OFFICE OF NAVAL RESEARCH

Contract N00014-67-A-0115-0005

Task No. NR 137-643

TECHNICAL REPORT NO. 2

A. Degradation and Mineralization of Petroleum by Two Bacteria Isolated from Coastal Waters

and

B. Degradation and Mineralization of Petroleum in Sea Water: Limitation by Nitrogen and Phosphorus

by

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Prepared for Publication

in

"Biotechnology and Bioengineering"

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New Brunswick, N. J. 08903

31 December 1971

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DOCUMENT CONTROL DATA - R & D

Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified

1. ORIGINATING ACTIVITY (Corporate author)	2a. REPORT SECURITY CLASSIFICATION
Rutgers University, New Brunswick, N.J.	Unclassified
	2b. GROUP

3. REPORT TITLE: A. Degradation and Mineralization of Petroleum by Two Bacteria Isolated from Coastal Waters, B. Degradation and Mineralization of Petroleum in Sea Water: Limitation by Nitrogen and Phosphorus

4. DESCRIPTIVE NOTES (*Type of report and inclusive dates*): Technical Report, Jan. 71-Dec. 71

5. AUTHOR(S) (*First name, middle initial, last name*): Ronald M. Atlas and Richard Bartha

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
Dec. 71	39	39

8a. CONTRACT OR GRANT NO.	9a. ORIGINATOR'S REPORT NUMBER(S)
N00014-67-A-0115-0005	
b. PROJECT NO. NR 137-843	TR. No. 2
c.	9b. OTHER REPORT NO(S) (*Any other numbers that may be assigned this report*)
d.	

10. DISTRIBUTION STATEMENT: This document has been approved for public release and sale; its distribution is unlimited

11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
	Office of Naval Research Arlington, Virginia 22217

13. ABSTRACT

A. Within the framework of a study on the oil biodegradation potential of the sea the ability of a *Flavobacterium* sp. and *Brevibacterium* sp. to metabolize a paraffinic crude oil and a chemically defined hydrocarbon mixture 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.

B. 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. Attempts to clean up oil spills with the aid of microorganisms should take into consideration the nutritional deficiencies of sea water.

DD FORM 1 NOV 65 1473 (PAGE 1)

S/N 0101-807-6801

14 KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Oil Slicks						
Crude Oil						
Petroleum						
Hydrocarbons						
Biodegradation						
Mineralization						
Microorganisms						
Marine environment						

Section A

INTRODUCTION

Petroleum is an increasingly menacing pollutant of our oceans (4). Microorganisms have been described as the principal agents of self-purification (21). While microbial metabolism of individual hydrocarbons has received extensive attention (9,10,14,16), analytical complexities have kept studies on crude oil biodegradation few in number, and mostly qualitative in nature (7,8,11,12,13,15,17,19,24). Using two bacteria isolated from the coastal waters of New Jersey, we have studied the degradation and mineralization of a natural petroleum and a model hydrocarbon mixture. Our aims were to establish the order in which individual components are degraded, and to quantitate the overall rate and extent of degradation and mineralization as mediated by these marine isolates.

MATERIALS AND METHODS

Media and Isolation. A paraffinic petroleum, Sweden crude (gift of the Sun Oil Co., Marcus Hook, Pa.) was sterilized by filtration through a 0.2 micrometer pore size Flotronics silver membrane (Selas, Spring House, Pa.). A mixture of n-hexadecane, n-octadecane, and n-nonadecane (Aldrich, purity 99%) was used as a greatly simplified model petroleum. The choice of these hydrocarbons was based on their predominance in "weathered" (evaporation-exposed) Sweden crude and on their good gas chromatographic resolution. The individually steam-sterilized hydrocarbons were mixed in equal volumes. Both the Sweden crude and the model mixture were added at 1% (v/v) concentration as the sole sources of carbon and energy to an artificial sea water medium (Difco Bushnell-Haas broth, supplemented with 3% NaCl and adjusted to pH 7.8 with NaOH). Hydrocarbon

degraders were selectively enriched in these liquid media that were inoculated with aseptically collected sea water or sediment. For isolation of individual microorganisms from these enrichments the modified Bushnell-Haas broth was solidified by addition of 1.5% agar, and the hydrocarbon substrate was added to a sterile filter paper disc in the cover of an inverted Petri plate. All incubations and experiments were conducted at 28°C. Characterization and tentative identification of the isolated strains was carried out according to Skerman (20). The microorganisms were maintained routinely on Difco Marine Agar 2216.

Analytical. Hydrocarbon residues from the culture medium were extracted by two 50 ml portions of diethyl ether. The combined extract was dried with anhydrous Na_2SO_4 and the ether was allowed to evaporate. The residue was extracted by three small portions of ether, and the final volume of the extract was adjusted to 10 ml. This extract was analyzed by gas-liquid chromatography using an F & M model 700 instrument equipped with dual flame ionization detectors and a model 7127-A recorder with disc integrator. The dual 1.8 m long, 3 mm OD stainless steel columns were packed with 10% Apiezon L on 60/80 mesh Chromosorb W. The operational parameters were: injection port 300 C, detector 300 C, carrier (helium) 40 cc/min, hydrogen 40 cc/min, oxygen 250 cc/min, injection volume 1 µl. For crude oil analysis, the oven temperature was kept at 140 C isothermal for 5 minutes following injection, and was then programmed at the rate of 10 C/min to 240 C and held. For analysis of the model petroleum the oven temperature was kept 200 C isothermal. Individual hydrocarbons were quantitated by integration of the corresponding

peak areas. Total crude oil degradation was estimated by integration of the area under the resolved chromatogram-portion over a 30 min period. Integration was started 5 min after injection to exclude the solvent response. Since separate studies, to be published elsewhere, have established that under the conditions of these experiments the bacteria were unable to degrade pristane, this component of the crude oil was used as an internal standard. Corrections were made for evaporative and non-biological losses as described later. The results were plotted as the corrected per cent reduction with time of the original gas chromatographic area response.

The major hydrocarbon compounds of the Sweden crude were identified by their retention times comparing them to those of analytical standards, and also by combination gas chromatography-mass spectrometry. The latter measurements were performed using a Perkin-Elmer model RMU-7 mass spectrograph. Operational parameters of the gas chromatograph were identical to those described for the F & M instrument, but part of the gas flow was diverted prior to the detector by a stream splitter, and was introduced through separator pumps into the mass spectrograph. As each major peak was detected, a scan was taken on the mass spectrograph. The obtained mass spectra were compared to those of known compounds (2).

Degradation and mineralization experiments. Twenty-four hour cultures grown in Difco Marine Broth 2216 were sedimented by centrifugation (4000 G for 15 min), were twice washed with, and finally suspended in, artificial sea water. One ml of this suspension containing in various experiments 0.6 to 1.0 mg protein was used for inoculation of 250 ml filter flasks containing 100 ml artificial sea water and 1 ml of either Sweden crude or model petroleum. The filter flasks were fitted with spargers, with appropriate cotton filters to maintain sterility, and were attached to the manifold of a gas train arrangement similar to

