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Quantitative Cell Lysis of Indigenous Microorganisms and Rapid Extraction of Microbial DNA from Sediment

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This study reports improvements in two of the key steps, lysis of indigenous cells and DNA purification, required for achieving a rapid nonselective protocol for extracting nucleic acids directly from sodium dodecyl sulfate (SDS)-treated sediment rich in organic matter. Incorporation of bead-mill homogenization into the DNA extraction procedure doubled the densitometrically determined DNA yield (11.8 μg of DNA · g [dry weight] of sediment^{-1}) relative to incorporation of three cycles of freezing and thawing (5.2 μg of DNA · g [dry weight] of sediment^{-1}). The improved DNA extraction efficiency was attributed to increased cell lysis, measured by viable counts of sediment microorganisms which showed that 2 and 8%, respectively, survived the bead-mill homogenization and freeze-thaw procedures. Corresponding measurements of suspensions of viable *Bacillus* endospores demonstrated that 2 and 94% of the initial number survived. Conventional, laser scanning epifluorescence phase-contrast, and differential interference-contrast microscopy revealed that small coccoid bacterial cells (1.2 to 0.3 μm long) were left intact after combined SDS and bead-mill homogenization of sediment samples. Estimates of the residual fraction of the fluorescently stained cell numbers indicated that 6% (2.2 × 10^8 cells · g [dry weight] of sediment^{-1}) of the original population (3.8 × 10^9 cells · g [dry weight] of sediment^{-1}) remained after treatment with SDS and bead-mill homogenization. Thus, lysis of total cells was less efficient than that of cells which could be cultured. The extracted DNA was used to successfully amplify *nahR*, the regulatory gene for naphthalene catabolism in *Pseudomonas putida* G7, by PCR. By scaling down the mass of sediment extracted to 0.5 g and by using gel purification and SpinBind DNA purification cartridges, the time required to extract DNA from whole sediment samples was reduced to 2 h.

Microbial ecologists, systematicists, and population geneticists have become increasingly interested in methods for complete, unbiased isolation of DNA (7, 9, 12, 16, 29, 30) and RNA (6, 8, 11, 19, 34, 36) from soils and sediments because such procedures promise to make the genomes of uncultured indigenous microorganisms available for molecular analysis. The ideal (2, 35, 36) is to circumvent the biases implicit in culture-based procedures by directly accessing the genes of naturally occurring microbial communities. But achieving this ideal requires overcoming a variety of interferences that diminish the quality, yield, and diversity of extracted nucleic acids. These interferences raise questions about the completeness of nucleic acid extraction, and about the representativeness of results based on the procedures.

The popular direct lysis approach to DNA extraction and purification (24) may be dissected into the following conceptual steps: (i) washing the material to remove soluble components that may impair manipulation of subsequently isolated DNA; (ii) disruption of cells in the material to release DNA or RNA from the cells; (iii) separation of the DNA or RNA from solids; and (iv) isolation and purification of the released DNA or RNA so that it can be used in various molecular procedures (i.e., PCR, digestion by restriction enzymes, hybridization reactions, or sequencing). A variety of methods integrating most or all of these steps have been published (7, 12, 20, 22, 28, 29, 31), yet, no study has demonstrated that the DNA or RNA was extracted from soil or sediment completely. Nor have criteria for complete extraction of DNA or RNA from native soil and sediment communities been established.

Procedures for lysis of microbial cells in soils and sediments have relied on one or more of the following treatments: lysozyme, heat, proteinase K, sodium dodecyl sulfate (SDS), achromopeptidase, hot phenol, guanidine thiocyanate, pronase, acetone, Sarkosyl, EDTA, freeze-thaw cycles, freeze-boil cycles, sonication, bead-mill homogenization, microwave heating, and mortar mill grinding. Ogram et al. (20) reported that a combination of SDS (incubated at 70°C) and bead-mill homogenization achieved a 90% lysis efficiency for cells native to marine and freshwater sediments, as determined by microscopic counts. Tsai and Olson (31) reported that an EDTA-lysozyme treatment followed by three freeze-thaw cycles reduced microscopic counts of cells added to sediment and subsoil samples by 95%. Similarly, Picard et al. (22) reported that three sonication microwave-thermal shock cycles achieved complete lysis of *Streptomyces* spores. More recently, Erb and Wagner-Döbler (7), using microscopic counts of two bacterial strains added to sterile sediments, concluded that six SDS, freeze-thaw treatments led to 99% lysis efficiency. While all of these reports were based on microscopic observations, descriptions of surviving-cell size distribution and morphology have yet to be presented. Furthermore, general criteria for lysis efficiency of microorganisms native to sediments have yet to be established. In this regard, several investigators (7, 22, 31) have made the questionable assumption that test microorganisms added to sediments were valid surrogates for native cells.

The rationale for the use of a lytic procedure is clear: complete disruption of cellular structure and release of nucleic acids is the objective. A goal of our research was to better understand the effectiveness of cell lysis procedures by deter-

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mining their effects on the diverse assemblage of cells in native microbial communities. In this investigation, we compared the effects of two of the most widely used physical lysis procedures, cycles of freezing and thawing and bead-mill homogenization, on DNA yield and viable-cell plate counts. Total counts and microscopic observations of acridine orange-stained samples were also used as criteria for lysis effectiveness. Finally, we simplified the protocol for extraction and purification of DNA from the sediment.

