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Preservation potential of ancient plankton DNA in Pleistocene marine sediments

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ABSTRACT

Recent studies have shown that ancient plankton DNA can be recovered from Holocene lacustrine and marine sediments, including from species that do not leave diagnostic microscopic fossils in the sediment record. Therefore, the analysis of this so-called fossil plankton DNA is a promising approach for refining paleoecological and paleoenvironmental information. However, further studies are needed to reveal whether DNA of past plankton is preserved beyond the Holocene. Here, we identified past eukaryotic plankton members based on 18S rRNA gene profiling in eastern Mediterranean Holocene and Pleistocene sapropels S1 (~9 ka), S3 (~80 ka), S4 (~105 ka), and S5 (~125 ka). The majority of preserved ~400- to 500-bp-long 18S rDNA fragments of microalgae that were studied in detail (i.e. from haptophyte algae and dinoflagellates) were found in the youngest sapropel S1, whereas their specific lipid biomarkers (long-chain alkenones and dinosterol) were also abundant in sediments deposited between 80 and 124 ka BP. The late-Pleistocene sediments mainly contained eukaryotic DNA of marine fungi and from terrestrial plants, which could have been introduced via the river Nile at the time of deposition and preserved in pollen grains. A parallel analysis of Branched and Isoprenoid Tetraethers (i.e. BIT index) showed that most of the organic matter in the eastern Mediterranean sediment record was of marine (e.g. pelagic) origin. Therefore, the predominance of terrestrial plant DNA over plankton DNA in older sapropels suggests a preferential degradation of marine plankton DNA.

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INTRODUCTION

The reconstruction of marine and lacustrine plankton is typically based on the analysis of microscopic remains of (planktonic) species such as diatoms (Kemp *et al.*, 1999), coccolithophorid algae (Castradori, 1993), cyst-forming dinoflagellates (dinocysts) (Marret & Zonneveld, 2003), and foraminifera (Principato *et al.*, 2006) or geochemical analysis of characteristic molecules that are preserved in the geological record, so-called lipid biomarkers, or chemical fossils (Peters *et al.*, 2005; Volkman *et al.*, 1998). However, microscopic remains are rarely produced by all species of a taxonomic group [e.g. calcifying vs. noncalcifying haptophyte algae (Sáez *et al.*, 2004)], and lipid biomarkers are often diagnostic only at higher taxonomic levels (Volkman *et al.*, 1998).

In recent years, the analysis of ancient plankton-derived DNA preserved in marine or lacustrine sediments (i.e. fossil plankton DNA) has provided valuable information for refining reconstructions environmental conditions and, *vice versa*, the impact of climate-induced hydrologic and environmental changes on past plankton succession dynamics (Bissett *et al.*, 2005; Boere *et al.*, 2009, 2011; Coolen, 2011; Coolen & Overmann, 1998, 2007; Coolen *et al.*, 2004, 2006, 2007, 2008, 2009; Corinaldesi *et al.*, 2011; D'Andrea *et al.*, 2006; Epp *et al.*, 2010; Manske *et al.*, 2008; Panieri *et al.*, 2010; Reid *et al.*, 2000; Theroux *et al.*, 2010). Fossil plankton DNA records are particularly useful for identifying past species, most notably phototrophic bacteria and protists, which did not produce or leave diagnostic cellular features in the fossil record or lack diagnostic fossil lipid biomarkers. On the other hand, fossil DNA surveys are subject to several factors that could bias quantitative and qualitative (i.e. species composition) results. Reasons for why species are missed in PCR-based fossil DNA surveys (Coolen *et al.*, 2007) include

interspecies variability in the level of natural fragmentation of DNA (Boere *et al.*, unpubl.results), interspecies heterogeneity in small subunit rRNA operon copy numbers, and/or PCR primer efficiency and coverage (e.g. Von Wintzingerode *et al.*, 1997; Fogel *et al.*, 1999; Klappenbach *et al.*, 2001; Zhu *et al.*, 2005; LaJeunesse *et al.*, 2005; Potvin & Lovejoy, 2009).

The majority of the fossil DNA studies focused on the relatively recent past (Holocene), with the exception of the recovery of genuine past photic zone-dwelling cyanobacteria from Mediterranean evaporites, which were deposited during the late Miocene (Messinian) Salinity Crisis ~5.9 Ma ago (Panieri *et al.*, 2010). In addition, ~400-bp-long 16S rDNA fragments of ancient chemocline bacteria (i.e. obligate anoxygenic photolithoautotrophic green sulfur bacteria) were recovered from up to 217-kyr-old eastern Mediterranean sapropels (Coolen & Overmann, 2007).

However, studies showing the potential for preservation of fossil DNA from eukaryotic plankton members, which thrived in the pelagic ecosystem at geological time scales beyond the Holocene, are lacking. Eukaryotic 18S rDNA was detected in up to 1-Ma-old deep subsurface sediments of the Peru Margin, but whether this DNA was stemming from pelagic taxa remains unclear because the recovered 18S rDNA was not sequenced (Schippers & Neretin, 2006).

The upper few meters of eastern Mediterranean sediments offer a unique opportunity to study the Pleistocene pelagic eukaryote composition from DNA that has been exposed to varying (post)depositional conditions. These sediments contain organic carbon (C_{org})-rich sediment intervals (sapropels), which were formed as a result of orbitally forced changes in climatic and hydrological conditions leading to increased primary production, development of water column anoxia, and increased preservation of C_{org} in the anoxic bottom waters (Cramp & O'Sullivan, 1999; de Lange *et al.*, 2008; Emeis *et al.*, 2003; Negri *et al.*, 2009; Rohling, 1994) including the preservation of labile compounds such as DNA (Boere *et al.*, 2011; Coolen & Overmann, 2007). The sapropels are embedded in fully oxidized C_{org}-lean marls, which were formed after bottom water reventilation reestablished in the eastern Mediterranean Sea and presumably provided less optimal conditions for the preservation of DNA (Boere *et al.*, 2011).

Recently, we performed a comparative multiproxy survey (fossil DNA, calcareous nannofossils, and lipid biomarkers) to test whether preserved genetic signatures provide an accurate view of haptophyte and dinoflagellate populations during deposition of the Holocene Eastern Mediterranean sapropel S1 (Boere *et al.*, 2011). 18S rDNA of dinoflagellates and haptophytes could be recovered from the C_{org}-rich S1, but the most abundant phylotypes represented species that were not part of the calcareous nannofossil assemblage (Boere *et al.*, 2011) or dinocyst composition (Zonneveld *et al.*, 2001). Perhaps the most plausible reason for this discrepancy is the recent understanding that the majority of DNA in marine sediments is extracellular and no longer associated with living or intact cells (Dell'Anno & Danovaro, 2005), and that this pool of DNA was preserved despite the presence of microbial nucleases (Corinaldesi *et al.*, 2008, 2011). Extracellular DNA in marine sediments can be protected from degradation into small fragments by microbial nucleases for substantial periods when it is adsorbed to mineral and organic matrices (e.g. Lorenz & Wackernagel, 1987; Pietramellara *et al.*, 2009; Romanowski *et al.*, 1991). More specifically, recent DNA adsorption kinetics experiments revealed that circular prokaryotic DNA is more effectively adsorbed to clay minerals than eukaryotic linear DNA (Nguyen & Elimelech, 2007; Poly *et al.*, 2000). Therefore, the earlier findings that prokaryotic DNA can be preserved in sediment records older than the Holocene might not necessarily be true for DNA of eukaryotic plankton members.

Using the same core as analyzed by Boere *et al.* (2011), we extended the stratigraphic survey of ancient dinoflagellates and haptophyte algae and their relative abundance based on preserved partial 18S rDNA fragments in Pleistocene eastern Mediterranean sapropels [i.e. S3 (~30 ka), S4 (105 ka), and S5 (125 ka)] and their intercalating oxidized marls. Because many lipid biomarkers are generally thought to be more recalcitrant than DNA, we performed a parallel quantitative survey of preserved dinosterol and long-chain alkenones as an independent measure for the amount of biomass derived from respectively dinoflagellates and haptophytes of the order Isochrysidales (e.g. Marlowe *et al.*, 1984; Volkman, 2003). Because the location of the studied core was relatively close to the outlet of the Nile, we also determined the Branched and Isoprenoid Tetraethers (BIT) index (Hopmans *et al.*, 2004) as a measure for the relative amount of terrestrial (fluvial) vs. marine sources of organic matter (OM) including DNA. To our knowledge, this is the first study in which Pleistocene eukaryotic plankton members are both quantified and identified based on preserved genetic signatures, and in which the presence of their fossil DNA is validated via the parallel independent analysis of geochemical proxies.

