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MMS 98-0057

Coastal Marine Institute

Assessment of PAH Composition of Diesel Fuel Sorbed to Marine Sediments and Their Toxicity to Aquatic Food Webs

MMS U.S. Department of the Interior
Minerals Management Service
Gulf of Mexico OCS Region

LSU Cooperative Agreement
Coastal Marine Institute
Louisiana State University

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ABSTRACT

In a 28-day microcosm study, we examined the effects of diesel-contaminated sediment on the sedimentary bacterial community of a Louisiana salt marsh that has been chronically exposed to petroleum hydrocarbons for decades. Diesel contaminants in microcosms as determined from polycyclic aromatic hydrocarbon (PAH) concentration ranged from 0.55 to 55 ppm (dry weight). Bacterial metabolism (incorporation of ^{14}C-acetate and ^{3}H-leucine) and bacterial abundance were not affected by diesel-contaminated sediment at any concentration. Bacterial degradation of ^{14}C-phenanthrene, however, increased in direct proportion to the amount of diesel-contaminated sediment added. Ambient sediment also exhibited significant capacity to degrade PAH. The half life of phenanthrene (based on ^{14}C-phenanthrene-degradation experiments) ranged from 137 days in ambient sediments to 4.5 days in sediment chronically exposed to high levels of diesel-contaminated sediments for 28 days. Two- and three-ring PAH, including naphthalenes, phenanthrenes, and dibenzothiophenes constituted the bulk of PAH composition of diesel and were rapidly metabolized. Alkylated PAH were also readily metabolized. The rapid removal of PAH suggests that even if the marsh were exposed to chronically high levels of petroleum hydrocarbons, chemical evidence of the contaminants would not be detected in sediments. Collectively, these results are consistent with the hypothesis that the bacterial community in this salt marsh has adapted to chronic exposure to petroleum hydrocarbons.

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LIST OF ABBREVIATIONS

AODC	acridine orange direct counts
Bq	Bequerel
dpm	disintegrations per minute
GC	gas chromatograph
gdw	gram dry weight
h	hour
LSU	Louisiana State University
LSC	liquid scintillation counter
LUMCON	Louisiana Universities Marine Consortium
MMS	Minerals Management Service
MS	mass spectrometer
nm	nanometer
PAH	polynuclear aromatic hydrocarbons
ppb	parts per billion
ppm	parts per million
QA	quality assessment
QC	quality control
µE	micro Einstein

Chapter 1. Introduction

It is estimated that 1.7-8.8 x 10^6 tons of petroleum hydrocarbons are released into the marine environment annually; ten percent or more of this input may be from refined petroleum such as fuel oils (National Research Council 1985a). Among the various refined petroleum products, diesel fuel is considered to be highly toxic because it is enriched in polycyclic aromatic hydrocarbons (PAH; approximately 30-40%, (National Toxicology Program 1986)), the most toxic component of petroleum hydrocarbons (Kennish 1992, Clark 1989). Because of its toxicity and widespread use in military, commercial, and recreational vessels, diesel fuel represents a potentially significant contaminant to aquatic environments. Most of the PAH released into aquatic environments (approximately 1.7 x 10^5 tons per year) accumulates in estuaries (Kennish 1992). As opposed to lighter fuels such as gasoline, many of the PAH in diesel are of a sufficiently high molecular weight that they do not readily evaporate (Clark 1989), but become associated with fine hydrophobic particles and are ultimately transported to the benthos (Connell & Miller 1984). Salt marshes are low-energy environments where these particles are likely to accumulate (Little 1987). Salt marshes are also highly productive and serve as nursery grounds for many commercially and economically important species. Because of these physical and biological characteristics, salt marshes are considered to be particularly susceptible to chronic and/or catastrophic inputs of petroleum hydrocarbons (National Research Council 1985b, Samiullah 1985) .

Several studies have examined the response of benthic microbial communities to individual PAH (Bauer & Capone 1985a, 1985b, Bauer *et al.* 1988, MacGillivray & Shiaris 1994) or crude oil (Alexander & Schwarz 1980, Griffiths *et al.* 1981a, 1981b, Heitkamp & Cerniglia 1988), but few have considered the effects of refined fuels (Jamison *et al.* 1976). Further, while it is recognized that microorganisms play a critical role in the breakdown of hydrocarbons, the impact of hydrocarbons on the metabolism and abundance of natural microbial communities is poorly understood (Bartha & Atlas 1987). Crude oil, for example has been shown to enhance (Bunch 1987), reduce (Griffiths *et al.* 1981a), or have no effect (Bauer & Capone 1985a, Wyndham 1985) on total abundance of sedimentary bacteria. Studies of individual aromatic compounds typically detect no significant influence on total bacterial abundance (Bauer & Capone 1985a). Bacterial communities also vary considerably in their metabolic response to petroleum hydrocarbons (Bauer & Capone 1985a, Bauer *et al.* 1988, Alexander & Schwarz 1980, Griffiths *et al.* 1981a, 1981b). Previous chronic exposure to hydrocarbons has been proposed as a partial explanation for variability in bacterial response to petroleum hydrocarbons (Griffiths *et al.* 1981b).

This report is part of a study in which microcosm experiments were performed to examine the effects of diesel fuel on the benthic food web of a coastal salt marsh. Future papers will consider the impact of diesel on microalgal activity and abundance, meiofaunal grazing, and meiofaunal community structure. Here, we examine the influence of diesel-contaminated sediments on the benthic bacterial assemblage in terms of abundance, metabolic activity, and capacity to degrade PAH.

Chapter 2. Materials and Methods

2.1 Study site.

The research was performed using sediments from Terrebonne Bay estuary (29° 15' N; 91° 21'W) near the Louisiana Universities Marine Consortium Laboratory (LUMCON) at Cocodrie, LA. Tidal range in the estuary is approximately 0.3 m and salinity ranges from 4-26 ppt. The estuary is a highly productive salt marsh that is dominated by the cord grass Spartina alterniflora. Sediment has a median grain size of 38 µm and is composed primarily of silts (41%) and clays (17%) (Chandler & Fleeger 1983). Organic content of sediment is approximately 2.5%. The study site is located in a region of intense hydrocarbon production and drilling activity, and commercial and recreational boat traffic is high. These combined factors lead to a high probability that the marsh experiences chronic exposure to both refined and crude hydrocarbons.

2.2 Experimental design.

The effects of diesel fuel on sedimentary bacteria were examined using intact, natural sediment collected in cylindrical microcosms from the study site. Microcosms were maintained in the LUMCON laboratory under controlled temperature and light conditions. Experimental treatments consisted of the daily addition to microcosms of small doses of diesel-contaminated, and bacterial responses were determined over a 28-day period.

Microcosm experiments were performed with a 2 x 4 x 5 factorial design, with two wet tables (as blocks), four exposure times, and five diesel treatments as factors. Each diesel x time combination was replicated twice in both wet tables. Microcosms were constructed of 15.2 cm i.d. PVC pipe with windows covered with Nitex mesh (62 µm) to allow exchange of water. At low tide on 22 May 1994, 80 microcosms of exposed unvegetated sediment were collected by hand from mud flats surrounded by Spartina alterniflora marsh. Microcosms were gently pushed into the sediment to a depth of 15 cm, mud was excavated from the outside of the microcosm, and a form-fitting base was placed on the bottom. Intact microcosms were removed from the mud flat and transported to the LUMCON facility. Forty microcosms were randomly assigned to both of two wet tables.

Microcosms were irrigated individually using a drip system. Ambient marsh water was filtered (5 µm) and pumped into a 1200 l holding tank. Water was aerated by continuous recirculation. Water was pumped from the holding tank to a 60-l head tank, which fed the drip system. Water was dripped into microcosms at a rate of approximately 1 l h^{-1}, sufficient to exchange the overlying water approximately once every hour. Oxygen measurements confirmed that this procedure maintained saturated O_2 concentrations in microcosms.