MATERIALS AND METHODS

Sediment samples. Sediment samples were obtained aseptically from a coal tar-contaminated site near South Glens Falls, New York. Sample characteristics and other details of the site have been described previously (12, 17, 18). Approximately 35 years ago, coal tar was buried in a single depositional event, and since that time, groundwater flow has distributed soluble coal tar constituents in a narrow contaminant plume through sandy subsurface sediments. The contaminated water, which contains naphthalene and phenanthrene, emerges in an organic matter-rich seep area at the foot of a hill slope, 400 m down-gradient from the original coal tar deposit. The methods described here primarily utilized the seep sediment, in which organic matter content was approximately 20% and the water content was approximately 50% (12). Other sandier sediments (approximately 1% organic matter and 20% water) were also used in this study: these subsurface sediments, designated "source," "upgradient," and "downgradient," were obtained from boreholes at the field site along a midline transect of the plume of groundwater contaminants (17, 18). In samples from source, upgradient, and downgradient locations, the concentrations of polycyclic aromatic compounds, especially naphthalene and phenanthrene, gradually diminished. Storage of seep and subsurface samples (in presterilized screw-cap glass jars) was at 4°C for periods up to 1 and 3 years, respectively. Any changes in microbial populations that may have occurred during storage were immaterial for the purposes of this investigation.

Cell lysis. The following general lysis protocol was used in all experiments. Equal weights (either 0.25 or 0.5 g) of wet sediment and phosphate buffer (100 mM, pH 8 [23]) were added sequentially to 2-ml screw-cap polypropylene microcentrifuge tubes (Laboratory Products Sales, Inc., Rochester, N.Y.) containing 2.5 g of 0.1-mm-diameter zirconia/silica beads (BioSpec Products, Bartlesville, Okla.) previously sterilized by autoclaving for 50 min at 120°C and 15 lb/in^2. Next, 0.25 ml of a 10% SDS solution (SDS-Tris-NaCl: 100 mM NaCl–500 mM Tris, pH 8–10% SDS) was added; the final concentration of SDS was approximately 4%. Each tube was shaken at high speed for 5 or 10 min in a bead-mill homogenizing unit (BioSpec Mini-Bead Beater). The selection of bead size and the proportion of beads to cell suspension were determined by following guidelines for disrupting bacterial cells provided by the manufacturer. The tubes were removed from the bead-mill and centrifuged for 3 min at 12,000 × *g*.

To compare the lysis efficiency of the bead-mill homogenization and freeze-thaw procedures, the sediment was mixed by adding 3 g of sediment to 3 ml of phosphate buffer in a 15-ml plastic centrifuge tube and mixing for 2 min on a vortex mixer; 0.5 ml of the mixed suspension (equivalent to 0.25 g of sediment) was immediately distributed to the 2-ml microcentrifuge tubes with and without prior addition of 0.1-mm beads as described above. A 0.5-ml suspension of *Bacillus* endospores in the phosphate buffer was also added to microcentrifuge tubes with and without beads. Endospores were harvested from a culture of *Bacillus subtilis* CU 1065 (Section of Microbiology, Cornell University) by culturing the bacterium on 5% PTYG agar medium (4, 5) and allowing extensive (approximately 40 days) desiccation to occur at 22°C. The spores were harvested by flooding the plate with the phosphate buffer. Microscopic examination showed that 100% of the *Bacillus* cells in the suspension had sporulated. Each tube received 0.25 ml of the 10% SDS–Tris–NaCl solution. The SDS-containing suspensions of the spores or sediment were then subjected to two different lysis procedures. In the freeze-thaw procedure, samples were rapidly frozen by immersion in liquid nitrogen (2 min) and then thawed in a 65°C water bath (5 min); this freeze-thaw cycle was carried out three times. The bead-mill homogenization procedure was carried out for 5 min as described above, with or without beads added. In this case, lysis efficiency was evaluated by triplicate viable-cell plate counts on 5% PTYG agar medium and microscopically as described below. The results were confirmed in three separate experiments, though data from only one are reported here.

Microscopic evaluation of cell lysis. Intact sediment samples or samples treated with SDS and subjected to the lysis procedures were stained with 0.01% acridine orange and examined with either a Zeiss Standard 18 microscope under phase-contrast and epifluorescence viewing or a Zeiss laser scanning microscope (model LSM-10) equipped for fluorescence, phase, and differential interference contrast imaging under 488-nm light from an argon laser. The LSM-10 is configured such that a single field of view can be examined by conventional transmitted and epifluorescence illumination or by comparable laser-scanning illumination. Both microscopes are fitted with × 100 oil immersion objective lenses with numerical apertures of 1.3 or 1.4. An acridine orange direct count (AODC) agar-smear procedure (5, 10) was used to assess the extent of lysis of the endospores and enumerate the total number of cells in the sediment. The computerized imaging and analysis systems of the LSM-10 were used to document the size distribution of microbial cells surviving the various lytic procedures. In enumerating cells in the sediment prior to implementing lysis procedures, the average count and standard deviation were computed from duplicate smears prepared from three independent subsamples of the sediment as described previously (5). In lysis experiments, the same general procedure was followed, except that only one smear from each sample was examined. In one instance, the number of surviving cells was estimated from a wet mount of a known volume of sample under a 22-mm^2 coverslip.

