Quantitative Relationship between Naphthalene Catabolic Gene Frequency and Expression in Predicting PAH Degradation in Soils at Town Gas Manufacturing Sites

James T. Fleming,*† John Sanseverino,‡ and Gary S. Sayler†

Center for Environmental Biotechnology, University of Tennessee, 10515 Research Drive, Suite 100, Knoxville, Tennessee 37932, and Technology Development, IT Corporation, Knoxville, Tennessee 37923

The inability to monitor in situ expression of biodegradative genes to predict rates of pollutant degradation or to evaluate the efficacy of engineering applications of bioremediation in complex environments such as contaminated soils has limited the routine acceptance of this technology in hazardous waste management. To overcome this limitation, an approach has been developed to measure catabolic gene expression in PAH-contaminated soils. In soils populated with as few as 10⁶ naphthalene degradative bacteria g⁻¹, in situ isolation and quantitation of mRNA levels was achieved for the NAH7 naphthalene dioxygenase (nahA) gene. NahA transcript levels correlated positively with [¹⁴C]naphthalene mineralization rates, soil naphthalene concentration, and nahA gene frequency.

Introduction

The chief compounds of concern in the coal tars, sludges, and oils that contaminate early manufactured gas plant (MGP) sites are polycyclic aromatic hydrocarbons (PAHs). These compounds pose a significant hazard because of their carcinogenicity and slow degradation in the environment. MGP sites may be ideally suited to reclamation by in situ bioremediation because an increasing number of PAHs including naphthalene (1), phenanthrene (2), anthracene (3), benzo[a]pyrene (4), benzo[a]anthracene (4), and pyrene (5) have been reported degradable by soil microorganisms. The immediate application of this technology is limited by the inability to predict degradative rates in situ due to a lack of knowledge about the expression of microbial biodegradative catabolic pathways in the environment and the rate limitations imposed by PAH desorption and bioavailability (6, 7). The application of nucleic acid based analytical methods, such as genetic probing, to environmental microbiology are well-suited to the quantitative monitoring of specific catabolic DNA sequences associated with the degradation of recalcitrant organic compounds. Characterization of the population genotype associated with a particular environment, therefore, constitutes a molecular diagnosis which permits the determination of the catabolic potential a site has to degrade the toxins present. Nucleic acids may be extracted in situ or directly from environmental samples, such as soils or sediments, thus avoiding the bias inherent in any method requiring cultivation of organisms (8–10). However, the development of models to predict biodegradative capabilities requires that, in addition to assessing the genetic potential of a particular indigenous population, methods must be developed to provide a direct measure of the instantaneous activity or expression of catabolic genes.

Activity studies and kinetic models based on mineralization of [¹⁴C]-labeled substrates in contaminated soils have required extensive mathematical interpretation based on a number of biological parameters such as cell population, predation, induction of active organisms, toxin accumulation, and depletion of inorganic nutrients to account for the many different types of curves obtained (11). Because all studied environmentally relevant bacterial catabolic genes are regulated at the transcriptional level, in situ quantitation of catabolic transcript levels followed by subsequent correlation to the particular catabolic cell population would assist in the interpretation and development of kinetic models from mineralization data and provide a good index of the instantaneous activity of soil bacteria. However, at the outset, such a procedure is replete with inherent technical difficulties. Even from pure culture, RNA is more difficult to isolate than DNA because of the ubiquitous presence of ribonucleases that quickly degrade single-stranded RNA (12). In addition, after in situ lysis, soil bacterial DNA may be irreversibly adsorbed to the soil matrix. Unlike DNA, the functional groups of the nitrogenous bases of single-stranded RNA may interact with soil particles and colloids.

