

A comparison of methods for total community DNA preservation and extraction from various thermal environments

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Abstract The widespread use of molecular techniques in studying microbial communities has greatly enhanced our understanding of microbial diversity and function in the natural environment and contributed to an explosion of novel commercially viable enzymes. One of the most promising environments for detecting novel processes, enzymes, and microbial diversity is hot springs. We examined potential biases introduced by DNA preservation and extraction methods by comparing the quality, quantity, and diversity of environmental DNA samples preserved and extracted by commonly used methods. We included samples from sites representing the spectrum of environmental conditions that are found in Yellowstone National Park thermal features. Samples preserved in a non-toxic sucrose lysis buffer (SLB), along with a variation of a standard DNA extraction method using CTAB resulted in higher quality and quantity DNA than the other preservation and extraction methods tested here. Richness determined using DGGE revealed that there was some variation within replicates of a sample, but no statistical difference among the methods. However, the sucrose lysis buffer preserved samples extracted by the CTAB method were 15–43% more diverse than the other treatments.

Keywords DGGE · DNA extraction · Environmental microbiology · Thermophile

Introduction

The impact of molecular studies on our knowledge of microbial diversity cannot be overstated. As a consequence, the entire field of environmental microbiology, from basic ecological research into the organization of microbial communities to bioprospecting for commercially relevant enzymes has changed. Even with recent advances in culturing efforts [19, 38], the majority of microbes in the environment still cannot be cultivated in the laboratory [34]. However, inability to maintain an organism in culture is no longer a major impediment to accessing its genetic diversity. Metagenomic studies similar to those that have been useful in exploring the diversity of uncultivated organisms have also been used to mine for enzymatic diversity [22]. The biotechnology applications that are currently targeting microbial metagenomic studies range from the search for new antibiotics to environmentally sound biocatalysts such as amylases [26, 35].

Thermal environments have been a particularly rich source of unique organisms [2, 13, 16, 20, 43], processes [5], novel enzymes [19, 39], and on-going research into the origin and diversity of microbes. Fundamental to any of these studies is maximizing the detectable diversity by optimizing the quality and quantity of DNA examined and minimizing the biases of the methods. There are layers of potential bias in molecular studies because of the sequential nature of the process, including those inherent in PCR [44] which have been well studied and will not be addressed in this paper. The first potential source of bias in the molecular study of environmental samples is determined by the method used to preserve the biomass. Cultivation based studies have shown that the groups of organisms that can be cultured from samples change drastically if the sample is not adequately preserved [15]. However, the impact of

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sample preservation on molecular diversity surveys is rarely examined in the literature (except see [17]).

Another possible source of bias includes DNA extraction methods and post-extraction purification. The effect of DNA extraction method on detectable diversity has been examined in soil [10, 11], marine sediments [27], compost [23, 45], and volcanic environments [18]. The results of these studies are mixed; certain methods are more effective for a particular type of sample than others. Additionally, no reports have examined the impact that the range of environmental pH extremes may have on extraction efficiency, which is of particular importance for thermal samples since they can come from springs on either end of the pH spectrum. While there are plenty of studies that examine effectiveness of extraction method on a particular type of sample, we aim to determine the method that is best for a wide variety of sample types.

Here, we evaluated sample preservation and DNA extraction methods, to identify a procedure that results in high molecular weight DNA that is relatively free from contaminants and maximizes detectable diversity. We compared three preservation and four nucleic acid extraction methods. The ideal preservation and extraction method would work well with a variety of samples that included high and low biomass, a wide range of pH, as well as different sample types including microbial mats, filamentous biofilms, and sediments. Additionally, this method should be quick to accommodate high throughput of large sample numbers and facilitate sample collection from the remote

backcountry where liquid nitrogen and dry ice are not practical. We included samples from the whole spectrum of environmental conditions that are found in Yellowstone National Park, USA (YNP) thermal features and our results are applicable to future studies in YNP, other extreme environments, and microbial surveys in general.