DNA purification. The supernatant from the lysis treatment (150 to 250 μl) was mixed 5:2 with a volume of 7.5 M ammonium acetate, and a precipitate was allowed to form for 5 min at 4°C. Then, the tube was spun for 3 min at 12,000 × *g* and 150 μl of the supernatant was concentrated and partially purified with a SpinBind DNA extraction cartridge (FMC BioProducts, Rockland, Maine). In a SpinBind cartridge, the DNA binds to a microporous silica membrane in the presence of chaotropic salts; after washing, the DNA can be eluted with water. The units were used according to the manufacturer's instructions, except that an EDTA-free ethanol wash buffer was employed and the DNA was eluted with 30 μl of warm (60°C) deionized water. The eluted DNA was loaded onto a 1% agarose gel containing 0.3 μg of ethidium bromide · ml^{-1} and subjected to electrophoresis (4 V/cm) for 20 min in Tris-acetate-EDTA (TAE) buffer according to a standard protocol (3). The resulting DNA bands were cut out of the gel and purified with a SpinBind cartridge according to the manufacturer's instructions for extraction from an agarose gel.

Quantification of DNA. The concentration of DNA after the

TABLE 1. Effect of freeze-thaw treatment and bead-mill homogenization on survival of culturable sediment bacteria and *Bacillus* endospores

Sample	Treatment[a]	CFU (±SD) $\cdot g^{-1}$ or ml^{-1}[b]	% Survival	Viable/total cell ratio (%)[c]
Sediment	None	$1.0 (\pm 0.2) \cdot 10^7$	100	0.3
	SDS + freeze-thaw	$7.8 (\pm 1.5) \cdot 10^5$	8	0.02
	SDS + 5-min bead-mill[d]	$1.5 (\pm 0.2) \cdot 10^5$	2	0.004
Endospores	None	$1.8 (\pm 0.1) \cdot 10^8$	100	ND[e]
	SDS + freeze-thaw	$1.7 (\pm 0.3) \cdot 10^8$	94	ND
	SDS + 5-min bead-mill	$3.5 (\pm 0.5) \cdot 10^6$	2	ND

[a] See Materials and Methods.
[b] CFU for sediment computed per gram dry weight; CFU for endospores computed per milliliter.
[c] Also see Table 2; AODC of untreated sample = $3.8 (\pm 0.3) \times 10^9$ cells $\cdot gdw^{-1}$.
[d] When beads were omitted from the homogenization procedure, the postlysis CFU count was $6.5 (\pm 1.0) \cdot 10^6$ (65% survival).
[e] ND, not determined.

final purification step was measured by densitometry as follows: 3.0 μl of each sample and 4, 2, 1, and 0.5 μl of Lambda DNA standards (Promega, Madison, Wis.) cut with *Hin*dIII (New England Biolabs, Beverly, Mass.) were electrophoresed on a 1% agarose gel in TAE running buffer as described above. The gel was photographed under a Spectroline 302-nm UV transilluminator (model TR-302) as previously described (37). A negative image of the gel was produced with a Polaroid MP4 Land camera using Polaroid type 55 film. Bands on the negative were scanned with a laser densitometer (Helena Laboratories, Houston, Tex.), and the DNA was quantified by interpolation from a calibration curve prepared from the densities of Lambda-*Hin*dIII-cut DNA standards.

PCR amplification of extracted DNA. The suitability of the isolated DNA to undergo enzymatic amplification reactions was tested by a nested PCR protocol (15, 27), using primers for *nahR*, the regulatory gene in the naphthalene catabolism gene cluster encoded on the NAH7 plasmid of *Pseudomonas putida* G7 (39). The outer primer sequences were 5′AACTGCGT GACCTGGATTTAA3′ and 5′CGCCGCCGGCTCGGCTG GTGT3′, corresponding to nucleotides 152 to 172 and 1244 to 1224 (39) of the *nahR* gene. The inner primer sequences were 5′GCCGCGCATCTGGCCGAGCCCGTCACTTCGG3′ and 5′CTGGAGGATGTGGCCAACGGCGGCGAAGTGC3′, corresponding to nucleotides 343 to 373 and 1200 to 1170 of the gene. The final product was 828 bp long. Reagents and conditions for carrying out the PCR were as previously described (12), except that the inner and outer reactions were prepared under "hot-start" conditions, with the deoxynucleoside triphosphates added after the tubes were heated to 80°C. The outer reaction mixture included 2 μl of SpinBind-purified sample and was cycled 1 time at 95°C for 5 min; 5 times at 94°C for 2 min, 65°C for 1 min, and 72°C for 1 min; 25 times at 95°C for 30 s, 65°C for 30 s, and 72°C for 30 s; and 1 time at 72°C for 5 min. For the inner amplification which followed, 5 μl of solution produced from the outer reaction mixture was used as template. Tubes were cycled 30 times at 95°C for 30 s and 70°C for 1 min and 1 time at 72°C for 5 min. The PCR products were detected by agarose gel electrophoresis in 1% agarose gels as described above. *P. putida* G7, used as a positive control in the PCR assay, was originally obtained from G. S. Sayler (University of Tennessee) and was grown at 30°C in 5% PTYG as previously described (12). Negative controls in the PCR assay were done with reagent only (i.e., no added DNA) and a blank derived from a peripheral piece of the DNA purification gel that was taken through purification and amplification procedures.

