In vitro study of microbial carbon cycling in subseafloor sediments

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Abstract

Over geological time, the majority of organic matter that escapes the internal cycling in the biosphere is deposited and preserved in marine sediments. Although the biodegradation of organic matter during early diagenesis beneath the seafloor has long been supported by geochemical evidence, it is only recently that the central parts of microbial carbon cycling, i.e. the microorganisms and their metabolic intermediates, have been intensively examined in subseafloor sediments.

Recent studies based on intact polar lipids (IPLs) contributed significantly to the proposition of a 'marine deep biosphere' dominated by live, heterotrophic archaea. In this study, an IPL-stable carbon isotope probing experiment was performed to evaluate the connection between sedimentary archaeal IPLs and benthic archaea. An analytical protocol was also developed to determine the isotopic composition of both the head groups and hydrocarbon chains of the archaeal glycolipids. Among the four ^{13}C-labeled substrates tested (bicarbonate, methane, acetate, and *Spirulina platensis* cells), only *S. platensis* cells resulted in significant labeling signals. The glycosidic headgroups exhibited stronger signals of ^{13}C incorporation than the hydrocarbon chains. These results suggest that marine benthic archaea are heterotrophic, and may generate IPLs via an anabolic shortcut that bypasses the energy-costly tetraether biosynthesis.

Hydrogen (H_2) is a metabolic intermediate that is poorly understood, although there has been a persistent interest in hydrogenotrophic processes in subseafloor sediments. In the present study, the first step to elucidate the H_2-fueled carbon cycling was to determine sedimentary H_2 concentrations with both the classical 'headspace equilibration technique' and a newly-developed extraction-based procedure. The H_2 concentrations obtained by both methods were orders of magnitude higher than the level predicted by thermodynamic calculations, and would be high enough to fuel some hydrogenotrophic trace volatile formation proposed in earlier studies. In the subsequent laboratory experiments, the supplementation of H_2 induced the formation of trace volatiles, mainly methylated sulfides. In the lake sediment, the formation of dimethyl sulfide by CO_2 reduction was found to be a biological process, whereas in marine sediments, the formation of thiols was an abiotic reaction. The carbon of the thiols was not from CO_2 but from another uncharacterized source.

Zusammenfassung

Der Hauptanteil des organischen Materials, welches sich den internen Kreisläufen in der Biosphäre entzieht, wird über geologische Zeiträume in marinen Sedimenten abgelagert und erhalten. Obwohl geochemische Hinweise schon seit längerer Zeit auf Biodegradation von organischem Material bei der Frühdiagenese unter dem Meeresboden hinweisen, wurde das Zentrum des mikrobiellen Kohlenstoffkreislaufs, also die Mikroorganismen und ihre metabolischen Intermediate, erst kürzlich im Detail in tief versenkten Sedimenten untersucht.

Neuere Studien basierend auf intakten polaren Lipiden (IPL) haben wesentlich zu unserem Verständnis einer 'marinen tiefen Biosphäre' beigetragen, welche von lebenden, heterotrophen Archaeen dominiert wird. In dieser Arbeit wurde ein Isotopenmarkierungsexperiment an stabilen Kohlenstoffisotopen von IPLs vorgenommen, um die Verbindung zwischen sedimentären intakten Archaeenlipiden und benthischen Archaeen zu untersuchen. Außerdem wurde ein analytisches Protokoll entwickelt, um die isotopische Zusammensetzung von beiden Teilen der archaeellen Glykolipide zu bestimmen: der Kopfgruppen und Kohlenwasserstoffketten. Von vier verschiedenen ^{13}C-markierten Substraten welche getestet wurden (Bikarbonat, Methan, Azetat und Zellen von *Spirulina platensis*) lieferte nur *S. platensis* ein signifikantes Markierungssignal. Die glykosidische Kopfgruppe hingegen zeigte ein wesentlich stärkeres Signal von ^{13}C Einbau als bei den Kohlenwasserstoffketten beobachtet. Diese Ergebnisse deuten darauf hin, dass marine benthische Archaeen tatsächlich heterotroph sind und dass ihre IPL-Synthese auf einer anabolischen Abkürzung basiert, welche die energieintensive Biosynthese der Tetraether umgeht.

Wasserstoff (H_2) ist ein bisher wenig verstandenes metabolisches Intermediat, obwohl intensive Anstrengungen unternommen wurden um die wasserstoffzehrenden Prozesse in tiefen Sedimenten zu verstehen. Als erster Schritt um den wasserstoffgetriebenen Kohlenstoffkreislauf zu untersuchen, wurden in dieser Arbeit Wasserstoffkonzentrationen in Sedimentproben mit zwei verschiedenen Techniken bestimmt: der klassischen 'Gasraum-Equilibrationsmethode' und einer neu entwickelten extraktionsbasierten Methode. Die Wasserstoffkonzentrationen die mit beiden Methoden bestimmt wurden waren mehrere Größenordnungen über den von thermodynamischen

Berechnungen vorhergesagten Mengen und wären groß genug um wasserstoffzehrende Prozesse anzutreiben die flüchtige Spurenbestandteile bilden und welche von vorherigen Studien vorhergesagt wurden. In den folgenden Laborexperimenten zeigte sich, dass die Zugabe von H_2 die Bildung von flüchtigen Spurenbestandteilen—hauptsächlich von methylierten Sulfiden—förderte. In einem Seesediment wurde die biologische Bildung von Dimethylsulfid durch CO_2-Reduktion gefunden, wohingegen in marinen Sedimenten die Bildung von Thiolen eine abiotische Reaktion darstellt. Der Kohlenstoff der Thiole stammte dort nicht von CO_2, sondern von einer anderen, bisher uncharakterisierten Quelle.

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Chapter 1
Introduction

The present-day habitable marine sediments are estimated to contain 400×10^{18} g of organic carbon (Lipp et al., 2008), a pool that is about one order of magnitude larger than the sum of carbon in the reactive surficial reservoirs (Killops and Killops, 2005). In the context of the global organic carbon cycle, marine sediments play an important role in linking the biosphere and lithosphere. They represent the major sink for materials that leak from the biological carbon cycle, and the deposited organic matter leads to the formation of natural gas, petroleum, coal, or metamorphic forms of carbon (Rullkötter, 2006). The amount of organic carbon hence stored in sedimentary rocks is estimated to be 1.5×10^{22} g C and represents 20% of major carbon reservoirs on Earth (Hedges and Keil, 1995; Falkowski et al., 2000).

However, marine sediments, even those under anoxic conditions, do not act as a loyal conveyor of organic materials from the biosphere to the lithosphere. Complex, low-temperature processes, collectively termed diagenesis, are taking place to alter organic matter during the early stages of burial. There are multiple lines of evidence suggesting that biological agents are mainly responsible for diagenetic transformation. In oxic sediments, the involvement of organisms is straightforwardly supported by the presence of benthic detritus feeders, such as protozoa (e.g., Gooday, 1993). In anoxic sediments, geochemical evidence provided the hints of biodegradation. For example, it was found that the potential of an organic compound to be degraded in sediment is generally proportional to their water solubility (see Table 4.5 in Rullkötter, 2006). Macromolecules that are easily hydrolyzed to water-soluble monomers are also poorly preserved. These are attributable to a better accessibility of water-soluble compounds and the macromolecules to microorganisms and their hydrolytic enzymes (Middelburg et al., 1993). The other important line of evidence comes from studies on redox species in anoxic sediments. Studies in the 1970s demonstrated that the oxidized chemical species, such as NO_3^-, Mn(IV), Fe(III), SO_4^{2-}, and CO_2, are transformed into their reduced forms in anoxic sediments with a characteristic vertical zonation (Froelich et al., 1979). Since organic matter is the major electron donor in marine sediment, the reduction of these chemical species has been interpreted to be an

expression of microbial terminal electron-accepting processes coupled to organic matter oxidation. The question is then, what is the dimension and composition of this anaerobic microbial biota, and its link to the geochemical observations?

A Marine Deep Biosphere in Subseafloor Sediments: A Mini-review and Open Questions

The questions raised by the geochemical studies led to the progress of geomicrobiology, which when combined with the classical geochemical approaches, formulates our understanding of the 'marine deep biosphere' in subseafloor sediments. There are already articles and one thesis available that broadly review the major findings of this rapidly developing research field (Parkes et al., 2000; Jørgensen and Boetius, 2007; Fry et al., 2008; J. S. Lipp, PhD thesis). Here, only the two issues relevant to this PhD project, together with the open questions left from previous studies, will be briefly summarized.

Issue 1: Who lives in sea floor?

Habitable marine sediments were estimated to contain $56 – 303 \times 10^{15}$ g of cellular carbon, accounting for 5-30% of the extant global biomass (Whitman et al., 1998; Parkes et al., 2000; Lipp et al., 2008). Experimental data show that except for a few cases (e.g., Parkes et al., 2005), the vertical distribution of cellular carbon is irrelevant to the zonation of redox species, but decreases simply with sediment depth (Parkes et al., 2000; Lipp et al., 2008). It is noteworthy that the reason for such a correlation between biomass and depth is not fully understood. Sediment age, a parameter that is indicative of organic matter quality and/or quantity, does no correlate as significantly with the content of biomass as depth (Parkes et al., 2000).

The community composition of such a tremendous subseafloor biota remains at issue. Interestingly, an agreement among the debates is that, despite the conspicuous geochemical gradients, typical microbial groups that are capable of undertaking terminal electron-accepting processes constitute only a minor fraction of the subsurface population (Teske, 2006; Jørgensen and Boetius, 2007; Fry et al., 2008; Biddle et al., 2008). Regarding the major inhabitants in the

sea floor, the core of the debate is: Are Bacteria or Archaea the major living prokaryotes in the marine deep biosphere? Studies based on different approaches—and sometime even the same approach—reached different conclusions. Earlier studies employing quantitative, real-time polymerase chain reaction suggest the dominance of living Bacteria over Archaea (Schippers et al., 2005; Inagaki et al., 2006). In contrast, more recent studies applying the same method give the opposite conclusion (Biddle et al., 2008; Lipp et al., 2008). Methods involving hybridization techniques also yielded contradictory results (Mauclaire et al., 2004; Schippers et al., 2005; Biddle et al., 2006; Lipp et al., 2008). A molecular probe-independent method, intact polar lipid (IPL) analysis, declared the winner to be Archaea (Lipp et al., 2008). However, due to the lower phylogenetic resolution of these archaeal IPLs and their lack of distinct stable carbon isotopic signatures (Biddle et al., 2006), the controversy of Bacteria versus Archaea evolved into a new debate (e.g., Schouten et al., 2008; Lipp and Hinrichs, 2009): Do the archaeal IPLs in marine sediments represent living Archaea, or are they fossil remains from planktonic Archaea?

The research community responded to these controversies. On the one hand, researchers became aware of the selectivity of their methods (Teske and Sørensen, 2008; Lever et al., 2009) and the bias introduced during the extraction steps (Lipp et al., 2008; Lipp et al., 2009). It is time to go back and evaluate the error in individual protocols before revisiting the issue. On the other hand, the fuzzy nature of the boundary between live and dead for microbes, particularly those in low-energy environments, was reiterated (Pearson, 2008). A prokaryotic cell may be at different physiological statuses ranging from metabolic active to moribund while maintaining the same classes of biomolecules. Although the quantities and sometimes also the quality of certain biomolecules vary with physiological statuses, quantification of these biomolecules is never an easy task, and can lead to contradictory results. Additionally, in subseafloor sediments where the rates of enzymatic activity are expected to be extremely low, it is unclear how rapidly a biomolecule disintegrates after cell death and lysis. Therefore, to resolve the controversies and to substantiate the present picture of the marine deep biosphere, other ideas and methods are required.

Issue 2: Fermentation, H_2, and hydrogenotrophy

Although the typical microbial groups that are involved in terminal electron-accepting processes are only minor fractions of the whole community and were barely enriched in laboratory (Batzke et al., 2007; Parkes et al., 2009), the traces they leave, i.e., the concentrations of redox species (e.g., D'Hondt et al., 2002, 2004) and in a few cases also the stable isotopes (e.g., Milkov, 2005; Böttcher et al., 2006), are well documented by geochemical measurements. The rates of certain processes have also been extensively measured by radiotracer assays (e.g., Hoehler et al., 2000; Parkes et al., 2005). A strong contrast to this ever-growing knowledge of terminal electron-accepting processes is our poor understanding of fermentation in subseafloor sediments.

Microbiological work showed that there are fermentative prokaryotes in the marine deep biosphere. Small subunit ribosomal RNA gene sequences closely related to the phylogenetic groups containing fermenting bacteria have been detected in subseafloor sediments, although these sequences constitute only a minor fraction of the clone libraries (Inagaki et al., 2006). The result based on culture-independent method was further supported by successful enrichment or isolation of fermenting bacteria (Batzke et al., 2007; Parkes et al., 2009). On the other hand, fermentation is a trait that also exists in Archaea (e.g., Kengen et al., 1994). Based on the stable carbon isotopic signatures of archaeal biomass, Biddle et al. (2006) hypothesized that the benthic archaea, most being novel phylotypes without cultured representatives, are heterotrophic. Since sequences of the uncultured benthic archaea have been also detected in methanogenic zone (Fry et al., 2008), it is likely that some of them are capable of fermentation.

Designing a reasonable radiotracer assay for studying fermentative rates requires a knowledge on the type and concentrations of substrates (carbohydrates, amino acids, purines, pyrimidines, etc.), which are among the least studied compound classes in marine deep biosphere research. A better understanding has been obtained for the fermentative products, particularly volatile fatty acids (VFAs). Data of VFA concentrations in subseafloor sediments have been published since the 1990s, showing that the VFA concentrations are kept very low ($< 15\ \mu mol\ L^{-1}$) in non-hydrothermal sediments (Wellsbury et al., 1997; Parkes et al., 2007a). Higher concentrations of VFAs have been found in sediments under special geochemical conditions (Wellsbury et al., 1997; Lorenson et al., 2006; Heuer et al., 2009). With the recently developed

technique that allows online carbon isotopic determination of VFAs (Heuer et al., 2006), it is possible for the first time to infer the metabolic pathways that produce or consume VFAs, and to link the inferred metabolic variation to geochemical zonation (e.g., Heuer et al., 2009).

In contrast to VFAs, there is very little data available for the other two classes of fermentative products, dissolved alcohols and H_2, in subseafloor sediments. A main reason is that the conventional sample preparation procedures for volatile compounds, such as simple porewater analysis for VFAs and headspace analysis for hydrocarbon gases, do not suffice for successful determination of dissolved alcohols and H_2. H_2 is of broader interest than dissolved alcohols due to the insights it provides into bioenergetics (reviewed in Chapter 5). Unlike other analytical methods for gas, the 'headspace equilibration technique' (Lovely and Goodwin, 1988; Hoehler et al., 1998), a well-established method for H_2 determination in sediment samples, does not target the in situ gas pool but aims at the steady-state H_2 level reached under laboratory incubation of sediment samples. The theory underlying this method associates closely with thermodynamics. This method was applied to the study of H_2 concentrations in subseafloor sediment during the Ocean Drilling Program Leg 201 in 2002, but the results often deviated from the values predicted by thermodynamic calculations (D'Hondt et al., 2003). This raised the concern about the applicability of this method for subseafloor sediments. Since then, H_2 data in marine sediments have been barely published.

Despite the lack of knowledge on in situ H_2 concentrations, there is a persistent interest in hydrogenotrophic activities in marine subsurface sediments. In principle, all the anaerobic terminal electron-accepting processes can be coupled to H_2 oxidation, but the inventory from hydrogenotrophy cannot be distinguished from those coupled with organic electron donors. The only exception is microbial CO_2 reduction coupled to hydrogenotrophy, as the products are usually highly ^{13}C-depleted and can be easily discerned from those derived from non-hydrogenotrophic pathways (Whiticar et al., 1986; Heuer et al., 2006). This principle has been applied to explain the isotopic variations of CH_4 (e.g., Parkes et al., 2007a) and acetate (Heuer et al., 2009). Recently, the interest in hydrogenotrophic activities has been extended to trace volatiles. For example, Sassen et al. (2006) detected highly ^{13}C-depleted ethane in marine sediments and proposed biological ethane formation probably via a pathway similar to

hydrogenotrophic methanogenesis. In addition to ethanogenesis, Hinrichs et al. (2006) further proposed hydrogenotrophic propanogenesis based on field data and geochemical modeling. With the development of a new experimental approach (see below), it is now possible to test these hypotheses in vitro and to see if there are other undiscovered hydrogenotrophic pathways.

In vitro studies: The New Concept and New Method

In vitro studies hold the potential to solve some of the open questions outlined above. In the past decades, the types of in vitro studies that have been most often implemented to study the marine deep biosphere are enrichment or isolation of microorganisms (D'Hondt et al., 2004; Biddle et al., 2005; Batzke et al., 2007; Parkes et al., 2009), and heating experiments with sediment slurries to simulate the biogeochemical processes during burial (Wellsbury et al., 1997; Parkes et al., 2007b). Meanwhile, a new concept and a new method for in vitro studies have been proposed. The new concept reshapes our ways of designing laboratory experiments, whereas the new method provides a greater flexibility and opportunity than the traditional approaches.

New concept: Adaptations to energy stress dictate the ecology of the Archaea

The hypothesis proposed by Valentine (2007) provides an important theoretical framework not only for understanding the biochemical and ecological differentiation between Bacteria and Archaea, but also for formulating appropriate experimental strategies for studying these two prokaryotic groups. The author supported his contention of the low-energy adaptation as a characteristic of Archaea by (1) the structural features of archaeal lipids, which were interpreted to maintain lower membrane permeability to ions for a more effective proton motive force and a less energy loss, and (2) the endurance of Archaea to extreme environments. Under moderate environmental conditions, the competition between Archaea and Bacteria intensifies, but most isolated mesophilic Archaea are known to possess a singularity in their catabolism to exclude or out-compete Bacteria for low-energy availability.

In deep biosphere research, there has been a general difficulty in isolating Archaea—not only those novel phylotypes without cultured representatives and clues of metabolism, but also the well known ones such as methanogens. So far, there are only two strains of mesophilic methanogens that have been isolated from subseafloor sediment (Mickucki et al., 2003; Kendall et al., 2006). Parkes et al. (2009), when reflecting on their failure to enrich any Archaea even with the state-of-the-art high-pressure culturing system, referred to this ecological hypothesis and asked if the medium they used intrinsically led to discrimination of Archaea. If Valentine's hypothesis is true, to obtain signals from mesophilic, heterotrophic archaea, addition of substrates should be zero or minimized, but this does not necessarily lead to any measurable results. A compromise between the theory and the approaches employed in the existing cultivation procedures would be required.

New method: Stable carbon isotope probing

In 1998, Boschker and colleagues published the first paper describing an experimental technique that aimed at linking specific biogeochemical processes to microbial populations in environmental samples by adding ^{13}C-labeled substrates. In the following ten years, such a technique, now called 'stable (carbon) isotope probing (SIP)', has been broadly applied in environmental microbiology. SIP can be applied to study different compound classes: DNA (Radajewski et al., 2000; Gallagher et al., 2005), RNA (Manefield et al., 2000), protein (Jehmlich et al., 2008), lipid (Blumenberg et al., 2005; Bühring et al., 2006; Wegener et al., 2008), and carbohydrates (Glaser and Gross, 2005).

Different SIP techniques require different extents of microbial growth and therefore support different strategies of substrate addition and/or labeling time. There are three factors that determine the required extent of microbial growth for an SIP technique: the standing stock of carbon in the target cellular constituent, the turnover rate of the constituent, and the required ^{13}C incorporation for clear detection. Table 1-1 summarizes the carbon pool sizes in major cell constituents and the required ^{13}C incorporation for SIP experimentation. The turnover rates of these constituents may vary among different organisms at different growth stages, but in general, RNA, proteins and carbohydrates (when in the form of glycogen) have higher turnover rates than

Table 1-1. Major constituents and the corresponding carbon pool size in a growing bacterial cell. The required extent of ^{13}C incorporation for stable isotope probing of each cell constituent is also listed

Cell constituent	Percentage in the total cellular dry weight (%)[a]	Carbon content in each constituent (%)[b]	Carbon pool size relative to the total cellular carbon (%)[c]	Required ^{13}C incorporation for detection (%)[d]
DNA	3.2	36	2.6	50
RNA	15.7	34	12	20
Carbohydrates	16.6	40	15	0.06
Lipids	9.4	63	14	0.06
Proteins	52.2	47	56	1-2

[a] Data from Russell and Cook (1995).

[b] Estimation of carbon content in each cell constituent: DNA and RNA were estimated based on the average carbon content of four deoxyribonucleotides and four ribonucleotides, respectively. Polysaccharides were estimated based on the carbon content of neutral monosaccharide. Lipids were estimated based on the average carbon content of phosphodiacylglycerol with palmitic and stearic acids as the core lipids. Proteins were estimated based on the average carbon content of the 20 amino acids.

[c] The values were calculated using the data of the percentage of each constituent in the total dry weight and the corresponding carbon content of each constituent.

[d] Data origins: Radajewski et al. (2003) for DNA- and RNA-SIP, and Jehmlich et al. (2008) for proteins. Values for carbohydrates and lipids were calculated assuming a shift in stable carbon isotopic values from -25 to +25‰. Note that the values for nucleic acids are for the whole DNA and RNA pools, whereas the values for carbohydrates, lipids, and proteins are for individual monosaccharides, lipids, and enzymes, respectively.

DNA and lipids. To achieve the goal of minimal substrate addition during SIP experimentation, it would be ideal to target cell constituents that have a small standing stock of carbon and a high turnover rate, such as RNA. However, RNA-SIP is limited by the analytical constraint, which requires up to 20% ^{13}C incorporation for physical separation of labeled RNA from unlabelled ones in density gradients (Radajewski et al., 2003). In contrast, although lipids are not replaced as often as RNA in cells, the strength of lipid-SIP is greatly enhanced by the analytical approach. Furthermore, lipids convey considerable taxonomic information, making lipid-SIP more preferable than carbohydrate-SIP. Therefore, lipid-SIP is more practical for combination with the strategy of minimal substrate addition, and hence, holds greater potential for probing Archaea in subseafloor sediment.

In addition to linking metabolisms to organisms, the SIP can also be applied to trace precursor-product relationships of carbon-bearing compounds, particularly those with only trace amounts. Traditionally, precursor-product relationships have been studied either by monitoring the kinetics of reactants and products (e.g., Oremland et al., 1988), or with ^{14}C-labeled substrates

(e.g., Zinder and Brock, 1978). ^{13}C-labeled substrates were used in a few cases, but more as a method to support the radiolabeling results (e.g., de Graaf et al., 1996). The kinetics of minor compounds can be easily obscured in a complex system (such as sediment) in which many processes are taking place. Hence, a ^{13}C-labeling approach will provide a less ambiguous assignment of precursor-product relationships. Although not enabling accurate rate determination and being less sensitive than radiotracers, the ^{13}C-labeling approach has the advantage that the samples can be screened with isotope ratio mass spectrometers coupled to chromatographic instruments. With online isotopic analysis, minor compounds can be easily monitored without the need of a laborious and complicated scheme to separate them from the major compounds. For some minor compounds which have chemical properties almost identical to the major compound, an effective separation by wet chemistry is infeasible. In this case, SIP in combination with chromatographic separation is the best solution to study their dynamics.

Objectives of This Study

The overarching goal of this study is to obtain a better understanding of the microbiological and biogeochemical processes in the marine deep biosphere via laboratory experimentation. The major questions for the microbiology part are:

1. *Are the archaeal IPLs found in subseafloor sediments 'live proxies' for marine benthic archaea?*
   So far the proposition of an Archaea-dominated marine deep biosphere is largely based on IPL studies. However, the linkage between the sedimentary archaeal IPLs and marine benthic archaea is not straightforward and needs to be evaluated.
2. *What are the marine benthic archaea doing?*
   The hypothesis of 'heterotrophic benthic archaea' is also based on proxies, that is, the natural carbon isotopic signatures of archaeal whole cells and IPLs. An SIP experiment can provide an unambiguous and straightforward link of processes to organisms.

The major questions for the biogeochemistry part are:

1. *What are the H_2 concentrations in subseafloor sediments?*

   H_2 concentrations were poorly constrained in subseafloor sediments. The classical headspace equilibration technique has been applied in an early study, but the results were not always reasonable. There is a concern regarding the validity of the assumption embedded in this method.

2. *Are there any trace volatiles produced via CO_2 reduction coupled to H_2 oxidation?*

   Recent geochemical studies proposed hydrogenotrophic ethano- and propanogenesis, but these processes have never been demonstrated in vitro. It is likely that there are other undiscovered hydrogenotrophic pathways that lead to formation of trace volatiles.

The thesis is therefore divided into two parts, with the first part focusing on the microbiological issues and the second part in the biogeochemical problems. The three chapters in the first part are telling one long story from different angles. Chapter 2 reports the quality of the refrigerated, legacy whole-round-core samples collected during previous deep-sea drilling programs. It is this type of sample that was used to start the SIP experiment. Chapter 3 describes an analytical protocol which allows intramolecular stable carbon isotopic analysis of intact archaeal glycolipids. This new protocol was later applied in the IPL-SIP study. The following Chapter 4 presents the results of an IPL-SIP experiment targeting benthic archaeal lipids. The second part deals with the topic of H_2-fueled carbon cycling. Chapter 5 presents the development and evaluation of a new analytical method that aims at determining in situ H_2 concentrations in sediments. Chapter 6 reports the first attempt of this PhD project to study trace volatiles and their relationship to CO_2/H_2 in lake sediment. The substrate-product relationship has been established with labeling experiments. Chapter 7 is only a data report. A pronounced and extensive phenomenon of H_2-induced trace volatile production has been detected during heating experiments with marine sediments, although the substrate-product relationship was not fully understood. Finally, all the major observations are briefly reiterated in Chapter 8, together with future perspectives.

Part I

Archaea-dominated marine deep biosphere?

Chapter 2

Effect of storage conditions on archaeal and bacterial communities in subsurface marine sediments

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Abstract

We have studied the effects of slow infiltration of oxygen on microbial communities in refrigerated legacy samples from ocean drilling expeditions. Storage was in heat-sealed, laminated foil bags with a N_2 headspace for geomicrobiological studies. Analysis of microbial lipids suggests that *Bacteria* were barely detectable *in situ* but increased remarkably during storage. Detailed molecular examination of a methane-rich sediment horizon showed that refrigeration triggered selective growth of ANME-2 archaea and a drastic change in the bacterial community. Subsequent enrichment targeting methanogens yielded exclusively methylotrophs, which were probably selected for by high sulfate levels caused by oxidation of reduced sulfur species. We provide recommendations for sample storage in future ocean drilling expeditions.

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Introduction

In recent years, the exploration of the subseafloor biosphere has greatly expanded. However, drilling operations and core storage techniques were originally developed for geological studies and have recently been under scrutiny for their application to biological studies. The Ocean Drilling Program (ODP) and Integrated Ocean Drilling Program (IODP) have implemented a monitoring routine to detect the potential of drilling-related contamination of sediment and hard-rock with non-indigenous microbial communities; the monitoring results inform subsequent sample selection and analysis (Smith et al., 2000; House et al., 2003; Lever et al., 2006).

A typical workflow of sample handling and storage for microbiological analysis is as follows (D'Hondt et al., 2003; Tréhu et al., 2003). Soon after core retrieval, samples for microscopy are taken from the freshly cut core section end on the catwalk, fixed for cell counts or hybridization analysis (e.g., Parkes et al., 2000; Schippers et al., 2005), and stored at -20°C. Core sections are then transferred to a cold room where they are sampled at a higher resolution. Fresh sediments are either taken for cultivation work initiated onboard (e.g. Batzke et al., 2007) or stored as syringe subcores for further processing. Lastly, intact whole-round cores (WRCs) with both ends capped are either frozen at -80°C for nucleic acid and lipid analyses (e.g., Biddle et al., 2006; Sørensen and Teske, 2006; Biddle et al., 2008; Lipp et al., 2008), or kept refrigerated in anoxically packed trilaminate bags to keep anaerobic microbes alive. We here evaluate the suitability of the refrigerated, anoxically packed WRC samples for geomicrobiological studies.

The refrigerated, anoxically packed WRC samples are intended for shore-based work. Due to the constraints on deep-sea drilling intensity and limited ship space for scientific participants, only a fraction of the research is typically initiated onboard. The legacy WRC samples provide an opportunity for a broader scientific community to participate in the deep biosphere research. Their role as backup materials also enables further laboratory investigation to solve questions arising from novel findings. However, little is known about the storage conditions of the refrigerated, anoxically packed WRC samples, and if and how microbial communities change during storage. Studies on terrestrial deep subsurface samples already demonstrated that there is a general trend of increase in viable counts and decrease in microbial diversity after sample storage

(Hirsch and Rades-Rohkohl, 1988; Brockman et al., 1992; Haldeman et al., 1994, 1995). By performing short-term sample manipulation experiments to constrain the impact of sample handling, Rochelle et al. (1994) have shown that the bacterial community in submarine sediments could change greatly in a short amount of time, especially when abruptly exposed to oxic conditions. However, their study focused only on *Bacteria*, whereas marine sediments have ample archaeal populations (Biddle et al., 2006; Lipp et al., 2008) that might respond differently. Their short-term storage experiments can neither be directly extrapolated to the condition of long-term storage of ODP/IODP refrigerated WRC samples, which are usually stored for months to years.

It is the goal of the present study to address the issue of the storage conditions of the refrigerated WRC samples from multiple angles. First, the efficiency of the anoxically packed bags to maintain anoxic conditions over longer storage periods was assessed. We measured the oxygen content in the bags of refrigerated samples and evaluated the influence this oxygen has on sediment geochemistry. Second, the potential consequences on both archaeal and bacterial communities when the bags leaked were demonstrated by biomarker analysis. Factors contributing to the changes of biomarker profiles during storage were discussed. Lastly, one refrigerated WRC sample from a subseafloor depth of 153 m, contaminated by oxygen and with an altered biomarker profile, was used for enrichment of strict anaerobes. Through multiple approaches including analyses of biomarkers, 16S rRNA genes and genes indicative for physiological groups, we provide a detailed and quantitative analysis of the archaeal and bacterial community change. Results from the experiment demonstrate how the storage condition could change the output of enrichment, suggesting the need of improving the current procedure for archiving live sediment samples and validation of existing legacy WRC samples prior to initiation of experiments.

Materials and Methods

Sample collection

Sediment core sections were retrieved during ODP Legs 201 (Peru Margin and Eastern Equatorial Pacific, Jan-Mar 2002), 204 (Hydrate Ridge, Jul-Sep 2002), 207 (Demerara Rise, Jan-Mar 2003), IODP Expeditions 307 (Porcupine Basin, Apr-May 2005) and 311 (Cascadia Margin, Aug-Oct 2005). WRC samples were either frozen at -80°C within a few hours after recovery or kept at 4°C in N_2-flushed and heat-sealed laminated foil bags. Pairs of frozen and refrigerated WRC samples from ODP Leg 204 and IODP Expedition 311 were taken within a difference in core depth of 0.5 to 1 m. Given the usually monotonous distribution of microbial communities in the sediment columns at the scale of hundreds of meters (Inagaki et al., 2006; Lipp et al., 2008), such a slight difference in sediment depth of paired WRC samples is considered negligible with respect to of the comparability of results. Materials for later analyses were taken aseptically from core interiors (at least 1 cm away from core liners).

Geochemical analysis

Gas analysis of the bag headspace was performed during Oct and Nov, 2007. A better accessibility to refrigerated WRC samples from IODP Expedition 311 allowed for a larger sample size than for the other cruises. To seal the package against ambient air during and after sampling, a self-adhesive septum was placed at the spot on the bag where the gas sample will be taken. By stitching the needle through the septum, 50-200 μL of headspace gas was withdrawn into a glass syringe pre-flushed with helium. Separation of oxygen from other permanent gases was attained by two independent Hewlett-Packard 6890 gas chromatographs equipped with either a homemade packed molecular sieve 5Å column (80 cm × 1/8”, 60-80 mesh, silicosteel) or a molecular sieve 5Å capillary column (25 m × 0.320 mm, film thickness 30 μm) and with helium as the carrier gas. A thermal conductivity detector was used in both instruments to quantify the oxygen signal.

To prepare samples for sulfate determination, bagged core sections were transferred into a glove bag flushed with nitrogen for one hour and opened. Redox strips were utilized to confirm that the atmosphere in the glove bag remained anoxic during sampling. Aliquots of sediment were transferred to pre-weighed Hungate tubes and mixed with defined volumes of anoxically prepared, sulfate-free artificial seawater. Sediment slurries sat for 2-4 hours at +4°C to allow

particles to settle down. Supernatant (300 μL) was taken by syringes with hypodermic needles and immediately fixed with 600 μL of 5% zinc acetate solution. The fixed samples were stored at -20°C until analysis. For determination of sulfate concentration by the photometric method (Tabatabai, 1974), fixed samples were centrifuged at 15,500 × g, and only the supernatant was used for analysis. Sediment dry weight was determined to calculate sulfate concentration in porewater.

Contents of total organic carbon (TOC) were determined on decalcified sediments after acidification of ~4 g of freeze-dried sediment with 6 N HCl. Dried residues were analyzed on a Leco CS200 analyzer.

Biomarker analysis

Intact polar lipid (IPL) analysis, a quick method allowing semi-quantitative and simultaneous determination of bacterial and archaeal lipids (e.g., Sturt et al., 2004; Rossel et al., 2008), was initially applied to characterize microbial communities of samples refrigerated for four months and for two years. Wet sediments from ODP Leg 204, freeze-dried sediments from IODP Expedition 311, and the methanogenic enrichment (see below) were analyzed. IPLs were extracted using a modified Bligh and Dyer method in four steps as described previously (Sturt et al., 2004). Before extraction, a known quantity of 1-*O*-hexadecyl-2-acetoyl-*sn*-glycero-3-phosphocholine was added as the internal standard to all samples. Total lipids were extracted four times with 2:1:0.8 (v/v/v) methanol/dichloromethane/buffer, where the buffer was 50 mmol L^{-1} phosphate at pH 7.4 in the first two steps (targeting bacterial cells) and 5% (w/v) trichloroacetate in the final two steps (targeting archaeal cells). After sonication for 10 min, the extraction mixture was heated up to 70°C using an automated microwave-assisted extraction system (MARS-X, CEM, USA), and centrifuged at 800 × g for 10 min. The combined supernatants were washed with water, and the organic phase was subsequently evaporated to dryness. A fraction of the total lipid extract was analyzed using high-performance liquid chromatography techniques described previously (Sturt et al., 2004). Because samples were analyzed only in the positive ionization mode, different core lipids types for phospholipids, i.e., diacyl glycerol (DAG) lipids and acyl/ether glycerol (AEG) lipids, were not distinguished. IPL concentrations were first

calculated from the peak areas of extracted mass chromatograms relative to that of the internal standard, followed by correction of ionization efficiency based on an external calibration series of commercial standards (Lipp et al., 2008). The detection limit was calculated on the basis of a minimum signal-to-noise ratio of three in the chromatogram, which corresponded to 10-50 ng IPL mL^{-1} sediment for ODP Leg 204 samples and 10-20 ng IPL g^{-1} dry sediment for samples from IODP Expedition 311.

