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The proposed research was designed to utilize a combination of laboratory and field studies to identify physical, chemical, genetic, and physiological influences that govern the accumulation and biodegradation of polycyclic aromatic hydrocarbons (PAHs). These and related compounds are among the chemicals whose environmental fate has been targeted by the U.S. Air Force Bioenvironmental Research Program. We have conducted a prior, independent study that has shown that, despite the presence of PAH mineralizing microorganisms, PAHs persist at a site where freshwater sediments are fed by PAH-contaminated ground water. Hypotheses to be tested address fundamental mechanisms for the persistence of environmental pollutants, these include: (1) the rate of delivery meets or exceeds the rate of biodegradation; (2) the PAHs are not available to microbial populations due to sorption onto the sediment organic matter, complexation reactions with dissolved organic carbon, or due to the physical arrangement of the sediment matrix which prevents contact between PAHs and microorganisms; (3) the microorganisms may be physiologically limited by the presence of preferred metabolic substrates or toxic or inhibitory substances, or by the lack of proper final electron acceptors, electron donors, or inorganic or organic nutrients; and (4) PAHs may persist simply due to restricted distribution and abundance of biodegradation genes in naturally occurring microbial populations. By working in an iterative manner between field observations and controlled laboratory determinations, we intend to systematically test the above hypotheses and thus identify constraints on microbiological processes that mineralize PAHs (naphthalene and phenanthrene) at the field site.

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(TO THE ATTENTION OF: Dr. Walter J. Kozumbo)

For AFOSR Project – 91-0436

GEOCHEMICAL, GENETIC, AND PHYSIOLOGICAL CONTROL OF POLLUTANT BIODEGRADATION

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1.0 BACKGROUND

This document reports results of field work and laboratory experiments carried out during the first year of a 3-year project (as originally conceived), entitled "Geochemical, Genetic, and Physiological Control of Pollutant Biodegradation". For a detailed literature review, project description and statement of work, the reader is referred to the original proposal in its entirety. A slightly modified version of the Abstract from the proposal that originally defined this project appears below. This synopsis is the most efficient way to familiarize the reader with project motivations and goals.

ABSTRACT

The proposed research was designed to utilize a combination of laboratory and field studies to identify physical, chemical, genetic, and physiological influences that govern the accumulation and biodegradation of polycyclic aromatic hydrocarbons (PAHs). These and related compounds are among the chemicals whose environmental fate has been targeted by the U.S. Air Force Bioenvironmental Research Program. We have conducted a prior, independent study that has shown that, despite the presence of PAH mineralizing microorganisms, PAHs persist at a site where freshwater sediments are fed by PAH-contaminated groundwater. Hypotheses to be tested address fundamental mechanisms for the persistence of environmental pollutants, these include: (1) the rate of delivery meets or exceeds the rate of biodegradation; (2) the PAHs are not available to microbial populations due to sorption onto the sediment organic matter, complexation reactions with dissolved organic carbon, or due to the physical arrangement of the sediment matrix which prevents contact between PAHs and microorganisms; (3) the microorganisms may be physiologically limited by the presence of preferred metabolic substrates or toxic or inhibitory substances, or by the lack of proper final electron acceptors, electron donors, or inorganic or organic nutrients; and (4) PAHs may persist simply due to restricted distribution and abundance of biodegradation genes in naturally occurring microbial populations. By working in an iterative manner between field observations and controlled laboratory determinations, we intend to systematically test the above hypotheses and thus identify constraints on microbiological processes that mineralize PAHs (naphthalene and phenanthrene) at the field site.

2.0 PROCEDURES AND RESULTS

During the 12 month period that has passed since initiation of this project, a variety of procedures for characterizing the field site for measuring microbial metabolism of PAHs, and for measuring PAH sorption reactions have been designed and implemented.

2.1. Field Measurements

The groundwater portion of the field study site, in South Glens Falls, NY has been relatively well characterized under the auspices of the Electric Power Research Institute (EPRI) (Madsen *et al.*, 1991). However, the portion of the site that is the object of study for this project, the seep, has not been extensively examined. Our first task was to develop a physical map, noting principal topographic, hydrologic, and chemical characteristics of the site. Figure 1 shows the relationship between the seep study site of this project and the groundwater portion of the study site (Madsen *et al.*, 1991). Figure 2 diagrams the major features of the seep area. The site is relatively small in scale. Care in sampling must be taken so as not to disturb the site's naturally occurring microbiological, chemical, and hydrologic gradients.

2.1.1. Sampling

Site sampling procedures varied according to the specific objectives. The overall rationale followed that prescribed by accepted authorities (e.g., APHA, 1985). Because the seep sediments are constantly being exposed to air-borne and water-borne microorganisms, asepsis is generally required only when attempting to discern differences in vertical distribution of microorganisms (within a profile, for example). Containers used varied from plastic garbage bags to carry large (- kg) portions of the seep, to small (50 cc) aseptic Whirl-Pak plastic bags, to sterile glass vials sealed with teflon faced septa, to sterile glass ampoules that were flame sealed on site within 5 minutes of obtaining a sample.

Sampling tools included clean shovels, flamed spoons and spatulas, sterile jars dipped into the flowing water, and 5 cm (in diameter) stainless steel tubing that was driven into the seep area to retrieve vertical cores. When the stainless steel cover was used, a portable electric drill prepared holes at various depths in the tubing so that a mini O_2 probe could be inserted within minutes after the core was obtained. After recording the O_2 reading, a rotary pipe cutter was used to expose the sediment at the same depth so that samples for naphthalene and

microbiological determinations could be performed. Three cm^3 samples for naphthalene determinations were placed within screw cap (teflon sealed) vials along with 2 ml chemical extractant (1:9; butanol:hexanes). Samples for microbiological analysis were transferred with a flamed spatula to sterile plastic Whirl pak bags. All samples were cooled on ice immediately at the site and kept cold until analyzed.

In anticipation of ice and snow cover during winter months, we visited the site shortly after the grant was initiated (October 1991) and used a spade to gather water logged sediments from the 1-20 cm depth that were stored at 4°C in plastic containers. These materials provided samples for chemical and microbiological experiments during the winter months. Whenever possible, however, additional site visits were completed so that experiments could also be performed on freshly-gathered seep materials. These additional site visits occurred in April, July, August, and September 1992.

2.1.2. On-Site Measurements

During 2 site visits, on-site measurements were performed because these most accurately reflect the (artifact free) biogeochemical status of the site. Field meters that were routinely used include pH (Orion), temperature (Orion), and O_2 (Microelectrodes, Londonderry, NH).

2.1.3. Other Measurements

Routine analysis of naphthalene and phenanthrene were carried out in E. L. Madsen's laboratory using as Perkin Elmer model 8500 gas chromatograph equipped with 0.25 mm x 25 m methyl-phenyl-5 glass capillary column and a Flame Ionization Detector. In L. Lion's laboratory, the same analyses were performed using a Hewlett Packard 5890 gas chromatograph equipped with a stainless steel packed column (10% SP-2100 on 100/120 Supelcoport packed column) and a flame ionization detector.

Nutrient analyses of the seep sediment were performed on dried, sieved samples of seep sediment by the Cornell University Soil Analysis Laboratory. GC/Mass spectrophotometry was performed in the Chemistry Department using a Hewlett-Packard model 5970 mass spectrometer using electron impact ionization at 70 eV.

PAHs were extracted from both water and sediment samples with either 100% hexanes or a mixture of butanol/hexanes (1:9) added to water (1:4) or to

sediment (2:3). Water samples from the site were added with extractant to glass ampoules, and sealed immediately. Sediment samples were added to screw cap bottles, mixed with extractant, and then immediately sealed with teflon faced septa.

Samples were thoroughly mixed, held, and allowed to settle for approximately 1 week prior to being opened so that 0.8 µl of extractant could be injected onto the GC for PAH analysis.

2.1.4. Results of Site Characterization Efforts

Table 1 shows the results of analyses designed to assess the general geochemical status of the site. The nutrient analyses show typical freshwater concentrations of a variety of inorganic constituents. There is no obvious indication of nutrient (N, P, Fe) limitation at the site.

Table 2 contains results of several GC analyses of both water flowing through the seep area and a sediment sample. It is clear that sealing ampoules on site immediately after sampling was effective in preventing volatilization loss of naphthalene. During the 8/8/92 site visit a clear naphthalene signal (6.7 ppm) was obtained from the sediment 15 cm deep. An example of chromatograms from the seep, as well as mass spectral confirmation of naphthalene are presented in Fig. 3.

Table 3 shows results of two coring efforts that provided discrete seep sediment samples from four depths. Each sample was analyzed for O_2 before segmenting the core, and later analyzed in the laboratory for naphthalene content and total microscopic bacterial counts. No phenanthrene was detected.

The most salient features of Tables 1-3 and Figure 3 are: (i) naphthalene is ubiquitous ; (ii) the sediment are anaerobic except at the very surface; (iii) nitrate, a nutrient and final electron acceptor, is absent; (iv) organic matter is high; (v) microorganisms are abundant.

2.2. Microbiological Measurements

2.2.1. Radiorespirometric Techniques

The principal indication of PAH metabolism used in this investigation was the conversion of ^{14}C present in an organic molecule to $^{14}CO_2$. This assay is routine in environmental microbiology research (Madsen *et al.*, 1991) but a few critical details are describe below. The ^{14}C labeled compounds (naphthalene and phenanthrene) were dissolved in water - no solvent carriers, that sometimes have undesirable chemical and microbiological side effects, were used. The radiolabeled materials were added to sediment samples within glass containers (serum bottles or screw cap vials) sealed with septa. Within each container was a small internal reservoir of 0.5 N NaOH used to trap $^{14}CO_2$. The NaOH was periodically removed, replenished (using a needle and syringe), and counted in a scintillation counter. Resultant data are displayed as time courses of cumulative $^{14}CO_2$ produced. All experimental treatments were prepared using triplicate assay vessels and were contrasted with a single poisoned control container handled identically to the live treatments except 1 ml of a 0.25 M $HgCl_2$/5% HCl solution was added. Metabolic activity in the poison controls was either low or absent and subtracted from activity found in the absence of poison.

Radiolabeled 1-^{14}C-naphthalene (specific activity 10.1 mCi/mmol, >98% radiopurity) and 9-^{14}C-phenanthrene (specific activity 13.1 mCi/mmol, >98% radiopurity) were purchased from Sigma Chemical Co. ^{14}C labeled materials were quantified using a Beckman Model LS 5000 scintillation counter using Ecoscint scintillation cocktail.

2.2.2 Nutrient Limitations Under Aerobic Conditions

In order to test the hypothesis that naphthalene degradation in seep sediments was limited by nutrients and/or inhibitory substances, nine treatments were prepared (in triplicate). Naphthalene mineralization was measured in 8 g portions of sediment amended with 1 ml deionized water that did or did not deliver nitrate + sulfate (100 ppm each), NH_4 + PO_4 (35 ppm; 5 ppm, respectively), a standard trace metals solution (Stanier *et al.*, 1966), a standard vitamin solution (Stanier *et al.*, 1966), non-radioactive naphthalene (3 ppm), acetate (30 ppm), and a head-space with O_2 concentration twice atmospheric.

Results are shown in Figure 4. All treatments, except the poison, converted approximately 54% of the added radioactivity to $^{14}CO_2$ within 18 hours; yet, no significant stimulation of aerobic naphthalene metabolism was found. This percentage of substrate mineralized is common for bacteria growing on naphthalene. Presumably the remaining 46% was incorporated into cell biomass.

Conclusions. The simple conclusion from data shown in Fig. 4 is that there were microorganisms present in the seep sediments that mineralized naphthalene at similar rates and to similar extents regardless of the various amendments. Thus, aerobic naphthalene metabolism at the site does not appear to be limited by (i) the absence of naphthalene degraders or (ii) toxic substances, or (iii) organic or inorganic nutrients.

2.2.3 Effect of Contact Time on Naphthalene Biodegradation: The Aging/Bioavailability Hypothesis

One of the primary hypotheses that this project is attempting to test is protection of PAHs by sorptive reactions that may reduce their bioavailability, hence impede PAH biodegradation. By experimentally manipulating the contact time between aseptically prepared PAHs and sediment, we hope that a pattern will emerge during subsequent mineralization experiments. Our hypothesis is that diffusion of PAHs into microporous sediment organic matter will render PAHs unavailable to sediment bacteria. As PAHs enter the coating more and more deeply with increasing exposure time, overall metabolism of PAHs should be inversely proportional to contact time. Because sorption reactions are related to the organic matter content of sorbent, the majority of the experiments described below utilized two types of sediment from the site: seep sediment (high in organic matter) and sandy aquifer sediment derived from upgradient area of the site (low in organic matter).

Our methodological approaches have been gradually refined with each successive experiment. A chronological treatment is given below. Table 4 summarizes the contact time/biodegradation experiments completed thus far.