RESULTS

One indication of the effectiveness of cell lysis procedures is cell viability. Therefore, we measured the change in viable bacteria (CFU) before and after bead-mill homogenization and freeze-thawing as an indicator of the extent of lysis. The data in Table 1 show that the bead-mill homogenization (2% survival) was more effective than freeze-thawing (8% survival) in reducing CFU of SDS-treated sediment bacteria. The CFU data only accounted for 0.3 to 0.004% of the total microscopic counts (Table 1); therefore, the survival of bacteria after the two cell lysis procedures was also tested with endospores of *B. subtilis*. Because of their resistance to physical disruption, endospores can serve as a model for other resistant microbial structures. The ineffectiveness of the freeze-thaw procedure in reducing the viability of a suspension of *B. subtilis* endospores was striking (94% survival [Table 1]) relative to the bead-mill homogenization, after which 2% of endospores remained viable. Lack of viability corresponded to the physical disruption of cell walls after bead-mill homogenization (Fig. 1). Phase-contrast microscopy showed that the phase-dense, refractile spores were completely ruptured after bead-mill homogenization (Fig. 1). The usual bright green fluorescence characteristic of DNA stained with acridine orange was missing in the ruptured spores and, therefore, had been released into the solution.

To further confirm that the reduced viability (Table 1) and ruptured cells (Fig. 1) were indicative of an extracellular release of DNA, we measured the yield of DNA from 0.5 g of sediment extracted and purified by several variations of the above lysis procedures: 10-min bead-mill homogenization as described above; three freeze-thaw cycles; or 5 min of bead-mill homogenization followed by three freeze-thaw cycles and then another 5 min of bead-mill homogenization. Initial qualitative examination of the yields from these three lysis methods was accomplished via 1% agarose gel electrophoresis (data not shown). On the basis of the fluorescence of intercalated ethidium bromide, there was no clear visual difference between the two treatments that utilized bead-mill homogenization. This suggested that bead-mill homogenization, alone, was as effective as a combination of freeze-thaw treatment and bead-mill homogenization in releasing DNA. In contrast, the intensity of the DNA band resulting from the freeze-thaw treatment alone was dimmest, thus corroborating the lower lysis efficiency of this treatment relative to bead-mill homogenization (Table 1). The DNA from all three lysis preparations was then concentrated with a SpinBind cartridge, employed in this study to improve DNA recovery over ethanol precipitation-DNA resuspension procedures used earlier (12). A portion of each