Methods and materials

Site description and sampling

Samples were collected from thermal features throughout YNP during the summer of 2002. A subset of fifteen samples was selected for this study that encompassed the full range of pH, temperature, and biomass types found in the park (YNP sample $n = 15$, see Table 1). The pH and temperature of the samples were split into three ranges, low (pH 0–4, temperature 40–60 °C), mid (pH 4.01–8, temperature 60.1–80 °C), and high (pH above 8.01, temperature above 80.1 °C). The samples were also categorized by the type of biomass collected: microbial mat, filaments, or sediment. Microbial mat samples are expected to be the highest biomass, for example, mats may contain up to 6.1×10^8 cells cm^{-3} and are also high in pigments and extracellular proteins and polysaccharides [4]. Filament samples represent an intermediate amount of biomass (maximum cell density in culture 4.6×10^7 cells ml^{-1} [33]) and are generally non-pigmented. The sediment samples are

Table 1 Sample site, description and environmental parameters

Sample ID	Sample area ^a	pH	T (°C)	Sample description	Northing ^b	Easting ^b
007-L	YNP Lower GB	7.54	79.9	Black powder sediment	4932385.831	517103.977
010-L	YNP Lower GB	3.55	90.6	Gray clay-like sediment	4933099.227	515316.123
022-L	YNP Lower GB	6.89	85.9	Black powder sediment	4933820.851	513269.383
045-L	YNP Lower GB	2.68	42.4	Brown foam, water, and sediment	4934500.042	513403.550
048-L	YNP Lower GB	3.39	48.8	Gray mud sediment	4934417.810	513990.150
058-L	YNP Lower GB	2.96	61.4	Yellow and tan powder sediment	4953191.966	522871.118
066-MV	YNP Mud Volcano	6.41	67.4	Orange mat and black filaments	4939784.796	544533.185
072-CH	YNP Crater Hills	5.47	55.6	Yellow powder sediment	4952741.659	523490.278
088-L	YNP Lower GB	8.46	52.8	Orange and green mat	4935485.818	515973.327
126-MM	YNP Mary Mountain	6.58	79.8	Gray powder sediment	4940597.962	532861.547
131-LS	YNP Lone Star GB	4.24	43.9	Yellow filaments	4916381.813	514106.247
139-LS	YNP Lone Star GB	2.49	54.8	Green mat and gray sediment	4919322.475	515169.854
171-S	YNP Shoshone GB	8.63	68.7	Orange and green mat	4911109.135	515791.782
184-S	YNP Shoshone GB	8.92	77.3	Tan filament	4911522.596	515932.872
190-S	YNP Shoshone GB	9.08	44.5	Layered orange mat	4911267.100	515925.080
BH	Jemez Bath House	7.15	76.0	Yellow filaments	3959977.588	347240.017
GS	Jemez Giggling Star Resort	6.45	53.4	Green mat	3959468.000	347235.000

^a Geyser Basin is abbreviated GB

^b Northing and Easting are given in UTM, grid 12N for YNP, 13N for Jemez Springs, NM, datum NAD83

expected to be the lowest biomass ($2.1\text{--}3.6 \times 10^6$ cells cm^{-3} [29]) and the highest clay content and heavy metal concentration [1]. Additionally, we collected samples from two neutral thermal springs in the Jemez Mountains, New Mexico. Samples (1–2.5 ml) were collected at each site with either sterile forceps or a syringe.

Sample preservation

Two replicate YNP samples were collected from each sampling site: one was preserved in an equal volume of sucrose lysis buffer (SLB) (20 mM EDTA, 200 mM NaCl, 0.75 M sucrose, 50 mM Tris-HCl, pH 9.0) [14] and the other replicate was preserved in an equal volume of GIT (5 M guanidine isothiocyanate, 50 mM Tris pH 7.4, 25 mM EDTA pH 8, 0.8% 2-mercaptoethanol)[6]. The YNP samples were held at ambient air temperature (10–26 °C) for up to 5 days before they were stored at –80 °C.