Enrichment of methanogens

Refrigerated sediment from Sample 1328-152.7 (in this paper we used simplified sample codes containing only site-average depth in mbsf; the official codes of samples examined for post-storage sulfate concentration and IPL contents are listed in Table 2-1) was used to prepare sediment slurry in an anoxic glove box. The original sediment was mixed with approximately the same volume of anoxic, sulfate-free artificial seawater (pre-reduced by addition of 0.5 mmol L^{-1} $Na_2S \cdot 9H_2O$; Widdel and Bak, 1992). No trace elements and vitamins were added. Aliquots of 60 mL sediment slurry were dispensed into 120-mL serum vials that were sealed with butyl stoppers and crimp caps. Sediment slurries were supplemented with one of the three substrates to the following final concentrations: H_2/CO_2 (v/v=90:10), 200 kPa; sodium acetate, 0.8 mmol L^{-1}; methanol, 0.8 mmol L^{-1}. Stock solutions of the aqueous substrates were prepared anoxically using the Hungate technique (Widdel and Bak, 1992). Each treatment was performed in duplicate. In addition to the autoclaved control, one flask without substrate amendment was used as the negative control. The sediment slurries were incubated at room temperature (21°C) with regular hand-shaking. Methane concentration in the headspace was measured using a ThermoFinnigan Trace GC Ultra gas chromatograph equipped with a flame ionization detector. Amended sediments were selected for further enrichment only when the methane concentration was significantly higher than that of the negative control after the first six months. Since only the methanol-treated sediment gave a clear response, we flushed the vial headspace to remove existing methane, added methanol to a final concentration of 0.8 mmol L^{-1}, monitored methane concentration until it leveled off, and repeated the procedure again. After five additional cycles of methanol treatment, the solid phase was harvested for IPL and molecular analyses.

Table 2-1. Oxygen contents in sample bags and sulfate concentrations from shipboard and shore-based measurements of some refrigerated samples from IODP Expedition 311 (after 708 days of storage). The samples for IPL analysis are also listed.

ODP/IODP sample code *Leg/Expedition-* Site and Hole-Core-Section, interval (cm)	Depth (mbsf)	O_2 content (%)	Shipboard SO_4^{2-} data[a] (mmol L^{-1})	SO_4^{2-} analyzed after storage[b] (mmol L^{-1})
IODP 311-				
1325B-1H-4,120-130	5.7	0.07	0.1	0.0
1326D-7X-2, 65-80*	138.2	0.08	1.7	1.7
1326D-19X-1, 56-71	252.8	0.05	0.0	4.5
1327C-3H-3, 55-70	19.2	20.5	0.0	16.4
1327C-19X-4, 55-70*	155.2	1.87	0.0	5.7
1327C-27X-4, 50-65	223.2	0.06	0.1	1.0
1328B-1H-3, 65-80	3.7	0.04	0.0	0.0
1328C-1H-5, 75-90	63.2	0.04	0.0	0.0
1328C-6H-7, 67-82	100.7	20.6	0.0	18.5
1328C-12X-3, 80-95*	152.7	20.5	0.2	15.3
1328C-20X-4, 70-85	230.3	0.05	1.0	0.0
1328C-26X-3, 90-105*	287.5	0.05	0.5	ND[c]
1329C-3H-4, 115-130	23.3	0.13	0.0	0.0
ODP 204-				
1249F-8H-1, 30-55*	30.7	ND	0.5	ND
1249F-10H-4, 75-100*	54.1	ND	1.3	ND
1250E-1H-1, 80-110*	0.80	ND	>1.7	ND
1251E-1H-1, 90-125*	0.95	ND	27.1	ND

[a] Analyzed by ion chromatography (Riedel et al 2006).
[b] Analyzed by the photometric method (Tabatabai 1974).
[c] Not determined.
* Used for IPL analysis.

Analysis of 16S rRNA and methyl-coenzyme M reductase A (mcrA) genes

Samples were taken from the 4°C, -80°C and enriched sub-samples of Sample 1328-152.7 and all were stored at -80°C prior to analysis. DNA was extracted from 5 g of each sample using the PowerSoil DNA extraction kit (MoBio Inc., Carlsbad, CA), according to manufacturer's instructions. Genes were amplified by PCR using the following primer sets and annealing temperatures: archaeal 16S rRNA genes, A21F/A915R, 58°C (DeLong, 1992); bacterial 16S rRNA genes, B8F/B1492R, 58°C (Teske et al., 2002), *mcrA* genes, mcrIRDF/mcrIRDR, 55°C (M. A. Lever, PhD thesis). PCRs were performed with 1 μL DNA for 25 cycles when amplifying the 16S genes of the enriched and 4°C samples. For the -80°C-sample, PCRs were performed with 5

μL DNA for 25 cycles for the archaeal 16S rRNA gene sequences; despite using 15 μL DNA for 35 cycles, amplifications with bacterial primers did not yield any product. The *mcrA* genes were amplified using 1 μL for 25 cycles for enriched and 4°C samples. The -80°C sample required using 5 μL DNA and a touchdown PCR cycle (ranging from 60-55°C for 20 cycles, then annealing at 55°C for 25 cycles). All PCR products were cloned using the TOPO-TA system (Invitrogen, Carlsbad, CA). Positive colonies were directly sequenced using M13 primers by Genewiz, Inc (Plainfield, NJ). Sequences were cleaned and joined using Sequencher (GeneCodes Corp, Ann Arbor, MI). Alignments were made in CLUSTAL-W (Thompson et al., 1994) and neighbor joining trees were made using MEGA-4 (Kumar et al., 2008). Sequences are deposited in GenBank under accession numbers GQ869574-GQ869642.

Quantitative PCR was performed using the mcrIRD primer set and 1, 5 and 10 μL DNA as template. All concentrations were run in duplicate and all data per sample were averaged together to report gene copy number and standard deviation per gram (dry weight) of sediment extracted. Plasmid was extracted from a clone in the aforementioned experiment and used as a positive control. Reactions were amplified on a MX3500P (Stratagene, La Jolla, CA) using QuantiFast SYBR green PCR kit (Qiagen, Valencia, CA) as per manufacturer instructions.

Statistical analysis

The significance of a difference in biomarker composition between paired frozen and refrigerated samples was tested using a *t* test with α set at 0.05.

Results and Discussion

Storage conditions of the anoxically packed samples

Refrigerated WRC samples from ODP/IODP cruises for shore-based microbiology work were packaged under N_2 gas in heat-sealed bags made of a lamination of film foil and polyethylene (Cragg et al., 1992; D'Hondt et al., 2007), much like bags used to package coffee

beans. The permeability (P) of a film to a gas is defined by the flux (F) of the gas through the film multiplied by the thickness (x) of the film and divided by the difference in partial pressure over the film (Δp) (Crank, 1975):

$$P = F \frac{x}{\Delta p} \qquad (1)$$

The flux is the amount of gas (n) passing through a membrane per area (A) and per time (t):

$$F = \frac{n}{A \times t} \qquad (2)$$

P can be therefore expressed as:

$$P = \frac{n \times x}{A \times t \times \Delta p} \qquad (3)$$

Although the exact permeability of the packing material used in ODP/IODP is not fully constrained, similar plastic films have an oxygen permeability of 0.009-0.010 mL cm m^{-2} d^{-1} bar^{-1} at 30-60% relative humidity and room temperature (Cragg et al., 1992; Hansen et al., 2000). These permeability data enabled us to calculate the amount of oxygen permeated into the bag after one year using equation (3) by assuming a 10-cm core section (diameter = 6.7 cm) packed in a welded bag with a film thickness of 132 μm and a membrane area of 405 cm^2 (length 23.3 cm × width 8.7 cm × 2 sides). If the volume of the bag headspace was equal to the core volume, the infiltrated oxygen would be less than 0.5% (v/v). The inclusion of oxygen scrubbers should further delay the oxygenation of the samples (Cragg et al., 1992).

To test whether the bags actually maintain this type of low oxygen headspace, we conducted a gas survey of WRC samples which had been taken and preserved in bags at 4°C (Fig. 2-1). In 52 samples, taken between 2002 and 2005, 32 bags were found to contain greater than 19% oxygen. Five additional bags contained between 1-19% oxygen and 15 bags contained less than 1% oxygen. Only limited amount of samples (from ODP Leg 207 and IODP Expedition 307) contained Anaerocults (Merck Ltd.) to additionally scavenge oxygen, yet bags from these expeditions were still found to contain oxygen.

Since the tested samples were retrieved from anoxic sediment, we investigated what geochemical effects these oxidations might have. Sulfate concentrations of 12 sediment samples below the sulfate-methane transition zone at the five sites drilled during IODP Expedition 311

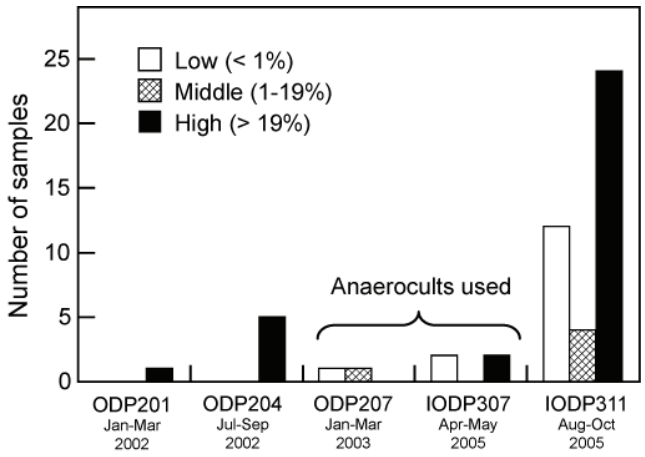

Figure 2-1. Oxygen content in the bags of refrigerated samples from selected ODP and IODP expeditions in 2002 to 2005. The samples were analyzed during Oct and Nov, 2007.

were analyzed. According to shipboard measurements, the highest sulfate concentration in these samples was 1.7 mmol L^{-1}, an exceptionally high value very likely due to drill fluid contamination (Riedel et al., 2006). The increase of sulfate concentration in samples containing <1% oxygen is negligible except in Samples 1326-252.76 and 1327-223.19, while 5 mmol L^{-1} and 16-18 mmol L^{-1} of sulfate were found in moderately and highly oxygenated samples, respectively (Table 2-1). This is an indication that oxygen has not just infiltrated the bags, but also interacted with the sediment. Since the concentration of dissolved sulfide was generally low in the samples examined (Riedel et al., 2006), the massive increase in sulfate is likely the consequence of pyrite oxidation by oxygen (Singer and Stumm, 1970). With the aid of ferrous ion, which is oxidized by oxygen to Fe^{3+} that attacks pyrite more effectively than oxygen, pyrite oxidation can take place abiotically. Other processes that can also contribute to sulfate formation, such as disproportionation of sulfur compounds, can not be ruled out. A detailed investigation of the geochemistry of sediment oxidation is beyond the scope of this study, but it has been demonstrated that other chemical species can also be influenced (e.g., Kraal et al., 2009), resulting in an environment similar to sediment at the oxic/anoxic interface.

These results indicated that the anoxically packed samples from scientific ocean drilling cruises were not stored in a satisfying way as expected. If the packing material does have the

stated low permeability of oxygen at 4°C that will allow only <0.5% oxygen in the bags after one year, we anticipate an age-dependent gradual increase of oxygen content, which was not the case. Since most bags were packed in the same way in different cruises, it is very likely that the working procedure was not optimized for the packaging. For example, a few bags from IODP Leg 311 had oxygen contents between 1% and 19% (Fig. 2-1), suggesting insufficient flushing time. Furthermore, small wrinkles or contaminants such as grease or particulates in the seal area could significantly reduce seal strength and integrity (Hernandez et al., 2000). Mutual scratching of the sharp bag edges during transport can even cause visible damages on the bags. Alternative procedures for sample storage must be sought in the future in order to maximize the sample's chemical integrity and maintenance of indigenous microbial populations.

Bacterial versus archaeal IPLs under storage

To investigate how the drastic change in redox condition during sample storage influences the composition of microbial communities, refrigerated sediments with varying contents of headspace oxygen, sulfate and TOC were selected. For comparison, frozen sediments from the nearby sample depth were also analyzed. TOC contents of the frozen and refrigerated samples were generally comparable.

Microbial community change is firstly demonstrated by IPL compositions of sediment cores from ODP Leg 204, Hydrate Ridge (Fig. 2-2). The set of WRC samples used for IPL analysis is different from that used for oxygen measurement (Table 2-1; Fig. 2-1). The latter sample set has been stored for five years before analysis, whereas the former was refrigerated for only four months, a period not uncommon between initial shipboard sampling and arrival at the home laboratory. Once in the laboratory, subsamples for IPL analysis were taken and frozen at -20°C. There are no oxygen or sulfate data available for these refrigerated WRC samples, but onboard analysis indicated varying *in situ* sulfate concentrations (Tréhu et al., 2003). The measured TOC contents are 1-2%. IPL analysis showed that the bacterial lipid concentrations increased significantly from below detection limit in onboard frozen samples to up to two orders higher than detection limit in all refrigerated samples examined (P values for one-tailed *t* tests of all paired samples are <0.05; Fig. 2-1). Surprisingly, there was a simultaneous increase of diglycosyl

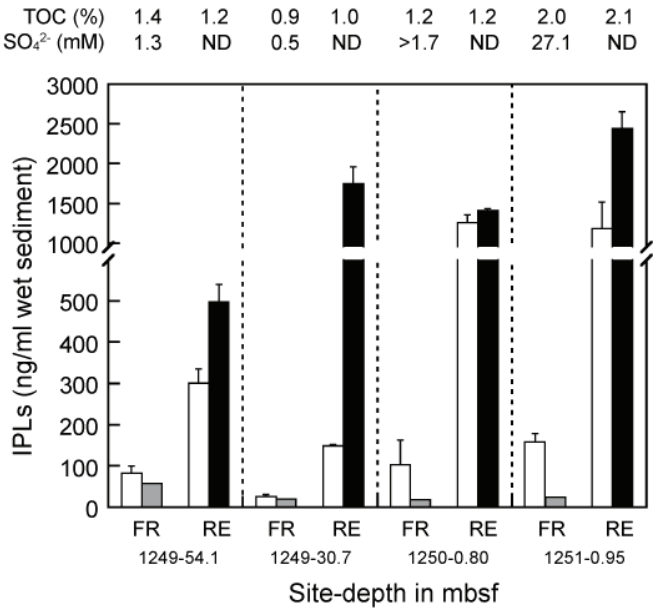

Figure 2-2. Changes of IPLs in subsurface sediments from ODP Leg 204, Hydrate Ridge, under different storage conditions. The refrigerated samples (RE) had been stored for four months before being sampled for IPL analysis. The contents of total organic carbon (TOC) and sulfate for frozen (FR) and refrigerated samples are listed. White: archaeal IPLs; black: bacterial IPLs; gray: limits of detection as estimates of bacterial IPLs. The error bars represent the standard error of measurements on duplicate extractions. ND: Not determined.

glyceroldialkylglyceroltetraethers (2Gly-GDGTs), the main archaeal IPLs found in marine subsurface sediment (Lipp et al., 2008). The increase of archaeal lipids was not as pronounced as for bacterial lipids but remains significant (P values of one-tailed *t* tests of all paired samples are <0.05).

The second set of subsurface sediments from IODP Expedition 311 was stored for two years (Fig. 2-3). The TOC contents are 0.4-0.8%, and the oxygen and sulfate concentrations of refrigerated samples were analyzed. When the oxygen content was low in the bags, as represented by Samples 1326-138.2 and 1328-287.5, the bacterial IPL contents are below detection limit in both frozen and refrigerated samples. In contrast, a conspicuous increase of bacterial IPLs was observed in the oxygenated, refrigerated samples (Samples 1327-155.2 and

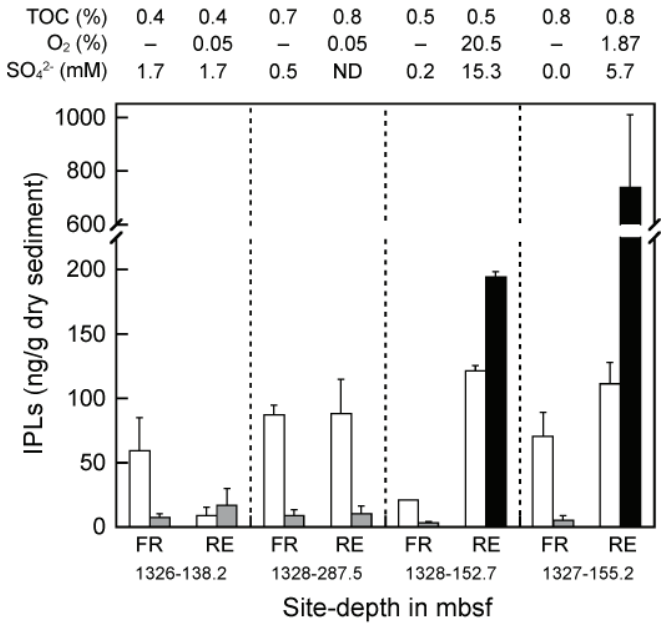

Figure 2-3. Changes of IPLs in subsurface sediments from IODP Expedition 311, Cascadia Margin, under different storage conditions. The refrigerated samples (RE) had been stored for two years before being sampled for IPL analysis. The contents of total organic carbon (TOC), oxygen and sulfate for frozen (FR) and refrigerated samples are listed. White: archaeal IPLs; black: bacterial IPLs; gray: limits of detection as estimates of bacterial IPLs. The error bars represent the standard error of measurements on duplicate extractions. ND: Not determined.

1328-152.7; P values for one-tailed *t* tests are both <0.05). The larger increase of bacterial IPLs in Sample 1327-155.2 compared to Sample 1328-152.7 is presumably attributed to the combined effect of oxygen availability and higher TOC content. Unlike the sediments from ODP Leg 204 (Fig. 2-2), the refrigerated samples from IODP Expedition 311 do not have higher archaeal IPLs content than the frozen ones (P values for one-tailed *t* tests are all >0.05), except in Sample 1328-152.7 (P=0.00, one-tailed *t* test).

It is highly unlikely that the prominent and consistent increase of bacterial IPLs results from the small offset of sample depths combined with a heterogeneous distribution of microorganisms for two reasons. First, Lipp et al. (2008) demonstrated that the *in situ* IPL pool in marine subsurface sediments is qualitatively monotonous, mostly dominated by archaeal IPLs. Second,

the paired WRC samples are not from sediment depths with steep geochemical gradients, which can lead to an abrupt change in lipid biomarkers (e.g., Orcutt et al., 2005). The best explanation is that *Bacteria* grew preferentially during sample storage, resulting in IPL profiles distinct from the *in situ* IPL pool that is dominated by archaeal membrane lipids (Lipp et al., 2008). Oxidation of sediment under the impaired storage condition provides part of the explanation for the observed IPL pattern, particularly in the deeper sediment samples from the IODP Expedition 311 (Fig. 2-3). In these samples, CO_2 becomes the primary electron acceptor, the energy yield of which is low compared to other catabolic processes. Valentine (2007) hypothesized that such a chronic energy stress is the main selective pressure that favors *Archaea* over *Bacteria*. When oxygen penetrated into the sample bags, it changed the redox condition, interacted with the sediment to form oxidized compounds that can be used as electron acceptors, and the energy stress was mitigated. Although we do not have the corresponding oxygen data for the IPL samples from ODP Leg 204, the marked increase of bacterial IPLs is a hint that the energy state may have changed after four months of storage. However, availability of new electron acceptors alone does not completely explain the quantitative change of the IPLs. For example, while Sample 1328-152.7 has a higher oxygen content in the bag headspace relative to Sample 1327-155.2, its bacterial IPL content is lower by a factor of 4. Other parameters that were not constrained in this study, such as the quality of organic matter and nutrient contents, may also influence the amount of biomass that could increase during storage.

Archaea responded to storage differently in these two sets of sediment. While in all the examined ODP Leg 204 samples archaeal IPLs increased after four months of storage, in the IODP Expedition 311 samples there are no significant changes in quantity except for Sample 1328-152.7. The higher TOC content and more labile organic matter in the shallower sediment of ODP Leg 204 may account for the difference, since the marine benthic archaea are proposed to be heterotrophic (Biddle et al., 2006) and their IPL contents appear to be broadly correlated with TOC (Lipp et al., 2008). Other reasons cannot be excluded, however, such as different archaeal species with varying doubling time at these two locations/depths. Strict factorial-design experiments are necessary to clarify the viability of these uncultured archaea and the factors controlling their growth.

Community change under storage and enrichment

To understand how the improper storage condition can affect the geomicrobiological studies performed on the legacy WRC cores, refrigerated Sample 1328-152.7 was used for enrichment of methanogens, a group of strict anaerobes. This sample was chosen because it originates from sulfate-free, methane-rich sediment, and the archaeal IPLs increased during storage are mostly archaeols (AR), a biomarker often affiliated with methanogens (Koga and Morii, 2005). Sediment samples with these conditions would be used for shore-based experiments concerning methanogenesis when the problem of oxygen infiltration is not recognized. After six months of incubation, methanogenesis was observed only in the methanol-amended sediment. Community composition in the frozen, refrigerated and the methanol-amended samples were characterized by analyses of IPLs (Fig. 2-4A; Table 2-2), detectable 16S rRNA sequences (Fig. 2-4B) and *mcrA* gene copy number (Table 2-2).

The archaeal IPLs show drastic compositional changes. In the frozen core, phosphatidyl archaeol (PA-AR) and 2Gly-GDGTs have equal proportions, while diglycosyl archaeol (2Gly-AR) becomes the predominant archaeal IPL under refrigerated and oxidative conditions. Abundant ARs with diverse head groups including phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidylglycerol (PS) and the appearance of hydroxyarchaeol (OH-AR) characterize the IPLs of this methanol-stimulated methanogen. PA-AR, found in the frozen sample, is less often reported for cultured archaea but is a major IPL of *Methanocaldococcus jannaschii* (Sturt et al, 2004). AR with sugar-head groups is a typical membrane lipid found in all major families of methanogens and many archaeal extremophiles (Koga and Morii, 2005), whereas OH-AR is a more specific marker for the families *Methanococcales* and *Methanosarcinales*, including the anaerobic methanotrophic archaea, ANME-2 and -3 (Rossel et al., 2008). AR combined with PE, PG, PI and PS can be each found in different lineages of *Archaea*; in combination they are present in members of *Methanosarcinales* (Koga and Nakano, 2008). GDGTs, the other type of archaeal lipids, occur extensively in different lineages of *Archaea*, but the uncultured benthic crenarchaeota are proposed to be the main producers of intact GDGTs in marine subsurface sediment (Lipp et al., 2008). GDGTs are also found to be the dominant IPLs in ANME-1 communities (Rossel et al.,

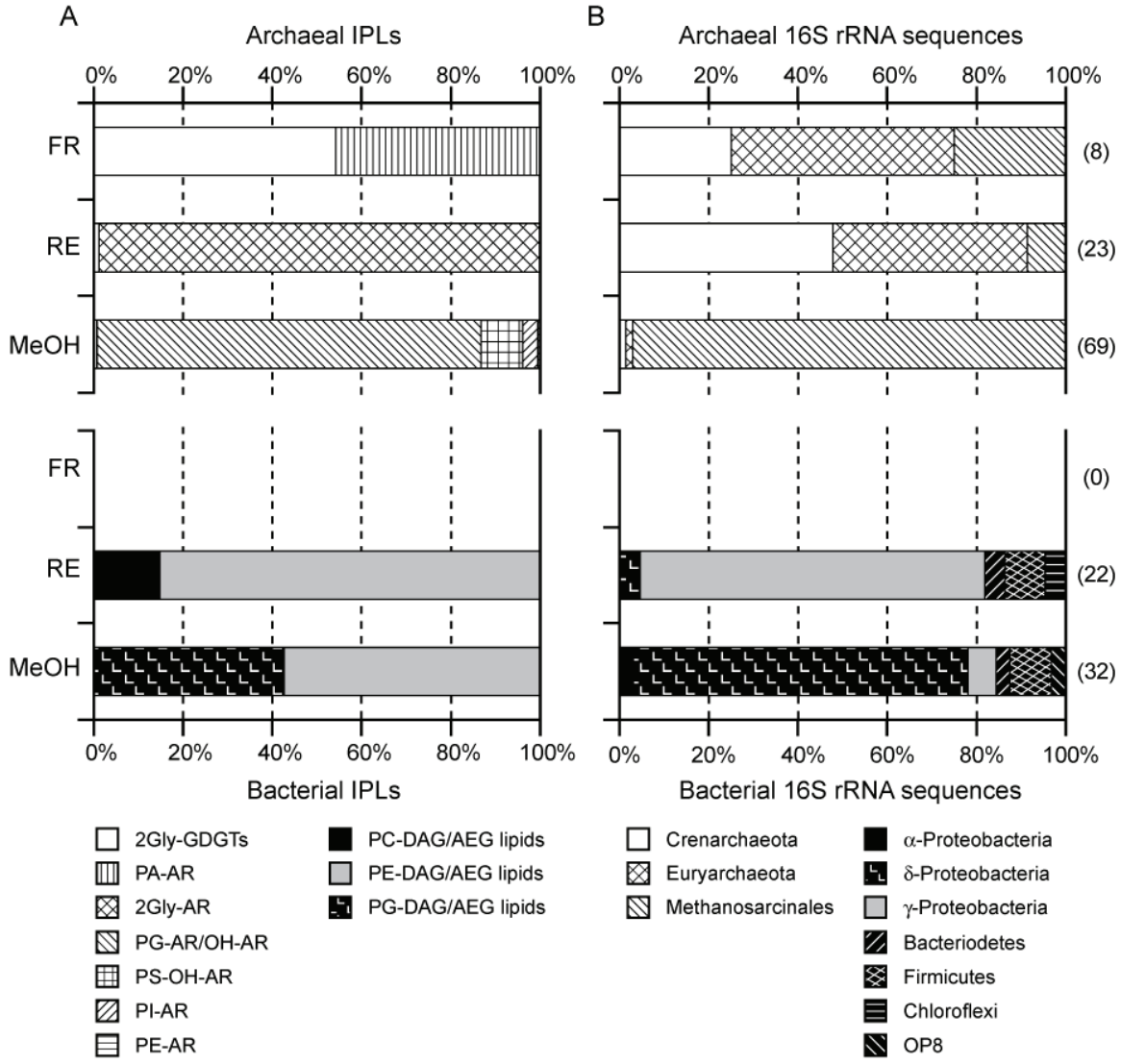

Figure 2-4. Comparison of microbial communities in subsurface sediment from Sample 1328-152.7 after different storage conditions or incubation with methanol at room temperature. FR: frozen onboard; RE: refrigerated. (A) Changes in archaeal and bacterial IPL composition. (B) Changes in 16S rRNA archaeal and bacterial clone library composition. The numbers in parentheses indicate the number of clones analyzed.

Table 2-2. Quantitative PCR detection of *mcrA* genes and intact polar archaeol and hydroxyarchaeol in Sample 1328-152.7 under different storage conditions and after incubation with methanol at room temperature. Numbers in parentheses are the coefficient of variation based on results of duplicate extractions. ND: not detected; n/a: not available

Treatment	Number of mcrA genes 10^3/g dry sediment		Intact polar lipids ng/g dry sediment			
			Archaeols		Hydroxyarchaeols	
Frozen at -80°C	16	(n/a)	10	(95%)	ND	(n/a)
Anoxically packed and refrigerated at 4°C	79	(12%)	121	(3%)	ND	(n/a)
Amended with methanol	1100	(42%)	798	(21%)	261	(23%)

2008). The differences of IPL diversity suggest a shift in active microbial populations, a change in the physiological status of the microbes, or the combination of both.

Signatures of 16S rRNA genes provide a complementary view on community change (Fig. 2-4B). At this taxonomic resolution, 16S rRNA sequences do not detect major differences in the archaeal population between frozen and refrigerated sediments, both of which contain sequences from crenarchaeota, euryarchaeota and *Methanosarcinales*. Apart from the taxonomic resolution, the apparent mismatch between IPL and 16S rRNA gene signatures could be a result of (i) differing turnover times of the pools of IPLs and DNA in combination with a relatively large pool of fossil archaeal DNA or, less likely, (ii) a shift in lipid distribution as a response of the *Archaea* to the changing chemical environment during sample storage. The methanol-amended enrichment shows dominant 16S rRNA sequences from a *Methanosarcinales* lineage, which agrees well with the IPL distribution.

To better characterize the active archaeal community, we selected *mcrA*, an indicator gene for methanogenic and methane-oxidizing archaea (Hallam et al., 2003; Friedrich, 2005), as the target for quantitative PCR analysis. Compared to the frozen sample, *mcrA* genes increased by 490% in the refrigerated sample, and by 7000% in the enrichment sample (Table 2-2). The shifts in contents of *mcrA* genes and archaeal-based IPLs (i.e., AR plus OH-AR) are comparable over three orders of magnitude. The cloned *mcrA* gene fragments were then sequenced to examine their phylogenetic relationship (Fig. 2-5). A phylogenetic progression is seen through the sample

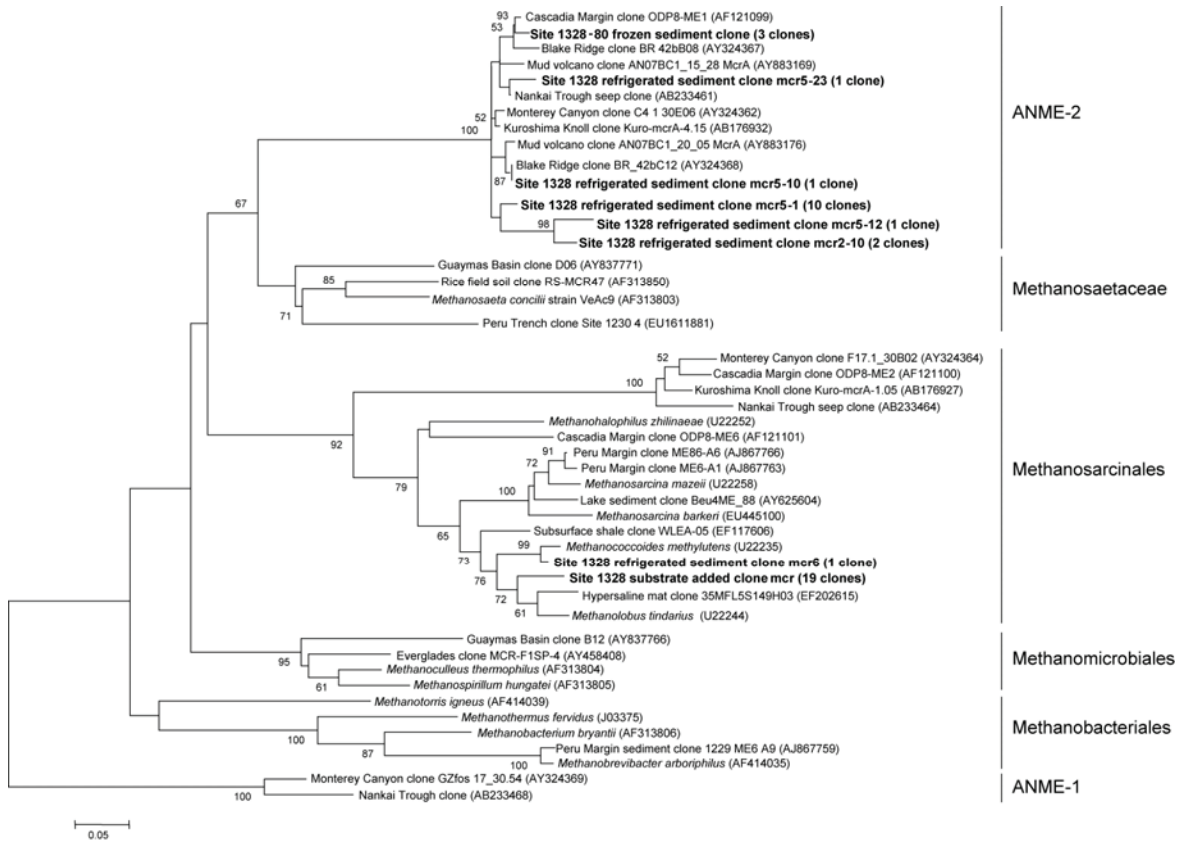

Figure 2-5. Phylogenetic tree showing fine-scale changes of detectable *mcrA* genes in Sample 1328-152.7 that was either frozen at -80°C, refrigerated at 4°C or incubated with methanol at room temperature. Detected genes are in bold. Bootstrap values over 50 are shown, 500 replicates were used.

storage conditions: A single ANME-2 phylotype was retrieved from the frozen sample; a greater diversity of sequences (ANME-2 and *Methanosarcinales*) was amplified from the refrigerated sample; the substrate-amended sample yielded a single phylotype that was related to *Methanolobus* and *Methanococcoides*, both methylotrophic genera of the *Methanosarcinaceae*. Based on the phylogenetic analysis and the quantitative PCR, the methylotrophic methanogens may have been rare sequences in the environment, but proliferated – even without substrate addition – during the period of refrigeration.

There is also a concurrent but less prominent shift of the bacterial IPLs. Starting with no detectable bacterial IPLs in the frozen sediment, DAG/AEG lipids with phosphatidylcholine (PC) and phosphatidylethanolamine (PE) headgroups appeared after sample storage, and finally PE- and PG-DAG/AEG lipids were the only detectable phospholipids in the methanol enrichment. Without precise information of core lipid structure, the taxonomic resolution of DAG/AEG lipids with phospho-head groups is quite limited, since these IPLs are broadly distributed in bacteria and eukaryotes. In contrast, a drastic difference is seen throughout the bacterial clone libraries (Fig. 2-6). The frozen sample had no detectable bacterial 16S rRNA signatures (Fig. 2-4B); no bacterial 16S rRNA clones were successfully retrieved from this sample despite multiple attempts. However, bacterial signals were amplified in the refrigerated and enriched samples. The bacterial community under refrigeration was dominated by 16S rRNA clones related to sulfur-oxidizing *Gammaproteobacteria* (17/22 clones). These organisms may be involved in the rapid rise in sulfate concentrations under storage. After substrate addition and incubation at 21°C, only a single of these gammaproteobacterial clones remained detectable (Fig. 2-6, Clone 10; 1/32 clones), but the majority of the library was now dominated by novel deltaproteobacterial phylotypes (24/32 clones) that did not appear in the library of the +4°C sample. *Firmicutes*, mostly clostridial relatives, were found in both clone libraries at lower abundance (Fig. 2-6).

