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BIODEGRADATION OF OIL SLICKS IN THE MARINE ENVIRONMENT

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The degradation of petroleum hydrocarbons by marine bacteria was studied with the ultimate aim of using this process in the cleanup of polluting oil. Hydrocarbon-utilizing marine bacteria were isolated, their growth requirements and metabolic pathways were studied. Enzymatic and regulatory mechanisms responsible for the recalcitrance or delayed utilization of certain hydrocarbons, e.g. polynuclear aromatics and highly branched iso-alkanes were identified. The abundance and distribution of hydrocarbon degrading

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20. bacteria was measured in New Jersey coastal waters. The limiting factors of oil biodegradation in the marine environment were studied. Apart of the nature of the oil itself, water temperature and mineral nutrients (N, P, Fe) were found to be the most important limiting factors. Given favorable water temperatures, the rate of oil biodegradation can be increased by an order of magnitude or more by supplying the above mineral nutrients to a floating oil slick in oil-soluble (oleophilic) form. This method of application prevents nutrient loss by dilution and does not trigger algal blooms. The patented procedure is considered to be a new cost-effective way to cleanup oceanic oil spills.

INTRODUCTION

The Office of Naval Research funded the Research Contract entitled, "Biodegradation of oil slicks in the marine environment" through the period of January 1, 1970 - August 31, 1976, for a total of 6.5 years. The total funding amounted to $102,000 or an average of approximately $16,000 per year. The U. S. Navy, being one of the heaviest users and transporters of oil products on the high seas, supported this contract in order to learn about the fate and effects of accidental and routine discharges of oil at sea, and to explore the use of biodegradation in the cleanup of oil slicks (Bartha, 1970). The specific aims of the study, as stated in the initial proposal, were to develop information on:

(a) the natural biodegradation rates of oil slicks in various marine environments;
(b) the identification of predominant degradation pathways, intermediary products and their biological activity;
(c) the evaluation of currently used emergency measures as to their effect on the ultimate biodegradation of the polluting oil;
(d) the development of new techniques for accelerating the biodegradation of oil slicks in the marine environment.

Throughout the contract period, we adhered to the above objectives closely. Our work proceded from the study of marine hydrocarbon degrading microorganisms, their substrate range and metabolic pathways to the environmental factors that control the biodegradation rate of marine oil slicks. The study culminated in showing that the supplementation of floating oil slicks with oil soluble forms of nitrogen, phosphorus and iron results in a high increase of the natural oil biodegradation rates, and that this procedure is applicable to the cleanup of floating oil slicks.

To date, the contract resulted in 16 scientific articles, one U. S. patent, 5 presentations at national meetings, several invited lectures at foreign and U. S. universities, and presentations at national and international workshops. Several additional papers are in various stages of preparation or publication. The contract funds supported by 4 Ph.D. candidates during most of their research work, and supported several other graduate students for shorter periods of time.

The summary of our results is presented under the following headings:

1) Marine hydrocarbon degrading microorganisms
2) Pathways and control mechanisms of hydrocarbon biodegradation
3) Rates of oil biodegradation in seawater and the influence of environmental factors
4) The effect of traditional cleanup techniques on oil biodegradation
5) Cleanup of oil slicks by stimulated biodegradation

This final report summarizes our most important results and conclusions only. For experimental details and discussion, reference is made to our publications. Details of our work are also available in our Technical Reports (1-5), but since these were frequently preprints of our publications, reference is made to them only if they contain data that did not subsequently appear in the scientific literature. Their complete list is attached following our publications along with a list of the resulting doctoral dissertations.

1) Marine hydrocarbon degraders

On sea salts media with individual hydrocarbons or with crude oils as the sole added carbon sources 40 strains of oil-degrading marine microorganisms have been isolated in pure culture. Preliminary identification placed them in the genera Micrococcus, Cryptococcus, Microbacterium, Pseudomonas, Flavobacterium, Protaminobacter, Brevibacterium, Bacillus, Caulobacter, Nocardia, Hansenula, Candida, Rhodotorula and Penicillium. The substrate range of these organisms was evaluated on 11 aliphatic, alicyclic and aromatic hydrocarbons. Aliphatic compounds supported the growth of most of the isolated cultures, but the substrate range of several organisms included also alicyclic and aromatic compounds (TR 1, 1970).

