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Chapter 4

Characterization of anaerobic bacterial chemoautotrophy in intertidal marine sediments

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Abstract

We estimated the rates of anaerobic chemoautotrophy and characterized the chemoautotrophic bacterial community by measuring dark-fixation into biomarkers in aerobic and anaerobic incubations of coastal sediments (Eastern Scheldt estuary, the Netherlands). Besides determining PLFA biomarker ^{13}C -labeling, we also quantified labeling in specific 16S rRNAs by using the Mag-SIP approach to further identify active groups. Rates of anaerobic chemoautotrophic carbon fixation were high and contributed substantially to the total chemoautotrophy rate but only in sediments where no free sulfide was detected. The rates of anaerobic chemoautotrophy were low in sulfidogenic sediments, which was probably due to the lack of oxidants that could drive anaerobic reoxidation processes. Both methods indicated that *Deltaproteobacteria* related to sulfate reducing bacteria were a major group in the anaerobic chemoautotrophic activity in all sediments studied next to *Gammaproteobacteria* that may be related to symbionts found in higher organisms. Our study shows that anaerobic chemoautotrophy is an important process in typical coastal sediments.

Introduction

Reoxidation of reduced intermediates such as sulfide and ammonium that are formed during anaerobic mineralization is an important process in coastal marine sediments (Jørgensen and Nelson, 2004). Oxygen is generally only found in the top millimeters to micrometers of the sediment as well as along burrows made by macrofauna (Glud, 2008). Carbon mineralization proceeds predominantly by anaerobic processes, primarily through sulfate reduction (Jørgensen and Nelson, 2004). However, in typical coastal sediments free sulfide is not found in the porewater in the top few centimeters of the sediment because it reacts with iron hydroxides to form iron sulfide or pyrite (Jørgensen and Nelson, 2004). Only in very active sediments or sediments containing little reactive iron, free sulfide can be found near the oxic top layer (Jørgensen and Nelson, 2004). Burial of reduced compounds is thought to be of minor importance since they are mostly transported to the more oxidized horizons either by diffusion or by bioturbation (Meysman *et al.*, 2006). It is estimated that reoxidation on average explains 50 to 70% of the sediment oxygen flux in shelf sediments and this value will be higher in intertidal areas that show higher mineralization rates because anaerobic mineralization will be more important (Soetaert *et al.*, 1996). Oxygen is the ultimate oxidant in the reoxidation processes, although intermediate anaerobic reoxidation steps involving nitrate or metal oxides may also be important (Jørgensen and Nelson, 2004).

Many of the known *Bacteria* and *Archaea* involved in reoxidation processes are chemo(litho)autotrophs, which gain energy from the oxidation of reduced (inorganic) compounds and use this to grow by CO₂ fixation (Canfield *et al.*, 2005). Chemoautotrophy is an important process in some marine ecosystems such as hydrothermal vents (Jannasch and Wirsen, 1979) and in the chemocline of anoxic marine basins (Jost *et al.*, 2008). However, the current consensus is that chemoautotrophy is a minor process in coastal sediments explaining probably less than 7% of the carbon cycle, which is attributed to the generally low growth yields of chemoautotrophic organisms and reoxidation by heterotrophic and mixotrophic bacteria or chemical reactions (Jørgensen and Nelson, 2004). However, studies that quantified chemoautotrophy by determining dark CO₂ fixation are rare for typical coastal marine sediments. We have found only two studies that quantified chemoautotrophy that both deal with shallow subtidal sediments in the Baltic Sea (Enoksson and Samuelsson, 1987; Thomsen and Kristensen, 1997).

We have recently measured chemoautotrophy rates and identified the active bacterial communities in two intertidal sediments by incubating sediment cores with stable isotope (¹³C) labeled bicarbonate and by measuring labeling of PLFA (Boschker *et al.*, 2010). We showed high rates of chemoautotrophy that explained up to 25% of the sediment carbon cycling. Substantial labeling was detected in PLFA that are generally assigned to sulfate reducing bacteria, suggesting that anaerobic chemoautotrophy may be important in coastal sediments (Boschker *et al.* 2010). Several sulfate reducing bacteria growing on hydrogen

gas or involved in disproportionation reactions of sulfur species are known to be chemoautotrophs (Rabus *et al.*, 2006), and both hydrogen and thiosulfate turnover are important processes in marine sediments (Jørgensen and Bak, 1991; Novelli *et al.*, 1988). In addition, high chemoautotrophy rates have been reported just below the aerobic top layer in subtidal marine sediments (Thomsen and Kristensen, 1997).

In the present study, we further studied the regulation of anaerobic chemoautotrophy in coastal marine sediments and characterized the chemoautotrophic bacterial community involved by measuring dark-fixation of ^{13}C -labeled bicarbonate into biomarkers under both aerobic and anaerobic conditions. Besides determining PLFA biomarker ^{13}C -labeling, we also quantified labeling in specific 16S rRNAs by using the Mag-SIP approach to further identify active groups (Miyatake *et al.*, 2009). We show that anaerobic chemoautotrophy can explain a large fraction of the total chemoautotrophic carbon fixation especially in sediments with low concentrations of free sulfide. Moreover, we demonstrate that *Deltaproteobacteria* related to sulfate reducers are prominent chemoautotrophs in the investigated marine sediments.