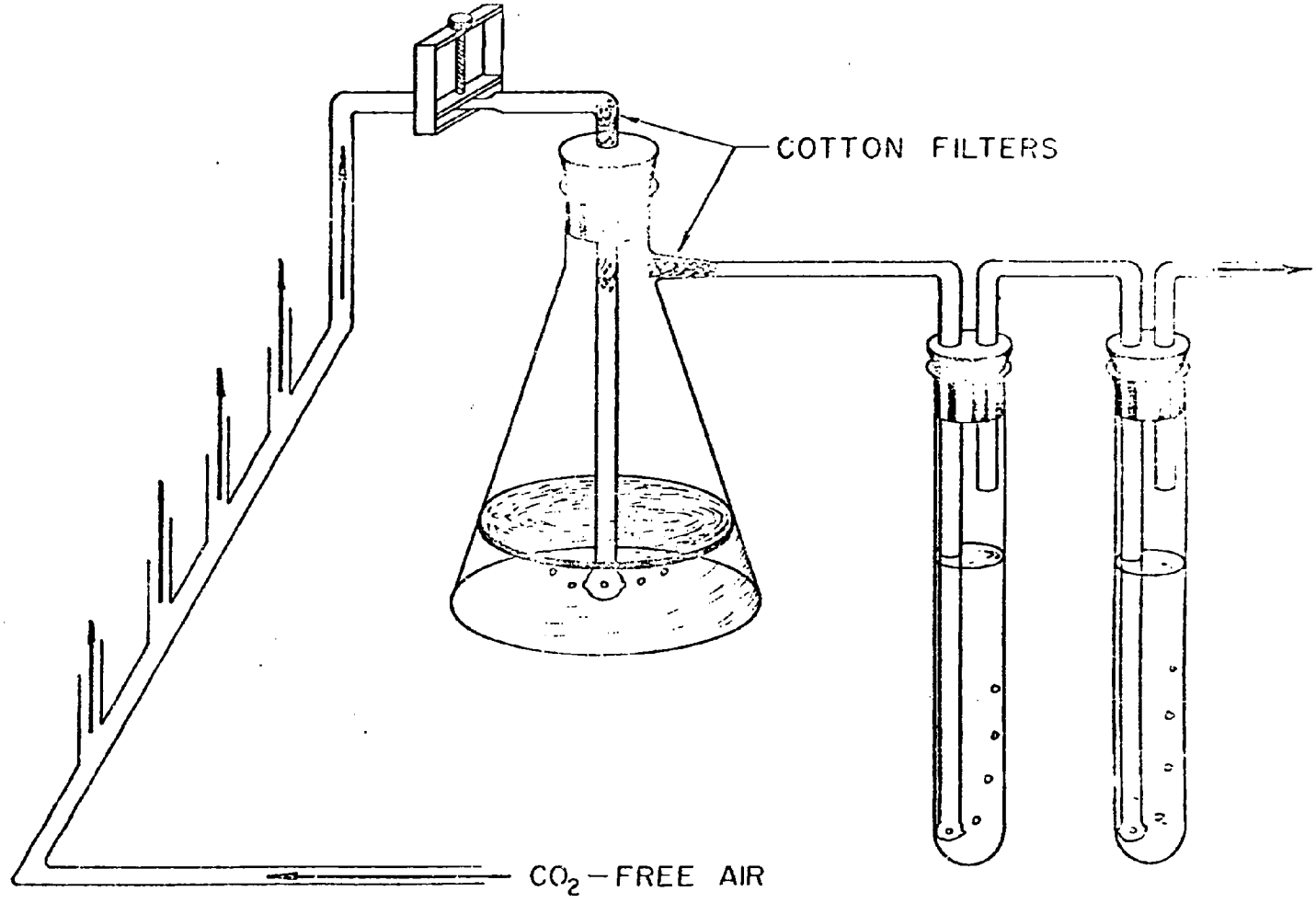

Fig. 1. Diagram of a gas train for measurement of petroleum mineralization. CO_2-free air passes through a manifold, regulator, and cotton filter into a filter flask containing mineral solution, petroleum, and micro-organisms. Evolved CO_2 is absorbed in two successive KOH-traps. The gas train is mounted on a rotary shaker.

the one used by Waksman and Starkey (21). A diagram of the arrangement is shown in Fig. 1.

A CO_2-free airstream of approximately 15 cc/min was passed through each flask. The CO_2 produced by mineralization of the hydrocarbons was absorbed in two successive traps, each containing 50 ml of 0.1 N KOH. The whole gas train arrangement was mounted on a rotary shaker and agitated at the rate of 200 rpm. Similarly treated cotton stoppered Erlenmeyer flasks were incubated on the rotary shaker without sparging; omission of sparging did not affect the conversion of oil.

Periodically, the CO_2 traps were changed and the absorbed CO_2 was determined by standard volumetric procedures. The CO_2 evolution was measured continuously and plotted cumulatively. The theoretical maximum of CO_2 production was determined by wet ashing of aliquots of model petroleum and weathered but not biodegraded Sweden crude oil (1). This procedure recovered 95% of the theoretical carbon content of the chemically defined hydrocarbons. Periodically, replicate flasks were sacrificed for extraction and measurement of residual oil. Sterile control flasks incubated and extracted under identical conditions were used to correct for oil losses by evaporation and chemical degradation during the experiments. The biodegradation data were corrected, accordingly, for non-biological losses. Biodegradation was expressed as a per cent decrease, compared to the petroleum remaining in the sterile control flasks at the time of sampling. All treatments and analyses were carried out in duplicates and the mean values were plotted.

RESULTS

Microorganisms. Two bacteria were selected for this study from 30 isolated microorganisms on the basis of their rapid growth and wide range

of hydrocarbon utilization. A short Gram-negative rod with Gram-positive inclusions was isolated from the brackish Arthur Kill at Seward, N.J. for its ability to grow on n-hexadecane as its sole source of carbon and energy. It is non-motile, non-fermentative, produces a yellow water insoluble pigment, requires 3% NaCl for optimal growth, and has been classified as a *Flavobacterium sp.* (20). The other organism is a short Gram-positive rod, and was isolated from the coastal waters at Sandy Hook, N.J. for its ability to grow on Sweden crude. It is non-motile, non-fermentative, non-acid-fast and produces a red, water insoluble pigment. It required the addition of 3% NaCl for growth, and has been classified as a *Brevibacterium sp.* (20).

Composition of Sweden crude oil. Fig. 2, part A shows the gas chromatographic pattern obtained for intact Sweden crude. The major peaks whose mass spectra were obtained are numbered. The m/e values for the parent ions are listed in Table 1. The breakdown pattern for each compound was also examined and compared to that of known compounds which had identical retention. The major hydrocarbon components of Sweden crude as identified by retention time and mass spectrometry are listed in Table 1.

Degradation and mineralization. Results of biodegradation and mineralization studies on Sweden crude oil using *Flavobacterium sp.* and *Brevibacterium sp.* are summarized in Fig. 3 and 4, respectively. Characteristic was a 2-4 day lag period at the start of the experiments, followed by rapid oil biodegradation. Mineralization was slower and slightly less extensive than biodegradation, the difference representing carbon tied up in degradation intermediates, and in microbial cells.

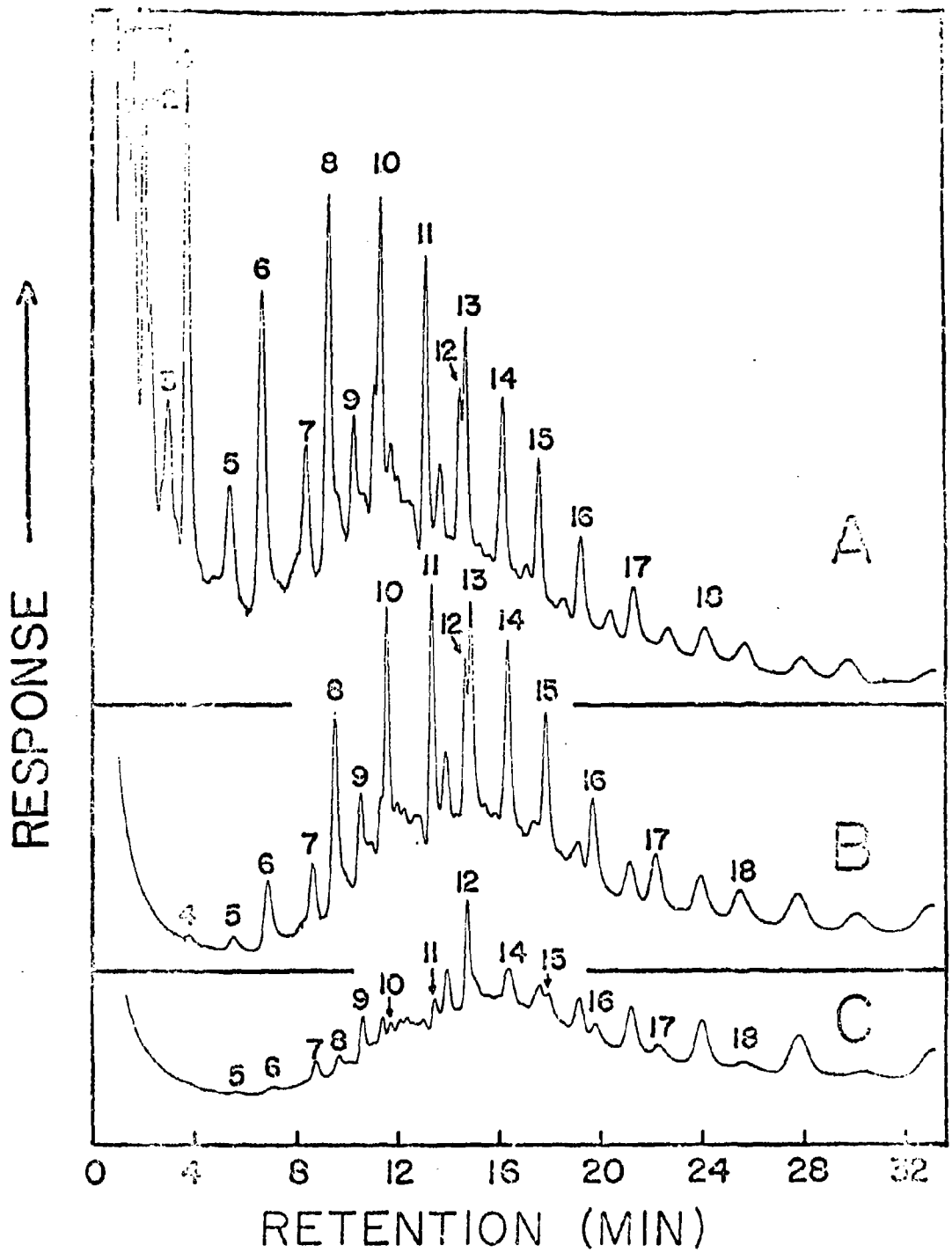

Fig. 2. C_{15} chromatograms of Sweden crude oil. A: intact sample; B: weathered (exposed to losses by volatility for 8 days); C: biodegraded by *Flavobacterium* sp. for 3 days. For identity of numbered peaks see Table 1. Operational conditions as described in the text.

