



Quantification of microbial communities in subsurface marine sediments of the Black Sea and off Namibia

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Organic-rich subsurface marine sediments were taken by gravity coring up to a depth of 10 m below seafloor at six stations from the anoxic Black Sea and the Benguela upwelling system off Namibia during the research cruises Meteor 72-5 and 76-1, respectively. The quantitative microbial community composition at various sediment depths was analyzed using total cell counting, catalyzed reporter deposition – fluorescence *in situ* hybridization (CARD–FISH) and quantitative real-time PCR (Q-PCR). Total cell counts decreased with depths from 10^9 to 10^{10} cells/mL at the sediment surface to 10^7 – 10^9 cells/mL below one meter depth. Based on CARD–FISH and Q-PCR analyses overall similar proportions of *Bacteria* and *Archaea* were found. The down-core distribution of prokaryotic and eukaryotic small subunit ribosomal RNA genes (16S and 18S rRNA) as well as functional genes involved in different biogeochemical processes was quantified using Q-PCR. *Crenarchaeota* and the bacterial candidate division JS-1 as well as the classes *Anaerolineae* and *Caldilineae* of the phylum *Chloroflexi* were highly abundant. Less abundant but detectable in most of the samples were *Eukarya* as well as the metal and sulfate-reducing *Geobacteraceae* (only in the Benguela upwelling influenced sediments). The functional genes *cbbL*, encoding for the large subunit of RuBisCO, the genes *dsrA* and *aprA*, indicative of sulfate-reducers as well as the *mcrA* gene of methanogens were detected in the Benguela upwelling and Black Sea sediments. Overall, the high organic carbon content of the sediments goes along with high cell counts and high gene copy numbers, as well as an equal abundance of *Bacteria* and *Archaea*.

Keywords: Benguela upwelling, Black Sea, CARD–FISH, deep biosphere, real-time PCR, sediments, subsurface

INTRODUCTION

The Black Sea and the Benguela upwelling system off the Atlantic coast of Namibia are both represent organic carbon-rich marine environments. Nevertheless, there are fundamental differences in the biogeochemistry and of these two settings. The Black Sea is the largest anoxic seawater basin on earth. It is characterized by high biological productivity in the oxygenated surface waters (Yilmaz et al., 2006). This and a deep anoxic water body below the chemocline at 100–150 m water depth (Neretin et al., 2007) provide suitable conditions for anaerobic microbial life and biogeochemical cycling in organic carbon-rich sediments. The sediments at water depths of 2000 m are under permanent anoxic and sulfidic conditions. Conversely, the Namibian continental margin underlies one of the most productive upwelling systems in the world. Periods of low oxygen, nitrate-rich, and sometimes sulfidic water, also leads to enhanced accumulation of organic carbon in the diatom-rich mud belt on the shelf. On the continental slope and rise the organic carbon flux to the underlying sediments is also relatively high. The sediments of these two marine areas have been well-studied for their biogeochemical processes (Niewöhner et al., 1998; Ferdelman et al., 1999; Brüchert et al., 2000, 2003, 2006; Fossing et al., 2000; Jørgensen et al., 2001, 2004; Emeis et al., 2004; Neretin et al., 2004; Knab et al., 2008; Dale

et al., 2009; Goldhammer et al., 2010, 2011; Riedinger et al., 2010; Holmkvist et al., 2011) but microbiological data are available for surface sediments mainly (Schulz et al., 1999; Thamdrup et al., 2000; Schulz and Schulz, 2005; Coolen et al., 2006a; Neretin et al., 2007; Coolen and Shtereva, 2009; Schubotz et al., 2009; Julies et al., 2010). Only few microbiological data are published for subsurface sediments of the Black Sea and the Benguela upwelling system (Leloup et al., 2007; Schäfer et al., 2007; Blazejak and Schippers, 2010, 2011).

Subsurface marine sediments are populated by numerous prokaryotes mainly belonging to uncultivated phylogenetic lineages (Parkes et al., 2000; Teske, 2006; Biddle et al., 2008; Fry et al., 2008; Teske and Sørensen, 2008). The abundance of particular phylogenetic and physiological prokaryotic groups, i.e., *Archaea* and *Bacteria*, methanogens or sulfate-reducers, in subsurface sediments at various sites has been quantified based on 16S rRNA and functional gene analysis by quantitative, real-time PCR (Q-PCR), FISH, and catalyzed reporter deposition–fluorescence *in situ* hybridization (CARD–FISH; Schippers et al., 2005, 2010; Biddle et al., 2006; Inagaki et al., 2006; Schippers and Neretin, 2006; Engelen et al., 2008; Nunoura et al., 2009; Webster et al., 2009; Breuker et al., 2011). While the detection of 16S rRNA genes may not be a good indicator for an active

microbial community, FISH and CARD–FISH analyses, targeting intact ribosomal RNA, suggest that an active microbial community is present. Eukaryotic DNA comprising, e.g., fungi (Edgcomb et al., 2011) has been quantified in marine sediments at few sites in much lower concentration than prokaryotic DNA (Schippers and Neretin, 2006; Schippers et al., 2010). However, eukaryotic DNA may be preserved as fossil DNA from any buried biota and might be indicative for former biological communities (Coolen et al., 2006a,b; Inagaki and Neilson, 2006; Boere et al., 2011).

