Cell wall components and intact membrane lipids as proxies for investigation of microbial communities in the deep biosphere

Dissertation

zur Erlangung des Doktorgrades

der Naturwissenschaften

- Dr. rer. nat. -

Am Fachbereich Geowissenschaften

Der Universität Bremen

vorgelegt von

Rong Zhu

Bremen

January 2014

1. Gutachter: Prof. Dr. Kai-Uwe Hinrichs

2. Gutachter: Prof. Dr. Wolfgang Bach

There is no royal road to science, and only those who do not dread the fatiguing climb of its steep paths have a chance of gaining its numinous summits.

Karl Marx

TABLE OF CONTENTS

THESIS ABSTRACT

Marine sediments have been estimated to host 0.6-30% of the total living biomass on Earth. Molecular biology has revealed the diversity and distribution of these benthic microorganisms. However, they are still poorly uncharacterized life forms, and consequently, their physiologies and roles in global biogeochemical processes remains elusive. In this thesis, three independent methods have been developed as gene- and cultivation-independent approaches aiming for deciphering microbial activity, community composition, and carbon cycling within the marine deep biosphere. These newly developed methods were tailored for analysis of biomarkers with different biochemical functions, i.e. amino sugars, phospholipids (PLs) and low-molecular weight compounds produced during pyrolysis of microbial biomass. Each resulting method was subsequently applied to fingerprinting of specific biomarkers in marine subseafloor sediments.

Pyrolysis-gas chromatography/mass spectrometry (py-GC/MS) is an efficient technique that can rapidly determine microorganisms with minimal sample pretreatment. Based on the analysis of different bacterial and archaeal cultures as well as reference substances, pyrolysis fingerprints consisting of benzyl nitrile 2-furanmethanol, indole, phenol and pyrrole were selected for tracking microbial signals in several mg-sized samples of marine sediments. Application of the py-GC/MS approach, microbial signals in the form of the pyrolysis fingerprints were detectable in marine sediments from different subseafloor depths (until ~400 m below the seafloor at the Canterbury Basin) and with different organic carbon contents (0.11-6.99%). However, microbial signals were still detectable in the horizons where the indigenous microbial cells were at least one order of magnitude lower than the minimum microbial cell density required for the py-GC/MS protocol. The assessed signals were therefore presumed to predominantly from fossil microbial biomass.

An alternative to direct pyrolysis of marine sediment involves the analysis of cell wall constituents, i.e. amino sugars. These compounds can potentially provide information on the types and quantities of microbes and, when subjected to compound-specific isotopic analysis, about the carbon metabolism of sedimentary microbes. To establish a robust protocol for the stable carbon isotopic analysis of amino sugars in marine sediments, various pretreatment steps have been systematically tested. Combination of the most effective steps, including a new

solid-phase extraction (SPE) protocol for post-hydrolysis clean-up and a new step for enrichment of amino sugars via preparative high performance liquid chromatography (HPLC), resulted in a GC-based method that enabled isotopic analysis of amino sugars at trace levels (limit of detection = 20 ng) and introduced negligible isotopic fractionation during sample preparation. Comprehensive information regarding the microbial community in subseafloor sediments was obtained by studying the downcore distributions and stable carbon isotopic compositions of amino sugars in ultra-deep subseafloor sediments from two settings, Peru Margin (ODP Leg 201 Site 1229) and Canterbury Basin (IODP Expedition 317 Site U1352), using the newly developed protocols. Significant differences in amino sugar distributions and ratios were observed between the two study sites, clearly reflecting different microbial sources to the organic matter for the respective sites. Compiling distributions of muramic acid (MurA) from different sites, we observed that the majority of MurA was not associated with active bacteria, which consequently challenged the application of MurA for assessment of bacterial populations in the deep biosphere. Notably, significant ^{13}C-depletion of MurA, observed in the sulfate-methane transition zones, suggested that MurA had isotopic imprints from indigenous bacteria that assimilate methane-derived carbon at these geochemical interfaces.

By contrast, analysis of PLs can reveal direct clues on the living microbial biomass in the deep biosphere. PL analysis in the deep subseafloor sediment is usually constrained by very low PL concentrations in combination with relatively high limits of detection, which are due to sedimentary matrices that cause ion suppression during LC-electrospray ionization (ESI)-MS analysis. Therefore, a cleanup protocol using a HybridSPE®-Phospholipid cartridge for purification of PLs was adapted for sedimentary lipid extracts. The new cleanup protocol improved the detection of sedimentary PLs by up to around threefold, with some PLs being only detectable after the cleanup protocol. Application of this protocol to oil-bearing hydrothermally heated sediments of the Guaymas Basin shed light on the presence of living microbial biomass up to maximum temperatures of ~90°C. Compared with samples from a non-hydrothermal reference site, distributions of PLs showed less diversity at the hydrothermal site, consistent with the presence of a comparatively simple thermophilic microbial community.

Analysis of different groups of microbial biomarkers using methods developed in

this thesis extended the toolbox for investigation of microbial processes and revealed new information on microbial communities in the subseafloor sediments. The combination of analysis of different biomarkers will provide more comprehensive insights for deciphering microbial communities in the deep marine biosphere.

ZUSAMMENFASSUNG

Marine Sedimente beherbergen zwischen 0,6 und 30% der gesamten lebenden Biomasse der Erde. Obwohl mit molekularbiologischen Methoden eine weite Verbreitung und große Diversität benthischer Mikroorganismen festgestellt wurde, sind diese Lebensformen bisher kaum charakterisiert. Die Physiologie dieser Organismen und ihre Bedeutung für die globalen biogeochemischen Stoffkreisläufe sind weiterhin unbekannt. In dieser Arbeit wurden drei separate Methoden entwickelt, die unabhängig von Genetik und Kultivierung zur Aufklärung der Aktivität und Zusammensetzung mikrobieller Gemeinschaften sowie deren Rolle im Kohlenstoffkreislauf in der tiefen marinen Biosphäre beitragen. Die neu entwickelten Methoden ermöglichen die Analyse von Biomarkern mit verschiedenen biochemischen Funktionen, z.B. Aminozucker, Phospholipide (PLs), und mittels Pyrolyse von mikrobieller Biomasse auch erzeugte niedermolekulare Verbindungen. Diese verfeinerte Methodik wurde auf tiefe marine Sedimente angewandt um spezifische Biomarker zu untersuchen.

Pyrolyse-Gaschromatographie/Massenspektrometrie (py-GC/MS) ist eine effiziente Methode zur Charakterisierung von Mikroorganismen ohne zeitaufwändige Probenvorbereitung. Basierend auf der Analyse verschiedener Bakterien- und Archaeenkulturen sowie Referenzsubstanzen wurden die Pyrolyseprodukte Benzonitril, 2-Furanmethanol, Indol, Phenol und Pyrrol ausgewählt um mikrobielle Signale in sehr kleinen Proben mariner Sedimente zu detektieren. Mit Hilfe dieser Technik konnten mikrobielle Signale in marinen Sedimenten in verschiedenen Tiefen (bis zu ca. 400 m unter dem Meeresboden in Proben des Canterbury Basin) und mit unterschiedlichem Gehalt an organischem Kohlenstoff (0.11-6.99%) nachgewiesen werden. Mikrobielle Signale waren selbst in Sedimenten detektierbar, deren Zellkonzentrationen eine Magnitude unterhalb der für die Pyrolyse theoretisch benötigten Zellkonzentration lag. Die untersuchten Signale stammen deshalb vermutlich überwiegend von fossiler mikrobieller Biomasse.

Eine Alternative zur direkten Pyrolyse mariner Sedimente ist die Analyse von Zellwand-Bestandteilen, d.h. Aminozuckern. Die Analyse von Aminozuckern liefert qualitative und quantitative Informationen über die vorhandenen Mikroben, während die komponentenspezifische Isotopenanalyse Rückschlüsse über den Metabolismus der Organismen ermöglicht. Zur Entwicklung einer Methode zur Messung der

Kohlenstoffisotopie von Aminozuckern wurden mehrere Aufbereitungsschritte systematisch getestet. Die optimierte Methode umfasst eine Aufreinigung hydrolysierter Proben mittels Festphasenextraktion sowie die Anreicherung von Aminozuckern durch präparative Flüssigchromatographie. Diese Methodik ermöglicht eine Isotopenanalyse von Spurenkonzentrationen von Aminozuckern mit einem Detektionslimit von 20 ng bei vernachlässigbarer Isotopenfraktionierung während der Probenaufbereitung. Mit dieser Methode wurden die Verteilung und die stabilen Kohlenstoffisotopenverhältnisse von Aminozuckern in ultra-tiefen Sedimenten aus Bohrkernen vom Kontinentalrand vor Peru (ODP Leg 201 Site 1229) und dem Canterbury Basin (IODP Expedition 317 Site U1352) untersucht. Signifikante Unterschiede in der Verteilung und den Verhältnissen von Aminozuckern wurden zwischen den beiden Lokationen festgestellt, welche unterschiedliche mikrobielle Quellen des organischen Materials widerspiegeln. Eine Zusammenstellung der Konzentrationen von Muraminsäure an verschiedenen Lokationen ergab keine Korrelation mit der Anzahl aktiver Bakterien. Dieses Ergebnis schränkt die Anwendbarkeit von Muraminsäure zur Abschätzung bakterieller Populationen in der tiefen Biosphäre ein. Muraminsäure zeigte eine signifikante ^{13}C-Abreicherung in der Sulfat-Methan-Übergangszone, die vermutlich auf die Assimilation von Kohlenstoff im Zusammenhang mit der Methanoxidation zurückzuführen ist.

Die Analyse von PLs ermöglicht eine direkte Analyse der lebenden Biomasse in der tiefen Biosphäre. Die Detektion von PLs in tief versenkten marinen Sedimenten über gekoppelte Flüssigchromatographie-Massenspektrometrie mittels Elektrosprayionisation wird jedoch durch sehr niedrige PL-Konzentrationen und Ionenunterdrückung durch die komplexe sedimentäre Matrix erschwert. Daher wurde ein existierendes Protokoll zur selektiven Aufreinigung wichtiger PLs mittels HybridSPE-Phospholipid-Kartuschen an die Matrixbedingungen in marinen Sedimenten angepasst. Dieses neue Protokoll verbessert nicht nur die Sensitivität der Detektion von PLs um einen Faktor von drei, sondern liefert auch ein umfassenderes Bild der Verteilung von PLs in marinen Sedimenten. Mit Hilfe dieser Methode konnte lebende mikrobielle Biomasse in ölführenden, hydrothermal aufgeheizten Sedimenten des Guaymas-Beckens bis zu einer Temperatur von 90 °C detektiert werden. Im Vergleich zu einer nicht aufgeheizten Referenzlokation enthielt die hydrothermale

Lokation eine vergleichsweise einfache, thermophile mikrobielle Gemeinschaft.

Die Analyse verschiedener Gruppen von Biomarkern mit Hilfe der Methoden, die in dieser Studie entwickelt wurden, erweitert die Möglichkeiten zur Untersuchung mikrobieller Prozesse und ermöglichte neue Einblicke in mikrobielle Gemeinschaften in tiefen marinen Sedimenten. Die Kombination verschiedener Biomarker ermöglicht somit eine fundierte Analyse mikrobieller Gemeinschaften in der tiefen Biosphäre.

LIST OF ABBREVIATIONS

AA	Alditol acetate
ACN	Acetonitrile
AEG	Acyletherglycerol
ANA	Aldononitrile acetate
ANME	Anaerobic methanotrophic archaea
AODC	Acridine orange direct count
AOM	Anaerobic oxidation of methane
AR	Archaeol
bd	Below the detection limit
DAG	Diacylglycerol
DCM	Methylene chloride
DEG	Dietherglycerol
DIC	Dissolved inorganic carbon
EA	Elemental Analyzer
EI	Electron impact
ESI	Electrospray ionization
FA	Fatty acid
FISH	Fluorescence-in-situ-hybridization
G-	Glycosidic-
GalN	Galactosamine
GC	Gas chromatography
GDGT	Glyceroldibiphytanylglyceroltetraethers
GL	Glycolipid
GlcN	Glucosamine
HPLC	High performance liquid chromatography
IPA	Isopropanol
IPL	Intact polar lipid
IRMS	Isotope ratio mass spectrometry
ManN	Mannosamine
3-*O*-Me-Glc	3-*O*-methyl-D-glucopyranose
MS	Mass spectrometry
mbsf	m below the seafloor

MSD	Mass selective detector
MurA	Muramic acid
OM	Organic matter
PC	Phosphocholine
PDME	Phosphatidyl-(N, N)-dimethylethanolamines
PE	Phosphoethanolamine
PG	Phosphatidylglycero
PME	Phosphatidyl-(N)-methylethanolamines
PS	Phosphoserine
PL	Phospholipid
PLFA	Phospholipid-derived fatty acid
Py	Pyrolysis
rRNA	Ribosomal RNA
SIM	Selective ion monitoring
SMTZ	Sulfate-methane transition zone
SPE	Solid-phase extraction
SST	Sea surface temperature
TAS	Total amino sugar
TFA	Trifluoroacetic acid
TLE	Total lipid extract
TMAH	Tetramethylammonium hydroxide
TOC	Total organic carbon
VFA	volatile fatty acid
VPDB	Vienna Pee dee Belemnite
wt.	Weight

LIST OF FIGURES

LIST OF TABLES

Chapter I

Introduction

I.1. GENERAL INTRODUCTION

I.1.1 The deep biosphere

I.1.1.1 Microbial communities in the marine deep biosphere

The habitable subseafloor sediments have been estimated to host microbial biomass in a range of 2.9-35 × 10^{29} cells, which adds up to 4.1-303 Pg of cellular carbon and accounts for 0.6-30% of the total living biomass on Earth (Parkes et al., 1994; Whitman et al., 1998; Parkes et al., 2000; Lipp et al., 2008; Kallmeyer et al., 2012). In global marine sediments, the distribution of microbial biomass is thought to logarithmically decrease with sediment depth (Fig. I.1a; Parkes et al., 2000; Lipp et al., 2008) and is strongly influenced by organic matter (OM) burial rate (Lipp et al., 2008; Kallmeyer et al., 2012).

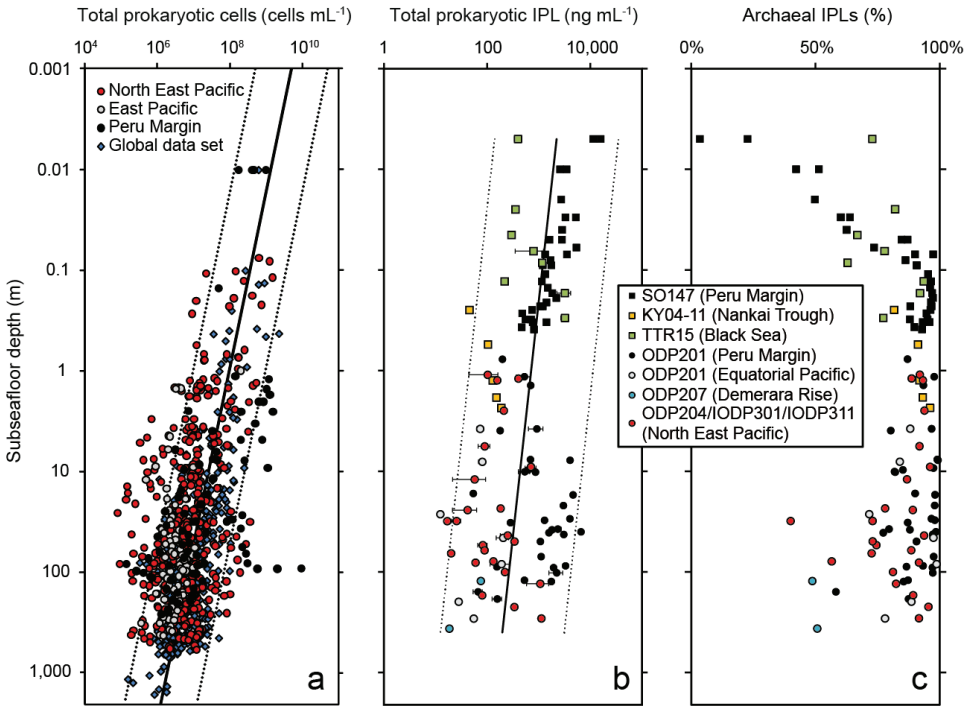


Fig. I.1. Depth profiles of (a) prokaryotic cell concentrations, (b) total IPLs concentrations, and (c) relative contribution of archaeal IPLs to total microbial IPLs. Cell concentrations were reproduced with data from Parkes et al., 2000; D'Hondt et al., 2004; Engelen et al., 2008; Webster et al., 2009; Roussel et al., 2008. (Figure was provided by Dr. Julius S. Lipp.)

The phylogenetic compositions of such tremendous microbial communities in subseafloor sediments remain unknown. On the basis of ribosomal RNA (rRNA) and DNA analysis, some studies have reported that *Bacteria* dominate the ocean margin sediments up to 400 m below the seafloor (msbf; Schippers et al., 2005; Inagaki et al.,

2006). Combined with analysis of intact polar lipids (IPLs) found in microbial cell membranes, other studies held a contrary view, suggesting that *Archaea* are the major inhabitant in the deep biosphere (Biddle et al., 2006; Lipp et al., 2008). Specifically, the archaeal IPLs, which can be indicative of living archaeal communities, accounted for more than 90% of the total IPLs between 0.1-1000 mbsf (Fig. I.1b, c; Lipp et al., 2008). In addition, recent studies using muramic acid (MurA) and dipicolinic acid as biomarkers revealed that bacterial endospores were on the order of 10^7 per cm^3 sediment in subseafloor sediments of the Aarhus Bay (Langerhuus et al., 2012) and the Peru Margin (Lomstein et al., 2012). Nowadays, researchers have become aware of the selectivity and/or bias of the methods employed due to the presence of fossil biomarkers (e.g. Lever et al., 2009; Lipp et al., 2009). In this context, the controversy of the dominance of *Bacteria* versus *Archaea* evolved into a new stage based on identifying and quantifying biomarkers of living biomass.

I.1.1.2 Turnover of microbial communities in the deep biosphere

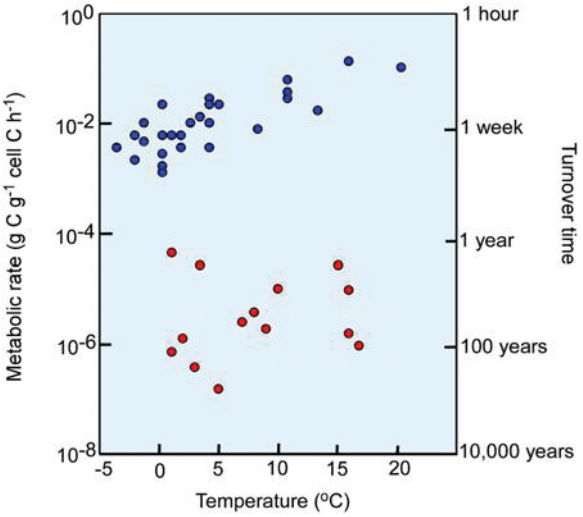


Fig. I.2. Metabolic rates and turnover times of microorganisms in different natural environments. Blue circles indicate nutrient-rich environments such as soil, lake water, or seawater. Red circles indicate nutrient-starved environments such as subsurface sediments. (Figure was taken from Jørgensen (2011).)

Models and measurements of marine subseafloor sediments have suggested that OM decreases logarithmically with age, or according to a power-law function (Middelburg, 1989; Rothman and Forney, 2007). Therefore, the large proportion of prokaryotes that is thought to persist under energy-limited conditions beneath the seafloor likely utilize old and recalcitrant OM (Parkes et al., 2000; Jørgensen, 2011). Based on microbiological approaches, the metabolic rate of benthic life is orders of magnitude lower than those on Earth's surface (D'Hondt et al., 2002; Jørgensen and

D'Hondt, 2006; Jørgensen, 2011). Specifically, the metabolic rate of microorganisms in surface ecosystems is typically in the range of 10^{-3} to 10^{-1} g C g^{-1} cell C h^{-1}, whereas that for deep subsurface bacteria is between 10^{-7} and 10^{-5} g C g^{-1} cell C h^{-1} (Fig. I.2). The estimated turnover time of prokaryotes in subsurface sediments therefore varies by several orders of magnitude, ranging from several years up to 100 ky (Parkes et al., 2000; Whitman et al., 1998; Biddle et al., 2006; Lipp et al., 2008).

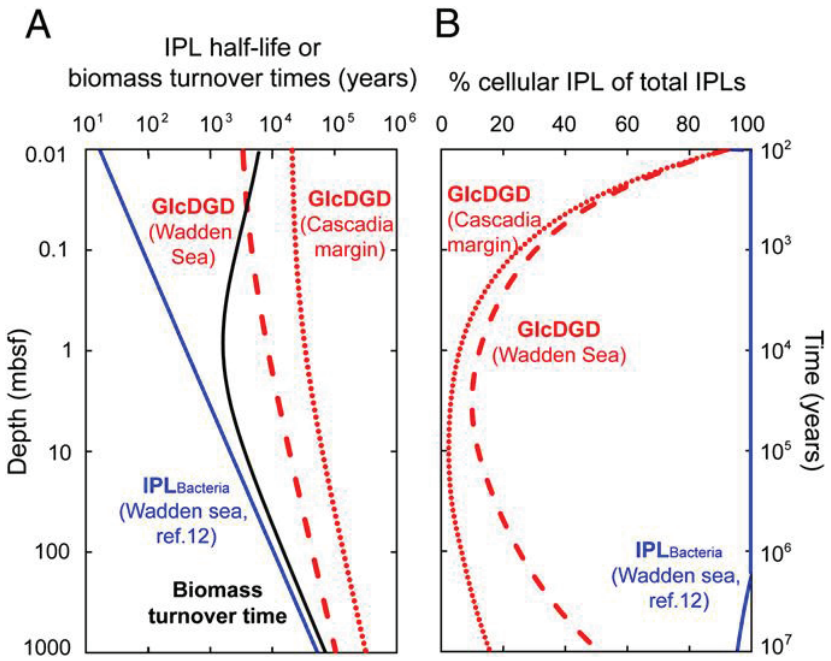


Fig. I.3. Turnover times of biomass and IPLs (A) and relative fractions of cellular IPLs for GlcDGD and bacterial IPLs (B) in subseafloor sediment. Modeled half-life of archaeal and bacterial IPLs based on degradation kinetics obtained from degradation experiment and data from Logemann et al. (2011). (Figure was taken from Xie et al. (2013).)

Recent investigations have applied a more thorough consideration on the fossil fractions of biomarkers in the benthic ecosystem. With a sensitive radiotracer assay, Xie et al. (2013) estimated the half-life of extracellular IPLs from the top to the bottom of a 1-km sediment column, which ranged from 0.017-53.4 ky for bacterial IPLs and 20-312 ky for archaeal IPLs (Fig. I.3a). Because the estimated turnover time of microbial biomass was 1.6 to 73 ky, the noncellular archaeal IPLs accounted for a substantial fraction (Fig. I.3b), suggesting that previous approaches based on IPLs probably overestimated the archaeal population. Using a D:L-amino-acid model, turnover time of active microbial biomass was estimated to be tens to thousands of years, whereas microbial necromass could be recycled over hundreds of thousands of years in subsurface sediments (Langerhuus et al., 2012; Lomstein et al., 2012). The

microbial necromass persisting over long timescales also indicates a major fossil component in sedimentary OM.

I.1.2. Biomarkers as molecular proxies

I.1.2.1 Biomarkers in microbial ecology

Biomarkers are complex organic compounds composed of carbon, hydrogen, and other elements that are found in petroleum, rocks, and sediments and show little or no change in structure from their parent organic molecules in living organisms (Peters et al., 2005). They can encode for particular kinds of living organisms (Eglinton and Calvin, 1967). In microbial studies, the most valuable biomarkers are taxonomically specific. They can be related to a certain group of organisms, carrying a characteristic signal from the environment in which they were synthesized (Killops and Killops, 2005). For instance, some branched phospholipid-derived fatty acids (PLFAs), such as 10me$C_{16:0}$, anteiso and iso $C_{15:0}$ and $C_{17:0}$, have been associated with sulfate-reducing bacteria (e.g. Boon et al., 1977; Taylor and Parkes, 1983; Kohring et al., 1994; Schubotz et al., 2009). Anaerobic methanotrophic archaea (ANME)-1 or ANME-2 dominated environments have been successfully identified by different patterns of IPL distribution, with diglycosidic glyceroldibiphytanylglyceroltetraethers (2G-GDGT) predominantly in the former group and phosphate-based archaeols and hydroxyarchaeols in the latter (Rossel et al., 2008). In addition, biomarkers that are present in relatively constant amounts in microbial biomass and rapidly degrade upon cell lysis can be used to estimate the size of a microbial population (Boschker and Middelburg, 2002). Accordingly, the significant abundance of bacterial endospores in the subseafloor sediments has been determined based on dipicolinic acid (Langerhuus et al., 2012; Lomstein et al., 2012), and microbial cell populations have been estimated based on IPL concentrations, using published conversion factors for lipid mass and cell numbers (Lipp et al., 2008).

I.1.2.2 Biomarkers as molecular proxies

On the basis of their different structures and compositions, biomarkers can provide widespread insights about the past and present environments, including energy requirements and ecology of the source organisms, and processes of

transportation and transformation of OM (Meyers, 1997). In the field of paleoceanography, several lipid biomarkers have been developed to reconstruct sea surface temperature (SST; Brassell et al., 1986; Prahl and Wakeham, 1987; Müller et al., 1998; Schouten et al., 2002; Kim et al., 2010), salinity (Rosell-Melé et al., 2002; Sicre et al., 2002; Schouten et al., 2006; van der Meer et al., 2007), OM inputs (Hopmans et al., 2004; Weijers et al., 2006), etc. Similarly, a wide range of low-molecular-weight biomarkers have been introduced to investigate microbial communities in the environment (e.g. Heuer et al., 2009; Carstens and Schubert, 2012; Lomstein et al., 2012). Amongst those, amino sugars have aroused increasing attention in soil science and aquatic samples during the past decades. Because they preserved in the form of microbial biopolymers with different stoichiometry (Schleifer and Kandler, 1972; Müller et al., 1986; Kandler et al., 1998; Madigan and Martinko, 2005; Liang et al., 2007), ratios of amino sugars have been frequently used as proxies of microbial community composition in soil (e.g. Guggenberger et al., 1999; Zhang et al., 1999; Glaser et al., 2004) and sources of OM in the marine realm (Benner and Kaiser, 2003; Niggemann and Schubert, 2006; Lomstein et al., 2009). In addition, the ratio of glucosamine/galactosamine (GlcN/GalN) has been used as an indicator of diagenetic alteration of marine OM, with lower ratios suggesting increased contributions of microbial-derived amino sugars in marine OM (Benner and Kaiser, 2003; Davis et al., 2009; Langerhuus et al., 2012).

I.1.3 Stable carbon isotopic analysis in biogeochemistry

There are two stable isotopes of carbon, ^{12}C and ^{13}C, which occur naturally at a proportion of approximately 99:l (Killops and Killops, 2005). The ratio of ^{13}C and ^{12}C in different compounds can be measured by isotope ratio mass spectrometry (IRMS) and is expressed using the δ notation: $\delta^{13}C = (R_{sample}/R_{standard} - 1) \times 1000‰$, with $R = {}^{13}C/{}^{12}C$ and $R_{standard} = 0.0112372 \pm 2.9 \times 10^{-6}$ (the reference standard is Vienna Pee dee Belemnite; VPDB).

The stable carbon isotopic compositions of naturally synthesized organic compounds depend on (l) the carbon source, (2) isotope effects associated with the assimilation of carbon, (3) isotope effects associated with metabolism and biosynthesis, and (4) cellular carbon budgets (Hayes, 1993). The different isotopic

compositions in microbial biomarkers can thus reveal important information about the carbon substrates and/or carbon fixation pathways of the source organisms. The stable carbon isotopic values of common carbon reservoirs found in nature are compiled in Fig. I.4 (Trumbore and Druffel, 1995). Determination of the stable carbon isotopic compositions of the carbon pools, in particular total organic carbon (TOC), dissolved inorganic carbon (DIC), and hydrocarbon gases like methane, provide essential information for investigating microbial metabolic and biosynthetic processes. Moreover, $\delta^{13}C$ analysis of metabolic intermediates, such as volatile fatty acids (VFAs), can allow for the reconstruction of carbon flow and can be used to identify pathways of microbial carbon turnover in subsurface environments (Heuer et al., 2006, 2009; Ijiri et al., 2012).

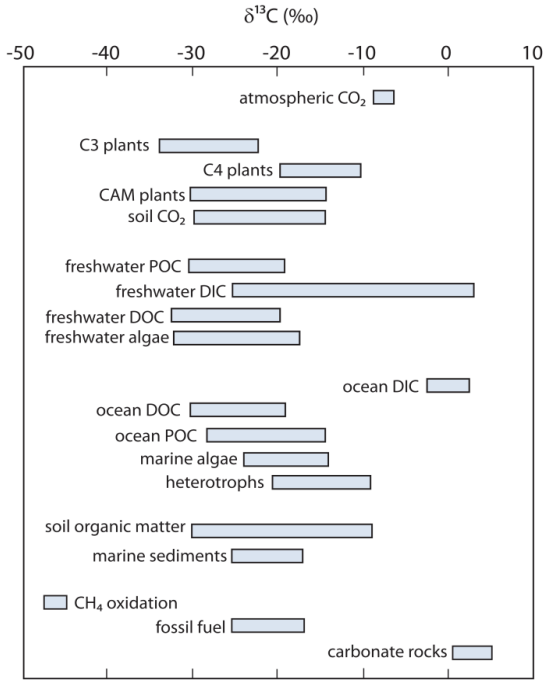


Fig. I.4. Overview on the range of $\delta^{13}C$ compositions for the major carbon reservoirs on earth. Different forms of carbon are characterized by widely varying $\delta^{13}C$ values as a result of fractionation processes. (Figure was adopted from Trumbore and Druffel (1995) by Dr. Julius S. Lipp.)

Biological uptake of carbon is usually associated with specific isotope fractionations that typically discriminate against the heavier stable isotope, i.e. ^{13}C, resulting in relatively ^{13}C-depleted products (Hayes, 2001). Isotopic fractionation factors are thus accounted for when evaluating the $\delta^{13}C$ compositions of biomarkers and elucidating the metabolism of their source organisms. For example, although the heterotrophic organisms often exhibit similar carbon isotopic ratios as their carbon sources (Blair et al., 1985; Coffin et al., 1990; Hullar et al., 1996), lipids are presumed to be generally depleted in ^{13}C by 3-6‰ with respect to the total biomass (e.g. Fig. I.5;

Hayes, 2001). Boschker et al. (1999) showed that specific bacterial PLFAs, such as i15:0 and a15:0, isolated from a mixed culture were depleted by about 4-6‰ relative to the substrate, and used this as a correction factor for determining the carbon sources of bacteria. However, other PLFAs were much more variable (-9 to +4‰; Boschker et al., 1999), and variable fractionation factors were observed for fatty acids from several bacterial strains growing on defined substrates (Abraham et al., 1998). In addition, more significant variations have been observed for archaea that assimilate carbon via the anaerobic oxidation of methane (AOM). For instance, archaeol lipids were 15-50‰ more depleted relative to the carbon substrate, methane (Elvert et al., 1999; Hinrichs et al., 1999; Hinrichs and Boetius, 2002).