2.2.3.1. Aging Experiment #1: Naphthalene with seep sediment aged in teflon sealed Ichem vials (similar to Pierce vials).

Procedures: 40 grams of γ-sterilized seep sediment were aseptically dispensed to 7 teflon-sealed Ichem vials. At times -20 d, -10 d, -8 d, -5

d, -2 d, -1 d, and 0 d, between 4 and $14 \cdot 10^4$ dpm of ^{14}C naphthalene were added in 16 ml of 0.005 M $CaSO_4$ along with non-radioactive naphthalene at a final concentration of 15 ppm (1/2 saturation). On day 0, the vials were opened, the supernatant was poured off and assayed for ^{14}C by scintillation counting, and 8 g (wet weight) quantities of the sediment were dispensed to 4 serum bottles (3 replicates inoculated and 1 poisoned with $HgCl_2$) sealed with butyl rubber septa containing internal plastic caps loaded with 2 N NaOH for $^{14}CO_2$ trapping. Periodically the NaOH was removed for scintillation counting and replaced. The inoculum was an enrichment of cells derived from seep sediment, grown on a liquid mineral salts/naphthalene mixture.

Results: Results of the first aging/mineralization experiment are shown in Fig. 5. Data shown represent the average of three inoculated replicate flasks minus the poisoned controls. The two most striking characteristics of the data in Fig. 5 are: (i) there is a greater % mineralized in the unaged (0) and briefly aged (1 day) treatments than in the others (aged between 2 and 20 days), which cluster together; and (ii) a very high proportion of the naphthalene was mineralized. As noted in Table 4, certain difficulties arose while trying to implement this first experiment.

The Ichem vials with teflon septa had imperfect seals but were reasonably (90%) effective in retaining phenanthrene as shown by tests measuring ^{14}C in solution over 33 days. Nonetheless, because of its greater volatility, it is probable that escape of ^{14}C-naphthalene diminished the amount of substrate actually delivered to the mineralization assay vials. If aging vials leaked, greatest leakage would have occurred in the older vials; thus, the trend shown in Fig. 5 could conceivably have been an artifact, imposed by use of improper vials during aging. Mineralization in older sediments may be lower simply because by the time the mineralizations were performed, the amount of ^{14}C to be metabolized was diminished.

The fact that mineralization reached high levels (106% for the sediments aged 1 day) probably represents minor inaccuracies, augmented by propagation of errors. When microorganisms are growing on organic substrates, established physiological principles predict about 50% assimilation of organic carbon and 50% conversion

to CO_2. Only when microorganisms are growing on accompanying carbon sources or not growing at all (just maintaining themselves) is mineralization expected to approach 100%. Neither the nature of the inoculum used nor accompanying carbon sources were characterized during this experiment. Therefore, a precise explanation for the high extent of mineralization is not possible.

As described in Table 4, there were some uncertainties in achieving a homogeneously mixed slurry. Lack of uniformly distributed ^{14}C naphthalene would contribute to large variabilities between replicate mineralization vials and detract from confidence in the data.

An additional difficulty that arose while carrying out the first "aging" experiment was the inability to be sure that naphthalene volatilization losses were negligible while preparing the aged sediment/naphthalene mixture for the mineralization assay. Once naphthalene is added to the complex subsurface matrix, we have no extraction technique that is 100% efficient for verifying the expected mass of naphthalene has not volatilized. We were careful to minimize periods when the vials were uncapped. Furthermore, naphthalene volatilization is expected to be minimal when in contact with hydrophobic sorption surfaces such as those in the seep sediment. But perfect mass balances are elusive

Conclusions:

a) Methods need to be improved.

b) Despite the weaknesses of methodology, data support the hypothesis that naphthalene bioavailability diminishes with increasing contact time with a sorbent.

2.2.3.2. Testing of Homogeneity of ^{14}C Naphthalene/Sediment Mixture

A possible weak link in the procedure described above is non-uniform distribution of the ^{14}C compound during the aging period and during dispensing of subsamples from Ichem vials into mineralization-assay vials.

Procedures to test homogeneity were implemented. 24 g of γ-sterilized sand and seep sediments were mixed with 16 ml of 0.005 M $CaSO_4$ containing ^{14}C naphthalene at a concentration of 0.6 ppm and sealed in glass ampoules. The ampoules were tumbled for 2 days then held stationary for 5 days before being opened. After opening, before

being disposed of, a subsample of clear supernatant was counted in a scintillation counter and its volume measured. Then 0.5 g portions of the sediments were mixed with 0.3 ml hexanes. The mixture was vortexed, centrifuged, and the ^{14}C in the hexane supernatant counted in a scintillation counter.

Results were as follows: Based on counts in the supernatant, we determined that 58% and 98.7% of the naphthalene sorbed to the sand and seep, respectively. The efficiency of hexanes for extracting the sorbed naphthalene from the sediments was quite low: 4.3% for the seep and 13% for the sand. But most importantly, when 8 subsamples were taken from each sealed ampoule, recovered dpm/g ranged from 100 to 173 dpm for the sand and 355 to 570 dpm for the seep. Thus, homogeneity was not achieved – inter-replicate variability was very large.

After completing two iterations of the above procedures, each time modifying various parameters, we arrived upon the following acceptable protocol. Using a 24 g sediment to 24 ml H_2O mixture in sealed ampoules and acetone as an extractant, 4 replicate subsamples were found to match within 9% of one another. Therefore, this improved protocol for aseptically aging naphthalene in the presence of sediments was used in subsequent experiments.

2.2.3.3. Aging Experiment #2: Naphthalene with seep and sand sediments aged in glass ampoules

Procedures were similar to experiment #1 except 24 g of sediment was mixed with 24 ml of water. At times - 21, -11, -2, -1 and 0 d, between 6 and $24 \cdot 10^4$ dpm of ^{14}C naphthalene was added along with non-radioactive naphthalene (3 ppm). Sorbents used included both sand and seep sediments. To eliminate the possibility of naphthalene loss during aging, aging was done in sealed glass ampoules. Because of the lower mass of sediment within each ampoule, the wet weights distributed to mineralization vials were reduced to 5 g. The hardware for the mineralization assay consisted of Pierce vials (screw cap 25 ml) sealed with teflon-backed silicone septa. Again, these septa were periodically punctured to remove, replace, and count ^{14}C in NaOH. The inoculum was not an enrichment culture. Rather stored seep sediment

was diluted (1 to 100) and a suspension was dispensed to each mineralization vial.

Results: Results of the second aging/mineralization experiment appear in Figs. 6A and B. The trend shown in Figs. 6A and B contrast with the one suggested in Fig Regardless of sorbent type (6A = sand, 6B = seep), the greatest rates and extents of naphthalene mineralization were found from the oldest (21- and 11-day treatments). But any simple relationship between contact time and naphthalene bioavailability seems to disappear in Fig. 6B in which treatments aged 1, 11, and 21 days all clustered together. Another characteristic of the data that contrasts with Fig. 5 is that a lower proportion of the naphthalene was metabolized from the sand sediments than the seep.

Discussion: In interpreting Figs. 6A and B, it is essential to appreciate the complex mechanisms that contribute to the net outcome of $^{14}CO_2$ production. The sorption/desorption and microbial attack/metabolism processes are, essentially, coupled "black boxes". One black box is the sediments. The other is the microbial community. Our naive hope was that by striving for uniformity, consistency, and control during experimental manipulations, a clean, simple relationship between sorptive contact time and naphthalene, bioavailabity would result. An initial reaction to Figs. 6A and B could be frustration and abandonment of the Aging/Bioavailability Hypothesis. But another approach is to examine the details — searching for explanations. Our overall goal is to understand the behavior of organic substances in groundwater environments. Toward that end, our specific objective is to prove or disprove the Aging/Bioavailability Hypothesis. Results of the second experiment may be perplexing, but they are insufficient to test the hypothesis.

Conclusions:

a) Despite methodological improvements, the Aging/Bioavailability Hypothesis has not been fully tested.

b) Variability in microbial physiology is probably the cause for discrepancies between results of Experiments 1 and 2. It is the microbiological component of the test system that is least understood.

c) A major difference between Experiments 1 and 2 was how the inoculum was prepared. In Experiment 1, the inoculum was an enrichment — the microbial community was given soluble naphthalene and the opportunity to adapt to this aqueous substrate prior to inoculation. Implicit in "adaptation" is a physiological or a compositional change in the microbial community. In Experiment 2, microorganisms native to the seep sediment were inoculated directly into the mineralization vials, before adaptation to aqueous naphthalene could be achieved. We feel that some microorganisms may prefer or be indifferent to sorbed naphthalene (Figs. 6A, B) while others may prefer it in an aqueous form. To further investigate this issue, aging Experiment 3 was implemented.

2.2.3.4. Aging experiment #3: Naphthalene with seep and sand sediments aged in glass ampoules inoculated with an enrichment culture.

Procedure: Essentially the same procedures were followed as for Experiment 2 except the aging period ranged from 0 to 28 days, and 4 rather than 5 g of wet sediment was dispensed to mineralization vials so that a portion could be extracted directly at t = 0 in an attempt to measure sorbed ^{14}C. The major variable that was changed, however, was use of a mineralization inoculum that was enriched on aqueous naphthalene.

Results: Results of the third aging experiment are shown in Fig. 7A and B. The data compare favorably with those in Fig. 5 and contrast with those in Figs. 6A and B. By changing the inoculum from "unenriched" to "enriched" (see rationale in Section 2.2.3.3), a clear relationship between rates/extents of mineralization and contact time was achieved. Regardless of sorbent (sand in 7A, seep in 7B), the greatest mineralization was observed on sediment aged 0 and 1 day. Furthermore, mineralization was generally diminished proportionately to the duration of the contact period between naphthalene and sorbent.

Results of extractions to verify ^{14}C present in mineralization assays. We sought to use an independent extraction method to verify our calculations for the amount of ^{14}C naphthalene delivered to each mineralization vial (total added minus total removed in the supernatant, times the proportion of total mass dispensed to each vial). To accomplish this, at the same time that 4 g wet sediment portions were

being dispensed to mineralization vials, 2 g were also added to 2 ml of a 1:1 mixture of CH_2Cl_2/acetone. After 1 hr of shaking, followed by centrifugation, 1 ml of the extractant was counted in the scintillation counter. Presuming that all of the possible ^{14}C was transferred to the organic phase, the amount of extracted, sediment-bound ^{14}C was calculated and compared to the same figure calculated by difference. Table 5 presents the results. Ratios (extracted ^{14}C to calculated ^{14}C) for the sand sediment ranged from 0.10 to 0.59, and were roughly inversely proportional to contact time. Ratios for the seep sediment ranged from 0.31 to 0.59 and showed no clear relationship to contact time.

The main lesson from data in Table 5 is that methodological limitations prevent us from being confident about mass balances in these tests. We are attempting to obtain a Supercritical Fluid Extraction apparatus that has a well proven record in removing PAHs from sediments. But in the absence of such a device, there is no simple reliable procedure that consistently recovers 100% of PAHs from complex sediment matrices. Had the ratios in Table 5 been close to unity, we would have been very confident in the denominators used in calculating the % mineralization figures that appear in Figs. 7A and B. The small ratios reported in Table 5 do not invalidate the mineralization data, but neither do they strengthen them. However, the lack of agreement does emphasize the methodological problems before us.

Conclusions:

a) The Aging/Bioavailability Hypothesis seems to hold true for microbial communities adapted towards aqueous phase naphthalene.

b) The above conclusion needs to be confirmed in subsequent experiments that simultaneously vary inocula. The findings also need to be strengthened by performing similar experiments with other PAHs such as phenanthrene.

c) Methodological improvements still need to be implemented that eliminate uncertainties about retaining volatile substrates while preparing and implementing the mineralization component of the tests.

2.2.3.5. Influence of non-sorbed naphthalene on aging experiment results.

In devising experimental approaches for testing the Aging/Bioavailability Hypothesis, there are unavoidable compromises. For instance, after exposing sand and seep sediments to aqueous radiolabeled naphthalene and transferring the sorbent to mineralization vials, some non-sorbed naphthalene was carried along in interstitial fluids. During subsequent mineralization tests, it was impossible to discern if the $^{14}CO_2$ was derived from the sorbed or non-sorbed pools. Rinsing off the unbound naphthalene was prevented because of its solubility and volatility.

