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10 **217,000 year-old DNA sequences of green sulfur bacteria in Mediterranean**

11 **sapropels and their implications for the reconstruction of the**

12 **paleoenvironment**

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1 **SUMMARY**

2 Deep-sea sediments of the eastern Mediterranean harbor a series of dark, organic carbon-rich

3 layers, so-called sapropels. Within these layers, the carotenoid isorenieratene was detected. Since

4 it is specific for the obligately anaerobic phototrophic green sulfur bacteria, the presence of

5 isorenieratene may suggest that extended water column anoxia occurred in the ancient

6 Mediterranean Sea during periods of sapropel formation. Only three carotenoids (isorenieratene,

7 β-isorenieratene and chlorobactene) are typical for green sulfur bacteria and thus do not permit

8 to differentiate between the ~80 known phylotypes. In order to reconstruct the paleoecological

9 conditions in more detail, we searched for fossil 16S rRNA gene sequences of green sulfur

10 bacteria employing ancient DNA methodology. 540 bp-long fossil sequences could indeed be

11 amplified from up to 217,000-year-old sapropels. In addition, such sequences were also

12 recovered from carbon-lean intermediate sediment layers deposited during times of an entirely

13 oxic water column. Unexpectedly, however, all the recovered 16S rRNA gene sequences

14 grouped with freshwater or brackish, rather than truly marine, types of green sulfur bacteria. It is

15 therefore feasible that the molecular remains of green sulfur bacteria originated from populations

16 which thrived in adjacent freshwater or estuarine coastal environments rather than from an

17 indigenous pelagic population.

18

1 **INTRODUCTION**

2 All known green sulfur bacteria and about half of the species of purple sulfur bacteria are

3 obligate anaerobic photolithoautotrophs which can only grow in the presence of light and

4 reduced sulfur compounds as photosynthetic electron donators (Pfennig and Trüper, 1989;

5 Overmann, 2001). Their specific physiological requirements render these bacteria suitable

6 indicator organisms for past environmental conditions (Passier *et al.*, 1999; Menzel *et al.*, 2002).

7 Eastern Mediterranean sediments harbor more than 1 cm-thick, organic carbon-rich layers,

8 so-called sapropels. Sapropels contain >2 % and up to 30% per weight of organic carbon and are

9 embedded in hemipelagic carbonate oozes (< 0.5 wt% organic C) (Kidd *et al.*, 1978). The

10 enhanced organic carbon preservation during sapropel formation has been explained by anoxia

11 of deep Mediterranean bottom water (Oceanic Anoxic Events; Rossignol-Strick, 1985; Rohling

12 and Hilgen, 1991; Passier *et al.*, 1999) or by increased marine primary production (Calvert,

13 1983; Calvert *et al.*, 1992). 1.8 to 3.0 million-year-old Pliocene sapropels were shown to contain

14 the carotenoid isorenieratene and its diagenetic derivatives (Passier *et al.*, 1999; Menzel *et al.*,

15 2002). Since isorenieratene occurs almost exclusively in green sulfur bacteria, it was concluded

16 that sulfidic bottom waters extended into the photic zone during sapropel formation and that the

17 Mediterranean Sea ecosystem underwent repeated and major oxic-anoxic shifts during the past 3

18 million years.

19 However, isorenieratene has also been detected in the actinobacteria *Streptomyces griseus*

20 and *Brevibacterium linens* (Krubasik and Sandmann, 2000; Krügel *et al.*, 1999). On the other

21 hand, certain strains of green sulfur bacteria do not contain any detectable amounts of those

22 carotenoids which were originally thought to be typical for this group (Glaeser *et al.* 2002).

23 Since green sulfur bacteria form a distinct and coherent phylogenetic lineage (Overmann and

24 Tuschak, 1997; Imhoff, 2003), 16S rRNA gene sequences provide an alternative means to trace

25 their occurrence and species composition in the environment (Overmann *et al.*, 1999). While

26 green sulfur bacteria contain only three specific carotenoids (isorenieratene, β-isorenieratene and

27 chlorobactene) (Overmann, 2001), 80 different 16S rRNA gene sequence types are recognized to

1   date (A. Manske and J. Overmann, submitted). The three carotenoids occur across all different

2   subgroups of the green sulfur bacteria (Imhoff, 2003), whereas species isolated from the marine

3   environment form a single well-separated sequence cluster (marine group 1) (Imhoff, 2003;

4   Manske *et al.*, 2005). Therefore, analyses of their fossil 16S rRNA gene sequences in subsurface

5   sediments would not only provide independent evidence for the occurrence of green sulfur

6   bacteria but also permit a more detailed reconstruction of their paleoenvironment. To date,

7   however, such a paleomicrobiological analysis has not been performed in the marine

8   environment.

9   Fully hydrated DNA spontaneously decays over only hundreds of years, mainly through

10   hydrolysis and oxidation (Shapiro, 1981; Lindahl, 1993; Hofreiter *et al.*, 2001). However, low

11   temperatures, high ionic strength, anoxic conditions and protection from enzymatic degradation

12   by adsorption extend the half-life of intact DNA by one or two orders of magnitude (Lindahl,

13   1993; Poinar *et al.*, 1996; Willerslev *et al.*, 2004a). So far, intact DNA of anoxygenic

14   phototrophic bacteria could be extracted from up to 9,100 year-old holocene lake sediments and

15   was analyzed by PCR amplification and sequencing (Coolen and Overmann, 1998). Recently,

16   amplification of fossil chloroplast DNA has been found to be reliable for samples which are

17   hundreds of thousands of years old (Willerslev *et al.*, 2003). Under favorable conditions of

18   preservation, genomic DNA of anoxygenic phototrophic bacteria thus may have persisted for

19   similar time periods in Mediterranean sapropels.

20   In the present study, fossil DNA sequences and carotenoids of ancient green sulfur bacteria

21   could be recovered from sapropels deposited between 8,000 and 217,000 years ago in the

22   Eastern Mediterranean. Phylogenetic analyses were used to infer the most likely origin of the

23   respective bacteria and provide a more detailed picture of the development of this marine

24   ecosystem.

25

1   **RESULTS**

2   *Vertical structure and age of sediment layers*

3   A four meter-long sediment core obtained at a water depth of 2155 m from the Eastern

4   Mediterranean southeast of Crete contained four different sapropels which were identified as

5   sapropels S_1, S_6, S_7, and S_8 based on geochemical evidence (Coolen *et al.*, 2002; Fig. 1). The

6   ages of these sapropels are 8,000 (S_1), 172,000 (S_6), 195,000 (S_7) and 217,000 (S_8) years (Emeis

7   *et al.*, 2000). The organic carbon content of the sapropels ranged between 2.3 and 8.5% (w/w),

8   whereas intermediate layers contained only up to 0.16 % (Coolen *et al.*, 2002). The intermediate

9   layer Z_1 is likely to represent a turbidite (K.-C. Emeis; pers. comm.).

10   For subsequent analyses, the top of the sediment (Z_0, 4-6 cm below surface), each sapropel

11   layer (S_1, 13-17 cm; S_6, 262-266 cm; S_7, 314-318 cm; and S_8, 364-368 cm), and three

12   intermediate hemipelagic layers between the sapropels (denoted Z_1, 62-66 cm; Z_6, 285-289 cm;

13   and Z_7, 333-337 cm) were selected (Fig. 1).

