Spring 1986

PHYSIOLOGY AND ESTUARINE ECOLOGY OF PHENANTHRENE-DEGRADING BACTERIA (MYCOBACTERIUM, MICROLAYER, METABOLITES, PAH, MARINAS, NEW HAMPSHIRE)

WILLIAM FRANCIS GUERIN
University of New Hampshire, Durham

INFORMATION TO USERS

This reproduction was made from a copy of a manuscript sent to us for publication and microfilming. While the most advanced technology has been used to photograph and reproduce this manuscript, the quality of the reproduction is heavily dependent upon the quality of the material submitted. Pages in any manuscript may have indistinct print. In all cases the best available copy has been filmed.

The following explanation of techniques is provided to help clarify notations which may appear on this reproduction.

1. Manuscripts may not always be complete. When it is not possible to obtain missing pages, a note appears to indicate this.

2. When copyrighted materials are removed from the manuscript, a note appears to indicate this.

3. Oversize materials (maps, drawings, and charts) are photographed by sectioning the original, beginning at the upper left hand corner and continuing from left to right in equal sections with small overlaps. Each oversize page is also filmed as one exposure and is available, for an additional charge, as a standard 35mm slide or in black and white paper format.*

4. Most photographs reproduce acceptably on positive microfilm or microfiche but lack clarity on xerographic copies made from the microfilm. For an additional charge, all photographs are available in black and white standard 35mm slide format.*

***For more information about black and white slides or enlarged paper reproductions, please contact the Dissertations Customer Services Department.**

U·M·I Dissertation Information Service

University Microfilms International
A Bell & Howell Information Company
300 N. Zeeb Road, Ann Arbor, Michigan 48106

Guerin, William Francis, Jr.

PHYSIOLOGY AND ESTUARINE ECOLOGY OF PHENANTHRENE-DEGRADING BACTERIA

University of New Hampshire PH.D. 1986

PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark __✓__.

1. Glossy photographs or pages ___✓___
2. Colored illustrations, paper or print _____
3. Photographs with dark background ___✓___
4. Illustrations are poor copy _____
5. Pages with black marks, not original copy _____
6. Print shows through as there is text on both sides of page _____
7. Indistinct, broken or small print on several pages ___✓___
8. Print exceeds margin requirements _____
9. Tightly bound copy with print lost in spine _____
10. Computer printout pages with indistinct print _____
11. Page(s) _____ lacking when material received, and not available from school or author.
12. Page(s) _____ seem to be missing in numbering only as text follows.
13. Two pages numbered _____. Text follows.
14. Curling and wrinkled pages _____
15. Dissertation contains pages with print at a slant, filmed as received _____
16. Other _____

_____

_____

PHYSIOLOGY AND ESTUARINE ECOLOGY

OF PHENANTHRENE-DEGRADING BACTERIA

BY

WILLIAM F. GUERIN, JR.
B.G.S., University of Michigan, 1975
M.S., University of South Florida, 1978

DISSERTATION

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of

Doctor of Philosophy

in

Microbiology

May, 1986

This dissertation has been examined and approved.

Galen E. Jones

Dissertation director, Galen E. Jones
Professor of Microbiology

Richard P. Blakemore

Richard P. Blakemore
Professor of Microbiology

Robert M. Zsigray

Robert M. Zsigray
Professor of Microbiology

W. Rudolf Seitz

W. Rudolf Seitz
Professor of Chemistry

Lawrence M. Mayer

Lawrence M. Mayer, Assoc. Professor
Geological Sciences and Oceanography
University of Maine

May 9, 1986

Date

ACKNOWLEDGMENTS

The work embodied by this dissertation would not have been possible without the aid and fellowship of dozens of people at UNH. I would like to thank Mark Hines for getting me started at UNH, Galen Jones for his confidence and continual support, and Joyce Tugel, Art Mathieson and Clayton Penniman at the Jackson Estuarine Lab for their years of comaraderie and interest. In the Chemistry Dept., Tiny Grant, Tom Gauthier and Rudy Seitz were instrumental in providing facilities and sharing their analytical expertise. I am grateful for the ship-board assistance and nutrient and DOC analyses provided by Ted Loder, Sue Welch and Greg Hancox of the Earth Science Dept. In the Marine Program office, Jane Pitroff and Meriel Bunker kept the operation running smoothly.

In the Microbiology Dept., special thanks go to Alberta Moulton and Bob Mooney for helping with the day to day details and to the many graduate students, past and present, whose friendship made being at work more enjoyable. I am grateful to the Microbiology faculty for their interest in my work and would especially like to thank Dick and Nancy Blakemore for their unselfish regard for my personal and professional life.

This work would not have been possible without the generous use of the HPLC afforded me by Mike Ikawa of the Biochemistry Dept. Linda Schick of the University of Maine's Darling Center provided CHN analyses and help with surface area measurements. I will always be indebted to Larry Mayer of the same institution for broadening my

biogeochemical horizons by teaching me to think small.

Inspiration over these past few years has come from several sources. Thanks to Olivier Donard for expanding my world through his photographic images, to John Updike for raising the science of biogeochemistry to new literary heights, and to Elvis Costello (and WUNH) for his music.

My deepest appreciation is extended to my wife, Molly, and my sons, Nathan and Martin, for the love, encouragement and support they have given over the course of this endeavor. Without them, I am sure, these pages would not have been written.

This work was supported by Hubbard funds provided through the Marine Program office and by grant no. NA-81-AA-D-0035 from the U.S. Dept. of Commerce's Sea Grant program.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

PHYSIOLOGY AND ESTUARINE ECOLOGY OF PHENANTHRENE-DEGRADING BACTERIA

by

WILLIAM F. GUERIN, JR.

University of New Hampshire, May, 1986

Using radiorespirometric, spectrophotometric and high performance liquid and thin layer chromatographic methods, the degradation and intermediary metabolism of the polycyclic aromatic hydrocarbon (PAH), phenanthrene, by estuarine enrichment and pure microbial cultures was examined.

A Mycobacterium species, strain BG1, able to use phenanthrene as sole carbon and energy source, was isolated from estuarine sediment. Phenanthrene degradation proceeded via the intermediates, 1-hydroxy-2-naphthoic acid (1H2NA) and protocatechuic acid. However, unlike other phenanthrene-degrading cultures, aromatic intermediates, including 1H2NA, did not accumulate. Consistent with the induction of meta pathway enzymes in phenanthrene-grown BG1 cells, phenanthrene degradation was stimulated in pyruvate-supplemented cultures (an end product of meta cleavage) and repressed in succinate-supplemented cultures (an end product of ortho cleavage). Phenanthrene-degrading cells possessed 3 plasmids (20.6, 57.5 and 76.7 megadaltons) likely responsible for degradation. Plasmids and the phenanthrene-degrading phenotype were absent in nutrient-grown cells.

Analogous to degradative reactions involving other PAH when

present in excess, enrichment cultures and cultures of most isolates derived from them accumulated near stoichiometric amounts of 1H2NA during phenanthrene degradation. In pure cultures, 1H2NA was not further degraded. In enrichment cultures, subsequent mineralization of 1H2NA led to secondary increases in biomass. Two-stage mineralization of phenanthrene was also evidenced by a biphasic $^{14}CO_2$ production in enrichment cultures spiked with low concentrations (0.5 mg L^{-1}) of ^{14}C-phenanthrene. Here, however, polar metabolites comprised less than 10% of the total initial activity.

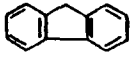
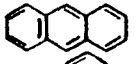
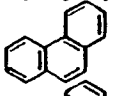
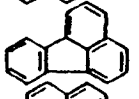
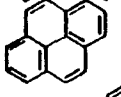
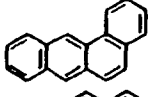
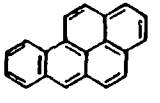
Phenanthrene-degrading bacteria were ubiquitous in the waters and sediments of the Great Bay Estuary, NH, and activities correlated positively with the degree of previous exposure to PAH. Particularly active were sediments collected near an oil refinery and water samples collected downstream from a dredging operation. Coal tar-derived PAH, analyzed in dredge and downstream sediments by capillary gas chromatography, were introduced into the river in high concentrations over several months. Phenanthrene degradation potentials were also high in areas influenced by pleasure and commercial boating activities. Surface microlayer samples from marinas were enriched with fluorescent hydrocarbons but often showed depressed phenanthrene degradative activities relative to underlying bulk waters. Sunlight, hydrocarbons or organotin compounds were possibly inhibitory.

INTRODUCTION

Phenanthrene ($C_{14}H_{10}$) is a three-ringed member of the class of compounds known as polycyclic aromatic hydrocarbons (PAH). These fluorescent, UV-absorbing compounds contain two or more fused aromatic rings in planar geometry. PAH are hydrophobic (lipophilic) with solubilities in distilled water ranging from 32 mg L^{-1} for naphthalene to 4 ug L^{-1} for benzo(a)pyrene (Table 1). Because of their high fugacities, PAH in aquatic environments are highly adsorptive. The extent of partitioning of PAH from water onto natural soils or sediments is governed by the compound's octanol-water partition coefficient (K_{ow}) and the organic carbon content of the sorbent. Dissolved organic matter, especially aromatic humic materials, compete with solid sorbents to some extent and help to solubilize PAH. Considering their high boiling points, PAH are also quite volatile.

PAH are naturally-occurring (diagenetic) components of fossil fuels and are universal products of the incomplete combustion of organic materials. Prior to the industrial revolution, PAH entered the environment *via* oil seeps and forest and prairie fires (Blumer and Youngblood, 1975). Anthropogenic inputs have drastically increased environmental PAH concentrations world-wide due to the deposition of air-borne flyash particulates, the global transport of crude and refined petroleum products and increased industrialization. The sedimentary record shows a sharp increase in PAH concentrations beginning around 1850, reaching a peak around 1950, and decreasing slightly since then due to the shift from coal to oil and gas as major

TABLE 1. Physical, chemical and biological properties of some polycyclic aromatic hydrocarbons.

Compound	Molecular Formula	Molecular Weight	Molecular Structure	Aqueous[a] Solubility	Vapor[b] Pressure	Log K_{ow}[c]	Carcinogenic Potential[d]
Two Rings							
Naphthalene	$C_{10}H_8$	128		31.7	0.23	3.36	---
Three Rings							
Fluorene	$C_{13}H_{10}$	166		1.685	4.76(-4)[e]	---	---
Anthracene	$C_{14}H_{10}$	178		0.045	1.95(-4)	4.54	---
Phenanthrene	$C_{14}H_{10}$	178		1.002	6.80(-4)	4.56	---
Four Rings							
Fluoranthene	$C_{16}H_{10}$	202		0.206	---	4.90	---
Pyrene	$C_{16}H_{10}$	202		0.132	6.85(-7)	5.17	---
Benzanthracene	$C_{18}H_{12}$	228		0.009	1.10(-7)	5.61	+/-
Five Rings							
Benzo(a)pyrene	$C_{20}H_{12}$	252		0.004[f]	5.49(-9)	6.04	+++

[a] values from May et al, 1978, except where noted, in mg L^{-1}.
[b] values from Readman et al, 1982, except where noted, in mm Hg; exponents in parentheses.
[c] log octanol/water partition coefficient values from Readman et al, 1982.
[d] ---, inactive; +/-, disputed; +++, high potential; from Phillips, 1983.
[e] from Boyle et al, 1984.
[f] from Dzombak and Luthy, 1984.

energy souces (Forstner and Muller, 1981; Gschwend and Hites, 1981). With the depletion of the latter, the return to coal for future energy needs promises to reverse this recent trend.

Concern about the environmental fate of PAH dates to the late 19th century when it was discovered that these compounds, particularly benzo(a)pyrene, were the components of soot and creosote responsible for the high incidence of cancer among chimney sweeps (Phillips, 1983). It has since been recognized that many environmentally induced cancers are caused by PAH (Phillips, 1983; Selkirk and MacLeod, 1982). Because of their toxicity (Calder and Lader, 1976; Spies et al, 1982; Trucco et al, 1983; Gilewicz et al, 1984), carcinogenicity (Brown et al, 1973; Jackim and Lake, 1978; Maccubbin et al, 1985), and potential for trophic biomagnification (Dobroski and Epifanio, 1980; Murray et al, 1981; Eadie et al, 1982; Landrum and Scavia, 1983; Trucco et al, 1983), PAH comprise 16 of the 96 organic chemicals listed as priority pollutants by the EPA (Ghisalba, 1983).

A major focus of the microbiological research on PAH metabolism has been to compare prokaryotic oxidation mechanisms with those carried out by eukaryotes. Since the partial oxidation products of PAH, and not the parent compounds, are the actual mutagens in mammalian systems (Phillips, 1983), bacteria would provide convenient models for studying PAH metabolism if they performed similar oxidative transformations. In fact, bacteria differ in that they form cis-dihydrodiols through dioxetane intermediates rather than trans-dihydrodiols through the mutagenic arene oxide (epoxide) intermediates of eukaryotes (Gibson et al, 1975; Jerina et al, 1976). A further distinction is that bacteria employ iron-containing dioxygenase enzymes which incorporate both atoms

of molecular oxygen into adjacent positions on the PAH substrate as a prerequisite to ring cleavage (Dagley, 1978). Eukaryotes employ monooxygenase enzymes which, as their names imply, incorporate one oxygen atom into the substrate (Cerniglia, 1981). The strategy of eukaryotic PAH metabolism is to solubilize the hydrophobic compounds by conjugating them with sulfate, glucuronide or other functional groups so they can be eliminated (Cerniglia et al, 1982; Selkirk and MacLeod, 1982).

Bacteria, in their roles as decomposers and remineralizers, have also been studied with regard to their PAH degrading abilities since 1927 (Tausson, cited in Cerniglia, 1984). Not surprisingly, since PAH are naturally occurring compounds, bacteria which can degrade them are ubiquitously distributed and represent diverse taxonomic groups. Several genera of bacteria have been described which can utilize two- and three-ringed PAH as their sole source of carbon and energy; PAH with four or more rings are attacked only slowly or not at all. Table 2 lists most of the studies of pure culture dissimilations of PAH published since 1957. The overwhelming majority of these have employed species of the genus, Pseudomonas, bacteria well known for their metabolic virtuosity (Clarke, 1982).

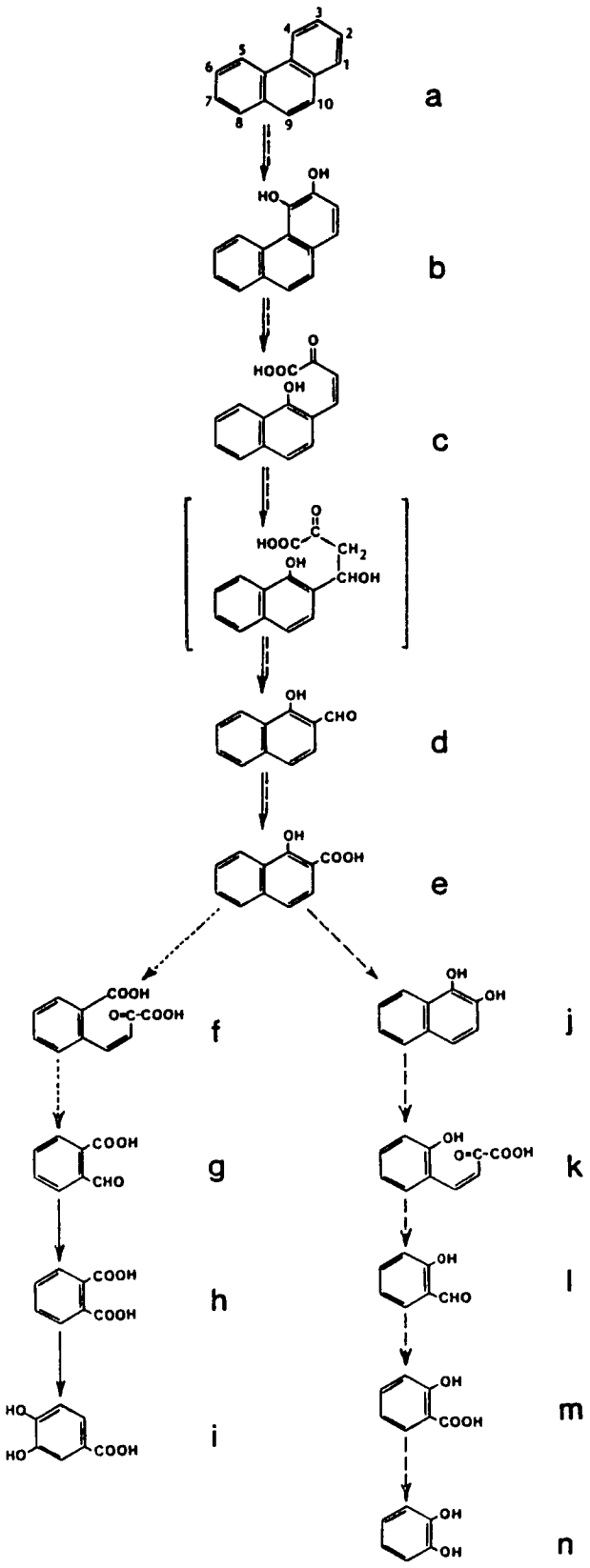
Pure culture studies with phenanthrene have elucidated the biochemical degradation pathway for this compound (Fig. 1). Early work showed phenanthrene to be degraded, through a series of intermediates, to 1-hydroxy-2-naphthoic acid (1H2NA, Fig. 1, compound e). Further degradation was postulated to occur as for naphthalene and anthracene, i.e., decarboxylation to give 1,2-dihydroxynaphthalene (compound j) which was then degraded via salicylate to catechol (compounds m and n,

TABLE 2. Pure culture studies of PAH degradation.

Compound(s)*	Organism(s)	Reference
PHN	Pseudomonas	Rogoff and Wender, 1957a
ANT	Pseudomonas	Rogoff and Wender, 1957b
NAH, ANT, PHN	Pseudomonas	Rogoff, 1962
NAH	Pseudomonas	Davies and Evans, 1964
NAH, ANT, PHN	Pseudomonas	Evans et al, 1965
NAH (co-oxidation)	Nocardia	Raymond et al, 1967
NAH, ANT, PHN	Pseudomonas	Dean-Raymond and Bartha, 1975
FLA, B(a)P	Pseudomonas	Barnsley, 1975
B(a)A, B(a)P	Beijerinckia	Gibson et al, 1975
ANT, PHN	Beijerinckia	Jerina et al, 1976
NAH, PHN	Pseudomonas, Aeromonas	Kiyohara and Nagao, 1978
NAH	Pseudomonas	Zuniga et al, 1981
PHN	Alcaligenes	Kiyohara et al, 1982
NAH, PHN	gram negative rods, cocci	Barnsley, 1983
PHN	Micrococcus	Ghosh and Mishra, 1983

* Compound abbreviations are as follows: NAH, naphthalene; ANT, anthracene; PHN, phenanthrene; FLA, fluoranthene; B(a)P, benzo(a)pyrene; B(a)A, benz(a)anthracene.

FIGURE 1. Pathway of bacterial phenanthrene degradation as outlined by Kiyohara et al. (1976), ——————; Barnsley (1983), ---------- ; and Evans et al. (1965), — — — — — — — — . a, phenanthrene; b, 3,4-dihydroxyphenanthrene; c, cis-4-(1-hydroxynaphth-2-yl)-2-oxobut-3-enoic acid; d, 1-hydroxy-2-naphthaldehyde; e, 1-hydroxy-2-naphthoic acid; f, o-carboxybenzalpyruvate; g, o-carboxybenzaldehyde; h, phthalic acid; i, protocatechuic acid; j, 1,2-dihydroxynaphthalene; k, o-hydroxybenzalpyruvate; l, salicylaldehyde; m, salicylic acid; n, catechol; [proposed intermediate].


a
b
c
d
e
f
g
h
i
j
k
l
m
n

respectively, Evans et al, 1965). Kiyohara et al. (1976) proved the existence of a different pathway in Aeromonas, Alcaligenes (Kiyohara et al, 1982), and in Pseudomonas (Kiyohara and Nagao, 1978), the same genus used in the earlier studies of Evans et al. (1965). According to this scheme, phenanthrene was degraded via 1H2NA, o-carboxybenzalpyruvate (Barnsley, 1983), and phthalic acid to protocatechuic acid (Fig. 1, compounds e, f, h and i, respectively). All subsequent work on bacterial phenanthrene degradation has substantiated the latter pathway. Both protocatechuic acid and catechol are central intermediates in aromatic catabolism (Dagley, 1978). These undergo either meta or ortho cleavage to yield non-aromatic products (Stanier and Ornston, 1973). Following the meta pathway, the complete mineralization of phenanthrene ($C_{14}H_{10}$) yields 3 molecules of pyruvate, one molecule of acetaldeyde and 3 molecules of CO_2.

Phenanthrene was chosen as a model PAH for this study because of its intermediate solubility, volatility and adsorptive behavior (Table 1). Phenanthrene is a major component of the PAH composites from many sources and, together with its alkylated homologs, is perhaps the single most abundant PAH in the environment (Neff, 1979). Phenanthrene is also a good model compound because it, like the carcinogenic PAH, possesses the so-called "bay region" formed by the angular ring arrangement (Table 1). Phenanthrene is non-carcinogenic and relatively non-toxic; this simplified handling precautions. Furthermore, and despite the extensive knowledge of the biochemistry of phenanthrene degradation, little was known about the biogeochemistry of this important PAH. As seen in Table 3, environmental studies of phenanthrene degradation are few in comparison to those for

TABLE 3. Studies on PAH biodegradation in aquatic environments.

Compounds Tested*	Habitat	Reference
NAH, PHN, ANT, B(a)A, DBA	Seawater	Sisler and ZoBell, 1947
NAH, B(a)A	Coastal seawater	Roubal and Atlas, 1978
NAH, MethylNAH, ANT, B(a)A, B(a)P	Marine mesocosms	Lee et al, 1978
NAH, ANT, B(a)A, B(a)P	Polluted and pristine stream sediments	Herbes and Schwall, 1978
ANT, FLA, B(a)A, B(a)P	Marine sediments	Gardner et al, 1979
PHN, PYR	Polluted and pristine freshwaters	Sherrill and Sayler, 1980
B(a)A	Marine mesocosms	Hinga et al, 1980
NAH	Oxidized and reduced salt marsh sediments	DeLaune et al, 1980
B(a)P	Oxidized and reduced estuarine sediments	DeLaune et al, 1981

TABLE 3. (cont.)

Compounds Tested*	Habitat	Reference
NAH, ANT, B(a)A	Stream water, sediments	Herbes, 1981
NAH, ANT, B(a)A	Marine and estuarine sediments	Atlas et al, 1981
B(a)A, DimethylB(a)A	Marine mesocosms	Lee et al, 1982
NAH, B(a)P	Marine (oil field) sediments	Saltzmann, 1982
NAH, B(a)P	Estuarine waters	Readman, et al, 1982
NAH	Lake water, sediment	Cooney et al, 1985
NAH, ANT	Marine sediments	Bauer and Capone, 1985

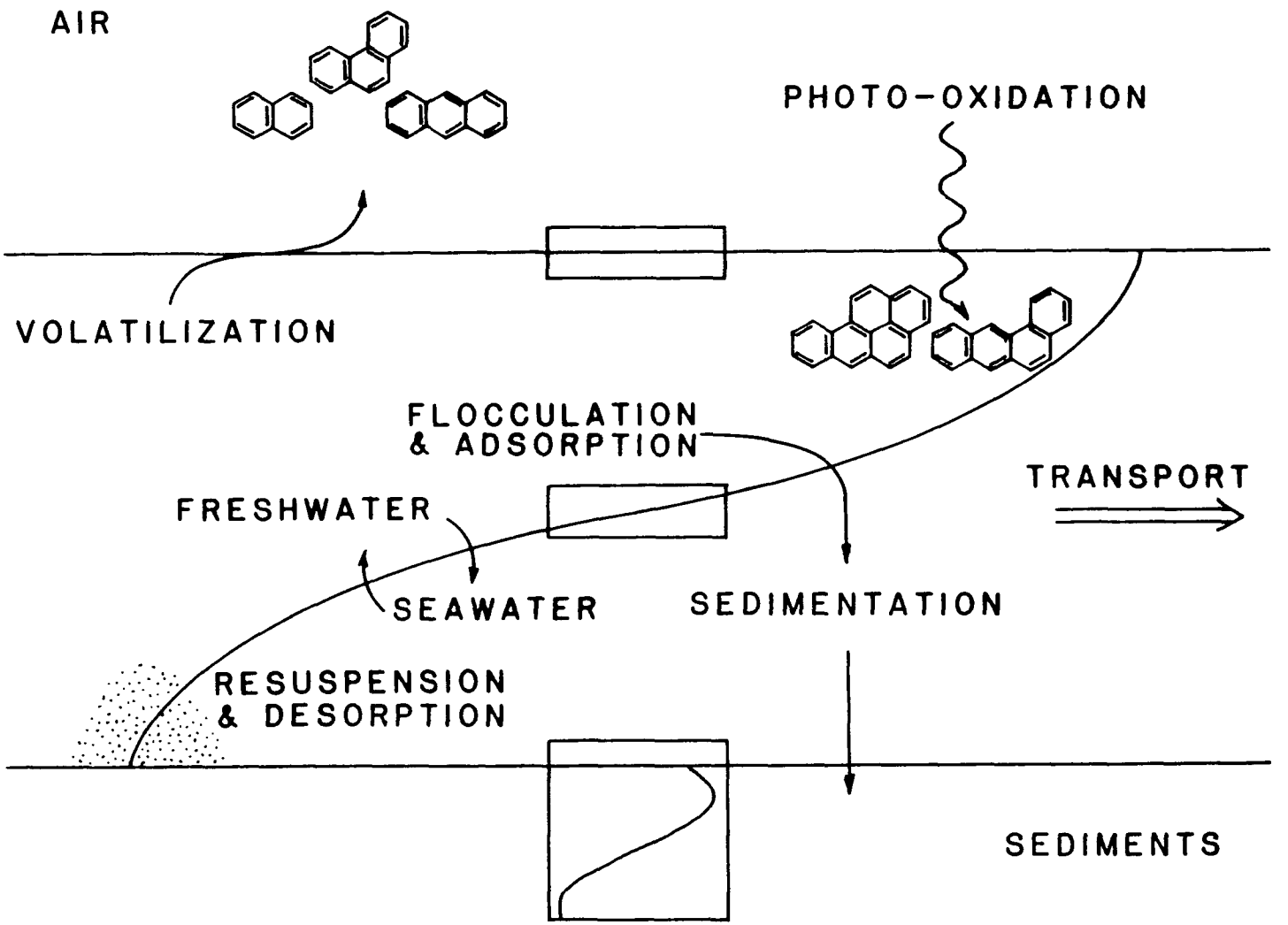
* Compound abbreviations are as follows: NAH, naphthalene; PHN, phenanthrene; ANT, anthracene; B(a)A, benz(a)anthracene; DBA, dibenz(a,h)anthracene; B(a)P, benzo(a)pyrene; FLA, fluoranthene; PYR, pyrene.

naphthalene, anthracene, benzanthracene and benzo(a)pyrene.

The purpose of this study was to gain some insight into the biogeochemical fate of phenanthrene (and PAH) in the estuarine environment, and in particular, the Great Bay Estuary, NH. This interest evolved out of the past occurrence of several major oil spills in the estuary and the recognition that PAH are among the most toxic and recalcitrant components of such spills. Figure 2 shows the complexity of interactions which may involve PAH in an estuarine environment. Physicochemical processes including volatilization, photooxidation, complexation with dissolved and colloidal organic matter and adsorption to biological and mineral particulates may influence the availability of PAH to biodegradative organisms. Additional fates for PAH include transport out of the estuary or burial in the sediments. In the sediments, PAH may persist because oxygen, which is an absolute requirement for degradation, may be absent. Oxygen limitation may be particularly important in estuarine and coastal sediments where, due to relatively high rates of organic matter deposition, anoxic conditions may develop just millimeters below the sediment/water interface. The abundance of mineralizable PAH in aquatic sediments suggests that oxygen limitation may be a major determinant of the environmental fate of these compounds.

Degradation of PAH in the environment is influenced by temperature, nutrient status, pH and salinity. Since the enzymes required for hydrocarbon metabolism are generally inducible, the availability of more easily assimilable carbon sources, or the presence of PAH in low concentrations, could suppress PAH metabolism (Alexander, 1981). Conversely, alternate substrates could act to support the

FIGURE 2. Estuarine interactions involving PAH. Boxes show compartments in which biodegradation potentials were assessed in this study including the estuarine surface microlayer, bulk water and sediments.

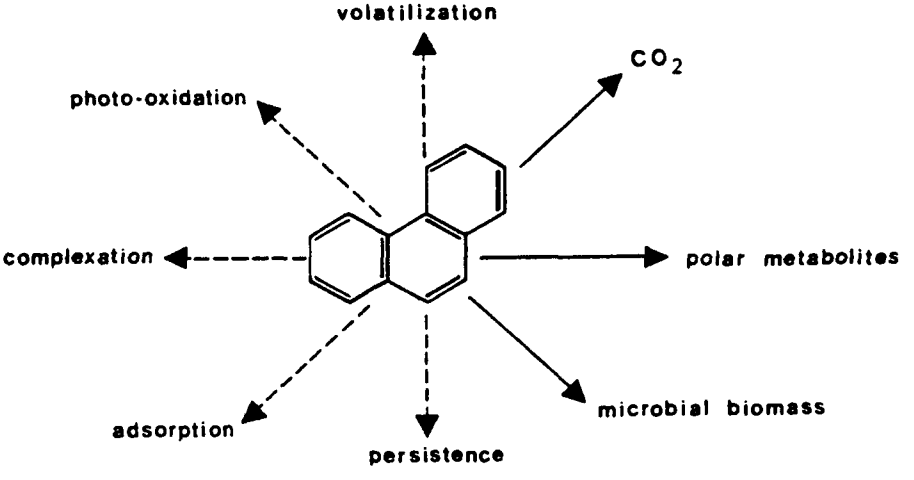

AIR
PHOTO-OXIDATION
VOLATILIZATION
FLOCCULATION
& ADSORPTION
TRANSPORT
FRESHWATER
SEAWATER
SEDIMENTATION
RESUSPENSION
& DESORPTION
SEDIMENTS

cometabolism of PAH which would otherwise go undegraded. Information on the biochemical pathways and metabolic intermediates of PAH degradations obtained through pure culture studies is invaluable. However, little of this information can be applied directly toward an understanding of the behavior of PAH in a complex estuarine environment due to insufficient knowledge of the physiology and regulation of PAH metabolism by microorganisms.

Before studying the ecology of phenanthrene degrading bacteria, it was necessary to develop methods for assessing bacterial PAH degradation in laboratory studies. In contrast to the situation for soluble substrates, routine methods for studying biodegradation of water-insoluble hydrocarbons are poorly developed (Gerike, 1984). As with all PAH, the study of phenanthrene biogeochemistry is fraught with analytical difficulties related to its low solubility and adsorptive behavior. To a great extent, these difficulties were surmounted and quantitative studies were made possible by the development of a growth medium in which abiotic losses of phenanthrene were avoided by the incorporation of surfactants.

During biodegradation assays, the carbon of phenanthrene may remain unchanged, be converted to biomass, be respired or be present in intermediate metabolic forms (Fig. 3). Measurements of substrate disappearance rates are of limited value as a sole index of biodegradation (Cook et al, 1983). It is desirable to corroborate substrate disappearance with some measure of cell growth. The necessity of working with particulate substrate suspensions precludes the use of turbidimetric and most enumerative measurements of growth due to attachment of degrading bacteria to the solid substrate. Manometric

FIGURE 3. Biological (solid arrows) and abiological (broken arrows) fates of phenanthrene in estuarine environments.


volatilization
CO_2
photo-oxidation
complexation
polar metabolites
microbial biomass
adsorption
persistence

methods suffer from the slow rate of particulate PAH mineralization during aquatic incubations.

A major emphasis of this study was to identify and assess the quantitative significance of metabolites produced during pure and mixed culture degradations of phenanthrene. Previous work had shown that the production of metabolites could be a significant fate for PAH (Herbes et al, 1977; Herbes and Schwall, 1978; Herbes, 1981). Since they are often more toxic than the parent compounds (Calder and Lader, 1976) and since they are chemically reactive (Wang and Li, 1977; Kirkbright et al, 1965; Bollag, 1983) much effort was spent in this study to develop new methods for analyzing bacterial PAH metabolites.

Using the Folin-Ciocalteau reaction as a (semi-)quantitative measure of metabolite concentration and HPLC as a qualitative tool for identifications, the intermediate metabolism (physiology) of phenanthrene degradation by pure and mixed microbial cultures was examined. In the course of this work, an unusual phenanthrene degradation pattern was observed in studies on an isolate (strain BG1) later identified as a Mycobacterium species. Unlike most other isolates which allow the accumulation of 1H2NA while degrading phenanthrene, BG1 brought about the complete mineralization of phenanthrene without accumulating 1H2NA regardless of the initial phenanthrene concentration. In mixed batch cultures, 1H2NA accumulated at high initial phenanthrene concentrations but was insignificant at low phenanthrene concentrations typical of estuarine environments. For environmental studies then, alternate methods for assessing biodegradation potentials were developed.

Radiorespirometric techniques for measuring the heterotrophic

activities of aquatic microbial communities have gained widespread acceptance in the past decade. Using a combination of flask and tube techniques, an extremely precise hybrid method for measuring the production of $^{14}CO_2$ from [9-^{14}C] phenanthrene was developed. This method was applied in ecological studies of the distribution of phenanthrene degradation potentials in the waters and sediments of the Great Bay Estuary. Simultaneous analyses of nutrients, organic matter, and particulate phases in the water along with analyses of sedimentary PAH revealed that phenanthrene degradation in the estuary was most active in areas with prior exposure to PAH. Marinas and a dredge site in the Cocheco River were found to be reservoirs of PAH-degrading bacterial populations.

To test the hypothesis that accumulation of hydrophobic PAH in the estuarine surface microlayer might enrich for an active PAH-degrading bacterial population, several surface microlayer and bulk water samples from throughout the estuary were analyzed. Microlayers showed consistent enrichments of organic matter, nutrients and hydrocarbons, but often a depressed activity of phenanthrene degrading bacteria. This observation was particularly true in polluted marina environments and may have been due to the accumulation of toxic organotin compounds or hydrocarbons in the microlayers.

REFERENCES

Alexander, M. 1981. Biodegradation of chemicals of environmental concern. Science 211:132-138.

Atlas, R.M., P.D. Boehm and J.A. Calder. 1981. Chemical and biological weathering of oil, from the Amoco Cadiz spillage, within the littoral zone. Est. Coast. Shelf Sci. 12:589-608.

Barnsley, E.A. 1975. The bacterial degradation of fluoranthene and benzo(a)pyrene. Can. J. Microbiol. 21:1004-1008.

Barnsley, E.A. 1983. Phthalate pathway of phenanthrene metabolism: Formation of 2'-carboxybenzalpyruvate. J. Bacteriol. 154:113-117.

Bauer, J.E. and D.G. Capone. 1985. Degradation and mineralization of the polycyclic aromatic hydrocarbons anthracene and naphthalene in intertidal marine sediments. Appl. Environ. Microbiol. 50:81-90.

Blumer, M. and W.W. Youngblood. 1975. Polycyclic aromatic hydrocarbons in soils and recent sediments. Science 188:53-55.

Bollag, J.-M. 1983. Cross-coupling of humus constituents and xenobiotic substances, pp. 127-141, In, R.F. Christman and E.T. Gjessing (ed.), Aquatic and Terrestrial Humic Materials. Ann Arbor Science, Ann Arbor, MI.

Boyle, T.P., S.E. Finger, J.D. Petty, L.M. Smith and J.N. Huckins. 1984. Distribution and rate of disappearance of fluorene in pond ecosystems. Chemosphere 13:997-1008.

Brown, E.R., J.J. Hazdra, L. Keith, I. Greenspan, J.B.G. Kwapinski and P. Beamer. 1973. Frequency of fish tumors found in a polluted watershed as compared to nonpolluted Canadian waters. Cancer Res. 33:189-198.

Calder, J.A. and J.H. Lader. 1976. Effect of dissolved aromatic hydrocarbons on the growth of marine bacteria in batch culture. Appl. Environ. Microbiol. 32:95-101.

Cerniglia, C.E. 1981. Aromatic hydrocarbons: Metabolism by bacteria, fungi adn algae. Rev. Biochem. Toxicol. 3:321-361.

Cerniglia, C.E., J.P. Freeman and R.K. Mitchum. 1982. Glucuronide and sulfate conjugation in the fungal metabolism of aromatic hydrocarbons. Appl. Environ. Microbiol. 43:1070-1075.

Cerniglia, C.E. 1984. Microbial metabolism of polycyclic aromatic hydrocarbons. Adv. Appl. Microbiol. 30:31-71.

Clarke, P.H. 1982. The metabolic versatility of pseudomonads. Antonie van Leeuwenhoek 48:105-130.

Cook, A.M., H. Grossenbacher and R. Hutter. 1983. Isolation and cultivation of microbes with biodegradative potential. Experientia 39:1191-1198.

Cooney, J.J., S.A. Silver and E.A. Beck. 1985. Factors influencing hydrocarbon degradation in three freshwater lakes. Microb. Ecol. 11:127-137.

Dagley, S. 1978. Determinants of biodegradability. Q. Rev. Biophys. 11:577-602.

Davies, J.I. and W.C. Evans. Oxidative metabolism of naphthalene by soil pseudomonads. Biochem. J. 91:251-261.

Dean-Raymond, D. and R. Bartha. 1975. Biodegradation of some polynuclear aromatic petroleum components by marine bacteria. Dev. Ind. Microbiol. 16:97-110.

DeLaune, R.D., G.A. Hambrick III and W.H. Patrick, Jr. 1980. Degradation of hydrocarbons in oxidized and reduced sediments. Mar. Poll. Bull. 11:103-106.

DeLaune, R.D., W.H. Patrick, Jr. and M.E. Casselman. 1981. Effect of sediment pH and redox conditions on degradation of benzo(a)pyrene. Mar. Poll. Bull. 12:251-253.

Dobroski, C.J., Jr. and C.E. Epifanio. 1980. Accumulation of benzo(a)pyrene in a larval bivalve via trophic transfer. Can. J. Fish. Aq. Aci. 37:2318-2322.

Dzombak, D.A. and R.G. Luthy. 1984. Estimating adsorption of polycyclic aromatic hydrocarbons on soils. Soil Sci. 137:292-308.

Eadie, B.J., P.F. Landrum and W. Faust. 1982. Polycyclic aromatic hydrocarbons in sediments, pore water and the amphipod Pontoporeia hoyi from Lake Michigan. Chemosphere 11:847-858.

Evans, W.C., H.N. Fernley and E. Griffiths. 1965. Oxidative metabolism of phenanthrene and anthracene by soil pseudomonads. Biochem. J. 95:819-831.

Forstner, U. and G, Muller. 1981. Concentrations of heavy metals and polycyclic aromatic hydrocarbons in river sediments: Geochemical background, man's influence and environmental impact. GeoJournal 5:417-432.

Gerike, P. 1984. The biodegradability testing of poorly water soluble compounds. Chemosphere 13:169-190.

Ghisalba, O. 1983. Chemical wastes and their biodegradation - an overview. Experientia 39:1247-1257.

Ghosh, D.K. and A.K. Mishra. 1983. Oxidation of phenanthrene by a strain of Micrococcus: Evidence of protocatechuate pathway. Curr. Microbiol. 9:219-224.

Gibson, D.T., V. Mahadevan, D.M. Jerina, H. Yagi and H.J.C. Yeh. 1975. Oxidation of the carcinogens benzo(a)pyrene and benzo(a)anthracene to dihydrodiols by a bacterium. Science 189:295-297.

Gilewicz, M., J.R. Guillaume, D. Carles, M. Leveau and J.C. Bertrand. 1984. Effects of petroleum hydrocarbons on the cytochrome P_{450} content of the mollusc bivalve Mytilus galloprovincialis. Mar. Biol. 80:155-159.

Gschwend, P.M. and R.A. Hites. 1981. Fluxes of polycyclic aromatic hydrocarbons to marine and lacustrine sediments in the northeastern United States. Geochim. Cosmochim. Acta 45:2359-2367.

Herbes, S.E., L.R. Schwall and G.A. Williams. 1977. Rate of microbial transformation of polycyclic aromatic hydrocarbons: A chromatographic quantification procedure. Appl. Environ. Microbiol. 34:244-246.

Herbes, S.E. and L.R. Schwall. 1978. Microbial transformation of polycyclic aromatic hydrocarbons in pristine and petroleum contaminated sediments. Appl. Environ. Microbiol. 35:306-316.

Herbes, S.E. 1981. Rates of microbial transformation of polycyclic aromatic hydrocarbons in water and sediments in the vicinity of a coal-coking wastewater discharge. Appl. Environ. Microbiol. 41:20-28.

Hinga, K.R., M.E.Q. Pilson, R.F. Lee, J.W. Farrington, K. Tjessem and A.C. Davis. 1980. Biogeochemistry of benzanthracene in an enclosed marine ecosystem. Environ. Sci. Tech. 14:1136-1143.

Jackim, E. and C. Lake. 1978. Polynuclear aromatic hydrocarbons in estuarine and nearshore environments. pp. 415-428, In, M.L. Wiley (ed.), Estuarine Interactions, Academic Press, New York.

Jerina, D.M., H. Selander, H.Yagi, M.C. Wells, J.F. Davey, V. Mahadevan and D.T. Gibson. 1976. Dihydrodiols from anthracene and phenanthrene. J. Am. Chem. Soc. 98:5988-5996.

Kirkbright, G.F., T.S. West and C. Woodward. 1965. Spectrofluorometric determination of submicrogram amounts of aluminum and beryllium with 2-hydroxy-3-naphthoic acid. Anal. Chem. 37:137-143.

Kiyohara, H. K. Nagao and R. Nomi. 1976. Degradation of phenanthrene through o-phthalate by an Aeromonas sp. Agric. Biol. Chem. 40:1075-1082.

Kiyohara, H. and K. Nagao. 1978. The catabolism of phenanthrene and naphthalene by bacteria. J. Gen. Microbiol. 105:69-75.

Kiyohara, H., K. Nagao, K.Kouno and K. Yano. 1982. Phenanthrene-degrading phenotype of Alcaligenes faecalis AFK2. Appl. Environ. Microbiol. 43:458-461.

Landrum, P.F. and D. Scavia. 1983. Influence of sediment on anthracene uptake, depuration, and biotransformation by the amphipod Hyalella azteca. Can. J. Fish. Aq. Sci. 40:298-305.

Lee, R.F., W.S. Gardner, J.W. Anderson, J.W. Blaylock and J. Barwell-Clarke. 1978. Fate of polycyclic aromatic hydrocarbons in controlled ecosystem enclosures. Environ. Sci. Tech. 12:832-838.

Lee, R.F., K. Hinga and G. Almquist. 1982. Fate of radiolabeled polycyclic aromatic hydrocarbons and pentachlorophenol in enclosed marine ecosystems. pp. 123-135, In, G.D. Grice and M.R. Reeve (ed.), Marine Mesocosms. Springer-Verlag, New York.

Maccubbin, A.E., P. Black, L. Trzeciak and J.J. Black. 1985. Evidence for polynuclear aromatic hydrocarbons in the diet of bottom-feeding fish. Bull. Environ. Contam. Toxicol. 34:876-882.

May, W.E., S.P. Wasik and D.H. Freeman. 1978. Determination of the solubility behavior of some polycyclic aromatic hydrocarbons in water. Anal. Chem. 50:997-1000.

Murray, H.E., L.E. Ray and C.S. Giam. 1981. Analysis of marine sediment, water and biota for selected organic pollutants. Chemosphere 10:1327-1334.

Neff, J.M. 1979. Polycyclic Aromatic Hydrocarbons in the Aquatic Environment. Applied Science Publishers, Ltd., London. 262 pp.

Phillips, D.H. 1983. Fifty years of benzo(a)pyrene. Nature 303:468-472.

Readman, J.W., R.F.C. Mantoura, M.M. Rhead and L. Brown. 1982. Aquatic distribution and heterotrophic degradation of polycyclic aromatic hydrocarbons (PAH) in the Tamar Estuary. Est. Coast. Shelf Sci. 14:369-389.

