Chemoautotrophic Carbon Fixation Rates and Active Bacterial Communities in Intertidal Marine Sediments

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Abstract

Chemoautotrophy has been little studied in typical coastal marine sediments, but may be an important component of carbon recycling as intense anaerobic mineralization processes in these sediments lead to accumulation of high amounts of reduced compounds, such as sulfides and ammonium. We studied chemoautotrophy by measuring dark-fixation of 13C-bicarbonate into phospholipid derived fatty acid (PLFA) biomarkers at two coastal sediment sites with contrasting sulfur chemistry in the Eastern Scheldt estuary, the Netherlands. At one site where free sulfide accumulated in the pore water right to the top of the sediment, PLFA labeling was restricted to compounds typically found in sulfur and ammonium oxidizing bacteria. At the other site, with no detectable free sulfide in the pore water, a very different PLFA labeling pattern was found with high amounts of label in branched i- and a-PLFA besides the typical compounds for sulfur and ammonium oxidizing bacteria. This suggests that other types of chemoautotrophic bacteria were also active, most likely Deltaproteobacteria related to sulfate reducers. Maximum rates of chemoautotrophy were detected in first 1 to 2 centimeters of both sediments and chemosynthetic biomass production was high ranging from 3 to 36 mmol C m⁻² d⁻¹. Average dark carbon fixation to sediment oxygen uptake ratios were 0.22±0.07 mol C (mol O₂)⁻¹, which is in the range of the maximum growth yields reported for sulfur oxidizing bacteria indicating highly efficient growth. Chemoautotrophic biomass production was similar to carbon mineralization rates in the top of the free sulfide site, suggesting that chemoautotrophic bacteria could play a crucial role in the microbial food web and labeling in eukaryotic polyunsaturated PLFA was indeed detectable. Our study shows that dark carbon fixation by chemoautotrophic bacteria is a major process in the carbon cycle of coastal sediments, and should therefore receive more attention in future studies on sediment biogeochemistry and microbial ecology.

Introduction

Reoxidation of reduced intermediates like sulfide and ammonium formed during anaerobic mineralization processes is an important process in coastal marine sediments. Oxygen is typically only found in the top millimeters of these sediments and along macrofauna burrows [1], and carbon mineralization proceeds in general by anaerobic processes primarily sulfate reduction. This results in the production and accumulation of large amounts of reduced compounds such as various forms of reduced sulfur and ammonium [2]. In typical coastal sediments, free sulfide in the porewater is however often only detected below a couple of centimeters as it quickly reacts with iron hydroxides forming iron sulfide (FeS) or pyrite (FeS₂) [3]. Only in very active sediments or sediments containing little reactive iron, free sulfide can be found near the oxic top layer [3]. Long term burial of reduced compounds is thought to be a minor process [3] and they are mostly transported to more oxidized horizons by either diffusion or bioturbation [4]. Oxygen is eventually the main oxidant of these reduced compounds although intermediate reoxidation steps by a variety of anaerobic pathways using nitrate or iron and manganese oxides may also be important [3]. It is estimated that reoxidation processes on average explain 70% of the sediment oxygen flux in shelf sediments [5] and this value is expected to be higher in active intertidal areas as anaerobic mineralization will be more important.

Many of the known prokaryotes involved in reoxidation processes are chemo(litho)autotrophs that use the energy gained from inorganic reactions to grow by fixing inorganic carbon in the dark [6]. Chemoautotrophic carbon fixation has been shown to be an important process in, for instance, extreme marine ecosystems such as hydrothermal vents [7,8] and in the chemocline of anoxic marine basins [9,10]. The current consensus is however that chemoautotrophy is a relatively minor process in coastal sediments due to the relatively low growth yields of chemoautotrophic organisms and the competition with chemical oxidation reactions [3]. In addition, true chemoautotrophic bacteria have to compete with mixotrophic and heterotrophic bacteria that are able to oxidize reduced sulfur compounds [11], which could be relevant especially in active coastal sediments receiving large amounts of organic matter. Studies where chemoautotrophy was actually quantified by determining dark carbon fixation rates are rare for
typical coastal marine sediments and we have only been able to locate four studies: two on shallow subtidal sediments from the Baltic [12,13], one study on an intertidal sand flat in the German Wadden Sea [14] and a recent study on three brackish coastal lake sediments in Brazil [15]. However, recent estimates suggest that up to 0.29 Pg C y$^{-1}$ could be potentially fixed by chemosynthetic microorganisms in near shore and shelf sediments worldwide compared to 0.92 Pg C y$^{-1}$ of mineralization [16], suggesting a major role in the sediment carbon cycle. Finally, the dominant chemosynthetic bacteria involved in sulfur oxidation are not well known in coastal marine sediments. A recent study identified an uncultured group of Gammaproteobacteria as important players [14], but there may be many other groups involved in the diversity of reoxidation processes that occur in marine sediments.