14   *Green sulfur bacterial carotenoids in late Pleistocene and Holocene sapropels*

15   Isorenieratene and β-isorenieratene could be detected in all four sapropel layers where they

16   reached concentrations between 56.4 and 1280 ng·(g dry weight sediment)$^{-1}$ (Fig. 1C). Initially,

17   both carotenoids were identified by HPLC based on their characteristic retention time and

18   absorption spectra (peaks at 454 and 482 nm and a shoulder at 428 nm). Analysis of these

19   compounds by LC-MS in the APCI positive ion mode yielded major signals at m/z values of 529

20   and 133. These masses match those of the protonated isorenieratene (i.e., $[M+H]^+$) and of

21   1,2,3,4-tetramethylbenzene, respectively, the latter representing a fragment typical of

22   isorenieratene and β-isorenieratene. Our mass spectrometric analysis thus provides the first

23   evidence for the presence of carotenoids of green sulfur bacteria in late Pleistocene and

24   Holocene sapropels.

25   In contrast, only traces of carotenoid compounds with the retention times and absorption

26   spectra of isorenieratene and β-isorenieratene were detected in intermediate layers.

1 Concentrations ranged from (1.4 ± 0.1) to (5.9 ± 0.3) ng·(g dry weight sediment)$^{-1}$ (Fig. 1C).

2 Although above the detection limit, these trace amounts did not allow further analyses by LC-

3 MS. Therefore, it cannot be completely ruled out that other carotenoids were present, though

4 carotenoids with retention times and absorption spectra identical to isorenieratene/β-

5 isorenieratene are currently not known.

6 *Detection and quantification of DNA of green sulfur bacteria*

7 After optimizing our extraction protocol, genomic DNA could be isolated from all eight

8 sediment layers (Fig. 1A). Between 0.7 and 5.0 µg of DNA were extracted per gram dry weight

9 of sediment from the four sapropels. These amounts significantly exceeded those obtained from

10 the intermediate layers (0.01 to 0.12 µg·(g dry wt)$^{-1}$) and were only matched by the value from

11 the sediment surface (1.04 µg·(g dry wt)$^{-1}$). Analysis by gel electrophoresis revealed that the

12 DNA mostly consisted of up to 23 - 30 kb long fragments (data not shown). The shortest

13 fragments detected measured 500 bp. This length distribution was even observed in extracts from

14 the 217,000-year-old Mediterranean sapropel S_8.

15 The specific primer combination and PCR conditions used in the present study permit the

16 selective amplification of 16S rRNA gene fragments of green sulfur bacteria (Overmann *et al.*,

17 1999). With the exception of the intermediate layer Z_6, all samples yielded amplification

18 products. The DNA extract of sapropel S_7 yielded only a faint amount of DNA of the correct

19 molecular size, but also some unspecific amplification products. Of all sapropel layers

20 investigated, S_7 contains the by far highest amount of kerogen (total organic carbon content of

21 8.5% as compared to ≤4.4% in other sapropels; Coolen *et al.*, 2002). Similarly, Z_6 contained the

22 highest amount of total organic carbon of all intermediate layers (0.16 versus ≤0.01%). Most

23 likely, the failure to obtain appropriate amplification products from these two layers resulted

24 from the unfavourable ratio of fossil DNA to organic kerogen.

25 Each amplification reaction was rigorously checked for contamination with extraneous DNA

26 by routinely including a set of procedural blanks and PCR controls (as detailed in the Materials

1 and Methods section). Yet, neither the controls for a contamination of PCR reagents, nor the

2 controls for a contamination during DNA extraction ever yielded amplification products (Fig. 2).

3   A specific dot blot hybridization protocol was used to quantify the amount of green sulfur

4 bacterial DNA in the different sediment layers after amplification (Fig. 3). Our original

5 amplification method was found to be highly sensitive but not suitable for quantification because

6 it did not yield a continuously increasing response with increasing concentrations of target DNA

7 used in the PCR. Calibration was possible, however, when an altered amplification PCR protocol

8 was employed (Fig. 3, standards). This technique permitted a detection of green sulfur bacteria

9 DNA if the latter constituted as little as 0.0065% of the total community DNA. Green sulfur

10 bacterial 16S rRNA gene sequences could be quantified in six of the sediment layers (Fig. 1C,

11 Fig.3). DNA extracted from the intermediate layers Z_6 and Z_7 did not yield detectable signals,

12 although the latter yielded a product with the original, more sensitive, PCR method.

13   Although most sapropel layers contained the highest absolute amounts of green sulfur

14 bacterial DNA (Fig. 1C), maxima of the relative amounts did not always coincide with the

15 presence or absence of sapropels (Fig. 1B). The highest percentage of green sulfur bacterial

16 DNA ($0.55 \pm 0.02\%$ of total community DNA) was determined for sapropel S_8, whereas the

17 second largest fraction of 16S rRNA genes of green sulfur bacteria (0.088% of total community

18 DNA) was determined for intermediate layer Z1 (Fig. 1B).

19 *Phylogenetic identification of the fossil green sulfur bacteria*

20 In order to identify individual sequences, the amplified 16S rRNA gene fragments were analyzed

21 further by denaturing gradient gel electrophoresis and sequencing. In order to be able to analyze

22 sequences from intermediate layer Z_7, PCR products obtained with the more sensitive

23 amplification method were used. However, only PCR reactions which yielded exclusively

24 specific products were analyzed, thereby excluding the amplification products from S_7.

25   A total of six different melting types could be recognized by DGGE fingerprinting (Fig. 4).

26 Nine of the DNA bands were excised and sequenced. The results confirm that sequences of

1   green sulfur bacteria had been selectively amplified from the extracted DNA. Seven of the

2   bands (A through G in Fig. 4) yielded unambiguous sequences. Of the latter, bands A, B, E and F

3   not only exhibited the same melting behavior during DGGE, but also contained the same

4   sequence. This phylotype was found to be identical to those of 11 other strains or environmental

5   sequences (Fig. 5) and fell into Group 3 among the green sulfur bacteria. Sequence type C was a

6   member of Group 4a and only present in the intermediate layer Z_1, yet could not be detected in

7   the adjacent sapropel S_1 or the surface layer Z_0. Two other sequences (D and G) fell into Group 3

8   (Fig. 5). Each represented a distinct, so far unknown sequence type (Fig. 5) and was found in

9   only a single sapropel layer but was missing in all other samples (Fig. 4).

10   *Adsorptive binding of DNA to the sediments*

11   Sediment material of the sapropel S_6 and the intermediate layer Z_6 was chosen for adsorption

12   assays since they contained comparatively low amounts of indigenous DNA compared to the

13   other layers (Fig. 1A). Since the concentration of indigenous DNA determined (1.44 µg·(g dry

14   weight sediment)$^{-1}$ in S_6) amounted to only a very minor fraction (0.003%) of the total

15   adsorption capacity, the DNA already present in the samples did not interfere with these

16   adsorption assays.

17   The sapropel exhibited an extraordinarily high maximum adsorption capacity S_{max} for DNA

18   ((79.3 ± 2.8) µmol double-stranded DNA (g dry wt)$^{-1}$; corresponding to 52.8 mg (g dry wt)$^{-1}$)

19   (Fig. 6, Table 1). This value is comparable to that of pure montmorillonite and is only surpassed

20   by purified humic acids (Table 1). Albeit lower, the adsorption capacity of Z_6 for double

21   stranded DNA was still significantly higher than that of soil (Table 1).

22

23

1 **DISCUSSION**

2 *Authenticity of the green sulfur bacterial DNA*

3 Analyses of ancient DNA are usually based on minute amounts of highly degraded template

4 molecules and thus are subject to a considerable risk of contamination (Cooper and Poinar,

5 2000). In order to avoid sources of extraneous DNA in the present study, subsampling of the

6 sediment cores was performed in a separate, PCR-product-free laboratory in which green sulfur

7 bacteria had never been worked with before, and a security level 2 laminar flow chamber

8 dedicated to work with low DNA template number samples was used for subsequent DNA

9 extraction and to set up amplification reactions. Each set of DNA extractions included controls

10 for contamination of the chemicals and vessels employed. None of these extraction controls

11 yielded an amplification product, indicating that no extraneous DNA from green sulfur bacteria

12 had been introduced during DNA isolation. Furthermore, each amplification run comprised two

13 reactions devoid of DNA template in order to control separately for a potential contamination of

14 PCR reagents and tubes. These negative controls also did not yield any PCR product. Finally,

15 quantification of fossil green sulfur bacterial DNA was performed in three independent parallels

16 to test reproducibility of the results.