Table 1. Some major components of Sweden crude oil as identified by gas chromatography and mass spectrometry

No.*	Parent ion m/e	Hydrocarbon	Retention of analytical standard (min)
1	142	n-decane	1.0
2	156	n-undecane	2.0
3	170	branched C_{12} paraffin	-
4	170	n-dodecane	4.0
5	184	branched C_{13} paraffin	-
6	184	n-tridecane	7.5
7	198	branched C_{14} paraffin	-
8	198	n-tetradecane	9.25
9	212	branched C_{15} paraffin	-
10	212	n-pentadecane	11.25
11	226	n-hexadecane	13.0
12	268	pristane	14.25
13	240	n-heptadecane	14.5
14	254	n-octadecane	16.0
15	268	n-nonadecane	17.5
16	282	n-eicosane	18.75
17	296	n-heneicosane	21.0
18	310	n-docosane	23.75

* Refers to Fig. 2.

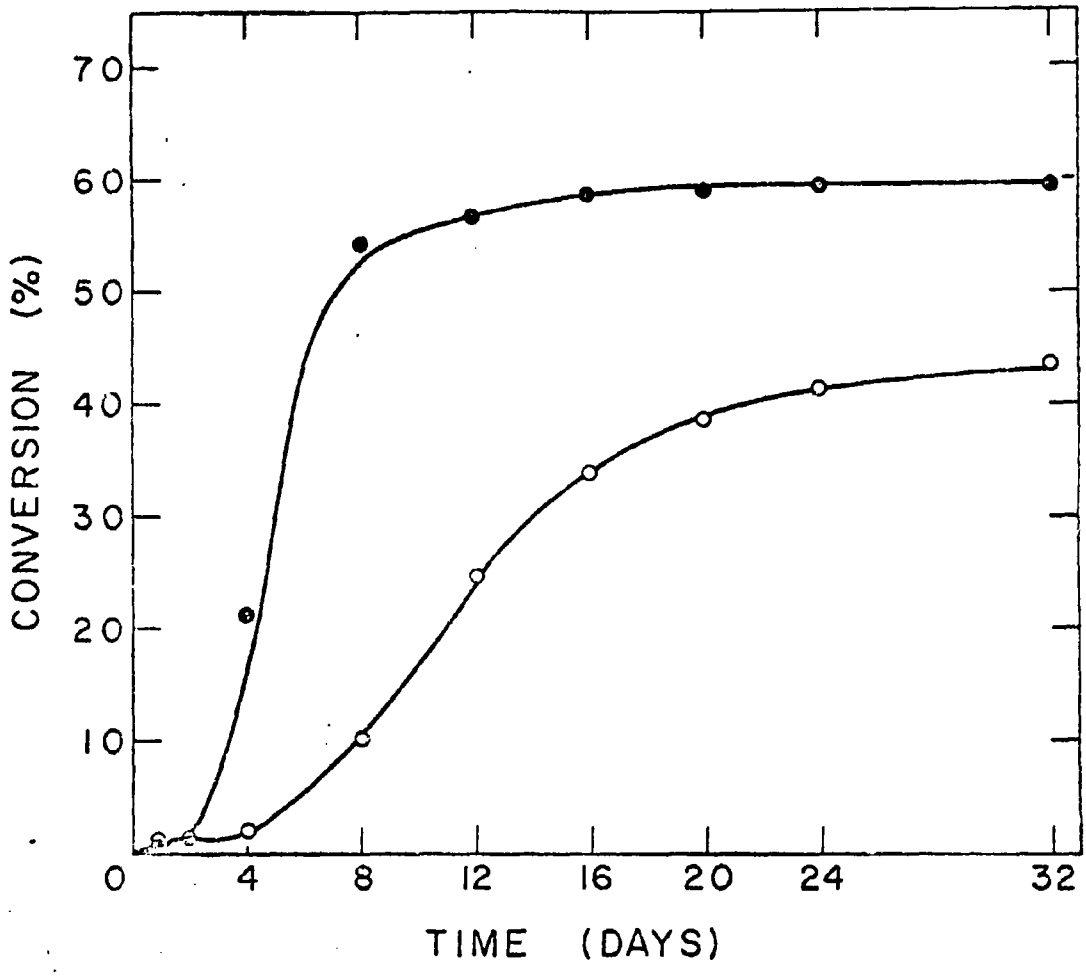

Fig. 3. Conversion of Sweden crude oil by *Flavobacterium sp.*
Symbols: biodegradation as measured by gas chromatograph (●);
mineralization as measured by CO_2 evolution (o)

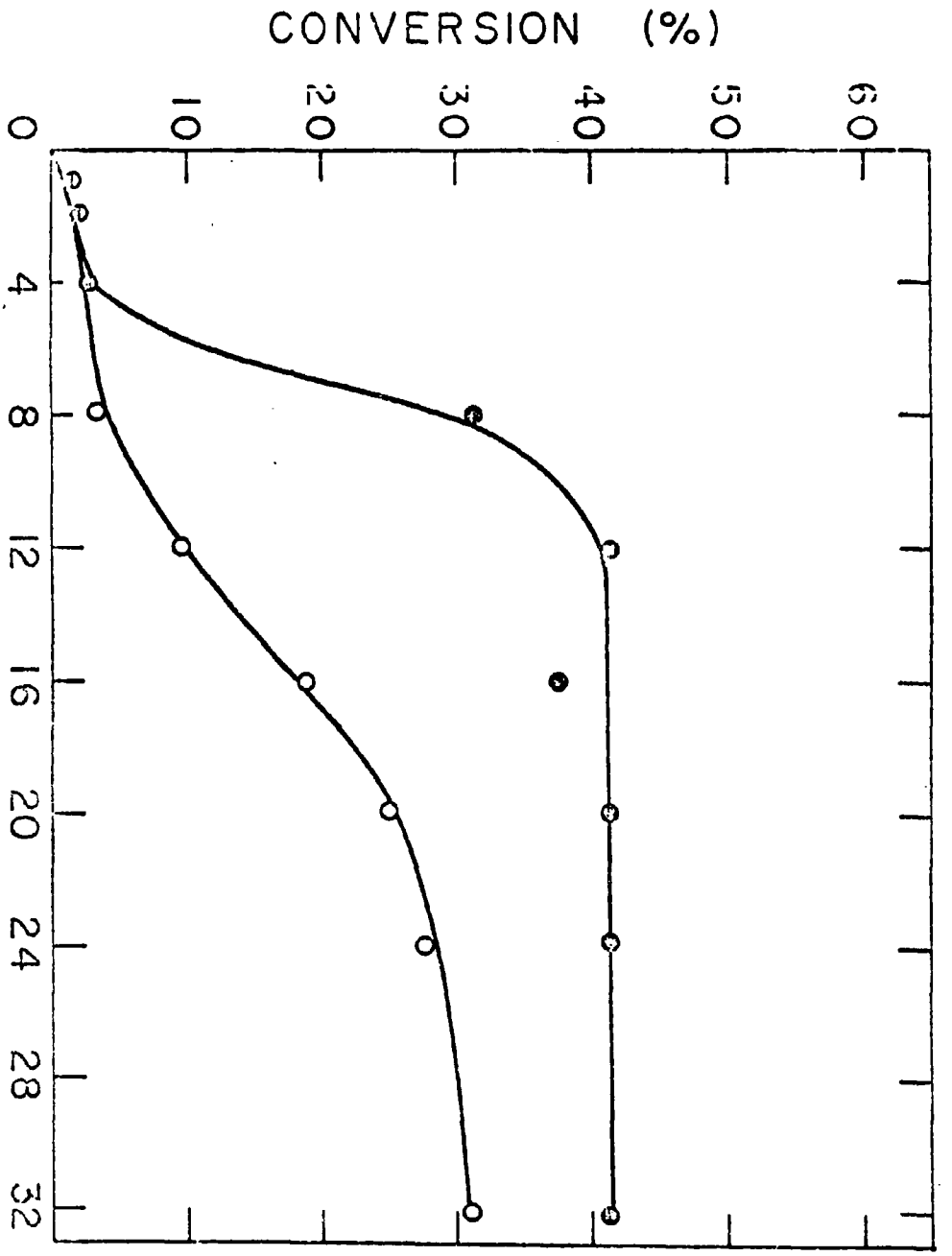

Fig. 4. Conversion of Sweden crude oil by *Brevibacterium sp.*

Symbols: biodegradation (•); mineralization (o).