Our enrichment experiment and the monitoring of community change demonstrated how improperly stored sediment can severely impair the cultivation output. Although low amounts of archaeol-based IPLs were already detected in the frozen sample, the archaeol IPL types in the refrigerated sample were structurally distinct and much concentrated. Surprisingly, such an enriched archaeol-bearing population is represented by ANME-2, which also exists in the frozen sediment (Fig. 2-5). The oxidation may have unintentionally created an environment ideal for anaerobic methane oxidizers – that is, methane-rich sediment plus sulfate derived from oxidation of reduced sulfur species. This population was lost in the subsequent incubation, which targeted methanogens rather than anaerobic methane oxidizers. The successful enrichment of a methanogenic phylotype related to *Methanococcoides* and *Methanolobus* species (Fig. 2-5) with methanol indicates that methanol-utilizing methanogens have survived the gradual oxidation of the sediment sample, the buildup of sulfate, and the growth of sulfate-reducing bacteria that outcompeted methanogens for acetate and/or hydrogen. In other words, the methanogen

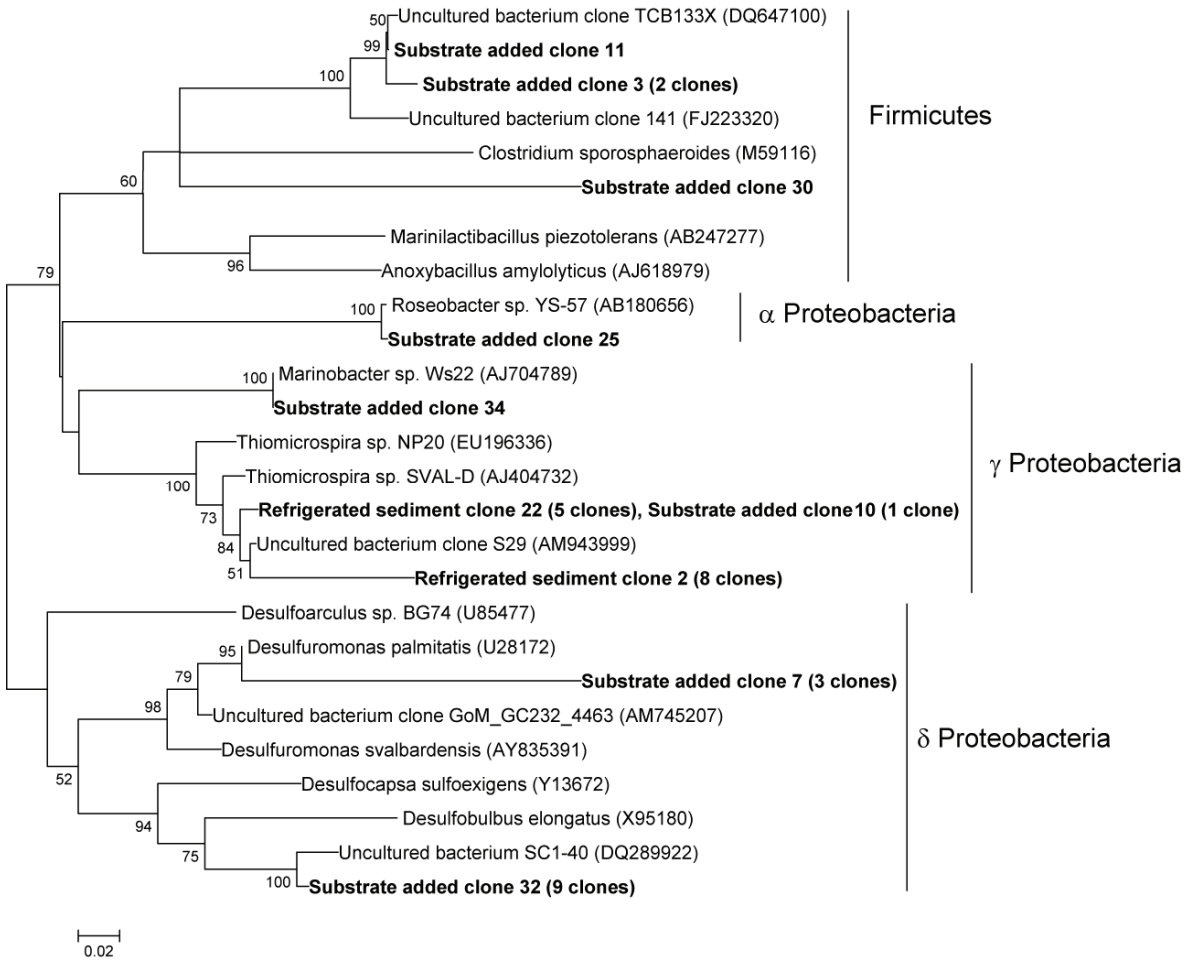

Figure 2-6. Phylogenetic tree of bacterial 16S ribosomal RNA genes detected in Sample 1328-152.7 that was either refrigerated at 4°C or incubated with methanol at room temperature. Detected genes are in bold. Bootstrap values over 50 are shown, 500 replicates were used. Not all bacterial clones are shown on the tree.

community has shifted towards genera that use methylated compounds, noncompetitive substrates that are not used by sulfate reducers or clostridial species (Oremland and Polcin, 1982). Therefore, the ability to recover strict anaerobes does not necessarily suggest that the samples have been stored appropriately.

Practical advice

For future scientific drilling expeditions in which the same packing procedures will be exercised, the quality of refrigerated WRC samples can be improved in two ways. First, the packing should be carefully implemented. Special attention should be paid to particles in the seal area. Wrinkles can be prevented by exerting tension in two perpendicular directions on the packing material during the heat sealing (Hernandez et al., 2000). Mutual scratching of the bags can be prevented by bubble wrap. Inclusion of oxygen scrubbers helps to improve but does not necessarily guarantee the maintenance of an anaerobic headspace (Fig. 2-1). Furthermore, the release of H_2, CO_2 and organic substances from oxygen scrubbers needs to be considered (Heizmann and Werner, 1989; Imhof and Heinzer, 1996). We also recommend including redox strips into the package to help users evaluate the storage condition. Second, the samples should not be left unattended for months and years. The gas phase and the oxygen scrubbers should be exchanged from time to time, even *en route* from the drilling vessel to home laboratories. One way to reduce the maintenance effort is to have packed WRC samples stored in groups in a bigger container such as an ammunition box (e.g., Musslewhite et al., 2007) and replace the gas therein. Otherwise, once in home laboratories, WRCs should be subsampled and stored in stoppered containers that are known to maintain the anoxic condition reliably for a longer period of time.

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Chapter 3

Intramolecular stable carbon isotopic analysis of archaeal glycosyl tetraether lipids

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Abstract

Glycolipids are prominent constituents in membranes of cells from all domains of life. For example, diglycosyl-glycerol dibiphytanyl glycerol tetraethers (2Gly-GDGTs) are associated with methanotrophic ANME-1 archaea and heterotrophic, benthic archaea, two archaeal groups of global biogeochemical importance. The hydrophobic biphytane moieties of 2Gly-GDGTs from these two uncultivated archaeal groups exhibit distinct carbon isotopic compositions reflective of the respective carbon sources. To explore whether the isotopic compositions of the sugar headgroups provide additional information on the metabolism of their producers, we developed a procedure to analyze the $\delta^{13}C$ values of glycosidically bound headgroups. Successful determination was achieved by (1) monitoring the contamination from free sugars during lipid extraction and preparation, (2) optimizing the hydrolytic conditions for glycolipids, and (3) derivatizing sugars into aldononitrile acetate derivatives, which are stable enough to withstand a subsequent column purification step. The aldononitrile acetate method gave sufficient isotopic accuracy for selected neutral monosaccharides that were later detected in environmental samples. First results of $\delta^{13}C$ values of sugars cleaved from 2Gly-GDGTs in one ANME-1 microbial mat and one sediment sample were obtained and compared with the $\delta^{13}C$ values of corresponding

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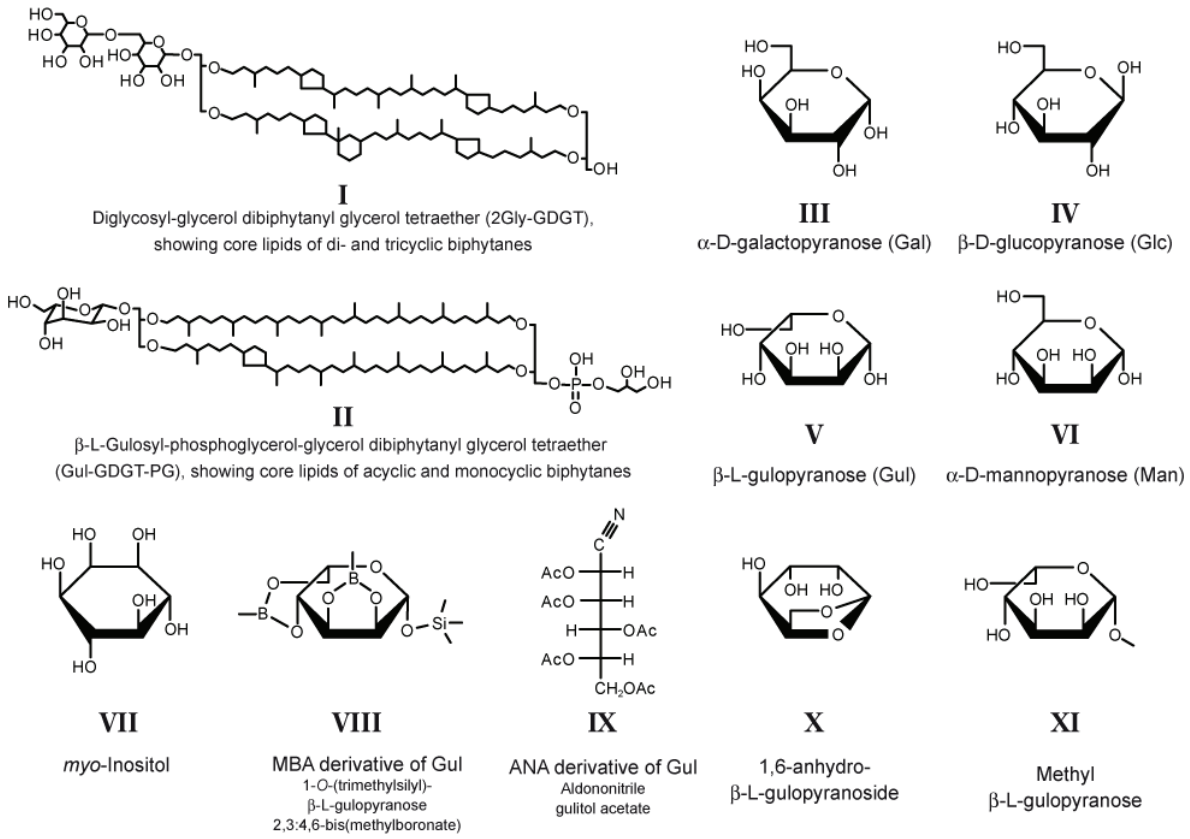
biphytanes. Sugar headgroups in the microbial mat are more ^{13}C-depleted than those in the sediment, but in both samples the dominant sugar headgroups have isotopic values heavier by 6-24‰ than the major biphytane. The varied extent of intramolecular isotopic difference in the two samples was tentatively explained by differences in carbon sources and biosynthetic pathways of the dominant archaea. Our results from archaeal glycolipids are in agreement with a general ^{13}C enrichment of carbohydrates compared to lipids. The analytical protocol, when combined with isotope labeling, can be a powerful tool to explore the turnover rates of different molecular moieties in uncultivated, glycolipid-producing archaea.

Introduction

Glycolipids are a structurally diverse group of membrane components with mono- or oligosaccharides as the polar headgroups. They are found in all domains of life. In eukaryotes, glycolipids are ubiquitous components and serve as major compounds in some specialized cells or organelles, e.g., nerve cells and chloroplasts (Curatolo, 1987). In prokaryotes, glycolipids are generally the predominant plasma membrane constituents in microbes without cell walls, e.g. mycoplasma (Shaw, 1970), and in those living in unusually harsh habitats, e.g. halophiles and thermoacidophiles (Curatolo, 1987). Recent studies on intact polar lipids (IPLs) in environmental samples further demonstrated the role of glycolipids as biomarkers for prokaryotes of global biogeochemical importance. In one study, diglycosyl-glycerol dibiphytanyl glycerol tetraethers (2Gly-GDGTs, **I**) were the major archaeal IPLs in a microbial mat dominated by ANME-1, an uncultivated archaeal group mediating anaerobic oxidation of methane (Rossel et al., 2008). The same type of glycolipids were also the major IPLs found in marine subsurface sediment (Lipp et al., 2008), where novel, uncultured and presumably heterotrophic benthic archaea are the main archaeal inhabitants (Biddle et al., 2006, 2008).

Despite the similarity in molecular composition, biphytanes moieties of 2Gly-GDGTs from the ANME-1 and benthic archaeal groups have distinct stable carbon isotopic compositions. While the benthic archaea have biphytane $\delta^{13}C$ values ranging from -17 to -37‰ (Biddle et al., 2006), negative $\delta^{13}C$ values ranging from -62 to -91‰ were reported for the same lipids in



Scheme 3-1. Structures of glycolipids, monosaccharides, polyol, and sugar derivatives mentioned in the text. MBA: methyl boronic acid; ANA: aldononitrile acetate.

ANME-1-dominated environmental samples (Aloisi et al., 2002; Blumenberg et al., 2004; Niemann and Elvert, 2008). These variations in lipid isotopes were generally explained by the difference in carbon source, namely, ^{13}C-depleted methane for ANME-1 and sedimentary organic carbon for benthic archaea. In contrast to the growing body of isotopic data for biphytanes, little is known about the δ^{13}C values of the sugar moieties of glycolipids, and the relationship between the lipid and carbohydrate carbon. Biochemical studies already showed that the biosynthetic pathways for lipids and carbohydrates diverge at an early stage, and generation of the respective precursors, acetate and pyruvate, varies among different carbon assimilation pathways (White, 2007). Therefore, the distinction of isotopic compositions of these two carbon pools holds great potential to provide deeper insights into the isotopic discrimination effects of the underlying enzymatic processes and to reveal information pertinent to decoding metabolic details (cf. Hayes,

2001). Additionally, such an intramolecular carbon isotopic analysis provides the unique opportunity to access both carbon pools in uncultured microorganisms, whereas simple comparison of polysaccharide-derived sugars and lipids, as has been practiced in pure culture studies (e.g., van der Meer et al., 2001; van Dongen et al., 2002), is not suitable for environmental samples because the former compounds are not specific biomarkers and do not enable linkage of isotopic information to particular organisms. Since both the ANME-1 and benthic archaeal groups have not been cultured, intramolecular carbon isotopic analysis of their 2Gly-GDGTs will allow simultaneous access to their carbohydrate and lipid pools.

Various techniques have been developed to analyze the carbon isotopic composition of monosaccharides by isotope ratio mass spectrometry (IRMS) coupled to either gas chromatography (GC) (Guerrant and Moss, 1984; Moers et al., 1993; van Dongen et al., 2001) or liquid chromatography (LC) (Krummen et al., 2004; Boschker et al., 2008). We focused on adaptation of the methyl boronic acid (MBA; van Dongen et al., 2001) and aldononitrile acetate (ANA; Guerrant and Moss, 1984) derivatization techniques for the compound-specific isotopic analysis of lipid-derived sugars. The former technique has the lowest carbon addition during derivatization and is consequently the candidate for maximum accuracy (van Dongen et al., 2001). The latter, when compared to the full alditol acetate method (Moers et al., 1993), has less carbon addition, is less labor-intensive, has good applicability with amino sugars (Glaser and Gross, 2005), and yields equally stable derivatives.

In biomass, sugars exist mostly in the form of polysaccharides, glycoproteins or glycolipids. Acid hydrolysis is necessary to recover monosaccharides for GC analysis. Satisfactory hydrolytic procedures exist for polysaccharides (e.g., Selvendran et al., 1979), and the hydrolytic conditions for glycoproteins have also been studied (Neeser and Schweizer, 1984). For glycolipids, mild methanolysis is the typical method to cleave off glycosidic headgroups (e.g., Jahn et al., 2004; Schouten et al., 2008). However, methanolysis is known to result in multiple products for one individual sugar (Neeser and Schweizer, 1984), leading to complex chromatograms, loss of taxonomic information, and increased detection limits for IRMS. Given the low proportion of sugar carbon per molecule of glycolipids, a systematic investigation on the hydrolysis of

glycolipids to optimize sugar recoveries is required for a practicable protocol for isotopic analysis.

Besides the hydrolytic procedure, there are two additional problems that need to be constrained for isotopic analysis of glycosidic headgroups. First, the bias originated during lipid extraction needs to be monitored. The extraction procedure employed for IPL analysis involves a moderately acid extraction step (cf. Sturt et al., 2004). It is unclear if glycosidic bonds are cleaved during this step, and if they are, whether the released monosaccharides will enter the organic phase called total lipid extract (TLE). Second, the sugar derivatives have to be purified from the polar matrix with which glycolipids coexist. Such a step is expected to help reduce the interference from the polar matrix for GC analysis.

The goal of this work is to develop an analytical procedure for stable carbon isotopic analysis of sugar headgroups from glycolipids in environmental samples. The analytical tasks are defined as follows: (1) Monitoring the presence and release of non-lipid monosaccharides during sample extraction and preparation prior to acid hydrolysis of glycolipids, (2) optimization of the hydrolytic conditions, and (3) purification of sugar derivatives. Both the MBA and ANA methods were applied to evaluate their compatibility with the hydrolysis and purification steps. The developed protocol was applied to assess intramolecular isotopic distribution of glycolipids from an ANME-1-dominated microbial mat from the Pakistan margin and a marine subsurface sediment sample from the Cascadia margin. Our results provide an understanding of isotopic discrimination during the biosynthesis of sugars and lipids in uncultivated archaea.

Experimental

Standards and environmental samples

L-Gulosyl-phosphatidylglycerol-glycerol dibiphytanyl glycerol tetraethers (Gul-GDGT-PG, >95% pure; **II**), the main polar lipids of *Thermoplasma acidophilum* (Swain et al., 1997), was purchased from Matreya, LLC (USA). Monosaccharides (**III**-**VI**) and myo-inositol (**VII**) with

purity >99% were purchased from Acros Organics (USA) or Sigma-Aldrich (Germany). The sediment sample was retrieved from a subsurface sulfate-methane transition zone at Hydrate Ridge, Cascadia margin (ODP 204-1245D-2H-3, 5-40 cm, 8.1 meter below seafloor; Tréhu et al., 2003). Molecular analysis showed that the main archaeal groups belong to the Marine Benthic Group B and South African Gold Mine Euryarchaeotic Group (J. F. Biddle and A. Teske, unpubl. data). The microbial mat sample was recovered from a cold seep at the Pakistan margin (*Meteor* M74-3, GeoB 12353, 1-2 cm; Bohrmann et al., 2008). Its IPL distribution is typical for ANME-1 archaea (cf. Rossel et al., 2008).

Lipid extraction and column separation

A schematic overview of the analytical procedure is provided in Fig. 3-1. In Step 1, wet environmental samples (ca. 50 g sediment and 6 g microbial mat material) or, in the case of method development, a mixture of monosaccharides was subjected to the modified Bligh and Dyer procedure, as previously described (Sturt et al., 2004). Samples were ultrasonicated four times with 2:1:0.8 (v/v/v) methanol/dichloromethane/buffer, where the buffer was 50 mmol L^{-1} phosphate (pH 7.4) in the first two steps and 50 mmol L^{-1} trichloroacetic acid (pH 2) in the final two steps. The combined supernatants were washed three times with Milli-Q water, and the organic phase was subsequently evaporated to dryness in a stream of N_2 and stored at -20°C until further processing. Aliquots of the TLE were analyzed by high performance liquid chromatography/electrospray ionization mass spectrometry (HPLC/ESI-MS) for determination of presence and types of glycolipids.

The second step was to separate 2Gly-GDGTs from core GDGTs without polar headgroups. The TLE from the microbial mat was separated into two fractions following a procedure modified from Oba et al. (2006) on a self-packed column of 1 g of silica gel using 10 mL ethyl acetate (eluting core GDGTs) and then 20 mL methanol:H_2O = 19:1 (v/v) (eluting 2Gly-GDGTs and other IPLs). The silica gel was activated at 110°C for at least 2 hrs and deactivated with water (final content = 5 mL H_2O per 100 g silica gel) prior to use. The separation procedure was also applied to the sugar standards to monitor the distribution of monosaccharides into both fractions after column separation. For the TLE from the sediment sample, 2Gly-GDGTs were

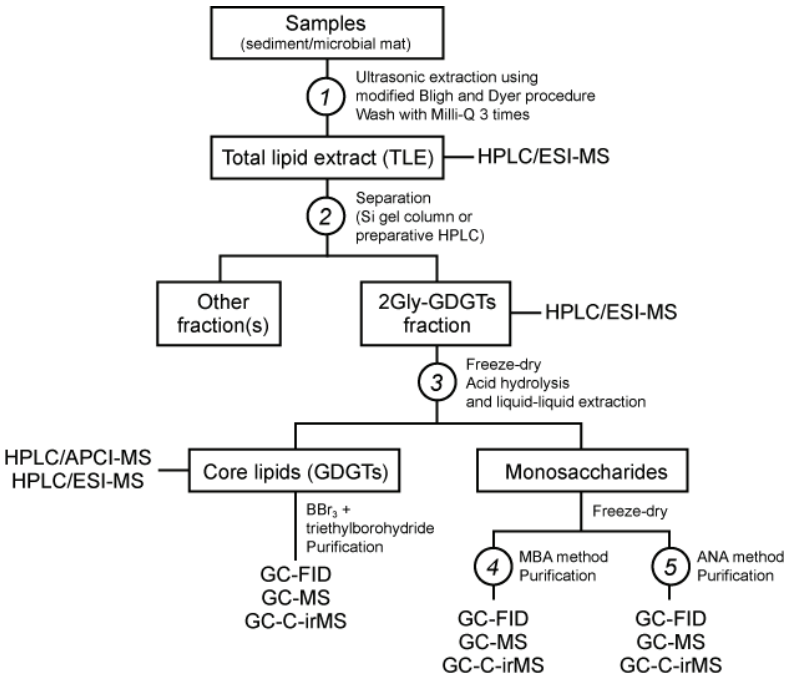


Figure 3-1. Schematic diagram of the analytical procedure. The numbers denote the analytical steps that have been investigated or optimized in this study.

purified by preparative HPLC using a LiChrosphere Si60 column (250 × 10 mm, 5 μm particle size, Alltech Associates, Inc., USA) and a fraction collector following established parameters (Biddle et al., 2006). The amount of TLE for each injection was kept below 25 mg. Fractions were analyzed by HPLC/ESI-MS to verify the presence of 2Gly-GDGTs. All lipid samples were freeze-dried for at least two hours to remove traces of methanol before acid hydrolysis.

Optimization of hydrolytic conditions for glycolipids

A series of experiments was performed to compare the sugar recoveries under different hydrolytic conditions (Step 3 in Fig. 3-1). The combinations of acids and the neutralization methods tested were: 1 M HCl in methanol, neutralized by evaporation; 1 M HCl, neutralized by evaporation, $NaHCO_3$ or NH_3; 1 M H_2SO_4, neutralized by $BaCO_3$; 50% or 98% trifluoroacetic acid (TFA), neutralized by evaporation. All hydrolyses were carried out at 70°C for 8 hours. 100 μg of Gul-GDGT-PG was used as model compound to evaluate the cleavage efficiency, whereas

10-20 μg gulose was used to examine the effect of different hydrolytic conditions on sugar recoveries. For Gul-GDGT-PG and environmental samples, liquid-liquid extraction was performed to separate the released monosaccharides from the lipids. The aqueous phase of the hydrolysates was reconstituted to 1 mL by adding Milli-Q water and vortexed with 1 mL of DCM. The DCM phase was collected by a glass syringe, and the washing step was performed for a total of 5 times by refluxing DCM. All the collected DCM phase was combined in a glass vial, evaporated to dryness, and stored at -20°C. This step was performed either before neutralization with base or after complete removal of TFA. The DCM fraction was checked by HPLC/ESI-MS and/or high performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry (HPLC/APCI-MS) for IPLs and core GDGTs, respectively (cf. Lipp and Hinrichs, 2009).

Sugars were derivatized by either the MBA or the ANA method for quantification. All the hydrolysis experiments used the same batches of lipid and sugar standards. Parallels in one experiment were analyzed by the same instrument, but different experiments were analyzed by either a flame ionization detector (FID) or a MS. To compare the results from different experiments, we had gulose treated only with Milli-Q water as the control for each experiment, and recoveries of the acid-treated gulose and Gul-GDGT-PG were normalized as relative yields compared to the control.

Derivatization of monosaccharides and purification of derivatives

All monosaccharides, either as sugar standards or released from glycolipids, were made anhydrous by freeze-drying overnight before derivatization. The MBA method and its optimization have been presented earlier by van Dongen et al. (2001) and Gross and Glaser (2004). In brief, 500 μL of a solution of 18 mg methylboronic acid in 1 mL pyridine was added to the sample, and the solution was kept at 60°C for 30 min. Subsequently, 100 μL of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was added and the mixture reacted at 60°C for 5 min. The solution was filtered over Na_2SO_4 with ethyl acetate, concentrated by evaporation under N_2, and reconstituted to a 250 or 500 μL using ethyl acetate prior to GC analysis. This method has been applied successfully for the analysis of carbon isotopic compositions of neutral

sugars with excellent accuracy (Gross and Glaser, 2004), but without a purification step (Step 4 in Fig. 3-1). Therefore, we performed an experiment in which a single batch of MBA derivatives (**VIII**) was split into aliquots for control without separation and another with column separation. The derivatives were purified using a self-packed silica gel column (0.5 g) following the procedure described above, and the column was eluted with 5 mL hexane followed by 5 mL ethyl acetate. The recoveries in these two fractions were normalized as relative yields of the control.

The ANA method has been described in Guerrant and Moss (1984). In short, aldononitrile derivatives were prepared by heating the sample in 300 μL of the derivatization reagent (32 mg hydroxylamine hydrochloride mL^{-1} and 40 mg 4-(dimethylamino)pyridine mL^{-1} in pyridine:methanol = 4:1) at 75°C for 30 min. Samples were subsequently acetylated by adding 1 mL of acetic anhydride at 75°C for 20 min. After addition of 1 mL DCM to the reaction mixture, the organic phase was washed twice with 1 mL of 1 M HCl and 3 times with 1 mL of Milli-Q water to remove excess derivatization reagents. Phase separation at the final washing step was aided by centrifugation at 800 × g for 3 min. The organic phase was evaporated to near dryness under N_2 at 40°C, and the samples were dissolved in 250 or 500 μL of a 1:1 mixture of ethyl acetate and hexane prior to GC analysis. This method has been used for the analysis of carbon isotopic compositions of amino sugars, although with an inferior accuracy compared to the MBA method (Glaser and Gross, 2005). However, the isotopic accuracy of this method for neutral sugars has not yet been evaluated, and the implementation of a purification step is required (Step 5 in Fig. 3-1). Therefore, in addition to carrying out an experiment aimed at purification, in analogy to that described above for the MBA derivatives, the isotopic values ANA derivatives (**IX**) of myo-inositol and four neutral sugars (galactose, glucose, gulose and mannose) were compared to the respective values determined by elemental analyzer-IRMS (EA-IRMS).

Preparation of hydrophobic moieties for GC analysis

Following acid hydrolysis to recover sugar headgroups, the hydrophobic moieties of tetraether lipids were prepared for GC-based isotopic analysis according to a published protocol (Jahn et al., 2004), with slight modification. Samples were treated with an excess of 1 M BBr_3 in

dry DCM at 60°C for 2 h under an atmosphere of argon to form alkylbromides. After drying under a stream of argon, the samples were amended with an excess of 1 M lithium triethylborohydride in tetrahydrofuran and kept at 60°C for 2 h in order to reduce the alkylbromides to the corresponding hydrocarbons. The reaction was quenched by adding a few drops of water, and the products were extracted into hexane. The hydrocarbons were purified with either a self-packed silica gel column (0.5 g) following the procedure described above or an aminopropyl solid phase extraction cartridge (0.5 g, Supelco). In both cases, 4-5 mL of hexane was used for the elution of hydrocarbons, and the purified samples were analyzed by GC.

Instrumentation

Liquid chromatography was performed using a ThermoFinnigan Surveyor HPLC system. Separation of IPLs was accomplished by a LiChrospher Diol column (150 mm × 2.1 mm, 5 μm particle size; Alltech, Germany) fitted with a guard column of the same material. IPLs were analyzed on a ThermoFinnigan LCQ DecaXP Plus electrospray ionization-ion trap multistage mass spectrometer (ESI-MS) in positive ion mode, using conditions described previously (Sturt et al. 2004). Core lipids (GDGTs) were separated on a Prevail Cyano column (150 mm × 2.1 mm, 3 μm particle size; Alltech, Germany) fitted with a guard column of the same material. GDGTs were determined by the same mass spectrometer using Atmospheric Pressure Chemical Ionization (APCI) under conditions described by Hopmans et al. (2000).

Gas chromatography was performed using a Trace GC 2000 or a Trace GC Ultra instrument (both from ThermoFinnigan GmbH, Germany). The injector temperature was set at 310°C. Separation of sugar derivatives and biphytanes was achieved using an Rxi-5ms column (30 m × 0.25 mm, 0.25 μm film thickness; Restek GmbH, Germany). The GCs were coupled to either a flame ionization detector (FID), a Trace MS mass spectrometer (MS), a Trace DSQ MS, or a Delta Plus XP isotope ratio mass spectrometer (IRMS) via a combustion interface (all from ThermoFinnigan GmbH, Germany). Helium was used as carrier gas with a flow rate of 1.1 mL min^{-1}. For lipid analysis, the oven temperature was set at 60°C at injection, held for 1 min, raised to 150°C at 10°C min^{-1}, further increased to 320°C at 4°C min^{-1}, and held isothermal for 22.5 min. For sugar analysis, the oven temperature was set at 70°C upon sample injection, raised to 180°C

at 4°C min^{-1}, and further increased at 10°C min^{-1} to 280°C where it was held for 2.5 min. To achieve better separation of ANA derivatives during GC-C-IRMS analysis, a different temperature program was used: the oven was set at 80°C upon sample injection, raised to 240°C at 6°C min^{-1}, increased to 300°C at 10°C min^{-1}, and held isothermal for 1 min. Behenic acid methyl ester was added to the sugar derivatives as the injection standard during isotopic analysis. Fig. 3-2 shows the GC-C-IRMS chromatograms of ANA derivatives from a standard solution and the microbial mat sample.

Stable carbon isotopic compositions of the pure neutral sugars and myo-inositol were determined independently by an elemental analyzer (EA) coupled to an IRMS (MAT 252, ThermoFinnigan GmbH, Germany). It was impracticable to prepare the syrup-like pure gulose for the EA-IRMS analysis. Hence we analyzed gulose in aqueous solution by flow injection into the IsoLink interface (ThermoFinnigan GmbH, Germany) coupled to a ThermoFinnigan Delta XP IRMS. Analysis of other neutral sugars with available reference values determined by EA-IRMS showed that the flow injection approach generated highly accurate $\delta^{13}C$ values of sugars. Acetic anhydride, after dissolved in Milli-Q water, was also analyzed by the flow injection approach.

Calculation

In general, we followed the procedure described in Gross and Glaser (2005) to calculate the isotopic values of sugar carbon and the total analytical error, with a few differences. First, Gross and Glaser (2005) evaluated the 'amount dependence' of F, a correction factor that compensates for any offset between EA-IRMS and GC-C-IRMS values, by derivatizing sugar standards at different concentrations and monitoring the corresponding isotopic shift. In the present study, F was determined only by one concentration (20 μg of each monosaccharide per batch) of the sugar standards. Second, they constrained any derivatization-related isotope fractionation by using a derivatization standard, which was absent in all the measurements presented in this study. Since carbon isotopic fractionation has also been observed with the ANA method (see below), we tentatively corrected this effect by a correction factor, which is the difference between the directly measured $\delta^{13}C$ value of acetic anhydride and the value derived from acetylated

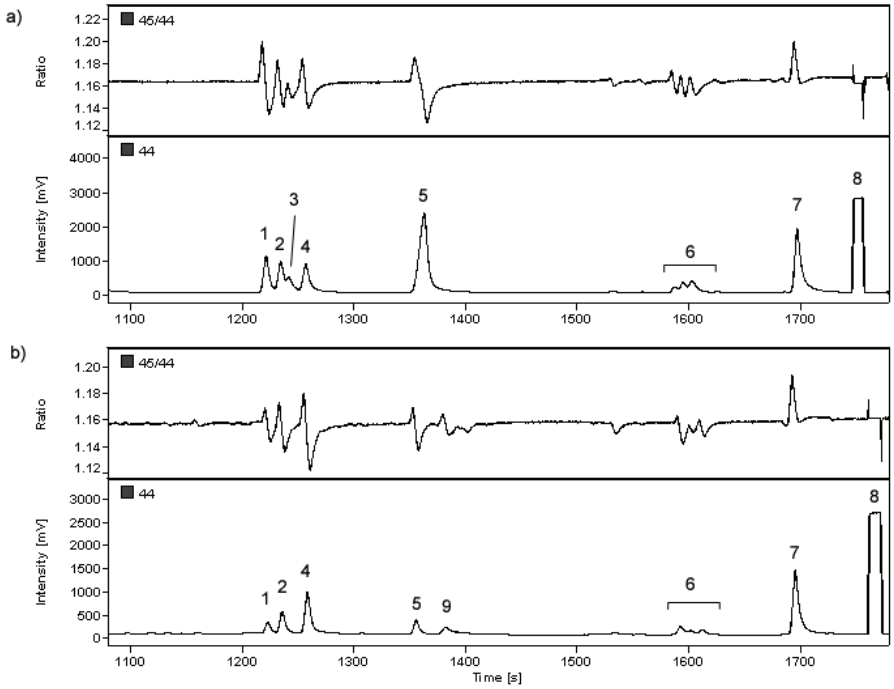


Figure 3-2. GC-C-IRMS chromatograms of (a) a standard mixture containing galactose, glucose, gulose, mannose and myo-inositol, and (b) sugar headgroups from diglycosyl-glycerol diphytanyl glycerol tetraethers in a microbial mat sample. Key to peak numbers: aldononitrile acetate derivatives of mannose (1), glucose (2), gulose (3), galactose (4), myo-inositol (5), disaccharides (6), behenic acid methyl ester as injection standard (7), reference CO_2 (8), and a unknown compound (9).

myo-inositol after mass balance calculation. In the near future these two problems will need to be addressed for more accurate isotopic determination of sugar headgroups.

Results and Discussion

Recoveries of free monosaccharides after extraction and separation of TLE

After a mixture of galactose, glucose, gulose, mannose and myo-inositol (100 μg each) was extracted with the Bligh and Dyer method, none of these sugars could be detected as ANA

Table 3-1. A summary on the recoveries of monosaccharides as aldononitrile acetate or methyl boronic acid derivatives after different sample preparation or clean-up steps. Monosaccharides were derivatized as aldononitrile acetate for quantification except those from the experiments for Step 4. Results are from duplicate experiments and are reported as percentages of the control (assigned as 100%). CV is the coefficient of variation, defined as the ratio of one standard error to the sample mean. ND: not detected; NA: not applicable.