The two Jemez Springs, New Mexico samples were collected from neutral thermal springs located at the Jemez Springs Bath House (BH) and Giggling Star Resort (GS). Three replicate samples were collected. Two of the replicates were preserved in SLB, one of these replicates was frozen immediately in liquid nitrogen and the other was held at 20 °C for 7 days before being frozen at –80 °C. The third replicate sample was collected and mixed with molten 2% agarose while in the field for extraction the noodle method (see below).

Extraction methods

We used four extraction methods: lysis by pulse boil, a CTAB (hexadecyltrimethylammonium) extraction, high molecular weight DNA agarose noodle [42], and the Mo Bio Soil DNA Purification kit, which combined with the preservation methods results in six treatments. With the pulse boil method, nucleic acids were extracted from 200 μl of the YNP and Jemez samples that were preserved in SLB (referred to here as “Boil”) [36]. Briefly, the samples were boiled at 96 °C then cooled to 4 °C three times in a thermocycler, sodium dodecyl sulphate (SDS) (to a final concentration of 2%) and proteinase K (final concentration 250 $\mu\text{g ml}^{-1}$) were added and the sample was incubated at 42 °C for 2.5 h, then incubated at 60 °C for 30 min, extracted once with phenol/chloroform then twice with chloroform, finally the DNA was precipitated and washed with ethanol.

We extracted nucleic acids from 200 μl of the YNP and Jemez SLB preserved samples using a variation of the CTAB method (referred to as CTAB S) [46]. Briefly, 2 volumes of 1% CTAB buffer (1% CTAB, 0.75 M NaCl, 50 mM Tris pH 8, 10 mM EDTA) and proteinase K (final concentration 100 $\mu\text{g ml}^{-1}$) were added to the SLB pre-

served samples; incubated for one hour at 60 °C, SDS (final concentration 2%) was added and incubated one hour at 60 °C, extracted once with phenol/chloroform then twice with chloroform, finally the DNA was precipitated with ethanol. The YNP samples that were preserved in GIT (referred to as CTAB G) were also extracted using the CTAB method with the modification of washing the sample three times with filter sterilized water before the addition of CTAB to remove the GIT from the sample. GIT is a protein denaturant and inhibits proteinase K activity if not removed.

SLB (Mo Bio S) and GIT (Mo Bio G) preserved YNP samples were extracted using the Mo Bio Soil DNA purification kit following manufacturer’s suggested protocol (Mo Bio Laboratories, Inc., Solana Beach, CA).

Nucleic acids were extracted from YNP SLB preserved samples and all Jemez samples using the high molecular weight noodle extraction method (noodle) [42]. Briefly, the sample was mixed with molten 2% agarose and cooled in 1 mL syringes forming agarose noodles, the noodles were incubated for 3 h at 37 °C in a lysis buffer (10 mM Tris, 50 mM NaCl, 50 mM EDTA, 0.2% SDS, 1% Sarkosyl, 1 mg ml^{-1} lysozyme) then incubated at 37 °C in ESP buffer (1% Sarkosyl, 0.1 M EDTA, 1 mg ml^{-1} proteinase K), the ESP buffer was changed once a day for a total of 4 days, the noodles were then stored at 4 °C in TE storage buffer (10 mM Tris and 50 mM EDTA). The purified nucleic acids were extracted from the agarose noodles by incubating at 60 °C to melt the agarose then adding agarose and incubating overnight at 37 °C. The nucleic acids were purified from the agarose/agarase slurry by extracting once with phenol/chloroform then twice with chloroform. The nucleic acids were precipitated with ethanol.

Measuring extraction success

Environmental DNAs were electrophoresed on an ethidium bromide stained 1.2% agarose gel. The size and quality (evidence of shearing, etc.) of the DNA was evaluated using a size standard DNA ladder (EZ Load 1 Kb, BioRad Laboratories).

The purity of the extracted DNA was quantified by calculating the ratio of the absorbance at 260 and 280 nm (A_{260}/A_{280}). Nucleic acids extracted from environmental samples are often contaminated with humic organic carbons, metals, and other compounds which cause the DNA concentration calculated from 260 nm absorbance to be unreliable. To circumvent this problem, we also quantified the DNA using PicoGreen dsDNA Quantitation Reagent (Molecular Probes, Inc., Eugene, OR, USA). Extracted DNA (5 μl) was mixed with a 1:200 solution of PicoGreen in 1X tris-acetate buffer (TAE) and absorbance was read in a fluorometer.