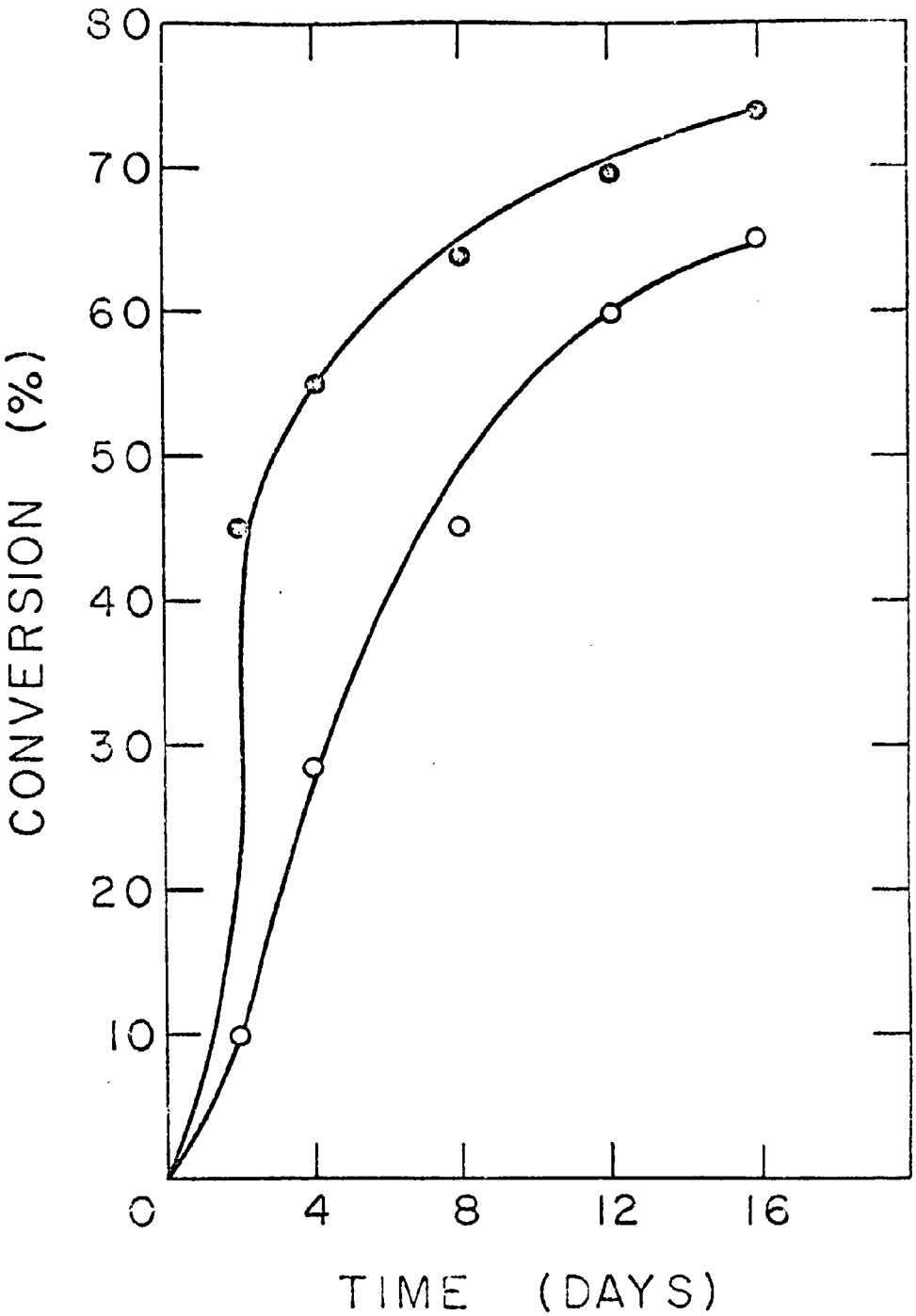

Fig. 5. Conversion of a mixture of n-paraffins (C_{16} , C_{18} , $C_{\ }$) by *Flavobacterium sp.* Symbols: biodegradation (●); mineralization (○).

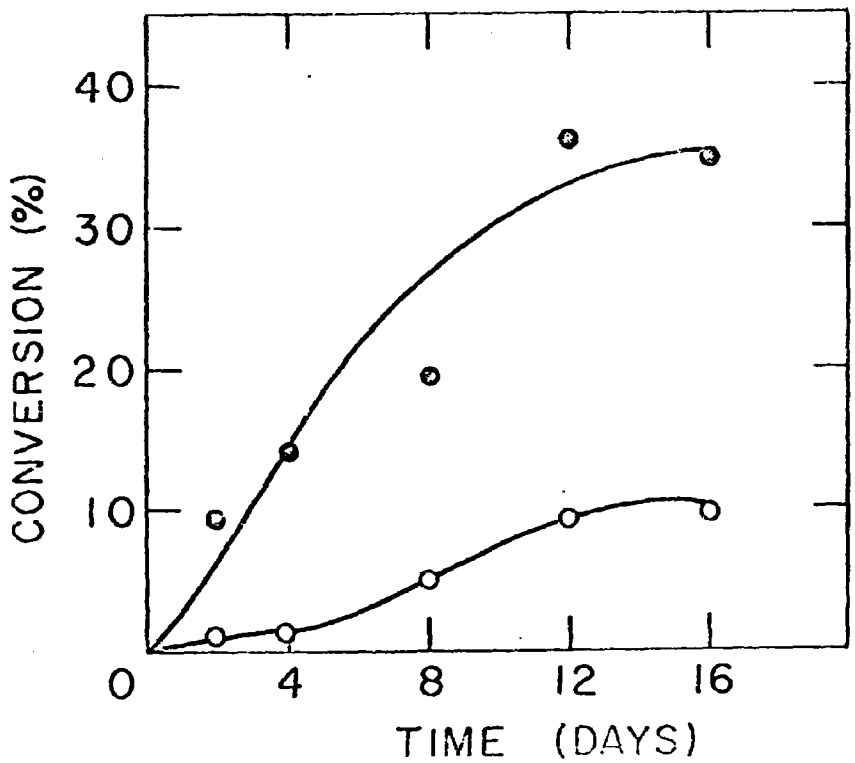

Fig. 6. Conversion of a mixture of n-paraffins (C_{16}, C_{18} , C_{19}) by *Brevibacterium sp.* Symbols: biodegradation (●); mineralization (o).

As reported by Miller and Johnson (18) the quantitative separation of cells from residual hydrocarbons becomes increasingly difficult as the chain length of the residues increases. In our experience, the tar-like residues of crude oil degradation fruther aggravate this problem. Since in the cleanup of polluting oil the cell yield is important only to the extent that it facilitates oil biodegradation, the abundant microbial growth was observed only visually, but was not quantiated in these experiements. The rate and the extent of petroleum biodegradation achieved by the *Flavobacterium sp.* was higher than that of the *Brevibacterium sp.*

Besides measuring the overall extent of crude oil biodegradation (57 and 40 per cent at 12 days for *Flavobacterium sp.* and *Brevibacterium sp.*, respectively), the gas chromatographic analysis discerned the crude oil components that were primarily subject to biodegradation. Comparison of the tracings A, B, and C in Fig. 2 shows that compounds 1 to 6 (up to and including *n*-tridecane) disappeared mainly due to their volatility. In various experiments, 32 to 36% by weight of the fresh Sweden crude oil was lost by volatility. This loss occurred during the first days of the incubation and did not measurably increase in the later stages of the experiment. The *n*-paraffins C_{14} through C_{22} were not lost significantly by volatility, but were subject to extensive biodegradation. Pristane (No. 12) and other branched chain paraffins (unnumbered peaks between No. 15-16, 16-17, and 17-18) were spared. Degradation of the *n*-paraffins in the measured range was simultaneous; no diauxie effects could be detected. This was confirmed by the results obtained on the model petroleum (Fig. 5 and 6). No lag period was evident in growth on model petroleum, indicating that the lag on Sweden crude was more likely to be due to its volatile toxic components than to a delay in enzyme induction. Degradation of the model petroleum by *Flavobacterium sp.* was more extensive (70% at 12 days)than that of the crude, but the efficiency of the *Brevibacterium sp.* showed no similar increase.

