Linking microbial community structure to biogeochemical function in coastal marine sediments: Stable isotope probing combined with magnetic bead capture

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Linking microbial community structure to biogeochemical function in coastal marine sediments

Stable isotope probing combined with magnetic bead capture

Tetsuro Miyatake

NIOO Thesis 79

Invitation
to the public defense
of the thesis

Linking microbial community structure to biogeochemical function in coastal marine sediments

by

Tetsuro Miyatake

on Tuesday
January 25, 2011
at 12:00 h

Agnietenkapel
Oudezijds Voorburgwal 231
1012 EZ, Amsterdam

Herewith I would like to invite you to the reception after the ceremony

by

Tetsuro Miyatake

Paranimphen:
Tanja C. W. Moerdijk-Poortvliet
Byron Mook
Linking microbial community structure
to biogeochemical function in coastal marine sediments

Stable isotope probing combined with magnetic bead capture
Promotor: Prof. dr. L. J. Stal

Co-Promotor: Dr. H. T. S. Boschker

Overige leden: Prof. dr. M. J. Teixeira de Mattos
Prof. dr. K. J. Hellingwerf
Prof. dr. J. Huisman
Prof. dr. M. Friedrich
Dr. S. Schouten
Prof. dr. A. J. M. Stams
Dr. H. G. van der Geest

Faculteit der Natuurwetenschappen, Wiskunde en Informatica
## Contents

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 1</td>
<td>General introduction</td>
<td>5</td>
</tr>
<tr>
<td>Chapter 2</td>
<td>Linking microbial community function to phylogeny of sulfate-reducing <em>Deltaproteobacteria</em> in marine sediments by combining stable isotope probing with magnetic bead capture hybridization of 16S rRNA</td>
<td>13</td>
</tr>
<tr>
<td>Chapter 3</td>
<td>Linking microbial community structure and function in marine intertidal sediment by Mag-SIP</td>
<td>33</td>
</tr>
<tr>
<td>Chapter 4</td>
<td>Characterization of anaerobic bacterial chemoautotrophy in intertidal marine sediments</td>
<td>51</td>
</tr>
<tr>
<td>Chapter 5</td>
<td>Tracing carbon flow from microphytobenthos to major phylogenetic groups in the bacterial community in an intertidal marine sediment</td>
<td>67</td>
</tr>
<tr>
<td>Chapter 6</td>
<td>General discussion</td>
<td>89</td>
</tr>
<tr>
<td>Summary</td>
<td></td>
<td>95</td>
</tr>
<tr>
<td>Samenvatting</td>
<td></td>
<td>99</td>
</tr>
<tr>
<td>要約</td>
<td></td>
<td>103</td>
</tr>
<tr>
<td>References</td>
<td></td>
<td>107</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td></td>
<td>119</td>
</tr>
</tbody>
</table>
Chapter 1

General introduction
**Biogeochemical cycles driven by microbial communities**

Global change is one of the biggest issues for humans not only scientifically but also economically. Considering the primal importance of microorganisms in the biogeochemical cycling of elements on earth, understanding their functions will deepen our understanding of ecosystems and will be critical in refining predictions about future environmental conditions. However, current ecosystem simulation models typical do not include microbial community composition, and often neither consider interactions within the community (Bardgett et al., 2008). An important reason for this is that the relationship between structure and function of microbial communities are complex and poorly understood. Therefore 'who is there,' and 'what are they doing,' is of great interest to microbial ecologists and biogeochemists.

**Carbon mineralization in continental shelf sediments**

The continental shelf plays a major role in the global carbon cycle in ocean, and has been recognized as a highly productive area with a primary production estimated at 5.2 Gt C yr\(^{-1}\) accounting for 15% of oceans primary production (Wollast, 1998). More importantly, most of the organic carbon burial (82%) takes place on the continental shelf, in particular in deltaic and other coastal sediments (Berner, 1982; Wollast, 1998). Local primary production and riverine organic matter are two major inputs of organic carbon in coastal areas.

Organic matter that is deposited on the shelf sediments is mineralized via a series of processes which are primary carried out by a diverse microbial community. The oxic zone is generally at most a couple of millimeters thick in typical coastal sediments (Brune et al., 2000). In this thin oxic zone, the heterotrophic microbial community consists of diverse microorganisms which employ a uniform metabolism, namely the aerobic respiration. Below the oxic zone, in the anaerobic but oxidized, sub-oxic zone, nitrate, manganese, and iron are the main electron acceptors (Braker et al., 2001; Edlund et al., 2008). The sub-oxic zone is also limited in most coastal sediments and much of the organic mineralization takes place in the anoxic zone where sulfate reduction predominates anaerobic carbon mineralization given the high concentrations of sulfate in marine waters. In shelf sediment, dissimilatory sulfate reduction accounts for on average about half of the total carbon mineralization (Jørgensen, 1982; Oenema, 1990). Thus, the carbon and sulfur cycles are strongly coupled in marine sediments. Sulfide burial is however limited in marine sediments and approximately 90% of sulfide formed by sulfate reduction is reoxidized again to sulfate by sulfur-oxidizing bacteria, many of which are chemoautotrophs that fix carbon dioxide for growth (Canfield et al., 2005). Chemoautotrophy is however thought to correspond to at most 7% of the carbon cycle in typical coastal sediments (Jørgensen and Nelson, 2004) but plays a more important role in specific environments such as hydrothermal vents and cold seeps (Arakawa et al., 2006; Jannasch et al., 1989). An overview of biogeochemical processes in marine sediments is shown in Fig. 1.1.
Carbon flow from primary producers to the heterotrophic microbial community

Primary production of microphytobenthos contributes significantly to the total primary production of estuarine and shallow water ecosystems where light reaches the sediment surface (MacIntyre et al., 1996; Underwood and Kromkamp, 1999). Epipelic diatoms typically dominate the microphytobenthos in intertidal marine sediments of temperate regions (Underwood 1994), and are known to exude large amounts carbohydrates in the form of extracellular polymeric substances (EPS) in order to migrate through the sediment and to stabilize the sediment surface avoiding resuspension (Paterson and Black, 1999). As a consequence, diatoms provide major carbon sources to benthic food web including heterotrophic bacteria in marine intertidal sediments (Smith and Underwood, 1998; van Oevelen et al., 2006).

Composition of carbohydrates exuded by diatoms changes depend on the conditions such as light irradiance, tidal cycle, and growth phase (Smith and Underwood, 1998; Underwood and Smith, 1998; van Duyl et al., 1999). High-molecular-weight exudates first need to be split into low-molecular-weight compounds by a variety of extracellular enzymes such as glucanase and glucosidase (van Duyl et al., 1999). Haynes et al. (2007) have reported a positive relation between β-glucosidase activity and the relative abundance of Gammaproteobacteria in experiments where EPS was added to surface sediment. Low-
molecular-weight exudates and products of hydrolysis by extracellular enzyme are utilized by all major groups in the community (Sundh, 1992) including diatoms (Smith, 1982). The microbial community structure of intertidal sediments (Bühring et al., 2005; Hunter et al., 2006; Rusch et al., 2003) and EPS formation by benthic diatoms (Goto et al., 2001; Haynes et al., 2007; Smith and Underwood, 1998) have both been extensively studied. There are however only a limited number of studies that directly traced carbon flows from diatoms to heterotrophic microbes (Bellinger et al., 2009; Middelburg et al., 2000), but none of these tried to identify responsible members in the microbial community.

**Linking microbial community structure and its function**

Since the late 1980s, the advent of cultivation-independent molecular methods has drastically increased our understandings of microbial community structure in natural ecosystems like marine sediments (e.g. Bowman and McCuaig, 2003; Kemp and Aller, 2004; Ravenschlag et al., 2001). The 16S rRNA gene has been used most widely as a marker to study microbial diversity and to show differences in community structure. However, 16S rRNA sequences alone provide little evidence about the physiology of microorganisms and their role in natural ecosystems, and more importantly most of the sequences detected in these studies belong to uncultured organisms with unknown physiologies. Functional genes encoding for key enzymes associated with biogeochemical processes such as dissimilatory sulfite reductase (Dhillon et al., 2003) and nitrite reductase (Braker et al., 2000) have provided more information on the role that certain microorganisms play. However, in many cases it remains difficult to directly link physiology with phylogeny for environmental samples containing substantial numbers of uncultured species as a reference to pure cultures is lacking and expression of functional genes is often poorly related to actual process rates (e.g. Severin and Stal, 2010). Our understanding of the functioning of microbial communities and the interactions between the different members therefore remains rather limited.

An elegant method for the simultaneous identification and the metabolic capabilities of microorganisms relies on isotope-based techniques. One of the more recent developments is in a combination of stable isotope labeling (\(^{13}\)C, \(^{15}\)N etc) and tracing isotope levels in biomarkers generally referred to as stable isotope probing (SIP: Boschker et al., 1998; Radajewski et al., 2000). An advantage of stable isotopes over radio isotopes is that they can be used directly in the field. Moreover, an advantage of SIP lies in the clear identification of specific sub-populations of microorganisms that metabolized specific compounds. Table 1.1 shows comparison of the currently available methods to simultaneously identify microorganisms and their functions by SIP. Phospholipid-derived fatty acids (PLFA) were the first type of biomarkers to be used in combination with stable isotope labeling. PLFA-SIP provides high sensitivity in terms of the amount of \(^{13}\)C label needed, but the phylogenetic resolution offered is generally low (Boschker et al., 1998). PLFA-SIP is however a quantitative technique that can be used to determine actual carbon
and nitrogen fluxes through specific groups in the microbial community (Drigo et al., 2010; Middelburg et al., 2000). The traditional DNA- and RNA-SIP methods are based on the separation of the ‘heavier’ $^{13}$C-labeled nucleic acid from unlabeled nucleic acid by density centrifugation (Manefield et al., 2002; Radajewski et al., 2000), and offer much higher phylogenetic resolution than PLFA-SIP as they are combined with various molecular fingerprinting techniques or clone libraries. SIP methods have now been applied in studies of a wide range of environments and substrates (Friedrich, 2006; Neufeld et al., 2007b; Whiteley et al., 2006). The density centrifugation technique is however a rather crude technique to determine stable isotope labeling and large amounts of labeled substrate is generally needed, which limits the application in natural ecosystems. Other developments are in novel single-cell methods, such as fluorescence in situ hybridization (FISH) coupled with Raman microscopy (Huang et al., 2007) and in halogen in-situ hybridization (HISH) coupled with nano-scale secondary-ion mass spectrometry (nanoSIMS) (Musat et al., 2008). However, each of these SIP methods has limitations, such as low phylogenetic resolution (PLFA-SIP), a requirement for high levels of isotope incorporation in the case of the traditional RNA- and DNA-SIP methods or the need to first isolate microbes from the sediment matrix (HISH-nanoSIMS). FISH-based methods also require relatively high cellular rRNA content and may be limited by the accessibility of probe target sites (Fuchs et al., 1998).

Table 1.1. Comparison of stable isotope ($^{13}$C) labeling methods to simultaneously identify microorganisms and their functions.

<table>
<thead>
<tr>
<th>Method</th>
<th>Target</th>
<th>Isotope detection limit in target (% $^{13}$C incorporation)</th>
<th>Proven phylogenetic resolution</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FISH-Raman Spectroscopy</td>
<td>Single cell</td>
<td>5</td>
<td>Family/genus level</td>
<td>Huang et al. (2007)</td>
</tr>
<tr>
<td>HISH-nanoSIMS</td>
<td>Single cell</td>
<td>0.01</td>
<td>Family level</td>
<td>Musat et al. (2008)</td>
</tr>
<tr>
<td>PLFA-SIP</td>
<td>Community PLFA</td>
<td>0.005</td>
<td>Class level</td>
<td>Boschker et al. (1998)</td>
</tr>
<tr>
<td>DNA-SIP</td>
<td>Active community DNA</td>
<td>10-30</td>
<td>Species/strain level</td>
<td>Radajewski et al. (2000)</td>
</tr>
<tr>
<td>RNA-SIP</td>
<td>Active community RNA</td>
<td>10-30</td>
<td>Species/strain level</td>
<td>Maneifield et al. (2002)</td>
</tr>
<tr>
<td>Mag-SIP</td>
<td>Community rRNA</td>
<td>0.01</td>
<td>Family level</td>
<td>Miyatake et al. (2009)</td>
</tr>
</tbody>
</table>
MacGregor et al. (MacGregor et al., 2002; MacGregor et al., 2006) developed an alternative SIP method that is based on stable isotope labeling combined with magnetic bead capturing of rRNA with specific molecular probes (Mag-SIP). A key feature of this method is the hybridization of target rRNA with a specific biotin-labeled oligonucleotide probe and capturing this hybrid with streptavidin-coated paramagnetic beads (Fig. 1.2). The beads are then collected with a magnet and the isolated rRNA is released for further phylogenetic and isotopic analysis. rRNA is more likely to reflect the phylogenetic composition of the metabolically active community, since its content per cell is generally positively related to growth (Kerkhof and Kemp, 1999; Moeseneder et al., 2005) although some prokaryotes maintain high numbers of ribosomes during starvation (Flärdh et al., 1992). When I started this research, the phylogenetic resolution of the Mag-SIP method was limited to the domain level. In order to obtain higher phylogenetic resolution, we further developed and improved the Mag-SIP method by lowering the amount of rRNA needed for isotope (13C) analysis thereby making it applicable to identify major phylogenetic groups within environmental samples at family level (Miyatake et al., 2009; Miyatake et al., 2010). Advantages of the improved Mag-SIP are a much higher phylogenetic resolution than PLFA-SIP and a much improved isotope detection limit than traditional DNA- and RNA-SIP methods. We applied the Mag-SIP approach in order to link microbial community structure and biogeochemical functions in typical coastal marine sediment.

![Figure 1.2. Schematic drawing of magnetic bead capturing](image-url)
**Aim and outline of this thesis**

The main aim of this thesis is to simultaneously elucidate active members of the microbial community and study their metabolic capabilities in marine sediments. We first further developed and improved the Mag-SIP method and subsequently applied it to study organic substrate utilization by major microbial groups in marine sediments in the first two experimental chapters. We also investigated the contribution of anaerobic chemoautotrophy to the sediment carbon cycle and identified the responsible groups in marine sediments by combining Mag-SIP with PLFA-SIP. Finally, carbon flow from microphytobenthos to the heterotrophic microbial community was studied for five days by applying an in-situ $^{13}$C pulse-chase method. We successfully elucidated specific sub-populations of microorganisms that metabolized specific compounds, and further determined the carbon flows within the microbial community of marine intertidal sediments.

The thesis consists of four experimental papers:

**Chapter 2: Linking microbial community function to phylogeny of sulfate-reducing Deltaproteobacteria in marine sediments by combining stable isotope probing with magnetic bead capture hybridization of 16S rRNA**

We further developed and improved the Mag-SIP method to link microbial function to phylogeny. Improvements of the method were mainly made in two areas. The first area was to improve the sensitivity of the isotope analysis of the captured rRNA and to reduce the carbon carryover in the protocol blanks in order and lower the amount of initial sediment sample needed to be able to target phylogenetic sub-groups within the microbial community. The second area was selection and testing of a nested set of probes to target the majority of the bacterial 16S rRNA in the community. We also illustrated the application of the improved Mag-SIP protocol to elucidate the substrate utilization of sulfate-reducing *Deltaproteobacteria* in an anaerobic marine sediment.

**Chapter 3: Linking microbial community structure and function in marine intertidal sediment by Mag-SIP**

The Mag-SIP method was used to investigate substrate utilization patterns by major members of the microbial community in two depth horizons of an intertidal marine sediment: the oxidized top layer and the fully anaerobic deeper layer of the sediment. There were strong contrasts in community structure and substrate utilization patterns between these two depth horizons.

**Chapter 4: Characterization of anaerobic bacterial chemoautotrophy in intertidal marine sediments**

Anaerobic chemoautotrophy rates in marine sediments and the chemoautotrophic bacterial community involved were characterized by combining PLFA- and Mag-SIP
analysis. Substantial anaerobic chemoautotrophy rates were detected, but the contribution of anaerobic chemoautotrophy to the total chemoautotrophy was highly variable between sediments. The anaerobic chemoautotrophy rates were comparatively low when free sulfide was detected. *Deltaproteobacteria* were a major group in the anaerobic chemoautotrophic activity in all sediments studied.

Chapter 5: *Tracing carbon flow from microphytobenthos to major phylogenetic groups in the bacterial community in an intertidal marine sediment*

Carbon flows from benthic primary producers to the heterotrophic microbial community were investigated. An in situ $^{13}$C-labeling approach was used and label incorporation into major carbon pools, intermediate metabolites, and biomarkers was traced for five consecutive days. Both the $^{13}$C-PLFA and rRNA data suggest that there was a fast transfer of label from diatoms to heterotrophic bacteria during the first 4 to 24 hours of the experiment, which was probably due to the exudation of low-molecular organic compounds by diatoms that could be directly utilized by heterotrophic bacteria. After this initial fast transfer of organic matter, labeling of the heterotrophic bacteria proceeded at a slower rate until the third day of the experiment, which coincided with the degradation of water-extractable extracellular polymeric carbohydrates initially produced by the diatoms. Labeling in heterotrophic bacteria closely tracked labeling in diatoms suggesting a closely coupled system.

Chapter 6: *General discussion*

The research presented in this thesis is discussed and integrated to reach overall conclusions and recommendations for further research.
Chapter 2

Linking microbial community function to phylogeny of sulfate-reducing Deltaproteobacteria in marine sediments by combining stable isotope probing with magnetic bead capture hybridization of 16S rRNA

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Abstract

We further developed the stable isotope probing, magnetic bead-capture method to make it applicable for linking microbial community function to phylogeny at the class and family level. The main improvements were a substantial decrease in the protocol blank and an approximately 10 fold increase in detection limit by using a micro-elemental analyzer coupled to isotope ratio mass spectrometry to determine $^{13}$C-labeling of isolated 16S rRNA. We demonstrated the method by studying substrate utilization by *Desulfobacteraceae*, a dominant group of complete oxidizing sulfate-reducing *Deltaproteobacteria* in marine sediments. Stable-isotope-labeled $[^{13}$C]glucose, $[^{13}$C]propionate, or $[^{13}$C]acetate were fed into an anoxic intertidal sediment. We applied a nested set of three biotin-labeled oligonucleotide probes to capture *Bacteria*, *Deltaproteobacteria* and finally *Desulfobacteraceae* rRNA by using hydrophobic streptavidin-coated paramagnetic beads. Target specificity of the probes was examined with pure cultures of target and non-target species and by determining the phylogenetic composition of the captured sediment rRNA. Specificity of the final protocol was generally very good as more than 90% of the captured 16S rRNA belonged to the target range of the probes. Our results indicated that *Desulfobacteraceae* were important consumers of propionate but not of glucose. However, results for acetate utilization were less conclusive due to lower and more variable labeling levels in captured rRNA. The main advantage of the method in this study over other nucleic acid based stable isotope probing methods is that $^{13}$C-labeling can be much lower, to the extent that even natural abundance $\delta^{13}$C ratios can be studied.
Introduction

Linking microbial phylogeny to community function provides us with insights into the roles that microorganisms play in global elemental cycling. In recent years, stable isotope tracing approaches combined with biomarkers have been widely applied to environmental studies (Boschker et al., 1998; Manefield et al., 2002; Radajewski et al., 2000). Tracking stable- or radio-isotope labeled atoms from particular substrates into components of microbial cells (biomarkers) can reveal which organisms are involved in the consumption of the substrate and also yield information on rates of specific biogeochemical transformation (Boschker et al., 1998).

Dissimilatory sulfate reduction is a major pathway for organic carbon mineralization in coastal marine sediments, accounting for on average 50% of the total carbon mineralization (Jørgensen, 1982; Oenema, 1990). Sulfate-reducing prokaryotes are a diverse and ubiquitous component of the bacterial community. The diversity of sulfate-reducing bacteria (SRB) in marine sediments has been investigated by clone libraries of 16S rRNA (Purdy et al., 1997) and dissimilatory sulfite reductase genes (Dhillon et al., 2003), and fluorescence in situ hybridization-related techniques (Mussmann et al., 2005; Ravenschlag et al., 2000). Desulfobacteraceae, a group of complete-oxidizing SRB belonging to the Deltaproteobacteria, have generally been found to be a major group of SRB in marine sediments.