Rogoff, M.H. and I. Wender. 1957a. The microbiology of coal I. Bacterial oxidation of phenanthrene. J. Bacteriol. 73:264-268.

Rogoff, M.H. and I. Wender. 1957b. 3-hydroxy-2-naphthoic acid as an intermediate in bacterial dissimilation of anthracene. J. Bacteriol. 74108-109.

Rogoff, M.H. 1962. Chemistry of oxidation of polycyclic aromatic hydrocarbons by soil pseudomonads. J. Bacteriol. 83:998-1004.

Roubal, G. and R.M. Atlas. 1978. Distribution of hydrocarbon-utilizing microorganisms and hydrocarbon biodegradation potentials in Alaskan continental shelf areas. Appl. Environ. Microbiol. 35:897-905.

Saltzmann, H.A. 1982. Biodegradation of aromatic hydrocarbons in marine sediments of three North Sea oil fields. Mar. Biol. 72:17-26.

Selkirk, J.K. and M.C. MacLeod. 1982. Chemical carcinogenesis: Nature's metabolic mistake. BioScience 32:601-605.

Sherrill, T.W. and G.S. Saylor. 1980. Phenanthrene biodegradation in freshwater environments. Appl. Environ. Microbiol. 39:172-178.

Sisler, F.D. and C.E. ZoBell. 1947. Microbial utilization of carcinogenic hydrocarbons. Science 106:521-522.

Spies, R.B., J.S. Felton and L. Dillard. 1982. Hepatic mixed-function oxidases in California flatfishes are increased in contaminated environments and by oil and PCB ingestion. Mar. Biol. 70:117-127.

Stanier, R.Y. and L.N. Ornston. 1973. The β-ketoadipate pathway. Adv. Microb. Physiol. 9:89-151.

Trucco, R.G., F.R. Engelhardt and B. Stacey. 1983. Toxicity, accumulation and clearance of aromatic hydrocarbons in Daphnia pulex. Environ. Poll. Ser. A. 31:191-202.

Wang, T.S.C. and S.W. Li. 1977. Clay minerals as heterogeneous catalysts in preparation of model humic substances. Z. Pflanzeneraehr. Bodenkd. 140:669-676.

Zuniga, M.C., D.R. Durham and R.A. Welch. 1981. Plasmid- and chromosome-mediated dissimilation of naphthalene and salicylate in Pseudomonas putida PMD-1. J. Bacteriol. 147:836-843.

CHAPTER ONE

CHAPTER ONE

MINERALIZATION OF PHENANTHRENE BY A *MYCOBACTERIUM* SPECIES

ABSTRACT

A *Mycobacterium* sp., designated strain BG1, able to utilize the polycyclic aromatic hydrocarbon, phenanthrene, as sole carbon and energy source was isolated from estuarine sediment following enrichment with the hydrocarbon. Unlike other phenanthrene degraders, this organism degraded phenanthrene *via* 1-hydroxy-2-naphthoic acid without accumulating this or other aromatic intermediates as shown using HPLC. Degradation proceeded via *meta* cleavage of protocatechuic acid. Addition of pyruvate, a product of *meta* cleavage, supported rapid mineralization of phenanthrene in broth culture; succinate, a product of *ortho* cleavage, supported growth but completely repressed the utilization of phenanthrene. Different non-ionic surfactants (Tweens) solubilized the phenanthrene to different degrees and enhanced phenanthrene utilization. The order of enhancement, however, did not correlate with increased solubility suggesting physiological as well as physicochemical effects of the surfactants. Plasmids of approximately 20.6, 57.5 and 76.7 megadaltons were detected in cells grown with phenanthrene but not in those which, after growth on nutrient media, had lost the phenanthrene-degrading phenotype. The involvement of plasmids, the possession of a hydrophobic cell wall or the colonization of particulate phenanthrene by mineralizing cultures may have given rise to the unusual degradation pattern observed.

INTRODUCTION

A variety of bacteria belonging to the genera Pseudomonas (Rogoff and Wender, 1957; Evans et al, 1965), Aeromonas (Kiyohara et al, 1976; Kiyohara and Nagao, 1978), Alcaligenes (Kiyohara et al, 1982), Micrococcus (Ghosh and Mishra, 1983), Beijerinckia (Kiyohara et al, 1983; Jerina et al, 1976), Nocardia (Peczynska-Czoch and Mordarski, 1984), Vibrio (West et al, 1984) and Flavobacterium (Colla et al, 1959 cited in Cerniglia, 1984) have been shown to degrade polycyclic aromatic hydrocarbons (PAH). Most studies of bacterial PAH degradation have been aimed at elucidating biochemical pathways and reaction mechanisms. In part, this has been achieved by isolating and identifying metabolic intermediates and determining their specific stereochemistries. Many of the degradative steps for PAH, however, have been inferred from simultaneous adaptation or sequential induction experiments and from enzyme activity assays (Evans et al, 1965; Kiyohara et al,1976). This is because most wild-type PAH degraders do not accumulate metabolic intermediates other than the o-hydroxy, carboxy derivatives formed upon cleavage of an end ring (Kiyohara et al, 1976; 1982; Kiyohara and Nagao, 1978; Rogoff and Wender, 1957; Wodzinski and Johnson, 1968; Evans et al, 1965; Ghosh and Mishra, 1983; Dean-Raymond and Bartha, 1975; Guerin and Jones, subm.). Thus, for phenanthrene, 1-hydroxy-2-naphthoic acid (1H2NA) accumulation is commonly observed during degradation by pure and mixed microbial cultures. In recent pure culture studies, 1H2NA has been shown to be degraded to protocatechuate (Kiyohara et al, 1976; Barnsley, 1983;

Ghosh and Mishra, 1983) rather than catechol (Evans et al, 1965) before entering the β-ketoadipate pathway or undergoing *meta* cleavage. While the biochemistry of phenanthrene degradation has been well established, the physiology has not. Little is known about the nature, extent or regulation of metabolites produced during bacterial growth on phenanthrene or how growth conditions affect the process. Such knowledge would be of industrial, environmental and scientific interest.

Here we describe a *Mycobacterium* sp., strain BG1, able to use phenanthrene as sole carbon and energy source. This organism, isolated from estuarine sediments, was atypical of phenanthrene degraders in that it mineralized the hydrocarbon without accumulating significant quantities of 1H2NA or other aromatic intermediates. Degradation did, however, proceed *via* 1H2NA and protocatechuate as proposed by Kiyohara et al.(1976). We examined the influences of alternate carbon sources and various non-ionic (Tween) surfactants on phenanthrene degradation and metabolite production by this organism. Evidence is presented indicating that phenanthrene degradation by strain BG1 was plasmid-mediated since loss of these elements in nutrient-grown cultures accompanied loss of the phenanthrene degrading phenoptype.

MATERIALS AND METHODS

Isolation

Strain BG1 was isolated from an enrichment culture using sediment from the Great Bay Estuary, NH, as inoculum. This enrichment contained 0.1% (vol/vol) of an Algerian crude oil rich in aromatics and 0.01% phenanthrene (Sigma). BG1 was purified by alternately streaking the sample on phenanthrene agar plates (0.01% phenanthrene introduced as a solution in acetone) and growing isolated colonies in Phenanthrene/Tween 80 (P/T80) broth (Guerin and Jones, subm.). Strain BG1 has been maintained in culture on phenanthrene since its isolation in 1980.

Identification and Characterization

Strain BG1 is a non-motile, non-sporulating, rod-shaped, pleomorphic bacterium which undergoes a rod to coccus cell cycle in aging broth cultures. On blood agar plates, it forms high-domed colonies with smooth edges. It is orange pigmented. BG1 stains poorly by Gram's method except that darkly staining granules are seen within the cells. The organism is acid-fast.

On the basis of the above characters, strain BG1 was presumed to be a *Mycobacterium* species. To confirm this identification, the cell wall lipids were analyzed by thin layer chromatography of whole cell methanolysates using the procedure outlined by Minniken et al.(1975). Lipids were extracted from BG1 and from several reference strains including members of the genera *Corynebacterium*, *Nocardia*, *Rhodococcus*, *Arthrobacter* and *Mycobacterium*, after growth in trypticase soy broth

supplemented with 0.01% asparagine. The only exception was M. intracellulare which was harvested from Lowenstein-Jensen slants. Lipids were visualized on TLC plates (20 x 20 x 0.025 cm) by spraying with a solution of $K_2Cr_2O_7$ (0.05% in 3.4N H_2SO_4) and charring (150°C for 1 h).

Phase contrast photomicrographs of living cells were taken on a Zeiss standard research microscope equipped with a 35mm camera and electronic flash attachment.

Isolation of plasmid DNA in strain BG1 was performed according to the method of Guerry et al. (1973). Prior to lysis with lysozyme/SDS, cell walls were weakened by repeated freezing and thawing. Plasmids were separated by vertical gel electrophoresis in 0.7% agarose. Plasmid molecular weights were calculated from their relative mobilities and the slope of the regression equation for log (relative mobility) vs. log (molecular weight) for a series of plasmid standards. Plasmid standards were isolated from E. coli strains PRC 104 (62 Md plasmid R1), PRC 116 (34 Md plasmid RP4), PRC 119 (25 Md plasmid S-a) and PRC 304 (5.5 Md plasmid RSF1030).

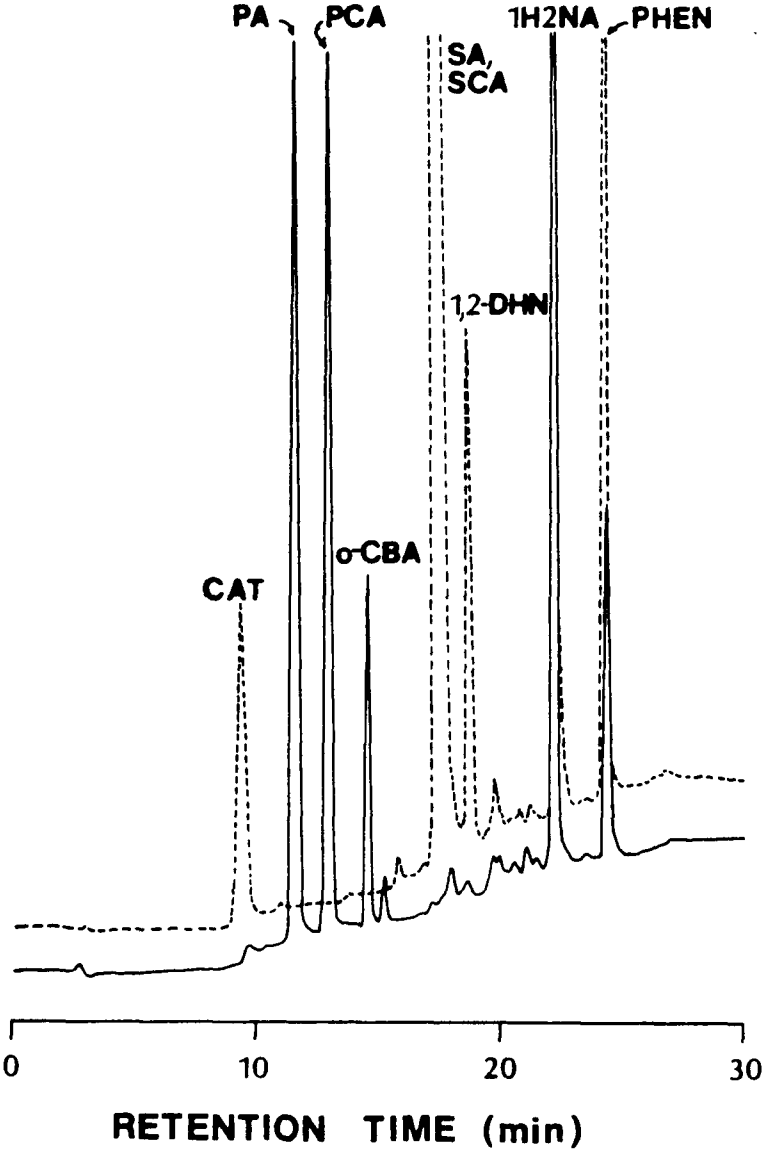
Characteristics of Growth of BG1 on Phenanthrene

Growth and metabolic activity of strain BG1 in P/T80 broth were followed using methods described previously (Guerin and Jones, subm.). These included solvent extraction of culture fluids to determine phenanthrene concentrations, measurements of protein (Lowry et al, 1951) as an estimate of biomass, and analysis of metabolic intermediates in culture supernatant fluids by a modification of the Folin-Ciocalteau (F-C) reaction for polyphenols. Aromatic acids,

aldehydes and amines also reduce the F-C reagent as do some non-aromatic and inorganic compounds (Box, 1983; Singleton, 1974). Because each compound exhibits a different molar absorptivity, the F-C reaction provides a semi-quantitative estimate of metabolite concentrations where many of these are present.

Soluble intermediates in culture supernatant fluids were also analyzed by high performance liquid chromatography (HPLC) using the direct injection procedure previously described (Guerin and Jones, subm.). Sample peak retention times were compared to those of authentic compounds known or suspected to be intermediates in phenanthrene degradation. A solution containing (mg L^{-1} distilled water) phenanthrene (0.2), 1H2NA (0.8), o-carboxybenzaldehyde (9.0), phthalic acid (10.1), and protocatechuic acid (3.0) contained all the aromatic intermediates of the phenanthrene degradation scheme proposed by Kiyohara et al. (1976) that were commercially available. A second standard solution containing the available intermediates of the pathway proposed by Evans et al. (1965) was composed of (mg L^{-1} distilled water) phenanthrene (0.5), 1H2NA (0.8), 1,2-dihydroxynaphthalene (concentration undetermined), salicylaldehyde (concentration undetermined), salicylic acid (10.4) and catechol (12.8). Several other known or suspected intermediates were not available commercially. Non-aromatic degradation products of phenanthrene gave low UV responses and eluted with, or shortly after, the injection peak. Figure 4 illustrates the separation of phenanthrene metabolites attainable with the HPLC procedure employed. Variability in sample peak and standard retention times was negligible in replicate analyses. Over the life of the HPLC column (several hundred analyses), however, column resolution and

FIGURE 4. HPLC chromatograms of phenanthrene degradation intermediates produced according to the pathways proposed by Evans et al. (1965, dotted line) and Kiyohara et al. (1976, solid line). Chromatogram peak designations as follows: PHEN, phenanthrene; 1H2NA, 1-hydroxy-2-naphthoic acid; 1,2-DHN, 1,2-dihydroxynahthalene; SA, salicylic acid; SCA, salicylaldehyde; o-CBA, o-carboxybenzaldehyde; PCA, protocatechuic acid; PA, phthalic acid; CAT, catechol.


PA
PCA
SA,
SCA
1H2NA
PHEN
1,2-DHN
o-CBA
CAT
0
10
20
30
RETENTION TIME (min)

selectivity slowly deteriorated and standard retention times decreased 10 to 15%. However, this deterioration was slow enough that no appreciable change in retention times occurred over the 20 or 30 d required for each experiment.

Influence of Alternate Carbon Sources on Phenanthrene Degradation

The influence of easily assimilable carbon sources on phenanthrene utilization by BG1 was tested by supplementing phenanthrene growth medium (no Tween 80 present) with sodium acetate, sodium pyruvate, sodium succinate, or glucose. Control flasks containing only phenanthrene or phenanthrene plus Tween 80 were also inoculated. In all flasks with two carbon sources (including Tween), the ratio of phenanthrene C: alternate C was 1:1. Hydrocarbon degradation in these flasks was followed by HPLC analysis of metabolites in culture supernatant fluids and of phenanthrene in CH_2Cl_2 extracts of culture aliquots. The latter measure proved to be unreliable due to adsorption of phenanthrene to the walls of flasks which did not contain Tween. None of the alternate carbon sources used were detectable by the UV monitor of the instrument. Likewise, no UV-absorbing intermediates or secondary metabolites were detected during HPLC analysis of inoculated controls containing the alternate carbon source but lacking phenanthrene.

Influence of Surfactants on Phenanthrene Degradation

To determine whether the solubilizing activity of different Tween surfactants influenced the availability of phenanthrene, and hence the growth rate of BG1, media were prepared which contained 100 mg L^{-1} each of phenanthrene and either Tween 20, 40, 60, 80 or 85. Flasks were

inoculated with early stationary phase cells of P/T80-grown BG1 to give an initial protein concentration of approximately 1 $\mu g\ ml^{-1}$ in the experimental flasks. Phenanthrene disappearance, biomass (protein) production and the production of F-C reactive intermediates were monitored in these flasks for 29 d. At 25 d, HPLC analysis of the supernatant fluids was also performed to determine if there were differences in the metabolites produced.

Using a procedure similar to that described by May et al. (1978) for determining the solubility of PAH, the solubilizing effects of Tweens on phenanthrene was investigated. Briefly, this involved pumping Tween solutions (0.5 and 1.0 mg L^{-1} distilled water) through a stainless steel column packed with phenanthrene-coated glass beads. The absorbance (250 nm) of the effluent was measured against the initial Tween solution and compared to a similarly prepared solution of phenanthrene in distilled water. The application of this technique to studying the solubilizing effects of marine dissolved organic matter on PAH has been presented recently (Whitehouse, 1985).

Distribution of Radioactivity in Metabolites After Incubation of BG1 Cells with [9-^{14}C] Phenanthrene

Aliquots (2.5 ml) of the P/T80 culture (25d) used in the above experiment, were transferred to acid-washed 15 ml Corex centrifuge tubes and spiked with 3.6×10^5 dpm [9-^{14}C] phenanthrene (19.3 mCi $mmol^{-1}$, Amersham Searle) in 2 μl acetone. The tubes were capped with serum stoppers and incubated at 18°C. After 1.25 h, one tube was acidified and $^{14}CO_2$ was trapped on phenethylamine-soaked filter paper suspended from the serum stopper in a plastic cup. After 18 h, the filter paper was added to a glass scintillation vial containing 9 ml

liquid scintillation cocktail (Scintiverse E, Fisher) and counted on a Beckman LS7000 liquid scintillation spectrometer. A second tube was centrifuged (12,100 x g, 10 min, 4°C) and 250 ul of the supernatant fluid was analyzed by HPLC. Thirty five 1 ml (min) elution fractions were collected in scintillation vials containing 9 ml scintillation cocktail and counted. Counts were converted to dpm by external standard quench correction. A second set of two tubes was harvested at 4.25 h and treated identically. The radiochemical purity of the labeled phenanthrene was 98% as determined by both TLC and HPLC.

RESULTS

Methanolysates of strain BG1 yielded a complex TLC spot pattern indicating the presence of four or more long carbon chain lipids (Fig. 5). Of the various reference strains tested, only mycobacteria gave similar multi-spot patterns of mycolic acid methyl esters (MAME, Minniken et al, 1975; Goodfellow et al, 1976) with M. intracellulare most closely resembling strain BG1. Low molecular weight fatty acid methyl esters (FAME) were also visualized on TLC plates. On the basis of its lipid composition, its failure to grow at 37°C and the production of dense growth in trypticase soy broth in 48 to 72 h at 20°C, strain BG1 was classified as a rapidly growing, saprophytic Mycobacterium species.

Analysis of plasmids in strain BG1 revealed the presence of three distinct bands in P/T80-grown cell preparations (Fig. 6). These had molecular weights of 20.6, 57.5 and 76.7 Md. After growth in nutrient medium, cells could no longer degrade phenanthrene and showed no evidence of plasmids.

During growth in media containing particulate phenanthrene suspensions, strain BG1 colonized the particles and proliferated until clusters of cells in the shape of the particles, but no hydrocarbon, remained. Figure 7 is a phase contrast photomicrograph showing this growth habit. The light area in the center of the photograph is the refracted light from a partially degraded phenanthrene particle encapsulated by BG1 cells.

FIGURE 5. Thin layer chromatograms of whole cell methanolysates showing the migration patterns of mycolic acid methyl esters (MAME) and fatty acid methyl esters (FAME) for strain BG1 and various reference strains. Lane 1, Mycobacterium rubrum (ATCC 14346); 2, M. gordonae (TCM 1318, UNH Microbiology Dept. collection); 3, M. smegmatis (TCM 1515, UNH); 4, Strain BG1; 5, M. scrofulaceum (UNH); 6, M. intracellulare (ATCC 13950).

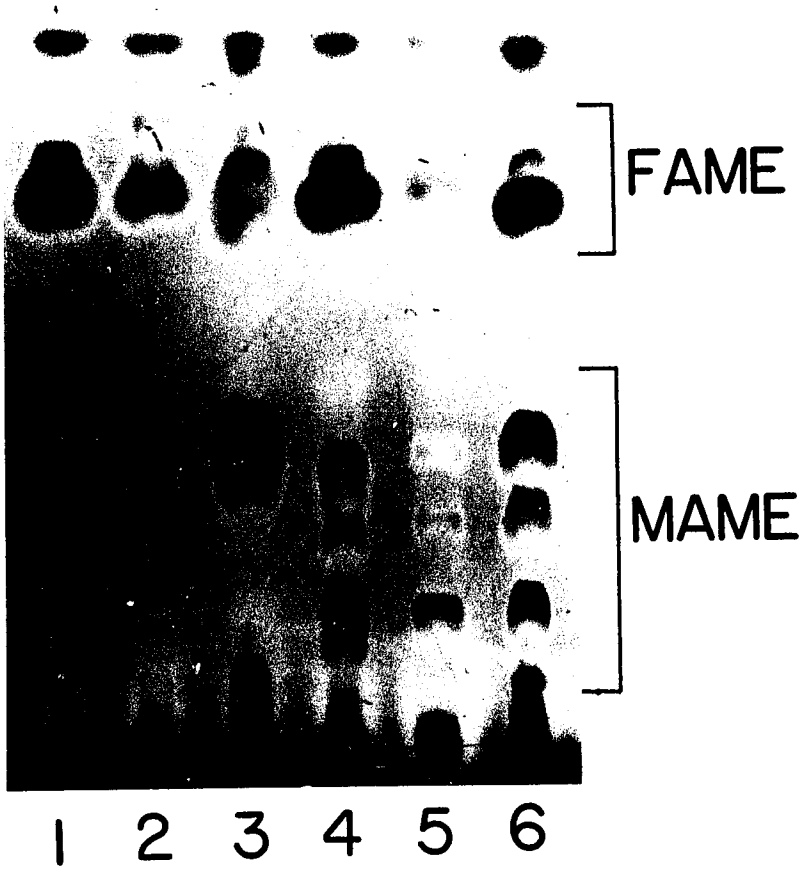

FAME
MAME
1 2 3 4 5 6

FIGURE 6. Vertical gel electrophoresis of plasmid preparations from (a) nutrient broth-grown BG1, (b) P/T80-grown BG1 and (c) various *E. coli* standard strains.

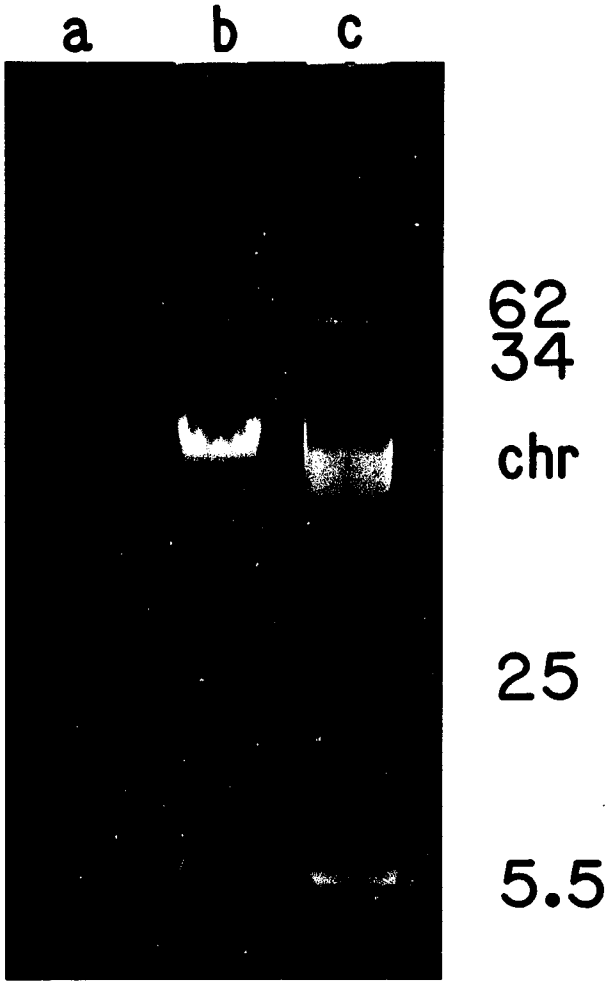
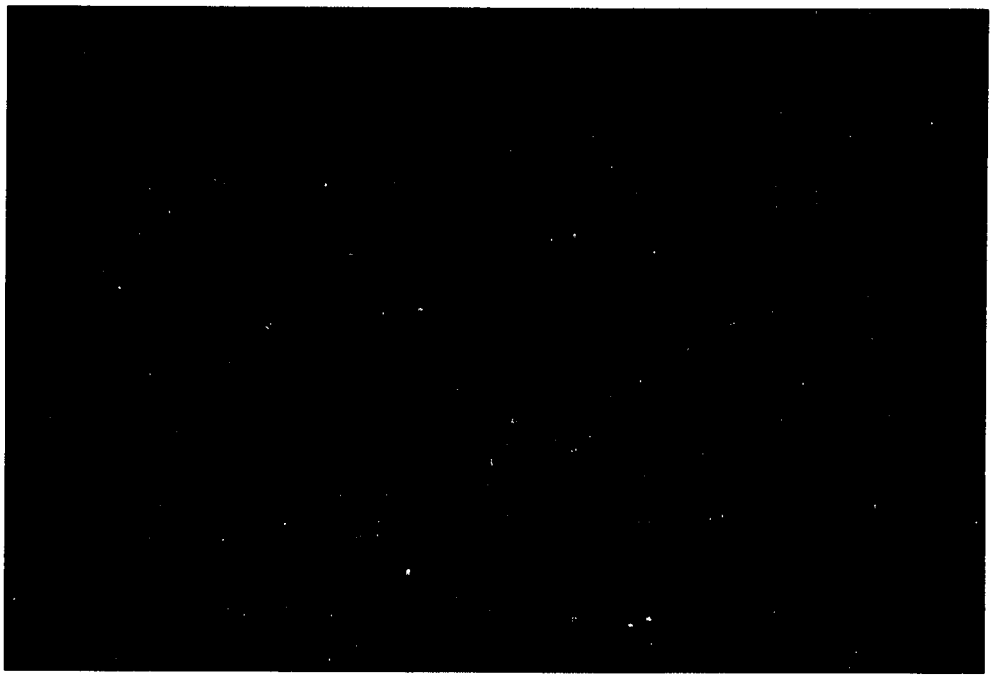

a
b
c
62
34
chr
25
5.5

FIGURE 7. Phase contrast photomicrograph of strain BG1 during log phase of growth on phenanthrene showing the colonization of the particulate substrate (light area in center of photograph) by cells.



Unlike other phenanthrene degrading isolates, strain BG1 mineralized this PAH without accumulating significant quantities of aromatic intermediates. Figure 8 shows a time course for phenanthrene degradation in P/T80 broth with an initial phenanthrene concentration of 200 $\mu g\ ml^{-1}$. Disappearance of the hydrocarbon was accompanied by exponential biomass (protein) production following a lag period of several days. F-C reactive metabolites began to increase at the onset of exponential cell growth, but then leveled off at a concentration of about 5 $\mu g\ ml^{-1}$ RE. These metabolites were slowly removed from the medium over the latter half of the incubation. The pattern (Fig. 8) of decreasing, then increasing phenanthrene concentration was a reproducible feature in P/T80 (or other surfactant) incubations, was also observed when cultures were made alkaline prior to extraction, and always occurred at the onset of rapid phenanthrene utilization. It was presumably a manifestation of sorption-desorption interactions between the inoculum and the substrate.

Concurrent HPLC analysis of BG1 culture supernatant fluids confirmed that only low concentrations of aromatic intermediates were formed during mineralization of phenanthrene. Figure 9 shows the HPLC spectra of an uninoculated control and of BG1 culture supernatants at d 4, 5, 7, 9, 11, 14 and 16. The general pattern observed is that of a progressive transformation of phenanthrene (rt = 25.6 min) to more polar products over the course of the incubation. Control cultures in which Tween 80 was the sole carbon source gave featureless chromatograms over the course of the experiment.

At the onset of phenanthrene degradation (d 4), an early metabolite (peak I) eluting at 24 min and corresponding exactly to the

FIGURE 8. Time course for phenanthrene degradation by strain BG1.

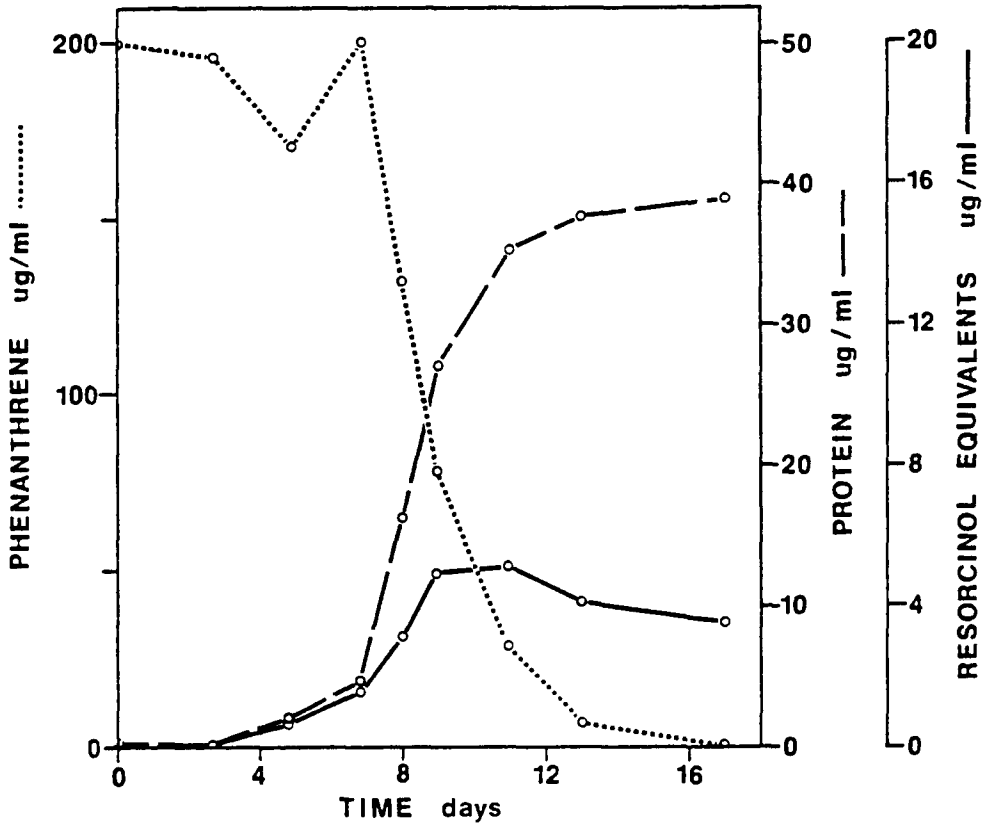
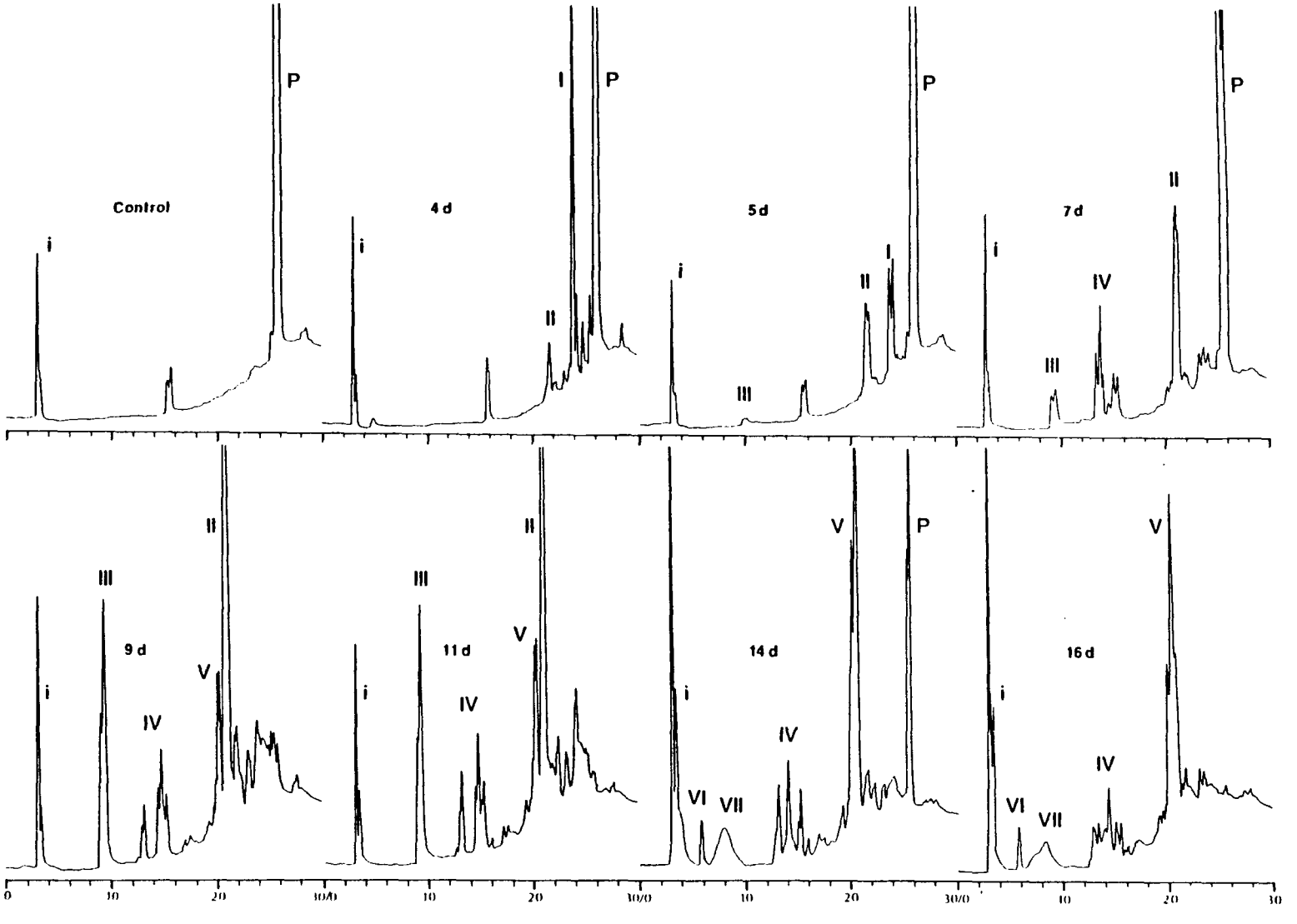

PHENANTHRENE ug/ml .....
PROTEIN ug/ml ——
RESORCINOL EQUIVALENTS ug/ml ——
200
100
0
50
40
30
20
10
0
20
16
12
8
4
0
0
4
8
12
16
TIME days

FIGURE 9. HPLC chromatograms of culture supernatants of strain BG1 grown in P/T80 broth during the incubation depicted in Fig. 8. Chromatograms at 4, 5, 7, 9, 11, 14 and 16 d and for an uninoculated control are shown. Retention time scale in min. Peak designations for reference to text.


Control
4 d
5 d
7 d
9 d
11 d
14 d
16 d
i
II
III
IV
V
VI
VII
I
P
0
10
20
30/0

retention time of 1H2NA was produced (Fig. 9). A peak of this size corresponded to a 1H2NA concentration of approximately 1 $\mu g\ ml^{-1}$ (Fig. 4). By d 5, peak I had diminished and two more polar products (peaks II and III) had appeared. A group of small peaks (IV) eluting between 13 and 16 min appeared on d 7 and became a permanent, yet variable feature of the chromatograms. These peaks, on d 9 and 11 corresponded in retention times to those of protocatechuate, phthalate and o-carboxybenzaldehyde. Peaks II and III increased in magnitude through d 9 at which time no phenanthrene was detectable in the supernatant. On d 11, peak II decreased while peak V (rt = 20 min) increased slightly in magnitude. By d 14, peak V had replaced peak II and peak III was no longer detected. Soluble phenanthrene (about 1 $\mu g\ ml^{-1}$) and two non-aromatic components of high polarity (peaks VI and VII) were present on d 14. At the end of the incubation, peak areas were reduced and, with the exception of the injection peak (i), chromatogram complexity decreased. Maximum chromatogram complexity on d 9 and 11 (Fig. 9) coincided with maximum F-C reactive intermediate concentrations (Fig. 8).

None of the authentic compounds tested had retention times corresponding to those of peaks II or V. Their retention times, however, were characteristic of dicyclic or alkyl-substituted monocyclic compounds. Peaks III, VI and VII had retention times of less than 10 min similar to those observed for non-aromatic compounds. Non-aromatic products of ring cleavage reactions include compounds such as β-ketoadipate, muconic seimaldehydes, muconate, pyruvate and succinate. With the exception of muconate, these exhibit low UV absorption. At the high concentrations needed to elicit a response in the UV detector, the

column's capacity factor is exceeded resulting in broad peaks for these compounds. As a result, peaks III, VI and VII could not be identified. At no time during the incubation were peaks corresponding in retention times to those of 1,2-dihydroxynaphthalene, salicylaldehyde, salicylic acid or catechol detected.

The same spectrum of metabolites was produced when BG1 was grown on phenanthrene alone or on phenanthrene together with an alternate, more easily assimilable carbon source. Of these, acetate, pyruvate and glucose increased the mineralization rate of phenanthrene such that mineralization was essentially complete 14 d after inoculation. In all incubations, the major metabolite observed in HPLC spectra was a 20 min peak corresponding to peak V in Fig. 9. In contrast to the above compounds, supplementation with succinate supported growth of BG1 but completely repressed phenanthrene utilization. No evidence of phenanthrene degradation was apparent even 64 d after inoculation of the phenanthrene plus succinate flask.

Tween 80 slightly increased the rate of phenanthrene degradation. However, the level of metabolite accumulation in the Tween 80 flask was highly exaggerated compared to that of the control flask or those supplemented with acetate, glucose or pyruvate. Metabolite concentrations were also much higher in this P/T80 broth (P-C:T80-C of 1:1, phenanthrene concentration 250 mg L^{-1}) than in the P/T80 medium represented in Figs. 8 and 9 (P-C:T80-C of 4:1, phenanthrene concentration 200 mg L^{-1}). The metabolites produced in P/T80 media differed quantitatively, though not qualitatively from those of cultures not receiving Tween.

While Tween 80 was incorporated into the growth medium for the

primary purpose of preventing losses of phenanthrene due to adsorption and volatilization, later work showed that this Tween was not the most conducive to phenanthrene utilization. Figure 10 shows time courses for phenanthrene degradation and protein and F-C reactive intermediate production by BG1 in flasks containing different Tween surfactants. The ratio of phenanthrene-C:Tween-C was approximately 2:1 in all flasks, with initial phenanthrene concentrations of about 100 mg L^{-1}. As seen in Fig. 10, the lag time preceeding rapid phenanthrene utilization varied significantly according to the Tween used in the medium. In general, the more lipophilic surfactants supported more rapid phenanthrene degradation. However, Table 4 shows that once initiated, the rate of phenanthrene utilization was similar in all flasks except that containing Tween 60. This Tween supported the highest phenanthrene utilization rate, protein production rate and protein yield from phenanthrene by BG1. BG1 did not grow at the expense of any of the Tweens when present as the sole carbon source; however, Tween 60 and Tween 40 were readily hydrolyzed as determined using the method of Slijkhuis et al. (1984). All of the Tweens enhanced the solubility of phenanthrene. Phenanthrene concentrations in Tween solutions were from 4 (Tween 20 at 0.5 mg L^{-1}) to 25% (Tween 85 at 1 mg L^{-1}) higher than distilled water concentrations with the low Hydrophile-Lipophile Balance (HLB) number surfactants being more effective. Figure 10 shows that with increasing rate of phenanthrene degradation, more of the substrate was incorporated into biomass (protein) at the expense of metabolite production. HPLC analysis of culture supernatant fluids at 25 d showed that, again, the same spectrum of metabolites were produced in all flasks.

FIGURE 10. Time courses for phenanthrene degradation by strain BG1 in media containing either Tween 20, 40, 60, 80 or 85. Phenanthrene disappearance, production of protein and production of metabolites are shown.

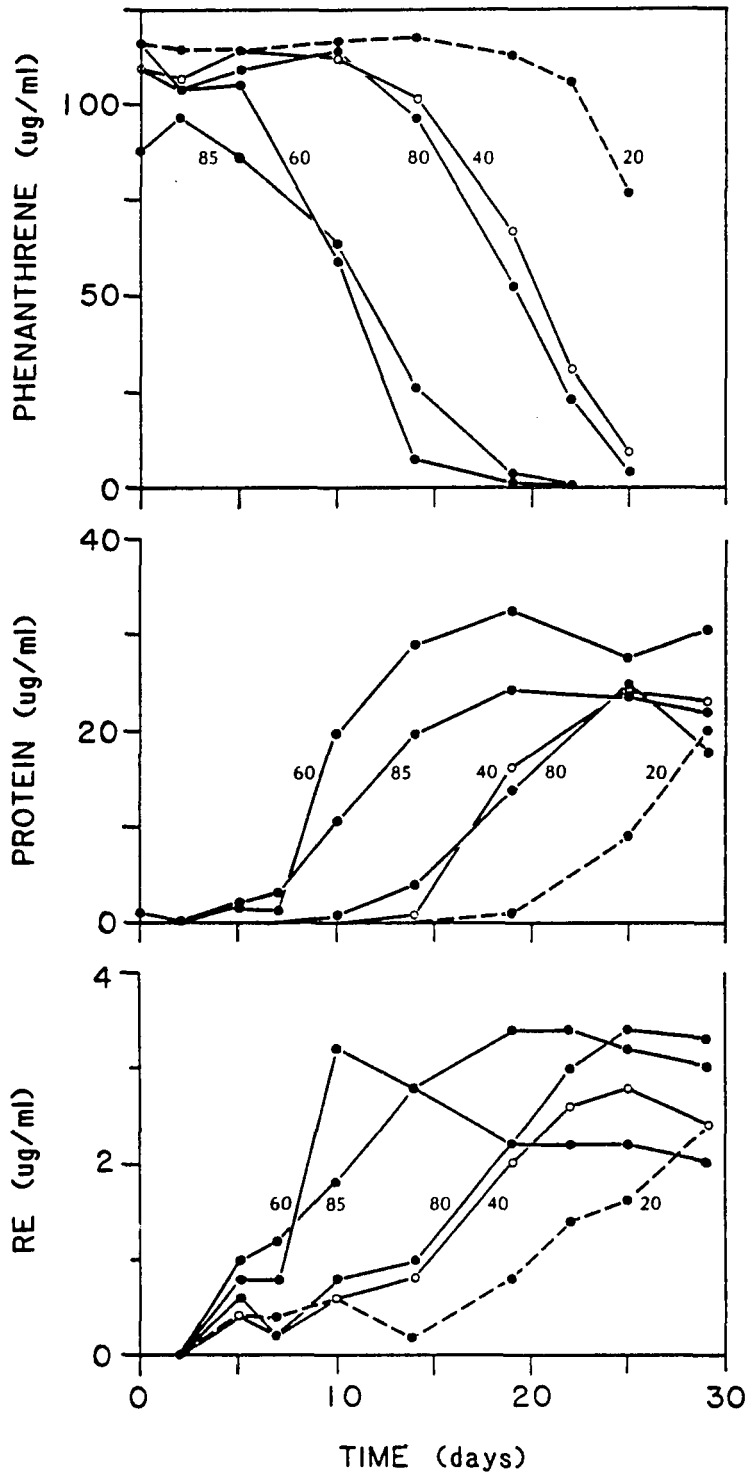

PHENANTHRENE (ug/ml)
100
50
0
85
60
80
40
20
PROTEIN (ug/ml)
40
20
0
60
85
40
80
20
RE (ug/ml)
4
2
0
60
85
80
40
20
0
10
20
30
TIME (days)

TABLE 4. Growth characteristics of Mycobacterium strain BG1 on phenanthrene with different Tween surfactants employed in the medium.