By measuring aqueous, non-sorbed ^{14}C in the aged supernatants and by accounting for the mass of water used in preparing and dispensing naphthalene-laden sediment, we were able to calculate the proportion of non-sorbed ^{14}C-naphthalene present in each mineralization assay completed in Experiments 1-3. Data show that when the seep sediment was used, between 0.7 and 3.5% of the ^{14}C naphthalene present was interstitial. In other words, between 99.3 and 96.5% of the labeled naphthalene was sorbed. Thus, the contribution of aqueous naphthalene to the $^{14}CO_2$ pool was small. However, when sand was the sorbent, the results were very different. The proportion of interstitial, non-sorbed ^{14}C naphthalene in assays using sand ranged from 17 to over 100% (figures >100% merely reflect the compounding of the variability in measurements). Because naphthalene metabolism by microorganisms may rely upon the aqueous, unbound pool (either at static equilibrium or through dynamic desorption reactions), insights into the mechanisms controlling metabolism can be gained by examining the relationship between non-sorbed naphthalene and the extent of naphthalene mineralization. Figs. 8 A-E plot the percent final naphthalene mineralization against percent of initial interstitial (unsorbed) naphthalene for Aging Experiments 1-3. A positive slope indicates that the non-sorbed naphthalene is more available than sorbed naphthalene for mineralization by microorganisms. Plots with no trends suggest no relationship or too much experimental variability and/or methodological problems. Three of the five plots in Fig. 8 (B, D, E) exhibit clear positive slopes. A fourth (Fig. 8C) shows a broad positive slope, while the data from the Aging Experiment #1 (Fig. 8A) shows uninterpretable

scatter; however, the results from this early experiment were most influenced by methodological problems.

The trends in Fig. 8 are consistent with the Aging/Bioavailability Hypothesis. Note that even the data from Aging Experiment #2 now support the hypothesis. Thus, it may not be "aging" per se that governs bioavailability, but merely the size of the non-sorbed naphthalene pool. Our original notion that the pool size was governed by contact time may or may not be true. The influence of inoculum type and the physiological capabilities of microbial communities on the non-sorbed pool size has yet to be determined.

2.2.3.6. Aging Experiment #4: Phenanthrene x seep and sand sediments aged in glass ampoules inoculated with seep and sand enrichment cultures.

Procedure: Essentially the same procedures were followed as for Experiment #3, except the aging period ranged from 0 to 20 days. Four g of seep sediment and 3 g of sand sediment were dispensed to mineralization vials. The two new major variables in this experiment were: change from naphthalene to phenanthrene and use of inocula derived from two different sources. Theoretically, the seep inoculum, being derived from material of high sorption capacity, would have a greater ability to metabolize sorbed phenanthrene.

Results: Results of the fourth aging experiment are shown in Figs. 9A and 9B. Because the inocula were "enriched" (i.e., grown on aqueous substances), according to results of Experiment #3, the microorganisms should not have been adapted to sorbed material. Therefore, an inverse relationship between aging and mineralization was anticipated. Moreover, sorbent effects should be magnified in these tests, because of phenanthrene's hydrophobic partitioning behavior.

A general inverse relationship between aging contact time and phenanthrene mineralization was found in the Fig. 9A data which depict $^{14}CO_2$ produced by the seep-derived inoculum from phenanthrene aged in the presence of sand. The amount of ^{14}C evolved from the 20 d treatment was 34%, which contrasts, with 48% which evolved from the 0 d treatment. More importantly, regardless of aging

period, no phenanthrene was mineralized when the seep sediment was sorbent.

In Fig. 9B data analogous to 9A are depicted for the enriched inoculum derived from sand. In this case, the inverse relationship between aging time and mineralization was weak. Though the day 20 treatment, did show the lowest extent of metabolism, it was the day 11 treatment (rather than day 0) that was metabolized to the greatest extent. But data in Fig. 9B do support the lack of availability of phenanthrene sorbed to the organic-rich seep sediment: No mineralization occurred regardless of aging time.

Conclusions. a) The Aging/Bioavailabity Hypothesis is consistent with data derived from sand sediment inoculated with an enrichment from seep sediment. However, the mixed enrichment derived from sand sediment mineralized phenanthrene in a manner that conflicted somewhat with the Aging/Bioavailability hypothesis. Reasons for these results are not perfectly clear, but probably reside in variabilities in properties of mixed unknown, microbial populations.

b) The lack of phenanthrene metabolism in the presence of seep sediment is striking and occurred regardless of inoculum type. Possible explanations include (i) the Aging/Bioavailability hypothesis (strong organic matter - phenanthrene interactions make the substrate unavailable), or (ii) phenanthrene-specific toxic effects of the seep sediments (unlikely, in length of section 2.2), or (iii) diauxic effects of the seep sediment (it may be that the inoculum preferentially metabolized alternative carbon compounds present in the sediment).

2.2.4. Anaerobic Metabolism of Naphthalene

Data in Tables 1 and 3 clearly show that anaerobic conditions predominate at depth in the seep sediments. Because dioxygenase enzyme complexes are the best characterized biochemical mechanisms of naphthalene metabolism (Haigler and Gibson, 1990), one obvious explanation for naphthalene's persistence at the site is simply lack of O_2. However, anaerobic metabolism of naphthalene under nitrate reducing conditions have also been reported (Mihelcic and Luthy, 1988a, b).

Procedures. To further explore the propensity for microorganisms at our study site to metabolize naphthalene under a variety of redox conditions. The following experiment was performed. In triplicate, 35 ml glass serum bottles, 4 g of seep sediments were amended with ^{14}C-naphthalene (80,000 dpm) and 1 ml of boiled deionized water with or without 30 ppm nitrate or sulfate. These bottles were prepared using anaerobic techniques in a Coy anaerobic hood containing a mixture of N_2:H_2 (98:2). After being sealed in the hood, the bottles were statically incubated in the dark at 24°C. One set of bottles, that received only water, was pressurized with pure O_2 to triple the atmospheric level. Thus, the treatments were: oxygen amendment (= aerobic), nonamended (= methanogenic), sulfate amended (= sulfate reducing), nitrate amended (= denitrifying).

Results. Results of time course experiments of $^{14}CO_2$ production are shown in Fig. 10. During the 16 day experiment, naphthalene was extensively converted to $^{14}CO_2$ in the vials to which oxygen was added. The reaction was quite rapid – occurring primarily within the first 3 days. The sulfate-reducing and methanogenic treatments showed no significant $^{14}CO_2$ production activity. However, $^{14}CO_2$ was produced in the treatments that received nitrate. The rate and final extent of denitrifying naphthalene mineralization were lower than for the aerobic treatment. Discovery of denitrifying naphthalene metabolism is exciting because it paves the way for a variety of far-reaching experiments. These results need to be approached with caution, however, because they have not yet been reproduced. We found poor agreement between replicate flasks in the denitrifying treatments. Although head space analysis has shown that air did not enter the flasks through a poor seal, other possible experimental errors need to be explored.

Conclusions. If naphthalene metabolism under methanogenic or sulfate reducing conditions were operating at the seep, each of these metabolic regimes would have responded to their respective treatments. Because denitrifying and aerobic mineralization of naphthalene were observed, lack of NO_3^- or O_2 are strongly implicated as the reason for naphthalene persistence at the study site.

2.3. Chemical Measurements

Organic carbon contents, naphthalene partition coefficients, and background naphthalene concentrations were determined for sediment samples collected April 1992. Naphthalene desorption kinetics for these sediments will be examined in the future.

2.3.1. Organic Carbon Analysis:

Procedure: Organic carbon content of the seep sediment at different depths was determined using the Walkley-Black Method (Allison, 1965), which involved the oxidation of organic material by dichromate, $Cr_2O_7^{-2}$.

Results: See Table 6.

Discussion: Because of the high organic carbon content of these sediments, particularly the top three layers, one would expect significant quantities of hydrophobic polyaromatic hydrocarbons (PAHs) to sorb to the sediment, significantly influencing pollutant behavior in the environment.

Conclusion: The seep sediments have relatively high organic contents, suggesting that they can act as sig ificant sinks (sorbents) for hydrophobic organic pollutants.

2.3.2. Batch Isotherm Experiments

Batch isotherm experiments were performed to determine the characteristic partition coefficient, K_p, of the PAH, naphthalene, between the sediment samples and the aqueous phase.

Procedure: The following experimental procedure for developing batch isotherms was adapted from Lion *et al.* (1991). Small (~15 mL) amber vials with teflon septa and aluminum crimp caps were used. The vials were cleaned in a chromic-sulfuric acid solution overnight, rinsed six times in 18.9 MΩ/cm deionized, distilled water, and air dried. The tare weights of the empty vials, crimp caps and septa were obtained (±0.0001 g). Sediment samples were air dried ≥ 24 hours and passed through a 2.0 mm sieve. Before samples were added to the tared vials, the sediment was tumbled to homogenize the material. For each isotherm, five variable amounts of sediment were added to the vials, with two duplicates of each amount. The sediment weights were recorded (±0.0001 g). By adding a constant quantity of pollutant (^{14}C-naphthalene) to each vial, five distinct aqueous concentrations were expected after equilibration. The dry weight of each sediment was separately determined by placing approximately 5 g of

sediment in an oven (105°C) for 24 hours and calculating weight loss and percent moisture. Electrolyte (0.005 M $CaSO_4$, 0.02% NaN_3) was added to the air-dried sediment in the vials at least 1 hour prior to adding the naphthalene. The electrolyte and sediment formed a slurry, allowing the formation of natural aggregates and displacing air within the sediment so the pollutant could be evenly distributed within the sediment sample.

A ^{14}C-naphthalene stock solution consisting of crystalline naphthalene in contact with a 0.005 M $CaSO_4$, 0.02% NaN_3 electrolyte was kept on a magnetic stir-plate at 4°C. 100 µL of the stock solution was added to 10 mL of scintillation cocktail and counted to determine the activity of the stock. An appropriate amount of ^{14}C-naphthalene was added to make the total activity in each vial approximately 40,000 DPM. The amount of stock added to each vial within an experiment was constant. Two control vials received stock samples in 10 mL of scintillation cocktail and were counted to determine the average activity of the stock added to the vials. In addition, 3 to 4 "blank" vials received stock but no sediment and were used to determine pollutant uptake by the vials and septa. The vials were typically filled in the following order: control 1, blank 1, half the randomly ordered vials containing sediment, blank 2, the remaining vials with sediment, blank 3, control 2.

After the ^{14}C-naphthalene was added, the vials were filled completely (zero head space) with the electrolyte solution, and the vials were crimped closed. Total aqueous volume added (electrolyte + stock) was determined gravimetrically (± 0.0001 g). The control vials were placed in the scintillation counter. The sealed vials were then rotated horizontally at 12 rpm and 25°±1°C for one week. Although sorption is often considered complete after 24 hours, the additional time was allowed because the sediment samples already contained significant amounts of the pollutant that may alter the sorption kinetics (Connaughton, 1992). Connaughton (1992) showed that no significant losses of naphthalene occurred within 10 days of sealing the vials.

After one week, the vials were centrifuged at ~830 xg and 25°C for 30 mins. The crimp caps and septa were removed and 1 mL of aqueous phase was added to 10 mL scintillation cocktail and counted.

Results: the results of the batch isotherm experiments were interpreted in two ways, each method giving a value for the partition coefficient, K_p. The

first method was based on a mass balance, resulting in the following equation (Lion *et al.*, 1991):

$$\frac{C_o V_o}{C_s V_s} = 1 + K_p \frac{M}{V_s}$$

where: C_o = initial pollutant concentration (DPM/mL)

V_o = initial volume of pollutant added (mL)

C_s = aqueous concentration at equilibrium (DPM/mL)

V_s = total aqueous volume in vial (mL)

M = sediment mass (g)

K_p = partition coefficient (mL/g)

K_p, the slope of the linearized equation above, was determined by plotting C_oV_o/C_sV_s *vs.* M/V_s. Figure 11 illustrates a typical set of results.

The second method for determining the partition coefficient was to average the K_ps of each individual data point, using:

$$K_p = \sum_{C_i} (S_i)/n = \text{Average } K_{pi}$$

This method has been shown to provide a more uniform distribution of the experimental variance (Connaughton, 1992) (see Table 7).

Discussion: There was good agreement between the values obtained using both data reduction methods, particularly for the D2 sediment, indicating either approach was valid. However, the standard deviation was much smaller for the Kp value determined graphically, suggesting this may be a better approach. The discrepancy in the D3 sediment could have been because the line for the D3 sediment did not fit the data nearly as well as it did for the D2 sediment. Because the samples with the highest mass-to-volume ratio in the D3 sediment had higher aqueous concentrations (lower CoVo/CsVs) than predicted by the line, there may have been a problem with separation of the aqueous and solid phases. If colloidal material originally in the sediment was not separated during centrifugation, it would be sampled

with the aqueous phase, resulting in artificially high aqueous concentrations (colloids have higher pollutant concentrations than would an equal volume of the aqueous phase). The D3 sediment had a higher organic carbon content than the D2 sediment (17.9% vs. 8.5%), so there was a greater chance that colloidal material which had sorbed some of the pollutant would remain suspended.

Because of the smaller standard deviation, the mass balance graphical method was used for determining K_p for the remaining sediments (see Table 8). As expected because of the lower organic carbon content, the K_p value for the D4 sediment was significantly lower than the partition coefficients for the D1 sediment, and the K_p for D1a was lower than the K_p for D1b (D1a was washed to remove "floatable" material and had a slightly lower organic carbon content).

