Ancient DNA derived from alkenone-biosynthesizing haptophytes and other algae in Holocene sediments from the Black Sea

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[1] Holocene sea surface temperatures (SST) of the Black Sea have been reconstructed using sedimentary C_{37} unsaturated alkenones assumed to be derived from the coccolithophorid haptophyte *Emiliania huxleyi*, whose fossil coccoliths are an important constituent of the unit I sediments. However, alkenones can also be biosynthesized by haptophyte species that do not produce microscopic recognizable coccoliths. A species-specific identification of haptophytes is important in such $U^{K'}_{37}$-based past SST reconstructions since different species have different alkenone-SST calibrations. We showed that 18S rDNA of *E. huxleyi* made up only a very small percentage (less than 0.8%) of the total eukaryotic 18S rDNA within the up to 3600-year-old fossil record obtained from the depocenter (>2000 m) of the Black Sea. The predominant fossil 18S rDNA was derived from dinoflagellates (*Gymnodinium* spp.), which are predominant members of the summer phytoplankton bloom in the modern Black Sea. Using a polymerase chain reaction/denaturing gradient gel electrophoresis method selective for haptophytes, we recovered substantial numbers of a preserved 458-base-pair (bp)-long 18S rDNA fragment of *E. huxleyi* from the Holocene Black Sea sediments. Additional fossil haptophyte sequences were not detected, indicating that the *E. huxleyi* alkenone-SST calibration can be applied for at least the last ~3600 years. The ancient *E. huxleyi* DNA was well protected against degradation since the DNA/alkenone ratio did not significantly decrease throughout the whole sediment core and 20% of ~2700-year-old fossil *E. huxleyi* DNA was still up to 23,000 base pairs long. We showed that fossil DNA offers great potential to study the Holocene paleoecology and paleoenvironment of anoxic deep-sea settings in unprecedented detail.

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1. Introduction

[2] Organic components in sediments, such as lipids and pigments, derived from specific organisms (i.e., biomarkers) form an archive of the past species composition of the water column and hence can be used to reconstruct the physical and chemical conditions caused by climate change [*Brassell*, 1993]. For example, long-chain (C_{37}, C_{38} and C_{39}) unsaturated methyl and ethyl ketones (alkenones) have been found to be characteristic of haptophyte microalgae, including the cosmopolitan coccolithophorid *Emiliania huxleyi* [*Volkman et al.*, 1980], which first appeared in the late Pleistocene [*Marlowe et al.*, 1990]. This species is considered to be the dominant source of alkenones in most contemporary marine sediments. Alkenones are of great interest to paleoceanographers because of the strong empirical relationship between the degree of unsaturation in alkenones and growth temperature, which forms the basis for their use as molecular proxies of past sea surface temperatures (SST) [*Bendle and Rosell-Melé*, 2004; *Brassell et al.*, 1986; *Conte et al.*, 2001; *Goni et al.*, 2004; *Prahl and Wakeham*, 1987; *Rosell-Melé*, 1998; *Sachs et al.*, 2000; *Sikes et al.*, 2005; *Volkman et al.*, 1995]. Apart from *E. huxleyi*, other haptophyte algae also biosynthesize alkenones and these algae often possess different relationships between the degree of unsaturation in alkenones and growth temperature [*Prahl and Wakeham*, 1987; *Versteegh et al.*, 2001; *Volkman et al.*, 1995] with implications for paleoceanographic interpretations. If these other species are coccolithophorids, preserved fossil coccoliths may indicate the species identity [*Villanueva et al.*, 2002] but there is also a group of alkenone-producing haptophytes that does not produce coccoliths.

[3] We recently showed for the Holocene sediments of postglacial Ace Lake (Vestfold Hills, Antarctica) by analysis of 18S rDNA of ancient haptophyte species that the sedimentary alkenones were derived from haptophytes related to noncoccolithophorid *Isochrysis* species [*Coolen et al.*, 2004]. This combined lipid-DNA biomarker approach also showed that Holocene salinity variations caused major changes in the abundance of different haptophyte species,

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each with different alkenone distributions. This study showed that potentially the analysis of fossil DNA can reveal important paleoceanographic information. However, in lakes most of the DNA of phototrophic organisms is already degraded before the decaying cells reached the sediment, although the portion of DNA buried within the Holocene sediments was well preserved and protected against further degradation [*Coolen and Overmann*, 1998; *Coolen et al.*, 2006]. These sulfidic lakes are relatively shallow (<25 m) and, therefore the DNA of decaying cells experience only a short travel distance and residence time before the cellular remains reach the sediment. This poses the question whether fossil DNA of water column dwelling algae may survive in the Holocene record of anoxic deep-sea environments, where cells have to travel a far greater distance and experience a longer residence time before they reach the sediment.

[4] To answer this question, we have chosen to analyze sediments of the Black Sea because the >2000-m-deep waters below ~70 m as well as the organic carbon-rich sediments are sulfidic and therefore are expected to provide excellent preservation conditions for fossil DNA. *Xu et al.* [2001] reported a predominance of $C_{37:2}$ and $C_{37:3}$ alkenones, indicative of *E. huxleyi* throughout the coccolith-bearing unit I sediments of the Black Sea. The latter study also described a novel $C_{36:2}$ ethyl ketone (hexatriaconta-16E,21E)-dien-3-one) which was found only in trace amounts in unit I sediments, but it was the most abundant alkenone in the older sediment layers of unit II where fossil coccoliths derived of *E. huxleyi* were absent. Since unit II sediments were deposited before *E. huxleyi* started to colonize the photic zone of the Black Sea [*Hay*, 1988], a different biological precursor for this compound was proposed. On the basis of these findings, we focused our study on whether fossil DNA evidence confirmed that *E. huxleyi* was indeed the only biological source of the alkenones or whether additional noncoccolithophorid haptophytes known to biosynthesize alkenones such as *Isochrysis galbana* [*Marlowe et al.*, 1984; *Rontani et al.*, 2004], thrived in the photic zone of the Black Sea during the Holocene. Fossil partial 18S rDNA fragments of ancient haptophytes were amplified by polymerase chain reaction (PCR) using general primers for the domain Eukarya [*Díez et al.*, 2001] as well as primers selective for haptophytes [*Coolen et al.*, 2004]. All amplicons were analyzed by denaturing gradient gel electrophoresis (DGGE) [*Coolen et al.*, 2004; *Muyzer et al.*, 1993].

[5] Since DNA of alkenone-biosynthesizing haptophytes was expected to be far less stable compared to these alkenone lipids, we also determined the haptophyte DNA/alkenone ratio in the sediment record and compared it to the haptophyte DNA/alkenone ratio found in the extant water column as well as in a culture of *E. huxleyi*. Fully hydrated fossil DNA in sulfidic lake sediments has been shown to be prone to fragmentation [*Coolen and Overmann*, 1998]. Thus we also studied the extent and onset of fragmentation of fossil DNA from the Black Sea deep-sea sediment record based on the quantitative distribution of fossil 458-bp-long *E. huxleyi* 18S rDNA by means of quantitative real-time PCR (Q-PCR) in various size classes of the extracted DNA. Since we expected to find shortened fossil DNA fragments, we decided that DGGE, which allows only the analysis of up to 600-bp-long fragments [*Muyzer et al.*, 1993], was the method of choice for the analysis of short ancient DNA fragments as this method is fast and far less laborious compared to the screening of a clone library.

2. Experimental Setup

2.1. Setting

[6] On the basis of stratigraphic analyses, we now know that the Black Sea was originally a freshwater lake but because of the postglacial rise of the global sea level, a seawater connection across the shallow sill of the Bosporus was established at least 7150 years ago. The first evidence of seawater intrusion dates earlier, however, some 9800 years ago [*Arthur and Dean*, 1998; *Jones and Gagnon*, 1994]. After the Bosporus connection, a stable pycnocline developed in the Black Sea between the brackish surface water, influenced by the large riverine inflow, and the more saline bottom water of Mediterranean origin. Owing to the stable stratification, anoxia developed below the pycnocline, and from ~7500 years ago an organic-rich sapropel started to accumulate. The coccolithophorid, *Emiliania huxleyi*, is believed to have invaded the Black Sea ~3450 years ago, and since then a varved coccolith ooze has been deposited [*Arthur and Dean*, 1998; *Calvert et al.*, 1987; *Hay et al.*, 1991; *Xu et al.*, 2001].

[7] In the chronological description of late Pleistocene and Holocene Black Sea sediments, three depositional periods are recognized [*Arthur and Dean*, 1998; *Ross and Degens*, 1974]: the modern coccolith ooze (unit I), the marine sapropel (unit II), and the deep limnic sediment (unit III). Unit I, the uppermost unit, is a laminated, organic carbon rich (3–7% total organic carbon, TOC), coccolith marl, with abundant *E. huxleyi* coccoliths [*Arthur et al.*, 1994]. The deepest section of unit I documents the first invasion of *E. huxleyi* and is briefly interrupted by a transition sapropel (TS) before returning to the deposition of coccolith marl that has continued to the present-day [*Hay*, 1988]. The Holocene sapropel of unit II has a lower carbonate content of approximately 16% but is richer in organic matter (5–20% TOC) considered to be mostly of marine origin [*Hay*, 1988]. The accumulation of organic carbon-rich sediments may be due to enhanced preservation during anoxia [*Arthur and Dean*, 1998; *Wilkin et al.*, 1997], enhanced primary productivity during the time of sapropel formation [*Calvert et al.*, 1987], lower sedimentation rate of other bulk components [*Calvert and Karlin*, 1998], or to a combination of these factors.

2.2. Sampling

[8] Particulate organic matter (POM) for phylogenetic analysis of the extant haptophyte communities as well as the alkenone composition, was collected on GFF filters from specific water depths of the photic zone at different locations in the Black Sea (Figure 1). Filters with POM of station 5 (Central Basin (43°06′33″N, 34°00′61″E) at depths of 10, 30, and 62 m) and station 7 (42°44′93″N, 37°30′00″E) (Eastern Basin at a depth of 30 m) were collected during the R/V *Knorr* cruise K172/8 in May

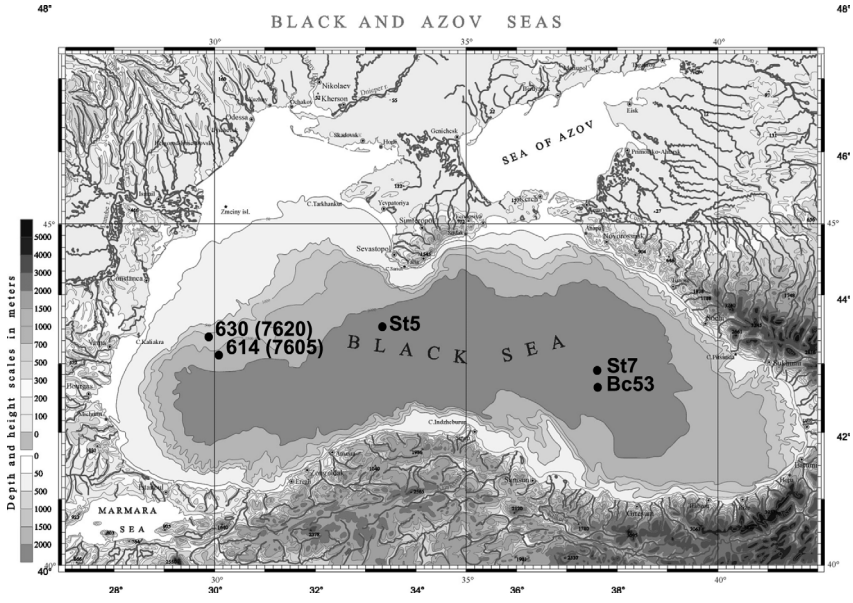

Figure 1. Sampling sites within the Black Sea. Location of the sampling sites for particulate organic matter (POM) (stations 6705 and 6720 within the Western Basin, which were sampled during the R/V *Meteor* 51/4 cruise in December 2001, and station 5 (ST5) of the Central Basin and station 7 (ST7) of the Eastern Basin, which were sampled during the R/V *Knorr* cruise K172/8 in May 2003) and core BC53, which was obtained in 1988 from the Eastern Basin during cruise 134/8 with the R/V *Knorr*.