Materials and methods

Description of field sites and sampling

Two field sites were selected in the Eastern Scheldt estuary (the Netherlands), which were both highly active but had major differences in sulfur chemistry. The Rattekaai (RK) site (51°26'21"N, 4°10'11"E) is situated at the entrance of a salt marsh creek where macroalgal debris (mainly *Ulva* derived) accumulates and is buried during winter. In May 2006, the sediment was highly sulfidic right below the sediment surface and samples were taken from patches where the sediment was covered with a white layer indicating elemental sulfur accumulation (RK06). In addition, sediment samples for laboratory experiments were collected near to the RK site on the adjacent intertidal sand flat that did not receive macroalgal debris. The second field site was situated in an open spot in a Pacific oyster (*Crassostrea gigas*) bed in the Zandkreek area (51°32'41"N, 3°53'22"E) and was sampled in October 2007 (ZK07) and April 2009 (ZK09). Sediment was non-sulfidic down to a depth of 20 mm for ZK07 and 50 mm for ZK09. Macrofauna was abundant at the Zandkreek site especially for ZK07 but was almost completely lacking at Rattekaai.

Sediments were sampled by using polycarbonate cores of two different sizes. The small cores (internal diameter 46 mm) contained injection ports made of silicon rubber at every 5 mm depth interval and were used for measuring chemoautotrophy. The larger cores (internal diameter 60 mm) were used for additional measurements of porewater profiles and as unlabeled control sediment. Sampling was done during low tide. The sediment cores were processed the same day for chemoautotrophy measurements and other analyses. Sediments for laboratory experiments were collected by scraping of the top centimeter of

the sediment which was subsequently sieved (1 mm diameter) in order to remove macrofauna.

Chemoautotrophy

Chemoautotrophy measurements were started by injecting 200 μl of 20 mM $\text{NaH}^{13}\text{CO}_3$ (99% ^{13}C ; Cambridge Isotope Laboratories, Andover, MA, USA) horizontally into the sediment cores at 5 mm depth intervals by using the line-injection method (Jørgensen, 1978). The ^{13}C -label was dissolved in artificial seawater lacking calcium or magnesium in order to avoid precipitation (Kester *et al.*, 1967). The label was made oxygen free by bubbling with nitrogen gas shortly before injection. Sediment cores were incubated for a day in strict darkness at 14°C (within 2°C of the in-situ sediment temperature). Cores were incubated without overlying water and the headspace was either air for the aerobic incubations or nitrogen gas for the anaerobic incubations. After incubation, sediment cores were sliced 1 cm each to a depth of 5 cm and sediment slices were quickly centrifuged (4500 rpm, 5 min) to collect porewater for analysis of ^{13}C -DIC and sulfide. Only the top centimeter was collected for the ZK09 sediment. Sediments for PLFA analysis were frozen at -20°C and lyophilized for further analysis. Samples for Mag-SIP were stored at -80°C. Unlabelled control cores were also processed in the same way.

Laboratory experiments

Experiments were done in 100 ml serum bottles that contained 10 g wet weight of sieved sediment and 35 ml of low nutrient seawater (summer sampled Atlantic Ocean water). Caps from the aerobic incubations were not completely closed to keep them aerated. Anaerobic incubations were closed with butyl rubber stoppers and the headspace was flushed with nitrogen gas for 5 min. Incubations were done in complete darkness at 14°C and bottles were shaken at 10 rpm on a rotary shaker to keep the waterphase homogeneous. Incubations were pre-incubated for 4-6 days and chemoautotrophic carbon fixation measurements were started by injecting 100 μl of 20 mM $\text{NaH}^{13}\text{CO}_3$ into the water phase (prepared as described above). Experiments were incubated for another day after which the overlying water collected and filtered over a syringe filter (0.45 μm , FP030, Schleicher and Schuell, Dassel, Germany) for ^{13}C -DIC, sulfide and pH analysis. Bottles with sediment were frozen at -20°C and lyophilized for subsequent PLFA analysis.

PLFA analysis and calculation of chemoautotrophic carbon-fixation rates

Lyophilized sediments were analyzed for PLFA concentrations and ^{13}C -labeling as previously described (Boschker, 2004). Briefly, PLFA were extracted and analyzed by gas chromatography - combustion - isotope ratio mass spectrometry (GC-c-IRMS, Thermo, Bremen, Germany) on an a-polar analytical column (HP5-MS, Agilent, Santa Clara, CA, USA). Excess ^{13}C in individual PLFA was calculated as in Middelburg *et al.* (Middelburg *et al.*, 2000), and divided by the atom percent excess ^{13}C in the DIC pool and the incubation

time to calculate actual chemoautotrophic PLFA synthesis rates. Total bacterial chemoautotrophy rates were determined as the sum of the synthesis rate of all PLFA typically found in *Bacteria* (12:0 to 19:0 range) and converted to chemoautotrophic biomass product by dividing by the typical PLFA content of aerobic bacteria (55 mmol PLFA-C/mol biomass C, (Brinch-Iversen and King, 1990; Middelburg *et al.*, 2000). Low labeling was found in poly-unsaturated PLFA typical for *Eukarya* (data not shown) suggesting that virtually all PLFA labeling was due to *Bacteria*. In the calculations, we therefore used the labeling data for all common bacterial PLFA in the 12:0 to 19:0 range and not just the specific bacterial biomarker PLFA (Middelburg *et al.*, 2000).

