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0273-2289/96/5758—0971$8.00

Estimating Biodegradative Gene Numbers at a JP-5 Contaminated Site Using PCR

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ABSTRACT

We have utilized a most-probable-number polymerase chain reaction (MPN-PCR) procedure to estimate gene numbers and biodegradative potential at a jet fuel (JP-5) contaminated site undergoing the first phase of bioremediation. Nucleic acid analysis was used to determine whether a lack of genetic potential for bioremediation was responsible for low levels of oxygen utilization at the site. Total community DNA was extracted and analyzed by PCR for genes (*nahAc*,*alkB*, and *xylE*) known to be involved in the degradation of certain JP-5 constituents. Results indicate that significant aromatic biodegradative potential exists at the site and outlying areas not subjected to engineered remediation, suggesting that physical and/or chemical factors are inhibiting oxygen delivery. *xylE* and *nahAc* were often present in significant portions of the microbial community, whereas *alkB* was rarely detected. This study illustrates the utility of molecular techniques in evaluating biodegradative potential in the field during active bioremediation.

Index Entries: PCR; bioremediation; jet fuel; JP-5; community DNA.

INTRODUCTION

Advances in nucleic acid extraction and detection techniques have recently allowed microbial ecologists to analyze more fully complex, mixed microbial communities in their natural environments independent of culturability (*1–4*). The ability to examine total community DNA removes inherent biases associated with traditional culture-based methods (*5*), and can circumvent postsampling changes in community structure associated with handling and storage of samples for metabolic activity assays (*6–8*). Recently, molecular techniques have been used to detect specific functional genes in soil and sediment communities (*9–12*).

Recent laboratory experiments (*13*) suggest that the scope of environmental contaminants amenable to *in situ* remediation is much greater than previously believed. However, the immediate application of remediation technologies (e.g., bioventing and air sparging) can be limited by an inability to predict degradative potential or effectively stimulate catabolic pathways *in situ*. Nucleic acid-based technologies are well suited for detecting specific catabolic DNA sequences in environmental samples and can provide a molecular diagnosis of the initial

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biodegradative potential of a site prior to bioremediation activities. In addition, nucleic acid techniques can monitor changes in microbial community structure in response to remediation activities, complementing classical measurement methods and providing a relatively unbiased assessment of *in situ* performance. Nevertheless, very few culture-independent, molecular studies have examined to what extent microbial populations change during bioremediation of contaminated sites.

Whereas gasoline and many other fuels are relatively easy to bioremediate *(14)* the jet fuel JP-5 is more recalcitrant, being composed of C_{10}-C_{15} alkanes and polyaromatic hydrocarbons *(15)*. At the Fallon Naval Air Station (Fallon, NV), an *in situ* respiration test within the study site's JP-5 plume showed low levels of oxygen utilization even though bioventing was being performed, whereas an *in situ* respiration test at a nearby location with little or no JP-5 contamination indicated very high levels of oxygen utilization *(15)*. This finding led to efforts to determine if microbial biodegradative potential existed at the former site, but was not being stimulated. The objective of this study was to apply community DNA extraction and polymerase chain reaction (PCR) methods to assess rapidly the levels of specific biodegradative genes at different locations within a field site contaminated with the jet fuel JP-5. Because no *a priori* information existed about population densities of fuel-degrading bacteria at the site, a PCR-based approach was used instead of DNA hybridization analysis to increase the sensitivity for detecting target genes residing in a small percentage of the microbial community.

METHODS

Site Description and Sample Collection

The study site was located on the Fallon Naval Air Station in Fallon, Nevada (Fig. 1). The first phase of bioremediation (bioslurping) is primarily a physical fuel-recovery process. Bioslurping combines low-flow rate bioventing (air injection) of the vadose zone with recovery of fuel from the capillary fringe and from the top of the water table using a vacuum system *(16)*. Once fuel recovery ceases, bioventing is increased to bioremediate the remaining contamination. The bioslurping field was approx 55 × 73 m and contained 48-wells. Sediment samples were collected at three locations: the east road ditch (ED), west of the road (WR), and at the bioslurping site. A sample was taken from the bioslurping site after 18 mo of operation. The ED sampling location was selected based on previous groundwater monitoring that indicated the region was contaminated with a mixture of fuels, including gasoline *(15)*. The WR sampling location was selected as a region outside the influence of bioslurping and contained lower levels of JP-5 than did the bioslurping site. *In situ* respiration tests *(17)* had been conducted previously at the bioslurping site and ED location.