We studied chemosynthetic rates in two intertidal sites with contrasting sulfur chemistry: a site where free sulfide was not detected in the top few centimeters of the sediment and a very active site where high concentrations of free sulfide were found right to the top of the sediment. The main substrates driving chemosynthetic rates were determined by incubating sediment cores with stable isotope labeled $^{14}$C bicarbonate and measuring labeling in phospholipid derived fatty acids (PLFA). This method both yields estimates of total chemosynthetic fluxes and provides an indication of the active bacterial community [17–19]. The diversity of Rubisco genes was studied to further indicate possible active chemosynthetic processes that use the Calvin cycle for carbon fixation. Finally, chemosynthetic rates were compared with diffusive oxygen fluxes and carbon mineralization rates.

Materials and Methods

Description of field sites

Two field sites in the Eastern Scheldt estuary (The Netherlands) were selected, which were expected to show high mineralization rates and have major differences in sulfur chemistry. The site in the Zandkreek area (51°38’41’’N, 3°53’22’’E) was situated next to a Pacific oyster (Crassostrea gigas) bed and was sampled in April 2005 (abbreviation ZK05) and October 2007 (ZK07). The Pacific oyster is an invasive species in the area that was introduced in the 1930s and is abundant in the top few millimeters of the Rattekaai sediment, especially in 2005 and to a lesser degree in 2006.

Sediment sampling

Undisturbed sediments were sampled with two sizes of polycarbonate core liners. The smaller cores (internal diameter 4.6 cm) contained silicon-filled injection ports at every 0.5 centimeter and were used for measuring chemosynthetic rates. The larger cores (internal diameter 6 cm) were used for additional measurements of porewater profiles and sediment characteristic, and for measuring mineralization rates. Sediments were sampled at low tide and therefore did not have overlying water. Cores were processed the same day for chemosynthetic rate measurements and other analyses.

Chemosynthetic rates

Chemosynthetic rate measurements were started by injecting 100 μl of 20 mM NaH$^{13}$CO$_3$ (99% $^{13}$C; Cambridge Isotope Laboratories, Andover, MA, USA) horizontally into the sediment cores at 0.5 cm depth intervals by using the line-injection method [2]. The $^{13}$C-label was dissolved in artificial seawater lacking calcium or magnesium in order to avoid precipitation [21]. The label was made oxygen free by bubbling with nitrogen gas shortly before injection. Sediment cores were incubated in the dark within 2°C of the in-situ temperature (see Table 1) for various periods of up to 4 days, and were ventilated daily by removing the top stopper for one minute (ZK) or incubated without top stoppers (RK) to circumvent the development of suboxic condition in the headspace. After incubation, sediment cores were sliced to a depth of 5 cm and sediment slices were quickly centrifuged (4500 rpm, 5 min) to collect porewater for concentration and $^{13}$C analysis of dissolved inorganic carbon (DIC). Sediments were subsequently frozen at −20°C and lyophilized before further analysis. Unlabelled, control cores were also processed.

PLFA analysis and calculation of chemosynthetic rates

Lyophilized sediments were analyzed for PLFA concentrations and $^{13}$C-labeling as described before [22,23]. In short, PLFA were extracted according to standard protocols and were analyzed by gas chromatography – isotope ratio mass spectrometry (GC-IRMS, Thermo, Bremen, Germany) on an a-polar analytical column (HP5-MS, Agilent, Santa Clara, CA, USA). Stable carbon isotope ratios are reported as δ$^{13}$C ratios on the VPDB scale. Excess $^{13}$C in individual PLFA was calculated as in Boschker et al [23] and divided by the atom percent excess $^{13}$C in the DIC pool to calculate actual PLFA synthesis rates. Only very minor labeling was found in poly-unsaturated PLFA typical for Eukarya (see Results) suggesting that PLFA labeling was primarily by Bacteria. We therefore used the labeling data for all common bacterial PLFA in the 12.0 to 20.0 range in our calculations and not just the specific bacterial biomarker PLFA [24]. Total bacterial chemosynthetic activities were determined by summing synthesis rates in all PLFA that were fixed in bacteria and converted to chemoautotrophic biomass production by dividing by the typical PLFA content of aerobic bacteria (55 mmol PLFA-C (mol biomass C)$^{-1}$ [24,25]). To study the differences in active chemoautotrophic bacterial communities, we performed a principle component analysis (PCA) on log-transformed PLFA $^{13}$C-labeling data (in Mol%) using the Statistica software package (StatSoft, Tulsa, USA).