We chose naphthalene degradation as a model system for studying PAH degradation because the pathway for naphthalene degradation is well-characterized and many of the genes encoding the enzymes have been cloned. Many Pseudomonas sp. containing plasmids encoding genes for naphthalene degradation have been isolated, and evidence suggests that the naphthalene genes are highly conserved (13). In addition, recent evidence has suggested that the nah genes also mediate the degradation of phenanthrene and anthracene (14). The 83-kilobase (kb) NAH7 plasmid from Pseudomonas putida G7 contains genes for 11 enzymes necessary for the degradation of naphthalene (13, 15). The structural genes are encoded in the two operons nah and sal; the first (nahABCDEF) encodes the enzymes that degrade naphthalene to salicylate, and the second (nahGHINLJK) encodes the enzymes that oxidize salicylate to acetalddehyde and pyruvate (13). Induction of both operons is positively controlled transcriptionally, not by naphthalene but by salicylate, the product of the nah operon (13). The nah operon is expressed at a low level without induction; in the presence of naphthalene, salicylate is produced which binds to the nahR gene product bound to the upstream promoter regions of both operons, thus transcriptionally activating the pathway. The half-life of the nahA mRNA, determined to be 12 min (unpublished observations), is long enough to permit isolation of undegraded transcripts from soil systems.

In this study, we have used nucleic acid hybridization techniques to determine the frequency and distribution of naphthalene genes in MGP soils and have developed a new approach to measure naphthalene gene expression by the isolation and quantitation of NAH7 mRNA levels.
The research has attempted to relate the observed mRNA levels to naphthalene gene frequency, [14C]naphthalene mineralization rates, and soil naphthalene concentrations.

**Experimental Methods**

**Chemicals and Reagents.** Chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO) and Baxter Chemical Co. (McGraw Park, IL). Naphthalene (scintillation grade, 99% pure) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Restriction endonucleases and competent cells were obtained from Bethesda Research Labs (Gaithersburg, MD). In vitro transcription kits and pBluescript vectors were purchased from Stratagene (La Jolla, CA). Ribonuclease protection assays were prepared by first linearizing pJF12 with BsaHI followed by transcription initiated from the T3 promoter using 3 μM [32P]UTP, 60 μM cold UTP, and 500 μM CTP, GTP, and ATP. Sense strand RNA was prepared by linearizing pJF12 with XhoI and initiating transcription from the T7 promoter. Both sense and antisense strands were gel purified on 5% acrylamide, 8 M urea gels. The amount of sense strand RNA transcribed was determined by the percent incorporation of [3H]UTP into the transcribed RNA and verified by spectrophotometry at 260 nm.

**Soil Samples.** Three MGP site soils and one creosote-contaminated soil were obtained under contract from the Gas Research Institute (Table I). All soils were received October 30, 1989, and stored at 4 °C in sealed glass bottles. Physical and chemical characterization including total soil naphthalene concentrations of MGP soils B, C, D, and the creosote soil G have been reported (16, 17).

**Bacterial Strains.** Pseudomonas putida G7 was obtained from Dr. David Gibson, University of Iowa. Uninduced liquid cultures of P. putida were grown in 150-mL Erlemeyer flasks with shaking at 28 °C in yeast extract–peptone–glucose (YPG) medium consisting of (gL⁻¹) yeast extract, 0.2; peptone, 2.0; NaH₂PO₄, 0.2; and D-glucose, 1.0. Induced P. putida cells were similarly grown at 28 °C in yeast extract–peptone–salicylate–sucinate (YPSS) medium consisting of (gL⁻¹) yeast extract, 0.2; peptone, 2.0; NaH₂PO₄, 0.2; sodium salicylate, 0.5; and sodium succinate, 2.7.

**Construction of Transcription Vectors.** A 558-bp KpnI fragment within the nahA gene (naphthalene dioxygenase), obtained by restriction of the NAH7 plasmid, was separated by agarose electrophoresis and cloned into the KpnI site of the pBluescript II SK (Stratagene, La Jolla, CA) multicloning region. The KpnI site within the N-terminal region of the β-galactosidase gene with opposable T3 and T7 RNA polymerase promoters. This nahA transcription vector, designated pJF12, was used to transform competent Escherichia coli J1M101. Transformants were selected as white colonies on plates containing X-Gal and IPTG.

A 2-kb HindIII–BglII fragment from P. pickettii pK01c, which encodes part of the phenol hydroxylase gene (18), was cloned into the HindIII–BamHI site of pBluescript II to construct pJF5.