The ED and WR sites were sampled prior to any remediation efforts and then resampled after vent wells were installed and vented continuously for 4-d at approx 20 standard ft^3/min. Postventing samples were taken 0.3–0.6 m from the vent well and within 1 m of the prevent samples. All samples were taken from the capillary fringe, 1.5–2.1 m below the surface, with a hand-driven 5-cm diameter bucket auger. Each sample was homogenized on a clean surface, and approx 200 g were placed into a whirl-pak bag. Each bag was wrapped and sealed with strapping tape. Samples were placed in a cooler with wet ice and shipped by overnight express to our lab in Richland, WA. On receipt, an aliquot was removed for microscopic acridine orange counts, and the remainder was frozen at –20°C.

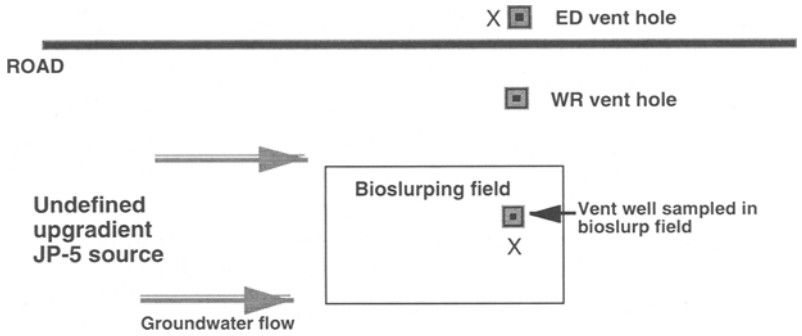

Fig. 1. Schematic representation of the Fallon site. Exact source and size of JP-5 plume is unknown. Picture is drawn approx to scale. ■, Sample location; X, *in situ* respiration test; ED, east ditch sample; WR, west of the road sample.

Total Petroleum Hydrocarbon (TPH) and Benzene-Toluene-Ethylbenzene-Xylenes (BTEX) Analysis

Samples were analyzed for the presence of BTEX and TPH. The samples were not collected and handled in a manner conducive to definitive quantitative analysis of contaminant levels. However, in order to verify their presence and to infer relative levels of contamination, the samples were analyzed for BTEX and TPH by two different laboratories (Battelle, Columbus, OH and Battelle, Richland, WA). Five-gram aliquots of frozen samples were placed in polypropylene vials, sealed, and refrozen for 2–7 d before analysis. The BTEX and TPH levels in each sample were determined by extraction with methanol and a heated purge and trap technique, which detects volatile and semivolatile compounds *(18)*. Following the methanol extraction and purge and trap processes, analysis was accomplished via gas chromatography (GC). TPH levels were reported as equivalent to hexane, based on the total peak area counts observed for each GC analysis, or by ranking the samples based on the GC peak area, which corresponded to alkanes.

Acridine Orange Direct Counts (AODC)

Ten grams of sediment were placed in 95 mL 0.1% pyrophosphate (pH 7.0) and shaken for 30 min on a reciprocating shaker at 150 rpm. Dilutions were performed in saline such that 1 mL containing the equivalent of 0.01 g sediment was added to 100 µL 0.1% acridine orange, and after 2 min, the solution was filtered through a 1.6-cm diameter, 0.2 µm pore size Nucleopore polycarbonate filter membrane. One milliliter sterile water was added to the membrane and filtered to remove excess acridine orange. Green- and red-fluorescing cells were counted in 20 randomly selected fields with a Nikon epifluorescent microscope using a 100× objective and 10× eyepiece.

Total DNA Extraction from Sediments

Total community DNA was isolated from 50 g sediment using a combination of direct lysis *(19)* and ballistic disintegration *(20)*. Briefly, freshly thawed sediments were transferred to sterile plastic bottles, to which 50 mL extraction buffer (0.2*M* sodium phosphate, 0.1*M* EDTA, pH 8.0) and 5.5 mL 20% sodium dodecyl sulfate

(SDS) were added. Samples were thoroughly mixed and frozen at –80°C. Individual samples were thawed for 30 min at 65°C with occasional mixing, and then combined with 50 g 0.1-mm glass beads in a bead-beater cup (BioSpec Products, Inc., Bartlesville, OK). Samples were blended for 30 s, transferred to sterile 250-mL centrifuge bottles, and the sediment/beads removed by centrifugation at 8000*g*, 20°C for 5 min. The resulting supernatant was transferred to a new tube, and the sediment re-extracted as described above with 35 mL fresh extraction buffer containing no SDS. Supernatants were combined, and the centrifugation/extraction was repeated once more (3× total) before the sediment was discarded. A reagent/bead "blank" was also extracted in identical fashion to test for possible crosscontamination during processing.

