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Combined lipid/DNA extraction method for environmental samples

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Abstract

Previously, separate methods have been developed for the extraction and purification of lipids and DNA from soils and sediments. This paper describes a new method for the isolation of both lipids and DNA from the same environmental sample. This combined method is based on the Bligh and Dyer lipid extraction technique. Upon phase separation, lipids partition into the organic phase and DNA partitions into the aqueous phase. DNA extraction and recovery from the solid phase is necessary under certain conditions. Preliminary experiments performed with ^{32}P-labeled DNA in the absence of soil showed that greater than 98% of the total DNA was present in the aqueous phase after the modified Bligh and Dyer extraction. Analysis of the DNA by polyacrylamide gel electrophoresis and autoradiography demonstrated that no degradation of DNA occurred during the lipid extraction procedure. Lipid extraction of lyophilized cells showed that DNA was released from *Pseudomonas putida* and *Bacillus subtilis* cells corresponding to 26±5 and 14±4% of the theoretical DNA yield, respectively. The combined lipid/DNA extraction method was applied to both lyophilized cells and wet cells added to soil. Analysis by DNA : DNA hybridization showed that approx. 40–50% of the DNA from cells added to soil was recovered after lipid extraction relative to samples treated only with conventional DNA extraction. Estimation of cell number per gram soil based on either lipid or DNA analysis showed good agreement with actual numbers added based on plate counts of the inocula. DNA extracts from samples which had been lipid-extracted also had lower amounts of humic material. Although some DNA was not recovered after lipid extraction, that which was recovered was of sufficiently high quality for hybridization analysis. This method shows utility for the co-recovery of both lipids and DNA from a single sample; this is particularly useful when a small sample size is all that is available or procurable.

Keywords: DNA; Lipid; Microbial community; Soil

1. Introduction

In the past, methods used for determining microbial communities have relied heavily on the culturing of microorganisms. It has been shown that these methods are only successful in culturing 10% or less of the population present in a given sample [1]. Because of the drawbacks of these classical mi-

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crobiological methods, it is necessary to examine the microbial community without utilizing culturing techniques. This can be accomplished using the techniques of nucleic acid analysis [2] and lipid analysis [3]. These methods have the advantage of assessing viable but nonculturable microorganisms by direct extraction of cellular components from environmental matrices.

Analysis of extracted DNA by DNA : DNA hybridization with specific gene probes can yield information on gene frequencies and population densities of specific microorganisms. By using more general probes such as a 23S rRNA gene probe for fluorescent pseudomonads [4] or a *nifKDH* probe [5] for nitrogen-fixers, one can gain insight into the community structure present in a sample. Likewise, lipid analysis can provide a wealth of information with respect to the biomass content, community structure, and physiological status of microorganisms. Since most microbial cell membranes contain phospholipids, the analysis of phospholipid fatty acid (PLFA) is a useful measure of viable biomass, since these compounds are turned over rapidly and do not persist following cell death. In addition, the analysis of PLFA can lead to the identification of specific genera (i.e., Type II methanotrophs by the signature fatty acid, *cis*-octadec-8-enoic) and in the identification of physiological stress (i.e., an increase in *trans*-monounsaturated fatty acids). Nucleic acid and lipid analyses provide complementary information and each approach can substantiate the results obtained from the other (i.e., biomass determination).

Since both methods rely on direct extraction of soil or sediment, it was advantageous to combine the two methods in order to obtain more information concerning the microbial ecology of the environmental sample. Upon liquid/liquid extraction and separation, the lipid components partition to the organic phase, whereas the nucleic acids partition to the aqueous phase. Recently, reports have stated that nucleic acids were released upon lipid extraction of sandy aquifer material [6]. However, the DNA which was recovered was fragmented, <100 bp in size, and the origin was uncertain, although it was deemed to be largely representative of extracellular DNA in the soil and not recovered from intact cells. In our study, a known amount of cells or lyophilized cell mass was added to a soil in order to make direct comparisons between DNA recoveries with and without prior lipid extraction. The aqueous and organic phases from the Bligh and Dyer extraction were evaluated for their DNA content. Recovered DNA was quantified by DNA : DNA hybridization analysis with comparison to known standards, therefore, this method was more specific than spectroscopic methods (UV absorbance or fluorescence).

The combined method presented here allows the researcher to maximize the amount of information that can be obtained from a single environmental sample. This method is especially useful when sample material is in small quantity and/or difficult to obtain. Furthermore, when population densities are small, a large volume of material may need to be extracted in order to obtain measurable quantities of lipids or DNA. In some cases, large sample sizes may be necessary to be representative of the environment being samples. By combining the two assays on such a sample, a single spatial point is identified for statistical comparisons.

