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Inorganic carbon fixation by sulfate-reducing bacteria in the Black Sea water column

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Summary

The Black Sea is the largest anoxic water basin on Earth and its stratified water column comprises an upper oxic, middle suboxic and a lower permanently anoxic, sulfidic zone. The abundance of sulfate-reducing bacteria (SRB) in water samples was determined by quantifying the copy number of the *dsrA* gene coding for the alpha subunit of the dissimilatory (bi)sulfite reductase using real-time polymerase chain reaction. The *dsrA* gene was detected throughout the whole suboxic and anoxic zones. The maximum *dsrA* copy numbers were 5×10^2 and 6.3×10^2 copies ml^{-1} at 95 m in the suboxic and at 150 m in the upper anoxic zone, respectively. The proportion of SRB to total *Bacteria* was 0.1% in the oxic, 0.8–1.9% in the suboxic and 1.2–4.7% in the anoxic zone. A phylogenetic analysis of 16S rDNA clones showed that most clones from the anoxic zone formed a coherent cluster within the *Desulfonema–Desulfosarcina* group. A similar depth profile as for *dsrA* copy numbers was obtained for the concentration of non-isoprenoidal dialkyl glycerol diethers (DGDs), which are most likely SRB-specific lipid biomarkers. Three different DGDs were found to be major components of the total lipid fractions from the anoxic zone. The DGDs were depleted in ^{13}C relative to the $\delta^{13}C$ values of dissolved CO_2 ($\delta^{13}C_{CO2}$) by 14–19‰. Their $\delta^{13}C$ values [$\delta^{13}C_{DGD(II–III)}$] co-varied with depth showing the least ^{13}C-depleted values in the top of the sulfidic, anoxic zone and the most ^{13}C-depleted values in the deep anoxic waters at 1500 m. This co-variation provides evidence for CO_2 incorporation by the DGD(II–III)-producing SRB, while the 1:2 relationship between $\delta^{13}C_{CO2}$ and $\delta^{13}C_{DGD(II–III)}$ indicates the use of an additional organic carbon source.

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Introduction

The Black Sea is the largest anoxic water basin on Earth with an upper oxic water column down to approximately 80–160 m and an anoxic and sulfidic lower part. The interface between the oxic and anoxic waters, often defined as 'suboxic zone' (Murray *et al*., 1995), is characterized by high bacterial numbers, enhanced microbial production (Pimenov *et al*., 2000) and enhanced rates of carbon, nitrogen, sulfur and manganese turnover (Jørgensen *et al*., 1991; Tebo, 1991; Kuypers *et al*., 2003; Schippers *et al*., 2005a; Murray and Yakushev, 2006; Pimenov and Neretin, 2006). Recently, the microbial communities involved in anammox (Kuypers *et al*., 2003), denitrification (Oakley *et al*., 2007), and aerobic and anaerobic methane oxidation (Durisch-Kaiser *et al*., 2005; Schubert *et al*., 2006), as well as green sulfur bacteria (Manske *et al*., 2005) in the Black Sea water column, were intensively investigated with molecular techniques.

Sulfate-reducing bacteria (SRB) are key players in anoxic basins (Volkov, 2000). In the first compiled organic carbon budget of the Black Sea, Deuser (1971) suggested that at least half of the total particulate carbon which is transported into the anoxic water column is oxidized by SRB. Analysing the alkalinity of the Black Sea water column and C:S stoichiometry, Volkov and colleagues (1998) found that sulfate reduction is almost entirely (95%) responsible for the total inorganic carbon production and a total sulfide production of 30–50 Tg per year (Neretin *et al*., 2001) in the anoxic zone. Despite the obvious importance of sulfate reduction for carbon remineralization in the Black Sea water column, the organisms involved have been poorly characterized.