**Nucleic Acid Probes.** In vitro transcribed RNA probes were prepared from pJF12 and pJF5 using an in vitro transcription kit (Stratagene, La Jolla, CA) according to the directions of the manufacturer. Anti-sense 32P-labeled nahA probes (10⁶ cpm/μg) for use in the ribonuclease protection assay were prepared by first linearizing pJF12 with BsaHI followed by transcription initiated from the T3 promoter using 3 μM [32P]UTP, 60 μM cold UTP, and 500 μM CTP, GTP, and ATP. Sense strand RNA was prepared by linearizing pJF12 with XhoI and initiating transcription from the T7 promoter. Both sense and antisense strands were gel purified on 5% acrylamide, 8 M urea gels. The amount of sense strand RNA transcribed was determined by the percent incorporation of [3H]UTP into the transcribed RNA and verified by spectrophotometry at 260 nm.

A 2-κb low specific activity (10⁶ cpm, μg⁻¹) in vitro transcribed 32P-labeled RNA was generated from the T3 promoter of pJF5 after linearizing the plasmid with XhoI. This probe was gel purified as described above and was used to estimate the efficiency of RNA extraction from soils.

**Enumeration of Soil Bacterial Populations.** The culturable soil bacterial population was determined for triplicate samplings of each soil with and without incubation in water. For the unincubated samples, 1 g of soil was added to 10 mL of a phosphate–saline buffer (pH 7.6) composed of (gL⁻¹) NaCl, 8.5; Na₂HPO₄, 1.2; Na₂HPO₄, 0.8; and sodium pyrophosphate, 1.0 in a 15-mL disposable centrifuge tube. The buffered soil slurries were then mixed on a vortex mixer for 2 min, serially diluted, and plated on nonselective 0.25 strength YEPG agar media (19, 20). Plates were incubated for 5–7 days at 28 °C. For the incubated samples, 1 g of soil was added to 0.5 mL of sterile deionized water in a 15-mL disposable centrifuge tube, loosely capped to allow aeration, and incubated with shaking at a 60° angle for 18 h at 27 °C. Following the incubation period, 4.5 mL of water and 5 mL of a concentrated phosphate–saline buffer (pH 7.6) were added to each tube to give the following final concentrations (gL⁻¹): NaCl, 8.5; Na₂HPO₄, 1.2; Na₂HPO₄, 0.8; and sodium pyrophosphate, 1.0. The samples were processed as described for the unincubated soils.

The naphthalene-degrading population was determined by colony hybridization using an anti-sense nahA probe generated from the T3 promoter of pJF12. Bacterial colonies on the Petri plates used for total population enumeration were transferred to nylon membranes and lysed by placing them on filter paper saturated with 0.4 N NaOH and 1.5 M NaCl, and the nucleic acids were fixed by baking at 80 °C for 1 h. Prehybridization, hybridization, and washing conditions for colony hybridization have been previously described (20). Washed filters were put on

---

**Table I. Comparative Soil Characteristics and PAH Concentrations in Soils Used in This Investigation**

<table>
<thead>
<tr>
<th>Soil Type</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>8.4</td>
<td>9.5</td>
<td>8.0</td>
<td>7.1</td>
</tr>
<tr>
<td>TOC</td>
<td>6 ± 1</td>
<td>33 ± 6</td>
<td>65 ± 91</td>
<td>112 ± 33</td>
</tr>
<tr>
<td>oil and grease</td>
<td>5.85 ± 1.39</td>
<td>5.3 ± 4.9</td>
<td>5.0 ± 0.9</td>
<td>61.7 ± 8</td>
</tr>
<tr>
<td>naphthalene</td>
<td>11 ± 7</td>
<td>1050 ± 553</td>
<td>52 ± 18</td>
<td>4400 ± 1000</td>
</tr>
<tr>
<td>phenanthrene</td>
<td>17 ± 23</td>
<td>341 ± 367</td>
<td>36 ± 20</td>
<td>9200 ± 1000</td>
</tr>
<tr>
<td>anthracene</td>
<td>33 ± 21</td>
<td>349 ± 214</td>
<td>29 ± 24</td>
<td>8800 ± 2000</td>
</tr>
<tr>
<td>pyrene</td>
<td>22 ± 18</td>
<td>184 ± 103</td>
<td>32 ± 12</td>
<td>3500 ± 560</td>
</tr>
<tr>
<td>benzo(a)pyrene</td>
<td>3 ± 1</td>
<td>67 ± 30</td>
<td>3 ± 2</td>
<td>330 ± 44</td>
</tr>
<tr>
<td>total PAHs</td>
<td>233 ± 149</td>
<td>3621 ± 186</td>
<td>34 ± 84</td>
<td>49 000 ± 28</td>
</tr>
<tr>
<td>silt/clay (%)</td>
<td>2</td>
<td>38</td>
<td>17</td>
<td>20</td>
</tr>
<tr>
<td>sands (%)</td>
<td>70</td>
<td>59</td>
<td>70</td>
<td>60</td>
</tr>
<tr>
<td>gravel (%)</td>
<td>28</td>
<td>3</td>
<td>12</td>
<td>20</td>
</tr>
</tbody>
</table>

