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RESEARCH ARTICLE

Quantitative PCR methods for RNA and DNA in marine sediments: maximizing yield while overcoming inhibition

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Abstract

For accurate quantification of DNA and RNA from environmental samples, yield loss during nucleic acid purification has to be minimized. Quantitative PCR (qPCR) and reverse transcription (RT)-qPCR require a trade-off between maximizing yield and removing inhibitors. We compared DNA and RNA yield and suitability for quantitative SYBR Green PCR and RT-PCR using the UltraClean and PowerSoil extraction kits and a bead-beating protocol with phenol/chloroform extraction steps. Purification methods included silica-column-based procedures from the MoBio kits, RNeasy MinElute, WizardPlus miniprep columns, and an acrylamide gel extraction. DNA and RNA purification with WizardPlus and RNeasy, respectively, led to significant losses of nucleic acids and archaeal 16S rRNA or 16S rRNA gene, as measured with RiboGreen or PicoGreen, and RT-qPCR or qPCR. Extraction and purification of DNA with the MoBio DNA UltraClean and DNA PowerSoil kits also decreased the yields slightly, relative to gel purification, in all sediments, except those from the deep sea in the Gulf of Mexico. Organic matter in humic-rich sediments may bind to these silica columns, reducing their nucleic acid-loading capacity. Purification with gel extraction cleans up organic-rich sediment samples sufficiently for quantitative analysis while avoiding the yield loss associated with commonly used silica columns.

Introduction

Real-time quantitative PCR (qPCR) and reverse transcription (RT)-qPCR are powerful methods for determining the copy numbers of individual genes within a sample. When applied to environmental nucleic acid extractions, these methods can provide valuable information about *in situ* microbial activity and are cornerstones of modern molecular ecological studies (Inagaki *et al.*, 2006; Schippers & Neretin, 2006; Wilms *et al.*, 2006; Sharma *et al.*, 2007). The comparability of such studies between environments, or even at different depths in the same sediment column, depends on nucleic acid yields and amplification efficiency for each particular sediment type. The sensitivity of qPCR and RT-qPCR to small amounts of inhibitors necessitates postextraction purification of nucleic acids from many environments, including organic-rich marine sediments. The cycle number at which the SYBR Green or TaqMan probe-bound fluorescence of amplicons can be detected (C_q for quantification cycle; Bustin *et al.*, 2009) is used to quantify the original template copy number. Even small amounts of inhibitor delay the C_q of each sample, causing erroneously low estimates of template copy number. This sensitivity warrants more stringent purification procedures than are required for endpoint PCR. PCR additives, such as bovine serum albumin or T4 protein, can alleviate inhibition (Kreader, 1996) but also disrupt qPCR/RT-qPCR by binding to template DNA or cDNA (Sharma *et al.*, 2007). Preparation of samples for RT-qPCR has the added complication that all DNA must be removed, because it will be amplified along with cDNA. Many studies have evaluated DNA and RNA purification protocols for sedimentary extracts in order to minimize PCR inhibition while maximizing the yield in endpoint PCR (Zhou *et al.*, 1996; Miller *et al.*, 1999; Hurt *et al.*, 2001; Lakay *et al.*, 2006). However, these purification methods must be evaluated for qPCR and RT-qPCR applications.

Previous studies have shown that commercially available DNA and RNA clean-up kits are sufficient for removing inhibitors for PCR and RT-PCR (Inagaki *et al.*, 2003;

Sørensen *et al.*, 2004; Biddle *et al.*, 2006; Lloyd *et al.*, 2006; Whitehouse & Hottel, 2007). The RNeasy MinElute Clean-Up (Qiagen, Valencia, CA), WizardPlus SV Miniprep (Promega, Madison, WI), and UltraClean or PowerSoil soil DNA extraction (MoBio, Carlsbad, CA) kits use silica columns to retain high-molecular-weight RNA or DNA while removing inhibiting molecules such as humic acids, salts, and proteins. We compared the silica columns using a gel extraction method that does not require the use of a silica column, and determined their effects on RNA and DNA extracted from estuarine and deep ocean sediments.