The treatments consisted of the addition to microcosms of sediment spiked with three levels of diesel (High, Medium, and Low), and two types of controls; in one

control (Cont1), no sediment was added to microcosms, in the second control (Cont2) "uncontaminated" sediment was added to microcosms. Four replicate microcosms (two from each wet table) of each of the five treatment levels (20 total microcosms) were harvested at each of four time intervals (0, 7, 14, and 28 days) following a previously determined randomization schedule.

2.3 Diesel-contaminated sediments.

Surficial sediments (top 2 cm) were collected from the marsh and processed following the procedure of Chandler (1986), which results in sterile sediment consisting of particles < 62 µm. Diesel fuel was obtained from a commercial vendor. Two liters of processed sediments and 600 ml of diesel were placed in an amber 4-l bottle and tumbled for 10 days. The bottle was then removed from the tumbler and sediment allowed to settle overnight. Diesel was aspirated from the bottle and 1 l of 15 ppt artificial seawater (ASW) was added. The mixture was tumbled again (overnight), allowed to settle, and the supernatant aspirated. This procedure was repeated three times (total of four rinses). The sediment-water slurry was transferred to 35-ml glass centrifuge tubes and centrifuged at 1700 x g for three min. The supernatant was removed and replaced with fresh ASW. Sediment and water were mixed thoroughly then recentrifuged. The supernatant was decanted again, and the process was repeated for a total of four rinses via centrifugation. Sediment was then recombined into a single batch and mixed to assure homogeneity. A sediment sample was removed from the batch, and total PAH (described below) was determined to be 687 ppm (dry weight). Contaminated sediment was then diluted with ambient sediment (processed as described above) to achieve PAH concentrations of 550, 55, and 5.5 ppm (dry weight). Diluted contaminated sediments were added to microcosms as described below with the objective of achieving final added concentrations in the top 1 cm of sediment of 55 (High), 5.5 (Medium), and 0.55 (Low) ppm.

At the beginning of the experiment, microcosms were dosed by adding sediment sufficient to create a 1-mm-thick layer of sediment on the microcosms surface. This was accomplished by loading 30-ml plastic syringes with 17.8 ml of contaminated (or control) sediment, then slowly dispensing the sediment into the water overlying the microcosm (after removal of drip tubes) in a uniform manner. Sediment settled onto the microcosm surface within approximately 1 h, at which time microcosm drip tubes were replaced. Within approximately 2 h, surface topography (tubes, burrows, and tracks) from resident meiofauna and macrofauna was apparent. On each subsequent day, microcosms were dosed with 1.8 ml of sediment, sufficient to create a 0.1-mm sediment layer on the surface of microcosms.

Total PAH in sediment used to dose High treatments, as well as sediment in the top 1 cm of Day-0 and Day-28 High and Medium treatments, were determined with an Iatroscan (Ackman *et al.* 1990). For Iatroscan analysis, 10-34 grams of sediment were extracted thrice in 70 ml dichloromethane, with 25 g of solvent-rinsed Na_2SO_4 added in the first extraction. ^{14}C-phenanthrene was added as an internal standard to determine

extraction efficiency. Combined extracts were passed through a column containing 0.5 g solvent-rinsed Na_2SO_4, collected in a 250 ml round-bottom flask, and concentrated by rotary evaporation to 1-2 ml. The concentrated extract was transferred with rinsing to a 13 x 100 mm tube then dried under N_2 and stored in the freezer until further analysis. The extract was dissolved in $CHCl_3$ and fractionated by solid phase extraction (SPE) chromatography on a silica column (500 mg, Whatman) with 5 ml $CHCl_3$. This fraction was dried under N_2, dissolved in toluene and fractionated by SPE chromatography on a silica column (500 mg) with 5 ml of toluene. The toluene fraction was dried then dissolved in a 50-100 µl $CHCl_3$ and duplicate 1-2 µl samples were spotted on a Chromarod (SIII). Chromarods were dried under active vacuum after each development described below. Chromarod developments were carried out at 35° C as follows: (i) toluene for 5 min., (ii) toluene for 5 min, (iii) hexane for 30 min. Rods were analyzed using an Iatroscan MK-5 TLC/FID analyzer. The PAH peaks were quantified by comparison to an external calibration curve generated using a standard consisting of a mixture of 16 PAH ranging from naphthalene (two rings) to benzo(g,h,i)perylene (six rings) (Supelco). Final concentrations were calculated with a correction for recovery of ^{14}C-phenanthrene.

Undiluted contaminated sediment, as well as the top 1 cm of sediment from two replicates of each treatment on Day 0 and Day 28 were analyzed by GC/MS for PAH content (Means & McMillin 1993). Sediments were extracted in glass ointment jars containing ~4 g wet sediment after removing ~0.5 g for moisture determination. Na_2SO_4 (30 g) was mixed into each sample and added to an empty container to create a reagent blank. Pesticide-grade dichloromethane (DCM, 40 ml) was added to each jar along with 15 µl of a mixture of deuterated PAH (Ultra Scientific, Inc. #US-108) at 40 ng/µl in hexane. Open jars were placed in an ice-cooled sonicating bath for 12 min. Solvent was decanted through solvent-rinsed Na_2SO_4 into a rotavap flask, and DCM extraction repeated twice more. Combined extracts were concentrated to ~1 ml, transferred with rinsing to a 4-ml vial, and further concentrated, with exchange to hexane, to 200 µl using a dried nitrogen stream. Activated fine-granular copper (MacLeod *et al.* 1985) was added in excess to remove sulfur interference.

Extracts were analyzed by GC/MS using a Hewlett-Packard 5890/5970B Mass Selective Detector (MSD) equipped with a 30 m by 0.25 mm i.d., 0.25 µm DB-5 film capillary column (J & W Scientific, Inc.). The GC was programmed from 50 to 300 °C using two temperature ramps over a period of 60 min. The MSD was operated in selected-ion mode. Response calibration was achieved using a mixture to authentic reference standards at 0.5 ng/µl, which included parent PAH (US 106, Ultra Scientific), deuterated compounds (see above), and 42 alkylated naphthalenes, dibenzothiophenes, and phenanthrenes (Chiron Laboratories A.S., Norway). 2-fluorobiphenyl was added to the extracts immediately prior to analysis to serve as an instrumental internal standard. Final concentrations were calculated with correction for recovery of the deuterated surrogate standards added during extraction.

2.4 Direct counts.

Bacterial abundance in the top 1 cm of sediment was determined from acridine orange direct counts (AODC, Carman 1993). This procedure included separation of bacteria from sediments by blending sediments in 0.01% sodium pyrophosphate. The resulting supernatant was stained with 0.04% acridine orange for two minutes, and bacteria were enumerated (Hobbie *et al.* 1977).

2.5 ^{14}C-acetate incorporation.

Bacterial activity was measured by administering ^{14}C-acetate into sediment cores (1.7 cm i.d.) and following the label into bacterial membrane lipids (phospholipids) and lipid storage products (poly-ß-hydroxyalkanoates - PHA; (Findlay & White 1987). Acetate was injected approximately 2 mm below the sediment-water interface through a silicon-sealed slit on the side of the core with a 50-µl syringe (Hamilton; Dobbs *et al.* 1989) 33.4 KBq [1,2-^{14}C]acetate (dissolved in 22 µl ASW; specific activity 4.0 GBq/mmol) were added to each core and incubated in the dark (to prevent photosynthetic fixation of respired $^{14}CO_2$) for 5 h. Water overlying the sediment was discarded, and the top 1 cm of sediment was extruded into a glass 50-ml tube containing 25 ml of modified Bligh-Dyer Solution (White *et al.* 1979). Bulk lipids were extracted and then fractionated into neutral, phospho-, and glycolipids (which contain PHA) (Guckert *et al.* 1985) and assayed for radioactivity. Controls were injected with ^{14}C-acetate and then immediately harvested as described above. Data were expressed as dpm ^{14}C incorporated after correction for controls.