---

* Cushy and Morgan, 1990. † Total organic carbon, g of TOC/kg of soil. ‡ Oils and grease, g/kg of soil. § Total soil naphthalene; mg of PAH/kg of soil.
X-ray (Kodak X-Omat RP) film with intensifying screens at -70 °C for 24-72 h.

**Isolation of P. putida G7 RNA from Pure Culture.** Total RNA was isolated from shake flask cultures of *P. putida* G7 using the RNA isolation method described by Oelmueller et al. (21). Uninduced cells were grown overnight in YEPG, and induced cells were grown overnight in YEPSS. RNA samples were ethanol precipitated and stored at -20 °C.

**In Situ Isolation of Soil Bacterial RNA.** Precautions were taken to ensure that the glassware, solutions, and reagents were free from ribonuclease (RNase) contamination. All solutions and plasticware used were treated with 0.1% diethylpyrocarbonate (DEPC) and autoclaved to inactivate ribonucleases. All glassware were baked overnight at 180 °C (12). Preliminary studies demonstrated the feasibility of mRNA recovery directly from soils inoculated with 10^8-10^10 bacteria/g of soil by in situ detergent lysis followed by exhaustive extraction of the soil (7, 22). However, more efficient mRNA recovery was obtained in situ from soil slurries using a modification of the hot phenol technique (21). Duplicate 10-g samples of each MGP and creosote soil were incubated in 5 mL of sterile water with shaking at 27 °C for 18 h. Sodium salicylate (Malnickrodt, Paris, KY) was added to one of the duplicate soil slurries to a concentration of 5 mM and allowed to incubate for 1 h. Each soil slurry was then immediately mixed with 20 mL of a buffer solution containing 50 mM sodium acetate, 50 mM sodium chloride, 5 mM EDTA, and 20% sodium dodecyl sulfate adjusted to a pH of 5.2 preheated to 60 °C. Twenty milliliters of a phenol/chloroform (50:50) solution preheated to 60 °C for 5 min was then immediately added to the slurry, and the slurry was shaken for 5 min at room temperature on a 'wrist action' shaker (Burrell, Pittsburgh, PA). The slurries were then cooled on ice for 5 min and centrifuged on a Beckmann TJ-6 centrifuge at 1 x 10^3 g for 10 min. The organic/sol phase was extracted and centrifuged two more times with 20 mL of extraction buffer in identical fashion. One-tenth volumes of 3 M sodium acetate were added to the combined aqueous extracts, and the nucleic acids were precipitated with the addition of 0.8 vol of 2-propanol and stored overnight at -20 °C. The following day the samples were centrifuged at 12000g for 45 min, the supernatants were poured off, and the pellet was dried at room temperature.

**Purification of in Situ Extracted Soil RNA.** The dried pellets were dissolved in 20 mM Tris and 1 mM EDTA and pelleted though a CsCl cushion (22) or applied to ion-exchange columns (Qiagen p-100, Chatsworth, CA) washed with 400 mM NaCl, and the RNA fraction was eluted with 900 mM NaCl and 6 M urea according to the manufacturer's protocol. The purified total RNA samples were precipitated, dissolved in DEPC-treated water, and treated with DNase (23) to remove any residual DNA. Whether purified by CsCl centrifugation or anion-exchange chromatography, the extracted RNA was dark brown in color, indicating contamination with soil humic materials.