Materials and methods

RNA and DNA extractions

Sediments were obtained with 1.5-m plunger-cores in the White Oak River (WOR) estuary in North Carolina in July 2005 (WOR-A) and December 2006 (WOR-E). Deep ocean sediments were obtained from the Peru Margin with Ocean Drilling Program Leg 201 at site 1229, hole D, subcore 1H-2, sample DNAT (2.25–2.30 m below the seafloor) at 150-m water depth (referred to as 1229) (D'Hondt *et al.*, 2003), and from the bacterial mat-covered sediment at the Gulf of Mexico site Mississippi Canyon 118 (referred to as GOM), 900-m water depth, using the Johnson Sea-Link submersible, dive 3570, core 1, 0–3 cm below the seafloor. Total RNA was extracted following previously described methods with 30 s of bead-beating in pH 5 phenol, followed by successive extractions with phenol, phenol/chloroform, and chloroform using 0.5–9 g of sediment (Biddle *et al.*, 2006). All glassware was baked overnight at 160 °C, all plasticware was autoclaved for 2 h, and all aqueous solutions were treated with 0.1% diethyl pyrocarbonate to inactivate RNAses. Total DNA was extracted from 0.7 to 10 g sediment using an identical method, except the extraction buffer and phenol were pH 8 instead of pH 5 (Fig. 1). DNA was also extracted using the UltraClean kit (MoBio; for WOR-A 43 and 49 cm) or the PowerSoil kit (MoBio; for WOR-A 1 cm and deep ocean sediments) following the manufacturer's instructions (Fig. 1). In all cases, extraction blanks underwent all extraction, purification, and measurement steps alongside the samples. Following phenol extraction and precipitation, the crude RNA and DNA extracts from WOR were dark brown and produced an opaque solution when dissolved in 80 μL water; further purification was required. The deep ocean samples were also brown, but translucent when dissolved in water. In contrast, the DNA extracts using the UltraClean and PowerSoil kits were nearly clear for all samples.

RNA and DNA purification

Phenol-extracted nucleic acids were purified using an RNeasy MinElute kit for RNA (Qiagen), a WizardPlus SV MiniPrep for DNA (Promega), or a gel extraction protocol for both RNA and DNA (Fig. 1). DNA extracted using the UltraClean and PowerSoil extractions was further purified either with the final silica column step in the manufacturer's protocol or by polyacrylamide gel electrophoresis (Fig. 1). For the gel purification of both RNA and DNA, 5 μL crude extract was mixed with sterile loading dye (0.1% bromophenol blue, 5 M urea) and electrophoresed on a denaturing acrylamide gel at 10 mA long enough for the brown, coextracted organic material to move about 0.5 cm away from the well, just ahead of the loading dye (~20–30 min) (Supporting Information, Fig. S1). The gel consisted of 4.5 mL 3.3% acrylamide stock (8 M urea, 0.3 M sucrose, 3.95 mL 19 : 1 acrylamide : bis-acrylamide solution, 3.95 mL 37.5 : 1 acrylamide : bis-acrylamide solution, 134 mM Tris (hydroxymethyl)aminomethane hydrochloride, 44.5 mM boric acid, 27.7 mM EDTA dihydrate) polymerized with 12 μL tetramethylethylenediamine and 22 μL ammonium

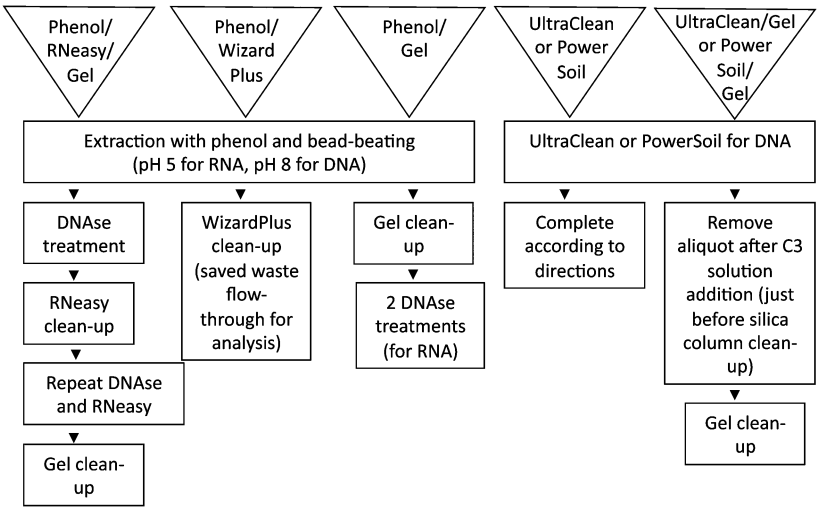

Fig. 1. Flowchart of each extraction/purification procedure, with the names of each procedure in the header arrows. UC, Ultra Clean; PS, PowerSoil; WP, WizardPlus.

