APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1987, p. 129–136
0099-2240/87/010129-08$02.00/0

Vol. 53, No. 1

Naphthalene Biodegradation in Environmental Microcosms: Estimates of Degradation Rates and Characterization of Metabolites

MICHAEL A. HEITKAMP, JAMES P. FREEMAN, AND CARL E. CERNIGLIA*

National Center for Toxicological Research, Food and Drug Administration, Jefferson, Arkansas 72079

Received 24 June 1986/Accepted 15 October 1986

Naphthalene biodegradation was investigated in microcosms containing sediment and water collected from three ecosystems which varied in past exposure to anthropogenic and petrogenic chemicals. Mineralization half-lives for naphthalene in microcosms ranged from 2.4 weeks in sediment chronically exposed to petroleum hydrocarbons to 4.4 weeks in sediment from a pristine environment. Microbiological analysis of sediments indicated that hydrocarbon-utilizing microbial populations also varied among ecosystems and were 5 to 12 times greater in sediment after chronic petrogenic chemical exposure than in sediment from an uncontaminated ecosystem. Sediment from an ecosystem exposed to agricultural chemicals had a mineralization half-life of 3.2 weeks for naphthalene and showed about a 30-fold increase in heterotrophic bacterial populations in comparison to uncontaminated sediments, but only a 2- to 3-fold increase in hydrocarbon-degrading bacteria. Analysis of organic solvent-extractable residues from the microcosms by high-pressure liquid chromatography detected polar metabolites which accounted for 1 to 3% of the total radioactivity. Purification of these residues by thin-layer chromatography and further analysis by gas chromatography-mass spectrometry indicated that *cis*-1,2-dihydroxy-1,2-dihydronaphthalene, 1-naphthol, salicylic acid, and catechol were metabolites of naphthalene. These results provide useful estimates for the rates of naphthalene mineralization in different natural ecosystems and on the degradative pathway for microbial metabolism of naphthalene in freshwater and estuarine environments.

Polycyclic aromatic hydrocarbons (PAHs) are compounds of environmental and human health concern since some PAHs have been shown to be toxic, mutagenic, or carcinogenic (30, 39, 40). Human exposure to PAHs may occur from emissions during the incomplete combustion of fossil fuels or due to accidental discharge into aquatic and terrestrial environments during the transport, use, and disposal of petroleum products (30, 35, 40, 41). Naphthalene, a dicyclic aromatic hydrocarbon, and its methylated derivatives are considered some of the most acutely toxic compounds in the water-soluble fraction of petroleum (1). Exposure to naphthalene has caused a decrease in hemoglobin concentration and inhibited oxygen consumption in various experimental organisms (15, 46).

The microbial metabolism of naphthalene has been studied extensively, and the rate, metabolic pathway, enzymatic reactions, and genetic regulations of naphthalene catabolism have been well documented (4–13, 16–18, 20, 31, 32, 34, 38, 47). However, there are few reports showing the rate and metabolism of naphthalene in experimental systems designed to model natural ecosystems (3, 26, 27, 36, 41). The degradation of chemicals in the environment can be affected by several factors which may differ among ecosystems, such as organic and inorganic nutrient levels, temperature, pH, previous chemical exposure, microbial adaptations, and oxygen tension (2, 22, 33, 45). In addition, many species of bacteria, cyanobacteria, filamentous fungi, and yeasts coexist in natural ecosystems and may act independently or in concert to metabolize aromatic hydrocarbons (5, 19, 20). The purpose of this investigation was to determine the rates of mineralization, the metabolic pathway, and the initial oxidation reactions for naphthalene catabolism in microcosms containing sediment and water collected from three well-characterized ecosystems which differed in past exposure to petrogenic and anthropogenic chemicals.

* Corresponding author.

MATERIALS AND METHODS

Chemicals. Naphthalene and phenanthrene were purchased from Aldrich Chemical Co., Milwaukee, Wis. [1,4,5,8-^{14}C]naphthalene with a specific activity of 5.10 mCi/mmol was purchased from Amersham Corp., Arlington Heights, Ill. *cis*-1,2-Dihydroxy-1,2-dihydronaphthalene (*cis*-naphthalene dihydrodiol) and *trans*-1,2,-dihydroxy-1,2-dihydronaphthalene (*trans*-naphthalene dihydrodiol) were obtained from David T. Gibson, University of Texas at Austin. Catechol, hexadecane, 1-naphthol, and salicylic acid were purchased from Chemical Service, Media, Pa. Solvents for high-pressure liquid chromatography (HPLC) and thin-layer chromatography (TLC) were purchased from Burdick and Jackson Laboratories, Muskegon, Mich. Solvents and chemical standards used in this study were of the highest purity available.

Design of naphthalene biodegradation studies. Biodegradation of naphthalene was monitored in a flowthrough microcosm test system which enabled monitoring of naphthalene mineralization (complete degradation to CO_2) and extraction and recovery of both volatile and nonvolatile metabolites as well as undegraded naphthalene (23, 29). Microcosms consisted of 0.5-liter glass minitanks (Foxboro/Analabs, North Haven, Conn.) containing 20 g of homogenized moist sediment and 180 ml of water collected from three different ecosystems which varied in their watershed development, nutrient levels, and history of previous exposure to chemical contaminants. The pH and sediment organic carbon concentrations for these ecosystems are as follows: DeGray Reservoir, a man-made impoundment in southwestern Arkansas, pH 7.2, 3.2%; Lake Chicot, a natural oxbow lake in southeastern Arkansas, pH 7.6, 6.6%; and Redfish Bay, a natural estuary near Port Aransas, Tex., pH 7.8, 3.5%. Microcosms

were exposed to 0.92 μCi (118 μg) of naphthalene for 8 weeks while being continuously purged with compressed air. The microcosms were mixed twice weekly, and the gaseous effluent from each microcosm was directed through a volatile-organic-trapping column containing 7 cm of polyurethane foam and 500 mg of Tenax GC (Alltech Associates Inc.), and a $^{14}CO_2$-trapping column containing 50 ml of monoethanolamine-ethylene glycol (3:7, vol/vol). The efficiency of polyurethane foam and Tenax GC for trapping volatile organics has been described previously (37, 48). Volatile-organic-trapping columns were replaced after 2, 4, and 8 weeks, and the $^{14}CO_2$-trapping columns were rejuvenated after 4 weeks by adding 8 ml of fresh trapping solution. In addition, the gaseous effluent from some microcosms was directed through secondary volatile-organic- and $^{14}CO_2$-trapping columns to ensure that the primary columns did not become saturated with naphthalene or volatile naphthalene metabolites. Naphthalene mineralization was monitored weekly by placing duplicate 1-ml portions of the $^{14}CO_2$-trapping solution into scintillation vials containing 15 ml of Fluoralloy-methanol (1:1, vol/vol) scintillation cocktail (Beckman Instrument Co., Fullerton, Calif.). Radioactivity was measured with a liquid scintillation counter (model 6881, Mark III; T.M. Analytic, Elk Grove Village, Ill.), and all values were corrected for trapping efficiency, quench, and background. Sterile microcosm controls were achieved by autoclaving for 30 min and adding 1 g of mercuric chloride. Batch experiments were conducted to isolate sufficient quantities of naphthalene metabolites for structure elucidation. In these experiments, five replicate microcosms were exposed to 0.92 μCi (118 μg) and 2 mg of naphthalene for 1 week, and then the supernatants were pooled and extracted as described below.

