GEOLOGICA ULTRAIECTINA

Mededelingen van de
Faculteit Geowetenschappen
Universiteit Utrecht

No. 330

Validation and application of fossil DNA as a recorder of past marine ecosystems and environmental conditions

Arjan Boere

ISBN: 978-90-5744-191-2

Validation and application of fossil DNA as a recorder of past marine ecosystems and environmental conditions

Validatie en toepassing van fossiel DNA
voor de beschrijving van mariene ecosystemen
en milieucondities uit het verleden

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. J.C. Stoof,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen
op vrijdag 29 oktober 2010 des middags te 2.30 uur

door

Arjan Christiaan Boere

geboren op 3 juni 1980 te Sint Maarten

Promotor: Prof. dr. ir. J.S. Sinninghe Damsté

Co-promotor: Dr. ir. M.J.L. Coolen

This work has been financially supported by the Research Council for Earth and Life Sciences of the Netherlands Organisation for Scientific Research (NWO-ALW).

Contents

1	**Introduction**	**1**
2	**Late-Holocene succession of dinoflagellates in an Antarctic fjord using a multi-proxy approach: paleometagenomics, lipid biomarkers, and palynomorphs.**	**11**
3	**Fossil DNA preservation varies among species in an anoxic basin (Ellis Fjord, Antarctica): Source-specific variability and its potential implications for fossil DNA-based paleoecological records.**	**37**
4	**Ancient DNA derived from alkenone-biosynthesizing haptophytes and other algae in Holocene sediments from the Black Sea.**	**53**
5	**Fossil DNA-inferred ecological and environmental changes during deposition of eastern Mediterranean sapropel S1.**	**81**
6	**Preservation potential of ancient DNA in Pleistocene marine sediments: Implications for paleoenvironmental reconstructions.**	**105**
7	**Synthesis and outlook**	**131**
	References	**137**
	Summary	**165**
	Samenvatting	**167**
	Dankwoord	**171**
	Curriculum Vitae	**175**

CHAPTER 1

Introduction

General introduction and thesis outline

The discovery of DNA and its consequences

The discovery in the 20th century of deoxyribonucleic acid (DNA) has revolutionized biology. The realization that simple series of four letters (i.e., the acronyms for four types of bases, which are attached to a phosphate-sugar backbone of the DNA molecule) encode all aspects of life has changed every aspect of biology. The invention of and developments in the polymerase chain reaction or PCR (Mullis and Faloona, 1987) were paramount in the developments in the field of molecular biology. PCR is a technique in which target DNA is copied *in vitro*, in a repeating series of heating steps, and is used as a standard in all molecular biological work. In theory, even a single strand of DNA could now be amplified into millions of copies within a few hours. Application of this technology allowed the analysis of the minute amounts of DNA (e.g., single cells), but also the analysis of whole communities in marine waters, without prior culturing of individual organism (i.e., metagenomics; Yooseph *et al.*, 2007). Besides the foundation of novel biological disciplines, such as genetics and biotechnology, the developing field of molecular biology also affected the more traditional disciplines. In particular the field of taxonomy and phylogeny, which studies the evolutionary relationships between organisms, changed with the introduction of molecular biology. In the absence of diagnostic morphological features, such as often accounts for micro-organisms, differences in nucleotide positions of important house-keeping genes, which are present in all living organisms, can be used to map the evolutionary relationships of species and allows the reconstruction of the tree of life (Fig. 1, Woese, 1987). For example, based on phylogenetic analysis of the gene encoding for 16S ribosomal RNA, a structural component of the small subunit (SSU) of the ribosome, archaea were recognized as a whole new domain of life (Woese, 1987). In addition, the changes (mutations) in DNA sequences roughly accumulate at fixed rates, and the observed differences between species can be used as a molecular clock to estimate the timing of important evolutionary events in earth's history (Gottschling *et al.*, 2008; Sinninghe Damsté *et al.*, 2004; van de Peer *et al.*, 1993). In this way, molecular biology not only affected biology, but indirectly also geology, and the earth sciences at large.

Sedimentary organic matter and biomarkers

Reconstruction of the history of life on earth is partly based on the analysis of organic matter (OM) preserved within terrestrial or marine sedimentary rocks. The majority of the OM in marine sediments originates from past planktonic organisms, most of which stem from the photic surface waters. The sequestered OM is a complex mixture of biochemical compounds, which reflect the environmental and oceanographic conditions under which they were formed. While it is transported to the bottom of the ocean, OM fuels a complex foodweb of pelagic and benthic consumers and, as a result, only a small part of the OM finally reaches the seafloor and is buried

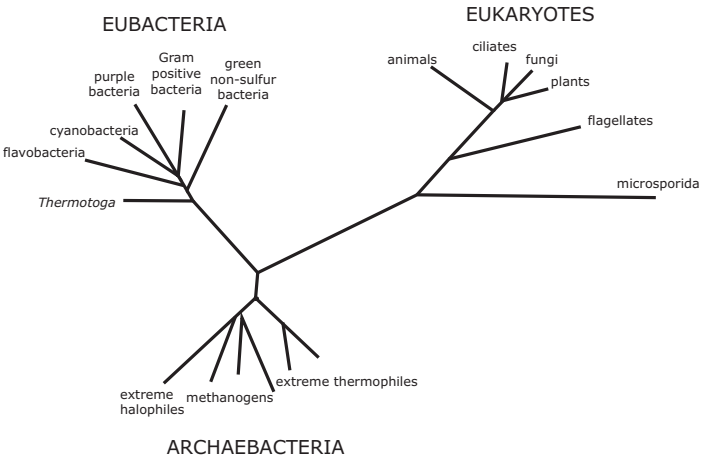


Figure 1: The original "universal phylogenetic tree determined from rRNA sequence comparisons" showing the distance between the Eukaryotes, the Eubacteria and the Archaeabacteria (later: "Archaea"). Figure redrawn from Woese (1987).

(Jørgensen and Boetius, 2007). In well-mixed and oxygenated waters, the majority of OM is efficiently remineralized in the upper few centimeters of the underlying surface sediments due to a combination of factors such as oxidation of OM, bioturbation and microbial decomposition (Middelburg, 1989; Rothman and Forney, 2007; Woulds *et al.*, 2007). In contrast, seasonal or permanent water column stratification often found in coastal areas, fjords, or closed basins, quickly results in oxygen-depleted or completely anoxic bottom waters, which promotes preservation and accumulation of fossil OM including refractory as well as more labile organic compounds (Hedges and Keil, 1995; Moodley *et al.*, 2005).

Organic compounds, such as lipids and pigments, preserved in aquatic sediments form a valuable archive of biological input from the overlying water column, especially if their occurrence in nature is restricted to a limited group of organisms, so they can be used as a 'biomarker' for the presence of this specific source organism. Under optimal preservation conditions, lipid biomarkers (or its diagenetic derivatives) can be preserved in sediments for up to billions of years (Brocks *et al.*, 2005; Brocks and Banfield, 2009; Kuypers *et al.*, 2001; Summons and Powell, 1986). Information encoded in the distribution and isotopic composition of these compounds can be used to infer past surface ocean productivity, and its response to climate variability (Brassell, 1993). For example, long-chain alkenones, specific lipid biomarkers of haptophyte algae (Volkman *et al.*, 1980), are of great interest to paleoceanographers because of the strong correlation between the degree of unsaturation in the C_{37} methylketones (expressed as $U_{37}^{K'}$) and growth temperature, paving their use as molecular proxies of past sea surface temperature (SST) (Brassell *et al.*, 1986; Eglinton *et al.*, 1992; Farrington *et al.*, 1988; McCaffrey *et al.*, 1990; Prahl and Wakeham, 1987). A range of

algal biomarkers have now been identified that provide insights into the distribution of past primary producers (e.g., Boon *et al.*, 1979; Brassell, 1993; Marlowe *et al.*, 1984; Sinninghe Damsté *et al.*, 2004; Volkman, 2003) and provide valuable information on the variations in surface ocean conditions that control phytoplankton assemblages and productivity. Lipid-based records are particularly valuable in cases where primary producers do not biomineralize or where their morphological remains are not well preserved in the sedimentary record, i.e., due to carbonate dissolution (Crudeli *et al.*, 2004; Principato *et al.*, 2006; van Os *et al.*, 1994).

The ability to measure isotopic compositions of lipid biomarkers proved to be useful in verifying the biological sources and physiological roles of lipids (e.g., Hayes *et al.*, 1990; Pearson *et al.*, 2001). Environmental information encoded within molecular isotopic signatures is now beginning to be uncovered, with a notable recent example being the use of hydrogen isotopic composition (δD, ‰) of aquatic photoautotrophs as a paleotracer of temperature and moisture-driven evaporation/precipitation balances in lake systems or salinity variations in the ocean (Englebrecht and Sachs, 2005; Huang *et al.*, 2002; Sauer *et al.*, 2001).

Although the development and application of lipids as molecular proxies continues to grow, the interpretation of these molecular stratigraphic records is often complicated by the limited specificity of many, if not most lipid biomarkers. For example, although alkenones encountered in Late Quaternary sediments are typically attributed to *Emiliania huxleyi*, several other haptophyte species are known alkenone-producers. Each of these appear to have unique (but still systematic) unsaturation vs. SST relationships (e.g., Volkman *et al.*, 1995), with obvious implications for paleoclimate reconstruction. Even in the case where lipids are believed to be highly specific, they may be biosynthesized by a number of phylogenetically closely-related species. For example, highly branched isoprenoid (HBI) alkenes are biosynthesized by several species within four diatom genera (Sinninghe Damsté *et al.*, 2004).

In general, our understanding of the validity and specificity of lipid biomarkers as tracers of planktonic organisms is still limited due to the low number of cultivated species available for characterization.

Fossil DNA as a biomarker?

Over the last two decades, the field of microbiology has witnessed a revolution in the understanding of the diversity of life in the oceans through the development and application of molecular biological techniques that directly probe the genetic machinery of life - DNA. The gene encoding for SSU rRNA (i.e., 16S rDNA in prokaryotes and 18S rDNA in eukaryotic cells) is the most widely used phylogenetic marker for identification of individual species in each of the three domains of life. In comparison, taxonomic differentiation at the species-level is impossible for the majority of lipid biomarkers. In contrast to lipids, the preservation of DNA in marine sediments on longer timescales was believed to be impossible, as a result of the inherently fragile nature of the DNA-molecule (Fig. 2) and the rapid microbial recycling. In hydrated form, DNA is also known to be prone to various abiotic (hydratation, oxidation, UV-

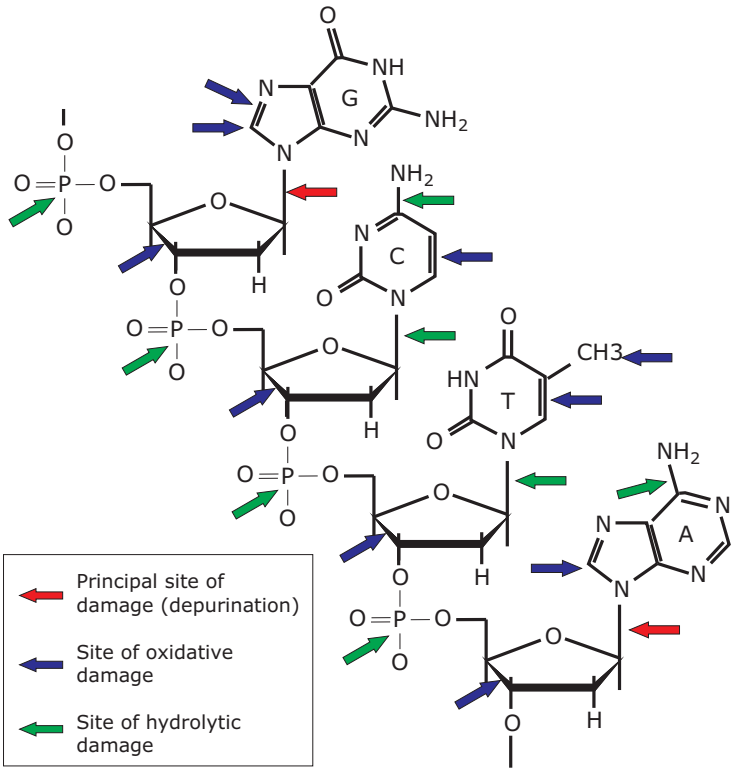


Figure 2: Sites of possible spontaneous chemical damage in a short stretch of DNA with the four bases guanine (G), cytosine (C), thymine (T) and adenine (A), illustrating the fragile nature of the DNA molecule. Figure from Hofreiter *et al.* (2001), modified after Lindahl (1993).

damage) and biotic (microbial nucleases) degradation processes, most notably leading to fragmentation and fewer template for PCR (Hebsgaard *et al.*, 2005; Lindahl, 1993; Willerslev and Cooper, 2005). In contrast, ancient DNA was shown to be well preserved in dry, cold, or enclosed ancient specimens such as amber, minerals, fossil bones, permafrost soils, and archeological artefacts (Cano and Borucki, 1995; Millar *et al.*, 2008; Mitchell *et al.*, 2005; Pääbo, 1989; Willerslev and Cooper, 2005).

Due to the lability of especially hydrated DNA, it was generally believed that labile biomolecules such as DNA would not survive burial within aquatic sediments, and that only extant species could be identified. Their utility as molecular proxies of paleoceanographic processes was therefore considered low. Contrary to this paradigm, a strong DNA adsorption to mineral and organic matrices in sediments is thought to be responsible for an enhanced preservation due to the protection against degradation by microbial nucleases. Either the DNA-degrading proteins (DNases) are

bound to reactive surfaces in the sediment and are inactivated, or the DNA itself is adsorbed and can no longer be reached by the nucleases (Lorenz and Wackernagel, 1987; Nguyen and Elimelech, 2007; Pietramellara *et al.*, 2009; Romanowski *et al.*, 1991; Schelble *et al.*, 2008). Estimates of the percentage of bound, and thus well-protected DNA, range from $50\% - 95\%$ of the total extracellular DNA (Danovaro *et al.*, 2006; Dell'Anno *et al.*, 2002). Moreover, DNA, and OM in general, is better preserved in stratified environments with anoxic bottom waters due to slower microbial activities, the absence of post-depositional oxidation of OM, and the absence of bioturbation. These, and additional factors such as low temperature, high pressure and high salinity, which also decrease the degradation rates of extracellular DNA in marine environments (Borin *et al.*, 2008; Coolen *et al.*, 2004a, 2007; Corinaldesi *et al.*, 2008; Danovaro *et al.*, 2005; Lindahl, 1993; Lorenz *et al.*, 1981; Panieri *et al.*, 2010; Romanowski *et al.*, 1991; Schelble *et al.*, 2008; Vreeland *et al.*, 2000), show that there is a considerable potential for the preservation of ancient or fossil DNA in sediments under various anoxic marine settings.

The analysis of DNA preserved in sediments ("Paleogenetics")

Several recent studies have demonstrated the successful detection and analysis of fossil DNA in a variety of marine and lacustrine sediments in the last decade. The first report about fossil DNA came from a meromictic (permanently stratified) anoxic lake (Mahoney Lake, BC, Canada; Coolen and Overmann, 1998). In C_{org}-rich Mahoney Lake sediments as old as the early Holocene ($\sim$11,000 yr before present), 16S rRNA gene sequences of obligate photoautotrophic purple sulfur bacteria were identified and compared to the group-specific carotenoid okenone (Coolen and Overmann, 1998). Since then, fossil DNA has also been successfully identified in lakes in the Vestfold Hills of eastern Antarctica, where several isolated saline lakes have become meromictic and developed anoxic bottom waters (Bird *et al.*, 1991; Gibson, 1999; Laybourn-Parry and Pearce, 2007). For instance, up to 10,450-year-old anoxic C_{org}-rich sediments of Ace Lake were shown to contain well preserved DNA and diagnostic lipid biomarkers of marine protists (diatoms and haptophytes). Using fossil DNA, it was possible to reconstruct the impact of postglacial hydrologic changes in Ace Lake, that evolved from a freshwater lake, to a marine inlet, and subsequently to the current isolated saline lake (Fig. 3), based on the Holocene ecology of haptophytes and diatoms (Coolen *et al.*, 2004b). In addition, a parallel stratigraphic analysis of lipids, carotenoids, and DNA was used to reconstruct micro-organisms that were involved in cycling of sulfur (anoxygenic photosynthetic green sulfur bacteria) and methane (Type I and II aerobic methanotrophic bacteria) in the, respectively, ancient sulfidic chemocline and the overlying suboxic zone of Ace Lake during its postglacial development (Coolen *et al.*, 2004a, 2006a, 2007, 2008). Sediments from a basin located within the nearby Ellis Fjord (i.e., small meromictic basin, SMB) were successfully analyzed to identify the precursor diatom species of characteristic highly branched isoprenoid lipids (Coolen *et al.*, 2007). Other meromictic lakes from both eastern and western Antarctica contained well preserved DNA of copepods (Bissett *et al.*, 2005),

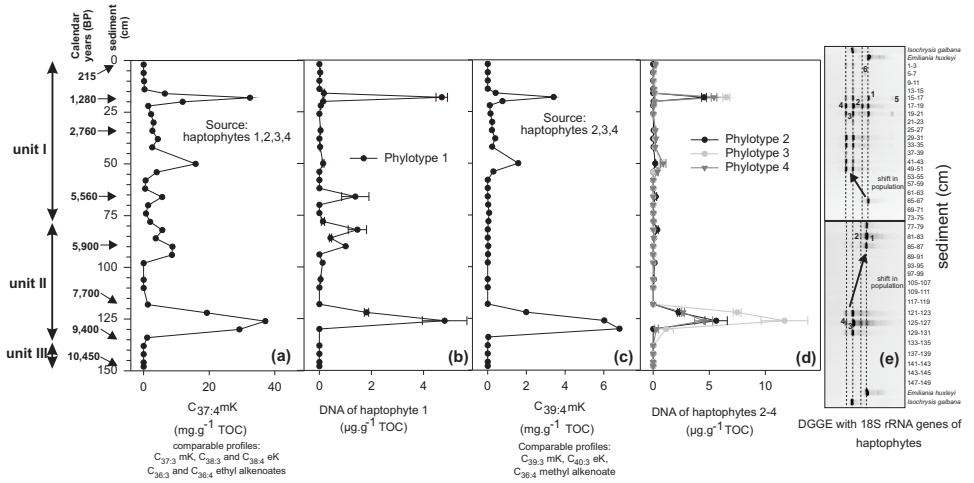


Figure 3: The postglacial development of Ace Lake, Antarctica reflected in the fossil DNA of haptophytes and associated $C_{36}-C_{40}$ long-chain alkenones. Ace Lake went from an freshwater basin (unit III) to a marine inlet (unit II), and finally became a lacustrine saline lake system (unit I). DNA of various groups of haptophytes were detected in the separate sedimentary intervals. Figure modified from figure 4 in Coolen *et al.* (2004a).

and by analyzing fossil DNA in meromictic lakes on Greenland, a new cluster of haptophytes was identified as the precursor haptophyte species of alkenones (D'Andrea *et al.*, 2006).

To date, only C_{org}-rich sediments underlying anoxic bottom waters have been used to reconstruct past planktonic communities, whereas the preservation of DNA is expected to be lower in, for example, deep-sea sediments that are low in C_{org} content as a result of extensive mineralization processes. In addition, it is currently unknown whether fossil plankton DNA is preserved in marine sediments at geological time scales beyond the Holocene era. Information is also lacking about whether fossil plankton DNA is equally well preserved in different lineages.

Scope and framework of the thesis

This thesis has two major objectives. The first objective was to validate fossil DNA as a paleoecology proxy, and the second objective was to reveal to what extent fossil DNA can aid in improving paleoenvironmental information inferred from traditional proxies. Hence, answers were sought for the following key questions:

1. Are their source-specific variations in the level of DNA preservation?

2. Which settings are suitable for fossil DNA analysis?

3. Does fossil DNA accurately reflect the past plankton composition?

4. Can fossil DNA be used to validate paleoecological and paleoenvironmental information from more traditional proxies?

5. How far back in time can we go? Is the analysis of fossil DNA of eukaryotic plankton members feasible for marine sediments deposited before the Holocene era?

Each chapter deals with different aspects in validating the fossil DNA proxy, whereas chapters 2, 4 and 5 also describe the use of fossil DNA as a paleoenvironmental proxy.

Chapter 2 describes a multiproxy approach (fossil DNA, sterols, and cysts) to study 2700 years of dinoflagellate succession in the permanently stratified and anoxic Antarctic Ellis Fjord. Overall, dinocysts were rare and the paleogenetics approach revealed the highest diversity of late-Holocene dinoflagellates in Ellis Fjord and was the only approach that recorded a shift in dinoflagellate composition at ∼1850 years ago indicative of a colder climate with more extensive ice cover.

Chapter 3 describes species-specific variation in the level of DNA preservation in Ellis Fjord and demonstrates that despite DNA being the best biomarker to reconstruct late-Holocene dinoflagellate succession in Ellis Fjord, 500 base pair (bp)-long fossil DNA fragments of a phylotype related to *Polarella glacialis*, in the absence of protective cysts, was degraded significantly faster as compared to similar-sized DNA of cyst-forming diatoms (*Chaetoceros*), whereas fossil DNA of past photosynthetic chemocline bacteria (green sulfur bacteria) was best preserved.

Chapter 4 shows that besides shallow anoxic lakes and fjords ($10-100$ m deep), the much deeper (>2 km deep) anoxic and sulfidic bottom waters and sediments of the Black Sea also provide good conditions for the preservation of fossil DNA. We demonstrated that fossil DNA and diagnostic lipid biomarkers (long-chain alkenones) of the calcareous haptophyte *Emiliania huxleyi* were equally well preserved for at least 3600 years. Alkenones are widely used to estimate ancient sea surface temperatures (SST), but the calibration of this paleothermometer is dependent on the source species. Fossil DNA showed that *E. huxleyi* has been the most important haptophyte species for the last ∼3600 yr, and that the SST-calibration based on *E. huxleyi* can be used for that period.

Chapter 5 describes a multiproxy approach to reveal the extent to which fossil DNA of planktonic protists was preserved in eastern Mediterranean sediments, and whether the paleogenetic data reflect known environmental changes that occurred during the formation of the Holocene sapropel S1 ($9.8-5.7$ ^{14}C kyr BP). A relatively high resolution stratigraphic sampling of a S1 sapropel recovered near the Nile fan (1630 m water column depth) and phylogenetic analysis of preserved ∼500-bp-long partial 18S rRNA genes of dinoflagellate and haptophytes revealed a number of distinct shifts in the paleocommunity, reflecting the changing paleoceanographic conditions during deposition. Low concentrations of lipid biomarkers diagnostic for dinoflagellates and haptophytes (i.e., dinosterol and long-chain alkenones), but no detectable 500-bp-long fossil rDNA fragments of these protists was present in the

C_{org}-depleted marls flanking the S1, thereby limiting paleogenetics of the relatively long DNA fragments to the C_{org}-rich sapropelic sediments.

Chapter 6 deals with the questions whether fossil planktonic microbial eukaryotes DNA can be recovered from marine sediments beyond the Holocene era. For this study we analyzed the general diversity of eukaryotes in the late-Pleistocene sapropels S3 (∼80 ka), S4 (∼100 ka) and S5 (∼125 ka). The parallel organic geochemical and molecular biological survey revealed a predominance of marine fungi and that late-Pleistocene DNA of terrestrial plants that entered the Mediterranean Sea via river run-off was better preserved than DNA of marine protists.

In summary, the studies revealed that despite species-specific variation in the level of preservation, the use of fossil DNA is a valuable tool to reconstruct qualitative Holocene plankton succession including non-fossilizing environmental indicator taxa species that would escape identification using more traditional paleoecological approaches. Fossil planktonic DNA stratigraphy seems to be most suitable in Holocene organic matter (OM)-rich sediments that were deposited as a result of restricted bottom water ventilation and/or increased export of primary produced organic matter since older comparable sediments mainly contained (probably viable) marine fungal DNA or DNA of terrestrial plants introduced from coastal catchment areas. Additional geochemical evidence for a preferred preservation of plant DNA in late-Holocene OM-rich sediments could nevertheless be promising to reconstruct coastal vegetation succession through time. Furthermore, sediments that experienced long-term exposure to oxidative mineralization of OM appear to be unsuitable for fossil DNA studies.

CHAPTER 2

Late-Holocene succession of dinoflagellates in an Antarctic fjord using a multi-proxy approach: paleometagenomics, lipid biomarkers, and palynomorphs.

Arjan C. Boere, Ben Abbas,
W. Irene C. Rijpstra, Gerard J. M. Versteegh,
John K. Volkman, Jaap S. Sinninghe Damsté,
Marco J. L. Coolen

Geobiology 7(3), 2009

Abstract

Recent work has shown that paleoenvironmental genomics, i.e. the development and application of genomic tools to analyze preserved genetic signatures in sedimentary records, is a promising approach to reconstruct the diversity and relative abundance of past planktonic communities. This, in turn, provides information about past ecological and environmental changes from climate variability or other perturbations. A major advantage of this approach is that individual species can be separately identified and quantified, including those that did not leave more traditional signatures (such as microfossils or specific lipid biomarkers) in the record. In this study, we determined which fossil dinoflagellate marker (i.e., 18S ribosomal DNA, sterol biomarkers or dinoflagellate cysts) provided the most detailed information about the late-Holocene succession of dinoflagellates in an Antarctic Fjord (Ellis Fjord, Vestfold Hills). The preserved rDNA revealed two key-intervals in the 2750-year-old sediment record. The dinoflagellate diversity was highest until ∼1850 cal. yr BP and included phylotypes related to known producers of the characteristic dinoflagellate sterol marker dinosterol. A lower concentration of dinosterol in sediments ca. <1850 cal yr BP coincided with a community shift towards a predominance of the Antarctic autotrophic sea-ice dinoflagellate *Polarella glacialis*, which is not a source of dinosterol. Remarkably, cultures of *P. glacialis* isolated from nearby sea-ice at Davis Station are known to produce other diagnostic sterols, but these were not recovered from the investigated core. In addition, conspicuous resting cysts of *P. glacialis* were not preserved in the analyzed sediments. Overall, dinocysts were rare and the paleoenvironmental genomics approach revealed the highest diversity of late-Holocene dinoflagellates in Ellis Fjord and was the only approach that recorded a shift in dinoflagellate composition at ∼1850 cal. yr. BP indicative of a colder climate with more extensive ice cover - this timing coincides with a period of known changing climate reported from this region.

Introduction

A standard approach to study past phytoplankton communities and their abundance is the microscopic determination and quantification of morphological remains. However, these paleoecological studies are restricted to taxa that leave easily identifiable morphological remains such as haptophyte coccoliths, frustules and cysts of diatoms, or dinoflagellate cysts (i.e., dinocysts), whereas no fossil record exists for most planktonic species. For example, diatom frustules are prone to dissolution and fragile species may not leave behind identifiable intact frustules (Kemp *et al.*, 1999). Also, many dinoflagellate taxa do not produce cysts (Head, 1996), or others produce calcareous cysts which are prone to aerobic degradation (Zonneveld *et al.*, 1997, 2001). In the absence of identifiable morphological remains, organisms can also be identified based on chemical fossils, so-called lipid biomarkers, including membrane lipids or pigments (Volkman, 2003). However, the specificity of sterol lipid biomarkers is

in many cases relatively low and rarely useful at the species-level (Volkman, 2003). In addition, many organisms lack specific lipid biomarkers or their lipid composition has not yet been studied. Recent studies showed that, under optimal preservation conditions, Holocene planktonic members could be identified at species-level based on preserved genetic signatures or "fossil DNA" (Coolen and Overmann, 1998; Coolen *et al.*, 2004a,b, 2006b, 2007, 2008; D'Andrea *et al.*, 2006; Manske *et al.*, 2008), even though there was no fossil record of microfossils. However, few attempts have been made to compare information about past planktonic community structures inferred from the combined analysis of fossil genes, lipids and palynomorphs (Coolen *et al.*, 2006b), and to test the fidelity of each of these paleoecological tools.

Dinoflagellates are ideal candidates to cross-validate each of these proxies. First of all, together with diatoms and coccolithophores, they form the dominant marine eukaryotic phytoplankton contributing to primary production in modern marine and estuarine settings (Marret and Zonneveld, 2003). In polar marine environments, diatoms are often numerically dominant, but in terms of total biomass dinoflagellates are often the most important constituents (Andreoli *et al.*, 1995). Secondly, dinoflagellates have been studied in both ancient and modern settings. Their organic, calcareous or siliceous walled cysts have long been used in biostratigrapy and, therefore, much is known about their distribution and their relationship to environmental conditions (e.g., Dale, 2001; de Vernal *et al.*, 2001; Marret and Zonneveld, 2003; Sangiorgi *et al.*, 2005; Sluijs *et al.*, 2005). However, not all dinoflagellates produce cysts and diagnostic calcareous cysts are in particular prone to dissolution especially in anoxic and sulfidic organic carbon-rich sediments (Zonneveld *et al.*, 2008). Thirdly, the lipid composition of the class Dinophyceae is relatively well known (see Volkman *et al.*, 1998, for a review). In particular, the structural variability of sterols in combination with the selective occurrence among the various orders of dinoflagellates qualifies sterols as valuable chemotaxonomic biomarkers (Leblond and Chapman, 2002; Volkman, 2003). However, some sterols are not fully diagnostic as they are also found in certain diatoms, haptophytes and unicellular red algae (Volkman, 2003). The sterol considered most characteristic for the dinoflagellates is 4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol, also referred to as "dinosterol". This biomarker is widely used to estimate the contribution of dinoflagellates to sedimentary organic matter (Boon *et al.*, 1979; Robinson *et al.*, 1984; Sangiorgi *et al.*, 2005; Mouradian *et al.*, 2007), despite the fact that dinosterol is not biosynthesized by all dinoflagellates and is synthesized in varying quantities by different dinoflagellate species (Volkman, 2003). Cross validation of the genomical, geochemical and palynological proxies should thus be possible by studying past dinoflagellate communities based on fossil DNA, sterols, and dinocysts, respectively.

Promising locations for cross-validating the three dinoflagellate proxies are found in the Vestfold Hills, Eastern Antarctica. This ice-free oasis is home to a number of saline lakes and fjords many of which are permanently stratified and contain anoxic or sulfidic bottom waters and sediments (Gallagher *et al.*, 1989; Gibson, 1999; Coolen *et al.*, 2004b, 2007). Low temperatures and long-term anoxia have provided excellent preservation conditions for deposited organic matter including lipid biomarkers

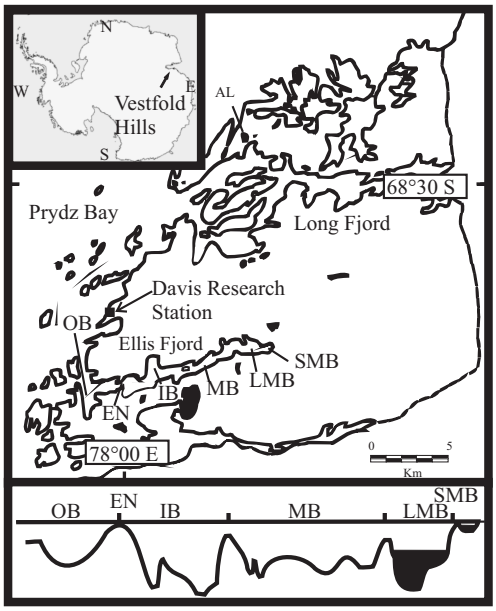


Figure 1: Map of the Vestfold Hills showing selected fjords and saline lakes (black) and a cross-section of Ellis Fjord showing the various basins. Black areas in the cross-section represent permanently stratified bottom waters. Map and cross-section are modified from Gallagher and Burton (1988). Abbreviations used: AL: Ace Lake; OB: Outer Basin; EN: Ellis Narrows; IB: Inner Basin; MB: Middle Basin; DMB: Deep Meromictic Basin; SMB: Small Meromictic Basin.

(Schouten *et al.*, 2001), and DNA in particular (Coolen *et al.*, 2004b,a, 2007, 2008). For example, in a previous general eukaryotic 18S rDNA-based survey in sediments of Ellis Fjord, one of the fjords in Vestfold Hills containing a series of permanently anoxic basins, Coolen *et al.* (2007) identified fossil 18S rDNA of the free-living autotrophic sea-ice dinoflagellate Polarella glacialis (Montresor *et al.*, 1999; Thomson *et al.*, 2004) among the various eukaryote DNA sequences. However, fossil 18S rDNA of *P. glacialis* could only be retrieved from the near-surface sediment layers of the Small Meromictic Basin (SMB) of Ellis Fjord with a general polymerase chain reaction (PCR) approach (Coolen *et al.*, 2007). This observation raised the question whether *P. glacialis* only recently colonized the sea-ice in the Ellis Fjord area or whether diagenetic processes prevented the recovery of dinoflagellate DNA from deeper and older sediments. *P. glacialis* does produce organic-walled resting cysts (Montresor *et al.*, 1999) and laboratory cultivars of this dinoflagellate isolated from sea-ice at nearby Davis Station (Fig. 1) produce a diagnostic sterol profile, but no dinosterol (Thomson *et al.*, 2004), potentially allowing a direct comparison between preserved genetic signatures, cysts, and sterols.

In this study, we developed dinoflagellate-specific primers in order to lower the

detection limit for preserved dinoflagellate 18S rDNA. This approach was then used to study the late-Holocene succession of dinoflagellates in Ellis Fjord's SMB and the results were compared with data from the more traditional geochemical (i.e., sterols) and palynological (i.e., dinocysts) proxies. In addition, we compared the timing of important dinoflagellate community shifts with the timing of late-Holocene eastern Antarctic climate changes and changing hydrological conditions (McMinn *et al.*, 2001; Roberts *et al.*, 2004).

Methods

Site description and sample collection

The Vestfold Hills in Eastern Antarctica is an ice-free oasis that is home to a number of saline lakes and fjords. The lakes have gradually become isolated from the ocean as a result of the post-glacial isostatic uplift of Antarctica (Gallagher and Burton, 1988). After isolation, a number of these lakes became meromictic (permanently stratified) basins with anaerobic and sulfidic bottom waters and organic carbon-rich sediments in which even labile molecules such as DNA from past plankton members are well preserved (Coolen *et al.*, 2004a,b, 2006a, 2007). Such meromictic and anoxic basins also occur in the ∼12-km-long Ellis Fjord (Fig. 1) including the shallow, distal-most Small Meromictic Basin (SMB), which was sampled for this work. In Ellis Fjord, exchange with the ocean is limited by the shallow sill at the mouth of the fjord (Ellis Narrows, <4 m depth) and prolonged sea-ice cover. SMB is in an advanced state of transition from a fjord system to a meromictic saline lake (Gallagher and Burton, 1988). In Ellis Fjord, the current (Grey *et al.*, 1997) and ancient (McMinn *et al.*, 2001; Sinninghe Damsté *et al.*, 2007) environmental conditions have previously been studied in detail. Unlike nearby Ace Lake, which shows a variable distribution of Holocene planktonic taxa as a result of different hydrological periods during its post-glacial development (Coolen *et al.*, 2004b,a), sedimentation within Ellis Fjord has remained stable over the past ∼4500 years (Bird *et al.*, 1991). In particular, previous fossil DNA analysis indicated that the SMB in Ellis Fjord apparently has not experienced any pronounced community shifts during at least the last ∼2700 years (Coolen *et al.*, 2007).

The samples from Small Meromictic Basin used in this study have been described in detail by Coolen *et al.* (2007) and Sinninghe Damsté *et al.* (2007). In brief, the samples were taken from the depositional center (13 m depth; 68.59702°S 78.22762°E) of the SMB in November 2000. Particulate organic matter (POM) samples were taken from six water depths using a 5 L Niskin bottle. The sampling depths (measured from the ice surface) in the water column were (1) oxic mixolimnion ($1.7-2.2$ m; $5.2-5.7$ m; $8.7-9.2$ m), (2) anoxic and sulfidic chemocline ($9.7-10.2$ m), and (3) anoxic and sulfidic monimolimnion ($10.7-11.2$ m, $12.5-13.0$ m). In addition, a 113-cm-long sediment core with a diameter of 5 cm was obtained using a gravity corer, and stored at −20°C. The upper 20 cm of the sediment core was sliced into 1 cm horizontal

sections and used for analysis. The sediment deeper than 20 cm was sliced into 2 cm sections and every other section was used for further analysis. Calibrated ^{14}C ages of six sediment layers of this core have been reported previously (Sinninghe Damsté *et al.*, 2007).

DNA extraction

The extraction of DNA from the particulate organic matter (POM) filters and the sediment core has been described by Coolen *et al.* (2007). The same DNA extracts were used in this study. In short, total DNA was extracted from ∼0.25 g of sediment using the UltraClean Soil DNA Kit (Mobio, Carlsbad, CA, USA). Filtered POM was extracted from pre-sliced whole filters according to Wuchter *et al.* (2006). Details concerning our standard steps taken to prevent contamination with foreign/modern DNA have also been described previously Coolen *et al.* (2006b).

Primer design

In order to select for dinoflagellate 18S rDNA, we developed a specific reverse primer (Dino-Rev: 5'-ACAAGACATGGATGCCCT-3') using the Probe design function implemented in ARB (Ludwig *et al.*, 2004). The combination of EukA (5'-AACCTGGTTGATCCTGCCAGT-3'; Medlin *et al.*, 1988) and Dino-Rev amplifies an approximately 465-base pair-long fragment. In order to prevent complete denaturation of the PCR products during subsequent analysis by denaturing gradient gel electrophoresis (DGGE), a GC-clamp (Muyzer and Smalla, 1998) was attached to the newly developed reverse primer. The specificity, optimal annealing temperature and running conditions of this dinoflagellate-specific primer set were checked by using genomic DNA of two dinoflagellate strains (*Alexandrium minutum* and *Scrippsiella* sp.; both in culture at the Royal NIOZ). These control strains were also run on DGGE, cut out and sequenced. None of the sequences recovered from the sediment was closely related to these control strains. Genomic DNA of the diatoms *Chaetoceros* sp. and *Dickeia ulvacea* served as a control for the primer specificity, and did not yield a product, or at least after many more cycles as opposed to the positive control templates.

Polymerase chain reaction (PCR) amplification of selective SSU rDNA

Amplification of dinoflagellate SSU rDNA by PCR was performed on an iCycler system (Biorad, Hercules, CA.). The amplification involved initial denaturing for 4 min at 95°C, followed by 38 cycles including denaturing (30 sec at 94°C), 40 sec of primer annealing at 63.5°Cand primer extension (40 sec at 72°C). A final extension was performed at 72°C (30 min). The reaction mixture was similar to the SYBR Green assay described previously (Coolen *et al.*, 2006b). The only difference was that a 10-fold dilution series with known copy numbers of the full length 18S rDNA of the

dinoflagellate Scrippsiella sp. obtained from the NIOZ culture collection was used to calibrate the copy numbers in the environmental samples. This control species was also analyzed on DGGE and subsequently sequenced to check for cross-contamination. In order to prevent contamination of the pristine sediments, DNA extracts, or PCR reagents, these calibration reactions were prepared in a PCR workstation located in a spatially separated post-PCR lab. Each PCR amplification series included reactions without DNA template in order to monitor contamination with foreign DNA during the pipetting of the reaction mixture components. In addition, we performed DNA extraction without the presence of sediment. One μl of those extracts were subjected to PCR as a control for contamination during the steps involved in DNA extraction and purification (i.e. "extraction controls").

The partial PCR-amplified dinoflagellate 18S rDNA was separated by DGGE (Muyzer and Smalla, 1998) using conditions as described by Coolen *et al.* (2006b). The gels contained a linear gradient of denaturant between 20 and 50%. In all cases, electrophoresis was run for 5 h at 12.5 V cm^{-1} and 60°C. All processes after electrophoresis including sequence analysis of excised DGGE fragments have been described previously (Coolen *et al.*, 2006b). A total of 40 bands with unique as well as identical melting positions were excised from the gel and sequenced.

Phylogenetic analysis of excised DGGE bands

Sequences were imported into the ARB software package (Ludwig *et al.*, 2004) and were aligned using the ARB FastAligner with full length 18S sequences of closest relatives provided in the SILVA database (Pruesse *et al.*, 2007) as well as additional sequences obtained from GenBank. Preliminary phylogenetic analyses (Distance Matrix, Maximum Likelihood and Maximum Parsimony) were run using ARB (version December 2007). Subsequently, a number of sequences (66 species, 1779 informative sites total) were selected to estimate the best model of nucleotide substitution using PAUP* 4.0b10 (Swofford, 2003) and MODELTEST3.7 (Posada and Crandall, 1998). Finally, the selected model (GTR+G+I) was implemented in a Bayesian analysis using MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001). With Perkinsus marinus defined as the outgroup, two heated four-chain Metropolis-coupled Monte Carlo Markov Chains were run in parallel for a total of 2,000,000 generations and were sampled every thousandth generation after which the two runs had converged (average standard deviation of split frequencies <0.02). The first 1,000 trees were discarded in order to obtain a reliable estimate of the posterior probabilities. Finally, a consensus tree was constructed of the retained trees which shows all observed bipartitions (i.e., contype=allcompat), although not all nodes had sufficiently supported (posterior probability<50). Only posterior probability values larger than 50% are shown at the nodes of the tree.

Sequence accession numbers

Sequences obtained from the 40 excised DGGE bands were grouped into 14 unique dinoflagellate phylotypes. The sequences of these phylotypes are deposited in the NCBI database under accession numbers FJ210850-FJ210863.

Lipid analysis

The sediment layers were freeze-dried and ultrasonically extracted with methanol, then dichloromethane (DCM)/methanol (1:1, v/v) and five times with DCM. All extracts were combined and the solvent was removed by rotary evaporation under vacuum. To an aliquot of the total extract an internal standard (6,6-d2-3-methyleicosane) was added and this mixture was methylated with diazomethane in diethyl ether and purified over a small pipette containing silica with ethyl acetate as an eluent. This total lipid fraction was subsequently silylated with BSTFA in pyridine and analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) as described elsewhere (Sinninghe Damsté *et al.*, 2007).

Individual sterols were quantified by mass spectrometry using specific mass fragment ions (m/z 359 for dinosterol, m/z 366 for $C_{27:2}\Delta^{5,22}$ sterol, m/z 329 for cholesterol, m/z 380 for $C_{28:2}\Delta^{5,22}$ sterol and m/z 386 for $C_{28:2}\Delta^{5,24}$ sterol). The intensity of these fragment ions was calibrated by comparison with the corresponding fragment ions in the mass spectra of standard sterols. Final quantification was performed by comparison with the internal standard (6,6-d2-3-methyleicosane, m/z 85).

Microscopic analysis

Fresh, wet sediment samples were examined under a light microscope using smear slides to obtain an insight in the nature of the material without any chemical treatment. Subsequently, the wet sediment samples ($2.8 - 5.5$ g) were treated with 38% HF and shaken for 2 h and then treated with HF for two days. Decantation was carried out three times, with a minimum interval of 7 h. Neither HCl nor alkaline solutions were applied to the samples. The samples were sieved over a 15 μm mesh steel sieve. Sieving over a 15 μm sieve may have led to the loss of the smaller cysts of *P. glacialis*. However, sieving over a 10 μm sieve was not an option since this fraction was unusable due to too high content of organic debris. To remove as much fine organic debris and fragment organic matter aggregates as possible, ultrasonic treatment and sieving alternated (up to 6 times with <1 min of ultrasonic treatment each time). After sieving, the samples were transferred to a 1.5 ml reaction vessel marked with 0.5 ml scale intervals. From a total volume of 1.0 ml, $15 - 40$ μl (depending on the total number of palynomorphs) of the homogenized suspension was put on a slide. The material was embedded in glycerin jelly and insulated from air by paraffin wax. To achieve dinocyst concentrations of at least one cyst per slide, the samples were sieved again over a 28 μm mesh steel sieve, keeping the >28 μm fraction. Concentration into 1.5 ml reaction vessels and slide preparation was performed as described above.

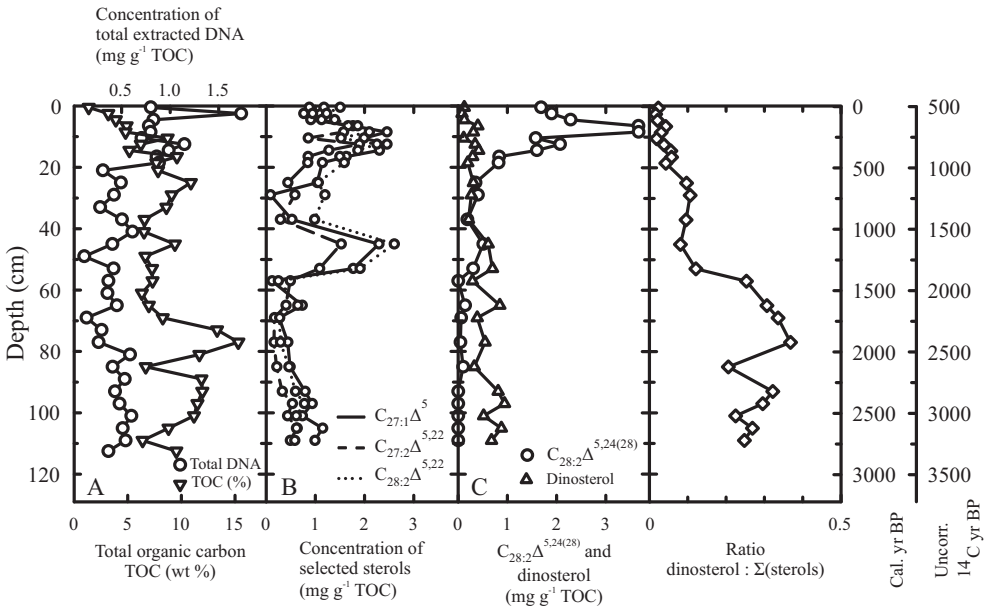


Figure 2: Depth distributions of various biomarkers within the sediments of SMB. A: Total organic carbon and total amount of extracted DNA (mg g^{-1} TOC). These values were obtained from Coolen *et al.* (2007). B: Concentrations of selected dominant fossil sterols: $C_{27:1}\Delta^5$ ("cholesterol") is commonly found in many microalgae, including diatoms and dinoflagellates. $C_{27:2}\Delta^{5,22}$ and -$C_{28:2}\Delta^{5,22}$ ("diatomsterol") are sterols that are commonly found in diatoms, but that are also known from other microalgae (Volkman, 2003). C: Distribution of the sterol $C_{28:2}\Delta^{5,24(28)}$, which is abundant in many diatoms, and the dinoflagellate-specific sterol dinosterol; D: Ratio of the concentration of dinosterol to the total concentration of quantified sterols. The calibrated ^{14}C timescale on the right axis is based on the age model determined for this core (Sinninghe Damsté *et al.*, 2007).

The slides were examined using a Zeiss Axioplan light microscope. Micrographs were made using a Zeiss Axiocam camera attached to the microscope and using Zeiss Axiovision software for image processing. In addition, we examined several smear slides for each sample in order to verify whether small cysts such as those of Polarella were lost during sieving using 15 μm and 28 μm mesh sieves. Several slides of palynologically processed material were examined.

Results

Total organic carbon (TOC) and total DNA in Late-Holocene sediment record

TOC values and total DNA concentrations were obtained from Coolen *et al.* (2007) (Fig. 2A) to facilitate a direct comparison of sterol data obtained during this study. The TOC content was lowest at the surface (1%) and steeply increased to ∼8% at 20 cm, then remained fairly stable at ∼8% between 20 and 70 cm, and finally increased to ∼12% below 70 cm. The concentration of extracted DNA, which represents both living biomass and preserved fossil DNA, was highest just below the surface at 2 cm (1.5 mg per gram TOC) and slightly lower in the upper 20 cm (∼1 mg g^{-1} TOC). The DNA concentration then dropped to relatively constant values of $0.3 - 0.4$ mg g^{-1} TOC in the remaining part of the core.

Primer specificity, number and distribution of phylotypes

The specificity of the dinoflagellate primer Dino-Rev, developed for this study, was checked in silico using the Silva database (Pruesse *et al.*, 2007). The primer showed an exact match (i.e., identical sequence) with 767 hits, 756 of which are dinoflagellates (∼50% of the dinoflagellate sequences in the database). When 3 mismatches are allowed, the primer matched with ∼81% of the dinoflagellates in the database, and with a small number of non-dinoflagellates (53 out of 1236 hits; 4%). Closely related alveolates (Syndiniales) represented the majority of the non-dinoflagellate hits.

Practical validation of the primer set came from the subsequent phylogenetic analysis of sequenced DGGE bands. A total of 40 DGGE bands with 18S rDNA were retrieved from the water column and sediment samples and sequenced. A number of those bands from different samples melted at identical positions in the gel but were nevertheless sequenced to verify whether they represented identical phylotypes. DGGE bands recovered from the water column and shallow sediment samples sometimes appeared as double bands (Fig. 3) This phenomenon is a known artefact in DGGE analyses, and is likely due to prematurely halted elongation of amplicons (Janse *et al.*, 2004). A number of these double bands were sequenced separately to confirm that they indeed represented the same phylotype. The 40 excised bands were eventually grouped into 14 unique phylotypes of dinoflagellates (i.e., with >98% sequence similarity).

Six unique phylotypes were recovered from the water column. Four of these sequences were found above the sulfidic chemocline (Fig. 3). In addition, one faint DGGE band (EF_Dino_05) was retrieved from the chemocline ($10.7 - 11.2$ m). DGGE bands were recovered from the deepest water column sample (e.g. EF_Dino_06), but visual inspection of the filter and the close relationship with dinoflagellates found in the sediment (EF_Dino_07) suggests that dinoflagellates from the surface sediments were trapped on the filter due to resuspension of the surface sediments.