2003. M. Kuypers (MPI-Bremen) kindly provided us a filter with POM collected during the R/V *Meteor* cruise M51/4 in December 2001 from the Western Basin (station 7605; 42°30′99″N, 30°14′27″E; 30 m). All filters were kept frozen at −20°C until DNA extraction.

[9] Alkenones and DNA of ancient haptophytes were analyzed from sulfidic Holocene sediments of box core BC 53. This core was obtained at a water depth of 2154 m within the Eastern Basin (42°39′73″N, 37°36′98″E) in 1988 during cruise 134/8 with the R/V *Knorr*. The core has continuously been stored at −20°C at the Woods Hole Oceanographic Institution until analysis at our PCR product-free fossil DNA laboratory at the Royal NIOZ. The frozen sediment core was sliced in horizontal fragments and 12 slices were chosen for lipid and DNA analysis: Holocene coccolith-bearing varved sediment layers of unit I (0–0.5 cm; 0.5–2.5 cm; 4.5–6.5 cm; 8.5–10.5 cm; 12.5–15.0 cm; 17.0–19.5 cm; 21.5–23.0 cm; 24.0–26.5 cm; 26.5–28.5 cm); the carbonate-poor transition sapropel (TS) (28.5–31 cm); the oldest coccolith-bearing varved sediment resulting from the first invasion of *E. huxleyi* (31.0–31.5 cm); and the top of the sapropel unit II (31.5–33.0 cm). The lowermost 6 cm of the core was a gray turbidite layer and was not analyzed.

2.3. Lipid Extraction for the Analysis of Alkenones

[10] Filters with POM were freeze-dried and ultrasonically extracted with methanol (3x), dichloromethane (DCM)/methanol (1:1, v/v) (3x) and DCM (3x), and all extracts were combined. The solvent was removed by rotary evaporation under vacuum. After addition of a standard (2,3-dimethyl-5-(1,1-d2-hexadecyl)thiophene) to an aliquot of the total extract, this fraction was methylated with diazomethane and subsequently chromatographed over a small SiO_2 column with ethyl acetate as eluent and derivatized with BSTFA (1% TMCS). The sediment samples were freeze-dried and extracted (Dionex ASE 200 Accelerated Solvent Extractor; DCM/methanol 9:1, v/v; 1000 psi; 100°C for 5 min; flush 100%; three cycles). The solvent was removed by rotary evaporation under vacuum. After addition of a standard (3-methyl-6-dideutero-heneicosane) to an aliquot of the total extract, this fraction was methylated with BF_3/methanol and subsequently chromatographed over a small SiO_2 column with ethyl acetate as eluent and derivatized with BSTFA (1% TMCS). All extracts were analyzed by capillary gas chromatography (GC) and GC-mass spectrometry (MS).

[11] GC was performed on a HP 5890 instrument, equipped with a fused silica column (25 m × 0.32 mm) coated with CP-Sil 5CB (0.12 μm film thickness) and He was used as carrier gas. The samples were injected at 70°C and the oven was programmed to 130°C at 20°C/min and then to 320°C at 4°C/min and hold for 10 min at 320°C.

[12] GC-MS was performed on a Thermo Electron TraceGC ultra interfaced with Trace DSQ mass spectrometer operating at 70 eV with a mass range of m/z 50–800 and a cycle time of 0.33 s. The GC column and conditions were the same as described above.

2.4. Total Organic Carbon (TOC)

[13] The TOC content and $\delta^{13}C$ of TOC was determined by elemental analysis (EA)/isotope-ratio-monitoring mass spectrometry (EA/irmMS). EA/irmMS analyses were performed on decalcified (by reaction with 1 N HCl for 18 hours) sediments using a Carlo Erba Flash elemental analyzer coupled to a Thermofinnigan Delta^{PLUS} irmMS

system. The TOC content (as a percentage) and $\delta^{13}C$ of TOC were determined using external standards of known carbon content and $\delta^{13}C$.

2.5. Extraction of Total DNA

[14] Total DNA was extracted from sections of GFF filters and from 0.25 g of the 12 selected sediment sections using the UltraClean™ Soil DNA Kit Mega Prep following the descriptions of the manufacturer (Mobio, Carlsbad, California). The sections of the filters used for DNA extraction contained POM from various amounts of filtered water: station 7605 (30 m (4 L)); station 5 (10 m (64 L), 30 m (94 L), 62 m (187 L)); and station 7 (30 m (55 L)). Prior to extraction, the filters were sliced with a sterile scalpel in order to enhance the extraction efficiency. We preferred to use this extraction kit for fossil DNA work since all reagents and disposable tubes are free of DNA, which substantially reduced the chance of contamination with foreign DNA.

[15] Because our study relied on the analysis of fossil 18S rDNA derived from ancient haptophytes by PCR amplification, it was of utmost importance to prevent any contamination of the sediment samples by foreign DNA. Therefore the DNA extractions were performed in our PCR product-free clean lab and inside a sterilized HEPA-filtered laminar flow bench. Prior to the extractions, the laboratory was cleaned with 6 wt % sodium hypochlorite and the laminar flow bench was sterilized by UV for 4 hours followed by sterilization of all surfaces with RNase Away (Molecular Bio Products, San Diego, California), a sterilizing agent which also destroys DNA. At all times, two layers of disposable gloves were worn and the second layer of gloves was replaced before vials with reagents from the MoBio kit were opened. Separate pipets and sterile DNA free filter tips were used in order to prevent the introduction of foreign DNA via aerosols during pipetting. As a control for contamination during DNA extraction, a parallel sample without sediment was subjected to the whole extraction and purification procedure (extraction control). The concentration of DNA for each extracted sediment sample was quantified with the fluorescent dye PicoGreen (MoBiTec, Göttingen, Germany). A subsample of the total DNA extracts from various sediment depths was subjected to agarose gel electrophoresis to determine the quality and fragment length of the DNA throughout the core. Undiluted, as well as 2, 5, 10, 20, and 50 times diluted DNA extracts were subjected to quantitative PCR (Q-PCR) reactions in order to determine whether PCR-inhibiting coextracted impurities within the DNA extracts were present.

[16] Extensive precautions against contamination with foreign DNA were also performed during the pipetting of PCR reagents. PCR reactions were prepared in the cleaned PCR product-free clean room inside a separate sterilized (UV + RNase Away) PCR work station. Separate pipets and filter tips were used for postextraction PCR. PCR ingredients and sterile disposable tubes were only opened inside the bench. Disposable gloves were worn at all times.

[17] Additional precautions against contamination with foreign (modern) DNA were performed during other experiments as described in the material and methods section.

2.6. Amplification of Ancient 18S rDNA

[18] All PCR reactions were performed in an iCycler (Biorad, Hercules, California). All reactions involved initial denaturing (5 min at 95°C), followed by 35–38 cycles including denaturing (30 s at 94°C), 40 s of primer annealing at temperatures described below, and primer extension (40 s at 72°C). A final extension was performed at 72°C. In an initial attempt to detect fossil 18S rDNA of haptophytes, the most abundant eukaryotic partial 18S rDNA was amplified using a primer combination as described previously [*Díez et al.*, 2001]. In addition, partial 18S rDNA solely found in haptophyte algae was selectively amplified using primers selective for haptophytes [*Coolen et al.*, 2004]. The annealing temperature was set to 64°C (eukaryotes) and 62.5°C (haptophyte-specific PCR). A 40-bp-long GC clamp [*Muyzer et al.*, 1993] was attached to the 5′-end of primer Euk 563r (eukaryotes) and Prym-887r (haptophyte-specific PCR), to prevent complete melting of the PCR products during DGGE. For other purposes, the same combinations without GC clamp were used. Each amplification reaction contained 0.25 m*M* of each deoxynucleotide (dNTP) (Amersham-Biosciences, Piscataway, New Jersey), 8 μg of Bovine Serum Albumin (BSA), 2 μL of 10 × PicoMaxx™ reaction buffer (Stratagene, LaJolla, California), 1 unit of PicoMaxx™ high-fidelity PCR system and 0.2 μM of primers (Thermo-Electron, Ulm, Germany). The reaction mixtures were adjusted to a final volume of 20 μL with DNA and DNase free, sterile water (Sigma, Saint Louis, Missouri). Each PCR amplification series included three reactions without DNA template, which served as a control for contaminations during the pipetting of the reaction mixture components. A fourth reaction with 0.4 μL of the extraction control was amplified by PCR as a control for contamination during the extraction of DNA from the sediment samples. A fifth reaction containing 4×10^6 copies of complete length 18S rDNA of the chlorophyte *Tetraselmis* was used to monitor the specificity of the PCR reactions.

2.7. Denaturing Gradient Gel Electrophoresis

[19] The PCR-amplified partial 18S rDNA of eukaryotes (603 bp including the 40 bp-long GC clamp) or haptophytes (498 bp including the 40-bp-long GC clamp), were separated by DGGE [*Muyzer et al.*, 1993] using the conditions as described by [*Coolen et al.*, 2004] with the slight modification that electrophoresis proceeded for 5 hours at 200 V and 60°C. Afterward, the gel was stained for 10 min by covering the gel with 4 mL of 1x TAE buffer (pH 8.3) containing 2 μL concentrated SybrGold (Molecular Probes, Eugene, Oregon). In order to prevent DNA damage by UV, we used a Dark Reader (Clare Chemicals Research Inc., Dolores, Colorado) which uses visible light instead of UV in order to visualize the SybrGold-stained DNA. DGGE fragments were sliced from the gel with a sterile scalpel and the DNA of each fragment was eluted in 50–75 μL sterile 10 m*M* Tris-HCl (pH 8.0) by incubation for 48 hours at 2°C. One μL of the eluted 18S rDNA fragments (approximately 10^7 copies) were reamplified using 25 cycles, and the primer combinations listed above, but this time

without GC clamp, in order to generate template DNA for the subsequent cycle sequencing reactions.

