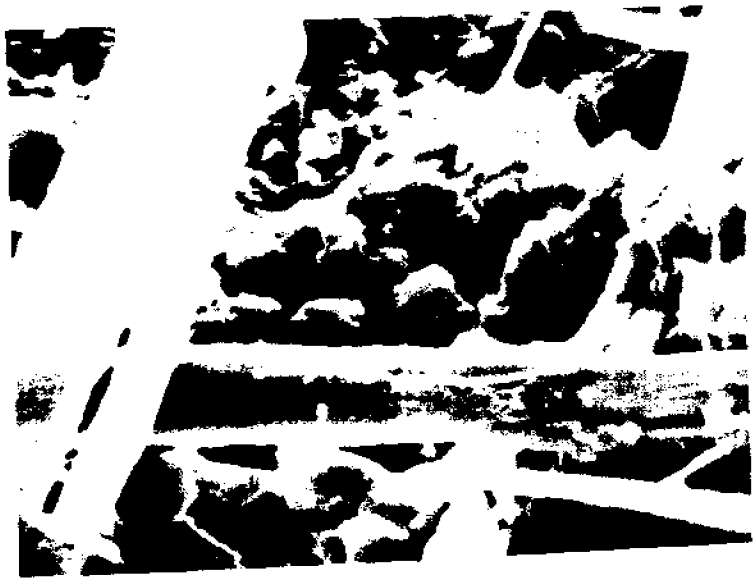

Figure 14. Scanning electron micrograph of microbial growth on a tar ball. 12,000 X.

Figure 15. Electron micrograph of bacteria developing on a tar ball. 93,000 X.

An unexpected ultrastructure modification, in addition to cytoplasmic pools of hydrocarbons, was the induction of intracytoplasmic membrane in hydrocarbon-grown bacteria. Figure 16 shows one type of membrane structure in *Acinetobacter* grown on hexadecane, while Figure 17 depicts a structural variation in intracytoplasmic membrane formation in hexadec-*1*-ene grown cells. These intracytoplasmic membrane structures appear as highly ordered trilaminar membranes extending throughout the cell. These membrane structures are present only when cells have been grown on hydrocarbons. A physical relationship between a hydrocarbon inclusion body and intracytoplasmic membrane is seen in Figure 18. The functional significance of this physical relationship is under investigation.

A growth phenomenon currently inexplicable concerns a gross cellular transformation in hydrocarbon-grown bacteria. At irregular intervals cultures of *Acinetobacter* on alkane or alk-*1*-ene transform into giant cells. Individual bacteria will become 4-10 times larger, greatly extended and elongated, and exhibit extensive intracytoplasmic membrane development. Figure 19 shows a thin-section of one of these giant cells obtained from a culture growing on hexadecane. This induced transformation has been observed to occur only when bacteria are grown on hydrocarbons. Subculturing of these transformed cells to new hydrocarbon-containing media maintains giant cell populations. Reversions to normal cell dimensions and shape occurs by subculturing to non-hydrocarbon nutritive media. A physical property associated with these transformed cultures is their buoyant density. These giant cells do not sediment in centrifugal fields as high as 40,000 x g but rather remain as a floating granular pellicle on the surface.

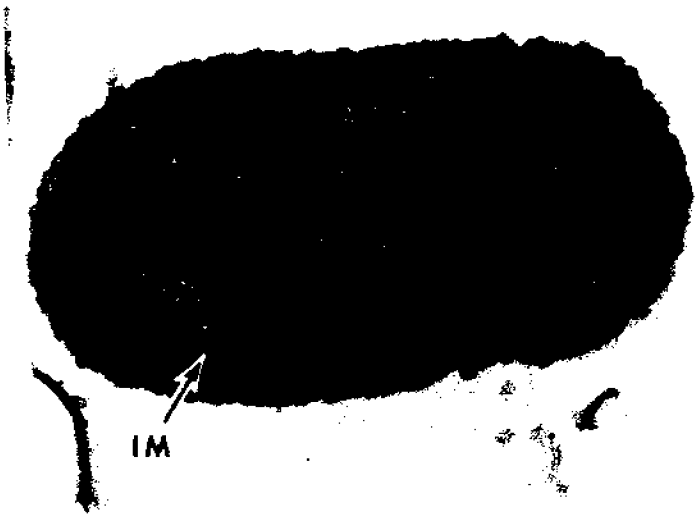

Figure 16. Intracytoplasmic membrane (IM) development in *Acinetobacter* sp grown on hexadecane. 53,000 X.

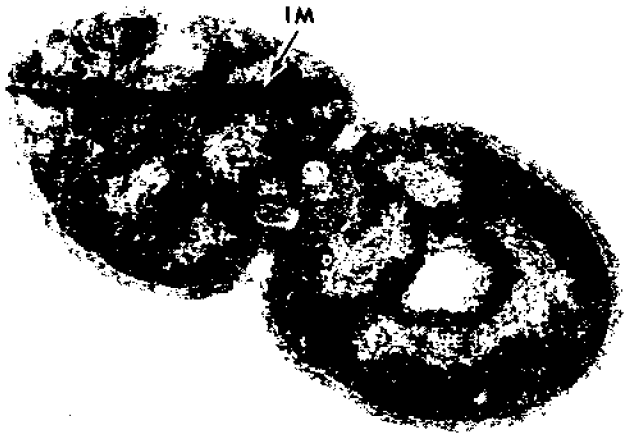

Figure 17. Intracytoplasmic membrane (IM) development in *Acinetobacter* sp grown on hexadec-1-ene. 58,000 X.

Figure 18. Electron micrograph of *Acinetobacter* sp grown on hexadec-1-ene. A physical relationship between hydrocarbon inclusion and intracytoplasmic membrane is demonstrated. 84,000 X

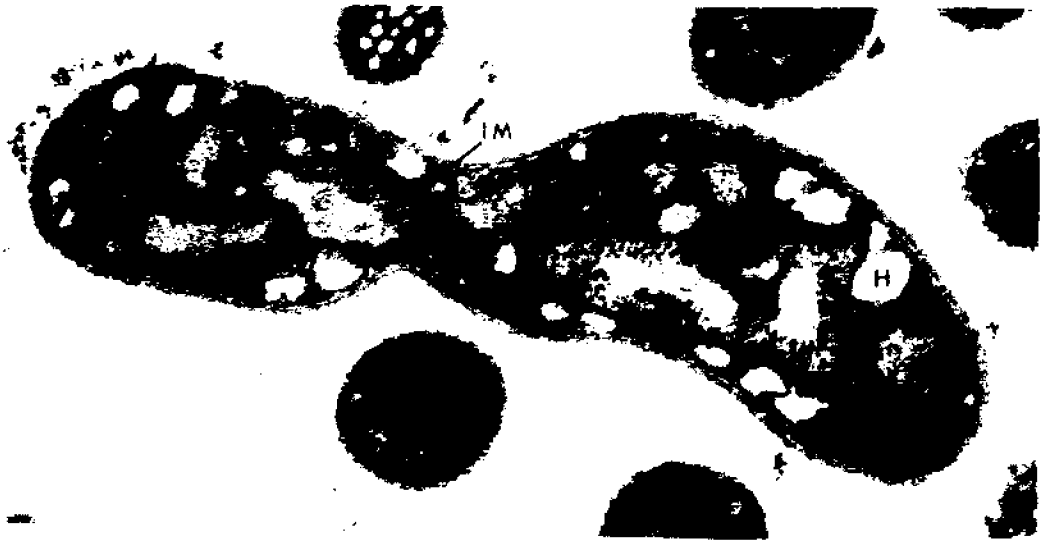

Figure 19. Electron micrograph of *Acinetobacter* sp transformed to large cells by repetitive growth on hexadecane. Intracytoplasmic membrane (IM) and hydrocarbon (H). 40,000 X.

The Relationship of Lipids to Hydrocarbon Oxidation.--A comparative study of lipids in relationship to hydrocarbon oxidation was quantitatively determined with *Acinetobacter* sp growing on hydrocarbon and non-hydrocarbon substrates. Table 1 shows the quantitative distribution of specific cellular lipds in *Acinetobacter* sp. Phospholipids doubled as a result of growth on hexadecane. Free fatty acid pools remain constant under the two growth conditions while significant differential concentrations of free fatty alcohol and wax ester were measured only in hexadecane grown cells. The presence of triglyceride is significant in terms of both qualitative and quantitative concentration.

TABLE 1

Quantitation of Cellular Lipids in *Acinetobacter* sp

Lipid Components	Micromoles/g dry cell weight	
	Non-hydrocarbon[a]	Hydrocarbon[b]
Phospholipids	60	120
Triglyceride	4.8	2.5
Free fatty acid	7.5	8.2
Free fatty alcohol	0	2.7
Wax ester	0	7.3

[a]Nutrient broth-yeast extract grown bacteria.

[b]Hexadecane-grown bacteria.

The quantitative aspects of extracellular lipid accumulation is shown in Table 2. Triglyceride, free fatty acid, free fatty alcohol, and wax ester are present in the culture broth of hexadecane-grown cells. Triglyceride, free fatty acid, and wax ester appear as major extracellular lipids in hexadecane cultures.

An analysis of bound and free fatty acid and fatty alcohol in the culture broth is shown in Figure 20. Fatty acid (bound and free) exhibited two maxima throughout the growth curve. The first maximum occurred during early to mid-exponential growth phase with a sharp decrease in the late exponential growth phase. The second maximum occurred coincident with the onset of the stationary growth phase with a subsequent rapid decrease to low concentrations. Fatty alcohol (bound and free) exhibited an identical response with two maxima occurring coincident with fatty acid. This study demonstrated that fatty acid and fatty alcohol exhibited peak concentration in early to mid-exponential growth phase. The significance of this cellular response to hydrocarbon may relate to the interaction of specific lipids as surfactants in the micellarization of hydrocarbon.

TABLE 2

Quantitation of Extracellular Lipids

Lipid Components	Micromoles/L of culture medium	
	Non-hydrocarbon[a]	Hydrocarbon[b]
Phospholipids	0	0
Triglyceride	2.4	25.6
Free fatty acid	4	60
Free fatty alcohol	0	0.5
Wax ester	0	280

[a]Nutrient broth-yeast extract grown cells.

[b]Hexadecane-grown cells.

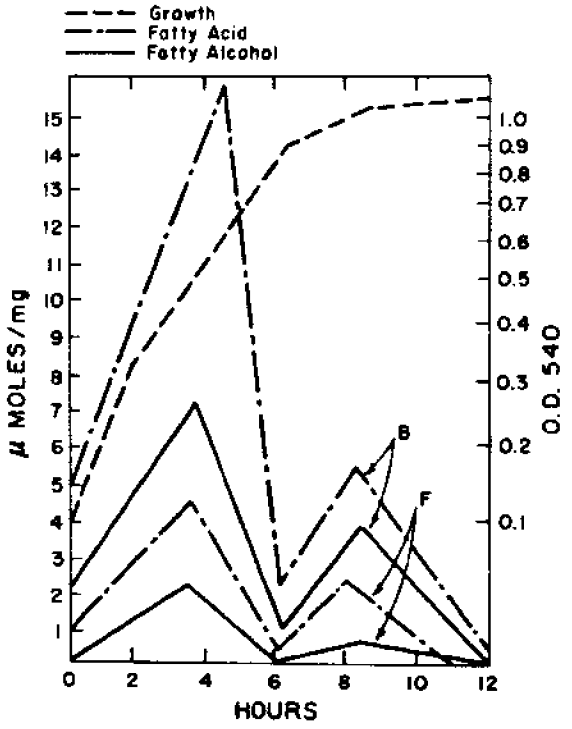

Figure 20. Analyses of bound (B) and free (F) fatty acid and fatty alcohol in the culture broth through the growth curve of *Acinetobacter* sp with hexadecane as substrate.

A growth curve response for total triglyceride is shown in Table 3. Total triglyceride from hexadecane-grown cells was significantly higher throughout the growth curve than with either acetate or nutrient broth-grown cells. Table 4 shows the fatty acid spectrum of triglyceride purified from cells grown on specified hydrocarbons. A direct correlation to substrate carbon number and chain length of fatty acid is apparent. The functional role of triglycerides in hydrocarbon metabolism is presently undetermined. Albeit, triglyceride as a specific surfactant in combination with a more polar lipid (e.g., fatty acid) may serve to form hydrocarbon in water macro- or micro-emulsions.

TABLE 3

Total Triglyceride throughout Growth Curve

Time (hrs)	Nanomoles/mg (dry cell wt)		
	Hexadecane	Acetate	Nutrient broth
0	54	21	14
1	48	18	11
2	44	17	5
4	32	12	3
6	24	7	2
8	20	6	1
10	16	5	1

TABLE 4

Fatty Acid Composition of Purified Triglyceride

Percent Fatty Acid Composition	Growth Substrate			
	NBYE	Pentadecane	Hexadecane	Heptadecane
14:0	1.96	4.12	2.64	
15:0		3.48		14.92
15:1		11.53		
16:0	70.33	11.87	75.10	1.98
16:1	5.04	16.20	12.06	2.23
17:0		17.45		49.97
17:1				26.74
18:0	6.56			4.16
18:1	16.11	18.42		
X		17.11		
Y			10.20	

DISCUSSION

These studies have served to direct attention to problems associated with petroleum which have been either totally ignored or unrecognized. The recent impact of petroleum in the environment tends to emphasize the cogency of providing concrete answers to these problems. Specific implications of this study are: a) cellular transformations induced by hydrocarbons; b) the sequestering of hydrocarbons by microorganisms with potential impact on food chain interrelationships; and c) the microbial production of specific solubilizing agents which aid or promote hydrocarbon metabolism.

The fate and effect of petroleum or petrolic byproducts on the environment has received cursory attention at best. A summary discussion of the effects of oil pollution on birds, mammals, fish, molluscs, plankton, plants, as well as marine communities has defined a few of the overall aspects of petroleum pollution (24). The examination of shellfish and marine sediments demonstrated the persistence of oil in sediments and shellfish several months following the pollution incident. Of significant interest was the retention of aromatic hydrocarbons by these shellfish (3). Recent studies have shown the rapid uptake of heptadecane, 1,2,3,4-tetrahydronaphthalene, toluene, naphthalene, and 3,4-benzopyrene by marine mussels. The data indicated that mussels did not metabolize these hydrocarbons but significant amounts were retained by the tissues (14).

We have observed that a microorganism undergoes a morphological transformation as a result of growth on chemically pure hydrocarbon. Whether this transformation is the result of synergism between hydrocarbon and a metabolic byproduct or a singular effect of hydrocarbon is unknown. Further evidence has demonstrated fine-structure modifications in the form of induced membrane synthesis. Quantitative biochemical analyses of the simple and complex lipids of *Acinetobacter* sp have demonstrated an increase in the cellular and extracellular lipids as a result of hydrocarbon metabolism. The pharmacological and toxicological properties of petroleum and petroleum byproducts on the aquatic environment are undetermined, although it is well known that many polycyclic aromatic hydrocarbons are carcinogenic. A hydrocarbon-oxidizing microorganism has responded physiologically and morphologically in a rather drastic manner to a hydrocarbon molecule which is tacitly assumed to be innocuous. The inescapable question of "why?" remains.

Sequestering of hydrocarbons by the microbial flora through active transport becomes of practical concern. Hydrocarbons which are pooled by the indigenous microbial flora could relate to the sequential transfer of hydrocarbons through the food chain. The phenomenon may serve as a mechanism for passage of potentially dangerous chemicals through the food chain webs to higher life forms. Compounds which can undergo such processes are: a) paraffinic and olefinic hydrocarbons; b) aromatic and polycyclic aromatic hydrocarbons; and c) halogenated hydrocarbons. We have investigated hydrocarbon-utilizing bacteria, yeasts, and fungi and note that all accumulate paraffinic and olefinic hydrocarbons in the cytoplasm. The critical question becomes just how far does hydrocarbon move through the food chain progression before it disappears, is converted into a potentially harmful intermediate, or is metabolized to non-toxic intermediates?

The conclusion appears warranted that *Acinetobacter* sp undergoes a

loss or alteration of biochemical control mechanisms for cell wall synthesis, membrane synthesis, and morphological shape when grown repetitively on hydrocarbons. The analogy to neoplastic transformations in higher cellular systems is evident. The "how" and "why" of what triggers a microbial cell to "pool" a hydrophobic hydrocarbon against a concentration gradient, to form complex intracellular membranes, and to undergo gross cellular transformations are undetermined.

It is well recognized that all microorganisms are incapable of hydrocarbon assimilation, presumably lacking the requisite enzymatic complement to effect such conversions. A study of hydrocarbon structure in relationship to microbial oxidizability has established a set of biological guidelines for determining the biodegradability of specific substituted hydrocarbons (22). Hydrocarbons as hydrophobic, water-insoluble compounds have circumstantially been considered as refractory to active transport processes. These considerations necessitated an extracellular modification of the hydrocarbon by the microbial agents with assimilation processes being effected on the byproducts of this activity. Our findings that hydrocarbons are pooled suggests a mechanism for active transport of hydrocarbons. The component parts and requirements for active transport of a water-insoluble substrate are ill-defined. However, if hydrocarbon-oxidizing microorganisms possess the ability to synthesize a biodetergent which promotes pseudo-solubilization of hydrocarbon, a mechanism for active transport against a concentration gradient can be effected. An analysis of cellular and extracellular lipids revealed that a number of specific lipids were present in a hydrocarbon-utilizing system that could fulfill this purpose. The explicit role of these lipids as solubilization agents for hydrocarbons has not been determined within this system. However, their known physical properties would suggest that an influential role could be served in maintaining a finite level of hydrocarbon in aqueous solution as macro- or micro-emulsions.

Future directions for analyzing the impact of petroleum on the aquatic environment can be formulated as a series of experimental questions: a) what is the diversity of microorganisms in the aquatic environment capable of hydrocarbon metabolism?; b) do differential kinetic parameters exist with respect to uptake of specific hydrocarbons?; c) what is the magnitude of hydrocarbon pooling in the microbial flora and what are the rates of hydrocarbon elimination from such microbial populations?; d) can internal pools of more than one hydrocarbon be maintained or do selective uptake and oxidation rates prevail depending on the hydrocarbons available?; e) do hydrocarbons induce cellular transformations throughout all microbial species?; f) what pharmacological and toxicological properties do specific hydrocarbons exert on higher cellular systems?

A serious problem facing hydrocarbon microbiology today is not so much microbial utilization of volatile petroleum fractions, but rather the biodegradation of resinous tars. These highly complex constituents of petroleum appear to be highly refractory to biological degradation so that further and immediate studies are needed to understand the apparent recalcitrant nature of these petroleum constituents.

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DISTRIBUTION AND ABUNDANCE OF OIL-OXIDIZING BACTERIA IN THE NORTH SEA

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Investigations include distribution of oil-oxidizing (and total heterotrophic bacteria) in the waters, surface films, sediments and beach sands of the North Sea in the vicinity of Helgoland and in the River Elbe estuary. Microbial concentrations in unpolluted samples, as great as 10^9 per liter of sediment, are reported.

INTRODUCTION

The existence of oil-decomposing bacteria has been known for many years. Comprehensive investigations using pure cultures under laboratory conditions have been conducted on the physiology of the degradation processes, especially in regard to the aliphatic paraffins. In contrast, our knowledge of the ecology of hydrocarbon-oxidizing bacteria is meager; there are only a relatively few papers dealing with their distribution in the sea (1,5,17,18, 20).

Due to the increasing problem of aquatic pollution, and the question of the limiting factors of oil degradation, considerable background data on distribution and abundance of the microorganisms involved in this process are urgently needed. Additionally, the question of seeding of oil slicks and beached oil to enhance degradation rates poses further problems.

It is recognized that the polluting oil consists of a complex mixture of many different hydrocarbons besides other organic compounds, and that microbial degradation is by mixed populations consisting of numerous species. Thus, populations of single microbial species cannot noticeably degrade crude oils (18). It is necessary to obtain basic data on the abundance of bacteria in the natural environment that are able to grow on crude oil or refined petroleum products rather than microorganisms that respond to individual hydrocarbons or special groups of compounds.

MATERIALS AND METHODS

Collections of water and sediment samples were made from the BAH (Biologische Anstalt Helgoland) vessels *Friedrich Heincke*, *Uthörn* and *Ellenbogen*. The majority of the water samples were taken at the cablebuoy (Kabeltonne) station near Helgoland. This site, between Helgoland and the adjacent sandy island, is in a channel with a strong (1-2 knots) tidal current. The

latter precluded influence of the island itself on the collections at the cablebuoy. Furthermore, samples at this station showed a bacterial flora comparable to that obtained at stations outside of the immediate Helgoland area. It should be noted that microbiological and chemical parameters have been monitored at Helgoland for the past 10 years.

One series of 17 stations was established between the light-vessel P 8 about 25 nautical miles WNW of Helgoland and the mouth of the River Elbe. This series of stations encompassed a distance of about 70 nautical miles. The salinity of water varied from about 1‰ (Elbe-Otterndorf stations 2, 10) to about 32‰ (light-vessel P 8 stations 1, 6). Water was collected aseptically at a depth of approximately 1 m using a modified ZoBell microbiological sampler (15). Locations of all sampling sites are shown in Figure 1.

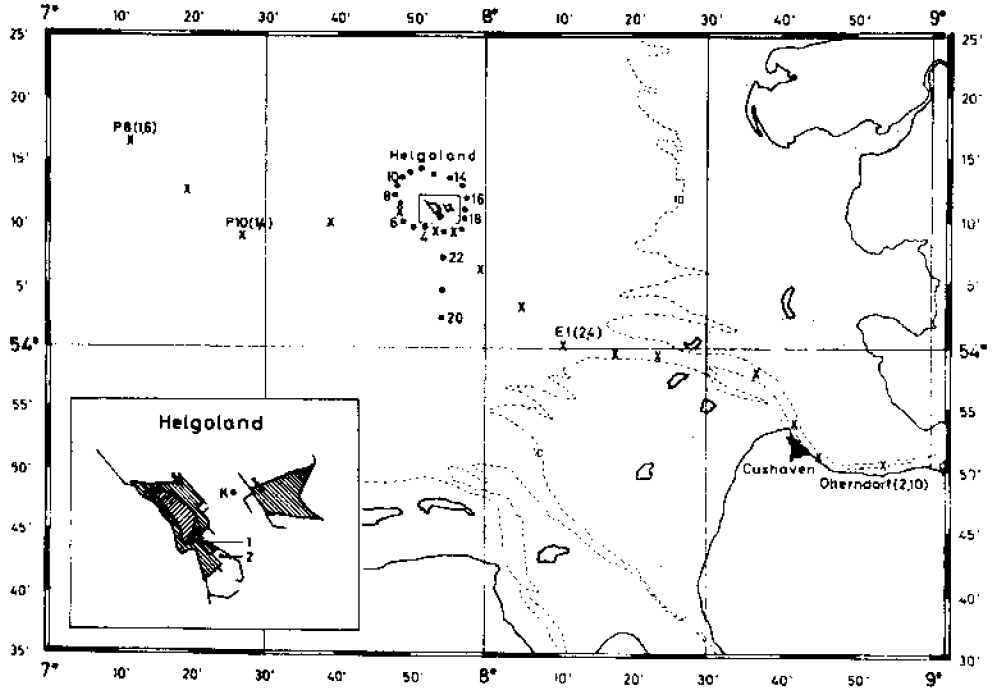

Figure 1. Location of Collection Stations in the North Sea. Crosses indicate water sampling station of the cruise between River Elbe Otterndorff (2, 10) to Light Ship P 8 (1, 6). Dots indicate the stations around Helgoland which were sampling sites for sediment. The insert map of Helgoland shows the site of the cablebuoy (Kabeltonne) station and locations of the beach sand collections.

Surface films of water were collected by use of a "fly screen" sampler made of stainless steel (2,11). Bottom samples were taken on two cruises around Helgoland using the van Veen grab sampler (14). The uppermost 5 mm of the sediment was taken using aseptic procedures. Beach sand samples were collected directly using sterilized bottles.

Biochemical oxygen demand (BOD) was determined after 21 days of incubation at 18 C using a modified Winkler method (8).

Sediment samples were treated as follows: 2 ml of the sediment was put into a sterile bottle containing 100 ml sterile aged seawater; 1 ml of a nontoxic, non-ionic emulsifier and one drop of a defoaming agent were added; the material was mixed using a high speed "Ultra Turrax." This homogenizer is an excellent tool to break up bacterial aggregates (3).

A dilution series was made with sterilized aged seawater (3 parts aged seawater to 1 part distilled water) so that each subsequent dilution contained 1/10 of the concentration of bacteria. From these dilution tubes, petri plates and prescription bottles (for MPN analysis) were inoculated. These methods, used in our laboratory for many years, have been described in detail elsewhere (5,6).

The following media were used:

a) Agar Medium 2216 E (9), made with a mixture of 3 parts aged seawater and one part distilled water. Bacteria isolated on this medium are designated "marine heterotrophic bacteria."

b) Agar Medium 2216 E, made with distilled water. The salinity of the inoculated medium was *ca* 2‰ due to the salinity of the inoculum. These bacteria are referred to here as "freshwater heterotrophic bacteria."

c) Liquid inorganic medium with oil for marine oil-decomposing bacteria. The only carbon and energy source in this medium was either Bunker oil medium or heavy gas oil. While heavy gas oil is a white distillation product oil, Bunker oil medium (syn. M. fuel oil) is viscous and black with a fairly high boiling point. One drop of oil was added to each prescription bottle. The medium consisted of: 0.5 g NH_4Cl; 0.5 g K_2HPO_4; 1 g Na_2HPO_4; 750 ml aged seawater and 250 ml distilled water.

d) Liquid inorganic medium with oil for freshwater oil-decomposing bacteria. The composition was similar to that of (c); however, only distilled water was used. In the medium the salinity was about 2‰ after inoculation.

The inoculated prescription bottles and petri plates were incubated at 18 C for 3 weeks. Before checking whether the bottles were positive (turbid due to growth of bacteria) or negative, they were acidified to dissolve inorganic precipitates.

RESULTS

Abundance of Bacteria at the Monitoring Station Cablebuoy (Kabeltonne).--During the period from March 1966 to April 1967, 57 water samples were taken at the station Kabeltonne. Monthly averages are plotted in Figure 2.

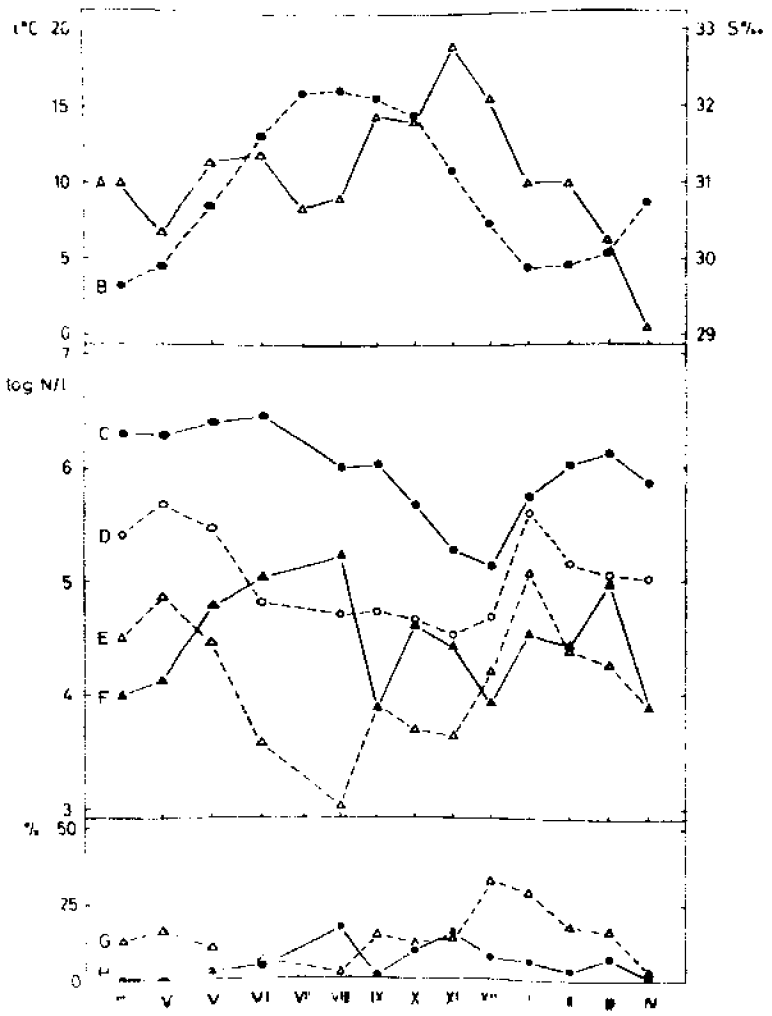

Figure 2. Monthly Averages of the Abundance of Bacteria at the Station Cablebuoy (Kabeltonne) during the time from March 1966 to April 1967.
A = salinity; B = temperature; C = marine-heterotrophic bacteria; D = freshwater-heterotrophic bacteria; E = freshwater-oil-oxidizing bacteria; F = marine oil oxidizing bacteria; G = percentage of freshwater-oil-oxidizing bacteria in regard to freshwater-heterotrophic bacteria; H = percentage of marine-oil-oxidizing bacteria in regard to marine heterotrophic bacteria.

Marine oil-oxidizing bacteria were present throughout the year in relatively high numbers, fluctuating between 930 cells/L to more than 460,000 cells/L in the individual sample. The average value of 57 samples was 53,860 cells/L.

The numbers of oil-oxidizing freshwater bacteria were only slightly lower, fluctuating between 910 cells/L and 460,000 cells/L. The average population for the 57 samples was 40,000 bacteria/L.

The numbers of heterotrophic bacteria were considerably higher than those for hydrocarbonoclastic bacteria. If we rate the concentration of the marine heterotrophic bacteria as 100%, the percentage of the marine oil-oxidizers averages monthly between 0.52 and 16.8%. The corresponding values for freshwater oil-oxidizing bacteria are 1.49% and 32.37%. While the percentages of the freshwater oil-oxidizing bacteria are somewhat higher, the absolute numbers are lower than those of the marine oil-oxidizing bacteria.

The numbers of bacteria change during the year for the four different groups. There is some conformity between marine and freshwater heterotrophs, but none between the oil-oxidizing bacteria and the heterotrophs. No definite yearly cycle is recognizable, which could be attributed to the season or to the temperature. This is somewhat contrary to our results normally obtained in monitoring heterotrophic bacteria during the last 10 years, taking samples every second day and calculating the overlapping 10 days averages. In most instances, good maxima were obtained in spring and in autumn, with low numbers in summer and winter. The reason for this may be that plotting monthly averages of 57 samples in 13 months smoothes the curves to such an extent that, together with the lower number of samples (taken at different tidal phases), maxima and minima that occurred only for a limited time are not apparent.

Distribution of Oil-Oxidizing Bacteria at a Section Elbe-Open Sea (Lightship P 8).--The distribution of heterotrophic and oil-oxidizing bacteria between the River Elbe and the open sea was determined together with the salinity and the BOD over a two-day cruise (RV *Uthörn*). The results are shown in Figure 3.

Salinity increases from 1‰ in the River Elbe to about 32‰ in the open sea, whereas BOD decreases from 9.39 mg/L (station Otterndorf) at stations 2 and 10 in the Elbe to 2.05 mg/L in the open sea. These data show that river water with a low salinity, and a high content of organic material, is mixed with water of the open sea which is high in salinity and low in organic material. That suggests that much of the organic material in the coastal zone enters the sea with the river water. The concentrations of all four groups of bacteria investigated show similar correlations with organic material except for numbers of the marine heterotrophic bacteria at stations of low salinities.

Bacterial populations decrease between Lightship P 8 and the station Elbe-Otterndorf:

	Range of Bacterial Cell Concentration
Freshwater heterotrophic bacteria (C)	1.3×10^{8} to 7.3×10^{4}
Marine heterotrophic bacteria (D)	8.2×10^{6} to 2.2×10^{5}
Freshwater oil-oxidizing bacteria (E)	3.0×10^{6} to 2.0×10^{2}
Marine oil-oxidizing bacteria (F)	2.4×10^{5} to 2.3×10^{3}

It is noteworthy that even at the stations having a high salinity quite distant from the estuary, numbers of freshwater oil-oxidizing bacteria are in most instances higher than those of the marine oil-oxidizing bacteria.

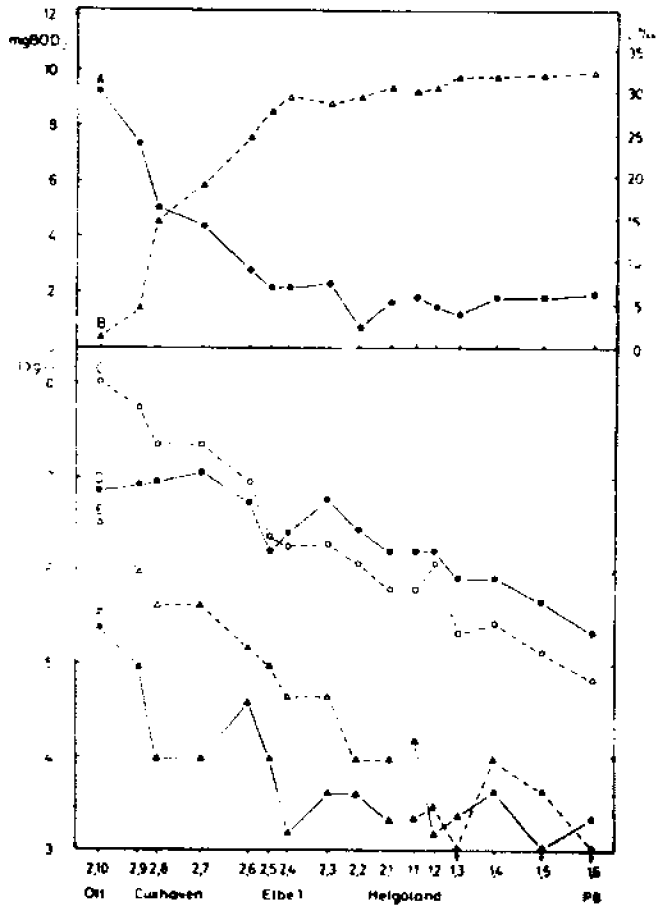

Figure 3. Distribution of Bacteria Between the River Elbe and the Open Sea (Lightship P 8).
A = BOD_{21}; B = salinity; C = marine heterotrophic bacteria; D = freshwater heterotrophic bacteria; E = freshwater oil-oxidizing bacteria; F = marine oil-oxidizing bacteria.

Fluctuations of the oil-decomposing bacteria are greater than those of the heterotrophic bacteria due to the lower accuracy of the MPN method.

Distribution of Oil-Oxidizing Bacteria and Yeasts in the Water Surface Films.--Due to the fact that oil pollutants normally form large slicks at the surface of waters, microorganisms present at the air-water interface come into closer contact with the pollutant than at a depth of 1 m. The latter is the depth at which "surface-samples" are normally taken. For this reason, some preliminary attempts were made to determine the numbers of oil-oxidizing bacteria in the surface film of water and compare these with concentrations at a depth of 1 m. Results are compiled in Table 1. In two cases, the number in the surface film was lower, in one instance the same, and in two cases higher. The highest value was more than 12-fold that at 1 m depth.

TABLE 1

Distribution of Oil-Oxidizing Bacteria in Surface Films and at a Depth of 1 m

Types of microorganisms	Date of sampling	Number in 1 L water at 1 m depth	Number in 1 L water of air-water interface	in % (1 m water depth=100%)
Marine oil-oxidizing bacteria	5/11/71	230,000	32,300	1%
	8/11/71	150,000	23,000	15%
	10/11/71	15,000	21,000	140%
	15/11/71	230,000	230,000	100%
	1/12/72	15,000	930,000	240%
	Average	140,000	247,260	176%
Yeasts	15/09/71	12	63	525%
	22/09/71	14	30	214%
	29/09/71	9	130	1444%
	27/10/71	24	80	333%
	3/11/71	41	570	1390%
	10/11/71	13	25	192%
	15/11/71	56	293	523%
	24/11/71	24	100	416%
	1/12/71	7	104	1485%
	Average	22	155	697.5%

In the lower portion of Table 1, examples of concentrations of heterotrophic yeasts are given. Although the oil-oxidizing ability of the yeasts was not checked, it is pertinent to present these data here. On an average, the number of yeasts was 7 times higher at the surface than at 1 m depth.

Thus, it appears that in the water, the highest number of oil-oxidizing yeasts probably occurs in the water-air interface.

Distribution of Oil-Oxidizing Bacteria in Sediments.--Twenty-one samples of sediments were collected around Helgoland from the BAH vessel RS *Friedrich Heincke*. The stations are given in Figure 1. The results for oil-oxidizing marine bacteria and for heterotrophic marine bacteria are plotted in Figure 4.

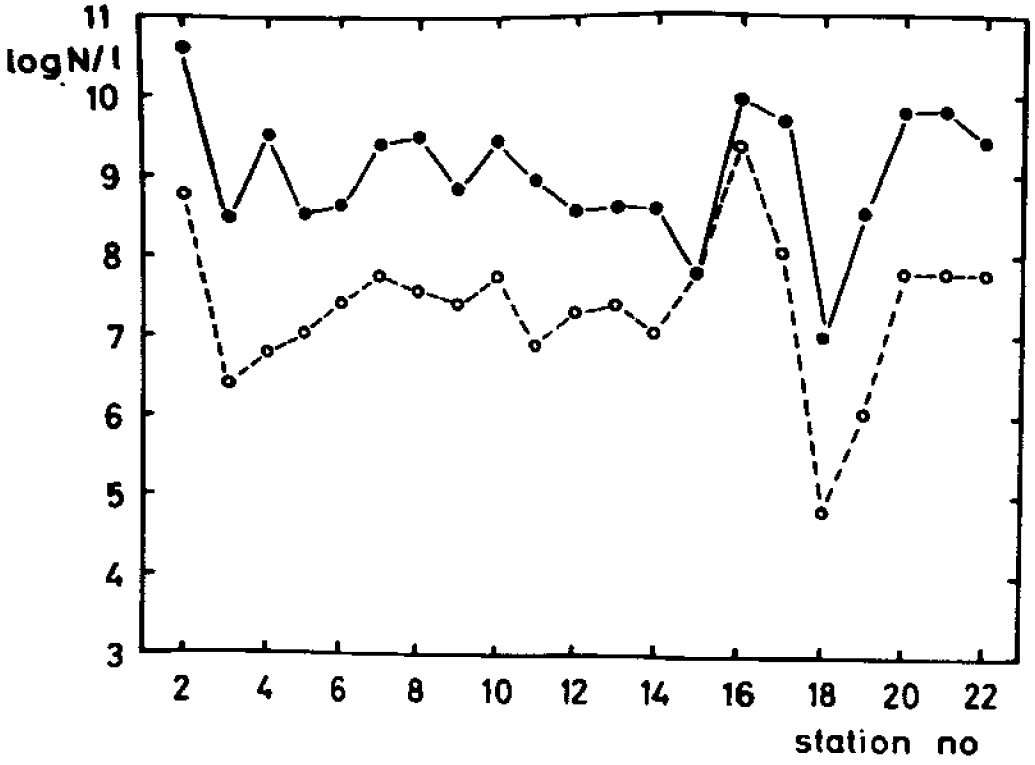

Figure 4. Abundance of Marine Heterotrophic Bacteria (solid line) at 21 Stations around Helgoland and of Marine Oil-Oxidizing Bacteria (dashed line).

The numbers of oil-oxidizing marine bacteria fluctuate between 5.75×10^4 and 2.3×10^9 per liter of sediment. The average of the 21 samples is 1.61×10^8 cells/L. The corresponding numbers for heterotrophic marine bacteria are 9.63×10^6 and 3.9×10^{10} cells/L, with an average of 4.0×10^9 cells/L. The average percentage of oil-oxidizing bacteria in regard to marine heterotrophic bacteria is 4.02%. The sediments investigated were dissimilar in regard to grain size, and consisted of fine sand with a high proportion of silt and clay up to samples composed of coarse sand and small stones. In all samples the grain size distribution was determined; in some samples, the hydrocarbon content was also determined. Preliminary calculations showed no significant correlation between the different parameters determined. It seems necessary to study additional aspects, i.e., Eh, organic bound carbon, inorganic

nutrients, microfauna, to understand the distribution patterns of heterotrophic marine bacteria and oil-oxidizing bacteria.

Distribution of Oil-Oxidizing Bacteria in Beach Sands.--During the course of the investigations, the bacterial content of many individual samples was determined. Table 2 gives examples for 6 samples taken at two different beaches. Location No. 1 was the north beach at Helgoland, location No. 2 was the north beach of the "Düne," the little island separated from Helgoland by a channel (see Fig. 1). In both locations, the sand has a similar grain size (medium) and was fairly clean; however, the north beach of Helgoland is protected against the prevailing westerly winds by the morphology of the island. At both locations, one sample was taken at the low-tide line, one at the high-tide line, and a third sample about 30 to 50 m behind the high-tide line. The low- and high-tide line samples were wet sand, while the third sample was dry.

TABLE 2

Distribution of Oil-Oxidizing Bacteria in Beach Sands

Location No.	Oil-oxidizing marine bacteria cells/L	Marine heterotrophic bacteria cells/L	Freshwater heterotrophic bacteria cells/L	in % (marine heterotrophic bacteria = 100%)
I. Helgoland north beach				
low-tide line	21.5×10^7	55.5×10^7	74.0×10^7	38.7%
high-tide line	115.0×10^7	53.5×10^7	145.0×10^7	215.0%
above high-tide line	21.5×10^7	635.0×10^7	590.0×10^7	3.4%
II. Helgoland "Düne" north beach				
low-tide line	4.65×10^7	173.0×10^7	54.0×10^7	2.7%
high-tide line	4.65×10^7	95.0×10^7	10.8×10^7	4.9%
above high-tide line	21.5×10^7	14.0×10^7	103×10^7	15.4%
Average	31.46×10^7	171×10^7	162.8×10^7	18 %

The two locations have different patterns of bacterial distribution (Table 2). Location No. 1 has the highest number of oil-oxidizing bacteria at the high-tide line. The percentage of these bacteria in regard to marine heterotrophic bacteria was more than twice the number of the heterotrophs (215%). The corresponding numbers of oil-oxidizing bacteria (high-tide line) at the "Düne" were lower--their highest value is above the high-tide line;

the corresponding numbers of heterotrophs were lower, so the percentage is higher than in Location No. 1.

DISCUSSION

The question of the occurrence of oil-oxidizing bacteria in the environment is important for ecological and applied reasons. Hungate (7), in discussing the criteria for adequacy of ecological analysis, states: "The number in which an organism occurs is the single most universal index for comparing the importance of a particular kind of bacterium in different habitats." For this reason he recommends: "Since numbers can be determined relatively easily by diluting the habitat before inoculating it to the enrichment medium, culture counts should always be part of the analysis of microbial ecosystems."

Simultaneously, with the determination of the bacterial group in question, other important environmental parameters and nutrients should be determined. However, only in balanced systems where continuously small amounts of organic substances are added can we expect a stable correlation between environmental parameters and microbial concentrations. To understand the role of bacteria in an oil spill occurring in a region usually free of pollution, it is necessary to follow the events from the very beginning over several weeks monitoring bacteria, oil and other parameters. Occasional sampling at some time after the spill has occurred, whose exact duration might be unknown, gives us very crude data. However, this is often the situation if we sample, for example, polluted sediments or polluted beach sand. In the marine environment such "convenient" circumstances like those investigated by Vorosilova and Dianova (12,13) seldom occur. These workers sampled the River Moskwa and Volga above the inlets of an oil refinery, in the pollution zone and far below this zone. They found 10-19,000 oil-oxidizing bacteria per ml above the inlet; however, more than 10^7 were enumerated below the inlet. The percentage of oil-oxidizing bacteria to bacteria which grew on peptone agar (heterotrophic freshwater bacteria) was calculated and found to be less than 5% above the inlet, in the oil-polluted zone up to 12,800%, and 100 km below this zone still 14%.

The results on the abundance of marine oil-oxidizing bacteria at the cablebuoy showed that all 57 samples contained oil-oxidizing bacteria. The average was 53,860 bacteria per liter with the percentage 0.52 to 16.8% of the total number of marine heterotrophic bacteria. Because it is highly unlikely that these large concentrations of bacteria belong to a single species, we can assume that bacteria with different potencies to break down various oil compounds are present throughout the year around Helgoland. Since under favorable conditions the generation time of bacteria can be as low as 30 minutes, only limiting factors of the environment will retard degradation. These factors limiting oil degradation are discussed elsewhere (4,17). In future investigations it should be worthwhile to take samples at closer intervals; also, the hydrocarbon content of the water should be examined.

The bacteriological data at Helgoland are supplemented by those of the cruise between the River Elbe and the open sea. It is especially important to resolve whether the oil-degrading bacteria are transported to the open sea from the polluted river, or whether they die off upon entry into the

marine environment. It is difficult to resolve this problem by just a single cruise that gives only the momentary picture of a specific time of the year. Synoptic cruise data are needed. However, it is still quite clear that seawater dilutes the concentration of all four bacterial groups as well as the BOD. Because the percentage of the decrease is different for the four groups it is evident that events other than dilution influence the four groups dissimilarly. There are at most stations more freshwater oil-oxidizing bacteria present; however, these decrease faster. The hydrography of the southern North Sea is very complicated and variable, and speculations about the factors which influence the distribution of bacteria can be made only if more data are used for correlation analyses.

The sediment samples collected around Helgoland give very different results. Bacterial numbers fluctuate about five orders of magnitude. It seems necessary to determine further environmental parameters to understand the reasons for such distribution patterns. Also it is necessary to differentiate between hydrocarbons which are introduced into the sediment by pollution and those of planktonic origin. Nevertheless, the data indicate the presence of a large bacterial population which is able to decompose different types of hydrocarbons.

Of considerable interest are the results obtained at the beaches in Helgoland at the "Düne." It was assumed that the highest values for oil-oxidizing bacteria would be at the high-tide line due to the possibility that surface films, which might contain traces of oil, would be deposited on the beach by wave action. This occurred at Location No. 1, protected against wind by the rock of Helgoland-Oberland. At Location No. 2, however, surprisingly the highest number of oil-oxidizing bacteria was found about 50 m behind and several meters above the high-tide level in a dry sand. Three reasons could be postulated for this:

a) Exhausts of planes from the landing strip at the "Düne." However, the number of starts and landings is very limited.
b) Hydrocarbons formed by the plants which grow at the sand in the close vicinity of the sampling station could promote the growth of oil-oxidizing bacteria. However, the sand was not mixed with plant debris, no roots were seen at the surface, and the density of the plants is very low.
c) Because the beach is exposed to NW to NE winds, the waves which hit the beach most of the year break and show white caps. It must be assumed that the surface film, with a great number of oil-oxidizing bacteria and liquid substances, is divided into small droplets and these are transported from the wind and are deposited some distance behind the high-tide line. The transportation of marine bacteria due to wind is described by ZoBell (16). This possibility seems to be the most realistic one.

Another interesting question is the concentration of oil-oxidizing bacteria in the air-water interface. For two years we have measured the lowering of the surface tension due to organic substances in collected surface films. In all cases, a considerably lower value was obtained for dyn/cm compared with the water at 1 m depth. Because oil pollution occurs in the former environment, further investigations will be concentrated in the surface film rather than in water samples from 1 m depth. We intend to quantify

the surface film to area, rather than to volume sampled by the fly screen, by using the adhesion force of oily substances to a Teflon plate (10).

We recognize that the pour plate method used does not permit all heterotrophic bacteria to grow. However, up to now there is no method available which routinely permits a better enumeration. Direct counts by light microscopy do not give reasonable results due to the small size of marine bacteria and the impossibility of distinguishing between small inorganic particles and bacteria. It is conceivable that the application of the scanning microscope offers new approaches. For this reason the term "total" heterotrophic bacteria has been omitted. Likewise, the numbers of oil-oxidizing bacteria in the samples probably are greater than were detected by the methodology described. As pointed out above, no single species is able to degrade oil to a noticeable degree. This means that at least several oil-oxidizing bacteria must be present in an individual laboratory reaction vessel to initiate biodegradation. However, the results of the heterotrophic bacteria and the oil-oxidizing bacteria, and the percentages, are in good agreement with data of other authors.