Pore water sampling and analysis

Pore water was sampled by slicing duplicate sediment cores from the unlabeled control incubations in an anaerobic glove-box filled with 3% hydrogen in nitrogen gas (Coy Laboratory Products, Ann Arbor, MI, USA) and centrifuging at 4500 rpm for 10 min at 14°C. Samples for sulfide analysis concentrations were immediately fixed in zinc acetate and subsequently analyzed (Cline, 1969). Samples (1 ml) for ¹³C-DIC were stored in headspace vials and analyzed by EA-IRMS (Moodley *et al.*, 2000). Water samples from the laboratory experiments were analyzed in the same way.

Mag-SIP analysis

Total RNA was extracted and specific 16S rRNA was captured and analyzed for ¹³C-labeling as described in detail in Miyatake *et al.* (2009). Briefly, total community RNA was extracted using the phenol-chloroform method (pH 5.1). For the Mag-SIP protocol, 20-40 µg total rRNA was hybridized with biotin-labeled probes (see below) and the 16S rRNA-probe hybrids were captured with hydrophobic streptavidin-coated paramagnetic beads (Dynabeads MyOne Streptavidin T1; Invitrogen, Carlsbad, CA, USA). Captured 16S rRNA was released from the beads, and approximately 500 ng of the captured material was used for stable isotope analysis by micro elemental analyzer–isotope ratio mass spectrometry (µEA-IRMS; DELTA V Advantage equipped with a LC Isolink interface; Thermo Fisher Scientific, Bremen, Germany). Captured rRNA was directly injected into the µEA-IRMS operating in bulk injection mode. Blanks without RNA were also processed with the Mag-SIP protocol and δ¹³C ratios of the captured material were corrected for blanks. Reported 16S rRNA labeling was expressed as the difference in δ¹³C between labeled samples and unlabeled controls (Δδ¹³C).

A nested set of biotin-labeled oligonucleotide probes was used with the Mag-SIP protocol (Table 4. 1). The probes EUB338 (Amann *et al.*, 1990) and DELTA495a (Loy *et al.*, 2002) were used to capture most *Bacteria* and *Deltaproteobacteria*, respectively, although they may not target all genera. The DELTA495a probe was used in combination with a competitor probe (cDELTA495a (Macalady *et al.*, 2006)) to avoid capture of *Gammaproteobacteria*, which have only one mismatch in a target region of DELTA495a.

Probe BG553 (Miyatake *et al.*, 2010) was used for *Gammaproteobacteria*, and unlabeled helper probes complementary to the consensus sequences upstream and downstream of the BG553 probe target sites were also used in order to increase yield. The BG553 probe also targets most *Betaproteobacteria*, but this group is rare in marine sediments (e.g. Bowman *et al.*, 2003) and was not detected in a 16S rRNA-derived cDNA clone library from the same sediment (Miyatake *et al.* 2010, this study).

We also used the Miyatake *et al.* (2010) clone library to study phylogenetic relationships of the active community. (Miyatake *et al.* (2010) only reported the relative distribution of major phyla.) Phylotypes ($\geq 97\%$ similarity in sequence) were aligned and the final bootstrapped Neighbor-joining tree with 1000 samplings was created in MEGA4 (Tamura *et al.*, 2007) using the Jukes-Cantor model.

Table 4. 1. 16S rRNA-targeted probes used in this study with the Mag-SIP protocol.

Probe	Sequence (5'-3')	% FA ¹	Specificity	Reference
EUB338	GCT GCC TCC CGT AGG AGT	25	Most <i>Bacteria</i>	(Amann <i>et al.</i> , 1990)
DELTA495a	AGT TAG CCG GTG CTT CCT	45	Most <i>Deltaproteobacteria</i> Most <i>Gemmatimonadetes</i>	(Loy <i>et al.</i> , 2002)
cDELTA495a	AGT TAG CCG GTG CTT CTT	45	Competitor of DELTA495a	(Macalady <i>et al.</i> , 2006)
BG553	CGC CCA GTA ATT CCG ATT	60	Most <i>Gamma-</i> and <i>Beta-</i> <i>proteobacteria</i>	(Miyatake <i>et al.</i> 2010)
BG553_up_help	AAC CGC CTR CGN RCG CTT TA	60	Helper probe for BG553	
BG553_down_help	AAC GCT YGC ACC CTM CTG ATT	60	Helper probe for BG553	

¹Percent formamide (FA) in hybridization buffer for hybridizations at 20°C

Results

Aerobic versus anaerobic chemoautotrophic carbon fixation rates

Aerobic and anaerobic chemoautotrophic fixation was studied by measuring ^{13}C -DIC incorporation into PLFA in the dark. The ^{13}C incorporation rates into PLFA were converted to actual chemoautotrophic biomass C production using the measured DIC ^{13}C -labeling and standard PLFA to biomass conversion factor as described in the methods section. The RK06 sediment showed a much higher activity than the ZK07 sediment (a factor 50 higher in aerobic incubations) and the depth distribution of chemoautotrophy was also different between the two sites (Fig. 4.1). The activity was highest in the top 1 cm of the sediment especially for the RK06 site where no chemoautotrophy was detected in deeper layers. Chemoautotrophy was still substantial in the 1-2 cm horizon of the ZK07 site and some activity was detected in the deepest layer sampled (3-5 cm). Only the top 1 cm of the sediment was used of the ZK09 sampling site and aerobic and anaerobic chemoautotrophic carbon fixation rates (0.131 ± 0.008 and $0.091 \pm 0.029 \mu\text{mol biomass-C g DW}^{-1} \text{d}^{-1}$, respectively) were somewhat lower than for ZK07. Anaerobic incubation caused a strong decrease in chemoautotrophic carbon fixation at RK06, whereas the anaerobic rates were similar to the aerobic rates for the ZK07 sediment to somewhat lower for ZK09 (Fig. 4. 1).