persulfate (4.4 mM) (Alm & Stahl, 2000). SYBR Gold staining of the acrylamide gel showed that genomic DNA and total RNA had migrated into the gel as a broad band just below the loading well. The gel area below the well was excised along with the well itself to retain all high-molecular-weight nucleic acids. Excised gel was crushed with a pipette against the walls of a 1.5-mL plastic tube containing an equal volume of 0.5 M ammonium acetate, 0.1% sodium dodecyl sulfate, and 0.1 mM EDTA. The solution was rotated end over end overnight (~16 h) at 37 °C. The supernatant was removed and retained; the remaining gel pieces were vortexed and extracted with a second equal volume of buffer, and both extracts were combined. The gel-eluted nucleic acids were precipitated in an equal volume of isopropanol, pelleted, and washed with 70% ethanol (adapted from Sambrook & Russell, 2001).

The RNeasy column was used following the manufacturer's instructions and visibly passed much of the dark-brown material as waste filtrate, although some of it remained absorbed to the column and required extra elution with 90 μL RNAse-free water. The WizardPlus column, which was also used following the manufacturer's instructions, passed all of the brown material as waste filtrate, leaving nothing visible on the column, and was subsequently eluted with 100 μL DNAse-free water. The waste filtrates were retained in separate tubes. The UltraClean and PowerSoil kits removed most of the brown material well before the silica column purification step, leaving only a light tan eluent. The gel extraction procedure resulted in a clear solution or a slightly tan eluent, as some of the brown matter was retained in the well.

RNA purified with RNeasy was treated with DNAse I (Ambion) for 30 min at 37 °C and filtered through another RNeasy column to remove enzymes and further inhibitors, and eluted with 15, 60, or 62 μL water as a translucent brown solution. RNA purified by gel extraction was treated twice for 30 min at 37 °C with TurboDNase I (Ambion), using TurboDNase I inactivating reagent (Ambion) each time.

A single sample, WOR-A 31 cm, was extracted and purified in a slightly different manner, starting with a phenol/RNeasy/gel extraction, but including a single RNeasy treatment, followed by two DNAse treatments, as in the phenol/gel procedure.

Quantification of total nucleic acids

Total RNA was measured using RiboGreen dye (Invitrogen) on the ND-3300 NanoDrop fluorescence spectrometer. Total DNA was measured using the QuantIt PicoGreen kit (Invitrogen) on a Stratagene Mx3005P real-time PCR machine, using the quantitative plate read mode. In both measurements, the fluorescence excitation and emission wavelengths were 492 and 516 nm, respectively. The RiboGreen (for RNA) and PicoGreen (for DNA) dyes are more sensitive than UV measurements because they specifically bind to nucleic acids and are resistant to binding to coextracted substances (Singer *et al.*, 1997; Jones *et al.*, 1998). RiboGreen does fluoresce in the presence of DNA; however, DNAse treatments are sufficient to eliminate this effect (Jones *et al.*, 1998). PicoGreen has much greater fluorescence with DNA than with RNA, but some background interference from RNA is possible (Singer *et al.*, 1997). Because DNA qPCR standards were made from purified plasmids containing target DNA sequences, RNA contamination should be minimal. Quantification with lambda DNA (Invitrogen) or RNA 250 (Ambion) standards was performed in triplicate. Commercial RNA standards were checked for integrity by gel electrophoresis; they have sometimes arrived from the supplier in seriously degraded condition (B.J. MacGregor, unpublished data). Because RiboGreen is an intercalating dye, each break in the RNA backbone removes a dye-binding site. The standard curve resulting from degraded standards will lead to overestimates of sample RNA concentrations.