2. Materials and methods

2.1. Experiments with exogenous ^{32}P-labeled DNA in the absence of soil

Initial experiments were performed to determine the distribution of DNA after the modified Bligh and Dyer extraction. A ^{32}P-labeled λ DNA fragment (500 bp) was generated using the λ control polymerase chain reaction (PCR) contained in the AmpliTaq kit (Perkin Elmer-Cetus, Norwalk, CT) and 5 μl [α-^{32}P]dCTP. The PCR product was purified from unincorporated radiolabel with a NucTrap push column (Stratagene, La Jolla, CA) and this fragment (10^8 total dpm) was added to buffer and subjected to lipid extraction as described by White et al. [3]. After phase separation, aliquots of the organic and aqueous phases were added to scintillation cocktail and analyzed on a liquid scintillation counter.

Similar experiments with ^{32}P-labeled λ DNA were conducted in the presence of bacterial cells since it was thought that DNAses could potentially be released from the cells contributing to decreased DNA recoveries. In addition, 1 mM Na_2EDTA was added

to one set of replicates to test whether DNA degradation would be inhibited by EDTA. Approx. 10^7 dpm ^{32}P-labeled λ DNA was added to the Bligh and Dyer extractions of $3.45 \pm 0.37 \times 10^{10}$ wet cells of *Pseudomonas putida*. After a 3 hr extraction period, phases were separated and 30 μl of each aqueous-phase sample was analyzed by polyacrylamide gel electrophoresis (PAGE). Samples were loaded onto a 5% polyacrylamide gel along with ^{32}P-end-labeled fragments from a *Dde*I digest of pBluescript II(KS) as a size marker. Due to the presence of methanol and trace amounts of chloroform in the aqueous phase, the gel was loaded dry and overlayed with 2% agarose to prevent diffusion of samples out of the wells. The running buffer was 1×TBE (0.1 M Tris-OH, 0.1 M boric acid, 1 mM EDTA), and electrophoresis was performed at 200 V for 2 h. The gel was dried and subjected to autoradiography.

Experiments were subsequently performed to determine the best method to recover and concentrate the DNA from the aqueous phase. Since this phase contained approx. 50% methanol, it was thought that precipitation of DNA may occur prior to addition of ethanol, typically used to precipitate DNA. The following four treatments were performed on the aqueous phase containing the ^{32}P-labeled λ DNA: (1) no addition; (2) addition of 0.1 volume 3 M sodium acetate; (3) sodium acetate plus 1 volume ethanol; (4) sodium acetate plus 2 volumes ethanol. After storage (−20°C, 1 h) and centrifugation (12 000×*g*, 4°C, 30 min), the amount of non-precipitated ^{32}P-labeled λ DNA was determined by liquid scintillation.

2.2. Experiments with lyophilized cells in the absence of soil

Experiments were performed on lyophilized *P. putida* and *B. subtilis* (Sigma) cells to determine if DNA was being released from whole cells into the aqueous phase following lipid extraction. A representative gram-negative and gram-positive were used to determine whether there were differences in recoveries for the two types of bacteria. Three replicates of known mass (5 mg) were extracted as described by White et al. [3]. After separation of phases, the aqueous phase was ethanol precipitated by addition of 0.1 volume 3 M sodium acetate and 2 volumes 100% ethanol and stored at −20°C overnight. After centrifugation at 15 000×*g* at 4°C for 30 min, the supernatant was decanted and the pellet was vacuum-dried. The pellet was resuspended in 8 ml TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and prepared for CsCl gradient centrifugation [7]. Samples were centrifuged at 20°C for 17 h at 285 000×*g*. Since no DNA bands were evident, the soluble tube contents were removed with a needle and syringe and butanol-extracted according to standard methods [7]. The DNA was subsequently concentrated by ethanol precipitation and resuspended in 1 ml of TE buffer. The UV absorbance at 260 nm was used to estimate the DNA content, since RNA and protein contaminants were removed in the centrifugation procedure.

2.3. Experiments with exogenous ^{32}P-labeled DNA in the presence of soil

In order to determine where DNA would partition upon lipid extraction when a soil phase was present, ^{32}P-labeled λ DNA (10^6 dpm) was added to three replicates of 10 g sandy loam soil (75.5% sand, 13.8% silt, 8.9% clay, 0.37% organic carbon) [8] which had been air-dried and passed through a 2 mm sieve. The soil was subjected to lipid extraction [3], after which the soil was pelleted and the supernatant was split into aqueous and organic phases. Aliquots of the organic and aqueous phases were added to scintillation cocktail and counted. The soil phase was subjected to the DNA extraction procedure (see Section 2.4).

2.4. Lipid and DNA extraction from lyophilized cells and soil

2.4.1. Sample preparation

To evaluate the effect of lipid extraction on DNA recovery, comparisons were made between samples extracted for both lipids and DNA and samples extracted only for DNA. For these studies, cells were added to soil to provide a marker for DNA hybridization analyses. *Pseudomonas fluorescens* 5R, which was isolated from a manufactured gas plant soil, was used. This strain contains a *nah*-type plasmid (pKA1) encoding enzymes involved in degradation of naphthalene to tricarboxylic acid cycle intermediates.