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MARINE HYDROCARBONOCLASTIC BACTERIA: TYPES AND RANGE OF OIL DEGRADATION

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Marine bacterial isolates were characterized on the basis of their ability to attack hydrocarbons in a complex synthetic mixture. It is possible to outline the oil degradation range of the organisms by properly balancing the various components. The degree of specialization and adaptation to hydrocarbon oxidation was determined indirectly with different nitrogen sources.

INTRODUCTION

Since the final objective is to degrade petroleum and various kinds of oil residues in the sea, it is necessary to know how hydrocarbonoclastic microorganisms, in particular marine forms, react to complex mixtures of hydrocarbons. Ability of an organism to separately oxidize tetradecane, hexadecane or naphthalene may establish its ability to assimilate those compounds, but it does not provide information on what happens when the same organism is challenged simultaneously with hundreds of different hydrocarbons.

Because of the complexity of crude oil it is difficult to experiment with the latter and obtain fundamental information on the fate of its components. It is possible, however, to build a small model of the oil system and carry out qualitative as well as quantitative studies of its microbial degradation.

MATERIALS AND METHODS

A synthetic oil containing representative members of the four major groups of petroleum hydrocarbons, i.e., normal paraffins, iso-paraffins, cyclo-paraffins and aromatics, has been previously formulated (5,6).

Different bacterial strains, isolated from marine environments, were exposed to a new hydrocarbon mixture in a synthetic marine medium (3) for a period of ten days at a temperature of 26 C. The disappearance of the individual hydrocarbons was followed by gas chromatography; this procedure has been described elsewhere (5,6).

From previous experiments (6) it appeared that by balancing the different components it might be possible to stimulate microbial degradation of a broad range of oil compounds. Composition of a "balanced" hydrocarbon mixture is illustrated in Table 1. The various components constitute the

following percentage by weight: normal paraffins, 26.81; iso-paraffins, 21.45; cyclo-paraffins, 12.26; aromatic hydrocarbons, 39.48.

TABLE 1

Percent Consumption of Hydrocarbons by Different Bacterial Strains, with Different Nitrogen Sources

BACTERIAL STRAIN	NITROGEN SOURCE	HYDROCARBON MIXTURE																						
		NORMAL PARAFFINS								ISO-PARAFFINS				CYCLO-PARAFFINS		AROMATICS								
		C_{13}	C_{14}	C_{16}	C_{24}	C_{25}	C_{26}	C_{28}	C_{30}	2,7,10-TRYMETHYLDODECANE "FARNESANE"	2,6,10,14-TETRAMETHYL PENTADECANE "PRISTANE"	2,6,10,14 TETRAMETHYLHEXADECANE "PHYTANE"	2,6,10,15,19,23-HEXAMETHYL TETRACOSANE "SQUALANE"	BICYCLOHEXYL	HEPTHYLCYCLOHEXANE	TETRAHYDRONAPHTHALENE "TETRALIN"	1,3,5-TRIETHYLBENZENE	2-ETHYLNAPHTHALENE	1,2-DIMETHYLNAPHTHALENE	ACENAPHTHENE	2,3,6-TRIMETHYLNAPHTHALENE	FLUORENE	FLUORANTHENE	PYRENE
TR4A	$(NH_4)_2SO_4$	26	22	24	13	11	10	7	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	KNO_3	33	29	35	14	12	11	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	ALANINE	47	36	55	26	28	26	24	18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	NO N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S83AIS	$(NH_4)_2SO_4$	0	0	0	0	0	0	0	0	0	0	0	0	0	0	59	0	70	10	12	0	9	0	0
	KNO_3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	53	0	57	8	10	0	6	0	0
	ALANINE	0	0	0	0	0	0	0	0	0	0	0	0	0	0	83	0	69	19	20	0	19	0	0
	NO N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S83IP7	$(NH_4)_2SO_4$	75	73	67	51	50	47	43	33	49	44	48	37	48	63	40	45	43	36	34	41	38	35	33
	KNO_3	97	96	95	93	92	91	96	95	88	82	89	81	83	93	38	66	60	49	38	62	51	67	71
	ALANINE	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	NO N	11	10	10	11	11	12	15	15	10	12	6	11	10	11	11	10	9	10	10	11	8	10	11
TR4N	$(NH_4)_2SO_4$	45	35	57	26	23	26	18	13	0	0	0	0	0	0	8	0	6	0	0	12	7	0	0
	KNO_3	40	34	49	31	28	28	24	17	16	11	19	13	13	19	12	12	10	8	6	10	10	13	7
	ALANINE	55	46	62	33	33	33	25	23	0	0	0	0	0	0	31	22	24	23	22	19	21	22	22
	NO N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TRIKIP	$(NH_4)_2SO_4$	62	57	57	41	38	37	28	25	32	29	35	29	30	39	0	15	0	0	0	8	0	10	9
	KNO_3	84	85	84	64	60	57	43	41	21	19	26	22	22	37	20	23	21	19	17	22	21	23	21
	ALANINE	56	52	55	40	36	36	29	26	25	22	33	25	21	30	0	11	0	0	0	6	0	10	0
	NO N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

RESULTS AND DISCUSSION

Activity of Microorganisms on Different Types of Hydrocarbons.--It has been shown that a preponderance of certain hydrocarbons determines the fate of other hydrocarbons (6). Since the great majority of hydrocarbon-oxidizing bacteria can readily digest normal paraffins, the amount of these in the oil may determine the extent to which iso-paraffins, cyclo-paraffins or even aromatics can be degraded. Within the normal paraffins, a sequential order of attack is apparent with the lighter compounds being attacked initially (5,6). Iso-paraffins present the same general pattern, although other factors may alter the sequential order of breakdown.

Table 2 shows phytane to be the iso-paraffin more readily attacked although its molecular weight is greater than that of either farnesane or pristane. This may be due to the great abundance in soils and water of phytol, a product of decomposition of chlorophyll, of which phytane is a precursor. Furthermore, the asymmetry in the molecule of phytane (Table 2) may perhaps facilitate its initial attack by microorganisms. A similar situation would apply for farnesane.

TABLE 2

Relationships Among Different Iso-Paraffins

Hydrocarbon	Basic structural formula	Average ratio of consumption
Farnesane $C_{15}H_{32}$	C C C CCCCCCCCCCC	87
Pristane $C_{19}H_{40}$	C C C C CCCCCCCCCCCCCCC	77
Phytane $C_{20}H_{42}$	C C C C CCCCCCCCCCCCCCCC	100
Squalane $C_{30}H_{62}$	C C C C C C CCCCCCCCCCCCCCCCCCCCCCCC	75

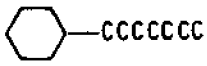
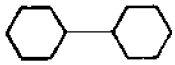
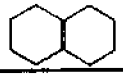
Oxidation of cyclo-paraffins seems to be linked with the presence of the paraffinic side chain (6). Table 3 shows the percentage of bacterial strains that attack heptylcyclohexane compared to bicyclohexyl and decalin (6). Although cyclo-paraffins, as a whole, may be more resistant to bacterial attack than normal paraffins, paraffinic side chains, if long enough, may be as biodegradable as a normal paraffin. This suggests that the extent of degradation of cyclo-paraffins in a crude oil would be dependent upon the presence of side chains and on the relative abundance of normal paraffins.

The possibility of degradation of cyclo-paraffins in a naphthenic crude should be greater than in a paraffinic crude. This may be of

importance, because of the greater potential toxicity of cyclo-paraffins to marine organisms (2). Studies of the effect of oil spills and the persistence of hydrocarbons in the marine environment should consider the type of oil spilled.

TABLE 3

Susceptibility of Different Cyclo-Paraffins to Degradation

Hydrocarbon	Basic structural formula	Percent of strains capable of oxidation
Heptylcyclohexane		88
Bicyclohexyl		62
Decalin		25

The activity of microorganisms on aromatic hydrocarbons seems to follow a much less predictable course. Much study is required before a general pattern can be recognized. The heavy aromatic fraction of crude oil is acknowledged to be persistent and potentially more toxic to marine life than any other residue.

Classification of Hydrocarbon Oxidizers.--As shown in Table 1, a hydrocarbon mixture containing a limited number of normal paraffins, in proportion to the other hydrocarbons, can serve as a model to outline the oxidizing capabilities of the microorganisms. It is clear from the diversity of results obtained with different bacteria that percentages of hydrocarbon consumption correlate with the actual enzymatic digestion of the particular hydrocarbon. It is possible that for some hydrocarbons, this is due to cooxidation, but apparently even cooxidation cannot extend the ultimate enzymatic capabilities of the organisms.

The results with the first three strains (Table 1) offer an idealized but real picture of three fundamentally different types. Previously (6) it was proposed that for the purpose of quick recognition, hydrocarbonoclastic bacteria could be broadly divided into three main types: a) those which attack primarily normal paraffins; b) those which preferentially oxidize aromatic hydrocarbons; c) those which can readily attack iso-paraffins, and apparently most other hydrocarbons.

The oxidizing abilities of the organisms, within the third type, may vary, as shown by strains SB3IP7, TR4N and TR1KIP; nonetheless, the results may still indicate the general activity of the organisms toward a complex mixture of hydrocarbons such as crude oil.

Effect of Different Nitrogen Sources.--Ammonia, nitrate and an amino acid were used separately as nitrogen sources to provide additional data for a further characterization of the various microorganisms.

The KNO_3 salt generally represented the best source of nitrogen, as far as rate of hydrocarbon consumption was concerned. Perhaps this may be due to the fact that, under reduced oxygen tension, KNO_3 may serve also as a hydrogen acceptor (7). Although oxygen measurements were not taken during the course of these experiments, conditions in the unshaken test tubes may have reached suboptimal levels for molecular oxygen concentration, during the average incubation period of ten days.

The amino acid alanine seemed to serve as a good indicator of the degree of heterotrophy of the organisms. If an organism can utilize an organic source of nitrogen (i.e., alanine), in all likelihood the same organism would be able to oxidize a more complex source of carbon than hydrocarbons. Such an organism is designated here as a "facultative" hydrocarbon-oxidizer. Contrariwise, an organism unable to utilize the amino acid and able to develop only with ammonia or nitrate would very likely only assimilate hydrocarbons. This type could be termed an "obligate" hydrocarbon-oxidizer, i.e., strain SB3IP7.

All of the strains in Table I, with the exception of SB3IP7, utilized alanine and grew in a peptone medium without hydrocarbons. Strain TR4A may be an example of a typical "facultative" organism, alanine being a better source of nitrogen than either nitrate or ammonia. All strains failed to utilize the hydrocarbons without a nitrogen source. The apparent carbon consumption of strain SB3IP7 in absence of nitrogen (Table I), reflects either an ability to fix atmospheric nitrogen or, more likely, an initial hydrocarbon attack accomplished through the utilization of traces of nitrogen transferred with the cells of the inoculum.

Obligate versus Facultative Hydrocarbon Oxidizers.--Strain SB3IP7 appears to be the only isolate with characteristics of an "obligate" hydrocarbon-oxidizer. It had been tentatively identified as an *Arthrobacter* sp. (4,5). Davis (I) isolated from oil field soils mycobacteria, viz., *Mycobacterium paraffinicum*, which appear to possess similar characteristics. *Mycobacterium paraffinicum* can attack a variety of paraffinic hydrocarbons, with the exception of methane, and is unable to utilize glycerol or peptone during an incubation period of 2-3 weeks (I).

These "obligate" hydrocarbon-oxidizers may be a promising tool for seeding and degradation of oil slicks in the sea. However, their competitive strength in the presence of other microorganisms may not be necessarily high. When strain SB3IP7 was mixed with other strains some inhibition of hydrocarbon oxidation was observed (6). Combination of various facultative strains, each with its own different degrading abilities could be more effective than a single strain with a broad range of oil degradation capabilities. Obligate hydrocarbon-oxidizers may be useful when emulsifiers or other carbon-containing compounds are added to an oil slick. These organisms, if not inhibited by the emulsifiers, would proceed with hydrocarbon degradation, leaving other carbon sources untouched.

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BIODEGRADATION OF OIL IN SEAWATER: LIMITING FACTORS AND ARTIFICIAL STIMULATION

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The limiting factors of petroleum biodegradation in seawater were systematically evaluated. In the surveyed coastal waters, hydrocarbon oxidizers were found to be abundant, but their substrate ranges relatively restricted. Besides lowering the biodegradation rates, low water temperatures caused long lag periods due to retention of volatile inhibitors in crude oil. Nitrogen and phosphorus were found to be severely limiting. Addition of these nutrients dramatically increased oil biodegradation both in laboratory and in field experiments. An oleophilic fertilizer formula for use on floating oil slicks is described.

Summarizing some identified or postulated causes that inhibit biodegradation of polluting oil in the sea, ZoBell (9) listed the low numbers of marine hydrocarbon degraders, their limited substrate range, toxic substances in crude oil, lack of sufficient aeration, lack of adequate dispersion, suboptimal temperatures, and insufficiency of essential mineral nutrients such as nitrogen and phosphorus. We have evaluated some of these limiting parameters in a quantitative manner in order to define practical approaches toward the stimulated biodegradation of oil as a pollution abatement technique.

MATERIALS AND METHODS

Marine hydrocarbon degraders were enriched for, isolated, and counted on an artificial sea water medium (Bushnell-Haas medium modified by addition of 3% NaCl and adjusted to pH 7.8) with 1% of filter-sterilized Sweden crude oil (gift of Sun Oil Co.) as the only carbon source. This medium was solidified, when required by 1.5% of agar.

Individual hydrocarbons of 96% or higher purity were obtained from Aldrich Chemical Co. and from Chemical Samples Co. Initial screening for substrate utilization was carried out on inverted agar plates with the steam-sterilized hydrocarbon added to a filter paper disc in the cover of the Petri dish. In case of selected strains, the results were verified in liquid culture consisting of 10 ml artificial sea water and 0.1 ml of the individual hydrocarbon. The cultures were inoculated with washed bacteria, equivalent

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to 0.1 mg protein, that were grown in Difco Marine Broth 2216 for 24 hr. Incubation was at 28 C for 14 days on a rotary shaker at 200 rpm. Levels of protein were determined by the Lowry method (7).

Biodegradation of petroleum and some of its representative hydrocarbon components was measured by CO_2-evolution and quantitative gas chromatography (2). Effect of temperature on oil degradation was studied using a series of adjustable-temperature waterbath shakers. Experimental details of laboratory and field experiments on oleophilic oil slick fertilizers will be described elsewhere (5).

RESULTS AND DISCUSSION

The abundance of oil-degrading microorganisms was measured in Raritan Bay as a function of the season, and of the distance from known oil pollution sources. Microbial numbers varied from 3400/L in the polluted Arthur Kill to 60/L toward the seaward fringes of the Bay (6). Seasonal fluctuations were evident, but extensive biodegradation of added Sweden crude oil was evident in each aseptically collected 100 ml water sample. In similar waters, the inoculation of spilt oil to accelerate its biodegradation (1) is probably unnecessary, but this measure may be beneficial in pelagic areas.

From the oil-polluted Arthur Kill and from New Jersey coastal waters, 30 strains of marine hydrocarbon degrading microorganisms were isolated. After initial screening on the basis of their substrate range and growth characteristics, a *Flavobacterium* sp. and a *Brevibacterium* sp. were selected for more intensive study. Table 1 shows the substrate range of these two organisms as determined by the protein yield after two weeks of incubation. The rate of mineralization of some representative petroleum hydrocarbons by the same organisms was monitored for 20 days using the CO_2-evolution technique. *n*-Paraffins (Figs. 1 and 2, curves A and B) served as the best substrates for both organisms, but *Flavobacterium* sp. exhibited higher rates of mineralization. Branching, as in the case of pristane, prevented utilization by *Flavobacterium* sp. but not by *Brevibacterium* sp. (Figs. 1 and 2, curves D). Quaternary carbon atoms prevented utilization by either organisms (Figs. 1 and 2, curves G). Utilization of alicyclic and aromatic compounds occurred when a sufficiently long side chain was present (Table 1; Figs. 1 and 2, curves C) but aromatic or alicyclic compounds without such side chains were spared by these organisms (Table 1; Figs. 1 and 2, curves E and F). Other marine bacteria isolated in our laboratory utilized aromatics such as naphthalene and methylnaphthalene, but no utilizers of simple alicyclics such as cyclohexane or decalin could be found. After an exhaustive search, Pelz and Rehm (8) obtained a similar negative result. It may be concluded that bacterial inocula for oil slicks will require numerous strains with complementing metabolic abilities. Some components of crude oil are refractory, and may become susceptible to biodegradation only after an initial abiotic conversion step.

Low water temperatures (5 and 10 C) predictably lowered oil degradation rates but, in addition, lag periods up to 35 days were found to precede the onset of measurable biodegradation. The predominant portion of this lag period was shown to be caused by volatile inhibitors present in crude petroleum that evaporate only slowly at low temperatures (3). We suggest that

biodegradation of spilt crude oil in arctic waters might be hastened through the removal of such volatile inhibitors by ignition, but experiments to prove this point are yet to be performed.

TABLE 1

Ability of Individual Hydrocarbons to Support Bacterial Growth

	Brevibacterium sp. mg protein increase/ml	*Flavobacterium* sp. mg protein increase/ml
I. Aliphatic		
A. Straight Chain		
1. hexadecane	0.21	0.16
2. heptadecane	0.17	0.13
3. docosane	0.03	0.02
B. Branched		
1. pristane	0.09	0.00
2. 2,2,4,4,6,8,8-heptamethylnonane	0.00	0.00
II. Alicyclic		
A. One Ring		
1. octylcyclohexane	0.03	0.02
2. pentadecylcyclohexane	0.06	0.05
B. Two Ring		
1. decalin	0.00	0.00
2. tetrahydronaphthalene	0.00	0.00
III. Aromatic		
A. One Ring		
1. phenyldecane	0.05	0.04
2. toluene	0.00	0.00
B. Two Ring		
1. diphenylmethane	0.00	0.00
2. 1-methylnaphthalene	0.00	0.00

In freshly collected sea water samples, mineralization of Sweden crude oil added at 1% (v/v) was minimal (3% in 18 days) unless nitrogen and phosphorus supplements were added. Optimal concentrations of these supplements (10 mM N and 0.35 mM P) increased biodegradation in the above time period to 70% (4). Since nitrogen and phosphorus salts would be rapidly diluted in the open sea and also might precipitate algal blooms, various oleophilic N- and P- sources were tested as oil slick fertilizers (5). A combination of octylphosphate and a slow-release paraffinized urea fertilizer in laboratory

experiments gave as good or better performance than nitrogen and phosphorus salts. The nutrient formula was evaluated on oil slicks floating on the surface of flow-through seawater tanks. In this field-like situation, treatment with the oleophilic fertilizer resulted in a 10-fold increase of the oil biodegradation rate. In contrast to nitrogen and phosphorus salts, the oleophilic fertilizer formulation failed to stimulate algal blooms in illuminated seawater samples. The cost of treatment of spilt oil with the described fertilizer formulation is estimated to be reasonable. Oleophilic fertilizers, alone or in combination with proper microbial inocula, have a high potential for practical use as auxiliary tools for oil pollution abatement.

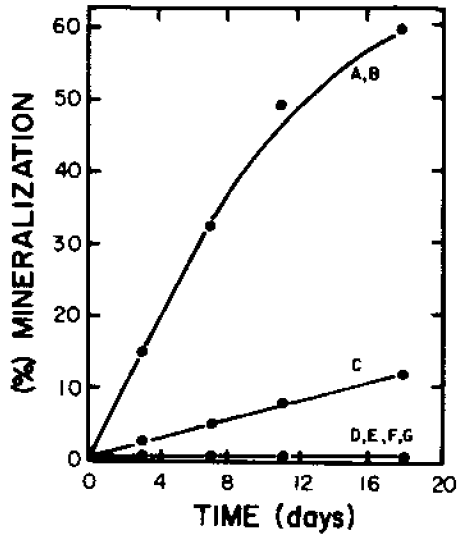

Figure 1. Mineralization (conversion to CO_2) of some representative petroleum hydrocarbons by *Flavobacterium* sp.: *n*-hexadecane (A), *n*-heptadecane (B), 1-phenyldecane (C), pristane (D), tetrahydronaphthalene (E), 1-methylnaphthalene (F), and 2,2,4,4,6,8,8-heptamethylnonane (G).

ACKNOWLEDGEMENTS

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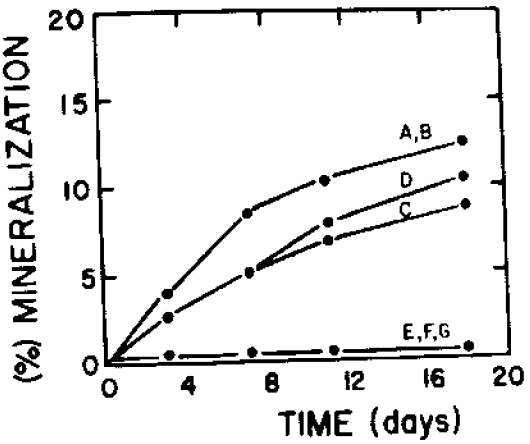

Figure 2. Mineralization (conversion to CO_2) of some representative petroleum hydrocarbons by *Brevibacterium* sp.: *n*-hexadecane (A), *n*-heptadecane (B), 1-phenyldecane (C), pristane (D), tetrahydronaphthalene (E), 1-methylnaphthalene (F), and 2,2,4,4,6,8,8-heptamethylnonane (G).

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BACTERIAL DEGRADATION OF MINERAL OILS AT LOW TEMPERATURES

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Psychrophilic oil-oxidizing bacteria were demonstrated in 13 samples of oil-polluted water, soil, and tundra muck collected from the North Alaska Slope. Within a week or two there was visual evidence that clear mineral oil was being degraded by bacteria at 8 and 4 C. Such activity became apparent after two to three weeks' incubation at -1.1 C, the freezing point of the medium. From oxygen uptake data it was calculated that bacteria oxidized mineral oil at rates ranging from 0.13 to 0.9 mg/liter/day at -1.1 C. Assuming an average generation time of 24 hours, it is calculated that a bacterial population of 10^8 reproducing cells per ml of mineral oil medium would consume oil for building biomass at a rate of 1.2 mg/liter/day at -1.1 C. Nine different crude oils were found to be slowly attacked by bacteria growing at -1.1 C.

It is well established that virtually all kinds of hydrocarbons, certain crude oils, and many refined petroleum products are vulnerable to microbial degradation under favorable conditions (4,11,12,14,15). Nearly 200 species of bacteria, yeasts, and filamentous fungi have been shown to metabolize certain hydrocarbons. The metabolic pathways of many are known (4,5,6, 9,10,12). Most of the observations on the microbial degradation of mineral oils have been made at temperatures extending from 20 to 30 C.

These studies were undertaken to determine the rate at which mineral oils are degraded by bacteria at low temperatures in soil and water at high latitudes and during the winter months. Attention has been focused on this problem by oil spills in cold water and by the discovery of oil fields in the North Sea, Siberia, and the North Alaska Slope (Fig. 1). Temperatures colder than 0 C are common in the Frigid Zones. Almost halfway to the equator, surface soil and coastal waters may be colder than 0 C each winter. Normal seawater freezes at -1.9 C. By volume, more than 90% of the sea is always colder than 5 C.

Medium grade motor oil dispersed in aqueous medium was found (8) to be oxidized by mesothermic bacteria about three times as rapidly at 25 C as at 10 C, with no evidence of action during six weeks at 5 C. Psychrophilic oil-oxidizing bacteria from Cook Inlet, Alaska, were reported (7) to be active at 5 C. Such bacteria from northern Alaska were shown (16) to oxidize mineral oil at -1 C. This is a continuation of those observations (16). Some of the results reported below have been independently corroborated by Agosti and Agosti (1), who collected the samples upon which these studies are based.

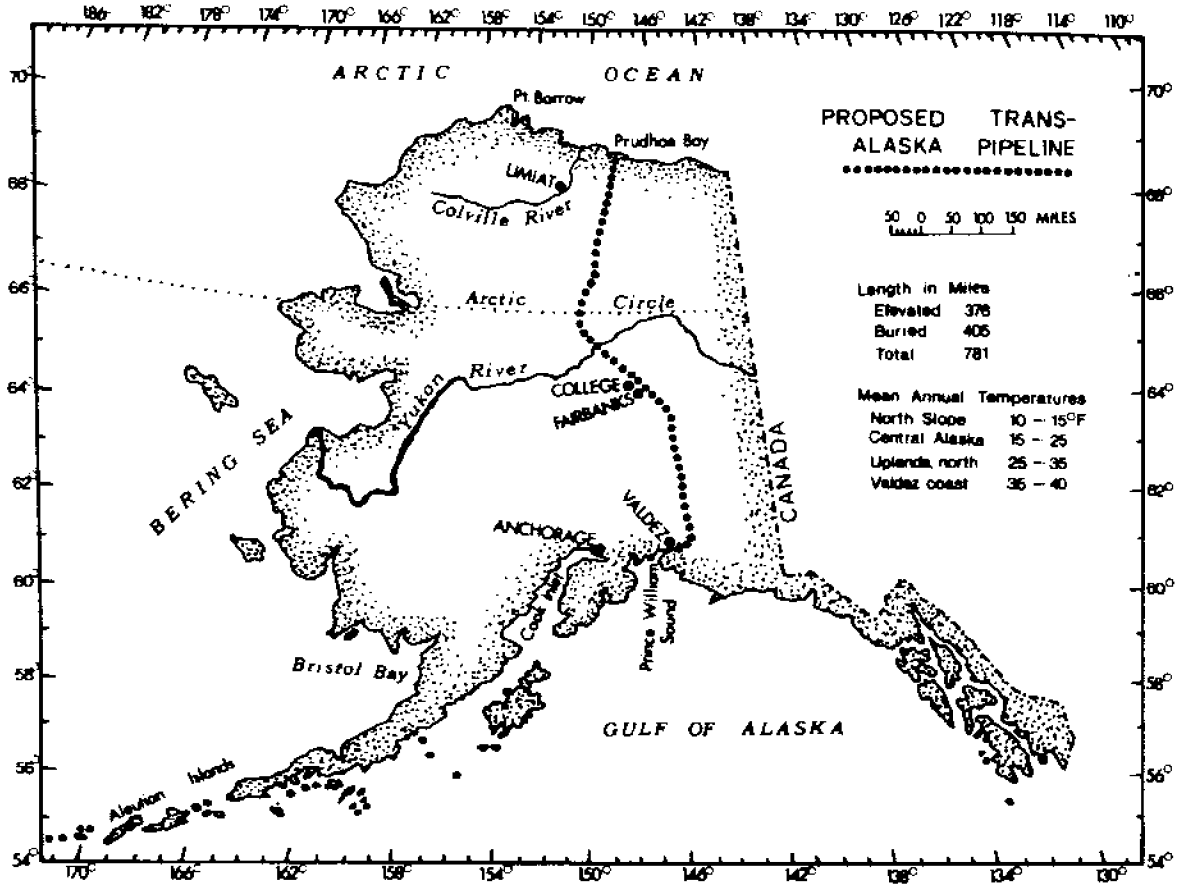

Figure 1. Alaska

MATERIALS AND METHODS

Samples.--Thirty samples of oil-soaked soil, tundra muck, and oil-polluted water were collected in the vicinity of Umiat and Prudhoe Bay about 650 miles north of Anchorage, Alaska. BP, Alaska provided transportation by jet plane between Anchorage and Prudhoe Bay and by chartered Twin Otter to Umiat (Fig. 1). At Umiat there is a nearly abandoned Naval Petroleum Reserve airfield. From there it was necessary to backpack a few miles to a small tundra lake and to various oil seeps along the Colville River.

The 40- to 200-gram samples were collected aseptically in wide-mouth, screw-capped plastic vials. They were packed in a Styrofoam blood-plasma carrying case to keep them cool. During the collection period, June 26 and 27, 1971, the air temperature ranged from 6 to 16 C, but the samples were much colder. In typical permafrost soils the temperature gradient within unfrozen soils extends from near 0 C to a maximum of 4 or 5 C in early summer (2). In the sampling region the mean annual air temperature is -12 C.

Sixteen of the samples were air-expressed from Anchorage to San Diego and then rushed to La Jolla, where they arrived within less than 40 hr after collection. The samples were kept cool and used at once to inoculate mineral salts medium.

Medium.--The following medium was designed to have a reaction of pH 7.0 after autoclave sterilization and an osmotic pressure of 8.7 atm to depress its freezing point to -1.1 C:

NaCl	6.0 g	M/100 $FeSO_4$	3 ml
KH_2PO_4	0.5 g	Dist. water	900 ml
$K_2HPO_4 \cdot 3H_2O$	1.0 g	Aged seawater	100 ml
NH_4NO_3	0.2 g		

Seawater provides calcium, magnesium, sulfate, carbonate, and various trace elements. The medium never freezes to solid ice when held in a refrigerated thermostat submerged in water containing the required quantity of glycerol (40 ml/liter) to depress the freezing point to -1.1 C.

The medium was dispensed in 160-ml screw-capped medicine bottles, 30 ml per bottle. Following sterilization, each bottle (except for controls) was treated with from 0.1 to 1.0 ml of either refined mineral oil or various crude oils, the amount depending on the kind of tests to be made. Owing to differences in the viscosities of oils and their adhesion to glass, it is difficult to measure exact amounts of oil with pipettes. Therefore, each bottle was weighed on a Mettler balance (accurate to 1 mg) before and after introduction of the oil.

In the initial enrichment cultures we used water-white mineral oil consisting largely of C_{12} to C_{20} alkanes as the source of energy for demonstrating the abundance and activity rates of oil-oxidizing bacteria in the soil and muck samples. Controls consisted of uninoculated medium treated with oil and inoculated mineral salts medium without oil. All bottles were incubated on their sides to provide maximum aeration of the medium in a quiescent state. Six replicates were inoculated with about 0.1 g of each sample for incubating in duplicate at 4, 8, and 25 C. Cultures which developed at 4 and 8 C were used to inoculate medium at these temperatures and also at -1.1 C. Tests were made at 25 C for comparative purposes.

Criteria for Biodegradation.--In preliminary tests reliance was placed largely on visual observations for evidence of microbial activity. Most conspicuous was increasing turbidity of inoculated medium and the emulsification of oil followed by its gradual disappearance. Direct microscopic counts (using a Petroff-Hausser counting chamber) and colony counts on precounts (using a Petroff-Hausser counting chamber) and colony counts on pre-solidified nutrient agar (3) demonstrated increases in bacterial populations. Terminally, saponification at 60 C followed by extraction with spectrographic-grade hexane, weighing, and gas chromatography provided information on how much oil was degraded.

In other experiments oxygen uptake was determined by manometric techniques as described by ZoBell and Prokop (17). More precise results on oxygen uptake were obtained in 160-ml BOD bottles filled to capacity with inoculated medium. Mineral salts medium without oil and sterile mineral oil medium were used as controls. In these experiments the oil was adsorbed on a mixture of ignited sand and purified asbestos fibers to provide for its dispersion

throughout the medium and to keep the oil from escaping when stoppering the full bottles.

RESULTS

Oil Oxidizers in North Alaska Samples.--Within a week at 25 C the emulsification of oil and increasing turbidity of the mineral salts medium indicated microbial activity in 13 of the 16 samples of oil-polluted water, soil, and tundra muck collected from the North Alaska Slope. Microbial activity was much slower in mineral oil medium incubated at 4 and 8 C (Table 1).

TABLE 1

Relative Amounts of Microbial Activity in Mineral Salts Oil Medium after Different Periods of Incubation at 4 and 8 C as Indicated by Visual Observations

Sample No.	Description of inoculum	7 days		14 days		28 days	
		4 C	8 C	4 C	8 C	4 C	8 C
1	Oil-seep soil near Umiat	0	0	+	++	+++	++++
2	Colville River foam & sediment	0	+	0	0	+	++++
3	Umiat oil seep, K-2	0	+	+	++	+++	++++
4	Oil-stained lake sediment	0	+	+	++	+++	++++
5	Umiat air-strip soil	0	0	+	++++	+++	++++
6	Oil seep near Prudhoe Bay	0	0	0	0	+	++
7	Soil from eroded River bank	0	0	++	+++	+++	++++
8	Brackish water, Prudhoe Bay	0	0	0	+	+	++++
11	Freshwater stream, snow water	0	0	0	0	0	+
12	Black swamp muck near Prudhoe	0	+	0	++	++	+++
14	Oil-soaked tundra muck	0	0	0	0	+	+++
15	Soil near Navy oil well	0	0	0	0	+	++
16	Oil-polluted swamp muck, Umiat	0	0	+	+	++	+++

0 = No evidence of activity; + = Visible turbidity;
++ = Increased turbidity with some emulsification of oil;
+++ = More turbid and most of the oil emulsified or degraded;
++++ = Very turbid and no visible oil.

Initially, most of the oil (about 0.2 ml per 30 ml medium) floats in irregular shapes and sizes of blobs or rafts. Some oil adheres to the walls of the glass bottles. Oil-oxidizing bacteria tend to disperse the oil and to cause its gradual disappearance. First, the blobs of floating oil commence to break up into smaller and smaller patches. Oil globules flatten out to form a uniform layer or film on the surface of the medium. Concurrently, oil is freed from glass surfaces. The oil gradually disappears from sight at rates which depend on its chemical composition, the incubation temperature, and other factors.

Although colony counts revealed some reproduction of bacteria in

mineral oil medium within two or three days at 4 C, there was no visual evidence of oil oxidation for nearly 14 days. Appreciable turbidity of the medium and emulsification of oil or its disappearance resulted from microbial activity in most of the samples after 28 days at 4 C and more so at 8 C. Colony counts at 8 C demonstrated the presence of from 10^6 to 10^9 bacteria per ml in all cultures scored with plus signs in Table 1.

Bacterial Activity at -1.1 C.--A 2-mm loopful of some of the enrichment cultures which developed at 8 and 4 C was used to inoculate mineral oil medium for incubation at -1.1 C. When inoculated to give from 10^3 to 10^4 colony-forming units per ml, bacterial growth and the emulsification of oil caused turbidity within two or three weeks.

In similarly inoculated mineral oil medium incubated at -1.1 C in BOD bottles the equivalent of from 1.3 to 8.4 mg of oxygen was consumed per liter per day. Theoretically, this much oxygen could cause the complete oxidation (to CO_2 and H_2O) of from 0.13 to 0.9 mg of oil, based on the assumption that the BOD of oil is 3. Actually the theoretical BOD of liquid and solid hydrocarbons ranges from 2.39 for naphthalene to 3.5 for octane (17).

Growth Curves and Generation Times.--Colony counts on nutrient agar medium demonstrated significant increases in bacterial populations in mineral oil medium within a day or two at -1.1 C (Fig. 2). The rate of reproduction was about twice as fast at 8 C as at -1.1 C. Results obtained at 4 C differed so little from results obtained at -1.1 C that it was impractical to draw growth curves for both on Fig. 2. Direct microscopic observations yield evidence that the tendency for lower temperature to retard reproduction is offset in part by the beneficial effects of solid surfaces provided by ice crystals (slush ice) in the medium at its freezing point.

The growth curves are based on results obtained with mixed enrichment cultures. The cultures designated S-3 and S-7 were originally enriched from samples No. 3 and 7 respectively, incubated and maintained at 4 C. Only three or four colonial and morphological types developed in media incubated at -1.1 C. Nearly twice as many types developed in oil media incubated at 4 C and more types developed at 8 C than at 4 C. After the fifth serial transfer, none of the colonies from the -1.1 C cultures was able to grow at 25 C. After five serial transfers at 4 C, culture S-3 failed to grow at 18 C. The cultures were sterilized in 10 minutes at 25 C.

When incubated at -1.1 C, the generation time of a pure culture P-3 was 26 hr during the first day, 16 to 18 hr during the next four days, and an average of 27.2 hr during the sixth to tenth day of incubation (Table 2). The apparent longer generation time during the first day is attributed to a 6- or 8-hr lag phase. Between the tenth and 30th day the population became stabilized at around 10^8 viable cells/ml. From five to fifty times more cells were detected by direct microscopic observations than by counting colonies which developed on nutrient agar incubated at 8 C.

Taking 12×10^{-11} mg as the average carbon content of each bacterial cell and assuming an average generation time of 24 hr, it is calculated that a bacterial population of 10^8 cells per ml of mineral oil medium would consume oil for building bacterial biomass at a rate of about 1.2 mg/liter/day at -1.1 C. If only half of the metabolized oil is converted into biomass and the other half is oxidized to CO_2, twice as much oil would be oxidized.

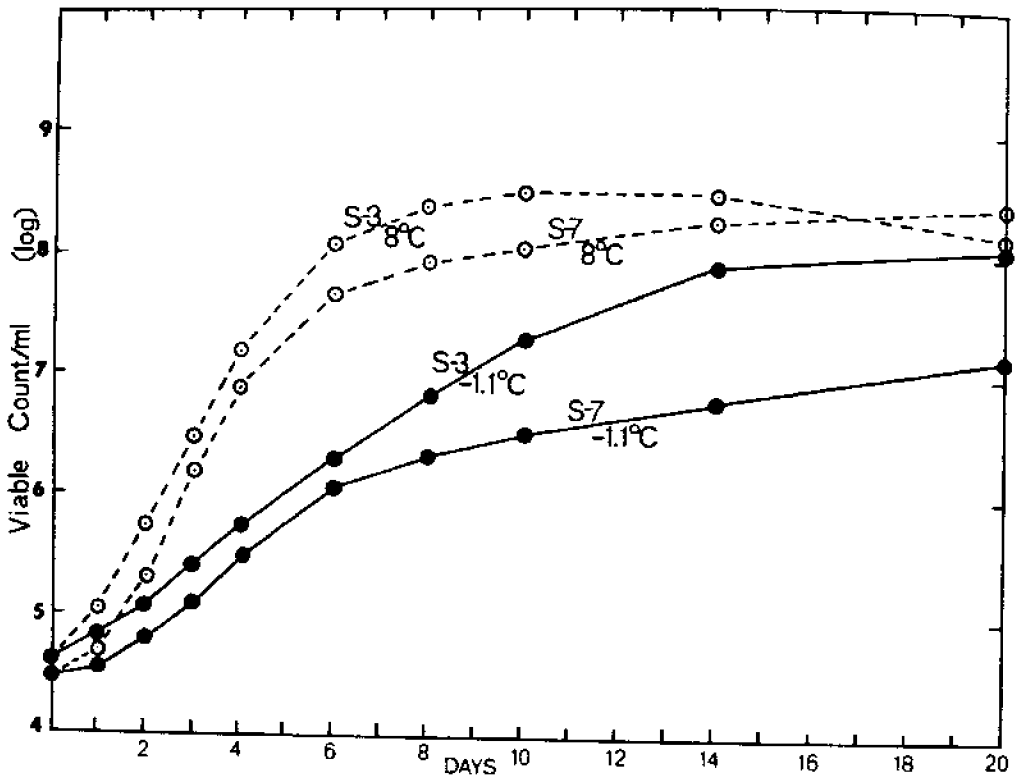

Figure 2. Growth Curves of Mixed Cultures (S-3 and S-7) in Mineral Salts Oil Medium at -1.1 and 8 C

Degradation of Crude Oils.--Nine different crude oils, freed of substances volatile at 60 C, were found to be slowly degraded by mixed cultures at low temperatures (Table 3). Approximately 0.2 ml of oil was added to 30 ml of mineral salts medium inoculated with enough oil-oxidizing bacteria which developed at 4 C to give a few thousand viable cells per ml. They were incubated in a quiescent state at different temperatures. The quantities of hexane-soluble, non-saponifiable material (considered to be oil) in the uninoculated controls and in the inoculated medium was determined at the end of the experiment. The percentage of oil degraded by bacteria was calculated from the difference.

Although none of the crude oils was completely oxidized after ten weeks at low temperatures, within a week or two emulsification of oil and increased turbidity of the medium indicated that all of the oils were being attacked.

TABLE 2

Generation Time of a Pure Culture, P-3, in Mineral Salts Medium Incubated at -1.1 C

Incubation period	Colony Count per ml	Generation time (hr)
0 (initial)	4,900	
1st day	9,400	26.1
2nd day	24,400	15.9
3rd day	66,000	16.8
4th day	168,000	18.4
5th day	340,000	19.2
6th to 10th day	5,300,000	27.2

TABLE 3

Percent of Various Crude Oils Degraded by Psychrophilic Bacteria in 30 ml of Mineral Salts Medium during Ten Weeks' Incubation

Sources of crude oil	Oil in sterile control (mg)	% of oil degraded at -1.1 C	4 C	8 C
North Alaska, Richfield	214	58	64	74
Prudhoe Bay, BP, Alaska	234	61	68	82
Cymric Field, Calif., Associated	198	24	28	46
Abgaio, Arabian, ARAMCO	207	40	43	51
Avoyelles Parish, La., Amerada	218	59	76	91
Barataria Bay, La., Texaco	172	48	54	73
Santa Fe Springs, Calif., Union	188	29	35	38
Lost Hills, Calif., Gen. Petroleum	223	27	30	43
La Guillas, Venezuela, Esso	195	32	37	48
Average	205.4	37.8	43.5	54.6

DISCUSSION

Whereas only from 38 to 91% of each crude oil was degraded in ten weeks at 8 C, virtually all of the purified mineral oil consisting largely of liquid alkanes was degraded under similar conditions. This may be partly due to the presence in certain crude oils of constituents which are more refractory to bacterial attack than alkanes. It may also be a reflection of the kinds of bacteria with which the crude oil media were inoculated. The results summarized in Table 3 were obtained with cultures which had been enriched at 4 C in medium containing purified mineral oil consisting largely of alkanes. The observations on crude oil should be repeated using a mixture of microbial strains known to oxidize aromatic, naphthenic, and asphaltic compounds as well as alkanes.

The rate at which crude oils are degraded by bacteria depends on the temperature of incubation, the chemical composition of the oil, and the varieties of oil-oxidizing bacteria which are active. In our mixed enrichment cultures many more varieties were found which grow at 4 and 8 C than at -1.1 C.

The rate of oxygen uptake is a sensitive indicator of how rapidly oil is oxidized by bacteria, but the amount of oxygen consumed tells little about the oxidation products. For example, whereas theoretically 14 molecules of oxygen could convert 28 hydrocarbon molecules into mono-hydroxy compounds:

$$28\ RCH_3 + 14\ O_2 \rightarrow 28\ RCH_2OH$$

14 molecules of oxygen would be required for the complete oxidation of one molecule of nonane:

$$C_9H_{20} + 14\ O_2 \rightarrow 9\ CO_2 + 10\ H_2O$$

In closed systems where oxygen becomes a limiting factor, the general tendency is for more hydrocarbon to be only partly oxidized to various hydroxy, carboxy, or other partially oxygenated compounds.

From data summarized in Table 3, it is calculated that crude oils were degraded at an average rate of 18 mg/liter of medium/day at -1.1 C. This rate is an order of magnitude higher than the rates calculated from oxygen uptake and bacterial generation times. This suggests that many of the constituents of oils were only partially oxidized rather than being quantitatively converted into bacterial biomass and CO_2.

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BACTERIAL DEGRADATION OF PETROLEUM MATERIALS IN LOW TEMPERATURE MARINE ENVIRONMENTS

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The microbial degradation of oil pollutants has generated considerable interest and an increasing quantity of data in the past few years. It is important to underline three basic considerations for assessing the potential of microbial-facilitated oil degradation.

First, the open sea, estuaries and coast lines are the domain of our concern with major emphasis on the last two zones. A study of major oil spills between 1956 and 1969 demonstrated that 75% of the spills were associated with vessels (mainly tankers) and that 80% of the spills occurred within 10 miles of the shore (2). Our past concern has centered on the warm waters such as those in the Gulf of Mexico. However, the problem is equally acute in temperate and polar regions and low temperature problems will become more apparent with increased northern east coast and north slope Alaska oil production and transportation. Also, the bulk of our ocean water temperatures fall into the psychrophilic range.

Secondly, we need to know and understand the natural potential of this environment for degradation of petroleum materials. ZoBell (3) stated in the 1969 API/FWPCA conference that it was not known whether marine psychrophiles contribute to the decomposition of petroleum. At the 1971 ASM meeting, Zobell (4) described degradation at temperatures of -2 C to 0 C. Kinney et al. (1) implied such action in their study of the Cook Inlet of Alaska. The critical points today are the rate and extent of such degradation and the range of component hydrocarbons involved

Thirdly, we must be concerned with toxicity. This involves the toxicity of the oil per se, the pathogenicity of marine hydrocarbon degraders and the toxicity of their degradation products. Sufficient data are not yet available for a proper evaluation of toxicity; however, there is an inherent danger of pathogenicity associated with the use of high density microbial cultures. This is particularly significant for most, if not all, marine hydrocarbon degraders are also able to utilize peptones as a source of carbon.

I believe that we need to obtain a significant spectrum of marine microorganisms capable of metabolizing petroleum materials in the psychrophilic temperature ranges. These organisms should include both obligate and facultative psychrophiles which can utilize aliphatic and aromatic hydrocarbons. It would also be desirable to obtain organisms which can utilize the other petroleum components such as the oxygenated compounds and complex nitrogen compounds. Hopefully, the isolated organisms would comprise several genera with a diversity of metabolic capabilities. The substrate utilization profiles

should be determined for each organism as well as their temperature ranges and growth optima, and rates at which they can metabolize various substrates at different temperatures. Once this basic information is available, an integrated toxicity-pathogenicity program should be initiated with selected organisms and mixtures of organisms. This program should be conducted by a team consisting of microbiologists, phytopathologists, zoologists and an analytical chemist. Each member of this team should have experience or backgrounds related to the marine ecosystem. Our research program is patterned after the above.

MATERIALS AND METHODS

Samples for study have been obtained from Isle Madame and Crichton Island in Chedabucto Bay, Nova Scotia, and several locations in Narragansett Bay, Rhode Island. Narragansett Bay has a history of chronic oil pollution in small spills of #2 Fuel Oil, Bunker C or #6 Fuel Oil, Navy Special and the more volatile distillates such as JP-5. Chedabucto Bay, on the other hand, reports one major spill of Bunker C oil in February 1970.

Sediments and water were quick frozen in liquid nitrogen for transport over long distances or held at water temperature on close sampling trips (Fig. 1). Samples were inoculated into Rila Salts solution adjusted to a salinity of the sampling environment. The basic Rila Salts solution was supplemented with 0.2 grs. of NH_4Cl and 1.0 grs. of potassium phosphate per liter (SRS). For enrichment each sample was inoculated into separate flasks of this medium containing 1 percent #1 and #6 Fuel Oils and Naphthalene as the sole source of carbon and energy. Replicate flasks were incubated with shaking at 0 C, 8 C and 24 C until evidence of growth initiation. Once visible growth or emulsification was observed, each developing population was streaked for isolation onto sea water nutrient (SWN) agar. The isolation plates were incubated at enrichment temperature and the characteristics of each colony type determined. A representative of each colony type was streaked to a segment of a Supplemented Rila Salts agar containing #1 Fuel Oil as the carbon source and again incubated at enrichment temperature. Only those organisms growing on #1 Fuel Oil were placed in stock culture. This isolation and screening sequence was repeated when the enrichment flask reached the point of maximum growth.

A representative of each hydrocarbon-utilizing colony from each enrichment substrate and from each temperature was selected for determination of its hydrocarbon utilization profile. This was done by preparing agar plates of supplemented Rila Salts with the following separate substrates: hexane, dodecane, hexadecane, 2,2-dimethylhexane, methylcyclohexane, P-xylene, hexylbenzene and naphthalene. Hexane, xylene and methylcyclohexane were provided in the vapor phase to lessen toxicity whereas the other hydrocarbons are emulsified in the agar. Each organism was streaked to four plates of the same medium to obtain the growth potential on each substrate at 0 C, 8 C, 16 C and 24 C. Growth was scored by visual examiantion at 2-day intervals up to a maximum of 20 days. Noble agar which will not support growth of these organisms was used for all solid media preparations.