Although the high organic carbon sediments had higher partition coefficients than the lower carbon sediments, the K_ps determined using batch isotherm experiments did not have a strong linear relationship with organic carbon content. The individually calculated K_{oc} values ($K_{oc}=K_p/f_{oc}$) varied, yielding a K_{oc} for the organic carbon in the sediment with a large standard deviation (Table 8). This suggested variability in the sorptive capacities of the organics, variable influence by the non-separated organics, or the possibility that different mechanisms are acting in the different sediments. Never the less, normalization for organic content explained much of the variance in K_p, as has previously been reported (Karickhoff *et al.*, 1979).

Conclusions:

a) Solids separation may have influenced calculated K_p values.

b) Organic carbon exhibits variable sorptive capacity.

c) Normalization for organic carbon content of sediments explains much of the variance in their naphthalene distribution coefficients.

2.3.3. Extraction of Naphthalene from Sediments:

A solvent extraction procedure was employed to determine pollutant concentrations in sediment samples. A variety of solvents, including butanol and hexane, have been reported as potential extractants; however, no known solvent is completely effective in all cases, so different solvents and mixtures were examined.

2.3.3.1. Comparison of PAH Extraction with Butanol and Hexane

Procedure: Approximately 5 g of homogenized, wet, D3 (contaminated) sediment were added to each of 6 tared amber vials with teflon septa and crimp caps. The dry weight of the sediment was separately determined by drying approximately 5 g of the sediment overnight at 105°C. Three to four mL of butanol were added to 3 vials and 3-4 mL of hexane were added to the remaining vials. The vials were then immediately sealed, shaken vigorously by hand for 1 min, and vortexed briefly. Actual solvent volumes added were determined gravimetrically. The vials were rotated horizontally at 12 rpm for 24 hours at 25°C. The vials were then centrifuged at ~ 830 xg, 25°C, for 30 mins. One to five μL of solvent phase was sampled through the septa and injected onto a GC column, with at least 2 injections from each vial. Conditions for GC analysis are described in Section 2.3.4.4.

Results: Because the extractions were carried out prior to GC calibration, the relative amount of naphthalene extracted (GC peak area) was compared. Only the vials with butanol as an extractant had any peaks in the region expected for naphthalene, so butanol was chosen as the extractant for determining background pollutant levels. Samples extracted with hexane showed only the solvent peaks. When hexane was added to the wet sediment samples solid aggregates formed which did not break up significantly during shaking and vortexing. This may partially account for the inability of hexane to extract the pollutants from the sediment matrix.

Conclusion: Butanol appears to serve as a satisfactory solvent for extraction of naphthalene from moist, contaminated seep sediments.

2.3.3.2. Background Naphthalene Concentrations

2.3.3.2.1. Naphthalene Extraction #1

Procedure: The naphthalene concentrations in the sediments collected in April 1992, were estimated using the extraction methods described above (roughly 5 g wet sediment to 3-4 mL butanol). Four samples of each sediment were pr ured. After 3-4 days, 5 µL of the liquid phase were injected onto the GC column and naphthalene concen ations were determined in µg naphthalene/g dry sediment.

Results: see Table 9.

Discussion: With the exception of the D1[1] sediment, n nthalene concentration varied fairly linearly with organic c on content and K_p (see Figs. 12, 13, 14). This is consistent with the anticipated role of sediment organic carbon in the partitioning of nonionic organic pollutants. One explanation for the comparatively low naphthalene concentrations in the D1 sediment could be the presence of more oxygen in the sediment at the surface than in the deeper layers. Since naphthalene is mineralized aerobically, the presence of oxygen could account for the lower surface concentration. Another explana on for the low naphthalene concentration in the surface sediment could be volatilization to the atmosphere. Very little volatilization would be expected from the deeper sediments. In addition, it possible that the contaminated plume was below the surface, so e surface sediments did not receive the same input pollutant concentrations as the deeper sediments. Little is presently known about the subsurface hydrology of the seep area.

Conclusion: Organic carbon content influenced sediment naphthalene levels.

2.3.3.2.2. N thalene Extr ction #2

Procedure: Replication f seep sediment extraction and measurement was performed on the April 1992 sediments. The same extraction procedure was used as in Naphthalene Extraction #1, except two extractants were examined: butanol

[1] When not specified as D1a or D1b, the "unwashed" (D1b) sediment was used.

and butanol/hexane. The earlier examination of butanol and hexane as extractants (used separately) showed butanol to be more effective. In addition, a 1:3 combination of butanol and hexane was also evaluated. A possible advantage of using a mixture of butanol and hexane was to reduce the amount of water injected onto the GC column. Because wet samples were being extracted and butanol is fairly miscible with water, the potential existed for significant amounts of water to become associated with the solvent phase and injected, damaging the GC column. By minimizing the amount of butanol and replacing it with hexane (which is immiscible with water), the amount of water which dissolves in the solvent was reduced. The comparison of butanol and butanol/hexane as extractants was performed to determine whether reducing the amount of butanol reduced the effectiveness of microextraction.

Results: See Table 10.

Discussion: In most cases, there did not appear to be much of a difference in extraction efficiency between butanol and the butanol/hexane mix. Therefore, the mix will be used in future extractions. One notable exception was the D2 sediment where it appeared that butanol alone was a better extractant; however, because of the large standard deviation (and small n), the difference may not be significant. Possible alternative explanations for the deviation are nonuniform sampling and incomplete mixing of the sample.

The result of extractions performed on the sediments in July are included in Table 10 for comparison. It is apparent, particularly in the D3 sediment, that the naphthalene levels decreased with time. Volatilization, mineralization, or both may have been responsible for the decreasing levels. It was not surprising that the largest change took place in the D3 sediment because that sample container was opened and sampled more often than the others, allowing oxygen to enter and naphthalene to volatilize.

Conclusions:

a) 1:3 butanol:hexane solvent mixture is an acceptable alternative to butanol only.

b) Naphthalene concentrations in sediments stored at 4°C are decreasing with time.

2.3.4. Equipment and Methods for Measuring Desorption Kinetics:

2.3.4.1. Gas Purge Apparatus

The apparatus to be used for the desorption experiments was comparable to that developed by Connaughton (1992) (see Fig 15).

Procedure: Nitrogen gas (3 L/min) was used to strip the contaminants from sediment that was suspended in 300 mL of the calcium sulfate/sodium azide electrolyte solution. Volatile pollutants were trapped on carbon (Carbotrap^{TM} C) as the gas stream passed through the traps that were changed intermittently. The pollutants were then thermally desorbed from the traps and swept through the GC for analysis (see section 2.3.4.4 for discussion of GC methods).

For GC analysis, the column was attached directly to the trap, which was inserted through the heating block of the GC injector port. N_2 gas was passed through the top of the heated traps to transfer volatile organics onto the GC column. Contaminated traps were sealed with Swage Lok® fittings until they could be analyzed.

2.3.4.2. Carbon Traps

The traps used in the apparatus described above consisted of a stainless steel tube (diameter = 0.25 inches) packed with 0.20 g silanized glass beads and 0.425 g Carbotrap^{TM} C (Supelco) held in place with glass wool. For thermal desorption of the naphthalene, the trap was placed into the GC so that the packed portion of the trap was within the injector port heating block (see Fig. 16).

2.3.4.3. Sample Preparation

The purpose for the gas purge apparatus was to measure desorption rates of naphthalene from sediment samples, with the ultimate goal of correlating this data with mineralization kinetics. An effort has been made to treat all samples identically whenever

possible. The following gas-purge procedure has been developed with these goals in mind.

Procedure: Because mineralization assays are performed on water-saturated sediments, field-wet sediments (as opposed to air dried samples used by Connaughton, 1992) will be used in the purging experiments. In addition, a comparison between desorption rates for field-aged and freshly added naphthalene is desired. Since an equilibration step will be required for introducing the fresh contaminant to the sediments, all field samples will be similarly equilibrated to eliminate variables other than the addition of naphthalene. The sediments will be "aged" in all-glass, flame sealed ampoules for at least one day in the calcium sulfate/azide electrolyte solution. The sediment/electrolyte ratio will be approximately 1:1.

2.3.4.4. Gas Chromatography

A Hewlett Packard 5890A gas chromatograph with a packed column (10% SP-2100 on 100/120 Supelcoport packed column) was used for analyzing samples trapped during the gas-purge desorption experiments.

2.3.4.4.1. Thermal Desorption Efficiency

Experiments were run to estimate the efficiency of the thermal desorption method for measuring the concentration of naphthalene trapped on carbon during gas-purge experiments.

Procedure: Two known concentrations of naphthalene dissolved in butanol were injected onto traps mounted in within the GC heating block. N_2 was passed through the trap for 15-30 mins to distribute the naphthalene onto the carbon within the trap. After the pollutant was distributed, the temperature program for trap analysis was initiated. The response area for the thermally desorbed naphthalene was compared to the calibrated area obtained by direct injection of the solvent on the GC column.

Results: Recoveries were 92.5% $\pm$ 1.6% and 85.9% $\pm$ 1.3% for 35 μg and 3.5 μg injections, respectively. Some of the unrecovered naphthalene may have been lost

during connection of the carrier gas line to the top of the trap after injection of the pollutant onto the trap. No detectable naphthalene remained on the traps after thermal desorption.

3.0 SUMMARY

This project has merged three research areas (field geochemistry, microbiology, and sorption chemistry) in order to understand the biogeochemistry of PAH compounds in a contaminated field site. Although one year of research effort has not explained why biodegradable PAHs anomalously persist at our field study site, substantial progress toward testing the relevant hypotheses has been made.

- Lack of naphthalene metabolism is caused neither by the absence of microbial metabolic capabilities, nutrient limitation, nor the presence of toxins in site samples (Section 2.2.2).
- Sorption of PAHs has been found to affect their bioavailability, hence biodegradation, in complex and varying ways (Section 2.2.3).
  - The notion that duration of sorption contact time completely governs PAH metabolism is simplistic. Under some circumstances mineralization of naphthalene does seem to be inversely proportional to sorption contact time (Section 2.2.3.4). But this relationship is demonstrable with only certain microbiological populations. Thus, the idiosyncrasies and diversities of microbial metabolism are probably the key in understanding the sorption/bioavailability hypotheses. The size of the nonsorbed naphthalene pool (Section 2.2.3.5) also seems to be a significant influence.
  - When the Sorption/Bioavailability hypothesis was tested with phenanthrene, the type of sorbent emerged as a critical influence: regardless of inoculum source, no mineralization was observed when seep sediment (high in organic matter) was sorbent. In contrast, when sand was the sorbent (low in organic matter) mineralization did occur.
- Under anaerobic conditions favoring methanogens and sulfate reducers, no naphthalene metabolism occurred during a 16 day experiment. When oxygen and nitrate were supplied to the same sediments and microbial populations, rapid naphthalene mineralization occurred (Section 2.2.4). Thus, simple oxygen and nitrate limitation has emerged as one of the most probable causes for PAH persistence at the field site. The presence of naphthalene mineralizing denitrifiers at this site, if confirmed, opens up a broad area of physiological and genetic investigations comparing individual aerobic and denitrifying bacteria.

4.0 FUTURE PLANS

During the second funding period for this project we intend to continue the established trend of testing hypotheses aimed at explaining the persistence of PAHs at our study site. The three-pronged approach fusing field observations with microbiology and chemistry will be refined in manners described below:

- Assays following metabolism of the PAHs already contaminating the sediments will be implemented (using extractions and GC analyses) and compared with $^{14}CO_2$ production assays using the same reaction vials.
  This type of comparison is important because it addresses the Aging/Bioavailability issue from another side. It could very well be that experiments employing freshly added radioisotopes are completely misleading because the actual contaminants are not freshly added.
- The finding that phenanthrene is not mineralized after being sorbed to seep sediments is worthy of exploration. The three relevant hypotheses (sorption, toxicity, diauxy) can be distinguished by showing that cells can metabolize other non-catabolite suppressing, non-sorbing compounds such as glucose in the sediments.
- The possibility that the Sorption/Bioavailability hypothesis is linked to microbial metabolism and diversity requires further exploration. Pure cultures derived from sorbed and *non-sorbed enrichment* conditions will be isolated and their metabolic behavior compared.
- Additional anaerobic studies are needed to confirm that NO_3^- can serve as an electron acceptor for oxidation of naphthalene at the site. These will utilize sediment slurries and measure the linkage between naphthalene and nitrate consumption. We will attempt to isolate pure cultures of denitrifying naphthalene degraders so that biochemical and genetic aspects of the process can be examined.
- Based on the physiological assays obtained thus far, oxygen and nitrate limitations may be the principal factors governing PAH persistence at the field site. Provided permission is granted from the site owners, we plan a replicated field assay comparing PAH persistence in field treatments that have enhanced and unenhanced oxygen and nitrate concentrations.
- The hypothesis that PAH biodegradation is governed by desorption kinetics will be tested by evaluating PAH desorption rates and comparing them to PAH mineralization rates under aerobic and denitrifying conditions.
- Understanding how naturally occurring populations respond, at the genetic level, to contaminants is important basic research applicable to all Air Force-impacted sites. The genetic variability of organisms in contaminated and uncontaminated seep

sediments will be explored by comparing DNA sequences (for naphthalene catabolism or ribosomal RNA) from a variety of bacterial isolates.