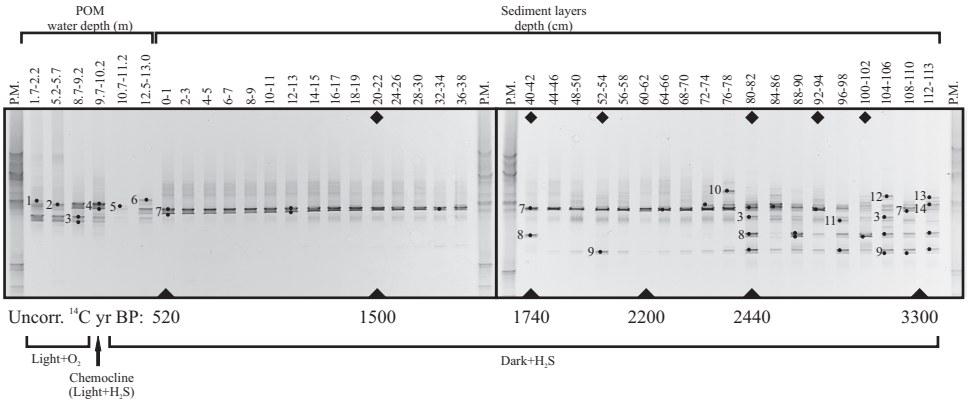


Figure 3: DGGE gels showing the distribution of PCR-amplified fragments of dinoflagellate 18S rDNA in samples from SMB water column and sediment core. The top of the sulfidic chemocline (9.7 m) in the water column was determined based on the presence of high levels of the carotenoids chlorobactene and isorenieratene of anoxygenic photoautolithotrophic green sulfur bacteria (Boere et al., unpublished results). A total of 40 bands (bands marked with a black dot) were excised, sequenced and subsequently grouped into 14 unique phylotypes (numbered EF_Dino_1 through 14). Excised bands at similar positions in the DGGE were found to represent the same phylotype. Samples marked with diamonds (♦; shown at top of gel) were analyzed for the presence of dinocysts. Black triangles (bottom of gel) indicate samples for which radiocarbon ages have been determined. The accompanying numbers indicate uncorrected ^{14}C age (yr BP) and were reported previously (Sinninghe Damsté *et al.*, 2007). Abbreviations used: POM: particulate organic matter; P.M.: Position Marker.

Nine different phylotypes were recovered from the sediment layers (Fig. 3), of which only a single phylotype was also recovered from the water column at the time of sampling (i.e. EF_Dino_03). This phylotype was recovered in sediment layers deeper than 80 cm. The sediments from the surface down to approximately 80 cm (notably excluding depths $40-42$ and $52-54$ cm) revealed only a single phylotype (EF_Dino_07). Below 80 cm, several additional phylotypes were present. Whereas most other phylotypes (e.g. EF_Dino_10) were only recovered from single sediment intervals, two of the additional phylotypes (EF_Dino_08 and EF_Dino_09) were present throughout the whole sediment interval below 80 cm (Fig. 3).

Phylogenetic position of dinoflagellate phylotypes recovered from the water column

The phylotypes EF_Dino_01 and EF_Dino_03, which were recovered from the upper water column, clustered with Gymnodinioid species isolated from the Ross Sea (i.e. *Dinophyceae* sp. W5-1 and RS-24; (Gast *et al.*, 2006) and from Prydz Bay adjacent

to Ellis Fjord (marine eukaryote clone E1-166; Piquet *et al.*, 2008, Fig4.). Phylotypes EF_Dino_02 and EF_Dino_04 are closely related and might represent the same phylotype. However, based on observed differences between the two sequences (9 out of 464 base pair differences; ∼98% similarity), they appeared as separate phylotypes in the phylogenetic tree (Fig. 4). Both are related to *Heterocapsa* species, which were also recovered from Prydz Bay (e.g. marine eukaryote clone E3-121; (Piquet *et al.*, 2008). The single sequence recovered from beneath the sulfidic chemocline (EF_Dino_05) cannot be assigned to any of the dinoflagellate clades with certainty. Each phylogenetic analysis performed here placed this species at a basal position in the tree (Fig. 4). In additional BLAST searches, its closest affinity is with uncultured species from other sulfidic fjords (Behnke *et al.*, 2006). The phylotype recovered from the bottom water column sample (EF_Dino_06) is virtually identical (1 bp difference) to the species that we recovered from the surface sediments, i.e. *P. glacialis* (see below).

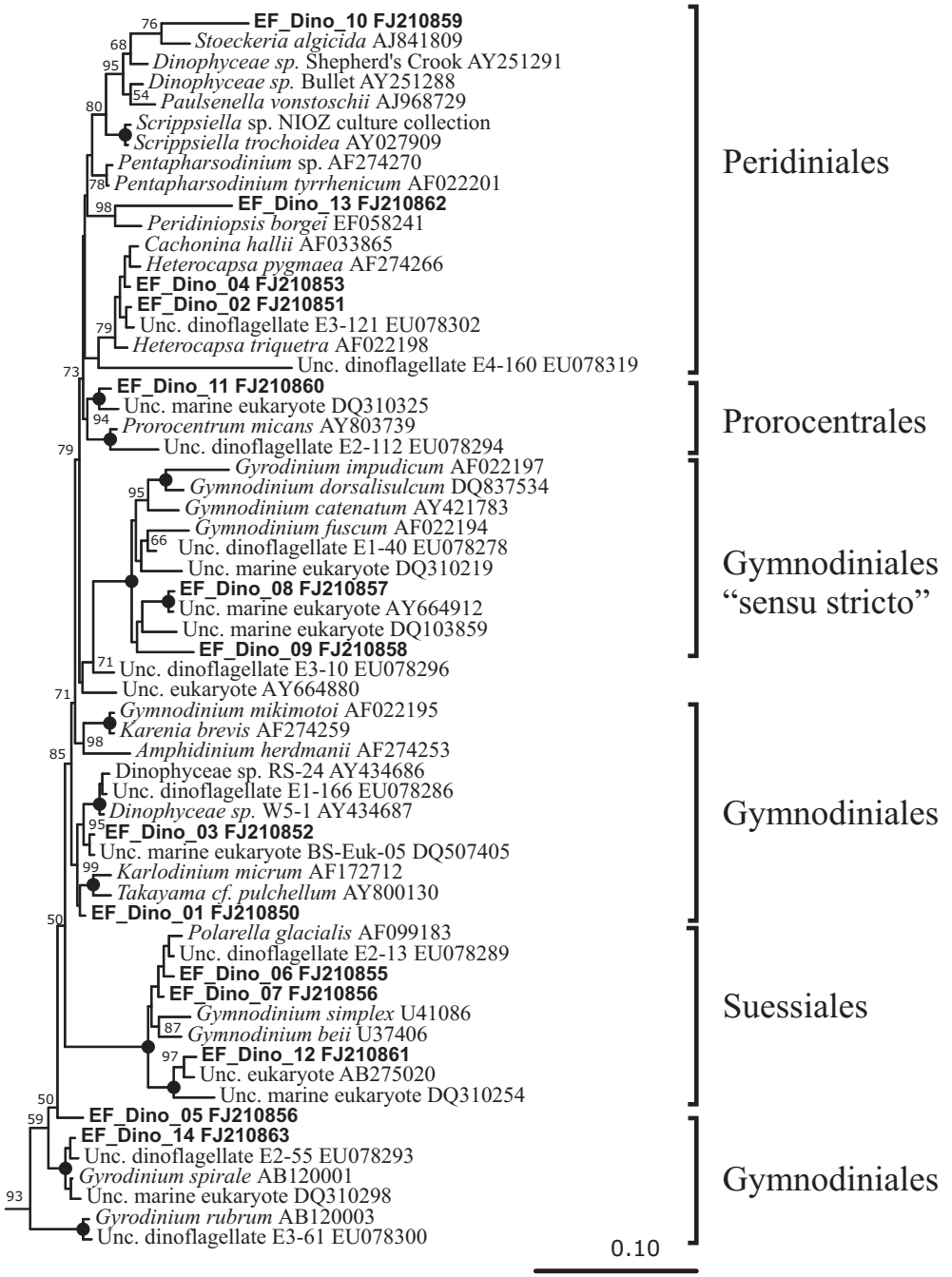
Phylogenetic position of dinoflagellate phylotypes recovered from the Late Holocene sediment record

The single sequence in the upper sediments (EF_Dino_07) clustered within the order Suessiales (Fig. 4). Based on genetic and chemotaxonomic data, this order comprises a number of species of *Gymnodinium* and *Symbiodinium* (Saldarriaga *et al.*, 2004; Thomson *et al.*, 2004). In addition, it contains the recently described dominant sea-ice species P. glacialis (Montresor *et al.*, 1999). The recovered phylotype is nearly identical to *P. glacialis* (1 bp difference).

The additional dominant phylotypes from the deeper sediments (EF_Dino_08 and EF_Dino_09) clustered within a well-supported group of *Gymnodiniales*, which is referred to as *Gymnodinium* sensu stricto (Saldarriaga *et al.*, 2004) (Fig. 4). Their closest affinity is with uncultured microeukaryotes that were isolated from the water columns of anoxic fjords (Behnke *et al.*, 2006; Zuendorf *et al.*, 2006). This cluster also contains the dominant dinoflagellate clone E1-40 recovered from Prydz Bay (Piquet *et al.*, 2008).

Other phylotypes, which were only found in single sediment layers (e.g. EF_Dino_-11, EF_Dino_14), clustered with uncultured dinoflagellates found in anoxic or sulfidic

Figure 4: Bayesian tree showing the phylogenetic position of partial 18S SSU rRNA gene sequences from Ellis Fjord found in this study. Relatives are selected from the Silva database (Pruesse *et al.*, 2007) and from additional BLAST-searches in the NCBI database. *Perkinsus marinus* was defined as outgroup (root not shown in tree). Recovered DGGE-bands were grouped into phylotypes (>98% similarity) for clarity and are displayed in Arial font. Only posterior probability values >50% are shown at the internal nodes. Nodes with a posterior probability of 100% are marked with a black dot. Higher taxonomy nomenclature is based on Saldarriaga *et al.* (2004).


EF_Dino_10 FJ210859
Stoeckeria algicida AJ841809
Dinophyceae sp. Shepherd's Crook AY251291
Dinophyceae sp. Bullet AY251288
Paulsenella vonstoschii AJ968729
Scrippsiella sp. NIOZ culture collection
Scrippsiella trochoidea AY027909
Pentapharsodinium sp. AF274270
Pentapharsodinium tyrrhenicum AF022201
EF_Dino_13 FJ210862
Peridiniopsis borgei EF058241
Cachonina hallii AF033865
Heterocapsa pygmaea AF274266
EF_Dino_04 FJ210853
EF_Dino_02 FJ210851
Unc. dinoflagellate E3-121 EU078302
Heterocapsa triquetra AF022198
Unc. dinoflagellate E4-160 EU078319
EF_Dino_11 FJ210860
Unc. marine eukaryote DQ310325
Prorocentrum micans AY803739
Unc. dinoflagellate E2-112 EU078294
Gyrodinium impudicum AF022197
Gymnodinium dorsalisulcum DQ837534
Gymnodinium catenatum AY421783
Gymnodinium fuscum AF022194
Unc. dinoflagellate E1-40 EU078278
Unc. marine eukaryote DQ310219
EF_Dino_08 FJ210857
Unc. marine eukaryote AY664912
Unc. marine eukaryote DQ103859
EF_Dino_09 FJ210858
Unc. dinoflagellate E3-10 EU078296
Unc. eukaryote AY664880
Gymnodinium mikimotoi AF022195
Karenia brevis AF274259
Amphidinium herdmanii AF274253
Dinophyceae sp. RS-24 AY434686
Unc. dinoflagellate E1-166 EU078286
Dinophyceae sp. W5-1 AY434687
EF_Dino_03 FJ210852
Unc. marine eukaryote BS-Euk-05 DQ507405
Karlodinium micrum AF172712
Takayama cf. pulchellum AY800130
EF_Dino_01 FJ210850
Polarella glacialis AF099183
Unc. dinoflagellate E2-13 EU078289
EF_Dino_06 FJ210855
EF_Dino_07 FJ210856
Gymnodinium simplex U41086
Gymnodinium beii U37406
EF_Dino_12 FJ210861
Unc. eukaryote AB275020
Unc. marine eukaryote DQ310254
EF_Dino_05 FJ210856
EF_Dino_14 FJ210863
Unc. dinoflagellate E2-55 EU078293
Gyrodinium spirale AB120001
Unc. marine eukaryote DQ310298
Gyrodinium rubrum AB120003
Unc. dinoflagellate E3-61 EU078300
0.10
Peridiniales
Prorocentrales
Gymnodiniales "sensu stricto"
Gymnodiniales
Suessiales
Gymnodiniales

fjord waters (Behnke *et al.*, 2006; Zuendorf *et al.*, 2006). The closest cultured relative of EF_Dino_11 and EF_Dino_14 are *Prorocentrum* sp. and the unarmored heterotroph *Gyrodinium spirale*, respectively (Fig. 4).

The phylogenetic positions of many of our phylotypes were well supported in the phylogenetic analyses. However, there are some concerns. Bayesian phylogenetic analysis tends to give a generally higher support than for example bootstrap values. This implies that taxa with low Bayesian posterior probability (PP) values should be considered with extra caution (Alfaro *et al.*, 2003). In general, many of the basal nodes have only low support values. In addition, higher taxonomic groups defined by others studies (e.g., Saldarriaga *et al.*, 2004) were not always observed or were not monophyletic. Reconstructing dinoflagellate molecular phylogeny has proven to be difficult and should ideally be performed with more than one gene (Saldarriaga *et al.*, 2004). However, our goal was to study late-Holocene dinoflagellate successions and to compare the paleoenvironmental genomic approach with more traditional dinoflagellate proxies rather than to explore the exact phylogeny of dinoflagellates. Generally, the phylogenetic positions of the dinoflagellates found in the SMB are generally well supported (e.g., PP>90), even though the backbone of the tree is not as well supported (Fig. 4). Only the clade including EF_Dino_02 and EF_Dino_04 is relatively poorly supported (PP=79).

Fossil sterols

We identified a number of dominant sterols (Fig. 2B-C), which are produced by several microalgal groups and are not necessarily diagnostic for dinoflagellates (Volkman, 2003). In particular cholest-5-en-3β-ol ("cholesterol", $C_{27:1}\Delta^5$ in Fig. 2B) is ubiquitous in microalgae and in aquatic fauna. The other major compounds cholesta-5,22E-dien-3β-ol ($C_{27:2}\Delta^{5,22}$) and 24-methylcholesta-5,22E-dien-3β-ol ("diatomsterol" or brassicasterol depending on C-24 stereochemistry, $C_{28:2}\Delta 5,22$) are also found in many microalgae but are often abundant in diatoms (Volkman, 2003) and serve as a general marker for paleoproductivity.

These three sterols all show a similar pattern with high concentrations (between 1 and 2.5 mg g^{-1} TOC) in the upper 20 cm and lower values (<1 mg g^{-1} TOC) at greater depth. Their concentrations show a distinct increase between 40 and 60 cm depth with values up to ca. 2.5 mg g^{-1} TOC before the concentrations drop again to around 0.2 mg g^{-1} TOC. A gradual increase to 0.5−0.6 mg g^{-1} TOC was observed for sediments deeper than 80 cm.

The sterol 24-methylcholesta-5,24(28)E-dien-3β-ol ($C_{28:2}\Delta^{5,24(28)}$; Fig. 2C) is sometimes used as a diagnostic biomarker for diatoms, although it has also been identified in some dinoflagellates and a few other algae (Volkman, 2003). The concentration of this sterol was highest (up to ∼4 mg g^{-1} TOC) in the subsurface sediments. Slightly elevated levels of this sterol (<1 mg g^{-1} TOC) were also found between 40 and 60 cm where the other sterols were also more abundant. In the deeper interval of the core, the concentration of this sterol was low compared to the other sterols and dropped to barely detectable levels in the deeper layers below 90 cm.

The only marker considered diagnostic for dinoflagellates that we analyzed was 4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol (i.e., dinosterol; Fig. 2C). The concentration of dinosterol in the uppermost subsurface layers was lower than the other sterols ($\sim$0.1 mg g^{-1} TOC) and increased to $\sim$0.3 mg g^{-1} TOC towards 40 cm depth. In the remainder of the core the concentrations and trend were similar for all investigated sterols with the dinosterol content showing an increasing trend with concentrations varying between 0.4 and 0.8 mg g^{-1} TOC.

If the dinosterol concentration is plotted relative to the total sterol concentration (Fig. 2D), it reflects the contribution of dinoflagellates to the total eukaryote sterol pool. Instead of the total sterol pool, which is a complex mixture of compounds with individual contributions varying with depth, the ratio of dinosterol to the sum of the five quantified sterols (including dinosterol) was plotted, because these five sterols make up an important part of the total sterols throughout the core. This dinosterol-to-quantified-sterols ratio shows a clear change around $60-80$ cm depth, below which dinosterol makes up a considerable part ($\sim$30%) of the total sterols, and above which it drops to low values ($<$0.1), as a result of both decreasing dinosterol and increasing total sterol concentrations.

Microscope analysis of palynomorphs

The fresh sediment samples were rich in diatoms and contain a high proportion of particulate and unstructured organic matter (Fig. 5 A-C). Often the microalgae were so well preserved that the chloroplasts were still intact with their original color. Diatoms, green algae (both filamentous and coccoid), and organic and siliceous algal debris dominated the material. Additionally, fragments of crustacean cuticles were found. Despite the excellent preservation of other microfossils as described above, dinoflagellate cysts, including the small ($10-15$ μm) spiny cysts of *P. glacialis* were not encountered in the smear slides of unsieved matter.

Chemical treatment and repeated ultrasonic treatment and sieving removed the siliceous and $<$15 μm fraction (Fig. 5). Although this procedure strongly concentrated the palynomorphs, the samples were still dominated by unstructured organic matter aggregates, which resisted the ultrasonic treatment, and by particulate organic matter of uncertain origin. The remains of green algae dominated the palynomorphs. Crustacean cuticles were also abundant. Less abundant to rare were various other types of zooclasts, acritarchs, haptophytes (*Cymatiosphaera* sp.), and organic foraminifera linings. The upper samples ($20-22$ and $40-42$ cm) were relatively rich in filamentous green algae (Fig. 5). Compared to the other organic matter, dinoflagellate cysts were rare so that, from a palynological point of view, dinoflagellates seem to represent only a tiny fraction of the export production (Fig. 5). Cysts smaller than $\sim$35 μm were not encountered in the larger than 15 μm fraction. To further concentrate the cysts this fraction was sieved using a pore size of 28 μm. Although this removed a considerable amount of the organic matter, organic debris remained fairly abundant and prevented proper concentrating of the cyst assemblages and most morphotypes occurred only once per slide or were absent. These low numbers make quantitative

Table 1: Sediment intervals that were selected for multiple dinoflagellate proxy analyses. Six depth intervals were analyzed for dinosterol content, the presence (+) or absence (-) of phylotypes related to assigned and non-assigned dinoflagellates, number of cysts (g wet sediment)$^{-1}$, and the presence (+) or absence (-) of the six cyst types. Only phylotypes that were found within these samples are shown. *No dinosterol concentrations were available for these samples; the concentration found in 2-cm sediment intervals just above and below the intervals are listed.

Depth (cm)	Dinosterol (mg g^{-1} TOC)	Phylotypes	EF_Dino_03	EF_Dino_07	EF_Dino_08	EF_Dino_09	EF_Dino_11	EF_Dino_12	Total cysts (g wet sed)$^{-1}$	Type 1	Type 2	Type 3	Type 4	Type 5	Type 6
20-22*	0.26		-	+	-	-	-	-	120	-	+	+	-	+	+
40-42*	0.41		-	+	+	-	-	-	130	+	+	+	-	-	-
52-54	0.70		-	+	-	+	-	-	180	+	+	+	-	-	-
80-82*	0.44		+	+	+	+	-	-	560	+	+	+	+	-	-
92-94	0.81		+	+	+	+	+	-	33	+	-	+	+	-	-
100-102	0.52		+	+	+	+	+	+	230	+	+	+	-	-	-

assessment of the assemblages unreliable. Moreover, it leads to underestimation of the diversity since rare taxa are likely to remain unobserved. Only three morphotypes were more abundant and occurred in most of the investigated samples (Table 1): Type 1 closely resembles Dinocyst sp. A described by Wrenn *et al.* (1998) (up to 4 specimens per slide in sample $50-52$ cm but absent above sample $40-44$; Fig. 5 D-F); Type 2, *Islandinium minutum* (up to 29 specimens in a slide from $80-82$ cm, absent in sample $90-92$ cm; Fig. 5G, H) and Type 3, *Echinidinium zonneveldii* (up to 19 specimens in a slide from $52-54$ cm; Fig. 5I). Only a single specimen was encountered of type 4 (Protoperidinium conicoides; Fig. 5J) in sample $92-94$ cm. Also, one single specimen of type 5 cf. Cryodinium meridianum (Fig. 5K) was observed (sample $20-22$ cm). Apart from these five morphotypes of dinoflagellate cysts, type 6 (3 specimens in sample $20-22$ cm; Fig. 5L) could not be assigned to any group of organisms due to a lack of identifying characters, and for that reason is assigned to the acritarchs. Judging by its size, shape, color and wall properties, the acritarch could be of dinoflagellate nature. Sample $92-94$ cm was extremely poor in dinocysts with only ~30 cysts g^{-1} wet sediment, whereas the cyst concentrations in other investigated samples varied from 120 to 230 cysts g^{-1} wet sediment. The sample from $80-82$ cm depth was the only sample with a higher cyst concentration (~560 cysts g^{-1} wet sediment). Due to the high water content of the samples, cyst concentrations reached $1.9 \times 10^2 - 3.1 \times 10^3$ cysts g^{-1} dry sediment.

Discussion

Current dinoflagellate community in the SMB water column

Our 18S rDNA survey revealed five dinoflagellate phylotypes in the oxygenated part of the water column at the time of sampling. In general, this low diversity of dinoflagellates is in accordance with earlier reports on fjords and saline lakes of the Vestfold Hills (Grey *et al.*, 1997; Rengefors *et al.*, 2008). Between May and October of 1993 a maximum of thirteen dinoflagellate species were counted in Ellis Fjord including three *Gymnodinium* and five *Protoperidinium* species (Grey *et al.*, 1997). As a result of restricted marine ingress into Ellis Fjord, the community within the Fjord is a subset of the open marine community and takes an intermediate position between the 34 described species of dinoflagellates in the open Antarctic ocean (Andreoli *et al.*, 1995), and four dinoflagellate species in saline lakes of Vestfold Hills (Perriss and Laybourn-Parry, 1997; Rengefors *et al.*, 2008). A truncation of the microbial community occurred during the formation of these saline lakes (Rengefors *et al.*, 2008).

Previous studies on Antarctic marine microbial eukaryote communities have generally been based on direct microscopic analysis or cultivation efforts (McMinn and Hodgson, 1993; Andreoli *et al.*, 1995; Grey *et al.*, 1997; Thomson *et al.*, 2004; Ryan *et al.*, 2006; Rengefors *et al.*, 2008). It is difficult to directly compare these morphological studies with our genomic results, as many of the previously described species are yet to be cultured and few 18S rDNA sequences are available (Piquet *et al.*, 2008). In addition, flagellates are often difficult to determine microscopically (Gast *et al.*, 2004) and thus many species may easily be missed, especially in the smaller fractions of the (phyto)plankton.

Only recently, culture-independent molecular techniques have been used to investigate the Antarctic coastal microbial eukaryote community (Gast *et al.*, 2004; Coolen *et al.*, 2007; Piquet *et al.*, 2008). In an experiment to investigate the effects of UV radiation on marine microbial eukaryotes, the microeukaryotic diversity was determined using DGGE and clone libraries from early spring through summer in $2002-2003$ in Prydz Bay. Dinoflagellates were found to be a dominant part of the community at all times (Piquet *et al.*, 2008). One of the three most dominant dinoflagellate phylotypes identified was related to *Heterocapsa triquetra*, similar to the dominant phylotypes EF_Dino_02 and EF_Dino_04 we found in the water column of SMB.

At first glance, the dinoflagellate diversity we detected in the water column of SMB seems low compared to Prydz Bay. However, our sampling in late spring only represented a snapshot of the total microbial eukaryote community occurring through the seasons. In fact, one of the experiments by Piquet *et al.* (2008) showed a community in which the *H. triquetra*-related phylotype was the most dominant dinoflagellate species. Our sampling might have recorded a similar condition.

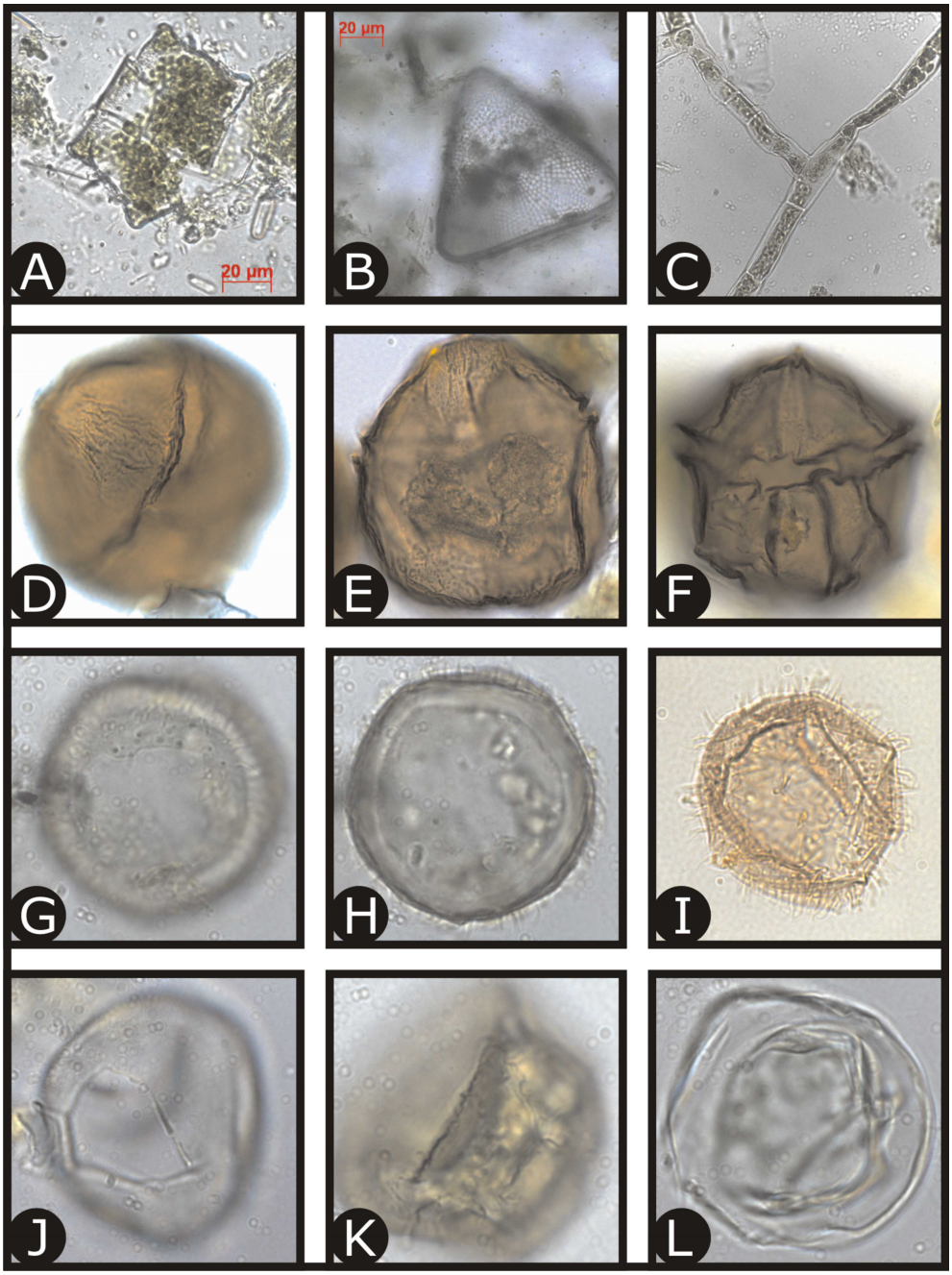

A
20 µm
B
20 µm
C
D
E
F
G
H
I
J
K
L

Figure 5: Selected palynomorphs from the Ellis Fjord sediments. The exceptionally good preservation is reflected in the retained color of the chlorophyll in diatoms (frustules) and green algae (A-C). D-L show organic walled cysts. D-F; Cyst type 1, cf. cyst sp. A (Wrenn *et al.*, 1998), diameter of the specimens $55 - 60$ μm diameter, sample $52 - 54$ cm; G; Cyst type 2, *Islandinium minutum* surface with archaeopyle, size 40 μm, sample $40 - 42$ cm, H; same specimen as G but in cross section; I, Cyst type 3, *Echinidinium zonneveldii* 45 μm, sample $40 - 42$ cm; J, Cyst type 4, *Protoperidinium conicoides*, 40 μm, sample $90 - 92$ cm; K, Cyst type 5 cf. *Cryodinium meridianum*, 40 μm; L, Cyst type 6, 40μm, sample $20 - 22$ cm (see results section for details).

Late-Holocene succession of dinoflagellates in Ellis Fjords' SMB

The highest preserved dinoflagellate diversity was found in the deepest part of the core. Some of these phylotypes only occurred in isolated sediment layers such as EF_Dino_10, which was present only at a depth of $76 - 78$ cm. Its closest named relative is *Stoeckeria algicida* in a cluster of *Pfiesteria*-like species (Jeong *et al.*, 2005). The latter are often associated with estuarine environments and form harmful algal blooms (HAB) and have been associated with fish mortality in Korea (Jeong *et al.*, 2005).

The phylotypes (e.g., EF_Dino_08, EF_Dino_09) in the deeper part of the core were related to species identified from the water column below the chemocline of the supersulfidic Framvaren and anoxic Mariager fjord (Behnke *et al.*, 2006; Zuendorf *et al.*, 2006). It is likely that these dinoflagellates were living in the anoxic part of the water column at the time of deposition since a number of recent publications reported the presence of previously unknown basal lineages of eukaryotes in anoxic bottom waters and surface sediments (Stoeck and Epstein, 2003; Behnke *et al.*, 2006; Zuendorf *et al.*, 2006). However, microbial eukaryotes actively living in (anoxic) subsurface sediments are to the best of our knowledge not yet reported. In addition, if these dinoflagellates were indigenous to the sulfidic sediments, we would have expected to find their sequences throughout the sediment record as opposed to a presence restricted to the deepest part of the core. It is thus more likely that the sequences represent ancient pelagic species and could be indicative for the presence of bottom water anoxia for up to $\sim$2700 yr.

Except for the occurrence of the aforementioned phylotypes, the dinoflagellate community found in the deep part of our core is similar to the modern species composition in Prydz Bay (Piquet *et al.*, 2008). The two dominant present-day phylotypes (besides *H. triquetra*) are related to *Gymnodinium catenatum* and *Gyrodinium spirale* (Piquet *et al.*, 2008). Our dominant sedimentary phylotypes (EF_Dino_08 and EF_Dino_09) clustered in the same group as *G. catenatum* (i.e., *Gymnodiniales* "sensu stricto" Saldarriaga *et al.*, 2004). In addition, phylotype EF_Dino_14, which was found in the deepest sample, is closely related to *Gyrodinium spirale*. This heterotrophic species constitutes an important part of under-ice microbial communities and preys on other dinoflagellates as well as on diatoms such as *Chaetoceros* (Ichinomiya *et al.*,

2007; Rengefors *et al.*, 2008). In short, the dominant community members preserved in the deep part of the core are similar to the current dominant coastal Antarctic dinoflagellates.

The dinoflagellate community was strikingly different in the upper part of the core. Our paleoenvironmental genomic survey revealed that the predominant dinoflagellate within the upper 80 cm of sediment (i.e., after 1850 cal. yr BP or 2440 ^{14}C yr. BP) was the autotrophic brine dinoflagellate *P. glacialis* (Montresor *et al.*, 1999). This species forms extensive summer blooms in both sea-ice and water column in various Antarctic marine (Stoecker *et al.*, 2000; Ryan *et al.*, 2006) and lake settings (Rengefors *et al.*, 2008). For instance, more than 10^6 living cells l^{-1} brine and up to 10^7 hypnozygotes (resting cysts) L^{-1} brine were found at McMurdo Sound in December and January (Stoecker *et al.*, 2000). The predominance in the fossil record is likely the result of the high flux of *P. glacialis* cells or cysts during these summer blooms.

Due to the high biomass influx of *P. glacialis*, it is likely that additional, less abundant, dinoflagellates escaped detection with our PCR/DGGE approach. For example, in our previous study, diatom sequences which comprised less than ∼5% of the total diatom 18S rDNA pool most likely escaped identification (Coolen *et al.*, 2007).

Comparison between fossil sterols and the paleoenvironmental genomic information

Sterols are found in all species of (micro)-algae and as such are valuable as quantitative marker for primary production, and in some cases as a diagnostic marker for identifying individual algal classes or sometimes species (Volkman, 2003). The trend of increasing levels of the more ubiquitous fossil sterols reported in this study, therefore, point towards a general increase in productivity towards the present. In contrast, the dinoflagellate-specific dinosterol showed the reverse trend, with highest concentrations in the deepest part of the core and comprising only a small fraction of the total sterols in the upper part (Fig. 2C, D). This dinosterol profile does suggest that the relative contribution of dinoflagellates to the preserved organic matter decreased towards the present.

However, our paleoenvironmental genomics approach showed that the decline in fossil dinosterol between 60 and 80 cm depth (Fig. 2) coincided with a shift from species (Fig. 3) related to known dinosterol producers such as *Heterocapsa*, *Prorocentrum* and *Gymnodinium* (Alam *et al.*, 1984; Mansour *et al.*, 1999; Volkman *et al.*, 1999) to *P. glacialis*, which does not produce dinosterol in cultures (Thomson *et al.*, 2004). This differential occurrence of dinosterol among dinoflagellate species is often recognized, but not explicitly taken into account (Sangiorgi *et al.*, 2005; Mouradian *et al.*, 2007). Thus, we conclude that the decrease in dinosterol in the upper sediment layers in this setting does not necessarily record a reduced relative contribution of dinoflagellates to sedimentary organic matter, but rather supports the change in community composition found in the fossil DNA profile, towards a situation where a non-dinosterol-producing species dominates the community.

Instead of dinosterol, the dominant sterol in two laboratory cultures of *P. glacialis*, which were isolated from McMurdo sound (the type strain) and from sea-ice near Davis Station, was found to be 27-nor-24-methylcholesta-5,22E-dien-3β-ol (making up 64% of the total sterols; Thomson *et al.*, 2004). The occurrence of this sterol is restricted to a small group of dinoflagellates and it has therefore been proposed as a potential chemotaxonomic biomarker for *P. glacialis* in sea-ice or in the water column (Thomson *et al.*, 2004). Surprisingly, we did not detect the 27-nor-24-methylcholesta-5,22E-dien-3β-ol in the SMB sediments, but we found cholesta-5,22E-dien-3β-ol in low amounts instead. In addition to 27-nor-24-methylcholesta-5,22E-dien-3β-ol, Thomson *et al.* (2004) found another relatively important sterol in their *P. glacialis* strain (4α,24-dimethylcholestanol; $\sim$20% of total sterols). This ratio (relatively high 4,24-dimethylcholestanol and low dinosterol content) is typical for a subgroup of *Gymnodinium* species, including *G. simplex* (Withers, 1987) and supports the phylogenetic position based on morphology and rDNA of *P. glacialis* in the order *Suessiales* (Mansour *et al.*, 1999; Saldarriaga *et al.*, 2004). Although 4α,24-dimethylcholestanol was found in very low quantities in our core, the low concentration prevents it from being used as unambiguous supporting evidence for the dominance of *P. glacialis*.

Thus, in general, the lipid biomarker analysis undertaken here did not support the late-Holocene dinoflagellate species distribution derived from the paleoenvironmental genomics approach, other than the predicted absence of dinosterol in the *P. glacialis* -dominated sediment. At this stage, we can only speculate about the reason why the specific sterols associated with *P. glacialis* in culture did not appear to be present in the late-Holocene sediment record. A possible explanation would be that dinoflagellate input might be masked by the other, much more abundant microalgal lipids because the general preserved lipid profiles are consistent with a source community of primary producers dominated by diatoms. Dinoflagellates are known to have rather huge genomes and can have high numbers of 18S rDNA per cell (Rizzo, 1987; LaJeunesse *et al.*, 2005), and there is no significant linear relation between dinoflagellate copy number and biomass (Godhe *et al.*, 2008). Thus, if the ratio of preserved rDNA to associated preserved lipid biomarkers is relatively high, it is possible to still find the fossil DNA, even though the contribution of preserved lipids to the total organic matter is low.

Another possibility is that the *P. glacialis*-specific C_{27}-sterols could no longer be identified because they have reacted with reduced inorganic sulfur species in the sulfidic, organic carbon-rich sediments (i.e., sulfurization). In SMB, the sulfurization process was previously shown to be completed within 500 years for a different class of compounds, highly branched isoprenoids (Sinninghe Damsté *et al.*, 2007). In sulfidic sediments of the nearby Ace Lake, steroids were completely sulfurized within 1000$-$3000 years (Kok *et al.*, 2000). Differential sulfurization was shown to occur among sterols, with a strong bias towards C_{27} sterols for yet unknown reasons (Kok *et al.*, 2000). Sulfurized compounds and their diagenetic products can be released by desulfurization of the lipid extracts (Sinninghe Damsté *et al.*, 2007). However, even after desulfurization of the extracts, we did not detect traces of 27-nor-24-methylcholesta-5,22E-dien-3β-ol or its diagenetic products. Therefore, this

explanation is highly unlikely.

A third possibility for the observed discrepancy between the fossil sterol composition and the sterol composition in *P. glacialis* cultivars is that lipid profiles within a species can vary at different growth stages or physiological states (Mansour *et al.*, 2003; Volkman, 2003). As most organic matter that reaches the sediment is probably derived from the non-motile cyst phase, direct comparison to published lipid profiles from free-living cells are confounded. Although earlier studies have reported the presence of sterols in dinocysts, no direct comparison was made with living cells (Kokinos *et al.*, 1998). In general, little is known about the difference in lipid profile between viable living cells and cyst stages, but large differences are likely to occur (Volkman, 2003; de Leeuw *et al.*, 2006). Interestingly, Thomson *et al.* (2004) found little difference in the sterol profiles between exponential and stationary phase cultures of *P. glacialis*, even though the latter contained a higher proportion of cysts and planozygote stages.

Unfortunately, no samples of pure cysts are available to test the difference in lipid composition between cysts and vegetative cells. The best approach would be to identify which phase in the life cycle of *P. glacialis* is buried in the sediment record by microscopic observation of sediment trap material collected in the sulfidic bottom waters of SMB. This material should then be analyzed for the presence of the diagnostic C_{27} sterols of *P. glacialis*. If the diagnostic sterols should be present in the sediment trap material but, according to our study, absent from the sediment, then this would indicate that post-depositional diagenesis of the sterols, such as sulfurization, occurred. If the sterols prove to be absent from the sediment trap material then it will also be necessary to analyze the sterol composition from the sea-ice of SMB directly and/or from a *P. glacialis* culture isolated from SMB.

Micropaleontology vs. paleoenvironmental genomics

The third approach to study the past dinoflagellate community composition was by analyzing preserved dinocysts, which can often be identified at the species level (Zonneveld *et al.*, 2008). The six dinoflagellate cyst types encountered are paleontological species. Because the cyst-theca relations are often unknown, placement of paleontological species in the (increasingly DNA-based) biological system is generally difficult, except at a higher taxonomic level. Judging by the shape and color of the morphotypes, most of the recovered cyst types are heterotrophic peridinioids. Such a dominance of heterotrophs is also known in the Arctic (see overview by Marret and Zonneveld, 2003). Although unambiguously linking the cyst data to the genomic dataset is impossible at this time, the cysts may very well turn out to match the recovered rDNA phylotypes.

On the basis of the paleoenvironmental genomics dataset we expected to find cysts of *Heterocapsa*, *Polarella*, *Stoeckeria* or the microreticulate cysts similar or equal to those of *Gymnodinium catenatum* or *Gyrodinium spirale*. For *Heterocapsa* and *Stoeckeria*, the absence of these cyst types is no surprise since their cysts may be hard to distinguish from the transparent remains of other groups of organism (e.g.,

Jeong *et al.*, 2005). Moreover, the cysts may not preserve well and there are closely related members (as is the case for G. catenatum and Gyr. spirale) for which a cyst stage is presently unknown. The resting cysts of *P. glacialis* have a very conspicuous morphology and have been reported in high abundance from several Antarctic marine settings (Stoecker *et al.*, 1997, 1998; Thomson *et al.*, 2006). However, just like the lipid analysis, palynology provided no evidence for *P. glacialis* from our core. In the original description of *P. glacialis* and its cysts, Montresor *et al.* (1999) report the lack of dinosporin, a compound involved in cyst durability (de Leeuw *et al.*, 2006; Zonneveld *et al.*, 2008). Within the order Suessiales, of which *P. glacialis* is the only living member, the ability to produce dinosporin was likely lost during the Jurassic (∼187 Mya) (Bucefalo Palliani and Riding, 2003). The vulnerable cysts have never been found deeper than a few centimeters in cores from the ocean around Antarctica (McMinn, 1995; Montresor *et al.*, 2003). In marine environments, aerobic degradation is considered the most important factor in the diagenetic breakdown of organic-walled cysts (Zonneveld *et al.*, 2008), whereas dinocysts are often well preserved in anoxic settings (Zonneveld *et al.*, 2001; Coyne and Cary, 2005). We did not observe *P. glacialis* cysts in our study. These $<15\mu$m cysts may well have been lost by the sieving procedure (over a sieve with 15 μm pore size). However, these cysts were also absent in the unsieved smear slides, which were not subject to size-fractionated bias. It is nevertheless possible that some cysts were present but could not be observed microscopically due to the strong dilution with amorphous organic matter in the slides, but an absence of the *P. glacialis* cysts in the permanently anoxic bottom waters of SMB could well be explained as a result of poor preservation. A possible reason for the poor preservation of *P. glacialis* cysts may be that upon release from the sea-ice, the cells excysted and the empty cysts were rapidly degraded in transit to the sediment.

Despite the otherwise excellent preservation of organic material, we found also relatively few organic-walled dinocysts. This is at first surprising because a cyst life-stage is a widely used strategy to survive periods of poor light regime and low temperatures during winter in many Antarctic microbial eukaryotes (McMinn *et al.*, 2001; Rengefors *et al.*, 2008). Endemic Antarctic species may have evolved other strategies for their dormancy period, as successful excystment after a period of dormancy is hindered in perpetually dark, cold, anoxic bottom waters (Anderson *et al.*, 1987; Coyne and Cary, 2005). For instance, a proposed life history for *P. glacialis* suggests that the spiny resting cyst is incorporated in the overlying sea-ice instead of residing in the sediment in a dormant stage (McMinn, 1996; Stoecker *et al.*, 1997, 1998). In this scenario, only a relatively small proportion of its cysts may have reached the sediment.

Similar survival strategies may also have been adopted by other Antarctic dinoflagellate species other than K, thus limiting cyst sequestration in the sediments of this ice-covered location. Furthermore, not all dinoflagellates follow the strategy of surviving adverse conditions by producing (preservable) cysts. Finally, not all dinoflagellate cysts are recognized as such. Several lack the morphological characteristics needed to assign them unambiguously to this group (and for this reason are assigned to the Acritarcha) such as our cyst type 6. Only by performing hatching experiments or

genetic analysis of single cells, would it be possible to confirm that these acritarchous morphotypes represent dinoflagellates (Dale *et al.*, 1978; Zonneveld *et al.*, 1997). Even for the relatively well studied coastal waters, new 'acritarchous' cysts are still regularly assigned to dinoflagellates in this way. The Ellis Fjord material contains such 'acritarchous' dinoflagellate-like cysts including the *Echinidinium* species (cyst Type 3, Fig. 5I). The dinoflagellate diversity as estimated from the palynomorph assemblages in the sediments is, therefore, likely to be underestimated.

Several studies have shown that cysts are preserved and can even remain viable for extended periods in anoxic conditions (Anderson *et al.*, 1987; Coyne and Cary, 2005). Dinocysts are clearly an important possible mechanism for the preservation of DNA in sediments. Although the morphotypes that were expected on the basis of the paleoenvironmental genomics dataset were not encountered, three novel dinoflagellate morphotypes were found in most samples, and a few other morphotypes were encountered in isolated layers. In this respect it is interesting to note that in two samples in which we found dinocysts (i.e., $40-42$ and $52-54$ cm) there was a concomitant presence of two Gymnodinioid-related phylotypes (EF_Dino_08 and EF_Dino_09). Upon the complete description of both cysts it will become possible to verify whether the cysts were indeed represented by these phylotypes.

Interestingly, Dinocyst sp. A (Fig. 5D-F) was often found with its cell content preserved, whereas this was rarely the case for the cyst cf. Islandinium minutum (Fig. 5G, H). The persistent presence of cell contents of Dinocyst sp. A implies that cysts that reached the sediment did not excyst subsequently and that re-seeding of the population was prevented in the anoxic conditions in SMB. Alternatively, cysts that did hatch were not preserved and thus not found in the analysis. Picking such well-preserved intact cysts and extracting its DNA, or performing direct single cell PCR (Takano and Horiguchi, 2004) may provide a shortcut to identify their position in the biological system without taking the laborious classical route of establishing a cyst-theca relation.

Possible climate-changes inferred from the late-Holocene dinoflagellate succession in SMB

Antarctic lakes respond rapidly to changing environmental conditions and are thus highly sensitive recorders of climate change (Quayle *et al.*, 2002). Although SMB is currently still connected to the ocean, it is in the first stages of its evolution from an open, marine inlet towards a series of isolated meromictic basins with a reduced marine influence (Gallagher *et al.*, 1989). Marine incursions are already restricted by the shallow (<2 m) sills, which has led to reduced mixing and the formation of anoxic bottom waters in SMB (as well as in the adjacent Deep Meromictic Basin). The sea-ice covering the inner basins of the fjord often reaches 2 m in thickness, causing the SMB to become fully isolated for 3 months per year or more (Gallagher *et al.*, 1989).

Based on the relative fossil distribution of sea-ice associated planktonic diatom assemblages it is possible to reconstruct the level of past sea-ice extension, e.g. a

more extensive ice cover during a colder Antarctic climate versus open water conditions during warmer climatic periods (McMinn *et al.*, 2001; Roberts *et al.*, 2004). For instance, changes in diatom assemblage have been associated with increased evaporation in the eastern Antarctic Windmill Islands (dated at ∼1850 corr. yr BP; Roberts *et al.*, 2004) and with an ice cap retreat in the Vestfold Hills' Long Fjord (dated at ∼1750 corr. yr BP; (McMinn *et al.*, 2000). The observed community shift from multiple dominant species to a community dominated by the sea-ice dinoflagellate *P. glacialis* (∼1850 cal. yr BP) would, therefore, point towards a more extensive sea-ice cover in SMB compared to the analyzed ∼850 years prior to this "event". This is in agreement with the findings of McMinn *et al.* (2001) who reported an increase in the abundance of the sea-ice diatom *Navicula glaciei* at an uncorrected ^{14}C age of 2300 yr BP in their study, a value which is close to the uncorrected ^{14}C age of ∼2350 yr BP for the increased dominance of fossil *P. glacialis* 18S rDNA that we observed in our study.

Conclusion

In summary, our combined genomical, organic geochemical and palynological survey revealed that fossil dinoflagellate DNA was preserved for at least ∼2700 years in organic carbon-rich, sulfidic sediments of SMB. This 18S rDNA-based survey revealed at least nine different past dinoflagellate species in SMB during this period. This approach revealed a pronounced shift at ∼1850 cal. yr. BP towards a dinoflagellate community dominated by the autotrophic sea-ice (brine) dwelling species *P. glacialis*. The parallel lipid biomarker survey revealed that dinosterol was the only dinoflagellate-diagnostic sterol in the analyzed sediments. A general decrease in dinosterol first suggested a decreasing contribution of dinoflagellates to the sedimentary organic matter towards the present-day. In contrast to this observation, the genomical approach revealed that the lower dinosterol concentration in more recent deposits was a result of the transition to *P. glacialis* as the main precursor of fossil dinoflagellate biomass, a species which in culture does not produce dinosterol. On the other hand, in culture experiments, a strain of *P. glacialis* from sea-ice at nearby Davis Station was previously shown to produce high levels of two diagnostic C_{27} sterols which were absent or minor constituents in the sediments. At this stage, we can only speculate that the absence of the latter diagnostic sterols could have been caused by (a) a relatively small contribution of dinoflagellates to the total preserved organic matter (b) a fast reaction with reduced sulfur species (i.e., sulfurization) or, more likely, that (c) these diagnostic lipids are not produced in *P. glacialis* during the phase of its life cycle when most cells become buried in the sediment record. Apart from the one known cyst-theca relationship for the observed palynomorphs, it was not possible to relate specific morphotypes to sequences derived from the paleoenvironmental genomics approach. For the other morphotypes it remains possible that further work will show that they match with at least some of the phylotypes identified by paleoenvironmental genomics.

The shift towards a predominance of *P. glacialis* at ∼1850 cal. yr BP reflects a more extensive ice cover compared to at least 850 years before this shift. This timing of this shift is in agreement with data on fossil sea-ice diatom assemblages in this region from other studies. Of the three dinoflagellate biomarkers studied here, phylogenetic analysis of sequenced fossil 18S rDNA provided the highest level of information about the past dinoflagellate composition (Table 1). It is however worth noting that the three approaches provide complementary information to each other. Species that do not produce preservable cysts can still be identified using fossil DNA. On the other hand, rare species which were present as cysts were not detected with the PCR/DGGE approach. The lipid approach provides a good indication of the relative importance of dinoflagellates as a source of preserved organic matter, but the lack of a universal dinoflagellate marker means that using dinosterol alone can lead to a biased interpretation of changes in dinoflagellate abundance over time. Our work has demonstrated that if DNA of past plankton members is preserved, paleoenvironmental genomics can serve as an important tool to validate or refine paleoecological and paleoenvironmental information inferred from more traditional proxies.

Acknowledgements

We thank Dr. Cornelia Wuchter, Peter Thompson and the crew of Australian Antarctic Division for help with obtaining the samples and core, and Anna Noordeloos for providing cultures of Alexandrium minutum and Scrippsiella sp. used to develop the dinoflagellate-specific primers. Two anonymous reviewers are thanked for helpful comments on an earlier version of the manuscript. This work was supported by grants from the Australian Antarctic Science Advisory Committee (1166 to J.K.V.), the Netherlands Organization for Scientific Research (NWO; 851.20.006 to J.S.S.D.; NWO grant 813.03.001 and NWO-VENI grant 016.051.014 to M.J.L.C.) and the German Science Foundation (DFG grant VE486/3-1) to GJMV.

CHAPTER 3

Fossil DNA preservation varies among species in an anoxic basin (Ellis Fjord, Antarctica): Source-specific variability and its potential implications for fossil DNA-based paleoecological records.

