Natural Selection of PAH-degrading Bacterial Guilds at Coal-Tar Disposal Sites

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Microbial activity patterns at buried coal-tar disposal sites have been under investigation for several years to determine the response of naturally occurring microflora to polycyclic aromatic hydrocarbons (PAHs) at the sites. At one site in upstate New York, data have shown enrichment of PAH-degrading bacteria in subsurface contaminated zones but not in uncontaminated zones. Similar work at a midwestern site showed that the same trends existed in a heterogeneous disposal site except that a borehole outside the plume showed some PAH-mineralization activity. Polymerase chain reaction amplification of DNA extracted from sediment samples from the New York site indicated the presence of naphthalene metabolism genes *nahAc* and *nahR*, similar to those found on the NAH7 plasmid of *Pseudomonas putida* G7. Significant sequence polymorphism was observed in amplified *nahAc* products, indicating that divergent homologs of *nahAc* were present in the native community. Protozoan numbers were elevated in sediment samples displaying relatively high PAH-degrading activity, suggesting that a food chain was established based on PAH-degrading bacteria. Removal of the coal-tar source at the site occurred in 1991. In 1992, sampling of three key borehole stations revealed that mixing and backfilling operations had introduced soil microorganisms into the source area and introduced ^{14}C-PAH-mineralization activity into the previously inactive pristine area. Thus removal of the source of the contaminants and restoration at the site have altered the microbial activity patterns outside the contaminant plume as well as in the source area. — Environ Health Perspect 103(Suppl 5):107–111 (1995)

Key words: naphthalene, phenanthrene, mineralization, PCR-detection, population genetics, *nahAc, nahR, Pseudomonas putida*

Introduction

The concept of bacterial guild* selection lies at the roots of modern microbial ecology. Historically, the seminal work of Winogradsky and Beijerinck on the ecology of soil microorganisms in the 19th and early 20th centuries set the stage for subsequent work on the physiological ecology of bacteria during the mid-20th century and, in turn, for the work of the present generation of microbial ecologists (*1–4*). While other areas of microbiology have flourished, the broad field of microbial ecology is still attempting to define itself. Nevertheless, the principle of environmental selection first promoted by Beijerinck [see Atlas and Bartha (*1*)] has long been employed in the laboratory for selective enrichment and isolation of individual species of bacteria. Furthermore, most microbiologists tacitly assume that extant environmental conditions, even those created by release of mixtures of toxic organic chemicals at Superfund sites, will select for bacterial guilds that can use the available carbon and energy sources. Provided the environmental conditions are favorable, it is further assumed that the selected guilds will transform the toxic compounds completely to harmless products. However, because of the complexity of most natural systems and because of a lack of rigorous field-oriented methods in microbiology, unequivocal verification that the laboratory-based principle is operative in nature has been difficult (*5*).

This article reviews our recent work to achieve this goal by inference from studies of the microbial ecology of two coal tar disposal sites (*5–7*). We examined microbial distribution, abundance, and activity patterns at both sites and showed that the potential was high for biodegradation of soluble polycyclic aromatic hydrocarbons (PAHs) in contaminated sediments at the sites. We also found evidence that PAH-degrading bacterial guilds serve as the food source for protozoa in the active zones. Here we present preliminary results describing the microbial activity patterns at the site in upstate New York that have developed since excavation procedures have removed the most highly contaminated sediments.

Description of Study Sites

Both sites were selected from a list of power company-owned coal-tar disposal sites that qualified as Superfund sites.

Site 24 is located in Glens Falls, New York, in a rural forested area. The soil and underlying sediments are native sandy alluvium. The contents of a coal-tar repository originally located at the local manufactured gas plant (MGP) were transported and buried below the depth of the water table (~3–5 m) more than 30 years ago. During the past 10 years, a plume of soluble coal-tar constituents was defined and monitored in groundwater [see (*5,8,9*) for more details].

This paper was presented at the Conference on Biodegradation: Its Role in Reducing Toxicity and Exposure to Environmental Contaminants held 26–28 April 1993 in Research Triangle Park, North Carolina. Manuscript updated: fall 1994; manuscript received: January 23, 1995; manuscript accepted: February 13, 1995.

This work was supported in part by grant 08-P2ES05950A Sub 147-84 from the National Institute of Environmental Health Sciences; contract 91-091 from the Electric Power Research Institute; grant AFOSR-91-0436 from the U.S. Air Force Office of Scientific Research; and grant NYS-CAT 92054 from the Cornell Center for Advanced Technology in Biotechnology, which is sponsored by the New York State Science and Technology Foundation, a consortium of industries, and the National Science Foundation.

