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13. ABSTRACT *(Maximum 200 words)*

This project has merged four-research areas (field geochemistry, sorption chemistry, microbiology, and molecular biology) in order to understand the behavior of low molecular weight polycyclic aromatic hydrocarbon (PAH) compounds in a contaminated field site. Naphthalene has been transported approximately 400 m via ground-water flow from buried subsurface coal-tar to an organic matter-rich seep area where the water energes at the foot of a hill at a field study sity. Gas chromatographic analyses of cores taken from the sediments show naphthalene concentrations in vertical profiles ranging from 0.9 to 45 ug/cm3. We have tested hypotheses for explaining why naphthalene and phenanthrene persists in seep sediments and found that neither toxicity, nor nutrient limitation, nor the absence of microorganisms or appropriate catabolic genes were the cause of PAH persistence. Despite clear laboratory indications of O2- limited naphthalene metabolism, when H2O2 was added to site sediments in situ in a randomized block design, no discernible naphthalene loss occurred. Reasons for lack of naphthalene loss during this field experiment may include spatial heterogeneity, selective metabolism of non-naphthalene substrates, protection by sorption or diffusion reactions, or in situ replenish-

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ment of naphthalene by migration into the sediment from hydrologically upgradient site locations. To investigate the possibility that sorption or diffusion reactions may cause decreased bioavailability, hence, PAH, persistence, sediments from the field site were sterilized using Y-irradiation and then exposed under aseptic conditions to 14C-labeled naphthalene for periods ranging from 0 to 28 days prior to being inoculated with PAH-degrading microorganisms. Resulting patterns in the extent and rate of PAH mineralization supported diminished bioavailability as a mechanism of PAH persistence because an inverse relationship to the duration of contact with the sorbent was found; however, this was highly dependent upon the means of inoculum preparation, the inoculum source, and the type of sediment used as sorbent.

FINAL TECHNICAL REPORT
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U.S. AIR FORCE OFFICE OF SCIENTIFIC RESEARCH

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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

Third Project Period: 30 March 1994 to 31 March 1995

Date: May 12, 1995

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1.0 BACKGROUND

This document reports results of laboratory and field experiments carried out during the final fully funded year of a $3^1/_2$-year project entitled "Geochemical, Genetic, and Physiological Control of Pollutant Biodegradation" (a six-month no-cost extension period was sandwiched between years 1 and 2). For a detailed literature review, project description and statement of work, the reader is referred to the original proposal in its entirety.

1.1. Project Motivation and Goals:

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

(from original proposal, 9/91)

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.

1.2. Synopsis of progress from first period (12-month):

The Synopsis, below, is taken from p. 26 of the first Technical Report for this project (30 September 1991 – 29 September 1992). This Summary appears here in order to give the reader an appreciation of the significance and rationale for new experimental results reported in this volume.

Synopsis

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.
- Sorption of PAHs has been found to affect their bioavailability, hence biodegradation, in complex and varying ways.
  - 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. 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 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. 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.

1.3 Synopsis of Progress from Second Period (6 month no-cost extension):

During the second period of the project (6-month no-cost extension of Year 1), weather precluded field work. Instead of visiting the site, measurements were performed on site-derived samples. Key areas of progress were:

– Isolation and characterization of a phenanthrene metabolizing bacterium whose behavior to sorbed phenanthrene mimics that of mixed populations in site-derived sediment samples.

– Discovery that mixed populations from the field-site sediments vary in their abilities towards metabolize sorbed phenanthrene, depending on sediment type and how the sediments were handled. Even phenanthrene sorbed to the organic-rich seep sediment can be metabolized. Thus, the role of physiological diversity in PAH metabolism has been confirmed and extended.

– Initiation of an approach to measure PAH metabolism by two independent analytical methods. This was designed to compare the fate of freshly-added and long-sorbed naphthalene. The insights promised by this type of experiment are very significant, but the details of PAH extraction and analysis procedures need to be improved before success is achieved.

– Establishment of serum-bottle-HPLC-based procedures for studying anaerobic metabolism of naphthalene. Results show that naphthalene mass balances can be assembled and that no denitrifying naphthalene metabolism could be detected. However, a clear oxygen limitation was demonstrated

1.4. Synopsis of progress from Third Period (12-month period):

The most recent progress report described five areas where research has been conducted.

1) The field site-derived phenanthrene-metabolizing bacterium, *Sphingomonas paucimobilis* RSP1 (described briefly in the second report), has been further characterized. The background physiological data presented here provide a sound foundation for future work aimed at understanding relationships between the fate of PAHs and the properties of this bacterium.

2) Investigations of microbial metabolism of sorbed PAHs have continued. These assays use a phenomenological approach which assess the responses of mixed cultures and

pure cultures to PAHs aged for varying periods under aseptic conditions in the presence of sediments sterilized by γ-irradiation.

3) Data addressing the physiological, taxonomic, and molecular responses of soil and sediment microorganisms to naphthalene has been obtained. This component of the report focused on a population genetics approach to the distribution of naphthalene metabolism genes at the coal-tar contaminated study site. A diversity of isolates capable of metabolizing naphthalene have been isolated from 2 spatially distinct locations at the field site (the contaminated seep area and an uncontaminated adjacent hillside soil). The isolated bacteria have been characterized taxonomically (via a variety of procedures including the commercial BIOLOG series of substrate utilization tests) and genetically (by PCR amplifying and sequencing a portion of the *nah*Ac and *nah* R catabolic genes).

4) The mobility of PAHs and bacteria capable of PAH-metabolizing in site sediments has been investigated. These data were obtained in the Fall of 1993 from a field experiment in which an array of polyurethane foam plugs and small sterile sand bags were installed in the seep portion of the study site. Periodically, subsets of the originally clean sorbent materials were removed from the field site. Chemical [gas chromatograph/mass spectrometry (GC/MS)] analyses were conducted on material sorbed to the urethane foam and numbers of phenanthrene and naphthalene metabolizing bacteria adhering to the sand sorbents were also assayed.

5) Development of methods for extracting DNA from sediments. This topic is concerned with how to obtain DNA directly from environmental samples so that tools of molecular biology can be applied to microbial communities present in field sites. The results of this study are In press, due to appear in the May issue of Applied and Environmental Microbiology. The manuscript is attached to this report.

2.0 RESULTS FROM FINAL 12-MONTH PERIOD

During the final 12-month period, progress in a number of related areas has been made. These include:

1) completion of field experiments examining hypotheses for explaining the persistence of naphthalene in the field study site;

2) completion of aging-bioavailability experiments using phenanthrene;

3) development and applications of methodologies for extracting a unique an intermediary metabolite that is indicative of real-time *in situ* naphthalene biodegradation in the field study site;

4) refinement of phylogenetic evidence for horizontal transfer of the naphthalene catabolic gene, NahAc, between bacteria in the field site.

Details about each of the above 4 topics appear in the form of separate manuscripts presented in Appendices 4.1-4.4. Sections 2.1-2.4 below, report the Abstract for each topic:

2.1 Oxygen limitations and aging as explanations for the field persistence of naphthalene in coal-tar contaminated surface sediments (by Eugene L. Madsen and Sharon Bilotta-Best)

ABSTRACT

Naphthalene has been transported approximately 400 m via ground-water flow from buried subsurface coal-tar to an organic matter-rich seep area where the water emerges at the foot of a hill at a field study site. Gas chromatographic analyses of cores taken from the sediments show naphthalene concentrations in vertical profiles ranging from 0.9 to 45 $\mu g/cm^3$. We have tested hypotheses for explaining why naphthalene persists in seep sediments. In aerobic laboratory flask assays conversion of ^{14}C- naphthalene to $^{14}CO_2$ occurred and was not stimulated by amendments with vitamins or inorganic nutrients. Thus, neither toxicity nor nutrient limitation were the cause of naphthalene persistence. With the exception of site surface waters, *in situ* sediment oxygen concentrations were below detection; this suggested that lack of this final electron acceptor could be a key reason for naphthalene persistence. Oxygen limitation was confirmed by measuring no conversion of ^{14}C-naphthalene to $^{14}CO_2$ in samples of seep sediments prepared anaerobically. Naphthalene also persisted in anaerobic nitrate-amended slurry-phase incubations of the sediment until O_2 was added. Despite these clear laboratory indications of O_2-limited naphthalene metabolism, when H_2O_2 was added to site sediments *in situ* in a randomized block design, no discernible naphthalene loss occurred. Reasons for lack of naphthalene loss during this field experiment may include spatial heterogeneity, selective metabolism of non-naphthalene substrates, protection by sorption or diffusion reactions, or

in situ replenishment of naphthalene by migration into the sediment from hydrologically upgradient site locations. To investigate the possibility that sorption or diffusion reactions may cause decreased bioavailability, hence, naphthalene persistence, sediments from the field site were sterilized using γ-irradiation and then exposed under aseptic conditions to ^{14}C-labeled naphthalene for periods ranging from 0 to 28 days. After aging, the sediments were dispensed to sealed vials, inoculated with mixed microbial populations from the study site, and $^{14}CO_2$ production was measured. Resulting patterns in the extent and rate of naphthalene mineralization revealed an inverse relationship to the duration of contact with the sorbent, but only when the mixed inoculum had been enriched on aqueous-phase naphthalene. We conclude that oxygen limitation is the most probable cause for lack of naphthalene biodegradation at our field study site. However, sorption or diffusion reactions may also play a role.

For supporting data and text see Appendix 4.1.

2.2 Regulation of microbial phenanthrene mineralization in sediment samples by sorbent-sorbate contact time, inocula, and gamma irradiation-induced sterilization artifacts (by Robert L. Sandoli, William C. Ghiorse, and Eugene L. Madsen)

ABSTRACT

Time-dependent sorption and/or diffusion reactions were proposed as a hypothesis for explaining the persistence of polycyclic aromatic hydrocarbons (PAHs) in sediments from a coal tar waste-contaminated field site. To test this hypothesis, ^{14}C-labeled phenanthrene was aged in both subsurface sand and organic matter-rich seep sediments that had previously been sterilized by gamma irradiation. After aging periods ranging from 0 to 28 days, the sediments were dispensed to replicate vials, inoculated with site-derived phenanthrene degrading microorganisms, with or without previous enrichment on phenanthrene , and cumulative $^{14}CO_2$ production was measured. Patterns of $^{14}CO_2$ evolved from the sand sediments showed that the extent and rate of mineralization of aged

phenanthrene was largely dependent upon the inoculum source (sand versus seep sediments). When both pure culture and mixed inocula originated from the seep sediments, phenanthrene mineralization was retarded in samples with longer aging periods. However, when the inoculum originated from the sand sediments, aging of the phenanthrene had only a slight or no effect on its rate or extent of mineralization. Preenrichment of phenanthrene-degrading microorganisms in either sediment type with aqueous-phase phenanthrene had little influence on subsequent mineralization of phenanthrene aged in the sand sediment.

Although microorganisms in the fresh organic matter-rich seep sediments readily converted ^{14}C-phenanthrene to $^{14}CO_2$, this activity was diminished or completely eliminated when gamma radiation-sterilized sediments were present. To explain the gamma irradiation-induced inhibition of phenanthrene metabolism, mineralization experiments were performed with both pure and mixed cultures. In these experiments, ^{14}C-glucose was readily converted to $^{14}CO_2$ in the presence of the irradiated seep sediments, and nonradioactive glucose added to fresh seep sediment failed to delay phenanthrene metabolism. Therefore, gamma irradiation-induced toxicity and utilization of preferred growth substrates were unlikely causes of the inhibition. Instead, gamma radiation-induced changes in the sorptive properties of the seep sediments seemed to be the cause. This was indicated both by an increase in the sorption-controlled distribution coefficient, K_d, for gamma-sterilized sediment and by tests showing that the extent of ^{14}C-phenanthrene mineralization was roughly proportional to the amount of fresh seep sediment added to the irradiated sediment.

For supporting data and text see Appendix 4.2.

2.3 Field extraction of a transient intermediary metabolite indicative of real time *In situ* naphthalene biodegradation (by M. S. Wilson and E. L. Madsen)

Abstract

Metabolic activity of naturally occurring microbial communities in soils, sediments, and waters often determines the fate of organic environmental contaminants. Yet this activity has traditionally resisted direct documentation. The metabolic pathway for bacterial naphthalene oxidation was used as a guide for selecting 1,2-dihydroxy-1,2,-dihydronaphthalene (1,2-DHDN) as a unique transient intermediary metabolite whose presence in samples from a contaminated field site would indicate real time *in situ* naphthalene biodegradation. When surface waters emerging from the site were processed in a manner designed to avoid sample-handling artifacts, the 1,2-DHDN intermediate was successfully concentrated, extracted, and identified by gas chromatography/mass spectrometry. This is an example of how knowledge and techniques of biochemistry, microbial physiology, and analytical chemistry can be focused toward measuring transformations catalyzed *in situ* by naturally occurring microbial communities in real time.

For supporting data and text see Appendix 4.3.

2.4 Phylogenetic evidence for horizontal transfer of the naphthalene catabolic gene *nah* Ac (Herrick, J. B. 1995. Ph.D. Thesis. Cornell University, Ithaca, NY)

ABSTRACT

Documenting the exchange of catabolic and other genes among bacteria inhabiting soil, sediment and aquatic habitats is of ecological significance and poses substantial methodological challenges. We here provide evidence of horizontal transfer of the naphthalene dioxygenase iron-sulfur protein gene *nahAc* among populations of naphthalene-degrading bacteria at a coal tar-contaminated site in upstate New York. In a previous study (Chapter 2), we described a phenotypically diverse group of Gram-negative naphthalene-mineralizing bacterial strains isolated from a coal tar-contaminated surface seep

sediment. These possessed PCR-amplifiable homologs of *nahAc*. In the present study, DNA sequencing was utilized to investigate relationships between these amplifiable *nahAc* genes. It was found that six of seven sequenced seep isolates had an identical *nahAc* allele (allele Cg1) while the seventh (allele Cg2) differed by only one of the more than 380 sequenced nucleotides. These two alleles differed substantially from the *nahAc* allele of the archetypal naphthalene-degrading strain *Pseudomonas putida* G7 (5.1% dissimilarity between Cg1 and G7) and from that of a recently isolated Washington State strain, *P. fluorescens* N1 (6.7% dissimilarity to Cg1). However, the Cg1 allele was very similar to the *nahAc* of type strain *P. putida* NCIB 9816-4 from the British Isles (0.5% dissimilarity) and identical to *nahAc* of *P. fluorescens* Nd9, isolated from the contaminated source area of the study site. Partial 16S rRNA gene sequences were obtained for the naphthalene-degrading strains studied. Those bacteria with the Cg1 *nahAc* allele had 16S rRNA sequences with dissimilarities ranging from 0% to 7.9%, suggesting that many of these isolates are much more distantly related than are their *nahAc* genes. The phylogenies of the two genes were not congruent: the Cg1 *nahAc* allele was distributed throughout the strains represented by the three major branches of the 16S rRNA phylogenetic tree. These data provide strong indirect evidence for the horizontal transfer of *nahAc* among the lineages of the naphthalene-degrading populations represented by our isolates. Genetic transfer of catabolic genes may play an important role in the evolution and adaptation of biodegradative bacterial populations to contaminants.

For supporting data and text see Appendix 4.4.

3.0 FUTURE PLANS

Although AFOSR-91-0436 has reached its official termination date (31 March 1995), data, information, and understanding obtained will serve as a foundation for a new AFOSR-sponsored project entitled, "Microbial Mechanisms Controlling the Fate of Fuel Components in Soil". This new project is designed to pursue a variety of research issues that include identification of the mobile catabolic genetic element present in our field study site, an examination of PAH catabolic diversity, and an exploration of microbial metabolism of a future Air Force fuel compound,

quadracyclane. Continued progress in these areas is also supported by AASERT Grant #93-NL-073, "Research Training for Understanding the Fate of Environmental Pollutants" (project period June 1, 1993 - May 31, 1996).

APPENDIX 4.1

OXYGEN LIMITATIONS AND AGING AS EXPLANATIONS FOR THE FIELD PERSISTENCE OF NAPHTHALENE IN COAL-TAR CONTAMINATED SURFACE SEDIMENTS

E.L. MADSEN* AND S. E. BILOTTA-BEST

Submitted to Environmental Toxicology and Chemistry

ABSTRACT

Naphthalene has been transported approximately 400 m via ground-water flow from buried subsurface coal-tar to an organic matter-rich seep area where the water emerges at the foot of a hill at a field study site. Gas chromatographic analyses of cores taken from the sediments show naphthalene concentrations in vertical profiles ranging from 0.9 to 45 $\mu g/cm^3$. We have tested hypotheses for explaining why naphthalene persists in seep sediments. In aerobic laboratory flask assays conversion of ^{14}C- naphthalene to $^{14}CO_2$ occurred and was not stimulated by amendments with vitamins or inorganic nutrients. Thus, neither toxicity nor nutrient limitation were the cause of naphthalene persistence. With the exception of site surface waters, *in situ* sediment oxygen concentrations were below detection; this suggested that lack of this final electron acceptor could be a key reason for naphthalene persistence. Oxygen limitation was confirmed by measuring no conversion of ^{14}C-naphthalene to $^{14}CO_2$ in samples of seep sediments prepared anaerobically. Naphthalene also persisted in anaerobic nitrate-amended slurry-phase incubations of the sediment until O_2 was added. Despite these clear laboratory indications of O_2-limited naphthalene metabolism, when H_2O_2 was added to site sediments *in situ* in a randomized block design, no discernible naphthalene loss occurred. Reasons for lack of naphthalene loss during this field experiment may include spatial heterogeneity, selective metabolism of non-naphthalene substrates, protection by sorption or diffusion reactions, or *in situ* replenishment of naphthalene by migration into the sediment from hydrologically upgradient site locations. To investigate the possibility that sorption or diffusion reactions may cause decreased bioavailability, hence, naphthalene persistence, sediments from the field site were sterilized using γ-irradiation and then exposed under aseptic conditions to ^{14}C-labeled naphthalene for periods ranging from 0 to 28 days. After aging, the sediments were dispensed to sealed vials, inoculated with mixed microbial populations from the study site, and $^{14}CO_2$ production was measured. Resulting patterns in the extent and rate of naphthalene mineralization revealed an inverse relationship to the duration of contact with the sorbent, but only when the mixed inoculum had been enriched on aqueous-phase naphthalene. We conclude that oxygen limitation is the most probable cause for lack of naphthalene biodegradation at our field study site. However, sorption or diffusion reactions may also play a role.

INTRODUCTION

The persistence of organic contaminant compounds in field sites can pose serious health hazards to both wildlife and humans that may be exposed to site-derived waters, soils, or sediments (British Medical Association, 1991; Fordham and Reagan, 1991; Malins *et al.*, 1987). Conversely, *in situ* microbial activity in field sites has the potential to mineralize contaminant organic compounds, thereby eliminating toxicological risks. For several years, we have been examining the fate of coal tar-derived polycyclic aromatic hydrocarbons (PAHs) at a field study site in upstate New York (Madsen *et al.*, 1991; Madsen *et al.*, 1992; Herrick *et al.*, 1993). Past and ongoing measurements using solvent extraction and gas chromatography procedures (Madsen *et al.*, 1991; Madsen *et al.*, 1995) indicate that PAH compounds at the field site occur in a groundwater plume that extends from a point source (a coal tar waste disposal pit) to a seep area, where groundwater and aquifer sediments are exposed by a drop in surface elevation (Figs. 1 and 2). Yet, mineralization assays, using model low molecular weight PAHs, indicate that microorganisms present in these seep sediments are capable of degrading freshly-added radiolabeled naphthalene and phenanthrene at rapid rates (Madsen *et al.*, 1991; Madsen *et al.*, 1992; Madsen *et al.*, 1995). Thus, we have identified an anomaly: despite an immense biodegradation potential at this site, PAHs persist in the sediments.

At least a portion of the naphthalene and phenanthrene in upgradient subsurface region of this field study site is microbiologically available because metabolic adaptation has occurred. Naphthalene and phenanthrene were metabolized by microorganisms in groundwater sediments from inside but not outside the contaminated plume (Madsen *et al.*, 1991). This observation, in combination with high numbers of protozoa in the groundwater contaminant plume, demonstrated *in situ* biodegradation of these contaminants (Madsen *et al.*, 1991). However, substantial amounts of the naphthalene in downgradient seep sediments at this field site appear to be protected. One simple yet profound way of explaining the lack of naphthalene metabolism is that in the field site's setting, delivery of final electron acceptors to microbial cells is too slow. Thus, experiments contrasting aerobic and anaerobic naphthalene metabolism are warranted.

The inhibition of contaminant biodegradation by diffusion and partitioning processes in soil and sediments has been investigated for many years (Manilal and Alexander, 1991; Ogram *et al.*, 1985; Steen *et al.*, 1980; Steinberg *et al.*, 1987). In some reports, the partitioning of organic compounds into organic-matter pools in soil has been found to be caused by sorption- desorption reactions (Guerin and Boyd, 1992; Scribner *et al.*, 1992) or formation of covalent bonds as bound residues (Bollag, 1992). However in many reports, the precise mechanisms of the contaminant binding has not been known. Furthermore, the relationship of bioavailability to the kinetics of these binding reactions is also not well understood. Insights into this relationship are crucial for determining the environmental fate and effects of contaminants. Recently, Hatzinger and Alexander (1995) have shown that model contaminant compounds, phenanthrene and 4-nitrophenol, became less extractable and more resistant to biodegradation as the contact time for diffusion and partitioning reactions increased. This inverse relationship between the bioavailability of contaminants and the duration of partitioning reactions, termed an "aging effect", may have significant implications for explaining the persistence of biodegradable compounds, generally, and may have specific bearing on the fate of naphthalene at our study site.

We have defined three hypotheses for explaining why the simplest of the PAH compounds, naphthalene, has persisted in sediment at our field study site: (1) the activity of the naphthalene-degrading microbial populations in the sediments are limited by either inorganic and organic nutrients or the accrual of toxic materials; (2) the native microbial populations are limited by the abundance of suitable final electron acceptors; and (3) sorption and/or partitioning reactions have protected the naphthalene by diminishing its bioavailability. This study was designed to test these hypotheses using a mixture of field and laboratory approaches. This is a companion paper accompanying a laboratory study that focuses on the influence of sorbate-sorbent contact time (aging) on phenanthrene metabolism in the same site-derived sediments (Sandoli *et al.*, 1995).