Arjan C. Boere, W. Irene C. Rijpstra,
John K. Volkman, Jaap S. Sinninghe Damsté,
Marco J. L. Coolen

submitted (Geobiology)

Abstract

Recent studies have shown that study of ancient plankton-derived DNA preserved in marine and lacustrine sediments is a promising approach for refining paleoenvironmental information. However, whether and to what extent the preservation of fossil plankton DNA differs between source organisms is poorly understood. Using a continuous and undisturbed 2700-year-old sediment record from a permanently stratified and anoxic basin in the Ellis Fjord, Antarctica we found that the amount of PCR-amplifiable fossil dinoflagellate 18S rDNA (500 base pairs; bp), derived mostly from a *Polarella*-related species, was up to 5 orders of magnitude lower in the deeper sediments than at the top centimetres. In contrast, similar-sized PCR-amplifiable diatom-derived 18S rDNA (predominantly from a cyst-forming *Chaetoceros* sp.) was only one order of magnitude less abundant. The relatively constant sedimentary C_{org}-content indicates that the amount of biomass exported to the sediment record has not changed significantly, and therefore we attribute these changes in abundance to DNA degradation over the ∼2700 years of deposition. The amount of fossil 16S rDNA from past chemocline-associated photosynthetic green sulfur bacteria (GSB) varied by up to two orders of magnitude between sediment horizons, but showed no obvious decreasing trend during the 2700 years of deposition. Assuming unaltered continuous degradation rates, we estimated that most of the fossil DNA of the investigated taxa in this environment would be degraded within ∼12 to ∼30 kyr. In good agreement with the quantitative data, the extent of post-depositional natural fragmentation into lower molecular weight size-classes (<2.2 kilobases) was found to be highest for dinoflagellates and lowest for GSB. Part of the diagnostic carotenoids of GSB was diagenetically altered as indicated by in increased ratio between PCR amplifiable 16S rDNA and the amount of intact chlorobactene derived from *C. phaeovibrioides* in the deeper sediments. This paper describes the possible causes behind the variation in the level of DNA preservation among the investigated planktonic taxa as well as consequences for the paleogenetics approach.

Introduction

In recent years, the analysis of ancient DNA derived from phyto- and bacterioplankton preserved in marine or lacustrine sediments (fossil DNA) has provided valuable information for refining reconstructions of Holocene and even late-Pleistocene environmental conditions and, vice versa, the impact of climate-induced hydrologic and environmental changes on past plankton succession dynamics (Coolen and Overmann, 2007; Coolen *et al.*, 2004b, 2006b; Boere *et al.*, 2009; Coolen *et al.*, 2009, 2008, 2007, 2006a; D'Andrea *et al.*, 2006; Manske *et al.*, 2008; Takeuchi *et al.*, 2009; Bissett *et al.*, 2005). Fossil DNA records are particularly useful in identifying past species, most notably phototrophic bacteria and protists, which did not produce or leave diagnostic cellular features in the fossil record or in the absence of diagnostic fossil lipid biomarkers.

However, a broad understanding of the factors controlling successful preservation of DNA in the sedimentary record is essential before paleogenetic analyses can be widely applied. Factors that are known to influence the fate of DNA in the sedimentary record include processes like microbial (nuclease) activity, adsorption of DNA to mineral and organic matrices, oxidative damage as well as various physical conditions such as temperature, salinity and pressure (Danovaro *et al.*, 2005; Schelble *et al.*, 2008; Coolen *et al.*, 2004a, 2007; Corinaldesi *et al.*, 2008; Romanowski *et al.*, 1991; Edgcomb *et al.*, 2009; Lorenz *et al.*, 1981; Lindahl, 1993; Vreeland *et al.*, 2000; Panieri *et al.*, 2010; Vreeland *et al.*, 2007; Borin *et al.*, 2008). However, whether and to what extent long-term preservation efficiency of fossil DNA differs between the various plankton precursor species remains poorly understood. For instance, DNA inside resting stages of certain plankton species (spores, cysts, but also plant seeds and pollen) is most likely better preserved than taxa that do not produce such resistant life stages (Gugerli *et al.*, 2005; Setlow, 1995; Coolen *et al.*, 2007; Lewis *et al.*, 2008; Stewart Johnson *et al.*, 2007).

The Small Meromictic Basin (SMB) in Ellis Fjord, eastern Antarctica, represents a suitable setting to study long-term taxon-specific variation in DNA preservation. Permanent stratification, bottom water anoxia and sulfidic conditions in SMB during at least for the last 3000 years (Bird *et al.*, 1991) resulted in the deposition of laminated sediments, which represent an undisturbed archive of past plankton species (Trull *et al.*, 2001). In addition, the amount of primary produced biomass exported to the sediment record has not changed significantly throughout the 2700 years of deposition as indicated by the relatively constant sedimentary C_{org}-content (Boere *et al.*, 2009). Recent paleogenetic studies on a well-dated core of SMB have already identified the long-term and continuous presence of the same predominant diatom (Coolen *et al.*, 2007) and dinoflagellate species (Boere *et al.*, 2009). The use of general primers targeting 18S ribosomal RNA genes (18S rDNA) and subsequent denaturing gradient gel electrophoresis (DGGE) fingerprinting resulted in the detection of *Chaetoceros* DNA throughout the ∼2700-year-old record. Cysts and cells of this species represented the majority of the fossil diatom remains in a parallel core analyzed from SMB (McMinn *et al.*, 2001). General primers targeting fossil dinoflagellate 18S rDNA resulted in the detection of a single abundant phylotype closely related to the sea-ice associated *Polarella glacialis* since the last 1850 years and a predominance of *Gymnodinium*-related phylotypes as putative sources of the dinoflagellate lipid biomarker dinosterol in the older sediments (Boere *et al.*, 2009).

Thus, the late-Holocene sediment record of SMB offers a unique opportunity to study variations in DNA preservation of species within different taxonomical groups that were exposed to the same environmental conditions for thousands of years. In the current study, we performed a parallel quantitative analysis of preserved partial 18S rDNA fragments (∼500 bp) and dinosterol to study differences in the preservation potential of the genetic vs. general lipid biomarker of dinoflagellates. In addition, we examined the extant and Holocene diversity of chemocline bacteria, i.e., the obligate anoxygenic photoautotrophic green sulfur bacteria (Chlorobiaceae or GSB), using denaturing gradient gel electrophoresis (DGGE) and sequencing. The amount of

preserved fossil 16S rDNA of GSB (525 bp) and diagnostic carotenoids (chlorobactene and isorenieratene) were compared to reveal differences in the preservation potential of these GSB markers. To estimate potential taxon-specific variability in the level of DNA preservation, we compared the rate of natural fragmentation of the fossil diatom, dinoflagellate, and GSB DNA.

Materials and Methods

Site description, sample collection, DNA extraction

A 113-cm-long sediment core spanning 2700 years of deposition (Sinninge Damsté *et al.*, 2007) was obtained in November 2001 from the depocentre (13 m) of the Small Meromictic Basin (SMB) of Ellis Fjord, Vestfold Hills, eastern Antarctica. For an elaborate site description, see Boere *et al.* (2009) and Coolen *et al.* (2007) and references therein.

Total DNA had previously been extracted from 34 one-cm intervals to study the succession of late-Holocene diatoms (Coolen *et al.*, 2007) and dinoflagellates (Boere *et al.*, 2009; Coolen *et al.*, 2007), using our standardized procedures to prevent and test for contamination with foreign DNA (e.g., Coolen *et al.*, 2006b). The same DNA extracts as used in the previous paleogenetic studies (Boere *et al.*, 2009; Coolen *et al.*, 2007) were used for this DNA degradation survey.

Quantitative polymerase chain reaction amplification of SSU rDNA

Quantitative real-time PCR (qPCR) amplification of small subunit (SSU) ribosomal RNA genes of GSB and dinoflagellates was performed using an iCycler system (Biorad, Hercules, CA.). Detailed information about the quantification of partial diatom 18S rDNA (516 bp) and the identification of the late-Holocene diatom population in SMB can be found elsewhere (Coolen *et al.*, 2007). PCR amplification involved initial denaturing for 4 min at 95°C, followed by 45 cycles including denaturing (30 sec at 94°C), 40 sec of primer annealing at 56.5°C (for GSB) or 63.5°C (for dinoflagellates), and a primer extension (40 sec at 72°C). A final product extension step was performed at 72°C (30 min). With the exception of the primers used in this study, the reaction mixture ingredients were similar to the SYBR Green assay described previously (Coolen *et al.*, 2007; Boere *et al.*, 2009). For the GSB assay, the primers GSB-619F (5'-GGGGTTAAATCCATGTGCT-3') and GSB-1144R (5'-CAGTTCARTTAGAGTCC-3') were used to amplify a fragment of 525 bp (Achenbach *et al.*, 2001). A ten-fold dilution series of full length 16S rDNA ($10^0 - 10^8$ copies) of *Chlorobium limicola* strain DSM245 served as a quantitative standard. For the dinoflagellate assay, the primers Euk-A (5'-AACCTGGTTGATCCTGCCAGT-3'; Medlin *et al.*, 1988) and (Dino-Rev: 5'-ACAAGACATGGATGCCCT-3'; Boere *et al.*, 2009) were used to amplify a fragment of 503 bp. A ten-fold dilution series

of full-length 18S rDNA ($10^0 - 10^8$ copies) of *Scrippsiella* sp. (obtained from the culture collection at the Royal NIOZ) was used as a standard. The amount of fossil SSU rRNA genes was expressed as copies per gram organic carbon (copies g^{-1} C_{org}) to compensate for variations in the sedimentary organic carbon content. In order to prevent contamination of the pristine sediments, fossil DNA extracts or PCR reagents, all calibration reactions were prepared in a PCR workstation located in a spatially separated post-PCR lab. The qPCR amplicons were subjected to a melting curve analysis (temperature range from 60°C to 96°C in 0.3°C temperature increments) in order to identify non-specific PCR products and subsequently the samples were subjected to agarose gel electrophoresis to check for primer dimers and other PCR artifacts.

Amplification of 16S rDNA for Denaturing Gradient Gel Electrophoresis (DGGE)

The partial PCR-amplified 16S rDNA of GSB was separated by DGGE (Muyzer and Smalla, 1998). PCR-product for this was amplified in a two-step protocol to increase the sensitivity of the assay. First, 20 μl reaction mixtures were run for 15 amplification cycles with the unmodified GSB-primers at an annealing temperature of 56.5°C, after which 1 μl was transferred to 50 μl of fresh reaction mixture with the same ingredients as used during the first amplification round accept that the 5' end of the reverse primer (GSB-1144r) contained a 40-bp GC-rich sequence (GC-clamp; (Muyzer and Smalla, 1998). This second reaction was run for 20 cycles at an annealing temperature of 55°C. The GC-clamp that was introduced during the second PCR run served to prevent complete denaturation of the PCR products in the DGGE. The DGGE (6% acrylamide gel) contained a linear gradient of denaturant between 20% and 70%. 100 ng of each GSB amplicon was loaded onto the gel and electrophoresis proceeded for 5 h at 200 V (12.5 V cm^{-1}) and 60°C. All procedures involved in the DGGE analysis of PCR-amplified diatom and dinoflagellate DNA as well as the steps involved to generate template DNA for standard Sanger sequencing of the excised DGGE fragments have been described previously (Coolen *et al.*, 2007; Boere *et al.*, 2009).

Natural fragmentation of fossil DNA

In order to determine the degree of taxon-specific fossil DNA fragmentation, 5 μl of total sedimentary DNA extracts from 6 depth intervals ($1 - 2$ cm, $19 - 20$ cm, $38 - 40$ cm, $58 - 60$ cm, $78 - 80$ cm and $110 - 112$ cm, corresponding to $\sim$0, $\sim$800, $\sim$1100, $\sim$1500, $\sim$1800 and $\sim$2700 calendar years before present (BP), respectively (Sinninghe Damsté *et al.*, 2007) was subjected to agarose gel electrophoresis (0.7% w/vol agarose; 1× Tris-acetate EDTA buffer, pH 8.0, both sterilized by autoclaving) for 45 min at 90 V. Two size markers (phage λ Hind III digest and precision molecular mass standard (PMMS) from Biorad) were used to estimate the length of the sedimentary DNA. Different size classes of sedimentary DNA were excised from the

gel using a flame-sterilized scalpel and tweezers as described previously (Coolen *et al.*, 2006b). The selected size classes were (1) >23 kilobases (kb), (2) $23 - 4.4$ kb, (3) $4.4 - 2.2$ kb, (4) $2.2 - 0.7$ kb and (5) <0.7 kb. DNA was subsequently recovered from the gel slices using a Centrilutor Micro-ElectroElutor system (Millipore) following the manufacturer's procedures. The DNA eluted from the gel slices was dissolved in 25 μl sterile 1 Tris-EDTA. As a control, empty pieces of gel were excised and co-eluted to monitor any contamination during the process. A subsample of each eluted DNA extract was subjected to a second round of gel electrophoresis to check for yield and correct DNA fragment length. Then, the number of preserved ∼500-bp-long plankton DNA in each DNA size class was determined by qPCR using the dinoflagellate and GSB primer combinations as described above. In addition, the amount of partial diatom-derived 18S rDNA preserved in each DNA size class was determined by qPCR using the primer combinations and conditions described previously (Boere *et al.*, 2009; Coolen *et al.*, 2007). All PCRs were run in duplicate.

Carotenoid analysis

Freeze-dried and ground sediment samples of 0.1 to 0.7 g were ultrasonically extracted with acetone (3×, 3 min). Samples were centrifuged at 3000 rpm for 5 min. The supernatants were decanted, combined and concentrated using rotary vacuum evaporation. The extracts were subsequently dried under a gentle flow of nitrogen. The residue was redissolved in dichloromethane and applied to a silica column and the apolar carotenoids eluted with dichloromethane, dried under nitrogen and the residue dissolved in acetone. Care was taken during the entire sample preparation procedure to avoid exposure of the samples to light and heat. This carotenoid fraction was then immediately analyzed on a HP 1100 series HPLC equipped with an auto-injector and photodiode array detector. Separation was achieved on a ZORBAX Eclipse XDB-C18 column (2.1 × 150 mm, 3.5 μm; Agilent Technologies, USA), maintained at 25°C, with a linear gradient from 65% solvent B to 80% solvent B in 45 min, with solvent A being methanol/water (4:1, v/v) and solvent B acetone/methanol/water (19:1:1, v/v/v). The flow rate was 0.3 ml/min. Detection was achieved by in-line UV-detection ($250 - 700$ nm). Chlorobactene and isorenieratene were quantified by comparing their UV responses at 462 nm (λ_{max} of chlorobactene in the mobile phase) and 454 nm (the λ_{max} of isorenieratene in the mobile phase) of known amounts of an authentic β-carotene standard (Aldrich) and correcting for the difference in extinction coefficients (Britton *et al.*, 1995).

In order to examine whether chlorobactene was accompanied by its diagenetic products (e.g., partly hydrogenated chlorobactane), ca. 3 g of two sediment sections ($37 - 39$ cm and $85 - 87$ cm) were ultrasonically extracted using mixtures of dichloromethane and methanol. An aliquot of the total lipid extract, with an internal standard (6,6-d2-2-methylheneicosane) was hydrogenated for 1 h with PtO_2 and a few drops of acetic acid. The resulting fraction was chromatographed over a column packed with Al_2O_3 to obtain an apolar fraction using hexane/dichloromethane (9:1, v/v) as an eluent and analysed by capillary gas chromatography (GC) and GC-mass

spectrometry (GC-MS) as described elsewhere (Coolen *et al.*, 2004b).

Results

Quantitative distribution of fossil dinoflagellate markers: 18S rDNA vs. dinosterol

The fossil dinoflagellate 18S rDNA content was highest in the top 2 cm of sediment (up to $\sim 10^{12}$ copies g^{-1} C_{org}, Fig. 1B), followed by a three orders of magnitude exponential decrease within the last ~800 years of deposition (upper ~20 cm of sediment). Dinoflagellate copy numbers continued to decrease, but at a slower rate (two orders of magnitude), in the sediments deposited between ~800 and ~2700 calendar years (i.e., $20 - 113$ cm) ago. Thus, the amount of dinoflagellate rDNA decreased by five orders of magnitude over the 2700 years of sediment deposition. Contrary to the exponential decrease of fossil dinoflagellate DNA, the dinoflagellate-specific lipid biomarker dinosterol (Fig. 1B) increased almost ten-fold with depth, from ~100 μg g^{-1} C_{org} at the surface to ~1000 μg g^{-1} C_{org} in the deepest sediments analyzed data modified from Boere *et al.* (2009)).

Quantitative distribution of fossil GSB markers: 16S rDNA vs. carotenoids

Whereas an exponential decrease in preserved dinoflagellate DNA had started to occur even in the top few cm of the core, the amount of similar-sized partial GSB 16S rDNA declined only between 20 and 35 cm (from 5×10^9 to 2×10^8 copies g^{-1} C_{org}). The fossil GSB DNA content strongly fluctuated between 35 and 80 cm, and increased again in the oldest sediment layers to levels found in the surface layers (Fig. 1C).

Three fossil GSB-specific carotenoids (chlorobactene, isorenieratene, β-isorenieratene) were identified and their abundance was compared with the concentration of the fossil GSB rDNA to determine any variations in the preservation potential of both types of GSB markers. Since the most abundant carotenoid in all analyzed sediments was chlorobactene and the other less abundant carotenoids showed a similar concentration pattern, only the concentration profile of chlorobactene is shown (Fig. 1C). Chlorobactene reaches a maximum concentration (23 mg g^{-1} C_{org}) at 20 cm, followed by a gradual decrease to ~0.5 mg g^{-1} C_{org} at 100 cm depth, and an increase again (~8 mg g^{-1} C_{org}) in the deepest 13 cm of sediment.

Extant and late-Holocene GSB community composition

DGGE analysis of partial GSB-derived 16S rDNA amplicons recovered from the sulfidic chemocline and the Holocene sediment layers resulted in a single predominant band (Fig. 1D). To confirm that all bands at the same horizontal position in the gel represented the same phylotype, a total of 10 bands, marked with a black dot

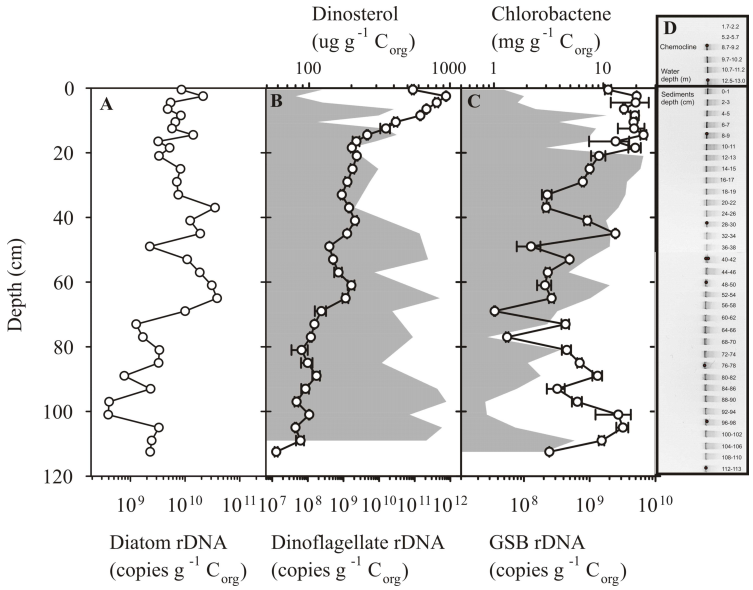


Figure 1: Concentrations of preserved genetic (∼500 bp) and lipid biomarkers of ancient plankton members in the anoxic sediments of Ellis Fjord. A) Partial diatom 18S rDNA (copies g^{-1} C_{org}) modified from (Coolen *et al.*, 2007) are shown for comparison. B) Number of partial 18S rDNA copies of dinoflagellates (copies g^{-1} C_{org}) and associated biomarker dinosterol (grey area, modified from Boere *et al.* (2009)). C) Partial 16S rDNA of green sulfur bacteria (GSB; copies g^{-1} C_{org}) and associated biomarker chlorobactene (grey area, this study). D) DGGE showing a single band of partial GSB 16S rDNA that was continuously present throughout the last ∼2700 yr and the sulfidic part of the photic zone. DGGE results for diatoms and dinoflagellates of the same core and samples can be found elsewhere (Coolen *et al.*, 2007; Boere *et al.*, 2009).

in Figure 1D, were excised from various depths and sequenced. All analyzed DGGE bands represented the same GSB 16S rDNA sequence with >98% sequence similarity (a maximum of 5 different nucleotide positions out of 478 total nucleotides) with *Chlorobium phaeovibrioides* strains 2631 (acc.nr. NR_029322) and DSM 261 (acc.nr. AJ290828; formerly assigned to *C. vibrioforme*; Imhoff and Thiel, 2010). Other, equally related environmental sequences were obtained from Antarctic saline lakes (clone PENDANT-30, acc.nr. AF142942; Bowman *et al.*, 2000). The partial 16S rDNA of the SMB strain differs from the sequence of the control strain that served as a standard during qPCR (20 mismatches and 8 gaps, 95% sequence similarity), thus ruling out cross contamination. The new *Chlorobium* sequence is deposited in Genbank under accession number HM182103.

Fragmentation of fossil DNA into low molecular weight size classes

The successful separation of total DNA extracts from six depth intervals into five individual DNA size-classes (>23 kb, 23−4.4 kb, 4.4−2.0 kb, 2.0−0.7 kb, and <0.7 kb) is shown in Fig. 2. For each size-class, the numbers of partial rDNA copies of diatoms (∼516 bp long fragment), dinoflagellates (503 bp) and GSB (525 bp) were determined in duplicate. Then, per depth, the copy numbers in the separate size-classes were summed, and the copy number in the separate size classes expressed as a percentage of the copies per depth (Fig. 3). In addition, the sum of copies (%) in all size classes except the >23 kb-class (i.e., the copies found in the degraded DNA fraction), is shown in the top right corners of each graph as a rough estimate of the fragmentation per depth. For fossil DNA of diatoms and dinoflagellates, the percentage of PCR-amplifiable 18S rDNA fragments in the largest analyzed DNA size-class (>23 kb) declined with increasing sediment age. For both groups of eukaryotic plankton, there was an increase in the amount of PCR-amplifiable 18S rDNA fragments in the second smallest DNA size class (0.7 to 2.2 kb). This DNA fragmentation trend most apparent for the dinoflagellates, since $20 - 40\%$ of the 18S rDNA copies were present in the largest DNA size-class (>23 kb) in sediments up to 60 cm deep, followed by a steep decline to only ∼3% of PCR-amplifiable dinoflagellate 18S rDNA in the largest DNA size class from the oldest analyzed sediments layer (Fig. 3). In addition, a small percentage of diatom and dinoflagellate 18S rDNA copies was also present in the smallest DNA size class (<700 bp).

In contrast, the percentage of PCR amplifiable 16S rDNA of GSB in the largest DNA size class remained fairly constant at $\sim 30 - 55\%$ throughout the core and the smallest DNA size class did not contain any PCR-amplifiable GSB 16S rDNA.

Discussion

Preservation of fossil dinoflagellate vs. diatom DNA

Our results show that the amount of fossil dinoflagellate-derived 18S rDNA decreased by five orders of magnitude over the 120 cm sediment interval (Fig. 1B), whereas the amount of similar-sized partial 18S rDNA fragments of diatoms decreased by only one order of magnitude over 2700 years of deposition (Coolen *et al.*, 2007). This difference can either be the result by an ecological shift to a dinoflagellate-dominated ecosystem in SMB over the course of the last ∼2700 years, or represent a variation in the preservation potential of fossil DNA of dinoflagellates vs. diatoms. At first glance, the decreasing trend in dinosterol in the upper part of the core would argue against a more dinoflagellate-dominated ecosystem in the recent past. However, a predominance towards a different dinoflagellate population with known dinosterol producers was previously found to be the explanation for the higher dinosterol concentration in the deeper sediments (Boere *et al.*, 2009). Thus, the genetic and lipid biomarkers record

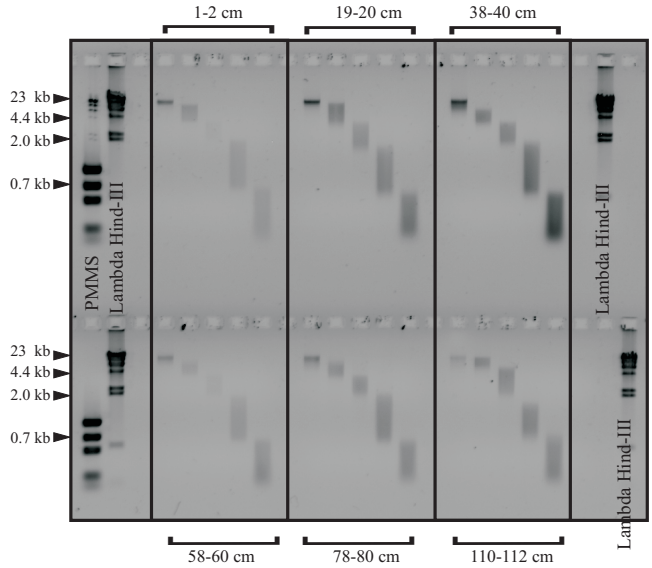


Figure 2: Agarose gel showing the separation of total DNA from 6 depths ($1-2$, $19-20$, $38-40$, $58-60$, $78-80$ and $110-112$ cm depth) into five high-to-low molecular weight DNA size-classes (>23 kb, $23-4.4$ kb, $4.4-2.2$ kb, $2.2-0.7$ kb and <0.7 kb, (cf. Coolen *et al.*, 2006b)). The various DNA size classes served as template to determine the number of preserved ∼500 bp-long rDNA copies as a measure for the level of natural fragmentation of plankton-specific DNA (shown in Fig. 3).

intrinsically different aspects of the dinoflagellate paleocommunity, and should not be used to directly compare (quantitatively) the individual results. Rather, they should be used as complementary tools.

Nevertheless, there is no evidence for an increasingly dinoflagellate-dominated ecosystem (Grey *et al.*, 1997; Laybourn-Parry and Pearce, 2007) and abundant diatom blooms still occur seasonally in the SMB (McMinn *et al.*, 2001; McMinn and Hodgson, 1993). In addition, the amount of primary produced biomass exported to the sediment record has not changed significantly throughout the ∼2700 years of deposition as indicated by the relatively constant sedimentary C_{org}-content (Boere *et al.*, 2009).

Assuming a relatively constant productivity over time in the Small Meromictic Basin, taxon-specific differences in the extent of post-depositional degradation more likely accounts for the more extensive downcore decline in dinoflagellate-derived DNA contents and the increased post-depositional fragmentation of dinoflagellate DNA as opposed to diatom DNA. This differential degradation rate is confirmed by the fragmentation experiment, which showed that a slightly higher percentage of dinoflagellate copies is found in the smaller size classes (e.g., 94% of dinoflagellate copies vs. 89% of diatom copies in the deepest analyzed sample). Our previous paleogenetics survey

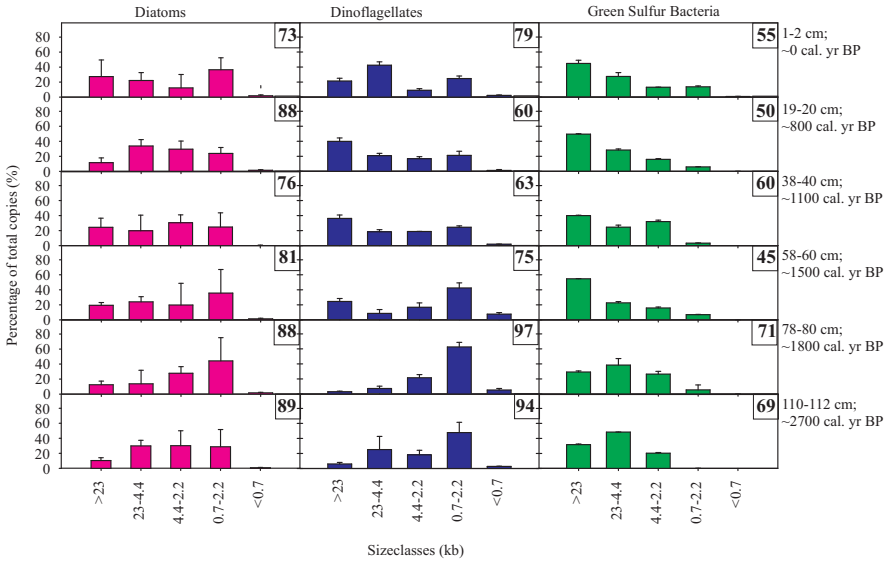


Figure 3: Distribution of the numbers of rDNA copies detected in the different size classes (Fig. 2). The numbers in the top right corners of the graphs are the sum of copies (%) found in the size classes other than >23 kb, i.e., the percentage of copies that is found in the degraded DNA fraction. Based on the distribution of PCR-amplifiable partial rDNA in the various DNA size classes studied it is clear that fossil GSB-derived DNA is preserved best and the dinoflagellate-derived DNA shows the most fragmentation.

revealed that the most abundant fossil diatom DNA in SMB was derived from *Chaetoceros* spp., with DNA from other diatom species being $2-4$ orders of magnitude less abundant (Coolen *et al.*, 2007). Both cysts and vegetative cells of *Chaetoceros* were previously found to be most abundant in the anoxic sediments of the basin (McMinn *et al.*, 2001) and were shown to remain viable for years (Lewis *et al.*, 1999). In contrast, the spiny resting cysts produced by *Polarella glacialis*, which was the dominant dinoflagellate identified from the fossil DNA pool in SMB during the last 1850 years (Coolen *et al.*, 2006b; Boere *et al.*, 2009), are very fragile and hardly ever recovered from sediments (Buck *et al.*, 1992; McMinn, 1995; Montresor *et al.*, 1999). Therefore, the differences in the preservation efficiency between 18S rDNA derived from *Chaetoceros* and *Polarella* in the fossil record of SMB can be explained by the differences in their cellular architecture.

Another aspect that might contribute to the observed differences in preservation is that dinoflagellate DNA is unusual (Rizzo, 1987; de la Espina *et al.*, 2005), for instance in lacking chromosome-stabilizing histones. This lack of histones may promote DNA degradation, as these proteins play an important role in the stability of DNA (Kelman and Moran, 1996). Many dinoflagellate species have extremely large genomes with

multiple (up to thousands of) rRNA gene copies (Hou and Lin, 2009; Hackett and Bhattacharya, 2006; LaJeunesse *et al.*, 2005; Rizzo, 1987). This would explain the extremely high copy numbers in the upper few cm of recently deposited sediment. In this part of the core, the number of copies of dinoflagellates even exceeds the total number of eukaryotic 18S rDNA copies (Fig. 1A; data from Coolen *et al.*, 2007). Factors that could have contributed to these biased qPCR results are not restricted to varying rRNA operon copy number, but include differences in cell lysis, primer efficiency and coverage (Klappenbach *et al.*, 2001; Zhu *et al.*, 2005; von Wintzingerode *et al.*, 1997; Potvin and Lovejoy, 2009; Fogel *et al.*, 1999). However, by using the same protocols for total DNA extraction and purification, as well as by using the same PCR ingredients including primers with high coverage, we minimized most of the qPCR-based bias for studying the relative abundance of species through time.

Preservation of 16S rDNA vs. carotenoids of green sulfur bacteria

Sulfur-oxidizing green sulfur bacteria (GSB; Chlorobiaceae) occur widely in the sulfidic chemoclines of stratified settings with sufficient light to promote anoxygenic photoautolithotrophic growth (Pfennig, 1989; Imhoff and Thiel, 2010; Larsen, 1953). The occurrence of carotenoids that are diagnostic for GSB (i.e., isorenieratene, β-isorenieratene and chlorobactene) in the fossil record is therefore considered as a proxy for past stratified conditions, sulfidic bottom waters, and photic zone anoxia (Menzel *et al.*, 2002; Passier *et al.*, 1999; Sinninghe Damsté and Koopmans, 1997; Sinninghe Damsté *et al.*, 1993). Variations in the amount of fossil biomarkers of GSB (carotenoids and perhaps DNA) reflect past changes in the hydrology that resulted in either a shoaling or deepening of the GSB-populated chemocline.

However, we observed a poor correlation between GSB rDNA and chlorobactene in the fossil record of SMB. Since only a single GSB-phylotype was detected, this particular species must have been the source organism of all the GSB-specific carotenoids, and similar trends would be expected if both markers are preserved equally well. Only between 20 and 70 cm depth, both biomarkers show a similar decreasing trend, but whereas the carotenoid concentration continues to decline below 70 cm, the GSB 16S rDNA content increases again. A similar situation was shown for fossil DNA of an identical phylotype and carotenoids in up to 10,450-year-old anoxic sediments of the nearby Ace Lake (Coolen *et al.*, 2006a). This finding was attributed to diagenetic processes leading to the formation of hydrogenated carotenoids, most notably chlorobactane (cf. Sinninghe Damsté and Koopmans, 1997). Since our current study also confirmed that post-depositional fragmentation of fossil GSB DNA was limited, fossil GSB DNA in the late-Holocene SMB sediments seems to be less prone to diagenetic alteration and represent a more reliable and more informative diagnostic marker for GSB than intact carotenoids in this setting. Small amounts of GSB 18S rDNA were nevertheless present in the lower molecular weight DNA size classes, but this may be explained by limited shearing of the DNA during DNA extraction (Coolen *et al.*, 2006b).

The reason for the efficient preservation of GSB DNA remains speculative, since we did not investigate whether fossil DNA of GSB was present in the form of endospores, vegetative cells, or in the form of extracellular DNA (Coolen *et al.*, 2007; Corinaldesi *et al.*, 2008; Lewis *et al.*, 2008). However, to the best of our knowledge, GSB do not produce protective resting stages such as cysts or endospores that would facilitate the preservation of DNA. Studies on ancient DNA in permafrost suggested that bacterial DNA is best preserved in intact viable cells rather than in spores, because intact bacterial cells continue to repair the DNA at slow rates for up to thousands of years (Lewis *et al.*, 2008; Stewart Johnson *et al.*, 2007). In accordance with this finding, certain GSB are able to maintain cell integrity and remain viable under prolonged dark conditions by metabolizing intracellularly stored polyglucose (Koizumi *et al.*, 2004; Sirevåg and Ormerod, 1977). Whether cells of *C. phaeovibrioides* in the SMB sediments are still viable and use these or similar mechanisms to maintain cell integrity remains speculative, but could in theory explain why the majority of its fossil DNA is still present in the form of high molecular weight DNA. In this respect, it is worth noting that fossil GSB DNA of green sulfur bacteria has been detected in up to 217-kyr-old eastern Mediterranean sapropels (Coolen and Overmann, 2007), whereas the presence of fossil DNA of marine microbial eukaryotes was mainly restricted to the youngest sapropel S1 (∼10 kyr cal. BP) and sporadically present in the late Pleistocene sapropels (Boere, submitted).

Since we employed a stringent and efficient DNA extraction protocol we cannot differentiate between intracellular and extracellular DNA (e.g., Corinaldesi *et al.*, 2008). A few recent studies have shown that the majority of DNA in marine sediments is present in the form of extracellular DNA (Corinaldesi *et al.*, 2008; Coolen *et al.*, 2007; Dell'Anno *et al.*, 2002; Danovaro *et al.*, 2006; Dell'Anno and Danovaro, 2005). Extracellular DNA in marine sediments can be protected against degradation by microbial nucleases for substantial periods of time when it is adsorbed to mineral and organic matrices (Romanowski *et al.*, 1991; Lorenz *et al.*, 1981; Lorenz and Wackernagel, 1987). More specifically, studies of DNA adsorption kinetics indicate that circular prokaryotic DNA is better adsorbed to clay minerals than eukaryotic linear DNA (Nguyen and Elimelech, 2007; Poly *et al.*, 2000). Further work is necessary to exactly understand which mechanisms are responsible for the observed enhanced preservation of fossil GSB DNA as opposed to the DNA of the two investigated important eukaryotic plankton groups, most notably that of the faster degrading dinoflagellate DNA.

Estimated maximum preservation age of fossil plankton DNA in SMB?

Using the age model for this SMB sediment record after Sinninghe Damsté *et al.* (2007) and assuming a constant degradation rate, the dinoflagellate DNA would be totally degraded within 12,000 years (∼450 cm depth) (r^2=0.84; Fig. 4). However, the concentration of fossil dinoflagellate DNA is expected to reach the detection limit for PCR (i.e., 10^3 to 10^4 copies g^{-1} C_{org}) earlier and this would effectively restrict

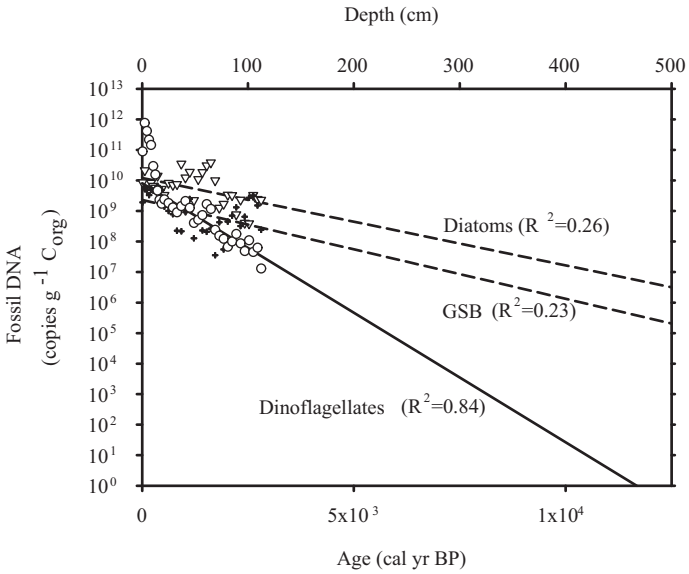


Figure 4: A regression line plotted through the number of dinoflagellate copies in the investigated samples shows a rather good fit (r2=0.84). Further extrapolation of this regression line, assuming a constant degradation rate and using the age model for this SMB sediment record from Sinninghe Damsté *et al.* (2007), yields an estimate for the maximum preservation age of dinoflagellate rDNA, i.e., $\sim 1.2 \times 10^4$ yr BP. A similar regression through the diatom and GSB copy numbers gives an estimated maximum preservation age of $\sim 3 \times 10^4$ yr BP, but have low r^2 values (i.e., 0.26 and 0.23, respectively).

the analysis of fossil dinoflagellate DNA to the late Holocene ($\sim 7 - 8$ kyr BP). The decline in fossil content of diatom and GSB DNA does not show a similar exponential trend. Taking into account the poor regression (r^2=0.26 and 0.23, Fig. 4), we roughly estimated that DNA from diatoms and GSB would be completely degraded within ~30,000 years, despite the good preservational characteristics of these sediments.

This maximum extrapolated preservation age of fossil plankton DNA in SMB is relatively short when compared to earlier (theoretical) estimated maximum timescales of fossil DNA preservation (100 kyr-1Myr, Willerslev and Cooper, 2005; Corinaldesi *et al.*, 2008) or empirical data (Coolen and Overmann, 2007; Coolen *et al.*, 2006a; Schippers and Neretin, 2006). Since the refractory nature of bulk organic matter generally increases with sediment depth and age (D'Hondt *et al.*, 2004; Jørgensen and Boetius, 2007) and bioavailability generally decreases (Middelburg, 1989; Rothman and Forney, 2007), our assumption that DNA degradation rates remain are likely incorrect. In that case, the fossil plankton DNA in SMB, and other locations, could be preserved substantially longer than based on our extrapolated estimates, especially if adsorption to sediment particles is involved.

Conclusions

Although much remains to be learned about the processes and factors governing the preservation of DNA in the geological record, we have presented here the first empirical evidence of taxon-specific variation in fossil DNA degradation rates. Of the three taxa investigated here, green sulfur bacterial DNA was best preserved, with the most limited post-depositional fragmentation in the late-Holocene Antarctic fjord sediments, followed by slightly less well-preserved and more fragmented fossil diatom-derived DNA (i.e., mainly from cyst-forming *Chaetoceros* sp.). In contrast, the amount of fossil dinoflagellate-derived DNA (i.e., from a *Polarella* species with poorly preserving cysts) decreased exponentially with depth concomitant with the highest degree of post-depositional fragmentation.

The data reported in this study highlight potential pitfalls in paleogenetic studies. Most notably, taxon-specific variable levels of post-depositional fragmentation of fossil DNA could negatively impact PCR efficiencies unless only relatively short (<400-bp-long) gene fragments are being analyzed in PCR-based surveys to study past plankton molecular succession dynamics. The analysis of smaller sized phylogenetic markers via pyro-tag sequencing (Sogin *et al.*, 2006; Amaral-Zettler *et al.*, 2009), would minimize PCR bias, lower the detection limit, and enable paleogenetic studies using much older sediment records. Our paleogenetic survey also found limitations in the use of diagnostic lipid biomarkers for green sulfur bacteria. A down-core increase in the ratio between fossil GSB 16S rDNA and chlorobactene was indicative of a higher degree of diagenetic alteration of intact carotenoids, and that DNA was a more robust and informative biomarker for GSB in this anoxic setting. It is thus important to use paleogenetic approaches in combination with other independent methods to cross-validate the results of the separate approaches and gain a better understanding of the paleoecological and paleoenvironmental information inferred from genetic and lipid biomarkers.

CHAPTER 4

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

Marco J. L. Coolen, Arjan C. Boere,
Ben Abbas, Marianne Baas,
Stuart G. Wakeham, Jaap S. Sinninghe Damsté

Paleoceanography, vol.21, PA1005, 2006
doi:10.1029/2005PA001188

Abstract

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.

Introduction

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.

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 non-coccolithophorid *Isochrysis* species (Coolen *et al.*, 2004a). This combined lipid-DNA biomarker approach also showed that Holocene salinity variations caused major changes in the abundance of different haptophyte species, 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.*, 2006b). 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 deepsea environments, where cells have to travel a far greater distance and experience a longer residence time before they reach the sediment.

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 coccolithbearing 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 (Diéz *et al.*, 2001) as well as primers selective for haptophytes (Coolen *et al.*, 2004a). All amplicons were analyzed by denaturing gradient gel electrophoresis (DGGE) (Coolen *et al.*, 2004a; Muyzer *et al.*, 1993).

Since DNA of alkenone-biosynthesizing haptophytes was expected to be far less stable compared to these alkenone lipids, we also determined the haptophyte DNA/al-

kenone 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 (qPCR) 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.

Experimental setup

Setting

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).

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.

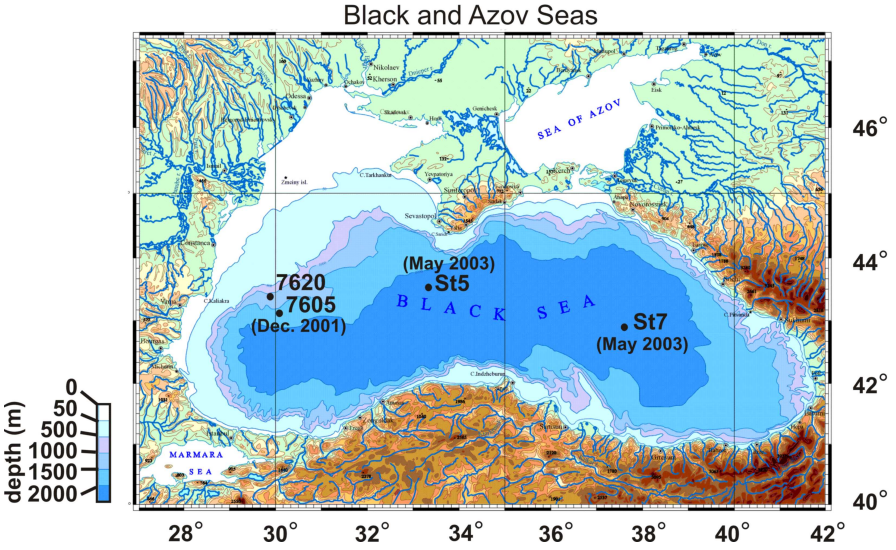


Figure 1: Sampling sites within the Black Sea. Location of the sampling sites for particulate organic matter (POM) (stations 6705 and 6720 within the Western Basin, which were sampled during the R/V *Meteor* 51/4 cruise in December 2001, and station 5 (ST5) of the Central Basin and station 7 (ST7) of 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*.

Sampling

Particulate organi 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 (Fig. 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 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.

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.

Lipid extraction for the analysis of alkenones

Filters with POM were freeze-dried and ultrasonically extracted with methanol (3×), dichloro-methane (DCM)/methanol (1:1, v/v) (3×) and DCM (3×), 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-dideuteroheneicosane) 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).

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.

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.

Total Organic Carbon (TOC)

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 $\delta^{13}C$.

Extraction of total DNA

Total DNAwas extracted from sections of GFF filters and from 0.25 g of the 12 selected sediment sections using the UltraClean^{TM} 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 DNAwork since all reagents and disposable tubes are free of DNA, which substantially reduced the chance of contamination with foreign DNA.

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 productfree 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 (qPCR) reactions in order to determine whether PCR-inhibiting coextracted impurities within the DNA extracts were present.

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.

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

Amplification of ancient 18S rDNA

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 (Diéz *et al.*, 2001). In addition, partial 18S rDNA solely found in haptophyte algae was selectively amplified using primers selective for haptophytes (Coolen *et al.*, 2004a). 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 50-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 mM of each deoxynucleotide (dNTP) (Amersham-Biosciences, Piscataway, New Jersey), 8 μg of Bovine Serum Albumin (BSA), 2 μl of 10× PicoMaxx^{TM} reaction buffer (Stratagene, LaJolla, California), 1 unit of PicoMaxx^{TM} 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.

Denaturing gradient gel electrophoresis

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.*, 2004a) 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 $50-75$ mL sterile 10 mM 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.

Sequencing of DGGE fragments

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.* (2004a).

Phylogenetic analysis

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.

Quantitative real-time PCR

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 SUBRGreen. Reaction mixtures (20 mL) contained 1 unit of Picomaxx^{TM} high-fidelity DNA polymerase, 2 μl of 10$\times$ Picomaxx PCR buffer (both Stratagene), 0.25 mM of each dNTP, 8 μg of BSA, 0.2 μM of primers, 50,000 times diluted SYBRgreen (Molecular Probes) (optimized concentration), a final concentration of 10 nM fluorescein, 3 mM 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 qPCR reaction.

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-bplong 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 qPCR along with the samples. 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.

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.

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.

Natural fragmentation of fossil DNA of *E. huxleyi*

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 Ultra-Clean 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.

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 1× 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 1× SybrGold (Molecular Probes) in sterile 1× TAE pH 8.0. In order to prevent DNA damage by UV, we used the Dark Reader (Clare Chemical Research, Inc.) to visualize the SybrGoldstained 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.

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 $\lambda\times$ *Hind*III digest and Position Molecular Mass Standard (PMMS, Biorad)) on each side of the agarose gel.

The 0.4 μl of the total extracts as well as of each individual fragment was subjected to qPCR 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 qPCR 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.

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 qPCR using the primers and conditions selective for haptophytes.

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 qPCR contained besides the natural amount of sedimentary *E. huxleyi* DNA, the DNA of 2×10^5 added *E. huxleyi* cells.

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.

Results

Calibration of sediment ages

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 coccolithbearing 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).

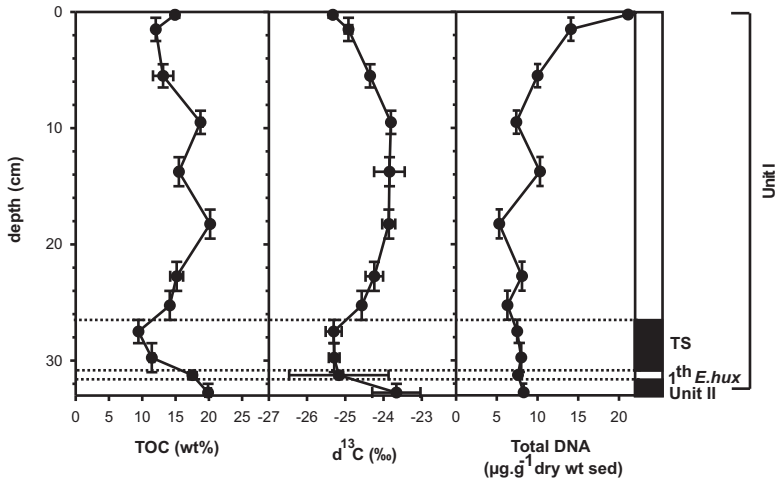


Figure 2: (a) Total organic carbon (TOC) content (wt%), (b) isotopic composition of bulk organic carbon (%), and (c) total DNA concentration (μg (g dry sediment)$^{-1}$ of the Holocene sediments of BC53. The lithology of the core is indicated at the right.

TOC, δ^{13}C of TOC, and total DNA concentrations

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 (Fig. 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 (Fig. 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 $\sim$8 μg (g dry sediment)$^{-1}$ throughout the remaining part of the core (Fig. 2C).

The 18S rDNA-based identification and quantification of eukaryotes

The PCR/DGGE analysis selective for eukaryotic 18S rDNA (Fig. 3) resulted in the identification of 13 unique phylotypes throughout the sediment core (Fig. 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* (Figs. 3 and 4).

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

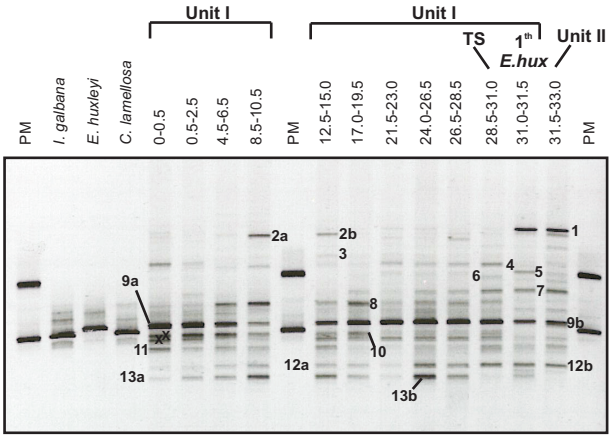


Figure 3: Denaturing gradient gel electrophoresis (DGGE) analysis of the predominant polymerase chain reaction (PCR)-amplified partial 18S rDNA of the domain of Eukarya obtained from the Holocene sediments of the Black Sea. DGGE bands that were sliced from the gel and subsequently sequenced are indicated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

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).