On the basis of the above substrate range tests and growth characteristics, a Flavobacterium sp. and a Brevibacterium sp. were selected for more intensive study. The substrate range of these two organisms was determined by the protein yield after two weeks of incubation. n-Paraffins 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. Quaternary carbon atoms prevented utilization by either organisms. Utilization of alicyclic and aromatic compounds occurred only when a sufficiently long side chain was present but aromatic or alicyclic compounds without such side chains were spared by these organisms (Bartha and Atlas, 1973).

The ability of the above Flavobacterium sp. and Brevibacterium sp. to metabolize Sweden (Texas) crude oil, a paraffinic-naphthenic petroleum, and a chemically defined "model petroleum" hydrocarbon mixture in a sea salts mineral medium was investigated. Major components of the crude oil were identified by combination gas chromatography and mass spectrometry. The rate and extent of total hydrocarbon biodegradation was measured. In addition, CO_2 evolution from the crude oil was continuously monitored in a shaker-mounted gas train arrangement. Degradation started after a 2 to 4 day lag period, and reached its maximum within two weeks. At this time up to 60% of the crude oil and 75% of the model hydrocarbon mixture, each added at the level of 1 ml per 100 ml artificial sea water, were degraded. Mineralization (conversion to CO_2) was slightly lower due to formation of products and bacterial cell material. n-Paraffins were preferentially degraded as compared to branched chain hydrocarbons. Biodegradation of n-paraffins in the range of C_{12} to C_{20} was simultaneous; no diauxie effects were observed (Atlas and Bartha, 1971, 1972a).

The extent of petroleum biodegradation by Flavobacterium sp. and Brevibacterium sp. increased when they were grown in dialysis culture. Fatty acids were shown to be produced during petroleum biodegradation and they were causally linked to the observed inhibition in absence of dialysis. The fatty acids formed showed a synergistic toxicity in the presence of crude oil. Short-chain fatty acids were more toxic than longer chain ones. The fatty acid inhibition is very significant in laboratory cultures, but is presumed to have little influence on the biodegradation of floating oil slicks, due to their rapid dilution in the large volume of sea water (Atlas and Bartha, 1973a).

After growing on petroleum and on nonhydrocarbon substrates, the ultrastructure of the two marine hydrocarbon utilizers was examined by transmission electron microscopy. Large electron-dense inclusions, which were located predominantly at the cell terminus, characterized species of Flavobacterium and Brevibacterium after growth on oil. Cells of Flavobacterium sp. had smaller

inclusions when grown on marine agar, while inclusion bodies were not found in Brevibacterium sp. grown on marine agar. Sudan black B staining indicated the inclusions are stored lipids. (Atlas and Heintz, 1973).

For counting the numbers of oildegrading microorganisms in aquatic environments a new technique was developed. The technique involved collection of hydrocarbon-degraders on millipore filters and incubation of these filters on sea salts agar containing emulsified petroleum. Using this improved enumeration technique, the abundance of oil degrading microorganisms was monitored in Raritan Bay during a one-year period. The determined numbers varied from a low of 20/l to a high of 3400/l of surface sea water. The abundance of oil-degrading microorganisms was positively correlated with existing patterns of low-level oil pollution and with the water temperature, but was independent of total microbial counts. All tested sea water samples contained an adequate microbial population to cause the extensive biodegradation of added Sweden crude oil within eighteen days. The number of oil-degrading microorganisms in sea water samples was found to be a useful and sensitive indicator of low-level oil pollution that escaped routine gas chromatographic detection. (Atlas and Bartha 1973 G).

Work on oil-degrading microorganisms performed in other laboratories was the subject of two comprehensive reviews (Atlas and Bartha, 1973c, Bartha and Atlas, 1977).

2) Pathways and control mechanisms of hydrocarbon biodegradation

Our principal concern here was with components of crude oil that are relatively resistant to biodegradation. We wished to learn about specific molecular configurations that render a hydrocarbon immune to enzymatic attack. We wished to learn the specific step in the biodegradation sequence where the given molecular configuration exerted a blocking effect. Finally, we wished to know why some hydrocarbons that support microbial growth when provided as single substrate, would be spared as a component in a complex crude oil.