qPCR and RT-qPCR

qPCR or RT-qPCR was used to determine the copy numbers of archaeal 16S rRNA or 16S rRNA genes present in different DNA and RNA fractions, using the Stratagene Mx3005P. DNA standards were prepared from TOPO 2.1 plasmids (Invitrogen) containing an insert of a near-complete, PCR-amplified archaeal 16S rRNA gene with no closely related cultured relatives from an environmental sample, and purified using the WizardPlus kit. RNA standards were *in vitro* transcribed from the same plasmid used for DNA, by cutting with SpeI (New England BioLabs), transcribing with T7 polymerase (TaKaRa), and purifying using the RNeasy MinElute kit. DNA standards were quantified with PicoGreen and RNA standards were quantified with RiboGreen on a Stratagene Mx3005P in the quantitative plate read mode. Primer concentrations were chosen to minimize the C_q of the standard, while also minimizing primer-dimers and nontarget amplification, as assessed through postamplification dsDNA melt curves. Primers A915f (DeLong, 1992) and A1059r (Yu *et al.*, 2005) were chosen because they had good coverage within the archaea and multiple mismatches to nontarget groups, as shown by the probe match tool of the ARB software (http://www.arb-home.de; Ludwig *et al.*, 2004) with the Spring 2007 Silva database (Pruesse *et al.*, 2007). Each 25-μL reaction contained 1 μL DNA or RNA template at the appropriate dilution, 12.5 μL QuantiFast PCR or RT-PCR master mix (Qiagen), 0.2 μL A915r (10 μM) and 0.24 μL A1059r (10 μM), and 0.25 μL QuantiFast reverse transcriptase (mixture of Sensiscript and Omniscript) for RT-qPCR. The RT-qPCR protocol included the following steps: 50 °C incubation for reverse transcription

for 10 min, 95 °C polymerase activation for 5 min, followed by 40 cycles of 95 °C denaturation for 10 s and 60 °C annealing for 45 s, followed by a melt curve from 95 to 55 °C. The qPCR protocol was identical, minus the initial 50 °C step. STRATAGENE MXPRO software was used to determine the C_q of each reaction and the efficiency of each standard curve (all were $> 90\%$). Extraction blanks as well as RNA samples without reverse transcriptase treatment all had C_qs more than five cycles higher than the samples, indicating negligible contributions of contamination from extraneous nucleic acids or from DNA. Primer-dimers were not formed during qPCR experiments, as shown by post-amplification melt curves for every run. All qPCR and RT-qPCR measurements used in the methods' comparisons were 100-fold dilutions made in triplicate, using a new dilution from a single extraction for each replicate. Therefore, the variability in the measurements takes dilution variability into account.

Results

Assessment of inhibition

The presence of inhibitors in purified nucleic acid extracts was tested by serially diluting the purified sample and measuring the copy numbers through qPCR or RT-qPCR for every dilution step. This is essentially identical to previously published methods (Gallup & Ackermann, 2006), where inhibitors are assumed to be diluted out when a log-linear relationship is achieved between C_q and the dilution factor. The WizardPlus-, UltraClean-, PowerSoil- and gel-purified DNA, as well as the gel-purified RNA, showed an alleviation of inhibition at 100-fold dilution, indicating that residual inhibitors could be compensated for by moderate dilution (factor 100) and no further purification was needed (Fig. S2).

The product of measured copy number and dilution factor for WOR RNA purified only by RNeasy, however, continued to increase to a 4000-fold dilution before the effect of inhibitors was neutralized (data not shown). At such a high dilution, the amount of template is decreased, potentially resulting in measurement error or primer-dimers and/or reaching the detection limit of the qPCR machine (Chandler, 1998). For this reason, measurements of the RNeasy group were made only on samples that had also been further purified using the gel method. The WizardPlus, UltraClean, and PowerSoil kits all yielded DNA pure enough for qPCR analysis (100-fold diluted) without further gel purification, as shown by dilution series qPCR (data not shown). In contrast to the dilution requirements of qPCR, endpoint PCR required only a 10-fold dilution to amplify enough material for a clone library (Lloyd, 2009), demonstrating the different requirements of endpoint and real-time methods.

Comparison of yields of purification methods

The total RNA resulting from the phenol/RNeasy/gel method was only 10–25% of the amount obtained by the phenol/gel method, even for WOR-A 31 cm, which underwent only a single RNeasy treatment compared with double RNeasy purification for the other samples (Fig. 2a). This difference was shown to be significant with a 99% confidence interval with a paired one-tailed *t*-test (Table 1). Archaeal 16S rRNA cDNA copies were 3–10-fold lower in samples that included the RNeasy step, a difference that was significant with a 97% confidence interval (Table 1), corroborating the total RNA results (Fig. 2b). The RNeasy kit has been shown to result in high yields of very pure RNA in plant and animal tissue as well as pure cultures of bacteria (Bonham & Danielpour, 1996; Nuyts *et al.*, 2001; Siju *et al.*, 2007), but our data show significant yield losses for estuarine sediments. These methods showed little variability in the quality of the resulting 16S rRNA, with high-quality intact 16S rRNA visible on acrylamide gels using each of these methods (Fig. 3a).