PLFA were the first type of biomarkers to be used in combination with stable isotope probing (SIP) (Boschker et al., 1998). PLFA-SIP provides high sensitivity in terms of the amount of $^{13}$C label needed, but the phylogenetic resolution offered is low and requires reference signatures of closely related culturable relatives (Boschker et al., 1998). The main advantage of DNA- and RNA-SIP is that they offer improved phylogenetic resolution (Manefield et al., 2002; Radajewski et al., 2000). These two methods are based on the separation of the ‘heavier’ $^{13}$C-labeled nucleic acid from unlabeled nucleic acid by density centrifugation. Subsequently, organisms incorporating the greatest proportion of label into their DNA or RNA are identified by various molecular fingerprinting techniques or by constructing clone libraries. RNA has a higher turnover rate than DNA, resulting in faster labeling, and incubation times can therefore be substantially shortened (Manefield et al., 2002; Rosset et al., 1966). RNA is also more likely to reflect the phylogenetic composition of the metabolically active community, since it is highly susceptible to chemical and enzymatic degradation, and its cellular levels are often tightly regulated (Kerkhof and Kemp, 1999; Moeseneder et al., 2005), although some prokaryotes maintain high numbers of ribosomes during starvation (e.g. Flärđh et al., 1992).

MacGregor et al. (MacGregor et al., 2002; MacGregor et al., 2006) developed a related approach, stable-isotope probing combined with magnetic-bead capture hybridization (here named Mag-SIP), which is based on the isolation of small subunit rRNA from particular phylogenetic groups and the detection of $^{13}$C-labeling levels by isotope ratio mass
spectrometry (IRMS). rRNA is captured by hybridization with specific biotin-labeled oligonucleotide probes followed by retrieval of hybridized target rRNA using streptavidin-coated magnetic beads. The main advantage of Mag-SIP over other nucleic acid based SIP methods is that in principle much lower labeling levels can be applied as label detection is based on IRMS methods (about 0.001% vs. >10% $^{13}$C, respectively). For instance, it has been shown that the method can be used to study the effects of oil pollution on natural $\delta^{13}$C ratios of bacterial communities in sediments (Pearson et al., 2008). Moreover, Mag-SIP is not based on PCR, as the isotope ratio of target rRNA is directly measured without amplification of nucleic acid, avoiding possible PCR artifacts. However, the large amounts (1-10 µg) of RNA needed for an accurate isotope ratio analysis by traditional elemental analyzer (EA)-IRMS has limited the use of Mag-SIP to general domain-specific probes (MacGregor et al., 2002; MacGregor et al., 2006). Recently, several methods such as liquid chromatography (LC) combined with IRMS and spooling-wire micro-combustion combined with IRMS have been introduced that allow isotopic analysis of much smaller samples than with the traditional EA-IRMS systems (Krummen et al., 2004; Sessions et al., 2005).

In this study, we used the wet oxidation interface of an LC-IRMS as a micro elemental analyzer (µEA)-IRMS (Krummen et al., 2004). The use of µEA-IRMS substantially lowers the detection limit of isotope ratio measurements in terms of the amount of rRNA needed for an analysis, but also calls for modifications of the Mag-SIP protocol in order to decrease protocol blanks (carbon from materials and reagents used in the protocol). We tested a nested set of three biotin-labeled oligonucleotide probes to capture 16S rRNA derived from Bacteria, Deltaproteobacteria and finally Desulfobacteraceae. Target specificity and stringency of these probes was tested against pure cultures of both target and non-target organisms. Moreover, phylogenetic analysis of captured 16S rRNA from environmental samples was done to check specificity and where necessary adjust probe stringency. Finally, we demonstrate Mag-SIP with a study on in situ substrate use by sulfate-reducing Deltaproteobacteria in intertidal, anoxic marine sediment. A generalized scheme for Mag-SIP is shown in Figure 2.1.
Figure 2.1. A generalized scheme for Mag-SIP. Control is sediment incubated without substrate.
Materials and methods

Probes

Biotin-labeled oligonucleotide probes and unlabeled helper and competitor probes (Table 2.1) were purchased from Isogen Life Science (De Meern, The Netherlands). EUB338 and DELTA495a are commonly used probes for Bacteria and Deltaproteobacteria, respectively, even though they do not target all the genera in the domain and DELTA495a also targets most Gemmatimonadetes (Lücker et al., 2007). The DELTA495a probe was used in combination with a competitor probe (cDELTA495a) to avoid capture of Gammaproteobacteria, which have only one mismatch in a target region of DELTA495a. DSS658 is a commonly used probe for Desulfobacteraceae (Manz et al., 1998), but also has only a single mismatch to many Gammaproteobacteria, resulting in poor specificity with the Mag-SIP protocol (results not shown). We therefore designed another specific probe (Dbact653) targeting a similar range of Desulfobacteraceae using the ARB software (Ludwig et al., 2004), which has less probability of capturing Gammaproteobacteria. In order to increase the yield, unlabeled 21-mer helper probes (Fuchs et al., 2000; MacGregor et al., 2002) complementary to the consensus sequences upstream and downstream of the Dbact653 probe target sites were also designed. Stringency and specificity of the probes were examined as described below.

Table 2.1. 16S rRNA-targeted probes used in this study

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence (5'-3')</th>
<th>% FA(^a)</th>
<th>Specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB338</td>
<td>GCT GCC TCC CGT AGG AGT</td>
<td>25</td>
<td>Most Bacteria</td>
<td>(Amann et al., 1990)</td>
</tr>
<tr>
<td>DELTA495a</td>
<td>AGT TAG CCG GTG CTT CCT</td>
<td>45</td>
<td>Most Deltaproteobacteria</td>
<td>(Loy et al., 2002)</td>
</tr>
<tr>
<td>cDELTA495a</td>
<td>AGT TAG CCG GTG CTT CTT</td>
<td>45</td>
<td>Competitor of DELTA495a</td>
<td>(Macalady et al., 2006)</td>
</tr>
<tr>
<td>Dbact653</td>
<td>TTC CCT CTC CCA TAC TCA</td>
<td>25</td>
<td>Most Desulfobacteraceae</td>
<td>This study</td>
</tr>
<tr>
<td>Dbact653_up_help</td>
<td>CCC CGG AAG TGC AYT TGA WAC</td>
<td>25</td>
<td>Helper probe for Dbact653</td>
<td>This study</td>
</tr>
<tr>
<td>Dbact653_down_help</td>
<td>GTG GAA TTC CTG GTG TAG AGG</td>
<td>25</td>
<td>Helper probe for Dbact653</td>
<td>This study</td>
</tr>
</tbody>
</table>

\(^a\)Percent formamide (FA) in hybridization buffer for hybridizations at 20°C
Pure cultures

Desulfococcus multivorans strain DSM 2059^T, a sulfate-reducing Deltaproteobacterium, was grown in DSM medium 197 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). D. multivorans is a target organism for all the probes used in this study. Desulfovibrio strain SB1, a target organism for the EUB338 and DELTA495a probes, was grown in DSM medium 63. E. coli DH10B was grown in LB broth (Difco; BD Biosciences, San Jose, CA, USA). E. coli DH10B is a target for EUB338 but not for the other probes. However, the DELTA495a probe has only a single mismatch in the target site with E. coli.

Sampling and ^13C labeling

Sediment samples were collected at an intertidal flat in the Rattekaai area of the Oosterschelde estuary (The Netherlands) in May 2008. Undisturbed sediment cores (diameter 5.2 cm) were transported immediately back to the laboratory. Equimolar ^13C amounts of uniformly labeled [^13C]glucose, [^13C]propionate, or [^13C]acetate (50, 100, and 150 mM, respectively; 99% ^13C; Cambridge Isotope Laboratories, Andover, MA, USA) were injected (19 injections of 17 µl each) into the top 5 cm of the sediment cores by the line injection method (Jørgensen, 1978). A total of four cores for each substrate were incubated for 24 h in the dark at in situ temperature (14°C). Sediment from the black, anoxic zone (2–5 cm depth) of cores was sectioned and stored at -80°C. Unlabeled control cores, incubated without labeled substrate, were also processed.

RNA isolation and electrophoresis

In order to extract total community RNA from sediment, a frozen sediment sample (20-25 g wet wt) was transferred into a sterile 80 mL glass bottle containing 7 g of sterile glass beads (5 g of 1 mm diameter, 2 g of 0.1 mm diameter; Sartorius, Göttingen, Germany), 20 ml of analysis grade phenol and 10 ml of extraction buffer (250 mM sodium acetate, 50 mM EDTA, 2.5% SDS, pH 5.1). The two sizes of glass beads were used in the amount and concentration which gave the highest yield of 16S rRNA (data not shown). Samples were vigorously agitated in an MSK-Zellhomogenisator (B. Braun Biotech International, Melsungen, Germany). For cultures, cells were collected by centrifugation and added to 1.5 ml centrifuge tubes with 0.5 g sterile glass beads (0.1 mm diameter; Sartorius), 800 µl of phenol, and 200 µl of extraction buffer (250 mM sodium acetate, 50 mM EDTA, 2.5% SDS, pH 5.1) followed by bead beating with Vortex-Genie 2 (Scientific Industries, Bohemia, NY, USA). RNA preparations from both sediment samples and pure cultures were purified by phenol-chloroform extraction and isopropanol precipitation (MacGregor et al., 2006; Stahl et al., 1988). Total community RNA from several sediment extractions was combined and used for later magnetic bead capture hybridization. All treatments were analyzed in duplicate. Total RNA was quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). In order to estimate the amount of 16S rRNA
among total community RNA, RNA extracts were visualized on a 5% polyacrylamide gel stained with ethidium bromide. The proportion of 16S rRNA among RNA extracts was analyzed by comparing band intensities with known amounts of capture isolated 16S rRNA by using the ImageJ software (http://rsb.info.nih.gov/ij/).

**Magnetic bead capture hybridization of pure culture RNA**

The capture protocol was first tested with pure cultures. The optimal hybridization stringency was determined for each probe by varying the formamide concentration in the hybridization buffer between 0 and 60%. The formamide concentration that gave about half of the 16S rRNA band intensity at 0% formamide was used further in the protocol. Both target and non-target cultures were tested at this formamide concentration to determine the specificity of the assay, and where necessary the formamide concentration was adapted.

For the assay, ten microliters of pure-culture RNA extract (1-2 µg RNA) and 90 µl of hybridization buffer (5× saline-sodium citrate (SSC) buffer, 0.1% N-laurylsarcosine, 0.1% NaCl, 0.02% SDS) containing the appropriate concentration of formamide were mixed in a 1.5 ml centrifuge tube. The hybridization mixture was incubated for 10 min at 70°C and then 30 min at room temperature (Mastrangeli et al., 1996). The probe (10-25 pmol) was added to the hybridization mixture at five times the molar concentration of the target 16S rRNA, and incubated overnight at 20°C on a rotator (Stuart SB3; Dynalab, Rochester, NY, USA). This ratio between probe and target gave the highest recovery (data not shown). Two types of beads with either a hydrophilic (Dynabeads M-280 Streptavidin; Invitrogen, Carlsbad, CA, USA) or a hydrophobic (Dynabeads MyOne Streptavidin T1; Invitrogen) surface were tested. Aliquots (25 µl per reaction tube) of beads were rinsed three times with an equal volume of 0.5× SSC using a magnetic particle concentrator (Dynal MPC-S; Invitrogen). Rinsed beads were resuspended in 0.1% blocking reagent solution (Roche Applied Science, Mannheim, Germany) and incubated for 1 h on a rotator. The blocking reagent solution was removed using a magnetic particle concentrator. The beads were resuspended in 0.5× SSC, and 25 µl was dispensed per reaction tube and incubated with probe-target hybrid at room temperature on a rotator for 2 hours (Bach et al., 1999; MacGregor et al., 2002). The concentration of the beads was 10 mg/ml and the binding capacity of the beads is 400 pmol/mg (according to the manufacturer), which was approximately four times more than the amount of probes added in the protocol. The magnetic particle concentrator was used to collect the beads on the tube wall, and the hybridization mixture containing the RNA not bound to the beads was removed. The beads were rinsed three times with 7.5× SSC, and captured 16S rRNA was eluted in Milli-Q water (Millipore, Billerica, MA, USA) at 90°C for 3 min (MacGregor et al., 2006). The eluted 16S rRNA was separated from the beads with the magnetic particle concentrator. Isolated 16S rRNA was precipitated with 1 volume of isopropanol and 0.2 volume of 7.5 M ammonium acetate, and finally dissolved in Milli-Q water. Captured 16S rRNA was visualized and quantified as described before.
Magnetic bead capture hybridization of total community RNA from sediment

Magnetic bead capture was performed as described above, except that different amounts of RNA extract and beads were used, and the blocking reagent treatment and 16S rRNA precipitation steps were modified to minimize protocol blanks (see Results). Total community RNA extract (40 µl containing 20-40 µg RNA) and 360 µl of hybridization buffer (5× SSC, 0.1% N-laurylsarcosine, 0.1% NaCl, 0.02% SDS) containing the appropriate formamide concentration (Table 2.1) were mixed per 1.5 ml centrifuge tube, then incubated for 10 min at 70°C and 30 min at room temperature. After overnight hybridization with the probes (30-50 pmol) at 20°C on a rotator, 100 µl of beads that had been rinsed two times after blocking reagent treatment were dispensed into each reaction tube and incubated for 2 hours. The probe was added at five times the molar concentration of the target 16S rRNA, which was estimated from the amount of the total community RNA and the proportion of target 16S rRNA clones among the total community clones. Eluted 16S rRNA was precipitated two times by 1 volume of isopropanol and 0.2 volume of 3 M NaCl, and finally dissolved in Milli-Q water that was not treated with diethyl pyrocarbonate (DEPC). DEPC resulted in high protocol blanks. Between 300 and 600 ng C of captured 16S rRNA was pooled for isotope ratio measurement. Captured 16S rRNA was freeze-dried and dissolved in freshly prepared Milli-Q water shortly before analysis by µEA-IRMS. Protocol blanks with no RNA extract were also prepared by the same protocol. A small fraction of the captured 16S rRNA was used for phylogenetic analysis.

13C analysis of captured 16S rRNA

Isotope ratio analysis was performed by µEA-IRMS consisting of a wet oxidation interface (LC IsoLink; Thermo Fisher Scientific) coupled on-line to an isotope ratio mass spectrometer (DELTA V Advantage; Thermo Fisher Scientific, Bremen, Germany) (Krummen et al., 2004). Samples (50 µl containing 300-600 ng C of RNA) were directly injected into this µEA-IRMS operating in bulk injection mode. Standard curves were made with phthalic acid ranging from 0-1000 ng of carbon. Linearity for 13C-enriched materials was previously tested (Boschker et al., 2008). Stable carbon isotope ratios were expressed as δ13C values calibrated against the international standard VPDB. The delta notation is defined as:

$$\delta^{13}C_{\text{sample}} (\%) = [(R_s/R_{st})-1] \times 1000$$  \hspace{1cm} (1)

where $R_s$ is the ratio of $^{13}$C in the sample and $R_{st}$ is the ratio of the international standard VPDB (0.0111797). The measured $\delta^{13}$C values were corrected for the protocol blank:
\[
\delta^{13} C_{\text{RNA}} (\text{‰}) = \left( \frac{\left( \delta^{13} C_{\text{sample}} \times C_{\text{sample}} \right) \left( \delta^{13} C_{\text{blank}} \times C_{\text{blank}} \right)}{C_{\text{sample}} - C_{\text{blank}}} \right) \]

(2)

where \( \delta^{13} C_{\text{sample}} \) is the \( \delta^{13} C \) value of the sample, \( C_{\text{sample}} \) is the amount of carbon in the sample, \( \delta^{13} C_{\text{blank}} \) is the \( \delta^{13} C \) value of the blank, and \( C_{\text{blank}} \) is the amount of carbon in the blank (Boschker, 2004).

**Clone libraries of total and captured 16S rRNA**

To check specificity of the capture protocol for each probe, aliquots (100-200 ng) of captured 16S rRNA from unlabeled sediment samples and total community RNA were reverse transcribed with reverse primer DXR518 (5'-CGTATTACCGCGGCTGCTGG-3') (Nogales et al., 1999) and Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen). The cDNA was amplified with 10 cycles of PCR using PCR primers 27F-DXR518 (Martinez et al., 2006; Mills et al., 2005). PCR products were quantified and ligated into pGEM T-easy vector, and transformed into *Escherichia coli* JM109 competent cells (Promega, Madison, WI, USA). Positive clones were re-amplified with M13 primers, and sequenced with the 27F primer on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence chromatographs were analyzed using the ChromasPro software version 1.3.3.0 (http://www.technelysium.com.au/chromas.html). Phylogenetic analysis was performed by using the fast aligner and treeing tools implemented in the ARB program package (Ludwig et al., 2004). Phylogenetic relationships were determined by inserting sequences from this study into an ARB tree composed of the Greengenes database (http://greengenes.lbl.gov/) to which additional *Deltaproteobacteria* clone sequences from marine environments had been added. The final bootstrapped neighbor-joining tree with 1000 samplings was created in MEGA4 (Tamura et al., 2007) using the Jukes-Cantor model.

**Nucleotide sequence accession numbers**

Nucleotide sequences obtained from total community, EUB338-captured, DELTA495a-captured, and Dbact653-captured 16S rRNA have been deposited in the Genbank/DDBJ/EMBL database under accession numbers FJ787045 to FJ787299.
Results

Modification of the Mag-SIP protocol for µEA-IRMS

The detection limit for $^{13}$C analysis is mainly determined by the sensitivity of the equipment used for isotopic analysis and the amount of blank carbon added during sample preparation (protocol blank). The sensitivity of the $^{13}$C-measurements in terms of the amount of carbon needed for accurate analysis was examined with a standard curve of phthalic acid. Approximately 50 ng C was required for an accurate isotope measurement using a µEA-IRMS, which is similar to other studies (Krummen et al., 2004). However, this low detection limit also meant that the capturing protocol blank had to be decreased substantially. The previously published assay gave a high protocol blank of approximately 300 ng of carbon. In order to lower blank from the magnetic bead capture protocol, we examined carbon carryover and its $\delta^{13}$C values for the different steps in the protocol. We concluded that blocking reagent, the ammonium acetate and alcohol in the final RNA precipitation step, DEPC treatment of Milli-Q water, and the type of vial used for the final step in the protocol all contributed to the protocol blank. Glass vials generally resulted in high protocol blanks as did several types of plastic centrifuge tubes (70-150 ng C). Standard 1.5 ml centrifuge tubes from Greiner Bio-One (Frickenhausen, Germany) gave the lowest protocol blank (approximately 15 ng C) and were used through the protocol. Protocol blanks were also lowered by approximately 200 ng C by including an additional rinsing step of blocking reagent-treated beads with 0.5× SSC, use of sodium chloride as the salt for RNA precipitation instead of ammonium acetate, additional rinse of RNA pellets with 70% ethanol, and subsequently freeze-drying RNA pellets. Finally, freshly prepared Milli-Q water not treated with DEPC was used to dissolve the captured 16S rRNA because DEPC treatment contributed approximately 20 ng C of protocol blank (MacGregor et al., 2002). The exclusion of DEPC might have lead to some rRNA fragmentation, but this was not evident from the gels and µEA-IRMS signals would not be influenced by fragmentation. Milli-Q water without DEPC treatment was added shortly before $^{13}$C analysis which also decreased the blank from air CO$_2$ dissolved into the sample (3-9 ng C after one day). The standard protocol for removing CO$_2$ by acidification and purging with helium could not be used because rRNA was precipitated by the acid (data not shown). As a result of these modifications, the protocol blank dropped to 40-50 ng C per 50 µl injection, which is close to the detection limit of the µEA-IRMS. A minimum of approximately 50 ng C of captured 16S rRNA was required for isotope ratio measurements. Consequently, Mag-SIP using µEA-IRMS required about 10 times less 16S rRNA for isotope ratio measurements than Mag-SIP using standard EA-IRMS. For the type of samples in this study, this improvement enabled us to use Mag-SIP for phylogenetic groups explaining 20% of the cDNA library, but in our experience we can go down to 2-5%.
Testing probe stringency and specificity

Binding strength of each probe was examined by varying the formamide concentration between 0 and 60%. From the result of hybridization with 16S rRNA of *D. multivorans*, a target of all the probes, initial formamide concentrations for EUB338, DELTA495a, and Dbact653 were determined as 25, 40, and 25%, respectively. Subsequently, specificity at these formamide concentrations was tested against *Desulfovibrio* sp. SB1, which is a target of EUB338 and DELTA495a but not Dbact653. 16S rRNA of *Desulfovibrio* sp. SB1 was captured by EUB338 and DELTA495a at the relative band intensity of 13.1 and 9.3%, respectively, but not by Dbact653 (0%) (Fig. 2.2A). Moreover, initial formamide concentrations were also tested against *E. coli*, which is targeted by EUB338 but not by the other probes. 16S rRNA of *E. coli* was captured by EUB338 but not by Dbact653. The DELTA495a probe has only one mismatch in target region of *E. coli*, and a faint band of DELTA495a-captured material was recognized on the gel at 40% formamide. The DELTA495a probe was further tested against *E. coli* at 40, 45 and 50% formamide. Captured material was recognized at 40% but not at 45 and 50% (Fig. 2.2B). Hence, formamide concentration for DELTA495a was increased to 45%.