Tween	HLB Number[a]	Alkyl Side Chain	Phenanthrene Utilization Rate[b]	Protein Production Rate[b]	Yield[c]
20	17 (most hydrophilic)	monolaurate ($C_{12}H_{24}O_2$)	0.38	0.117	n.d.
40	16	monopalmitate ($C_{16}H_{32}O_2$)	0.37	0.130	0.21
60	15	monostearate ($C_{18}H_{36}O_2$)	0.46	0.271	0.28
80	14	monooleate ($C_{18}H_{34}O_2$)	0.38	0.080	0.23
85	10 (most lipophilic)	trioleate ($C_{18}H_{34}O_2)_3$	0.38	0.109	0.25

[a] Hydrophile-Lipophile Balance Numbers.
[b] $\mu g\ ml^{-1}\ h^{-1}$ calculated from linear portions of curves.
[c] $(\mu g\ protein)(\mu g\ phenanthrene)^{-1}$.
n.d. not determined.

Short term incubations of P/T80 culture aliquots (25 d, Fig. 10) with [9-^{14}C] phenanthrene confirmed that only low levels of metabolites were accumulated by strain BG1 and that HPLC peaks represented phenanthrene degradation products and not secondary metabolites or exuded UV-absorbing materials. Figure 11 shows the distribution of radioactivity in HPLC elution fractions of the supernatant 1.25 h after addition of labeled phenanthrene to a P/T80 culture aliquot. The 4.25 h sample gave an identical chromatogram and a similar radioactivity elution pattern. Almost none of the added activity was detected as phenanthrene (rt = 28.7 min) but some was present in peaks corresponding to early metabolites. Table 5 shows the fractional distribution of radioactivity in respired, intermediate and bound forms for the 1.25 and 4.25 h samples. Samples for HPLC were not acidified prior to analysis. Therefore, the elution pattern of $NaH^{14}CO_3$ in 75% artificial seawater was determined and corresponded exactly to that of the large polar peak eluting between 3 and 14 min in the sample chromatograms. However, since pyruvate and other highly polar products of phenanthrene degradation showed similar retention times, data for HPLC fractions 1 to 14, 15 to 35 and total (1 to 35) are given in Table 5. The decrease in the total HPLC activity from 1.25 to 4.25 h was accounted for by the increase in activity detected as $^{14}CO_2$ in indepen dent analyses between 1.25 and 4.25 h. The calculated percentage of added ^{14}C present in bound form was constant at about 44% in both samples. Regardless of whether fractions 1 to 14 represented $^{14}CO_2$ or non-aromatic metabolites, only 7 to 8% of the total radioactivity was present as aromatic intermediates after short term incubations. Most of this activity was present in early eluting peaks, particularly,

FIGURE 11. HPLC chromatogram of 250 µl of supernatant from a stationary phase P/T80 broth culture of strain BG1 and distribution of ^{14}C in 1 ml elution fractions 1.25 h after addition of [9-^{14}C] phenanthrene (3.6 x 10^5 dpm per 2.5 ml). Large peak eluting at 9 min. is acetone used as carrier in ^{14}C-phenanthrene addition. Mobile phase: 100% H_2O for 3 min, 0-100% methanol (linear) over 20 min, held at 100% methanol.

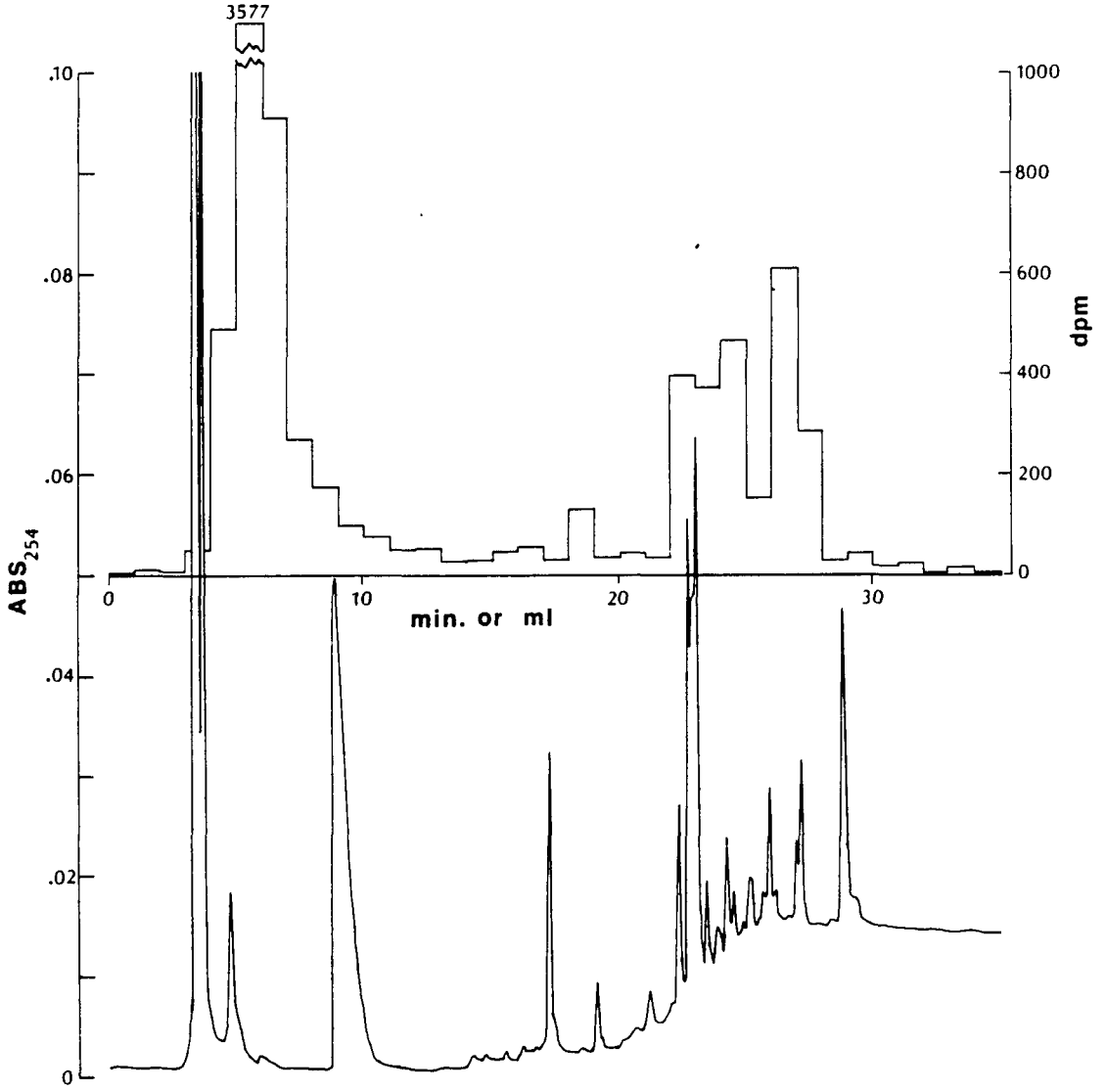

3577
.10
.08
.06
.04
.02
0
ABS254
1000
800
600
400
200
0
dpm
0
10
20
30
min. or ml

TABLE 5. Distribution of radioactivity in respired, intermediate and bound form in short-term labeling experiments with BG1 and elution pattern of $NaH^{14}CO_3$-spiked 75% artificial seawater control.

Sample	% Total Radioactivity in				
		HPLC Eluate			
	$^{14}CO_2$	Fractions 1-14	Fractions 15-35	Total	Bound*
BG1 (1.25 h)	33	16.0	7.8	23.9	43.1
BG1 (4.25 h)	38	11.1	6.9	18.0	44
$NaH^{14}CO_3$	25.5**	74.5	0	74.5	

* 100 - ($^{14}CO_2$ + HPLC total)
** By difference

the 23 min peak corresponding to peak V in Fig. 9. The HPLC profile of the P/T80 culture supernatant without addition of ^{14}C-phenanthrene was identical to that shown in Fig. 11 except for the absence of the acetone peak (rt = 9 min). Likewise, radiochemical impurities did not contribute to the distribution of radioactivity observed.

DISCUSSION

Mycobacteria are well known for the roles they play in pathogenic processes but are comparatively obscure in their roles as saprophytes (Collins et al, 1984; Krulwich and Pelliccione, 1979; Kubica and Good, 1981; Williams et al, 1984). Yet the list of substances which mycobacteria can degrade includes steroids (Mulheirn and Van Eyk, 1981) as well as aliphatic (Murphy and Perry, 1983; King and Perry, 1975; Bushnell and Haas,1941; Foster, 1962), aromatic (Peczynska-Czoch and Mordarski, 1984) and heterocyclic (Krulwich and Pelliccione, 1979) hydrocarbons. The potential importance of mycobacteria in hydrocarbon degradation can be appreciated by considering that a common method of enrichment for mycobacteria has been to provide paraffin as the carbon source in a simple salts solution (Kubica and Good, 1981). The hydrophobic cell wall of mycobacteria should allow intimate contact between cells and hydrocarbon substrates. Mycobacteria incorporate aliphatic hydrocarbons intact into their cell lipids (King and Perry, 1975; Murphy and Perry, 1983).

This is the first report of a Mycobacterium able to use phenanthrene (or other PAH) as sole source of carbon and energy. Strain BG1 was atypical of other phenanthrene degraders, however, in that aromatic intermediates, and in particular, 1H2NA, did not accumulate to appreciable levels during degradation. Degradation did, however, proceed via 1H2NA and protocatechuic acid as shown by HPLC analysis of metabolites. HPLC also permitted the detection of several unidentified non-aromatic compounds which must have been present in high

concentrations considering their generally low UV extinction coefficients. The major aromatic intermediates of phenanthrene degradation by strain BG1 (peaks II and V, Fig. 9) did not match the retention times of any of the authentic compounds available. Peaks with identical retention times were also the major metabolites produced by a Micrococcus species isolated from the estuary and cultured in a phenanthrene medium without Tween (Guerin and Sanseverino, unpublished data). Radiolabel experiments (Fig. 11) and HPLC retention times (Fig. 4) suggest that these peaks were dicyclic or substituted monocyclic aromatic intermediates. Attempts to identify these intermediates remain hindered by their low levels of production.

Low levels of metabolite production by phenanthrene-degrading cultures of strain BG1 may be related to a physical interaction between the cell and the substrate. When cultured with phenanthrene, BG1 grew and proliferated while attached to the hydrocarbon particles (Fig. 7). Adsorption of phenanthrene into the lipid-rich cell wall of BG1 could act to retard the diffusive loss of early polar metabolites such as 1H2NA. The resulting higher localized concentrations of metabolites might more effectively induce synthesis of enzymes involved in subsequent reactions and thus maintain dissolved intermediate concentrations at low levels. Consistent with this interpretation was the finding that increasing concentrations of Tween in the medium resulted in increased concentrations of phenanthrene metabolites. Tween 80 is commonly employed in mycobacterial growth media to disrupt the hydrophobic interaction between cells that causes "cording" (Davis et al, 1973).

Surfactants also act to emulsify liquid hydrocarbons (Lupton and

Marshall, 1978) and to improve mass transfer of solid hydrocarbons (Cox and Williams, 1980) thereby increasing their availability to microorganisms. Addition of surfactants increased yields of cis-1,2-dihydroxy-1,2-dihydronaphthalene from naphthalene by a Pseudomonas mutant (Cox and Williams, 1980). Likewise, the rate of aliphatic hydrocarbon (C_{10} to C_{20}) degradation by an Acinetobacter strain was enhanced by the addition of Tweens at concentrations as high as 1 g L^{-1} (Lupton and Marshall, 1978). Degradation (and emulsification) increased with decreasing HLB numbers of the surfactants with Tween 85 being the most effective and Tween 20 the least effective. The results presented here are consistent with these findings and support the conclusions of Wodzinski and Coyle (1974) that phenanthrene mineralization rate is solubility limited. Thus surfactants with low HLB numbers solubilized phenanthrene and promoted its more rapid mineralization in comparison to high HLB number surfactants (Table 4). The more rapid mineralization of phenanthrene in P/T85 medium compared to P/T80 medium must have been due to the greater solubilizing activity of Tween 85 since the aliphatic moieties in both Tweens is oleic acid. However, since mineralization was most rapid in P/T60 (intermediate HLB number) medium, additional physiological factors must also be important. Perhaps, like many mycobacteria (Schaefer and Lewis, 1965; Hedgecook, 1968), BG1 incorporates the aliphatic groups of Tweens into lipid components intact (without metabolizing them) and the saturated stearate (C_{18}) moiety of Tween 60 may have been a better building block than the monounsaturated groups of Tweens 80 (monooleate) and 85 (trioleate). The palmitate and laurate groups of Tweens 40 and 20, respectively, were even poorer supplements for phenanthrene degradation

by and growth of strain BG1 (Table 4).

An alternate explanation for the low level of metabolite accumulation by strain BG1 could be that phenanthrene degrading enzyme synthesis was highly regulated in this organism. As the 1H2NA (peak I, Fig. 9) concentration reached approximately 1 mg L^{-1} in the culture broth, it was immediately consumed with the concomitant appearance of more polar metabolites. The concentration of 1H2NA was maintained at undetectable levels (< 10 $\mu g\ L^{-1}$) throughout the remainder of the incubation, suggestive of some kind of induction mechanism. Similarly, the metabolite represented by peak III in Fig. 9 appeared only transiently during the incubation. Its subsequent disappearance was followed by the appearance of two more polar products, peaks VI and VII. Growth curves of BG1 on phenanthrene showed normal batch culture kinetics typical of microbial growth on simple, dissolved substrates. Likewise, $^{14}CO_2$ production curves for BG1 growing with [9-^{14}C] phenanthrene were smooth and sigmoidal (data not shown) with 50% conversion to $^{14}CO_2$ and no suggestion of the two stage mineralization pattern observed in cultures which accumulate 1H2NA (Guerin and Jones, subm.). Plasmids may have been involved in regulating degradation.

Plasmids are involved in the degradation of many monocyclic and polycyclic aromatic hydrocarbons. Such involvement is often inferred from the frequent loss of degradative functions in organisms isolated on hydrocarbons after culture on alternate carbon sources or nutrient media (Clarke, 1982; Kiyohara et al, 1983; Cook et al, 1983; Guerin, unpublished). Plasmid-mediated degradations of aromatic hydrocarbons almost invariably occur *via meta* cleavage (Zuniga et al, 1981; Chakrabarty, 1972; 1976; Dunn and Gunsalus, 1973) while some

substrates, eg., benzoate, are degraded *via ortho* cleavage by chromosome-encoded enzymes. Growth of plasmid-bearing isolates on the latter compound selects for cured strains (Clarke, 1982; Haas, 1983; Kiyohara et al, 1983).

Plasmids have been shown to be responsible for the bacterial degradation of biphenyl (Kiyohara et al, 1983), dibenzothiophene (Monticello et al, 1985), naphthalene (Chakrabarty, 1976), salicylate (Zuniga et al, 1981) and phenanthrene (Kiyohara et al, 1983; Foght and Westlake, Abstr. Q20, Am. Soc. Microbiol. Ann. Mtg., Las Vegas, Nev., 1985). The biochemical relatedness of naphthalene (NAH) and salicylate (SAL) degradation plasmids has been established (Haas, 1983). As seen in Table 6, molecular weight determinations place these and other biodegradative plasmids into a few narrow size ranges - 20 to 21, 55 to 58 and 73 to 79 Md - or multiples of them. The related functions of at least certain aspects of these plasmids, eg., the generalized metabolic approach to PAH ring cleavages (Guerin and Jones, subm.), allow speculation that they share some homologous regions. In analogy to the plasmid-mediated degradation of the naphthalene metabolite, salicylate (Zuniga et al, 1981), 1H2NA degradation may also be plasmid-encoded. Inefficient transfer of extrachromosomal genetic elements among biodegradative populations might partially explain the accumulation of 1H2NA during pure and mixed culture degradations of phenanthrene (Guerin and Jones, subm.).

Results with strain BG1 conform to these observations. Degradation of phenanthrene by this organism was most likely plasmid-mediated since both features, plasmids and the phenanthrene degrading phenotype, were lost by cultures grown on nutrient media. Phenanthrene degradative

TABLE 6. Molecular weights (thousands) of various aromatic biodegradative plasmids.

Compound	Organism	Plasmid Molecular Weight(s) (Megadaltons)				Reference
Phenanthrene	Mycobacterium	20.6	57.5	76.7		This work
	Beijerinckia	20.8		(73.5)[a]	147	Kiyohara et al, 1983
	Flavobacterium	30		76		Foght and Westlake, 1985
Naphthalene	Pseudomonas	38				Painceira et al, 1985
	Pseudomonas	40				Chakrabarty, 1976
Salicylate	Pseudomonas		55			Chakrabarty, 1976
	Pseudomonas		(55)		110	Zuniga et al, 1981
Dibenzothiophene	Pseudomonas		55			Monticello et al, 1985
Toluene	Pseudomonas			75		Chakrabarty, 1976
Nicotine	Arthrobacter			(78.9)		Brandsch and Decker, 1984

[a] Values in parentheses are half-weights of large (>100,000 MW) plasmids which may exist as dimers.

functions were apparently highly regulated in this organism since aromatic intermediates, and in particular 1H2NA, did not accumulate. Phenanthrene-grown cells of BG1 gave negative Rothera reactions indicating *meta* cleavage by this organism. In addition, phenanthrene degradation was enhanced in medium containing pyruvate but was completely repressed in medium supplemented with succinate. This observation indicated that phenanthrene mineralization was compatible with the utilization of pyruvate, a *meta* cleavage product, but was incompatible with the utilization of succinate, a product of *ortho* cleavage. Succinate was found to repress the plamid-mediated degradation of dibenzothiophene by a *Pseudomonas* species (Monticello et al, 1985). Similar results were obtained with *Rhizobium japonicum* in which succinate completely repressed phenol utilization but only partially inhibited p-hydroxybenzoate respiration (Rohm and Werner, 1985). The latter substrate is metabolized *via* the *ortho* cleavage pathway by *R. japonicum* (Muthukumar et al, 1982) and by *Pseudomonas putida* (Clarke, 1982). In *P. putida*, however, phenol is degraded independently *via* the *meta* cleavage pathway (Feist and Hegeman, 1969). The operation of a different, *meta* cleavage pathway for phenol degradation in *R. japonicum* is thus suggested by its repression by succinate and by the finding that the addition of pyruvate elicited a more rapid and extensive mineralization of phenol after a short lag period (Rohm and Werner, 1985). No search for plasmids in this *R. japonicum* strain was conducted. The extension of these results on the simultaneous utilization of aromatic hydrocarbons and ring cleavage end products may help to identify ring cleavage pathways for other

hydrocarbons and organisms and possibly be used as a screen for plasmid-bearing isolates.

REFERENCES

Barnsley, E.A. 1983. Phthalate pathway of phenanthrene metabolism: Formation of 2'-carboxybenzalpyruvate. J. Bacteriol. 154:113-117.

Box, J.D. 1983. Investigation of the Folin-Ciocalteau phenol reagent for the determination of polyphenolic substances in natural waters. Water Res. 17:511-525.

Brandsch, R. and K. Decker. 1984. Isolation and partial characterization of plasmid DNA from Arthrobacter oxidans. Arch. Microbiol. 138:15-17.

Bushnell, L.D. and H.F. Haas. 1941. The utilization of certain hydrocarbons by microorganisms. J. Bacteriol. 41:653-673.

Cerniglia, C.E. 1984. Microbial metabolism of polycyclic aromatic hydrocarbons. Adv. Appl. Microbiol. 30:31-71.

Chakrabarty, A.M. 1972. Genetic basis of the biodegradation of salicylate in Pseudomonas. J. Bacteriol. 112:815-823.

Chakrabarty, A.M. 1976. Plasmids in Pseudomonas. Ann. Rev. Genet. 10:7-30.

Clarke, P.H. 1982. The metabolic versatility of pseudomonads. Antonie van Leeuwenhoek 48:105-130.

Collins, C.H., J.M. Grange and M.D. Yates. 1984. Mycobacteria in water. J. Appl. Bacteriol. 57:193-211.

Cook, A.M., H. Grossenbacher and R. Hutter. 1983. Isolation and cultivation of microbes with biodegradtive potential. Experientia 39:1191-1198.

Cox, D.P. and A.L. Williams. 1980. Biological process for converting naphthalene to cis-1,2-dihydroxy-1,2-dihydronaphthalene. Appl. Environ. Microbiol. 39:320-326.

Davis, B.D., R. Dulbecco, H.N. Eisen, H.S. Ginsberg, W.B. Wood, Jr. and M. McCarty. 1973. Microbiology. Harper & Row, Hagerstown, Maryland.

Dean-Raymond, D. and R. Bartha. 1975. Biodegradation of some polynuclear aromatic petroleum components by marine bacteria. Dev. Ind. Microbiol. 16:97-110.

Dunn, N.W. and I.C. Gunsalus. 1973. Transmissable plasmid coding early enzymes of naphthalene oxidation in Pseudomonas putida. J. Bacteriol. 114:974-979.

Evans, W.C., H.N. Fernley and E. Griffiths. 1965. Oxidative metabolism of phenanthrene and anthracene by soil pseudomonads. Biochem. J. 95:819-831.

Feist, C.F. and G.D. Hegeman. 1969. Phenol and benzoate metabolism by Pseudomonas putida: Regulation of tangential pathways. J. Bacteriol. 100:869-877.

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

Ghosh, D.K. and A.K. Mishra. 1983. Oxidation of phenanthrene by a strain of Micrococcus: Evidence of protocatechuate pathway. Curr. Microbiol. 9:219-224.

Goodfellow, M., M.D. Collins and D.E. Minnikin. 1976. Thin-layer chromatographic analysis of mycolic acid and other long-chain components in whole-organism methanolysates of coryneform and related taxa. J. Gen. Microbiol. 96:351-358.

Guerry, P., D.J. LeBlanc and F. Falkow. 1973. General method for the isolation of plasmid deoxyribonucleic acid. J. Bacteriol. 116:1064-1066.

Haas, D. 1983. Genetic aspects of biodegradation by pseudomonads. Experientia 39:1199-1213.

Hedgecook, L.W. 1968. Nutritional characteristics of the atypical mycobacteria. J. Bacteriol. 96:306-313.

Jerina, D.M., H. Selander, H. Yagi, M.C. Wells, J.F. Davey, V. Mahadevan and D.T. Gibson. 1976. Dihydrodiols from anthracene and phenanthrene. J. Am. Chem. Soc. 98:5988-5996.

King, D.H. and J.J. Perry. 1975. The origin of fatty acids in the hydrocarbon-utilizing microorganism Mycobacterium vaccae. Can. J. Microbiol. 21:85-89.

Kiyohara, H., K. Nagao and R. Nomi. 1976. Degradation of phenanthrene through o-phthalate by an Aeromonas sp. Agr. Biol. Chem. 40:1075-1082.

Kiyohara, H. and K. Nagao. 1978. The catabolism of phenanthrene and naphthalene by bacteria. J. Gen. Microbiol. 105:69-75.

Kiyohara, H., K. Nagao, K. Kouno and K. Yano. 1982. Phenanthrene-degrading phenotype of Alcaligenes faecalis AFK2. Appl. Environ. Microbiol. 43:458-461.

Kiyohara, H., M. Sugiyama, F.J. Mondello, D.T. Gibson and K. Yano. 1983. Plasmid involvement in the degradation of polycyclic aromatic hydrocarbons by a Beijerinckia species. Biochem. Biophys. Res. Comm. 111:939-945.

Krulwich, T.A. and N.J. Pelliccione. 1979. Catabolic pathways of coryneforms, nocardias, and mycobacteria. Ann. Rev. Microbiol. 33:95-111.

Kubica, G.C. and R.C. Good. 1981. The genus Mycobacterium (except M. leprae). p 1962-1984, In M.P. Starr, H. Stolp, H.G. Truper, A. Balows, H.G. Schlegel (ed.), The Prokaryotes, vol. II. Springer-Verlag, NY.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

Lupton, F.S. and K.C. Marshall. 1978. Effectiveness of surfactants in the microbial degradation of oil. Geomicrobiology J. 1:235-247.

May, W.E., S.P. Wasik and D.H. Freeman. 1978. Determination of the aqueous solubility of polynuclear aromatic hydrocarbons by a coupled column liquid chromatographic technique. Anal. Chem. 50:175-179.

Minnikin, D.E., L. Alshamaony and M. Goodfellow. 1975. Differentiation of Mycobacterium, Nocardia, and related taxa by thin-layer chromatographic analysis of whole-organism methanolysates. J. Gen. Microbiol. 88:200-204.

Monticello, D.J., D. Bakker and W.R. Finnerty. 1985. Plasmid-mediated degradation of dibenzothiophene by Pseudomonas species. Appl. Environ. Microbiol. 49:756-760.

Mulheirn, L.J. and J. Van Eyk. 1981. Microbiological oxidation of steroid hydrocarbons. J. Gen. Microbiol. 126:267-275.

Murphy, G.L. and J.J. Perry. 1983. Incorporation of chlorinated alkanes into fatty acids of hydrocarbon-utilizing mycobacteria. J. Bacteriol. 156:1158-1164.

Muthukumar, G., A. Arunakumari and A. Mahadevan. 1982. Degradation of aromatic compounds by Rhizobium spp. Plant and Soil 69:163-169.

Painceira, M.T., J.C. Alonso, A.N. Sarachu and O. Grau. 1985. A 38 Md plasmid associated with naphthalene utilization in Pseudomonas. Microbios Lett. 28:41-46.

Peczynska-Czoch, W. and M. Mordarski. 1984. Transformation of xenobiotics, p. 287-336. In M. Goodfellow, M. Mordarski and S.T. Williams (ed.), The Biology of The Actinomycetes. Academic Press, London.

Rogoff, M.H. and I. Wender. 1957. The microbiology of coal I. Bacterial oxidation of phenanthrene. J. Bacteriol. 73:264-268.

Rohm, M. and D. Werner. 1985. Regulation of the β-ketoadipate pathway in Rhizobium japonicum and bacteroids by succinate. Arch. Microbiol. 140:375-379.

Schaefer, W.B. and C.W. Lewis, Jr. 1965. Effect of oleic acid on growth and cell structure of mycobacteria. J. Bacteriol. 90:1438-1447.

Singleton, V.L. 1974. Analytical fractionation of the phenolic substances of grapes and wine and some practical uses of such analyses. Adv. Chem. 137:184-211.

Slijkhuis, H., J.W. Van Groenestijn and D.J. Kylstra. 1984. Microthrix parvicella, a filamentous bacterium from activated sludge: Growth on Tween 80 as carbon and energy source. J. Gen. Microbiol. 130:2035-2042.

West, P.A., G.C. Okpokwasili, P.R. Brayton, D.J. Grimes and R.R. Colwell. 1984. Numerical taxonomy of phenanthrene-degrading bacteria isolated from the Chesapeake Bay. Appl. Environ. Microbiol. 48:988-993.

Whitehouse, B. 1985. The effects of dissolved organic matter on the aqueous partitioning of polynuclear aromatic hydrocarbons. Est. Coast. Shelf Sci. 20:393-402.

Williams, S.T., S. Lanning and E.M.H. Wellington. 1984. Ecology of actinomycetes. p. 481-528. In M. Goodfellow, M. Mordarski and S.T. Williams (ed.), The Biology of the Actinomycetes. Academic Press, London.

Wodzinski, R.S. and M.J. Johnson. 1968. Yields of bacterial cells from hydrocarbons. Appl. Microbiol. 16:1886-1891.

Wodzinski, R.S. and J.E. Coyle. 1974. Physical state of phenanthrene for utilization by bacteria. Appl. Microbiol. 27:1081-1084.

Zuniga, M.C., D.R. Durham and R.A. Welch. 1981. Plasmid- and chromosome-mediated dissimilation of naphthalene and salicylate in Pseudomonas putida PMD-1. J. Bacteriol. 147:836-843.

CHAPTER TWO

CHAPTER TWO

TWO-STAGE MINERALIZATION OF PHENANTHRENE BY ESTUARINE ENRICHMENT CULTURES

ABSTRACT

The polycyclic aromatic hydrocarbon (PAH), phenanthrene, was mineralized in two stages by soil, estuarine water and sediment microbial populations. At high concentrations, phenanthrene was degraded with concomitant production of biomass and accumulation of Folin-Ciocalteau-reactive aromatic intermediates. Subsequent consumption of these intermediates resulted in a secondary increase in biomass. Analysis of intermediates by HPLC, TLC and UV absorption spectrometry showed 1-hydroxy-2-naphthoic acid (1H2NA) to be the predominant product. A two-stage mineralization pattern was also observed by monitoring $^{14}CO_2$ production from low concentrations (0.5 mg L^{-1}) of radiolabeled phenanthrene. Incremental $^{14}CO_2$ was produced upon the mineralization of ^{14}C-1H2NA. The production of metabolites during PAH biodegradation is discussed with regard to its possible adaptive significance and its methodological implications.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH), are a class of fused ring aromatic compounds which are of great concern because of their toxicity, carcinogenicity, potential for trophic biomagnification, and resistance to biodegradation (Phillips, 1983; Maccubbin et al, 1985). As products of combustion and as components of fossil fuels, PAH are ubiquitous environmental pollutants and their abundance is correlated with proximity to urban and industrial centers (Windsor and Hites, 1979; Lake et al, 1979). Estuaries are prime repositories for PAH due to urbanization and the flocculation of river-borne, humic- and particle-associated PAH induced by seawater mixing(Jackim and Lake, 1978; Olsen et al, 1982). Total PAH concentrations in urban estuarine sediments may exceed 100 $\mu g\ g^{-1}$ (Windsor and Hites, 1979) while dissolved concentrations of individual compounds generally lie in the part per trillion range or several orders of magnitude below saturation (Readman et al, 1982). The return to coal combustion as a major future energy source promises to reverse the recent trend of decreasing environmental PAH burdens brought on by the conversion from coal to oil and gas in the 1950's.

Phenanthrene ($C_{14}H_{10}$), though not particularly hazardous itself (Phillips, 1983), is a good model compound for studying PAH biogeochemistry because it is intermediate in solubility and physical behavior (May et al, 1978; Readman et al, 1982). As a result of detailed biochemical studies (Jerina et al, 1976; Barnsley, 1983; Evans et al, 1965; Kiyohara et al, 1976), the degradative pathways for

phenanthrene are well known. Common to all published reports is the initial conversion of phenanthrene to 1-hydroxy-2-naphthoic acid (1H2NA), which often accumulates in the growth medium and turns it an orange color (Dean-Raymond and Bartha, 1975; Evans et al, 1965; Ghosh and Mishra, 1983; Kiyohara et al, 1976, 1982; Kiyohara and Nagao, 1977, 1978; Rogoff and Wender, 1957a; Wodzinski and Johnson, 1968). In comparison to the analogous accumulation of salicylic acid (SA) during naphthalene degradation (Klausmeier and Strawinski, 1957; Hosler, 1963; Ishikura et al, 1968; Murphy and Stone, 1955; Shamsuzzaman and Barnsley, 1974; Wodzinski and Johnson, 1968) however, virtually nothing is known about the factors regulating the accumulation or subsequent utilization of 1H2NA during phenanthrene degradation.

Although 1H2NA accumulation is characteristic of most pure cultures degrading phenanthrene, in a companion paper we describe a Mycobacterium sp. which mineralizes phenanthrene via 1H2NA but without accumulating the metabolite. We undertook the present study to determine whether (or which) results with pure cultures could be applied to processes of phenanthrene degradation by mixed cultures. The purpose was to identify and assess the quantitative significance of metabolic intermediates produced by consortia of estuarine microorganisms degrading phenanthrene. With this information, it might then be possible to evaluate the efficacy of biodegradation methods which measure substrate disappearance or $^{14}CO_2$ evolution from labeled phenanthrene, but which do not consider the potentially important compartment of partially oxidized, soluble transformation products.

MATERIALS AND METHODS

Media

Two types of phenanthrene growth media were employed in this study. These differed fundamentally in their phenanthrene concentrations, and consequently in the physical states of the substrate. Low concentration phenanthrene solutions were prepared by pumping 75% artificial seawater (ASW, Kester et al, 1967) through a column (4.6 x 250 mm stainless steel) packed with phenanthrene-coated glass beads (60/80 mesh) at a flow rate of 0.5 ml min^{-1}. The glass beads were prepared as described by May et al. (1978). A column of this size generated phenanthrene solutions ranging from about 70 to 90% saturation. After dilution with 75% ASW to give a half-saturated phenanthrene solution (0.5 mg L^{-1} as determined by absorbance at 250 nm), 1.5 mg NH_4NO_3, 1.5 mg Tween 80 and 1 ml of 0.05 M phosphate buffer (pH 7.5) per liter were added. The pH was adjusted to 7.5 and the solution was sterilized by filtration (0.2 um Nuclepore). Aliquots of 190 ml were aseptically dispensed into sterile 500 ml Erlenmeyer flasks modified by the addition of 8mm (o.d.) pyrex sidearms fitted with serum stoppers (Fedorak et al, 1982). Flasks were inoculated with 10 ml of untreated estuarine water or a 10^{-2} dilution of sediment and spiked with 10 µl (~1 µCi) of a [9-^{14}C] phenanthrene in acetone solution (sp. act. 19.3 mCi $mMole^{-1}$, Amersham-Searle, 98% radiochemical purity by TLC and HPLC) and stoppered. Alternatively, 200 ml water samples were spiked with 10 µl of acetone in which the labeled phenanthrene (~1 µCi) was diluted with unlabeled phenanthrene to result

in a final sample concentration of 0.5 μg ml^{-1}. The total initial activity in each flask was determined from duplicate 1 ml aliquots taken immediately after inoculation. Enrichments were incubated at 18°C in the dark on a rotary shaker (140 rpm).

Media containing phenanthrene at concentrations in excess of its solubility were prepared in two parts. Part A was prepared by adding 150 ml distilled water (Milli Q system, Millipore), 80 mg NH_4NO_3 and 1 mg $FeCl_3 \cdot 6H_2O$ to 750 ml 100% ASW. The solution was filtered through 0.4 um Nuclepore filters, and the pH was adjusted to 7.5. Aliquots (90 ml) were dispensed into 250 ml Erlenmeyer flasks and autoclaved. Part B was prepared by blending 200 mg of dry heat-sterilized (75-85°C, 24 h) phenanthrene (94.4%C) with 100 ml of sterile distilled water containing 80 mg Tween 80 (59%C) and 300 ul of 0.05 M phosphate buffer (pH 7.5). Two 2.5 min blending periods with a 5 min interval to allow reintroduction of the bubble-suspended phenanthrene into the aqueous phase resulted in a fine dispersion. Ten ml aliquots of suspension B were added to flasks containing part A to produce a Phenanthrene/Tween 80 (P/T80) broth with a P-C:T80-C ratio of 4:1 and an approximate C:N:P ratio of 100:10:2. The latter ratio varied, as did the initial phenanthrene concentration, due to evaporative losses during autoclaving, to volatilization of hydrocarbon during sterilization, and to errors inherent in taking aliquots from particulate suspensions. Volumes were sometimes doubled for incubations in 500 ml Erlenmeyer flasks and phenanthrene concentrations were varied, as indicated, while maintaining a constant P-C:T80-C ratio.

Analyses

Degradation of radiolabeled phenanthrene at 0.5 mg L^{-1} was

followed by periodic analysis of the $^{14}CO_2$ produced from respiration. Duplicate 1% aliquots of the cultures (2 ml water, 4 ml headspace) were collected by syringe and injected into evacuated 15 x 125 mm test tubes capped with butyl rubber serum stoppers and containing 1 ml of 2N HCl. The CO_2 liberated was trapped on a 6 x 1 cm strip of pleated Whatman no. 1 filter paper soaked with 0.2 ml of phenethylamine (National Diagnostics) and suspended from the serum stopper in a plastic cup. After 24 h of degassing with shaking, the filter papers were removed and swirled in 9 ml Scintiverse E (Fisher) and 5 ml of 95% ethanol (used to rinse the plastic cup) in 20 ml scintillation vials. Samples were counted (2 min preset time, 2% preset error) on a Beckman LS7000 liquid scintillation counter. Counts were converted to dpm by use of the automatic quench compensation feature of the instrument. H number vs. percent efficiency curves were constructed using ^{14}C-toluene internal standards with acetone as a quenching agent. Careful attention to flask sampling details and scrupulous cleaning of test tubes (combustion at 475°C for 6 h) and stopper-cup assemblies between uses resulted in extremely precise measures of $^{14}CO_2$ production. Precision among triplicate tubes averaged ±0.48% (± 1 sd) of the total initial activity (n=87, range 0.03 to 2.19). To conserve supplies, samples were routinely run in duplicate. Control tubes yielded apparent $^{14}CO_2$ activities amounting to 2 to 3% of the total initial activity due to trapping of volatilized ^{14}C phenanthrene. Four to five days after spiking with radiolabeled phenanthrene, radioactivity in control tubes dropped to background levels due to adsorption. Samples, uncorrected for controls, sometimes manifested this volatilization artifact early in the incubations.

Radioactivity in the above flasks was separated into polar (including 1H2NA) and nonpolar (phenanthrene) fractions by sequential 5 ml CH_2Cl_2 extractions of a 5 ml culture aliquot made alkaline by the addition of 1 drop 2N NaOH in 15 ml glass-stoppered centrifuge tubes. Tubes were centrifuged (10 min, 2500 g) after shaking for 1 min to give an upper aqueous layer, a lower organic layer and an intervening pellicle of cells and debris. The first 5 ml of CH_2Cl_2 was removed by pasteur pipet to a scintillation vial. After the second extraction, 4 ml of upper aqueous layer was removed with a glass syringe and transferred to a separate scintillation vial containing 1 ml of 2N HCl. The second 5 ml of CH_2Cl_2 was then withdrawn and combined with the first extract and the vials were left to evaporate (or degas) in the hood overnight. The following day, 1 ml of 100% ethanol was used to dissolve the organic soluble residue and 9 ml of scintillation cocktail were added to each set of vials. Vials were counted as described above to give measures of undegraded and partially degraded phenanthrene.

The nature of the water soluble activity was also assessed by HPLC analysis of culture supernatant fluids after centrifugation (12,100 x g, 10 min, 4°C). One ml elution fractions, corresponding to the retention times of known phenanthrene metabolites, were collected in scintillation vials containing 9 ml cocktail. Further details of the HPLC procedure are given below and in Guerin and Jones (subm.).

Degradation of particulate phenanthrene was monitored by several methods. Substrate disappearance was determined by extracting duplicate 1 to 5 ml aliquots of the cultures in acid washed and solvent rinsed 60 ml separatory funnels with 2 x 10 ml portions of CH_2Cl_2 (Baker, Photrex grade). Extracts were pooled in similarly cleaned 25 ml graduated

cylinders and 50-100 mg of 100/200 mesh silca gel (Supelcosil, Supelco) was added to clear the extracts of T80/H_2O emulsions. The extract was brought to volume before measuring the absorbance at 250 nm on a Beckman DU-8 spectrophotometer in 1 cm quartz cuvets. Tween 80 served to stabilize the medium; no losses of phenanthrene from uninoculated controls were detected.

Due to the attachment of cells to phenanthrene particles, enumeration of cells by plate counts or by direct epifluorescence microscopic counts proved unsuitable. Therefore, biomass was estimated as protein by the method of Lowry et al. (1951) on a Bausch and Lomb Spectronic 88 using bovine serum albumin as a standard. Cells were harvested in acid washed Corex centrifuge tubes (12,100 x g, 10 min, 4°C); supernatant fluids were saved for analysis of polar metabolites.

Analysis of phenanthrene metabolic intermediates was carried out using two methods. Since many of the known phenanthrene metabolites are hydroxylated aromatic compounds, culture supernatant fluids were analyzed for the presence of phenolics by a modification of the Folin-Ciocalteau reaction as outlined by Box (1983). Absorbance at 750 nm was measured on a Bausch and Lomb Spectronic 88 after centrifugation to remove the precipitate that forms in the application of this method to seawater. The salt precipitate did not affect the linearity of the calibration. Resorcinol was used as a standard and all values were expressed in $\mu g\ ml^{-1}$ resorcinol equivalents (RE).

Phenanthrene degradation products were also analyzed by reverse phase HPLC. Culture supernatant fluids (12,100 x g, 10 min, 4°C) were injected directly into a Beckman Model 332 Gradient Liquid Chromatograph with dual model 110 pumps, a model 420 microprocessor

controller/programmer and fitted with a 4.6 x 250 mm 5 um C-18 Adsorbosphere column (Applied Sciences). A linear elution gradient (0-100% methanol at 5% min^{-1}) was used with a starting solvent of 100% H_2O adjusted to pH 2.75 with H_3PO_4. The solvents and the H_3PO_4 were HPLC grade (Baker). Flow rate was 1 ml min^{-1} and detection was by absorbance at 254 nm in a Hitachi model 100-40 spectrophotometer fitted with an Altex model 155-00 20 µl flow-through cell. Detector sensitivity was set to give full scale deflection on a Heath recorder at an absorbance of 0.1. Peak retention times were compared to those of authentic standards which remained constant over the course of an experiment. No adverse effects on column performance were noted using this direct injection procedure. Controls containing Tween 80 as the sole carbon and energy source gave featureless HPLC spectra and no F-C reaction.

To confirm their identity, metabolites were isolated by ethyl acetate extraction (2 x 50 ml) of acidified culture supernatant fluids (100 ml). Extracts were dried over anhydrous Na_2SO_4 and concentrated under high purity N_2 gas. Concentrates and standards were applied to preparative TLC plates (2 mm x 20 cm x 20 cm) and developed in a solvent system of petroleum ether:toluene:acetone:acetic acid (80:20:10:4). Fluorescent spots were scraped from the plates, eluted in acetone and the UV absorption spectra recorded on a Beckman DU-8 spectrophotometer in quartz cuvets.

Inocula

As inocula, samples of sediment, beach tar, surface microlayer and bulk water were collected from locations around the Great Bay Estuary, NH. A petroleum contaminated soil sample collected from the UNH bus

depot was also used in these studies. Several phenanthrene-degrading isolates were obtained from these enrichments on the basis of their ability to form clearing zones in a crystalline phenanthrene overlay on agar plates (Kiyohara et al, 1982). These isolates were subsequently tested by the methods described to depict their patterns of phenanthrene degradation and metabolite formation.

RESULTS

All estuarine and soil enrichment cultures followed the same general pattern of degradation when phenanthrene was supplied in particulate suspension. Figure 12 shows the time courses for duplicate incubations designated AP-31 and AP-32 with initial phenanthrene concentrations of 220 and 260 mg L^{-1}, respectively. Sediment from a boat ramp at Adam's Point was used as the initial inoculum. Time zero in Fig. 12 represents the time of the second transfer of the mixed culture to fresh P/T80 broths. The pre-adaptation of this culture to growth on phenanthrene led to the rapid disappearance of the hydrocarbon during the first stage of growth. Phenanthrene disappearance was accompanied by an increase in biomass and the production of F-C reactive intermediates (RE) resulting in an orange coloration of the growth medium. At 10 d, F-C reactive product concentrations were maximal and protein concentrations plateaued or decreased slightly. Further increases in protein concentrations occurred during the second stage of degradation during which metabolites were consumed. AP-32 lagged 7 days behind AP-31 during this stage. After the disappearance of metabolites from the medium, protein concentrations began to decrease.

Figure 13 shows the time course for phenanthrene degradation in primary enrichments using surface microlayer and bulk water microbial populations from a marina in Prescott Park (PP), Portsmouth, NH as inocula. Initial phenanthrene concentrations were about 40 mg L^{-1}. The microlayer sample (mean thickness of 45 um collected with a glass

FIGURE 12. Time course for phenanthrene degradation by parallel sediment enrichment cultures AP-31 (open circles) and AP-32 (solid circles).