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 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.

qPCR with the same primer combination selective for the domain of Eukarya (but

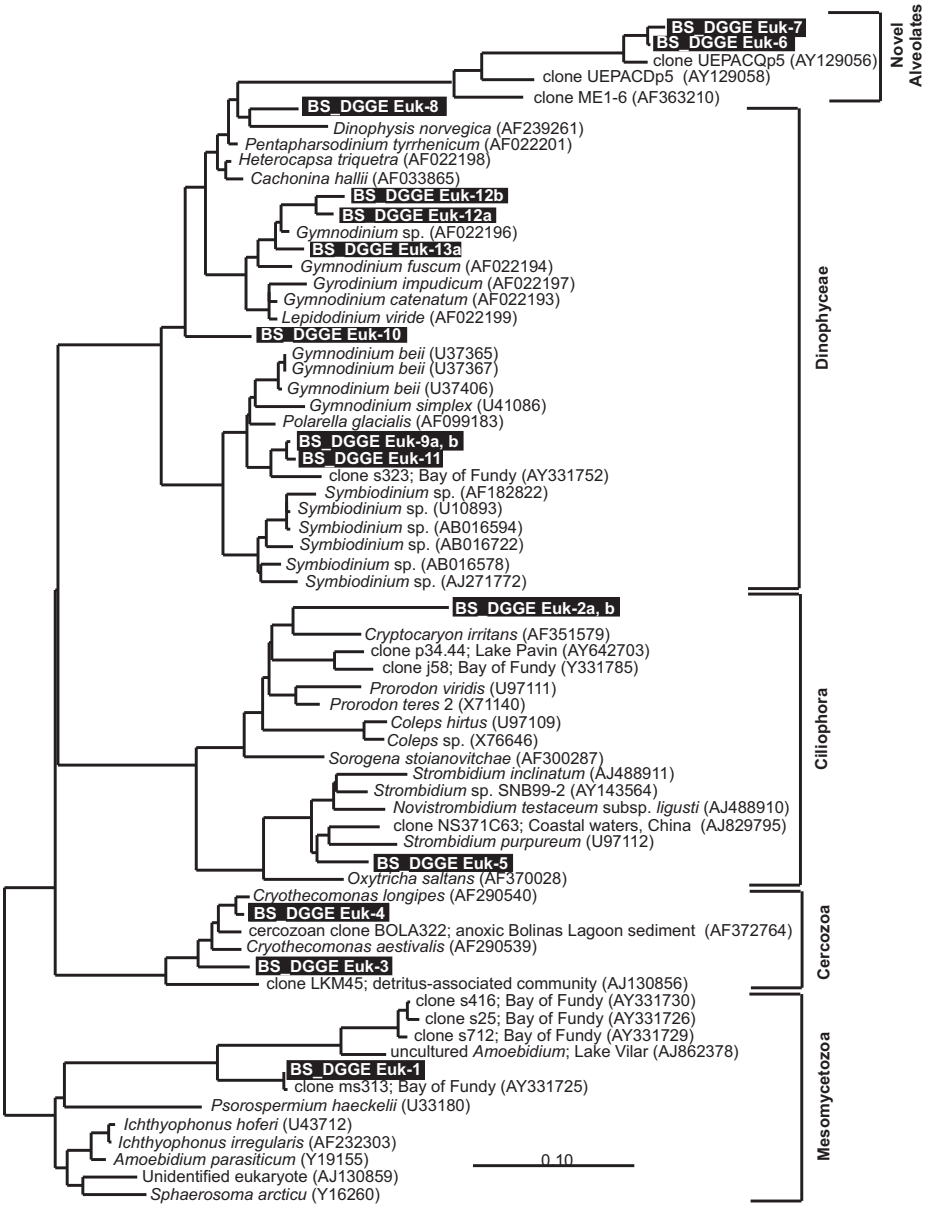

BS_DGGE Euk-7
BS_DGGE Euk-6
clone UEPACQp5 (AY129056)
clone UEPACDp5 (AY129058)
clone ME1-6 (AF363210)
Novel Alveolates
BS_DGGE Euk-8
Dinophysis norvegica (AF239261)
Pentapharsodinium tyrrhenicum (AF022201)
Heterocapsa triquetra (AF022198)
Cachonina hallii (AF033865)
BS_DGGE Euk-12b
BS_DGGE Euk-12a
Gymnodinium sp. (AF022196)
BS_DGGE Euk-13a
Gymnodinium fuscum (AF022194)
Gyrodinium impudicum (AF022197)
Gymnodinium catenatum (AF022193)
Lepidodinium viride (AF022199)
BS_DGGE Euk-10
Gymnodinium beii (U37365)
Gymnodinium beii (U37367)
Gymnodinium beii (U37406)
Gymnodinium simplex (U41086)
Polarella glacialis (AF099183)
BS_DGGE Euk-9a, b
BS_DGGE Euk-11
clone s323; Bay of Fundy (AY331752)
Symbiodinium sp. (AF182822)
Symbiodinium sp. (U10893)
Symbiodinium sp. (AB016594)
Symbiodinium sp. (AB016722)
Symbiodinium sp. (AB016578)
Symbiodinium sp. (AJ271772)
Dinophyceae
BS_DGGE Euk-2a, b
Cryptocaryon irritans (AF351579)
clone p34.44; Lake Pavin (AY642703)
clone j58; Bay of Fundy (Y331785)
Prorodon viridis (U97111)
Prorodon teres 2 (X71140)
Coleps hirtus (U97109)
Coleps sp. (X76646)
Sorogena stoianovitchae (AF300287)
Strombidium inclinatum (AJ488911)
Strombidium sp. SNB99-2 (AY143564)
Novistrombidium testaceum subsp. ligusti (AJ488910)
clone NS371C63; Coastal waters, China (AJ829795)
Strombidium purpureum (U97112)
BS_DGGE Euk-5
Oxytricha saltans (AF370028)
Ciliophora
Cryothecomonas longipes (AF290540)
BS_DGGE Euk-4
cercozoan clone BOLA322; anoxic Bolinas Lagoon sediment (AF372764)
Cryothecomonas aestivalis (AF290539)
BS_DGGE Euk-3
clone LKM45; detritus-associated community (AJ130856)
Cercozoa
clone s416; Bay of Fundy (AY331730)
clone s25; Bay of Fundy (AY331726)
clone s712; Bay of Fundy (AY331729)
uncultured Amoebidium; Lake Vilar (AJ862378)
BS_DGGE Euk-1
clone ms313; Bay of Fundy (AY331725)
Psorospermium haeckelii (U33180)
Ichthyophonus hoferi (U43712)
Ichthyophonus irregularis (AF232303)
Amoebidium parasiticum (Y19155)
Unidentified eukaryote (AJ130859)
Sphaerosoma arcticu (Y16260)
Mesomycetozoa
0.10

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.

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 (Fig. 5C).

Biomarkers of haptophytes

Fossil alkenones of haptophytes

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 (Fig. 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)$^{-1}$) (Fig. 5A). $C_{37:4}$ mK was not detected in core BC53.

Fossil 18S rDNA of haptophytes

qPCR 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 (Figs. 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 (Figs. 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 (Fig. 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 (Fig. 5D).

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

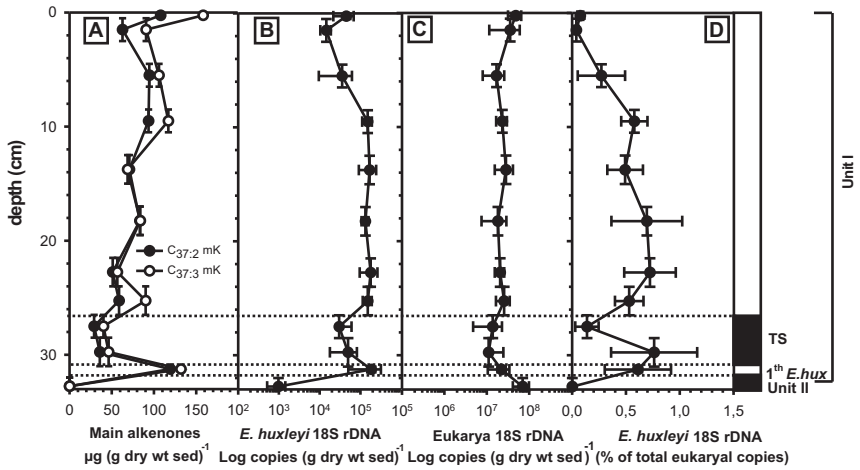


Figure 5: Abundance of haptophyte-specific biomarkers in the Holocene sediments of the Black Sea. (a) Main haptophyte-derived alkenones ($C_{37:2}$ methyl ketone [mK] and $C_{37:3}$ mK) (b) Number of 18S rDNA copies of *E. huxleyi*. (c) Number of most predominant 18S rDNA copies of the domain Eukarya.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

unique phylotypes (Figs. 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 (Fig. 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 (Fig. 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 producers from non-coccolithophorid, alkenone biosythesizing haptophyte species (the genera *Isochrysis* and *Chrysotila*) (Fig. 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 (Fig. 7).

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)$^{-1}$ (Fig. 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)$^{-1}$) (Figs. 5B and 6), whereas alkenones were below

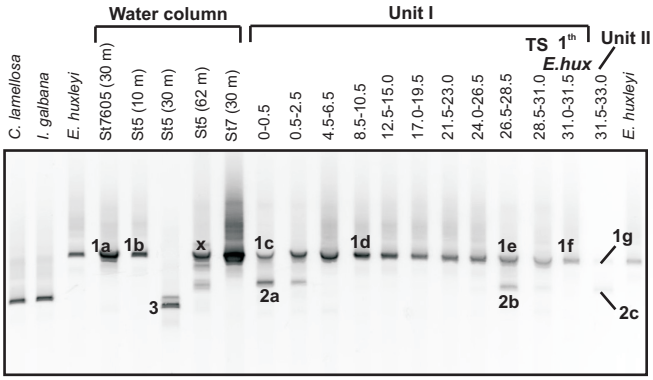


Figure 6: DGGE analysis of PCR-amplified partial 18S rDNA of haptophyte algae obtained from the water column (POM) as well as the Holocene sediments of the Black Sea. DGGE bands that were sliced from the gel and subsequently sequenced are indicated with numbers. For comparative analysis, PCR products 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.

the detection limit (Fig. 5A).

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 (Figs. 5A and 5B).

Ratio between *E. huxleyi* 18S rDNA and alkenones in contemporary samples and Holocene Black Sea sediments

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)$^{-1}$).

Fragmentation of fossil DNA of *E. huxleyi*

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

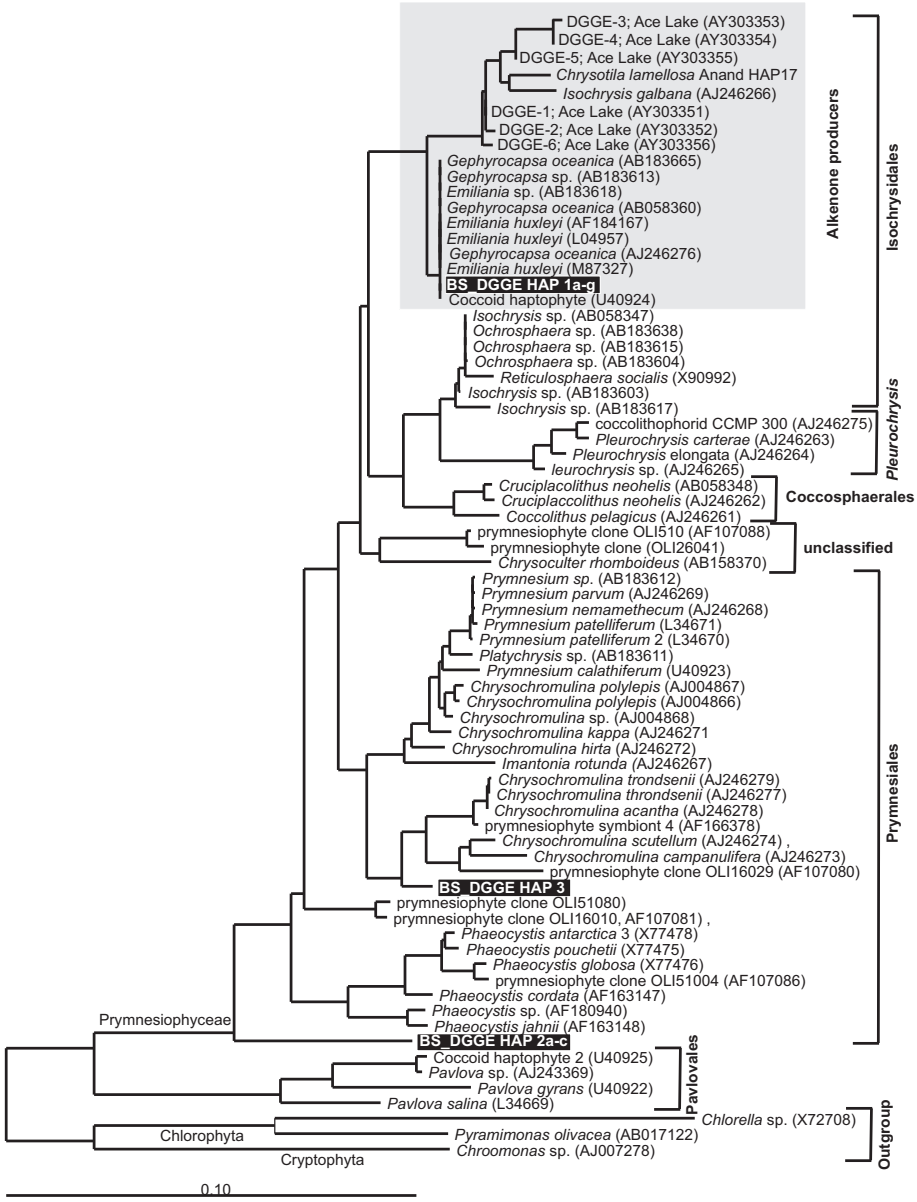

DGGE-3; Ace Lake (AY303353)
DGGE-4; Ace Lake (AY303354)
DGGE-5; Ace Lake (AY303355)
Chrysotila lamellosa Anand HAP17
Isochrysis galbana (AJ246266)
DGGE-1; Ace Lake (AY303351)
DGGE-2; Ace Lake (AY303352)
DGGE-6; Ace Lake (AY303356)
Gephyrocapsa oceanica (AB183665)
Gephyrocapsa sp. (AB183613)
Emiliania sp. (AB183618)
Gephyrocapsa oceanica (AB058360)
Emiliania huxleyi (AF184167)
Emiliania huxleyi (L04957)
Gephyrocapsa oceanica (AJ246276)
Emiliania huxleyi (M87327)
BS_DGGE HAP 1a-g
Coccoid haptophyte (U40924)
Isochrysis sp. (AB058347)
Ochrosphaera sp. (AB183638)
Ochrosphaera sp. (AB183615)
Ochrosphaera sp. (AB183604)
Reticulosphaera socialis (X90992)
Isochrysis sp. (AB183603)
Isochrysis sp. (AB183617)
coccolithophorid CCMP 300 (AJ246275)
Pleurochrysis carterae (AJ246263)
Pleurochrysis elongata (AJ246264)
leurochrysis sp. (AJ246265)
Cruciplacolithus neohelis (AB058348)
Cruciplaccolithus neohelis (AJ246262)
Coccolithus pelagicus (AJ246261)
prymnesiophyte clone OLI510 (AF107088)
prymnesiophyte clone (OLI26041)
Chrysoculter rhomboideus (AB158370)
Prymnesium sp. (AB183612)
Prymnesium parvum (AJ246269)
Prymnesium nemamethecum (AJ246268)
Prymnesium patelliferum (L34671)
Prymnesium patelliferum 2 (L34670)
Platychrysis sp. (AB183611)
Prymnesium calathiferum (U40923)
Chrysochromulina polylepis (AJ004867)
Chrysochromulina polylepis (AJ004866)
Chrysochromulina sp. (AJ004868)
Chrysochromulina kappa (AJ246271
Chrysochromulina hirta (AJ246272)
Imantonia rotunda (AJ246267)
Chrysochromulina trondsenii (AJ246279)
Chrysochromulina throndsenii (AJ246277)
Chrysochromulina acantha (AJ246278)
prymnesiophyte symbiont 4 (AF166378)
Chrysochromulina scutellum (AJ246274) ,
Chrysochromulina campanulifera (AJ246273)
prymnesiophyte clone OLI16029 (AF107080)
BS_DGGE HAP 3
prymnesiophyte clone OLI51080)
prymnesiophyte clone OLI16010, AF107081) ,
Phaeocystis antarctica 3 (X77478)
Phaeocystis pouchetii (X77475)
Phaeocystis globosa (X77476)
prymnesiophyte clone OLI51004 (AF107086)
Phaeocystis cordata (AF163147)
Phaeocystis sp. (AF180940)
Phaeocystis jahnii (AF163148)
Prymnesiophyceae
BS_DGGE HAP 2a-c
Coccoid haptophyte 2 (U40925)
Pavlova sp. (AJ243369)
Pavlova gyrans (U40922)
Pavlova salina (L34669)
Chlorella sp. (X72708)
Chlorophyta
Pyramimonas olivacea (AB017122)
Chroomonas sp. (AJ007278)
Cryptophyta
0.10
Alkenone producers
Isochrysidales
Pleurochrysis
Coccosphaerales
unclassified
Prymnesiales
Pavlovales
Outgroup

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).

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 (Fig. 8). PCR amplifiable fossil 0.46-kbplong 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 (Fig. 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 fragment size of ancient *E. huxleyi* occurred at depths between 19.0 and 26.5 cm.

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 (Fig. 9E). In comparison to the ground $24-26.5$ cm sample (Fig. 9D), the nonground sample (Fig. 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 (Fig. 9F), fragmentation was significantly smaller compared to the ground pristine sediment (Fig. 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 (Figs. 9A-9D and 9F), the influence of grinding on the observed fragmentation should also have been the same.

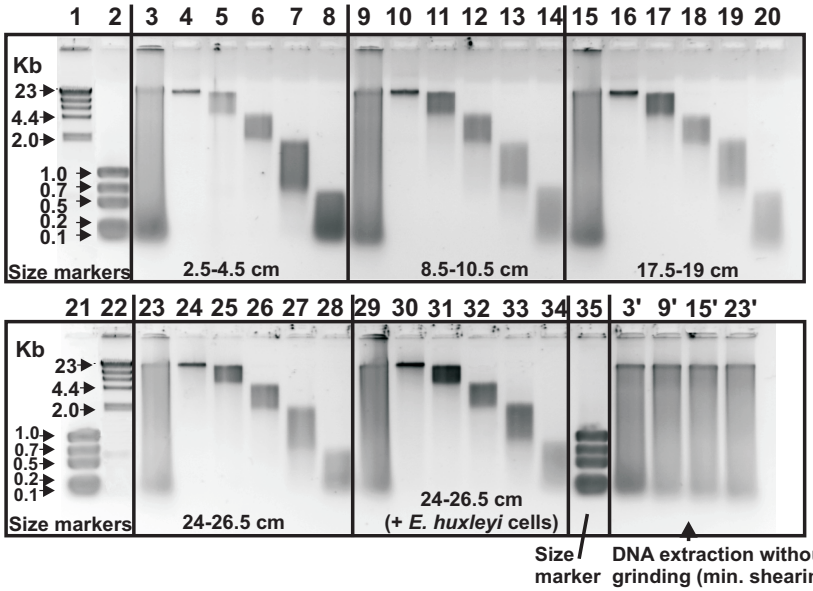


Figure 8: Agarose gel with total DNA extracts from selected Holocene unit I sediments and separated individual size classes of the extracted DNA. Fragment length rulers are lanes 1 and 22 (phage $\lambda\times$ *Hind*III digest) and lanes 2 and 21 (Precision Molecular Mass Standard (PMMS) BioRad). The 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)).

Discussion

Predominant sedimentary eukaryotic 18S rRNA genes

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* (Figs. 3 and 4). In a previous study, *Gymnodinium* 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).

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.

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

The remaining sequences which were retrieved with the nonselective primers for the eukaryotic domain were related to heterotrophs such as ciliates, cercozoa (represent-

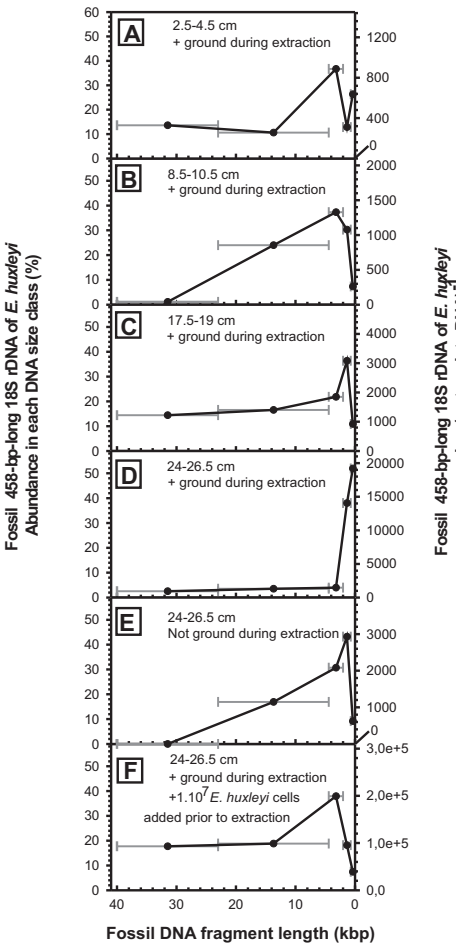


Figure 9: Fragmentation of fossil DNA of ancient *E. huxleyi*. (left) Percentage and (right) number of fossil 458-bp-long rDNA fragments per ng fossil template DNA within varying fragment size classes (less than 0.7 kbp, $0.7-2.2$ kbp, $2.2-4.4$ kbp, $4.4-23$ kbp, and $23-40$ kbp). The sediment sections 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 (Fig. 9F). An aliquot of these samples (Figs. 9A-F) was subjected to qPCR using haptophyte-specific primers to quantify the number of *E. huxleyi* copies. In the case of Figure 9F this qPCR 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 BP (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.

ing faint DGGE bands) and a sequence related to a novel lineage of fungi/metazoan (Fig. 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) (Fig. 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 (Fig. 2) where 18S rDNA of this species was below the detection limit (Fig. 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).

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.

Fossil DNA of haptophytes in the Holocene Black Sea sediments

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.*, 2004a). 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 coccolithbearing 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 (Fig. 5).

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 (Figs. 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 qPCR, 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 qPCR 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.

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.*, 2004a, 2006a; Coolen and Overmann, 1998). This showed that in the various investigated sediments the contamination with vertically transported DNA was negligible.

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.

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.

Fate of fossil DNA of *E. huxleyi* in the Holocene Black Sea sediment record

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 (Fig. 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.

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.*, 2006b). Even in the presence of 8 mM 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.*, 2006b). 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.

Even fossil DNA in sulfidic sediments is, however, not likely to completely escape degradation. Coolen and Overmann (1998) studied fossil 16S rDNA of Mahoney Lakes 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 mM, 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 (Fig. 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 (Fig. 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 (Fig. 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.

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.*, 2006b). 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.*, 2006b) 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.

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.

Conclusion

The 18S rDNA of the coccolithophorid, alkenone-biosynthesizing haptophyte *E. huxleyi* which colonized the Black Sea's 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 qPCR 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.

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 coccolithbearing 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*.

This work showed for the first time that, in addition to traditional proxies, fossil DNA of ancient photic-zonedwelling 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.

Acknowledgments

We would like to thank Marcel Kuypers (Max Planck Institute for Marine Microbiology, Bremen, Germany) for providing a filter with POM collected from 30 m water depth at the western part of the Black Sea (station 6705). We thank Anna Noordeloos (Royal NIOZ) for providing a culture of *E. huxleyi* strain from Oslo Sea and Jean- François Rontani (Centre dOcéanologie de Marseille, France) for providing a culture of *Chrysotila lamellosa* HAP 17. In addition, we would like to acknowledge Judith van Bleijswijk of the Royal NIOZ for assistance with phylogenetic analysis. This work was supported by a grant from the Netherlands Organization for Scientific Research (NWO) (Open Competition Program 813.13.001 to M.J.L.C.) and NSF grant OCE0117824 to S.G.W., which we greatly appreciate.

CHAPTER 5

Fossil DNA-inferred ecological and environmental changes during deposition of eastern Mediterranean sapropel S1.

Arjan C. Boere, W. Irene C. Rijpstra,
Gert J. de Lange, Elisa Malinverno,
Jaap S. Sinninghe Damsté, Marco J. L. Coolen

in review (Paleoceanography)

Abstract

A multiproxy approach was used to reveal the extent to which fossil DNA of planktonic protists (i.e., dinoflagellates and haptophytes) was preserved in eastern Mediterranean sediments, and whether the paleogenetic data reflect known environmental changes that occurred during the formation of sapropel S1 ($9.8 - 5.7$ ^{14}C kyr BP or $10.8 - 6.1$ kyr cal. BP). Sequencing analysis of preserved ∼500-bp-long 18S rRNA gene fragments in S1 sediments ($65 - 22$ cm sediment depth) recovered near the Nile fan revealed a predominance of dinoflagellate phylotypes until ∼8 ^{14}C kyr BP, which were previously only detected in anoxic Black Sea sediments. In the same section of the core, the most abundant haptophyte 18S rRNA gene sequence showed highest sequence similarity with uncultivated haptophytes previously shown to grow mixotrophically as predators of picocyanobacteria in a lab-controlled RNA-stable isotopic probing (RNA-SIP) experiment. Predatory consumption of picocyanobacteria could have been an adaptation of this previously overlooked haptophyte to thrive in an oligotrophic photic zone, which was separated from deep nutrient-rich waters as a result of water column stratification during early S1 deposition. A sequence closely related to *Emiliania huxleyi* was identified, but none of the identified sequences was identical to less abundant haptophyte species present in the calcareous nannofossil assemblage. Low concentrations of lipid biomarkers diagnostic for dinoflagellates and haptophytes (i.e., dinosterol and long-chain alkenones), but no detectable 500-bp-long fossil rDNA fragments of these protists was present in the C_{org}-depleted marls flanking the S1, thereby limiting paleogenetics of the relatively long DNA fragments to the C_{org}-rich S1 sediments.

Introduction

The geological record offers our best opportunity for understanding how biological systems function over long timescales and under varying paleoenvironmental conditions. Understanding these ecosystem responses to change is critical for biologists in trying to understand how organisms interact and adapt to environmental changes, and for geologists seeking to use these biology-geology relationships in order to reconstruct past climate conditions from sediment records. For example, enumeration of microscopic fossilizing calcareous and silicifying protists has become a standard paleoecological approach in the field of paleoclimatology (Castradori, 1993; Meier *et al.*, 2004; Zonneveld *et al.*, 2008). However, the identification of morphological remains is not always straightforward, as many taxa lack diagnostic features preserved upon fossilization. For example, dinocysts are not produced by all dinoflagellate species, they can be preferentially degraded in the sediment and in many cases, fossil cysts cannot be linked to those of extant species (Boere *et al.*, 2009; Head, 1996; Zonneveld *et al.*, 2008). Likewise, nannofossils of calcareous haptophytes serve as paleoenvironmental proxies, but many important taxa do not calcify (Coolen *et al.*, 2004b; Edvardsen *et al.*, 2000), or species produce coccoliths only during some phases of their life cy-

cle (i.e., Frada *et al.*, 2006) and cannot be identified from a micropaleontological approach. Lipid-based records can be particularly valuable in the absence of diagnostic cellular features in the sedimentary record. Nevertheless, the interpretation of these molecular stratigraphic records is often complicated by the limited specificity of many lipid biomarkers (Volkman *et al.*, 1998; Volkman, 2003). For instance, dinosterol is widely used to estimate the relative dinoflagellate contribution to sedimentary organic matter (OM) and for reconstructing paleoenvironmental conditions (Boon *et al.*, 1979; Robinson *et al.*, 1984; Volkman *et al.*, 1998), although there are important quantitative differences between dinosterol and dinocyst data (Mouradian *et al.*, 2007; Sangiorgi *et al.*, 2005). In addition, haptophyte-specific lipid biomarkers (long-chain alkenones) are of great interest to paleoceanographers because of the strong empirical relationship between the degree of unsaturation in alkenones ($U_{37}^{K'}$ -unsaturation parameter) and growth temperature, which forms the basis for their use as a molecular proxy of past sea surface temperatures (SSTs) (e.g., Brassell *et al.*, 1986; Prahl and Wakeham, 1987). However, identification of fossil sources for species-specific calibration of alkenone-inferred SST values (e.g., Versteegh *et al.*, 2001) is not always straightforward as both calcifying (*Emiliania huxleyi* and *Gephyrocapsa oceanica*) and non-fossilizing species (e.g., *Isochrysis* spp. and *Chrysotila lamellosa*) can be planktonic sources of fossil alkenones (Marlowe *et al.*, 1984; Rontani *et al.*, 2004; Versteegh *et al.*, 2001; Volkman *et al.*, 1995).

There is thus a need for biomarkers with greater source-specificity that can be used to complement and enhance interpretations based on existing methods. The field of molecular biology offers a most promising approach that is just starting to gain wider utility: the use of ancient DNA preserved in the sedimentary record (fossil DNA) to reconstruct past ecosystems. For example, fossil DNA has been used recently to reconstruct the Holocene succession of planktonic protists (haptophytes, dinoflagellates, diatoms), zooplankton (copepods), and photosynthetic bacterioplankton (cyanobacteria, and sulfidic chemocline-derived purple- and green sulfur bacteria) in shallow lakes and fjords (Bissett *et al.*, 2005; Coolen *et al.*, 2004b,a, 2006a, 2007, 2008; Coolen and Overmann, 1998; D'Andrea *et al.*, 2006). Fossil DNA stratigraphy also aided in a better understanding of the Holocene ecology and environmental conditions of the world's largest permanently stratified anoxic basin, the Black Sea (Coolen *et al.*, 2006b, 2009; Manske *et al.*, 2008). The permanent water column stratification and bottom water anoxia in these settings is generally thought to have enhanced the preservation of DNA in the underlying undisturbed, organic carbon (C_{org})-rich, laminated sediments (Coolen *et al.*, 2006b; Coolen and Overmann, 1998; Corinaldesi *et al.*, 2008). A major benefit of using fossil genetic signatures as highly specific"biomarkers" is the ability to identify also important paleoenvironmental indicator species that leave no other diagnostic features in the sediment record (Bissett *et al.*, 2005; Boere *et al.*, 2009; Coolen and Overmann, 1998; Coolen *et al.*, 2007), and to validate paleoenvironmental information inferred from more traditional proxies (Coolen *et al.*, 2004b, 2009; D'Andrea *et al.*, 2006).

Eastern Mediterranean sapropels are dark sedimentary units with C_{org} contents of >2 wt% that occur intercalated within C_{org}-poor (∼0.2 wt%) pelagic-hemipelagic

carbonate oozes (Kidd *et al.*, 1978), and represent a potentially rich, but largely untapped archive for the application of paleogenetics (Coolen and Overmann, 2007). These C_{org}-rich sedimentary intervals have formed repeatedly in the eastern Mediterranean Sea, in response to precession cycle-triggered maxima in northern Hemisphere solar radiation and intensified African monsoons (de Lange *et al.*, 2008; Rohling and Thunell, 1999). The oceanic environmental conditions that led to the formation of eastern Mediterranean sapropels have been studied mainly based on isotopic, geochemical and micropaleontological data (e.g., Castradori, 1993; de Lange *et al.*, 2008; Giunta *et al.*, 2003; Principato *et al.*, 2006; Thomson *et al.*, 1999, 2004). Especially the youngest sapropel S1 ($9.8 - 5.7$ ^{14}C kyr BP or $10.8 - 6.1$ kyr cal. BP), which was deposited during the Early Holocene climate optimum, has been the subject of extensive study (de Lange *et al.*, 2008; Gennari *et al.*, 2009; Meier *et al.*, 2004; Negri and Giunta, 2001, e.g.,). An increasingly warmer and wetter climate at the start of the Holocene triggered an increased discharge of freshwater and nutrients from the Nile, which could have resulted in water column stratification and the formation of bottom water anoxia (e.g., de Lange *et al.*, 2008; Emeis *et al.*, 2003; Rohling, 1989), but an enhanced primary production and OM transport also must have played a role in the accumulation of C_{org}-rich sapropels (Calvert, 1983; de Lange and ten Haven, 1983). Low bottom water oxygen concentrations during S1 deposition are indicated by (1) low amounts or absence of benthic foraminifera (Principato *et al.*, 2006); (2) formation of pyrite with isotopically depleted δ^{34}S indicative of anoxic sulfidic conditions in sediments and near-bottom water (Passier *et al.*, 1996); a (3) rapid degradation of S1 organic matter when exposed to oxygen (Moodley *et al.*, 2005); and (4) an excellent preservation of delicate organic dinoflagellate cysts (Cheddadi and Rossignol-Strick, 1995b; Zonneveld *et al.*, 2001). A suite of geochemical proxies (e.g., C_{org} content and barite fluxes) recently provided comprehensive evidence that the whole eastern Mediterranean basin was been predominantly anoxic below ∼1.8 km during the 4 kyr of S1 deposition, whereas more frequent ventilation events resulted in lower C_{org} contents in S1 sediments at shallower depths (de Lange *et al.*, 2008). In addition, an increase in paleofluxes of the calcifying haptophyte *Florisphaera profunda* coupled with a decrease in the accumulation rate of upper- and middle zone coccolithophorids, suggests an ecological depth-separation of the water column, characterized by higher nutrient availability at depth and nutrient-depleted surface waters during early to mid S1 deposition ($9.7 - 6.5$ ^{14}C kyr BP) (Principato *et al.*, 2006). In the present eastern Mediterranean (i.e., non-sapropel times), an extensive remineralization of C_{org} is caused by a well mixed and oxygenated water column and due to the regular formation of new deep and bottom waters in the Adriatic and Aegean Seas (Bethoux, 1993; Casford *et al.*, 2003; Roether and Well, 2001).

In this study we used a comparative multiproxy survey (fossil DNA, calcareous nannofossils, and lipid biomarkers) to test whether preserved genetic signatures provide an accurate view into haptophyte and dinoflagellate populations during the deposition of the youngest sapropel (S1) and in the C_{org}-depleted marls flanking the S1, and if we could identify important environmental indicator species that did not fossilize and escaped previous microscopic identification. Furthermore, we explored

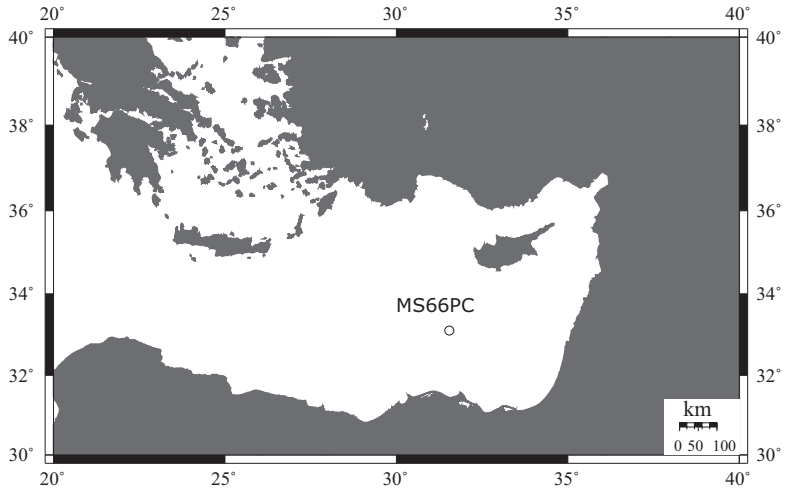


Figure 1: Map of the Mediterranean Sea showing the location of core MS66PC investigated in this study.

whether fossil DNA stratigraphy as a paleoecological tool can contribute to an increased understanding of the Holocene environmental conditions during deposition of the eastern Mediterranean Sea S1 sapropel.

Material and methods

Site description and sample collection

During the 2004 MIMES cruise on the R/V Pelagia, a piston core (MS66PC) was recovered from near the Nile fan, Eastern Mediterranean (location: 33N1.9' 31E47.9') at a water depth of 1630 m (Fig. 1). The 625 cm-long core was sub-sampled onboard and the sediments were placed in sterile 50 ml Greiner tubes and frozen at $-40°$C immediately. Sub-samples were taken at one cm resolution from the S1. Two cm intervals at every $10-20$ cm were sub-sampled from the carbonate marls enclosing the sapropel. A total of 18 samples were selected for lipid geochemistry and paleogenetics from the sapropel S1 ($\sim 22-65$ cm below sea floor), and three subsamples from the enclosing marls above and below the sapropel.

Geochemical analyses

Inorganic bulk element analyses was performed on freeze-dried subsamples at the University of Utrecht by Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) after total digestion (Reitz *et al.*, 2006; Reitz and de Lange, 2006). Organic

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

Molecular Biology

Precautions to prevent contamination

Extensive measures were taken to prevent contamination of samples and reagents with foreign DNA as described in previous work (Coolen *et al.*, 2004b, 2009). At the NIOZ, all DNA extractions and pre-PCR processes were performed in HEPA-filtered, and UV-sterilized PCR cabinets inside a PCR-product free lab dedicated to ancient DNA work. All surfaces, pipettes and consumables were decontaminated with RNAse-Away^{tm} (Sigma-Aldrich, St Louis, MO, USA) prior to use. Common sense practices such as frequently exchanging disposable vinyl gloves and the use of filter tips during pipetting of reactions also significantly reduce the change of contamination with foreign nucleic acids. Multiple controls were subjected to the whole extraction (extraction controls; e.c.) and PCR procedure (non-template controls; NTC) alongside the samples to monitor possible contamination. No PCR-products or any other "high copy number" DNA sources were ever introduced in the cabinets dedicated to fossil DNA analyses, and all downstream applications after PCR were performed in a physically separated laboratory.

DNA extraction

Total DNA was extracted using the PowerMax^{tm} Soil DNA isolation kit (MoBio laboratories, Carlsbad, Ca), according to the manufacturers guidelines. The resulting DNA was concentrated using a cold ethanol precipitation, and the DNA pellet was dissolved in 100 μl of sterile und nucleases-free Tris-EDTA (TE) buffer (pH 8.0) (Ambion). The integrity and yield of DNA in the extracts were checked by agarose gel electrophoresis. Nucleic acid yield was quantified by fluorescence (PicoGreen; MoBiTec, Göttingen, Germany) and found to be between $2 - 15$ ng μl^{-1} within the sapropel layers, and less than 2 ng μl^{-1} in the enclosing marls.

Polymerase Chain Reactions (PCR) conditions

One μl of the purified DNA extracts was subjected to real-time quantitative PCR (qPCR) using primers Prym-429F and Prym-887R (Coolen *et al.*, 2004b) for the selective amplification of a 458 bp-long region of haptophyte 18S rDNA. A 503-bp-long 18S rDNA region of dinoflagellate 18S rDNA was amplified using a general eukaryote forward primer (Euk-1f, Medlin *et al.*, 1988) and a dinoflagellate-specific reverse primer (Dino-Rev: 5'-ACAAGACATGGATGCCCT-3'; Boere *et al.*, 2009). The qPCR reactions were run on an Icycler IQ^{tm} real-time PCR detection system (Bio-Rad, Hercules, CA, USA) (NIOZ) and a Mastercycler ep realplex (Eppendorf, Hauppauge, NY) at WHOI, using a SYBRGreen I assay. Each 50 μl reaction contained the following reagents and concentrations: 2.5 units PicoMaxx^{tm} High Fidelity PCR

System and 1× PicoMaxx buffer (Stratagene, LaJolla, CA., USA), 0.25 mM of each dNTPs, 50 μg Bovin Serum Albumin (BSA), 1 mM $MgCl_2$, 1× SYBRGreen I, 10 nM Fluorescein (only with the iCycler), and 0.2 mM of each of the primers, and DNA-free PCR water (Sigma-Aldrich, St Louis, MO, USA) was added to 50 μl. All PCR programs contained an initial denaturing step at 96°C for 4 min, followed by a number of amplification cycles outlined below comprising a denaturing step (94°C, 40 sec), an annealing step (T_a = 62°C for haptophytes and T_a = 63.5°C for dinoflagellates, 40 sec), an elongation step (72°C, 60 sec) and a photo step (80°C, 20 sec). A final elongation step (72°C, 10 min) was added for the quantification reaction. A 10-fold dilution series of reactions containing from 10^0 to 10^7 copies of full-length 18S rDNA of *Emiliania huxleyi* was used as a standard to quantify the absolute number of haptophyte 18S rDNA copies in our samples. To quantify the fossil dinoflagellates rDNA, full-length rDNA of *Scrippsiella* sp. was used. The qPCR data was expressed as number of (partial) gene copies per gram of total organic carbon (copies g^{-1} C_{org}) in order to compensate for variable organic content between the analyzed sediment intervals.

Quality and length of the produced PCR amplicons was checked by a melting curve analysis and by standard agarose gel electrophoresis followed by ethidium bromide staining. In the melting curve analysis, 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.

Denaturing Gradient Gel Electrophoresis (DGGE)

Amplicons for subsequent DGGE analysis were generated in a second (semi-nested) amplification, by adding 1 μl of the amplicons from the first PCR series to 20 μl reaction mixtures with fresh PCR reagents, with the same forward primer but a modified reverse primer. A GC-clamp, i.e., a 40 base pair long GC-rich tag was attached to this reverse primer. The addition of a GC-clamp prevents complete dissociation of the two strands and loss of amplicons during DGGE analysis (Muyzer *et al.*, 1993). The resulting re-amplified PCR-products were checked and quantified on agarose gel, and ∼100 ng per sample was brought on a DGGE gel which contained a $20\% - 60\%$ gradient of denaturing chemicals (urea and formamide). After running for 5 h at 200 V (12.5 V cm^{-1}), the gel was stained for ∼20 min with SYBRGold (Invitrogen, Carlsbad, CA) and visualized using a Dark Reader (Clare Chemicals Research, Dolores, CO, USA). Subsequently, 39 bands of dinoflagellate 18S rDNA and 21 bands of haptophyte 18S rDNA were excised and sequenced, both including a number of bands from similar position in the gel in order to check if they indeed represent the same phylotypes (e.g., following Coolen *et al.*, 2006b).

Sequencing and phylogenetic analyses

Sequencing of the excised DGGE bands was done by Macrogen Inc. (Seoul, Korea). Related species were identified by a BLAST search on the NCBI homepage

(www.ncbi.nlm.nih.gov). The sequences were then imported into the ARB package (Ludwig *et al.*, 2004) and were aligned using the FAST aligner utility with the approximately 50.000 pre-aligned eukaryote sequences in the SILVA database (release 93, Pruesse *et al.*, 2007). The alignment was subsequently checked and refined manually. Similar bands were grouped into operational taxonomic units (phylotypes) using an 98% sequence similarity cut-off value using the program DOTUR (Schloss and Handelsman, 2005). The 15 unique phylotypes (9 dinoflagellates and 6 haptophytes) recovered in this study are deposited in the NCBI database under accession numbers FJ717393 - FJ717407.

These consensus sequences were exported from ARB, and along with selected relatives, analysed in MEGA4 (Tamura *et al.*, 2007). A Neighbour Joining analysis was run, using the Jukes-Cantor model and assuming uniform rates among sites. A consensus tree which shows only splits with bootstrap support >50% (1000 bootstrap replicates) was finally generated.

Lipid geochemistry

Wet sediments (ca. 5 g) were freeze-dried and subsequently ultrasonically extracted 5 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 fraction using DCM and DCM/MeOH (1:1, v/v) as the eluent, respectively. To the apolar fractions an internal standard (6,6-d2-3-methyl-eicosane) was added and they were analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS). 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 (BSTFA) 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 (FID) 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 was stayed 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 3 scans per second and an ionization energy of 70 eV.

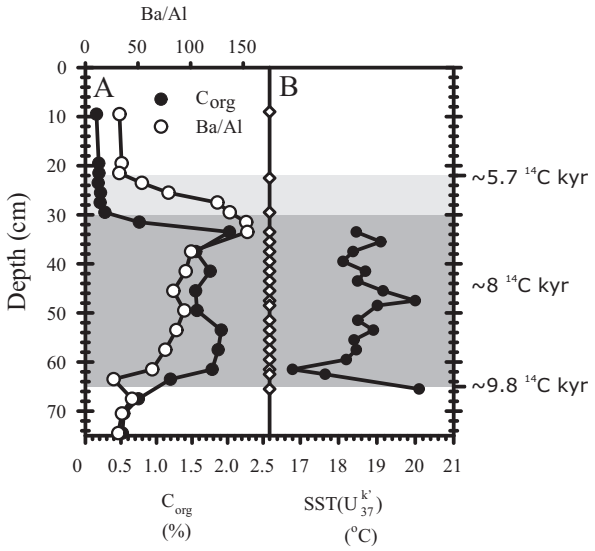


Figure 2: Sedimentary profiles of core MS66PC. A. Organic carbon (C_{org}) content in core MS66PC as percentage of the total sediment weight (•) and the Ba /Al ratio (∘). B. Sea surface temperature (SST) estimated for the sapropel S1 based on the alkenone-based U^{K}_{37}-paleothermometer. The approximate interval of the sapropel S1 is shown as grey shaded area, and the part of S1 that was re-oxidized after re-ventilation of the bottom waters is shown in lighter grey. The ^{14}C ages of the onset and termination of the original sapropel are shown on the right hand side (de Lange *et al.*, 2008). Depths that were used in the paleogenetic analysis are marked with a diamond on the middle y-axis.

Microscopical analysis of calcareous nannofossils.

Five samples were selected for micropaleontological analyses of calcareous nannofossils (sample depths 36.5, 40.5, 44.5, 52.5 and 58.5 ± 0.5 cm). Samples were prepared following the standard smear slide techniques and analyzed with a polarised light microscope (PLM) at 1250×. Semi-quantitative analyses of nannofossil species occurrence and abundance were performed for each slide through a count of approximately 300 specimens. For the identification of rare species, a fixed area of 150 fields of view was examined.

Results

Organic carbon (C_{org}), Ba/Al

C_{org} values were low in sediments between 75 and 68 cm (∼0.5 wt%) and in the upper 30 cm (∼0.2 wt%). Elevated amounts of C_{org} ranging between 1.5 and 2%

were measured between 63 and 32 cm (Fig. 2A). The Ba/Al ratio gradually increased with decreasing sediment depth and reached a maximum value of ∼150 at 30 cm (Fig. 2A). This ratio was also elevated in the C_{org}-depleted sediments between 30 and 22 cm, indicative that this part of the core was re-oxidized after the return of a well-ventilated water column (cf. de Lange *et al.*, 2008; Thomson *et al.*, 1999), and that the S1 in our core is located at depths between 65 and 22 cm.

Quantitative analysis of preserved dinoflagellate and haptophyte 18S rRNA genes

Throughout S1, the amount of preserved 500-bp-long dinoflagellate 18S rDNA fragments exceeds that of similar-sized haptophyte 18S rDNA by one order of magnitude (Figs. 3, 4). The dinoflagellate 18S rDNA content was highest at 53 cm (2.2×10^7 copies g^{-1} C_{org}) with 2 to 10 times lower concentrations in the deeper and shallower intervals of S1 (Fig. 3). Preserved haptophyte 18S rDNA ($10^5 - 10^6$ copies g^{-1} C_{org}) was often near the detection limit ($\sim 10^5$ copies g^{-1} C_{org}), especially between 42 and 30 cm, which hampers the reproducibility of the quantitative results and leads to relatively large error bars (Fig. 4). Both dinoflagellate and haptophyte 18S rRNA gene sequences were below the detection limit in the re-oxidized part of S1 ($30 - 22$ cm) as well as in the C_{org}-poor marls flanking sapropel S1.

Dinoflagellate communities during the development of sapropel S1

We were unable to amplify 500-bp-long dinoflagellate rRNA gene fragments from the total DNA pool extracted from the oxidized part of the S1 between 30 and 22 cm (Figs. 2, 3). However, the development of the dinoflagellate paleocommunity during the deposition of the C_{org}-rich part of sapropel S1 shows three distinct intervals in the DGGE (intervals D-I through D-III; Fig. 3). A total of 39 dinoflagellate bands were excised from the gel of which 36 bands were successfully sequenced. The sequenced bands were aligned and grouped into phylotypes using a 98% sequences similarity value. The nine resulting phylotypes (dinoflagellate phylotypes A-I) and selected relevant sequences available through NCBI with highest similarities to phylotypes A-I were used in a Neighbour Joining phylogenetic analysis (Fig. 5). Interval D1 (65 cm) was the only C_{org}-depleted sediment layer that, despite the extremely low dinoflagellate DNA content (Fig. 3A), revealed a single phylotype (phylotype E; FJ717397; Fig. 3B). The amount of preserved dinoflagellate DNA was much higher in the C_{org}-rich early to mid S1 (varying between 10^6 and 10^7 copies g^{-1} C_{org}; Interval D-II, 62−45 cm), but despite more DNA template for PCR, the dinoflagellate diversity (phylotypes C, D, and I) was not much higher than in the C_{org}-depleted marl sample directly below S1. Within Interval D-II, phylotype C (FJ717395) represented the most abundant species with lower contributions of phylotypes D (FJ717396) and I (FJ717401). Sequences with the highest similarities with phylotypes C (BS-Euk-DGGE-08; 100%), and phylotype D (BS-Euk-DGGE-10; 98%, Fig. 5) were recovered

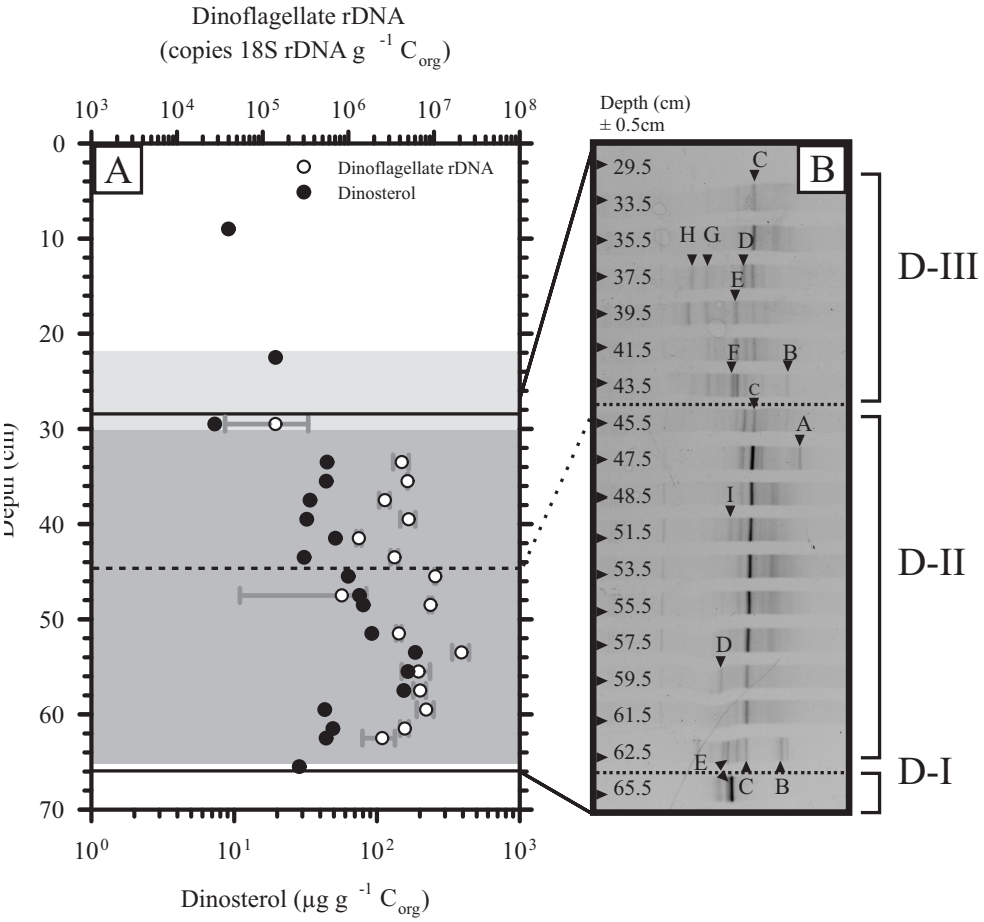


Figure 3: A. Preserved dinoflagellate DNA, expressed as number of copies of the partial 18S rDNA, normalized per gram of total organic carbon (copies g^{-1} C_{org}; open circles) and concentration of dinoflagellate biomarker dinosterol (g g^{-1} C_{org}; closed circles). B. DGGE with fossil DNA preserved in the Holocene sapropel S1 showing changes in the dinoflagellate community. Partial 18S rDNA was amplified using a dinoflagellate-specific primer set (Euk-1A and Dino-Rev) and separated using DGGE. A total of 39 bands were excised and sequenced, sequences were clustered (using a 98% cut-off value) into nine unique phylotypes (dinoflagellate phylotype A-I). Depth values are the average of 1 cm thick sediment slices (± 0.5 cm).

from anoxic and sulfidic Holocene Black Sea sediments (Coolen *et al.*, 2006b). The highest diversity of dinoflagellates (phylotypes B-E and F-H), was found in interval D-III (45 − 33 cm). Within this youngest C_{org}-rich interval of S1, phylotype C became less abundant and was below detection limit in some of the analyzed sediment layers. The majority of the phylotypes that were only detected in interval D-III have not been identified from stratified anoxic settings or they were related to sequences found in normal oceanic settings (Figs. 3, 5). For example, phylotypes A (FJ717393) and B (FJ717394) have no known close relatives, but are distantly related to species derived from the water column at 500 meter depth in the Sargasso Sea (eukaryote clone SSRPB60; Not *et al.*, 2007). The closest related sequence of phylotypes F (99%) and I (98%) was an uncultured clone isolated from surface seawater (clone BTQB20030806.0094, Fig. 5).