Cells were grown in yeast extract-polypeptone-glucose (YEPG) media, harvested and washed in phosphate-buffered salts (PBS) media (g/l: 8.0 NaCl; 0.2 KCl; 1.15 Na_2HPO_4; 0.2 KH_2PO_4; pH 7.0) prior to lyophilization. Lyophilized cells (10 mg) were added to 10 g of sandy loam soil. The cells and soil were mixed briefly to ensure that cells were evenly distributed prior to extraction. Three 10 g replicates were subjected to the modified Bligh and Dyer lipid extraction prior to DNA extraction while three 10 g replicates were subjected only to DNA extraction. The combined lipid/DNA extraction procedure is outlined in Fig. 1.

2.4.2. Lipid extraction

Samples (10 g) were placed into 250 ml Pyrex centrifuge bottles with Teflon-lined caps. The first extraction phase consisted of the addition of 10 ml methanol, 5 ml chloroform and 4 ml phosphate buffer (50 mM K_2HPO_4, pH 7.4). After sonication for 2 min, the bottles were incubated with intermittent shaking for 2 h. The bottles were subsequently centrifuged for 30 min at 8000×g. The supernatant was poured into a separatory funnel and the sedimented soil was stored at −20°C prior to DNA extraction. Separation of the phases was achieved by addition of 5 ml each of chloroform and water. The separatory funnel was vigorously shaken and the phases were allowed to separate overnight. The lower organic phase was used for lipid analysis and the aqueous phase was evaluated for DNA content.

The total lipid contained in the lower organic phase was fractionated using silicic acid chromatography to separate the polar lipids from the other lipid

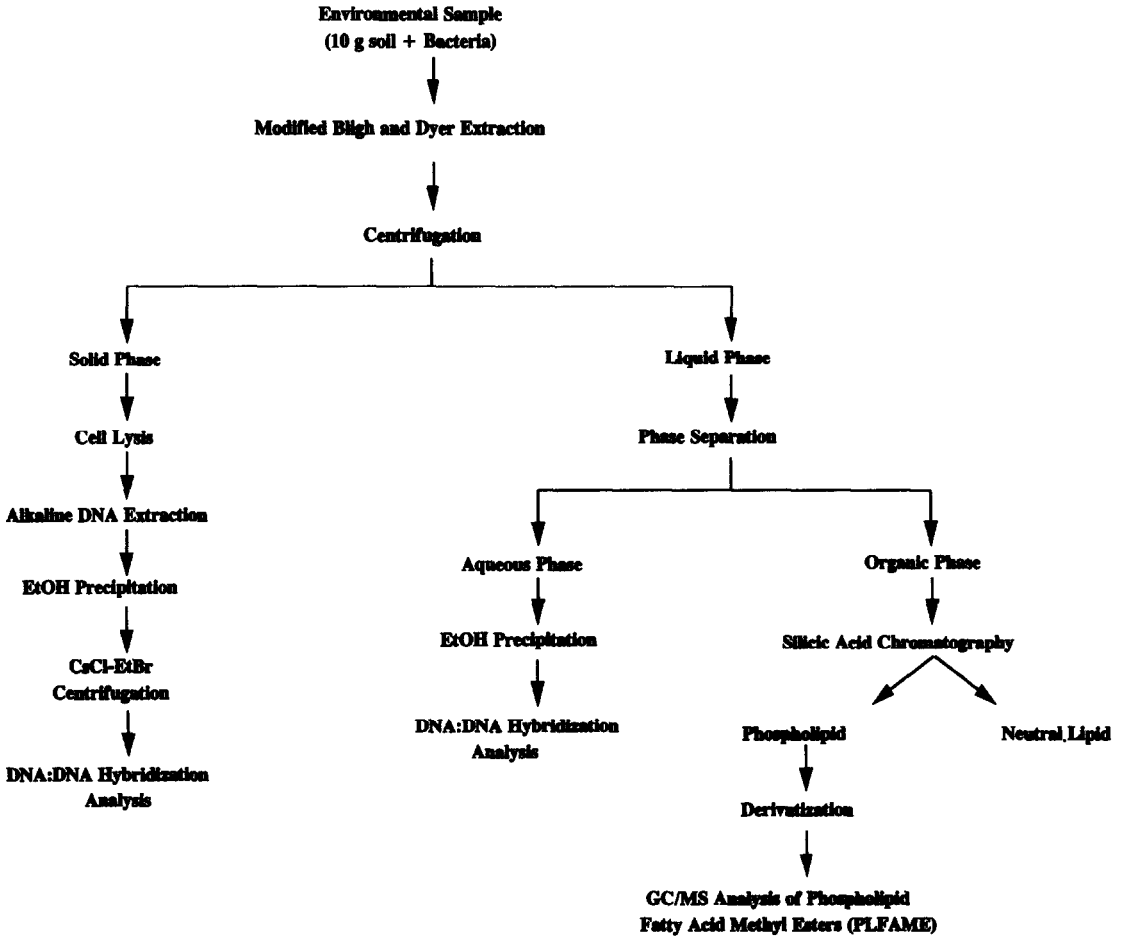

Fig. 1. Flow chart of the combined lipid/DNA extraction procedure.