**Efficiency of in Situ Contaminated Soil RNA Extraction.** The efficiency of the in situ RNA isolation procedure was determined as the percent recovery of a 32P-labeled pJF6 RNA internal standard. An in vitro 32P-UTP labeled transcript was generated from the T3 promoter of pJF6, purified on a 5% acrylamide, 8 M urea gel, eluted, and an identical amount (typically 5 x 10^5 cpm) added to each soil slurry and scintillation vial to compensate for decay of the 32P-labeled nucleotide. After purification and DNase treatment of the purified soil RNA, an aliquot of the RNA solution was used for scintillation counting to determine the percent recovery of the initial cpmS originally added (Figure 1). The mRNA values for each soil extraction as determined by the RPA were individually normalized on the basis of

**Quantitation of RNA by Ribonuclease Protection Assay.** The ribonuclease protection assay, as outlined in Figure 1, was performed using a RPAII kit, (Ambion Inc., Austin, TX) according to the manufacturer's recommendations. In situ extracted MGP soil bacterial RNA was hybridized with 2.0 x 10^5 cpm of 32P-labeled anti-sense nahA pJF12 RNA probe overnight at 45 °C in hybridization buffer. A nahA sense strand standard curve was created by hybridizing the 32P-labeled anti-sense transcript with increasing amounts of nahA sense transcripts. After RNase digestion, protected fragments were electrophoresed on 5% acrylamide and 8 M urea gels, and the gel(s) was (were) exposed on a single piece of X-ray film (Kodak X-Omat RP) for 48-72 h. The molecular weight band corresponding to the sense strand protected fragment was quantified against the standard bands using a Visage 110 (Millipore, Bedford, MA) computerized scanner. For absolute quantitation of a particular RNA sample, it was necessary to run preliminary gels to determine the approximate concentration of soil mRNA so the correct standard curve range could be chosen.

The mRNA values for each soil extraction as determined by the RPA were individually normalized on the basis of

**Figure 1.** Schematic for in situ soil bacterial mRNA isolation and the quantitation of nahA mRNA by the ribonuclease protection assay.
The percent recovery of $^{32}$PpJF5 from each extraction. No single correction factor was used for the soil samples.

**Estimation of Water-Soluble Naphthalene.** The water-soluble naphthalene component of the total soil naphthalene determined by use of the tar-water partition coefficient ($K_{ow}$) was estimated (24). The tar phase, rather than the particulate phase, is the dominant force in determining partition in these soils due to the high organic carbon content. The following relationship between the octanol-water partition coefficient ($K_{ow}$) and $K_{tw}$ has been reported for MGP soils (24):

$$\log K_{tw} = 1.13 \log K_{ow} + 0.33$$

(1)

The log $K_{ow}$ value for naphthalene used in the calculation was 3.37 (25).

**Mineralization Assays.** Naphthalene mineralization assays were performed by placing 2 g of each soil sample in 1 mL of minimal salts medium in a 25-mL EPA vial with a Teflon-lined silicone septum cap (Pierce, Rockford, IL). A 7.5-mL glass vial containing 0.5 mL of 0.4 M NaOH to trap CO$_2$ was added to each larger vial. Each sample was amended with 100 000 dpm of [14C]naphthalene (0.36 µg g$^{-1}$) in 10 µL of acetone. A series of vials, typically 40, were prepared for each soil sample, and the vials were shaken vertically at 100 rpm (stroke 2 cm) in the dark at 25°C for periods of 24–72 h. The assay was terminated by the injection of 0.5 mL of N$_2$H$_4$SO$_4$ into the outer sample vial. Duplicate vials were terminated at 2-h intervals for the first 12 h and at 4-h intervals thereafter. The vials were then allowed to sit for 30 min before 500 µL of the NaOH solution from the inner vial by duplicate samples was removed and added to 10 mL of Beckman Ready-Protein scintillation cocktail. 14CO$_2$ was quantified using liquid scintillation counting on a Beckman LS6501 scintillation counter. The H$_2$ method was used for automatic quench compensation, and dpm conversion from cpm was based on quench curves using 14C standards. The contents of the outer vial was extracted twice with 5 mL of hexane:2-propanol (4:1). [14C]Naphthalene remaining in the vial was determined by the addition of 0.5 mL aliquots of the hexane-phase extracts to Econofluor (DuPont, NEN products, Boston, MA) followed by scintillation counting. 14C-Labeled aqueous metabolites were estimated by performing a third extraction with 5 mL of distilled water followed by scintillation counting of 0.5 mL of the water extract. Soil populations at the various time points of the mineralization experiment were not enumerated.

