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A comparison of DNA extraction methods for biodiversity studies of eukaryotes in marine sediments

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ABSTRACT: There is increasing interest in understanding the diversity of eukaryotic microorganisms living in marine sediments, particularly to assess the effects of anthropogenic activities. Sequencing technologies generate high-resolution data for biodiversity studies that are useful for environmental monitoring. However, there are challenges in coupling classical monitoring with new sequencing technology and, consequently, there is a requirement for stringent optimization and standardization of any new protocol. Sample preparation is a critical factor because errors introduced during this step will severely affect further analyses and conclusions. This is particularly important in studies where biodiversity between different samples is compared, such as in environmental monitoring programs. Several protocols for extracting genomic DNA from soil and sediment samples have been developed, but most are optimized for prokaryotes in terrestrial soils and may therefore not be optimally adapted for investigating benthic marine eukaryotes. In this study, we compared existing and modified genomic DNA extraction methods on 2 different marine sediments with the aim to find an optimal protocol for processing a high number of sediment samples for further sequencing analysis. The protocols were evaluated based on quantity and quality of genomic DNA and recovered biodiversity. Results indicated significant variations in overall genomic DNA yield and purity among protocols. Further, our data suggest an effect of genomic DNA extraction procedure on eukaryotic diversity profiles, particularly for sediments with a high content of silt and clay.

KEY WORDS: 18S rRNA gene · Benthic ecosystems · Biodiversity · Genomic DNA extraction · Sequencing

INTRODUCTION

The high diversity of eukaryotic microorganisms found in benthic environments indicates that they play a key role in maintaining ecosystem function (Bik et al. 2012). Indeed, a growing number of studies are investigating the diversity of eukaryotic microorganisms in marine sediments (Park et al. 2008, Lecroq et al. 2011, Pawlowski et al. 2011, Quaiser et al. 2011, Bik et al. 2012). Until 2 decades ago, the most common way to study the diversity of environmental samples was through culturing or microscopy. Today, sequencing techniques can generate a large number of sequence reads from one single sample, enabling high-resolution biodiversity analysis (Shendure & Ji 2008). Although sequencing methods are powerful, a critical assumption is that the PCR amplicons generated from the genomic DNA prior to sequencing accurately reflect species diversity.

Due to interactions between cells and sediment particles and the high content of potential enzyme-inhibitors in extracted samples (Lorenz et al. 1981, De Flaun & Mayer 1983, Ogram et al. 1987), it is difficult to obtain high quality DNA from marine sediments (Lovell & Piceno 1994). In general, protocols for genomic DNA extractions of soil and sediment samples can be divided into 2 categories: cell recovery (Fægri et al. 1977, Torsvik & Goksoyr 1978) and

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direct lysis (Ogram et al. 1987). Cell recovery methods separate cells from sediment particles prior to lysis and therefore reduce the presence of inhibitors in the final extract. However, genomic DNA yields using this approach are often low (Steffan et al. 1988), which can be problematic since many next generation sequencing platforms often require large amounts of library DNA. Direct lysis has been documented to obtain higher genomic DNA yields than cell recovery methods, but the procedure often co-extracts salts and organic compounds, which can inhibit downstream PCR and restriction assays (Ogram et al. 1987, Porteous & Armstrong 1991, Tsai & Olson 1992, Tebbe & Vahjen 1993, Leff et al. 1995). A method to improve the quality of the extract is to use phenol, which separates nucleic acids from other cellular components (Kirby 1956). Humic acids are common organic compounds in soil and sediments that are often co-extracted using direct lysis methods. These compounds are difficult to remove due to their structural similarity with DNA (Harry et al. 1999) and methods developed to separate humic acids from genomic DNA are often associated with significant loss of material (Moré et al. 1994, Zhou et al. 1996). An alternative method is to use bovine serum albumin (BSA) as an additive to the PCR or digestive enzyme mixture (Cho et al. 1996). This will maintain high genomic DNA yields and, because of its capacity for hydrophobic interactions and its high lysine content, BSA is able to bind lipids and anions (Loomis 1974), thereby preventing the co-extracted contaminants from binding and inactivating polymerases and digestive enzymes (Cho et al. 1996).