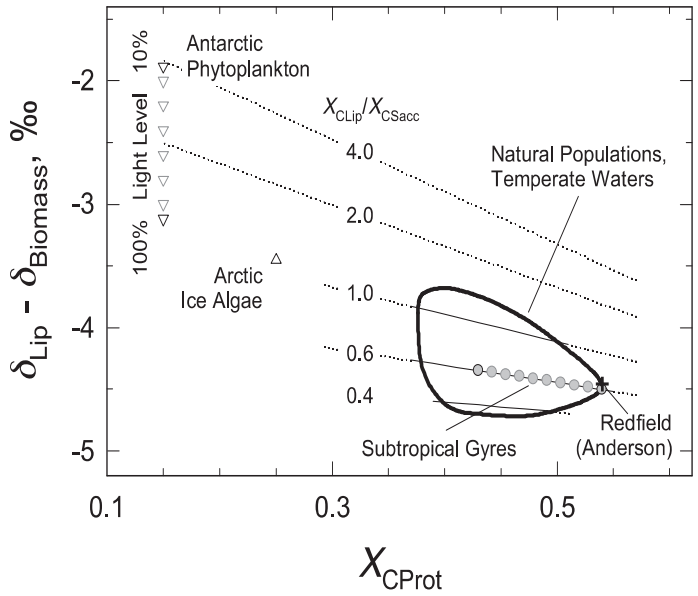


Fig. I.5. Depletion of ^{13}C in lipids relative to biomass as a function of cellular composition. X_{CProt}, X_{CLip} and X_{CSacch} are the mole fractions of carbon in proteins, lipids, and carbohydrates, respectively. The indicated relationships are based on isotopic mass-balance requirements and on concepts outlined by Laws (1991). The cross marked "Redfield (Anderson)" indicates the position of cells with C/N/P = 106/16/1 but with lower (and much more realistic) abundances of H and O than those specified by the conventional Redfield formula (Anderson, 1995). (Figure was taken from Hayes (2001).)

I.2. METHODS

I.2.1 Amino sugars approach

I.2.1.1 Significance of amino sugars in the environment

Amino sugars are significant sedimentary components that are predominantly

derived from microorganisms and invertebrates. The four common amino sugars, GlcN, GalN, mannosamine (ManN) and MurA (Fig. I.6), account for up to 12% of TOC in grassland soils in North America (Amelung et al., 1999) and ~2% of TOC in coastal Peruvian surface sediments (Niggemann and Schubert, 2006). MurA is a diagnostic bacterial biomarker because it is exclusively derived from bacterial cell wall peptidoglycan, which comprises alternating units of MurA and GlcN in acetylated forms (Schleifer and Kandler, 1972; Madigan and Martinko, 2005; Fig. I.7).

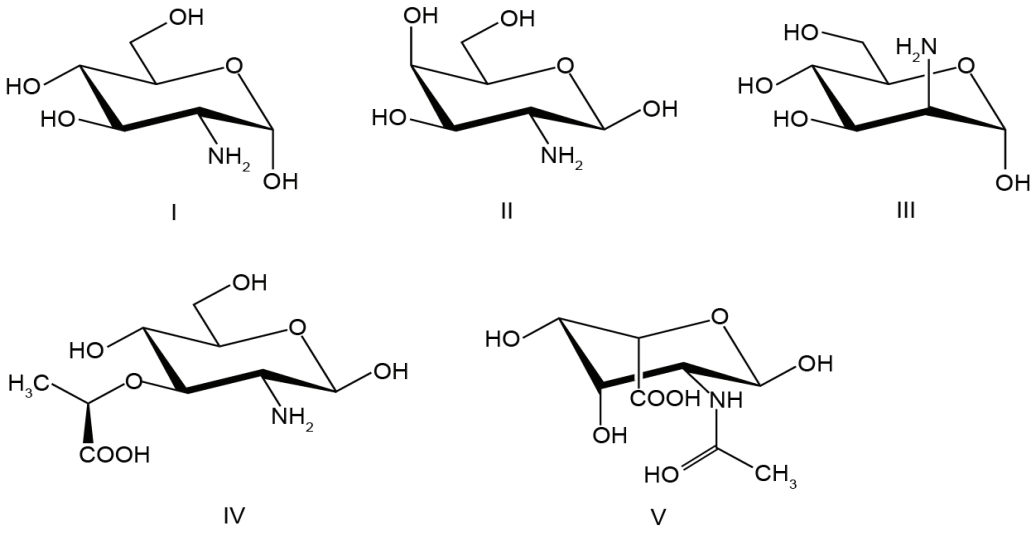


Fig. I.6. Structures of amino sugars: glucosamine (I), galactosamine (II), mannosamine (III), muramic acid (IV) and *N*-acetyaltalosaminuronic acid (V).

Peptidoglycan of gram-positive and gram-negative bacteria is similar in chemical composition but significantly different in quantities, accounting for as much as 90% of the cell walls in the former while only about 10% in the latter (Madigan and Martinko, 2005; Fig. I.7). Archaea do not produce peptidoglycan, but the cell walls of some species of archaea, such as *Methanobacteriales* and *Methanopyrus*, contain a similar polysaccharide layer called pseudopeptidoglycan, in which MurA is substituted by acetyaltalosaminuronic acid (Fig. I.6V; Kandler and König, 1998). Because MurA is presumed to rapidly degrade after cell death (King and White, 1977; Moriarty, 1977), it has been successfully applied as a labile biomarker, correlating well with bacterial concentrations in the water column (Mimura and Romano, 1985; Carstens et al., 2012). However, the turnover of MurA in environmental samples is controlled by several factors, e.g. incorporation with complex sample matrices, different ratios of gram-positive/gram-negative bacteria, and can thus vary distinctly (Nagata et al., 2003). Therefore, some studies have demonstrated that MurA was

associated with bacterial debris rather than living bacteria in the water column (Benner and Kaiser, 2003; Kaiser and Benner, 2008) or surface marine sediments (Niggemann and Schubert, 2006; Lomstein et al., 2009). Investigation of MurA has been recently extended to the marine deep biosphere by Lomstein et al. (2012). Through assessment of MurA and dipicolinic acid (i.e. a specific marker derived from endospores), they estimated endospore populations in the order of 10^7 cells cm^{-3} sediment in sediments from the Peru Margin, revealing a significant but previously overlooked MurA reservoir in subseafloor sediments.

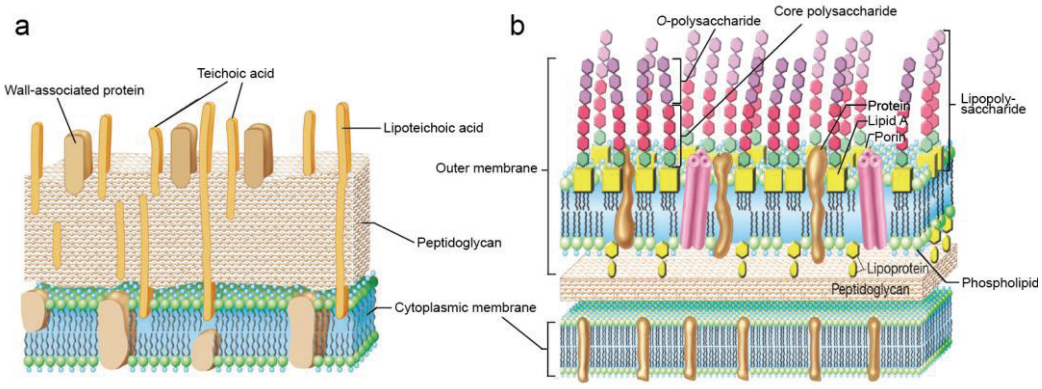


Fig. I.7. The overall structure of Gram-positive (a) and Gram-negative (b) bacteria cell walls and membranes. (Figure was modified from Madigan and Martinko, 2005.)

Contrary to MurA, the three hexosamines have diverse microbial sources. GlcN and GalN are typically the major amino sugars found in environmental samples and are derived primarily from prokaryotes in the forms of peptidoglycan, lipopolysaccharides, and pseudopeptidoglycan, etc. (Schleifer and Kandler, 1972; Kandler et al., 1998; Madigan and Martinko, 2005). Moreover, GlcN is also present as chitin in most fungal cell walls and invertebrate exoskeletons (Müller et al., 1986; Liang et al., 2007; Davis et al., 2009). ManN is widely distributed in glycoproteins and glycolipids of prokaryotes, fungi, mammals and plants (Pickering, 1965; Amir et al., 1966; Glaser et al., 2004; Indorf et al., 2011), but usually with minor abundance or even undetectable (Zhang and Amelung, 1996; Guggenberger et al., 1999; Benner and Kaiser, 2003; Niggemann and Schubert, 2006; Carstens et al., 2012). On the basis of their different distributions, ratios between different amino sugars have been employed as indicators of microbial communities in environmental samples (Guggenberger et al., 1999; Zhang et al., 1999; Glaser et al., 2004). For instance, values of GlcN/GalN between 1-2.5 indicate a predominant contribution from

heterotrophic prokaryotes (Benner and Kaiser, 2003; Davis and Benner, 2005; Kawasaki and Benner, 2006); GlcN/ManN ratios of 2-46 are indicative of bacterial origins, while algae are between 8-18 (Benner and Kaiser, 2003; Glaser et al., 2004).

I.2.1.2 Stable carbon isotopic analysis of amino sugars in marine subseafloor sediments

Stable carbon isotopic compositions ($\delta^{13}C$) of individual amino sugars can reveal carbon metabolism and/or lifestyles of their microbial producers. Over the last decade, incubations with ^{13}C-labeled substrates have been used to investigate biosynthesis and/or turnover kinetics of amino sugars in soils (Glaser and Gross 2005; Decock et al., 2009; Bai et al., 2012). However, the isotopic composition of amino sugars in marine sediment remains poorly explored, partly because of the much lower concentration of amino sugars in marine sediment compared to soils. In this regard, we have systematically evaluated various pretreatment steps and established a robust protocol for gas chromatography (GC)-based $\delta^{13}C$ analysis of amino sugars in marine sediments (Chapter II).

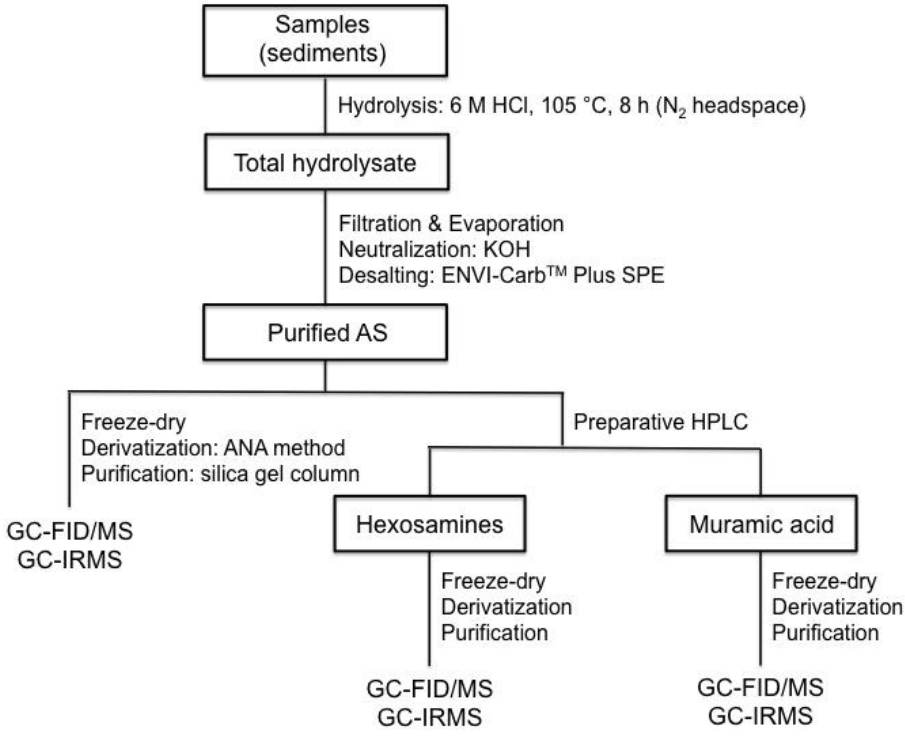


Fig. I.8. Schematic of the optimized procedure for isotope analysis of amino sugars.

The optimized pretreatment protocol for $\delta^{13}C$ analysis of amino sugars includes

three major steps: acid hydrolysis, purification and derivatization (Fig. I.8). In brief, amino sugars were released from marine sediment by hot HCl. The hydrolysate then underwent a series of purification steps, including filtration through a combusted glass fiber filter, evaporation under vacuum, neutralization with KOH solution, and desalting using an ENVI-Carb^{TM} Plus SPE cartridge. After freeze-drying overnight, the extracted amino sugars were transformed to aldononitrile acetate (ANA) derivatives according to the method of Guerrant and Moss (1984) and further purified using a self-packed silica gel column (Lin et al., 2010). Myo-inositol was spiked into the sample subsequent to acid hydrolysis as an internal standard for quantification, while 3-*O*-methyl-D-glucopyranose was added prior to lyophilization as a second standard to compensate for the isotopic fractionation that occurs during derivatization. For samples that exhibited low signals of MurA and/or ManN, due to high background generated by sedimentary matrix or high abundance of the adjacent peak of GalN, amino sugars were separated into two fractions (i.e. hexosamine and MurA fractions) via a preparative high performance liquid chromatography (HPLC) equipped with an Econosphere NH_2 column. Amino sugars in the preparative HPLC fractions could be concentrated to a desired level for $\delta^{13}C$ analysis after conversion to ANA derivatives. The minimum threshold for $\delta^{13}C$ analysis of amino sugars in marine subseafloor sediments with the resulting protocol is ~20 ng.

I.2.2 Phospholipids approach

I.2.2.1 Phospholipids as taxonomic biomarkers for active microorganisms

Phospholipids (PLs) are principal constituents of microbial cytoplasmic membranes that account for ~5% of the cell dry weight for *Thermodesulfotobacterium*, for example (Langworthy et al., 1983). PLs typically consist of phosphate-based head group(s), glycerol backbone(s) and hydrophobic chain(s) that are linked to the glyceryl moiety via ester or ether bonds (Fig. I.9). The widespread head groups include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidyl-(N)-ethanolamine (PME), and phosphatidyl-(N, N)-ethanolamine (PDME) and phosphatidylglycerol (PG), etc. The dominant core structures are diacylglycerol (DAG), acyletherglycerol (AEG) and dietherglycerol (DEG). Bacterial and eukaryotic PLs differ in the length of their fatty acid (FA) side chains and degree

of unsaturation. For example, bacterial PLs typically consist of chain lengths of 14-24 carbon atoms with one unsaturation (Zhang and Rock, 2008).

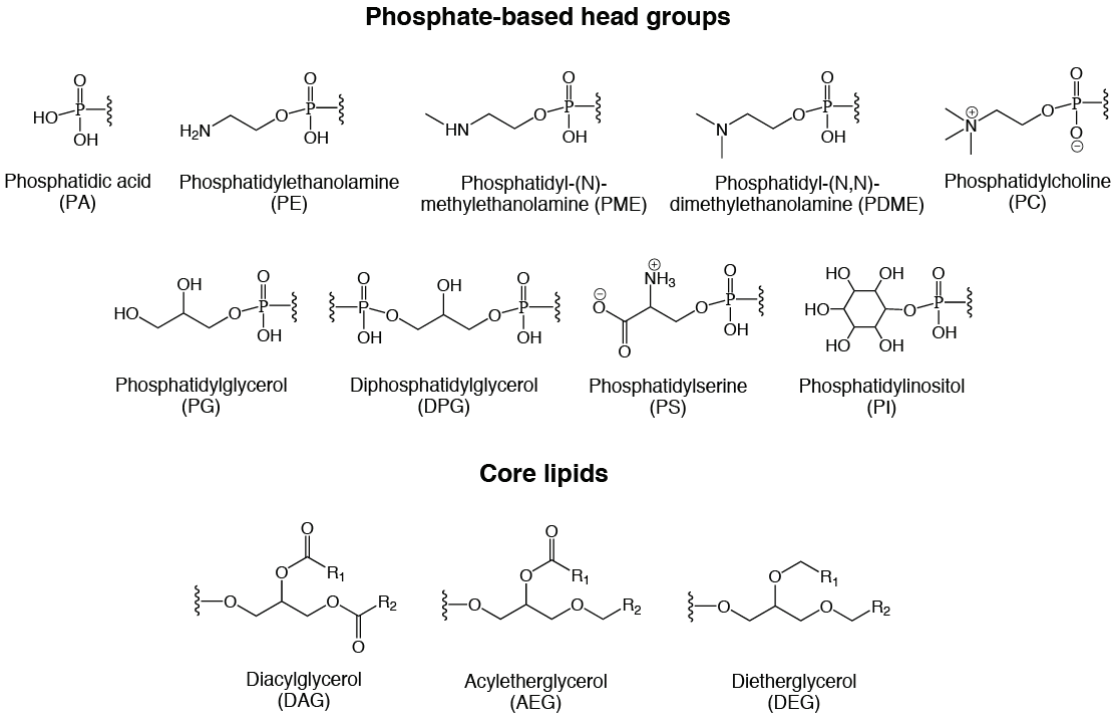


Fig. I.9. Structures of phosphate-based head groups and core lipids.

PLs have been assigned to different groups of organisms and can be indicative of specific environmental factors (Goldfine, 1984; Langworthy and Pond, 1986; Kaneda et al., 1991; Itoh et al., 2001), with chemotaxonomic information primarily encoded in the FA side chains (Fang et al., 2000; Zink et al., 2003; Koga and Morii, 2005; Mills et al., 2006; Wakeham et al., 2007). For example, sulfate-reducing bacteria could synthesize some characteristic branched FAs, such as anteiso and iso $C_{15:0}$ and $C_{17:0}$, which were observed to be genus specific (Boon et al., 1977; Dowling et al., 1986; Kohring et al., 1994). Moreover, studies in surface marine sediments demonstrated that PLs decomposed rapidly after cell lysis (White et al., 1979; Harvey et al., 1986; Logemann et al., 2011). Intact PLs thus reflect the presence of vegetative cells and have been widely investigated as a culture- and gene-independent approach for decoding microbial community composition and total biomass in a variety of aquatic environments (Bobbie and White, 1980; Vestal and White, 1989; Zelles et al., 1992; Sundh et al., 1997; Lipp et al., 2008; Schubotz et al., 2009; Peterse et al., 2011). By combining this approach with stable carbon isotopic analysis of the FA side chains, some recent studies have assessed the isotopic fractionation associated with the incorporation of carbon substrates into microbial lipids (Londry et al., 2004; Schubotz

et al., 2011; Bühring et al., 2012; Kellermann et al., 2012), opening a promising analytical window to investigate benthic microbial metabolisms in the deep biosphere.

I.2.2.2 Analysis of PLs in marine sediments

PLs are conventionally analyzed using GC-mass spectrometry (MS) by characterizing their FA derivatives after hydrolytic cleavage of the polar head groups and ester linkages to the glycerol backbones (White et al., 1979; DeLong and Yayanos, 1986; Frostegård et al., 1993; Elvert et al., 2003; Mills et al., 2006). With LC-electrospray ionization (ESI)-MS, intact PLs can be directly measured, without breaking PLs into FA components, thereby providing more comprehensive taxonomic information for deciphering microbial communities and a quantitative overview of microbial populations in environmental samples (Fang and Barcelona, 1998; Rütters et al., 2002; Sturt et al., 2004; Schubotz et al., 2009). However, LC-based analyses of PLs are subject to significant interference due to co-eluting compounds within the sample matrix, which reduces PL ionization efficiency and consequently detection (Wörmer et al., 2013). We therefore implemented a cleanup strategy for sedimentary PL analysis using a HybridSPE®-Phospholipid cartridge (Chapter IV), which has been recently introduced for isolation of PLs from biological samples, such as serum and plasma (Pucci et al., 2009; Jiang et al., 2011; Moriarty et al., 2012). The HybridSPE®-Phospholipid cartridge is filled with zirconia-coated silica sorbents, which have strong affinity to phosphate moieties due to Lewis acid-base interaction (Fig. I.10), while the retention of a wide range of basic, neutral and acidic compounds onto the cartridge is minimal.

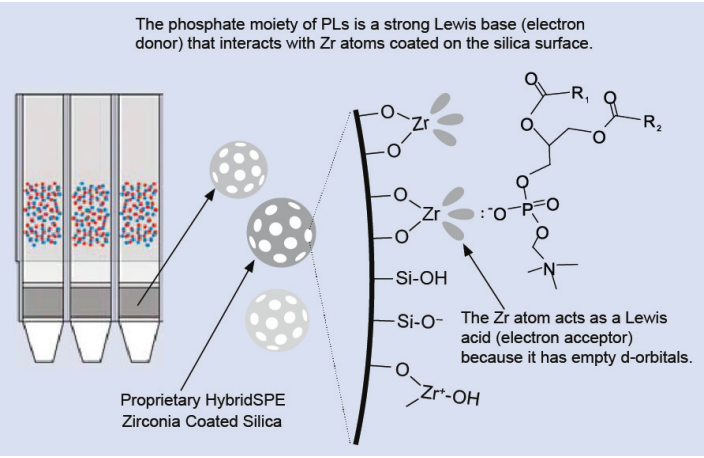


Fig. I.10. Mechanism of the HybridSPE®-Phospholipid cartridge for retention of phospholipids (Figure was modified from Sigma Technical Report).

The optimized PL analysis procedure is summarized as follows. In brief, sediment samples were extracted using a modified Bligh and Dyer method described by Sturt et al. (2004). The resulting total lipid extracts (TLEs) were re-dissolved in MeOH:HCO_2H (99:1, v:v), loaded onto the preconditioned SPE cartridges and eluted under vacuum. The cartridges were then washed with 3 ml MeOH containing 1% NH_4CO_2H (wt.%) followed by 3 ml MeOH. PLs in the TLEs were selectively retained on the cartridges and thus separated from the majority of sedimentary extracts, including free fatty acids and glycolipids. PLs were then eluted from the cartridges using three different eluents, including 6 ml IPA containing 10% (wt.%) NH_4OH, followed by 6 ml IPA containing 15% (wt.%) NH_4OH and finally 6 ml DCM:IPA:NH_4OH (5:9:6, v:v:v) under vacuum. All three eluents were collected and combined as the PL fraction and analyzed via LC-ESI-MS after Wörmer et al. (2013). The cleanup protocol can strongly improve the detection of PLs, thereby offering more accurate and comprehensive description of PL fingerprints in marine sediments.

I.2.3 Pyrolysis approach

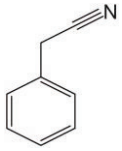
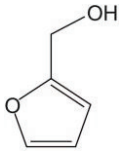
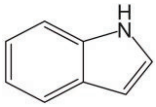
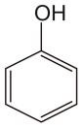
I.2.3.1 Pyrolysis - a promising technique for investigation of biomarkers

Pyrolysis (py)-GC is a well-established technique by which nonvolatile organic macromolecules can be thermally decomposed to low-molecular fingerprints in the absence of oxygen (Challinor, 2001) and can potentially result in side reactions that form new compounds (e.g. ring structures) during pyrolysis (White et al., 2004). It is advantageous due to the minimal requirements for sample preparation and sample size (submilligram), rapid analysis and low cost (Meuzelaar et al., 1974; Stankiewicz et al., 1997; Barshick et al., 1999). Therefore, py-GC has been routinely used in many fields such as art materials (Chiavari et al., 1998; van den Berg et al., 1998), fuel sources (Inan et al., 1998; Weng et al., 2003) and forensic science (Hida et al., 1995; Kochanowski and Morgan, 2000). A variety of biological substances have been investigated using pyrolysis techniques over the past dacades, including DNA (Jarman, 1980; Poinar and Stankiewicz, 1999), proteins (Tsuge and Matsubara, 1985; Langhammer et al., 1986; Knicker et al., 2001), chitin (Lal and Hayes, 1984; van der Kaaden et al, 1984; Sato et al., 1998), sporopollenin, algaenan and dinosporin (see review by de Leeuw et al., 2006). With multivariate statistic analysis of the diagnostic

fragments (e.g. principal component analysis), different strains of microorganisms can be identified (Smith et al, 1987; Barshick et al., 1999; Miketova et al., 2003; Snyder et al., 2004).

I.2.3.2 Detection of microbial biomass by py-GC/MS

Table I.1. Structures and biological sources of the selected pyrolysis fingerprints for tracking microbial biomass in marine sediments.

Compound	Formula	Structure	Biological source
Benzyl nitrile	C_8H_7N		DNA, protein
2-Furanmethanol	$C_5H_6O_2$		DNA, peptidoglycan
Indole	C_8H_7N		peptidoglycan, protein
Phenol	C_6H_6O		DNA, peptidoglycan, protein
Pyrrole	C_4H_5N		peptidoglycan

The conventional approaches for qualitative and quantitative assessment of microbial biomass in marine sediments usually require a tedious sample pretreatment procedure, such as extraction of the biomarkers using solvents. In contrast, the pyrolysis technique can provide an alternative route via studying the characteristic fragments of the target compounds in marine sediments. During method development, we analyzed several biological substances (e.g. peptidoglycan, DNA, protein) and isolated prokaryotic biomass (Chapter VI) by py-GC/MS to monitor characteristic pyrolysis fragments of microbial biomass. After lyophilization over night, the well-homogenized samples were directly loaded into a quartz tube for pyrolysis analysis. A GC column with high polarity was applied in this study, which enabled separation of polar compounds without derivatization. Five pyrolysis fragments, i.e.

benzyl nitrile (from DNA and protein), 2-furanmethanol (from DNA and peptidoglycan), indole (from peptidoglycan and protein), phenol (from DNA, peptidoglycan and protein) and pyrrole (from peptidoglycan) were selected as indicators for microbial biomass in sediments (Table I.1). Naphthalene-D8 was spiked into the sample prior to pyrolysis as an internal standard for quantification. This approach enabled rapid detection and quantification of microbial biomass in sediment samples.

I.3. OBJECTIVES OF THE THESIS

On the basis of previous lipid-based studies, bacterial signals have been rarely detected in the deep marine subseafloor sediments, presumably due to the reduced biomass density and complex sedimentary matrices. Therefore, the major objectives of this thesis were to develop and apply novel methods for the analysis of microbial biomarkers, and consequently deciphering microbial communities in the deep biosphere. The methods introduced in this thesis include two targeting specifically bacterial biomarkers and one bulk chemical technique for microbial signals as assemblages. The thesis thus can be divided into three parts according to different groups of biomarkers studied and techniques employed.

The first part of the thesis, including Chapter II and Chapter III, focus on quantitative and stable carbon isotopic analysis of four major amino sugars, i.e. GlcN, GalN, ManN and MurA, in marine sediments. Chapter II reports an optimized sample pretreatment procedure for GC-based stable carbon isotopic analysis of amino sugars at trace levels (limit of detection = 20 ng of amino sugar). This optimized protocol was applied for analysis of amino sugars in subseafloor sediments from two contrasting sites, ODP Leg 201 Site 1229 from the Peru Margin and IODP Expedition 317 Site U1352 from the Canterbury Basin (Chapter III). The scientific questions addressed in this part are:

(1) Can we use MurA as a biomarker to estimate active bacterial cells in marine sediments?

Previous lipid-based studies suggested that archaea constitute the dominant microbial group in the deep biosphere. However, the IPL-based

approach may be biased due to the preferential preservation of archaeal lipids. MurA is a diagnostic biomarker that is exclusively present in bacterial cell walls; in the water column its abundance is well correlated with bacterial cell concentrations. Therefore, MurA can be potentially applied for detecting and quantifying bacterial communities in marine sediments.

(2) Can amino sugars be used to determine microbial biomass concentrations and/or to distinguish different OM types in subseafloor sediments?

Amino sugars are predominantly of microbial origin. Ratios of different amino sugars have been used to differentiate microbial communities in soils and to identify OM from different sources in aquatic and surface sediment samples. I therefore hypothesize that distinct downcore profiles of amino sugar concentrations and ratios characterize the two study sites, which feature contrasting TOC content, OM type and microbial abundance.

(3) Can we gain information regarding indigenous microbial metabolisms through stable carbon isotopic analysis of amino sugars?

The stable carbon isotopic compositions of bacterial and archaeal lipids have revealed the carbon metabolism and/or lifestyles of the source organisms in environmental samples. By analogy, I hypothesize that the $\delta^{13}C$ signatures of amino sugars can reflect in-situ biogeochemical processes in deep biosphere.

The second part of the thesis, consisting of Chapters IV and V, aims to decipher microbial communities in marine sediments by analysis of PLs using LC-ESI-MS. Chapter IV reports a novel cleanup protocol for the analysis of PLs in sediment samples, which significantly improved PL detection. This cleanup protocol enabled isolation of intact PLs from the majority of sedimentary matrices, thus providing an opportunity for PL analysis in oil-bearing sediments from the Guaymas Basin hydrothermal field with comparatively low interference (Chapter V). Sediments from an adjacent non-hydrothermal site were analyzed for reference. The major scientific

questions addressed in this section are:

(1) What is the upper temperature limit of the microbial population that is sustained in the hydrothermal environment?

Previous studies have reported the maximum growth temperature for hyperthermophilic archaea was up to ~120 °C (Takai et al., 2008). Because PLs decompose rapidly upon cell lysis, their detection can be indicative of the presence of living microorganisms. Combined with sedimentary temperature measurements, this analysis can be used to infer the maximum temperature threshold sustainable by bacteria in the Guaymas Basin hydrothermal field.

(2) Does temperature affect PL distribution patterns between the hydrothermal and non-hydrothermal sites?

Bacteria can react to the variable environmental conditions by modifying their fatty acid chains (e.g. chain length, unsaturation, cyclisation, and methyl branches) and/or the polar head group. Therefore, PL compositions are expected to differ between the hydrothermal site compared to the non-hydrothermal site.

In the third part of the thesis, i.e. Chapter VI, a py-GC/MS based protocol is introduced for the rapid determination of microbial biomass in marine sediments. Subseafloor sediments of different TOC contents and from different depths were analyzed using this protocol. The scientific questions addressed in Chapter VI are:

(1) Are pyrolysis fingerprints indicative of microbial biomass in marine sediments?

Py-GC/MS has been widely applied for the analysis of a variety of biological substances, resulting in diagnostic pyrolysis fragments for the isolated biopolymers or microbial linages. Therefore, characteristic pyrolysis fragments may be indicative of microbial signals in marine sediments.

(2) To what extent can the py-GC/MS technique be used for the rapid detection of microbial signals in marine subseafloor sediments?

Compared with the traditional geochemical methods for analysis of

microbial biomarkers (e.g. cell counts, IPLs), py-GC/MS requires only minimal sample preparation and sample sizes. Because microbial populations are less abundant in deep marine sediment, the sensitivity of the py-GC/MS technique must be carefully evaluated.

I.4. CONTRIBUTIONS TO PUBLICATIONS

Chapter II – full manuscript

Optimizing sample pretreatment for compound-specific stable carbon isotopic analysis of amino sugars in marine sediment

Rong Zhu, Yu-Shih Lin, Julius S. Lipp, Travis B. Meador, Kai-Uwe Hinrichs

R.Z. designed the project with help from Y.-S.L. and J.S.L. and K.-U.H. R.Z. optimized the method with help from Y.-S.L., J.S.L. and T.B.M. R.Z. extracted samples for method evaluation, $\delta^{13}C$ analysis and interpreted the $\delta^{13}C$ results with guidance from Y.-S.L. and T.B.M. R.Z. wrote the paper with input from all co-authors. *Biogeosciences*. Accepted for online discussion.