![Figure 2.2](image_url)

Figure 2.2. Relative band intensity of the probe-captured 16S rRNA from pure cultures. (A) *Desulfovibrio* sp. SB1 16S rRNA captured by EUB338, DELTA495a, and Dbact653 (lanes EUB, DELTA, and Dbact, respectively) on a 5% acrylamide gel. Lane labeled *D. SB1* was total RNA extracted from *Desulfovibrio* sp. SB1 pure culture. Relative capture efficiencies were 13.1, 9.3, and 0%, respectively. Note that *Desulfovibrio* sp. SB1 is not a target of Dbact633. (B) *E. coli* 16S rRNA captured by DELTA495a (1 mismatch) on a 5% acrylamide gel. Formamide concentration was tested at 40, 45, and 50% (lanes 40, 45, and 50, respectively). Lane labeled *E. coli* was total RNA extracted from *E. coli* pure culture.
In order to further check the target specificity of the probes for sediment rRNA extracts, clone libraries derived from the total community RNA and the 16S rRNA captured by each probe were examined. Although the result may not be quantitative, this provided a final check of capture specificity for real samples containing a variety of rRNA. Initially we used hydrophilic magnetic beads as described in the original protocol. However, Cyanobacteria/chloroplast-like clones were frequently observed in clones of Dbact653- captured rRNA (11-14% of the clones). The capturing of these Cyanobacteria/chloroplast-like clones was unexpected as they had four to five mismatches in the target region of the probe, suggesting that there may be non-specific binding of rRNA directly to the bead surface. We therefore tested beads with a hydrophobic surface, which also have a higher specific binding capacity as capturing efficiency is in general less than with hydrophilic beads. With these hydrophobic beads, frequency of the Cyanobacteria/chloroplast-like clones was decreased to less than 2% of the clones in Dbact653- captured rRNA. Hence, hydrophobic beads were used in further experiments.

For the total community RNA, Desulfobacteraceae accounted for approximately 20% of the clones and other Deltaproteobacteria were relatively rare (3%, Fig 2.3). Cyanobacteria/chloroplast and Gammaproteobacteria clones were also major groups. Frequencies of Desulfobacteraceae and other Deltaproteobacteria in EUB338-captured rRNA appeared to be lower than in total community RNA, which may be due to the limited number of clones sequenced. Specificity of the DELTA495a probe (in combination with the competitor probe) was very good, with 90% of the clones belonging to the target group. Most clones captured by DELTA495a affiliated to the Desulfobacteraceae and the remainder mainly belonged to a variety of other Deltaproteobacteria (Fig 2.3 and 2.4). More than 93% of clones captured by Dbact653 in combination with helper probes were indeed affiliated to the Desulfobacteraceae (Fig 2.3 and 2.4). These results showed that specificity should be evaluated on actual samples as well as pure cultures, and that the final protocol was highly specific.
Figure 2.3. Frequencies of clones obtained from total community RNA and 16S rRNA captured with specific probes. Total numbers of clones sequenced are indicated as n. Bar beside each probe column indicates the expected target range of the probe. DELTA495a was used in combination with its competitor and helper probes were used with Dbact653.

Figure 2.4. Neighbor-joining tree showing the affiliations of clones to selected sequences of the Deltaproteobacteria. Bootstrap values represent 1000 replicates and only values greater than 50% are reported. Clone sequences from this study are in boldface. Clones with designations containing TRNA, EUB, DELTA, and Dbact are derived from total community RNA, EUB338-captured, DELTA495a-captured, and Dbact653-captured 16S rRNA, respectively. The numbers in brackets are accession numbers. Escherichia coli was used as the outgroup. The scale bar indicates 10% estimated phylogenetic divergence. For each group, a representative environmental clone is indicated. The table indicates number of clones derived from total community RNA, EUB338-captured, DELTA495a-captured, and Dbact653-captured 16S rRNA affiliating into each group.
$^{13}$C incorporation into captured 16S rRNA

To examine label incorporation into 16S rRNA from different labeled substrates, $\delta^{13}$C values of captured 16S rRNA were measured by $\mu$EA-IRMS. Sediment samples were incubated for 24 hours with labeled [$^{13}$C]glucose, [$^{13}$C]propionate, or [$^{13}$C]acetate. Unlabeled control cores were also incubated under the same conditions. Approximately 10 $\mu$g of total community RNA were extracted from 1 g (dry wt) sediment. In order to get sufficient amounts of captured 16S rRNA, 5-10 g (dry wt) sediment per assay were used for EUB338 captures and 15-20 g for other probes. Between 300 and 600 ng C of captured 16S rRNA was used per isotope ratio measurement, well above the detection limit of $\mu$EA-IRMS.

Figure 2.5 illustrates the increase in $\delta^{13}$C ratios between labeled sediments and unlabeled, control sediments ($\Delta\delta^{13}$C) for the different captured 16S rRNA fractions. Unlabeled controls had $\delta^{13}$C values between -15 and -20 ‰, within the typical range for marine heterotrophic bacteria (Boschker and Middelburg, 2002; Coffin et al., 1990). Labeling with glucose was higher than with propionate, and labeling decreased going from Bacteria to Desulfobacteraceae 16S rRNA. In contrast, $^{13}$C incorporation of propionate was about two times higher in Desulfobacteraceae than in all Bacteria. Together these results suggest that Desulfobacteraceae incorporated more propionate than other members of the community, and that they were relatively less important for glucose incorporation. Incorporation with acetate was even lower than with propionate, and there were no significant differences in acetate incorporation among probes.

![Figure 2.5. Increase in $\delta^{13}$C ratios ($\Delta\delta^{13}$C) of captured 16S rRNA from sediments incubated with [$^{13}$C]glucose, [$^{13}$C]propionate, or [$^{13}$C]acetate. Total community RNA was captured using EUB338, DELTA495a, and Dbact653 probes and analyzed by $\mu$EA-IRMS. DELTA495a was used in combination with its competitor and helper probes were used with Dbact653. Note that differences in $\delta^{13}$C value between duplicate analysis of unlabeled controls was less than 2 ‰, and that therefore all treatments were significantly labeled.](image-url)
Discussion

Several culture-independent approaches have been developed to identify the major microbial groups responsible for environmental processes. SIP based on PLFA, DNA, or RNA (Boschker et al., 1998; Manefield et al., 2002; Radajewski et al., 2000) has become an attractive method in recent years. However, each of these SIP methods has inherent limitations in terms of phylogenetic resolution or $^{13}$C sensitivity. The major advantage of Mag-SIP is that it combines excellent phylogenetic resolution through specific probes with the highest possible $^{13}$C sensitivity through IRMS analysis. It has been shown that Mag-SIP can be applied to study carbon sources used by Bacteria by studying small variations in natural $^{13}$C abundance (this paper, (Pearson et al., 2008)). Another advantage of Mag-SIP is its independence from PCR bias (Acinas et al., 2005; Qiu et al., 2001). In this paper, we improved the Mag-SIP protocol by substantially lowering the amount of rRNA needed through µEA-IRMS analysis and by lowering the carbon blank from the sample preparation protocol. As a consequence of these modifications, the protocol blank dropped to 40-50 ng C per sample and about 50 ng C of captured 16S rRNA was sufficient for isotope ratio analysis. This detection limit was an order of magnitude lower than in previous Mag-SIP studies that used traditional EA-IRMS (MacGregor et al., 2002; MacGregor et al., 2006). Similar sensitivities were obtained by Pearson et al. (Pearson et al., 2008) who used a spooling wire interface to determine natural stable isotope ratios in total bacterial 16S rRNA. These improvements enabled us for the first time to apply Mag-SIP at family-level resolution, and we demonstrated the method in an experiment on substrate utilization by sulfate-reducing *Deltaproteobacteria* in anoxic marine sediment.

We tested a nested set of three biotin-labeled oligonucleotide probes with a stepwise narrowing of the target from Bacteria to Deltaproteobacteria and further to *Desulfobacteraceae*. Through changing the hybridization stringency and by including unlabelled helper and competitor probes, the protocol enabled the isolation of target rRNA at a high specificity of 90% or more from complex environmental samples. An advantage of the probe capture technique, not shared by many other probe-based methods, is that phylogenetic composition of the isolated rRNA can be further studied. In general, the cDNA clone libraries of rRNA captured with the more specific probes were a representative subset of the more general probes (Fig 2.4). However, the composition of the clone library from the EUB338-captured material appeared to be different from the total RNA clone library. Some of these differences were expected as the EUB338 probe does not target *Planctomycetes* (Neef et al., 1998), which were present in the cDNA library but not in the EUB338 library. This could be circumvented by including other versions of the EUB338 probe that target these groups (Daims et al., 1999). The abundance of *Deltaproteobacteria* in the EUB338-captured clones was lower than expected from the cDNA library (Fig 2.3 and 2.4). Although most *Deltaproteobacteria* are targeted by the EUB338 probe, this effect could be due to either differences in accessibility of the probe to binding site or to PCR and
cloning artifacts (Acinas et al., 2005; Qiu et al., 2001) including a stochastic effect as a relatively low number of clones were sequenced for each clone library. For the Mag-SIP protocol, the newly developed Desulfovibrio probe Dbact653 showed much better specificity than the commonly used DSS658 probe, which targets a similar range of organisms (Manz et al., 1998). The basic idea behind the application of this nested set of probes was that an increase in labeling (Δδ^{13}C ratios) with decreasing target range would indicate that the target belonged to a ^{13}C-substrate utilizing member of the microbial community.

To demonstrate the Mag-SIP protocol, we applied it to a study of substrate utilization by sulfate-reducing bacteria in marine sediment. Sulfate-reducing bacteria play an important role in the final degradation steps during anaerobic organic matter mineralization in marine sediments (Jørgensen, 1982). They are thought to mainly use fermentation products such as propionate and acetate produced by fermenting bacteria, whereas direct consumption of more complex substrates such as glucose is generally very limited (Widdel and Hansen, 1992). The three labeled substrates tested, namely [^{13}C]glucose, [^{13}C] propionate, and [^{13}C]acetate, indeed resulted in differential labeling among rRNA captured by the three nested probes, suggesting that these substrates were mostly specifically utilized by sub-groups within the microbial community. The incorporation was mainly compared within a substrate, as it was not known if yields were the same for each substrate or if all substrate had been consumed completely. With [^{13}C]glucose, the decreasing trend in labeling levels with decreasing target range suggested that Deltaproteobacteria and especially Desulfobacteraceae were not the main consumers of glucose. Some labeling was however detected in Desulfobacteraceae. Although we cannot directly reject that some members of Desulfobacteraceae were actively using glucose, this labeling is most likely due to uptake of fermentation products produced by other glucose-utilizing species. We can not completely disregard the less-likely possibility that some of the detected labeling was due to unspecific rRNA binding (< 10% of total captured). This potential problem may be circumvented by further increasing stringency at the cost of rRNA recovery or by using a more complete probe set targeting all main groups in the community. In contrast to glucose, the increase in labeling from total bacterial rRNA to Desulfovibrio with propionate, and to a lesser degree with acetate, showed that Desulfobacteraceae were the major players in the consumption of these fermentation products. Both propionate and acetate are considered as important substrates for SRB including the Desulfobacteraceae in marine sediments (Purdy et al., 1997; Sørensen et al., 1981). Several dominant clades among the Desulfobacteraceae related to Desulfoarcina are typically detected in sediment clone libraries (Fig 2.4) (Mussmann et al., 2005; Ravenschlag et al., 2000). By using Mag-SIP with specific probes for these clades, it should be possible to further determine whether they play different roles in carbon mineralization. In general, the results of the labeling study are in agreement with the predicted roles of sulfate-reducing Deltaproteobacteria in marine sediments.
We demonstrated the utility of Mag-SIP using µEA-IRMS to link microbial community function to phylogeny at the family level. While Mag-SIP is a target-based approach developed to study the substrate range of important environmental phylogenetic groups, the approach can also be used to study the role of different groups by applying a nested design of probes as in this study. Although the improvement of the protocol in this study substantially lowered the amount of sample needed, Mag-SIP is still best suited for high biomass samples such as active sediments, soils, and bioreactors. Improving the sensitivity of the Mag-SIP protocol further, both in terms of the amount of rRNA and the amount of $^{13}$C label needed, will be difficult as a certain amount of carbon is needed for an accurate $^{13}$C analysis by IRMS and the transfer of carbon from the sample to the IRMS is already highly efficient (Krummen et al., 2004). However, an LC-IRMS based detection method for rRNA or its constituents instead of the bulk analysis as performed in this study may further decrease in protocol blanks as rRNA will be separated from contaminants by chromatography (Application Note 30055; Thermo Scientific, Bremen, Germany). Further improvement of the method would be in the development of a more comprehensive probe set to target a wider range of dominant Bacteria within microbial communities, and the 23S rRNA with its higher phylogenetic resolution may also provide interesting options (Hunt et al., 2006). Other interesting possibilities lay in the combination of Mag-SIP with novel single-cell methods such as fluorescence in situ hybridization (FISH) coupled with Raman microscopy (Huang et al., 2007) or with nanometer-scale secondary-ion mass spectrometry (NanoSIMS) (Behrens et al., 2008). Although the proportion of rRNA from particular organisms among total community RNA may not always represent their actual population size (Fegatella et al., 1998; Kerkhof and Kemp, 1999), this method may allow us to identify active groups in microbial communities and perform food web studies with limited disturbance of the community structure.

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Chapter 3

Linking microbial community structure and function in marine intertidal sediment by Mag-SIP

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Abstract

Substrate utilization by major microbial groups was investigated by using stable isotope probing combined with magnetic bead capture hybridization (Mag-SIP) in marine intertidal sediment. Sediment cores were incubated with different \(^{13}\)C-labeled substrates, and sectioned into surface (0-2 cm) and deeper (2-5 cm) layers. The surface layer contained the aerobic zone and the top of the anaerobic zone, whereas the deeper layer represented the sulfate-reduction zone. Specific 16S rRNA was isolated with a nested set of five probes targeting about 80\% of the active community, and analyzed for \(^{13}\)C-labeling. We also determined total and active microbial community structure by constructing clone libraries for the 16S rRNA genes and the 16S rRNA. The sequences of 16S rRNA genes and rRNA were partially overlapped, and were dominated by *Deltaproteobacteria* (only in the deeper layer), *Gammaproteobacteria*, *Cyanobacteria*, and chloroplast sequences. In the deeper layer, *Gammaproteobacteria* dominated glucose consumption, and sulfate-reducing *Deltaproteobacteria*, specifically *Desulfovibrioaceae*, were relatively important in acetate, propionate, and amino acid degradation. Surprisingly, *Cyanobacteria* and chloroplast sequences explained a substantial part of the rRNA library in the deeper layer, and our results indicate that these phototrophs may survive by glucose utilization under dark, anaerobic conditions. In the surface layer, all substrates were more evenly utilized by all major groups, indicating limited specialization at the phylogenetic level of this study. There were therefore strong contrasts in community characteristics and substrate utilization patterns between the two depth horizons, which are discussed in terms of general community functioning and the known characteristics of the microbial groups.
Introduction

Coastal sediments are generally characterized by high inputs of organic matter, and play a prominent role in the marine carbon cycle (Middelburg and Soetaert, 2005). Most of the organic matter is rapidly mineralized by a diverse microbial community along a vertical sequence of electron acceptors (Braker et al., 2001; Edlund et al., 2008). Oxygen is generally found only in the top few millimeters of the sediment. Here, aerobic respiration is the dominant pathway and is primarily carried out by a diverse heterotrophic microbial community. Sulfate reduction is the main anaerobic pathway for organic carbon mineralization in coastal sediments (Canfield et al., 1993; Jørgensen, 1982). Under anaerobic conditions, a consortium of microbes is thought to be involved in organic matter degradation, with fermenting bacteria producing intermediates such as propionate and acetate which are degraded to carbon dioxide by sulfate-reducing bacteria. Sulfate reducers are specialized with respect to the utilization of these fermentation products (Widdel and Hansen, 1992). In addition, several sulfate reducers can only carry out an incomplete oxidation of these substrates, whereas others such as many Desulfobacteraceae are capable of complete oxidation to carbon dioxide (Canfield et al., 2005).

Microbial communities and their activities have been widely studied in marine sediments. The development of cultivation-independent molecular methods since the late 1980s has led to an explosion of papers on microbial diversity and community structure (Bowman and McCuaig, 2003; Kemp and Aller, 2004; Ravenschlag et al., 2001). One of the more recent developments in the study of microbial community structure and function is a combination of $^{13}$C-labeling and tracing isotope levels in biomarkers generally referred to as stable isotope probing (SIP) (Boschker et al., 1998; Manefield et al., 2002; Radajewski et al., 2000). Traditional SIP methods have been used in marine sediments (Boschker et al., 1998; Webster et al., 2006). Other developments are in novel single-cell methods, such as fluorescence in situ hybridization (FISH) coupled with micro-autoradiography (Sintes and Herndl, 2006) or with Raman microscopy (Huang et al., 2007) and nano-scale secondary-ion mass spectrometry (nanoSIMS) (Musat et al., 2008). However, each of these SIP methods has limitations, such as low phylogenetic resolution or a requirement for high levels of isotope incorporation. FISH-based methods also require relatively high cellular rRNA content and accessibility of probe target sites (Fuchs et al., 1998). Recently, we further developed a method that is based on stable isotope probing combined with magnetic bead capture hybridization of rRNA (Mag-SIP) by lowering the amount of rRNA needed and thereby making it applicable to major phylogenetic groups within environmental samples (MacGregor et al., 2002; Miyatake et al., 2009). Advantages of Mag-SIP are a combination of high sensitivity for $^{13}$C-incorporation in target rRNA and high phylogenetic resolution.

In the present study, we applied the Mag-SIP protocol to study substrate utilization patterns by major groups in the microbial community of an intertidal sediment. Sediment
cores were incubated with different $^{13}$C-labeled substrates, and were sectioned into surface (0-2 cm) and deeper (2-5 cm) layers. The aerobic/anaerobic boundary is mostly situated a few mm from the sediment surface in coastal sediments (Brune et al., 2000), so the surface layer is expected to contain both the aerobic and the top of the anaerobic zone, whereas the deeper layer represents the sulfate reduction zone (Oenema, 1990). 16S rRNA of major groups was captured with specific oligonucleotide probes and $^{13}$C-labeling levels were determined by micro elemental analyzer-isotope ratio mass spectrometry (µEA-IRMS) (Miyatake et al., 2009). We also constructed clone libraries for the 16S rRNA genes and for the reversed-transcribed 16S rRNA as an alternative method to identify major active populations in both layers (Martinez et al., 2006; Mills et al., 2005). The results show different community characteristics and substrate utilization patterns between the surface and deeper layers, and are discussed in terms of general community functioning and the known characteristics of the detected microbial groups.

**Materials and methods**

**Sampling and $^{13}$C labeling**

Sediment samples were collected at an intertidal flat in the Rattekaai area (51°26.341’N, 4°10.040’E) of the Oosterschelde bay (The Netherlands) in May 2008. The sampling location was characterized by extensive intertidal sand flats. Undisturbed sediment cores (internal diameter 5.2 cm) were sampled at low tide and immediately transported back to the laboratory. Approximately equimolar $^{13}$C amounts of uniformly labeled $[^{13}C]$D-glucose, $[^{13}C]$sodium propionate or $[^{13}C]$sodium acetate (50, 100 and 150 mM, respectively; 99% $^{13}$C; Cambridge Isotope Laboratories, Andover, MA, USA) were injected with the line injection method (19 injections of 17 µl each) into the top 5 cm of the sediment (Jørgensen, 1978). A $[^{13}C]$-labeled algal-derived amino acid mixture (13 mM; 98% $^{13}$C; Cambridge Isotope Laboratories) was also injected with the same method. Four cores for each substrate were incubated for 24 h in the dark at in-situ temperature (14°C), followed by sectioning in 0-2 cm (surface layer) and 2-5 cm (deeper layer) from the surface. From each core, twenty to twenty-five grams wet weight of each section were stored at -80°C. Unlabeled control cores (no labeled substrates added) were also processed.