**Determination of Mineralization Rates.** The specific activity of total naphthalene was determined by dividing the microcuries of [14C]naphthalene added to the slurry by the calculated number of micromoles of the water-soluble naphthalene component of the total naphthalene. Mineralization rates were determined by plotting the number of micromoles of 14CO$_2$ produced vs time at 2-h intervals for the first 12 h and at 4-h intervals for the remainder of the experiment. The slope of the linear portion ($r^2 \geq 0.95$) of each curve was used as an estimate of the mineralization rate.

**Results and Discussion**

**Bacterial Populations.** The total bacterial population of each soil was initially determined without an incubation period. Total heterotrophic bacterial populations for the unincubated soils ranged from 1.1 ± 0.3 x 10$^8$ to 3.4 ± 0.8 x 10$^8$ cfu (g of soil)$^{-1}$ (Table II). A series of soil samples were also incubated for 18 h at 27°C in a soil slurry prior to population enumeration and mRNA isolation to approximate the soil slurry conditions created during the mineralization experiments, thus permitting a better comparison of the results for these two sets of experiments. The total heterotrophic bacterial populations of the three incubated MGP soils and the creosote-contaminated soil ranged from 2.8 ± 0.2 x 10$^9$ to 3.4 ± 0.6 x 10$^8$ cfu (g of soil)$^{-1}$ (Table II). Soil populations of incubated and unincubated samples were compared using a Student's t-test for means. The populations of all four soils differed significantly (P = 0.05) from those of the unincubated soils.

Plates from the incubated soil samples were subsequently transferred to nylon membranes, and colonies were probed for the presence of the naphthalene dioxygenase gene (nahA). The nahA positive populations ranged from 4.0 ± 1.73 x 10$^9$ to 3.2 ± 0.4 x 10$^7$ cfu g$^{-1}$ representing 1–35% of the total bacterial populations. NahA positive colonies correlated poorly, though positively, with soil naphthalene levels may be due to the presence of naphthalene-degrading bacteria nonhomologous to the NAH7 genotype that do not probe positive with nahA gene probes.
Mineralization Assays. The $^{14}$C-label was followed through the carbon dioxide production, the aqueous metabolites in the water phase, the remaining $[^{14}$C$]$-naphthalene that partitioned into the organic phase, and the immobilized $^{14}$C that remained with the soil permitting the overall percent recovery to be calculated. The immobilized phase was assumed to be irreversibly sorbed or trapped in the soil at the conclusion of the mineralization experiment. That each of the four soils was catabolically active was confirmed by the observation of $[^{14}$C$]$naphthalene mineralization (Table II). Total $^{14}$C recovery varied from soil-to-soil and ranged from 64 to 92%. Mass balance for soil G could not be determined. All of the contaminated soils studied mineralized $[^{14}$C$]$naphthalene to $^{14}$CO$_2$ with percentages ranging from 17.5% to 81.1% (Table II).

First-order mineralization rates were determined by calculating the water-soluble naphthalene and calculating the specific activity for $[^{14}$C$]$naphthalene. The water-soluble naphthalene concentrations ranged from $2.9 \times 10^{-1}$ to $8.0 \times 10^{-4}$ µg mL$^{-1}$ (Table II). The obtained mineralization rates ranged from $3.2 \times 10^{-5}$ to $7.8 \times 10^{-1}$ µg of $[^{14}$C$]$naphthalene (g of soil)$^{-1}$ h$^{-1}$ (Table II).

Because the $[^{14}$C$]$naphthalene used was labeled at the C1 carbon, complete mineralization of labeled naphthalene to CO$_2$ cannot be proven on the basis of the data presented here. However, the symmetry of the naphthalene molecule suggests that, statistically, 50% of the added [1-$^{14}$C]naphthalene will be metabolized as $[5-$14C$]$naphthalene. For the case of soils D and G which showed 73 and 81.1% recovery of $^{14}$CO$_2$, respectively, we can be confident that at least both rings of the naphthalene molecule were opened. On the other hand, attack of both naphthalene rings in soils B and C, which showed 33.8 and 17.5% recovery of $^{14}$CO$_2$, respectively, cannot be assumed.