Different protocols for genomic DNA extraction utilize a variety of chemical compounds, enzymes and physical techniques for lysis of cells, separation of nucleic acids from other cellular compounds and purification of DNA. Eukaryotic microorganisms are a highly diverse group with large variations in cell and cell wall composition. Different lysis methods might therefore yield overall variation in lysis efficiency of cells of different taxonomic origin. High yields and purity of genomic DNA are critical when analyzing diversity of environmental samples and several studies have compared differences in DNA yield and purity among protocols for genomic DNA extraction from marine sediments (Leff et al. 1995, Miller et al. 1999). Some previous studies have reported significantly higher yields of genomic DNA using bead mill homogenization for physical disruption (Krsek & Wellington 1999, Miller et al. 1999). However, it is also important to assess the biological diversity obtained from different extraction protocols. Previous studies comparing methods for DNA extraction from marine sediments and the resulting diversity profiles have mostly considered the prokaryotic fraction (Martin-Laurent et al. 2001, Luna et al. 2006, Carrigg et al. 2007). These studies demonstrated that the method used for extracting genomic DNA affected the diversity patterns of prokaryotic organisms as well as the yield and purity of genomic DNA. A similar study comparing commercially available kits for extracting DNA from eukaryotic microorganisms in terrestrial soil found large variations in lysis efficiency and DNA yield and purity, as well as in the diversity patterns obtained (Mahmoudi et al. 2011). However, no studies have assessed the effect of genomic DNA extraction protocols on the biodiversity determined by sequencing methods and further taxonomical analyses. All studies investigating biodiversity have used gene fragment profiling techniques such as denaturating gradient gel electrophoresis (DGGE), amplified ribosomal DNA restriction analysis (ARDRA), terminal restriction fragment length polymorphism (TRFLP) and ribosomal intergenic spacer analysis (RISA) (Krsek & Wellington 1999, Bürgmann et al. 2001, Martin-Laurent et al. 2001, Luna et al. 2006, Carrigg et al. 2007, Thakuria et al. 2008, Mahmoudi et al. 2011).

The aim of the present study was to find an optimal protocol for extracting genomic DNA from marine sediments and to assess whether genomic DNA extraction protocols exert an effect on eukaryotic diversity profiles. This was done to set standards for sediment processing for next generation sequencing as part of routine environmental monitoring. Eight different genomic DNA extraction protocols were evaluated based on yield and purity. The most suitable protocols were further compared by cloning and sequencing of the PCR amplified 18S rRNA fragments to investigate variation in eukaryotic biodiversity yields from the different extraction methods.

MATERIALS AND METHODS

Sampling

The sediments in this study were collected as part of an ongoing offshore environmental monitoring program in the North Sea carried out by Det Norske Veritas (DNV) and Molab, during a cruise in Region III (Oseberg/Troll) in May 2010 (DNV 2011). For each station in this region, approximately 50 to 100 g of sediment was collected using a sediment grabber and transferred to a 250 ml plastic container (Kautex

Textron) and fixed with 96% ethanol (Harry et al. 2000). This resulted in a final concentration of approximately 80% ethanol due to the water content of the sediment. The samples were stored at –20°C until further analysis. Two different marine sediment samples were used in this study: Sample S was a coarse sand sediment from the offshore Huldra oil field (sample 05) at 126 m depth, with a silt and clay content of 1.9%; Sample C was a clay sediment from the offshore Troll A oil field (sample 07) at 490 m depth, with a silt and clay content of >90%.

Genomic DNA extraction

Eight different DNA extraction methods were tested in replicates of 3 on Samples S and C. The methods were selected based on their success in previous studies (Zhou et al. 1996, Bürgmann et al. 2001, Luna et al. 2006, Whitehouse & Hottel 2007, Thakuria et al. 2008, Mahmoudi et al. 2011). For all methods, we used 0.25 g of sediment as input to the extraction procedure. Ethanol was removed from the sediment prior to genomic DNA extraction by centrifugation at 6000 × *g* followed by pipetting.

Method 1. Following the protocol developed by Zhou et al. (1996), sediment samples were mixed with an extraction buffer containing 1% hexadecyltrimethylammonium bromide (CTAB) and 50 µg Proteinase K in screw cap reaction tubes. After shaking horizontally at 500 rpm for 30 min at 37°C, 75 µl of 20% sodium dodecyl sulfate (SDS) was added and the samples were incubated at 65°C for 2 h before centrifugation (6000 × *g*, 10 min) to remove non-biological materials. This procedure was repeated twice and supernatants were pooled and extracted using chloroform:isoamylalcohol (24:1) to separate proteins and lipids from the genomic DNA. The aqueous phase containing DNA was precipitated by adding 0.6 volumes of isopropanol, followed by centrifugation at 16 000 × *g* for 30 min. The resulting DNA pellet was washed with 70% cold ethanol and re-suspended in 1 × TE buffer (Invitrogen) in a final volume of 50 µl.

Method 2. Based on Method 1, but with 375 mg silica beads (Biospec Products) (0.1 and 1 mm size, in a 1:1 ratio) added before CTAB and Proteinase K incubation. The beads were added prior to the shaking to enhance cellular lysis. Bead beating was performed at 2500 rpm for 15 min using a Digital Vortex Mixer (VWR).

Method 3. Based on Method 2, but with an additional phenol-chloroform step prior to the chloroform:isoamylalcohol extraction. This was done to evaluate whether an additional phenol step would yield genomic DNA with a higher purity (Kirby 1956).

Method 4. Following the protocol developed by Bürgmann et al. (2001), the sediment sample was mixed with 375 mg silica beads (0.1 and 1 mm size, in a 1:1 ratio) and a DNA extraction buffer containing 0.2% CTAB and 1 mM dithiotreitol (DTT) before being placed on a bead beater at 2500 rpm for 2 min. The mixture was centrifuged (16 000 × *g* for 5 min) to remove non-biological material including the silica beads. The aqueous phase was removed and extracted using phenol and chloroform, followed by 2 rounds of chloroform:isoamylalcohol (24:1) treatments. The final aqueous phase was mixed with 20% polyethylene glycol 6000 (Merck) and incubated at 37°C for 1 h. The DNA was precipitated at 16 000 × *g* for 30 min before the DNA pellet was washed with cold 70% ethanol and re-suspension in 50 µl TE buffer.