Pooled supernatants (~130 mL total) were placed in large dialysis tubing (Spectrapor; 12,000 to 14,000 mol-wt cutoff; 28.6-mm diameter; Spectrum Medical Industries, Inc., Los Angeles, CA) and dialyzed extensively (5–6 buffer changes over 24 h) against sterile TE (10 m*M* Tris, 1 m*M* EDTA, pH 7.8) to remove salts and residual hydrocarbons. To reduce dialysate volumes, bags were then packed in solid polyethylene glycol (PEG; 15,000–20,000 average mol-wt; Sigma, St. Louis, MO) overnight at 4°C. Sediment extracts were then transferred to sterile 50-mL Oak Ridge tubes, and nucleic acids were precipitated overnight by addition of 0.2 volumes 10*M* ammonium acetate and 1 vol isopropanol. Nucleic acids were collected by centrifugation at 10,000*g* for 15 min at room temperature, and the resulting pellet washed once in 70% ethanol. Pellets were dried under vacuum, resuspended in sterile TE, and further purified by CTAB extraction as described by Xia et al. *(21)*. Nucleic acids were resuspended in a final volume of 50 µL TE. DNA averaged 4–8 kbp in length, which is adequate for PCR amplification. Coextraction of humic compounds and other soil constituents prevented quantitation by conventional UV absorption or fluorometry, so DNA recovery was estimated by gel electrophoresis against known amounts of pure DNA standard *(22)*. Extraction efficiency was estimated by comparing DNA yield to the theoretical DNA yield derived from acridine orange direct counts (AODC), using 8.3 fg DNA/cell as the DNA content for an "average" soil bacterium *(23)*.

Primer Design

PCR primers were designed for three genes known to be involved in hydrocarbon degradation; alkane hydroxylase *(alkB)*, catechol–2,3-dioxygenase *(xylE)*, and naphthalene dioxygenase *(nahAc = doxB = ndoB = pahAc)*. *AlkB* primers were based on the single available sequence from *Pseudomonas oleovorans (24)*. The *alkB* forward primer (alkB.f) was 5'-CTG-GAT-TCC-GCT-CCA-GAG-TAC and the reverse primer (alkB.r) 5'-AAT-AAG-GAC-ACC-ACC-AGC-CCA. The cosmid pGEc–29 *(25)* served as a positive template control, producing a PCR product of approx 1085 bp. Primers for *xylE* were based on a manual alignment of *Pseudomonas putida* catechol–2,3-dioxygenase genes available on the ENTREZ data base, but could not be made sufficiently degenerate to amplify similar genes in other gram-negative or gram-positive organisms. Sequences for the *xylE* primers were (xylE.f) 5'-AGG-NGT-(A,T)AT-GCG-ICC-IGG-CCA-(C,T)GT for the forward primer and (xylE.r) 5'-TCG-TG(A,G)-TA(A,G)-AAG-AT(G,C)-GCC-TTG-CC for the reverse primer. Parentheses indicate mixed base positions. Plasmid pLV1012 *(26)* served as a positive control, producing a PCR product of approx 900 bp. *NahAc* primer sequences

were (nahAc1) 5'-GTT-TGC-AGC-TAT-CAC-GGC-TGG-GGC (forward primer) and (nahAc3) 5'-TTC-GAC-AAT-GGC-GTA-GGT-CCA-GAC (reverse primer), and share 100% sequence identity with other polyaromatic hydrocarbon oxygenase genes *(27–29)*. Plasmid pSAD15–47 *(28)* was the positive control, yielding a PCR product of approx 700 bp. All primers were synthesized by Keystone Laboratories, Inc. (Menlo Park, CA).

Most Probable Number PCR (MPN-PCR)

An estimate of gene density in environmental samples can be achieved using most probable number PCR (MPN-PCR) *(30,31)*. Ten-fold serial dilutions of sediment extracts were prepared in TE (to 10^{-5} dilution) and a single PCR reaction was performed at each dilution. Primers (0.5 µL each from 20 µ*M* working solutions in 0.1X TE pH 7.8) and 1 µL template DNA were held at 80°C in 40 µL total volume under a 50 µL oil overlay ("hot start") before 60 µL enzyme master mix were added. Final reaction conditions were: 10 m*M* Tris-Cl, pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 200 µ*M* each dNTP, 0.1 µ*M* each primer, 2.5 U LD-*Taq* DNA polymerase (Perkin-Elmer, Foster City, CA), and 1.5 µg T4 gene 32 protein (Boehringer Mannheim, Indianapolis, IN). Cycling conditions were 5 cycles at (1.5 min, 94°C; 30 s, 55°C; 2 min, 72°C) followed by 35 cycles at (1 min, 94°C; 30 s, 65°C; 2 min, 72°C with a 5 s extension/cycle) using a Perkin-Elmer 480 thermal cycler. A final 20 min, 72°C extension was performed before samples were held at 4°C. The same cycling conditions were used for all three primer pairs (alkB, xylE, nahAc). Five types of PCR reactions were conducted to estimate minimum gene detection limits and sediment inhibition of amplification. They were:

1. 1 µL sediment extracts at the five dilutions;
2. Positive control plasmid templates (10^2 copies for *nahAc* and *alkB*, 10^3 copies for *xylE*) run "clean" (no added sediment extract);
3. Positive control plasmids spiked into 10^1 and 10^2 dilutions of the sediment extracts to test for inhibition of amplification *(32)* ;
4. A no template reaction to test for contamination of PCR components; and
5. One microliter from the blank extract to test for possible cross–contamination of samples during processing. Results from reactions 2–5 were used to define the PCR conditions.

