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

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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) C₂₅:₂ 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 C₂₅:₂ 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.


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 C₂₅:₂ HBI alkene in the surface sediments. C₂₅ HBI alkenes are produced only by species of the pennate diatom genera Navicula, Haslea, and Pleurosigma as well as some...
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 (14C 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 *Pleurosira*, *Haslea*, or *Rhizosolenia* spp.; however, frustules of the sea ice diatom *Navicula glaciei* 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.

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

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.

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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Primer Sequence</th>
<th>Target (18s rDNA)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Euk-A</td>
<td>1 – 20°</td>
<td>5’-ACC TGG TTG ATC CTA CGA CCA GT-3’</td>
<td>eukaryotes</td>
<td>Medlin et al. [1988]</td>
</tr>
<tr>
<td>II</td>
<td>Euk-563r</td>
<td>548 – 563°</td>
<td>5’-[GC-clamp] ACC AGA CTG GCC CTC C-3’</td>
<td>eukaryotes</td>
<td>Amann et al. [1990]</td>
</tr>
<tr>
<td>III</td>
<td>Dia-516r</td>
<td>497 – 516°</td>
<td>5’-[GC-clamp] 5’CTT ATT CTA ATT GCC AGA CC-3’</td>
<td>diatoms (general)</td>
<td>Coolen et al. [2004b]</td>
</tr>
<tr>
<td>IV</td>
<td>Pen-1024f</td>
<td>1024 – 1041°</td>
<td>5’-[GC-clamp] CTA TGC CGA CAA GGG ATT-3’</td>
<td>pennate diatoms</td>
<td>this work</td>
</tr>
<tr>
<td>V</td>
<td>Pen-1041r</td>
<td>1024 – 1041°</td>
<td>5’-[GC-clamp] AAT CCC TTG TCG GCA TAG-3’</td>
<td>pennate diatoms</td>
<td>this work</td>
</tr>
<tr>
<td>VI</td>
<td>HBI-II-nh363r</td>
<td>1346 – 1365°</td>
<td>5’-AAG AAG ACA GCA GAC ATG-3’</td>
<td>HBI-II (Navicula plus Haslea)</td>
<td>this work</td>
</tr>
<tr>
<td>VII</td>
<td>HBI-II-p635f</td>
<td>635 – 653°</td>
<td>5’-CTT GGG TTT CGG TGT TGC-3’</td>
<td>HBI-II (Pleurosigma)</td>
<td>this work</td>
</tr>
<tr>
<td>VIII</td>
<td>HBI-I-Rb460f</td>
<td>460 – 475°</td>
<td>3’-AAT AAC AAT GCC GGR T-5’</td>
<td>HBI-I (Rhizosolenia)</td>
<td>this work</td>
</tr>
<tr>
<td>IX</td>
<td>HBI-I-Rh321r</td>
<td>1125 – 1144°</td>
<td>5’-[GC clamp] GGG TCT CGT TCG TTA ACG CA-3’</td>
<td>HBI-I (Rhizosolenia)</td>
<td>this work</td>
</tr>
</tbody>
</table>

*aSaccharomyces cerevisiae* positions.

*bSequence of 40 bp GC clamp: (5’CGC CCG CCG CCC GCG CCC GGT CCG CCC CCC CCC CCG G-G-3’). Additional information about primer combinations for the group-selective PCR amplification of 18S rDNA can be found in Table 2.

*cMore information about the phylogeny of diatoms that biosynthesize HBI-alkenes is provided by Sinninghe Damsté et al. [2004].

*dRhizosolenia setigera* (AY485461) positions.

Sediment layers deeper than 20 cm were sliced into 2-cm horizontal sections with a gap of 2 cm between sections. Sinninghe Damsté et al. [2007] reported the following 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

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, Göttinngen, 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

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 Dicz 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-
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 Pterygogram 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 Pterygogram 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 phylelepta (AY485456) (ranging between 10^-2 and 10^3) 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...
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.

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 [Diez 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...
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* (AJ404886) (Figure 3). This species has previously been isolated from sea ice (Ross Sea, Antarctica) and forms cysts

**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.
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.
3.2. Selective Search for 18S rDNA Found in Diatoms

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 (AY485446) (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.

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. glaciei or C. costatum in the sediment layers where the relative abundance of their frustules was high.

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 seseviscensis (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
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\textsubscript{25} HBI alkenes were produced only by species of the pennate diatom genera Navicula, Haslea, and Pleurosoma 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\textsubscript{25-2} HBI alkene. Accordingly to find the biological source of the C\textsubscript{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 Pleurosoma 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

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**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).
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 good 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


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*...
Nucleic Acids sp. nov., a colourless nanoflagellate

COOLEN ET AL.: FOSSIL HOLOCENE PHYTOPLANKTON DNA

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Science 71

Paleoceanography Org.

Guinardia

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Chem.

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´ (2004), Temperature-

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Gene

gen. nov. (Protista

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Pyramimonas australis

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Appl. Environ.

Holocene

Appl. Environ.

71

Appl. Environ. Micro-

67

sp. nov. (Pra-

61x328

Dı

Dı

Dell’Anno, A., C. Corinaldesi, S. Stavrakakis,

Dell’Anno, A., S. Bompadre, and R. Danovaro

(2005), Simultaneous recovery of extracellular

and intracellular DNA suitable for molecular

studies from marine sediments, Appl. Environ.

Microbiol. 71, 1–6.


Corinaldesi, C., R. Danovaro, and A. Dell’Anno

(2005), Simultaneous recovery of extracellular

and intracellular DNA suitable for molecular

studies from marine sediments. Limnol.

Oceanogr., 49, 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 Mediterranea-

nean, Appl. Environ. Microbiol., 71, 6070–

6076.

Dizè, B. C., Pedrós-Alõí, T. L. Marsh, and

R. Massana (2001a), Application of denatur-

ing gradient gel electrophoresis (DGGE) to

study the diversity of marine picoeukaryotic

assemblages and comparison of DGGE with other molecular techniques, Appl. Environ.


Dizè, B. C. Pedrós-Alõí, and R. Massana

(2001b), Study of genetic diversity of eukar-

yotic picoplankton in different oceanic regions

by small-subunit rRNA gene cloning and se-

quencing, Appl. Environ. Microbiol., 67,

2932–2941.


Hartnett, H. E., R. G. Keil, J. I. Hedges, and

A. H. Devol (1998), Influence of oxygen

tissue exposure time on organic carbon preserva-


Hebting, Y., P. Schaeffer, A. Behrens, P. Adam, G. Schnepf, R. H. Campbell, S. M. Bernasconi, and P. Albrecht (2006), Bio-


Ludwig, W. et al. (2004), ARB: A software en-

vironment for sequence data, Nucleic Acids


McMinn, A., and D. Hodgson (1993), Summer phytoplankton succession in Ellis Fjord, eastern


McMinn, A., N. Bleakley, K. Steinburner, D. Roberts, and L. Treonery (2000), Effect of permanent sea ice cover and different nutrient regimes on the phytoplankton succession of fjords of the Vestfold Hills Oasis, eastern Ant-


Miyake, H. A., K. R. Buck, P. A. Bolt, and


Wuchter, C., S. Schouten, M. J. L. Coolen, and J. S. Sinninghe Damsté (2004), Temperature-


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