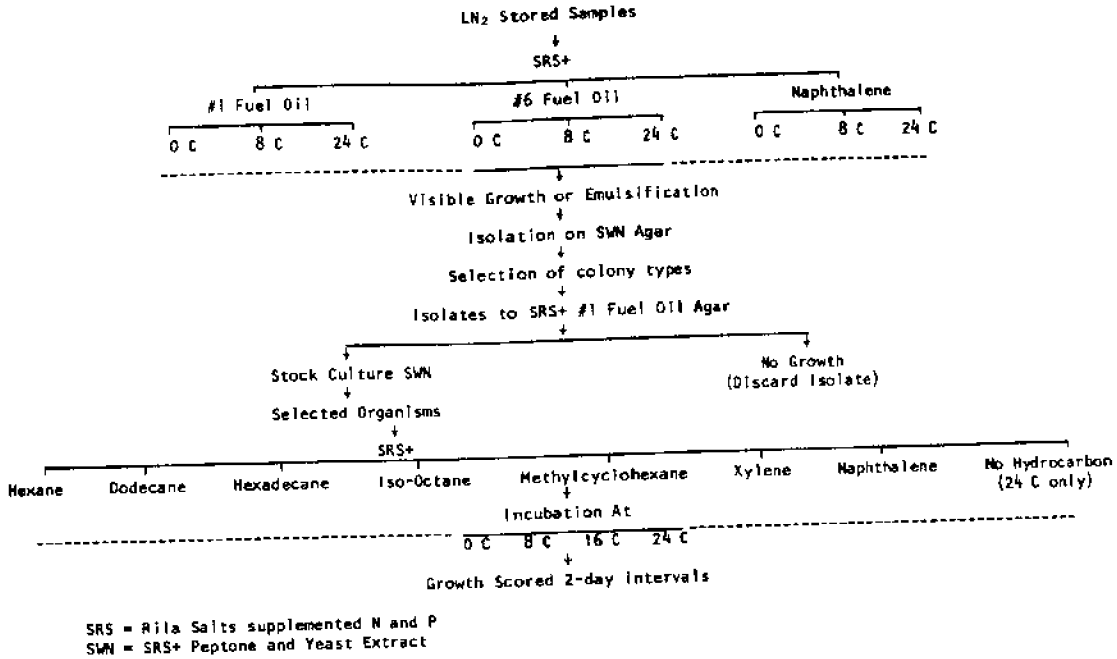

Figure 1. Procedure for Isolation and Screening of Hydrocarbon-Utilizing Marine Bacteria

RESULTS AND DISCUSSION

Early in our studies we found that sediments were a better source of organisms than water column samples. Not only were bacteria present in larger numbers in the sediments but a wider spectrum of genera were obtained from sediment samples. This seems particularly true for Chedabucto Bay which has a faster flushing rate than Narragansett Bay.

To date, we have isolated 49 hydrocarbon-utilizing bacteria from Chedabucto Bay and 72 organisms from Narragansett Bay. The capacity of all of these isolates to grow on hydrocarbons was confirmed by development on #1 Fuel Oil prior to screening studies. The Chedabucto Bay samples were obtained from a 16 to 17 C environment, whereas the Narragansett Bay samples were taken at temperatures of 2.5 to 5 C.

Table 1 shows the percentage of naphthalene enrichment isolates from Chedabucto Bay which utilize the various substrates at 25 C, 16 C and 8 C after eight days of incubation. Sixty-one percent of these isolates are able to utilize naphthalene at 25 C or 16 C within this time frame and a smaller percentage were able to utilize the other aromatic substrates. However, we find the percentage of isolates able to utilize dodecane and hexadecane approaching 100% and even a high percentage are able to utilize iso-octane. From this we concluded that aromatic utilizers are also able to utilize aliphatic substrates. An examination of the profile of individual isolates

confirms this conclusion. The higher percentage of aliphatic utilizers than aromatic utilizers also suggests that not all aliphatic-utilizing bacteria are able to attack an aromatic substrate. This fact is, of course, well documented for the non-marine hydrocarbon-utilizing bacteria. Examination of similar data in Table 2 for Chedabucto Bay #1 Fuel Oil enrichment isolates demonstrates that they have a lesser capacity for utilization of naphthalene and xylenes but not for hexylbenzene. The lower aromatic content of #1 Fuel Oil may have prevented the selection of many aromatic-utilizing bacteria present in the samples. The organisms utilizing hexylbenzene may be growing at the expense of the aliphatic side chain rather than the aromatic nucleus.

TABLE 1

Percent Substrate Utilization of 13 Chedabucto Bay Naphthalene Isolates after 8 Days at Different Screening Temperatures

Substrate	25 C	16 C	8 C
Hexane	15[a]	38	30
Dodecane	92	100	77
Hexadecane	92	92	15
Iso-octane	77	100	85
Methylcyclohexane	54	70	15
Xylene	8	30	30
Hexylbenzene	46	30	30
Naphthalene	61	61	30

[a]Percent utilizers, 13 isolates.

TABLE 2

Percent Substrate Utilization of 6 Chedabucto Bay #1 Fuel Oil Isolates after 8 Days at Different Screening Temperatures

Substrate	25 C	16 C	8 C
Hexane	17[a]	50	0
Dodecane	100	84	84
Hexadecane	84	66	17
Iso-octane	66	84	33
Methylcyclohexane	84	33	33
Xylene	0	0	0
Hexylbenzene	50	50	17
Naphthalene	33	33	0

[a]Percent utilizers, 6 isolates.

In Table 3 are presented profile data for kerosene enrichment isolates from Narragansett Bay. This profile is much like that of the #1 Fuel Oil isolates from Chedabucto Bay. There are fewer aromatic hydrocarbon-utilizing bacteria among these isolates, of which the greatest percentage utilize dodecane, hexadecane and iso-octane. Again we find a number of hexylbenzene utilizing bacteria which may be growing at the expense of the aromatic side chain.

TABLE 3

Percent Substrate Utilization of Narragansett Bay Kerosene Isolates after 8 Days at Different Screening Temperatures

Substrate	25 C	16 C	8 C	0 C
Hexane	12[a]	12	25	25
Dodecane	88	88	88	38
Hexadecane	75	75	63	25
Iso-octane	75	75	38	13
Methylcyclohexane	38	38	38	38
Xylene	0	0	0	0
Hexylbenzene	25	25	25	25
Naphthalene	0	12	12	0

[a]Percent utilizers.

One may obtain a variety of aromatic hydrocarbon-utilizing bacteria. Better results are obtained if a pure aromatic substrate is used for enrichment rather than a mixture of aliphatic and aromatic hydrocarbons. Our data indicate that aromatic hydrocarbon-utilizing bacteria are capable of growth on aliphatic hydrocarbons, but that the majority of the bacteria isolated on aliphatic or mixed substrates utilize aliphatic but not aromatic hydrocarbons. Further, the hydrocarbon profile data show that marine bacteria exist which can utilize aliphatic straight and branched chain hydrocarbons, cyclic hydrocarbons, and aromatic hydrocarbons in the psychrophilic temperature range.

Figure 2 shows the effect of hydrocarbon profile screening temperature on growth of the Chedabucto Bay isolates. As mentioned earlier these isolates were obtained from a 16-17 C environment. Dodecane and naphthalene were chosen to demonstrate the temperature effect as they were representative of the effects observed with other aliphatic and aromatic substrates. Most of the organisms grew on dodecane promptly at 16 C. After only two days, 21 of 30 isolates were scored as growing on dodecane. Also, these isolates grew well at 24 C, but not as many initiated growth early at this temperature. The response of the isolates at 8 C indicates that most are able to grow at this temperature but that growth initiation and/or growth rate are slow. Five of the isolates initiated development after 10 days at 0 C which indicates some of the organisms were capable of slow growth at this temperature. Essentially the same observations are noted for the isolates on naphthalene. The numbers of organisms growing on the aromatic substrate are less but the

general trend is the same, viz., good initiation at 16 and 24 C but the slow initiation of growth at 8 C and no growth on aromatic substrates even after 10 days at 0 C.

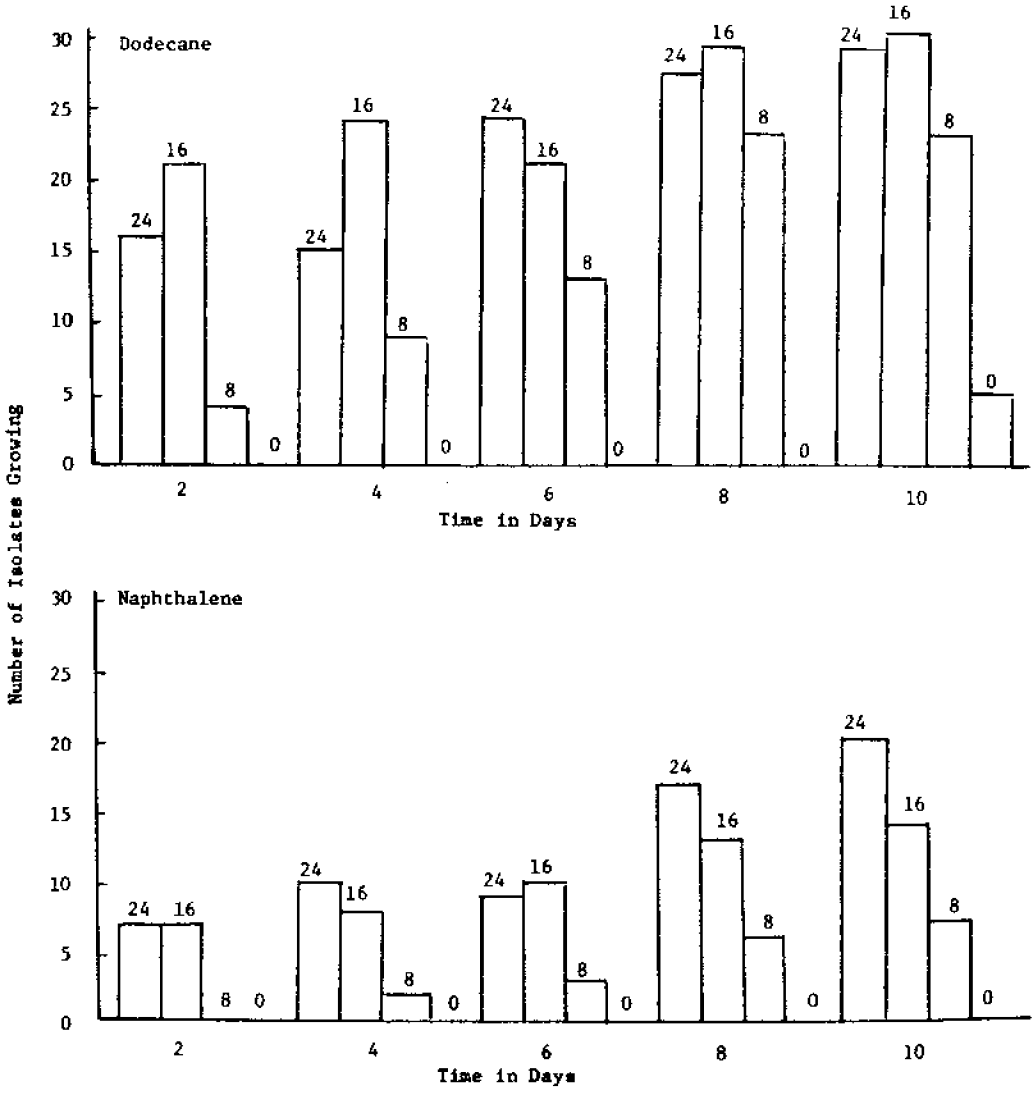

Figure 2. Number of Chedabucto Bay Isolates Growing on Dodecane and Naphthalene Seawater Agars at 24, 16 and 0 C

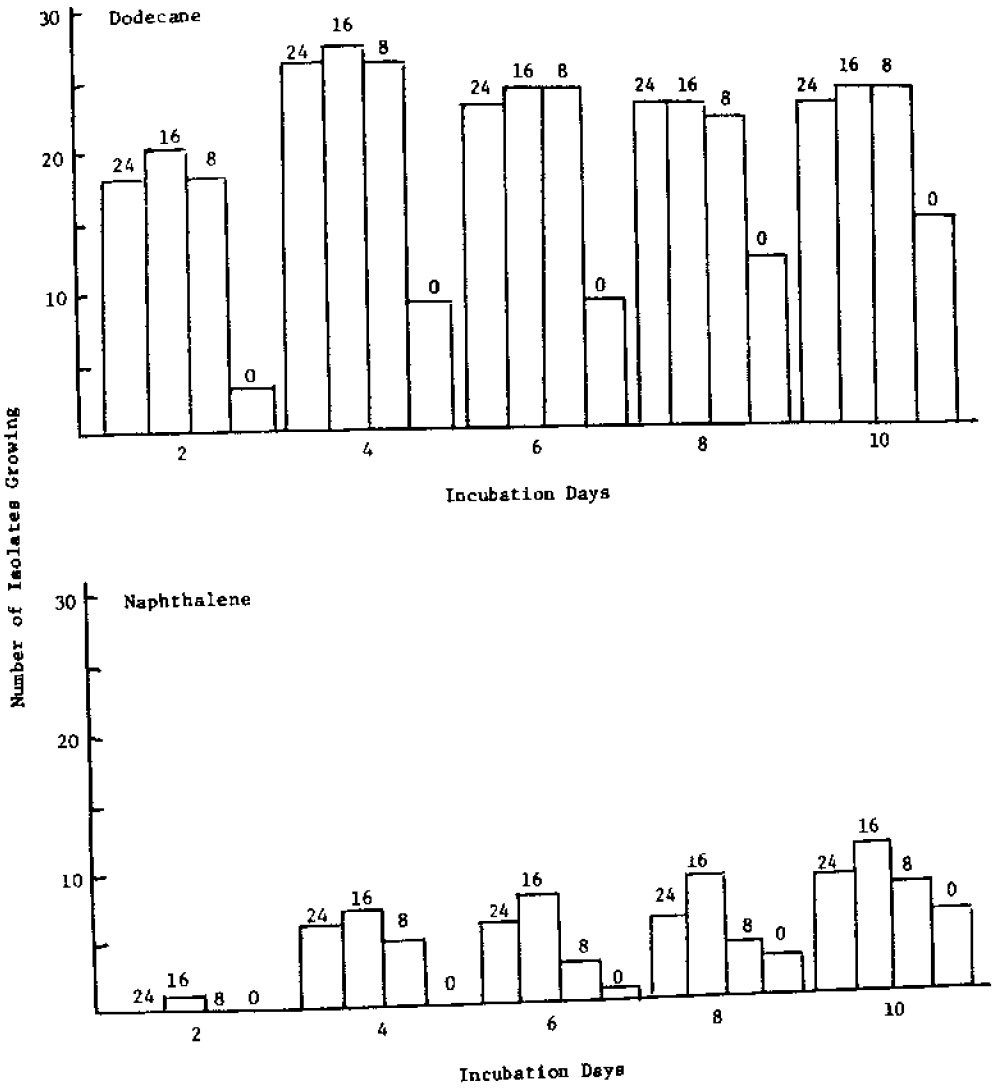

Figure 3. Number of Narragansett Bay Isolates Growing on Dodecane and Naphthalene Seawater Agars at 24, 16, 8 and 0 C

Examination of the Narragansett Bay isolates (Figure 3) demonstrates that these organisms initiate growth more readily on both dodecane and naphthalene at 8 C and 0 C. Furthermore, a slightly greater number of these isolates use naphthalene at 16 C than at 24 C. Utilization of dodecane appears within 2 days incubation at 0 C with some of these organisms, whereas 10 days was required for the Chedabucto Bay isolates. It is significant that the Narragansett Bay isolates were obtained from a 2-5 C environment.

As expected, these isolates from a colder environment (winter

Narragansett Bay) initiate growth earlier at 0 C and 8 C than organisms from a borderline psychrophilic environment such as Chedabucto Bay in mid-summer. The organisms from Narragansett Bay have a wide temperature span for growth as do those from Chedabucto Bay. None of these isolates could be considered as obligate psychrophiles but I would class them as facultative psychrophiles or psychro-tolerant in that they initiate growth and develop a population within a few days at low temperatures.

The data presented are not complete and represent an analysis of only certain facets of our work. Currently, we are studying rate of growth and substrate utilization at different temperatures with selected isolates from both sampling sites. This winter (1973-74), after Narragansett Bay has returned to psychrophilic temperatures we will continue isolations but with technique modifications which should yield obligate marine psychrophiles that can utilize the aromatic hydrocarbons of petroleum. We would also like to resample Chedabucto Bay in the spring while its waters and sediments are in the psychrophilic temperature range. Preliminary examination of the isolates indicates an assortment of genera represented among the organisms from both locations. Our data demonstrate that marine facultative psychrophiles (psychro-tolerant bacteria) can be isolated which readily utilize a variety of aliphatic, cyclic and aromatic hydrocarbons. We feel we will be able to demonstrate the existence of obligate marine psychrophiles with similar degradative potential.

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HYDROCARBON BIODEGRADATION IN ALASKAN WATERS

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Populations of hydrocarbon-oxidizing organisms were of the order of 1/cc in Alaska's Cook Inlet and Port Valdez, less in the Arctic Ocean. Distribution decreased with salinity in Cook Inlet and with depth in Port Valdez. In situ oxidation of ^{14}C-dodecane (91 µg C/liter) started within hours and proceeded at a rate of 1 µg/liter-day. Storage of Cook Inlet crude oil for four years in sea water at 10 C effected removal of most visible components. Mixing had a major effect on oil slick stability. The solubilization process was little affected by added silt, a major component of many Alaskan estuaries. Isolated organisms had unique preferences for various components of kerosene, emulsified crude oil and responded in a normal way to incubation temperature, E_a 14.5 to 16 Kcal/mole. Some effects of crude oil inhibition are discussed. Calculations show that motility, particularly in combination with chemotaxis, is necessary for rapid slick inoculation.

INTRODUCTION

Hydrocarbon biodegradation depends on the association of oil nutrients and appropriate organisms. In the case of marine spills, the nutrient ratios, inoculum size, mixing conditions, temperature, oil solubility and vapor pressure all affect the half lives of residual components. In Alaska's Cook Inlet we estimated some of the oil removal terms (9). Tide-driven flushing was 90% complete in 10 months. Evaporation removed about half of the C-12 hydrocarbons from slicks in 8 hours and was found to be a major removal mechanism for this and lighter fractions. Large slicks had half lives of the order of a day due to the 20-30 foot tides and 5 knot currents. In this communication we report observations on the distribution of hydrocarbon-oxidizing organisms, some characteristics of several organisms isolated, rates of hydrocarbon oxidation observed and some estimates of inoculation frequency.

MATERIALS AND METHODS

Plate Counts of In Situ Populations.--Microbiological samples were taken from rubber bulb type bacteriological water samplers (CM^2 Inc., Mountain View, Calif.), evacuated, and triggered to fill at depth. Samplers were autoclaved (121 C, 15 min) prior to hydrographic casts. Sea water in 0.1 to 50-ml samples was transferred in a glove box to discourage airborne contamination and then filtered. Resulting filters (0.45 µm pore size) of organisms

collected were transferred to agar plates and incubated at 10 C for subsequent enumeration.

Agar Media.--Agar for plates had the following composition per liter: agar, 15 g; NaCl, 30 g; Na_2HPO_4, 2g; $(NH_4)_2SO_4$, 60 mg; KCl, 40 mg; $MgSO_4$, 25 mg; $CaCl_2 \cdot 2H_2O$, 5 mg; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 5 mg; $ZnSO_4 \cdot 7H_2O$, 0.7 µg; $CuSO_4 \cdot 5H_2O$, 25 ng; $MnSO_4 \cdot 7H_2O$, 1.5 ng; $CoSO_4 \cdot 7H_2O$, 1.5 ng; MoO_3, 0.5 ng; vitamins B_1, B_{12} and biotin, 10^{-10} moles. Half of the agar mixture contained 1 g/liter of both yeast extract and succinic acid, and the other half of the medium had no further additions. The pH was adjusted to 7.3 in both media, which were then autoclaved for 15 min at 15 psi.

At each station, sea water samples were collected and filtered from depths of 0, 5, 10, 25 and 50 m. For each depth at each dilution there were four plates with filtered samples; two yeast extract-succinic acid plates, and two plain agar plates. Sample volumes passed through the filters were 0.1, 1.0 and 10.0 ml for the yeast extract-succinic acid plates and 1.0, 10.0 and 50.0 ml for the plain agar plates. Samples were passed through filters and the filters were incubated on the agar surfaces. Crude oil was added to one of each pair of the two types of agar plates by dropping it onto sterile filter paper situated on the inner top of the culture dish. The plates were inspected periodically for the appearance of colonies on the filters.

Minimum Sample Volume for Oil Slick Disruption.--A nutrient supplemented sea water medium was prepared by adding 100 mg/liter $(NH_4)_2SO_4$, 4 mg/liter Na_2HPO_4, and 10^{-5} moles/liter EDTA to 20 liters of sea water, which was taken in October 1971 from a depth of 10 meters in Valdez Narrows. The pH was adjusted to 7.3 and the carboy was autoclaved for 25 min at 15 psi. After the medium cooled, 20 ml of Prudhoe Bay crude oil, sterilized at 121 C in sealed ampoules, was added to the carboy, which was vigorously shaken for several minutes and left to equilibrate for 24 hours during which time the oil phase separated out on top. Without disturbing the oil phase, 100 ml of oil-equilibrated medium was siphoned into each of 200 sterile 250-ml screw cap bottles. Care was taken to keep the medium sterile during transfer.

Sea water samples of 1, 10, or 100 ml were introduced into the bottles. After incubation for one week, the bottles were charged with an oil slick by adding three drops, or about 30 µl, of the sterile Prudhoe Bay crude oil. Adenosine triphosphate resulting from oil metabolized was measured according to Stroehler and McElroy (13).

In Situ Microbial Oxidation Rates.--Either ^{14}C-1-dodecane or an amino acid mixture uniformly labeled with ^{14}C was added to 1-liter sea water samples. After substrate addition, all samples were incubated in situ from 0 to 35 days. After incubation, the CO_2 was extracted from the sample, collected in alkali, and precipitated as $BaCO_3$. The radioactivity of the $BaCO_3$ precipitate was measured to quantify the amount of substrate respired during incubation. The amount of particulate and dissolved ^{14}C-substrate remaining after each incubation period was also measured.

Uniformly labeled solutions were prepared of: 1) ^{14}C-amino acid mixture, specific activity 54 mCi/matom-C (Amersham Searle, Arlington Heights, Ill.), containing 2.5 µCi and 550 ng C/ml; and 2) ^{14}C-dodecane, specific

activity 1.86 mCi/mmole (ICN Tracerlab, Waltham, Mass.), containing 826 nCi and 75.5 µg dodecane in 0.125 µl. Radiochemicals were autoclaved and stored in sealed ampoules.

Samples of coastal water (30 m total depth) were pumped from 10 m to fill dark incubation bottles. The pump consisted of a PVC plastic hand bilge pump fixed to 10 m of 2.5 mm O.D. polypropylene tubing. The tubing was weighted to remain at depth. The whole system was flushed in 95% ethyl alcohol before use and then with large volumes of sea water from sample depth. Samples were divided into two experimental sets as follows: a) bottles with amino acid mixture, each containing 2.5 µCi and 550 ng C in 1 liter of sea water; and b) bottles with dodecane, each containing 825 nCi and 75.5 µg dodecane in 1 liter of sea water.

Immediately after the addition of substrates, the samples were lowered and left to incubate for up to 35 days. An arrangement of buoys, nylon line, and anchors kept the bottles submerged at 10 m during incubation.

Microbial Growth Rates.--Maximum growth rates of isolated organisms were determined using the hydrocarbon liquid medium at pH 7.5 with the addition of 0.1% kerosene. Rates of CO_2 evolution were monitored by recording the infrared absorption of the effluent gas on a calibrated CO_2 analyzer. Gas flow was regulated by passing compressed CO_2-free air through a flow restriction of small glass beads packed into capillary tubing, and quantitated by passage through a wet test meter. Absorption by volatile hydrocarbons was corrected out by subtracting the response of a parallel sterile control. These two reactors were 500-ml magnetically stirred indented conical flasks. Maximum growth rates in nutrient broth were measured by following the rate of change of absorbency at 565 nm in a medium containing 8 g/liter nutrient broth, 10 g/liter glucose and 20 g/liter NaCl, pH 7.65.

Chromatography.--Gas chromatography was performed on the pentane extracts of isolated cultures grown on hydrocarbons. The mineral salts medium was supplemented with 4 g/liter kerosene and inoculated. After growth and extraction with a half volume of pentane, the pentane phase was filtered, concentrated 50:2 ml and sealed in an ampoule for subsequent chromatography. Parallel uninoculated controls were carried through the procedure and used for a peak height reference.

RESULTS

Bacterial Populations.--Hydrocarbon-oxidizing organisms were easier to obtain from Cook Inlet than from Valdez or arctic coastal areas. Colonies developing on membrane filters through which water samples had been passed could be cultured on glass filters over otherwise nutrient-free liquid media exposed to crude oil vapors. Their concentrations were essentially constant with respect to depth, responding to the high order of tidal mixing reported (9). Even the surface film, collected on a wire screen, contained populations similar to those in deeper portions of the water column. Average populations of hydrocarbon oxidizers are shown in Figure 1 representing about 10% of the total colony-forming units developing on nutrient agar plates.

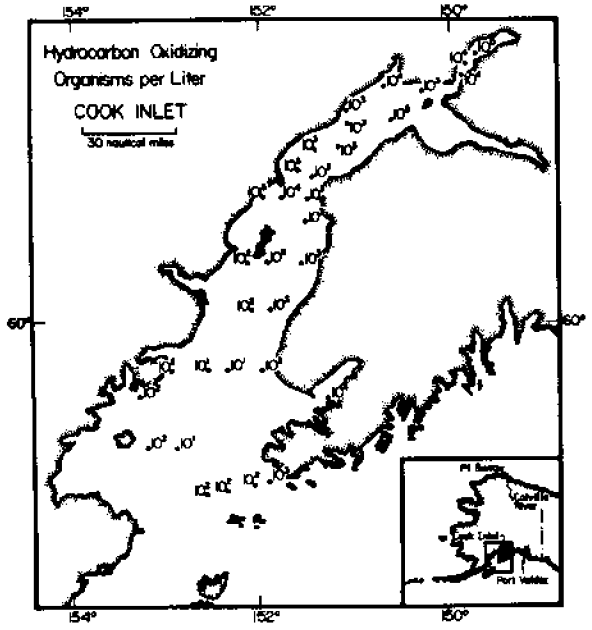

Figure 1. Average distribution of hydrocarbon-oxidizing organisms per liter in Cook Inlet in July

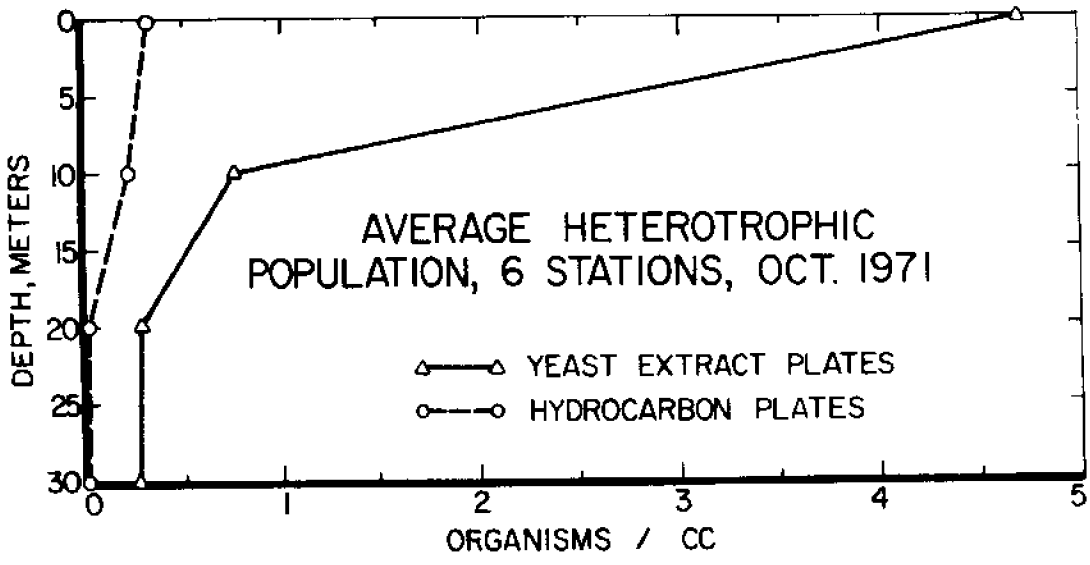

Figure 2. Average heterotrophic populations from six stations in Port Valdez that would grow on hydrocarbon vapors and mineral salts agar plates or on complete medium plates; with respect to depth

Usual membrane filter counts were taken throughout Port Valdez. Populations of organisms developing on agar plates with oil or with glucose-succinate carbon sources are shown in Figure 2. Of those colonies developing on oil plates, 177 were transferred to yeast extract plates with oil, yeast extract without oil, mineral salts medium with oil and without crude oil added. Hydrocarbon vapors were not found to be inhibitory to the development of colonies. The crude oil vapors stimulated the growth of colonies from plates designed to select for hydrocarbon oxidizers in only 2 out of 177 cases over the agar base alone. This technique suggested general bacterial population levels but gave little evidence of a vigorous hydrocarbon-oxidizing flora.

Degradation of oil slicks is widely observed to produce visible changes in the consistency of the slick. Also the responsible microflora can be detected by transfer to nutrient agar plates or by measuring the adenosine triphosphate produced by the organisms at the expense of the oil. These techniques were used in conjunction with "most probable number" techniques for estimating the hydrocarbon-oxidizing microflora in Port Valdez.

Bottles containing 100 ml of sterilized sea water were inoculated with various volumes of water samples from Port Valdez. The water was pre-equilibrated with crude oil and supplemented with nitrogen, phosphate and a chelate. These additives were all shown to increase the degree of positive samples compared to controls, but did not affect the sample volume required to initiate biodegradation. Effects on the oil slick, whether or not it was coagulated compared to the smooth consistency of sterile controls, adenosine triphosphate content, and colony-producing ability of the disruption bottles were measured. Thus, if a bottle produced large amounts of ATP or if the slick was materially altered, organisms collected in the volume of water sample added were attributed to inoculation. The oil slick must have served as the major carbon source. Some 34 replicate tests of these various techniques gave fairly good reproducibility and precision, showing acceptably linear response with respect to dilution. General populations measured by this technique were quite similar to those measured by the plate count techniques. Total populations tested by the ability of developing cultures to appear on inoculated nutrient agar plates were about an order of magnitude above hydrocarbon oxidizers. As judged from samples at four depths from six stations during spring and fall, the density of hydrocarbon oxidizers at most points in Port Valdez was sufficient for each cubic centimeter of water to initiate biodegradation. Smaller volumes were generally inadequate. These observations do not differ substantially from the Cook Inlet results. Complete data are reported elsewhere (11).

In Situ Oxidation Rates.--The occurrence of hydrocarbon biodegradation as an ongoing process in Port Valdez was confirmed by incubating at depth, 32 separate 10-meter water samples to which ^{14}C-dodecane had been added. $^{14}C\text{-}CO_2$ appeared in these during the first few hours of incubation. The oxidation rate sustained was about 1 µg hydrocarbon/liter-day from an initial concentration of 91.1 µg C/liter (Fig. 3). For comparison, parallel samples using radioactive amino acids produced carbon dioxide at a rate of about 0.2 ng/liter-day from an initial concentration of 0.65 µg C/liter.

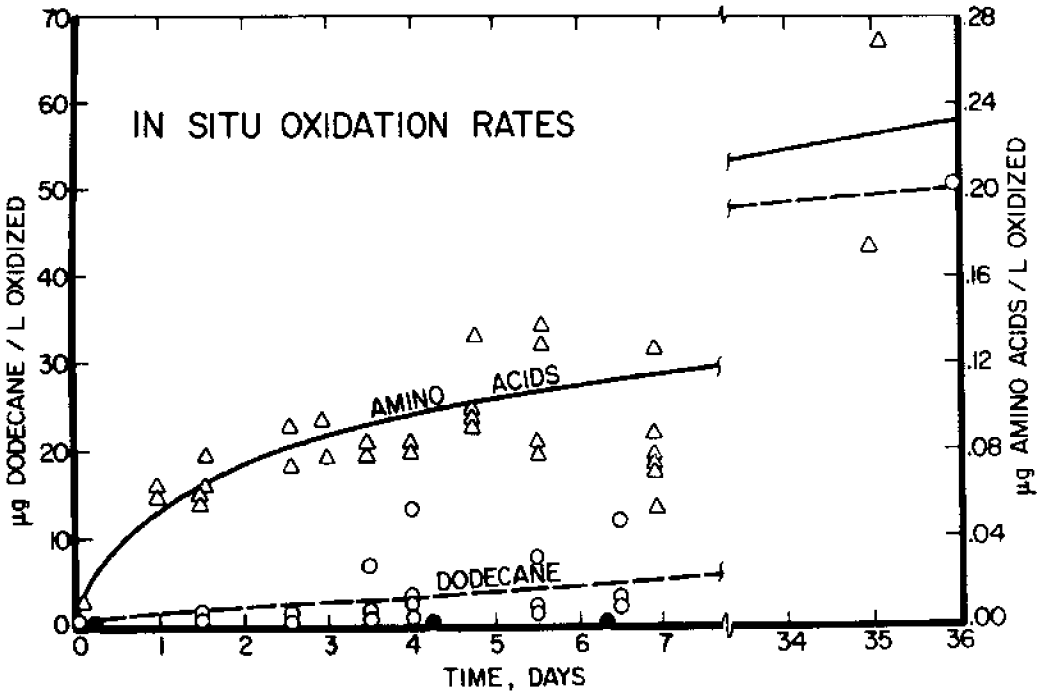

Figure 3. Carbon dioxide liberated from radioactive dodecane and from an amino acid mixture incubated in Port Valdez

A series of plate enumerations in the Colville River delta area of the Arctic Ocean during summer failed to reveal any hydrocarbon oxidizers. Amino acid oxidation rates off Point Barrow were of the order of 0.01 µg/liter-day; glucose, an order of magnitude less (1). While these data are limited, there appears to be significantly less microbial activity in summer arctic water than in western Alaska. Since a heterotrophic microflora does exist, one would expect the process of oil biodegradation to proceed, however slowly.

Much of the Alaskan coastal water carried fairly heavy suspended sediment loads. Suspended sediment in Cook Inlet runs as high as 1500 mg/liter, rendering a few centimeters of this water opaque. Port Valdez normally contains 5-10 mg/liter of this material. Suspended sediment loads in the Colville River delta area of the Arctic Ocean run to 10 mg/liter during the spring run-off (A. S. Naidu, personal communication). The effect of this silt on oil slick dissipation was observed by inoculating 3 carboys of synthetic sea water with a mixed culture. All contained surface slicks of 1.9 mg/cm^2. Two contained Cook Inlet sediment at 100 mg/liter, one of which was "poisoned" with 1 mg/liter $HgCl_2$. The unpoisoned slicks solidified and stopped moving with the stirred medium on the third day. Both microbially active slicks broke and mixed down in on the 14th day, showing that added silt had no effect on the stability of the slick. During this time and in the succeeding 45 days no change was observed in the poisoned control.

The importance of mixing was observed by adding a very thin oil slick, 10 $\mu g/cm^2$, to a carboy of synthetic sea water enriched with 10 mg/liter nitrogen and 1 mg/liter phosphorus and inoculating as before. Although rapid dissipation of the slick was anticipated due to the comparatively high nutrient concentrations, it was found to be stable throughout 50 days of observation. The only obvious consistency changes were the development of opalescence and the appearance of aerial hyphae from surface mold. Generally oil slick stability is enhanced greatly by the absence of turbulent mixing energy, but little affected by the presence of suspended sediments.

To establish general characteristics of northern hydrocarbon-oxidizing organisms, a number of hydrocarbon-oxidizing organisms were isolated (Table 1) and examined for their potential to use oil slicks. Culture 179 came from a winter plate near an oil spill in Cook Inlet. Cultures 181 and 197 were from a beach near the 1969 Santa Barbara oil spill. The remaining organisms were from points and depths distributed throughout Cook Inlet in July. Maximum growth rates, grams of cells/(grams of cells x hr), are reported for these isolates growing on mineral salts with kerosene the sole source of carbon, and on nutrient broth. These rates are related to temperature by their activation energies E_a calculated from growth rates at 10 C and 25 C according to the Arrhenius equation.

TABLE 1

Characteristics of Hydrocarbon-Oxidizing Organisms

Isolate No.	Gram Reaction	Cell Dimensions (μM)	Cell Shape	μ_{max} Broth hr^{-1}	μ_{max} Kerosene hr^{-1}	E_a Kcal/mole	Emulsification	Type*
54	+	0.6 x 1.0	rod	0.23	--	--	3	*Mycobacterium*
72	+	0.5 x 2.0	rod	0.35	0.20	16.0	2	*Nocardia*
80	+	1.0 x 1.2	coccoid	0.35	0.087	--	2	*Arthrobacter*
114	+	0.6 x 3.0	rod	0.35	0.020	16.0	3	*Nocardia*
179	+	0.6 x 3.0	rod	0.35	--	15.2	3	*Nocardia*
181	-	0.5	cocci	0.15	--	--	1	*Micrococcus*
197	+	0.5 x 20	branched filaments	--	--	--	--	*Streptomyces*
198	+	0.6 x 3	rod	0.35	--	14.5	2	*Mycobacterium*

*Most closely related genus.

All of the hydrocarbon-oxidizing organisms reported here had striking emulsification characteristics. Culture 115, for example, stabilized twice its dry weight of crude oil as an emulsion. Culture tubes otherwise wetted by crude oil presented clean walls when shaken with a few mg of these organisms. Accordingly, these cultures were frequently masked from microscopic view when presented with an oil emulsion because the organisms enter into the oil phase. The presence of cells was sometimes apparent by distortion of the otherwise spherical droplets and by CO_2 evolution of these emulsions. Culture

broth from which the organisms had been centrifugally separated retained emulsification properties, suggesting the production of an extracellular emulsifying agent. Relative emulsification capacity is shown in Table 1.

The isolates were examined for hydrocarbon specificity using kerosene as the sole source of carbon. A representative gas chromatogram is shown in Figure 4 together with an unreacted control. Each of the isolates was characterized by a different group of components after fermentation. Table 2 shows the fate of the larger peaks numbered in Figure 4. None of the lesser components were observed to change in concentration. These are probably aromatic. The frequently metabolized peaks 10, 17, 22, 29, 32 and 34 are probably normal saturated straight chain hydrocarbons as deduced from kerosene analysis and hydrocarbon standards. All organisms isolated produced a unique spectra of remaining hydrocarbons from kerosene so that: a) individual hydrocarbon components can be separately categorized by organisms; b) several hydrocarbons, but not all, are used by each species; and c) a group of organisms is required for complete hydrocarbon removal. Table 3 shows that liter quantities of Cook Inlet water contained sufficient flora to remove about 90% of the visible portions of an oil slick in 30 days. Storage of the samples for 4 years resulted in samples with the lowest level of oil added completely clear except for traces of very small black specks entrapped in mycelial matter.

TABLE 2

Utilization of Kerosene Components by Marine Microorganisms Isolated

	Peak														
	5	10	11	16	17	21	22	26	29	30	32	33	34	P	D
	Carbon Number[a]														
	11	12			13		14		15		16		17	10	12
Organism	Degree of Utilization[b]														
54	+	+		-	+	-	+	-	+	-	+	-	+	+	
72		+	+	-	+	-	+	+	-	+			-		
80		+		+	+	-	+	-	+	-	+	-		+	+
114		+					+	-	+	-	+		+		+
179	I	-	-	-	+	-	+	-	+	-	+	-	-		-
181	I	-	-	-	-	-	-	-	-	-	-	-	-	+	-
197	I	-	-	-	-	-	-	-		-	-	-	-		
198		+		-	+	-	+	-	+	-	+	-	+	+	

[a]Carbon number shows number of carbons in normal hydrocarbon columns: P is 2,6,10,14-tetramethylpentadecane (pristane); D, dodecane; I, peak size increased.

[b]+ = peak utilized; - = peak not utilized.

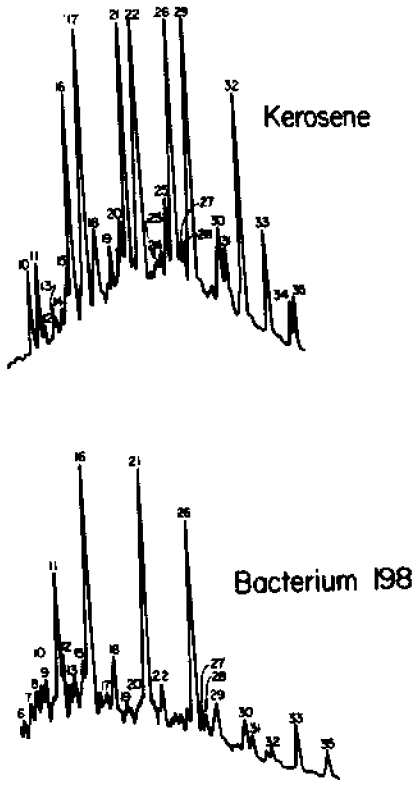

Figure 4. Gas chromatographs of remaining hydrocarbons after fermentation together with a kerosene control. Large numbers identify cultures used and small numbers identify kerosene components.

TABLE 3

Oil Oxidation in Cook Inlet Water Samples

Added crude oil mg/L	Dissolved organic carbon, mg/L 0 days	6 days	5 day population, colonies/L	Residue 12 mo, mg/L
1.9	2.6	8	1×10^9	<1
21.0	5.3	8	-	<1
48.0	9.1	15	9×10^8	10

Solubility.--Radioactivities of distilled water-equilibrated pure radioactive hydrocarbons indicated solubilities in good agreement with those of McAuliffe (10). However, solubility measurements were suspect because of particles generated from the Pyrex carboys used. These were visible in a beam of collimated light and could be eliminated by storage of water, previously passed through macromolecular separation filters, in polypropylene containers. This procedure resulted in water free of Tyndall effect. Equilibration for these studies was done with great care to avoid emulsion formation, convectively mixing the water with floating dodecane by warming one side of the polypropylene carboy. While solubility studies are continuing, present indications are that particles, such as suspended clays as well as silicates from Pyrex carboys, can absorb significant quantities of dissolved hydrocarbons from aqueous solution.

Oil Toxicity.--Three mechanisms appear to be dominant in short term oil toxicity toward microorganisms. One results from the coalescence of organisms with oil droplets on contact so that their microenvironment becomes an oil rather than the usual aqueous phase. The second is metabolic response to hydrocarbons in the C-5 to C-8 range (4). The third is interference with the process of chemotaxis (14). Many heterotrophs grow on low molecular weight hydrocarbons when supplied at low concentrations, although hydrocarbon concentrations approaching aqueous saturation inhibit growth. This is probably due to solution of phospholipid membrane components in locally high hydrocarbon concentrations collected by partitioning against the hydrophobic cell surface. Alteration in the important capacity to perform metabolite specific osmotic work would be expected. Different species might be expected to differ in their tolerance thresholds toward these solvents. We observed reduction in population of a randomly mixed continuous culture of heterotrophic organisms by additions of crude oil to the feed (unpublished data). The following experiment was designed to test oil toxicity on a specific membrane function, that of phosphate active transport into a marine occurring yeast (5). This system has been found to be exceedingly sensitive toward various naturally occurring inhibitors. Table 4 shows that saturated aqueous solutions of the lower molecular weight solvents, all constituents of crude oil, produce grossly inhibited transport rates. However, in the several experiments shown, no response was noted with crude oil additions. This would indicate that the mole fraction of solvent components in our crude samples was insufficient to develop the dissolved aqueous concentrations necessary for major membrane disorientation in this system.

Perturbation susceptibility of an algal culture to crude oil was tested by introducing oil into the continuous culture supply medium while monitoring reactor population concentrations. Population was maintained constant by adjusting the feed rate equal to the maximum growth rate of the culture (0.041 hr^{-1}). The culture was then growing at its maximum rate. The population (*Selenastrum capricornutum*) was 1.1×10^5/ml or about 0.7 mg/liter as shown in Table 5. The steady state algal culture was unchanged in population after perturbation with oil as shown. Thus, the maximum growth rate of the organisms under conditions of both light and nutrient saturation was not reduced by soluble crude oil components as they exist when the growth medium is partitioned against the many components of crude in the oil phase. Bacterial populations in the unialgal culture increased as shown. Numbers of

different heterotrophic species comprising the total population, however, decreased markedly. This was apparent from the number of colony types appearing on plates. Similar reductions in species number, but an increase in heterotrophic biomass, is the anticipated response of the microbiological community to an oil spill.

TABLE 4

Inhibition of Phosphate Transport in a Marine Yeast by Various Dissolved Hydrocarbons

Component	Flux, μM PO_4 per g cells per min	% Control
Control	13.0	100
Prudhoe Bay crude oil	13.0	100
Toluene	0.0	0
Xylene	0.3	2.3
Naphthalene	4.0	31.0
Cyclohexane	7.0	54.0
Benzene	9.0	69.0
Pentane	12.0	92.0
Dodecane	12.5	96.0

TABLE 5

Response of Algal Continuous Culture to Perturbation by Oil Added to the Culture Medium

Culture time (in hrs)	Conditions	Algae/cc	Bacteria/cc
480	oil free	1.1×10^5	--
490	oil free	1.0×10^5	--
504	oil free	1.1×10^5	5×10^4
528	oil added	1.1×10^5	--
540	oil added	1.2×10^5	--
548	oil added	1.2×10^5	12×10^4

Hydrocarbons in the oil phase were carefully omitted in these experiments. Where present as an emulsion, the probability exists of separating organisms' surfaces from aqueous contact. We noted earlier the ability of some hydrocarbon-oxidizing organisms to pass into and metabolize inside the the oil phase. The three microorganisms commonly used in our laboratory are

a hydrocarbon-oxidizing bacterium (isolate 198), a marine occurring yeast (*Rhodotorula rubra*), and a fresh water alga (*S. capricornutum*). All three organisms collect around oil droplets upon microscopic examination in accord with the observations of Ahearn (paper presented at Workshop on Microbial Degradation of Oil Pollutants, Atlanta, Ga., December, 1972). The extent of their preference for the oil phase varies greatly and is in the order listed. The bacterium is particularly oleophilic in that where an oil phase is present in a growing culture, cells are difficult to collect from the underlying medium. Oil droplets in the yeast culture are densely coated with organisms to the exclusion of nearby medium. The algae can be seen in contact with crude oil droplets when medium and oil are shaken together.

It appears that effects of oil emulsions on microbial processes would be due in part to their propensity for hydrophobic surface adhesion and concomitant alteration of the organisms' immediate external chemical environment. Microbial surfaces, normally exposed to aqueous chemistry, contact oil when present. In that such organisms are sustained by functional transport systems on their surfaces which concentrate required metabolites, part of the effects of emulsified oil on microbial metabolism might be one of physical exclusion of the aqueous medium. Local oil concentration around the organisms would then increase by a factor of several million.

DISCUSSION

The fate of oil spills is one of disruption and emulsification, dispersion and finally biodegradation. The latter is prevented only in the unlikely event of deposition in anoxic sediments. Mixing is the dominant driving force of dispersal in marine systems. Resulting particles are near natural buoyancy and remain suspended (7), probably until oxidation is complete. Cook Inlet, a large turbulent estuary with a flushing time of about a year (9), engulfs oil spills without visible or detectable effects. The extant heavy suspended sediment loads appear to be of little consequence in slick dissipation. Mechanisms for rapid dispersal and biodegradation are indigenous to Cook Inlet and Port Valdez. Port Valdez has not yet been exposed to major oil spills. An abundance of hydrocarbon-oxidizing organisms are present in Port Valdez but this site is a much more quiescent system than is Cook Inlet. One estimate of the flushing time of the former small harbor is 30 days (R. Muench, personal communication). Spills are likely to remain at least as long as in open ocean systems such as the week periods observed in the Santa Barbara (8) and Torrey Canyon (12) incidents. Arctic oil spill stability is difficult to predict at this point. Clearly the two major dissipation mechanisms, mixing and biodegradation, are potentially less effective in the arctic than at other points around the Alaskan coast.