fractions. The polar lipid fraction containing the phospholipids was then subjected to a mild alkaline methanolysis [9]. This resulted in cleavage of the fatty acids from the phosphoglyceride backbones, creating fatty acid methyl esters (PLFAME). The resultant fatty acid methyl esters were separated and quantified using a ramped temperature program with an IBM GC/9630 Gas Chromatograph equipped with an FID. Quantitation was performed by comparing the peak areas of each fatty acid to the peak area of an internal standard (nonadecanoic acid methyl ester, 50 pmol/μl) Fatty acid identifications were made by comparing the sample retention times to those of a standard fatty acid mixture. Peak areas were integrated using Nelson Chromatography Software (Perkin Elmer, Norwalk, CT). Cell numbers were calculated from the fatty acid concentration assuming that each phospholipid contains 2 fatty acids per molecule, and that the average cell contains approximately 21.12×10^6 phospholipid molecules per cell [10].

2.4.3. DNA extraction and purification

For samples which were extracted for lipids, the aqueous phase volume (approx. 30 ml) was determined before recombining with the soil phase and transferring to 250 ml polypropylene bottles for DNA extraction. The liquid volume was brought to 40 ml with addition of 1 M Na_2HPO_4 (4 ml), 25% (w/v) sodium dodecyl sulfate, SDS (1.3 ml) and water (4.7 ml) to yield a final solution of 0.1 M Na_2HPO_4 (pH 8.0). For samples which were not extracted for lipids, 25 ml of 0.12 M Na_2HPO_4 (pH 8.0) and 5 ml of 5% (w/v) SDS were added. From this point forward, samples were treated identically.

Samples were incubated at 70°C for 1 h with shaking every 5–10 min. Samples were placed in small bead beater chambers (Biospec Products, Bartlesville, OK) and bead-beats (5 g, 0.1 mm diam.) were added with subsequent beating for 5 min with an ice pack for cooling. The samples were returned to 250 ml bottles and bead beater chambers were rinsed two times with 5 ml 0.12 M Na_2HPO_4. The combined material was centrifuged at $5500\times g$ at 10°C for 25 min. The supernatant was poured into a new 250 ml centrifuge bottle and extraction of the pelleted soil was repeated with addition of 15 ml 0.12 M Na_2HPO_4 (pH 8.0) with heating at 70°C for 15–20 min. Samples were centrifuged as before and the supernatants were combined. Extraction of the pelleted soil was repeated an additional time with subsequent centrifugation and combining of supernatants. The pooled supernatants were centrifuged at $15\,000\times g$ at 4°C for 1 h. The resulting supernatant was ethanol precipitated with addition of 2 volumes of 100% ethanol and stored at −20°C for 4 h to overnight.

After centrifugation at $15\,000\times g$ at 4°C for 30–60 min, the supernatant was removed and the pellet was dried under vacuum. The pellet was resuspended in 7 ml TE buffer with heating at 50°C to dissolve completely. Samples were placed into Spectra/Por dialysis tubing (20.4 mm, 6000–8000 MW cut-off) and dialyzed against TE at 4°C. Dialyzed samples were precipitated by addition of 0.1 volume 3 M sodium acetate and 2 volumes 100% ethanol and stored at −20°C for 2 h to overnight. After centrifugation and vacuum-drying, the pellet was resuspended in 8 ml TE and prepared for CsCl gradient centrifugation. DNA bands were sampled via needle and syringe from the CsCl gradients and butanol-extracted [7]. An equal volume of water was added prior to ethanol precipitation performed as described above. Precipitated DNA was recovered by centrifugation at $12\,000\times g$ at 4°C for 1 h. The pellet was vacuum-dried and resuspended in 2 ml TE buffer.

2.4.4. DNA quantitation

Samples were analyzed for *nahA* [11] gene frequency by using a DNA slot-blot procedure whereby single-stranded DNA is fixed to Biotrans™ nylon membranes (ICN, Irvine, CA). DNA extracts were diluted 10-fold in TE prior to applying to membranes. It appeared that more concentrated samples had lower hybridization signals due to binding of humic materials to the membranes, therefore blocking binding of DNA. More consistent results were obtained when samples were diluted prior to fixing to membranes and probing. Dilutions of samples and *nahA* DNA standards were prepared in 0.4 M NaOH (0.5 ml final volume) and boiled for 10 min. Samples and standards were applied to slots in a slot-blot apparatus holding a pre-wetted nylon membrane. A vacuum was applied until slots were dry. After rinsing slots with 0.5 ml 0.4 M NaOH and vacuum-drying, the blot was removed and baked for 1 h at 80°C. The blot was then rinsed briefly in 2×SSC

(0.3 M NaCl, 34 mM trisodium citrate, pH 7.0) and allowed to air-dry.