Sulfate-reducing microorganisms which are frequently found in near-surface sediments (Knoblauch et al., 1999; Sahn et al., 1999; Ravensschlag et al., 2000; Schippers et al., 2010), or methanogenic Archaea, were rarely detected in deep sediments (Parkes et al., 2005; Biddle et al., 2006; Inagaki et al., 2006; Teske, 2006; Teske and Sørensen, 2008; Webster et al., 2009). In addition to 16S rRNA gene analyses, sulfate-reducers and methanogenic lineages of *Archaea* have been detected and quantified detecting their functional genes. Such genes encode for the dissimilatory sulfite reductase (*dsrA*), adenosine 5'-phosphosulfate reductase subunit A (*aprA*), and methyl coenzyme M reductase (*mcrA*, Parkes et al., 2005; Schippers and Neretin, 2006; Leloup et al., 2007; Wilms et al., 2007; Colwell et al., 2008; Engelen et al., 2008; Nunoura et al., 2009; Webster et al., 2009; Schippers et al., 2010; Blazejak and Schippers, 2011). The frequent occurrence of genes involved in sulfate reduction and methanogenesis indicates that microbial communities actively perform carbon and sulfur cycling under reduced redox conditions.

Despite heterotrophy seems to be dominant in subsurface sediments (D'Hondt et al., 2004; Biddle et al., 2006), autotrophy may play a role and has scarcely been investigated. There are different pathways of CO₂ fixation, of which the Calvin–Benson–Bassham (CBB) cycle is the best described. The *cbpL* gene encoding for the large subunit of the form I “red-like” enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) occurs in autotrophic *Proteobacteria* that fix CO₂ via the CBB cycle (Selesi et al., 2007; Badger and Bek, 2008). This gene was so far only quantified in terrestrial (Breuker et al., 2011), but not in subsurface marine sediments.

In this study, the quantitative microbial community composition at six marine sediment stations from the Black Sea and in the Benguela upwelling area off Namibia at various sediment depths up to 10 m below seafloor (mbsf) was analyzed using total cell counting, CARD–FISH for the quantification of living *Bacteria*

and *Archaea*, and 12 different Q-PCR assays for the quantification of particular phylogenetic or functional groups.

MATERIALS AND METHODS

SEDIMENT DESCRIPTION

Samples were collected at six marine stations during two research vessel expeditions (Table 1). The M72-5 R/V Meteor cruise to the Black Sea took place in May/June 2007 (Figure 1). The sampled Black Sea sediments were permanently anoxic due to the overlaying anoxic water column. Sulfate reduction and methanogenesis are generally the predominant terminal biogeochemical processes for organic matter degradation (Jørgensen et al., 2004; Leloup et al., 2007). The sediments underlying the anoxic water column had an upper layer composed of laminated coccolith ooze with high organic carbon content, while an underlying sapropel is characterized by even higher organic content (Brumsack, 1989). At station 22 a sapropel layer occurred at a depth of 8 mbsf. This sapropel deposited 130 ka ago after the Eem glacial.

The M76-1 R/V Meteor cruise to the Benguela upwelling area off Namibia took place in April/May 2008 (Figure 2). In the coastal upwelling area off Namibia, sediments were sampled along a transect course. This transect commenced at the deep seafloor at the lower continental margin at 3795 m water depth (station 8, GeoB 12808). This first core consisted of carbonate-rich deep sea clay, was oxic or suboxic with signs of bioturbation. The second station was situated at the upper continental margin at 1940 m water depth (station 3, GeoB 12803) was oxic and characterized by foraminifera and bioturbation. The third station was held at the shallow shelf in a mudbelt beneath a zone of high productivity (station 10, GeoB 12810) where foraminifera and dark olive colored mud characterized the sediment. A high abundance of *Thioploca* and *Beggiatoa*-like filaments from the surface down to 10–12 cm was found.

SEDIMENT SAMPLING

Sediment samples were taken from the near-surface sediment down to a depth of up to ca. 0.5 mbsf employing a multicorer (MUC). Deeper sediments down to 10 mbsf were sampled using a gravity corer (GC). The GC cores were quickly cut into 1 m sections onboard and immediately stored at 4°C. For further section-wise processing within several hours, each 1 m section was subsequently split lengthwise into half-core sections. During sampling, the outer surface of the core was carefully removed to avoid contamination with seawater.

Table 1 | Sampled sediment stations and total organic carbon [TOC, data from S. Eckert and B. Schnetger (Black Sea), Y. Lin and K.-U. Hinrichs (Namibia)].

Cruise	Area	Station	Sampling tool	Position	Water depth (m)	Sampling depth (mbsf)	Number of analyzed samples	TOC (%)
Black Sea		6	MUC and GC	43°25.91'N 32°16.48'E	2027	8.8	19	nd
M72-5		20	MUC and GC	43°57.25'N 35°38.46'E	2048	5.8	19	nd
		22	GC	42°13.53'N 36°29.55'E	840	9.7	19	0.3–4.4
Benguela	Mudbelt	10	MUC and GC	24°03.19'S 14°15.69'E	120	3.4	16	4.1–13.4
Upwelling	Upper slope	3	MUC and GC	25°45.60'S 13°04.20'E	1940	5.4	15	3.8–8.7
M76-1	Lower slope	8	MUC and GC	26°22.18'S 11°53.49'E	3795	5.4	19	1–1.8



FIGURE 1 | Black Sea sediment stations of the cruise M72-5 analyzed in this study.

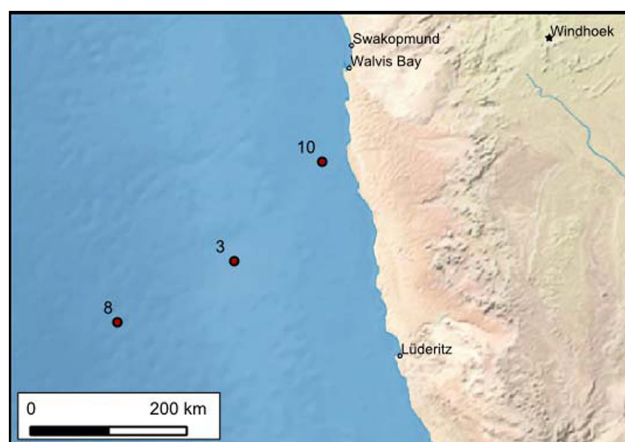


FIGURE 2 | Benguela upwelling transect sediment stations off Namibia of the cruise M76-1 analyzed in this study.