The results of all incubations are summarized in Fig. 4.2 showing the contribution of anaerobic chemoautotrophic carbon fixation to the rate of carbon fixation as measured in the aerobic incubations. The contribution of anaerobic chemoautotrophy was variable between incubations and ranged between 4% for experiment B2 and 110% for the ZK07 sediment. The ZK07 data were not different from 100% suggesting that basically all chemoautotrophy could be explained by anaerobic processes.

Much of the variation in the contribution of anaerobic carbon fixation appeared to be inversely related to the sulfide concentration as measured either in the porewater of the sediment cores or in the overlying water of the experiments. Free sulfide was detectable in RK06 sediment cores ($0.96 \pm 0.20 \text{ mM}$) and in experiment B2 ($0.22 \pm 0.06 \text{ mM}$); the other three incubations showed no detectable sulfide (Fig. 4.2). Highest contributions of anaerobic chemoautotrophy were found in incubations without detectable sulfide.

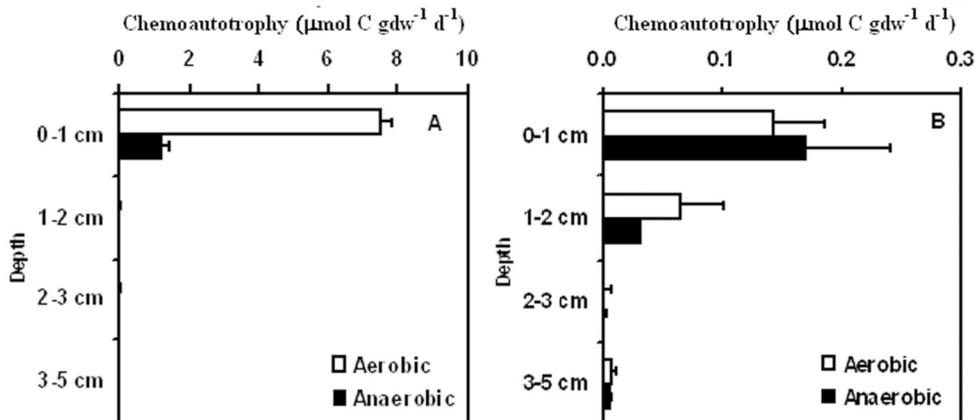


Figure 4.1. Depth distribution of total aerobic and anaerobic chemoautotrophy as estimated from dark ^{13}C -fixation into PLFA for RK06 (A) and ZK07 (B) intertidal sediments.

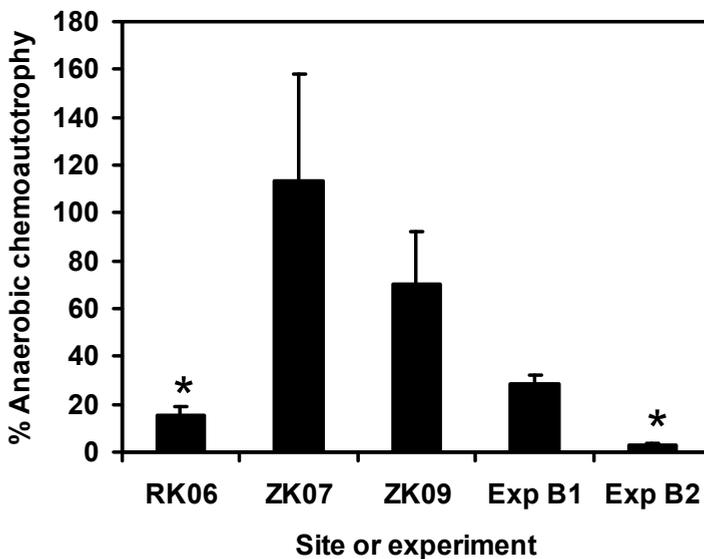


Figure 4.2. Anaerobic chemoautotrophy as percentage of the chemoautotrophy as measured under aerobic conditions in the headspace of the incubations. Results are from incubations of sediment cores (RK06, ZK07 and ZK09) and from two laboratory experiments (Exp. B1 and B2). The incubations marked * showed measurable free sulfide concentrations in either the sediment porewater (cores) or the overlying water (experiments).