**Nucleic acid extraction from the sediment**

Total community RNA was extracted using the phenol-chloroform method (pH 5.1) as described in Miyatake et al. (2009). For every treatment, RNA extracted from frozen sediment samples of two cores were combined as a total community RNA sample for subsequent magnetic bead capture hybridization. All treatments were processed in duplicate. Total community RNA was quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). In order to estimate the amount of 16S rRNA
among total community RNA, RNA extracts were ran on a 5% polyacrylamide gel electrophoresis and were visualized by staining with ethidium bromide. DNA was extracted using the same method except that the extraction buffer and phenol had a pH of 7.0.

**Phylogenetic analysis of total community RNA, captured 16S rRNA, and total community DNA**

In order to identify major microbial groups in the community, we constructed both 16S rRNA genes and rRNA clone libraries from the two sediment horizons. In addition, clone libraries were constructed from 16S rRNA captured with each probe in order to check the specificity of the capture protocol (see below). Aliquots of total community (approximately 100 ng) and captured RNA (25 to 50 ng) were reverse transcribed with reverse primer DXR518 (5′-CGTATTACCGGCTGCTGG-3′) (Nogales et al., 1999) and Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The reverse transcripts were amplified with 10 cycles of PCR using primers 27F-DXR518 (Martinez et al., 2006; Mills et al., 2005). DNA extracted from the sediment was amplified directly using the same primers. PCR products were ligated into pGEM T-easy vector, and transformed into *Escherichia coli* JM109 competent cells (Promega, Madison, WI, USA). Positive clones were re-amplified with M13 primers, and sequenced with the 27F primer on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence chromatograms were manually checked using the Chromas Lite software (http://www.technelysium.com.au/chromas_lite.html). Phylogenetic analysis was performed by using the fast aligner and treeing tools implemented in the ARB program package (Ludwig et al., 2004). Phylogenetic relationships were determined by inserting sequences from this study into an ARB tree composed of the Greengenes database (http://greengenes.lbl.gov/) to which additional *Deltaproteobacteria* clone sequences from marine environments had been added. Phylotypes (≥ 97% similarity in sequence) were defined and the final bootstrapped neighbor-joining tree with 1000 samplings was created in MEGA4 (Tamura et al., 2007) using the Jukes-Cantor model.

**Oligonucleotide probes**

Based on the rRNA clone library, biotin-labeled oligonucleotide probes targeting major groups and unlabeled helper and competitor probes (Table 3.1) were selected and purchased from Ocimum Biosolutions (IJsselstein, The Netherlands). The nested set of probes targeted approximately 80% of the different rRNA sequences recovered from the two sediment layers. The testing of the probes for total bacterial rRNA (EUB338), *Deltaproteobacteria* (DELTA495a), and *Desulfobacteraceae* (Dbact653) is described in Miyatake et al. (2009). The DELTA495a probe also targets most *Gemmatismonadetes* (Lücker et al., 2007). It was used in combination with a competitor probe (cDELTA495a) to avoid capture of *Gammaproteobacteria*, which have only a single mismatch in the probe target region. In order to increase the yield, unlabeled 21-mer helper probes (Fuchs et al., 2000; MacGregor
et al., 2002) complementary to the consensus sequences upstream and downstream of the Dbact653 probe target sites were also used. Two probes targeting Cyanobacteria/chloroplast (CYA361 and CYA762) (Schönhuber et al., 1999) were tested with the Mag-SIP protocol. CYA361 was selected as it gave higher yields with sufficient coverage of clones in the rRNA library. Gammaproteobacteria are a generally abundant in marine sediments (Bowman and McCuaig, 2003; Ravenshlag et al., 2001). However, the commonly used probe for Gammaproteobacteria (GAM42a) (Manz et al., 1992) could not be used directly without further adaptation and testing of the protocol as it targets the 23S rRNA. Using the ARB software, we therefore designed another specific probe (BG553) and matching helper probes that target the 16S rRNA of most Beta/Gammaproteobacteria (Table 3.1). In this study, this probe is basically Gammaproteobacteria specific as we did not detect any Betaproteobacteria-related sequences in any of the clone libraries, including the rRNA library derived from the rRNA captured with this probe. Betaproteobacteria are typically a minor component of the microbial community in marine sediments (Bowman and McCuaig, 2003; Hunter et al., 2006). Optimal formamide concentrations (Table 3.1) in terms of capture efficiency versus specificity were determined as previously described (Miyatake et al., 2009) and gave more than 90% of target specificity as checked with clone libraries from captured 16S rRNA.
Table 3.1. 16S rRNA-targeted probes used in this study

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence (5'-3')</th>
<th>% FA&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB338</td>
<td>GCT GCC TCC CGT AGG AGT</td>
<td>25</td>
<td>Most Bacteria</td>
<td>(Amann et al., 1990)</td>
</tr>
<tr>
<td>DELTA495a</td>
<td>AGT TAG CCG GTG CTT CCT</td>
<td>45</td>
<td>Most Deltaproteobacteria</td>
<td>(Loy et al., 2002)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Most Gemmatimonadetes</td>
<td></td>
</tr>
<tr>
<td>cDELTA495a</td>
<td>AGT TAG CCG GTG CTT CT</td>
<td>45</td>
<td>Competitor of DELTA495a</td>
<td>(Macalady et al., 2006)</td>
</tr>
<tr>
<td>Dbact653</td>
<td>TTC CCT CTC CCA TAC TCA</td>
<td>25</td>
<td>Most Desulfobacteraceae</td>
<td>(Miyatake et al., 2009)</td>
</tr>
<tr>
<td>Dbact653_ up_help</td>
<td>CCC CGG AAG TGC AYT TGA WAC</td>
<td>25</td>
<td>Helper probe for Dbact653</td>
<td>(Miyatake et al., 2009)</td>
</tr>
<tr>
<td>Dbact653_ down_help</td>
<td>GTG GAA TTC CTG GTG TAG AGG</td>
<td>25</td>
<td>Helper probe for Dbact653</td>
<td>(Miyatake et al., 2009)</td>
</tr>
<tr>
<td>CYA361</td>
<td>CCC ATT GCG GAA AAT TCC</td>
<td>20</td>
<td>Most Cyanobacteria and many chloroplast</td>
<td>(Schönhuber et al., 1999)</td>
</tr>
<tr>
<td>BG553</td>
<td>CGC CCA GTA ATT CCG ATT</td>
<td>60</td>
<td>Most Betaproteobacteria Most Gammaproteobacteria</td>
<td>This study</td>
</tr>
<tr>
<td>BG553_ up_help</td>
<td>AAC CGC CTR CGN RCG CTT TA</td>
<td>60</td>
<td>Helper probe for BG553</td>
<td>This study</td>
</tr>
<tr>
<td>BG553_ down_help</td>
<td>AAC GCT YGC ACC CTM CTG ATT</td>
<td>60</td>
<td>Helper probe for BG553</td>
<td>This study</td>
</tr>
</tbody>
</table>

<sup>1</sup>Percent formamide (FA) in hybridization buffer for hybridizations at 20°C

**Hybridization and magnetic bead capturing**

Ribosomal RNA from specific groups of microorganisms was isolated with the Mag-SIP protocol as described in Miyatake et al. (2009). In brief, 20–40 µg total RNA was hybridized with a specific biotin-labeled probe and the 16S rRNA-probe hybrids were captured with streptavidin coated hydrophobic paramagnetic beads (Dynabeads MyOne Streptavidin T1; Invitrogen). Captured 16S rRNA was released from the beads, and approximately 500 ng of the captured material used for stable isotope analysis. Small aliquots of captured material were used for clone library construction.
Isotope ratio analysis of captured 16S rRNA was performed by µEA-IRMS consisting of a wet oxidation interface (LC IsoLink; Thermo Fisher Scientific, Bremen, Germany) coupled on-line to an isotope ratio mass spectrometer (DELTA V Advantage; Thermo Fisher Scientific) (Krummen et al., 2004) as described in Miyatake et al. (2009). Samples (50 µl) were directly injected into this µEA-IRMS operating in bulk injection mode. Standard curves ranging from 0-1000 ng of carbon were made with phthalic acid. Stable carbon isotope ratios were expressed as δ13C values calibrated against the international standard Vienna Pee Dee Belemnite (VPDB). The delta-notation is defined as:

$$\delta^{13}C_{\text{sample}} (\text{‰}) = [(R_s/R_{st})-1] \times 1000$$  \hspace{1cm} (1)

, where $R_s$ is the ratio of $^{13}C/^{12}C$ in the sample and $R_{st}$ is the ratio of the international standard VPDB (0.0111797). The measured $\delta^{13}C$ values were corrected for the protocol blank:

$$\delta^{13}C_{\text{RNA}} (\text{‰}) = \left[ \frac{(\delta^{13}C_{\text{sample}} \times C_{\text{sample}}) - (\delta^{13}C_{\text{blank}} \times C_{\text{blank}})}{C_{\text{sample}} - C_{\text{blank}}} \right]$$  \hspace{1cm} (2)

, where $\delta^{13}C_{\text{sample}}$ is the $\delta^{13}C$ value of the sample, $C_{\text{sample}}$ is the amount of carbon in the sample, $\delta^{13}C_{\text{blank}}$ is the $\delta^{13}C$ value of the blank, and $C_{\text{blank}}$ is the amount of carbon in the blank (Boschker, 2004).

**Nucleotide sequence accession numbers**

Nucleotide sequences obtained from total community RNA, DNA, and probe-captured 16S rRNA have been deposited in the Genbank/DDBJ/EMBL database under accession numbers GQ449821 to GQ450274.
Results

Comparison of 16S rRNA gene and rRNA clone libraries

Clone libraries were constructed from both 16S rRNA genes and rRNA for the sediment surface (0-2 cm) and deeper (2-5 cm) layers. Between 80 and 100 clones were obtained for each of the four clone libraries. Figure 3.1 shows phylogenetic relationships of 16S rRNA genes and rRNA clone sequences from both layers. Clones with 97% sequence similarity were considered to represent similar phylotypes, so only representative clones are shown. Approximately 35 µg of DNA and 15 µg of RNA per 1 g (dry weight) sediment were extracted from the surface layer, and somewhat lower amounts from the deeper layer (approximately 15 µg g⁻¹ of DNA and 10 µg g⁻¹ of RNA).

The clones obtained were often most closely related to environmental clones derived from other marine sediment studies (Fig. 3.1). Diversity, in terms of the number of clones detected at the ≥ 97% level, appeared to be approximately similar between the surface layer (53 phylotypes, both 16S rRNA genes and rRNA clones) and the deeper layer (46 phylotypes), within the limitations of the study. The rRNA libraries were somewhat less diverse than the 16S rRNA gene libraries, with 35 and 37 16S rRNA gene phylotypes, and 27 and 23 rRNA phylotypes for the surface and the deeper layer, respectively. The 16S rRNA gene libraries were rather different between the surface and the deeper layers, suggesting a major difference in community structure (Fig. 3.1), although some 16S rRNA gene phylotypes were found in both layers. The rRNA phylotypes were dominated by several abundant phylotypes in the Deltaproteobacteria (only in the deeper layer), Gammaproteobacteria, Planctomycetes, Cyanobacteria and chloroplast. Many but not all 16S rRNA gene phylotypes were matched by rRNA phylotypes recovered from the same layer. Most clones affiliated with Cyanobacteria and chloroplast were detected in both layers, and were mainly found in a limited number of highly abundant phylotypes. The chloroplast clones were mostly related to diatoms. For the rRNA libraries, there was a clear difference in Deltaproteobacteria phylotypes between the two layers. Desulfobacteraceae clones were recovered much more frequently from the deeper layer, and there was a shift from Desulfuromusa- to Desulfocapsa-related clones between the surface and the deeper layer. The lower number of rRNA clones from Desulfobacteraceae in the surface layer was mainly due to the absence of Desulfococcus- and Desulfosarcina-related clones, while sequences related to clone OS02-TRNA-76 were more abundant both in the rRNA library (Fig. 3.1) and in probe-captured rRNA, where they accounted for about 80% of the clones in the surface layer (data not shown). The rRNA phylotypes belonging to the Gammaproteobacteria were rather diverse in comparison with other groups, and many of the dominant phylotypes were found in both layers. They were most closely related to environmental clones, although some were distantly related to Thiomicomicrospira.
Figure 3.1. (Continue to the next page) Neighbor-joining tree showing the affiliation of clones obtained from 16S rRNA genes and 16S rRNA to closely related sequences. Clones designated OS2 are derived from DNA and RNA in the surface layer, and clones designated OS25 are from the deeper layer. Clones with designations containing DNA and TRNA are derived from DNA and total community RNA, respectively. Numbers and letters in parenthesis indicate number of clones in a phylotype (≥ 97% similarity in sequence) and type of sequences (D for DNA-derived, R for RNA-derived). The RNA clones from the 2-5 cm layer are derived from Miyatake et al. (2009). Bootstrap values represent 1000 replicates and only values greater than 50% are reported. The scale bar indicates 10% estimated phylogenetic divergence.
Figure 3.1. Cont.
In order to get a comprehensive overview, the distribution of clones belonging to major microbial groups in the 16S rRNA gene and rRNA libraries in both layers was compared (Fig. 3.2). There were obvious differences in community structure between the surface and the deeper layer. *Desulfobacteraceae* clones were found in similar proportions (6%) in 16S rRNA gene libraries of both layers, but were more abundant in the rRNA library of the deeper layer (19%) than of the surface layer (2%). *Deltaproteobacteria* not belonging to the *Desulfobacteraceae* were more abundant in the deeper layer (7%) than in the surface layer (4%) in the 16S rRNA gene libraries, whereas the opposite was true for the rRNA libraries (deeper layer 3%, surface layer 7%). *Gammaproteobacteria* clones were found in almost the same numbers in both 16S rRNA gene and rRNA library of both layers. *Cyanobacteria* and chloroplast had more or less the same proportion in the 16S rRNA gene library of both layers (5-9%), but were generally much more abundant in the rRNA libraries of both layers (19 and 30% for *Cyanobacteria*, 14% for chloroplast of the deeper layer). Clones belonging to the *Bacteroidetes*, *Alphaproteobacteria* and others were hardly detected in the rRNA libraries even though they were sometimes abundant in the 16S rRNA gene libraries. *Planctomycetes* were not covered by our Mag-SIP probes, and were more abundant in the rRNA than 16S rRNA gene libraries of both layers. Finally, the proportion of 16S rRNA gene phylotypes that were also found in the rRNA library is also indicated in Figure 3.2, and was in total lower in the surface layer than in the deeper layer (29 and 39%, respectively). Interestingly, all *Cyanobacteria* and chloroplast 16S rRNA gene phylotypes in both layers and all 16S rRNA gene phylotypes from the *Desulfobacteraceae* in the deeper layer were found in rRNA library, suggesting that the majority of the members belonging to these groups were viable and growing.

The specific probes used in this study targeted *Deltaproteobacteria*, *Desulfobacteraceae*, *Gammaproteobacteria*, *Cyanobacteria* and chloroplast, and the clones from these groups accounted for approximately 80% of the rRNA sequences in both sediment layers. We also produced rRNA libraries from the rRNA isolated with the different probes in the Mag-SIP protocol. Specificity of all the probes was better than 90%. There was in general good agreement between the phylogenies of the captured rRNA and total rRNA clone libraries. Sufficient rRNA for isotope analysis could be isolated with all probes from both layers. *Desulfobacteraceae* explained only 1-2% of the rRNA library from the surface layer (Fig. 3.2), showing that also relatively minor populations can be targeted by the Mag-SIP protocol.
Figure 3.2. Proportion of clones affiliated with major groups among each library derived from 16S 16S rRNA genes or 16S rRNA in either the surface layer (0-2 cm) or the deeper layer (2-5 cm). Total numbers of clones sequenced are indicated as n. Shaded part of the bars in 16S rRNA gene libraries indicates proportion of clones which were also found in the rRNA library (at the level of ≥ 97% sequence similarity) in each layer.

Substrate incorporation by major groups within the microbial community

There were clear contrasts in $^{13}$C-label incorporation of rRNA captured by the different probes between the substrates and sediment layers (Fig 3.3). For the surface layer of the sediment, differences in labeling levels among the major microbial groups were relatively small (within a factor of 2) and often rather similar to total bacterial rRNA labeling. Labeling levels detected in *Gammaproteobacteria* were higher than total bacterial rRNA with both $^{13}$C-glucose and -propionate, and the same was true for *Deltaproteobacteria* and *Desulfobacteraceae* for $^{13}$C-propionate. Label incorporation in *Cyanobacteria/chloroplast* was detected with all substrates in the surface layer, but to a somewhat lower degree than the other groups. Labeling levels for other probe-substrate combinations were similar to each other.

Differences in label incorporation between substrates and microbial groups were much more pronounced in the deeper layer representing the anaerobic sulfate-reducing zone of the sediment. For glucose-labeled sediment, *Gammaproteobacteria* clearly showed higher incorporation than the other groups, but they were relatively less important for the other substrates. *Desulfobacteraceae* were in general more highly labeled with propionate, acetate, and amino acid than the other groups. Labeling in *Cyanobacteria/chloroplast* was
detected only with glucose and to a much lesser extent with propionate, suggesting that these phototrophic organisms were able to utilize glucose under anaerobic conditions in the dark. Overall, although some similarities in label distribution were therefore found between the surface and the deeper layer, the differences in labeling between phylogenetic groups and substrates were much more pronounced in the deeper layer.

Figure 3.3. The increase in $\delta^{13}C$ ratios between labeled sediments and unlabeled control sediment ($\Delta\delta^{13}C$) for the different captured-16S rRNA fractions. The sediment were incubated with [13C]glucose, [13C]propionate, [13C]acetate, or [13C]amino acid. Data for 13C-amino acid labeled sediment were normalized to the amount of 13C as added with 13C-acetate. Some of the results from the deeper layer are derived from Miyatake et al. (2009); the results with probes BG553 and CYA361 are new as are all results from the 13C-amino acid labeling experiment. Unlabeled controls had $\delta^{13}C$ values between -15 and -20 ‰, within the typical range for marine phytobenthos and Bacteria (Boschker and Middelburg, 2002; Coffin et al., 1990) and differences in $\delta^{13}C$ value between duplicate analysis of unlabeled controls was less than 2 ‰.
Discussion

We used two complementary approaches to study active microbial communities in intertidal marine sediment. First, we compared 16S rRNA gene- and rRNA-derived clone libraries to indicate potential active groups. Second, we used Mag-SIP to study $^{13}$C labeling of major phylogenetic groups within the microbial community in order to determine substrate utilization patterns. Despite some prominent exceptions (Fegatella et al., 1998; Flärdh et al., 1992), rRNA content is generally related to growth rate and is highest in growing cells (Kerkhof and Kemp, 1999; Moeseneder et al., 2005). Clone libraries made from rRNA are therefore generally considered to emphasize the metabolically active populations, whereas 16S rRNA gene libraries are more representative of the numerically abundant population (Duineveld et al., 2001; Gentile et al., 2006; Mills et al., 2005). In our study, the distribution of clones derived from DNA and RNA only partially overlapped, and this is in agreement with previous studies (Mengoni et al., 2005; Nogales et al., 2001).

There were clear contrasts in microbial community structure (at the level of $\geq 97\%$ sequence similarity) and label distribution of rRNA captured by the different probes between the surface (0-2 cm) and the deeper (2-5 cm) layers. The surface layer contained the aerobic zone and the top of the anaerobic zone, whereas the deeper layer represented the dissimilatory sulfate reduction-dominated zone (Oenema, 1990). The Mag-SIP study revealed clear differences in substrate use between the different major phylogenetic groups. In the deeper layer, glucose was primarily incorporated by Gammaproteobacteria, whereas Deltaproteobacteria, more specifically Desulfobacteraceae, were involved in acetate and propionate consumption (Fig. 3.3). Under anaerobic sulfate-reducing conditions, a consortium of microbes is thought to be involved in organic matter degradation, with fermenting bacteria producing intermediates such as propionate and acetate that are degraded to carbon dioxide by sulfate-reducing bacteria (Canfield et al., 2005). Our results are in agreement with this model of anaerobic mineralization and suggest that, among the heterotrophic bacteria, the major active phylogenetic groups are also the main functional groups in sulfate-reducing marine sediment.