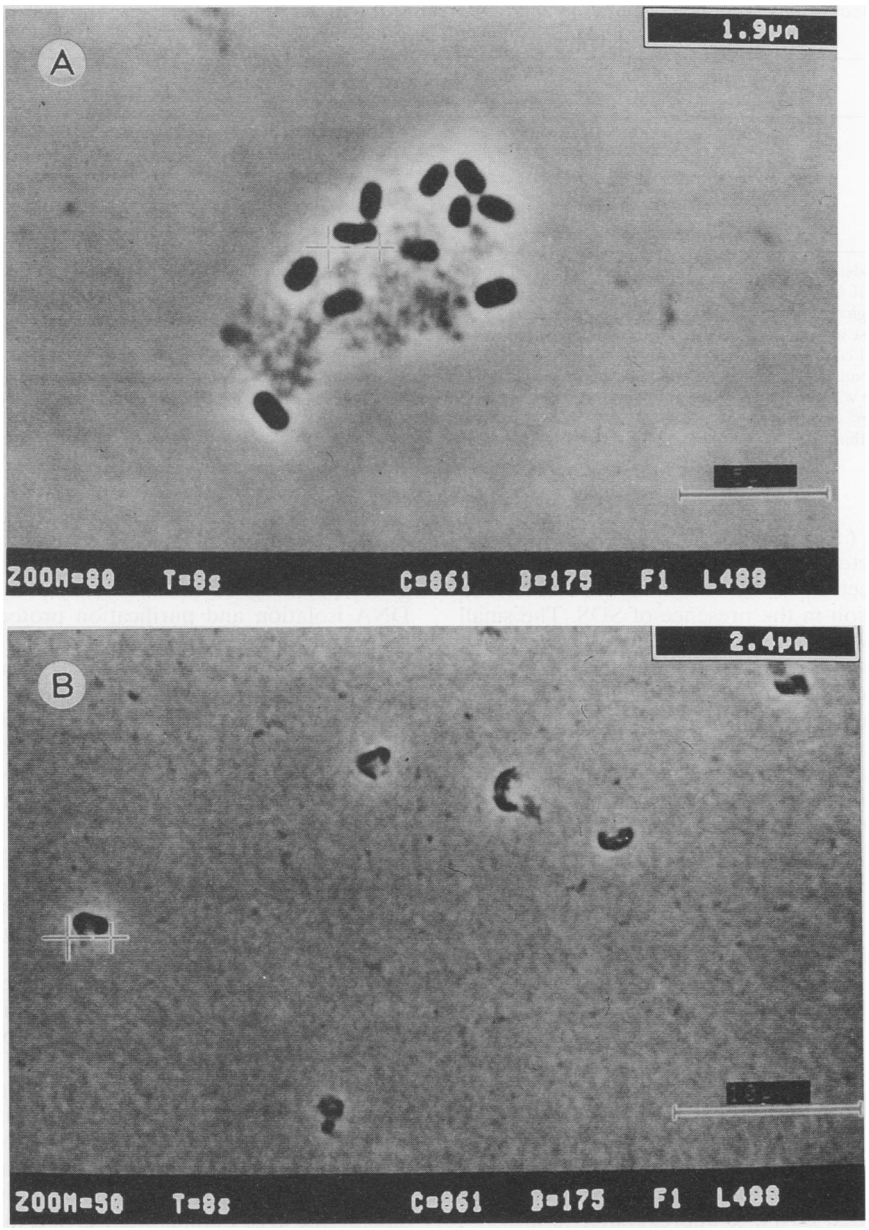

FIG. 1. Phase-contrast micrographs of *B. subtilis* spores before (A) and after (B) bead-mill homogenization. Dimensions in the upper righthand corner of each micrograph denote the distance between the crosses marking the ends of cells.

concentrated DNA preparation was next electrophoretically purified on a 1% agarose gel. Each DNA band was excised from the gel, processed a second time with the SpinBind cartridge, and visualized on an agarose gel, and then a negative image of each band was scanned with a laser densitometer to quantify the DNA. Yields from the bead-mill homogenization (alone), freeze-thaw treatment (alone), and the two lysis treatments combined were 11.8, 5.2, and 11.0 mg of DNA · g (dry weight) of sediment^{-1}, respectively. Thus, freeze-thaw treatment, alone, released one-half as much DNA as from the two bead-mill homogenization treatments, whose DNA yields were virtually indistinguishable.

Microscopic examination of the sediment provided an additional means of assessing the response of native microorganisms to lytic procedures. Because the data in Table 1 and the above DNA yields clearly demonstrated that freeze-thawing was a less effective cell lysis method than bead-mill homogenization procedures, only the latter and several variations (aimed at discerning the role of SDS in the procedure) were investigated. Table 2 reports the total bacterial numbers (AODC), approximate size distribution, and morphological diversity of microorganisms in sediments before and after SDS treatment, bead-mill homogenization, or both treatments. Prior to lysis, the sediment sample contained a rich and varied collection of both eukaryotic and prokaryotic cells which spanned a wide range of cell sizes (Table 2). In general, the various size fractions diminished as the severity of lytic procedures increased. The key observation shown in Table 2 is that

TABLE 2. Effect of bead-mill homogenization and SDS treatment on total bacterial numbers, bacterial size distribution, and morphological diversity in sediment

Treatment[a]	AODC (±SD) · gdw^{-1}[b]	AODC remaining (%)	Approx size range[c] (μm)	Morphological diversity[c]
Untreated	3.8 (±0.3) · 10^{9}[d]	100	>10–0.3	I
5-min bead-mill	1.9 (±0.5) · 10^{9}	50	2.0–0.3	III
10-min bead-mill	1.0 (±0.2) · 10^{9}[e]	26	2.0–0.3	III
SDS	4.9 (±0.4) · 10^{8}	13	5.0–0.3	II
SDS + 10-min bead-mill	2.2 (±1.6) · 10^{8}[f]	6	1.2–0.3	III

[a] See Materials and Methods; note that the SDS reagent was included in the bead-mill homogenization procedure reported in Table 1.
[b] gdw, grams (dry weight) of sediment.
[c] Size range and morphological diversity of fluorescent cells in at least 15 microscopic fields observed during AODC counting by conventional and laser scanning epifluorescence phase-contrast and differential interference-contrast microscopy. I, large and small filaments, rods, cocci, sarcina-like clusters of microcolonies. II, sarcina-like clusters and small coccoid cells only. III, small coccoid cells only.
[d] A factor of 380 greater than the untreated sample CFU described in Table 1.
[e] The AODC of this sample was estimated by determining the number of green fluorescent cells per ×1,000 field of 10 μl of a 1:8 diluted sample containing acridine orange spread under a 22-mm^2 coverslip.
[f] A factor of 1,467 greater than the SDS- and bead-mill-treated CFU described in Table 1.

approximately 6% [2.2 (±1.6) × 10^8 cells · g (dry weight) of sediment^{-1}] of the bacteria, mostly small coccoid cells in the sediment, were unaffected by the most severe treatment, bead-mill homogenization in the presence of SDS. The small cells which resisted lysis were observed by laser scanning epifluorescence microscopy (Fig. 2). It is important to note that the epifluorescence images are produced electronically in black and white by using a green analyzer filter and photomultiplier detector. Therefore, the degree of brightness of an object in these images was directly related to green fluorescence. It is also important to note that the ratio of viable to total counts (CFU/AODC) of the original sample was 0.3% before treatment (Table 1). After treatment with SDS and 10 min of bead-mill homogenization, the CFU/AODC ratio was 0.07% (inverse of factor in footnote *f* of Table 2). Thus, the net effect of these combined treatments was to lyse the larger cells that were more likely than the small cells to grow on the plate count medium.

PCR amplification of sediment-derived DNA. In addition to examining the efficacy of cell lysis procedures, this study also pursued the goal of achieving a rapid overall procedure for extracting and purifying DNA from sediment. By scaling down the total amount of sediment processed, from 1 (12) to 0.25 or 0.5 g, we were able to perform all of the above procedures in microcentrifuge tubes. This, in combination with utilization of SpinBind units, shortened the total processing time, from crude sediment to purified DNA, to approximately 2 h.