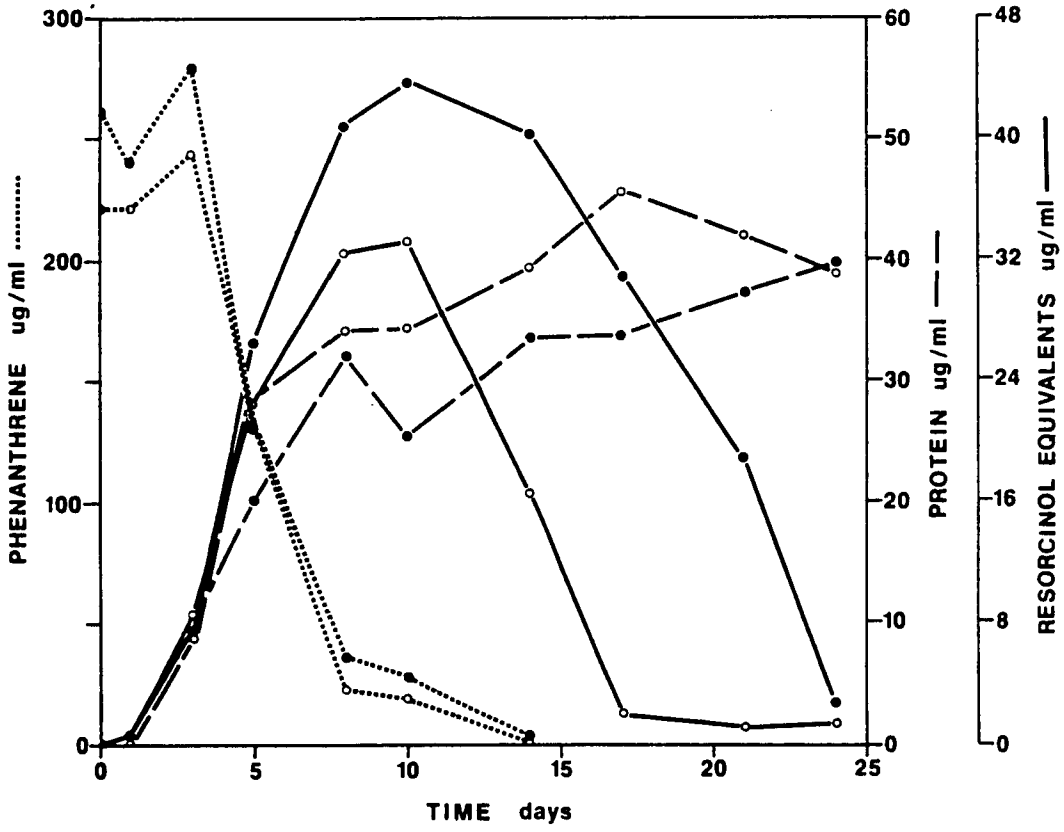

PHENANTHRENE ug/ml .....
PROTEIN ug/ml ——
RESORCINOL EQUIVALENTS ug/ml ——
TIME days
300
200
100
0
60
50
40
30
20
10
0
48
40
32
24
16
8
0
0
5
10
15
20
25

FIGURE 13. Time course for phenanthrene degradation by PP surface microlayer (top) and bulk water (bottom) microbial populations. Breaks in the phenanthrene (broken line) and RE (solid line) curves at 10 d in the microlayer incubation are extrapolations based on subsequent data points. Protein denoted by dotted line.

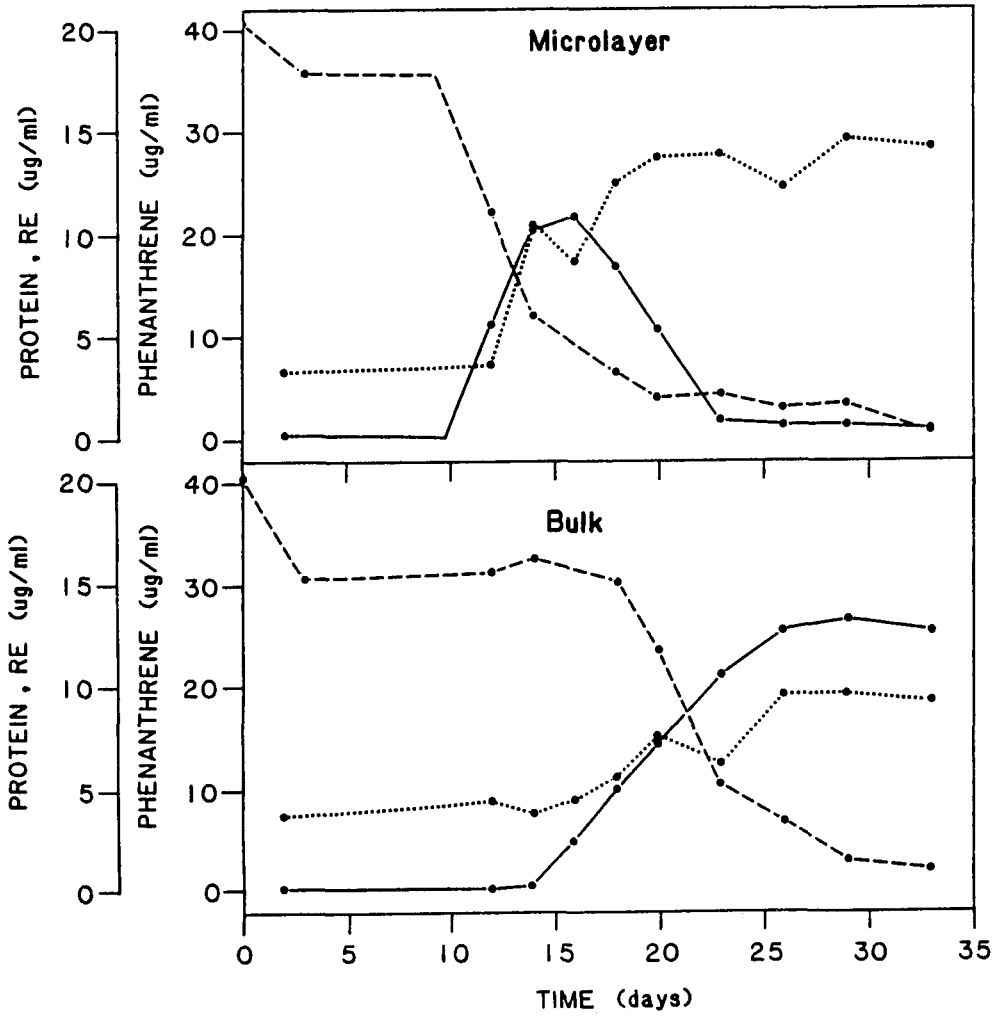
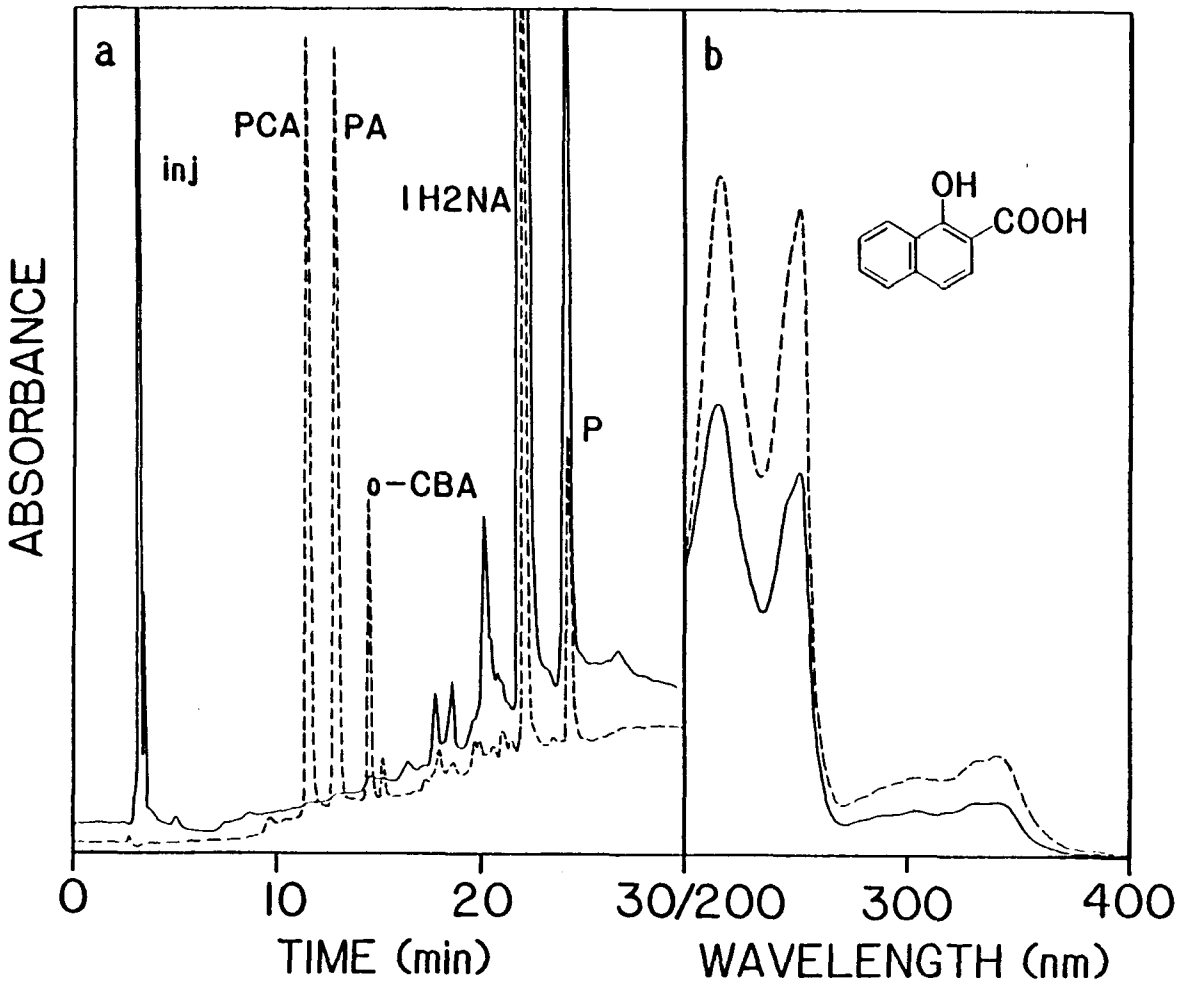

Microlayer
Bulk
PROTEIN , RE (ug/ml)
PHENANTHRENE (ug/ml)
TIME (days)
0
5
10
15
20
25
30
35
40

plate) was enriched threefold in total viable heterotrophs relative to the underlying bulk water and showed a shorter lag period prior to phenanthrene degradation. Phenanthrene and metabolites had disappeared in the microlayer sample by 23 d with a maximum protein yield of about 15 $\mu g\ ml^{-1}$. Degradation by the bulk water population occurred more slowly with metabolites just beginning to disappear at 33 d and maximum protein concentrations of about 10 $\mu g\ ml^{-1}$. Secondary increases in protein concentrations followed temporary decreases coinciding with the depletion of phenanthrene (less than 10 $\mu g\ ml^{-1}$ at 16 d) and F-C reactive intermediates (26 d) during the microlayer incubation, and the depletion of phenanthrene (23 d) during the bulk water incubation.

Analysis of culture supernatant fluids by HPLC during the course of these incubations revealed that the onset and disappearance of F-C reactivity was due to the production and subsequent consumption of a single component whose retention time matched that of 1H2NA. Other UV-absorbing intermediates were present only in minor amounts. Figure 14a shows the HPLC chromatogram for the PP bulk water sample corresponding to d 34 of the incubation shown in Fig. 13. The retention time of the major peak in this sample matched that of 1H2NA. Likewise, on TLC plates, acidified culture extracts gave a blue fluorescent spot with the R_f value of 1H2NA. The identity of the major metabolite was confirmed by matching the UV absorption spectrum of authentic 1H2NA (Sigma) with that of the TLC-isolated product (Fig. 14b).

Since only a single compound was responsible for F-C reactivity in these enrichments, RE could be used to provide a quantitative measure of metabolite production after intercalibration with 1H2NA. A maximum of 33 and 36 moles % of the initial phenanthrene could be accounted

FIGURE 14. Analysis of metabolites in PP bulk water sample (Fig. 13, bottom) after 34 d of incubation. a) HPLC chromatograms for 34 d culture supernatant fluid (solid line) and a standard mixture made up of authentic compounds dissolved in distilled water (broken line). PCA, protocatechuic acid; PA, phthalic acid; o-CBA, o-carboxybenzaldehyde; 1H2NA, 1-hydroxy-2-naphthoic acid; P, phenanthrene. b) UV absorption spectra of authentic 1H2NA (broken line) and the TLC-isolated product after ethyl acetate extraction of acidified 34 d bulk water sample (solid line).

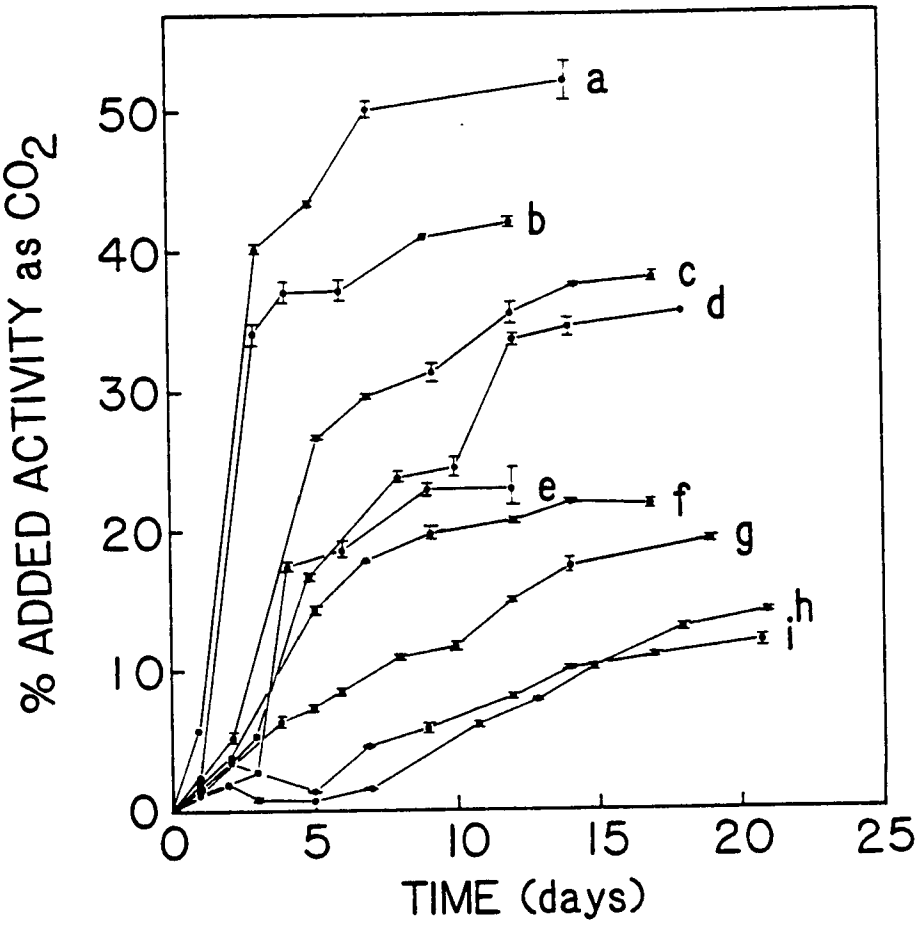

a
b
inj
PCA
PA
1H2NA
o-CBA
P
OH
COOH
ABSORBANCE
0
10
20
30/200
300
400
TIME (min)
WAVELENGTH (nm)

for as 1H2NA in sediment enrichments AP-31 and AP-32, respectively, and a maximum of 59 and 72 moles % was present as 1H2NA in the PP microlayer and bulk water enrichments, respectively.

During incubations with [9-^{14}C] phenanthrene, degradation beyond 1H2NA is necessary before $^{14}CO_2$ is evolved. A degradation pattern in which some of the phenanthrene carbon accumulates as 1H2NA (now labeled at C-3 or C-4) and is subsequently mineralized, would then be expected to show $^{14}CO_2$ evolution curves similar to the growth (protein) curves in Figs. 12 and 13. Such a two stage pattern of mineralization was evidenced in the stepped $^{14}CO_2$ evolution curves shown in Fig. 15 for several water and sediment samples collected from the estuary over a one year period. In general, this stepped pattern was more pronounced in the more active samples, i.e., those with a short lag period, a high initial rate of mineralization, and a high final percent mineralization. Because of the precision of the $^{14}CO_2$ measurement, however, two stage patterns were also discerned in low activity samples.

At the low phenanthrene concentrations (0.5 mg L^{-1}) employed in radiolabel experiments, water-soluble products did not account for more than about 10% of the total initial activity. In some cases, the water-soluble radioactivity showed production and subsequent consumption curves similar to the RE curves in Figs. 12 and 13, while in others, the activity increased slowly over time. After 7 d incubation of 8 water samples, approximately one half (0.46 $\pm$ 0.12, $\bar{x} \pm 1$ s.d.) of the water soluble radioactivity eluted in the HPLC fraction corresponding in retention time to 1H2NA. In accordance with these figures, the incremental $^{14}CO_2$ produced during the second stage of mineralization

FIGURE 15. Two-stage mineralization curves showing evolution of $^{14}CO_2$ during low concentration (0.5 mg L^{-1}) incubations with [9-^{14}C] phenanthrene for several surface microlayer (d, h), bulk water (a, c, f, g, i) and sediment (b, e) samples. Error bars for curves d, g and h are ± 1 s.d. around the mean of triplicate determinations. All others represent the ranges of duplicate determinations.


% ADDED ACTIVITY as CO_2
0
10
20
30
40
50
0
5
10
15
20
25
TIME (days)
a
b
c
d
e
f
g
h
i

ranged from approximately 1 to 11 % of the total initial activity (Fig. 15). In control and experimental flasks, CH_2Cl_2-extractable radioactivity decreased exponentially over time.

Isolates were obtained from enrichments on the basis of their ability to form clearing zones in a crystalline phenanthrene overlay after colony development on phenanthrene (0.02%) agar plates. Usually, clearing of the phenanthrene overlay caused a brown or orange coloration of the agar below due to diffusion of soluble intermediates. When grown in P/T80 broth, these isolates, designated phenanthrene "degraders," accumulated 1H2NA (up to 60 $\mu g\ ml^{-1}$) without a parallel increase in biomass. Eventually, 1H2NA production stopped even though abundant particulate phenanthrene remained. This was not attributable to acid inhibition since the pH in the naturally-buffered seawater medium never dropped below 7. Figure 16 shows the HPLC chromatograms of P/T80 culture supernatant fluids for isolates obtained from enrichments inoculated with petroleum-contaminated soil (BD3-E and BD2-Y) estuarine sediment (APBS61B) or beach tar (TBB-C). The organisms, all gram negative rods, showed the production of high concentrations of 1H2NA as the sole or major metabolite while degrading phenanthrene.

One organism, a yellow pigmented, non-motile, gram negative rod, was tested for its ability to degrade phenanthrene beyond 1H2NA by monitoring $^{14}CO_2$ production from labeled phenanthrene over a range of initial concentrations (0.084 to 68.5 mg L^{-1}) with KNO_3 or NH_4Cl as N sources. The results, given in Table 7, showed that phenanthrene mineralization by this phenanthrene "degrader" was concentration dependent. At concentrations below 2 or 3 mg L^{-1}, 40 to 47% of the added radioactivity was present as $^{14}CO_2$ after 19 d and intermediates

FIGURE 16. HPLC chromatograms of four phenanthrene-degrading isolates showing the production of the predominant intermediate, 1H2NA.

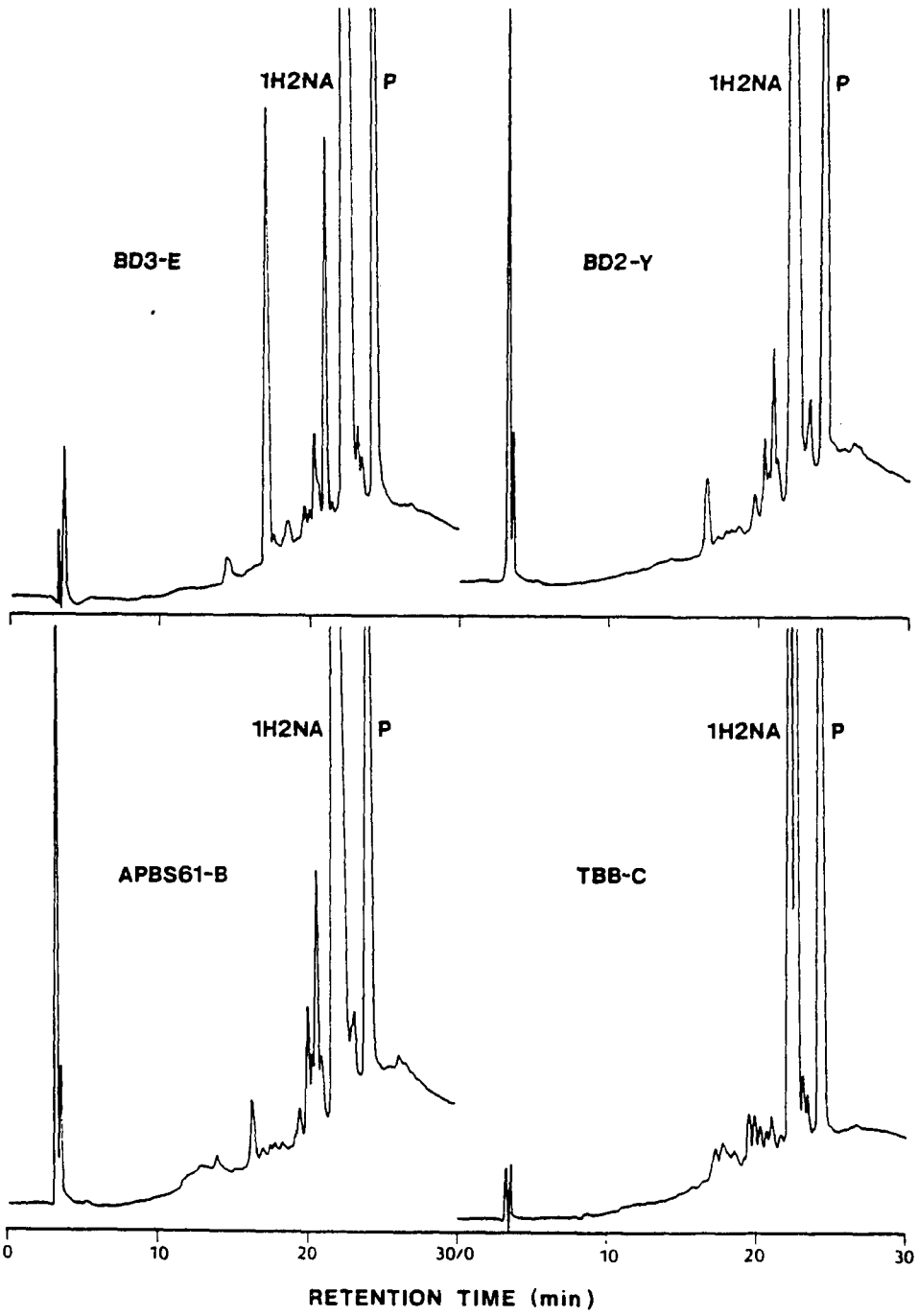

1H2NA
P
BD3-E
1H2NA
P
BD2-Y
1H2NA
P
APBS61-B
1H2NA
P
TBB-C
0
10
20
30/0
10
20
30
RETENTION TIME (min)

TABLE 7. Effects of nitrogen source and phenanthrene concentration on the percent distribution of radioactivity between CO_2, water-soluble, and CH_2Cl_2-extractable fractions after 19 d growth of isolate PC-1 on [9-^{14}C] phenanthrene. RE concentrations at 19 d are also shown.

Nitrogen Source	Initial Phenanthrene Concentration[a]	Percent of Initial Radioactivity In			19 d RE
		CO_2	Water-Soluble	CH_2Cl_2-Extractable	
NH_4Cl	0.084	42.3	3.9	3.8	<1
	0.38	42.4	4.2	3.2	<1
	2.65	40.0	4.7	4.5	<1
	68.5	25.3	6.6	17.0	4.6
KNO_3	0.084	46.8	3.5	3.5	<1
	0.38	41.9	3.4	3.8	<1
	2.65	44.0	5.9	4.5	<1
	68.5	30.1	7.1	13.0	6.4

[a] Initial phenanthrene concentrations (ug ml^{-1}) were varied by dilution with unlabeled compound.

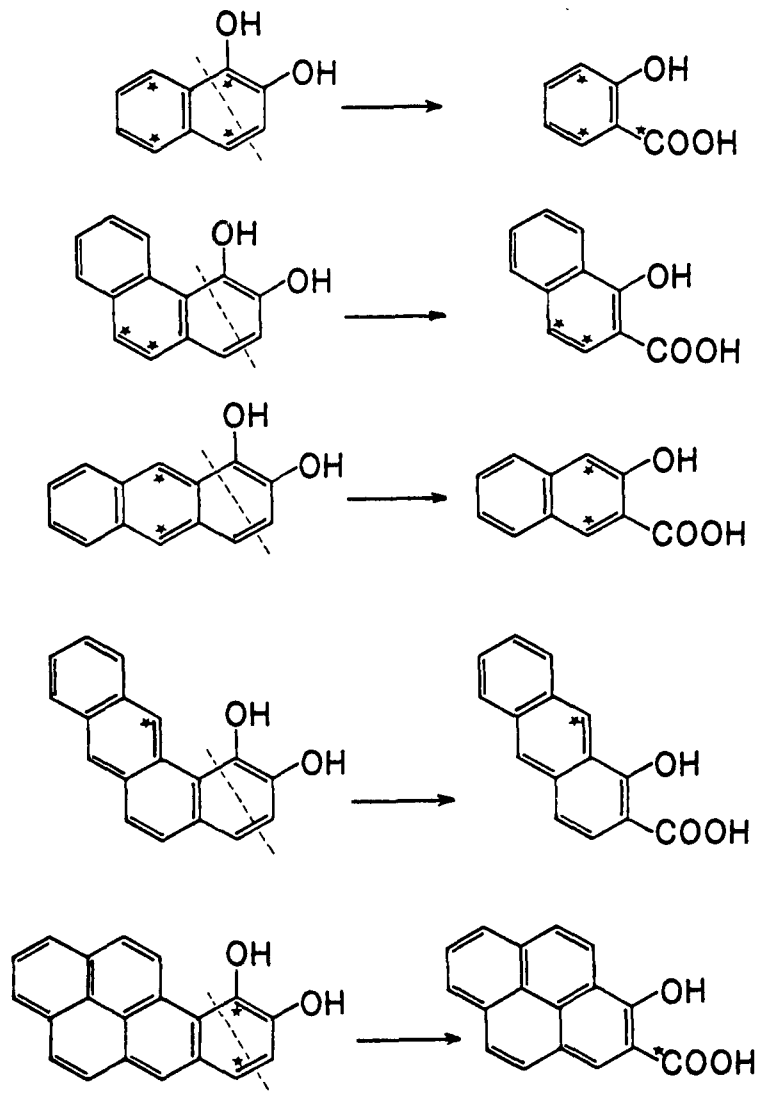
did not accumulate. At 68.5 mg L^{-1}, $^{14}CO_2$ production was lower (25 to 30% of the initial activity) and intermediate concentrations remained high even after 3 weeks. The 19 d RE estimate of metabolite accumulation was higher than the estimate based on water soluble radioactivity due to incomplete equilibration between the unlabeled particulate phenanthrene (> 1 mg L^{-1}) and the ^{14}C-phenanthrene added in acetone. Likewise, solvent extractable ^{14}C was probably an underestimate of the actual percent of the initial phenanthrene undegraded after 19 d. Slightly higher production of $^{14}CO_2$ and intermediates were noted at high initial phenanthrene concentrations when KNO_3 rather than NH_4Cl was the source of N.

Occasionally, isolates were obtained which degraded phenanthrene without significant accumulation of metabolites (including 1H2NA) regardless of the initial phenanthrene concentration. These isolates, designated as phenanthrene "mineralizers," attained high cell densities and utilized all of the phenanthrene provided to them. The characteristics of one such organism, a Mycobacterium sp., are given in a companion paper (Guerin and Jones, submitted).

DISCUSSION

Metabolite accumulation is a common characteristic of PAH (but not benzene) catabolism by bacteria (Abbott and Gledhill, 1971). The most common intermediates produced from PAH are the o-hydroxy, carboxy compounds which result from cleavage of a terminal ring. Figure 17 shows the initial dihydroxylation sites and end ring cleavage products of naphthalene, phenanthrene, anthracene, benzanthracene and benzo(a)pyrene. The well-studied transformations involving naphthalene and phenanthrene have shown salicylate and 1H2NA, respectively, to be the major or sole transformation products. Work with anthracene has been limited but 2-hydroxy-3-naphthoic acid (2H3NA) has been identified as a major intermediate (Evans et al, 1965; Rogoff and Wender, 1957b). The recent identification of 1-hydroxy-2-anthranoic acid as the dominant metabolite of benz(a)anthracene degradation by a Beijerinckia sp. (W. Mahaffey, Abstr. K166, Am. Soc. Microbiol. Ann. Mtg., Las Vegas, Nev. March, 1985) was the first report of ring cleavage of a PAH with more than three rings. While the 9,10-dihydroxy derivative of benz(a)pyrene is the major early transformation product of this compound (Gibson et al, 1975), the ring cleavage product depicted in Fig. 17 is hypothetical. End ring cleavage, however, may account for reports of the slow production of $^{14}CO_2$ from [7,10-^{14}C] benzo(a)pyrene by mixed cultures (Gardner et al, 1979; DeLaune et al, 1981; Readman et al, 1982; Saltzmann, 1982) and, recently, by a fungus (Bumpus et al, 1985). The similarities of these reactions suggest a generalized approach to PAH transformations by bacteria.

FIGURE 17. Initial reactions in the bacterial dissimilation of (from top to bottom) naphthalene, phenanthrene, anthracene, benzanthracene and benzo(a)pyrene. Major sites of dihydroxylation and planes of ring cleavage (dotted lines) are shown on the left side. On the right side are shown the o-hydroxy, carboxy intermediates frequently reported to accumulate during PAH degradations. The ring cleavage product for benz(a)pyrene is hypothetical. Stars indicate the most common positions of ^{14}C-labeling in commercially available PAH and the bacterial metabolites of them.


OH
OH
OH
COOH
OH
OH
OH
COOH
OH
OH
OH
COOH
OH
OH
OH
COOH
OH
OH
OH
COOH

Because pyruvate is readily utilized by most organisms, its production during the end ring cleavage reactions depicted in Fig. 17, would violate at least one condition required to call these reactions cometabolic (Hulbert and Krawiec, 1977). However, the production of intermediates which can not be further metabolized by an organism through the coincidental action of low specificity dioxygenase enzymes is common to such (cometabolic) reactions (Foster, 1962; Perry, 1979). The production of salicylate and 2-hydroxy-3-naphthoic acid (2H3NA) from naphthalene and anthracene, respectively, by a Corynebacterium sp. growing on hexadecane and glucose, respectively, has been attributed to cometabolism (Dalton and Stirling, 1982). Reports of $^{14}CO_2$ evolution from end ring-labeled benzo(a)pyrene may be another example of such a process, and not necessarily evidence of complete mineralization (Fig. 17). However, "cometabolism" does not describe the accumulation of salicylate (Ishikura et al, 1968), 1H2NA (Kiyohara et al, 1976, 1982), or 2H3NA (Evans et al, 1965) by organisms capable of the complete dissimilation of the respective parent PAH. While it is not clear why an organism with the potential to mineralize a hydrocarbon does not do so in a coordinated manner without accumulating metabolites, the extensive work on naphthalene metabolism may shed some light on this problem.

Much of the interest in naphthalene metabolism is directed at maximizing production of salicylate and other intermediates (Cox and Williams, 1980) for chemical and industrial uses. Factors shown to increase salicylate yields were: 1) keeping the pH of the medium above levels where acid effects were detrimental (Klausmeier and Strawinski, 1957); 2) keeping the pH below 8 where further degradation of the

salicylate was promoted (Hosler, 1963); 3) the presence of an anion exchanger or dialysis to keep dissolved salicylate concentrations low (Abbott and Gledhill, 1971); and 4) the presence of naphthalene in excess to inhibit salicylate degradation (Hosler, 1963). Shamsuzzaman and Barnsley (1974), working with a Pseudomonas sp., reported that the molar ratio of salicylate produced to naphthalene consumed was 0.53. Others have reported values as high as 0.87 (Klausmeier and Strawinski, 1957). Hosler (1963) noted that during early stages of naphthalene degradation, the cells grew efficiently with little salicylate accumulation; as degradation proceeded, stoichiometric salicylate production from naphthalene occurred and cell growth at the expense of the ring cleavage product (pyruvate) was poor. [It was recently reported that a Pseudomonas sp. accumulated salicylate and grew poorly when NH_4^+ or NH_4NO_3 was supplied as the nitrogen source (Aranha and Brown, 1981). When grown with NO_3^-, salicylate was detected in trace amounts only early in the incubation and the culture attained high cell densities. Oxygen consumption and CO_2 production were also sevenfold higher using NO_3^- suggesting a much more efficient use of the substrate. Thus, a Pseudomonas behaved as a naphthalene "degrader" when grown with NH_4^+ and a naphthalene "mineralizer" when grown with NO_3^-. The phenomenon described by these authors does not apply to naphthalene degraders in general, however (Walker and Wiltshire, 1953), nor to the analogous accumulation of 1H2NA during phenanthrene degradation (Table 7)].

With many organisms, the salicylate that is produced during naphthalene dissimilation is later consumed as the primary substrate is exhausted (Klausmeier and Strawinski, 1957; Murphy and Stone, 1955;

Ishikura et al, 1968; Shamsuzzaman and Barnsley, 1974). Diauxic growth curves corresponding to the successive utilization of naphthalene and salicylate by P. aeruginosa have been presented (Ishikura et al, 1968). At the point of maximum salicylate concentration, cell counts decreased preceding a second period of growth during which the salicylate was utilized (Murphy and Stone, 1955). Morphological changes associated with this shift in metabolism have been noted (Hosler, 1963).

When present in excess, then, naphthalene is not treated by most organisms as a single substrate to be mineralized by consecutive enzymatic reactions. Rather, under these substrate sufficient conditions, naphthalene is "seen" as a readily available source of pyruvate and the salicylate that is produced assumes the role of a second substrate. Once the naphthalene is depleted, and provided the acidic biproduct does not attain inhibitory concentrations, organisms may then synthesize a new suite of enzymes, or derepress existing ones for the efficient utilization of salicylate (Harder and Dijkhuizen, 1982).

A similar approach to phenanthrene degradation is the most likely explanation for the accumulation of 1H2NA by pure cultures and for the two stage mineralization pattern observed here for mixed microbial cultures. At high phenanthrene concentrations (>0.5 mg L^{-1}), enrichment cultures are dominated by phenanthrene "degraders" which, although perhaps able to mineralize the hydrocarbon, degrade it only as far as 1H2NA, making the ring cleavage product, pyruvate, available for growth. In comparison to phenanthrene "mineralizers," which derive three molecules of pyruvate and one of acetaldehyde from the coordinate mineralization of phenanthrene via meta cleavage (Dagley, 1978), these

phenanthrene "degraders" must process much more substrate to acquire an equivalent amount of energy. The result is that 1H2NA is produced more rapidly than it is consumed during the first stage of mineralization and thus accumulates. Only upon depletion of the phenanthrene is 1H2NA, as the second substrate, degraded, allowing a second stage of protein production to ensue. A third stage of growth (apparent during the PP microlayer incubation of figure 13) may follow as later, non-aromatic intermediates, which would go undetected in the F-C and HPLC analyses, are utilized.

At low phenanthrene concentrations, 1H2NA accumulates to a lesser extent. Under these nutrient limiting conditions, concurrent utilization of phenanthrene and 1H2NA allows phenanthrene "degraders" to more efficiently mineralize the substrate (Table 7). Alternatively, phenanthrene "mineralizers" may be more competitive at low substrate concentrations because of their greater inherent efficiency of substrate utilization. The result in either case is that 1H2NA does not accumulate in high molar proportion to the phenanthrene degraded and a more subtle, two-stage pattern of mineralization is sometimes, but not always discerned.

The two-stage mineralization pattern observed at high phenanthrene concentrations was characteristic of all the samples examined regardless of the extent of PAH pollution and hence pre-exposure (preadaptation) of the community to phenanthrene. In comparison to the PP samples (Fig. 13), the more rapid phenanthrene degradation and lower molar conversion to 1H2NA by the AP samples (Fig. 12) may have been due to selection for a more efficient phenanthrene degrading population during repeated transfer of the AP sample to fresh medium. The rapid

transformation of PAH to polar intermediates may have adaptive significance. In an open system, the advantage of this mode of metabolism is that hydrophobic aromatic compounds which could potentially disrupt membrane functions (Abbott and Gledhill, 1971) are converted to water soluble products which are free to diffuse or be advected away. Incomplete mineralization of PAH may be the prokaryotic equivalent of the eukaryotic detoxification strategy of solubilization of PAH by conjugation (Cerniglia et al, 1982). In soil, sedimentary and aquatic environments, the reactivity of the aromatic acid and phenolic products would favor their interaction or condensation with natural humic materials which contain structural units of similar composition (Schnitzer, 1978; Bollag, 1983). Thus, PAH may be rendered innocuous without being completely mineralized.

Of the few studies concerned with PAH biodegradation by environmental samples, most do not consider polar transformation products as a separate compartment. Notable exceptions are the works of Herbes and coworkers. Herbes et al (1977) noted almost complete conversion of ^{14}C-naphthalene and ^{14}C-anthracene to polar products during short-term incubations with mixed cultures of environmental isolates. With water samples, polar ^{14}C was the dominant anthracene transformation product, but accounted for only a small percentage of the added radioactivity (Herbes, 1981). From sediments, $^{14}CO_2$ and bound ^{14}C (perhaps a humic acid-2H3NA adduct) were the major products recovered after incubations with labeled anthracene (Herbes and Schwall, 1978). Transformation to polar products may also be implicated by the slow rates of $^{14}CO_2$ production from labeled anthracene despite its rapid removal from a marine enclosure (Lee et al, 1978) and from

sediment slurries (Bauer and Capone, 1985). We are unaware of any other studies dealing with phenanthrene degradation by natural samples.

Herbes and Schwall (1978) presented $^{14}CO_2$ evolution curves for naphthalene closely resembling the stepped curves for phenanthrene observed here. Using a procedure similar to the one described here for seawater samples, Reichardt et al. (1981) also presented $^{14}CO_2$ evolution data from [U-^{14}C] biphenyl and monochlorobiphenyls (labeled uniformly on one ring) which showed stepped curves. (Chlorinated) Biphenyl degradation is often accompanied by accumulation of (chloro)benzoic acid or other ring cleavage products (Cerniglia, 1981) and, although no significant radioactive pool of water soluble transformation products was detected, the analyses were performed at the end of the incubation (14% conversion to CO_2), after the incremental $^{14}CO_2$ was produced.

Transformation of PAH to polar intermediates has important implications regarding the methodology used to measure PAH biodegradation. Clearly, the disappearance of parent compounds during incubations with natural samples should not be equated with mineralization. Conversely, $^{14}CO_2$ evolution from interior ring-labeled PAH (Fig. 17) will provide an underestimation of transformation rates at high test concentrations. For phenanthrene at low concentrations (<1 mg L^{-1}) however, metabolites constitute a minor fraction of the ^{14}C added and $^{14}CO_2$ production is a reasonable index of degradation. Nevertheless, from the shapes of the mineralization curves depicted here (Fig. 15), it is clear that biodegradation rate data derived by connecting a time zero and some later $^{14}CO_2$ evolution data point, may be erroneous. Use of the procedure described here for monitoring

mineralization of ^{14}C-PAH provides information and precision unattainable with other methods.

REFERENCES

Abbott, B.J. and W.E. Gledhill. 1971. The extracellular accumulation of metabolic products by hydrocarbon-degrading microorganisms. Adv. Appl. Microbiol. 14:249-388.

Aranha, H.G. and L.R. Brown. 1981. Effect of nitrogen source on end products of naphthalene degradation. Appl. Environ. Microbiol. 42:74-78.

Barnsley, E.A. 1983. Phthalate pathway of phenanthrene metabolism: formation of 2'-carboxybenzalpyruvate. J. Bacteriol. 154:113-117.

Bauer, J.E. and D.G. Capone. 1985. Degradation and mineralization of the polycyclic aromatic hydrocarbons anthracene and naphthalene in intertidal marine sediments. Appl. Environ. Microbiol. 50:81-90.

Bollag, J.-M. 1983. Cross-coupling of humus constituents and xenobiotic substances, p. 127-141. In R.F. Christman and E.T. Gjessing (ed.), Aquatic and Terrestrial Humic Materials. Ann Arbor Science, Ann Arbor, MI.

Box, J.D. 1983. Investigation of the Folin-Ciocalteau phenol reagent for the determination of polyphenolic substances in natural waters. Water Res. 17:511-525.

Bumpus, J.A., M. Tien, D. Wright and S.D. Aust. 1985. Oxidation of persistent environmental pollutants by a white rot fungus. Science 228:1434-1436.

Cerniglia, C.E. 1981. Aromatic hydrocarbons: metabolism by bacteria, fungi and algae. Rev. Biochem. Toxicol. 3:321-361.

Cerniglia, C.E., J.P. Freeman and R.K. Mitchum. 1982. Glucuronide and sulfate conjugation in the fungal metabolism of aromatic hydrocarbons. Appl. Environ. Microbiol. 43:1070-1075.

Cox, D.P. and A.L. Williams. 1980. Biological process for converting naphthalene to cis-1,2-dihydroxy-1,2-dihydronaphthalene. Appl. Environ. Microbiol. 39:320-326.

Dagley, S. 1978. Determinants of biodegradability. Q. Rev. Biophys. 11:577-602.

Dalton, H. and D.I. Stirling. 1982. Co-metabolism. Phil. Trans. R. Soc. Lond. 297:481-496.

Dean-Raymond, D. and R. Bartha. 1975. Biodegradation of some polynuclear aromatic petroleum components by marine bacteria. Dev. Ind. Microbiol. 16:97-110.

DeLaune, R.D., W.H. Patrick, Jr. and M.E. Casselman. 1981. Effect of sediment pH and redox conditions on degradation of benzo(a)pyrene. Mar. Poll. Bull. 12:251-253.

Evans, W.C., H.N. Fernley and E. Griffiths. 1965. Oxidative metabolism of phenanthrene and anthracene by soil pseudomonads. Biochem. J. 95:819-831.

Fedorak, P.M., J.M. Foght and D.W.S. Westlake. 1982. A method for monitoring mineralization of ^{14}C-labeled compounds in aqueous samples. Water Res. 16:1285-1290.

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

Gardner, W.S., R.F. Lee, K.R. Tenore and L.W. Smith. 1979. Degradation of selected polycyclic aromatic hydrocarbons in coastal sediments: Importance of microbes and polychaete worms. Water, Air, Soil Poll. 11:339-347.

Ghosh, D.K. and A.K. Mishra. 1983. Oxidation of phenanthrene by a strain of Micrococcus: evidence of protocatechuate pathway. Curr. Microbiol. 9:219-224.

Gibson, D.T., V. Mahadevan, D.M. Jerina, H. Yagi and H.J.C. Yeh. 1975. Oxidation of the carcinogens benzo(a)pyrene and benzo(a)anthracene to dihydrodiols by a bacterium. Science 189:295-297.

Harder, W. and L. Dijkhuizen. 1982. Strategies of mixed substrate utilization in microorganisms. Phil. Trans. Roy. Soc. Lond. (B) 297:459-480.

Herbes, S.E., L.R. Schwall and G.A. Williams. 1977. Rate of microbial transformation of polycyclic aromatic hydrocarbons: a chromatographic quantification procedure. Appl. Environ. Microbiol. 34:244-246.

Herbes, S.E. and L.R. Schwall. 1978. Microbial transformation of polycyclic aromatic hydrocarbons in pristine and petroleum-contaminated sediments. Appl. Environ. Microbiol. 35:306-316.

Herbes, S.E. 1981. Rates of microbial transformation of polycyclic aromatic hydrocarbons in water and sediments in the vicinity of a coal-coking wastewater discharge. Appl. Environ. Microbiol. 41:20-28.

Hosler, P. 1963. Kinetic study of the salicylic acid fermentation. Biotech. Bioengr. 5:243-251.

Hulbert, M.H. and S. Krawiec. 1977. Cometabolism: A critique. J. Theor. Biol. 69:287-291.

Ishikura, T., H. Nishida, K. Tanno, N. Miyachi and A. Ozaki. 1968. Microbial production of salicylic acid from naphthalene. Agric. Biol. Chem. 32:12-20.