2.6 ^{3}H-leucine incorporation.

Cores of sediment (1.7 cm i.d.) were collected from microcosms on Day 28 only and injected with 405 KBq (22 µl) of L-[4,5- ^{3}H]leucine (American Radiolabeled Chemicals, Inc.; 2.2 GBq/mM) as described above for ^{14}C-acetate incubations. Sediments were incubated for 45 min, after which the water overlying the sediment was discarded and the top 1 cm of sediment was extruded into a plastic bag. Sediment was then frozen and stored in liquid nitrogen until further processing. Controls were injected with ^{3}H-leucine and immediately frozen. Incorporation of ^{3}H into protein was determined after an acid/base hydrolysis procedure to separate protein from other macromolecules (Carman *et al.* 1988). Data were expressed as dpm ^{3}H incorporated after correction for controls.

2.7 PAH metabolism.

Bacterial metabolism of PAH was examined with a modified version of the procedure described by MacGillivray & Shiaris (1994). Microcosm sediment was sampled with a 3-cc syringe core, and the top 1 cm (1 cm^3) was extruded into sterile,

35-ml serum bottles. Nine ml of 0.45-µm filtered marsh water were added to bottles. Nine ml of 2% formaldehyde were added to controls. [9-^{14}C]phenanthrene (Sigma, 307 MBq/mmol) was solubilized in ethanol, and 10 µl (26.3 KBq; 0.085 µmol) were added to serum bottles. Serum bottles were capped with a rubber stopper through which a small plastic cup containing fluted Whatman #1 filter paper was inserted. Bottles were placed on a shaker table and incubated in the dark at 27 °C for 72 h. Phenethylamine (0.1 ml) was injected into the wick, and 1.0 ml of 1 N HCl was added. Acidified samples were incubated over night, after which wicks were removed and assayed for radioactivity. Data were expressed as the percent ^{14}C-phenanthrene converted to CO_2 after subtraction of control values. Radioactivity recovered in control wicks averaged 1.3% (range 0.6 to 2.5%) of the total radioactivity added. Radioactivity in controls ranged from an average of 51% of radioactivity in all experimental values on Day 0 to < 4% of radioactivity in High treatments on Days 14 and 28.

Chapter 3. Results

3.1 PAH composition.

Absolute concentrations of major PAH classes are summarized in Table 1. The most abundant classes of PAH in diesel-contaminated sediment were naphthalenes, phenanthrenes, and dibenzothiophenes (DBT). Alkylated PAH made up 93% of the total PAH. The high proportions of naphthalenes, phenanthrenes, DBT and alkylated PAH are typical of refined petroleum hydrocarbons (National Research Council 1985c). To examine compositional differences in PAH among treatments, proportional PAH abundances were calculated (Steinhauer & Boehm 1992). The concentration of each compound was expressed as a fraction of the compound with the highest concentration. E.g., in diesel-contaminated sediment used to dose microcosms, C2-naphthalenes had the highest concentration (Table 1) and all other PAH were expressed as a fraction of C2-naphthalene concentration (Fig. 1). In comparison to diesel-contaminated sediment, ambient sediment was relatively depleted in 2- and 3-ring PAH, and most PAH was in the form of 4- and 5-ring compounds (Fig. 1)

Composition of PAH in Day-0 microcosms was variable, but generally reflected that of the added diesel-contaminated sediment (Table 1, Fig. 2). In High (Fig. 2c) and Medium (Fig. 2b) treatments, naphthalene and C1-, C2-, and C3-naphthalenes were proportionately less abundant than they were in diesel-contaminated sediments (Fig. 1). The loss of these lower-molecular-weight compounds in microcosms was probably the result of rapid sediment-water exchange and volatilization. Low treatments (Fig. 1a) consisted of only a minor PAH addition to microcosms, and thus the proportional abundances of PAH were similar to those of controls, i.e., very little naphthalene (parent or alkylated) and relatively high abundances of 4- and 5-ring compounds. The relatively high concentrations of phenanthrenes and C2-DBT were, however, evidence of the addition of diesel-contaminated sediments.

PAH composition in Day-28 microcosms differed substantially from Day-0 samples, and the degree of change differed among treatments (Fig. 2). In Day-28 Low treatments, the proportional abundance of phenanthrenes and C2-DBT was greatly reduced relative to Day-0 Low samples (Fig. 2a), and the profile closely resembled Day-0 control sediments (Fig. 1).

The alkylated naphthalenes that were abundant in Day-0 Medium treatments were completely eliminated by Day-28 (Fig. 2b). The proportional abundances of phenanthrenes and 4- and 5 ring compounds increased substantially by Day-28.

In High treatments, the compositional change of PAH from Day-0 to Day-28 was less dramatic than in Medium and Low treatments (Fig. 2c). Naphthalenes were reduced, but not eliminated as in Medium treatments. Phenanthrenes and DBT replaced naphthalenes as the dominant compounds, and, with the possible exception of pyrene, four- and five-ring PAH remained a minor component of total PAH composition.

Table 1.

Analyses of PAH in microcosm sediments. "Cont1" and "Cont2" refer to control microcosms; "Low", "Med", and "High", refer to three levels of additions of diesel-contaminated sediments (see text). Values for GC/MS represent the average ppb of two replicates. Values under Iatroscan represent the mean (S.D.) ppb of four replicates. "nd" = not detected.

	Day-0					Day-28					
Analysis	Cont1	Cont2	Low	Med	High	Cont1	Cont2	Low	Med	High	Dies. Sed
GC/MS											
C1-Naphthalenes	nd	3	nd	90	47	3	2	2	nd	6	44,054
C2-Naphthalenes	nd	4	nd	395	186	1	2	1	nd	8	143,537
C3-Naphthalenes	nd	10	nd	1412	944	10	8	10	nd	132	141,640
C4-Naphthalenes	nd	11	nd	3717	3273	9	7	8	nd	608	84,171
C1-Dibenzothiophenes	1	0	2	105	155	1	3	nd	nd	10	23,930
C2-Dibenzothiophenes	8	4	40	426	1693	8	15	15	15	1175	52,212
C1-Phenanthrenes	7	2	37	209	280	6	8	49	49	581	53,680
C2-Phenanthrenes	7	3	80	420	1314	8	13	68	68	1189	56,659
C3-Phenanthrenes	8	4	91	346	1210	9	19	82	82	1534	38,957
Pyrene	53	51	93	103	145	21	31	29	97	212	3839
Total Naphthalenes	nd	28	nd	5614	4450	24	20	21	nd	752	413,403
Total Dibenzothiophenes	9	5	40	531	1848	8	18	5	15	1385	76,142
Total Phenanthrenes	21	9	208	974	2803	24	40	11	199	3304	149296
Total Parent PAH	240	217	258	549	349	330	245	210	374	999	48,548
Total Alkylated PAH	30	42	247	7119	9101	56	77	38	214	5442	638,841
%Alkylated	13	16	49	93	96	15	24	15	36	84	93
Total PAH	269	259	505	7668	9450	385	323	248	587	6441	687,389
Iatroscan											
Total PAH				30,000 (3900)	79,000 (51,000)				12,900 (3000)	30,400 (16,100)	