2.8. Sequencing of DGGE Fragments

[20] Primers and dNTPs were removed using the QIAquick PCR Purification Spin Kit (Qiagen, Hilden, Germany) and the amount of DNA was quantified with the fluorescent dye PicoGreen (MoBiTec, Germany). Cycle sequencing reactions were performed as described by [*Coolen et al.*, 2004].

2.9. Phylogenetic Analysis

[21] Sequence data were compiled using ARB software [*Ludwig et al.*, 2004] and aligned with complete length sequences of closest relatives obtained from the GenBank database [*Benson et al.*, 2004] using the ARB FastAligner utility. Matrices of similarity, distance and phylogenetically corrected distance values were generated using the neighbor joining and maximum parsimony option in ARB. Sequences obtained in this study have been deposited in the GenBank sequence database under accession numbers DQ234281 to DQ234297.

2.10. Quantitative Real-Time PCR

[22] Real time PCR was performed in an iCycler system (Biorad) in order to study the relative quantitative distribution of fossil 18S rDNA copies of eukaryotes and specifically haptophyte algae. To quantify the 18S rDNA copy numbers, the PCR conditions and primers (without GC clamp) were used as described above. Accumulation of newly amplified double stranded rDNA was followed online as the increase in fluorescence because of the binding of the fluorescent dye SybrGreen. Reaction mixtures (20 μL) contained 1 unit of Picomaxx™ high-fidelity DNA polymerase, 2 μL of 10x Picomaxx PCR buffer (both Stratagene), 0.25 m*M* of each dNTP, 8 μg of BSA, 0.2 μ*M* of primers, 50,000 times diluted SYBRgreen (Molecular Probes) (optimized concentration), a final concentration of 10 n*M* fluorescein, 3 m*M* of $MgCl_2$ (optimized concentration) and ultra pure sterile water (Sigma). Even after 45 cycles, all control reactions stayed negative which also indicated that the formation of primer dimers was negligible. Nevertheless, the fluorescent signal was read in each cycle during an additional step holding the temperature at 80°C for 25 s in order to maximize the chance that a-specific products such as primer dimers were melted and not quantified. Known amounts of template DNA (total DNA extracts ranging between 1 and 25 ng) from each sample was added to each Q-PCR reaction.

[23] Calibration of the samples was performed as follows. Genomic DNA of *E. huxleyi* strain Oslo Fjord served as a template to generate 563-bp-long PCR fragments using the primers for the eukaryotal domain and to generate 458-bp-long PCR fragments using the haptophyte specific primer set. Primers and salts were removed from these PCR products using Qiaquick Spin Columns (Qiagen) and the exact DNA concentrations were determined fluorometrically (Picogreen, Molecular Probes) in order to calculate the number of 18S rDNA copies.μL^{-1} of purified PCR products. For the calibration of the samples, between 3.10^{-2} and 3.10^{7} copies of the 563-bp-long fragment (quantification of most predominant eukarya) and between 1.10^{-2} and 1.10^{7} copies of the 458-bp-long fragment (quantification of haptophytes) were subjected to Q-PCR along with the samples.

[24] The quantification of the number of 18S rDNA copies of *E. huxleyi* or the domain Eukarya was repeated from duplicate total DNA extracts and the second quantification series were calibrated with freshly prepared standards.

[25] Control reactions were performed and included a reaction without DNA as a control for contamination during pipetting. A second reaction contained 0.4 μL of DNA extracted with the MoBio kit but without addition of sediment as a control for contamination with foreign DNA during the DNA extraction procedure. A third reaction contained 1×10^{8} copies of the complete 18S rDNA of the chlorophyte *Tetraselmis* as a control for the specificity of the haptophyte selective amplification reactions.

[26] In order to prevent any contamination of the pristine sediments, fossil DNA extracts, or PCR reagents with PCR products via aerosols, the calibration reactions were prepared in a PCR workstation located in a spatially separated post-PCR laboratory where no ancient DNA analysis was performed.

2.11. Natural Fragmentation of Fossil DNA of *E. huxleyi*

[27] Nucleic acids were extracted from 4 g of wet sediment of varved, coccolith-bearing unit I sediment layers: 2.5–4.5 cm, 8.5–10.5 cm, 17.5–19.0, and the oldest unit I layer located just above the transition sapropel (24–26.5 cm). For this experiment, the previously described simultaneous DNA and RNA extraction method [*Hurt et al.*, 2001] was used since this method allowed the extraction of up to 40-kb-long DNA fragments, whereas only up to 10-kb-long fragments can be recovered with the UltraClean Soil DNA Kit (MoBio). A grinding step was included in the extraction method used [*Hurt et al.*, 2001] for efficient cell lysis. The grinding was performed in a similar way for all samples; equal amounts of sterile, heat-sterilized sand (5 hours at 450°C) was added to the sediments and grinding occurred in a sterile, baked mortar with liquid nitrogen (the nitrogen was transported in a heat-sterilized container (5 hours at 450°C)). The samples were ground with a pestle using 30 circular movements. This grinding procedure was repeated twice for all samples.

[28] A fraction of the total DNA extracts was subjected to agarose gel electrophoresis (33 min at 90 V). In order to minimize contamination with foreign DNA, the sterile 1.5% (w/vol) agarose gel was run with sterile 1x TAE pH 8.0 buffer. The electrophoresis chamber was first treated with RNAse Away (Molecular Bio Products) and rinsed with nucleic acid and Nuclease free water (Sigma) and exposed with UV for 30 min prior to use. Afterward, the gel was stained for 20 min in a sterile chamber with 1x SybrGold (Molecular Probes) in sterile 1x TAE pH 8.0. In order to prevent DNA damage by UV, we used the Dark Reader (Clare Chemical Research, Inc.) to visualize the SybrGold-stained DNA. Using a sterile set of a scalpel and tweezers, sedimentary DNA size classes (23–40 kb, 4.4–23 kb, 2.2–

4.4 kb, 0.7–2.2 kb and less than 0.7 kb) were sliced from the gel and the DNA was eluted using a Centrilutor electrophoresis system (Millipore) following the procedures according to the manufacturer.

[29] Cross contamination was prevented by leaving one lane between each sample during initial gel electrophoresis and by slicing the largest DNA size class first, followed by slicing the shorter DNA size classes. In between slicing, the scalpel and tweezers were heat-sterilized using a gas flame. The exact fragment lengths of the isolated DNA size classes were again subjected to agarose gel electrophoresis to determine their exact sizes using fragment size rulers (phage $\lambda \times$ *Hin*dIII digest and Position Molecular Mass Standard (PMMS, Biorad)) on each side of the agarose gel.

[30] The 0.4 μL of the total extracts as well as of each individual fragment was subjected to Q-PCR using the method selective for haptophytes as described earlier. The concentration of partial 458-bp-long amplification products of the fossil 18S rDNA of *E. huxleyi* found per fossil DNA size class was calculated (copies (ng dry wt sediment)$^{-1}$) and represented the fragmentation of ancient DNA of *E. huxleyi* in the fossil record. The Q-PCR products were reamplified (10 cycles) using primers (including the GC clamp) selective for haptophytes for subsequent DGGE analysis. Sequence analysis of the resulting DGGE bands was used to verify whether solely *E. huxleyi* was quantified in each DNA size class.

[31] As a control for (cross) contamination during this experiment, pieces of "empty" gel were excised along with the various DNA-containing fractions and subjected to all steps performed during this experiment. Along with the samples, 0.4 μL of the controls were subjected to Q-PCR using the primers and conditions selective for haptophytes.

[32] In order to estimate the amount of DNA which was sheared because of grinding compared to natural shearing of the ancient DNA, the above experiment was also performed using an aliquot (4 g) of wet sediment 24–26.5 cm (1) without shearing and (2) to which 10^7 intact cells of *E. huxleyi* were added. The aliquot of this extract which was subjected to Q-PCR contained besides the natural amount of sedimentary *E. huxleyi* DNA, the DNA of 2.10^5 added *E. huxleyi* cells.

[33] In order to prevent any cross contamination of modern DNA of *E. huxleyi*, the addition of *E. huxleyi* cells to the sediment aliquot was performed in the post-PCR laboratory and after all ancient DNA experiments at pristine sediments were completed.

3. Results

3.1. Calibration of Sediment Ages

[34] The lithology of core BC53 was determined in 1988 by X-radiography. The upper 28.5 cm represented the coccolith-bearing varved sediment layers of unit I, followed by the presence of an olive green sapropelic layer defined as the transition sapropel between 28.5 and 31.0 cm. The transition sapropel was separated from the upper 2 cm of olive green sapropel of unit II by a thin layer of coccolith-bearing sediment at 31.0–31.5 cm. This layer was deposited during the first invasion of *E. huxleyi*. AMS radiocarbon (^{14}C) studies revealed that the first invasion of *E. huxleyi* occurred ~3450 years before present (BP) [*Hay et al.*, 1991].

3.2. TOC, δ^{13}C of TOC, and Total DNA Concentrations

[35] The TOC content varied between 11 and 20% in the coccolith-bearing sediment layers of unit I, dropping to 10% in the transition sapropel (TS) and then increasing to 20% within the upper layer of the sapropel of unit II (Figure 2a). The δ^{13}C values of the bulk organic matter varied between −25.3 and −24‰ in unit I. δ^{13}C in the TS was slightly more depleted compared to the coccolith layers but slightly enriched in the unit II sapropel layer (Figure 2b). The total DNA concentration was highest in the top sediment layer (20 μg (g dry sediment)$^{-1}$), then declined to a relatively stable concentration of ~8 μg (g dry sediment)$^{-1}$ throughout the remaining part of the core (Figure 2c).

3.3. The 18S rDNA-Based Identification and Quantification of Eukaryotes

[36] The PCR/DGGE analysis selective for eukaryotic 18S rDNA (Figure 3) resulted in the identification of 13 unique phylotypes throughout the sediment core (Figure 4). Despite the abundance of morphological remains (coccoliths) of *E. huxleyi* in Black Sea unit I sediments, none of the sequenced predominant eukaryotic DGGE fragments represented *E. huxleyi* (Figures 3 and 4).

[37] The most predominant DGGE fragment 9, which was found throughout unit I and in the upper part of unit II, melted at the same position as the DGGE fragment resulting from the Sargasso Sea strain of *E. huxleyi*. However, sequence analysis revealed that the closest cultivated relative of fragment 9 and the related fragment 11 were dinoflagellates of the genus *Gymnodinium* (94–97.2% homology). Six out of the 13 recovered eukaryotic phylotypes represented dinoflagellates. DGGE fragment 12a, 12b and 13 were related to a separate cluster of *Gymnodinium* species (94.6–97.4% homology), whereas the closest relative of fragment 8 was *Dinophysis norvegica* (95.0% homology). Sequence 12 was present in all analyzed sediment layers, whereas sequence 13 was absent from the unit II sapropel layer as well as the transition sapropel. DGGE fragment 1 was the only sequence unique to the unit II sapropel and dropped below detection limit soon after the interval representing the first invasion of *E. huxleyi*. Sequence 1 clusters within a novel lineage of fungi/metazoa with sequences obtained from the oxygenated marine surface waters of the Bay of Fundy with clone ms313 as its closest relative [*Savin et al.*, 2004] (99.8% sequence homology).