MATERIALS AND METHODS

Field Site Characterization

Spatial (Fig. 1), hydrologic (Fig. 2), and chemical characteristics of the field study site have been described in detail elsewhere (Madsen *et al.*, 1991, 1992; Murarka *et al.*, 1992). Vertical sediment cores were obtained by using a sledge hammer to drive two steel pipes (rigid electrical conduit pipe 5.5 cm OD, 1.5 m in length) through the water saturated organic muck and sand lenses into the relatively dense underlying aquifer sand. This denser material sealed the end of the pipe, allowing recovery of intact vertical core samples. By measuring the depth of pipe insertion and the height of removed core, the degree of sediment compression was calculated and used to compute pre-compression sampling depths.

Accurate determination of *in situ* O_2 concentrations was of primary concern during coring operations. All O_2 determinations were completed in the field within 5 min of core removal using a portable O_2 probe (Microelectrodes, Inc., Londonderry, NH) that was inserted either directly into surface sediments or through holes drilled into the side of the coring barrel. As the drill bit was removed, the probe was inserted and it prevented the entry of atmospheric O_2 into the core because the periphery of the hole was sealed by the slurry-like muds. Calibration of the probe was accomplished using N2-purged water (0% O_2) and air-saturated water (21% O_2). While still at the field site, the steel pipes were segmented at selected depths using a rotary hand pipe cutter. As the segments were removed, a flamed stainless steel spatula was used to scrape off the top layer of mud to expose undisturbed sediment beneath. A flamed stainless steel scoop (3 cm^3 fixed volume) was next used to immediately transfer 3 cm^3 of this mud at a 20-ml precleaned glass vial (Ichem) followed by the addition of 2 ml butanol:hexanes (1:9) extractant prior to being sealed with a screw cap Teflon-backed septum and placed on ice. Surface water from the site was gathered using glass pipettes. Four ml volumes of water were transferred to 10 ml sterile glass ampules. These were immediately amended with 1 ml of the butanol/hexanes extractant and sealed with a butane torch prior to being transported back to the laboratory. GC analysis of naphthalene is described below. Total microscopic microorganisms were determined by fluorescence microscopy as described by Beloin *et al.* (1988).

Mineralization assays

Procedures were those of Madsen *et al.* (1995). Under aseptic conditions, 4 to 8 g (wet weight) of contaminated seep sediment was placed in 25 ml screw cap vials (Pierce, Rockford, IL) containing a glass marble. Two ml sterile deionized water and 0.05 µCi [1-^{14}C] naphthalene (10.1 mCi/mM, >98% radiopurity; Sigma Radiochemicals, St. Louis, MO, from crystals allowed to

dissolve in sterile deionized water) were added to the sediment. A shell vial (15 x 45 mm, Kimble, Vineland, NJ) containing 0.4 ml of 0.5 N NaOH as a CO_2 trapping solution was placed on top of the marble inside the 25-ml vial which was then sealed with a Teflon-faced silicone septum (Pierce) and a screw cap ring. The vials were incubated in triplicate at room temperature on a rotary platform set to 30 rpm. At several time intervals after sealing the vials, the trapping solution inside was withdrawn with a needle and syringe through the septum. Trapping solution was replaced and portions of the ^{14}C trapped were counted with liquid scintillation counter (Beckman Instruments Inc., Fullerton, CA, Model LS 5000 CE).

Assays were designed to test the response of sediment microorganisms to a variety of nutrient amendments. The amendments included inorganic compounds [nitrate and sulfate (at a final concentration of 100 ppm each), ammonium + phosphate (35 ppm, 5 ppm, respectively), a standard trace metal solution (Stanier *et al.*, 1966)], a standard vitamin solution (Stanier *et al.*, 1966) or carbon sources (non-radioactive naphthalene (3 ppm) or acetate at 30 ppm]. In order to allow for the possibility that microsites in the test flasks may be O_2 limited, a treatment with headspace at twice atmospheric was also prepared.

Anaerobic incubations

Four gram of seep sediments, in triplicate 35 ml glass serum bottles, were amended with ^{14}C-naphthalene 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 (Ann Arbor, MI) containing a mixture of N2:H2 (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 treatment conditions were: oxygen amendment (= aerobic), nonamended (= methanogenic), sulfate amended (= sulfate reducing), nitrate amended (= denitrifying).

Anaerobic slurry phase naphthalene metabolism assays were prepared and implemented using HPLC analysis as described by Madsen and Bollag (1989) and Madsen *et al.* (1988). The anaerobically prepared mineral salts naphthalene-saturated medium was stirred and transferred in 75 ml aliquots, under anaerobic conditions, to 12 120-ml serum bottles. The 12 bottles were split into 4 treatments (3 replicates each) as follows: (1) the anaerobic medium uninoculated [the medium, alone, where no activity was expected]; (2) the anaerobic medium, inoculated [the "live" treatment where activity was expected]; (3) was like #2, but autoclaved [to prove the activity can be eliminated by heat and pressure]; (4) was like #2, but poisoned with $HgCl_2$/HCl [to prove the activity could be eliminated with known biotic inhibitors]. HPLC analyses of 1 ml fluid subsamples were used to follow loss of naphthalene from solution.

The oxygen content of headspace gases was determined by injecting 100 µl onto a Perkin Elmer model 8500 gas chromatograph equipped with a thermal conductivity detector and a molecular sieve 5A-filled column (5.5 m x 0.3 cm; Supelco). Oven temperature was constant at 40°C.

Determination of naphthalene in site sediments and waters

Vials and ampules containing extractant and site sediment and water samples were stored at 4°C until injection of 1 µl into a Hewlett-Packard 5971A GC/MS equipped with a Nukol glass capillary column 30 m x 0.25 mm ID, 0.25 µ film thickness; (Supelco, Bellefonte, PA). The unit was equipped with a 5791 mass selective detector operated in scanning mode at a voltage of 2047,

temperature at 300°C, and scanning mass range of 10-180 m/z. The injector temperature was 250°C. The oven temperature began at 40°C held for 1 min and then raised to 200°C at a rate of 10°C/min.

Field Assay to Test O_2 Limitations

A randomized block design was implemented in which 40 open-ended glass tubes [11 x 1.7 cm (height x O.D.)] were inserted on 10 cm centers directly in a flat 0.5 m^2 area of the seep. Each cylinder was gently forced into the organic mud to a depth of 9 cm. This allowed 2 cm of the cylinder to extend above sediment surface. One half of the cylinders were amended with 1.33 ml of a NaBr solution (12.5 g/l; used as an internal tracer to account for dilution of solutes contained by the tubes) while the other half were amended with the NaBr solution plus a freshly-prepared H_2O_2 stock solution so that the final H_2O_2 concentration in the 18 ml tube volume was 250 ppm. At sampling times, four tubes and their contents from each of the two treatments were removed from the field site, emptied into glass vials, immediately amended with 4.5 ml of hexanes/acetone extractant (1:1), sealed with Teflon lined caps, shaken, put on ice for transport back to the laboratory, and stored at 4°C for GC/MS analysis of naphthalene. Bromide was measured in aqueous dilutions prepared from the sediments retrieved from the field site using a specific bromide ion electrode (Orion Instruments) using protocols and standards recommended by the manufacturer.

Aging Naphthalene With Sediments

Procedures were those described by Sandoli (1994) and Sandoli *et al.* (1995). Twenty-four grams of γ-sterilized (2.5 Mrad from a ^{60}Co source in Ward Laboratory, Cornell University) seep sediment was aseptically dispensed to 20 sterile 60-ml glass ampules. At times, between 28 and 0 d, 10^5 dpm of aqueous ^{14}C naphthalene was added in 24 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 ampules were opened, the supernatant was poured off and assayed for ^{14}C by scintillation counting, and 5 g (wet weight) quantities of the sediment were dispensed to 25-ml screw capped glass vials for the mineralization assay described above. The inoculum was either an unenriched 1:100 dilution of seep sediment dispensed (1.0 ml) to each vial or an enrichment (1 g to 100 ml mineral salts + naphthalene 30 ppm) of cells derived from seep sediment, grown on a liquid mineral salts/naphthalene mixture for 1 day prior to being dispensed (0.5 ml) to each vial.

RESULTS

Field Site

Figs. 1 and 2 portray the spatial arrangements and hydrologic characteristics, respectively, of the study area. In the upland (Western) portion of the site (Fig. 1), where coal-tar waste was buried approximately 35 years ago, contaminated zones of sediment and groundwater are accessible only by removing sediment cores through the use of drilling equipment. Details of aquifer characteristics and site microbiology have been described previously (Madsen *et al.*, 1991; Madsen *et al.*, 1992). In the lowland (Eastern) portion of the site (Fig. 2), the contaminated groundwater emerges at the foot of a hillside where it contacts organic matter-rich freshwater sediments. Henceforth this shall be referred to as the "seep area". These seep sediments contain a variety of coal tar-derived compounds, especially naphthalene, methylnaphthalenes, and indenes (Connaughton *et al.*, 1993; Madsen *et al.*, 1994, 1995). Several previous reports (Madsen *et al.*, 1991; Madsen *et al.*, 1992) have shown that aerobic microorganisms native to the subsurface

sediments are capable of mineralizing both naphthalene and phenanthrene, yet naphthalene has been repeatedly detected by GC and GC/MS analysis in seep sediments. The vertical profile of oxygen, naphthalene, and microorganism concentrations in two independent cores that were removed from the seep area are shown in Table 1.

It is clear from data in Table 1 that surface waters of the site are aerobic; however, only centimeters below the surface, oxygen was below detection. The vertical distribution of both naphthalene and microbial cells were variable with depth and ranged from 0.9 to 45 ppm and log 9.0 to 9.9 cells/cm^3, respectively.

Nutrient limitation assay under aerobic conditions

In order to test the hypothesis that naphthalene persistence in seep sediments may be caused by limiting amounts of nutrients and/or inhibitory substances, naphthalene mineralization assays were performed using seep sediments amended with a variety of potentially limiting nutrients. Results of the mineralization assays appear in Fig. 3. No significant amounts of $^{14}CO_2$ were generated in the poisoned controls. However, all live treatments converted approximately 54% of the added radioactivity to $^{14}CO_2$ within 18 hrs. Relative to the unamended treatment, no significant stimulation of aerobic naphthalene mineralization was detected in treatments amended with the inorganic and organic nutrients shown in Fig. 3. We conclude that microorganisms were present in the seep sediments and mineralized naphthalene at similar rates and to similar extents, regardless of the various amendments. Thus, aerobic naphthalene metabolism in site sediments does not appear to be limited by (i) the absence of naphthalene degrading microorganisms, or (ii) the presence of toxic substances, or (iii) simultaneous availability of carbon sources, or (iv) organic or inorganic nutrients.

Anaerobic Naphthalene Metabolism Assays

Data in Table 1 clearly show that anaerobic conditions predominate at depth in the seep sediments. Because dioxygenase enzyme complexes that require molecular oxygen as a cosubstrate 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 was also been reported (Mihelcic and Luthy, 1991, 1988a, b).

To further explore the propensity for microorganisms at our study site to metabolize naphthalene under a variety of redox conditions, assays analogous to the aerobic naphthalene mineralization (Fig. 3) were performed under anaerobic conditions designed to favor methanogens, sulfate reducing bacteria, and denitrifiers. An O_2-amended treatment was also prepared. Results of the 16 day incubation in which $^{14}CO_2$ production was monitored revealed that 77% of the naphthalene was converted to $^{14}CO_2$ in the vials to which oxygen was added, but not in the other treatments (data not shown). Thus, no significant naphthalene mineralization was detected in treatments designed to encourage denitrifying, sulfate reducing or methanogenic processes.

Because naphthalene metabolism under denitrifying conditions had been previously reported (Mihelcic and Luthy, 1988a, b; 1991), an additional attempt using slurried sediments was implemented. The fate of naphthalene in all four denitrifying treatments is shown in Fig. 4. During the first 12 days of monitoring, the naphthalene concentration remained constant in all treatments. Thus, no evidence for denitrifying naphthalene metabolism was obtained. To prove that there were aerobic naphthalene-degrading cells present in the inoculum, on day 12, the headspace gas in two

of the triplicate vials in each treatment was pressurized with O_2. GC analyses of headspace gasses confirmed that the O_2 concentration rose from <.2% (below the detection limit) to 30%; thus, the oxygen pressurization was effective. The third vial in each triplicate was left untreated to serve as an anaerobic control. Addition of O_2 to the uninoculated, autoclaved, and poisoned treatments had no effect on naphthalene concentrations: regardless of O_2 additions, naphthalene concentrations remained constant. However, in the live bottles after O_2 addition, the naphthalene concentration plummeted rapidly (Fig. 4). This loss of naphthalene did not occur in the live bottle that not receive O_2. Furthermore, the experiment yielding the data in Fig. 4 was repeated and found to be reproducible.

Data in Fig. 4 provided no evidence for denitrifying naphthalene metabolism in sediments from the seep area at the study site. To the contrary, data in Fig. 4 unequivocally prove that O_2 was limiting naphthalene metabolism in the seep-derived microbial community. This relates directly to data in Table 1, which shows that O_2 was absent under field conditions at the study site. If naphthalene metabolism under methanogenic or sulfate reducing or denitrifying conditions were operating at the seep, each of these metabolic regimes would have responded to their respective treatments. Because only aerobic metabolism of naphthalene was observed, lack of O_2 was strongly implicated as the reason for naphthalene persistence at the study site. Thus, provision of O_2 to these sediments in field plots at the study site should theoretically eliminate the naphthalene contamination.

Field Assay to Test O_2 Limitations

The clear stimulation of anaerobically-incubated naphthalene-degrading microorganisms by O_2 injections (Fig. 4) suggested that a major cause for the persistence of naphthalene in the seep study area is the absence of oxygen at depth (Table 1). A randomized block design field assay was designed to test this hypothesis by periodically removing tube-enclosed sediments from the field site and assaying the retrieved slurries for bromide and naphthalene. Results are shown in Table 2. Over the 21 day field experiment, recovered bromide concentrations diminished by a factor of 2.5, presumably due to dilution caused both by rainfall and surface water flow. Naphthalene recovered from these tube-enclosed sediments during this experiment were highly variable and roughly constant in both the H_2O_2-amended and unamended treatments (Table 2). Thus, the H_2O_2 treatment failed to enhance naphthalene disappearance in the field site.

Assessing the Influence of Sorption Reaction on Naphthalene Mineralization

One of the hypotheses that this investigation sought to test is protection of PAHs by sorptive and diffusion reactions that may reduce their bioavailability, hence impede PAH biodegradation. By experimentally manipulating the contact time between aseptically prepared PAHs and sediment, we sought an emergent pattern during subsequent mineralization experiments. Our hypothesis was that diffusion of PAHs into microporous sediment organic matter would render PAHs unavailable to sediment bacteria.

Results of the aging/mineralization experiment in which the inoculum was a dilution of unenriched seep microorganisms appear in Fig. 5. The greatest rate and extent of naphthalene mineralization were found from the oldest (21- and 11-day treatments). No simple relationship between the duration of contact time and naphthalene bioavailability occurred, as treatments aged 1, 11, and 21 days all clustered together.

Results of the aging-mineralization experiment in which the inoculum was enriched in aqueous naphthalene are shown in Fig. 6. The data contrast strikingly with those in Fig. 5. By changing the inoculum from "unenriched" to "enriched", a clear relationship between rates/extents of mineralization and contact time was achieved. 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 the sediment. Thus, the aging-bioavailability hypothesis seemed to apply to the sediment microbial community, after being physiologically adapted towards aqueous phase naphthalene.

DISCUSSION

The anomalous persistence of the readily biodegradable low molecular weight PAH, naphthalene, was investigated by testing a series of hypotheses using both laboratory and field methodologies. Freshly added ^{14}C-naphthalene was extensively converted to $^{14}CO_2$ in aerobic laboratory-flask assays regardless of a variety of organic and inorganic amendments. Thus, neither toxicity nor nutrient limitation were responsible for the absence of *in situ* naphthalene metabolism. Naphthalene metabolism was absent from anaerobic incubations which used both $^{14}CO_2$ trapping and HPLC analysis of slurry-phase sediments. However, O_2-amended anaerobic treatments rapidly metabolized the substrate. Therefore, O_2 limitation, as supported by O_2-probe analyses of field cores, was implicated as the explanation for field persistence of naphthalene. However, a randomized block design H_2O_2-amended field experiment failed to stimulate naphthalene loss *in situ* from the sediments, relative to the unamended controls. Reasons for lack of stimulation of aerobic naphthalene biodegradation by the added H_2O_2 are uncertain, but may include: (1) spatial heterogeneity of naphthalene in the field sediments, (2) O_2 respiration at the expense of non-naphthalene substrates in seep sediments, (3) rapid release of O_2 from H_2O_2 without its utilization by microorganisms, (4) lack of O_2 limitation, and (5) the naphthalene in the sediment may not have been accessible (bioavailable) to microbial attack.

This investigation failed to uncover any evidence for anaerobic naphthalene metabolism. Absence of evidence for anaerobic naphthalene metabolism is not proof that the process does not occur on site — it merely means that the procedures used were unsuccessful. Several reasons for this can be proposed: (i) the mineral salts medium either lacked key nutrients or contained inhibitory substances; (ii) the organisms that carry out the process may be active only in a true 3-dimensional matrix; by attempting to measure the reaction in a dilute slurry, habitat disruption may have eliminated activity; (iii) the naphthalene concentration may have been too low to support growth; and (iv) the denitrifying and other anaerobic naphthalene degraders were truly absent. It is very clear, however, that aerobic metabolism is a robust metabolic process that is likely to dominate any other naphthalene transformation whenever O_2 is available to sediment microorganisms. In fact, a unique product of dioxygenase attack on naphthalene has recently been isolated from field site waters (Wilson and Madsen, 1995).

The final approach reported here for explaining naphthalene protection from microbial metabolism was the aging-bioavailability hypothesis. This postulates that time-dependent sequestration reactions have rendered the naphthalene unavailable for microbial attack. In interpreting the data shown here (Figs. 5 and 6), it is essential to appreciate the complex mechanisms that contribute to the net outcome of $^{14}CO_2$ production. The sorption-desorption and

microbial contact-metabolism processes are, essentially, coupled "black boxes". One black box is the sediments. The other is the microbial community. The approach taken here was to seek a simple relationship between sorptive contact time and naphthalene mineralization as a measure of its bioavailability.

The aging-bioavailability hypothesis is consistent with data derived from sediment inoculated with an enrichment culture from seep sediment (Fig. 6). However, when an unenriched inoculum was utilized, the resultant $^{14}CO_2$ production curves showed an indifference to the duration of contact time between sorbent and substrate (Fig. 5). Reasons for these results are uncertain, but probably reside in variabilities in physiological properties of mixed unknown, microbial populations. It is the microbiological component of the test system that was least understood. A major difference between the two aging experiments was how the inocula were prepared. In the first experiment (Fig. 5), microorganisms native to the seep sediment were inoculated directly into the mineralization vials, before adaptation to aqueous naphthalene could be achieved. Whereas in the second experiment, 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. We feel that some microorganisms may prefer or be indifferent to sorbed naphthalene while others may prefer it in an aqueous form. This type of physiological specialization for naphthalene metabolism has been described previously by Guerin and Boyd (1992). Had we employed aging periods for duration that matched those of Hatzinger and Alexander (1995), perhaps the naphthalene would have been more strongly or deeply sequestered, hence become less bioavailable.

Methodologies for aging of chemicals with sediments used here contrast with those used by Hatzinger and Alexander (1995) in various respects. For instance, unlike Hatzinger and Alexander (1995) who dissolved their hydrophobic substrate in an organic solvent (CH_2Cl_2), we added our test substrate at its saturated aqueous-phase concentration in deionized water. Furthermore, our aging vessels were glass ampules rather than the screw-cap test tubes used by Hatzinger and Alexander (1995). In addition, Hatzinger and Alexander (1995) reported that their aged chemicals were readily extracted (high recovery efficiency) from the soils tested. Whereas our attempts to use solvents to extract naphthalene from the organic matter-rich sediments from our field site were less satisfactory (data not shown). Thus, it is clear that the implementation of aging-bioavailability studies necessarily requires that certain logistical decisions and arbitrary choices about both materials and procedures be made when devising experimental approaches. Differing details of the methodological approaches may influence the outcome of these experiments, which are designed to explore the mechanisms by which the interactions between matrix solids and chemicals alters their bioavailability.

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TABLE 1. Vertical Profiles of Naphthalene, Oxygen, and Microorganims in Two Cores from the Organic Matter-Rich Seep Area of the Study Site

Core	Depth (cm)	O_2[a] (mg/l)	Naphthalene (ppm)[b]	Total Microbial Counts (log)[c]
1	0[d]	4	1.0	nd[e]
	3.5	0	2.0	9.7
	7.6	0	11	9.2
	13	0	27	9.2
	29	0	3.6	9.9
2	0[d]	4	0.9	nd[e]
	8.6	0	18	9.6
	18	0	39	9.1
	30	0	20	9.0
	51	0	45	9.4

[a] O_2 was measured by dipping the oxygen probe into on-site-flowing surface water or by inserting the probe into an intact core immediately after its removal. See Materials and Methods for details.

[b] Sample extracted with 1:9 (butanol:hexanes) immediately after core was removed from seep. Units are μg naphthalene per cm^3 of sediment. Naphthalene concentration for water samples represents an average of 3 replicate 4 ml samples gathered, extracted, and sealed in glass ampules on site. See Materials and Methods for details.

[c] Total microscopic bacterial counts performed using fluorescence microscopy.

[d] Surface water

[e] nd = not determined.

TABLE 2. Naphthalene Concentrations in Field Experiment in Which Sediments Contained by Open-ended Glass Cylinders Were Amended with 250 ppm H_2O_2

	Naphthalene in sediment (ppm)[1]	
Time (day)	$-H_2O_2$	$+H_2O_2$
0	5.5 ± 2.6	5.2 ± 1.7
1	4.3 ± 0.9	5.9 ± 3.5
5	12.2 ± 2.4	6.2 ± 2.8
12	8.0 ± 2.0	12.9 ± 8.9
21	12.3 ± 0.2	7.6 ± 1.7

[1] Average of 4 replicate sediments. All concentrations of naphthalene normalized to 50 g wet weight of recovered sediment.