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*The term "guild" is used here to describe a group of species sharing a common resource, in this case, a soluble polycyclic aromatic hydrocarbon, naphthalene, as a carbon-energy source [see (*1–3*) for further discussion of the concept of guild structure]. Guild is used here in preference to the more commonly used term in the microbial ecology literature, population. The term population in the general ecology literature usually refers to a group of genetically related species. In most cases, the genetic makeup of the members of a given bacterial guild is not known. Indeed, it is likely that several different species of PAH-degrading bacteria make up the guilds we are studying at the upstate New York field site.

The location of Site 97 is typical of urban coal-tar disposal sites. It lies under the parking lot of a post office, which was formerly an MGP site, in a midwestern city close to the Mississippi River. The coal tar contaminant plume and the geohydrology of Site 97 have been defined, but they are not as well characterized as Site 24 (*7*).

Microbiological Sampling and Analyses

Our sampling strategy was designed to integrate key microbial analyses with geohydrologic and pollutant chemical analyses of fresh subsurface core samples taken at prescribed depths of the sediment profile inside and outside contaminated zones. This required considerable planning and coordination of field operations to assure that equivalent samples or the same samples could be analyzed for microbial abundance [total bacteria, heterotrophic colony-forming units (CFU), protozoa, numbers] and activity (^{14}C-naphthalene, ^{14}C-phenanthrene, ^{14}C-parahydroxybenzoate mineralization), as well as PAH concentration. Details of our sampling and analytical methods and procedures have been described previously (*5,7*). Recently, we have also employed the polymerase chain reaction and other molecular techniques in studies of the distribution of indigenous bacterial genes at Site 24 (*6,10*).

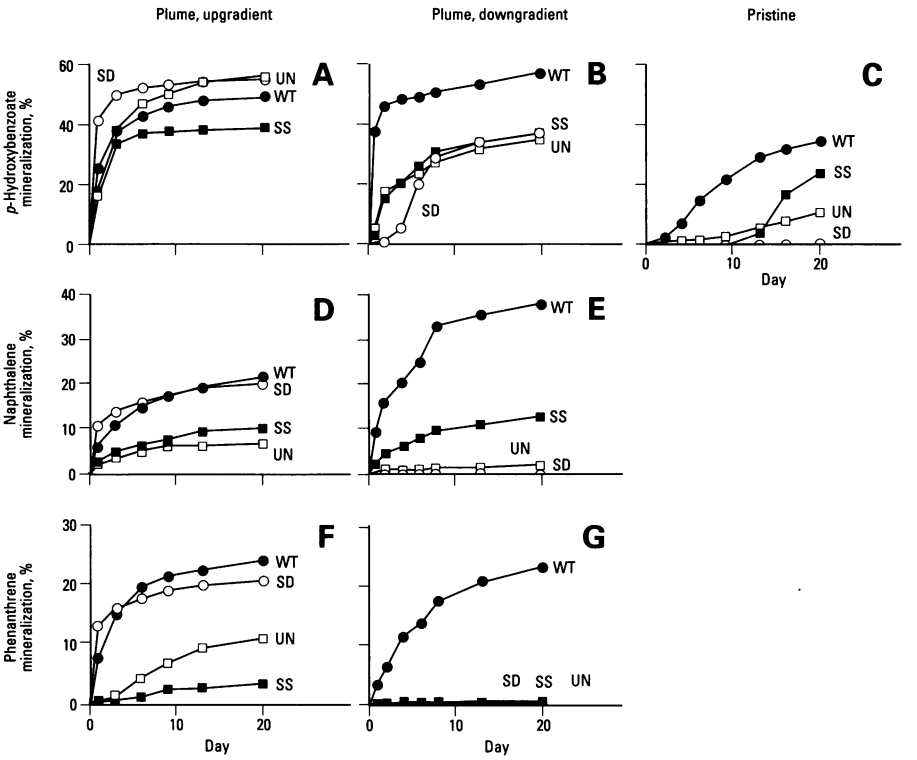

Figure 1. Mineralization of organic compounds by subsurface sediment samples. Boreholes were drilled at locations shown. Sediments were obtained employing aseptic techniques from equivalent geologic strata in four zones within each borehole: unsaturated (UN), water table (WT), shallow saturated (SS), and deep saturated (SD). The samples were amended with ^{14}C-labeled *p*-hydroxybenzoate (PHB, top row), naphthalene (NAP, middle row), and phenanthrene (PHEN, bottom row). Plots *A–G* are time courses of cumulative $^{14}CO_2$ trapped in triplicate flasks. No $^{14}CO_2$ was evolved in control flasks containing autoclaved, $HgCl_2$-poisoned sediments. Absence of data indicates no mineralization activity. From Madsen et al. (*5*).