The purpose of this study was twofold: (i) to characterize the sulfate-reducing community using quantitative real-time polymerase chain reaction (Q-PCR) and 16S rRNA gene cloning and sequencing, and (ii) to gain an insight into the carbon fixation metabolism of SRB in the

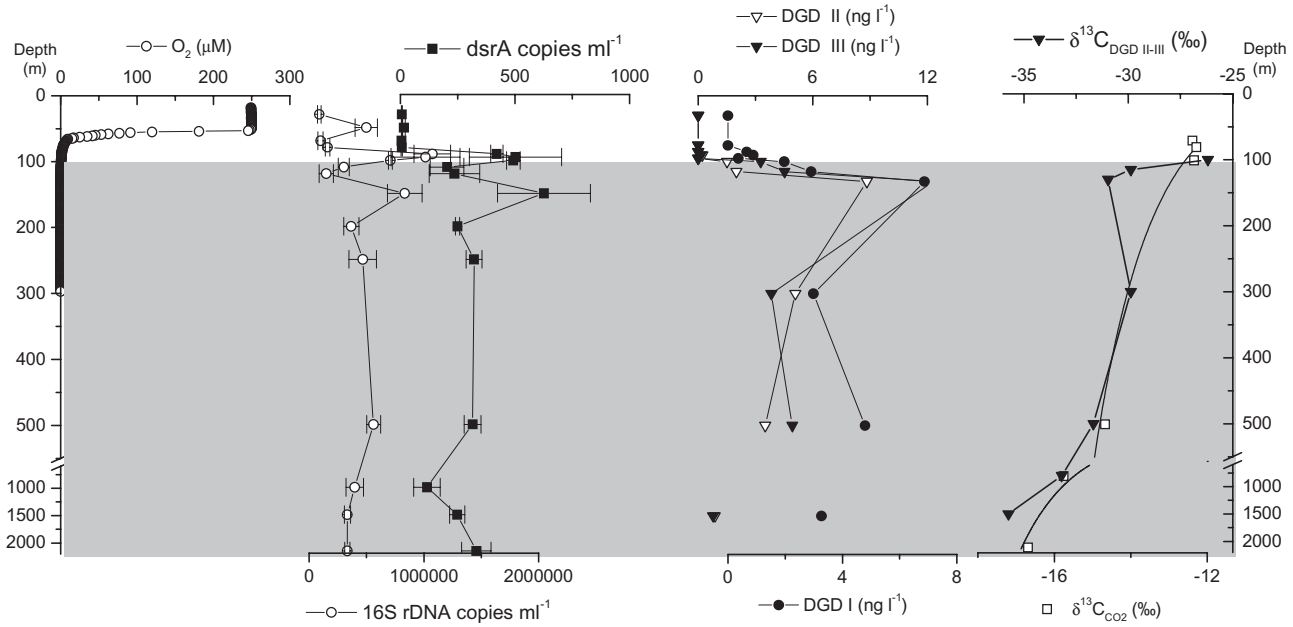

Fig. 1. Depth distribution of dissolved oxygen, the *dsrA* and 16S rDNA gene copy numbers, amounts of three types of dialkyl glycerol diethers (DGDs) as well as the carbon isotopic composition of DGDs and of dissolved CO_2 in the Black Sea water column at Station GeoB 7605. Oxic zone: 0 to ~80 m; suboxic zone: ~80 to ~100 m; anoxic, sulfidic zone: deeper than ~100 m (grey area).

Black Sea water column by profiling the carbon isotopic composition of specific lipid biomarkers.

Results and discussion

Samples were obtained during the M51-4 cruise of *R/V Meteor* in December 2001 at Station GeoB 7605 (42°30.71′N, 30°14.69′E) located in the western central basin. The station had a characteristic vertical distribution of physicochemical parameters. Dissolved oxygen concentrations were below detection from 80 m downwards and hydrogen sulfide was detected below 100 m (Manske *et al*., 2005). Thus, the suboxic zone could be defined between 80 and 100 m. The maximum prokaryotic cell numbers were observed from the upper suboxic zone down to 130 m and reached 3×10^5 cells ml^{-1} at 95 m (Manske *et al*., 2005). The vertical distribution of 16S rDNA copy numbers of *Bacteria* and *Archaea* assessed using Q-PCR was described by Schubert and colleagues (2006). Prokaryotic 16S rDNA copy numbers in the oxic part of the water column were below 7×10^4 copies ml^{-1} and sharply increased in the suboxic zone for both *Bacteria* and *Archaea* up to 2×10^5 copies ml^{-1} and 2.3×10^4 copies ml^{-1} respectively. In the anoxic zone below 300 m, the 16S rDNA copy numbers of *Bacteria* and *Archaea* were about 4×10^4 copies ml^{-1} and $2–3 \times 10^2$ copies ml^{-1} respectively.