Chapter III – full manuscript

Distribution and isotopic composition of amino sugars in contrasting subseafloor sediments

Rong Zhu, Julius S. Lipp, Yu-Shih Lin, Kai-Uwe Hinrichs

R.Z., J.S.L. and K.-U.H. designed the project. J.S.L. and K.-U.H. collected the samples and analyzed for TOC, TN, $\delta^{13}C$-DIC, $\delta^{13}C$-TOC and $\delta^{13}C$-CH_4. R.Z. extracted samples and performed quantification and $\delta^{13}C$ analysis of amino sugars with technical guidance from Y.-S.L. R.Z. interpreted the results with help from J.S.L. and Y.-S.L. R.Z. wrote the paper with input from all co-authors. In preparation for *Geochimica et Cosmochimica Acta*.

Chapter IV – full manuscript

Improved sensitivity of sedimentary phospholipid analysis resulting from a novel extract cleanup strategy

Rong Zhu, Thomas W. Evans, Lars Wörmer, Yu-Shih Lin, Chun Zhu, Kai-Uwe Hinrichs

R.Z. designed the project with help from Y.-S.L., L.W., C.Z. and K.-U.H. R.Z. and T.W.E. extracted samples and optimized the method. R.Z. wrote the paper with input from all co-authors. Published in *Organic Geochemistry*, vol. 65, page 46-52, 2013. doi: 10.1016/j.orggeochem.2013.10.002

Chapter V – Data report

Distributions of phospholipids in oil-bearing hydrothermal field of the Guaymas Basin

Rong Zhu, Yu-Shih Lin, Kai-Uwe Hinrichs

R.Z. and Y.-S.L. designed the project. Y.-S.L. and K.-U.H. collected the samples. Y.-S.L. extracted the samples and measured TOC contents. R.Z. performed sample clean-up and analyzed the samples. Because more analyses, such as cell counting, are currently in progress at the University of North Carolina, we only briefly report our PL data here. R.Z. wrote the chapter with input from all co-authors.

Chapter VI – full manuscript

Detection of microbial biomass in subseafloor sediment by pyrolysis-GC/MS

Rong Zhu, Gerard J. M. Versteegh, Kai-Uwe Hinrichs

R.Z., G.J.M.V. and K.-U.H. designed the project. R.Z. performed the experiments with technical guidance from G.J.M.V. R.Z. wrote the paper with input from all co-authors. In preparation for *Journal of Analytical and Applied Pyrolysis*.

I.5. REFERENCES

Abraham, W.R., Hesse, C., Pelz, O., 1998. Ratios of carbon isotopes in microbial lipids as an indicator of substrate usage. Applied and Environmental Microbiology 64, 4202-4209.

Amelung, W., Flach, K., Zech, W., 1999. Neutral and acidic sugars in particle-size fractions as influenced by climate. Soil Science Society of America Journal 63, 865-873.

Amir, S., Barker, S., Butt, W., Crooke, A., Davies, A., 1966. Administration of N-acetyl-D-mannosamine to mammals. Nature 211, 976-977.

Anderson, L.A., 1995. On the hydrogen and oxygen content of marine phytoplankton. Deep-Sea Research 42, 1675-1680.

Bai, Z., Bode, S., Huygens, D., Zhang, X., Boeckx, P., 2012. Kinetics of amino sugar formation from organic residues of different quality. Soil Biology and

Biochemistry 57, 814-821.

Barshick, S., Wolf, D., Vass, A., 1999. Differentiation of microorganisms based on pyrolysis-ion trap mass spectrometry using chemical ionization. Analytical Chemistry 71, 633-641.

Benner, R., Kaiser, K., 2003. Abundance of amino sugars and peptidoglycan in marine particulate and dissolved organic matter. Limnology and Oceanography 48, 118-128.

Biddle, J.F., Lipp, J.S., Lever, M.A., Lloyd, K.G., Sørensen, K.B., Anderson, R., Fredricks, H.F., Elvert, M., Kelly, T.J., Schrag, D.P., 2006. Heterotrophic Archaea dominate sedimentary subsurface ecosystems off Peru. Proceedings of the National Academy of Sciences of the United States of America 103, 3846-3851.

Blair, N., Leu, A., Muñoz, E., Olsen, J., Kwong, E., Des Marais, D., 1985. Carbon isotopic fractionation in heterotrophic microbial metabolism. Applied and Environmental Microbiology 50, 996-1001.

Bobbie, R.J., White, D.C., 1980. Characterization of benthic microbial community structure by high-resolution gas chromatography of fatty acid methyl esters. Applied and Environmental Microbiology 39, 1212-1222.

Boon, J., de Leeuw, J., Hoek, G., Vosjan, J., 1977. Significance and taxonomic value of iso and anteiso monoenoic fatty acids and branded beta-hydroxy acids in Desulfovibrio desulfuricans. Journal of Bacteriology 129, 1183-1191.

Boschker, H., De Brouwer, J., Cappenberg, T., 1999. The contribution of macrophyte-derived organic matter to microbial biomass in salt-marsh sediments: Stable carbon isotope analysis of microbial biomarkers. Limnology and Oceanography 44, 309-319.

Boschker, H., Middelburg, J., 2002. Stable isotopes and biomarkers in microbial ecology. FEMS Microbiology Ecology 40, 85-95.

Brassell, S., Brereton, R., Eglinton, G., Grimalt, J., Liebezeit, G., Marlowe, I., Pflaumann, U., Sarnthein, M., 1986. Palaeoclimatic signals recognized by chemometric treatment of molecular stratigraphic data. Organic Geochemistry 10, 649-660.

Bühring, S., Schubotz, F., Harms, C., Lipp, J., Amils, R., Hinrichs, K.-U., 2012. Lipid signatures of acidophilic microbial communities in an extreme acidic environment-Río Tinto, Spain. Organic Geochemistry 47, 66-77.

Carstens, D., Köllner, K.E., Bürgmann, H., Wehrli, B., Schubert, C.J., 2012. Contribution of bacterial cells to lacustrine organic matter based on amino sugars and D-amino acids. Geochimica et Cosmochimica Acta 89, 159-172.

Challinor, J.M., 2001. Review: the development and applications of thermally assisted hydrolysis and methylation reactions. Journal of Analytical and Applied Pyrolysis 61, 3-34.

Chiavari, G., Gandini, N., Russo, P., Fabbri, D., 1998. Characterisation of standard tempera painting layers containing proteinaceous binders by pyrolysis (/methylation)-gas chromatography-mass spectrometry. Chromatographia 47, 420-426.

Coffin, R.B., Velinsky, D., Devereux, R., Price, W., Cifuentes, L., 1990. Stable carbon isotope analysis of nucleic acids to trace sources of dissolved substrates used by estuarine bacteria. Applied and Environmental Microbiology 56, 2012-2020.

D'Hondt, S., Rutherford, S., Spivack, A.J., 2002. Metabolic activity of subsurface life in deep-sea sediments. Science 295, 2067.

Davis, J., Benner, R., 2005. Seasonal trends in the abundance, composition and bioavailability of particulate and dissolved organic matter in the Chukchi/Beaufort Seas and western Canada Basin. Deep Sea Research Part II: Topical Studies in Oceanography 52, 3396-3410.

Davis, J., Kaiser, K., Benner, R., 2009. Amino acid and amino sugar yields and compositions as indicators of dissolved organic matter diagenesis. Organic Geochemistry 40, 343-352.

de Leeuw, J.W., Versteegh, G.J., van Bergen, P.F., 2006. Biomacromolecules of algae and plants and their fossil analogues. Plant Ecology 182, 209-233.

Decock, C., Denef, K., Bodé, S., Six, J., Boeckx, P., 2009. Critical assessment of the applicability of gas chromatography-combustion-isotope ratio mass spectrometry to determine amino sugar dynamics in soil. Rapid Communications in Mass Spectrometry 23, 1201-1211.

DeLong, E.F., Yayanos, A.A., 1986. Biochemical function and ecological significance of novel bacterial lipids in deep-sea procaryotes. Applied and Environmental Microbiology 51, 730-737.

Dowling, N.J., Widdel, F., White, D.C., 1986. Phospholipid ester-linked fatty acid biomarkers of acetate-oxidizing sulphate-reducers and other sulphide-forming bacteria. Journal of General Microbiology 132, 1815-1825.

Eglinton, G., Hamilton, R.J., 1967. Leaf epicuticular waxes. Science 156, 1322-1335.

Elvert, M., Suess, E., Whiticar, M.J., 1999. Anaerobic methane oxidation associated with marine gas hydrates: Superlight C-isotopes from saturated and unsaturated C_{20} and C_{25} irregular isoprenoids. Naturwissenschaften 86, 295-300.

Elvert, M., Boetius, A., Knittel, K., Jørgensen, B.B., 2003. Characterization of specific membrane fatty acids as chemotaxonomic markers for sulfate-reducing bacteria involved in anaerobic oxidation of methane. Geomicrobiology Journal 20, 403-419.

Fang, J., Barcelona, M.J., 1998. Structural determination and quantitative analysis of bacterial phospholipids using liquid chromatography/electrospray ionization/mass spectrometry. Journal of Microbiological Methods 33, 23-35.

Fang, J., Barcelona, M.J., Semrau, J.D., 2000. Characterization of methanotrophic bacteria on the basis of intact phospholipid profiles. FEMS Microbiology Letters 189, 67-72.

Frostegård, Å., Tunlid, A., Bååth, E., 1993. Phospholipid fatty acid composition, biomass, and activity of microbial communities from two soil types experimentally exposed to different heavy metals. Applied and Environmental Microbiology 59, 3605-3617.

Glaser, B., Gross, S., 2005. Compound-specific $\delta^{13}C$ analysis of individual amino sugars - a tool to quantify timing and amount of soil microbial residue

stabilization. Rapid Communications in Mass Spectrometry 19, 1409-1416.

Glaser, B., Turrión, M., Alef, K., 2004. Amino sugars and muramic acid-biomarkers for soil microbial community structure analysis. Soil Biology and Biochemistry 36, 399-407.

Goldfine, H., 1984. Bacterial membranes and lipid packing theory. Journal of Lipid Research 25, 1501-1507.

Guerrant, G., Moss, C., 1984. Determination of monosaccharides as aldononitrile, O-methyloxime, alditol, and cyclitol acetate derivatives by gas chromatography. Analytical Chemistry 56, 633-638.

Guggenberger, G., Frey, S., Six, J., Paustian, K., Elliott, E., 1999. Bacterial and fungal cell-wall residues in conventional and no-tillage agroecosystems. Soil Science Society of America Journal 63, 1188-1198.

Harvey, H.R., Fallon, R.D., Patton, J.S., 1986. The effect of organic matter and oxygen on the degradation of bacterial membrane lipids in marine sediments. Geochimica et Cosmochimica Acta 50, 795-804.

Hayes, J., 1993. Factors controlling ^{13}C contents of sedimentary organic compounds: Principles and evidence. Marine Geology 113, 111-125.

Hayes, J.M., 2001. Fractionation of carbon and hydrogen isotopes in biosynthetic processes. Reviews in Mineralogy and Geochemistry 43, 225-277.

Heuer, V., Elvert, M., Tille, S., Krummen, M., Mollar, X.P., Hmelo, L.R., Hinrichs, K.-U., 2006. Online $\delta^{13}C$ analysis of volatile fatty acids in sediment/porewater systems by liquid chromatography-isotope ratio-mass spectrometry. Limnology and Oceanography: Methods 4, 346-357.

Heuer, V.B., Pohlman, J.W., Torres, M.E., Elvert, M., Hinrichs, K.-U., 2009. The stable carbon isotope biogeochemistry of acetate and other dissolved carbon species in deep subseafloor sediments at the northern Cascadia Margin. Geochimica et Cosmochimica Acta 73, 3323-3336.

Hida, M., Mitsui, T., Minami, Y., Fujimura, Y., 1995. Classification of hashish by pyrolysis-gas chromatography. Journal of Analytical and Applied Pyrolysis 32, 197-204.

Hinrichs, K.-U., Hayes, J.M., Sylva, S.P., Brewer, P.G., DeLong, E.F., 1999. Methane-consuming archaebacteria in marine sediments. Nature 398, 802-805.

Hinrichs, K.-U., Boetius, A., 2002. The anaerobic oxidation of methane: New insights in microbial ecology and biogeochemistry. In Ocean Margin Systems (eds. Wefer, G. et al.), Springer Verlag, Berlin-Heidelberg, 457-477.

Hopmans, E.C., Weijers, J.W., Schefuß, E., Herfort, L., Sinninghe Damsté, J.S., Schouten, S., 2004. A novel proxy for terrestrial organic matter in sediments based on branched and isoprenoid tetraether lipids. Earth and Planetary Science Letters 224, 107-116.

Hullar, M., Fry, B., Peterson, B., Wright, R., 1996. Microbial utilization of estuarine dissolved organic carbon: a stable isotope tracer approach tested by mass balance. Applied and Environmental Microbiology 62, 2489-2493.

Ijiri, A., Harada, N., Hirota, A., Tsunogai, U., Ogawa, N.O., Itaki, T., Khim, B.-K., Uchida, M., 2012. Biogeochemical processes involving acetate in sub-seafloor

sediments from the Bering Sea shelf break. Organic Geochemistry 48, 47-55.

Inagaki, F., Nunoura, T., Nakagawa, S., Teske, A., Lever, M., Lauer, A., Suzuki, M., Takai, K., Delwiche, M., Colwell, F.S., 2006. Biogeographical distribution and diversity of microbes in methane hydrate-bearing deep marine sediments on the Pacific Ocean Margin. Proceedings of the National Academy of Sciences of the United States of America 103, 2815-2820.

Inan, S., Yalçin, M.N., Mann, U., 1998. Expulsion of oil from petroleum source rocks: inferences from pyrolysis of samples of unconventional grain size. Organic Geochemistry 29, 45-61.

Indorf, C., Dyckmans, J., Khan, K.S., Joergensen, R.G., 2011. Optimisation of amino sugar quantification by HPLC in soil and plant hydrolysates. Biology and Fertility of Soils 47, 387-396.

Itoh, Y., Sugai, A., Uda, I., Itoh, T., 2001. The evolution of lipids. Advances in Space Research 28, 719-724.

Jarman, M., 1980. Pyrolysis of deoxyribonucleic acid: isolation and mass spectrometry of individual pyrolysis products. Journal of Analytical and Applied Pyrolysis 2, 217-223.

Jiang, H., Zhang, Y., Ida, M., LaFayette, A., Fast, D.M., 2011. Determination of carboplatin in human plasma using HybridSPE-precipitation along with liquid chromatography-tandem mass spectrometry. Journal of Chromatography B 879, 2162-2170.

Jørgensen, B.B., 2011. Deep subseafloor microbial cells on physiological standby. Proceedings of the National Academy of Sciences of the United States of America 108, 18193-18194.

Jørgensen, B.B., D'Hondt, S., 2006. A starving majority deep beneath the seafloor. Science 314, 932-934.

Kaiser, K., Benner, R., 2008. Major bacterial contribution to the ocean reservoir of detrital organic carbon and nitrogen. Limnology and Oceanography 53, 99-112.

Kallmeyer, J., Pockalny, R., Adhikari, R.R., Smith, D.C., D'Hondt, S., 2012. Global distribution of microbial abundance and biomass in subseafloor sediment. Proceedings of the National Academy of Sciences of the United States of America 109, 16213-16216.

Kandler, O., König, H., 1998. Cell wall polymers in Archaea (Archaebacteria). Cellular and Molecular Life Sciences 54, 305-308.

Kaneda, T., 1991. Iso-and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. Microbiological Reviews 55, 288-302.

Kawasaki, N., Benner, R., 2006. Bacterial release of dissolved organic matter during cell growth and decline: Molecular origin and composition. Limnology and Oceanography, 2170-2180.

Kellermann, M.Y., Wegener, G., Elvert, M., Yoshinaga, M.Y., Lin, Y.S., Holler, T., Mollar, X.P., Knittel, K., Hinrichs, K.-U., 2012. Autotrophy as a predominant mode of carbon fixation in anaerobic methane-oxidizing microbial communities. Proceedings of the National Academy of Sciences of the United States of America 109, 19321-19326.

Killops, S., Killops, V., 2005. Introduction to organic geochemistry. Blackwell Publishing, Oxford.

Kim, J.-H., Van der Meer, J., Schouten, S., Helmke, P., Willmott, V., Sangiorgi, F., Koç, N., Hopmans, E.C., Damsté, J.S.S., 2010. New indices and calibrations derived from the distribution of crenarchaeal isoprenoid tetraether lipids: Implications for past sea surface temperature reconstructions. Geochimica et Cosmochimica Acta 74, 4639-4654.

King, J.D., White, D.C., 1977. Muramic acid as a measure of microbial biomass in estuarine and marine samples. Applied and Environmental Microbiology 33, 777-783.

Knicker, H., Río, J.d., Hatcher, P.G., Minard, R.D., 2001. Identification of protein remnants in insoluble geopolymers using TMAH thermochemolysis/GC-MS. Organic Geochemistry 32, 397-409.

Kochanowski, B.K., Morgan, S.L., 2000. Forensic discrimination of automotive paint samples using pyrolysis-gas chromatography-mass spectrometry with multivariate statistics. Journal of Chromatographic Science 38, 100-108.

Koga, Y., Morii, H., 2005. Recent advances in structural research on ether lipids from archaea including comparative and physiological aspects. Bioscience, Biotechnology, and Biochemistry 69, 2019-2034.

Kohring, L.L., Ringelberg, D.B., Devereux, R., Stahl, D.A., Mittelman, M.W., White, D.C., 1994. Comparison of phylogenetic relationships based on phospholipid fatty acid profiles and ribosomal RNA sequence similarities among dissimilatory sulfate-reducing bacteria. FEMS Microbiology Letters 119, 303-308.

Lal, G., Hayes, E., 1984. Determination of the amine content of chitosan by pyrolysis-gas chromatography. Journal of Analytical and Applied Pyrolysis 6, 183-193.

Langerhuus, A.T., Røy, H., Lever, M.A., Morono, Y., Inagaki, F., Jørgensen, B.B., Lomstein, B.A., 2012. Endospore Abundance and d: l-Amino Acid Modeling of Bacterial Turnover in Holocene Marine Sediment (Aarhus Bay). Geochimica et Cosmochimica Acta 99, 87-99.

Langhammer, M., Lüderwald, I., Simons, A., 1986. Analytical pyrolysis of proteins. Fresenius' Journal of Analytical Chemistry 324, 5-8.

Langworthy, T.A., Holzer, G., Zeikus, J.G., Tornabene, T.G., 1983. Iso-and Anteiso-Branched Glycerol Diethers of the Thermophilic Anaerobe *Thermodesulfotobacterium commune*. Systematic and Applied Microbiology 4, 1-17.

Langworthy, T.A., Pond, J.L., 1986. Archaebacterial ether lipids and chemotaxonomy. Systematic and Applied Microbiology 7, 253-257.

Laws, E.A., 1991. Photosynthetic quotients, new production and net community production in the open ocean. Deep-Sea Research 38, 143-167.

Lever, M.A., Heuer, V.B., Morono, Y., Masui, N., Schmidt, F., Alperin, M.J., Inagaki, F., Hinrichs, K.-U., Teske, A., 2010. Acetogenesis in deep subseafloor sediments of the Juan de Fuca Ridge Flank: a synthesis of geochemical, thermodynamic, and gene-based evidence. Geomicrobiology Journal 27, 183-211.

Liang, C., Zhang, X., Balser, T.C., 2007. Net microbial amino sugar accumulation process in soil as influenced by different plant material inputs. Biology and Fertility of Soils 44, 1-7.

Lin, Y.S., Lipp, J.S., Yoshinaga, M.Y., Lin, S.H., Elvert, M., Hinrichs, K.-U., 2010. Intramolecular stable carbon isotopic analysis of archaeal glycosyl tetraether lipids. Rapid Communications in Mass Spectrometry 24, 2817-2826.

Lipp, J.S., Hinrichs, K.-U., 2009. Structural diversity and fate of intact polar lipids in marine sediments. Geochimica et Cosmochimica Acta 73, 6816-6833.

Lipp, J.S., Morono, Y., Inagaki, F., Hinrichs, K.-U., 2008. Significant contribution of Archaea to extant biomass in marine subsurface sediments. Nature 454, 991-994.

Logemann, J., Graue, J., Köster, J., Engelen, B., Rullkötter, J., Cypionka, H., 2011. A laboratory experiment of intact polar lipid degradation in sandy sediments. Biogeosciences 8, 2547-2560.

Lomstein, B., Niggemann, J., Jørgensen, B., Langerhuus, A., 2009. Accumulation of prokaryotic remains during organic matter diagenesis in surface sediments off Peru. Limnology and Oceanography 54, 1139-1151.

Lomstein, B.A., Langerhuus, A.T., D'Hondt, S., Jørgensen, B.B., Spivack, A.J., 2012. Endospore abundance, microbial growth and necromass turnover in deep sub-seafloor sediment. Nature 484, 101-104.

Londry, K., Jahnke, L., Des Marais, D., 2004. Stable carbon isotope ratios of lipid biomarkers of sulfate-reducing bacteria. Applied and Environmental Microbiology 70, 745-751.

Madigan, M.T., Martinko, J.M., 2005. Brock Biology of Microorganisms 11th edn., Prentice Hall, Upper Saddle River, USA.

Meuzelaar, H.L., Kistemaker, P.G., Posthumus, M.A., 1974. Recent advances in pyrolysis mass spectrometry of complex biological materials. Biological Mass Spectrometry 1, 312-319.

Meyers, P.A., 1997. Organic geochemical proxies of paleoceanographic, paleolimnologic, and paleoclimatic processes. Organic Geochemistry 27, 213-250.

Middelburg, J.J., 1989. A simple rate model for organic matter decomposition in marine sediments. Geochimica et Cosmochimica Acta 53, 1577-1581.

Miketova, P., Abbas-Hawks, C., Voorhees, K.J., Hadfield, T.L., 2003. Microorganism Gram-type differentiation of whole cells based on pyrolysis high-resolution mass spectrometry data. Journal of Analytical and Applied Pyrolysis 67, 109-122.

Mills, C.T., Dias, R.F., Graham, D., Mandernack, K.W., 2006. Determination of phospholipid fatty acid structures and stable carbon isotope compositions of deep-sea sediments of the Northwest Pacific, ODP site 1179. Marine Chemistry 98, 197-209.

Mimura, T., Romano, J.C., 1985. Muramic acid measurements for bacterial investigations in marine environments by high-pressure liquid chromatography. Applied and Environmental Microbiology 50, 229-237.

Moriarty, D.J.W., 1977. Improved method using muramic acid to estimate biomass of bacteria in sediments. Oecologia 26, 317-323.

Moriarty, M., Lee, A., O'Connell, B., Lehane, M., Keeley, H., Furey, A., 2012. The Application and validation of HybridSPE-Precipitation Cartridge Technology for the Rapid Clean-up of Serum Matrices (from Phospholipids) for the clinical analysis of serotonin, dopamine and melatonin. Chromatographia 75, 1257-1269.

Müller, P.J., Kirst, G., Ruhland, G., von Storch, I., Rosell-Melé, A., 1998. Calibration of the alkenone paleotemperature index $U^{K'}_{37}$ based on core-tops from the eastern South Atlantic and the global ocean (60° N-60° S). Geochimica et Cosmochimica Acta 62, 1757-1772.

Müller, P.J., Suess, E., André Ungerer, C., 1986. Amino acids and amino sugars of surface particulate and sediment trap material from waters of the Scotia Sea. Deep Sea Research Part A. Oceanographic Research Papers 33, 819-838.

Nagata, T., Meon, B., Kirchman, D., 2003. Microbial degradation of peptidoglycan in seawater. Limnology and Oceanography 48, 745-754.

Niggemann, J., Schubert, C., 2006. Sources and fate of amino sugars in coastal Peruvian sediments. Geochimica et Cosmochimica Acta 70, 2229-2237.

Parkes, R.J., Cragg, B.A., Bale, S., Getlifff, J., Goodman, K., Rochelle, P.A., Fry, J.C., Weightman, A.J., Harvey, S., 1994. Deep bacterial biosphere in Pacific Ocean sediments. Nature 371, 410-413.

Parkes, R.J., Cragg, B.A., Wellsbury, P., 2000. Recent studies on bacterial populations and processes in subseafloor sediments: a review. Hydrogeology Journal 8, 11-28.

Peters, K.E., Walters, C.C.C., Moldowan, J.M., 2005. The biomarker guide: Biomarkers and isostopes in the environment and human history. 1. Cambridge University Press.

Peterse, F., Hopmans, E.C., Schouten, S., Mets, A., Rijpstra, W.I.C., Sinninghe Damsté, J.S., 2011. Identification and distribution of intact polar branched tetraether lipids in peat and soil. Organic Geochemistry 42, 1007-1015.

Pickering, B., 1965. Mannosamine in the cell wall of Clostridium welchii (type A). Nature 206, 400-401.

Poinar, H.N., Stankiewicz, B.A., 1999. Protein preservation and DNA retrieval from ancient tissues. Proceedings of the National Academy of Sciences of the United States of America 96, 8426-8431.

Prahl, F., Wakeham, S., 1987. Calibration of unsaturation patterns in long-chain ketone compositions for palaeotemperature assessment. Nature 330, 367-369.

Pucci, V., Di Palma, S., Alfieri, A., Bonelli, F., Monteagudo, E., 2009. A novel strategy for reducing phospholipids-based matrix effect in LC-ESI-MS bioanalysis by means of HybridSPE. Journal of Pharmaceutical and Biomedical Analysis 50, 867-871.

Rosell-Melé, A., Jansen, E., Weinelt, M., 2002. Appraisal of a molecular approach to infer variations in surface ocean freshwater inputs into the North Atlantic during the last glacial. Global and Planetary Change 34, 143-152.

Rossel, P.E., Lipp, J.S., Fredricks, H.F., Arnds, J., Boetius, A., Elvert, M., Hinrichs, K.-U., 2008. Intact polar lipids of anaerobic methanotrophic archaea and associated bacteria. Organic Geochemistry 39, 992-999.

Rothman, D.H., Forney, D.C., 2007. Physical model for the decay and preservation of marine organic carbon. Science 316, 1325-1328.

Rütters, H., Sass, H., Cypionka, H., Rullkötter, J., 2002. Phospholipid analysis as a tool to study complex microbial communities in marine sediments. Journal of Microbiological Methods 48, 149-160.

Sato, H., Mizutani, S., Tsuge, S., Ohtani, H., Aoi, K., Takasu, A., Okada, M., Kobayashi, S., Kiyosada, T., Shoda, S., 1998. Determination of the degree of acetylation of chitin/chitosan by pyrolysis-gas chromatography in the presence of oxalic acid. Analytical Chemistry 70, 7-12.

Schippers, A., Neretin, L.N., Kallmeyer, J., Ferdelman, T.G., Cragg, B.A., Parkes, R.J., Jørgensen, B.B., 2005. Prokaryotic cells of the deep sub-seafloor biosphere identified as living bacteria. Nature 433, 861-864.

Schleifer, K., Kandler, O., 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriological Reviews 36, 407.

Schouten, S., Hopmans, E.C., Schefuß, E., Sinninghe Damsté, J.S., 2002. Distributional variations in marine crenarchaeotal membrane lipids: a new tool for reconstructing ancient sea water temperatures? Earth and Planetary Science Letters 204, 265-274.

Schouten, S., Ossebaar, J., Schreiber, K., Kienhuis, M., Langer, G., Benthien, A., Bijma, J., 2006. The effect of temperature, salinity and growth rate on the stable hydrogen isotopic composition of long chain alkenones produced by *Emiliania huxleyi* and *Gephyrocapsa oceanica*. Biogeosciences 3, 113-119.

Schubotz, F., Lipp, J.S., Elvert, M., Hinrichs, K.-U., 2011. Stable carbon isotopic compositions of intact polar lipids reveal complex carbon flow patterns among hydrocarbon degrading microbial communities at the Chapopote asphalt volcano. Geochimica et Cosmochimica Acta 75, 4399-4415.

Schubotz, F., Wakeham, S.G., Lipp, J.S., Fredricks, H.F., Hinrichs, K.-U., 2009. Detection of microbial biomass by intact polar membrane lipid analysis in the water column and surface sediments of the Black Sea. Environmental Microbiology 11, 2720-2734.

Sicre, M.A., Bard, E., Ezat, U., Rostek, F., 2002. Alkenone distributions in the North Atlantic and Nordic sea surface waters. Geochemistry, Geophysics, Geosystems 3, 1 of 13-13 of 13.

Smith, C.S., Morgan, S.L., Parks, C.D., Fox, A., Pritchard, D.G., 1987. Chemical marker for the differentiation of group A and group B streptococci by pyrolysis-gas chromatography-mass spectrometry. Analytical Chemistry 59, 1410-1413.

Snyder, A.P., Dworzanski, J.P., Tripathi, A., Maswadeh, W.M., Wick, C.H., 2004. Correlation of mass spectrometry identified bacterial biomarkers from a fielded pyrolysis-gas chromatography-ion mobility spectrometry biodetector with the microbiological gram stain classification scheme. Analytical Chemistry 76, 6492-6499.

Stankiewicz, B.A., Briggs, D.E.G., Evershed, R.P., 1997. Chemical composition of Paleozoic and Mesozoic fossil invertebrate cuticles as revealed by pyrolysis-gas chromatography/mass spectrometry. Energy & fuels 11, 515-521.

Sturt, H.F., Summons, R.E., Smith, K., Elvert, M., Hinrichs, K.-U., 2004. Intact polar membrane lipids in prokaryotes and sediments deciphered by high-performance liquid chromatography/electrospray ionization multistage mass spectrometry-new biomarkers for biogeochemistry and microbial ecology. Rapid Communications in Mass Spectrometry 18, 617-628.

Sundh, I., Nilsson, M., Borga, P., 1997. Variation in microbial community structure in two boreal peatlands as determined by analysis of phospholipid Fatty Acid profiles. Applied and Environmental Microbiology 63, 1476-1482.

Takai, K., Nakamura, K., Toki, T., Tsunogai, U., Miyazaki, M., Miyazaki, J., Hirayama, H., Nakagawa, S., Nunoura, T., Horikoshi, K., 2008. Cell proliferation at 122°C and isotopically heavy CH_4 production by a hyperthermophilic methanogen under high-pressure cultivation. Proceedings of the National Academy of Sciences of the United States of America 105, 10949-10954.

Taylor, J., Parkes, R.J., 1983. The cellular fatty acids of the sulphate-reducing bacteria, Desulfobacter sp., Desulfobulbus sp. and Desulfovibrio desulfuricans. Journal of General Microbiology 129, 3303-3309.

Trumbore, S.E., Druffel, E.R.M., 1995. Carbon isotopes for characterizing sources and turnover of nonliving organic matter. In: Role of Nonliving Organic Matter in Earth's Carbon Cycle (eds. Zepp, R.G., Sonntag, C.), John Wiley & Sons, Chichester.

Tsuge, S., Matsubara, H., 1985. High-resolution pyrolysis-gas chromatography of proteins and related materials. Journal of Analytical and Applied Pyrolysis 8, 49-64.

van den Berg, J.D., Boon, J.J., van den Berg, K.J., Fiedler, I., Miller, M.A., 1998. Identification of an original non-terpenoid varnish from the early 20th century oil painting "The white horse"(1929), by H. Menzel. Analytical Chemistry 70, 1823-1830.