FIGURE LEGENDS

Figure 1. Plan view of contaminated study area showing spatial relationships between the seep area to other upland portions of the site.

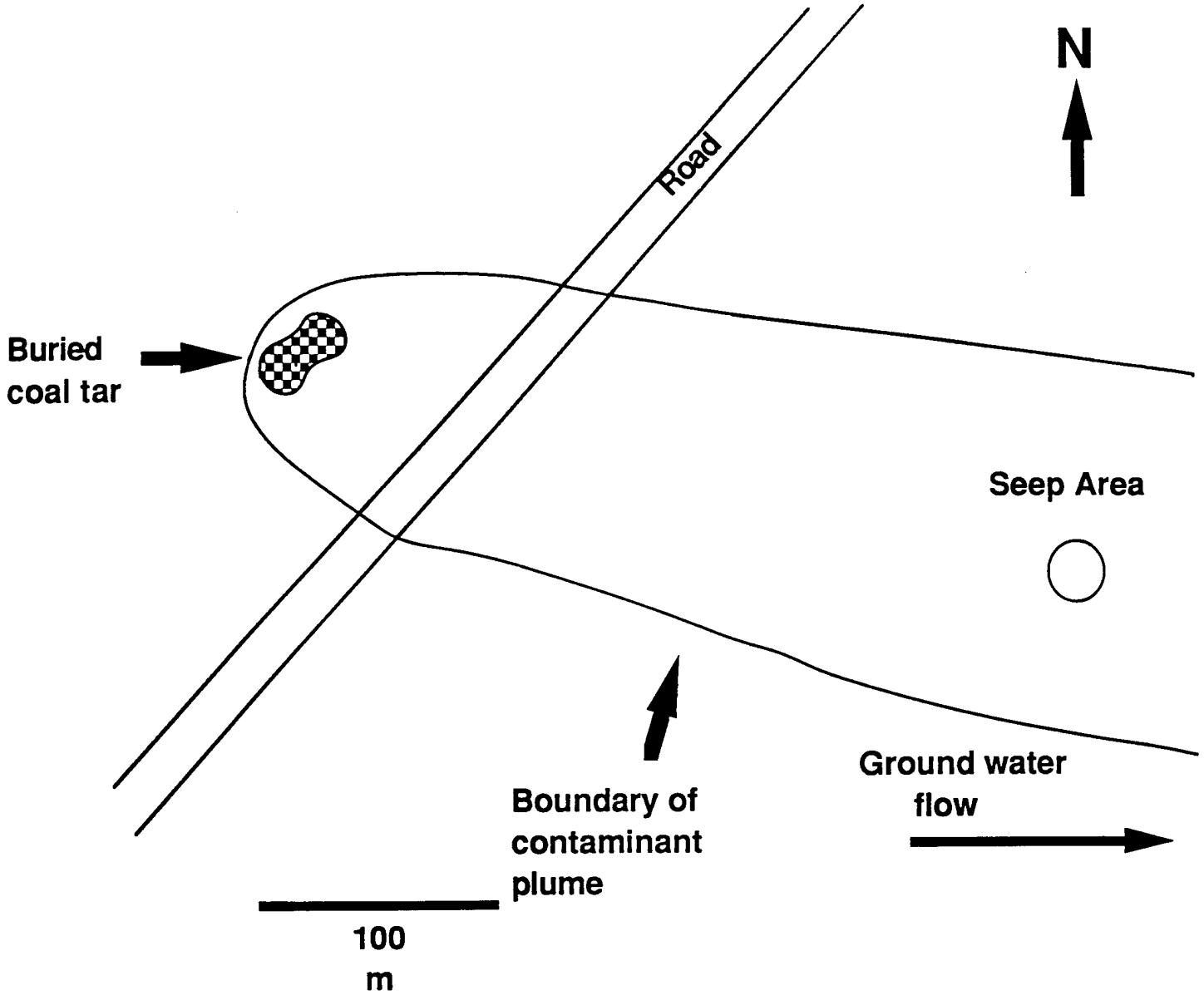
Figure 2. Diagram of study site in vertical cross section showing source of coal-tar contaminants, ground-water flow, and sampling sites.

Figure 3. Aerobic mineralization of naphthalene in seep sediments with and without amendments of a variety of potentially limiting inorganic and organic nutrients.

Figure 4. Slurry -phase anaerobic incubation of aqueous naphthalene in Teflon-sealed serum bottles. The medium contained mineral salts and KNO3 to encourage denitrifying microorganisms. Treatments were the live (inoculated), uninoculated, autoclaved, and poisoned with $HgCl_2$/HCl. After 12 days, the head-space in two of the three replicate bottles for each treatment was pressurized with oxygen.

Figure 5. Cumulative mineralization of naphthalene aseptically aged with seep sediments and subsequently inoculated with an unenriched suspension from the seep. Aging times from 0 to 21 days. Each data point represents the average from three replicate flasks. Error bars represent standard deviation.

Figure 6. Cumulative mineralization of naphthalene aseptically aged with seep sediments and subsequently inoculated with aqueous enrichment suspensions from the seep. Aging times ranged to 0 to 28 days. Each data point represents the average from three replicate flasks. Error bars represent standard deviation.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m

Fig. 1

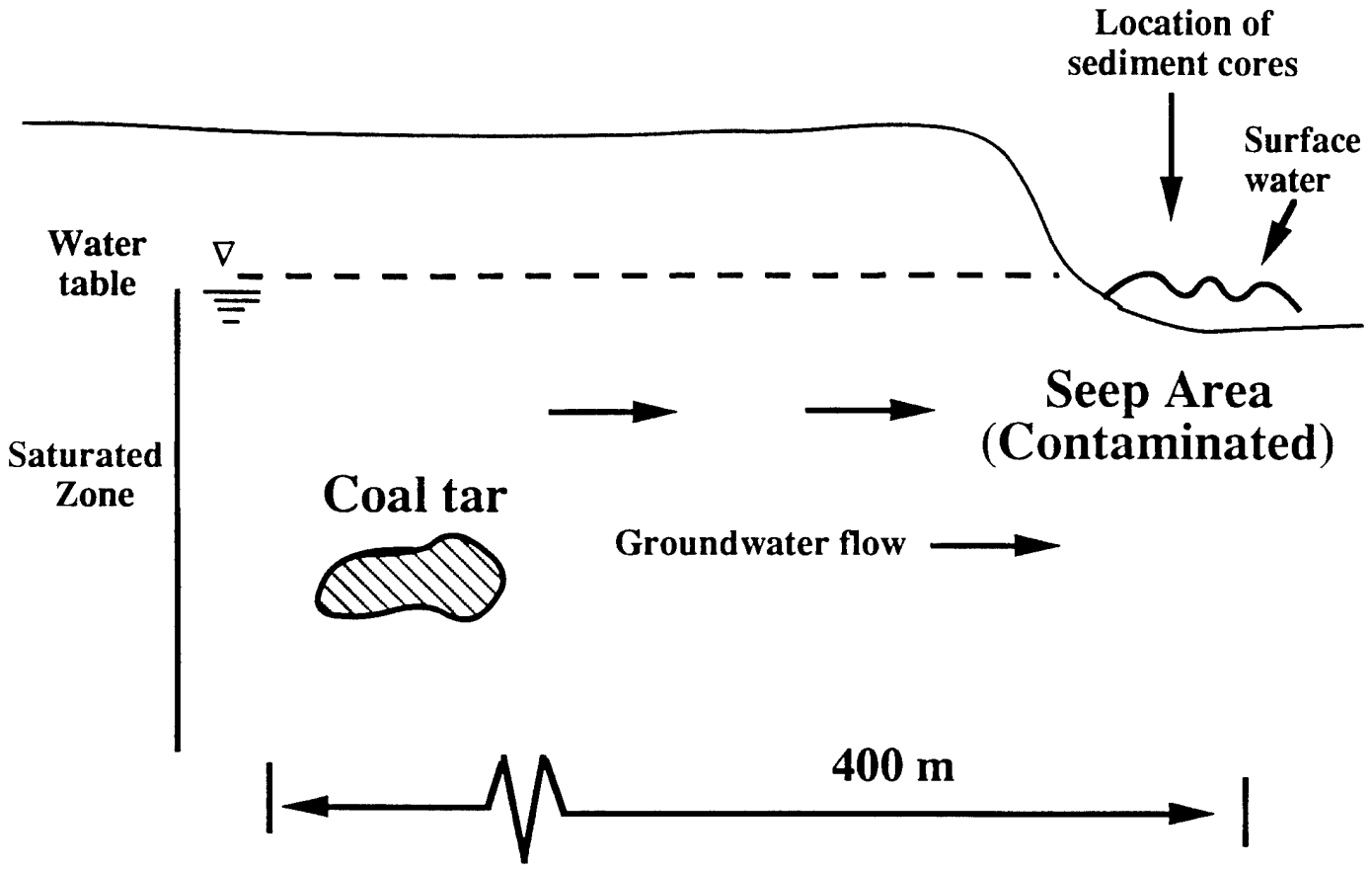
Upland portion of site
Location of sediment cores
Surface water
Water table
Saturated Zone
Seep Area (Contaminated)
Coal tar
Groundwater flow
400 m

Fig 2

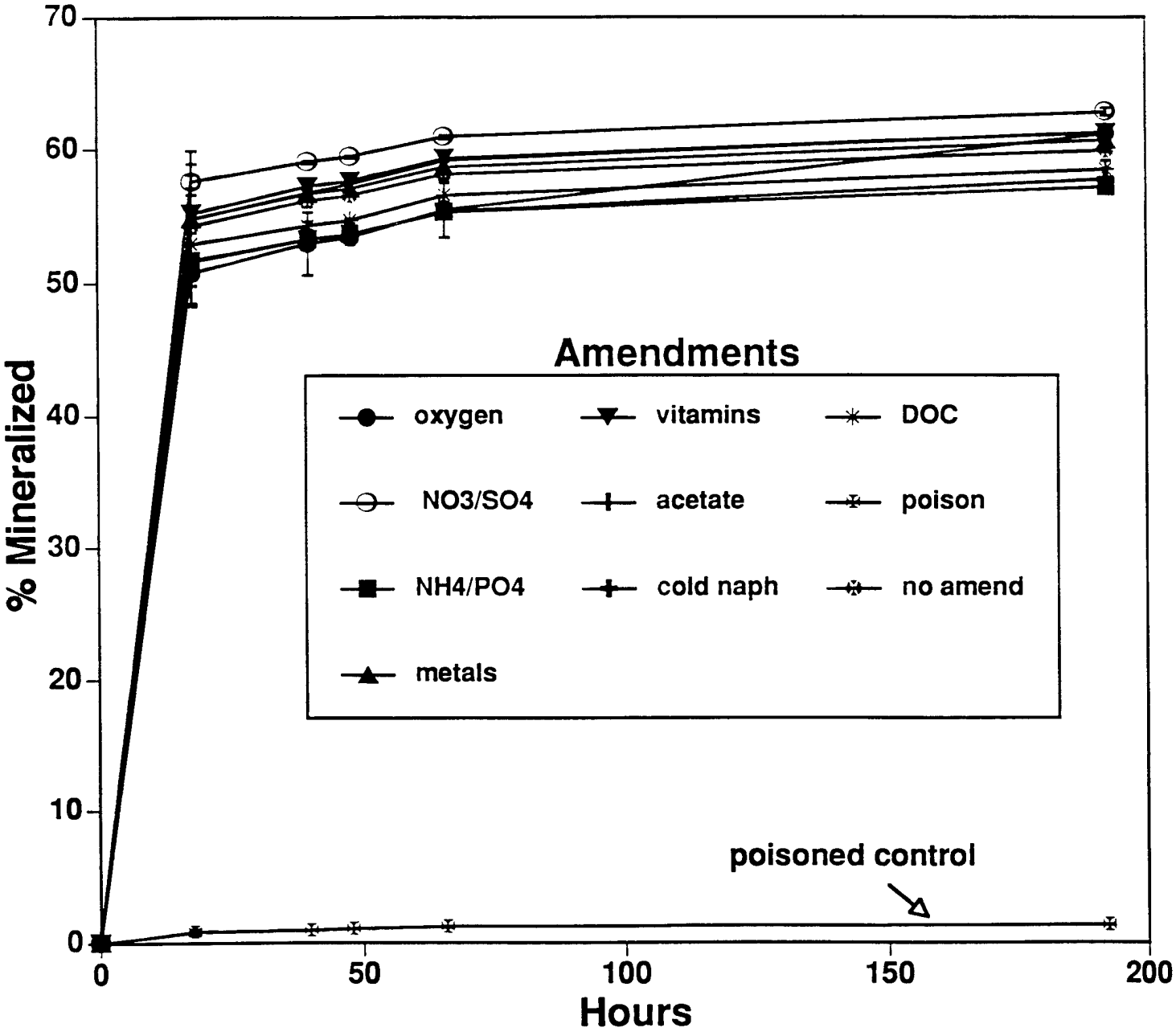
70
60
50
40
30
20
10
0
% Mineralized
0
50
100
150
200
Hours
Amendments
oxygen
vitamins
DOC
NO3/SO4
acetate
poison
NH4/PO4
cold naph
no amend
metals
poisoned control

Fig 3

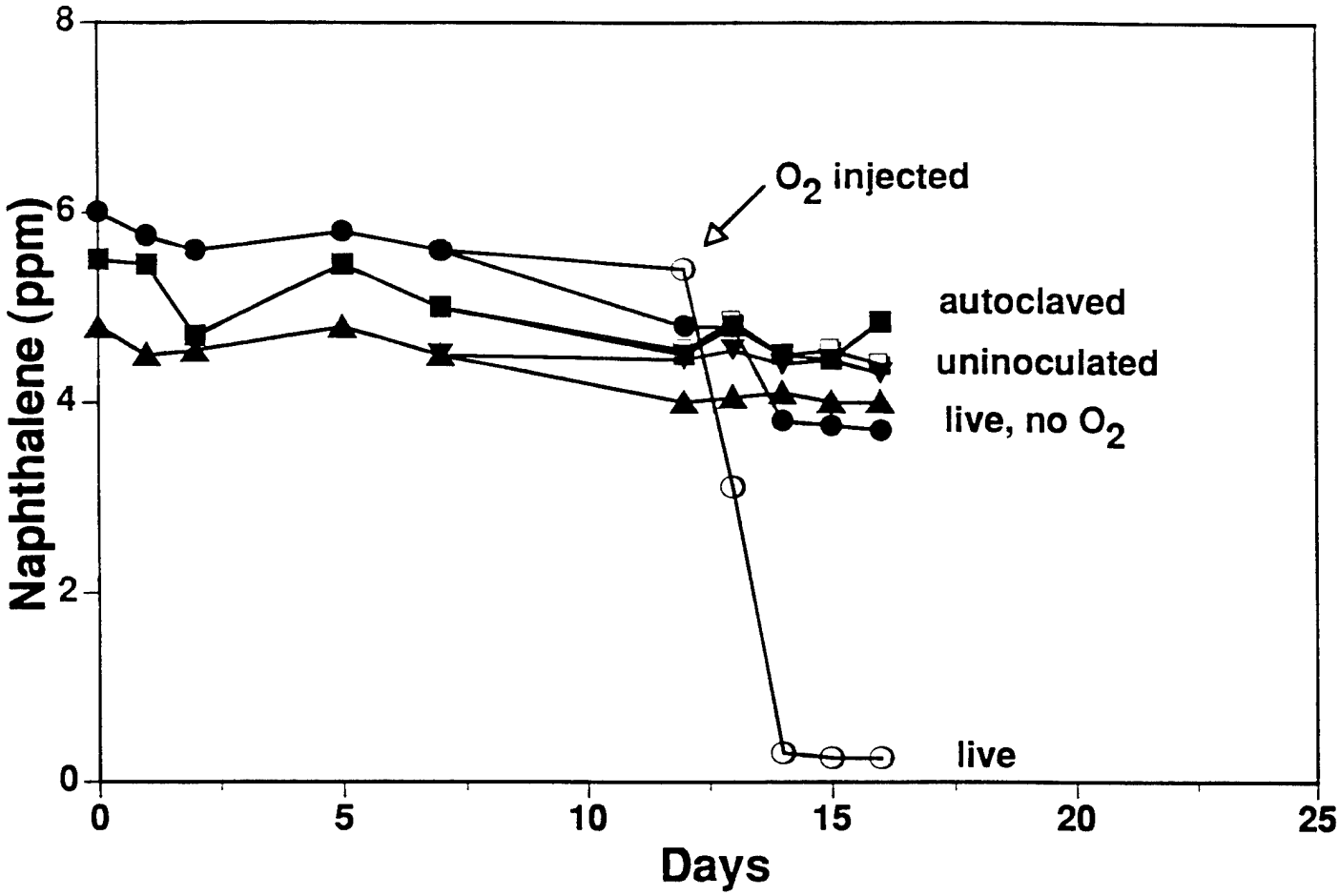
8
6
4
2
0
Naphthalene (ppm)
0
5
10
15
20
25
Days
O_2 injected
autoclaved
uninoculated
live, no O_2
live

Fig 4

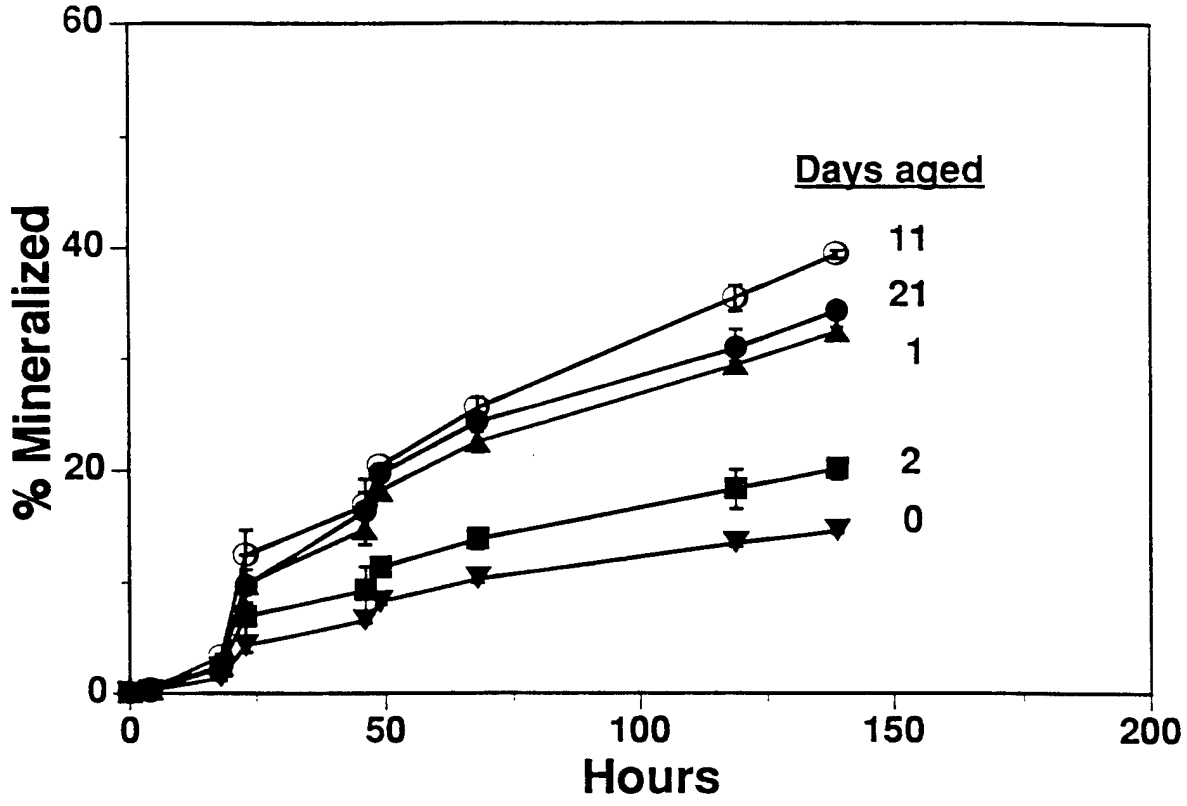
60
40
20
0
% Mineralized
0
50
100
150
200
Hours
Days aged
11
21
1
2
0

Fig 5

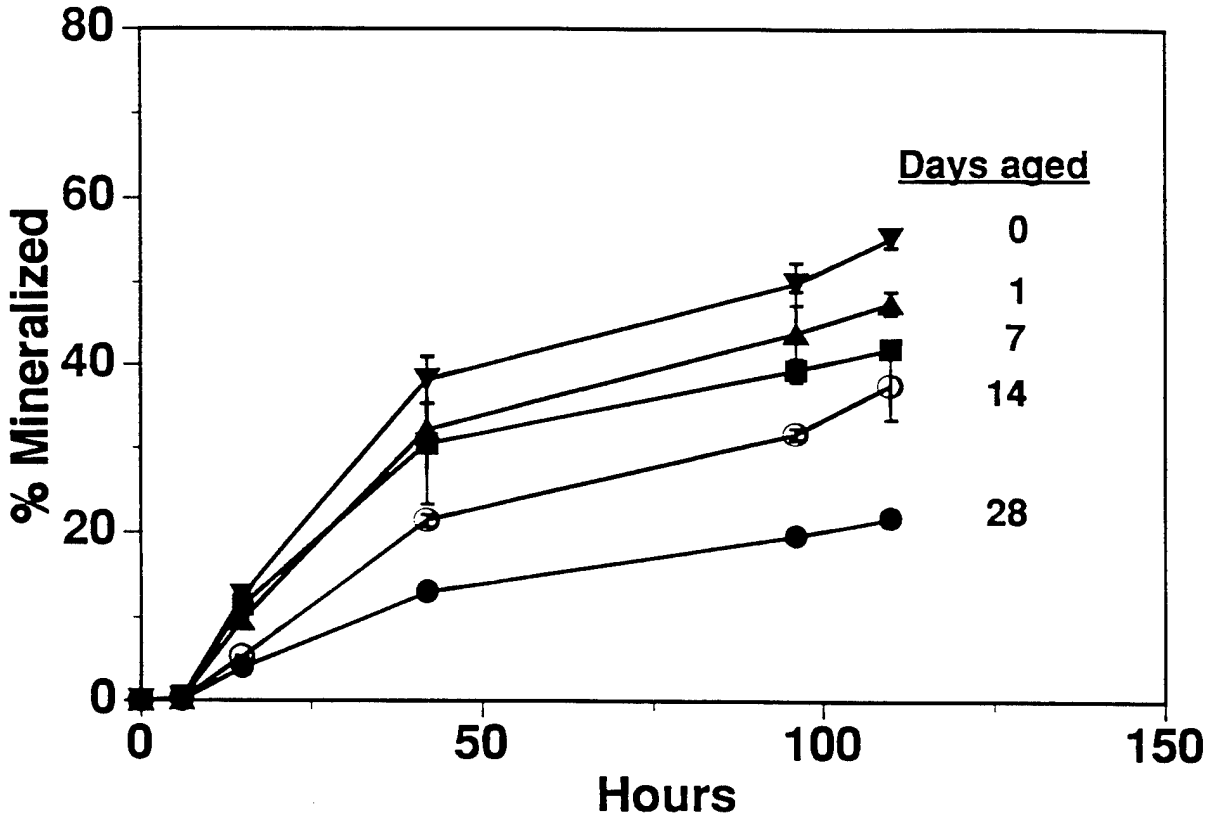

Fig. 6

APPENDIX 4.2

Regulation of Microbial Phenanthrene Mineralization in Sediment Samples by Sorbent-Sorbate Contact Time, Inocula, and Gamma Irradiation-Induced Sterilization Artifacts

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ABSTRACT

Time-dependent sorption and/or diffusion reactions were proposed as a hypothesis for explaining the persistence of polycyclic aromatic hydrocarbons (PAHs) in sediments from a coal tar waste-contaminated field site. To test this hypothesis, ^{14}C-labeled phenanthrene was aged in both subsurface sand and organic matter-rich seep sediments that had previously been sterilized by gamma irradiation. After aging periods ranging from 0 to 28 days, the sediments were dispensed to replicate vials, inoculated with site-derived phenanthrene degrading microorganisms, with or without previous enrichment on phenanthrene , and cumulative $^{14}CO_2$ production was measured. Patterns of $^{14}CO_2$ evolved from the sand sediments showed that the extent and rate of mineralization of aged phenanthrene was largely dependent upon the inoculum source (sand versus seep sediments). When both pure culture and mixed inocula originated from the seep sediments, phenanthrene mineralization was retarded in samples with longer aging periods. However, when the inoculum originated from the sand sediments, aging of the phenanthrene had only a slight or no effect on its rate or extent of mineralization. Preenrichment of phenanthrene-degrading microorganisms in either sediment type with aqueous-phase phenanthrene had little influence on subsequent mineralization of phenanthrene aged in the sand sediment.