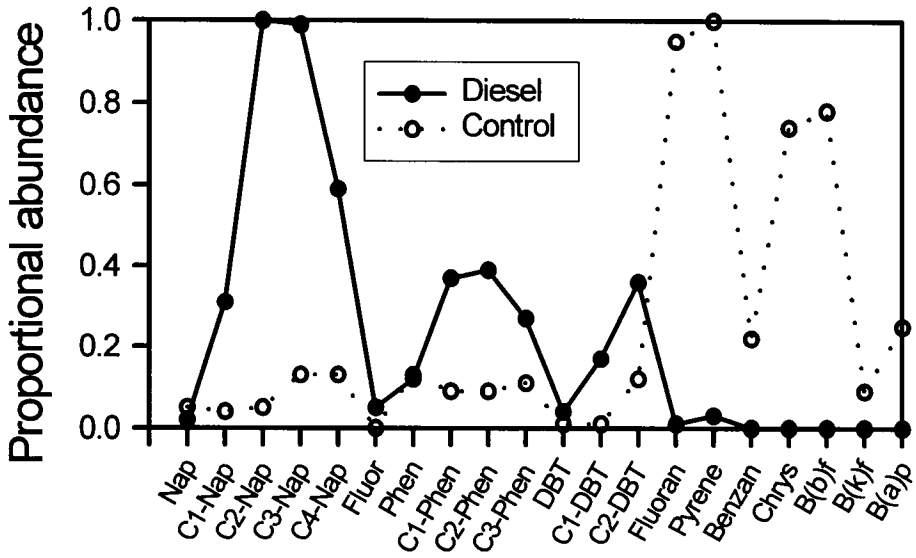

Figure 1. Proportional abundance of major groups of PAH in diesel-contaminated and control (ambient) sediment. Nap = naphthalene, Fluor = fluorene, Phen = phenanthrene, DBT = dibenzothiophene, Fluoran = fluoranthene, Benzan = benzanthracene, Chrys = chrysene, B(b)f = benzo(b)fluoranthene, B(k)f = benzo(k)fluoranthene, B(a)P = benzo(a)pyrene. C1, C2, C3, and C4: alkylated homologs of parent compounds containing from 1 to 4 methyl side chains, respectively. Values are averages of 2 replicates.

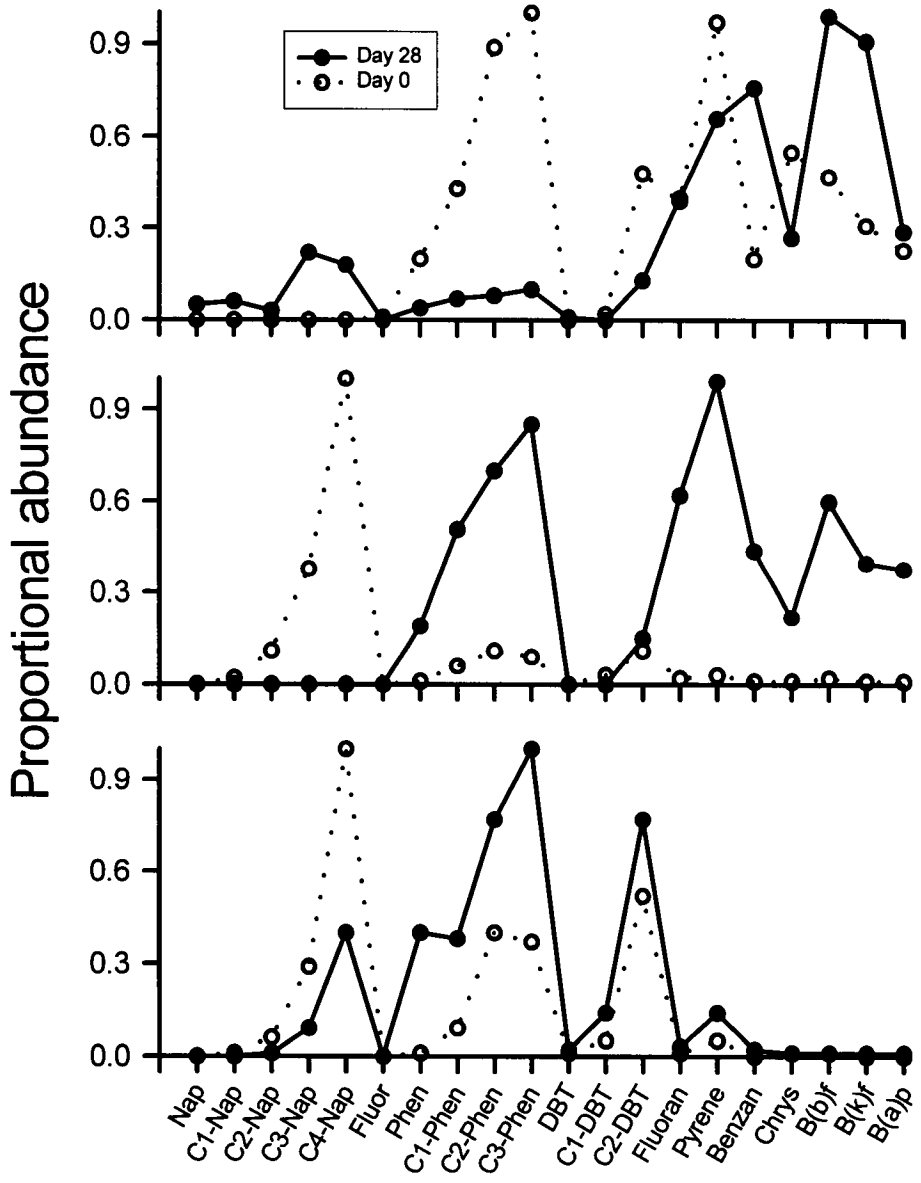

Figure 2. Change in proportional abundance of major groups of PAH in (A) Low, (B) Medium, and (C) High treatments over the 28 d study period. Abbreviations as in Fig. 1. Values are averages of 2 replicates.

3.2 PAH concentration.

Average total PAH (from GC/MS measurements) in control microcosms (Cont1 and Cont2) was 0.26 ppm on Day-0, and 0.35 ppm on Day-28. Total PAH in Day-0 Low, Medium, and High treatments were 0.59, 7.7, and 9.4 ppm respectively (Table 1). Concentrations in Low and Medium treatments were similar to expected concentrations (calculated PAH concentration of contaminated sediment plus ambient concentration), but the value in High treatments did not represent the 10x increase over Medium treatments that was expected. As discussed below, this apparent discrepancy may have resulted from uneven distribution of diesel-contaminated sediments in Day-0 microcosms.

In Medium treatments, concentration of all PAH containing $\leq$ 3 rings decreased from Day-0 to Day-28, whereas the concentration of 4/6 four- and five-ring compounds increased (Table 1). With a few notable exceptions, changes in PAH concentrations in High treatments were similar to those observed in Medium treatments (Table 1). The concentrations of all alkylated naphthalenes decreased and, with the exception of C2-alkylated forms, the concentrations of all phenanthrenes and DBT decreased. As in Medium treatments 4/6 four- and five-ring PAH increased in abundance over time in High treatments.

The concentration of three-ring compounds decreased in Low treatments from Day-0 to Day-28 (Table 1). In contrast to Medium and High treatments, however, the concentrations of four- and five-ring compounds in Low treatments did not change substantially over time.