Many reports have shown that soil and sediments contain humic or other substances that may remain associated with extracted DNA, thus preventing its subsequent analysis (13, 29, 33). To determine if the DNA yielded from sediment samples was pure enough to allow subsequent molecular analysis, we performed a variety of tests. The first was designed to ascertain the effectiveness of gel electrophoresis in DNA purification. A 1-μl volume containing 45 *P. putida* G7 cells (determined by plate counts) was added to the PCR mixture along with 2 μl of sediment-derived DNA that had twice been passed through the SpinBind cartridges, with and without gel electrophoretic purification in between (the particular sediment subsample used here lacked amplifiable *nahR*). After completion of the nested PCR procedure on both preparations, *nahR* was amplified from *P. putida* G7 cells only with the electrophoretic purification (data not shown). Thus, we confirmed our previous results (12) indicating that the sediment contained PCR-inhibitory substances whose removal required a gel-electrophoretic purification step.

Prior work has shown that sediment samples from a variety of locations in our coal tar-contaminated field site contain genes homologous to *nahA* (12) and *nahR* (27). Using the DNA isolation and purification protocol described here, we repeatedly examined the quality of the DNA so obtained. Figure 3 shows the PCR products that resulted from four different sediment samples from our study site. Because PCR is sensitive both to concentrations of inhibitory substances and to the concentration of target DNA sequences, we amplified directly after the final elution from the SpinBind unit (Fig. 3, lanes 1, 3, 5, and 7) and after a 10-fold dilution (Fig. 3, lanes 2, 4, 6, and 8). The DNAs extracted from seep sediment (used to develop the protocols described in this study [Fig. 3, lanes 1 and 2]) and source sediment (Fig. 3, lanes 7 and 8) were susceptible to PCR amplification of the *nahR* gene, regardless of dilution. However, DNA preparations from the other sediment samples displayed differing responses to dilution. *nahR* was not amplified from the diluted upgradient sediment DNA (Fig. 3, lane 4)—possibly indicating a low titer of target DNA. In contrast, the DNA preparation from the downgradient sediment yielded a relatively weak amplification band in the undiluted sample Fig. 3, lane 5)—possibly indicating that the electrophoresis and SpinBind purification steps failed to completely remove substances inhibitory to the PCR. As an additional negative control treatment in the experiment whose results are shown in Fig. 3, a piece of the purification gel from outside the DNA bands was carried through the PCR procedure and failed to yield the amplified product (data not shown). When additional subsamples of the sediments used (Fig. 3) were repeatedly carried through the DNA extraction, purification, and PCR procedures, amplification of the *nahR* genes was not always consistent. This inconsistency was noted previously in the amplification of *nahAc* from the upgradient and downgradient samples (12). The reason for this variability is uncertain, but the variation may have been caused by heterogeneity inherent in the physical, chemical, and microbiological characteristics of field site-derived sediments.

DISCUSSION

This report has articulated the role of cell lysis as the first in a series of procedures required for achieving efficient, nonselective access to the genes in naturally occurring sediment microbial communities. But, perhaps more importantly, we have presented criteria for evaluating the effectiveness of the lysis step. These criteria were loss of cell viability, total DNA

