Identification of organic matter sources in sulfidic late Holocene Antarctic fjord sediments from fossil rDNA sequence analysis

Marco J. L. Coolen,[1,2] John K. Volkman,[3] Ben Abbas,[1] Gerard Muyzer,[1,4] Stefan Schouten,[1] and Jaap S. Sinninghe Damsté[1]

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[1] The 18S ribosomal DNA (rDNA) isolated from sulfidic Holocene sediments and particulate organic matter in the water column of the stratified Small Meromictic Basin (SMB) in Ellis Fjord (eastern Antarctica) was analyzed to identify possible biological sources of organic matter. Previous work had shown that the sediments contained numerous diatom frustules and high contents of a highly branched isoprenoid (HBI) $C_{25:2}$ alkene (which is a specific biomarker of certain species of the diatom genera *Navicula*, *Haslea*, *Pleurosigma,* or *Rhizosolenia*), so we focused our search on preserved fossil 18S rDNA of diatoms using sensitive polymerase chain reaction (PCR) approaches. We did not find diatom-derived fossil 18S rDNA using general eukaryotic primers, and even when we used primers selective for diatom 18S rDNA, we only identified a *Chaetoceros* phylotype, which is known to form cysts in the SMB but is not a likely source of the $C_{25:2}$ HBI. When we used PCR/denaturing gradient gel electrophoresis methods specific to phylotypes within the HBI-biosynthesizing genera, we were able to identify three phylotypes in the sediments related to HBI-producing strains of the genera *Haslea* and *Navicula.* The ancient DNA data thus provided a limited, but valuable, view of the diversity of late Holocene primary producers with a particular bias to specific components of the biota that were better preserved such as the *Chaetoceros* cysts. This use of paleogenetics also revealed unexpected possible sources of organic matter such as novel stramenopiles for which no specific lipid biomarkers are known and thus would not have been identified based on traditional lipid stratigraphy alone.

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1. Introduction

[2] Bacterial mineralization of primary produced organic matter is generally highly effective leading to a small fraction of organic matter produced in the water column actually being preserved in the geosphere. However, anoxic environments [*Hebting et al.*, 2006] and many continental margin systems act as depositional centers accumulating large amount of organic matter deposited to the seafloor in deeper sediment layers [*Hartnett et al.*, 1998]. In such circumstances, DNA can also be well preserved but the extent to which it is degraded will vary in different environments because of complex interactions between microbial remineralization, adsorption and chemical processes [e.g., *Coolen and Overmann*, 1998; *Coolen et al.*, 2004b; 2006b; *Dell'Anno et al.*, 2002, 2005].

[3] Recently, it was shown that in deep-sea sediments, extracellular DNA accounted for up to 70% of the total DNA pool [*Corinaldesi et al.*, 2005] and more than 50% of the extracellular DNA was recalcitrant to enzymatic degradation [*Dell'Anno et al.*, 2002]. However, the source of the extracellular DNA was not determined in these studies.

[4] The sequence analysis of DNA preserved in sediments can provide considerable information about the biological sources of organic matter in particular depositional environments [*Coolen et al.*, 2004a, 2004b, 2006a, 2006b; *D'Andrea et al.*, 2006], but there is still little information about the extent to which the preserved DNA reflects the exact ancient species composition and the abundance.

[5] The Holocene sediments of the Small Meromictic Basin (SMB) in Ellis Fjord, Antarctica are sulfidic and rich in organic carbon. For these reasons, the SMB in Ellis Fjord represents a model system in which to study the preservation of organic compounds including DNA. Our previous work on lipid biomarkers in these sediments [*Sinninghe Damsté et al.*, 2007] showed a diversity of compounds indicative of mixed algal and bacterial sources. Of particular interest was the high abundance of a $C_{25:2}$ HBI alkene in the surface sediments. C_{25} HBI alkenes are produced only by species of the pennate diatom genera *Navicula*, *Haslea*, and *Pleurosigma* as well as some

[1]Department of Marine Biogeochemistry, Royal Netherlands Institute for Sea Research, Den Burg, Netherlands.
[2]Also at Department of Marine Chemistry and Geochemistry, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts, USA.
[3]CSIRO Marine and Atmospheric Research, Hobart, Tasmania, Australia.
[4]Now at Kluyver Laboratory for Biotechnology, Delft University of Technology, Delft, Netherlands.

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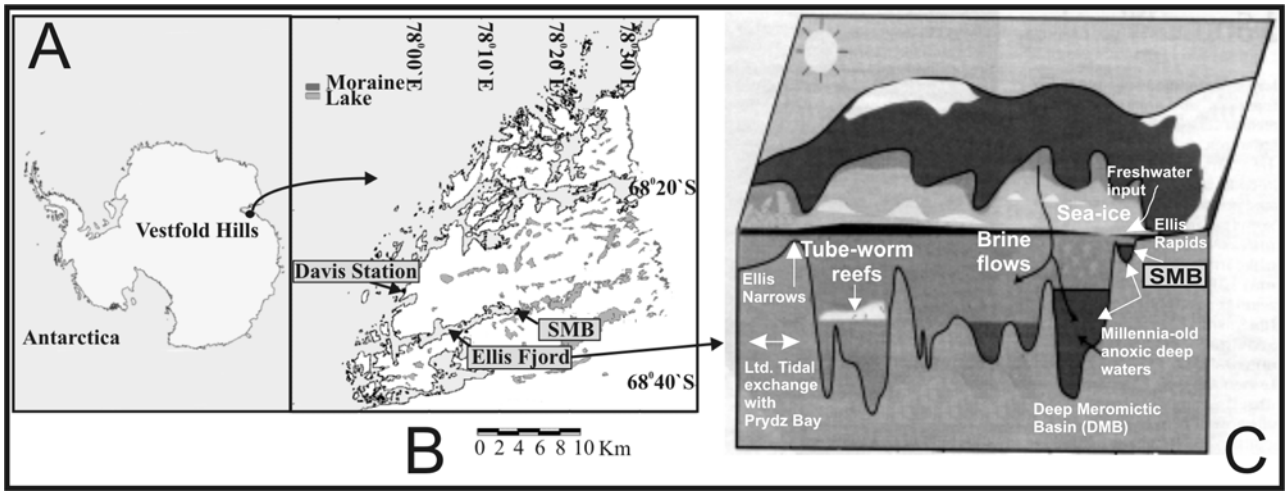

Figure 1. (a) Location of the Vestfold Hills, eastern Antarctica. (b) Map of the Vestfold Hills showing the location of Ellis Fjord and the Small Meromictic Basin (SMB) within Ellis Fjord where all samples were obtained. (c) Cartoon of Ellis Fjord with the stratified SMB, which contains millennia-old anoxic deep waters. Figure 1c was modified after *Trull et al.* [2001].

species of the centric genus *Rhizosolenia* [*Sinninghe Damsté et al.*, 2004]. The abundance of this biomarker declined rapidly with sediment depth as the alkene became increasingly sulfurized, but the total amounts of sulfurized and nonsulfurized HBI alkene remained high indicating that its diatom source was an important contributor of organic matter during the whole period represented by the sediment core (^{14}C age of 3300 years at 111 cm [*Sinninghe Damsté et al.*, 2007]). A previous study of diatom frustules preserved in Holocene sediments of the SMB by *McMinn et al.* [2001] did not identify frustules of *Pleurosigma*, *Haslea*, or *Rhizosolenia* spp.; however, frustules of the sea ice diatom *Navicula glacei* were relatively abundant [*McMinn et al.*, 2001] and this diatom could be the source for the HBI alkene. However, in anoxic, sulfidic organic-carbon-rich sediments, diatom frustules are prone to dissolution [*Michard et al.*, 1994] and some species might not have been recognized by microscopic observation.