The metabolism of polynuclear aromatic hydrocarbons was studied using gas chromatographic, thin-layer chromatographic, infrared, and mass spectrometric techniques. Of six bacterial strains isolated from oil-polluted estuarine water, all grew on naphthalene, 2-methylnaphthalene, and 2-ethylnaphthalene as sole sources of carbon and energy. Only one strain grew on 1-methylnaphthalene, but all exhibied some growth on 1-ethylnaphthalene. Dimethylnaphthalenes (DMN) failed to support growth, but 1,3-DMN; 2,3-DMN; and 2,6-DMN were matabolized by naphthalene-grown resting cells as evidenced by the accumulation of metabolic products. The 1,4-DMN and 1,5-DMN were metabolized by only two out of the six strains. Of several polyaromatic compounds tested, phenanthrene and anthracene were metabolized by three out of six organisms. Only one naphthalene-grown strain was able to metabolize other polyaromatic compounds, including acenaphthylene, biphenyl, fluorene, and tetralin. The specificity of salicylate hydroxylase was found to govern the utilization of several naphthalene derivatives, while inability to oxidize naphthoic acids prevented growth on dimethylnaphthalenes (Raymond and Bartha, 1974; Dean-Raymond and Bartha, 1975).

Branched- and straight-chain alkanes are metabolized by Brevibacterium erythrogenes by means of two distinct pathways. Normal alkanes (e.g., n-pentadecane) were degraded, after terminal oxidation, by the beta-oxidation system operational in fatty acid catabolism. Branched alkanes like pristane (2,6,10,14-tetramethylpentadecane) and 2-methylundecane were degraded as dicarboxylic acids, which also underwent beta-oxidation. Pristane-derived intermediates were observed

to accumulate, with time, as a series of dicarboxylic acids. This decarboxylic acid pathway was not observed in the presence of normal alkanes. Release of $^{14}CO_2$ from [1-^{14}C] pristane was delayed, or entirely inhibited, in the presence of n-hexadecane, whereas CO_2 release from n-hexadecane remained unaffected. These results suggested an inducible dicarboxylic acid pathway for degradation of branched-chain alkanes. Induction of this pathway is repressed in the presence of n-alkanes (Pirnik et al., 1973,1974). The effects of methyl-branching on biodegradation and on induction of the diacid versus the monoacid pathway was explored on additional hydrocarbons, and the previous findings were confirmed and elaborated upon. When methyl branches did not interfere with β-oxidation, they were released as propionyl-SCoA (Coenzyme A thioester). Fixation of radioactive carbon dioxide in methylmalonic acid during growth at the expense of branched alkanes established the degradative pathway for propionate in this organism.

Growth at the expense of alkylcyclohexanes, when the alkyl side chain was C_8 or longer, resulted in the accumulation of high levels of cyclohexanecarboxylic acid (from odd-carbon alkylcyclohexanes) or cyclohexylacetic acid (from even-carbon alkylcyclohexames) in culture fluids, and extensive incorporation of ω-cyclohexyl fatty acids into cellular lipids. The cyclohexyl moiety did not interfere with β-oxidation, but was metabolically unavailable to the organism.

The overall fatty acid composition of B. erythrogenes reflected the growth substrate. Acetate-grown cells contained palmitate, oleate and palmitoleate as the major fatty acids, while propionate-grown cells contained pentadecanoate, pentadecenoate and heptadecenoate. When branched alkanes were offered as sole carbon sources, the above fatty acids were found in association with branched acids derived from the substrate alkane. Dicarboxylic acids were not found among the cellular lipids. The dicarboxylic acid degradative pathway effectively limited the availability of branched fatty acids for assimilation into cellular lipids. (Pirnik and Bartha, unpublished results).

3) The rate of oil biodegradation in seawater and the influence of environmental factors

Calculations based on our own results as well as on measurements in other laboratories have shown that hydrocarbons can be degraded in seawater at rates up to 2500 g/m^3/day. These rates reflected the activity of the natural microbial population of seawater, but environmental conditions were artificially optimized. The above rates contrast sharply with the in situ hydrocarbon biodegradation potentials at 0.001-0.05 g/m^3/day (Bartha and Atlas, 1977). The contrast suggests that the biodegradation rate of oil by the natural microbial population in seawater could be considerably increased through the identification and at least partial alleviation of limiting environmental conditions. Among these, special attention was given to temperature and mineral nutrients.