[38] DGGE fragments 2 and 5 represented ciliates. DGGE fragment 2 was only abundant between 8.5 and 15 cm and its closest cultivated relative was *Cryptocaryon irritans*. *Strombidium purpureum* was the closest cultivated relative of DGGE fragment 5 and appeared only in the sediment layer which was deposited during the first invasion of *E. huxleyi*. DGGE fragment 4 first appeared in the transition sapropel and younger sediment layers of unit I and its closest relative was the cercozoa *Cryothecomonas longipes* (98% sequence homology). The faint DGGE fragment 3

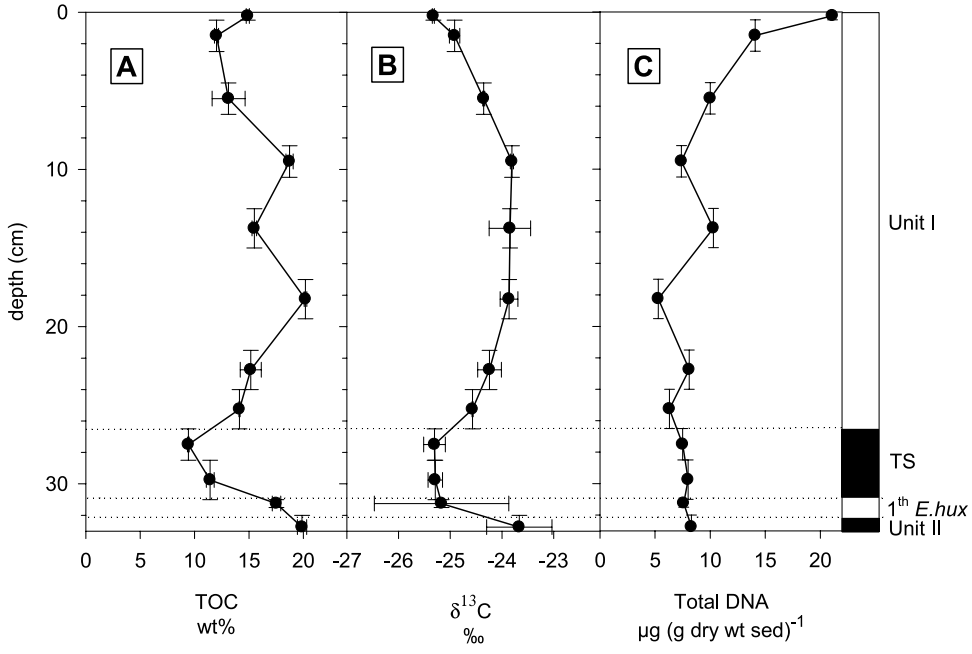

Figure 2. (a) Total organic carbon (TOC) content (wt %), (b) isotopic composition of bulk organic carbon (‰), and (c) total DNA concentration (µg g^{-1} dry sediment) of the Holocene sediments of BC53. The lithology of the core is indicated at the right.

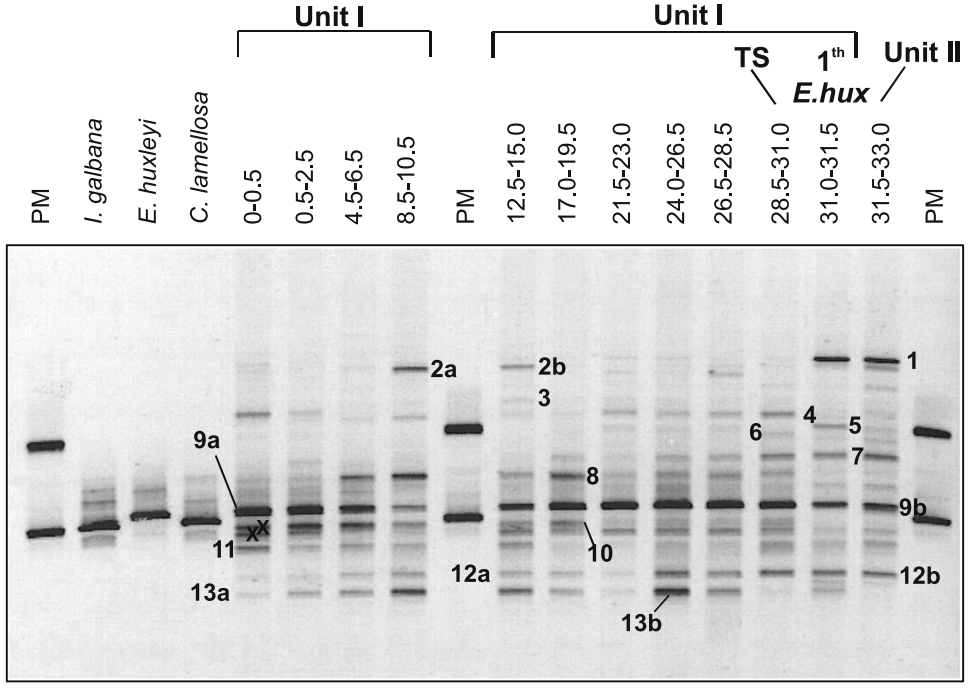

Figure 3. Denaturing gradient gel electrophoresis (DGGE) analysis of the predominant polymerase chain reaction (PCR)-amplified partial 18S rDNA of the domain of Eukarya obtained from the Holocene sediments of the Black Sea. DGGE bands that were sliced from the gel and subsequently sequenced are indicated with numbers.

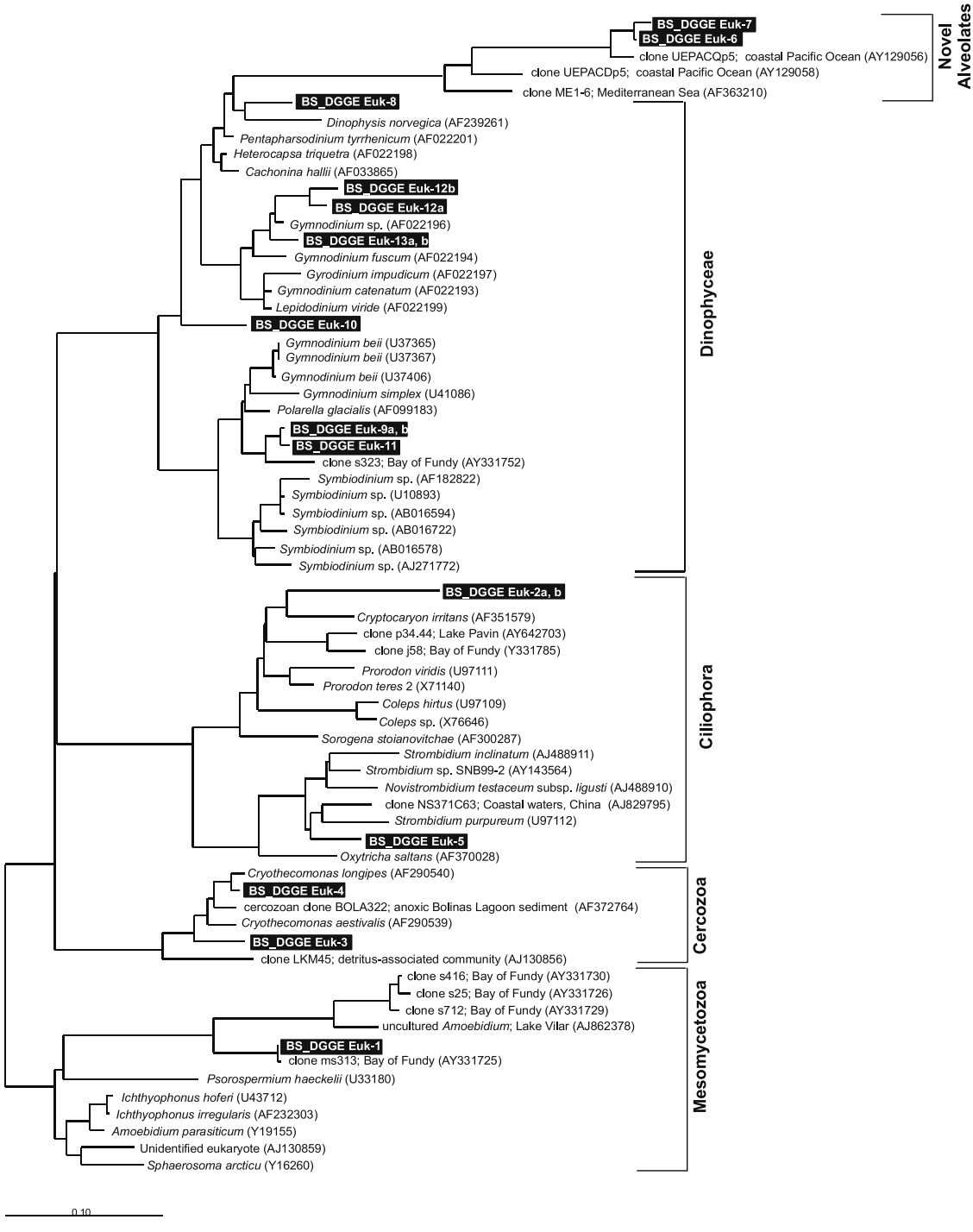

Figure 4. Phylogenetic tree showing the relationship of predominant 18S rDNA sequences of eukaryotes retrieved from the water column (POM) and Holocene sediment layers of the Black Sea (white text in black rectangles) to reference sequences obtained from the GenBank database. The latter sequences were determined from the DGGE analysis shown in Figure 3. DGGE bands with identical melting positions within the gel appeared to contain identical sequences except for sequences 12a and 12b. As a result, phylotype BS_DGGE Euk 9 was found throughout the fossil record.