The phenol/WizardPlus method yielded only 1–2% of the total DNA obtained by the phenol/gel method (Fig. 4a). The UltraClean and PowerSoil methods gave much higher yields, as was expected, because they are designed to purify genomic DNA from organic-rich sediments. However, these methods still only yielded 9–55% of the amount obtained by the phenol/gel method for WOR and 1229 sediments (Fig. 4a). Mirroring the PicoGreen results for extracted DNA, summarized in Fig. 4a, 15- to 50-fold fewer archaeal 16S rRNA gene copies were detected in phenol/WizardPlus samples than in the phenol/gel samples (Fig. 4b, see WOR-A 43 and 49-cm samples). Archaeal 16S rRNA gene copy numbers determined using the UltraClean and PowerSoil methods were 2–12-fold lower than those measured in the phenol/gel group. An important exception to these trends was the GOM sample, whose phenol/gel-extracted DNA concentration was below the detection limit for fluorescence quantification using PicoGreen (Fig. 4a) and yielded very low qPCR results despite three extraction attempts (Fig. 4b). PicoGreen analysis of pre-gel-extracted DNA from this sample showed that the yield loss occurred during extraction, not in the purification step (data not shown). When the Gulf of Mexico samples are removed, the phenol/gel-extracted DNA copies are significantly higher than those of the silica-column containing MoBio kits (Table 1). The differences in the total amounts of DNA, however, were not significant. This shows that the yield losses from the MoBio kits were much less than those seen in the RNA extractions from RNeasy columns.

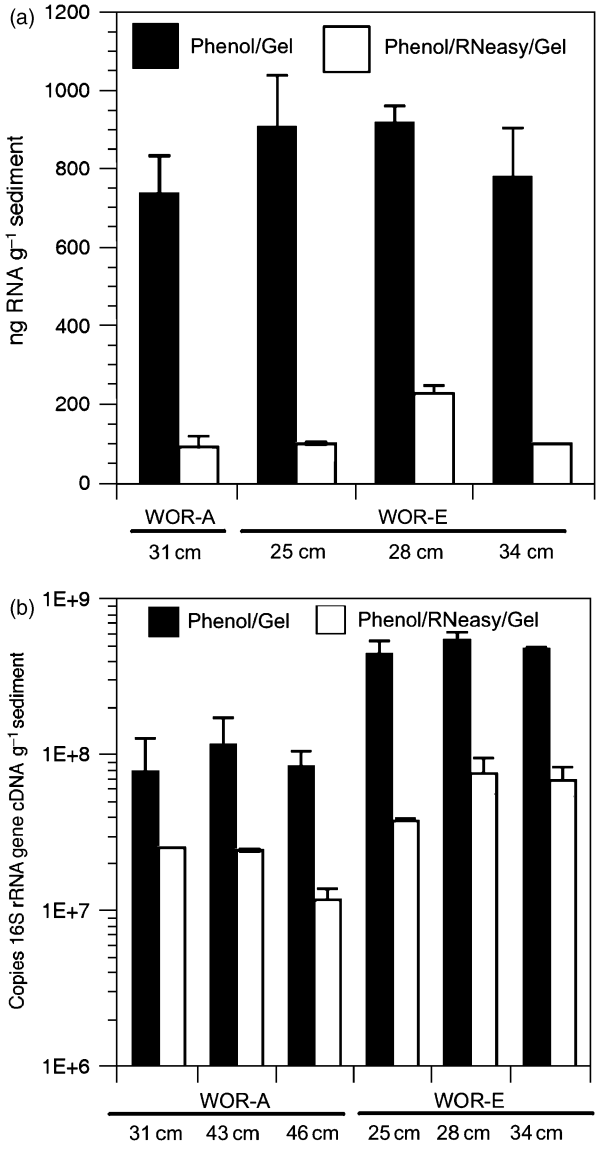

Fig. 2. Comparisons of RNA yield using different RNA purification methods listed in Fig. 1. RNA recovery from sediment samples from WOR cores A and E; depths below sediment surface are given for individual subsamples within each core. (a) RiboGreen quantification of ng RNA g^{-1} whole sediment. All error bars represent the SD of triplicate sample measurements, except WOR-E 28 cm phenol/gel, where the difference between duplicate measurements is shown. (b) RT-qPCR of archaeal 16S rRNA cDNA g^{-1} whole sediment. Error bars for phenol/gel values represent the SD of triplicate sample measurements; for phenol/RNeasy/gel results, the difference between duplicate measurements is shown. The phenol/RNeasy/gel result for sample WOR-A 31 cm is a single measurement.