In laboratory experiments, freshly collected seawater samples were treated with petroleum and were incubated at controlled temperatures between 5 and 20°C. Biodegradation was monitored by the measurement of CO_2 evolution and by quantitative gas chromatographic analysis. Low water temperatures not only resulted in slower degradation rates, but caused increasing lag periods that preceded the onset of measurable biodegradation. A substantial portion of these lag periods was eliminated when, instead of fresh petroleum, a "weathered" sample was used. The results suggest that some volatile components of petroleum that are inhibitory to

oil-degrading microorganisms evaporate only very slowly at low temperatures, and thus retard biodegradation. Unfortunately, the modification of seawater temperature as a means of increasing oil biodegradation rates is not considered to be practical (Atlas and Bartha, 1972).

Both petroleum and seawater are deficient in important mineral nutrients such as nitrogen, phosphorus and iron, and the lack of these nutrients can limit the biodegradation rate of polluting oil. Biodegradation and mineralization of petroleum, added at 1% (v/v) to freshly collected sea water, were measured using gas-liquid chromatographic, residual weight, and CO_2-evolution techniques. Only 3% of the added petroleum was biodegraded and 1% was mineralized in unamended sea water after 18 days of incubation. Added individually, nitrate (10^{-2} M) or phosphate (3.5×10^{-4} M) supplements caused little improvement, but when added in combination, they increased petroleum biodegradation and mineralization to 70% and 42%, respectively. (Atlas and Bartha, 1972 c, 1972 d, Bartha and Atlas, 1972 a, Bartha and Atlas, 1973). Iron had a positive effect in clean but not in polluted seawater (Dibble and Bartha, 1976 a, 1976 b).

4) The effect of traditional cleanup techniques on oil biodegradation

Dispersants and oil herders are used in the cleanup of oil spills, at least in some situations. It was of interest to know what, if any, effect such treatments have on the ultimate biodegradation of the oil. Six currently used oil dispersants and two oil herders were tested as to their effect on the biodegradation of crude oil. All tested compounds significantly increased the rate but not the effect of petroleum biodegradation. The beneficial effect was appapently due to the increased surface of oil droplets and an absence of toxicity to oil degrading microorganisms at the recommended concentrations.

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, little was 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.

Several bacterial inocula are on the market for the purpose of dispersing and degrading oil and grease. The accompanying technical and promotional literature describes these inocula as highly effective, both in fresh and salt water, but no controlled studies with these materials were published in the scientific literature. The two commercial bacterial inocula we had opportunity to test (Ecolo-Gest and DBC Bacteria), when used as directed, failed to increase the biodegradation rate in natural sea water samples. When tested in sterile seawater, these inocula showed inferior performance as compared to the natural microflora of seawater, and were judged to be ineffective for marine applications (Atlas and Bartha, 1973 d).

5) Cleanup of oil slicks by stimulated biodegradation

The strong positive effect of mineral nutrient supplements on oil biodegradation, described in section 3, suggested that this process could be utilized in the stimulated biodegradation of oil(Bartha and Atlas, 1972 b, 1977). It was recognized, however, that the use of water soluble nutrients would be wasteful in case of

floating oil slicks, and would most likely have an eutrophication side effect. For this reason, oil soluble sources of these mineral nutrients were selected and tested, initially in laboratory experiments, and later in a field-like situation. In the latter case, miniature oil slicks were floated on the surface of a flow-through seawater aquarium. A combination of paraffinized urea and octylphosphate promoted oil biodegradation both in the laboratory and in the field experiments in a highly effective manner. The biodegradation rate in the field increased approximately twelve-fold due to treatment with the oleophilic fertilizer compounds. At the same time, paraffinized urea and octylphosphate failed to cause algal blooms, while equimolar amounts of nitrogen and phosphorus salts caused a two orders of maguitude increase in algal density (Atlas and Bartha, 1973 e). The process was judged to have practical value in the cleanup of floating oil slicks and was patented (Bartha and Atlas, 1976).

In subsequent experiments, iron was shown to have an added stimulatory effect on oil biodegradation when supplied in combination with nitrogen and phosphorus. The added stumulation was obvious in clean but not in polluted seawater. Iron octoate was selected and shown to be effective as an oleophilic iron source in combination of paraffinized urea and octylphosphate. The biodegradation of nutrient-supplemented South Louisiana crude oil reached 73% in 3 days. The rate and extent of this biodegradation compares favorably to physical cleanup methods which are much more costly and require ideal weather conditions. At favorable water temperatures, stimulated biogegradation by means of oleophilic fertilizers is judged to be a highly cost-effective and weather-independent means of cleanup for slicks of South Louisiana crude oil and for polluting oils of similar composition.

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