

# Reports

## Extraction of high molecular weight DNA from microbial mats

Benjamin S. Bey, Erin B. Fichot, Gargi Dayama, Alan W. Decho, and R. Sean Norman

*Department of Environmental Health Sciences, Arnold School of Public Health, University of South Carolina, Columbia, SC, USA*

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Due to the presence of inhibitors such as extracellular polymeric substances (EPSs) and salts, most microbial mat studies have relied on harsh methods of direct DNA extraction that result in DNA fragments too small for large-insert vector cloning. High molecular weight (HMW) DNA is crucial in functional metagenomic studies, because large fragments present greater access to genes of interest. Here we report improved methodologies for extracting HMW DNA from EPS-rich hypersaline microbial mats. The protocol uses a combination of microbial cell separation with mechanical and chemical methods for DNA extraction and purification followed by precipitation with polyethylene glycol (PEG). The protocol yields  $>2 \mu\text{g}$  HMW DNA ( $>48 \text{ kb}$ ) per gram of mat sample, with  $A_{260:280}$  ratios  $>1.7$ . In addition, 16S rRNA gene analysis using denaturing gradient gel electrophoresis and pyrosequencing showed that this protocol extracts representative DNA from microbial mat communities and results in higher overall calculated diversity indices compared with three other standard methods of DNA extraction. Our results show the importance of validating the DNA extraction methods used in metagenomic studies to ensure optimal recovery of microbial richness.

Hypersaline microbial mats are layered, organosedimentary structures that provide unique systems to examine community dynamics and biogeochemical cycling (1–7). Given the high level of interactions occurring within microbial mats, they provide ideal model systems for metagenomic studies aimed at understanding complex microbial interactions. However, unlike soil, sediment, or water samples, microbial mats contain high levels of salts and extracellular polymeric substances (EPSs) (3), rendering the isolation of high-quality DNA extremely difficult. Currently, most molecular-based microbial studies use harsh methods of DNA extraction directly from samples, such as microbial mats (8) and soils/sediments (9–13). While these methods are effective, there are two major limitations that preclude a detailed understanding of microbial mat functioning: (i) it is not known how well the final DNA represents the microbial community and (ii) the DNA is sheared into small fragments that are sufficient for microbial diversity studies but too small for large-insert metagenomic studies.

In this study, we developed methods to extract microbial community DNA from hypersaline microbial mat samples and compared it with commonly used DNA extraction methods. Our goals were to extract DNA (i) with comparable efficiency and presumed minimal bias inherent to bead

beating methods; (ii) with high molecular weight (HMW) by using gentle approaches that minimally shear DNA; and (iii) with high quality for downstream applications. Results suggest that removing bacterial and archaeal cells from sediment is an essential first step in DNA extraction from hypersaline mats. While this study focused on microbial mats as a model system, our results show the importance of validating DNA extraction methods used in metagenomic studies to ensure optimal microbial richness recovery. Furthermore, since microbial mats represent one of the most troublesome sample types for nucleic acid recovery, our approach can be utilized for other recalcitrant samples. Finally, this study highlights the importance of extensive DNA extraction quality control prior to any metagenomic studies based on clone library construction or pyrosequencing.

### Materials and methods

#### Sample sites and collection

Microbial mats were collected from three hypersaline ponds in the Bahamas: Salt Pond (24°01'N, 74°27'W), French Pond (23°57'N, 74°31'W), and Darby Pond (23°50'N, 76°12'W). Samples were collected to a depth of 6 cm and frozen at  $-20^\circ\text{C}$  for transport to the laboratory

where they were maintained at  $-80^\circ\text{C}$  until processing.