Linear and nonlinear regression analyses were used to model naphthalene mineralization in microcosms from each ecosystem. The highest correlation coefficients (0.94 to 0.98) were obtained with a nonlinear kinetics model (C. M. Metzler and D. L. Weiner, NONLIN84 Users Guide, Statistical Consultants, Inc., Lexington, Ky., 1984) designed to model experiments with bolus chemical input, no lag phase, and exponential chemical degradation rates. All mineralization values were corrected for naphthalene losses due to volatility, adsorption onto sediment, or permanent incorporation into sediment microorganisms. These statistical analyses enabled the calculation of mineralization half-lives for naphthalene in microcosms from each ecosystem.

Extraction and chemical analysis procedures. Volatile ^{14}C-residues in the polyurethane foam were measured by placing 1-cm slices into scintillation vials containing 15 ml of Scintisol (Isolab Inc., Akron, Ohio), and radioactivity was measured as described above. The volatile ^{14}C-residues were identified in some microcosms by extracting the polyurethane foam with 5 ml of ethyl ether and direct chemical analysis by capillary column gas chromatography-mass spectrometry (GC-MS). Radiolabeled organic residues adsorbed to the Tenax GC and to sediments were combusted to $^{14}CO_2$ in a Packard model B306 Tricarb sample oxidizer (Packard Instrument Co., Downers Grove, Ill.) and counted by liquid scintillation spectrometry.

Direct counting of a 1-ml portion of the microcosm supernatant in a scintillation vial containing 12 ml of Scintisol indicated that most of the ^{14}C-residues remaining after 1 week were in the aqueous phase. Therefore, supernatants from the metabolite enrichment experiments (five microcosms) were pooled after 1 week and extracted with 3 equal volumes of ethyl acetate. The supernatants were acidified to pH 4.0 with a known volume of 0.5 N HCl to enhance the recovery of possible acidic metabolites and reextracted with 3 equal volumes of ethyl acetate. The extracts were pooled, dried with anhydrous Na_2SO_4, and evaporated in vacuo at 35°C to 10 ml, transferred to calibrated glass vials, and evaporated to 500 μl under a gentle stream of dry argon.

Ethyl acetate-extractable residues were separated and purified by TLC and HPLC. All TLC analyses were performed with 500-μM Silica Gel GF plates (Analtech, Newark, Del.), and separation was achieved with either hexane-benzene (1:1, vol/vol), chloroform-acetone (8:2, vol/vol), or hexane-acetone-acetic acid (80:20:1, vol/vol/vol) solvent systems. Autoradiograms to locate ^{14}C-metabolites were produced by exposing XAR-2 X-ray film (Eastman Kodak Co., Rochester, N.Y.) to TLC plates of [^{14}C]naphthalene extracts for 4 weeks. Radioactive spots containing naphthalene metabolites and undegraded naphthalene were scraped from TLC plates, and the compounds were eluted with acetone-methanol (1:1, vol/vol) and further analyzed by capillary column GC-MS. All HPLC analyses were performed with two model 100A pumps (Beckman Instruments) and a Hitachi model 100-40 variable-wavelength absorbance detector adjusted to 254 nm. A 5-μm C_{18} Ultrasphere ODS column (4.6 mm by 25 cm; Altex Scientific, Berkeley, Calif.) was used, and separation was achieved with a methanol-water linear gradient (50 to 100%, vol/vol; 30 min) at a flow rate of 1 ml/min. Quantitative analysis of [^{14}C]naphthalene extracts by HPLC was determined by collecting the effluent 0.5-ml fractions into scintillation vials containing 10 ml of Scintisol, and radioactivity was determined as described above.

Naphthalene metabolites were analyzed after TLC cleanup with a GC-MS system (model 4023; Finnigan Instruments, San Jose, Calif.) equipped with a quadrupole mass filter and a DB5 capillary column (0.25 mm [inside diameter] by 30 m; J & W Scientific, Rancho Cordova, Calif.). Metabolites were derivatized by methylation with diazomethane (43) or acetylation with acetic anhydride and pyridine (28). Analyses were performed in the electron impact mode with an electron energy of 70 eV. Samples were injected into the GC at 60°C, held isothermally for 2 min, and programmed to rise to 225°C at 10°C/min.

Enumeration of microorganisms. Sediments from each ecosystem were diluted with phosphate buffer (0.05 M; pH 7.8) prior to each experiment, and the number of total heterotrophic, aliphatic hydrocarbon-degrading, and PAH-degrading microorganisms was determined by a miniaturized most-probable-number (MPN) method (23) or by standard plating techniques. For the MPN analyses, each sediment was serially diluted, and 50-μl inocula were added to eight replicate wells containing 100 μl of culture medium. Heterotrophic microorganisms were cultured on casein-peptone-starch (CPS) medium (14), and aliphatic hydrocarbon-degrading microorganisms were grown on a 2% agar mixture of minimal basal salts (MBS) medium (44) after the sterile addition of 10 μl of hexadecane to each well. PAh-degrading microorganisms were cultured on standard petri dishes containing a 2% agar mixture of MBS medium after the surfaces were coated lightly with an acetone spray of dilute phenanthrene and dried overnight at 35°C to volatilize the acetone. Naphthalene was not used as the PAH substrate due to its volatility. Growth of heterotrophic and hydrocarbon-degrading microorganisms was determined by visual detection of colonies or turbidity, and final populations were determined by direct counting from petri dishes or were computed from a standard eight-tube MPH table (42). Sterile

phosphate buffer and sediment samples were plated on CPS and MBS agar, respectively, to test for contamination of plates and buffer and for microbial growth on MBS agar in the absence of added nutrients. The sediment in the 10^{-1} dilution of each sediment was dried and weighed, and final microbial population values were expressed as 10^4 microorganisms per g of dry sediment.

