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RESEARCH NOTES

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CO-OXIDATION OF PETROLEUM HYDROCARBONS BY ESTUARINE MICROORGANISMS

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Co-oxidation is a little understood means of microbial degradation of petroleum hydrocarbons. In co-oxidation, microbes growing on one compound, the growth substrate, are able to oxidize a second compound, the cosubstrate. This project examines the role of co-oxidation in the degradation of polycyclic aromatic hydrocarbons (PAH's), one of the most recalcitrant petroleum compounds now found in the Chesapeake Bay.

Cooney and Shiaris first developed an assay procedure for detecting bacteria and fungi that degrade PAH's in the presence or absence of organic substrates. Using this assay, they found preliminary evidence that co-oxidation may be occurring among complex hydrocarbon mixtures such as those found in the sediments of the estuary.

For their experiments, they chose phenanthrene as cosubstrate, a PAH difficult to degrade because of its high molecular weight. When they exposed water and sediment samples to phenanthrene and potential growth substrates, they found no degradation in the water samples. Some degradation of phenanthrene, however, did occur among sediment samples in the presence of a yeast-peptone substrate. According to Cooney, this is "strong, indirect evidence" that co-oxidation may be occurring in estuarine sediments. Since the difficult-to-degrade compounds inevitably sink to the estuary floor, co-oxidizing micro-organisms could be a factor in petroleum degradation.

-- The Editors

INTRODUCTION

Recently, co-oxidation and the more general phenomenon of cometabolism have been the subject of several reviews and critiques. It is generally accepted that cometabolism may play an important role in the degradation of persistent compounds, such as aromatic pollutants, in aquatic ecosystems. Yet, the extent of cometabolism of these compounds under *in situ* conditions is not known. Even less is known of how cometabolism may be manipulated to enhance biodegradation in aquatic environments.

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Polycyclic aromatic hydrocarbons (PAH's) are a class of persistent compounds that may be degraded by cometabolic mechanisms in the environment. PAH's are the subject of increasing concern as natural products and pollutants of marine ecosystems. Recent reviews have summarized the growing list of toxic and mutagenic effects caused by some PAH's to marine biota and potentially to man.

The more water soluble aromatic hydrocarbons (naphthalenes and phenanthrenes) have been implicated as the most toxic fraction of petroleum spills. PAH's are subject to degradation in the marine environment through a variety of mechanisms; the most prevalent are photooxidation, chemical oxidation, and biological transformation. However, since the majority of natural PAH-degrading mechanisms require molecular oxygen, they may persist indefinitely in oxygen-poor waters and sediments. In addition, the large negative resonance energy of the aromatic nucleus renders PAH's resistant to microbial degradation.

Little is known of the biodegradative fate of PAH's in marine systems. PAH's are often incompletely oxidized by microorganisms, leading to the general assumption that many PAH's are degraded in natural systems by a process of cometabolism. Work from several laboratories has shown that degradation of a number of hydrocarbons can proceed by cometabolic mechanisms. Most of these investigations, however, utilized selected pure cultures and highly purified substrates under optimal laboratory conditions. Only two recent reports provide compelling evidence that chlorinated pollutants are cometabolized *in situ*. Therefore, studies are lacking which assess the extent of PAH transformation and the fate of PAH's by natural populations of microbiota in aquatic sediment.

Hence, this study was undertaken to determine if the ability to cometabolize aromatic hydrocarbons is prevalent among estuarine microorganisms.

MATERIALS AND METHODS

Ten sites were examined in the central and northern Chesapeake Bay and its tidal tributary rivers from February through July, 1980. At each site, three separate sediment grabs were taken with a Van Veen sampler. With a sterile spatula, the surface 1 cm of the sediment (approximately 150 g net weight) was collected into glass bottles with aluminum foil-lined screw caps.

At each sampling site, the following physico-chemical parameters were determined: pH, sediment and water temperature, water depth, salinity (Beckman Model RS5-3 meter), and dissolved oxygen (YSI dissolved oxygen probe). Sediment dry weight was determined by standard gravimetric methods.

Phenanthrene agar (PA) was prepared by spreading phenanthrene on the surface of pre-poured and air-dried agar plates. Phenanthrene was overlayed on the agar surface by pipetting 0.2 ml of a phenanthrene solution in acetone (5 g liter^{-1}) while plates were spinning on a turntable to distribute the solution evenly over the agar surface. Phenanthrene agar plates were dried overnight at 30 C to evaporate the acetone. Naphthalene and chrysene plates were prepared in the same manner with a 1 g liter^{-1} solution in acetone.