EXPERIMENTAL

Setting and sampling

During the 2004 MIMES cruise on the R/V *Pelagia* as part of the MEDIFLUX project, a piston core (MS66PC) was recovered from near the deep-sea Nile fan, Eastern Mediterranean (location: 33N1.9′ 31E47.9′) at a water depth of 1630 m (Boere *et al.*, 2011; Fig. S1). The total length of the core was 6 m and included the sediment–water interface. A sediment section located between 137 and 174 cm was lost during sampling. Visual inspection showed that the core contained sapropels S1, S3, S4, and S5, an observation that was corroborated by $\delta^{18}O$ measurements on bulk carbonate.

Inside a closed container on board of the R/V *Pelagia*, the core was split in half and processed using aseptic conditions to avoid cross contamination: The upper one cm of exposed sediment from the working core half was scraped off using sterilized knives to reveal visibly undisturbed depositional laminations. Samples for DNA and lipid analyses were taken at 1 cm resolution from the sapropel intervals and at a lower resolution (i.e. 2 cm intervals every 10–20 cm) from the oxidized marls by pressing headless (ends cut) sterile plastic syringes into the sediments. Hereby, the top 1 cm of the sediment in each syringe was omitted, and a distance of 2 cm between the core liner and subsampled sediment was maintained while using this syringe sampling method to avoid cross contamination (Coolen *et al.*, 2009). Subsamples were transferred to sterile disposable 50-mL centrifuge tubes and stored frozen −40 °C until further analysis. Subsamples at 1 cm resolution were also obtained for inorganic geochemistry and stored at 4 °C.

DNA extraction

The extraction of DNA was performed in clean laboratories dedicated for ancient DNA work at the Royal Netherlands Institute for Sea Research (NIOZ) and at the Woods Hole Oceanographic Institution (WHOI). Extensive measures were taken to prevent contamination of samples and reagents used during DNA extraction with foreign DNA as described in detail previously (Boere *et al.*, 2011; Coolen *et al.*, 2009). At the Royal NIOZ, a total of 28 samples from depths spanning early, mid, and final deposition of sapropels S1 [six samples], S3 [4], S4 [5], and S5 [7] as well as the C_{org}-lean sediments between sapropels [6], were selected for fossil DNA and lipid analysis. Per sample depth, approximately 10–20 g wet sediment was available. The entire sample was defrosted, briefly homogenized by vortexing, and 5–10 g wet sediment was used for DNA extraction. The remaining sample fraction was refrozen and used for lipid biomarker analysis.

Fifteen additional samples from this core (S1 [3], S3 [1], S4 [2], S5 [3], and from the intercalating sediment marls [6]) were shipped to the ancient DNA-dedicated laboratory at WHOI for the qualitative and quantitative survey of the eukaryotic 18S rDNA pool. This served as an additional, independent validation of the data obtained at the NIOZ and to check for possible laboratory-specific contaminations.

Total DNA was extracted using the PowerMax™ Soil DNA isolation kit (MoBio laboratories, Carlsbad, CA, USA), according to the manufacturer's guidelines. The resulting DNA was concentrated with a cold ethanol precipitation, redissolved in 100 μL 1× sterile Tris-EDTA buffer (Ambion) and frozen at −80 °C until further use. The integrity and yield of DNA in the extracts were checked by gel electrophoresis (Fig. S2). Nucleic acid yield was quantified by fluorescence (PicoGreen; MoBiTec, Göttingen, Germany).

At both laboratories, extraction controls (EC) were subjected to the entire extraction procedure, with ultraclean PCR water (Sigma-Aldrich, St Louis, MO, USA) in place of sediment. Aliquots of those samples were subjected to PCR (see below) to monitor for contamination during the DNA extraction.

Quantitative Polymerase Chain Reaction (qPCR)

The amount of DNA (from eukaryotes in general, haptophytes, and dinoflagellates) in each sediment interval was determined by qPCR and expressed as number of 18S rDNA copies per nanogram of extracted DNA to normalize for possible variations in DNA extraction efficiencies between samples. All qPCRs were run on an iCycler IQ™ real-time PCR detection system (Bio-Rad, Hercules, CA, USA) at the NIOZ or a Realplex qPCR cycler (Eppendorf, Westbury, NY, USA) at WHOI, using a SYBR®Green I (Invitrogen, Carlsbad, CA, USA) assay. Each reaction contained the following reagents and concentrations: 5 μL 1× Picomaxx buffer and 2.5 units Picomaxx enzyme (Stratagene, LaJolla, CA, USA), 0.25 mM of each nucleotide, 50 μg bovin serum albumin, 1 mM $MgCl_2$, 1× SYBR®Green I, 10 nM fluorescein, and 0.2 μM final concentration of each of the primers listed in Table S1, and Ultra Clean PCR water (Sigma-Aldrich) added to 50 μL. The PCR ingredients used at WHOI differed slightly in that 4.5 mM $MgCl_2$, 0.5× SYBR®Green and no fluorescein were used. Three different primer sets were used for targeting similar sized (400–500 bp long) 18S rDNA fragments of general eukaryotes, haptophytes, and dinoflagellates, respectively (Table S1).

All PCR programs contained an initial denaturing step at 96 °C for 4 min, followed by 32 or 40 cycles of denaturing (94 °C, 40 s), 40 s of annealing at varying temperatures (Table S1), elongation (72 °C, 60 s), and imaging of newly formed SYBR®Green-stained dsDNA (80 °C, 20 s). A final elongation step (72 °C, 10 min) was added for the quantification reaction. All reactions were stopped within the exponential phase that was achieved after 32 or after 40 cycles depending on the amount of initial template in each sample. Amplification was followed by a melting curve analysis, in which fluorescence in each sample was measured during a stepwise increase of the temperature from 60 °C to 96 °C in 0.5 °C temperature increments. Between 10^0 and 10^7 copies of full-length 18S rDNA of *Emiliania huxleyi* was used as a standard series to quantify the number of general eukaryote 18S rDNA and haptophyte 18S rDNA copies in our samples. A standard series from 10^0 to 10^7 copies of full-length rDNA of *Scrippsiella* sp. served to quantify the fossil dinoflagellate 18S rDNA. PCR efficiencies were found to be >92%, and the quality and length of the produced PCR amplicons was checked by standard agarose gel electrophoresis.

Prior to these qPCR assays, each DNA extract was checked for the presence of PCR-inhibiting impurities, which would

otherwise have lowered the efficiency and accuracy of the qPCRs (e.g. Coolen *et al.*, 2009). For this, 50-μL PCRs were assayed with 1 μL of sample DNA plus 10^6 copies of full-length *E. coli* 16S rDNA and general bacterial primers. The addition of *E. coli* 16S rDNA was performed in a separate general-use laboratory because PCR products with high copy number template DNA are not allowed in both ancient DNA laboratories. The threshold cycles of each reaction with DNA of an individual sample were then compared to a control reaction that only contained *E. coli* 16S rDNA. This assay revealed slightly delayed threshold cycles indicative of minor inhibition for some samples. In these cases, PCR inhibition could be completely eliminated by using 5–10 times diluted template DNA in the qPCR assays (Coolen *et al.*, 2009).

Denaturing gradient gel electrophoresis (DGGE) and sequencing of bands

To generate template for subsequent DGGE analysis of the samples analyzed at the NIOZ, 1 μL of the qPCRs from the samples and controls served as template for a second round of PCR (two-step PCR approach) with 15 amplification cycles and fresh PCR reagents (20 μL reaction mixtures). These reactions contained the same forward primers, but with modified reverse primers to which a GC-clamp, i.e. a 40-bp-long GC-rich tag was attached (called 'GC-primer' from now on). The addition of a GC-clamp prevents complete dissociation of the two strands and loss of amplicons during DGGE analysis (Muyzer *et al.*, 1993).

This two-step approach served also as a test for the specificity of the qPCRs because the DGGE bands generated during the second round of PCR were subsequently sequenced. In addition, all field samples and controls analyzed at the NIOZ were directly amplified with GC primers (i.e. one-step PCR approach) with a total of 42 amplification cycles, and the diversity of the sequenced DGGE bands was compared with the results obtained via the two-step PCR approach. The direct use of GC primers generally results in reduced PCR efficiencies (85% or less) and was therefore avoided for qPCR purposes. The samples analyzed at WHOI were only analyzed using this one-step PCR approach. For the PCRs with GC primers, the final extension step was prolonged to 30 min following Janse *et al.* (2004).