Ten-microliter aliquots of the reaction products were analyzed on 1% agarose gels, containing 30 ng/mL ethidium bromide, in 1X TAE (40 m*M* Tris-acetate, 2 m*M* EDTA) running buffer. PCR products were detected visually by UV fluorescence at 300 nm and photographed. An estimate of gene densities in sediment samples was derived from:

1. The PCR results in the dilution series of the sediment extract;
2. The detection level of PCR conditions; and
3. The estimated extraction efficiency.

For example, the presence of a positive PCR signal through the 10^4 dilution of the sediment extract, a detection level of 300 gene copies, and a 1% extraction efficiency would result in $10^4 \times 300 \times (100\%/1\%) = 3 \times 10^8$ estimated gene copies/gram of starting sediment. An estimate of the minimum gene density (per gram of starting sediment) required to observe a PCR signal from the initial sediment was calculated

as $P_{AODC} - (D_{DL} - D_{IO})$. P_{AODC} is the population based on acridine orange direct counts, D_{DL} is the dilution where the number of theoretical genomes (determined from actual DNA yield) in the PCR reaction becomes <the detection level of the PCR conditions, and D_{IO} is the dilution where inhibition by the sediment extract is overcome. For example, if the AODC is 10^9, the PCR detection level is exceeded at the 10^4 dilution, and humic inhibition is removed at 10^1 dilution, then the minimum gene density in the original sediment required to observe a PCR product would be approx 10^6/gram.

RESULTS

DNA extraction and PCR analyses for five samples, the blank extract, and controls required <3 d of hands-on time and <5 d of cumulative time. Thus, these methods allowed rapid analysis of samples. Control reactions with no added template never produced PCR products, indicating that PCR components never became contaminated with genomic DNA or amplification products. However, amplification of *xylE* and *nahAc* did occur in control reactions containing undiluted blank extract when PCR conditions were conducive to a 30-copy detection limit. In combination, the no-template and blank extract results suggested crosscontamination during processing. PCR products could be generated from 30 copies of *alkB* and *nahAc*, but a 10–fold higher template concentration (300 copies) was required to produce a *xylE* PCR product; the higher concentration was likely the result of degeneracy in the *xylE* primers. Positive controls, spiked into sediment extract, showed that the PCR was usually inhibited by the sediment extracts at the 10^1 dilutions, although the level of inhibition varied for each sediment and primer set (Table 1). To ensure that PCR products were authentic and not the result of crosscontamination, the number of amplification cycles was reduced to 40 for all three primer sets, such that approx 300 copies of *alkB* and *nahAc* , and approx 3000 copies of *xylE* were required to produce a PCR product detectable by agarose gel electrophoresis and ethidium bromide staining. Under these conditions, *xylE* and *nahAc* could not be detected from the blank extract at the 10^1 dilution, the initial dilution tested for all sediment extracts. The detection level set by the PCR conditions, differences in extraction efficiency between samples, and differences in the PCR detection level for different primer sets affected the minimum number of gene copies required to detect a PCR product; this detection limit was specific for each sediment and primer set, ranging from $\geq 10^3$ to $\geq 10^6$ copies/gram sediment (Table 2).

Amplification signals were obtained for the *xylE* and *nahAc* genes in all samples (Table 1). Estimates of gene densities suggest that a high percentage of the microbial population contained naphthalene and catechol biodegradative genes at the ED site before venting (Table 2), assuming one gene/organism. Levels of *nahAc* genes (10^5 to 10^9 copies/gram) were more variable among the different samples than for *xylE* (10^7 to 10^9 copies/gram). Estimated densities of *nahAc* and *xylE* genes were in the order: ED > bioslurping site = WR. After venting at the ED and WR locations, neither *xylE* nor *nahAc* appeared to increase above their preventing levels. The *alkB* amplicon was detected at the bioslurping site (10^6/gram) and at the WR location after venting (10^5/gram), but in no other samples. Occasionally, PCR products different in size from the positive controls were observed (for example, Fig. 2). Whether or not these alternate products were derived from target (or closely related) genes was not determined.