– In order to understand the effect of contact time on desorption rates, experiments will be performed to determine desorption rates of field-aged versus freshly added naphthalene from sediment collected April 1992.

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TABLE 1. CHARACTERISTICS OF SEEP STUDY SITE

Nutrient analysis of Oct 1991 Sample (mg/kg)		Field analysis during July 1992 visit	Field analysis during August 1992 visit
P	0.5	Mud Surface	Mud Surface
K	22	pH 7.5	pH 6.9
Mg	119	Temp 17.5°C	Temp 18°C
Ca	948	O_2 4 mg/l	O_2 4 mg/l
Fe	2.0	15 cm Depth	20 cm depth
Al	12.2	pH 6.9	pH 5.7
Mn	6.3	Temp 17.4°C	Temp 16.1°C
Zn	0.2	O_2 3.4 mg/l	O_2 0 mg/l
Cu	0	Surface Flowing Water	Surface Flowing Water
NO_3	0	pH 7.2	pH 7.6
NH_3	3.5	Temp 19.5°C	Temp 15.6°C
		O_2 4 mg/l	O_2 4 mg/l
Organic Matter (by loss on ignition)	3.1%		
pH	6.2		

TABLE 2. NAPHTHALENE IN SEEP SAMPLES

Sampling Date	Type of Sample	Naphthalene Concentration (ppm)	Comment
7/29/92	Flowing water in seep	0.97	1 ml Hexanes to 4 ml water in sealed ampoules prepared in triplicate
8/8/92	Flowing water in seep	1.0	"
	Flowing water ~10 m downstream from seep	0.41	", but in duplicate
	Seep sediment, 15 cm depth	6.7	Sample gathered in screw-cap jar and stored at 4°C. Later, 6 g seep sediment mixed with 2 ml hexanes stored 10 days at 4°C

TABLE 3. VERTICAL PROFILES OF NAPHTHALENE, OXYGEN, AND MICROORGANISMS IN TWO CORES FROM THE SEEP AREA

Core	Depth (cm)	O_2[a]	Naphthalene (ppm)[b]	Total Microscopic Counts (log)[c]
1	3.5	0	2.0	7.6
	7.6	0	11	7.2
	13	0	27	6.9
	29	0	3.6	7.4
2	8.6	0	18	7.7
	18	0	39	7.8
	30	0	20	7.5
	51	0	45	7.8

[a] O_2 measured in intact core immediately after removal. After drilling hole into saturated sediment, an O_2 probe was inserted and the hole was sealed by water and sediment.

[b] Sample extracted with 1:9 (butanol:hexanes) immediately after core was removed from seep. The stainless steel core was divided at the depth where O_2 was measured. Sediment was mixed with extractant in screw cap teflon-lined vials. Units are μg naphthalene per cm^3 of sediment.

[c] Preliminary total microscopic bacterial counts performed using Direct Fluorescence Microscopy.

TABLE 4. SUMMARY OF METHODS DEVELOPMENT IN AGING/BIODEGRADATION EXPERIMENTS

			Methodological Details				
Expt.	Chemical	Principal Variables	Aging vessel and naphthalene concentration	Inoculum	Mineralization vessels and ratio of wet sediment to other fluid in slurry	Weaknesses	Conclusion
#1	Naphthalene	aging period = 0-20 d sediment = seep	Ichem vial, 15 ppm nap	5 g seep added to 100 ml minimal medium saturated with naphthalene shaken for 2 days	serum bottles x butyl septa, 2 ml of inoculum added to 8 g wet sediment	1a) During aging, mixing may not have been sufficient to attain homogenous distribution of ^{14}C. Also, ^{14}C may have escaped. 1b) During mineralization, stopper and CO_2 trap may have sorbed the naphthalene. 1c) ^{14}C naphthalene may have volatilized from sediments during preparation for the mineralization assay.	Need to improve mixing, improve mineralization assay and minimize naphthalene volatilization losses.
#2	Naphthalene	aging period = 0-21 d sediment = sand and seep	glass ampoule, 3 ppm nap	1 g seep to 100 ml 0.005M $CaSO_4$ No enrichment	Pierce vial/Teflon septa, 1 ml of inoculum added to 5 g wet sediment	2a) point 1c above not solved 2b) During mineralization assays the septa may leak after being punctured.	Despite methodological improvements, results contrasted strikingly with those of experiment #1.
#3	Naphthalene	aging period = 0-28 d sediment = sand and seep	glass ampoule, 3 ppm nap	2 g seep added to 150 ml minimal medium saturated with naphthalene shaken overnight	Pierce vial/teflon septa, 0.5 ml of inoculum added to 4 g wet sediment	3a) See 2a 3b) see 2b 3c) Dissolved naphthalene may have accompanied the inoculu	With more refined procedures, confidence in results grows. Trend suggests that mineralization is governed by contact time, but the inoculum may also be critical.
#4	Phenanthrene	aging period = 0-20 d sediment = sand and seep	glass ampoule, 1 ppm phen	2 ml of enrichment culture from either seep or sand. Medium contained phenanthrene yeast extract, peptone, and glucose	Pierce vial/teflon septa, 2 ml of inoculum to 3 g (sand) or 4 g (seep)	4 a) see 2a, phenanthrene is, however, less volatile b) see 2b c) see 3c	See #3

TABLE 5. RATIO OF EXTRACTED ^{14}C NAPHTHALENE TO CALCULATED ^{14}C NAPHTHALENE (BY DIFFERENCE BETWEEN ACTIVITIES ADDED AND REMOVED IN SUPERNATANT FLUID) WHEN THE MINERALIZATION PORTION OF AGING EXPERIMENT #3 WAS INITIATED.

Sediment	Aging Period (d)	Ratio (Extracted/Calculated)
Sand	28	0.10
	14	0.14
	7	0.19
	0	0.59
Seep	28	0.31
	14	0.44
	7	0.59
	1	0.49
	0	0.56

TABLE 6. ORGANIC CARBON CONTENT AND APPROXIMATE DEPTH OF SEDIMENTS COLLECTED 4/92

Sediment[1]	Approximate Depth (cm)	% Organic-C ± SD
D1a[2]	0 - 5	17.7 ± 2.4 (n=4)
D1b	0 - 5	18.3 ± 1.7 (n=4)
D2	5 - 10	8.5 ± 0.1 (n=3)
D3	15	17.9 ± 1.0 (n=4)
D4	30 - 35	2.1 ± 0.2 (n=3)

[1]All samples were air dried for ≥ 24 hours and passed through a 0.84 mm sieve before testing.

[2]Sediment was placed in 0.005M $CaSO_4$ solution to remove "floatable" material which might interfere with later experiments by limiting solids/liquid separation. The sample was then air dried and sieved.

TABLE 7. COMPARISON OF NAPHTHALENE PARTITION COEFFICIENT VALUES FOR SEDIMENT SAMPLES USING TWO METHODS OF DATA REDUCTION

Sediment	K_p value ± SD	Method
D2	92.5 ± 14.5	average K_{pi}
	90.7 ± 3.82	graphical (mass balance)
D3	182 ± 30.5	average K_{pi}
	169 ± 9.17	graphical (mass balance)

TABLE 8. NAPHTHALENE PARTITION COEFFICIENTS DETERMINED USING LINEARIZED MASS BALANCE EQUATION

Sediment	K_p±SD	K_{oc}
D1a[3]	98.8± 7.17	558
D1b	112± 8.85	611
D2	90.7± 3.82	1070
D3	169± 9.17	946
D4	25.5± 1.24	1210
overall:		879±285

[3]Centrifuged at ~330 x g for 30 minutes

TABLE 9. RESULTS OF NAPHTHALENE EXTRACTION #1

Sediment		Naphthalene concentration (µg / g)	± SD
D1b	(0-5 cm)	4.59	0.12 (n=4)
D2	(5-10 cm)	38.9	7.15 (n=4)
D3	(~ 15 cm)	92.9	4.18 (n=4)
D4	(30-35 cm)	3.78	0.89 (n=4)

TABLE 10. RESULTS OF NAPHTHALENE EXTRACTION #2

Sediment	Naphthalene concentration (µg/g)	Extractant (date)
D1	4.424±0.174 (n=2)	butanol (9/92)
	4.903±0.477 (n=2)	butanol/hexane (9/92)
	4.591±0.120 (n=4)	butanol (7/92)
D2	38.65±15.07 (n=2)	butanol (9/92)
	25.86±6.315 (n=2)	butanol/hexane (9/92)
	38.94±7.147 (n=4)	butanol (7/92)
D3	38.22±1.430 (n=2)	butanol (9/92)
	39.15 (n=1)	butanol/hexane (9/92)
	92.85±4.185 (n=4)	butanol (7/92)
D4	1.883±0.104 (n=2)	butanol (9/92)
	2.251±0.049 (n=2)	butanol/hexane (9/92)
	3.779±0.889 (n=4)	butanol (7/92)

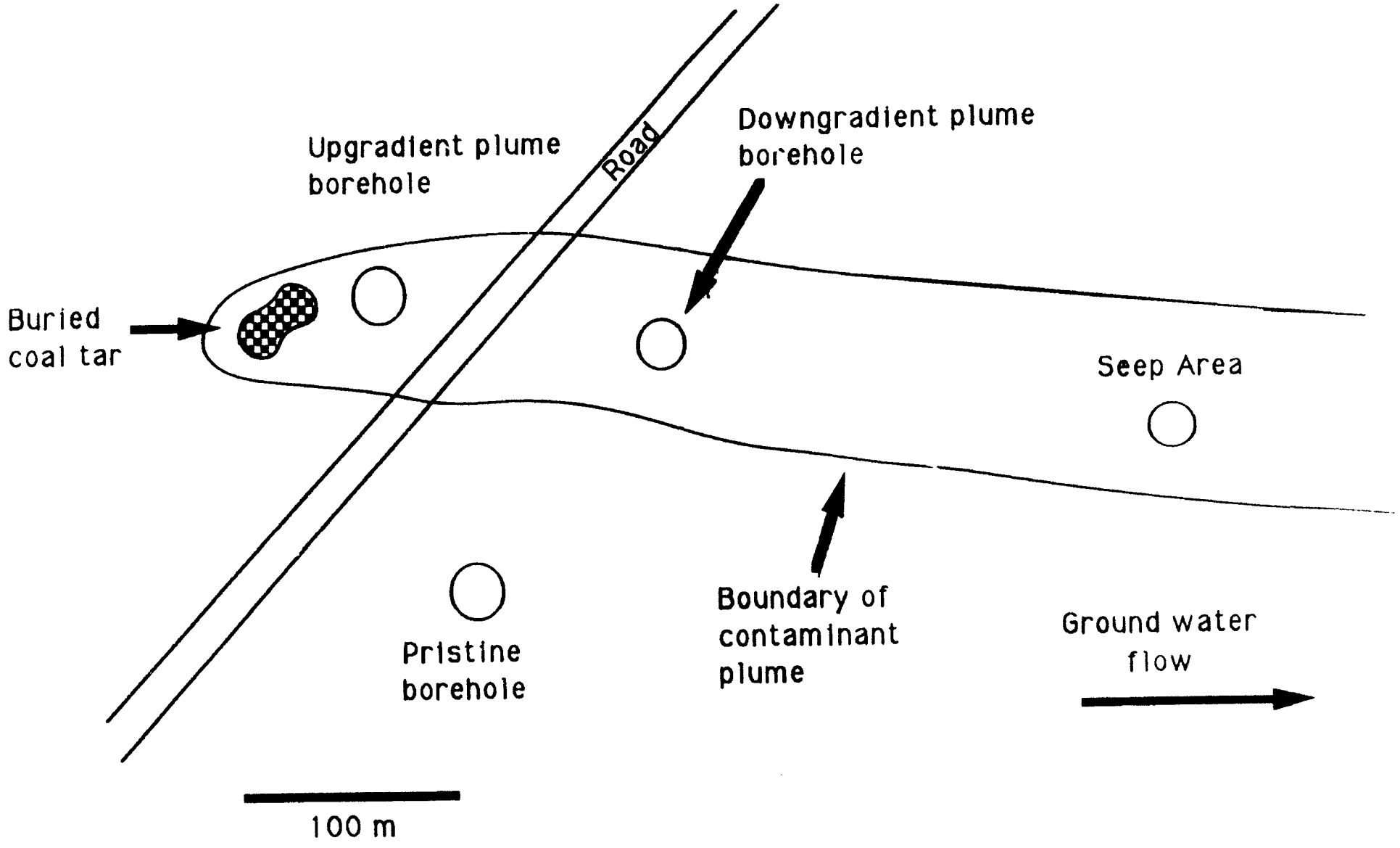


Figure 1. Relationship of seep study area to other portion of the contaminated site.