Haptophyte communities during the development of sapropel S1

Similar-sized partial haptophyte 18S rRNA gene fragments were also only recovered from C_{org}-depleted marl just below the S1 as well as the C_{org}-rich part of the S1 (Fig. 4). Seventeen out of twenty-one excised haptophyte DGGE bands could successfully be sequenced and were grouped into 6 phylotypes based on a 98% sequence similarity value (haptophyte phylotypes J-O, Figs. 4 and 6). Haptophyte DNA was close to the detection limit in the deepest analyzed sample from the C_{org}-depleted marl just below the S1. Whereas the fossil dinoflagellate DNA survey recorded a unique community in this "pre-S1 marl", the haptophyte community in this layer and in the lower part of the S1 was identical. We, therefore, identified two intervals with unique haptophyte populations as opposed to the three intervals with unique dinoflagellate populations. According to the fossil DNA survey, a major shift in the haptophyte community occurred at 43 cm in our sediment record (Fig. 4B), which coincided with the observed major shift in the dinoflagellate population (Fig. 3B). Haptophyte phylotype O (FJ717407), which co-occurred with dinoflagellate phylotype C between 65 and 43 cm (Interval H-I), showed greatest sequence similarity (99.5%) with uncultivated haptophytes that, according to RNA stable isotopic probing (RNA-SIP) experiments, were capable of mixotrophic growth as predators on picocyanobacteria (Frias-Lopez *et al.*, 2009). Phylotype O was below detection limit in interval H-II (<43 cm).

Despite several attempts, haptophyte sequences of the correct fragment length (458 bp) were only recovered from interval H-II at depths between 37.5 ± 0.5 cm and 33.5 ± 0.5 cm: Phylotype K (FJ717403) occurred in both intervals and showed 98% sequence similarity with the alkenone-producing coccolithophorids *E. huxleyi* and *Gephyrocapsa oceanica*. Phylotype L was only recovered from interval H-II and showed 98% sequence similarity to a clone recovered from the North Atlantic (Diéz *et al.*, 2001).

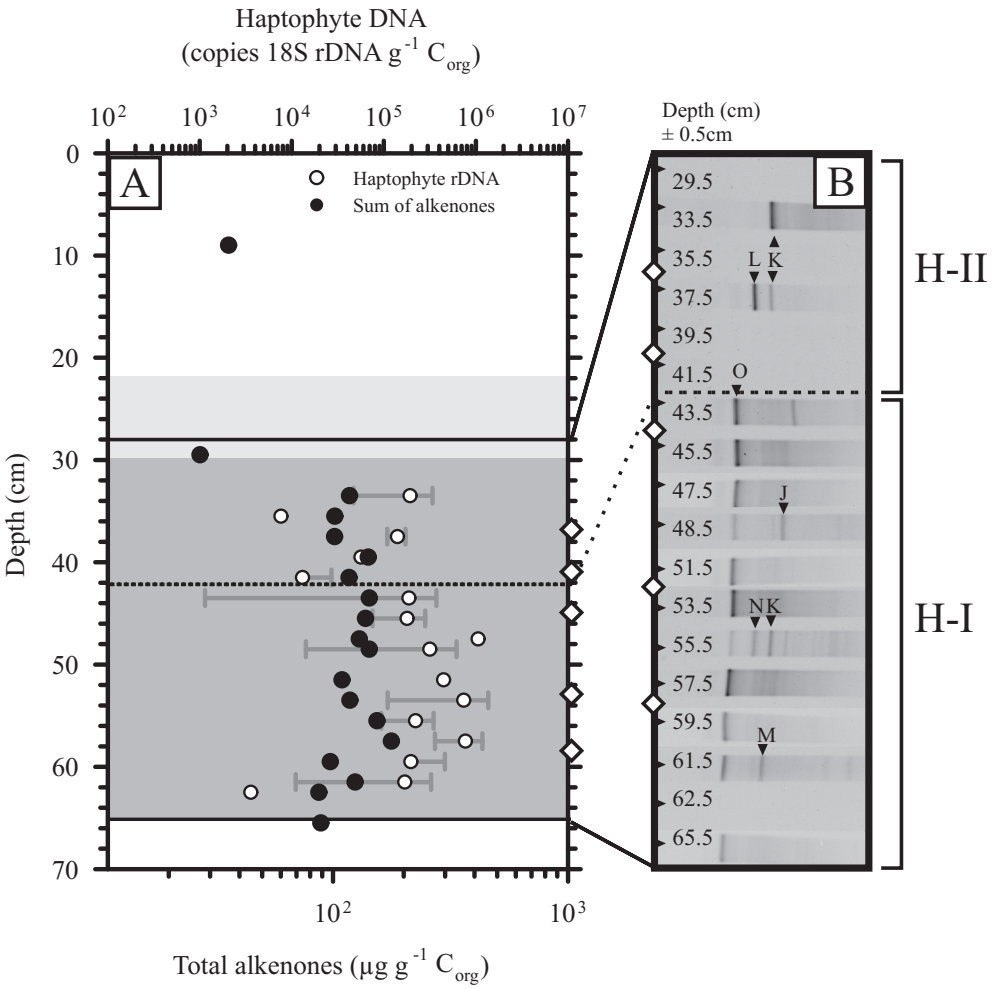


Figure 4: A. Preserved haptophyte DNA, expressed as the number of copies of partial 18S rDNA, normalized per gram C_{org} (copies g^{-1} C_{org}; ○) and the sum of dominant alkenones $C_{37:2}$, $C_{37:3}$ and $C_{38:3}$ (μg g^{-1} C_{org}, ●). The five samples that were analysed for nannofossil association are marked on the right axis with white diamonds (◇) B. DGGE with fossil rDNA preserved in the Holocene sapropel S1 showing changes in the haptophyte paleocommunity. Partial 18S rDNA was amplified using haptophyte-specific primers Prym-429F and Prym-887R and separated using DGGE. A total of 17 bands were excised and sequenced, and sequences were clustered (using a 98% cut-off value) into 6 unique phylotypes (haptophytes phylotypes J-O). Depths are given as the average of 1 cm thick sediment slices (± 0.5 cm).

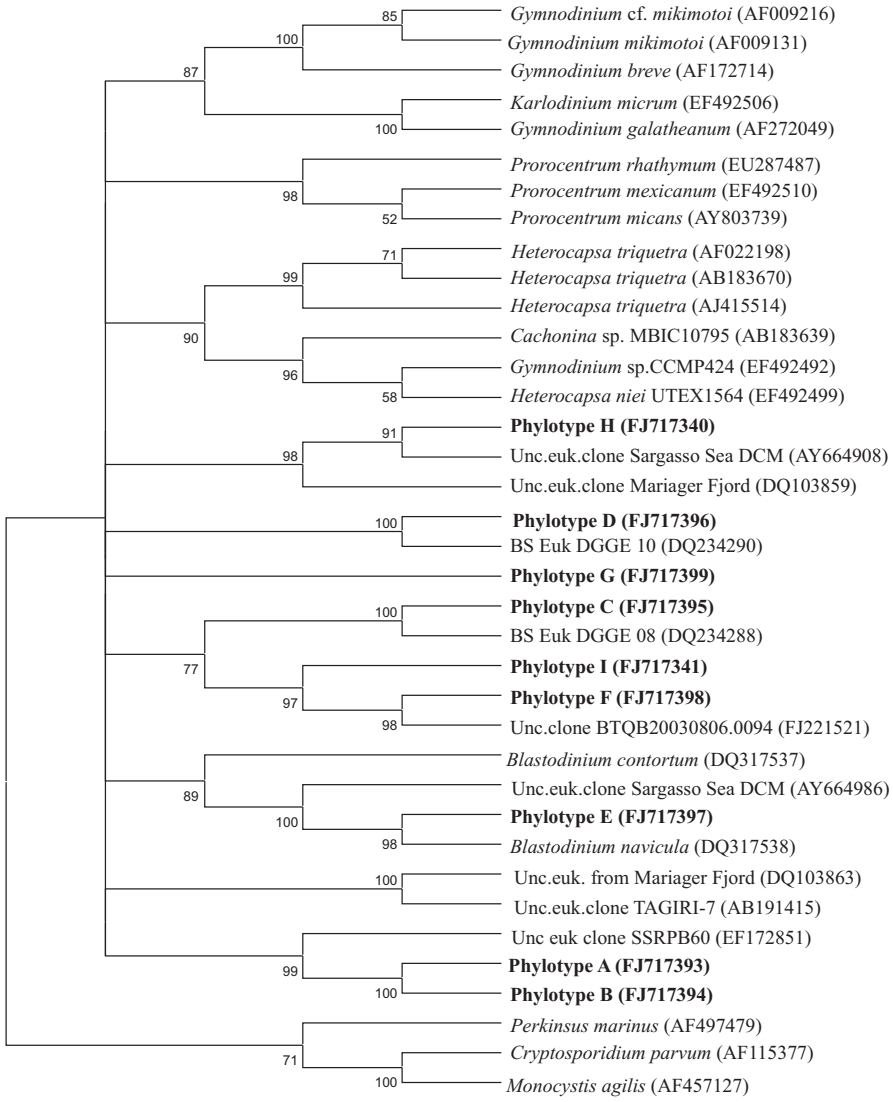


Figure 5: Phylogenetic positions of recovered dinoflagellate phylotypes. The bootstrap consensus tree was build using the Neighbour Joining method (JC model, uniform substitution rates) in the program MEGA4. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Phylotypes recovered in this study are shown in bold.

Fossil lipid biomarkers diagnostic for dinoflagellates and haptophytes.

Dinosterol (4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol), the lipid biomarker of many but not all dinoflagellates (Volkman *et al.*, 1998), was identified in all analyzed layers of sapropel S1 (Fig. 2). The average concentration was $\sim 50-90$ μg g^{-1} C_{org} throughout sapropel S1, except for an interval close to the basal part of the sapropel ($\sim$52 and 58 cm) where dinosterol reached maximum concentrations of up to $\sim$200 μg g^{-1} C_{org}. Dinosterol concentrations were significantly lower ($<10-20$ μg g^{-1} C_{org}) in the organic carbon-lean enclosing marls and in the re-oxidized part of S1.

The concentration of fossil haptophyte-derived long-chain alkenones was fairly constant throughout sapropel S1 ($100-150$ μg g^{-1} C_{org}; Fig. 4). The predominant alkenones were the two- and three-times unsaturated C_{37} methyl ketones used in $U_{37}^{K'}$ paleothermometry ($C_{37:2}$ and $C_{37:3}$ methly ketones (mK); together comprising $\sim$60% of the total alkenone content). C_{38} alkenones represented the remaining $\sim$40% of the total alkenone content. As for dinosterol, alkenones were less abundant ($0-20$ μg g^{-1} C_{org}) in the C_{org}-poor marls and in the re-oxidized part of S1.

$U_{37}^{K'}$-based sea surface temperature (SST) estimates gradually increased from 17 to 19°C between early and mid sapropel deposition ($63-48$ cm depth; Fig. 2B) and a peak in SST (20°C) was observed at $\sim$46 cm. The SST the dropped slightly again and remained relatively constant at $\sim$18°C above 44 cm. No reliable SST estimate could be obtained from the C_{org}-poor marls below and above sapropel S1 since alkenone concentrations were generally low and $C_{37:3}$ was below the detection limit.

Identification of calcareous microfossils in sapropel S1

In the youngest of the analyzed sapropel S1 sample (36.5 cm), the calcareous nannofossil assemblage was dominated by *Emiliania huxleyi* (55%), followed by *F. profunda*(33%) with a contribution of minor species (Fig. 7). The heavily calcified morphotype of *E. huxleyi* (as described by Crudeli *et al.*, 2004) (Fig. 8) was present in all samples but most abundant at 52.5 cm and at 36.5 cm (Fig. 7). Within the older part of the sapropel, *F. profunda* ranges from 28 to 37%, but becomes more abundant than *E. huxleyi* at 36.5 cm. *A. robusta* is another important locally-important species, which is abundant in the older part of the sapropel (5 to 16%) but absent in the youngest analyzed interval at 36.5 cm.

Overall, minor species include: *Rhabdosphaera clavigera*, *Calciosolenia brasiliensis*, *Syracosphaera pulchra*, *Umbellosphaera tenuis*, *Syracolithus* spp., *Helicosphaera* spp., *Umbilicosphaera sibogae*, *Coronosphaera* spp., *Discosphaera tubifera* and rare *Calcidiscus leptoporus*, *Pontosphaera* spp., *Scyphosphaera apsteinii*. Isolated occurrences of medium and large morphotypes of the pentaliths of *Braarudosphaera bigelowii* were detected in sediments up to 47.5 cm (Fig. 8), but these pentaliths were not found above 47.5 cm.

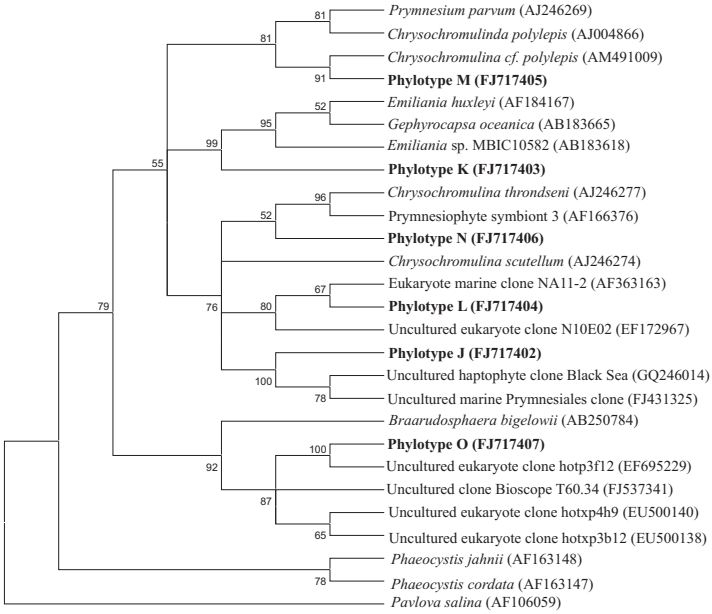


Figure 6: Phylogenetic positions of recovered haptophyte phylotypes. The bootstrap consensus tree was build using the Neighbour Joining method (JC model, uniform substitution rates) in the program MEGA4. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Phylotypes recovered in this study are shown in bold.

Discussion

Our parallel stratigraphic analysis of dinoflagellate (fossil DNA and dinosterol) and haptophyte (fossil DNA, long-chain alkenone biomarkers, and nannofossils) markers enabled us to cross validate the presence of each paleoecological proxy prior, during, and after eastern Mediterranean S1 deposition. We will first discuss the suitability of fossil DNA as a paleoecology proxy for dinoflagellates and haptophytes followed by a discussion of paleoenvironmental information inferred from the fossil DNA signatures and how well this correlates with existing views on the environmental conditions in the eastern Mediterranean Sea during the Holocene.

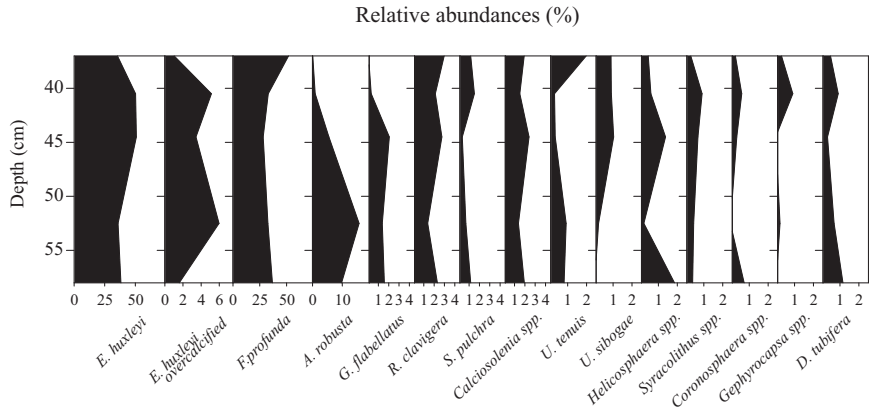


Figure 7: Relative abundances (%) of the most dominant calcareous nannoplanktonic species.

Validating fossil DNA as a proxy for Holocene Mediterranean dinoflagellates.

Whereas all earlier mentioned fossil DNA studies focused on reconstructing the ancient plankton ecology in permanently stratified, anoxic and sulfidic lake and marine settings, this is the first attempt of using paleogenetics to study plankton DNA that was deposited in the presence of bottom water anoxia (early S1) (e.g., de Lange *et al.*, 2008; Principato *et al.*, 2006) as well as in the presence of a well-mixed water column and fully oxygenated bottom waters and surface sediments (i.e., during deposition of C_{org}-depleted sediments Bethoux, 1993; Casford *et al.*, 2003; Roether and Well, 2001).

C_{org}-depleted sediments flanking the S1 as well as the oxidized upper part of the S1 contained low concentrations of dinosterol, but only the oxidized marl that was deposited just before the onset of the formation of the C_{org}-rich part of the S1 (65.5 ± 0.5 cm; interval D-I), still contained traces of detectable 500-bp-long dinoflagellate 18S rDNA. Most likely, a fraction of the fossil DNA in this layer survived degradation due to a much shorter post-depositional exposure to oxidative damage (Lindahl, 1993) as opposed to the DNA in all other analyzed C_{org}-depleted layers.

A single dinoflagellate (phylotype E) with up to 99% sequence similarity to 18S rRNA gene sequences of known marine copepod parasites of the genus *Blastodinium* (Skovgaard, 2005) was detected in interval D-I. This phylotype branches closely enough with *Blastodinium* sequences to assume that it represents a past copepod parasite of this genus (Skovgaard *et al.*, 2007). The detection of phylotype E is of paleoecological interest since it shows the potential of using paleogenetics to identify past ancient parasite/host interactions. On the other hand, the presence of phylotype E does not provide paleoenvironmental information.

PCR/DGGE selects for the most abundant phylotypes present (Coolen *et al.*,

2007), but the actual detected dinoflagellate diversity in interval D-I must be underestimated due to the low number of preserved 18S rDNA copies that were available for PCR amplification. A parallel analysis of preserved calcareous and organic-walled dinoflagellates cysts of this core was not performed, but up to 5 million calcareous dinoflagellate cysts per gram sediment, with *Thoracosphaera hemii* and *Sphaerodinella tuberose* variants as the most abundant species, were reported in a comparable "pre-S1 layer" (Zonneveld *et al.*, 2001). Organic-walled cysts were several orders of magnitude less in abundance (Zonneveld *et al.*, 2001). Clearly, the paleogenetics approach did not detect any of those species in our pre-S1 interval. This could either be due to the degradation of DNA, even inside intact cysts, or that our extraction method failed to lyse DNA from the cysts. The former explanation is more likely since the stringent DNA extraction method includes vigorous chemical and mechanical (i.e., bead beating) cell disruption steps and *Blastodinium* related species represented by phylotype E is known to form dinospores (free-living stage) or to occur as a theca-covered multicellular form (parasitic stage) (Skovgaard *et al.*, 2007). Moreover, we successfully used this DNA extraction approach in prior studies and were able to extract large amounts of DNA from, for example, *Chaetoceros* diatom cysts (Coolen *et al.*, 2007). Assuming that the fossil phylotype E was derived from the parasitic theca-covered multicellular form of *Blastodinium*, this life cycle has little resemblance to typical dinoflagellate morphologies (Skovgaard *et al.*, 2007), which then could have been a reason why this species escaped prior microscopical identification from the eastern Mediterranean Holocene sediment record. A preferred sequestration of DNA of the parasitic multicellular and difficult to determine life cycle of *Blastodium* is likely due to a higher DNA content in a multicellular stage as opposed to single celled free-living dinospores, a protective theca covering, and an effective transport to the sediment upon death of a large and fast sinking host.

The highest dinosterol concentrations and up to 2×10^7 copies g^{-1} C_{org} of predominantly phylotype C were found in interval D-II, but the genetic and lipid biomarker depth profiles were not identical (Fig. 3). It has recently been reported that shifts in dinoflagellate communities from dinosterol-producing species to species that do not produce dinosterol may lead to misinterpretation of fossil dinosterol profiles (Boere *et al.*, 2009). On the other hand, it is possible that some of the species found in the fossil cyst assemblage of comparable Holocene eastern Mediterranean sediments (Zonneveld *et al.*, 2001), but which were missed with our paleogenetics approach, were sources of dinosterol. For example, *Scrippsiella regalis* represented one of the less abundant cysts (Zonneveld *et al.*, 2001), and *Scrippsiella* species were proven to be sources of dinosterol (Harvey *et al.*, 1988; Mansour *et al.*, 2003). It is thus possible that differences between the amounts of dinosterol vs. 18S rDNA (Fig. 3), was caused by a preferential degradation of DNA from alternate sources of dinosterol. In addition, it should be mentioned that the number of genomic 18S rRNA gene copies varies in eukaryotes, especially among dinoflagellates (Godhe *et al.*, 2008; LaJeunesse *et al.*, 2005). However, the dinoflagellate species composition dramatically changed between intervals D-II and D-III whereas the number of rDNA copies only changed by less than a factor of five between sediment horizons. Therefore, the variation in ge-

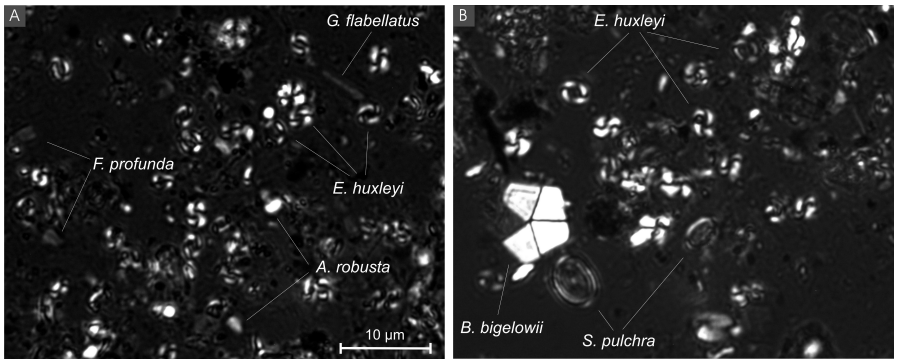


Figure 8: (A) The typical nannofossil association in the samples from the lower part of the S1 sapropel. (B) The isolated occurrence of the large morphotype of *B. bigelowii* within the *E. huxleyi*-dominated nannofossil association.

nomic and cellular 18S rDNA copy numbers between species must have been limited. Despite these discrepancies, the observed fossil DNA-inferred dinoflagellate species succession through time does nevertheless record environmental changes as discussed later.

Validating fossil DNA as a proxy for Holocene Mediterranean haptophytes.

All analyzed sediments including the C_{org}-poor marl layers and the oxidized upper part of S1 contained long-chain alkenones indicative of the preservation of ancient haptophyte biomass in all sediment horizons (Fig. 4). However, PCR-amplifiable partial fossil haptophyte 18S rDNA could only be recovered from the C_{org}-rich part of the S1 and from the C_{org}-poor layer just below the S1. All alkenone-producing haptophytes cluster within the order *Isochrysidales* and comprise the calcifying *Emiliania huxleyi* and its closely related ancestor *Gephyrocapsa oceanica*, both with identical full-length 18S rRNA gene sequences, as well as non-fossilizing and phylogenetically more diverse species (i.e., *Isochrysis* spp. and *Chrysotila lamellosa*; Marlowe *et al.*, 1984; Rontani *et al.*, 2004; Versteegh *et al.*, 2001; Volkman *et al.*, 1995). None of the detected haptophyte sequences were related to the non-calcifying alkenone producing haptophytes, and phylotype K was not identical (98% sequence similarity) to the closely related calcifying alkenone producers *E. huxleyi* and *G. oceanica* (Figs. 4, 6). According to the nannofossil dataset, *E. huxleyi* comprised on average $50-60\%$ of the coccolith assemblage in all five selected sediment horizons spanning early, mid, and late sapropel deposition (Fig. 7), and other studies have also shown that the calcareous nannofossil association preserved in sapropel S1 sediments is usually dominated by *E. huxleyi* (Castradori, 1993; Negri and Giunta, 2001; Principato *et al.*, 2003; Thomson *et al.*, 2004). The failure to identify a phylotype with identical sequence to *E. huxleyi* 18S

rDNA is surprising since genetic markers of *E. huxleyi* including 18S rDNA was found to be well preserved in anoxic Holocene coccolith-bearing sediments of the Black Sea, but also in the underlying organic-rich Black Sea sapropel where coccoliths were most likely absent as a result of post-depositional dissolution (Coolen *et al.*, 2006b, 2009). These differences in the level of preservation of DNA vs. coccoliths of *E. huxleyi* could reflect differences in (post)depositional environmental conditions between the Mediterranean Sea during S1 deposition and the Black Sea such as the extent of carbonate dissolution as a result of variation in organic matter decay related to reduced pore water $[CO_3^{2-}]$, and variations in the level of exposure to sulfidic conditions.

Our fossil DNA survey did not identify any of the additional calcareous haptophyte species that were represented in the nannofossil assemblage. For instance, we were unable to detect the 18S rRNA gene sequence of the recently cultured *Algirosphaera robusta* (Medlin *et al.*, 2008; Probert *et al.*, 2007), despite the fact that this haptophyte represented a significant portion ($\sim 10 - 15\%$) of the calcareous nannofossil assemblage in some samples. The deep photic zone dwelling *Florisphaera profunda* (Principato *et al.*, 2006; Thomson *et al.*, 2004), estimated to represent $20 - 25\%$ of the calcareous nannofossil composition in our core (Fig. 7), was also not identified by the paleogenetic approach.

On the other hand, the paleogenetic approach revealed sequences of non-calcifying species or those that were not previously described from nannofossil determination studies, including *Chrysochromulina* spp. (Fig. 6) and, most notably, a potentially important environmental taxon represented by phylotype O as outlined in the next paragraph.

Fossil DNA-inferred paleoenvironmental conditions during S1 deposition.

The most striking observation from our paleogenetic dataset is the dramatic shift in both dinoflagellate and haptophyte composition at ∼44 cm (Figs. 3, 4). The timing of this ecological shift is synchronous with the disappearance of the fossil pentaliths of *Braarudosphaera bigelowii* (Fig. 8), which has been previously defined as the beginning of "ecozone" C2 at 8 ^{14}C kyr BP from other sapropel cores (Giunta *et al.*, 2003; Principato *et al.*, 2003).

From the onset of S1 deposition (9.7 ^{14}C kyr BP) until the ecological shift at ∼8 kyr BP, the fossil DNA stratigraphy recorded a predominance of dinoflagellate phylotype C with 100% sequence similarity to a predominant clone found in the anoxic, sulfidic sediments of the Black Sea (BS_DGGE_Euk8, Coolen *et al.*, 2006b). Further studies are required to determine the geographic distribution of this phylotype, and whether it could be a potential marker for stratified and anoxic settings. If so, the predominance of phylotype C as well as a relatively constant C_{org} content of ∼2 wt% (Fig. 2) could be indicative of the presence of bottom water anoxia until ∼8 ^{14}C kyr BP at our coring site near the Nile fan at a depth of 1630 m. However, this is slightly in contradiction with recent geochemical evidence revealed that basin-wide only waters below 1800 m faced near-to complete anoxia during S1 deposition (de Lange *et al.*,

2008). Interval D-III (> 44 cm; $\sim 8 - 5.7$ kyr BP; Fig. 3) is marked by a different dinoflagellate community more related to clones recovered from marine surface waters and an absence or less predominant presence of phylotype C. This could be indicative of a reduced riverine input of freshwater and increasing sea surface salinities with newly formed oxygenated deep waters sinking to greater depths than during early sapropel deposition (de Lange *et al.*, 2008).

According to the fossil DNA results, the haptophyte community in the eastern Mediterranean Sea until ∼8 kyr BP was dominated by a haptophyte species (Phylotype O) with 99.5% sequence similarity to uncultivated haptophytes that, according to RNA stable isotopic probing (RNA-SIP) experiments, were capable of mixotrophic growth as predators of picocyanobacteria (Frias-Lopez *et al.*, 2009). The adoption of mixotrophy offers a survival strategy under oligotrophic oceanic conditions (Raven, 1997). For example, Arenovski *et al.* (1995) presented experimental evidence of a decrease in the abundance of mixotrophic phototrophs under nutrient enrichment conditions, suggesting that phagotrophy is used under low dissolved nutrient concentrations. Whether the haptophyte represented by phylotype O was indeed involved in phagotrophy during oligotrophic conditions in the Holocene eastern Mediterranean Sea solely based on high 18S rRNA gene sequence similarities is speculative, but evidence for the presence of oligotrophic surface waters during early S1 deposition was also provided from fossil nannofossil compositions (Principato *et al.*, 2006). The latter authors showed a marked increase in paleofluxes of *Florisphaera profunda* coupled with a decrease in the accumulation rate of upper- and middle photic zone coccoliths, suggesting an ecological depth-separation of the water column, probably characterized by higher nutrient availability at depth and nutrient-depleted surface waters during early to mid S1 formation (Principato *et al.*, 2006).

Potential use of paleogenetics in improving $U_{37}^{K'}$-paleothermometry

Haptophyte algae are important to paleoceanographers because of the use of their specific alkenones in reconstructing sea surface temperature (Prahl and Wakeham, 1987). As different species of haptophytes have a different functional response to growth temperature and thus a different U_{37}^{K}-temperature calibration (Prahl and Wakeham, 1987; Versteegh *et al.*, 2001; Volkman *et al.*, 1995), it is important for paleotemperature reconstructions to know which haptophyte species were present, and thus which calibration to apply. Previous studies have shown that fossil DNA enabled the identification of novel non-calcifying haptophytes as the major or only sources of fossil alkenones in less saline environments (Coolen *et al.*, 2004b, 2006b, 2009; D'Andrea *et al.*, 2006). Our paleogenetics survey did not reveal any sequences related to non-calcifying alkenone sources, and based on the nannofossil composition in our core as well as cores analyzed by others (Negri *et al.*, 2003; Principato *et al.*, 2006), *E. huxleyi* is expected to have been the major source of alkenones during S1 deposition. We, therefore, did not find evidence for the need of a species-specific calibration of the alkenone-based SST estimates during S1 deposition. The SST values

based on the general $U_{37}^{K'}$ calibration (Fig. 2, Prahl and Wakeham, 1987) are well within the range estimated for the sapropel S1 from other sediment cores from the Eastern Mediterranean (Emeis *et al.*, 2003).

Conclusion

Our paleogenetic approach recorded a major shift at ∼8 kyr BP in previously unidentified dinoflagellate and haptophyte communities during the deposition of the eastern Mediterranean sapropel S1 located near the Nile fan at a depth of 1630 m, as a result of known paleoenvironmental changes. The major dinoflagellate phylotype C in S1 sediments older than ∼8 kyr BP was previously also found in anaerobic and sulfidic Holocene Black Sea sediments. Whether this in indicative of a certain extent of bottom water anoxia during early S1 deposition can be confirmed if future studies reveal that phylotype C is widely distributed in stratified anoxic settings. A shift towards surface water dwelling species and a decrease or absence of phylotype C in S1 intervals younger than ∼8 ^{14}C kyr BP could be indicative of a frequent return of well mixed oxygenated waters and a breakdown of stratification. The most abundant, previously unrecognized haptophyte sequence in sediments deposited before ∼8 ^{14}C kyr BP had 99.5% sequence similarity with uncultivated haptophytes previously shown to grow mixotrophically as predators of picocyanobacteria in a lab-controlled RNA-stable isotopic probing (RNA-SIP) experiment. Predatory consumption of picocyanobacteria could have been an adaptation of the past haptophyte population thriving in an oligotrophic photic zone, which was separated from deep nutrient-rich waters as a result of water column stratification during early S1 deposition.

A species closely related to *Emiliania huxleyi* was identified, but none of the identified sequences were identical to species present in the nannofossil assemblage. Low concentrations of lipid biomarkers diagnostic for dinoflagellates and haptophytes (i.e., dinosterol and long-chain alkenones), but no detectable 500-bp-long fossil rDNA fragments of these protists were present in the C_{org}-depleted marls flanking the S1, thereby limiting paleogenetics of the relatively long DNA fragments to the C_{org}-rich S1 sediments. We suggest that the use of massive parallel pyrotag sequencing (Amaral-Zettler *et al.*, 2009; Sogin *et al.*, 2006) of PCR-amplified shorter hypervariable regions of phylogenetic marker genes could enhance the detection of fossil plankton DNA in a wider variety of sediments. Our study also shows the importance of including fossil DNA as a paleoecology proxy to improve paleoecological and paleoenvironmental assessments based on traditional proxies.

Acknowledgements

We would like to thank Alina Stadnitskaia, Shauna ní Flaithearta, and the captain of the R/V Pelagia for obtaining the core and subsequent sub-sampling on board of the ship. Marianne Baas, Jort Ossebaar and Sanela Gusic are thanked for analytical

assistance. This work was supported by grants and prizes from the Netherlands Organization for Scientific Research (NWO Open Competition grant 813.03.001 to M.J.L.C.; NWO Pass2 grant to G.J.d.L.; Spinoza prize to J.S.S.D.) as well as NSF-OCE Chemical Oceanography grant 0825020 to M.J.L.C.

CHAPTER 6

Preservation potential of ancient DNA in Pleistocene marine sediments: Implications for paleoenvironmental reconstructions.

Arjan C. Boere, W. Irene C. Rijpstra,
Gert J. de Lange, Jaap S. Sinninghe Damsté,
Marco J. L. Coolen

submitted (Earth and Planetary Science Letters)

Abstract

Recent studies have shown that Holocene, anoxic, organic carbon (C_{org})-rich marine and lacustrine sediments may contain an archive of genetic signatures derived from planktonic (micro-)organisms. In order to reveal to what extent ancient DNA of planktonic eukaryotes is preserved beyond the Holocene period we identified the microbial eukaryotic (protistan) composition based on preserved 18S rDNA in eastern Mediterranean Holocene and Pleistocene sapropels S1 (∼9 ka), S3 (∼80 ka), S4 (∼105 ka) and S5 (∼125 ka). Most of the fossil DNA of specific pelagic protists that were studied in detail (i.e., haptophyte algae and dinoflagellates) was degraded to shorter than 500-base pair-long fragments between ∼10 and 80 ka after deposition, whereas their specific lipid biomarkers (long-chain alkenones and dinosterol) were still present in sediments older than ∼80 ka. These sediments mainly contained eukaryotic DNA from marine fungi. Besides fungal DNA, the sediments older than 80 ka also contained ancient DNA from terrestrial plants which were most likely introduced via the river Nile. A parallel analysis of Branched and Isoprenoid Tetrathers (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 ancient terrestrial plant DNA suggests a preferred degradation of >10 ka-old marine protist DNA. We conclude that although Pleistocene anoxic C_{org}-rich marine sediments only contain traces of protist DNA, they could nevertheless represent rich archives of ancient DNA from past terrestrial vegetation.

Introduction

The reconstruction of marine and lacustrine paleoenvironmental conditions is typically based on the analysis of microscopic remains of (planktonic) species (e.g., diatoms (Kemp *et al.*, 1999), coccolithophorid algae (Castradori, 1993), cyst-forming dinoflagellates (dinocysts) (Marret and 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 (Peters *et al.*, 2005; Volkman *et al.*, 1998). However, microscopic remains are rarely produced by all species of a taxonomic group (e.g., calcifying and non-calcifying haptophyte algae Sáez *et al.*, 2004) and lipid biomarkers are often diagnostic only at higher taxonomic levels (Volkman *et al.*, 1998).

Various recent studies reported the successful use of ancient DNA preserved in sediments (i.e., "fossil DNA" as an additional paleoecological tool for identifying past planktonic species at the species level. These studies focused on fossil DNA of obligate photoautotrophs such as haptophyte algae, diatoms, pelagic cyanobacteria, green sulfur bacteria (Chlorobiaceae) and pelagic zooplankton (i.e., copepods) in shallow anoxic lake or fjord settings (Coolen *et al.*, 2006b; D'Andrea *et al.*, 2006; Coolen *et al.*, 2004a; Coolen and Overmann, 1998, 2007; Coolen *et al.*, 2006a; Bissett *et al.*, 2005; Boere *et al.*, 2009; Coolen *et al.*, 2004a; Coolen and Overmann, 2007; Bissett *et al.*,

2005; Coolen *et al.*, 2004b), as well as in the permanently stratified Black Sea (Coolen *et al.*, 2006b, 2009; Manske *et al.*, 2008). The sediments at all these locations were deposited during the Holocene and under anoxic and/or cold conditions, and therefore provided excellent conditions for the preservation of organic matter, including DNA.

The oldest fossil DNA found in marine sediments so far represented ∼400-bp-long 16S rRNA gene fragments of obligate anoxygenic photolithoautotrophic green sulfur bacteria (i.e., Chlorobiaceae). DNA of these ancient chemocline bacteria was identified from up to 217-kyr-old eastern Mediterranean sapropels (Coolen *et al.*, 2007). Eastern Mediterranean sapropels are C_{org}-rich sediment layers that were formed as a result of orbitally-forced changes in climatic and hydrological conditions which led to increased primary production, development of water column anoxia, and increased preservation of C_{org} in the anoxic bottom waters (Emeis *et al.*, 2003; Cramp and O'Sullivan, 1999; Negri *et al.*, 2009; Rohling, 1994; de Lange *et al.*, 2008). The sapropels are embedded in fully oxidized C_{org}-lean marls, which were formed after bottom water reventilation re-established in the eastern Mediterranean Sea. Therefore, eastern Mediterranean sediments provide the unique opportunity to study the preservation potential of DNA from a variety of plankton members under presumably optimal (during sapropel deposition) as compared to less optimal conditions (during the deposition of the intercalating marls) and at time scales exceeding the Holocene period.

Here, we studied the preservation of DNA from past planktonic eukaryote communities in the eastern Mediterranean Sea during the deposition of up to 124 ka-old sapropels (i.e., S1 [$5.7 - 9.8$ ka], S3 [∼30 ka], S4 [105 ka], and S5 [125 ka]) and the intercalating marls. Using quantitative real-time polymerase chain reaction (qPCR) assays, we quantified the number of eukaryote 18S rDNA copies of various groups using multiple primer sets: one assay targeted eukaryotes in general, one assay specifically targeted haptophyte algae (Prymnesiophyceae) and a third assay targeted dinoflagellates (Dinophyceae). A subsequent phylogenetic analysis of sequenced denaturing gradient gel electrophoresis bands (DGGE, e.g., Muyzer *et al.*, 1993) was performed to identify the eukaryote sequences present within the sediment. In order to corroborate the quantitative molecular results, we performed a parallel quantitative analysis of long-chain (C_{37}-C_{39}) unsaturated methyl- and ethyl ketones (alkenones) and the steroid dinosterol as diagnostic lipid biomarkers for haptophytes and dinoflagellates, respectively. 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 fluvially transported continental organic matter. To our knowledge, this is the first study in which eukaryote sequences from Mediterranean subsurface sediments, including those derived from truly ancient plankton members, are both quantified and identified, and in which their presence is corroborated with an independent analysis of diagnostic lipids in order to cross-validate both approaches. The combined information will ultimately enable us to gain a better understanding of the marine microbial community as it was present during deposition of the sapropels.

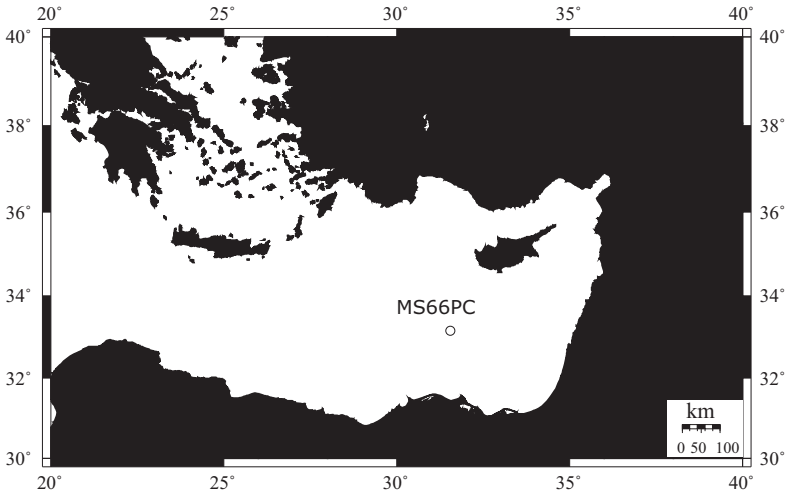


Figure 1: Map of the Mediterranean Sea showing the location of core MS66PC.

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 (Fig. 1). The length of the core was 6 m and included the surface-water interface. A section of approximately 37 cm (137 − 174 cm below the sediment surface) was lost during sampling. Visual inspection showed that the core contained sapropels S1, S3, S4 and S5, an observation that was corroborated by δ^{18}O measurements.

The core was split in two halves, one half was subsampled on board, continuously at 1cm intervals for inorganic geochemistry; samples were stored at 4°C. Simultaneously, samples for DNA and lipid analyses were taken at the same resolution in sapropel intervals and at lower resolution in the enclosing sediment marls; typically in the order of a 2 cm thick slice every 10−20 cm. The sediments were stored in sterile 50-ml Greiner tubes and frozen immediately. The sediment samples were constantly kept at −20°C at the Royal Netherlands Institute for Sea Research (Royal NIOZ) until they were processed.

Total organic carbon and elemental analyses

After freeze-drying and powdering the samples in the Utrecht laboratory, inorganic bulk element was analyzed by Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) after total digestion (Reitz *et al.*, 2006; Reitz and de Lange,

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; Reitz and de Lange, 2006).

Precautions to prevent and monitor contamination with modern DNA

Contamination is a serious issue in the field of ancient or fossil DNA. Therefore, extensive measures were taken in sampling and handling of the sediments, as described previously (Coolen *et al.*, 2004a). Briefly, all DNA extractions and pre-PCR processes performed at the Royal NIOZ were done in a separate lab that is dedicated to fossil DNA work. In addition, all extractions and pipetting of PCR reagents were performed in a closed PCR-cabinet, which is routinely sterilized with an incorporated UV-light for at least 30 min prior to and after working in the cabinet. All surfaces, pipettes and consumables were decontaminated with RNAse-Away^{tm} (Molecular Bio Products, San Diego, CA.) prior to use. Routinely, gloves are worn at all time. Multiple controls (extraction controls; EC) were subjected to the whole extraction procedure, replacing the sediment with ultra clean PCR water (Sigma-Aldritch, St Louis, MO). Non-template control (NTC) PCR reaction mixtures containing all reagents except the template DNA were run alongside the samples to monitor possible contamination during pipetting. No PCR-products were ever introduced in the PCR-cabinet dedicated to fossil DNA and all downstream applications after PCR were performed in a physically separated laboratory space.

Fifteen samples from depths adjacent to the samples analyzed at Royal NIOZ (above S1 [1 sample], S1 [3], S3 [1], S4 [2], S5 [3] and from the intercalating sediment marls [5]) were analyzed for eukaryotic and haptophyte DNA in the ancient DNA dedicated laboratory at the Woods Hole Oceanographic Institution (WHOI). Here, those samples were subjected to the same paleogenetic analyses as described below as an additional, independent validation of the data obtained at the NIOZ and to check for possible laboratory-specific contaminations.

DNA extraction

At the Royal NIOZ, a total of 28 samples from depths spanning early, mid and final deposition of sapropels S1 [6 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. In addition, ECs were subjected to the whole process, without adding sediment. Per sample depth, approximately $10 - 20$ g wet sediment was available. To compare the results of both approaches, 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.

Total DNA was extracted using the PowerMax^{tm} Soil DNA isolation kit (MoBio laboratories, Carlsbad, Ca, USA), according to the manufacturers guidelines. The resulting DNA was concentrated with a cold ethanol precipitation, re-dissolved in

Table 1: 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.

Primer set	Primer sequence (5'−3')	Position (*E. coli*)	Frag. length (bp)	T_a	Specificity	Ref.
Euk-1F[a]	ACCTGGTTGATCCTGCCAGT	7−26	461 − 579[c]	64	Eukaryotes	I
Euk-516R[b]	ACCAGACTTGCCCTCC	502−516				II
Prym-429F	GCGCGTAAATTGCCCGAA	358−374	425 − 427[c]	62	Prymnesiophyceae	III
Prym-887R[b]	GGAATACGAGTGCCCCTGAC	664−680				
Euk-1F[a]	ACCTGGTTGATCCTGCCAGT	7−26	463 − 467[c]	63.5	Dinophyceae	I
Dino-Rev[a]	ACAAGACATGGATGCCCT	414−431				IV

a Primer Euk-1F is identical to EukA (Medlin *et al.*, 1988), except that it has one less base (A) on the 3' side.
b GC-clamp sequence: (5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC-3') Muyzer *et al.* (1993). In all sets, the GC-clamp for DGGE is attached to the reversed primers.
c Fragment length is giving as sequence length without the primers
I Medlin *et al.* (1988)
II Amann *et al.* (1990)
III Coolen *et al.* (2004a)
IV Boere *et al.* (2009)

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. Nucleic acid yield was quantified by fluorescence (PicoGreen; MoBiTec, Göttingen, Germany) and found to be in the range between $2 - 15$ ng μl^{-1} within the sapropel layers, but below the detection limit in the enclosing marls.

Polymerase Chain Reaction (PCR)

To quantify the amount of eukaryote DNA present in the sediments, expressed as the number of 18S rDNA copies g^{-1} C_{org}, we run a series of quantitative real-time PCR (qPCR) assays (see Table 1). All qPCR reactions were run on an iCycler IQ^{tm} 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 SYBRGreen I (Invitrogen, Carlsbad, CA) assay. Each reaction contained the following reagents and concentrations: 5 μl 1× Picomaxx buffer and 2.5 units Picomaxx enzyme (Stratagene, LaJolla, CA.), 0.25 mM of each nucleotide, 50 μg Bovin Serum Albumin (BSA), 1 mM $MgCl_2$, 1× SYBRGreen I, 10 nM Fluorescein, and 0.2 mM of each of the primers (Table 1), and Ultra Clean PCR water (Sigma-Aldritch, St Louis, MO) added to 50 μl. The PCR ingredients used at WHOI differed slightly in that 4.5 mM $MgCl_2$, 0.5× SYBRGreen and no fluorescein were used. Three different primer sets, which amplified DNA fragments of $400 - 500$ basepairs, were used for targeting 18S rDNA of general eukaryotes, haptophytes and dinoflagellates, respectively (Table 1).

All PCR programs contained an initial denaturing step at 96°C for 4 min, fol-

lowed by a number of amplification cycles outlined below comprising a denaturing step (94°C, 40 s), an annealing step (annealing temperature T_a, 40s: for the T_a of individual assays, see Table 1), an elongation step (72°C, 60s) and a photo step (80°C, 20 s). A final elongation step (72°C, 10 min) was added for the quantification reaction. 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. In addition, quality and length of the produced PCR amplicons was checked by standard agarose gel electrophoresis with ethidium bromide staining.

To generate template for subsequent DGGE analysis of the samples analyzed at the NIOZ, one μl of the qPCR reactions from both samples and controls served as template for a second (semi-nested) amplification with fresh PCR reagents (20 μl reaction mixtures) containing the same forward primer but with modified reverse primers (called "GC-primer" from now on) to which a GC-clamp, i.e. a 40 base pair long GC-rich tag was attached. The addition of a GC-clamp prevented complete dissociation of the two strands and loss of amplicons during DGGE analysis (Muyzer *et al.*, 1993).

All field samples analyzed at the NIOZ were also directly amplified with GC-primers (i.e., one step PCR approach) in order to test for possible variations in the recovered eukaryote population as compared to the semi-nested PCR approach. The samples analyzed at WHOI were only analyzed using this one-step PCR approach.