Additional measurements

Oxygen profiles were determined with oxygen microelectrodes (Unisense Ox100, Aarhus, Denmark), which were lowered with a micromanipulator into the sediment until no oxygen was detected. Two profiles were recorded for each duplicate sediment core (four
Soil DNA Isolation kit according to protocol (MoBio, Carlsbad, CA, USA). Soil DNA was extracted from 0.5 g of wet sediment using the MoBio UltraClean DNA Isolation kit. DNA was amplified using degenerative primers targeting Rubisco sequences [33], which was important as Beggiatoa-like bacteria were found at the RK site but were not covered by previously published primer sets developed for chemoautotrophic bacteria. The primer set also targets some of the lower branching sequences and 13C-content by elemental analyzer - IRMS [29].

Sediment porewater was sampled by slicing duplicate sediment cores in an anaerobic glove-box filled with 3% hydrogen in nitrogen gas (Coy Laboratory Products, Ann Arbor, MI, USA) and slices were centrifuged at 4500 rpm for 10 min at in situ temperature. Samples for sulfide analysis were immediately fixed in zinc acetate and analyzed according to Cline [28]. Samples for ammonium and anion analysis were frozen, and analyzed on a QuAAtro segmented flow analyzer (Seal Analytical, Norderstedt, Germany) and suppressed high performance ion chromatography (SPEX, Metuchen, NJ, USA), and results were then translated to protein sequences, and compared to known sequences (T3, T7, 571, 898E) were removed from sequences, then translated to protein sequences, and compared to known sequences using BLAST. Protein sequence alignments and phylogenetic analysis was done in MEGA V [34]. Sequences have been deposited in the GenBank database under accession numbers JQ659214 to JQ659253.

### Results

In spring 2005, both sites (RK05 and ZK05) were studied in an initial test to determine if chemoautotrophy rates could be quantified by 13C-DIC labeling of PLFA in the dark. Sites were sampled again in spring 2006 (RK06) and autumn 2007 (ZK07), when a more extensive sampling program was executed.

### Sediment biogeochemistry

Oxygen penetrated significantly deeper in ZK sediment (1–2 mm) than RK sediment (0.2–0.5 mm, Table 1). At RK06, high concentrations of free sulfide were found in the very top layer of the sediment, whereas sulfide only started to accumulate below 2 cm sediment depth at ZK07 (Fig. 1). Sulfide and ammonium concentrations were more than 10 times higher for RK06 than for ZK07 throughout the sediment column (Fig. 1). In 2005, porewater samples were also taken at both sites and analyzed for sulfide, but samples were taken two weeks before chemoautotrophy measurements and not at exactly the same location, which is consistent with the differences observed in 2006.

### Table 1. Sediment in-situ temperature, sediment characteristics, oxygen consumption rates, carbon mineralization rates, chemoautotrophy rates and yields (averages ± standard deviations, N = 2) for the coastal marine sediments in this study.

<table>
<thead>
<tr>
<th>Site/Year</th>
<th>Temp. °C</th>
<th>POC (%)</th>
<th>C/N</th>
<th>O2 penetration depth (mm)</th>
<th>O2 flux (mmol m⁻² d⁻¹)</th>
<th>C mineralization (mmol C m⁻² d⁻¹)</th>
<th>Chemoautotrophy (mmol C m⁻² d⁻¹)</th>
<th>Yield C/O2 (mol C (mol O2)⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RK05</td>
<td>14</td>
<td>-</td>
<td>-</td>
<td>0.45±0.10</td>
<td>17.2±3.0</td>
<td>-</td>
<td>5.5±1.9</td>
<td>0.32±0.11</td>
</tr>
<tr>
<td>RK06</td>
<td>17</td>
<td>2.0</td>
<td>10.9</td>
<td>0.23±0.06</td>
<td>192±41</td>
<td>197±36</td>
<td>36.3±4.8</td>
<td>0.19±0.03</td>
</tr>
<tr>
<td>ZK05</td>
<td>14</td>
<td>-</td>
<td>-</td>
<td>1.7±0.1</td>
<td>15.0±0.4</td>
<td>-</td>
<td>2.6±0.3</td>
<td>0.17±0.02</td>
</tr>
<tr>
<td>ZK07</td>
<td>13</td>
<td>0.6</td>
<td>7.7</td>
<td>0.95±0.06</td>
<td>15.5±1.6</td>
<td>105.9±19.1</td>
<td>2.9±0.2</td>
<td>0.18±0.01</td>
</tr>
</tbody>
</table>