The third parameter that was qualified was the ability to amplify 16S rRNA gene from the environmental genome of each extraction replicate. Dilutions of the genomic DNA were used as template. The reaction included (1X Promega buffer with 15 mM MgCl₂, bovine serum albumin (0.04% final), 2.5 U Taq DNA polymerase (Promega U.S.) 2.5% Igepal CA-630 (Sigma–Aldrich), 10 μM each dATP, dGTP, dCTP, dTTP (BioLine USA, Inc.), 20 μM Bacterial specific primers [24] 338FGC (CGCCC GCCGCGCCC CGCGCCCGTCCC GCCGCCCCCGCCCTCTACGGGAGGCAGCAG) and 519R (ATTACCGCGGCTGCTGG)). The PCR reaction (50 μl) was incubated in a thermocycler (ABI GeneAmp 2700) for 5 min at 94.0 °C then for 30 cycles of 30 s at 94.0 °C, 30 s at 50.0 °C and 30 s at 72.0 °C. The reaction was incubated at 72.0 °C for 7 min for final extension.

Richness

Differences in relative species richness among the preservation and extraction methods were determined using denaturing gradient gel electrophoresis (DGGE). Ten microlitre of PCR product (approximately 500 ng) was run on a 6% (wt/vol) acrylamide gel with 1X TAE (40 mM Tris, 20 mM acetate, and 1 mM EDTA) with a denaturing gradient of 20% (8% (vol/vol) formamide and 8.4% (wt/vol) urea) to 60% (24% formamide and 25.2% urea). The gels were run in a BioRad DCode Universal Mutation Detection System (BioRad Laboratories, Hercules, CA) at 180 V for 3.5 h. The gels were stained in 1X TAE containing SybrGreen (100 μl L⁻¹) for 30 min then destained in 1X TAE for 15 min. The gels were photographed under UV light and DGGE bands were identified and analyzed using Kodak 1D software. For our analyses, only bands with a minimum intensity of 72% were recognized (program default). Bands were distinguished based on migration distance within each gel, as determined by the software using the DGGE product from *Escherichia coli* as a standard [9]. Although re-amplification and sequencing of individual DGGE bands can be particularly important in resolving heteroduplex fragments, we did not sequence the bands we detected in this study because we were interested in detecting the greatest number of bands possible. The effectiveness of methods within each sample was calculated by dividing the number of bands detected within a treatment by the total number of unique bands found within a sample. This calculation of relative species richness was computed for samples where at least two of the treatments resulted in successful amplification (YNP samples $n = 12$).

Statistical analysis

Statistical analyses were performed using Minitab software version 13 and SPSS 11 (for Mac OS X). The purity of the

DNA among treatments was compared using the absolute value that resulted from subtracting the A_{260}/A_{280} from 1.8, the A_{260}/A_{280} of pure DNA [37]. Univariate ANOVA was used to detect statistical differences in the quantity and purity of the DNA and relative species richness (dependent variables) among the treatments and by pH, temperature, and biomass type (fixed factors). For analysis of the DGGE bands, only samples where at least two of the treatments amplified were included, otherwise a statistical comparison would not be possible. The Bonferroni test (which is more powerful for small sample sizes than Tukey's test) was performed to identify treatments that were significantly different. Rarefaction curves were calculated in EstimateS version 8.0.0.

Results

Sample preservation

We extracted more DNA from the samples preserved in SLB than from the GIT replicates (paired t test $p = 0.01$). The extracted DNAs were quantified using the PicoGreen reagent. Using our protocol, the PicoGreen assay has a dsDNA detection limit of 500 pg ml⁻¹. We also compared the amount of DNA recovered from the Jemez samples that were frozen or mixed with agarose in the field with those that were held at ambient temperature; there was no statistical difference between the two replicates (paired t test $\alpha = 0.05$). Even when samples cannot be frozen immediately, the DNA is adequately preserved in SLB.