Microbiological samples for the quantification of microorganisms were taken with a sterilized 5 mL syringe of which the Leur head was cut off before. The uncontaminated center of the sediment core was sampled. For total cell counts, 0.5 mL sediment was fixed in 1 mL of a cold 4% formaldehyde–PBS [phosphate buffered saline (ultra), 150 mM sodium phosphate, 150 mM NaCl, pH 7.2] for 4–15 h at 4–10°C, washed twice with cold PBS using a mini-centrifuge (Eppendorf) at 13000 rpm for 10 min each, and finally stored at –20°C in 1 mL PBS–ethanol (1:1) in 2 mL vials (Eppendorf), transported to BGR frozen with dry ice as air-freight, and stored in BGR at –20°C. For DNA extraction and quantification with Q-PCR, several grams of fresh sediment were frozen immediately after sampling at –20°C in screw capped vials.

TOTAL CELL COUNTS AND CARD–FISH

Total cell numbers were determined in formaldehyde fixed samples by staining with SYBR Green® II following two different protocols. In analogy to acridine direct counts (AODC) we use the term SYBR Green® direct counts (SGDC). While cells were counted in the sediment matrix as described by Weinbauer et al., 1998, SGDC1), cells were detached from sediment particles before counting using the protocol of Kallmeyer et al., 2008, SGDC2). CARD–FISH analysis was carried out as described previously (Pernthaler et al., 2002; Schippers et al., 2005) and filters were hybridized for *Archaea* and *Bacteria* using probes ARCH915 or EUB338 I–III as a mixture. As a negative hybridization control the probe NON338 was applied.

QUANTITATIVE, REAL-TIME PCR ANALYSIS

The quantitative composition of the total (active and inactive) microbial community was analyzed using Q-PCR of extracted DNA. High-molecular-weight DNA was extracted from 0.5 g of a frozen sediment sample employing a modified Fast DNA Spin Kit for Soil (Bio101) protocol (Webster et al., 2003). Sterilized quartz sand treated in a muffle furnace for organic carbon removal was used as negative control in the extraction procedure. Extracted DNA was amplified by Q-PCR using the device ABI Prism 7000 (Applied Biosystems) and different master mixes from the companies Applied Biosystems, Eurogentec or Invitrogen. Each DNA extract was measured in triplicate. After each Q-PCR, melting curves were measured for SYBR Green® I assays. The copy numbers of the 16S rRNA gene were quantified for prokaryotes, *Archaea* (Takai and Horikoshi, 2000), *Bacteria* (Nadkarni et al., 2002), *Crenarchaeota* (Ochsenreiter et al., 2003), the JS-1- and *Chloroflexi*-related bacteria (Blazejak and Schippers, 2010), and the metal and sulfate-reducing *Geobacteraceae* (Holmes et al., 2002). The 18S rRNA gene of *Eukarya* was determined as previously described (Schippers and Neretin, 2006). Functional genes were quantified as described elsewhere: *mcrA* (*mcrA* assay 1, Wilms

et al., 2007; *mcrA* assay 2, Steinberg and Regan, 2009), *dsrA* (Schippers and Neretin, 2006); *aprA* (Blazejak and Schippers, 2011), and *cbbL* for (RuBisCO; Selesi et al., 2007).

RESULTS

Results of the quantitative microbial community composition in subsurface marine sediments of the Black Sea and the Benguela upwelling area off Namibia are shown in **Figures 3** and **4**, respectively.

BLACK SEA

Total cell counts and CARD-FISH

Total cells stained with SYBR Green[®] were counted following two different protocols. Depth profiles of total cell counts are shown in **Figure 3** (left). For both protocols, the maximal cell counts were detected near the sediment surface at all three Black Sea stations. The total cell counts sharply declined within the first meter sediment depth, and slightly decreased further below. The method comparison showed that the highest cell counts for all depths were obtained using the protocol without detaching cells from sediment particles (SGDC1, Weinbauer et al., 1998). For this protocol, maximum cell counts declined from 10^9 to 10^{10} cells/mL at the sediment surface to 10^7 – 10^8 cells/mL below 1 mbsf. In comparison, the cell counts obtained from the cell detachment protocol (SGDC2, Kallmeyer et al., 2008) were about one order of magnitude lower within the first 1 mbsf but showed similar values than SGDC1 below one meter depth. An increase of cell counts could be observed at 8–9 mbsf at station 22 where the layer of the organic carbon rich sapropel was discovered.

Catalyzed reporter deposition – fluorescence *in situ* hybridization cell counts above the detection limit of 10^5 cells/mL were obtained from the sediment surface down to 2.5 mbsf for the stations 6 and 20, and in the whole sampling depth of more than 8 mbsf for station 22. *Bacteria* and *Archaea* occurred in overall equal numbers.