[6] To identify the biological source of the HBI alkene we set out to determine whether fossil DNA from diatoms is well preserved in these organic carbon-rich sulfidic sediments by means of a phylogenetic analysis of 18S rDNA sequences. Our previous work on organic-rich anoxic sediments from a nearby saline lake in the Vestfold Hills, Ace Lake, had revealed sequences for a great variety of algal species. However, diatoms are not abundant in that ecosystem [*Coolen et al.*, 2004b]. We used general as well as selective (and therefore highly sensitive) PCR approaches. The amplicons were separated by denaturing gradient gel electrophoresis (DGGE) for subsequent phylogenetic analysis of sequenced DGGE fragments. The number of 18S rDNA copies of potential diatom sources of HBI alkene was determined by quantitative real-time PCR (QPCR). The ratio between 18S rDNA copies and the sulfurized and nonsulfurized HBI alkene provided information about the survival of both types of molecules and showed that in this instance the fossil 18S rDNA of diatoms provided a quantitative "biomarker" for some components of the diatom populations.

2. Materials and Methods

2.1. Sampling

[7] Water samples were collected in November 2000 using a 50-cm-long 5-L Niskin bottle from various positions in the water column of the depositional center (13 m depth; 68.59702°S 78.22762°E) of the Small Meromictic Basin (SMB) in Ellis Fjord, Vestfold Hills, eastern Antarctica (Figure 1). The general features of this area and its attraction for geochemical study are described by *Trull et al.* [2001]. The sampling positions (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 with dense accumulation of obligate photolithotrophic green sulfur bacteria (9.7–10.2 m), and (3) anoxic and sulfidic monimolimnion (10.7–11.2 m, 12.5–13.0 m). In order to collect particulate organic matter (POM) from the water samples, we first filtered through 0.7 μm pore size glass fiber filters (GFF, Millipore) to collect larger cells followed by filtration through 0.2 μm pore size polycarbonate (PC) filters (Millipore) in order to retain also the small prokaryotic cells. Owing to the use of this two-step filtration we were able to filter volumes between 0.25 to 15 L before particles completely clogged the filters. The filters were stored at −40°C prior to DNA or lipid extraction. The extracts from both the GFF and PC filters at each water depth were pooled.

[8] Using a gravity corer, a 113-cm-long sediment core with a diameter of 5 cm was obtained from the depositional center of the SMB. Immediately after sampling, the sediment core was kept in the dark and stored at −20°C at Davis Station in Antarctica and at −40°C at the Royal NIOZ in Netherlands. The upper 20 cm of sediment was sliced into 1-cm horizontal sections and used for analysis.

Table 1. Information About the Polymerase Chain Reaction Primers Used During This Study

Primer	Forward Primer	Reverse Primer	Primer Sequence	Target (18S rDNA)	Reference
I	Euk-A	1–20[a]	5'-ACC TGG TTG ATC CTG CCA GT-3'	eukaryotes	*Medlin et al.* [1988]
II	Euk-563r	548–563[a]	5'-[GC-clamp][b] ACC AGA CTT GCC CTC C-3'	eukaryotes	*Amann et al.* [1990]
III	Dia-516r	497–516[a]	5'-[GC-clamp][c] 5'CTC ATT CCA ATT GCC AGA CC-3'	diatoms (general)	*Coolen et al.* [2004b]
IV	Pen-1024f	1024–1041[a]	5'-[GC-clamp][c] CTA TGC CGA CAA GGG ATT-3'	pennate diatoms	this work
V	Pen-1041r	1024–1041[a]	5'-[GC-clamp][c] AAT CCC TTG TCG GCA TAG-3'	pennate diatoms	this work
VI	HBI-II-nh1363r	1346–1363[a]	5'-AAG AAG CCA GCA GTC AGT-3'	HBI-II (*Navicula* plus *Haslea*)[d]	this work
VII	HBI-II-p635f	635–653[a]	5'-CTT GGG TTT CGG TGT TGC-3'	HBI-II (*Pleurosigma*)[d]	this work
VIII	HBI-I-Rh460f	460–475[e]	3'-AAT AAC AAT GCC GGR T-5'	HBI-I (*Rhizosolenia*)[d]	this work
IX	HBI-I-Rh1321r	1125–1144[e]	5'-[GC clamp][c] GGG TCT CGT TCG TTA ACG CA-3'	HBI-I (*Rhizosolenia*)[d]	this work

[a]*Saccharomyces cerevisiae* positions.
[b]Sequence of 40 base pairs (bp)-long guanine and cytosine (GC-rich) clamp: (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3').
[c]Sequence of 40 bp GC clamp: (5'CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC C-3'). Additional information about primer combinations for the group-selective PCR amplification of 18S rDNA can be found in Table 2.
[d]More information about the phylogeny of diatoms that biosynthesize HBI-alkenes is provided by *Sinninghe Damsté et al.* [2004].
[e]*Rhizosolenia setigera* (AY485461) positions.

Sediment layers deeper than 20 cm were sliced into 2-cm horizontal sections with a gap of 2 cm between intervals. *Sinninghe Damsté et al.* [2007] reported the following ^{14}C ages (uncorrected for any minor reservoir effect) for various sediment depths: 0–1 cm: 520 years B.P., 20–22 cm: 1500 years B.P., 40–42 cm: 1740 years B.P., 60–62 cm: 2200 years B.P., 80–82 cm: 2440 years B.P., 110–112 cm: 3300 years B.P.

2.2. Extraction of Total DNA

[9] Total DNA was extracted from 0.25 g of sediment using the UltraClean Soil DNA Kit following the descriptions of the manufacturer (Mobio, Carlsbad, California, USA). Because our study relied on the analysis of fossil 18S rDNA derived from ancient diatoms by PCR amplification, it was of utmost importance to prevent any contamination of the sediment samples by foreign DNA. Details concerning the extraction of DNA and the extensive steps taken to prevent contamination with foreign/modern DNA have been described previously [*Coolen et al.*, 2006b]. As a control for contamination during DNA extraction, a parallel sample without sediment was subjected to the whole extraction and purification procedure (extraction control). Filtered POM was extracted from presliced whole filters according to *Wuchter et al.* [2004]. 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 and the filtered POM was subjected to agarose gel electrophoresis to determine the quality and fragment length of the DNA throughout the core. A QPCR using general bacterial primers was run for undiluted, as well as 2, 5, 10, 20, and 50 times diluted DNA extracts. PCR-inhibiting, coextracted impurities within the DNA extracts were examined by comparing the threshold cycles of the diluted vs. undiluted samples.