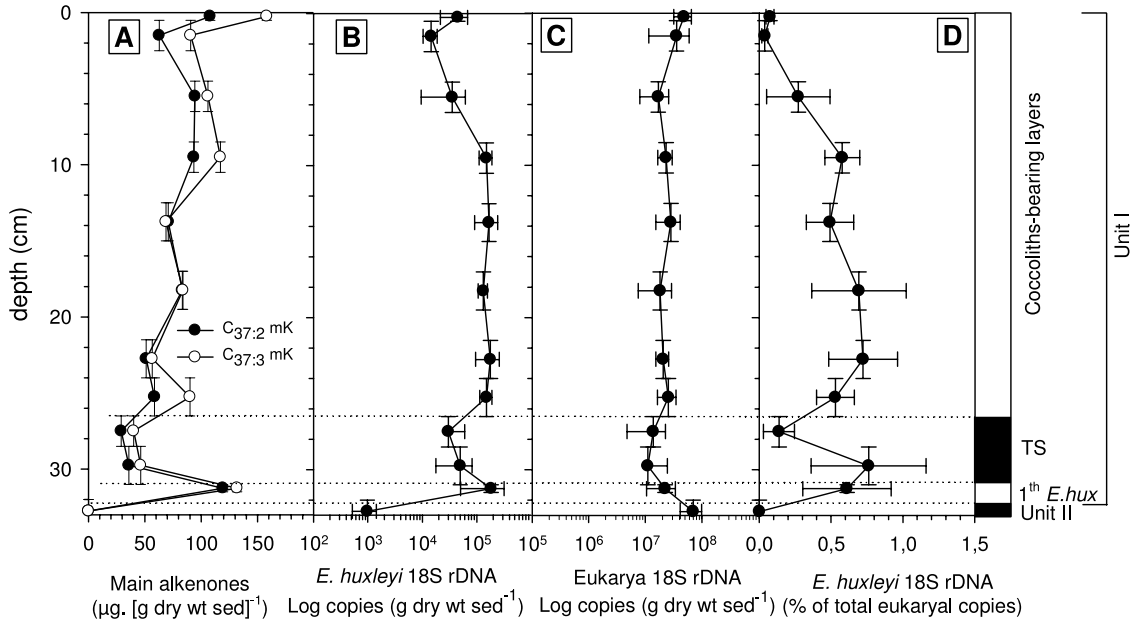

Figure 5. Abundance of haptophyte-specific biomarkers in the Holocene sediments of the Black Sea. (a) Main haptophyte-derived alkenones ($C_{37:2}$ methyl ketone [mK] and $C_{37:3}$ mK) (b) Number of 18S rDNA copies of *E. huxleyi*. (c) Number of most predominant 18S rDNA copies of the domain Eukarya. (d) Percentage of *E. huxleyi* 18S rDNA (of the total eukaryal 18S rDNA copies present). The lithology of the core is indicated at the right.

appeared only in one sediment layer and its closest relative was *Cryothecomonas aestivales* (95% sequence similarity). All attempts to sequence the DGGE fragments just above DGGE fragment 11, indicated with a cross, failed.

[39] Q-PCR with the same primer combination selective for the domain of Eukarya (but without GC clamp) revealed that the number of 18S rDNA copies was on average 10^7 per gram of dry sediment within unit I and was 10^8 in the top layer of unit II (Figure 5c).

3.4. Biomarkers of Haptophytes

3.4.1. Fossil Alkenones of Haptophytes

[40] The methyl and ethyl ketones with 2–3 double bonds and 37 ($C_{37:2}$ mK and $C_{37:3}$ mK) or 38 carbon atoms ($C_{38:2}$ mK/eK and $C_{38:3}$ mK/eK) were the predominant alkenones found throughout the sediments of unit I, whereas all alkenones were below detection limit in the top sapropel layer of unit II (Figure 5a). Within the sediments of unit I, $C_{37:3}$ mK dominated over $C_{37:2}$ in most of the analyzed samples and their concentrations were highest at the surface and in the sediment layer which was deposited during the first invasion of *E. huxleyi* (100 to 150 µg (g dry sediment)$^{-1}$). Their concentration was lowest in the transition sapropel (30 to 48 µg (g dry sediment)$^{-1}$) (Figure 5a). $C_{37:4}$ mK was not detected in core BC53.

3.4.2. Fossil 18S rDNA of Haptophytes

[41] Q-PCR analysis using the primer set selective for haptophytes showed that 18S rDNA of haptophytes made up only a very minor fraction of the predominant eukaryotic 18S rDNA (Figures 5b and 5d). The 18S rDNA copies of haptophytes in the unit I layers were outnumbered by a factor of 150 to 2500 and a factor of 86,000 in the unit II sapropel by 18S rDNA of the other identified eukaryotic species (Figures 4 and 5c). The percentage of *E. huxleyi* 18S rDNA compared to the total eukaryotic 18S rDNA (mainly dinoflagellate DNA) in the fossil record was highest (0.3–0.7%) between 4 and 26.5 cm of unit I and only ~0.05% in the top 4 cm and ~0.13 in the transition sapropel (Figure 5d). The percentage of haptophyte DNA in the unit II sapropel was minimal, being only 0.001% of the total number of eukaryotic 18S rDNA present (Figure 5d).

[42] In order to decrease the detection limit for rare sequences of fossil haptophytes we therefore employed the same haptophyte-selective primer set during PCR but with a GC clamp for subsequent DGGE analysis. The specific amplification of 18S rDNA of haptophytes and separation by DGGE resulted in the identification of three unique phylotypes (Figures 6 and 7). DGGE fragment 1 was found basin-wide in the present-day water column at the analyzed depths between 10 and 62 m as well as throughout the Holocene sediments of unit I and in very low abundance (as determined from the faint band) in unit II. This DGGE fragment melted at the same position in the DGGE as the partial 18S rDNA of the *E. huxleyi* culture (Figure 6). Sequence analysis revealed that the predominant DGGE fragment 1 was identical to extant alkenone-biosynthesizing *E. huxleyi* species and the closely related *Gephyrocapsa* species (Figure 7). The 458-bp-long fragment of the 18S rDNA is apparently identical among the alkenone-biosynthesizing coccolithophorid haptophyte species (the genera *Emiliania* and *Gephyrocapsa*), whereas the fragment is suitable to distinguish these coccolithophorid alkenone

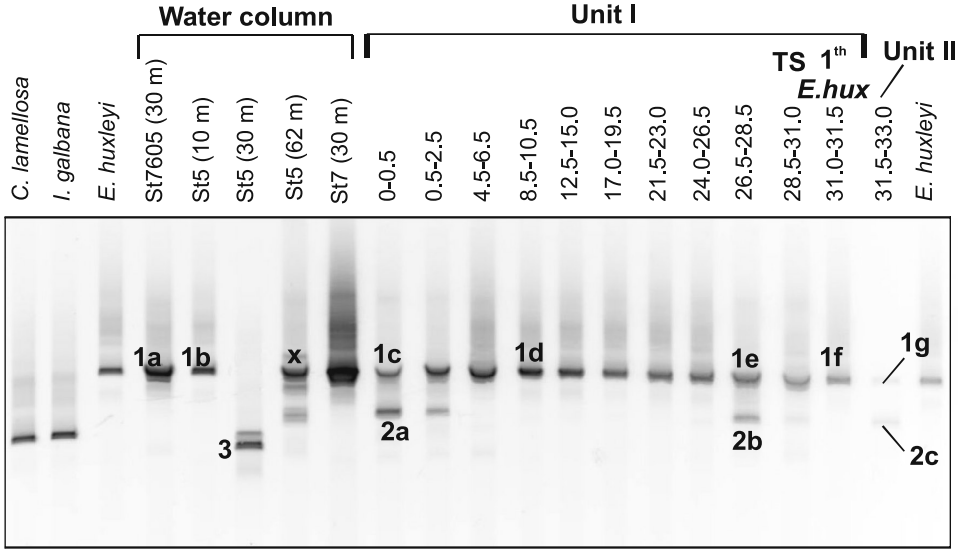

Figure 6. DGGE analysis of PCR-amplified partial 18S rDNA of haptophyte algae obtained from the water column (POM) as well as the Holocene sediments of the Black Sea. DGGE bands that were sliced from the gel and subsequently sequenced are indicated with numbers. For comparative analysis, PCR products of three reference haptophyte strains (*Emiliania huxleyi* strain Oslo Sea, *Isochrysis galbana* CCMP 1323, and *Chrysotila lamellose* HAP 17) were separated by DGGE along with the samples.

producers from noncoccolithophorid, alkenone biosynthesizing haptophyte species (the genera *Isochrysis* and *Chrysotila*) (Figure 7). The sequences of DGGE fragments 2 and 3 clustered within the order of Prymnesiales which do not contain species that are known to biosynthesize alkenones (Figure 7).

[43] The highest number of 18S rDNA copies of *E. huxleyi* was found in the coccolith layers which were deposited during the first invasion of *E. huxleyi* and the 10–25 cm section of unit I (on average 1×10^5 copies (g dry sediment)$^{-1}$ (Figure 5b). The number of *E. huxleyi* 18S rDNA copies was 5 times lower in the transition sapropel as well as in the upper 5 cm of unit I and reached the detection limit in the upper unit II layer. Nevertheless, within the upper layer of unit II, we detected 18S rDNA of *E. huxleyi* (6×10^2 copies (g dry sediment)$^{-1}$) (Figures 5b and 6), whereas alkenones were below the detection limit (Figure 5a).

[44] The correlation between the amount of fossil alkenones and the number of 18S rDNA copies of *E. huxleyi* was low ($r^2 = 0.13$). However, both type of biomarkers showed similarities in their barely detectable levels in unit II, the steep increase in concentration concomitant with the first deposition of coccoliths, a drop in concentration in the transition sapropel and again an increase in concentration during the deposition of coccoliths up to the present (Figures 5a and 5b).

3.5. Ratio Between *E. huxleyi* 18S rDNA and Alkenones in Contemporary Samples and Holocene Black Sea Sediments

[45] The ratio between the number of 18S rDNA copies of *E. huxleyi* and the amount of $C_{37:2} + C_{37:3}$ mK within POM collected from the extant photic zone of the Black Sea was 11×10^4 copies (μg $C_{37:2} + C_{37:3}$ mK)$^{-1}$ at 10 m and 14×10^4 copies (μg $C_{37:2} + C_{37:3}$ mK)$^{-1}$ at 30 m, This ratio was 1 order of magnitude higher in cells from *E. huxleyi* strain Oslo Sea. In the sediments this ratio was 3 to 4 orders magnitude lower but the ratio remained constant with depth ($16 \pm 6 \times 10^1$ copies (μg $C_{37:2} + C_{37:3}$ mK)$^{-1}$).