Jackim, E. and C. Lake. 1978. Polynuclear aromatic hydrocarbons in estuarine and nearshore environments. in Estuarine Interactions, Martin L. Wiley (ed.), Academic Press. pp.415-428.

Jerina, D.M., H. Selander, H. Yagi, M.C. Wells, J.F. Davey, V. Mahadevan and D.T. Gibson. 1976. Dihydrodiols from anthracene and phenanthrene. J. Am. Chem. Soc. 98:5988-5996.

Kester, D.R., I.W. Duedall, D.N. Connors and R.M. Pytkowicz. 1967. Preparation of artificial seawater. Limnol. Oceanogr. 12:176-179.

Kiyohara, H., K. Nagao and R. Nomi. 1976. Degradation of phenanthrene through o-phthalate by an Aeromonas sp. Agric. Biol. Chem. 40:1075-1082.

Kiyohara, H. and K. Nagao. 1978. The catabolism of phenanthrene and naphthalene by bacteria. J. Gen. Microbiol. 105:69-75.

Kiyohara, H., K. Nagao and K. Yano. 1982. Rapid screen for bacteria degrading water-insoluble, solid hydrocarbons on agar plates. Appl. Environ. Microbiol. 43:454-457.

Kiyohara, H., K. Nagao, K. Kouno and K. Yano. 1982. Phenanthrene-degrading phenotype of Alcaligenes faecalis AFK2. Appl. Environ. Microbiol. 43:458-461.

Klausmeier, R.E. and R.J. Strawinski. 1957. Microbial oxidation of naphthalene I. Factors concerning salicylate accumulation. J. Bacteriol. 73:461-464.

Lake, J.L., C. Norwood, C. Dimock and R. Bowen. 1979. Origins of polycyclic aromatic hydrocarbons in estuarine sediments. Geochim. Cosmochim. Acta. 43:1847-1854.

Lee, R.F., W.S. Gardner, J.W. Anderson, J.W. Blaylock and J. Barwell-Clarke. 1978. Fate of polycyclic aromatic hydrocarbons in controlled ecosystem enclosures. Environ. Sci. Tech. 12:832-838.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

Maccubbin, A.E., P. Black, L. Trzeciak and J.J. Black. 1985. Evidence for polynuclear aromatic hydrocarbons in the diet of bottom-feeding fish. Bull. Env. Contam. Toxicol. 34:876-882.

May, W.E., S.P. Wasik and D.H. Freeman. 1978. Determination of the aqueous solubility of polynuclear aromatic hydrocarbons by a coupled column liquid chromatographic technique. Anal. Chem. 50:175-179.

Murphy, J.F. and R.W. Stone. 1955. The bacterial dissimilation of naphthalene. Can. J. Microbiol. 1:579-588.

Olsen, C.R., N.H. Cutshall and I.L. Larsen. 1982. Pollutant-particle associations and dynamics in coastal marine environments: a review. Mar. Chem. 11:501-533.

Perry, J.J. 1979. Microbial cooxidations involving hydrocarbons. Microbiol. Rev. 43:59-72.

Phillips, D.H. 1983. Fifty years of benzo(a)pyrene. Nature 303:468-472.

Readman, J.W., R.F.C. Mantoura, M.M. Rhead and L. Brown. 1982. Aquatic distribution and heterotrophic degradation of polycyclic aromatic hydrocarbons (PAH) in the Tamar Estuary. Est. Coast. Shelf Sci. 14:369-389.

Reichardt, P.B., B.L. Chadwick, M.A. Cole, B.R. Robertson and D.K. Button. 1981. Kinetic study of the biodegradation of biphenyl and its monochlorinated analogues by a mixed marine microbial community. Environ. Sci. Tech. 15:75-79.

Rogoff, M.H. and I. Wender. 1957a. The microbiology of coal I. Bacterial oxidation of phenanthrene. J. Bacteriol. 73:264-268.

Rogoff, M.H. and I. Wender. 1957b. 3-Hydroxy-2-naphthoic acid as an intermediate in bacterial dissimilation of anthracene. J. Bacteriol 74:108-109.

Saltzmann, H.A. 1982. Biodegradation of aromatic hydrocarbons in marine sediments of three North Sea oil fields. Mar. Biol. 72:17-26.

Shamsuzzaman, K.M. and E.A. Barnsley. 1974. The regulation of naphthalene oxygenase in pseudomonads. J. Gen. Microbiol. 83:165-170.

Schnitzer, M. 1978. Humic substances: chemistry and reactions, p. 1-64. In M. Schnitzer and S.U. Khan (Ed.), Soil Organic Matter. Elsevier, Amsterdam.

Walker, N. and G.H. Wiltshire. 1953. The breakdown of naphthalene by a soil bacterium. J. Gen. Microbiol. 8:273-276.

Windsor, J.G. and R.A. Hites. 1979. Polycyclic aromatic hydrocarbons in Gulf of Maine sediments and Nova Scotia soils. Geochim. Cosmochim. Acta. 43:27-33.

Wodzinski, R.S. and M.J. Johnson. 1968. Yields of bacterial cells from hydrocarbons. Appl. Microbiol. 16:1886-1891.

CHAPTER THREE

CHAPTER THREE

ESTUARINE ECOLOGY OF PHENANTHRENE-DEGRADING BACTERIA

ABSTRACT

Phenanthrene degrading bacteria were ubiquitously distributed in the waters and sediments of the Great Bay Estuary, NH, as determined using a ^{14}C-phenanthrene mineralization assay. Similar levels of activity were observed in water samples collected in March and June when these were incubated at 18°C even though ambient water temperatures were 1-4°C and 10-22°C, respectively. This observation indicated the constant presence of a mesophilic phenanthrene-degrading bacterial population in the estuary. Highest biodegradation activities were associated with PAH-contaminated waters downstream from a dredging operation which introduced high concentrations of coal tar PAH (polycyclic aromatic hydrocarbons) into the Cocheco River. High activities also occurred in areas receiving PAH inputs from pleasure and commercial boating activities. Low correlations were obtained between biodegradation rates and concentrations of nutrients, particulates and organic matter. Somewhat better agreements between ^{14}C-phenanthrene mineralization potentials and plate counts on a phenanthrene/toluene agar (PTA) medium were observed. PTA counts showed highly significant correlations with dimethyltin (DMT)-resistant plate counts indicating that marinas, as sources of both PAH hydrocarbons and antifouling organotin compounds, profoundly influenced the microbiology of the estuary. Variability in the phenanthrene degradation potentials

exhibited by estuarine water and sediment samples appeared to be overwhelmingly due to the degree of previous exposure to PAH.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH) are a naturally occurring class of fused ring aromatic compounds whose primary pre-industrial route of entry into the environment was through oil seeps and forest fires (Blumer and Youngblood, 1975). As products of combustion and as components of fossil fuels, modern day inputs of PAH are numerous and plentiful (Neff, 1979). Estuaries, particularly those in close proximity to urban/industrial centers, are prime repositories for PAH (Lake et al, 1979; Olsen et al, 1982). PAH enter estuarine environments through atmospheric fallout of flyash particulates (Heit, 1985; Prahl et al, 1984), runoff from streets and highways (Herrmann, 1981; Hoffman et al, 1984), municipal and industrial wastewater discharges (Barrick, 1982), riverine transport (Prahl et al, 1984; Herrmann and Hubner, 1982), major oil spillages (Neff, 1979) and chronic low-level inputs associated with commercial and pleasure boating activities. Due to the multiplicity of inputs, each with its own PAH compositional signature, it is usually difficult to pinpoint the source of hydrocarbons in a particular environment. The problem is exacerbated by the variable volatility, solubility and biodegradability of the various compounds. Sediments, nevertheless, provide an historical record which shows a dramatic post-industrial (ca. 1850) increase in the environmental burden of PAH (Hites et al, 1977; Forstner and Muller, 1981).

Adsorption is a major process governing the fate of PAH in aquatic environments. High molecular weight PAH with four or more rings and octanol-water partition coefficients (K_{ow}s) above ~10^5 show strong

correlations with suspended particulates in estuarine systems (Readman et al, 1982). Concentrations of lower molecular weight PAH with two or three rings correlate poorly with particulate materials due to the higher solubility (lower K_{ow}) and volatility of these compounds. At equilibrium with the average suspended particulate load of coastal environments (10 to 20 mg L^{-1}, 6% carbon), compounds with K_{ow} values $\leq 10^5$ exist predominantly (80 to 90%) in the dissolved form (Pavlou and Dexter, 1979). As the particulate levels increase, such as at the sediment/water interface, adsorption becomes much more important. Here, however, higher concentrations of dissolved organic matter would compete with the organic coatings on particulates and help to solubilize PAH (Gjessing and Berglind, 1981; Wijayaratne and Means, 1984; Whitehouse, 1985).

Phenanthrene is a major component of the PAH assemblage found in sediments and in the various input sources (Neff, 1979; Barrick, 1982; Hoffman et al, 1984; Ogan et al, 1979). It is an excellent model compound for the study of PAH biogeochemistry due to its intermediate solubility (1.0 $\mu g\ ml^{-1}$, May et al, 1978), volatility (vapor pressure, 6.8×10^{-4} mm Hg, Readman et al, 1982) and association with particulate phases ($K_{ow} = 3.7 \times 10^4$, Karickhoff et al, 1979). Phenanthrene is also intermediate in its biodegradability, being less easily degraded than naphthalene but more easily degraded than anthracene and larger ring compounds (Fedorak and Westlake, 1981). In addition, phenanthrene is the smallest of the angular PAH possessing the so-called bay region which is characteristic of the carcinogenic members of this class of hydrocarbons (Phillips, 1983). Phenanthrene is non-carcinogenic (Phillips, 1983) and relatively non-toxic (Calder and Lader, 1976;

Husseini and Stretton, 1981). Despite these features, phenanthrene is little studied in comparison to some other PAH including naphthalene, anthracene, benzanthracene and benzo(a)pyrene.

Bacteria are the principal agents of PAH degradation in nature (Alexander, 1981) although fungi (Cerniglia, 1981; Fedorak et al, 1984), microalgae (Lindquist and Warshawsky, 1985), and higher organisms (Stegeman, 1981) bring about limited transformations of these compounds. Two and three ring PAH are completely mineralizable whereas four ring and larger compounds are not suitable as sole carbon and energy sources for bacteria (Cernglia, 1984). The mineralization of PAH by some, if not all organisms, is dependent on the possession of degradative plasmids some of which (*NAH, SAL*) have been characterized (Chakrabarty, 1976).

As with the catabolism of any organic substrate, the availability of nutrients, the temperature and the pH also affect the mineralization of PAH by bacteria. Unlike some other substrates, however, oxygen is an absolute requirement for PAH dissimilation because the diatomic O_2 molecule is incorporated directly into the substrate as a prerequisite to ring cleavage (Dagley, 1978). The oxidation-reduction (redox) states of natural environments are thus critical in determining the persistence of PAH (DeLaune et al, 1980, 1981; Gardner et al, 1979; Bauer and Capone, 1985; Hughes and McKenzie, 1975). The availability of alternate carbon sources may also be important in supporting the cometabolism of PAH (Shiaris and Cooney, 1983; Dalton and Stirling, 1983; Rubin and Alexander, 1983; Shimp and Pfaender, 1985a); alternatively, these sources may act to prevent induction of PAH degrading enzymes (Ward and Brock, 1975; Martens, 1982; Shimp and

Pfaender, 1985b).

Compound related factors such as PAH concentration and pre-exposure to the compounds determine the degradative activities of microbial populations in nature (Wyndham and Costerton, 1981). These factors act by selection for species with degradative abilities, induction of enzymes required for degradation, and proliferation of extrachromosomal elements encoding degradative enzymes (Spain and Van Veld, 1983). Though PAH are ubiquitous in trace amounts, their concentrations may be below levels necessary to supply the maintenance energy requirements of active biodegradative populations (Boethling and Alexander, 1979; Alexander, 1981; Rubin and Alexander, 1983). However, PAH degrading bacteria are also ubiquitous (Mulkins-Phillips and Stewart, 1974b; Sisler and ZoBell, 1947) and through enrichment and adaptation can become active. Thus bacterial populations from PAH contaminated sites exhibit more rapid turnover of PAH than their counterparts from pristine environments (Herbes, 1981; Herbes and Schwall, 1978; Lee et al, 1978; Mulkins-Phillips and Stewart, 1974a; Saltzmann, 1982; Walker and Colwell; 1975; Cooney et al, 1985; Sherrill and Saylor, 1980; Bauer and Capone, 1985b).

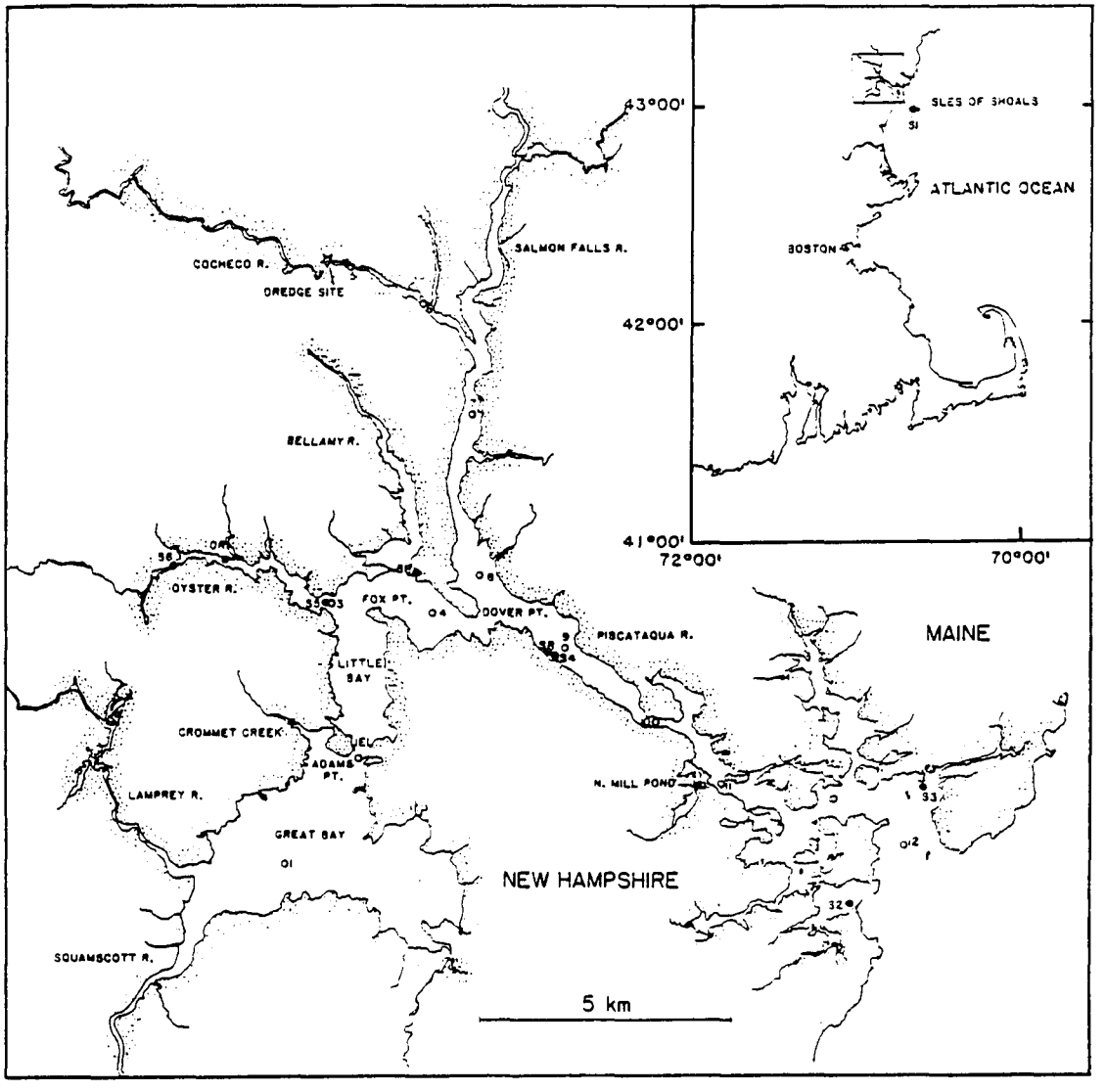
In this paper, I examined the activity and distribution of phenanthrene-degrading bacteria in the waters and sediments of a north temperate estuary. Distributions were then related to the physical, chemical and biological characteristics of the environment.

MATERIALS AND METHODS

Study Area

The Great Bay estuarine ecosystem (Fig. 18) is one of the largest estuaries on the eastern seaboard of the United States (Mathieson and Hehre, 1986), with a watershed of over 900 square miles and a tidal area of 11000 acres. Semidiurnal tides with an amplitude of 2.5 m create strong currents of up to 6 knots through the narrows adjacent to Dover, Fox and Adams Points. Most of the tidal prism flowing up the Piscataqua River is channeled past Dover Pt. and serves to flush Little and Great Bays (collectively, the Bay). Seawater also intrudes up the Piscataqua River and beyond the confluence of the Salmon Falls and Cocheco Rivers but this flow is a minor fraction of the total tidal flow. Seven rivers empty into the system with a combined mean flow of 1370 cfs of which 1.9% is sewage. This flow accounts for less than 2% of the total water volume exchanged during each tidal cycle. The residence time of water in the Bay has been estimated at 20 to 30 d (Loder et al, 1983). Most of the rivers feeding the estuary are dammed to produce discontinuous salinity gradients. The main stem of the Piscataqua River (from Dover Pt. to the sea) is heavily industrialized with oil refineries, boatyards, marinas, a coal-fired power plant, and several municipalities including the city of Portsmouth, NH along its banks. Minor industries and sewage treatment facilities in the smaller towns and cities surrounding the estuary also impact on the overall water quality of the system.

FIGURE 18. Map of the Great Bay Estuary, New Hampshire-Maine, showing locations of sediment samples collected 8-31-84 (JEL, OR, BB, and SR) and 10-17-84 (S1-S6) and water samples (1-12) collected in March and June, 1985. The location of a dredging operation in the Cocheco River and of sediments used in PAH analyses (stars) are also indicated.


43°00'
42°00'
41°00'
72°00'
70°00'
ATLANTIC OCEAN
BOSTON
SLES OF SHOALS
COCHECO R.
DREDGE SITE
SALMON FALLS R.
BELLAMY R.
OYSTER R.
FOX PT.
DOVER PT.
PISCATAQUA R.
MAINE
LITTLE BAY
CROMMET CREEK
ADAMS PT.
LAMPREY R.
GREAT BAY
N. MILL POND
NEW HAMPSHIRE
SQUAMSCOTT R.
5 km

Sample Collection

Water and sediment samples were collected from locations throughout the estuary as shown in Fig. 18. Intertidal sediment samples were collected 8-31-84 from the JEL (Jackson Estuarine Laboratory, UNH), OR (Oyster River), BB (Bellamy River Bridge) and SR (Sprague Refinery) sites by aseptically scooping the top 0.5 cm into sterile beakers. Samples were returned to the laboratory on ice and processed within 3 h of collection. Sediment samples S1 through S6 were collected 10-17-84 from aboard the R/V Jere Chase using a Shipek grab sampler. Oxidized surface sediment (top 1 cm) out of contact with the sampler was transferred to sterile bottles and kept on ice until returning to the laboratory within 12 h.

Surveys of the estuary were performed in March and June, 1985. Surface water samples from 12 stations (Fig. 18) were collected in a stainless steel bucket from the bow of the R/V Jere A. Chase to avoid contamination. Subsamples for microbiological analyses were taken aseptically, additional subsamples were taken for chemical analysis, and the bucket was wiped thoroughly with 100% ethanol between samples.

Bacterial Enumerations

Immediately after collection, water samples from stations 1 through 12 were serially diluted in 9 ml blanks of 75% artificial seawater (ASW, Kester et al, 1967) and several dilutions were spread plated in quadruplicate onto estuarine nutrient agar (ENA) and phenanthrene/toluene agar (PTA). Upon return to the laboratory, plates were placed in a dark 18°C incubator for 2 (ENA) or 3 (PTA) weeks before counting.

Water samples were also plated onto estuarine nutrient agar (ENA)

which had been supplemented with dimethyl tin (15 mg L^{-1} $(CH_3)_2SnCl_2$ as Sn). Plates were incubated for 2 weeks at 18°C in the dark and counted. Counts of dimethyl tin (DMT)-resistant bacteria were compared to counts on unsupplemented ENA.

Numbers of bacteria in sediment samples S1 through S6 were also enumerated on PTA after dilution with 75% ASW. Dilution and plating of these samples was carried out upon retun to the laboratory, as much as 12 h after collection.

The seasonal distribution of phenanthrene-degrading bacteria in the water and sediments at the JEL, OR, BB and SR sites was the topic of a Master's thesis by J. Sanseverino (UNH, Dept. of Microbiology, 1984). Phenanthrene degrading bacteria in these samples were enumerated on a 0.01% phenanthrene agar by counting the clearing zones in a crystalline phenanthrene overlay sprayed on the plates after inoculation. While not directly comparable to the estimates made on the other samples, they did allow comparisons between the four sediments to be made.

Phenanthrene Biodegradation Potentials

One cm^3 portions of well-mixed sediment samples were taken with sterile syringe corers and diluted ten fold in 75% ASW. One ml aliquots of the dilutions were used to inoculate 100 ml of a 0.5 mg L^{-1} solution of phenanthrene in 75% ASW which also contained (per L); NH_4NO_3 (1.5 mg), 0.05M phosphate buffer (pH 7.5, 1 ml), $FeCl_3 \cdot 6H_2O$ (0.2 mg) and Tween 80 (0.85 mg) to minimize adsorption and volatilization of the phenanthrene. The pH of the medium was adjusted to 7.5 before sterilization by filtration (0.2 µm Nuclepore). Flasks were spiked with

5 µl of a [9-^{14}C] phenanthrene (Amersham-Searle, 19.3 mCi mmole^{-1}, 98% radiochemical purity by HPLC and TLC) in acetone solution and total initial activity (~10,000 dpm ml^{-1}) was assessed by counting duplicate 1 ml aliquots of the medium. Incubations were carried out in stoppered 250 ml Erlenmeyer flasks fitted with 8 mm sampling ports for periodic withdrawal of water and headspace for measurement of $^{14}CO_2$ (Fedorak et al, 1982).

Phenanthrene biodegradation potentials of undiluted water samples were determined by aseptically transferring 200 ml aliquots of water samples to 500 ml Erlenmeyer flasks fitted with 8 mm sampling ports. Samples were spiked with 10 µl of an acetone solution of [9-^{14}C] phenanthrene which had been diluted with unlabeled phenanthrene to give a total assay concentration of 1.09 mg L^{-1}. Total initial activites in the flasks were about 10,000 dpm ml^{-1} as determined by counting duplicate 1 ml aliquots immediately after spiking. Mineralization of phenanthrene was followed in the same manner as for sediment samples. Details of the procedure have been previously described (Guerin and Jones, submitted).

Effects of Nutrients (N and P) and Alternate Carbon Sources on Phenanthrene Biodegradation

During the June survey, eight 100 ml aliquots of water from station 6 were transferred to 250 ml Erlenmeyer flasks. Flasks then received 1 ml of a solution containing 200 ug C as acetate, succinate, pyruvate, glucose, Tween 80 or humic acid (Aldrich). One flask received 0.5 ml of 0.05 M phosphate buffer (pH 7.5) plus 0.5 ml NH_4NO_3 (1 mg ml^{-1} distilled water) and one flask received 1 ml sterile, distilled water as a control. All flasks received 5 µl of a ^{14}C-labeled

phenanthrene solution (total assay concentration of 1.09 mg L^{-1}) to result in a phenanthrene-C:alternate-C ratio of 1:2. Total initial activity was immediately determined and flasks were incubated and $^{14}CO_2$ production was monitored as described above.

Chemical Analyses

Water samples were filtered (0.4 μm Nuclepore) on board ship and aliquots of the filtrate were immediately frozen for later dissolved organic carbon (DOC) and nutrient analyses. Samples for DOC were stored in precombusted (475°C, 6 h) screw-cap test tubes at -20°C for up to 9 mos before analysis on a Barnsted Photochem DOC analyzer. Nutrients (NH_4^+, NO_3^- + NO_2^-, PO_4^{3-}) in duplicate samples were analyzed on a Technicon Autoanalyzer according to the procedures of Glibert and Loder (1977).

Additional unfiltered water samples were transferred to 1 L polyethylene bottles and stored on ice until their return to the laboratory. There, samples were filtered through precombusted, preweighed Whatman GF/C glass fiber filters (effective retention 1.2 μm). Filters were dried (90°C overnight), reweighed, combusted (475°C, 6 h) and weighed again to give suspended particulate matter (SPM) and particulate organic matter (POM) concentration estimates. Salinities of the filtrates were determined on a Guildline Autosal Salinometer Model 8400. The absorbance of filtrates at 250 nm was measured on a Beckman DU-8 spectrophotometer in 1 cm quartz cuvets.

The concentration and speciation of tin compounds (inorganic, mono-, di-, tri- and tetramethyl and mono- and tributyl tins) was also determined on March and June water samples and will be presented

elsewhere (Donard and Guerin, in preparation.).

Organic matter content of all sediments was determined as the weight loss of dried (90°C, overnight) sediments after combustion at 475°C for 6 h. In addition, sediments S1 through S6 were analyzed in duplicate for organic C and N on a Carlo Erba Elemental Analyzer Model 1106. The specific surface areas of all sediments were determined by the cetyl pyridinium bromide (CPB) adsorption technique described by Mayer and Rossi (1981) and by the BET (nitrogen adsorption) method using a Quantachrome Corp. Monosorb instrument.

Polycyclic aromatic hydrocarbon analysis of sediments was carried out using the extraction and cleanup procedure of Giger and Schaffner (1978) with slight modifications. Analysis was by capillary gas chromatography on a Shimadzu GC-9A instrument with FID detector and a programmable integrating recorder. Compounds were identified by matching sample peak retention times with those of authentic standards. While coupled gas chromatography-mass spectrometry would allow more definitive identifications, such an instrument was not available to us. Confidence in our identifications is strengthened, however, by the remarkable similarity of our chromatograms to those of others (eg., Giger and Schaffner, 1978; Prahl and Carpenter, 1983) who employed this method. The data presented here are part of a more complete study of the distribution of PAH in the Great Bay Estuary and correlations of concentrations with grain size and organic content parameters (Guerin and Gauthier, in prep.).

RESULTS AND DISCUSSION

Phenanthrene Degradation in Sediment Samples

Table 8 lists the physical and chemical characteristics of the sediments employed in this study. Sediments in the Great Bay Estuary range from muds in the salt marshes, tidal flats and sheltered coves to sands in the main tidal channels. This is reflected in the surface area data of Table 8 which shows values ranging from 6 to 7 $m^2\ g^{-1}$ in the Piscataqua River (SR and S4) to 17 $m^2\ g^{-1}$ at S2 (CPB data). The surface area data for S1 were variable using the CPB and BET methods due to the calcareous nature of this sediment; otherwise, agreement between the two measures was good (r^2 = 0.966 for all Great Bay samples except S1, n = 20). Sediments showed a surface area-dependent variability in organic matter content (r^2 = 0.863, n = 14, using CPB data) typical of estuarine and marine sediments (Mayer et al, 1985). The C:N ratio in sediments S2 through S6 increased monotonically with distance upstream reflecting the predominance of terrestrially-derived organic materials in the rivers.

Phenanthrene (and other PAH) concentrations in sediments throughout the estuary were unrelated to surface area or organic matter content; rather, concentrations were dependent on proximity to point sources (Guerin and Gauthier, in prep.). High concentrations at S4 were due to inputs of petroleum products from the adjacent Sprague Oil Refinery. The sediments here contained sand sized agglomerates of mineral particles embedded in a tar-like residue. High concentrations in sample S2 were most likely derived from the nearby marina on the

TABLE 8. Characteristics of the Great Bay sediments used in this study.

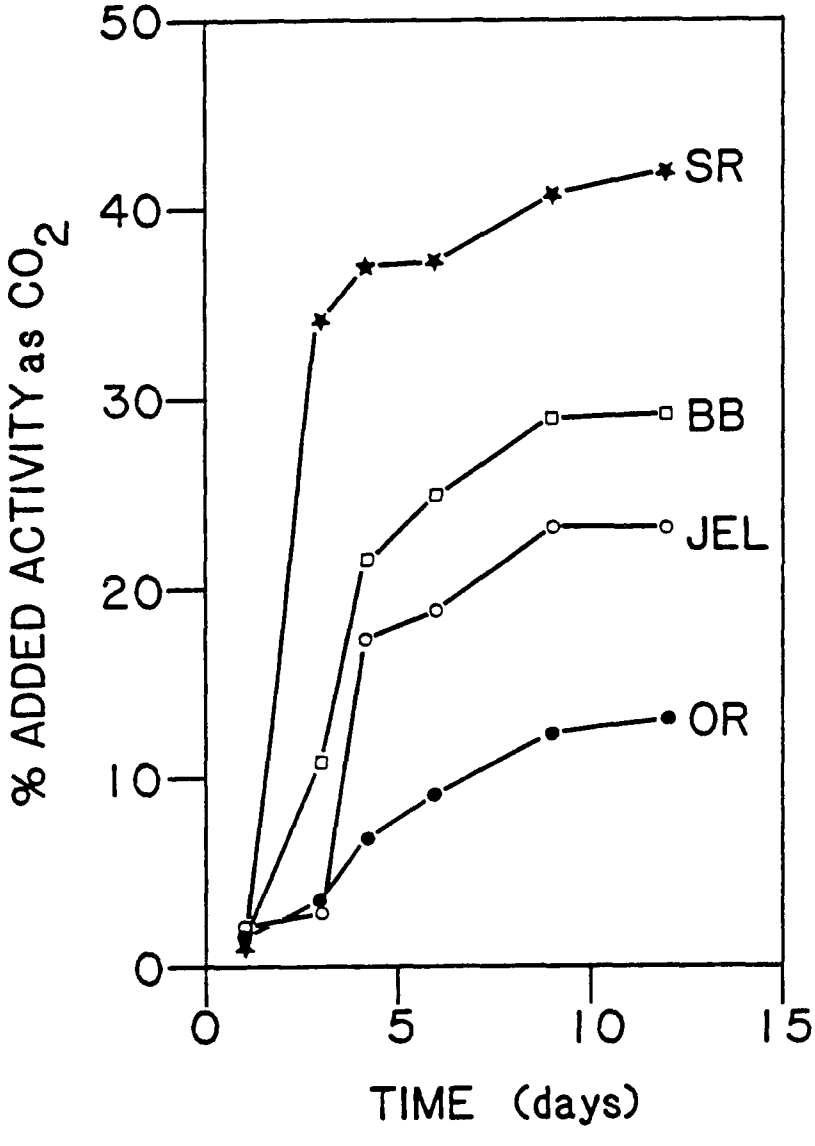
Sample	Weight Loss (%)	CPB Area (m^2/g)	BET Area (m^2/g)	% C	% N	C:N	Phenanthrene (ng/g dry weight)
JEL	1.1	5.6	n.d.[a]	n.d.	n.d.	n.d.	290[b]
OR	5.9	12.5	9.5	n.d.	n.d.	n.d.	c
BB	4.6	9.6	7.2	n.d.	n.d.	n.d.	n.d.
SR	2.8	6.6	2.7	n.d.	n.d.	n.d.	d
S1	2.6	36.4	3.3	n.d.	n.d.	n.d.	146
S2	10.6	16.6	12.1	3.26	0.44	7.41	443
S3	7.3	14.5	9.7	2.31	0.28	8.25	167
S4	3.1	7.0	4.4	0.86	0.09	9.56	691
S5	7.4	13.2	13.1	2.30	0.23	10.00	179
S6	8.6	13.8	11.5	2.75	0.24	11.46	337

a not determined
b determined on sample collected at a later date
c compare S5 value
d compare S4 value

sheltered side of New Castle Island. Appreciable PAH concentrations in the offshore S1 sample were perhaps due to the deposition of fine-grained materials and associated hydrocarbons eroded from the estuary by the strong tidal currents (Young et al, 1985). The high CPB surface area measurement supports this hypothesis and agrees with the findings of Boehm and Farrington (1984).

Figure 19 shows the phenanthrene mineralization curves for four sediments. Among the JEL, OR, BB and SR sediments examined by Sanseverino (1984), phenanthrene degrading bacteria were detected only in the OR sample (5.0×10^2 ml^{-1}), the one with the highest surface area and organic matter content. This sample, however, was the least active in terms of ^{14}C-phenanthrene mineralization activity, with a 12 d mineralization efficiency (^{14}C-phenanthrene to $^{14}CO_2$) of 13%. Sediment from the PAH-contaminated SR site brought about rapid and extensive mineralization of phenanthrene (42% efficiency at 12 d) despite its low surface area, organic matter content and absence of culturable phenanthrene degraders. This sample and the JEL sediment both showed distinctive two-stage mineralization curves characteristic of mixed culture degradations in which accumulation of the intermediate, 1-hydroxy-2-naphthoic acid (1H2NA) occurs. The BB sample also showed active phenanthrene mineralization. Although phenanthrene concentration data for this location were unavailable, it appeared that the level of prior exposure to phenanthrene (i.e., concentration) determined the potential of the sediment populations to degrade the hydrocarbon in the assays. Accordingly, the phenanthrene biodegradation potential was highest in the sample (SR) collected from the industrialized part of the Piscataqua R. The microbiological data of

FIGURE 19. Time courses of $^{14}CO_2$ evolution from ^{14}C-phenanthrene by sediment samples collected 8-31-84.

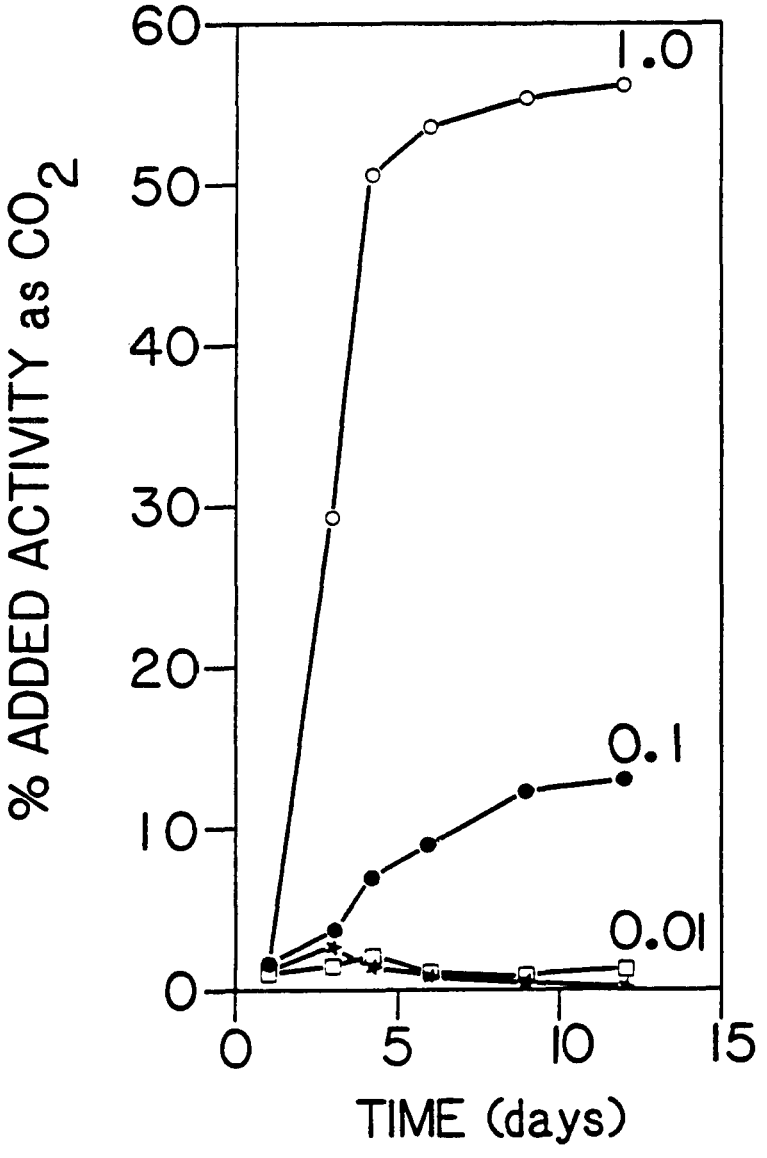

50
40
30
20
10
0
% ADDED ACTIVITY as CO_2
SR
BB
JEL
OR
0
5
10
15
TIME (days)

Sanseverino (1984) correlated poorly with the mineralization results obtained.

Mineralization of ^{14}C-phenanthrene in the assays was dependent on the size of the inoculum. Figure 20 shows the $^{14}CO_2$ evolution curves when 1.0, 0.1 or 0.01 cm^3 of OR sediment was used as inoculum in mineralization assays. The heavily-inoculated flask showed essentially no lag period before the complete utilization of the added phenanthrene (50-60% CO_2, 40-50% biomass-C typical for growth substrates (Fewson, 1985) and for pure cultures growing on phenanthrene (Guerin, unpublished data)). Almost no mineralization was observed in the flask receiving 0.01 cm^3 OR sediment as inoculum. The fortuitous choice of a 0.1 cm^3 inoculum in sediment assays (Fig. 19) provided an intermediate level of activity which allowed comparisons among samples to be made.

The variable activity observed with different sized inocula has been observed in other systems (Paris et al, 1981; Kool, 1984) and highlights the desirability of normalizing biodegradation results to some biological parameter. For expression of the heterotrophic activities of aquatic microbial communites, the use of specific (per cell, determined by epifluorescence direct count) activities has been suggested (Wright, 1978). Such an expression is useful when the substrate of interest, eg., glucose, is utilizable by the majority of organisms comprising a microbial community. However, it is difficult to accurately enumerate the small proportion of a sediment population which can degrade phenanthrene (Ward and Brock, 1976). Inasmuch as specific surface area ultimately governs the organic matter content and microbial numbers in sediments, it is sufficient to recognize that biodegradation activity was higher in the less populated (Sanseverino,

FIGURE 20. Time courses of $^{14}CO_2$ evolution from ^{14}C-phenanthrene using different amounts (cm^3) of OR sediment as inoculum. Apparent $^{14}CO_2$ activity resulting from volatilization and trapping of ^{14}C-phenanthrene in an uninoculated control flask is also shown (stars).


60
50
40
30
20
10
0
1.0
0.1
0.01
0
5
10
15
% ADDED ACTIVITY as CO_2
TIME (days)

unpublished), low specific surface area (SR) sample than the more populated, high specific surface area (OR) sample. This further emphasizes the importance of prior exposure as a determinant of the phenanthrene degrading activity of sediments.

^{14}C-Phenanthrene mineralization potentials decreased landward in sediment samples S1 through S6 (Fig. 21). Numbers of culturable heterotrophs on PTA showed a similar decrease up estuary with the exception of a low count in the offshore sample, S1 (Table 9). Nutrients were added (arrow "a", Fig. 21) and samples were switched from a fed batch to a batch culture mode (arrow "b", Fig. 21) before appreciable mineralization was observed in samples S3 through S6. The low mineralization efficiencies at incubation times as long as 100 d in these samples contrasted with results obtained with intertidal sediments collected 2 mos prior (JEL, OR, BB and SR sediments, above). This difference may have been due to seasonal effects or the development of anaerobiosis in the samples between the time of collection and sample processing. An alternate explanation relates to the organic matter of the sediments examined. Increasing C:N ratios with distance up estuary (Table 8) reflected the increasing contribution by carbon-rich terrigenous organic material. Therefore, although nitrogen was added to the assay medium, the lower C:N ratio of the organic matter from the more marine sediments may have been a better supplement in supporting phenanthrene degradation. In addition, the more aromatic character of the terrigenous organic material associated with the non-marine sediments may have more effectively sequestered the added phenanthrene (Whitehouse, 1985), making it less available to the microorganisms.

FIGURE 21. Time courses of $^{14}CO_2$ evolution from ^{14}C-phenanthrene for the six sediment samples collected 10-17-84. Arrows indicate times of nutrient addition ("a", 10 mg yeast extract plus 10 mg glucose) and conversion from fed batch to batch culture ("b"). Note break in time scale on x axis.

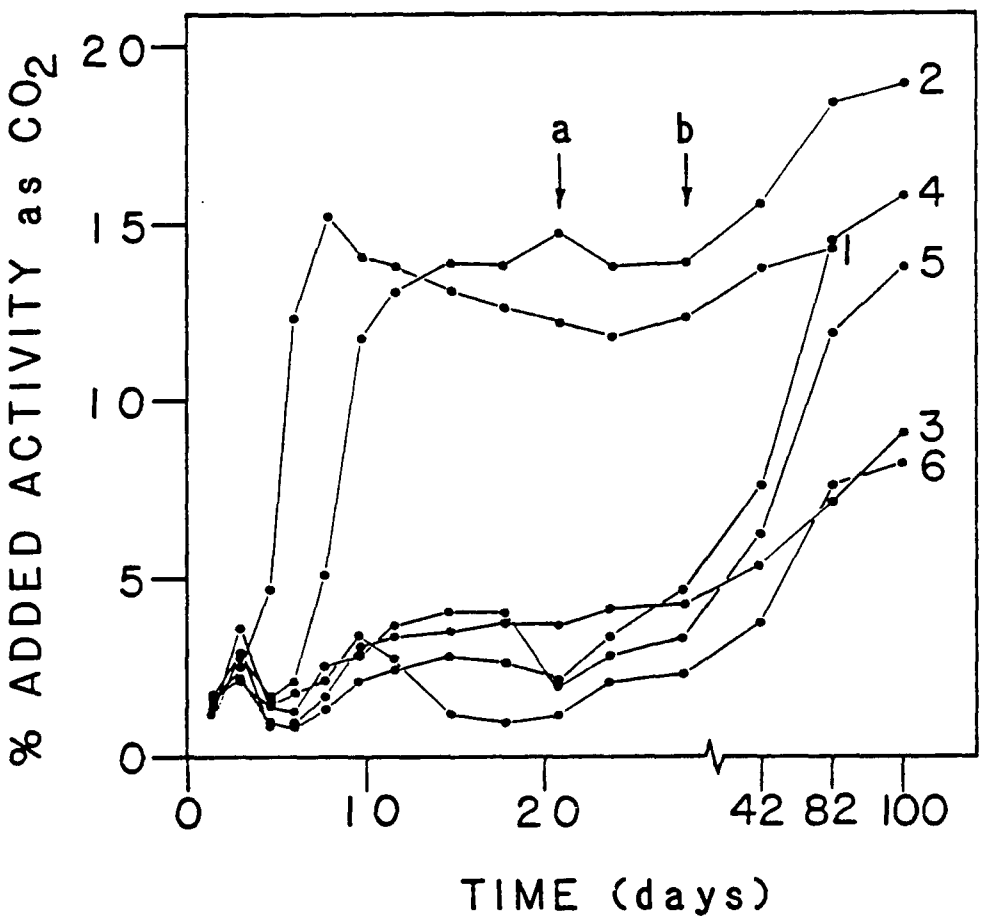

% ADDED ACTIVITY as CO_2
20
15
10
5
0
a
b
2
4
1
5
3
6
0
10
20
42
82
100
TIME (days)

TABLE 9. PTA plate count data for sediment samples S1 through S6.

Sample	Log (PTA cfu per ml)
S1	6.43 (±0.72)*
S2	7.54 (±0.25)
S3	7.43 (±0.04)
S4	7.27 (±0.41)
S5	7.00 (±0.17)
S6	6.95 (±0.49)

* Numbers in parentheses represent ± 1 s.d. about the mean of quadruplicate counts.

Phenanthrene Degradation in Water Samples

The chemical characteristics of the water at the 12 sampling stations in March and June are shown in Table 10. The March sampling trip was preceded by rains and a spring thaw. Zero salinities at stations 5, 6 and 7 and relatively low salinities (16 to 22‰) at the Bay stations (1 through 4) were evidence of the high runoff at this time. High SPM and POM were also found. Low flow conditions during the June sampling trip were reflected in the relatively high and constant salinities observed in the Bay. Seawater intrusion up the Cocheco River resulted in a salinity of 3.5‰ at station 5. This station was several hundred m downstream of a primary sewage effluent outfall in the city of Dover, NH as indicated by the high POM, DOC and nutrient concentrations observed here and further downstream at station 6. A secondary sewage treatment plant in the town of Durham, NH on the Oyster River (near S6, Fig. 18) resulted in elevated concentrations of these components at station 3 as well. The impact of the Durham plant on the water chemistry at station 3 was more pronounced in March than in June when University classes were no longer in session. The greater discharge rate of the Cocheco River diluted the Dover sewage effluent in March; the impact of this plant on the water chemistry at stations 5, 6 and perhaps 7, was more pronounced in June.