There was no significant difference among the preservation methods in the richness we detected (paired t test $\alpha = 0.05$). However, the 16S rRNA gene from the samples that were preserved in SLB were amplified by PCR more successfully; 80.5% of SLB preserved samples PCR amplified compared to just 50% of the GIT preserved samples.

DNA extraction

The CTAB extraction on SLB preserved samples resulted in the greatest yield of DNA. The results of the quantification are displayed in Fig. 1a. ANOVA showed a statistically significant difference in DNA yield among the extraction methods ($p < 0.001$). Post hoc multiple comparison analysis indicates that the CTAB S extracted samples resulted in the highest quantity of DNA.

DNA purity, as measured by A_{260}/A_{280} ratio, was greatest in samples extracted using the Mo Bio extraction kits, however, the kit results in less DNA and smaller fragments (presumably from shearing and the filter column used in the kit [3]) (Fig. 1b). There was no significant difference in the purity of the DNA extracted

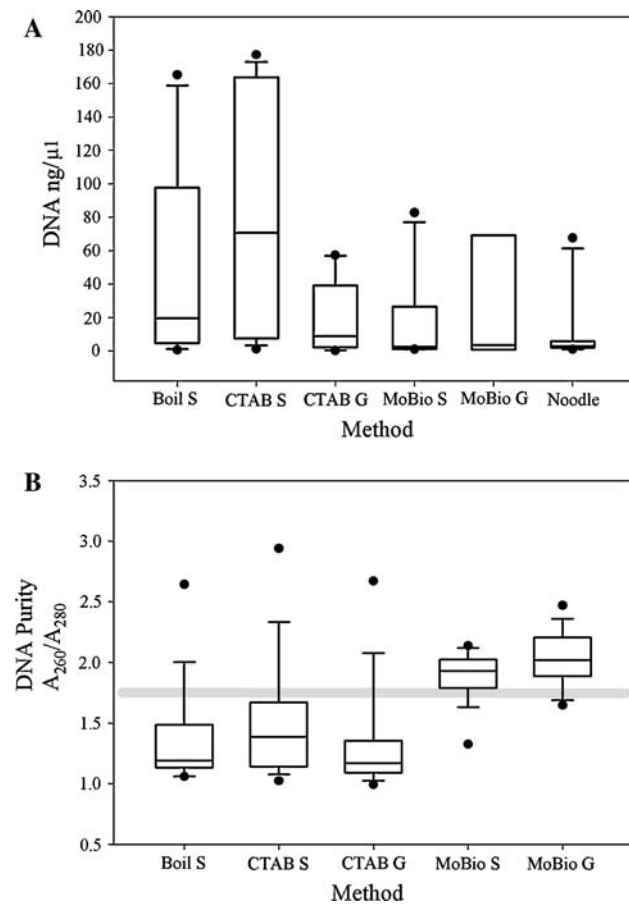


Fig. 1 **a** Mean DNA concentration as measured using PicoGreen assay for each preservation and extraction method with 95% confidence intervals, outliers are filled circles. **b** Mean DNA purity with 95% confidence interval and outliers. Pure DNA has a A_{260}/A_{280} ratio of 1.8, shown as the gray bar. The concentrations of the DNAs from the noodle extractions were not high enough to be detected by the fluorometer

with the Boil, CTAB S, or CTAB G methods (ANOVA $\alpha = 0.05$). The noodle method did not result in sufficient DNA quantity to obtain reliable absorbance data, therefore it was not included in the DNA purity comparison. Most of the amplification success we observed was in sample extracted with either the CTAB method (89% amplified) or the Mo Bio kit (95% amplified) (ANOVA and Bonferroni test $p < 0.001$).

The sequence diversity detected by DGGE, number of bands, varied within the replicates of the samples (Fig. 2). The only statistically significant difference we detected was between the CTAB S and the noodle method ($p = 0.014$). However, CTAB S resulted in 15–43% more bands than the other treatments (Fig. 3). There were no differences at the fixed effects level (pH, temperature, or biomass type), nor were any interactions (e.g., pH X method) among the factors detected. This suggests that CTAB S is appropriate for a wide range of sample types.