3.6. Fragmentation of Fossil DNA of *E. huxleyi*

[46] To investigate the natural fragmentation of fossil DNA in Holocene sediment layers of coccolith-bearing unit I, we quantified the distribution of partial 458-bp-long 18S rDNA fragments of haptophytes in various size classes of the extracted fossil DNA. Even the oldest analyzed sediment sample of the coccolith-bearing unit I sediments (24–26.5 cm) contained high molecular weight DNA of 23 to 40 kbp long (Figure 8). PCR amplifiable fossil 0.46-kbp-long fragments of the 18S rDNA of *E. huxleyi* were found in all of the analyzed DNA size classes ranging from 23–40 kbp to less than 0.7 kbp (Figure 9). 14% of the *E. huxleyi* partial 18S rDNA copies (330 copies ng template DNA^{-1}) was found in the 23 to 40 kbp size class of the youngest of all analyzed samples (sediment between 2.5–4.5 cm), whereas 3% of all *E. huxleyi* partial 18S rDNA was still present in the 23 to 40 kbp DNA size class of sample 24–26.5 cm (940 copies ng template DNA^{-1}). Between 24 and 31% of the 0.46-kbp-long *E. huxleyi* 18S rDNA was present in 4- to 40-kbp-long DNA recovered from sediment layers down to 19.5 cm, followed by a sharp decrease to only 6% within the 23–40 kbp DNA size class in the oldest sediment layer 24–26.5 cm. The percentage of 458-bp-long *E. huxleyi* rDNA within the shortest DNA fragment size class (less than 0.7 kbp) was found to be highest within layer 24–26.5 cm (52%). This showed that a substantial decline in

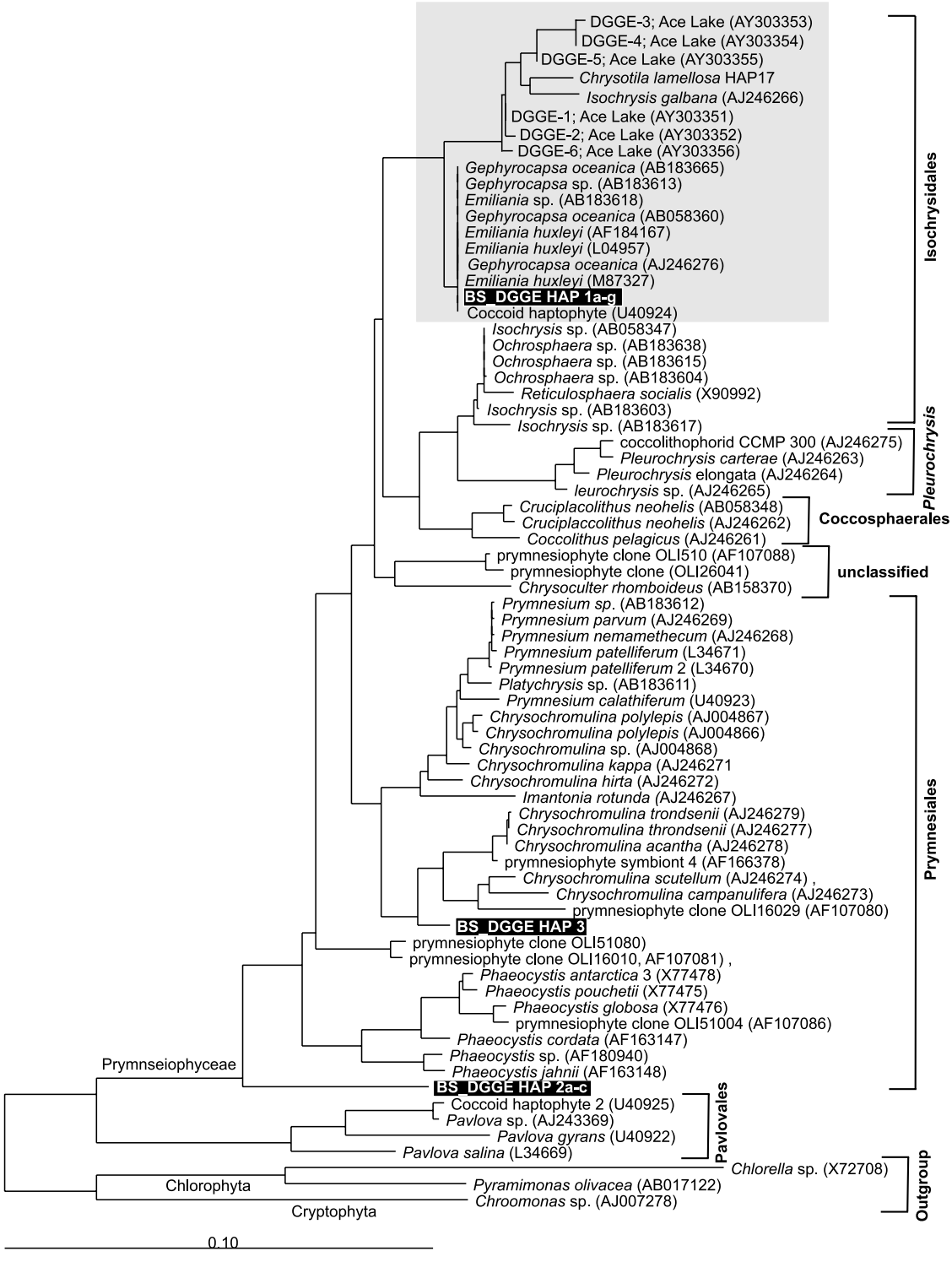

Figure 7. Phylogenetic tree showing the relationship of 18S rDNA sequences of haptophyte algae retrieved from the water column (POM) and Holocene sediment layers of the Black Sea (white text in black rectangles) to reference sequences obtained from the GenBank database. The grey box indicates the alkenone-biosynthesizing haptophytes of the order Isochrysidales. The haptophyte sequences from the Black Sea were determined from the DGGE represented in Figure 6. DGGE bands with identical melting positions within the gel appeared to contain identical sequences. For example, sequences BS_DGGE HAP 1a and 1b of the extant water column were identical to sequences found in the fossil record (BS_DGGE HAP 1c–1g).

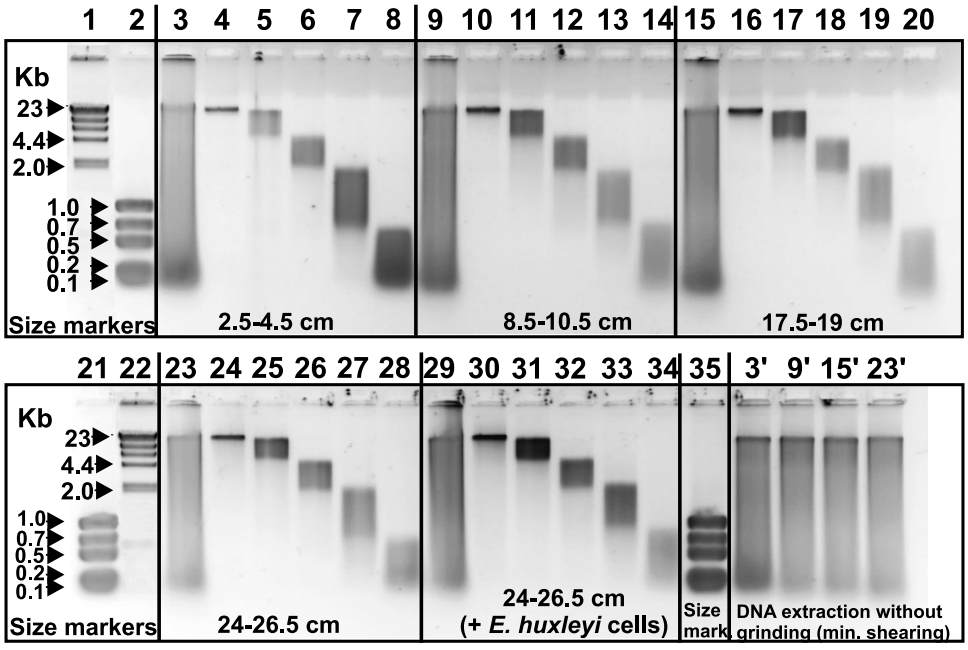

Figure 8. Agarose gel with total DNA extracts from selected Holocene unit I sediments and separated individual size classes of the extracted DNA. Fragment length rulers are lanes 1 and 22 (phage $\lambda \times$ *Hin*dIII digest) and lanes 2 and 21 (Precision Molecular Mass Standard (PMMS) BioRad). The fragment sizes of these DNA size rulers are denoted on the left and served to estimate the length of total DNA extracts as well as the individual size classes of the extracted DNA. The various total DNA extracts and individual DNA size classes were obtained from sediment layers 2.5–4.5 cm (lanes 3–8), 8.5–10.5 cm (lanes 9–14), 17.5–19 cm (lanes 15–20), and 24–26.5 cm (lanes 23–28). The total DNA extracts contained fragments between 0.1 kilo base pairs (kbp) and 40 kbp (lanes 3, 9, 15, 23, and 29). The individually analyzed DNA size classes were between 23 and 40 kbp long (lanes 4, 10, 16, 24, and 30), 4.4–23 kbp (lanes 5, 11, 17, 25, and 31), 2.2–4.4 kbp (lanes 6, 12, 18, 26, and 32), 0.7–2.2 kbp (lanes 7, 13, 19, 27, and 33), or 0.7 kbp and less (lanes 8, 14, 20, 28, and 34). In a different experiment, 10^7, *E. huxleyi* cells were added to an aliquot of sediment layer 24–26.5 cm prior to DNA extraction. The latter fresh cells of *E. huxleyi* added to layer 24–26.5 cm were expected to contain high molecular weight DNA, and the quantitative distribution of their partial 18S rDNA in the separate DNA size classes was used to monitor the level of DNA shearing due to grinding of the sample (lanes 29–34). Total DNA was also extracted from the above sediments without the grinding step to estimate whether the low molecular weight DNA was natural or was a result of DNA shearing due to grinding (lanes 3′ (2.5–4.5 cm), lane 9′ (8.5–10.5 cm), lane 15′ (17.5–19 cm), and lane 23′ (24–26.5 cm)).

fragment size of ancient *E. huxleyi* occurred at depths between 19.0 and 26.5 cm.

[47] We also tested whether the grinding step during the extraction procedure resulted in fragmentation of DNA. To this end, we quantified fossil 18S rDNA copies of *E. huxleyi* within extracted DNA size classes from sediment layer 24–26.5 cm which had not been subjected to grinding (Figure 9e). In comparison to the ground 24–26.5 cm sample (Figure 9d), the nonground sample (Figure 9e) showed a lower degree of DNA shearing. On the other hand, after known numbers of *E. huxleyi* cells with intact genomes were added to the 24–26.5 cm layer followed by grinding (Figure 9f), fragmentation was significantly smaller compared to the ground pristine sediment (Figure 9d). Without grinding, 20% of the 0.46-kbp-long 18S rDNA fragments were present in the 4.4 to 23 kbp fossil DNA size class. Since grinding was performed in a similar way for all samples (Figures 9a–9d and 9f), the influence of grinding on the observed fragmentation should also have been the same.

4. Discussion

4.1. Predominant Sedimentary Eukaryotic 18S rRNA Genes

[48] During the initial attempt to identify fossil 18S rDNA of ancient photic-zone-dwelling alkenone-producing haptophytes, we used general primers for the eukaryotic domain which selected for the most predominant 18S rDNA present. Despite the abundance of coccoliths of *E. huxleyi* in the sediment, fossil 18S rDNA of haptophytes was not detected using this general PCR approach. Instead, the most predominant fossil eukaryotic 18S rDNA sequences were derived from dinoflagellates and showed the highest similarity with those of species of the genera *Gymnodinium* and *Dinophysis* (Figures 3 and 4). In a previous study, *Gymno-*

dinium was found to comprise up to 74% of the biomass (16–23% of the phytoplankton cells) in the western and eastern regions of the southern Black Sea during the summer of 1996 [*Eker-Develi and Kideys*, 2003].