2.3. Amplification of 18S rDNA by PCR for Subsequent DGGE Analysis

[10] All PCR reactions were performed in an iCycler (Biorad, Hercules, California). Partial 18S rDNA of the domain Eukarya was amplified by PCR using primer combination **A** (Tables 1 and 2) and conditions as described by *Díez et al.* [2001a]. In order to detect the main diatom sequences, partial 18S rDNA unique to diatoms was amplified by PCR using primer combination **B** (Tables 1 and 2) and conditions as described previously [*Coolen et al.*, 2004b]. Partial 18S rDNA of species within the HBI-II clusters (pennates) was selectively amplified by PCR using new combinations of primers **C** (*Navicula* and *Haslea*), and **D** (*Pleurosigma*), while the HBI-I cluster (*Rhizosolenia*) was amplified with combination **E** (Tables 1 and 2). A two-

Table 2. Primer Combinations Used in This Study Plus Information on the Selectivity of the Primers, Fragment Lengths of the Amplicons and the Genomic DNA (15 ng) of Species That Served as Positive Controls During PCR, and the Genomic DNA of Species That Served as Controls for the Specificity of the QPCR Reactions[a]

Primer Set	Primers	Target (18S rDNA)	Fragments Excluding GC Clamp[b]	Positive Controls	Controls for the Specificity of the PCR Reactions
A	I + II	eukaryotes	563	*Rhizosolenia setigera* (AY485461)	NR[c]
B	I + III	diatoms (general)	516	*Rhizosolenia setigera* (AY485461)	*Tetraselmis* sp.
C	IV + VI	HBI-II (*Navicula* plus *Haslea*)	324	*Navicula phyllepta* (AY485456)	*Dickieia ulvacea* (AY485462)
D	VII + V	HBI-II (*Pleurosigma*)	406	*Pleurosigma intermedium* (AY485514)	*Dickieia ulvacea* (AY485462)
E	VIII + IX	HBI-I (*Rhizosolenia*)	684	*Rhizosolenia setigera* (AY485461)	*Navicula phyllepta* (AY485456)

[a]Additional information about the primers can be found in Table 1.
[b]Base pairs are given.
[c]NR indicates not required for these non-selective primers.

step (nested) PCR in which an aliquot of the first PCR product was reamplified using fresh PCR ingredients was performed to lower the detection limit of the selective amplification reactions for HBI-biosynthesizing clusters of diatoms (see Results section). The first amplification involved: initial denaturing for 4 min at 96°C, followed by 30 cycles including denaturing (30 sec at 94°C), 40 sec of primer annealing at 62.5°C (combination C), 59°C (combination D), or 60°C (combination E) and primer extension (40 sec at 72°C). A final extension was performed at 72°C (10 min). One μl of the PCR products was added to a mixture with fresh PCR ingredients and then reamplified using 18 cycles and conditions as described above. During the latter reamplification reactions, a 40-base pairs (bp)-long GC clamp [*Muyzer et al.*, 1993] was introduced to the PCR product in order to prevent complete melting of the PCR products during subsequent DGGE analysis (Table 1). The additional PCR ingredients have been described previously as have our extensive procedures for avoiding DNA contamination [*Coolen et al.*, 2006b].

[11] Each PCR amplification series included reactions to monitor contamination with foreign DNA during the pipetting of the reaction mixture components or during the extraction of DNA from the sediment samples. 10^5 copies of complete length 18S rDNA of closely related diatoms that did not cluster with HBI-biosynthesizing diatoms were used to monitor the specificity of the different (nested) PCR reactions (Table 2).

2.4. Sequence Analysis of DGGE Fragments

[12] The partial PCR-amplified 18S rDNA of eukaryotes, diatoms, and pennate diatoms of the HBI-II clusters was separated by DGGE [*Muyzer et al.*, 1993] 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 proceeded for 5 h at 200 V and 60°C. All processes after electrophoresis including sequence analysis of excised DGGE fragments have been described by *Coolen et al.* [2006b].

2.5. Phylogenetic Analysis and Primer Design

[13] Sequence data were compiled using ARB software [*Ludwig et al.*, 2004] and aligned with complete length sequences of closest relatives obtained from the National Centre for Biotechnology Information (NCBI) database http://www.ncbi.nlm.nih.gov/) using the ARB FastAligner utility. Matrices of similarity and phylogenetically corrected distance values were generated using the maximum parsimony option in ARB. Primers Pen-1024f and Pen-1041r (targeting 91% of available sequences of pennate diatoms), primer HBI-II-nh1348r (targeting all available sequences of *Navicula* and *Haslea* species known to biosynthesize HBIs), primer HBI-II-p635f (targeting all available sequences of *Pleurosigma* species that are known to biosynthesize HBIs) and primers HBI-I-Rh460f and HBI-I-Rh1321r (targeting all available sequences of *Rhizosolenia* species known to biosynthesize HBIs) were designed using the PROBE DESIGN option of the software program of ARB. All available sequences of diatoms plus 81 additional sequences from our diatom culture collection were used to design suitable primers selective for all available sequences of HBI producers within the pennate diatoms. The sequences reported in this study have been deposited in the National Centre for Biotechnology Information (NCBI) database http://www.ncbi.nlm.nih.gov/) under accession numbers DQ507401 to DQ507420.

2.6. Real-Time Quantitative PCR

[14] Real time PCR was performed in an iCycler system (Biorad) in order to study the relative quantitative distribution of the most abundant fossil 18S rDNA copies of diatoms (primer set selective for most diatoms [*Coolen et al.*, 2004b]) and specifically HBI-biosynthesizing diatoms of the genera *Navicula* and *Haslea* (primer sets Pen-1024f and HBI-II-nh1363r). We did not quantify 18S rDNA of *Pleurosigma* or *Rhizosolenia* since phylotypes of these genera were not identified from the Ellis Fjord samples. To quantify the 18S rDNA copy numbers, the PCR conditions and primers (without GC clamp) were used as described above. The accumulation of newly amplified double stranded rDNA was measured by the increase in fluorescence due to the binding of the fluorescent dye SYBRGreen to the double stranded amplification products. One microliter template DNA (total DNA extracts ranging between 1 and 25 ng) from each sample was added to each QPCR reaction. The additional ingredients of the reaction mixtures have been described previously [*Coolen et al.*, 2006b].

[15] For the calibration of samples, known copy numbers of 18S rDNA of *Navicula phyllepta* (AY485456) (ranging between 10^{-2} and 10^7) were subjected to real time PCR. Similar control reactions as described above were performed to monitor contamination from foreign DNA or to monitor the specificity of the reactions.

[16] 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 lab where no ancient DNA analysis was performed.

[17] Quantitative PCR products were subjected to agarose gel electrophoresis in order to identify nonspecific PCR products. The introduction of a GC clamp during a second PCR run for subsequent phylogenetic analysis of sequenced DGGE fragments was the only difference between the qualitative and quantitative PCR approaches in this study. Therefore the phylogenetic analysis of sequenced DGGE fragments provided proof for the specificity of the QPCRs.

3. Results and Discussion

3.1. Search for Predominant Eukaryotic 18S rDNA

[18] In order to identify possible eukaryotic sources for organic matter in the sediments of the Small Meromictic Basin (SMB) in Ellis Fjord, Antarctica (Figure 1), we performed a PCR with nonselective primers for the domain Eukarya to isolate 18S rDNA from the water column and sediments. The 18S rDNA amplicons were analyzed by DGGE and resulted in about 12 unique major fragments (Figure 2a). Phylogenetic analysis of the sequenced DGGE fragments revealed that the major 18S rDNA fragments represented copepoda, cercozoa, alveolata, chlorophyta and