After checking the quality of PCR products on agarose gel, ~100 ng DNA of each reaction was run on DGGE. The polyacrylamide gels (6%, wt/vol) contained a denaturing gradient of 20–50% (where 100% denaturing reagent equals 7 M urea and 40% formamide) for eukaryotes and haptophytes. For dinoflagellates, a gradient of 20–60% was used. Gels were run for 5 h at 200 V (12.5 V cm^{-1}) in 1× TAE buffer at a temperature of 60 °C. Subsequently, the gel was stained for 30 min with 1× SYBR Gold (Molecular Probes, Eugene, OR, USA), rinsed with Milli-Q, and visualized using a Dark Reader (Clare Chemical Research Inc., Dolores, CO, USA). Bands melting at unique positions in the gel were excised using flame-sterilized scalpels, and the DNA in each band was eluted in 50 μL 1× TE-buffer for 24 h. In addition, bands that melted at identical vertical positions in the gel were analyzed to assess intra-band sequence diversity. Subsequently, the bands were re-amplified using the original primer set and purified using the Genscript QuickClean PCR purification kit (Genscript, Piscataway, NJ, USA). Purified PCR products were sent to Macrogen Inc. (Seoul, Korea) for standard Sanger sequencing.

Phylogenetic analyses

The phylogenetic affinity of recovered sequences was initially identified by BLAST searches using the NCBI website (http://www.ncbi.nlm.nih.gov/). Sequences were subsequently imported into the SILVA database (SSURef 93; (Pruesse *et al.*, 2007) using the ARB software package (version December 2007; Ludwig *et al.*, 2004)). The sequences were aligned to the SILVA SSU reference alignment using the FastAligner tool in ARB. The alignment was checked and refined manually, and sequenced DGGE bands were grouped into operational taxonomic units or phylotypes based on 98% sequence similarity cutoff value using the program DOTUR (Schloss & Handelsman, 2005). Phylogenetic analyses included distance matrix and maximum likelihood approaches applying several position variability filters. Finally, a Bayesian phylogenetic inference analysis was performed on all recovered haptophyte and dinoflagellate phylotypes and on closely related relatives. This analysis was performed in the program MRBAYES (version 3.1.2, http://mrbayes.csit.fsu.edu) using two metropolis-coupled Markov chains, each comprising four chains, which were run for 1 000 000 generations. Trees were sampled every 1000th generation. The first 100 trees (10%) were discarded as burn-in, and the remaining 900 trees were assembled into a consensus tree.

Sequences recovered at Royal NIOZ with general euk aryote primers are deposited in NCBI under accession numbers FJ785836-FJ785859 (archaea), FJ785860-FJ785896 (fungi), and FJ785897-FJ785984 (remaining part of sequences obtained by the general eukaryotic assay). The haptophyte- and dinoflagellate-specific sequences are deposited under accession numbers: FJ796994-FJ797011 and FJ797012-FJ797032, respectively. Sequences obtained by the general eukaryotic assay at WHOI were deposited under accession numbers FJ834307-FJ834324.

Total organic carbon, elemental composition, and lipid geochemistry

The inorganic bulk elemental composition was analyzed from freeze-dried sediment by inductively coupled plasma-atomic emission spectrometry (ICP-AES) after the three-step digestion to ensure total dissolution as described previously

(Reitz *et al.*, 2006). Organic carbon and $CaCO_3$ contents were measured on a Fisons Instruments CNS NA analyzer using dry combustion at 1030 °C (Reitz *et al.*, 2006).

Parallel lipid analyses were performed on the samples that were analyzed for fossil DNA at the Royal NIOZ. Sediments (~10 g) were freeze-dried and ultrasonically extracted five times with dichloromethane/methanol (DCM/MeOH; 2:1, v/v). The solvent was removed by rotary evaporation under vacuum. The extracts were methylated with diazomethane and separated by column chromatography on Al_2O_3 into apolar (containing alkenones) and polar fractions using respectively DCM and DCM/MeOH (1:1, v/v) as eluents. An internal standard (6,6-d2-3-methyl-eicosane) was added to the apolar fractions prior to gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS) analyses. The same internal standard (6,6-d2-3-methyl-eicosane) was added to half of the polar fraction, and this total lipid fraction was subsequently silylated with bis(trimethyl)trifluoracetamide at 60 °C for 20 min and analyzed by GC and GC/MS.

To quantify the biomarker concentrations, GC analyses were performed using a Hewlett-Packard 5890 instrument equipped with a flame ionization detector and an on-column injector. A fused silica capillary column (25 m × 0.32 mm i.d.) coated with CP-Sil 5 (film thickness 0.12 μm) was used with helium as carrier gas. The oven was programmed at a starting (injection) temperature of 70 °C and programmed to 130 °C at 20 °C/min and then to 320 °C at 4 °C/min, at which it remained for 10 min. The different fractions were analyzed by GC/MS using a Finnigan Trace GC Ultra coupled to a Finnigan Trace DSQ mass spectrometer. GC conditions and column were as described above. The column was directly inserted into the electron impact ion source of the DSQ quadrupole mass spectrometer, scanning a mass range of m/z 50-800 at three scans per second and an ionization energy of 70 eV.

The Branched and Isoprenoid Tetraether (BIT) index was measured by dissolving the other half of the polar fractions in hexane/n-propanol (99:1, v/v) to a concentration of 2 mg mL^{-1} and filtering over a 0.45-μm, 4-mm-diameter PTFE filter. This was analyzed using high-performance liquid chromatography/atmospheric pressure positive ion chemical ionization mass spectrometry (HPLC/APCI-MS) as described by Hopmans *et al.* (2004).

RESULTS

Elemental composition, sedimentary organic carbon, and DNA content

The location of the sapropels in the core was determined based on the organic carbon (C_{org}) content and the ratio between the elements barium and aluminum (Ba/Al ratio) (Fig. 1A). The C_{org} content was highest (4%) in the deepest sapropel S5, and ~2 wt% in sapropels S1, S3, and S4. C_{org} was <0.4 wt% in the intercalating marls (Fig. 1A). The Ba/Al ratio was most elevated in the S5 (ca. 200) and in the younger sapropels (ca. 100–150), with values of ~30–40 in the intercalating marls (Fig. 1A). A few cm above the C_{org}-rich sapropels S3, and especially above S1, the Ba/Al ratio remains high, which is indicative of postdepositional reoxidation of the upper few cm of these sapropels (de Lange *et al.*, 2008). Postdepositional re-oxidation was not apparent for S4 and S5 sapropels, but a temporary decrease in C_{org} content and Ba/Al ratio can be observed during mid S4 and S5 deposition, which has been interpreted as an interruption of sapropel deposition (de Rijk *et al.*, 1999).

DNA concentrations were highest in the S1 interval (8–40 μg g^{-1} C_{org}) and between 0.5 and 8 μg g^{-1} C_{org} in the Pleistocene sapropels. DNA concentrations were generally below the detection limit with the picogreen method (<0.1 μg g^{-1} C_{org}) in the majority of the analyzed intercalating marls with the exception of one layer at ~100 cm (between the S1 and S3) and at ~500 cm (between the S4b and S5) (Fig. 1D). Agarose gel electrophoresis revealed that the majority of the bulk sediment DNA was of high molecular weight even in the oldest sapropels with a smear of fragmented DNA down to 200 bp or less, whereas the intercalating marls and extraction control did not yield visible amounts of DNA on the gel (Fig. S2).

Quantitative analysis of eukaryotic DNA in the Holocene to late-Pleistocene sediments

Eukaryotic 18S rDNA was quantified throughout the Pleistocene eastern Mediterranean sediments by qPCR with a general eukaryote primer set without GC-clamp (Table S1). All samples were analyzed in duplicate and yielded reproducible results. The abundance of eukaryotic 18S rDNA was highest in all four sapropels and varied only up to one order of magnitude between the analyzed intervals (5×10^6–3×10^7 copies μg^{-1}) (Fig. 1E). Similar amounts of eukaryotic 18S rDNA were measured in the two samples from the intercalating marl between S1 and S3, whereas eukaryotic 18S rDNA copy numbers were 1–2 orders of magnitude lower in intercalating marls just above or below the S4 and S5 (Fig. 1E).