Supply of utilizable hydrocarbons was not exhausted at the time when degradation of the crude and the model petroleum had leveled off. Since the modified Bushnell-Haas medium was heavily buffered with phosphate and the spent medium gave a positive reaction with Nessler reagent, availability of phosphorous and nitrogen did not limit the degradation. At the conclusion of the experiments, the pH of the medium remained within 0.3 units of the initial value. Among other possible explanations, inhibition by accumulating metabolic products may account for the incompleteness of *n*-paraffin degradation by the bacterial cultures.

DISCUSSION

Between easily recycled natural products and certain extremely recalcitrant man-made organics such as chlorinated hydrocarbon insecticides, chlorinated biphenyls, and plastics, petroleum and petroleum products as pollutants occupy an intermediary position. They are natural products which, however, seldom enter the biosphere in large quantities except as a consequence of man's activities. It is, therefore, not surprising that marine and other aquatic ecosystems are poorly equipped to handle large influxes of oil, and this inefficiency greatly aggravates our current oil pollution problems.

The term "degradation" has been applied in the pollution field so broadly as to become rather confusion. As an example, a pesticide may be described as "degraded" when it has lost its activity on the target organisms, even though the product of this"degradation" may be of higher molecular weight than the parent compound (6). Since similar paradoxes may well exist in the case of petroleum (tar formation), "degradation" is interpreted here empirically as disappearance of the original hydrocarbon by processes other than volatility. In contrast, "mineralization"

designates complete recycling to inorganic compounds, and is a more meaningful term in relation to pollution problems. It eliminates the possibility of beleiving that a particular compound has been detoxified when it actually has been replaced by an equally undesirable product. Both degradation and mineralization were measured in the foregoing experiments.

Measurement of crude oil biodegradation by integrating the total resolved portion of the gas chromatogram is subject to limitations that should not be overlooked. Hydrocarbons with the lowest and with the highest carbon numbers may escape detection, and detector sensitivity to compounds of different classes will vary. Since in this study biodegradation was largely restricted to n-paraffins of the medium range, these errors were negligible. Good correlation was obtained between the integration value and the residual weight of oil (5), and mineralization as measured by CO_2 evolution was always a lower but a proportional value. The great advantage of this technique is that besides giving a good estimate of the total petroleum biodegradation, the chromatograms contain a wealth of information pertaining to the fate of its individual components. The relative recalcitrance of pristane and its value as internal standard has also been reported by other workers (15, 17).

The possible use of hydrocarbon utilizing microorganisms for accerlerated degradation of accidentally spilled oil is receiving serious consideration (3). Miget *et al.* (17) and Kator *et al.* (15) have studied the effectiveness of mixed marine enrichment cultures for this purpose. There is no doubt that mixed enrichments can degrade a highly complex substrate such as petroleum more effectively than any single microorganism, but the practical use of an enrichment of unknown composition is likely to encounter licensing difficulties because of its potential side effects

on marine oils. It may be necessary to construct an effective mixed culture from certain microorganisms with wide and complementing substrate ranges.

Emphasizing the importance of mixed microbial cultures in petroleum biodegradation, ZoBell in his recent review of the field (25) stated that he had "never found a single microbial species which could noticeably degrade any crude oil." In this light, the performance of the isolated bacteria, especially that of the *Flavobacterium sp.*, seems remarkable.

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ACKNOWLEDGEMENTS

This work was supported in part by the Office of Naval Research. The authors thank Mrs. Van Es and Dr. J. D. Rosen for their help in mass spectrometric studies, and Dr. R. L. Raymond for his advice and his aid in obtaining crude oil samples. Paper of the Journal Series, New Jersey Agricultural Experiment Station.

Section I

INTRODUCTION

The yearly influx of petroleum pollutants into the oceans has been estimated to be as high as ten million tons (4). Apparently, most of this petroleum eventually undergoes microbial degradation (14), but at a rate that is too slow to provide immediate relief in massive pollution incidents. Numerous reasons have been proposed to explain the slow rate of microbial oil degradation in the sea, e.g. the usually low counts of hydrocarbon-degrading microorganisms, toxicity of some petroleum components, the limited oil-water interface, insufficiency of dissolved oxygen, suboptimal temperatures, and lack of essential mineral nutrients (15).

Artificial stimulation of biodegradation is under consideration either as a primary or as a supplementary cleanup measure for accidental oil spills, and inoculation with effective petroleum-degrading microorganisms has been proposed (2). Inoculation alone may prove to be of limited benefit if petroleum degradation in sea water is limited also by the lack of essential nutrients. Considering the composition of crude oil and sea water, nitrogen and phosphorous are the most likely nutrients to be limiting. Indeed, this suggestion has been made earlier, but was substantiated mostly by visual observation and other non-rigorous criteria (5,6,7,8,9,10,12). Furthermore, the required levels of nitrogen and/or phosphorous supplements were not established.

In this study, we have measured the rate and extent of the degradation and mineralization of a crude oil by the indigenous population of freshly collected sea water samples, and have determined the effect of various levels of phosphorous and nitrogen supplements on these processes.

MATERIALS AND METHODS

Analytical. Total nitrogen and total phosphorous were determined in the collected sea water samples according to Miller and Miller (12) and Strickland and Parsons (13), respectively. Carbon dioxide produced in the process of petroleum mineralization was measured as described in the preceeding article (3). The carbon content of the crude oil was determined by wet ashing (1), and the CO_2 evolved as a consequence of microbial oxidation was plotted cumulatively as per cent of this theoretical maximum. The extent of biodegradation as measured by gas chromatography(3) was determined only upon the conclusion of the experiment. The biodegradation percentage derived from the gas chromatographic measurement was correlated both with the CO_2 production and with the residual weight of the petroleum. For the latter measurement, an aliquot of the dry ether extract (3) was allowed to evaporate in a pre-weighed dish overnight at room temperature, and the weight of the petroleum residue was determined using a P. Bunge Model 100 V analytical balance.

Degradation and mineralization experiments. Sea water was collected in the month of April off the east shore of Sandy Hook, N.J., a relatively unpolluted area. Total microbial counts were performed by plating dilutions on Difco Marine Agar 2216. In order to restrict microorganisms to the indigeneous population, collection, handling and incubation were performed using aseptic techniques, and the sample was processed on the day of the collection. Samples of sea water, 100 ml by volume, plus 1 ml (800 mg) amounts of filter-sterilized Sweden crude oil (3) were introduced into the filter flask components of the shaker-mounted gas train assembly

described in the preceeding article (3). Supplements were added as sterile concentrated stock solutions in a total volume of 1.0 ml. The equal amount of distilled water was added to unamended samples. Nitrogen was added either as KNO_3 (final concentrations in the sea water 1 X 10^{-3}, 5 X 10^{-3}, and 1 X 10^{-2} M) or as NH_4NO_3 (3.7 X 10^{-4}, 1.85 X 10^{-3}, and 3.7 X 10^{-3} M). Phosphate was added as Na_2HPO_4 (3.5 X 10^{-7}, 7 X 10^{-5}, and 7 X 10^{-4} M). The three KNO_3 and NH_4NO_3 concentrations were added either as the only supplements, or in combination with the highest (7 X 10^{-4}M) Na_2HPO_4 concentration. Similarly, the three Na_2HPO_4 concentrations were added either as the only supplements, or in combination with the highest (1 X 10^{-2} M) KNO_3 concentration. The following controls were included: millipore-sterilized (Nalgene, 0.2 micrometer pore size) sea water with oil (*a*), natural sea water without oil (*b*), natural sea water without oil but amended with the highest concentration of KNO_3 (1 X 10^{-2} M) (*c*), or Na_2HPO_4 (7 X 10^{-4} M) (*d*), or both (*e*), and natural sea water with oil, but without mineral supplements (*f*). The flasks were incubated at 28 C. Two replicate flasks were used for each treatment and the mean values were plotted.

RESULTS

The sea water sample used in these experiments had a salinity of 2.85% and a pH of 8.3. The viable microbial count was 300/ml. Total nitrogen and phosphorous were 98 and 7 micrograms per 100 ml sea water, respectivcly. These concentrations were more than an order of magnitude below the lowest levels of supplementation, and thus considered negligible.