In the reactions with GC-primers, the final extension step was prolonged to 30 min following Janse *et al.* (2004). The semi-nested approach comprised either 32 or 40 amplification cycles with normal primers, followed by 15 re-amplification cycles with GC-primers. In the direct amplification approach, the PCRs were run for $42 - 46$ cycles.

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 rDNA.

Denaturing gradient gel electrophoresis (DGGE) and sequencing of bands.

After visually checking the PCR products on agarose gel, ∼100 ng DNA of each reaction was run on DGGE. The polyacrylamide gel (6%, wt/vol) contained a denaturing gradient of 20% to 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 5h at 200V (12.5 $V.cm^{-1}$) in 1× TAE buffer at a temperature of 60C. Subsequently, the gel was stained for 30 m with 1× SYBRGold (Molecular Probes, Eugene, Oregon, USA), rinsed with Milli-Q and visualized using a Dark Reader transilluminator (Clare Chemical Research Inc., Dolores, Co, USA). Unique bands, and a number of duplicate bands from similar positions in the gel,

were excised using flame-sterilized scalpels and eluted in 50 μl 10 mM TRIS for 24 h. Subsequently, the bands were re-amplified using the original primer set and purified using the Genscript QuickClean PCR purification kit (Genscript, Piscataway, NJ). Purified PCR-products were sent to Macrogen Inc. (Seoul, Korea) for standard Sanger sequencing.

Comparison between direct and nested general eukaryote PCR assays.

The use of GC-clamp primers generally resulted in less efficient PCR reaction efficiencies and was not suitable for qPCR. Therefore, we adopted a (semi-)nested or 2-step PCR approach: qPCR was first performed without GC-primers. Then, one μl of the qPCR products was subjected to a second round of PCR in which a 40-bp-long GC-rich clamp was incorporated for subsequent analysis by DGGE. The less efficient one-step PCR assay performed directly with GC-primers, nevertheless yielded amplicons of the correct length. In general, the resulting eukaryotic community was comparable between both PCR approaches, with one notable exception. In the two-step approach, a number of shorter DGGE bands (app. 300 bp) were found which were completely absent in the direct approach. These particular bands proved difficult to sequence, but the ones that were sequenced and assembled successfully showed closest affinity to uncultivated archaeal species recovered from tidal flat systems (Wilms *et al.*, 2006) and from marine benthic communities in the Pacific Ocean (Lauer *et al.*, unpublished; Table 2).

Phylogenetic analyses

The phylogenetic affinity of recovered sequences was initially identified by BLAST searches using the NCBI website (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 (OTUs) or phylotypes based on a 98% sequence similarity cut-off value using the program DOTUR (Schloss and Handelsman, 2005). Phylogenetic analyses included Distance Matrix and Maximum Likelihood approaches applying several position variability filters. Finally, a Bayesian phylogenetic inference analysis was done on all recovered haptophyte and dinoflagellate phylotypes and on closely related relatives. This analysis was performed in the program MrBayes (version 3.1.2) 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 eukaryote primers are deposited in NCBI under accession numbers FJ785836-FJ785859 (archaea), FJ785860-FJ785896

Table 2: Number of specific eukaryote phylotypes in the various sections of the investigated core. 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 non-template controls are marked with an asterisk (*). Abbreviations: Unc.: uncultured; S1−S5: sapropel S1−S5; i.s.: non-sapropelic intercalating sediment intervals; EC: extraction ctrl; ntc: non-template PCR control

Phylogenetic affilliation		i.s.	S1	i.s.	S3	i.s.	S4	i.s.	S5	EC	ntc
Protists											
Alveolata	*Dinoflagellata*		5	1					1		
	Apicomplexa		1								
	Unc. *Alveolata*		5				1				
Stramenopiles	*MAST*-12		1								
Haptophyta				1	1						
Amoebozoa									1		
Rhizaria	*Cercozoa*		6								
	Radiolaria			1	1						
Novel basal lineage			1								
Fungi	*Ascomycota*	1	2		1		3	1	2	1	
	Basidiomycota								2		
Metazoa	*Arachnida*				1		1				
	*Insecta**										1
	Copepoda		2								
	Enteropneusta		1								
	*Mammalia**				1				2		1
Chloroplastidae	*Prasinophytae*		1								
	Plantae				1	2	4		5		
Archaea			1								
	Unc. *Archaea*		2		2	1	3	1	2		

(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.

Analysis of biomarkers

Parallel lipid analyses were performed on the samples which were analyzed for fossil DNA at the Royal NIOZ. Sediments (∼10 g) were freeze-dried and ultrasonically extracted 5 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 fraction using DCM and DCM/MeOH (1:1, v/v) as eluent, respectively. To the apolar fractions an internal standard (6,6-d2-3-methyl-eicosane) was added and they were analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS). 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 (BSTFA) 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 (FID) 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 3 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 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

Location of the sapropels.

The location of the sapropels in the core was determined based on the organic carbon (C_{org}) content and the barium/aluminum ratio (Ba/Al) (Fig. 2A). The C_{org} content was highest (4%) in the deepest sapropel S5, and ∼2 wt% in sapropels S1, S3, and S4. C_{org} values in the intercalating sediments reached maximum values of 0.4%. The Ba/Al ratio was higher in the S5 (ca. 200) as compared to the other sapropels (ca.$100 - 150$), and about $30 - 40$ in the intercalating marls. A few cm above the C_{org}-rich sapropels S3, and especially above S1, the Ba/Al ratio remains high, which is indicative of post-depositional reoxidation of the upper few cm of these sapropels (de Lange *et al.*, 2008). In contrast, post-depositional re-oxidation is not apparent for S4 and S5 sapropels but both show a temporary decrease in C_{org} content and Ba/Al ratio in the middle of the sapropels, which has been interpreted as an interruption of sapropel deposition (de Rijk *et al.*, 1999).

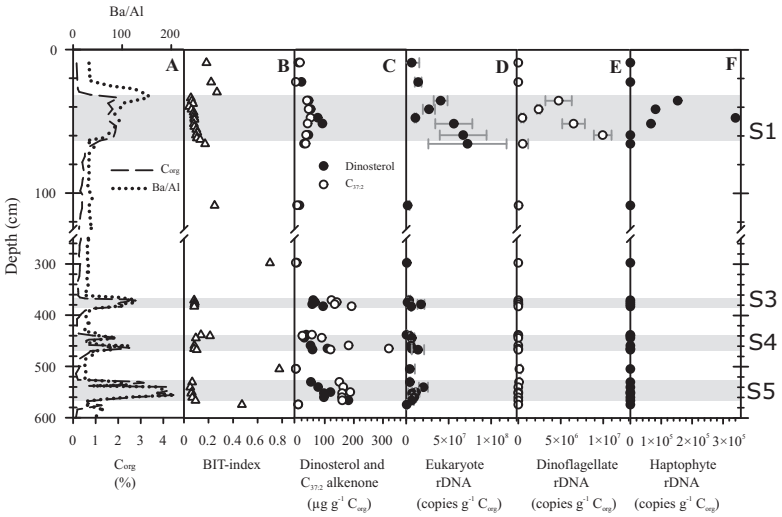


Figure 2: Bulk, biomarker and DNA profiles of the investigated core (MS66PC). The position of the four sapropels (S1, S3, S4, and S5) are shown as grey shaded areas in the graphs. A) Organic carbon content (C_{org}; wt%) and Ba/Al ratio. The core was analyzed at 1 cm interval resolution for these parameters. 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 in higher resolution. C) Concentrations of selected lipid biomarkers dinosterol, which is used as a biomarker for the input of dinoflagellates to sedimentary biomass, and the $C_{37:2}$ alkenone concentration, which is specific to certain haptophytes. D, E, F) Number of 18S rDNA copies quantified using real-time quantitative PCR with (D) general eukaryote primers Euk1f / Euk516R, (E) a dinoflagellate specific primer set Euk1f / DinoRev, and (F) a haptophyte specific primer set Prym448/Prym884. Error bars represent the standard deviation of two replicates. Note the different scales for rDNA of the various taxa. The break in the depth axis represents the sediment interval $125 - 250$ cm below sea floor.

Quantitative distribution of eukaryote 18S rDNA

Eukaryotic 18S rDNA was quantified throughout the Pleistocene eastern Mediterranean sediments by qPCR with a the general eukaryote primer set without GC-clamp (Table 1). All samples were analyzed in duplicate and yielded reproducible results. The number of eukaryotic 18S rDNA copies was highest in S1 (7×10^7 copies g^{-1} C_{org}), and these values were only ∼5 times lower in the older sapropels S3 through S5 (∼1 to 2×10^7 copies g^{-1} C_{org}) (Fig. 2D). Eukaryotic 18S rDNA was also detected in most of the intercalating marls, but the average copy numbers were $1 - 2$ orders of magnitude lower (∼1×10^5 copies g^{-1} C_{org}) than within the sapropels.

In addition, we specifically quantified the amount of preserved 18S rDNA of dinoflagellates and haptophyte algae throughout the late-Pleistocene sediment record

(Figs. 2E, F). Dinoflagellate rDNA comprised ∼10% of the total eukaryote 18S rDNA (maximum 1×10^7 copies g^{-1} C_{org}; Fig. 2E) in S1. Below the S1, 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. However, the latter results could not be reproduced in separate analyses, suggesting that the number of preserved dinoflagellate 18S rDNA fragments in the >80 ka-old sapropel layers was close to the detection limit.

Quantification of rDNA copy numbers using the haptophyte-specific primer set revealed that ∼400-bp-long haptophyte 18S rDNA fragments represented 1% or less (up to 3×10^5 copies g^{-1} C_{org}) of the total number of eukaryotic 18S rDNA copies in the S1 (Fig. 2F). 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 in the Pleistocene sediments below sapropel S1.

Molecular phylogeny of the sedimentary Eukaryotic phylotypes

The amplicons from the quantitative PCR assays were reamplified with GC-primers (Table 1) and subjected to DGGE. Excised DGGE-bands were subsequently sequenced for phylogenetic analysis. The closest BLAST hits, including accession number and sequence similarity (%), of recovered phylotypes are shown in Supplementary Table 1. 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 2 provides an overview of the distribution of these higher taxonomic groups per depth interval.

The majority of eukaryotic phylotypes in the S1 represented marine protists of which Alveolata (a group comprising dinoflagellates, apicomplexa and "unclassified alveolata") represented the largest group. Other protist groups included cercozoa, a prasinophyte and a protist belonging to the MAST-12 cluster (cf. clone DSGM-40, Takishita *et al.*, 2005, 2007, Table 1). One phylotype ("unknown affiliation", Table 2) is related to an environmental phylotype (uncultured marine eukaryote clone CCI73, AY179972) that was isolated from an anoxic water-sediment interface and is currently without known close relatives (Stoeck and Epstein, 2003).

Besides protists, a number of metazoan phylotypes were found in sapropel S1, including two copepods and a phylotype related to acorn worms (Enteropneusta) (Table 3). Two additional phylotypes recovered from sapropel S1 were related to marine fungi. One of these (cf. *Pichia guilliermondi*) is found throughout the core (see below) whereas a fungal phylotype related to *Metschnikowia* sp. (Ascomycetes) was found only in S1.

Most eukaryotes that were consistently present throughout the core below S1 belong to marine fungi, in particular *P. guilliermondi* (Table 2). Other marine fungal phylotypes, related to *Rhodosporidium*, were only found in sapropel S5 (Fung-02

and Fung-07). Besides marine fungi, the numerically most abundant phylotypes (13 phylotypes) recovered from below the sapropel S1 were related to terrestrial plants (Chloroplastidae), representing a plants of widely different groups (Table 3).

Preserved partial 18S rRNA gene sequences of marine protists could only occasionally be recovered from the Pleistocene sediments: Two phylotypes (EUK-24 and EUK-42) related to dinoflagellates were recovered from the intercalating marl between S1 and S3 as well as from S5. These sequences were not found in S1, and were only distantly related to known dinoflagellate species (Fig. 3A). S4 harbored a single sequence related to uncultured alveolates from the photic zone of the Sargasso Sea (AY664058; Armbrust, unpublished). A sequence with 99% sequence similarity to the alkenone-producing coccolithophorid *Emiliania huxleyi* was recovered from the intercalating marl between S1 and S3 as well as in S3. The latter sediments also revealed a sequence related to siliceous nannoplanktonic radiolarian species (Table 2). Furthermore, the S5 contained DNA of an unassigned amoebozoa (EUK-25).

Controls for possible contaminations during sampling and handling

In order to monitor possible contamination of the samples containing the fossil 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, non-template 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 WHOI laboratory using a similar qPCR-DGGE-sequencing approach.

Both ECs and NTCs at Royal NIOZ remained negative in most runs, and products that were formed in the controls were regarded as contamination, including beetle (Coleoptera) and spider (Arachnida) sequences. These sequences were not detected in the investigated sediment samples. However, mammalian DNA was found both in non-template controls and in some of the sediment samples. In addition, one of the extraction controls yielded a fungal species (*Pichia guilliermondi*) that was also found in the sediments. As this same fungal sequence was never found in the NTCs, the EC was likely contaminated with a sample during the extraction procedure. The ECs analyzed at WHOI did not show a product, but one of the NTCs showed a plant sequence, closest related to a pine tree (*Pinus*).

The parallel analysis at WHOI in general showed a similar preserved eukaryote community. The direct general eukaryote GC-primer assay yielded 18 phylotypes, nine of which were found in the S1 samples (Table 3). Eight of the nine S1 phylotypes were related to marine (microbial) eukaryotes and were identical to sequences found at the Royal NIOZ (Table 3). The ninth unique sequence was related to a fly (Insecta; 95% similarity). The phylotypes found in the samples from below the S1 were related to the same fungal phylotype (cf. *P. guilliermondii*) found in the sediments analyzed at Royal NIOZ, or to higher plants. Four out of the five phylotypes related to higher

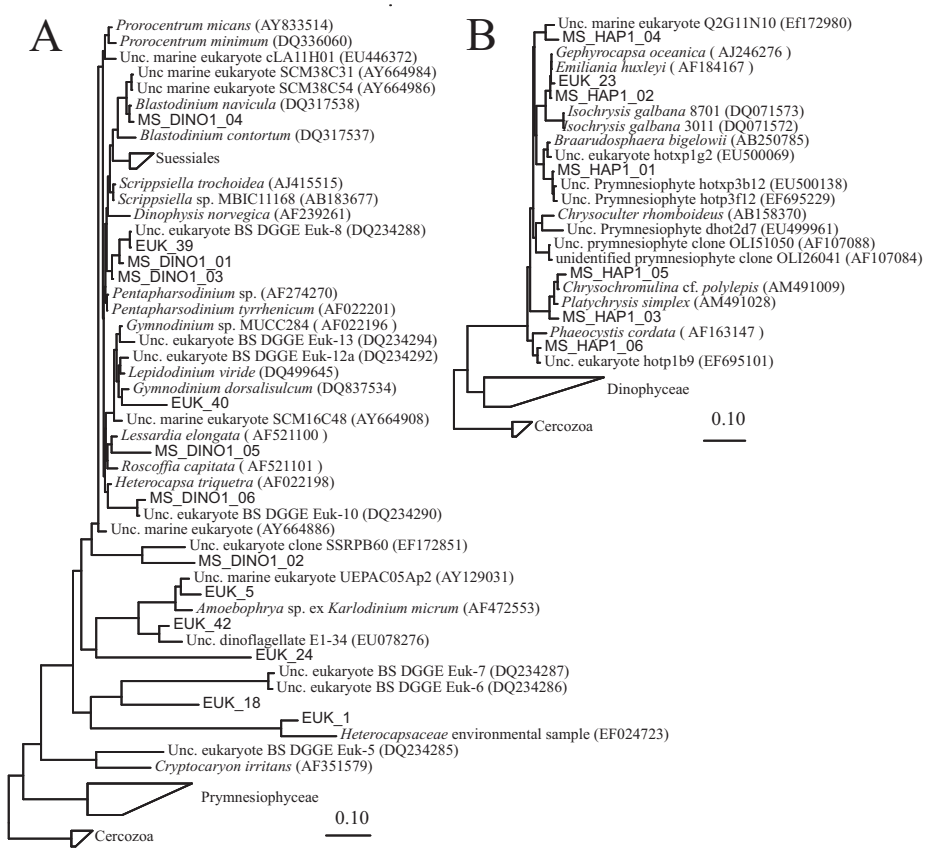


Figure 3: Bayesian inferred phylogenetic trees showing the relationship between fossil rDNA sequences found using group-specific (haptophytes and dinoflagellate) primers, and sequences related to these taxonomic groups recovered with the general eukaryote primers. Both trees were obtained in one single 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. Abbreviation: unc.: uncultured; i.s.: intercalating, non-sapropel sediment layer.

plants were different from those found in the samples analyzed at the Royal NIOZ. As adjacent samples representing different time intervals were analyzed, this would indicate that the preserved plant DNA composition was rather variable over time.

Dinoflagellate and haptophyte related phylotypes.

Besides using general eukaryote primers to identify preserved microbial eukaryotes, we also used group-specific primers for dinoflagellates and haptophytes. These more specific primers lowered the detection limit for their rDNA and resulted in the identification of additional phylotypes (Fig. 3).

For the dinoflagellates, the use of specific primers resulted in the identification of 6 phylotypes within sapropel S1 (Fig. 3). Only one sequence (MS_Dino1_01) was similar to a phylotype identified by the general eukaryote primer (EUK_39). A closely related phylotype was also recovered from fossil rDNA preserved in the anoxic Black Sea sediment (DQ234288; Coolen *et al.*, 2006b) with the genus *Pentapharsodinium* as closest cultured relatives. The other five dinoflagellate phylotypes were not detected with the general eukaryote primers. They all cluster within the dinoflagellate crown group, whereas the phylotypes from the eukaryote assay cluster with the more basal dinoflagellates (i.e, *Syndiniales* Guillou *et al.*, 2008).

The haptophyte-specific PCR assay resulted in the identification of 5 phylotypes within S1 that were missed with the general PCR assay (Fig. 3B). These phylotypes represent various orders of the Prymnesiophyceae (Fig. 3B). The only phylotype that was recovered by both assays (EUK_23, MS_Hap1_02; Fig. 3B) was related to *Emiliania huxleyi*.

As mentioned in the quantitative analysis, dinoflagellate DNA was only detected in isolated sediment horizons. Attempts to re-amplify these amplicons with GC-primers generally resulted in formation of bands of incorrect lengths. Only one amplicon of the correct length could be sequenced below the S1. This phylotype (MS_DINO1_-04) was identical to phylotype found in the S1 and was related to the *Blastodinium navicula*, a known dinoflagellate parasite on copepods.

Table 3: (Next page) Phylogenetic affinity of recovered general eukaryote phylotypes. Shown are the higher taxonomic grouping, closest BLAST hit name, closest BLAST hit accession number and average similarity to closest BLAST Hit (in %). The number of sequences shows how many of the sequenced DGGE bands are placed within that phylotype. Presence within the 4 sapropel layers (S1 through S5) or the intercalating sediments is indicated with a plus (+) sign. Sequences found at WHOI are shown in a separate column. Abbreviations: Unc.: uncultured; i.s.: Nonsapropelic intermediate sediment interval; e.c.: extraction control; ntc: non-template control PCR reaction.

Phylogenetic affiliation	OTU name	BLAST hit name	Acc. Nr.	Sim. (%)	nr of seq.	NIOZ S1	NIOZ S3	NIOZ S4	NIOZ S5	NIOZ i.s.	NIOZ e.c.	NIOZ ntc	WHOI sap	WHOI i.s.	WHOI ntc
Protista															
Dinophyceae	EUK-1	Heterocapsaceae env sample clone	EF024723	92	5	+							+		
	EUK-10	*Lepidodinium viride*	DQ499645	94	2	+									
	EUK-18	*Apicoporus parvidiaboli sack*	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										
Apicomplexa	EUK-30	Unc. eukaryote clone DSGM-13	AB275013	96	1	+									
Unc. Alveolata	EUK-5	Unc. marine eukaryote clone UEPAC05ap2	AY129031	97	3	+									
	EUK-7	Unc. marine eukaryote clone N 1B242	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	+									
	?	Unc. marine alveolata	AF290078	97	1								+		
	?	Unc. eukaryote clone DSGM-22	AB275022	95	1								+		
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	EU287798	90	1		+								
Haptophyta	EUK-23	*Emiliania huxleyi*	AF184167	99	2		+			+					
Amoebozoa	EUK-25	*Hartmannella vermiformis* clone 4391	DQ084364	100	1				+						
MAST-12	EUK-35	Unc. marine eukaryote clone DSGM-40	AB275040	98	1	+									
Unknown affinity	EUK-15	Unc. marine eukaryote clone CCI73	AY179972	86	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				+						

Phylogenetic affiliation	OTU name	BLAST hit name	Acc. Nr.	Sim. (%)	nr of seq.	NIOZ S1	NIOZ S3	NIOZ S4	NIOZ S5	NIOZ i.s.	NIOZ e.c.	NIOZ ntc	WHOI sap	WHOI i.s.	WHOI ntc
Metazoa															
Arachnida	EUK-21	*Schwiebea pseudotsuqae*	EU152499	97	1			+							
	EUK-34	*Sitticus palustris*	DQ665749	97	1		+								
Insecta	EUK-33	*Discoderus cordicollis**	AF012472	98	1							+			
	Euk_07_mc	*Laphystia tolandi*	EF650144	95	1								+		
Copepoda	EUK-22	*Drepanopus forcipatus*	AF462321	99	1	+									
	EUK-26	*Haloptilus ocellatus*	AY118069	96	2	+									
Enteropneusta	EUK-17	*Saccoglossus pusillus*	AF236800	90	1	+									
Chordata	EUK-6	*Homo sapiens**	EU647001	99	6		+		+			+			
Chloroplastidae															
Prasinophyceae	EUK-29	Nannochloris sp. MBIC10749	AJ402345	98	1	+									
	EUK_13_mc	Nannochloris sp. ANR-9	AY220081	99	1								+		
Plantae	EUK-4	Unc. eukaryote clone Elev 7261	EF025039	99	3		+	+	+						
	EUK-11	Unc. streptophyte clone	AY496507	99	1				+						
	EUK-12	*Zea mays*	AF168884	100	1					+					
	EUK-14	*Albizzia 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	n								+		+
	Euk_18_mc	*Sassafras albidum*	U52031	99	1								+		
	Euk_16_mc	*Lolium multiflora*	AY846367	99	1								+		
	Euk_14_mc	Unc. eukaryote clone	EU326599	100	1								+		
Archaea															
	ARCH-1	Unc. archaeon partial 16S	AM072592	87	9	+	+	+	+						
	ARCH-2	Unc. euryarchaeote clone 1aD3	AY800210	90	6			+	+	+					
	ARCH-3	Unc. archaeon partial 16S	AM072592	88	4		+		+		+	+			
	ARCH-4	Unc. archaeon clone MD2896-A116	EU385665	92	2	+		+							
	ARCH-5	Unc. euryarchaeote partial 16S	AM072593	96	1			+							
	ARCH-6	Unc. euryarchaeote partial 16S	AM072593	92	1	+									
	ARCH-7	Unc. euryarchaeote clone 1aD3	AY800210	92	1			+							

Biomarker lipids

A parallel lipid biomarker analysis on the sediments that were analyzed for fossil DNA at the Royal NIOZ was also performed to compare the results of our palaeogenetic approach with that of a more conventional method. Biomarker lipids specific to important algal groups were quantified, in particular the sterol dinosterol (4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol) for dinoflagellates and long-chain alkenones specific for haptophytes (Fig. 2C). For clarity, only the concentration profile of the $C_{37:2}$ alkenone is shown in Fig. 2C to compare with the haptophyte rDNA data (Fig. 2F). Other alkenones (i.e., $C_{37:3}$ and $C_{38:2}$), however, show a similar trend.

Overall, the dinosterol and alkenone concentrations varied between 50 and 200 μg g^{-1} C_{org} in the sapropels, with lowest concentrations in the S1 (i.e., $\sim$50 μg g^{-1} C_{org}). Alkenone concentrations were generally higher than dinosterol concentrations, except in the S1, where concentrations of both biomarkers were comparable (Fig. 2C). Lower, but still detectable concentrations were present in the intercalating sediments ($<$10 μg g^{-1} C_{org}; Fig. 2C).

In order to estimate the contribution of continental organic matter in our core, we determined the ratio between the soil-derived derived branched tetraether lipids and marine pelagic 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 organic matter 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. 2B).

Discussion

Preservation of protist rDNA in eastern Mediterranean sapropels

In the core investigated here, the presence of preserved rDNA of marine protists (i.e., unicellular marine eukaryotes) that are unambiguously derived from the water column is, with some notable exceptions, restricted to the early Holocene sapropel S1. Although 18S rDNA was also detected in the deeper sapropel layers using general eukaryote primers, phylogenetic analysis showed that most of these eukaryote phylotypes did not unambiguously represent ancient marine protists, as discussed below. In addition, the number of rDNA copies in the deeper sapropels S3$-$S5 was $\sim$4 times lower than in the youngest sapropel S1. In an attempt to lower the detection limit for fossil DNA of microbial eukaryotes unambiguously stemming from the ancient pelagic environment, we used group-specific primer sets for dinoflagellates and haptophytes and validated this paleogenetic data from this approach with a quantitative compari-

son of characteristic lipid biomarkers (i.e., dinosterol and long-chain alkenones). The use of these group-specific primers only yielded reliable PCR products within the S1 sapropel and (almost) none in the deeper sediments. In contrast, the investigated lipid biomarkers showed highest concentrations in the deeper sapropels S3 through S5, and lower concentrations in sapropel S1.

Fossil 18S rDNA preserved in Holocene sapropel S1

Despite the relatively restricted maximum preservation of DNA, the fossil DNA that we found can be informative for reconstructing paleoenvironmental conditions. The assemblage of protists found within sapropel S1 using the general eukaryote primers comprises a number of informative phylotypes originating from the photic zone. As these species require light to photosynthesize and, thus, they cannot live in the dark anoxic sediments and/or bottom waters that prevailed during sapropel formation (Coolen and Overmann, 1998; Teske, 2007), it is likely that fossil DNA of these obligate photoautotrophic algae found in sediments was sequestered in the sediment from the overlying photic zone at the time of deposition.

Protistan phylotypes derived from the photic zone.

Only few phylotypes we recovered from the S1 sapropel using the general primers were closely related to unambiguously pelagic algae, such as a photoautotrophic *Nannochloris* species (class Prasinophyceae). Surprisingly, no diatom phylotypes were recovered, while diatoms are often considered to be important species contributing to organic matter in sapropels (Kemp *et al.*, 1999). The fossil DNA of obligatory photoautotrophic haptophytes was not detected within the S1 using the general eukaryote primer set, even though the parallel analysis of their characteristic alkenone lipids indicates their continuous presence within all the sapropels. In order to lower the detection limit of our approach, we also applied a haptophyte-specific primer set. General primers often fail to detect whole groups as a result of primer bias (Stoeck *et al.*, 2006; Potvin and Lovejoy, 2009) and recent studies stressed the increased phylogenetic resolution when group-specific primers are used to detect important ecological groups (Richards and Bass, 2005; Viprey *et al.*, 2008; Coolen *et al.*, 2004a). Now, using the haptophyte specific primer set, we were indeed able to detect haptophyte phylotypes derived from the photic zone within S1, such as *E. huxleyi* and *Braarudosphaera bigelowii* (Hagino *et al.*, 2005). These particular species produce calcareous coccoliths that are often found in the S1 sapropel and have been used for paleoenvironmental reconstructions before (Principato *et al.*, 2006; Giunta *et al.*, 2003). A higher resolution analysis of the preserved haptophyte community within sapropel S1 and its paleoenvironmental implications will be published elsewhere.

Protistan phylotypes possibly derived from the photic zone.

The group Alveolata, which includes the dinoflagellates, is numerically the most important group of protistan phylotypes that we detected within S1 (Table 1, Table 3). For some algal taxa from this group, in particular dinoflagellates, it is not so straightforward to infer a strictly photoautotrophic lifestyle. The group is considered part of marine eukaryote phytoplankton but can be autotrophic, heterotrophic, mixotrophic or parasitic (Marret and Zonneveld, 2003; Stoecker *et al.*, 1997; Stoecker, 1999). In addition, the known diversity of marine alveolata has greatly expanded in recent years due to analyses of anoxic marine sediments, and it has become clear now that many basal alveolate lineages are capable of living in anoxic marine conditions and sediments (Edgcomb *et al.*, 2009; Takishita *et al.*, 2007; Dawson and Pace, 2002; Behnke *et al.*, 2006; López-García *et al.*, 2003; Stoeck *et al.*, 2007; Alexander *et al.*, 2009; Coolen and Shtereva, 2009). Because these novel anoxic lineages are uncultured so far, little is known about their physiological and environmental limitations (Kolodziej and Stoeck, 2007; Teske, 2007) and, therefore, these taxa are relatively uninformative for paleoenvironmental reconstructions, other than merely indicating the presence of anoxic conditions.

The detection limit for preserved dinoflagellate DNA was lowered considerably by using the more specific primer set (Boere *et al.*, 2009), that preferably selects for the dinoflagellate "core-group" (Guillou *et al.*, 2008; Edgcomb *et al.*, 2009). The distribution of the recovered phylotypes in the resulting phylogenetic tree (Fig. 3A) indeed shows that many of the phylotypes found with the general primers (e.g., EUK-1, EUK-5, EUK-18, EUK-24, EUK-42) cluster with the more basal lineages related to dinoflagellates (i.e., Syndiniales, (Guillou *et al.*, 2008)), whereas the phylotypes found with the specific primers (e.g., MS_DINO_01 through MS_DINO_06) cluster within the "core" group (Fig. 3). This core-group comprises mainly well-known species derived from the water column, thus, species that are potentially informative for reconstructing paleoenvironmental conditions. The use of specific primers results in this case in more useful data. The paleoceanographic implications of these dinoflagellate species from S1 will be discussed in more detail elsewhere, along with the haptophyte phylotypes.

Non-protist rDNA within S1

Besides the DNA of protists, we also detected DNA of certain metazoan species within sapropel S1, such as DNA of calanoid copepods (i.e., zooplankton). Although copepods can live under extremely low oxygen conditions (Auel and Verheye, 2007; Wishner *et al.*, 2008), these species are unambiguously of pelagic origin. Their DNA in the subsurface sediment of the Mediterranean must have been deposited there during the sapropel formation and is therefore considered to be of genuine ancient origin. Resistant copepod resting eggs (Skovgaard, 2005) would significantly increase the chance that fossil copepod DNA is preserved. The use of copepods in paleoenvironmental studies has been limited because their cuticles are hard to distinguish, but

fossil copepod DNA has previously been used to identify cryptic species in Holocene Antarctic lake sediments (Bissett *et al.*, 2005) as well as the Black Sea (Coolen and Shtereva, 2009).

The presence of DNA of a benthic worm (Enteropneusta, Cameron *et al.*, 2000) within sapropel S1 is would indicate occurrence of burrowing in or into sapropels. The fact that we found only a single occurrence of worm DNA indicates that bioturbation was limited, as previously reported (Löwemark *et al.*, 2006; Basso *et al.*, 2004).

18S rDNA in Pleistocene sediments below sapropel S1

Within the investigated eastern Mediterranean sapropels, the presence of microbial eukaryote sequences that originate from the ancient water column, is largely restricted to sapropel S1. Only isolated occurrences of protistan phylotypes were found within the deeper parts of the core using the general eukaryote primers. The use of group-specific primer sets did not change this. Both the haptophyte and the dinoflagellate primer sets only yielded PCR products of correct length within the S1, and none in the deeper sapropel layers or intermediate layers (or not reproducible between replicate runs). In addition, the two samples from below the S1, which revealed DNA of *Emiliania huxleyi* using the general primers, did not yield a similar PCR-product with the haptophyte-specific primers. These results probably indicate that the concentration of protistan DNA preserved in the deeper layers is very close to the detection limit, whereas it is still considerably higher in the S1. Several non-protistan eukaryote phylotypes were, however, abundantly recovered from the deeper layers.

Fungi

The most common phylotypes from the deeper sediments are related to yeasts or unicellular fungi. The fungal phylotype that was found in most sapropels, as well as in the intercalating sediments (i.e., related to *Pichia guilliermondi*) is a widespread facultative marine yeast (Kutty and Philip, 2008; Gadanho and Sampaio, 2005; Bass *et al.*, 2007). Other fungal phylotypes we found are related to obligate marine strains, notably *Metschnikowa*, *Rhodosporidium* and *Rhodotorula* species (Kutty and Philip, 2008). In contrast to terrestrial fungi, marine fungi were until recently believed to comprise a minor part of the eukaryote community in deep-sea environments (Bass *et al.*, 2007). The introduction of cultivation-independent molecular approaches revealed, however, the widespread occurrence and the importance of fungi in marine environments (Edgcomb *et al.*, 2002, 2009; Bass *et al.*, 2007; López-García *et al.*, 2003; Takishita *et al.*, 2006; Alexander *et al.*, 2009; Burgaud *et al.*, 2009). It is therefore unclear if the fungal DNA we detected are of ancient origin. As marine fungi, including yeasts, may be growing actively in the subsurface sediments (Damare *et al.*, 2006; Damare and Raghukumar, 2008), these phylotypes may not be of ancient origin but represent extant subsurface biomass.

On the other hand, fungi are capable of long-term preservation of their DNA in the form of spores (Lydolph *et al.*, 2005), and these spores have been proposed as

a possible paleoenvironmental indicator (Raghukumar *et al.*, 2004). Although the use of fungal spores for this goal is in its infancy, the analysis of their (preserved) DNA would be an interesting approach to escape the cultivation bias associated with ancient preserved spores (Raghukumar *et al.*, 2004; Damare *et al.*, 2006).

Terrestrial plant DNA

Besides fungi, phylotypes related to terrestrial plants were numerically abundant and outnumbered marine protist phylotypes in the Pleistocene sediments. This suggests an extensive fluvial transport into the Mediterranean Sea and preservation of terrestrial plant material during sapropel formation (Rossignol-Strick, 1999; Cheddadi and Rossignol-Strick, 1995a,b; Kholeif and Mudie, 2009). On the other hand, the low BIT-index found in sapropels indicates that most organic matter is derived of marine instead of terrestrial origin (Hopmans *et al.*, 2004; Menzel *et al.*, 2006). The predominance of terrestrial plant DNA over marine protist DNA with increasing sediment depth and age, therefore, suggests that protist DNA is more prone to degradation as compared to terrestrial plant DNA. Many plant parts, such as seeds and pollen, have evolved for the protection of DNA, and are therefore, theoretically, an ideal source of ancient DNA (Schlumbaum *et al.*, 2008; Parducci *et al.*, 2005; Bennett and Parducci, 2006). Our dataset suggest that Pleistocene anoxic sediments such as the eastern Mediterranean sapropels could represent a rich archive of ancient plant DNA. On the other hand, airborne pollen (and fungal spores) are an omnipresent source of contamination during sampling and handling of the sediments (Boreson *et al.*, 2004). Several studies on marine protists have excluded plant sequences from the analyses as possible contaminating, non-target DNA (Viprey *et al.*, 2008; Stoeck *et al.*, 2007; Behnke *et al.*, 2006). Therefore, any paleoclimatic inferences based on the presence and identification of fossil plant ribosomal DNA alone should be treated with caution.

Unexpected detection of Archaea

Another interesting, but unexpected result of the semi-nested general eukaryote assay is the consistent detection of archaeal phylotypes in nearly all samples, phylotypes which were never detected in the one-step approach. Other studies have observed this same amplification of non-target DNA bias in sediments from mud flats (Wilms *et al.*, 2006), aquifers (Euringer and Lueders, 2008) and deep sea anoxic basins (Jeon *et al.*, 2008; Alexander *et al.*, 2009). Archaea are an numerically important group in subsurface sediments (Coolen *et al.*, 2002; Jørgensen and Boetius, 2007; D'Hondt *et al.*, 2002), with 16S rDNA concentrations several orders of magnitude higher than eukaryote rDNA (Schippers *et al.*, 2005; Schippers and Neretin, 2006). Apparently, the initial amplification step results in a high ratio of non-target archaeal sequences to target eukaryote sequences. This ratio becomes so high that even GC-primers which select against archaea will amplify archaeal rDNA.

These results, however, show that merely quantifying 18S rDNA in sediments, without identifying the amplified DNA, may result in wrong conclusions. In our case,

archaeal DNA was likely co-amplified in the S3 to S5 layers during the eukaryote qPCR (Fig. 2D) and resulted in an overestimation of the eukaryote 18S rDNA present in this section of the core. Likewise, a significant fraction of the rDNA quantified in up to 5 times older sediments of the Peru margin using eukaryote primers (Schippers *et al.*, 2005; Schippers and Neretin, 2006) might also have been of archaeal, rather than of eukaryote origin. Alternatively, they might have measured fungal DNA, although this is contradicted by the fact that attempts to detect eukaryotes using CARD-FISH were unsuccessful (Schippers *et al.*, 2005; Schippers and Neretin, 2006). Even though the (post)depositional and preservation conditions of the Peru Margin and the Mediterranean Sea during sapropel deposition are quite different, the near total lack of DNA of ancient species in the deeper part of our core ($\sim 80 - 120$ kyr), sheds doubt on the considerably longer preservation of eukaryote rDNA in the up to 1 Ma-old Peru Margin sediments (Schippers *et al.*, 2005; Schippers and Neretin, 2006). Therefore, we urge to not only quantify preserved fossil DNA in paleogenetic studies, but to complement such an approach with a qualitative approach in order to ensure one is indeed looking at preserved eukaryote DNA.

Why do we find DNA in S1 but not in the deeper sediments?

The fact that we found fossil DNA in the most shallow sapropel S1, but not in the deeper sapropels, supports earlier findings that DNA can be protected against degradation for thousands of years in marine sediments (Coolen *et al.*, 2004a, 2007) but that ongoing post-depositional degradation limits the maximum preservation, whereas lipids are much better protected once they have reached the sediment (Corinaldesi *et al.*, 2008; Coolen *et al.*, 2002, 2006b, 2004a). No target rDNA was detected in the younger sediment marls above the S1 sapropel, which indicates that DNA is only preserved in the C_{org}-rich sapropel layers, and not in the hemipelagic C_{org}-poor intercalating marls. This is likely due to the fact that the sedimentary organic matter (including DNA) was subjected to oxic degradation during deposition of the marls but not during deposition of the sapropels as a consequence of the changing redox conditions of the bottom waters. Although the processes responsible for preservation of DNA are still not well understood, anoxic conditions are an important factor influencing the successful preservation of DNA in sediments (Coolen *et al.*, 2004a, 2006b; Willerslev and Cooper, 2005).

Since the majority of the fossil protist DNA was found in the S1 ($\sim$10 ka), and protist DNA was not detected in or below S3 ($\sim$80 ka), we estimate that most of the fossil protist DNA was degraded between $\sim$10 and $\sim$80 ka. This is relatively fast in comparison to the theoretical maximum preservation age estimates for ancient DNA in paleontological and archeological artefacts ($\sim$100 kyr $-$ 1 Myr (Mitchell *et al.*, 2005; Willerslev and Cooper, 2005)) and in marine sediments (Corinaldesi *et al.*, 2008).

After deposition, biological degradation and remineralization of DNA in sediments is limited by the adsorption of either the DNA or the nucleases to the sediment matrix (Lorenz and Wackernagel, 1987; Romanowski *et al.*, 1991; Demanèche *et al.*, 2001; Pietramellara *et al.*, 2009). Nevertheless, various processes could lead to (fur-

ther) fragmentation of fossil DNA (Coolen and Overmann, 1998; Coolen *et al.*, 2006a; Hansen *et al.*, 2006; Willerslev and Cooper, 2005) and could have hampered the efficient PCR amplification of the ∼500-bp-long 18S rDNA regions especially in the sediments older than the S1.

In addition, methodological issues could have resulted in an underestimation of the past eukaryotic diversity in the Mediterranean sapropels and the OC-poor intermediate layers. For example, it is possible that certain dinoflagellate and haptophyte taxa might have escaped amplification when using selective primers during PCR and only clearly visible and more intense DGGE bands can be successfully excised and sequenced. This kind of bias could most likely be omitted when using other techniques, notably tag-encoded FLX or Titanium pyrosequencing of environmental PCR products (Sogin *et al.*, 2006). Pyrotag sequencing of shorter amplicons could furthermore lower the detection of the presumably more degraded and fragmented DNA in sedimentary records exceeding the Holocene era.

Interestingly, a previous study of Mediterranean sapropels reported the successful amplification of fossil 16S rDNA of green sulfur bacteria (Chlorobiacaea) in older sapropels (Coolen *et al.*, 2007), whereas attempts to amplify rDNA of green sulfur bacteria in our core failed (data not shown). The different results between that study and the present study may be due to differences between the cores. For instance, the core of Coolen *et al.* (2007) was taken from a deeper location (2250 m water column depth versus 1630 m for our core). Organic matter in the S1 is better preserved at greater water depths, as indicated by the generally increased C_{org} concentrations in sapropels deposited at greater depths (de Lange *et al.*, 2008; Murat and Got, 2000). In addition, earlier studies of Pliocene Mediterranean sapropels have shown that concentrations of isorenieratene, a pigment derived from the anoxygenic photosynthetic green sulfur bacteria, are lower in sapropels close to the Nile, as a result of deepening of the chemocline due to freshwater input from the Nile (Menzel *et al.*, 2002).

Conclusions

Our results show that rDNA of microbial eukaryotes can be present in anoxic deep-sea subsurface sediments and that, based on the phylogenetic affiliation with photoautotrophic species living in the photic zone, at least part of this DNA is of ancient origin and may thus be used in reconstructing paleoenvironmental conditions. Many of the recovered phylotypes are, however, related to protistan species living in anoxic marine settings, and further study should focus on answering the question of how much of this sedimentary DNA is actually of ancient origin or if it represents the presence of living microbial eukaryotes in the subsurface by targeting markers for active living biomass (e.g., rRNA or intact polar lipids). In our study, the presence of eukaryote rDNA that is unambiguously derived from the ancient water column is restricted to the Holocene sapropel S1. As DNA was strictly found within the periodical C_{org}-rich sapropels, and not within the intercalating marls, only a very broad maximum age limit for the preservation of eukaryote DNA can be inferred, at somewhere between

∼10 kyr (S1) and ∼80 kyr (S3). Sediments with continuously favorable conditions for DNA preservation in combination with more sensitive molecular approaches, notably tag-encoded FLX or Titanium amplicon pyrosequencing, are required to further constrain this maximum preservation age of fossil DNA in marine sediments

Acknowledgements

We would like to thank Alina Stadnitskaia, Shauna ní Flaithearta, and the captain and crew of the R/V Pelagia for obtaining and subsampling the core. Marianne Baas, Jort Ossebaar and Sanela Gusic are thanked for initial lipid analyses and C_{org} measurements, respectively. This work was supported by grants and prizes from the Netherlands Organization for Scientific Research (NWO Open Competition grant 813.03.001 and NWO-VENI grant 016.051.014 to M.J.L.C., Spinoza prize to J.S.S.D.) as well as financial support from the U.S. National Science Foundation grant OCE Chemical Oceanography grant 0825020 to M.J.L.C.

CHAPTER 7

Synthesis and outlook

Synthesis and outlook

The reconstruction of past environmental circumstances is partly based on preservation of microscopical and chemical remains in marine sediments. Microscopical remains comprise, for instance, calcareous and silica shells and organic- or calcareous cysts. In addition, lipid-based records are particularly valuable in cases where primary producers do not biomineralize or where their morphological remains are not well preserved in the sedimentary record. Several recent studies have successfully demonstrated that preserved genetic signatures (i.e., fossil DNA) can be used to identify ancient plankton members, including important environmental indicator species that did not produce or leave diagnostic markers. To date, only C_{org}-rich sediments underlying anoxic bottom waters have been used to reconstruct past planktonic communities, whereas the preservation of DNA is expected to be lower in, for example, deep-sea sediments that are low in C_{org} content as a result of extensive biodegradation. In addition, it remains unknown at what geological time scales fossil plankton DNA is preserved in marine sediments. Information is also lacking about whether fossil plankton DNA is equally well preserved among different organisms.

The objectives of this thesis were to validate fossil DNA as a paleoecological proxy, and to reveal to what extent fossil DNA can aid in improving paleoenvironmental information inferred from traditional proxies. For this goal, fossil DNA of important planktonic eukaryotes and prokaryotes, including environmental indicator species without fossilizing diagnostic features, were analyzed from multiple locations and from sediments deposited under various environmental conditions. Where possible, the paleogenetic data was cross-validated by a parallel analysis of cellular fossils and/or diagnostic lipid biomarkers.

Dinoflagellates are a numerically important group of protists in marine and freshwater environments, and roughly 10% of the described species are known to produce calcareous or organic-walled cysts, which can be preserved in the geological record. Furthermore, dinosterol, a lipid biomarker that is produced by many, but not all dinoflagellates, can be identified from the fossil record. Therefore, dinoflagellates are excellent model species to cross-validate the various proxies. The Small Meromictic (permanently stratified) Basin (SMB) in Ellis Fjord, Vestfold Hills, Antarctica, which was previously shown to contain fossil diatom DNA, was revisited to reconstruct the succession of dinoflagellates using a multiproxy approach (i.e., DNA, dinocysts, and sterols). PCR-amplification of partial (500-bp-long) dinoflagellate 18S rDNA, denaturing gradient gel electrophoresis (DGGE), and Sanger sequencing of individual DGGE bands, revealed the succession of several dinoflagellate species during the late-Holocene development of the SMB, whereas dinocysts were rare and could thus not be used to reconstruct the history of the basin. The fossil DNA-based dinoflagellate paleocommunity revealed a community shift at ∼1850 cal. yr BP, which coincided with a decrease in dinosterol since that period. PCR/DGGE analysis identified the sea-ice dinoflagellate *Polarella glacialis* as the most abundant species after ∼1850 cal.yr BP. Since *P. glacialis* is not a source of dinosterol, the analysis of dinosterol alone does

not provide an accurate view on the actual input of dinoflagellate biomass in the past. Despite the excellent preservation of other organic matter, the organic-walled resting cysts of *P. glacialis* were not discovered in the analyzed sediments, making fossil DNA the most informative qualitative marker in this setting.

Fossil DNA stratigraphy also revealed a constant presence of anoxygenic photosynthetic green sulfur bacteria (GSB), indicative of permanent water column stratification with anoxic bottom waters and a sulfidic chemocline extending up into the photic zone in the Ellis Fjord basin during at least the last ∼2700 years. PCR/DGGE selective for partial 16S rDNA of GSB revealed the continuous presence of single GSB, related to *Chlorobium phaeovibrioides*, as the source of fossil carotenoids.

Since the planktonic community and the environmental conditions were relatively constant for the last 2700 years, the sediment record of the SMB in Ellis Fjord offered a unique opportunity to study species-specific variations in the level of DNA preservation through time. First, the number of preserved ∼500-bp-long SSU rRNA gene fragments per gram C_{org} was determined by means of quantitative PCR. This approach revealed that the amount of PCR-amplifiable fossil dinoflagellate 18S rDNA (500 base pairs; bp), predominated by *Polarella glacialis*, decreased up to 5 orders of magnitude within ∼2700 years of deposition. In contrast, similar-sized PCR-amplifiable diatom 18S rDNA (predominantly from a cyst-forming *Chaetoceros* sp.) decreased in abundance only one order of magnitude within this time frame. The amount of fossil 16S rDNA of *Chlorobium phaeovibrioides* varied by up to two orders of magnitude between samples, but showed no obvious decreasing trend during the 2700 years of deposition.

In agreement with the quantitative data, the level of post-depositional natural fragmentation into lower molecular weight size-classes (<2.2 kilobases) was found to be highest for dinoflagellates and lowest for GSB. Assuming unaltered continuous degradation rates, most of the fossil DNA of the investigated taxa in this environment would be degraded within ∼12 to ∼30 kyr.

The differences in the preservation efficiency between 18S rDNA derived from *Chaetoceros* (known to be present as cysts in the SMB sediment record) and *Polarella* (no preserved cysts) can be best explained by the differences in their cellular architecture. On the other hand, *Chlorobium* spp. are not known to form protective resting stages and further studies are required to test why *Chlorobium* DNA is so well preserved in the sedimentary record. For example, can these green sulfur bacteria maintain cell integrity and remain viable for hundreds or even thousands of years in the absence of light?

Most paleogenetic studies including those reported in this thesis were performed using sediments of cold and C_{org}-rich laminated sediments of lakes and fjords (e.g., British Columbia, Antarctica, Greenland) that were deposited in the permanent presence of bottom water anoxia. Moreover, all these environments were shallow with relatively short residence time of dead and decaying cells in the water column before the cells and their DNA content became sequestered in the underlying sediment record. In this thesis, fossil DNA of eukaryotes and that of haptophyte algae in particular, was analyzed from Holocene sediments of the world's largest permanently stratified

and anoxic basin, the Black Sea with depths exceeding 2000 m at the depositional centre. A core with sediments deposited during the last ∼3600 years was obtained from the depositional centre of the eastern basin of the Black Sea for fossil DNA and lipid analysis. Domain and group-specific qPCR and phylogenetic analysis of PCR amplified and DGGE-separated partial 18S rDNA revealed that although haptophytes presented a small portion of the total DNA pool, fossil DNA of *Emiliania huxleyi*, was successfully identified in the Black Sea from sediments up to ∼3600 year old. A considerable part of the fossil haptophyte DNA in the Black Sea sediments was shown to be present at relatively high molecular weight DNA (>23 kilobases), indicative that a significant amount of DNA from photosynthetic algae became buried in the deep-sea sediments and was well preserved. Whereas also non-calcifying species of haptophytes can be sources of haptophytes, the paleogenetics approach revealed that *E. huxleyi* was the only source of long-chain alkenones in the Black Sea during the last ∼3600 years. The alkenone unsaturation index is widely being used as a proxy for sea surface temperatures (SST), but the calibration of this paleothermometer is dependent on the source species. This work, therefore, confirmed that the SST-calibration based on *E. huxleyi* can be applied during that period in the Black Sea.