RESULTS

Description of ecosystems used for naphthalene degradation studies. The ecosystems selected for this study varied in their previous exposure to petrogenic and anthropogenic chemicals. Redfish Bay, Tex., is an estuarine ecosystem which is known to be chronically exposed primarily to petroleum hydrocarbons. In contrast, Lake Chicot, Ark., receives no known exposure to petrogenic chemicals, but is chronically exposed to pesticide, herbicide, and fertilizer runoff from a watershed which is 90% heavily farmed. DeGray Reservoir, Ark., was selected as a pristine, uncontaminated ecosystem since it has excellent water quality, 70% of its watershed is undeveloped, and it receives no significant agricultural, urban, or industrial contamination. A description and a detailed comparison of the limnological and physical characteristics of these ecosystems has been presented previously (23, 24). Since the degradation of chemicals in the environment is determined by autochthonous microorganisms, we used microcosms containing sediment and water from each of these ecosystems to determine how the rate, extent, and metabolic pathway of naphthalene degradation varied among different natural ecosystems.

Naphthalene mineralization in microcosms from various ecosystems. Naphthalene was readily degraded to CO_2 by the natural microbiota in all the sediment-water microcosms with no apparent lag phase (Fig. 1). However, the rate of mineralization differed according to the source of the environmental sample. For example, over three times more naphthalene mineralization was observed after 1 week in microcosms from Redfish Bay than in the other two ecosystems. Naphthalene mineralization was higher throughout the experiment in microcosms from Redfish Bay, followed by Lake Chicot and DeGray Reservoir, in that order. Statistical modeling of these data by nonlinear regression analysis calculated the following rate and half-lives for naphthalene mineralization: Redfish Bay, 2.9% day^{-1}, 2.4 weeks; Lake Chicot, 2.2% day^{-1}, 3.2 weeks; and DeGray Reservoir, 1.7% day^{-1}, 4.4 weeks. These rates of naphthalene metabolism and calculated half-lives are consistent with the known histories of previous chemical exposure for these ecosystems and suggest that chronic exposure to petrogenic chemicals and, to a lesser extent, agricultural chemicals can cause enhanced degradation rates for aromatic hydrocarbons.

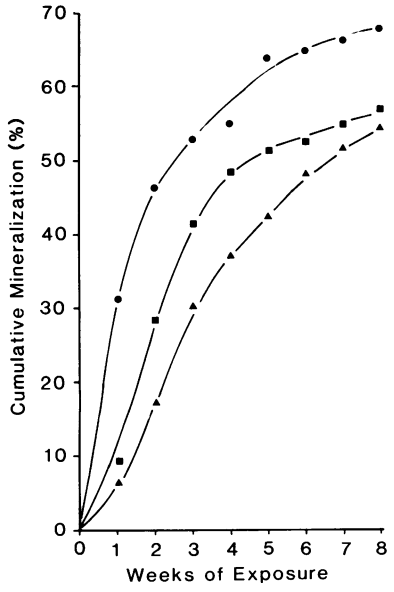

FIG. 1. Mineralization of naphthalene in microcosms from Redfish Bay (●), Lake Chicot (■), and DeGray Reservoir (▲).

TABLE 1. Heterotrophic and hydrocarbon-degrading microbial populations in sediment from each ecosystem

Organism type	Population (10^4 organisms/g [dry wt] of sediment)		
	DeGray Reservoir	Lake Chicot	Redfish Bay
Heterotrophic	5.0	163.0	6.3
Hydrocarbon degrading			
Hexadecane	1.1	2.4	5.4
Phenanthrene	0.1	0.3	1.2

The cumulative mineralization of naphthalene appeared to reach a plateau after about 60 to 70% mineralization in all the microcosms. This resulted from depletion of naphthalene in the microcosms, since analysis of the volatile-organic-trapping columns indicated that 12 to 15% of the naphthalene was lost by volatilization during the first 2 weeks of incubation. Combustion of the ^{14}C-residues contained in the sediments to $^{14}CO_2$ indicated that 5 to 8% of ^{14}C-residues remained in the sediments after 8 weeks of exposure to naphthalene. These residues were probably either adsorbed onto sediments and thus biologically unavailable or permanently incorporated into microorganisms. GC-MS analysis of extracts from the volatile-organic-trapping columns detected the presence of naphthalene. No volatile metabolites were found. In addition, we did not detect volatile residues in the secondary volatile-organic-trapping columns, which indicated that the primary trapping columns did not become saturated with residues during the time course of the experiments. These results indicate the importance of using volatile-organic-trapping columns in degradation studies with volatile chemicals to ensure that $^{14}CO_2$-trapping solutions do not contain undegraded or partially degraded chemical residues which could cause overestimation of mineralization. We detected no abiotic degradation of naphthalene in static, sterile microcosms from each ecosystem.

Heterotrophic and hydrocarbon-utilizing sediment microorganisms. Since differences in the rates of naphthalene mineralization were observed among the ecosystems, we enumerated sediment populations of heterotrophic and hydrocarbon-utilizing microorganisms to determine whether these rate differences resulted from higher total microbial populations or from selective increases in hydrocarbon-degrading microorganisms (Table 1). Sediment from Lake Chicot had a heterotrophic microbial population which was about 25 times higher than sediments from DeGray Reservoir or Redfish Bay. However, the differences in the population of hydrocarbon-degrading microorganisms were related to the different rates of naphthalene mineralization among the ecosystems. For example, the number of microorganisms in Redfish Bay sediment which were capable of growing solely on hexadecane was only 15% lower than the total observed heterotrophic population. The hexadecane-