17 Whereas parallel extraction trials for the identical sediment layer were not feasible due to the

18 restricted availability of deep sea sediment samples, a comparison of the results from the

19 consecutive sediment horizons further confirms that the DNA genuinely originated from these

20 samples. Sequences of green sulfur bacteria were not only detected on a single occasion, but

21 occurred in seven out of eight different sediment layers. Furthermore, amplification of green

22 sulfur bacterial sequences was reproducible for the same extract (compare standard deviations

23 for quantification of green sulfur bacterial amplification products in Fig. 1C).

24 Three of the four sequence types obtained (Z1-C, S6-D, S8-G) were detected in only a single

25 sample. It appears unlikely that each of the three sediment samples Z1, S6 and S8 was

26 contaminated with yet another, different type of green sulfur bacterium. Two of these sequence

27 types (S6-D, S8-G) have neither been isolated as a clone nor from a culture before. It is also

1   extremely unlikely that two samples were contaminated individually, yet by two different

2   sequences which are not available in any laboratory world wide. Our phylogenetic analysis of the

3   recovered sequences thus provides independent evidence for the conclusion that the green sulfur

4   bacterial DNA is indigenous to the Mediterranean sediments.

5   *Fossil origin and mechanisms of persistence of green sulfur bacterial DNA*

6   Compared to the multitude of studies targeting higher organisms, studies of ancient bacteria have

7   been limited to a few pathogenic species (*Mycobacterium tuberculosis*, *M. leprae*, *Yersinia pestis*

8   and *Treponema pallidum*) or to intestinal bacteria, and cover a time period of only the last 5,400

9   years (Rollo, 1998). Information on past bacterial communities in the environment is very sparse

10   (Coolen and Overmann, 1998; Coolen et al., 2006; Willerslev *et al.*, 2004b) which can be

11   attributed to the difficulty to distinguish ancient from modern bacterial DNA.

12   All cultured representatives of the green sulfur bacteria are obligately anaerobic

13   photolithoautotrophs and, accordingly, require the simultaneous presence of light and sulfide for

14   growth. The family *Chlorobiaceae* comprises all known green sulfur bacteria plus numerous

15   environmental sequences (Overmann and Tuschak, 1997; Overmann *et al.*, 1999; A. Manske and

16   J. Overmann, submitted). Similar to their cultured relatives, all so-far-uncultured members of

17   this group have been exclusively detected in illuminated sulfidic environments like the

18   chemocline of lakes, lagoons or benthic microbial mats. As the sequences recovered in the

19   present study clearly fall within this group, the available evidence indicates that they originated

20   from obligately anaerobic photolithoautotrophs. All our attempts to enrich green sulfur bacteria

21   by different cultivation methods (Overmann, 2001; Manske *et al.*, 2005) failed, supporting the

22   view that viable green sulfur bacteria do not exist in these sediments. In conclusion, the green

23   sulfur bacterial sequences obtained in the present study are highly unlikely to originate from

24   bacteria growing within the sediments. Rather, the cells grew outside the sediments and the

25   aphotic portion of the water column and were subsequently deposited in Mediterranean deep sea

26   sediments.

1   DNA spontaneously decays into 100 - 600 bp short fragments after its deposition in

2   lacustrine sediments (Coolen and Overmann, 1998). Based on our data, green sulfur bacterial

3   genome fragments at least 540-bp in size persisted in Mediterranean sapropels over 217,000

4   years. Survival of ancient DNA in a lake sediment could be demonstrated for a time period of

5   9,100 years (Coolen and Overmann, 1998) due to the short lifespan of this ecosystem. The

6   partial 16S rRNA gene fragments obtained in this study are among the oldest authenticated

7   ancient bacterial sequences available to date. For animals and plants, the oldest authenticated

8   records come from 60,000-year-old remains of brown bears (Barnes *et al.*, 2002), and from

9   ~400,000-year-old chloroplast DNA (Willerslev *et al.*, 2003), respectively, both recovered from

10   Alaskan permafrost. The age of the fossil 16S rRNA gene sequences of green sulfur bacteria

11   detected in the present study falls well within this time frame. Based on the relatively high *in situ*

12   temperature of 14°C, however, the efficient preservation of fossil DNA in eastern Mediterranean

13   sediments must be attributed to factors other than low temperature.

14   Besides low temperatures, the persistence of fossil DNA is significantly extended by high

15   ionic strength, anoxic conditions, and rapid dehydration and adsorption of DNA (Lindahl, 1993;

16   Poinar *et al.*, 1996; 2003; Willerslev *et al.*, 2004a). The ionic strength and anoxic conditions in

17   eastern Mediterranean sediments are comparable to other sediment environments. Adsorption to

18   clay and other mineral surfaces significantly decreases the degradation rates for organic

19   compounds in soils (Jones and Edwards, 1998), and marine sediments (Keil *et al.*, 1994) by up to

20   five orders of magnitude. Adsorptive binding to hydroxyapatite retards the spontaneous decay of

21   DNA (Lindahl, 1993). In addition to spontaneous hydrolysis, microbial DNase activity leads to

22   rapid degradation of free DNA in water and sediments (Lorenz and Wackernagel, 1994), but is

23   also effectively prevented by adsorption even in sandy sediments which bind DNA much less

24   efficiently than the sapropels (Romanowski *et al.*, 1991; Crecchio and Stotzky, 1998). Based on

25   our results, the outstanding adsorption capacity of eastern Mediterranean sediments, in particular

26   of the sapropel matrix, represents the major reason for the efficient preservation of fossil DNA of

1   green sulfur bacteria. Potentially, sapropels may also represent important archives of fossil DNA

2   from other microorganisms.

3   *Implications for the reconstruction of past ecosystems*

4   To date, isorenieratene and its degradation products have been used as an indicator of past water

5   column anoxia (Passier *et al.*, 1999; Menzel *et al.*, 2002). Because of their very limited diversity,

6   carotenoids do not permit a differentiation between species with contrasting physiology and

7   ecology. In the eastern Mediterranean, isorenieratene has previously been shown to occur in $\geq$1.8

8   million-year-old Pliocene sapropels (Passier *et al.*, 1999; Menzel *et al.*, 2002). However, only

9   four of the 83 currently recognized sapropels (Emeis *et al.*, 2000) have been investigated so far.

10 It was therefore unknown whether isorenieratene represents a typical constituent of the

11 sapropels. The present study provides the first instance of occurrence of isorenieratene in marine

12 deposits which are sufficiently young to also harbor 16S rRNA gene sequences of green sulfur

13 bacteria. The latter are high resolution fossil biomarkers which permit an improved assessment

14 of their origin and hence a more detailed reconstruction of the paleoenvironment.