In contrast, differences in labeling between groups and substrates were much smaller in the surface layer of the sediment, indicating limited specialization in substrate range between groups. The surface layer also contained the top of the anaerobic sediment and it may well be that the small differences in labeling detected were actually due to incorporation by Bacteria in the anaerobic part of the surface layer. It could be that the variety of electron acceptors (e.g. oxygen, nitrate, metal oxides and sulfate) available to the microbial community in the surface layer allowed them to use a wider range of organic substrates. It is also possible that the dominant subclades within the main phyla, as detected in this study and generally found in environmental studies (Bowman and McCuaig, 2003; Edlund et al., 2008), would show differences in substrate range, but our results suggest that
the diverse microbial community found in the surface of marine sediments is functionally redundant in terms of carbon utilization.

*Gammaproteobacterial* sequences were the most abundant in the clone libraries (Fig. 3.2) and were responsible for much of the glucose incorporation, especially in the deeper layer (Fig. 3.3). Their labeling with glucose in $\Delta\delta^{13}C$ was approximately four times higher in the deeper layer than in the surface layer (Fig. 3.3), which was unexpected as similar amounts of label were added. This is most likely due to less dilution of $^{13}C$ label into a lower bacterial biomass of the deeper sediment layer, or decreased competition for glucose with other members of the microbial community. Although *Gammaproteobacteria* such as *Escherichia coli* and some *Shewanella* species are known for anaerobic growth by fermentation of glucose (Bowman et al., 1997; Ivanova et al., 2001; Lovley, 1991), their sequences are only distantly related to *Gammaproteobacteria* clones from this study, with approximately 90% sequence similarity. Approximately one-third of the *Gammaproteobacteria* clones in this study are most closely related to sulfur-oxidizing symbionts and their free-living relatives, which are thought to be chemoautotrophs involved in the oxidative part of the sulfur cycle (Distel et al., 1994; Nercessian et al., 2005). It is however difficult to draw conclusions on which of the diverse *Gammaproteobacteria* detected were the dominant glucose users.

*Deltaproteobacteria* were a major group, as expected for marine sediment where sulfate reduction is important, and a clear difference in the active subclades of the community was detected between the sediment layers studied (Fig. 3.1). In the deeper layer, *Desulfobacteraceae* clones related to *Desulfosarcina* and *Desulfococcus* were abundant. This *Desulfosarcina-Desulfococcus* group is ubiquitous and sometimes predominates microbial communities in anaerobic coastal sediments (Edgcomb et al., 1999; Ravenschlag et al., 2000; Sahm et al., 1999). The *Desulfosarcina-Desulfococcus* group are generally complete oxidizing members of the sulfate-reducing bacteria that are able to use a wide range of substrates, including acetate and propionate, but typically do not utilize carbohydrates (Canfield et al., 2005; Widdel and Hansen, 1992). Our labeling results for the deeper layer are generally in agreement with this physiology, as *Desulfobacteraceae* played major role in acetate and propionate (and amino acid) utilization. Webster et al. (2006) applied traditional DNA-SIP to study the use of $^{13}C$-glucose and acetate in anaerobic intertidal sediment and their results partially agree with our study, as *Desulfobacteraceae* were indicated as major consumers of acetate. However, they also found that glucose was used by the majority of the microbial community, whereas we found clear dominance of *Gammaproteobacteria*. This may be due to differences in methods applied such as lower substrate concentrations and shorter incubation time used in this study. For *Desulfobacteraceae*, the relatively minor labeling with glucose may suggest some direct incorporation but is also in agreement with the use of labeled fermentation products produced by for instance *Gammaproteobacteria*, which were dominant glucose utilizers (Fig. 3.3). In contrast, the Mag-SIP results from the surface sediment layer suggest that *Deltaproteobacteria,*
including *Desulfobacteraceae*, were able to use glucose as well as all other substrates (Fig. 3.3). The rRNA clone library from the surface layer was dominated by a cluster related to *Desulfuromusa* in the other *Deltaproteobacteria* and a cluster only related environmental clones in the *Desulfobacteraceae* (Fig. 3.1). The genus *Desulfuromusa* and its relatives includes sulfur- and metal oxide-reducing bacteria as well as fermentative organisms capable of utilizing glucose (Liesack and Finster, 1994). The dominant cluster in the *Desulfobacteraceae* detected in the surface layer is commonly found in marine sediments, and is closely related to subclade IB as defined by Klepac-Ceraj et al. (Klepac-Ceraj et al., 2004) and to the sulfate-reducing endosymbionts found in *Olavius* species (Dubilier et al., 2001). They are generally treated as sulfate reducers, and our results suggest that they may have a wide substrate range and may even be able to use other electron acceptors beside sulfate as generally found in the top layer of marine sediments.

Interestingly, *Cyanobacteria* and chloroplast clones - expected in the surface layer - were also highly abundant in the deeper dark anaerobic layer. All *Cyanobacteria* clones were affiliated with *Oscillatoriales* and the chloroplast clones with diatoms. Moreover, all the *Cyanobacteria* 16S rRNA gene phylotypes and most chloroplast 16S rRNA gene phylotypes were also found in the rRNA library in both layers, implying that they were viable and growing even in the lower dark and anaerobic layer. An alternative explanation is that ribosomal content of these phototrophs decreases only slowly relative to protein synthesis rate even if they are starving or in stationary phase for prolonged periods of time, as has been found in some heterotrophic bacteria (Givskov et al., 1994). However, *Cyanobacteria* and diatoms were capable of utilizing all the tested 13C-substrates in the surface layer and incorporated glucose in the deeper layer, which clearly shows that they were metabolically active in both layers. Active heterotrophic growth of *Cyanobacteria* and diatoms on glucose under aerobic conditions in the dark is well known (Lewin and Hellebust, 1976; Smith and Underwood, 2000). Our results suggest that they may also be able to survive for prolonged periods by utilizing glucose, probably via fermentation, under the anaerobic conditions found deep within marine sediments. Based on 16S rRNA libraries and on the incorporation of bromodeoxyuridine into DNA, viability and growth of *Cyanobacteria* in dark anaerobic conditions has also been observed in Baltic Sea sediment (Edlund et al., 2008). Under anaerobic conditions, many *Cyanobacteria* are able to gain energy from fermentation of storage carbohydrates accumulated during photoautotrophic growth, but fermentation of external organic substrates in free-living *Cyanobacteria* is poorly documented (Stal and Moezelaar, 1997). Heterotrophic growth in these phototrophs is probably used to survive dark periods either during the night or during longer periods when they are mixed into the sediment by for instance bioturbation. Our results suggest that they may remain viable and active in the dark anaerobic conditions probably to be able to respond quickly when light becomes available. Many of the *Cyanobacteria* and diatoms in marine sediments may therefore be mixotrophs, suggesting that the functional distinction between phototrophic primary producers and heterotrophic bacteria is blurred.
Similar to other studies (Bowman and McCuaig, 2003; Edlund et al., 2008), several dominant 16S rRNA phylotypes were generally detected within the major phylogenetic groups targeted with the Mag-SIP protocol in this study. It would be interesting to further extend the Mag-SIP protocol to these dominant phylotypes. This would involve developing and testing new probe sets, for which the 23S rRNA may also offer interesting options (Fuchs et al., 2001). With a limited number of probe sets, it would be possible to cover the dominant rRNA clones. For instance, three probes would be necessary to cover most of the *Desulfobacteraceae* and two additional probes would cover most of the *Deltaproteobacteria* in the studied sediment (assuming that relevant probes can be designed). The Mag-SIP protocol is likely sensitive enough for this, as we showed that we were able to isolate 16S rRNA for $^{13}$C-analysis from groups accounting for 1-2% of the total 16S rRNA clones. Other possible developments lie in the combination of Mag-SIP with novel single-cell methods such as nanometer-scale secondary-ion mass spectrometry (NanoSIMS) (Behrens et al., 2008; Musat et al., 2008). The Mag-SIP approach therefore is a very valuable asset to study relationships between microbial community structure and functioning in a range of environments.

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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 toplayer (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 CO$_2$ 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 CO$_2$ 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 ($^{13}$C) 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 NaH\(^{13}\)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 NaH\(^{13}\)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 (≥ 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.

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

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 ± 0.029 $\mu$mol biomass-C g DW$^{-1}$ 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 ± 0.20 mM) and in experiment B2 (0.22 ± 0.06 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, 16:1ω7c, 16:1ω5, 16:0 and 18:1ω7c. 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 18:1ω9c 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, 16:1ω7c, 16:1ω5, 16:0 and 18:1ω7c) 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, 16:1ω7c, 16:1ω5) 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 $^{13}$C-incorporation into individual PLFA are corrected for $^{13}$C-DIC labeling levels and therefore show total synthesis rates from inorganic carbon.

To further identify active anaerobic bacterial chemoautotrophs, we measured $^{13}$C 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 $^{13}$C-labeled samples and ranged between 10 and 28‰ $\Delta^{13}$C (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. $\Delta \delta^{13}C$ 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 chemosynthetic 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 chemosynthetic bacteria at the ZK09 site and that both groups were involved in the anaerobic chemosynthetic 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 chemosynthetic activity are commonly found in sulfate reducing *Deltaproteobacteria*. Some of these PLFA, like i17:1ω7, a17:1ω7 and 17:1ω8c, 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 chemosynthetic 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 chemosynthetic 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 chemosynthetic activity, 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 *Gammaproteobacteria* (14:0, 16:1ω7c, 16:0 and 18:1ω7c; 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\textsubscript{3} and MnO\textsubscript{2} as oxidants under anaerobic conditions (Canfield et al., 2005).

To conclude, we detected substantial anaerobic chemoautotrophy rates by measuring dark \textsuperscript{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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Chapter 5

Tracing carbon flow from microphytobenthos to major phylogenetic groups in the bacterial community in an intertidal marine sediment

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Abstract

Carbon flow from benthic diatoms to the heterotrophic bacterial community was traced in a marine intertidal sediment for five consecutive days. An in situ pulse-chase experiment was done in which $^{13}$C-labelled bicarbonate was sprayed onto the sediment surface during low tide and $^{13}$C-label incorporation in major carbon pools, intermediate metabolites, and biomarkers was monitored. PLFA and rRNA were used as biomarkers in order to identify responsible members of the microbial community at class- and family-level phylogenetic resolution. Diatoms were the predominant primary producer at the study site, and *Gammaproteobacteria*, *Bacteroidetes*, and *Deltaproteobacteria* were major groups in the heterotrophic microbial community based on pigment analysis, PLFA analysis, and a 16S rRNA clone library. Both the $^{13}$C-PLFA and $^{13}$C-rRNA data suggest that there was a fast transfer of label from diatoms to heterotrophic bacteria during the first 24 hours of the experiment, which was probably due to the exudation of low-molecular organic compounds by diatoms that could be directly utilized by heterotrophic bacteria. After this initial fast transfer of organic matter, labeling of the heterotrophic bacteria proceeded at a slower rate until the third day of the experiment, which coincided with the degradation of water-extractable extracellular polymeric carbohydrates initially produced by the diatoms. Labeling in heterotrophic bacteria closely tracked labeling in diatoms suggesting a closely coupled system, and all heterotrophic bacterial groups benefited about equally from the organic matter released by the diatoms indicating limited specialization.
Introduction

Marine intertidal areas are highly productive ecosystems, and primary production of microphytobenthos contributes significantly to the total primary production of estuarine and shallow water ecosystems (MacIntyre et al., 1996; Underwood and Kromkamp, 1999). Benthic diatoms are typically the dominant microphytobenthos in marine intertidal sediments in temperate regions (Underwood and Paterson, 2003), and they are known to exude large amounts of carbohydrates in the form of extracellular polymeric substances (EPS) in order to migrate through the sediment and to stabilize the sediment surface avoiding resuspension (Paterson and Black, 1999). As a consequence, diatoms provide major carbon sources to benthic food web including heterotrophic bacteria in marine intertidal sediments (Smith and Underwood, 1998; van Oevelen et al., 2006).

Diatoms exude different types of carbohydrates depending on environmental conditions and the nutrient status, which vary in structure and composition (de Brouwer and Stal, 2001; Smith and Underwood, 2000). Low-molecular-weight compounds exuded by diatoms can be directly utilized by the heterotrophic microbial community (Sundh, 1992), whereas high-molecular-weight compounds such as EPS first need to be broken down by extracellular enzymes (Goto et al., 2001). The microbial community structure of intertidal sediments (Bühring et al., 2005; Hunter et al., 2006; Rusch et al., 2003) and EPS formation by benthic diatoms (Goto et al., 2001; Haynes et al., 2007; Smith and Underwood, 1998) have both been extensively studied. However, there are only a limited number of studies that directly linked carbon flows from diatoms to heterotrophic microbes and in addition identified responsible members in the microbial community. Middelburg et al. (2000) and Evrard et al. (2008) have reported rapid transfer of carbon from microphytobenthos into bacterial biomass by combining in situ $^{13}$C pulse-chase labeling and PLFA biomarker analysis to differentiate between labeling in algae and heterotrophic bacteria. Bellinger et al. (2009) extended these studies by showing that EPS-derived carbohydrates were major intermediates in the transfer of organic matter between benthic diatoms and heterotrophic bacteria. Given the diverse microbial community found in intertidal sediments (Bühring et al., 2005; Hunter et al., 2006; Rusch et al., 2003), it is expected that specialized bacteria will be present that are involved in the degradation of EPS and other materials produced by benthic diatoms.

The phylogenetic resolution of PLFA biomarkers is however limited, and bacterial populations involved in the coupling with benthic diatoms can not be identified in much detail (Boschker and Middelburg, 2002). In general, 16S rRNA provides much higher phylogenetic resolution than possible with PLFA, and is widely used to identify microbial communities in their natural environment. MacGregor et al. (2002) developed a method where 16S rRNA from defined phylogenetic groups is isolated with specific oligonucleotide probes and paramagnetic beads for subsequent stable isotope analysis (Mag-SIP).
Miyatake et al. (2009; 2010) further improved the Mag-SIP method and were able to study organic substrate utilization by major microbial groups down to the family level in marine sediments.

In the present study, we used the in situ $^{13}$C pulse-chase method to trace carbon flow in an intertidal benthic diatom mat for five days. In situ stable-isotope labeling approaches have the advantage over laboratory based incubations that environmental conditions such as waves, tidal currents, sediment mixing, and advective pore water flow are not disturbed (Middelburg et al., 2000). We applied both the Mag-SIP method and PLFA biomarkers for simultaneous identification of major active microbial groups and to quantify their $^{13}$C-label incorporation rates. Furthermore, we used recently developed high-performance liquid chromatography combined with isotope ratio mass spectrometry (HPLC-IRMS) methods to trace $^{13}$C in intermediate metabolites namely total and EPS-derived carbohydrates and low-molecular-weight carboxylic acids (LCA) including volatile fatty acids. We also constructed a cDNA clone library derived from 16S rRNA to determine the metabolically active microbial population structure in relation to the specific oligonucleotide probe set used in the Mag-SIP protocol. In this study, we identified major intermediates involved in the coupling between benthic diatoms and heterotrophic bacteria and show that all studied groups within the heterotrophic bacterial community appear to be equally important in this coupling.

Materials and methods

Study site, $^{13}$C labeling, and sampling

From April 14th to 19th 2009, an in situ $^{13}$C-labeling experiment was done at an intertidal flat in the Zandkreek area (51°33’N, 3°53’E) of the Oosterschelde bay (The Netherlands). The sampling site was located 0.15 m below the mean tidal level, and exposed period was approximately 6 h per tidal cycle (Fig. 5.1A). The sediment was relatively sandy containing only 6.8% silt (<63 µm particle size), and a thick diatom mat was clearly visible on the surface. Salinity and water temperature were constant at around 28.5 and 15°C, respectively, during the experiment period. The weather was mostly sunny with high light levels (Fig 5.1A) and an average mean air temperature of 16.2°C. Almost no rainfall occurred during the experiment except for some minor drizzle on the third day.

During the first day of the experiment, shortly after exposure of the site during low tide, two 0.25-m$^2$ frames were inserted into the sediment to a depth of 8 cm in order to constrain the labeling and sampling area. The two frames were treated as duplicates. Initial, unlabelled control samples from the sediment and porewater were taken just outside the frames as described below. The in situ labeling experiment was started by spraying the surface of the sediment in each frame with 250 ml of $[^{13}$C] sodium bicarbonate (99% $^{13}$C; Cambridge Isotope Laboratories) with ambient salinity to obtain a final concentration of 1 g
$[^{13}\text{C}]$ sodium bicarbonate m$^2$ (Middelburg et al., 2000). The first sampling of the labeled sediment was performed after 4 h at the end of the low tide (the pulse-labeling period), and the two frames were sampled in a time-course of 12 h, 1 day, 2 days, 3 days, and 5 days (the chase period). The frames were divided in a 10 by 10 cm sampling grid.

Figure 5.1. (A) Light irradiance during the experiment period. Shaded part is indicating submerged period of the sampling site. Sampling times are indicated with arrows. Excess $^{13}\text{C}$ in (B) DIC and (C) TOC are shown as a function of time. Error bars represent standard deviations.
At each sampling time, porewater and sediment samples were collected and mixed from two randomly chosen positions within the sampling grid of each frame. First, porewater (two times 1 ml) was sampled with the porous polymer sippers (Rhizon Soil Moisture Sampler; Eijkelkamp Agrisearch Equipment, Giesbeek, The Netherlands) inserted into the upper 1.5 cm of the sediment. For $^{13}$C-DIC analysis, 1 ml of mixed pore water sample per frame was dispensed into air-tight headspace vials. The remainder of the porewater sample was used for $^{13}$C-LCA analysis. Subsequently, sediment was sampled by inserting a core liner (inside diameter 10 cm) to a depth of 5 cm, and top 1.5 cm of the sediment was collected. The sampling hole inside the core was refilled with unlabelled sediment collected just outside the sampling frames and the core liner was removed. The two sediment samples taken in each frame were homogenized, and divided into 8 samples of 20-25 g each for Mag-SIP analysis, one 45 g sample for total organic carbon (TOC), PLFA and total carbohydrates (bulk carbohydrate) measurements, and a 5 g sample for pigment analysis. Additionally, approximately 2 g of the homogenized sediment sample was directly processed for EPS carbohydrate extraction by adding it to 4.5 ml Milli-Q water as described in De Brouwer and Stal (2001). All samples were immediately transferred to the laboratory on ice. In the laboratory, EPS carbohydrate samples were shaken gently for 1 h at 30°C. The supernatant was collected after centrifugation at 4000 ×g for 15 min, and stored for carbohydrate analysis. This is the water-extractable fraction of EPS carbohydrates (wEPS). We also extracted the EDTA-extractable EPS fraction (De Brouwer and Stal, 2001), but these samples were not compatible with the HPLC-IRMS method used for carbohydrate analysis. Sediment samples for Mag-SIP and pigment analysis were stored in -80°C before analysis, and all other samples were stored at -20°C.

**Analytical procedures**

Sediment samples for PLFA, TOC, pigment, and bulk carbohydrate analysis were freeze-dried. Lipids were extracted from 3 g of freeze-dried sediment using a modified Bligh and Dyer extraction (Boschker *et al.*, 1999), from which the PLFA fraction was isolated on silica columns. The PLFA fraction was derivatized to volatile fatty-acid methyl esters, and carbon content and isotopic composition were measured by gas chromatography–combustion-isotope ratio mass spectrometry (Middelburg *et al.*, 2000). Carbon content and isotopic composition of TOC was analyzed by using an elemental analyzer–isotope ratio mass spectrometry (EA-IRMS) after the removal of carbonate with hydrochloric acid (Boschker *et al.*, 1999). For DIC analysis, pore water samples were acidified by adding 0.1 ml of 19 mol l$^{-1}$ phosphoric acid (Miyajima *et al.*, 1995), and headspace gas was injected into EA-IRMS in order to determine concentration and isotopic composition of DIC. Pigments were extracted from freeze-dried sediment with aceton (90%, buffered with 5% ammonium acetate), and analyzed by reverse-phase HPLC (Dijkman and Kromkamp, 2006).
Carbon content and isotopic composition of LCA were analyzed by HPLC-IRMS (Isolink interface and DELTA V Advantage IRMS; Thermo Fisher Scientific, Bremen, Germany) equipped with an Aminex HPX-87H cation-exchange column (Bio-Rad, Hercules, California, United States of America (U.S.A.)). The eluent was 8 mmol l⁻¹ sulfuric acid at a flow rate of 0.4 ml min⁻¹ (Krumbock and Conrad, 1991).

Carbohydrate analysis of bulk sediment and wEPS extracts was done by HPLC-IRMS (Boschker et al., 2008). Freeze-dried sediment and extracts were hydrolized to monosaccharide with 1.1 mol l⁻¹ sulfuric acid for 1 h at 120°C. The hydrolysate was neutralized with strontium carbonate, and the precipitate was removed by centrifugation. Monosaccharide concentrations and isotope ratios were analyzed by HPLC-IRMS (see above) equipped with a Carbopac PA20 (Dionex Benelux, Amsterdam, The Netherlands). The eluent was 1 mmol l⁻¹ sodium hydroxide at a flow rate of 0.3 ml min⁻¹.