Measurements of total PAH by Iatroscan were generally higher than those determined from GC/MS, especially in the Day-0, High treatment. There are various possible explanations for this discrepancy. The first may be related to calibration. The Iatroscan method that we used separated all PAH into a single peak. The PAH composition of our standard did not precisely match that of samples (the composition of which was variable), and variable detector responses to different compounds could have resulted in an overestimation of total PAH. Nevertheless, the measured total PAH concentration in Day-0, High treatments as determined from Iatroscan analysis (69.7 ppm) was reasonably close to the calculated expected concentration of 55 ppm. The apparent discrepancy between Iatroscan and GC/MS could also have been the result of the high degree of variability that was detected in Day-0, High treatments. For example, Iatroscan values from individual replicates of Day-0, High treatments were 169.2, 97.9, 6.0, and 5.8 ppm. This variability could have been the result of an uneven distribution of diesel-contaminated sediments in microcosms immediately after contaminated sediments were added to microcosms. The two GC/MS samples corresponded to the 6.0 and 169.2 ppm Iatroscan samples, and yielded values of 4.9 and 14.0 ppm respectively. Variability in Day-0 Iatroscan data from the High treatment could also have been a consequence of a loss of naphthalenes during processing of samples. We have observed that non-alkylated naphthalene is lost during the process of Chromarod development. Since naphthalene was less abundant after Day 0, this source of variability was probably less important after Day 0. Collectively, however, PAH as determined by GC/MS was a good predictor of total PAH as determined by

Iatroscan (r^2 = 0.89, data not shown). Thus, at a minimum, the Iatroscan provided a good relative indication of total PAH concentration.

Iatroscan data indicated that PAH in High treatments accumulated over the first week, then decreased by approximately one half by Day-14, and again by one half by Day-28 (Fig. 3). Total PAH in Medium treatments, decreased by a factor of approximately seven by Day-7 and remained relatively constant thereafter (Fig. 3). These trends were qualitatively consistent with GC/MS data, which indicated that total PAH in Day-28 Medium treatments was only slightly greater than Day-0 controls, while total PAH in Day-28 High treatments was higher than Day-0 controls by a factor of 10 or more (Table 1). Thus, removal rate of PAH in Medium (and Low) treatments was equal to or exceeded the rate of addition. The removal rate of PAH from High treatments, however, was not sufficient to reduce PAH concentrations to background levels.

3.3 Bacterial abundance.

Bacterial abundance in microcosms ranged from 0.27 to 2.8 x 10^9 cells/gdw (gram dry weight) throughout the experiment (Fig. 4). Bacterial abundance was not significantly affected by diesel-contaminated sediment ($p = 0.178$) , and there was no trend that was even suggestive of an effect. Bacterial abundance did vary significantly among days ($p < 0.0001$), with greatest numbers being detected on Day 7.

3.4 Bacterial activity.

As with bacterial abundance, bacterial activity as determined by ^{14}C-acetate incorporation into phospholipids (Fig. 5a), or the phospholipid:PHA (poly-ß-hydroxyalkanoates) ratio of ^{14}C-acetate incorporation (Findlay & White 1987) were not significantly influenced by diesel-contaminated sediment ($p = 0.674$ and 0.739, respectively; Fig. 5b). Similarly, ^{3}H-leucine incorporation into protein (measured on Day-28 only) was remarkably consistent among treatments (Fig. 6, $p = 0.742$).

3.5 PAH metabolism.

In contrast to bacterial abundance and assays of bacterial activity, bacterial degradation of ^{14}C-phenanthrene was sensitive to diesel-contaminated sediment (Fig. 7). In Day-0 microcosms, degradation of ^{14}C-phenanthrene was low but detectable (0.9 to 1.3% of total available over a 72-h period), and did not differ among treatments. Degradation rates of ^{14}C-phenanthrene remained relatively low in both controls (Cont1 and Cont2) over the entire course of the experiment (range 0.8 to 2.0%). Dose-dependent enhancement of phenanthrene degradation in all diesel treatments (Low, Medium, and High) occurred from Day 7 through Day 28. The enhancement of ^{14}C-phenanthrene degradation was statistically significant in Medium and High treatments when performing ANOVA on the entire data set, and when considering Days 7 through 28 individually ($p < 0.0001$). Degradation of ^{14}C-phenanthrene in Low treatments was significantly higher than in controls only on Day 28. After Day 7, the enhancement of

^{14}C-phenanthrene degradation in Low and Medium treatments remained constant or was slightly diminished. ^{14}C-phenanthrene degradation in High treatments continued to increase throughout the experimental period.

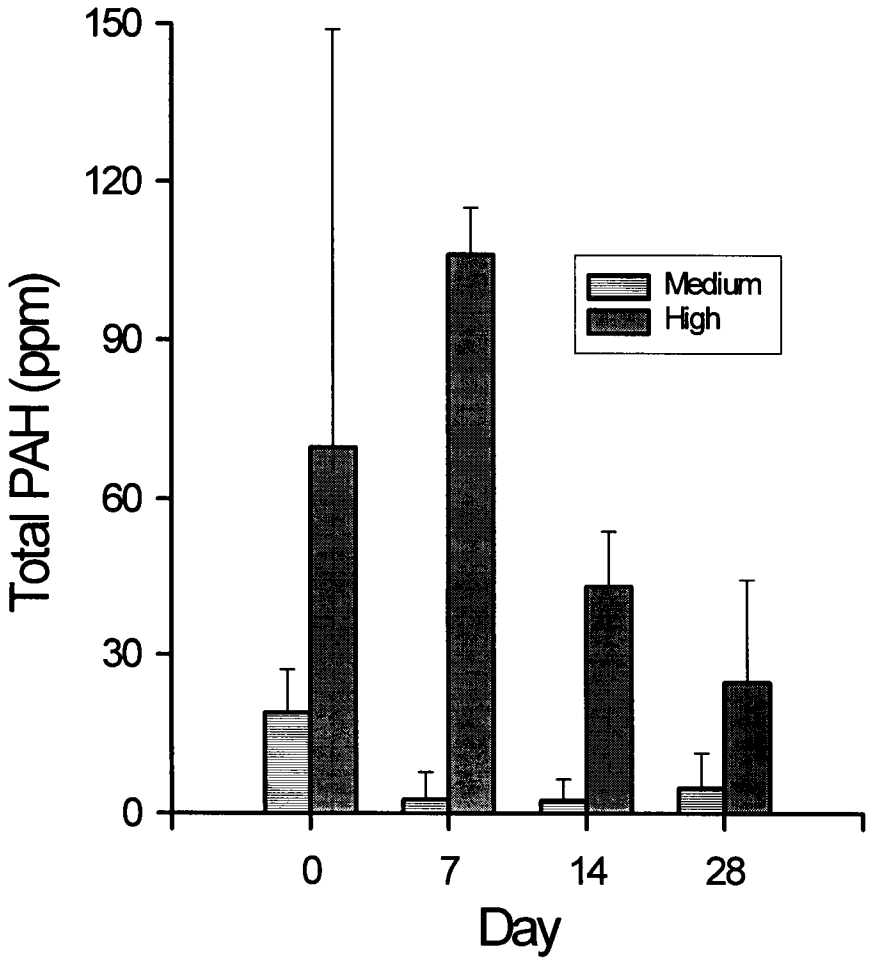

Figure 3. Total PAH in High and Medium treatments as determined by Iatroscan. Values are means + 1 SD (n=4).