Active chemoautotrophic bacterial communities

Label distribution in individual PLFA can be used to indicate differences in active bacterial communities and for a first identification of active groups. The PLFA labeling patterns for the RK06 and ZK07 sediments are shown for both aerobic and anaerobic incubations in Fig. 4.3. There were clear differences in label distribution between the different sediments and incubations. The labeling pattern for the aerobic incubation of RK06 was simple with major amounts of label in 14:0, 16:1 ω 7c, 16:1 ω 5, 16:0 and 18:1 ω 7c. In contrast, the pattern for the aerobic incubations of ZK07 was different with many more PLFA being labeled. Major differences between the two sediments were a much higher labeling of the methyl-branched PLFA i15:0, a15:0, i17:1w7 and a17:0 and of 18:1 ω 9c at the ZK07 sediment. Results for the ZK09 sediments were similar to ZK07 (data not shown). This clearly suggested that different groups of chemoautotrophic bacteria were active at the two sites.

Although total chemoautotrophy was similar for the aerobic and anaerobic incubations of ZK07 (Fig. 4. 2), there was a clear shift in label distribution between aerobic and anaerobic incubations in many PLFA (i15:0, a15:0, 15:1, 15:0, i17:1w7, a17:1w7, 17:1w8c, cy17:0 and 17:0) as they incorporated more ¹³C label under anaerobic than under aerobic conditions (Fig. 4.3). In contrast, the PLFA that gained most label under aerobic conditions for RK06 (14:0, 16:1w7c, 16:1w5, 16:0 and 18:1w7c) were all much less labeled under anaerobic conditions (Fig. 4. 3). However, although total labeling was much lower under anaerobic conditions for RK06 (Fig. 4.2), labeling in some PLFA (i15:0, a15:0 and 15:0) was not much altered under anaerobic conditions (Fig. 4.3). It is also interesting to note that some of the PLFA that were less labeled under anaerobic conditions (14:0, 16:1 ω 7c, 16:1 ω 5) were the same for RK06 and ZK07. The results from ZK09 were similar to ZK07 except that the stimulation of the labeling in the same set of PLFA was somewhat less for ZK09 (data not shown). Results for the low free-sulfide experiment B1 were similar to the whole core ZK incubations in that inhibition in the same set of mainly methyl-branched and other 15- and 17-PLFA was limited (data not shown). Data for the high free-sulfide experiment B2 were not reliable due to the very low labeling under anaerobic conditions. Together these results suggest that the anaerobic chemoautotrophic community had a similar PLFA composition which is characterized by methyl-branched and other 15- and 17-PLFA in all sediments, but that depending on sulfide concentrations the contribution of this group to the total chemoautotrophy varied strongly between sediments.

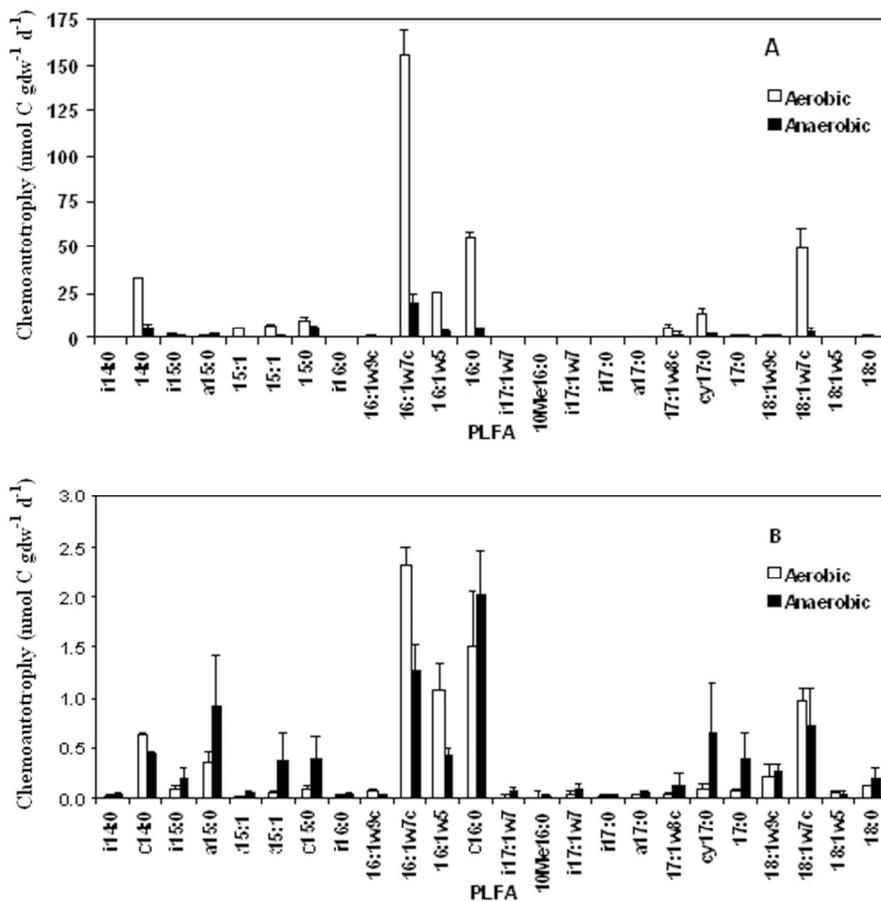


Figure 4.3. Chemoautotrophic synthesis rates of individual PLFA for the (A) RK06 and (B) ZK07 intertidal sediment cores in the surface layer (0-1 cm) of the sediment. Results from ¹³C-incorporation into individual PLFA are corrected for ¹³C-DIC labeling levels and therefore show total synthesis rates from inorganic carbon.