Summary of Significant Results

The ^{14}C-mineralization results [Figure 1, from (*5*), and Figure 2, from (*7*)] showed that at both sites the ^{14}C-naphthalene and ^{14}C-phenanthrene mineralization potential was highest inside the plume of contamination in aquifer zones contaminated by moderate amounts of soluble hydrocarbons. Generally, most activity was found in the samples taken from the water table interface zone. Samples from the unsaturated or saturated zones showed variable activity depending on their oxygen status and concentration of PAHs. As predicted from past experience (*11*), the unsaturated zone of the sediment profile frequently was the least active, although this finding was more variable in the shallow surficial aquifers of this study.

The increased activity at the water table interface inside the contaminant plume at Site 24 was reflected by increased protozoan abundance, especially in the downgradient region [Figure 3, (*12*)]. The numbers of protozoa detected in the downgradient water table interface sample were higher than those of many surface soils (*5*). Bacterial numbers were also elevated in these samples, but bacterial biomass estimates alone were not a good indicator of high activity zones. Only when mineralization potential, bacterial biomass, and protozoan numbers were compared were convincing activity patterns revealed (Figures 1, 3).

A major difference between the Site 24 and Site 97 results was the ^{14}C-mineralization activity pattern in the profile of the borehole located outside the contaminated zone (*7*). At Site 24 there was no mineralization activity on ^{14}C-naphthalene or ^{14}C-phenanthrene by sediment samples from outside of the contaminated plume; and ^{14}C-hydroxybenzoate was mineralized as expected. However, at Site 97 the equivalent uncontaminated borehole showed moderate activity on both PAHs, albeit the mineralization rate and extent were reduced outside of the zone of contamination. Further examination of microbiological data from Site 97 showed the presence of microflora typically associated with surface soil in subsurface zones, indicating that the subsurface contained buried soil. We concluded that the buried soil or other displaced buried materials used for backfill during past construction operations was responsible for the moderate activity in the borehole located outside the contaminated zone at Site 97 (*7*).

A recent investigation has detected naphthalene-degrading bacteria in uncontaminated hillside forest soil adjacent to a distant downgradient area of Site 24 where groundwater emerges at the foot of a hill. This finding, in combination with that from Site 97, suggests that PAH mineralization activity may be widespread in surface soil and buried surface soil adjacent to coal-tar contaminated sites. Thus, our initial finding of no activity outside the plume at Site 24 may be an exception. Indeed, as discussed below, construction activities at Site 24 during source removal have left the formerly inactive borehole outside the plume with a moderate amount