Mag-SIP analysis and clone library construction

A nested set of oligonucleotide probes covering about 80% of clones of the microbial community (Table 5.1) was used with the Mag-SIP protocol (Miyatake et al., 2010). The probes EUB338 (Amann et al., 1990) and DELTA495a (Loy et al., 2002) were used for most Bacteria and Deltaproteobacteria, respectively, even though they do not target all the genera in the domains. 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. The family Desulfobacteraceae were covered with probe Dbact653 (Miyatake et al., 2009) and probe BG553 was used for Gammaproteobacteria (Miyatake et al., 2010). Both the probes Dbact653 and BG553 were used in combination with unlabeled helper probes complementary to the consensus sequence upstream and downstream of the probe target in order to increase yield (Fuchs et al., 2000; MacGregor et al., 2002). The probe CYA361 (Schönhuber et al., 1999) was used for Cyanobacteria/chloroplast.

For the Mag-SIP protocol, sediments were extracted with the chloroform-phenol method and 20-40 µg total rRNA was hybridized with biotin-labeled probes and the 16S rRNA-probe hybrids were captured with streptavidin-coated hydrophobic paramagnetic beads (Dynabeads MyOne Streptavidin T1; Invitrogen, Carlsbad, California, U.S.A.). Captured 16S rRNA was released from the beads, and approximately 500 ng of the captured rRNA used for stable isotope analysis by micro elemental analyzer–isotope ratio mass spectrometry (µEA-IRMS; Isolink interface with DELTA V Advantage IRMS; Thermo Fisher Scientific). Samples (in 50 µl) were directly injected into this µEA-IRMS operating in bulk injection mode. The protocol carbon blank was also determined by performing the Mag-SIP protocol without RNA extract. A detailed description of the Mag-SIP protocol and the ¹³C-rRNA analysis is found in Miyatake et al. (2009).
Table 5.1. 16S rRNA-targeted probes used in this study

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence (5’-3’)</th>
<th>% FA(^1)</th>
<th>Specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB338</td>
<td>GCT GCC TCC CGT AGG AGT</td>
<td>25</td>
<td>Most Bacteria</td>
<td>(Amann et al., 1990)</td>
</tr>
<tr>
<td>DELTA495a</td>
<td>AGT TAG CCG GTG CTT CCT</td>
<td>45</td>
<td>Most Deltaproteobacteria</td>
<td>(Loy et al., 2002)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Most Gemmatimonadetes</td>
<td></td>
</tr>
<tr>
<td>cDELTA495a</td>
<td>AGT TAG CCG GTG CTT CTT</td>
<td>45</td>
<td>Competitor of DELTA495a</td>
<td>(Macalady et al., 2006)</td>
</tr>
<tr>
<td>Dbact653</td>
<td>TTC CCT CTC CCA TAC TCA</td>
<td>25</td>
<td>Most Desulfobacteraceae</td>
<td>(Miyatake et al., 2009)</td>
</tr>
<tr>
<td>Dbact653_up_help</td>
<td>CCC CGG AAG TGC AYT TGA WAC</td>
<td>25</td>
<td>Helper probe for Dbact653</td>
<td>(Miyatake et al., 2009)</td>
</tr>
<tr>
<td>Dbact653_down_help</td>
<td>GTG GAA TTC CTG GTG TAG AGG</td>
<td>25</td>
<td>Helper probe for Dbact653</td>
<td>(Miyatake et al., 2009)</td>
</tr>
<tr>
<td>CYA361</td>
<td>CCC ATT GCG GAA AAT TCC</td>
<td>20</td>
<td>Most Cyanobacteria and many chloroplast</td>
<td>(Schönhuber et al., 1999)</td>
</tr>
<tr>
<td>BG553</td>
<td>CGC CCA GTA ATT CCG ATT</td>
<td>60</td>
<td>Most Beta- and Gamma- proteobacteria</td>
<td>(Miyatake et al., 2010)</td>
</tr>
<tr>
<td>BG553_up_help</td>
<td>AAC CGC CTR CGN RCG CTT TA</td>
<td>60</td>
<td>Helper probe for BG553</td>
<td>(Miyatake et al., 2010)</td>
</tr>
<tr>
<td>BG553_down_help</td>
<td>AAC GCT YGC ACC CTA CTG ATT</td>
<td>60</td>
<td>Helper probe for BG553</td>
<td>(Miyatake et al., 2010)</td>
</tr>
</tbody>
</table>

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

In order to further identify active microbial groups in the community and determine the coverage of the target-specific oligonucleotide probes used in the Mag-SIP protocol, a cDNA clone library derived from community rRNA was constructed as described in Miyatake et al. (2009). In short, RNA was extracted from the sediment (0 h sample), and was reverse transcribed with reverse primer DXR518 (Nogales et al., 1999). The reverse transcripts were amplified by PCR using primers 27F-DXR518 (Martinez et al., 2006; Mills et al., 2005). PCR products were ligated into pGEM T-easy vector (Promega, Madison, Wisconsin, U.S.A.), and transformed into *Escherichia coli* JM109 competent cells. Positive clones were re-amplified with M13 primers, and sequenced with the 27F primer on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, California, U.S.A.). Sequence chromatographs were manually checked using the Chromas
Lite software (http://www.technelysium.com.au/chromas_lite.html). Phylogenetic analysis was performed by using the fast aligner and treeing tools implemented in the ARB program package (Ludwig et al., 2004). Phylogenetic relationships were determined by inserting sequences from this study into an ARB tree composed of the Greengenes database (http://greengenes.lbl.gov/) to which additional Deltaproteobacteria clone sequences from marine environments had been added. Nucleotide sequences obtained in this study have been deposited in the Genbank/DDBJ/EMBL database under accession numbers GU215079-GU215169.

**Calculations**

Stable carbon isotope ratios were expressed as $\delta^{13}C$ values calibrated against the international standard Vienna Pee Dee Belemnite (VPDB). The delta notation is defined as:

$$\delta^{13}C_{\text{sample}} (\text{‰}) = \left[ \frac{\text{R}_{\text{sample}}}{\text{R}_{\text{st}}} - 1 \right] \times 1000$$

, where $\text{R}_{\text{sample}}$ is the ratio of $^{13}C$ in the sample and $\text{R}_{\text{st}}$ is the ratio of the international standard VPDB (0.0111797).

Captured rRNA was measured by bulk injection and therefore a correction for protocol blank was necessary. Correction of $\delta^{13}C_{\text{rRNA}}$ value for the protocol blank is done as:

$$\delta^{13}C_{\text{rRNA}} (\text{‰}) = \left[ \frac{\left( \delta^{13}C_{\text{sample}} \times C_{\text{sample}} \right) - \left( \delta^{13}C_{\text{blank}} \times C_{\text{blank}} \right)}{C_{\text{sample}} - C_{\text{blank}}} \right]$$

, where $C_{\text{sample}}$ is the amount of carbon in the sample, $\delta^{13}C_{\text{blank}}$ is the $\delta^{13}C$ value of the protocol blank, and $C_{\text{blank}}$ is the amount of carbon in the protocol blank (Boschker, 2004). $^{13}C$-label incorporation in rRNA is indicated as the increase of $\delta^{13}C$ value between background (unlabeled sample) and labeled samples ($\Delta\delta^{13}C; \text{‰}$).

The absolute amount of $^{13}C$ incorporated into different carbon pools over the background was expressed as Excess $^{13}C$ and calculated from $\delta^{13}C_{\text{sample}}$ as:

$$\text{Excess }^{13}C \ (\text{mol }^{13}C \ \text{g}^{-1}\text{dw}) =$$

$$\left[ \left( \frac{\left( \delta^{13}C_{\text{sample}} / 1000 + 1 \right) \times \text{R}_{\text{st}}}{\left( \delta^{13}C_{\text{sample}} / 1000 + 1 \right) \times \text{R}_{\text{st}} + 1} \right) - \left( \frac{\left( \delta^{13}C_{\text{background}} / 1000 + 1 \right) \times \text{R}_{\text{st}}}{\left( \delta^{13}C_{\text{background}} / 1000 + 1 \right) \times \text{R}_{\text{st}} + 1} \right) \right] \times C_{\text{sample}}$$

, where $\delta^{13}C_{\text{background}}$ is the $\delta^{13}C$ value of the unlabeled sample and $C_{\text{sample}}$ is the pool size in mol $C \ \text{g}^{-1}\text{dw}$ of sediment.
Excess $^{13}$C into bacterial biomass was estimated from the label in bacterial-biomarker PLFA as:

$$\text{Excess}^{13}\text{C-bacterial biomass (mol}^{13}\text{C g}^{-1}\text{dw)} = \sum \text{Excess}^{13}\text{C}_{\text{PLFA}_{\text{bact}}} / (0.056 \times 0.28)$$

where $^{13}\text{C}_{\text{PLFA}_{\text{bact}}}$ is $^{13}$C in bacterial-biomarker PLFA ($i_{14}:0$, $i_{15}:0$, $a_{15}:0$, $i_{16}:0$, and $18:1\omega 7c$), 0.056 represents the average PLFA concentration in *Bacteria* as carbon and 0.28 is the average fraction of these specific bacterial PLFA biomarkers in bacteria-dominated marine sediments (Middelburg *et al.*, 2000). Excess $^{13}$C into algal biomass was calculated from the difference between excess $^{13}$C into all PLFA and excess $^{13}$C into bacterial-biomarker PLFA and also corrected for typical PLFA content in diatoms:

$$\text{Excess}^{13}\text{C-algal biomass (mol}^{13}\text{C g}^{-1}\text{dw)} = \left(\sum \text{Excess}^{13}\text{C}_{\text{PLFA}_{\text{all}}} - \sum \text{Excess}^{13}\text{C}_{\text{PLFA}_{\text{bact}}} \right) / 0.035$$

where $^{13}\text{C}_{\text{PLFA}_{\text{all}}}$ is $^{13}$C in all individual PLFA measured in this study and 0.035 represents the average PLFA concentration in diatoms (Middelburg *et al.*, 2000). Both algal and bacterial biomass carbon per g dw of sediment were also calculated as above but with concentrations of biomarker PLFA instead of excess $^{13}$C.

**Results**

**Incorporation of $^{13}$C in major carbon pools and intermediate metabolites**

Concentrations of DIC and TOC in the sediment were almost constant at 4.0 ± 0.4 µmol C ml$^{-1}$ and 309 ± 20 µmol C g$^{-1}$dw, respectively, throughout the experimental period. As expected, $^{13}$C-label incorporation in DIC was the highest after 4 h and sharply decreased during the first day of the experiment followed by more gradual decrease till the end of the experiment when almost no $^{13}$C label remained (Fig. 5.1B). Label incorporation in TOC was almost constant at approximately 200 nmol $^{13}$C g$^{-1}$dw from the first labeled sample (4 h) to day 5 (Fig. 5.1C). There was a small peak in TOC labeling after 12 hours and some label in the TOC pool was lost at the very end of the experiment. These data appeared to suggest rapid incorporation of the $^{13}$C-DIC label by the benthic primary producers during the labeling pulse followed by minimal losses for the remainder of the experiment.

The bulk carbohydrate concentration remained relatively constant at 27.1 ± 1.7 µmol C g$^{-1}$dw throughout the experiment period (Fig. 5.2A). Glucose and galactose were the two major components and their contributions remained constant (approximately 30 and 15%),
respectively). The concentration of wEPS accounted for approximately 10% of bulk carbohydrate pool, and was variable during the experiment (Fig. 5.2B). Lower concentrations of wEPS carbohydrates were either for the night sampling (12 h) or for the 3d sampling. Glucose was the main carbohydrate detected in the wEPS (approximately 65%).

The limited turnover of $^{13}$C-label in the TOC pool during the trace period was not seen in the bulk carbohydrate and wEPS data. Excess $^{13}$C in bulk carbohydrate and wEPS were highest at 12 and 4 hours after labeling, respectively, and decreased in time (Fig. 5.2C, D). Interestingly, more than 90% of $^{13}$C labeling was recovered in glucose for both pools. Excess $^{13}$C in the wEPS initially explained about a half of $^{13}$C in bulk carbohydrate pool. Label decreased faster in the wEPS than in bulk carbohydrate and had almost disappeared at the end of the experiment. Label incorporation in other carbohydrates beside glucose was much lower and remained more-or-less constant. The decrease in $^{13}$C-label incorporation in bulk carbohydrate between 4 h and day 5 was therefore mainly due (by about 75%) to the decrease in wEPS derived glucose.

**Figure 5.2.** Monosaccharide composition and concentrations of (A) bulk and (B) wEPS carbohydrates are shown together with excess $^{13}$C of (C) bulk and (D) wEPS carbohydrates.
LCA in the porewater were the most dynamic intermediate pool analyzed for this experiment. LCA detected in this study were formate, acetate, oxalate, malate, lactate, and succinate. Lactate and succinate could not be separated sufficiently in the HPLC-IRMS chromatograms and are therefore reported together. The propionate peak overlapped with an unknown peak. Significant changes in concentration were observed for lactate/succinate and acetate. Other LCA detected in this study had low and constant concentrations during the experiment (20-30 nmol C g\(^{-1}\) dw), hence only lactate/succinate and acetate are plotted in Figure 5.3. The concentration of lactate/succinate was higher than acetate, and showed two distinct peaks in the beginning of the experiment and at day 3. The concentration of acetate was highest at 0 h followed by sharp decrease at 4 h, then gradually increased to day 5. \(^{13}\)C-label incorporation was only detected for lactate/succinate and acetate, and was higher in lactate/succinate than in acetate. Excess \(^{13}\)C in lactate/succinate and acetate also showed two distinct peaks after 4 h and day 1 or 2 (Fig. 5.3B). Note that 12-h sample was the only sample which was collected in the dark (Fig. 5.1A).

Figure 5.3. (A) Concentrations and (B) excess \(^{13}\)C of LCA (lactate/succinate and acetate). Error bars represent standard deviations.
Microbial community composition

Thick diatom mats were clearly visible on the surface of the sediment throughout the experiment period. Chlorophyll $a$ concentrations were $17.2 \pm 0.9 \, \mu g \, g^{-1}dw$, and the concentration of fucoxanthin, a marker for diatoms (Dijkman and Kromkamp, 2006; Wright and Jeffrey, 1987), was $6.1 \pm 0.3 \, \mu g \, g^{-1}dw$ throughout the experiment indicating high diatom biomass. The 16S rRNA clone library, which is considered to reflect the metabolically active community, also showed a high proportion of benthic diatoms (44% of the sequences) whereas Cyanobacteria clones were less important (6%, Fig. 5.4). Moreover, concentrations of poly-unsaturated fatty acid typically found in diatoms were much higher (e.g. $86.5 \pm 9.3$ and $495.5 \pm 110.4$ nmol C g$^{-1}dw$ for 16 PUFA and 20:5ω3, respectively) than representative biomarker PLFA for Cyanobacteria (e.g. $51.8 \pm 10.1$ and $30.9 \pm 5.5$ nmol C g$^{-1}dw$ for 18 PUFA and 18:2ω6c, respectively) suggesting a much higher biomass of diatoms than Cyanobacteria (Fig. 5.5). The rRNA clone library showed that Gammaproteobacteria (21% of the sequences), Bacteroidetes (8%), and Deltaproteobacteria (7%) were major heterotrophic bacterial groups in the community (Fig. 5.4). EUB338 probe used as a general bacterial probe in this study covered most of clones in the clone library as it only missed the Planctomycetes. The combination of other specific probes covered 77% of the clones targeted with the general bacterial EUB338 probe but did not cover the Bacteroidetes.

Figure 5.4. Proportion of clones affiliated with major phylogenetic groups in the clone library derived from reversed-transcribed 16S rRNA. Total numbers of clones sequenced are indicated as $n$. 

79
Incorporation of $^{13}$C into biomarkers

Label ($^{13}$C) incorporation in PLFA biomarkers and specific rRNA was measured to trace the carbon transfer from diatoms to heterotrophic bacteria. Total amount of RNA extracted from per unit of sediment was constant throughout the experiment period (10.9 ± 1.5 µg RNA g$^{-1}$dw), as were the PLFA concentrations (3.22 ± 0.35 µmol C g$^{-1}$dw) although these showed a small decrease between 12 and 24 hours (Fig 5.6A). The PLFA content in diatoms and Bacteria is relatively constant, and standard conversion factors can be used to infer actual amounts of label in their biomass (Middelburg et al., 2000). However, rRNA data can not be used in this way because rRNA content may be very variable depending on species and growth conditions (Flärdh et al., 1992; Kerkhof and Kemp, 1999). In addition, capturing efficiency of the different probes may also vary. Therefore, $^{13}$C incorporation in rRNA was expressed only as $\Delta\delta^{13}$C, which is a measure of specific $^{13}$C-label incorporation into the target group relative to the biomass of this group and therefore related to growth rate and rRNA turnover.

Based on PLFA data, excess $^{13}$C in diatom biomass rapidly increased in the first 12 hours and decrease somewhat till day 1, which was again followed by an increase until day 3 (Fig. 5.6B). This is also true for excess $^{13}$C in bacterial biomass but to lesser degree as label incorporation appeared to level off at 12 h but then increased again until day 3. Notice that hardly any $^{13}$C-DIC label remained in the sediment after the first day (Fig. 5.1),

Figure 5.5. Concentrations of individual PLFA in unlabeled sediment (0 h). Error bars represent standard deviations.
suggesting that the secondary increase in label incorporation in both diatoms and *Bacteria* was based on the consumption of a $^{13}$C-labeled organic pool produced during the first 4 h of the experiment. This second maximum in diatom and bacterial biomass $^{13}$C-label incorporation coincided with the decrease in label incorporation in the wEPS glucose (Fig. 5.2D). There was no detectable label incorporation of PLFA in the dark incubations (data not shown), indicating that dark fixation by for instance chemoautotrophic bacteria was minimal. The PLFA data therefore suggested a rapid transfer of organic carbon from diatoms to *Bacteria* in the first 12 hours, followed by a slower secondary utilization of primarily $^{13}$C labeled wEPS glucose by both diatoms and *Bacteria*.

**Figure 5.6.** (A) concentrations of biomass carbon and (B) excess $^{13}$C in diatoms and in *Bacteria* calculated from concentrations of biomarker PLFA and $^{13}$C-label incorporation of PLFA, respectively. The bacterial-biomarker PLFA used are i14:0, i15:0, a15:0, i16:0, and 18:1ω7c. Biomass carbon and $^{13}$C-label incorporation of diatoms were calculated from the difference between total PLFA and bacterial PLFA. Error bars represent standard deviations.
Label incorporation in rRNA captured with probe set generally also showed two peaks on day 1 and day 3 (Fig. 5.7A). Unlabeled controls of rRNA had δ^{13}C values between -15 and -20 ‰, within the typical range for marine phytoplankton and *Bacteria* (Boschker and Middelburg, 2002; Coffin et al., 1990). The Δδ^{13}C value of diatom/Cyanobacteria rRNA will present primarily label incorporation by diatoms as *Cyanobacteria* biomass was low. Remarkably, Δδ^{13}C values of all captured rRNA reached very similar levels and showed almost the same trend during the experiment. For instance, the difference in Δδ^{13}C pattern between total *Bacteria* (EUB338-captured rRNA) and *Desulfobacteraceae* (Dbact653-captured rRNA), which comprised only 3% of the clone library, was small. The only exception was that *Deltaproteobacteria* rRNA showed a significantly delay in labeling during the second part of the experiment with lower labeling on day 3 than the other captured rRNAs and labeling peaked only at the end of the experiment on day 5.