#### Microbial cell removal

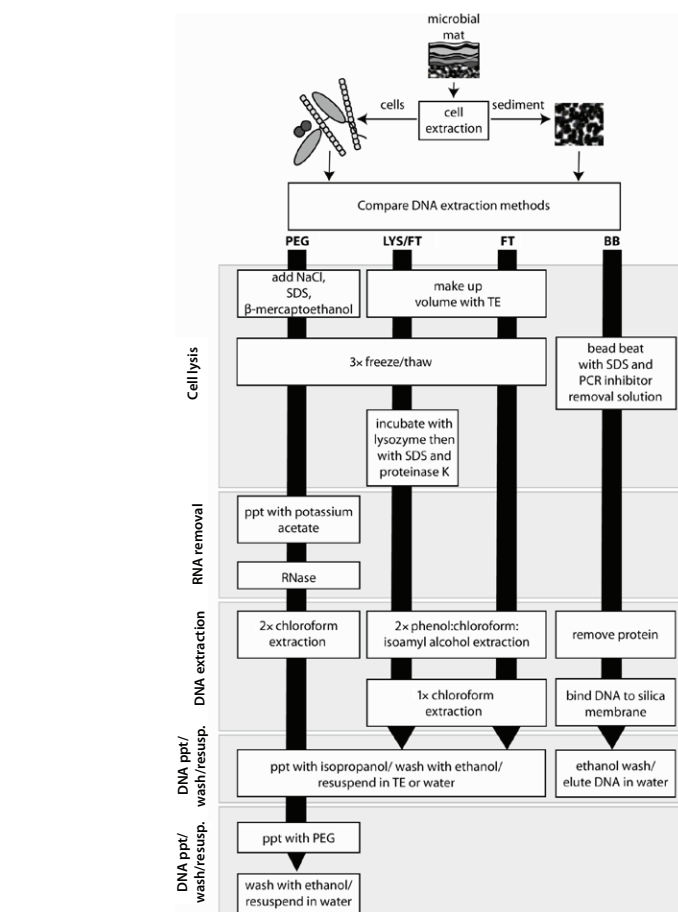
Microbial cells were extracted from 30 g (wet weight) of homogenized mat samples to separate the cells from the background sediment matrix. First, samples were blended three times in 100 mL 1 M NaCl (BDH Chemicals, Dubai, UAE) using a Waring blender (Waring Laboratory Science, Torrington, CT, USA) at medium speed for 1 min with intermittent cooling for 1 min at  $-20^\circ\text{C}$  to limit temperature-induced cell lysis (8,9). To enhance cell desorption from sediments, the final blended slurry was adjusted to 250 mL with 1 M NaCl and shaken (150 rpm) at room temperature for 30 min. It should be noted that this salinity is sample-specific and can be adjusted for different samples. The slurry was centrifuged at low speed ( $500\times g$  for 15 min at  $4^\circ\text{C}$ ) to separate particulates from microbial cells. The supernatant, containing microbial cells, was transferred into a 250-mL centrifuge bottle, and the sediment from the previous cell extraction was blended again, shaken, and centrifuged four additional times in 250 mL 1 M NaCl, as described earlier. Observation by epifluorescent microscopy of extracted cells and spent-sediment suggested that cyanobac-

teria were represented in the cell extracts. Supernatants from each of the five blending steps were combined, and microbial cells were pelleted by high-speed centrifugation (25,000× *g* for 15 min at 4°C). To reduce inhibitors of downstream applications, the cell pellet was resuspended in 250 mL 2% sodium hexametaphosphate (EMD Chemicals, Gibbstown, NJ, USA), a sequestrant/chelator of compounds such as humic acids, free DNA, and antibiotics (10,11). The resuspended cells were shaken at room temperature for 30 min at 150 rpm and centrifuged at 25,000× *g* for 15 min at 4°C. The cell pellet was washed with 250 mL Tris-EDTA (TE) buffer, pH 8.0, containing 50 mM EDTA, shaken at room temperature for 10 min, and centrifuged at 25,000× *g* for 15 min at 4°C. The final cell pellet was resuspended in 15 mL TE (10 mM EDTA), and 200-μL aliquots were stored at -80°C for subsequent DNA extractions.

### DNA extraction

Mat sections were processed according to Figure 1. Microbial cells separated from the mat matrix were used for DNA extraction according to the following protocols: (i) the method described in this paper, modified freeze-thaw/polyethylene glycol (PEG); (ii) freeze-thaw, lysozyme (LYS/FT) method; (iii) freeze-thaw (FT) method; and (iv) a commercial kit that uses bead beating (BB). Wide-bore pipet tips were used in all DNA transfer steps to reduce shearing effects.