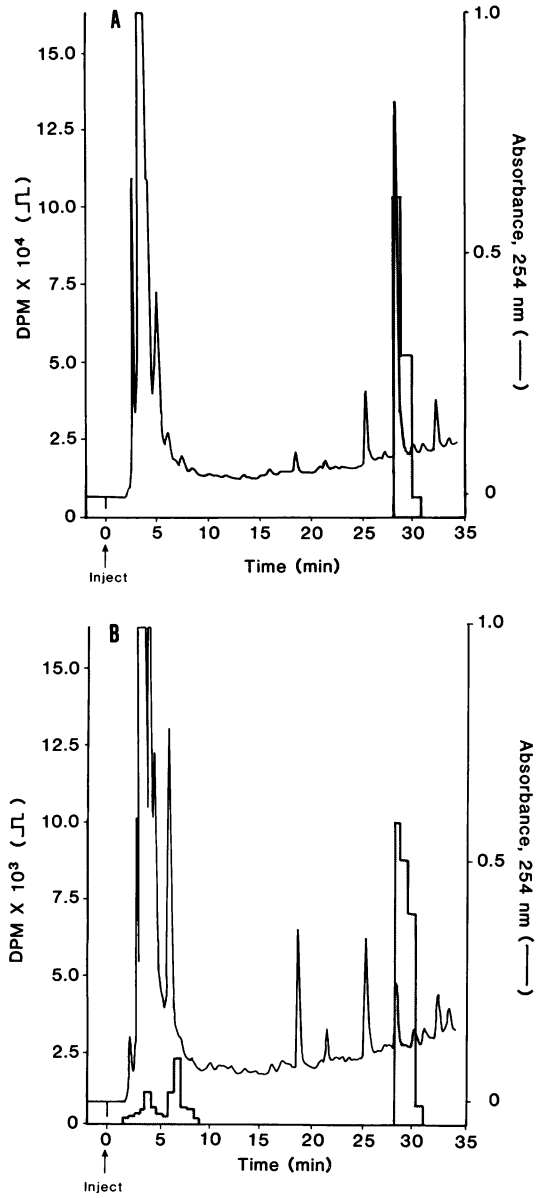

FIG. 2. HPLC elution profiles showing UV absorbance and radioactivity of organic-extractable naphthalene residues in microcosms from Redfish Bay, Tex., after 1 week in a sterile control (A) and after 1 week of degradation (B).

degrading microbial population in sediments from DeGray Reservoir and Lake Chicot was overall lower than that in Redfish Bay sediment and represented a much smaller fraction of the indigenous heterotrophic microbial population. Similar differences among the ecosystems were observed for phenanthrene-degrading microorganisms, although aromatic hydrocarbon-degrading microbial populations were overall much smaller than aliphatic hydrocarbon-degrading populations. These results indicate that chronic exposure to petrogenic chemicals may not increase the total number of heterotrophic microorganisms, but does selectively increase hydrocarbon-degrading microbial populations, resulting in enhanced mineralization rates for naphthalene.

Naphthalene degradation products. The combined radioactivity of ^{14}C-intermediates detected in microcosms exposed to [^{14}C]naphthalene for 1 week accounted for about 1 to 3% of the total radioactivity. Most of the [^{14}C]naphthalene in the microcosms was either completely mineralized to $^{14}CO_2$ or remained as undegraded naphthalene. In experiments to enrich for naphthalene metabolites, extracts of pooled supernatants from five replicate microcosms which were incubated with naphthalene for 1 week were analyzed by HPLC and compared with extracts of sterilized controls. Figure 2 shows the UV and radioactivity HPLC elution profile for sterilized and pooled 7-day extracts from Redfish Bay microcosms. The extract from the sterile control (Fig. 2A) showed a single radioactive chromatographic peak with a retention time of 28.5 min, which was identical to that of authentic naphthalene. However, the extract from the Redfish Bay microcosms (Fig. 2B) showed, in addition to a peak which corresponded to undegraded naphthalene, radioactive peaks at 4.5 and 6.0 min, which corresponded to the elution times for the suspected naphthalene metabolites catechol and naphthalene dihydrodiol, respectively.

Since the elution of the detected metabolites from the reverse-phase HPLC column indicated that they were polar intermediates, we used preparative TLC (prep-TLC) with a benzene-hexane solvent system to isolate the polar metabolites from undegraded naphthalene and other nonradioactive, nonpolar biogenic chemicals. Authentic standards of catechol, salicylic acid, and naphthalene dihydrodiol remained near the origin of the prep-TLC plate in this chromatographic system. When we applied the [^{14}C]naphthalene extract in an identical manner, the radioactivity remained at the origin. When a second solvent system consisting of hexane-acetone-acetic acid was used, the ^{14}C-residues which we scraped and extracted from the origin of the first prep-TLC plate migrated similarly to authentic naphthalene dihydrodiol, catechol, salicylic acid, and 1-naphthol, which had R_f values of 0.18, 0.25, 0.31, and 0.52, respectively. These ^{14}C-residues were then scraped, extracted, derivatized, and individually analyzed by GC-MS.

Figure 3 shows the mass spectrum of a [^{14}C]naphthalene metabolite which had an R_f of 0.18 and capillary column

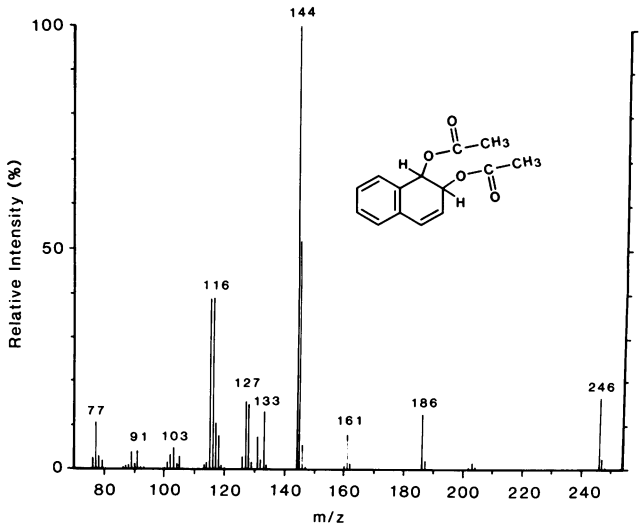

FIG. 3. Mass spectrum of a diacetylated derivative of naphthalene dihydrodiol (structure shown inset) extracted from Redfish Bay microcosms which had a capillary column GC-MS retention time of 14.68 min.