Although microorganisms in the fresh organic matter-rich seep sediments readily converted ^{14}C-phenanthrene to $^{14}CO_2$, this activity was diminished or completely eliminated when gamma radiation-sterilized sediments were present. To explain the gamma irradiation-induced inhibition of phenanthrene metabolism, mineralization experiments were performed with both pure and mixed cultures. In these experiments, ^{14}C-glucose was readily converted to $^{14}CO_2$ in the presence of the irradiated seep sediments, and nonradioactive glucose added to fresh seep sediment failed to delay phenanthrene metabolism. Therefore, gamma irradiation-induced toxicity and utilization of preferred growth substrates were unlikely causes of the inhibition. Instead, gamma radiation-induced changes in the sorptive properties of the seep sediments seemed to be the cause. This was indicated both by an increase in the sorption-controlled distribution coefficient, K_d, for gamma-sterilized sediment and by tests showing that the extent of ^{14}C-phenanthrene mineralization was roughly proportional to the amount of fresh seep sediment added to the irradiated sediment.

Key words: phenanthrene , biodegradation, sorption, gamma irradiation, sediment

INTRODUCTION

Reactions between hydrophobic organic contaminant compounds and soil, organic matter-rich sediment, or organic matter-poor aquifer matrix materials have important implications for understanding and predicting the transport and fate of environmental contaminants (Daniel and Trautwein, 1994; Fetter, 1993; Jury and Roth, 1990; Pinder and Abriola, 1986).The mechanisms of interaction between contaminant compounds and matrix solids can vary from formation of covalent bonds (Bollag, 1992) to hydrophic partitioning (Freeman and Chueng, 1981; Brusseau and Rao, 1989; Lion, 1990) to surface adsorption (Brusseau, 1993; Scribner *et al.*, 1992; Steen *et al.*, 1980) to diffusion into micropores (Ball and Roberts, 1991; Steinberg *et al.*, 1987). Regardless of the mechanism by which contaminants are sequested in solid matrices, the net result is decreased bioavailability of the contaminant. These mechanisms have been examined from both chemical (Pignatello *et al.*, 1993) and microbiological (Angley *et al.*, 1992; Manilal and Alexander, 1991; Ogram *et al.*, 1985; Scow and Alexander, 1992; Steen *et al.*, 1980) viewpoints. A variety of investigations have concluded that the fraction of model organic compound that is resistant to rapid release (Connaughton *et al.*, 1993; McCall and Agin, 1985) and to microbial attack (Hatzinger and Alexander, 1995) may be inversely proportional to the duration of contact with matrix solids. An understanding of this "aging" process can be approached using quantitative models [either discontinuous (Brusseau and Rao, 1991; Karickhoff, 1980; Karickhoff and Morris, 1985) or continuous (Connaughton *et al.*, 1993)] or simply by hypothesizing that kinetically-governed diffusion and hydrophobic reactions cause the partitioning of organic compounds evermore deeply in the microporous inorganic and organic structure of the matrix materials.

One of the most important implications of chemical aging processes in soil and other natural materials is the possibility that the sequestered toxic chemicals are inaccessible to microbiological attack. Analytical methods for measuring the aged organic chemical pollutants in environmental samples routinely use solvent extraction techniques, while biodegradation activity is routinely determined by following $^{14}CO_2$ production from ^{14}C-labeled compounds freshly added to the environmental samples. A problem arises when comparing the results from the two methods because more accessible freshly added ^{14}C-labeled hydrophobic compounds such as polycyclic aromatic hydrocarbons (PAHs) may behave differently from unlabeled PAHs whose matrix residence times are longer, yet whose presence may be revealed by solvent extraction. As part of an ongoing program to discern the environmental factors and biogeochemical mechanisms governing fate of coal tar-derived organic environmental pollutants at a field study site, we examined how sorption and related reactions influence the susceptibility of phenanthrene to microbial metabolism. In particular, we set out to examine limits of the "aging-bioavailability hypothesis" (Hatzinger and Alexander, 1995; Madsen and Bilotta-Best, 1995) by testing key factors that may affect the results of the ^{14}C-mineralization experiments. A companion paper (Madsen and Bilotta-Best, 1995) focuses on the fate of naphthalene at the same coal tar-contaminated field study site.

MATERIALS AND METHODS

Field site, sediments, and bacteria

Spatial (Fig. 1), hydrogeologic, and chemical characteristics of the field study site have been described in detail elsewhere (Madsen *et al.*, 1991, 1992; Madsen and Bilotta-Best, 1995; Murarka *et al.*, 1992). Sandy, uncontaminated subsurface sediment (organic matter ≈ 1%; Connaughton *et al.*, 1993; Herrick *et al.*, 1993) from near the source area of the field study site (Fig. 1; ~2.0-2.5 m depth) and organic matter-rich surface sediment (organic matter ≈ 13%; Connaughton *et al.*, 1993; Herrick *et al.*, 1993) from the "seep area" (~10-20 cm depth) were aseptically sampled and stored at 4°C. Each sediment was mixed and sieved wet (5 mm) to create homogeneous samples. Sediment subsamples were air dried, sieved again (2 mm), dispensed to 50-ml screw cap glass tubes, and gamma-irradiated (2.5 Mrad from the ^{60}Co source in Ward Laboratory, Cornell University) to generate uniform, sterile material for use in aging experiments. The remainder of the field samples was stored in the original saturated state at 4°C.

Bacteria used as inocula in these studies, *Sphingomonas paucimobilis* RSP1 and an unidentified gram-negative isolate, RSA2, were isolated from field material on a mineral salts-agar based medium (Stanier *et al*, 1966) supplemented with phenanthrene crystals as described (Sandoli, 1994). The bacterial cultures have been further characterized by Sandoli (1994). Inocula were grown for 15 days in shake-flask cultures (23°C, 150 rpm) containing the mineral salts medium and phenanthrene. Cells were harvested by centrifugation and washed twice in 5 mM $CaSO_4$ as described by Sandoli (1994).

Aging-bioavailability and mineralization experiments

The generalized scheme for the aging-bioavailability experiments is depicted in Fig. 2. Twenty four ml of a phenanthrene solution containing equal volumes of sterile ^{14}C-phenanthrene-saturated deionized water (13.1 mCi/mmole, >98% radiopurity, Sigma Chemical Co., 1.7 μCi total added radioactivity), and sterile, unlabeled phenanthrene-saturated 5mM $CaSO_4$ were combined with 24 g of dried, sieved, gamma-irradiated sediments in 60-ml glass ampules (Wheaton, Millville, NJ). The ampules were flame-sealed, tumbled horizontally at low speed for 1-3 hours to promote even distribution of the phenanthrene in the saturated slurries, and stored ("aged") vertically in the dark at 23°C for various time periods. Dates for preparing the slurries were selected so that all of the ampules containing aged material could be opened on the same day (Day 0) to initiate the mineralization assays.

On Day 0, ampules were snapped at the neck and the aqueous phase above the settled sediment material was withdrawn, its volume noted, and a portion of it was counted in a Beckman model LS 5000CE scintillation counter (Beckman Instruments Inc., Fullerton, CA). The bodies of the ampules were then opened by etching the glass with a file above the sediment level, placing a drop of water on the etched glass, and touching a heated glass rod to the wetted etch, thereby cracking the glass.

The sediments in the ampules were processed as follows. (i) Precisely measured but variable weights (approximately 4 g) of sediment were dispensed to tared aluminum pans for wet and dry weight analyses to determine their water content. (ii) The remainder of the saturated sediments were aseptically dispensed to replicate 25-ml mineralization vials (Pierce,

Rockford, IL) which received various inocula. (iii) Poison controls received 0.5-1.0 ml of 0.4M $HgCl_2$ solution made in 5% HCl. (iv) Small $^{14}CO_2$ trap vials (shell vials; Kimble, Vineland, NJ) containing 0.4 ml of 0.5M NaOH were placed inside 40-ml screw cap glass vials (Pierce), which were then sealed with a Teflon-faced silicone septum. (v) Mineralization ($^{14}CO_2$ production) was monitored periodically by using a 22-gauge needle fitted onto a 1-ml syringe to withdraw the $^{14}CO_2$ trapping solution (0.5 m NaOH) and to transfer it to scintillation cocktail for counting. The trapping solution was immediately replaced with fresh trapping solution after each sampling. The cumulative mineralization values reported in the graphs were corrected for ^{14}C measured in poison controls, which was approximately 50 dpm for each sampling of each control. (vi) The radioactivity contained in each of the mineralization vials was determined (based on knowledge of radioactivity originally added to each ampule and of that removed from the aqueous phase of each ampule) in order to calculate the percentages of ^{14}C-phenanthrene converted to $^{14}CO_2$ (mineralized).

Tests evaluating alteration of organic matter-rich sediments by γ-irradiation

To examine the possibility of toxin formation by gamma irradiation, glucose metabolism was examined by adding both unlabeled (10 ppm) and radiolabeled glucose (^{14}C-glucose[U-^{14}C]; 99% radiopurity, 279 mCi/mmol, ICN Irvine, CA; .05 μCi added per vial) and site-derived inocula to 5-g portions of gamma-irradiated seep sediment in triplicate. To examine the possibility that alternative carbon sources, perhaps byproducts of the γ-irradiation treatment, may have been present in the seep sediment material and been metabolized in preference to the exogenously added phenanthrene, the influence of a glucose supplement (a well established preferentially metabolized carbon source) on the mineralization of ^{14}C-phenanthrene by microorganisms in the seep material was investigated. Procedures involved combining fresh and/or gamma-irradiated seep sediments in mineralization vials followed by addition of a washed suspension of a site-derived phenanthrene-degrading bacteria, *Pseudomonas paucimobilis* strain RSP1 or RSA2 (Sandoli, 1994) grown on a mineral salts medium (Stanier *et al.*, 1968) supplemented with sterile phenanthrene crystals.

Sorption-isotherms

Procedures for measuring sorption isotherms for the seep sediments both before and after sterilization by gamma irradiation were those of Connaughton *et al.* (1993). Quadruplicate glass ampules were amended with varying masses of each sediment and a constant mass of ^{14}C phenanthrene. The ampules were sealed and equilibrated for 4 days prior to centrifugation and scintillation-counting of ^{14}C phenanthrene remaining unsorbed. Standard isotherm plots of the mass of phenanthrene sorbed versus the mass of sorbent in each ampule allowed the distribution coefficient, K_d, to be calculated.

RESULTS

Bioavailability of phenanthrene after aging with sand sediments

Mineralization of phenanthrene after aseptic storage with sand sediments is shown in Figs. 3 and 4. The inocula added in experiments which produced data shown in Fig. 3

consisted of an undefined mixed microbial suspension from enrichment cultures of coal tar waste-contaminated seep (Fig. 3A) or sand (Fig. 3B) sediments from the site. The inocula were from a two-day enrichment culture composed of each sediment diluted into minimal media supplemented with phenanthrene and then supplemented with a glucose-based medium (Balkwill and Ghiorse, 1984), to boost cell numbers overnight. This enrichment was designed to provide washed cells representing enhanced phenanthrene-degrading populations from the indigenous microbial communities.

Panel A of Figure 3 shows that the inverse relationship between mineralization and aging time which is predicted by the aging-bioavailability hypothesis was consistent when inoculated with the enrichment culture of seep material. Mineralization in the 20-day aged treatments reached an average of only 34% at the end of the sampling period, in contrast to 44% measured in the 0-day treatments. The extent of mineralization in the treatments aged 1, 5, and 11 days, although very similar to each other, fell between the values for the 20- and 0-day treatments. Unlike the results shown in Panel A of Fig. 3, mineralization of ^{14}C-phenanthrene by the enrichment culture of sand material was not strongly affected by aging (Fig. 3B). Despite the longest-aged (20 day) sediment showing the smallest percent of mineralization, the other aging times did not display a consistent decreasing rate or extent of mineralization with increasing aging time.

Because the means of inoculum preparation had been influential in related studies examining microbial metabolism of aged naphthalene from the same site sediments (Madsen and Bilotta-Best, 1995), we implemented experiments analogous to those whose data are shown in Fig. 3, but varied the means of inoculum preparation. Fig. 4 shows results of phenanthrene mineralization assays inoculated with unenriched sand sediment from the seep area of the study site (Fig. 4A); a mixture of the phenanthrene-mineralizing strains, RSA2 and *Sphingomonas paucimobilis* RSP1 (Panel B) and unenriched sand sediment from the study site (Panel C). Mineralization patterns in Fig. 4 show that aging of ^{14}C-phenanthrene did not consistently affect mineralization by the various inocula. Aging did not have a significant impact on mineralization by the sand inoculum (Panel C). Vials containing aged and unaged ^{14}C-phenanthrene-sand sediment mixtures achieved the same extent of mineralization (~52%) at similar rates. However, aging reduced mineralization by the seep inoculum (Panel A; unaged, 50%; aged, 34%), and by the RSP1/RSA2 mixed culture inoculum (Panel B; unaged, 34%; aged, 25%). A significant lag (one to two days) in mineralization was observed with the pure culture inocula, probably because the cells were added in low numbers. (approximately 10^4 cells per mineralization vial). For uncertain reasons, the extent of mineralization by the pure culture inocula was generally less than that shown by the sediment inocula.

Bioavailability of phenanthrene aged with organic matter-rich seep sediments

Mineralization assays analogous to those shown in Figs. 3 and 4 were also conducted using mixtures of ^{14}C-phenanthrene aged in the presence of gamma-irradiated seep sediment. Invariably these assays revealed a complete elimination of mineralization activity. Regardless of inoculum, no $^{14}CO_2$ was evolved from ^{14}C-phenanthrene that had been aged with the gamma ray-sterilized organic matter-rich seep sediment. This absence of mineralization conflicted with our previous data (unpublished) showing rapid $^{14}CO_2$ evolution from ^{14}C-phenanthrene added

to seep sediment that was not previously sterilized. Thus, γ-irradiation sterilization artifacts were clearly introduced. Several experiments were conducted to investigate the nature of these artifacts.

The absence of $^{14}CO_2$ evolution from phenanthrene aged with γ -irradiated sediment suggested that sorption reactions characteristic of high organic carbon sorbents may have completely inhibited phenanthrene metabolism. Alternatively, the lack of phenanthrene metabolism may be explained by the possibility that seep material contained toxic byproducts of the gamma-irradiation or that other carbon sources (perhaps produced in the sediment using gamma-irradiation), served to repress expression of phenanthrene catabolic genes. This possibility could be termed a diauxic effect.

Fig. 5 shows the results of the experiments conducted to discriminate between the three possible explanations for the influence of gamma ray sterilization on the seep sediment. Panel A shows that strain RSP1 rapidly converted 37% of the amended ^{14}C-glucose to $^{14}CO_2$ in the presence of the gamma-irradiated seep. Clearly then, the gamma-irradiated seep material itself was not toxic to glucose metabolism by RSP1. The gamma ray-induced formation of toxic substances was therefore an unlikely explanation for the elimination of phenanthrene metabolism in the aged sterile sediments. Panel B of Fig. 5 shows that mineralization of ^{14}C-phenanthrene by microorganisms indigenous to the untreated seep material progressed at comparable rates and to similar extents (45-55%) both in the presence and absence of 10 ppm glucose, a compound that is well known to cause catabolite repression. This suggests that the mixed microbial community was not sensitive to catabolite repression and, therefore, that it is unlikely that alternative carbon sources produced in the seep material by gamma-irradiation was the cause of inhibited phenanthrene mineralization in the aged sterile sediments.

Panel C of Fig. 5 shows mineralization of ^{14}C-phenanthrene from fresh seep sediment, γ-irradiated sediment, and a mixture of the two. It is clear that the gamma-irradiated seep material protected the ^{14}C-phenanthrene from mineralization by a sediment-derived cell suspension and RSP1 cells, yet this bacterium was metabolically active in a pattern resembling a sediment-derived inoculum when the sorbent was sand rather than seep sediment (Figs. 4A, B). In addition, Panel C shows that 47% mineralization was achieved when the ^{14}C-phenanthrene was added to the untreated seep sediment, thereby confirming the capacity of indigenous microorganisms in the untreated seep material to catabolize phenanthrene. Interestingly, Panel C also shows that both the rate and extent of mineralization in the untreated seep material were significantly reduced (only 5% conversion to $^{14}CO_2$ after 9 days), despite supplemental inoculation of this material with strain RSP1, when the gamma-irradiated sediment was also present. The most likely explanation for the inhibition of mineralization is that the small amount (1:6 volume, dry weight) of gamma-irradiated seep sediment severely reduced phenanthrene bioavailability. Gamma-irradiated seep was added to these vials to ensure that sediment:liquid ratios [i.e., the degree of saturation] were consistent in all of the vials in the experiment so as not to alter the equilibrium concentrations of phenanthrene in each type of sediment. Thus, in light of the fact that the formation of γ ray-induced toxins and preferentially

metabolized substrates were unlikely explanations (Panels A and B of Fig. 5), the results shown in Fig. 5C suggest that gamma-irradiation may have altered properties of seep sediment for sorbing phenanthrene in a manner which resulted in diminished phenanthrene bioavailability.

Results of pre- and post-gamma irradiation isotherm determination for seep sediment revealed that gamma irradiation caused a 32% increase in the affinity of phenanthrene binding, as indicated by K_d. The gamma irradiated seep sediment exhibited a K_d of 1389 ml/g (r^2 value of 0.89), while prior to irradiation, the value of K_d was 1056 ml/g ($r^2 = 0.79$).

DISCUSSION

Sorption and/or other time-related diffusion reactions were investigated as a means for explaining the persistence of phenanthrene in sediments taken from our coal tar waste-contaminated field study site. Experiments were conducted to test the hypothesis that the aging of phenanthrene, a compound chosen to represent the PAH class of compounds, in sediments may allow sequestration reactions to reduce the bioavailability of this compound. A corollary of this hypothesis is that the fraction of sequestered, hence unavailable, phenanthrene should be proportional to the duration of this aging period. The aging-bioavailability experiments described here showed that mineralization (bioavailability) of ^{14}C-phenanthrene was reduced in aged mixtures of ^{14}C-phenanthrene with sand sediment inoculated with pure and mixed microbial cultures derived from sediments originating in the seep area of the study site (Figs. 3A and 4A, B). However, when a mixed microbial inoculum from the sand sediment of the study site was used to assess phenanthrene bioavailability, aging had no (Fig. 4C) or little (Fig. 3B) effect. These observations fall midway between those of Guerin and Boyd (1992), who concluded that different microbial populations may respond differently to sorbed substrates, and those of Hatzinger and Alexander (1995), whose data on extraction and mineralization of aged compounds in soil showed a strict relationship between aging time and sorbate bioavailability. The principal finding of the present study is that the aging-bioavailability hypothesis holds true for some combinations of sorbent, sorbate, and microbial inocula, but not others.

The design of the aging-bioavailability experiments described here required the elimination of biological activity in the sediment materials which were used. A number of methods have been routinely employed to eliminate biological activity or to completely sterilize soils and sediments (Brock, 1978). These methods include autoclaving and exposure to methyl bromide, sodium azide, ethylene or propylene oxides, and gamma irradiation. The use of any sterilization method will alter the chemical and physical properties of a soil or sediment; this is an inescapable fact. Gamma-irradiation is one of the least destructive sterilization methods, and consequently, is thought to impose the fewest artifacts on soils and sediments used in laboratory experiments (Wolf and Skipper, 1994). However, a variety of investigators have demonstrated that gamma-irradiation may considerably alter the physical and chemical properties of soils (Eno and Popenoe, 1964; Salonius *et al.*, 1967, Wolf *et al.*, 1989). As was clear from results described here in experiments using organic matter-rich seep sediments, the effects of gamma irradiation can severely alter the outcome of biodegradation experiments.