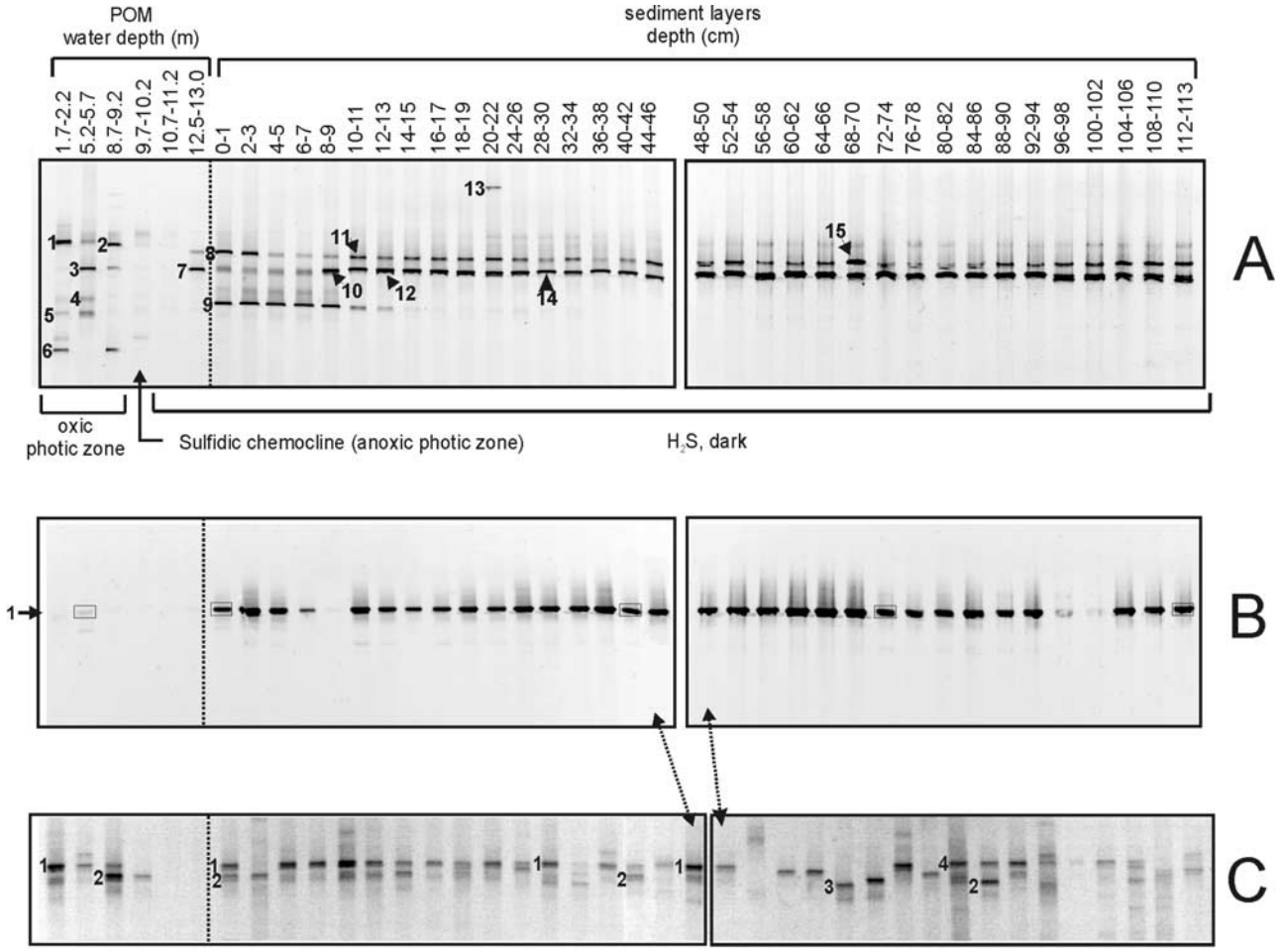

Figure 2. (a) DGGE with PCR-amplified 18S rRNA genes of the domain Eurkarya obtained from the water column and sediment samples of SMB. Fifteen fragments were excised from the gel and sequenced and labeled SMB_Euk DGGE 1–15. The phylogeny is shown in Figure 3. Fragments 8, 11, and 15 all melted at a similar position in the DGGE and were found to have the same sequence. (b) DGGE with PCR-amplified 18S rRNA genes of diatoms recovered from the water column and sediment samples of SMB. Only one major fragment (labeled 1) was found in all analyzed samples. The fragment was sliced from various positions in the DGGE (sliced fragments are marked with rectangles) and was found to have a similar sequence (labeled SMB_Dia DGGE 1 in Figure 4) to *Chaetoceros socialis*, which is not a likely source of HBIs. (c) DGGE with 18S rRNA genes of diatoms related to HBI-biosynthesizing species of the genera *Navicula* and *Haslea* recovered from the water column and sediment samples of SMB. The major fragments (labeled 1–4) were sequenced, and their phylogeny is shown in Figure 4.

stramenopiles (Figure 3). Note that some fragments with similar melting behavior were sampled from different depths (Figure 2a) to check that they represented the same phylotype. From the POM in the oxygenated photic zone of the water column we identified organic matter from a calanoid copepod related to *Calanus pacificus*, a dinoflagellate related to *Polarella* or *Gymnodinium*, a ciliate, and a green microalga related to *Pyramimonas australis* (Figures 2a and 3). A sequence related to the green alga *Tetraselmis* was only found from the waters just above the sediment, but this water sample might have been "contaminated" from resuspended material from the water/sediment interphase.

[19] From the anoxic sediments three abundant and unique phylotypes were retrieved. One of the sequences (DGGE band 10 and 14) showed 99% sequence homology to a novel stramenopile clone ME1-22. The latter is a heterotrophic flagellate which predominates in the North Atlantic, Mediterranean, as well as in Antarctic marine waters [*Díez et al.*, 2001b]. None of the closest relatives have been described morphologically and their role in the marine environment remains unknown. This sequence was not recovered from the water column POM samples from Austral summer which indicates that these organisms did not thrive in the water column of Ellis Fjord at that time. Why their sequences were so abundant in the fossil record