15   All but one (Z1C) of the 16S rRNA gene sequences recovered were phylogenetically

16 affiliated with typical freshwater or brackish water species, whereas no single representative of

17 the marine group 1 could be detected. Secondly, the presence of green sulfur bacterial 16S rRNA

18 genes in intermediate layers was unexpected since the latter were deposited under a fully

19 oxygenated water column (Schmiedl *et al.*, 1998). Theoretically, DNA could have reached

20 intermediate layers through vertical percolation after deposition in the sapropel layers. However,

21 vertical migration of fossil DNA fragments should be independent of their actual base sequence

22 and, as a result, sequence types present in sapropels should also be detectable in the adjacent

23 intermediate layers. Yet, phylotype C was detected in intermediate layer Z_1, but not in the

24 adjacent S_1 (compare Fig. 4). This indicates that the strong adsorption to the sediment particles

25 has effectively immobilized the fossil DNA of green sulfur bacteria. Thirdly, a long-distance

26 transport of green sulfur bacterial biomarkers has been shown for North Atlantic deep-sea

1   sediments (Rosell-Melé *et al.*, 1997). Indeed, a turbidite origin was proposed for the intermediate

2   layer Z1 (K.-C. Emeis, pers. comm.) which contained the by far highest fraction of green sulfur

3   bacterial DNA among all organic carbon lean intermediate layers. It thus appears feasible that at

4   least the DNA of green sulfur bacterial phylotypes affiliated with typical freshwater species may

5   have originated from environments like coastal lagoons where these bacteria frequently form

6   dense blooms.

7   In conclusion, our results suggest that not all green sulfur bacteria deposited in the ancient

8   Mediterranean Sea during the last 217,000 years were autochthonous but instead are more likely

9   to have originated from Mediterranean coastal environments. Our cumulative evidence casts

10   some doubts on the hypothesis of the presence of green sulfur bacteria in the open waters of the

11   eastern Mediterranean and, by inference, questions the alleged past photic zone anoxia. A future

12   isolation and physiological characterization of the two previously unknown phylotypes detected

13   in the present study will help to more precisely assign a certain habitat, hence origin, to the fossil

14   remains of green sulfur bacteria present in the sapropel layers.

15

1   **EXPERIMENTAL PROCEDURES**

2   *Sampling and sample preparation*

3   A gravity core (#69-2SL) was obtained during cruise 40 leg 4 of the R/V Meteor on January 30,

4   1998, at position 33°51,53'N and 24°54,46'E southeast of Crete. The core was collected at a

5   depth of 2155 m and was subsampled employing the aseptic techniques developed for the

6   recovery of fossil DNA from sediment samples (Coolen and Overmann, 1998; 2000). It was cut

7   longitudinally which left behind a potentially contaminated surface. This surface was rapidly

8   frozen with powdered dry ice and subsequently lifted off. Through the freshly exposed surface, 5

9   cm^3 subsamples were retrieved aseptically using sterile plastic syringes which had their ends cut

10   off and were immediately stored in sterile vials at -80^0C until extraction.

11   *Precautions and controls to prevent contamination*

12   Preparation of sediment samples was performed directly after retrieval of sediment cores in the

13   laboratory on board of the R/V Meteor, employing the aseptic DNA techniques established

14   previously (Coolen and Overmann, 1998; Coolen *et al.*, 2006). Green sulfur bacteria had never

15   been introduced or worked with in these premises. Further precautions against contamination of

16   the samples with foreign DNA included the use of a laminar flow hood dedicated to low

17   template number samples. Prior to each use, the hood was UV-sterilized for 4 hours and all

18   surfaces were subsequently sterilized with sodium hypochlorite. Nucleic acids-free disposable

19   plastics were used throughout and autoclaved before use. All solutions were prepared in fresh

20   double quartz-distilled water, sterile filtered, and autoclaved. As a control for contamination

21   during DNA-extraction, two procedural blanks without sediment were subjected to the whole

22   extraction and purification procedure along with the sediment samples. One µl of each of these

23   extraction controls (corresponding to the average volume of sediment extracts used for

24   amplifications) was included in subsequent PCR amplifications. As additional controls, each

25   amplification included reactions without DNA template to independently control for

26   contamination of PCR reagents.

1 *Pigment analysis*

2 Pigments were extracted in the dark from 10 grams of freeze-dried and finely ground sediment

3 samples. Acetone/methanol (7/2, v/v), acetone, and dichloromethane were used for successive

4 extractions, followed by alkaline hydrolysis of the sediment with 6% KOH in methanol (Glaeser

5 *et al.*, 2002). To the latter, distilled water and dichloromethane were added and after phase

6 separation, the organic phases were combined and concentrated by rotary evaporation. The

7 extracts were subsequently dried under a flow of nitrogen. Individual pigments were quantified

8 by reverse-phase HPLC (Glaeser *et al.*, 2002) employing a NovaPak C_{18} end-capped 60Å 4 μm

9 4.6 × 250 mm column (Waters) with a Spherisorb 5 ODS 2 4.6 × 10 mm guard column (Waters).

10 The detection limit of this method is 1.0 ng isorenieratene·(g dry weight sediment)$^{-1}$.

11 For mass spectroscopy, the residue was redissolved in dichloromethane and applied to a

12 silica column. The apolar carotenoids were eluted with dichloromethane, dried under nitrogen

13 and the residue dissolved in acetone. This carotenoid fraction was then immediately analyzed on

14 a HP 1100 series LC/MS equipped with an auto-injector and photodiode array detector.

15 Separation was achieved on a ZORBAX Eclipse XDB-C_{18} column (2.1 × 150 mm, 5 μm; Agilent

16 Technologies, USA), maintained at 25ºC, with a linear gradient from 100% solvent A to 100%

17 solvent B in 50 min at a flow rate of 0.6 ml·min^{-1}. Solvent A was methanol/water (4:1, v/v) and

18 solvent B acetone/methanol/water (19:1:1, v/v/v). Total run time was 60 minutes. Detection was

19 achieved by in-line UV-detection (250–700 nm) and positive ion APCI (Atmospheric Pressure

20 Chemical Ionization) of the eluent in either scanning or Single Ion Monitoring (SIM) mode.

21 Conditions for APCI-MS were as follows: nebulizer pressure 60 psi, vaporizer temperature

22 325°C, drying gas (N_2) flow 7 L·min^{-1} and temperature 350°C, capillary voltage 3000V, corona 4

23 μA. Positive ion spectra were generated by scanning *m/z* 100-1000. In SIM mode *m/z* 529

24 (protonated molecule of isorenieratene) was monitored. Isorenieratene was quantified by

25 comparing its UV response at 454 nm (the λ_{max} of isorenieratene in the mobile phase) to known

26 amounts of an authentic β-carotene standard (Aldrich) and correcting for the difference in

27 extinction coefficients (Britton, 1995).

1 *Extraction of genomic DNA*

2 Each sediment sample was aliquoted in ten 2 ml bead-beat vials and 0.7 g of glass beads

3 (0.1 mm diameter) and 0.9 ml lysis buffer (100 mM Tris-HCl, 500 mM sodium-EDTA and

4 1 wt% SDS; pH 8.0) were added to each vial. The sediment samples were pre-heated for 5

5 minutes at 70°C in a water bath. Cell-lysis was accomplished by bead-beating at 5,000 rpm for

6 80 sec (Biospec Mini Bead-Beater; Bartlesville, Oklahoma, USA) followed by another

7 incubation at 70°C for 30 min. Following centrifugation of the samples in a microfuge (2 min;

8 14,000 rpm), the supernatants were transferred to a 45 ml teflon centrifuge tube. For extraction

9 of extracellular, adsorbed DNA, each pellet was resuspended in 0.8 ml of 0.12 M sodium

10 phosphate buffer (pH 8.0) containing 1% wt/vol SDS, followed by a second round of preheating,

11 bead-beating and heating. Following centrifugation, the supernatants were recovered. Finally,

12 each subsample was washed three times with 0.8 ml of 0.12 M sodium phosphate buffer (without

13 SDS). For each sediment, the supernatants of all subsamples were pooled, yielding a total

14 volume of about 40 ml.