**Modified freeze-thaw/polyethylene glycol (PEG).** To the 200-μL aliquots of microbial cells, 200 μL 5 M NaCl, 200 μL 10% SDS (Fisher Scientific, Fair Lawn, NJ, USA), and 100 μL β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) were added to initiate cell lysis. For further cell lysis, the sample was gently mixed and subjected to three rounds of FT (12–14), which included submersion in liquid nitrogen for 2 min followed by thawing at 65°C for 5 min. Following the final thaw, samples were incubated for an additional 10 min at 65°C. To precipitate cell debris, proteins, and any remaining polysaccharides, 200 μL 5 M potassium acetate, pH 5.5 (Fisher Scientific) were added, and the samples were incubated on ice for 10 min followed by centrifugation at 10,000× *g* for



**Figure 1. Flowchart describing DNA extraction methodology.** DNA was indirectly extracted from the microbial cells within mat samples using four methods: (i) PEG method developed in this study; (ii) LYS/FT method; (iii) FT method; and (iv) BB method. For each extraction method, steps pertaining to cell lysis; RNA removal; DNA extraction; and DNA precipitation (ppt), washing, and resuspension are outlined.

5 min at 4°C. Fifteen micrograms of RNase (Epicentre Biotechnologies, Madison, WI, USA) were added to the supernatant followed by incubation at 37°C for 1 h. The lysate was extracted twice with an equal volume of chloroform (Fisher Scientific), and the DNA was precipitated for 30 min on ice with an equal volume of isopropanol (Fisher Scientific). Precipitated DNA was centrifuged at maximum speed for 10 min at 4°C, and the pellet was washed with 1 mL 70% ethanol (Fisher Scientific). DNA was then resuspended in 500 μL TE and further purified away from contaminating polysaccharides by the addition of 500 μL 20% PEG 8000 (Promega, Madison, WI, USA)

followed by centrifugation at maximum speed for 10 min at 4°C. The final DNA pellet was washed with 70% ethanol, resuspended in 30 μL molecular-grade water, and stored at -80°C.

**Lysozyme/SDS/proteinase K/FT (LYS/FT).** To the 200-μL cell aliquots, TE was added to adjust the volume to 556 μL. Samples were subjected to three rounds of FT as described in the PEG method. Next, 1 mg lysozyme (EMD Chemicals, Gibbstown, NJ, USA) was added, and the samples were incubated at 37°C for 1 h. Following incubation, 30 μL 10% SDS and 0.2 mg proteinase K (Ambion, Austin, TX, USA) was added,

**Table 1. Assessment of the quality and quantity of DNA extracted from three hypersaline mat systems using four different methods**

Extraction method	Darby Pond			French Pond			Salt Pond		
	ng/g	A <sub>260/280</sub>	A <sub>260/230</sub>	ng/g	A <sub>260/280</sub>	A <sub>260/230</sub>	ng/g	A <sub>260/280</sub>	A <sub>260/230</sub>
PEG	2050.0	1.7	0.8	2050.0	1.8	0.8	2187.5	1.8	1.1
LYS/FT	1112.5	1.5	0.5	1025.0	1.6	0.6	837.5	1.8	0.6
FT	650.0	1.5	0.5	700.0	1.6	0.6	512.5	1.6	0.4
BB	312.5	1.4	0.2	395.8	1.4	0.2	229.2	1.3	0.3

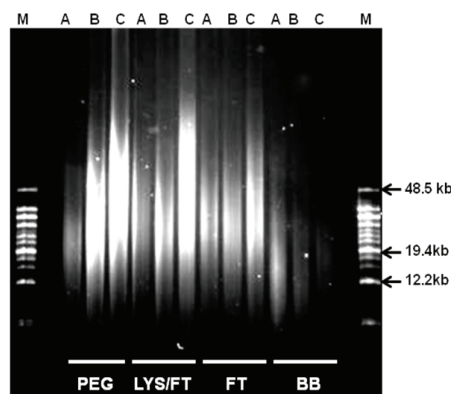
and the samples were incubated at 37°C for 2 h. The samples were extracted twice with 750  $\mu$ L phenol-chloroform-isoamyl alcohol (P:C:I; 25:24:1; Fisher Scientific) and once with an equal volume of chloroform. DNA was precipitated for 30 min on ice with an equal volume of isopropanol, centrifuged at maximum speed for 10 min, washed with 750  $\mu$ L 70% alcohol, and air-dried. The DNA was resuspended in 30  $\mu$ L molecular-grade nuclease-free water and stored at -80°C.