Sample preparation step Treatment	Average (%)	CV (%)
Step 1. Recovery of monosaccharides after lipid extraction		
Control: Glc, Gal, Gul, Man and Ino, 100 μg of each	100	<21
Organic fraction after the lipid extraction procedure	ND	NA
Step 2. Silica gel column chromatography of monosaccharides		
Control: Glc, 20 μg	100	7
Silica gel, 1 g; fraction 1 (ethyl acetate, 10 mL)	ND	NA
Silica gel, 1 g; fraction 2 (methanol, 10 mL)	34	2
Silica gel, 1 g; fraction 3 (Milli-Q, 10 mL)	1	0
Step 4. Silica gel column chromatography of MBA derivatives		
Control: Gal, 10 μg	100	29
Silica gel, 0.5 g; fraction 1 (hexane, 5 mL)	ND	NA
Silica gel, 0.5 g; fraction 2 (ethyl acetate, 5 mL)	ND	NA
Step 5. Silica gel column chromatography of ANA derivatives		
Control: Glc, 20 μg	100	4
Silica gel, 0.4 g; hexane:ethyl acetate=1:1, 5 mL	103	9

derivatives in the TLE (Table 3-1). When glucose (20 μg) was subjected to the three-fraction silica gel column chromatography and derivatized by the ANA method, 34±2% was recovered in the methanol fraction, and 1% was recovered in the aqueous fraction (Table 3-1). Therefore, washing of the TLE with aqueous solutions is an essential step because our tests have shown that, if not removed prior to silica gel column separation, free monosaccharides can co-elute with 2Gly-GDGTs and mix with the glycosidic headgroups released by acid hydrolysis.

Acid hydrolysis

Table 3-2 provides an overview of the recoveries of gulose and glycolipid-gulose under different hydrolytic conditions. Sometimes only one derivatization method was employed to assess the yield of one hydrolytic condition. The standard derivatives, that is, pyranose with two

Table 3-2. A summary on the recoveries of sugar derivatives from gulose and L-gulosyl-phosphatidylglycerol-glycerol dibiphytanyl glycerol tetraethers (Gul-GDGT-PG) under different acid hydrolysis and neutralization conditions. Monosaccharides were derivatized with either methyl boronic acid (MBA) or aldononitrile acetate (ANA) method for quantification; the recoveries are reported as relative yields compared to the control (gulose in H_2O) for each individual experiment. Results are reported as the mean and ±1 standard error from duplicate experiments. NE: no experiment performed; –: no byproduct detected; +: with byproduct; ++: byproduct predominates.

Solution for control and acid hydrolysis	Neutralization method	Derivatization method	Recovery		Byproduct
			Gulose, 10-20 μg (%)	Gul-GDGT-PG, 100 μg (%)	
H_2O (control)	None	MBA or ANA	100	NE	–
TFA, 98%	Evaporation	ANA	65 ± 14	16 ± 2	+
TFA, 50%	Evaporation	ANA	NE	55 ± 10	+
HCl in methanol, 1 M	Evaporation	MBA	12 ± 3	NE	++
	Evaporation	ANA	4 ± 3	NE	++
HCl, 1 M	Evaporation	MBA	4 ± 3	NE	–
	Evaporation	ANA	2 ± 0	NE	+
	$NaHCO_3$	MBA	4 ± 0	NE	–
	NH_3	ANA	97	39 ± 5	+
H_2SO_4, 1 M	$BaCO_3$	MBA	25 ± 7	8 ± 8	–

MBA groups and one TMS group for the MBA method, and aldononitrile with five O-acetyl groups for the ANA method, were used to calculate the yields. Hydrolysis of Gul- GDGTs-PG was performed when the hydrolytic conditions gave >20% of recovery for gulose.

Among all the hydrolytic conditions, TFA gave the highest yield of gulose from Gul-GDGT-PG. It is not surprising, since TFA has several properties that are ideal for hydrolysis of glycolipids: It is identical to an organic solvent, is acidic enough to cleave glycosidic bonds, is less oxidizing than H_2SO_4, and can be neutralized easily by evaporation. Nevertheless, concentrated TFA (98%) gave a yield of only 65±14% for gulose ANA derivatives. In addition to the ANA derivatives, we observed 1,6-anhydrogulopyranose (**X**) and disaccharides (Fig. 3-2a), both being fully acetylated. When concentrated TFA was applied to Gul-GDGT-PG, we further

detected diglycosyl trifluoroacetylated-GDGTs (2Gly-GDGTs-TFA), compounds that do not exist in the glycolipid standard. These observations are consistent with the previous finding that TFA has higher potential for catalyzing formation of new glycosidic bonds (Neeser and Schaweizer, 1984), either within one monosaccharide molecule or with other molecules. When the TFA concentration was reduced to 50%, the yields of ANA derivatives from Gul-GDGT-PG increased to 55±10%. 2Gly-GDGTs-TFA were no longer detectable, but trace amounts of acetylated disaccharides were still present. It is noteworthy that both 98% and 50% TFA caused a collapse of HPLC/APCI-MS chromatograms. Boschker et al. (2008), when preparing sugar samples with TFA for isotopic analysis by HPLC-C-IRMS, reported a similar problem. Therefore, a separate aliquot of IPL sample should be kept for methanolysis when the core lipid composition is of interest.

Results from the other hydrolytic conditions were briefly summarized below. Methanolysis in combination with evaporation, the typical method to recover core lipids (Hopmans et al., 2000), gave very low recoveries of gulose as MBA or ANA derivatives. One reason for such low recoveries is the formation of methyl gulopyranoside (**XI**), which prohibited subsequent formation of MBA and ANA derivatives. When 1 M HCl was used in combination with evaporation or bases, the recoveries remained low except when NH_3 was used. We also detected the byproduct 1,6-anhydrogulopyranose in all the 1M HCl-treated samples, suggesting that formation of such a structure is not related to a particular acid but to the conformational behavior of gulose under acidic conditions (Mills, 1955). However, detection of 1,6-anhydrogulopyranose was associated with the ANA method, indicating that the MBA method has a more rigorous stereochemical requirement for sugars. The combination or 1 M HCl and NH_3 gave excellent recoveries for gulose, yet the yield of gulose from Gul-GDGT-PG was still lower than that obtained with 50% TFA. Dilute H_2SO_4 in combination with $BaCO_3$ has been often employed to recover monosaccharides from cells or tissues (e.g., van Dongen et al., 2002; Boschker et al., 2008). However, the yields of gulose MBA derivative in gulose and Gul-GDGT-PG standards were both low; no byproducts were detected by GC.

Purification of monosaccharide derivatives

The liquid-liquid extraction after acid hydrolysis (Step 3 in Fig. 3-1) separated apolar and moderately polar compounds from the monosaccharides. Subsequently, further removal of water-soluble polar matrix is required for purification of sugar derivatives. For this purpose, silica gel column chromatography was performed after derivatization. Using the MBA method, no galactose-MBA derivative could be detected in either the hexane or the ethyl acetate fraction (Table 3-1). In contrast, the ANA derivative of glucose was fully recovered by elution with the hexane:ethyl acetate mixture. These results are consistent with the reported higher stability of ANA derivatives (at least six months at 4°C) than MBA derivatives (at least one month at <0°C) (Guerrant and Moss, 1984; van Dongen et al., 2001).

Isotopic accuracy of neutral sugars derivatized by the ANA method

The higher stability of the ANA derivatives makes the ANA method more favorable than the MBA method for glycolipid studies. Before proceeding to isotopic analysis of environmental samples, we investigated the isotopic accuracy of the ANA derivatives of neutral sugars and myo-inositol. In all cases, the ANA derivatives were significantly more ^{13}C-depleted than predicted by stoichiometric calculation on the order of -12 to -16‰, suggesting carbon isotopic fractionation during the derivatization step. An earlier study using the full alditol acetate method also reported isotopic fractionation on the carbon added to sugars and attributed the fractionation to the acetylation step (Macko et al., 1995). After mass balance calculation, the $\delta^{13}C$ value of the acetyl groups attached to myo-inositol was -59.5‰, more negative than the directly measured value by -22‰. With the assumption that such an offset applied to all the other neutral monosaccharides, we calculated the $\delta^{13}C$ values of the sugars from the measured values of their ANA derivatives. The results matched moderately well with the EA-IRMS values (Fig. 3-3). In the cases of gulose and galactose, the large offset between the EA-IRMS and GC-C-IRMS values propagated from a difference of <3‰ between the stoichiometrically predicted and measured values of their ANA derivatives. For environmental samples, such an offset will be corrected by the correction factor F described in Gross and Glaser (2005).

Intramolecular isotopic analysis of 2Gly-GDGTs in environmental samples

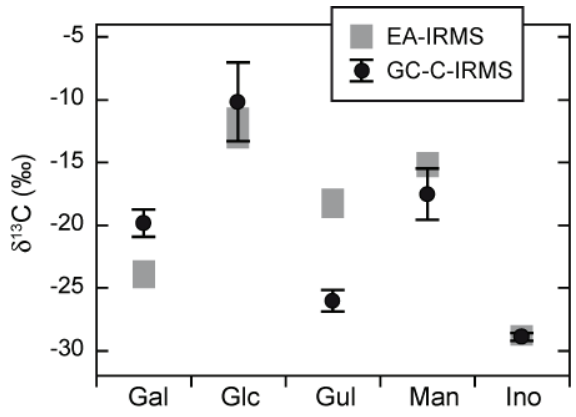


Figure 3-3. Comparison of $\delta^{13}C$ values of neutral monosaccharides and myo-inositol determined by EA-IRMS and GC-C-IRMS. Results of EA-IRMS measurements are presented as the mean and ± 1 standard error of duplicate or triplicate measurements. The mass balance equation and error propagation procedure described in Glaser and Gross (2005) were implemented to back calculate the $\delta^{13}C$ values of sugars from their aldononitrile acetate derivatives and the overall error. The carbon isotopic fractionation effect during acetylation was constrained by the $\delta^{13}C$ values of myo-inositol ANA derivatives; see text for details. Abbreviations: Man, mannose; Glc, glucose; Gul, gulose; Gal, galactose; Ino, myo-inositol.

We applied the optimized protocol to analyze the $\delta^{13}C$ values of sugars cleaved from 2Gly-GDGTs in the microbial mat and the sediment sample. Freeze-dried lipid fractions containing 2Gly-GDGTs were hydrolyzed with 50% TFA. After a liquid-liquid extraction step, the aqueous fraction was freeze-dried and derivatized by the ANA method. The ANA derivatives were purified on a silica gel column before being analyzed by GC. The biphytanes were prepared from the GDGTs according to a previously described method (Jahn et al., 2004), and analyzed by the GC-C-IRMS. Our results showed that the microbial mat sample has acyclic and bicyclic biphytanes that are more ^{13}C-depleted than those in the sediment sample (Fig. 3-4a). This pattern is in agreement with previous observations (Blumenberg et al., 2004; Biddle et al., 2006; Niemann and Elvert, 2008). The isotopic values of sugar moieties in these two samples followed the same trend, with $\delta^{13}C$ values being much more negative in the microbial mat sample (-21 to -60‰) than in the sediment sample (-14 to -18‰).

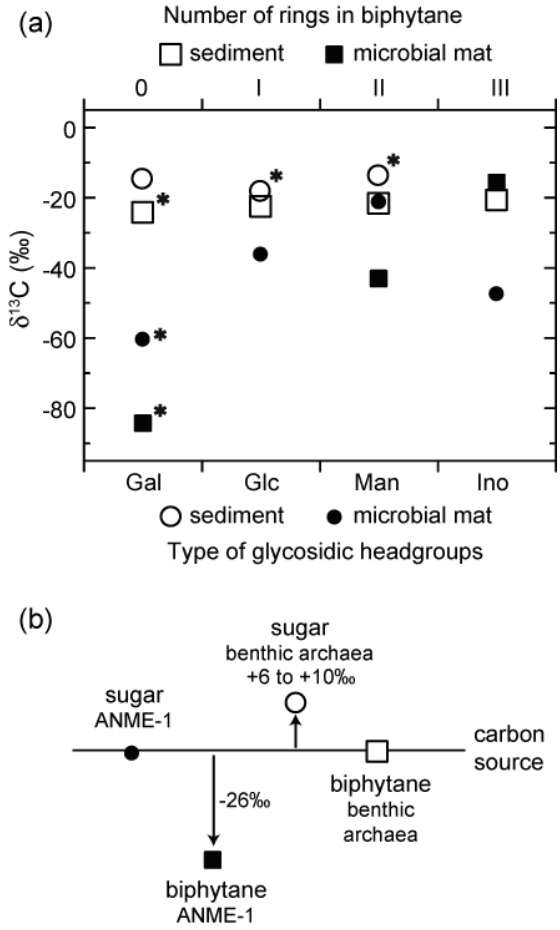


Figure 3-4. (a) Stable carbon isotopic values of biphytanes and sugars cleaved from diglycosyl-glycerol diphytanyl glycerol tetraethers in a marine subsurface sediment sample and an ANME-1 archaea dominated microbial mat. Glycolipids were hydrolyzed by 50% TFA, and the glycosidic headgroups were derivatized as aldononitrile acetate. Standard error of three repeated GC-C-IRMS measurements was about 1‰ (equivalent to a total error of 2.7‰ after error propagation). Monocyclic biphytane and myo-inositol were below detection limit in the microbial mat and in the sediment sample, respectively. The major glycolipid components in both samples are marked with asterisks. See the caption of Fig. 3-3 for abbreviations for glycosidic headgroups. (b) Schematic diagram showing the isotopic ordering of the major glycolipid components relative to the inferred carbon source for both archaeal groups.

A closer look at the isotopic data and the relationships to major carbon pools in the environments reveals additional information on the isotopic discrimination during biosynthesis of sugars and lipids in these two uncultivated archaeal groups (Fig. 3-4b). In the microbial mat, the isotopic difference between the major glycolipid constituents (galactose and acyclic biphytane) is 24‰. Methane, dissolved inorganic carbon (DIC) and total organic carbon (TOC) from the same depth has a carbon isotopic value of -70, -46 and -43‰, respectively (M. Yoshinaga, unpubl. data). Wegener et al. (2009) observed that methanotrophs assimilate carbon not only from methane but also from CO_2. If we assumed an equal contribution from both carbon pools, our data suggest: (1) isotope discrimination during biosynthesis contributes considerably (up to 26‰) to the ^{13}C-depletion in biphytanes of ANME-1, and (2) the major sugar headgroups in ANME-1 glycolipids have a carbon isotopic composition identical to that of the pooled carbon source (-58‰). The sediment sample offers a good contrast to the ANME-1 mat. The isotopic difference between the major glycolipid constituents (glucose, mannose and acyclic biphytane) is only 6-10‰. There are no carbon isotopic data available for methane and dissolved inorganic carbon at the exact sediment depth (Claypool et al., 2006; Torres and Rugh, 2006); the $\delta^{13}C$ value of TOC is -24‰ (Y.S. Lin, unpubl. data). Biddle et al. (2006) hypothesized that marine benthic archaea are heterotrophic and assimilate sedimentary organic carbon. If the hypothesis holds for our sediment sample, our data suggest that there is barely any isotopic fractionation during isoprenoid biosynthesis in benthic archaea, but the major sugar headgroups are ^{13}C-enriched relative to the carbon source by 6-10‰.

A ^{13}C enrichment in carbohydrates compared to lipids and/or carbon sources has been observed in algae and plants that fix CO_2 with the Calvin-Benson cycle (van Dongen et al., 2002), and in the anaerobic photoautotrophic bacterium *Chloroflexus aurantiacus* (van der Meer et al., 2001), which uses the 3-hydroxypropionate cycle for carbon fixation. Although there have been diverse opinions regarding the mechanisms for such a ^{13}C enrichment in sugars, in general, decarboxylation and carboxylation are considered as key processes that make pyruvate (the building block of sugars via gluconeogenesis) relatively ^{13}C-enriched compared to acetyl-CoA (the building block of lipids). In phototrophic organisms, CO_2 is first fixed as glucose. Glucose is metabolized to pyruvate, which is further converted to acetyl-CoA through decarboxylation. The decarboxylation of pyruvate by pyruvate dehydrogenase has been associated with a kinetic

isotopic effect (Melzer and Schmidt, 1987; van Dongen et al., 2002), leading to ^{13}C depletion in the carboxyl atom of acetyl-CoA. On the other hand, in organisms such as *C. aurantiacus* and microbes that fix CO_2 through pathways other than the Calvin-Benson cycle, pyruvate is derived directly or indirectly from C2-compounds such as acetyl-CoA and glyoxylate, with carboxylation steps involved in-between. Therefore, the ^{13}C enrichment of sugars in *C. aurantiacus* has been partially attributed to the incorporation of inorganic carbon (van der Meer et al., 2001).

In the present study, the previous knowledge of ^{13}C enrichment in cellular sugars relative to lipids is extended to two archaeal groups with the following preliminary explanations. It is very likely that the methanotrophic archaea follow the anabolic pathway similar to their methanogen relatives, which use acetyl-CoA as the starting material to synthesize cellular carbon (Whitman et al., 2006). If this is true, the heavier sugar isotopic values relative to lipids would be a consequence of inorganic carbon incorporation during carboxylation of acetyl-CoA. This would also partially explain the relative large intramolecular isotopic difference between the sugar and biphytane moieties in ANME-1 glycolipids, since DIC is isotopically heavier than the inferred carbon pool (methane plus DIC) by 12‰. Isotopic fractionation during other biosynthetic steps may have also contributed to the large isotopic difference, but is beyond the scope of the present study. Less biochemical information is available for heterotrophic benthic archaea. Some thermophilic heterotrophic archaea utilize the complete citric acid cycle, but pyruvate is decarboxylated by pyruvate ferredoxin-oxidoreductase rather than the typical pyruvate dehydrogenase (Verhees et al., 2003). If the enzyme pyruvate ferredoxin-oxidoreductase would be present in marine benthic archaea, a kinetic isotopic effect of 6-10‰ would be expected for the reaction catalyzed by this enzyme in order to explain the ^{13}C enrichment of glycosidic headgroups relative to lipids and carbon source in our case study.

Conclusions

We have developed an analytical procedure for the analysis of stable carbon isotopic compositions of glycosidic headgroups of intact membrane lipids. The major yield-limiting step, i.e., the acid hydrolysis of glycolipids, has been overcome by using 50% TFA. The ANA method

is the derivatization method of choice due to the higher stability of the derivatives that enables further column purification and longer storage. By applying this procedure, we determined the isotopic values of sugar headgroups from glycolipids from two environmentally important marine archaeal groups. There are intramolecular isotopic difference between the sugar and biphytane moieties, with the former more ^{13}C-enriched by 6-24‰ than the latter. The method opens a new analytical window for the examination of carbon isotopic relationships between sugars and lipids in uncultivated organisms. Furthermore, combined with stable isotope probing techniques, this method will enable comparison of turnover rates for different molecular moieties. This will improve our understanding of the physiological functions of glycolipids in uncultured microorganisms.

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Chapter 4

Stable carbon isotope probing of intact polar lipids from benthic archaea in marine subsurface sediment

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Abstract

Recent studies based on intact polar lipids (IPLs) have suggested that Archaea make a significant contribution of the extant biomass on Earth, but the linkage between the archaeal IPLs and marine benthic archaea have not been demonstrated by in vitro studies. With a combined goal of elucidating the substrate specificity of marine benthic archaea, an intact polar lipid-stable carbon isotope probing (IPL-SIP) experiment was performed on a subseafloor sediment sample with ^{13}C-labeled bicarbonate, methane, acetate, or *Spirulina platensis* cells. After prolonged incubation for up to 468 days, the hydrophobic moieties of the archaeal IPLs showed minimal label incorporation. The strongest shift in carbon isotopic values (up to 4‰) was detected in crenarchaeol-derived tricyclic biphytane in the sediment slurries supplemented with ^{13}C-labeled *S. platensis*. In contrast, under the same labeling condition, close to 5% of the mannose cleaved from the archaeal glycosyl tetraether lipids was ^{13}C-labeled. Our results suggest that archaeal IPLs were being generated in the sediment containing benthic archaeal sequences. The unbalanced ^{13}C uptake between the glycosidic head groups and the hydrocarbon chains implies

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the presence of an anabolic shortcut that enables benthic archaea to regenerate glycolipids while bypassing the energy-costly tetraether biosynthesis.

Introduction

Over geological time, the majority of organic matter that escapes the internal cycling in the biological carbon cycle is deposited and preserved in marine sediments (Hedges and Keil, 1995). Although the biodegradation of organic matter during early diagenesis beneath the seafloor has long been supported by geochemical evidence (e.g., Froelich et al., 1979), it is only recently that the dimensions of such a 'marine deep biosphere' and its link to biodegradation have been demonstrated. Marine subsurface sediments are estimated to harbor a vast prokaryotic ecosystem, which may comprise 5-30% of the extant global biomass (Whiteman et al., 1998; Parkes et al., 2000; Lipp et al., 2008). This ecosystem is also distinctive in its community composition compared with other environments because of its predominance of novel, uncultured archaeal groups (Biddle et al., 2008). These archaea are proposed to have a heterotrophic lifestyle (Biddle et al., 2006) and may play an important role in the organic matter remineralization in marine sediments.

However, the proposition of a vast benthic community dominated by living archaea that assimilate sedimentary organic compounds has been solely based on evidence from culture-independent approaches. Approaches targeting 16S ribosomal RNA (rRNA) and quantitative analysis of rRNA gene copies from extracted DNA demonstrated the presence of live benthic archaea (e.g., Biddle et al., 2006, 2008); quantification of intact polar lipids (IPLs) suggested the dominance of Archaea over Bacteria (Lipp et al., 2008); stable carbon isotopic values of whole cells and IPLs led to the hypothesis that benthic archaea are heterotrophic. Nevertheless, there are uncertainties inherent in each of these culture-independent approaches, and the issue of 'who lives in sea floor' is still an ongoing debate (cf. Pearson, 2008). One critical point that remains to be clarified is the linkage between 16S rRNA sequences and archaeal IPLs detected in subseafloor sediments. The linkage is not a straightforward one because the overlying water column hosts a sizeable and viable archaeal population (Karner et al., 2001), which has a

distinct phylogenetic identity but a related IPL composition (Schouten et al., 2008; Schubotz et al., 2009) and identical carbon isotopic values of some core lipids (Hoefs et al., 1997). Although IPLs degrade rapidly in experiments (Harvey et al., 1986), little is known about their persistence in environments with extraordinarily low rates of enzymatic activity. Since the results from IPLs formulate a significant piece of the current picture of marine deep biosphere, it is essential to evaluate the representability of archaeal IPLs in subseafloor sediments as the biomarkers for marine benthic archaea.

In principle, biomarkers can be linked directly to their producers in isolates or enrichments, and cultivation work can be planned based on the hypothesis that benthic archaea are heterotrophs (Biddle et al., 2006). Nevertheless, endeavors to isolate (Batzke et al., 2007) or enrich (Parkes et al., 2009) heterotrophs in marine subsurface sediments have all failed to promote archaeal growth. Only heterotrophic bacteria were culturable. One explanation is that cultivation using medium with high substrate concentrations discriminated against the low-energy adaptation, recently suggested to be a characteristic of Archaea (Valentine, 2007). If this is the case, heterotrophic archaea in moderate environments will be extremely difficult to enrich or isolate with the conventional cultivation approaches, which intrinsically favor the growth of Bacteria over Archaea.

Stable isotope probing (SIP) experiments provide a promising alternative to link molecules to organisms with additional information on substrate specificity. The principle is to supplement ^{13}C-labeled substrates and to track the uptake of the label into biomolecules (Boschker et al., 1998). With carefully selected sediment samples in which the existence of living planktonic archaea can be excluded, the relationship between archaeal biomarkers and benthic archaea can be assessed. In this report, we present the results from an SIP experiment performed on a sediment sample retrieved from a subsurface sulfate-methane transition zone (8 m below the seafloor) at Hydrate Ridge, Cascadia margin. Our goals were to examine the linkage between archaeal biomarkers and benthic archaea, and to constrain the substrate that leads to ^{13}C assimilation. Since a very low growth rate has been proposed for benthic archaea (Biddle et al., 2006), we targeted only archaeal IPLs in the SIP experiment, so that the problem of isotope dilution caused by apolar core lipids from unspecified fossil sources can be circumvented and the

sensitivity of the SIP experiment improved. The polar head groups of IPLs also provided additional information on the metabolic activity of the organisms.

Experimental Procedures

Set-up of the incubation

The sediment sample used for the incubation experiment was retrieved from a subsurface sulfate-methane transition zone at Hydrate Ridge, Cascadia margin (ODP 204-1245D-2H-3, 5-40 cm, 8 meter below seafloor; Tréhu et al., 2003). In an anoxic glove box, the sediment was mixed with approximately the same volume of anoxic sulfate reducer medium prepared according to Widdle and Bak (1992) with some modifications. The medium contained only 5 mmol L^{-1} $NaHCO_3$, had lower concentrations of NH_4Cl (50 μmol L^{-1}) and KH_2PO_4 (15 μmol L^{-1}), and was not enriched with trace elements and vitamins. The total DIC concentration in the aqueous phase of the slurry was estimated to be around 10 mmol L^{-1} after taking the reported alkalinity value of the sample into account (Tréhu et al., 2003). An aliquot of 120 mL homogenized sediment slurry was transferred to a 156 mL serum vial, which was sealed with a thick butyl stopper and crimp capped. After addition of the ^{13}C-labeled substrates, the headspace was pressurized to 300 kPa with methane. All the vials were incubated in the dark at 12°C and shaken by hand regularly.

The ^{13}C-labeled substrates were supplemented in the following ways: $H^{13}CO_3^-$ and [2-^{13}C]acetate were added from anoxically prepared stock solutions, $^{13}CH_4$ was added by injecting the gas, and [^{13}C]*S. platensis* was prepared as particles suspended in the anoxic medium described above and injected by a plastic syringe fitted with a thicker needle (gauge 21). [2-^{13}C]acetate and [^{13}C]*S. platensis* were both added to a final concentration of 800 μmol total C L^{-1} assuming a 50% contribution of C to the weight of the lyophilized *S. platensis* cells. All the substrates were only 10% labeled during the first two rounds of feeding. For the remaining four rounds of feeding, we used 99%-labeled $^{13}CH_4$, [2-^{13}C]acetate, and [^{13}C]*S. platensis*. The ^{13}C content in the $H^{13}CO_3^-$-supplemented samples was also raised to 50% with a simultaneous increase of the DIC concentration to ca. 20 mmol L^{-1}.

The entire course of incubation can be divided into three stages based on the time of harvest (Fig. 4-1). Samples from the first stage were completely used for IPL analysis. The sediment slurry was centrifuged, and the solid phase was stored at -20°C in a glass container until lipid extraction. For the second and third stages, an aliquot of sediment slurry was immediately taken after the vial was opened, and stored in a Falcon tube at -80°C for 16S rRNA gene analysis. The remaining slurry was processed for IPL analysis by the same procedure as described above.

Analysis of DIC and $\delta^{13}C_{DIC}$

Samples for DIC analyses were taken from the supernatant of the sediment slurry by a plastic syringe fitted to a long needle. Aliquots of samples were stored in 2 mL glass vials and frozen at -20°C till analysis. DIC concentrations were analyzed by a Shimadzu TOC-VCPN with a nondispersive infrared detector. For the determination of $\delta^{13}C_{DIC}$, a liquid sample was injected into a sealed glass tube, which contained 100 μL of phosphoric acid and was evacuated and purged five times with helium. After equilibration for > 5 hr, the released CO_2 gas was analyzed using a GasBench II automated sampler interfaced to an IRMS (MAT 252, ThermoFinnigan GmbH, Germany). The instrumental precision was 0.1‰ (one standard deviation). $\delta^{13}C_{DIC}$ values higher than +1000‰ were obtained with an isotope dilution approach. A sample was mixed with a $NaHCO_3$ standard solution at 2-3 different volume ratios. The $\delta^{13}C_{DIC}$ values of these mixtures were plotted against the content ratios of DIC in the standard to that in the sample. The $\delta^{13}C_{DIC}$ value of the sample was then estimated from this plot by setting the ratio at 0.

Extraction, analysis, and purification of IPLs

IPLs were extracted using a modified Bligh and Dyer method in four steps as described previously (Sturt et al., 2004). Before extraction, a known quantity of 1-*O*-hexadecyl-2-acetoyl-*sn*-glycero-3-phosphocholine was added as the internal standard to all samples. Total lipids were ultrasonicated four times with 2:1:0.8 (v/v/v) methanol/dichloromethane/buffer, where the buffer was 50 mmol L^{-1} phosphate at pH 7.4 in the first two steps and 50 mmol L^{-1} trichloroacetate at pH 2.0 in the final two steps. The combined

supernatants were washed three times with Milli-Q water, and the organic phase called the total lipid extract (TLE) was subsequently evaporated to dryness in a stream of N_2 and stored at -20°C until further processing. A fraction of the TLE was analyzed using high performance liquid chromatography/electrospray ionization mass spectrometry (HPLC/ESI-MS) described previously (Sturt et al., 2004). Because samples were analyzed only in the positive ionization mode, different core lipids types for phospholipids, i.e., diacyl glycerol (DAG) lipids and acyl/ether glycerol (AEG) lipids, were not distinguished. IPL concentrations were first calculated from the peak areas of extracted mass chromatograms relative to that of the internal standard, followed by correction of ionization efficiency based on an external calibration series of commercial standards (Lipp et al., 2008). Only compounds with a signal-to-noise ratio higher than 3 were reported.

2Gly- and H341-GDGTs in the remaining TLE were subsequently purified by preparative HPLC and a fraction collector following established parameters (Biddle et al., 2006). The amount of TLE for each injection was kept below 25 mg. Fractions were analyzed by HPLC/ESI-MS to verify the presence of 2Gly- and H341-GDGTs.

Preparation and isotopic analysis of biphytanes and sugar derivatives

Two published protocols were employed to prepare biphytanes from the purified 2Gly- and H341-GDGTs. The protocol described in Biddle et al. (2006), involving ether cleavage via HI treatment and a subsequent reduction of the iodides by $LiAlH_4$, was applied to samples from the first harvest. For samples from the later harvests, we followed the procedure described in Jahn et al. (2004) by cleaving the ether bonds with BBr_3 and reducing the bromides with lithium triethylborohydride. Out tests using a GDGT standard showed that the method of Jahn et al. (2004) gave a higher yield of biphytanes and was hence more appropriate for accurate determination of biphytane isotopic values.

To prepare both the biphytanes and derivatives of sugars from the purified 2Gly- and H341-GDGTs of samples from the third harvest, we applied the protocol recently developed by Lin et al. (Chapter 3, this volume). The fraction containing 2Gly- and H341-GDGTs was

freeze-dried and hydrolyzed with 50% TFA. After a liquid-liquid extraction step, the core GDGTs were subjected to the procedure of Jahn et al. (2004) for biphytane preparation. The aqueous fraction was freeze-dried and derivatized into aldononitrile acetate following the procedure described in Guerrant and Moss (1984). The sugar derivatives were purified on a silica gel column before being analyzed by GC.

For GC-C-IRMS measurements, a Delta Plus XP IRMS was used, connected via a Combustion Interface III to a Trace GC 2000 (all from ThermoFinnigan GmbH, Germany), equipped with an Rxi-5ms column (30 m × 0.25 mm, 0.25 μm film thickness; Restek GmbH, Germany). 2,6,10,15,19,23-Hexamethyltetracosane and behenic acid methyl ester were used as the injection standards for isotopic analysis of biphytanes and sugar derivatives, respectively. Stable carbon isotope values were given in the δ-notation against Vienna PeeDee Belemnite (V-PDB). The instrumental precision was 1‰ (one standard deviation).

16S ribosomal RNA gene analysis

DNA was extracted from 0.5 g sediment using the MoBio® PowerSoil DNA extraction kit (Carlsbad, CA). Small subunit ribosomal genes were amplified using bacterial primers 8F and 1492R (Teske et al., 2002) and archaeal primers 21F and 915R (DeLong, 1992). PCR products were verified on 1.5% TBE agarose gels and successful amplifications were cloned into the TOPO-TA system (Invitrogen, Carlsbad, CA) and transformed into *Escherichia coli*. Clones were screened by blue/white screening and those with inserts were selected for direct colony sequencing by Genewiz, Inc. (Plainsfield, NJ). Sequences were cleaned using Sequencher (GeneCodes Corp, Ann Arbor, MI) and initial identifications were made by BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/) and confirmed through alignment and neighbor-joining trees in the ARB software platform (http://www.arb-home.de).

Results

Carbon isotopic values of dissolved inorganic carbon (DIC)

The turnover of added organic substrates was monitored by the carbon isotopic values of dissolved inorganic carbon ($\delta^{13}C_{DIC}$). The results (Fig. 4-1) show that methane oxidation was almost negligible, but other organic substrates were utilized, resulting in significant enrichment of the DIC pools. It is noteworthy that sediment slurries supplemented with [^{13}C]*Spirulina platensis*, which had twice the amount of organic ^{13}C compared with those in [2-^{13}C]acetate, yielded less ^{13}C-DIC. To evaluate the degree of remineralization of both substrates at the end of incubation, we divided the measured $\delta^{13}C_{DIC}$ values by the calculated $\delta^{13}C_{DIC}$ values, assuming complete remineralization of the added ^{13}C. This calculation estimated that only 31% and 11% of

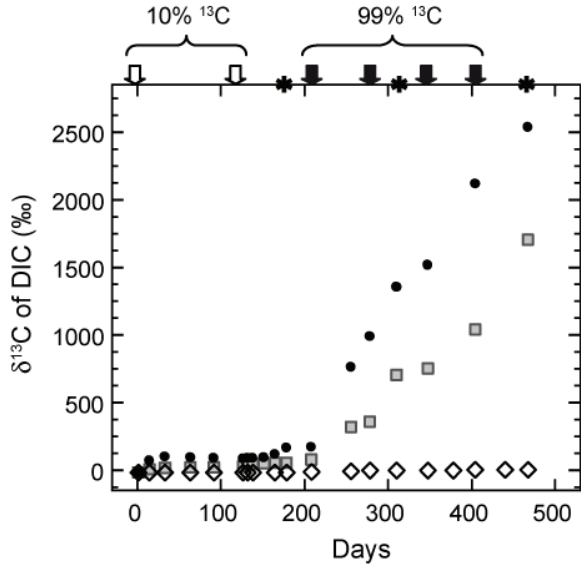

Figure 4-1. Stable carbon isotopic values of dissolved inorganic carbon (DIC) of the sediment slurries amended with $^{13}CH_4$ (diamonds), [2-^{13}C]acetate (dots), or [^{13}C]*Spirulina platensis* (squares). Results for the first 324 days are presented as the mean of duplicate vials; the ±1 standard error of duplicate vials is <2‰ for the $^{13}CH_4$-added samples, <60‰ for the [2-^{13}C]acetate-added samples, and <30‰ for the [^{13}C]*S. platensis*-added samples. Arrows indicate the time of feeding with ^{13}C-labeled substrates. The ^{13}C content of added substrates increased from 10% to 99% during the course of incubation. The percentage of ^{13}C in bicarbonate-added sediment slurries was also adjusted from 10% to 50%. Asterisks denote the time of harvesting the solid phase for IPL and/or molecular analysis.

the labeled acetate and *S. platensis* entered the remineralized carbon pool, respectively.

Archaeal and bacterial IPL profiles

The temporal changes in the microbial community after supplementation with different substrates were monitored at a low taxonomic resolution by IPL analysis. The ionization efficiencies of IPLs in samples with similar matrices were identical among the consecutive runs, and commercial standards were used to control the variations in the ionization efficiency of different IPLs over time. However, a coefficient of variation of up to 50% was observed for IPL quantification when the same batch of the time-zero sample was analyzed twice, with the duplicate measurements made only 10 days apart. To retain most of the total lipid extract for isotopic analysis, the IPL analysis of each incubated sediment sample was performed only once. Therefore, the temporal changes in the IPL contents presented in Fig. 4-2 should be interpreted with caution. We assumed a similar coefficient of variance of 50% in each single run, and two IPL concentrations were considered different only when they differed by a factor of > 2.