The blot was prehybridized in a solution consisting of 0.5 M Na_2HPO_4, 1 mM EDTA, and 7% SDS (pH 7.2) at 65°C overnight. Radiolabeled single-stranded DNA probe was generated by PCR (GeneAmp, Perkin Elmer Cetus) with AmpliTaq DNA polymerase using double-stranded *nahA* DNA as template [12], a single oligonucleotide primer, cold dATP, dTTP and dGTP, and [α-^{32}P]dCTP. The probe was purified by elution through a push column and quantified by liquid scintillation counting. Approx. 10^8 dpm were added to the prehybridization solution and allowed to hybridize at 65°C overnight. The blots were washed at 65°C for 30 min three times in high stringency buffer consisting of (g/l): 0.59 NaCl, 2.42 Tris-OH, 0.37 EDTA and 5.0 SDS (pH 7.0–8.0). Blots were subsequently autoradiographed and hybridization signals were quantitated by a Visage 110 digital imager (Millipore, Ann Arbor MI) with reference to a standard curve of integrated optical densities calculated from the same blot.

2.4.5. Lipid and DNA extraction from wet cells and soil

The effect of lipid extraction on the recovery of DNA from wet bacterial cells and soil was also evaluated. In this case, a known quantity of cells was added so that comparisons could be made with cell number estimates obtained from both lipid and DNA analyses. Samples were prepared as follows: *Pseudomonas putida* B2 was grown to an A_{546} of approx. 1.0 on Luria broth with kanamycin at 50 μg/ml. This strain contains a single copy of *todC1* [13] on the chromosome which encodes for the α-subunit of toluene dioxygenase. This strain was used since the presence of one copy per cell is more consistent than the estimated 4 copies per cell [14] for the *nahA* gene frequency. Furthermore, the presence of a kanamycin antibiotic resistance gene on a plasmid in this strain helped ensure that the starting inocula was homogeneous. Cells were harvested by centrifugation at 5500×*g* for 10 min at 4°C. After washing 2 times with phosphate-buffered salts media, cells were concentrated about 40-fold and 2 ml were added to 10 g of air-dried sieved soil, a sufficient volume to just wet the soil. From a dilution series of the inoculum, it was determined that $4.1\pm0.7\times10^9$ CFU were added per g dry soil. The cell/soil mixture was frozen at −50°C until the extractions were performed. As described above, three replicates were subjected to the modified Bligh and Dyer extraction with subsequent DNA extraction and three replicates were subjected only to DNA extraction. The Bligh and Dyer extraction was performed essentially as described above except that the extraction for lipids was allowed to proceed for 3 hr. Centrifugation (8000×*g*, 15 min) was used to facilitate phase separation. The aqueous phase was kept separate from the soil phase since it had been determined that any DNA present could be recovered by ethanol precipitation. DNA extracts were analyzed as described except that PCR-generated single-stranded *todC1* probe (approx. 2.0×10^7 dpm) was used for hybridization. The aqueous phase and the cellular material from the aqueous/organic interface from the Bligh and Dyer extractions were analyzed for the presence of *todC1* DNA as well. Samples were ethanol precipitated, resuspended in TE buffer and applied to membranes with subsequent hybridization with the *todC1* probe.

2.4.6. Analysis of DNA degradation

Aliquots of DNA extracts from samples treated with and without lipid extraction were subjected to 1% agarose gel electrophoresis at 70 V in 1×TBE. A 1 kb ladder (Gibco BRL, Grand Island, NY) was used as a size marker. The gel was stained with ethidium bromide and the DNA was visualized under UV irradiation.

3. Results and discussion

3.1. Experiments with ^{32}P-labeled DNA in the absence or presence of soil

It was determined from scintillation counting that when soil was not present, essentially all of the ^{32}P-labeled λ DNA (>98% of added counts) was recovered in the aqueous phase after lipid extraction. Essentially no DNA was precipitated without the addition of a 0.1 volume of 3 M sodium acetate and at least one volume of ethanol. Addition of 2 volumes of ethanol resulted in complete DNA precipitation. Therefore, this treatment was performed

on the aqueous and interface phase samples recovered from the Bligh and Dyer extraction. Analysis of the aqueous phase samples by PAGE showed that there was no degradation of λ DNA during lipid extraction even in the presence of a high bacterial cell density (Fig. 2). There was no smearing below the 500 bp λ DNA fragment showing a lack of degradation products. The inclusion of EDTA in the extraction buffer did not appear to be necessary.

Conversely, when soil was present, the ^{32}P-labeled λ DNA was almost completely associated with the soil phase after lipid extraction. Less than 2% of the DNA was recovered in the aqueous phase and no DNA was recovered in the organic phase. Upon DNA extraction of the soil, approx. 80% of the counts were recovered in the pooled crude DNA extracts with about 8% remaining with the soil. Although no comparisons were made with treatments which were not subjected to lipid extraction, these results show that DNA in the presence of soil organic matter was not partitioning to the organic phase.