Phenanthrene-degrading isolates were maintained on agar slants. The medium was prepared by adding 0.1 g yeast extract, 0.1 g peptone, 0.1 g dextrose, 0.01 g phenanthrene, and 18 g agar to a liter of estuarine salts solution (pH 7.0).

A standard spread plating technique was used in conjunction with the replica plating method to enumerate phenanthrene degraders and potential phenanthrene

cometabolizers. Water or sediment samples were diluted serially in filter-sterilized estuarine water blanks and plated onto ESWA plates (5 plates per dilution). After three days incubation at 25°C, the majority of visible colonies were less than 1 to 2 mm in diameter. Longer incubation times resulted in larger colonies and spreading colonies which were unsuitable for replica plating.

Plates containing between 300 and 1000 colony-forming units (CFU) were used for replica plating. Each plate was replicated onto four plates containing PA plus one of the following growth substrates at a concentration of 0.1 g liter^{-1}: (1) yeast extract plus peptone (1:1, wt/wt), (2) sodium benzoate and (4) nondetergent motor oil plus kerosene (1:1, vol/vol). A fifth plate containing no additional growth substrate was included to determine phenanthrene utilizers. Plates were incubated at 25°C and examined daily for two weeks for zones of phenanthrene clearing. Organisms in colonies able to degrade phenanthrene without an additional growth source were scored as phenanthrene-utilizers. Organisms which degraded phenanthrene only on the presence of an additional growth source were scored as phenanthrene-cometabolizers.

Several experiments were performed to assess the suitability of the replica plating technique for enumerating phenanthrene degraders and potential phenanthrene cometabolizers.

RESULTS AND DISCUSSION

Replica Plating Techniques

Estuarine salt water agar (ESWA), which promoted small colonies (1-2 mm or less) up to one week incubation, was chosen for the replica plating technique. Zones of phenanthrene clearing were easily discerned on PA plates. Phenanthrene degradation on replica plates and by pure cultures was first observed after two or three days of incubation; and the majority of phenanthrene degraders produced zones of clearing within five days. To insure maximum counts, plates were monitored daily for two weeks and colonies were selected for isolation at that time. (Unsuccessful attempts were made to utilize napththalene and chrysene as polynuclear aromatic hydrocarbon sources in the medium. Naphthalene rapidly evaporated from the agar surface. Chrysene formed a good overlay, but chrysene degraders were not detected in three sediment samples which were positive for phenanthrene degraders. Either chrysene degraders were absent or the rate of chrysene degradation was too slow to form a zone of clearing in the 2-week incubation period.)

Neither the phenanthrene overlay itself nor the acetone used as a carrier solvent for the phenanthrene affected total plate counts on ESWA. Apparently, growth of estuarine microorganisms is not markedly affected by relatively high levels of phenanthrene.

To examine the suitability of the replica plating technique for detecting cometabolizers and utilizers, 75 phenanthrene-degraders were streaked onto ESWA plates for isloation and maintained on ESWA slants. Five of these isolates were chosen for more rigorous examination.

Table 1 presents quantitative data on the biodegradation of phenanthrene by the five isolates in liquid media. Each of the five isolates was capable of

Table 1 Biodegradation of Phenanthrene by Bacterial Isolates in an Estuarine Salts Solution Supplemented with Phenanthrene and Alternate Carbon Sources[1]