Quantitative microbial community analysis by Q-PCR

Q-PCR results for 16S and 18S rRNA and functional genes at the three Black Sea stations are shown in **Figure 3** (middle and right, respectively). The Q-PCR data on archaeal and bacterial 16S rRNA gene copy numbers matched well with the total cell counts (SGDC1). In overall agreement with the CARD-FISH data, *Archaea* were found in similar copy numbers compared with *Bacteria* at all three sites. At station 22 between 2 and 8 mbsf, *Bacteria* could not be detected although the bacterial candidate division JS-1 and the classes *Anaerolineae* and *Caldilineae* of the phylum *Chloroflexi* were detectable in this depth range. At the other depths at all three stations these specific bacterial groups occurred in similar gene copy numbers than the *Bacteria* (data for station 20 from Blazejak and Schippers, 2010). Likewise, the *Crenarchaeota* frequently occurred in high gene copy numbers compared with *Archaea*. In some samples, the copy numbers of *Crenarchaeota* were even higher than those of *Archaea*. *Eukarya* were only detectable down to 1 mbsf at stations 6 and 22. However, they were detected throughout the whole core of station 20. Their 18S rRNA gene copy numbers were always lower than the prokaryotic 16S rRNA gene copy numbers.

Similar depth profiles were obtained for the functional genes (**Figure 3**, right). However the investigated genes were not detectable between 1 and 7 mbsf of station 22. Below, in the sapropel layer between 8 and 9 mbsf, they were again present. This was in good agreement with increased 16S rRNA gene copy numbers and increased SGDC1. Genes encoding for enzymes of sulfate-reducers (*dsrA* and *aprA*) were the most abundant (data for station 20 from Blazejak and Schippers, 2011). The *mcrA* gene of methanogens was detected at the surface of all three stations and on the bottom of the stations 20 and 22. The *cbbL* gene was found only in the near-surface sediment and in two deeper layers of the station 20.

BENGUELA UPWELLING AREA OFF NAMIBIA

The quantitative distribution of the microorganisms at the three stations along the transect course of the Benguela upwelling area (**Figure 4**) was more heterogeneous than for the three Black Sea stations. At the Benguela upwelling sediment station 10 (**Figure 4** bottom) a high abundance of *Thioploca* and *Beggiatoa*-like filaments was found at the near-surface sulfidic sediment of the shallow shelf. The other two stations on the continental slope at about 2000 m water depth (station 3, **Figure 4** top) and at about 4000 m water depth (station 8, **Figure 4** middle) were oxic or suboxic and bioturbated. The transect course followed a trend of decreasing organic carbon content (TOC, **Table 1**). However the TOC values were always above 1%, thus the sediments at all stations can be characterized as organic-rich and eutrophic.

Total cell counts and CARD-FISH

Depth profiles of total cell counts are shown in **Figure 4** (left). For both, SGDC1 and SGDC2, the maximal cell counts were found near the sediment surface at all three Benguela upwelling stations. Like for the Black Sea stations, the total cell counts sharply declined within the first 1 mbsf, and slightly decreased further below at the stations 3 and 8. At the station 10, no further decline of cell numbers was observed between 0.5 and 3.5 mbsf, the maximum sampling depth of this station. The maximum cell counts in the near-surface sediment were highest for the shelf station 10 with 10^{10} cells/mL and between 10^9 and 10^{10} cells/mL for the two continental slope stations. While SGDC1 remained above 10^9 cells/mL at 3.5 mbsf at station 10, the numbers were about an order of magnitude lower at the same depth for the two slope stations. The method comparison shows that SGDC1 was always slightly higher than SGDC2 for all three stations at all depths.

Catalyzed reporter deposition – fluorescence *in situ* hybridization signals were observed at almost all stations and depths. However, *Archaea* were detectable in equal numbers compared with *Bacteria* only in the near-surface sediment. In the deeper layers only *Bacteria* (besides two samples with *Archaea*) were detected in significantly lower numbers than the corresponding SGDC1.

Quantitative microbial community analysis by Q-PCR

For the Benguela upwelling sediments, Q-PCR analyses of the 16S and 18S rRNA and the functional genes are depicted in **Figure 4** (middle and right, respectively). The Q-PCR results for archaeal and bacterial 16S rRNA gene copy numbers were in good agreement with the total cell counts (SGDC1). In contrast to the CARD-FISH data, *Archaea* were found in similar copy numbers compared with the *Bacteria* at all three stations (as for the Black Sea results).

The archaeal copy numbers were higher than the bacterial ones in the deeper layers of station 10.

As found in the Black Sea sediments, the 16S rRNA gene copy numbers of the bacterial candidate division JS-1 and the classes