Table 1
Qualitative MPN-PCR Detection of Catabolic Genes[a]

Sample	Site	Gene	Dilution of sediment extract 10^1	10^2	10^3	10^4	10^5
	ED	*xylE*	H[b]	+	+	+	+/–[c]
1		*nahAc*	H	+	+	+	+
		alkB	H	–	–	–	–
	ED	*xylE*	H	H	+	+	–
16	after	*nahAc*	+	+	+	+	+
	venting	*alkB*	H	H	–	–	–
	WR	*xylE*	H	+	+	–	–
6		*nahAc*	H	+	+/–	–	–
		alkB	H	–	–	–	–
	WR	*xylE*	H	+	+	–	–
12	after	*nahAc*	H	+	–	–	–
	venting	*alkB*	H	+/–	–	–	–
	Bioslurping site	*xylE*	+	+	–	–	–
9		*nahAc*	+	+	+/–	–	–
		alkB	+	+/–	–	–	–

[a]+/– Results were counted as positive when estimating gene densities.
[b]H = Inhibition of the reaction, probably owing to humic compounds, as judged by extracts spiked with 300 copies of the positive control plasmid.
[c]+/- Indicates an observed PCR product that was less intense than the signal generated from the positive control plasmid.

Table 2
Estimated Catabolic Gene Densities/Gram Sediment[a]

Sample	Estimated Gene Density[b] *xylE*	*nahAc*	*alkB*	AODC
ED	2×10^9	2×10^9	ND[c]	1×10^9
	(3×10^6)	(3×10^5)	(3×10^5)	
ED after venting	1×10^8	1×10^8	ND	2×10^9
	(5×10^6)	(5×10^3)	(5×10^5)	
WR	2×10^7	2×10^6	ND	2×10^8
	(7×10^5)	(7×10^4)	(7×10^4)	
WR after venting	1×10^7	1×10^5	1×10^5	3×10^8
	(1×10^4)	(1×10^3)	(1×10^3)	
Bioslurping site	2×10^7	2×10^7	2×10^6	6×10^8
	(2×10^5)	(2×10^4)	(2×10^4)	

[a] The minimum gene density required for detection in each sample is indicated in parentheses.
[b]Gene density (copies per gram) calculated as (detection limit) × (highest dilution with positive PCR signal), normalized to 100% extraction efficiency. *See text* for example calculation.
[c]PCR product not detected.

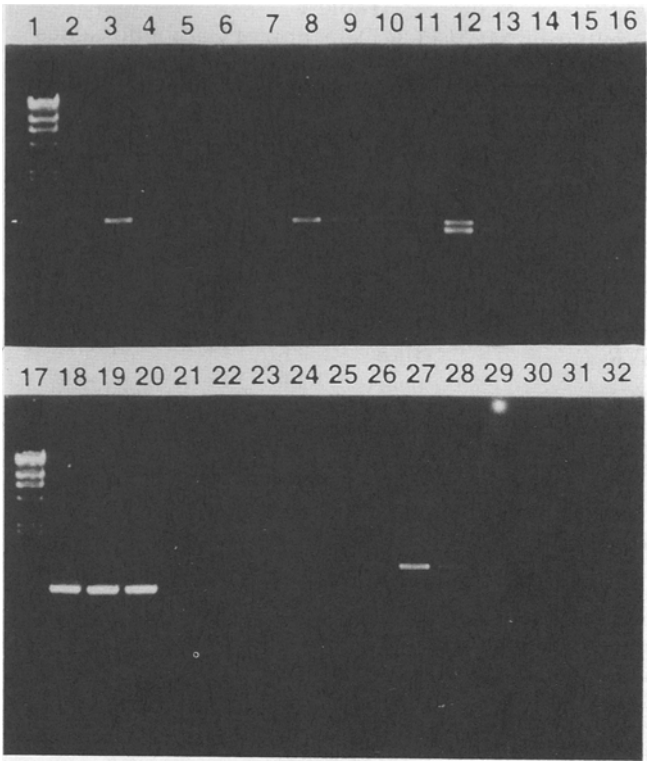

Fig. 2. Biodegradative gene detection at the bioslurping site (sample 9) using the polymerase chain reaction. Lane assignments are: 1 and 17, λ × HindIII; 2–10 are *xylE* PCR products in the order no template, 10^1–10^5 dilution of sediment extract, 10^1 dilution of sediment extract containing *xylE* positive control plasmid, 10^2 dilution of sediment extract containing *xylE* positive control plasmid, and *xylE* positive control plasmid alone; lanes 11–20 are in the same order as 2–10, except they show *nahAc* PCR products; lanes 21–29 are also the same, except they show *alkB* PCR products; lanes 30–32 are blank. In this sample, there was no humic inhibition of the PCR at 10^1 or 10^2 dilution of sediment extract. Band intensities were reduced during photo reproduction.

AODC values were high in all sediment samples, approx 10^8 to 10^9 microorganisms/gram (Table 2). Total bacterial counts were highest at the ED location, where the *in situ* respiration test showed high levels of oxygen utilization. The bioslurping site, where bioslurping and bioventing had been occurring for 18 mo, had higher AODC values than the WR location. The 4-d bioventing at the ED and WR locations appeared to increase slightly the total bacterial populations, but values were not statistically significant. Estimates of DNA extraction efficiency (theoretical DNA yield from AODC compared to actual DNA yield) ranged from 1 to 10% (data not shown).