Method 5. Based on Method 4, but following modifications according to Thakuria et al. (2008) using a polyvinylpolypyrrolidone (PVPP) spin column for additional purification. The spin columns were prepared by adding 300 µl of sterile 10% PVPP and centrifuged at 14 000 × *g* for 1 min to create a matrix. The sediment samples were mixed with silica beads and buffers as described in Method 4. After centrifugation the supernatant was passed through the PVPP spin column. The eluate was mixed with 50 µl 5 M CH_3CO_2K and incubated on ice for 5 min before centrifugation at 16 000 × *g* at 4°C for 30 min. The supernatant was mixed with an equal volume of 20% polyethylene glycol 6000 and incubated for 1 h at 37°C. The sample was further centrifuged for 30 min at 16 000 × *g* and dissolved in 400 µl TE. The DNA was extracted using phenol:chloroform:isoamylalcohol (25:24:1; pH 6.8), followed by 2 additional steps of chloroform:isoamyl (24:1) treatments. The DNA was precipitated with 2.5 volumes of ethanol and the DNA pellet was washed with 70% cold ethanol and eluted in 50 µl TE buffer.

Method 6. Sediment samples were vortexed with 500 µl of 10% Chelex before incubation at 100°C for 20 min. The tubes were gently mixed after 10 min. After centrifugation at 6000 × *g* for 1 min the supernatant was transferred to a new tube and extracted with an equal volume of chloroform:isoamylalcohol (24:1). The DNA was precipitated from the aqueous phase using 0.6 volume of isopropanol (1 h, room temperature) followed by centrifugation at 16 000 × *g* for 30 min. The pellet was washed with cold 70% ethanol and eluted in 50 µl TE.

Method 7. This method used the UltraClean® Soil DNA isolation kit (MoBio), following the manufac-

turer's protocol and recommendations. Homogenization buffer and beads were added to the sediments and then vortexed. An SDS-based lysis solution was then added and the solution was vortexed for 15 min to enhance cell lysis. After bead beating, the solution was centrifuged to remove cell debris. The supernatant was collected and added to a spin column with a silica membrane, which binds genomic DNA. The DNA was washed and finally eluted in 50 µl buffer.

Method 8. This method used the PowerSoil® DNA isolation kit (MoBio), following the manufacturer's protocol and recommendations. The protocol is essentially as described for Method 7, but with added purification step on the collected supernatant prior to the spin column. Purified genomic DNA was eluted in 100 µl buffer.

Quantification and purity assessments

The quantities of genomic DNA from each of the different extraction protocols were estimated using Quant-iT™ PicoGreen® dsDNA quantitation kit (Invitrogen) together with a fluorospectrophotometer (ND 3000, Nanodrop Technologies). Prior to quantification, the samples were diluted 10- and 100-fold in 1 × TE. Both dilutions were quantified. TE was used as a blank and bacteriophage λ DNA (Invitrogen) for the standard curve. The DNA yield was calculated as µg DNA g^{-1} sediment. Purity of the genomic DNA extracts was assessed using a Nanodrop ND 1000 Spectrophotometer. The ratios of 260:230 and 260:280 absorbance (A) were calculated as a measure of purity. Nucleic acids have absorbance maxima at 260 nm, proteins at 280 nm, while salts and organic compounds (e.g. humic acids) have maxima at 230 nm (Yeates et al. 1998). Ratios $A_{260:280}$ of 1.8 and $A_{260:230}$ of 2.0 indicate highest quality of genomic DNA.

Optimization of BSA concentration in the PCR reactions

To inactivate possible inhibitors from the genomic DNA extracts, different concentrations of BSA (Fermentas) (0, 0.2, 0.4, 1.0, and 1.6 µg $µl^{-1}$) were added to the PCR mastermix. PCR reactions were performed using 0.01 U Dynazyme (Finnzymes), 1 × buffer (Finnzymes), 0.1 µM dNTP (Finnzymes), and 0.5 µM each of the eukaryotic universal primers F-566 and R-1200 (Hadziavdic et al. 2014), generating a 650 bp fragment of the 18S rRNA gene. Aliquots of 2.5 µl of the DNA extract obtained using Method 8 from sediment Samples S and C were used as templates. Positive and negative controls were included. PCR amplification was carried out with a thermal cycler (C1000™ Thermal Cycler, BioRad) with the following cycle program: 95°C for 5 min, 30 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 1 min, and a final extension step of 72°C for 10 min. Amplifications were visualized on a 1.5% agarose gel electrophoresis, with the DNA stain GelRed™ (Biotium). GeneRuler T DNA Ladder Mix (Fermentas) was run as a size marker.