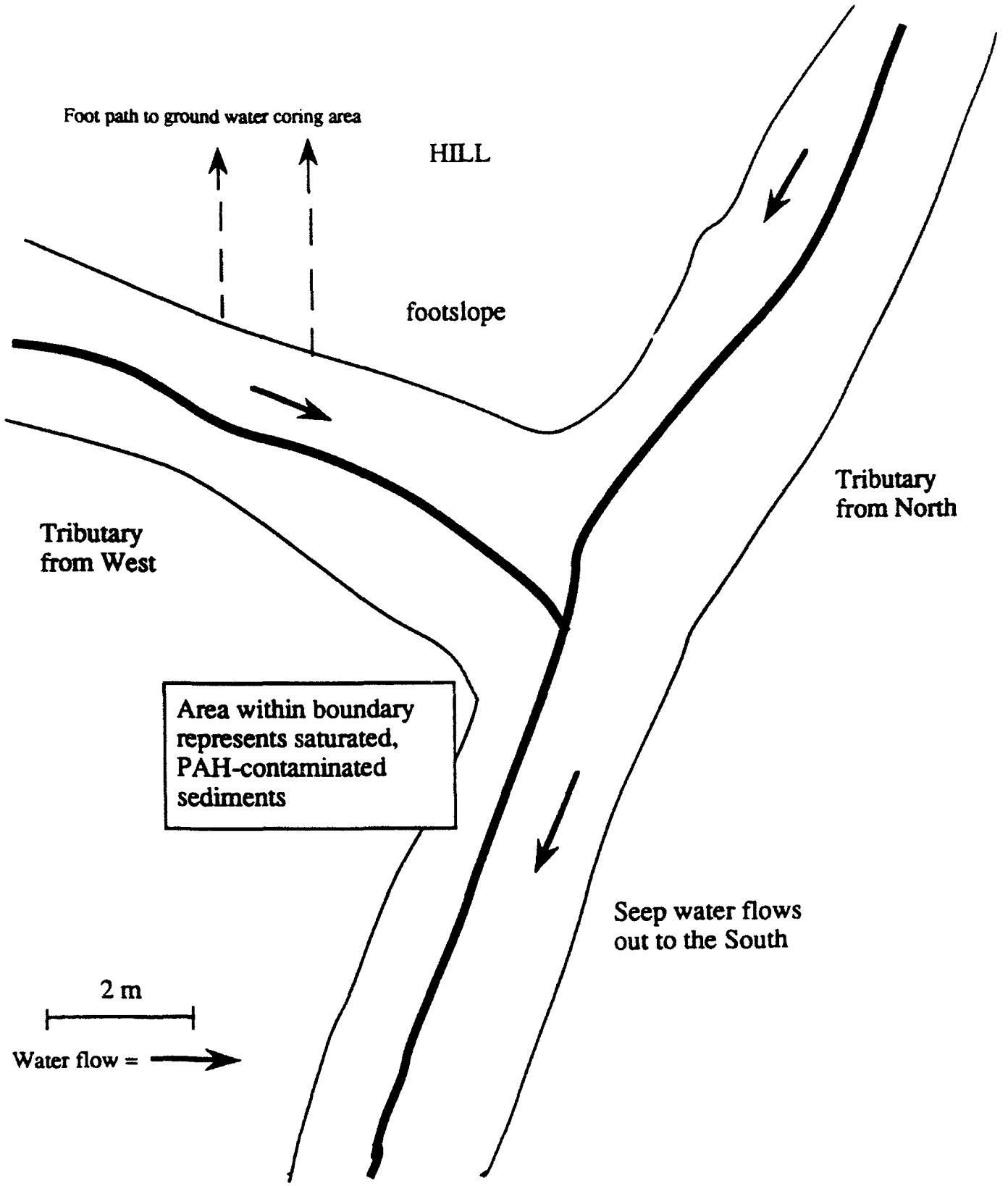
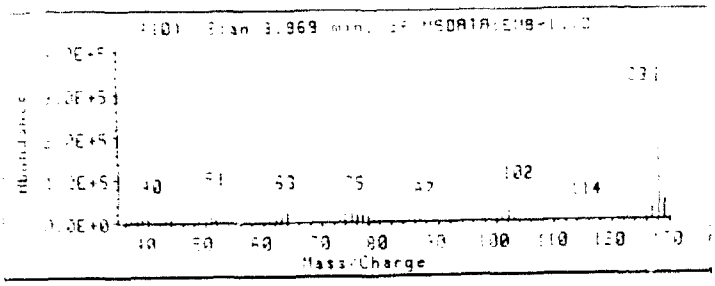
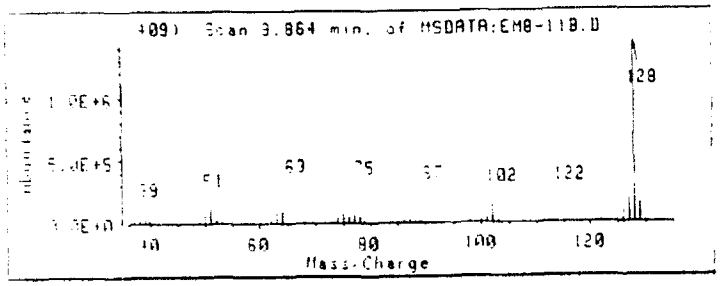


Figure 2. Diagram of seep area. Samples were obtained primarily from the point where the two tributaries meet.



A



B

C

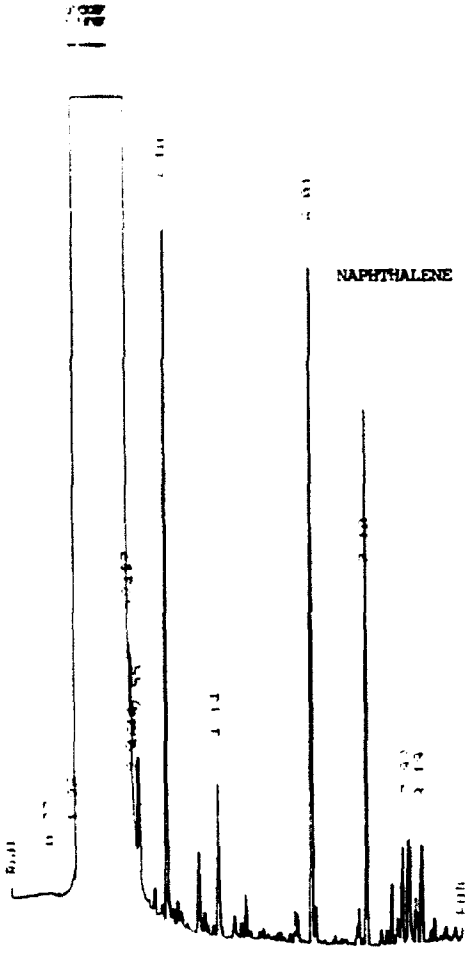


Figure 3. GC/Mass spectrometric identification of naphthalene in water and Seep sediments. Fragmentation pattern from seep and water analysis (top), from authentic sample. Bottom panel shows gas chromatogram of compounds extracted from seep sediment (naphthalene eluted at 6.01 min.).

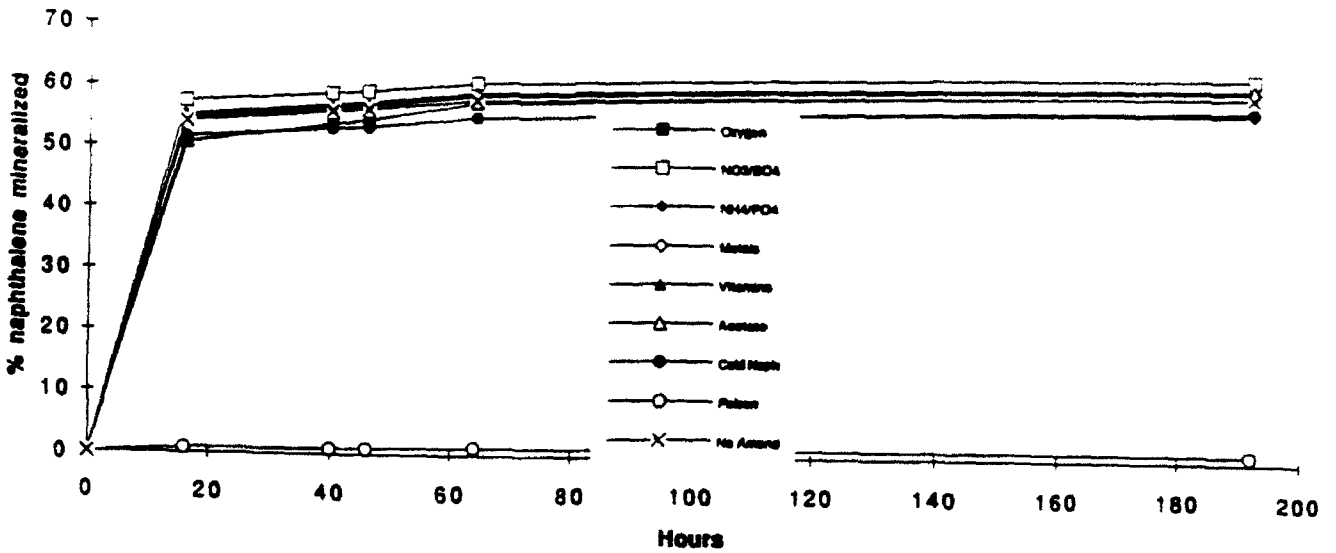


Figure 4. Mineralization and naphthalene in seep sediments amended with a variety of organic and inorganic nutrients.

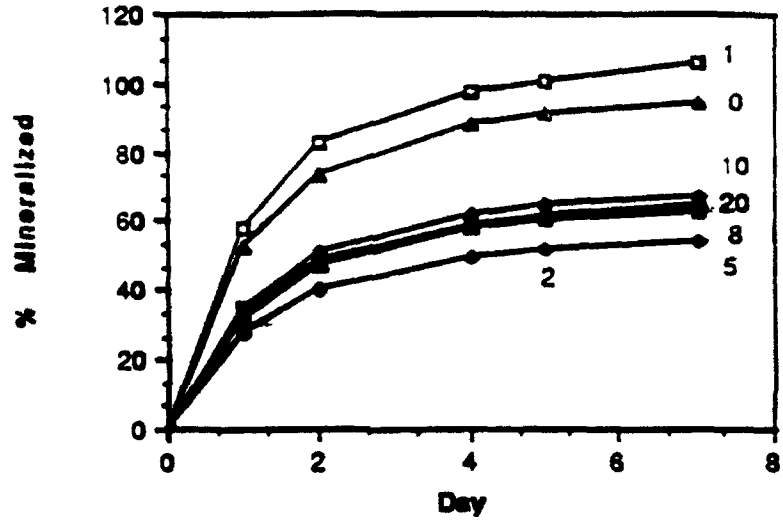


Figure 5. Mineralization of naphthalene aseptically aged with seep sediments and subsequently inoculated with an enrichment culture from seep sediment. Aging times ranged to 0 to 20 days.