Isolate	Alternate Carbon Source[2]	Growth (turbidity)[3]	Phenanthrene Recovery (mg)[4]	Phenanthrene Degradation (% of sterile control)	Total Number of Potential Metabolites[5]
Sterile control	none	0	4.76 ± 0.24	0	0
SPE 18	none	0	4.25 ± 0.66	10.7	0
	yeast extract and peptone	++++	5.22 ± 0.26	0	0
	glucose	N.D.[7]	N.D.[7]	-	N.D.[7]
	benzoate	+	3.71 ± 0.50	22.1[6]	4
	oil	0	5.33 ± 0.18	0	N.D.[7]
	naphthalene	0	5.18 ± 0.33	0	N.D.[7]
B1	none	0	3.84 ± 0.67	19.1	0
	yeast extract and peptone	++++	1.80 ± 0.70	62.2[6]	4
	glucose	++	3.26 ± 0.28	31.5[6]	4
	benzoate	0	3.21 ± 0.52	32.6[6]	4
	oil	+	3.63 ± 0.96	23.7[6]	N.D.[7]
	naphthalene	+	2.73 ± 0.87	42.7[6]	N.D.[7]
B3	none	+	5.11 ± 0.03	0	0
	yeast extract and peptone	+	1.34 ± 0.41	71.8[6]	7
	glucose	+++	1.10 ± 0.29	76.9[6]	9
	benzoate	+	2.84 ± 0.68	40.3[6]	3
	oil	+	3.27 ± 0.38	31.3[6]	N.D.[7]
	naphthalene	N.D.[7]	N.D.[7]	-	N.D.[7]
G20	none	++	3.02 ± 1.00	36.6	1
	yeast extract and peptone	++++	2.07 ± 0.79	56.5[6]	10
	glucose	+++	2.07 ± 0.36	56.5[6]	8
	benzoate	+	4.89 ± 1.07	0	0
	oil	+	3.33 ± 0.54	30.0[6]	N.D.[7]
	naphthalene	N.D.[7]	N.D.[7]	-	N.D.[7]
I1	none	0	4.29 ± 1.21	9.9	0
	yeast extract and peptone	+++	4.45 ± 1.46	6.5	N.D.[7]
	glucose	+++	2.02 ± 0.91	57.6[6]	6
	benzoate	0	3.75 ± 0.80	21.2	0
	oil	N.D.[7]	N.D.[7]	-	N.D.[7]
	naphthalene	+	1.89 0.49	60.2[6]	N.D.[7]

[1] Duplicate Erlenmeyer flasks each contained 100 ml medium plus 50 mg phenanthrene liter^{-1} and were incubated for 2 weeks at 25°C without shaking.

[2] Final concentration, 100 mg liter^{-1}.

[3] The notation used indicates: 0, no turbidity; +, slight turbidity; ++, low turbidity; +++, moderate turbidity; ++++, heavy turbidity.

[4] 5 mg Phenanthrene was initially placed in each flask.

[5] The number of spots observed on TLC plates spotty with ethyl acetate extracts of spent medium. Spots from naphthalene and oil plus kerosene degradation interfered with potential phenanthrene metabolites in extracts from those media.

[6] Significantly different from sterile control ($\alpha < 0.05$).

[7] N.D., not determined.

significant phenanthrene biodegradation under appropriate nutrient conditions. Furthermore, the extent of biodegradation was dependent on the alternate growth substrate. Each isolate displayed a distinct pattern of phenanthrene in the five liquid media. The presence of potential phenanthrene metabolites on TLC plates prepared from the organic extract of the spent liquid medium (Table 1) confirmed the degradation of phenanthrene by the five isolates. Potential phenanthrene metabolites on plates from media containing either oil plus kerosene, or naphthalene were obscured by comigrating spots on the TLC plates from the metabolism of these compounds.

The ability of isolates to degrade phenanthrene under three different experimental conditions is summarized in Table 2. As indicated by replica plating, none of the five isolates was capable of degrading phenanthrene in liquid medium without an additional growth source. Four of the five isolates were not capable of degrading phenanthrene on PA plates unless an additional growth source was provided; isolate G20 was the exception. All the isolates, both in liquid culture or on PA plates degraded phenanthrene when supplied with an appropriate growth substrate. Subsequent growth in a liquid medium containing phenanthrene did not confirm phenanthrene degradation in all cases (Table 1). For example, isolate SPE18 grew well in phenanthrene plus yeast extract and peptone. but significant phenanthrene degradation was not detected. These results confirm that the replica plate method is a reliable approach for estimating the number of phenanthrene cometabolizers in estuarine sediments.

There was, however, little correlation between the pattern of degradation observed initially on replica plates and the degradation of phenanthrene by pure cultures inoculated into liquid medium and streaked on PA plates after the isolates had been transferred several times on laboratory media (Table 2). There was only a 52% correlation between results obtained by the replica plate technique and liquid cultures, and only 41% correlation between replica plates and PA plates inoculated after several transfers on laboratory media. However, an 83% correlation was found between degradation tests of pure cultures on liquid medium and PA plates.