Biodiversity analysis

To investigate whether extraction method influences taxonomic recovery, PCR amplified 18S rRNA gene fragments obtained from the 5 genomic DNA extraction methods (1, 2, 3, 7 and 8) that yielded sufficient amounts of DNA were cloned using the StrataClone PCR Cloning kit (Agilent Technologies). Triplicate genomic DNA extracts from Samples S and C were PCR amplified using the universal eukaryotic primers F-566 and R-1200 (Hadziavdic et al. 2014). PCR assays were conducted as described above using 1 µg $µl^{-1}$ of BSA. To evaluate amplification products, 5 µl of the final PCR reactions were visualized by 1.5% agarose gel electrophoresis stained with GelRed™ (Biotium). PCR products from the 3 replicas of each DNA extraction method were pooled before cloning. From each clonal library, 96 clones were sequenced at Molecular Cloning Laboratories (MCLAB), San Francisco, USA.

Statistics and data analysis

All statistical analyses were performed using R v.2.15.0 software (the R Foundation for Statistical Computing; R Development Core Team 2008). All treatments were run in triplicate with means and standard deviations calculated. Homogeneity of variance in the datasets was tested using Levene's test (Levene 1960). Shapiro-Wilk's test was used to assess normal distribution of data (Shapiro & Wilk 1965) to fulfill the criteria for an ANOVA. In cases with no significant differences in these tests ($p > 0.05$), 1-way analysis of variance (ANOVA) was used to evaluate significant differences in DNA yield and purity between the treatments. Significant results in the ANOVA were assessed post hoc by Tukey's HSD for pairwise comparison of treatments. In cases where homogeneity in variances and normal distribution

were rejected ($p < 0.05$), non-parametric Kruskal-Wallis tests were used to assess differences (Kruskal & Wallis 1952). Sequences were edited and further checked for chimeras using the Chimera Uchime algorithm (Edgar et al. 2011) in mothur v. 1.31.2 (Schloss 2009). The sequences were aligned using Clustal Omega and clustered into operational taxonomic units (OTUs) using the furthest neighbor algorithm in mothur with 97% similarity cutoff (Schloss & Handelsmann 2005). Diversity was estimated using the Shannon-Weaver index (Shannon & Weaver 1949) and ACE nonparametric richness estimations (Chao & Lee 1992), calculated in R with the OTU data as input. All sequences were taxonomically assigned using the Ribosomal Database Project (RDP) taxonomy assigner implemented in QIIME (Wang et al. 2007) against the Silva database v. 108 with eukaryotic sequences (Quast et al. 2013).

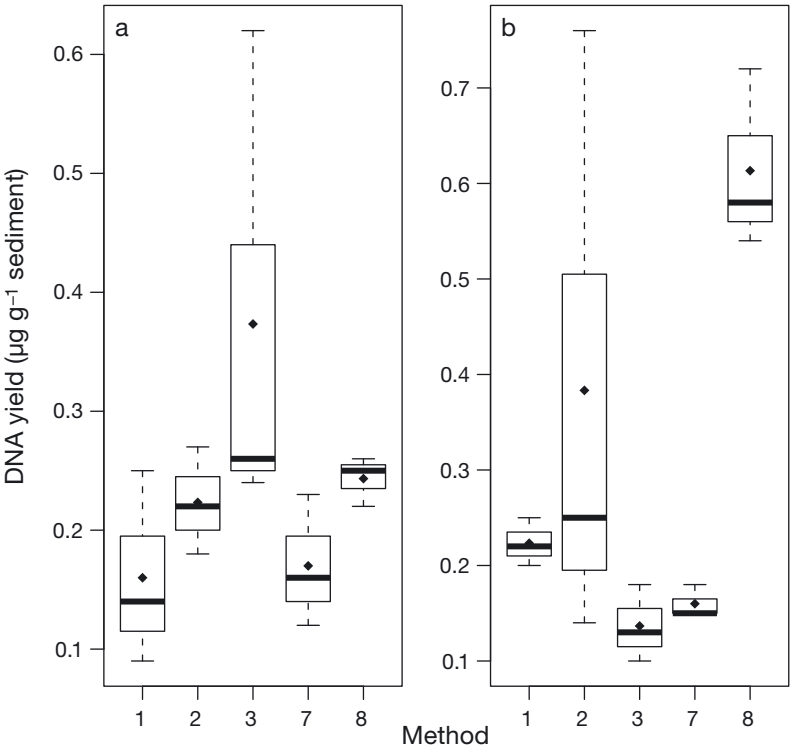

Fig. 1. Boxplot of the genomic DNA yields from (a) sand (Sample S) and (b) clay (Sample C) sediments extracted using Methods 1, 2, 3, 7 and 8. Diamonds indicate mean values, bold horizontal lines the median value, the boxes define the quartiles and the whiskers indicate variability outside the upper and lower quartiles with minimum and maximum data values

RESULTS

Yield and purity of genomic DNA

From the 8 DNA extraction protocols tested in this study, Methods 1, 2, 3, 7 and 8 generated yields ranging from 0.16 to 0.37 µg g^{-1} sediment from Sample S and 0.14 to 0.61 µg g^{-1} sediment from Sample C (Fig. 1). Methods 4, 5 and 6 did not yield significant amounts of genomic DNA, and were excluded from statistical analyses.