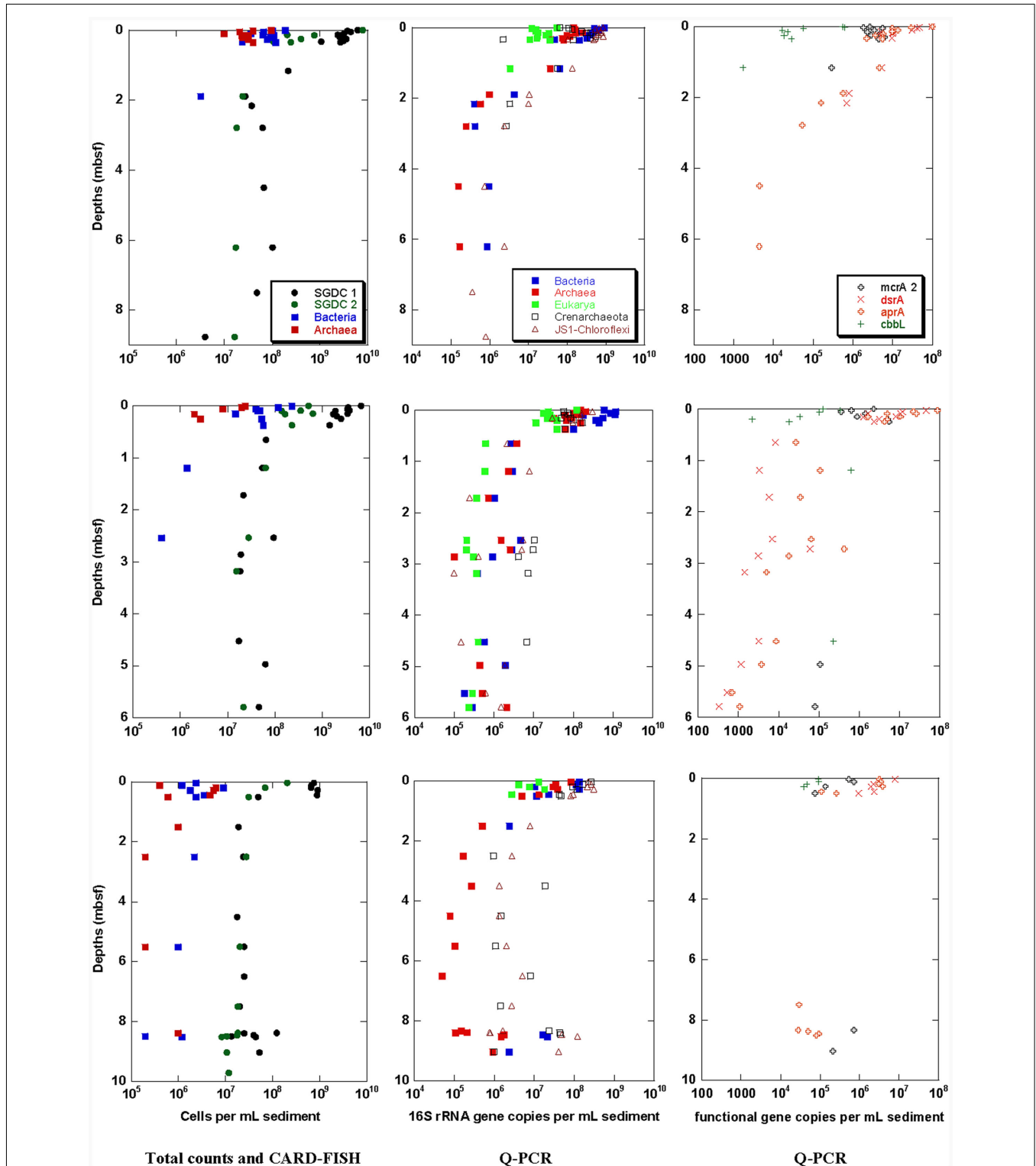


FIGURE 3 | Black Sea sediment samples of stations 6 (top), 20 (middle), and 22 (bottom). Total cell counts obtained with two different methods (SGDC1 after Weinbauer et al., 1998, SGDC2 after Kallmeyer et al., 2008) and CARD-FISH numbers for *Bacteria* and *Archaea* (left), and Q-PCR quantification of 16S or 18S rRNA genes of *Bacteria*, *Archaea*, *Eukarya*, *Crenarchaeota*, and JS-1-*Chloroflexi* (middle) and the functional genes *mcrA*, *dsrA*, *aprA*, and *cbbL* (right).