No measurable CO_2 was produced from filter-sterilized sea water with petroleum (*a*) or from natural sea water without petroleum (*b*)

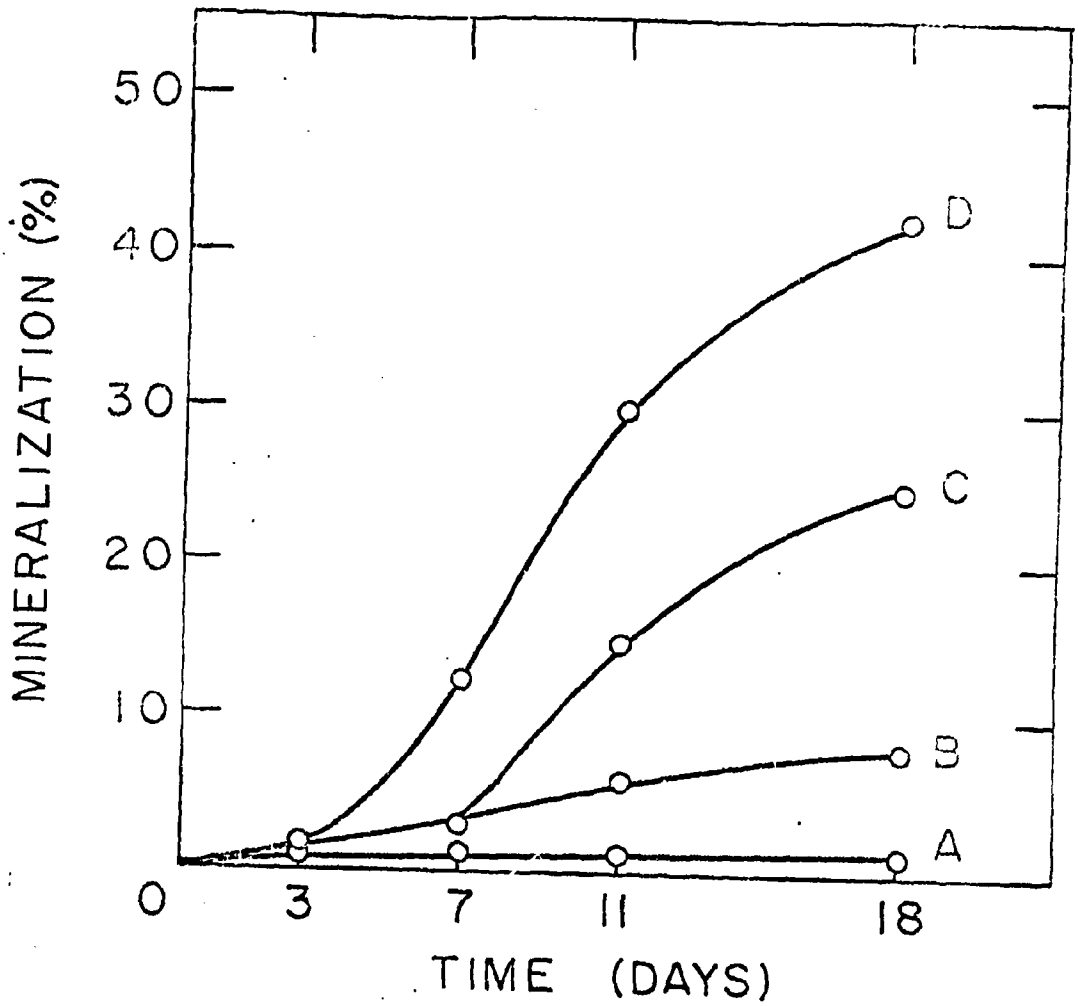

Fig. 1. Effect of supplements on the mineralization of petroleum by the natural population of sea water as measured by CO_2 evolution. All samples were supplemented with 7 X 10^{-4}M Na_2HPO_4. In addition, B, C, and D received 1 X 10^{-3}, 5 X 10^{-3}, and 1 X 10^{-2} M KNO_3, respectively.

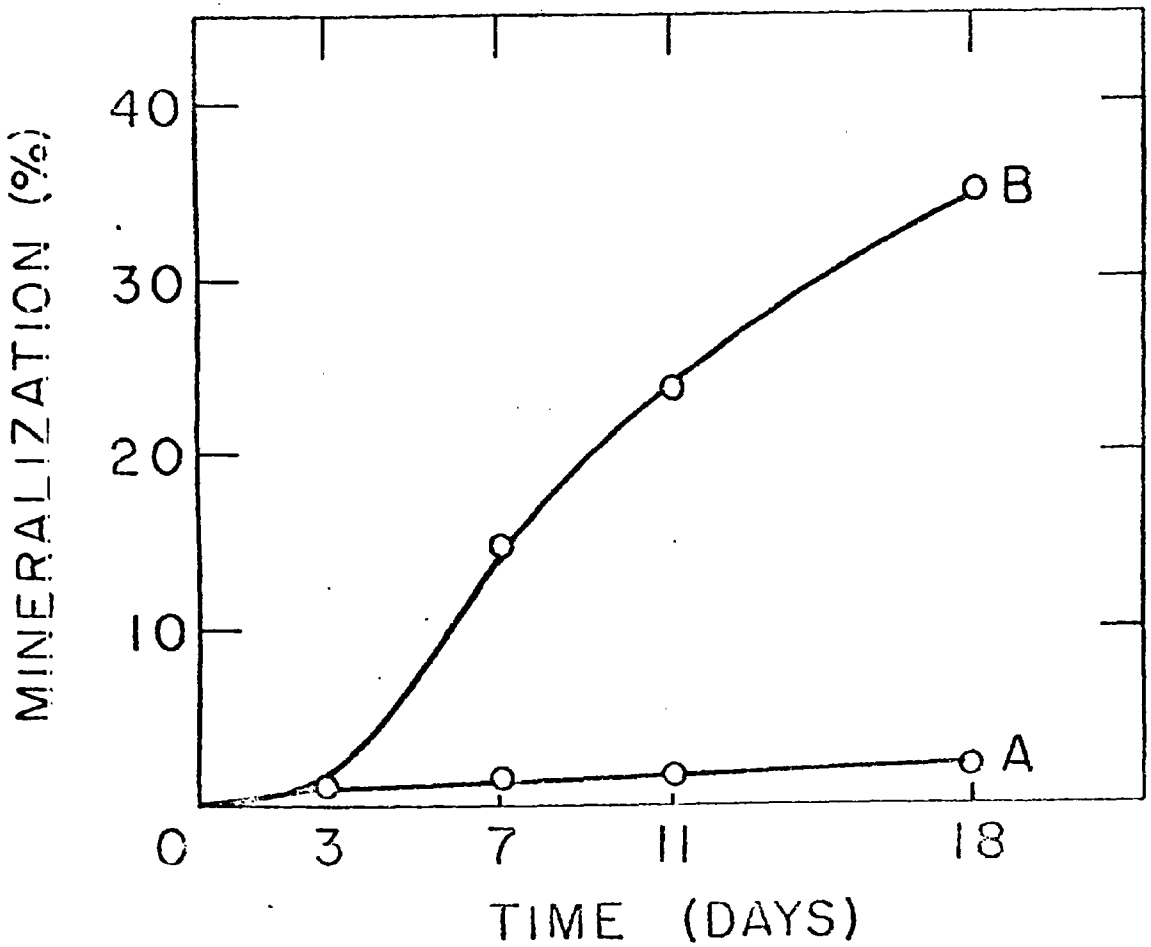

Fig. 2. Effect of supplements on the mineralization of petroleum in sea water. Both samples were supplemented with 3.7×10^{-3} M NH_4NO_3. In addition, B received 7×10^{-4} M Na_2HPO_4.