15   Organic carbon in Mediterranean sapropels contains long chains of polymethylenic carbon

16 (Petsch *et al.* 2001), which copurified with the genomic DNA and inhibited subsequent PCR. For

17 removal of the kerogen, several consecutive purification steps were required. Most of the

18 kerogen could be removed from the crude DNA extracts by adding 3 grams of autoclaved, acid

19 washed polyvinylpolypyrrolidone (PVPP; Zhou *et al.*, 1996) to each sample. After an incubation

20 for 20 min on a rotary shaker, the samples were centrifuged for 20 min at 20,400 x g (Beckman

21 J2-HS, München, Germany), and the supernatants were transferred to a new sterile centrifuge

22 tube. Extractions with phenol, phenol/chloroform/isoamylalcohol, and chloroform followed

23 (Sambrook *et al.*, 1989) and the DNA was recovered by standard ethanol precipitation. Finally,

24 the resulting pellet was washed with sterile double distilled water using a Centricon 50

25 ultrafiltration unit (Amicon; Witten, Germany) and purified with the Wizard PCR-preps DNA-

26 purification kit (Promega, Mannheim, Germany). The DNA concentrations in the extracts were

27 quantified by fluorescent dye binding with PicoGreen (MoBiTec, Göttingen, Germany).

1 *Amplification of 16S rRNA genes of green sulfur bacteria*

2 16S rRNA sequences of green sulfur bacteria were selectively amplified with eubacterial primer

3 341f and the group-specific primer GSB822r, using the previously published cycling conditions

4 (Overmann *et al.*, 1999). The phylum green sulfur bacteria consists of a crown group of closely

5 related green sulfur bacteria *sensu strictu* (the family *Chlorobiaceae*) as well as an increasing

6 number of deep-branching phylotypes which are all uncultured and hence of unknown

7 physiology. However, all members of the crown group investigated to date are typical

8 unicellular, obligate photolithoautotrophs. We reassessed the specificity of primer GSB822 by

9 also including the novel database entries for 16S rRNA gene sequences. Of a total of 86

10 sequences of *Chlorobiaceae* tested, 11 showed a base substitution (A for T) at the 3´-end of the

11 target sequence: 5´-AATACTAGATGTTGG(A instead of T)CAT-3´. In three other phylotypes,

12 different base substitutions were found. All but one of these sequences fell into group 4a (Fig. 5),

13 while the remaining was a member of group 3. In contrast, all truly marine phylotypes contained

14 the probe target sequence. Based on this analysis, the PCR method was therefore found to be

15 suitable to detect in particular the marine members of the green sulfur bacteria.

16 For separation by DGGE, a 40 bp-long GC-clamp has to be added at one end of the PCR

17 product during amplification, employing primer GC 341f which carries the GC clamp (5'-

18 CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC-3') at its 5´-end (Muyzer *et*

19 *al.*, 1998)). Initial experiments revealed a significantly reduced detection limit of green sulfur

20 bacterial 16S rRNA genes when GC341f was employed directly for amplification. Consequently,

21 the GC clamp was added by performing 15 additional cycles with 1 µl of the primary

22 amplification and with 0.5 pmoles each of primers GC341f and GSB822r.

23 In order to control for the specificity of the amplification conditions, genomic DNA of

24 *Chlorobium phaeobacteroides* strain MN1 (Overmann *et al.*, 1992) and *Chl. phaeovibrioides*

25 DSMZ 269[T] were used as positive controls. Since the *Bacteroidetes* represent the sister group of

26 the phylum green sulfur bacteria, DNA of *Cytophaga* sp. strain 2b served as a negative control.

27 Purity of the PCR reagents was checked by including two reactions without DNA template in

1   each amplification trial. Possible contaminations during DNA extraction were checked by

2   including reactions spiked with one µl of each of the extraction controls (see above).

3   *Denaturing gradient gel electrophoresis (DGGE)*

4   PCR-products were separated by DGGE (Muyzer *et al.*, 1998) in a Bio-Rad D Gene system

5   (Biorad, München, Germany). PCR samples were applied directly onto 6% (wt/vol)

6   polyacrylamide gels (acrylamide/N,N'-methylene bisacrylamide ratio, 37:1 [w/w]) in 1 x TAE

7   buffer (pH 7.4) which had been prepared from sterile solutions and were cast aseptically between

8   sterilized glass plates. The gels contained a linear gradient of 30% to 70% denaturant (100%

9   denaturant correspond to 7 M urea plus 40% [v/v] formamide). Electrophoresis proceeded for 5 h

10   at 200 V and 60°C. Afterwards, gels were stained for 20 min with sterile ethidium bromide

11   solution and photographed. Finally, individual fragments were excised with a sterile scalpel,

12   the DNA was eluted in sterile 1 x TAE (pH 8.0) by electrophoresis (3 h, 200 V) in Centricon 50

13   concentrators inserted into a Centrilutor Micro Electroelutor (Amicon, Witten, Germany).

14   *Sequencing and phylogenetic analysis*

15   One µl of the eluted DNA was reamplified with primers 341f and GSB840r. Primers and

16   deoxyribonucleoside triphosphates were removed using the QIAquick PCR Purification Spin Kit

17   (Qiagen, Hilden, Germany). After cycle sequencing with the SequiTherm EXCEL Long-Read

18   Sequencing Kit-LC (Biozym, Hess. Oldendorf, Germany) and employing primers 341f and

19   GSB840r, sequence data were collected with a LiCor-4000 automated sequencer (Lincoln,

20   Nebraska, USA).

21   Each sequence was checked for chimeras employing the CHECK_CHIMERA option of the

22   ribosomal database project (RDP). The 16S rRNA gene sequences were then analyzed using the

23   ARB phylogeny software package (Ludwig *et al.*, 2004). The Fast Aligner V1.03 tool was used

24   for automatic alignment and the resulting alignments were corrected based on the 16S rRNA

25   secondary structure information for *Chlorobium vibrioforme* DSMZ 260[T], as available through

26   The Comparative RNA Web (CRW) Site (www.rna.icmb.utexas.edu; Cannone *et al.*, 2002).

1   Phylogenetic trees were constructed including 16S rRNA gene sequences of available strains and

2   environmental sequences. First, sequences longer than 1100 bp were used for the calculation,

3   employing the MAXIMUM LIKELIHOOD algorithm (Fast DNA_ML). The shorter

4   environmental sequences were inserted afterwards without changing overall tree topology

5   employing the PARSIMONY INTERACTIVE tool implemented in the ARB software package.

6   The 16S rRNA gene sequences obtained during the present study have been deposited in

7   GenBank under accession numbers AF298531-AF298537.

8   *Quantification of DNA of green sulfur bacteria by dot blot hybridization*

9   For dot blot quantification, 16S rRNA gene sequences of green sulfur bacteria were amplified

10   with an altered PCR protocol. Instead of a step down, primer annealing was performed at a

11   constant temperature of 52°C for 40 s and 31 cycles. Samples were denatured for 7 min at 100°C

12   and vacuum blotted onto positively charged nylon membranes (Boehringer Mannheim,

13   Germany). The membrane was baked (25 min at 120°C) and pre-hybridized in 10 ml of DIG

14   Easy Hyb buffer (Boehringer). Afterwards, 150 pmole of a highly specific probe for green sulfur

15   bacteria (5'- TGCCACCCCTGTATC-3'; *E. coli* positions 532 to 546; Tuschak *et al.*, 1999), 5'

16   labeled with dig-11-dUTP (MWG-Biotech, Germany), was added and hybridization was carried

17   out for 12 h. After hybridization, the blot was washed twice for 5 min in 2 x SSC (150 mM

18   NaCl, 15 mM Na-citrate, pH 7.0) plus 0.1% SDS, followed by two stringent washing steps

19   (15 min in 0.1xSSC/0.1% SDS). The hybridization signal was detected by chemiluminescence

20   with the DIG Luminescent Detection Kit (Boehringer). Lumi-Film (Boehringer) was exposed for

21   30 min, developed and the image digitized with a flatbed scanner. For quantification of the

22   individual dots, the ZERO-Dscan software (Scanalytics, Billerica, USA) was employed.