Van der Kaaden, A., Boon, J., De Leeuw, J., De Lange, F., Schuyl, P., Schulten, H., Bahr, U., 1984. Comparison of analytical pyrolysis techniques in the characterization of chitin. Analytical Chemistry 56, 2160-2165.

van der Meer, M.T., Baas, M., Rijpstra, W.I.C., Marino, G., Rohling, E.J., Sinninghe Damsté, J.S., Schouten, S., 2007. Hydrogen isotopic compositions of long-chain alkenones record freshwater flooding of the Eastern Mediterranean at the onset of sapropel deposition. Earth and Planetary Science Letters 262, 594-600.

Vestal, J.R., White, D.C., 1989. Lipid analysis in microbial ecology. Bioscience 39, 535-541.

Wakeham, S.G., Amann, R., Freeman, K.H., Hopmans, E.C., Jørgensen, B.B., Putnam, I.F., Schouten, S., Sinninghe Damsté, J.S., Talbot, H.M., Woebken, D., 2007. Microbial ecology of the stratified water column of the Black Sea as revealed by a comprehensive biomarker study. Organic Geochemistry 38, 2070-2097.

Weijers, J.W., Schouten, S., Spaargaren, O.C., Sinninghe Damsté, J.S., 2006. Occurrence and distribution of tetraether membrane lipids in soils: Implications for the use of the TEX_{86} proxy and the BIT index. Organic Geochemistry 37, 1680-1693.

Weng, R.-F., Huang, W.-L., Kuo, C.-L., Inan, S., 2003. Characterization of oil generation and expulsion from coals and source rocks using diamond anvil cell pyrolysis. Organic geochemistry 34, 771-787.

White, D., Davis, W., Nickels, J., King, J., Bobbie, R., 1979. Determination of the sedimentary microbial biomass by extractible lipid phosphate. Oecologia 40, 51-62.

White, D.M., Garland, D.S., Beyer, L., Yoshikawa, K., 2004. Pyrolysis-GC/MS fingerprinting of environmental samples. Journal of Analytical and Applied Pyrolysis 71, 107-118.

Whitman, W.B., Coleman, D.C., Wiebe, W.J., 1998. Prokaryotes: the unseen majority. Proceedings of the National Academy of Sciences of the United States of America 95, 6578-6583.

Wörmer, L., Lipp, J.S., Schröder, J.M., Hinrichs, K.-U., 2013. Application of two new LC-ESI-MS methods for improved detection of intact polar lipids (IPLs) in environmental samples. Organic Geochemistry 59, 10-21.

Xie, S., Lipp, J.S., Wegener, G., Ferdelman, T.G., Hinrichs, K.-U., 2013. Turnover of microbial lipids in the deep biosphere and growth of benthic archaeal populations. Proceedings of the National Academy of Sciences of the United States of America 110, 6010-6014.

Zelles, L., Bai, Q., Beck, T., Beese, F., 1992. Signature fatty acids in phospholipids and lipopolysaccharides as indicators of microbial biomass and community structure in agricultural soils. Soil Biology and Biochemistry 24, 317-323.

Zhang, X., Amelung, W., 1996. Gas chromatographic determination of muramic acid, glucosamine, mannosamine, and galactosamine in soils. Soil Biology and Biochemistry 28, 1201-1206.

Zhang, X., Amelung, W., Yuan, Y., Samson-Liebig, S., Brown, L., Zech, W., 1999. Land-use effects on amino sugars in particle size fractions of an Argiudoll. Applied Soil Ecology 11, 271-275.

Zhang, Y.-M., Rock, C.O., 2008. Membrane lipid homeostasis in bacteria. Nature Reviews Microbiology 6, 222-233.

Zink, K.G., Wilkes, H., Disko, U., Elvert, M., Horsfield, B., 2003. Intact phospholipids-microbial ‘‘life markers’’ in marine deep subsurface sediments. Organic Geochemistry 34, 755-769.

Chapter II

Optimizing sample pretreatment for compound-specific stable carbon isotopic analysis of amino sugars in marine sediment

Rong Zhu[1], Yu-Shih Lin[1, 2], Julius S. Lipp[1], Travis B. Meador[1], Kai-Uwe Hinrichs[1]

In press in *Biogeosciences Discussion*

doi: 10.5194/bgd-11-1-2014

[1] Organic Geochemistry Group, MARUM-Center for Marine Environmental Sciences & Department of Geosciences, University of Bremen, 28359 Bremen, Germany

[2] Department of Oceanography, National Sun Yat-Sen University, No.70 Lienhai Rd, Kaohsiung 804, Taiwan

Correspondence to: R. Zhu (rongzhu1018@gmail.com)

II.1. ABSTRACT

Amino sugars are quantitatively significant constituents of soil and marine sediment, but their sources and turnover in environmental samples remain poorly understood. The stable carbon isotopic composition of amino sugars can provide information on the lifestyles of their source organisms and can be monitored during incubations with labeled substrates to estimate the turnover rates of microbial populations. However, until now, such investigation has been carried out only with soil samples, partly because of the much lower abundance of amino sugars in marine environments. We therefore optimized a procedure for compound-specific isotopic analysis of amino sugars in marine sediment employing gas chromatography-isotope ratio mass spectrometry. The whole procedure consisted of hydrolysis, neutralization, enrichment, and derivatization of amino sugars. Except for the derivatization step, the protocol introduced negligible isotopic fractionation, and the minimum requirement of amino sugar for isotopic analysis was 20 ng, i.e. equivalent to ~8 ng of amino sugar carbon. Our results obtained from $\delta^{13}C$ analysis of amino sugars in selected marine sediment samples showed that muramic acid had isotopic imprints from indigenous bacterial activities, whereas glucosamine and galactosamine were mainly derived from organic detritus. The analysis of stable carbon isotopic compositions of amino sugars opens a promising window for the investigation of microbial metabolisms in marine sediments and the deep marine biosphere.

II.2. INTRODUCTION

Amino sugars are significant sedimentary components that are mostly derived from microorganisms and invertebrates. For example, the four major amino sugars, glucosamine (GlcN), galactosamine (GalN), mannosamine (ManN) and muramic acid (MurA; Fig. II.1), accounted for up to 12% of total organic carbon (TOC) in grassland soils in North America (Amelung et al., 1999) and ~2% of TOC in coastal Peruvian surface sediments (Niggemann and Schubert, 2006). As amino sugars are preserved in the form of biopolymers such as peptidoglycan, chitin, and lipopolysaccharides, the amount of amino sugars has been frequently used as a proxy for microbial contributions to soil organic matter, and the ratios between different amino sugars have been employed as indicators of microbial community compositions (e.g.

Guggenberger et al., 1999; Zhang et al., 1999; Glaser et al., 2004). In marine environments, amino sugars could be of microbial or animal origin. Prokaryotic biomass is thought to be the major source of GlcN and GalN in marine sediment (e.g. Niggemann and Schubert, 2006; Langerhuus et al., 2012) and seawater (Benner and Kaiser, 2003); however, most amino sugars are not likely associated with peptidoglycan (Aluwihare et al., 2005; Aluwihare and Meador, 2008). Recently, the investigation of amino sugars has been extended to the marine deep biosphere by Lomstein et al. (2012), who used MurA as a tool for indirectly quantifying endospores in subseafloor sediment.

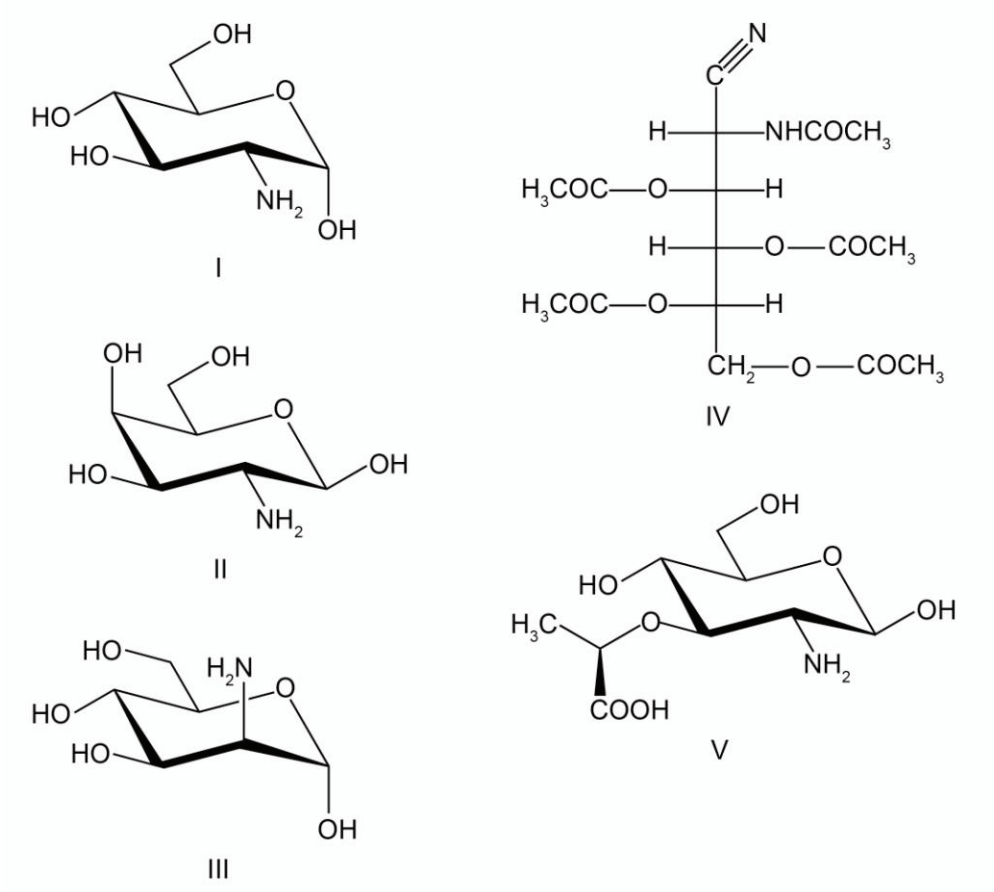


Fig. II.1. Structures of amino sugars and aldononitrile acetate (ANA) derivative: glucosamine (I), galactosamine (II), mannosamine (III), ANA derivative of glucosamine (IV) and muramic acid (V).

The stable carbon isotopic composition ($\delta^{13}C$) of individual cellular biomarkers can reveal the carbon metabolism and/or lifestyles of the source organisms in the natural environment (e.g. Lin et al., 2010; Schubotz et al., 2011), and incubations with ^{13}C-labeled substrates have been used to probe microbial communities by tracking the production of labeled biomarkers over time (e.g. Veuger et al., 2006; Lin et al., 2012;

Kellermann et al., 2012). The latter technique has been widely employed in soil science to investigate the formation and/or turnover kinetics of amino sugars (e.g. Glaser et al., 2005; Decock et al., 2009; Bai et al., 2012). However, to date, the isotopic composition of amino sugars in marine sediment remains poorly explored, partly because of the much lower concentration of amino sugars in marine sediment compared to soils, which results in the need for an efficient pretreatment procedure to enable precise isotopic determination.

Compound-specific $\delta^{13}C$ analysis of amino sugars is commonly performed via isotope ratio mass spectrometry (IRMS) after separation of compounds by either gas chromatography (GC; Glaser and Gross, 2005) or liquid chromatography (LC; Bodé et al., 2009). Compared with LC-IRMS, the GC-based method is advantageous as it provides separation of amino sugars in a single measurement and is less sensitive to the sample matrix (McCullagh, 2010; Morrison et al., 2010; Rinne et al., 2012). Substantial effort has been made during the past few decades to optimize the pretreatment procedure for GC-based quantification of amino sugars, which requires three major steps: acid hydrolysis, purification, and derivatization, with hydrolysis being the key step for releasing amino sugars from biopolymers. Besides the most frequently used hydrolysis protocol, namely 6 M hot hydrochloric acid (HCl; Zhang and Amelung, 1996), less destructive procedures involving either hot trifluoroacetic acid (TFA; Neeser and Schweizer, 1984) or sulfuric acid (H_2SO_4; Fox et al., 1983) have been proposed for the simultaneous extraction of neutral and amino sugars. Different purification protocols, such as neutralization with a base solution (Zhang and Amelung, 1996), precipitation of excess acid (Cowie and Hedges, 1984; Neeser and Schweizer, 1984), and deionization of hydrolysate (Cowie and Hedges, 1984), to name a few, have been used to reduce the content of acid and salts that are known to interfere with amino sugar derivatization. Conversion of amino sugars for GC analysis has been achieved via derivatization into alditol acetates (AA; Fox et al., 1983), aldononitrile acetates (ANA; Guerrant and Moss, 1984), or O-methyloxime acetates (Neeser and Schweizer, 1984). However, to perform isotopic analysis of amino sugars at trace levels, a systematic evaluation of these various methods with regard to the product recoveries is necessary.

The goal of this study is to devise a pretreatment protocol for GC-based isotopic analysis of amino sugars in subseafloor sediments. Since deep sediment samples are

generally severely limited in size and typically contain substantially lower TOC than the surface sediments from the Peru Margin (Niggemann and Schubert, 2006), which represent the high end-member of TOC content in modern marine sediments, a protocol for sensitive analysis of amino sugars in small sample size is crucial. Hence, we tackled three major analytical tasks. (1) We systematically evaluated existing pretreatment methods for amino sugar analysis to select the method that gave the highest recoveries of products from marine sediments. (2) We introduced the use of a new type of solid-phase extraction (SPE) in the pretreatment protocol and demonstrated enhanced recoveries compared with existing methods. (3) We developed a preparative high performance liquid chromatography (HPLC) method to enrich amino sugars to a desired concentration for proper isotopic assessment. We then applied our protocol to determine the stable carbon isotopic compositions of amino sugars from the selected subseafloor sediment samples.

II.3. EXPERIMENTAL

II.3.1. Standards and environmental samples

The amino sugar standards, derivatization reagents, and all other chemicals used in this study were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany) or Merck KGaA (Darmstadt, Germany). The Supelclean™ ENVI-Carb™ Plus SPE cartridges and accessories were obtained from Sigma-Aldrich Chemie GmbH or Carl Roth GmbH (Karlsruhe, Germany).

A batch of surface sediment sample for method optimization was collected from the upper tidal flat of the Wadden Sea near Wremen, Germany, in February 2010 (53° 38' 0N, 8° 29' 30E). In addition, four marine sediment samples of different types were selected for stable carbon isotopic analysis of individual amino sugars. The seep sample was recovered from the continental margin off Pakistan (Site GeoB 12315-9, 1-2 cm below the seafloor, cmbsf) during the RV Meteor cruise M74/3 in November 2007. It was located within the lower part of the oxygen minimum zone and associated with dense microbial mats from sulfide-oxidizing bacteria (Fischer et al., 2012). Two subseafloor samples were retrieved from the Marmara Sea (Site GeoB 15104-2) and the Black Sea (Site GeoB 15105-2) during the RV Meteor cruise M84/1 (DARCSEAS) in February 2011 (Zabel et al., 2013). The sample from the Marmara

Sea was collected at 2.88 mbsf and was located in a sapropel layer deposited under suboxic bottom-water condition (Çağatay et al., 2000). The Black Sea sample was collected at 1.57 mbsf, where the sediment was highly diluted by terrigenous components and showed a lack of bioturbation. The deepest sediment sample was recovered from the Peru Margin (Ocean Drilling Program Leg 201, 1229A-3H-2, 16.6 mbsf) and had high TOC content (3.8%).

II.3.2. Hydrolysis tests

Hydrolysis tests were performed in triplicate in 40-ml glass tubes by adding different acids to ~5 g of freeze-dried Wadden Sea sediment. The tubes were filled with nitrogen before being sealed with Teflon-coated screw caps to prevent oxidation of sugars at high temperature during hydrolysis. The evaluated hydrolytic conditions were as follows.

(a) The sample was kept at room temperature for 2 h after the addition of 12 M H_2SO_4, followed by dilution to 1.2 M H_2SO_4 and heating at 105 °C for 3 h (modified from Cowie and Hedges, 1984).
(b) The sample was hydrolyzed with 4 M TFA at 105 °C for 4 h (Amelung et al., 1996).
(c) The sample was treated with 6 M HCl at 105 °C for 8 h (Zhang and Amelung, 1996).

After hydrolysis, the samples were cooled to room temperature, spiked with 100 µg of myo-inositol as an internal standard, and centrifuged at 800 × *g* for 5 min. After being passed through combusted glass microfiber filters (GF/F, 70mm Φ, Whatman™), the hydrolysates were evaporated to dryness with a rotary evaporator (45 °C, under vacuum) and re-dissolved in 4 ml of MilliQ water.

II.3.3. Neutralization and desalting

Samples hydrolyzed with H_2SO_4 were neutralized by adding finely ground barium hydroxide ($Ba(OH)_2$; Cowie and Hedges, 1984), whereas TFA-treated samples were freeze-dried overnight to remove the acid. Hydrolysates liberated with HCl were initially subjected to the procedure described by Zhang and Amelung (1996) with

slight modification, i.e. the acidic solution was adjusted to pH 6.5-7.0 with 1 M potassium hydroxide (KOH), evaporated to dryness under N_2, re-dissolved in 2 ml methanol (MeOH) and amino sugars were collected in the supernatant after centrifugation. Because treatments with HCl gave the best recoveries (see below), we went on to explore other neutralization and desalting methods that are compatible with HCl. A standard mixture containing 20 µg of each amino sugar was treated with 1 ml of 6 M HCl and the following three methods were performed in triplicate to compare the yields with that of Zhang and Amelung (1996).

(a) Silver carbonate (Ag_2CO_3) was added gradually to the mixture to neutralize the HCl. The silver chloride (AgCl) precipitate was removed by centrifugation (cf. Neeser and Schweizer, 1984).

(b) After neutralization with 1 M KOH solution, the mixture was desalted by percolating the solution through a glass column filled with 3 g of pre-conditioned Dowex 50WX8 cation exchange resin (100-200 mesh), as described by Amelung et al. (1996). The column was washed with 10 ml of MilliQ water to remove excess salts and amino sugars were subsequently eluted with 10 ml of 2 M ammonium hydroxide (NH_4OH).

(c) We evaluated for the first time the applicability of Supelclean™ ENVI-Carb™ Plus SPE cartridges in carbohydrate analysis. ENVI-Carb™ Plus is a microporous amorphous carbon molecular sieve developed for the extraction of highly polar compounds from water. Its predecessor, Supelclean™ ENVI-Carb™, has been used to extract oligosaccharides in aqueous samples (Itoh et al., 2002). Prior to use, the SPE cartridge was pre-conditioned sequentially with 10 ml methylene chloride (DCM), 20 ml MeOH, and 15 ml MilliQ water. The hydrolysate containing amino sugars was neutralized with 1 M KOH and desalted by pulling through the SPE cartridge coupled with a SPE manifold (Carl Roth GmbH, Karlsruhe, Germany) under vacuum. The SPE cartridge was then dried for 10 min and eluted under vacuum in the reversed direction with 5 ml MeOH followed by 5 ml DCM/MeOH (1:1, v:v) to recover the amino sugars.

All the desalted products were evaporated under a stream of N_2, lyophilized overnight, and converted to GC-amenable derivatives for analysis. Quantities and recoveries of individual amino sugars were determined via calibration curves that

were generated from pure standards. The Wadden Sea sediment was also used to assess the efficiencies of the neutralization and desalting methods for environmental samples.

II.3.4. Derivatization and purification

Amino sugar standards were transformed to AA or ANA derivatives in triplicate following the methods of Fox et al. (1983) or Guerrant and Moss (1984), respectively. After being converted to ANA derivatives, amino sugars extracted from the environmental samples were further purified with a self-packed silica gel column (0.5 g; Kieselgel, 0.06-0.2 mm; Carl Roth GmbH, Karlsruhe, Germany; cf. Lin et al., 2010). The best recovery (>95% of each amino sugar as ANA derivative) was achieved by elution with 8 ml hexane/ethyl acetate (1:4, v:v). The eluent was evaporated to dryness under a N_2 stream and re-dissolved in hexane/ethyl acetate (1:1, v:v) prior to analysis.

II.3.5. Preparation of amino sugar-enriched fractions

A mixture of amino sugar standards and desalted hydrolysate of the Wadden Sea sediment sample were employed for developing separation of amino sugars via preparative HPLC. The sample was re-dissolved in acetonitrile (ACN)/H_2O (7:3, v:v) prior to injection. The flow rate was 1 ml/min, and the eluent gradient ramped steadily from 100% eluent A to 100% eluent B over 15 min, then held at 100% eluent B for 15 min, followed by 10 min re-equilibration with 100% eluent A. Eluent A was composed of ACN/H_2O/NH_4OH/formic acid (90:10:0.1:0.2, v:v:v:v); eluent B was ACN/H_2O/NH_4OH/formic acid (30:70:0.1:0.2, v:v:v:v). NH_4OH and formic acid were included for online monitoring of the separation of amino sugars by MS and impeding amino sugars partially retained on the stationary phase of the column.

Amino sugars were thereafter separated into two fractions, the first (F1; 5.0-7.6 min) containing the three hexosamines and the second (F2; 7.6-9.6 min) MurA. Fractions collected during preparative HPLC were evaporated to dryness and converted to GC-amenable derivatives to monitor separation efficiency and isotopic fractionation effect.

II.3.6. Accessible amino sugar concentrations for GC-IRMS analysis

To determine the accessible concentration range of amino sugars for compound-specific $\delta^{13}C$ analysis, 10-140 ng of the two minor amino sugars found in natural marine environments (i.e. ManN and MurA) were converted to ANA derivatives and injected as pure standards into the GC-IRMS in triplicate. Additionally, isotopic analysis was validated for realistic conditions with matrix obtained from analysis of marine sediment samples. For this purpose, the $\delta^{13}C$ of pure ManN and MurA was measured after addition to the preparative HPLC fractions obtained from separation of sedimentary preparations that were either ManN-free or MurA-free.

II.3.7. Instrumentation

Separation and online detection of amino sugars were achieved with an Agilent 6130 MSD single quadrupole mass spectrometer coupled to an Agilent 1200 Series HPLC system via a multimode ion source in electrospray ionization mode (Agilent Technologies Deutschland GmbH, Böblingen, Germany) equipped with an Econosphere NH_2 column (250 mm × 4.6 mm, 5 mm particle size; Alltech Associates Inc., Deerfield, IL, USA).

Quantification of amino sugars was accomplished using an Agilent 6890N GC instrument coupled to an Agilent 5973 inert Mass Selective Detector (MSD) with an electron impact (EI) source, whereas compound-specific isotopic analysis of amino sugars was performed with a Trace GC Ultra instrument coupled to a Delta Plus XP isotope ratio mass spectrometer via a Combustion Interface III (Thermo Finnigan MAT GmbH, Bremen, Germany). Two columns with different polarities, an Optima 17MS column (30 m × 0.25 mm, 0.25 μm film thickness; Macherey-Nagel GmbH & Co. KG, Düren, Germany) and an Rxi-5ms column (30 m × 0.25 mm, 0.25 μm film thickness; Restek GmbH, Bad Homburg, Germany), were applied to optimize separation of sugar derivatives.

The ANA method produced the most stable derivatives and satisfying results (see below); the corresponding GC operation conditions were specified here in detail. Separation of ANA derivatives on the Optima 17 MS column was modified from

Glaser and Gross (2005). The injector temperature was 250 °C and helium was used as carrier gas at a constant flow rate of 1 ml/min. The GC temperature program was initiated at 80 °C and held for 4 min, then increased to 250 °C at 30 °C/min and held for 10 min, and finally raised to 280 °C at 3 °C/min and held for 5 min. The GC parameters for the Rxi-5ms column were as follows: injector temperature, 250 °C; carrier gas, helium; flow rate, 1.1 ml/min. The oven temperature was kept at 70 °C for 1 min, ramped to 230 °C at 20 °C/min, held for 20 min, and further increased at 20 °C/min to 300 °C and held for 5 min. The mass spectrometer was programmed in selective ion monitoring (SIM) mode to target specific mass fragment ions of the derivatives for quantification. The selected ions were m/z 187 and 289 for GlcN, GalN and ManN; m/z 236 and 356 for MurA.

Stable carbon isotopic compositions of the pure amino sugars, internal standards and the acetylation reagent were determined independently by a Flash 2000 Organic Elemental Analyzer coupled to a Delta V Plus isotope ratio mass spectrometer (EA-IRMS) via a Conflo IV interface (Thermo Finnigan MAT GmbH). Quadruplicate measurements by EA-IRMS generated highly accurate $\delta^{13}C$ values of each compound.

II.3.8. Calculations

Isotopic compositions were expressed using the δ notation: $\delta^{13}C = (R_{sample}/R_{standard} - 1) \times 1000‰$, with $R = {}^{13}C/{}^{12}C$ and $R_{standard} = 0.0112372 \pm 2.9 \times 10^{-6}$; the reference standard was Vienna Pee Dee Belemite. We determined the isotopic values only for ANA derivatives of amino sugars, which exhibited the highest yields. The $\delta^{13}C$ values and the corresponding standard errors (s_{Total}) of individual amino sugars were calculated following the procedure described by Glaser and Gross (2005), with slight modification. 3-*O*-methyl-D-glucopyranose (3-*O*-Me-Glc) was applied as an internal standard to correct the isotopic fractionation during acetylation of the hydroxyl groups (Lin et al., 2010). The determination of *F*, a compound-specific correction factor, was based on analysis of ANA derivatives of seven external standard solutions in the concentration range of 1-20 μg per batch for ManN and MurA and 1-60 μg per batch for the other standards.

Table II.1. Amino sugars liberated from surface sediment by different hydrolytic procedures. The sample used in this test was collected from the Wadden Sea. All the amino sugars were quantified by GC-MS in SIM mode (n = 3); the ions selected for detection were m/z 187 and 289 for GlcN, GalN and ManN; m/z 236 and 356 for MurA.

Hydrolysis and neutralization methods	Reference	GlcN (μg/g dw)	GalN (μg/g dw)	ManN (μg/g dw)	MurA (μg/g dw)
12 M H_2SO_4 - 1.2 M H_2SO_4; $Ba(OH)_2$	Cowie and Hedges, 1984	8.8 ± 1.1	4.7 ± 0.8	0.7 ± 0.1	0.1 ± 0.2
4 M TFA; 105 °C, 4 h; evaporation	Amelung et al., 1996	1.2 ± 0.1	1.0 ± 0.4	0.3 ± 0.5	0.2 ± 0.1
6 M HCl; 105 °C, 8 h; KOH -MeOH	Zhang et al., 1996	17.6 ± 0.8	15.7 ± 0.6	1.5 ± 0.2	0.5 ± 0.2
6 M HCl; 105 °C, 8 h; KOH - SPE	This study	34.8 ± 0.3	26.0 ± 0.7	2.2 ± 0.3	0.7 ± 0.02

Abbreviations: GlcN, glucosamine; GalN, galactosamine; ManN, mannosamine; MurA, muramic acid.

Table II.2. Recoveries of HCl-treated amino sugar standards after different neutralization and desalting procedures (n = 3).

Methods		Reference	GlcN (%)	GalN (%)	ManN (%)	MurA (%)
KOH - MeOH		Zhang et al., 1996	95.4 ± 4.7	86.5 ± 4.2	77.3 ± 9.6	97.8 ± 10.7
Ag_2CO_3		Neeser and Schweizer, 1984	26.4 ± 6.7	12.0 ± 1.0	82.8 ± 6.4	7.0 ± 0.6
Dowex 50WX8	H_2O	Amelung et al., 1996	7.0 ± 1.5	8.7 ± 1.9	8.5 ± 1.8	7.1 ± 0.2
Dowex 50WX8	NH_4OH	Amelung et al., 1996	104.5 ± 29.9	63.7 ± 23.9	71.6 ± 23.7	107.7 ± 28.5
KOH - SPE		Our proposed method	103.6 ± 6.6	92.7 ± 5.5	89.4 ± 4.9	95.0 ± 2.4

II.4. RESULTS AND DISCUSSION

II.4.1. Hydrolytic conditions for releasing amino sugar monomers

Table II.1 summarizes the results of the hydrolysis tests. Hydrolysis with 6 M HCl provided the highest recoveries of amino sugars from the Wadden Sea surface sediment sample, followed by H_2SO_4 and TFA. GlcN and GalN were the most abundant amino sugars, with concentrations being one order of magnitude higher than those of ManN and MurA. Based on these results, further work aimed to optimize the neutralization and desalting steps that followed the hot HCl treatment. We observed that the HCl method yielded lower recoveries of neutral sugars compared with the other two protocols (data not shown), suggesting that partial destruction of neutral sugars occurred under the harsh hydrolysis condition. The different responses of amino sugars and neutral sugars to strong acids may be attributable to structural differences in the oligo- or polysaccharides as which they are preserved.

II.4.2. Comparison of neutralization and desalting methods after hydrolysis by HCl

Recoveries of amino sugar standards ranged from 77% to 98% using the procedure proposed by Zhang and Amelung (1996), i.e. neutralization with 1 M KOH and desalting with MeOH (Table II.2). We did not observe significant differences in amino sugar recoveries using standard compounds after employing either the ENVI-Carb^{TM} Plus SPE cartridge or the method described by Zhang and Amelung (1996). However, for surface sediment samples, recoveries using the former method exceeded those of the latter by a factor of 1.5 to 2 (Table II.1), suggesting that the SPE method is less sensitive to the presence of sedimentary matrices. Moreover, we noted that the SPE cartridge allowed for excellent recoveries of neutral hexoses such as glucose, galactose, and mannose (data not shown).

The other two methods tested in this study yielded less satisfying results. Ag_2CO_3 reacts with HCl to form CO_2 and AgCl, which has very low solubility in water and eliminates the need for a desalting step. However, with the exception of ManN, major losses of amino sugars were observed (Table II.2), presumably owing to interactions between amino sugars and the AgCl-Ag_2CO_3 mixture. Cation exchange using the Dowex 50WX8 cation exchange resin allows for separation of amino sugars (in the NH_4OH fraction) from neutral sugars (in the H_2O fraction). Recoveries of

amino sugars by this method were highly variable (Table II.2), partly because of the incomplete evaporation of NH_4OH, which might affect the efficiency of ANA derivatization.