With this criterion, we summarized the observations as follows. The archaeal IPL contents did not change over the course of the incubation for the substrate tested, except at the third harvest of the sample to which $H^{13}CO_3^-$ was added. In this sample, the archaeal IPL content was only 48% of the time-zero level. The composition of the polar head groups of the archaeal IPLs was also constant over time. The glycerol dibiphytanyl glycerol tetraethers (GDGTs), with an unknown head group 18 Da greater in mass than that of diglycosyl-GDGTs (H341-GDGTs hereafter), were the major archaeal IPLs, followed by diglycosyl-GDGTs (2Gly-GDGTs) and GDGTs with an unknown head group 18 Da greater in mass than that of triglycosyl-GDGTs (data not shown).

Bacterial IPLs were already present in the refrigerated sample that was used to start the experiment (cf. Lin et al, in press), but their contents did not change significantly after incubation. The only exception was in the third harvest of the [^{13}C]*S. platensis*-added sample, in which the bacterial IPLs were three times more abundant than in the time-zero sample. The bacterial IPL composition of the time-zero sample included diacyl glycerols or acyl/ether glycerols

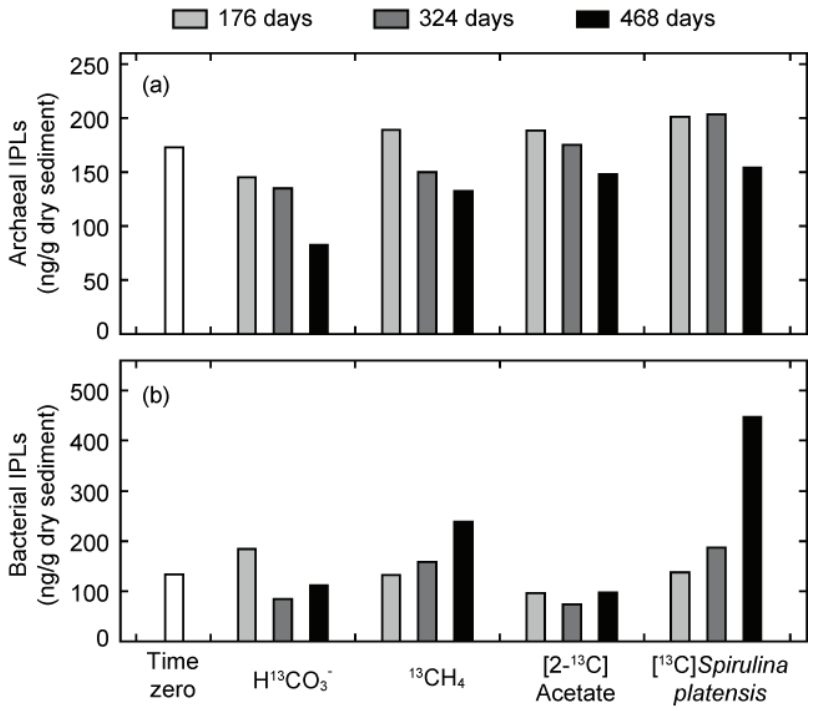

Figure 4-2. Contents of archaeal (a) and bacterial intact polar lipids (IPLs) (b) in the sediment slurries. Results are from single measurement except for the time-zero values, which are the average of duplicate measurements.

(DAGs/AEGs) with phosphatidylcholine as the major head group. During the course of incubation, the proportions of phosphatidylethanolamine and phosphatidylglycerol increased and eventually became the predominant phospholipids in the [^{13}C]*S. platensis*-added samples (data not shown). IPL remains from [^{13}C]*S. plantensis* do not account for the increase of these phospholipids, because this cyanobacterial strain possesses predominantly monoglycosyl-DAGs (MG-DAGs), diglycosyl-DAG (DG-DAGs), and sulphoquinovosyl-DAG (Hudson and Karis, 1974). Their only phospholipids, phosphatidylglycerol-DAGs, could be distinguished from the bacterial ones due to their high ^{13}C content that gave a distinct pattern in mass spectra.

Stable carbon isotopic values of biphytanes

We directed our focus to 2Gly-GDGTs and H341-GDGTs, both of which are the most abundant and consistently occurring IPLs in marine subsurface sediments (Lipp and Hinrichs,

2009). The hydrophobic moieties from the mixture of 2Gly-GDGTs and H341-GDGTs contained biphytanes ranging from zero to three rings. The $\delta^{13}C$ values of the acyclic and monocyclic biphytanes in the supplemented sediment slurries did not differ significantly from those in the time-zero sample (data not shown). The $\delta^{13}C$ values of the dicyclic biphytane in the [^{13}C]*S. platensis*-supplemented sample increased by 1.5‰ at the first harvest, but did not differ from the time-zero value at the later harvests (data not shown). The $\delta^{13}C$ values of the tricyclic biphytane ($\delta^{13}C_{BiP3}$), the hydrocarbon that originates from crenarchaeol, showed slight ^{13}C enrichment after labeling (Fig. 4-3). The heaviest values were observed in the sediment slurry supplemented with [^{13}C]*S. platensis*, where the $\delta^{13}C_{BiP3}$ values increased to -19.6‰ after 176 days of incubation and reached -16.6‰ after 324 days. However, the trend in enrichment did not continue in the sample incubated for 468 days, and the heaviest $\delta^{13}C_{BiP3}$ value (-16.6‰) is at the positive end of the natural isotopic values (-17 to -37‰) reported for biphytanes in marine subsurface sediments (Biddle et al., 2006). When evaluated with a one-tailed *t* test, the difference between the [^{13}C]*S. platensis*-supplemented (second and third harvests) and the time-zero samples was significant at

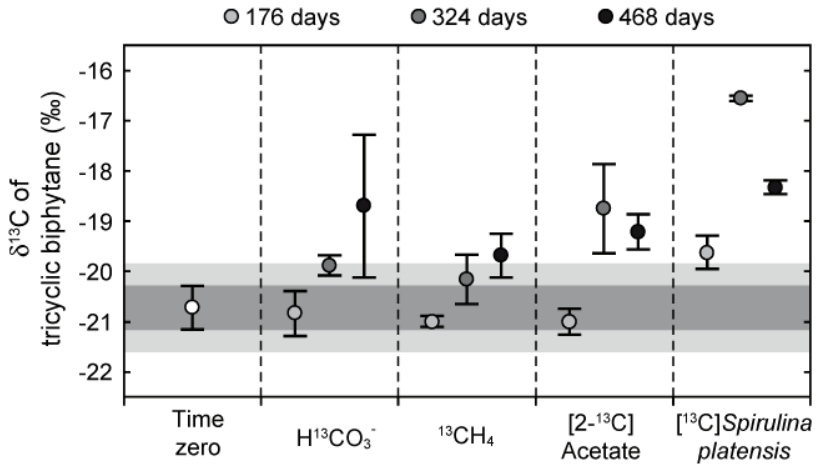

Figure 4-3. Stable carbon isotopic values of crenarchaeol-derived tricyclic biphytane cleaved from the mixture of diglycosyl-glycerol dibiphytanyl glycerol tetraethers (2Gly-GDGTs) and H341-GDGTs in the sediment slurries. Results are presented as the mean and one standard error of duplicate or triplicate isotopic measurements. The dark-gray and light-gray shaded areas mark the range of ±1 and ±2 standard error of time-zero isotopic values, respectively.

the 0.1% level. For the other substrates, the $\delta^{13}C_{BiP3}$ values were different from the time-zero value only at the 1% level (second and third harvests of the [2-^{13}C]acetate-added sample) or even higher (for the $H^{13}CO_3^-$- and $^{13}CH_4$-added samples).

Stable carbon isotopic values of the glycosidic head groups

In the time-zero sample, the hexoses detected included mannose, glucose, and galactose; their $\delta^{13}C$ values were in the range of -10 to -20‰ (Fig. 4-4). The instrumental uncertainty in gas chromatography-combustion-isotope ratio mass spectrometric (GC-C-IRMS) analysis, after error propagation, accounted for most of the variations in the $\delta^{13}C$ values of the glycosidic head groups in the time-zero sample. Based on a single GC-C-IRMS measurement, the $\delta^{13}C$ values of the glycosidic head groups in the sediment slurries supplemented with $H^{13}CO_3^-$, $^{13}CH_4$ and [2-^{13}C]acetate did not differ significantly from the time-zero values.

Previous studies showed that the cyanobacterial MG- and DG-DAGs contain predominantly galactose (Hudson and Karis, 1974), but the polysaccharides and lipopolysaccharides contain glucose and mannose at lower levels (Filali Mouhim et al., 1983; Mikheiskaya et al., 1983; Vonshak, 1997). To ensure that the glycosidic head groups cleaved from the archaeal glycolipids were not contaminated with those released from the cyanobacterial cell constituents, we first used preparative high-performance liquid chromatography to separate the majority of remaining MG- and DG-DAG from the target fraction containing 2Gly-GDGTs and H341-GDGTs. We also treated the TLE of *S. platensis* with the same purification procedure and analyzed the detectable sugars after acid hydrolysis in the fraction where 2Gly-GDGTs and H341-GDGTs eluted. The result showed that galactose and glucose were present, but mannose was no detectable. Therefore, we focused only on the $\delta^{13}C$ value of mannose in the [^{13}C]*S. platensis*-supplemented sample. Surprisingly, the $\delta^{13}C$ value of mannose reached 4000‰, indicating that ~5% of the glycolipid-mannose was ^{13}C-labeled.

Community composition of the Archaea

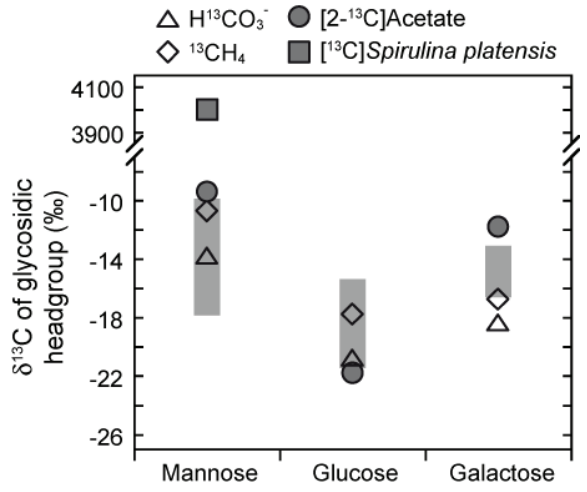

Figure 4-4. Stable carbon isotopic values of glycosidic headgroups cleaved from the mixture of diglycosyl-glycerol dibiphytanyl glycerol tetraethers (2Gly-GDGTs) and H341-GDGTs in the sediment slurries incubated for 468 days. The gray bars mark the range of ±1 standard error of the time-zero isotopic values after triplicate measurements. The isotopic values of the [^{13}C]*Spirulina platensis*-added sample are the mean of triplicate measurements, whereas the results of the other treatments are from single measurement. Glucose and galactose in the [^{13}C]*Spirulina platensis*-added sample were omitted from discussion; see text for details.

Analysis of the 16S rRNA gene showed that in the time-zero sample, the archaeal community contained the crenarchaeotal Marine Benthic Group B and the South African Goldmine Euryarchaeotal Group (Fig. 4-5). These two archaeal lineages are widespread in marine subsurface sediments (Teske and Sørensen, 2008). After prolonged incubation, the yield of archaeal sequences varied among the sediment slurries supplemented with different substrates. The addition of [^{13}C]*S. platensis* resulted in the highest yield with diverse archaeal phylotypes, whereas supplementation with [2-^{13}C]acetate gave the lowest yield. No sequences affiliated to Marine Group 1 were detected.

Discussion

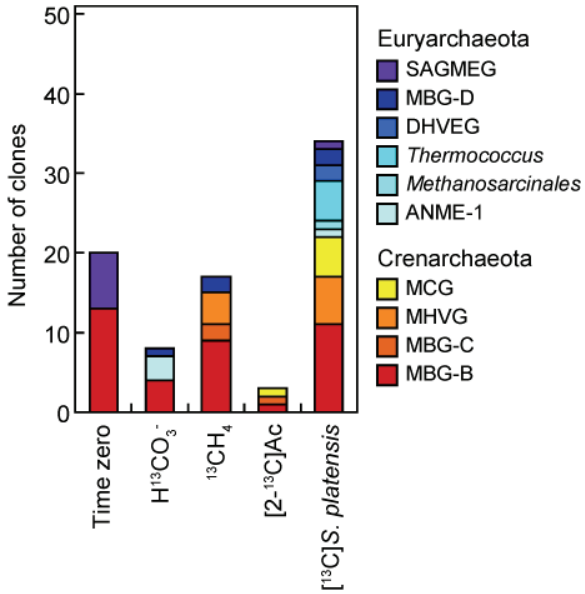

Figure 4-5. Archaeal 16S rRNA gene clone library composition in the time-zero sample and the sediment slurries incubated for 468 days. Abbreviations for the archaeal lineages: SAGMEG, South African Goldmine Euryarchaeotal Group; MBG-B/C/D, Marine Benthic Group-B/C/D; DHVEG, Deep-Sea Hydrothermal Vent Euryarchaeotal Group; ANME-1, Anaerobic Methanotrophic Archaea 1; MCG, Miscellaneous Crenarchaeotal Group; MHVG, Marine Hydrothermal Vent Group.

In our long-term IPL-SIP experiment, we showed that among the four tested substrates, only the whole cells of *S. platensis* generated significant signals, in which the hydrophilic polar head groups of 2Gly-GDGTs and H341-GDGTs were more strongly labeled than the hydrophobic lipid moieties. Note that the two glycolipid moieties have different taxonomic resolutions: while the tricyclic biphytane is associated with Crenarchaeota, the mannose can be derived from both Euryarchaeota and Crenarchaeota (Jahn et al., 2004; Koga and Morii, 2005). Nevertheless, no increase in archaeal IPL content was detected, suggesting either limited growth or no net increase of archaeal biomass. Analysis of the 16S rRNA genes showed that the archaeal community consisted of only typical benthic archaeal lineages. The potential contribution of Marine Group 1, the phylogenetic group containing the sequences of planktonic archaea (DeLong, 1992) that are sometimes also found in marine sediments (Teske and Sørensen, 2008), can be excluded. Taken

together, these data suggest that 2Gly-GDGTs and H341-GDGTs were being generated in the sediment containing benthic archaeal sequences. The strong labeling signal in the polar head groups and the weak signal in the core lipids demonstrated the strength of IPL-SIP in probing slow-growing microorganisms.

A closer look at the responses of the sediment slurries to the four tested substrates provides further insight into the metabolic functionality of marine benthic archaea. Unlike the results of previous studies, which showed that marine planktonic archaea can assimilate inorganic carbon (Wuchter et al., 2003; Könneke et al., 2005), our data indicate that $H^{13}CO_3^-$ was not incorporated into the IPLs of benthic archaea. The lack of clear labeling signals with $H^{13}CO_3^-$ is consistent with the observation that the $\delta^{13}C$ values of biphytanes cleaved from intact polar GDGTs in marine sediments do not covary with the $\delta^{13}C$ values of DIC, which have a wide range from -30 to 20‰ (Biddle et al., 2006). $^{13}CH_4$ was neither oxidized (Fig. 4-1) nor assimilated, even though the sediment was retrieved from a subsurface sulfate-methane transition zone, where the anaerobic oxidation of methane should have been taking place in situ. This can be attributed to the loss of either the methane-oxidizing activity or population before the experiment was initiated, or to the lack of such a catabolic ability in the cosmopolitan benthic archaea detected in our clone libraries (Fig. 4-5).

The difference in the results obtained with [2-^{13}C]acetate and [^{13}C]*S. platensis* is intriguing. Acetate is the building block of isoprenoidal lipids; it can also be used for carbohydrate biosynthesis after being carboxylated to pyruvate. However, there was no labeling signals in the biphytanes (Fig. 4-3) and glycosidic head groups (Fig. 4-4) in the [2-^{13}C]acetate-supplemented samples. There are two plausible explanations. (1) The dominant microorganisms in natural environments tend to maintain their competitive advantage by having a higher affinity to substrates that are usually kept at low concentrations (e.g., Sakai et al., 2007). However, in our experiment, acetate was supplemented as single spikes at concentrations much higher than in situ levels (cf. Heuer et al., 2009). Other microorganisms with lower affinities for acetate could have been stimulated and consumed most of the added acetate. In contrast, during the decomposition of *S. platensis*, acetate, as a fermentation product, may have been released continuously and kept at a low level by microbial syntrophy. Over time, the cumulative amount of acetate accessible to

microorganisms adapted to low concentrations could be eventually higher. This explanation echoes the hypothesis that adaptation to chronic energy stress is a crucial characteristic of Archaea (Valentine, 2007). (2) Alternatively, marine benthic archaea do not metabolize or assimilate acetate from the ambient environment but pursue a typical heterotrophic lifestyle using polypeptides or saccharides, both of which are common substrates for cultures of heterotrophic Archaea (Verhees et al., 2003) and could be supplied by the bulk biomass of *S. platensis*. Although *S. platensis* cells showed a lower degree of remineralization than acetate (Fig. 4-1), they seemed to result in the net increase of bacterial biomass (Fig. 4-2), and the bacterial community was dominated by heterotrophs (data not shown). We are currently investigating the second possibility by further SIP experiments using ^{13}C-labeled leucine and glucose. Despite these uncertainties, our current data support the hypothesis of a heterotrophic metabolism of marine benthic archaea (Biddle et al., 2006).

The mixture of 2Gly-GDGTs and H341-GDGTs in the [^{13}C]*S. platensis*-supplemented sample showed unbalanced ^{13}C uptake into the hydrophilic and hydrophobic moieties. The more strongly labeled mannose suggests the formation of new intact GDGTs by glycosidic bonds between labeled mannose and existing GDGTs. The possibility of extracellular glycosidic-bond formation is low. First, extracellular ^{13}C-labled mannose released from *S. platensis* must be first activated to uridine-diphosphate mannose to be recognized by hexosyltransferases. Second, hexosyltransferases are specific to the stereoconfiguration of the glycerol moiety of the membrane lipids (Karlsson et al., 1997; Morii et al., 2007). Therefore, the extracellular hexosyltransferases from *S. platensis* or bacteria can not catalyze the formation of glycosidic bonds in 2Gly-GDGTs and H341-GDGTs. Third, it is unlikely that the hexosyltransferases from the remains of planktonic archaea are able to maintain the structural integrity necessary for proper functioning at a sediment depth of 8 m below the seafloor, from where our sample was retrieved. Yet the possibility that enzymes were released from recently lysed cells of benthic archaea cannot be excluded. In this case, our observation of an unbalanced ^{13}C uptake should be interpreted only as a consequence of extracellular enzymatic reactions, without physiological implications. The presence of hexosyltransferases from benthic archaea, even though from earlier generations, still supports our principal conclusion that 2Gly-GDGTs and H341-GDGTs in subseafloor sediments can be originated from indigenous archaea.

Conversely, the option of the unbalanced ^{13}C uptake as a biological signature should be considered equally possible. What are the biochemical implications of this finding? According to the current model of intact GDGT biosynthesis (summarized in Koga and Morii, 2007; Fig. 4-6), the glycosidic moiety is attached after GDGT is formed (VI to VIII in Fig. 4-6). GDGT is, in turn, synthesized by the fusion of two molecules of archaeol with phospho-head groups already attached (IV to V in Fig. 4-6). The attachment of the phospho-head groups occurs at an earlier stage and has been found to involve the unsaturated intermediate di-*O*-geranylgeranyl-glycerol-1-phosphate (Ia in Fig. 4-6), rather than glycerol with saturated hydrocarbon chains (Ib in Fig. 4-6), which can be present at higher concentrations as a result of the degeneration of older lipids. In other words, according to the model for glycosyl-GDGTs biosynthesis, an even distribution of ^{13}C-labeled alkyl and sugar moieties would be expected, as both parts must be synthesized de novo.

Our IPL-SIP results are apparently incompatible with the predictions of this model, prompting the questions: To what extent do Archaea in natural environments synthesize novel membrane lipids according to the pathway outlined in the model, and how do they maintain the membrane integrity? The model is based on studies of pure cultures, which were grown under optimal conditions and free from competition with other coexisting microbes. Cultured GDGT-producing Archaea, even at late stationary phase (Morii and Koga, 1993) or after isolation from oligotrophic environments (Schouten et al., 2008), maintain considerable levels of phospho-GDGTs in addition to glycosyl-GDGTs, as can be expected based on the model. This is in strong contrast to the findings of the environmental samples, in which the archaeal IPL pools were in many cases dominated by glycosyl-GDGTs (Rossel et al., 2008; Lipp et al., 2008; Schubotz et al., 2009). Two explanations may reconcile these contrasting results. First, the Archaea in these environments rarely undertake the entire lipid biosynthesis that would result in higher levels of phospholipids. Phospho-GDGTs may be synthesized only at trace levels each time, and are transformed very rapidly into glycosyl-GDGTs. This would explain our inability to detect phospho-GDGTs in environmental samples. Second, it is possible that Archaea, under natural conditions, may utilize other anabolic shortcuts that help them bypass the de novo biosynthesis of glycosyl-GDGTs. Based on the unbalanced ^{13}C uptake observed in our IPL-SIP

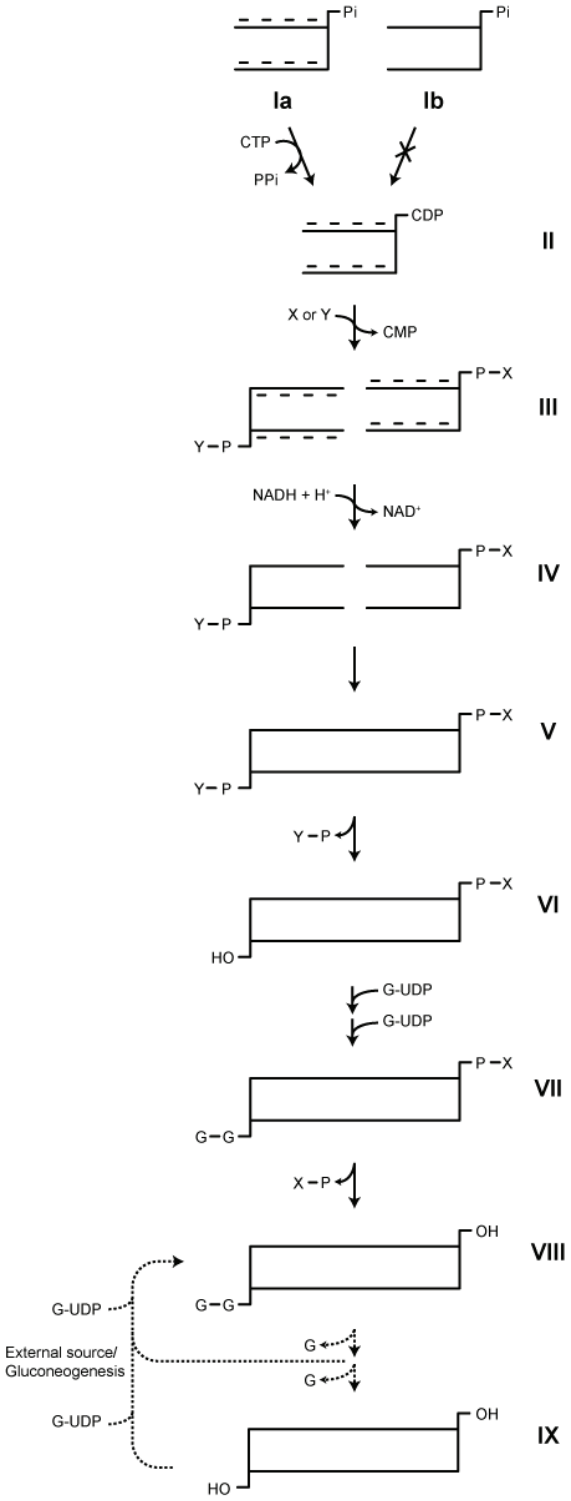

Figure 4-6. Biosynthesis of diglycosyl-glycerol dibiphytanyl glycerol tetraether (VIII). Arrows with solid line: established pathway based on studies of isolated archaea (Koga and Morii, 2007; Morii et al., 2007); arrows with dash line: proposed pathway based on the result of stable carbon isotope probing of archaeal intact polar lipids in environmental samples. G and P denote glycosidic and phosphate-containing head groups, respectively. X and Y represent the polar heads attached to the phospholipids. Abbreviations: CDP, cytidine diphosphate; CMP, cytidine monophosphate; CTP, cytidine triphosphate; NADH, nicotinamide adenine dinucleotide; PPi, diphosphate; UDP, uridine diphosphate.

experiment, we propose a shortcut that involves the formation of glycosyl GDGTs (VIII in Fig. 4-6) via reaction between existing core GDGTs (IX in Fig. 4-6) and sugars that are newly synthesized or derived from external sources. This would enable the Archaea to undertake membrane maintenance at low energy cost: for a generation of one molecule of 2Gly-GDGT, for example, the ATP requirement can be reduced greatly from 36 molecules for de novo synthesis to two molecules with this shortcut. Because glycosidic head groups are normally exposed on the outer surface of the cytoplasmic membrane (Morii and Koga, 1994), the glycosidic bonds may be subject to acidic hydrolysis caused by the localized reduction in pH during the periodic buildup of the proton gradient required for ATP synthesis. This shortcut may be an important mechanism by which Archaea in natural environments repair such damage with a reasonable energy investment. In the future, more IPL-SIP studies of natural archaeal ecosystems or starvation experiments on pure cultures will be necessary to verify the presence of such a shortcut and to identify the details of its mechanism.

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Part II

H_2-fueled carbon cycling

Chapter 5

Evaluating the extraction-based technique for determination of in situ hydrogen concentrations in marine subsurface sediment

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Abstract

Molecular hydrogen (H_2) is a key metabolic intermediate that couples organic matter degradation and terminal electron-accepting processes. The concentration of H_2 provides insights into the bioenergetics of anaerobic microorganisms and is hence an attractive parameter for understanding the subseafloor ecosystem. Generally, sedimentary H_2 concentrations were determined by the headspace equilibration technique. However, the extremely low microbial activity in marine subsurface sediment obliges the need of complementary methods that do not attempt to resume the in situ steady state during laboratory incubation. We report the evaluation of a new protocol that aims at determining the in situ H_2 concentrations in subseafloor sediment. This protocol involves an extraction step in which a slurry sample is equilibrated with a H_2-free headspace. Contamination by atmospheric H_2 through needle punctures was found to be the major source of background H_2 for this method. The method detection limit was estimated to be 35 nmol L^{-1} for our experimental setup. This method was applied in parallel to the headspace equilibration technique to determine H_2 concentrations in marine sediments where a subsurface sulfate-methane transition zone was penetrated or close to penetration. H_2 concentrations

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obtained by both methods differed by one to two orders of magnitude, but are both much higher than the thermodynamically predicted values for sulfate reducing sediment, implying a relaxation of coupling between H_2-producing and H_2-consuming activities at these sediment depths. We suggest applying both the extraction-based and headspace equilibration techniques to obtain a more complete view of the H_2 geochemistry in subseafloor sediment.

Introduction

Marine sediments contain one of the largest global reservoirs of organic carbon on Earth (Hedges and Keil, 1995) and maintain a deep biosphere consisting of viable (Schippers et al., 2005), ubiquitous (Teske, 2006), diversity-limited (Inagaki et al., 2006), and mostly uncultured prokaryotes with poorly understood physiologies and activities. Downcore distributions of redox-related chemical species suggest the presence of ongoing terminal electron-accepting processes, but the metabolic rates are several orders of magnitude lower than those detected in surface ecosystems (D'Hondt et al., 2002; J. Kallmeyer, PhD thesis). A recent study on volatile fatty acids and their isotope geochemistry provides additional evidence that degradation of organic matter coupled to reduction of inorganic carbon to both methane and acetate are taking place in subsurface sediment (Heuer et al., 2009). However, the role of elemental hydrogen (H_2), a key metabolic intermediate that couples organic matter degradation and terminal electron-accepting processes, remains poorly constrained.

Two principal processes supply H_2 within sediment in low-temperature marine sediment. At continental margins where sediment receives a continuous supply of organic matter, H_2 is generated as an end product during fermentation of organic compounds (cf. Stams and Plugge, 2009). The other is water radiolysis, a ubiquitous process that supplies H_2 (D'Hondt et al., 2009); its relative contribution increases in organic-poor sediment (Blair et al., 2007). On the other hand, the ability to utilize H_2 is widely distributed among microorganisms that use different electron acceptors, including oxygen (O_2), Fe(III), Mn(IV), sulfate (SO_4^{2-}), carbon dioxide (CO_2), and several low-molecular-weight organic compounds (Cord-Ruwisch et al., 1988). Studies on H_2 coupling between producers and consumers in cultures have given rise to the development of a

series of concepts regarding the bioenergetics of microorganisms. First, hydrogen production by fermenters is subject to inhibition by product accumulation (Stams and Plugge, 2009). When fermenters are cocultured with H_2 consumers, the latter maintains a low H_2 concentration that favors H_2 generation. Therefore, anaerobes tend to form syntrophic communities linked by interspecies H_2 transfer (Stams and Plugge, 2009). Second, in the above-mentioned syntrophic communities, a threshold H_2 concentration is maintained to keep both H_2 production and consumption thermodynamically permissive. When several researchers calculated the Gibbs free energy (ΔG) at the observed H_2 threshold, they obtained very small negative ΔG values of around -15 kJ per reaction, which were interpreted as the critical minimal energy necessary for microbial survival (Seitz et al., 1988; Conrad and Wetter, 1990; Lu et al., 2004). Finally, the H_2 threshold values usually decrease with increasing redox potentials of the H_2-consuming processes, for example in the order of methanogenesis > sulfate reduction > iron and manganese reduction > nitrate reduction. (Table 5-1). As a consequence, H_2 concentration has been proposed as a proxy for sedimentary redox potentials, which cannot be reliably measured with redox electrodes because of multiple analytical problems (Lovely and Goodwin, 1988). This is explained by a difference in the H_2 affinity, which correlates with the yield of ΔG per molecule of H_2 oxidized, among different H_2 consumers (Cord-Ruwisch et al., 1988). It should be noted that the typical values listed in Table 5-1 are based on culturing studies performed at 20°-30°C. H_2 thresholds generally decrease with temperature, parallel to the values predicted by thermodynamics (Conrad and Wetter, 1990). In summary, the insights into bioenergetics provided by H_2 concentration data, as well as its application as a redox indicator, make H_2 a particularly attractive parameter for the understanding of microbial redox processes in sediment.

One method of determining the H_2 concentrations in sediment samples is to analyze the gaseous H_2 in equilibrium with dissolved H_2 during the incubation of samples for periods that ensure the establishment of a steady state between the production and consumption of H_2. This method was initially applied to freshwater sediments amended with an excess of different electron acceptors and incubated at the in situ temperature (20°C; Lovely and Goodwin, 1988). The results matched excellently with the H_2 threshold values reported for cultures (Table 5-1). Hoehler et al. (1998) further extended this headspace equilibration technique to downcore H_2 measurement in coastal marine sediments by incubating the sediments at in situ temperatures of

Table 5-1. Threshold or steady-state H_2 concentrations determined in cultures or environmental samples with the major redox processes controlled or identified. The dissolved H_2 concentrations were determined by using the headspace equilibration technique. The incubations were performed in the temperature range of 15°-30°C

Redox process	Type of culture or sample	Dissolved H_2 (nmol L^{-1})[a]	Reference[b]
Oxygen reduction	Knallgas bacteria	0.5 – 6.2	1, 2
Nitrate reduction	Nitrate reducers	<0.05	3
	Freshwater sediments (Potomac River, MD, USA)	<0.05	4
	Marine sediments (Cape Lookout Bight, NC, USA)	0.03	5
Mn(IV) reduction	Mn(IV) reducers	0.3	6
	Freshwater sediments (Potomac River, MD, USA)	<0.05	4
Fe(III) reduction	Fe(III) reducers	0.3 – 0.6	6, 7
	Freshwater sediments (Potomac River, MD, USA)	0.2	4
Sulfate reduction	Sulfate reducers	2 – 13	3, 8
	Freshwater sediments (Potomac River, MD, USA)	1 – 1.5	4
	Marine sediments (Cape Lookout Bight, NC, USA)	1.6	5
Methanogenesis	Methanogens	6 – 70	3, 9, 10
	Freshwater sediments (Potomac River, MD, USA)	7 –10	4
	Marine sediments (Cape Lookout Bight, NC, USA)	13	5
Acetogenesis	Acetogens	70 – 1300	3, 8, 10
	Marine sediments (Cape Lookout Bight, NC, USA)	133	5

[a] When the original data for isolates were presented in the unit of molar fraction or Pa, we converted the values into dissolved concentration (nmol L^{-1}) according to the data of pressure, temperature and salinity described in the articles.

[b] References: 1 = Conrad et al. (1983); 2 = Häring and Conrad (1991); 3 = Cord-Ruwisch et al. (1988); 4 = Lovely and Goodwin (1988); 5 = Hoehler et al. (1998); 6 = Lovely et al. (1989); 7 = Klüber and Conrad (1993); 8 = Krumholz et al. (1999); 9 = Lovely (1985); 10 = Kotsyurbenko et al. (2001).

15°-27°C without an extra addition of electron acceptors. The H_2 profiles agreed well with the H_2 levels predicted by thermodynamic calculations (Table 5-1). In 2002, the same approach was applied to marine subsurface sediments retrieved during the Ocean Drilling Program Leg 201 at the Peru continental margin (D'Hondt et al., 2003). However, the measured H_2 concentrations disagreed with the thermodynamically predicted values in many cases. For example, at Site 1231, the sediment at 20-30 m below the seafloor had no detectable nitrate, an accumulation of Fe(II) and Mn(II), and no decrease in sulfate concentration in the interstitial water. Nevertheless, the H_2 concentrations reached as high as 100 nmol L^{-1}, a value that is orders of magnitude higher than the H_2 threshold of nitrate, iron and manganese reducers at the in situ temperature of 4°C. In sediments where a deep methanogenic zone was penetrated (e.g., Site 1229), the measured H_2

concentrations were barely higher than 1 nmol L^{-1} (D'Hondt et al., 2003), while our thermodynamic calculation predicted a H_2 level of 4 nmol L^{-1} for methanogenesis with a ΔG value of -15 kJ per mol methane produced at an in situ temperature of 11°C.

The discrepancy between the results from the Ocean Drilling Program Leg 201 and earlier studies questions the validity of the assumption of the headspace equilibration technique when the method is applied to marine subsurface sediment samples. The assumption is that a steady state representative of in situ equilibrium between production and consumption of H_2 can be re-established during laboratory incubation. Under steady-state conditions, the H_2 concentrations depend solely upon the physiological characteristics of H_2-consuming organisms (Lovely and Goodwin, 1988). However, in marine subsurface sediments, multiple lines of evidence suggest that the microorganisms mediating terminal electron-accepting processes constitute only a small fraction of the total communities (Batzke et al., 2007; Biddle et al., 2008; Fry et al., 2008) and metabolize at very low rates (D'Hondt et al., 2002; Parkes et al., 2005; J. Kallmeyer, PhD thesis). It is difficult to envisage a steady state being reached within an acceptable time frame in laboratory given the low microbial activities; when it does after prolonged incubation, it is not known whether the steady state is representative of the in situ condition.