While sampling in the Cocheco River (stations 5 and 6) in March, patches of oily surface material were observed floating downstream but the source of the material was not determined at the time. Several days later it was learned that a dredging operation was being conducted upstream in the city of Dover, NH and that an extensive bed of hydrocarbon-laden sediments had been disturbed. The hydrocarbons

TABLE 10. Chemical characteristics of water samples collected in March and June (in parentheses) from the Great Bay Estuary. All nutrient concentrations expressed in units of μmoles/L.

Station	Temp (°C)	Salinity (‰)	$NO_3^-+NO_2^-$	NH_4^+	PO_4^{-3}	DOC (mg/L)	SPM (mg/L)	POM (mg/L)
1	2(18)	16.8(29.9)	8.08(2.52)	4.43(3.17)	0.84(0.74)	2.9(2.0)	20.2(12.7)	2.1(2.1)
2	4(19)	20.3(29.7)	6.36(1.56)	2.97(1.82)	0.67(0.59)	2.8(2.4)	23.0(13.6)	2.3(2.4)
3	4(18)	16.6(29.9)	11.57(1.48)	8.05(2.28)	1.10(0.62)	3.5(2.6)	23.8(12.9)	2.2(2.0)
4	4(18)	21.8(30.0)	6.93(2.31)	2.93(3.19)	0.67(0.75)	1.8(---)	21.1(11.5)	2.2(1.9)
5	1(22)	0 (3.5)	11.46(32.1)	10.68(75.0)	0.98(6.37)	5.6(7.2)	126.4(11.6)	12.3(6.5)
6	1(22)	0 (13.1)	11.65(19.3)	9.80(58.2)	1.24(4.90)	7.0(5.1)	100.8(15.9)	13.7(4.7)
7	2(22)	0 (20.3)	10.03(9.18)	6.27(12.6)	0.83(1.25)	6.4(4.0)	49.4(12.1)	5.8(2.5)
8	3(19)	10.9(27.8)	9.13(4.88)	5.77(6.46)	0.75(0.99)	4.0(---)	27.8(9.4)	2.6(1.6)
9	4(18)	23.4(30.3)	6.76(2.62)	3.70(3.55)	0.69(0.84)	1.3(3.7)	15.8(9.8)	1.5(1.5)
10	4(15)	28.6(30.9)	5.48(3.78)	2.66(3.08)	0.77(0.75)	1.2(---)	16.6(9.8)	1.4(1.7)
11	4(14)	29.9(31.2)	5.25(1.96)	1.96(2.84)	0.78(0.70)	1.2(2.6)	15.8(9.2)	1.4(1.9)
12	4(10)	32.5(32.1)	5.07(0.59)	1.53(1.13)	0.83(0.40)	1.0(2.4)	13.5(7.4)	1.1(1.8)

released from the dredge site formed an oily slick covering hundreds of m^2 and this material was being broken up and transported downstream. Water samples were not analyzed directly for PAH content. However, the presence of dissolved aromatic hydrocarbons in the water at station 5 was indicated by its high absorbance (250 nm) to DOC ratio (Fig. 22).

Samples of the oily dredge material and sediments from downstream sites were collected and analyzed for PAH content. The PAH chromatograms of both materials were similar and, based on their high fluorene content and other characteristics (Lao et al, 1975) were concluded to be derived from coal tar wastes from a 19th century gas plant which occupied the river bank near the dredge site. The gas chromatograms for a Cocheco River sediment PAH extract is shown in Fig. 23 along with chromatograms for a relatively polluted sediment collected below a storm water drain in the city of Portsmouth, NH (N. Mill Pond) and a pristine sediment collected in a salt marsh near Adams Point (Crommet Creek). The Cocheco River extract, diluted ten-fold relative to the others, was rich in 2, 3, 4 and 5 ring PAH with concentrations of phenanthrene and the potent carcinogen, benzo(a)pyrene of 35.5 and 27.1 $\mu g\ g^{-1}$ (dry weight), respectively. These are among the highest PAH concentrations ever reported. The dredging operation was completed at the end of March, 1985 but hydrocarbons continued to be released from the sediments and from the river banks where the dredge was brought ashore for several months. During the June sampling trip, the smell of aromatic hydrocarbons (reminiscent of mothballs) could be detected more than 1 km downstream of the dredge. The input of hydrocarbons from the dredge was undoubtedly responsible for the high phenanthrene biodegradation rates

FIGURE 22. Linear regression lines between absorbance (250 nm) of March (open symbols) and June (closed symbols) water samples and dissolved organic carbon (DOC) contents showing the high absorbance at station 5.

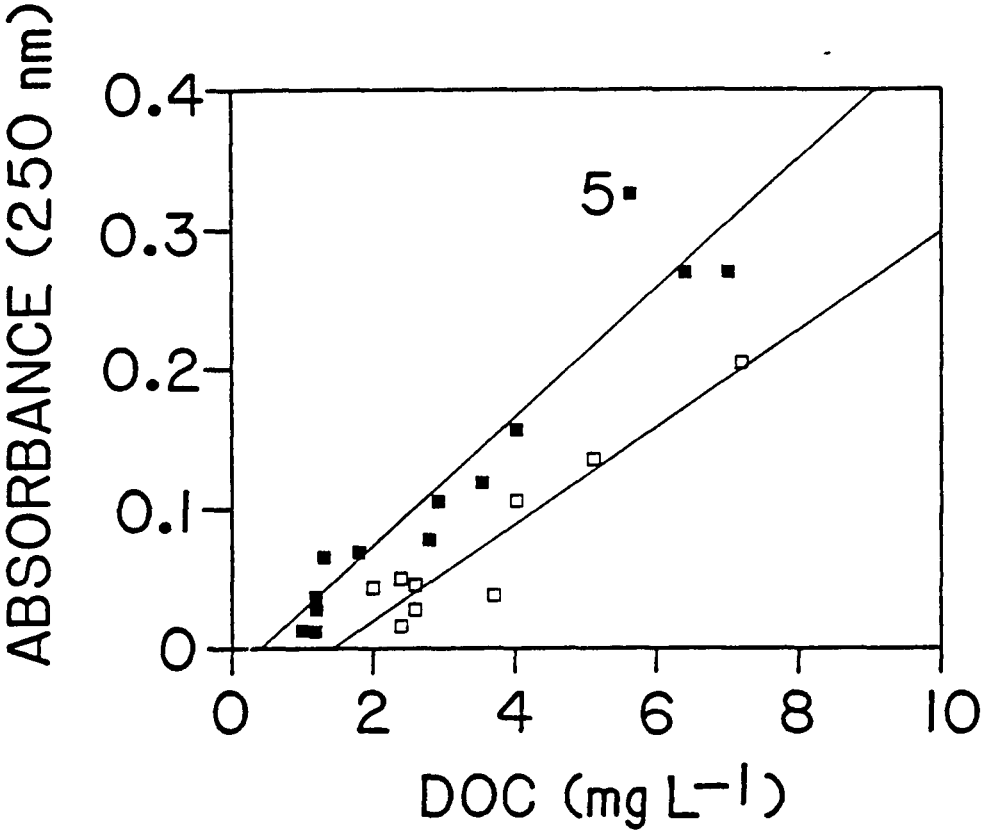
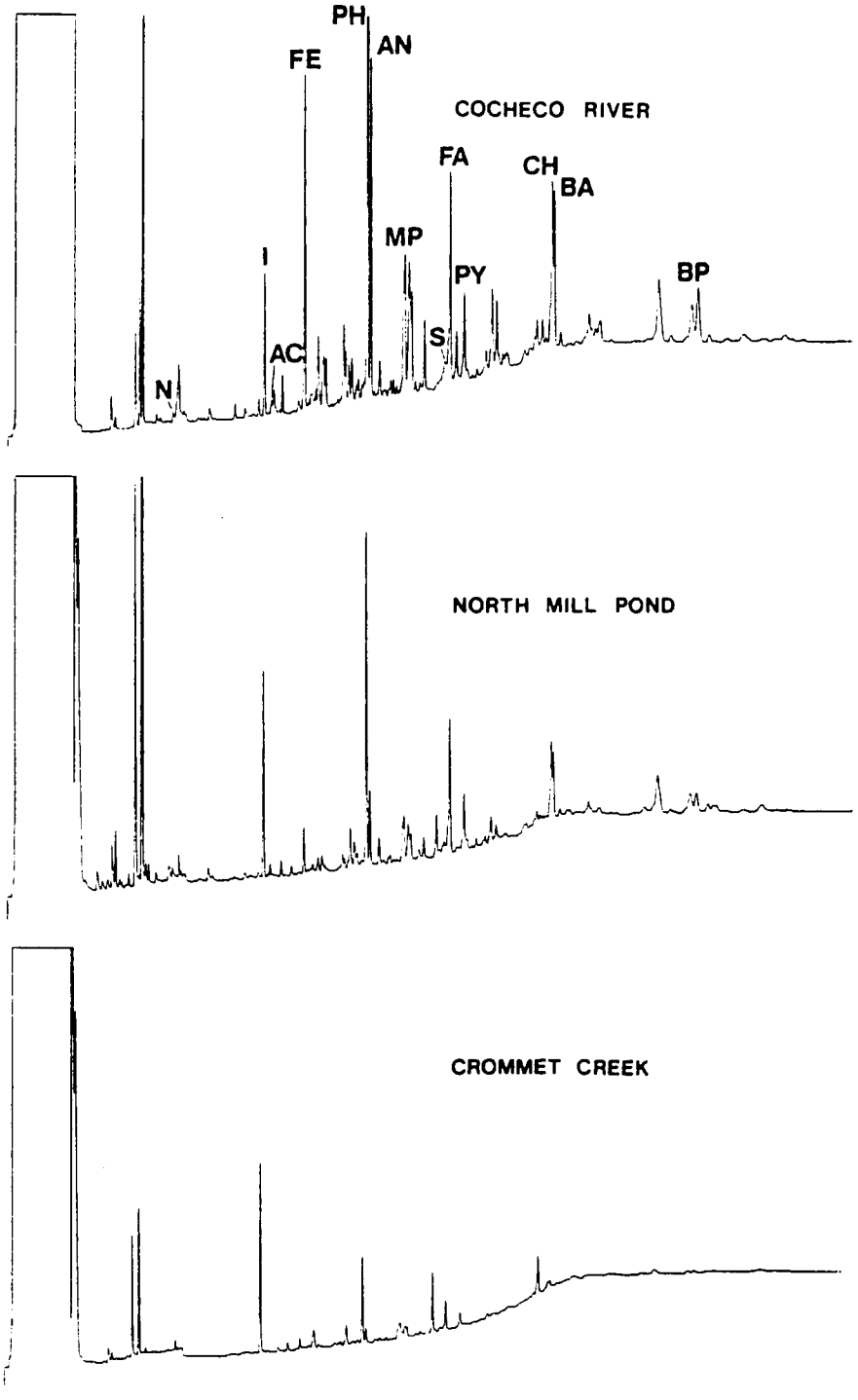

ABSORBANCE (250 nm)
0.4
0.3
0.2
0.1
0
5
0
2
4
6
8
10
DOC (mg L⁻¹)

FIGURE 23. Capillary gas chromatograms of sediment PAH extracts from the dredge-impacted Cocheco River, the relatively polluted N. Mill Pond (city of Portsmouth, NH) and the pristine Crommet Creek. Peak identities as follows: N, naphthalene; I, injection standard (hexamehylbenzene); AC, acenaphthene; FE, fluorene; PH, phenanthrene; AN, anthracene; MP, methylphenanthrenes; S, extraction standard (dimethylphenanthrene); FA, fluoranthene; PY, pyrene; CH, chrysene; BA, benzanthracene; BP, benzo(a)pyrene. Note that the Cocheco River extract was diluted ten fold relative to the other samples.

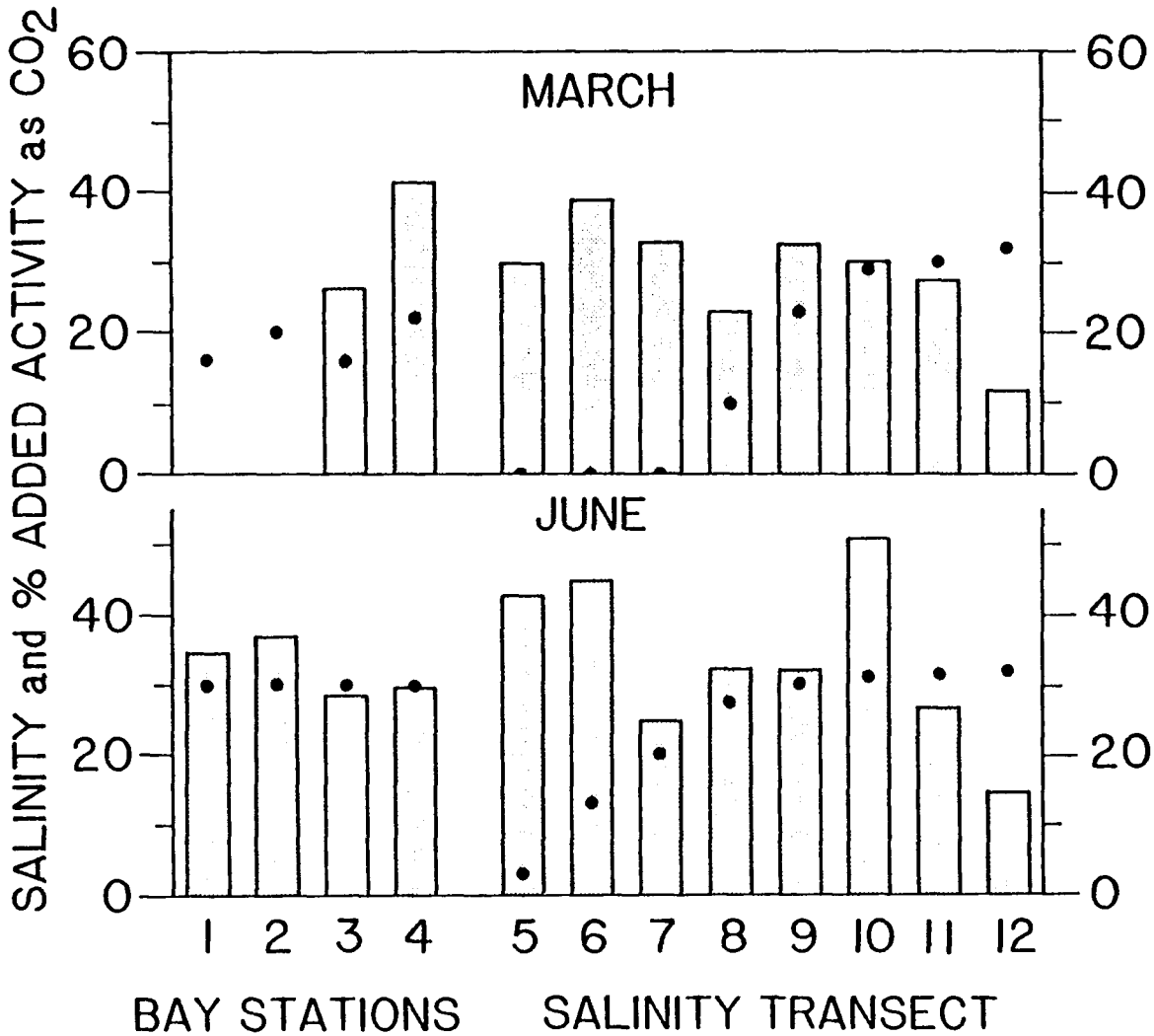

PH
AN
FE
COCHECO RIVER
FA
CH
BA
MP
I
PY
BP
S
AC
N
NORTH MILL POND
CROMMET CREEK

observed in the Cocheco River samples.

^{14}C-phenanthrene mineralization efficiences (^{14}C-phenanthrene to $^{14}CO_2$ after 500 h incubation) were high in the Cocheco River samples (stations 5 and 6) in March and June (Fig. 24). Mineralization occurred in these samples without an appreciable lag period and attained efficiencies of up to 43%. High runoff during the March sampling trip caused a downstream shift of activity such that extensive degradation was also observed in the station 7 sample. Low runoff in June served to confine activity to the Cocheco River samples. The salinity data in Fig. 24 reflect these hydrodynamic differences.

Because all incubations were conducted at 18°C, the influence of temperature on PAH degradation was not evaluated in this study; rather, the distribution of mesophilic phenanthrene degrading bacteria in the estuary was assessed. The data in Fig. 24 reveal that, in general, approximately equal levels of activity were observed in the March and June samples indicating the presence of a mesophilic phenanthrene-degrading population at both times of year. Similar results were obtained by Cooney et al.(1985) who found hydrocarbon degradation by lake water and sediment populations to remain fairly constant throughout the year when incubations were carried out at 27°C despite seasonal water temperature fluctuations from 0 to 27°C. Likewise, Sherrill and Saylor (1980) found fairly constant rates of phenanthrene degradation by lake water populations throughout the year when samples were incubated at 25°C. Hydrocarbonoclastic and other heterotrophic activities are temperature-limited <u>in situ</u> despite the constant presence of mesophilic biodegradative populations (Mulkins-Phillips and Stewart, 1974a; Bauer and Capone, 1985b; Ward and Brock, 1976).

FIGURE 24. ^{14}C-phenanthrene mineralization efficiencies (500 h conversion to $^{14}CO_2$) and salinities (solid dots) of the 12 water samples collected in March and June, 1985.


SALINITY and % ADDED ACTIVITY as CO_2
MARCH
JUNE
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12
BAY STATIONS
SALINITY TRANSECT

Stations 9, 10 and 11 were situated in the industrialized portion of the Piscataqua River. The relatively high activities observed at these stations was no doubt related to anthropogenic influences. However, since the tidal currents in this portion of the estuary are so strong, and the mixing so extensive, biodegradative activity was spatially homogeneous and showed little relationship with possible point sources of PAH. An exception was the extremely active sample from station 10 in June.

The differences between the March and June sample sets were due primarily to hydrodynamic conditions, and not seasonal effects. The high phenanthrene degradation potential at station 10 in June, however, was probably due to seasonal effects. In this section of the Piscataqua River, the operation of numerous seasonal marinas results in chronic low-level inputs of PAH in the warm summer months. Thus, pre-exposure to PAH allowed the station 10 sample to bring about extensive mineralization of phenanthrene without a lag period. The seasonal impact of the marinas was also supported by the elevated levels of butyl tin compounds found in the Piscataqua R. during June (Donard and Guerin, in prep.). Concentrations of NO_3^- + NO_2^- were slightly elevated at station 10 in June (Table 10).

In March, salinities in Great Bay and Little Bay (stations 1-4) varied from 16 to 22‰ with low values near the mouths of the Oyster and Lamprey Rivers. In June, salinities were uniform at about 30‰ at all four stations. Since the residence time of the water in the Bay is 20 to 30 d and the water column is chemically homogeneous, a microbial flora adapted to a fairly restricted range of physicochemical conditions would seem to be favored. It is perhaps significant then,

that the mid-estuarine maxima in phenanthrene degrading activity observed during both sampling trips along the salinity gradient between stations 5 and 12 occured at those stations whose salinities most closely matched those of the Bay (stations 1-4). In March, this occurred at station 9 (salinity = 23‰) and in June at stations 8 and 9 (salinity = 28 and 30‰, respectively). With the exception of an anomalously high activity at station 10 in June, and disregarding the dredge impacted samples, biodegradation rates decreased with both increasing and decreasing salinities about these maxima. No explanation can be given for the variability in phenanthrene mineralization efficiencies observed in the Bay stations (1-4), although station 4 was near a large marina.

The addition of N + P enhanced and the addition of alternate carbon sources depressed the ^{14}C-phenanthrene mineralization efficiencies of sample aliquots collected from station 6 in June. Figure 25 shows the $^{14}CO_2$ evolution curves for a control and the various treatments. The addition of N + P to station 6 water resulted in a 15% increase in the amount of $^{14}CO_2$ evolved at 500 h. Humic acid, succinate, pyruvate and acetate depressed $^{14}CO_2$ production by 21 to 23% relative to the control. Less significant reductions were observed in the glucose and Tween 80 amended samples. These results are discussed below in the context of the ambient nutrient and DOC concentrations.

Plate counts on ENA and PTA showed fairly uniform numbers of heterotrophs throughout the estuary in March (Fig. 26). The high river discharge and wash-in of terrestrial microbes probably dampened much of the spatial heterogeneity in the distribution of bacteria in the estuary. However, as with phenanthrene mineralization efficiencies,

FIGURE 25. Time courses of $^{14}CO_2$ evolution from ^{14}C-phenanthrene for June, 1985 station 6 water samples amended with nutrients (N + P) or organic substrates. Abbreviations for carbon additions are: C, control; T80, Tween 80; G, glucose; HA, humic acid; S, succinate; A, acetate; P, pyruvate. Carbon source additions resulted in a phenanthrene-C:alternate-C of 1:2.

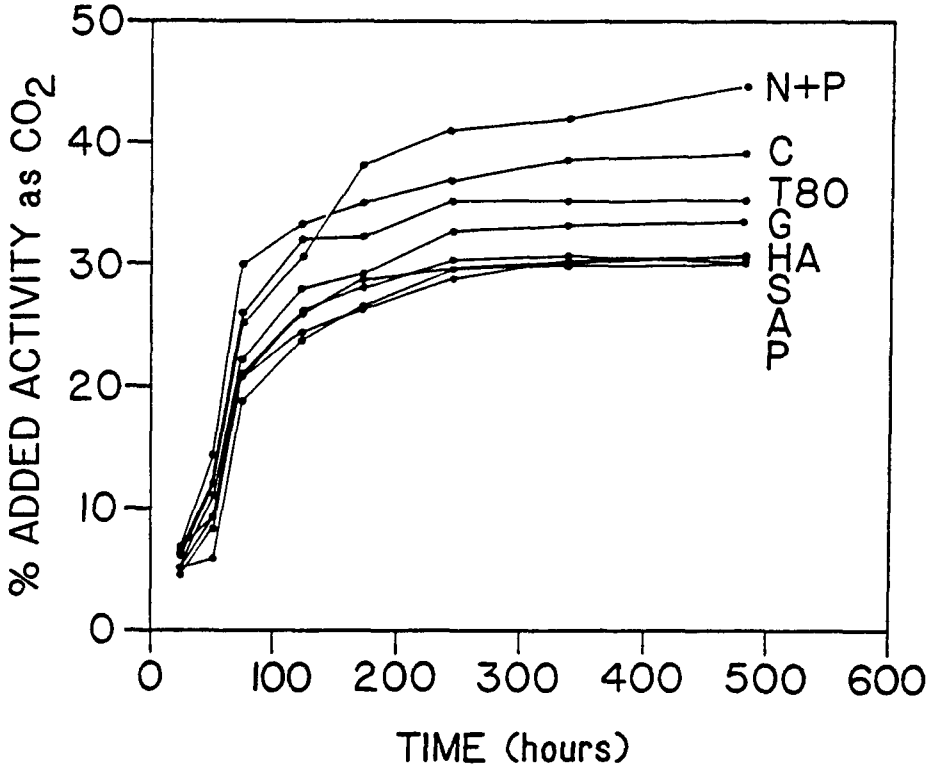
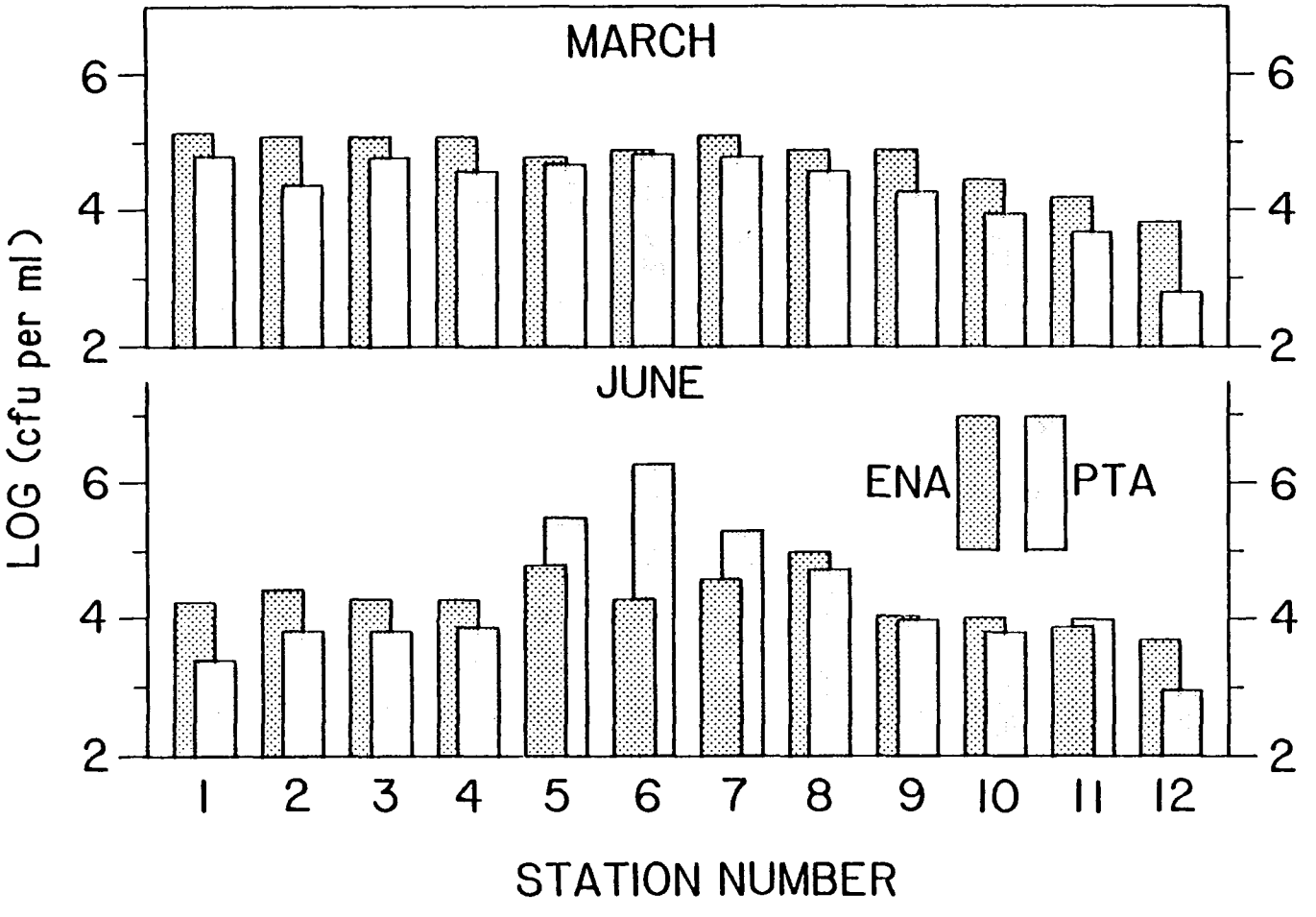

50
40
30
20
10
0
% ADDED ACTIVITY as CO_2
0
100
200
300
400
500
600
TIME (hours)
N+P
C
T80
G
HA
S
A
P

FIGURE 26. Distribution of viable heterotrophic bacteria, as determined by plate counts on ENA and PTA, in the Great Bay Estuary in March and June, 1985.


MARCH
JUNE
ENA
PTA
LOG (cfu per ml)
STATION NUMBER
2
4
6
1
2
3
4
5
6
7
8
9
10
11
12

there was a seaward decrease in colony forming units (CFU) on both ENA and PTA. Somewhat lower numbers of ENA culturable organisms and higher numbers of PTA culturable organisms in the dredge-impacted Cocheco R. samples (stations 5 and 6) caused a smaller discrepancy between the two counts at these stations than was generally observed. The depressed ENA count and elevated absolute numbers of DMT-resistant bacteria at these stations also resulted in a high proportion of tin-resistant to total heterotrophic bacteria.

A low ENA count at station 5 was also observed in June when ENA counts throughout the estuary exhibited more spatial variability. Remarkably, counts on PTA were higher than those on ENA for stations 5, 6, 7 and 11 in June. The elevated PTA counts at stations 5 and 6 corresponded with the high phenanthrene degrading activities observed (Fig. 24).

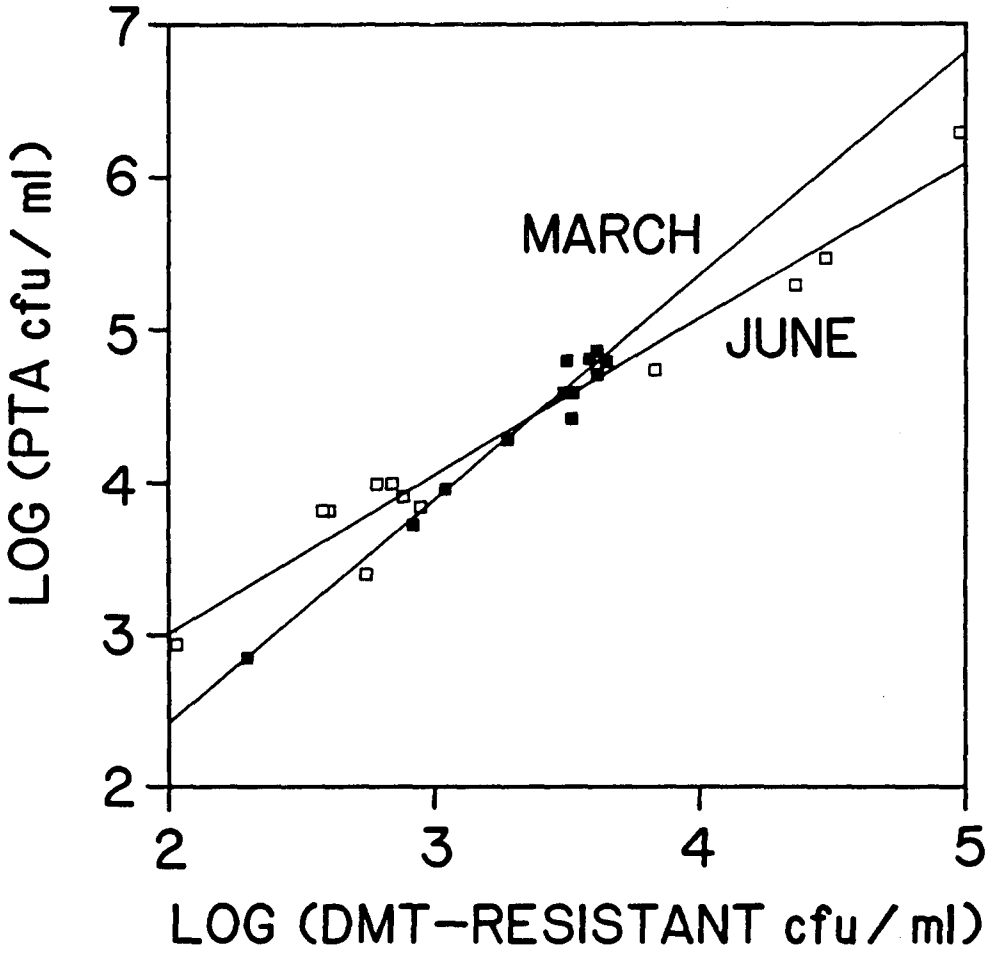
The mid-estuarine maxima in phenanthrene biodegradation potentials (stations 8 and 9, Fig. 24) were not reflected in the PTA plate count data (Fig. 26). However, PTA was prepared with 75% ASW and it was observed in another study (Guerin, in preparation) that significantly higher counts on both ENA and PTA were obtained for a low salinity (3.5‰) sample on media prepared with 25% ASW versus 75% ASW; the reverse was true for a high salinity (33.5‰) sample. The lack of correspondence between the plate count data and the mineralization results has been observed by others (Roubal and Atlas, 1978) and is not surprising in light of the fact that only the latter provides the opportunity for phenanthrene utilization by mixed consortia of organisms.

Again in June, ENA counts were low at the Cocheco River stations

and most of these were enteric organisms derived from the sewage treatment plant upstream. Since no samples were taken upstream of the dredge site, the apparent depression of microbial numbers downstream could not be positively related to the dredging. However, the dredge material was rich in low molecular weight aromatic hydrocarbons (Fig. 23) to which toxic effects have been ascribed elsewhere (Calder and Lader, 1976; Bauer and Capone, 1985a).

Highly significant correlations between PTA counts and DMT-resistant counts were noted throughout the estuary. Figure 27 shows the data for the March and June sampling trips. Although the slopes of the lines differ, the data fit the regression lines quite well ($r = 0.915$ in March, $r = 0.958$ in June). The observed relationships were not an artifact of measuring subsets of the same population since, 1) DMT-resistant bacteria comprised only a small percentage of the total viable count on ENA (2 to 6% in March, 2 to 9% in June), 2) the correlation between PTA counts and ENA counts was low in June ($r = 0.62$) with several stations giving higher numbers on PTA than on ENA, and 3) PTA counts were at least 10-fold higher than DMT-resistant counts. Walker and Colwell (1974) observed a high incidence of mercury resistance among estuarine bacteria isolated on the basis of their ability to utilize petroleum. Conversely, a high percentage of isolates obtained on the basis of their mercury resistance demonstrated petroleum degradative abilities. In the contaminated sediments from which these isolates were obtained, the organisms received simultaneous exposure to mercury and petroleum hydrocarbons. Simultaneous selection for tin resistant and hydrocarbon degrading bacteria could occur in the marinas which abound in the Great Bay Estuary. Here, the leaching of

FIGURE 27. Linear regression lines showing the relationships between dimethyltin-resistant organisms and viable heterotrophs on PTA among the March (closed symbols) and June (open symbols), 1985 water samples.


7
6
5
4
3
2
2
3
4
5
MARCH
JUNE
LOG (PTA cfu / ml)
LOG (DMT-RESISTANT cfu / ml)

tin from anti-fouling marine paints and the chronic release of aromatic hydrocarbons occur together, especially during the warm months.

Table 11 shows the results of linear regression analyses between various biological and chemical parameters (independent variables) and phenanthrene mineralization efficiencies at 500 h (dependent variables) for the March and June data sets. Correlation coefficients between ^{14}C-phenanthrene mineralization efficiencies and initial ^{14}C-phenanthrene mineralization rates (calculated from the 120 h $^{14}CO_2$ production figures) were 0.95 and 0.91 for the March and June data, respectively. These figures indicate that the adaptiveness of the microbial community, its preparedness to mineralize phenanthrene, was the feature measured by the procedure employed. The preference of the 500 h parameter to express mineralization potentials is based on the stepped $^{14}CO_2$ evolution curves sometimes observed for mixed cultures degrading phenanthrene in two stages. A similar correspondence between initial mineralization rate and maximum % PAH mineralized was observed by Bauer and Capone (1985b). Plate counts on PTA accounted for 52% of the variability in phenanthrene mineralization efficiencies in March, but only 28% in June (r values squared). A phenanthrene-based agar medium was found by Mallory and Saylor (1983) to be relatively non-selective. This is most likely due to the low solubility of the hydrocarbon. Oligotrophic bacteria growing on impurities in agar would be just as likely to be selected as phenanthrene degraders.

Correlations with nutrient concentrations indicated variable influences of these parameters on phenanthrene degradation with coefficients (r values) ranging from 0.12 (PO_4^{-3} in March) to 0.57 (PO_4^{-3} in June). The chance simultaneous inputs of primary sewage effluent and

TABLE 11. Correlation coefficients (r values) with ^{14}C-phenanthrene mineralization efficiencies (500 h conversion to $^{14}CO_2$) in March and June water samples as the dependent variables and various biological and chemical parameters as the independent variables.

	Mineralization Efficiencies in	
Independent Variable	March	June
Initial Mineralization Rate[a]	0.95	0.91
LOG (PTA cfu/ml)	0.72	0.53 (0.17)[b]
SPM	0.34	0.38 (0.14)
POM	0.42	0.50 (-0.19)
DOC	0.37	0.71 (0.11)
NH_4^+	0.33	0.55 (-0.01)
$NO_3^- + NO_2^-$	0.33	0.34 (0.18)
PO_4^{-3}	0.12	0.57 (0.22)

[a] calculated by connecting time zero and 120 h data points.
[b] values in parentheses are coefficients with data from stations 5 and 6 excluded from the regression analysis.

PAH hydrocarbons upstream of stations 5 and 6 obscured the ecological significance of prior exposure to PAH as a selection pressure resulting in high mineralization efficiencies, and exaggerated the importance of nutrient concentrations in determining the activity of the samples. When the Cocheco River data (stations 5 and 6) were deleted from the analyses, the coefficients dropped by one half or more (values in parentheses). Similarly, the influences of DOC, POM and SPM on mineralization were overestimated by inclusion of the (effluent) data from stations 5 and 6. This is not to say, however, that the nutrient status of the water at stations 5 and 6 (Table 10) had a negligible effect on the mineralization efficiencies observed there. The impact of a small seasonal marina (for which the dredging operation provided a turning basin) across the river from the sewage treatment plant should also not be ignored. Surface microlayer and bulk water samples collected at this site 8 mos prior to the dredging actively degraded phenanthrene. The effects of the PAH introduced as a result of the dredging operation, however, were far more pervasive and far-reaching than these inputs.

There are many data on the influence of nutrients on the hydrocarbon degradation potentials of marine, estuarine and freshwaters (for reviews, see Atlas, 1981; Colwell and Walker, 1977; Rosenburg and Gutnick, 1981). Phosphorous is occasionally cited as being more limiting than nitrogen (Mulkins-Phillips and Stewart, 1974a) especially in surface seawater where PO_4^{-3} concentrations are extremely low. Addition of nutrients to water samples can stimulate degradation. Aromatic hydrocarbons are more readily degraded than the more reduced alkanes under low nutrient conditions (Cooney et al, 1985, Fedorak and

Westlake, 1981; Roubal and Atlas, 1978). In the Great Bay Estuary, nutrient concentrations did not determine the relative activities of phenanthrene degrading bacteria (Table 11) and were probably not limiting at the level of carbon enrichment chosen. The stimulation of $^{14}CO_2$ production by the addition of N + P to the June station 6 sample, however, was puzzling in light of the already eutrophic nature of the water here (Table 10).

Dissolved organic carbon (DOC) has been reported to both promote and delay hydrocarbon degrading activities. Phenanthrene mineralization by Great Bay water samples was independent of the carbon concentrations in the water as evidenced by the low correlations observed with DOC and POM (Table 11). Alternate carbon source experiments showed the addition of these to uniformly depress $^{14}CO_2$ production from ^{14}C-phenanthrene in water samples from station 6 in June (Fig. 25). However, these data report only the production of respired carbon from phenanthrene and not the total respiration of the available carbon by the community. No additonal lag period was observed in carbon amended versus control samples, indicating that at the low substrate levels added, these were utilized simultaneously by the community (Harder and Dijkhuizen, 1982; Schmidt and Alexander, 1985). Perhaps in a less well-adapted population, diauxic utilization of the substrates would be observed. Work with mixed substrate utilization by pure and mixed microbial cultures has shown that changing the average oxidation state of the carbon supplied causes shifts in the ratio of respired to incorporated carbon (Babel and Muller, 1985). Therefore, little can be concluded from the alternate carbon source data presented. The effect these treatments would have on phenanthrene mineralization by an oligotrophic

microbial community may have been different.

Suspended particulate matter (SPM) concentrations were also unimportant in determining the distribution of phenanthrene degrading activity. This observation agrees with the results of Sherrill and Saylor (1980) and of Readman et al. (1982). The latter investigators found naphthalene degradation to correlate poorly with particulate levels in the Tamar Estuary. Naphthalene, phenanthrene and anthracene distributions were also unrelated to particle distributions. Concentrations of the more insoluble benzo(a)pyrene, as well as benzo(a)pyrene degradation rates were strongly correlated with particulate concentrations in the estuary. Because PAH are reactive compounds subject to adsorption, photo-oxidation, volatilization, complexation and biodegradation, dissolved concentrations of naphthalene and benzo(a)pyrene were not correlated with their rates of biodegradation.

The lack of adsorption as an important factor governing the fate of phenanthrene or the distribution of phenanthrene degrading bacteria in the estuarine environment can also be inferred from the results of the experiment on the effects of inoculum size on the rate of degradation shown in Fig. 20. A large sediment inoculum was expected to show a short lag period because of the large number of organisms introduced, but to be limited in the final mineralization efficiency by a higher degree of adsorption to the inoculum. A smaller inoculum, by this reasoning, would show a longer lag period but a higher final mineralization efficiency. However, increasing particulate concentrations in the samples did not result in adsorption-limited degradation of phenanthrene. Rather, the larger inoculum, consistent

with the model of Paris et al. (1981), allowed the more rapid turnover of phenanthrene. Results with acenaphthene (solubility 2.4 $\mu g\ ml^{-1}$, Rossi and Thomas, 1981) also showed no inhibition of degradation by high solids levels (Hall et al, 1986). In laboratory studies, even with 50% adsorption of phenanthrene to organic-conditioned montmorillonite, no diminution of mineralization by pure cultures was observed relative to particle-free controls (Guerin, unpublished data). For the more soluble PAH then, adsorption, to the extent that it does occur, does not limit microbial degradation. This situation contrasts with that for other xenobiotic compounds such as (2,4-dichlorophenoxy)acetic acid (Ogram et al, 1985) and perhaps the larger, more hydrophobic PAH.

The selection of PAH-degrading bacteria by exposure to the compounds has been amply illustrated in laboratory and field studies (see Introduction). Dissolved concentrations of PAH were not determined in this study but the influence of the dredging operation in the Cocheco River on the adaptation of phenanthrene degrading bacteria downstream was obvious. Hydrocarbon pre-exposure due to boating activities in the Piscataqua River was the likely explanation for the high activity observed at station 10 in June.

The high activity of water and sediment samples collected in the industrialized portion of the Piscataqua River also implicates pre-exposure as an important determinant of biodegradative activities. At the mouth of the estuary, degradation potentials decreased due to dilution with water previously unexposed to PAH. During two summer cruises in the Gulf of Maine, no phenanthrene degrading activity was observed in surface microlayer or bulk water samples (Guerin, unpublished data). Microlayer and bulk water samples collected at the

mouth of the Merrimack River (MA) and in the Great Bay Estuary, on the other hand, actively degraded phenanthrene even when diluted 20 fold. In the undiluted samples assayed in this study, significant mineralization of phenanthrene occurred throughout the estuary, largely as a result of pre-exposure to PAH through human activities.

REFERENCES

Alexander, M. 1981. Biodegradation of chemicals of environmental concern. Science. 211:132-138.

Atlas, R.M. 1981. Microbial degradation of petroleum hydrocarbons: An environmental perspective. Microbiol. Rev. 45:180-209.

Atlas, R.M., P.D. Boehm and J.A. Calder. 1981. Chemical and biological weathering of oil, from the Amoco Cadiz spillage, within the littoral zone. Est. Coast. Shelf Sci. 12:589-608.

Babel, W. and R.H. Muller. 1985. Mixed substrate utilization in microorganisms: Biochemical aspects and energetics. J. Gen. Microbiol. 131:39-45.

Barrick, R.C. 1982. Flux of aliphatic and polycyclic aromatic hydrocarbons to central Puget Sound from Seattle (Washington) primary sewage effluent. Environ. Sci. Tech. 16:682-692.

Bauer, J.E. and D.G. Capone. 1985a. Effects of four aromatic organic pollutants on microbial glucose metabolism and thymidine incorporation in marine sediments. Appl. Environ. Microbiol. 49:828-835.

Bauer, J.E. and D.G. Capone. 1985b. Degradation and mineralization of the polycyclic aromatic hydrocarbons anthracene and naphthalene in intertidal marine sediments. Appl. Environ. Microbiol. 50:81-90.

Blumer, M. and W.W. Youngblood. 1975. Polycyclic aromatic hydrocarbons in soils and recent sediments. Science 188:53-55.

Boehm, P.D. and J.W. Farrington. 1984. Aspects of the polycyclic aromatic hydrocarbon geochemistry of recent seidments in the Georges Bank region. Environ. Sci. Tech. 18:840-845.