In addition, we specifically quantified the amount of preserved partial 18S rDNA of dinoflagellates and haptophyte algae throughout the late-Pleistocene sediment record (Fig. 1F,G). Dinoflagellate 18S rDNA comprised ~10% of the total eukaryote 18S rDNA (maximum 8×10^5 copies μg^{-1} extracted DNA) in the S1 with 20-fold lower concentration just below the S1 (Fig. 1F). In the Pleistocene intervals, correctly sized dinoflagellate 18S rDNA amplicons were only found in two horizons: in sapropel S3 at 378 cmbsf and in sapropel S5 at 530 cmbsf at concentrations up to two orders of magnitude lower than in the S1 (Fig. 1F). The dinoflagellate 18S rDNA content in the S3 at 378 cm was just above

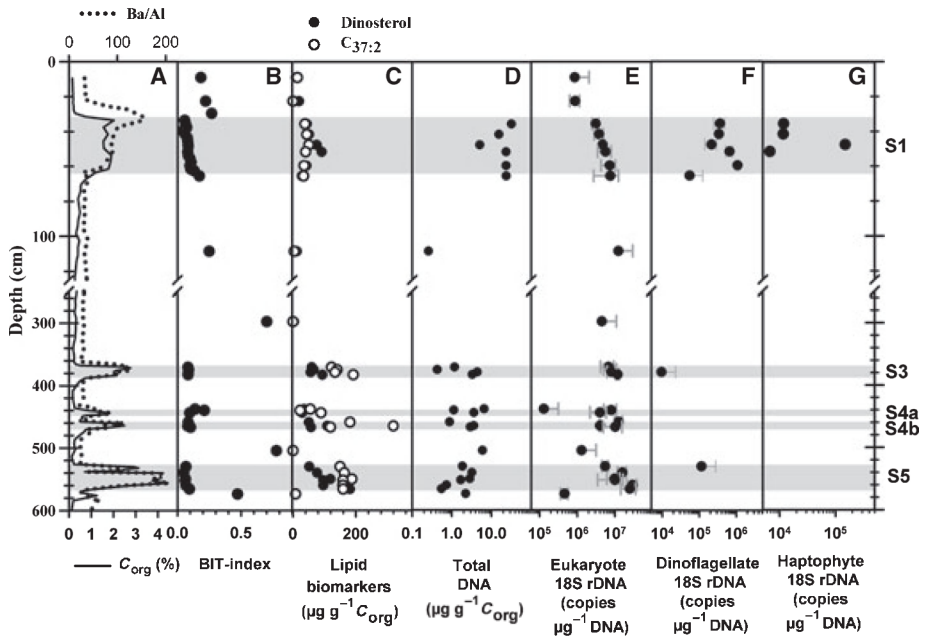


Fig. 1 Geochemical, lipid biomarker, and DNA profiles of the investigated core (MS66PC). Gray-shaded areas mark the vertical positions of the four sapropels (S1, S3, S4a+b, and S5). (A) Organic carbon content (C_{org}; wt %) and Ba/Al ratio. The core was analyzed at 1-cm interval resolution for these parameters. (B) Branched and Isoprenoid Tetraethers (BIT) index. The same samples that were analyzed for lipids and DNA were used for the BIT index, except for sapropel S1, which was analyzed at higher resolution. (C) Concentrations of selected lipid biomarkers for dinoflagellates (dinosterol) vs. haptophytes ($C_{37:2}$ methyl ketone). (D) Total extracted DNA concentration in microgram per gram organic carbon. (E, F, G) 18S rDNA copy numbers as revealed by qPCR with (E) general eukaryotic primers Euk1f/Euk516R, (F) a dinoflagellate-specific primer set Euk1f/DinoRev, and (G) a haptophyte-specific primer set Prym448/Prym884. The qPCR data were expressed as copy numbers per microgram extracted DNA to normalize for possible variations in the DNA extraction efficiencies between samples. Error bars represent the standard deviation of two replicates. The break in the depth axis represents the sediment interval 125–250 cm below sea floor.

the detection limit ($\sim 5 \times 10^3$ copies μg^{-1} extracted DNA). QPCR using haptophyte-specific primers revealed that ~400-bp-long haptophyte 18S rDNA fragments represented 1% or less (up to 2×10^5 copies μg^{-1} extracted DNA) of the total number of eukaryotic 18S rDNA copies in the S1 (Fig. 1G). These quantitative results were not reproducible, suggesting that these values were close to the detection limit. Despite the low copy numbers, reamplification of the qPCR with GC primers for DGGE and subsequent phylogenetic analysis of sequenced DGGE bands yielded unambiguous haptophyte sequences as outlined below. Haptophyte DNA was below the detection limit $<10^3$ copies μg^{-1} extracted DNA in the Pleistocene sediments.

Eukaryotic phylotypes recovered from the Holocene to late-Pleistocene sediments

The amplicons from the qPCR assays were reamplified with GC primers (Table S1) and subjected to DGGE. Excised DGGE bands were subsequently sequenced for phylogenetic analysis. The recovered phylotypes were, where possible, clustered at higher taxonomic levels, and the number of phylotypes per higher level was determined per sediment interval (either sapropel or intercalating sediment interval). Table 1 provides an overview of the distribution of these higher taxonomic groups per depth interval. The closest BLAST hits, including accession number and sequence similarity (%), of recovered phylotypes are shown in the more detailed overview Table 2. Bands melting at identical positions in the gel were shown to contain identical sequences and therefore no intra-band sequence diversity.

The majority of eukaryotic phylotypes in the S1 represented marine protists of which Alveolata (a superphylum comprising dinoflagellates, ciliates, apicomplexa, and 'unclassified alveolata') represented the largest group. Other protists included cercozoa, chlorophytes (green algae) related to *Nannochloris*, and a stramenopile belonging to the MAST-12 cluster (cf. clone DSGM-40 (Takishita *et al.*, 2005, 2007); Table 1). One phylotypes ('unknown affiliation', Table 1) is related to the uncultured marine clone CCI73 (AY179972), which was isolated from an anoxic water–sediment interface and is currently without known close relatives (Stoeck & Epstein, 2003).

Besides protist DNA, metazoan phylotypes were found in sapropel S1, most notably zooplankton (copepods; Euk-22 and Euk-26) and a phylotype related to acorn worms (*Enteropneusta*; Euk-17) (Table 2). Two additional phylotypes recovered from sapropel S1 were related to marine fungi. One of these (cf. *Pichia guilliermondii*; FUNG-1) was found throughout the core (outlined below), whereas a fungal phyl-

Table 1 Number of specific eukaryote phylotypes in the various sections of the investigated core

Phylogenetic affiliation		i.s.	S1	i.s.	S3	i.s.	S4	i.s.	S5	EC	ntc
Protista											
Alveolata	Dinophyceae		5	1					1		
	Apicomplexa		1								
	Unc. Alveolata		5				1				
Haptophyta	Isochrysidales			1	1						
Rhizaria	Cercozoa		6								
	Radiolaria			1	1						
Stramenopiles	MAST-12		1								
Unikonta	Amoebozoa								1		
Novel basal lineage			1								
Metazoa											
Arthropoda	Arachnida				1		1				
	Copepoda		2								
	Insecta*										1
Chordata	Mammalia*				1				2		1
Hemichordata	*Enteropneusta*		1								
Chloroplastida	Chlorophytes		1								
	Land plants				1	2	4		5		
Fungi	Ascomycota	1	2		1		3	1	2	1	
	Basidiomycota								2		
Archaea	Unc. Archaea		2		2	1	3	1	2		

The first column shows higher taxonomic grouping nomenclature. The number of phylotypes found per higher taxonomic group is shown for each sediment interval. Sapropel layers are shown in shaded columns. Phylotypes that were found in nontemplate controls are marked with an asterisk (*).
Unc., uncultured; Sap, sapropel; i.s., Nonsapropelic intercalating sediment; e.c., extraction control; ntc, nontemplate control. Compare Table 2 for more detailed phylogenetic information.

otype (FUNG-4) related to *Metschnikowia sp.* (Ascomycetes) was found only in S1.

Preserved partial 18S rDNA of marine protists could only occasionally be recovered from the Pleistocene sediments: Two phylotypes (EUK-24 and EUK-42) distantly related to named dinoflagellate species (Fig. 2A) were recovered from the intercalating marl between S1 and S3 as well as from S5, but not from S1 (Table 2). S4 harbored a single sequence (Euk-32) related to uncultured alveolates from the photic zone of the Sargasso Sea (AY664058; Fig. 2A, Tables 1 and 2) (Armbrust *et al.*, 2008). A sequence (Euk-23) with 99% sequence similarity to the alkenone-producing coccolithophorid *E. huxleyi* (Fig. 2B) was recovered from the intercalating marl between S1 and S3 as well as in S3 (Tables 1 and 2). The latter sediments also revealed two sequences related to siliceous nannoplanktonic radiolarian species (Euk-13 and Euk-20; Tables 1 and 2). Furthermore, the S5 contained DNA of an unassigned amoebozoan (EUK-25).