1Data for 0–1 cm sediment depth. 2 Data integrated over 0–5 cm sediment depth.
especially important for the RK site due to its patchy nature. However, the contrast between the RK and ZK sites was similar with high concentrations of free sulfide at RK05 right to the top of the sediment core and no detectable sulfide in top 5 cm of the ZK05 sediment (results not shown). Porowater concentrations of DIC and SO\(_4^{2-}\) showed little variation with depth for ZK07 strongly indicating bio-irrigation, whereas DIC increased and SO\(_4^{2-}\) decreased with depth for RK06 indicating carbon mineralization by sulfate reduction (Fig. 1).

Diffusive sediment oxygen consumption rates as determined from microelectrode profiles were very high for RK06 with 192 mmol m\(^{-2}\) d\(^{-1}\) and were approximately 15 mmol m\(^{-2}\) d\(^{-1}\) for all other samplings (Table 1). The difference between the two RK samplings is probably due to the patchy nature of the site, even though visually similar black sediments with a whitish top layer were sampled in both years. For RK06, anaerobic carbon mineralization rates were about twice as high in the top centimeter (6.8 ± 0.5 μmol C cm\(^{-3}\) d\(^{-1}\)) than in the 1–5 cm layer (3.2 ± 0.8 μmol C cm\(^{-3}\) d\(^{-1}\)), whereas both sediment layers showed similar carbon mineralization rates for ZK07 (0–1 cm, 1.6 ± 0.9 μmol C cm\(^{-3}\) d\(^{-1}\); 1–5 cm, 2.3 ± 0.3 μmol C cm\(^{-3}\) d\(^{-1}\)). Integrated over the upper 5 cm, anaerobic carbon mineralization rates were 197 and 106 mmol C m\(^{-2}\) d\(^{-1}\) at RK06 and ZK07, respectively (Table 1). Ammonium production-based mineralization rates agreed with carbon mineralization rates at all sediment depths and ammonium-based mineralization rates in the top layer (0–1 cm) were 8.0 ± 2.0 and 1.1 ± 0.3 μmol C cm\(^{-3}\) d\(^{-1}\) for RK06 and ZK07, respectively.

Chemooautotrophy rates

Dynamics of PLFA labeling with \(^{13}\)C-bicarbonate were studied in detail for RK06. Substantial labeling could already be detected in the 0–0.5 cm horizon after 4 hours of incubation and, although there was some variation, total PLFA labeling increased linearly with time for up to 4 days (R\(^2\) = 0.77, n = 8). Similar results were obtained for RK05 and ZK05 as calculated chemooautotrophy rates were similar after 2 and 4 days of incubation (data not shown). For RK06, the \(^{13}\)C-enrichment in the DIC pool in the 0–0.5 cm of the sediment decreased from 1800 ± 120 % Δ\(^{13}\)C (\(^{13}\)C enrichment of the DIC pool of 1.9%) approximately after 4 hours to 550 ± 110 % Δ\(^{13}\)C (0.5% \(^{13}\)C) after four days, probably because of exchange with atmospheric carbon dioxide and dilution with DIC produced during organic matter mineralization. Cores from RK06 could not be kept closed at the top because sub-oxic conditions developed within one day due to the very high oxygen consumption rates. The reported chemooautotrophy rates have been corrected for this change in DIC labeling with incubation time.

Chemooautotrophy was generally limited to the top centimeter of the sediment especially at the RK site (Fig. 2). For RK06, the main activity (95 ± 1%) was found in the top 0–0.5 cm horizon, which contains the oxic top layer, and below 1 cm depth no activity could be detected. Similar results were obtained for RK05 when depth profiles were determined after 2 and 4 days. Interestingly, chemooautotrophy rates recorded in the top layer of RK06 were similar (7.2 μmol C cm\(^{-3}\) d\(^{-1}\), Fig 2) to the anaerobic carbon mineralization rates (6.8 μmol C cm\(^{-3}\) d\(^{-1}\), see above), suggesting balanced CO\(_2\) production and consumption. For the ZK sediment, highest chemooautotrophy rates were also found in the top layer of the sediment, but activity remained relatively high down to 2 cm depth for both sampling dates.