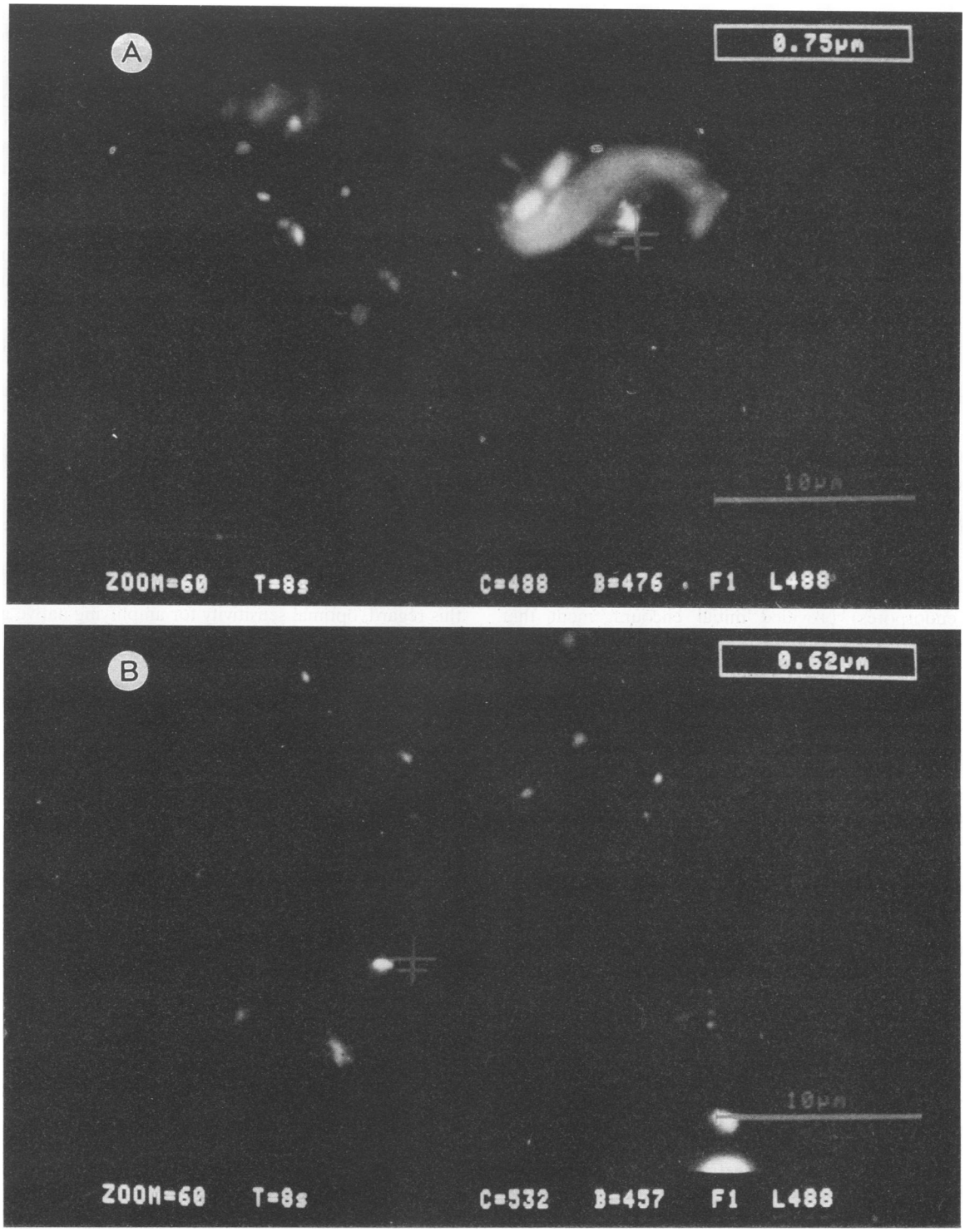

FIG. 2. Laser scanning epifluorescence micrographs of acridine orange-stained native cells in sediment before (A) and after (B) bead-mill homogenization. Note the presence of both large and small cells in the center of the prelysis micrograph. Only very small cells remained after lysis. Dimensions in the upper right-hand corner of each micrograph denote the distance between the crosses adjacent to cells or particles.

yield, and microscopic examination of sediment-derived cells for total direct counts and morphological diversity changes. By all four criteria, bead-mill homogenization was shown to be more efficient in lysing cells than freeze-thawing. Furthermore, the quality of the DNA subsequently extracted from the sediment was verified by PCR amplification of a native naphthalene catabolic gene.

Precedent has been set for using the behavior of an indicator microorganism, often seeded into sediments prior to determining the efficiency of lysis or DNA recovery, as a basis for

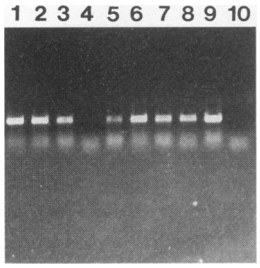

FIG. 3. Amplification of *nahR* from four sediment samples with nested primer PCR. For lanes 1, 3, 5, and 7, a 2-μl concentrated sample was used as a template for PCR amplification; and for lanes 2, 4, 6, and 8, a 2-μl 1:10 dilution of the same samples was used. Lanes: 1 and 2, seep sediment; 3 and 4, upgradient sediment; 5 and 6, downgradient sediment; 7 and 8, source sediment; 9, positive control (part of a *P. putida* G7 colony added to the PCR mixture); 10, PCR negative control (reagent only).

extrapolating to the behavior of indigenous cells (7, 22, 31). Similarly, in this study, viable counts of *Bacillus* endospores and native sediment bacteria were examined as a means for testing cell lysis procedures. A consistent proportion of surviving cells (2%) (Table 1) shared by the two very different microbial assemblages (total sediment microorganisms and *Bacillus* endospores) provided initial encouragement that DNA released from sediment by bead-mill homogenization would be completely representative of the sediment microbial community. However, the total viable counts derived from the sediment represented only 0.3% (prelysis) (Table 1) and 0.07% (postlysis) (Table 2, footnote *f*) of the total microscopic count. This total microscopic count necessarily included unknown proportions of nonviable but intact cells and both cultured and uncultured viable cells. Thus, an astonishingly large component of the sediment microbial community studied here was characterized only according to microscopically discernible traits such as cell size and morphology (Table 2). The SDS, bead-mill homogenization treatment disrupted indigenous cells in a biased manner by leaving the smallest size fraction (1.2 to 0.3 μm long) (Table 2; Fig. 2) intact. Until this resistant portion of the sediment microbial community can be lysed (perhaps by using smaller beads and additional chemical lytic agents), the ideal of accessing all of the indigenous genes will be thwarted. Furthermore, it is clearly unwise to use added indicator microorganisms, or even viable indigenous cells as a basis for drawing inferences about the susceptibility of the uncultured microbial community to cell lysis procedures.