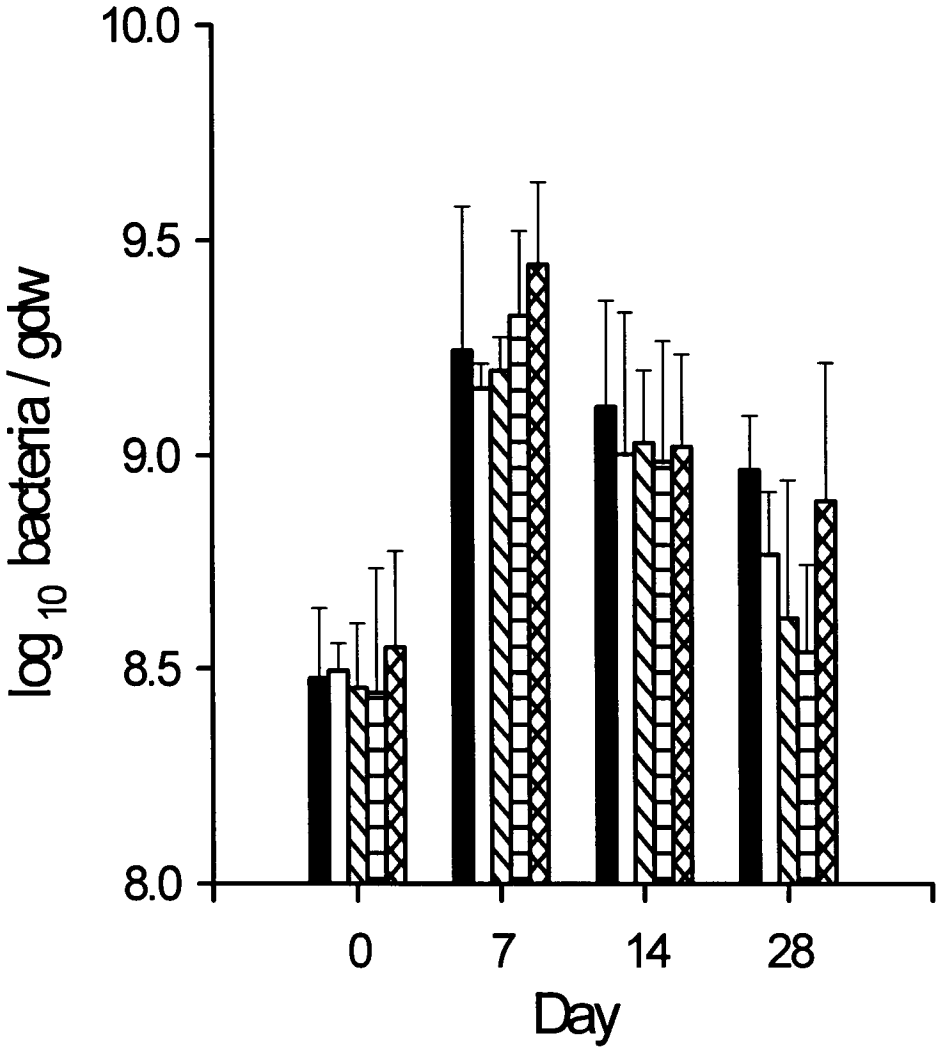

Figure 4. Bacterial abundance per gram dry weight (gdw) in sediments exposed to a range of diesel contamination over a 28-day period. Solid bars = Cont1; Open bars = Cont2; Diagonally hatched bars = Low; Horizontally hatched bars = Medium; Cross-hatched bars = High. Values are means ± 1 S.D. (n = 4).

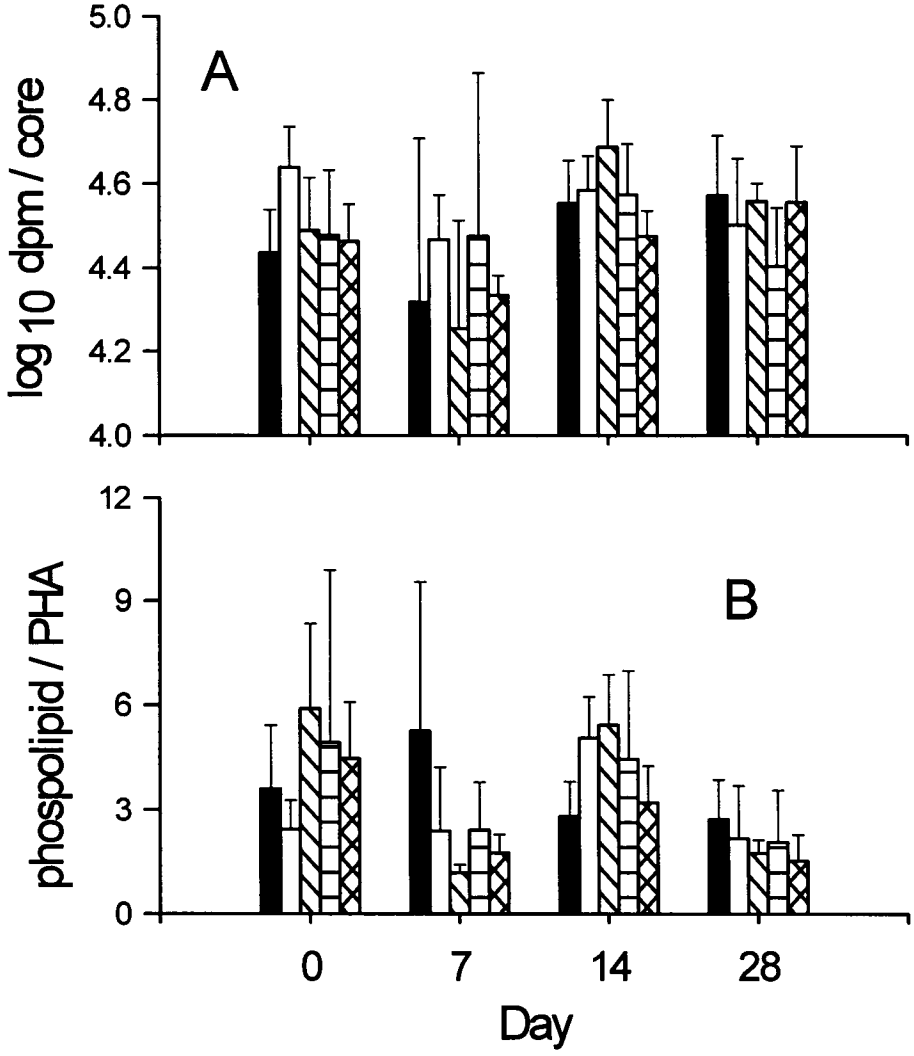

Figure 5. ^{14}C-acetate metabolism in sediments exposed to a range of diesel contamination over a 28-day period. (A) Incorporation of ^{14}C into polar lipids. (B) Phospholipid/PHA ratio of ^{14}C incorporation. lid bars = Cont1; Open bars = Cont2; Diagonally hatched bars = Low; Horizontally hatched bars = Medium; Cross-hatched bars = High. Values are means $\pm$ 1 S.D. (n = 4).