GC-MS retention time of 14.68 min. This diacetylated metabolite had a molecular ion (M^+) at *m/z* 246 and fragment ions at *m/z* 186 $[M - 60]^+$ and at *m/z* 144 $[M - 102]^+$. These fragments probably resulted from an acetic acid loss followed by a ketene loss. The MS fragmentation pattern and GC retention time indicated that this metabolite was naphthalene dihydrodiol. Since *cis*- and *trans*-naphthalene dihydrodiol are produced as the initial oxidation product of naphthalene by bacteria and fungi, respectively (4, 6, 10, 11), we used capillary column GC-MS and TLC to elucidate the configuration of the metabolite isolated from the Redfish Bay microcosms. Separation of diacetylated derivatives of *cis*- and *trans*-naphthalene dihydrodiol standards was achieved on capillary column GC-MS (Fig. 4A), with the *trans*-isomer eluting 7 s later than the *cis*-isomer (14.80 min). MS fragmentation patterns were similar between the diacetylated *cis*- and *trans*-isomers, with the exception that *trans*-naphthalene dihydrodiol showed a much weaker M^+ at *m/z* 246 than *cis*-naphthalene dihydrodiol. Identical capillary GC-MS analyses showed that derivatized naphthalene dihydrodiol extracted from Redfish Bay microcosms (Fig. 4B) had the same retention time as the derivatized *cis*-isomer and produced a strong M^+ ion at *m/z* 246. The *cis*-configuration of naphthalene dihydrodiol was further confirmed by coinjection with standards onto capillary column GC-MS: coinjection with the *cis*-isomer standard produced a single enhanced peak; coinjection with the *trans*-isomer standard produced two separate peaks, each with an M^+ at *m/z* 246 (data not shown). Furthermore, TLC separation of underivatized *cis*- and *trans*-naphthalene dihydrodiol standards was achieved by repeated development of a prep-TLC plate in the chloroform-acetone solvent system. Prep-TLC analysis of underivatized metabolite showed that after repeated development, the naphthalene dihydrodiol from Redfish Bay microcosms chromatographed similarly to the *cis*-isomer standard. These results indicate that *cis*-naphthalene dihydrodiol was formed from naphthalene in Redfish Bay microcosms as a minor metabolite and that the *trans*-isomer was not detected.

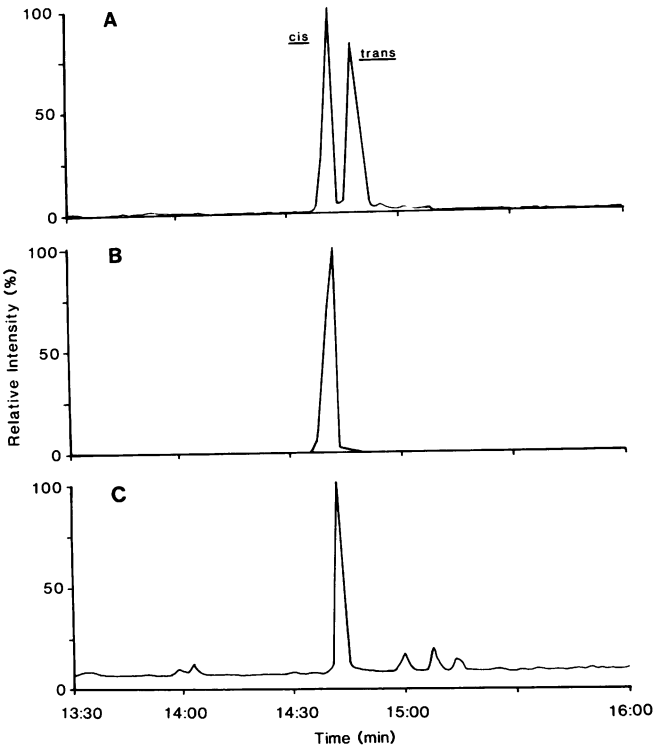

FIG. 4. Capillary column GC-MS traces of diacetylated derivatives of authentic naphthalene dihydrodiol standards and TLC-purified naphthalene dihydrodiol metabolites. (A) *cis*- and *trans*-naphthalene dihydrodiol standards; (B) *cis*-naphthalene dihydrodiol extracted from Redfish Bay microcosms; (C) *cis*-naphthalene dihydrodiol extracted from Lake Chicot microcosms.

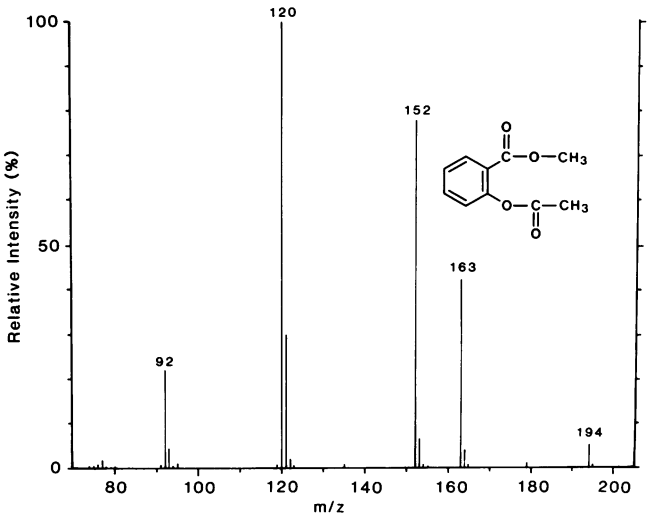

FIG. 5. Mass spectrum of an acetylated and methylated derivative (structure shown inset) of salicylic acid extracted from Redfish Bay microcosms, purified by prep-TLC and analyzed by GC-MS (10.15-min retention time).

Derivatization experiments with authentic salicylic acid showed that diazomethane (43) efficiently methylated the carboxylic acid moiety and that acetic anhydride and pyridine (28) efficiently acetylated the aromatic hydroxyl moiety. It is noteworthy that in methanol, the acetyl on the aromatic ring was completely displaced within 12 h by a methyl substituent to produce dimethylated salicylic acid. However, since acetylated salicylic acid extracts in acetone were stable, acetone was used as the solvent in all analyses for salicylic acid. Figure 5 shows the mass spectrum of a methylated and acetylated ^{14}C-metabolite which had a capillary column GC-MS retention time (10.15 min) and TLC migration (R_f of 0.31, underivatized) identical to those of authentic salicylic acid. This derivatized metabolite had an M^+ at *m/z* 194, fragmentation ions at *m/z* 163 $[M - 31]^+$ and *m/z* 152 $[M - 42]^+$, and a base peak at *m/z* 120. The fragmentation ions may be the result of molecular losses of CH_3O and ketene and combined losses of ketene and methanol. The capillary column GC-MS retention time and MS fragmentation pattern indicated that this metabolite was an acetylated and methylated salicylic acid and not diacetylated catechol, which also produces an M^+ at *m/z* 194, but has a capillary column GC-MS retention time of 9.58 min and a different MS fragmentation pattern.