23   Genomic DNA of *Chlorobium phaeovibrioides* DSMZ 269[T] was used for calibration. The

24   homology of the 16S rRNA gene sequences of other Bacteria at the target site of probe 532 is

25   low, which reflects the large phylogenetic distance of green sulfur bacteria to other bacteria

26   (Overmann and Tuschak, 1997). Therefore we chose DNA of *Cytophaga* strain 2b (which,

1 however shows 5 mismatches) as a negative control for the specificity of the hybridization. For

2 each sample, quantification was conducted in three independent parallels. The quantification was

3 based on the assumption that genome sizes of green sulfur bacteria and the number of rRNA

4 operons are similar. Based on the currently available information, these assumptions appear to be

5 largely valid since genome sizes of green sulfur bacteria show comparatively little variation in

6 length (1.97 to 3.13 Mb) and typically comprise one (33% of the genomes) or two (66% of the

7 genomes) *rrn* operons (http://genome.jgi-psf.org/draft_ microbes/).

8 *Adsorption capacity for DNA of the sapropels*

9 In order to study the potential protection of fossil DNA by adsorption, equilibrium adsorption

10 isotherms were determined. Herring sperm DNA (Boehringer Mannheim) at concentrations

11 between 1 and 10 $\mu g \cdot ml^{-1}$ was incubated for 12 hours with aliquots (5 mg dry weight$\cdot ml^{-1}$) of

12 sapropel S_6 and the intermediate layer Z_6. Adsorption of DNA was observed to reach a

13 maximum and therefore yielded the best fit with the Langmuir equation (Ogram *et al.*, 1987)

14 according to:

$$S = S_{max} \cdot \frac{K \cdot C_e}{(1 + K \cdot C_e)} \tag{1}$$

16 Equation (1) was fitted to the data points of the amount S of DNA adsorbed ($\mu mol \cdot$(g dry wt)$^{-1}$)

17 and the concentration C_e of the substance remaining in solution (in $\mu mol \cdot ml^{-1}$). This yielded an

18 estimate for the maximum adsorption capacity S_{max} ($\mu mol \cdot$(g dry wt)$^{-1}$) and the Langmuir affinity

19 coefficient K (ml$\cdot \mu mol^{-1}$). For comparison, equilibrium adsorption isotherms of DNA for

20 montmorillonite and cellulose were also measured.

21

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8

9

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33

1   **FIGURE LEGENDS**

2   **Fig. 1. A.** Total DNA content (●) at eight consecutive depths of core #69-2SL. The vertical

3   positions of the sapropel layers (black rectangles) and of sampling depths are denoted on

4   the right. **B.** DNA of green sulfur bacteria as fraction of total DNA (■). **C.** Absolute

5   amounts of green sulfur bacterial DNA as determined by dot blot quantification (▲) and

6   of the carotenoid isorenieratene (o). Horizontal bars indicate one standard deviation.

7   **Fig. 2.** Amplification products generated with primers GC341f and GSB822. Controls for

8   contamination of PCR reagents (no template), for contamination with extraneous DNA

9   (extraction controls, see Experimental procedures) and for primer specificity (*Cytophaga*

10   sp. strain 2b) were included in each PCR run. *Chlorobium phaeovibrioides* DSMZ 269^T

11   and *Chlorobium phaeobacteroides* strain MN1 served as positive controls. A negative

12   image of an ethidium bromide-stained agarose gel is shown.

13   **Fig. 3.** Dot blot quantification of fossil DNA after amplification of 2 ng each of sample DNA.

14   The standard curve was obtained by using different amounts (given in pg) of genomic

15   DNA of *Chlorobium phaeovibrioides* DSMZ 269^T as template. As negative controls, two

16   independent PCR amplifications were conducted either without DNA template (n.t.), with

17   1 µl of the extraction controls (Extr., compare Experimental Procedures), or with 2 ng of

18   genomic DNA of *Cytophaga* strain 2b (*Cyt*).

19   **Fig. 4.** Separation of green sulfur bacterial 16S rRNA gene fragments by DGGE. For

20   comparison, amplification products from the two brown-colored marine strains

21   *Chlorobium phaeovibrioides* DSMZ 269^T and *Chl. phaeobacteroides* MN1 (Overmann *et*

22   *al.*, 1992) were included. DNA bands used in the subsequent phylogenetic analyses are

23   denoted by labels A through G. Asterisks denote sequences which did not yield

24   unambiguous sequences. Six different melting types are marked with arrows.

25   **Fig. 5.** Phylogenetic position of the seven fossil partial 16S rRNA gene sequences of green

26   sulfur bacteria (given in bold face; labeling refers to layer of origin plus the band no.

27   according to Fig. 4) recovered from sapropels and intermediate layers. Currently

28   recognized groups are indicated (Imhoff, 2003; denoted in rectangles). Groups comprising

29   salt tolerant or salt-requiring strains marked by black rectangles. Bar indicates 0.1 fixed

30   point mutations per nucleotide. Numbers at nodes give bootstrap values out of 100

31   resamplings for phylogenetic trees calculated by Maximum Likelihood / Maximum

32   Parsimony / Neighbor Joining methods. *C.*, *Chlorochromatium*; *Cba., Chlorobaculum*,

33   *Chl., Chlorobium*; *Chp.*; *P. Pelochromatium*; *Pld., Pelodictyon*.

34

1   **Fig. 6.** Equilibrium adsorption isotherms of double-stranded herring sperm DNA bound to

2   sapropel S6 (●) and the intermediate layer Z6 (o). Bars indicate one standard deviation.

3   Lines indicate the curves fitted to the data according to the Langmuir equation (eq. 1, see

4   text).

5

1   **Table 1.** Parameters of the Langmuir equilibrium adsorption isotherms for DNA binding to
2   different adsorbers
3

Adsorber	S_{max} [1] $\mu mol \cdot (g\ dry\ wt)^{-1}$	K $(ml \cdot \mu mol^{-1})$	Reference
Humic acids (pH 4)	250.9 ± 72.4	7.2 ± 3.8	Crecchio and Stotzky, 1998
Humic acids (pH 3)	112.1 ± 19.1	9.2 ± 4.4	Crecchio and Stotzky, 1998
Sapropel S_6 (50 mM $MgCl_2$)	79.3 ± 2.8	447 ± 63	this work
Intermediate layer Z_6 (50 mM Tris-HCl)[2]	18.0 ± 1.9	209 ± 116	this work
Ca^{2+}-montmorillonite (0.5 mM $CaCl_2$)	64.0 ± 12.1	286 ± 129	Paget et al., 1992
Mg^{2+}-montmorillonite (50 mM $MgCl_2$)	59.6 ± 6.3	41.8 ± 16.6	Barghorn and Overmann, unpubl.
Mg^{2+}-montmorillonite (2.0 mM $MgCl_2$)	14.2 ± 0.7	4099 ± 1063	Paget et al., 1992
Mg^{2+}-montmorillonite (0.5 mM $MgCl_2$)	12.6 ± 0.7	293 ± 48	Paget et al., 1992
Na^+-montmorillonite (25 mM NaCl)	1.76 ± 1.18	451 ± 706	Paget et al., 1992
Mg^{2+}-cellulose (50 mM $MgCl_2$)	0.755 ± 0.103	74.6 ± 23.1	Barghorn and Overmann, unpubl.
Memphis-soil	0.023	673	Ogram et al., 1987