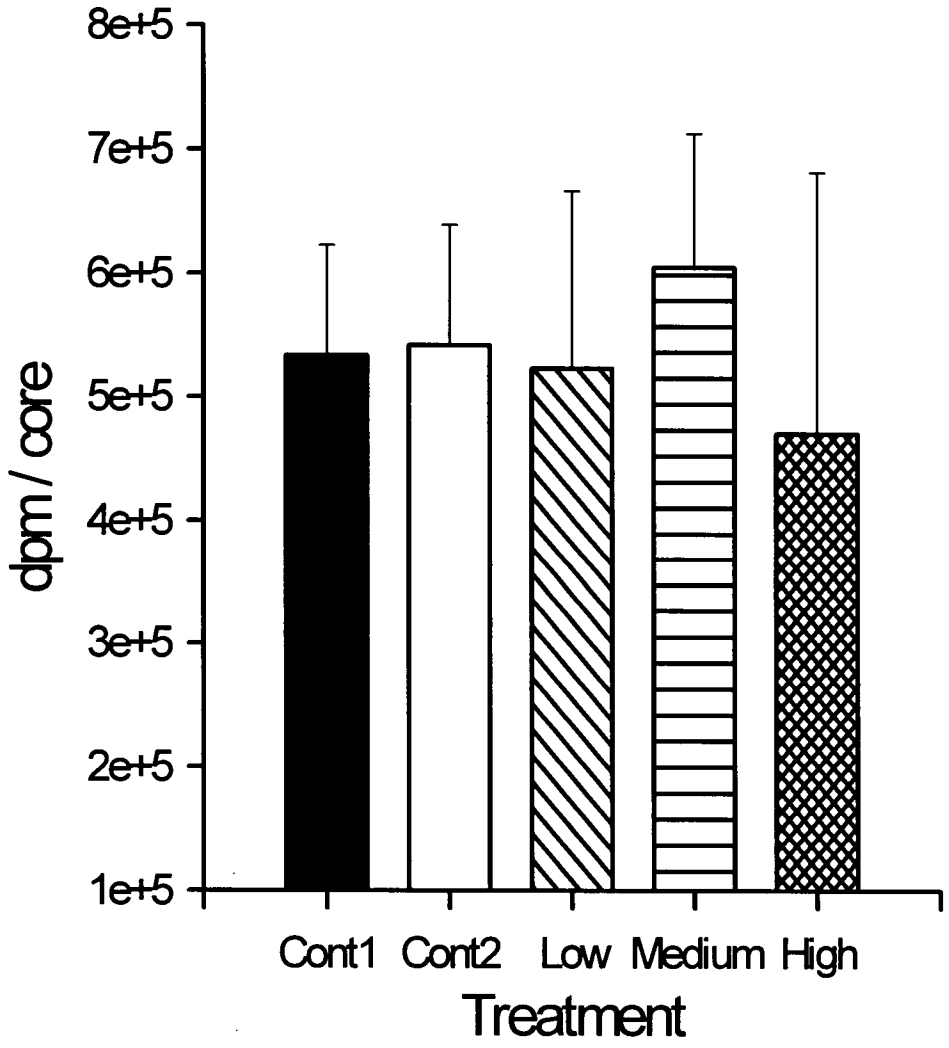

Figure 6. ^{3}H-leucine incorporation into protein in sediments exposed to a range of diesel contamination on day-28 of experiment. Solid bars = Cont1; Open bars = Cont2; Diagonally hatched bars = Low; Horizontally hatched bars = Medium; Cross-hatched bars = High. Values are means $\pm$ 1 S.D. (n = 4).

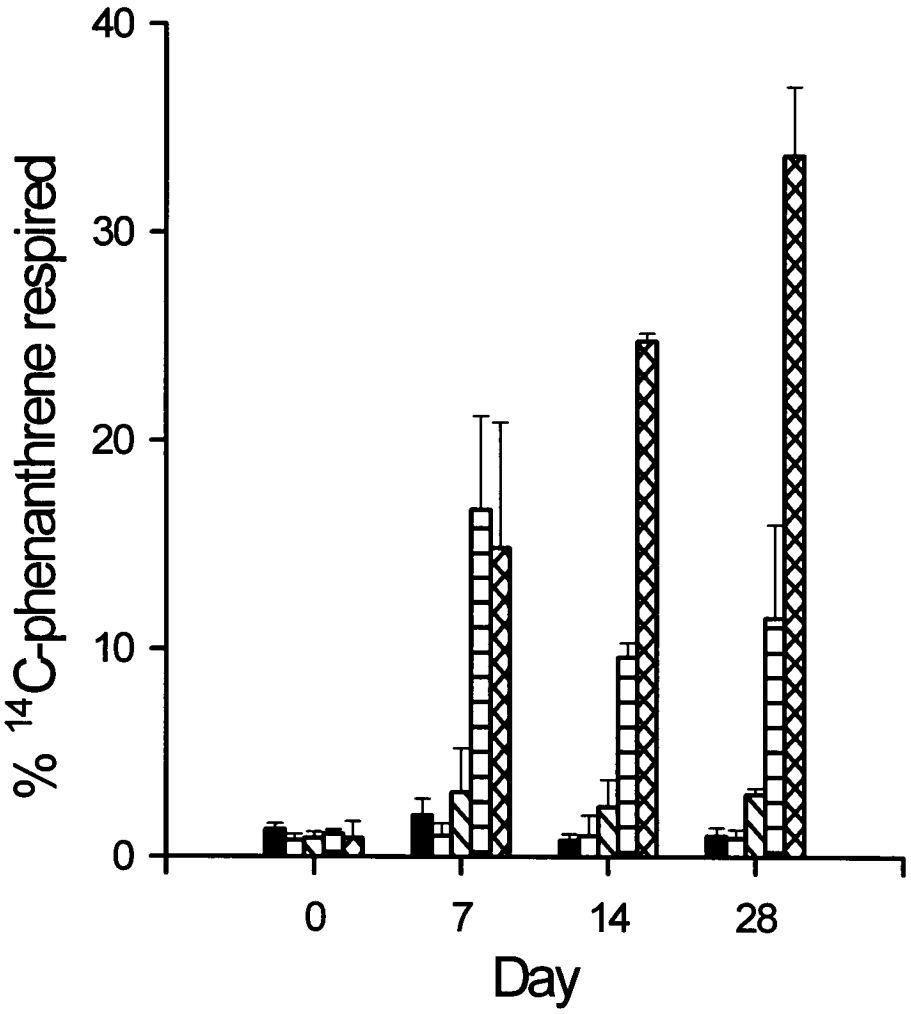

Figure 7. Conversion of ^{14}C-phenanthrene to $^{14}CO_2$ in sediments exposed to a range of diesel contamination over a 28-day period. Sediments were incubated for 72 h at 27 °C. Solid bars = Cont1; Open bars = Cont2; Diagonally hatched bars = Low; Horizontally hatched bars = Medium; Cross-hatched bars = High. Values are means $\pm$ 1 S.D. (n = 4).

Chapter 4. Discussion

Our observations on the effects of diesel on Gulf of Mexico sedimentary bacteria appear to be generally consistent with previous studies of individual PAH or crude oils. Even at the highest doses (ca. 55 ppm PAH), diesel-contaminated sediment had no detectable influence on bacterial incorporation of ^{14}C-acetate or ^{3}H-leucine, or on bacterial abundance. In a microcosm study similar in design to that reported here, PAH-contaminated sediments from a produced-water site in the Gulf of Mexico also failed to elicit a change in sedimentary bacterial abundance or metabolism of ^{14}C-acetate (Carman *et al.* 1995). Nor did diesel-contaminated sediments have an influence on the physiological condition of the bacterial community as indicated by the relative incorporation of ^{14}C-acetate into phospholipids and PHA. Our experimental manipulations could have potentially produced both a physical (addition of sediment) and chemical (addition of hydrocarbons) disturbance to benthic microorganisms. Nevertheless, no evidence of disturbance was detected.

Failure to detect changes in bacterial abundance or metabolic activity, however, does not mean that the bacterial community was unaffected by addition of hydrocarbons. Indeed, Baker & Griffiths (1984) proposed that evolved resistance to environmental contaminants may be responsible for variability in responses of sedimentary microorganisms to petroleum hydrocarbons. Further, Griffiths et al. (1981b) proposed that the insensitivity of Gulf of Mexico bacteria to petroleum hydrocarbons is the result of adaptation to chronic exposure from years of oil-production activities in the area.