Discussion

Sample preservation

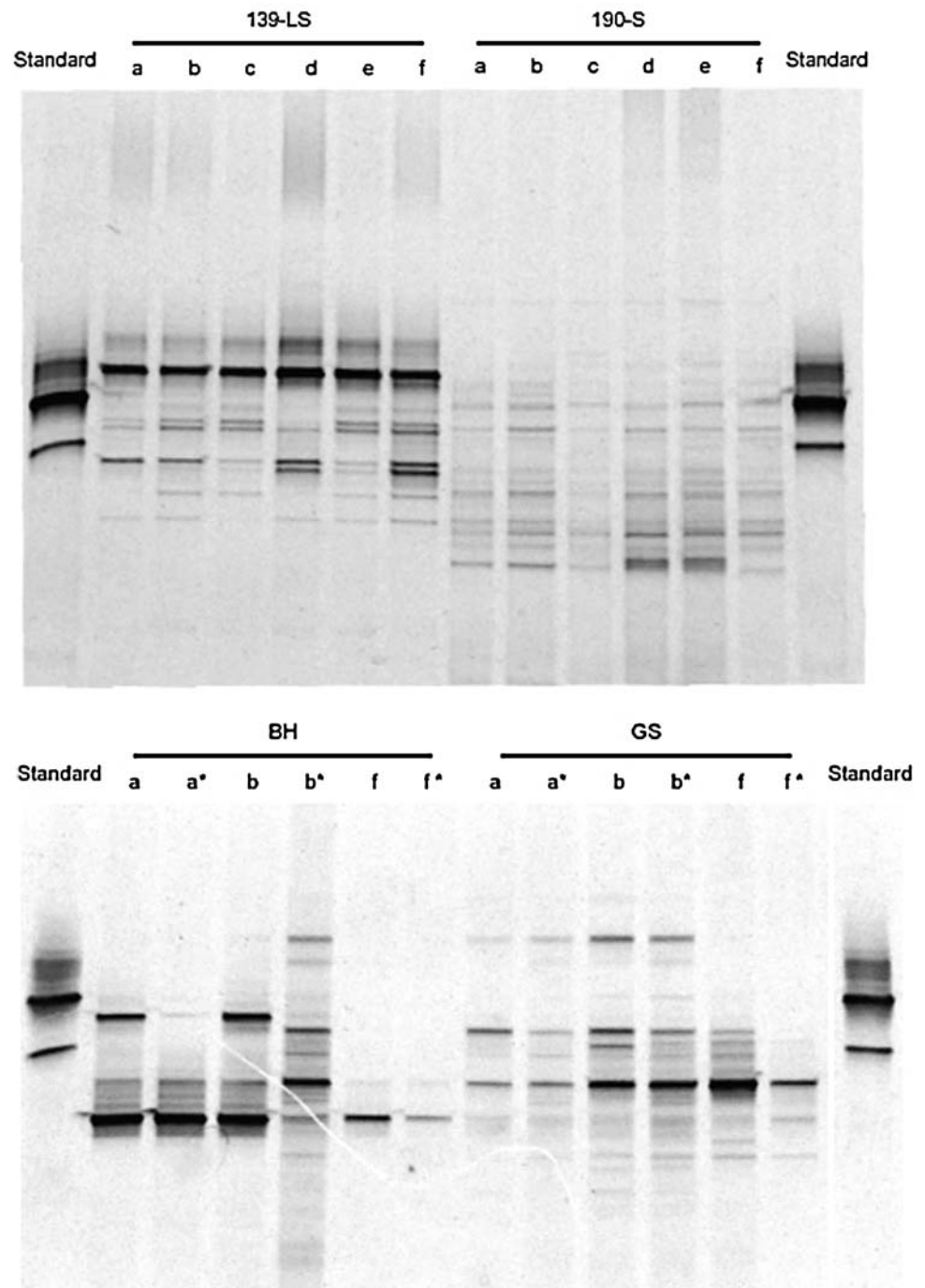
SLB preserved our extreme environment samples better than the GIT solution. This was likely due to more effective lysing, as the SLB initiates the lysis process while the sample is being stored. Additionally, the alkaline SLB raised the pH within the sample vials, potentially slowing the degradation of the DNA in low pH samples. Raising the pH of acidic samples is likely to have two positive impacts on preserving the DNA in those samples. First, DNA degrades in acidic solutions due to depurination [12], raising the pH of the solution reduces the rate of this reaction. Second, DNA binding to clay minerals increases as the pH decreases [21]. Even when samples could not be frozen immediately, the DNA is adequately preserved in SLB. This result is especially important because of restrictions on carrying and shipping dry ice and for samples that are collected from remote areas where it is logistically very difficult to freeze samples immediately and maintain them frozen. The non-toxic nature of SLB may be an additional attraction for field microbiologists, and for those shipping samples either commercially or hand carrying on airplanes. Although this study did not assess the relative effectiveness of the different methods in extracting RNA, we have successfully produced cDNA from samples preserved in SLB using reverse transcriptase PCR (unpublished data).