The DNA yield obtained from Sample S was not significantly affected by the extraction method ($p > 0.05$, Table 1). However, significant differences between methods were observed in Sample C ($p < 0.05$). For this sample, while post hoc Tukey HSD analysis revealed no difference in genomic DNA yields between Methods 1, 2, 3 and 7 ($p > 0.05$), there was significantly higher genomic DNA yield using Method 8 compared to Methods 3 and 7 ($p = 0.0244$ and 0.0324, respectively). However, there was no significant difference between Method 8 and Methods 1 and 2 ($p > 0.05$).

DNA extracts obtained using the 5 methods (1, 2, 3, 7 and 8) had $A_{260:280}$ ratios ranging from 0.69 to 1.62 (Table 2). In general there was higher variability

Table 1. ANOVA comparison of genomic DNA yields from sand (Sample S) and clay (Sample C) sediments using DNA extraction Methods 1, 2, 3, 7 and 8. Values were log transformed

	SS	df	MS	*F*	p
Sample S					
Treatment	1.32	4	0.33	2.37	0.12
Error	1.39	10	0.14		
Sample C					
Treatment	4.31	4	1.08	6.18	<0.01
Error	1.74	10	0.17		

Table 2. Absorbance ratios $A_{260:280}$ and $A_{260:230}$, indicating genomic DNA purity (mean ± SD) in extracts from sand (Sample S) and clay (Sample C) sediments using DNA extraction Methods 1, 2, 3, 7 and 8

Method	Sample S		Sample C	
	$A_{260:280}$	$A_{260:230}$	$A_{260:280}$	$A_{260:230}$
1	1.26 ± 0.52	0.48 ± 0.36	1.21 ± 0.67	0.67 ± 0.21
2	0.69 ± 0.46	1.63 ± 1.58	1.18 ± 0.24	0.83 ± 0.19
3	1.50 ± 0.44	0.75 ± 0.59	1.62 ± 0.21	0.54 ± 0.11
7	1.16 ± 0.11	1.65 ± 0.23	1.52 ± 0.13	1.50 ± 0.12
8	1.30 ± 0.14	1.66 ± 0.15	1.45 ± 0.07	1.41 ± 0.22

Table 3. ANOVA comparison of genomic DNA purity from sand (Sample S) and clay (Sample C) sediments using DNA extraction Methods 1, 2, 3, 7 and 8. Significant p-values are shown in bold type

	SS	df	MS	*F*	p
$A_{260:280}$					
Sample S					
Treatment	1.09	4	0.27	1.93	0.18
Error	1.41	10	0.14		
Sample C[a]					
Treatment	0.22	4	0.05	1.03	0.44
Error	0.53	10	0.05		
$A_{260:230}$					
Sample S					
Treatment	3.55	4	0.89	3.52	**<0.05**
Error	2.52	10	0.25		
Sample C					
Treatment	2.28	4	0.57	18.66	**<0.001**
Error	0.31	10	0.03		

[a]Values were log transformed

between the replicates in the manual methods (Methods 1, 2 and 3) compared to the commercial kits (Methods 7 and 8). However, while there were no significant differences in the $A_{260:280}$ ratio, there were differences in the $A_{260:230}$ ratio for both Samples S and C among the 5 methods (Table 3). Post hoc Tukey HSD analysis revealed that the differences in $A_{260:230}$ ratios separated the 5 methods into 2 groups: the 2 commercial kits (Methods 7 and 8) generated $A_{260:230}$ values significantly closer to 2.0 compared to the manual Methods 1, 2 and 3 ($p < 0.05$). These results suggest that all methods removed proteins in equal amounts, while the 2 commercial kits were able to remove higher levels of salts and organic compounds such as humic acids.

Optimization of BSA concentration in PCR reactions

When working with environmental DNA, additional purification of the eluate may be necessary to remove inhibitors and retrieve PCR amplifiable DNA. We included BSA in the PCR mastermix for extracts from Method 8 and adjusted the concentration needed to optimize PCR amplifications. Untreated samples exhibited a strong PCR inhibitory effect, with failure to amplify genomic DNA templates extracted from Sample C and a weak band for Sample S (Fig. 2). A gradient of BSA concentrations (0.2 to 1.6 µg µl^{-1}) was added to the genomic DNA templates from Method 8 in the PCR assay. The most efficient amplification of PCR products was observed at 1 µg µl^{-1} addition, a concentration subsequently used in all further PCR assays.

Biodiversity analyses

To test whether different DNA extraction protocols affected eukaryotic biodiversity profiles, clone libraries were constructed. Genomic DNA extracts from sediment Samples S and C obtained by Methods 1, 2, 3, 7