An alternative method is to determine the dissolved H_2 concentration directly without attempting to reproduce steady-state conditions in laboratory. This is not a trivial task because of the low in situ H_2 concentrations and possible sampling artifacts. Table 5-2 summarizes the H_2 concentrations determined directly from some sediment samples with known geochemical conditions. The following two approaches are known to have minimal sampling artifacts for sediment samples: determination of the molar fraction of H_2 in void gas (Lorenson et al., 2006) or determination of dissolved H_2 by a gas diffusion probe (Krämer and Conrad, 1993). However, the former approach is constrained by the visibility (transparent core liners are a prerequisite) and the presence of gas voids in sediment cores, and the concentration data cannot be converted to dissolved H_2 concentrations for thermodynamic calculation. The latter technique has a reported instrumental detection limit of merely 7 nmol L^{-1}, but the value could be eventually higher given the lower gas diffusion coefficients in sediment. Additionally, the need to change the gas

Table 5-2. H_2 concentrations determined directly from sediment samples

Geochemical condition	Sediment sample	Sampling Method[a]	Dissolved or void gas H_2	Reference[b]
Oxic zone	Sediments off Baja, Mexico	A	< detection limit (11 nmol L^{-1})	1
	Marine subsurface sediments in the South Pacific Gyre	B	< detection limit (2 – 229 nmol L^{-1})	2
Sulfate reduction zone	Princess Louisa Inlet, British Columbia, Canada	A	2 – 25 nmol L^{-1}	1
	Buzzards Bay, MA, USA	A	2 – 25 nmol L^{-1}	3
	Town Cove, MA, USA	A	< 10 nmol L^{-1}	3
	Carmans River Estuary, Long Island, USA	A	20 – 30 nmol L^{-1}	4
Methanogenic zone	Skan Bay, AK, USA	A	40 – 50 nmol L^{-1}	1
	Carmans River Estuary, Long Island, USA	A	100 – 290 nmol L^{-1}	4
	Lake Mendota, WI, USA	C	20 – 40 nmol L^{-1}	5
	Lake Constance, Germany	D	10 – 60 nmol L^{-1}	6
	Hydrate Ridge, offshore Oregon, USA (ODP Leg 204)	E	20 – 920 ppmv	7

[a] Sampling methods: A = Sediments slurried in headspace vials that are sealed in a N_2-flushed glove bag. Headspace gas transferred after 20 min into pre-evacuated vials. B = Sediments extruded into headspace vials that are filled to the top with a solution. Headspace introduced by a syringe. C = Identical to A, but samples are processed in special glass flasks. D = A gas diffusion probe; E = Void gas measurement.

[b] References: 1 = Novelli et al. (1987); 2 = D'Hondt et al. (2009); 3 = Novelli et al. (1988); 4 = Michener et al. (1988); 5 = Conrad et al. (1985); 6 = Krämer and Conrad (1993); 7 = Lorenson et al. (2006).

permeable membrane frequently also makes this method less preferable for large batches of environmental samples.

Other approaches involve an extraction step in which a slurry sample is equilibrated with a H_2-free headspace (Conrad et al., 1985; Novelli et al., 1987; D'Hondt et al., 2009). When applied to samples with high microbial activities, these extraction-based approaches pose the potential problem of obtaining overestimations for the dissolved H_2 concentrations. This is because H_2 partitions preferentially into the gaseous phase, resulting in a lower dissolved H_2 concentration, and hence favoring excess H_2 production (Krämer and Conrad, 1993). Nevertheless, this type of technique would be most applicable to systems with a long H_2 residence time due to the lack of a dynamic microbial turnover (Hoehler et al., 1998). Since the marine deep biosphere is characterized by low microbial activities, we consider the extraction-based technique appropriate

and the most promising approach for delineating the distribution of in situ H_2 concentrations in subsurface sediment.

Not all the published extraction-based techniques can be easily adapted to deep biosphere research. The procedure developed by Conrad et al. (1985) requires slurry-like samples and a large sample volume of 30-50 mL, while the deeply buried sediments retrieved by coring are usually rigid and material-limited. In the protocol of Novelli et al. (1987), sediment cores need to be sampled in a glove bag. This is feasible for short multicorer and box cores but impracticable for gravity cores and the core types employed in drilling programs, as these long cores are usually cut into sections on deck and sampled immediately to minimize loss of gas by diffusion. Based on the method of Novelli et al. (1987), we tried to develop a new procedure that is dedicated for H_2 determination in deeper sediment. During the course of our method development, a similar method was reported in D'Hondt et al. (2009) and was applied to study in situ H_2 concentrations in subseafloor sediment samples. However, they report a very wide range in the detection limit (2-229 nmol L^{-1}), which makes it difficult to resolve the reported variation of H_2 concentration in sediment (Table 5-2) with their method. They selected distilled water to slurry marine sediment samples, but the potential artifacts were not assessed. Furthermore, they employed this procedure for South Pacific Gyre sediment, which lies under surface water that has a very low primary productivity, and the penetrated sediment was fully oxygenated with no evidence of anaerobic redox reactions at most stations. Since little is known about H_2 concentrations in oxygenated marine sediment compared to anoxic sediments, it is difficult to evaluate their data quality, and hence to know the applicability of the method in deep biosphere research.

In this study, we presented the results of multiple tests performed to evaluate a newly developed extraction-based technique, which has a similar procedure as that employed by D'Hondt et al. (2009). The evaluation contained two complementary parts. In the first part, we performed laboratory experiments to diagnose the source of the background H_2. These investigations allowed us to undertake the subsequent detection limit calculation and procedure improvement. The response of sediment to different types of solutions was also examined. In the second part, this extraction-based method, together with the headspace equilibration technique (Hoehler et al., 1998), was applied to measure the downcore distribution of H_2 in organic-rich

marine sediments where the sulfate-methane transition zone (SMTZ) was either penetrated or reached. We showed that both techniques generated H_2 profiles that differed in concentration level by one to two orders of magnitude, but had several similarities in the overall trend.

Materials and Procedures

Instrumentation

H_2 concentration was determined by gas chromatography with mercury oxide detection, using a Peak Performer 1 (Peak Laboratories, LLC, USA). The instrument was calibrated with a 10 ppm H_2 primary standard (Air Liquide, Germany) on a daily basis. Typically, more than 3 mL and 1 mL of gas sample was injected to thoroughly flush the 1 mL and 0.1 mL sample loops, respectively, and the tubing between the injection port and the loop. The instrumental detection limit, evaluated statistically by a serial dilution of the primary standard with H_2-free N_2, the bypass gas out of the Peak Performer 1, is about 8 ppb.

Extraction-based technique

Our procedure was identical to that described in D'Hondt et al. (2009) with some modifications. A sediment sample of 2-3 mL was extruded into a 22 mL headspace vial, which was immediately filled with a solution to the top, sealed with a thin butyl stopper, and crimp capped. The sampling and preparation steps typically took less than 1 min to minimize the diffusion loss of gas. The choice of solution and its preparation were investigated in the present study (see below). A blank was a vial filled with the same solution but without sediment. The thin butyl stoppers were favored over other flat-bottomed stoppers because of their concave-down shape at their bottom side that allows gas bubbles to escape easily. A headspace was created by displacing 5-7 mL of the aqueous phase with an equal volume of H_2-free N_2. Once the headspace reached the intended volume, the gas-in needle was first removed, and the liquid-out needle, connected to a syringe, was allowed to equilibrate with the overpressure in the vial headspace; the volume offset in the liquid-out syringe was catalogued. The vial was then vortexed, turned

upside-down, and allowed to sit for 20 min to let H_2 diffuse out of the interstitial water and equilibrate with the headspace. The choice of 20 min instead of 24 h equilibration time (D'Hondt et al., 2009) was made according to the recommendation of Novelli et al. (1987). For H_2 analysis, the headspace gas was displaced into a N_2-flushed plastic syringe by injecting into the vial the same volume of the solution used to prepare sediment slurries. Needles for transferring solution in or out of the vials had a gauge of 23 and a length of $2^3/_8$ or $3^1/_8$ in. Needles for transferring gas in or out of the vials have a gauge of 26 and a length of 1 in. Care was taken not to evacuate the headspace during the gas sampling step; otherwise, we observed that atmospheric H_2 could be sucked into the vial through the stopper, leading to erroneously high H_2 signals.

Headspace equilibration technique

The procedure published in Hoehler et al. (1998) was followed to determine H_2 concentrations in incubated sediment samples. In brief, a sediment sample of 2-3 mL was extruded into a 22 mL headspace vial, immediately sealed with a thick black butyl stopper, crimp capped, and flushed with N_2 (purity = 99.999%) for at least 1 min. The samples were incubated in the dark at the in situ temperature of 4°C in our research area and analyzed every 1-3 days until an approximate steady state was reached. To avoid evacuating the headspace by repeated removal of headspace gas, 1 mL of H_2-free N_2 was injected into the headspace immediately following the removal of headspace gas to ensure a constant headspace gas pressure.

Sediment sample collection, processing and analysis

H_2 determination was performed on board during expedition M76/1 (April – May 2008) of the RV *Meteor* at the continental margin off the coast of Namibia. After retrieval, the multicorer cores were immediately processed on deck by extruding the sediment upwards by measured increments and sampling the freshly exposed sediment surface. The gravity cores were first cut into 1 m segments and syringe samples were taken from every cut segment base for gas analysis. The gravity cores were then transferred to a 4°C cold room, where further samples for gas analysis were taken within a few hours after the core recovery. Small sampling ports (ca. 2 × 3 cm) were cut into the core liner to retrieve the sample. Typically, every sediment surface was

penetrated by several 3 mL cut-off plastic syringes for the following gas analyses: H_2 by the extraction-based technique, H_2 by the headspace equilibration technique, and dissolved CH_4. The samples were extruded into individual headspace vials and sealed according to the specified procedures.

Dissolved CH_4 concentrations were analyzed following the previously published protocol (D'Hondt et al. 2003). Porosity was measured on sediment samples using the approach of Blume (1997).

Calculation

H_2 concentrations in the interstitial water were calculated differently from the data generated by the extraction-based and the headspace equilibration techniques. The first step for both methods was to convert H_2 concentrations in the headspace from molar fractions to molar concentrations ($[H_2]_g$):

$$[H_2]_g = \chi_{H2} \times P \times R^{-1} \times T^{-1} \qquad (1)$$

where $[H_2]_g$ is expressed as nmol L^{-1}, χ_{H2} is the molar fraction of H_2 in the headspace gas (in ppb, obtained from chromatographic analysis), P is the total gas pressure (in atm) in the headspace, R is the universal gas constant, and T is the incubation temperature in degrees Kelvin. To calculate the porewater H_2 concentrations determined from the incubated sediment by the headspace equilibration technique ($[H_2]_{incub}$), the following equation was used:

$$[H_2]_{incub} = \beta \times [H_2]_g \qquad (2)$$

Here $[H_2]_{incub}$ is expressed in nmol L^{-1}. β is an experimentally determined solubility constant corrected for temperature and salinity (Crozier and Yamamoto, 1974). The value of β is 0.01737 for seawater (salinity = 35 parts per thousand) at 4°C. To calculate the porewater H_2 concentrations determined by the extraction-based technique ($[H_2]_{extract}$), another equation was used:

$$[H_2]_{extract} = ([H_2]_g \times V_g + [H_2]_{aq} \times V_{aq}) \times V_{sed}^{-1} \times \phi^{-1} \qquad (3)$$

All H_2 concentrations were expressed in nmol L^{-1}. $[H_2]_g$ was calculated using Equation 1. $[H_2]_{aq}$, the H_2 concentration in the aqueous phase, was obtained using Equation 2 with $[H_2]_{incub}$ replaced by $[H_2]_{aq}$. The β values for pure water and 3.5% NaCl at 25°C are 0.01744 and 0.01499,

respectively (Crozier and Yamamoto, 1974). In the case where saturated NaCl solution (salinity = 35%) was used as the solution, the β value corrected for the "salting-out effect" was estimated by the Sechenov equation with the Sechenov constant calculated by the empirical model described in Weisenberger and Schumpe (1996). We obtained a β value of 0.00423 for H_2 in saturated NaCl at 25°C. This value was used for the calculation of $[H_2]_{aq}$. V_g represents the volume of the headspace and V_{aq} the volume of the aqueous phase, including the porewater and the solution added. V_{sed} is the volume of the sediment sample, and ϕ is the sediment porosity.

To predict the H_2 values dictated by the thermodynamics of terminal electron-accepting processes in marine sediments, we calculated the ΔG° of hydrogenotrophic sulfate reduction and methanogenesis under the in situ condition of pressure = 10 MPa, temperature = 4°C using the software package SUPCRT92 (Johnson et al., 1992) and the thermodynamic data from Shock and Helgeson (1990). With the free energy of nonstandard state (ΔG) set at -15kJ/reaction, we computed the corresponding H_2 concentrations by recasting the equation $\Delta G = \Delta G° + R \cdot T \cdot \ln Q$ and solving the H_2 term in Q, which is the activity quotient of the reactants and reaction products. The activity of chemical species was approximated by molar concentrations without correcting for the solution's ionic strength. Other data required for thermodynamic calculation, i.e., the concentrations of sulfate, sulfide, dissolved inorganic carbon, and pH, will be published elsewhere (Goldhammer et al. in prep.).

Assessment

Background H_2 in solution

The solution used to fill headspace vials is critical to the extraction-based technique for three reasons. First, the solution may carry background H_2 which would lead to overestimation of the sample signal. The extraction-based technique employed in this study would be particularly sensitive to background H_2 in solution because the volume ratio of added solution to sediment sample is much higher than other published extraction-based methods (Conrad et al., 1985; Novelli et al., 1987). Second, the solution should not interact with any component of the system,

including the septa or stopper, the hypodermic needles, the sediment, among others, to produce or consume H_2. Third, an ideal solution would be one that also acts to stop or retard the microbial reactions in the sediment, so that the headspace-induced excess H_2 production described above could be avoided.

A saturated $HgCl_2$ solution has been applied to determine H_2 concentrations in water samples (e.g., Scranton et al., 1984). However, Novelli et al (1987) report that both $HgCl_2$ and $CdCl_2$ have either failed to inhibit bacterial activity or created artifacts in sediment samples. Conrad et al. (1985) also observe that addition of NaOH (final concentration = 0.5 mmol L^{-1}) or glutaraldehyde (final concentration = 2.5%) resulted in an increase in the H_2 concentration. For safety reasons it is preferable not to use hazardous chemicals because some spillage onto the work bench is inevitable when filling up the headspace vials to the top. D'Hondt et al. (2009) used distilled water for sample processing. In addition to distilled water, we included 3.5% and 35% NaCl solutions into our following tests. 3.5% NaCl solution represents the salinity of seawater and was considered pertinent for processing marine sediment; saturated NaCl solution has a salting-out effect and can inhibit biological activity in normal marine sediments where the microbial groups are adapted to seawater salinity.

We first measured the background H_2 concentration in the three solutions after different treatments. The dissolved H_2 concentrations in the selected solutions at equilibrium with the atmospheric H_2 partial pressure (530 ppb; Novelli et al., 1999) can be calculated using Equations 1 and 2. The values are 0.4, 0.3, and 0.1 nmol L^{-1} for distilled water, 3.5% NaCl and 35% NaCl, respectively (Table 5-3). The measured concentrations in fresh solutions (i.e., fresh distilled water from the laboratory tap and freshly dissolved NaCl crystals in distilled water in a glass bottle) were 5-45 times higher than these values. There are three potential sources for such a high H_2 background:

(1) The solutions contained extra H_2 and did not reach equilibrium with the atmosphere. For example, atmospheric H_2 may have been stripped into the salt solution to form microbubbles when the solution was shaken vigorously to dissolve the salt crystals (Krämer and Conrad, 1993).

Table 5-3. The background H_2 concentrations in distilled water, 3.5% NaCl and 35% NaCl after different treatments and the extracted H_2 concentrations from sediment

Group of tests Calculation or experiment	Distilled water	3.5% NaCl	35% NaCl
1. Background H_2 in solutions			
a. Calculated $[H_2]_{aq}$ when the solution is equilibrated with H_2 in the atmosphere (530 ppb)[1]	0.4	0.3	0.1
b. Freshly prepared solution, without bubbling	1.9 ± 1.2	1.4 ± 0.7	4.4 ± 0.6
c. Equilibrated with the atmosphere for >5 hrs	1.5 ± 1.1	1.5 ± 0.6	2.3 ± 1.6
d. Stir for 3 hrs	1.6 ± 1.2	1.7 ± 0.8	0.7 ± 0.1
e. Treatment c + bubbled with N_2 for >20 min	2.0 ± 1.4	0.8 ± 0.1	1.2 ± 0.9
f. Treatment c + stored in a 50-mL plastic syringe for <5 min	1.0 ± 1.3	0.6 ± 0.6	1.3 ± 1.2
g. Treatment c + stored in a 50-mL plastic syringe for 20-30 min	0.8 ± 0.4	1.5 ± 0.6	1.6 ± 0.5
h. Treatment c/d + minimizing the gas stripping by having a tubing attached to the syringe tip	1.0 ± 0.8	1.5 ± 1.2	2.1 ± 1.2
3. Interaction of solutions with sediment and cultures			
a. Tidal flat sediment from the North Sea. Solution blank see Experiment 1f	4.6 ± 1.9	16.8 ± 3.8	4.1 ± 3.1

[1] The global average H_2 concentration in the atmosphere is from Novelli et al. (1999). The $[H_2]_{aq}$ was calculated using the Bunsen constants (Crozier and Yamamoto, 1974) for distilled water and 3.5% NaCl. The salting-out effect of 35% NaCl was estimated using the procedure described in Weisenberger and Schumpe (1996).

(2) Atmospheric H_2 may have been stripped into the solution when the headspace vial was being filled to the top.

(3) Atmospheric H_2 may have contaminated the headspace of the vials.

These possibilities were examined in the following tests.

The background H_2 concentrations in 35% NaCl solution decreased to half and one sixth of its original value, respectively, when the freshly prepared solution was left under atmosphere for >5 h without any physical disturbance (shaking or stirring) or was stirred for 3 hrs. This is an indication that a fresh 35% salt solution contains excess H_2 and needs to be equilibrated with the atmosphere before use. The background H_2 concentrations did not decline further when the 5 hr equilibration was followed by bubbling with N_2 for >20 min. These treatments made no significant difference in the other two solutions.

The processing of sediment cores and the preparation of solutions typically took place in separate sites on the ship. To convey solutions between these two locations, we used 50 mL plastic syringes with its Luer tip fitted with a two-way plastic valve. The background H_2 concentrations did not increase after 20-30 min of storage in the syringe (Table 5-3).

Finally, we tried to minimize the gas stripping when filling up headspace vials by attaching 7 cm long plastic tubing to the tip of the two-way plastic valve fitted to the 50 mL syringe. To fill a headspace vial, the end of the tubing was placed as close to the bottom of the vial as possible, and was maintained at the interface between the headspace and the liquid phase while the liquid level was increased. The background H_2 concentrations after this treatment did not decrease for any of the three solutions, suggesting that the contribution of gas stripping during this step was negligible.

In summary, the background H_2 concentration in distilled water and the 3.5% NaCl solution did not change under different treatment. The background in the 35% NaCl solution could be significantly lowered by stirring. The measured solution background in equilibrated solutions is around 1.4±1 nmol L^{-1}.

Contamination of the container headspace by atmospheric H_2

So far we have only considered the background H_2 concentrations in the solutions. The container headspace could also be contaminated directly by atmospheric H_2 in two possible ways. The first process, the permeation of H_2 through the glass wall, can be evaluated when the gas permeability constant is known. The temperature dependence of the permeability constant of a gas through a solid (Φ) is defined as follows (Souers et al., 1978):

$$\Phi = \Phi_0 \times T \times e^{(-Q/T)} \qquad (4)$$

Φ is expressed in mol cm cm^{-2} min^{-1} atm^{-1}, Φ_0 and Q are gas- and solid-dependent constants, and T is the absolute temperature. Assuming that the headspace vials used in this study were made of the most common soda-lime glass, we used the Φ_0 and Q values for H_2 provided in Souers et al. (1978) and obtained an Φ value of 2.5×10^{-5} *p*mol cm cm^{-2} min^{-1} atm^{-1} at 25°C. The next step was

to calculate the amount of gas molecules that permeated into the container after a defined period of time by solving the recast general gas permeability equation:

$$n = \Phi \times A \times t \times \Delta P \times d^{-1} \qquad (5)$$

where n is the amount of gas molecules, A is the contact area between the solid and the gas, t is the length of the permeation time, ΔP is the difference in partial pressure of the gas between both sides of the solid, and d is the thickness of the solid. For a 22 mL headspace vial that has a wall thickness of 0.11 cm, a contact area to air of 13 cm^2 for a 5 mL headspace and a 20 min permeation time, the amount of H_2 molecules entering from the air into the container is 3×10^{-8} *p*mol, which would result in an increase of the solution background by only 2×10^{-9} nmol L^{-1}. We concluded that the contribution from such gas permeation to the background H_2 concentration was negligible.

Another possible source of H_2 contamination to the container headspace is via gas leakage. Since the vials were turned upside-down during the 20 min equilibration time, it is unlikely that leakage through the septa took place during this period. Instead, we speculated that leakage could have taken place upon puncturing the needles through the gray butyl stoppers. We tried to test for this by the following experiment (Fig. 5-1). A series of empty 11 mL headspace vials was sealed with stoppers, crimp capped, evacuated and flushed three times with H_2-free N_2 (step a). These headspace vials were punctured by gauge 23 and 26 needles, twice by each, at either 0 min (step c) or immediately before measurement (steps e). The needles used to puncture the septa were connected to N_2-flushed syringes. The control vials had no additional needle punctures (step b). All the vials were allowed to sit with their tops immersed in distilled water during the waiting time (steps b, d and e). Gas samples were withdrawn from the vials by injecting the same volume of distilled water (step g). The results showed that the headspace H_2 concentrations were much higher in vials with extra needle punctures, and the degree of increase was not related to the 20 min equilibration time, verifying our conjecture that leakage takes place at the time when a septum is punctured (Table 5-4). Assuming that such a H_2 leakage is independent of the volume of the gaseous phase, the total amount of H_2 in the 11 mL headspace vials was divided by a solution volume of 17 mL (a 22 mL headspace vial with 5 mL of headspace). The dissolved H_2 concentrations calculated from the amount of H_2 detected in the leakage experiments (Table 5-4) already explain the detected solution background values (Table 5-3).

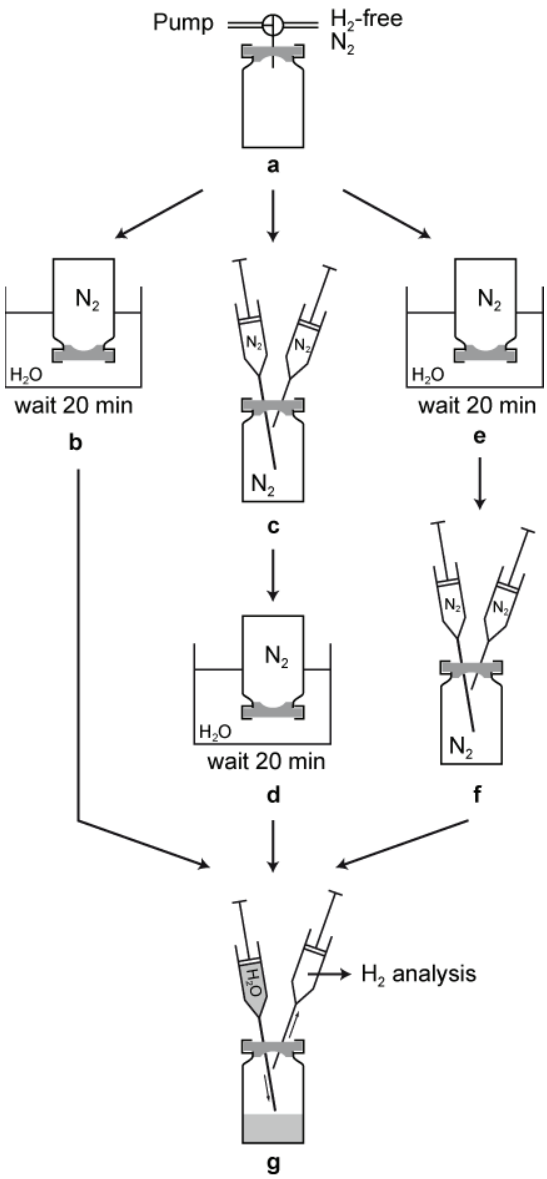

Figure 5-1. Schematic diagram of the H_2 leakage experiment. See text for details.

Evaluation of detection limit

To evaluate the detection limit of the extraction-based technique, we needed to first calculate the blank H_2 concentration, which is the amount of H_2 detected in sediment-free samples divided by a porewater volume representative of real samples. Our analyses suggest that

Table 5-4. Test of H_2 leakage into 11-mL solution-free headspace vials. Assuming H_2 leakage is independent of the volumes of gaseous phase, the amount of H_2 detected in the 11-mL headspace vials was divided with an aqueous phase volume of 17 mL (22 mL headspace vial with a 5 mL headspace) to acquire the corresponding dissolved H_2 concentrations

Group of tests Treatment	H_2 in headspace, ppb	Corresponding dissolved $[H_2]$ in solution, nmol L^{-1}
2. Contamination of container headspace by air		
a. Control, wait 20 min	26 ± 4.9	0.7 ± 0.1
b. Puncture the septum with needles of gauge 23 and 26, twice of each, wait 20 min	155 ± 101	3.4 ± 2.7
c. Wait 20 min, puncture the septum with needles of gauge 23 and 26, twice of each	116 ± 94	2.4 ± 2.5

the background H_2 mainly comes from leakage through the septa rather than from solution. When a fully equilibrated solution is used, the background H_2 is independent of the volume of solution. The measured average solution background was 1.4±1 nmol L^{-1} H_2, which is roughly equivalent to 24±17 pmol H_2 per sample vial (a 22 mL vial with 17 mL solution). When this amount of background H_2 was divided by 2.1 mL of porewater (3 mL sediment with a porosity of 0.7) and the error propagated accordingly, we obtained a blank H_2 concentration of 11±8 nmol L^{-1}. A statistically significant sample signal would be 35 nmol L^{-1} following the conventional equation Detection Limit = Blank + (3 × 1σ). The only way to decrease the blank and to improve the detection limit is to increase the volume of the sediment sample. For example, when the volume of sediment doubles, the blank would be 6±4 nmol L^{-1} and the detection limit then becomes 20 nmol L^{-1}. Changing the volumes of vials and headspace will not affect the blank since the amount of background H_2 is independent of these parameters.

For clarification, in the following text we present blank-corrected H_2 concentrations for sediment samples with an additional term describing the significance or the raw data (> or < detection limit).

Interaction of solutions with sediments

A major difference among distilled water, 3.5% NaCl, and 35% NaCl is that they will exert different osmotic pressures on cells. Under extreme osmotic pressures, the activity of cells can be slowed or stopped, as is often observed in the Na^+ concentration gradient tests performed on new isolates (e.g., Sowers and Ferry, 1983). Therefore, we expect a difference in the H_2 concentrations obtained with 3.5% NaCl and the other two solutions in sediment samples, since 3.5% NaCl solution is unlikely to inhibit the metabolism of marine microorganisms and headspace-induced excess H_2 production is likely to occur.

The effect of solutions on sedimentary H_2 concentrations was evaluated using tidal flat sediment from the North Sea. The sample was retrieved from a depth of 10-20 cm below the surface where the sediment appeared dark gray, a color indicative of sulfide minerals precipitated in the course of sulfate reduction. The obtained H_2 concentrations were around 4 nmol L^{-1} when the sediment was treated with distilled water or 35% NaCl, but were four times higher (17 nmol L^{-1}) when 3.5% NaCl was used (Table 5-3). Although these values are all below the detection limit, the values obtained with 3.5% NaCl are significantly higher than those obtained with the other two solutions. The result is in agreement with our expectation that the headspace-induced excess H_2 production would take place in the sediment treated with 3.5% NaCl solution. However, for subseafloor sediment where the H_2-producing organisms are presumably less active, it is likely that sediment mixed with 3.5% NaCl solution will not necessarily give higher H_2 concentrations.

It is unclear whether distilled water and 35% NaCl stop the H_2-producing and H_2-consuming activities at a different speed, a speculation put forward by Krämer and Conrad (1993) to explain the high H_2 concentrations when sediment samples were treated with killing reagents. Testing these two solutions with pure cultures of different metabolic activities and at different growth stages will be necessary to clarify the possibility of such an artifact.

Application to marine subsurface sediment samples

During the expedition M76/1, we determined H_2 concentrations in sediment samples using both the extraction-based technique and the headspace equilibration technique of Hoehler et al.

(1998). Since we did not observe any artifacts associated with the use of a 35% NaCl solution during laboratory evaluation, we used this solution to prepare the samples so that the concern of headspace-induced excess H_2 production could be minimized. Since the headspace equilibration technique is limited by the incubation time available onboard, we present H_2 profiles only from two stations (GeoB 12802 and 12803) that were drilled during the early stage of the cruise and where the SMTZ at both sites was penetrated or was close to penetration (Fig. 5-2).

At both stations, the two methods generated H_2 profiles with concentration ranges differing from each other by one to two orders of magnitude, but there are several similarities observable in the overall trend. The extraction-based technique gave higher estimates of H_2 concentrations. More than 50% of the data presented in the two profiles are above the detection limit. The downcore distribution of the extractable H_2 pool at both stations does not have an obvious correlation with the sulfate and methane profiles; instead, both have a minimum of 20 nmol L^{-1} (< detection limit) at the sediment surface and a maximum of around 200-240 nmol L^{-1} within the sulfate reduction zone. At GeoB 12802, H_2 concentrations of the samples taken in the cool room followed the profile defined by the samples taken on deck. At GeoB 12803, measurements on the cool-room samples taken at a higher resolution at 300-400 cm below the seafloor (cmbsf) delineated a H_2 minimum located at 370 cmbsf, but no apparent excursion at the same depth was found in the profiles of other geochemical parameters (sulfate, sulfide, methane, dissolved inorganic carbon, and pH).

The profiles generated by the headspace equilibration technique did not always follow the H_2 range predicted by thermodynamic calculation. The sediment surface at both stations had the lowest H_2 values. In the sulfate reduction zone at GeoB 12802, the H_2 concentrations were much higher than the predicted values by one to two orders of magnitude. The maximum at 27 cmbsf coincided with the maximum in the extractable pool. Whereas the H_2 level in the extractable pool was below the detection limit in sediment deeper than 300 cmbsf at GeoB 12802, the equilibrated H_2 values increased downward and seemed to match the thermodynamically predicted values. The excursion of 14.6 nmol L^{-1} at 260 cmbsf, close to the SMTZ, was not observed in the extractable pool. Such a high H_2 concentration would be a strong driving force for both methanogenesis and homoacetogenesis, but acetate concentration at this depth was below the

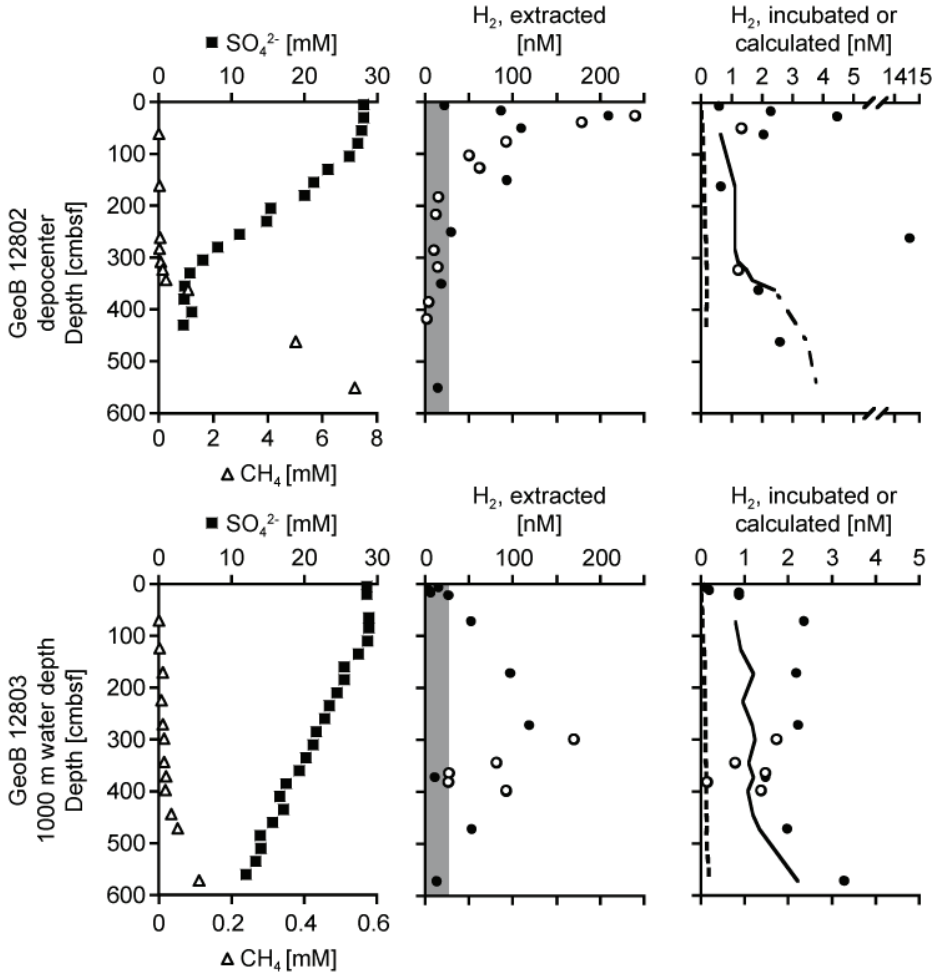

Figure 5-2. Depth profiles of sulfate, methane and hydrogen concentrations in sediment interstitial waters at GeoB 12802 and 12803, offshore Namibia. Solid and open dots represent H_2 samples take on deck and in the cold room, respectively. The shaded areas mark the detection limit of the extraction-based technique. The sediment samples at GeoB 12802 and 12803 were incubated at 4°C for 25 and 23 days, respectively. Dash and solid lines are the predicted H_2 values for hydrogenotrophic sulfate reduction and methanogenesis, respectively. There are no data of dissolved inorganic carbon (DIC) concentration available for the bottom 200 cm sediment at GeoB 12802, and calculation of H_2 values for methanogenesis at these depths (dash-dotted line) was based on the measured DIC value at 362 cm. The ΔG value for thermodynamic calculation was set at -15 kJ/reaction.

detection limit (V. Heuer, unpubl. data). At GeoB 12803, where we reached only the upper SMTZ, the H_2 concentrations were all substantially higher than the predicted values except at 382 cmbsf, where a H_2 minimum of 0.16 nmol L^{-1} was observed. Interestingly, this minimum not only agrees with the thermodynamically predicted values, but also coincides with the minimum in the extractable H_2 pool.