Waste filtrate that passed through the WizardPlus column was retained, gel-purified, and found to contain roughly as many archaeal rRNA gene copies as the phenol/gel extractions (Fig. 4b). Thus, the majority of the DNA extracted by the phenol method was not retained on the WizardPlus silica column, but would have been lost in the waste filtrate fraction. Likewise, the subsample portion of UltraClean- or PowerSoil-extracted DNA that was gel purified instead of being passed through the UltraClean or PowerSoil columns yielded total DNA and archaeal 16S rRNA genes similar to the amounts obtained using the phenol/gel method. Thus, much of the yield loss in the WizardPlus, UltraClean, and PowerSoil kits, relative to the phenol/gel method, occurred in the final column purification step. DNA extracted using the phenol/bead-beating method appeared to be degraded on an agarose gel (Fig. 3b) relative to DNA extracted without bead-beating.

Table 1. *P*-values for paired *t*-test comparing extractions with and without silica columns

		Total RNA or DNA	Confidence interval	Copies 16S rRNA cDNA or DNA g^{-1} sediment	Confidence interval
RNA	Phenol/gel vs. RNeasy	0.0001	99%	0.0132	97%
DNA	Phenol/gel vs. MoBio	0.2038	NS	0.2052	NS
DNA	Phenol/gel vs. MoBio (no GOM)	0.1271	NS	0.0376	95%

NS, not significant.

Discussion

When performing qPCR and RT-qPCR of environmental samples, the absence of inhibition must first be demonstrated by finding the asymptote of measurements of total DNA or cDNA copies in a dilution series. In each case examined, a similar inhibitor-free asymptote was reached, but purifications involving silica columns [RNeasy MinElute Clean-Up (Qiagen), WizardPlus SV Miniprep (Promega), UltraClean Soil DNA extraction (MoBio), and PowerSoil DNA extraction (MoBio)] resulted in significant RNA or DNA yield loss relative to acrylamide gel-purified samples. This yield loss was observed even though the amount of RNA and DNA present in these sediments was well below the binding capacity of the WizardPlus (20 μg), RNeasy (100 μg), and UltraClean and PowerSoil (20 μg) columns. Most likely, coextracted charged organic compounds such as humic acids (indicated by the brown color of the nucleic acid extracts before purification) compete with nucleic acids for silica-binding sites, causing much of the nucleic acids to pass through. Alternatively, inhibitors present in the extract may have bound to the nucleic acids, preventing their retention on the silica filter.

Unlike the WOR estuary and the Peru Margin, sediments from the Gulf of Mexico yielded the most RNA and DNA using the UltraClean kit, and did not appear to have a large

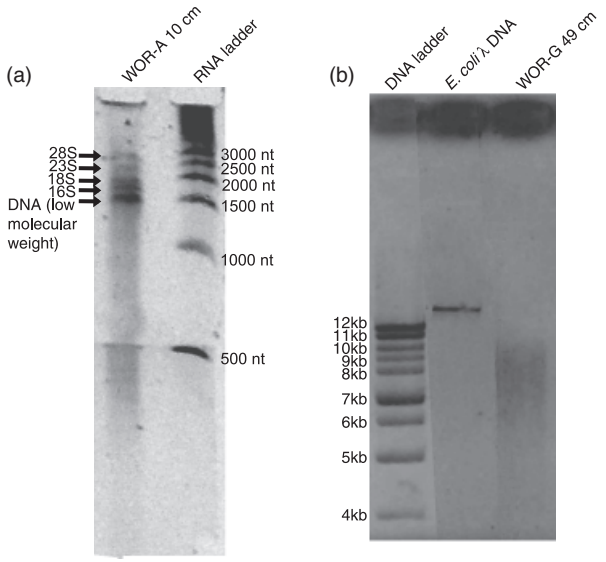

Fig. 3. (a) RNA extracted with phenol/bead-beating, RNeasy, and acrylamide gel clean-up showing intact RNA subunits next to the RNA Millenium Markers (Ambion), stained with SYBR Gold on a denaturing gel with 20% acrylamide overlying 80% acrylamide (transition visible near 500 nucleotides). (b) DNA extracted with phenol/bead-beating and acrylamide gel clean-up showing degraded DNA next to genomic DNA from lambda *Escherichia coli* and a 1-kb DNA ladder (Invitrogen). Sample was from July 2008 core G, and was stained with ethidium bromide and run on a nondenaturing 1.5% agarose gel. Lanes were digitally cut from a single gel picture and moved next to each other to avoid lanes from another experiment.