To further identify active anaerobic bacterial chemoautotrophs, we measured ¹³C labeling of specific 16S rRNAs by using the Mag-SIP method for the ZK09 sediment (Fig. 4.4). Only the ZK sediment was studied because it had the highest contribution from anaerobic chemoautotrophy. Labeling was detected in all captured 16S rRNA fractions from ¹³C-labeled samples and ranged between 10 and 28‰ Δδ¹³C (Fig. 4.4). As with the ZK07 and ZK09 PLFA data, labeling in the total bacterial 16S rRNA was similar under aerobic and anaerobic conditions. Labeling in *Gammaproteobacteria* was lower under anaerobic condition, whereas labeling of *Deltaproteobacteria* was not affected.

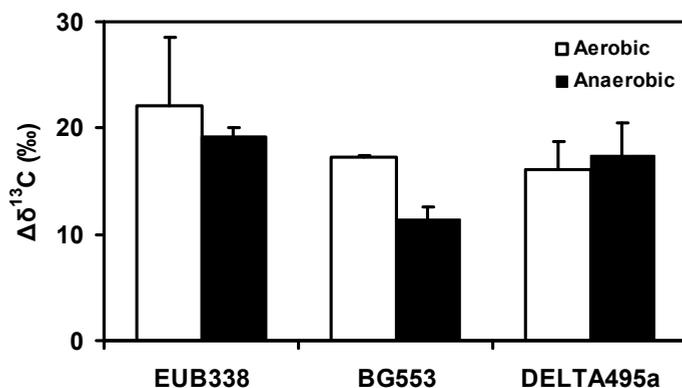
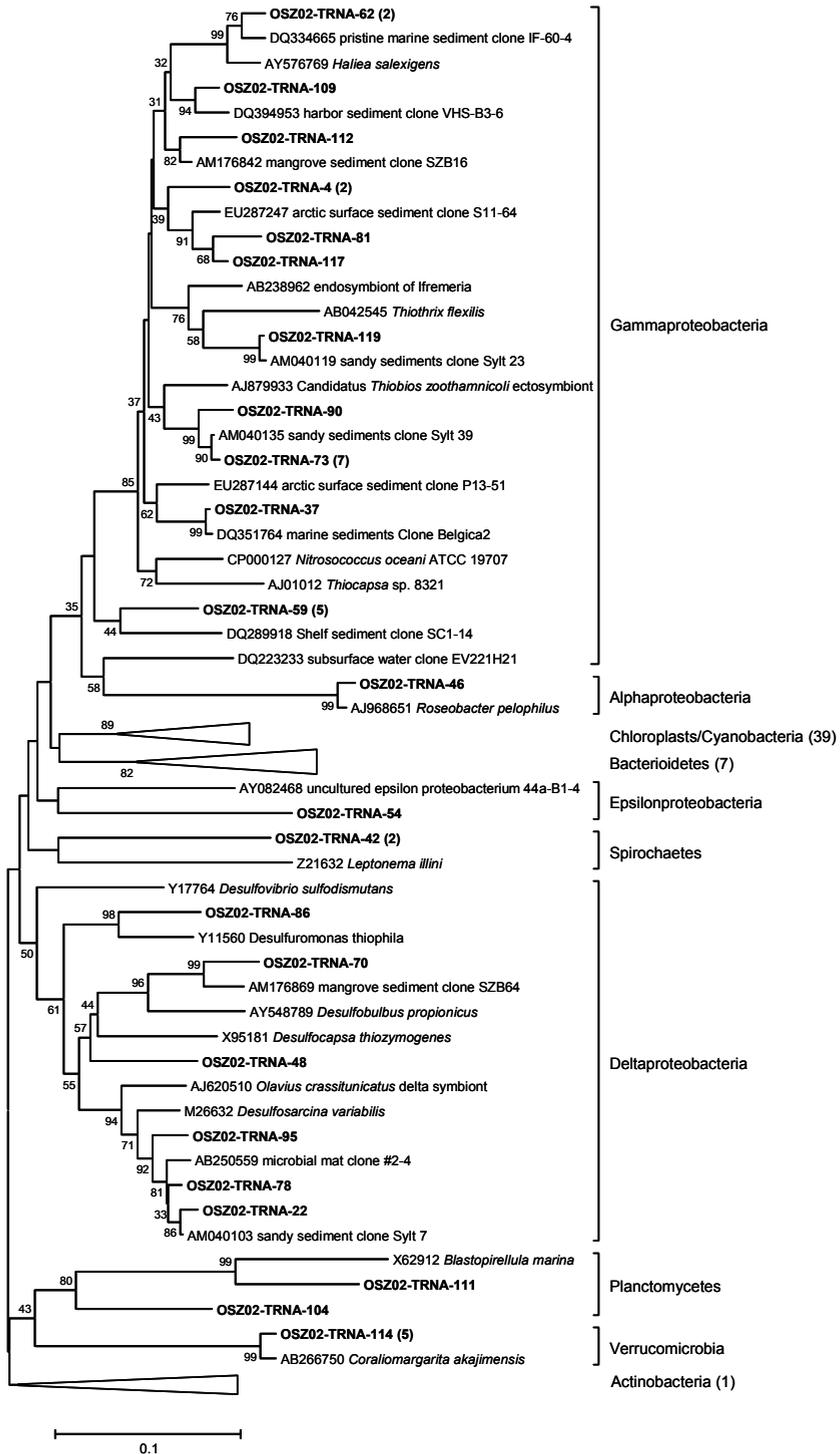


Figure 4.4. $\Delta\delta^{13}\text{C}$ values of rRNA captured with the nested set of probes used in the Mag-SIP protocol. Probes used were EUB338 for most *Bacteria*, BG553 for *Gammaproteobacteria* and DELTA495a for *Deltaproteobacteria*.