Discussion

The extraction-based technique was first evaluated in laboratory to diagnose the sources of background H_2. The importance of characterizing the source of background H_2 is two-fold: First, it informs us of the correct way to calculate the blank and the detection limit of the sampling procedure. Second, it indicates the strategy required to improve the detection limit. Our experiments demonstrated that 35% NaCl solution needs to be equilibrated with the atmosphere to remove excess H_2 before being used to fill the headspace vials. When an equilibrated solution is used, most of the background H_2 is introduced into the vials upon puncturing the septa with needles. The detection limit is therefore independent of the volume of added solution but related to the frequency of needle puncture, the type of septa, and the volume of porewater. The detection limit for the analytical condition employed in this study was 35 nmol L^{-1}, meaning that the method would not be able to resolve the H_2 variation if the sedimentary H_2 concentrations were fully controlled by thermodynamics.

Surprisingly, more than 50% of the H_2 values obtained by the extraction-based technique were above the detection limit. The results from the headspace equilibration technique also showed the H_2 concentrations to be usually higher than the thermodynamically predicted value, especially in the sulfate reduction zone. Furthermore, some subsurface H_2 maxima in the extractable pool (e.g., at GeoB 12802) corresponded to a higher H_2 value obtained after only a few weeks of incubation. All these lines of evidence suggest that the in situ H_2 concentrations are unlikely to be thermodynamically controlled by the terminal electron-accepting processes, but rather imply a relaxation of coupling between H_2-producing and H_2-consuming activities. This interpretation agrees with the microbiological observation that microbes mediating terminal

electron-accepting processes in marine subsurface sediment have a much lower activity than those in the surface ecosystem (D'Hondt et al., 2002; J. Kallmeyer, PhD thesis). On the other hand, thermodynamics will also set an upper H_2 limit for fermenters which are known to be inhibited by their own metabolic end product (Stams and Plugge, 2009). Since concentrations of volatile fatty acids were below the detection limit of around 3-5 μmol L^{-1} (Heuer et al., 2006) in samples from these two stations, we assumed a concentration of 1 μmol L^{-1} for butyrate, propionate and acetate, and calculated the corresponding H_2 concentration under the condition $[HCO_3^-]$ = 3 mmol L^{-1}, pH = 7.5, and ΔG = 0. The results showed that fermentation of butyrate and propionate remains exergonic with H_2 concentrations up to 48 and 33 nmol L^{-1}, respectively. The measured H_2 values in the incubated sediments were far below these thresholds, suggesting that fermentation would have been able to proceed despite the weak coupling with the H_2 consumers. The maximal value of 200-240 nmol L^{-1} acquired by extraction suggests that there were H_2 sources other than fermentation (e.g., water radiolysis), or that H_2 pools other than the dissolved fraction were extracted. Our data did not enable us to distinguish these possibilities.

On the other hand, there were a few cases where the measured H_2 concentrations were close to the thermodynamically predicted values. One example is the near-surface sediment. The uppermost sediment samples collected during the cruise were actually taken from 4-9 cmbsf. In most stations visited during the expedition, these uppermost sediment samples yielded the lowest H_2 concentrations using both techniques. It is unlikely that fermenters were relatively inactive in these near-surface samples, since the labile organic substrate content is normally highest in the uppermost sediment. Therefore, the low H_2 concentrations observed at shallow depths are attributed to a more active population of H_2 consumers maintaining a tight coupling with fermenters. Another example is the subsurface minimum at GeoB 12803. If only the H_2 profiles generated by the headspace equilibration method were available for interpretation, one may speculate that the minimum was caused by microorganisms activated during laboratory incubation. However, since this minimum was also present in the extractable pool, it conceivably reflects some in situ extreme in H_2 cycling. This particular sediment interval behaved similarly during laboratory incubation. Nevertheless, during the entire cruise, a subsurface minimum was found only at GeoB 12803, suggesting that this is not a common situation. We are investigating

the microbial community at this sediment depth to see if sequences affiliated with H_2-consuming organisms can be found to explain this local H_2 minimum.

Comments and Recommendations

We demonstrated the opportunities and constraints of the extraction-based technique. For future campaigns, we suggest the choice of one or both of the two methods, depending on (1) the expected microbial activity in the sediment, and (2) the duration of the expedition. For example, in sediments where high microbial activities are expected, such as those near hydrothermal vents, gas seeps, or at shallow subseafloor depths (tenths of cm), the headspace equilibration technique is the recommended method since the likelihood of achieving steady-state conditions within a short period of incubation is higher. Furthermore, according to our evaluation, the usually low steady-state H_2 concentrations cannot be adequately determined by the extraction-based approach according to our evaluation. However, for sediments with low microbial activities, such as those that cover the vast ocean basins, we suggest that the extraction-based technique be used as the major approach for shorter cruises of up to one month, and that it can be a complementary approach onboard for longer cruises. For the shorter cruises, the incubation of sediment samples can be initiated on board, but monitoring of the H_2 concentrations needs to be continued following the cruise to ensure that an apparent steady state has been reached. If there is sufficient manpower, we highly recommend the application of both H_2 determination methods. The extraction-based technique provides a ‘snapshot’ of in situ distribution that probably cannot be reproduced under laboratory conditions but can be biased by the higher uncertainties inherent to the method, whereas the headspace equilibration technique hints at whether the sediment obeys to thermodynamic control and provides insight into understanding the snapshot generated by the other method.

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Chapter 6

Microbial formation of methylated sulfides in the anoxic sediment of Lake Plußsee, Germany

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Abstract

In anoxic environments, volatile methylated sulfides including methanethiol (MT) and dimethyl sulfide (DMS) link the cycles of inorganic and organic carbon and sulfur. However, the mechanistic relationships between CO_2, H_2 and methylated sulfides are not fully understood. During examination of the hydrogenotrophic microbial activity at elevated temperature in anoxic sediment of Lake Plußsee, DMS levels rose six-fold when supplemented with both H_2 and bicarbonate, whereas MT levels declined slightly. Methanogenesis was suppressed in the presence of H_2, but acetate accumulation increased 2.7-fold. The observed accumulation of DMS and MT could not be further enhanced by addition of methyl-group carrying potential reactants such as syringic acid in combination with sodium sulfide. Addition of 2-bromoethanesulfonate inhibited DMS formation and caused slight MT accumulation. MT and DMS had average $\delta^{13}C$ values of –55‰ and –62‰, respectively. Labeling with $NaH^{13}CO_3$ indicates that incorporation of bicarbonate-derived C into DMS occurred through methylation of MT. Labeling with $H_2{}^{35}S$ demonstrated a slow process of hydrogen sulfide methylation that accounted for <10% of the observed accumulation rate of DMS. Our data suggest: (1) methanogens are involved in DMS formation from bicarbonate, (2) the major source of the ^{13}C-depleted MT is neither bicarbonate nor methoxylated aromatic compounds. Other possibilities for isotopically light MT, such as

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demethylation of ^{13}C-depleted DMS or other organic precursors, were discussed. The observed DMS-forming process may be relevant for other anoxic environments that have similar physiochemical conditions such as hydrothermal vents or sulfate-methane transition zones in marine sediments.

Introduction

Among volatile organic sulfur compounds, methylated sulfides including dimethyl sulfide (DMS) and, to a lesser extent, methanethiol (MT) are the most abundant components. The biogeochemical processes of methylated sulfides in ocean surface waters have received particular attention because of the connection between DMS and climate (Charlson et al., 1987). In anoxic environments, DMS and MT link carbon and sulfur cycles in versatile manners. In contrast to complex organic sulfur compounds formed during early diagenesis that are refractory to biodegradation (Ferdelman et al., 1991), DMS and MT remain reactive and available for microbial processes. Their role as intermediates during remineralization of organic matter has been elaborated in earlier studies and is briefly summarized below and in Fig. 6-1. Decomposition of S-methyl compounds such as dimethylsulfoniopropionate (DMSP) and amino acids initially yields DMS or MT (Kiene et al., 1990), both of which can be further catabolized by coupling with terminal electron-accepting processes. Isolated microorganisms that are known to degrade methylated sulfides include denitrifying bacteria (Visscher and Taylor, 1993), sulfate reducing bacteria (Tanimoto and Bak, 1994) and methanogens (Lomans et al., 1999a). During degradation of DMS, MT usually accumulates transiently as an intermediate (Lomans et al., 1999b). Whether methylated sulfides can be used to synthesize protein-sulfur during anabolism in anoxic environments is not clear, although evidence exists that pelagic marine bacterioplankton preferentially assimilate methylated sulfides over sulfate or hydrogen sulfide (Kiene et al., 1999).

In addition to these ‘traditional’ sources and sinks, more unconventional processes exist in which methylated sulfides couple both organic and inorganic carbon to the sulfur cycle, either biologically and/or abiotically (Fig. 6-1). First, O-methyl groups can be transferred microbially to

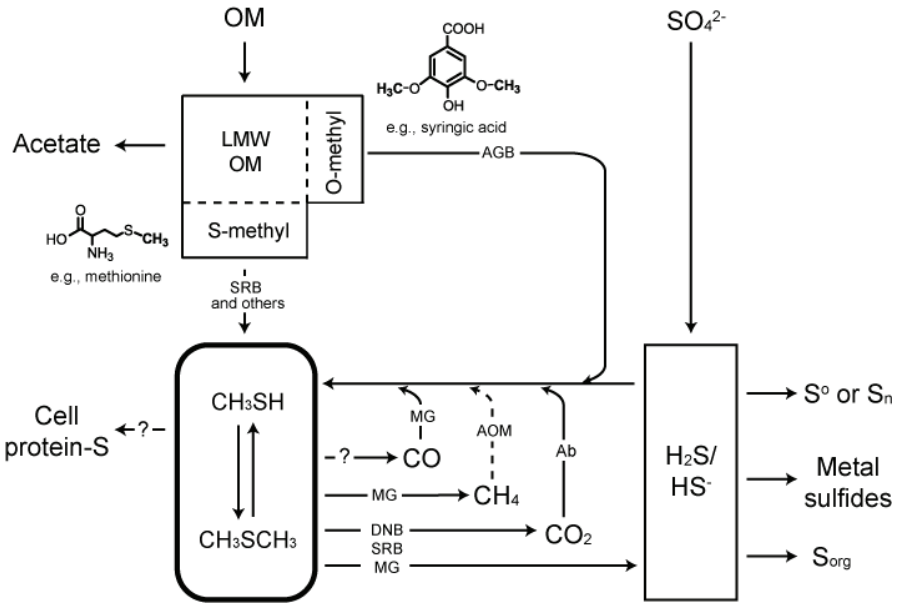

Figure 6-1. A schematic diagram summarizing geochemical processes linked by methylated sulfides in anoxic environments. Arrows with dashed line: hypothesized processes, arrows with a question mark: processes that have not been examined. See text for detailed discussion. The structures of syringic acid and methionine are also shown as examples of compounds with O- and S-methyl groups, respectively. Abbreviations: Ab, abiotic process; AGB, acetogenic bacteria; AOM, anaerobic oxidation of methane; DNB, denitrifying bacteria; LMW, low molecular weight; MG, methanogens; OM, organic matter; S_{org}: sulfurized OM formed during early diagenesis; SRB, sulfate reducing bacteria.

H_2S/HS^- (ΣH_2S hereafter) to form MT, with additional methylation under certain circumstances yielding DMS (Lomans et al., 2002). Known O-methyl donors include methanol (van Leerdam et al., 2006) and methoxylated aromatic compounds such as lignin monomers (Lomans et al., 2002). This process is proposed to be the main mechanism contributing to methylated sulfides in DMSP-limited freshwater sediments, as evidenced by the strong correlation between concentrations of methylated sulfides and ΣH_2S in lake sediment (Lomans et al., 1997). Second, based on the study of trace methane oxidation of *Methanosarcina acetivorans*, Moran et al. (2007) hypothesized that during anaerobic oxidation of methane (AOM), methane is converted to methylated sulfides, which are subsequently oxidized by sulfate reducers. It is currently not known if and how such a

coupling exactly takes place in AOM organisms. Third, conversion of CO to MT and DMS has also been observed in *M. acetivorans* (Moran et al., 2008), and represents the first finding of microbially mediated incorporation of inorganic carbon into methylated sulfides. The process probably proceeds via stepwise reduction of CO as coenzyme-bound intermediates to form a methyl group and could be coupled to energy conservation via a chemiosmotic mechanism. However, this process has not yet been studied using sediment samples, and the lack of knowledge on CO abundance in environment hampers evaluating the significance of this process in natural settings. Lastly, small alkyl-S molecules, except DMS, can be abiotically synthesized from CO_2 and ΣH_2S in the presence of iron monosulfide under anoxic conditions (Heinen and Lauwers, 1996). This process is accelerated at temperatures higher than 50°C. The abiotically synthesized alkylated sulfides are considered as building blocks for larger organic molecules in the primitive earth (Huber and Wächtershäuser, 1997). Jointly, these biological and abiotic reactions compete for ΣH_2S with other reactions such as the formation of elemental sulfur, polysulfur, metal sulfides and complex organic sulfur compounds.

The finding of methyl sulfides formation from CO by *M. acetivorans* raises the question whether CO_2 (or bicarbonate at neutral pH), the most abundant inorganic carbon species in nature, can be converted to MT and DMS by microbial activity. Although CO_2 is not a growth substrate of *M. acetivorans*, it is produced in vivo via oxidation of CO and then carried into the reductive steps by methanofuran, a coenzyme shared by autotrophic methanogens (Oelgeschläger and Rother, 2008). In anoxic environments, reduction of CO_2 to a methyl group is mostly coupled to hydrogenotrophy, such as autotrophic methanogenesis and acetogenesis. Previous studies on the effect of H_2 on methylated sulfides have yielded inconclusive results. Lomans et al. (1999b) found that after prolonged incubation of anoxic lake sediment under H_2, degradation of methylated sulfides decreased dramatically. In contrast, the addition of H_2 did not affect thiol methylation potential in wetland sediments (Stets et al., 2004).

In an experiment in which we studied hydrogenotrophic reactions as a function of temperature in the anoxic sediment of the eutrophic Lake Plußsee, we noticed that DMS production was stimulated by the addition of H_2 and bicarbonate at 55°C. This observation points to the potential presence of a hitherto unrecognized pathway of DMS formation that may be

relevant in buried sediments at elevated temperature. Our study seeks to establish that this reaction is in fact mediated biologically and to further examine the link among CO_2, H_2, and methylated sulfides. We present the experiments performed to characterize the mechanism and constrain the microbial group responsible for the production of methylated sulfides in sediment slurry incubations. The effect of H_2 and bicarbonate on methylated sulfides and other major hydrogenotrophic products was examined, followed by tests aimed at verifying whether the observed DMS formation is related to methyl transfer during O-demethylation from organic substrates. We used a specific inhibitor to obtain a first indication of the involved enzymatic pathway, which was later supported by the natural carbon isotopic abundance of methylated sulfides. The flow of atoms into MT and DMS was further tracked by isotope labeling using ^{13}C- and ^{35}S-labeled compounds.

Materials and Methods

Study site and sampling

Lake Plußsee (54°10'N, 10°23'E) is a well-studied eutrophic lake located in northern Germany. It has a stable thermal stratification in summer and regularly occurring anoxia in the hypolimnion, leading to high ΣH_2S concentrations in the bottom water (up to 50 μmol L^{-1}; Eller et al., 2005). Sediment samples used for this study were taken in July 2006 from the deepest part of the lake, i.e., at 28 m using a small multicorer from a rowing boat. After sampling, the upper 15 cm of triplicate sediment cores was homogenized, placed into an air-tight bottle without gas headspace and stored in the dark at 4°C for another three months before being used for incubation experiments.

Slurry preparation

Sediment slurries were prepared anoxically by homogenizing approximately one volume of sediment with one volume of sterilized distilled water. After autoclavation, the distilled water was sparged with N_2 for at least one hour to remove dissolved oxygen. $NaHCO_3$ was added to buffer pH after sparging (final concentration = 1–3 mmol L^{-1}) and $Na_2S{\cdot}9H_2O$ as reducing agent

(final concentration = 50 μmol L^{-1}). Aliquots of 8 or 10 mL of sediment slurry were dispensed into 16 mL Hungate tubes and sealed with butyl rubber stoppers. The tubes were evacuated three times and flushed with N_2 or H_2, and were pressurized to 200 kPa after sediment amendments. All the tubes were incubated at 55°C in the dark without shaking.

Substrate and inhibitor amendments

Substrate and inhibitor concentrations are reported for a liquid phase that is ca. 95% (w/w) of the sediment slurry. $NaHCO_3$ was added to 10 mmol L^{-1} from a CO_2-stabilized stock solution. Other tested substrates (reported in final concentrations) include: $Na_2S{\cdot}9H_2O$, 200 μmol L^{-1}; MT, 50 μmol L^{-1}; syringic acid, 100 μmol L^{-1}. 2-Bromoethanesulfonic acid (BES, 20 mmol L^{-1}) was applied to inhibit methanogens.

Stable isotope labeling

^{13}C-labeled $NaHCO_3$ was spiked in the middle of an experiment started with the addition of both H_2 and 10 mmol L^{-1} $NaHCO_3$ at time zero, so that the concentrations of methylated sulfides in the headspace of 'labeling time zero' tubes were high enough for isotope measurement. ^{13}C-labeled $NaHCO_3$ was added to less than 5% of the background level, which was assumed to be 5 mmol L^{-1} at the time point of addition. Tubes for carbon isotope determination were stored at -20°C and heated to 60°C for 20 min prior to analysis.

Radioisotope labeling

In the labeling experiment with $H_2{}^{35}S$, $Na_2S{\cdot}9H_2O$ was not added to the sterilized and purged distilled water in order not to decrease the specific activity of dissolved inorganic sulfide. Instead, the distilled water was added with resazurin (final concentration = 1 mg L^{-1}) and reduced slowly with freshly-prepared sodium dithionite solution until the liquid became colorless. 25-mL aliquots of sediment slurry were poured through a funnel into 120 mL serum vials and amended with H_2 and 10 mmol L^{-1} $NaHCO_3$. Autoclaved slurries (120°C, 25 min) were used as control to account for non-biological reaction and/or processing artifacts.

Radioactive sulfide was produced biologically and purified to remove S-bearing byproducts (T. Holler, unpubl. data). The specific activity was 50.9 MBq $mmol^{-1}$ in the form of $Zn^{35}S$. For each individual serum vial, we amended $H_2{}^{35}S$ gas released from a defined volume of $Zn^{35}S$ suspension (equivalent to 59.5 kBq), which was acidified by concentrated H_3PO_4 in a N_2-flushed 3.3-mL Veroject tube. Due to the excellent solubility of H_2S in acidic solution (Douabul and Riley, 1979), a large volume ratio of headspace to aqueous phase is essential to keep most of the $H_2{}^{35}S$ in the gaseous phase. In this study, the ratio was headspace:$Zn^{35}S$:H_3PO_4 = 2.91:0.3:0.09 (v/v/v). The released gas was displaced into a N_2-flushed plastic syringe by injecting an equal volume of saturated NaCl in the Veroject tube. By counting the remaining radioactivity in the Veroject tubes, the radioactivity recovered in the gaseous phase was estimated to be 79±2% of the total dosage. The actual radioactivity reaching the sediment slurry could be lower owing to (1) the unavoidable small gas bubble left in the Veroject tube, (2) the diffusion loss during gas transfer, (3) the adsorption of hydrogen sulfide to the black gasket at the end of the syringe plunger, and/or (4) the adsorption of hydrogen sulfide on the butyl stoppers used to seal the serum vials. The injected $H_2{}^{35}S$ should have resulted in a final ΣH_2S concentration of 37 μmol L^{-1} if there was no partitioning into solid phases.

To allow equilibrium of $H_2{}^{35}S$ in the serum vials, the time-zero sampling was performed after two hours of incubation. The clear supernatant of the sediment slurry was taken by a plastic syringe fitted with a hypodermic needle, filtered (0.2 μm), and the filtrate was injected directly into a 5% zinc acetate solution for determination of radioactivity in the dissolved fraction and concentration of ΣH_2S. The samples for ΣH_2S concentration were stored at –20°C until analysis. The remaining sediment slurry was treated with 20% of zinc acetate and 1 mmol L^{-1} NaOH and sat at 4°C for two hours. The purpose was to remove H_2S, MT and carbon disulfide (Adewuyl and Carmichael, 1987) from the gaseous phase. We interpreted the remaining volatile radioactivity to represent DMS, which was trapped using the method slightly modified from Kiene and Linn (2000). The original butyl stopper on the serum vial was exchanged with a butyl stopper attached with an Eppendorf centrifuge vial (1.5 ml) containing a strip of Gelman AR glass fiber filter immersed in 1 mL of freshly prepared 3% H_2O_2 solution. The serum vials were placed in the dark at room temperature and the sediment slurries were stirred for >6 hours. The traps were then removed, and the strips placed in 5 mL scintillation vials with scintillation fluid

for determination of ^{35}S-radioactivity. Tests with 50 μmol L^{-1} DMS showed that after trapping, DMS decreased to a level below the detection limit of the GC-FID. ^{35}S-DMS was not available to determine the exact trapping efficiency, but the trapping efficiency for $H_2{}^{35}S$ in bicarbonate-buffered solution was better than 90%. The sediment slurries fixed with NaOH and zinc acetate were subjected to two-step cold distillation (Fossing and Jørgensen, 1989) to investigate the distribution of radioactivity in acid volatile sulfide (AVS: H_2S + FeS) and chromium reducible sulfur (CRS: S^0 + FeS_2). *N*, *N*-dimethylformamide was applied in the second step to improve the yield of elemental sulfur (Kallmeyer et al., 2004).

The average production rate of DMS derived from doubly methylated ΣH_2S ($Rate_{\Sigma H_2S+2Me}$) during a labeling period can be calculated by

$$Rate_{\Sigma H_2S+2Me} = \frac{a_{trap}}{a_{dis}} \times \{\Sigma H_2S\} \times \frac{1}{t} \qquad (1)$$

Here $Rate_{\Sigma H_2S+2Me}$ is expressed as μmol (L of slurry)$^{-1}$ d^{-1}. a_{trap} is the radioactivity of the trapped pool, a_{dis} is the activity in the dissolved pool of the sediment slurry, $\{\Sigma H_2S\}$ is the concentration of ΣH_2S per volume of sediment slurry (μmol (L of slurry)$^{-1}$), and t is the number of days. Since $\Sigma H_2{}^{35}S$ is very likely to disproportionate into other aqueous sulfur species (Elsgaard and Jørgensen, 1992), a_{dis} can be an overestimation of the $\Sigma H_2{}^{35}S$ pool, leading to underestimated values of $Rate_{\Sigma H_2S+2Me}$.

Analytical techniques

Concentrations of methane and methylated sulfides were determined by headspace analysis. Tubes were only removed from the oven shortly before gas sampling, to make sure that the gas temperature was close to 55°C. An aliquot of 100 μL gas was taken from a headspace of 8–10 mL using a Hamilton gas-tight syringe for on-column injection via a programmable temperature vaporizing (PTV) inlet at splitless mode. A gas chromatograph (Trace GC Ultra, ThermoFinnigan) equipped with a CP-PoraBOND Q (Varian Inc.) column and a flame ionization detector (FID) was used to quantify the compounds. The column temperature was programmed from 60°C (1 min isothermal) to 240°C (2 min isothermal) at a rate of 40°C/min. The distribution coefficients for DMS and methanethiol at 55°C are 6.9 and 4.3, respectively (Przyjazny et al., 1983).

Calibration was made by standards prepared anoxically from chemicals, and the limits of detection (LOD) were 0.4 μmol L^{-1} for methanethiol and 0.5 μmol L^{-1} for DMS under the described analytical conditions.

The same model of GC was coupled to a ThermoFinnigan Delta Plus XP isotope ratio mass spectrometer (IRMS) via a Finnigan combustion interface-III for carbon isotope analysis. One milliliter of gas was injected into the split/splitless (SSL) inlet set at a split ratio of 0.1. A column and temperature program identical to those described above was used. Values of $\delta^{13}C$ relative to that for Vienna-PDB are defined by the equation $\delta^{13}C$ (‰) = $(R_{sample}/R_{standard} - 1) \times 1000$ with R = $^{13}C/^{12}C$ and $R_{standard} = 0.0112372 \pm 2.9 \times 10^{-6}$. Internal precision of $\delta^{13}C$ was better than ±0.1‰ (1 standard deviation). We used two types of standards to evaluate the accuracies of methylated sulfides isotopes at low concentrations: (1) gaseous standards were prepared by injecting small amount of liquid chemicals into helium-flushed, water-free headspace vials that were allowed to equilibrate at 60°C. Isotopic values of the gaseous standards were not influenced by partition of methylated sulfides into aqueous solution and were taken as 'real' values. (2) Solution standards of different concentrations were prepared under a helium headspace with a defined volume of water. By comparing the isotopic values of the solution standards with those of the gaseous standards, we found that at concentrations lower than 5 μmol L^{-1}, there was a positive shift for methanethiol (up to 4.5‰) and a negative shift for DMS (up to 2.5‰). Since a strict relationship between the correction factor and concentration was not established, we did not correct the $\delta^{13}C$ values of methanethiol, which had concentrations lower than the threshold during the whole course of the incubation experiments.

Radioactivity was determined by liquid scintillation counting (Packard 2500 TR) with a counting window of 4 to 167 keV without luminescence correction. The scintillation cocktail Lumasafe Plus (Lumac BV, Holland) was mixed with the zinc acetate-fixed dissolved fraction and the glass fiber strips. The counting efficiency was better than 95%. Counting time was 10 min for all samples. The concentration of hydrogen sulfide was determined colorimetrically by the methylene blue method.

For acetate analysis, sediment slurries in Hungate tubes were centrifuged at 800 × g for 10 min, and 1 mL supernatant was taken by a plastic syringe fitted with a 21-gauge needle and filtered through a 0.45-μm Rotilabo Teflon syringe filter. The filtrates were stored at –20°C until analysis. Acetate was measured using a high performance liquid chromatograph (HPLC) equipped with a Nucleogel Column (Machery-Nagel Inc.) and a photodiode array (PDA) detector. Calibration curves were generated using standards prepared gravimetrically from sodium acetate. The detection limit for acetate was 10-15 μmol L^{-1}.

Statistical analysis

The LOD of instrumental methods was assessed statistically by the equation LOD = Y_B + 3 $S_{y/x}$, where Y_B represents the signal of instrumental background and was estimated by the intercept of a calibration curve, and $S_{y/x}$ stands for the deviation of Y values (see Miller and Miller, 2005, for detailed description). The effect of individual treatments was tested using a one- or two-tailed *t* test with α set at 0.05.

Results

Addition of substrates

In the first experiment, we examined the effects of H_2 and bicarbonate on the dynamics of DMS and MT at 55°C. We also monitored the concentrations of methane and acetate, the major carbon pools that can potentially compete for H_2 and bicarbonate in this system. Compared to the control without any addition (Fig. 6-2a), there was no or only minor stimulation of DMS accumulation when H_2 or bicarbonate was added as single substrates (Figs. 6-2b, 6-2c). However, simultaneous addition of both H_2 and bicarbonate resulted in a six-fold increase of DMS (Fig. 6-2d). The average accumulation rate was 2.1 μmol DMS (L of slurry)$^{-1}$ d^{-1} between days 3 and 9. Subsequent tests with the same treatment yielded a range of maximal accumulation rates from 1.3 to 2.4 μmol DMS (L of slurry)$^{-1}$ d^{-1}. In the presence of H_2 and bicarbonate, DMS concentration in the autoclaved sediment was significantly lower than that in the live sediment. For MT, the

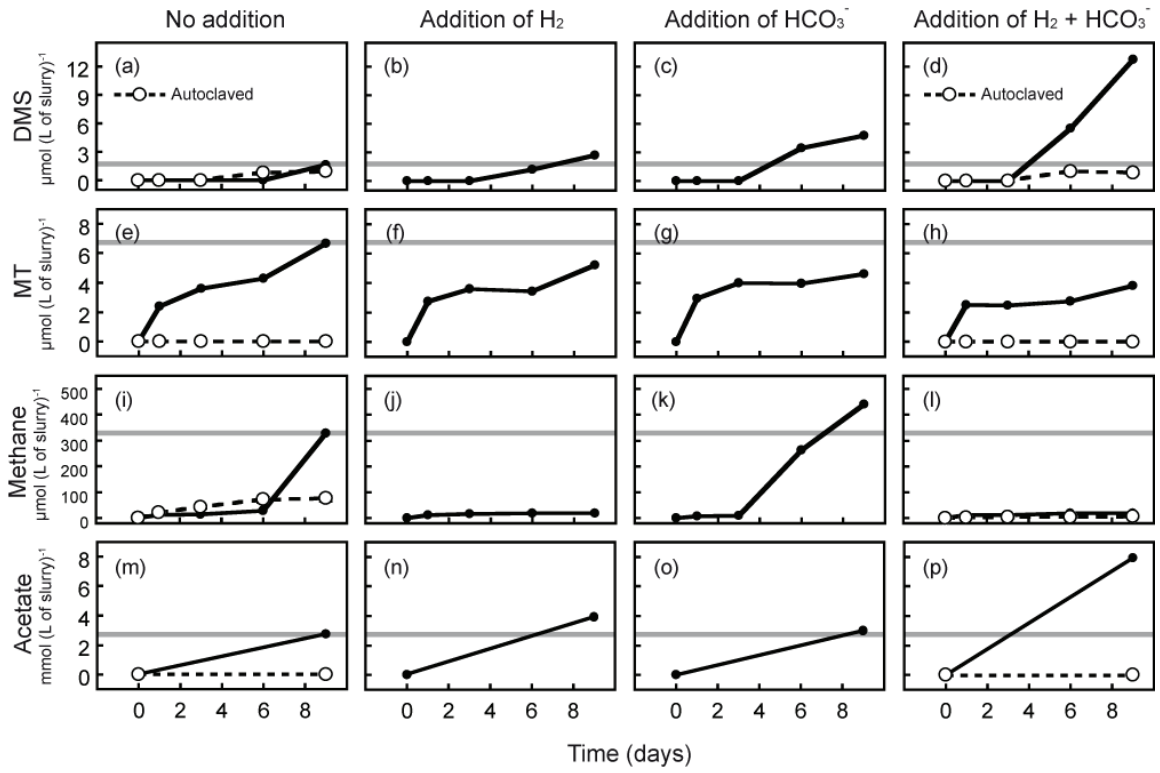

Figure 6-2. Effects of H_2 and bicarbonate on the formation of dimethyl sulfide (DMS), methanethiol (MT), methane, and acetate in the sediment slurries of Lake Plußsee. Gray lines mark the final concentration of each compound in the parallel in which no substrate was added. Data are from a single sample tube.

final concentration after nine days of incubation was highest in the control (Fig. 6-2e) and lowest when both H_2 and bicarbonate were added (Fig. 6-2h), but there was no apparent stoichiometric relationship between the kinetics of DMS and MT accumulation. MT concentration was below LOD in the autoclaved sediments (Figs. 6-2e, 6-2h). Methane accumulation was suppressed by addition of H_2 (Figs. 6-2j, 6-2l) but slightly enhanced by amendment of bicarbonate (Fig. 6-2k). The acetate concentrations in time-zero and autoclaved sediments were both below 10 μmol L^{-1}. The response of acetate formation to addition of H_2 and/or bicarbonate showed a similar pattern to that of DMS formation: H_2 and bicarbonate as single substrates caused a negligible or a minor stimulation of acetate (Figs. 6-2n, 6-2o); in combination they resulted in a 2.7-fold increase of acetate (Fig. 6-2p). Note that the concentration of acetate was much higher than DMS, MT and

methane by one to three orders of magnitude at the end of incubation. Because of the pronounced stimulation of DMS formation in the presence of both H_2 and bicarbonate, these two compounds were always supplemented in the following tests that targeted mainly DMS.

To test whether the observed DMS formation is related to the activity of O-demethylation, we examined the effects of sodium sulfide, methanethiol and syringic acid in the presence of H_2 and bicarbonate. Experimental concentrations of each of these compounds were in the range that generated microcosm response in previous studies of freshwater sediment (Lomans et al., 1997; Stets et al., 2004). However, none of these compounds significantly improved DMS formation after 12 days of incubation (Table 6-1). Neither did MT concentration increase significantly after addition of sodium sulfide or syringic acid.

Inhibition tests

Reduction of bicarbonate with H_2 in anoxic sediment is commonly associated with activities of either acetogens or methanogens. Acetogenesis was very active in our incubated sediment but cannot specifically be inhibited to examine the corresponding effects on formation of methylated sulfides. Nevertheless, the role of methanogens can be evaluated by BES, a specific inhibitor for methanogenic pathways (Chidthaisong et al., 2000). Relative to the positive control (Fig. 6-3), DMS formation was inhibited by 60% ($P = 0.024$, one-tailed *t* test), whereas methanethiol accumulation increased slightly ($P = 0.044$, one-tailed *t* test). The inhibition of methane formation by BES was not significant ($P = 0.182$, one-tailed *t* test). BES had no effect on the

Table 6-1. Concentrations of DMS and MT after 12 days of incubation with inorganic and organic substrates. Results from duplicate tubes.

Treatment	DMS μmol (L of slurry)$^{-1}$	MT μmol (L of slurry)$^{-1}$
$H_2 + HCO_3^-$	11.1 ± 0.3	2.8 ± 0.1
$H_2 + HCO_3^-$ + sodium sulfide, 200 μmol L^{-1}	9.6 ± 1.0	4.4 ± 1.6
$H_2 + HCO_3^-$ + methanethiol, 50 μmol L^{-1}	9.1 ± 0.6	14.8 ± 4.2
$H_2 + HCO_3^-$ + syringic acid, 100 μmol L^{-1}	6.8 ± 0.5	3.8 ± 0.4

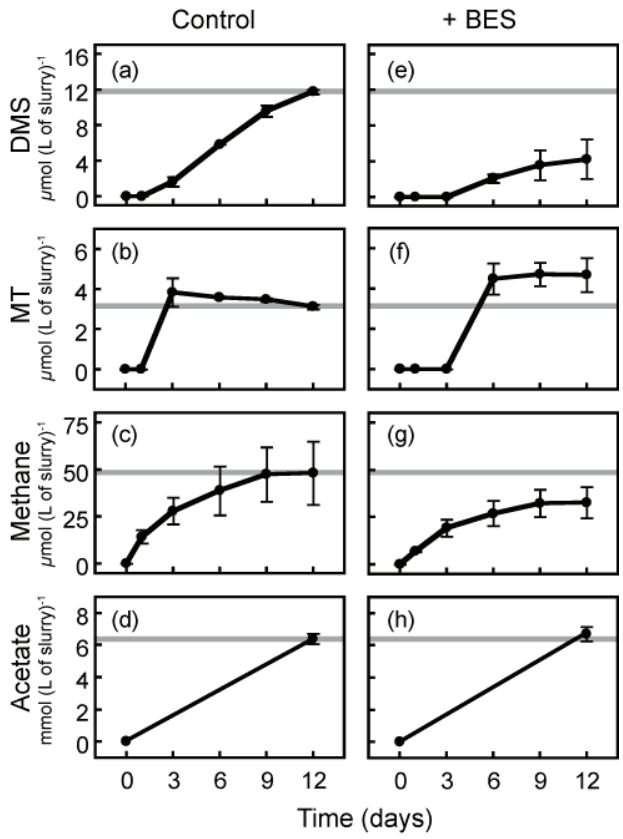

Figure 6-3. Effects of 20 mmol L^{-1} 2-bromoethanesulfonate (BES) on formation of dimethyl sulfide (DMS), methanethiol (MT), methane and acetate in the sediment slurries of Lake Plußsee. Gray lines mark the final concentration of each compound in the control in which no inhibitor was added. Error bars represent ±1 standard deviation of duplicate tubes.

formation of acetate.