It is quite possible that the addition of [1-$^{14}$C]naphthalene to the soils may have induced growth over the time period of the experiment, because the amount of labeled naphthalene added to the soils (0.37 pg/mL) exceeded the calculated water-soluble endogenous naphthalene by as much as several orders of magnitude (Table II). This consideration points out an inherent weakness of mineralization assays: the microbial population is unavoidably perturbed by the addition of labeled substrate. Because degradative rates are affected by adsorption to soil matrix components (29), exogenously added labeled substrate may represent a fast-reacting pool of unabsorbed substrate and, thus, be a limited model for the actual availability of an endogenous pollutant (27).

Quantitation of Contaminated Soil mRNA. One difficulty involved with in situ extraction procedures, as opposed to methods that involve initial separation of cells from the soil matrix, is that soil organic materials, such as humic acids, copurify with nucleic acids, even when a cesium chloride (CsCl) gradient centrifugation step is used. Although we found ion-exchange chromatography to yield a more purified sample compared to CsCl purification, the extracted RNA was still quite contaminated with humic materials (data not shown). Because of the UV absorbance properties of these humic contaminants, the usual method of optically quantifying RNA was not possible. We chose, therefore, to quantify nahA mRNA directly using the ribonuclease protection assay (RPA). Unlike Northern or slot blots, which involve hybridization to mRNA immobilized to a membrane, the RPA involves a solution hybridization step, thus eliminating concern about poor adherence of contaminated RNA to nylon membranes. In this procedure, a solution containing soil RNA is allowed to hybridize under optimum conditions to a labeled in vitro transcribed anti-sense RNA probe complementary to the gene of interest. The resulting double-stranded RNA hybrid is subjected to ribonuclease digestion which degrades any nonhybridized single-stranded probe. The 'protected' double-stranded hybrid may then be quantified by comparison with protected in vitro transcribed sense strand standards (Figure 1). This method has a reported sensitivity of 0.1 pg of specific RNA (29) and is more conductive to absolute mRNA quantitation from soil extractions than are polymerase chain reaction (PCR) based methods (29) because soil contaminants may interfere with the PCR enzymatic steps (30). The sensitivity of the method is dependent on the specific activity of the anti-sense probe used; in our hands the limit of detection was 1 pg of nahA RNA using a probe with a specific activity of $10^8$ cpm/µg.

The recoveries of the $[^{32}$P$]pJF5$ RNA probe were variable ranging from 1.6 to 50% (Table II), depending primarily on the choice of final RNA purification method. The average recovery using CsCl gradient centrifugation was 22.1 ± 24.2% ($n = 3$), while the average recovery using anion-exchange chromatography was 5.0 ± 4.3% ($n = 4$). While CsCl centrifugation gave a higher recovery, anion-exchange chromatography resulted in a more highly purified product giving cleaner protected fragments with the RPA (Figure 2). Not enough data was obtained to observe trends between particular soil type, degree of soil contamination, and recovery efficiencies. The choice of the phenol hydroxylase RNA (pJF5) for the internal standard, other than for the reason that it showed no hybridization with nahA, was purely arbitrary. The assumption that recovery of the added in vitro transcribed pJF5 is necessarily related to the recovery of endogenous soil bacterial RNA may be invalid. The percent recovery may be a function of transcript length, secondary structure, and actual sequence, though this was not examined by the experiment.

The amount of mRNA recovered from each MGP soil and the creosote-contaminated soil, as determined by the ribonuclease protection assay, was individually normalized on the basis of the percent recovery of added $[^{32}$P$]pJF5$ RNA for each soil extraction to calculate the nahA mRNA (g of soil)$^{-1}$. NahA transcript levels for the uninduced soils ranged from 2 to 75 pg (g of soil)$^{-1}$ (Table II). Uninduced soil nahA mRNA levels correlated linearly with soluble soil naphthalene concentrations ($r^2 = 0.919$) (Figure 3). While labeled RNA recoveries were variable depending, in part, on the purification procedure used, calculated nahA transcript levels obtained using either CsCl centrifugation or anion-exchange chromatography were comparable (Table II, soils C and G). NahA transcript levels also correlated positively with $^{14}$C mineralization rates ($r^2 = 0.957$) (Figure 4).