[49] Some dinoflagellate species are known to produce cysts (dinocysts) and one would assume that DNA within a cyst would be better preserved compared to DNA from a noncyst-forming species. Within unit I and unit II sediments obtained from the anoxic sulfidic part of the southern Black Sea, the number of dinocysts varied between 2000 and 8000 per gram of sediment [*Mudie et al.*, 2002]. However, species of the genera *Gymnodinium* and *Dinophysis* were not found to produce cysts in the Black Sea. On the other hand, dinoflagellate species of other genera which were found as cysts (dinocysts) in unit I and unit II layers of Black Sea sediments [*Mudie et al.*, 2001, 2002] were not identified with our nonselective PCR approach. Therefore our results showed that even after 3600 years of deposition, the fossil 18S rDNA pool of *Gymnodinium* species related to noncyst-forming dinoflagellates still outnumbered the number of 18S rDNA copies of dinocysts. Dinosterol, a known biomarker of dinoflagellates thriving in the Black Sea [*Boon et al.*, 1979], was also abundant in the unit I and II sediments (data not shown). *Gymnodinium* species are known to produce dinosterol [*Mansour et al.*, 1999, 2003] and the predominance of fossil 18S rDNA related to *Gymnodinium* species would suggest that these dinoflagellates were major biological precursors for dinosterol in the Black Sea sediments.

[50] The biomass of *E. huxleyi* was lower than for dinoflagellates but its cell number was highest of all phytoplankton between March and October of 1996 [*Eker-Develi and Kideys*, 2003]. Diatoms made up the bulk of phytoplankton biomass in spring and autumn with comparable cell numbers to *E. huxleyi* in spring [*Eker-Develi and Kideys*, 2003]. Even though *E. huxleyi* and diatoms are predominant phytoplankton members of the modern Black Sea and the nonselective primers do have target sites to the 18S rDNA sequences of *E. huxleyi* as well as most of the

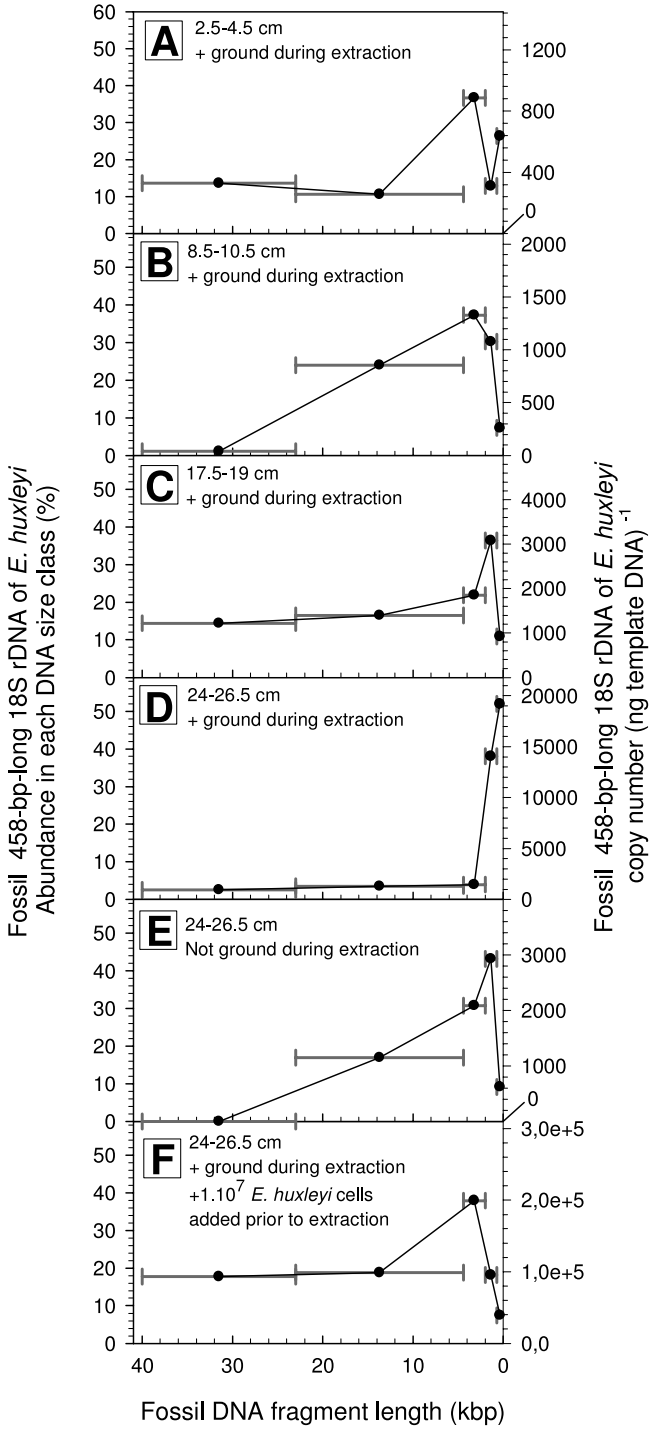

Figure 9. Fragmentation of fossil DNA of ancient *E. huxleyi*. (left) Percentage and (right) number of fossil 458-bp-long rDNA fragments per ng fossil template DNA within varying fragment size classes (less than 0.7 kbp, 0.7–2.2 kbp, 2.2–4.4 kbp, 4.4–23 kbp, and 23–40 kbp). The sediment sections used for this test were (a) 2.5–4.5 cm, (b) 8.5–10.5 cm, (c) 17.5–19 cm, and (d–f) 24–26.5 cm. A grinding step was included for efficient cell disruption and lysis except for Figure 9e. The latter experiment was performed to monitor the effect of grinding on the distribution of fossil partial 18S rDNA among the different DNA fragment lengths. Intact *E. huxleyi* cells (10^7) were added to a 4-g aliquot of the 24- to 26.5-cm sediment layer prior to DNA extraction in order to monitor the effect of grinding on the shearing of modern high molecular weight DNA (Figure 9f). An aliquot of these samples (Figures 9a–9f) was subjected to Q-PCR using haptophyte-specific primers to quantify the number of *E. huxleyi* copies. In the case of Figure 9f this Q-PCR reaction contained, besides the natural presence of fossil DNA of *E. huxleyi*, the DNA of 2×10^5 of the added cells. We do not have ^{14}C ages of the sediment layers used in this fragmentation experiment. However, the deepest layer analyzed here (24–26.5 cm) where a significant natural fragmentation of fossil DNA started to occur was located 6 cm above the oldest varved coccolith layer (first invasion of *E. huxleyi*), which is known to be deposited 3450 ± 120 years B.P. [*Hay et al.*, 1991]. Assuming that the sedimentation rate was constant during the last ~3450 years, the estimated ^{14}C age of layer 24–26.5 cm is 2700 years.

known diatom sequences, none of the identified 18S rDNA sequences were found to be haptophytes or diatoms (Figures 3 and 4). This suggests that there are unknown mechanisms that cause a species- or group-specific preservation of fossil DNA.

[51] The remaining sequences which were retrieved with the nonselective primers for the eukaryotic domain were related to heterotrophs such as ciliates, cercozoa (representing faint DGGE bands) and a sequence related to a novel lineage of fungi/metazoan (Figure 3). The latter sequence (DGGE band 1) showed 99.8% sequence homology with clone ms313 obtained from oxygenated water of the Bay of Fundy [*Savin et al.*, 2004] (Figure 4). The latter sequence was predominant only in the unit II layer and disappeared soon after *E. huxleyi* started to colonize the photic zone. Whether the biological precursor of the sequence was also adapted to thrive in the sapropel layer of unit II is unlikely as the TOC concentration did not vary greatly between the sapropel and the oldest coccolith layer or the transition sapropel (Figure 2) where 18S rDNA of this species was below the detection limit (Figure 3). Most likely, this not-yet-described species was deposited during times of sapropel (unit II) deposition. Its abrupt absence after *E. huxleyi* started to colonize the Black Sea points toward less favorable conditions possibly related to a changing salinity. Fungal remains (spores) have indeed been described from southern Black Sea sediments and found to be highest in the sapropel. The presence of fungal spores was associated with a greater freshwater/terrestrial input at the times of sapropel deposition [*Mudie et al.*, 2002].

[52] These results showed that fossil DNA of various ancient photic zone associated microorganisms survived in the Holocene sulfidic sediment record of the Black Sea. However, the mechanisms controlling the deposition of cells and the preservation of their fossil DNA is not similar for every cell type. We, furthermore, conclude that nonselective primers for, for example, the domain Eukarya cannot be used to identify specific and less abundant sequences of ancient phytoplankton.

4.2. Fossil DNA of Haptophytes in the Holocene Black Sea Sediments

[53] Since our initial attempts to identify fossil DNA of haptophytes from Holocene Black Sea sediments using a nonselective PCR for 18S rDNA of Eukarya failed, we tried to lower the detection limit by using a PCR approach selective for haptophytes [*Coolen et al.*, 2004]. Using selective primers during (Q)-PCR, we were indeed able to lower the detection limit of 18S rDNA of haptophytes to around 0.1% of the total 18S rDNA pool in unit I sediments and even to 0.001% of the total 18S rDNA pool in unit II. Using this selective and sensitive approach, a phylotype with 100% sequence similarity to sequences of extant *E. huxleyi* strains was detected in all analyzed coccolith-bearing layers of unit I, concomitant with the presence of diunsaturated and triunsaturated C_{37} to C_{39} methyl and ethyl ketones believed to be derived from *E. huxleyi*. The amount of fossil 18S rDNA of *E. huxleyi* showed a comparable concentration profile as for the fossil $C_{37:2}$ mK and $C_{37:3}$ mK (Figure 5).

[54] Despite the fact that alkenone concentrations were below the detection limit and coccoliths of *E. huxleyi* have not been reported from unit II sediments, our highly sensitive PCR-based approach resulted also in the detection of trace amounts of 18S rDNA of *E. huxleyi* in the upper 2 cm of the sapropel unit II (Figures 6 and 7). This indicated that low numbers of *E. huxleyi* started to colonize the Black Sea prior to the deposition of the oldest, very well distinguishable varved coccolith-bearing layer, and that coccoliths in the unit II sapropel were perhaps prone to dissolution which often occurs in TOC-rich sediments [*Van Os et al.*, 1994]. Using Q-PCR, we here reached the absolute detection limit with 200 copies of *E. huxleyi* 18S rDNA per gram dry sediment. This amount was equal to only $\sim 1-5$ copies in the Q-PCR reaction for which 38 cycles were needed to generate visible amounts of PCR products. A contamination with modern *E. huxleyi* can be ruled out as no visible PCR products were generated even after 45 cycles from the various control reactions. In addition, haptophyte sequence 2 which was detected also from the unit II layer, only appeared in the upper layers of sediment, and therefore a cross contamination during sampling of adjacent sediment layers is also not likely.

[55] Vertical transport of exogenous DNA (e.g., by groundwater seepage over time) has been proposed to bias the true age of ancient DNA in the fossil record [*Hoehler*, 2005; *Inagaki et al.*, 2005]. However, DNA is known to be strongly adsorbed by the sediment matrix [*Khanna and Stotzky*, 1992; *Lorenz and Wackernagel*, 1987; *Paget et al.*, 1992]. Our present combined DNA and lipid analysis showed that PCR-amplified 18S rDNA of *E. huxleyi* as well as its alkenones were barely detectable from the top 2 cm of the unit II sediment, whereas this layer was located just below the oldest varved coccolith-bearing layer of core BC 53 where both the DNA and alkenones of *E. huxleyi* became abundant. This indicated that DNA of *E. huxleyi* was indeed strongly adsorbed to the sediment matrix and that vertical transport of nucleic acids via pore water was negligible. Also during our previous studies dealing with fossil DNA in sulfidic sediments, we never found PCR amplifiable rDNA of, for example, haptophytes or photosynthetic sulfur bacteria in sediment layers where their specific biomarkers (respectively alkenones or carotenoids) were absent, whereas their rDNA could be amplified from layers with a concomitant presence of the specific lipid biomarkers [*Coolen et al.*, 2004; *Coolen et al.*, 2006; *Coolen and Overmann*, 1998]. This showed that in the various investigated sediments the contamination with vertically transported DNA was negligible.