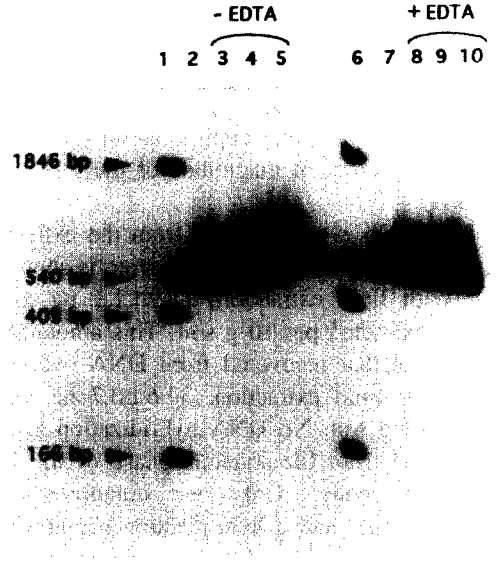

Fig. 2. Autoradiogram of ^{32}P-labeled PCR-generated 500 bp λ DNA fragment electrophoresed on a 5% polyacrylamide gel. Lanes 1 and 6: ^{32}P-end-labeled fragments from a *Dde*I digest of pBluescript II (KS); Lanes 3–5: Replicates of λ DNA exposed to the 3-h lipid extraction; Lanes 8–10: Replicates of λ DNA exposed to the 3 h lipid extraction in the presence of 1 mM Na_2EDTA; Lanes 2 and 7: Control, the same amount of λ DNA not exposed to lipid extraction.

3.2. Experiments with lyophilized cells in the absence of soil

Analysis of the aqueous phase after lipid extraction by UV absorbance showed 260/280 nm ratios of 1.9 or greater for the *P. putida* DNA samples. For the *B. subtilis* samples the values were slightly lower, 1.7 to 1.8, but still representative of relatively pure DNA. Based on the absorbance values λ=260 nm, 26±5% of the total theoretical DNA yield was recovered from the *P. putida* cell. For *B. subtilis*, 14±4% of the theoretical total DNA was recovered in the aqueous phase. These results are based on a theoretical value of 3.1 g DNA/100 g dry cell weight [15] which was determined for *E. coli* during balanced growth at 37°C. It appeared that more DNA was recovered from *P. putida* than from *B. subtilis*. These results suggest that the lipid extraction results in differential release of DNA into the aqueous phase for gram-positive and gram-negative bacteria. These data support the need to perform DNA extraction of the sample in order to recover comparable amounts of DNA from gram-positive and gram-negative bacteria.

3.3. DNA extraction methodology

The DNA extraction procedure (modified from [2]) without polyethylene glycol precipitation and phenol/chloroform extraction worked well with these samples. With a higher organic carbon content soil, it may be more difficult to visualize the DNA band after CsCl gradient centrifugation. By sampling the entire liquid volume, there is a lower chance of loss of DNA. Although not included in this study, an external DNA fragment, such as λ DNA can be added in order to account for DNA extraction efficiency [16,17].

For the experiment with lyophilized cells, the entire liquid volume was sampled since it was difficult to visualize a distinct band, whereas for the experiment with wet cells a band was sampled via a needle and syringe. The lipid extraction had the effect of removing some soil humic components which are also extracted with the DNA extraction procedure. It was easier therefore to visualize the DNA band after CsCl centrifugation in the lipid-extracted treatments.

Table 1
Cell number determination for *P. fluorescens* 5R and *P. putida* B2 from lipid analysis, DNA analysis and plate counts of the inoculum

Treatment	Quantity of DNA (ng/10 g soil)	Estimate of cell density from DNA analysis (cells/10 g soil)	Estimate of cell density from lipid analysis (cells/10 g soil)
Lyophilized cells (5R)			
With lipid extraction	22.8±6.2	5.20×10^{10}	
Without lipid extraction	42.4±4.4	9.67×10^{10}	
Wet cells (B2)			
With lipid extraction	11.9±1.1	$3.34\pm0.11\times10^{9}$	$3.31\pm0.46\times10^{10}$
Without lipid extraction	32.6±0.7	$2.98\pm0.06\times10^{10}$ [a]	
Plate counts		$4.1\pm0.7\times10$	

[a]Value represents average of two replicates. All other values based on average of three replicates.

3.4. DNA analysis

The results from DNA : DNA hybridization analyses of extracted DNA are summarized in Table 1. Hybridization analyses of DNA extracted from lyophilized cells and soil showed that after lipid extraction, 22.8±6.2 ng *nahA* DNA was extracted from 10 mg lyophilized cells and 10 g soil. This amount was 54% of that extracted from cells and soil which had not been previously extracted for lipids, 42.4±4.4 ng *nahA* DNA. Fig. 3 shows the autoradiogram of the slot blot hybridized with the *nahA* probe. Soils for which no cells were added did not show any hybridization signals (data not shown).