Oil particle inoculation from indigenous populations may be sufficiently slow to allow considerable dispersal before biodegradation can occur. The rate at which particles P are inoculated to form inoculated particles P_i is given by

$$\frac{dP_i}{dt} = k \times O \qquad (1)$$

where X and O are numbers of bacteria and oil particles/cm^3, respectively. The second order rate constant can be estimated by observing bacteria-phage infection rates. We measured this collision rate in order to estimate the influence of turbulent diffusion on collision frequency (unpublished data). We chose the T-4 *Escherichia coli* bacteria-phage system. Higher stirring rates decreased the infection rate, apparently due to the rather fragile T-4 attachment system. However, this did show that turbulence had sheer effects within mμ distances from particles. Rate constants were near those predicted from diffusivities and those measured in the literature (3) of about 10^{-10} cm^3 min^{-1}. Thus, at a concentration of one bacterium and one phage-sized particle per ml, 2000 years would be required for the first collision. Considering the collision frequency of larger particles, the velocity due to Brownian motion (6) is

$$\frac{(\Delta X)}{\Delta t} = \frac{R\,T}{N_o\,6\,\pi\,z\,r} \qquad (2)$$

where ΔX is distance traveled per unit time and r is particle radius. This gives even slower particle collision frequencies for larger particles, 10^6 years for μm-sized particles and 10^3 years for μm-sized particles with a millimeter-sized target. Motility, however, greatly enhances the frequency of probable collisions to once per 600 hours if moving randomly at 100 μM per second and once per minute if oriented by chemotactic mechanisms. Cultures 197 and 198 of Table 1 appear to be motile and exhibit chemotactic response toward oxygen.

Growth cannot be accelerated in excess of maximum growth rates at the temperature of the water. Growth rates are reduced to about 10% of their 25 C values near the freezing point of water. This means nutrient saturation (adequate supply of nitrogen, phosphorus, oxygen and water) occurs at about 10% of the 25 C concentrations since nutrient diffusion is essentially unaffected by temperature. Thus, based on either the large body of literature on nutrient limitation or on collision frequency calculations, adequate nitrogen and phosphate at the cell surface are probably of the order of 10^{-7} and 10^{-8}M for nitrogen and phosphate respectively (5).

Toxic effects toward microorganisms, aside from physical smothering, appear to be difficult to document. Coating or oiling occurs with aquatic microorganisms in much the same way it does with sea birds. This has demonstrable local effects. Barsdate (2) has reported reduced primary productivity in an arctic pond subjected to a controlled oil spill during the year following oil addition. However, even very sensitive microbial transport processes appear to function normally when the organisms are not in direct contact with the oil phase and when large mole fractions of the low molecular weight components (C-4 to C-8) are avoided. Effects on chemotactic response are apparently a possibility. One probable local effect of oil addition to the marine system might likely be the enrichment of the hydrocarbon-oxidizing heterotrophic microflora with a concomitant increase in total population and decrease in the number of predominating species.

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MICROBIAL ECOLOGY AND THE PROBLEM OF PETROLEUM DEGRADATION IN CHESAPEAKE BAY

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Petroleum degradation in the marine environment is one of the many processes effected by microorganisms. Control of this process will be achieved only through an understanding of microbial ecology. Information obtained on the ecology of mercury-metabolizing bacteria in Chesapeake Bay has provided interesting comparisons with the petroleum-degrading microbial populations. Petroleum degradation studies are being done to obtain a seasonal incidence, as well as species distribution of petroleum-degrading microorganisms in Chesapeake Bay. From analysis of water and sediments collected at two stations in Chesapeake Bay it was found that the concentration of petroleum in an oil polluted site in Baltimore Harbor was *ca* five times greater than in Eastern Bay. The numbers of petroleum-degrading microorganisms, measured by direct and replica plating, in the water and sediment samples were related to the concentration of oil in each sample. Total yields of petroleum-degrading microorganisms grown on an oil substrate were greater for those organisms exposed to oil in the natural environment. Microorganisms isolated from water and sediment samples collected in Baltimore Harbor grew on substrates representative of the aliphatic, aromatic and refractory hydrocarbons. From analyses of species distribution, it was observed that a hydrocarbon-utilizing fungus, *Cladosporium resinae*, and actinomycetes were predominant among the hydrocarbon-utilizing isolates. Microbial degradation of petroleum in Chesapeake Bay appears to be mediated by the autochthonous microbial flora. The objective of work in process is to determine whether a seasonal fluctuation in the petroleum-degrading microflora occurs in the Bay.

INTRODUCTION

Many of the environmental problems facing society today are microbiological problems, in the sense that the primary level of activity is within the microbial community. Mercury and petroleum, as contaminants and/or pollutants of the environment, are metabolized by microbial species found in the natural environment. Enhancement of the activities of the autochthonous species in hastening degradation or cycling of these materials is a logical avenue of pursuit. However, without requisite knowledge of microbial systems ecology and quantitation of such parameters in the microbial populations, as well as microbial species interactions, attempts to harness "microbial power" on a large scale will not succeed.

Basic knowledge of the total viable microbial populations, population shifts with season or with introduction of a new metabolite, and of the kinds of microorganisms composing the natural microbial flora is not available currently. Problems in the acquisition of such ecological data include lack of appreciation of the principles of microbial ecology as well as the sheer magnitude of characterizing the individual species units of the microbial communities.

In our studies of the microbial ecology of Chesapeake Bay, we have attempted to characterize the aerobic heterotrophic bacterial flora of the water, sediment and animals in several Bay locations. The incidence of mercury-tolerant bacteria, for example, is seen to fluctuate on an annual basis according to season (23). The problem of isolating, identifying and classifying the microbial species of Chesapeake Bay has been approached using numerical taxonomy methodology (20) and molecular genetic taxonomy. Fatty acid profiles of microorganisms also offers promise as a rapid diagnostic tool, particularly for petroleum-degrading microorganisms.

One of the aims of our research program is to identify and enumerate the genera of petroleum-degrading microorganisms in Chesapeake Bay, in order to assess the potential of microorganisms to degrade petroleum in vitro and in vivo, as well as to examine the type of petroleum attacked (31). In the current work, the composition of oil in water and sediments in Chesapeake Bay is being analyzed and seasonal fluctuations of heterotrophic and petroleum-utilizing microorganisms are being determined.

Chesapeake Bay is the largest and most important estuary, with respect to aquatic fauna, in the mid-Atlantic coastal area (21). It provides a feeding ground for fish (13) and shellfish (29), a recreational facility for public use, and a repository for industrial and domestic waste (18). Since 90% of the pollution of water and waterways is of petroleum origin (100,000,000 tons per annum), and because construction of superports for oil tankers is being considered for Chesapeake Bay, the assessment of the microbial potential for degradation of petroleum in the Bay is important and timely. Baseline data on the natural microbial flora are necessary to ascertain the effects of oil on biological communities (9,36).

The full extent of the effects of petroleum on biological ecosystems is unclear, although petroleum persists in the natural environment (7,8,10, 11). The use of microorganisms (4,19) or mechanical devices (27) has been suggested for control of oil pollution, but rigidly controlled tests must be conducted before microbial seeding of oil polluted areas can be done on a large scale.

MATERIALS AND METHODS

Sampling.--Water samples for bacterial enumeration and chemical analyses were collected using a Niskin Sampler (24), at a depth of 1 meter from the bottom. Aseptic procedures were followed in transferring the samples from the sampler to sterile bottles prior to analysis. The top layer of sediment, i.e., the mud-water interface, was sampled using a Ponar Sampler and an Ekman Dredge (Wildlife Supply Co., Saginaw, Michigan). Immediately upon obtaining the water and sediment samples, appropriate dilutions were prepared and inoculations were performed aboard ship.

Media.--An estuarine salts solution consisting of 10.0 g NaCl, 2.3 g $MgCl_2$ and 0.3 g KCl per liter of distilled water was used to prepare the dilution blanks and for the diluent in the basal medium. The pH of the salts solution was adjusted to 7.0 for the bacterial counts and to pH 5.5 for isolation of yeasts and molds. The basal medium employed for the determination of total viable counts (TVC) consisted of: 2.0 g glucose; 5.0 g casamino acids; 1.0 g yeast extract; and 20.0 g Difco purified agar (Difco Laboratories, Detroit, Michigan) per liter of estuarine salts solution. The culture medium used for the petroleum-degrading microorganisms ("motor oil medium") was composed of: 10.0 g NaCl; 0.5 g $MgSO_4$; 1.0 g NH_4NO_3; 1 drop $FeCl_3$ (25 g/ml); 20.0 g Difco purified agar; 10.0 g oil powder; and a specific ratio of KH_2PO_4 and K_2HPO_4 per liter of distilled water (see below). The oil powder was prepared by adding 10 g silica gel to 10 g motor oil (non-detergent) dissolved in 30 ml of diethyl ether. The ether was evaporated and the resulting powder was added to the basal medium prior to autoclaving. From a sterile solution of KH_2PO_4 or K_2HPO_4 (10 g per 100 ml distilled water), a specific ratio of each salt was added, after autoclaving, to obtain the desired pH, e.g., pH 5.5 for molds and yeasts. To obtain pH 5.5, 9.4 ml of KH_2PO_4 solution and 0.6 ml K_2HPO_4 solution were added per liter. The pH of the basal medium used to isolate bacteria was 7.0, obtained by addition of 3.0 ml of KH_2PO_4 solution and 7.0 ml K_2HPO_4 solution per liter. Antibiotics were also added to the basal and to the petroleum media after autoclaving, viz., fungizone (10 µg/ml) to the bacterial culture medium and streptomycin (50 µg/ml) for isolation of fungi. To estimate growth of microorganisms on refractory petroleum compounds, the compound, e.g., naphthalene or perylene, was substituted for the motor oil.

The liquid culture medium employed consisted of either sterile Chesapeake Bay water or salts solution (10.0 g NaCl, 0.5 g $MgSO_4$, 1.0 g NH_4NO_3 per liter of distilled water and the appropriate ratio of KH_2PO_4:K_2HPO_4 to achieve the desired pH). Antibiotics were added as described above for preparation of solid media. Liquid hydrocarbons and the motor oil were sterilized separately by filtration. The salts solution was sterilized by autoclaving and was subsequently overlaid with 1% (v/v) hydrocarbon or oil. Solid hydrocarbons (1% w/v) were added to the media prior to autoclaving. With some cultures a mixture of hydrocarbons or "model petroleum," consisting of 1% of the total volume, was used as the carbon source.

Isolation and Identification of Microorganisms.--Heterotrophic bacteria, molds and yeasts were isolated by direct plating of appropriate dilutions of water and sediment onto basal medium, adjusted to the required pH and supplemented with the appropriate antibiotics. Petroleum-utilizing microorganisms and those capable of degrading refractory hydrocarbons were also isolated by direct plating of water and sediments onto media containing oil or the refractory compounds. As above, the medium was supplemented with antibiotics and adjusted to the appropriate pH. The laboratory aboard the Johns Hopkins University vessel, *R/V Ridgely Warfield*, was used for microbiological analysis of the samples immediately upon retrieval of the samples. For studies not performed aboard ship, samples were processed upon return to the shore laboratory. The time interval between samples and analysis was < 6 hr. Samples that were processed ashore were kept at 10 C in transit. Plate counts for heterotrophs were tabulated after 7 days incubation at 20±2 C. Plate counts for organisms utilizing oil or refractory compounds were made after 21 days incubation at 20±2 C.

When replica plating was done, heterotrophic bacteria were transferred to hydrocarbon or motor oil media and the plates were examined after incubation for 21 days at 20±2 C.

Isolated colonies of microorganisms demonstrating degradation of oil or of refractory compounds were transferred to basal agar slants and were stored at 20 C. Petroleum-degrading microorganisms were identified following procedures described elsewhere (Walker and Colwell, in preparation).

Culture Systems.--To measure microbial growth on oil, hydrocarbons or model petroleum in 250-ml flasks containing 100 ml salts solution or sterile Chesapeake Bay water were inoculated with sediment or water samples. The inoculated flasks were placed on a reciprocal shaker set at 60 1¼-in strokes per min. Unless stated otherwise, cultures used in this part of the study were incubated at 20±2 C. Growth was followed by visual inspection, turbidimetry ("Spectronic 20," Bausch and Lomb, Rochester, New York), or direct plate counts. Dry weight determinations were obtained by filtering the spent culture medium through pre-weighed Whatman No. 1 filter paper, washing the cells retained on the filter with hexane to remove residual oil, and drying the filters to constant weight at 100 C.

Chemical Analysis.--Water samples (2l) were extracted twice with 800 ml benzene. The sediment samples (100 g) were extracted twice with 200 ml benzene or chloroform. Extracts were concentrated by rotary evaporation at 35 C. Methods used in determination of the chemical composition of the petroleum present in the samples will be described elsewhere (Walker and Colwell, in preparation).

Physical and Chemical Parameters.--To correlate the microbial populations in the samples tested with environmental conditions observed, selected physical and chemical measurements were made at the time of sampling. Dissolved oxygen (DO) and temperature were measured using a YSI Model 51 A DO meter (Yellow Springs Instrument Co., Yellow Springs, Ohio) specifically designed for use with estuarine and seawater samples. Percent saturation of DO was calculated using the nomogram prepared by Dawson (14). Transparency was measured using a standard Sekki Disc. The pH of water and sediment was taken using a Corning portable pH meter and salinity was recorded with a Model RS53 Beckman salinometer (Beckman Instruments, Cedar Grove, New Jersey).

Chemicals.--All of the solvents employed were of reagent grade, spectro-quality for GLC analysis. Hydrocarbons purchased from Chemical Samples Company (Columbus, Ohio) were 99+% pure, according to manufacturer's specification.

RESULTS AND DISCUSSION

Two stations in Chesapeake Bay were sampled during the preliminary phase of this study. One of the sites was Eastern Bay, a relatively unpolluted and commercially productive region, found to contain stable bacterial populations in the water and sediment (20). Baltimore Harbor, the other site sampled, is relatively oil-polluted (Table 1). Twenty-five percent of the traffic passing into Baltimore Harbor involves oil tankers (22). Although

this may be relatively low compared with the ports of Providence, Rhode Island (91%), and San Francisco, California (84%), it nevertheless presents a problem because of tank washings and accidental spills. These can account for the higher percent of petroleum extracted from water and sediments compared with samples from Eastern Bay (Table 1).

TABLE 1

Yield of Petroleum from Chesapeake Bay Water and Sediments[a]

Source	Percent petroleum extracted with	
	Chloroform	Benzene
Eastern Bay Water	0.002%	0.002%
Eastern Bay Sediment	0.1 %	0.1 %
Colgate Creek Water	0.05 %	0.08 %
Colgate Creek Sediment	0.4 %	0.5 %

[a]Yield expressed as (w/v) for water and (w/w) for sediment.

The aerobic heterotrophic flora of the water and sediment of Eastern Bay has been studied on a seasonal basis since 1964 (20). Many species of several genera are present, in a finely-tuned ecological balance. Eastern Bay is commercially productive for shellfish while the Baltimore area of Chesapeake Bay no longer provides shellfish harvests. Also, Marumsco Bar, on the Eastern Shore of the Bay, is not commercially productive for shellfish. Here, the bacterial flora is dominated by *Vibrio* spp., including some pathogenic species (20). Hence, an imbalance in the microbial flora has occurred in the Marumsco Bar region of the Bay.

Concentrations of mercury-resistant bacteria are higher in Baltimore Harbor than in Eastern Bay (23). A particularly noteworthy aspect of these populations is that there is a seasonal incidence of mercury-resistant bacteria, with large populations being observed in the spring. Also, *Pseudomonas* spp. clearly predominate the mercury-resistant populations of bacteria in Baltimore Harbor.

Water and sediment samples were collected from an area south of Parsons Island in Eastern Bay and from Colgate Creek in Baltimore Harbor. Environmental parameters measured at the time of sampling are listed in Table 2.

The higher concentration of oil in water and sediment from Colgate Creek (Table 1) correlated with higher numbers of petroleum-degrading bacteria (Table 3). Appropriate controls were run to ensure that the oil medium did not support growth of non-petroleum utilizing microorganisms. A higher concentration of petroleum degraders were associated with the oil-polluted site (Table 3). Results from replica plating (Table 4) confirmed the results of direct plating (Table 3) for petroleum-degrading bacteria. Dodecane was used

as a carbon source to determine whether toxic components in the oil inhibited microbial growth. Slightly higher numbers of petroleum utilizers were found on the oil medium, compared with dodecane. This observation most likely is a reflection of the large number of different hydrocarbons in oil capable of supporting bacterial growth. Although percent recovery was higher on replica plating than on direct plating, the trends with respect to percent of petroleum utilizers from each source were the same.

TABLE 2

Physical and Chemical Parameters from Chesapeake Bay Sampling Stations

Parameter	Station and date			
	Eastern Bay		Colgate Creek	
	10/6/72	1/28/72	10/5/72	11/15/72
Air Temperature (C)	22.0	10.0	21.0	8.5
Dissolved Oxygen (ppm)	8.3	11.4	8.1	10.3
Water Temperature (C)	19.5	7.0	20.5	11.0
Percent Saturation	90.0	95.0	90.0	93.0
pH	7.9	6.8	7.3	7.0
Salinity (0/00)	7.1	12.1	9.2	9.5
Depth (m)	9.0	9.0	10.7	10.7
Transparency (m)	1.6	1.6	0.9	1.0

TABLE 3

Total Aerobic Bacterial Populations from Eastern Bay and Colgate Creek Samples in October 1972

Bacteria	Total Viable Bacterial Populations from			
	Eastern Bay Water	Eastern Bay Sediment	Colgate Creek Water	Colgate Creek Sediment
Heterotrophs	1.5×10^3	7.9×10^4	1.5×10^4	1.1×10^5
Petroleum Degraders	5.0×10^1	3.0×10^3	6.0×10^2	9.0×10^3
% Petroleum Degraders	3.3	3.8	4.0	8.2

TABLE 4

Percent Heterotrophic Bacteria Utilizing Oil or Hydrocarbon after Replica Plating[a]

Source of heterotrophs	Motor oil medium	Dodecane medium
Eastern Bay Water	26.7	18.3
Eastern Bay Sediment	48.3	46.5
Colgate Creek Water	40.0	40.0
Colgate Creek Sediment	75.0	55.0

[a]Results are expressed as the average of two separate replica platings. Replicates did not differ by more than 10%.

Further sampling demonstrated that greater numbers of petroleum-degrading bacteria and fungi were present at the Colgate Creek site, when the data for Eastern Bay were compared (Table 5). About 84.6 to 98.2% of the bacterial population and 66.6% of the fungal population utilized naphthalene as a carbon source, thus confirming the previous report that naphthalene supports growth of these organisms (31).

Oil-degrading microorganisms have been isolated from a number of harbor water and sediment samples, as well as from bays throughout the world (35). However, studies of the ecology and seasonal distribution of petroleum-degrading microorganisms are lacking. A routine monthly sampling is now underway in our laboratory to evaluate the results obtained to date with respect to the complete annual cycle of petroleum-degrading microorganisms in Chesapeake Bay. Furthermore, very few reports describing the ecology and interaction of petroleum-degrading bacteria, yeasts and fungi, one of the goals of this research, are available (16).

Results in Table 6 describe the growth of different microbial populations after 7 days incubation in a motor oil medium. The growth yields of microorganisms isolated from each of the two sites tested, and harvested by filtration, correlated with the percent petroleum degraders recovered on initial examination (Tables 3 and 4). The decrease in pH of the culture medium inoculated with petroleum-degrading microorganisms from Colgate Creek may be due to acid production or the replacement of the NH_4^+ ion of NH_4NO_3 with H^+, yielding HNO_3. Chemical analysis of the oil degradation products is in progress.

Growth of microbial populations from Colgate Creek on hydrocarbons is described in Figure 1. Samples from the area were used, since it is highly probable that those microbial populations would be exposed to such compounds in situ. All hydrocarbon mixtures supported growth and higher yields were obtained with cultures inoculated with sediment (Fig. 1). However, benzene, alone, did not support growth and naphthalene supported only slight growth (31). The former observation confirmed results of an earlier report that naphthalene is generally more susceptible to degradation than benzene (34). For dodecane plus benzene, the following may occur: i) dodecane degradation, ii) cooxidation (25) or iii) stimulation of dodecane oxidation by benzene (33).

TABLE 5

Total Aerobic Microbial Populations Isolated from Eastern Bay and Colgate Creek Samples in November 1972

Microorganism	Eastern Bay Water	Eastern Bay Sediment	Colgate Creek Water	Colgate Creek Sediment
Bacteria				
Heterotrophs	5.0×10^1	7.0×10^3	5.6×10^5	1.3×10^6
Petroleum Degraders	5.0×10^0	4.5×10^3	5.0×10^4	3.0×10^5
% Petroleum Degraders	12.5	64.2	8.9	23.0
Naphthalene Degraders			5.5×10^5	1.1×10^6
% Naphthalene Degraders			98.2	84.6
Fungi				
Heterotrophs	5.0×10^0	2.0×10^1	1.5×10^2	3.0×10^3
Petroleum Degraders	5.0×10^0	5.0×10^0	1.0×10^1	1.0×10^3
% Petroleum Degraders	100	25	6.6	33.3
Naphthalene Degraders			1.0×10	1.0×10^3
% Naphthalene Degraders			66.6	66.6

TABLE 6

Growth Yields and pH of Cultures Grown on Motor Oil in Salts Solution

Source of inoculum	pH[a]	Dry weight (mg/ml)[b]
Eastern Bay Water	6.0	3.4
Eastern Bay Sediment	6.0	7.3
Colgate Creek Water	5.5	7.1
Colgate Creek Sediment	4.8	10.2

[a] Initial pH = 6.2.

[b] After seven days incubation.

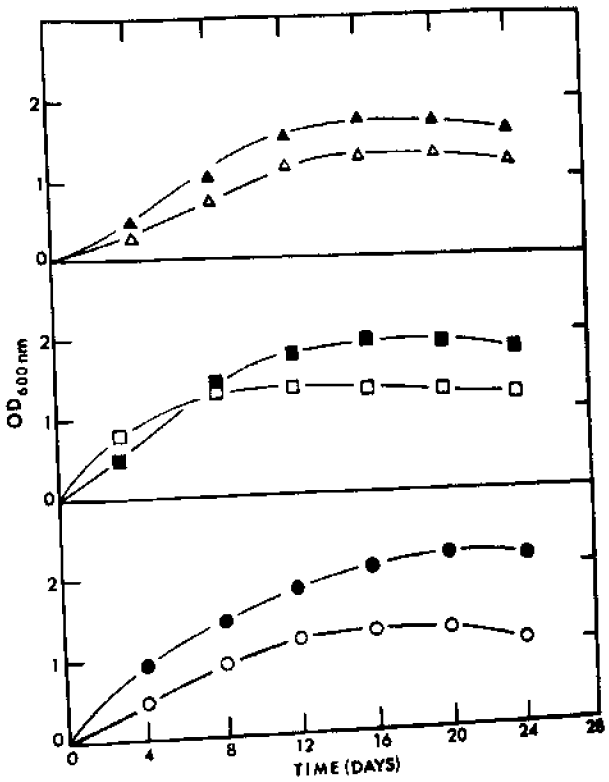

Figure 1. Growth of Microorganisms on Dodecane (circles), Dodecane plus Benzene (8:2, v/v; squares) and Dodecane, Benzene and Naphthalene (7:2;1, v/v/w; triangles). Cultures were inoculated with Colgate Creek sediment (closed figures) and Colgate Creek water (open figures).

Growth curves of microbial populations on oil are shown in Figures 2 and 3. Higher yields were recorded for cultures in a medium containing an estuarine salts solution than in media with Chesapeake Bay water. Since the latter was not supplemented with nutrient, phosphate and nitrate may have been limiting. Diauxie was observed for Eastern Bay water and sediment populations grown in media prepared with the salts solution. Maximum growth yields of cultures from Colgate Creek samples was *ca* 1 x 10^2 organisms per ml for all cultures tested. Morphology of the organisms growing on oil has been followed using scanning electron microscopy (30) and have been observed to be located preferentially on the surfaces of the oil.

Studies show that seawater supports significant growth of petroleum-degrading microorganisms only when supplemented with nitrogen and/or phosphorus (6,16). In this work, the synthetic estuarine salts solution and Chesapeake Bay water were intended to approximate the optimum (in vitro) and "natural" (in situ) conditions for petroleum degradation, respectively. Our results suggest that microorganisms are capable of growth on petroleum under "natural" conditions.

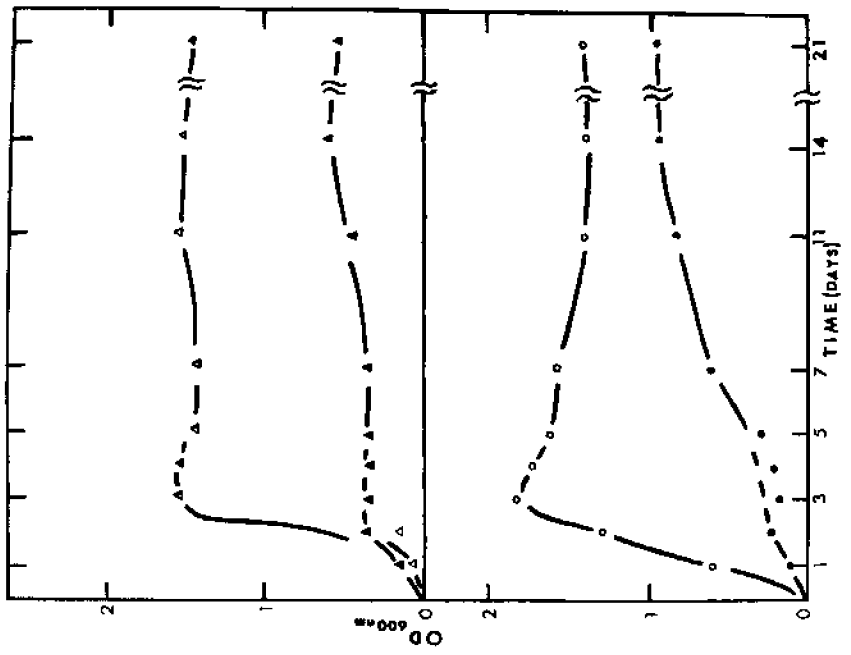

Figure 3. Growth Curve of Microorganisms from Colgate Creek water (triangles) and sediment (circles) cultured on motor oil medium made up with salts solution (open symbols) and Chesapeake Bay water (closed symbols).

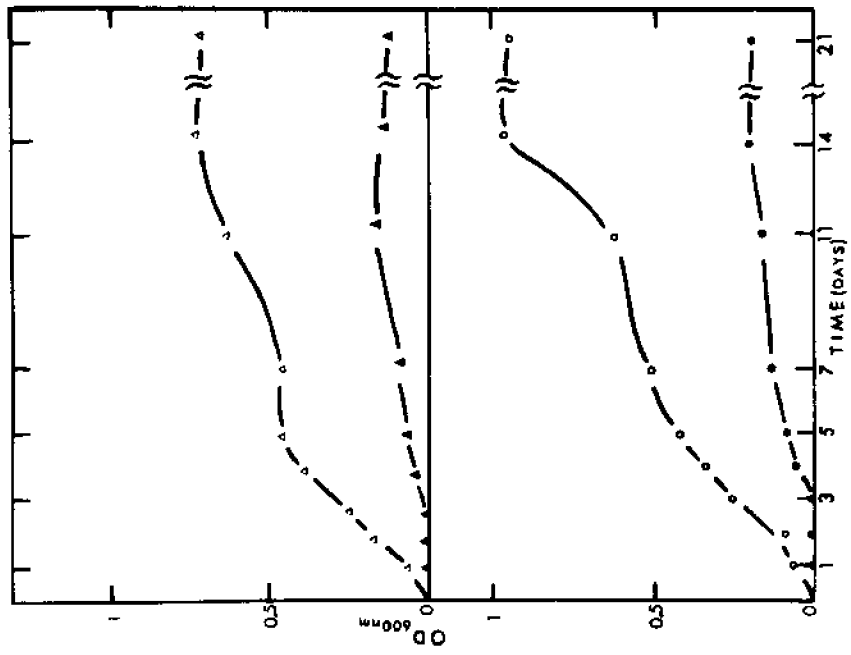

Figure 2. Growth Curve of Microorganisms from Eastern Bay water (triangles) and sediment (circles) cultured on motor oil medium made up with salts solution (open symbols) and Chesapeake Bay water (closed symbols).

Cladosporium resinae may well be one of the most important petroleum-degrading fungi in the natural environment (32). Five species of *Cladosporium* were isolated from Colgate Creek and Eastern Bay water and sediment samples, as well as three *Penicillium* spp., an *Alternaria* sp., a *Trichoderma* sp. and several unidentified fungi. Preliminary results suggest that several of the *Cladosporium* species are *C. resinae*. The isolates of yeasts and bacteria have not yet been identified. Data obtained suggest that between fifty and seventy percent of the bacterial isolates are actinomycetes.

In summary, the results indicate that a petroleum-degrading microbial population exists in the sediment of Colgate Creek. Clearly, microbial populations capable of degrading petroleum are present in Chesapeake Bay. Through extended studies of the microbial ecology of Chesapeak Bay, it is hoped that the distribution and significance of these microorganisms can be confirmed.

ACKNOWLEDGEMENTS

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ALKANE DEGRADATION IN BEACH SANDS

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An estimation of the rate of *n*-alkane degradation was made in beach sands from Coal Oil Point, California. Bulk sand samples from a beach chronically polluted with oil from natural seeps were extracted and analyzed gas chromatographically and gravimetrically for hydrocarbon content. Calculations indicate that a maximum degradation of 0.32 g of *n*-alkanes occurs per day for 100 m^2 of beach. The oil input on the day of sampling was estimated to be 5 g/100 m^2/day. From these figures it is seen that the daily maximum oxidation, including bacterial degradation, evaporation and autoxidation accounts for only 6% removal of the alkane content of the sand.

In recent years, considerable interest has developed to "seed" oil slicks with bacteria as a final clean-up after mechanical removal procedures. The present study was undertaken to determine the rate at which *n*-alkanes are degraded under natural conditions, and to provide base-line data for comparison of seeding experiments. There are few accessible locations where steady oil seepage occurs naturally, which have some mechanism for the estimation of the time period over which the observed hydrocarbon degradation takes place. One area that fulfills these criteria is Coal Oil Point, California. The beach at Coal Oil Point receives a daily input of crude oil from a number of naturally occurring offshore seeps (1) and experiences periods of active sand transport, i.e., away from the area between December and May, and deposition between June and November (3,5). By measuring normal alkane concentrations in the sands at increasing depths, the rate at which these hydrocarbons were oxidized could be estimated. In this instance, the rate of sand deposition, which is known for the area, served as a "clock" to give an appropriate time scale.

The oil seeps at Coal Oil Point account for an average release of 50-70 barrels of crude oil per day (1). ZoBell (6) reported that an average of 21.5 lbs of oily material per 500 ft^2 were deposited each year on the beach. In the current communication, preliminary estimates of the rate of *n*-alkane degradation in beach sands are noted under natural conditions. However, for a closer approximation, a more exhaustive sampling and analytical program over a longer time span is needed.

MATERIALS AND METHODS

Bulk sand samples, collected as 6" x 8" x 1" blocks, were taken at four beach locations designated A-D (Table 1). For hydrocarbon analysis, the sand was allowed to air dry and one microliter of ^{14}C-hexadecane was added to the samples for the determination of extraction efficiency. Extraction was accomplished with three separate washings using 250 ml of chloroform-methanol (3:1) each time. The sand was vigorously shaken in the solvent for 10 min in an acid-washed 1 L Erlenmeyer flask followed by a 24 hr soaking period for the first extraction and 1 hr for the remaining extractions. The three washings were combined and concentrated in a rotary evaporator. The chloroform-methanol remaining in the concentrated sample was evaporated under pre-purified nitrogen, the residue redissolved in hexane and quantitatively applied to a silica gel column as described by Meinshein and Kenny (4) for separation of the aliphatic fraction. A 21 cm glass column packed with Woelm (activity grade 1) activated silica gel was used. After elution, excess hexane was evaporated under nitrogen and the alkane fraction maintained in about 0.5 ml hexane for analysis in a temperature programmed gas chromatograph (Bendix Chroma-lab 2100 with a 5% Dexsil 300 on 60/80 mesh Chromosorb G column treated with dimethyldichlorosilane). Normal alkanes were identified by comparison of retention times with standards and their relative abundance determined as the percentage of *n*-alkane peak area to total chromatograph area.

TABLE 1

Locations of Bulk Sand Samples Collected at Coal Oil Point, California

Sample No.	Distance from water (feet)	Interval depth below sand surface (inches)
A-2	0	0-1
B-2	7.5[a]	0-1
B-4	7.5	2-3
B-6	7.5	4-5
C-2	17.5	0-1
C-4	17.5	4-5
C-6	17.5	10-11
D-2	37.5	0-1
D-4	37.5	2-3
D-6	37.5	6-7
D-8	37.5	12-13
D-10	37.5	18-19

[a]Occasional high waves came within 3 ft of Station B at the time of sample collection.

The hydrocarbon remaining after gas chromatography was transferred to dried and tared aluminum pans, the hexane evaporated and the remaining material weighed to 10^{-1} mg on a Mettler H160 balance. The weights were corrected for extraction efficiency and for the materials removed for other analytical purposes.

Sand samples for bacterial enumeration were collected using a flamed spatula at the same depths as the sand for hydrocarbon analysis. One gram of this sand was shaken vigorously in a 250 ml bottle containing 100 ml of sea-water. Dilutions were made of the suspension and surface-spread on an agar medium composed of $(NH_4)_2SO_4$, 10 ppt; K_2HPO_4, 50 ppm; yeast extract, 0.01%; agar, 1.5% in sea water. The hydrocarbon source consisted of Fisher paraffin oil and commercial kerosene mixed 1:1 and added to the cover of the inverted Petri plate.

RESULTS

Twelve bulk sand samples were collected at various depths from the four Coal Oil Point locations. The distance from the water line to the sampling locations at the time of collection and the depths through the sand column are shown in Table 1. The results of our analyses are presented in Table 2 and indicate that the stations nearest the water (locations A and B) have greater hydrocarbon burdens than the stations further back on the beach (locations C and D). The concentration of *n*-alkanes was found to decrease with depth within the sand column at the point of more recent oil deposition (Table 2, col. 5, Station B). At a depth of 5 inches at Station B, approximately 85% of the *n*-alkane component was degraded. Station C shows a more uniform hydrocarbon burden with increasing depth. The relatively high value of C-4 was due to the presence of tar veins which were observed at the time of collection. Below a depth of 7 inches at Station D, a large increase in alkane content was found. This increase occurred where the sand showed a change in texture and was first observed to be flooded. Johnston (2), demonstrated that in the interstitial water of beach sands, the oxygen may be depleted in the presence of crude oil. Under such conditions hydrocarbon degradation will not occur, and the enrichment of alkanes observed in samples D-8 and D-10 may be the result of the development of anoxic conditions.

The total bacterial population was uniform for all samples. In view of the alkane degradation at Station B relative to Stations C or D, we would have expected to see greater variation in the magnitude of the bacteral numbers between these locations. While the population of hydrocarbon-utilizing bacteria is large, the consistency of the data indicates a better method is needed to assess the activity of these organisms.

DISCUSSION

Sand samples were collected on a beach at the water line (Station A); at a point beyond the wave reach but within the normal intertidal zone (Station B); at a point beyond the intertidal zone as estimated by the position of deposited kelp (Station C); and at a point on the backbeach near the cliffs (Station D). Station A was receiving oil input at the time of sampling and will serve as an index of the amount of oil reaching the beach on

the particular sampling day. Station B had received oil the day of sampling and a comparison of the changes in *n*-alkane concentration at this location to the concentration observed at Station A can be used to estimate the percentage of the daily oil input degraded.

TABLE 2

Hydrocarbon Analysis for Bulk Sand Samples from Coal Oil Point, California, and Bacterial Populations Determined through Direct Enumeration

1 Sample No.	2 Weight of Sand Extracted (gm)	3[a] Adjusted Weight Hexane Eluate (gm)	4 % Normal Hydrocarbons of total Chromatograph of Hexane Eluate	5 Micrograms Normal Hydrocarbons/100 gm Sand	6 Hydrocarbon-Oxidizing Bacteria (cells/ml)
A-2	429	0.0012	100	300	1.8×10^6
B-2	290	0.0018	39	234	1.5×10^6
B-4	580	0.0024	19	76	1.1×10^6
B-6	446	0.0010	16	32	2.3×10^6
C-2	529	0.0019	16	64	3.4×10^6
C-4	474	0.0022	18	90	1.4×10^5
C-6	474	0.0021	13	52	6.0×10^5
D-2	425	0.0011	13	39	9.6×10^5
D-4	486	0.0012	13	26	1.1×10^7
D-6	525	0.0027	7	35	9.7×10^6
D-8	491	0.0037	68	345	2.7×10^6
D-10	471	0.0020	49	196	3.3×10^6

[a]Corrected for extraction efficiency.

Referring to the data for Station B in Table 2, 158 µg of *n*-alkanes were removed between samples B-2 and B-4. Each of these samples had an average thickness of 2.5 cm when collected, and it is assumed that the observed hydrocarbon loss occurred linearly between the centers of the sample blocks, i.e., between 1.25 and 6.25 cm, or over a net distance of 5 cm. Sedimentation rates determined by Kolpack (3) indicate that approximately 1 meter of sand is deposited on the beach at Coal Oil Point during the six-month period from June to November. From this datum a deposition rate of 0.6 cm of sand per day is calculated. At this rate the 5 cm interval between samples B-2 and B-4 corresponds to a time interval of 8.3 days during which 158 µg of *n*-alkanes were removed per 100 g of sand. The degradation rate of the alkanes can also be expressed on an areal basis. Each sand sample had an average weight of 500 g and represented an area of 300 cm^2 when collected. A 100 g aliquot would then correspond to a beach area of 60 cm^2 and the 158 µg of *n*-alkanes degraded per 100 g of sand would correspond to the hydrocarbon loss of the oil deposited on 60 cm^2 of beach over a time interval of 8.3 days. Assuming a linear degradation rate, this reduces to a daily degradation rate

of 0.32 g of *n*-alkanes per 100 m^2 of beach.

The input of oil for Station A was calculated to be 5.0 g *n*-alkanes per 100 m^2 per day. The degradation rate calculated for the first 7 cm of sand at Station B accounts for only 6% removal of the daily input of *n*-alkanes. Thus the oil input at Coal Oil Point exceeds the degradation rate. Other factors such as sand and oil transport away from the beach would appear to be more important than the immediate action of microbes in the removal of beach oil at this location.

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HYDROCARBONS OF SUSPECTED POLLUTANT ORIGIN IN AQUATIC ORGANISMS OF SAN FRANCISCO BAY: METHODS AND PRELIMINARY RESULTS

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Large amounts of petroleum-derived hydrocarbons enter the waters of San Francisco Bay each year, yet little is known concerning the fate and effects of pollutant hydrocarbons on estuarine organisms and, indirectly, on the human environment of the Bay area. Investigation into fate and effects of petroleum-derived hydrocarbons was initiated by analyzing the hydrocarbon content of selected Bay animals to determine if this served as an indicator of chronic hydrocarbon pollution in Bay food chains. Initial results have been obtained using solvent extracts of sponge, mussel, and crab tissues. Closely related animals were obtained from relatively clean waters along the northern California coast to provide "unpolluted" control extracts for comparison. To date, it has been shown that Bay organisms have a significantly higher content of hydrocarbons than the clean water organisms'. Methods tested included gas chromatography (GC), thin layer chromatography (TLC), fluorescence spectrometry, and high pressure liquid chromatography (HPLC). Comparative results between the different methods suggest thin layer chromatography to be the method of choice.

INTRODUCTION

The group of embayments and river deltas of the San Francisco Bay region form one of California's few estuarine ecosystems. The "Bay" is presently about 450 square miles in area with approximately 270 miles of shoreline. The current population of the Bay area is nearly 5 million; the domestic and industrial waste water effluent is approaching 800 million gallons per day. The ports of San Francisco, Oakland, and other cities host a brisk marine trade, and naval facilities at several Bay locations are moderately active. Commercial shipyards as well as other industries are located around the Bay margins; these are often built on reclaimed land, the development of which has reduced the surface area of the Bay by about 30% in the last 100 years. Among the most prominent of the industries on the Bay shore and on its tributaries are the oil refineries of several companies which receive tanker-transported crude oil. Additionally, the Bay is the site of popular boating and sport-fishing, with small boat marinas widely scattered around its shores. All of this suggests a high level of usage placed on the environment regarding waste disposal, transportation, and recreation. The natural functions of the

Bay ecosystem, in relation to the uses placed on it by man, have been largely ignored.

Hydrocarbon Inputs.--In 1971, oil pollution of the Bay received nationwide recognition when a tanker collision released approximately 800,000 gallons of crude oil. According to U.S. Coast Guard estimates, an additional 90,000 gallons of oil were spilled in the remainder of 1971 in miscellaneous instances. These totals, although unusually high that year, represented only the acute hydrocarbon inputs. Chronic low-level inputs at a number of loci around the Bay margin are of serious import in assays of environmental stress. The Bay Area Regional Water Quality Control Board estimates that 10-100 tons of oil and grease probably enter the Bay ecosystem each day. The fate and effects of hydrocarbons reaching the Bay are largely unknown.

A simplified model of hydrocarbon flow through the Bay ecosystem includes compartments representing input and output from the system with intermediate storages in water, organisms and sediments. Storrs et al. (18) reported widely ranging amounts (10^2-10^4 mg/kg) of hexane-soluble residues in Bay sediment samples. Hydrocarbons may be lost from the system by tidal and river flushing and microbial decomposition. Cobet and Guard (9) have begun to demonstrate the potential of microorganisms in decomposition of residual oil fractions in Bay beaches. To date, the literature has dealt primarily with studies of overt oil spills in which the amount and composition of the spilled oil was known (2,4). However, subtle effects on organisms of crude oil constituents at extremely high dilution have been recently described (14).

Although hydrocarbon pollution may exist as microscopic and dissolved particulate matter, we chose to assay macroorganisms first, hypothesizing that these might serve as integrative indicators of dispersed hydrocarbon pollution. Filter-feeding and scavenging organisms accumulate these fat-soluble pollutants through normal feeding activities and/or direct tissue uptake from the water. Of long-term significance was the problem of determining if sufficient petroleum residues were concentrated in the food chain sufficient to interfere with physiological functions of the organisms and their natural waste processing abilities. Of immediate interest was the need to determine the type and quantities of pollutant hydrocarbons in certain organisms such as crabs and fishes used for human consumption.

Since very little work has been done on the chronic buildup of petroleum residues in organisms in the natural environment, an important problem in the current research was the selection of methods of hydrocarbon analysis. We attempted to simplify existing research methods for development of a straightforward technique to be used to provide survey information useful in public health and environmental evaluations.

Since a range of naturally occurring hydrocarbons exist (5,6,16) it was necessary to make relative discrimination between naturally-occurring and pollutant hydrocarbons. Methodology was needed which would remove oleophilic compounds, other than hydrocarbons, from extracts prior to hydrocarbon determination. Since petroleum-derived hydrocarbons are extremely ubiquitous, it was of great importance to prevent contamination in our determinations by exogenous hydrocarbons, i.e., paint fumes, lubricants, etc.

METHODS AND MATERIALS

Organism Sampling.--Organisms were collected from San Francisco Bay and selected points on the California coastline based on the degree of exposure of the water to waste hydrocarbons. The species collected, location, and feeding mechanisms of the animals are summarized in Table 1. Samples were collected by diving, and placed in clean containers during collection to avoid extraneous contamination. Samples were returned to the laboratory, and tissues of interest were obtained by dissection with care taken to prevent exposure of internal tissues to contaminants residing on animal surfaces. Samples were placed in solvent-washed containers and deep frozen until dissected for extraction procedures. All dissections were carried out using instruments rinsed in acetone and chloroform to avoid contamination by hydrocarbons in lubricants.

TABLE 1

Organisms Sampled for Content of Petroleum Hydrocarbon Residues

Common name	Species	Trophic position	Location of collection[a]	Habitat[b]
Sponges	*Leucosolenia* sp	suspension feeders	SFB, NSC pier	st, p
	Ophlatispongia sp?		SFB, NSC pier	st, b
	Tethya aurantia		FR	st, b
Mussels	*Mytilus edulis*	suspension feeders	SFB various	it,st,p
	Mytilus californianus		FR, VB	it, b
Edible crabs	*Cancer antennarius*	scavenging, multivorous	SFB, NSC pier (note 1)	st, b
	Cancer magister			
Starfish	*Pisaster brevispinus*	carnivorous on sessile and slow moving species	SFB, BP	st, b
	Pisaster ochraceus		SFB*, FR	st, p
Sea slugs	*Diaulula sandiegensis*	benthic grazers	SFB, NSC pier	st, b
	Anisodoris nobilis		FR	
Sea squirts	*Ciona intestinalis***	suspension feeders	SFB**, NSC pier	p
Leopard shark	*Triakis semifasciatus***	carnivorous motile species	SFB**	n

[a] SFB - San Francisco Bay
NSC - Naval Supply Center (Oakland)
FR - Fort Ross
BP - Berkeley Pier
VB - Vandenberg Air Force Base (location of natural oil seepage)

*Presidio, near Golden Gate.
**To be collected at as yet undetermined clean water stations.

[b] st - subtidal
it - intertidal
p - piling
b - bottom
n - free swimming

Note 1: Obtained from fishermen returning to Fishermans Wharf, San Francisco.

Tissue Extraction and Column Chromatography.--Extractions of tissue samples were carried out following the procedure of Clark and Finley (8). This method includes two 16 hour Soxhlet extractions, one with 50:50 methanol-benzene, and the other employing pure benzene. All solvents were double-distilled (Vigreaux column) to avoid contaminant concentration in subsequent procedures.

Tissue extracts were concentrated by rotary evaporation, dried using solvent washed anhydrous magnesium sulfate, and taken up in either pentane or hexane. Extracts were then transferred to weighing bottles and evaporated to constant tare weight.

Our column chromatography procedures employed a ratio of 1 g organic extract to 100 g column adsorbent. For GC procedures below, extracts in pentane were passed first through 300 g of alumina, washed in double-distilled solvent and activated at 240 C. The column was maintained at 5 C to prevent degassing of the pentane. Approximately 120 ml pentane was used to elute the entire hydrocarbon fraction. This eluate was concentrated by rotary evaporation and passed through a column containing 50 g washed, activated silica gel; UV detection at 260 nm was used to separate the initial fraction containing alkanes and olefins from the subsequent fraction containing aromatics. Approximately 100 ml of solvent was needed to elute the alkane-olefin fraction. For TLC procedures, lipids (polar, fat-soluble compounds including fatty acids, fats, waxes, phospholipids, steroids), pigments and other extraneous biochemicals were removed from the crude extracts by passage through 50 ml silica gel columns at room temperature using hexane as solvent. Approximately 350 ml hexane was used to obtain the colorless hydrocarbon fraction. Excess solvent was removed from the samples by rotary evaporation prior to analysis, with samples adjusted to standard volumes of 2 ml or less.

Gas Chromatography (GC).--Some analyses were performed using the gas chromatographic analytical procedures of Blumer et al. (2) as detailed by Clark (8). This method was designed to obtain alkane profiles of extracts for interorganism comparisons (7). A Hewlett-Packard Model 700 gas chromatograph, with a 4 m column packed with OV-101, was used. The sensitivity of this method was approximately 1 ppm for each alkane in the sample.

A sample of shark oil (*Triakis semifasciatus*) was analyzed by this method during development of methodology and attempts were made to analyze sponge and mussel extracts.

Thin Layer Chromatography (TLC).--A TLC method modified after Kirchner et al. (13) was tested as a possible determinant for patterns of hydrocarbon content between Bay and clean water organisms. The method separates extracts into alkane, alkene and aromatic fractions at a sensitivity of about 1 µg of individual compounds in the analyzed sample.

Glass plates (5 x 20 cm) were coated to a nominal thickness of 250 µ with silica gel "G" using an adjustable applicator (Brinkmann Instrument Co.). The plates were dried, activated at 110 C for at least 30 min, cleared with a single vertical migration of chloroform, and then air dried. Known amounts of prepared tissue extracts (5-10 µl) were spotted on the plates 1-1.5 cm apart and 2 cm above the edge of the plate. Two samples were usually spotted on a single plate, leaving an unused blank position for background correction. Sample spots were migrated vertically for 10 cm using hexane as solvent. The plates were air dired, observed for fluorescent spots with UV light, and then sprayed with concentrated sulfuric acid containing a trace of selenium. Compounds on the plates were finally visualized by charring at 200 C. Figure 1 illustrates a conceptual TLC plate with loci of model compounds.