In contrast, marine fungi, in particular *P. guilliermondii* (Table 1), were consistently present throughout the core below S1. Marine fungi related to *Rhodosporidium* and *Rhodotorula* were only found in sapropel S5 (FUNG-2 and FUNG-7). S4 contained fungal sequences related to uncultured Trichocomaceae (FUNG-5) and *Acremonium* (FUNG-6) (Table 2). Besides marine fungi, the numerically most abundant phylotypes (13 phylotypes) that were recovered from below the sapropel S1 were related to a wide variety of land plants (Table 2).

Controls for monitoring contaminations during sampling and handling

To monitor possible contamination of the samples with foreign DNA, a set of blanks and controls were processed in parallel to the samples. Extraction controls (ECs) were subjected to the same DNA extraction protocol as the samples were, but the sediment replaced with PCR water. In addition, during PCR preparation, nontemplate controls (NTCs) containing all PCR ingredients except template DNA were run alongside the samples. To check for laboratory-specific contamination, another selection of 15 pristine samples was independently extracted and analyzed at the WHOI laboratory using a similar qPCR-DGGE-sequencing approach.

The ECs that were subject to the two-step PCR approach at the NIOZ revealed the presence of archaeal 16S rDNA (ARCH-3), whereas insect and human DNA were occasionally recovered from the NTCs (Table 2). The archaeal and human sequences were also recovered from some of the investigated sediment samples and regarded as contamination. In addition, one of the extraction controls yielded the fungal phylotype related to *P. guilliermondii* which was also recovered from the sedimentary DNA extracts. Because this same fungal sequence was never found in the NTCs, the EC was likely contaminated with a sample during the extraction procedure.

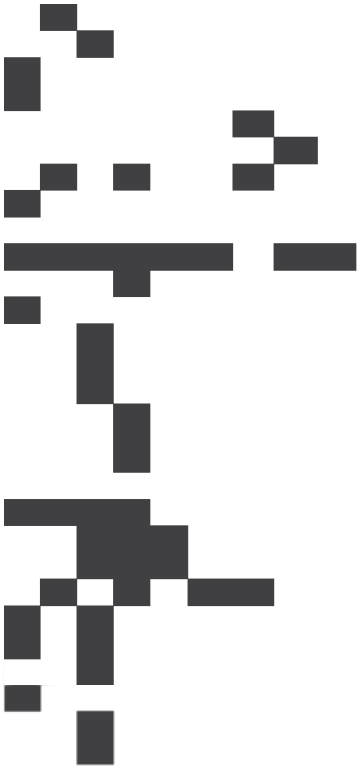
The parallel analysis at WHOI with a different set of samples of S1–S5 and intercalating sediment intervals from core MS66PC revealed a comparable pattern in preserved eukaryotic DNA with sometimes 100% sequence similarity to phylotypes found in the samples analyzed at the NIOZ. The one-step PCR assay with general eukaryotic primers yielded 14 phylotypes. Five of these phylotypes were related to marine protists and were related or identical to sequences found at the Royal NIOZ (Table 2). The phylotypes from sediments analyzed at WHOI older than the S1 contained the same fungal phylotype (*P. guilliermondii*) found in the sediments analyzed at Royal NIOZ, or sequences from land plants. *P. guilliermondii* was the only sequence recovered from the intercalating C_{org}-poor marls, and nonspecific amplification of archaeal 16S rDNA was not apparent from the one-step PCR approach directly with GC primers at WHOI (Table 2). The ECs analyzed during the one-step PCR approach at WHOI remained negative, but one of the NTCs showed a *Picea*-related plant sequence (Table 2). For reasons discussed below, phylotype Euk-12 with identical sequence to *Zea*

Table 2 Phylogenetic affinity of recovered phylotypes using general eukaryote primers

Phylogenetic affiliation		OTU	Closest BLAST hit	NCBI Acc.No.	Similarity (%)	No. of clones	NIOZ S1	NIOZ S3	NIOZ S4	NIOZ S5	NIOZ i.s.	NIOZ e.c.	NIOZ ntc	WHOI sap	WHOI i.s.	WHOI e.c.	WHOI ntc
Protista																	
Alveolata	Apicomplexa	EUK-30	Unc. eukaryote clone DSGM-13	AB275013	96	1	■										
	Dinophyceae	EUK-1	Heterocapsaceae env sample clone Elev_18S_1214	EF024723	92	5	■							■			
		EUK-10	*Lepidodinium viride*	DQ499645	94	2	■										
		EUK-18	Unc. eukaryote clone SCM16C28	AY664886	87	1	■										
		EUK-24	Unc. marine eukaryote clone cLA11H01	EU446372	83	1					■						
		EUK-39	Unc. eukaryote DGGE band Euk-8	DQ234288	100	2	■							■			
		EUK-40	*Lepidodinium viride*	DQ499645	93	3	■										
		EUK-42	Unc. marine eukaryote clone e1-34	EU078276	96	1				■							
	Unc. Alveolata	EUK-5	Unc. marine eukaryote clone UEPAC05ap2	AY129031	97	3	■										
		EUK-7	Unc. marine eukaryote clone NS51B242	AJ829831	98	8	■										
		EUK-8	Unc. eukaryote clone DSGM-23	AB275023	99	2	■										
		EUK-9	Unc. eukaryote clone DSGM-23	AB275023	97	2	■										
		EUK-32	Unc. marine eukaryote clone SCM28c137	AY664058	99	1			■								
		EUK-38	Haplosporidian parasite	AY449715	90	1	■										
Chlorophyta	Prasinophyceae	EUK-29	*Nannochloris* sp. MBIC10749	AJ402345	98	1	■										
		Euk 13 mc	*Nannochloris* sp. ANR-9	AY220081	99	1								■			
Haptophyta	Isochrysidales	EUK-23	*Emiliania huxleyi*	AF184167	99	2		■			■						
Rhizaria	Cercozoa	EUK-2	Unc. eukaryote DGGE band Euk-2	DQ234282	100	7	■							■			
		EUK-3	Unc. marine eukaryote clone e4-41	EU078309	96	5	■										
		EUK-19	Unc. eukaryote DGGE band Euk-4	DQ234284	98	1	■										
		EUK-41	Unc. eukaryote clone DSGM-53	AB275053	98	1	■							■			
		EUK-43	Unc. marine eukaryote clone NAMAKO-16	AB252756	99	1	■										
		EUK-44	Unc. eukaryote clone DSGM-53	AB275053	96	1											
	Radiolaria	EUK-13	Unc. marine eukaryote clone SSRPB05	EF172840	98	1				■							
		EUK-20	Unc. Polycystinea clone OLI011-75m.62	EU287798	90	1		■									
Stramenopiles	MAST-12	EUK-35	Unc. marine eukaryote clone DSGM-40	AB275040	98	1	■										
Unikonta	Amoebozoa	EUK-25	*Hartmannella vermiformis* clone 4391	DQ084364	100	1				■							
Unknown affinity		EUK-15	Unc. marine eukaryote clone CCI73	AY179972	86	1	■										
Embryophytes																	
(Land plants)		EUK-4	Unc. eukaryote clone Elev 7261	EF025039	99	3		■	■	■							
		EUK-11	Unc. streptophyte clone UF-89	AY496507	99	1				■							
		EUK-12	*Zea mays*	AF168884	100	1					■						
		EUK-14	*Albizia julibrissin*	U42536	99	1				■							
		EUK-16	*Poncirus trifoliata*	AF206997	99	1				■							
		EUK-27	*Allium thunbergii*	AF168825	99	1			■								
		EUK-28	*Musa basjoo*	EF376005	99	1			■					■			
		EUK-31	*Lygodium japonicum*	AB001538	99	1					■						
		EUK-36	*Cucumis sativus*	AF206894	98	3				■							
		EUK-37	Unc. eukaryote clone S30	EU326607	99	4			■								
		Euk 02 mc	*Picea morrisonicola* *	AB026939	100	1								■			■
		Euk 18 mc	*Sassafras albidum*	U52031	99	1								■			
		Euk 16 mc	*Lolium multiflora*	AY846367	99	1								■			
		Euk 14 mc	Unc. eukaryote clone 1	EU326599	100	1								■			

Table 2 (Continued)