Fig. 3. Autoradiogram of a DNA slot-blot hybridized with a ^{32}P-labeled *nahA* probe under high stringency conditions. DNA was extracted from soil to which lyophilized *P. fluorescens* 5R had been added. Results are shown for DNA extraction with or without initial lipid extraction. The volumes shown correspond to the amount of the extracts loaded. The DNA standards were prepared from a *nahA* clone (gift from B. Ensley). The 0.3, 0.1 and 0.03 ng *nahA* standards were used to generate the standard curve (r^2 =0.999) and the 5 μl samples were used for quantitation.

Assuming that there are approx. 4 copies of *nahA* per cell and since *nahA* is 1 kb in size, 42.4 ng *nahA* DNA per 10 g soil corresponds to approx. 9.67×10^{9} cells per 10 g soil. The detection limit for this analysis was approx. 2.0×10^{6} cells. Based on the assumption that the dry weight of a cell is approx. 2.8×10^{-10} mg as determined for *E. coli* [15], 10 mg of lyophilized cells should have yielded 3.57×10^{10} cells per 10 g soil. Although the calculated cell density is based on some approximations, it is still within half an order of magnitude of the theoretical cell density.

Similar results were obtained from the extraction of wet cells and soil. The autoradiogram is shown in Fig. 4. With lipid extraction, 11.9±1.1 ng *todC1* DNA was extracted per 10 g soil. This amount was about 37% of that recovered from DNA extraction without prior lipid extraction, 32.6±0.7 ng *todC1* DNA per 10 g soil. No DNA hybridization signals were detected from the aqueous phase of the lipid extraction procedure. Only one organic/aqueous interphase sample had a hybridization signal which corresponded to <0.2% of that recovered from the soil phase.

Since *todC1* DNA is 1 kb in size and there is only one copy per cell, 32.5 ng *todC1* DNA per 10 g soil corresponds to approx. 2.98×10^{10} cells per 10 g soil, determined from plate counts of the inoculum. For the samples extracted for lipids prior to DNA extraction, 11.9 ng *todC1* DNA per 10 g soil

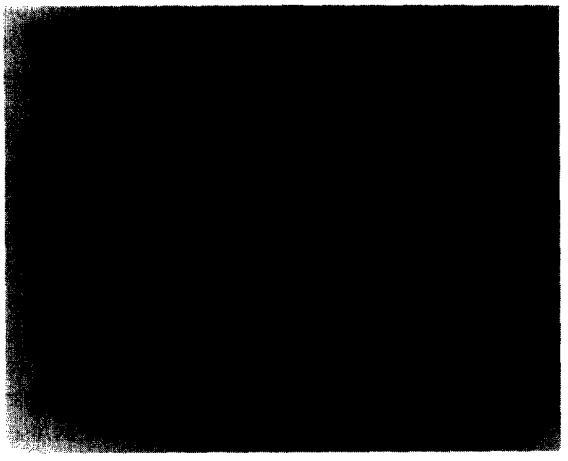

Fig. 4. Autoradiogram of a DNA slot blot hybridized with a ^{32}P-labeled *todC1* probe under high stringency conditions. DNA was extracted from soil to which *P. putida* B2 had been added. Results are shown for conventional DNA extraction and extraction from the solid phase after lipid extraction. No DNA hybridization signals were detected from the aqueous phase of the lipid extraction procedure. The volumes shown correspond to the amount of the extracts loaded. The DNA standards were prepared from pDTG601 containing the genes encoding toluene dioxygenase ([13]). The 0.3, 0.1 and 0.03 ng *todC1* standards were used to generate the standard curve (r^2 =0.998) and the 1 or 5 μl samples were used for quantitation.

corresponds to approximately 1.09×10^{10} cells per 10 g soil. The detection limit for this analysis was approx. 9.0×10^{6} cells.

3.5. Lipid analysis

The fatty acid profiles obtained were typical of members of the *Pseudomonas* genus, with no fatty acids present that would indicate an indigenous population of soil bacteria. A significant extant population was not expected, since the soil used had a fairly low organic carbon content and had been stored in a dried, sieved state for more than 2 years. Lipid analysis showed that there was no interference from the soil chemical parameters. The total picomoles of fatty acid were used to estimate cell number in each soil sample. Sample 1 contained 2.44×10^{7} pmol of fatty acid per 10 g soil. This corresponds to 3.45×10^{10} cells in 10 g of soil [18]. Sample 2 contained 2.59×10^{7} pmol total fatty acid, corresponding to 3.69×10^{10} cells in the sample. Sample 3 contained a total of 1.98×10^{7} pmol fatty acid, corresponding to 2.8×10^{10} cells.

The cell number values obtained from both lipid analysis (3.3×10^{10}) and DNA analysis (2.98×10^{10}) compared favorably with the original plate count numbers (4.1×10^{10}) of the inoculum, which shows the utility of each technique for estimation of microbial biomass.