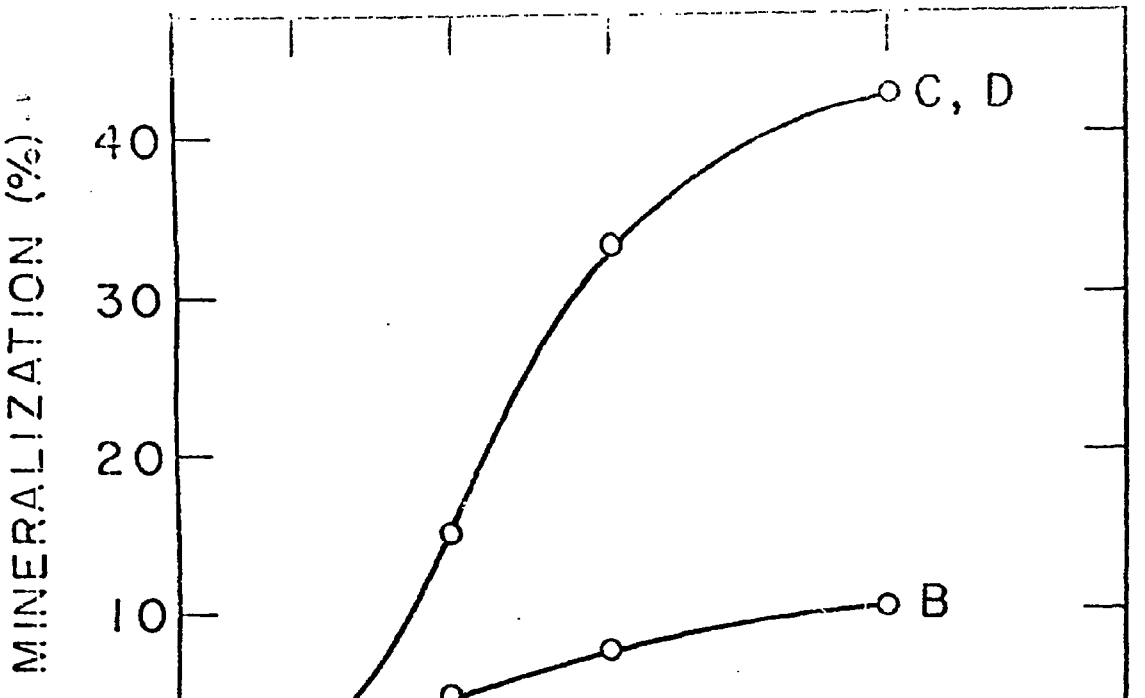

Fig. 3. Effect of supplements on the mineralization of petroleum in sea water. All samples were supplemented with 1 X 10^{-3}M KNO_3. In addition, B, C, and D received 7 X 10^{-5}, 3.5 X 10^{-4}, and 7 X 10^{-4}M Na_2HPO_4, respectively.

even when supplemented with mineral nutrients (*c*, *d*, *e*). Natural sea water with added petroleum but no mineral supplements (*f*) produced CO_2 at a very low rate; only 1% of the theoretical maximum was attained during 18 days of incubation.

When added individually, even the highest concentrations of Na_2HPO_4 , NH_4NO_3, or KNO_3 failed to promote petroleum degradation significantly (curve A, Fig. 1, 2, and 3). In the presence of the highest phosphate concentration, increasing levels of KNO_3 led to increased percentages of petroleum mineralization (Fig. 1, B, C, and D). It was not critical whether nitrogen was supplied in the form of nitrate or ammonia, since NH_4NO_3 gave a response substantially greater than its nitrate content could have elicited (Fig. 2, B). In the presence of the highest KNO_3 concentration, increasing levels of phosphate supplementation led to increasing percentages of petroleum mineralization (Fig. 3, B, and C). Apparently, 3.5×10^{-4}M phosphate saturated the experimental system, since a further increase of phosphate to 7×10^{-4} failed to augment mineralization.

The gas chromatographic measurement of petroleum biodegradation supported the results of the CO_2 evolution studies. Fig. 4 compares gas chromatograms of Sweden crude oil incubated for 18 days in sterile sea water (*A*), in unsupplemented natural sea water (*B*), and in sea water supplemented with 7×10^{-4} M Na_2HPO_4(*C*) or 1×10^{-2} M KNO_3 (*D*) or both (*E*). As compared to *A*, chromatograms *B*, *C*, and *D* exhibit only minor changes. In contrast, *E* demonstrates extensive biodegradation. All identified hydrocarbons have disappeared, including the highly branched pristane (No. 12) that had resisted the attack of the isolated cultures used in the preceding publication (3). The small residual peaks in chromatogram *E* do not represent the remnants of the major peaks visible in

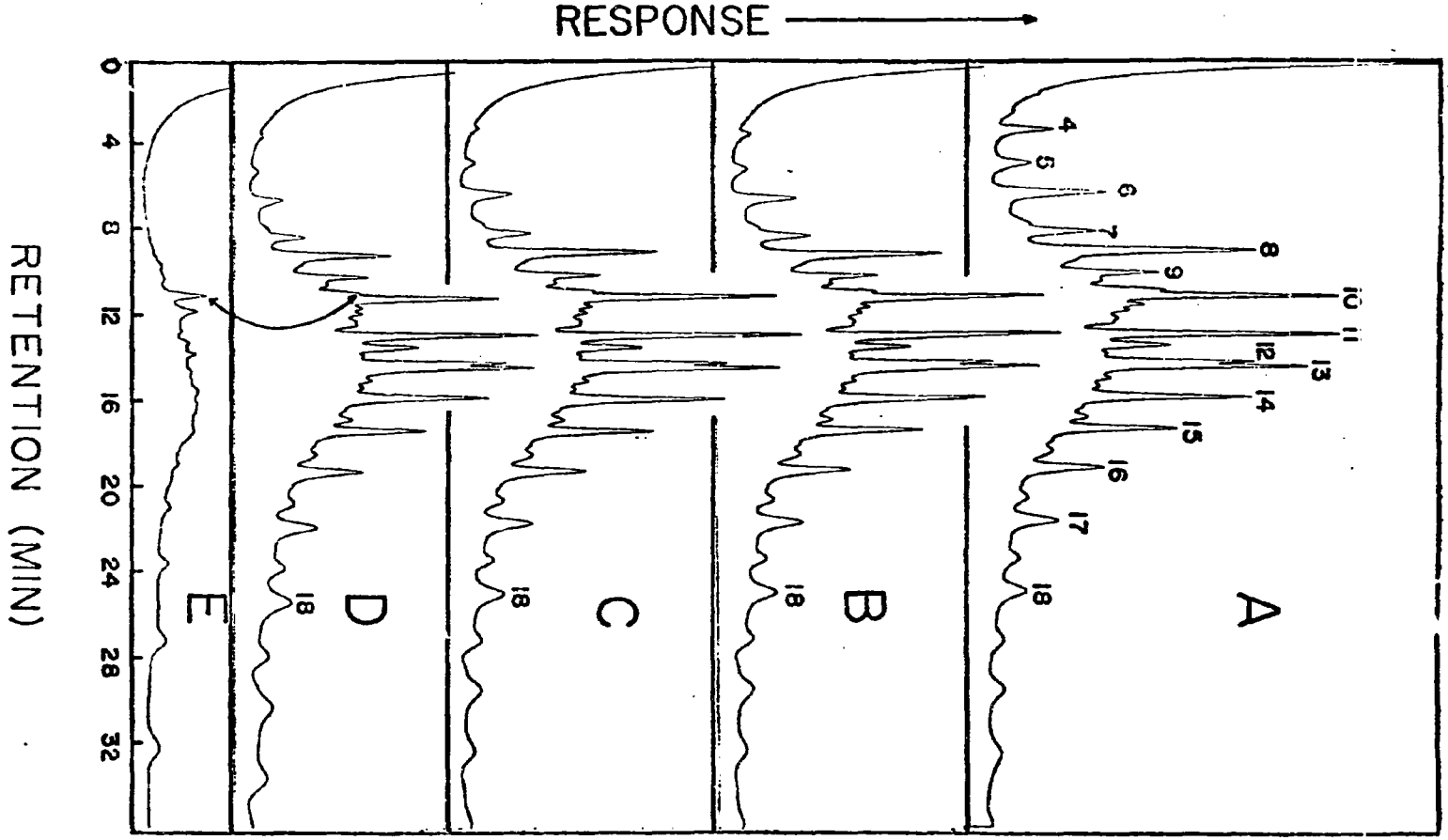

Fig. 4. Gas chromatograms of Sweden crude oil, incubated for 18 days under the following conditions: in filter-sterilized sea water (A), in natural sea water (B), in sea water supplemented with 7×10^{-4} M Na_2HPO_4 (C), 1×10^{-2} M KNO_3 (D), and both (E). For identity of the numbered peaks see Table 1 in the preceeding article (3).