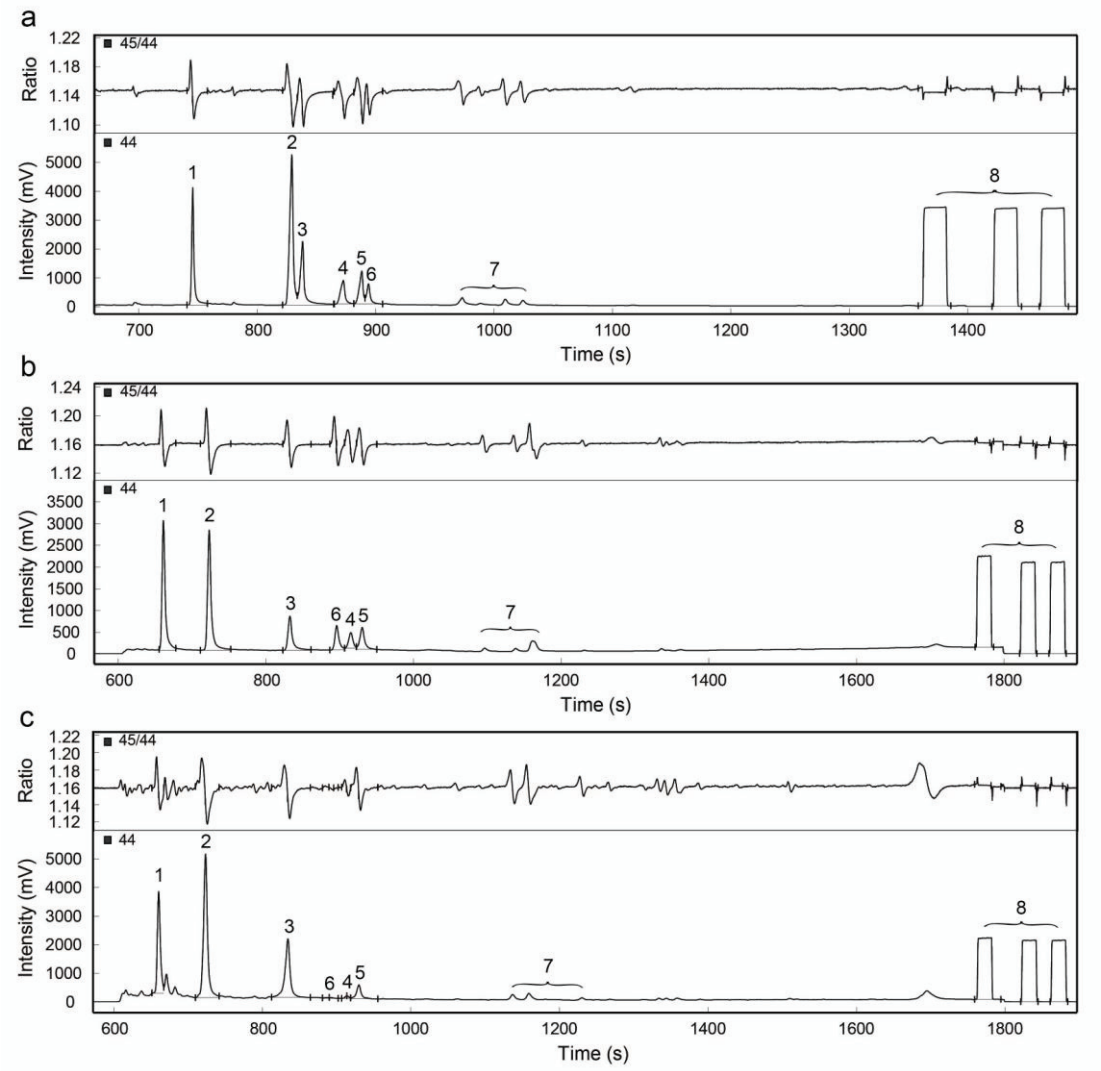


Fig. II.2. GC-IRMS chromatograms of a standard mixture containing 100 ng of each amino sugar separated by an Rxi-5ms column (a) and an Optima 17MS column (b), respectively, and a marine sediment sample from the Peru Margin separated by an Optima 17MS column (c). Key to peak numbers: aldononitrile derivatives of 3-*O*-methyl-D-glucopyranose (internal standard) (1), myo-inositol (2), glucosamine (3), mannosamine (4), galactosamine (5), muramic acid (6), unknown compounds (7) and reference CO_2 gas (8).

II.4.3. Derivatization of amino sugars

Although the AA derivatization method has been employed previously to investigate amino sugars (Fox et al., 1995; Fox et al., 1996), it was rather time consuming (~1 d; cf. Fox et al., 1983), and the derivative of MurA exhibited poor

stability compared with the corresponding hexosamine derivatives (data not shown). The addition of *N*-methylimidazole as a catalyst helped to accelerate the acetylation of hexosamines, but it failed to produce an appreciable signal peak for MurA derivative (Whiton et al., 1985). By contrast, the acetylation time of the ANA derivatization method was reasonably low (20 min), and the derivatives remained stable at -20 °C for up to 1 yr, as confirmed by repeated injections of amino sugar ANA derivatives over the course of 12 months (data not shown). Each amino sugar, including MurA, yielded a single, well-resolved chromatographic peak during GC analysis with either an Rxi-5ms or an Optima 17MS column, when the injected amount was < 120 ng per amino sugar. We therefore concluded that the ANA method was superior to the AA approach for GC-based analysis of amino sugars and subsequently implemented this step in our method optimization. Like Glaser and Gross (2005), we also noted that the elution order of ANA derivatives differed between the two GC columns (Fig. II.2a, b). The availability of two alternative separations was advantageous for determining the $\delta^{13}C$ values of the minor components, which co-eluted with major peaks when using the Optima 17MS column, but were separable when using the Rxi-5ms column for environmental samples.

Table II.3. Recoveries and $\delta^{13}C$ values of amino sugar standards after separation by preparative HPLC. Standard errors of GC-IRMS measurements (s_{GC}) are reported for individual amino sugars and the total analytical error of $\delta^{13}C$ values (s_{Total}) was calculated according to error propagation (n = 3).

Fraction	Amino sugars	Recovery (%)	GC-IRMS		
			$\delta^{13}C_{AS,\,GC}$ (‰)	s_{GC} (‰)	s_{Total} (‰)
1	GlcN	86.6 ± 1.5	-20.6	0.26	1.57
	GalN	113.8 ± 3.0	-20.1	0.36	1.73
	ManN	70.8 ± 2.8	-19.8	0.75	1.44
2	MurA	105.8 ± 6.7	-20.9	0.35	1.02

II.4.4. Performance of the preparative HPLC procedure

MurA was the primary target compound when developing the preparative HPLC protocol because it serves as a specific biomarker for bacteria but is typically low in abundance in subseafloor sediments (Fig. II.2c). ManN, another minor amino sugar, could not be isolated from GlcN and GalN using this approach. Nevertheless,

preparative HPLC was beneficial for isotopic analysis of ManN due to partial removal of the sample matrix, which allowed us to concentrate the sample to a small volume for GC injection. We observed minimal losses of GalN and MurA after preparative HPLC, but recoveries of GlcN and ManN were only 87% and 71%, respectively (Table II.3). The F2 and the waste fractions contained less than 1% of the original GlcN and ManN, suggesting that GlcN and ManN were either lost via preferential adsorption on the column or derivatization of these two hexosamines was particularly sensitive to the presence of ammonium formate originating from the HPLC eluents.

II.4.5. Summary of the protocol and isotope data assessment

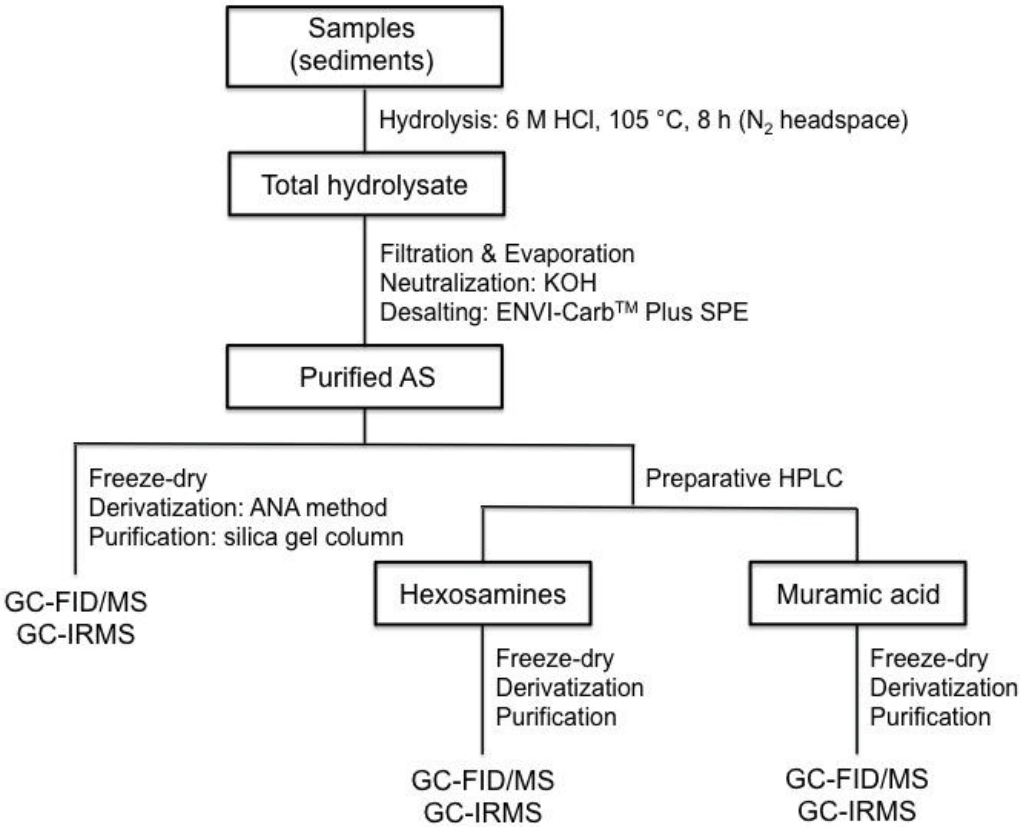


Fig. II.3. Schematic of the optimized procedure for isotope analysis of amino sugars. Each step has been validated and/or optimized in this study.

Figure II.3 summarizes the optimized pretreatment procedure. In brief, amino sugars are released from marine sediments by hot HCl. The hydrolysate then undergoes a series of purification steps, including filtration with combusted glass fiber filters, evaporation under vacuum, neutralization with KOH solution, and desalting with the ENVI-Carb™ Plus SPE cartridge. After freeze-drying overnight, amino

sugars in the extract are transformed to ANA derivatives and further purified by a self-packed silica gel column. The recoveries of the whole procedure, estimated from parallel tests (n = 3) using a mixture of amino sugar standards, were 111.6 ± 1.9%, 95.0 ± 9.2%, 47.0 ± 3.8% and 102.5 ± 2.6% for GlcN, GalN, ManN, and MurA, respectively. The lower recovery of ManN may result from successive preferential losses during each step and is probably related to its steric structure, but the underlying mechanism is not yet fully understood.

Table II.4. $\delta^{13}C$ values of amino sugar standards analyzed by EA-IRMS ($\delta^{13}C_{AS,\,EA}$) or according to pretreatment procedure described in Fig. II.2 prior to GC-IRMS analysis ($\delta^{13}C_{AS,\,GC}$). s_{EA} and s_{GC} stand for standard errors of EA- or GC-IRMS measurements, respectively. s_{Total} is the total analytical error of $\delta^{13}C$ values of individual amino sugars calculated according to error propagation (n = 3).

Compounds	EA-IRMS Pure standard		GC-IRMS Hydrolyzed standard		
	$\delta^{13}C_{AS,\,EA}$ (‰)	s_{EA} (‰)	$\delta^{13}C_{AS,\,GC}$ (‰)	s_{GC} (‰)	s_{Total} (‰)
3-*O*-Me-Glc	-21.62	0.02	-21.6	0.32	0.95
Ino	-30.25	0.02	-29.2	0.65	1.54
GlcN	-20.02	0.03	-19.9	0.41	1.34
GalN	-20.06	0.02	-20.1	0.29	1.36
ManN	-20.32	0.05	-20.1	0.98	1.32
MurA	-20.77	0.02	-20.8	0.30	0.84
Acetic anhydride	-38.55	0.03	-	-	-

It is established that derivatization with acetic anhydride to ANA derivatives is associated with an isotope effect (Glaser and Gross, 2005; Decock et al., 2009); this effect can be corrected with the use of a derivatization standard such as 3-*O*-Me-Glc (e.g. Lin et al., 2010). After processing authentic amino sugar standards using our proposed preparation procedure (Fig. II.3) and correcting for the isotope effect, the $\delta^{13}C$ values measured by GC-IRMS ($\delta^{13}C_{AS,\,GC}$) were in good agreement with those obtained by EA-IRMS ($\delta^{13}C_{AS,\,EA}$; Table II.4), indicating negligible isotopic fractionation during the other sample pretreatment steps. The standard errors of repeated injections for GC-IRMS were less than 1‰, but the total errors were up to 1.4‰, i.e. about 1‰ greater than those reported for the HPLC-based method developed for soils (Bodé et al., 2009). The total errors impose constraints on the

isotopic resolving power of our method and should be taken into account during data interpretation.

For samples that exhibited low signals of MurA and/or ManN due to the high background generated by sedimentary matrix or high abundance of the adjacent peak of GalN (i.e. the Peru Margin sample), amino sugar extracts were separated into two fractions (i.e. hexosamine fraction and MurA fraction) via the preparative HPLC procedure described above (Fig. II.3), which could be further concentrated for GC-IRMS analysis. Tests using authentic amino sugar standards confirmed that this additional step did not introduce significant isotopic fractionation, as the $\delta^{13}C$ values (Table II.3) deviated from those obtained by EA-IRMS (Table II.4) by less than 0.6‰.

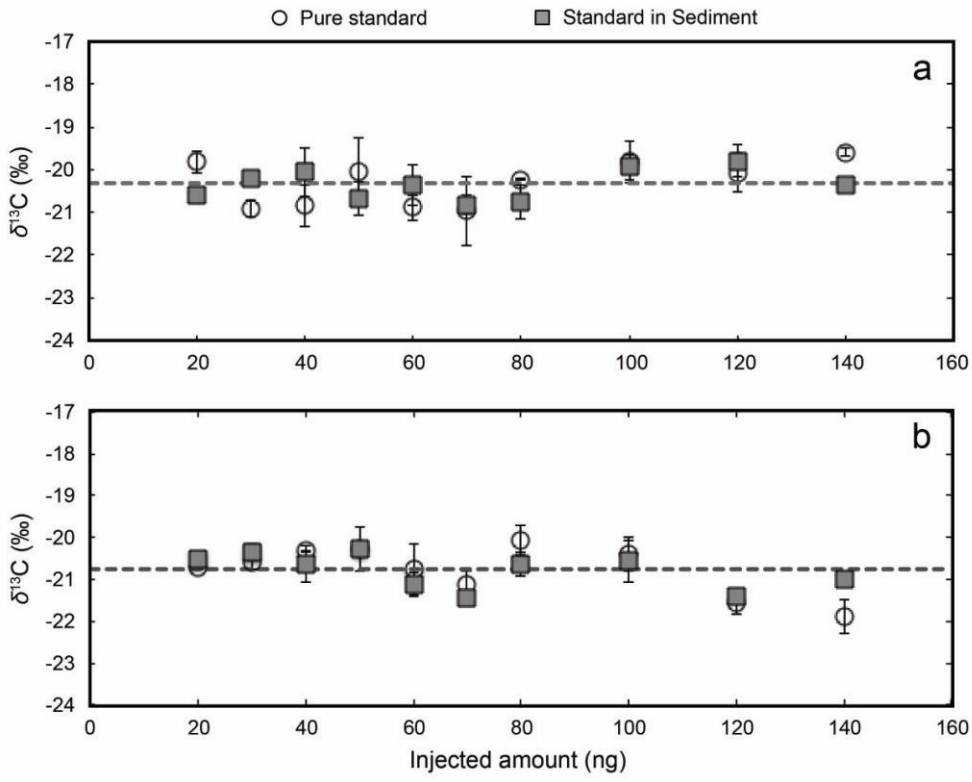


Fig. II.4. Stable carbon isotopic analysis of ManN (a) and MurA (b) in a range of 20 to 140 ng. Open circles represent the $\delta^{13}C_{AS,\ GC}$ values of pure standards. Solid squares designate the $\delta^{13}C_{AS,\ GC}$ values of the same standards spiked into sediment extract that did not contain the corresponding amino sugar. The dashed lines are the isotopic values of ManN and MurA determined by EA-IRMS, respectively (n = 3). The error bars represent for the total analytical errors.

The $\delta^{13}C$ values of ANA derivatives of ManN and MurA were consistent with the EA-IRMS reference values within the range of 20 to 140 ng of non-derivatized compound, i.e. equivalent to ~8-56 ng of amino sugar carbon (Fig. II.4), regardless of the presence of sedimentary matrix; we note that these two amino sugars are usually

expected to have the lowest concentration in environmental samples (e.g. Guggenberger et al., 1999; Niggemann and Schubert, 2006; Carsten et al., 2012). At quantities below 20 ng, indistinct peaks precluded proper evaluation of the isotopic composition. The minimum threshold of 20 ng of amino sugar on GC-IRMS is one order of magnitude lower than the values reported for HPLC-IRMS (Bodé et al., 2009) and enables the isotopic analysis of amino sugars at trace levels, as required by their low abundances in subseafloor sediment.

II.4.6. Abundance and isotopic composition of individual amino sugars in marine sediment

Figure II.5 summarizes the concentrations and $\delta^{13}C$ values of individual amino sugars from four marine sediment samples determined according to the protocol described in Fig. II.3. GlcN, accounting for 43.7-63.0% of the total amino sugars, was the most abundant amino sugar in all investigated samples, followed by GalN (29.0-39.7%) and ManN (4.4-12.4%), while MurA was always < 5%. The $\delta^{13}C$ values of GlcN and GalN were similar and fell between $\delta^{13}C_{TOC}$ and $\delta^{13}C_{DIC}$ in sediment collected from the Black Sea, Marmara Sea, and Peru Margin. In the cold seep sample from the Pakistan Margin, GalN was depleted in ^{13}C by 9‰ compared to GlcN. This sample was collected from a microbial mat composed of anaerobic methanotrophic archaea (ANME) group 2 and associated bacterial partners (Yoshinaga et al., 2012). These organisms were likely the predominant sources of TOC in the seep sample, which also exhibited the lowest $\delta^{13}C_{TOC}$ (-40.0‰). ManN exhibited $\delta^{13}C$ values ranging from -31.5‰ to -10.9‰ and showed no consistent trend relative to $\delta^{13}C_{TOC}$ and $\delta^{13}C_{DIC}$, or to the other amino sugars. MurA spanned the widest range in $\delta^{13}C$ (from -46.0‰ to -13.6‰), and was more depleted than $\delta^{13}C_{TOC}$ in the Pakistan Margin, Black Sea, and Peru Margin samples.

GlcN and GalN are typically the major amino sugars found in sediment and preserved in the form of peptidoglycan, lipopolysaccharides, and pseudopeptidoglycan from prokaryotes (Schleifer and Kandler, 1972; Kandler et al., 1998; Madigan and Martinko, 2005). Moreover, GlcN has also been detected in most fungal cell walls and invertebrate exoskeletons as chitin (Müller et al., 1986; Liang et al., 2007; Davis et al., 2009). It is generally believed that the hexosamines persist after cell death and are stable in soils (Glaser et al., 2004). Assuming a similar behavior in

marine sediment, the hexosamines are likely derived from a diverse assemblage of pelagic and sedimentary organisms and have been preserved in the sediment. The lack of a significant discrepancy between the $\delta^{13}C$ values of GlcN and GalN (usually < 3.5‰) implies a common, likely allochthonous source in the Black Sea, Marmara Sea, and Peru Margin sediments. The distinct isotopic compositions of GlcN (-28.2‰) and GalN (-37.2‰) in the Pakistan Margin cold seep sample can be best explained by an increasing fraction of these amino sugars from autochthonous microbes that utilize ^{13}C-depleted carbon for biosynthesis, as known from other biomolecules at similar sites (e.g. Lin et al., 2010; Schubotz et al., 2011). This process, and others, such as organic matter diagenesis or zones of enhanced microbial activity in specific sediment horizons, could be further examined by application of this method to define the heterogeneity of $\delta^{13}C$ signatures of individual hexosamines in the downcore sediment profiles.

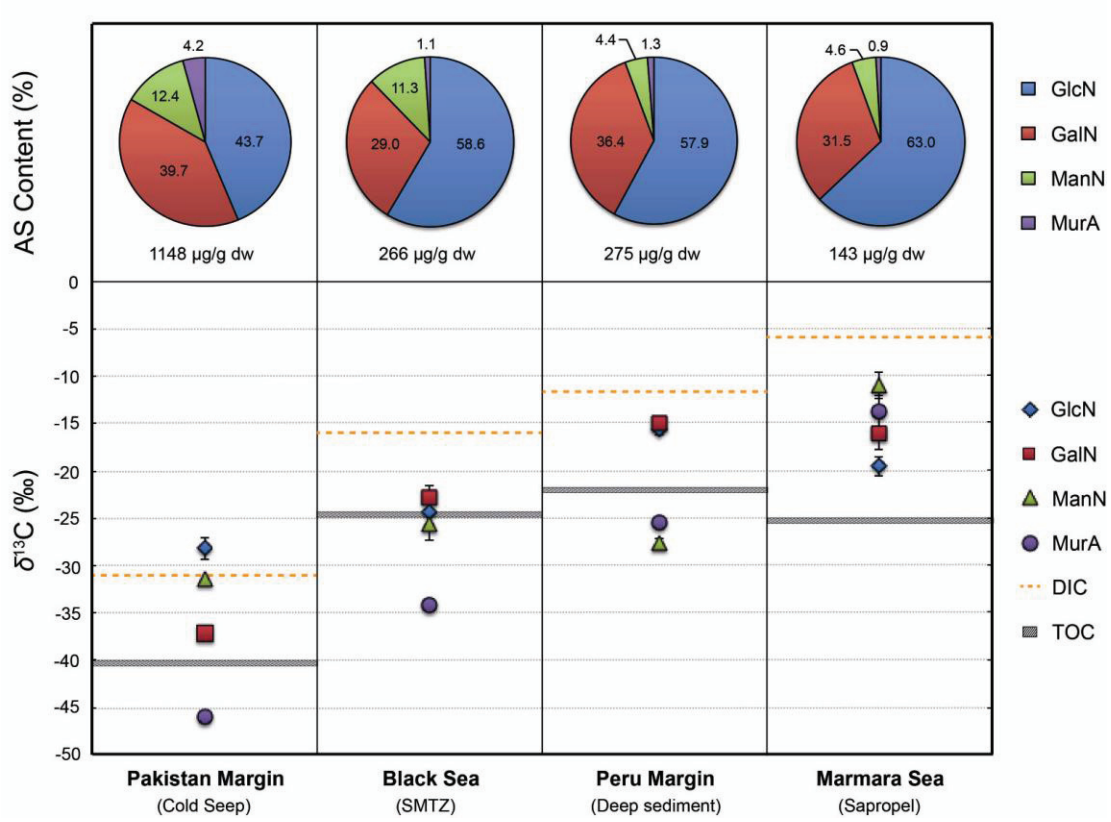


Fig. II.5. Concentrations and stable carbon isotopic values of amino sugars released from selected marine sediment samples. The depositional settings of the sediment samples are indicated in parentheses. Error bars indicate the total analytical errors (s_{Total}) of $\delta^{13}C$ values of individual amino sugars calculated according to error propagation. The isotopic values of dissolved inorganic carbon (DIC) and total organic carbon (TOC) are also plotted for comparison.

In contrast to the hexosamines with their diverse origin, MurA is a diagnostic bacterial biomarker because it is exclusively derived from peptidoglycan in bacterial cell walls (Schleifer and Kandler, 1972) and is presumed to rapidly degrade after cell death (Moriarty, 1977). The relatively high abundance of MurA (4.2% of total amino sugars) and its strong ^{13}C-depletion ($\delta^{13}C$ of -46.0‰) in the Pakistan Margin sample are consistent with our expectations of cold seep sediment that hosts microbial biomass fueled by methane (Hinrichs et al., 1999). Likewise, the ^{13}C-depleted MurA in the Black Sea sample is probably an indigenous signal resulting from bacteria that utilize relatively ^{13}C-depleted carbon at this depth (1.57 mbsf), which is close to the sulfate-methane transition zone, based on this site's methane profile (Zabel et al., 2013). Although compound-specific isotopic analysis of amino sugars is more demanding than that of membrane lipids, these observations of putative indigenous bacterial signals in the form of MurA make this compound a valuable addition to the targets selected for isotopic analysis of microbial biomass in the deep marine biosphere, which so far has been based on lipids and intact cells and biased towards detection of signals of the Archaea (cf. Biddle et al., 2006; Schubotz et al., 2011).

II.5. CONCLUSION

Methods for the carbon isotopic analysis of amino sugars have been developed for soils but not for marine sediments, where amino sugar concentrations tend to be lower. We tested various steps in the workflow of amino sugar analysis in order to establish a robust protocol for the stable carbon isotopic analysis of amino sugars in marine sediments. A combination of the most effective steps, including a new SPE protocol for the post-hydrolysis clean-up and a new step for enrichment of amino sugars via preparative HPLC, resulted in a protocol optimized for GC-based isotopic analysis of amino sugars at trace levels (limit of detection = 20 ng; equivalent to ~8 ng of amino sugar C). Moreover, use of the protocol did not introduce significant isotopic fractionation during sample preparation, except for the derivatization step, which can be accounted for with a derivatization standard. Applying this protocol, we determined for the first time the carbon isotopic composition of amino sugars in marine sediment samples. The stable carbon isotopic values of hexosamines indicated a major contribution from organic detritus, whereas MurA was more sensitive to an indigenous and active bacterial community. This method thus enables investigation of

the stable carbon isotopic compositions of amino sugars to infer microbial metabolism in the deep marine biosphere.

II.6. ACKNOWLEDGEMENTS

Samples were retrieved from a field trip to the Wadden Sea (Germany), during the RV Meteor cruises M74/3 and M84/1 (DARCSEAS), and Leg 201 of the Ocean Drilling Program (ODP), which is sponsored by the US National Science Foundation and participating countries under management of Joint Oceanographic Institutions (JOI), Inc. We thank the participating scientists and ship crews for sample recovery, Marcus Elvert and Marcos Yoshinaga for valuable advice and discussion, and Jessica Arndt, Raika Himmelsbach, Xavier Prieto Mollar, and Jenny Wendt for technical assistance. This study was primarily supported by the Deutsche Forschungsgemeinschaft (DFG) through Grant HI 616/11-1 (Cell Surf) to K.-U.H. R.Z. was sponsored by the China Scholarship Council (CSC) and the Gottfried-Wilhelm Leibniz Program of the DFG (through the Leibniz Price to K.-U.H.).

II.7. REFERENCES

Aluwihare, L.I., Repeta, D.J., Pantoja, S., and Johnson, C.G.: Two chemically distinct pools of organic nitrogen accumulate in the ocean, Science, 308, 1007-1010, 2005.

Aluwihare, L.I. and Meador, T.B. (Eds.): Chemical composition of marine dissolved organic nitrogen, in: Nitrogen in the marine environment, Elsevier, Oxford, UK, 95-140, 2008.

Amelung, W., Cheshire, M.V., and Guggenberger, G.: Determination of neutral and acidic sugars in soil by capillary gas-liquid chromatography after trifluoroacetic acid hydrolysis, Soil Biol. Biochem., 28, 1631-1639, 1996.

Amelung, W., Zhang, X., Flach, K.W., and Zech, W.: Amino sugars in native grassland soils along a climosequence in North America, Soil Sci. Soc. Am. J., 63, 86-92, 1999.

Bai, Z., Bode, S., Huygens, D., Zhang, X., and Boeckx, P.: Kinetics of amino sugar formation from organic residues of different quality, Soil Biol. Biochem., 57, 814-821, 2012.

Benner, R. and Kaiser, K.: Abundance of amino sugars and peptidoglycan in marine particulate and dissolved organic matter, Limnol. Oceanogr., 48, 118-128, 2003.

Biddle, J.F., Lipp, J.S., Lever, M.A., Lloyd, K.G., Sørensen, K.B., Anderson, R., Fredricks, H.F., Elvert, M., Kelly, T.J., Schrag, D.P., Sogin, M.L., Brenchley, J.E., Teske, A., House, C.H., and Hinrichs K.-U.: Heterotrophic Archaea dominate

sedimentary subsurface ecosystems off Peru, Proc. Natl. Acad. Sci. USA, 103, 3846-3851, 2006.

Bodé, S., Denef, K., and Boeckx, P.: Development and evaluation of a high-performance liquid chromatography/isotope ratio mass spectrometry methodology for ^{13}C analyses of amino sugars in soil, Rapid Commun. Mass Spectrom., 23, 2519-2526, 2009.

Çağatay, M., Görür, N., Algan, O., Eastoe, C., Tchapalyga, A., Ongan, D., Kuhn, T., and Kuşcu, İ.: Late Glacial-Holocene palaeoceanography of the Sea of Marmara: timing of connections with the Mediterranean and the Black Seas, Mar. Geol., 167, 191-206, 2000.

Carstens, D., Köllner, K.E., Bürgmann, H., Wehrli, B., and Schubert, C.J.: Contribution of bacterial cells to lacustrine organic matter based on amino sugars and D-amino acids, Geochim. Cosmochim. Acta, 89, 159-172, 2012.

Cowie, G.L. and Hedges, J.I.: Determination of neutral sugars in plankton, sediments, and wood by capillary gas chromatography of equilibrated isomeric mixtures, Anal. Chem., 56, 497-504, 1984.

Davis, J., Kaiser, K., and Benner, R.: Amino acid and amino sugar yields and compositions as indicators of dissolved organic matter diagenesis, Org. Geochem., 40, 343-352, 2009.

Decock, C., Denef, K., Bodé, S., Six, J., and Boeckx, P.: Critical assessment of the applicability of gas chromatography-combustion-isotope ratio mass spectrometry to determine amino sugar dynamics in soil, Rapid Commun. Mass Spectrom., 23, 1201-1211, 2009.

Fischer, D., Sahling, H., Nöthen, K., Bohrmann, G., Zabel, M., and Kasten, S.: Interaction between hydrocarbon seepage, chemosynthetic communities, and bottom water redox at cold seeps of the Makran accretionary prism: insights from habitat-specific pore water sampling and modeling, Biogeosciences, 9, 2013-2031, 2012.

Fox, A., Morgan, S.L., Hudson, J.R., Zhu, Z.T., and Lau, P.Y.: Capillary gas chromatographic analysis of alditol acetates of neutral and amino sugars in bacterial cell walls, J. Chromatogr. A, 256, 429-438, 1983.

Fox, A., Wright, L., and Fox, K.: Gas chromatography-tandem mass spectrometry for trace detection of muramic acid, a peptidoglycan chemical marker, in organic dust, J. Microbiol. Methods, 22, 11-26, 1995.

Fox, A., Krahmer, M., and Harrelson, D.: Monitoring muramic acid in air (after alditol acetate derivatization) using a gas chromatograph-ion trap tandem mass spectrometer, J. Microbiol. Methods, 27, 129-138, 1996.

Glaser, B., Turrión, M., and Alef, K.: Amino sugars and muramic acid-biomarkers for soil microbial community structure analysis, Soil Biol. Biochem., 36, 399-407, 2004.

Glaser, B. and Gross, S.: Compound-specific $\delta^{13}C$ analysis of individual amino sugars – a tool to quantify timing and amount of soil microbial residue stabilization, Rapid Commun. Mass Spectrom., 19, 1409-1416, 2005.

Guerrant, G. and Moss, C.: Determination of monosaccharides as aldononitrile, O-methyloxime, alditol, and cyclitol acetate derivatives by gas chromatography, Anal. Chem., 56, 633-638, 1984.

Guggenberger, G., Frey, S., Six, J., Paustian, K., and Elliott, E.: Bacterial and fungal cell-wall residues in conventional and no-tillage agroecosystems, Soil Sci. Soc. Am. J., 63, 1188-1198, 1999.

Hinrichs, K.-U., Hayes, J.M., Sylva, S.P., Brewer, P.G., and DeLong, E.F.: Methane-consuming archaeabacteria in marine sediments, Nature, 398, 802-805, 1999.

Itoh, S., Kawasaki, N., Ohta, M., and Hayakawa, T.: Structural analysis of a glycoprotein by liquid chromatography-mass spectrometry and liquid chromatography with tandem mass spectrometry: Application to recombinant human thrombomodulin, J Chromatogr. A, 978, 141-152, 2002.

Kandler, O. and König, H.: Cell wall polymers in Archaea (Archaebacteria), Cell Mol. Life Sci., 54, 305-308, 1998.