3.6. Quality of extracted DNA

A visual inspection of the extracted DNA by agarose gel electrophoresis and EtBr-staining is shown in Fig. 5. Three times more of the lipid-extracted samples were loaded relative to the non-lipid-extracted samples. The gel showed that the samples subjected to lipid extraction had less total DNA, confirming the hybridization data. However, the lipid extraction did not appear to affect the molecular weight distribution of the recovered DNA. Plasmid DNA (above 12.2 kb band of ladder) is

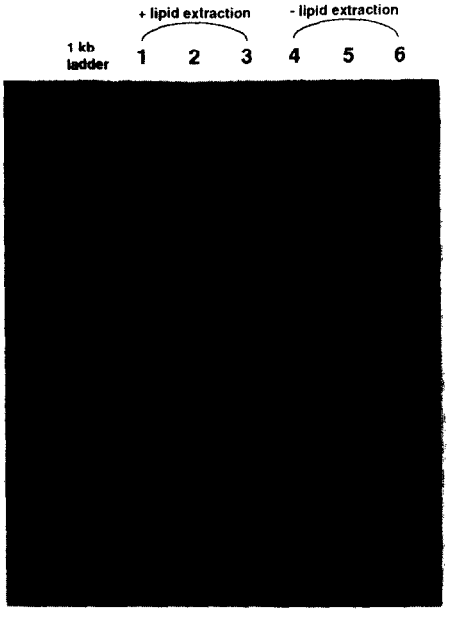

Fig. 5. An ethidium-bromide stained agarose gel loaded with DNA extracted from soil with or without initial lipid extraction. Lane 1. 1 kb DNA ladder; Lanes 2–7: DNA extracted from soil to which *P. putida* B2 had been added (lanes 2–4, plus lipid extraction, 15 μl of sample loaded; lanes 5–7: minus lipid extraction, 5 μl of sample loaded).

apparent in extracts from samples treated with and without lipid extraction.

3.7. Effect of lipid extraction on DNA recovery

This study showed that DNA of sufficient quality for DNA : DNA hybridization analysis was recovered from samples which were previously extracted for lipids. However, the yield was approx. 40–50% of that recovered by conventional DNA extraction. For these experiments where a large biomass was added, the amount of DNA recovered was well above the detection limits for DNA hybridization analyses. For samples which contain lower biomass, PCR amplification may be required. The DNA may be further purified by using PVPP [19], Elutip-d columns [20], or glass milk purification [21], should such purification be necessary.

Initially it was thought that the lipid extraction procedure resulted in release of DNA to the aqueous phase and that this DNA was degraded or lost by some other means prior to its isolation. Our results showed that DNA is released from cells into the aqueous phase after lipid extraction. However, experiments with cells and ^{32}P-labeled λ DNA in the absence of soil showed that no degradation occurred over the course of the lipid extraction period (Fig. 5). Furthermore, when soil was present less than 2% of the ^{32}P-labeled λ DNA partitioned to the aqueous phase of the lipid extract and about 80% of the counts were obtained by DNA extraction of the soil. Therefore, the loss of DNA was not likely due to degradation in the aqueous phase.

The most plausible explanation for the loss of DNA relates to the decrease in the amount of soil organic matter recovered in the DNA extracts. Organic matter in the form of humic acids are extracted during the DNA extraction procedure. Samples which had been extracted for lipids prior to DNA extraction were consistently lighter in color, suggestive of a decreased organic matter content. In the absence of soil, lipid extraction resulted in extraction of DNA from *P. putida* into the aqueous phase. It is possible that in the presence of soil the lipid extraction also releases DNA from the cells and that this DNA is then tightly associated with the organic matter and suffers the same fate; either it is extracted with the lipids or it remains associated with the soil during DNA extraction. However, we did show that exogenous λ DNA in the presence of soil organic matter is not partitioning into the organic phase of the lipid extraction.

Contrary to the soil-free experiments with lyophilized cells, none of the aqueous phase samples from the lipid extraction of cells and soil showed DNA to be present by hybridization analysis. The detection limit was approx. 9×10^6 cells (0.03% of that recovered from the soil phase). In only one of the three replicates was there any DNA detected in an organic/aqueous interface sample, accounting for less than 0.2% of that recovered from the soil phase. From sands or sandy aquifer material, such as used by Hard et al. [6], less DNA may be associated with the solid phase after lipid extraction. Ogram et al. [22] showed that DNA adsorption coefficients were one to two orders of magnitude lower for sand than for any of the soils tested. The fact that Hard et al. [6] recovered small fragments (<100 bp) is supported by the work of Ogram et al. [22] who reported that larger DNA fragments are preferentially sorbed to sand. Regardless of DNA adsorption, the method used in this work (modified from [2]) resulted in efficient extraction of DNA from a sandy loam soil, resulting in a range of fragment sizes, as well as intact plasmid DNA. These data demonstrate the necessity of performing the DNA extraction protocol in order to release the adsorbed DNA as well as to recover DNA more uniformly from both gram-negative and gram-positive bacteria. The combination of a lipid and a DNA analysis, as described here, can provide a powerful tool for the direct examination of native microbial communities in environmental samples.

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