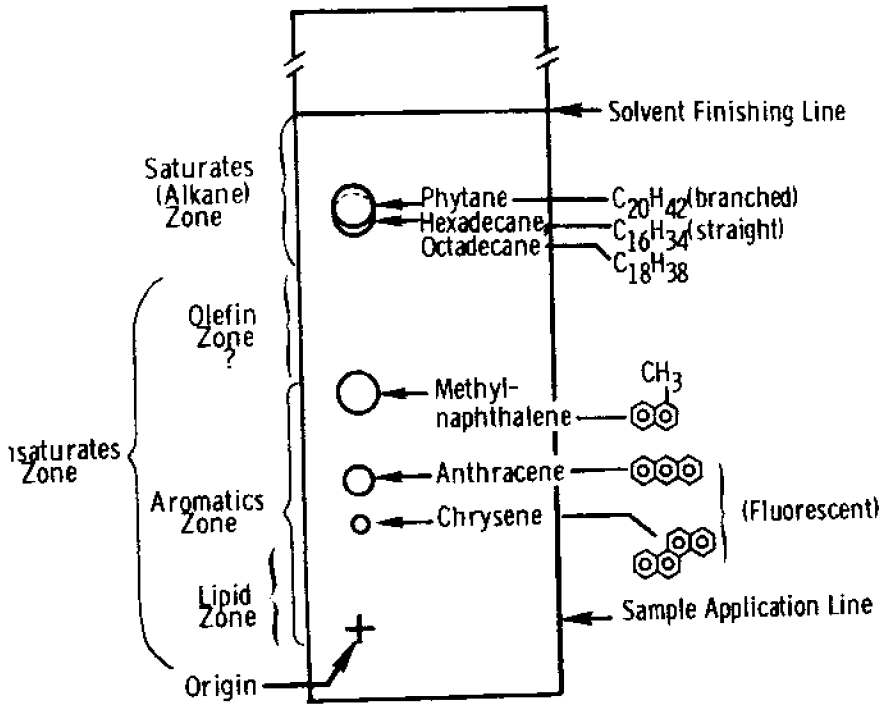

Figure 1. Thin layer chromatography plate (x1) showing idealized positions of several marker compounds and approximate zonal locations of hydrocarbon types migrated with hexane.

Hydrocarbon spots were scanned densitometrically using a Model 520 Photovolt Co. photometer and a Model 52-C transmission density unit in preliminary attempts at quantitative estimation of recovered residues. A corresponding blank portion of each plate was scanned to correct for absorption due to the silica gel. The resulting differential optical density was graphically related to distance along the TLC plate to provide a quantitative estimate of each visualized hydrocarbon type. Control determinations established that linear (*n*-octadecane) and branched (phytane) aliphatic hydrocarbons (R_f 7-10) gave the same area vs weight response curve when visualized under identical conditions (Fig. 2A). A general response curve for aromatic hydrocarbons was not established. Hydrocarbon spots in the "aromatic" region of the TLC plate (R_f 2-6.5) are tentatively expressed in terms of anthracene, for which a standard curve has been established (Fig. 2B).

A measure of the amount and type, saturate (alkane) and unsaturate (aromatic), of hydrocarbon in the sample was obtained by calculating the areas under the peaks on the graph and comparing these with the above area vs weight curves.

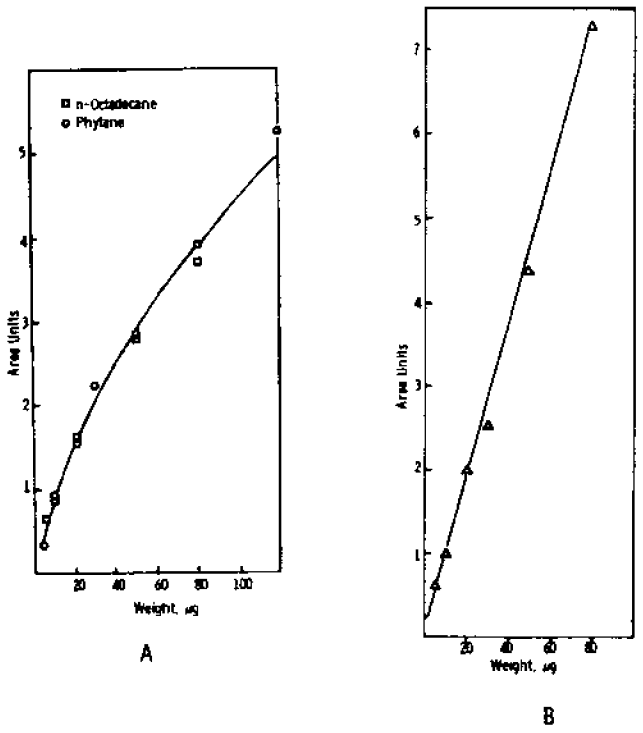

Figure 2. TLC Response Curves: A. *n*-octadecane and phytane; B. anthracene.

Spectrofluorometry.--Some spectrofluorometric analyses of oil slick samples and extracts of organisms from areas of chronic oil slick occurrence have been made using methods similar to Zitko (20) and Thruston and Knight (19). Organism extracts and authentic residual fuel oils diluted in CCl_4 have been analyzed with a Model SF-1 Baird Atomic spectrofluorometer (mercury vapor lamp) scanning the emission spectrum between 300 and 550 nm using excitation at 363 nm. Emission spectra have been compared between new and aged slicks, and mussels from polluted and unpolluted regions. This method may differentiate polluted and unpolluted organism extracts by showing higher proportions of fluorescent aromatic compounds in polluted extracts. Zitko (20) has obtained a sensitivity of 1 µg/ml for "bunker fuel" in extracts of organisms affected by an acute oil spill of known composition.

High Pressure Liquid Chromatography (HPLC).--Analysis of tissue extracts by HPLC was performed using a 2.1 by 150 mm column packed with "Vydac Reverse Phase" (Chromatronix, Inc., Berkeley), with a Chromatronix Model 230 UV detector at 245 and 280 nm. The samples were eluted with a gradient of initially 26% aqueous methanol programmed at 4% per min to 100% methanol at a flow rate of 6 ml per min. However, analysis time is greatly reduced compared to gravity liquid chromatography and quantitative determinations are more readily obtained than in thin layer chromatography.

The application of high pressure liquid chromatography to the analysis

of pollutants in tissue extracts is based on the hypothesis that aromatic hydrocarbons may be used as indicators of pollution since tissue extracts should not contain any of these compounds. With properly selected conditions, it is possible to separate the aromatic hydrocarbons from the alkanes, lipids and other polar compounds. The aromatic hydrocarbons can be detected readily by a UV-detector.

RESULTS AND DISCUSSION

Extraction and Sample Preparation.--Soxhlet extractions of animal tissues were performed with mixed success. Several times, the mucoid nature of the tissue mass clogged the Soxhlet cups and it was necessary to change cups and add glass beads to the samples. Additionally, the methanol caused precipitation of proteins which hindered Soxhlet extractions. It became apparent that extraction procedures would require modification in continuing work to shorten times between sample collection and instrumental analysis as well as to reduce the risk of contamination due to added sample manipulation. One method investigated was the grinding of a weighed amount of sample in a mortar with solvent (CCl_4) and clean sand, using several changes of solvent until a colorless extract was obtained. For example, a 7 g sample of mussel gonad using 100 g sand required four 10 ml solvent washes. Water and extracted tissue remained in a bolus with the sand, and only a minor $MgSO_4$ drying step was required. Continuing experimentation will determine the efficiency of this recovery technique using known amounts of hydrocarbons added to the tissue sample.

Column Chromatography.--Difficulty was experienced in reproducing results of Clark and Finley (8) involving separation of alkane, olefinic, and aromatic compounds on an alumina-silica gel column using pentane as eluent. Aromatic compounds were detected in early phases of column elution where only the alkanes were expected to appear. By collecting only the earliest fractions which showed no UV absorbance at 260 nm, the alkane fractions were often too small to analyze in our instrumental system which is sensitive only to about 1 ppm of any given alkane in the injected sample. Although greater instrumental sensitivity would alleviate this problem, it would only provide qualitative results regarding the alkanes. We assume that important amounts of the alkanes were left behind in the aromatic-containing fraction. These would thus escape quantitative detection. Aromatics mixed with the alkanes would produce peaks on the chromatograms leading to erroneous qualitative estimations.

Gas Chromatography.--The GC method has been used by a number of investigators to follow hydrocarbon residues which were taken up by organisms as the result of acute oil spills of known origin (2,20). Samples of the authentic spill were available for comparison with organism extracts, and relative changes could be observed over given time periods. In our experimentation, the GC method provided a high differentiation between the alkane hydrocarbons. A shark liver extract from San Francisco Bay suggested the presence of approximately 1 ppm each of about 30 compounds in the chromatograms where C_{13} through C_{37} alkanes were expected to appear. Mixed results were obtained with sponge and mussel extracts, leaving questions of repro-

ducibility of results. Difficulties experienced in column chromatography (above) in obtaining a purely alkane fraction and the high risk of contamination in the procedures (8) coupled with the fact that alkanes are most rapidly biodegraded (12) have suggested that more emphasis be placed on techniques such as thin layer chromatography. The GC methods are not readily adaptable for survey work, and it should be noted that previous workers using this technique have been limited to single species or sample types (1,2,10,11).

Thin Layer Chromatography.--Preliminary results obtained with the TLC method suggest its use in differentiating between "polluted" and "unpolluted" organism extracts. Figures 3, 4, 5 and 6 illustrate TLC plates run to compare clean water organism extracts with those from areas of suspected chronic hydrocarbon input. In all cases the extracts of "polluted" organisms show comparatively greater amounts of hydrocarbons than appear in extracts of unpolluted organisms. Significantly, no aromatic compounds were observed in extracts of unpolluted organisms. Preliminary quantitative estimates obtained by densitometric scanning of the TLC plates are listed in Table 2. Control studies are in progress to determine percentage recovery of known amounts of hydrocarbon content in these organisms. The mussel (unpolluted *Mytilus californianus*) data compare favorably with those of Lee et al. (15) who reported approximately 1 mg naturally-occurring hydrocarbons per mussel (this includes straight and branched chain hydrocarbons from C_{16} to C_{26}, with no aromatic hydrocarbons occurring). Order of magnitude agreement is also noted in comparing our results with those of Ehrhart (10) in his studies of polluted oysters.

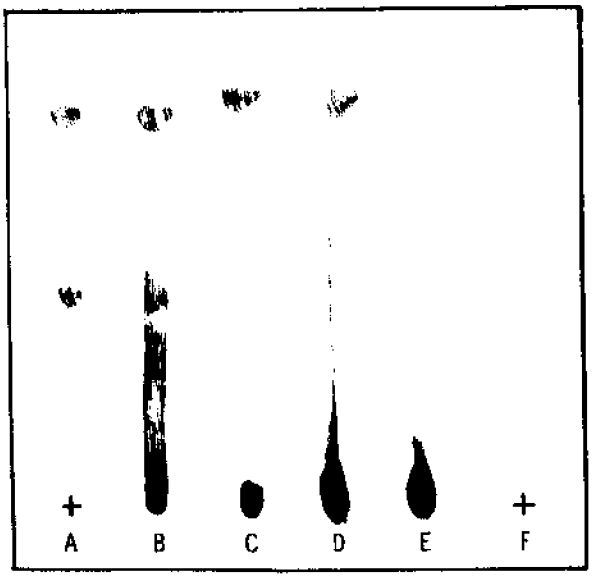

Figure 3. Thin layer chromatography plate to show positions of compounds as follows: A - 5 mg hexadecane (top), 5 mg methyl naphthalene (middle); B - 50 mg Chevron Bunker fuel; C, D - lipid extracts of *Mytilus edulis* gonad from the Naval Supply Center pilings, Oakland; E - lipid extract from *Mytilus californianus* gonad, Fort Ross area.

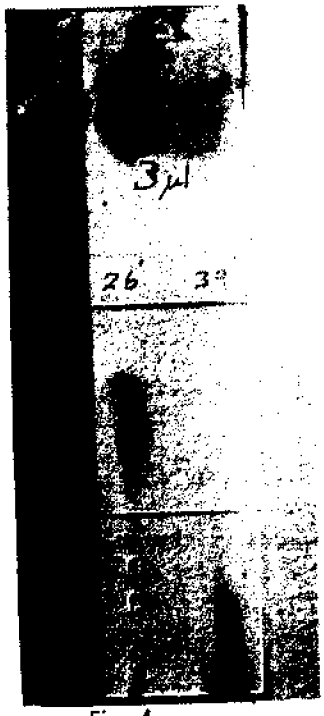

Fig. 4.

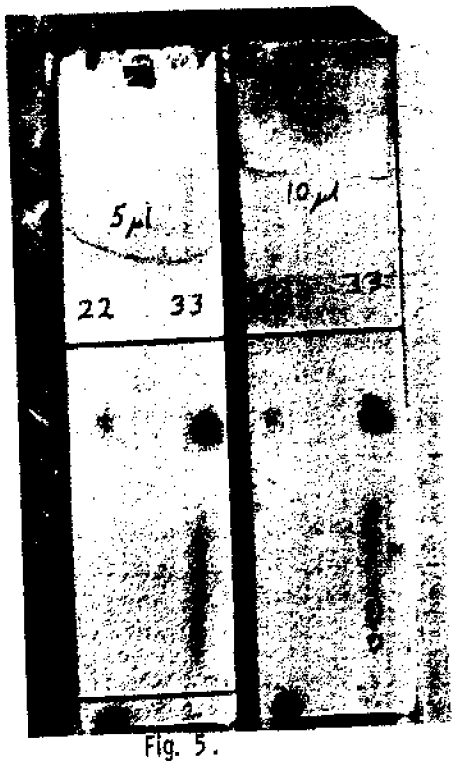

Fig. 5.

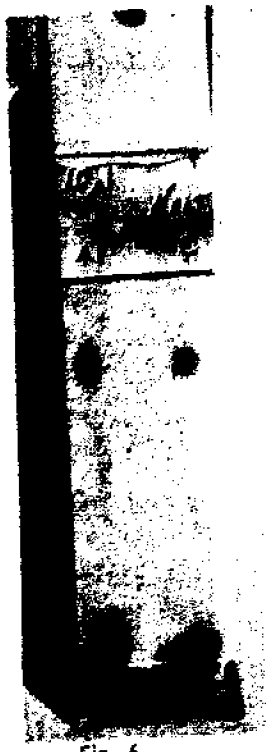

Fig. 6.

Figures 4-6. Thin layer chromatography plates with organism extracts developed in hexane and visualized by use of concentrated H_2SO_4 and heating. Dotted circles on chromatograms represent locations of fluorescing compounds visualized using UV light. Amounts marked at top of plates represent amounts spotted from 0.5-1.0 ml concentrates of 350 ml column eluates (see Table 2). "Neat C" in Fig. 6 represents an unrelated sample.

Fig. 4. Sample 26, a San Francisco Bay sponge extract, versus sample 39, a clean water sponge from the Fort Ross area.

Fig. 5. Sample 33, a San Francisco Bay mussel extract, versus sample 22, a clean water mussel from the Fort Ross area.

Fig. 6. Sample 41, a San Francisco Bay crab extract.

See Table 1 for organism identifications.

TABLE 2

Preliminary Densitometric Results from Thin Layer Chromatographic Determinations of Hydrocarbon Residues in Selected Marine Organisms

Sample No.	Species	Tissue Sampled	Tissue wet Weight g	Extract Weight g	Hydrocarbons % of Total Extract		Approx. total Hydrocarbons in Wet tissue sample ppm
					Paraffinics	Aromatics[b]	
Sponges							
26	*Ophlatispongia* sp	entire	75.9	0.610	0.704	1.310	158
39	*Tethya aurantia*	entire	95.0	1.526	<0.004	<0.004	<1
Mussels							
33	*Mytilus edulis*	(5) entire	46.4	1.375	1.545	1.183	850
32	*Mytilus californianus*	(3) entire	136.1	1.336	0.385	<0.02	<38
Crabs							
41	*Cancer antennarius*	(6) gonadal and digestive gland	45	3.505	1.332	0.351	106

[a] See Table 1. [b] Calculated as anthracene (see Fig. 2B).

The basis for the TLC quantitative procedure rests on the following assumptions:

1) That, in general, the two main types of hydrocarbon, saturates and unsaturates (olefins and aromatics), consistently appear in well-defined separate regions (Fig. 1) of the thin layer chromatogram. While a further division between olefins and aromatics (Fig. 1) may very well be possible, this is probably of little practical importance since the presence of olefins in aged oil slicks is very unlikely. The separation may become somewhat blurred in certain extreme cases, e.g., aromatics highly alkylated with long carbon chains.

2) That the degree of intensity of charring in the H_2SO_4 visualization procedure varies in a regular manner with amount of hydrocarbons. This is amply confirmed by the curves for 2-octadecane/phytane and anthracene (Figs. 2A and 2B).

3) That the charring behavior is similar for a given type of hydrocarbon. This appears to be true for alkanes, as suggested by the curves in Fig. 2A. A similar situation has not yet been established for aromatic hydrocarbons. Accordingly, the figures calculated for aromatic content are provisionally expressed as anthracene for which an acceptable weight vs optical density curve has been obtained. It should be noted that there is a lower limit, in terms of hydrocarbon molecular weight, which can be detected and measured by this procedure as is common in most TLC procedures. This lower (volatility) limit has not been established for hydrocarbons but probably occurs in the C_8-C_{10} range, e.g., toluene and xylene could not be detected. In the present instance, the effect of this limitation is minimal since the weathered oil spills responsible for chronic pollution will generally have already lost the

bulk of their volatile components.

Although confidence intervals for quantitative accuracy of the procedure have not yet been established, its simplicity and speed make it an attractive method for surveying a wide variety of samples.

Spectrofluorometry.--Spectrofluorometry of the polluted and unpolluted *Mytilus* samples analyzed by TLC (Fig. 5) showed differences in pattern and intensity of fluorescence emission. This (Figure 7) suggests differences in content of fluorescent substances, probably polynuclear aromatics, which may have been taken up as pollutants. Fluorescence spectra of oily material collected from the water surface at the NSC pier are shown in Figure 8. This may indicate that over a period of a few days fluorescent compounds in the slick were broken down by microbiological action; nevertheless, they were lost from the slick. A remarkable comparison was obtained between the fluorescence spectrum of the "incoming oil slick" (Fig. 8) and the total lipid extract of Mussel "A" (Fig. 7) collected from a nearby piling. It must be emphasized that the slick material (Fig. 8) is representative of chronic oil pollution seen almost daily at the collecting site. Origin of the slick material is unknown, and could be anywhere in the Bay north of the Naval Supply Center, as prevailing winds, tidal flow, and as yet to be defined circulation patterns tend to collect Bay surface detritus at the NSC wharf.

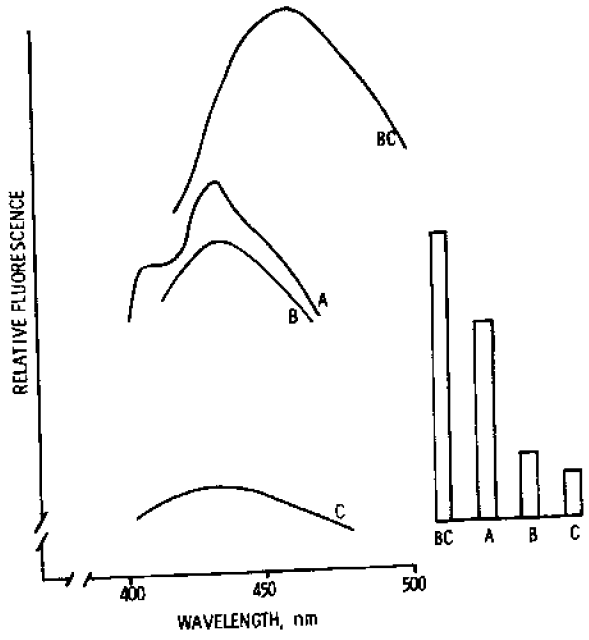

Figure 7. Fluorescence spectra of CCl_4 extracts of *Mytilus edulis* (A,B)*, *Mytilus californianus* (C)* and a residual fuel (BC). Histogram compares relative peak heights when samples are calculated to standard concentrations.
*See Table 1 for organism identifications.

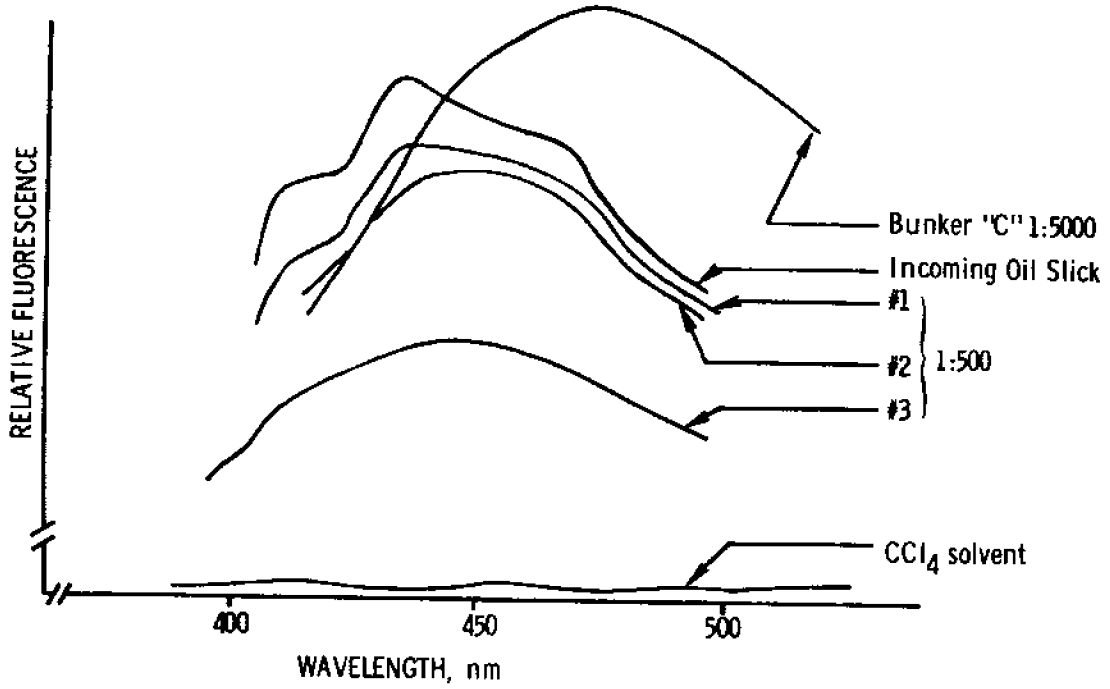

Figure 8. Fluorescence spectra of material obtained from a slick collected at the NSC pier 29 Sept. 1972 (#1), 28 Sept. 1972 (#2), and 26 Sept. 1972 (#3). Spectra of incoming oil slick and of a residual fuel oil (Bunker "C") are included for comparison.

The spectrofluorometric technique is an interesting qualitative method in these studies, although of little quantitative value. Zitko (20) was able to analyze the hydrocarbon content of oil-polluted organisms after an oil spill primarily because the type of spilled oil was known and could be used as a reference standard. We have included the spectrum of a bunker fuel (Standard Oil of California) since this is a type which is a potential source of oil pollution in the Bay. Known petroleum products undergo various changes upon entering seawater, thus the use of oily matter from chronically-occurring slicks may be a useful background standard for oil in tissues of piling organisms. Further experimentation is needed to obtain meaningful quantitative data using this method.

High Pressure Liquid Chromatography.--This method offers the same possibilities for separating complex mixtures into constituent classes of compounds, i.e., saturated aliphatic hydrocarbons, aromatic hydrocarbons and lipids, as gravity liquid chromatography or thin layer chromatography. However, analysis time is greatly reduced compared to gravity liquid chromatography and no pre-separation is required as for the thin layer chromatography procedure. The application of high pressure liquid chromatography to the analysis of pollutants in tissue extracts is based on the hypothesis that aromatic hydrocarbons may be used as indicators of pollution since tissues of marine organisms contain only very small amounts of biogenic aromatic hydro-

carbons. With properly selected conditions it is possible to separate the aromatic hydrocarbons from the alkanes, lipids and other polar compounds. The aromatic hydrocarbons can be detected readily by a UV detector.

The HPLC chromatogram (at 254 nm) of tissue extracts of polluted and unpolluted mussels is presented in Figure 9. The initial large peak results from the lipids which are eluted most rapidly in reversed phase chromatography. Subsequent peaks have retention times corresponding to aromatics and alkylated aromatics. For example, 2-methylnaphthalene has a retention time of 9 min. The alkanes are not detected by a UV detector.

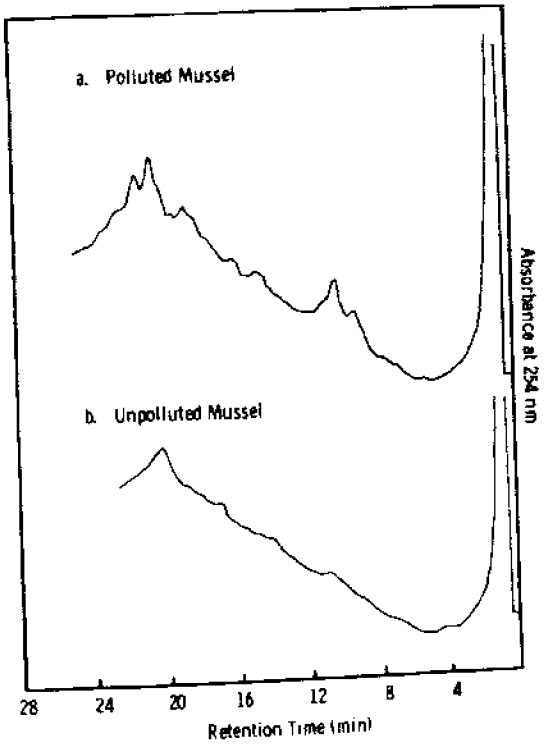

Figure 9. Comparisons of high pressure liquid chromatograms of *Mytilus californianus* collected at clean water area near Fort Ross (a), and near Vandenberg Air Force Base (b), in a region of chronic natural petroleum seepage.

The tissue extract from the oiled mussel contains several aromatic compounds which are not detected in the clean water mussel extract. These samples are of particular interest as this is the only comparison between two organisms of the same species (*Mytilus californianus*). Other comparisons were made at the genus level or higher.

CONCLUSIONS

Early results suggest measurable differences between the hydrocarbon content of animals from chronically-polluted waters and that of their counterparts from clean waters. Although we have as yet no basis for general conclusions from data obtained to date, the first results bear out the hypothesis that such differences would indeed be found. Continued findings of this type in the general survey of organisms listed in Table 1 will better elucidate the extent of this type of tissue contamination. The experimental design and methods employed in TLC determinations strongly suggest that the hydrocarbons recovered are, in fact, pollutant in origin rather than naturally occurring. This further suggests that continued development of these techniques may result in a widely applicable standard method in oil pollution research.

The tentative numbers for hydrocarbon content reported in Table 2 are significant for comparative purposes, as originally intended in using the TLC method. As research progresses, the densitometric method should provide increasingly valid results for absolute estimates of fractions. Using the values for alkane hydrocarbon content of the *Mytilus edulis* sample in Table 2 (1.545% of "total extract" or approximately 20 mg), with the dry weight of the five animals approximately 25% of the wet weight (approximately 2.3 g per mussel), the total amount of hydrocarbon per dry g of polluted mussel was about 1.7 mg. This compared favorably with the reported value of 1.0 mg heptadecane per dry gram in mussels incubated in 6.2 mg heptadecane per liter seawater for 48 hours (15).

Assuming a firm basis is established showing an unusually large hydrocarbon burden in Bay organisms, further steps in the research will be to determine: (a) its effects on the physiology, population dynamics, and ecosystems functions of the animals involved, and (b) the public health and other human-oriented aspects of the problem. In the former category, large hydrocarbon burdens may explain the unusually sparse distribution of mussels (*Mytilus edulis*) on inner Bay pilings and also the absence of starfish (*Pisaster brevispinus*) as noticed during our collecting activities. In the latter category, large numbers of crabs (*Cancer antennarius*) (see Fig. 6) are taken from the Bay for human consumption by Sport fishermen. Obviously, it is of public health interest to determine the types and toxicities of the hydrocarbons occurring in these animals.

The "polluted" animals which have been analyzed were apparently in healthy condition although no behavioral or physiological measurements were conducted. Thus, the question of physiological effect of hydrocarbon pollutants is unanswered. More important, perhaps, is the fact that these organisms indeed survived to adulthood and thus acted as indicators of pollutant buildup. Specific experiments suggested for future research include transplanting of "clean" individuals into polluted water areas with determination of time of buildup of pollutants in the tissues. Conversely, "polluted" organisms should be transplanted to clean waters and rates of elimiantion of hydrocarbon pollutants from tissues determined. In this manner, coupled with information on uptake rates and discharge rates from literature (15), a steady state model may be constructed. Such a model could be used to approximate concentrations of hydrocarbon pollutants in the water, and analyze the dynamics of their transfer through animal populations in the Bay.

Of ultimate interest, however, is the total community response to chronic hydrocarbon input. This includes information on partitioning of the hydrocarbons among different members of a benthic community and symbiotic interactions between macro- and microorganisms in processing the hydrocarbons. Other points of interest include effects of chronic petroleum inputs on community metabolism, and quantitative and qualitative information on the fate of biologically intractable hydrocarbon residues. This type of experimentation is highly adapted to the use of microcosm techniques.

ACKNOWLEDGEMENTS

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THE IMPACT OF OIL ON MARSHLAND MICROBIAL ECOSYSTEMS

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The vast productivity of wetland regions along the Louisiana coast, and their proximity to oil-producing sites, necessitates a more comprehensive understanding of the significance of alterations in the microbial community concurrent with oil intrusion and massive depositions of petroleum effluents. Investigations are concerned with examination of such effects on the microbial ecology and the basic food web of the *Spartina* marshland ecosystem.

INTRODUCTION

The real and potential toxicity of pollutant oils for freshwater, brackish and marine invertebrates and vertebrates has been variously documented after oil spills, and is the subject of an increasing number of communications. Certainly, the significance of the problem has been well emphasized. In contrast, there are comparatively few studies on the effects of oil pollutants on inshore plant-dominated communities and especially their complex microbial ecosystems. Publicized oil spills have occurred mostly in coastal areas, characterized by cliffs and pocket beaches. The effects of a major oil spill in a broad shallow salt-marsh estuary, such as that along the Louisiana coast, are largely speculative.

Normally, estuarine energy flow systems are able to absorb fluxes induced by environmental changes; however, impact of stress factors of significant magnitude, or within a restricted time frame, i.e., a sudden oil spill, can deleteriously alter the balance of the system. The extent of alteration is largely unknown, but indications are that under certain conditions, it can lead to irreversible changes in the habitat. Thus, pertinent information is needed on the biotic and abiotic factors that control normal and induced microbial degradative processes and the effect of these activities on the food web kinetics of the estuary.

A major portion of our current investigations has been concerned with *Spartina alterniflora*-dominated marshlands along the southeastern Louisiana coast (4,5,12,13,14,15,16,18). These wetlands, comprising a range of ecologically diverse areas, from fresh water to marine, are among the most productive ecosystems known. The significance of such regions to estuarine and marine food webs cannot be underestimated. In Louisiana alone, seven million of the

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state's 28 million acres consist of coastal marshes, swamps and estuaries. Over two million acres of estuaries are considered important habitat areas for fish and wildlife and comprise vital nursery grounds for shrimp and a range of other economically valuable species.

Our overall program, portions of which are summarized here, involve analyses of the biodegradation of *Spartina*, including detrital-microbial interrelationships and the overall role of bacteria, molds and yeasts in transfer of energy-rich substrates into subsequent levels of the marine food web.

RESULTS

Baseline studies, described elsewhere (4,15,18), have demonstrated the unique mycological characteristics of the Barataria Bay (Fig. 1) marsh. Significant concentration of yeasts, notably sporogenous taxa, exceeding 90,000 viable cells/cm^3 sediment are not uncommon within the oxidized portion of the *Spartina* rhizosphere. Densities of this magnitude are comparable to those found in a productive meadow, and in general are indicative of a region of high productivity and considerable organic deposition. One of the predominant species, *P. spartinae* (4), is characterized by a relatively strong utilization of the cellulose breakdown product, cellobiose. In addition to its presence in rhizosphere sediments, *P. spartinae* occurs in high concentrations *within* the fluid and cavities of the plant culm and on the stem surface. Evidence indicates that the inner culm is a virtual microcosm of biological activity. *Kluyveromyces drosophilarum*, a species with a repressed cellobiase (β-glucosidase) predominates in the uppermost portion of the plant rhizosphere. Although species of *Pichia* and *Kluyveromyces* have been isolated occasionally from marine environments, these marsh collections comprise a heretofore unreported concentration of the genera in terms of total cell biomass. In many instances, species of the two sporogenous genera constituted *over 70%* of the total yeast population isolated. Evidence indicates that *P. spartinae* plays an important role in turnover of plant carbohydrates in the carbon cycle of the marsh.

In general, the principal yeast species of the *Spartina* community show discrete distribution patterns. In addition to the yeast population, the stems and sheath areas of the plant, particularly in the decaying portions, support populations of cellulolytic fungi of the genera *Leptosphaeria* and *Lulworthia*. The fungal biota appears essential for optimal mineralization and conversion of the plant substrates. The role of fungi in *Spartina* transformation has been discussed elsewhere (12). Molds colonizing external plant surfaces differ from those isolated from within the culm. Fungal attack is correlated with seasonal development and subsequent decomposition of the plant. Mutualistic associations of β-glucosidase-active yeasts and molds on *Spartina* breakdown have been suggested (16).

As noted in Table 1, exposure of marsh areas to controlled additions of oil significantly alters the composition of the yeast community. Soluble oil fractions and volatile components (6) may influence this pattern. Shifts in the yeast population toward an asexual hydrocarbonoclastic flora, not unlike that recovered in the area of the Gulf of Mexico oil spill, have been documented. Cellulose agar prepared with water exposed to crude oils fails

to support significant cellulase activity by the cellulolytic fungus *Trichoderma virde*. Previous studies (3) indicated that the cellobiose-utilizing species, *P. spartinae*, was inhibited by vapors of certain aromatic hydrocarbons. Such phenomena could serve to retard cellulose decomposition and the production of a microbial food source for herbivores. Biodegradation of cellulose and chitin, as well as other complex polymeric substrates, by bacteria, yeasts and molds is fundamental to energy input into the marsh system.

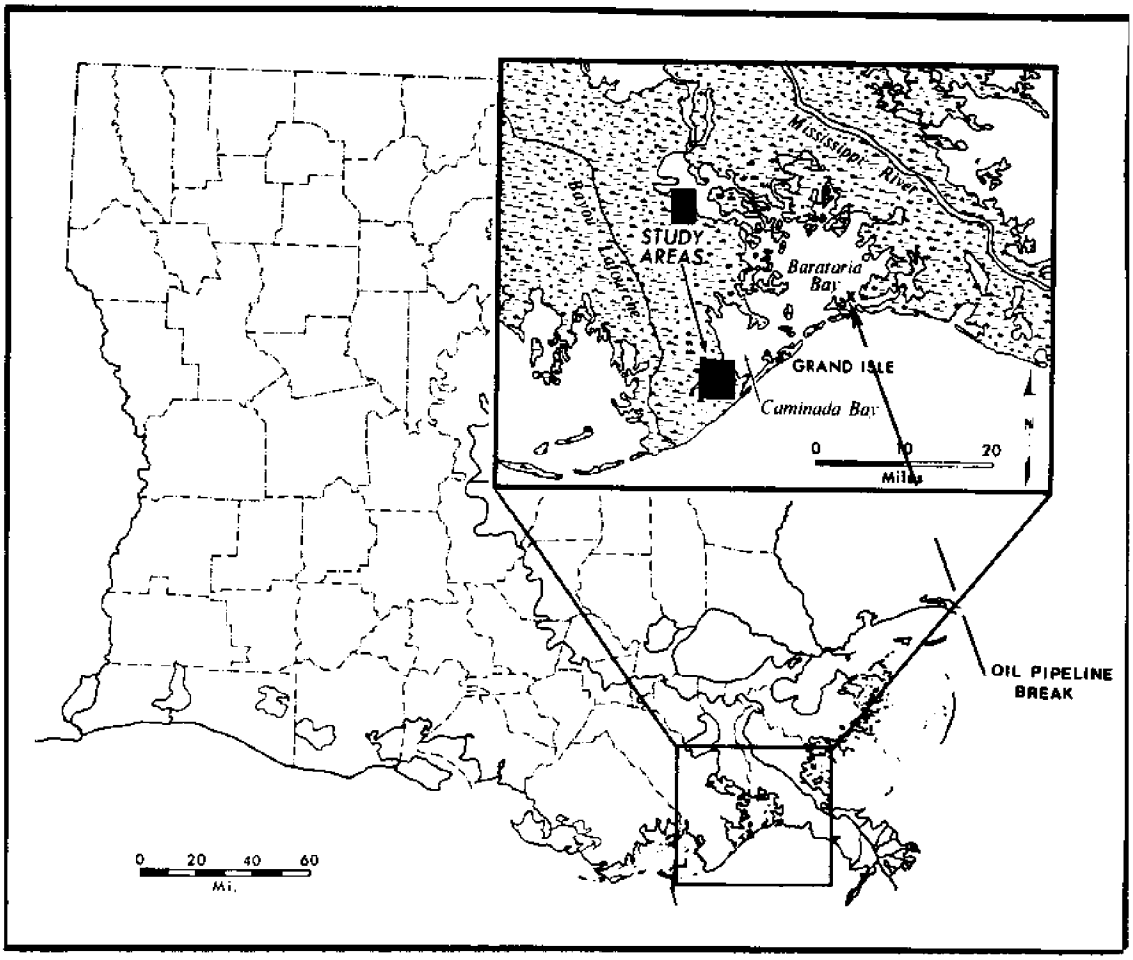

Figure 1. Map of Louisiana Coastal Region with Insert of Detailed Barataria Bay Study Areas

The response of *Pichia ohmeri*, *Trichosporon* sp and *Rhodotorula* (including *Rhodosporidium* sp) to oil enrichment is specially noteworthy. *Pichia ohmeri*, generally not isolated from the marshlands prior to introduction of oil, is physiologically similar to *Candida guilliermondii*, an established hydrocarbonoclastic species. Isolates of *P. ohmeri* grew more rapidly on crude oil than did any of the other marshland yeasts tested. Selected

representatives of *Pichia* and *Trichosporon* demonstrated emulsification capabilities, while certain of the *Trichosporon* strains were oleophilic, eventually filling the interior of the oil globules and exhibiting a filamentous growth consisting of short bulbous hyphae. The *Trichosporon* isolates, although relatively slow-growing, utilized a wide spectrum of hydrocarbons including aromatics.

TABLE 1

Yeast Populations in Marshland Sediments Before and After One-Year Controlled Enrichment with Oil

Species	% of Total Population[a]	
	Before	After
Pichia spartinae	15-40	<10
P. saitoi	20-30	<10
Kluyveromyces drosophilarum	10-25	<10
P. ohmeri	<10	20-30
Trichosporon sp	<10	15-30
Rhodotorula sp	<10	20-30
Cryptococcus sp	<15	<10
Sporobolomyces sp	<15	<10
Mean Population[b]	7800	17400

[a]Adapted in part from Ahearn and Meyers, 1972 (3).

[b]Based on the number of colony forming units (CFU)/cm^3 sediment, 72 samples analyzed quarterly.

Hydrocarbonoclastic yeasts, *Candida tropicalis* and *Endomycopsis lipolytica* (syn. *C. lipolytica*), from sites chronically polluted with oil, were introduced into marshland plots seeded with oil. These strains demonstrated rates of oxygen uptake, with 1% v/v crude oil as the substrate, of nearly 400 mg of oxygen/liter. This rate is approximately twice that found for the most active indigenous marsh flora (*Pichia ohmeri*) responding to oil enrichment (2). Seeding experiments showed that *C. tropicalis* and *E. lipolytica* introduced into oil-enriched plots either as a paste of actively growing cells or as lyophilized cells could be established as an active part of the flora for up to 2 months of testing. The hydrocarbonoclastic yeasts appeared to survive best during the fall-winter season and remained restricted to the hydrocarbon-saturated plots. Ecological factors, such as effects of higher temperatures, the role of predator invertebrates and competitive flora, in failures to establish the hydrocarbonoclastic species in the marshland throughout the year remain to be clarified.

Bacteriological aspects of the marsh also have been examined, especially organisms active in transformation of chitin. Predominant species of marsh bacteria have comprised representatives of the chitinoclastic genus *Beneckea*, representing a significant bacterial biomass active in chitin

turnover. Concentrations as large as 10^5 cells/gm sediment are not uncommon. Evidence indicated that the total bacterial biomass as well as species diversity of chitinoclastic bacteria may serve as indicators of chitin turnover, reflecting meiofaunal development in the marsh and crustacean migration into the estuary. Measurements of in situ chitinoclastic activities showed maximal substrate turnover of 35×10^2 mg/month occurring at water-sediment interfaces (8,9). Studies of activities of selected chitinoclastic bacteria disclosed utilization rates of 30-40 mg chitin/24 hrs/10^{10} bacteria. Whereas the few species of *Beneckea* examined in our work have been non-hydrocarbonoclastic, more extensive studies of this type are planned to fully assess the impact of oil deposition on the major microbial components of the *Spartina* ecosystem. Elsewhere (C. Knowles, Personal communication), studies on a marine *Beneckea* (*B. naturiegens*, syn. *Pseudomonas natriegens*) have indicated an ability to readily metabolize a wide range of organic compounds, including aromatic and aliphatic hydrocarbons as well as heterocycles.

DISCUSSION

Disruption and alteration of chitin and cellulose turnover can significantly alter the basic stage of the food web, either shifting the productivity of the area to a marginal level, or allowing development of allochthonous microorganisms, with an effect on the biomass kinetics of the locality. The development of hydrocarbonoclastic microorganisms in response to oil intrusion has been observed, but apparently strains with dispersant properties and enzymatic capacities for rapid attack of a broad spectrum of hydrocarbons are rare. Indeed, a single organism with these combined capacities and simple growth requirements may not exist. In the absence of rapid biodegradation it is reasonable to expect that the physical coating of microbial substrates with oil will block active sites for enzymatic attack on detritus. Moreover, after volatilization and initial photochemical oxidation, the substrate, now coated with a bitumen fraction, may become anaerobic and recalcitrant. Oil-coated or oil-saturated detritus may also pose real problems in acceptability and transformation by the detritivore population. As noted by Meyers and Hopper (17), a complex balance exists between the meiobenthos and its associated microbial biota.

The estuarine salt marsh habitat is a eutrophic high net-yield system with the most important unit of primary production being the marsh grass. Production of *Spartina alterniflora* in Barataria Bay (in boundary zones between tide-affected waters and the marsh) is the highest ever measured for a salt marsh, i.e., net production 2800 g dry wt/m^2/yr (10). Decomposition of this substrate into detritus is of vast significance, for the trophic structure of tidal estuaries and their associated periphyton and benthic communities is heavily dependent on an input of both autochthonous and allochthonous detritus (20,21). The importance of benthic algal systems and detrital microbial communities on dead *Spartina* in penaeid shrimp nutrition has been shown recently by Condrey et al. (7).

The detrital surface, through both microbial and adsorptive processes, develops an organic film, effectively converting the largely cellulosic plant particle into a protein-rich substrate. Changes in production and modification of the detritus through oil pollution could seriously affect the

marshland ecosystem. Since transformation of detritus is mediated by a host of microorganisms, any deleterious effect on this population has significant implications. In our Barataria Bay studies, oil-induced yeast populations ar not as metabolically active in regard to carbohydrate metabolism as the indigenous mycota, thus a significant reduction in the nutrient regeneration in the estuary could be expected, especially in view of the inability of the dominant marsh yeasts to significantly assimilate crude oil.

The considerations raised here on the impact of oil pollutants on the marshland ecosystem are by no means theoretical. Within a two-year period, two major well blowouts have occurred off coastal Louisiana (19,22). Fortunately, the greater part of the spilled oil was swept away from the marshes by current and wind action. Current considerations (11) of a deepwater port ("Superport," an oil-receiving and super tanker terminal) off the coast of Louisiana has raised many questions. Among these are the major environmental stresses concerning possible oil spill damage and utilization of coastal wetlands and estuaries as support facility areas. Estimates of the magnitude of oil involved are calculated at approximately 4×10^6 barrels per day (1.5×10^8 barrels/yr or an order of magnitude comparable to the total 1968 production from both onshore and offshore Louisiana!

Increased spillage of petroleum products, particularly waste oils, into these areas, and projected development of the Superport facilities, with concurrent environmental risks, indicates the need to critically examine microbial ecosystem patterns with oil as a major stress factor. The effect of concentrations of organic substances within the aquatic environment on both the incidence and activities of yeasts and molds has been readily noted (1).

The reality of the situation has recently been emphasized by a pipeline break in 1972 (comprising 300,000 gallons of spilled oil within a 24-hr period) in the Barataria Pass (Fig. 1) region. While outgoing tides and northeast winds carried the major portion of the oil offshore rather than within the marsh system, portions of the oil still were transported into Barataria Bay and deposited at several sites. This has allowed close monitoring and evaluation of the microbial ecology of the affected sites to ascertain species patterns and successions. The ignition of the oil on the marsh sediment has permitted an examination of the impact of burning and oil residues on the macro- and microbiota. Preliminary studies of the areas affected by the pipeline break and the subsequent burning show the comparative inefficiency of burning oil after its landfall. Microbial populations after one week of the oil deposition indicate a lowering of "baseline" concentrations. Continual monitoring over the past six months has demonstrated the obvious presence of crude oil and its location within the subsurface microaerophilic to anaerobic zones of the sediment. The importance of this reduced zone to the nutrition of *Spartina* is well established. Comparisons of the dry weight of the above-ground material with that of the roots (to a 53 cm depth) indicated that approximately 92% of the *Spartina* is in the below-ground rhizoidal system (Patrick, Personal communication). Furthermore, studies of mineralization of ammonium nitrogen in marsh soils of Barataria Bay (over a 4-month period) have shown rates as great as 3.8 to 5.5 g/week/m^2. An understanding of the effects of oil intrusion on the microbial systems of the *Spartina* rhizosphere is necessary.

ACKNOWLEDGMENTS

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PRODUCTION AND CHARACTERIZATION OF EMULSIFYING FACTORS FROM HYDROCARBONOCLASTIC YEAST AND BACTERIA

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A hydrocarbonoclastic yeast (*Candida*) and bacterium (*Pseudomonas*) were found to release into the culture medium heat-stable factor(s). The latter were effective over a wide range of pH in emulsifying hydrocarbons and crude oils in water.

The chemical conversion of oil pollutants to carbon dioxide (mineralization) and water-soluble, non-toxic organic oxidation products is an esthetically pleasing decontamination process which is being performed continuously by microorganisms in natural environments chronically polluted with oil. The time required by the naturally occurring biological systems to assimilate the sudden surges in pollution levels resulting from man's activities, however, is frequently unsatisfactorily long. We are therefore interested in gaining an appreciably greater level of control over the location, rate, and perhaps end products of this biological wet combustion process. We have noted that a common (perhaps universal) characteristic of the growth of hydrocarbonoclastic microorganisms in liquid media containing hydrocarbons is the production of hydrocarbon-water emulsion early in the growth phase. Because of the potential value of biodegradable, non-toxic emulsifying factors for accelerating the growth and metabolic processes of microorganisms on oil pollutants, we have examined the production and some pertinent characteristics of emulsifying factors from a yeast (*Candida petrophilum*, ATCC 20226) and a bacterium (*Pseudomonas aeruginosa*, NCIB 9904) grown on hexadecane.