Previous studies have suggested that the noodle method, for very high molecular weight DNA [42], and storage in absolute ethanol, for samples where immediate freezing is not possible [17], adequately preserve DNA. However, based on the results from this study, the noodle preservation method does not work well with the samples we collect which often have low biomass in a dense extracellular matrix. In many samples, the concentration of DNA recovered using the noodle method was an order of magnitude less than recovered using the CTAB protocol. Similarly, we did not test ethanol preservation because the ethanol must be nearly absolute to prevent DNA degradation [8, 28] which would not be possible for samples that contain significant amounts of water. Therefore, we do not think that either the noodle method or absolute ethanol preservation are appropriate for thermal environmental samples.

DNA extraction efficiency and purity

When considering preservation alone, among the techniques we tested, SLB with or without freezing preserved environmental DNA best. However, we also were interested in the effect of extraction method on the amount and quality of extracted DNA and detectable diversity. Extracting pure DNA from environmental samples is nearly as

Fig. 2 Example DGGE of PCR products on 6% acrylamide gel with 20–60% urea/formamide denaturing gradient, stained with SybrGreen [Treatments: **a** Boil, **b** CTAB S, **c** CTAB G, **d** Mo Bio S, **e** Mo Bio G, **f** noodle]. Samples marked with an *asterisk* were frozen in the field



important as extraction efficiency and is one of the most difficult technical problems in using molecular techniques on environmental samples. Most DNA extraction procedures co-extract humic organic carbon, pigments, heavy metals, and other contaminants. These contaminants play havoc with PCR reactions and can degrade the DNA during storage. We found that CTAB extraction combined with SLB preserved samples resulted in the most DNA. The greater efficiency of the CTAB extraction method has previously been found in several environments, marine

sediments [27], basalt rock [18], and by one of us in caterpillar intestinal tracts and filtered water (unpublished data) indicating that this method is effective on a wide range of sample types. An extraction comparison on compost microbial communities found no difference in the amount of DNA recovered by the CTAB method, but it resulted in the highest percentage of cells lysed [45]. It is likely that the combination of proteinase K and hot SDS lyses more cells, including cell types more resistant to lysis (e.g., gram-positive bacteria) [32]. We found two exceptions where larger