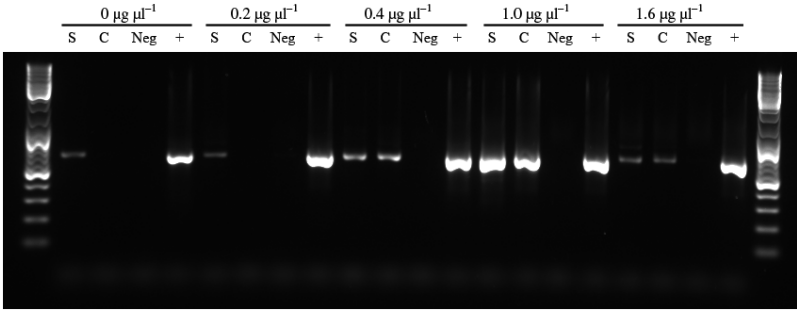

Fig. 2. Inclusion of bovine serum albumin (BSA) to optimize PCR amplification of DNA samples extracted from sand (Sample S) and clay (Sample C) sediments. 5 µl PCR products were visualized on a 1.5% agarose gel. Neg: negative control; +: positive control. BSA concentrations (0, 0.2, 0.4, 1.0, and 1.6 µg µl^{-1}) are indicated at the top. On both sides of the gel is the GeneRuler T DNA Ladder Mix, added as a size marker

Table 4. Number of clones, OTUs clustered with a 97% similarity cut-off level and diversity estimates for genomic DNA from sand (Sample S) and clay (Sample C) sediments obtained by selected DNA extraction methods

Method	No. of sequences	No. of OTUs (97%)	Shannon diversity index	ACE OTU richness estimate (±SE)
Sample S				
1	62	38	3.32	170.6 ± 7.4
2	86	47	3.33	268.5 ± 10.1
3	84	39	2.79	151.0 ± 4.5
7	86	35	2.62	181.9 ± 9.1
8	88	50	3.52	248.1 ± 10.9
Sample C				
1	83	32	2.78	157.0 ± 9.0
2	54	28	3.21	35.2 ± 2.9
3	86	26	2.20	47.3 ± 2.9
7	94	54	3.56	231.9 ± 7.2
8	90	61	3.86	306.5 ± 12.9

and 8 were amplified using universal 18S rRNA gene primers. The 10 amplified gene fragments were cloned and 96 colonies were analyzed per clone library by sequencing. Table 4 lists the number of good-quality sequences retrieved from each clone library, the number of OTUs, diversity estimates and richness predictions. Shannon-Weaver diversity estimates for the clone libraries demonstrated larger variation in diversity between DNA extraction methods performed on Sample C (2.20 to 3.86) compared to Sample S (2.62 to 3.52). This trend was also supported by the nonparametric abundance-based coverage estimator (ACE), which predicted a range of OTU numbers from 35 to 307 for Sample C and 151 to 268 for Sample S depending on DNA extraction method. Some methods generated a lower number of reads than others, i.e. only 63 high quality sequences were retrieved from Sample S using Method 1 and only 54 sequences were retrieved from Sample C using Method 2. This could bias the richness estimate since it is known that ACE underestimate true richness at low sample sizes (Hughes et al. 2001).

Fig. 3 shows the taxonomic composition in sediment Samples S and C retrieved by extraction Methods 1, 2, 3, 7 and 8 based on sequence data. Our data suggest that the major taxonomic groups in Sample S included Alveolata, Metazoa, Stramenopiles and Rhizaria (Fig. 3a). Sequences affiliating with these groups were retrieved from all genomic DNA extraction methods. In Sample C, sequences from the same groups were also detected with all 5 methods (Fig. 3b).

Time and costs

For the applicability of sequencing for biodiversity analysis in routine environmental surveys, where hundreds to thousands of samples are being processed, factors such as time and cost of processing also need to be considered. Table 5 lists the processing time for 20 samples and cost per sample, using

Table 5. Sample preparation costs and labor time for selected DNA extraction methods. Prices are listed without VAT according to Norwegian price levels

Method	Preparation time (h per 20 samples)	Costs (Euro per sample)
1	6.0	0.73
2	6.5	1.04
3	7.0	1.43
7	2.0	4.43
8	2.0	5.21