4
5   [1] Molarity of DNA calculated as molarity of nucleotides.
6   [2] Due to precipitation in the presence of Mg^{++}, adsorption was determined in 50 mM Tris-HCl.
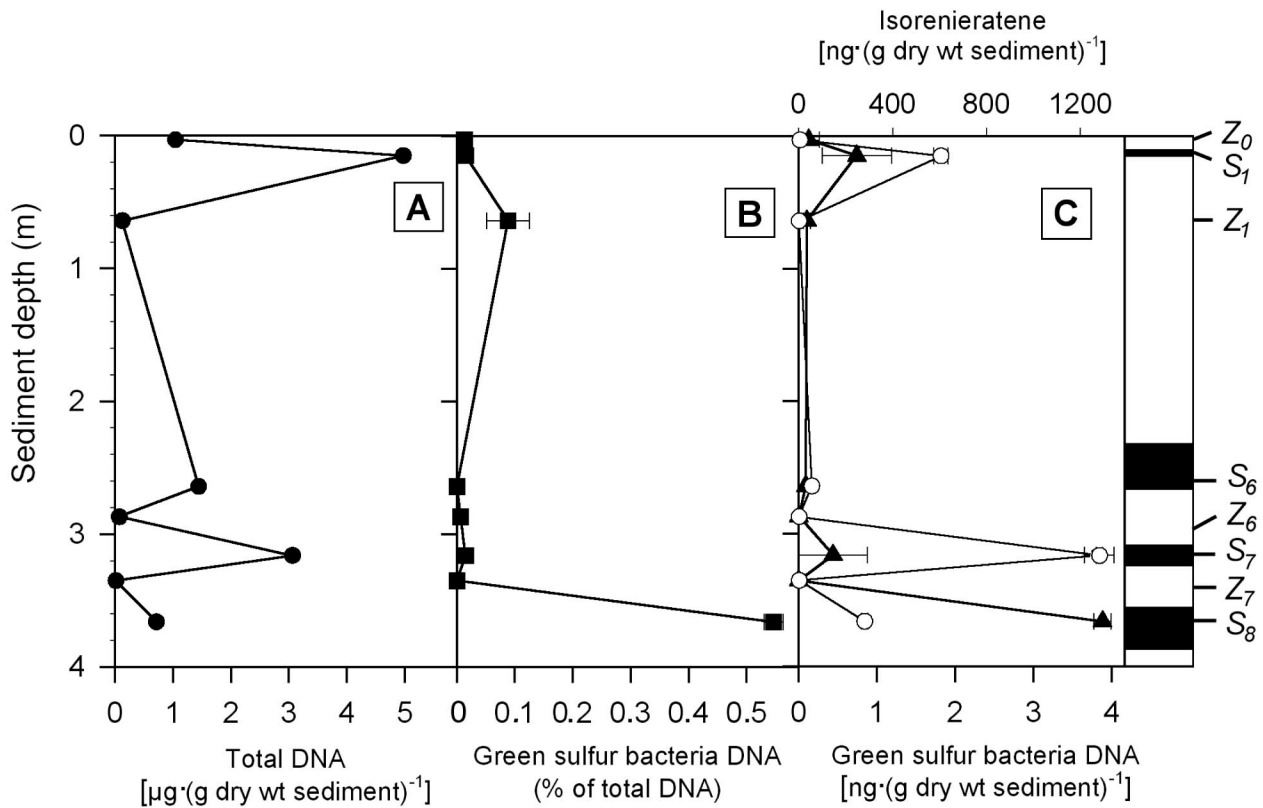
7