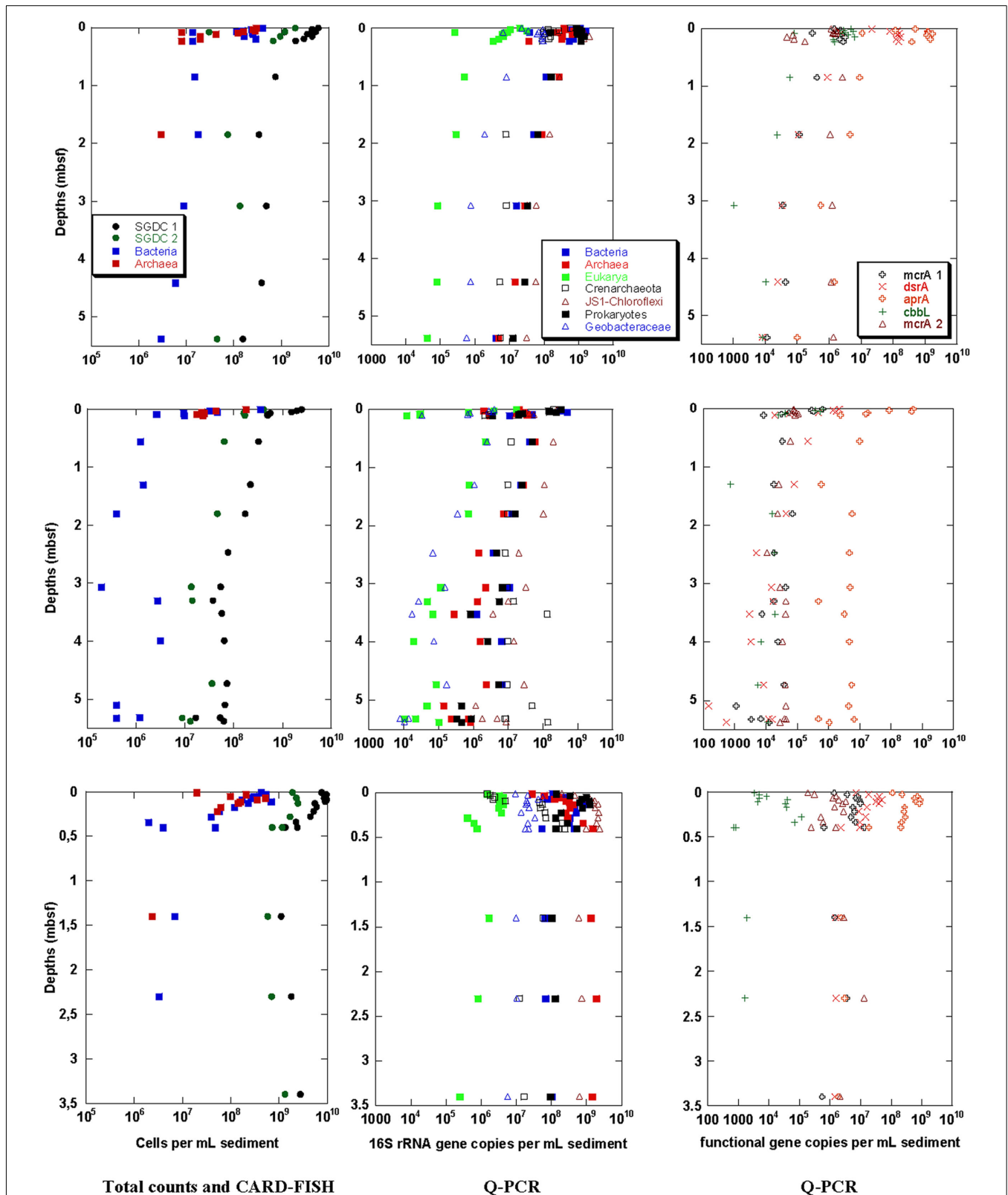


FIGURE 4 | Benguela upwelling sediment samples of stations 3 (top), 8 (middle), and 10 (bottom). Total cell counts obtained with two different methods (SGDC1 after Weinbauer et al., 1998, SGDC2 after Kallmeyer et al., 2008) and CARD-FISH numbers for *Bacteria* and

Archaea (left), Q-PCR quantification of 16S rRNA genes of *Prokaryotes*, *Bacteria*, *Archaea*, *Eukarya*, *Crenarchaeota*, and *JS1-Chloroflexi* (middle) and the functional genes *mcrA* 1 and 2, *dsrA*, *aprA*, and *cbbL* (right).

Anaerolineae and *Caldilineae* of the phylum *Chloroflexi* as well as those of the *Crenarchaeota* occurred in similar numbers of 10^6 to 10^9 copies/mL than the 16S rRNA gene copy numbers of *Prokaryotes*, *Bacteria*, and *Archaea*. The metal and sulfate-reducing family *Geobacteraceae* occurred at all three stations at all sediment depths in lower 16S rRNA gene copy numbers. The least abundant phylogenetic group was the *Eukarya* which nevertheless occurred in all analyzed samples.

Similar depth profiles like for the phylogenetic groups (and SGDC1) were obtained for the functional genes. All functional genes were detected in almost all analyzed samples. Among the functional groups, sulfate-reducers were most abundant, especially their functional gene *aprA* occurred with maximum numbers of 10^9 copies/mL sediment. In contrast to the Black Sea sediments, both *mcrA* assays 1 and 2 of methanogens provided successful gene amplification, and *mcrA* and *cbbL* occurred not only in the near-surface sediment but also in the deeper subsurface sediment.

DISCUSSION

Microbial communities in the subsurface marine sediments of the Black Sea and the Benguela upwelling area off Namibia have been analyzed. Total cell counts, CARD-FISH data as well as Q-PCR analysis exhibited a high abundance of prokaryotes in the eutrophic, organic carbon-rich sediments at all six stations. A similar high abundance of prokaryotes analyzed by the same methods has been detected in organic carbon-rich sediments of the Peru margin (ODP Leg 201, D'Hondt et al., 2004; Schippers et al., 2005; Inagaki et al., 2006), gas-hydrate bearing sediments from the Cascadia margin (ODP Leg 204; Inagaki et al., 2006), northeast Pacific ridge-flank sediments (IODP Exp. 301; Engelen et al., 2008), tidal flat sediments of the North Sea (Wilms et al., 2007), and sediments off Sumatra (Schippers et al., 2010). Less organic carbon-rich marine sediments exhibited, as expected, a lower abundance of prokaryotes as shown for the equatorial Pacific (ODP Leg 201; D'Hondt et al., 2004; Schippers et al., 2005), the Porcupine Seabight (IODP Exp. 307; Webster et al., 2009), and the Gulf of Mexico (IODP Exp. 308; Nunoura et al., 2009). Oligotrophic, organic carbon-poor marine sediments contain generally very low cell numbers as shown for the South Pacific Gyre (D'Hondt et al., 2009), and the Western flank of the Mid-Atlantic Ridge at 23°N (North Pond; A. Breuker and A. Schippers, unpublished). A compilation of mean cell numbers determined by total cell counting, Q-PCR, and CARD-FISH in subsurface marine sediments of various sites is given in **Table 2**.

In this study, a decrease of cell numbers with sediment depth was observed for all stations as described for subsurface marine sediments (Parkes et al., 1994, 2000; D'Hondt et al., 2004). However, in the sapropel layer at 8–9 mbsf of the Black Sea station 22, the cell as well as the gene copy numbers again increased by more than an order of magnitude, explainable by the increased organic carbon content of the sapropel serving as substrate for the microorganisms. Such an increase in biomass at distinct geologically different sediment layers has been described for other sediments as well (Inagaki et al., 2003; Parkes et al., 2005), and in particular for sapropels (Coolen et al., 2002).

In the sediments of this study, total cell counts were determined after staining with SYBR Green® following two different protocols. Cell numbers directly counted in the sediment matrix (SGDC1)

were verified by comparing the counts with those of the SGDC2 protocol in which the cells were detached from sediment particles before counting. The general depth trends of SGDC1 could be confirmed with SGDC2. However, the cell detachment method revealed somehow lower cell numbers as previously shown for subsurface marine sediments off Sumatra (Schippers et al., 2010) and terrestrial sediments in the Chesapeake area (Breuker et al., 2011). The difference in cell numbers between these two protocols was never higher than one order of magnitude in our study. A comparison of the total cell counts with the 16S rRNA gene copy numbers of the *Bacteria* and *Archaea* obtained by Q-PCR gives a good match for SGDC1 as well as for SGDC2, indicating a high reliability of the data for these quantification methods. CARD-FISH cell numbers were always considerably lower than SGDC 1. This indicates that either the majority of the cells was inactive, which seems to be unlikely in the organic-rich sediments, or more likely, insufficient cell wall permeabilization in the CARD-FISH protocol and mismatches of the archaeal CARD-FISH probe ARCH915 with predominant environmental gene sequences (Teske and Sørensen, 2008) prevented the detection of many living cells, especially *Archaea*.