Depth integrated (0–5 cm), whole-core chemooautotrophy rates ranged from 3–36 mmol C m\(^{-2}\) d\(^{-1}\) (Table 1). Rates measured at RK06 were very high, about 6 times higher than for RK05, which is probably due to the patchy nature of the site and differences between years (Table 1). Rates for ZK05 and ZK07 were however in the same range and always lower than for the RK site. Whole-core chemooautotrophy rates generally scaled with diffusive oxygen uptake rates and whole sediment chemooautotrophy to oxygen consumption ratios were relatively similar for all sites ranging from 0.17 to 0.32 mol C (mol O\(_2\))\(^{-1}\) (Table 1).

Active chemooautotrophic bacterial communities

As we determined \(^{13}\)C-dark fixation rates into biomarker PLFA, the results can also be used to describe and compare active communities of chemooautotrophic bacteria. As an example, Fig. 3 shows the PLFA concentration and labeling data as obtained during this study after one day of incubation for the top layer (0–0.5 cm) of RK06. The PLFA detected were typical for intertidal sediments with high amounts of both bacterial and eukaryote-specific biomarkers; the latter were probably mainly derived from the diatoms growing on the sediment surface as they were dominated by 16:0 and 20:5n3 that occur in high amounts in diatoms (Fig. 3A; [35]). Detection of PLFA labeling is based on the increase in δ\(^{13}\)C ratios and these were well above detection limits in many PLFA (Fig. 3B; 0.6 to 2% Δ\(^{13}\)C detection limit depending on compound). The Δ\(^{13}\)C ratios were highly variable between PLFA ranging from 0 to 110% after one day (Fig. 3B), suggesting that a specific sub-group of the total bacterial community was active. At RK06, PLFA that gained most \(^{13}\)C label were 14:0, 16:0, 17:0, 18:0 and 18:1 that together explaining 83±4% of the total incorporation into PLFA (Fig. 3C). There were also minor amounts of label recovered in 14:1, 15:1, 15:0, 17:0 and cy17:0. Branched, i- and a-PLFA and eukaryote PLFA like 20:4 and 20:5n3 gained very little label even though they were a dominant feature in the PLFA concentration pattern (Fig. 3A & 3C).

The PLFA labeling pattern for the two ZK samplings was very different from the RK site strongly suggesting that different chemooautotrophic communities were active at the two sites. For comparison, the labeling pattern of ZK05 is also presented in Fig. 3D as it showed the largest differences from RK06. Main differences were a much higher labeling in all branched i- and a-PLFA and in several mono-unsaturated PLFA (15:1, 16:1t, 17:1t, 18:0 and 18:1t) and uneven numbered saturated PLFA (15:0, cy17:0 and 17:0) for both RK samplings.

To study the differences in PLFA labeling patterns further we performed a PCA analysis for all sediment layers where significant chemooautotrophy was detected (depth ranges down to 2 cm; Fig. 4). The first PCA axis explained 42% of the variation found in the data set, whereas the second axis added another 15%. Clustering was mainly based on 16:1\(\delta c, 18:1\) and uneven numbered PLFA for the first axis similarly as seen in Fig. 3 and 16:0 and 16:1t versus 14:0, 14:1 and cy17:0 for the second axis. Clustering of sediment samples was mostly determined by sampling site and sampling year with both RK samplings clustering closely together and more dispersal amongst the samples from the ZK site was observed (Fig. 4A). Some additional variation was found with sediment depth but only for RK06, where the top layer data (0–1 cm) clustered more closely together with the RK data whereas the deeper layers were shifted towards the ZK05 samples (Fig. 4A). Distribution of label among PLFA did not change substantially with incubation time, suggesting that active chemooautotrophic communities remained similar for up to 4 days. The PCA analysis therefore also showed major difference in active chemooautotrophic bacterial communities between the high free-sulfide RK and low free-sulfide ZK sediments.
There was also indirect evidence of transfer of dark-fixed carbon to fauna: some $^{13}$C-labelling was detected in bulk sediment PLFA characteristic of Eukarya and therefore fauna, 18:2$\omega6c$ for both sites and 20:4$\omega6$ and 20:5$\omega3$ for the ZK site (Fig 3C and D).

Rubisco type IA diversity

To further characterize the chemoautotrophic community, we used a novel primer set to construct clone libraries for the Rubisco Type IA large-subunit gene for both sites in 2008. For the RK site, 17 clones related to Type IA Rubisco were recovered and site ZK yielded 23 clones. We also found a limited number of clones related to unicellular cyanobacteria derived type IB Rubisco especially for the RK site (not shown).