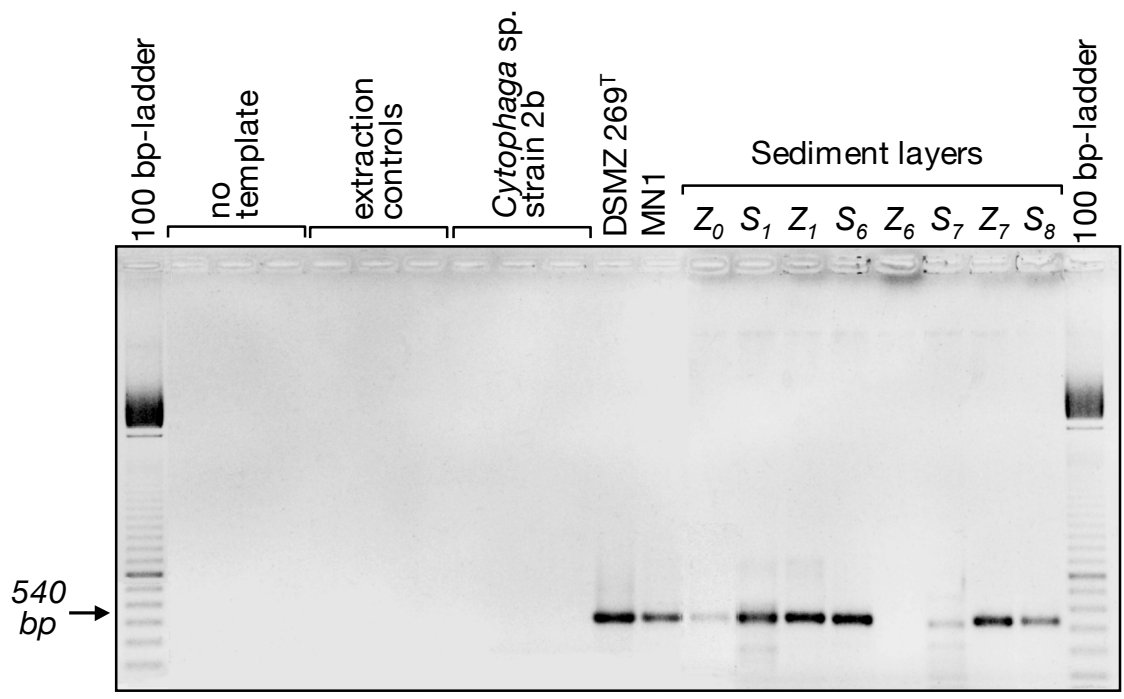Fig.1
Coolen and Overmann (2006)
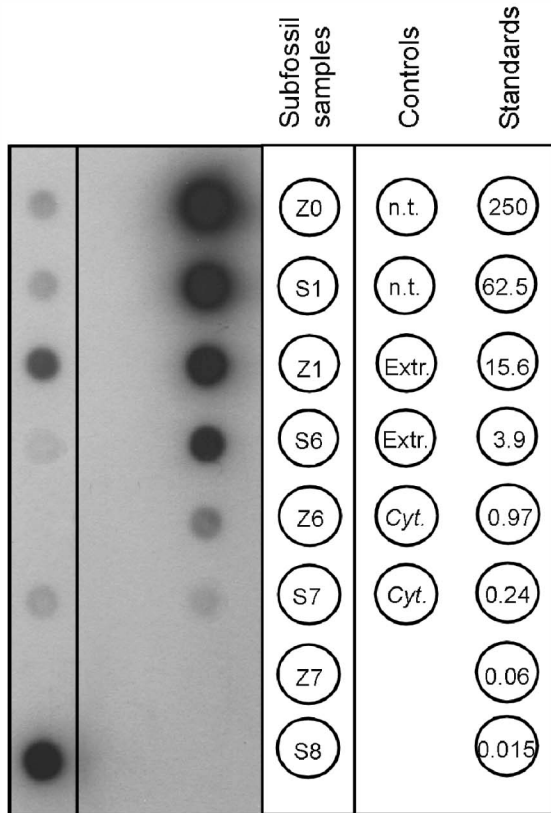
Fig. 2
Coolen and Overmann (2006)
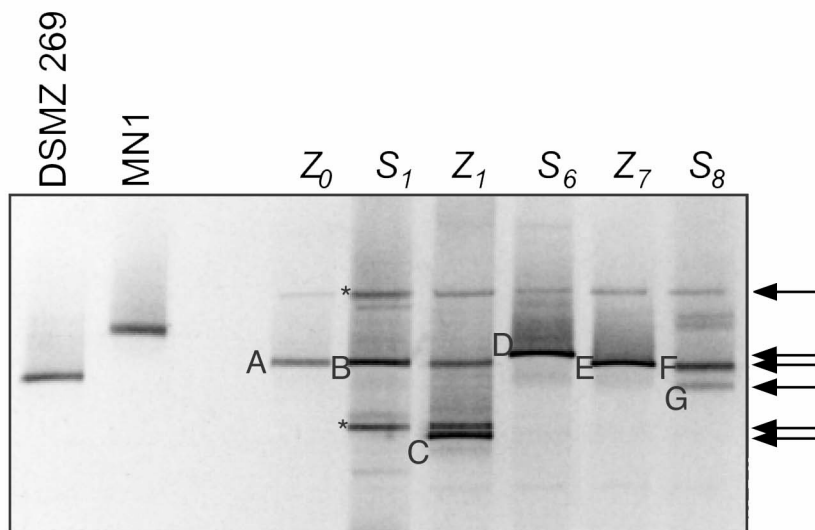
Fig. 3
Coolen and Overmann (2006)
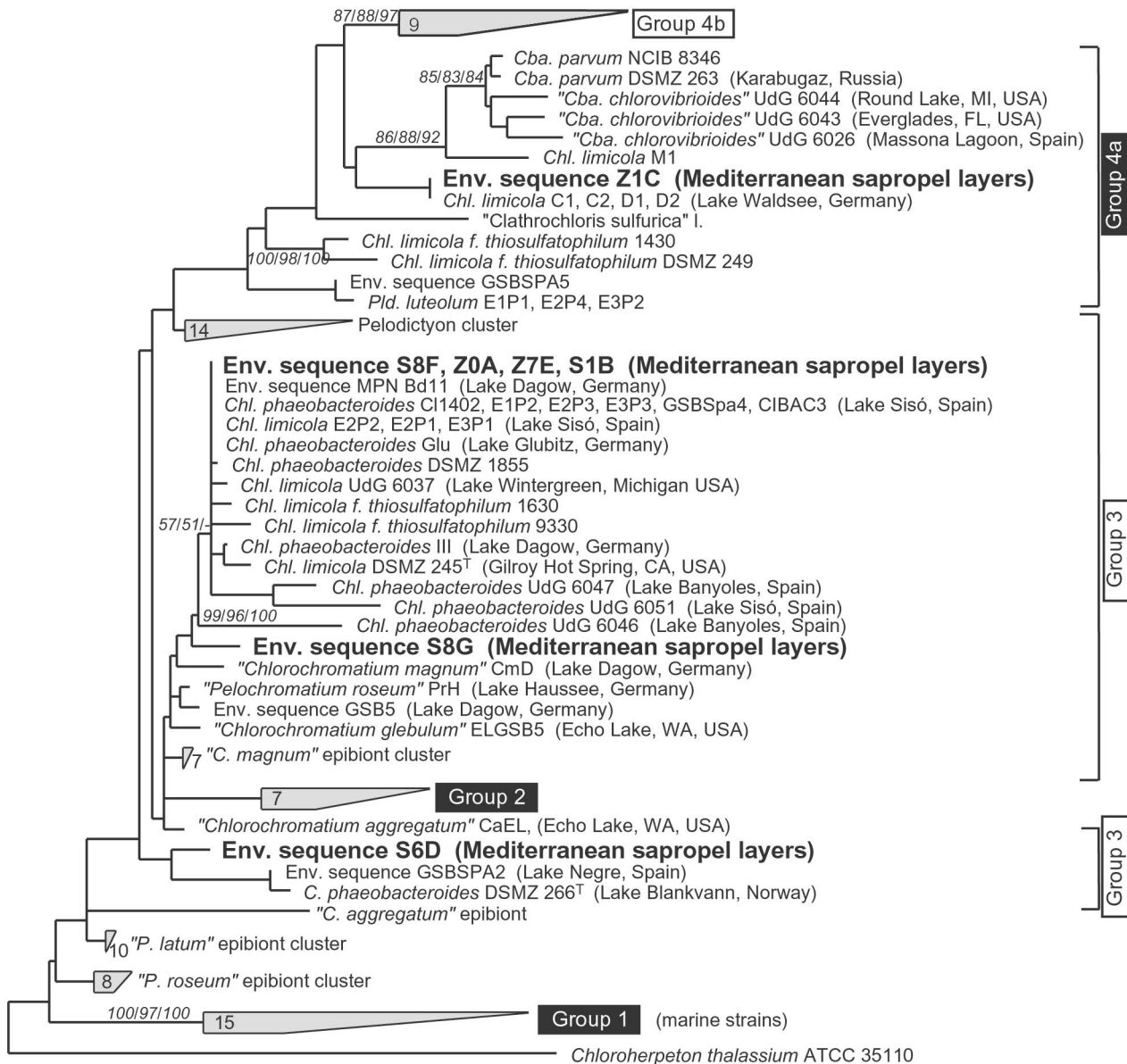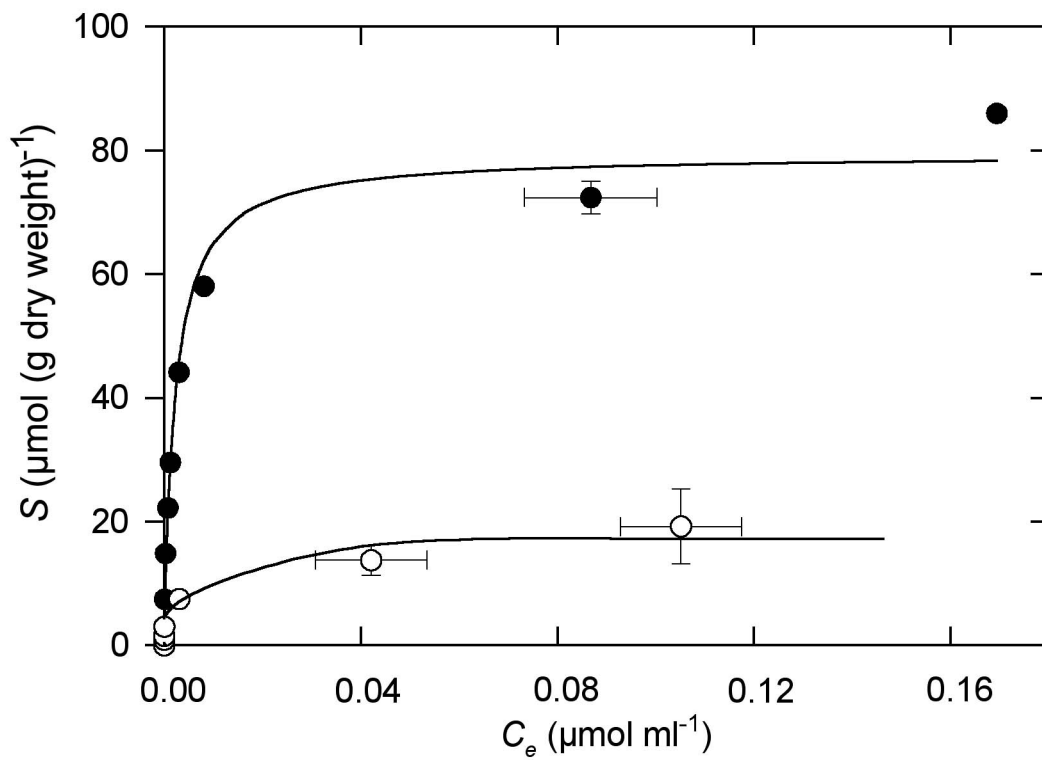
Fig. 4
Coolen and Overmann (2006)

Fig. 5
Coolen and Overmann (2006)

Fig. 6
Coolen and Overmann (2006)