The depth distribution of dissolved oxygen, the abundance of *dsrA*, 16S rDNA and dialkyl glycerol diethers (DGDs) as well as the carbon isotopic composition of DGDs and CO_2 is shown in Fig. 1. The number of *dsrA* gene copies was below 20 copies ml^{-1} in the oxic part and increased sharply together with the 16S rDNA copy numbers in the chemocline. The *dsrA* depth distribution had two maxima, the upper maximum in the suboxic zone at 95 m with 5.0×10^2 copies ml^{-1} and the lower maximum in the upper anoxic zone with 6.3×10^2 copies ml^{-1} at 150 m water depth. In the entire anoxic zone the number of *dsrA* copies varied between 1.2×10^2 and 3.3×10^2 copies ml^{-1} representing 1–5% of total bacterial abundance. These numbers are comparable to earlier estimates of 3–7% obtained by using fluorescence *in situ* hybridization (FISH) (Durisch-Kaiser *et al*., 2005). For comparison, the *dsrA* copy numbers in the upper sediment at this station assessed with a similar Q-PCR method were in the range from 10^6 to 10^7 copies ml^{-1} (A. Schippers and L. Neretin, unpublished data).

Using the sulfate reduction rates of Albert and colleagues (1995) for the central western Black Sea, we calculated cell-specific sulfate reduction rates in the water column. The rates varied between 1.4 and 6.8 fmol cell^{-1} day^{-1} at 2000 and 200 m, respectively, and were compared with those previously reported for marine sediments and cultures. Sedimentary cell-specific sulfate reduction rates vary between 0.01 and 0.14 fmol cell^{-1} day^{-1} (Sahm *et al*., 1999; Ravenschlag *et al*., 2000; Schippers *et al*., 2005b; Jørgensen *et al*., 2006; Teske, 2006). Cell-specific rates for SRB in laboratory cultures were considerably higher and varied between 0.3 and 434 fmol cell^{-1} day^{-1} (Knoblauch *et al*.,

1999; Detmers *et al*., 2001). This study provided the first direct *in situ* quantification of SRB in the Black Sea water column using functional gene abundance.

Polymerase chain reaction amplification of 16S rDNA fragments of the sulfate-reducing community at six water depths spanning the entire anoxic water column revealed that most clones (18) formed a coherent cluster closely related to *Desulfosarcina variabilis* and *Desulfonema magnum* (Fig. 2). The close relatives of clones 518, 522, 529, 644 and 647 (i.e. sequences with the Accession No. AF354161 and AJ535234) were among known anaerobic hydrocarbon-degrading bacteria and their occurrence in methane-rich sediments suggests that they may be involved in anaerobic methane oxidation in the water column. Recently, both archaeal ANME-1 and ANME-2 groups were detected in the Black Sea water column using FISH and sequencing techniques (Durisch-Kaiser *et al*., 2005; Schubert *et al*., 2006). The detection of aforementioned phylotypes, together with the earlier terminal restriction fragment length polymorphism (T-RFLP) analyses of the Black Sea microbial communities (Vetriani *et al*., 2003), suggests that anaerobic aromatic hydrocarbon degraders could play an important role in the Black Sea water column. Interestingly, only one clone within the *Desulfovibrio* group was obtained, while previously this group was supposed to be abundant in the Black Sea anoxic zone (Sorokin, 2002).

The presence of non-isoprenoidal DGDs in the Black Sea water column is reported for the first time here. Three different DGDs were found to be major components of the total lipid fractions from the anoxic zone. One of these compounds (DGD I) was identified as a DGD with an anteiso $C_{15}H_{31}$ group at both the *sn*-1 and *sn*-2 position respectively (Pancost *et al*., 2001). The other two compounds (DGD II and III) are unknown DGDs both containing a $C_{16}H_{31}$ and a $C_{16}H_{33}$ group. The three DGDs showed a depth distribution similar to that for the *dsrA* copy numbers, with no detectable DGDs in the oxic zone, a sharp increase in the base of the suboxic zone and a peak of up to 12 ng of DGDs per liter of sea water in the upper part of the anoxic zone (~130 m, Fig. 1).

