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# **Chapter 1**

## **General introduction**

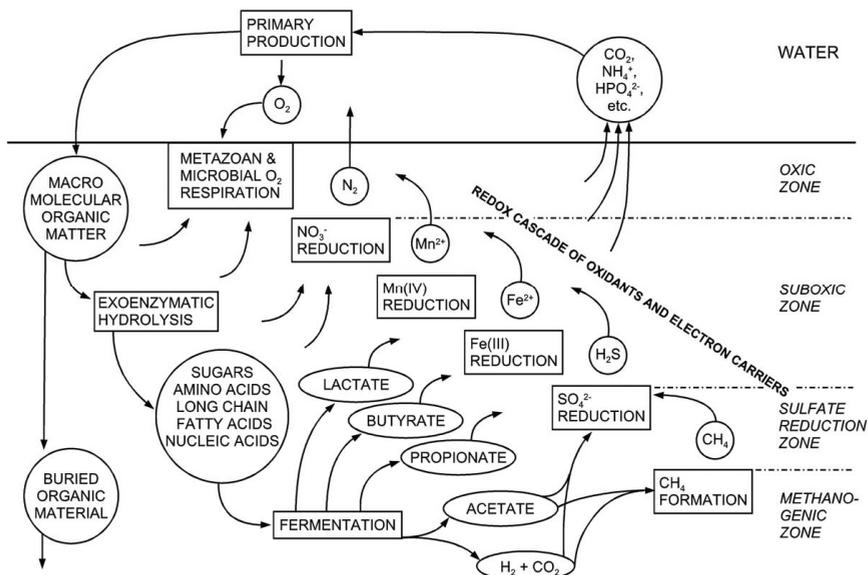
### ***Biogeochemical cycles driven by microbial communities***

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<sup>-1</sup> 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 (Bernier, 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.



**Figure 1.1.** Pathways of organic matter degradation in marine sediments and their relation to the geochemical zonations and the consumption of oxidants (Jørgensen, 2006).

### ***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). Epipellic 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  $\beta$ -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 (Bühning *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.

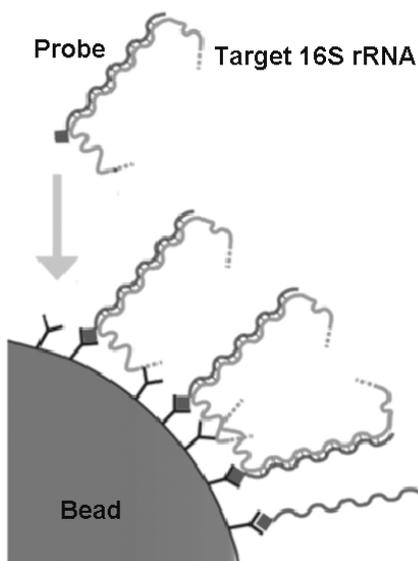
An elegant method for the simultaneous identification and the metabolic capabilities of microorganisms relies on isotope-based techniques. One of the more recent developments is in a combination of stable isotope labeling ( $^{13}\text{C}$ ,  $^{15}\text{N}$  etc) and tracing isotope levels in biomarkers 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  $^{13}\text{C}$  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’  $^{13}\text{C}$ -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 ( $^{13}\text{C}$ ) labeling methods to simultaneously identify microorganisms and their functions.

Method	Target	Isotope detection limit in target (% $^{13}\text{C}$ incorporation)	Proven phylogenetic resolution	Reference
FISH-Raman Spectroscopy	Single cell	5	Family/genus level	Huang <i>et al.</i> (2007)
HISH-nanoSIMS	Single cell	0.01	Family level	Musat <i>et al.</i> (2008)
PLFA-SIP	Community PLFA	0.005	Class level	Boschker <i>et al.</i> (1998)
DNA-SIP	Active community DNA	10-30	Species/strain level	Radajewski <i>et al.</i> (2000)
RNA-SIP	Active community RNA	10-30	Species/strain level	Manefield <i>et al.</i> (2002)
<b>Mag-SIP</b>	Community rRNA	0.01	Family level	Miyatake <i>et al.</i> (2009)

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 (Flårdh *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 ( $^{13}\text{C}$ ) 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

### ***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  $^{13}\text{C}$  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  $^{13}\text{C}$ -labeling approach was used and label incorporation into major carbon pools, intermediate metabolites, and biomarkers was traced for five consecutive days. Both the  $^{13}\text{C}$ -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.