The phylogenetic relationship for the clones from both sites together with other environmental clones and related microorganisms is shown in Fig. 5. Type IA clones were found in two main clades labeled type IA 1 and IA 2 (Fig. 5). Rubisco type IA 1 clones were most closely related to uncultured faunal endosymbionts belonging to the Gammaproteobacteria and marine sediment clones from a variety of other studies. Whereas sequences in clade IA 2 were related to various chemoautotrophic sulfur and ammonium oxidizing bacteria and also to other environmental sediment clones. *Beggiatoa* Rubisco type IA also clustered in clade IA 2, but although clones from both RK and ZK were found in this clade they were not closely related to *Beggiatoa* suggesting that they belonged to other groups of chemoautotrophic bacteria. In general rather similar sequences were recovered from both sites, although ZK clones were relatively more abundant in clade IA 1 and RK clones dominated clade IA 2. The results suggest that chemoautotrophic communities, which use the Calvin cycle for carbon fixation, were relatively similar at both sites and that *Beggiatoa* could not be detected at least for the 2008 sampling.

Discussion

Rates of chemoautotrophic carbon fixation

We detected very high rates of chemoautotrophic dark fixation in the top layers of two intertidal sediments especially for the
Sulfur oxidizing bacteria are expected to be the main chemoautotrophs in these intertidal sediments; the contribution from ammonium oxidizing bacteria should be less important because about 6 times more sulfide than ammonium is produced during anaerobic carbon mineralization given the typical C:N ratio for marine organic matter [42]. In addition, nitrifying prokaryotes also tend to have lower growth yields per mol of substrate oxidized than sulfur oxidizers [43]. The chemoautotrophy data from our study scaled well with measured diffusive oxygen fluxes with an average whole system yield (± SD) of 0.22 ± 0.07 mol C (mol O2)^{-1} (Table 1), which is very similar to the typically reported maximum growth yields for aerobic sulfur oxidizing bacteria of 0.23 ± 0.11 mol C (mol O2)^{-1} [44–50]. The similarity in C:O2 yield between intertidal sediments and sulfur oxidizing bacterial cultures can only be explained if most of the sediment oxygen consumption was indeed used for reoxidation of reduced sulfur and if reoxidation was predominantly performed by obligate sulfur-oxidizing chemoautotrophic bacteria growing close to their maximum reported yields. In addition, chemoautotrophic bacteria should have effectively competed with chemical oxidation processes such as the oxidation of free sulfide with oxygen, as has been shown in gradient systems where oxygen and sulfide are found in close proximity similar to the RK site [3]. Furthermore, even though these coastal sediments receive very high organic matter inputs, our data suggest that the activity by heterotrophic and mixotrophic sulfur-oxidizing bacteria was also limited as this

Figure 2. Depth distribution of chemoautotrophy as estimated from 13C-DIC PLFA labeling in dark incubations for the RK and ZK sediments.
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should have led to lower C:O₂ yields. Competition between autotrophic and heterotrophic or mixotrophic sulfur oxidizing bacteria depends to a large extent on the sulfur/organic substrate ratio with low ratios supporting heterotrophic sulfur oxidation [11,51], but this apparently didn’t play a role in the studied sediments possibly due to strong competition for organic substrates by other heterotrophic bacteria. Our results suggest that dark fixation rates as determined by ¹³C-bicarbonate labeling of PLFA yield realistic chemoaotrophy rates in relation to sediment biogeochemistry and that chemoaotrophic bacteria were growing with high efficiency independent of sediment sulfur chemistry.