Phylogenetic affiliation		OTU	Closest BLAST hit	NCBI Acc.No.	Similarity (%)	No. of clones	NIOZ S1	NIOZ S3	NIOZ S4	NIOZ S5	NIOZ i.s.	NIOZ e.c.	NIOZ ntc	WHOI sap	WHOI i.s.	WHOI e.c.	WHOI ntc
Metazoa																	
Arthropoda	Arachnida	EUK-34	*Sitticus palustris*	DQ665749	97	1											
		EUK-21	*Schwiebea pseudotsuqae*	EU152499	97	1											
	Copepoda	EUK-22	*Drepanopus forcipatus*	AF462321	99	1											
		EUK-26	*Haloptilus ocellatus*	AY118069	96	2											
	Insecta	EUK-33	*Discoderus cordicollis**	AF012472	98	1											
		Euk 07 mc	*Laphystia tolandi*	EF650144	95	1											
Chordata	Mammalia	EUK-6	*Homo sapiens**	EU647001	99	6											
Hemichordata	Enteropneusta	EUK-17	*Saccoglossus pusillus*	AF236800	90	1											
Fungi																	
Ascomycota		FUNG-1	*Pichia guilliermondii*	EU784644	99	29											
		FUNG-3	*Lewia infectoria*	U43465	99	2											
		FUNG-4	*Metschnikowia*	AB023475	95	1											
		FUNG-5	Unc. Trichocomaceae clone 8-2-5	EU085016	99	1											
		FUNG-6	*Acremonium* sp CSSF-1	AB167384	99	1											
Basidiomycota		FUNG-2	*Rhodosporidium azoricum*	AB073269	99	3											
		FUNG-7	*Rhodotorula marina*	AB126645	99	1											
Archaea																	
		ARCH-1	Unc archaeon clone 280NJ33E	AM072592	87	9											
		ARCH-2	Unc euryarchaeote clone 1aD3	AY800210	90	6											
		ARCH-3	Unc archaeon clone 280NJ33E	AM072592	88	4											
		ARCH-4	Unc archaeon clone MD2896-A116	EU385665	92	2											
		ARCH-5	Unc archaeon clone 280NJ34E	AM072593	96	1											
		ARCH-6	Unc archaeon clone 280NJ34E	AM072593	92	1											
		ARCH-7	Unc euryarchaeote clone 1aD3	AY800210	92	1											



Shown are the higher taxonomic grouping, name of the recovered phylotype (OTU: operational taxonomic unit, based on 98% similarity), closest BLAST hits (names, accession numbers, and similarity %). The number of clones refers to the number of sequenced DGGE bands that were placed within that phylotype. Presence within the sapropel layers (S1 through S5) or the intercalating marls is indicated with black cells. Sequences found at WHOI are shown in separate columns. Phylotypes that were found in nontemplate controls are marked with an asterisk (*).
Unc., uncultured; Sap, sapropel; i.s., Nonsapropelic intercalating sediment; e.c., extraction control; ntc, nontemplate control.

mays, which was only recovered from the intercalating marl between S3 and S4, represents a likely contamination even though this sequence was not recovered from the control PCRs.

Dinoflagellate and haptophyte-related phylotypes

Besides using general eukaryote primers to identify the most abundant eukaryote phylotypes present, we also used group-specific primers to target only partial 18S rDNA of past dinoflagellates and haptophytes. These more specific primers lowered the detection limit for their 18S rDNA and resulted in the identification of additional phylotypes (Fig. 2).

The use of dinoflagellate-specific primers resulted in the identification of six phylotypes within sapropel S1 (Fig. 2). Only one sequence (MS_Dino1_01) was similar to a phylotype identified by the general eukaryote PCR assay (EUK-39). The most closely related sequence was also recovered from anoxic Black Sea sediments (DQ234288; Coolen *et al.*, 2006) with the genus *Pentapharsodinium* as closest cultured relatives. The other five dinoflagellate phylotypes were not detected with the general eukaryote PCR assay. These additionally retrieved sequences all cluster within the dinoflagellate crown group, whereas the phylotypes from the general eukaryote PCR assay cluster with the more basal dinoflagellates (i.e. Syndiniales; Guillou *et al.*, 2008).

The haptophyte-specific PCR assay resulted in the identification of five phylotypes within S1 that were missed with the general PCR assay (Fig. 2B). The only phylotype that was recovered by both assays (EUK_23, MS_Hap1_02; Fig. 2B) was related to the marine alkenone-producing and calcifying haptophyte *E. huxleyi*. Another sequence of interest is MS_Hap1_01 with 99.5% sequence similarity to uncultivated Prymnesiophyte clones that, according to RNA stable isotopic probing (RNA-SIP) experiments, were capable of mixotrophic growth as predators of picocyanobacteria (Frias-Lopez *et al.*, 2009).

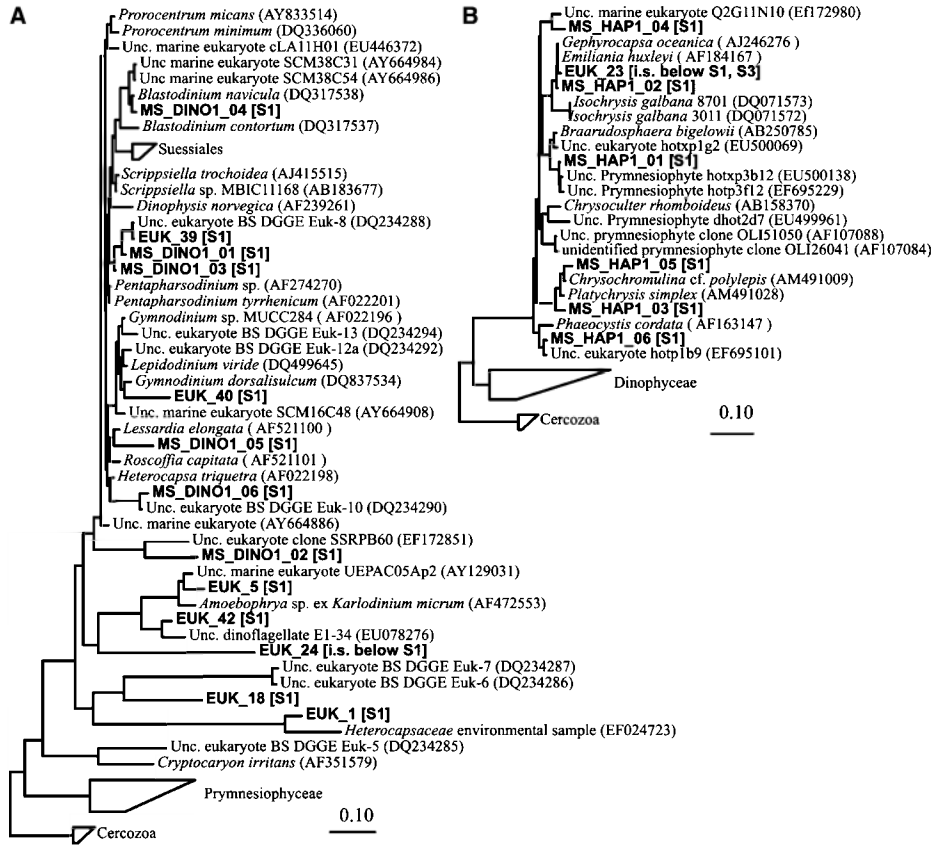


Fig. 2 Bayesian inferred phylogenetic trees showing the relationship between fossil 18S rDNA sequences found using group-specific PCR assays for haptophytes 'HAP' and dinoflagellates 'DINO', and sequences related to these taxonomic groups recovered with the general eukaryote PCR assay ('EUK'). Both trees were obtained in one single MrBayes run, and the subtrees of interest are shown separately. (A) Subtree with dinoflagellate sequences. (B) Subtree with haptophyte sequences. Phylotypes found in this study are shown in bold face. The sediments layers in which the unique phylotypes were found are indicated in square brackets, e.g. [S1]. Abbreviation: unc. (uncultured); i.s. (intercalating, C_{org}-depleted sediment).

Biomarker lipids

A parallel quantitative analysis of the sterol dinosterol (4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol) and long-chain alkenones was performed as a measure for the amount of biomass derived from dinoflagellates (Volkman, 2003 and references therein) vs. haptophytes (Marlowe *et al.*, 1984) of the order Isochrysidales (most notably *E. huxleyi*). A comparable quantitative profile of DNA would be expected unless the fossil plankton DNA underwent extensive degradation with increasing sediment depth and age.