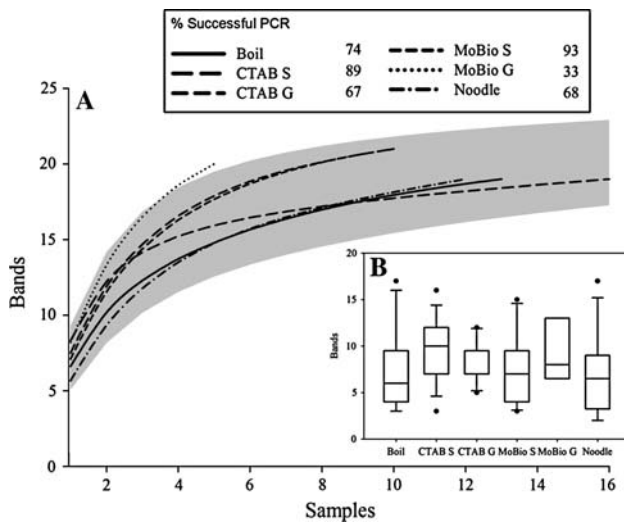


Fig. 3 a Rarefaction curves of DGGE bands detected with each extraction method. The 95% confidence interval for the diversity of all samples and extraction methods combined is in *gray* to demonstrate that there was no significant difference in diversity detected by each method. **b** Mean diversity detected as number of DGGE bands with 95% confidence intervals, outliers are *filled circles*

quantities of DNA were not recovered by the CTAB method. The PicoGreen analysis on two samples, 066-MV and 139-LS, indicates that more DNA was extracted using the Mo Bio kit than was extracted with the Boil and CTAB methods. The Boil and CTAB extracted DNA from these two samples was dark brown, which was most likely caused by pigments that were co-extracted with the DNA. The Mo Bio kit, which produced clear DNA extracts, was effective at removing these organics because it is the only method that incorporated a post-extraction purification [3]. Work on basalt rock samples found that only Mo Bio extracted DNA could be amplified [18], which is contrary to our results. We suspect that the co-extracted pigments interfered with PicoGreen fluorescence, but both of these samples were successfully amplified with all of the extraction methods. The co-extracted pigments from the Boil and CTAB methods do not appear to inhibit the PCR reaction. It should be noted that some of the samples with no detectable DNA, as determined using the PicoGreen fluorometer quantification, still had enough DNA to amplify with PCR (theoretical detection limit for PCR amplification <1 pg ml⁻¹[41]).

Richness

Although the CTAB S treatments consistently had more bands than any of the other methods (15–43%), the only statistical difference detected was between the CTAB S and the noodle extraction method. We also tested for fixed effects such as pH, temperature, and biomass type, but did

not detect any statistical differences. However, there were three samples that we were unable to amplify using two or more of the extraction treatments, those samples could not be included in the richness analyses. It is interesting to note that all three of these samples were sediments from low pH systems (<pH 3.5), indicating that there may be a sample type or geochemistry effect on extraction efficiency or amplification success. This result could have been due to inefficient extraction of DNA, co-extraction of PCR inhibitors, or too little target DNA in the original sample (i.e., if the community was dominated by Archaea to the exclusion of Bacteria). For those types of extremely recalcitrant samples, it may be necessary to crush the sample using a sterile mortar before the enzymatic lysis step [18]. However, CTAB worked on a majority of the samples and we suggest it should be used as a good general extraction method. If unsuccessful, alternate approaches should be used that may more effectively lyse the biomass or better remove humics.

There are many methods for assessing microbial community richness, generally based on the 16S rRNA gene, such as clone libraries, DGGE, and T-RFLP (terminal restriction fragment length polymorphisms). These analyses have greatly increased our understanding of microbial ecology and revealed novel enzymes. DGGE is an attractive method for microbial ecology studies because of the relatively rapid ease with which a community profile can be generated. DGGE can resolve minor base differences among samples; therefore, distinct bands may represent sequences that differ by less than 1% [31]. Depending on the nature of the study being performed, 1% may or may not be significant to the research question. Conversely, we are aware that individual bands could be heteroduplexes [7, 40], comprised of several different sequences, however, because our methods were standardized across samples, this effect does not figure in our conclusions. We repeated the PCR amplification and DGGE for selected samples to ensure reproducibility in the number of bands detected (data not shown, but available on request).

It is standard practice that investigators informally evaluate methods to determine which are appropriate for their study. However, few of these studies have been published [11] and often, statistical analyses are not used to determine the most appropriate methods [9, 18]. Additionally, other published studies did not evaluate the effect of extraction method on the diversity detected, only the quantity of DNA [25, 30]. This study presents a framework for the systematic comparison and evaluation of methods by statistical analysis. The current interest in the organisms that inhabit extreme environments makes this study, which used samples from a wide variety of thermal environments, of importance to thermal biology researchers, and applicable to other microbial studies in general.

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