Investigators using γ-ray sterilization procedures need to be aware of potentially misleading artifacts. We recommend an initial screening of both chemical and microbiological properties of gamma irradiated sediments prior to their extensive use in sorption and/or biodegradation studies. It is interesting to note, however, that the specificity of artifacts may be great. For instance, aging-bioavailability procedures identical to those described here were also carried out using naphthalene (rather than phenanthrene) as sorbate (Madsen and Bilotta-Best, 1995), with no apparent adverse consequences. The gamma ray-induced sorption artifacts described here add to a growing literature (Dalton *et al.*, 1989; Dao *et al.*, 1988; Wolf *et al.*, 1989) that have demonstrated how sterilization procedures can influence sorptive properties of soil.

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FIGURE LEGENDS

Figure 1. Plan view of contaminated study area showing locations where sandy and organic matter-rich sediments were collected.

Figure 2. Schematic diagram of the procedures used to age phenanthrene with sediments and later implement the mineralization assays.

Figure 3. Mineralization of ^{14}C-phenanthrene after ^{14}C-phenanthrene sand sediment mixtures that had been aged from 0 to 20 days and inoculated with microorganisms from aqueous-phase phenanthrene enriched seep (A) and sand (B) sediment material from the field site. Each point is an average of triplicate determinations. Error bars represent standard deviations.

Figure 4. Mineralization of ^{14}C-phenanthrene from aged (28 day) and unaged ^{14}C-phenanthrene sand sediment mixtures after inoculation with unenriched field-saturated seep (A) and (C) sand sediments from the field site. The inoculum used to generate data in panel B was a mixed culture of strains *Sphingomonas paucimobilis* RSP1 and RSA2. Each point is an average of triplicate determinations. Error bars represent standard deviations.

Figure 5. Mineralization of ^{14}C-glucose by bacterium *S. paucimobilis* RSP1 in the presence of gamma-irradiated seep material (A); of ^{14}C-phenanthrene in nonsterile seep sediment with or without a 10 ppm glucose supplement (B); of ^{14}C-phenanthrene in the presence of nonsterile seep sediment, in gamma-irradiated seep inoculated with both a seep-derived cell suspension and bacterium *S. paucimobilis* RSP1, and in a mixture (1:6) of γ-irradiated and untreated seep sediment inoculated with *S. paucimobilis* RSP1 (C). Each point is an average of triplicate determinations. Error bars represent standard deviations.

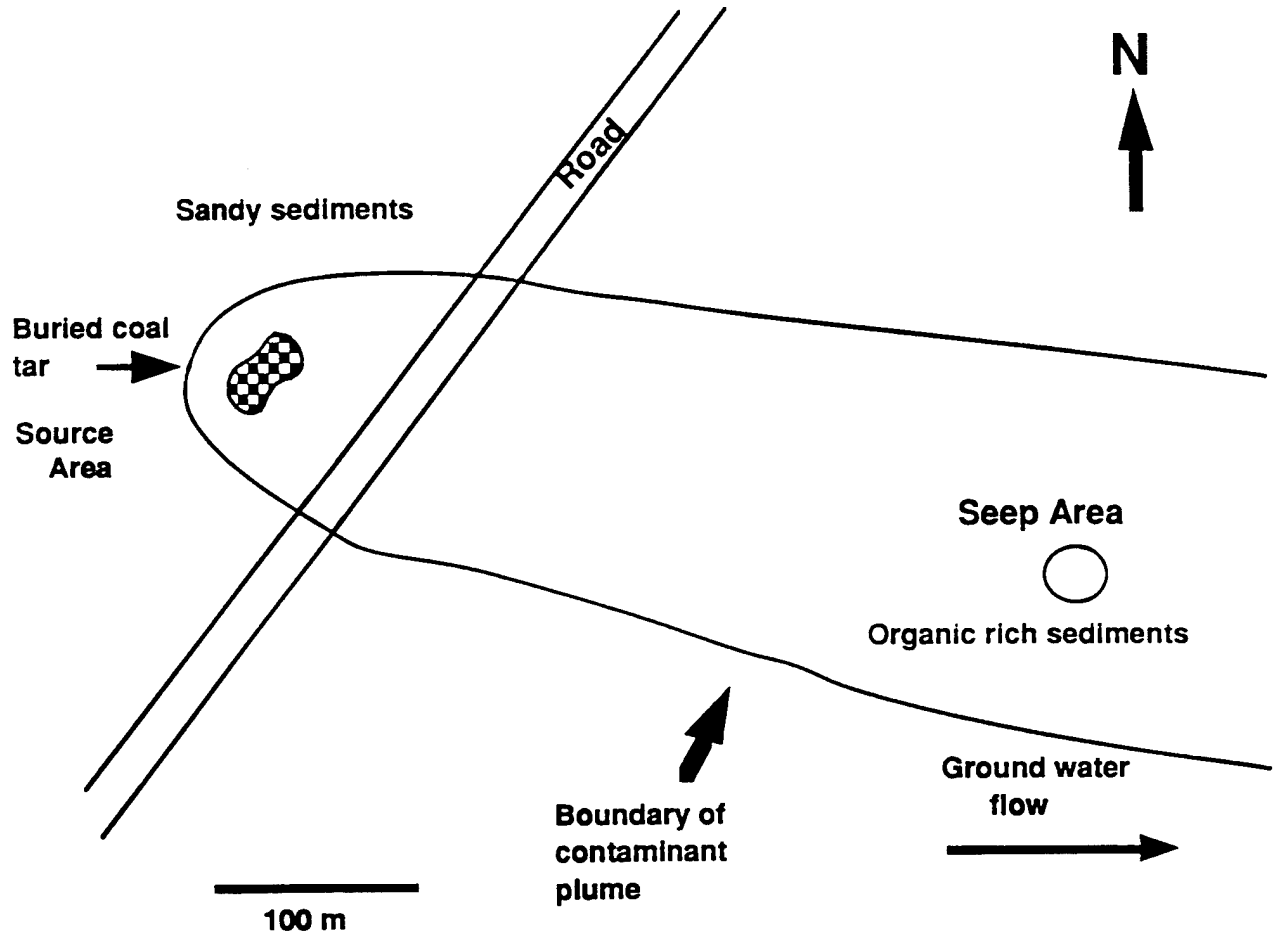

N
Road
Sandy sediments
Buried coal tar
Source Area
Seep Area
Organic rich sediments
Boundary of contaminant plume
Ground water flow
100 m

Fig 1

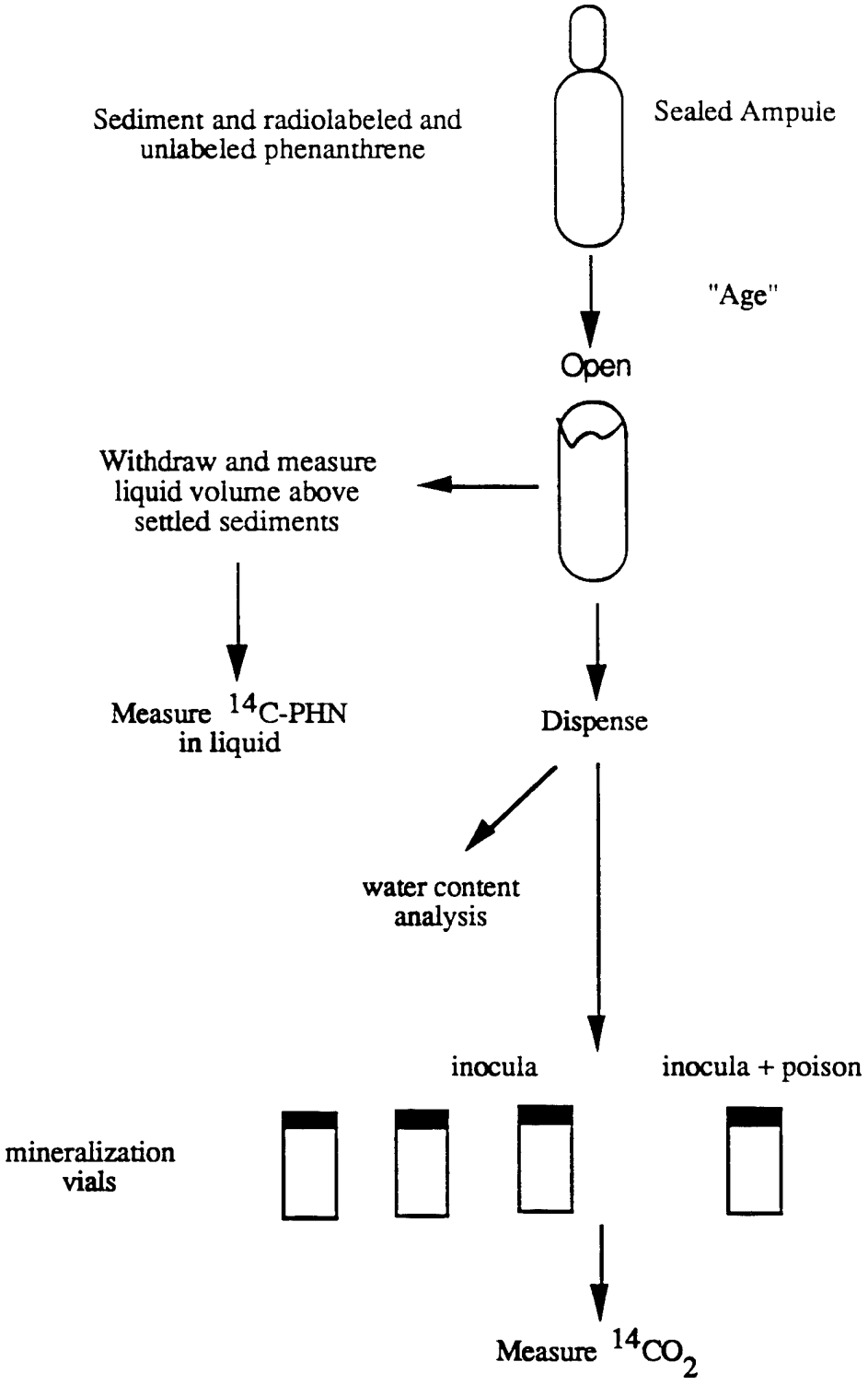
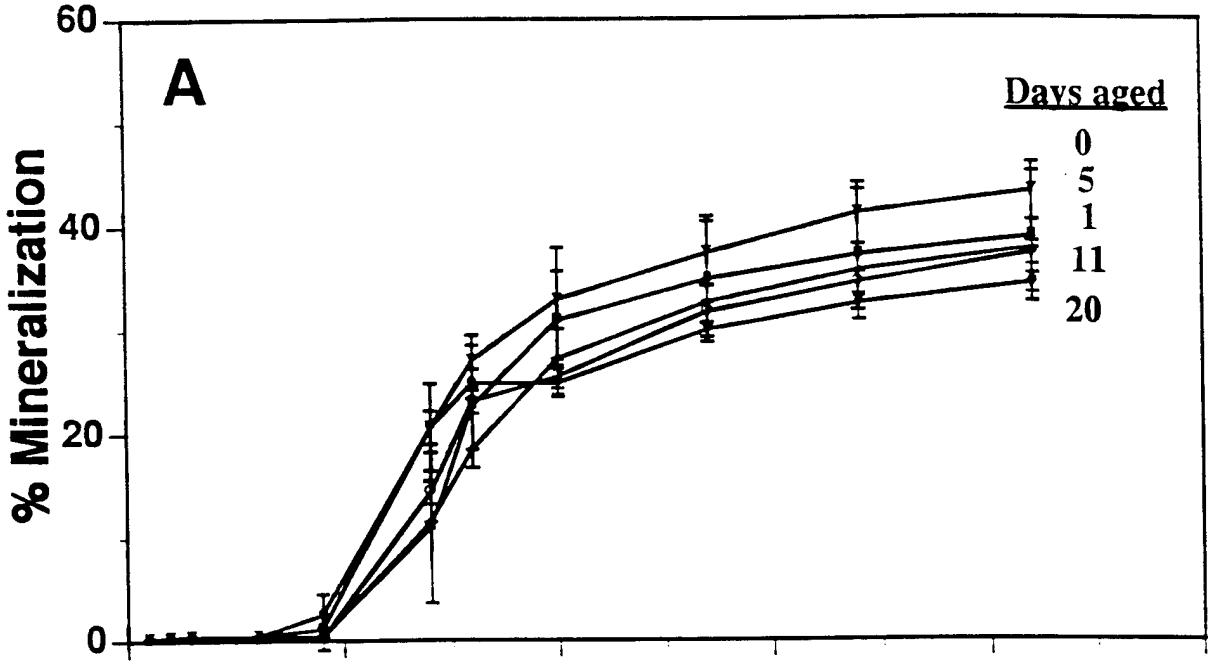

Sediment and radiolabeled and unlabeled phenanthrene
Sealed Ampule
"Age"
Open
Withdraw and measure liquid volume above settled sediments
Measure ^{14}C-PHN in liquid
Dispense
water content analysis
inocula
inocula + poison
mineralization vials
Measure $^{14}CO_2$

Fig. 2


A
Days aged
0
5
1
11
20
% Mineralization
60
40
20
0

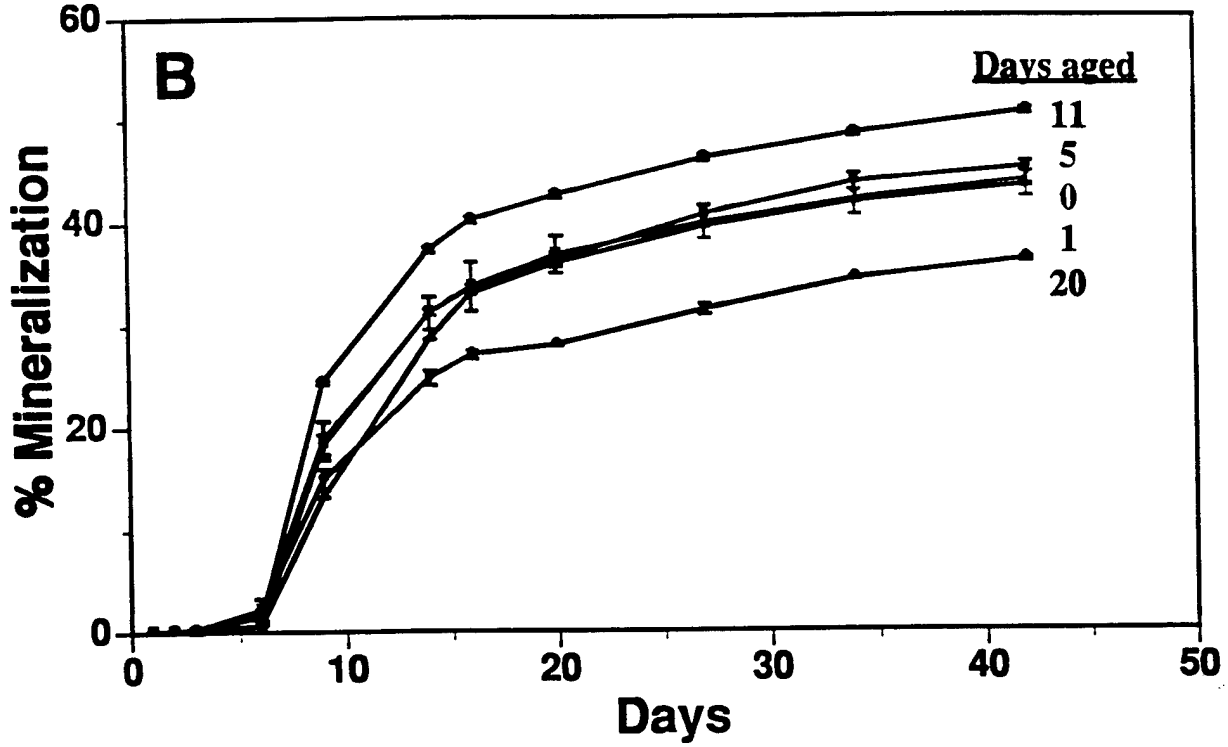

B
Days aged
11
5
0
1
20
% Mineralization
60
40
20
0
0
10
20
30
40
50
Days

Fig 3

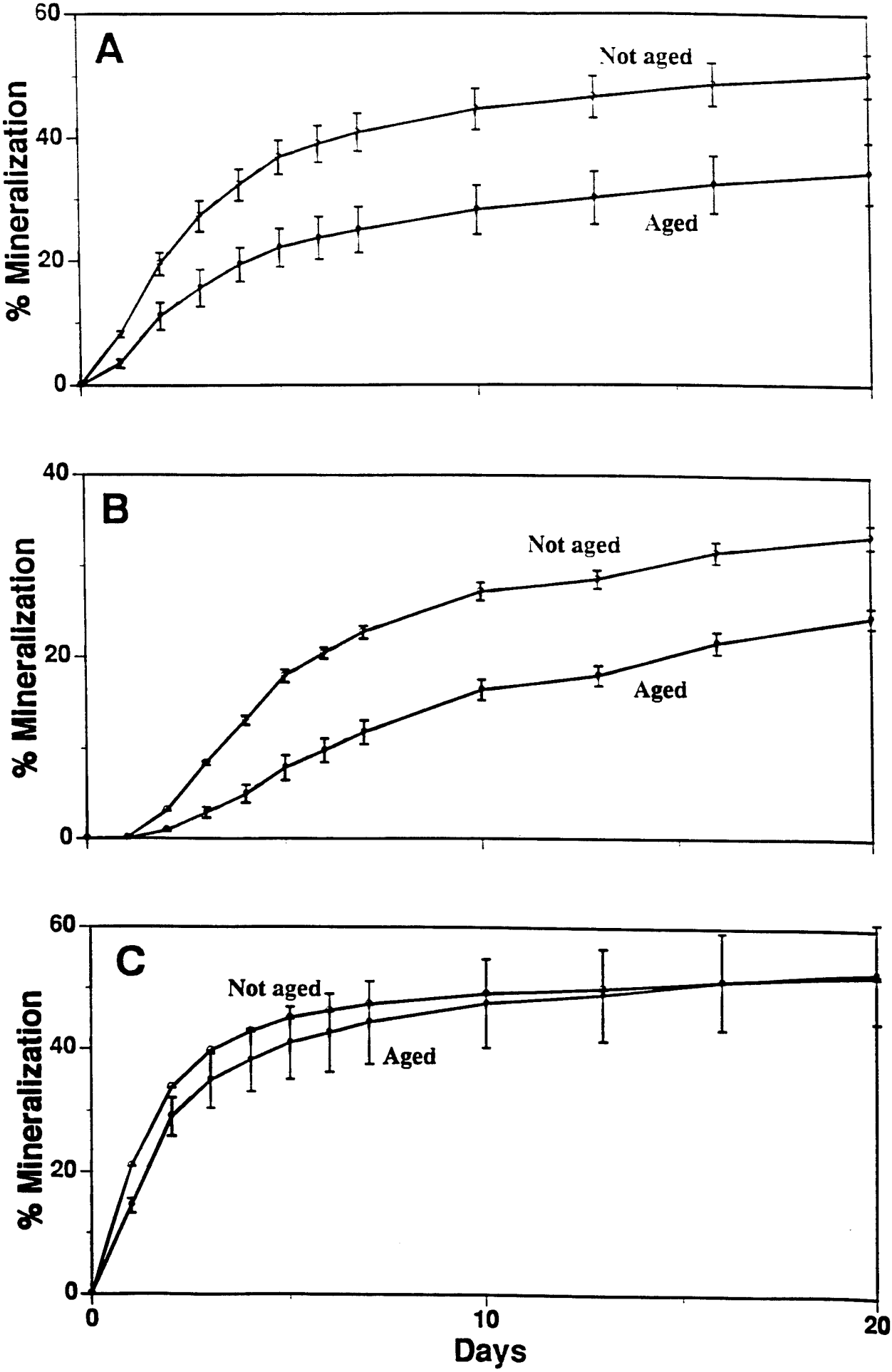

A
Not aged
Aged
% Mineralization
60
40
20
0
B
Not aged
Aged
% Mineralization
40
20
0
C
Not aged
Aged
% Mineralization
60
40
20
0
0
10
20
Days

Fig 4

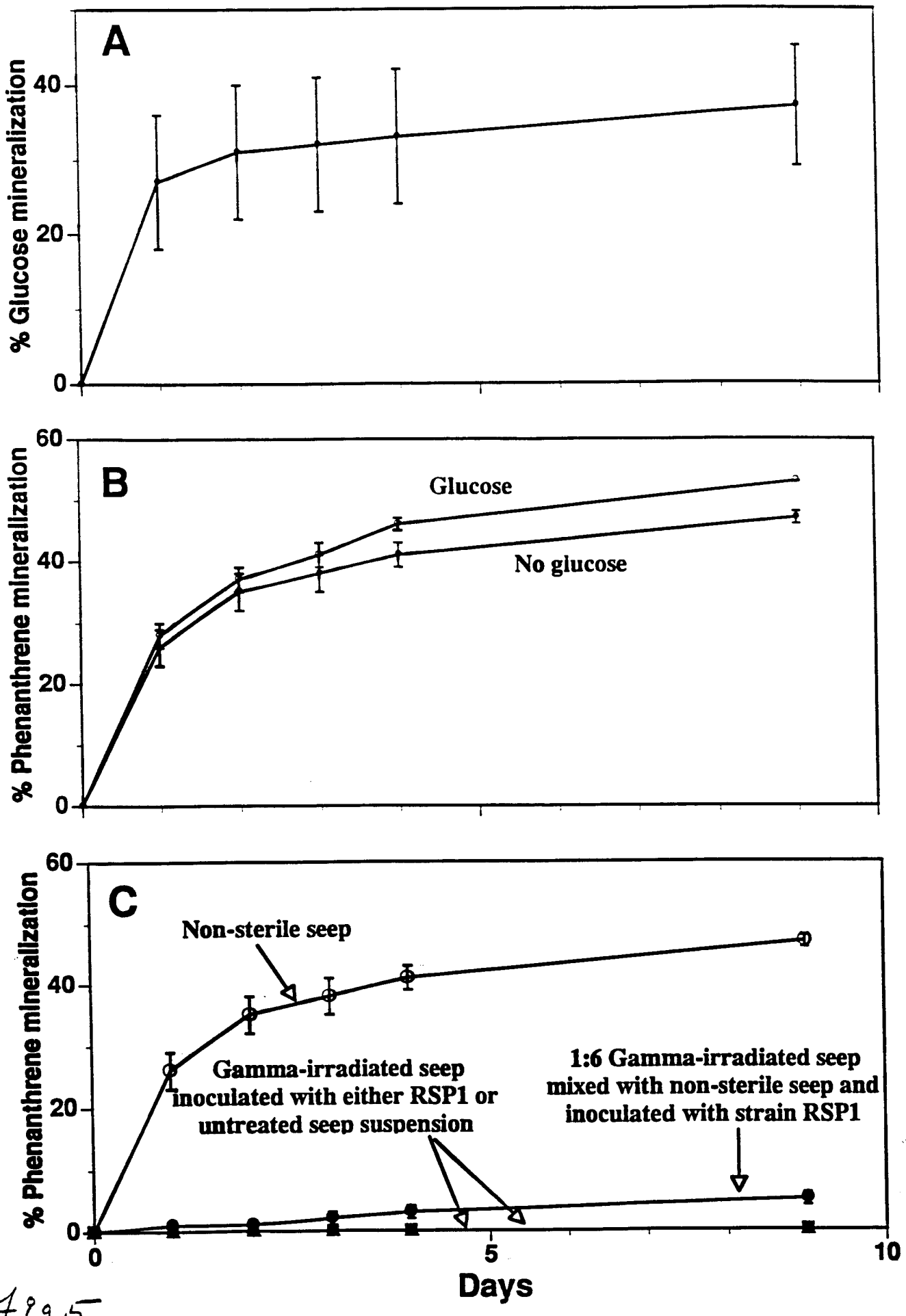

A
% Glucose mineralization
0
20
40
B
% Phenanthrene mineralization
0
20
40
60
Glucose
No glucose
C
% Phenanthrene mineralization
0
20
40
60
Non-sterile seep
Gamma-irradiated seep inoculated with either RSP1 or untreated seep suspension
1:6 Gamma-irradiated seep mixed with non-sterile seep and inoculated with strain RSP1
0
5
10
Days

Fig 5

APPENDIX 4.3

FIELD EXTRACTION OF A TRANSIENT INTERMEDIARY METABOLITE INDICATIVE OF REAL TIME *IN SITU* NAPHTHALENE BIODEGRADATION

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Submitted to Environmental Science and Technology

Key Words: microbial metabolism, dihydrodiol, bioremediation, dioxygenase, mass spectrometry, coal tar.