Based on the 16S-rRNA based cDNA clone library reported by Miyatake et al. (2010), a phylogenetic tree was constructed to show the relationship between clones and relate sequences focusing on the two Mag-SIP captured groups (Fig. 4.5). *Gammaproteobacteria* (21% of the sequences) were a diverse group mostly related to environmental sequences not closely related to *Bacteria* cultured. However, a substantial number of *Gammaproteobacteria* sequences (35%) was related to chemoautotrophic symbionts found in higher organisms (Fig. 4.5). *Deltaproteobacteria* explained 7% of the community 16S rRNA and were dominated by sequences placed in the *Desulfobulbaceae* and in the *Desulfobacteraceae*. The combined results of the Mag-SIP assay and the rRNA clone library therefore suggested that, beside *Gammaproteobacteria*, *Deltaproteobacteria* probably related to sulfate reducing bacteria were important chemoautotrophs at the ZK09 site and that both groups were involved in the anaerobic chemoautotrophy rates detected in this study.

Figure 4.5. Neighbor-joining tree showing the affiliation of 16S rRNA clones to closely related sequences. Clones with designations containing TRNA are derived from total community RNA in this study. Numbers in parenthesis indicate number of clones in a phylotype ($\geq 97\%$ similarity in sequence). Bootstrap values represent 1000 replicates and only values greater than 50% are reported. The scale bar indicates 10% estimated phylogenetic divergence.



Discussion

We detected substantial anaerobic chemoautotrophy rates by measuring dark ^{13}C -fixation into microbial biomarkers in typical, highly active coastal sediments. The contribution of anaerobic rate to the total chemoautotrophic carbon fixation rate was highly variable between sediments and was comparatively high when free sulfide was not detected. Concentrations of free sulfide are generally very low in the first centimeters of coastal marine sediments (Jørgensen and Nelson, 2004; Oenema, 1990), suggesting that anaerobic chemoautotrophy may be a common phenomenon in typical coastal sediments such as the ZK site. Sulfide formed during sulfate reduction is either quickly oxidized when oxidants such as O_2 , NO_3^- or MnO_2 are available or it precipitates as iron sulfides when reactive FeOOH is present (Jørgensen and Nelson, 2004). Free sulfide will therefore only accumulate if oxidants and reactive FeOOH are not available, which was apparently the case at the very active RK site. Many anaerobic reoxidation processes such as the oxidation of iron sulfides with nitrate or MnO_2 that may support chemoautotrophy depend on the availability of oxidants. Hence, the accumulation of free sulfide in the porewater indicates a lack of oxidants that could support anaerobic chemoautotrophy explaining the low contribution from anaerobic chemoautotrophy at the RK site and in experiment B2.

Another explanation for the low anaerobic chemoautotrophy in the presence of free sulfide is that high sulfide concentrations are toxic for many organisms (Bagarinao, 1992; Chapman *et al.*, 2002). However, it seems unlikely that this was the main cause in our study as sulfide concentrations stayed below 1 mM and the pH was around 8, which means that most of the sulfide would be in the less toxic dissociated form that can not diffuse into the cell. Finally, anaerobic chemoautotrophic bacteria capable of disproportionation of elemental sulfur, where elemental sulfur is fermented to sulfide and more oxidized forms of sulfur, are inhibited by free sulfide as the process becomes energetically unfavorable (Janssen *et al.*, 1996). Sulfur disproportionation can be important in coastal marine sediments and the process can only occur when sulfide is scavenged by FeOOH (Canfield and Thamdrup, 1996). However, other disproportionation reactions with thiosulfate and sulfite as substrates are not sensitive to sulfide accumulation (Bak and Pfennig, 1987). The only other publication on anaerobic chemoautotrophy rates in marine sediments, also reported high rates just below the depth where oxygen disappeared from the porewater (Thomsen and Kristensen, 1997). Similarly, in stratified marine waters, anaerobic chemoautotrophy is also typically high and often exceeds aerobic chemoautotrophic carbon fixation rates (Jost *et al.*, 2008; Taylor *et al.*, 2001).

We used both PLFA and Mag-SIP to study active chemoautotrophic microorganisms in marine sediments. Both methods indicated that *Deltaproteobacteria*, related to sulfate reducing bacteria, were a major group in the anaerobic chemoautotrophic activity in all sediments studied especially at the low free-sulfide ZK site. The Mag-SIP results indicated the activity of chemoautotrophic *Deltaproteobacteria*, which was not inhibited under

anaerobic conditions. Furthermore, many *Deltaproteobacteria* sequences recovered in this study fell in clades that are mostly considered to be sulfate or sulfur reducing bacteria. Finally, the methyl-branched and other 15- and 17-PLFA associated with anaerobic chemoautotrophic activity are commonly found in sulfate reducing *Deltaproteobacteria*. Some of these PLFA, like i17:1 ω 7, a17:1 ω 7 and 17:1 ω 8c, have been indicated as potential specific biomarkers for incomplete oxidizing sulfate reducers and related organisms (Dowling *et al.*, 1986; Oude Elferink *et al.*, 1998; Taylor and Parkes, 1983). Labeling in the typical sulfate reducer PLFA was actually stimulated under anaerobic conditions in the ZK07 sediment, suggesting that aerobic and anaerobic chemoautotrophic bacteria to some degree competed for the same reduced inorganic substrates.