TPH and BTEX were present in all samples (data not shown). Measured concentrations were probably much lower than actual concentrations because of sample handling and storage procedures; concentrations prior to bioslurping were commonly thousands of ppm for TPH and tens of ppm for naphthalene *(15)*. Sample BTEX concentrations were in the order: ED before venting > ED after venting > WR before venting > bioslurping site > WR after venting. TPH was greatest in the ED

before venting sample and approx fourfold lower in the remaining samples. Venting decreased BTEX levels at the ED and WR locations and TPH levels at the ED location, but removal by physical processes may have been a more important factor than microbial degradation.

DISCUSSION

Research Approach

Molecular methods were used to determine the biodegradative potential of a microbial community by rapidly assessing levels of specific biodegradative genes in samples contaminated with hydrocarbons. We used PCR instead of standard DNA hybridizations (slot- or Southern blots), because it was anticipated that PCR would detect target genes with much greater sensitivity. Calculated gene densities, however, should be considered as estimates only. Factors that influence calculations of gene density include primer bias/selectivity, gene copy number/organism, presence of eukaryotic DNA in the extract, difficulty in accurately quantifying recovered DNA, and method of PCR product detection (agarose gels, nested PCR, hybridizations with internal primers). Given the difficulty in accurately quantifying each variable, gene density estimates differing by a factor of 10 probably have little biological significance. In contrast, gene density estimates differing by 10^2 or 10^3/gram sediment are likely to be biologically relevant and have implications for remediation efforts.

Although maximum DNA extraction efficiency and maximum PCR sensitivity are important in many studies, their benefits for field assessment of biodegradative potential must be considered. Gene probe technology has not been widely used during actual remediation attempts owing, in part, to time-consuming and therefore costly sample analysis (*33*). In light of these issues, we accepted a reduced level of sensitivity and/or accuracy in several areas to increase sample processing speed and efficiency. First, we used a brief, but vigorous lysis procedure rather than applying a more gentle extraction technique or extracting cells prior to lysis. Our extraction efficiency ranged from 1–10% based on theoretical DNA yield calculated from observed microscopic direct counts. Reported extraction efficiencies range from >90% for seeded sediments (for example, *10*, *30*, *32*) to <20% for unseeded sediments (*34*). Our estimated extraction efficiency, then, was in agreement with expectation and consistent with previous reports from unseeded sediments. Second, because microbial biomass was found to be high by AODC, we extracted a single large sample (50 g) to speed sample processing and to compensate for an expected lower extraction efficiency. A large sample also reduces bias owing to microbial spatial variability. Third, DNA was not extensively purified because the inhibitory effects of humic compounds on PCR can usually be removed through a 1:10 or 1:100 dilution (e.g. *27*), and further purification techniques reduce DNA yield. Loss of sensitivity from dilution was judged as acceptable for the objectives of this study. Fourth, 10-fold dilutions of sediment extracts and template in control reactions were used. Two- or threefold dilutions could have been used, and a three-well or five-well MPN-PCR format would have provided more accurate PCR extinction values. In combination, these changes could be used to estimate gene density in samples better and reduce the actual detection limit for a particular gene. Fifth, because all three primer sets were used under the same cycling conditions to reduce processing time (and therefore cost), and because process-level contamination

was eliminated by reducing sensitivity, the detection levels for *alkB* and *xylE* were higher than necessary. Had we elected to perform separate, optimum cycling conditions for each primer set, detection levels for *alkB* and *xylE* would have improved by at least an order of magnitude. Finally, secondary detection methods applied to amplification products (hybridization with internal primers or nested PCR) were not performed to reduce overall processing costs and time, at the expense of increased sensitivity and confirmation of amplicon identity. Independent confirmation of PCR product identity would be useful, especially in those cases where alternate amplification products are observed.

Interpretation of Results

Acridine orange direct counts of bacteria showed microbial populations in all samples were 100- to 1000-fold higher than would be expected for non-contaminated subsurface sediments ($\geq 10^8$ at the site vs 10^5–10^7 for pristine sediments in arid environments *[35,36]*). Total bacterial population samples from the ED and WR locations before the 4-d bioventing test indicate that microorganisms were growing on fuel components and that some level of intrinsic remediation was occurring. The ED location had the highest bacterial population, aromatic hydrocarbon level, and *nahAc* and *xylE* genes (10^9/gram), indicating that the microbial community had been enriched for cells containing these degradative pathways and that natural attenuation of aromatic hydrocarbons was occurring *(14)*. Detection of *alkB* at the bioslurping site and at the WR location after venting suggests that *alkB* -bacteria may be enriched by oxygen addition, but very low gene densities and preliminary dodecane mineralization data (not shown) suggests that other alkane-degrading gene products were responsible for most of the biological TPH loss.