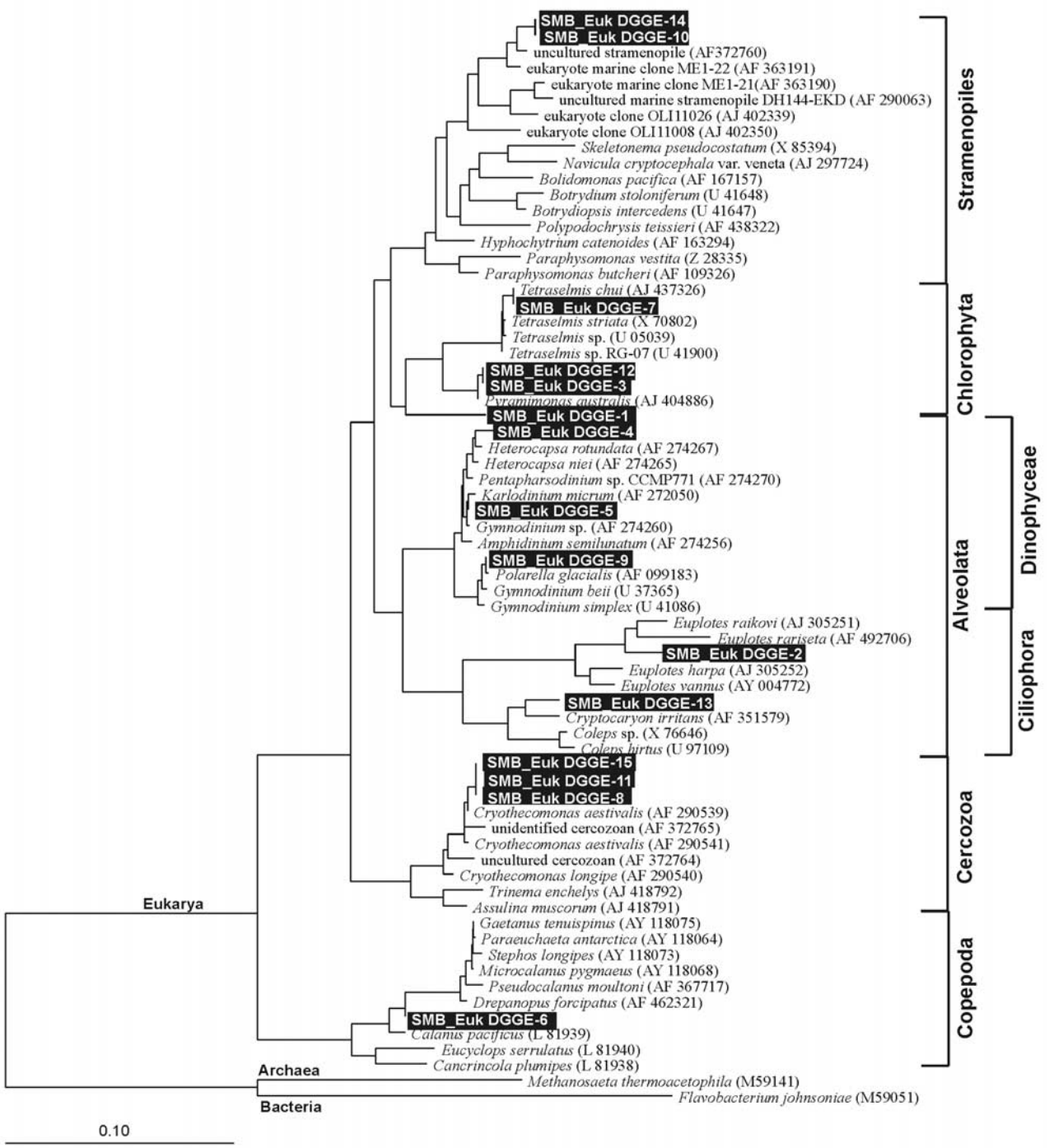

Figure 3. Phylogenetic tree showing the relationship between the major eukaryotic 18S rDNA sequences (white text in black rectangles) as depicted in the DGGE of Figure 2a together with their closest relatives from the National Centre for Biotechnology Information (NCBI) database.

remains speculative, but possible explanations could be: (1) a preferred transport of cells toward the sediment, (2) a preferred preservation of their fossil DNA compared to other cell types, (3) or a high copy number of 18S rDNA per genome.

[20] A second major DGGE fragment (SMB_Euk DGGE-12, Figure 2a) melting slightly below the DGGE fragment of the novel stramenopile was recovered throughout the core as well as from the POM sample between 5.2 and 5.7 m water depth (SMB_Euk DGGE-3). Its partial 18S rDNA sequence showed 100% similarity to the 18S rDNA sequence of the chlorophyte *Pyramimonas australis* (AJ 404886) (Figure 3). This species has previously been isolated from sea ice (Ross Sea, Antarctica) and forms cysts

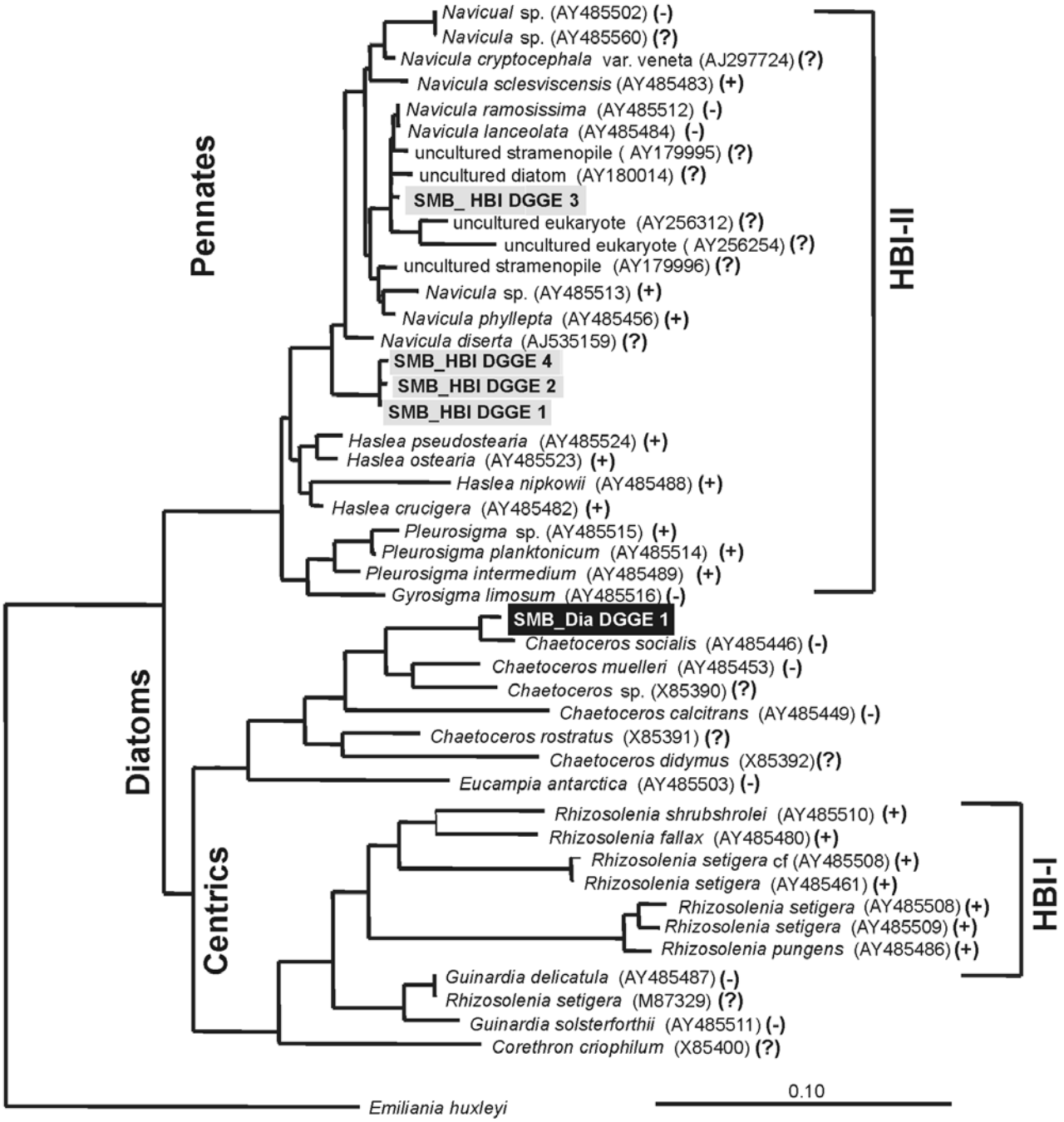

Figure 4. Phylogenetic tree showing the relationship of 18S rDNA sequences of diatoms retrieved from the water column and sediment samples of SMB with closest relatives from the NCBI database. Sequences of cultivated closest relatives that have been shown to biosynthesize HBIs [*Sinninghe Damsté et al.*, 2004] are indicated with a plus sign, whereas sequences from diatoms that do not biosynthesize HBIs are indicated with a minus sign [*Sinninghe Damsté et al.*, 2004]. Sequences with a question mark indicate diatoms that have not been tested for the ability to biosynthesize HBIs. SMB_HBI DGGE fragments 1, 2, and 4 (black text in shaded rectangles, as depicted in the DGGE of Figure 2c) were affiliated with the HBI-II cluster of *Navicula* plus *Haslea.* The affiliation of phylotype SMB_Dia DGGE-1 obtained using the general diatom primer set with *Chaetoceros* spp. (white text in black rectangle, depicted in the DGGE of Figure 2b) is shown for comparison.