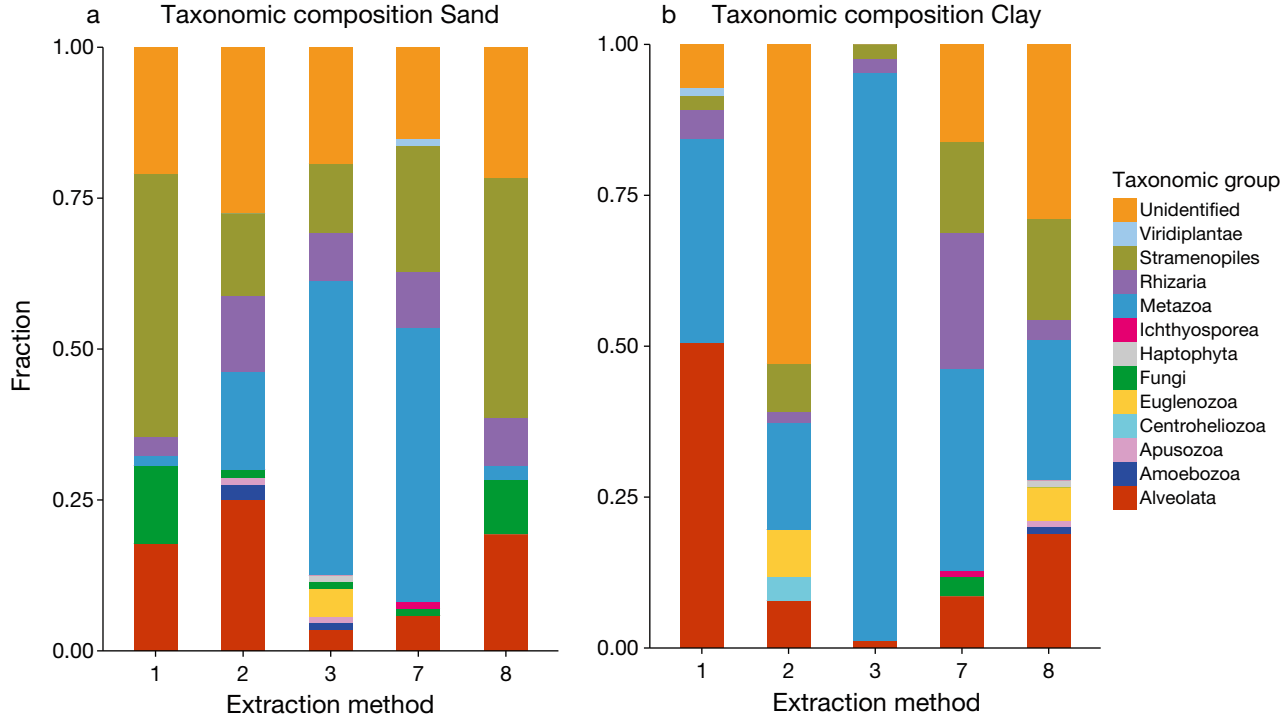

Fig. 3. Distribution of taxonomic groups based on operational taxonomic units (OTUs) retrieved from clone libraries of (a) sand (Sample S) and (b) clay (Sample C) sediments extracted with Methods 1, 2, 3, 7 and 8

Methods 1, 2, 3, 7 and 8. Preparation times of buffers and other reagents for the manual laboratory methods were not considered. The commercial kits had the advantage of short preparation time (2 h) compared with the manual extraction methods (6 to 7 h). The commercial kits were, however, also more expensive than manual protocols.

DISCUSSION

Sequencing technology has enhanced our knowledge in microbial ecology and allowed investigation of highly complex and diverse communities (Logares et al. 2012). Although high-resolution methods are becoming readily available they need great care in sample preparation as sub-optimal protocols can severely bias conclusions regarding biodiversity. This is particularly important in environmental monitoring programs, which are subject to accreditations and standardization of procedures. In this study, we therefore evaluated 8 different genomic DNA extraction protocols on marine sediments.

Method 1, developed by Zhou et al. (1996), was included due to its success in previous studies in which extraction methods for marine sediments were compared (Luna et al. 2006). This protocol was further modified to improve lysis of the sample (Method 2) and purification of the DNA (Method 3). These 3 methods were the only manual methods able to obtain sufficient amounts of genomic DNA. Method 6 was tested for its simplicity and cost effectiveness (Martín-Platero et al. 2010), while Methods 4 and 5 were protocols that had previously been optimized for soils (Bürgmann et al. 2001, Thakuria et al. 2008). The latter 2 protocols were similar in several steps to Methods 1, 2 and 3, in which the first step involved the use of a homogenization buffer containing CTAB and the second step involved precipitation by phenol: chloroform:isoamylalcohol followed by an ethanol wash. There were 2 main differences between the methods: (1) the use of PVPP spin-columns and (2) repeated incubation with homogenization buffers followed by centrifugation. A decrease in DNA yields when implementing PVPP spin columns in extraction protocols has been reported previously (Zhou et al. 1996, Krsek & Wellington 1999). This would however, only explain the low yield from Method 5, and not from Method 4, for which the PVPP spin-column cleaning steps were absent. Our results indicate that repeated homogenization steps using the CTAB buffer followed by centrifugation as carried out in Methods 1, 2 and 3 might be necessary to obtain efficient lysis of the cells prior to extraction of genomic DNA from marine sediments. We also evaluated the commercial MoBio UltraClean DNA extraction kit (Method 7), which has been tested in several previous studies involving prokaryotes (Martin-Laurent et al. 2001, Dong et al. 2006, Luna et al. 2006, Carrigg et al. 2007, Whitehouse & Hottel 2007, Thakuria et al. 2008) and reported to give the highest yield of genomic DNA from soil samples compared to other commercially available kits (Whitehouse & Hottel 2007) including the MoBio PowerSoil (Method 8). The latter protocol has commonly been used for sediment extractions in diversity studies (Park et al. 2008, Lecroq et al. 2011, Pawlowski et al. 2011, Quaiser et al. 2011). In particular Mahmoudi et al. (2011) demonstrated that MoBio PowerSoil yielded the highest quality DNA compared to other commercially available tools and also yielded several consistent bands on a DGGE fingerprinting analysis.

Our results indicated no significant difference between the yields obtained with Method 1, in which bead beating was not included in the protocol, and Methods 2 and 3, in which bead beating was performed in the first step of the procedures. It is known that extracting DNA from sediments with a high content of clay is difficult due to the interaction between microbial cells and soil colloids (Lorenz et al. 1981, Hurt et al. 2001). Our results support these findings as the choice of extraction method did not have a significant effect on genomic DNA yields from Sample S, while the choice of extraction method did significantly affect yields from Sample C, with Method 8 obtaining the highest concentration of genomic DNA (Fig. 1).