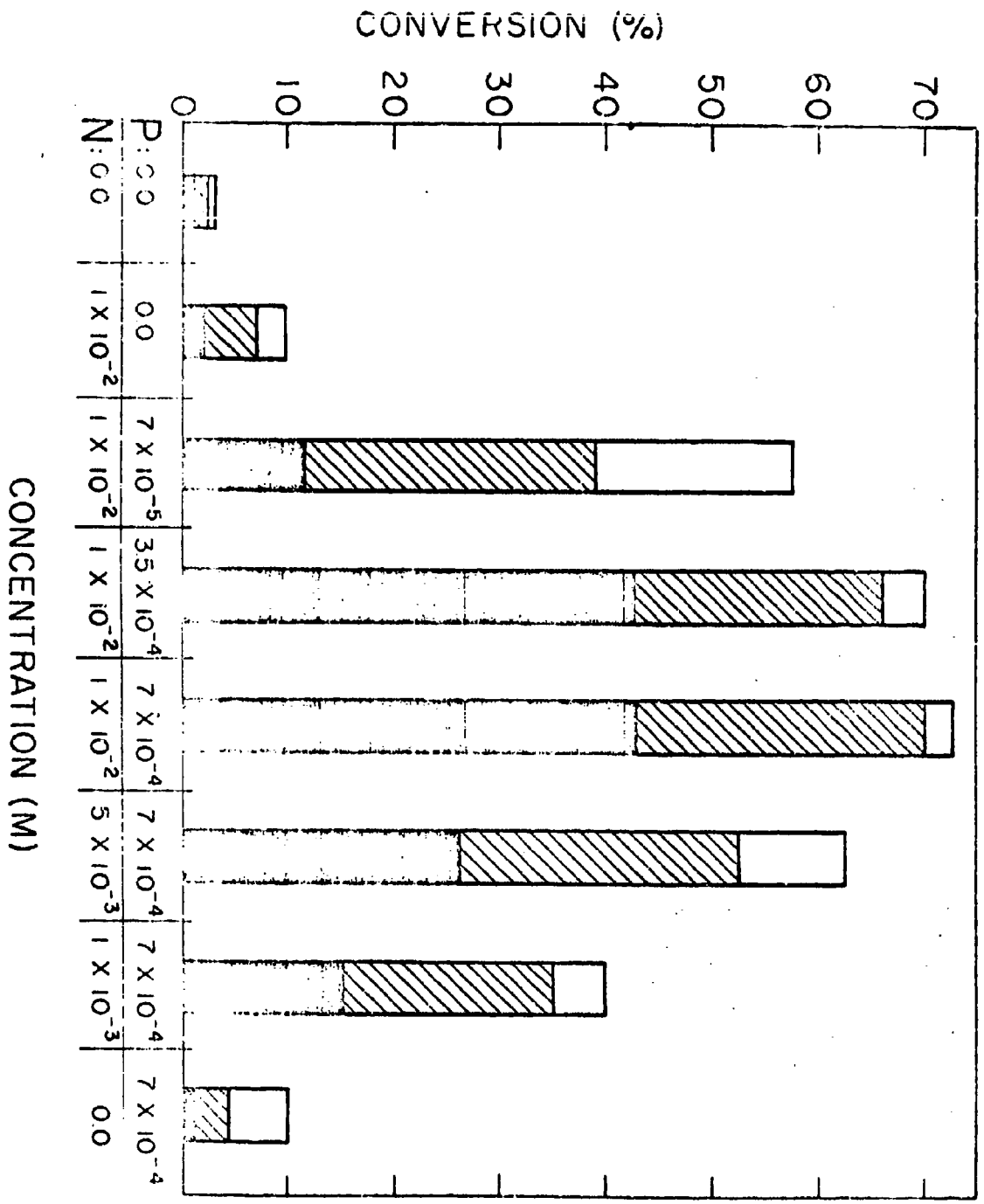

Fig. 5. Conversion of petroleum in sea water supplemented by various concentrations of Na_2HPO_4 and KNO_3 during 18 days of incubation. Solid portions of the histograms represent mineralization percentage as measured by CO_2 evolution. The upper limits of the cross hatched and empty portions of the histograms represent biodegradation percentages as measured by gas chromatography and by residual weight, respectively.

A, *B*, *C*, and *D*, but have appeared only after biodegradation has stripped away larger overlapping peaks. Both n-paraffins and branched paraffins were subject to biodegradation by the indigeneous population of the coastal sea water sample.

The extent of petroleum biodegradation and mineralization in response to mineral supplements is summarized in Fig. 5. Measurements of residual weight gave the highest values; additional evaporative losses prior to weighing may have introduced a positive error into the apparent biodegradation percentage. Integration of the area under the gas chromatograms gave a close but somewhat lower value. Mineralization, due to the carbon tied up in products and cell material, gave lower figures. At 18 days, biodegradation reached 70% and mineralization reached 42% in the optimally supplemented samples.

DISCUSSION

Stimulated biodegradation of petroleum products in response to nitrogen, phosphorous and potassium supplements was observed by Iz'yurova (8, 9) and removal of the mineral supplements from solution by the hydrocarbon-degrading microorganisms was noted. Brown *et al.* (5) and Brown and Tischer (6) reported increased petroleum disappearance from artificial sea water supplemented by phosphate and either nitrate or ammonia. This report was based on visual observation only, and the concentrations of the mineral supplements were not specified. Gunkel (7) reported increases in the rate but not in the extent of petroleum disappearance upon supplementation of natural sea water by unspecified amounts of phosphate and nitrate. The most probable numbers of petroleum degrading microorganisms were also increased. Using gas chromatographic techniques, Petit and Barthelemy (12) observed increased biodegradation of individual

n-paraffins in a petroleum fraction exposed to the natural microflora of sea water, when the latter was supplemented with 0.1% $(NH_4)_2HPO_4$. No additional concentrations or separate N- and P- sources were tested. Kator *et al.* (10) reported a modest stimulation of petroleum degradation under simulated field conditions in response to inoculation with hydrocarbon-degrading mixed microbial enrichments. Inoculation was combined with addition of $(NH_4)_2SO_4$ to the natural sea water underlaying the oil slick. Phosphorous was not added, though the authors stated that it could have been limiting.

Our results are in general agreement with the cited reports, and they identify the low concentrations of phosphate and nitrate in natural sea water as the principal limiting parameters of petroleum biodegradation, presuming reasonable aeration and temperatures favorable for microbial activity. Additional experiments will be required to decide whether in nitrogen- and phosphorous-enriched sea water the extent of petroleum biodegradation is limited by the recalcitrance of hydrocarbons only, or whether under these conditions additional mineral nutrients become limiting. The relative shortness of the observed petroleum degradation lag period is in agreement with the reported prevalence of hydrocarbon-degrading microorganisms in the neritic zone; the same may not be the case in pelagic areas (15).

Measurement of biodegradation in a complex mixture such as petroleum automatically raises the question of valid analytical criteria. All methods available for routine measurements are subject to various positive and negative errors, and give only relative values. As an example, in residual weight measurements some volatile fractions are lost, but this weight loss may be partially compensated for by oxygen

incorporation into the non-volatile fractions. To assure the validity of our conclusions, we have measured petroleum conversion by three independent criteria: CO_2 evolution, quantitative gas chromatography, and residual weight. The three parameters showed good correlation. Biodegradation as measured by gas chromatography gave a more conservative (and presumably more accurate) estimate than gravimetric analysis. Biodegradation figures in the rest of the discussion are based on the gas chromatographic measurements.

While mineralization (Fig. 5, solid portion of the histograms) showed a pronounced dependence on the concentration of the nitrogen and phosphorous supplements, an almost constant 25% of the added petroleum was converted to cell material and products (cross hatched portion of the histograms) as long as some of each mineral supplement was provided. Theorethical calculations assuming an 8% nitrogen and 1% phosphorous content of the dry weight show that at the lowest and the highest level of mineral supplementation 17.5 and 175 mg (dry weight) microbial biomass could have been produced, although approximately 200 mg of petroleum were degraded but not mineralized in both cases. Reliable measurements of microbial biomass and protein were not possible because of the interference of the strongly adhering oil. It seems reasonable to assume that either nitrogen or phosphorous deficiency will tend to produce cells with abnormally high lipid stores and low metabolic activity. The same condition may also cause the accumulation of extracellular intermediary metabolites. In contrast, sufficiency of nitrogen and phosphorous will favor the formation of protein-rich cells with high metabolic activity, and will also promote the mineralization of of intermediary degradation products.

In agreement with the experimental results, the theoretical calculations also show that at the highest level of supplementation, phosphorous was present in excess and nitrogen may have been limiting. As calculated, the phosphorous and nitrogen contents of 100 ml unsupplemented sea water would support less than 0.1 mg (dry weight) microbial biomass. This fact appears to be a major cause for the slow rate of petroleum biodegradation in the sea, especially when vigorous circulation does not occur in the water column underneath the oil slick.

As physical removal or burning of accidentally spilled crude oil is seldom feasible, and dispersion or sinking may adversely affect marine life (4), artificially stimulated biodegradation is being considered as a possible alternative (2). For the success of this approach it is essential that the biodegradation-limiting parameters in sea water should be properly identified. Our results indicate, that in a nutritionally unfavorable environment reliance on inoculation alone would be ecologically unsound , and of little, if any, benefit. Any "seeding" operation should be connected with application of an appropriate "fertilizer". A hydrophobic binder may achieve the selective retention of the mineral supplements within the .oil slick.

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ACKNOWLEDGEMENTS

This work was supported in part by the Office of Naval Research. Paper of the Journal Series, New Jersey Agricultural Experiment Station.