So far DGDs were described only in a few environmental settings such as cyanobacterial mats, marine sediments and cold-seeps with anaerobic oxidation of methane (AOM) activity (e.g. Zeng *et al*., 1992; Hinrichs *et al*., 2000; Pancost *et al*., 2001). Sulfate-reducing bacteria are generally believed to be the main source for DGDs in the marine environment (e.g. Hinrichs *et al*., 2000; Pancost *et al*., 2001). Based on structural information of DGDs such as the presence of cyclopropyl groups, Pancost and colleagues (2001) proposed that DGDs, similar to those found in this study, could be derived from mesophilic SRB. The concentration profiles of DGD (I–III) in the Black Sea water column are very similar to the SRB abundance profile, suggesting that these DGDs indeed derive from sulfate reducers (Fig. 1).

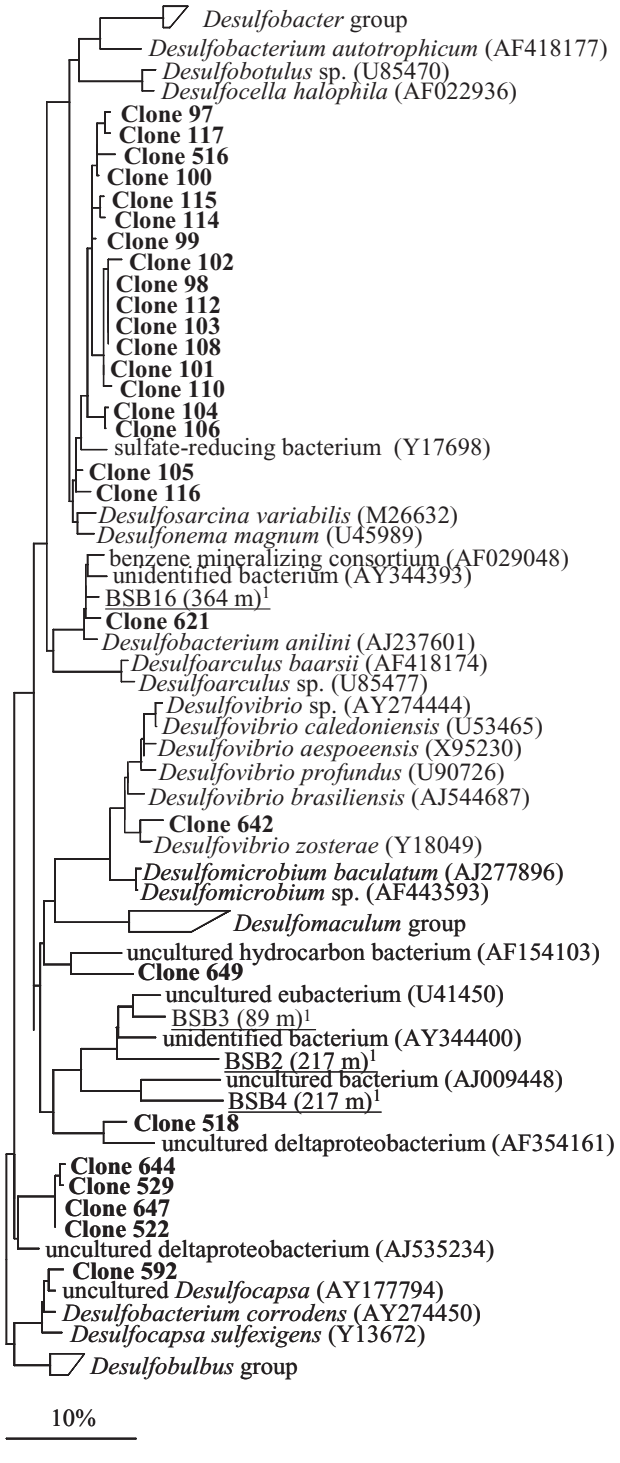

Fig. 2. Maximum likelihood phylogenetic tree of 16S rDNA gene sequences of SRB. Bold clones are from this study. [1]BSB clones are also from the Black Sea water column (Vetriani *et al*., 2003).