The microorganisms in this study were grown in shake culture at 30 C in basal salts medium--pH 5.5 with glycerol for the yeast, and pH 7.0 with succinate for the bacterium. These conditions were used to produce emulsifying factors by the organisms inoculated into basal salts with 0.5% *n*-hexadecane as carbon source. After incubation for 30 hours, cells were removed by centrifugation and the chilled supernatant was filtered through cheesecloth to remove the remaining hydrocarbon. This crude culture supernatant was used for tests of toxicity and stability of the emulsifying activity to temperature, pH and hydrolytic enzymes.

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Preliminary toxicity tests with the water flea (*Daphnia magna*) have indicated an LD_{50} concentration after 48 hours exposure, of 40% for the yeast culture supernatant and 8% for the pseudomonas supernatant. Such concentrations of toxins are unlikely to be attained at oil spills on large bodies of water.

The stability of the emulsifying factors to extremes of temperature and pH and their susceptibility to enzymes and biodegradation were tested by means of a semi-quantitative assay for emulsifying power (minimum concentration giving stable emulsion with water and hydrocarbon and/or width of emulsion band produced by a known concentration of emulsifying factor). The results of these tests indicated essentially complete stability to boiling water temperature for at least 30 minutes for both preparations, and a pH-activity optimum of about 6.5. Significant emulsifying power was exhibited by the yeast preparation over the pH range 0.2 to 11, and by the bacterium from 2 to 9 (Figure 1).

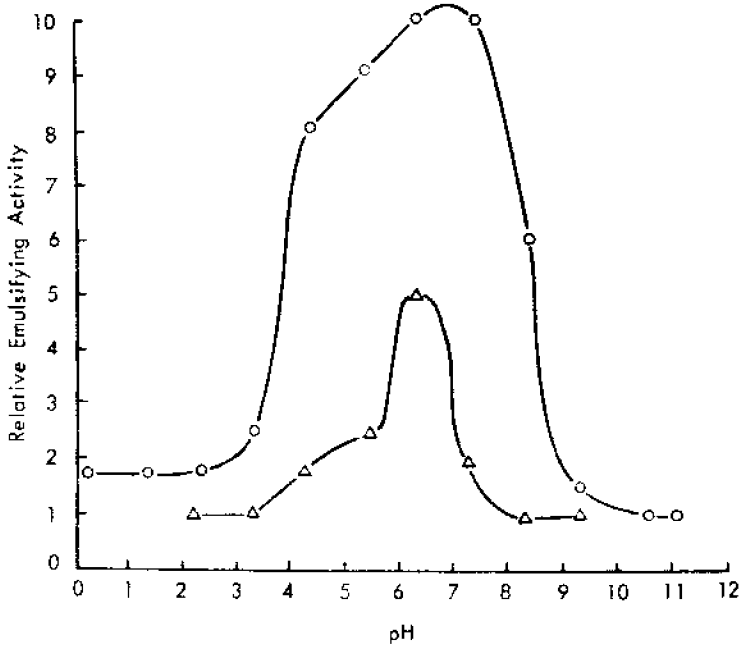

Figure 1. pH Dependence of Emulsifying Activity

Partially purified emulsifying factor preparations were placed in water to 2 mg/ml. The pH values were read with a glass electrode. Changes were made by the addition with stirring of HCl or NaOH solutions. Emulsifying activity tests were made with *n*-heptane.

The activity of both preparations was susceptible to pronase (a broad-spectrum protease) but neither was affected by trypsin or chymotrypsin (Table 1). The pseudomonas preparation showed some susceptibility to hog pancreatic lipase but the activity of neither preparation was decreased by wheat germ lipase. This enzyme susceptibility data and a preliminary test with an *E. coli* growth culture indicate the biodegradability and low toxicity levels of these preparations.

TABLE 1

Enzyme Susceptibility

Enzyme	Incubation Time: 1 min. (← Undiluted →)	1.5 hrs. (← Undiluted →)	6.5 hrs. (3-fold)	6.5 hrs. (9-fold)
Pseudomonas Factor				
Lipase (Hog)	3+	3+	2+	1+	(+)
Lipase (Wheat)	3+	3+	3+	2+	1+
Pronase	(+)	?	?	?	0
Trypsin	3+	2+	2+	1+	(+)
Chymotrypsin	3+	3+	3+	1+	1+
Yeast Factor					
Lipase (Hog)	3+	2+	(+)	(+)	(+)
Lipase (Wheat)	3+	3+	2+	2+	1+
Pronase	(+)	?	(+)	?	0
Trypsin	3+	2+	1+	1+	(+)
Chymotrypsin	3+	3+	2+	2+	2+

The partially purified emulsifying factor preparations were placed in water to 1 mg/ml and adjusted to pH 7.5. The test enzymes were added to 0.2 mg/ml. After various periods of incubation at room temperature, aliquots were removed for emulsifying activities with *n*-heptane.

Efforts toward the purification and chemical characterization of the active emulsifying factor(s) in these preparations have been initiated, with the aim of evaluating the feasibility of large-scale chemical or biological production of non-toxic biodegradable emulsifying factors for enhancing the rate and/or reducing the lag time in the microbial degradation of oil pollutants.

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THE RELATIVE CHANGES IN *n*-ALKANE COMPOSITION IN SURFACE WATER SLICKS

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Hydrocarbon oxidation by bacteria has been the subject of numerous investigations. However, the activity of hydrocarbon oxidizing bacteria in natural environments and the rate at which hydrocarbons are assimilated is not as well documented. Hydrocarbon metabolism in soil and sea water samples has been followed using either a Warburg or other forms of respirometric devices (2,3,4,5). Buswell and Jurtshuk (1) measured microbial hydrocarbon oxidation using an oxygen electrode. Another approach which would permit estimation of hydrocarbon assimilation is to quantitatively follow changes in the composition and extractable weight of an oil slick relative to time, flow or position of the slick. The use of an internal standard or a more refractory naturally occurring hydrocarbon such as phytane could be used to normalize the data or account for dispersion. Preliminary findings are reported here on the changes of *n*-alkane composition in surface slicks in Barataria Bay, Louisiana.

At the locations studied in Barataria Bay, the net movement of water is to the south away from the gas wells in the north, and through Barataria Pass. By sampling away from the producing areas and toward the Pass, qualitative changes in the *n*-alkanes in surface slicks was followed. Samples of surface slicks were sampled with 1.5 ft^2 sheets of Whatman #1 filter paper, pre-extracted with chloroform to remove any contaminants. At each of the sampling sites (Figure 1), four sheets of filter paper were carefully applied to the water with metal tongs. These papers were then stored in glass jars for subsequent analysis. Before hydrocarbon extraction, ^{14}C-hexadecane was added to each paper to indicate extraction efficiency. The papers were shredded, and soaked in 350 ml of chloroform. The chloroform was removed in a rotary evaporator and the sample purified by the method of Meinschein and Kenny (1957). The hexane eluate was brought up to 2.50 ml, and 0.5 ml removed for ^{14}C counting. The remaining sample was concentrated and chromatographed on a Bendix Chromolab Series 2100 GLC, and the results for each *n*-alkane expressed as a percentage of the total *n*-alkane peak areas. The extraction efficiencies were used to correct the weight of extracted material. For this experiment, C-18 was assumed to remain unchanged at all of the sampling sites and the other *n*-alkanes normalized to it to permit comparison of relative changes. The data are shown in Table 1.

Station 7 was the scene of a small spill and thus the total quantity and composition of the material collected is not comparable to the remaining stations. Station 2 was at the mouth of the Bay and the sample

was collected with on-shore winds. Such conditions may have served to introduce surface material from the off-shore wells accounting for the enrichment of C-20, C-22 and C-28 relative to stations 3 to 6.

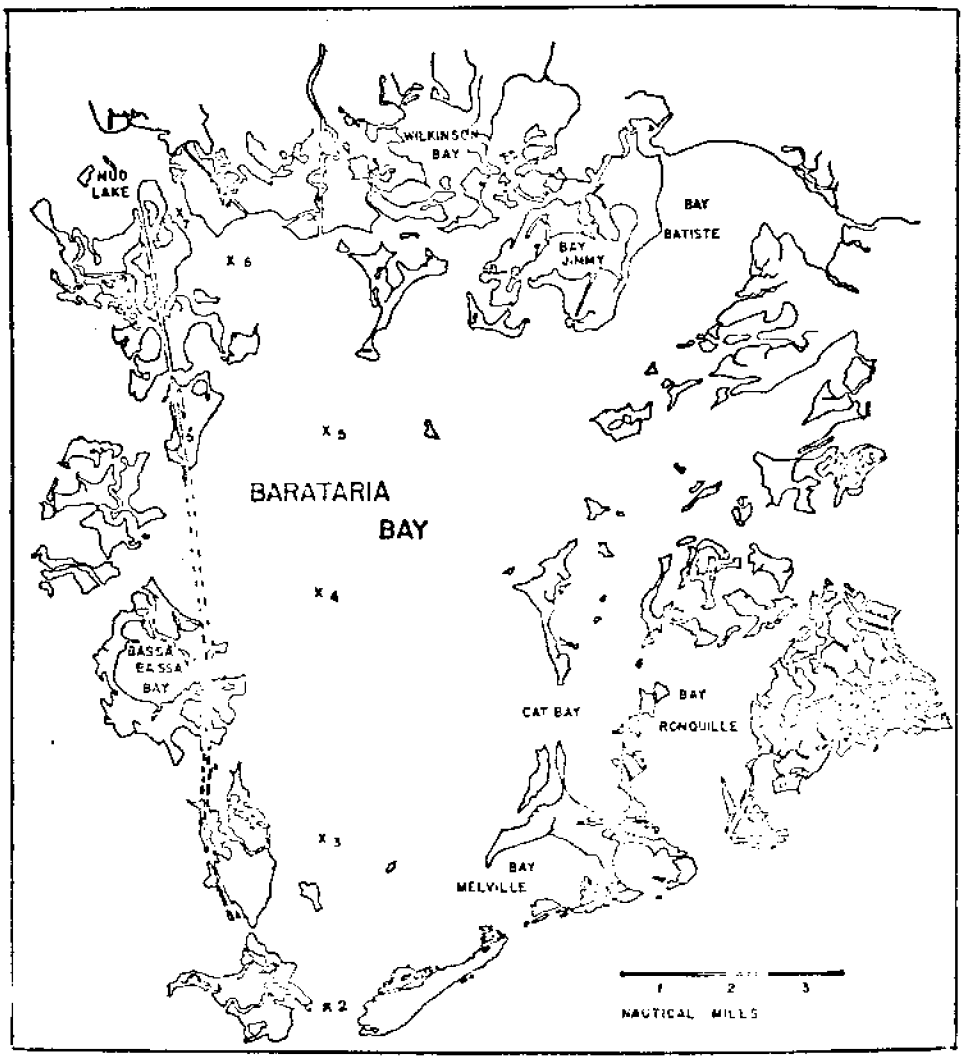

Figure 1. Sampling Locations at Barataria Bay, Louisiana

Station 5 was 200 ft south of a large gas well and the low values for C-16 and C-17 and the higher values for C-19 to C-28 may be the result of hydrocarbon addition from the well. For comparative purposes, therefore, we will use only the data from stations 3, 4, and 6 to follow relative changes in the *n*-alkane composition of surface slicks. At this time we have no estimate on the movement of water with time in the test area and thus must limit our discussion to qualitative changes only.

TABLE I

Relative Concentration of *n*-Alkanes in Surface Water Slicks[a]

Carbon Number	Sample Location					
	2	3	4	5	6	7
14	12.6	-	15.3	-	54.5	8.9
15	-	-	-	-	62.8	-
16	20.3	24.8	36.2	12.2	43.5	17.8
17	71.6	76.0	96.0	61.5	93.0	64.5
18	100	100	100	100	100	100
19	72.6	56.4	60.0	89.0	55.6	44.5
20	48.5	28.4	38.3	55.3	31.2	33.5
21	18.1	11.8	23.7	27.6	18.0	28.0
22	36.7	12.6	22.6	38.8	25.6	13.2
23	18.1	10.5	19.3	28.2	22.4	35.6
24	40.0	8.7	21.5	55.3	25.6	28.0
25	10.3	6.4	15.7	16.0	19.8	19.1
26	6.1	5.4	13.2	13.8	18.6	11.0
27	6.1	5.4	11.7	-	17.3	-
28	33.3	6.2	11.7	34.5	16.0	11.9
29	6.1	5.4	11.7	-	15.4	8.5
30	2.4	1.0	3.0	-	9.0	-
31	-	-	6.6	-	11.0	-
32	-	-	3.0	-	7.7	-

[a]Concentration expressed as percent peak area of each *n*-alkane normalized to C-18 plus phytane. In each of the chromatographs C-18 + phytane was the dominant peak and accounted for between 15.1% at station 6 to 27.9% at station 2 of the total *n*-alkane peak area.

The general trend at stations 3, 4, and 6 shows alkanes with a chain length between C-17 and C-20 to be relatively unchanged, whereas C-14, C-16 and C-21 to C-32 show net decreases moving toward the south. This decrease amounted to 50 to 80% for alkanes over C-21. If dispersion were the principal factor in reducing the hydrocarbon density, we would have expected to find little change in the alkane composition. Evaporation would affect the lower molecular weight material, and thus the changes in concentration of the long chain alkanes must presumably be the result of bacterial degradation.

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ESTUARINE MICROBES AND ORGANOCHLORINE PESTICIDES (A BRIEF REVIEW)[1]

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Little is known about microbiological degradation of organochlorine pesticides in the estuarine and oceanic environments. Since mircoorganisms are probably the main instruments of pesticide breakdown, and possibly offer an array of mechanisms by which pollution may be reduced, research is needed to learn the pathways of microbial degradation in the marine environment.

Table 1 lists a number of microorganisms, chiefly soil and aquatic, with demonstrated ability to partially degrade organochlorine pesticides in various environments. An excellent review of the interaction between halogenated pesticides and microorganisms has been given by Pfister and Matsumura (28). Lichtenstein and Schulz (18) found that soil bacteria converted aldrin to its more stable epoxide, dieldrin; the peak of dieldrin formation occurred 56 days after treatment. A bacterium, *Proteus vulgaris*, isolated from the gut of a mouse, converted DDT to DDD (4) and some soil actinomycetes degrade polychloro-nitrobenzene (PCNB) and dechlorinate DDT (8). Most of the reports listed in Table 1 were concerned with pure cultures and few involved more than one or two step transformations. However, other investigators have reported extensive degradation by soil microorganisms leading to speculation that biodegradation could result in mineralization of organochlorine compounds in the presence of certain microbial assemblages and environmental parameters. Bixby et al. (6) reported a soil fungus, *Trichoderma koningi*, which degraded dieldrin to carbon dioxide with cleavage of the chlorinated ring structure. Focht (10) reported that an aquatic fungus, isolated from sewage effluent, metabolized chlorinated bacterial degradation products to water, carbon dioxide, and hydrochloric acid.

Many soil microorganisms also occur in water, thus environmental relationships and microbial associations similar to those in soil may exist. The essential differences between terrestrial and aquatic environments relative to microbial activity appear to be: (a) usually fewer nutrients per unit mass and less biochemical activity are found in water than in soil, and (b) usually fewer adsorptive surfaces for microbial growth in water than in soil. Reports have indicated that fresh surface waters do not have a characteristic bacterial flora (12). However, certain microorganisms, such as *Beneckea* and *Caulobacter*, have been designated as typical marine or estuarine genera. Differences between estuarine, freshwater, and soil ecosystems make

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TABLE 1

Microorganisms Known to Metabolize Organochlorine Pesticides

Genera	Pesticides	Environment	Reference (see Literature Cited)
Bacteria			
Arthrobacter	Endrin, DDT	Soil-aerobic	Patil et al., 1970 (25)
Bacillus	Endrin, DDT	Soil-aerobic	Patil et al., 1970 (25)
Clostridium	Lindane	Aquatic-anaerobic	McRae et al., 1969 (19)
Escherichia	DDT	Aquatic-anaerobic	Mendel and Walton, 1966 (22)
Hydrogenomonas	DDT	Aquatic-anaerobic	Focht, 1972 (10)
Klebsiella	DDT	Aquatic-anaerobic	Wedemeyer, 1966 (32)
Micrococcus	Endrin, Aldrin, DDT	Soil-aerobic	Patil et al., 1970 (25)
Proteus	DDT	Aquatic-aerobic	Barker et al., 1965 (4)
Pseudomonas spp.	Endrin, Aldrin, DDT	Soil-aerobic	Patil et al., 1970 (25)
Pseudomonas spp.	Heptachlor	Aquatic-aerobic	Bourquin et al., 1971 (7)
Pseudomonas spp.	Dieldrin	Soil-aerobic	Matsumura et al., 1968 (21)
Unidentified	Dieldrin, Aldrin, Endrin, DDT	Marine-aerobic	Patil et al., 1972 (27)
Unidentified	Lindane, Aldrin	Soil-aerobic	Lichtenstein and Schulz, 1959 (18)
Actinomycetes			
Nocardia	DDT, PCNB	Soil-aerobic	Chacko et al., 1966 (8)
Streptomyces	PCNB	Soil-aerobic	Chacko et al., 1966 (8)
Fungi			
Aspergillus	PCNB	Soil-aerobic	Chacko et al., 1966 (8)
Fusarium	DDT	Aquatic-aerobic	Focht, 1972 (10)
Mucor	Dieldrin	Soil-aerobic	Anderson et al., 1970 (3)
Trichoderma	Dieldrin	Soil-aerobic	Bixby et al., 1971 (6)
Yeast			
Saccharomyces	DDT	Aquatic-anaerobic	Kallerman and Andrews, 1968 (16)
Algae			
Chlamydomonas	Lindane	Aquatic-aerobic	Sweeney, 1968 (31)

the estuarine area a unique environment for study of microbial degradation. Because of this uniqueness, data from soil and freshwater ecosystems cannot necessarily be extrapolated to estuarine systems. Therefore, research is needed to learn more about degradation pathways in the marine environment.

Several investigators have reported that degradation of pesticides by aquatic microorganisms is similar to degradation by soil microorganisms. Miles et al. (24) reported that soil microorganisms metabolized heptachlor to 1-hydroxy-2,3-epoxychlordene. Bourquin et al. (7) reported similar results, and proposed a pathway for microbial transformation of heptachlor in the aquatic environment. Metabolism of DDT occurs in soil, freshwater, and lake sediments (26). The similarity of transformations of these compounds may be due to similarity of microflora in the different environments. However, microbial differences as well as environmental factors exist between aquatic and terrestrial ecosystems. For example, most cultivated soils to which insecticides are applied are more aerobic than aquatic sediments. Although DDT is converted to DDD and other products in anaerobic systems, it is stable in aerobic systems (2,13).

Estuarine sediment is a reservoir for pesticides transported by rivers. Because organochlorine pesticides are strongly sorbed on soil and other particulate material (30), including microorganisms (17), they are found on suspended particulates in rivers and are incorporated into estuarine sediment (2). These sediments are often enriched by decomposing organic matter and are anaerobic beneath the surface. Although the rate of carbon turnover due to microbial activity in the sea may not be substantially different from that in fresh water, estuaries are areas of rapid microbial transformations (35). The latter play an important role in estuarine nutrition.

Biodegradation of organochlorine pesticides in estuarine or oceanic environments has been little studied despite the known persistence of the pesticides. Patil et al. (27) studied microbial metabolic transformations of DDT, dieldrin, aldrin, and endrin in samples of marine water, bottom sediments, and surface films. Transformations of DDT and cyclodiene insecticides occurred in samples with biological materials such as surface films, plankton, and algae, but not in waters from the open ocean. Pure cultures of marine microorganisms also metabolized the pesticides. In general, patterns of degradation that have been observed in terrestrial and aquatic ecosystems closely resemble those found for the marine environment (27). For example, production of 6,7-t-dihydroxy-dihydro-aldrin was the major metabolite found in soil fungi (20), in an aquatic bacterium (33), in algal cultures (5), and in pure cultures of marine algae, bacteria, and surface films (27). Similar results were obtained from aldrin, endrin, and DDT, except that algal cultures appeared to convert DDT to a "DDOH-like compound" (2,2-bis (p-chlorophenyl)). The strong degradation activity associated with surface films is significant. Surface films are areas of high biological activity (9) and concentrators of dissolved organics (34) and pesticides (29). Such films provide the environment necessary for selection of hydrocarbon-degrading microorganisms and a relatively high nutrient concentration for proliferation of cells. Presence of pollutants, crude oil or pesticides, in an already enriched area of microbial activity may select for hydrocarbon-degrading microorganisms.

The organically-enriched estuarine environment provides an opportunity for study of co-metabolism of pesticides by microorganisms. As noted by Focht

and Alexander (11), "Co-metabolism is the adventitious biological transformation of organic compounds which provides neither energy nor structural components to the organism." Many bacteria break down certain compounds while metabolizing other substrates but do not utilize the co-substrate as a source of energy or carbon for growth. Relevance of this phenomenon to natural soil ecosystems was noted by Horvath and Alexander (15). Focht and Alexander (11) demonstrated degradation of DDT by sewage bacteria that grew on diphenylmethane, an analogue of DDT. Co-metabolism has not been reported in the estuarine environment. Natural conditions, however, predispose estuaries to such metabolism because of the large microbial communities that exhibit a wide variety of physiological activities. These include degradation of relatively recalcitrant large molecular weight compounds such as complex polysaccharides and some petroleum products (1,23).

Synergism within the microbial ecosystem, including bacteria, yeasts and fungi, in the estuarine environment is another factor to consider when studying breakdown of pesticides. In the soil, microorganisms act synergistically to degrade molecules considered resistant to attack by single species (14). However, in estuarine and oceanic environments, such complex interrelationships among microorganisms have not been investigated adequately. Studies of interactions of estuarine microbial assemblages can provide important data for understanding microbial degradation in estuarine systems.

Estuaries, periodically flushed by tides, provide the near shore portions of the open ocean with many organic nutrients in solution or in the form of partially degraded organic detritus. Organic matter from estuaries and other biologically productive waters often forms a slick, or calm streak, on a rippled sea. As noted, surface slicks are areas of high biological activity and could provide nutrients necessary for co-metabolic transformation of pesticides in off-shore marine locales. Microbiology of naturally occurring surface slicks and oil slicks caused by spills or seepage is an important but neglected area of estuarine research.

Pathways of microbial attack upon chlorinated hydrocarbons in the estuarine environment need to be investigated since breakdown by microorganisms is probably the main natural process of pesticide degradation. Although microbiological processes might reduce environmental pollution attributed to use of persistent pesticides, detailed studies of degradative pathways are required to assess the degree of hazard caused by breakdown products. Required information on microbial degradation of organochlorine pesticides in estuaries will be supplied when we answer the following questions:

What types of microorganisms are involved in transformation of organochlorine pesticides? Are they the same types that predominate in organic detritus formation or are they species selected by exposure to pesticide pollution?

What is the degree of degradation of specific compounds?

Does co-metabolism occur in the estuary, and is it a means of degrading pesticides?

What effects do additional hydrocarbons, such as oil, have on microbial degradation of pesticides in the estuary?

Is synergistic activity within the estuarine microflora a factor in microbial degradation of pesticides?

What is the role of microbial intracellular accumulation and adsorption in biodegradation and/or biological magnification of pesticides?

What environmental factors in the estuary prevent or inhibit or accelerate microbial breakdown of pesticides?

What types of pesticides are easily degraded?

What are the effects of degradation products on estuarine macro- and microflora and fauna?

Considering all the above questions, are similar reactions, selections and effects occurring in the water column and in the sediments?

Such studies will provide data for an accurate picture of the role of estuarine microorganisms on the fate of organic pollutants. Such data are needed to formulate water quality criteria for pesticide regulation in the estuarine environment.

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MEASUREMENT OF BASELINE LEVELS OF ENTERIC BACTERIAL ACTIVITY IN RIVER WATER

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This investigation indicates that natural populations and selected laboratory strains of enteric bacteria, including pathogens, have the capacity to metabolize substrates present in the Oconee River, including autoclaved river water. These organisms, however, lacked the ability to increase in numbers in continuous culture with river water and suspended detritus recovered above a secondary sewage treatment facility, but demonstrated positive growth rates with substrates recovered below the plant. Data also showed that the sands and clays forming the stream bottom had the capacity to sorb substrates from the overlayering water, and that sediment eluates stimulated the respiration rate of bacteria. This suggests that the stream bottom can provide a suitable environment for the growth of bacteria and perhaps controls basal nutrient concentration in the water itself.

INTRODUCTION

Reports concerning the hazardous nature of petroleum pollutants and some oil-dispersants toward aquatic life are well known and have been reviewed by Smith (27), Carthy and Arthur (4), LaRoche (14), and by others. However, comparatively few investigators have examined the possibility that some organisms able to oxidize hydrocarbons might also be capable of causing disease in man or other animals. The question deserves consideration not only from a public health standpoint, but also in regard to possible effects toward fauna within the aquatic environment variously contaminated with oil pollutants.

Prior to examination of possible pathogenicity of hydrocarbonoclastic microorganisms, base line information is needed to determine survival and growth of pathogenic bacteria in the natural, aquatic environment. This report summarizes two different procedures used to measure the metabolism and growth of potentially pathogenic, non-hydrocarbonoclastic, enteric bacteria in fresh water recovered near a sewage outfall. These experiments include attempts to relate the types of organic material in and on the bottom of a warm, fresh water stream to their ability to support growth and multiplication of pathogenic and nonpathogenic enteric bacteria. Once specific knowledge has been obtained concerning the utilization of such substrates, we would be in a better position to understand the roles of the aquatic environment and its bacterial biota in a polluted stream. Such data could also aid in classification of rivers and streams and form a foundation for construction of

model systems to predict the theoretical pollution limits of a given body of water.

MATERIALS AND METHODS

Chemical and Bacterial Assays

Site Selection.--Three sites were selected on the north branch of the Oconee River that flows through Athens, Georgia. Site one, about 4 mi northeast of the city, is an area of agricultural and undeveloped land. Site two is located near the center of Athens, well within the city limits. The river at this point has received water from various storm drains and creeks that drain the residential areas. No domestic sewage is known to be added to the river in this area. Site three is about 750 meters below one of the two Athens municipal sewage plants. The sewage at this facility receives both primary and secondary treatment, and the effluent is chlorinated and allowed to settle prior to addition into the stream.

Assay Procedures.--Chemical and physical parameters of the Oconee River were monitored by techniques approved by the American Public Health Association and published in *Standard Methods for the Examination of Water and Wastewater* (1). Glucose and total hexose were measured by the Glucostat (Worthington Biochem. Corp.) and anthrone (16) procedures, respectively, and protein content was determined by the Folin-Ciocalteau and Biuret procedures (5). Enteric bacteria enumerations were made according to the techniques of Morrison and Fair (17), and species identification made with the use of Bergey's Manual (3), the taxonomic aid, *The Identification of Enterobacteriaceae* (6) and then confirmed by NCDC, Atlanta, Georgia.

Organic-Free Water and Glassware.--Since many prototophic enteric bacterial species are reported to be capable of growth in basal salts solution without added organic material (2,20,26), distilled-deionized water that was carbon-nitrogen free, as determined by C-H-N analyses, was used in all phases of the study. Laboratory glassware was soaked in hot sulfuric acid-dichromate solution overnight, rinsed repeatedly in carbon-free, deionized water and capped with paper prior to autoclaving. With these precautions, control experiments using the test organism in a basal salts solution (without carbon source) demonstrated no increase in bacterial cell densities.

Respiration studies

Organisms.--One strain each of *Escherichia coli*, ATCC 11775; *Enterobacter aerogenes* (*Aerobacter aerogenes*), ATCC 12658; *Proteus rettgeri*, *Arizona arizonae* (*Paracolobactrum arizonae*), *Shigella flexneri* AI, NCDC (Atlanta, Georgia); and *Salmonella senftenberg*, CPHS (Ottawa, Canada), was grown in Trypticase Soy Broth (BBL) at 30 C for 16 hours. Cells were then harvested by centrifugation; washed 3 times in sterile, carbon-free, deionized water; incubated at 30 C for 4 hr to exhaust endogenous reserves; and stored at 4 C for 18 hr before each experiment.

Experimental Substrates.--River water and stream bottom sediments were collected from each study site. The river water was immediately

sterilized at 121 C for 15 min and then frozen after samples were removed for chemical analysis. Sediments from each site were divided into 30 g lots, and each aliquot was washed three consecutive times with 50 ml volumes of carbon-free deionized water. These slurries were agitated for 30 min with a magnetic mixer and then clarified by centrifugation. Each washing was collected, sterilized in the autoclave, and frozen for later use.

After determining optimal pH and buffer ionic strength for elution, 30 g aliquots of washed sediment from each site were eluted with 50 ml of 0.3 M sodium phosphate buffer (5), pH 7.0, in separate experiments, and with river water from the site where the sediment was collected. These eluates were also autoclaved at 121 C for 15 min and then frozen for later respiration studies and chemical analysis.

Bacterial Respiration Studies with Eluted Sediments.--Cell suspensions of each rested bacterial culture were prepared in deionized water and standardized to 0.9 optical density at 540 nm with a Spectronic 20 (Bausch and Lomb) colorimeter-spetrophotometer. Dry cell weights were obtained by comparing the optical density with a standard curve which was prepared with cells washed with deionized water and then dried overnight at 105 C. Respiration studies were carried out with the use of a Biological Oxygen Monitoring System (Cole-Parmer, Chicago, Illinois) in which 4 ml of a particular substrate were placed into the monitor with 2 ml of the standardized bacterial suspension, and oxygen uptake was measured for 15 min. Individual experiments for each organism and substrate were run in duplicate at temperatures of 30 C, 20 C, and 5 C. Control substrates consisting of deionized water, river water from each site, varying dilutions of minimal salts-glucose medium (dilute minimal medium x 10), and minimal medium containing 0.1, 0.2, and 0.3 M phosphate were run in each temperature series. Temperature of incubation was controlled within ±0.2 C with a Lauda-Brinkman K-2R Circulator (Westbury, New York) and respiration was calculated as mg atoms O/hr/mg dried cell weight (20) after correcting for endogenous activity.

Chemostat Studies

Organisms.--In addition to the organisms employed in the respiration studies, bacteria naturally present in the river water from Site 3 were also used in some chemostat experiments. For these studies, 1.7 liters of water was centrifuged at 8,500 x g for 30 min in a Sorvall RC2-B centrifuge. The resulting pellet was resuspended in carbon-free, deionized water and centrifuged in conical tubes at 2,000 x g for 5 min to sediment the heavier particulates and leaving most of the microorganisms free in the supernatant. After aspirating the supernatant into another conical tube, the microorganisms were sedimented at 11,000 x g for 10 min, washed in carbon-free, deionized water and prepared for direct use by the same procedure outlined for the stock cultures.

Experimental Substrates.--River water was collected from each study site. The river water was immediately sterilized at 121 C for 15 min and then frozen after samples were removed for basal nutrient analysis. No attempt was made to use filter-sterilized water because of the high solid content present. As a control substrate, a dilute glucose-mineral salts medium was prepared of the following composition per liter: K_2HPO_4, 7.0 mg; KH_2PO_4,

3.0 mg; $(NH_4)_2SO_4$, 1.0 mg; $MgSO_4 \cdot 7H_2O$, 0.1 mg; glucose, 10.0 mg. The glucose in this medium was autoclaved separately from the rest of the medium and added aseptically when both the glucose and the salt solutions had cooled to room temperature.

Continuous Culture System.--A chemostat similar in design to that reported by Herbert et al. (11) was constructed to yield a working volume of 1.7 liters. The dilution rates were controlled by varying the height of the pressure head and the speed of the medium-waste peristaltic pump (Buchler Instruments, Fort Lee, N.J.). The temperature of the chemostat was controlled with a Lauda-Brinkman K-2/R Circulator (Westbury, N.Y.), and back contamination of the medium from the chemostat was prevented by heating a portion of the medium inlet-tube with a 0.1 ohm/cm nichrome wire connected to a regulated power supply. This system yielded satisfactory dilution rates as low as 0.005 hr^{-1} over extended periods of time.

Bacterial Growth Studies with River Water.--Two different procedures were used to study enteric bacterial growth in dilute nutrient systems. The first series of experiments employed stock cultures of enteric bacteria while later studies utilized organisms from the river. All individual experiments were run in triplicate, and the rate of bacterial decline in numbers was resolved by linear regression (28).

Rested cell suspensions of bacterial cultures were prepared in carbon-free, deionized water and standardized to 0.9 optical density at 540 nm as noted earlier. In separate experiments, 0.1 ml of each bacterial suspension was introduced into chemostats containing river water from each study site. Initial experiments with *E. coli* were run at varying dilution rates, but ultimately 0.012 hr^{-1} was used in all subsequent experiments unless otherwise noted. Samples were taken from each chemostat daily and the organisms present were enumerated on M-Endo broth (Difco) by the membrane filter procedure at 30 C.

To determine specific growth rates of enteric bacteria in river water from the three study sites, bacteria present in 1.7 liters of river water from Site 3 were recovered by centrifugation and prepared for use by the procedure outlined earlier. These organisms were then introduced into chemostats in which minimal medium and river water from each site were used as nutrient sources. A dilution rate of 0.012 hr^{-1} was maintained in these experiments and the incubation temperature was 30.0 C. Samples were also taken from these chemostats on a daily basis, but organisms present were enumerated on M-Plate count broth and M-Endo broth (Difco) on membrane filters at both 30.0 and 44.5 C. Growth rates were calculated by the procedure of Jannasch (12).

RESULTS AND DISCUSSION

The basal nutrient concentration of the autoclaved river water and bottom sediment from the three study locations is presented in Table 1. These concentrations increased gradually as the river water flowed down stream (Sites 1 and 2) and reached a maximum below the sewage effluent (Site 3). The carbohydrate and protein colorimetric procedures used allow for the detection of a variety of compounds, some of which may not be readily

degradable by some enteric bacteria. Attempts to elute measurable quantities of the basal nutrients from the sediments with river water above those already present in the river water were unsuccessful.

TABLE 1

Basic Nutrient Analysis of Oconee River Water and Extracts of River Bottom Sediment from the Three Study Sites

Nutrient assayed[a]	Site	River water	Bottom sediment	
			Washed[b]	Buffer eluted[d]
Ammonium nitrogen	1	1.6	0.6	9.0
	2	2.3	0.2	8.5
	3	4.7	1.0	25.0
Folin protein	1	2.9	1.5	45.0
	2	3.2	0.2	63.0
	3	5.1	0.2	120.0
Carbohydrate	1	1.6	1.0	12.0
	2	2.4	0.6	22.5
	3	2.8	2.4	36.0
Orthophosphate	1	1.5	0.0	-[d]
	2	2.0	0.0	-
	3	4.1	0.4	-
pH	1	6.9	7.0	7.0
	2	7.0	7.0	7.0
	3	7.0	7.0	7.0

[a]Concentrations expressed in mg/l of sample.

[b]Concentration of nutrients present in the 3rd successive washing of deionized water.

[c]Sediment eluted with 0.3 M phosphate buffer (pH 7.0).

[d]Phosphate concentrations were 0.3 M.

Microbial metabolism of the substrates in the river water and those in buffer eluted bottom sediments is shown in Tables 2 through 4 and in Figure 1. Tables 2, 3, and 4 expressed the respiration rates of the non-pathogenic enterics at 30, 20, and 5 C with the test substrates from Sites 1, 2, and 3, respectively. In Figure 1 are compared the respiration rates of the nonpathogenic enterics with the pathogenic species of *S. flexneri*, *S. eenftenberg*, and *A. arizonae* in buffer-eluted sediments and river water taken at Site 3.

TABLE 2

Respiration of Various Enteric Bacteria in Oconee River Water and in Extracts of River Bottom Sediments from Site 1

Organism	Temp. C	River water[a]	Bottom sediment	
			Washed[b]	Buffer eluted[c]
Escherichia coli	30	0.19[d]	0.34	1.98
	20	0.00	0.00	0.44
	5	0.00	0.00	0.44
Enterobacter aerogenes	30	0.22	0.46	1.60
	20	0.00	0.09	1.13
	5	0.00	0.16	0.00
Proteus rettgeri	30	0.26	0.12	1.64
	20	0.00	0.00	1.38
	5	0.00	0.00	0.00

[a]Respiration rates expressed as mg atoms oxygen (O)/hr/mg dry cell weight.

[b]After third successive wash.

[c]0.3 M phosphate buffer (pH 7.0).

[d]All respiration rates have been corrected for endogenous activity.

Mean specific growth rates at 30, 20 and 5 C for the selected enteric bacterial strains in river water from Site 3 are given in Table 5. In these chemostat studies, all six organisms demonstrated positive specific growth rates at 30 C, but growth was slight or undetectable at the lower two temperatures. Table 6 shows the mean specific growth rates of natural bacterial populations in Oconee River water (Site 3) and in dilute minimal medium. No growth was observed with these organisms nor with the selected enterics in water from Sites 1 and 2 (Fig. 2).

It is known that various bacterial species are capable of growth and reproduction in extremely dilute nutrient concentrations of laboratory media (2,8,15), but most of these organisms are not involved in pathogenesis of man or higher animals. The enterobacteriaceae, however, not only contains species which serve as indicators of fecal pollution, but others, such as *Salmonella, Shigella,* and *Arizona*, that are responsible for serious intestinal disease. Data obtained in this study (Table 1) indicate that the basal nutrients for the test organisms were in short supply. River water taken below the sewage plant effluent was slightly higher in nitrogen, Folin-protein, carbohydrate, and phosphate content than was water from the other two sites.

TABLE 3

Respiration of Various Enteric Bacteria in Oconee River Water and in Extracts of River Bottom Sediments from Site 2

Organism	Temp. C	River water[a]	Bottom sediment	
			Washed[b]	Buffer eluted[c]
Escherichia coli	30	0.43[d]	0.23	1.81
	20	0.21	0.52	1.68
	5	0.00	0.00	0.18
Enterobacter aerogenes	30	0.53	0.37	1.26
	20	0.00	0.00	1.27
	5	0.00	0.24	0.10
Proteus rettgeri	30	0.25	0.00	1.68
	20	0.00	0.21	0.80
	5	0.00	0.00	0.00

[a]Respiration rates expressed as mg atoms oxygen (0)/hr/mg dry cell weight.

[b]After third successive wash.

[c]0.3 M phosphate buffer (pH 7.0).

[d]All respiration rates have been corrected for endogenous activity.

There is, however, a certain amount of difficulty in attempting to directly compare basal nutrient data from one site with that from another, or even that from distant localities. Even though the colorimetric procedures used in this study are capable of detecting a wide range of possible nutrients, it is conceivable that other compounds could be present in the water that could either stimulate or inhibit growth and still remain undetected. However, on the basis of the growth studies, the enteric bacteria employed here were able to grow in river water from below the sewage plant which implies that conditions were more favorable in the water from this site than the other two sites.

Table 1 also demonstrates that bottom sediments from the Oconee River can be washed relatively free from loosely associated material and that a very high concentration of ammonia nitrogen, protein, and carbohydrate can be sorbed onto the river bottom sediments. This observation becomes even more significant because three successive washings with deionized water and trial elution with river water failed to remove the tightly bound material. Elution, though, was accomplished with buffer of ionic strengths which might be common to only the most severely polluted aquatic environments. These data suggest that the basal nutrients were very tightly sorbed on the sands and clays forming the stream bottom sediments, and that they may not be readily available for metabolism by aquatic microorganisms. We have tested

this concept with BOD studies in our laboratory and found that washed sediments had no stimulating effect on the oxygen demand. This is in agreement with Weber and Coble (30), who found that cationic pesticides, subject to microbial degradation, could be adsorbed on various clays and were then no longer subject to decomposition or even readily available for plant uptake. Perhaps this would be true for hydrocarbons also.

TABLE 4

Respiration of Various Enteric Bacteria in Oconee River Water and in Extracts of River Bottom Sediments from Site 3

Organism	Temp. C	River water[a]	Bottom sediment	
			Washed[b]	Buffer eluted[c]
Escherichia coli	30	0.58[d]	0.22	2.58
	20	0.33	0.10	1.73
	5	0.00	0.00	0.34
Enterobacter aerogenes	30	0.45	1.34	4.25
	20	0.00	0.90	1.13
	5	0.00	0.00	0.10
Proteus rettgeri	30	0.36	0.36	3.23
	20	0.17	0.12	1.07
	5	0.00	0.00	0.00

[a]Respiration rates expressed as mg atoms oxygen (0)/hr/mg dry cell weight.

[b]After third successive wash.

[c]0.3 M phosphate buffer (pH 7.0).

[d]All respiration rates have been corrected for endogenous activity.

Respiration of organisms in the aquatic environment has been used as a means of estimating in-situ activity (18,22,25). Previous studies (9) have suggested that a respiration rate of about 0.5-3.5 mg atoms 0/hr/mg dried cell weight could be achieved if the carbon (hexose and protein) analyses of the river water and extract bottom sediments represent readily oxidizable substrates. Similar rates would be expected with the prepared natural substrates at both the 30 and 20 C incubation temperatures, but respiration at 5 C should be minimal or nonexistent. Results of the respiration rate studies using river water and extracted sediments confirmed this hypothesis (Tables 2, 3, and 4) and reflected the basal nutrient concentration. Respiration rates above endogenous levels for all organisms tested in river water were lowest at Site 1, but as nutrient concentration increased in the water from Sites 2 and 3, respiration rates at both 20 and 30 C approached the predicted values (Tables 3 and 4). Sediment eluted with phosphate buffer in

all cases yielded respiration rates far exceeding those observed with river water. With the exception of *Enterobacter aerogenes* at Site 1, both *Escherichia coli* and *Enterobacter aerogenes* could use the substrates present in the eluates from all sites at 5 C. Sediments eluted with buffer from below the sewage plant demonstrated the highest respiration rates achieved at 30 C with rates at 20 and 5 C approximating those at the other two sites. When these rates were compared with those for the pathogenic species, e.g., *Shigella flexneri*, *Salmonella senftenberg*, and *Arizona arizonae*, equivalent respiration was observed (Fig. 1). These data indicate that both pathogenic as well as nonpathogenic bacteria could use substrates present in the river water and those adsorbed on the bottom sediments after relatively mild laboratory treatment.

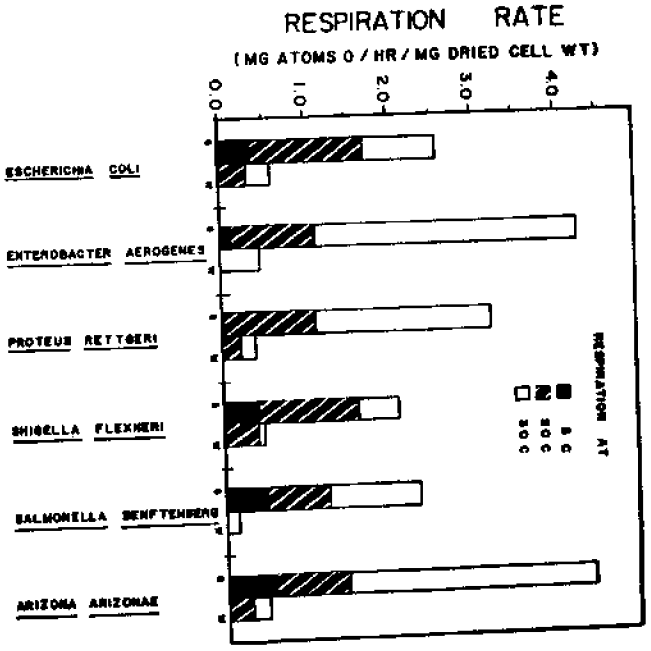

Figure 1. Respiration Rates of Selected Pathogenic and Nonpathogenic Enteric Bacteria in 0.3 M phosphate buffer extracted sediments (s) and river water (w) from Site 3

Results (Fig. 2), which demonstrated the lack of growth of *E. coli* in river water from Sites 1 and 2, were confirmed with the remaining five stock cultures. Growth, however, was observed for all six cultures, including pathogens, in water from Site 3 (Table 5). Maximal specific growth rates were observed in Site 3 water at the 30 C incubation temperature for all organisms tested, while generation times ranged between 33.3 and 90.0 hrs at this temperature. *E. coli* and *E. aerogenes* demonstrated generation times of 34.5 and 33.3 hrs, respectively, while the remaining *Proteus*, *Arizona*, *Salmonella* and *Shigella* sp. reproduced at a rate 2 to 3 times greater than the

coliforms. Generation times of the six test organisms were extended at 20 and 5 C, and death was observed for *S. senftenberg* at 20 and 5 C and for *E. aerogenes* and *P. rettgeri* at 5 C. These results are consistent with those of Postgate et al. (20) who observed generation times of approximately 100 hrs ($D = 0.0101\ hr^{-1}$ at 37 C) for *E. aerogenes* in media where the carbon source was rate limiting. In later studies, though, generation times of 38.9 hrs ($D = 0.0145$ at 37 C) were also observed (21) and agree in general with the data from the respirations studies.

TABLE 5

Growth Rates[a] of Selected Enteric Bacteria in Oconee River Water from Site 3

Organism	Temp. C	Wash out Rate (hr^{-1})	Growth Rate (hr^{-1})	Generation Time (hrs)
Escherichia coli	30	0.017	0.029	34.5
	20	-0.009	0.003	333.3
	5	-0.011	0.001	1,000.0
Enterobacter aerogenes	30	0.018	0.030	33.3
	20	-0.006	0.006	166.6
	5	-0.018[b]	-	-
Proteus rettgeri	30	-0.002	0.012	83.3
	20	-0.001	0.010	100.0
	5	-0.017	-	-
Arizonae arizona	30	-0.001	0.011	90.0
	20	-0.011	0.001	1,000.0
	5	-0.011	0.001	1,000.0
Salmonella senftenberg	30	0.001	0.013	76.9
	20	-0.020	-	-
	5	-0.032	-	-
Shigella flexneri	30	0.001	0.013	76.9
	20	-0.007	0.005	200.0
	5	-0.010	0.002	500.0

[a]Dilution rate was $0.012\ hr^{-1}$ in each case.

[b]A washout rate of $-0.012\ hr^{-1}$ or less is defined as death of the population (12).

The mean specific growth rate for the coliform bacteria naturally present in the Oconee River was $0.006\ hr^{-1}$ (Table 6) with an equivalent generation time of approximately 166 hours. Although the fecal coliform bacteria, as estimated by the 44.5 C population, demonstrated a negative growth rate which can be defined as death of the population (21), both the 30 and 44.5 C population of heterotrophic bacteria were capable of growth in the

water. When these data were compared with those obtained with dilute minimal medium, the heterotrophs as well as the enteric and coliform bacteria were capable of growth.

TABLE 6

Average Growth Rates of Native Bacterial Populations in Site 3 Oconee River Water and Dilute Minimal Medium

Nutrient Source	Bacterial Group	Growth Rate, hr^{-1}	
		30 C Population	44.5 C Population
Minimal Medium 1:1,000 dilution	Heterotrophs	0.017	0.019
	Enterics	0.018	0.015
	Coliforms	0.019	0.012
River Water (Site 3)	Heterotrophs	0.007	0.013
	Enterics	0.007	d[a]
	Coliforms	0.006	d

[a]Death of the culture.

In all chemostat experiments using bacteria recovered from the river, bacterial numbers were observed to oscillate with a periodicity of approximately 100 hours. Although such growth has been termed "cryptic" by other investigators (21,23), it is doubtful whether sufficient energy could be derived from the dead and lysed cells to allow for replenishment of the loss in population, especially in a chemostat where wastes are being constantly removed. A more plausible explanation could involve the slime layer that is produced on the glass walls of the chemostat. Studies (13,24) have shown that mixed bacterial cultures capable of growing in water or dilute nutrient systems can produce slime layers on bare artificial surfaces. Sanders (24) has shown that the slime layer can slough off when the underlying layers become sufficiently anaerobic to alter the metabolism of the bacteria present. Since slime production is a function of time, it is conceivable that the slime could be sloughed at 100 hr intervals. This process contributes bacteria and added nutrients for metabolism by the suspended populations. Such a hypothesis is consistent with previous reports of bacterial and nutrient sorption to negatively charged surfaces (9,29).