DNA extracts from environmental samples such as marine sediments are likely to contain components that affect purity (Ogram et al. 1987). The $A_{260:280}$ ratio, which is influenced by co-extracted proteins, was generally rather low but not significantly affected by the extraction method for either of the sediments. The procedure in Method 2 was further modified by addition of a phenol step (Method 3). Our results demonstrated that adding phenol to the DNA extraction protocols yielded higher mean values, but did not statistically improve the quality of DNA (Table 2).

All of the tested methods involved direct lysis of cells (Ogram et al. 1987). In contrast to cell recovery methods where the cells are separated from the sediments prior to lysis (Fægri et al. 1977, Torsvik & Goksoyr 1978), direct lysis often yields higher amounts of genomic DNA as well as extracted humic acids and other contaminants (Ogram et al. 1987, Tsai & Olson

1992, Leff et al. 1995). The $A_{260:230}$ ratio, influenced in part by humic acids often found in marine sediments, was better in the DNA extracts obtained using commercial kits compared to manual methods (Tables 2 & 3). These results are in agreement with earlier studies (Webster et al. 2003, Xu et al. 2011). Our data further suggest that it is more difficult to obtain pure genomic DNA extracts for the sediment with high silt and clay content.

Several methods for removing potential inhibitors from nucleic acids have been developed (Jackson et al. 1997, Dong et al. 2006, Persoh et al. 2008). However, such methods are often associated with a high loss of DNA (Moré et al. 1994, Zhou et al. 1996). Therefore, we tested whether we could quench inhibition and improve PCR amplifications through the addition of BSA in the mastermix. Our data indicate that addition of BSA at a concentration of 1 µg μl^{-1} in the PCR mastermix generated viable products and reduced the influence of inhibitors in the extract (Fig. 2). It should however be noted that this concentration was only optimized for extracts obtained with Method 8.

Luna et al. (2006) compared 3 DNA extraction methods and demonstrated variations in genomic DNA yields, but with no differences in bacterial ribotype profiles. Our results on the other hand, indicated that extraction method did not affect the yield of genomic DNA from Sample S (Table 1), nor did it severely impact the eukaryotic diversity profiles of this sediment (Table 4). For Sample C, however, the extraction method did affect both DNA yield and diversity estimates. Methods 2 and 3 in particular gave low ACE richness estimates. ACE is a nonparametric estimator based on mark-release-recapture (MMR) (Hughes et al. 2001). It considers the skewness of OTUs in our sample. Hence the low ACE values in Methods 2 and 3 were generated because of the relatively high number of reads per OTU and few singletons. In Method 8 on the other hand, the sequence reads were distributed among more OTUs and in addition contained a higher number of singletons. The taxonomic analysis further indicated variations between the extraction methods, particularly for Sample C (Fig. 3). The most abundant groups (Alveolata, Metazoa and Stramenopiles) were detected by all 5 methods in both Samples S and C, as was the lower abundance group Rhizaria. Fungi were detected by all 5 methods in Sample S. The variation seen between these major groups as a consequence of extraction method suggests that extraction method is important and will influence the results. The less abundant groups on the other hand (Amoebozoa, Apusozoa, Centroheliozoa, Euglenozoa, Haptophyta, Ichthyosporea and Viridiplantae) were not detected by all methods. This could be a result of differences between the various procedures to lyse cells and extract DNA from eukaryotes. It is also possible that the variation detected in the less dominant taxonomic groups was influenced by high diversity within samples and insufficient coverage from clone libraries. This may explain why no method was able to retrieve sequences affiliated with all taxonomical groups detected in the combined extracts of each sample. An alternative to clone libraries in combination with Sanger sequencing of the 650 bp 18S rRNA fragment (Hadziavdic et al. 2014) is 454 pyrosequencing. This method would yield higher sequencing depth. However a full diversity characterization of the sediments was not the primary objective but rather identification of different extraction efficiencies, which was identified based on the major taxonomical group. Overall, our data indicate that extraction Method 3 obtained sequences from more taxonomical groups than the other methods for Sample S, while Method 8 obtained sequences from more taxonomical groups than the other methods for Sample C. Our data further indicate that Method 8 generated the largest number of OTUs from both sediments.

Our results suggest that selection of DNA extraction method for sand sediments was less critical than for sediments with a high content of silt and clay. Overall, Method 8 (MoBio PowerSoil DNA extraction kit) generated a good combination of DNA yield, purity and diversity coverage of eukaryotes from both sediments tested in this study. This method is also time efficient and is therefore well-adapted to pipelines for diversity surveys (Table 5). Indeed, the Earth Microbiome Project has standardized its protocols using the MoBio PowerSoil kit for all its DNA extractions (www.earthmicrobiome.org/emp-standard-protocols/dna-extraction-protocol/). This emphasizes the importance of using a single optimized procedure throughout comparative diversity studies of eukaryotes in marine sediments, since different extraction methods can influence final biodiversity assessments, particularly when different sediment types are included.

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