Bioventing at the ED and WR locations did not result in a noticeable increase in *nahAc* or *xylE* gene densities. The 4-d continuous venting should have been adequate to promote aerobic growth near the vent wells (assuming other nutrients were not limiting), but may not have been long enough to cause significant shifts in the microbial community structure. The apparent reduction in *nahAc* gene numbers at the ED location after venting is more likely a result of the confounding factors described above, rather than an actual population decrease.

DNA hybridizations in slot-blot or Southern blot format require $\geq 10^5$–10^6 target copies for detection, assuming DNA preparations are relatively clean and free of inhibitory components. Our estimates of gene density indicate that a standard hybridization approach would have been practical for detecting *nahAc* and *xylE* sequences at the ED location. Even under ideal conditions, however, standard hybridizations would have been too insensitive to detect *alkB*, unless 100% of available DNA was recovered in a form suitable for DNA hybridization. Owing to high levels of humic contamination in our sediment extracts, *nahAc* and *xylE* may have also gone undetected at the bioslurping site and the WR location had we used gene probe methods.

One major drawback to current molecular techniques is the scarcity of sequence data for biodegradative genes. We selected *nahAc, xylE,* and *alkB* because sequence information was readily available and their gene products are representative of various biochemical pathways that might be involved in JP-5 biodegradation. For example, *nahAc* represents a class of highly conserved polyaromatic hydrocarbon dioxygenase genes *(27–29)* that degrade naphthalene and other, more

recalcitrant, polyaromatics. *xylE* represents a pathway involved in biodegradation of simple aromatics and (potential) intermediates in polyaromatic hydrocarbon degradation. The *alkB* gene was selected to represent an alkane-degradative pathway. However, published *nah* sequences are limited to aerobic strains of *P. putida* and *xylE* sequences are so divergent that consensus primers only targeted *Pseudomonas* strains. Only one sequence was available for alkane hydroxylase, and this too was restricted to an aerobic pseudomonad with a substrate range of C_6–C_{10} alkanes. The primary alkanes at the site were C_{10}–C_{15}, so little overlap existed between the substrate range of the target gene and the primary substrates at the site. The limited ability to detect alkane degradative genes, then, was most likely owing to primer specificity arising from the lack of sequence information for alkane degradative genes. Similarly, organisms harboring aromatic hydrocarbon catabolic genes different than *nahAc* and *xylE* would not have been detected. As more DNA sequences for biodegradative genes become available, the accuracy and utility of *in situ* detection and enumeration should also increase.

To the best of our knowledge, this study is the first report using PCR methods to estimate biodegradative gene numbers in samples obtained during field-scale *in situ* remediation. Major conclusions of the study are that:

1. The DNA extraction and PCR analyses allowed rapid analysis of samples (< 3 d of hands-on time) consistent with relatively low analysis costs;
2. Microscopic direct counts suggest intrinsic remediation is occurring;
3. The microbial population at the ED site has been naturally enriched in organisms carrying genes for aromatic hydrocarbon degradation;
4. *alkB*-containing organisms were not a major component of the microbial population; and
5. Poor bioremediation performance (as indicated by the *in situ* respiration test) appears to be a result of physical and/or chemical problems rather than a lack of genetic potential for aerobic biodegradation.

ACKNOWLEDGMENTS

We thank Jeff Kittel for installing the two vent wells at the site and collecting the sediment samples. We also thank Al Pollack for chemical analyses, Siegrid Lu for microscopic counts, and Margaret Romine for xylE primer design. This work was supported by an internal grant from Battelle Memorial Institute.

REFERENCES

1. Atlas, R. M., Sayler, G., Burlage, R. S., and Bej, A. K. (1992), *BioTechniques* **12,** 706–717.
2. Sayler, G. S. and Layton, A. C. (1990), *Annu. Rev. Microbiol.* **44,** 625–648.
3. Steffan, R. J. and Atlas, R. M. (1991), *Annu. Rev. Microbiol.* **45,** 137–161.
4. Liesack, W. and Stackebrandt, E. (1992), *Biodiversity and Conservation* **1,** 250–262.
5. Ward, D. M., Bateson, M. M., Weller, R., and Ruff-Roberts, A. L. (1992), *Adv. Microb. Ecology* **12,** 219–286.
6. Brockman, F. J., Li, S. W., Fredrickson, J. K., Ringelberg, D. B., Kieft, T. L., Spadoni, C. M., McKinley, J. P., and White, D. C. (1995), *Appl. Environ. Microbiol.* Submitted.
7. Brockman, F. J., Kieft, T. L., Fredrickson, J. K., Bjornstad, B. N., Li, S. W., Spangenburg, W., and Long, P. E. (1992), *Microb. Ecology* **23,** 279–301.
8. Haldeman, D. L., Amy, P. S., White, D. C., and Ringelberg, D. B. (1994), *Appl. Environ. Microbiol.* **60,** 2697–2703.