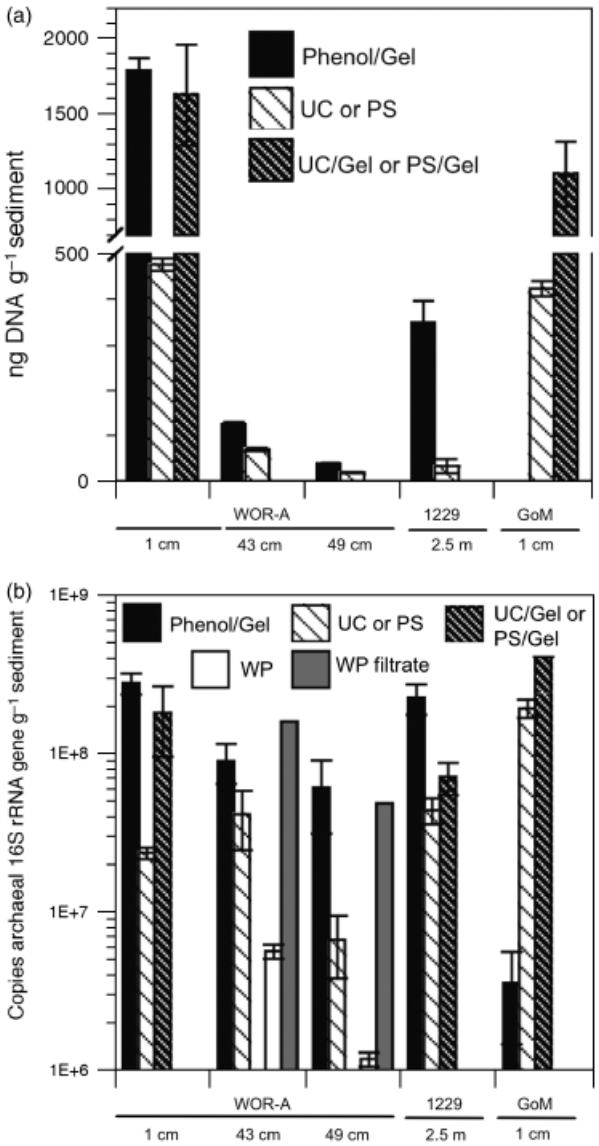

Fig. 4. Comparisons of different DNA extraction/purification methods. (a) PicoGreen-measured ng DNA g^{-1} whole sediment. Error bars for WOR-A depths 43 and 49 cm represent the average SD in the triplicate standard curve; for the PowerSoil/gel measurements, the difference between two measurements is shown; all other error bars are the SD of triplicate sample measurements. Only samples WOR-A (43 and 49 cm) underwent the phenol/WizardPlus procedure; these measurements were below 3 ng g^{-1} sediment and are not visible on the plot. For WOR-A 49 cm and 1229, measurements of the UltraClean/gel and PowerSoil/gel groups were below the PicoGreen detection limit. WOR-A 43 cm did not undergo the UltraClean/gel procedure. (b) qPCR-determined copies of archaeal 16S rRNA genes g^{-1} whole sediment. Error bars represent the SD of triplicate sample measurements. For duplicate sample measurements, such as UltraClean/gel, PowerSoil/gel, and WOR-A 43 cm phenol/WizardPlus, the differences are shown, and phenol/WizardPlus filtrate values are from a single measurement. UC, UltraClean; PS, PowerSoil; WP, WizardPlus.

yield loss due to the final silica column step of that kit. The organic matter in these methane seep sediments is predominantly microbially recycled fossil hydrocarbons (Lapham *et al.*, 2008), whereas photosynthetically derived organic carbon dominates in the other two samples, which may help account for the difference. These results emphasize that no single extraction method is optimal for all sediment types (Zhou *et al.*, 1996; Alm & Stahl, 2000).