**Freeze-thaw (FT).** The 200- $\mu$ L cell aliquots were adjusted to 500  $\mu$ L with TE and then subjected to three rounds of FT as described earlier. Samples were extracted twice with 750  $\mu$ L P:C:I (25:24:1) followed by a final extraction with an equal volume of chloroform. DNA was precipitated and washed as described in the LYS/FT method. The DNA was resuspended in 30  $\mu$ L molecular-grade nuclease-free water and stored at -80°C.

**Bead beating (BB).** The 200- $\mu$ L cell aliquots were subjected to continuous BB using the Vortex Genie 2 (MoBio Laboratories, Carlsbad, CA, USA) for 10 min, and the DNA was eluted through a silica column according to the manufacturer's instruction (UltraClean Soil DNA kit; MoBio Laboratories). This method was also used to extract DNA from 100 mg spent-sediments after bacterial cell extraction. The spent-sediment DNA was used to verify the effectiveness of our method in the removal of representative microbial cells.

#### DNA quantification, purity, and size

DNA concentration was measured with a fluorometer using a Quant-iT dsDNA Assay kit (Invitrogen, Carlsbad, CA, USA). Purity of the extracted DNA was measured with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Further purity and size determination of 700 ng each DNA extract were examined using pulse-field gel electrophoresis on a 1% agarose gel with 0.5 $\times$  TBE buffer using a CHEF Mapper XA System (Bio-Rad Laboratories, Hercules, CA, USA). Run-time parameters included an initial switch time of 0.35 s, a final switch time of 7.67 s, and 120° included angle for a gradient of



**Figure 2. Molecular weight characterization of extracted DNA by pulse-field gel electrophoresis.** DNA was extracted from (A) Darby Pond, (B) French Pond, and (C) Salt Pond using the PEG, LYS/FT, FT, and BB DNA extraction methods. The marker (M) used was a 48.5-kb ladder.

6.0 V/cm at a linear ramping factor. The gel was stained with 1 $\times$  SYBR Green I nucleic acid stain for 30 min and imaged on a BioSpectrum AC Imaging System (UVP, Upland, CA, USA).

#### Denaturing gradient gel electrophoresis

The V3 hypervariable region of the bacterial 16S rRNA genes was amplified using primers 341F (with 40 bp GC clamp) and 534R (15). Each PCR reaction contained 1 ng DNA template, 1 $\times$  PCR buffer, 1 mM MgCl<sub>2</sub>, 0.2 mM each primer, 0.2 mM nucleotide mixture, and 0.5 U *Taq* DNA polymerase (Invitrogen). Amplification was carried out on a DNA Engine thermal cycler (Bio-Rad Laboratories) with the cycling conditions of 95°C for 4 min, 19 touchdown cycles at 94°C for 1 min, 65°C (-0.5°C per cycle) for 1 min, 72°C for 45 s, followed by an additional 14 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 45 s, and a final extension at 72°C for 5 min. PCR amplicons were analyzed by denaturing gradient gel electrophoresis (DGGE; Bio-Rad Laboratories) on a 10% polyacrylamide gel with a denaturant gradient of urea and formamide ranging from 40% to 60%. Electrophoresis was carried out for 16 h at 70 V and 60°C. The gel was stained for 1 h with SYBR Green I DNA stain and imaged with a Storm fluores-

cence imaging system (GE Healthcare Biosciences, Piscataway, NJ, USA).