Figure 6 shows the mass spectrum of a ^{14}C-metabolite extracted from Redfish Bay microcosms which after acetylation eluted from capillary column GC-MS at 9.58 min. This metabolite had an M^+ at *m/z* 194, a fragmentation ion at *m/z* 152 $[M - 42]^+$, and a base peak at *m/z* 110 $[M - 84]^+$. Notably, the absence of a fragmentation ion at *m/z* 163 and the presence of a base peak at *m/z* 110 indicate that this

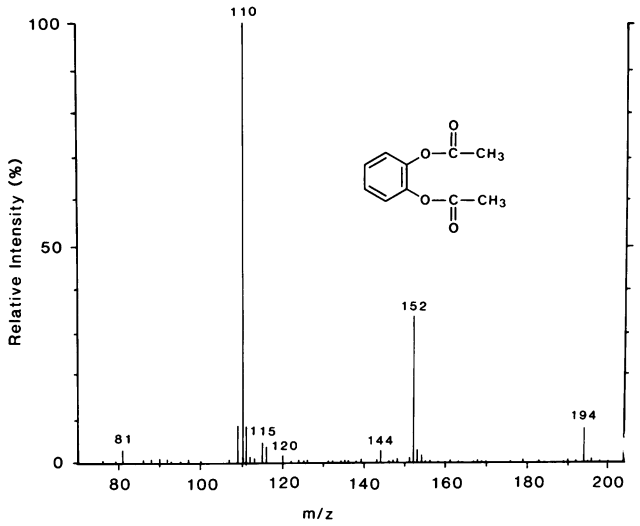

FIG. 6. Mass spectrum of a diacetylated derivative (structure shown inset) of catechol extracted from Redfish Bay microcosms, purified by prep-TLC and analyzed by GC-MS (9.58-min retention time).

metabolite is diacetylated catechol and not acetylated and methylated salicylic acid. Catechol is known to be an immediate precursor to ring cleavage of aromatic hydrocarbons by microorganisms (5, 20).

The mass spectrum of a derivatized metabolite (R_f of 0.52) which migrated similarly to 1-naphthol on prep-TLC is shown in Fig. 7. This metabolite had a capillary column GC-MS retention time (12.36 min) and an M^+ ion (*m/z* 186) which were identical to those of authentic acetylated 1-naphthol. We observed fragmentation ions at *m/z* 144 $[M - 42]^+$ and at *m/z* 115 $[M - 71]^+$, identical to authentic 1-naphthol. These fragments probably resulted from a $COCH_3$ loss followed by a COH loss.

Similar chemical analyses on organic extracts from Lake Chicot microcosms detected the presence of the *cis*-isomer of naphthalene dihydrodiol (Fig. 4C) and catechol as metabolites (data not shown). However, 1-naphthol and salicylic acid were not detected in extracts from Lake Chicot microcosms.

DISCUSSION

Differences in the physical, chemical, and microbial characteristics of ecosystems can affect the disposition and persistence of chemicals in the environment. Consideration of these differences as well as the complexity of natural ecosystems and the physical and chemical properties of toxicants have led to the use of multicomponent microcosms as laboratory models to predict the toxic effects and degradation of chemicals in the environment (21). This investigation provides a comparison of the metabolic pathway and mineralization rates of naphthalene by natural microorganisms in microcosms containing sediment and water from pristine, contaminated, freshwater, and estuarine environments.

The absence of significant lag phases for naphthalene mineralization in microcosms from the various ecosystems indicates that naphthalene was readily degraded by indigenous microorganisms. Our results indicate that the rate of naphthalene mineralization in these microcosms was determined primarily by the presence of elevated hydrocarbon-degrading microbial populations and may not be directly related to elevated populations of heterotrophic bacteria or sediment organic carbon content. The half-life and mineralization rate that we calculated for naphthalene in Redfish Bay microcosms (2.4 weeks or about 17 days, 2.9% day^{-1}) compares favorably with previously reported rates of naphthalene mineralization in sediments and water from estuarine and marine ecosystems. For example, Hambrick et al. (22) reported that 50% of the naphthalene added to continuously mixed suspensions of sediment and water from an estuarine site near an oil field in Louisiana was mineralized after about 15 to 20 days at 30°C. In a recent study, Bauer and Capone (3) reported a 1- to 3-day lag phase for naphthalene mineralization in intertidal marine sediment, followed by a maximum mineralization rate of 1 to 4% per day, resulting in 32% of the naphthalene being mineralized after 21 days at 25°C. Very rapid mineralization rates for naphthalene have been reported for some sediments which are chronically exposed to very high concentrations of degradable hydrocarbons. For example, Herbes and Schwall (27) reported a turnover time of 7.1 h for naphthalene in sediments from a small, heavily petroleum-contaminated stream which flowed through an oil-holding facility in Tennessee. In another study, Herbes (26) reported a 13-h turnover time for naphthalene in sediments exposed to coal-coking wastewater discharge. In the present study, the mineralization rates that we report for naphthalene in microcosms are representative of naphthalene degradation in larger aquatic ecosystems which are chronically exposed to the more diluted concentrations of hydrocarbons that normally occur downstream from point sources of petroleum contamination.

The approximate twofold increase in the mineralization half-life of naphthalene we observed in microcosms from DeGray Reservoir suggests that naphthalene will persist

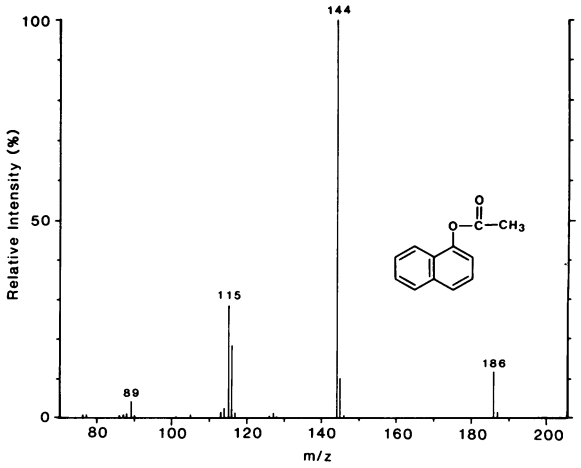

FIG. 7. Mass spectrum of an acetylated derivative of naphthol (structure shown inset) extracted from Redfish Bay microcosms, purified by prep-TLC and analyzed by GC-MS (12.36-min retention time).

longer and perhaps have greater toxicity in pristine freshwater ecosystems which are not microbially adapted to the presence of petroleum hydrocarbons. The half-lives calculated for naphthalene mineralization in this study provide useful estimates for the rates of microbial degradation of naphthalene in different ecosystems and also indicate the significance of microbial adaptations which occur as a result of chronic exposure to low levels of petroleum hydrocarbons and, to a lesser extent, agricultural chemicals. Since the environmental toxicity of chemicals is determined by both the concentration and duration of their exposure to environmental organisms, similar differences in the mineralization half-lives of high-molecular-weight, recalcitrant, and potentially carcinogenic PAHs would also have a significant effect on their persistence and toxicity in the environment. Furthermore, this study indicates that selective microbial adaptations from chronic exposure to petroleum hydrocarbons result in disproportionate increases in hydrocarbon-degrading microbial populations, but may not increase total heterotrophic microbial populations in the sediments. The physiological, enzymatic, and genetic characteristics of these adapted microbial populations warrant further investigation.