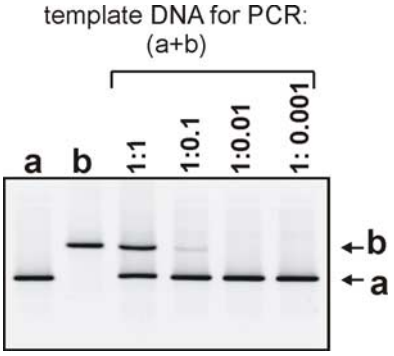

Figure 5. DGGE analysis of amplified 18S rDNA from known amounts of genomic DNA of *Chaetoceros socialis* (AY 485446) with genomic DNA of *Navicula sclesviscensis* (AY 485483) (ratios of 1:1 1:0.1, 1:0.01, etc.) showing that the DGGE fragment of *Navicula* (labeled a) could no longer be detected when its genomic template DNA was 2 orders of magnitude less abundant than the *Chaetoceros* template DNA (labeled b).

[*Moro et al.*, 2002], which likely explains the preferential preservation of its fossil DNA.

[21] A third major 18S rDNA fragment found in all the sediment samples (DGGE fragments 8, 11 and 15) showed 99.6% sequence similarity to the diatom parasite *Cryothecomonas aestivalis* (AF292539). This unusual flagellate is frequently found in the North Sea phytoplankton feeding on the centric diatom *Guinardia delicatula* [*Drebes et al.*, 1996]. This genus is also known from submarine calderas [*Takishita et al.*, 2005] and sea ice [*Thomsen et al.*, 1991]. *Cryothecomonas* species typically feed on cells in the size range 2–4.5 μm [*Thomsen et al.*, 1991], using their pseudopodium that penetrates the diatom cell and phagocytizes the host cytoplasm [*Drebes et al.*, 1996]. *Cryothecomonas* species have close fitting multilayered theca, which may explain their enhanced preservation in these sediments. *Crypthecomonas* species (including *C. armigera* and an unknown species) represented on average over 50% of the biomass of heterotrophic protists in ice edge sea ice brine samples from McMurdo Sound, Antarctica during December [*Stoecker et al.*, 1993], demonstrating a major role of *Crypthecomonas* in the microbial food web there. Our DNA data suggest that they may play a similarly important role in Ellis Fjord.

[22] Using primers selective for eukaryotic 18S rDNA during PCR, no sequences of diatoms were recovered from the water column in summer or from the Holocene sediment layers (Figures 2a and 3). If diatoms made up at least 1% of the total eukaryotic community, they should have been identified by this PCR/DGGE approach [*Muyzer et al.*, 1993]. In our previous studies, the use of primers specific to species or groups improved the sensitivity and allowed the identification of traces of ancient DNA in Holocene sediments [*Coolen and Overmann*, 1998; *Coolen et al.*, 2004a, 2004b, 2006a, 2006b], so this prompted us to undertake a more selective approach.

3.2. Selective Search for 18S rDNA Found in Diatoms

[23] In order to enhance our detection for 18S rRNA genes of diatoms we performed a PCR with primers selective for most of the available diatoms including all sequences of known HBI-biosynthesizing diatoms. This resulted in the identification of one diatom phylotype (SMB_Dia DGGE-1) (Figures 2b and 4) with 94.5% homology to its closest relative *Chaetoceros socialis* (AY 485446) (Figure 4). This phylotype was found in all analyzed sediments. Very small amounts of PCR products of diatoms were recovered from the water column POM samples and so the whole PCR product was subjected to DGGE (Figure 2b). The same *Chaetoceros* phylotype (Figure 4), albeit in low amounts, was also found in the POM from the oxygenated photic zone at a depth between 5.2 and 5.7 m (Figure 2b). The poor yield of diatom 18S rDNA amplicons in POM from the water column was most likely due to the fact that at the time of sampling the SMB was seasonally disconnected from the remaining fjord because of its intact ice cover. After the ice cover starts to melt, benthic sea ice diatoms are liberated into the water column and once the influence of the melting ice has diminished, open water blooms of *Chaetoceros* dominate the fjords in the Vestfold Hills [*McMinn and Hodgson*, 1993; *McMinn et al.*, 2000]. The intact ice cover at the time of sampling also explains why the 18S rDNA of these sea ice diatoms was below the detection limit in the water column POM samples. The poor recovery of 18S rDNA amplicons of diatoms from the water column samples again indicates that diatoms were not a major fraction of the eukaryotic community in the water column at the time of sampling.

[24] The predominance of the *Chaetoceros* phylotype in the Holocene sediment layers of SMB is in accordance with the results of *McMinn et al.* [2001] who reported that cysts of *Chaetoceros* accounted for up to 80% of diatom frustules within the anoxic, sulfidic Holocene sediment layers of SMB. These authors also reported 7 fast-ice diatom taxa, 14 benthic sea ice diatoms, and 23 planktonic species. *Navicula glaciei* composed up to 40% of the counted frustules in the top 20 cm of the core, and on average about 5% of the total counts down core. From sediment layers between 50 to 100 cm, *Cocconeus costatum* composed up to 50% of the counted frustules. Therefore we expected to find, in addition to 18S rDNA of *Chaetoceros*, at least the 18S rDNA of *N. glacei* or *C. costatum* in the sediment layers where the relative abundance of their frustules was high.

[25] This directed us to find out whether our inability to detect other species might be an experimental limitation related to the high predominance of 18S rDNA of *Chaetoceros*. To test this, we mixed known amounts of genomic DNA of *Chaetoceros socialis* (AY485446) and *Navicula sclesviscensis* (AY485483) (ratios of 1:1, 1:0.1, 1:0.01 etc.) and performed DGGE analysis of their amplified 18S rDNA. This experiment showed that we could no longer identify the DGGE fragment of *Navicula* when its genomic template DNA added to the PCR mixture was 2 orders of magnitude less abundant than the *Chaetoceros* template DNA (Figure 5). In other words, the absence of sequences of minor diatoms in the SMB sediments could be due to the fact that the PCR/DGGE is biased to identify