#### 16S rRNA gene tag pyrosequencing

The V3 hypervariable region of bacterial and archaeal 16S rRNA genes was amplified using the 454-pyrosequencing adapter primer sets 341f-A (5'-GCCTCCCTCGCGCCAT-CAGCCTACGGGAGGCAGCAG-3') and 534r-B (5'-GCCTTGCCA-GCCCGCTCAGATTACCGCG-GCTGC-TGG-3') (15), and Arc 344f-A (5'-GCCTCCCTCGCGCCATCA-GACGGGGCGCAGCAGGCGCA-3') and Arc 517r-B (5'-GCCTTGCCAGC-CCGCTCAGATTACCGCGGCT-GCTGG-3') (2). The 454 adapter sequences are underlined. PCR conditions were 3 min at 94°C; 30 cycles of 45 s at 94°C, 45 s at 55°C, and 45 s at 72°C; and then 10 min at 72°C. Amplicons were sequenced on a Genome Sequencer FLX System (Roche, Branford, CT, USA) at the University of South Carolina Environmental Genomics Facility (EnGenCore, LLC, Columbia, SC, USA). Sequences were cleaned using the following bioinformatics pipeline: (i) sequences with low quality scores (<30) and short read lengths (<100) were removed and (ii) sequence data were dereplicated and the remaining sequences checked for chimeras using Bellerophon (16). High-quality and nonchimeric sequences were aligned using MUSCLE (17), and a distance matrix was calculated using PHYLIP (18). Diversity indices (Shannon-Weaver and Simpson), richness estimators (Chao1 and ACE), and rarefaction curves (97% identity level) were generated using the program MOTHR (19).

#### Results and discussion

Many methods exist for extracting DNA from soil and sediment microbial communities. These are categorized into direct (20–25) and indirect methods (8,9,26–28). Preliminary studies indicated that while DNA could be directly extracted from microbial mat samples, the DNA contained contaminants that inhibited downstream PCR-based applications. Thus, extracting HMW DNA from hypersaline microbial mats for metagenomic studies presents a

**Table 2. Diversity and richness indices calculated from pyrosequencing data for OTUs recovered from extracted bacterial cells versus spent-sediment.**

Indices	Cell PEG			Cell BB			Sediment BB		
	Darby Pond	French Pond	Salt Pond	Darby Pond	French Pond	Salt Pond	Darby Pond	French Pond	Salt Pond
Simpson	0.9860	0.9738	0.9887	0.9636	0.9069	0.8032	0.8757	0.5752	0.8873
Shannon-Weaver	5.39	4.56	5.47	4.47	3.86	2.86	3.46	1.92	3.07
Chao1	2259.3	578.60	1447.00	624.12	768.43	288.55	572.50	251.05	492.06
ACE	4458.4	894.41	2433.83	1039.13	1145.8	513.13	1200.70	405.99	888.91

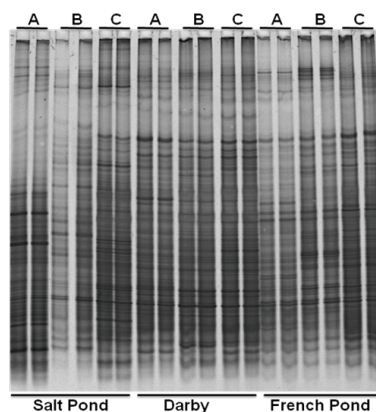
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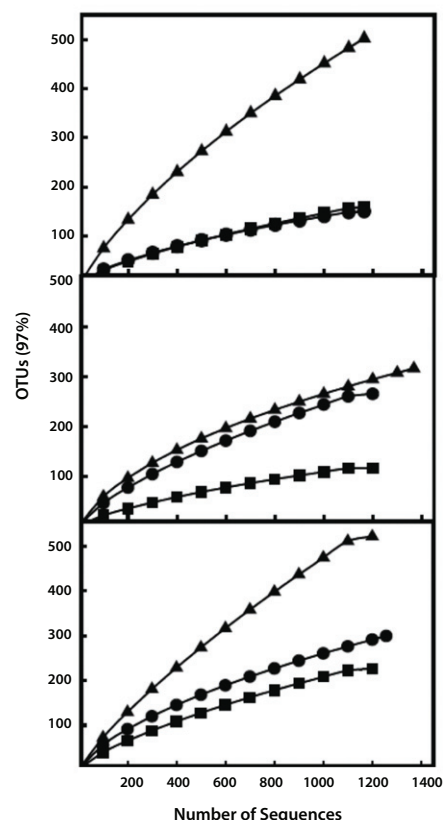
**Figure 3.** Effect of DNA extraction method on observed microbial community composition as determined by 16S rRNA gene amplification and DGGE analysis. Samples from Salt Pond, Darby Pond, and French Pond were either extracted from cell-extracted spent-sediment with (A) BB or indirectly extracted from the microbial mat-separated cells with (B) BB or (C) PEG. Duplicate PCRs were run in adjacent lanes.

challenge. Indirect methods, which involve the separation of cells from background matrix prior to DNA extraction, are increasingly used for environmental samples to minimize difficulties posed by organic and inorganic contaminants. Here, we used an indirect method, coupled with combined and modified DNA extraction protocols, to extract HMW DNA with high quality and high quantity from three different hypersaline microbial mats.