Abstract

Metabolic activity of naturally occurring microbial communities in soils, sediments, and waters often determines the fate of organic environmental contaminants. Yet this activity has traditionally resisted direct documentation. The metabolic pathway for bacterial naphthalene oxidation was used as a guide for selecting 1,2-dihydroxy-1,2,-dihydronaphthalene (1,2-DHDN) as a unique transient intermediary metabolite whose presence in samples from a contaminated field site would indicate real time *in situ* naphthalene biodegradation. When surface waters emerging from the site were processed in a manner designed to avoid sample-handling artifacts, the 1,2-DHDN intermediate was successfully concentrated, extracted, and identified by gas chromatography/mass spectrometry. This is an example of how knowledge and techniques of biochemistry, microbial physiology, and analytical chemistry can be focused toward measuring transformations catalyzed *in situ* by naturally occurring microbial communities in real time.

Introduction

Of the many biogeochemical processes catalyzed by microorganisms in field sites, one of particular relevance to contemporary society is the biodegradation of environmental contaminants (*1, 2*). Because biodegradation processes have the potential to eliminate the toxicity of organic contaminants where they reside in soil, sediments, and water, it can be crucial to know if and when the reactions are actually in progress in real time. In this regard, the methodological limitations of environmental microbiology have major practical implications for safeguarding human health and environmental quality. Within the last several years significant conceptual and technological improvements in environmental microbiology have been made which have advanced our understanding of how to demonstrate microbial biodegradation activity in field sites (*3-6*). Detection of unique intermediary metabolites in site-derived samples is perhaps the most elegant of the variety of criteria that have recently become accepted as evidence for the occurrence of *in situ* contaminant biodegradation.

The conclusions that can be drawn from the detection of intermediary metabolites in field samples are governed by a combination of both our understanding of the biochemistry of the metabolic process and the degree to which sample-handling methods have precluded artifactual results. For instance, some products of microbial metabolic transformations are not themselves subject to extensive enzymatic attack. The presence of these stable compounds in field samples serves as evidence that the parent compound has been altered *in situ*, but at uncertain times and locations in the past (*7-10*). Other intermediary metabolites are so unstable and fleeting, both chemically and physiologically, that their detection is best explained by the metabolic process having been actively in progress *in situ* at the time and place of sample removal from the field site. But, given the propensity for microorganisms to respond, sometimes instantaneously, to environmental changes implicit in field-site sample removal (*11, 12*), the utmost care must be taken in preventing metabolic change in the microbial community during the interim between sample removal and metabolite analysis. Thus, studies that use unstable intermediary metabolites to directly and unequivocally document *in situ* real time biodegradation of environmental contaminants are rare (*13, 14*). The objective of this study was to use the metabolic pathway for bacterial naphthalene oxidation as a guide for selecting 1,2-dihydroxy-1,2,-dihydronaphthalene

(1,2-DHDN) as a unique transient intermediary metabolite whose presence in samples from a contaminated field site would indicate real time *in situ* naphthalene biodegradation. Naphthalene is a component of a variety of pollutant mixtures, being the major constituent of coal tar (*15*) and a predominant constituent of the fraction of crude oil used to produce diesel and jet fuels (*16*).

Materials and Methods

Biosynthesis of 1,2-dihydroxy-1,2-dihydronaphthalene (1,2-DHDN). Authentic 1,2-DHDN was prepared using a protocol (*17*) and a bacterium, *Escherichia coli* JM109(pDTG601), provided by G. J. Zylstra of Rutgers University. This bacterium contains the cloned toluene dioxygenase genes from *Pseudomonas putida* F1 under the control of the *lac* promoter (*18*). An overnight culture of the strain was diluted 1:50 into 250 mls MSB medium containing 20 mM glucose, 1 mM thiamine, and 100 µg ampicillin/ml, and shaken at 37°C at 250 rpm. When the OD at 600 nm reached 0.5, Isopropyl-1-thio-ß-D-galactoside was added to 1 mM and shaking continued for 1 hr. Cells were harvested at 5000 g, resuspended in 125 mls of 50 mM KPO_4 buffer, pH 7.25, with 20 mM glucose. One-hundred mg of naphthalene in N,N-dimethylformamide (DMF) was added to the suspension which was shaken at 250 rpm at 30°C overnight. The suspension was extracted with two equal volumes of ethyl acetate, dried with anhydrous sodium sulfate and evaporated to dryness under nitrogen. One-hundred µg of the resulting powder was dissolved in 1 ml ethyl acetate and derivatized with either N-methylbis(trifluoroacetamide) (BSTFA) or 1-butane boronic acid. The trimethylsilyl derivative produced a spectrum identical with that prepared from an authentic standard provided by C. E. Cerniglia (National Center for Toxicological Research, Jefferson, AR); and the 1-butane boronic acid derivative produced a spectrum identical to one provided by Drs. S. Resnick, D. T. Gibson, and J. D. Haddock (University of Iowa).

Production of 1,2-DHDN by site-derived enrichment cultures. Contaminated sediment was enriched for naphthalene degrading bacteria using 25g of the sediment in 50 mls sterile mineral salts broth (MSB) (*19*) to which naphthalene crystals were added aseptically. After shaking at 22°C for 3 days, the bacteria in these enrichments were then separated from sediment solids (*20*), washed twice in 50 mM phosphate buffer to remove metabolites present, and resuspended in a volume equal to that of the original sediment. Twenty five µl of 100 mM naphthalene in DMF was then added to 5 mls of the cell suspensions and metabolism allowed to proceed for 5 min. Metabolites were extracted with 10 mls of ethyl acetate, dried under nitrogen to 100 µl, derivatized with 10 µl of a solution of 10 mg 1-butane boronic acid/ ml of ethyl acetate, and analyzed by GC/MS.

Extraction and analysis of 1,2-DHDN from site waters. Two liter water samples were collected at the site by bailing surface water using a sterile glass beaker and immediately amended with sodium azide (1 mg/ml) in order to inhibit bacterial respiration. Samples were put on ice at the sampling location and kept on ice during processing at the field laboratory. Samples were passed through Whatman #1 filters to remove large particles, and aromatic compounds in the sample were concentrated using Supelclean Envi-Chrom P (Supelco, Inc., Bellefonte PA, USA) solid phase extraction (SPE) tubes. Tubes were conditioned with 5 ml ethyl acetate, methanol, and water rinses, and samples were processed at a flow rate of approximately 100 ml/minute. Compounds were eluted drop-wise with 2-5 ml of ethyl acetate which had been

neutralized with 1 M sodium hydroxide. These extracts were derivatized with 25 µl of a solution of 10 mg 1-butaneboronic acid /ml ethyl acetate. The derivatized extracts were stored on ice in glass vials with Teflon-lined caps during transport, a 4-h drive, to the main laboratory. On the same day, extracts were concentrated to 100 µl under a gentle nitrogen stream, and analyzed by GC/MS.

Two types of control experiments were performed. In the first type of control, solutions of distilled water with relevant naphthalene concentrations were processed in a manner identical to the site water samples. These controls were designed to ensure that the detection of 1,2-DHDN was not due to a processing or analysis artifact. In the second type of control, we gave serious consideration to the hypothesis that the 1,2-DHDN was created while water-borne cells and accompanying naphthalene were being processed in the SPE procedure. Though the sodium azide inhibitor should have precluded this occurrence, we examined 1,2-DHDN formation in solid phase extraction tubes using a known naphthalene-metabolizing bacterium, *Pseudomonas putida* G7, added at a cell density which matched that of the entire waterborne microbial community [$7 \cdot 10^4$ cells/ml as determined by epifluorescence microscopy (*21*)]. P. *putida* PpG7 cells were grown to mid-log phase on the complex medium 5% PTYG (*21*), and diluted into a mineral salts buffer to the desired cell density. Four liters of the cell suspension were placed on ice, amended with 1 g sodium azide and 10 µg naphthalene per liter, and processed in a manner similar to the site-derived water samples.

Gas Chromatography/Mass Spectrometry analyses. Nine-tenths µl of sample was injected into a Hewlett-Packard Model 5890 Series II gas chromatograph equipped with a 30 m x 0.25 mm x 0.25 µm film thickness, HP-5 (5% phenyl methyl silicone; Hewlett-Packard) fused silica capillary column connected to a Hewlett-Packard Model 5971A quadrupole mass selective detector operated at an electron energy of 70 eV and a detector voltage of 2000 to 3000. A splitless injection was used, with a 1-min delay before septum purge. Helium at a linear gas velocity of 30 cm/s was the carrier gas. The injector and detector temperatures were 250° and 300°C, respectively. The ion source pressure was maintained at 1.0×10^{-5} Torr. The GC temperature profile was 40°C for 1 min, 10°C/min to 250°C.

Results and Discussion

Choice of metabolite. The initial enzymatic attack on naphthalene by all bacteria examined to date occurs via an aromatic ring dioxygenase which inserts the two atoms of an oxygen molecule to form *cis*-1,2-DHDN (*22*) (Fig. 1). This dihydrodiol then undergoes further intracellular reactions leading eventually to CO_2 and microbial biomass. 1,2-DHDN has several characteristics which make it an attractive choice as an indicator of naphthalene metabolism: it is unique to the metabolism of naphthalene; it is a chemically and biochemically unstable (*22, 23*) compound that is created and consumed intracellularly; and this compound can be stabilized for chemical analysis through the use of boronic acid gas-chromatography derivatizing agents (*24, 25*). Because the biochemical steps of pollutant metabolism are not always universal between microorganisms (*26, 27*), we performed experiments designed to ensure that the pure culture-derived metabolic pathway shown in Fig. 1 was relevant to the sediment microorganisms native to our naphthalene-contaminated study site. Using freshly-gathered sediments, a 3-day enrichment culture was prepared to increase the number of naphthalene degrading

microorganisms present and simultaneously to induce expression of naphthalene catabolic genes. Microbial cells were then separated from the sediments, washed free of accumulated metabolites, and assayed for the production of fresh metabolites from naphthalene. A large peak was present in the chromatograms from these assays which had an identical retention time (Fig. 2A, B) and mass spectrum (Fig. 3A, B) as that of an authentic 1,2-DHDN standard. Thus, naphthalene was metabolized by microorganisms indigenous to the site according to the expected biochemical pathway. Furthermore, absence of the 1,2-DHDN metabolite from washed cell preparations that were immediately extracted and from those that were incubated for 3 days in the absence of added naphthalene made it clear that preconcentration would be required for direct detection of 1,2-DHDN in site-derived samples.

Field extraction of metabolite. We then attempted to use solid-phase extraction and gas chromatography/mass spectrometry (GC/MS) procedures to concentrate, isolate, and detect 1,2-DHDN in the water flowing through sediments at the contaminated study site. A location at the confluence of two slow-flowing streams which drain from the site was chosen for sampling. Here the oxygen content was found to be 4-6 mg/liter, as determined by insertion of an oxygen electrode. To minimize artifacts often associated with sample handling and storage, a field laboratory was established. Steps for avoiding changes in the native aquatic microbial community during sample processing included (i) addition of a respiratory inhibitor, sodium azide (*28*), immediately after gathering the water; (ii) immediately placing the water sample on ice; (iii) minimizing sample disturbance; and (iv) minimizing the period of time that elapsed between sample removal, derivatization of the metabolite, and GC/MS analysis. We were able to complete the sample gathering, extraction, and derivatization portion of the analysis within 30 min; the GC/MS analysis was completed in as few as 6 and never more than 12 hours. The 1,2-DHDN intermediary metabolite was present in chromatograms produced from all water samples collected on three dates in October of 1994 (Fig. 2C) and absent in negative controls. The mass spectrum of the field extracted 1,2-DHDN (Fig. 3C) matched the authentic standard (Fig. 3A). We conclude from this that microorganisms within the microbial community present in site water and adjacent sediments were actively engaged in naphthalene metabolism at the time of sample removal.

Because the absolute purity of our authentic 1,2 DHDN standards was uncertain and because rapid processing of water samples forced departure of the elution rate recommended by the manufacture of the SPE tubes, precise quantification of 1,2-DHDN in site waters was not possible. However, assuming 25% purity of the standard, 50% binding onto the extraction adsorbent, a concentration factor of $2 \cdot 10^4$ (2 liters reduced to 0.1 ml), we estimate ambient levels of 1,2-DHDN to have been approximately 1 ng/ml in site waters. This was a factor of 10^{-3} to 10^{-4} below the ambient concentrations of naphthalene in site water and sediments.

Significance. Successful demonstration and documentation of biodegradation activity is constrained largely by the methodological limitations of environmental microbiology. Direct field documentation of real time *in situ* microbial activity has been pursued by microbial ecologists for decades, but has only been routinely achievable by the subset of these scientists concerned with fluxes of gaseous materials to and from the atmosphere (*29*). The key methodology which allows accurate measurement of gaseous fluxes is the placement of chambers onto soil or water surfaces in field sites, followed by periodic sampling and analysis of

headspace gases for biologically induced changes in analytes such as CH_4, CO_2, O_2, and N_2O (*30-34*). Data produced in this manner are valid because the chambers employed leave the microbial communities of interest and their habitats intact. Few or no artifacts are imposed. However, the microbial metabolic processes which determine the fate of most organic pollutant compounds generally do not involve gaseous intermediary metabolites. Detection of the nongaseous intermediary metabolite reported here required removal of samples from the field, therefore, we adopted procedures designed to prevent the concentration of 1,2-DHDN from changing during the time that elapsed between sample removal and completion of the analysis. Because of its fleeting nature and unique structure, detection of 1,2-DHDN in carefully handled water samples documents real time *in situ* naphthalene metabolism by the microorganisms indigenous to the field study site. The physical, chemical, hydrologic, and microbiological processes which determine the environmental fate of coal tar-derived environmental contaminants have been examined at this study site for several years (*35, 36*). Documentation of real time aerobic biodegradation of naphthalene in waters exiting the site adds to our understanding of how naturally occurring microbiological processes may destroy organic pollutants, thus diminishing their potential adverse effects on downstream receptors.

Acknowledgements

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FIGURE LEGENDS

Figure 1. Early steps in the metabolic pathway for naphthalene (*22*).

Figure 2. Gas chromatograms of 1,2-dihydroxy-1,2-dihydronaphthalene showing resolution and retention time for this compound prepared as an authentic standard (A); extracted from liquid media containing naphthalene and inoculated with a mixture of site-derived microorganisms after 3 days of enrichment culture in the laboratory (B); and concentrated and extracted directly from field site-derived waters (C). Panels A and B are total ion chromatographs in which the mass detector registers masses of all ion fragments. Panel C shows the abundance of the molecular ion (m/z = 228) characteristic of 1,2-DHDN.

Figure 3. Mass spectra of authentic 1,2-dihydroxy-1,2-dihydronaphthalene (A); the metabolite produced by the 3-day enrichment culture inoculated with a mixture of site-derived microorganisms (B); the metabolite concentrated and extracted directly from site-derived waters (C). All spectra were gathered using the total ion scanning mode of the mass spectrometer. The match quality (as determined by the instrument manufacturer's algorithm for spectral comparison) between panels B and A and panels C and A were 99 and 94, respectively.

Figure 1)