In order to directly compare label incorporation in rRNA and PLFA, Δδ^{13}C values of several representative biomarker PLFA for microphytobenthos (16 PUFA, 18 PUFA, 18:2ω6c, and 20:5ω3) and biomarker PLFA for heterotrophic bacteria (i14:0, i15:0, a15:0, i16:0, and 18:1ω7c) are presented in Fig. 5.7B and C. Biomarker PLFA Δδ^{13}C values for diatoms (16 PUFA and 20:5ω3) and *Cyanobacteria* (18 PUFA and 18:2ω6c) showed different trends (Fig. 5.7B). Labeling in biomarker PLFA for *Cyanobacteria* initially increase sharply followed by a gradual decrease till the end of the experiment. In contrast, diatom biomarker PLFA labeling showed a fast increase until 12 hours followed by a more gradual increase until day 3 and then decreased. Although Δδ^{13}C values of biomarker PLFA for *Cyanobacteria* were higher than for diatoms, excess ^13^C in diatom PLFA was much higher because diatoms were the dominant primary producers at the study site (data not shown). Representative biomarker PLFA for *Bacteria* generally showed similar labeling patterns as biomarker PLFA for diatoms with a fast increase during the first day of the experiment followed by a slower labeling until labeling peaked after 3 days (Fig. 5.7C). Furthermore, highest Δδ^{13}C values in the different captured rRNA and biomarker PLFA showed a similar range. These results suggest that photosynthetically fixed carbon by diatoms was evenly utilized by different members accounted substantial part of the heterotrophic bacterial community.
Figure 5.7. $\Delta\delta^{13}C$ values of (A) rRNA captured with the nested set of probes used in the Mag-SIP protocol, (B) representative biomarker PLFA for microphytobenthos (diatoms; 16 PUFA and 20:5ω3: Cyanobacteria; 18 PUFA and 18:2ω6c), and (C) representative biomarker PLFA for Bacteria. The variation in $\Delta\delta^{13}C$ values between duplicate frames was rather limited with maximum differences of 31% in rRNA and 34% in PLFA.
Discussion

In this study, carbon flows from benthic diatoms to heterotrophic microbial community were traced in detail by studying $^{13}$C-labeling dynamics of intermediate metabolites and major groups within microbial community. We demonstrated, for the first time, that Mag-SIP can be used to trace carbon flows in benthic diatom mats. Other in situ $^{13}$C-labeling studies have used PLFA and D-alanin as biomarkers, which have a phylogenetic resolution that is limited to domains (Evrard et al., 2008; Middelburg et al., 2000; Veuger et al., 2006). Moreover, $^{13}$C-labelling dynamics in total and EPS-derived carbohydrates and LCA intermediates were studied using recently developed HPLC-IRMS methods. Although the relationship between diatom carbohydrate exudates and the heterotrophic microbial community have been intensively studied in intertidal sediments (Bellinger et al., 2009; Hanlon et al., 2006; Haynes et al., 2007), studies including $^{13}$C-labeling incorporation of LCA in pore water are scarce. A conceptual model of carbon-flows during the experiment is presented in Fig. 5.8. We found two main mechanisms operating on different time scales by which organic carbon was transferred from benthic diatoms to heterotrophic bacteria.

![Conceptual model of carbon flow in marine intertidal sediment](image)

**Figure 5.8.** Conceptual model of carbon flow in marine intertidal sediment. Solid arrows indicate short-term carbon flow and dotted arrows indicate long-term carbon flow. Numbers correspond to period of carbon flow after labeling.
Both the $^{13}$C-PLFA and rRNA data suggest that there was a fast transfer of label from diatoms to heterotrophic bacteria during the first 24 hours of the experiment (Fig. 5.6 and 5.7), which was probably due to the exudation of low-molecular-weight organic compounds by diatoms that could be directly utilized by heterotrophic bacteria. Size distribution and composition of diatom exudates change depend on the conditions such as light irradiance, tidal cycle, and growth phase (Smith and Underwood, 1998; Underwood and Smith, 1998; van Duyl et al., 1999). During photosynthesis, photo-assimilated carbon is stored as intracellular glucan, which consist of 90% glucose (Underwood et al., 2004), and concurrently excess carbon leaches out from the cells as water-extractable carbohydrates (de Brouwer and Stal, 2001; Smith and Underwood, 2000). Up to 80% of water-extractable carbohydrates exuded by diatoms in illuminated and exposed period in intertidal sediments can be mono- and oligo-saccharides (Smith and Underwood, 2000; Underwood et al., 1995; Underwood and Smith, 1998), which may explain the very fast initial labeling of heterotrophic bacteria. This fast labeling coincided with a sharp peak in LCA labeling (Fig. 5.3) indicating that some low-molecular-weight exudates, possibly glucose, produced by the diatoms was quickly fermented by Bacteria to LCA. Generally, LCA such as acetate and lactate play important role as intermediate fermentation products in anaerobic bacterial communities (Jørgensen, 2006). Alternatively, diatoms might also be able to do fermentation when growing under anaerobic conditions, although we are unaware of studies in this direction.

After this initial fast transfer of organic matter, labeling of the heterotrophic bacteria proceeded at a slower rate until the third day of the experiment (Fig. 5.6 and 5.7), which coincided with the degradation of wEPS-derived carbohydrates initially produced by the diatoms (Fig. 5.2D). After 4 to 12 hours, label recovered in wEPS explained 70% of the total sediment carbohydrate labeling and between 20 and 25% of the total TOC labeling (Fig. 5.1 and 5.2); clearly suggesting that wEPS was a major intermediate pool in the transfer of organic matter from diatoms to heterotrophic bacteria. Glucose showed the highest $^{13}$C-label incorporation among carbohydrates and its $^{13}$C-label incorporation was the highest at 4 h after labeling then almost disappeared at day 3 (Fig. 5.2C and D). Glucose is the first compound synthesized by phototrophic organisms and it is therefore not surprising it was the highest labeled compounds as also found in other studies (Bellinger et al., 2009). Bacteria can utilize high-molecular-weight compounds ($\geq 600$ Da) like wEPS only after they are split into low-molecular-weight compounds by a variety of extracellular enzymes, which may explain the slower labeling between day 1 and 3. There were also indications that diatoms were able to reabsorb some of the wEPS themselves, as the fast decrease in labeling in wEPS coincided with a secondary increase in diatom labeling until day 3 when almost no labeled DIC was left in the sediment. This suggests that diatoms may use wEPS as an external carbon storage as has also been shown for pure cultures of diatoms (de Brouwer and Stal, 2001).
We used both PLFA- and Mag-SIP to determine labeling dynamics in major microbial groups in the benthic diatoms mat. The PLFA content of bacterial biomass is relatively constant (Middelburg et al., 2000) compared to rRNA which allowed us to estimate excess $^{13}$C per unit of biomass with PLFA-SIP but not with Mag-SIP. With Mag-SIP, on the other hand, much higher phylogenetic resolution can be achieved than with PLFA-SIP and is only limited by the selection of specific probes (Miyatake et al., 2009; Miyatake et al., 2010). We found that labeling levels in rRNA and PLFA were in a similar range, although there are indications that labeling in rRNA was more dynamic than in PLFA (Fig 5.7). The rRNA pool in *Bacteria* is under active metabolic control and shows a rapid turnover in *Bacteria* (MacGregor et al., 2006), which may explain its slightly faster increase and decrease in labeling during the first day of the experiment. In contrast, MacGregor et al. (2006) compared PLFA- and Mag-SIP in a study where sediments were directly labeled with $^{13}$C-labeled organic substrates and showed higher and faster label incorporation in bacterial PLFA than in 16S rRNA. However, no correction for protocol blanks was applied to the Mag-SIP data in this study, which may explain some of the differences.

Interestingly, our results also suggest that all major groups within the heterotrophic bacterial community depended almost equally on diatom derived organic matter especially in during the first day of the experiment (Fig. 5.7). Labeling of rRNA captured by each probe were almost the same during the experiment (Fig. 5.7A). Only labeling in *Deltaproteobacteria* showed a slower labeling rate than other groups but only between day 1 and 5, which may be due to the utilization of fermentation products by this group of mainly sulfate reducing bacteria produced by other members of the community under anaerobic conditions (Miyatake et al., 2010). This mostly even labeling between different bacterial groups may be explained by a combination of diatom primary production dominating heterotrophic carbon cycling, a wide variety of exudates produced by diatoms (although our results indicate that glucose is most important) or a lack of specialization in the diverse heterotrophic bacterial community. For instance, Miyatake et al. (2010) have shown even utilization of glucose (and other organic substrates) by most major groups in microbial community in surface layer (0-2 cm) of an intertidal sediment in the same bay, suggesting limited specialization. This fast and even utilization by all members of the community suggests that the microbial community is either a closely coupled system or most major members grow at very similar rates.

The highest $\Delta\delta^{13}$C values of diatom PLFA, predominant primary producer at the study site, and bacterial PLFA were in almost the same range at 50-150 ‰ in this study. This is consistent with the study by Bellinger et al. (2009), but not with the studies by Middelburg et al. (2000) and Evrard et al. (2008) who showed approximately five-fold higher labeling in terms of $\Delta\delta^{13}$C values in diatom PLFA than in bacterial PLFA. Another difference between these studies is that Middelburg et al. (2000) and Evrard et al. (2008) showed a much faster decay in total labeling stocks that the other two experiments. A possible reason for this difference is that current experiment and the one by Bellinger et al. (2009) were
done earlier in spring when thick diatom mats are clearly visible on the sediment. In our study, macrofauna had not yet fully developed and there was therefore less grazing pressure on diatom mats. Hence, the ecosystem was in a climax situation with high biomass and limited growth, and a closer coupling between the diatoms and the heterotrophic bacterial community.

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Chapter 6

General discussion
A major challenge that microbial ecologists face is to identify which microorganisms carry out specific processes in natural environments. One of the most recent developments is a combination of stable isotope labeling (\(^{13}\text{C}, {^{15}\text{N}}\) etc) and tracing isotope levels into biomarkers generally referred to as stable isotope probing (SIP). Separation and stable isotope analysis of PLFA or nucleic acids (DNA or RNA) reveals both phylogenetic and functional information about the microorganisms responsible for the metabolism of a particular substrate. However, the traditional SIP methods have inherent limitations. In this thesis, I further developed and improved the Mag-SIP method in order to compensate for limitations in the traditional SIP methods. We applied the Mag-SIP method in combination with other methods in order to link microbial community structure to biogeochemical functions in marine sediments.

**Utility of the Mag-SIP method**

Each of traditional SIP methods has its limitations. For instance, PLFA-SIP provides high sensitivity in terms of the amount of \(^{13}\text{C}\) label needed (0.005 \% \(^{13}\text{C}\)), but the phylogenetic resolution offered is low and limited to major groups like *Bacteria* versus *Eukaryotes*. Specific biomarker PLFA have only been described for certain groups such as the sulfate reducing bacteria and methanotrophic bacteria (Boschker *et al.*, 1998). Although DNA- and RNA-SIP offer higher phylogenetic resolution than PLFA-SIP as they can be combined with clone libraries or various molecular fingerprinting techniques, they both require high levels of isotope incorporation into the nucleic acids (10-20\% \(^{13}\text{C}\)) as separation of labeled from unlabeled materials is based on density-gradient centrifugation (Manefield *et al.*, 2004). It was therefore worth developing a new method that has both higher sensitivity in terms of isotope incorporation than DNA- and RNA-SIP combined with better phylogenetic resolution than PLFA-SIP. The Mag-SIP method (MacGregor *et al.*, 2002) in principle offers these characteristics and we greatly improved the method by applying HPLC-IRMS for isotope analysis yielding a more sensitive method in terms of the amount of rRNA needed and by substantially reducing protocol blank. The current protocol offers a 0.005\% detection limit in terms of \(^{13}\text{C}\) incorporation into target rRNA and the phylogenetic resolution is only limited by probe design. In terms of the amount of rRNA needed, the method is sensitive enough to target 1\% of the microbial community in active marine sediments (Chapter 2; Miyatake *et al* 2009). It therefore provides higher phylogenetic resolution than PLFA-SIP and requires less label incorporation than DNA- and RNA-SIP thereby avoiding incubation biases (Neufeld *et al.*, 2007a). Moreover, improvement of the Mag-SIP in this thesis substantially lowered the amount of sediment sample needed (about 10 fold) compared to the original method (Chapter 2, Miyatake *et al* 2009) and enable us to use more specific probes which was limited to use of domain-specific probes in the original method (MacGregor *et al.*, 2002; MacGregor *et al.*, 2006). Overall, the improved Mag-SIP method is suitable for studies on substrate utilization, carbon uptake, and cross-feeding between members of the microbial community with
minimum incubation bias in laboratory or in situ studies. In addition, the method can be used to study natural abundance of carbon and possibly nitrogen stable isotopes in target nucleic acids, which can be used for instance to study carbon sources used by phylogenetic groups in natural ecosystems (Boschker and Middelburg, 2002).

However, there are a number of limitations with the Mag-SIP method. Although some prokaryotes maintain high numbers of ribosome during starvation (Flärdh et al., 1992), rRNA content per cell is generally strongly related to the growth rate and phase of the microorganisms (Kerkhof and Kemp, 1999; Moeseneder et al., 2005). Hence, the method tends to be biases for fast growing sub-population, and active but non-growing sub-population may be missed. Moreover, we still need relatively large amounts of rRNA for stable isotope measurement (approximately 500 ng) and the method is therefore mainly suitable for active and high-biomass ecosystem such as coastal sediments, biofilms and bioreactors (e.g. wastewater treatment plant).

We operated the HPLC-IRMS in bulk-injection mode without separating isolated rRNA from impurities in this thesis. A possible improvement of the method is therefore to combine Mag-SIP with a chromatographic HPLC-IRMS method that would probably further enhance sensitivity by further lowering protocol blanks. However, such a method would require a substantial amount of method development work as published HPLC methods for rRNA or rRNA nucleosides can not directly be transferred to the HPLC-IRMS because eluents must be carbon free (no organic buffers and solvents).

Further improvements lie in a more complete probe set tested with the Mag-SIP protocol. There are protocols available for Archaea and Eukarya (MacGregor et al. 2002), and we developed a nested probe set that covers approximately 75% of the bacterial 16S rRNA community in typical marine sediments. However, we still miss some major phyla such as the Bacteroidetes and the Planctomycetes, and the coverage of the probe set within major phyla could be improved. We were able to develop a specific probe for the family Desulfobacteraceae, typically found in marine sediment clone libraries. Based on 16S rRNA clone libraries, there are however still several dominant environmental phylotypes (3 clades) found within this family and other major groups in the Deltaproteobacteria such as the Desulfobulbaceae for which specific probes could be designed (Chapters 2, 3 and 5). The use of probes targeting 23S rRNA (Hunt et al., 2006) is another option since, due to its length, 23S rRNA has more options to design more specific probes and contains more carbon atoms per molecule than 16S rRNA. Other possible developments lie in the combination of Mag-SIP with novel single-cell methods such as fluorescence in situ hybridization (FISH) coupled with Raman microscopy (Huang et al., 2007) and in halogen in-situ hybridization (HISH) coupled with nano-scale secondary-ion mass spectrometry (nanoSIMS) (Musat et al., 2008). The Mag-SIP method provides information about activity of the whole microbial community or sub-populations, therefore, by combining with single-cell methods, we can get further insights on physiology and role of individual cells in the community. The Mag-SIP approach therefore is a very valuable asset to study relationships
between microbial community structure and functioning in a range of environments. The improved Mag-SIP method was subsequently used to study different aspects in the microbial ecology of intertidal marine sediments.

**Substrate utilization and carbon uptake of major groups in microbial community**

A study on organic substrate utilization (Chapter 3) revealed clear contrasts in label distribution of rRNA captured by the different probes in two depth horizons of a marine sediment. In the surface layer (0-2 cm), all substrates were evenly utilized by all major groups except that labeling was somewhat lower by *Cyanobacteria*, indicating limited specialization in substrate utilization. This even substrate utilization could be caused by the addition of relatively high amount of labeled substrates leading uncompetitive uptake or rapid cross-feeding among all members of the community. However, our results suggest that all major groups within the microbial community were at least capable of utilizing a variety of organic substrates. Limited specialization in diverse communities is often seen for instance in phytoplankton communities that often also show high diversity while only competing for a few limiting nutrients or light (e.g. Scheffer *et al.*, 2003).

In the lower sediment layer (2-5 cm), glucose consumption was dominated by *Gammaproteobacteria*, and sulfate-reducing *Deltaproteobacteria*, specifically the family *Desulfobacteraceae*, were important in acetate, propionate, and amino acid utilization. This is in agreement with the model of anaerobic mineralization where substrates like glucose are first fermented to organic acids and these fermentation products are then finally mineralized by sulfate reducing bacteria (Canfield *et al.*, 2005). Our results therefore suggest that, among the heterotrophic bacteria, the major active phylogenetic groups are also the main functional groups in sulfate-reducing marine sediment and broadly agree with the known physiologies in the Gamma- and Deltaproteobacteria as discussed in Chapter 3.

Glucose uptake and active growth of *Cyanobacteria* and diatoms in the lower layer (dark and anaerobic conditions) were also observed (Chapter 3). This result suggests that *Cyanobacteria* and diatoms may be able to survive for prolonged periods by utilizing glucose probably via fermentation under anaerobic conditions. Viability and growth of *Cyanobacteria* in dark anaerobic condition has also been observed in Baltic Sea sediment (Edlund *et al.*, 2008). However, although many *Cyanobacteria* are able to gain energy from fermentation of storage carbohydrates accumulated during photoautotrophic growth under anaerobic conditions, fermentation of external organic substrates in free-living *Cyanobacteria* is poorly documented (Stal and Moezelaar, 1997). Oxygen supply by bioturbation is one of the possibilities but it seems unlikely that this was the main cause. Numbers of macrofauna were low during the sampling that took place in May (results not shown). Further research is needed to understand the physiology of *Cyanobacteria* and diatoms under dark, anaerobic conditions.

In Chapter 4, anaerobic chemoautotrophy was investigated by studying dark-fixation of inorganic $^{13}$C into biomarkers in two different coastal sediments, one with free sulfide and
another with no sulfide detected. The proportion of anaerobic chemosynthesis rate to the total chemosynthesis rate was comparatively low when free sulfide was detected. The result of PLFA-SIP showed major difference in label distribution between the two sites clearly suggesting different groups of chemosynthetic bacteria were active. The Mag-SIP results further elucidated that sulfate-reducing \textit{Deltaproteobacteria} were more important players in anaerobic chemosynthesis than \textit{Gammaproteobacteria}. The results indicate that these sulfate-reducing \textit{Deltaproteobacteria} are probably capable of disproportionation of sulfur species (Rabus \textit{et al.}, 2006). Overall, we detected substantial anaerobic chemosynthesis in typical coastal sediments and therefore the importance of anaerobic chemosynthesis in coastal sediments should therefore receive more attention in future studies. Moreover, sulfate-reducing \textit{Deltaproteobacteria} are also known to use the reversed TCA cycle or the reductive Acetyl-CoA pathway for inorganic carbon fixation (Canfield \textit{et al.}, 2005) but these pathways have not been studied in any detail in coastal sediments. Studying the diversity and expression of key genes involved in these pathways by applying genomic or proteomic approaches may enhance our knowledge about this important process in future. It also seems likely the chemosynthetic and heterotrophic activities detected may reside in different abundant environmental phylotypes within the \textit{Deltaproteobacteria}, several of which were detected in our clone libraries (Chapter 2, 3, 4) and the same groups are commonly found in other studies on marine sediments (Edlund \textit{et al.}, 2008; Webster \textit{et al.}, 2006). This can be tested by developing new Mag-SIP probes for these commonly found environmental phylotypes.

\textit{Carbon pathways from diatoms to the heterotrophic microbial community}

Carbon flow from primary producers to the heterotrophic microbial community was traced in a marine intertidal sediment for five consecutive days (Chapter 5). Most of the photoassimilated carbon was exuded within a day in form of either low-molecular-weight organic compounds or wEPS. Both the PLFA and rRNA $^{13}$C-labeling data suggest that there was a fast transfer of label from diatoms to heterotrophic bacteria during the first 4 to 24 hours of the experiment, which was probably due to the exudation of low-molecular-weight organic compounds by diatoms that could be directly utilized by heterotrophic bacteria. After this initial fast transfer of organic matter, labeling of the heterotrophic bacteria proceeded at a slower rate until the third day of the experiment, which coincided with the degradation of wEPS-derived carbohydrates initially produced by the diatoms. After 4 to 12 hours, label recovered in wEPS explained 70% of the total sediment carbohydrate labeling and between 20 and 25% of the total TOC labeling; clearly suggesting that wEPS was a major intermediate pool in the transfer of organic matter from diatoms to heterotrophic bacteria.

Additionally, $\Delta\delta^{13}$C values of rRNA captured by each probe were almost the same during the experiment even though \textit{Desulfobacteraceae} comprised only 3% of the clone library. Most of photoassimilated carbon by diatoms was transferred to the heterotrophic
microbial community in form of glucose in colloidal carbohydrates. This even utilization by all members of the community suggests that the microbial community here is closely coupled system where most major members either grow at very similar rates or recycle carbon very fast. For future study, application of shorter sampling intervals and use of more specific probes are favorable. Despite $\Delta \delta^{13}C$ values of rRNA captured by each probe did not show clear difference in this study, it is evident from our results that combination of Mag-SIP and other methods is a useful tool to investigate carbon flows and to identify the responsible organisms in the environment.