For each mat system, the PEG method yielded DNA with the highest purity, while BB resulted in the lowest purity based on 260/280 and 260/230 ratios (Table 1). The low 260/230 ratios are likely due to small amounts of EPS carryover, which did not inhibit downstream enzyme-based applications, as indicated by subsequent successful PCR amplification of bacterial 16S rRNA genes. Furthermore, the PEG method yielded the highest amount of DNA [2050, 2050, and 2188 ng/g sediment (wet) from Darby Pond, French Pond, and Salt Pond, respectively], followed by LYS/FT with concentrations ~50% of that obtained by the PEG method. The FT and BB methods yielded the lowest amounts of DNA. These data show that the PEG method results in the highest concentration and quality of DNA extracted from the mat systems.

Pulse-field gel electrophoresis shows that the PEG, LYS/FT, and FT methods yielded higher MW DNA than the BB method, regardless of source material (Figure 2). DNA extracted by BB was noticeably sheared, producing DNA <20 kb in comparison to the larger-sized DNA (>48 kb) obtained with the PEG, LYS/FT, and FT methods.

While some cell lysis is likely to occur during cell removal, the step of separating cells



**Figure 4.** Rarefaction analysis of microbial communities from microbial mats. (A) Salt Pond, (B) French Pond, and (C) Darby Pond. The number of observed phylotypes at 97% identity (species) is plotted as a function of the cumulative number of 16S rRNA gene tag sequences. (▲) OTUs recovered using DNA extracted from bacterial cells with PEG; (●) OTUs recovered using DNA extracted from bacterial cells with BB; and (■) OTUs recovered using DNA extracted from spent-sediment with BB.

from the sediment was found to be a crucial first step in reducing levels of salts and EPS from mat samples. EPS is an adhesive matrix that can bind DNA (6,29), which limits DNA yield (30). Coprecipitation of EPS with extracted DNA may also occur, resulting in inhibition of subsequent downstream enzymatically mediated processes and applications, such as PCR and cloning. Additional cell washes with sodium hexametaphosphate and TE buffer used during cell extraction further reduced EPS and other contaminants (10,11), as seen by successful extraction of DNA and use in PCR-DGGE.

Another advantage of indirect DNA extraction methods is that cell removal from background material allows for gentle lysis, yielding DNA that is larger than DNA directly extracted from samples using harsh methods (31). In the current study, cell extraction removed contaminating EPS and allowed a gentle lysis of cells, yielding HMW DNA >48 kb in size from all mat samples. While larger DNA fragments can

be obtained by lysing cells embedded within an agar matrix, the efficiency of cell lysis is reduced, resulting in the introduction of an additional bias in subsequent analyses of complex microbial communities.

The ability to utilize extracted DNA in downstream applications was tested by PCR amplification of bacterial 16S rRNA genes. For each extraction method and site, amplification was successful prior to the 16S rRNA gene analysis using DGGE shown in Figure 3. Since it is practically impossible to remove all microbial cells from the mat samples, it was important to verify the representativeness of microbial communities removed by cell extraction. Although relevant to the ultimate interpretation of metagenomic data, most studies have avoided this step.

We used DGGE and pyrosequencing of 16S rRNA genes to verify the representativeness of bacterial communities removed from microbial mats. While DGGE is a low-resolution technique that underestimates true microbial richness, it is a rapid and inexpensive way to verify the representativeness of extracted bacterial communities. DGGE results showed that the PEG method developed in this study produces DNA that is representative of the community that remains tightly bound to the sediment matrix (Figure 3). It also showed that bacterial cell separation and DNA extraction were appropriate for representative bacterial community extraction as suggested by similar bands in cell and spent-sediment DNA from mat systems.