Certainly data reported in this study cannot be extrapolated directly to the natural aquatic environment to conclude unequivocally that enteric bacteria, including pathogens, are capable of growth in fresh water. There are, however, data to indicate that these organisms are able to grow in situ under very limited nutrient conditions (8,10). This study has demonstrated the presence of sufficient nutrients in autoclaved river water, taken below a sewage outfall, to support limited bacterial growth.

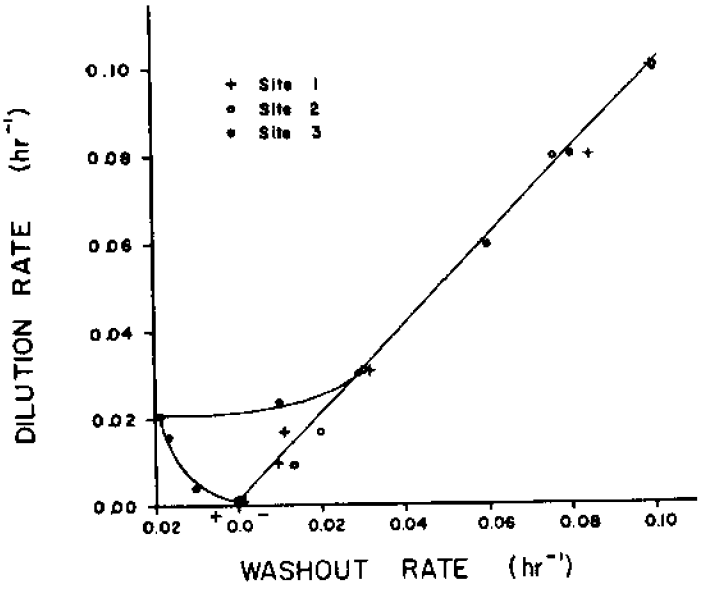

Figure 2. Washout Rate versus Dilution Rate for *Escherichia Coli* in Oconee River Water from Sites 1, 2, and 3 at 30 C *E. coli* was capable of growth where the dilution rate minus the washout rate equals a positive growth rate (μ). (1/μ = culture generation time)

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SUBLETHAL EFFECTS OF THE WATER SOLUBLE COMPONENT OF OIL: CHEMICAL COMMUNICATION IN THE MARINE ENVIRONMENT

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Crude oil and petroleum products contain water soluble components that are potent inhibitors of chemoreceptors in marine organisms. Since many species rely on chemoreception for location of food and sexual partners, disruption of chemoreception in the marine environment by oil spills can have profound effects on survival and reproduction. These effects on species survival will not be revealed in the usual toxicity (LD) studies of pollutants.

INTRODUCTION

Present studies of the biological effects of oil pollution have focused on the lethal effects, either attempts to establish changes in the biota following a spill or by exposure of adult organisms to pollutants in the laboratory. Few studies have been concerned with the sublethal physiological effects of oil pollution or with the water-soluble components of the oil (3). Some studies have even emphasized that, following a spill, those components of the oil that evaporate or are dissolved in the water are no longer a pollution problem (2).

In our laboratory we have been studying the functions of chemical communication in the marine environment. We find that for most species the chemical sense is the dominant one. Chemical cues are needed not only for detection of food or a suitable substrate or niche, but also provide the signal for gamete fusion in many species, the trigger for metamorphosis during development in some species, homing and aggregation behavior, and in many species the location and selection of a sexual partner. Social structure in groups of some species of fish is also mediated through chemical cues (5). Among the crustacea, the female may release a sex pheromone to signal her presence and physiological state. In the crabs we have studied (4) the lowest concentrations of the pheromone (below 10^{-13}M) stimulate a search behavior in the males. At higher concentrations, the pheromone triggers a "mating stance" in the males. On approach of the male to the female, behavioral evidence suggests that the male then releases a sex pheromone that may have the function of an aphrodisiac. The male then seizes the female and protects

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her during ecdysis. The pair copulates after ecdysis and the male protects the female while her new carapace hardens. In addition to the initial female sex pheromone which functions to attract the male, the female releases two additional sex pheromones. These two pheromones, besides functioning as attractants, may signal the receptiveness of the female. One is released only before ecdysis while the other is released after ecdysis.

Utilizing both behavioral response and electrophysiological recording, Case and Gwilliam have shown that the "feeding response" of crabs is triggered by a number of amino acids and that the acidic amino acids are the most effective (1). The "feeding response" is characterized, in its initial stage, by a rapid lateral movement of the mouthparts.

METHODS

In these studies we have utilized a local intertidal crab, *Pachygrapsus crassipes*. The two bioassays are based on the response of the males to the female sex pheromone, crustecdysone, and the response of both sexes to a feeding stimulant, taurine.

When a male *P. crassipes* is presented with a 10^{-8} M solution of crustecdysone, a "mating stance" is exhibited within a period of three minutes. Small aquaria were constructed for this bioassay by forming a large transverse indent in each of several 2000 ml beakers just above the base. Except for a viewing window opposite the indent the beakers were painted flat black. The niche beneath the indent simulates a rock crevasse and a layer of 3 mm glass spheres provides a normal substrate. The central space is sufficiently large for the crab to display a normal "mating stance" while requiring a minimal volume of sea water solutions of the pheromone. The crabs were exposed to the water soluble extracts of crude oils or petroleum products for varying periods of time, rinsed five times and then placed in the bioassay beakers and stimulated with 1000 ml of 10^{-8} crustecdysone.

A study of the response of *P. crassipes* to various amino acids led to the selection of a 20 µl aliquot of a 3 mM solution of taurine for the standard stimulus for the "feeding response." This stimulus was administered in the region of the antennules of the crab from a 20 cm syringe needle attached to a repeating syringe. In the control crabs this stimulus always released a strong "feeding response." After exposure to the "polluted" water, the crabs were rinsed five times and placed in 1000 ml beakers for the assay. The stimulus was presented to each crab five times at five-minute intervals and the response was scored as negative, partial or positive, based on the extent of the movement of the mouthparts. Four experimental crabs and one control were assayed for each exposure condition. The crabs were tested one hour after exposure and each day until recovery. The initial experiments revealed an inverse relationship between the concentration of the "pollutant" and the length of exposure in the expression of the inhibition of chemoreception. To establish consistency a 24 hr exposure was selected for our standard bioassay. It was felt that this exposure might best reflect the conditions occurring during an oil spill in the marine environment.

We are unable to state the concentration of the total "water solubles" to which the crabs were exposed, but the method of preparation assures that it was low. To obtain the "water solubles" in low concentration and to avoid the

problems inherent in separating an emulsion of oil in sea water, a thin film of oil was extracted. In initial studies two crude oils were utilized, a Sisquoc sand and a California crude from the lower Miocene. Thin films of these two crude oils were prepared by swirling dilute diethyl ether solutions in 2000 ml beakers until the solvent evaporated. The crude oil content of these films was about 0.2 ml. Each of the beakers was filled with sea water and stirred slowly overnight. In later experiments dialysis was utilized, as suggested by Galtsoff (3), to obtain the water soluble fraction of petroleum products. Spectrofluorometric studies have indicated that the attainment of equilibrium is quite slow with simply dialysis, requiring two to three days to attain equilibrium.

During the course of this study it was learned that the Navy was developing oil/water separators for use aboard ship. These separators employ centrifugation followed by percolation of the aqueous phase through a "coalescer" to remove the colloidally-dispersed emulsion. The design of these "coalescers" is based on the surface properties of a packing of fine glass fibers. The water effluent of these separators would be expected to be "saturated" with the water-soluble components of the initial petroleum products (i.e., the concentration of each water-soluble component in the aqueous phase will reflect the partition coefficient of that component in the equilibrium partition between sea water and the organic phase). Samples of the effluent of each of these separators were obtained and subjected to the "feeding response" bioassay. A sample of Navy Special Fuel Oil was also obtained and an extract prepared of the water-soluble components by dialyzing 5 ml of the oil against 2000 ml of sea water.

RESULTS

The exposure of the crabs to the water-soluble extracts of the two crude oils completely inhibited both the "feeding response" to the taurine stimulus and the "mating stance" response of the males when exposed to the sex pheromone. It was found that this inhibition was persistent. Ten of the crabs that exhibited inhibition of the "feeding response" following exposure to the "polluted" water were tested daily for recovery. The recovery period varied from three to six days, eight recovering in four or five days. The sea water extracts of the crude oils were diluted 10:1 and 100:1. Each of these diluted extracts was effective in inhibiting chemoreception in the crabs following a 24 hr exposure. If it is assumed that 1% of the crude oil films was extracted into the sea water, we can conclude that the threshold concentration of total water solubles that is effective in inhibiting chemoreception in *P. crassipes* is below 10^{-8}.

As in all behavioral bioassays, in the "feeding response" bioassay of chemoreception following inhibition by petroleum extracts there are partial responses that are difficult to score. A full response is expressed by a rapid and persistent lateral movement of the mouthparts following a pulse stimulus; however, often during the recovery of the crabs responses were observed ranging from one or two movements of the mouthparts, sometimes involving only one side, to rapid movement persisting for a few seconds. In an attempt to quantitate this, we scored any movement of the mouthpart persisting for less than five seconds as a partial response with a value of one and assigned a value of three for a full response (Table 1). Unscored are the

other changes in the behavior of the crabs. After exposure the crabs often exhibited movements that might be described as restless or confused; occasionally the proper presentation of the stimulus was difficult due to this excessive movement. The dialysate of the Navy Special Fuel Oil exhibited strong inhibitory properties. Two of the crabs exposed to this extract did not recover their chemosensory capacity until the eleventh day after exposure.

TABLE 1

Effect of Water-Soluble Extracts of Oil on Feeding Response of Crabs to Taurine

Oil Extract[a]	Day 0	1	2	3	4	5	6	7	8	9	10	11
A	6[b]	2	17	11	11	15	13	8	19(1)	22	22	43(2)
B	15	6	1	9	6	13	17	19	41(1)	33(1)	45(1)	58(1)
C	12	21(1)	21	22(1)	41	31	43(1)	52				

[a]A, dialysate of Navy Special Fuel Oil; B, oil/water separator effluent; C, 1000:1 dilution of B.

[b]Cumulative response of mouth parts of four crabs to five stimulations with 20 µl of 3 mM taurine; 3--full response, 1--partial, 0--negative. Numbers in parentheses are number of crabs judged to have recovered. In "A", one crab escaped the second day; in "C", one crab molted after the fifth day.

The attempt to bioassay the effluent of the oil/water separator being developed on the west coast indicated that the water was toxic to the crabs. Investigation revealed a high bacterial count in this water (10^8 cells/ml). Inquiry of the contractor indicated that they had repeatedly cycled a 25,000 gallon supply of sea water through "pollution" and "separation" for several months and that the sample of effluent provided had not come directly from the coalescer but from a large storage reservoir for the effluent that had contained varying amounts of effluent for most of this period. The bacteria were not characterized, but the evidence suggests a massive enrichment culture. Observations also suggest that some of the metabolic byproducts of hydrocarbonoclastic bacteria could have contributed to the toxicity of this water. No attempt was made to characterize the biologically active compounds in this sample.

The effluent of the oil/water separator being developed on the east coast resembled the dialysate of the Navy Special Fuel Oil in its content of substances capable of inhibiting the chemoreceptors of *P. crassipes*. The crabs exposed to this water were strongly inhibited and did not recover their capacity until the eighth to eleventh day after exposure. Like the dialysate, this water was diluted 1000:1 and was still effective in inhibiting the chemoreceptors; however, the crabs exposed to this concentration of "pollutant"

recovered in one to six days and the "partial responses" were more frequent during the recovery period (Table 1).

What are the compounds in the water-soluble fraction that are responsible for this persistent inhibition? Aromatic hydrocarbons are known to have a limited solubility in sea water and we therefore examined the effects of a number of monoaromatic and polynuclear aromatic compounds. We found that all of the monoaromatic hydrocarbons were effective as inhibitors of chemoreception in crabs but that the effect was transient, the crabs recovering in about 30 minutes to one hour. The polynuclear hydrocarbons, however, exhibited an effect that paralleled our observations with the "water-soluble" component. Crabs exposed to solutions of naphthalene and binaphthyl were inhibited for 8 to 11 days, those exposed to anthracene were inhibited for 13 days. The threshold for this effect was not sharp, but with naphthalene and binaphthyl at 100, 10 and 1 µg/liter concentrations the duration of the inhibition decreased and there was greater variability between the recovery time for different crabs. At 1 µg/liter of naphthalene the crabs started to recover after 3 days.

DISCUSSION

These observations suggest that the sublethal effects of the water-soluble components of petroleum products may alter species survival. The basic elements of species survival are reproduction and growth. It has been shown that both of these elements, because of the major role of chemoreception in the marine environment, are sensitive to minute concentrations of aromatic hydrocarbon pollution.

ACKNOWLEDGMENTS

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SOME ACUTE EFFECTS OF LOW-BOILING PETROLEUM FRACTIONS ON THE CELLULAR STRUCTURES OF FISH GILLS UNDER FIELD CONDITIONS

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Short chain hydrocarbons and arene compounds in crude oil have a relatively greater dispersability and solubility in sea water than the longer chained fractions. Short chained fractions are often considered insignificant in environmental pollution as they are rapidly dissipated by volatilization and possibly by microbial degradation. Recently, Takahashi (5) reported that oil fractions may interfere with the chemoreception in arthropods by rendering them unable to detect pheromones secreted by others of their species.

Personal investigations as well as repeated references in the literature indicate the gills of fish to be excellent indices of the effects of external irritants and toxins upon fish populations in the field (2).

MATERIALS AND METHODS

Representative specimens from seven species of fish were randomly selected from areas around the spill site off the Louisiana coast southeast of East Timbalier Island (Fig. 1) and from Aransas Bay, Texas. Test specimens and their area of collection are given in Table 1.

The first gill arch was removed from each specimen and fixed in a formalin-based preservative. The gills were embedded in a soft paraffin and thin sections, 4-6μ, were prepared with a rotary microtome. These sections were stained with Delafield's heamatoxylin and eosin and a modified Azan-Mallory trichromic stain. All sections were observed under a compound microscope using normal and polarized light and significant areas were photographed. All comparisons were made using high-contrast prints and black and white projections.

RESULTS

In the higher fishes, the gill pouch is located along either side of the head and opens directly from the pharynx to the outside. Contained within the gill pouch are a series of gill arches, covered with a mucous membrane that is raised into numerous horizontal ridges called filaments (3). As se

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In Figure 2a, the basic histological structure of the branchial filaments consists of a low, single-layered epithelium overlying a basal structure called the lamella. The blood vessels are lacunar or subsinusoidal in shape and are supported by connective tissue cells called pillar cells. Mucous secreting cells are common and scattered along the lamellar structure (4). Numerous acidophilic secretory cells are also present and are presumed to be the site of production of the carbonic anhydrase-like enzymes (1). These acidophilic cells are located on the side of the lamellar structure supplied by the afferent capillaries. In addition, fish adapted to life in salt water possess an "excretory vesicle" at the free surface of the epithelial cells. When damaged the branchial filament takes on a clean appearance due to the loss of much of the epithelial tissue.

TABLE 1

Test Specimens and Site of Collection

Species	Collection Site[a]
Ancyclopsetta quadrocellata	Aransas Bay SPNW-17
Micropogon undulatus	Aransas Bay SPNW-16
Etropus crossotus	Aransas Bay SPN-8
Prionotus tribulus	Aransas Bay
Chloroscombrus chrysurus	SPN-8
Chaetodipterus faber	SPN-11

[a]See Fig. 1 for location of SPN sites.

The specimens examined in this study exhibited the anomalies expected, although the different species varied somewhat in the degree of damage. The flounder, *Ancyclopsetta*, showed a loss of epithelial and mucous cells, as well as a considerable reduction in the number of acidophilic cells (Fig. 3). The flounder from the control site exhibited a ragged gill filament with loosely arranged cells between adjacent filaments (Fig. 4). The clean appearance of the filaments in Fig. 3 was caused by sloughing of the epithelial tissue and the remaining portion of the filament appears to be swollen, causing the "blurred" effect.

The specimens of *Micropogon* showed changes comparable to those observed in the flounder, although some mucous cells remain and fewer acidophilic cells are missing from the filaments of the specimen taken from the spill site (Figs. 5 and 6). This may be due to the relatively greater tendency of *Micropogon* to migrate away from areas of stress, while the bottom-dwelling flounder would be more likely to experience a higher degree of exposure to external irritants. This hypothesis was checked by examining specimens of the sole, *Etropus*, another bottom-dwelling fish and two species of free-swimming fish, *Chloroscombrus* and *Chaetodipterus*. As seen in Figs. 7 and 8, the sole showed almost identical changes as those seen in the flounder,

while the specimen from the control area exhibited no damage, *Chloroscombrus* and *Chaetodipterus* (Figs. 9 and 10) were examined and found to be almost completely normal.

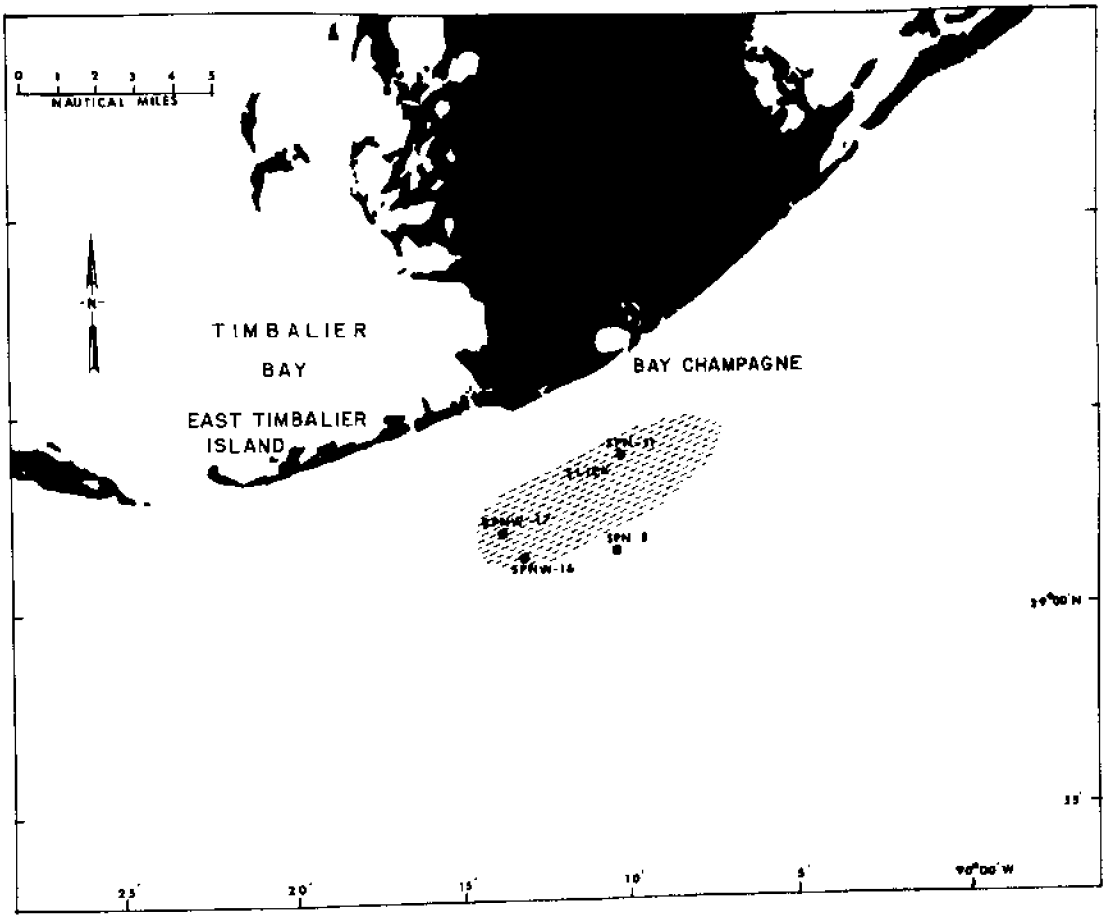
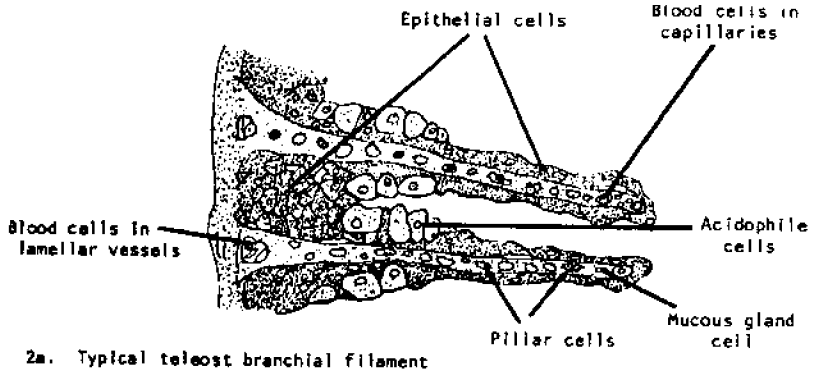


Figure 1. Trawl Station Location Map

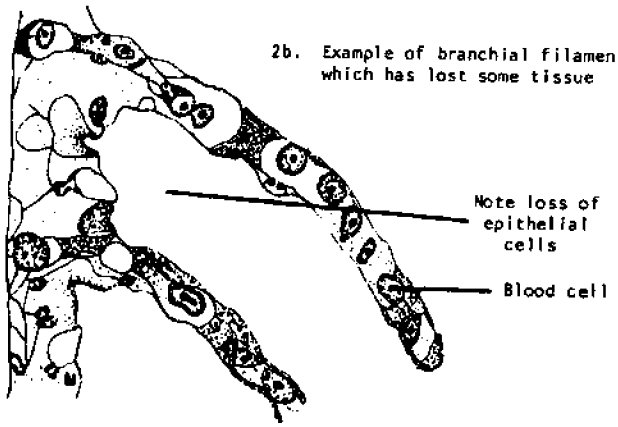
DISCUSSION

Fish taken from the area of the oil slick showed gill damage in the form of cell loss. The alterations of branchial filaments discussed herein may produce certain physiological malfunctions. The loss of sufficient acidophiles would reduce the production of carbonic anhydrase-like enzyme. This enzyme deficiency could lead to pH changes in the blood and tissue fluids (acidosis or alkalosis) through a reduction in the rate of dissociation of carbonic acid into CO_2 and hence malfunction of the blood-buffer system.

Loss of mucous cells may result in the clogging of the filaments with detritus. Resultant irritation of the filament may produce epithelial sloughing as that seen in test specimens.



2a. Typical teleost branchial filament



2b. Example of branchial filament which has lost some tissue

Figure 2. Histological Structure of Branchial Filaments

These data are not conclusive with regard to the impact of petroleum fractions on pelagic megafauna because of the migratory nature of these animals; however, the observed damage raises certain critical questions which deserve further investigations under controlled laboratory conditions. Laboratory studies using sublethal quantities of oil and histological techniques should be incorporated into other studies on the fate and effects of marine oil spills and chronic low-level oil pollution. Standard TLM tests coupled with autopsies of terminal animals and histological procedures are also indicated. Such studies would establish direct cause-effect relationships.

Figure 3. Normal Branchial Filament from *Ancyclopsetta*. Note cells between each filament (arrows), mucous cells (M), pillar cells (P), and acidophiles (A).

Figure 4. Branchial Filament from *Ancyclopsetta* taken near SPNW-17. Note loss of epithelial cells (sloughing) between the filaments, giving a rather "clean" appearance. Also, note swollen filaments (S).

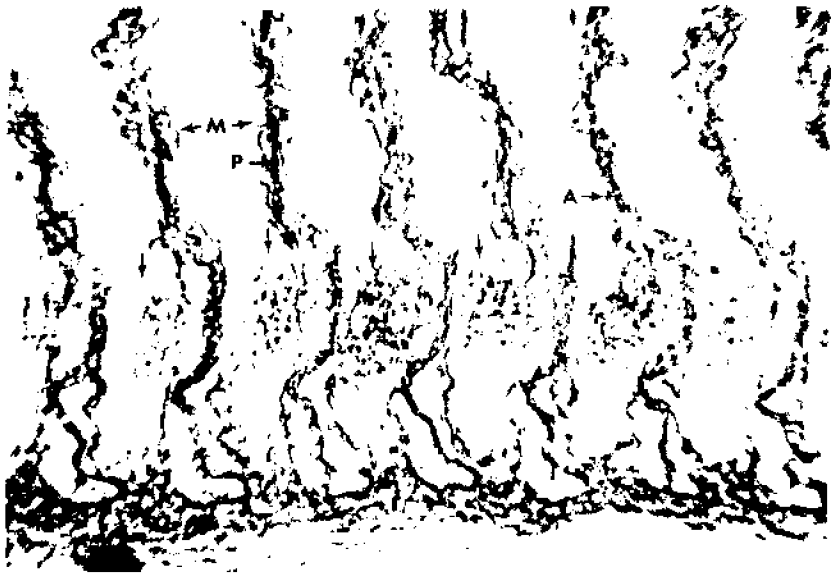


Figure 5. Normal Filaments from *Micropogon*. Structures seen are identical to those in Fig. 2a. Epithelial cells (arrows), mucous cells (M), pillar cells (P), and acidophiles (A).

Figure 6. Filaments of *Micropogon* from Spill Site. Note washed-out appearance due to sloughing (arrows) and swollen filaments (S).

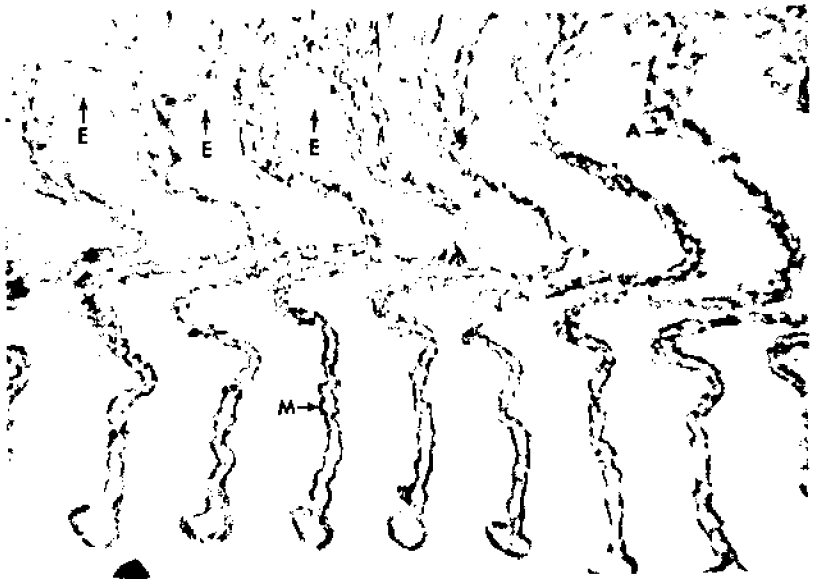


Figure 7. Normal *Etropus* from Aransas Bay. Note many mucous cells (M), acidophiles (A), epithellum (E).

Figure 8. *Etropus* from SPN-8. Note swelling (arrows), fewer mucous cells (M).

Figure 9. *Chloroscombrus* from SPN-8. All cells intact. Swollen filaments (S), acidophiles (A), pillar cells (P), and mucous cells (M).

Figure 10. *Chaetodipterus* from SPN-11. All cells intact. Acidophiles (A), mucous (M), pillar cells (P).

The damage to gills reported herein appears to have resulted from short term exposure to freshly spilled oil. The data, however, are subject to the following limitations: (1) the actual composition of the oil in question is not known since its origin was not determined, however, its odor indicated a short residence time; (2) no data are available at this time concerning such critical subjects as recovery from gill damage; and (3) certain questions arise concerning the migration of the specimens examined. There appears, however, to be an inverse relationship between the amount of gill damage and the speed with which the fish in question usually swims, suggesting a direct relationship between damage and time of exposure (escape time).

ACKNOWLEDGMENT

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INHIBITION OF BACTERIAL CHEMORECEPTION BY HYDROCARBONS

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A wide variety of chemical and physical water pollutants is shown to inhibit microbial chemotaxis. Such chemotaxis inhibition may have direct effects on microbial predator-prey relationships or on the rate of degradation of organic substrates in the sea.

INTRODUCTION

Intraspecies chemical communication in animals is a well-known phenomenon. Its importance in providing signals to determine sexual behavior, territory definition, prey location, and social structure has been demonstrated by Wilson (6). Similarly, bacterial intraspecies and interspecies responses may be dependent upon chemical communication. Adler (1) observed that *Escherichia coli* is attracted to a broad spectrum of organic compounds, most of which were metabolized. Chet, Fogel, and Mitchell (3) observed that motile bacterial predators on the fungus *Pythium*, or on the marine diatom *Skeletonema*, are attracted specifically to exudates of the prey. Such motile bacteria thus exhibit an apparently beneficial chemically-based response to suitable food sources; they are capable of moving in a non-random fashion due to chemotaxis.

The rate of degradation of organic material, and thus the transfer of bound energy, in most ecosystems is catalyzed by bacteria. This rate has been assumed to be dependent only upon bacterial enzyme activity. However, significant populations of bacteria must be maintained in the vicinity of the organic source to maintain enzyme concentrations. Bacterial chemotaxis may provide such a mechanism and may thus increase the rate of degradation of organic matter.

Bacterial predator-prey relationships may also be dependent on chemotaxis. Mitchell (4) has shown that many marine bacteria are predacious on other microbial forms such as phytoplankton, bacteria, and fungi. The populations of these predators and prey are both generally less than 10^4/ml. If one assumes that a single collision is necessary for direct predator-prey interaction, or that a minimum interaction sphere is necessary because of the threshold concentration essential for enzyme activity, then such predator-prey interaction can be viewed in terms of collisions of single gas molecules. The frequency of random collisions, assuming populations of 10^4/ml, is too

small to result in significant die-off in natural waters. Again, bacterial chemotaxis may act to make predator motion non-random and thereby increase collision frequency. This would explain observed rapid die-off rates of such bacteria as *Escherichia coli* in sea water.

In this report is described the effect of a broad spectrum of hydrocarbons on bacterial chemotaxis. Those hydrocarbons which affect chemotaxis at concentrations found in the environment may be ecologically significant in that they may alter predator-prey interactions or the rate of biodegradation of organic substrates without affecting gross bacterial physiology.

METHODS

The motile marine bacteria used in this study were isolated on seawater nutrient agar (Difco) and were cultured routinely in seawater nutrient broth (Difco). The main test organism was an isolate of a motile marine *Pseudomonas* sp.

Chemotactic response was evaluated using the method described by Adler (2) of bacteria capture in microcapillaries. The pollutants were formulated as 10^{-1} M or 20,000 ppm solutions in seawater; these stock solutions were diluted in seawater to yield concentrations as low as 10^{-7} M or 0.002 ppm. The test bacterium was added to each of the dilutions at a concentration of approximately 10^6 cells per ml. These suspensions were immediately placed on a microscope slide. A 0.8% solution of nutrient broth (the attractant) was placed in a 5 µm capillary tube, sealed at one end with agar, and placed in the bacterial suspension. A control capillary containing only seawater was also placed in the suspension. The capillaries were covered with a cover slip. Bacterial attraction was observed microscopically to the mouth of the capillary containing nutrient broth. After ten minutes, the capillares were removed from the suspension. Quantitative estimates of the numbers of bacteria attracted to the nutrient broth were obtained by plating the contents of the capillaries and comparing the number of bacteria captured. The concentration of hydrocarbon which significantly lowered the number of bacteria captured in the capillary containing seawater and no attractant was considered to be the threshold concentration for pollutant toxicity. At concentrations less than this threshold value, the population difference between the control and the attractant capillaries was considered to be an estimate of the sub-lethal effect on chemotaxis of that pollutant concentration.

RESULTS AND DISCUSSION

One marine bacterium, studied in detail, showed a wide variation in chemotactic response in the presence of the hydrocarbons. Table 1 shows the effect expressed as an index of chemotaxis inhibition. The CI_{50} is defined as the concentration of the hydrocarbon which reduced the chemotactic response by 50% without affecting gross bacterial physiology. It was possible to reverse the chemotaxis inhibiting effects of these hydrocarbons by washing the bacteria in fresh seawater. This implies that chemotaxis inhibition is the result of a reversible blocking of chemoreceptors.

TABLE 1

Chemotaxis Inhibition Index for Some Common Hydrocarbons Using a Motile Marine Isolate

Pollutant	Concentration for CI_{50}[a] (ppm)
2,4-D	1.0
o-Chlorobiphenyl	0.5
Kuwait crude oil	10
Kerosene	12
Benzene	0.1
Squalene	1.0
Aniline	1000
Phenol	120
Formalin	115
Chloroform	15

[a]These concentrations result in 50% chemotaxis inhibition without affecting the gross physiology of the bacterium.

The hydrocarbon concentration ranges which affect chemotaxis by 50% are at least a factor of 10 greater than would be expected in natural ecosystems on a routine basis. Table 2 shows the effect of lower concentrations of *o*-chlorobiphenyl on chemotaxis. The concentration of this water pollutant, found by the Corps of Engineers in Massachusetts waters in 1972 (0.004 ppm), decreases bacterial chemotaxis by approximately five percent.

TABLE 2

The Effect of *o*-Chlorobiphenyl on Chemotaxis of a Motile Marine Bacterium

Concentration, ppm	Chemotaxis Inhibition, %
0.	0
0.005	5
0.02	17
0.05	23
0.2	33
0.5	50

The effect of pollutants on chemotaxis has important short- and long-term implications. In the vicinity of a pollutant release, the latter can seriously affect the microbial community by altering predator-prey relationships or degradation rates without directly affecting the gross physiology of

the bacterium. As the spill is dissipated and the pollutant is diluted, the extent of inhibition of chemotaxis will be decreased. Mitchell, Fogel, and Chet (5) observed that if bacteria are washed clean of pollutants, their original chemotactic response level returns. Apparently, the chemoreceptors are blocked by the pollutant. By decreasing the pollutant concentration through dilution, an increased number of chemoreceptors again become active. If, however, a significant concentration of the pollutant remains, chemotaxis inhibition becomes a long-term ecological problem. A portion of the bacterial population will become less efficient in locating food sources, adaptation phenomena may occur, and new, less sensitive, species may develop. The microbial community, both in species diversities and population numbers, must change in response to this new physio-chemical environmental constraint. The impact of this change may be reflected either in the stability of the microbial community or that of the higher organisms as further stress is placed upon the ecosystem by the pollutants.

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DEGRADATION OF CRUDE OIL BY YEASTS AND ITS EFFECTS ON *LESBISTES RETICULATUS*

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High densities of hydrocarbonoclastic yeasts have been isolated from oil-enriched fresh and marine waters (2,5). Certain strains, mainly from sites affected by chronic oil pollution, have demonstrated relatively high rates of oil oxidation and emulsification and the ability to utilize various hydrocarbons (2,4). These observations have suggested that yeasts may possibly be utilized to facilitate degradation of oil pollutants. If high concentrations of microorganisms are dispersed as inocula "seed" into the environment, careful attention should be given to the potential pathogenicity and toxicity of their metabolites for the natural biota. Two hydrocarbonoclastic yeasts, *Candida tropicalis* and *Endomycopsis lipolytica* (perfect stage of *C. lipolytica*) selected for the aforementioned hydrocarbon-utilization activities, have been examined in combination with crude oils for their effects on the mortality of the common guppy, *Lesbistes reticulatus*.

MATERIALS AND METHODS

In all instances, the yeasts were grown in 100 ml of a glucose-peptone-yeast extract medium (GPY) for 72 hours. The cells were separated from the medium by centrifugation and washed three times in sterile aquarium water. An inoculum of 4×10^9 cells of each organism was added to test aquaria containing four liters of water and 1% (v/v) of oil. Both high asphalt-low volatile content Mississippi crude oil and low asphalt-high volatile Louisiana crude oil were tested. Five guppies, *L. reticulatus*, were added to each aquarium and monitored daily for feeding activity and survival. The fish were maintained on a commercial fish food which was added to the surface of the water at a break in the oil slick. All experiments were conducted in triplicate. Control aquaria contained crude oil and fish, crude oil alone, yeasts alone, and fish alone.

Water samples were removed from the tanks at one-day intervals for the first two weeks and periodically thereafter to determine the viable population of yeasts. Samples were streaked on casein media for differentiation of the caseinolytic species, *E. lipolytica* (3) from *C. tropicalis*.

To compare the dispersant properties of the yeasts, actively growing cells from GPY medium, autoclaved cells, and lyophilized cells in concentrations ranging from 10^{10} to 10^{50} cells, were added to 16 x 125 mm tubes containing 0.01% (v/v) crude oil in tap water (10 ml total volume). After agitation, samples were removed from tubes showing optimal emulsification and the average diameters of the dispersed oil droplets were determined.

RESULTS AND DISCUSSION

The addition of yeasts to the aquaria with oil caused partial but immediate emulsification of the oil. The degree of emulsification increased during the 30-day test periods with an obvious disruption of the surface slick and reduction of oil adhesion to the sides of the aquaria. The surface slick of the high asphalt Mississippi crude oil was less extensively affected than that of the low asphalt Louisiana crude. Microscopic examination showed that emulsified oil droplets were surrounded by yeasts, with the lighter colored droplets eventually being penetrated by yeasts. The aquaria were well aerated and the resulting turbulence always kept a part of the oil droplets in suspension. Nevertheless, in still tanks with oil and yeasts, the surface slick was still significantly disrupted and, in contrast to tanks without yeast, significant water was lost through evaporation. In still tanks, the yeasts occurred mainly as hyphae growing in oily surface patches.

The numbers of yeasts isolated periodically from the aquaria are presented in Table 1. The yeasts, after an initial reduction in colony forming units (cfu), maintained a more or less stable population in the aquaria for over 30 days. Higher populations of yeasts were found with the Louisiana crude than with the Mississippi crude. In both instances, *C. tropicalis* was the predominant organism. The guppies survived an average of 11 days in aquaria with Louisiana crude. With the addition of yeasts, the fish survived an average of 12 days. In Mississippi crude, the fish survived an average of 25 days, whereas, with yeasts added, survival was reduced to an average of 13 days. In tanks without oil or yeasts the guppies survived an average of 25 days, whereas with the addition of yeasts the fish survived an average of 28 days. In tests using these same two crude oils with the recommended amount of a biodegradable chemical dispersant, the emulsification was not stable after 96 hours and the oil droplets gradually coalesced. Moreover, 100% fish mortality occurred in less than 2 days.

Emulsification of the crude oils was accomplished by all yeast preparations (Table 2). Cells of *E. lipolytica*, particularly lyophilized and actively growing preparations, appeared to give the better emulsifications with Louisiana crude, whereas *C. tropicalis* gave best results with Mississippi crude. With both oils, however, the most stable emulsification with permanent disruption of surface film, was produced by *C. tropicalis*. Oil added to cell-free GPY culture broth of *C. tropicalis* reacted as if placed on a chemical oil herder.

Water samples from tanks with yeast and crude oil were examined microscopically. In nearly every field examined, numerous amoebae, flagellates, ciliates, *Daphnia* and other micro-invertebrates were observed feeding on the yeasts as well as ingesting small oil globules. In aquaria with oil, but lacking yeasts, approximately one microscopic field in 50 contained invertebrates. The concentration of invertebrate predators in the natural environment in areas enriched with yeasts has been noted (5).

Our closed system laboratory experiments suggest that metabolites or by-products produced during yeast decomposition and emulsification of certain high asphalt crudes may be harmful to fish. In contrast, a readily emulsified and generally less recalcitrant oil may have its toxicity reduced by the addition of yeasts. The extrapolation of these closed system studies to events

in the open environment have not been yet undertaken.

TABLE 1

Growth of Yeasts on Crude Oil

	C. tropicalis		*E. lipolytica*	
	0 days	3-30 days	0 days	3-30 days
	Colony Forming Units (cfu)/L x 10^5			
Mississippi Crude Oil				
Mean	114	9	123	5
Range		1-44 (24)[a]		<1-21 (24)
Louisiana Crude Oil				
Mean	170	12	146	8
Range		<1-51 (24)		<1-47 (24)
Yeasts, No Oil				
Mean	237	2	112	4
Range		<1-10 (23)		<1-19 (23)

[a]Number of samples

TABLE 2

Average Diameter of Crude Oil Droplets Emulsified by Yeasts[a]

	Louisiana Crude				Mississippi Crude			
	C. tropicalis		*E. lipolytica*		*C. tropicalis*		*E. lipolytica*	
Condition of Cells	Diam[b]	Range[c]	Diam[b]	Range[c]	Diam[b]	Range[c]	Diam[b]	Range[c]
	Size in Micrometers							
Autoclaved	101	20-580	82	20-385	63	10-395	55	15-230
Lyophilized	80	15-405	58	10-250	44	10-200	51	10-530
Active	83	15-450	51	10-180	54	10-515	94	10-335
Control (no yeast)	165	50-925			574	20-2870		

[a]Measured by ocular micrometer after agitation; sample from suspended material.

[b]Mean diameter, 25 globules; globules larger than 1 mm not considered.

[c]Size range of globules; globules larger than 1 mm not considered.

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EFFECTS OF SOME COMMERCIAL OIL HERDERS, DISPERSANTS AND BACTERIAL INOCULA ON BIODEGRADATION OF OIL IN SEAWATER

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The effects on petroleum biodegradation of several oil herders, dispersants and commercial bacterial inocula were tested. The oil herders and dispersants significantly increased the rate of mineralization but not the extent of petroleum biodegradation. The beneficial effect is apparently due to the increased surface of oil droplets and an absence of toxicity to oil degrading microorganisms at the recommended concentrations. The two commercial bacterial inocula tested failed to improve either the rate or the extent of oil biodegradation when tested in natural seawater. In sterile seawater, these inocula showed inferior performance compared to the natural microflora of seawater, and were judged to be ineffective for marine applications.

Data on the safety and effectiveness of available oil pollution control products are basic to their correct use. While numerous studies have been conducted on the toxicity of such products to vertebrates and invertebrates (5,6,7), little is known about their effects on the indigenous microflora of the sea. Since microbial degradation is the major natural process for the ultimate destruction of polluting oil, it is essential that this process not be interfered with. The recommended concentrations of two oil herders, six dispersants and two bacterial inocula were tested for their effects on the biodegradation of Sweden crude oil in freshly collected seawater samples.

MATERIALS AND METHODS

A seawater sample was collected in early May off the east shore of Sandy Hook, New Jersey. One hundred milliliter aliquots of the seawater were introduced into a gas train arrangement previously described (2). Oil dispersants or oil herders were added in quantities of 0.05 ml either alone, with phosphate and nitrate, with crude oil, or with phosphate, nitrate and crude oil. Sweden crude oil (gift of Sun Oil Company) was added in 1 ml (800 mg) amounts. The composition of Sweden crude, a paraffinic petroleum, has been described previously (2). Phosphate and nitrate were added from a concentrated solution to give final concentrations of 10 mM NO_3^- and 0.5 mM $PO_4^{\equiv}$. These levels of N and P were previously found to be optimal for the degradation of 800 mg of Sweden crude (3). The oil dispersants tested were Corexit

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7664 (Enjay Chem., New York, N.Y.), Corexit 8666 (Enjay Chem., New York, N.Y.), (gifts of Esso Research, Linden, N.J.), Magnus (Magnus Chemical, Garwood, N.J.), BP 1100 (BP North America Inc., New York, N.Y.), Marine Cleaner 8555 (Enjay Chemical, New York, N.Y.), and Pyraxon (Guardian Chem. Corp., Long Island City, N.Y.) (gifts of EPA, Edison Water Quality Control Lab). The oil herders tested were: Shell oil herder #3 (Shell Oil Company, Tulsa, Okla.) and Smith herder (Werner G. Smith, Inc., Cleveland, Ohio) (gifts of EPA, Edison Water Quality Control Lab., New Jersey).

Two commercial petroleum-degrading bacterial inocula, Ekolo-Gest (National Chem. Corp., Lakewood, N.J.) and DBC bacteria (Gerald Bauer Corp., Calif.) were also tested (gifts of EPA, Edison Water Quality Control Lab., New Jersey). Ekolo-Gest is also marketed under the name Petrobac. The viable numbers of microorganisms in the inocula were checked by plating serial dilutions on marine agar 2216 and on oil agar. The techniques for enumeration of total and oil-degrading microorganisms have been described elsewhere (4).

The bacterial inocula were added to the seawater samples in quantities of 8 mg, alone, with 800 mg of Sweden crude oil, or with 800 mg Sweden crude oil and additional nutrients. The additional nutrients were added either as 0.5 mM $PO_4^{\equiv}$ and 10 mM NO_3^- or as 72 mg of the nutrient source supplied with the bacterial inocula. The bacterial inocula were also added in quantities of 8 mg to sterile artificial seawater medium with 800 mg Sweden crude oil.

All combinations were incubated at 28 C on a rotary shaker at 200 rpm. The flasks were aerated with CO_2-free air at a rate of 15 ml/min. The CO_2 produced from the mineralization of the crude oil and/or the oil dispersant, oil herder or commercial petroleum-degrading bacterial inoculum was monitored continuously (2). After 28 days of incubation the residual oil was extracted with diethyl ether and the percent biodegradation was determined by gas-liquid chromatography as previously described (2).

RESULTS

All of the chemical dispersants and oil herders tested increased the rate of oil mineralization when the seawater was amended with nitrate and phosphate (Fig. 1). None of the oil herders or dispersants were able to replace the need for phosphorus and nitrogen supplementation and no CO_2 production was detected when only the crude oil, dispersant or herder and seawater were incubated without added nitrogen and phosphorus. No CO_2 production was detected from the dispersants or herders incubated with seawater without added crude oil, even when supplemented with phosphate and nitrate.

The most extensive enhancement of mineralization occurred with the two oil herders and with BP 1100 (Fig. 1, G, H, I). These three substances resulted not only in an increase in rate but also in the overall CO_2 production. The extent of biodegradation showed some variation but no significant enhancement as a result of treatment with any of the dispersants or oil herders (Table 1). In all cases, about 70% of the Sweden crude oil was biodegraded within 28 days.

The commercial bacterial inocula tested were totally ineffective in stimulating biodegradation, both in terms of the extent of degradation (Table 2) and the rate or extent of mineralization (Fig. 2). When the inocula were

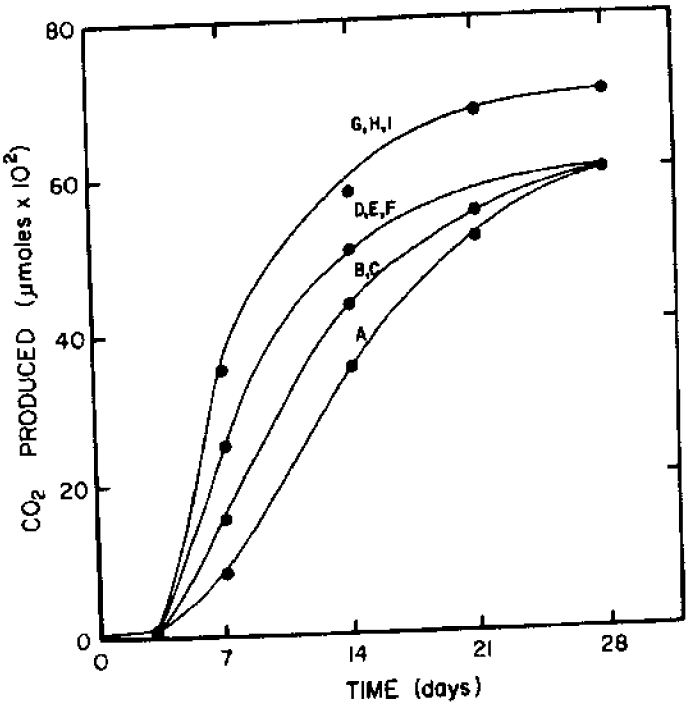

Figure 1. Effects of Some Commercial Dispersants and Oil Herders on Crude-Oil Mineralization by Microorganisms in Natural Seawater. A--Control; B--Corexit 7664; C--Pyraxon; D--Magnus; E--Marine cleaner 8555; F--Corexit 8666; G--BP 1100; H--Smith herder; I--Shell oil herder #3.