Eastern Mediterranean sapropels are dark sedimentary units with C_{org} contents of >2 wt% that occur intercalated within C_{org}-poor (∼0.2 wt%) pelagic-hemipelagic carbonate oozes, and represented a potentially rich, but largely untapped archive for the application of paleogenetics. These C_{org}-rich sedimentary intervals have formed repeatedly in the eastern Mediterranean Sea, in response to precession cycle-triggered maxima in northern Hemisphere solar radiation and intensified African monsoons. Whether fossil DNA of ancient planktonic protists could be recovered from the youngest sapropel ($9.8 - 5.7$ ^{14}C kyr BP) was determined from a piston core obtained at a water depth of 1630 m near the Nile fan. Sequencing analysis of preserved ∼500-bp-long 18S rRNA gene fragments in S1 sediments revealed a predominance of dinoflagellate phylotypes, which were also detected in the anoxic Black Sea sediments, below ∼8 ^{14}C kyr BP. In the same section of the core, the most abundant haptophyte 18S rRNA gene sequence showed highest sequence similarity with uncultivated haptophytes previously shown to grow mixotrophically as predators of picocyanobacteria. Predatory consumption of picocyanobacteria could have been an adaptation of this previously overlooked haptophyte to thrive in an oligotrophic photic zone, which was separated from deep nutrient-rich waters as a result of water column stratification during early S1 deposition. Such a paleogenetics-inferred scenario is in agreement with prior nannofossil-based paleoecological studies, which argued for the presence of an ecological depth-separation of the water column, characterized by higher nutrient availability at depth and nutrient-depleted surface waters during early to mid S1 formation. A sequence closely related to *Emiliania huxleyi* was identified, but none of the identified sequences was identical to less abundant haptophyte species present in the calcareous nannofossil assemblage. Low concentrations of lipid biomarkers diagnostic for dinoflagellates and haptophytes (i.e., dinosterol and long-chain alkenones) were detected in the C_{org}-depleted marls flanking the S1, but no detectable 500-bp-long fossil rDNA fragments of these protists was found. This indicates that paleogenetics

of the relatively long DNA fragments is restricted to the C_{org}-rich S1 sediments.

The presence of fossil eukaryote DNA was also investigated in older sapropels (S3, S4 and S5; with ages of ∼80, ∼105 and ∼125 kyr BP, respectively) as a first attempt to study if fossil DNA of ancient planktonic protists could be recovered from marine sediments beyond the Holocene. Eukaryote DNA was detected in all sapropel layers, but identification of the discovered phylotypes showed that fossil DNA of protists unambiguously derived from the photic zone (including several species alveolates and dinoflagellates as most numerous representatives) was largely restricted to the Holocene S1 sapropel. Instead, marine fungi and terrestrial plants, comprised the majority of PCR-amplified 18S rDNA using general primers targeting all eukaryotes. Most likely, DNA of terrestrial vegetation that was introduced via the river Nile is better preserved than DNA of planktonic protists since a parallel analysis of Branched vs. Isoprenoid Tetraether (BIT)-index showed that the majority of the OM in the sapropels was of marine instead of terrestrial origin. In addition, despite a large abundance of long-chain alkenones and dinosterol, ∼500-bp-long 18S rDNA amplicons of haptophytes and dinoflagellates were below the detection limit in the late-Pleistocene sapropels, indicating that the fossil DNA of these groups was degraded, but that the lipid biomarkers were still well preserved in the deeper sapropel layers.

In summary, the studies reported in this thesis revealed that despite species-specific variation in the level of preservation, the use of fossil DNA is a valuable tool to qualitatively reconstruct the Holocene plankton succession including non-fossilizing environmental indicator species that would escape identification using more traditional paleoecological approaches. Fossil planktonic DNA stratigraphy seem to be most suitable in Holocene C_{org}-rich sediments that were deposited during periods of restricted bottom water ventilation and/or increased export of primary produced organic matter, since older comparable sediments mainly contained marine fungal DNA or DNA of terrestrial plants introduced from terrestrial catchment areas. Furthermore, sediments that experienced long-term exposure to oxidative mineralization of OM appear to be unsuitable for fossil DNA studies.

Outlook

Rapid developments in molecular biological techniques will provide new advances for paleogenetics, most notably in the emerging field of next generation sequencing. Pyrosequencing of shorter (e.g., 100-bp-long) PCR amplified hypervariable regions of suitable phylogenetic markers could lower the detection limit of degraded ancient DNA in sediments, and may push the age limits of successful paleogenetic analysis further back in time. Currently, the SSU RNA gene is often used for classification, for a number of reasons (e.g., conserved regions for primer design, wide occurrence among species and access to a large public rRNA gene database for data comparison). Although the rDNA indeed reflects phylogenetic diversity, it often does not reveal the metabolic diversity of organisms. Future paleogenetic approaches should also target functional genes that both serve as phylogenetic markers and as metabolic markers

to reconstruct species involved in ancient biogeochemical cycling processes or those species that carried the genetic machinery to biosynthesize certain lipids.

When comparing fossil DNA to other approaches, such as microfossil analysis, a mismatch between both approaches was observed. Many species known from microfossil assemblages are now extinct, or, even when these species are living in the water column, they have not yet been cultured. Also, in the case of dinoflagellates, the cyst-theca relationships are not always known, and a direct coupling between fossil cyst and extant species can therefore be difficult. For all such species, no rDNA sequences are available for comparison and, therefore, no direct comparison can be made from combined paleogenetic and microfossil results. Future focus on culturing species that are important in paleoceanography (e.g., coccolithophores such as *Florisphaera profunda*) will hopefully improve such comparison.

This last observation highlights once again that paleogenetics can best be used in conjunction with other scientific disciplines, either to provide reciprocal confirmation of results or to provide complementary data. When incorporated in multidisciplinary studies, the analysis of fossil DNA has already shown great potential to help reconstruct paleoenvironmental conditions, and will likely continue to do so as our knowledge about fossil DNA continues to grow. As a result, DNA will continue to have an impact on geology (and the earth sciences in general) in the 21st century, just as it changed the field of biology in the previous century.

References

References

Achenbach, L.A., J. Carey and M.T. Madigan. Photosynthetic and phylogenetic primers for detection of anoxygenic phototrophs in natural environments. *Applied and Environmental Microbiology*, vol. 67(7): 2922–2926, 2001.

Alam, M., R. Sanduja, D.A. Watson and A.R. Loeblich. Sterol distribution in the genus *Heterocapsa* (Pyrrhophyta). *Journal of Phycology*, vol. 20(3): 331–335, 1984.

Alexander, E., A. Stock, H.W. Breiner, A. Behnke, J. Bunge, M.M. Yakimov and T. Stoeck. Microbial eukaryotes in the hypersaline anoxic L'Atalante deep-sea basin. *Environmental Microbiology*, vol. 11(2): 360–381, 2009.

Alfaro, M.E., S. Zoller and F. Lutzoni. Bayes or bootstrap? A simulation study comparing the performance of Bayesian Markov chain Monte Carlo sampling and bootstrapping in assessing phylogenetic confidence. *Mol.Biol.Evol.*, vol. 20(2): 255–266, 2003.

Amann, R.I., B.J. Binder, R.J. Olson, S.W. Chisholm, R. Devereux and D.A. Stahl. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Applied and Environmental Microbiology*, vol. 56(6): 1919–1925, 1990.

Amaral-Zettler, L.A., E.A. McCliment, H.W. Ducklow and S.M. Huse. A method for studying protistan diversity using massively parallel sequencing of V9 hypervariable regions of small-subunit ribosomal RNA genes. *Plos One*, vol. 4: e6372, 2009, doi: 10.1371/journal.pone.0006372.

Anderson, D.M., C.D. Taylor and E.V. Armbrust. The effects of darkness and anaerobiosis on dinoflagellate cyst germination. *Limnology and Oceanography*, vol. 32(2): 340–351, 1987.

Andreoli, C., C. Tolomio, I. Moro, M. Radice, E. Moschin and S. Bellato. Diatoms and dinoflagellates in Terra-Nova Bay (Ross Sea-Antarctica) during austral summer 1990. *Polar Biology*, vol. 15(7): 465–475, 1995.

Arenovski, A.L., E.L. Lim and D.A. Caron. Mixotrophic nanoplankton in oligotrophic surface waters of the Sargasso Sea may employ phagotrophy to obtain major nutrients. *Journal of Plankton Research*, vol. 17(4): 801–820, 1995.

Arthur, M.A., W.A. Dean, E.D. Neff, B.J. Hay, J. King and G. Jones. Varve calibration records of carbonate and organic carbon accumulation over the last 2000 years in the Black Sea. *Global Biogeochemal Cycles*, vol. 8(2): 195–217, 1994.

Arthur, M.A. and W.E. Dean. Organic-matter production and preservation and evolution of anoxia in the Holocene Black Sea. *Paleoceanography*, vol. 13(4): 395–411, 1998.

Auel, H. and H.M. Verheye. Hypoxia tolerance in the copepod *Calanoides carinatus* and the effect of an intermediate oxygen minimum layer on copepod vertical distribution in the northern Benguela Current upwelling system and the Angola-Benguela Front. *Journal of Experimental Marine Biology and Ecology*, vol. 352(1): 234–243, 2007.

Bass, D., A. Howe, N. Brown, H. Barton, M. Demidova, H. Michelle, L. Li, H. Sanders, S.C. Watkinson, S. Willcock and T.A. Richards. Yeast forms dominate fungal diversity in the deep oceans. *Proceedings of the Royal Society London B: Biological Sciences*, vol. 274(1629): 3069–3077, 2007.

Basso, D., J. Thomson and C. Corselli. Indications of low macrobenthic activity in the deep sediments of the eastern Mediterranean Sea. *Scientia Marina*, vol. 68: 53–62, 2004.

Behnke, A., J. Bunge, K. Barger, H.-W. Breiner, V. Alla and T. Stoeck. Microeukaryote community patterns along an O_2/H_2S gradient in a supersulfidic anoxic fjord (Framvaren, Norway). *Applied and Environmental Microbiology*, vol. 72(5): 3626–3636, 2006.

Bendle, J. and A. Rosell-Melé. Distributions of U_{37}^{K} and $U_{37}^{K'}$ in the surface waters and sediments of the Nordic Seas: Implications for paleoceanography. *Geochemistry, Geophysics, Geosystems*, vol. 5(11): Q11013, 2004, doi:10.1029/2004GC000741.

Bennett, K.D. and L. Parducci. DNA from pollen: principles and potential. *Holocene*, vol. 16(8): 1031–1034, 2006.

Benson, D.A., I. Karsch-Mizrachi, D.J. Lipman, J. Ostell and D.L. Wheeler. GenBank: update. *Nucleic Acids Research*, vol. 32 Database issue: D23–D26, 2004.

Bethoux, J.P. Mediterranean sapropel formation, dynamic and climatic viewpoints. *Oceanologica Acta*, vol. 16(2): 127–133, 1993.

Bird, M.I., A.R. Chivas, C.J. Radnell and H.R. Burton. Sedimentological and stable-isotope evolution of lakes in the Vestfold Hills, Antarctica. *Palaeogeography, Palaeoclimatology, Palaeoecology*, vol. 84(1-4): 109–130, 1991.

Bissett, A., J.A.E. Gibson, S.N. Jarman, K.M. Swadling and L. Cromer. Isolation, amplification, and identification of ancient copepod DNA from lake sediments. *Limnology and Oceanography: Methods*, vol. 3: 533–542, 2005.

Boere, A.C., B. Abbas, W.I.C. Rijpstra, G.J.M. Versteegh, J.K. Volkman, J.S Sinninghe Damsté and M.J.L. Coolen. Late-Holocene succession of dinoflagellates in an Antarctic fjord using a multi-proxy approach: paleoenvironmental genomics, lipid biomarkers and palynomorphs. *Geobiology*, vol. 7(3): 265–281, 2009.

Boon, J.J., W.I.C. Rijpstra, F. de Lange, J.W. de Leeuw, M. Yoshioka and Y. Shimizu. Black sea sterol- a molecular fossil for dinoflagellate blooms. *Nature*, vol. 277: 125–127, 1979.

Boreson, J., A.M. Dillner and J. Peccia. Correlating bioaerosol load with PM2.5 and PM10cf concentrations: a comparison between natural desert and urban-fringe aerosols. *Atmospheric Environment*, vol. 38(35): 6029–6041, 2004.

Borin, S., E. Crotti, F. Mapelli, I. Tamagnini, C. Corselli and D. Daffonchio. DNA is preserved and maintains transforming potential after contact with brines of the deep anoxic hypersaline lakes of the Eastern Mediterranean Sea. *Saline Systems*, vol. 4(10): doi:10.1186/1746–1448–4–10, 2008.

Bowman, J.P., S.M. Rea, S.A. McCammon and T.A. McMeekin. Diversity and community structure within anoxic sediment from marine salinity meromictic lakes and a coastal meromictic marine basin, Vestfold Hills, Eastern Antarctica. *Environmental Microbiology*, vol. 2(2): 227–237, 2000.

Brassell, S.C., Applications of biomarkers for delineating marine paleoclimatic fluctuations during the Pleistocene. In: *Organic Geochemistry. Principles and Applications* (eds. M.H. Engel and S.A. Macko), 34, pp. 699–738, Plenum Press, New York-London, 1993.

Brassell, S.C., G. Eglinton, I.T. Marlowe, U. Pflaumann and M. Sarnthein. Molecular stratigraphy: a new tool for climatic assessment. *Nature*, vol. 320(6058): 129–133, 1986.

Britton, G., S. Liaanen-Jensen and H. Pfander, *UV/Visible Spectroscopy*. Birkhauser Verlag, Basel, 1995.

Brocks, J.J., G.D. Love, R.E. Summons, A.H. Knoll, G.A. Logan and S.A. Bowden. Biomarker evidence for green and purple sulphur bacteria in a stratified Palaeoproterozoic sea. *Nature*, vol. 437(7060): 866–870, 2005.

Brocks, J.J. and J. Banfield. Unravelling ancient microbial history with community proteogenomics and lipid geochemistry. *Nature Reviews Microbiology*, vol. 7: 601–609, 2009.

Bucefalo Palliani, R.B. and J.B. Riding. *Umbriadinium* and *Polarella*: an example of selectivity in the dinoflagellate fossil record. *Grana*, vol. 42(2): 108–111, 2003.

Buck, K.R., P.A. Bolt, W.N. Bentham and D.L. Garrison. A dinoflagellate cyst from Antarctic Sea ice. *Journal of Phycology*, vol. 28(1): 15–18, 1992.

Burgaud, G., T. Le Calvez, D. Arzur, P. Vanderkoornhuyse and G. Barbier. Diversity of culturable marine filamentous fungi from deep-sea hydrothermal vents. *Environmental Microbiology*, vol. 11(6): 1588–1600, 2009.

Calvert, S.E. Geochemistry of Pleistocene sapropels and associated sediments from the Eastern Mediterranean. *Oceanol.Acta*, vol. 6(3): 255–267, 1983.

Calvert, S.E., J.S. Vogel and J.R. Southon. Carbon accumulation rates and the origin of the Holocene sapropel in the Black Sea. *Geology*, vol. 15: 918–921, 1987.

Calvert, S.E. and R.E. Karlin. Organic carbon accumulation in the Holocene sapropel of the Black Sea. *Geology*, vol. 26(2): 107–110, 1998.

Cameron, C.B., J.R. Garey and B.J. Swalla. Evolution of the chordate body plan: New insights from phylogenetic analyses of deuterostome phyla. *Proceedings of the National Academy of Sciences*, vol. 97(9): 4469–4474, 2000.

Cano, R.J. and M.K. Borucki. Revival and identification of bacterial spores in 25-Million-year- old to 40-Million-year-old Dominican amber. *Science*, vol. 268(5213): 1060–1064, 1995.

Casford, J.S.L., E.J. Rohling, R.H. Abu-Zied, C. Fontanier, F.J. Jorissen, M.J. Leng, G. Schmiedl and J. Thomson. A dynamic concept for eastern Mediterranean circulation and oxygenation during sapropel formation. *Palaeogeography, Palaeoclimatology, Palaeoecology*, vol. 190: 103–119, 2003.

Castradori, D. Calcareous nannofossils and the origin of eastern Mediterranean sapropels. *Paleoceanography*, vol. 8(4): 459–471, 1993.

Cheddadi, R. and M. Rossignol-Strick. Eastern Mediterranean Quaternary paleoclimates from pollen and isotope records of marine cores in the Nile cone area. *Paleoceanography*, vol. 10(2): 291–300, 1995a.

Cheddadi, R. and M. Rossignol-Strick. Improved preservation of organic matter and pollen in Eastern Mediterranean sapropels. *Paleoceanography*, vol. 10(2): 301–309, 1995b.

Conte, M.H., J.C. Weber, L.L. King and S.G. Wakeham. The alkenone temperature signal in western North Atlantic surface waters. *Geochimica et Cosmochimica Acta*, vol. 65(23): 4275–4287, 2001.

Coolen, M.J.L. and J. Overmann. Analysis of subfossil molecular remains of purple sulfur bacteria in a lake sediment. *Applied and Environmental Microbiology*, vol. 64(11): 4513–4521, 1998.

Coolen, M.J.L., H. Cypionka, A.M. Sass, H. Sass and J. Overmann. Ongoing modification of Mediterranean Pleistocene sapropels mediated by prokaryotes. *Science*, vol. 296(5577): 2407–2410, 2002.

Coolen, M.J.L., E.C. Hopmans, W.I.C. Rijpstra, G. Muyzer, S. Schouten, J.K. Volkman and J.S. Sinninghe Damsté. Evolution of the methane cycle in Ace Lake (Antarctica) during the Holocene: Response of methanogens and methanotrophs to environmental change. *Organic Geochemistry*, vol. 35(10): 1151–1167, 2004a.

Coolen, M.J.L., G. Muyzer, W.I.C. Rijpstra, S. Schouten, J.K. Volkman and J.S. Sinninghe Damsté. Combined DNA and lipid analyses of sediments reveal changes in Holocene haptophyte and diatom populations in an Antarctic lake. *Earth and Planetary Science Letters*, vol. 223: 225–239, 2004b.

Coolen, M.J.L., A.C. Boere, B. Abbas, M. Baas, S.G. Wakeham and J.S. Sinninghe Damsté. Ancient DNA derived from alkenone-biosynthesizing haptophytes and other algae in Holocene sediments from the Black Sea. *Paleoceanography*, vol. 21(1): PA1005, 2006a, doi:10.1029/2005PA001188.

Coolen, M.J.L., G. Muyzer, S. Schouten, J.K. Volkman and J.S. Sinninghe Damsté, Sulfur and methane cycling during the Holocene in Ace Lake (Antarctica) revealed by lipid and DNA stratigraphy. In: *Nato Science Series: IV: Earth and Environmental Sciences*, pp. 41–65, Springer Netherlands, Dordrecht, 2006b.

Coolen, M.J.L. and J. Overmann. 217 000-year-old DNA sequences of green sulfur bacteria in Mediterranean sapropels and their implications for the reconstruction of the paleoenvironment. *Environmental Microbiology*, vol. 9(1): 238–249, 2007.

Coolen, M.J.L., J.K. Volkman, B. Abbas, G. Muyzer, S. Schouten and J.S. Sinninghe Damsté. Identification of organic matter sources in sulfidic late Holocene Antarctic fjord sediments from fossil rDNA sequence analysis. *Paleoceanography*, vol. 22: pa2211, 2007, doi:10.1029/2006PA001309.

Coolen, M.J.L., H.M. Talbot, B.A. Abbas, C. Ward, S. Schouten, J.K. Volkman and J.S Sinninghe Damsté. Sources for sedimentary bacteriohopanepolyols as revealed by 16S rDNA stratigraphy. *Environmental Microbiology*, vol. 10(7): 1783–1803, 2008.

Coolen, M.J.L., J.P. Saenz, L. Giosan, N.Y. Trowbridge, P. Dimitrov, D. Dimitrov and T.I. Eglinton. DNA and lipid molecular stratigraphic records of haptohyte succession in the Black Sea during the Holocene. *Earth and Planetary Science Letters*, vol. 284(3-4): 610–621, 2009.

Coolen, M.J.L. and G. Shtereva. Vertical distribution of metabolically active eukaryotes in the water column and sediments of the Black Sea. *FEMS Microbiology Ecology*, vol. 70(3): 525–539, 2009.

Corinaldesi, C., F. Beolchini and A. Dell'Anno. Damage and degradation rates of extracellular DNA in marine sediments: implications for the preservation of gene sequences. *Molecular Ecology*, vol. 17(17): 3939–3951, 2008.

Coyne, K.J. and S.C. Cary. Molecular approaches to the investigation of viable dinoflagellate cysts in natural sediments from estuarine environments. *Journal of Eukaryote Microbiology*, vol. 52(2): 90–94, 2005.

Cramp, A. and G. O'Sullivan. Neogene sapropels in the Mediterranean: a review. *Marine Geology*, vol. 153(1-4): 11–28, 1999.

Crudeli, D., J.R. Young, E. Erba, G.J. de Lange, K. Henriksen, H. Kinkel, C.P. Slomp and P. Ziveri. Abnormal carbonate diagenesis in Holocene-late Pleistocene sapropel-associated sediments from the Eastern Mediterranean; evidence from *Emiliania huxleyi* coccolith morphology. *Marine Micropaleontology*, vol. 52: 217–240, 2004.

Dale, B. The sedimentary record of dinoflagellate cysts: looking back into the future of phytoplankton blooms. *Scientia Marina*, vol. 65(Suppl.2.): 257–272, 2001.

Dale, B., C.M. Yentsch and J.W. Hurst. Toxicity in resting cysts of the red-tide dinoflagellate *Gonyaulax excavata* from deeper water coastal sediments. *Science*, vol. 201: 1223–1225, 1978.

Damare, S., S. Raghukumar and S. Raghukumar. Fungi in deep-sea sediments of the Central Indian Basin. *Deep-Sea Research Part I: Oceanographic Research Papers*, vol. 53(1): 14–27, 2006.

Damare, S. and C. Raghukumar. Fungi and macroaggregation in deep-sea sediments. *Microbial Ecology*, vol. 56: 168–177, 2008.

D'Andrea, W.J., M. Lage, J.B.H. Martiny, A.D. Laatsch, L.A. Amaral-Zettler, M.L. Sogin and Y. Huang. Alkenone producers inferred from well-preserved 18S rDNA in Greenland lake sediments. *Journal of Geophysical Research*, vol. 111: G03013, 2006, doi:10.1029/2005JG000121.

Danovaro, R., C. Corinaldesi, A. Dell'Anno, M. Fabiano and C. Corselli. Viruses, prokaryotes and DNA in the sediments of a deep-hypersaline anoxic basin (DHAB) of the Mediterranean Sea. *Environmental Microbiology*, vol. 7(4): 586–592, 2005.

Danovaro, R., C. Corinaldesi, G.M. Luna and A. Dell'Anno, Marine Organic Matter: Biomarkers, Isotopes and DNA. In: *Hdb.Env.Chem.*, vol. 2N, chap. Molecular tools for analysis of DNA in marine environments, pp. 105–126, Springer, Berlin / Heidelberg, 2006.

Dawson, S.C. and N.R. Pace. Novel kingdom-level eukaryotic diversity in anoxic environments. *Proceedings of the National Academy of Sciences*, vol. 99(12): 8324–8329, 2002.

Dell'Anno, A., S. Bompadre and R. Danovaro. Quantification, base composition, and fate of extracellular DNA in marine sediments. *Limnology and Oceanography*, vol. 47(3): 899–905, 2002.

Dell'Anno, A. and R. Danovaro. Extracellular DNA plays a key role in deep-sea ecosystem functioning. *Science*, vol. 309(5744): 2179–2179, 2005.

Demanèche, S., L. Jocteur-Monrozier, H. Quiquampoix and P. Simonet. Evaluation of biological and physical protection against nuclease degradation of clay-bound plasmid DNA. *Applied and Environmental Microbiology*, vol. 67(1): 293–299, 2001.

D'Hondt, S., S. Rutherford and A.J. Spivack. Metabolic activity of subsurface life in deep-sea sediments. *Science*, vol. 295(5562): 2067–2070, 2002.

D'Hondt, S., B.B. Jørgensen, D.J. Miller, A. Batzke, R. Blake, B.A. Cragg, H. Cypionka, G.R. Dickens, T. Ferdelman, K.-U. Hinrichs, N.G. Holm, R. Mitterer, A. Spivack, G. Wang, B. Bekins, B. Engelen, K. Ford, G. Gettemy, S.D. Rutherford, H. Sass, C.G. Skilbeck, I.W. Aiello, G. Guèrin, C.H. House, F. Inagaki, P. Meister, T. Naehr, S. Niitsuma, R.J. Parkes, A. Schippers, D.C. Smith, A. Teske, J. Wiegel, C.N. Padilla and J.L.S. Acosta. Distributions of microbial activities in deep subseafloor sediments. *Science*, vol. 306(5705): 2216–2221, 2004.

Diéz, B., C. Pedrós-Alió, T.L. Marsh and R. Massana. Application of denaturing gradient gel electrophoresis (DGGE) to study the diversity of marine picoeukaryotic assemblages and comparison of DGGE with other molecular techniques. *Applied and Environmental Microbiology*, vol. 67(7): 2942–2951, 2001.

Edgcomb, V.P., D.T. Kysela, A. Teske, A.D. Gomez and M.L. Sogin. Benthic eukaryotic diversity in the Guaymas Basin hydrothermal vent environment. *Proceedings of the National Academy of Sciences*, vol. 99(11): 7658–7662, 2002.

Edgcomb, V.P., W. Orsi, C. Leslin, S.S. Epstein, J. Bunge, S. Jeon, M.M. Yakimov, A. Behnke and T. Stoeck. Protistan community patterns within the brine and halocline of deep hypersaline anoxic basins in the eastern Mediterranean Sea. *Extremophiles*, vol. 13(1): 151–167, 2009.

Edvardsen, B., W. Eikrem, J.C. Green, R.A. Andersen, SY Moon-van der Staay and L.K. Medlin. Phylogenetic reconstructions of the Haptophyta inferred from 18S ribosomal DNA sequences and available morphological data. *Phycologia*, vol. 39(1): 19–35, 2000.

Eglinton, G., S.A. Bradshaw, A. Rosell, M. Sarnthein, U. Pflaumann and R. Tiedemann. Molecular record of secular sea surface temperature changes on 100-year timescales for glacial terminations I, II and IV. *Nature*, vol. 356: 423 – 426, 1992.

Eker-Develi, E. and A.E. Kideys. Distribution of phytoplankton in the southern Black Sea in summer 1996, spring and autumn 1998. *Journal of Marine Systems*, vol. 39(3-4): 203–211, 2003.

Emeis, K.-C., H. Schulz, U. Struck, M. Rossignol-Strick, H. Erlenkeuser, M.W. Howell, D. Kroon, A. Mackensen, S. Ishizuka, T. Oba, T. Sakamoto and I. Koizumi. Eastern Mediterranean surface water temperatures and δ^{18}O composition during deposition of sapropels in the late Quaternary. *Paleoceanography*, vol. 18(1): 1005, 2003, doi: 10.1029/2000PA000617.

Englebrecht, A.C. and J.P. Sachs. Determination of sediment provenance at drift sites using hydrogen isotopes and unsaturation ratios in alkenones. *Geochimica et Cosmochimica Acta*, vol. 69(17): 4253–4265, 2005.

de la Espina, S.M.D., E. Alverca, A. Cuadrado and S. Franca. Organization of the genome and gene expression in a nuclear environment lacking histones and nucleosomes: the amazing dinoflagellates. *European Journal of Cell Biology*, vol. 84(2-3): 137–149, 2005.

Euringer, K. and T. Lueders. An optimised PCR/T-RFLP fingerprinting approach for the investigation of protistan communities in groundwater environments. *Journal Microbiological Methods*, vol. 75(2): 262–268, 2008.

Farrington, J.W., A.C. Davis, J. Sulanowski, M.A. McCaffrey, M. McCarthy, C.H. Clifford, P. Dickinson and J.K. Volkman. Biogeochemistry of lipids in surface sediments of the Peru upwelling area at 15°C. *Organic Geochemistry*, vol. 13(4-6): 607–617, 1988.

Fogel, G.B., C.R. Collins, J. Li and C.F. Brunk. Prokaryotic genome size and SSU rDNA copy number: Estimation of microbial relative abundance from a mixed population. *Microbial Ecology*, vol. 38(2): 93–113, 1999.

Frada, M., F. Not, I. Probert and C. de Vargas. $CaCO_3$ optical detection with fluorescent in situ hybridization: a new method to identify and quantify calcifying microorganisms from the oceans. *Journal of Phycology*, vol. 42: 1162–1169, 2006.

Frias-Lopez, J., A. Thompson, J. Waldbauer and S.W. Chisholm. Use of stable isotope-labelled cells to identify active grazers of picocyanobacteria in ocean surface waters. *Environmental Microbiology*, vol. 11(2): 512–525, 2009.

Gadanho, M. and J.P. Sampaio. Occurrence and diversity of yeasts in the Mid-Atlantic ridge hydrothermal fields near the Azores Archipelago. *Microbial Ecology*, vol. 50(3): 408–417, 2005.

Gallagher, J.B. and H.R. Burton. Seasonal mixing of Ellis Fjord, Vestfold Hills, East Antarctica. *Estuarine and Coastal Shelf Science*, vol. 27(4): 363–380, 1988.

Gallagher, J.B., H.R. Burton and G.E. Calf. Meromixis in an Antarctic fjord: a precursor to meromictic lakes on an isostatically rising coastline. *Hydrobiologia*, vol. 172: 235–254, 1989.

Gast, R.J., M.R. Dennett and D.A. Caron. Characterization of protistan assemblages in the Ross Sea, Antarctica, by denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology*, vol. 70(4): 2028–2037, 2004.

Gast, R.J., D.M. Moran, D.J. Beaudoin, J.N. Blythe, M.R. Dennett and D.A. Caron. Abundance of a novel dinoflagellate phylotype in the Ross Sea, Antarctica. *Journal of Phycology*, vol. 42(1): 233–242, 2006.

Gennari, G., F. Tamburini, D. Ariztegui, I. Hajdas and S. Spezzaferri. Geochemical evidence for high-resolution variations during deposition of the Holocene S1 sapropel on the Cretan Ridge, Eastern Mediterranean. *Palaeogeography, Palaeoclimatology, Palaeoecology*, vol. 273(3-4): 239–248, 2009.

Gibson, J.A.E. The meromictic lakes and stratified marine basins of the Vestfold Hills, East Antarctica. *Antarctic Science*, vol. 11(2): 175–192, 1999.

Giunta, S., A. Negri, C. Morigi, L. Capotondi, N. Combourieu-Nebout, K.C. Emeis, F. Sangiorgi and L. Vigliotti. Coccolithophorid ecostratigraphy and multi-proxy paleoceanographic reconstruction in the Southern Adriatic Sea during the last deglacial time (Core AD91-17). *Palaeogeography, Palaeoclimatology, Palaeoecology*, vol. 190: 39–59, 2003.

Godhe, A., M.E. Asplund, K. Harnstrom, V. Saravanan, A. Tyagi and I. Karunasagar. Quantification of diatom and dinoflagellate biomasses in coastal marine seawater samples by real-time PCR. *Applied and Environmental Microbiology*, vol. 74(23): 7174–7182, 2008.

Goni, M.A., M.P. Woodworth, H.L. Aceves, R.C. Thunell, E. Tappa, D. Black, F. Muller-Karger, Y. Astor and R. Varela. Generation, transport, and preservation of the alkenone-based U_{37}^{K} sea surface temperature index in the water column and sediments of the Cariaco Basin (Venezuela). *Global Biogeochem.Cy.*, vol. 18(2): 1–21, 2004.

Gottschling, M., S.S. Renner, K.J.S. Meier, H. Willems and H. Keupp. Timing deep divergence events in calcareous dinoflagellates. *Journal of Phycology*, vol. 44(2): 429–438, 2008.

Grey, J., J. Laybourn-Parry, R.J.G. Leakey and A. McMinn. Temporal patterns of protozooplankton abundance and their food in Ellis Fjord, Princess Elizabeth Land, eastern Antarctica. *Estuarine and Coastal Shelf Science*, vol. 45(1): 17–25, 1997.

Gugerli, F., L. Parducci and R.J. Petit. Ancient plant DNA: review and prospects. *New Phytologist*, vol. 166(2): 409–418, 2005.

Guillou, L., M. Viprey, A. Chambouvet, R.M. Welsh, A.R. Kirkham, R. Massana, D.J. Scanlan and A.Z. Worden. Widespread occurrence and genetic diversity of marine parasitoids belonging to *Syndiniales* (*Alveolata*). *Environmental Microbiology*, vol. 10(12): 3349–3365, 2008.

Hackett, J.D. and D. Bhattacharya, The genomes of dinoflagellates. In: *Genomics and Evolution of Microbial Eukaryotes*, pp. 48–63, Oxford University Press, New York, 2006.

Hagino, K., H. Okada and H. Matsuoka. Coccolithophore assemblages and morphotypes of *Emiliania huxleyi* in the boundary zone between the cold Oyashio and warm Kuroshio currents off the coast of Japan. *Marine Micropaleontology*, vol. 55(1-2): 19–47, 2005.

Hansen, A.J., D.L. Mitchell, C. Wiuf, L. Panikert, T.B. Brand, J. Binladen, D.A. Gilichinsky, R. Ronn and E. Willerslev. Crosslinks rather than strand breaks determine access to ancient DNA sequences from frozen sediments. *Genetics*, vol. 173(2): 1175–1179, 2006.

Harvey, H.R., S.A. Bradshaw, S.C.M. O'Hara, G. Eglinton and E.D.S. Corner. Lipid composition of the marine dinoflagellate *Scrippsiella trochoidea*. *Phytochemistry*, vol. 27(6): 1723–1729, 1988.

Hay, B.J. Sediment accumulation in the central western Black Sea over the past 5100 years. *Paleoceanography*, vol. 3(4): 491–508, 1988.

Hay, B.J., M.A. Arthur, W.A. Dean, E.D. Neff and S. Honjo. Sediment deposition in the Late Holocene abyssal Black Sea with climatic and chronological implications. *Deep-Sea Research I*, vol. 38(2): 1211–1235, 1991.

Hayes, J.M., K.F. Freeman, B.N. Popp and C.H. Hoham. Compound-specific isotopic analyses: A novel tool for reconstruction of ancient biogeochemical processes. *Organic Geochemistry*, vol. 16(4-6): 1115–1128, 1990.

Head, M.J, *Modern dinoflagellate cysts and their biological affinities.*, vol. Vol. 3. American Association of Stratigraphic Palynologists Foundation, Salt Lake City, Ut, 1996.

Hebsgaard, M.B., M.J. Phillips and E. Willerslev. Geologically ancient DNA: fact or artefact? *Trends in Microbiology*, vol. 13(5): 212–220, 2005.

Hedges, J.I. and R.G. Keil. Sedimentary organic matter preservation: an assessment and speculative synthesis. *Marine Chemistry*, vol. 49(2-3): 81–115, 1995.

Hoehler, T.M. Cretaceous Park? A commentary on microbial paleomics. *Astrobiology*, vol. 5(2): 95–99, 2005.

Hofreiter, M., V. Jaenicke, D. Serre, A. von Haeseler and S. Pbo. DNA sequences from multiple amplifications reveal artifacts induced by cytosine deamination in ancient DNA. *Nucleic Acids Research*, vol. 29(23): 4793–4799, 2001.

Hopmans, E.C., J.W.H. Weijers, E. Schefuß, L. Herfort, J.S. Sinninghe Damsté and S. Schouten. A novel proxy for terrestrial organic matter in sediments based on branched and isoprenoid tetraether lipids. *Earth and Planetary Science Letters*, vol. 224(1-2): 107–116, 2004.

Hou, Y.B. and S.J. Lin. Distinct gene number-genome size relationships for eukaryotes and non-eukaryotes: gene content estimation for dinoflagellate genomes. *Plos One*, vol. 4: e6978, 2009, doi:10.1371/journal.pone.0006978.

Huang, Y., B. Shuman, Y. Wang and T. Webb III. Hydrogen isotope ratios of palmitic acid in lacustrine sediments record late Quaternary climate variations. *Geology*, vol. 30(12): 1103–1106, 2002.

Huelsenbeck, J.P. and F. Ronquist. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*, vol. 17(8): 754–755, 2001.

Hurt, R.A., X. Qiu, L. Wu, Y. Roh, A.V. Palumbo, J.M. Tiedje and J. Zhou. Simultaneous recovery of RNA and DNA from soils and sediments. *Applied and Environmental Microbiology*, vol. 67(10): 4495–4503, 2001.

Ichinomiya, M., M. Honda, H. Shimoda, K. Saito, T. Odate, M. Fukuchi and A. Taniguchi. Structure of the summer under fast ice microbial community near Syowa Station, eastern Antarctica. *Polar Biology*, vol. 30: 1285–1293, 2007.

Imhoff, J.F. and V. Thiel. Phylogeny and taxonomy of *Chlorobiaceae*. *Photosynthesis Research*, vol. 104(2-3): 123–136, 2010, doi:10.1007/s11120-009-9510-7.

Inagaki, F., H. Okada, A.I. Tsapin and K.H. Nealson. The paleome: A sedimentary genetic record of past microbial communities. *Astrobiology*, vol. 5(2): 141–153, 2005.

Janse, I., J. Bok and G. Zwart. A simple remedy against artifactual double bands in denaturing gradient gel electrophoresis. *Journal Microbiological Methods*, vol. 57(2): 279–281, 2004.

Jeon, S.-O., T.-S. Ahn and S.-H. Hong. A novel archaeal group in the phylum Crenarchaeota found unexpectedly in an eukaryotic survey in the Cariaco Basin. *Journal of Microbiology*, vol. 46(1): 34–39, 2008.

Jeong, H.J., J.S. Kim, J.Y. Park, J.H. Kim, S. Kim, I. Lee, S.H. Lee, J.H. Ha and W.H. Yih. *Stoeckeria algicida* n. gen., n. sp. (Dinophyceae) from the coastal waters off Southern Korea: Morphology and small subunit ribosomal DNA gene sequence. *Journal of Eukaryote Microbiology*, vol. 52(4): 382–390, 2005.

Jones, G.A. and A.R. Gagnon. Radiocarbon chronology of Black Sea sediments. *Deep-Sea Research Part I-Oceanographic Research Papers*, vol. 41(3): 531–557, 1994.

Jørgensen, B.B. and A. Boetius. Feast and famine - microbial life in the deep-sea bed. *Nature Reviews Microbiology*, vol. 5(10): 770–781, 2007.

Kelman, Z. and M.A. Moran. Degradation of ancient DNA. *Current Biology*, vol. 6(3): 223–223, 1996.

Kemp, A.E.S., R.B. Pearce, I. Koizumi, J. Pike and S.J. Rance. The role of mat-forming diatoms in the formation of Mediterranean sapropels. *Nature*, vol. 398(6722): 57–61, 1999.

Khanna, M. and G. Stotzky. Transformation of *Bacillus subtilis* by DNA bound on montmorillonite and effect of DNase on the transforming ability of bound DNA. *Applied and Environmental Microbiology*, vol. 58(6): 1930–1939, 1992.

Kholeif, S.E.A. and P.J. Mudie. Palynological records of climate and oceanic conditions in the late Pleistocene and Holocene of the Nile cone, Southeastern Mediterranean, Egypt. *Palynology*, vol. 33: 1–24, 2009.

Kidd, R.B., M.B. Cita and W.B.F. Ryan. Stratigraphy of eastern Mediterranean sapropel sequences recovered during DSDP leg 42A and their paleoenvironmental significance. *Initial Reports of DSDP*, vol. 42: 421–443, 1978.

Klappenbach, J.A., P.R. Saxman, J.R. Cole and T.M. Schmidt. rrndb: the Ribosomal RNA operon copy number database. *Nucleic Acids Research*, vol. 29(1): 181–184, 2001.

Koizumi, Y., H. Kojima and M. Fukui. Dominant microbial composition and its vertical distribution in saline meromictic Lake Kaiike (Japan) as revealed by quantitative oligonucleotide probe membrane hybridization. *Applied and Environmental Microbiology*, vol. 70(8): 4930–4940, 2004.

Kok, M.D., W.I.C. Rijpstra, L. Robertson, J.K. Volkman and J.S. Sinninghe Damsté. Early steroid sulfurisation in surface sediments of a permanently stratified lake (Ace Lake, Antarctica). *Geochimica et Cosmochimica Acta*, vol. 64(8): 1425–1436, 2000.

Kokinos, J.P., T.I. Eglinton, M.A. Goi, J.J. Boon, P.A Martoglio and D.M. Anderson. Characterization of a highly resistant biomacromolecular material in the cell wall of a marine dinoflagellate resting cyst. *Organic Geochemistry*, vol. 28(5): 265–288, 1998.

Kolodziej, K. and T. Stoeck. Cellular identification of a novel uncultured marine stramenopile (MAST-12 clade) small-subunit rRNA gene sequence from a Norwegian estuary by use of fluorescence in situ hybridization-scanning electron microscopy. *Applied and Environmental Microbiology*, vol. 73(8): 2718–2726, 2007.

Kutty, S.N. and R. Philip. Marine yeasts - a review. *Yeast*, vol. 25(7): 465–483, 2008, doi:10.1002/yea.1599.

Kuypers, M.M.M., P. Blokker, J. Erbacher, H. Kinkel, R.D. Pancost, S. Schouten and J.S. Sinninghe Damsté. Massive expansion of marine archaea during a mid-Cretaceous oceanic anoxic event. *Science*, vol. 293(5527): 92–94, 2001.

LaJeunesse, T.C., G. Lambert, R.A. Andersen, M.A. Coffroth and D.W. Galbraith. *Symbiodinium* (Pyrrhophyta) genome sizes (DNA content) are smallest among dinoflagellates. *Journal of Phycology*, vol. 41(4): 880–886, 2005.

de Lange, G.J. and H.L. ten Haven. Recent sapropel formation in the eastern Mediterranean. *Nature*, vol. 305(5937): 797–798, 1983.

de Lange, G.J., J. Thomson, A. Reitz, C.P. Slomp, M.S. Principato, E. Erba and C. Corselli. Synchronous basin-wide formation and redox-controlled preservation of a Mediterranean sapropel. *Nature Geoscience*, vol. 1(9): 606–610, 2008.

Larsen, H. On the microbiology and biochemistry of the photosynthetic green sulfur bacteria. *Kongelige Norske Videnskabernes Selskabs Skrifter*, vol. 1: 1–185, 1953.

Laybourn-Parry, J. and D.A. Pearce. The biodiversity and ecology of Antarctic lakes: models for evolution. *Philosophical Transactions of the Royal Society B-Biological Sciences*, vol. 362(1488): 2273–2289, 2007.

Leblond, J.D. and P.J. Chapman. A survey of the sterol composition of the marine dinoflagellates *Karenia brevis*, *Karenia mikimotoi*, and *Karlodinium micrum* distribution of sterols within other members of the class Dinophyceae. *Journal of Phycology*, vol. 38(4): 670–682, 2002.

de Leeuw, J.W., G.J.M. Versteegh and P.F. van Bergen. Biomacromolecules of algae and plants and their fossil analogues. *Plant Ecology*, vol. 182(1-2): 209–233, 2006.

Lewis, J., A.S.D. Harris, K.J. Jones and R.L. Edmonds. Long-term survival of marine planktonic diatoms and dinoflagellates in stored sediment samples. *Journal of Plankton Research*, vol. 21(2): 343–354, 1999.

Lewis, K., S. Epstein, V.G. Godoy and S.-H. Hong. Intact DNA in ancient permafrost. *Trends in Microbiology*, vol. 16(3): 92–94, 2008.

Lindahl, T. Instability and decay of the primary structure of DNA. *Nature*, vol. 362(6422): 709–715, 1993.

López-García, P., H. Philippe, F. Gail and D. Moreira. Autochthonous eukaryotic diversity in hydrothermal sediment and experimental microcolonizers at the Mid-Atlantic Ridge. *Proceedings of the National Academy of Sciences*, vol. 100(2): 697–702, 2003.

Lorenz, M.G., B.W. Aardema and W.E. Krumbein. Interaction of marine sediment with DNA and DNA availability to nucleases. *Marine Biology*, vol. 64: 225–230, 1981.

Lorenz, M.G. and W. Wackernagel. Adsorption of DNA to sand and variable degradation rates of adsorbed DNA. *Applied and Environmental Microbiology*, vol. 53(12): 2948–2952, 1987.

Löwemark, L., Y. Lin, H.F. Chen, T.N. Yang, C. Beier, F. Werner, C.Y. Lee, S.R. Song and S.J. Kao. Sapropel burn-down and ichnological response to late Quaternary sapropel formation in two similar to 400 ky records from the eastern Mediterranean Sea. *Palaeogeography, Palaeoclimatology, Palaeoecology*, vol. 239(3-4): 406–425, 2006.

Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Förster, I. Brettske, S. Gerber, A.W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. König, T. Liss, R. Lüßmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode and K.-H. Schleifer. ARB: a software environment for sequence data. *Nucleic Acids Research*, vol. 32(4): 1363–1371, 2004.

Lydolph, M.C., J. Jacobsen, P. Arctander, M.T.P. Gilbert, D.A. Gilichinsky, A.J. Hansen, E. Willerslev and L. Lange. Beringian paleoecology inferred from permafrost-preserved fungal DNA. *Applied and Environmental Microbiology*, vol. 71(2): 1012–1017, 2005.

Manske, A.K., U. Henge, J. Glaeser and J. Overmann. Subfossil 16S rRNA gene sequences of green sulfur bacteria in the Black Sea and their implications for past photic zone anoxia. *Applied and Environmental Microbiology*, vol. 74(3): 624–632, 2008.

Mansour, M.P., J.K. Volkman, A.E. Jackson and S.I. Blackburn. The fatty acid and sterol composition of five marine dinoflagellates. *Journal of Phycology*, vol. 35(4): 710–720, 1999.

Mansour, M.P., J.K. Volkman and S.I. Blackburn. The effect of growth phase on the lipid class, fatty acid and sterol composition in the marine dinoflagellate *Gymnodinium* sp in batch culture. *Phytochemistry*, vol. 63(2): 145–153, 2003.

Marlowe, I.T., J.C. Green, A.C. Neal, S.C. Brassell, G. Eglinton and P.A. Course. Long-Chain (n-C_{37}-C_{39}) alkenones in the *Prymnesiophyceae*. Distribution of alkenones and other lipids and their taxonomic significance. *British Phycological Journal*, vol. 19(3): 203–216, 1984.

Marlowe, I.T., S.C. Brassell, G. Eglinton and J.C. Green. Long-Chain alkenones and alkyl alkenoates and the fossil coccolith record of marine sediments. *Chemical Geology*, vol. 88(3-4): 349–375, 1990.

Marret, F. and K.A.F. Zonneveld. Atlas of modern organic-walled dinoflagellate cyst distribution. *Review of Palaeobotany and Palynology*, vol. 125(1-2): 1–200, 2003.

McCaffrey, M.A., J.W. Farrington and D.J. Repeta. The organic geochemistry of Peru margin surface sediments: I. A comparison of the U_{37}^{K} alkenone and historical El Niño records. *Geochimica et Cosmochimica Acta*, vol. 54: 1671–1682, 1990.

McMinn, A. and D. Hodgson. Summer phytoplankton succession in Ellis Fjord, eastern Antarctica. *Journal of Plankton Research*, vol. 15(8): 925–938, 1993.

McMinn, A. Why are there no post-Paleogene dinoflagellate cysts in the Southern Ocean? *Micropaleontology*, vol. 41(4): 383–386, 1995.

McMinn, A. Preliminary investigation of the contribution of fast-ice algae to the spring phytoplankton bloom in Ellis Fjord, eastern Antarctica. *Polar Biology*, vol. 16(4): 301–307, 1996.

McMinn, A., N. Bleakley, K. Steinburner, D. Roberts and L. Trenerry. Effect of permanent sea ice cover and different nutrient regimes on the phytoplankton succession of fjords of the Vestfold Hills Oasis, eastern Antarctica. *J.Plankton Res.*, vol. 22(2): 287–303, 2000.

McMinn, A., H. Heijnis, K. Harle and G. McOrist. Late-Holocene climatic change recorded in sediment cores from Ellis Fjord, eastern Antarctica. *Holocene*, vol. 11(3): 291–300, 2001.

Medlin, L., H.J. Elwood, S. Stickel and M.L. Sogin. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene*, vol. 71(2): 491–499, 1988.

Medlin, L.K., A.G. Sáez and J.R. Young. A molecular clock for coccolithophores and implications for selectivity of phytoplankton extinctions across the K/T boundary. *Marine Micropaleontology*, vol. 67(1): 69–86, 2008.

Meier, K.J.S., K.A.F. Zonneveld, S. Kasten and H. Willems. Different nutrient sources forcing increased productivity during eastern Mediterranean S1 sapropel formation as reflected by calcareous dinoflagellate cysts. *Paleoceanography*, vol. 19(1): PA1012, 2004, doi:10.1029/2003PA000895.

Menzel, D., E.C. Hopmans, P.F. van Bergen, J.W. de Leeuw and J.S. Sinninghe Damsté. Development of photic zone euxinia in the eastern Mediterranean Basin during deposition of Pliocene sapropels. *Marine Geology*, vol. 189(3-4): 215–226, 2002.

Menzel, D., E.C. Hopmans, S. Schouten and J.S. Sinninghe Damsté. Membrane tetraether lipids of planktonic Crenarchaeota in Pliocene sapropels of the eastern Mediterranean Sea. *Palaeogeography, Palaeoclimatology, Palaeoecology*, vol. 239: 1–15, 2006.

Middelburg, J.J. A simple rate model for organic-matter decomposition in marine sediments. *Geochimica et Cosmochimica Acta*, vol. 53(7): 1577–1581, 1989.

Millar, C.D., L Huynen, S. Subramaniam, E. Mohandesan and D.M. Lambert. New developments in ancient genomics. *Trends.Ecol.Evol.*, vol. 23(7): 386–393, 2008.

Mitchell, D., E. Willerslev and A. Hansen. Damage and repair of ancient DNA. *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 571(1-2): 265–276, 2005.

Montresor, M., G. Procaccini and D.K. Stoecker. *Polarella glacialis*, gen. nov., sp. nov. (Dinophyceae): *Suessiaceae* are still alive! *Journal of Phycology*, vol. 35(1): 186–197, 1999.

Montresor, M., C. Lovejoy, L. Orsini, G. Procaccini and S. Roy. Bipolar distribution of the cyst-forming dinoflagellate *Polarella glacialis*. *Polar Biology*, vol. 26(3): 186–194, 2003.

Moodley, L., J.J. Middelburg, P.M.J. Herman, K. Soetaert and G.J. de Lange. Oxygenation and organic-matter preservation in marine sediments: Direct experimental evidence from ancient organic carbon-rich deposits. *Geology*, vol. 33(11): 889–892, 2005.

Mouradian, M., R.J. Panetta, A. de Vernal and Y. Glinas. Dinosterols or dinocysts to estimate dinoflagellate contributions to marine sedimentary organic matter? *Limnology and Oceanography*, vol. 52(6): 2569–2581, 2007.