In general, the dinosterol and alkenone concentrations varied between 50 and 200 μg g^{-1} C_{org} in the sapropels, with lowest concentrations in S1 (i.e. ~50 μg g^{-1} C_{org}). Alkenone concentrations exceeded dinosterol concentrations in most samples, except in S1, where concentrations of both biomarkers were comparable (Fig. 1C). Lower, but still detectable concentrations were present in the intercalating sediments (<10 μg g^{-1} C_{org}; Fig. 1C). For clarity, only the concentration profile of the $C_{37:2}$ alkenone is shown in Fig. 1C to compare with the haptophyte 18S rDNA data (Fig. 1F). Additional detected alkenones (i.e. $C_{37:3}$ and $C_{38:2}$) revealed a similar downcore trend.

To estimate the contribution of continental OM in our core, we determined the ratio between soil-derived branched tetraether lipids and marine-derived crenarchaeol (i.e. BIT index; [Hopmans *et al.*, 2004;]). This BIT- index was low (<0.1–0.2) within all sapropel layers, indicating that most of the sedimentary OM was of marine (pelagic) origin and that the terrestrial input into the Mediterranean was relatively limited during these intervals. Within sapropel S1, a slight decrease in BIT-index values from the bottom of the sapropel to the top was found, possibly reflecting the increased terrestrial run-off at the onset of sapropel formation. Intercalating marls occasionally showed high BIT-index values (up to 0.8 around 500 cm depth, Fig. 1B).

DISCUSSION

Eukaryotic DNA in the Holocene eastern Mediterranean S1

The presence of detectable amounts of preserved fossil ~500-bp-long partial 18S rDNA fragments from organisms stemming from the ancient water column in core MS66PC

was mainly restricted to the early Holocene sapropel S1. For example, the use of general primers resulted in the detection of photoautotrophic protists such as the chlorophyte *Nannochloris* sp. (class *Prasinophyceae*). Such protists must originate from the ancient photic zone because they required light for photosynthesis, which is absent from the dark anoxic sediments (Coolen & Overmann, 1998; Teske, 2007). The S1 sediments also contained fossil DNA of metazoans such as calanoid copepods (i.e. zooplankton). Although copepods can live under extremely low oxygen conditions (Auel & Verheye, 2007; Wishner *et al.*, 2008), these species are unambiguously of pelagic origin. Copepods could serve as sensitive recorders of past salinity, temperature regimes, or change in currents, but they have been excluded from paleoenvironmental studies because their preserved morphological remains, merely resting eggs or cuticles, are often difficult to distinguish. However, fossil copepod DNA has recently been used to identify cryptic species in Holocene Antarctic lake sediments (Bissett *et al.*, 2005) and the Black Sea (Coolen & Shtereva, 2009) and offers potential to include copepods in future paleoenvironmental studies. A single occurrence of preserved DNA (Euk-17) from a benthic worm (*Enteropneusta*; Cameron *et al.*, 2000), which cannot be identified microscopically because of the lack of fossilizing remains, is supportive for limited bioturbation that occurred during S1 deposition (Basso *et al.*, 2004; Löwemark *et al.*, 2006).

The majority of the recovered sequences from the S1 belong to the superphylum Alveolata (Tables 1 and 2). Many species of the Alveolata have complex lifestyles and can be autotrophic, heterotrophic, mixotrophic, and even parasitic (Stoecker *et al.*, 1997; Stoecker, 1999). Many of the Alveolata phylotypes that were recovered from S1 intervals using general primers (e.g. EUK-1, -5, 18, -24, -42 in Fig. 2A) cluster with basal lineages related to dinoflagellates (i.e. Syndiniales; Guillou *et al.*, 2008). Recent cultivation-independent molecular surveys revealed that many basal alveolate lineages can be found in sulfidic bottom waters of stratified aquatic settings (Alexander *et al.*, 2009; Behnke *et al.*, 2006; Coolen & Shtereva, 2009; Dawson & Pace, 2002; Edgcomb *et al.*, 2009; López-García *et al.*, 2003; Stoeck *et al.*, 2007; Takishita *et al.*, 2007). To date, the novel anoxic lineages are uncultured, and nothing is known about their physiological and environmental requirements or limitations (Kolodziej & Stoeck, 2007; Teske, 2007). However, because the sequences found in S1 are related to Alveolata from anoxic, sulfidic waters, our sequences could be markers for such conditions during S1 deposition.

Surprisingly, no sequences of phototrophic diatoms were recovered, although diatoms are often considered to be important species contributing to OM in sapropels (Kemp *et al.*, 1999). Similarly, despite the presence of diagnostic long-chain alkenones, no haptophyte sequences were recovered from S1 intervals using the general eukaryotic PCR assay (Tables 1 and 2). The use of general primers often fails to detect important phyla (Potvin & Lovejoy, 2009; Stoeck *et al.*, 2006), which can be overcome by the use of more specific (group-specific) primers during PCR (Coolen *et al.*, 2004; Richards & Bass, 2005; Viprey *et al.*, 2008). In our case, using primers targeting only haptophyte 18S rDNA enabled the detection of the alkenone-producing calcified *E. huxleyi* in the S1 interval (MS_HAP1_02). Another interesting finding is phylotype MS_Hap1_01, which is related to uncultivated haptophytes that were not part of the nannofossil assemblage (Boere *et al.*, 2011) and which were experimentally shown to grow mixotrophically as predators of picocyanobacteria (Frias-Lopez *et al.*, 2009), an adaptation that promotes growth in oligotrophic marine waters (Raven, 1997) such as most likely prevailed during early S1 deposition (e.g. Principato *et al.*, 2006).

Eukaryote DNA in Pleistocene eastern Mediterranean sediment intervals

The use of group-specific primers for dinoflagellates and haptophyte algae yielded reliable PCR products predominantly within the S1 sapropel and rarely in the deeper sediments. In contrast, the concentration of diagnostic general lipid biomarkers of both plankton groups (i.e. dinosterol and long-chain alkenones) was higher in the deeper sapropels S3 through S5 than in the S1. This result, in addition to the fact that the majority of protist sequences were also recovered from the S1 using general eukaryotic primers, suggests that most of the investigated protist DNA was degraded to smaller than 400- to 500-bp-long fragments between 10 and 80 ka BP and for that reason escaped amplification by PCR. For analyzing fossil plankton DNA in sediments older than the Holocene and deposited under less favorable conditions, we therefore suggest targeting a shorter, hypervariable region of the 18S rDNA such as the ~130-bp-long V9 region of 18S rDNA, which was recently introduced for amplicon 454 pyrosequencing (Amaral-Zettler *et al.*, 2009).

Instead, fungi were among the most frequently sequenced partial 18S rRNA genes from the deeper sediments. The ascomycete *P. guilliermondii*, which is closely related to phylotype FUNG-1 found in most sapropels as well as in the intercalating C_{org}-depleted sediments, is a widespread facultative marine yeast (Bass *et al.*, 2007; Gadanho & Sampaio, 2005; Kutty & Philp, 2008). Other recovered fungal phylotypes were related to obligate marine strains, notably *Metschnikowa*, *Rhodosporidium*, and *Rhodotorula* species (Kutty & Philp, 2008). Marine fungi were until recently believed to comprise a minor part of the eukaryotic community in deep-sea environments (Bass *et al.*, 2007), but their widespread occurrence was recently confirmed by several cultivation-independent molecular approaches (Alexander *et al.*, 2009; Bass *et al.*, 2007; Burgaud *et al.*, 2009; Edgcomb *et al.*, 2002, 2009, 2011; López-García *et al.*, 2003; Takishita *et al.*, 2006). It

remains unclear whether the fungal DNA in core MS66PC is of ancient origin because marine fungi, including yeasts, may be growing actively in subsurface sediments (Damare *et al.*, 2006; Edgcomb *et al.*, 2011; Damare & Raghukumar, 2008). On the other hand, fungi are capable of long-term preservation of their DNA in the form of spores (Lydolph *et al.*, 2005), and the distribution of fungal spores in Pleistocene Indian Ocean deep-sea sediments has been linked to environmental changes in the overlying water column at the time of sediment deposition (Raghukumar *et al.*, 2004). Alive or preserved, intact fungal DNA and that of prokaryotes could be part of the high molecular weight DNA size class in the up to 124-ka-old sediments as shown in Fig. S2. The use of fungal spores for paleoenvironmental reconstructions is in its infancy, but fossil DNA would be a useful alternate approach to escape cultivation bias associated with ancient preserved spores (Damare *et al.*, 2006; Raghukumar *et al.*, 2004).