TABLE 1

Effect of Some Oil Dispersants and Herders on Crude Oil Biodegradation

Treatment	% Biodegradation
Control (seawater)	70
BP 1100	75
Corexit 7664	67
Corexit 8666	70
Magnus	70
Marine Cleaner 8555	68
Pyraxon	72
Shell Oil Herder #3	70
Smith Oil Herder	75

added to natural seawater, the rate of crude oil mineralization was identical to the uninoculated case (Fig. 2, A, B, C). No mineralization occurred when the inoculum was added without a source of nitrogen and phosphorus. The nutrient source supplied with the Ekolo-Gest inoculum provided sufficient nitrogen and phosphorus to result in identical oil conversion as when 10 mM nitrate and 0.5 mM phosphate were added. The viable numbers of microorganisms were 1×10^9/g Ekolo-Gest inoculum and 5×10^7/g DBC inoculum. The viable number of oil degraders were 3.1×10^4 in the Ekolo-Gest inoculum and 87/g in the DBC inoculum.

TABLE 2

Effect of Seeding with Some Commercial Oil-Degrading Inocula on Crude-Oil Biodegradation

Treatment	% Biodegradation
Seawater control	70
Seawater + DBC bacteria	68
Seawater + Ekolo-Gest	65
DBC bacteria	0
Ekolo-Gest	23

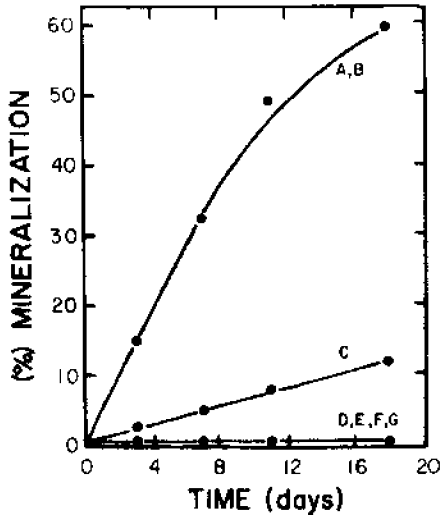

Figure 2. Effects of Seeding with Some Commercial Oil-Degrading Inocula on Crude-Oil Mineralization. A--Natural seawater (control); B--Natural seawater + DBC inoculum; C--Natural seawater + Ekolo-Gest inoculum; D--artificial seawater + Ekolo-Gest; E--artificial seawater + DBC inoculum.

When added to artificial seawater as the sole source of microorganisms, the DBC inoculum did not result in mineralization of the oil (Fig. 2 D) and the Ekolo-Gest inoculum resulted in a rate of mineralization that was much slower than that of natural seawater (Fig. 2 E). The Ekolo-Gest and DBC inocula biodegraded 23 and 0 percent of the crude oil respectively within 28 days (Table 2), which was much lower than the amount biodegraded by the natural microbial population of seawater during the same time period. Additionally, it was revealed by gas-chromatographic analysis that, unlike the natural microbial population of seawater, the microorganisms in the Ekolo-Gest inoculum were unable to degrade any of the branched components of the oil.

DISCUSSION

Treatment of oil spillages with methods that are essentially cosmetic should not interfere with the ultimate removal of the polluting oil from the marine ecosystem. For this reason, the effects of some such measures on the natural removal process of microbial degradation were tested. In addition, the effectiveness of some commercial products designed to stimulate this natural removal process were evaluated.

The term "biodegradation" is used within the context of this study to denote the disappearance of the original petroleum components. The term "mineralization" describes the complete degradation of the organic petroleum components to inorganic substances. The mineralization of hydrocarbons results in the formation of carbon dioxide and water. Although some geologists view petroleum and petroleum hydrocarbons as "minerals," hydrocarbons are properly classified as "organic" by chemical convention.

There are a great number of chemical formulations that have been developed and marketed as oil dispersants (1). Some of them have been found to be toxic to marine life or to increase the toxicity of spilt oil (5,6,7). The dispersants tested in this study were of a variety of types including water-based, petroleum-solvent based, aromatic, and non-aromatic (1). The concentrations used were within the range of concentrations recommended by the manufacturers for treatment of an oil spill.

No toxicity to the microbial population was detected for any of the dispersants tested. Rather, adding a dispersing agent resulted in more rapid mineralization of the oil. A similar enhancement of oil biodegradation by chemical dispersants has been reported by Robichaux and Myrick (8) who tested six dispersants, BDOWH 2, BDOWE 8, BDOWE 7, BDOWB 1, BDOWH 10, and BDOWH X. All six formulations were composed of anionic and nonionic chemicals in organic solvents. Robichaux and Myrick found that addition of these dispersants increased the rate of oxygen uptake by seawater that had been supplemented with phosphate, ammonium nitrogen, and a mixed microbial culture. The increased rate of oil biodegradation in response to the addition of chemical dispersants probably is the result of increased surface area, which makes the oil more accessible.

Recently, oil herders have received much publicity as a method for dealing with spilt oil. On calm waters, oil herders spread on the surface, pushing back the oil and thus facilitating physical collection of the oil. If agitated, however, oil herders may act as dispersants. The two oil herders

tested resulted in a marked increase in the rate of oil mineralization. This effect may have been the result of dispersion of the oil, analogous to the enhancement found with the dispersing agents.

Several commercial microbial inocula have been marketed for combatting oil spills. The microorganisms are sold in a freeze-dried state for "seeding" the oil spill and are supplied with a nutrient source for fertilizing and with a dispersant for increasing the surface area. Unfortunately, none of the inocula tested stimulated oil biodegradation. In fact, the DBC inoculum was totally unable to degrade the crude oil used in this study, and the Ekolo-Gest inoculum degraded the oil at a much slower rate than natural seawater. This does not exclude the possibility of using microbial seeding as one measure for dealing with spilt oil. It does, however, point up the need to test products to insure that they do the job for which they are sold.

Microbial seeding with effective petroleum-degrading microorganisms would be of most value when an oil spill occurs where the natural oil-degrading microbial population is low. Such areas would probably include the mid-oceans and the cold oceans of the world. In coastal waters, microbial seeding would probably have to be done with active rather than with freeze-dried inocula so as to minimize the lag period prior to the onset of oil biodegradation.

ACKNOWLEDGMENT

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BACTERIAL SEEDING TO ENHANCE BIODEGRADATION OF OIL SLICKS

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Evaluations have been made to determine the feasibility of adding selected hydrocarbon-oxidizing bacteria and required nutrient salts to an oil slick to enhance biodegradation of the polluting hydrocarbon. Fifty active mixed cultures were isolated. Culture characteristics including cell morphology, temperature tolerance, and resistance to chemical dispersants were determined. Biodegradation of adsorbed vs. non-adsorbed oil was compared, and media for mass cultivation of organisms was developed.

Simulated field, or tank, experiments showed the effectiveness of microbial seeding varied more with the type and quantity of crude oil used than with such factors as inoculum density or nutrient salt concentration. Initial 24-hr oil losses were generally twice as large in bacterial seeded tanks relative to uninoculated controls. Biological Oxygen Demand and Total Organic Carbon analyses of tank water indicated little metabolic product pollution of the water.

INTRODUCTION

Our environment has been exposed to naturally occurring hydrocarbons such as submarine seeps, and plant and animal oils through much of geologic time. During this period natural processes such as microbial decomposition and photochemical oxidation have prevented a significant accumulation of hydrocarbons in the ocean (29).

About fifty years ago, with the conversion from coal to oil as a major source of power and manufacturing, increasing amounts of oil and oil products have been released to the environment. Much of this is returned to the sea where it evaporates, emulsifies, sinks, or is beached ashore, all the while being slowly decomposed by sunlight and microorganisms.

Blumer (1) stated that tar on the sea surface now exceeds the amount of surface plant life, and estimated that oil influx to the oceans from shipping losses alone amounts to about 10^{12} grams/year. Other sources, i.e., sewage, manufacturing, and incomplete or 2-cycle combustion, may add considerably to this value. More alarming is the fact that a single accident can account for most of this spillage. For example, the Santa Barbara mishap released an estimated 10^{10} grams of crude oil into the sea, and the "Torry Canyon" lost 10^{11} grams of oil. When vast quantities of crude oil are so

released, the detrimental effects on terrestrial or marine life are acute.

Stringent enforcement of government regulations dealing with industrial pollution and shipping procedures can reduce careless or negligent oil pollution; however, there still remains the problem of adequate cleanup methods for accidental spills which, considering the volume of oil transported over water (approximately 10^{15} grams/year), will inevitably occur.

Proposed methods for cleaning up accidental spills in the marine environment include physical removal, chemical dispersal, adsorption to solids, ignition of the oil on the sea surface, and seeding the area with hydrocarbon-oxidizing bacteria (23). Each of these methods has obvious limitations, depending primarily on the spill location, the condition of the sea, and the type and quantity of oil spilled. An optimal procedure for cleaning up oil spills would involve actual physical removal of oil from the sea surface. An array of cleaning equipment, from suction pumps to magnetic oil adsorbents, have recently been introduced. The efficacy of physical removal, however, is largely dependent on the condition of the sea and the extent and thickness of the oil slick (26). Burning the oil on the sea surface has met with limited success due to the rapid transfer of heat to the water and the loss of volatile components through evaporation and dilution (24). The use of chemical dispersants has been severely limited because of their toxicity to marine life (11,18,19,21). The most universally accepted method of oil removal is still adsorption onto straw and recovery (7), but this is time consuming, costly, and often inefficient once the oil reaches the shoreline (22).

Considerable research has been devoted to the study of microbial hydrocarbon metabolism (28,4,13,25,3,2), but few if any investigations have been carried out to determine the possibility of cleaning up oil spills by seeding with hydrocarbon-degrading microorganisms and nutrients (23,16).

In areas subjected to chronic oil pollution, such as Cook's Inlet, Alaska, populations of 10^4 hydrocarbon-oxidizing bacteria per liter (about 10% of the total microbial population) are estimated to be present the year around (10). However, in unpolluted areas, oil-degrading microbial populations are usually low; furthermore, it has been estimated that periods of one to two weeks are necessary for bacteria to colonize the oil-water interface, and another two or three months for decomposition (16). ZoBell (29) noted that out of several hundred 10 to 50 ml samples of seawater collected beyond the continental shelf, fewer than 5% contained bacteria able to oxidize liquid hydrocarbons. However, he states that oil-oxidizers in the vicinity of coastal settlements or oilfields ranged from <1 to 10^8 cells per ml of seawater or gram of mud. In addition to the number of naturally-occurring oil-degrading microorganisms, the rate of natural decomposition is affected by water temperature, agitation, type of oil, and nutrient concentration. Removal of oil from the sea surface by microorganisms can be accomplished primarily through one, or both, of the following mechanisms: (1) direct oxidation for carbon and energy to CO_2 and cell material, or (2) conversion of oil to surfactants such as fatty acids which emulsify more resistant crude oil components (12).

The purpose of this research has been to determine the feasibility of seeding an oil spill area with selected cultures of bacteria and nutrients to accelerate natural degradation and emulsification of the polluting oil. In view of this being a feasibility study of an applied research problem,

numerous experiments were carried out in order to assess the potential of bacterial seeding as an oil cleanup method. The paper has been divided into two sections in order to preserve continuity, and to show an orderly sequence of experimental work: (1) The isolation and characterization of oil-degrading microorganisms, as well as laboratory experiments using closed flask systems, and (2) the development of seeding and sampling techniques for, and results of, experiments carried out in large outdoor ponds used to simulate environmental conditions.

Controlled laboratory studies were used to determine the characteristics and oil-degrading properties of isolated cultures. Investigations were designed with future field application in mind and included such studies as (a) the effects of temperature, aeration, and agitation on rates of microbial degradation of oil; (b) a determination of conditions to bring about more immediate oxidation of oil (i.e., lag time decrease); and (c) development of optimal conditions for mass cultivation of seed organisms. The potential lethal effect of sunlight (and UV radiation) on microorganisms exposed at the oil-water interface was investigated. In addition, the toxicity of commercial dispersants was determined, and biodegradability of adsorbed vs. nonadsorbed oil was compared.

The simulated field experiments were generally designed to verify lab results. Techniques were developed to measure changes in the quantity and composition of seeded and control crude oils. These included bacterial assay procedures, chromatographic analyses, surface tension measurements, total organic carbon determinations, and oil weight differences. The low oil to seawater ratio used for all simulated field experiments permitted an evaluation of possible metabolic product pollution, i.e., secondary pollution. The observed and measured changes resulting from microbial degradation of five test crude oils in these tank experiments were considered to constitute a reliable basis from which to project the limitations as well as the potential of bacterial seeding as an oil cleanup method under actual field conditions.

Since many similar studies have been presented by numerous investigators within the past year (6,17,27), only data which will enhance this body of literature will be given here. Additional experimental data are reported elsewhere (8,9,14,15).

METHODS

Isolation of Cultures.--Bacterial cultures which rapidly degraded crude oils were isolated from a variety of soils and water, continuously exposed to hydrocarbons for several years. Isolation methods, employing nutrient salt-enriched seawater (ESW) overlain with a hydrocarbon substrate, have been previously reported (14).

All flasks used in isolation and laboratory experiments were modified with indentations around their bases to increase physical emulsification of the oil when samples were incubated on a rotating shaker table. Laboratory experiments were conducted in closed flasks containing 50 ml ESW and capped with aluminum foil unless otherwise noted.

Laboratory Experiments.--Ultraviolet radiation was provided by a 20-watt germicidal lamp installed 18 inches above exposed samples in a sterile

incubation box. Both UV radiation and sunlight intensity were measured with a YSI model 51 radiometer.

Adsorbents tested were commercially available clays and chemicals, as well as oats straw. In some experiments montmorillonite was first boiled for 15 min in 1 N NH_4Cl, then rinsed with distilled water, dried, and ground to a fine powder before being applied to an oil film.

Simulated Field (Tank) Experiments.--Various homemade and commercial oil booms were used in an attempt to contain bacteria-seeded oil films in offshore field experiments. Unfortunately none of the containment devices proved satisfactory. High winds and wave action invariably either destroyed the booms or washed the oil out in the course of an experiment. Large plywood epoxy-coated tanks (4' x 4' x 2') with provision for flow through circulation of fresh seawater via submersible pumps were therefore used for field tests. Experimental details concerning application of cells and nutrient salts, sampling procedures, and analytical techniques employed in these studies using a variety of crude oils have been reported elsewhere (9,15).

RESULTS

Isolation, Maintenance, and Characterization of Oil-Degrading Microorganisms.--Of the 50 mixed cultures initially isolated, two distinct types of hydrocarbon-degrading bacteria were observed. Certain of the mixed cultures grew and remained at the oil-water interface, leaving a flaky residue of oil which quickly floated to the surface after the flask was shaken. The aqueous phase of these reaction flasks remained clear. More often, however, microbial oxidation resulted in extensive emulsification of the oil, which only slowly came to the surface when left undisturbed. The aqueous phase contained numerous cells as evidenced by turbidity and microscopic examination. Since the majority of mixed cultures exhibited this second type of growth, and because the resulting oil emulsification was more extensive, these cultures were used for most laboratory and simulated field experiments.

Fifty mixed cultures originally isolated were preserved for laboratory and field use by freeze-drying. Twenty of these stock cultures were reconstituted in ESW plus oil after five months to check viability and oil-degrading characteristics. Fifteen of the twenty cultures grew as they had prior to lyophilization, i.e., remaining at the oil-water interface leaving a flaky residue, or resulting in more extensive oil emulsification with numerous cells in the aqueous phase. Of the remaining five cultures, three degraded crude oils differently (type one to type two), while two did not survive or had lost their ability to degrade crude oil.

Repeated attempts to isolate pure cultures which consistently degraded crude oils were unsuccessful. Often single isolates from a mixed population were unable to effectively degrade crude oil even when recombined. Therefore, since mixed bacterial populations consistently degraded crude oils in a characteristic manner, and were easily maintained on ESW+agar slants, they were used for both laboratory and field experiments. Data given here refer specifically to the BHMO culture mentioned below, a mixture of *ca* 14-15 morphological types probably comprising a number of different species.

Crude Oils and Pure Hydrocarbons Oxidized by a Single Mixed Culture.--One active mixed culture, BHMO (BH--Boston Harbor-isolated; MO--mineral oil-grown), was tested for its ability to oxidize a variety of crude oils and pure hydrocarbons. One-tenth ml of a 24-hr ESW plus oil culture, centrifuge-havested and resuspended with sterile seawater to an O.D. of 1.0, was added to ESW flasks containing 1% of the test substrate. Optical density of the samples, containing emulsified oil and cells, was determined after 3 days. Results are presented in Table 1.

Although chemical analysis (i.e., heptane, methanol, CCl_4 eluates) of the compounds in each crude oil were not determined, the most rapid and extensive emulsification of oil was observed to occur in those crudes which had a low viscosity and smelled strongly of gasoline.

Sunlight and Ultraviolet Radiation.--Sterile Louisiana crude oil was added to three of four white enamel trays filled with 0.45μ membrane-filtered seawater. The fourth tray served as a control with no oil film. Each tray was inoculated by applying a dense (1.0 O.D.) cell suspension of BHMO cells to the surface. Samples were collected from the interface with disposable syringes initially and after three hours exposure to bright sunlight (8×10^5 ergs/cm^2-sec). Results are shown in Table 2.

The lower initial concentration of cells in the control tray without oil was due to the dilution of bacteria throughout the water, whereas cells applied to the oil films remained principally at the oil-water interface. Exposure for three hrs to bright sunlight apparently was not lethal to these microorganisms.

The UV screening effect of oil films was determined by exposing cells at the oil-water interface to radiation from a 20-watt germicidal lamp. A sterile loop was used to collect samples from the oil-water interface. Results are seen in Table 3.

Concentration of Cells at the Oil-Water Interface.--Sunlight and UV studies had indicated that seeded bacteria remained concentrated at the oil-water interface. The following tests were designed to quantify these observations. In the first experiment, a 1.0 O.D. BHMO cell suspension was sprayed onto the surfaces of three oil films (Venezuelan, Louisiana, and California crudes), each approximately 10μ thick. The cells were applied using a chromatographic atomizer with the pressure adjusted so the oil films were not broken. Accurate direct microscopic counts of cells remaining at the oil-water interface could not be determined as most of the cells adhered to oil droplets. However, it was estimated that at least 100 to 1000 times more cells were concentrated at the interface than were in the seawater. Gentle spraying consistently resulted in higher concentrations of cells at the oil-water interface than did vigorous spray intensities which broke the oil films.

In a similar experiment seawater was first inoculated with hydrocarbon-oxidizing bacteria, then the oil was added. The flasks were vigorously agitated for one minute and samples from the interface and from the water phase were examined microscopically. Again, the bacteria concentrated at the interface were 100-fold over numbers in the water phase.

TABLE 1

Oxidation of Crude Oils and Pure Hydrocarbons by Mixed Culture BHMO

Crude Oil Well location No.[b]	Score[c]
5	+
6	+++
7	+++
8	++
10	+
13	+
15	0
16	-
17	+
18	+
19	-
20	+
21	+++
23	+
25	+
26	-
27	++
28	+
29	+
30	++
Pure Hydrocarbons	
pristane	+++
butylamine	-
toluene	-
dodecane	+
cyclohexane	-
hexadecane	+
cyclooctane	-
2,2,4 trimethylpentane	-
decane	+

[a] Flasks were incubated at 30 C and agitated at 160 rpm for 3 days.

[b] Well locations are listed in the appendix to Table 1.

[c] 0 = no measurable turbidity in the water but the oil becomes flaky; - = O.D. less than 0.01; + = O.D. 0.01 to 0.10; ++ = O.D. 0.10 to 0.80; +++ = O.D. greater than 0.80.

TABLE 2

Effect of Sunlight on Survival of BHMO Cells[a]

Sample	Time[b]	
	0 hr	3 hr
#1 oil--exposed	10^6	10^6
#2 oil--exposed	10^6	10^6
#3 oil--not exposed	10^6	10^7
#4 no oil--exposed	10^4	10^3

[a]Cells were applied in a gentle spray which did not break the surface oil film. Film thickness was calculated to be approximately 1μ in tray one, and approximately 2μ in trays two and three.

[b]Values refer to the numbers of viable hydrocarbon-degrading cells at the oil-water interface as determined by dilution tube technique.

Inoculum as a Nutrient Source.--The following experiment was designed to determine whether application of a large number of cells, without additional nutrient salts, would facilitate rapid oxidation of crude oil. BHMO cells grown in ESW plus Kuwait crude oil were centrifuge-harvested, resuspended in sterile seawater to an O.D. of 8.10, and used as inoculum. Flasks were prepared by adding different amounts of inoculum to sterile non-enriched seawater to give a final volume of 50 ml. Control flasks contained identical volumes of heat-killed cells. One-tenth ml sterile Kuwait crude oil was added to each sample, and the flasks incubated at 25 C and 160 rpm. Results are shown in Table 4. As noted, the degree of oil emulsification in unenriched seawater resulting from microbial utilization of cell nutrients, relative to emulsification resulting from surfactants in the inoculum (control samples), was neither large nor predictable.

Adsorbents: Effects on Microbial Oxidation of Oil.--An experiment was designed to compare the fate of adsorbed vs. non-adsorbed oil in inoculated ESW beakers. Ammonium stearate, straw, montmorillonite, kaolin, and Fullers Earth were tested. The clays were treated with Siliclad (TM for a water-soluble silicon solution manufactured by Clay-Adams Co.), dried, and ground to a fine powder before being applied. Enough material was added to respective beakers to adsorb the oil film as completely as possible. Two of the adsorbents (kaolin and Fullers Earth) were relatively ineffective in soaking up the oil. Therefore, quantitative data on the comparative rates of crude oil oxidation could not be determined, since non-adsorbed oil was readily metabolized.

TABLE 3

The Effect of Ultraviolet Radiation on Oil-Oxidizing Microorganisms[a]

Crude Type	Sample	Irradiation Time[b] 0 min	1 min	5 min
Louisiana crude	1--no oil film	+	-	-
	2--1μ oil film	+	-	-
	3--2μ oil film	+	+	-
	4--3μ oil film	+	+	+
California (E-76) crude	1--no oil film	+	-	-
	2--1μ oil film	+	+	+
	3--2μ oil film	+	+	+
	4--3μ oil film	+	+	+
Venezuelan crude	1--no oil film	+	-	-
	2--1μ oil film	+	+	+
	3--2μ oil film	+	+	+
	4--3μ oil film	+	+	+

[a]One ml of a 0.5 O.D. BHMO culture was added to each 30 ml sample of sterile seawater contained in 100 mm diameter petri plates. Oil was added, and the samples were gently swirled to concentrate cells at the interface.

[b]Survival was determined by inoculating the loop samples into ESW dilution tubes overlain with the respective sterile crude oil. Results were scored positive if oil emulsification occurred after 3 days incubation at 22 C.

Extensive oil emulsification was observed in inoculated control samples containing no adsorbent, and in the beakers in which the oil had been only poorly adsorbed (i.e., kaolin and Fullers Earth). Materials which were most effective in adsorbing oil (ammonium stearate, montmorillonite, and straw) showed minimal bacterial degradation.

These three adsorbents were further tested for their effects on microbial degradation of hydrocarbons. Crude oil films in pairs of inoculated ESW beakers were adsorbed with exactly three times their weight of ammonium stearate or montmorillonite, or five times their weight of straw. One of each pair was "poisoned" with $HgCl_2$. Inoculated controls contained only ESW and oil. All beakers were incubated at 22 C and 160 rpm. Optical Density (O.D.) and Total Organic Carbon (T.O.C.) were determined for samples at 24, 60 and 108 hours. Results are presented in Table 5 with bacterial populations shown in Table 6.

TABLE 4

The Effect of Cell Concentration on the Microbial Oxidation of Kuwait Crude Oil in Non-Enriched Seawater

Inoculum Volume	Optical Density[a] 48 hours	Change in O.D. after 1 min[b]
0.5 ml	0.50	0.04
0.5 ml (duplicate)	0.63	0.06
0.5 ml (control)	0.45	0.08
3.0 ml	0.71	0.02
3.0 ml	0.83	0.05
3.0 ml (control)	0.63	0.00
5.0 ml	1.62	0.00
5.0 ml	1.62	0.00
5.0 ml (control)	1.77	0.04
10.0 ml	2.82	0.00
10.0 ml	2.36	0.00
10.0 ml (control)	3.60	0.00
0.00 ml (control)	0.43	0.03
0.5 ml in 50 ml ESW	5.84	0.00

[a]Flasks were vigorously shaken and samples withdrawn immediately.

[b]Stability of the emulsions was indicated by the change in optical density of vigorously agitated samples after settling for one minute.

These results showed that adsorbed oil was slowly degraded relative to oil floating on the surface. This was presumably due to the greatly decreased surface area of the adsorbed oil. While the O.D. values for straw indicated rates and extent of degradation comparable to control values, it was not possible to separate microbial growth and emulsification of oil resulting from the utilization of dissolved organics leached from the straw from oxidation of the oil itself. The T.O.C. data more accurately reflected degradation of the oil itself, as indicated by the much larger carbon values relative to O.D. in the inoculated controls as compared to the straw-adsorbed samples.

Since straw is such a widely used oil spill cleanup material, the following experiment was designed to quantify the microbial oxidation of crude oil adsorbed to straw. Straw was added to pairs of beakers containing accurately weighed amounts of oil overlying sterile ESW. One of each pair was poisoned with $HgCl_2$. One pair in each series contained only cells and oil and served as inoculated controls. Beakers were incubated at 22 C and 160 rpm. After three days the contents of each beaker (straw and water)

extracted with benzene, the solvent evaporated, and the residue weighed. Results seen in Table 7 are presented as the percent difference between inoculated and control (poisoned) samples of benzene extractable material.

TABLE 5

The Effects of Adsorbents on the Rate and Extent of Oil Oxidation by BHMO

Oil	Adsorbent[b]	Optical Density (Total Organic Carbon)[c] 24 hrs	60 hrs	108 hrs
Bunker C	Montmorillonite	0 (-.13)	0.0 (1.28)	2.5 (6.64)
Bunker C	Ammonium stearate	0 (1.51)	0.0 (3.79)	2.5 (4.05)
Bunker C	Straw	14 (4.32)	25.5 (21.36)	27.0 (27.41)
Bunker C	Control	0 (0.04	5.0 (17.80)	31.5 (75.06)
California	Montmorillonite	0 (-2.65)	0 (2.60)	1.0 (12.04)
California	Ammonium stearate	1.0 (3.14)	1.0 (15.14)	5.0 (14.53)
California	Straw	17.5 (-26.76)	12.5 (-2.31)	9.0 (18.42)
California	Control	0 (4.17)	8.0 (22.71)	24.0 (50.11)

[a]Beakers were inoculated with 0.1 ml of a 0.5 O.D. BHMO culture.

[b]Montmorillonite was treated with Siliclad and dried.

[c]Optical density and total organic carbon (ppm carbon) values are presented as the difference between inoculated and control ("poisoned") samples in each pair. Samples were withdrawn from the middle of each beaker after settling for 10 min. Negative T.O.C. values represent samples in which the control value exceeded the inoculated value.

Under these closed flask conditions, the adsorbed Kuwait crude was microbially degraded as was the non-adsorbed oil. However, the more viscous Bunker C fuel was only slightly oxidized when adsorbed to straw, relative to the control.

Simulated Field (Tank) Experiments.--The experimental parameters for a typical tank experiment are given in Table 8 and results presented in Figure 1. Data on Figure 1 are surface oil samples collected using the glass slide technique (9).

Samples taken the first day showed the characteristic rapid rate of oil loss in the inoculated, nutrient-enriched tank, No. 5. Loss of oil in tank No. 2, containing lyophilized cells only, without nutrients, was comparable to oil loss in the control tank, No. 4. Only one of the two pumps was operated in tank No. 1 during the first day. The difference in evaporative losses between tanks No. 1 and No. 4 reflects the importance of surface turbulence in promoting evaporation.

TABLE 6

Oil-Degrading Bacterial Populations Developing in ESW Beakers Containing Adsorbed Oil

Oil	Adsorbent	Bacterial numbers[a] 24 hrs	108 hrs
Bunker C	Montmorillonite	10^3	10^4
Bunker C	Ammonium stearate	10^4	10^3
Bunker C	Straw	10^5	10^3
Bunker C	Control	10^4	10^6
California	Montmorillonite	10^4	10^4
California	Ammonium stearate	10^5	10^4
California	Straw	10^5	10^5
California	Control	10^6	10^8

[a]Populations were estimated using the MPN technique.

TABLE 7

Microbial Oxidation of Oil Adsorbed on Straw

Oil	Adsorbent	Percent loss of oil
Kuwait	Straw	42.2
Kuwait	Treated straw[a]	51.9
Kuwait	None (control)	38.1
Bunker C	Straw	3.3
Bunker C	Treated straw	13.0
Bunker C	None (control)	27.9

[a]Straw chopped up and leached in successive changes of boiling seawater. Although much of the water soluble material was removed, additional leaching took place during the experiment as evidenced by a yellow coloration of the water.

By the third day, oil in all tanks had a parchment-like texture and extremely uneven distributions. Quantitative sampling was not possible. However, the asphaltic residue in each tank was easily collected completely by moving a glass slide through the film (9). The residual oil in each tank was thus collected and presented as third day samples in Figure 1. The data show a greater loss of oil in the inoculated, nutrient-enriched tanks (Nos. and 3) relative to the control (No. 4), and the nutrient-deficient (No. 2) tanks.

TABLE 8

Experimental Parameters for a Typical Tank Experiment (No. 6) Using Venezuelan Crude Oil[a]

Tank No.	Application	Treatment
1	Initial	50 ml Venezuelan crude only
	1 day	200 ml BHMO culture (10^{13} cells/ml)
	1 day	200 ml 10% Ammonium sulfate solution
2	Initial	50 ml Venezuelan crude oil 2 gm lyophilized BHMO culture re-suspended in 200 ml seawater
3	Initial	50 ml Venezuelan crude oil 200 ml BHMO culture (10^{13} cells/ml) 200 ml 10% Ammonium sulfate solution
	1 day	200 ml 10% Ammonium sulfate solution
4	Initial	50 ml Venezuelan crude only 200 ml 10% Ammonium sulfate solution
	1 day	200 ml 10% Ammonium sulfate solution

[a]Fresh seawater was added at the rate of 1 L/min which replaced the volume of each tank twice a day. Ammonium-N concentration was approximately 9 mg/l initially, in all tanks, and 5 mg/l after one day and 4 mg/l after three days in tanks 1, 3, and 4. Film thickness was calculated to be approximately 50μ in each tank.

The residual oil which could not be collected with the slides left a thin film on each tank. It was observed that the oil films in the seeded tanks (Nos. 1, 2, and 3) were no longer visible after two days, whereas an iridescent film remained visible in the control tank (No. 4) for several days.

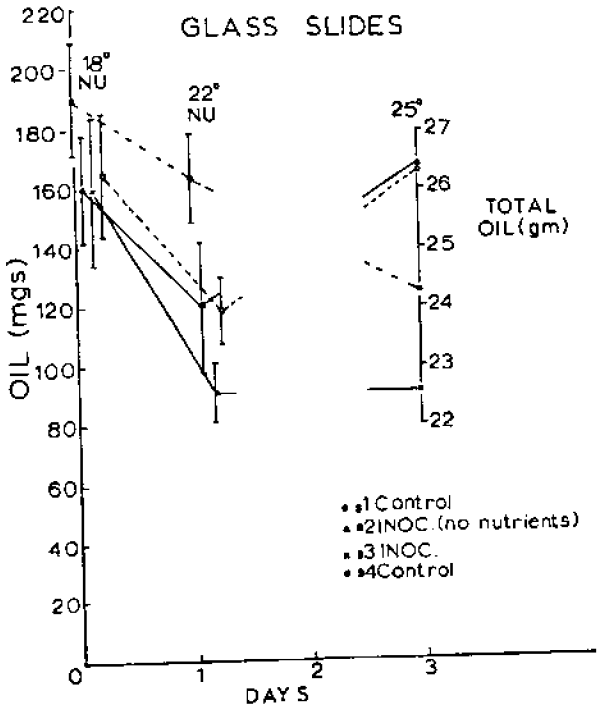

Figure 1. Glass Slide Sample Data for Unevaporated Venezuelan Crude Oil Experiment No. 6 (Table 8). Vertical lines represent standard deviations for each sample point. Surface temperatures are noted at each sample point. NU refers to nutrient application. The glass slide technique has been described elsewhere (9).

DISCUSSION

Mixed bacterial cultures more effectively degraded a variety of crude oils and pure hydrocarbons than did single isolates from these mixed populations. This observation is consistent with the conclusions of several authors (20,4,3,29) that the complexity of crude oils requires a diversity of microorganisms capable of attacking various oil components as well as metabolic products.

Lyophilized mixed cultures displayed a 4 to 5 day lag period before actively degrading oil, in contrast to a one-day lag for cells from ESW plus oil agar slants. Since laboratory experiments showed that cell concentration was an important factor in reducing lag time in ESW plus oil flasks (15), the low number of cells surviving lyophilization (5) might explain the increased lag time before active oil degradation occurred. These results indicated that, although bacteria highly active for hydrocarbons could be easily "stockpiled" by lyophilization, a more active culture in terms of immediate

oil degradation could be obtained in large quantities within 24 hours by mass cultivation in industrial fermenters.

Data from sunlight and UV radiation tests showed that oil-degrading cells at the air-water interface were not affected by intense sunlight, and were afforded protection from lethal UV radiation when trapped at the oil-water interface.

Laboratory experiments with oil-adsorbing materials demonstrated that the rate of microbial degradation of crude oils is greatly decreased when oil is adsorbed, relative to an oil film floating on the surface. This decrease is presumably due to the decreased surface of oil exposed to bacterial action as the adsorbed oil aggregates. These results contrast with reports of increased bacterial activity on suspended particulate materials, which was attributed to the concentration of dissolved organics on the particles, and the stability of the microenvironment which afforded surface area for efficient enzyme activity (30).

In dealing with an oil spill, however, the concentration of available carbon on surfaces is less important than in an unpolluted environment where carbon may be low. The natural dispersion of oil due to wind and waves forms oil droplets which themselves become sites for increased bacterial activity without the oil being adsorbed on particles.

Simulated field experiments using tanks showed a general trend for all crudes tested. An initial rapid loss of oil occurred in both seeded and control tanks due to evaporation of low molecular weight components in the crude oils. Additional oil loss during the first day in the inoculated tanks due to microbial oxidation resulted in a total oil loss for the first 24 hrs approximately twice as large as control tank losses. Rates of oil loss in inoculated and control tanks after the first day were usually similar, indicating that sampling techniques could no longer differentiate between oil lost due to evaporation and that lost as a result of microbial oxidation of the residual weathered oil. These observations are similar to laboratory results from ESW flask experiments where a decrease in the rate of oil degradation coincided with depletion of biologically labile low molecular weight *n*-paraffins, C-12 to C-20 (9).

Seeded oil films lost their cohesiveness and "sticky" property within the first day following application of cells and nutrient salts. These observations complemented earlier laboratory studies which showed that oil emulsified via bacterial activity in ESW flasks lost its "stickiness," or ability to coat beach sand (15).

Laboratory experiments using ESW flasks resulted in extensive emulsification of the test crude even though sometimes as little as 25% of the crude oil was converted to non-extractable (with benzene) products (15). Tank experiments, however, indicated little emulsification of degraded oil either at the surface or in the water column. Biochemical oxygen demand (BOD) data and Total Organic Carbon (T.O.C.) analyses of water samples showed only slight increases in carbon in the water, suggesting that little emulsification of the surface oil, or solution of water soluble metabolic products occurred during the experimental time periods.

The general conclusion drawn from the tank experiments, therefore, was that microbial seeding could measurably enhance crude oil degradation

within a 24-48 hr time period provided a sufficient nutrient salts concentration could be maintained at the oil-water interface. However, the nutrient salts concentrations required are 100 to 1000 times the values typically reported for inshore waters.

Since laboratory results and tank experimental data indicated that an addition of nutrient salts was required to effect observable microbial degradation of an oil slick within 24 to 48 hrs, a search was begun for a method of supplying these salts at the oil-water interface. The possibility of periodic application of water-soluble salts was first considered. However, a preliminary experiment carried out at the laboratory boat basin on a calm day showed that a concentrated nutrient salt solution, gently sprayed onto the water, was no longer detectable at the air-water interface after 15 min.

The next approach was to evaluate materials which could be used to encapsulate water-soluble nutrient salts, and which would also remain at the oil-water interface. After screening several potential materials, ordinary paraffin wax was examined as a potential encapsulating material. Paraffin wax is relatively inexpensive ($0.10 per pound), has not been shown to be toxic, and adsorbs oil. Thus the wax-salt composite ideally should both adsorb the oil and provide a constant source of nutrient salts to increase the rate of microbial degradation. Several wax-salt composites are presently being tested for their nutrient release capabilities and oil adsorbing properties.

Although laboratory experiments showed adsorbed oil to be more slowly degraded relative to finely dispersed oil droplets or a floating oil film, perhaps certain of the more biologically refractory components in crude oils (e.g., high molecular weight polynuclear aromatics) might be more rapidly degraded if adsorbed onto a wax-salt composite than if dispersed in nutrient-poor water. Research is presently being carried out to determine the fate of selected high molecular weight crude oil components when the oil is adsorbed to wax-salt composites in non-nutrient enriched seawater.

At the present time, microbial seeding of oil slicks might be realistically viewed: a) as a useful cleanup method to be used alone on relatively thin oil films covering large areas where physical removal is not possible or practical, or b) to be used in conjunction with other cleanup operations such as mechanical removal, chemical dispersion, adsorption, or burning--perhaps as a final treatment to cleaning up residual oil. Using microorganisms to treat only thin films would also lessen requirements for additional nitrogen and phosphorus.

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APPENDIX TO TABLE 1

Locations of Crude Oil Wells

Container No.	Sample Designation	Age	Formation	Field	Location
1	L2-127	Pleistocene	"B" Sand	Ship Shoal Blk 32	Terrebonne Parish, La.
2	L2-132	Pleistocene		274 A Platform	Houma Par., La.
3	L2-133	Pliocene	Bul. (1) A-5	S.S. 208	Vermillion Par., La.
4	T2-76	Pliocene	West Flank	High Island	Galveston Co., Texas
5	T2-86	Miocene	S.E. Flank	Spindletop	Jefferson Co., Texas
6	L2-175	Miocene		W. Hackberry	Cameron Par., La.
7	T2-568	Oligocene	N-1 (A-4)	E. Hastings	Brazoria Co., Texas
8	L2-179	Oligocene	Mang Howell	W. Hackberry	Cameron Par., La.
9	L2-180	Eocene	Wilcox-Minter	Five Mi. Bayou	Avoyelles Par., La.
10	T2-409	Eocene	Yegue	Hockley Dome	Harris Co., Texas
11	W4-168	Paleocene	Ft. Union	Big Piney	Sublette Co., Wyo.
12	M8-107	Cretaceous	Muddy	Bell Creek	Powder River Co., Mont.
13	M6-211	Cretaceous	Rodessa	Shangelo Crk.	Smith Co., Miss.
14	M6-214	Jurassic	Smackover	Nancy	Clarke Co., Miss.
15	C3-39	Jurassic	M. Brush Basin	Rangley	Rio Blanco Co., Colo.
16	C2-30	Triassic	D-Zone	So. Sturgin Lake	Alberta, Canada
17	N8-78	Triassic	Spearfish	Newberg	Bottineau Co., N.D.
18	T2-554	Permian	Wolfcamp	Wildcat	Ward Co., Texas
19	W4-318	Permian	Minnelusa	Wildcat	Crook Co., Wyo.
20	N5-127	Pennsylvanian		Ranger Lake	Lea Co., N. Mex.
21	N8-59	Pennsylvanian	Tyler	Medora	Billings Co., N.D.
22	N8-75	Mississippian	Madison	NW Hope	Bottineau Co., N.D.

APPENDIX TO TABLE 1 (cont'd)

Container No.	Sample Designation	Age	Formation	Field	Location
23	K1-29	Missis-sippian	Warsaw	W. Fralick	Kiowa Co., Kans.
24	N5-138	Devonian		Knowles	Lea Co., N. Mex.
25	C2-145	Devonian	Keg River	Utikuma Lake	Alberta, Canada
26	T2-603	Silurian	Fusselman	Wildcat	Dawson Co., Texas
27	C2-148	Silurian	Guelph	Clay Creek	Lambton, Ont., Canada
28	N5-218	Ordovician	Ellenburger	Fowler	Lea Co., N. Mex.
29	O2-147	Ordovician	Simpson (2nd Wilcox)	Moore	Cleveland Co., Okla.
30	K2-58	Cambrian	Rome Sand	Wildcat	Boyd Co., Ky.

DISTRIBUTION OF HYDROCARBON OXIDIZING BACTERIA IN SOME PACIFIC OCEAN WATER MASSES

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One hundred seventy-three water samples from twenty-seven hydrographic stations in the California current, the Eastern Gyre of the Pacific Ocean and Hawaiian waters yielded forty-one cultures of hydrocarbon-utilizing bacteria.

Oil pollution of oceans and coastlines is a continuous and increasingly urgent environmental problem. The terminal degradation of petroleum must be accomplished by microbial action, thus, the amount of oil degradation may be assumed to be proportional to the numbers of microorganisms present.

During the 1971 summer cruise of the Stanford University *R/V Proteus*, from Monterey, California, to the waters surrounding the Hawaiian Islands, a survey was undertaken to study the relative concentration of hydrocarbon-utilizing microorganisms in this oceanic sector.

A total of 173 water samples were collected in Niskin bacteriological samplers and Nansen bottles at 27 hydrographic stations at 10 sites equidistant between Monterey and the Hawaiian Islands and at areas adjacent to the islands of Hawaii, Maui and Oahu. Specimens were taken at varying depths from surface to 1000 meters; however, since this survey was coordinated with other scientific studies the samples obtained at different hydrographic stations were not always from uniform depths.

All samples were immediately taken from the collection devices and transferred into autoclaved glass stoppered bottles. Portions of each sample were filtered through 0.45 μm porosity membranes which were implanted on peptone and hydrocarbon agar media. Incubation was at room temperature, i.e., from 18 C at Monterey Bay, California, to 29 C in the Hawaiian Islands. Colonies of bacteria growing on peptone agar were enumerated at 24 hours of incubation and discarded. Hydrocarbon-grown cultures were similarly examined at 24-hr intervals for 10 days.

Hydrocarbon media for isolation and maintenance contained per liter of distilled water: NH_4Cl, 0.1%; $MgSO_4$, 0.5%, $CaCl_2$, 0.005%; $FeCl_3$, 0.005%; $NaHPO_4H_2O$, 0.036%; K_2HPO_4, 0.07%; NaCl, 3.0%; agar, 2.0%; *n*-hexane, *n*-heptane and isoocane (2-2-4 methyl pentane) were added as single carbon sources to this melted, cooled medium just before use. Peptone-containing medium was Medium 2216 of ZoBell (3). All ingredients were autoclaved except the hydrocarbons which were sterilized by passage through 0.45 micron membrane filters.

Hydrocarbon-utilizing bacteria, when compared with peptone utilizers, were sparsely but widely distributed in the waters surveyed. All 173 water samples had peptone-utilizing bacteria but only 41 water samples contained hydrocarbon-utilizing bacteria. It is known that yeasts and fungi exist in oceanic waters, and that they may be involved in hydrocarbon degradation, but none were obtained from any of the 173 water samples with the isolation techniques used (2).

In the California Current east of 130 W longitude hydrocarbon-utilizing bacteria were absent from 14 samples taken at two hydrographic stations, although one hydrocarbon-utilizer was obtained from the surface water of Monterey Bay. Between 130 and 150 W longtiude in the Eastern Gyre of the Pacific Ocean, 10 of the 46 water samples at 6 hydrographic stations contained hydrocarbon-oxidizing bacteria. The 113 samples from 17 stations close to the Hawaiian Islands yielded hydrocarbon-oxidizing bacteria in 30 samples. Hydrocarbon-utilizing bacteria were usually found at only one or two depths. However, at 3 stations, 2 near the Hawaiian Islands and 1 in the Eastern Gyre of the Pacific Ocean, culturally identical hydrocarbon-utilizing bacteria were present throughout the upper water column. Although samples were collected to depths of 1000 m at five stations, hydrocarbon-oxidizing bacteria were not found below 250 m.

Rarely were there more than 5 colonies of hydrocarbon-oxidizing bacteria on the isolation filters even when peptone-utilizing organisms from the same water sample were present in such high concentration that colonies were too numerous to count. All hydrocarbon-utilizing bacteria grew luxuriantly on peptone medium but produced only small, slow growing colonies on hydrocarbon agars. Of the 23 different hydrocarbon-utilizing cultures selected for further examination, 15 were small gram negative rods, 7 were gram positive rods and one was a gram positive coccus.

Growth occurred on the medium with the branched chain compound isooctane as a carbon source as readily as it occurred on the medium with the straight chain compounds. Utilization of benzene, biphenyl and napthalene as single carbon sources demonstrated the ability of all 15 of the bacterial hydrocarbon-utilizers tested to use the benzene ring and compounds composed of the benzene ring as substrates.

A greater number of hydrocarbon-utilizing bacteria possibly may have been obtained during this study by use of longer chain hydrocarbons as single carbon sources since short chain hydrocarbons, such as the ones used here, are toxic to some organisms (1).

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ABSTRACT

LIPID BIOSYNTHESIS IN HYDROCARBON-UTILIZING ORGANISMS

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n-Alkanes can be directly oxidized to fatty acids which are then incorporated into triglycerides and phospholipids within the growing cell. Elongation of chain-length only occurs with alkanes below C_{13} so that it is feasible to consider "tailor-making" certain of the more expensive plant lipids such as those containing a high proportion of myristic acid (C_{14}) by use of appropriate microorganisms. Besides a study into the factors influencing lipid formation yeasts growing in continuous culture on *n*-alkanes, we are also examining the metabolic state of such yeasts. The effect which alkanes and alkane products have on the regulation and control of intermediary metabolism is under current investigation. We have noted that inhibition of glucose transport and metabolism, as well as *de novo* fatty acid biosynthesis, occurs in some organisms exposed to *n*-alkanes.

ABSTRACT

OIL BIODEGRADATION INVESTIGATIONS AT THE UNIVERSITY OF WESTERN ONTARIO

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Studies on the biodegradation of oil at the University of Western Ontario are being conducted in biochemical engineering. In addition, small activities are located in the Departments of Bacteriology and Immunology, and Plant Science. Although we have conducted numerous studies on the paraffinic hydrocarbons, efforts during the last two or three years have been concentrated on the following topics: Biodegradation of Bunker C Oil; The microbial emulsification of Bunker C oil; Flocculation reactions produced by microorganisms causing oil to sink; Chemical changes in Bunker C Oil which occur during biodegradation.

One emulsifying agent produced by *Corynebacterium hydrocarboclastus* has been purified and found to be highly reactive. This emulsifying agent has many important properties. It acts as a surfactant on detergent and will even flocculate clays and certain other colloidal materials. More recently we have initiated projects on the movement of oil on the surface of water but underneath an ice cover. In addition, we are studying the biodegradation of asphaltenes and polycyclic hydrocarbons which are present in Bunker C or Grade 6 fuel oil. Attention will be directed for some time toward the biodegradation of high molecular weight hydrocarbons both in pure and mixed form. Since we have also isolated some other emulsifying agents from hydrocarbons which are synthesized by microbes, this latter area will continue to be emphasized.

PROGRAM OF THE WORKSHOP

MORNING PRESENTATIONS

December 4

Welcome Address, Charles B. Vail
Dean, School of Arts and Sciences
Georgia State University

Impact of Oil on Marshland Microbial Ecosystems,
Donald G. Ahearn

December 5

Microbes and Petroleum, A Reappraisal of Dynamic Interrelationships,
W. R. Finnerty

December 6

Status and Future Prospects for Controlled Biodegradation of Oil,
C. E. ZoBell

PANEL MEETINGS

Bacterial degradation of oil, the range and mechanisms of enzymatic activity
P. H. Pritchard, Chairman

Fungal degradation of oil, the range and mechanisms of enzymatic activity
J. J. Cooney, Chairman

Toxicity and pathogenicity of hydrocarbonoclastic microorganisms
C. W. Hendricks, Chairman

Environmental considerations in microbial degradation of oil
J. D. Friede, Chairman

WORKSHOP

MICROBIAL DEGRADATION OF OIL POLLUTANTS
Georgia State University Atlanta, Georgia
December 4-6, 1972

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