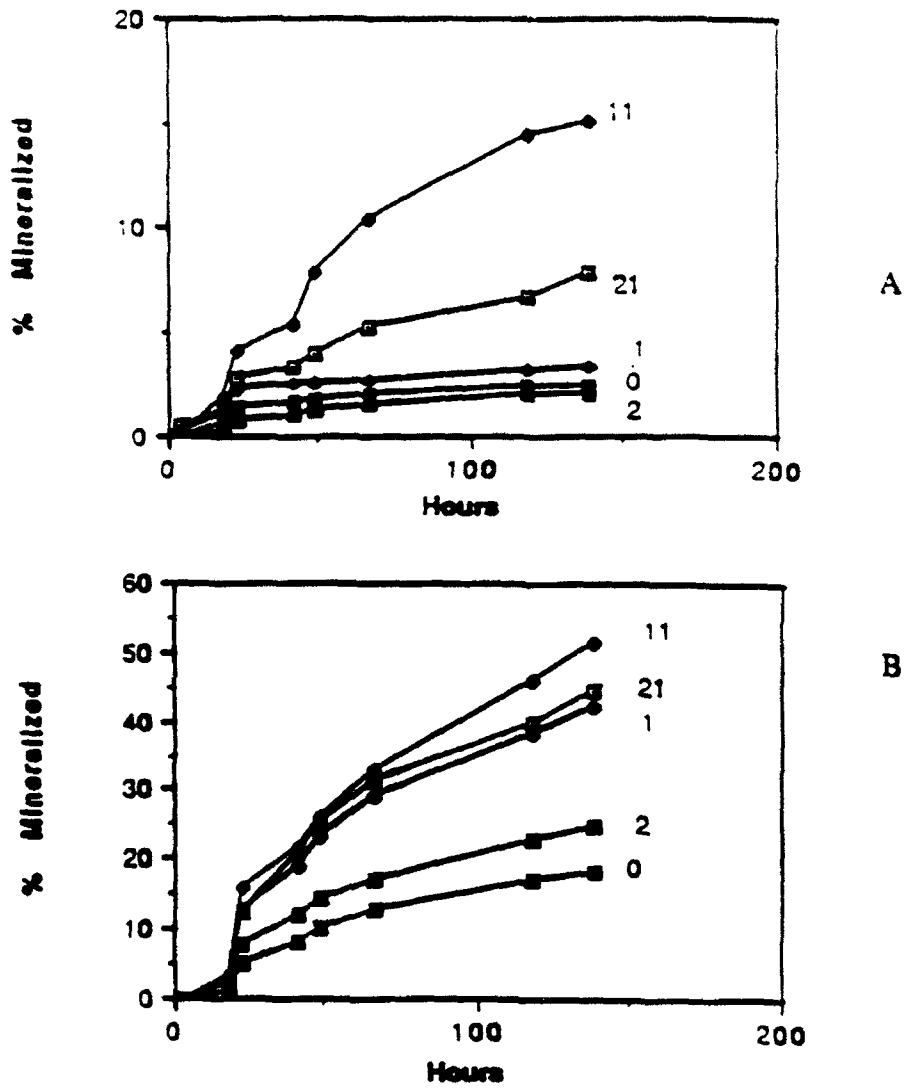


Figure 6. Mineralization of naphthalene aseptically aged with sand (A) and seep (B) sediments and subsequently inoculated with an unenriched suspension from the seep. Aging times from 0 to 21 days.

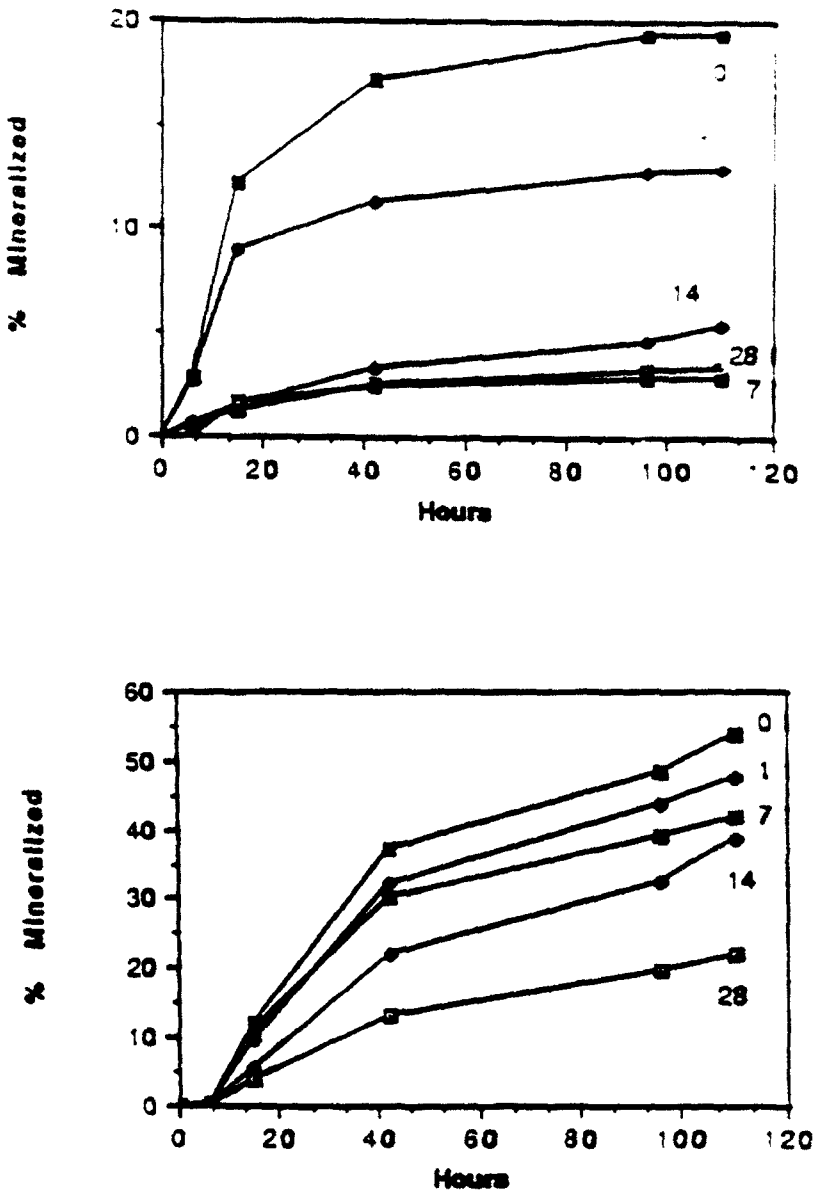


Figure 7. Mineralization of naphthalene aseptically aged with sand (A) and seep (B) sediments and subsequently inoculated with an enrichment culture from the seep. Aging times ranged to 0 to 28 days.

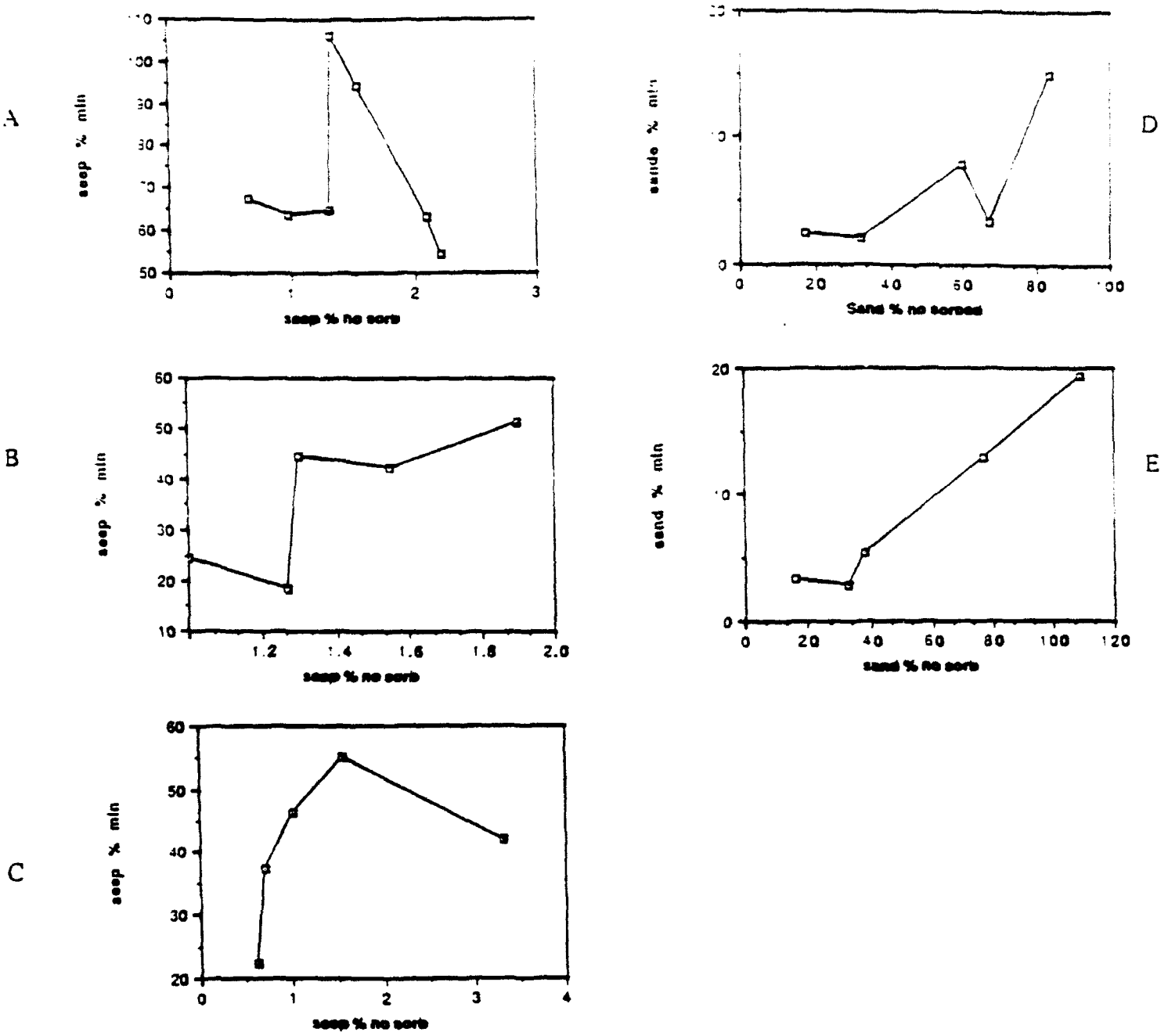


Figure 8. Relationship between percent non-sorbed naphthalene and extent of mineralization for Aging Experiments 1-3. A. Seep sediment, Experiment #1; B. Seep sediment, Experiment #2; C. Seep sediment, Experiment #3; D. Sand, Experiment #2; E. Sand, Experiment #3.

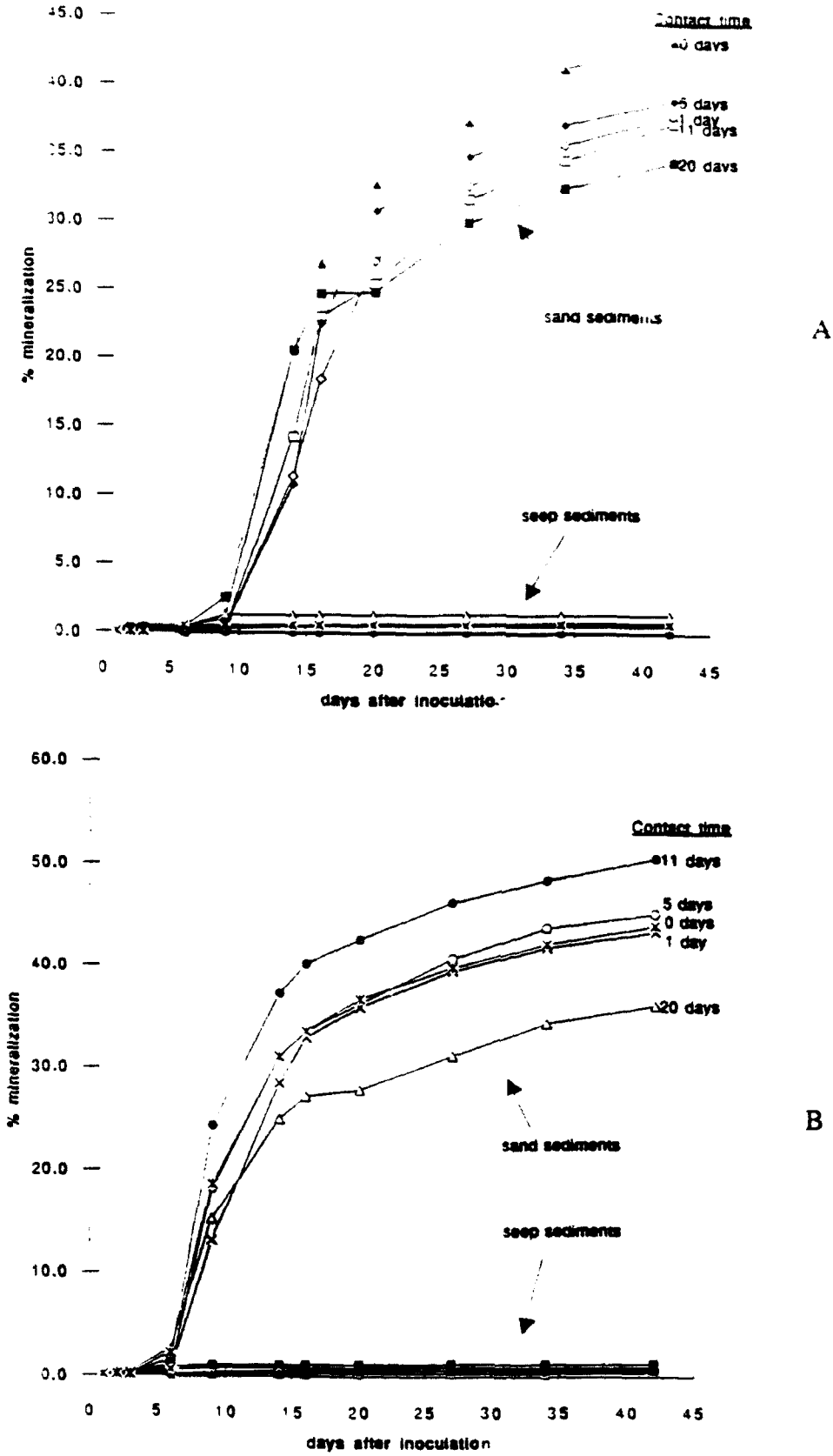


Figure 9. Mineralization of phenanthrene aseptically aged with sand and seep sediments and subsequently inoculated with enriched suspensions from either the seep (A) or sandy upgradient sediment (B).