Kellermann, M.Y., Wegener, G., Elvert, M., Yoshinaga, M.Y., Lin, Y.S., Holler, T., Mollar, X.P., Knittel, K., and Hinrichs, K.-U.: Autotrophy as a predominant mode of carbon fixation in anaerobic methane-oxidizing microbial communities, Proc. Natl. Acad. Sci. USA, 109, 19321-19326, 2012.

Langerhuus, A.T., Røy, H., Lever, M.A., Morono, Y., Inagaki, F., Jørgensen, B.B., and Lomstein, B.A.: Endospore abundance and d: l-amino acid modeling of bacterial turnover in holocene marine sediment (Aarhus Bay), Geochim. Cosmochim. Acta, 99, 87-99, 2012.

Liang, C., Zhang, X. and Balser, T.C.: Net microbial amino sugar accumulation process in soil as influenced by different plant material inputs, Biol. Fertil. Soils, 44, 1-7, 2007.

Lin, Y.S., Lipp, J.S., Yoshinaga, M.Y., Lin, S.H., Elvert, M., and Hinrichs, K.-U.: Intramolecular stable carbon isotopic analysis of archaeal glycosyl tetraether lipids, Rapid Commun. Mass Spectrom., 24, 2817-2826, 2010.

Lin, Y.S., Lipp, J.S., Elvert, M., Holler, T., and Hinrichs, K.-U.: Assessing production of the ubiquitous archaeal diglycosyl tetraether lipids in marine subsurface sediment using intramolecular stable isotope probing, Environ. Microbiol., 15, 1634-1646, 2012.

Lomstein, B.A., Langerhuus, A.T., D'Hondt, S., Jørgensen, B.B., and Spivack, A.J.: Endospore abundance, microbial growth and necromass turnover in deep sub-seafloor sediment, Nature, 484, 101-104, 2012.

Madigan, M.T. and Martinko, J.M. (eds.): Brock Biology of Microorganisms 11th edn., Prentice Hall, Upper Saddle River, USA, 2005.

McCullagh, J.S.O.: Mixed-mode chromatography/isotope ratio mass spectrometry, Rapid Commun. Mass Spectrom., 24, 483-494, 2010.

Moriarty, D.: Improved method using muramic acid to estimate biomass of bacteria in sediments, Oecologia, 26, 317-323, 1977.

Morrison, D.J., Taylor, K., and Preston, T.: Strong anion-exchange liquid chromatography coupled with isotope ratio mass spectrometry using a Liquiface interface, Rapid Commun. Mass Spectrom., 24, 1755-1762, 2010.

Müller, P.J., Suess, E., and André Ungerer, C.: Amino acids and amino sugars of surface particulate and sediment trap material from waters of the Scotia Sea, Deep Sea Res., 33, 819-838, 1986.

Neeser, J. and Schweizer, T.: A quantitative determination by capillary gas-liquid chromatography of neutral and amino sugars (as O-methyloxime acetates), and a study on hydrolytic conditions for glycoproteins and polysaccharides in order to increase sugar recoveries, Anal. Biochem., 142, 58-67, 1984.

Niggemann, J. and Schubert, C.: Sources and fate of amino sugars in coastal Peruvian sediments, Geochim. Cosmochim. Acta, 70, 2229-2237, 2006.

Quaiser, A., Zivanovic, Y., Moreira, D., and López-García, P.: Comparative metagenomics of bathypelagic plankton and bottom sediment from the Sea of Marmara, ISME J., 5, 285-304, 2010.

Rinne, K.T., Saurer, M., Streit, K., and Siegwolf, R.T.W.: Evaluation of a liquid chromatography method for compound-specific $\delta^{13}C$ analysis of plant carbohydrates in alkaline media, Rapid Commun. Mass Spectrom., 26, 2173-2185, 2012.

Schleifer, K. and Kandler, O.: Peptidoglycan types of bacterial cell walls and their taxonomic implications, Bacteriol. Rev., 36, 407-477, 1972.

Schubotz, F., Lipp, J.S., Elvert, M., and Hinrichs, K.-U.: Stable carbon isotopic compositions of intact polar lipids reveal complex carbon flow patterns among hydrocarbon degrading microbial communities at the Chapopote asphalt volcano, Geochim. Cosmochim. Acta, 75, 4399-4415, 2011.

Veuger, B., van Oevelen, D., Boschker, H.T.S., and Middelburg, J.J.: Fate of peptidoglycan in an intertidal sediment: An in situ ^{13}C-labeling study, Limnol. Oceanogr., 51, 1572-1580, 2006.

Yoshinaga, M.Y., Wörmer, L., Elvert, M., and Hinrichs, K.-U.: Novel cardiolipins from uncultured methane-metabolizing Archaea, Archaea, http://dx.doi.org/10.1155/2012/832097, 2012.

Zabel, M. and cruise participants: RV METEOR, Cruise Report M84/L1, Biogeochemistry and methane hydrates of the Black Sea; Oceanography of the Mediterranean; shelf sedimentation and cold water carbonates 2011, DFG Senatskommission für Ozeanographie, pp. 39, 2013.

Zhang, X. and Amelung, W.: Gas chromatographic determination of muramic acid, glucosamine, mannosamine, and galactosamine in soils, Soil Biol. Biochem. 28, 1201-1206, 1996.

Zhang, X., Amelung, W., Yuan, Y., Samson-Liebig, S., Brown, L., and Zech, W.: Land-use effects on amino sugars in particle size fractions of an Argiudoll, Appl. Soil Ecol., 11, 271-275, 1999.

Chapter III

Distribution and isotopic composition of amino sugars in contrasting subseafloor sediments

Rong Zhu[a, *], Julius S. Lipp[a], Yu-Shih Lin[a, b], and Kai-Uwe Hinrichs[a]

In preparation for *Geochimica et Cosmochimica Acta*

[a] Organic Geochemistry Group, MARUM Center for Marine Environmental Sciences & Department of Geosciences, University of Bremen, Leobener Str., 28359 Bremen, Germany

[b] Department of Oceanography, National Sun Yat-Sen University, No.70 Lienhai Rd, Kaohsiung 804, Taiwan

* Corresponding author. Email address: rongzhu1018@gmail.com (R. Zhu)

III.1. ABSTRACT

Amino sugars are significant constituents in microbial biopolymers and have been successfully employed to fingerprint microbial communities and track microbial-mediated transformation of organic matter (OM) in soils and aquatic systems. Analysis of stable carbon isotopic compositions of individual amino sugars can provide important clues on metabolisms of their microbial producers. With a recently optimized sample preparation protocol that allows for quantification and isotopic analysis of amino sugars in complex sedimentary matrices, we studied distributions of amino sugars in subseafloor sediments from two locations, ODP Leg 201 Site 1229 at the Peru Margin and IODP Expedition 317 Site U1352 at the Canterbury Basin, and report for the first time detailed downcore profiles of stable carbon isotopic compositions ($\delta^{13}C$) of individual amino sugars in deeply buried marine sediments. Significant differences are observed in amino sugar distributions and ratios between the two study sites, which can reflect varied OM contents and inputs at the respective sites. By compiling published muramic acid (MurA) data together with our data, we estimate that intracellular MurA usually accounts for less than 15% of the total MurA in subseafloor sediments; consequently MurA may not be used as a strict biomarker for quantification of bacterial populations in marine sediments. Notably, $\delta^{13}C$ of MurA and mannosamine were lower in the sulfate-methane transition zones from both sites, suggesting that the source microbial biomass is partially assimilating methane-derived carbon in these horizons. This study provides new insights into microbial life in deeply buried marine sediments and extends our knowledge of amino sugar dynamics in this important carbon reservoir.

III.2. INTRODUCTION

Amino sugars are often presumed to be abundant components of marine organic matter (OM). The most common amino sugars, i.e. glucosamine (GlcN), galactosamine (GalN), mannosamine (ManN) and muramic acid (MurA), account for ~3% of total organic carbon (TOC) in coastal Peruvian surface sediments (Lomstein et al., 2009). Being predominantly originated from microbial biopolymers, amino sugars have been routinely applied to investigate contributions of microorganisms to soil OM (SOM), whilst their ratios have been used to distinguish microbial

community structures, i.e. the relative proportions of fungi and bacteria to SOM (Zhang et al., 1999; Kandeler et al., 2000; Glaser et al., 2004; Simpson et al., 2004; Liang et al., 2007a). Combined with the stable-isotope-probing approach, amino sugars have provided important clues on the transformation of organic carbon and nitrogen in soils (Glaser and Gross, 2005; Liang et al., 2007b; Roberts et al., 2007; Decock et al., 2009; He et al., 2011; Bai et al., 2012). However, investigation of amino sugar isotopic compositions at natural abundance is still missing, impeding our understanding of biogeochemical cycling of amino sugars relative to other biomarkers with well characterized isotope systematics, such as lipids (e.g. Hinrichs et al., 1999; Elvert et al, 2000; Pancost et al., 2000; Orphan et al., 2001; Lin et al., 2010; Schubotz et al., 2011).

During the past decades, amino sugars have received increasing attention in biogeochemical studies of the marine realm (Ittekkot et al., 1984a, b; Gupta et al., 1997; Dauwe and Middelburg, 1998; Benner and Kaiser, 2003; Davis and Benner, 2005; Kaiser and Benner, 2008; Carstens et al., 2012). Earlier work has been predominantly focusing on water column samples, but a few recent studies started to explore the information carried by amino sugars in marine sediments with respect to the endospore population (Lomstein et al., 2012) and sources, transformation and accumulation of OM (Niggemann and Schubert, 2006; Lomstein et al., 2009; Carstens and Schubert, 2012). Since MurA is exclusively derived from peptidoglycan in bacterial cell walls, with its proportion in gram-positive bacteria around one order of magnitude higher than in gram-negative bacteria (Schleifer and Kandler, 1972; Madigan and Martinko, 2005), of particular interest is MurA-based estimation of microbial population in environmental samples. Such an application is based on the observation that MurA is rapidly decomposed by microorganisms in environmental samples, as demonstrated via ^{14}C-labelling experiments using surface marine sediments (King and White, 1977; Moriarty, 1977). In water column studies, MurA concentrations were correlated with total bacterial cell numbers (Mimura and Romano, 1985; Carstens et al., 2012). Nevertheless, the validity of MurA as a proxy for bacterial biomass is influenced by several critical factors, including different compositions of gram-positive vs. gram-negative bacteria and complex sample matrices that can potentially incorporate MurA, and consequently bias the estimates (Nagata et al., 2003). Substantial proportions of MurA are therefore suggested to be

associated with bacterial debris rather than living bacteria in both water column samples (Benner and Kaiser, 2003; Kaiser and Benner, 2008) and surface sediments (Niggemann and Schubert, 2006; Lomstein et al., 2009).

In the present study, downcore distributions and stable carbon isotopic compositions of individual amino sugars in deep subseafloor sediments were investigated simultaneously for the first time. We chose sediment samples from two locations, Ocean Drilling Program (ODP) Leg 201 Site 1229 (high TOC contents and marine OM dominance; subseafloor depth of up to ~190 m below the seafloor; mbsf) and Integrated Ocean Drilling Program (IODP) Expedition 317 Site U1352 (organic-lean and terrestrial OM dominance; up to 1900 mbsf), to compare amino sugar signatures in contrasting sedimentary environments. Moreover, proportions of intracellular MurA as well as the bacterial contribution to the amino sugar pool were estimated in different marine regions. Our results revealed significant variations in microbial origins, transformation and metabolisms of amino sugars between the two study sites and provided insights into the microbial communities in ultra-deep marine sediments.

III.3. MATETIAL AND METHODS

III.3.1. Study sites

Sediment samples were collected from two locations, ODP Leg 201 Site 1229 (January - March, 2002) and IODP Expedition 317 Site U1352 (November, 2009 - January, 2010). Site 1229 is located on the continental shelf off the coast of Peru, at a water depth of 150.5 m. The sediments are organic-rich and affected by a sulfate-bearing brine that is present below the seafloor of this area (D'Hondt et al., 2003). Site U1352 is situated on the upper slope of the Canterbury Basin, New Zealand, at a water depth of 344 m. It is one of the deepest drilled IODP sites that have been systematically sampled for molecular biological and chemical analysis so far, thus providing the opportunity to explore microbial life in the ultra-deep subseafloor up to 1900 mbsf. The sediments at this site are organic-lean and originate from terrestrial sources (Fulthorpe et al., 2011). All the sediment samples examined in this study were taken as whole-round cores and were immediately frozen on board and stored at -20°C until extraction and analysis.

III.3.2. Analyses

III.3.2.1. Biogeochemical parameters

For analysis of TOC and total nitrogen (TN) contents as well as δ^{13}C-TOC, sediment samples were decalcified with 10% HCl, washed with MilliQ water and freeze-dried. Aliquots of the samples were weighed and measured in triplicate with a Flash 2000 Organic Elemental Analyzer coupled to a Delta V Plus isotope ratio mass spectrometer (EA-IRMS) via a Conflo IV interface (Thermo Finnigan MAT GmbH, Bremen, Germany).

Stable carbon isotopic composition of dissolved inorganic carbon (DIC) at Site U1352 was measured by Finnigan GasBench-II headspace autosampler equipped with a Finnigan MAT 252 IRMS using the method from Torres et al. (2005). δ^{13}C analysis of methane (CH_4) was performed with a Thermo Finnigan Trace GC Ultra coupled to a Delta V Plus IRMS via a Conflo IV interface according to the protocol previously described by Ertefai et al. (2010). δ^{13}C values of DIC and CH_4 at Site 1229 were obtained from Meister et al. (2007) and Hinrichs et al. (2006), respectively.

III.3.2.2. Amino sugars

Amino sugars were extracted from marine sediments following the procedure described by Zhu et al. (in press). In brief, freeze-dried samples were hydrolyzed with 6 M HCl at 105°C for 8 h under N_2 headspace. The hydrolysate then underwent a series of purification steps, including filtration with combusted glass fiber filters, evaporation under vacuum, neutralization with 1 M KOH solution (until pH 6.5-7.0) and desalting with an ENVI-Carb^{TM} Plus SPE cartridge (Sigma-Aldrich Chemie GmbH, Munich, Germany). After freeze-drying overnight, the extract was transformed to aldononitrile acetate (ANA) derivatives according to the method from Guerrant and Moss (1984) and further purified with a self-packed silica gel column to remove the interference of polar compounds (Lin et al., 2010). Myo-Inositol was spiked for quantification purposes to the samples as an internal standard after acid hydrolysis; the relative standard errors of triplicate analysis were 2-4% for GlcN, ManN and MurA and 9% for GalN. 3-*O*-methyl-D-glucopyranose was added prior to lyophilization as a second standard in order to correct for isotopic fractionation

introduced via derivatization (cf. Lin et al., 2010); the total analytical errors of individual amino sugars were < 1.4‰.

Table III.1. Concentraions of TOC, TN, TASs and relative proportions of individual amino sugars at ODP Leg 201 Site 1229.

Depth (mbsf)	TOC (%)	TN (%)	TASs (μg/g sed)	GlcN (%)	GalN (%)	ManN (%)	MurA (%)
1.40	6.99	0.57	407.5	52.4	43.4	3.0	1.2
3.95	6.07	0.47	354.0	51.7	40.7	6.7	1.0
7.77	3.11	0.22	237.3	54.2	40.3	5.1	0.4
16.64	3.80	0.23	274.6	77.9	36.4	4.4	1.3
30.69	4.83	0.44	656.3	59.8	31.2	8.6	0.4
32.42	4.20	0.32	281.9	53.9	35.9	9.1	1.0
39.95	2.54	0.14	355.6	57.0	30.7	12.0	0.4
42.49	2.28	0.17	60.1	58.0	33.0	8.7	0.3
49.88	3.03	0.18	82.1	56.7	37.5	5.2	0.7
70.54	1.07	0.07	51.2	60.5	32.3	6.6	0.6
87.09	3.58	0.21	113.8	49.5	37.6	12.7	0.3
88.90	1.15	0.09	15.7	61.0	31.9	5.9	1.3
90.75	1.01	0.06	34.6	59.0	32.8	6.9	1.3
102.19	2.82	0.19	199.3	59.0	33.2	6.9	1.0
121.10	1.59	0.10	33.7	56.6	38.2	4.5	0.7
157.70	0.47	0.03	24.6	51.8	34.2	12.9	1.1
185.88	0.39	0.02	26.9	48.2	24.7	26.1	1.1

Abbreviations: TOC, total organic carbon; TN, total nitrogen; TASs, total amino sugars; GlcN, glucosamine; GalN, galactosamine; ManN, mannosamine; MurA, muramic acid; mbsf, meter below the seafloor.

Quantification of amino sugars was accomplished using an Agilent 6890N GC instrument coupled to an Agilent 5973 inert Mass Selective Detector (MSD) with an electron impact (EI) source (Agilent Technologies Deutschland GmbH, Böblingen, Germany). An Rxi-5ms column (30 m × 0.25 mm, 0.25 μm film thickness; Restek GmbH, Bad Homburg, Germany) was used for chromatographic separation and the MS was programmed in selective ion monitoring (SIM) mode to target specific mass fragments of the derivatives (Zhu et al., in press). Compound-specific stable carbon isotope analysis of individual amino sugars was performed with a Trace GC Ultra

instrument coupled to a Delta Plus XP isotope ratio mass spectrometer via a Combustion Interface III (Thermo Finnigan MAT GmbH, Bremen, Germany). Two columns with different polarities, an Optima 17MS column (30 m × 0.25 mm, 0.25 μm film thickness; Macherey-Nagel GmbH & Co. KG, Düren, Germany) and an Rxi-5ms column, were applied to obtain reliable $\delta^{13}C$ values.

For samples that exhibited low signals of MurA and/or ManN and were influenced by high abundance of the adjacent GalN peak, aliquots of desalted extracts were separated into two fractions, the hexosamine fraction and MurA fraction, via an Agilent 1200 Series preparative high performance liquid chromatography (HPLC) system (Agilent Technologies Deutschland GmbH, Böblingen, Germany) equipped with an Econosphere NH_2 column (250 mm × 4.6 mm, 5 mm particle size; Alltech Associates Inc., Deerfield, IL, USA; Zhu et al., in press). Both fractions were transformed to ANA derivatives, purified with silica gel column and subsequently analyzed on GC-IRMS.

III.4. RESULTS

III.4.1. Total amino sugars

The total amino sugars (TASs, sum of GlcN, GalN, ManN and MurA) strongly correlated with contents of TOC ($r^2 = 0.61$, $p < 0.001$; n = 47) and TN ($r^2 = 0.70$, $p < 0.001$; n = 47) at double logarithmic scales (Fig. III.1). Specifically, amino sugars were enriched in the organic-rich reservoir at Site 1229 from the Peru Margin, where the TOC and TN contents were 0.39-6.99% and 0.02-0.57%, respectively (Table III.1). Concentrations of the TASs covered a range of 16-656 μg/g dry sediment; the corresponding values of amino sugar-derived carbon (AS-C%) and nitrogen (AS-N%) accounted for 0.06-0.56% of the TOC and 0.14-1.98% of the TN, respectively (Fig. III.2a). AS-C% and AS-N% generally co-varied throughout the core, with maximum values occurring in both sulfate-methane transition zones (SMTZs), and an increase towards the bottom of the core.

In contrast, the TOC and TN contents at Site U1352 from the Canterbury Basin were generally below 0.48% and 0.05%, respectively, with average values an order of magnitude lower than those at Site 1229 (Table III.2). 0.3-205 μg/g sediment of TASs

were recovered at this ultra-deep, organic-lean site, which accounted for 0.01-1.72% of the TOC and 0.02-3.14% of the TN, respectively (Fig. III.2b). The maximum values of AS-C% and AS-N% occurred in the upper sediment column, concentrations then decreased steeply with depth, with the exceptions of two- to four-fold increases in the SMTZ and at ~300 mbsf.

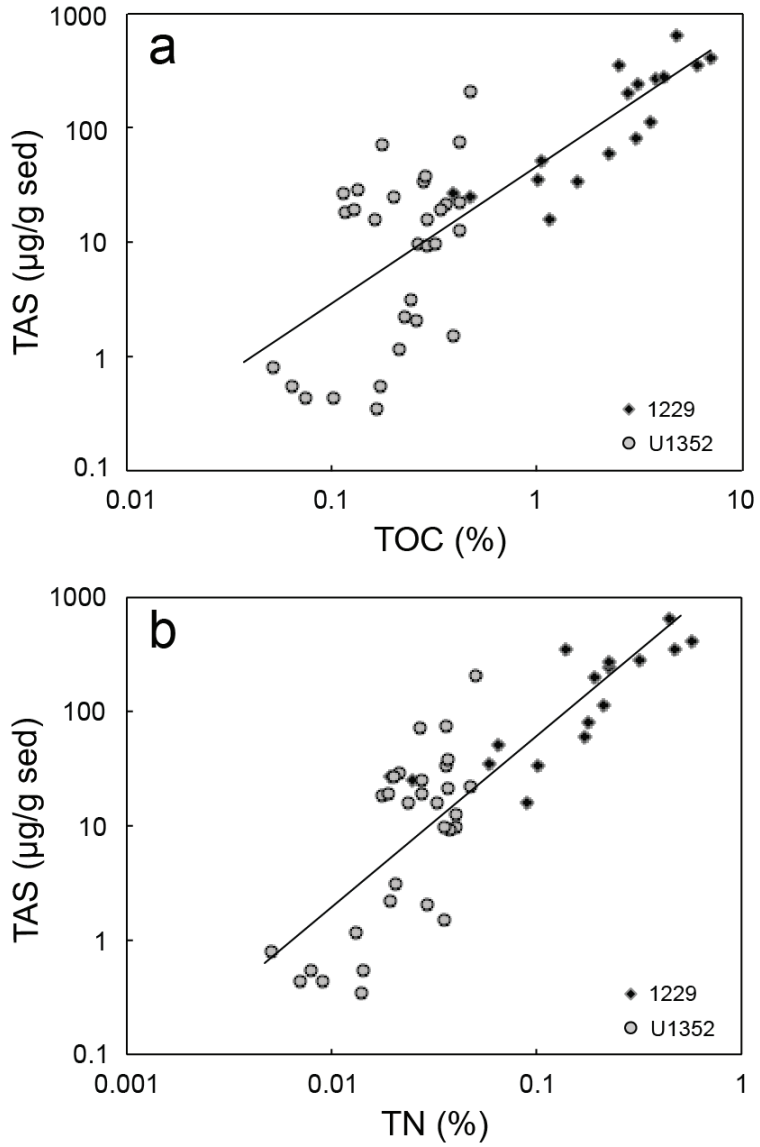


Fig. III.1. Correlation of concentrations of TASs vs. TOC (a) and TN (b). Regression lines: (a) log(TAS) = 1.2034 log(TOC) + 1.6661 (r^2 = 0.61, $p < 0.001$; n = 47); (b) log(TAS) = 1.4803 log(TN) + 3.2888 (r^2= 0.70, $p < 0.001$; n = 47).

III.4.2. Distribution of individual amino sugars

To compare distributions of amino sugars from the two contrasting sites, concentrations of individual amino sugars were reported relative to the TOC contents

(Fig. III.3). At Site 1229, GlcN was the most abundant amino sugar, with concentrations of 835-8120 μg/g TOC and accounting for 48.2-61.0% of the TASs (Fig. 3a; Table III.1). GalN and ManN covered ranges of 437-4303 μg/g TOC and 80-1778 μg/g TOC, making up 24.7-43.4% and 3.0-26.1% of the TASs, respectively (Fig. III.3a, b; Table III.1). Concentration of MurA ranged from 8.9 to 91.5 μg/g TOC and only contributed 0.3-1.3% to the TAS pool (Fig. III.3b; Table III.1). The three hexosamines (GlcN, GalN and ManN) generally co-varied along the core, with conspicuous peaks (ca. two- to four-fold increases) occurring at 30-40 mbsf and ~100 mbsf compared to the adjacent horizons. MurA exhibited greater variability in the downcore profile. In addition to the increases in the two SMTZs, signals of MurA were also significantly enhanced in the uppermost and bottom samples, with the maximum value at ~17 mbsf.

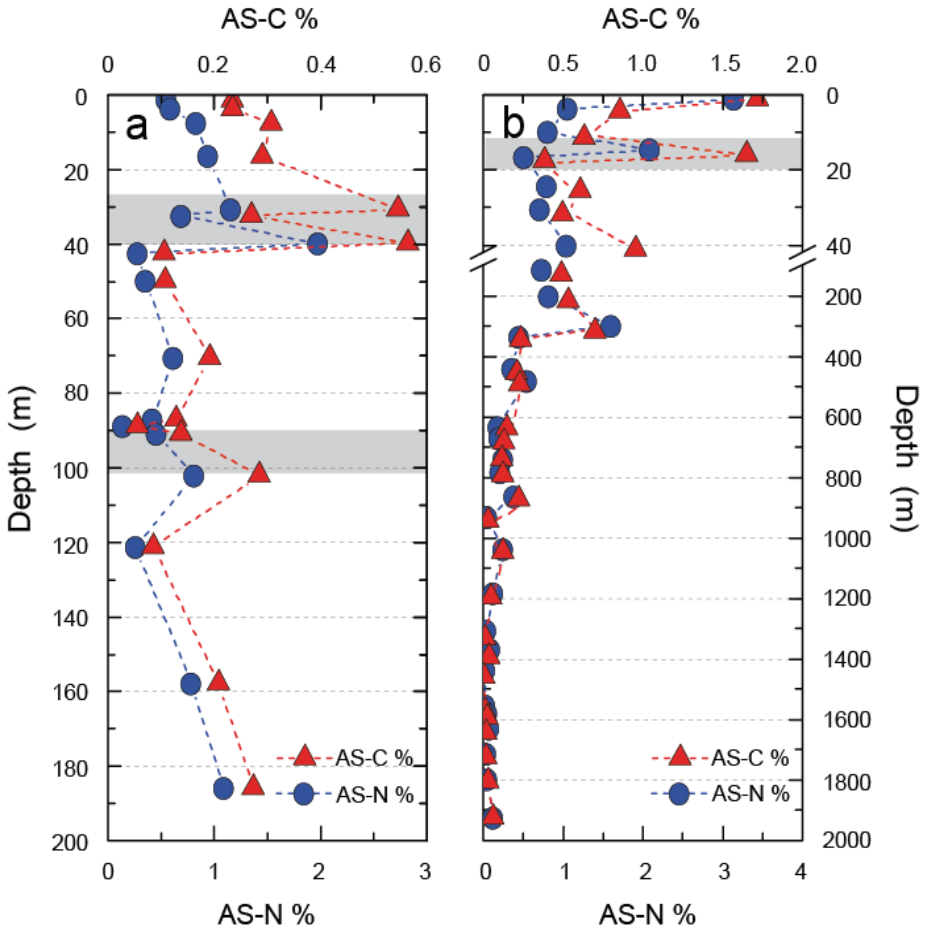


Fig. III.2. Depth profiles of amino sugar carbon and nitrogen accounted for total organic carbon (AS-C%) and total nitrogen (AS-N%) at ODP Leg 201 Site 1229 from the Peru Margin (a) and IODP Exp. 317 Site U1352 from the Canterbury Basin (b). The gray bars designate the depth interval of SMTZs.

Table III.2. Concentrations of TOC, TN, TASs and relative proportions of individual amino sugars at IODP Exp. 317 Site U1352.

Depth (mbsf)	TOC (%)	TN (%)	TASs (μg/g sed)	GlcN (%)	GalN (%)	ManN (%)	MurA (%)
1.05	0.48	0.05	205.0	57.3	36.0	6.1	0.7
3.86	0.13	0.02	28.8	57.6	35.3	6.4	0.7
10.10	0.12	0.02	18.4	52.6	39.8	6.8	0.8
14.75	0.18	0.03	72.0	59.7	33.6	6.2	0.6
16.60	0.16	0.02	15.6	66.7	25.9	6.8	0.7
24.30	0.13	0.02	29.3	48.9	42.1	8.3	0.7
30.60	0.20	0.03	25.0	54.1	36.7	8.1	1.1
40.05	0.11	0.02	26.9	53.8	38.2	7.2	0.8
115.58	0.28	0.04	34.0	59.7	32.8	6.9	0.5
203.10	0.29	0.04	38.2	67.2	26.5	5.8	0.5
298.25	0.42	0.04	73.6	45.6	24.1	29.7	0.5
335.90	0.36	0.04	21.2	60.4	22.2	17.1	0.3
441.50	0.42	0.05	22.0	53.7	14.6	31.4	0.3
479.90	0.34	0.03	19.2	36.1	23.6	39.3	1.0
633.35	0.26	0.04	9.8	77.3	14.2	8.1	0.4
668.95	0.29	0.04	9.4	63.3	11.9	24.4	0.4
737.75	0.42	0.04	12.5	74.4	10.7	14.5	0.4
783.46	0.32	0.04	9.8	74.0	10.5	15.1	0.5
864.61	0.29	0.03	16.0	68.0	14.2	16.7	1.1
930.84	0.26	0.03	2.0	45.8	17.6	35.9	0.7
1183.70	0.24	0.02	3.1	61.8	12.7	25.0	0.5
1305.02	0.39	0.04	1.5	58.7	12.3	29.0	bd
1367.00	0.23	0.02	2.2	47.6	7.1	44.4	0.9
1435.70	0.16	0.01	0.3	36.6	28.4	35.0	bd
1553.57	0.17	0.01	0.6	37.9	17.3	44.8	bd
1576.94	0.08	0.01	0.4	31.6	26.0	42.4	bd
1630.05	0.21	0.01	1.2	46.5	12.7	40.8	bd
1711.30	0.10	0.01	0.4	29.9	30.2	40.0	bd
1795.78	0.06	0.01	0.5	31.1	33.7	35.2	bd
1922.20	0.05	0.01	0.8	25.1	26.1	48.8	bd

Abbreviation: bd, below detection limit.

Distributions of amino sugars were slightly different at Site U1352, with an exception of ManN, which exhibited more scatters in the downcore profile. GlcN, ranging from 76.3 to 24500 μg/g TOC, made up 25.1-77.3% of the TASs and dominated in the upper 1400 m of the core (Fig. III.3c; Table III.2). Concentration of

GalN was in the range of 46.8 to 15372 µg/g TOC. The percentages of GalN in the TASs remained fairly constant (avg. 33.7 ± 5.9%) in the top 300 m of the core, and subsequently dropped to 13.4 ± 5.5% between 300 and 1400 mbsf, where proportions of GlcN were substantially increased (up to 77%), and finally reached to 23.5 ± 7.9% in the deepest samples. ManN was determined as 73-5190 µg/g TOC (Fig. III.3d). Notably, it only accounted for ~7% of the TASs in the top 300 m, but made up to 48.8% of the TASs in the deeper sediments and dominated the TAS pool below 1400 mbsf (Table III.2). To our knowledge, such high proportions of ManN have never been documented in environmental samples before. MurA was detectable down to a depth of ~1300 mbsf, with concentration of 5.2-292 µg/g TOC and contributed < 1.1% of the TASs. In contrast to Site 1229, all amino sugars at Site U1352 were more abundant in the shallower sediment and decreased sharply by almost one order of magnitude below 4 m. A four-fold increase occurred in the SMTZ, followed by two distinct peaks at ~40 and ~300 mbsf. Amino sugar contents slightly decreased from 400 mbsf toward the bottom of the core, and remained at low levels below ~1200 mbsf, with an exception of ManN, which exhibited substantial scatter in the deeper sediments.