Boethling, R.S. and M. Alexander. 1979. Effect of concentration of organic chemicals on their biodegradation by natural microbial communities. Appl. Environ. Microbiol. 37:1211-1216.

Calder, J.A. and J.H. Lader. 1976. Effect of dissolved aromatic hydrocarbons on the growth of marine bacteria in batch culture. Appl. Environ. Microbiol. 32:95-101.

Cerniglia, C.E. 1981. Aromatic hydrocarbons: Metabolism by bacteria, fungi and algae. Rev. Biochem. Toxicol. 3:321-361.

Cerniglia, C.E. 1984. Microbial metabolism of polycyclic aromatic hydrocarbons. Adv. Appl. Microbiol. 30:31-71.

Chakrabarty, A.M. 1976. Plasmids in Pseudomonas. Ann. Rev. Genet. 10:7-30.

Colwell, R.R. and J.D. Walker. 1977. Ecological aspects of microbial degradation of petroleum in the marine environment. CRC Crit. Rev. Microbiol. :423-445.

Cooney, J.J., S.A. Silver and E.A. Beck. 1985. Factors influencing hydrocarbon degradation in three freshwater lakes. Microb. Ecol. 11:127-137.

Dagley, S. 1978. Determinants of biodegradability. Q. Rev. Biophys. 11:577-602.

DeLaune, R.D., G.A. Hambrick III and W.H. Patrick, Jr. 1980. Degradation of hydrocarbons in oxidized and reduced sediments. Mar. Poll. Bull. 11:103-106.

DeLaune, R.D., W.H. Patrick, Jr. and M.E. Casselman. 1981. Effect of sediment pH and redox conditions on degradation of benzo(a)pyrene. Mar. Poll. Bull. 12:251-253.

Fedorak, P.M. and D.W.S. Westlake. 1981. Microbial degradation of aromatics and saturates in Prudhoe Bay crude oil as determined by glass capillary gas chromatography. Can. J. Microbiol. 27:432-443.

Fedorak, P.M., J.M. Foght and D.W.S. Westlake. 1982. A method for monitoring mineralization of ^{14}C-labeled compounds in aqueous samples. Water Res. 16:1285-1290.

Fedorak, P.M., K.M. Semple and D.W.S. Westlake. 1984. Oil-degrading capabilities of yeasts and fungi isolated from coastal marine environments. Can. J. Microbiol. 30:565-571.

Fewson, C.A. 1985. Growth yields and respiratory efficiency of Acinetobacter calcoaceticus. J. Gen. Microbiol. 131:865-872.

Forstner, U. and G. Muller. 1981. Concentrations of heavy metals and polycyclic aromatic hydrocarbons in river sediments: Geochemical background, man's influence and environmental impact. GeoJournal 5:417-432.

Gardner, W.S., R.F. Lee, K.R. Tenore and L.W. Smith. 1979. Degradation of selected polycyclic aromatic hydrocarbons in coastal sediments: Importance of microbes and polychaete worms. Water, Air, Soil Poll. 11:339-347.

Giger, W. and C. Schaffner. 1978. Determination of polycyclic aromatic hydrocarbons in the environment by glass capillary gas chromatography. Anal. Chem. 50:243-249.

Gjessing, E.T. and L. Berglind. 1981. Adsorption of PAH to aquatic humus. Arch. Hydrobiol. 92:24-30.

Glibert, P.L. and T.C. Loder. 1977. Automated analysis of nutrients in seawater. A manual of techniques. Woods Hole Oceanographic Institution Technical Report WHOI-77-47.

Hall, W.S., T.J. Leslie and K.L. Dickson. 1986. Effects of suspended solids on the biotransformation of acenaphthene. Bull. Environ. Contam. Toxicol. 36:286-293.

Harder, W. and L. Dijkhuizen. 1982. Strategies of mixed substrate utilization in microorganisms. Phil. Trans. Roy. Soc. Lond. (B) 297:459-480.

Heit, M. 1985. The relationship of a coal fired power plant to the levels of polycyclic aromatic hydrocarbons (PAH) in the sediment of Cayuga Lake. Water, Air, and Soil Poll. 24:41-61.

Herbes, S.E. and L.R. Schwall. 1978. Microbial transformation of polycyclic aromatic hydrocarbons in pristine and petroleum-contaminated sediments. Appl. Environ. Microbiol. 35:306-316.

Herbes, S.E. 1981. Rates of microbial transformation of polycyclic aromatic hydrocarbons in water and sediments in the vicinity of a coal-coking wastewater discharge. Appl. Environ. Microbiol. 41:20-28.

Herrmann, R. 1981. Transport of polycyclic aromatic hydrocarbons through a partly urbanized river basin. Water, Air, Soil Poll. 16:445-467.

Herrmann, R. and D. Hubner. 1982. Behaviour of polycyclic aromatic hydrocarbons in the Exe Estuary, Devon. Neth. J. Sea Res. 15:362-390.

Hinga, K.R., M.E.Q. Pilson, R.F. Lee, J.W. Farrington, K. Tjessem and A.C. Davis. 1980. Biogeochemistry of benzanthracene in an enclosed marine ecosystem. Environ. Sci. Tech. 14:1136-1143.

Hites, R.A., R.E. Laflamme and J.W. Farrington. 1977. Sedimentary polycyclic aromatic hydrocarbons: The historical record. Science 198:829-831.

Hoffman, E.J., G.L. Mills, J.S. Latimer and J.G. Quinn 1984. Urban runoff as a source of polycyclic aromatic hydrocarbons to coastal waters. Environ. Sci. Tech. 18:580-587.

Hughes, D.E. and P. McKenzie. 1975. The microbial degradation of oil in the sea. Proc. Roy. Soc. Lond. (B) 189:375-390.

Husseini, R.H. and R.J. Stretton. 1981. The antibacterial activity of some phenanthroline and phenanthrene compounds. Microbios. Lett. 16:85-94.

Karickhoff, S.W., D.S. Brown and T.A. Scott. 1979. Sorption of hydrophobic pollutants on natural sediments. Water Res. 13:241-248.

Kool, H.J. 1984. Influence of microbial biomass on the biodegradability of organic compounds. Chemosphere 13:751-761.

Lake, J.L., C. Norwood, C. Dimock and R. Bowen. 1979. Origins of polycyclic aromatic hydrocarbons in estuarine sediments. Geochim. Cosmochim. Acta 43:1847-1854.

Lao, R.C., R.S. Thomas and J.L. Monkman. 1975. Computerized gas chromatographic-mass spectrometric analysis of polycyclic aromatic hydrocarbons in environmental samples. J. Chromatogr. 112:681-700.

Lee, R.F., W.S. Gardner, J.W. Anderson, J.W. Blaylock and J. Barwell-Clarke. 1978. Fate of polycyclic aromatic hydrocarbons in controlled ecosystem enclosures. Environ. Sci. Tech. 12:832-838.

Lee, R.F., K. Hinga and G. Almquist. 1982. Fate of radiolabeled polycyclic aromatic hydrocarbons and pentachlorophenol in enclosed marine ecosystems. pp. 123-135, In, G.D. Grice and M.R. Reeve (ed.), Marine Mesocosms. Springer-Verlag, New York.

Lindquist, B. and D. Warshawsky. 1985. Stereospecificity in algal oxidation of the carcinogen benzo(a)pyrene. Experientia 41:767-769.

Loder, T.C., J.A. Love, C.E. Penniman and C.D. Neefus. 1983. Long-term environmental trends in nutrient and hydrographic data from the Great Bay Estuarine System, New Hampshire-Maine. UNH Marine Program Report No. UNH-MP-D/TR-SG-83-6.

Mallory, L.M. and G.S. Sayler. 1983. Heterotrophic bacterial guild structure: Relationship to biodegradative populations. Microb. Ecol. 9:41-55.

Martens, R. 1982. Concentrations and microbial mineralization of four to six ring polycyclic aromatic hydrocarbons in composted municipal waste. Chemosphere 11:761-770.

May, W.E., S.P. Wasik and D.H. Freeman. 1978. Determination of the solubility behavior of some polycyclic aromatic hydrocarbons in water. Anal. Chem. 50:997-1000.

Mayer, L.M. and P.M. Rossi. 1981. Application of the cetyl pyridinium bromide (CPB) technique to the determination of specific surface areas of Maine coastal sediments: Relationship of surface area with other sediment textural factors. Land and Water Resources Center Technical Report B-016-ME-1, University of Maine at Orono, 23 pp.

Mayer, L.M., P.T. Rahaim, W. Guerin, S.A. Macko, L. Watling and F.E. Anderson. 1985. Biological and granulometric controls on sedimentary organic matter of an intertidal mudflat. Est. Coast. Shelf Sci. 20:491-503.

Mulkins-Phillips, G.J. and J.E. Stewart. 1974a. Effect of environmental parameters on bacterial degradation of Bunker C oil, crude oils and hydrocarbons. Appl. Microbiol. 28:915-922.

Mulkins-Phillips, G.J. and J.E. Stewart. 1974b. Distribution of hydrocarbon-utilizing bacteria in northwestern Atlantic waters and coastal sediments. Can. J. Microbiol. 20:955-962.

Neff, J.M. 1979. Polycyclic Aromatic Hydrocarbons in the Aquatic Environment. Applied Science Publishers, Ltd., London. 262 pp.

Ogan, K., E. Katz and W. Slavin. 1979. Determination of polycyclic aromatic hydrocarbons in aqueous samples by reversed-phase liquid chromatography. Anal. Chem. 51:1315-1320.

Ogram, A.V., R.E. Jessup, L.T. Ou and P.S.C. Rao. 1985. Effects of sorption of biological degradation rates of (2,4-dichlorophenoxy)acetic acid in soils. Appl. Environ. Microbiol. 49:582-587.

Olsen, C.R., N.H. Cutshall and I.L. Larsen. 1982. Pollutant-particle associations and dynamics in coastal marine environments: A review. Mar. Chem. 11:501-533.

Paris, D.F., W.C. Steen, G.L. Baughman and J.T. Barnett, Jr. 1981. Second-order model to predict microbial degradation of organic compounds in natural waters. Appl. Environ. Microbiol. 41:603-609.

Pavlou, S.P. and R.N. Dexter. 1979. Distribution of polychlorinated biphenyls (PCB) in estuarine ecosystems. Testing the concept of equilibrium partitioning in the marine environment. Environ. Sci. Tech. 13:65-71.

Phillips, D.H. 1983. Fifty years of benzo(a)pyrene. Nature 303:468-472.

Prahl, F.G. and R. Carpenter. 1983. Polycyclic aromatic hydrocarbon (PAH)-phase associations in Washington coastal sediment. Geochim. Cosmochim. Acta 47:1013-1-23.

Prahl, F.G., E. Crecelius and R. Carpenter. 1984. Polycyclic aromatic hydrocarbons in Washington coastal sediments: An evaluation of atmospheric and riverine routes of introduction. Environ. Sci. Tech. 18:687-693.

Readman, J.W., R.F.C. Mantoura, M.M. Rhead and L.Brown. 1982. Aquatic distribution and heterotrophic degradation of polycyclic aromatic hydrocarbons (PAH) in the Tamar Estuary. Est. Coast. Shelf Sci. 14:369-389.

Rosenberg, E. and D.L. Gutnick. 1981. The hydrocarbon-oxidizing bacteria. pp. 903-912 In, The Prokaryotes, Vol. I. M.P. Starr, H. Stolp, H.G. Truper, A. Balows and H.G. Schlegel (ed.). Springer-Verlag, Berlin.

Rossi, S.S. and W.H. Thomas. 1981. Solubility behavior of three aromatic hydrocarbons in distilled water and natural seawater. Environ. Sci. Tech. 15:715-716.

Rubin, H.E. and M. Alexander. 1983. Effect of nutrients on the rates of mineralization of trace concentrations of phenol and p-nitrophenol. Environ. Sci. Tech. 17:104-107.

Saltzmann, H.A. 1982. Biodegradation of aromatic hydrocarbons in marine sediments of three North Sea oil fields. Mar. Biol. 72:17-26.

Schmidt, S.K. and M. Alexander. 1985. Effects of dissolved organic carbon and second substrates on the biodegradation of organic compounds at low concentrations. Appl. Environ. Microbiol. 49:822-827.

Sherrill, T.W. and G.S. Saylor. 1980. Phenanthrene biodegradation in freshwater environments. Appl. Environ. Microbiol. 39:172-178.

Shiaris, M.P. and J.J. Cooney. 1983. Replica plating method for estimating phenanthrene-utilizing and phenanthrene-cometabolizing microorganisms. Appl. Environ. Microbiol. 45:706-710.

Shimp, R.J. and F.K. Pfaender. 1985a. Influence of easily degradable naturally occurring carbon substrates on biodegradation of monosubstituted phenols by aquatic bacteria. Appl. Environ. Microbiol. 49:394-401.

Shimp, R.J. and F.J. Pfaender. 1985b. Influence of naturally occurring humic acids on biodegradation of monosubstituted phenols by aquatic bacteria. Appl. Environ. Microbiol. 49:402-407.

Sisler, F.D. and C.E. ZoBell. 1947. Microbial utilization of carcinogenic hydrocarbons. Science 106:521-522.

Spain, J.C. and P.A. VanVeld. 1983. Adaptation of natural microbial communities to degradation of xenobiotic compounds: effects of concentration, exposure time, inoculum, and chemical structure. Appl. Environ. Microbiol. 45:428-435.

Stegeman, J.J. 1981. Polynuclear aromatic hydrocarbons and their metabolism in the marine environment. pp. 1-60, In, H.V. Gelboin and P.O.P. Ts'o (ed.), Polycyclic Hydrocarbons and Cancer, Vol. 3. Academic Press, New York.

Walker, J.D. and R.R. Colwell. 1974. Mercury-resistant bacteria and petroleum degradation. Appl. Microbiol. 27:285-287.

Walker, J.D. and R.R. Colwell. 1975. Some effects of petroleum on estuarine and marine microorganisms. Can. J. Microbiol. 21:305-313.

Ward, D.M. and T.D. Brock. 1976. Environmental factors influencing the rate of hydrocarbon oxidation in temperate lakes. Appl. Environ. Microbiol. 31:764-772.

Whitehouse, B. 1985. The effects of dissolved organic matter on the aqueous partitioning of polynuclear aromatic hydrocarbons. Est. Coast. Shelf Sci. 20:393-402.

Wijayaratne, R.D. and J.C. Means. 1984. Sorption of polycyclic aromatic hydrocarbons by natural estuarine colloids. Mar. Environ. Res. 11:77-89.

Wright, R.T. 1978. Measurement and significance of specific activity in the heterotrophic bacteria of natural waters. Appl. Environ. Microbiol. 36:297-305.

Wyndham, R.C. and J.W. Costerton. 1981. Heterotrophic potentials and hydrocarbon biodegradation potentials of sediment microorganisms within the Athabasca Oil Sands deposit. Appl. Environ. Microbiol. 41:783-790.

Young, R.A., D.J.P. Swift, T.L. Clarke, G.R. Harvey and P.R. Betzer. 1985. Dispersal pathways for particle-associated pollutants. Science 229:431-435.

CHAPTER FOUR

CHAPTER FOUR

PHENANTHRENE DEGRADATION BY ESTUARINE SURFACE MICROLAYER AND BULK WATER MICROBIAL POPULATIONS

ABSTRACT

Paired estuarine surface microlayer and bulk water samples from pristine and contaminated sites in the Great Bay Estuary, NH, were examined with regard to numbers of bacteria, ^{14}C-phenanthrene biodegradation potentials and organic and inorganic chemical characteristics. Microlayer samples were generally enriched in nutrients (N and P) and dissolved organic matter concentrations. Several microlayer samples gave spectral (peaks in UV absorption at 250 nm and non-humic associated fluorescence) and chemical (butyl tin compound enrichment) evidence of hydrocarbon contamination by marinas. Despite these consistent enrichments, ^{14}C-phenanthrene biodegradation potentials were sometimes lower in microlayer than in bulk water samples. The cause of the apparent inhibition of microlayer samples was undetermined. Phenanthrene-degrading bacteria, enumerated by counting clearing zones in a crystalline phenanthrene overlay after colony development on a phenanthrene/toluene agar (PTA) medium, was the best microbiological predictor of the more costly and time consuming ^{14}C assay.

INTRODUCTION

Aquatic surface microlayers constitute the organically enriched uppermost micrometer of fresh and salt waters (Sieburth et al, 1976; Hunter and Liss, 1981). They are important features which influence the exchange of materials (gases, volatile organics, dust and salts) across the air/water interface. Once thought to be composed predominantly of lipid-like materials (Larsson et al, 1974), recent evidence suggests that carbohydrates and proteins are quantitatively more significant components (Kattner et al, 1985; Henrichs and Williams, 1985; Hunter and Liss, 1981). In addition, organic and inorganic particulates (Lion et al, 1979), trace elements (Hardy et al, 1985; Lion and Leckie, 1981; Duce et al, 1972), polychlorinated biphenyls (PCB's, Duce et al, 1972; Larsson et al, 1974), pesticides (DDT, DDE, etc., Larsson et al, 1974), hydrocarbons (Meyers and Kawka, 1982; Gearing and Gearing, 1982; Osovitc et al, 1985; Hansen, 1975) and inorganic nutrients (Hatcher and Parker, 1974; Horrigan et al, 1981) are often enriched in microlayers over their corresponding bulk water concentrations. The degree of enrichment depends largely on the thickness of the sample collected which in turn varies from less than 1 μm to almost 1 mm depending on the sampler employed (Norkrans, 1980).

Polycyclic aromatic hydrocarbons (PAH) are also enriched in surface microlayers. These naturally fluorescent, fused-ring aromatic hydrocarbons are components of fossil fuels and products of combustion and may enter the aquatic environment as spilled oil, atmospheric fallout and street and highway runoff. Concern about their

environmental fate stems from their recalcitrance, toxicity, carcinogenicity and potential for trophic biomagnification. PAH are extremely hydrophobic and bind tenaciously to such microlayer components as humic materials (Gjessing and Berglind, 1981) and estuarine colloids (Wijayaratne and Means, 1984) as well as mineral (Karickhoff et al, 1979) and biological (Steen and Karickhoff, 1981) particulates. Readman et al. (1982) found that the distribution of four-ringed and larger PAH in the Tamar Estuary was highly correlated with the distribution of particulate material. However, naphthalene, anthracene and phenanthrene, because of their relatively higher solubilities and vapor pressures did not correlate with particulate materials; their estuarine distributions were governed principally by volatilization and biodegradation. The surface microlayer may act as a physical and chemical impediment to the volatilization of low molecular weight PAH and provide a site for their biodegradation by bacteria. In fact, Marty et al. (1978, cited in Neff, 1979) found 4-fold enrichments of total hydrocarbons in microlayers of the tropical Atlantic Ocean with a preferential enrichment of aromatic hydrocarbons. Furthermore, phenanthrene was the single most abundant compound comprising 40% of the aromatic fraction; methyl phenanthrenes were also abundant.

Phenanthrene degrading bacteria are ubiquitous in estuarine environments and may be particularly active in surface microlayers where PAH are abundant and where O_2, nutrients (Hatcher and Parker, 1974) and supporting substrates (Henrichs and Williams, 1985) are also enriched. The almost invariable enrichment of total and culturable heterotrophic bacteria in surface microlayers has been amply documented (Fuhs, 1982; Norkrans, 1980; Dutka and Kwan, 1978; Dietz et al, 1976).

However, such enrichment may be the result of a physical attraction between the bacteria and the interface (Dahlback et al, 1981; Marshall, 1976) and not the result of *in situ* reproduction of indigenous microorganisms. Low specific (per cell) adenylate energy charge (Dietz et al, 1976; De Souza-Lima and Romano, 1983; Norkrans, 1980) and heterotrophic potential (Dietz et al, 1976) and high total count : viable count ratios (Norkrans, 1980) in the microlayer indicate a highly stressed microbial population. Daytime depletion and nightime enrichment of phytoneuston (Carlson, 1982a) and the inhibition of neustonic nitrifying bacteria by light (Horrigan et al, 1981) suggest that harmful radiation may contribute to the stressed condition of marine microlayer organisms. However, other factors including enrichments of toxic metals, toxic organics and photo-activated chemical species may be contributory. Here, as part of a more extensive study on their estuarine ecology, I compare the phenanthrene biodegradation potentials of bacteria from estuarine surface microlayer and bulk water environments.

MATERIALS AND METHODS

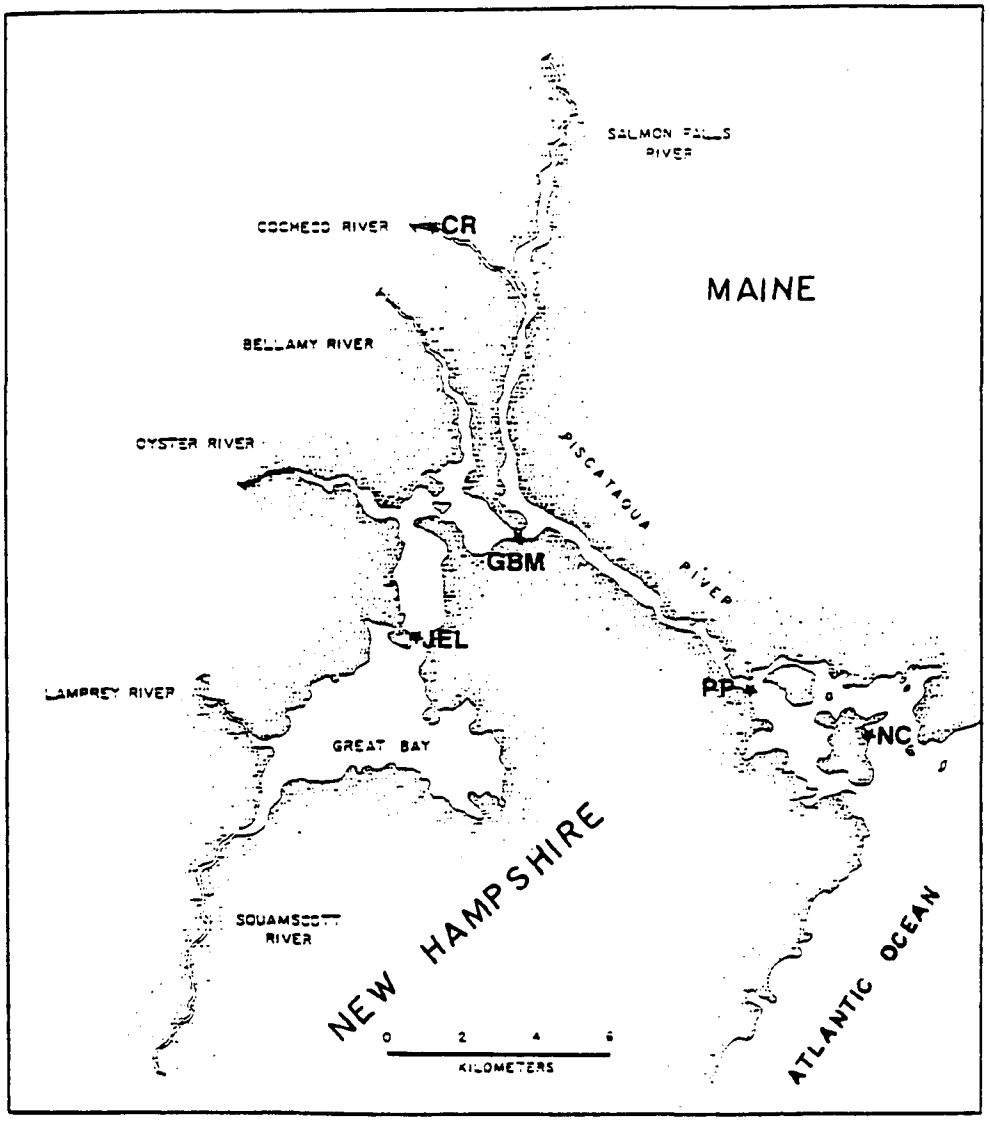
Study Site

With over 11,000 acres of tidewater, the Great Bay estuarine ecosystem (Fig. 28) is one of the largest estuaries on the eastern seaboard of the United States (Mathieson and Hehre, 1986). Seven rivers feed the estuary, draining an area of about 930 square miles. Six of these rivers are dammed to produce discontinuous salinity gradients. The semi-diurnal tidal amplitude is approximately 2.5 m resulting in current velocities up to 6 knots along the Piscataqua River and through the narrows adjacent to Adams Point, Fox Point and Dover Point. The water column is vertically homogenous throughout most of the estuary (Loder and Glibert, 1980) and the sediments are graded from predominantly mud to sand seaward. The Great Bay is a multiple use estuary and surrounding farmland, sewage treatment plants, shipbuilding yards, marinas, an oil terminal and several municipalities including the city of Portsmouth, NH influence the chemistry of the water. Sedimentary phenanthrene concentrations range from about 100 ng g^{-1} in the far reaches of Great Bay to almost 200 $\mu g\ g^{-1}$ in the Cocheco River (Guerin et al, in prep.) where the sediments are laden with coal tar wastes from a 19th century gas plant.

Sampling

Surface microlayer and bulk water samples were collected between July and November, 1984 at the five stations in Great Bay shown in Fig. 28. Microlayers were sampled at high tide from docks (JEL, GBM, PP), from the bow of a fiberglass dingy (CR) or by wading in 1 m of

FIGURE 28. Map of the Great Bay Estuary, New Hampshire-Maine, showing surface microlayer sampling sites.


SALMON FALLS RIVER
COCHECO RIVER
CR
MAINE
BELLAMY RIVER
OYSTER RIVER
PISCATAQUA RIVER
GBM
JEL
LAMPREY RIVER
PP
NC
GREAT BAY
NEW HAMPSHIRE
SQUAMSCOTT RIVER
ATLANTIC OCEAN
0 2 4 6
KILOMETERS

water (NC). A sterile neoprene windshield wiper was used to scrape the sample from a sterile glass plate (20 x 20 cm) which was dipped repeatedly until 200 to 300 ml of sample was collected in a sterile, pre-combusted 500 ml brown glass bottle. Bulk water samples were obtained from depths of 40 to 50 cm. Water temperature, sea state and the number of dips were recorded and samples were returned to the laboratory on ice.

Enumeration of Bacteria

One ml aliquots of samples were added to screw-cap test tubes containing 4% formalin in 9 ml of 75% artificial seawater (ASW, Kester et al, 1967) and refrigerated in the dark. Cells were stained with DAPI (4',6-diamidino-2-phenylindole, Sigma) and counted after filtration onto an Irgalan black-stained 0.2 μm Nuclepore filter on a Zeiss research microscope with epifluorescence attachment (Porter and Feig, 1980). Total counts were obtained by averaging the number of cells in a minimum of 10 random fields and applying appropriate correction factors for field area, effective filter area and sample volume. Blank values were subtracted from all counts and were minimized by filtering (0.4 μm) all solutions.

Viable heterotrophs were enumerated after 2 weeks incubation (18°C) of serially-diluted samples spread-plated in quadruplicate on estuarine nutrient agar (ENA) of the following composition (per 1 75% ASW): glucose (0.5 g), yeast extract (0.5 g), peptone (0.5 g), NH_4NO_3 (0.1 g), actidione (or cycloheximide, a fungicide, 0.1 g), agar (15 g). The pH was adjusted to 7.5, the medium was autoclaved (20 min., 121°C). When cool, 2 ml 0.05 M phosphate buffer (pH 7.5) was added before

pouring plates.

Samples were plated on phenanthrene/toluene agar (PTA) prepared by adding 250 µl of a solution of phenanthrene in toluene (250 mg ml^{-1}) to hot (75°C) medium containing (per l 75% ASW): $FeCl_3 \cdot 6H_2O$ (3 mg), NH_4NO_3 (0.1 g), actidione (0.1 g), agar (15 g), pH 7.5. The medium was autoclaved and when cooled, 1 ml 0.05 M phosphate buffer was added. Quadruplicate plates were incubated for 3 weeks at 18°C before counting. After counting, plates were sprayed with a solution (100 mg ml^{-1}) of phenanthrene in dimethyl sulfoxide (DMSO). The DMSO evaporated to leave a crystalline phenanthrene overlay on the agar surface. Clearing zones in the overlay (OCZ) were counted after an additional 10 d incubation at 18°C.

Phenanthrene Biodegradation Potential

For the measurement of phenanthrene biodegradation potential, 10 ml of samples were added to 190 ml of an artificial seawater medium containing phenanthrene at a concentration of 0.5 mg L^{-1} in modified 500 ml Erlenmeyer flasks (Fedorak et al, 1982). Flasks were spiked with 10 µl (~1 µCi) of a solution of [9-^{14}C] phenanthrene (sp. activity 19.3 mCi $mMol^{-1}$, Amersham-Searle, 98% radiochemical purity) in acetone. Initial activity (dpm ml^{-1}) was immediately determined on duplicate 1 ml aliquots. Flasks were stoppered and incubated at 18°C in the dark on a rotary shaker (140 rpm). At intervals, aliquots were withdrawn in triplicate for the determination of respired $^{14}CO_2$. Precision of the analyses averaged ±0.5% of the total initial activity (n=87, range 0.03 to 2.51). For some samples, the disappearance of CH_2Cl_2-extractable ^{14}C-phenanthrene from the medium and the appearance of water soluble

^{14}C-labeled transformation products was also monitored. Details of the methods can be found in Guerin and Jones (subm.). At the beginning of the incubations, flask contents removed for analysis (water plus headspace, 2 to 3% of total volume) at each sampling time were replaced by uninoculated medium and sterile air from a control flask (fed batch mode). As the labeled phenanthrene in the control flask disappeared after a few days due to adsorption, withdrawn sample was no longer replaced and incubations were switched to batch mode. To determine if phenanthrene degradation by the samples was nutrient limited, 1 ml of a nutrient solution (10 mg yeast extract, 10 mg glucose per ml distilled water) was added later in the incubations.

For the PP samples, phenanthrene biodegradation potentials were also assessed by inoculating 10 ml of microlayer and bulk water samples into a phenanthrene/Tween 80 (P/T80) medium with an initial phenanthrene concentration of about 40 mg L^{-1}. The disappearance of phenanthrene and the production of biomass and metabolic products were determined by methods described previously (Guerin and Jones, submitted).

Chemical Analyses

Samples were filtered through pre-combusted (475°C, 6 h) Whatman GF/C glass fiber filters (1.2 μm effective retention). Salinity of the filtrates was measured by refractometry and pH was determined with a Fisher Accumet pH meter. Nutrient concentrations (NO_3^- + NO_2^-, NH_4^+, PO_4^{-3}) were determined in duplicate on a Technicon Autoanalyzer according to the methods of Glibert and Loder (1977). Several nonspecific measures of bulk organic matter were obtained. Ultraviolet absorption spectra and readings at 250 and 280 nm were recorded on a Beckman DU8

spectrophotometer in 1 cm quartz cuvets. Dissolved organic carbon (DOC) was measured on a Barnsted Photochem DOC Analyzer. Relative concentrations of polyphenolic materials in water samples were measured by the Folin-Ciocalteau reaction using the reagents and proportions recommended by Box (1981) on a Beckman model 35 spectrophotometer in 5 cm cuvets. Resorcinol was used as a standard and all values are expressed in $\mu g\ ml^{-1}$ resorcinol equivalents (RE).

Relative concentrations of humic (bulk organic) materials were also estimated as the fluorescence of samples (λ_{exc} = 350 nm, λ_{em} = 450 nm) on a Perkin-Elmer MPF-44E spectrofluorometer after adjusting the instrument gain to give a constant fluorescence for a 15 $\mu g\ L^{-1}$ quinine sulfate standard. Additional fluorescence spectra were recorded using excitation and emission wavelengths found to give maximal responses during manual scans of microlayer samples. These spectra were uncorrected for wavelength dependent variations in sensitivity and response.

RESULTS AND DISCUSSION

Using the glass plate, the average thickness of the microlayers sampled ranged from 45 to 81 μm (Table 12), within the range reported by others using this method (see Carlson, 1982b). None of the samples were collected entirely within visible surface slicks although patches of natural (PP and NC) and oily (GBM, CR and PP, all collected in or near marinas) slick material comprised minor fractions of some samples. No differences in salinity or pH were discerned between microlayer and bulk water samples.

Nitrate plus nitrite and ammonia were enriched in the surface microlayers in all but the CR sample (Table 13). Here, high nitrogen and phosphorous concentrations reflected the proximity of the sampling site to the submerged outfall of a sewage treatment plant in the city of Dover, NH. The PP microlayer was strongly enriched in nitrogen but slightly depleted in phosphorous relative to the bulk water. The PP bulk water sample had nutrient concentrations more typical of this part of the estuary in October (Loder et al, 1983). The GBM and JEL samples likewise showed microlayer enrichment of nitrogen and depletion of phosphorous while just the reverse was true for the CR sample. The NC microlayer was enriched in all nutrients analyzed.

Microlayer samples were consistently enriched in bulk organic matter relative to the underlying water as shown by their higher absorbance (250 and 280 nm), fluorescence, RE and DOC values (Table 14). All of these measures provide estimates of the humic-like materials which comprise a major fraction of the organic matter in

TABLE 12. Physical and chemical characteristics of microlayer samples.

Sample[a]	Date	Time[b]	Water Temperature	Salinity	pH	Surface Conditions	Average Thickness
GBM	7/23/84	+1	17°C	29.5‰	7.8	calm	71 µm
JEL	7/23/84	+1.5	22°C	26.7‰	7.75	turbulent	81 µm
NC	8/22/84	0	18.5°C	33.5‰	7.9	ripples	71 µm
CR	8/22/84	+1.5	23°C	3.5‰	7.2	turbulent	76 µm
PP	11/11/84	0	12.5°C	33.5‰	--	calm, some slick	45 µm

[a] Sample site abbreviations are as follows:GBM, Great Bay Marina; JEL, Jackson Estuarine Laboratory (UNH); NC, New Castle Island; CR, Cocheco River; PP, Prescott Park, city of Portsmouth, NH.

[b] Time denoted as hours past (+) slack high tide (0).

TABLE 13. Nutrient concentrations[a] and enrichment factors[b] (in parentheses) in Great Bay Estuary surface microlayer (s) and bulk water (ss) samples.

Sample		$NO_3^- + NO_2^-$		NH_4^+		PO_4^{-3}	
GBM	S	4.8	(1.37)	7.5	(1.92)	0.89	(0.82)
	SS	3.5		3.9		1.08	
JEL	S	3.2	(1.14)	--		0.56	(0.98)
	SS	2.8		--		0.57	
NC	S	3.9	(2.79)	5.3	(1.26)	1.16	(2.11)
	SS	1.4		4.2		0.55	
CR	S	22.2	(0.95)	6.7	(0.32)	2.92	(1.55)
	SS	23.3		20.8		1.88	
PP	S	52.8	(13.2)	14.8	(3.52)	0.74	(0.92)
	SS	4.0		4.2		0.80	

[a] All concentrations expressed in μMoles L^{-1}.
[b] Enrichment factors calculated as ratios of microlayer to bulk water concentrations.

TABLE 14. Organic matter characteristics of Great Bay estuarine surface microlayer and bulk water samples. Enrichment factors for microlayer samples shown in parentheses.

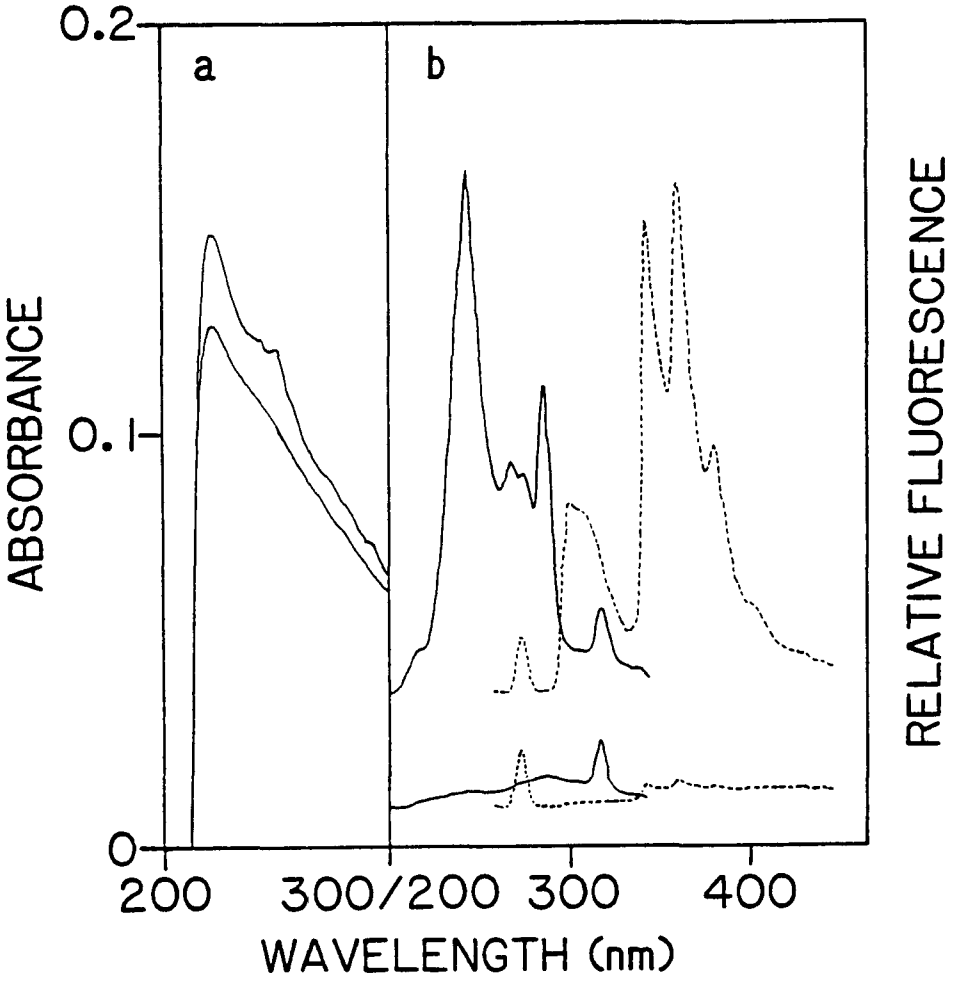
Sample		DOC[a]		Polyphenols[b]		Fluorescence[c]		A_{250}		A_{280}		$A_{250}:A_{280}$
GBM	S	2.0	(1.54)[d]	0.25	(0.81)	87	(1.18)	.091	(1.25)	.053	(1.13)	1.72
	SS	1.3		0.31		74		.073		.047		1.55
JEL	S	4.8	(2.09)	0.47	(1.31)	161	(1.53)	.101	(1.04)	.073	(0.97)	1.38
	SS	2.3		0.36		105		.097		.075		1.29
CR	S	4.0	(0.93)	0.56	(1.08)	217	(1.08)	.231	(1.26)	.154	(1.12)	1.50
	SS	4.3		0.52		201		.184		.138		1.33
NC	S	2.6	(1.37)	0.16	(1.23)	71	(1.58)	.068	(2.19)	.040	(1.54)	1.70
	SS	1.9		0.13		45		.031		.026		1.19
PP	S	4.4	(3.67)	0.02		--		.035	(2.69)	.022	(2.75)	1.59
	SS	1.2		n.d.		--		.013		.008		1.62

[a] Dissolved Organic Carbon expressed in mg L^{-1}.
[b] Expressed in mg L^{-1} Resorcinol Equivalents (RE).
[c] Arbitrary units with λ_{exc} = 350 nm, λ_{em} = 450 nm.
[d] Enrichment factors expressed as ratio of microlayer concentration to bulk water concentration.

natural waters. RE is sensitive to the polyphenolic component of this material and may originate from either terrestrial or macroalgal sources (Carlson and Mayer, 1983), the latter contributing more during the low-runoff, summer months. Absorbance at 280 nm correlates strongly with polyphenolic concentrations in other north temperate estuaries (Carlson and Mayer, 1980). Fluorescence has been used as a tracer for dissolved organic matter but because of different fluorescence efficiencies per unit DOC for low-molecular-weight fulvic acids and high-molecular-weight humic acids (Stewart and Wetzel, 1981), fluorescence is not suitable as an independent measure of organic matter concentrations. This is particularly true in estuarine settings where high-molecular-weight materials are more prone to salt-induced flocculation selectively removing them from estuarine waters (Sholkowitz, 1976; Fox, 1983). On the other hand, humic acids are also more surface active and tend to partition into the surface microlayer in preference to fulvic materials (Hayase and Tsubota, 1983). Regardless, plots of fluorescence (350/450 nm) vs absorbance (280 nm), fluorescence vs RE and absorbance vs RE all showed highly significant correlations (data not shown).

Several microlayer samples gave small peaks in absorbance at 250 nm and had relatively high $A_{250}:A_{280}$ ratios suggesting enrichments of aromatic relative to functionalized aromatic hydrocarbons (Table 14). These samples (GBM, CR microlayer, NC microlayer and PP) were also found to exhibit fluorescence excitation maxima at 250 nm. Figure 29 shows the absorbance and fluorescence excitation and emission spectra for the GBM microlayer and bulk water samples. Whereas absorbance and fluorescence (350/450 nm) measurements (Table 14) indicated small

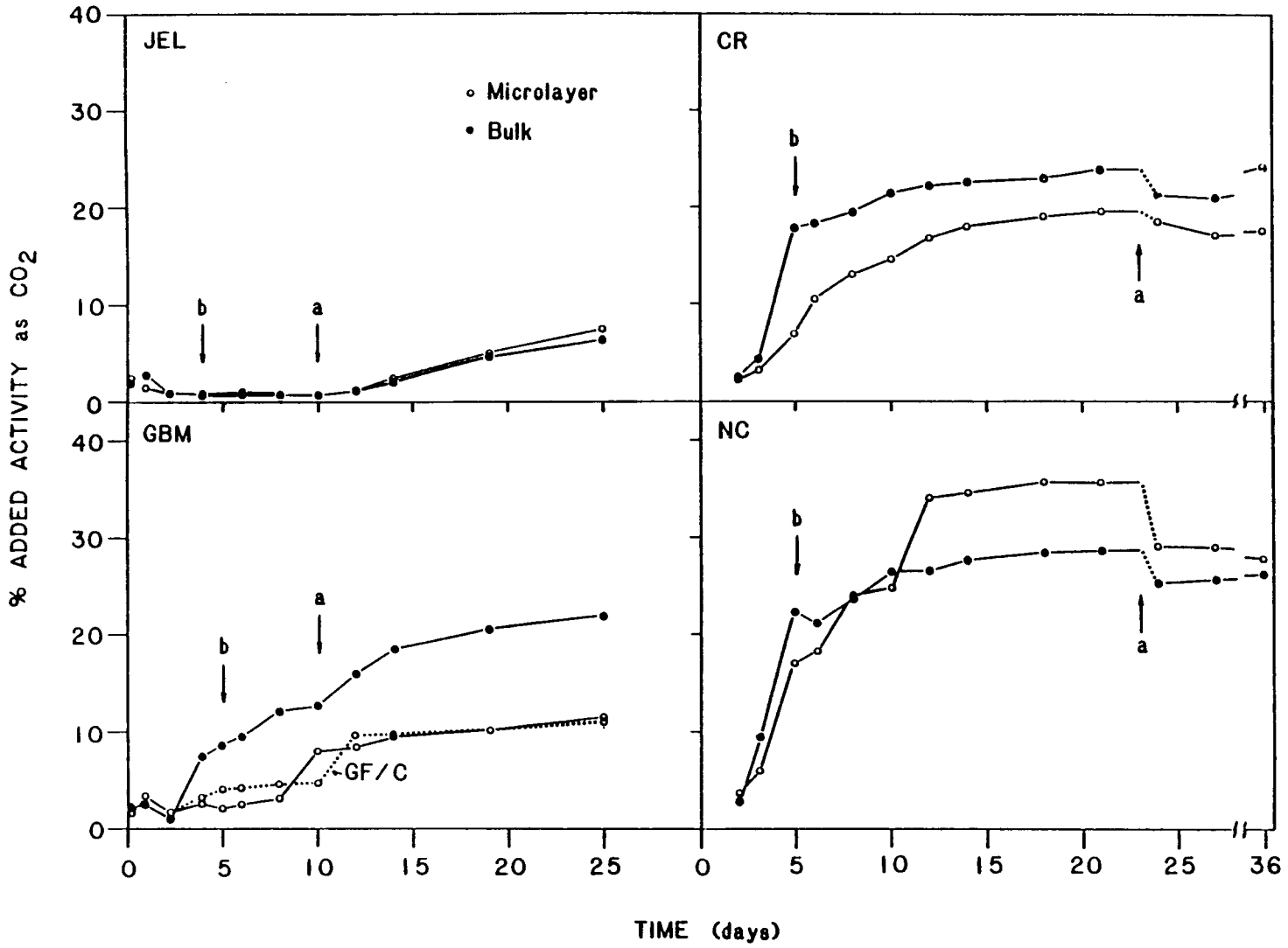
FIGURE 29. Ultraviolet absorption spectra (a) and fluorescence excitation (λ_{em} = 363 nm, solid line) and emission (λ_{exc} = 251 nm, broken line) spectra (b) of the GBM surface microlayer (upper curves) and bulk water (lower curves) samples.