(To evaluate the high occurrence of false positive and negative reactions, an experiment was designed for assessing the reproducibility of the replica plating technique. The number of phenanthrene-degrading microorganisms was consistent in six replications on the same medium; thus it was concluded that the initial replica plates were highly reproducible, and the occurrence of apparent false negative/positive phenanthrene degraders was due to other causes.)

The replica plate method has several advantages over enrichment techniques which have been employed commonly for examining cometabolizers. First, it allows screening of a large number of colonies. Thus, the relative numbers of phenanthrene utilizers and cometabolizers in the total plate count can be determined. Second, the test for phenanthrene degradation is performed on microorganisms only once removed from the natural environment. Third, it is faster and less tedious than methods which require individual screening of large numbers of pure cultures.

The method has limitations. First, plate count procedures underestimate the number of viable organisms; although it is assumed that enumerations of phenanthrene utilizers and cometabolizers are underestimated to the same degree as the total plate counts. Second, the growth substrates used may not include all compounds capable of supporting cometabolism. Third, the method does not distinguish organisms which use yeast extract plus peptone as a carbon source from organisms which obtain micronutrients from yeast extract or peptone. Thus, some of the organisms scored as cometabolizers with yeast extract plus peptone as alternate growth substrates may actually utilize phenanthrene except for vitamin or cofactor deficiencies. This is not the case when glucose, benzoate or oil plus kerosene are employed as growth substrates.

Nevertheless, the method appears useful as a means of screening large numbers of microorganisms and for estimating the relative numbers of phenanthrene degraders and cometabolizers in natural populations.

Phenanthrene Microorganisms in the Chesapeake Bay

The phenomenon of cometabolism is commonly associated with recalcitrant compounds that do not readily serve as sole carbon and energy sources for heterotrophic microorganisms. From an environmental perspective, the transformation and eventual removal of persistent and hazardous compounds from the environment may be the most important consequence of cometabolism. Therefore, a greater understanding of the process may result in the ability to enhance the degradation of chemical pollutants.

Phenanthrene degraders comprised more than 0.1% of the total plate count at only three of the sample sites. But at each of these sites phenanthrene cometabolizers comprised 34 to 67% of the phenanthrene degraders.

Phenanthrene-degrading organisms were not detected in water samples, even at sites where the sediments contained phenanthrene degraders. Thus, phenanthrene degraders and hence microbial phenanthrene-degrading activity appears to be localized predominantly in the sediments of the central and northern Chesapeake Bay.

Each of the four sediment samples which contained 0.1% or more phenanthrene degraders was examined further to obtain information about potential cooxidation. Of 355 phenanthrene degraders detected, 54% utilized phenanthrene as a sole carbon and energy source. Therefore, the remaining 46% were potential phenanthrene cometabolizers. Almost all possible combinations of the four growth substrates were observed to promote phenanthrene degradation. This indicates that a variety of microorganisms with distinct metabolic patterns may be involved in the cometabolism of phenanthrene. Also, a variety of growth substrates can support or stimulate cometabolism. The growth substrates examined stimulated phenanthrene degradation in the descending order: yeast extract plus peptone > benzoate > glucose > oil plus kerosene.

Polynuclear aromatic hydrocarbon recovery based on an internal biphenyl standard ranged between 80.7% and 86.1% for all sediments analyzed. Intrasite variation among replicates indicated considerable variability.

The ten sites were broadly classified into three groups, based on hydrocarbon concentrations when analyzed by the Student-Neuman-Keules (SNK) multiple range test ($\alpha < 0.01$): heavily polluted (Baltimore Harbor), moderately contaminated with hydrocarbons, (Patapsco River, Solomons Harbor, and Patuxent River) relatively unpolluted with respect to hydrocarbons (Gunpowder River, Chester River, West River North, Choptank River and North, and Smith Island). The phenanthrene plus anthracene

concentration from the Baltimore Harbor site, which was located next to an oil refinery, was markedly higher than concentrations at the other sites.