The proportions of *Bacteria* and *Archaea* in marine sediments have shown to be highly variable in different sediments and sediment layers. Based on CARD-FISH and Q-PCR analyses overall similar proportions of *Bacteria* and *Archaea* have been determined for sediments studied here. An almost equal abundance of *Bacteria* and *Archaea* has also been found for the Porcupine Seabight (IODP Exp. 307; Webster et al., 2009), the northeast Pacific ridge-flank (IODP Exp. 301; Engelen et al., 2008), and Sumatra forearc basins (Schippers et al., 2010). By contrast, using Q-PCR it has been discovered that *Bacteria* dominated other sediments such as the Sea of Okhotsk (Inagaki et al., 2003), the Gulf of Mexico (IODP Exp. 308; Nunoura et al., 2009), the Peru continental margin, and the equatorial Pacific sediments (ODP Leg 201; Schippers et al., 2005; Inagaki et al., 2006; Schippers and Neretin, 2006), as well as gas-hydrate bearing sediments from the Cascadia margin (ODP Leg 204; Inagaki et al., 2006). The dominance of *Bacteria* for the Peru continental margin was confirmed by CARD-FISH (Schippers et al., 2005). Unlike nucleic acid based methods (CARD-FISH and Q-PCR), the analysis of intact polar lipids (IPL) of prokaryotic cell membranes unveiled *Archaea* as prevailing prokaryotes in deeply buried sediments (Biddle et al., 2006; Lipp et al., 2008). These conflicting results may be explained by insufficient quantitative extraction protocols, or primer mismatches (Teske and Sørensen, 2008) and/or a different preservation of DNA and IPL in deeply buried sediments (e.g., stabilized on clay surfaces or organic matter; Coolen et al., 2006a,b; Inagaki and Nealson, 2006; Schippers and Neretin, 2006; Boere et al., 2011). Recently, Schouten et al. (2010) as well as Logemann et al. (2011) reported the preservation of archaeal IPL biomarkers in marine sediments indicating that IPL biomarkers do not necessarily detect living *Archaea* and putting their proposed dominance in the deep biosphere into question. Obviously, it is not yet understood which factors control the proportions of *Bacteria* and *Archaea* in marine sediments.

Particular phylogenetic and physiological groups, inhabiting Black Sea and Benguela upwelling sediments, were revealed by Q-PCR in this study. The sediments were clearly dominated by prokaryotes since the abundance of eukaryotic 18S rRNA genes

Table 2 | Compilation of mean cell numbers (N/mL) in the depth range of 1–10 and 10–200 mbsf in subsurface marine sediments; nd, not determined.

Expedition	Total counts	Q-PCR	CARD-FISH	Total counts	Q-PCR	CARD-FISH	Reference
	1–10 mbsf			10–200 mbsf			
~20 ODP and other sites	10 ⁷ –10 ⁹	nd	nd	10 ⁶ –10 ⁸	nd	nd	Parkes et al. (1994, 2000), Bird et al. (2001), Wellsbury et al. (2002), Inagaki et al. (2003), Kallmeyer et al. (2008), Morono et al. (2009), Roussel et al. (2009)
ODP Leg 201 Equatorial Pacific	10 ⁷	nd	10 ⁶	10 ⁶ –10 ⁷	nd	10 ⁶	D'Hondt et al. (2004), Schippers et al. (2005)
ODP Leg 201 Peru margin	10 ⁷ –10 ⁸	10 ⁷	10 ⁶	10 ⁷	10 ⁶	10 ⁶	D'Hondt et al. (2004), Schippers et al. (2005), Inagaki et al. (2006)
ODP Leg 204 Cascadia margin	10 ⁷	10 ⁷	nd	10 ⁶	10 ⁶	nd	Inagaki et al. (2006), Nunoura et al. (2008)
IODP 301 Juan de Fuca	10 ⁸ –10 ⁹	10 ⁶ –10 ⁸	nd	10 ⁸	10 ⁶	nd	Engelen et al. (2008)
IODP 307 Porcupine Seamount				10 ⁶ –10 ⁷	10 ⁵ –10 ⁶	10 ⁵ –10 ⁶	Webster et al. (2009)
IODP 308 Gulf of Mexico	10 ⁵ –10 ⁶	10 ⁵ –10 ⁶	nd	10 ⁴ –10 ⁵	10 ⁴ –10 ⁵	nd	Nunoura et al. (2009)
IODP 313 New Jersey Shallow Shelf	10 ⁶	10 ⁵ –10 ⁶	nd	10 ⁵ –10 ⁶	10 ⁵ –10 ⁶	nd	A. Breuker, S. Stadler, and A. Schippers (unpublished)
North Sea tidal flat	10 ⁷ –10 ⁸	10 ⁷	nd				Wilms et al. (2007)
SO 189 Forearc off Sumatra	10 ⁷ –10 ⁸	10 ⁷ –10 ⁸	nd				Schippers et al. (2010)
South Pacific Gyre	10 ³ –10 ⁴	nd	nd				D'Hondt et al. (2009)
MSM 11-1 "North Pond"	10 ⁵ –10 ⁶	10 ⁴ –10 ⁶	nd				A. Breuker and A. Schippers (unpublished)
M 72-5 Black Sea	10 ⁷ –10 ⁸	10 ⁵ –10 ⁶	10 ⁵ –10 ⁶				This study
M 76-1 Benguela Upwelling	10 ⁷ –10 ⁹	10 ⁶ –10 ⁸	10 ⁶ –10 ⁷				This study
ICDP Chesapeake Bay terrestrial sediments	10 ⁶ –10 ⁷	10 ⁶ –10 ⁷	10 ⁶	10 ⁶	10 ⁶	nd	Breuker et al. (2011)