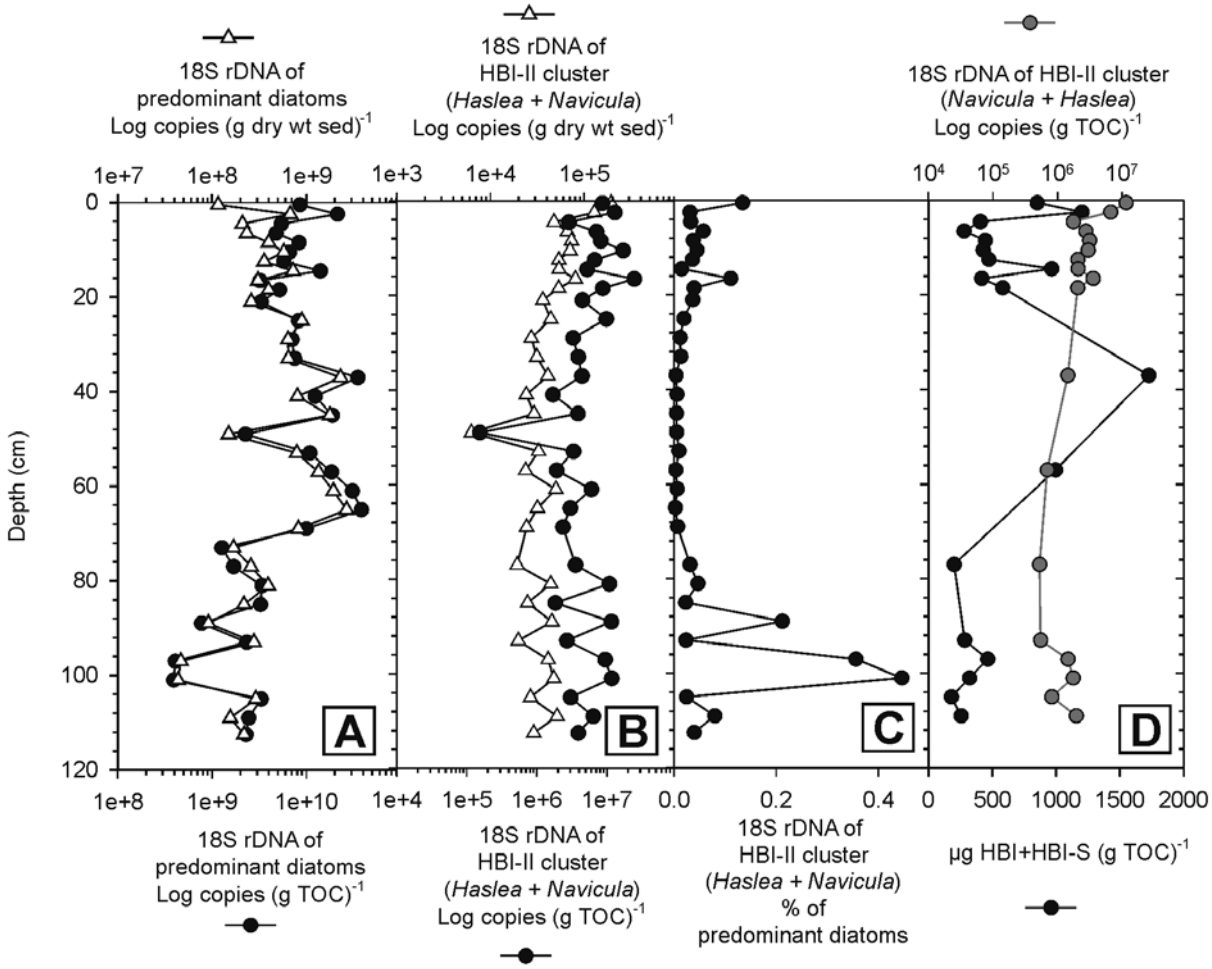

Figure 6. Depth distribution of (a) 18S rDNA of the predominant diatom (*Chaetoceros*) and (b) the HBI-II cluster (*Navicula* plus *Haslea*) (both expressed as log copies per gram total organic carbon (TOC) and as log copies per gram dry weight sediment), (c) 18S rDNA of the HBI-II cluster (*Navicula* plus *Haslea*) as a percentage of the total diatom 18S rDNA, and (d) plot of HBI alkenes (free and sulfurized forms) per gram of TOC (lipid data from *Sinninghe Damsté et al.* [2007]) versus content of 18S rDNA of the HBI-II cluster (*Navicula* plus *Haslea*) (expressed as log copies per gram TOC).

only the major diatom phylotypes. In addition, the DNA inside the *Chaetoceros* cysts is likely to be better protected against degradation compared to extracellular DNA or to the intracellular DNA of diatoms which do not form cysts.

[26] HBIs were below the detection limit in POM from the water column, including the POM sample where the *Chaetoceros* phylotype was found. In a previous study in which 123 diatom cultures were tested for the biosynthesis of HBI alkenes, *Sinninghe Damsté et al.* [2004] showed that C_{25} HBI alkenes were produced only by species of the pennate diatom genera *Navicula*, *Haslea*, and *Pleurosigma* as well as by species of the centric genus *Rhizosolenia*, but not by *Chaetoceros*. Therefore, at this stage of the project, we had still not detected the possible fossil source of the $C_{25:2}$ HBI alkene. Accordingly to find the biological source of the $C_{25:2}$ HBI, we increased the sensitivity of the PCR approach by PCR amplification of 18S rDNA solely found in pennate diatoms of the genera *Navicula*, *Haslea*, and *Pleurosigma* using newly developed highly selective primers (Table 1).

3.3. Selective Search for 18S rDNA of HBI-Producing Pennate Diatoms

[27] PCR with up to 35 cycles failed to yield 18S rDNA amplicons of HBI-producing diatoms within the *Navicula* and *Haslea* genera from the water column POM samples or from the sediments of SMB. Since the use of a GC clamp can result in primer dimer formation causing a lower yield of the desired 18S rDNA amplicons we decided to first amplify the 18S rDNA of diatoms of the HBI-II cluster using primers without GC clamp. However, despite this and demonstrably less production of primer dimers, we still could not generate amplicons of HBI-producing diatoms from the water column POM and sediments within 35 cycles. In contrast, a detectable fluorescent PCR product

occurred after 35 cycles using the control strain, the pennate diatom *Dickieia ulvacea* (AY485462).

[28] It is well accepted that the number of cycles during PCR should be kept below 30 since the efficiency of DNA polymerase diminishes and artifacts may be formed [*Qiu et al.*, 2001]. This led us to use a nested-PCR approach involving fewer cycles (30) during the first PCR amplification, plus the addition of fresh PCR ingredients and the introduction of a GC clamp during the second amplification reaction of 18 cycles. This double PCR run approach resulted in the efficient and very selective amplification of trace amounts of fossil 18S rDNA for which the primers were selective (Figures 2c and 4). When the GC clamp was introduced during the first PCR run with 35 cycles, the detection limit for 18S rDNA of HBI-producing diatoms within the *Navicula* and *Haslea* genera was $\sim 2 \times 10^3$ copies within the reaction mixture. However, by using the double PCR run approach we lowered the detection limit to ~20 to 50 *Navicula*/*Haslea* 18S rDNA copies within the reaction mixture which equals a detection limit of $\sim 5 \times 10^3$ copies per gram TOC or $\sim 7 \times 10^2$ copies per gram dry weight sediment. The control reactions did not result in detectable amounts of PCR products under these conditions whereas measurable amounts of PCR products developed between 40 and 46 cycles for the POM samples of the photic zone of SMB as well as most of the Holocene sediment layers.

[29] The same strategy was then used for the PCR amplification of 18S rDNA of the genera *Pleurosigma* as well as *Rhizosolenia* (Tables 1 and 2) which only resulted in amplification products for the positive controls used. This strongly indicated that the possible source of the $C_{25:2}$ HBI was an as yet uncultivated diatom clustering between HBI-biosynthesizing *Navicula* and *Haslea* species (SMB_HBI DGGE 1, 2, and 4; Figure 4). We did not expect to find *Rhizosolenia* DNA since these species produce C_{30} HBIs and none were found in the SMB sediments [*Sinninghe Damsté et al.*, 2007].

[30] We then compared the two measures of the abundance of HBI-containing diatoms in the sediment record: their recovered DNA (measured as 18S rDNA copies g^{-1} TOC and per gram dry weight sediment) and total HBIs in the sediment (free plus sulfurized; lipid data from *Sinninghe Damsté et al.* [2007]). There was a general correspondence in both the top 20 cm and below 80 cm (Figure 6d), but there was no correlation at the intermediate depths where HBI abundances were quite high (overall correlation $r^2 = 0.07$). Clearly, only a small proportion of the DNA derived from HBI-biosynthesizing diatoms is preserved and the amounts detected in the sediment probably cannot be used as a quantitative measure of the HBI-containing diatoms. The amount of DNA from the HBI-II cluster is 2 to 4 orders of magnitude less abundant than the amount of 18S rDNA from the predominant *Chaetoceros* diatom (Figures 6a–6c). Moreover, the abundance of the latter shows marked changes with depth (Figure 6c) and it seems that the DNA abundance in this case is a good measure of the abundance of diatomaceous (*Chaetoceros* derived) organic matter in the sediments. This apparent divergence almost certainly reflects the fact that the *Chaetoceros* species formed resistant cysts whereas the cells of the HBI producers were readily lysed and degraded. The role of the diatom parasite *Cryothecomonas*, an important eukaryote detected by our fossil DNA approach (see above), in enhancing diatom cell degradation in this environment is unknown.