Recovery of nahA mRNA from soil B, while initially successful, was not reproducible, suggesting that a soil cell population of greater than approximately $4 \times 10^4$ inducible cells g$^{-1}$ is required for quantitation by RPA of soil-extracted mRNA. The absolute number of cells required is, of course, dependent on the level of induction. However, the inability to show further nahA induction of
these soils suggests that they may have been fully induced, and this is a reliable limit for PAH-contaminated soils.

**In Situ Induction of nahA mRNA.** For soils C, D, and G with 7.9 × 10^{-2}, 2.0 × 10^{-2}, 2.9 × 10^{-2} μg of soluble naphthalene mL^{-1}, respectively, the nahA operon was not further induced with added salicylate. This finding suggests that addition of [14C]naphthalene probably did not further induce the nahA positive bacteria, and thus the obtained mineralization rates are representative of unperturbed expression. While the bacteria in these soils may have been fully induced, and thus not affected by added inducer, an alternative explanation for lack of nahA transcript inducibility may be due to the presence of inhibitory contaminants. Cyanide (CN) was present in all the soils studied but at relatively low concentrations of 1–12 μg kg^{-1} (16). While all the soils were contaminated with metals such as Al, Ag, Ar, Cd, Cr, Cu, Fe, Pb, Hg, Ni, and Zn, the concentrations were roughly comparable (16). Each of the four soils was catabolically active as determined by positive [14C]naphthalene mineralization rates (Table II). In addition, lack of inducibility was apparently not due to limiting soil nutrients such as nitrogen or phosphorous because all the soils incubated in sterile water showed growth without addition of nutrients.

**Conclusions**

The ability to predict the degradative fate of pollutants in contaminated environments requires quantitative information about site-specific microbial populations and their biodegradative activities in situ. We have developed an in situ soil mRNA quantitation procedure that permits study of the instantaneous activity of soil microorganisms and allows in situ soil bioremediation treatment interventions to be monitored on the transcriptional level. However, the correlation of a single transcript with the integrated activities of multioperon catabolic pathways and the function of the enzymes they encode seems unlikely. This is especially the case in the environment where parameters such as limiting nutrients, adverse pH, and toxic chemical effects may differentially effect operon transcription and enzyme activities. In addition, different catabolic pathways may be differentially affected by these environmental parameters. However, the strong corre-
lation between nahA mRNA and [14C]naphthalene mineralization rates, taken here as the definitive measure of catabolic activity, suggests that transcriptional analysis may also be a valid index of catabolic activity. The prominence of naphthalene dioxygenase in the degradation of PAHs and the coincidental regulation of both nah operons by salicylate may make a good case for MGP soils as a model system for the assessment of catabolic activity by transcriptional analysis. In this regard, transcriptional analysis may provide complimentary information to that obtained by mineralization studies where the addition of labeled substrate may actually induce the system under study.

Due to the substantial time lag between soil sampling, chemical analysis, delivery, storage, and the experiments performed in our laboratory, these results are not truly representative of the soils under field conditions because the concentrations of naphthalene and specific catabolic microbial populations may have been altered over time. While the conditions under which these soils were studied here more closely approximate a soil treatment process rather than conditions found in unperturbed field samples, the method should be applicable to the study of field soils. For in situ study, soil samples could be quickly frozen on site and transported to the laboratory for analysis. In situ RNA extraction might be useful to assess the endogenous degradative ability under natural field conditions as it responds to bioavailable pollutant substrates or engineered treatment regimes such as hydraulic flow, nutrients, electron acceptors, or metabolic inducers.

Literature Cited

(2) Evans, W. C.; Fernley, H. N.; Griffiths, E. Biochem. J. 1965, 91, 15P–16P.
(17) Blackburn, J. W.; DiGrazia, P. M.; Sanseverino, J. Treatability and scale-up protocols for polynuclear aromatic hydrocarbon bioremediation of manufactured gas plant soils; Gas Research Institute Report 5087-253-1490; Gas Research Institute, Chicago, IL, 1991.

Received for review May 18, 1992. Revised manuscript received January 4, 1993. Accepted February 16, 1993.