Conclusions

We further developed and improved the Mag-SIP method by lowering detection limit ten-fold in comparison to the original published method. With the nested set of probes that was developed, we were able to measure $^{13}C$-isotope labeling in 75% of the microbial community with a detection limit of 1% target sub-population within the total active microbial community. Substrate utilization of all major groups in the microbial community in two depth horizons of marine intertidal sediment was successfully monitored by the Mag-SIP method. The importance of anaerobic chemoautotrophy rate in coastal sediments and responsible microbial groups were also identified in this thesis. Moreover, carbon flow from primary producers to the heterotrophic microbial community in marine sediment surface was elucidated by combining the Mag-SIP method with other techniques. Taken together, we demonstrated the utility of the Mag-SIP method in microbial ecology and identified the microorganisms that are responsible for a number of important processes in coastal sediments.
Summary
The aim of this thesis is to link microbial community structure and its biogeochemical functions by using 16S rRNA based stable isotope ($^{13}$C) probing (SIP) in marine sediments. The 16S rRNA gene has been used most widely as a marker to study microbial diversity and to show differences in community structure. However, 16S rRNA sequences alone provide little evidence about the physiology of microorganisms, and more importantly most of the sequences detected in these studies belong to uncultured organisms with unknown physiologies. Functional genes encoding for key enzymes associated with biogeochemical processes have provided more information on the role of certain microorganisms. However, in many cases it remains difficult to directly link physiology with phylogeny for environmental samples containing substantial numbers of uncultured species, and expression of functional genes is often poorly related to actual process rates. Our understanding of the functioning of microbial communities and the interactions between the different members therefore remains rather limited.

A method for the simultaneous identification and the metabolic capabilities of microorganisms is a combination of stable isotope labeling ($^{13}$C, $^{15}$N etc) and tracing isotope levels in biomarkers generally referred to as SIP. First, we further developed and improved a SIP method that uses oligonucleotide probes and paramagnetic beads in order to capture 16S rRNA from specific phylogenetic groups for stable isotope analysis (Mag-SIP). Compare to other traditional SIP methods, the advantage of this method lies in a combination of high phylogenetic resolution and low requirement of isotope incorporation into target microorganisms. We then applied the Mag-SIP method to study substrate utilization, anaerobic chemoautotrophy, and carbon transfer among major microbial groups in intertidal marine sediments.

Development and improvement of the Mag-SIP method were examined in Chapter 2. Application of µEA-IRMS provided higher sensitivity than EA-IRMS that was used in the original method. Furthermore, considerable efforts were made to substantially reduce the protocol carbon blank. Consequently, the improved Mag-SIP method required about 10 times less 16S rRNA for isotope ratio measurements than the original method. This allowed us to apply a nested set of probes with a stepwise narrowing of the target from Bacteria to Deltaproteobacteria and further to Desulfobacteraceae; a family that is thought to be the main group of sulfate reducing bacteria in marine sediments. Target specificity of each probe was examined by constructing clone libraries of captured 16S rRNA, and specificity of the final protocol was generally very good as more than 90% of the sequences belonged to the target group. The utility of the Mag-SIP method was demonstrated by studying substrate utilization of sulfate-reducing Deltaproteobacteria in intertidal, anoxic marine sediment. The results indicated that Desulfobacteraceae were important consumers of propionate but not of glucose.
Substrate utilization of major microbial groups in two depth horizons of marine sediment was investigated by adding five different $^{13}$C-labeled substrates in Chapter 3. The Mag-SIP method clearly showed contrasts in community characteristics and substrate utilization patterns between the two depth horizons. In the surface layer, all substrates were more evenly utilized by all major groups, indicating limited specialization at the phylogenetic level of this study. Whereas in the deeper layer, *Gammaproteobacteria* dominated glucose consumption, and sulfate-reducing *Deltaproteobacteria*, specifically *Desulfobacteraceae*, were relatively important in acetate, propionate, and amino acid utilization. The results for the deeper layer were consistent with known physiology of the major groups detected in clone libraries. Additionally the Mag-SIP method suggested that *Cyanobacteria* and diatoms may survive for prolonged periods under the anaerobic dark conditions by utilizing glucose, probably via fermentation. The fermentation of external organic substrates in free-living *Cyanobacteria* and diatoms is poorly documented.

Anaerobic chemoautotrophy rates and active bacterial communities were studied in two marine intertidal sediments in Chapter 4. Dark-fixation of $[^{13}$C]sodium bicarbonate was measured in aerobic and anaerobic incubations. The result of PLFA-SIP showed clear difference in label distribution between two sites suggesting different groups of chemoautotrophic bacteria were active. This was many due to differences in importance of anaerobic chemoautotrophy between the two sites. Anaerobic chemoautotrophy rates were comparatively low when free sulfide was detected. The Mag-SIP method further elucidated that sulfate-reducing *Deltaproteobacteria* were more important in anaerobic chemoautotrophy than *Gammaproteobacteria*. The results of Chapter 4 further suggest that anaerobic chemoautotrophy is an important process in typical coastal sediments, and should therefore receive more attention in future studies.

Carbon flow from benthic diatoms to the heterotrophic bacterial community was investigated in Chapter 5. An in situ pulse-chase experiment was done in which $[^{13}$C]sodium bicarbonate was sprayed onto the sediment surface during low tide and $^{13}$C-label incorporation in major carbon pools, intermediate metabolites, and biomarkers were monitored for five days. PLFA- and Mag-SIP were used to trace $^{13}$C-label incorporation in biomarkers. Diatoms were the predominant primary producer at the study site. The results suggest that there are two major carbon pathways from microphytobenthos to the microbial community, which have different turnover rates. Both the $^{13}$C-PLFA and $^{13}$C-rRNA data suggest that there was a fast transfer of label from diatoms to heterotrophic bacteria during the first 24 hours of the experiment, which was probably due to the exudation of low-molecular organic compounds by diatoms. After this initial fast transfer of organic matter, labeling of the heterotrophic bacteria proceeded at a slower rate until the third day of the experiment, which coincided with the degradation of water-extractable extracellular polymeric carbohydrates initially produced by the diatoms. Additionally, the patterns of
$^{13}$C-label incorporation of rRNA suggest that the microbial community at the study site is closely coupled system where most major members grow at very similar rates.

In conclusion, microbial community structure and its biogeochemical functions in marine sediments can be successfully linked by using the improved Mag-SIP method in combination with other approaches. In this thesis, we were able to provide detailed information on the microorganisms responsible for the utilization of major substrates in marine sediments. It was shown that a number of important groups in the microbial community played a broader role than expected. Some surprising results are that oxygenic phototrophs such as benthic diatoms and *Cyanobacteria* are apparently capable of surviving under dark, anaerobic conditions through glucose fermentation, and that sulfate-reducing *Deltaproteobacteria* are important players in the high anaerobic chemoautotrophy rates detected in intertidal sediments.
Samenvatting
Micro-organismen (bacteriën, microalgen etc.) vormen een zeer diverse groep in natuurlijke ecosystemen en spelen een centrale rol in vele processen in de elementencycli. Het doel van deze studie was om door middel van het labelen van 16S rRNA met het stabiele isotoop $^{13}$C ('stable isotope probing'; SIP) verbanden aan te tonen tussen de microbiële soortensamenstelling en de biogeochemische processen in kustsedimenten. In de microbiële ecologie wordt het 16S rRNA algemeen gebruikt om de diversiteit en soortensamenstelling van micro-organismen te beschrijven. Het merendeel van de 16S rRNA sequenties die in natuurlijke ecosystemen gevonden worden is echter afkomstig van micro-organismen die nog niet geïsoleerd en beschreven zijn. Er is daarom weinig bekend over het metabolisme van deze micro-organismen en daarmee over hun rol in biogeochemische processen. Functionele genen die coderen voor bepaalde enzymen geven meer informatie over de rol van micro-organismen. Het blijft echter moeilijk om een direct verband te leggen tussen een organismer een microbiële proces omdat er vaak geen eenduidig verband bestaat tussen genexpressie en processen. Er is daardoor weinig bekend over het functioneren van microbiële gemeenschappen en de interacties tussen micro-organismen onderling en met hun omgeving.

SIP, een verzameling van technieken waarbij biomarkers worden gelabeld met stabiele isotopen ($^{13}$C, $^{15}$N), biedt de mogelijkheid om tegelijkertijd micro-organismen te identificeren (de biomarker) en hun metabolische activiteiten te karakteriseren (label opname). In dit proefschrift is eerst een SIP-methode ontwikkeld die gebruikt maakt van oligonucleotide probes en paramagnetische bolletjes om het 16S rRNA van specifieke fysogenetische groepen te isoleren en om die vervolgens te analyseren op de isotopen samenstelling. Het voordeel van deze methode (Mag-SIP) ten opzichte van andere SIP methoden is de combinatie van een hoge fysogenetische resolutie en de mogelijkheid om lage hoeveelheden label te kunnen detecteren waardoor artefacten als gevolg van de toevoeging van gelabelde substraten worden vermeden.

De Mag-SIP methode werd vervolgens toegepast op het sediment van slikplaten in de getijdenzone om te onderzoeken welke groepen van micro-organismen betrokken zijn bij chemoheterotrofe en chemoautotrofe processen, en om de koolstofstromen tussen groepen van micro-organismen te meten.

In hoofdstuk 2 wordt de ontwikkeling en verfijning van de Mag-SIP methode beschreven. De isotopenanalyse van het geïsoleerde 16S rRNA werd verbeterd waardoor er ongeveer 10 keer minder 16S rRNA nodig is voor de isotopenmeting. Dit maakte het mogelijk om een gehele set van specifieke probes te ontwikkelen die een stapsgewijze verfinning van de fysogenetische groepen van de Bacteria, via de Deltaproteobacteria naar de Desulfobacteraceae mogelijk maakten. In kustsedimenten is de familie Desulfobacteraceae vermoedelijk betrokken bij de sulfaatreductie; het belangrijkste afbraakproces van organisch materiaal. De specificiteit van elke probe werd bepaald door het maken van kloonbibliotheek van het geïsoleerde 16S rRNA. De specificiteit van het
uitgebreide protocol was uitstekend omdat meer dan 90% van de klonen tot de doelgroep behoorden. De Mag-SIP methode werd vervolgens gebruikt in een studie naar de substraatopname door sulfaatreducerende *Deltaproteobacteria* in zuurstofloos kustsediment. Hieruit bleek dat propionzuur maar niet glucose een belangrijk substraat was voor de *Desulfobacteraceae*.

Substraatgebruik door de belangrijkste fylogenetische groepen van micro-organismen werd vergeleken in twee sedimentlagen van 0-2 cm en 2-5 cm diep (hoofdstuk 3). Kloombibliotheken en Mag-SIP resultaten lieten duidelijke verschillen zien tussen de twee sedimentlagen. In de toplaag van het sediment gebruikten alle onderzochte groepen micro-organismen de aangeboden $^{13}$C-gelabelde substraten. In de diepere zuurstofloze laag domineerden daarentegen *Gammaproteobacteria* de glucoseopname terwijl sulfaatreducerende *Deltaproteobacteria* en vooral *Desulfobacteraceae* betrokken waren bij de opname van azijnzuur, propionzuur en aminozuren. De oxygeen fototrofe micro-organismen *Cyanobacteria* en diatomeeën waren dominant aanwezig in beide sedimentlagen. Met de Mag-SIP methode werd aangetoond dat *Cyanobacteria* en diatomeeën in staat bleken om alle substraten te gebruiken in de toplaag van het sediment, terwijl ze in de diepere sedimentlaag mogelijk overleven door de vergisting van glucose. Vergisting van externe organische substraten door *Cyanobacteria* en diatomeeën is tot nu toe nog maar weinig onderzocht.

Anaerobe chemotrofie en de betrokken bacteriën werden bestudeerd in twee inter-getijde sedimenten (hoofdstuk 4). Donker fixatie van natrium$[^{13}$C]$\text{bicarbonaat werd gemeten in aerobe en anaerobe incubaties. Tussen de twee locaties werd met de PLFA-SIP methode een duidelijk verschil in de labelverdeling over de verschillende biomarkers gevonden. Dit suggereert dat verschillende chemotrofe groepen van micro-organismen op de twee locaties actief waren. Het verschil tussen de twee locaties werd vooral bepaald door het belang van anaerobe chemotrophy. In de aanwezigheid van vrije sulfide was anaerobe chemotrophy niet erg belangrijk. Vervolgens werd met de Mag-SIP methode aangetoond dat de sulfaatreducerende *Deltaproteobacteria* belangrijker waren voor de anaerobe chemotrophy dan de *Gammaproteobacteria*. De resultaten uit hoofdstuk 4 suggereren dat anaerobe chemotrophy een belangrijk proces is in kustsedimenten en daarom meer aandacht verdient in toekomstige studies.

**Hoofdstuk 5** behandelt de koolstofstromen van benthische diatomeeën naar heterotrofe bacteriën. In een in situ pulse-chase experiment werd natrium$[^{13}$C]$\text{bicarbonaat verdeeld over het sedimentoppervlak tijdens laag tij. Vervolgens werd gedurende 5 dagen de $^{13}$C-opname in de belangrijkste koolstofreservoirs, in intermediaire metabolieten en in microbiële biomarkers gevolgd. De inbouw van $^{13}$C-label in micro-organismen werd bestudeerd met de PLFA-SIP en Mag-SIP methoden. Diatomeeën waren de belangrijkste
primaire producenten en de overdracht van $^{13}$C-label naar heterotrofe bacteriën verliep via twee processen die zich onderscheiden door hun processnelheid. Zowel de $^{13}$C-PLFA als $^{13}$C-rRNA data suggererden een snelle overdracht van label van de diatomeeën naar heterotrofe bacteriën gedurende de eerste 24 uur. Dit was vermoedelijk het gevolg van de uitscheiding van laagmoleculaire organische stoffen door de diatomeeën. Deze aanvankelijke snelle overdracht werd gevolgd door een langzamer proces. Dit viel samen met de afbraak van water-extraheerbare extracellulaire polymere koolhydraten (EPS) afkomstig van diatomeeën. Uit de $^{13}$C-label incorporatie in het 16S rRNA bleek dat de microbiële gemeenschap in biofilms van diatomeeën een sterk verweven ecosysteem is waarin de meeste groepen vergelijkbare groeisnelheden hebben.

Met de in dit proefschrift beschreven Mag-SIP methode kunnen, in combinatie met andere technieken, de microbiële soortensamenstelling en biogeochemische functies aan elkaar gerelateerd worden. Hierdoor kon gedetailleerde informatie verkregen worden over de micro-organismen die betrokken zijn bij een aantal belangrijke processen in kustsedimenten. Van een aantal groepen werd aangetoond dat hun rol breder is dan algemeen werd aangenomen. Een aantal verrassende resultaten zijn ondermeer dat de fototrofe diatomeeën en Cyanobacteria zeer wel in staat zijn om ook onder zuurstofloze condities organische substraten op te nemen en dat in mariene sedimenten sulfaatreducerende bacteriën uit de Deltaproteobacteria belangrijke chemoautotrofe micro-organismen zijn.
要 約

海洋堆積物中の微生物群の構成とその生物地球化学的機能の特定

Mag-SIP 法の改良と応用
この学位論文の主题は、16S rRNA を基に安定同位体^{13}C を使った海洋堆積物中の微生物群の構成とその生物地球化学的機能の特定である。16S rRNA 遺伝子は、微生物の多様性と微生物群の構成の違いの研究に広く用いられている。しかし、16S rRNA 遺伝子自体からは微生物の生理機能に関する情報は得られず、生理機能がすでに知られている微生物の 16S rRNA 遺伝子を得ることはまれである。生理機能に介在する酵素の遺伝子は微生物の生理機能の特定についてより多くの情報をもたらしてくれるが、多くの環境試料中では微生物の分類と酵素遺伝子を照らせ合わせることは難しいとともに、酵素遺伝子の存在とその機能の発現が一致しないことが多い。したがって、我々の微生物群内のそれぞれのグループが持つ生理機能と相互作用に対する知識は未だ限られたものである。それを知る一手段として、近年、放射性同位体によるバイオマーカーの標識とその測定を用いた SIP 法が広く用いられている。我々はまず初めに、SIP の一手法である Mag-SIP 法の更なる開発と改良を行った。この方法の利点は、これまでの SIP 法に比べて系統学的精度と要求される同位体含有量の組み合わせが良いことである。さらに、この Mag-SIP 法と他の方法の組み合わせを用いて潮間帯海洋堆積物中の微生物群の物質代謝、化学合成独立栄養による炭素の取り込み、微生物間の炭素の移動に関する研究を行った。

第2章では、Mag-SIP 法の開発と改良に関する研究を行った。まず μEA-IRMS の採用により EA-IRMS を用いる旧来の方法より高精度な測定を可能としたともに、手法に潜在的に含まれる炭素量を減らすことにより、旧来の方法に比べて10分の1の試料での測定を実現した。我々はまた、細菌全体から Delta-proteobacteria、さらに Desulfobacteraceae へと段階的に選択性を狭めたプローブの組み合わせを用いた。それぞれのプローブの選択性の精度は、捕集物からクローンライブラリーを作成することにより確認され、90％以上と良好であった。Mag-SIP 法の実用性は、無酸素潮間帯堆積物中の硫酸還元性 Delta-proteobacteria の物質代謝の研究により検証された。これにより、Desulfo bacteraceae は堆積物中のブドウ糖の代謝には寄与していないが、プロピオン酸の代謝には大きく寄与していることが分かった。

第3章では、2層に分けられた海洋堆積物中の微生物群の5種類の安定同位体^{13}C で標識された物質に対する代謝について研究した。Mag-SIP 法は、二つの層の微生物群の特性と物質代謝の違いを鮮明に浮き彫りにした。表層では、全ての物質が全ての主だったグループによって同様に代謝され、限られた特異性が示唆された。一方その下の層では、Gammaproteobacteria が優先的にブドウ糖を代謝したのに対し、酵酸、プロピオン酸、アミノ酸は Delta-proteobacteria 特に
Desulfobacteraceae によって主に代謝された。この結果は、この章で作成されたクローニングライブラリーの構成から予測された微生物群の特性と一致している。また、Mag-SIP 法は藻類と珪藻類が暗い無酸素堆積物中でブドウ糖を多分醗酵により代謝しながら生存していることを示唆する。しかし、無酸素中での藻類の細胞外物質の醗酵についての研究は未だあまりなされていないのが現状である。

第 4 章では、化学合成独立栄養の割合とそれに関与する微生物群の研究を 2 箇所の潮間帯海洋堆積物中について行った。暗闇での$[^{13}C]$sodium bicarbonate の同化が、有酸素と無酸素の条件下での培養で測定された。全化学合成独立栄養に対する無酸素化学合成独立栄養の比率は、遊離酸素存在下において低かった。PLFA-SIP の結果は、2 章所における明確な標識同位体の分布の違いを示し、それぞれ違ったグループが化学合成独立栄養の活性を持つことを示唆した。さらに Mag-SIP 法は、硫酸還元 Deltaproteobacteria が無酸素の化学合成独立栄養において Gammaproteobacteria より重要な役割を担っていることを明らかにした。第 4 章の結果は、無酸素化学合成独立栄養が一般的な海洋堆積物中において今まで考えられていたより大きな役割を担っていることを示唆しており、今後の研究の進展が望まれる。

第 5 章では、基礎生産者から従属栄養微生物群への炭素の流れについて研究した。$[^{13}C]$sodium bicarbonate が堆積物表面に噴霧され、貯留炭素、中間生成物、生物指標化合物中への安定同位体の取り込みが経時的に測定された。生物指標化合物中への安定同位体の取り込みの測定には PLFA-SIP と Mag-SIP 法が用いられた。調査地点での主な基礎生産者は藻類で、詳細にわたって安定同位体の推移が記録された。以上の結果から、基礎生産者から従属栄養微生物群への速度の異なる二つの炭素の搬送経路が推察された。この速度の違いは、藻類の浸出物の組成の違いからくるものと考えられる。速い経路は低分子物質を介して、遅い経路は高分子物質を介するものと思われる。さらに、それぞれの主なグループの rRNA に取り込まれた安定同位体の結果から、主要グループは栄養的に互いに連携しており、ほぼ同じ速度で成長していることが示唆された。

総括すると、Mag-SIP 法と他の方法を組み合わせることにより、海洋堆積物中の微生物群の構成とその生物地球化学的機能は成功裏に特定された。我々はこの学位論文で、特定の物質の代謝に関わる微生物に関する詳細な情報を提供することに成功した。
References


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