The stable carbon isotopic composition of the DGD I could not be determined due to co-elution with other

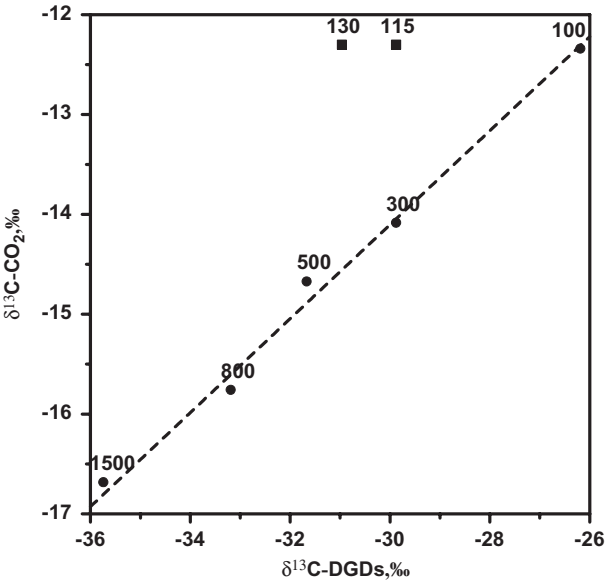

Fig. 3. The relationship between $\delta^{13}C_{CO2}$ and $\delta^{13}C_{DGD}$ in the Black Sea water column at Station GeoB 7605. Point labels are sampling depths (m). Two samples in the lower chemocline falling outside of the general fit curve are shown with squares.

compounds. Because DGDs II and III were not baseline separated, the combined carbon isotopic composition was determined. The combined isotopic signals were depleted in ^{13}C relative to the $\delta^{13}C$ values of dissolved CO_2 by 14–19‰ (Fig. 1). Intriguingly, the $\delta^{13}C$ values of DGD (II–III) and dissolved CO_2 co-varied with depth with both records showing the least ^{13}C-depleted values in the top of the sulfidic zone and the most ^{13}C-depleted values in the deep anoxic waters (1500 m). Relative to CO_2 ^{13}C-depleted carbon isotopic values of DGD II–III at 115 m and 130 m were apparently the only noteworthy exception and a possible explanation is given below.

When these two samples are excluded, the $\delta^{13}C$ values of DGD II–III and dissolved CO_2 show a strong linear correlation ($R^2 > 0.99$), with a ~2‰ change in $\delta^{13}C$ values of DGD II–III for every 1‰ change in the isotopic composition of dissolved CO_2 (Fig. 3). Such high correlation may indicate that the DGD(II–III)-producing SRB are at least partly growing autotrophically. Autotrophic growth with hydrogen as an electron donor has been reported for several SRB cultures and sediments (Sørensen *et al*., 1981; Brysch *et al*., 1987). However, the 1:2 relationship between $\delta^{13}C_{CO2}$ and $\delta^{13}C_{DGD(II–III)}$ indicates that SRB did not solely use inorganic carbon for biomass synthesis (autotrophic growth) in the anoxic Black Sea waters. A possible explanation for the observed 1:2 relationship is the utilization of an additional carbon source, for example, acetate. Most facultatively autotrophic sulfate reducers use the acetyl-CoA pathway during autotrophic growth (Brysch *et al*., 1987) and the CO_2 incorporation into bacterial biomass is associated with a large ^{13}C fractionation (Londry and Des Marais, 2003; Londry *et al*., 2004). Heterotrophic or mixotrophic growth of the same SRB is associated with smaller isotope effects (Londry and Des Marais, 2003; Londry *et al*., 2004). A mixed SRB population using CO_2 and short-chain fatty acids as carbon sources could therefore explain the observed 1:2 relationship between $\delta^{13}C_{CO2}$ and $\delta^{13}C_{DGD(II–III)}$. Due to the large differences in isotopic fractionation between different SRB species, the proportion of inorganic and organic carbon incorporated into SRB biomass cannot be estimated at this point (Londry and Des Marais, 2003; Londry *et al*., 2004). However, our stable carbon isotope data provide evidence for either a change in SRB community or an increase in autotrophic versus heterotrophic/mixotrophic growth with water depth in the Black Sea.