Stable carbon isotopic compositions at non-labeling and ^{13}C labeling conditions

The stable carbon isotopic ratio of methylated sulfides provides additional evidence on the enzymatic pathways involved in their formation. $\delta^{13}C$ values of DMS were as depleted as –62‰ on day 6, and increased slightly to –56‰ at the end of incubation (Fig. 6-4). The analyzed $\delta^{13}C$ values of MT were –55‰ and –53‰ on days 6 and 12, respectively, but the actual $\delta^{13}C$ values of

MT should have been more negative, since the MT concentrations were below the threshold value of 5 μmol L^{-1}. At both time points CH_4 had $\delta^{13}C$ values of around –49.8‰.

In parallel, we performed a ^{13}C-labeling experiment with $NaH^{13}CO_3$ to investigate the incorporation of bicarbonate into DMS. If bicarbonate was used directly to form one of the methyl groups in DMS rather than exerting an indirect effect that favored DMS formation (e.g., via buffering the aqueous solution), we would expect a rapid labeling after $NaH^{13}CO_3$ was added. The result of ^{13}C labeling supports the idea of direct labeling: one day after addition, the $\delta^{13}C$ values of DMS became enriched by nearly 40‰ and ended at 119‰ (Fig. 6-4). MT showed a much slower but significant (P = 0.002, one-tailed *t* test) enrichment, but reached only –24‰ by the end of the experiment. ^{13}C-enrichment of CH_4 was merely 9‰, suggesting that the activity of methanogenesis via reduction of CO_2 was low.

Labeling with $H_2{}^{35}S$

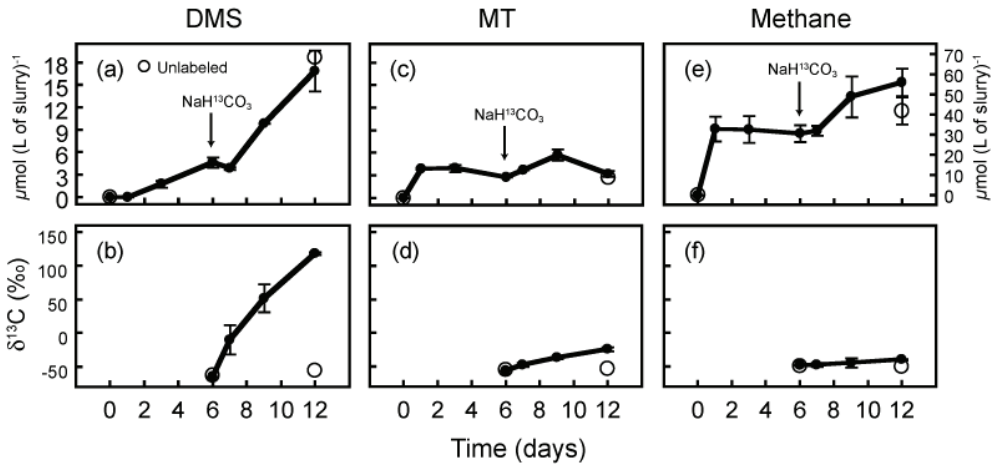

Figure 6-4. Concentrations and carbon isotopic values of dimethyl sulfide (DMS), methanethiol (MT) and methane in sediment slurries with or without addition of $Na^{13}HCO_3$. The labeled compound was added on day 6 of the incubation. Error bars represent ±1 standard deviation of duplicate tubes.

The result of the ^{13}C-labeling experiment indicates that DMS originates from MT, but the source of MT remains unclear. The effects of sodium sulfide and syringic acid on MT formation were insignificant (Table 6-1), and the slightly enriched methanethiol during ^{13}C labeling is not a conclusive proof for methylation of hydrogen sulfide by reduced bicarbonate. By the labeling experiment with $H_2{}^{35}S$, we sought to better characterize the reaction of $\Sigma H_2{}^{35}S$—via the intermediate MT—to DMS. The amount of MT quantified in this experiment may derive from the reactions of H_2S with inorganic carbon or non-S-methyl compounds including O-methyl pools (Fig. 6-1).

At time zero (after two hours of incubation), the dissolved fraction contained less than 10% of total added radioactivity (Fig. 6-5). Not surprisingly, a significant amount of $H_2{}^{35}S$ entered the solid phase AVS (most likely FeS) and CRS fractions via isotope exchange (Fossing et al., 1992). Assuming that only the dissolved fraction could be responsible for DMS formation, we used the specific activity of the dissolved fraction to calculate the rate of DMS formation (see Eq. 1). The trapped radioactivity in non-autoclaved samples was much higher than in the autoclaved control ($P = 0.008$, one-tailed *t* test), suggesting (1) only a minor component of incompletely fixed $H_2{}^{35}S$ vapor was trapped, and (2) biological formation of DMS. In the non-autoclaved samples, the trapped radioactivity increased most rapidly between days 3 and 7 and finally reached 2.5% of the dissolved radioactivity. We obtained an average $Rate_{\Sigma H_2S+2Me}$ of 0.06 μmol (L of slurry)$^{-1}$ d^{-1} over the whole period of incubation and a higher rate of 0.1 μmol (L of slurry)$^{-1}$ d^{-1} between days 3 and 7. The rates are consistent with the reported rates of sulfide-dependent MT production in freshwater sediment with low hydrogen sulfide concentration (Lomans et al., 1997), but represent <10% of the ambient DMS production rate determined by the kinetics of total DMS formation in this study.

Discussion

Our experimental data showed that both DMS and MT were of biogenic origin and depleted in ^{13}C. However, both compounds responded differently to addition of substrates and inhibitors, and exhibited different patterns of isotope label incorporation. Only DMS showed a clear

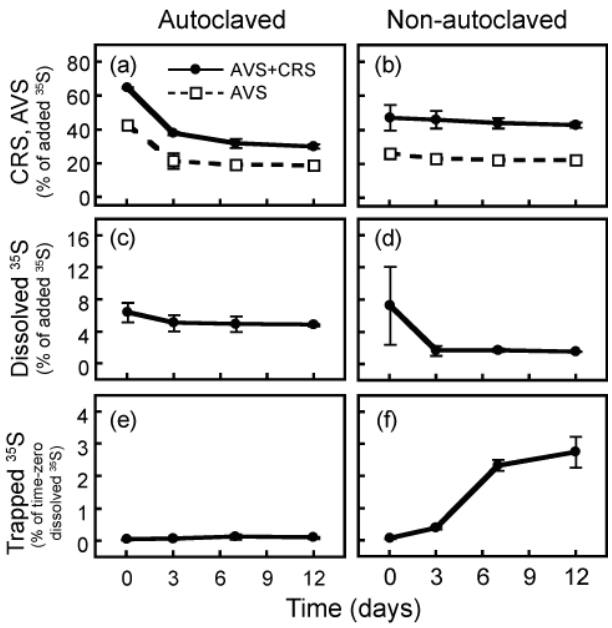

Figure 6-5. Distribution of radioactivity in the sediment slurries of Lake Plußsee when incubated with H_2, bicarbonate and $H_2{}^{35}S$. Time-zero values were measured two hours after addition of radiotracer. AVS: acid volatile sulfide (H_2S + FeS); CRS: chromium reducible sulfur (S^o + FeS_2). The dissolved ^{35}S was measured from filtered slurry supernatant. The trapped ^{35}S was interpreted to represent labeled DMS. Error bars represent ±1 standard deviation of duplicate bottles.

response to addition of H_2 and bicarbonate and a clear signal of inorganic ^{13}C incorporation. Therefore, DMS and MT formation will be discussed separately, followed by a note on the implication of these processes in anoxic environments.

Microbial DMS formation

The distinct effects of H_2 and bicarbonate to stimulate DMS formation, the slight accumulation of MT when DMS production was inhibited, as well as the more rapid incorporation of inorganic ^{13}C into DMS compared to MT, suggest the following reaction:

$$HCO_3^- + H^+ + 3H_2 + CH_3SH \rightarrow CH_3SCH_3 + 3H_2O \qquad (2)$$

$$\Delta G^{\circ\prime} = -159.8 \text{ kJ per reaction}$$

The standard-state free energy for the proposed reaction was calculated using thermodynamic data for aqueous species listed in Scholten et al. (2003). Our observation indicates that inorganic carbon in anoxic aquatic environments can be incorporated into the pools of methylated sulfides. The production rate is consistent with the previously observed kinetics in other freshwater environments (Lohman et al., 1997; Stets et al., 2004). Addition of MT did not further stimulate DMS formation (Table 6-1), suggesting that this process is not limited by MT. DMS formation is not related to O-demethylation because supplement of syringic acid did not stimulate DMS production. The $\delta^{13}C$ value of DMS in experiments without label addition is as depleted as –62‰ (Fig. 6-4). According to Eq. 2, one of the methyl groups is from MT and the other from reduced bicarbonate. The measured $\delta^{13}C$ value of MT in experiments without label addition is around -54‰. A simple mixing model provides a rough estimate of –70‰ for the $\delta^{13}C$ value of the methyl group from reduced bicarbonate. This negative value is consistent with a kinetic isotopic effect typical for H_2/CO_2 metabolizers such as autotrophic methanogens (Whiticar, 1999) or acetogens (Gelwicks et al., 1989).

Suppression of DMS production by BES further suggested that coenzyme M (CoM-SH) is involved in DMS formation, and our anoxic system limits the involved organisms to methanogens. The enzymatic steps of *M. acetivorans* to produce methylated sulfides have been discussed by Moran et al. (2008), but in our case the direct precursor of the coenzyme-bound methyl group is not CO, but CO_2 (Fig. 6-6). Normally, CoM-SH receives the methyl group from N^5-methyl-H_4MPT:CoM-SH methyltransferase (Mtr) complex to form methyl-CoM, which releases methane by forming heterodisulfide with coenzyme B (CoB-SH) under the catalysis of methyl-CoM reductase (Mcr). Under the condition when DMS is produced, the methyl group is transferred from methyl-CoM to MT. As Moran et al. (2008) pointed out, such a shortcut to regenerate CoM-SH is feasible, owing to the low energy barrier in the activation step of methanogenic DMS consumption by methylthiol:CoM methyltransferase (Mts) ($\Delta G^{\circ\prime}$ = 0.35 kJ per reaction; Tallant et al., 2001). The overall energy yield for the methanogenic DMS production is theoretically lower than normal methanogenesis, as the H^+-pumping step of heterodisulfide reduction is bypassed and energy conservation is restricted to the Na^+-pumping Mtr complex (Hedderich and Whitman, 2006).

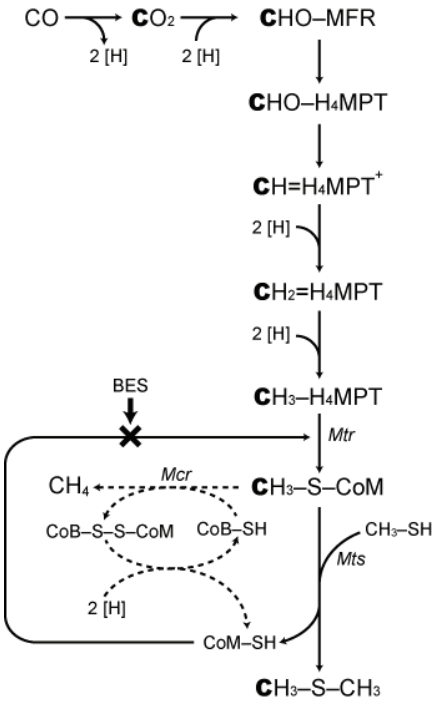

Figure 6-6. A simplified scheme of methanogenic DMS formation. The formation of DMS from CO is reported in Moran et al. (2008). The carbon atoms highlighted in bold type refer to the process proposed in this study, i.e., formation of DMS from CO_2. The dashed arrows are pathways that are bypassed when DMS rather than methane is produced. The enzymatic step inhibited by 2-bromoethanesulfonic acid (BES) is marked with a cross. Abbreviations: MFR, methanofuran; H_4MPT, tetrahydromethanopterin; CoM-SH, coenzyme M; CH_3-S-CoM, methyl coenzyme M; CoB-SH, coenzyme B; Mtr, N^5-methyl-H_4MPT:CoM-SH methyltransferase; Mcr, methyl-CoM reductase; Mts, methylthiol:CoM methyltransferase.

Why methanogens should respire in a suboptimal way in terms of energy production remains unclear. In the case of *M. acetivorans*, Moran et al. (2008) attributed the production of methylated sulfides to the high CO concentration (300 kPa) in the culturing condition. CO is likely to inhibit Mcr, causing CoM-SH to be sequestered and energy production stopped. Transferring the methyl group to hydrogen sulfide or MT can be a shortcut to regenerate CoM-SH while bypassing Mcr. In our incubation, the headspace CO concentrations were lower than 5 ppm (data not shown) and should not have a marked inhibitory effect. However, under the high H_2 partial pressure, methanogenesis in our system was also suppressed, a phenomenon already

reported in previous studies of the same lake sediment (Nüsslein and Conrad, 2000; Heuer et al., in press). Nüsslein and Conrad (2000) further showed that methanogenesis was more active by addition of 4% H_2 rather than 80%, suggesting that the methanogenic population is adapted to low H_2 concentration. Transferring the methyl group to an external reactant is probably a response of the stressed methanogens, when external methyl acceptors are available. However, DMS formation was not detected at lower temperatures (4°, 27 and 40°C) when we supplemented high partial pressure of H_2 and bicarbonate. This can be due to a much slower DMS production rate, a deficient supply of endogenous MT, or a coupling between DMS formation and degradation at lower temperatures. An alternative ecological explanation for the DMS formation would be that it is mediated by a methanogenic population that is activated by elevated temperature. For example, a temperature-induced community change has been reported by Fey et al. (2001), who observed a shift from acetoclastic to H_2-dependent methanogenic community at temperature higher than 40°C in rice field soil. Our current experimental data do not enable us to distinguish among these possibilities. Further investigation on lake sediments will be necessary to constrain the ecological condition under which this process occurs.

Microbial MT formation

Autoclaved controls confirmed that MT in our system is also of biological origin, and the natural carbon isotopic values are very negative (<–50‰). In contrast to some previous studies which suggest that methyl transfer from methoxylated aromatic compounds to hydrogen sulfide is a main source of MT in freshwater sediment (e.g., Lomans et al., 1997), addition of sodium sulfide and syringic acid to our microcosm failed to stimulate MT formation. By labeling with $H_2{}^{35}S$ we were able to quantify the inventory of methylated ΣH_2S that finally entered the DMS pool. The result is consistent with other experiments that that the slow production of MT from either O-methyl pools or inorganic carbon cannot be easily discerned by our analytical protocols. The minor supply of MT derived directly from ΣH_2S does not support the accumulation rate of DMS that is supposed to derive from MT (Eq. 2). Taken together, our experiments suggest that the DMS production does not proceed via MT directly from an inorganic source of sulfide; however, we can not yet from these experiments identify the mechanism of MT formation.

Nevertheless, the carbon isotopic composition of MT at natural abundance levels is intriguing and deserves further discussion. The main question is: How can we explain the negative carbon isotopic values of MT? Hydrogenotrophic bicarbonate reduction is usually considered the main process that generates ^{13}C-depleted methyl group. If the slight enrichment of MT in the ^{13}C-labeling experiment reflects the signal of bicarbonate incorporation (Fig. 6-4), the contribution of bicarbonate reduction must be very minor, otherwise the small carbon pool of MT and its inferred rapid turnover should have allowed a pronounced labeling signal. Alternatively, the slight enrichment of MT can also be explained by demethylation of ^{13}C-labeled DMS. Such a process would also recruit the MT pool with ^{13}C-depleted methyl group produced during bicarbonate reduction under the natural (non-labeling) condition. However, ^{13}C- or ^{35}S-labeled DMS was not available to confirm the presence of DMS demethylation experimentally. A second possible source of ^{13}C-depleted methyl group is methoxylated aromatic compounds: Keppler et al. (2004) reported that the methyl pool in lignin has δ^{13}C values as negative as –66‰. However, our results from the substrate tests and $H_2{}^{35}$S-labeling experiment suggest that this source is unlikely to have a major contribution. A last potential source that has received little attention but cannot be ruled out is the S-methyl pool of amino acids. MT accumulated rapidly after addition of methionine in the Plußsee sediment (data not shown), but we have no information on the pool size of free methionine and its endogenesis from enzymatic hydrolysis of macromolecules. If the δ^{13}C values of the methyl pool in methionine is identical to that of S-adenosylmethionine (SAM), a coenzyme that derives from methionine and has δ^{13}C values of <–39‰ for its methyl pool (Weilacher et al., 1996), methionine could be another source for a moderately ^{13}C-depleted methyl pool. Additionally, methionine and SAM are involved in biosynthesis of many O- and S-methyl pools in organic matter, including DMSP and lignin monomers. Direct isotopic analysis of the methyl group in methionine will be essential in the future to better constrain the propagation and distribution of δ^{13}C signatures of C1 compounds in nature, including methylated sulfides.

Implications for anoxic environments

The incorporation of bicarbonate into DMS may be relevant to several anoxic environments, including hydrothermal vents. As summarized in Fig. 6-1, abiotic synthesis of alkylated sulfides

has been demonstrated in the laboratory and is considered relevant for hydrothermal environments. A recent study on in situ measurements further demonstrated considerable amounts of MT in a hydrothermal area (Reeves and Seewald, 2009). The supply of MT, the usually high chemical potential of H_2 and bicarbonate, and, possibly, elevated temperature, qualify hydrothermal systems as candidate ecosystems in which methanogenic DMS production could take place. Another relevant setting is the globally important sulfate-methane transition zones (SMTZ) in marine sediments associated with anaerobic methanotrophic activity (e.g., Hoehler et al., 2000). It is a particular zone where sulfate is exhausted and methane starts to build up. Additionally, this zone is often accompanied by peak ΣH_2S and bicarbonate concentrations. Laboratory experiments demonstrated that during the transition from sulfate reduction to methanogenesis, there is a decoupling of H_2 production and consumption and hence a temporary accumulation of H_2 (Hoehler et al., 1999). Isotopic evidence for acetogenesis via CO_2 reduction in an extended sediment interval just below the SMTZ at Cascadia Margin is also consistent with elevated H_2 concentration in situ (Heuer et al., 2009). If there are sources of MT, e.g., transmethylation from lignin monomer to ΣH_2S, the SMTZ qualifies as an additional environment where methanogenic DMS is thermodynamically favorable.

In conclusion, our work provides multiple lines of evidence for a novel microbial pathway of DMS production in anoxic lake sediment. This pathway connects DMS to bicarbonate and H_2 and is mediated by methanogens. Subsequent steps would have to characterize the physiological conditions at which methanogens favor production of DMS and other methylated sulfides rather than methane, and to explore the environmental relevance of this novel pathway. On the other hand, our data cannot identify the mechanism of MT formation despite various experimental attempts. This illustrates a more complicated biogeochemistry of MT, which will remain a great challenge for future research.

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Chapter 7

Data report: H_2-induced formation of methanethiol in marine sediments

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Abstract

Accumulation of methanethiol was detected in anoxic marine sediments under laboratory conditions. Formation of methanethiol was dependent on H_2, but ^{13}C-labeled bicarbonate was not incorporated into this volatile sulfide. It is a slow, abiotic reaction, and can be accelerated by elevated temperatures. The abiotically produced methanethiol was consumed by biological sinks.

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Introduction

Methanethiol and dimethyl sulfide are the most abundant volatile organic sulfur compounds. They have been detected in diverse environments including surface seawater (Kiene, 1996), stratified lakes (Fritz and Bachofen, 2000), marine and lake sediments (Kiene, 1991; Lomans et al., 2002), and hydrothermal fluids (Reeves and Seewald, 2009). Additionally, they are involved in various biological and chemical processes, coupling both the organic and inorganic carbon to the sulfur cycle (reviewed in Chapter 6, this volume). Because of their biological and chemical reactivity, the ambient concentrations of both compounds are usually low, and elucidation of their sources and sinks largely depends on laboratory experimentation.

Our recent work demonstrated that under laboratory conditions, methylation of methanethiol by reduced bicarbonate resulted in dimethyl sulfide formation in the anoxic sediment of a eutrophic lake (Chapter 6, this volume). Radiotracer experiments further showed that dissolved sulfide was also incorporated into dimethyl sulfide. Methanogens were found to be responsible for such an unusual DMS-forming process. According to the equation proposed for the process, the sulfate-methane transition zone in marine sediments qualifies another environment where the methanogenic DMS formation can take place. However, only the surface and near-surface marine sediments have been examined for the concentrations of methanethiol and dimethyl sulfide (Whelan et al., 1980; Kiene and Taylor, 1988), and no laboratory experiments have been performed to explore biogeochemical processes involving volatile methylated sulfides in subseafloor sediments. Here we report our first attempt to study the relationship among H_2, bicarbonate and volatile methylated sulfides in marine sediments. We characterized the laboratory conditions that led to the formation of volatile methylated sulfides in marine sediments, and carried out ^{13}C-labeling experiments to examine the potential precursor of the observed methylated sulfides.

Materials and methods

Table 7-1. Marine sediments tested in this study. All sediment slurries were supplemented with H_2 and bicarbonate. Results are the mean and standard error of duplicate tubes. ND: not detected.

Location	Cruise, station	Depth (cmbsf)	Status	Duration of incubation (days)	Incubation temperature (°C)	Methanethiol (μmol per L of slurry)
Wadden Sea	-	10-20	Live	6	27	ND
Black Sea	*Meteor* M 72/5, 6-MUC2	0-48	Live	8	25	0.8 ± 0.4
	Meteor M 72/5, 9-GC2	158-168*	Live	14	27	2.0 ± 0.1
			Sterilized	14	27	1.9 ± 0.0
	Meteor M 72/5, 22-GC5	586-596*	Sterilized	49	27	ND
Arabian Sea	*Meteor* M 74/2, GeoB 12204-6	120*	Live	16	27	2.5 ± 0.8

* From the sulfate-methane transition zone.

Table 7-1 lists the location and subseafloor depth of marine sediments tested in this study. Sediment slurries were prepared in a N_2-flushed glove bag by homogenizing approximately one volume of sediment with one volume of a sterile, sulfate-free mineral salts solution. The mineral salts solution contained 3 mmol L^{-1} of $NaHCO_3$ and was reduced with dithionite prior to mixing with the sediment. Aliquots of 9 mL of sediment slurry were dispensed into 16 mL Hungate tubes and sealed with butyl rubber stoppers. Heat-killed controls were autoclaved twice at 121°C for 30 min. A H_2 headspace was established by evacuating and flushing the tubes three times with H_2 (final pressure = 100 kPa). $NaHCO_3$ in the H_2-supplemented tubes was brought to a final concentration of 10 mmol L^{-1}. The negative pressure in the headspace due to consumption of H_2 was compensated by inserting a needle attached to a H_2-filled plastic syringe. For sediments retrieved from the sulfate-methane transition zone, $Na_2S{\cdot}9H_2O$ was added to a final concentration of 10 mmol L^{-1}. $NaH^{13}CO_3$ was added to ~5% of the $NaHCO_3$ pool.

Concentrations and stable carbon isotopic values of volatile methylated sulfides and other hydrocarbon gases were monitored using a gas chromatograph coupled to either a flame ionization detector or an isotope ratio mass spectrometer via a combustion interface following established analytical procedures (Chapter 6, this volume). The temperature-corrected distribution coefficients for volatile methylated sulfides were calculated using the equations in Przyjazny et al. (1983). Values of $\delta^{13}C$ relative to that for Vienna-PeeDee Belemnite are defined

by the equation $\delta^{13}C$ (‰) = ($R_{sample}/R_{standard} - 1$) × 1000 with R = $^{13}C/^{12}C$. The significance of difference between two treatments was tested using a *t* test with α set at 0.05.

Results and discussion

Methanethiol was detected in three out of the five sediment samples when H_2 and bicarbonate was supplemented (Table 7-1). Trace level of dimethyl sulfide was also detected, but the concentration was not influenced by addition of H_2 and bicarbonate (data not shown). The live and sterilized sediment slurries from Station 9-GC2 showed an identical extent of methanethiol accumulation (P = 0.14, two-tailed *t* test), suggesting that this compound was produced via an abiotic process.

The condition favoring methanethiol formation was studied further by long-term experiments with the sediment from Station 9-GC2. We first examined the effect of H_2 on methanethiol formation (Fig. 7-1). After 265 days of incubation at 27°C, CH_4 accumulated in sediment slurries with and without H_2 addition (Fig. 7-1), and was highly ^{13}C-labeled ($\delta^{13}C$ = 114 ± 48‰) in the sample supplemented with H_2 and $NaH^{13}CO_3$. CH_4 concentrations in sterilized sample did not increase over time and were too low to be displayed clearly in Fig. 7-1. Methanethiol was not detectable in the live sediment with a N_2 headspace. In the live sediment with a H_2 headspace, the concentration of methanethiol remained low after prolonged incubation. In contrast, methanethiol concentration increased by one order of magnitude in the sterilized, H_2-supplemented sample after 265 days of incubation (P = 0.03, one-tailed *t* test). This striking contrast suggests that there is a biological sink for the slowly released methanethiol in the live sediment.

Temperature exerted a pronounced influence on the production kinetics of methanethiol (Fig. 7-2). After 14 days on incubation with a H_2 headspace, the concentrations of methanethiol in sterilized sediment from Station 9-GC2 were proportional to the temperature. After prolonged incubation, the sterilized sediment incubated at 40°C had a methanethiol concentration of 98 ± 32 μmol (L of slurry)$^{-1}$. Because the production kinetics was not monitored regularly during the

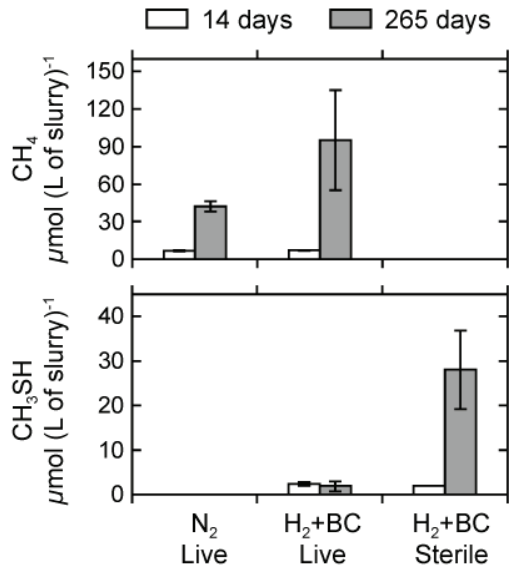

Figure 7-1. The concentrations of CH_4 and CH_3SH in the Black Sea sediment (*Meteor* M72-5, 9-GC2, 158-168 cmbsf) after different treatments. The sediment slurries were incubated at 27°C. Error bars represent the ±1 standard error of duplicate tubes. BC: bicarbonate.

course of incubation, it is unclear whether the production still continued or had leveled off due to depletion of substrates.

So far, the characterized conditions that favor methanethiol formation, i.e., addition of H_2 and higher temperature, are consistent with a previously described reaction of abiotic thiol synthesis. Heinen and Lauwers (1996), when simulating reactions in hydrothermal systems, observed formation of volatile thiols by incubating FeS with H_2S and CO_2 at elevated temperatures under anoxic conditions. We tested if such an abiotic mechanism explained our observation by labeling the sediment sample from Station 9-GC2 with $NaH^{13}CO_3$. However, no ^{13}C uptake was found in methanethiol, which had constant $\delta^{13}C$ values between -20 and -30‰ (Fig. 7-2). There are two possible explanations. First, the methanethiol is formed via an abiotic reaction identical to that proposed by Heinen and Lauwers (1996), but the source of inorganic carbon is not CO_2/bicarbonate but CO. This can be tested by a ^{13}C-labeling experiment with ^{13}CO. Alternatively, the methanethiol is released from a pool associated with sediment particles. Kiene (1991) observed that added methanethiol disappeared rapidly from the dissolved fraction of

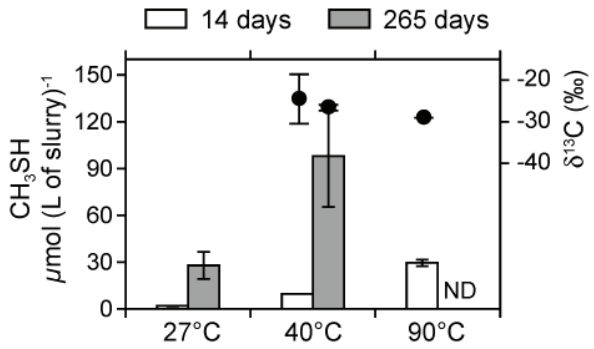

Figure 7-2. The concentrations and stable carbon isotopic values of CH_3SH in the Black Sea sediment (*Meteor* M72-5, 9-GC2, 158-168 cmbsf) incubated at three temperatures. The sediment slurries were sterilized and supplemented with H_2 and $H^{13}CO_3^-$. Error bars represent the ±1 standard error of duplicate tubes. ND: not determined.

sediment slurries, probably due to association with the solid phase. He further showed that the bound fraction of methanethiol could be released by treating the sediment with tributylphosphine, a specific disulfide cleaving reagent. This approach should also help to identify the source of methanethiol in marine sediments.

In the future, further efforts should be spent to constrain the role of H_2 in controlling the kinetics of methanethiol formation. The highest H_2 concentration reported for subseafloor sediments is only 0.8 kPa (Parkes et al., 2007b). If H_2 concentrations lower than this value are sufficient to induce this process in laboratory, this pool of methanethiol may serve as a continuous trickle to fuel a methylotrophic community in the marine deep biosphere.

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Chapter 8
Conclusions and future perspectives

Conclusions: A Marine Deep Biosphere with Glycolipid-producing Archaea and Decoupled H_2 Metabolisms

The studies performed in this PhD project have addressed the questions pertaining to the microbiological and biogeochemical conditions in the marine deep biosphere, and provide new insights into the bioenergetic status of the ecosystem. The major conclusions are:

- *Archaeal IPLs are being generated by indigenous archaea in sediments.* In the IPL-SIP experiment, weak and strong ^{13}C incorporation was found in the hydrocarbon chains and the glycosidic headgroups of archaeal glycolipids, respectively. This unbalanced ^{13}C uptake between the hydrocarbon chains and the glycosidic headgroups implies the presence of an anabolic shortcut that allows the Archaea to maintain their membrane integrity with a minimal energy investment.
- *Benthic archaea are heterotrophic.* The above-mentioned results were obtained only in the sediment slurries supplemented with ^{13}C-labeled lyophilized cyanobacterial cells. Other labeled substrates, including bicarbonate, methane and acetate, did not lead to any significant labeling signals.
- *Sedimentary H_2 concentrations are higher than predicted.* Dissolved H_2 concentrations, determined in samples prepared by two different preparation procedures, were found to be orders of magnitude higher than the level predicted by thermodynamic calculations, especially in sulfate reducing sediments. The observation of much higher H_2 concentrations implies a relaxation of coupling between H_2-producing and H_2-consuming activities at these sediment depths.
- *Formation of volatile methylated sulfides can be stimulated by H_2.* In the Plußsee sediment, formation of dimethyl sulfide is a microbial process. The ^{13}C-labeling experiment confirmed that CO_2 contributes to one of the carbon on dimethyl sulfide. Such an unusual process was interpreted as a response of stressed methanogens. In

marine sediments, formation of methanethiol is an abiotic process. The amount of methanethiol produced was proportional to temperature and addition of H_2, and the methanethiol was later consumed by biological sinks. The carbon source for the methanethiol was not CO_2 but another uncharacterized source.

Two novel methods have been developed in this PhD project to provide the analytical grounds that are necessary to solve the major questions. These analytical methods are:

- *Intramolecular stable carbon isotopic analysis of intact glycolipids.* A method that enables examination of carbon isotopic relationships between sugars and lipids in uncultivated organisms. Archaeal glycolipids from an ANME-1-dominated microbial mat and a subseafloor sediment sample were analyzed. In these samples, the major glycosidic headgroups were more ^{13}C-enriched than the major hydrocarbon chain by 6-24‰.
- *An extraction-based technique for determination of in situ H_2 concentrations.* A method that allows rapid determination of H_2 concentration in subseafloor sediment samples. The major background H_2 for this method comes from H_2 leakage through needle punctures. The detection limit was estimated to be 35 nmol L^{-1}.

Perspectives: From the Laboratory to the Field

Although the in vitro experiments designed and the new methods developed in this PhD project have helped to answer some of the open questions left from earlier generations of marine deep biosphere research, there is still considerable space for improvement. The results of the in vitro experiments also highlight the need of applying new methods for in situ studies. Below are a few perspectives that emerged during the course of this PhD project:

- *In vitro labeling experiments*. Some published lipid-SIP works were performed in situ (e.g., Bühring et al., 2006). The major advantage of in situ experiments compared to in vitro studies is: No change of the physicochemical conditions to which the

microorganisms have been adapted. This can be essential for some microorganisms such as piezophiles. During the course of this PhD project, an in situ ^{13}C-labeling experiment targeting also marine benthic archaea has been carried out by another research group (Takano et al., in prep.). However, because the in situ incubation apparatus has been inserted into the seabed from the sediment surface and a body of bottom water was also enclosed, the likelihood of contaminating signals from planktonic archaea could not be fully excluded. In situ experimentation of deeper sediments may require the aid of other unconventional engineering techniques, such as lateral borehole drilling.

- *Gas chromatography-combustion-continuous counting.* A striking fact in the study of Takano et al. (in prep.) is that, in their in situ ^{13}C labeling experiment, they also obtained very weak labeling signals in the hydrocarbon chains of the archaeal glycolipids. It is likely that the growth rates of certain microorganisms in natural environments are simply below or around the detection limits of SIP. Radiotracer assays should provide the requested sensitivity, but the widely applied liquid scintillation counting does not offer an easy solution for online analysis. In fact, an online technique for ^{14}C analysis of gas chromatograph-amenable compounds has been present since 1968 (Martin, 1968; Nelson and Zeikus, 1974; Czerkawski and Breckenridge, 1975), but has received little attention. For probing the unseen, starved and probably slow-growing majority in natural environments, such type of techniques deserves further exploration.
- *Solid phase microextraction*. Another focus of this PhD project is trace volatiles. These compounds can be easily detected in laboratory experiments, but determination of their in situ concentrations remains a challenge. As these compounds partition highly into liquid phase and tend to co-elute with other hydrocarbon gases during gas chromatography, their presence in subseafloor sediments may have been overlooked. Solid phase microextraction, a method that has been applied in environmental sciences to study trace volatiles, has not been applied in the marine deep biosphere research. In addition to volatiles, such a technique has been also employed to study dissolved alcohols and holds great potential to provide insights into fermentative processes in subseafloor sediments.

- *Linking molecules to organisms/processes*. The reasonable next step following the demonstration of heterotrophic benthic archaea is to know what exactly they are doing with organic matter. Traditionally, this is not possible without a first step of enriching or isolating the organisms. However, with the recent development of imaging-assisted mass spectrometric techniques, it is now possible to infer the connection between molecules and the physiology of cells based on their topological relationship (e.g., Lane et al., 2009). Although these techniques are still at their infancy, they provide a new and promising direction for new research ideas.

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Bremen, 07. Oktober 2009

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