Mudie, P.J., A.E. Aksu and D. Yasar. Late Quaternary dinoflagellate cysts from the Black, Marmara and Aegean seas: variations in assemblages, morphology and paleosalinity. *Marine Micropaleontology*, vol. 43(1-2): 155–178, 2001.

Mudie, P.J., A. Rochon, A.E. Aksu and H. Gillespie. Dinoflagellate cysts, freshwater algae and fungal spores as salinity indicators in Late Quaternary cores from Marmara and Black seas. *Marine Geology*, vol. 190(1-2): 203–231, 2002.

Mullis, K.B. and F.A. Faloona. Specific synthesis of DNA in vitro via a polymerase catalyzed chain reaction. *Methods in Enzymology*, vol. 155: 335–350, 1987.

Murat, A. and H. Got. Organic carbon variations of the eastern Mediterranean Holocene sapropel: a key for understanding formation processes. *Palaeogeography, Palaeoclimatology, Palaeoecology*, vol. 158(3-4): 241–257, 2000.

Muyzer, G., E.C. De Waal and A.G. Uitterlinden. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology*, vol. 59(3): 695–700, 1993.

Muyzer, G. and K. Smalla. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoeck International Journal General and Molecular Microbiology.*, vol. 73(1): 127–141, 1998.

Negri, A. and S. Giunta. Calcareous nannofossil paleoecology in the sapropel S1 of the eastern Ionian sea: paleoceanographic implications. *Palaeogeography, Palaeoclimatology, Palaeoecology*, vol. 169(1-2): 101–112, 2001.

Negri, A., C. Morigi and S. Giunta. Are productivity and stratification important to sapropel deposition? Microfossil evidence from late Pliocene insolation cycle 180 at Vrica, Calabria. *Palaeogeography, Palaeoclimatology, Palaeoecology*, vol. 190: 243–255, 2003.

Negri, A., A. Ferretti, T. Wagner and P.A. Meyers. Phanerozoic organic-carbon-rich marine sediments: Overview and future research challenges. *Palaeogeography Palaeoclimatology Palaeoecology*, vol. 273(3-4): 218–227, 2009.

Nguyen, T.H. and M. Elimelech. Adsorption of plasmid DNA to a natural organic matter-coated silica surface: Kinetics, conformation, and reversibility. *Langmuir*, vol. 23(6): 3273–3279, 2007.

Not, F., R. Gausling, F. Azam, J.F. Heidelberg and A.Z. Worden. Vertical distribution of picoeukaryotic diversity in the Sargasso Sea. *Environmental Microbiology*, vol. 9(5): 1233–1252, 2007.

van Os, B.J.H., L.J. Lourens, F.J. Hilgen, G.J. de Lange and L. Beaufort. The formation of Pliocene sapropels and carbonate cycles in the Mediterranean: Diagenesis, dilution, and productivity. *Paleoceanography*, vol. 9(4): 601–617, 1994.

Pääbo, S. Ancient DNA - extraction, characterization, molecular-cloning, and enzymatic amplification. *Proceedings of the National Academy of Sciences*, vol. 86(6): 1939–1943, 1989.

Paget, E., L.J. Monrozier and P. Simonet. Adsorption of DNA on clay-minerals: protection against DNase I and influence on gene transfer. *FEMS Microbiol.Lett.*, vol. 97(1-2): 31–39, 1992.

Panieri, G., S. Lugli, V. Manzi, M Roveri, B.C. Schreiber and K.A. Palinska. Ribosomal RNA gene fragments from fossilized cyanobacteria identified in primary gypsum from the late Miocene, Italy. *Geobiology*, vol. 8(2): 101–111, 2010.

Parducci, L., Y. Suyama, M. Lascoux and K.D. Bennett. Ancient DNA from pollen: a genetic record of population history in Scots pine. *Molecular Ecology*, vol. 14(9): 2873–2882, 2005.

Passier, H.F., J.J. Middelburg, B.J.H. van Os and G.J. de Lange. Diagenetic pyritisation under eastern Mediterranean sapropels caused by downward sulphide diffusion. *Geochimica et Cosmochimica Acta*, vol. 60(5): 751–763, 1996.

Passier, H.F., H.-J. Bosch, I.A. Nijenhuis, L.L. Lourens, M.E. Böttcher, A. Leenders, J.S. Sinninghe Damsté, G.J. de Lange and J.W. de Leeuw. Sulphidic Mediterranean surface waters during Pliocene sapropel formation. *Nature*, vol. 397(6715): 146–149, 1999.

Pearson, A., A.P. McNichol, B.C. Benitez-Nelson, J.M. Hayes and T.I. Eglinton. Origins of lipid biomarkers in Santa Monica Basin surface sediment: A case study using compound-specific δ^{14}C analysis. *Geochimica et Cosmochimica Acta*, vol. 65(18): 3123–3127, 2001.

van de Peer, Y., J.-M. Neefs, P. de Rijk and R. de Wachter. Reconstructing evolution from eukaryotic small ribosomal subunit RNA sequences - Calibration of the molecular clock. *Journal of Molecular Evolution*, vol. 37(2): 221–232, 1993.

Perriss, S.J. and J. Laybourn-Parry. Microbial communities in saline lakes of the Vestfold Hills (eastern Antarctica). *Polar Biology*, vol. 18(2): 135–144, 1997.

Peters, K.E., C.C. Walters and J.M. Moldowan, *The biomarker guide: Biomarkers and isotopes in petroleum exploration and earth history*, vol. 2nd. Cambridge University Press, New York, 2005.

Pfennig, N., Autotrophic Bacteria. In: *Autotrophic Bacteria* (eds. H.G. Schlegel and B. Bowien), 6, chap. Ecology of phototrophic Purple and Green Sulfur Bacteria, pp. 97–116, Springer, New York, 1989.

Pietramellara, G., J. Ascher, F. Borgogni, M.T. Ceccherini, G. Guerri and P. Nannipieri. Extracellular DNA in soil and sediment: fate and ecological relevance. *Biology and Fertility of Soils*, vol. 45(3): 219–235, 2009.

Piquet, A.M.T., H. Bolhuis, A.T. Davidson, P.G. Thomson and A.G.J. Buma. Diversity and dynamics of Antarctic marine microbial eukaryotes under manipulated environmental UV radiation. *FEMS Microbiology Ecology*, vol. 66(2): 352–366, 2008.

Poly, F., C. Chenu, P. Simonet, J. Rouiller and L.J. Monrozier. Differences between linear chromosomal and supercoiled plasmid DNA in their mechanisms and extent of adsorption on clay minerals. *Langmuir*, vol. 16(3): 1233–1238, 2000.

Posada, D. and K.A. Crandall. MODELTEST: testing the model of DNA substitution. *Bioinformatics*, vol. 14(9): 817–818, 1998.

Potvin, M. and C. Lovejoy. PCR-based diversity estimates of artificial and environmental 18S rRNA gene libraries. *Journal of Eukaryotic Microbiology*, vol. 56(2): 174–181, 2009.

Prahl, F.G. and S.G. Wakeham. Calibration of unsaturation patterns in long-chain ketone compositions for palaeotemperature assessment. *Nature*, vol. 330: 367–369, 1987.

Principato, M.S., S. Giunta, C. Corselli and A. Negri. Late Pleistocene-Holocene planktonic assemblages in three box-cores from the Mediterranean Ridge area (west-southwest of Crete): palaeoecological and palaeoceanographic reconstruction of sapropel S1 interval. *Palaeogeography, Palaeoclimatology, Palaeoecology*, vol. 190: 61–77, 2003.

Principato, M.S., D. Crudeli, P. Ziveri, C.P. Slomp, C. Corselli, E. Erba and G.J. de Lange. Phyto- and zooplankton paleofluxes during the deposition of sapropel S1 (eastern Mediterranean): Biogenic carbonate preservation and paleoecological implications. *Palaeogeography, Palaeoclimatology, Palaeoecology*, vol. 235(1-3): 8–27, 2006.

Probert, I., J. Fresnel, C. Billard, M. Geisen and J.R. Young. Light and electron microscope observations of *Algirosphaera robusta* (Prymnesiophyceae). *Journal of Phycology*, vol. 43(2): 319–332, 2007.

Pruesse, E., C. Quast, K. Knittel, B.M. Fuchs, W.G. Ludwig, J. Peplies and F.O. Glckner. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Research*, vol. 35(21): 7188–7196, 2007.

Quayle, W.C., L.S. Peck, H. Peat, J.C. Ellis-Evans and P.R. Harrigan. Extreme responses to climate change in Antarctic lakes. *Science*, vol. 295(5555): 645–645, 2002.

Raghukumar, C., S. Raghukumar, G. Sheelu, S.M. Gupta, B. Nagender Nath and B.R. Rao. Buried in time: culturable fungi in a deep-sea sediment core from the Chagos Trench, Indian Ocean. *Deep-Sea Research I*, vol. 51(11): 1759–1768, 2004.

Raven, J.A. Phagotrophy in phototrophs. *Limnology and Oceanography*, vol. 42(1): 198–205, 1997.

Reitz, A. and G.J. de Lange. Abundant Sr-rich aragonite in eastern Mediterranean sapropel S1: Diagenetic vs. detrital/biogenic origin. *Palaeogeography, Palaeoclimatology, Palaeoecology*, vol. 235(1-3): 135–148, 2006.

Reitz, A., J. Thomson, G.J. de Lange and C. Hensen. Source and development of large manganese enrichments above eastern Mediterranean sapropel S1. *Paleoceanography*, vol. 21(3): PA3007, 2006, doi:10.1029/2005PA001169.

Rengefors, K., J. Laybourn-Parry, R. Logares, W.A. Marshall and G. Hansen. Marine-derived dinoflagellates in Antarctic saline lakes: Community composition and annual dynamics. *Journal of Phycology*, vol. 44(3): 592–604, 2008.

Richards, T.A. and D. Bass. Molecular screening of free-living microbial eukaryotes: diversity and distribution using a meta-analysis. *Current Opinions in Microbiology*, vol. 8(3): 240–252, 2005.

de Rijk, S., A. Hayes and E.J. Rohling. Eastern Mediterranean sapropel S1 interruption: an expression of the onset of climate determination around 7 ka BP. *Marine Geology*, vol. 153: 337–343, 1999.

Rizzo, P.J., Biochemistry of the dinoflagellate nucleus. In: *Botanical Monographs, Vol.21*, pp. 143–171, Blackwell Scientific Publications, Oxford, 1987.

Roberts, D., A. McMinn, H. Cremer, D.B. Gore and M. Melles. The Holocene evolution and palaeosalinity history of Beall Lake, Windmill Islands (East Antarctica) using an expanded diatom-based weighted averaging model. *Palaeogeography, Palaeoclimatology, Palaeoecology*, vol. 208(1-2): 121–140, 2004.

Robinson, N., G. Eglinton, S.C. Brassell and P.A. Cranwell. Dinoflagellate origin for sedimentary 4α-methylsteroids and 5α(H)-stanols. *Nature*, vol. 308: 439–442, 1984.

Roether, W. and R. Well. Oxygen consumption in the Eastern Mediterranean. *Deep-Sea Research Part I-Oceanographic Research Papers*, vol. 48(6): 1535–1551, 2001.

Rohling, E.J. Late Quaternary changes in Mediterranean intermediate water density and formation rate. *Paleoceanography*, vol. 4(5): 531–545, 1989.

Rohling, E.J. Review and new aspects concerning the formation of eastern Mediterranean sapropels. *Marine Geology*, vol. 122(1-2): 1–28, 1994.

Rohling, E.J. and R.C. Thunell. Five decades of Mediterranean palaeoclimate and sapropel studies. *Marine Geology*, vol. 153(1-4): 7–10, 1999.

Romanowski, G., M.G. Lorenz and W. Wackernagel. Adsorption of plasmid DNA to mineral surfaces and protection against DNase I. *Applied and Environmental Microbiology*, vol. 57(4): 1057–1061, 1991.

Rontani, J.F., B. Beker and J.K. Volkman. Long-chain alkenones and related compounds in the benthic haptophyte *Chrysotila lamellosa* Anand HAP 17. *Phytochemistry*, vol. 65(1): 117–126, 2004.

Rosell-Melé, A. Interhemispheric appraisal of the value of alkenone indices as temperature and salinity proxies in high-latitude locations. *Paleoceanography*, vol. 13(6): 694–703, 1998.

Ross, D.A. and E.T. Degens. Recent sediments of Black Sea. *AAPG Bulletin*, vol. 20: 183–199, 1974.

Rossignol-Strick, M. The Holocene climatic optimum and pollen records of sapropel 1 in the eastern Mediterranean, 9000-6000 BP. *Quaternary Science Reviews*, vol. 18(4-5): 515–530, 1999.

Rothman, D.H. and D.C. Forney. Physical model for the decay and preservation of marine organic carbon. *Science*, vol. 316: 1325–1328, 2007.

Ryan, K.G., E.N. Hegseth, A. Martin, S.K. Davy, R. O'Toole, P.J. Ralph, A. McMinn and C.J. Thorn. Comparison of the microalgal community within fast ice at two sites along the Ross Sea coast, Antarctica. *Antarctic Science*, vol. 18(4): 583–594, 2006.

Sachs, J.P., R.R. Schneider, T.I. Eglinton, K.H. Freeman, G. Ganssen, J.F. McManus and D.W. Oppo. Alkenones as paleoceanographic proxies. *Geochemistry, Geophysics, Geosystems*, vol. 1: Paper number 2000GC000059, 2000.

Sáez, A.G., I. Probert, J.R. Young, B. Edvardsen, W. Eikrem and L.K. Medlin, Coccolithophores. From molecular processes to global impact. In: *Coccolithophores. From molecular processes to global impact.* (eds. H.R. Thierstein and J.R. Young), chap. A review of the phylogeny of the Haptophyta, pp. 251–269, Springer Verlag, Berlin, 2004.

Saldarriaga, J.F., F.J.R.M. Taylor, T. Cavalier-Smith, S. Menden-Deuer and P.J. Keeling. Molecular data and the evolutionary history of dinoflagellates. *European Journal of Protistology*, vol. 40(1): 85–111, 2004.

Sangiorgi, F., D. Fabbri, M. Comandini, G. Gabbianelli and E. Tagliavini. The distribution of sterols and organic-walled dinoflagellate cysts in surface sediments of the North-western Adriatic Sea (Italy). *Estuarine and Coastal Shelf Science*, vol. 64(2-3): 395–406, 2005.

Sauer, P.E., T.I. Eglinton, J.M. Hayes, A. Schimmelmann and A.L. Sessions. Compound-specific D/H ratios of lipid biomarkers from sediments as a proxy for environmental and climatic conditions. *Geochimica et Cosmochimica Acta*, vol. 65(2): 213–222, 2001.

Savin, M.C., J.L. Martin, M. LeGresley, M. Giewat and J. Rooney-Varga. Plankton diversity in the Bay of Fundy as measured by morphological and molecular methods. *Microbial Ecology*, vol. 48(1): 51–65, 2004.

Schelble, R.T., J.A. Hall, K.H. Nealson and A. Steele. DNA perseverance of microorganisms exposed to silica: an experimental study. *Geobiology*, vol. 6(5): 503–511, 2008.

Schippers, A., L.N. Neretin, J. Kallmeyer, T.G. Ferdelman, B.A. Cragg, R.J. Parkes and B.B. Jorgensen. Prokaryotic cells of the deep sub-seafloor biosphere identified as living bacteria. *Nature*, vol. 433(7028): 861–864, 2005.

Schippers, A. and L.N. Neretin. Quantification of microbial communities in near-surface and deeply buried marine sediments on the Peru continental margin using real-time PCR. *Environmental Microbiology*, vol. 8(7): 1251–1260, 2006.

Schloss, P.D. and J. Handelsman. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Applied and Environmental Microbiology*, vol. 71(3): 1501–1506, 2005.

Schlumbaum, A., M. Tensen and V. Jaenicke-Despres. Ancient plant DNA in archaeobotany. *Vegetation History and Archaeobotany*, vol. 17(2): 233–244, 2008.

Schouten, S., W.I.C. Rijpstra, M. Kok, E.C. Hopmans, R.E. Summons, J.K. Volkman and J.S. Sinninghe Damsté. Molecular organic tracers of biogeochemical processes in a saline meromictic lake (Ace Lake). *Geochimica et Cosmochimica Acta*, vol. 65(10): 1629–1640, 2001.

Setlow, P. Mechanisms for the prevention of damage to DNA in spores of *Bacillus* species. *Annual Reviews of Microbiology*, vol. 49: 29–54, 1995.

Sikes, E.L., T. O'Leary, S.D. Nodder and J.K. Volkman. Alkenone temperature records and biomarker flux at the subtropical front on the Chatham Rise, SW Pacific Ocean. *Deep-Sea Research I*, vol. 52: 721–748, 2005.

Sinninghe Damsté, J.S., S.G. Wakeham, M.E.L. Kohnen, J.M. Hayes and J.W. de Leeuw. A 6,000-year sedimentary molecular record of chemocline excursions in the Black Sea. *Nature*, vol. 362(6423): 827–829, 1993.

Sinninghe Damsté, J.S. and M.P. Koopmans. The fate of carotenoids in sediments: An overview. *Pure and Applied Chemistry*, vol. 69(10): 2067–2074, 1997.

Sinninghe Damsté, J.S., G. Muyzer, B. Abbas, S.W. Rampen, G. Masse, W.G. Allard, S.T. Belt, J.-M. Robert, S.J. Rowland, J.M. Moldowan, S.M. Barbanti, F.J. Fago, P. Denisevich, J. Dahl, L.A.F. Trindade and S. Schouten. The rise of the rhizosolenid diatoms. *Science*, vol. 304(5670): 584–587, 2004.

Sinninghe Damsté, J.S., W.I.C. Rijpstra, M.J.L. Coolen, S. Schouten and J.K. Volkman. Rapid sulfurisation of highly branched isoprenoid (HBI) alkenes in sulfidic Holocene sediments from Ellis Fjord, Antarctica. *Organic Geochemistry*, vol. 38: 128–139, 2007.

Sirevåg, R. and J.G. Ormerod. Synthesis, storage and degradation of polyglucose in *Chlorobium thiosulfatophilum*. *Archives of Microbiology*, vol. 111(3): 239–244, 1977.

Skovgaard, A. Infection with the dinoflagellate parasite *Blastodinium* spp. in two Mediterranean copepods. *Aquatic Microbial Ecology*, vol. 38(1): 93–101, 2005.

Skovgaard, A., R. Massana and E. Saiz. Parasitic species of the genus *Blastodinium* (Blastodiniphyceae) are peridinioid dinoflagellates. *Journal of Phycology*, vol. 43(3): 553–560, 2007.

Sluijs, A., J. Pross and H. Brinkhuis. From greenhouse to icehouse; organic-walled dinoflagellate cysts as paleoenvironmental indicators in the Paleogene. *Earth-Science Reviews*, vol. 68(3-4): 281–315, 2005.

Sogin, M.L., H.G. Morrison, J.A. Huber, D.M. Welch, S.M. Huse, P.R. Neal, J.M. Arrieta and G.J. Herndl. Microbial diversity in the deep sea and the underexplored "rare biosphere". *Proceedings of the National Academy of Sciences*, vol. 103(32): 12115–12120, 2006.

Stewart Johnson, Sarah, Martin B. Hebsgaard, Torben R. Christensen, Mikhail Mastepanov, Rasmus Nielsen, Kasper Munch, Tina Brand, M. Thomas P. Gilbert, Maria T. Zuber, Michael Bunce, Regin Rnn, David Gilichinsky, Duane Froese and Eske Willerslev. Ancient bacteria show evidence of DNA repair. *Proceedings of the National Academy of Sciences*, vol. 104(36): 14401–14405, 2007, doi: 10.1073/pnas.0706787104.

Stoeck, T. and S. Epstein. Novel eukaryotic lineages inferred from small-subunit rRNA analyses of oxygen-depleted marine environments. *Applied and Environmental Microbiology*, vol. 69(5): 2657–2663, 2003.

Stoeck, T., B. Hayward, G.T. Taylor, R. Varela and S.S. Epstein. A multiple PCR-primer approach to access the microeukaryotic diversity in environmental samples. *Protist*, vol. 157(1): 31–43, 2006.

Stoeck, T., A. Zuendorf, H.-W. Breiner and A. Behnke. A molecular approach to identify active microbes in environmental eukaryote clone libraries. *Microbial Ecology*, vol. 53(2): 328–339, 2007.

Stoecker, D.K. Mixotrophy among dinoflagellates. *Journal of Eukaryotic Microbiology*, vol. 46(4): 397–401, 1999.

Stoecker, D.K., D.E. Gustafson, C.T. Baier and M.M.D. Black. Primary production in the upper sea ice. *Aquatic Microbial Ecology*, vol. 21(3): 275–287, 2000.

Stoecker, D.K., D.E. Gustafson, M.M.D. Black and C.T. Baier. Population dynamics of microalgae in the upper land-fast sea ice at a snow-free location. *Journal of Phycology*, vol. 34(1): 60–69, 1998.

Stoecker, D.K., D.E. Gustafson, J.R. Merrell, M.M.D. Black and C.T. Baier. Excystment and growth of chrysophytes and dinoflagellates at low temperatures and high salinities in Antarctic sea-ice. *Journal of Phycology*, vol. 33(4): 585–595, 1997.

Summons, R.E. and T.G. Powell. *Chlorobiaceae* in Palaeozoic seas revealed by biological markers, isotopes and geology. *Nature*, vol. 319: 763–765, 1986.

Swofford, D.L., *PAUP* 4.0: Phylogenetic Analysis Using Parsimony (*and other methods).* Sinauer Associates, Sunderland, MA, 2003.

Takano, Y. and T. Horiguchi. Surface ultrastructure and molecular phylogenetics of four unarmored heterotrophic dinoflagellates, including the type species of the genus *Gyrodinium* (Dinophyceae). *Phycological Research*, vol. 52(2): 107–116, 2004.

Takeuchi, M., T. Komai, S. Hanada, H. Tamaki, S. Tanabe, Y. Miyachi, M. Uchiyama, T. Nakazawa, K. Kimura and Y. Kamagata. Bacterial and archaeal 16S rRNA genes in late Pleistocene to Holocene muddy sediments from the Kanto Plain of Japan. *Geomicrobiological Journal*, vol. 26(2): 104–118, 2009.

Takishita, K., H. Miyake, M. Kawato and T. Maruyama. Genetic diversity of microbial eukaryotes in anoxic sediment around fumaroles on a submarine caldera floor based on the small-subunit rDNA phylogeny. *Extremophiles*, vol. 9: 185–96, 2005.

Takishita, K., M. Tsuchiya, J.D. Reimer and T. Maruyama. Molecular evidence demonstrating the basidiomycetous fungus *Cryptococcus curvatus* is the dominant microbial eukaryote in sediment at the Kuroshima Knoll methane seep. *Extremophiles*, vol. 10(2): 165–169, 2006.

Takishita, K., M. Tsuchiya, M. Kawato, K. Oguri, H. Kitazato and T. Maruyama. Genetic diversity of microbial eukaryotes in anoxic sediment of the saline meromictic lake Namako-ike (Japan): On the detection of anaerobic or anoxic-tolerant lineages of eukaryotes. *Protist*, vol. 158(1): 51–64, 2007.

Tamura, K., J. Dudley, M. Nei and S. Kumar. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular and Biological Evolution*, vol. 24(8): 1596–1599, 2007.

Teske, A., Cellular origin, Life in extreme habitats and astrobiology. In: *Cellular origin, Life in extreme habitats and astrobiology*, chap. Enigmatic archaeal and eukaryotic Life at hydrothermal vents and in marine subsurface sediments, pp. 519–533, Springer, Netherlands, 2007.

Thomson, J., D. Crudeli, G.J. de Lange, C.P. Slomp, E. Erba, C. Corselli and S.E. Calvert. *Florisphaera profunda* and the origin and diagenesis of carbonate phases in eastern Mediterranean sapropel units. *Paleoceanography*, vol. 19(3): PA3003, 2004, doi:10.1029/2003PA000976.

Thomson, J., D. Mercone, G.J. de Lange and P.J.M. van Santvoort. Review of recent advances in the interpretation of eastern Mediterranean sapropel S1 from geochemical evidence. *Marine Geology*, vol. 153(1-4): 77–89, 1999.

Thomson, P.G., A. McMinn, I. Kiessling, M. Watson and P.M. Goldsworthy. Composition and succession of dinoflagellates and chrysophytes in the upper fast ice of Davis Station, East Antarctica. *Polar Biology*, vol. 29(4): 337–345, 2006.

Trull, T.W., J.A.E. Gibson, K.L. Beaumont, J. Bowman, H.R. Burton, A.T. Davidson, A. Guichard, J.M. Kirkwood, A. McMinn, P.D. Nichols, J.H. Skerratt, R.E. Summons, K.M. Swadling and J.K. Volkman. Site holds promise for ecosystem and biogeochemical investigations. *Eos*, pp. 305–307, 2001.

de Vernal, A., M. Henry, J. Matthiessen, P.J. Mudie, A. Rochon, K.P. Boessenkool, F. Eynaud, K. Grosfjeld, J. Guiot, D. Hamel, R. Harland, M.J. Head, M. Kunz-Pirrung, E. Levac, V. Loucheur, O. Peyron, V. Pospelova, T. Radi, J.L. Turon and E. Voronina. Dinoflagellate cyst assemblages as tracers of sea-surface conditions in the northern North Atlantic, Arctic and sub-Arctic seas: the new 'n=677' data base and its application for quantitative palaeoceanographic reconstruction. *Journal of Quarternary Science*, vol. 16(7): 681–698, 2001.

Versteegh, G.J.M., R. Riegman, J.W. de Leeuw and J.H.F. Jansen. $U_{37}^{K'}$ values for *Isochrysis galbana* as a function of culture temperature, light intensity and nutrient concentrations. *Organic Geochemistry*, vol. 32: 785–794, 2001.

Villanueva, Joan, Jose A. Flores and Joan O. Grimalt. A detailed comparison of the $U_{37}^{K'}$ and coccolith records over the past 290 kyears: implications to the alkenone paleotemperature method. *Organic Geochemistry*, vol. 33(8): 897–905, 2002.

Viprey, M., L. Guillou, M. Ferreol and D. Vaulot. Wide genetic diversity of picoplanktonic green algae (Chloroplastida) in the Mediterranean Sea uncovered by a phylum-biased PCR approach. *Environmental Microbiology*, vol. 10(7): 1804–1822, 2008.

Volkman, J.K., G. Eglinton, E.D.S. Corner and T.E.V. Forsberg. Long-chain alkenes and alkenones in the marine coccolithophorid *Emiliania huxleyi*. *Phytochemistry*, vol. 19: 2619–2622, 1980.

Volkman, J.K., S.M. Barrett, S.I. Blackburn and E.L. Sikes. Alkenones in *Gephyrocapsa oceanica*: Implications for studies of paleoclimate. *Geochimica et Cosmochimica Acta*, vol. 59(3): 513–520, 1995.

Volkman, J.K., S.M. Barrett, S.I. Blackburn, M.P. Mansour, E.L. Sikes and F. Gelin. Microalgal biomarkers: A review of recent research developments. *Organic Geochemistry*, vol. 29(5-7): 1163–1179, 1998.

Volkman, J.K., W.I.C. Rijpstra, J.W. de Leeuw, M.P. Mansour, A.E. Jackson and S.I. Blackburn. Sterols of four species of dinoflagellates from the genus *Prorocentrum*. *Phytochemistry*, vol. 52: 659–668, 1999.

Volkman, J.K. Sterols in microorganisms. *Applied Microbiology and Biotechnology*, vol. 60(5): 495–506, 2003.

Vreeland, R.H., W.D. Rosenzweig and D.W. Powers. Isolation of a 250 million-year-old halotolerant bacterium from a primary salt crystal. *Nature*, vol. 407: 897–900, 2000.

Vreeland, R.H., J. Jones, A. Monson, W.D. Rosenzweig, T.K. Lowenstein, M. Timofeeff, C. Satterfield, B.C. Cho, J.S. Park, A. Wallace and W.D. Grant. Isolation of live Cretaceous (121-112 million years old) halophilic Archaea from primary salt crystals. *Geomicrobiological Journal*, vol. 24(3-4): 275–282, 2007.

Wilkin, R.T., M.A. Arthur and W.A. Dean. History of water-column anoxia in the Black Sea indicated by pyrite framboid size distributions. *Earth and Planetary Science Letters*, vol. 148: 517–525, 1997.

Willerslev, E. and A. Cooper. Ancient DNA. *Proceedings of the Royal Society London B: Biological Sciences*, vol. 272(1558): 3–16, 2005.

Wilms, R., H. Sass, B. Kopke, H. Kster, H. Cypionka and B. Engelen. Specific bacterial, archaeal, and eukaryotic communities in tidal-flat sediments along a vertical profile of several meters. *Applied and Environmental Microbiology*, vol. 72(4): 2756–2764, 2006.

von Wintzingerode, F., U.B. Gubel and E. Stackebrandt. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiology Reviews*, vol. 21(3): 213–229, 1997.

Wishner, K.F., C. Gelfman, M.M. Gowing, D.M. Outram, M. Rapien and R.L. Williams. Vertical zonation and distributions of calanoid copepods through the lower oxycline of the Arabian Sea oxygen minimum zone. *Progress in Oceanography*, vol. 78(2): 163–191, 2008.

Withers, N., Dinoflagellate sterols. In: *The biology of dinoflagellates* (ed. F.J.R. Taylor), vol. 21, pp. 316–359, Blackwell Scientific Publications, Oxford, 1987.

Woese, C.R. Bacterial Evolution. *Microbiological Reviews*, vol. 51(2): 221–271, 1987.

Woulds, C., G.L. Cowie, L.A. Levin, J.H. Andersson, J.J. Middelburg, S. Vandewiele, P.A. Lamont, K.E. Larkin, A.J. Gooday, S. Schumacher, C. Whitcraft, R.M. Jeffreys and M. Schwartz. Oxygen as a control on seafloor biological communities and their roles in sedimentary carbon cycling. *Limnology and Oceanography*, vol. 52(4): 1698–1709, 2007.

Wrenn, J.H., M.J. Hannah and J.I. Raine. Diversity and palaeoenvironmental significance of Late Cainozoic marine palynomorphs from the CRP-1 core, Ross Sea, Antarctica. *Terra Antartica*, vol. 5: 553–570, 1998.

Wuchter, C., B. Abbas, M.J.L. Coolen, L. Herfort, J. van Bleijswijk, P. Timmers, M. Strous, E. Teira, G.J. Herndl, J.J. Middelburg, S. Schouten and J.S. Sinninghe Damsté. Archaeal nitrification in the ocean. *Proceedings of the National Academy of Sciences*, vol. 103(33): 12317–12322, 2006.

Xu, L., C.M. Reddy, J.W. Farrington, G.S. Frysinger, R.B. Gaines, C.g. Johnson, R.K. Nelson and T.I. Eglinton. Identification of a novel alkenone in Black Sea sediments. *Organic Geochemistry*, vol. 32(5): 633–645, 2001.

Yooseph, S., G. Sutton, D.B. Rusch, A.L. Halpern, S.J. Williamson, K. Remington, J.A. Eisen, K.B. Heidelberg, G. Manning, W.Z. Li, L. Jaroszewski, P. Cieplak,

C.S. Miller, H.Y. Li, S.T. Mashiyama, M.P. Joachimiak, C. van Belle, J.M. Chandonia, D.A. Soergel, Y.F. Zhai, K. Natarajan, S. Lee, B.J. Raphael, V. Bafna, R. Friedman, S.E. Brenner, A. Godzik, D. Eisenberg, J.E. Dixon, S.S. Taylor, R.L. Strausberg, M. Frazier and J.C. Venter. The Sorcerer II Global Ocean Sampling expedition: Expanding the universe of protein families. *Plos Biology*, vol. 5(3): 432–466, 2007.

Zhu, F., R. Massana, F. Not, D. Marie and D. Vaulot. Mapping of picoeucaryotes in marine ecosystems with quantitative PCR of the 18S rRNA gene. *FEMS Microbiology Ecology*, vol. 52(1): 79–92, 2005.

Zonneveld, K.A.F., G.J.M. Versteegh and G.J. de Lange. Preservation of organic-walled dinoflagellate cysts in different oxygen regimes: a 10,000 year natural experiment. *Marine Micropaleontology*, vol. 29(3-4): 393–405, 1997.

Zonneveld, K.A.F., G.J.M. Versteegh and G.J. de Lange. Palaeoproductivity and post-depositional aerobic organic matter decay reflected by dinoflagellate cyst assemblages of the Eastern Mediterranean S1 sapropel. *Marine Geology*, vol. 172(3-4): 181–195, 2001.

Zonneveld, K.A.F., G.J.M. Versteegh and M. Kodrans-Nsiah. Preservation and organic chemistry of Late Cenozoic organic-walled dinoflagellate cysts: A review. *Marine Micropaleontology*, vol. 68: 179–197, 2008.

Zuendorf, A., J. Bunge, A. Behnke, K.J.A. Barger and T. Stoeck. Diversity estimates of microeukaryotes below the chemocline of the anoxic Mariager Fjord, Denmark. *FEMS Microbiology Ecology*, vol. 58(3): 476–491, 2006.

Summary

Summary

The majority of planktonic species, including those that are informative in the reconstructions of past marine environmental conditions, do not produce diagnostic features (e.g., cysts, spores, or lipid biomarkers) and would therefore escape identification from the fossil record using traditional paleoecological tools (microscopy or lipid biomarker geochemistry). However, several studies have recently demonstrated that fossil DNA of planktonic species can be preserved for thousands of years and can be used for species-specific characterization using molecular biological techniques. The objectives of this thesis were to investigate the potential fate of fossil DNA and to what extent it can be used as a qualitative and quantitative biomarker for paleoecological and paleoenvironmental reconstructions. For this goal, fossil DNA of several groups of planktonic protists was analyzed in various marine settings, and validated by comparing the results to microscopical identification and lipid biomarker analyses.

A Holocene sediment core from Ellis Fjord, Antarctica, spanning 2700 years of deposition was used to study taxon-specific variation in the level of preservation of fossil DNA. The results showed that post-depositional fragmentation of DNA was highest for dinoflagellates, followed by diatoms and lowest for phototrophic green sulfur bacteria (GSB) stemming from the ancient sulfidic chemocline. Dinoflagellate cysts were rare in the sediment record and despite an exponential decline in fossil dinoflagellate DNA, paleogenetics was the only approach that revealed an important shift in dinoflagellate communities around 1850 years ago, indicative of colder climate and an increased ice cover. In similar aged anoxic sediments from the much deeper (2000 m) permanently stratified Black Sea, a significant fraction (∼30%) of fossil DNA of the calcifying haptophyte *Emiliania huxleyi*, an important species involved in global oceanic C and N cycling and a source of alkenones as a proxy for past oceanic sea surface temperatures (SST), appeared to escape fragmentation and was relatively well preserved for up to 3600 years. Fossil DNA revealed an absence of additional haptophyte species as sources of fossil alkenones indicating that no species-specific calibration of alkenone-SST is required for at least the last 3600 years. Finally, fossil DNA was studied in up to 124,000-year old eastern Mediterranean sediments to reveal the extent of DNA preservation beyond the Holocene era in organic carbon-rich sapropel layers as compared to the organic carbon-poor oxidized sediments. Despite the observation that a significant amount of plankton DNA could only be recovered from the youngest Holocene sapropel S1 (∼5 to 9 kyr BP), the paleogenetics approach recorded a simultaneous community shift of non-fossilized haptophytes and dinoflagellates reflecting the changing hydrological conditions during sapropel formation.

In summary, the studies reported in this thesis show that the use of fossil DNA is a promising tool for reconstructing Holocene paleoenvironmental conditions in settings where poor bottom water ventilation resulted in the deposition of organic-rich sediments.

Samenvatting

Samenvatting

Overblijfselen van planktonische protisten, zoals cysten, endosporen of karakteristieke chemische verbindingen (zogenaamde biomarkers), worden veelvuldig gebruikt voor de reconstructie van milieuomstandigheden in het verleden. Lang niet alle soorten laten herkenbare sporen na en kunnen met deze meer traditionele paleoecologische aanpak (bijvoorbeeld microscopie of organische geochemie) dan ook niet terug gevonden worden. Verschillende recente studies hebben echter aangetoond dat het fossiel DNA van deze planktonische soorten ook duizenden jaren bewaard kan blijven en met behulp van moleculair-biologische technieken kan elke afzonderlijk soort worden gedentificeerd. Het doel van dit proefschrift is om te onderzoeken in welke mate fossiel DNA gebruikt kan worden als zowel een kwalitatieve als kwantitatieve biomarker voor de reconstructie van milieuomstandigheden in het verleden ("paleogenetica"). Daartoe werd het fossiel DNA van verschillende groepen van planktonische ééncelligen geanalyseerd in sedimenten van verschillende mariene locaties, en werden deze resultaten waar mogelijk vergeleken met microscopische identificatie en organisch geochemische analyses.

Een Holocene boorkern uit Ellis Fjord, Antarctica, met daarin sedimenten tot ∼2700 jaar oud, werd geanalyseerd om de variaties in de preservatie van fossiel DNA tussen soorten onderling te bestuderen. De resultaten toonden aan dat fragmentatie van DNA nadat het in het sediment is terechtgekomen het hoogst was voor DNA van dinoflagellaten, gevolgd door dat van diatomeeën en uiteindelijk door DNA van foto-autotrofe groene zwavelbacteriën, afkomstig van de sulfidische chemocline in het fjord. Karakteristieke cysten van dinoflagellaten waren zeldzaam in het onderzochte sediment en konden daarom niet gebruikt worden voor klimaatreconstructies. Ondanks een exponentiële afname van de hoeveelheid fossiel dinoflagellaten DNA was de paleogenetische analyse de enige aanpak die een opvallende verschuiving in de soortensamenstelling liet zien rond 1850 jaar geleden, toen er een kouder klimaat en een toename in de ijsbedekking intrad.

In zuurstofloze sedimenten van vergelijkbare leeftijd uit de veel diepere Zwarte Zee (2000 m diepte) bleek een aanzienlijke fractie (∼30%) van het fossiel DNA van de calcificerende haptophyt *Emiliania huxleyi* tot ∼3600 jaar oud nog relatief goed bewaard gebleven. *E. huxleyi* is een belangrijke soort die betrokken is bij de wereldwijde oceanische koolstof en stikstof cycli, en is daarnaast een bron van alkenonen, moleculen die als proxy worden gebruikt voor de reconstructie van oceaantemperaturen in het verleden. Uit het fossiel DNA profiel bleek de afwezigheid van andere haptophyte soorten die als mogelijke bron konden dienen van de fossiele alkenonen, met als conclusie dat er voor deze periode geen andere, soort-specifieke kalibratie voor de relatie tussen de alkenonen en de oppervlakte watertemperatuur gebruikt moet worden. Ten slotte werd fossiel DNA bestudeerd in tot 124.000 jaar oude sedimentlagen uit de oostelijke Middellandse Zee om te onderzoeken of fossiel DNA ook bewaard blijft in organisch koolstof-rijke lagen (sapropelen) die ouder zijn dan het Holoceen. Hieruit bleek dat alleen in de jongste sapropel uit het Holoceen (S1, ∼5

tot 9 duizend jaar oud) voldoende fossiel DNA bewaard is gebleven, maar hierin vertoonde het fossiel DNA wel een duidelijke verschuiving in de levensgemeenschappen van zowel niet-gefossiliseerde haptophyten als dinoflagellaten, als reactie op de veranderende hydrologische omstandigheden tijdens de sapropel formatie.

Samenvattend laat deze studie zien dat, hoewel de ouderdom voornamelijk beperkt is tot het Holoceen, het gebruik van fossiel DNA een veelbelovend instrument is voor de reconstructie van milieuomstandigheden in het verleden in mariene locaties waar een slechte water ventilatie geleid heeft tot de afzetting van organisch koolstof rijke sedimenten.

Dankwoord

Voor de voltooing van dit proefschrift ben ik dank verschuldigd aan vele collega's, vrienden, kennissen etc. Ik zal me hier moeten beperken tot de belangrijkste, en zal er daarbij helaas een groot aantal vergeten...

Allereerst ben ik dank verschuldigd aan Marco Coolen, voor het mogelijk maken van mijn onderzoek. Het begeleiden van een eerste OIO is niet makkelijk, zeker niet als dat op afstand via telefoon en mail moet gebeuren, maar met jouw steun en toewijding heb ik het ondanks alles toch gered. Speciale dank ook voor de gastvrijheid tijdens mijn verblijf in Woods Hole. Daarnaast ook dank aan Jaap Sinninghe Damsté, voor de kans om mijn onderzoek op het NIOZ te doen, en voor het geduld en het begrip voor de situatie waarin ik het onderzoek uitvoerde.

Hoewel ik bij MBT (later BGC) werkte, heb ik mijn tijd voornamelijk in het moleculair lab doorgebracht en het zijn dan ook de mensen van het MolLab die me het meest geholpen en gesteund hebben. Biologen zijn over het algemeen aardige en leuke mensen (...is mij wel eens verteld) en dat blijkt zeker ook te gelden voor moleculair biologen. Met name de "harde kern" van het lab ben ik veel dank verschuldigd. Gedurende de eerste ∼2 jaar is met name Ben Abbas erg belangrijk voor me geweest om me op weg te helpen, als steunpaal, begeleider, mentor, criticus, geweten en vooral ook als vriend.
Daarnaast natuurlijk Elda Panoto, waar ik bijna de gehele periode het kantoor mee heb mogen delen. Dank je wel voor alle gezelligheid en voor alle steun bij mijn "palliatieve onderzoek", voor het weglachen van mijn OIO-dip en voor het samen onder de knie krijgen van ARB en de andere bomen-bouw-programma's.
Harry Witte, Texelaar in hart en nieren, jouw aanwezigheid en steun in het lab hebben me erg geholpen, en met name je nuchtere blik op alles.
Judith van Bleijswijk, jij bent voor mij een echte bioloog: het tomeloze enthousiasme dat jij uitstraalt voor alles wat met biologie te maken heeft (zolals schudden met een fles zeewater op de WC, zodat je de zeevonk kunt zien oplichten), is de reden dat ik ooit biologie ben gaan studeren: zeldzaam om te vinden, maar geweldig om mee te maken.
En Anneke Bol, als vaste kern in het lab, altijd in voor een praatje, over muziek of wat dan ook. Het was altijd gezellig als jij er was. Blijf vooral jezelf, want stiekem ben jij de spil waar het hele lab om draait.
Verder zijn er natuurlijk de andere OIO's, studenten en stagiaires die voor kortere of langere tijd het leven op het MolLab verlevendigden: Pedro Frade (onze DGGE-master) met al zijn studenten (een poging: Floris, Francisca, Annemarie, João, Catarina 1 t/m?), Maaike (toch ook een beetje mijn student), Héléne, Thomas, Daniele, Eva, Anne, Arnaud, Manon, Stefan, Julie, Jazzpère etc etc,... Ongelooflijk hoeveel mensen er in zo'n relatief korte tijd doorheen gegaan zijn.
Daarnaast een speciale dank voor Hans Malschaert, onze LINUX-wizard. Ik heb behoorlijk wat tijd achter de ARB computers gezeten en zonder jouw steun zou dat met

nog veel meer gevloek gepaard zijn gegaan, dan nu al het geval was.

Aangezien ik uiteindelijk ~~weinig~~ geen tijd in het prachtige MBT-lab heb mogen doorbrengen heb ik de meeste mensen van MBT / BGC vooral meegemaakt tijdens de koffiepauzes/lunches, dagjes-uit, festiviteiten etc. Hierbij dank aan de hele afdeling voor de gezelligheid die we gedeeld hebben gedurende de afgelopen 5 jaar, maar een aantal MBT-ers wil ik daarnaast graag in het bijzonder noemen:
Allereerst dank aan Irene Rijpstra, voor alle chemische analyses die je voor me gedaan heeft en voor het geduld dat je daarbij met me gehad hebt.
Michiel Kienhuis, in de eerst plaats dank voor (ja, toch) de hulp bij "computerdingen", maar natuurlijk vooral de gestage stroom BS-mailtjes. Jort en Sharyn, dank voor de vele gezellige koffie- en lunch-pauzes en voor een heugelijke avond met Penderyn. Een Bik smile gaat naar Margot, voor het invoeren van al mijn pdf's en voor jou als persoonlijkheid (niet per sé in die volgorde). Met praktisch alle OIO's/postdocs die na mij begonnen, heb ik in ieder geval kortstondig het grote kantoor beneden gedeeld, en zo heb ik een hoop "ex-kantoor-genoten" verzameld: allemaal anders, en allemaal gezellig en/of interessant (*in order of appearance*): Andrea (2 hele dagen, maar who's counting), Maaike, Francien, Jerome, Kim, Adam, Veronica, Petra, Raquel, Darci, Sabine, Antje...

Een speciale alinea is gereserveerd voor de huisgenoten in mijn buitenhuisje aan de Herenstraat 11. Allereerst Johan Weijers: de beste huisgenoot die je kunt wensen. Punctueel, ruimt op, wast af, lapt de ramen als hij zich verveelt, rookt niet. En je hebt gelijk: als je in het lab kan staan, kun je ook een taart bakken.
Jayne Rattray, sinds jij vertrokken bent, mis ik de authentieke toad-in-the-hole en natuurlijk de gehaktschotel op woensdag, eigenlijk ben je wat dat betreft meer een Nederlander dan ik. Blijf vooral praten tijdens het 8-uur journaal!
Angela Pitcher, there is never a dull moment when you're around. I am glad that you decided to move back to Texel to come live with us in the Herenstraat. Thanks for all the pizzas, and for being a super-roomie.
En dan natuurlijk Thorsten Bauersachs, je bent wel geen Man-van-Noord, maar het was fantastisch om een huis te delen, samen voetbal te kijken, muziek te luisteren, door weer en wind naar Oudeschild te fietsen,... etc. Helaas heb ik je net niet meer vloeiend Nederlands kunnen leren.

Daarnaast zijn er nog meer mensen die mijn verblijf op Texel en het NIOZ op één of andere manier hebben opgeleukt. Bijvoorbeeld de collega's waarmee ik het aquarium schoonmaakte (Hans Witte, Bert Aggenbach, Carmen Blaauboer, Maarten Brugge, Gerhard Cadee), waardoor ik in ieder geval af en toe echt het gevoel had dat ik op een zeeonderzoek-instituut werkte door tot mijn oksel in het zeewater te zitten. De MEE-literatuur discussie groep van Allert, waar ik af en toe (okee, veel te weinig) bij mocht zitten en altijd erg interessant vond. Daarnaast de los-vaste kern van mensen waarmee ik regelmatig op donderdag in de 12 Balcken heb gezeten, en door wie ik goed heb leren pipetteren met een kater: Micha, Tanya, Pedro, Jasper, Cees, Lukas,

Furu, Craig, Matthijs, Allert, Jenny, Isla, etc. En ten slotte Willem en Els van Het Kompas in den Hoorn, voor menig gezellig avondje. Beter een goede buur met een whiskey-cafe,...

Hoe mooi mijn tijd op Texel ook is geweest, Amsterdam bleef toch mijn thuis. Gedurende mijn hele OIO-schap ben ik daarom vrijwel ieder weekend heen en weer gereisd naar Amsterdam. Speciaal wil ik hierbij Marian Keuning noemen, voor onze therapeutische vrijdagse trein-biertjes; sinds je gestopt bent bij het NIOZ smaakten ze toch een stuk minder lekker.

Ik wil mijn ouders, schoonouders, familie en vrienden (Joke, Wim, Olga, Lex, Michiel, Charlotte, Roel, Andrea, Marc, Ilse, Hilbert, Glenn, Neel, Daan, Karla en Paul,...) bedanken voor hun steun en liefde, ook al was het voor niet iedereen helemaal duidelijk wat ik nou precies deed op het NIOZ. Door het heen en weer reizen waren de weekenden vaak te kort om regelmatig met vrienden en familie te kunnen afspreken. Gelukkig wilden iedereen in de zomer ook wel een keer naar Texel komen en die gezellige weekendjes Texel zal ik gaan missen in de toekomst. Maar nu woon ik weer gewoon in Amsterdam en hoop ik dat we weer wat vaker samen kunnen afspreken. Ik heb namelijk nog een boek liggen vol met dingen die jullie met mij willen gaan doen...

Als aller-aller-laatste wil ik Marieke bedanken, zonder wie de afgelopen jaren een stuk zwaarder zouden zijn geweest, en dan in het bijzonder de laatste loodjes. Dank je wel voor alles...

Curriculum Vitae

Arjan Christiaan Boere werd geboren op 3 juni 1980, in Sint Maarten (Noord-Holland) en groeide op in Sint Maartensbrug. Aansluitend aan het behalen van zijn VWO-diploma in 1998 aan het Bernardus Alfrink College in Schagen, ging hij biologie studeren aan de Universiteit van Amsterdam (UvA). Binnen de afstudeerrichting algemene biologie liep hij stages in de richtingen paleontologie en (populatie-)genetica. Na zijn afstuderen in 2003 volgde hij de eerstegraads lerarenopleiding aan het Insituut voor de Lerarenopleiding (ILO) in Amsterdam, die hij in 2004 afrondde.
Begin 2005 begon Arjan als OIO bij het koninklijk NIOZ op de afdeling Marine Biogeochemistry and Toxicology (MBT) aan onderzoek naar het gebruik van fossiel DNA als biomarker in paleoceanographie, waarbij moleculaire biologie en organische (geo)chemie werden gecombineerd. Hij werd in dit onderzoek begeleid door dr. ir. Marco Coolen en prof. dr. ir. Jaap Sinninghe Damsté. De resultaten van dit onderzoek zijn beschreven in dit proefschrift.
Aansluitend aan het promotieonderzoek werkte Arjan als toegevoegd onderzoeker mee aan een onderzoek naar de genetische karakterisering van specifieke marine virussen bij de afdeling Biologische Oceanografie van het koninklijk NIOZ onder begeleiding van dr. Corina Brussaard en dr. Joaquín Martínez Martínez.
In juni 2010 is Arjan begonnen als product specialist bij BIOKÉ in Leiden.

Scientific assessment committee

Prof. dr. J.W. de Leeuw
Department of Marine Organic Biogeochemistry
Royal Netherlands Institute for Sea Reasearch, the Netherlands
and
Department of Earth Sciences - Geochemistry, Faculty of Geosciences
Utrecht University, the Netherlands

Dr. ir. S. Schouten
Department of Marine Organic Biogeochemistry
Royal Netherlands Institute for Sea Reasearch, the Netherlands

Prof. dr. Y. Huang
Department of Geological Sciences
Brown University, Providence, RI, U.S.A.

Prof. dr. A. Pearson
Department of Earth and Planetary Sciences
Harvard University, Cambridge, MA, U.S.A.