0.2
0.1
0
a
b
ABSORBANCE
RELATIVE FLUORESCENCE
200
300/200
300
400
WAVELENGTH (nm)

enrichments of humic-like materials in the GBM microlayer, fluorescence excitation (λ_{em} = 363 nm) and emission (λ_{exc} = 251 nm) spectra showed substantial enrichments of fluorescent aromatic hydrocarbons here. Furthermore, these spectra resembled those for phenanthrene in the positioning of some, but not all peaks. Enrichment of hydrophobic aromatic materials in the GBM microlayer may have been responsible for crowding of surface-active polyphenolic materials out with the result that this sample was the only one to show microlayer depletion of RE (Table 14). With the exception of the NC microlayer, all samples with high A_{250}:A_{280} ratios were collected within or near marinas. Evidence that the NC sample was also impacted by marina inputs was indicated, however, by extremely high mono- and tri-butyl tin concentrations in the microlayer (Donard et al, 1985). These tin compounds are the active antifouling components of marine paints.

Despite enrichments of aromatic hydrocarbons in marina-impacted microlayers, the phenanthrene degradation potentials of these samples were often lower than those of the corresponding bulk waters. Figure 30 shows the $^{14}CO_2$ evolution curves for paired microlayer and bulk water samples from 4 sites in the Great Bay Estuary. Activity in the GBM and CR samples was greater in the bulk water than in the microlayer. The NC sample also showed a slightly greater initial activity in the bulk water but the 20 d mineralization efficiency (^{14}C-phenanthrene to $^{14}CO_2$)was higher in the microlayer. The JEL sample was inactive but showed some mineralization after the addition of nutrients at 10 d (arrows "a"). Nutrient addition also stimulated $^{14}CO_2$ production in the GBM bulk water sample but had no apparent effect on the microlayer samples. Sterile filtration of the latter through a Whatman GF/C glass

FIGURE 30. $^{14}CO_2$ evolution curves from ^{14}C-phenanthrene using surface microlayer and bulk water samples from the JEL, GBM, CR and NC sites as inocula. The mineralization curve for filtered (GF/C) GBM microlayer water is shown by the dotted line (lower left). Arrows ("a") indicate time of addition of nutrients or ("b") the conversion from fed batch to batch culture. Note break in time axis.


JEL
CR
GBM
NC
Microlayer
Bulk
GF/C
b
a
% ADDED ACTIVITY as CO_2
TIME (days)
0
10
20
30
40
0
5
10
15
20
25
36

fiber filter (effective retention, 1.2 μm) resulted in no diminution of activity and indicated that phenanthrene degraders were not particle associated. A similar conclusion was reached regarding naphthalene, but not benzo(a)pyrene degraders in the Tamar Estuary (Readman et al, 1982). Nutrient additon to CR and NC samples at 23 d caused a decrease in $^{14}CO_2$ in the flasks, perhaps due to heterotrophic CO_2 fixation. The percent mineralization before nutrient addition varied from 0 for both JEL samples to 36% for the NC microlayer. As previously noted (Guerin and Jones, subm.), samples with high mineralization efficiencies were also those which exhibited short lag periods and high initial mineralization rates. Arrows ("b") in Fig. 30 indicate that medium withdrawn for analysis was not replaced with fresh medium after this time, i.e., cultures were run in batch mode. The breaks in some of the mineralization curves occurring at the arrows were probably not related to this switch from fed batch to batch culture for the following reasons: 1) similar breaks in $^{14}CO_2$ curves are observed in samples run entirely in batch mode, 2) a small percentage of the culture volume (3% in these cases) was removed at each sampling point, and 3) little phenanthrene remained in the replacement broth (control) flask after 5 d due to adsortion.

Adsorbed phenanthrene in the experimental flasks was biologically available to some extent as evidenced by the continued production of $^{14}CO_2$ in these and other samples even after the disappearance of CH_2Cl_2-extractable ^{14}C-phenanthrene from the medium. In all flasks, as in controls (not shown), CH_2Cl_2-extractable radioactivity decreased exponentially with time (Fig. 31) due to adsorption to flask walls and contents. This adsorption made it impossible to distinguish between

FIGURE 31. Disposition of ^{14}C in organic, aqueous and CO_2 phases during incubation of PP microlayer (a) and bulk water (c) samples with ^{14}C-phenanthrene. Arrows ("a" and "b") are explained in the text and in caption to figure 3. Bottom panels show the results of simultaneous incubations of PP microlayer (b) and bulk water (d) samples with unlabeled phenanthrene. Solid lines represent protein, broken lines represent phenanthrene, and dotted lines represent Folin-reactive intermediates (expressed as resorcinol equivalents).

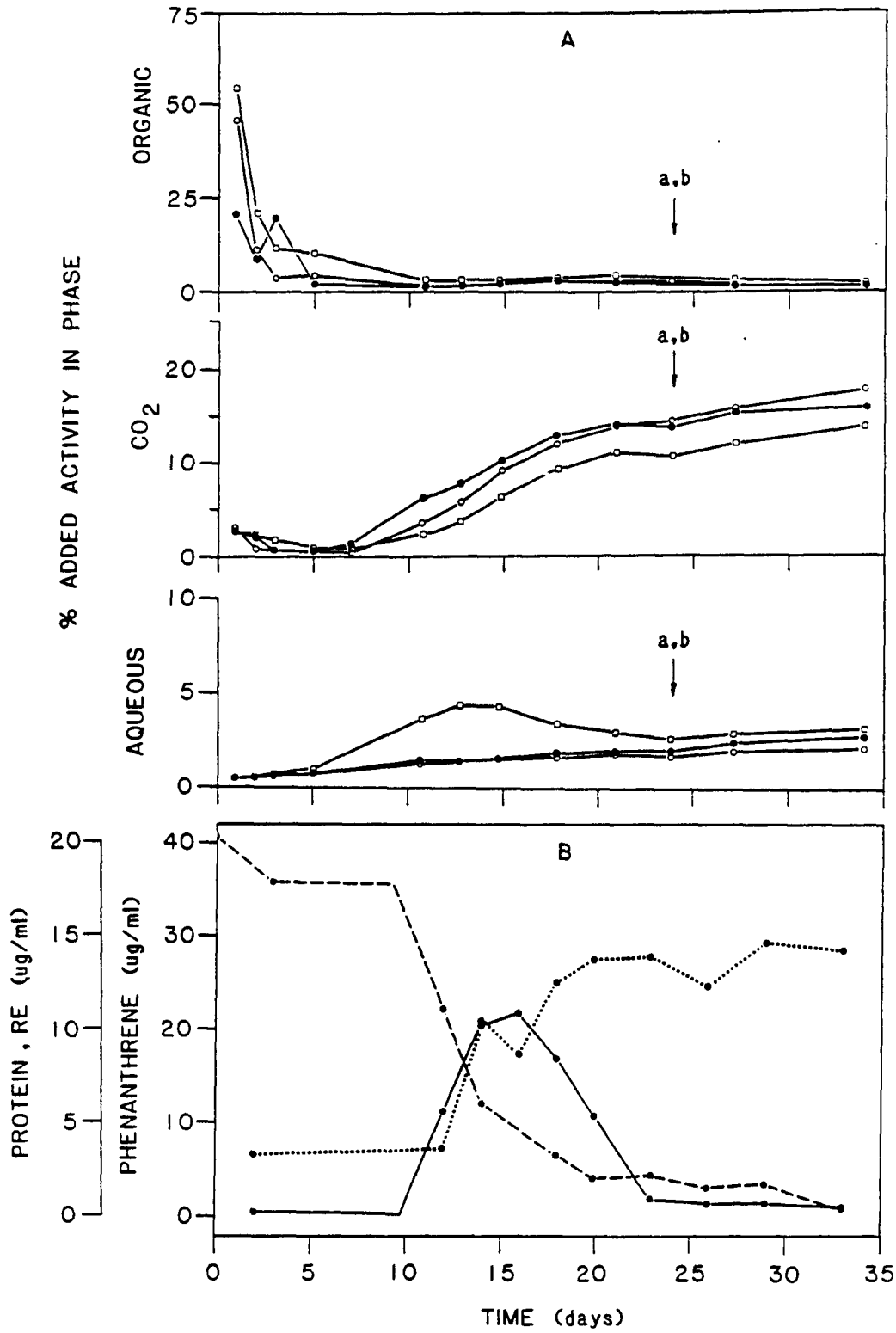
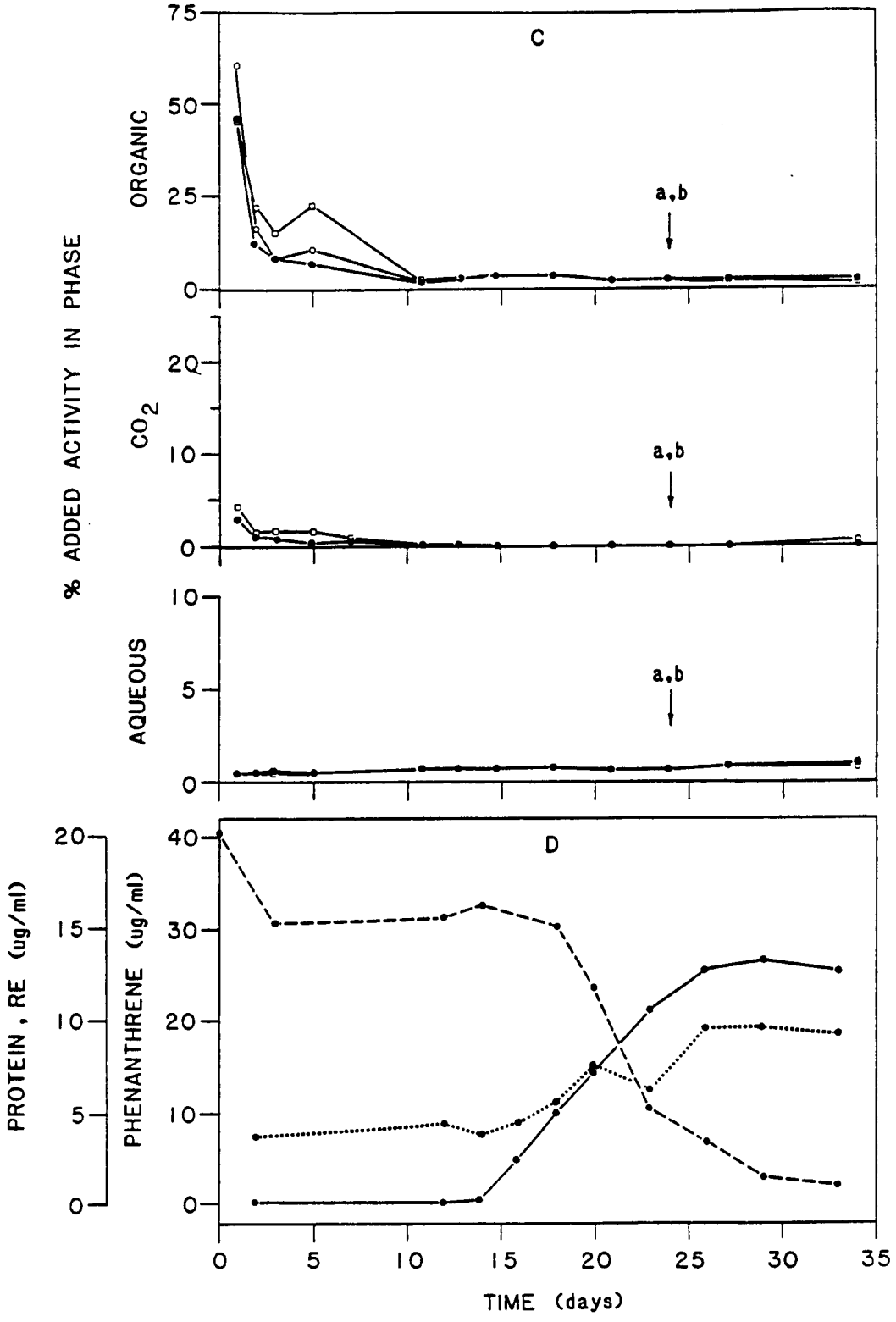

A
ORGANIC
a,b
CO_2
AQUEOUS
% ADDED ACTIVITY IN PHASE
B
PROTEIN, RE (ug/ml)
PHENANTHRENE (ug/ml)
TIME (days)

FIGURE 31. (cont.)


C
ORGANIC
CO_2
AQUEOUS
% ADDED ACTIVITY IN PHASE
a,b
D
PHENANTHRENE (ug/ml)
PROTEIN, RE (ug/ml)
TIME (days)

bound, unaltered ^{14}C-phenanthrene and ^{14}C incorporated into biomass, thus precluding a complete mass balance. However, the distribution of radioactivity in solvent extractable, water soluble and respired forms was measured during triplicate incubations of PP microlayer and bulk water samples with ^{14}C-phenanthrene (Fig. 31).

Figure 31a,c illustrates the results of triplicate incubations of PP microlayer and bulk water samples with ^{14}C-phenanthrene and shows that besides providing precise estimates of mineralized phenanthrene, the method also reproducibly estimates the degradation potential of a given water sample. The $^{14}CO_2$ evolution curves for the three microlayer flasks were similar, each showing a lag period of about 7 d and 24 d mineralization efficiencies between 11 and 14%. Addition of nutrients at 24 d (arrow "a") led to moderate increases in mineralization. The replicate flask with the lowest mineralization efficiency showed a production and consumption curve for water soluble radioactive intermediates typical of mixed phenanthrene degrading cultures (Guerin and Jones, subm.). 1-Hydroxy-2-naphthoic acid is the major water soluble intermediate formed in such incubations. The other two microlayer samples accumulated water soluble activity at a slow rate over the course of the incubations.

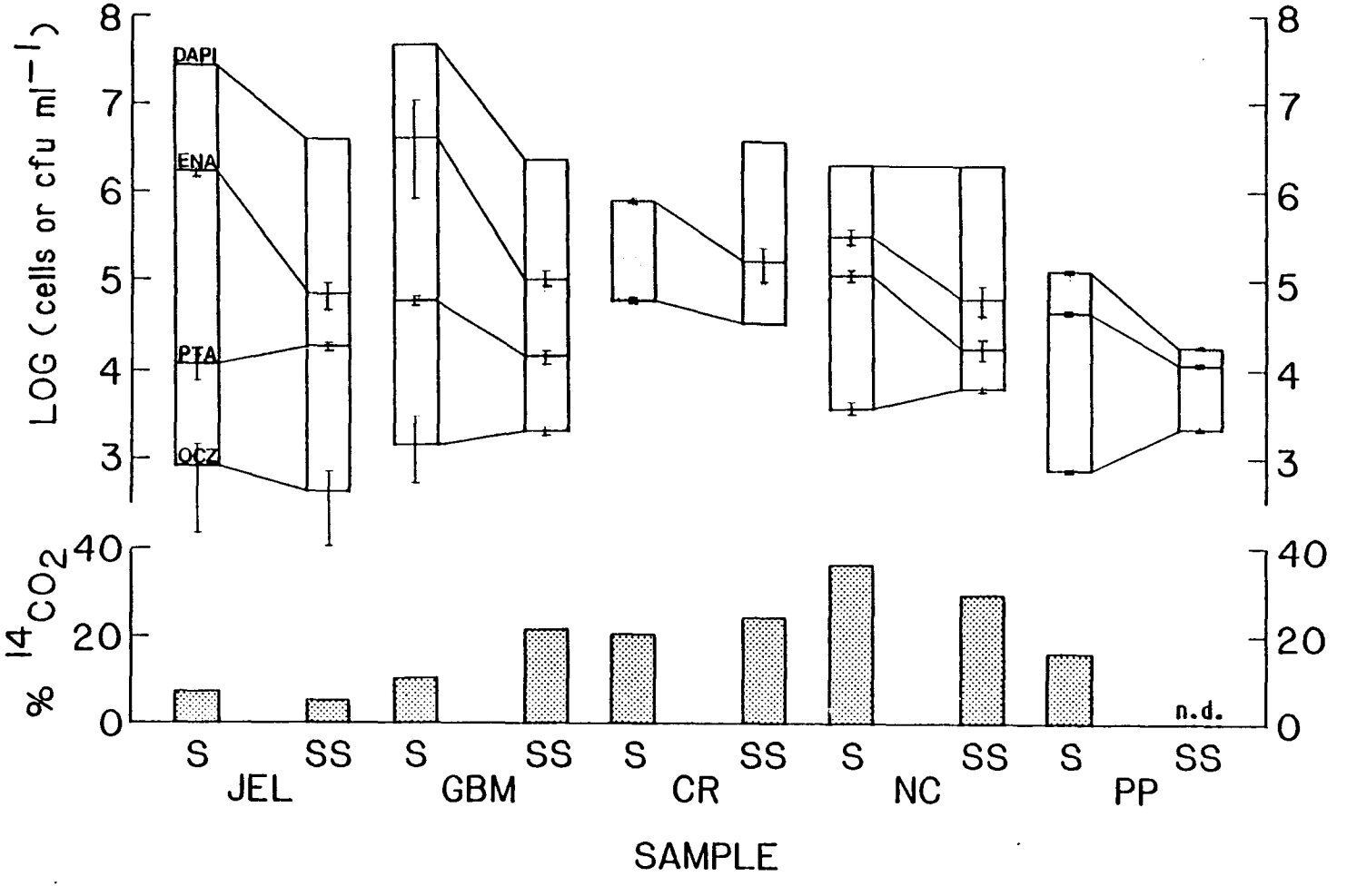
The PP bulk water samples were uniform in showing no phenanthrene degrading activity nor were they stimulated by the addition of nutrients at 24 d. However, 10 ml samples were also used to inoculate phenanthrene/Tween 80 medium with initial phenanthrene concentrations of 40 mg L^{-1} (Fig. 31b,d). Both the PP microlayer and bulk water samples degraded the phenanthrene in these flasks in the two-stage pattern typical of mixed cultures at high phenanthrene concentrations.

Degradation occurred with a shorter lag period, a lower peak metabolite (RE) concentration and a higher protein yield in the microlayer sample (Guerin and Jones, subm.). Phenanthrene degradation and microbial growth were more sluggish in the bulk water sample.

When bulk water samples from JEL or elsewhere in the estuary are assayed for phenanthrene biodegradation potential by adding (^{14}C-spiked) phenanthrene to undiluted water samples, all show substantial $^{14}CO_2$ production indicating the ubiquity of phenanthrene degrading bacteria in some numbers. Since biodegradation rates are proportional to the size of the biodegrading population, the fact that significant ^{14}C-phenanthrene mineralization was observed in many of the samples assayed here, despite a 20-fold dilution, indicates that highly active phenanthrene degrading populations occupy portions of the estuary in the warm summer and early fall months. The use of small inocula and low substrate concentrations in this study perhaps allowed finer resolution of differences in the phenanthrene biodegradation potentials in inter-sample comparisons while still providing high intra-sample reproducibility.

Bacterial counts were of limited help in explaining the phenanthrene mineralization results observed. DAPI direct epifluorescence counts were consistently higher in microlayer samples on a per volume basis, although only slightly so in the NC sample (Fig. 32). Viable heterotrophs on ENA were also more numerous in microlayer samples and gave numbers one to two orders of magnitude lower than DAPI counts. Similar to the observations of Fehon and Oliver (1979), microlayers contained a higher proportion of pigmented cells than the corresponding bulk water. In the low salinity CR sample, the

FIGURE 32. Log (cells or cfu ml^{-1}) as estimated by DAPI direct counts, plate counts on ENA or PTA, and overlay clearing zones (OCZ) on PTA for paired surface microlayer (S) and subsurface bulk water (SS) samples from five locations in the Great Bay Estuary. Error bars represent ± 1 s.d. about the mean of quadruplicate counts. On the bottom are shown the final ^{14}C-phenanthrene mineralization efficiencies for the samples.


LOG (cells or cfu ml⁻¹)
8
7
6
5
4
3
DAPI
ENA
PTA
OCZ
% ¹⁴CO₂
40
20
0
n.d.
S
SS
JEL
GBM
CR
NC
PP
SAMPLE

microlayer and bulk water contained ($\bar{x} \pm 1$ s.d.) 54±10% and 32±16% pigmented colony forming units (CFU), respectively. For the marine NC sample, 42±19% and 15±6% of the microlayer and bulk water CFU, respectively, were pigmented. Among the summer samples, the numbers of organisms by these estimates were lowest in the NC sample which showed the highest phenanthrene degrading activity. Interestingly, the discrepancy between DAPI direct counts and ENA viable counts was greater in bulk waters than in microlayers in samples where both estimations were made (Fig. 32). By this criterion (Norkrans, 1980), bulk water populations appeared more stressed than microlayer populations.

Viable plate counts on PTA were generally highest in samples with high phenanthrene mineralization activity. However, the relative abundance of organisms growing on PTA in microlayer vs. bulk water samples was often the reverse of their phenanthrene biodegradation potentials, thus limiting the usefulness of this parameter to less specific comparisons. Due to the volatility of toluene and the insolubility of phenanthrene, PTA probably provided as much selection for oligotrophic bacteria as for aromatic hydrocarbon degraders (Mallory and Saylor, 1983). Counts on PTA were sometimes as much as 100 fold lower than counts on ENA, the difference being greatest in microlayer samples. Colony size on PTA was generally quite small.

Overlay clearing zones (OCZ) on PTA plates provided a reasonable correlation with phenanthrene biodegradation potentials expressed as the maximum fraction of ^{14}C-phenanthrene mineralized. While not always predicting the "right" relationship between microlayer and bulk water (eg., higher OCZ in NC and PP bulk waters), the OCZ per ml, when

plotted against percent mineralization, gave a correlation coefficient of 0.7 (n = 8, Fig. 33). This translates into a coefficient of determination (r^2) which allows 50% of the variability in degradation potential to be explained by differences in OCZ number. Perhaps this is as good as can be expected considering that these cultural methods do not allow for species interactions or the expression of degradation potential by auxotrophic organisms which are certain to be involved in phenanthrene degradation in nature and in mixed cultures (Harder, 1981).

Part of the lack of correlation between ^{14}C-phenanthrene biodegradation potentials and plate count data may be related to the salinity of the media employed. Twenty fold dilutions of the inocula in ^{14}C biodegradation assays, while serving to normalize the chemical environment and accentuate biological differences, may have drastically affected the activities of mesohaline organisms. Table 15 shows the effects of salinity of the medium on the viable plate counts of low salinity (CR) and marine (NC) microlayer and bulk water samples. Counts on both ENA and PTA were higher on media prepared with 25% ASW for the CR sample and higher on media prepared with 75% ASW for the NC sample. The routine use of media prepared with 75% ASW in both the ^{14}C-phenanthrene mineralization assays and plate count enumerations may have more significantly underestimated the activities of low salinity than of high salinity samples. Furthermore, differential inhibition imposed by high salinity may have affected liquid and solid phase cultures.

In conjunction with additional tin data, the temporal and spatial distributions of phenanthrene degrading activity in the Great Bay

FIGURE 33. Relationship between the final ^{14}C-phenanthrene mineralization efficiencies and the number of overlay clearing zones after colony development on PTA for four paired surface microlayer and bulk water samples.

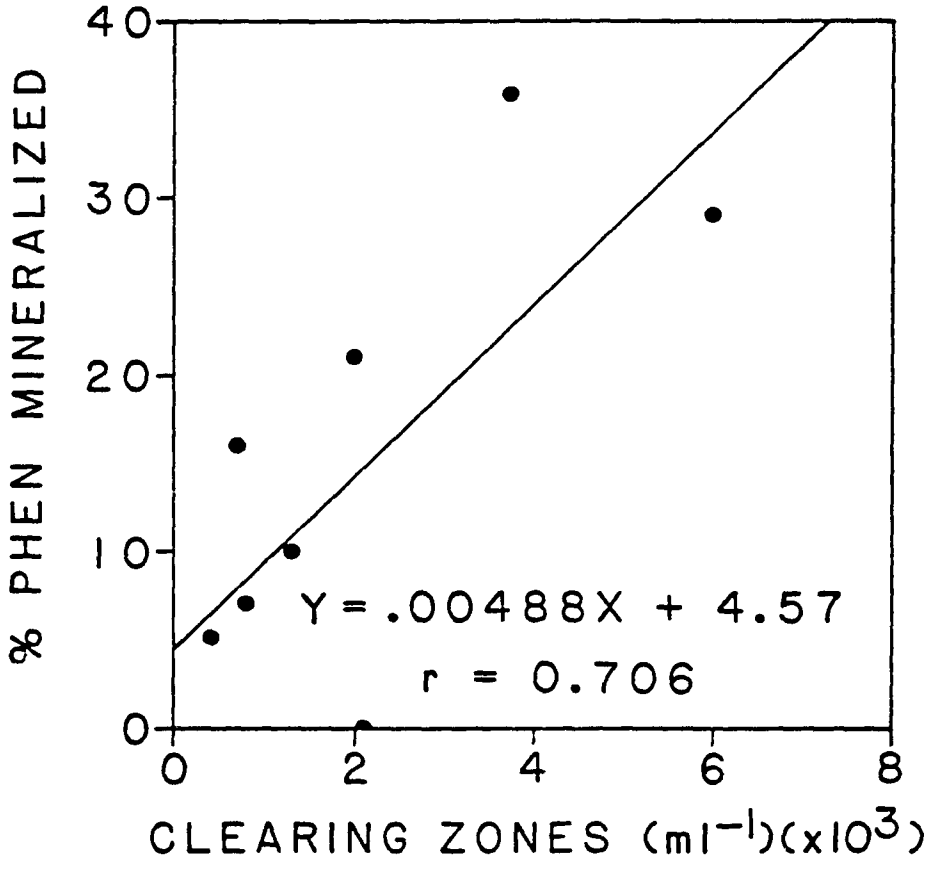

40
30
20
10
0
% PHEN MINERALIZED
Y = .00488X + 4.57
r = 0.706
0
2
4
6
8
CLEARING ZONES (ml^{-1})(x10^3)

TABLE 15. Numbers of culturable heterotrophs in marine and estuarine water samples as influenced by the salinity of the medium.

Sample	Salinity	Depth*	Medium	CFU per ml on agar prepared with 25% ASW	CFU per ml on agar prepared with 75% ASW
CR	3.5 ‰	S	ENA	**7.4×10^5**	2.3×10^4
			PTA	**5.7×10^4**	1.4×10^4
		SS	ENA	**1.5×10^5**	6.0×10^4
NC	33.5 ‰	S	ENA	1.2×10^4	**3.2×10^5**
			PTA	3.6×10^4	**1.1×10^5**
		SS	ENA	9.6×10^3	**6.4×10^4**
			PTA	3.8×10^3	**1.7×10^4**

* S, Surface microlayer; SS, Subsurface bulk water.

Estuary strongly implicate marinas as reservoirs of hydrocarbons and hydrocarbonoclastic bacteria (Guerin and Donard, unpublished data). Although the surface microlayers of such environments are hydrocarbon-enriched, they are often depleted with respect to bacteria which can degrade them. Others have made similar observations. Passman et al. (1979) found no significant differences in the hydrocarbonoclastic activities of microlayer and bulk water microbial populations in the North Atlantic but found that the activities of both covaried geographically. Fehon and Oliver (1977, cited in Norkrans, 1980) found that bulk water bacteria were more active crude oil degraders than the bacterioneuston in a highly polluted area. The predominant bacterial species in surface films have been found to be proteolytic or amylolytic rather than hydrocarbonoclastic (Bartha and Atlas, 1977). However, Rambeloarisoa et al. (1984) found extremely active hydrocarbon degraders in sea surface foams formed at the outlet of a petroleum refinery.

In addition to the potentially lethal effects of radiation, other factors may limit the numbers or activity of hydrocarbon degrading bacteria in estuarine surface microlayers. In marinas, the presence of toxic hydrocarbons in oil, gas or condensed exhaust (Hodson et al, 1977) or the accumulation of toxic organotin compounds in the surface film (Hallas and Cooney, 1981; Walsh, 1985) may contribute. The formation of reactive photooxidation products from hydrocarbon surface films may also be important (Larson et al, 1977). Unlike the situation in pelagic environments, microbial hydrocarbon degradation in the estuarine surface microlayers examined did not appear to be limited by the availability of nutrients or carbon substrates.

REFERENCES

Bartha, R. and R.M. Atlas. 1977. The microbiology of aquatic oil spills. Adv. Appl. Microbiol. 22:225-266.

Box, J.D. 1983. Investigation of the Folin-Ciocalteau phenol reagent for the determination of polyphenolic substances in natural waters. Water Res. 17:511-525.

Carlson, D.J. and L.M. Mayer. 1980. Enrichment of dissolved phenolic material in the surface microlayer of coastal waters. Nature 286:482-483.

Carlson, D.J. 1982a. Phytoplankton in marine surface microlayers. Can. J. Microbiol. 28:1226-1234.

Carlson, D.J. 1982b. A field evaluation of plate and screen microlayer sampling techniques. Mar. Chem. 11:189-208.

Carlson, D.J. and L.M. Mayer. 1983. Relative influences of riverine and macroalgal phenolic materials on UV absorbance in temperate coastal waters. Can. J. Fish. Aq. Sci. 40:1258-1263.

Cosovic, B., V. Zutic, V. Vojvodic and T. Plese. 1985. Determination of surfactant activity and anionic detergents in seawater and sea microlayer in the Mediterranean. Mar. Chem. 17:127-139.

Dahlback, B., M. Hermansson, S. Kjelleberg and B. Norkrans. 1981. The hydrophobicity of bacteria - an important factor in their initial adhesion at the air-water interface. Arch. Microbiol. 128:267-270.

De Souza-Lima, Y. and J.-C. Romano. 1983. Ecological aspects of the surface microlayer. I. ATP, ADP, AMP contents, and energy charge ratios of microplanktonic communities. J. Exp. Mar. Biol. Ecol. 70:107-122.

Dietz, A.S., L.J. Albright and T. Tuominen. 1976. Heterotrophic activities of bacterioneuston and bacterioplankton. Can. J. Microbiol. 22:1699-1709.

Donard, O.F.X., W.F. Guerin, F.T. Short, S. Rapsomanikis and J.H. Weber. 1985. Alkyltins in the Great Bay Estuarine Ecosystem (New Hampshire). Proceedings International Conference on Heavy Metals in the Environment. Athens, Greece.

Duce, R.A., J.G. Quinn, C.E. Olney, S.R. Piotrowicz, B.J. Ray and T.L. Wade. 1972. Enrichment of heavy metals and organic compounds in the surface microlayer of Narragansett Bay, Rhode Island. Science 176:161-163.

Dutka, B.J. and K.K. Kwan 1978. Health-indicator bacteria in water-surface microlayers. Can. J. Microbiol. 24:187-188.

Fedorak, P.M., J.M. Foght and D.W.S. Westlake. 1982. A method for monitoring mineralization of ^{14}C-labeled compounds in aqueous samples. Water Res. 16:1285-1290.

Fehon, W.C. and J. D. Oliver. 1979. Taxonomy and distribution of surface microlayer bacteria from two estuarine sites. Estuaries 2:194-197.

Fox, L.E. 1983. Geochemistry of humic acids during estuarine mixing. pp. 407-427, In R.F. Christman and E.T. Gjessing (ed.), Aquatic and Terrestrial Humic Substances. Ann Arbor Science, Ann Arbor, MI.

Fuhs, G.W. 1982. Microbiota in surface films: An historical perspective. J. Great Lakes Res. 8:312-315.

Gearing, P.J. and J.N. Gearing. 1982. Transport of no. 2 fuel oil between water column, surface microlayer and atmosphere in controlled ecosystems. Mar. Environ. Res. 6:133-143.

Gjessing, E.T. and L. Berglind. 1981. Adsorption of PAH to aquatic humus. Arch. Hydrobiol. 92:24-30.

Glibert, P.L. and T.C. Loder. 1977. Automated analysis of nutrients in seawater. A manual of techniques. Woods Hole Oceanographic Institution Technical Report WHOI-77-47.

Hallas, L.E. and J.J. Cooney. 1981. Effects of stannic chloride and organotin compounds on estuarine microorganisms. Dev. Ind. Microbiol. 22:529-535.

Hansen, H.P. 1975. Photochemical degradation of petroleum hydrocarbon surface films on seawater. Mar. Chem. 3:183-195.

Harder, W. 1981. Enrichment and characterization of degrading organisms. pp. 77-96, In T. Leisinger, R. Hutter, A.M. Cook, and J. Nuesch (ed.), Microbial Degradation of Xenobiotics and Recalcitrant Compounds. Academic Press, London.

Hardy, J.T., C.W. Apts, E.A. Crecelius and N.S. Bloom. 1985. Sea surface microlayer metals enrichment in an urban and rural bay. Est. Coast. Shelf Sci. 20:299-312.

Hatcher, R.F. and B.C. Parker. 1974. Microbiological and chemical enrichment of freshwater-surface microlayers relative to the bulk-subsurface water. Can. J. Microbiol. 20:1051-1057.

Hayase, K. and H. Tsubota. 1983. Sedimentary humic acid and fulvic acid as surface active substances. Geochim. Cosmochim. Acta 47:947-952.

Henrichs, S.M. and P.M. Williams. 1985. Dissolved and particulate amino acids and carbohydrates in the sea surface microlayer. Mar. Chem. 17:141-163.

Hodson, R.E., F. Azam and R.F. Lee. 1977. Effects of four oils on marine bacterial populations: Controlled ecosystem pollution experiment. Bull. Mar. Sci. 27:119-126.

Horrigan, S.G., A.F. Carlucci and P.M. Williams. 1981. Light inhibition of nitrification in sea-surface films. J. Mar. Res. 39:557-565.

Hunter, K.A. and P.S. Liss. 1981. Organic sea surface films. pp. 259-298, In E.K. Duursma and R. Dawson (ed.), Marine Organic Chemistry, Elsevier, Amsterdam.

Karickhoff, S.W., D.S. Brown and T.A. Scott. 1979. Sorption of hydrophobic pollutants on natural sediments. Water Res. 13:241-248.

Kattner, G., K. Nagel, K. Eberlein and K.-D. Hammer. 1985. Components of natural surface microlayers and subsurface water. Oceanologica Acta 8:175-183.

Kester, D.R., I.W. Duedall, D.N. Connors and R.M. Pytkowicz. 1967. Preparation of artificial seawater. Limnol. Oceanog. 12:176-179.

Larson, R.A., L.L. Hunt and D.W. Blankenship. 1977. Formation of toxic products from a #2 fuel oil by photooxidation. Environ. Sci. Tech. 11:492-496.

Larsson, K., G. Odham and A. Sodergren. 1974. On lipid surface films on the sea.I. A simple method for sampling and studies of composition. Mar. Chem. 2:49-57.

Lion, L.W., R.W. Harvey, L.Y. Young and J.O. Leckie. 1979. Particulate matter. Its association with microorganisms and trace metals in an estuarine salt marsh microlayer. Environ. Sci. Tech. 13:1522-1525.

Lion, L.W. and J.O. Leckie. 1981. The biogeochemistry of the air-sea interface. Ann. Rev. Earth Planet. Sci. 9:449-486.

Loder, T.C. and P.M. Glibert. 1977. Automated analysis of nutrients in seawater. A manual of techniques. Woods Hole Oceanographic Institution Technical Report WHOI-77-47.

Loder, T.C. and P.M. Glibert. 1980. Nutrient variability and fluxes in an estuarine system. pp. 111-122, In V.S. Kennedy (ed.), Estuarine Perspectives. Academic Press, New York.

Mallory, L.M. and G.S. Sayler. 1983. Heterotrophic bacterial guild structure: Relationship to biodegradative populations. Microb. Ecol. 9:41-55.

Marshall, K.C. 1976. Interfaces in Microbial Ecology. Harvard University Press, Cambridge, Mass.

Mathieson, A.C. and E.J. Hehre. 1986. A synopsis of New Hampshire seaweeds. Rhodora (in press).

Meyers, P.A. and O.E. Kawka. 1982. Fractionation of hydrophobic organic materials in surface microlayers. J. Great Lakes Res. 8:288-298.

Neff, J.M. 1979. Polycyclic Aromatic Hydrocarbons in the Aquatic Environment. Applied Science Publishers, Ltd., London. 262 pp.

Norkrans, B. 1980. Surface microlayers in aquatic environments. Adv. Microbial Ecol. 4:51-85.

Passman, F.J., T.J. Novitsky and S.W. Watson. 1979. Surface microlayers of the North Atlantic: Microbial populations, heterotrophic and hydrocarbonoclastic activities. pp. 214-226, In A.W. Bourquin and P.H. Pritchard (ed.), Microbial Degradation of Pollutants in Marine Environments. EPA-600/9-79-012.

Porter, K.G. and Y.S. Feig. 1980. The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanog. 25:943-948.

Rambeloarisoa, E., J.F. Routani, G. Giusti, Z. Duvnjak and J.C. Bertrand. 1984. Degradation of crude oil by a mixed population of bacteria isolated from sea-surface foams. Mar. Biol. 83:69-81.

Readman, J.W., R.F.C. Mantoura, M.M. Rhead and L. Brown. 1982. Aquatic distribution and heterotrophic degradation of polycyclic aromatic hydrocarbons (PAH) in the Tamar Estuary. Est. Coast. Shelf Sci. 14:369-389.

Sieburth, J.McN., P.-J. Willis, K.M. Johnson, C.M. Burney, D.M. Lavoie, K.R. Hinga, D.A. Caron, F.W. French III, P.W. Johnson and P.G. Davis. 1976. Dissolved organic matter and heterotrophic microneuston in the surface microlayers of the North Atlantic. Science 194:1415-1418.

Sholkovitz, E.R. 1976. Flocculation of dissolved organic and inorganic matter during the mixing of river water and seawater. Geochim. Cosmochim. Acta 40:831-845.

Steen, W.C. and S.W. Karickhoff. 1981. Biosorption of hydrophobic organic pollutants by mixed microbial populations. Chemosphere 10:27-32.

Stewart, A.J. and R.G. Wetzel. 1981. Asymmetrical relationships between absorbance, fluorescence, and dissolved organic carbon. Limnol. Oceanog. 26:590-597.

Walsh, G.E., L.L. McLaughlan, E.M. Lores, M.K. Louie and C.H. Deans. 1985. Effects of organotins on growth and survival of two marine diatoms, Skeletonema costatum and Thalassiosira pseudonana. Chemosphere 14:383-392.

Wijayaratne, R.D. and J.C. Means. 1984. Sorption of polycyclic aromatic hydrocarbons by natural estuarine colloids. Mar. Environ. Res. 11:77-89.

CONCLUSIONS

This work was undertaken with the purpose of gaining a better understanding of the microbiological fate of polycyclic aromatic hydrocarbons (PAH) in estuarine environments. Although a considerable body of knowledge concerning the biochemistry of PAH degradation by pure cultures of bacteria existed, its application to environmental processes was questionable. Specifically, it had been found in most pure culture studies with phenanthrene that 1-hydroxy-2-naphthoic acid (1H2NA) accumulated as a major degradation product. If this was also characteristic of phenanthrene degradation by mixed environmental samples, then environmental fate assessments based on rates of phenanthrene disappearance or the production of $^{14}CO_2$ from interior ring-labeled phenanthrene would be erroneous. The first two chapters of this thesis addressed questions concerning the physiology of phenanthrene degradation by pure and mixed microbial cultures.

The problem of extrapolating results obtained with pure cultures to processes mediated by mixed microbial populations was complicated by the isolation of an unusual phenanthrene degrading bacterium. Mycobacterium strain BG1, isolated from estuarine sediment mineralized phenanthrene without accumulating 1H2NA or other aromatic intermediates. Mineralization was most likely a plasmid-encoded function in this organism and in many other isolates which lost the degradative phenotype after subculture in nutrient or defined media lacking phenanthrene. The question then became not only **whether**, but also **which** results with pure cultures could be extended to mixed

cultures.

Mixed microbial cultures, like most pure cultures, degraded phenanthrene with the concomitant accumulation of 1H2NA. Unlike pure cultures, however, 1H2NA was subsequently degraded by mixed cultures after the phenanthrene was exhausted. The resultant two-stage pattern of phenanthrene mineralization was common in all enrichment cultures examined whether sediment, water or soil was used as the inoculum. An examination of the literature on the microbial degradation of other two-, three-, four- and five-ringed PAH revealed a commonality in the metabolism, perhaps cometabolism, of these compounds. That is, o-hydroxy, carboxy intermediates accumulate during the end ring cleavage of naphthalene, anthracene, benz(a)anthracene and, presumably, benzo(a)pyrene in a manner directly analagous to that observed for phenanthrene.

Metabolite (1H2NA) production by mixed microbial cultures was concentration dependent. At low initial phenanthrene concentrations (0.5 to 1 $\mu g\ L^{-1}$), little of the intermediate was produced and $^{14}CO_2$ production was a good index of degradative activity. It should be stressed here however, that although only low concentrations of intermediates were present, concentrations may have been sufficient in the batch culture systems employed, to induce synthesis of enzymes involved in intermediate degradation. In natural aquatic environments, intermediates would be free to diffuse or be advected away from degrading cells and thereby never attain concentrations sufficient to trigger their degradation. A principal fate for PAH in nature may be the incorporation of water soluble intermediates into the pool of structurally-related dissolved organic materials.

The second two chapters of this thesis contained the results of field studies aimed at determining the activity and distribution of phenanthrene degrading bacteria in the estuarine environment. In chapter three, it was established that previous exposure to PAH hydrocarbons, to the extent that it selected for a PAH-adapted microbial community, was of overwhelming importance in determining the potential of a given water or sediment sample to degrade phenanthrene under assay conditions. By comparison, the nutrient status, particulate matter concentration, dissolved organic carbon content, salinity and pH were of minor importance. Of particular interest was the microbial population downstream from a dredging operation in the Cocheco River which degraded phenanthrene with little or no lag period. The disturbance of a coal tar-laden bed of sediments in the river resulted in the release of enormous quantities of PAH and the development of an active PAH-degrading microbial population downstream. Marinas were also hot spots of phenanthrene-degrading activity due to PAH inputs related to boating activities.

The multiplicity of point and non-point inputs of PAH in the Great Bay Estuary ensured that PAH-degrading bacteria were ubiquitous. Phenanthrene degradation potentials decreased seaward and no activity was observed in samples collected in the Gulf of Maine during two summer cruises. While similar trends in the estuarine distribution of xenobiotic degradative activities have been ascribed to direct detrimental effects of salinity on freshwater organisms, it is likely, in the case of PAH, that the degradation gradient observed was more a reflection of the extent of dilution of the estuarine water sample by seawater previously unadapted to PAH. Indeed, evidence for a mid-

salinity optimum in phenanthrene degrading activity was obtained during estuarine transects on two separate occasions.

As a special case, an examination of the phenanthrene degrading activity of paired surface microlayer and bulk water microbial populations was conducted. Surface microlayers were consistently enriched in nutrients, dissolved organic carbon and numbers of viable heterotrophs culturable on nutrient media. Several microlayer samples, collected in or near marinas, were also enriched with fluorescent, presumably PAH, hydrocarbons. These showed no consistent enrichment of phenanthrene degrading activity suggesting inhibition of the microlayer population by some unidentified chemical or physical component. Interestingly, marina surface microlayer samples were highly enriched in organotin compounds employed as active agents in marine antifouling paints. A highly significant relationship was also observed between numbers of bacteria growing on phenanthrene/toluene agar and numbers of bacteria resistant to dimethyltin chloride in water samples collected during two surveys of the estuary. Marinas, as sources of both tin and PAH, thus appear to influence profoundly the microbiolgy of the Great Bay Estuary.