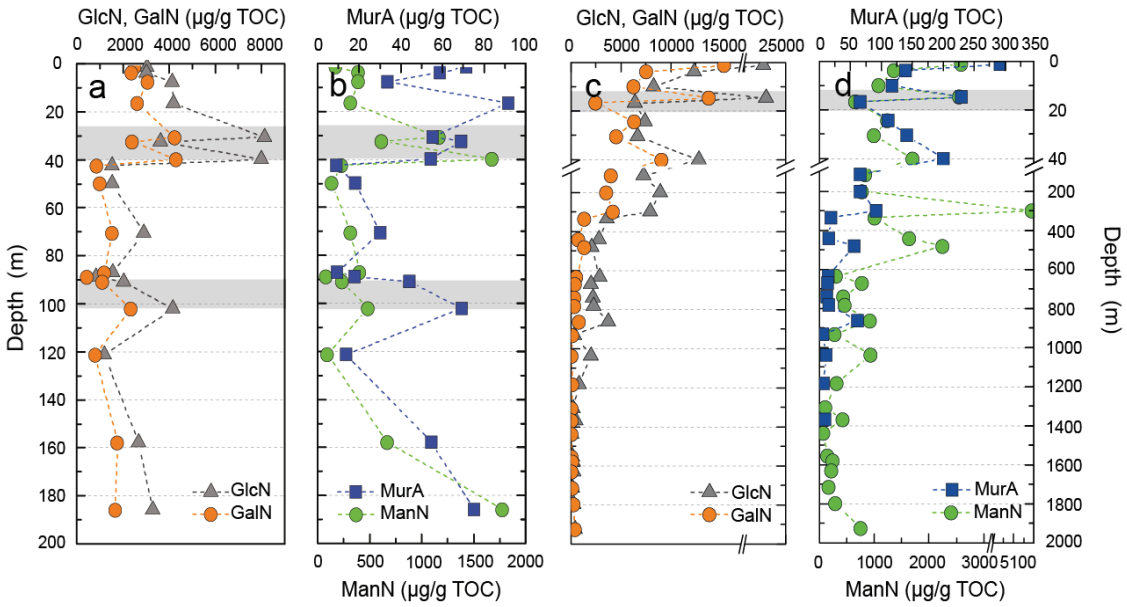


Fig. III.3. Depth profiles of amino sugar concentrations at ODP Leg 201 Site 1229 from the Peru Margin (a, b) and IODP Exp. 317 Site U1352 from the Canterbury Basin (c, d). The gray bars designate the depth interval of SMTZs.

III.4.3. Amino sugar ratios

Amino sugar molar ratios, which have been considered diagnostic for the source organisms (see below), showed distinct inter-core variations (Fig. III.4). At Site 1229, ratios of GlcN/GalN covered a narrow range of 1.2-2 (avg. 1.6 ± 0.3; Fig. III.4a). The GlcN/GalN ratios were slightly higher near the two SMTZs and in the bottom samples, whereas remained comparatively constant in the other horizons. In contrast, ratios of GlcN/GalN spanned a broad range of 0.9-7.0 (avg. 3.1 ± 3.7) at Site U1352, with higher values (> 3) usually observed between 500 and 1400 mbsf (Fig. III.4c). Ratios of GlcN/ManN ranged from 1.9 to 13.2 (avg. 8.4 ± 3.9) at Site 1229 and from 0.5 to 11.6 (avg. 4.2 ± 3.6) at Site U1352, respectively (Figs. III.4a, c). The downcore profile of GlcN/ManN at Site 1229 showed a high degree of scatter, with the maximum and minimum values registered in the uppermost and bottommost samples, respectively. Conversely, ratios of GlcN/ManN at Site U1352 generally decreased with depth, with only a few exceptions (e.g. at 200 and ~600 mbsf). The GlcN/MurA ratios ranged from 59.9 to 242 (avg. 120.8 ± 66.6) at Site 1229 and from 51.4 to 282.7 (avg. 157.7 ± 75.7) at Site U1352, respectively, and exhibited strong scatter at both sites (Fig. III.4b, d). Like the ratios of GlcN/GalN, the GlcN/MurA ratios were significantly increased near the two SMTZs at Site 1229 and in the interval of 500-1300 mbsf at Site U1352.

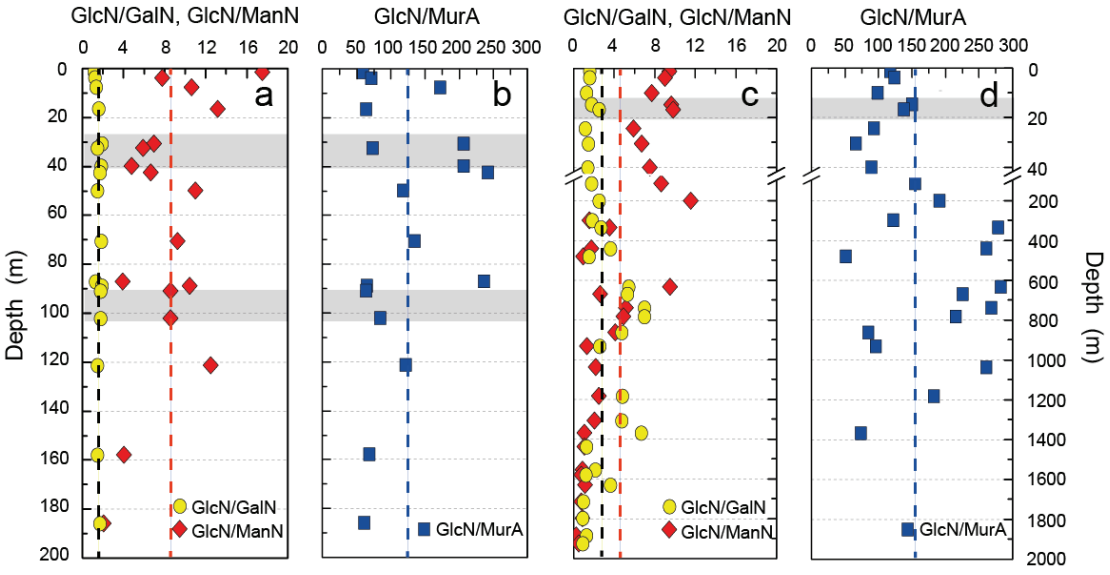


Fig. III.4. Ratios of amino sugars at ODP Leg 201 Site 1229 from the Peru Margin (a, b) and IODP Exp. 317 Site U1352 from the Canterbury Basin (c, d). The yellow dots, red diamonds and blue squares represent the ratios of GlcN/GalN, GlcN/ManN and GlcN/MurA, respectively, and the individual average values are indicated by the dashed lines. The gray bars designate the depth interval of SMTZs.

III.4.4. Amino sugar stable carbon isotopic compositions

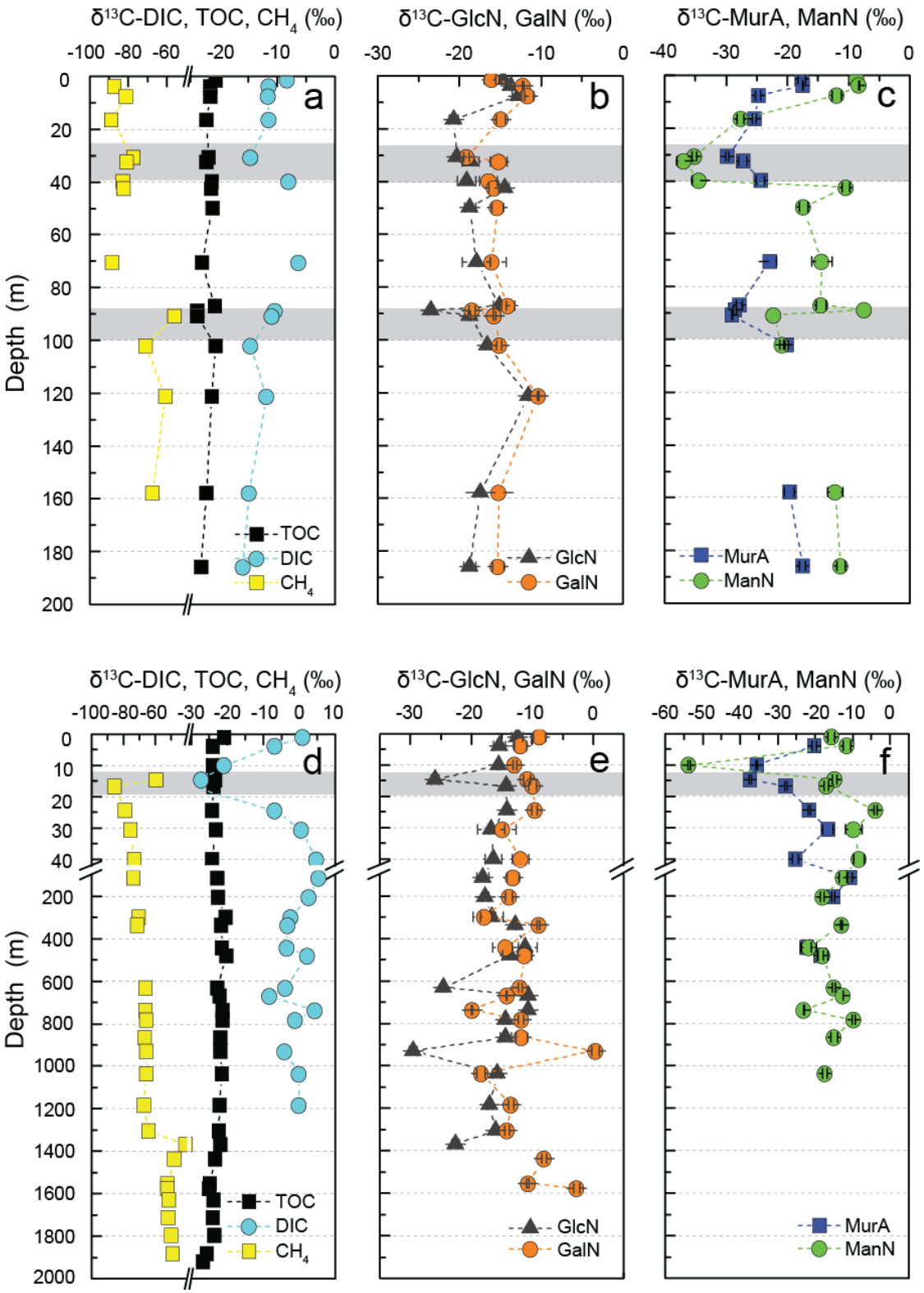


Fig. III.5. Stable carbon isotopic compositions of DIC, TOC, CH_4 and individual amino sugars at ODP Leg 201 Site 1229 from the Peru Margin (a, b, c) and at IODP Exp.317 Site U1352 from the Canterbury Basin (d, e, f). The gray bars designate the depth interval of SMTZs.

Figure III.5 summarizes the stable carbon isotopic compositions of individual amino sugars, CH_4, and TOC and DIC of both sites. At Site 1229, $\delta^{13}C$ values of TOC were fairly constant throughout the core with an average value of -21.9 ± 1.0‰, while values of $\delta^{13}C$-DIC and $\delta^{13}C$-CH_4 ranged from -15.9‰ to -6.4‰ and -88.7‰ to -55.8‰, respectively (Fig. III.5a). $\delta^{13}C$-GlcN and $\delta^{13}C$-GalN co-varied in the downcore distribution and the values were usually between -20‰ and -10‰ with only a few exceptions (Fig. III.5b). The $\delta^{13}C$ values of minor amino sugars, MurA and ManN, spanned wider ranges from -30‰ to -18‰ and -37‰ to -7‰, respectively (Fig. III.5c), with strong ^{13}C-depletion observed in the two SMTZs. At Site U1352, the $\delta^{13}C$-TOC values were almost constant throughout the core (avg. 22.9 ± 1.5‰; Fig. III.5d). Above 1400 mbsf, values of $\delta^{13}C$-CH_4 were generally in the range of -70‰ to -60‰, with exceptions of samples in the SMTZ (-80‰). Below 1400 mbsf, the $\delta^{13}C$-CH_4 values shifted toward -50‰. The $\delta^{13}C$-DIC values also showed a negative excursion in the SMTZ, while the values oscillated around the mean value of -0.3 ± 4.0‰ below 40 mbsf. $\delta^{13}C$ compositions of individual amino sugars were predominantly in the range of -25‰ to -5‰ (Fig. III.5e, f). Negative excursions in amino sugar $\delta^{13}C$ values were also observed in layers close to the SMTZ, with the respective ^{13}C-depletion of MurA and ManN being stronger than that of GlcN and GalN.

III.5. DISCUSSION

III.5.1. Contributions of TASs to organic matter pools

With 0.01-1.7% of TOC and 0.02-3.1% of TN, the TASs represent a minor to substantial proportion of the OM from both sites. The proportions are comparatively lower than previously published data in sediment samples from several oceanic regions, where the proportions of amino sugars are 0.4-5.0% of TOC and 1.2-8.6% of TN (Dauwe and Middelburg, 1998; Gupta et al., 1997; Jennerjahn and Ittekkot, 1999; Niggemann and Schubert, 2006). In the uppermost sample (1.4 mbsf) from Site 1229, the proportions of AS-C% and AS-N%, i.e. 0.23% and 0.55%, respectively, are substantially lower than those from surface sediments between 0.5-47 cmbsf at the Peru Margin (avg. 1.7% for AS-C% and 2.9% for AS-N%; Niggemann and Schubert, 2006). Such significant differences suggest that amino sugars in sediment depth

shallower than 0.5 m are predominantly derived and accumulated from water column material, and preferentially degraded relative to refractory OM downwards. Conspicuous peaks of AS-C% and AS-N% near the upper SMTZ are consistent with increased prokaryotic cell numbers (D'Hondt et al., 2003), indicating that microbial biomass is a significant contributor to the TAS pool in this horizon. Triggered by upward-diffusing sulfate-bearing brine and downward-diffusing methane, sediment within the second, deeper SMTZ provides sufficient energy to sustain elevated densities of microorganisms in deep sediments, where microbial cell numbers were up to thousand-fold higher compared to the adjacent horizons (D'Hondt et al., 2003; Biddle et al., 2006). Nonetheless, proportions of AS-C% and AS-N% do not increase correspondingly in the second SMTZ, which is partially in agreement with previously reported intact polar lipid (IPL) concentrations and fluorescence-in-situ-hybridization (FISH) counts of archaea and bacteria (Biddle et al., 2006). Possible explanations for the different records of amino sugars in the two SMTZs include diverse source organisms and/or varied turnover rates of individual amino sugars relative to cells at the respective depths.

Sediments at Site 1229 are influenced by high primary production rates, leading to high TOC contents and a dominance of marine OM type (D'Hondt et al. 2003), whereas sediments from Site U1352 are organic-lean and characterized by high influx of terrestrial OM derived from erosion (Fulthorpe et al., 2011). Different sedimentary characteristics are presumed to influence turnover rates of amino sugars and result in different downcore profiles of AS-C% and AS-N%. At Site U1352, amino sugars are distinctly enriched at the sample depth of 1.05 m (Fig. III.2b), presumably resulting from mixed signals of microbial debris accumulated from the water column and elevated microbial cells in the shallow sediments. Proportions of AS-C% and AS-N% subsequently decrease with depth, indicating preferential degradation of TASs relative to other OM constituents on geological timescales. Conspicuous peaks of AS-C% and AS-N% in the SMTZ are partially consistent with the elevated prokaryotic cell numbers (Ciobanu et al., in review), indicative of an increased contribution of active microbial biomass to the TAS pool in this horizon. Some environmental factors, such as adsorption by mineral surfaces, protection and incorporation by refractory macromolecules, can lead to selective preservation of organic compounds in marine sediments (Middelburg et al., 1993). In this regard, we tentatively infer that

accumulation of amino sugars at ~300 mbsf may primarily result from comparatively higher TOC content and/or changes of lithology compared to the adjacent depths (Fulthorpe et al., 2011). Moreover, higher porosity and clay content in this horizon is potentially preferable for microbial inhabitants and consequently may have lead to higher amino sugar concentrations (Albrechtsen and Winding, 1992). Towards the bottom of Site U1352, the TOC and TN contents approach the detection limit (Table III.2). The TASs account for minor proportions of the organic carbon and nitrogen pools, which are consistent with limited nutrients and pore space that potentially constrain microbial activities in ultra-deep sediments.

III.5.2. Sources of amino sugars in deeply buried marine sediments

Amino sugars account for considerable proportions of sedimentary OM, with concentrations of TASs being significantly correlated with TOC and TN (Fig. III.1). Therefore, amino sugars are not only valuable nutrient sources for heterotrophic organisms (Dauwe and Middelburg, 1998), but also feasible indicators for OM origin on the basis of their varying AS compositions in several potential sources (Benner and Kaiser, 2003; Niggemann and Schubert, 2006; Carstens and Schubert, 2012). GlcN and GalN are typically derived from prokaryotes in the forms of peptidoglycan, lipopolysaccharides, pseudopeptidoglycan, etc. (Schleifer and Kandler, 1972; Kandler et al., 1998; Amelung et al., 1999; Madigan and Martinko, 2005); GlcN is additionally present as chitin in most fungal cell walls and invertebrate exoskeletons (Müller et al., 1986; Liang et al., 2007a; Davis et al., 2009). Ratios of GlcN/GalN, for example, have been used as indication of diagenetic alteration of marine OM, with substantially decreased ratios suggesting increased contributions of microbial amino sugars in marine OM (Benner and Kaiser, 2003; Davis et al., 2009; Langerhuus et al., 2012). In addition, ratios of GlcN/GalN can also distinguish the relative contribution of different source organisms, e.g. a value between 1 and 2.5 is presumed to indicate a predominant source of heterotrophic prokaryotes (Benner and Kaiser, 2003; Davis and Benner, 2005; Kawasaki and Benner, 2006). Therefore, the GlcN/GalN ratios of 1.2-2 from Site 1229 (Fig. III.4a) indicate a major prokaryotic source of amino sugars to marine OM, which is in agreement with previous findings in surface sediments from the Peru Margin (Niggemann and Schubert, 2006; Lomstein et al., 2009). At Site U1352, downcore ratios of GlcN/GalN exhibit significant variations. The GlcN/GalN

ratios are relatively lower (usually < 3) in samples above 500 m and deeper than ~1400 m, whereas the ratios rise up to 7 in between, indicating the presence of stronger terrestrial material input or debris of phytoplankton and zooplankton in this interval (Fig. III.4c). Interestingly, these different zones of GlcN/GalN coincide with two geological unconformities, which are located at ~500 mbsf and ~1400 mbsf, respectively. In addition, decreased proportions of GalN partially co-occur with increased GlcN proportions between 500-1400 mbsf (Table III.2). Such variations presumably indicate different biological inputs of these two amino sugars, e.g. increased contributions of chitin-producing invertebrates and/or fungi, which may also be part of the deep biosphere (Coolen et al., 2013; Orsi et al., 2013).

ManN is a common amino sugar that is widely distributed in glycoproteins and glycolipids of prokaryotes, fungi, mammals and plants (Pickering, 1965; Amir et al., 1966; Glaser et al., 2004; Indorf et al., 2011), but has been a minor component or even undetectable in previous investigations from soils, water column samples and near surface sediments (e.g. Zhang and Amelung, 1996; Guggenberger et al., 1999; Benner and Kaiser, 2003; Niggemann and Schubert, 2006; Carstens et al., 2012). GlcN/ManN ratios from bacteria and algae are in the range of 2 to 46 and 8 to 18, respectively (Benner and Kaiser, 2003; Glaser et al., 2004). At Site 1229, the maximum value of GlcN/ManN in the uppermost sample suggests the presence of phytoplankton debris, which is presumably accumulated from water column material due to high primary production rate (Müller and Suess, 1979; Fig. III.4a). The decrease of GlcN/ManN ratios downwards suggests that algal material is preferentially decomposed, while prokaryotes become more predominant (Niggemann and Schubert, 2006).

At Site U1352, concentrations of ManN do not decrease significantly with depth unlike the other hexosamines, which resulted in unusually high proportions of ManN (up to 48.8%) and lowered GlcN/ManN ratios in the ultra-deep sediments (Table III.2; Fig. III.4c). To our knowledge, such elevated ManN proportions have never been documented before. Given the potential sources of ManN and the predominantly terrestrial OM input at Site U1352, tentative explanations for this phenomenon include preferential preservation of ManN over the other hexosamines, accumulation of refractory ManN-bearing polymers from terrestrial habitats, and/or some biochemical processes such as enzyme-mediated isomerization. Additional studies

need to be carried out using ultra-deep sediments from other regions to test these possibilities.

MurA is a diagnostic bacterial biomarker that is exclusively derived from bacterial cell walls and makes up peptidoglycan alternating with GlcN at a ratio of 1:1 (Schleifer and Kandler, 1972; Madigan and Martinko, 2005). Nonetheless, GlcN/MurA ratios of 2-14 have been determined from natural and cultivated bacterial assemblages (Benner and Kaiser, 2003), demonstrating that the majority of GlcN in active bacteria is not associated with peptidoglycan. Moreover, GlcN is relatively resistant to decomposition after cell lysis (Dauwe and Middelburg, 1998), resulting in concentration of extracellular GlcN in soils being two orders of magnitude higher than in intact cells (Glaser et al., 2004). Consequently, GlcN/MurA ratios assessed in environmental samples usually exhibit large scatter, for examples, 4.5-25 in soils (Glaser et al., 2004), 18-146 in marine particulate and dissolved OM (Benner and Kaiser, 2003), and 13-68 in surface marine sediments (Niggemann and Schubert, 2006). In our cases, ratios of GlcN/MurA also cover broad ranges at both sites (Figs. III.4b, d). A previous study on plant tissue revealed that GlcN/MurA ratios were lowest when bacterial activity was maximal and sharply increased when decomposition rates declined (Tremblay and Benner, 2006). If this observation applies to marine sediment, we expect to see a general increase of GlcN/MurA ratios with depth, and excursions towards lower values in the SMTZs, where microbial activity is typically enhanced relative to horizons above and below. However, we did not observe such a pattern. Among the three SMTZs, two possessed greater GlcN/MurA ratios compared to the adjacent horizons, which could signal accumulation of GlcN as a result of prolonged enhanced activity during persistent fixation of an SMTZ at a given sediment horizon (cf. Contreras et al., 2013). We thus conclude that the GlcN/MurA ratio has limited diagnostic power for deep marine sediment. This is attributable to the presence of complex factors controlling the abundance of individual amino sugars in this environment, including the varied biosynthetic preference of GlcN and MurA by different microorganisms at different physiological states, selective preservation or decomposition of GlcN and MurA during early diagenesis, and, most important, varied allochthonous inputs during sedimentation. The distinctly elevated ratios of GlcN/MurA (usually > 150) between 500 and 1300 mbsf at Site U1352 manifests the last point, showing that enhanced

GlcN concentrations, probably as a consequence of increased contributions of chitin-producing organisms during this period, is the main reason that drives the GlcN/MurA ratios to higher values.

III.5.3. Bacterial contributions to the amino sugar pool

Over the past decades, decomposition and preservation of MurA have been discussed controversially compared to the other three hexosamines in the marine realm (Moriarty, 1977; Benner and Kaiser, 2003; Niggemann and Schubert, 2006; Kaiser and Benner, 2008). It is therefore critical to understand to what extent MurA represents extant as opposed to fossil bacteria in marine sediments. We compiled published MurA concentrations from different regions (cf. Niggemann and Schubert, 2006; Lomstein et al., 2009; Carstens and Schubert, 2012; Lomstein et al., 2012; Zhu et al., in press) as well as this data set as a function of depth below the water-sediment interface at double-logarithmic scales (Fig. III.6a). MurA concentrations range from 0.01 to 360 µg/g sediment over a sampling depth interval from 0.005 to 1367 mbsf, while in more deeply buried samples MurA was not detectable. Among those sediments of diverse types, a significant correlation between MurA content and sample depth is observed ($r^2 = 0.75$, n = 176).

On the basis of the 1:1-ratio of GlcN/MurA in peptidoglycan (Schleifer and Kandler, 1972; Madigan and Martinko, 2005), bacterial cell wall-derived amino sugars only account for 0.5-2.2% of the TASs at Site 1229 and 0.5-2% of the TASs at Site U1352. The proportions at both sites are lower than those in surface sediments by a factor of two to three (e.g. Niggemann and Schubert, 2006; Lomstein et al., 2009) and up to one order of magnitude lower than in water column samples (e.g. Benner and Kaiser, 2003; Kaiser and Benner, 2008; Carstens et al., 2012).

To estimate proportions of MurA derived from active microbial biomass ($MurA_{intracellular}$) in marine sediments, a cell-specific conversion factor is employed to convert cell numbers to the $MurA_{intracellular}$ contents in the respective samples. Given that details on the community compositions at these sites are not constrained, we base our calculations on the following set of assumptions: We assume a ratio of 35:65 for gram-positive:gram-negative bacteria as done previously for the Peru Margin (Lomstein et al., 2012) on the basis of the apparent dominance of gram-negative

bacteria in marine bacterial communities (Moriarty and Hayward, 1982; Lomstein et al., 2009); based on this ratio, we use an average conversion factor of 5.45 fg MurA per cell, which is determined from previously reported MurA contents in gram-positive and gram-negative cells (Millar and Casida, 1970; Moriarty, 1975; Moriarty, 1977; Mimura and Romano, 1985) and consistent with values previously estimated by Niggemann and Schubert (2006) and Lomstein et al. (2012); given controversial reports regarding the community composition on the domain level (e.g. Schippers et al., 2005; Lipp et al., 2008), we assume a 50/50 contribution of cells from both domains, consistent with the assumption in a previous model (cf. Xie et al., 2013; Fig. III.6).

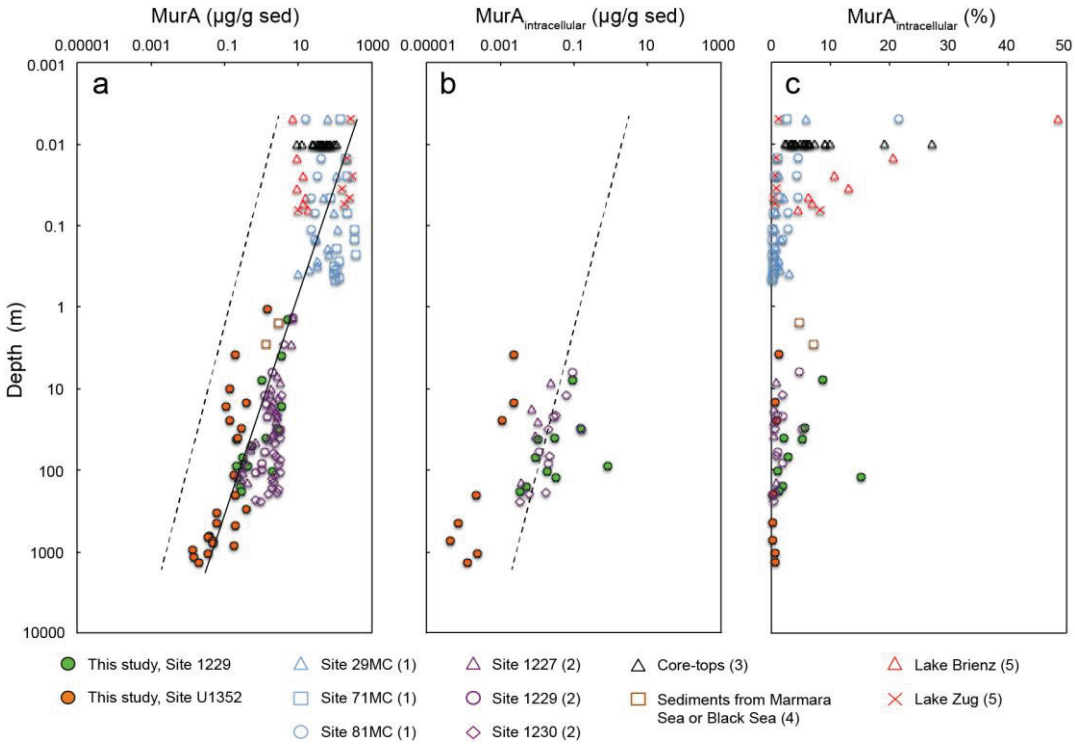


Fig. III.6. Depth profiles of total MurA concentrations (a), intracellular MurA (MurA$_{intracellular}$) concentrations (b), and proportions of MurA$_{intracellular}$ in total MurA (c) in different environmental samples. The solid regression line of the total MurA: log(depth) = -1.3580 log(MurA) + 1.2604 (r^2 = 0.75, n = 176). The dashed line represents the distribution of intracellular MurA, which is derived from the global regression of microbial population (6) and the cell-specific conversion factor (see the text). Concentrations of MurA$_{intracellular}$ in (b) are estimated from cell count data in the respective samples (7, 8) and the cell-specific conversion factor. Note that cell numbers for estimating MurA$_{intracellular}$ (%) in (c) are either calculated from the global regression line (for samples from references 1, 3, 4, 5) or from cell count data (for samples from references 2 and this study; see the text). Since cell counts data are not generated for all the samples, data points of the calculated MurA$_{intracellular}$ are less than the measured total MurA concentrations. References cited in caption and figure: (1) Niggemann and Schubert, 2006; (2) Lomstein et al., 2012; (3) Lomstein et al., 2009; (4) Zhu et al., in press; (5) Carstens and Schubert, 2012; (6) Parkes et al., 2000; (7) D'Hondt et al., 2003; (8) Ciobanu et al., in review.

Concentrations of $MurA_{intracellular}$ derived from conversion of the global regression line of cellular counts (Parkes et al., 2000; Fig. III.6a, dashed line) are around one to three orders of magnitude lower than the determined MurA concentrations at the respective depth intervals (Fig. III.6a, solid line). At Site 1229, concentrations of $MurA_{intracellular}$ estimated from the reported cell numbers (D'Hondt et al., 2003) are generally consistent with the simulated global $MurA_{intracellular}$ distribution (Fig. III.6b). Distributions of $MurA_{intracellular}$ from adjacent depths at Site 1229 and two other neighboring sites (data from Lomstein et al., 2012), i.e. Site 1227 and Site 1230, exhibit a comparable downcore trend. In contrast, the corresponding estimation of $MurA_{intracellular}$ concentrations at Site U1352 are around one to two orders of magnitude lower than the simulated global $MurA_{intracellular}$ distributions, presumably due to extremely low microbial density (Ciobanu et al., in review) resulting from insufficient nutrients and limited pore space in this ultra-deep, organic-lean reservoir. Lacking cellular concentration data, we estimated the concentrations of $MurA_{intracellular}$ in shallow marine and lacustrine sediments (i.e. data from Niggemann and Schubert, 2006; Lomstein et al., 2009; Carstens and Schubert, 2012; Zhu et al., in press) using the global cellular counts (Parkes et al., 2000). All these estimated $MurA_{intracellular}$ concentrations were expressed as percentages of $MurA_{intracellular}$ of the total MurA (Fig. III.6c). Contrary to previous findings in surface sediments according to which the majority of MurA is degraded on timescales of days (King and White, 1977; Moriarty, 1977), the estimated $MurA_{intracellular}$ mostly accounts for < 15% of the total MurA in sediments of various types and from different regions. Although intra-core differences of $MurA_{intracellular}$ are notable, the proportions of $MurA_{intracellular}$ generally decrease with increasing sample depth. This strongly suggests that turnover rate of MurA is low in deeply buried sediments. Bacterial endospores, which cannot be quantified by the acridine orange direct counts (AODC) method, are a potentially important MurA reservoir (Lomstein et al., 2012). It has been reported that the abundances of endospores are on the order of 10^7 per cm^3 sediment in Aarhus Bay (Langerhuus et al., 2012) and the Peru Margin (Lomstein et al., 2012). If similar abundances of endospores are also present in other regions, bacterial endospore-derived MurA would account for substantial proportions of the total MurA in sediments. Given the long dormant period an endospore presumably persists in the subseafloor, endospore-derived MurA has to be considered as a relative refractory component and is consequently not included the estimation of $MurA_{intracellular}$. In this

context, $MurA_{intracellular}$ estimated from different regions indicates that the majority of MurA is preserved in bacterial endospore or bacterial debris and/or is incorporated into sedimentary matrix, and consequently resistant to decomposition.