Despite the fact that SDS, bead-mill homogenization failed to disrupt small cells native to the sediment, it is appropriate to use the data presented here to estimate total sediment DNA and the overall efficiency of the extraction procedure. If we presume that prokaryotes were the predominant reservoir of sediment DNA and that each of the $3.8 \cdot 10^9$ prokaryotic cells · g (dry weight) of sediment^{-1} contained a single stationary-phase genome weighing $5 \cdot 10^{-15}$ g (based on data for *Escherichia coli* [38]), then 1 g (dry weight) of the sediment contained 19 μg of DNA. This value agrees reasonably well with the total sediment DNA estimated by Ogram et al. (20) (27 μg · g dry weight^{-1}) and with the ranges of total soil DNA (20 to 50 μg · g dry weight^{-1}) reported by Picard et al. (22), Selenska and Klingmüller (26), and Steffan et al. (28). Factors contributing to variability in total DNA estimates include those imposed by different extraction methodologies, as well as microbiological idiosyncrasies of particular samples stemming from physiological influences such as soil or sediment type, climate, and the content of water, oxygen, and organic matter, etc. The DNA yielded when SDS, bead-mill homogenization was combined with the extraction protocol described here (11.8 μg · g [dry weight] of sediment^{-1}) represents 62% of the 19 μg of total theoretical DNA. Many of the assumptions contributing to this efficiency figure are uncertain; nonetheless, this estimated yield is reasonably high. It is perhaps remarkably high in light of the fact that much of the DNA from cells 1.2 to 0.3 μm in length was not released (Table 2) and that the steps subsequent to cell lysis (especially separation of the DNA from sediment particles) were not carefully scrutinized. Only after each step has been thoroughly examined and optimized can DNA extraction biases be reduced and efficiency be increased. It should be noted that even if an extraction efficiency of 99.9% were achieved, with 10^9 cells per g this would still leave 10^6 organisms per g unsampled. Thus, even when the lysis efficiency is relatively high, minor members of the community may remain intact and, consequently, their DNA may escape detection. Although there is no clear solution to this dilemma, we feel that continued striving towards the combination of unbiased genome sampling and enhanced sensitivity afforded by PCR may partially mitigate such detection limit problems.

PCR detection of genes native to sediment requires that the ratio of target sequence be high relative to accompanying sediment-derived materials that may inhibit the denaturation, annealing, and DNA synthesis stages of PCR (29, 32, 33). In this regard, optimal sensitivity for amplifying native genes can only be achieved by separating the DNA from inhibitory substances. Recently, Abbaszadegan et al. (1) have shown that Sephadex G-100 and Chelex 100 resins successfully removed PCR-inhibitory substances from groundwater concentrates. Perhaps ironically, nontarget DNA itself has also recently been shown to mask the PCR amplification of target sequences in low abundance (29). This study has confirmed that purification of DNA extracts is required for successful PCR amplification of indigenous genes (*nahR*). But even in such purportedly pure DNA preparations, lack of amplification in undiluted DNA extract (Fig. 3) suggested that inhibitory substances still remained in the mixture. The need to dilute DNA extracts prior to PCR amplification has been reported earlier for electrophoretic purification of DNA extracted from environmental samples (7, 22, 33), and it is the simultaneous dilution of the target sequence that may ultimately limit the sensitivity of the method.

Scale and its equivalent, sample size, are other issues to be considered in performing and interpreting experiments examining molecular characteristics of naturally occurring microbial communities. The small-scale (0.5 g) processing of sediment reported here substantially diminished the logistical and time constraints on DNA extraction. But facile processing of small samples raises questions about how accurately such small samples represent microbial communities as they occur in the landscape. Not enough is known about the chemical, physical, and microbiological spatial heterogeneity of soils (21) and sediments to allow data from 0.5-g samples to be the basis for extrapolation to larger (i.e., kilogram) or very large (i.e., landscape) scales. Moreover, the amplifiability of genes present in 0.5-g samples undoubtedly reflects the variable spatial distribution of both the target DNA sequence and sediment-derived substances that inhibit PCR (see the discussion above). Because these determinants for successful gene amplification may vary independently, interpreting the results of such assays may prove challenging.

Recently, Erb and Wagner (7) used DNA extraction and PCR amplification techniques to obtain a polychlorinated

biphenyl catabolic gene directly from a German freshwater sediment. A comparison of restriction digests failed to detect any divergence between a sediment-derived *bphABC* gene fragment and that of the type strain, *Pseudomonas* sp. strain LB400 (7). In contrast, by hybridizing DNA extracted from soils with a variety of gene probes Holben et al. (14) have recently demonstrated that the genetic basis in soil microbial communities for 2,4-dichlorophenoxyacetic acid catabolism was broader than that of plasmid pJP4. Similarly, we reported significant restriction fragment length polymorphism relative to *P. putida* G7 in the *nahAc* genes in DNA extracted from the same coal tar-contaminated field site examined here (12). In order to learn more about the distribution of related naphthalene catabolic gene sequences, procedures in this study utilized a different, nested set of oligonucleotide primers, specifically designed to amplify an 828-bp fragment of the *nahR* gene (27, 39). *nahR* is a member of the *lysR* family of regulatory genes that are widely distributed among gram-negative bacteria (25). Detection of *nahR* in the DNA extracted from the sediment provides two types of information. First, amplification of this gene allowed the quality of sediment-derived DNA to be evaluated. Because PCR amplification was possible, we concluded that the rapid extraction and purification procedures developed in this investigation were successful. But, perhaps more interestingly, detecting *nahR* in DNA extracted from this field study site lends additional momentum to ecological inquiries which utilize DNA sequence information from pure culture-derived functional genes to explore gene distribution and variation in nature.

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