Chemoautotrophy rate data are available from only four other studies on coastal marine sediments, all of which measured total dark fixation rates by determining ¹⁴C-DIC incorporation into POC [12–15]. It should be noted that two of these studies are based on laboratory incubations with homogenized sediments.
Thomsen and Kristensen [12] reported maximum rates of approximately 0.35 μmol C cm$^{-2}$ d$^{-1}$ (averaged over the same depth as in the present study) similar to site ZK, whereas rates found by Enoksson and Samuelsson [13] and Lenk et al [14] are lower at about 0.12 μmol C cm$^{-2}$ d$^{-1}$. The data collected by Thomsen and Kristensen [12] yield a C:O$_2$ ratio of 0.24 mol C (mol O$_2$)$^{-1}$ similar to our data, whereas the chemoautotrophy rates reported by Enoksson and Samuelsson [13] are substantially higher than expected from their oxygen consumption rates (C:O$_2$ ratio about 1.2 mol C (mol O$_2$)$^{-1}$). The C:O$_2$ ratios from Enoksson and Samuelsson [13] cannot be readily explained in relation to the reported growth yields of sulfur reducing bacteria [44–50], suggesting that the chemoautotrophy rates for this sediment may have been substantially underestimated possibly due to the incomplete removal of $^{13}$C-DIC label. Santoro et al [15] found much lower yields of about 0.025 mol C (mol O$_2$)$^{-1}$ for three brackish coastal lake sediments. Lenk et al [14] also reported $^{13}$C-POC based chemoautotrophy rates of 4.16±0.03 mmol C m$^{-2}$ d$^{-1}$ for an intertidal flat consisting of permeable sands, which related well to sediment sulfide fluxes [14,52]. Lenk et al [14] did not report oxygen consumption rates. However, potential oxygen fluxes of approximately 70 mmol O$_2$ m$^{-2}$ d$^{-1}$ have been reported for the same sand flat [53], suggesting a relatively low C:O$_2$ yield of about 0.06 mol C (mol O$_2$)$^{-1}$. Oxygen fluxes may have been overestimated because they are potential rates based on aerobic incubations with sediments from different horizons that may not always be oxic. The depth distributions of chemoautotrophy of Enoksson and Samuelsson [13] and Lenk et al. [14] were different from our study as they both found substantial rates deeper in the sediment up to a depth of 10 cm. Lenk et al [14] studied a permeable sediment where oxidants such as oxygen are transported deep into the sediment by advective porewater flows [53], which may explain the high chemoautotrophy rates deeper in the sediment. The chemoautotrophy rates in our study at the RK06 (3.2 to 6.8 μmol C cm$^{-3}$ d$^{-1}$) are the highest reported so far for coastal marine sediments.

Furthermore, our results suggest that chemoautotrophically fixed carbon may also be an important food source in the microbial food web in these typical coastal sediments. Chemoautotrophy rates were similar to carbon mineralization rates in the top layer of the RK06 sediment (Fig. 2 and Results). Net consumption of DIC related to the chemoautotrophy has been indicated in the top layer of active coastal sediments [12,54]. This suggests that the production by chemoautotrophs dominated the microbial food web and that heterotrophic bacterial secondary production was less important (if one assumes a growth efficiency of 50% for heterotrophic bacteria). Santoro et al [15] measured growth of both chemoautotrophic and heterotrophic bacteria in three brackish lakes and found that chemoautotrophy could explain up to 50% of the heterotrophic bacterial growth. Additionally, chemosynthetically produced biomass may potentially be an important source of energy fueling the benthic food web. We indeed detected limited labeling in eukaryotic faunal-derived PLFA for both sediments (Fig 3C & D), which suggests that sediment fauna may in part be feeding on chemoautotrophic bacteria. Chemoautotrophic bacteria support the food web in many extreme marine ecosystems like hydrothermal vents and mud volcanoes with limited organic matter inputs [8,55]. Based on our results, the role of chemoautotrophic carbon fixation in the benthic food web of coastal marine sediments should receive more attention.

**Communities of chemoautotrophic bacteria**

We detected clear difference in PLFA labeling patterns for the ZK and RK sediments suggesting that the active chemoautotrophic bacterial communities were substantially different (Fig. 3 and 4). The classical sulfur and ammonium oxidizing bacteria predominantly contain even-numbered saturated and monounsaturated PLFA such as 14:0, 16:1ω7c, 16:0 and 18:1ω7c [17,55–60]. For the RK sediment, most of the label was indeed recovered in these typical PLFA and the PLFA labeling pattern for instance closely resembles the PLFA composition of *Beggiaota...*
especially for RK05 (Fig. 3 and [55,56]). Some types of sulfur oxidizing thiobacilli also contain uneven numbered PLFA like cy17:0 [17,57], and label was indeed recovered in these PLFA especially at both ZK samplings and at RK06 suggesting that these groups may also be active in coastal sediments. In view of the high free sulfide and ammonium concentrations in the porewater, the PLFA labeling at the RK site was therefore in agreement with the activity of typical chemoautotrophic sulfur and ammonium oxidizing bacteria.