[56] Even though 18S rDNA of *E. huxleyi*, which is not known to form resting stages, made up a relatively small number compared to the total 18S rDNA pool, the above results showed that the detection limit for ancient DNA in the fossil record can be lowered significantly by using group-specific primers during PCR. This study provides strong evidence that DNA of ancient photosynthetic algae survived degradation in a Holocene deep-sea sediment record with an overlaying sulfidic water column of more than 2000 m and was archived in the sedimentary record. This shows that even in deep-sea environments, the analysis

of fossil DNA is a promising tool to identify ancient water column derived microorganisms at the species level, and hence to reconstruct ancient paleoenvironments in unprecedented detail.

[57] Using our selective and sensitive DNA approach to analyze fossil 18S rDNA of ancient haptophytes, we found that no alkenone biosynthesizing haptophyte other than *E. huxleyi* colonized the Black Seas photic zone during the last 3600 years of deposition. This showed that for this time frame no species-specific calibration of the $U^{K'}_{37}$-based SST proxy [*Prahl and Wakeham*, 1987; *Versteegh et al.*, 2001; *Volkman et al.*, 1995] is required for the Black Sea. Unfortunately, core BC53 which we used for our analyses, did not penetrate deeper into the unit II sapropel, where *Xu et al.* [2001] reported a novel $C_{36:2}$-ethyl ketone (hexatriaconta-16E,21E)-dien-3-one) as an abundant alkenone. Since unit II sediments were deposited before *E. huxleyi* started to colonize the photic zone of the Black Sea [*Hay*, 1988], the authors assumed a possibly different biological precursor for this compound [*Xu et al.*, 2001], which could have been traced by our fossil DNA approach.

4.3. Fate of Fossil DNA of *E. huxleyi* in the Holocene Black Sea Sediment Record

[58] The fossil 18S rDNA/alkenone ratio of *E. huxleyi* was found to be 4 orders of magnitude lower than in cultured cells of *E. huxleyi* (strain Oslo Sea) and 3 orders of magnitude lower compared to particulate organic matter (POM) from the photic zone of the Black Sea. Remarkably, this ratio did not increase with increasing burial in the sediment (Figure 5). This indicated that haptophyte DNA was degraded much faster than the alkenones during the senescence of cells and subsequent transport to the sediment but that the DNA was somehow protected from further degradation in the late Holocene sediments. The lower DNA/alkenone ratio in POM than in intact cells seems to indicate that in the POM there is already a fraction of partially degraded haptophyte cell material.

[59] In previous work, we analyzed the 16S rDNA/isorenieratene ratio of obligate anoxygenic photosynthetic green sulfur bacteria (GSB) [*Pfennig*, 1989] within POM collected from various depths of the sulfidic part of the water column of the stratified Antarctic Ace Lake and compared this ratio with the ratio found in the Holocene sediment record [*Coolen et al.*, 2006]. Even in the presence of 8 m*M* H_2S, a strong decline within this ratio was observed within cells thriving in the photic sulfidic chemocline and within cells collected from the dark monimolimnion where light for anoxygenic photosynthesis was absent. However, as observed for the DNA to alkenone ratio in our present study, the ratio of DNA to isorenieratene of the GSB did not further decrease in the dark, sulfidic, and up to 9-kyr-old sediment layers of Ace Lake. Therefore the intracellular 16S rDNA of the GSB was degraded mainly within the water column before the cells became buried within the sediment record [*Coolen et al.*, 2006]. During the residence time in the dark sulfidic waters, the DNA in these decaying GSB was most likely attacked by intracellular nucleases and the presence of H_2S did not play a substantial role in protecting the intracellular DNA from being degraded. However, once adsorbed by the sediment matrix, extracellular DNA has been shown to be far less prone to attack by nucleases [*Khanna and Stotzky*, 1992; *Lorenz and Wackernagel*, 1987; *Paget et al.*, 1992]. Likely, nucleases might also be trapped in the sediment matrix and cannot reach and attack a substantial part of the adsorbed DNA. Our data from the Black Sea are in good agreement with this idea.

[60] Even fossil DNA in sulfidic sediments is, however, not likely to completely escape degradation. *Coolen and Overmann* [1998] studied fossil 16S rDNA of Mahoney Lake's predominant obligate anoxyphototrophic purple sulfur bacterium (PSB) *Amoebobacter purpureus*. They showed that even in the presence of extremely high and lethal in situ levels of H_2S of up to 60 m*M*, Holocene sediments older than 4 ka only contained up to 600-bp-long fragments and that the oldest (10-kyr-old) sediment only contained up to 400-bp-long fragments of the 16S rDNA. This revealed substantial fragmentation of fossil DNA and, because of the absence of high molecular weight (HMW) DNA indicative for the absence of intact and metabolically active prokaryotic cells, hydrolysis was deemed the most likely route for the observed fragmentation [*Coolen and Overmann*, 1998]. The up to 2.7-kyr-old Black Sea sediments contained in addition to short DNA fragments, also HMW DNA (Figure 8) and this indicated the presence of extant prokaryotes that most likely participated or perhaps still participate in the microbial degradation of fossil DNA. Nevertheless, in the 0–19 cm section of the sediment the 458-bp-long fossil *E. huxleyi* 18S rDNA occurred for a substantial part in the high molecular weight DNA size class (Figure 9). However, after 2700 years of deposition, 80% of the 458-bp-long fossil *E. huxleyi* 18S rDNA occurred in the extracted DNA size classes smaller than 4.4 kbp (Figure 9e), signifying a substantial fragmentation. Therefore the degree and onset of fragmentation of fossil DNA seems comparable in the Mahoney Lake and the Black Sea sedimentary record, despite a possible microbial degradation of fossil DNA, and a lower H_2S concentration in the Black Sea.

[61] An important question following from these findings is at what point natural fragmentation of fossil DNA starts to limit the PCR amplification of genes since this would determine the ultimate sedimentary age at which the potentially useful method of fossil DNA analysis for paleoenvironmental work can be applied. We found that the ratio between the total number of 458-bp-long fragments of *E. huxleyi* and the concentration of C_{37} alkenones did not further decrease with increasing sediment depth and age. This demonstrated that in the Black Sea sedimentary record, natural fragmentation in the first 30 cm of the record, spanning approximately 2700 years of deposition, did not yet limit the PCR amplification efficiency. In the Holocene sulfidic Ace Lake sedimentary record, the fossil 499-bp-long 16S rDNA of ancient GSB was even less prone to diagenetic alteration relative to its specific carotenoid chlorobactene as evidenced from the substantially increased fossil GSB-16S rDNA/chlorobactene ratios with increasing sediment depth [*Coolen et al.*, 2006]. This was due to rapid transformation of the carotenoid chlorobactene, which is much more prone to diagenetic reactions than the alkenones in the Black Sea

core. Therefore, from the previous study performed on Ace Lake [*Coolen et al.*, 2006] as well as our present study, it seems that in a lacustrine as well as in deep-sea Holocene sulfidic sediments, 450- to 500-bp-long fossil DNA fragments can be used for qualitative species-specific phylogenetic analysis and the quantitative analysis of ancient water column derived species.

[62] However, it is likely that the ancient DNA will become even more fragmented in older sediments and this will ultimately significantly decrease the PCR amplification efficiency. On the basis of the degree of fragmentation observed from Holocene sulfidic sediments, we expect that sediments older than Pleistocene cannot be used for ancient DNA-based phylogenetic studies. However, in order to determine this limitation, additional DNA fragmentation experiments on older sulfidic sediments should be performed.

5. Conclusion

[63] The 18S rDNA of the coccolithophorid, alkenone-biosynthesizing haptophyte *E. huxleyi* which colonized the Black Seas photic zone during the late Holocene, made up only a very small percentage of the total eukaryotic 18S rDNA within the fossil record (between 0.03 and 0.8% in the coccolith-bearing unit I and only 0.001% in the unit II sapropel). The predominant fossil 18S rDNA sequences, as revealed by Q-PCR using nonselective primers for the domain Eukarya, were derived from dinoflagellates related to *Gymnodinium* species which are predominant members of the summer phytoplankton bloom in the modern Black Sea. However, using a PCR method, selective for 18S rDNA of haptophyte species, we recovered preserved 458-bp-long 18S rDNA fragments of *Emiliania huxleyi* from Holocene sulfidic sediments from the depocenter of the Black Sea. Additional 18S rDNA sequences related to other alkenone biosynthesizing haptophytes were not detected, indicating that the *E. huxleyi* alkenone-SST calibration can be applied for at least the last 3600 years for the Black Sea. The ratio between fossil haptophyte 18S rDNA and alkenones, which are known to survive for long time periods in sediments, was 3 orders of magnitude lower compared to this ratio found in the extant water column of the Black Sea and 4 orders of magnitude lower compared to this ratio found in a *E. huxleyi* culture.

[64] During an experiment in which we quantified the number of 458-bp-long fossil 18S rDNA of *E. huxleyi* in individual size classes of the extracted fossil DNA, it was shown that significant fragmentation of ancient haptophyte DNA occurred only after ~2700 years of deposition. However, even after this time period, 200 copies of the partial *E. huxleyi* 18S rDNA per ng of fossil DNA (i.e., 3% of the total *E. huxleyi* 18S rDNA copies) were still present in the 23- to 40-kbp-long DNA fragment class and 20% of the copies were found in the 4.4- to 23-kbp-long DNA fragment size class. The presence of long DNA fragments of *E. huxleyi* and the fact that the haptophyte DNA to alkenone ratio did not significantly decrease with increasing sediment depth and age, indicated that the fossil DNA was well protected against degradation for at least 2700 years. In the unit II sapropel located just below the oldest coccolith-bearing sediment layer, biomarkers for *E. huxleyi* (alkenones, and 18S rDNA) were below or just at the detection limit. This indicated that the DNA of *E. huxleyi* was adsorbed by the sediment matrix and that vertical migration of DNA, which would otherwise have biased the true age of the ancient DNA, was very restricted. Toxic levels of H_2S, and a strong adsorption of the DNA to the sediment matrix as indicated by the low recovery of the DNA of *E. huxleyi*, are the most likely factors resulting in the excellent preservation of the fossil DNA of *E. huxleyi.*

[65] This work showed for the first time that, in addition to traditional proxies, fossil DNA of ancient photic-zone-dwelling microorganisms offers great potential to study the paleoecology and paleoenvironment in deep-sea settings in unprecedented detail. We are currently employing similar techniques to study the fate of DNA of other groups of microorganisms in the Holocene Black Sea sediments.

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