For each samplingsite, the overall banding pattern produced by each DNA extraction method is similar; however, not all bands [operational taxonomic units (OTUs)] are clearly identified with each extraction method. Compared with the BB method, PEG-extracted DNA increased OTU richness by 41% (Salt Pond), 38% (Darby Pond), and 28% (French Pond), based on band number. The extra cleaning steps in the PEG method were designed to decrease inhibitory compounds that may play a role in the enhanced microbial richness found using this method. In addition, when using the PEG method, OTU richness was 6% (Salt Pond), 28% (Darby Pond), and 37% (French Pond) higher in the cell extract than in the spent-sediment. Since it is inevitable that some microbes remain in the sediment following extraction, it is important to note that OTU richness in the cell extract was not compromised. In fact, removing the cells from the sediment matrix resulted in higher OTU richness, indicating that an effective bacterial cell removal protocol preceding DNA lysis and purification with PEG may enhance the ability to extract DNA from microbial community members.

Overall, this suggests that the PEG method produced DNA that was representative of the microbial community.

To provide estimates of community richness and diversity recovery using the PEG method, archaeal and bacterial 16S rRNA gene amplicons were pooled and sequenced, and OTU richness and diversity were compared with that obtained from the cell BB method. In all mats (Salt Pond, French Pond, and Darby Pond), the PEG method showed the highest OTU recovery by rarefaction analysis (Figure 4). Diversity indices and richness estimators were calculated to complement the results from the rarefaction curves and DGGE. Both Simpson's and Shannon-Weaver's diversity calculations indicate that the PEG method resulted in the highest estimated diversity recovery (Table 2). While species richness estimates (Chao1 and ACE) for Darby Pond and Salt Pond show higher recovery with the PEG versus cell BB method (similar to DGGE and rarefaction analyses), sequencing in the French Pond revealed the opposite trend, with higher species richness found after cell BB compared with PEG extraction (Table 2). Notwithstanding, the DNA extracted with PEG is of larger size, higher quantity, and higher quality than that extracted with cell BB.

While variability in the extraction efficiency of predominant community members (e.g., cyanobacteria) may influence the measured diversity, assuming that cell extraction is not biased toward a particular community, we hypothesized that the microbial diversity in the spent-sediment would be less than or equal to the diversity in cell extracts. Indeed, rarefaction curves show fewer OTUs (Figure 4), lower estimated richness, and lower diversity (Table 2) with spent-sediments from all three ponds, particularly when comparing with PEG extraction methods.

While all DNA extraction methods have limitations, this study suggests that microbial richness can be underrepresented in some studies that rely on direct extraction of DNA from difficult environmental samples or on DNA extraction protocols that are inefficient in removing environmental-specific inhibitors. Furthermore, and consistent with our results, Mayr and colleagues (32) found that the physiological profile of soil bacteria was unaffected following cell extraction, suggesting that extracted microbes can be representative of the indigenous microbial community.

The requirements for DNA used in large-insert vector-based metagenomic studies are high quantity, high quality (purity), HMW, and representativeness of the metagenome. To date, no method has successfully yielded DNA

with these properties from microbial mats, an ecosystem with copious EPS production. While Lee and colleagues (33) successfully extracted DNA from an EPS-producing bacteria, the resultant DNA was sheared and inappropriate for large-insert vector cloning. Our results showed that combining bacterial cell separation and PEG extraction protocols provided the best results, followed by LYS/FT, FT, and BB. The applicability of DNA extracted using the PEG protocol was demonstrated by successful construction of a metagenomic library from the Salt Pond microbial mat using the Epicentre CopyControl Fosmid Library Production kit. The library contained >1000 clones with an average insert size of 40 kb and is currently being screened for functional gene identification (data not shown).

The ability to generate large-insert metagenomic libraries from microbial mats will facilitate the discovery of novel biosynthetic pathways and ultimately lead to a more comprehensive understanding of this unique ecosystem. Furthermore, based on the results of this study, it is clear that a crucial first step for all metagenomic studies is to verify the representativeness of extracted DNA.

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## Competing interests

The authors declare no competing interests.

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Address correspondence to R. Sean Norman, University of South Carolina, Department of Environmental Health Sciences, 921 Assembly St., PHRC 401, Columbia, SC 29208, USA. e-mail: rsnorman@sc.edu