In contrast, major amounts of label were also found in branched i- and α-PLFA at the ZK site, which have not been reported in the classical chemoautotrophic sulfur oxidizing and nitrifying bacteria. However, several groups of sulfate reducing bacteria contain large amounts of branched PLFA [61–63]. In addition, significant amounts of label were recovered in i17:1ω7c, 17:1ω8c and cy17:0/17:1ω6c that have been suggested as specific biomarkers for certain groups of sulfate reducing bacteria [61–63], suggesting that sulfate reducing bacteria may be important chemoautotrophs in the ZK sediment. Chemoautotrophic growth can be found in two groups of sulfate reducing bacteria namely those that use hydrogen gas as substrate [64] and sulfur disproportionating bacteria that gain energy from inorganic fermentation of substrates such as thiosulfate and elemental sulfur to sulfate and sulfide [65,66]. Both hydrogen turnover and disproportionation reactions have been shown to be important processes in anaerobic marine sediments [67,68] and they have been indicated to possibly support chemoautotrophy in coastal sediments [12]. For the ZK07 sediment, the labeling in these branched PLFA became progressively more important with depth (Fig. 4), suggesting that it may indeed be associated with these anaerobic processes. Branched PLFA have also been found in anammox bacteria [69], but these also contain 10Me16:0 which showed no or limited labeling in our study (Fig. 3) suggesting that they were not important as chemoautotrophs. This is in agreement with studies that found that denitrification and not anammox is the dominant nitrate consuming process in active coastal sediments [70,71]. The high labeling in branched PLFA as detected at the low free-sulfide ZK site therefore indicates that anaerobic chemoautotrophs related to sulfate reducers may be important in coastal sediments.

We also studied the diversity of chemoautotrophic bacteria that use the Calvin cycle for carbon fixation by constructing clone libraries of the Rubisco type IA gene. Clones detected fell in two main clades: one related to uncultured endosymbiotic sulfur-oxidizing Gammaproteobacteria (clade IA 1) and another related to free living chemoautotrophic sulfur and ammonium oxidizing bacteria (clade IA 2, Fig. 5). Although clade IA 1 sequences were most closely related to endosymbionts, they are also commonly found in related uncultured free-living bacteria [14,72]. Two of these Rubisco clones are usually also detected in other environmental studies on marine sediments [32,73]. Lenk et al [14] also showed that free-living Gammaproteobacteria related to sulfur-oxidizing endosymbionts were important for dark carbon fixation in a permeable tidal flat sediment. The differences in Rubisco clone distribution between the two sediments are most likely due to differences in the availability of reduced sulfur compounds namely free sulfide at the RK site and iron sulfides at the ZK site. However, it seems unlikely that these differences in clone distribution explain the contrasting PLFA labeling patterns from the two sites. First, the differences in Rubisco clone distribution appear to be less site-dependent compared to the PLFA labeling patterns that showed major differences between sites. Second, the detected clones are related to bacteria that typically do not contain i- and a-branched PLFA as found for the chemoautotrophs from the ZK site. As discussed before, the PLFA composition of both the typical chemoautotrophic sulfur and ammonium oxidizers in clade IA 2 and Gammaproteobacteria like the ones detected in clade IA 1 is usually dominated by straight saturated and unsaturated compounds similar to the labeling patterns at the RK site [17,57,58]. Third, sulfate reducing bacteria typically fix inorganic carbon by either the reversed TCA-cycle or the reductive acetyl-CoA pathway [64] and are therefore not targeted in our Rubisco assay. The dominance of Gammaproteobacteria among the Rubisco clones is therefore also agreement with the PLFA labeling patterns especially for RK and to lesser degree for ZK.

To conclude, we detected very high rates of chemoautotrophy in two active intertidal sediments and our results indicate that chemoautotrophic carbon fixation is an important part of the carbon cycle of coastal sediments. Clear differences were found in active chemoautotrophic bacterial communities probably caused by differences in reduced sulfur compounds available in the two sediments. Our study suggests that anaerobic bacteria related to sulfate reducers played an important role at the low-sulfide ZK site while typical sulfur-oxidizers, probably Gammaproteobacteria, were more important at the free-sulfide RK site. Chemoautotrophic Archaea such as the ammonium-oxidizing Crenarcheota widely found in marine sediments [74] were not considered in the present study, but their activity could be studied by determining 13C labeling in Archaeal ether-lipids [41]. The number of coastal sediments where chemoautotrophic carbon fixation rates have been determined is still very low especially compared to studies on other aspects of the sedimentary carbon cycle. Besides our study we are aware of only four other studies where chemoautotrophy rates were quantified in coastal marine and brackish lake sediments [12–15]. Hence, chemoautotrophy in coastal marine sediments as driven by reoxidation processes should receive more attention in future studies given its importance in the carbon cycle of coastal sediments and the potential role in the benthic food web.

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Author Contributions

Conceived and designed the experiments: HTSB DVC LM. Performed the experiments: HTSB DVC LM. Analyzed the data: HTSB DVC LM. Contributed reagents/materials/analysis tools: HB TWCMP. Wrote the paper: HTSB DVC. Method development: HB.
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