Chemoautotrophic anaerobic growth has been described in a number of sulfate reducing bacteria and related organisms and is supported by either the oxidation of hydrogen gas with sulfate or by disproportionation reactions of different sulfur species such as sulfite, thiosulfate and elemental sulfur (Janssen *et al.*, 1996; Rabus *et al.*, 2006). Thomson and Kristensen (1998) also detected high anaerobic chemoautotrophy rates in a marine sediment and showed that it was stimulated by both the addition of hydrogen gas and thiosulfate. However, the depth distribution of the chemoautotrophy rates (this study; Thomsen and Kristensen, 1997) suggests that anaerobic rates are most likely supported by disproportionation reactions. Disproportionation of thiosulfate is an important process in marine sediments, similar to the distribution of anaerobic chemoautotrophy, and highest activities are generally found in the suboxic top few centimeters of the sediment (Jørgensen and Bak, 1991). Thiosulfate is thought to be an intermediate in the anaerobic oxidation of iron sulfide and pyrite with metal oxides, which is restricted to the suboxic zone in sediments (Schippers and Jørgensen, 2001). In contrast, hydrogenotrophic growth by sulfate reducing bacteria is a strictly anaerobic process, and hydrogen turnover is closely connected to organic matter degradation and typically found throughout the sediment column (Hoehler *et al.*, 1998; Novelli *et al.*, 1988). This argument is especially true for the RK05 site, where we showed that anaerobic organic matter mineralization is very high in the 1-5 cm horizon (Boschker *et al.* 2010), but chemoautotrophic carbon fixation was not detected (Fig. 4.1). The results therefore indicate that *Deltaproteobacteria* probably capable of disproportionation reactions may be important candidates for a substantial fraction of the anaerobic chemoautotrophy in coastal marine sediments.

The Mag-SIP approach showed that the activity of chemoautotrophic *Gammaproteobacteria* was lower in anaerobic incubations. This also agrees with the observation that PLFA typically found in chemoautotrophic and other *Gammaproteo-bacteria* (14:0, 16:1 ω 7c, 16:0 and 18:1 ω 7c; Kerger *et al.*, 1986; Knief *et al.*, 2003; Lipski *et al.*, 2001; Ratledge and Wilkinson, 1988; Zhang *et al.*, 2005) often showed considerably less labeling under anaerobic conditions in our study. Many of the detected *Gammaproteobacteria* sequences fell in several clades that are closely related to chemoautotrophic bacteria found as symbionts in higher organisms. This suggests that chemoautotrophy rates detected in this

study may have been in part associated with meio- and/or macrofauna. Some fauna may indeed be supported by chemoautotrophic carbon in similar types of sediment (Dubilier *et al.*, 2001; Ott *et al.*, 2004). However, the same *Gammaproteobacteria* clades are typically highly abundant in marine sediments and there are strong indications that the majority of them are free living bacteria that may be recruited as symbionts by higher organisms (Aida *et al.*, 2008; Bright and Bulgheresi, 2010). Sulfur oxidizing *Epsilonproteobacteria* are commonly associated with anaerobic chemoautotrophy in stratified water bodies (Glaubitz *et al.*, 2009), but were not detected in our clone library suggesting that they were probably not important. Anammox bacteria could be another possible candidate as anaerobic chemoautotrophs, but very limited labeling was detected in the 10Me16:0 PLFA found as a major membrane fatty acid in these *Bacteria* (Sinninghe Damste *et al.*, 2005). There are many possible reoxidation processes that may drive the anaerobic chemoautotrophy detected in *Gammaproteobacteria*. Many reduced sulfur compounds found in marine sediments such as iron sulfide, pyrite, sulfur and thiosulfate can be oxidized by *Bacteria* with NO_3^- and MnO_2 as oxidants under anaerobic conditions (Canfield *et al.*, 2005).

To conclude, we detected substantial anaerobic chemoautotrophy rates by measuring dark ^{13}C -fixation into microbial biomarkers in typical, highly active coastal sediments. *Deltaproteobacteria* related to sulfate reducing bacteria were major players in all studied sediments beside *Gammaproteobacteria*. Interestingly, chemoautotrophic sulfate reducers do not use the Calvin cycle for carbon fixation, but are known to use other pathways namely either the reversed TCA cycle or the reductive Acetyl-CoA pathway (Canfield *et al.*, 2005). Our results therefore suggest that these pathways may be important in marine sediments, but they have not been studied in any detail in these ecosystems. Recently, genomic information from chemoautotrophic sulfate reducers has become available (Dubilier *et al.*, 2001; Strittmatter *et al.*, 2009), which may guide future molecular ecological studies into the role of these carbon fixation pathways in marine sediments.

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