Table 3 contains the partial-moment correlation matrix of the ratio of phenanthrene degraders: total plate count, hydrocarbon concentrations, and other physicochemical parameters at the sites. The ratio of phenanthrene degraders to total plate count was positively correlated (α <0.01) with total hydrocarbon, phenanthrene plus anthracene, and pyrene concentrations, indicating that hydrocarbon-polluted sediments are enriched with a phenanthrene-degrading microbial population. It is interesting that most chronic polynuclear aromatic hydrocarbon pollution occurs near urban centers where domestic pollution rich in potential growth substrates also occurs. Thus, organic compounds from domestic sewage may support cometabolism of polynuclear aromatic hydrocarbons.

While phenanthrene tends to persist in aquatic ecosystems, as compared to the more readily-degradable analogue naphthalene it can serve as a sole carbon source for some microorganisms. But degradation of phenanthrene by bacterial isolates, even under optimal conditions, is relatively slow. PHA cooxidizers comprised a measurable fraction of the total plate count at the sites which contained the largest amount of hydrocarbons. Thus, cometabolism may play a prominent role in the biological transformation of phenanthrene in sediments.

Table 2. Phenanthrene Degradation by Bacterial Isolates on PA Replica Plates, PA Plates, and Liquid Media

Bacterial Isolate	Alternate Growth Source	Phenanthrene Degradation		
		PA Replica[1] Plates	Liquid Medium	PA Plates[2]
SPE18	none	-	-	-
	yeast extract + peptone	+	-	-
	glucose	N.D.[3]	N.D.[3]	-
	benzoate	N.D.[3]	+	+
	oil + kerosene	N.D.[3]	-	-
B1	none	-	-	-
	yeast extract + peptone	-	+	+
	glucose	-	+	+
	benzoate	+	+	+
	oil + kerosene	-	+	+
B3	none	-	-	-
	yeast extract + peptone	+	+	+
	glucose	+	+	+
	benzoate	-	+	-
	oil + kerosene	-	+	+
C20	none	-	-	+
	yeast extract + peptone	-	+	+
	glucose	+	+	-
	benzoate	-	-	-
	oil + kerosene	-	+	+
I1	none	-	-	-
	yeast extract + peptone	+	-	-
	glucose	+	+	+
	benzoate	+	-	+
	oil + kerosene	-	N.D.[3]	-

[1] Initial replica plate prior to bacterial isolation.
[2] Bacteria isolates streaked onto PA plates.
[3] N.D., not determined.

Table 3. Partial-Moment Correlation Coefficient (r^2) Matrix for Physical-Chemical Parameters and PHE Degraders in Chesapeake Bay Sediments

	TEMP	pH	DO	SAL	TPC	PHE	PYR	OIL
TEMP	1.000							
pH	0.355*	1.000						
DO	0.053	0.591**	1.000					
SAL	0.113	0.006	0.118	1.000				
TPC	-0.552**	-0.154	-0.417**	-0.428**	1.000			
PHE	-0.631**	0.367*	-0.372*	-0.155	0.433**	1.000		
PYR	-0.672**	0.106	-0.235	-0.061	0.447**	0.921**	1.000	
OIL	-0.688**	0.176	-0.033	0.583	0.895**	0.895**	0.895**	1.000
RATPHE	-0.684**	-0.224	0.063	0.071	0.245	0.531	0.660**	0.593**

Degrees of freedom = 40

* Significant at $\alpha \leq 0.05$

** Significant at $\alpha \leq 0.01$

Abbreviations: TEMP, temperature; DO, dissolved oxygen; SAL, salinity; TPC, total plate count; PHE, phenanthrene concentration; PYR, pyrene concentration; OIL, neutral hydrocarbon concentration, RATPHE, ratio of PHE degraders: Total plate count.

CONCLUSIONS

The major conclusions of this study are:

1. Replica plating methods are useful for the detection, enumeration, and isolation of phenanthrene degrading and cometabolizing microorganisms.

2. Microorganisms capable of utilizing and cometabolizing phenanthrene are found predominantly in sediments and not in the water column in the Chesapeake.

3. A significant proportion of the estuarine microorganisms that are capable of degrading phenanthrene are potential phenanthrene cooxidizers.

4. Of the growth substrates utilized to support cooxidation of phenanthrene, the order of effectiveness was: yeast extract plus peptone > benzoic acid > glucose > oil plus kerosene.

5. Chesapeake Bay sediments with high concentrations of hydrocarbons were enriched in numbers of phenanthrene-degrading microorganisms.

6. Microbial cometabolism may contribute significantly to phenanthrene transformation in estuarine sediments.