The sediment samples from the WOR estuary that are the focus of the current study are fairly high in total organic carbon (~4–6%) (Kelley *et al.*, 1990), including terrigenous inhibitors such as dark-colored humic acids. However, similar recovery and inhibition problems persist in the organic-rich continental margin sediments of the Peru Margin. Poor binding of nucleic acids to silica columns in the presence of humic acids has been observed in other studies of DNA extraction and purification techniques (Zhou *et al.*, 1996; Miller *et al.*, 1999; Martin-Laurent *et al.*, 2001; Luna *et al.*, 2006); however, silica columns are frequently used to obtain qPCR-amplifiable DNA from samples similar to those used in the current study (Inagaki *et al.*, 2003, 2004; Schippers *et al.*, 2005; Sørensen & Teske, 2006). For the subsurface sediments from ODP drilling site

1229, hole D, on the Peru Margin, the archaeal 16S rRNA gene copy number we derived from the PowerSoil-extracted sample (subcore 1H-2, 2.25–2.30 mbsf; 4.36×10^7 copies g^{-1} sediment) was remarkably similar to those obtained by Schippers & Neretin (2006) with the FastDNA spin kit (1.15×10^7 copies g^{-1} sediment), using a bulk density conversion of 1.5 g cm^{-3} (D'Hondt *et al.*, 2003) and averaging the 16S rRNA gene copy numbers from adjacent subcores 1H-1 and 1H-3 at 0.15 and 3.70 mbsf (D'Hondt *et al.*, 2003; A. Schippers, pers. commun.). The FastDNA spin kit includes a similar silica column purification method as the PowerSoil kit. The small difference of factor 3.5 between these 16S rRNA gene copy numbers may be explained by primers with different bias against highly abundant subsurface archaeal groups (Teske & Sørensen, 2008) or by differences in the SYBR Green qPCR technology vs. the TaqMan method used in the former work (Schippers & Neretin, 2006). The phenol/gel procedure, however, increases DNA yield by an order of magnitude (2.23×10^8 copies g^{-1} sediment). For this case, the choice of the purification method impacts gene quantification more than different qPCR technologies and primers.

Although yield is of critical importance in quantitative applications, the quality of the nucleic acids must also be considered. Although the acrylamide gel was a superior purification procedure, the phenol/bead-beating extraction resulted in degraded DNA, while RNA remained intact (Fig. 3). This is a well-documented phenomenon (Osborn & Smith, 2005). Because genomic DNA is much larger than intact ribosomes, it is more susceptible to shearing during bead-beating. Therefore, a non-bead-beating protocol is preferred for DNA extraction.

Conclusions

The WizardPlus, UltraClean, and PowerSoil silica columns alone removed enough PCR inhibitors to allow for PCR amplification, and their relative speed (a few minutes vs. 2 days for the gel purification) makes them attractive options for nonquantitative PCR analysis. However, the yield loss of over an order of magnitude in archaeal 16S rRNA genes and cDNA shows that using these columns for quantification of environmental nucleic acids could result in systematic underestimates. Even for nonquantitative methods, such as clone libraries, yield loss could result in rare microbial groups falling below the detection limit. Other nonsilica purification methods, such as Sepharose or ion-exchange columns, gel troughing, electroelution from gel slices, or the MoBio PowerSoil RNA extraction kit (with the DNA elution buffer), may offer further alternatives to the gel extraction protocol presented here (Miller *et al.*, 1999; Martin-Laurent *et al.*, 2001; Lakay *et al.*, 2006; Harnpicharnchai *et al.*, 2007). If quantitative recovery of nucleic acids is intended for samples rich in humic organic substances, the use of silica columns for extract purifications risks significantly decreased nucleic yields, and gel purification should be considered as a more effective alternative.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. (a) Visible color picture of preparative acrylamide gel showing that most of the brown humic acids run ahead of the blue loading dye (which itself runs well ahead of small RNA molecules). (b) SYBR Gold-stained preparative acrylamide gel showing stained humic acids running off the bottom of the gel, unstained loading dye, and stained RNA/DNA.

Fig. S2. Sample dilution and qPCR inhibition. RT-qPCR copy numbers of 16S rRNA cDNA (product of measured copy number and dilution factor) at different dilutions of template from WOR cores A and E at the listed depths below sediment surface.

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