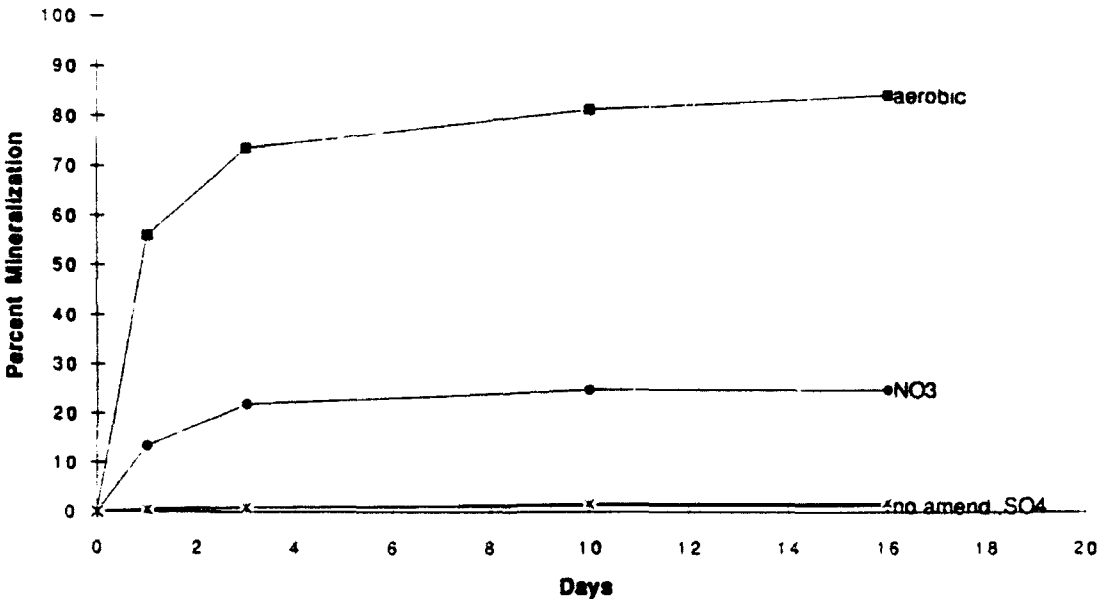


Figure 10. Metabolism of naphthalene by seep sediments under conditions designed to favor aerobes, methanogens, sulfate-reducers, and denitrifiers

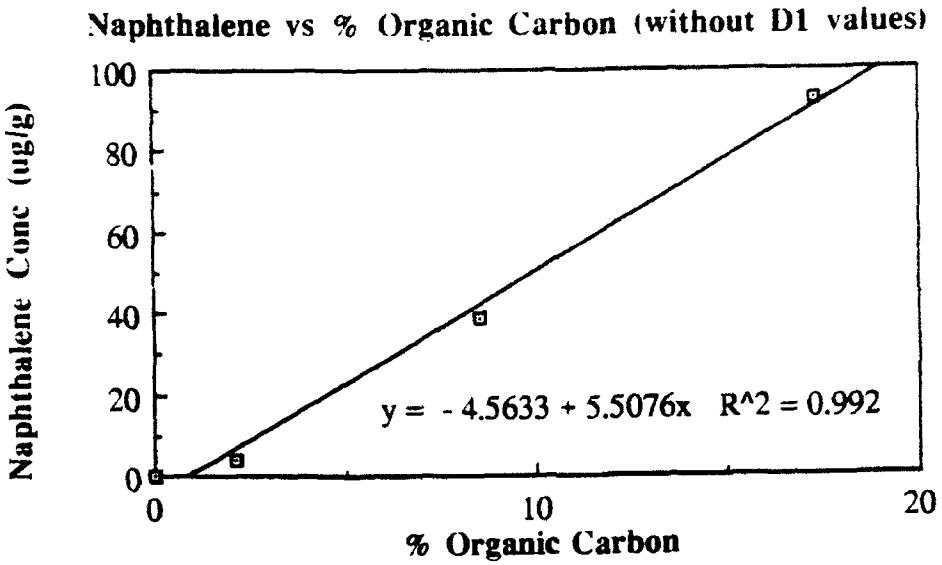


Figure 12.Naphthalene concentration versus organic carbon content

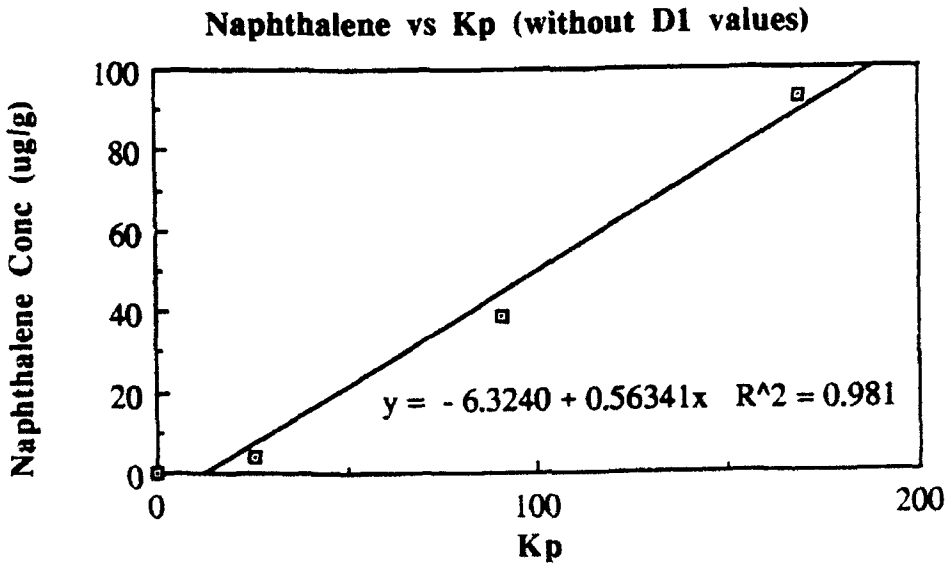


Figure 13. Naphthalene concentration versus partition coefficient

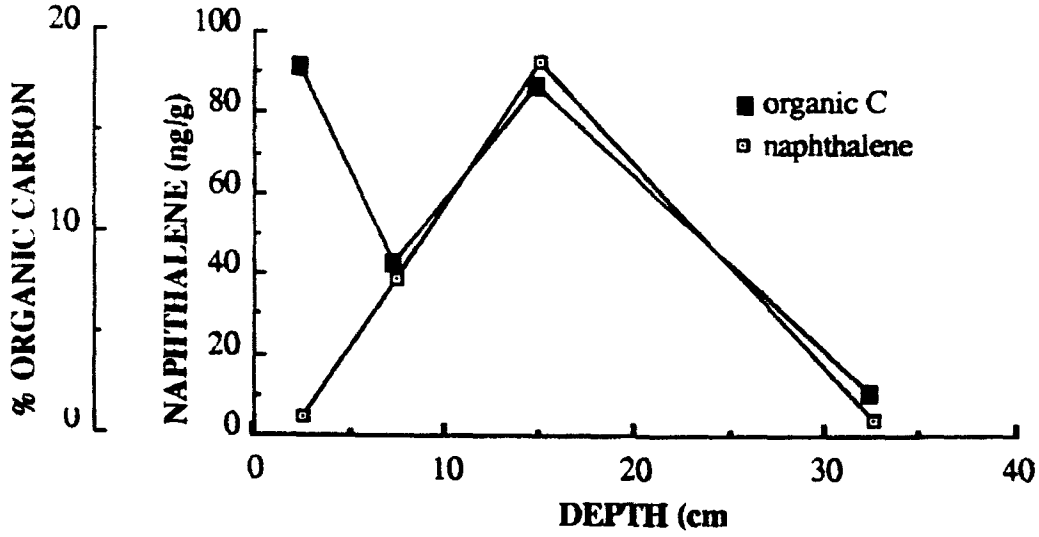


Figure 14. Variation in naphthalene concentration and organic carbon content with depth.

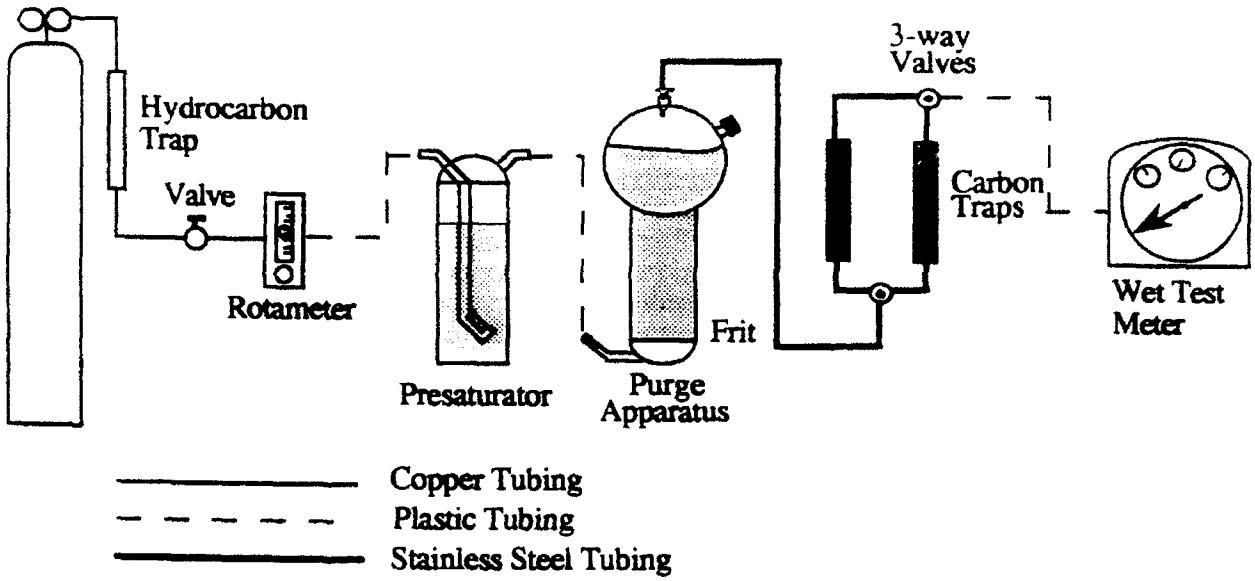


Figure 15. Diagram of gas purge apparatus

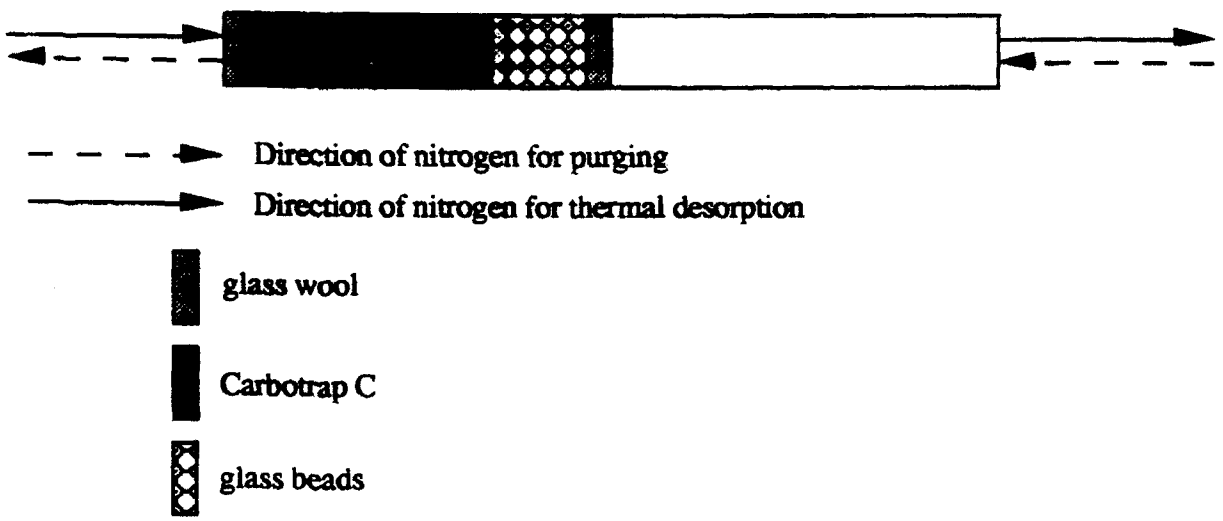


Figure 16. Diagram of traps used in gas purge experiments