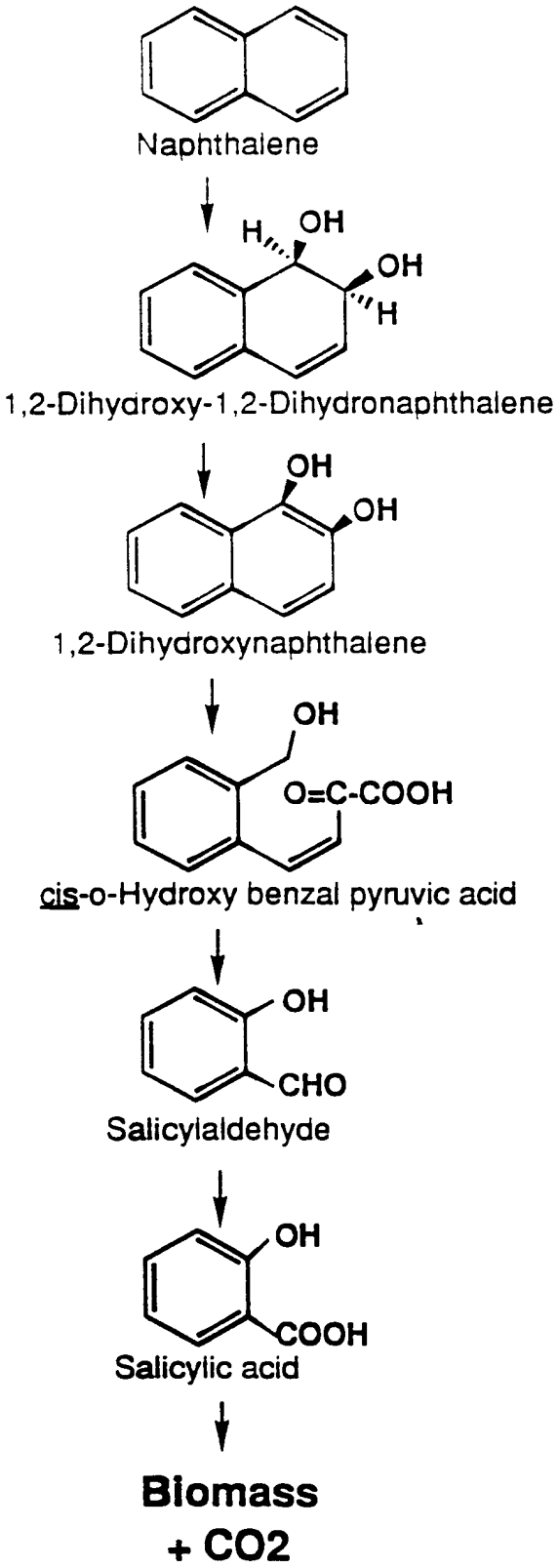
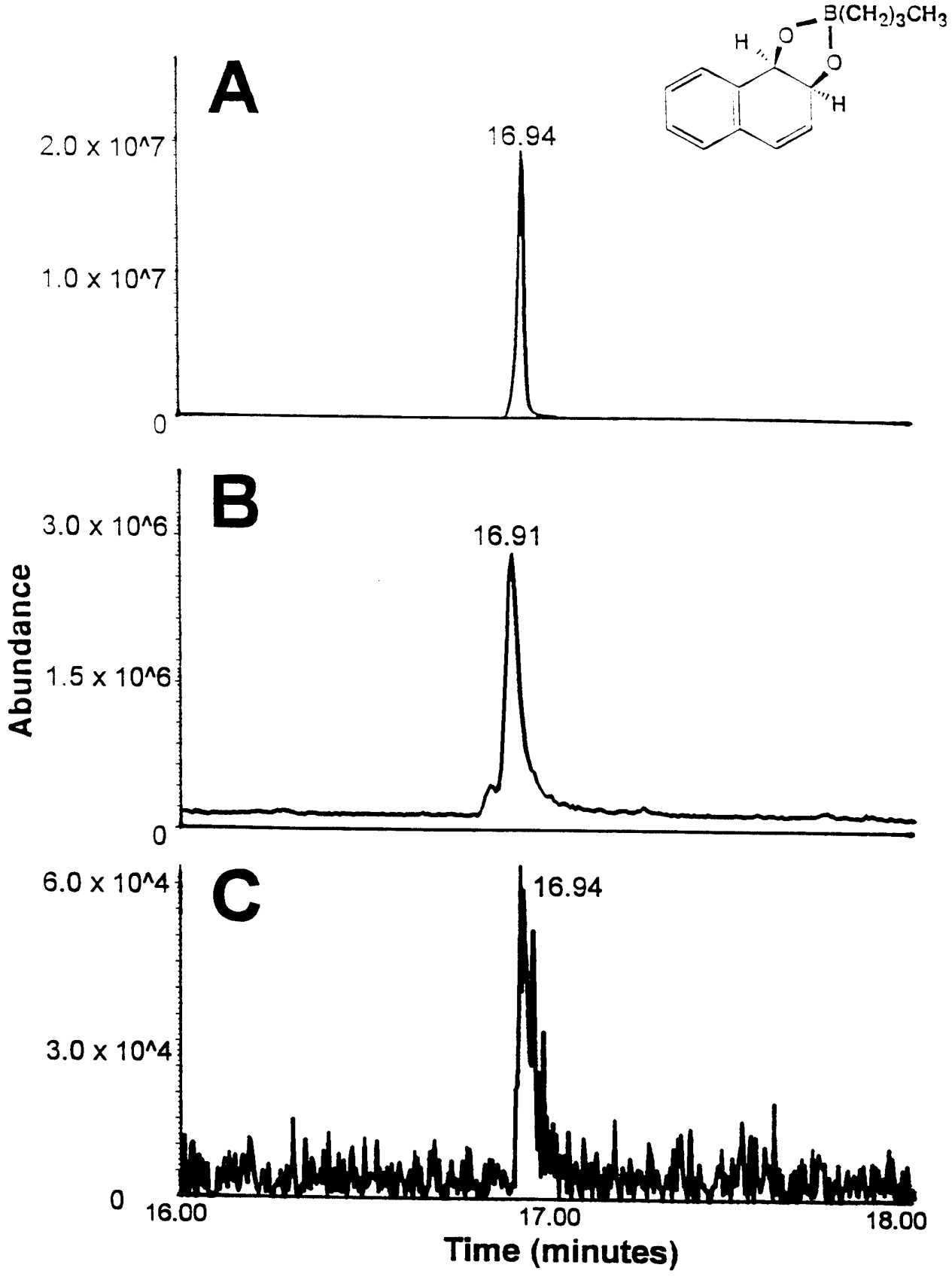
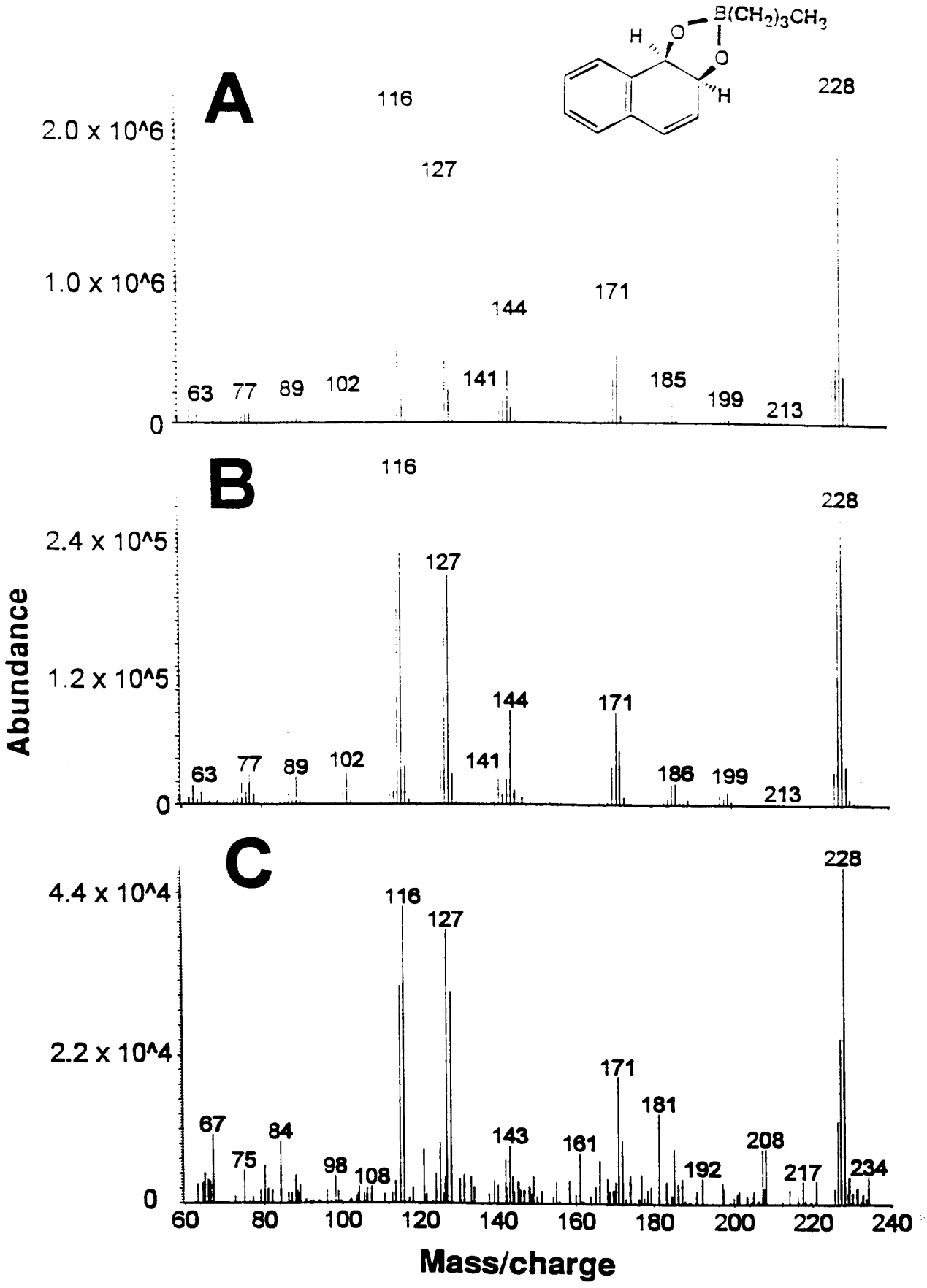

Naphthalene
H
OH
OH
H
1,2-Dihydroxy-1,2-Dihydronaphthalene
OH
OH
1,2-Dihydroxynaphthalene
OH
O=C-COOH
cis-o-Hydroxy benzal pyruvic acid
OH
CHO
Salicylaldehyde
OH
COOH
Salicylic acid
Biomass
+ CO2

Figure 2)


A
2.0 x 10^7
1.0 x 10^7
0
16.94
B(CH2)3CH3
H
O
O
H
B
3.0 x 10^6
1.5 x 10^6
0
16.91
Abundance
C
6.0 x 10^4
3.0 x 10^4
0
16.94
16.00
17.00
18.00
Time (minutes)

(Figure 3)


A
B
C
2.0 x 10^6
1.0 x 10^6
0
2.4 x 10^5
1.2 x 10^5
4.4 x 10^4
2.2 x 10^4
Abundance
116
127
144
171
228
63
77
89
102
141
185
199
213
186
67
84
75
98
108
143
161
181
192
208
217
234
60
80
100
120
140
160
180
200
220
240
Mass/charge
B(CH2)3CH3
O
H

APPENDIX 4.4

PHYLOGENETIC EVIDENCE FOR HORIZONTAL TRANSFER OF THE NAPHTHALENE CATABOLIC GENE *NAH Ac*

(Herrick, J. B. 1995. Ph.D. Thesis. Cornell University, Ithaca, NY)

ABSTRACT

Documenting the exchange of catabolic and other genes among bacteria inhabiting soil, sediment and aquatic habitats is of ecological significance and poses substantial methodological challenges. We here provide evidence of horizontal transfer of the naphthalene dioxygenase iron-sulfur protein gene *nahAc* among populations of naphthalene-degrading bacteria at a coal tar-contaminated site in upstate New York. In a previous study, we described a phenotypically diverse group of Gram-negative naphthalene-mineralizing bacterial strains isolated from a coal tar-contaminated surface seep sediment. These possessed PCR-amplifiable homologs of *nahAc*. In the present study, DNA sequencing was utilized to investigate relationships between these amplifiable *nahAc* genes. It was found that six of seven sequenced seep isolates had an identical *nahAc* allele (allele Cg1) while the seventh (allele Cg2) differed by only one of the more than 380 sequenced nucleotides. These two alleles differed substantially from the *nahAc* allele of the archetypal naphthalene-degrading strain *Pseudomonas putida* G7 (5.1% dissimilarity between Cg1 and G7) and from that of a recently isolated Washington State strain, *P. fluorescens* N1 (6.7% dissimilarity to Cg1). However, the Cg1 allele was very similar to the *nahAc* of type strain *P. putida* NCIB 9816-4 from the British Isles (0.5% dissimilarity) and identical to *nahAc* of *P. fluorescens* Nd9, isolated from the contaminated source area of the study site. Partial 16S rRNA gene sequences were obtained for the naphthalene-degrading strains studied. Those bacteria with the Cg1 *nahAc* allele had 16S rRNA sequences with dissimilarities ranging from 0% to 7.9%, suggesting that many of these isolates are much more distantly related than are their *nahAc* genes. The phylogenies of the two genes were not congruent: the Cg1 *nahAc* allele was distributed throughout the strains represented by the three major branches of the 16S rRNA phylogenetic tree. These data provide strong indirect evidence for the horizontal transfer of *nahAc* among the lineages of the naphthalene-degrading populations represented by our isolates. Genetic transfer of catabolic genes may play an important role in the evolution and adaptation of biodegradative bacterial populations to contaminants.

INTRODUCTION

Horizontal gene transfer -- the transmission of DNA fragments between different organismal lineages -- has been intensively studied in bacteria since the discovery of conjugation by Lederberg and Tatum (Lederberg, 1946). Natural gene transfer in terrestrial and aquatic environments has attracted increasing attention, particularly in the context of the debate over environmental release of genetically engineered microorganisms (Miller, 1989). Horizontal gene transfer is thought to be important in the evolution and genetic diversity of bacterial populations (Saye, 1989; Atlas, 1993) and may have a significant impact as well on natural bacterial communities and ecosystem function (Lorenz, 1993).

It is thought that genetic transfer can play an important role in the acclimation of bacterial communities to pollutants (Leahy, 1990; van der Meer, 1992), a phenomenon in which an increase in the rate of catabolism of a contaminant is observed after previous exposure of the community to the compound (Spain, 1980; Barkay, 1988). It has been reported that bacterial communities previously acclimated to naphthalene or salicylate (an intermediate in the naphthalene pathway) harbor an increased number of nah-related genes relative to unacclimated communities (Ogunseitan, 1991; Sayler, 1985). Since naphthalene catabolic genes have often been found to be located on self-transmissible plasmids (Sayler, 1990), it is possible that the observed increases in nah gene concentration are due not only to the growth of particular nah gene-containing populations (vertical or within-lineage gene transmission) but also to an increase in the transfer of genes horizontally (between-lineage) on plasmids.

Due to the low frequency of natural gene transfer and to the uncontrolled aspects of field investigations, it is all but impossible to directly detect transfer events in the natural habitats of microorganisms. Most studies have therefore focused on demonstrating that the potential for horizontal transmission exists in nature. Thus, lateral transmission of bacterial genes has been detected between natural isolates in pure culture; between isolates added back to sterile aquatic, soil, and sediment microcosms; and, more recently, between added strains and the natural flora of microcosms (Trevors, 1987; Klingmüller, 1990; Sandaa, 1994; Neilson, 1994; Brokamp, 199; Klingmüller, 1991; Stotzky, 1989; Saye, 1989).

An alternative, complementary approach is to use molecular population genetic methods to provide evidence that horizontal transfer has actually taken place between naturally-occurring populations of bacteria. In one such approach, the phylogenies of specific genes are compared in order to detect incongruent, independent patterns of gene distributions between organisms (Valdes, 1992; Guttman, 1994). For example, Kapur *et al.* (Kapur, 1992) sequenced the exotoxin gene speC in 15 *Streptococcus pyogenes* clones. They found that individual speC alleles occurred in multiple clonal lineages and interpreted this pattern as evidence of the horizontal transfer of the alleles. In another recent study, Guttman and Dykhuisen (Guttman, 1994) compared the genealogies of four genes -- *sppA, gapA, pabB,* and *zwf* -- in 12 natural *E. coli* isolates. By noting inconsistencies in the phylogenetic histories of some of the genes, they found strong evidence of three distinct transfer events having occurred in one group of strains.

The phylogenetic relationships of bacterial populations and genes indigenous to terrestrial and aquatic habitats are rarely as defined as those for well-characterized species such as *E. coli.* Nonetheless, incongruities between inferred organism and gene genealogies -- e.g. conservation in a particular gene coupled with diversity in the gene's bacterial hosts -- have been used as evidence

of horizontal DNA transmission (van der Meer, 1992). In one such study, Ka *et al.* (Ka, 1994) suggested that horizontal gene transfer on mobile catabolic plasmids explained their observation that a phenotypically diverse group of 2,4-D degraders hybridized strongly to *tfd* probes at high stringency. Rosello-Mora *et al.* (Rossello-Mora, 1994) speculated that similar *nahA* (naphthalene dioxygenase) gene homologs (detected by DNA hybridization) found in different genomovars of *Pseudomonas stutzeri* arose through horizontal gene transfer. And in a previous study, we demonstrated that a phenotypically diverse group of naphthalene-degrading bacteria harbored PCR-amplifiable homologs of *nahAc*, the gene encoding the α subunit of the iron-sulfur protein component of naphthalene dioxygenase.

In the present study, we have used nucleotide sequences from two genes in bacteria derived from a coal tar-contaminated field site and a comparative phylogenetic approach to test for evidence of horizontal gene transfer. This research was a portion of a larger study in which we have developed and utilized molecular methods to study the distribution and variation in *nah* genes and naphthalene catabolism at this polluted site (Moré, 1994). By comparing the partial DNA sequences of the *nahAc* and 16S rRNA genes from a number of isolates from the site and elsewhere, we found that nearly all of the site *nahAc* sequences were not only closely related but identical, whereas their 16S rRNA gene sequences were in some cases highly diverged. Incongruities were found between the phylogenetic trees for the two genes. We conclude that horizontal transfer of *nah* genes has occurred among the clonal lineages of the naphthalene-degrading bacteria isolated from our study site. It is possible that the lateral spread of naphthalene and other biodegradative genes among natural populations of bacteria plays an important role in the their adaptation to pollutants.

METHODS

Study site, sampling, and isolation of naphthalene-degrading bacteria. Bacteria were isolated from samples obtained from a coal tar-contaminated area located near Glens Falls in upstate New York. This site has been studied intensively and details of site history and sample characteristics have been published previously (Madsen, 1992; Herrick, 1993; Madsen, 1991; Moré, 1994). Sampling locations and procedures were also described previously.

Bacterial strains. Strains of bacteria used in this study are shown in Table 3.1. *P. putida* G7 and *P. putida* NCIB 9816-4 were generous gifts from Drs. Gary Sayler and Gerben Zylstra, respectively. *P. fluorescens* N1 was isolated in our laboratory from naphthalene-MPN tubes inoculated with surface soil from the Hanford Nuclear Site. *P. fluorescens* Nd9 was isolated in our laboratory from subsurface material obtained from the contaminated source area of the Glens Falls study site. The remaining strains listed in Table 3.1 were isolated previously from sediment samples taken aseptically approximately 10 cm deep in a seep outflow region approximately 400 m from the source of the contaminant plume at the same site (Madsen, 1991; Herrick, 1993). Metabolism of naphthalene by each of the isolates in Table 3.1 except *P. putida* NCIB-9816 was confirmed by assaying 14C-naphthalene mineralization in sealed flasks as described previously. Isolates were identified and characterized using standard microbiological procedures, the API-NFT kit (API Analytab Products, Plainview, NY), and the Biolog identification system (Bochner,

Table 3.1. Bacterial strains used in this study

Straina	Source
Pseudomonas putida G7	G. Sayler
P. putida NCIB 9816-4	G. Zylstra
P. fluorescens N1	Hanford Nuclear Site, Richland, WA
P. fluorescens Nd9	EBOS 24 site source material
P. putida Cg1	EBOS 24 site contaminated seep
P. fluorescens Cg2	EBOS 24 site contaminated seep
Gram-negative rod Cg4	EBOS 24 site contaminated seep
P. fluorescens Cg5	EBOS 24 site contaminated seep
Pseudomonas sp. Cg7	EBOS 24 site contaminated seep
Gram-negative rod Cg8	EBOS 24 site contaminated seep
P. mendocina Cg11	EBOS 24 site contaminated seep
Gram-negative rod Cg15	EBOS 24 site contaminated seep
Pseudomonas sp. Cg21	EBOS 24 site contaminated seep

[a] Species designations for all but *P. putida* G7 and *P. putida* NCIB 9816-4 were determined using the Biolog bacteria identification system.

1989), as well as cluster analysis of Biolog profiles using the UPGMA-based Biolog program MlClust as previously described.

Restriction mapping of *nahAc* genes. DNA was prepared for polymerase chain reaction (PCR) amplification and restriction digestion by first growing the purified isolates and type strains at room temperature on Stanier's Mineral Salts B (MSB) medium (Stanier, 1966) plus naphthalene vapor for three to seven days. A small number of cells from a single colony was transferred into 10 µl of ddH2O using a sterile inoculating needle and the cells subsequently heated at 95°C for 5 min to aid in cell lysis. PCR amplification of a 701-bp fragment of *nahAc* (the entire *nahAc* gene is 1349-bp in length) was carried out in a 100 µl volume using primers *nahAc*-1 and *nahAc*-3 (Table 3.2). Reagents were as previously described (Herrick, 1993) but with primer concentrations of 0.25 µM and 2.0 U *Taq* polymerase (Gibco-BRL, Gaithersburg, MD and Promega Corp., Madison, WI), and using "hot-start" conditions, with the deoxynucleoside triphosphates added after the tubes were heated to 80°C. Tubes were cycled 30 times at 92°C for 40 s and 65°C for 1 min 30 s; and 1 time at 75°C for 5 min. All PCR amplifications were carried out on an MJ Research (Watertown, MS) MinicyclerTM and all primers were synthesized at the Oligonucleotide Synthesis Facility at Cornell University.

Five replicate 100 µl reaction volumes were pooled for each isolate. Primers and nucleotides were removed and samples concentrated using a Centricon-100 (Amicon, Beverly, MA) microconcentrator according to the manufacturer's instructions. Restriction digestion of the amplified, concentrated PCR products was carried out using the 4-cutter restriction enzymes *Bst*UI, *Hha*I, *Hin*fI, *Msp*I, *Nla*IV, *Scr*FI, and *Taq*I (New England Biolabs, Beverly, MA) as per

the manufacturer's specifications. The digests were visualized and sized on an ethidium bromide-stained 3% MetaPhor high-sieving agarose gel (FMC, Rockland, ME) run at 5V/cm constant voltage.

DNA sequencing of *nahAc* and 16S rRNA genes. *NahAc* DNA was prepared for sequencing by first amplifying from plated colonies as above using primers *nahAc*-F and *nahAc*-R (Table 3.2). Reagents and conditions were as described above but with primer concentrations of 0.15 μM and using 1.0 U *Taq* polymerase. Tubes were cycled 5 times at 94°C for 2 min, 60°C for 1 min, and 74°C for 1 min; 25 times at 94°C for 30 s, 60°C for 30 s, and 74°C for 1 min; and 1 time at 74°C for 5 min. Two 100 μl reactions were pooled and concentrated as above, agarose gel-purified by electrophoresis in molecular grade preparative agarose (Bio-Rad Laboratories, Hercules, CA), and extracted from the agarose using a Spin-Bind DNA extraction cartridge (FMC BioProducts, Rockland, ME) according to the manufacturer's specifications. Extracted DNA was diluted to 25 ng/μl and sequenced using nested primers *nahAc*-1 and *nahAc*-5 (Table 3.2) and the *Taq* DyeDeoxy™ terminator cycle sequencing procedure on an Applied Biosystems (Foster City, CA) 373 DNA sequencer at the Cornell DNA Sequencing Facility. Both the DNA strands were sequenced.

16S DNA was prepared for sequencing using PCR amplification from colonies (as described above) using primers 16Sp-5 and 16Sp-3 (Table 3.2). Reagents and conditions were as previously described (Herrick, 1993) with the following exceptions: (i) primer concentration was 0.3 μM, (ii) 2.0 U *Taq* polymerase were used, and (iii) "hot-start" conditions were employed as described above. DNA was pooled, concentrated, purified, and extracted as above. Automatic DNA sequencing was also carried out as above using primer 16Sp-5 and nested primer 16Sp-339R. Both the DNA strands were sequenced; however, due to difficulties encountered sequencing from a non-nested primer (16Sp-5), the plus strand was sequenced twice in all cases for verification. Sequence data from both the 16S and *nahAc* genes was compiled using version 1.0.3 of the SeqEd DNA sequence analysis program (Applied Biosystems, Foster City, CA)

DNA sequence alignment. NahAc and 16S rRNA gene sequences were aligned manually. No complications were encountered in aligning the *nahAc* sequences, since there was general conservation of sequence and differences apparently consisted entirely of single nucleotide substitutions. Alignment of one portion of the 16S sequence -- helix 6 (Larsen, 1993), comprising positions 61 to 106 -- was more complex, however, due to its evidently rapid rate of substitution relative to the rest of the molecule. This variable region was therefore aligned using a group of 18 aligned sequences retrieved from the Ribosomal Database Project (RDP) database (Larsen, 1993) and using the predicted *E. coli* 16S rRNA secondary structure of Gutell *et al.* (Gutell, 1994). These complete alignments were used for the simple similarity comparisons described below. Phylogeny reconstruction, on the other hand, depends upon the assumption of strict "positional homology" (Swofford, 1990), i.e. nucleotides at a given position in all compared sequences should be descended from a common ancestral nucleotide at that position. Positions of ambiguous alignment within helix 6 were therefore excluded from phylogenetic analyses.