The first step in naphthalene degradation is the incorporation of molecular oxygen into the benzenoid nucleus, with the subsequent formation of a dihydrodiol (5, 20). Detection of the *cis*-isomer of naphthalene dihydrodiol and not the *trans*-isomer as an initial oxidation product in this study indicates that procaryotic pathways for naphthalene metabolism predominated in the microcosms. It is well known that pure cultures of bacteria oxidize naphthalene via dioxygenase enzymes to produce a *cis*-naphthalene dihydrodiol (4, 17, 31, 32, 47) which can be further degraded to salicylic acid, catechol, and ring cleavage products (16, 34). Since we isolated *cis*-naphthalene dihydrodiol and its ring cleavage products from our environmental samples, these results represent the first example of the existence of this pathway for naphthalene metabolism in sediments from freshwater and estuarine ecosystems.

1-Naphthol was identified as a minor compound in Redfish Bay microcosms. 1-Naphthol has been reported as a fungal metabolite of naphthalene in which an unstable naphthalene-1,2-oxide undergoes rearrangement via the NIH shift to form 1- and 2-naphthol (6, 7, 10, 11, 13). In addition, 1-naphthol has also been reported as a metabolite of naphthalene in cyanobacteria and a *Bacillus* sp. (8, 12, 38). It is unclear in this study whether the minor amount of 1-naphthol identified in microcosms from Redfish Bay occurred from nonenzymatic dehydration of *cis*-naphthalene dihydrodiol or from an oxidative pathway.

The ubiquitous distribution of filamentous fungi, yeasts, and algae and their capacity to metabolize aromatic hydrocarbons (5, 20) suggest that they may play an important role in the degradation of PAHs in the environment. In contrast to procaryotes, fungi do not utilize naphthalene as a sole source of carbon and energy, but do utilize monooxygenase enzymes to produce epoxides which may be converted to *trans*-dihydrodiols by epoxide hydrolase enzymes (6). Fungi may either eliminate polar transformation products directly as phenols or further metabolize them with conjugative enzymes to produce sulfate, glucoside, or glucuronide conjugates (9). Since we did not detect any *trans*-naphthalene dihydrodiol or conjugates of naphthalene metabolites in this study, the data indicate that bacteria play a predominant role in naphthalene catabolism in the ecosystems described in this report. However, aquatic fungi and algae as well as bacteria may play an important role in the environmental metabolism of naphthalene in open waters.

ACKNOWLEDGMENTS

We dedicate this study to the late Chase Van Baalen, University of Texas Marine Science Institute, Port Aransas, Tex., for his inspiration, counsel, and assistance in our studies on the microbial metabolism of PAHs. We also thank David Pack for assistance in collection of environmental samples and John Young for assistance with nonlinear regression analyses.

LITERATURE CITED

1. **Anderson, J. W., J. M. Neff, B. A. Cox, H. E. Tatem, and G. M. Hightower.** 1974. Characteristics of dispersions and water-soluble extracts of crude and refined oils and their toxicity to estuarine crustaceans and fish. Mar. Biol. (Berlin) **27**:75–88.
2. **Atlas, R. M.** 1981. Microbial degradation of petroleum hydrocarbons: an environmental perspective. Microbiol. Rev. **45**: 180–209.
3. **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.
4. **Catterall, F. A., K. Murray, and P. A. Williams.** 1971. The configuration of the 1,2-dihydroxy-1,2-dihydronaphthalene formed by the bacterial metabolism of naphthalene. Biochim. Biophys. Acta **237**:361–364.
5. **Cerniglia, C. E.** 1984. Microbial metabolism of polycyclic aromatic hydrocarbons. Adv. Appl. Microbiol. **30**:31–71.
6. **Cerniglia, C. E., J. R. Althaus, F. E. Evans, J. P. Freeman, R. K. Mitchum, and S. K. Yang.** 1983. Stereochemistry and evidence for an arene oxide-NIH shift pathway in the fungal metabolism of naphthalene. Chem. Biol. Interact. **44**:119–132.
7. **Cerniglia, C. E., and S. A. Crow.** 1980. Metabolism of aromatic hydrocarbons by yeasts. Arch. Microbiol. **129**:9–13.
8. **Cerniglia, C. E., J. P. Freeman, and F. E. Evans.** 1984. Evidence for an arene oxide-NIH shift pathway in the transformation of naphthalene to 1-naphthol by *Bacillus cereus*. Arch. Microbiol. **138**:283–286.
9. **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.
10. **Cerniglia, C. E., and D. T. Gibson.** 1977. Metabolism of naphthalene by *Cunninghamella elegans*. Appl. Environ. Microbiol. **34**:363–370.
11. **Cerniglia, C. E., and D. T. Gibson.** 1978. Metabolism of naphthalene by cell extracts of *Cunninghamella elegans*. Arch. Biochem. Biophys. **186**:121–127.
12. **Cerniglia, C. E., D. T. Gibson, and C. Van Baalen.** 1980. Oxidation of naphthalene by cyanobacteria and microalgae. J. Gen. Microbiol. **116**:495–500.
13. **Cerniglia, C. E., R. L. Hebert, P. J. Szaniszlo, and D. T. Gibson.** 1978. Fungal transformation of naphthalene. Arch. Microbiol. **117**:135–143.
14. **Collins, V. G., and L. G. Willoughby.** 1962. The distribution of bacteria and fungal spores in Blelham Tarn with particular reference to an experimental overturn. Arch. Mikrobiol. **43**:294.
15. **Darville, R. G., and J. L. Wilhm.** 1984. The effect of naphthalene on oxygen consumption and hemoglobin concentration in *Chironomus attenuatus* and an oxygen consumption and life cycle of *Tanytarus dissimilis*. Environ. Toxicol. Chem. **3**:135–141.
16. **Davies, J. I., and W. C. Evans.** 1964. Oxidative metabolism of naphthalene by soil pseudomonads. Biochem. J. **91**:251–261.
17. **Ensley, B. D., and D. T. Gibson.** 1983. Naphthalene dioxygenase: purification and properties of a terminal oxygenase component. J. Bacteriol. **155**:505–511.
18. **Ensley, B. D., D. T. Gibson, and A. L. LaBorde.** 1982. Oxidation of naphthalene by a multicomponent enzyme system from *Pseudomonas* sp. strain NCIB 9816. J. Bacteriol. **149**:948–954.