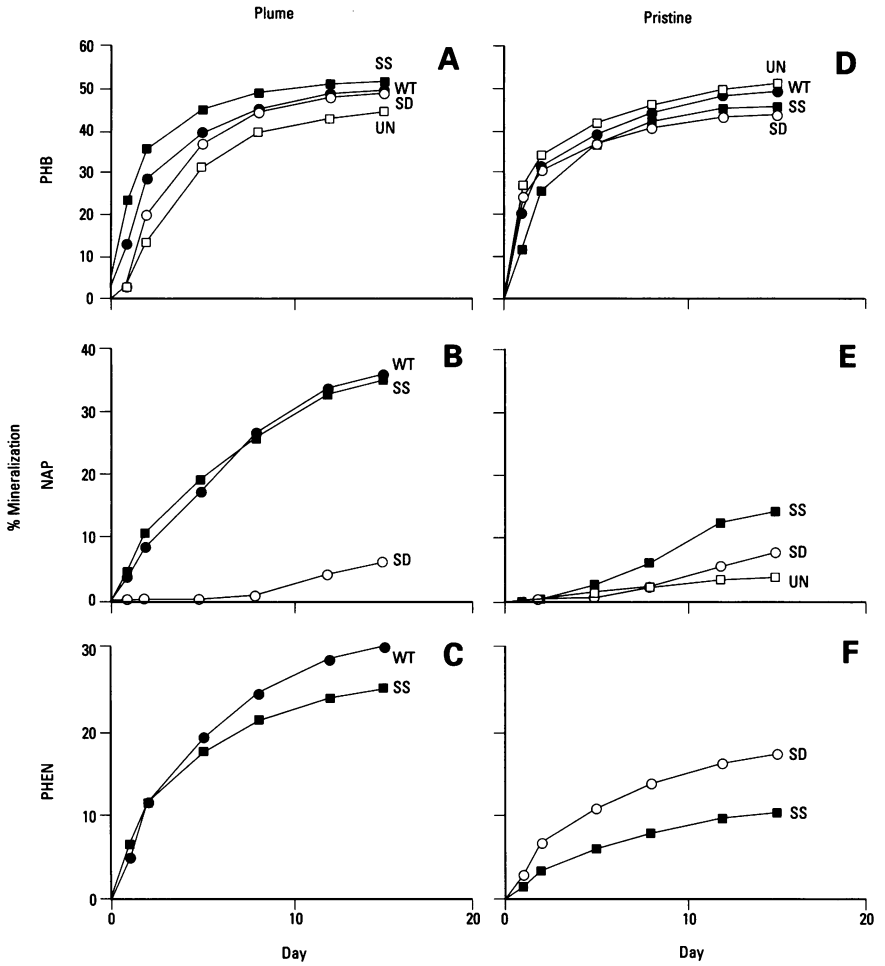

Figure 2. Mineralization of organic compounds [PHB, *para*-hydroxybenzene (*A,D*); NAP, naphthalene (*B,E*); PHEN, phenanthrene (*C,F*)] by subsurface sediment samples relative to autoclaved $HgCl_2$ control (% = 0) from the urban site. Boreholes were drilled at contaminated and uncontaminated sites as shown. Depths examined were unsaturated (UN), water table (WT), shallow saturated (SS), and deep saturated (SD) zones. Absence of data indicates no mineralization activity. From Madsen et al. (*7*).

of activity similar to that seen in the Site 97 borehole outside the plume.

In addition to the traditional assays for microbial distribution, abundance, and ^{14}C-mineralization activities, we are applying molecular approaches for studying the distribution of genetic potential for naphthalene metabolism (*6,9*). In this work we have successfully amplified portions of the *nahAc* (*6*) and *nahR* (*9*) genes in DNA extracted directly from Site 24 sediment samples. By restriction endonuclease fragment length polymorphism (RFLP) analysis, the *nahAc* genes were found to be similar, but not identical, to the *nahAc* gene of the NAH7 plasmid carried by *Pseudomonas putida* G7, a widely studied naphthalene-degrading bacterial strain. Significant sequence polymorphism in the amplified *nahAc* products indicated that divergent homologs of *nahAc* are present in the PAH-degrading guilds at Site 24. Recent work with isolates from several areas at Site 24 show that many of the naphthalene-mineralizing bacteria isolated from contaminated sediments also contain *nahAc* homologs, even those bacteria that are only distantly related, as judged by an array of taxonomically useful Biolog and API-NFT phenotypic tests. High-density four-cutter restriction maps of the *nahAc* genes from these isolates are identical to each other and to *P. putida* NC1B 9816 (*13*), yet they differ from that of *P. putida* G7 and an isolate from the highly contaminated source area at the site. We speculate that finding a highly conserved catabolic gene dispersed through taxonomically diverse bacterial populations from the seep area may be indicative of horizontal gene transfer.

Preliminary data suggest that naphthalene-mineralizing isolates from contaminated surface and subsurface sediments fall into a diverse array of *Pseudomonas fluorescens*-type bacteria, whereas soil-derived isolates from the adjacent uncontaminated hillside appear to be tightly clustered around another unrelated pseudomonad, *Pseudomonas gladioli*. All of