DNA sequence and phylogenetic analysis. Simple per cent dissimilarities were used for pairwise comparisons of sequences and also as a measure of evolutionary distance for distance matrix-based phylogenetic analyses. This method, which does not correct for superimposed substitutions, is a reasonable approach when, as here, one is not concerned with distant

Table 3.2. Oligonucleotides used in this study

Primer	Sequencea	Locationb
nahAc		
nahAc-F	5'-CGCGGAACTGGCTTTTTCTCACTCA	152-176
nahAc-R	5'-ACCGAAACCAAGGTTTGAAAGCAGA	1221-1197
nahAc-1	5'-GTTTGCAGCTATCACGGCTGGGGCTTCGGC	343-372
nahAc-3	5'-TTCGACAATGGCGTAGGTCCAGACCTCGGT	1044-1015
nahAc-5	5'-GGAGGTCATTTGCAAGCCTG	783-764
16S		
16Sp-5'	5'-ccgaattcgtcgacaacAGAGTTTGATCMTGG	8-21
16Sp-3'	5'-cccgggatccaagcttTACCTTGTTACGACTT	1504-1492
16Sp-339R	5'-TGCTGCCTCCCGTAGGAG	339-322

[a] Linkers containing restriction sites are indicated in lowercase letters; M denotes A or C.
[b] Numbering for *nahAc* primers is from the sequence published by Simon *et al.* (Simon, 1993) and for the 16S primers from Brosius *et al.* (Brosius, 1978).

phylogenetic relationships and divergence between sequences is small. Uncorrected similarities/dissimilarities are particularly appropriate when applied to closely-related protein-coding sequences such as *nahAc* (Swofford, 1990). However, evolutionary distances corrected for superimposed substitutions by the Jukes and Cantor (Jukes, 1969), Kimura (Kimura, 1980), and maximum likelihood (Felsenstein, 1981) models were also determined for both the 16S rRNA and *nahAc* genes using the program DNADIST in PHYLIP version 3.5c for the Power Macintosh (Felsenstein, 1993). These distances were compared to the uncorrected, dissimilarity-based distances and in all cases found to be nearly identical. Calculations of per cent dissimilarity (d) were carried out using the formula $d = (1 - S) \cdot 100$ where S is the fractional sequence similarity, or number of aligned nucleotide positions containing identical residues divided by the total number of nucleotide positions (Swofford, 1990). Positions with gaps or ambiguous nucleotides were ignored.

Evolutionary distances, both uncorrected and corrected, were converted to dendrograms using the least-squares distance algorithm of Fitch and Margoliash (Fitch, 1967) employed by the FITCH program in PHYLIP. Species input order was randomized, with 20 input orders examined, and the "global" branch-swapping option employed. Output from FITCH was converted into unrooted trees using the PHYLIP program DRAWTREE. Trees derived from least-squares analysis were compared with dendrograms generated by maximum parsimony analysis [PAUP version 3.0s (Swofford, 1991)], using the branch-and-bound algorithm.

RESULTS AND DISCUSSION

***NahAc* gene sequence similarity.** A diverse group of Gram-negative naphthalene-mineralizing isolates from the contaminated seep area of our study site was previously found, by

polymerase chain reaction (PCR) amplification, to contain homologs to the *Pseudomonas putida* G7 naphthalene dioxygenase gene *nahAc*. Successful PCR amplification implied that there was sequence conservation among these *nahAc* homologs, at least within the primer binding regions. Initially, fine scale four-cutter restriction mapping was used to compare the sequences of the *nahAc* alleles amplified from six randomly-chosen seep isolates -- Cg1, Cg2, Cg4, Cg5, Cg7, and Cg8 -- both with each other and with the archetypical naphthalene degrading strains *P. putida* G7 and *P. putida* NCIB 9816. Restriction maps (not shown) using the enzymes *Bst*UI, *Hha*I, *Hin*fI, *Msp*I, *Nla*IV, *Scr*FI, and *Taq*I for the six seep strains examined were identical to each other and to that of strain NCIB 9816. However, 10 of 21 sites differed relative to strain G7.

In order to determine precisely how similar the seep isolates' *nahAc* genes were, we sequenced a 373-bp portion of the *nahAc* gene from seven of the contaminated seep-derived strains, the two strains *P. putida* G7 and NCIB 9816, as well as from *P. fluorescens* N1, isolated in our laboratory from Washington state, and *P. fluorescens* Nd9, isolated from the contaminated source area of the study site (Table 3.1). The seven seep strains were chosen based upon their phenotypic dissimilarity from one another as judged by cluster analysis of their Biolog carbon-source utilization profiles. Surprisingly, base for base identity was found among the *nahAc* fragments of six out of seven of the seep isolates, as well as in Nd9 (Table 3.3). The *nahAc* sequence of *P. fluorescens* Cg2 differed from the other six by only one base, a thymine substituted for an adenine at position 328 in Table 3.3. Moreover, these two closely-related alleles (referred to hereafter as the Cg1 and Cg2 alleles) differed substantially from the *P. putida* G7 and *P. fluorescens* N1 *nahAc* alleles, as did these alleles from one another. The difference in the alleles extended to the deduced amino acid sequence. The deduced protein sequence for the Cg1 *nahAc* allele differed from that of *P. putida* G7 in six of 124 amino acids. In addition, the deduced amino acid sequence of *P. fluorescens* N1 differed from that in *P. putida* G7 by four amino acids. Nine of the 11 *nahAc* gene sequences presented in Table 3.3 were either identical or differed by only one nucleotide. In interpreting these results, it is essential to consider the possibility that the data were influenced by laboratory-imposed artifacts. These can include inadvertent transfer of post PCR-amplified target between DNA preparations (Orrego, 1990). However, we feel that the validity of our data is assured by 4 different lines of evidence: (i) great care was taken to isolate pre- from post-amplification reactions (see Materials and Methods) in order to prevent amplification product carryover, a common source of PCR contamination (Orrego, 1990); (ii) sequence for an individual isolate was often obtained from more than one initial template, i.e. at different times from newly-amplified template *nahAc*, and still found to be identical; (iii) as previously mentioned, not all of the sequences obtained were identical (e.g. from Cg2 and N1), even though these were amplified and sequenced along with isolates having the Cg1 allele; and (iv) the most likely *nahAc* allele to be replicated due to product carryover would be that of *P. putida* G7, by far the most common *nahAc* sequence in our laboratory (since it is used as a positive control for all *nahAc* amplifications); yet the G7 allele was not found at all. Thus, we do not believe that the identity of sequence which we have observed in these isolates is due to laboratory error.

Comparative divergence of 16S rRNA and *nahAc* genes. If lateral spread of nah genes has occurred in the lineages of these naphthalene-degrading isolates, one would expect the transferred genes to be more closely related to each other than would their host cells. Moreover,

Table 3.3. Polymorphic sites in partial *nahAc* alleles from naphthalene-mineralizing bacterial strains. The sequence of *nahAc* from *Pseudomonas putida* G7 is the reference sequence.

	1 1 1 1 1 1 1 1 1 1 2 2 2 2 2 3 3 3 3 3 3 3 3
	3 3 6 9 1 2 3 4 6 8 8 8 9 9 0 2 3 6 8 0 2 3 3 4 6 6 6 7
Strain[a]	1[b] 9 0 9 0 6 1 0 2 5 2 3 6 9 2 8 4 2 4 6 5 0 8 0 3 6 3 6 8 2
PpG7	T T C G C G C T T C G A G C C A A C C C A G T G A G C A T G
Cg1	C .[c]T T T . . C C . . G A T G G C . . . . . A . G T T G C A
Cg2	C . T T T . . C C . . G A T G G C . . . . . G T T G C A
Cg4	C . T T T . . C C . . G A T G G C . . . . . A . G T T G C A
Cg5	C . T T T . . C C . . G A T G G C . . . . . A . G T T G C A
Cg11	C . T T T . . C C . . G A T G G C . . . . . A . G T T G C A
Cg15	C . T T T . . C C . . G A T G G C . . . . . A . G T T G C A
Cg21	C . T T T . . C C . . G A T G G C . . . . . A . G T T G C A
Nd9	C . T T T . . C C . . G A T G G C . . . . . A . G T T G C A
NCIB9816	. . T T T . . C C . . G A T G G C . . . . . G T T G C A
N1	C C . . . A G . . T C G A . . . . A T T T C A A C T . . . .

[a] Full names of strains are listed in Table 3.1.
[b] Numbering is relative to the 373-bp region of *nahAc* sequenced in this study.
[c] Period denotes a nucleotide which is identical to that of *P. putida* G7 at the indicated position.

the phylogeny of the cells and transferred genes would not be congruent. We therefore examined the strains' 16S rRNA gene sequences and compared the relative divergence of this gene in the isolates to that of *nahAc*. The 16S rRNA gene was selected due to the following characteristics (Woese, 1987): (a) there is no evidence of horizontal transfer of the 16S rRNA gene or portions thereof, (b) it is highly conserved, with significant sequence identity retained even across domain and kingdom boundaries, and (c) its evolution is considered to be representative of the evolution of the organism containing it. Thus, the detection of sequence divergence in this highly-conserved chromosomally-located gene, coupled with sequence identity in the *nahAc* gene, would constitute strong evidence for recent horizontal transfer of *nahAc* between bacteria indigenous to the field study site.

Using primers 16Sp-5 and 16Sp-339R (Table 3.2), we sequenced a portion of the 5' end (ca. 300 bp) of the 16S gene in five of the six seep isolates having an identical *nahAc* allele, as well as in *P. fluorescens* Cg2, *P. putida* G7 and NCIB 9816, and *P. fluorescens* N1 and Nd9. The 16S rRNA gene has alternating regions of high and low sequence conservation (Woese, 1987) and, as expected, the sequences for all the strains were identical in nearly all of the very highly conserved portions of the gene fragment (nt numbers 1 to 48, 75 to 123, and 140 to 305, Table 3.4). There was considerable dissimilarity within two less-conserved regions of the fragment known as V1 and

TABLE 3.4. Polymorphic sites in partial 16S rRNA alleles from naphthalene-mineralizing bacterial strains. The 16S rRNA gene sequence from *Pseudomonas putida* G7 is the reference sequence.

Strain[a]	4 5 5 5 5 5 5 5 6 6 6 6 6 6 6 7 7 7 7 7 9 2 2 3 3 3 3 3 5 8[b] 1 2 3 4 5 6 7 8 3 4 5 6 7 8 9 0 1 2 3 4 6 4 9 0 1 2 7 9 3
PpG7	A -[c] A G A A G A G C T C T T C G A T - T C G C T T T C G A G
Cg1	.[d] . . A G . . . . . C T . . . . . C . . . G .
Cg2	T . . . . G A . . . . T C . . T T G . A G . T C . C G C G .
Cg4	C C . C G G . T A T A . C . G . T G G C G A T . . C G C G T
Cg5	. . . A G G . . . . . C T . . . . . C . . . G .
Cg11	C C . C G G . T A T A . C . G . T G G C G A T . . C G C G T
Cg21	T . . . . G A . . . . T C . . T T G . A G . T . . C G C G .
Nd9	T . . . . G A . . . . T C . . T T G . A G . T . . C G C G .
NCIB9816	. . . A G . . . . . C T . . . . .
N1	. . . A G G . . . . . C C T . . . . . C . . . G .

[a]Full names of strains are listed in Table 3.1.

[b]Numbering is relative to the portion of the 16S rRNA gene sequenced in this study. Nucleotide number one corresponds to nucleotide 23 on the *E. coli* 16S rRNA gene sequence of Brosius *et al.* (Brosius *et al.*, 199_).

[c]Designates a gap inserted to align sequences.

[d]Designates a nucleotide which is identical to that of *P. putida* G7 at the indicated position.

V2 (Larsen, 1993) corresponding to nt 39 to 80, and 96 to 213, respectively (Table 3.4). These polymorphic sites all fell within helices 6 (*E. coli* positions 61 to 106 and nt 39 to 84 in Table 3.4) and 9 (*E. coli* positions 144 to 178 and nt 118 to 152 in Table 3.4) of the *E. coli* rRNA secondary structure model of Larsen *et al.* (Larsen, 1993).

A tabulation of pair-wise comparisons between *nahAc* and 16S rRNA gene sequences among site-derived and other naphthalene-degrading bacteria appears in Table 3.5. Dissimilarities in the 16S rRNA sequence ranged from zero to as high as 7.87% in the strains examined. Three pairs of strains, Cg1/Cg5, Cg4/Cg11 and Cg21/Nd9 had identical 16S rRNA gene sequences in the region examined; however, the three 16S alleles differed substantially from one another (Tables 3.4 and 3.5). On the other hand, each of these six strains has the identical Cg1*nahAc* allele (Tables 3.3 and 3.5). A comparison among strains of relative sequence divergence for *nahAc* and 16S rRNA alleles (Table 3.5) is particularly insightful.

In two strains not indigenous to our field study site (*P. fluorescens* N1 and *P. putida* G7), *nahAc* has diverged from *P. putida* Cg1 a great deal (6.7% and 5.09% for G7 and N1, respectively), while the corresponding dissimilarity is much smaller in the 16S rRNA gene (1.97% and 0.66% for the same strains, respectively). This pattern might be expected if, as expected, the 16S rRNA gene is evolving less rapidly than is *nahAc* and if horizontal transfer has not occurred,

TABLE 3.5. Per cent dissimilarities in the 16S rRNA alleles (above diagonal) and the *nahAc* alleles (below diagonal) of naphthalene-mineralizing bacteria. Per cent dissimilarities (d) were calculated using hte formula $d = (1 - S) \cdot 100$ where S is the fractional similarity between two taxa (see Materials and Methods). Full names of strains are given in Table 3.1.

	Cg1	Cg2	Cg4	Cg5	Cg11	Cg15	Cg21	Nd9	9816	N1	PpG7
Cg1		6.89	7.87	0.00	7.87	ND	6.56	6.56	0.66	0.66	1.97
Cg2	0.27		5.57	6.89	5.57	ND	0.33	0.33	6.89	6.89	5.57
Cg4	0.00	0.27		7.87	0.00	ND	5.25	5.25	7.87	7.21	7.87
Cg5	0.00	0.27	0.00		7.87	ND	6.56	6.56	0.66	0.66	1.97
Cg11	0.00	0.27	0.00	0.00		ND	5.25	5.25	7.87	7.21	7.87
Cg15	0.00	0.27	0.00	0.00	0.00		ND	ND	ND	ND	ND
Cg21	0.00	0.27	0.00	0.00	0.00	0.00		0.00	6.56	5.90	5.25
Nd9	0.00	0.27	0.00	0.00	0.00	0.00	0.00		6.56	5.90	5.25
NCIB 9816	0.54	0.27	0.54	0.54	0.54	0.54	0.54	0.54		1.31	1.31
N1	6.70	6.97	6.70	6.70	6.70	6.70	6.70	6.70	7.24		2.62
PpG7	5.09	5.09	5.09	5.09	5.09	5.09	5.09	5.09	4.83	4.56	

at least not relatively recently. By contrast, an examination of gene sequences from bacteria originating within our field site which contain the highly conserved Cg1 and Cg2 *nahAc* alleles - (strains Cg2, Cg4, Cg5, Cg11, Cg21, and Nd9) reveals a broad range of dissimilarities in the 16S rRNA gene (0% to 7.87% dissimilarity versus Cg1). This suggests that many of these isolates are more diverged (from Cg1, in this example) than are their *nahAc* genes.Comparative phylogenies of the *nahAc* and 16S rRNA genes. Phylogenetic trees were generated using the least-squares distance method of Fitch-Margoliash (Fitch, 1967) and maximum parsimony analysis (Swofford, 1991). Fig. 3.1 shows the least-squares distance trees for each gene; parsimony analysis yielded trees with identical topologies (branching orders) to those shown. The 16S rRNA gene tree shown is based upon a conservative alignment having portions of the variable helix 6 region removed from analysis (nt nox. 48, 51, 52, 68, 69, 70-74, Table 3.5) due to alignment ambiguities (see Material and Methods). Since our purpose for this study was not to fully resolve the phylogeny of these strains but simply to contrast the gross phylogeny of the 16S rRNA gene in these taxa with that of *nahAc*, we elected to use a more conservative alignment based solely on regions in which the aligned nucleotides were clearly homologous (Swofford, 1991).

Comparing Figs. 3.1A and 3.1B reveals that the single Cg1*nahAc* allele is distributed throughout the bacterial strains in the three major 16S rRNA branches. Thus, the phylogenies of the two genes are not congruent and there is no apparent *nahAc* allele-rRNA genotype relationship. An alternative hypothesis for explaining the observed identity in the *nahAc* gene is that natural selection has acted to maintain an ancestral *nahAc* DNA sequence present in the phylogenetically diverse clonal lineages represented by the populations which we sampled. One can envision

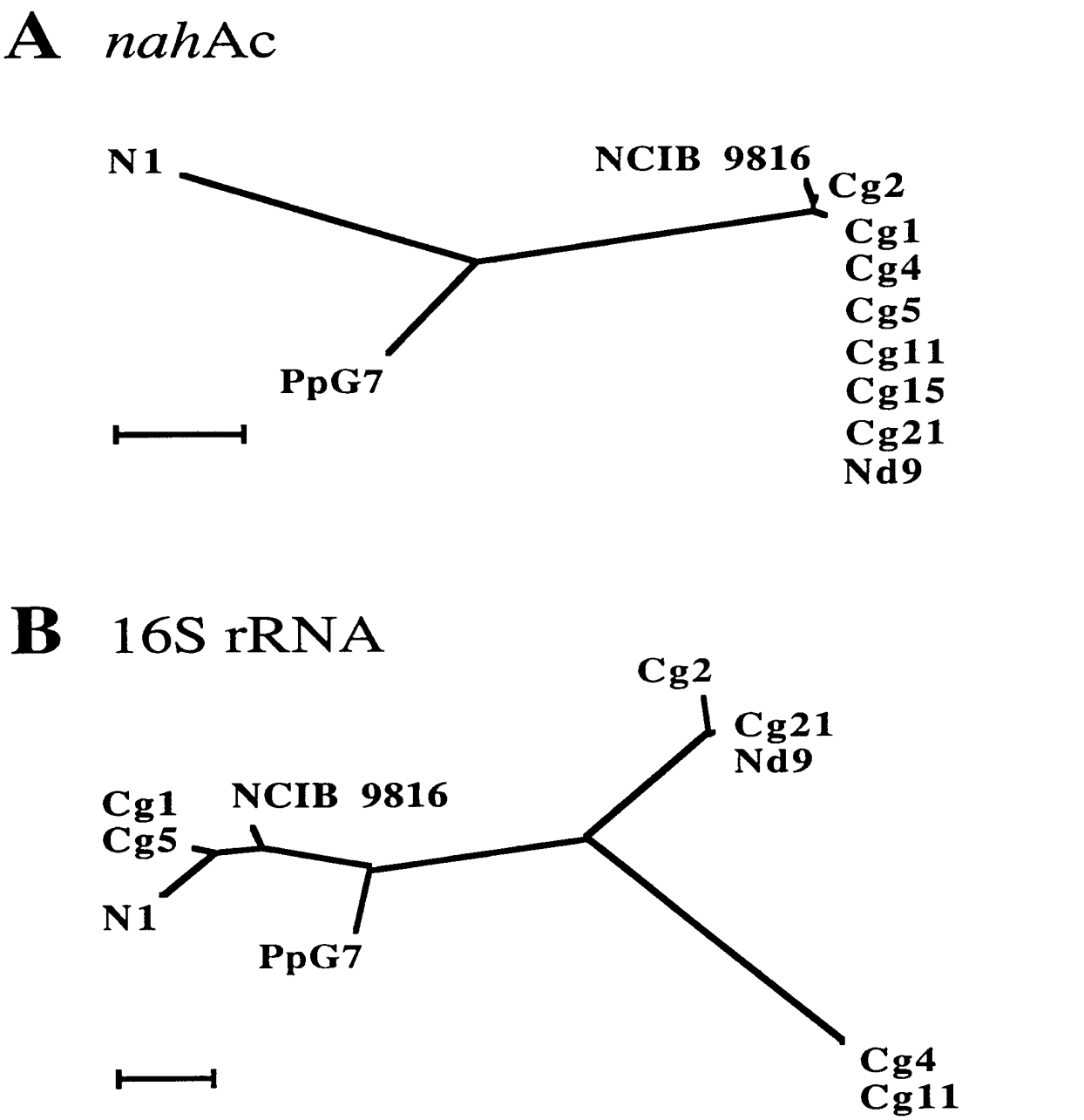


Figure 3.1. Phylogenetic trees of *nahAc* (A) and 16S rRNA (B) gene sequences within bacteria examined in this study. Phylogenetic analysis was done using the least-squares distance algorithm of Fitch and Margoliash (Fitch, 1967) and based upon a matrix of dissimilarity distances uncorrected for superimposed substitutions (see Materials and Methods).

selective pressure against alternative amino acids due to their possible detrimental effects on the function of the naphthalene dioxygenase enzyme. However, the selective constraints on nucleotide substitution at synonymous (silent) sites are generally weak to nonexistent, although codon bias can exert some influence on the evolution of synonymous sites (Hartl, 1989]. Synonymous sites in protein-coding genes such as *nahAc* are generally considered to be relatively neutral with regard to selection and therefore should easily accumulate substitutions even with selective pressure acting to maintain an adapted protein sequence. Because no synonymous site substitution was observed among the Cg1 *nahAc* alleles, we reject the hypothesis for selective maintenance of an ancestral protein form among these bacteria.

Conclusions. Comparative gene sequencing has provided evidence that an allele of the naphthalene dioxygenase gene *nahAc* has been laterally spread among naphthalene degrading bacteria from our field study site. This indirect, population genetic approach complements previous laboratory studies which have demonstrated that catabolic and other genes can be transferred horizontally among bacteria inhabiting soils, sediments, and aquatic habitats (Trevors, 1987; Klingmüller, 1990). The likely vehicle for the deduced gene transfer would be self-transmissible plasmids, since nearly all naphthalene degradation genes examined to date have been plasmid-borne (Sayler, 1990). We have found large plasmids in all of the strains in Table 3.1 and are currently in the process of determining whether their *nah* genes are indeed borne on plasmids and, if so, the relatedness and phylogenies of the plasmids themselves. By so doing, we hope to gain insight into the mode and extent of lateral transfer of *nah* catabolic genes at contaminated sites and thereby to better understand the adaptation of native biodegradative bacterial populations to contaminants in the field.

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