comprised only 3% and <1% of the number of prokaryotic 16S rRNA genes for the Black Sea and Namibia sediments, respectively. This can be attributed to the small pore space in subsurface sediments which impedes growth of large eukaryotic cells. Similar Q-PCR results were obtained for the organic carbon-rich sediments off Sumatra (Schippers et al., 2010) and for Peru margin sediments (Schippers and Neretin, 2006). In the latter sediments, mainly fungi have been identified as dominant *Eukarya* (Edgcomb et al., 2011). In the near-surface anoxic and sulfidic sediments of the Black Sea the following eukaryotic groups were detected via DGGE and 18S rRNA gene sequencing: copepods, rotifers, haptophytes, dinoflagellates, and ciliates (Coolen and Shtereva, 2009). Data on the composition of *Eukarya* in the deeper, subsurface sediment are not available.

As previously shown for the sediments off Sumatra, the Peru margin and the Black Sea station 20 (Blazejak and Schippers, 2010), the bacterial candidate division JS-1 and the classes *Anaerolineae* and *Caldilineae* of the phylum *Chloroflexi* were at least as highly abundant as the *Bacteria* for the other five stations of this study as well. This confirms their dominant role in subsurface marine sediments (Webster et al., 2004; Teske, 2006; Fry et al., 2008). *Crenarchaeota* were previously identified as dominant members in the archaeal 16S rRNA gene clone libraries (Teske, 2006; Fry et al., 2008; Teske and Sørensen, 2008). This is consistent with our finding that this group could be quantified by Q-PCR in comparable (or higher) copy numbers than the *Archaea* in subsurface marine sediments of the Black Sea and

the Benguela upwelling sediments. Moreover, uncultured *Crenarchaeota* as well as *Chloroflexi* have also been identified in one subsurface sediment core (station GeoB 3703) from the Benguela upwelling area (Schäfer et al., 2007). In many samples of our study more *Crenarchaeota* than *Archaea*, and more JS-1 and *Chloroflexi* than *Bacteria* were detected. This discrepancy may be explained by PCR bias due to different PCR efficiencies for the particular Q-PCR assays, primer mismatches of the general 16S rRNA gene primers with predominant environmental gene sequences (Teske and Sørensen, 2008), and probably different 16S rRNA gene copy numbers per cell which are unknown for the particular specific archaeal and bacterial groups analyzed in this study.

Functional genes were quantified by Q-PCR to demonstrate the importance of particular physiological prokaryotic groups. The functional genes *dsrA* and *aprA* of sulfate-reducers and the gene *mcrA* of methanogens were highly abundant in the Black Sea as well as in the Namibia sediments. This finding is not surprising because sulfate reduction, methanogenesis, and anaerobic methane oxidation were shown to be important biogeochemical processes in these sediments (Niewöhner et al., 1998; Ferdelman et al., 1999; Fossing et al., 2000; Jørgensen et al., 2001, 2004; Brüchert et al., 2003, 2006; Knab et al., 2008; Riedinger et al., 2010; Holmkvist et al., 2011). Q-PCR based *dsrA* quantification in another Black Sea subsurface sediment core also revealed that sulfate-reducers were highly abundant throughout the whole sampling depth of 4.6 mbsf (Leloup et al., 2007). A *dsrA* clone library

in the same study showed mostly sequences affiliated with the *Desulfobacteraceae*.

The detection of the functional gene *cbbl* coding for the large subunit of the form I “red-like” RuBisCO was different for the Black Sea and the Benguela upwelling samples. While in the latter the gene was detected in lower copy numbers in most samples and all depth, *cbbl* was mainly detectable only in near-surface sediments of the Black Sea. Overall, autotrophy via the RuBisCO pathway seems to play some role in these samples, despite its high organic carbon content supporting heterotrophy as shown for the organic carbon-rich sediments of the Peru margin (D’Hondt et al., 2004; Biddle et al., 2006). A higher abundance of the same *cbbl* gene than in this study was recently detected in the organic carbon-poor terrestrial subsurface sediments in the Chesapeake Bay area, VA, USA (Breuker et al., 2011). There, in agreement with the lower organic carbon content, autotrophy seems to be more important than in the marine sediments of this study.

CONCLUSION

The microbial communities in the subsurface marine sediments of the Black Sea and the Benguela upwelling area off Namibia have been quantitatively analyzed at six stations using total cell

counts, CARD-FISH and 12 different Q-PCR assays. A high abundance of *Prokaryotes* and overall similar proportions of *Bacteria* and *Archaea* were discovered in the eutrophic, organic carbon-rich sediments. *Crenarchaeota* and the bacterial candidate division JS-1 and the classes *Anaerolineae* and *Caldilineae* of the phylum *Chloroflexi* were as highly abundant. In agreement with the reported ongoing sulfate reduction and methanogenesis, the functional genes *dsrA* and *aprA* of sulfate-reducers and the gene *mcrA* of methanogens were highly abundant as well, suggesting a vital microbial community performing these processes. The detection of the *cbbl* gene shows the occurrence of autotrophic microorganisms.

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