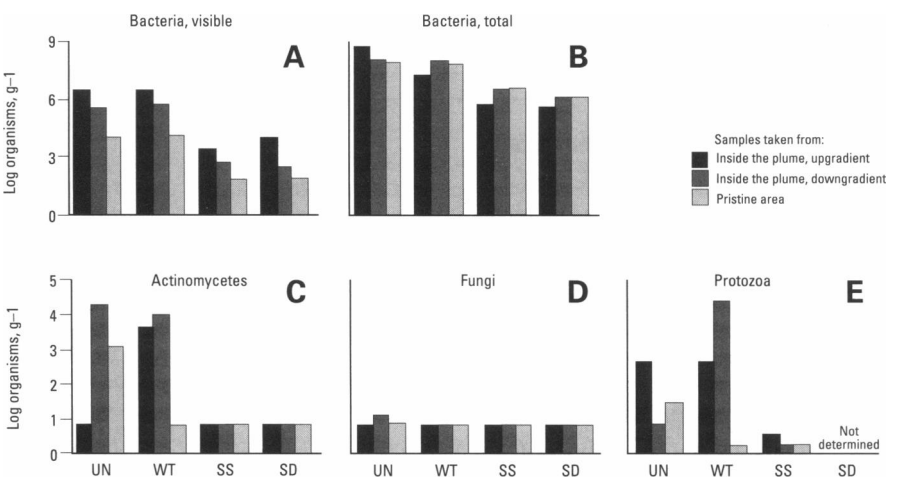

Figure 3. Comparison of microbiological abundances in sediments at four depths within three boreholes. The four depths examined were the unsaturated (UN), water table (WT), shallow saturated (SS), and deep saturated (SD) zones. Each cluster of three bars represents numbers of microorganisms from samples inside the plume, upgradient; inside the plume, downgradient; and in the pristine area. Viable bacteria (*A*), actinomycetes (*C*), and fungi (*D*) were determined by the plate count method. Total bacteria (*B*) were determined by epifluorescence microscopy. Protozoa (*E*) were enumerated using *Enterobacter aerogenes* as prey bacteria. From Madsen et al. (*5*).

the isolates mineralize naphthalene, yet only the sediment isolates contain *nahAc* genes similar to that of *P. putida* G7. Despite the fact that the isolates from the hillside forest soil were able to mineralize naphthalene, our PCR detection methods have been unable to amplify *nahAc* from any hillside-derived bacterium. This may possibly indicate previously unknown genetic diversity for PAH catabolism.

We recognize that the use of selective media for isolation of the strains for this study probably did not allow many of the members of a given naphthalene-degrading guild to be obtained. However, despite this unavoidable culturable bias, these results clearly indicate that the naphthalene-degrading guild structure of contaminated and uncontaminated areas at Site 24 are each composed of several species of bacteria and that the guilds in the contaminated zones are different from those in the uncontaminated surface soil.

Source Removal at Site 24

Site 24 was chosen as a demonstration site for remediation based on model calculations which predicted that if the source were removed, the site could be restored by natural microbial processes within a reasonable time. Source removal began in May 1991 and was completed 5 months later in September 1991. The entire volume of contaminated source material was excavated and hauled away to a soil remediation facility where the major coal-tar constituents were combusted and the residual sediment used as part of a paving mixture. Sandy soil from a local sandpit was used to replace the source material in a massive backfilling operation at the site. Contaminated groundwater was pumped and treated to remove soluble coal-tar constituents (*9,14*). After backfilling, the entire source area was restored to the original grade and an array of monitoring wells were installed. Subsequent chemical analyses of groundwater showed that PAH contaminants were successfully removed. Site 24 was delisted as a Superfund site. Groundwater in the plume area at the site still contains significant concentrations of soluble PAHs. Monitoring will continue for 10 years.

In October 1992, we sampled three key borehole stations to determine the initial impact of source removal on microbial distribution and activity patterns. As expected, the source area was most affected. The new activity and microbial distribution patterns resembled those previously seen at Site 97, where mixing of surface soil with subsurface sediment was suspected based on the occurrence of high numbers of typical soil microorganisms (e.g., endospore-forming bacteria) (*7*). Within the plume, the downgradient borehole (Figures 1,3) changed the least. ^{14}C-mineralization activity and protozoan numbers were still elevated in samples from the water table interface zone in the downgradient borehole. On the other hand, the pristine borehole station located outside the contaminant plume, which previously showed no ^{14}C-PAH mineralizing activity (Figure 1), now showed significant activity in previously inactive zones. Clearly, the restoration activities, including bulldozing of surface soil and movement of heavy equipment, which were carried out on the surface in the pristine borehole area for 5 months, impacted the sandy aquifer site. This area of New York State experiences large amounts of rain and snowfall each year. It is likely that recharge from the disturbed surface soil can account for the altered activity pattern in the pristine borehole zone.

Future Work at Site 24

We plan to continue working at Site 24, focusing on the distribution and *in situ* activities of the PAH-degrading bacteria at the site. A major effort is being made to develop new microscale methods (e.g., fluorescent antibody labeling and microautoradiography) for determining the distribution of PAHs and detecting specific bacterial types and their activities at the microscale (*15*). This will allow for better models of the microbial kinetics controlling *in situ* biodegradation rates. We are also studying the effects of sorption–desorption phenomena on bioavailability of PAHs. Sorption–desorption kinetics are believed to be an extremely important factor regulating biodegradation of many organic contaminants.

Ultimately, we plan to combine these approaches with the conventional approaches described above to give a much more comprehensive and accurate description of the activities of the PAH-degrading guilds and their potential effects on PAH biodegradation at Site 24.

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