Sequences from terrestrial plants were also more frequently recovered than sequences of marine protists in the Pleistocene sediments (Table 2), whereas the low BIT indices of all analyzed sapropels indicated that most of the buried OM was marine derived (Hopmans *et al.*, 2004; Menzel *et al.*, 2006). Therefore, the increased ratio in terrestrial plant vs. plankton DNA with increasing sediment depth and age suggests a preferred preservation of terrestrial plant DNA over marine plankton DNA. In particular, seeds and pollen, as the plant's reproductive organs, are intended for long-term DNA preservation and are often protected with a sturdy or lignified exocarp. The outer walls of pollen grains are extremely resistant to chemical and physical attack, and they might be indefinitely preserved in anaerobic sediments (Schlumbaum *et al.*, 2008 and references therein). Pollen are thus considered to be an excellent source for DNA (Bennett & Parducci, 2006; Parducci *et al.*, 2005; Schlumbaum *et al.*, 2008). For example, ancient plastid DNA has recently been recovered from up to 11 000-year-old pollen in lake sediments (Magyari *et al.*, 2011; Parducci *et al.*, 2005), and ancient plant DNA has also been extracted from enzymatically digested plant biomass in fossil feces (Poinar *et al.*, 2001). In contrast, the majority of plankton species, including 85% of the dinoflagellates, do not form protective resting stages, and the majority of DNA in marine sediments was shown to be extracellular (Dell'Anno & Danovaro, 2005), and therefore likely less well protected against (a)biotic degradation processes than DNA inside pollen grains.

Holocene and Pleistocene eastern Mediterranean sapropels obtained west of Greece were shown to contain between 20 000 and 70 000 pollen grains per gram sediment (e.g. Rossignol-Strick & Paterne, 1999). It is to be expected that the pollen content in our core is also high because of the close proximity to the Nile as a source of terrestrial organic matter input. Some of the recovered terrestrial plant sequences are related to sequences from genera that occur in northern Africa or more broadly in tropical regions of the world such as *Albizia* (Euk14), *Lygodium* (Euk31), and *Lolium* (Euk_16_mc). However, the relatively conserved 18S rDNA is not widely used as a suitable marker for plant barcoding. A more suitable marker for genus to species-level plant barcoding is the very short (90 bp) chloroplast P6 loop within the *trn*L intron, which was recently used to study the diet of various herbivores from heavily degraded DNA isolated from feces (Valentini *et al.*, 2009).

It is important to stress that paleoclimatic inferences based on the presence and identification of fossil plant gene sequences should be treated with caution because airborne pollen are considered to be a source of contamination during sampling and handling of the sediments (Boreson *et al.*, 2004; Parducci *et al.*, 2005), and some researchers automatically excluded plant sequences from their analyses (Behnke *et al.*, 2006; Stoeck *et al.*, 2007; Viprey *et al.*, 2008). On the other hand, the chance of contamination through airborne pollen in the middle of the Mediterranean Sea and inside a sealed container should have been minor in comparison to studies that involved ancient DNA from pollen recovered from lake sediments surrounded by forest (Parducci *et al.*, 2005). More importantly, there was sufficient heterogeneity in the terrestrial plant DNA composition between sediment intervals, whereas the same DNA profile would be expected if the surface of our aseptically prepared core was contaminated with airborne pollen. By way of exception, the *Zea mays* sequence in the marl between S3 and S4 was most likely introduced from the corn-derived packing material used for the shipment of the PowerMax Soil DNA isolation kit buffer S5 (MoBio laboratories). With the exception of one sequence related to *Picea* (spruce; Euk_02_mc) found in the 'no template DNA' control at WHOI, the controls for contamination with plant DNA during DNA extraction and for the preparation of PCR mixtures at the NIOZ, where most of the samples were analyzed, remained negative for terrestrial plant DNA. Therefore, Pleistocene eastern Mediterranean sediments seem to represent an archive of higher plant DNA to study past vegetation changes. This would help palynologists to identify plant sources of difficult to classify pollen grains. Furthermore, the laborious pollen stratigraphic analysis could be minimized with prior knowledge about the expected species composition as revealed by a preliminary fossil higher plant DNA survey.

Unexpected amplification of archaeal 16S rDNA

Archaeal PCR products were nonspecifically recovered from all sapropel intervals as well as some of the intercalating marls with the two-step PCR approach. Despite the high number of cycles (42) involved, direct amplification with GC primers did not result in the nonspecific recovery of archaeal sequences. This high number of cycles was necessary to compensate for the lower PCR efficiency when using these GC-clamp primers.

On the other hand, this approach was less sensitive because many more phylotypes were recovered from the samples analyzed with the two-step approach with eukaryotic primers at the NIOZ.

Other studies have observed the amplification of nontarget DNA bias in sediments from mud flats (Wilms *et al.*, 2006), aquifers (Euringer & Lueders, 2008), and deep-sea anoxic basins (Alexander *et al.*, 2009; Jeon *et al.*, 2008). Archaea are abundant in subsurface sediments (Biddle *et al.*, 2006; Coolen *et al.*, 2002; Inagaki *et al.*, 2005), and prokaryotic 16S rDNA was shown to outnumber eukaryote 18S rDNA by several orders of magnitude in Pleistocene Peru Margin sediments (Schippers *et al.*, 2005; Schippers & Neretin, 2006). For more reliable qPCR assays, especially when targeting low copy DNA, it is therefore important to reveal the identity of the amplified DNA through sequencing. In our study, the nonspecific co-amplification of archaeal 16S rDNA using general eukaryotic primers most likely resulted in an overestimation of the amount of preserved eukaryote 18S rDNA.

CONCLUSIONS

Our results showed that 400- to 500-bp-long 18S rDNA of planktonic (microbial) eukaryotes and zooplankton can be recovered from Holocene C_{org}-rich deep-sea subsurface sediments, but 18S rDNA from plankton groups that were analyzed in detail (i.e. from haptophyte algae and dinoflagellates) was sporadically amplified with both general and group-specific primers from sapropels deposited between 80 and 124 ka ago. This was unexpected because of the coinciding presence of preserved diagnostic general lipid biomarkers for both algal groups with concentrations in the Pleistocene sapropels exceeding those measured in the Holocene S1. This suggests that the majority of the planktonic DNA was degraded into smaller than 400- to 500-bp-long fragments between ~10 and 80 ka after deposition and escaped amplification by PCR. For analyzing fossil plankton DNA in sediments older than the Holocene and deposited under less favorable conditions, we suggest targeting a shorter, hypervariable region of the 18S rDNA such as the ~130-bp-long V9 region of 18S rDNA, which was recently introduced for amplicon 454 pyrosequencing (Amaral-Zettler *et al.*, 2009). Instead, the majority of the recovered 18S rDNA sequences in the Pleistocene sediments were derived from marine fungi as well as terrestrial plants. However, most of the sedimentary OM was of marine rather than terrestrial origin according to the BIT index, which suggests a preferred degradation of >10-ka-old marine plankton DNA over terrestrial plant DNA. Higher plant DNA is protected against degradation inside fossil pollen grains and because contamination of our samples with foreign DNA was unlikely, the Pleistocene eastern Mediterranean sediments seem to represent an archive of ancient higher plant DNA. This would help palynologists to identify plant sources of difficult to classify pollen grains. Furthermore, the laborious pollen stratigraphic analysis could be minimized with prior knowledge about the expected species composition as revealed by a preliminary fossil plant DNA survey. Resolving the past higher plant composition at up to genus or species-level taxonomic resolution would require further study and the use of faster evolving genes such as the short chloroplast P6 loop within the *trn*L intron. Lastly, the use of the two-step PCR approach to target low copy template ancient DNA was far more sensitive than the one-step PCR directly with GC primers, but this increased the chance of introducing PCR artifacts such as the nonspecific amplification of presumably abundant archaeal 16S rDNA or the specific amplification of low copy contaminants. For more reliable qPCR assays, especially when targeting low copy DNA, it is therefore important to determine the identity of the amplified DNA by sequencing.

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SUPPORTING INFORMATION

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

Fig. S1. Map of the Mediterranean Sea showing the location of core MS66PC.

Fig. S2. Agarose gel with total DNA extracts from selected intervals spanning sapropels S1, S3, S4, and S5 as well as oxidized C_{org}-poor intercalating sediments (i.s.).

Table S1. PCR assay specifications, including name and sequence of used primers, *E. coli* position of the primers, the amplicon size, annealing temperatures (T_a) used in the analyses, target phylogenetic group and references where the primers were first described.

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