19. **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.
20. **Gibson, D. T.** 1982. Microbial degradation of hydrocarbons. Environ. Toxicol. Chem. **5**:237–250.
21. **Giesy, J. P. (ed.).** 1980. Microcosms in Ecological Research. Department of Energy Symposium Series 52 (Conf-781101). National Technical Information Service, Va.
22. **Hambrick, G. A., R. D. DeLaune, and W. H. Patrick.** 1980. Effect of estuarine sediment pH and oxidation-reduction potential on microbial hydrocarbon degradation. Appl. Environ. Microbiol. **40**:365–369.
23. **Heitkamp, M. A., and C. E. Cerniglia.** 1986. Microbial degradation of *tert*-butylphenyl diphenyl phosphate: a comparative microcosm study among five diverse ecosystems. Toxicity Assessment Bull. **1**:103–122.
24. **Heitkamp, M. A., J. P. Freeman, and C. E. Cerniglia.** 1986. Biodegradation of *tert*-butylphenyl diphenyl phosphate. Appl. Environ. Microbiol. **51**:316–322.
25. **Heitkamp, M. A., and B. T. Johnson.** 1984. Impact of an oil field effluent on microbial activities is a Wyoming River. Can. J. Microbiol. **30**:786–792.
26. **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.
27. **Herbes, S. E., and L. R. Schwall.** 1978. Microbial transformation of polycyclic aromatic hydrocarbons in pristine and petroleum-contamination sediments. Appl. Environ. Microbiol. **35**:306–316.
28. **Holder, C. L., W. A. Korfmacher, W. Slikker, H. C. Thompson, and A. B. Gosnell.** 1985. Mass spectral characterization of doxylamine and its rhesus monkey urinary metabolites. Biomed. Mass Spectrometry **12**:151–158.
29. **Huckins, J. N., J. D. Petty, and M. A. Heitkamp.** 1984. Modular containers for microcosm and process model studies on the fate and effects of aquatic contaminants. Chemosphere **13**:1329–1341.
30. **Jacob, J., W. Karcher, J. J. Belliardo, and P. J. Wagstaffe.** 1986. Polycyclic aromatic compounds of environmental and occupational importance. Fresenius Z. Anal. Chem. **323**:1–10.
31. **Jeffrey, A. M., H. J. C. Yeh, D. M. Jerina, T. R. Patel, J. F. Davey, and D. T. Gibson.** 1975. Initial reactions in the oxidation of naphthalene by *Pseudomonas putida*. Biochemistry **14**:575–584.
32. **Jerina, D. M., J. W. Daly, A. M. Jeffrey, and D. T. Gibson.** 1971. *cis*-1,2-Dihydroxy-1,2-dihydronaphthalene: a bacterial metabolite from naphthalene. Arch. Biochem. Biophys. **142**:394–396.
33. **Johnson, B. T., M. A. Heitkamp, and J. R. Jones.** 1984. Environmental and chemical factors influencing the biodegradation of phthalic acid esters in freshwater sediments. Environ. Pollut. Ser.B **8**:101–118.
34. **Kiyohara, H., and K. Nagao.** 1978. The catabolism of phenanthrene and naphthalene by bacteria. J. Gen. Microbiol. **105**:69–75.
35. **Laflamme, R. E., and R. A. Hites.** 1978. The global distribution of polycyclic aromatic hydrocarbons in recent sediments. Geochim Cosmochim. Acta **42**:289–303.
36. **Lee, R. F., and C. Ryan.** 1983. Microbial and photochemical degradation of polycyclic aromatic hydrocarbons in estuarine waters and sediments. Can. J. Fish. Aquat. Sci. **40**(Suppl. 2): 86–94.
37. **Marcelin, G.** 1979. Use of Tenax-GC in single sample analysis of gas-liquid mixtures. J. Chromatogr. **174**:208–211.
38. **Narro, M. L., C. E. Cerniglia, D. T. Gibson, and C. Van Baalen.** 1985. The oxidation of aromatic compounds by microalgae, p. 249–254. *In* I. S. Kulaev, E. A. Dawes, and D. W. Tempest (ed.), Environmental regulation of microbial metabolism. Academic Press, Ltd., London.
39. **National Academy of Sciences.** 1972. Particulate polycyclic organic matter. National Academy Press, Washington, D.C.
40. **National Academy of Sciences.** 1983. Polycyclic aromatic hydrocarbons: evaluation of sources and effects. National Academy Press, Washington, D.C.
41. **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. Estuarine Coastal Shelf Sci. **14**:369–389.
42. **Rowe, R., R. Todd, and J. Waide.** 1977. Microtechnique for most-probable-number analysis. Appl. Environ. Microbiol. **33**:675–680.
43. **Rushing, L. G., J. R. Althaus, and H. C. Thompson.** 1982. Simultaneous determination of trimelletic anhydride and its trimellitic acid impurity by GC/FID. J. Anal. Toxicol. **6**:290–293.
44. **Skerman, V. B. D.** 1967. A guide to the identification of the genera of bacteria, 2nd ed. The Williams & Wilkins Co., Baltimore.
45. **Spain, J. C., P. H. Pritchard, and A. W. Bourquin.** 1980. Effects of adaptation on biodegradation rates in sediment/water cores for estuarine and freshwater environments. Appl. Environ. Microbiol. **40**:726–734.
46. **Struble, V. G., and H. J. Harmon.** 1983. Molecular basis for inhibition of mitochondrial respiration by naphthalene. Bull. Environ. Contam. Toxicol. **31**:644–648.
47. **Treccani, V., N. Walker, and G. H. Wiltshire.** 1954. The metabolism of naphthalene by soil bacteria. J. Gen. Microbiol. **11**:341–348.
48. **Turner, B. C., and D. E. Glotfelty.** 1977. Field air sampling of pesticide vapors with polyurethane foam. Anal. Chem. **49**: 7–10.