There is a distinct deviation from the general linear correlation between the $\delta^{13}C$ values of CO_2 and DGDs, with distinctly ^{13}C-depleted $\delta^{13}C$ values for DGDs, in the top of the anoxic zone ~130 m (Fig. 3), which coincides with a maximum in SRB abundance (Fig. 1). This zone is also characterized by maxima in short-chain fatty acids and sulfate reduction rates, which have been attributed to lytic release of readily degradable organic matter from particulate material settling into the anoxic waters from the upper oxic and suboxic zones (Albert *et al*., 1995). Schubert and colleagues (2006) have shown that anaerobic methane oxidation occurs in this zone. The sharp decrease in $\delta^{13}C$ values of the DGDs (Fig. 1) and the occurrence of ANME-1- and ANME-2-related SRB phylotypes at this depth could indicate that the DGD-producing SRB are involved in the anaerobic oxidation of methane. Alternatively, the isotopic shift could be explained by the growth of SRB on ^{13}C-depleted organic matter of chemosynthetic origin (Jørgensen *et al*., 1991; Kodina *et al*., 1996) or an increase in the relative contribution of CO_2-derived carbon to the SRB biomass.

Experimental procedures

Sampling

The Conductivity-Temperature-Depth (CTD) and dissolved oxygen profiles as well as water samples were obtained using a CTD (SeaBird) rosette equipped with 10 l Niskin water bottles. Particulate organic matter for lipid analyses was collected from specific water depths by *in situ* filtration of large volumes (~1000 l) of water through 292 mm diameter, precombusted (at 450°C) glass fibre filters (GFF; nominal pore size of 0.7 μM) using *in situ* pumps (Challenger Oceanic). Filters were kept frozen (–20°C) until lipid extraction. For DNA analyses, a total of 3–5 l of water was filtered through Sterivex (pore size of 0.2 μM) filter cassettes (Millipore, Bedford, MA, USA) using a Millipore peristaltic pump. Sterivex filters were stored at –20°C before DNA extraction.

Lipid analysis

Lipid extraction and analysis was performed as previously described (Schubert *et al*., 2006). The precise nature and position of the rings/double bonds in the DGDs was not assessed. As filtration through 0.7 μm pore size filters may lead to an under-sampling of prokaryotes, the calculated DGD concentrations represent minimum values.

DNA extraction and purification

Total DNA was extracted from Sterivex filters following the procedure of Massana and colleagues (1997). DNA was further purified using Microcon YM-100 filters (Millipore, Bedford, MA, USA) and quantified with a NanoDrop ND-1000 UV/Vis Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

16S rDNA and dsrA *quantification with Q-PCR*

Copy numbers of the 16S rRNA gene of prokaryotes and of the dissimilatory sulfite (bi)reductase gene (*dsrA*) were measured using Q-PCR as described elsewhere (Schippers and Neretin, 2006).

Phylogenetic analysis of SRB

DNA samples (*c*. 10 ng) from six different water depths were used as template for a two-step nested PCR. In the first step, the primer pair GM3F/GM4R was used to amplify the nearly complete sequence of 16S rRNA of the domain *Bacteria* (Muyzer *et al*., 1995). Subsequently, this product was used as a template for PCR using six SRB group-specific primers (Daly *et al*., 2000). Three selected PCR products from each depth at 125, 250 and 300 m were purified (QIA quick PCR purification kit, Qiagen, Hilden, Germany) and cloned (TOPO TA Cloning Kit, Invitrogen, Karlsruhe, Germany). The obtained clones were screened for the presence of inserts and the positive clones were sequenced. The obtained sequences were analysed using the ARB database (Ludwig *et al*., 2004). Phylogenetic trees were calculated by maximum parsimony based on long 16S rRNA sequences (> 1300 bp). The sequences were inserted into the reconstructed tree without allowing changes in the overall topology.

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