4. Conclusions

[31] This study shows that DNA from different sources is degraded at different rates in the sulfidic, anoxic sediments of Ellis Fjord, Antarctica and is biased to particular species that have better preservational properties. Even though previous lipid biomarker studies had shown a high abundance of HBI-producing diatoms we had to go to extraordinary lengths using specific primers and enhanced procedures to identify 18S r-DNA from these species. In contrast, DNA from *Chaetoceros* cysts was well preserved and provided a good indicator of the abundance of this diatom over the past 3300 years. Thus, in this instance, the fossil DNA data provided a limited view of the diversity of organic matter sources, but rather indicated specific components of the biota that were better preserved. However, the DNA analyses proved to be particularly valuable in identifying unexpected sources of organic matter (such as the diatom parasite *Cryothecomonas* or the novel stramenopile) and in identifying specific species as likely sources of particular lipids (e.g., the HBI alkenes).

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References

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

Coolen, M. J. L., and J. Overmann (1998), Analysis of subfossil molecular remains of purple sulfur bacteria in a lake sediment, *Appl. Environ. Microbiol.*, *64*, 4513–4521.

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

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

Coolen, M. J. L., G. Muyzer, S. Schouten, J. K. Volkman, and J. S. Sinninghe Damsté (2006a), Sulfur and methane cycling during the Holocene in Ace Lake (Antarctica) revealed by lipid and DNA stratigraphy, in *Past and Present*

Marine Water Column Anoxia, *NATO Sci. Ser., Ser. IV*, vol. 64, edited by L. N. Neretin. pp. 41–65, Springer, New York.

Coolen, M. J. L., A. Boere, B. Abbas, M. Bass, S. G. Wakeham, and J. S. Sinninghe Damsté (2006b), Fossil DNA derived from alkenone-biosynthesizing haptophytes and other algae in Holocene sediment from the Black Sea, *Paleoceanography*, *21*, PA1005, doi:10.1029/2005PA001188.

Corinaldesi, C., R. Danovaro, and A. Dell'Anno (2005), Simultaneous recovery of extracellular and intracellular DNA suitable for molecular studies from marine sediments, *Appl. Environ. Microbiol.*, *71*, 46–50.

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

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

Dell'Anno, A., C. Corinaldesi, S. Stavrakakis, V. Lykousis, and R. Danovaro (2005), Pelagic-benthic coupling and diagenesis of nucleic acids in a deep-sea continental margin and an open-slope system of the eastern Mediterranean, *Appl. Environ. Microbiol.*, *71*, 6070–6076.

Díez, B., C. Pedrós-Alió, T. L. Marsh, and R. Massana (2001a), Application of denaturing gradient gel electrophoresis (DGGE) to study the diversity of marine picoeukaryotic assemblages and comparison of DGGE with other molecular techniques, *Appl. Environ. Microbiol.*, *67*, 2942–2951.

Díez, B., C. Pedrós-Alió, and R. Massana (2001b), Study of genetic diversity of eukaryotic picoplankton in different oceanic regions by small-subunit rRNA gene cloning and sequencing, *Appl. Environ. Microbiol.*, *67*, 2932–2941.

Drebes, G., S. F. Kuhn, A. Gmelch, and E. Schnepf (1996), *Cryothecomonas aestivalis* sp. nov., a colourless nanoflagellate feeding on the marine centric diatom *Guinardia delicatula* (Cleve) Hasle, *Helgol. Meeresunters.*, *50*, 497–515.

Hartnett, H. E., R. G. Keil, J. I. Hedges, and A. H. Devol (1998), Influence of oxygen exposure time on organic carbon preservation in continental margin sediments, *Nature*, *391*, 572–574.

Hebting, Y., P. Schaeffer, A. Behrens, P. Adam, G. Schmitt, P. Schneckenburger, S. M. Bernasconi, and P. Albrecht (2006), Biomarker evidence for a major preservation pathway of sedimentary organic carbon, *Science*, *312*, 1627–1631.

Ludwig, W., et al. (2004), ARB: A software environment for sequence data, *Nucleic Acids Res.*, *32*, 1363–1371.

McMinn, A., and D. Hodgson (1993), Summer phytoplankton succession in Ellis Fjord, eastern Antarctica, *J. Plankton Res.*, *15*, 925–938.

McMinn, A., N. Bleakley, K. Steinburner, D. Roberts, and L. Trenerry (2000), 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.*, *22*, 287–303.

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

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

Michard, G., E. Viollier, D. Jezequel, and G. Sarazin (1994), Geochemical study of a crater lake—Pavin Lake, France: Identification, location and quantification of the chemical reactions in the lake, *Chem. Geol.*, *115*, 103–115.

Moro, I., N. La Rocca, L. Dalla Valle, E. Moschin, E. Negrisolo, and C. Andreoli (2002), *Pyramimonas australis* sp. nov. (Prasinophyceae, Chlorophyta) from Antarctica: Fine structure and molecular phylogeny, *Eur. J. Phycol.*, *37*, 103–114.

Muyzer, G., E. C. de Waal, and A. G. Uitterlinden (1993), Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA, *Appl. Environ. Microbiol.*, *59*, 695–700.

Qiu, X., L. Wu, H. Huang, P. E. McDonel, A. V. Palumbo, J. M. Tiedje, and J. Zhou (2001), Evaluation of PCR-generated chimeras, mutations, and heteroduplexes with 16S rRNA gene-based cloning, *Appl. Environ. Microbiol.*, *67*, 880–887.

Sinninghe Damsté, J. S., et al. (2004), The rise of the rhizosolenid diatoms, *Science*, *304*, 584–587.

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

Stoecker, D. K., K. R. Buck, and M. Putt (1993), Changes in the sea-ice brine community during the spring-summer transition, McMurdo Sound, Antarctica. 2. Phagotrophic protists, *Mar. Ecol. Prog. Ser.*, *95*, 103–113.

Takishita, K., H. Miyake, M. Kawato, and T. Maruyama (2005), Genetic diversity of microbial eukaryotes in anoxic sediment around fumaroles on a submarine caldera floor based on the small-subunit rDNA phylogeny, *Extremophiles*, *9*, 185–196.

Thomsen, H. A., K. R. Buck, P. A. Bolt, and D. L. Garrison (1991), Fine-structure and biology of *Cryothecomonas* gen. nov. (Protista incertae sedis) from the ice biota, *Can. J. Zool.*, *69*, 1070–11,048.

Trull, T. W., et al. (2001), Site holds promise for ecosystem and biogeochemical investigations, *Eos Trans. AGU*, *82*, 306,311.

Wuchter, C., S. Schouten, M. J. L. Coolen, and J. S. Sinninghe Damsté (2004), Temperature-dependent variation in the distribution of tetraether membrane lipids of marine Crenarchaeota: Implications for TEX_{86} paleothermometry, *Paleoceanography*, *19*, PA4028, doi:10.1029/2004PA001041.

B. Abbas, S. Schouten, and J. S. Sinninghe Damsté, Department of Marine Biogeochemistry, Royal Netherlands Institute for Sea Research, P.O. Box 59, 1790 AB Den Burg, Netherlands.

M. J. L. Coolen, Department of Marine Chemistry and Geochemistry, Woods Hole Oceanographic Institution, 360 Woods Hole Road, Woods Hole, MA 02543, USA. (mcoolen@whoi.edu)

G. Muyzer, Kluyver Laboratory for Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC, Delft, Netherlands.

J. K. Volkman, CSIRO Marine and Atmospheric Research, GPO Box 1538, Hobart, Tasmania 7001, Australia.