Cerniglia & Heitkamp (1989) proposed that microbial adaptation to PAH contamination occurs as a two-step process: (1) acutely toxic low-molecular-weight PAH (such as naphthalene) eliminates sensitive microbes, and (2) resistant microbes that can metabolize PAH undergo a period of increased growth/activity. Our observations are partially consistent with this hypothesis. First, we observed no evidence of acute (or chronic) toxicity. Neither bacterial metabolic activity nor bacterial abundance were significantly influenced by diesel after short (Day-0 or Day-7) periods of exposure. Thus, any mortality or suppression of activity that might have occurred was below the sensitivity of our techniques. It is worth noting, however, that with approximately 10^9 bacteria per gram of sediment, it is possible to eliminate or add millions of bacteria and not detect the change in a typical direct-count procedure. The second part of Cerniglia & Heitkamp's hypothesis is generally supported by our observations. Bacterial metabolism of ^{14}C-phenanthrene dramatically increased over time in a dose-dependent pattern, implying that a PAH-degrading assemblage of bacteria developed in response to the presence of diesel-contaminated sediments. Again, however, we detected no significant change in total bacterial abundance that was related to diesel contamination, suggesting that (i) growth of PAH-degrading bacteria was offset by mortality of other bacteria, (ii) existing bacteria have the capacity to metabolically switch to PAH degradation, or (iii) the total number of PAH-degrading bacteria was insignificant relative to the total bacterial community.

Although ^{14}C-phenanthrene degradation rates were relatively low at Day 0, degradation was nevertheless detectable (0.30 to 0.43% day^{-1}). Using estuarine sediments from New York, Bauer & Capone (1985b) observed that ^{14}C-anthracene (another three-ring aromatic) degradation was only 0.01% day^{-1} even after four days of exposure at 100 ppm. Thus, it would appear that ambient bacteria in this Louisiana salt marsh exhibit some significant level of preadaptation to PAH. Nevertheless, even modest (0.55 ppm) additions of diesel elicited significant elevations of phenanthrene degradation. In the Low and Medium treatments, the rate of ^{14}C-phenanthrene degradation peaked by one week, and remained constant thereafter. ^{14}C-phenanthrene degradation in High treatments, however, continued to increase throughout the experiment, and reached a rate of 11.2% day^{-1} by Day-28. For comparison, Bauer & Capone (1985b) reported a maximum degradation rate for anthracene of 3.9% day^{-1}. Further, the maximum phenanthrene degradation rates reported here are comparable to the maximum rates of naphthalene (a much more labile PAH) degradation (10% day^{-1}) reported by Bauer & Capone (1985b).

The continued acceleration of phenanthrene degradation in High treatments apparently occurred because the supply of hydrocarbons outpaced the rate at which they were metabolized. Specifically, PAH in Day-28, High treatments were still highly enriched in alkylated PAH, including naphthalenes, phenanthrenes, and DBT, indicating the presence of unmetabolized petroleum hydrocarbons.

We also observed that alkylated PAH, which are generally diagnostic of petroleum hydrocarbons, were readily removed from sediments. Relatively little information is available concerning the metabolism of alkylated versus parent PAH. Cerniglia & Heitkamp (1989) observed that 2-methylnaphthalene was metabolized much slower than naphthalene or phenanthrene. Our data suggest that even highly methylated naphthalene (C4) was readily metabolized, as were alkylated forms of other PAH (i.e., phenanthrenes and dibenzothiophenes). In Low and Medium treatments, parent and alkylated naphthalenes, phenanthrenes, and DBT were removed completely or almost completely over the 28-day study period. The rate of decrease in parent phenanthrene and DBT in Low and Medium treatments was lower than the rates of decrease in alkylated forms. In the High treatment, parent phenanthrene and DBT increased by approximately a factor of 15 and 1.4 respectively, whereas accumulation of alkylated phenanthrenes and DBT was generally much lower (Table 1). This implies that the removal of PAH was not simply a desorption phenomenon (Means *et al.* 1980, Means & Wijayaratne 1984, Means & McMillin 1993, Means 1995); if such were the case, higher molecular weight (alkylated) compounds would have been removed more slowly. Thus, microbial degradation must have contributed significantly to the removal of two- and three-ring parent and alkylated PAH, and alkylated PAH showed no evidence of being disproportionately resistant to microbial degradation.

Previous studies have suggested that DBT may provide a reliable marker for petroleum-hydrocarbon contamination because they are found in all types of petroleum (Steinhauer *et al.* 1994, Clark 1989), including diesel (Williams *et al.* 1986), and they are considered to be resistant to photochemical (Andersson 1993) and microbial (Sinkkonen 1989) degradation. In the present study, however, DBT were at least as susceptible to microbial degradation as were phenanthrenes. Over the 28-day study,

essentially all DBT were removed from Low and Medium treatments, thus leaving no evidence of diesel contamination. Fayad & Overton (1995) also observed high rates of DBT degradation in sediments contaminated during the 1991 Gulf War. In particular, they observed that C2- and C3-DBT were degraded more quickly than C1-DBT, an observation that is consistent with our data (Table 1). Our data also show that, in High treatments, the rate of accumulation of DBT was much less than that of phenanthrenes. Thus, our data indicate that DBT were metabolized by sedimentary bacteria at a high rate, and that DBT would not accumulate in these sediments unless very high rates of input were maintained.

Chapter 5. Conclusions

Collectively, our data indicate that the Louisiana salt marsh bacterial community studied here is symptomatic of one that has been chronically exposed to petroleum hydrocarbons: bacterial abundance and general assays of bacterial metabolism are insensitive to additions of diesel, ambient bacteria can metabolize PAH at substantial rates, and the PAH-degrading portion of the community responds quickly to additions petroleum hydrocarbons. It is possible that the PAH-degrading bacterial community maintains ambient sedimentary PAH concentrations at relatively low levels. In Low and Medium treatments, no significant accumulation of PAH could be detected over the 28-day period relative to ambient sediment. Further, PAH concentration in High treatments were reduced by approximately 43% over the 28-day experiment, despite the daily addition of diesel-contaminated sediment.

These observations of the bacterial response to diesel contamination have implications for understanding how ecosystems respond to contamination by crude or refined petroleum hydrocarbons. One possibility, is that rapid bacterial metabolism of petroleum hydrocarbons could ultimately reduce exposure of other benthic organisms to potentially toxic compounds. We have observed that the meiofaunal/microbial foodweb in this salt marsh is relatively resistant to petroleum-hydrocarbon contamination from produced water (Carman *et al.* 1995), or diesel (Carman, unpublished). Further study will be required to determine if the apparent insensitivity of this benthic food web is because the fauna themselves are resistant to hydrocarbons, or if they rely on bacterial detoxification of petroleum contaminants.

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As the Nation's principal conservation agency, the Department of the Interior has responsibility for most of our nationally owned public lands and natural resources. This includes fostering sound use of our land and water resources; protecting our fish, wildlife, and biological diversity; preserving the environmental and cultural values of our national parks and historical places; and providing for the enjoyment of life through outdoor recreation. The Department assesses our energy and mineral resources and works to ensure that their development is in the best interests of all our people by encouraging stewardship and citizen participation in their care. The Department also has a major responsibility for American Indian reservation communities and for people who live in island territories under U.S. administration.

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As a bureau of the Department of the Interior, the Minerals Management Service's (MMS) primary responsibilities are to manage the mineral resources located on the Nation's Outer Continental Shelf (OCS), collect revenue from the Federal OCS and onshore Federal and Indian lands, and distribute those revenues.

Moreover, in working to meet its responsibilities, the **Offshore Minerals Management Program** administers the OCS competitive leasing program and oversees the safe and environmentally sound exploration and production of our Nation's offshore natural gas, oil and other mineral resources. The MMS **Royalty Management Program** meets its responsibilities by ensuring the efficient, timely and accurate collection and disbursement of revenue from mineral leasing and production due to Indian tribes and allottees, States and the U.S. Treasury.

The MMS strives to fulfill its responsibilities through the general guiding principles of: (1) being responsive to the public's concerns and interests by maintaining a dialogue with all potentially affected parties and (2) carrying out its programs with an emphasis on working to enhance the quality of life for all Americans by lending MMS assistance and expertise to economic development and environmental protection.