9. Walia, S., Khan, A., and Rosenthal, N. (1990), *Appl. Environ. Microbiol.* **56,** 254–259.
10. Erb, R. W. and Wagner-Döbler, I. (1993), *Appl. Environ. Microbiol.* **59,** 4065–4073.
11. Fleming, J. T., Sanseverino, J., and Sayler, G. S. (1993), *Environ. Sci. Technol.* **27,** 1068–1074.
12. Ka, J. O., Holben, W. E., and Tiedje, J. M. (1994), *Appl. Environ. Microbiol.* **60,** 1116–1120.
13. Ogunseitan, O. A. (1994), in *Process Engineering for Pollution and Control and Waste Minimization,* Wise, D. L. and Trantolo, D. A., eds., Marcel Dekker, NY, pp. 171–192.
14. National Research Council (1993), In Situ *Bioremediation, When Does It Work?* National Academy Press, Washington, DC.
15. Kittel, J. A. (1995), Personal communication.
16. Kittel, J. A., Hinchee, R. E., Hoeppel, R., and Miller, R. (1994), in *Proceedings of the 1994 Petroleum Hydrocarbons and Organic Chemicals in Groundwater: Prevention, Detection, and Remediation,* Groundwater Publishing, Dublin.
17. Miller, R. N., Vogel, C. C., and Hinchee, R. E. (1991), in In Situ *Bioreclamation: Applications and Investigations for Hydrocarbon and Contaminated Site Remediation,* Hinchee, R. E. and Olfenbuttel, R. F., eds., Butterworth-Heinemann, Boston, pp. 283–302.
18. US Environmental Protection Agency (1986), Vol. v. 1B, method 5030, Office of Solid Waste and Emergency Response, Washington, DC.
19. Ogram, A., Sun, W., Brockman, F. J., and Fredrickson, J. K. (1995), *Appl. Environ. Microbiol.* **61,** 763–768.
20. More´, M. I., Herrick, J. B., Silva, M. C., Ghiorse, W. C., and Madsen, E. L. (1994), *Appl. Environ. Microbiol.* **60,** 1572–1580.
21. Xia, X., Bollinger, J., and Ogram, A. (1995), *Mol. Ecology* **4,** 17–28.
22. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989), *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
23. Brock, T. D. and Madigan, M. T. (1991), *Biology of Microorganisms,* Prentice-Hall, Englewood Cliffs, NJ.
24. Kok, M., Oldenhuis, R., van der Linden, M. P. G., Raatjes, P., Kingma, J., van Lelyveld, P. H., and Witholt, B. (1989), *J. Biol. Chem.* **264,** 5435–5441.
25. Eggink, G., Lageveen, R. G., Altenburg, B., and Witholt, B. (1987), *J. Biol. Chem.* **262,** 17,712–17,718.
26. Winstanley, C., Morgan, J. A. W., Pickup, R. W., Jones, J. G., and Saunders, J. R. (1989), Appl. *Envrion.* Microbiol. **55,** 771–777.
27. Herrick, J. B., Madsen, E. L., Batt, C. A., and Ghiorse, W. C. (1993), *Appl. Environ. Microbiol.* **59,** 687–694.
28. Denome, S. A., Stanley, D. C., Olson, E. S., and Young, K. D. (1993), *J. Bacteriology* **175,** 6890–6901.
29. Takizawa, N., Kaida, N., Torigoe, S., Moritani, T., Sawada, T., Satoh, S., and Kiyohara, H. (1994), *J. Bacteriology* **176,** 2444–2449.
30. Recorbet, G., Picard, C., Normand, P., and Simonet, P. (1993), *Appl. Environ. Microbiol.* **59,** 4289–4294.
31. Picard, C., Ponsonnet, C., Paget, E., Nesme, X., and Simonet, P. (1992), *Appl. Environ. Microbiol.* **58,** 2717–2722.
32. Tebbe, C. C., and Vahjen, W. (1993), *Appl. Environ. Microbiol.* **59,** 2657–2665.
33. Brockman, F. J. (1995), *Mol. Ecology* **4,** 567–578.
34. Steffan, R. J., Goksoyr, J., Bej, A. K., and Atlas, R. M. (1988), *Appl. Environ. Microbiol.* **54,** 2908–2915.
35. Kieft, T. L., Amy, P. S., Brockman, F. J., Fredrickson, J. K., Bjornstad, B. N., and Rosacker, L. L. (1993), *Microb. Ecology* **26,** 59–78.
36. Colwell, F. S. (1989), *Appl. Environ. Microbiol.* **55,** 2420–2423.