III.5.4. Isotopic compositions of individual amino sugars and benthic microbial community

Amino sugars are relatively resistant to degradation and consequently persist after cell death (Glaser et al., 2004; Liang et al., 2008). The stable carbon isotopic compositions of individual amino sugars determined in the present study therefore is likely to represent mixture of signals from diverse fossil sources as well as extant cells. At both study sites, the $\delta^{13}C$ values of GlcN and GalN ranged from -20‰ to -10‰ (Figs. III.5b, e). Both compounds are generally slightly enriched in ^{13}C relative to TOC (Figs. III.5a, d), as commonly observed for carbohydrates in sediments and soils (Boschker et al., 2008; Indorf et al., 2012). In the majority of samples from both sites, GlcN is somewhat depleted in ^{13}C relative to GalN while a tendency to parallel downcore variations of δ values of both compounds is noticeable; some exceptions from this parallel trend are especially aparent at Site U1352. The parallel nature is best explained by related sources and/or similar modes of biogeochemical cycling of these compounds. Notably, at Site 1229 larger $\delta^{13}C$ offsets between GlcN and GalN (> 5‰) are only observed near the deeper SMTZ, consistent with the horizon where the contrasting distributions of TASs and ratios of GlcN/MurA were observed, indicating an increasing fraction of GlcN from autochthonous microbes that utilize ^{13}C-depleted carbon for biosynthesis. At Site U1352, distinct differences between $\delta^{13}C$-GlcN and $\delta^{13}C$-GalN in the SMTZ indicate that GlcN and GalN either originate from diverse microbial producers or result from different isotopic fractionation effects during biosynthesis compared to adjacent horizons. In addition, significant discrepancies of $\delta^{13}C$-GlcN and $\delta^{13}C$-GalN also occur in a few samples between 500-1400 mbsf, presumably due to different diagenetic processes and/or different sources of GlcN and GalN.

Although $MurA_{intracellular}$ constitutes only a minor proportion of the total MurA in our samples, the more labile nature of MurA compared to GlcN and GalN (Ogawa et al., 2001; Tremblay and Benner, 2006) as well as its exclusive bacterial origin

(Schleifer and Kandler, 1972; Madigan and Martinko, 2005) suggest that its isotopic values will more likely record signatures from endogenous bacteria. MurA is generally depleted in ^{13}C relative to the other amino sugars (Fig. III.5). This depletion is amplified in the three SMTZs, which is consistent with an increased contribution of ^{13}C-depleted bacterial biomass in these horizons. Since certain biosynthetic processes (e.g. sugar polymerization) within different microorganisms may lead to slightly different $\delta^{13}C$ signatures (Macko et al., 1990), interpretation of $\delta^{13}C$-MurA variations in marine sediments is complex. As $\delta^{13}C$-TOC values remained fairly constant at both sites, the covariation between $\delta^{13}C$-DIC and $\delta^{13}C$-MurA suggest that a fraction of subseafloor bacteria is autotrophic. Given the strong influence of the SMTZs on the covariation between MurA and DIC, this relationship could be due to CO_2 assimilation of bacterial members of anaerobic methane-oxidizing consortia, in analogy to the archaeal members (Kellermann et al., 2012), direct assimilation by methane-derived C by unknown bacteria or heterotrophic consumption of fresh microbial biomass formed during anaerobic oxidation of methane.

In contrast to GlcN and GalN, ManN exhibits higher variability in $\delta^{13}C$ compositions as a function of depth (Figs. III.5c, f). Significant ^{13}C-depletion in the upper SMTZ at Site 1229 and SMTZ at Site U1352 clearly indicates a proportion of ManN producers are potentially involved in assimilation of ^{13}C-depleted carbon formed during anaerobic oxidation of methane. Compared to MurA, interpretation of $\delta^{13}C$-ManN data is more complicated. Previous analysis of $\delta^{13}C$-ManN did not reveal a clear ^{13}C-depletion in a cold seep sample (Zhu et al., in press). However, a methanogenic source of ManN (Veiga et al., 1997), or by inference an origin from closely related prokaryotic methane-oxidizers, cannot be ruled out, as it has been demonstrated as an essential component during bacterial and archaeal biosynthesis of UDP-*N*-acetylmannosaminuronate, which is an important constituent of bacterial lipopolysaccharides and archaeal S-layer protein, as well as for the biosynthesis of methanogenic coenzyme B (Namboori and Graham, 2008; Yurist-Doutsch et al., 2008). We point out that MurA and ManN appear to be more sensitive biomarkers for microbes involved in methane cycling in deep subseafloor sediments than archaeal lipids that did not show appreciable ^{13}C-depletion in the SMTZs at Site 1229 (Biddle et al., 2006).

III.6. ACKNOWLEDGEMENTS

Samples for this research were retrieved from ODP Leg 201 and IODP Expedition 317. ODP Leg 201 is sponsored by the U.S. National Science Foundation (NSF) and participating countries under management of Joint Oceanographic Institutions (JOI), Inc., while IODP Expedition 317 is sponsored by the U.S. National Science Foundation (NSF), Japan's Ministry of Education, Culture, Sports, Science and Technology, and other IODP members. We thank the participating crews and scientists for sample recovery; M. Elvert for precious advices; X. Prieto Mollar, J. Arndt, J. Wendt and R. Himmelsbach for technical assistance. This study was supported by the DFG-Research Center/Excellence Cluster 'The Ocean in the Earth System' (MARUM), grant H1 616/11-1(Cell Surf) to K.-U.H. R.Z. is sponsored by the China Scholarship Council (CSC) and the Gottfried-Wilhelm Leibniz Program of the DFG (through the Leibniz Price to K.-U.H.).

III.7. REFERENCES

Albrechtsen H.-J. and Winding, A. (1992) Microbial biomass and activity in subsurface sediments from Vejen, Denmark. *Microb. Ecol.* **23**, 303-317.

Amelung W., Zhang X., Flach K.W. and Zech W. (1999) Amino sugars in native grassland soils along a climosequence in North America. *Soil Sci. Soc. Am. J.* **63**, 86-92.

Amir S., Barker S., Butt W., Crooke A. and Davies, A. (1966) Administration of *N*-acetyl-D-mannosamine to mammals. *Nature* **211**, 976-977.

Bai Z., Bodé S., Huygens D., Zhang X. and Boeckx P. (2012) Kinetics of amino sugar formation from organic residues of different quality. *Soil Biol. Biochem.* **57**, 814-821.

Benner R. and Kaiser K. (2003) Abundance of amino sugars and peptidoglycan in marine particulate and dissolved organic matter. *Limnol. Oceanogr.* **48**, 118-128.

Biddle J.F., Lipp J.S., Lever M.A., Lloyd K.G., Sørensen K.B., Anderson R., Fredricks H.F., Elvert M., Kelly T.J., Schrag D.P., Sogin M.L., Brenchley J.E., Teske A., House C.H. and Hinrichs K.-U. (2006) Heterotrophic Archaea dominate sedimentary subsurface ecosystems off Peru. *Proc. Natl. Acad. Sci. USA* **103**, 3846-3851.

Boschker H.T.S., Moerdijk-Poortvliet T.C.W., van Breugel P., Houtekamer M., Middelburg J.J. (2008) A versatile method for stable carbon isotope analysis of carbohydrates by hihg-performance liquid chromatography/isotope ratio mass spectrometry. *Rapid Commun. Mass Spectrom.* **22**, 3902-3908.

Carstens D., Köllner K.E., Bürgmann H., Wehrli B. and Schubert, C.J. (2012) Contribution of bacterial cells to lacustrine organic matter based on amino sugars and D-amino acids. *Geochim. Cosmochim. Acta* **89**, 159-172.

Carstens D. and Schubert C.J. (2012) Amino acid and amino sugar transformation during sedimentation in lacustrine systems. *Org. Geochem.* **50**, 26-35.

Ciobanu M., Burgaud G., Dufresne A., Breuker A., Rédou V., Maamar S.B., Gaboyer F., Vandenabeele-Trambouze O., Lipp J.S., Schippers A., Vandenkoornhuyse P., Barbier G., Jebbar M., Godfroy A. Microorganisms persist at record depths in the subseafloor of the Canterbury Basin. *ISME J.*, in review.

Coolen M.J.L., Orsi W.D., Balkema C., Quince C., Harris K., Sylva S.P., Filipova-Marinova M. and Giosan L. (2008) Evolution of the plankton paleome in the Black Sea from the deglacial to anthropocene. *Proc. Natl. Acad. Sci. USA* **110**, 8609-8614.

Dauwe B. and Middelburg J. (1998) Amino acids and hexosamines as indicators of organic matter degradation state in North Sea sediments. *Limnol. Oceanogr.* **43**, 782-798.

Davis J. and Benner R. (2005) Seasonal trends in the abundance, composition and bioavailability of particulate and dissolved organic matter in the Chukchi/Beaufort Seas and western Canada Basin. *Deep-Sea Res. II* **53**, 3396-3410.

Davis J., Kaiser K. and Benner R. (2009) Amino acid and amino sugar yields and compositions as indicators of dissolved organic matter diagenesis. *Org. Geochem.* **40**, 343-352.

Decock C., Denef K., Bodé, S., Six, J. and Boeckx P. (2009) Critical assessment of the applicability of gas chromatography-combustion-isotope ratio mass spectrometry to determine amino sugar dynamics in soil. *Rapid Commun. Mass Spectrom.* **23**, 1201-1211.

D'Hondt S.L., Jørgensen B.B., Miller D.J. and Shipboard Scientific Party (2003) *Proc. ODP Init. Repts.* **201**. Ocean Drilling Program, Texas A & M University, College Station, TX. doi:10.2973/odp.proc.ir.201.2003.

Elvert M, Suess E., Greinert J. and Whiticar M.J. (2000) Archaea mediating anaerobic methane oxidation in deep-sea sediments at cold seeps of the eastern Aleutian subduction zone. *Org. Geochem.* **31**, 1175-1187.

Ertefai T.F., Heuer V.B., Mollar X.P., Vogt C., Sylva S.P., Seewald J. and Hinrichs K.-U. (2010) The biogeochemistry of sorbed methane in marine sediments. *Geochim. Cosmochim. Acta* **74**, 6033-6048.

Fulthorpe C.S., Hoyanagi K., Blum P. and the Expedition 317 Scientists (2011) *Proc. IODP* 317: Tokyo (Integrated Ocean Drilling Program Management International, Inc.). doi:10.2204/iodp.proc.317.2011.

Glaser B. and Gross S. (2005) Compound-specific $\delta^{13}C$ analysis of individual amino sugars – a tool to quantify timing and amount of soil microbial residue stabilization. *Rapid Commun. Mass Spectrom.* **19**, 1409-1416.

Glaser B., Turrión M. and Alef K. (2004) Amino sugars and muramic acid--biomarkers for soil microbial community structure analysis. *Soil Biol. Biochem.* **36**, 399-407.

Guerrant G. and Moss C. (1984) Determination of monosaccharides as aldononitrile, O-methyloxime, alditol, and cyclitol acetate derivatives by gas chromatography. *Anal. Chem.* **56**, 633-638.

Guggenberger G., Frey S., Six J., Paustian K. and Elliott E. (1999) Bacterial and fungal cell-wall residues in conventional and no-tillage agroecosystems. *Soil Sci. Soc. Am. J.* **63**, 1188-1198.

Gupta L., Subramanian V. and Ittekkot V. (1997) Biogeochemistry of particulate organic matter transported by the Godavari River, India. *Biogeochem.* **38**, 103-128.

Gupta L.P. and Kawahata H. (2000) Amino acid and hexosamine composition and flux of sinking particulate matter in the equatorial Pacific at 175°E longitude. *Deep-Sea Res. I* **47**, 1937-1960.

He H., Zhang W., Zhang X., Xie H. and Zhuang, J. (2011) Temporal responses of soil microorganisms to substrate addition as indicated by amino sugar differentiation. *Soil Biol. Biochem.* **43**, 1155-1161.

Hinrichs K.-U., Hayes J.M., Sylva S.P., Brewer P.G. and DeLong E.F. (1999) Methane-consuming archaeabacteria in marine sediments. *Nature* **398**, 802-805.

Hinrichs K.-U., Hayes J.M., Bach W., Spivack A.J., Hmelo L.R., Holm N.G., Johnson C.G. and Sylva S.P. (2006) Biological formation of ethane and propane in the deep marine sufsurface. *Proc. Natl. Acad. Sci. USA* **103**, 14684-14689.

Indorf C., Dyckmans J., Khan K.S. and Joergensen, R.G. (2011) Optimisation of amino sugar quantification by HPLC in soil and plant hydrolysates. *Biol. Fertil. Soils* **47**, 387-396.

Indorf C., Stamm F., Dyckmans J., Joergensen R.G. (2012) Determination of saprotrophic fungi turnover in different substrates by glucosamine-specific $\delta^{13}C$ liquid chromatography/isotope ratio mass spectrometry. *Fungal Ecol.* **5**, 694-701.

Ittekkot V., Degens E.T. and Honjo, S. (1984a) Seasonality in the fluxes of sugars, amino acids, and amino sugars to the deep ocean: Panama Basin. *Deep-Sea Res.* **31**, 1071-1083.

Ittekkot V., Deuser W.G. and Degens E.T. (1984b) Seasonality in the fluxes of sugars, amino acids, and amino sugars to the deep ocean: Sargasso Sea. *Deep-Sea Res.* **31**, 1057-1069.

Jennerjahn T. and Ittekkot V. (1999) Changes in organic matter from surface waters to continental slope sediments off the Sao Francisco River, eastern Brazil. *Mar. Geol.* **161**, 129-140.

Kaiser K. and Benner R. (2008) Major bacterial contribution to the ocean reservoir of detrital organic carbon and nitrogen. *Limn. Oceanogr.* **53**, 99-112.

Kaiser K. and Benner R. (2009) Biochemical composition and size distribution of organic matter at the Pacific and Atlantic time-series stations. *Mar. Chem.* **113**, 63-77.

Kandeler E., Tscherko D., Bruce K., Stemmer M., Hobbs P.J., Bardgett R.D. and Amelung W. (2000) Structure and function of the soil microbial community in microhabitats of a heavy metal polluted soil. *Biol. Fertil. Soils* **32**, 390-400.

Kandler O. and König H. (1998) Cell wall polymers in Archaea (Archaebacteria). *Cell Mol. Life Sci.* **54**, 305-308.

Kawasaki N. and Benner R. (2006) Bacterial release of dissolved organic matter during cell growth and decline: Molecular origin and composition. *Limnol. Oceanogr.* **51**, 2170-2180.

Kellermann M.Y., Wegener G., Elvert M., Yoshinage M.Y., Lin Y.-S., Holler T., Mollar X.P., Knittel K. and Hinrichs K.-U. (2012) Autotrophy as a predominant mode of carbon fixation in anaerobic methane-oxidizing microbial communities. *Proc. Natl. Acad. Sci. USA* **109**, 19321-19326.

King J.D. and White D.C. (1977) Muramic acid as a measure of microbial biomass in estuarine and marine samples. *Appl. Environ. Microbiol.* **33**, 777-783.

Langerhuus A.T., Røy H., Lever M.A., Morono Y., Inagaki F., Jørgensen B.B. and Lomstein B.A. (2012) Endospore Abundance and d:l-Amino Acid Modeling of Bacterial Turnover in Holocene Marine Sediment (Aarhus Bay). *Geochim. Cosmochim. Acta* **99**, 87-99.

Liang C., Zhang X. and Balser T.C. (2007a) Net microbial amino sugar accumulation process in soil as influenced by different plant material inputs. *Biol. Fertil. Soils* **44**, 1-7.

Liang C., Zhang X., Rubert K.F. and Balser T.C. (2007b) Effect of plant materials on microbial transformation of amino sugars in three soil microcosms. *Biol. Fertil. Soils* **43**, 631-639.

Liang C., Fujinuma R. and Balser T.C. (2008) Comparing PLFA and amino sugars for microbial analysis in an Upper Michigan old growth forest. *Soil Biol. Biochem.* **40**, 2063-2065.

Lin Y.S., Lipp J.S., Yoshinaga M.Y., Lin S.H., Elvert M. and Hinrichs K.-U. (2010) Intramolecular stable carbon isotopic analysis of archaeal glycosyl tetraether lipids. *Rapid Commun. Mass Spectrom.* **24**, 2817-2826.

Lipp J.S., Morono Y., Inagaki F. and Hinrichs K.-U. (2008) Significant contribution of Archaea to extant biomass in marine subsurface sediments. *Nature* **454**, 991-994.

Lomstein B., Niggemann J., Jørgensen B. and Langerhuus A. (2009) Accumulation of prokaryotic remains during organic matter diagenesis in surface sediments off Peru. *Limn. Oceanogr.* **54**, 1139-1151.

Lomstein B.A., Langerhuus A.T., D'Hondt S., Jørgensen B.B. and Spivack A.J. (2012) Endospore abundance, microbial growth and necromass turnover in deep sub-seafloor sediment. *Nature* **484**, 101-104.

Macko S.A., Helleur R., Hartley G. and Jackman P. (1990) Diagenesis of organic matter - A study using stable isotopes of individual carbohydrates. *Org. Geochem.* **16**, 1129-1137.

Madigan M.T. and Martinko J.M. (2005) *Brock Biology of Microorganisms* 11th edn. Prentice Hall, Upper Saddle River, USA.

Meister P., Mckenzie J.A., Vasconcelos C., Bernasconi S., Frank M., Gutjahr M. and Schrag D.P. (2007) Dolomite formation in the dynamic deep biosphere: results from the Peru Margin. *Sedimentology* **54**, 1007-1031.

Middelburg J.J., Vlug T., and van der Nat F. Jaco W.A. (1993) Organic matter mineralization in marine systems. *Glob. Plan. Ch.* **8**, 47-58.

Millar W.N. and Casida Jr L.E. (1970) Evidence for muramic acid in soil. *Can. J. Microbiol.* **16**, 299-304.

Mimura T. and Romano J.C. (1985) Muramic acid measurements for bacterial investigations in marine environments by high-pressure liquid chromatography. *Appl. Environ. Microbiol.* **50**, 229-237.

Moriarty D.J.W. (1975) A method for estimating the biomass of bacteria in aquatic sediments and its application to trophic studies. *Oecologia* **20**, 219-229.

Moriarty D. J.W. (1977) Improved method using muramic acid to estimate biomass of bacteria in sediments. *Oecologia* **26**, 317-323.

Moriarty D.J.W. and Hayward A.C. (1982) Ultrastructure of bacteria and the proportion of Gram-negative bacteria in marine sediments. *Microb. Ecol.* **8**, 1-14.

Müller P.J. and Suess E. (1979) Productivity, sedimentation rate, and sedimentary organic matter in the oceans-I. Organic carbon preservation. *Deep-Sea Res.* **26A**, 1347-1362.

Müller P.J., Suess E. and André Ungere C. (1986) Amino acids and amino sugars of surface particulate and sediment trap material from waters of the Scotia Sea. *Deep-Sea Res.* **33**, 819-838.

Nagata T., Meon B. and Kirchman D. (2003) Microbial degradation of peptidoglycan in seawater. *Limn. Oceanogr.* **48**, 745-754.

Namboori S.C. and Graham D.E. (2008) Acetamido sugar biosynthesis in the euryarchaea. *J. Bacteriol.* **190**, 2987-2996.

Niggemann J. and Schubert C. J. (2006) Sources and fate of amino sugars in coastal Peruvian sediments. *Geochim. Cosmochim. Acta* **70**, 2229–2237.

Ogawa H., Amagai Y., Koike I., Kaiser K. and Benner R. (2001) Production of refractory dissolved organic matter by bacteria. *Science* **292**, 917-920.

Orphan V.J., House C.H., Hinrichs K.-U., McKeegan K.D. and DeLong E.F. (2001) Methane-consuming archaea revealed by directly coupled isotopic and phylogenetic analysis. *Science* **293**, 484-487.

Orsi W., Biddle J.F. and Edgcomb V. (2013) Deep sequencing of subseafloor eukaryotic rRNA reveals active fungi across marine subsurface provinces. *PloS one* **8**, e56335.

Pancost R.D., Damsté J.S.S., de Lint S., van der Maarel M.J.E.C., Gottschal J.C. and the Medinaut Shipboard Scientific Party (2000) Biomarker evidence for widespread anaerobic methane oxidation in Mediterrannean sediments by a consortium of methanogenic archaea and bacteria. *Appl. Environ. Microbiol.* **66**, 1126-1132.

Parkes R.J., Cragg B.A. and Wellsbury P. (2000) Recent studies on bacterial populations and processes in subseafloor sediments: a review. *Hydrogeol. J.* **8**, 11-28.

Pickering B. (1965) Mannosamine in the cell wall of *Clostridium welchii* (type A). *Nature* **206**, 400-401.

Roberts P., Bol R. and Jones D.L. (2007) Free amino sugar reactions in soil in relation to soil carbon and nitrogen cycling. *Soil Biol. Biochem.* **39**, 3081-3092.

Schippers A., Neretin L.N., Kallmeyer J., Ferdelman T.G., Cragg B.A., Parkes R.J. and Jørgensen B.B. (2005) Prokaryotic cells of the deep sub-seafloor biosphere identified as living bacteria. *Nature* **433**, 861-864.

Schleifer K.H. and Kandler O. (1972) Peptidoglycan types of bacterial cell walls and their taxomonic implications. *Bacteriol. Rev.* **36**, 407-477.

Schubotz F., Lipp J.S., Elvert M. and Hinrichs K.-U. (2011) Stable carbon isotopic compositions of intact polar lipids reveal complex carbon flow patterns among hydrocarbon degrading microbial communities at the Chapopote asphalt volcano. *Geochim. Cosmochim. Acta* **75**, 4399-4415.

Simpson R.T., Frey S.D., Six J. and Thiet R.K. (2004) Preferential accumulation of microbial carbon in aggregate structures of no-tillage soils. *Soil Sci. Soc. Am. J.* **68**, 1249-1255.

Torres M.E., Mix A.C. and Rugh W.D. (2005) Precise $\delta^{13}C$ analysis of dissolved inorganic carbon in natural waters using automated headspace sampling and continuous flow mass spectrometry. *Limnol. Oceanogr. Methods* **3**, 349-360.

Tremblay L. and Benner R. (2006) Microbial contributions to N-immobilization and organic matter preservation in decaying plant detritus. *Geochim. Cosmochim. Acta* **70**, 133-146.

Veiga M.C., Jain M.K., Wu W.-M., Hollingsworth R.I. and Zeikus J.G. (1997) Composition and role of extracellular polymers in methanogenic granules. *Appl. Environ. Microbiol.* **63**, 403-407.

Xie S., Lipp J.S., Wegener G., Ferdelman T.G. and Hinrichs K.-U. (2013) Turnover of microbial lipids in the deep biosphere and growth of benthic archaeal populations. *Proc. Natl. Acad. Sci. USA* **110**, 6010-6014.

Yurist-Doutsch S., Chaban B., BanDyke D.J., Jarrell K.F. and Eichler J. (2008) Sweet to the extreme: protein glycosylation in Archaea. *Mol. Microbiol.* **68**, 1079-1084.

Zhang X. and Amelung W. (1996) Gas chromatographic determination of muramic acid, glucosamine, mannosamine, and galactosamine in soils. *Soil Biol. Biochem.* **28**, 1201-1206.

Zhang X., Amelung W., Yuan Y., Samson-Liebig S., Brown L. and Zech W. (1999) Land-use effects on amino sugars in particle size fractions of an Argiudoll. *Appl. Soil Ecol.* **11**, 271-275.

Zhu R., Lin Y.-S., Lipp J.S., Meador T. and Hinrichs K.-U. Optimizing sample pretreatment for compound-specific stable carbon isotopic analysis of amino sugars in marine sediment. *Biogeosciences Discussion.* In press. doi: 10.5194/bgd-11-1-2014.

Chapter IV

Improved sensitivity of sedimentary phospholipid analysis resulting from a novel extract cleanup strategy

Rong Zhu[a, *], Thomas William Evans[a], Lars Wörmer[a], Yu-Shih Lin[a, b], Chun Zhu[a], Kai-Uwe Hinrichs[a]

Published in *Organic Geochemistry*

Vol. 65, page 46-52, doi: 10.1016/j.orggeochem.2013.10.002

[a] Organic Geochemistry Group, MARUM Center for Marine Environmental Sciences & Department of Geosciences, University of Bremen, Leobenerstr., 28359 Bremen, Germany

[b] Department of Oceanography, National Sun Yat-Sen University, No. 70 Lienhai Rd, Kaohsiung 804, Taiwan

[*] Corresponding author. *Email address*: rongzhu@uni-bremen.de (Rong Zhu).

IV.1. ABSTRACT

The detection of intact phospholipids (PLs) in sedimentary extracts using liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) is commonly limited by ion suppression caused by complex matrices; the effect typically cannot be satisfactorily reduced using traditional column chromatographic cleanup procedures. We therefore implemented a cleanup strategy recently introduced for serum and plasma samples and adapted it for sedimentary lipid extracts. It involves selective retention of PLs on a zirconia-coated silica phase, available as a HybridSPE®-Phospholipid cartridge. Compared with conventional silica gel liquid chromatography, the HybridSPE-based protocol strongly enhances the detection of PLs using LC-ESI-MS as a result of more effective removal of complex environmental matrices, with negligible PL loss. The strength of the protocol was demonstrated by analysis of marine sediment samples from various depths and of different total organic carbon (TOC) content, for which we observed 1.2-2.7 times more intense signals from sedimentary PLs compared with the untreated reference. This new cleanup protocol significantly improves PL fingerprinting of microbial communities in sediments.

IV.2. INTRODUCTION

Phospholipids (PLs) consist of phosphate-based head group(s), glycerol backbone(s) and hydrophobic chain(s) that are linked to the glyceryl moiety via ester or ether bonds. They are principal constituents of microbial cytoplasmic membranes. As they bear chemotaxonomic information (Fang et al., 2000; Zink et al., 2003; Sturt et al., 2004; Koga and Morii, 2005) and decompose rapidly upon cell lysis (White et al., 1979; Harvey et al., 1986; Logemann et al., 2011), PLs have been widely employed as a culture- and gene-independent approach for decoding microbial community composition and total biomass (e.g. Bobbie and White, 1980; Vestal and White, 1989; Zelles et al., 1992; Sundh et al., 1997; Lipp et al., 2008; Peterse et al., 2011).

PLs are conventionally analyzed using gas chromatography-mass spectrometry (GC-MS) through characterizing their ester-linked fatty acid (FA) derivatives after hydrolytic cleavage of the polar head groups and ester linkages between acyl groups

and the glycerol backbones (e.g. White et al., 1979; Delong and Yayanos, 1986; Guckert et al., 1986; Frostegård et al., 1993; Elvert et al., 2003; Mills et al., 2006). However, this GC-based approach requires a PL faction purified from free and matrix-bound FAs, a precondition that needs critical evaluation case by case. With liquid chromatography-electrospray ionization-MS (LC-ESI-MS), intact PLs are directly determined without breakdown of the compounds (e.g. Fang and Barcelona, 1998; Rütters et al., 2002; Sturt et al., 2004), thereby providing more comprehensive information for deciphering signals from microbial communities in environmental samples. Unfortunately, LC-ESI-MS-based PL analysis suffers significant interference from the co-eluting sample matrix, which reduces PL ionization efficiency and consequently detection (Wörmer et al., 2013). Indeed, few studies have successfully identified intact PLs in deeply buried marine subseafloor sediments (e.g. Zink et al., 2003; Fredricks and Hinrichs, 2007), where the reduced biomass density creates low signal/high matrix conditions unfavorable for PL detection.

Silica gel columns have been widely used for fractionating samples and reducing sample matrix for the analysis of intact polar lipids (IPLs) in environmental samples (e.g. Rütters et al., 2002; Zink and Mangelsdorf, 2004; Pitcher et al., 2009; Schubotz et al., 2009; Liu et al., 2011; Rossel et al., 2011). However, a substantial loss of PLs (up to 80%) has been observed after elution from a silica gel column, presumably because phosphatidic head groups are selectively adsorbed to the silica (Lengger et al., 2012). Thus, it is critical to develop a reliable PL cleanup protocol for effective removal of the sedimentary matrix with minimum PL loss.

Studies using HybridSPE®-Phospholipid cartridges have recently demonstrated successful isolation of PLs from biological samples, such as serum and plasma (Pucci et al., 2009; Jiang et al., 2011; Moriarty et al., 2012). The solid phase's silica gel in the cartridges is coated with zirconia and exhibits high affinity for PLs due to the Lewis acid-base interaction between zirconia and phosphate moieties, while the retention of a wide range of basic, neutral and acidic compounds is minimal. Subsequently, the retained PLs can be recovered with a strong basic solution. We have adapted this approach for applications with environmental samples and compared results with those obtained with conventional silica gel chromatography. The results demonstrate better recovery and greatly improved detectability of PLs in various sediments using the approach.

IV.3. MATERIAL AND METHODS

IV.3.1. Material

In order to examine any possible cleanup discrimination among different PLs, we investigated the performance of the HybridSPE®-Phospholipid cartridge for diverse PLs with (i) different polar head groups (e.g. phosphocholine, PC; phosphoethanolamine, PE; phosphatidylglycerol, PG; phosphoserine, PS), (ii) varying side chain length (e.g. C_{16}, C_{18}, C_{21}) and (iii) varying core structure type (e.g. diacylglycerol, DAG; dietherglycerol, DEG; archaeol, AR). PL standards were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA), Matreya LLC (Pleasant Gap, PA, USA) and Sigma-Aldrich (Munich, Germany). Isopropanol (IPA), dichloromethane (DCM), HCO_2H (98%), NH_4OH (> 25%), and NH_4CO_2H were from Sigma-Aldrich. The HybridSPE®-Phospholipid cartridges (500 mg; 6 ml) and the CHROMABOND® SiOH glass columns (500 mg; 6 ml) were from Supelco Chemical Co. (Munich, Germany) and Macherey-Nagel GmbH (Düren, Germany), respectively. The vacuum manifold was from Carl Roth GmbH (Karlsruhe, Germany). Sediment samples from different burial depth, ranging from surface (0-1 cm) to subsurface (113.60 m below seafloor, mbsf), and of different total organic carbon (TOC) content (0.3 to 5.5%) were used for method development (listed in Table IV.1).

Table IV.1. Sample details.

No.	Region	Station	Depth (mbsf[a])	TOC (%)	Sample weight[b] (g)
1	Wadden Sea	53° 38' 0N 8° 29' 30E[c]	Surface	0.3	44.9
2	Peru Margin	ODP Leg 201 Site 1227[d]	8.05; 12.43; 17.15; 92.72; 113.60[e]	5.5[f]	27.4
3	Eastern Mediterranean Sea	GeoB 15103-2	3.73; 4.43; 5.59; 5.97[e]	1.5[f]	27.6
4	White Oak River Basin	WORB 2010-10	0.24	4.0	35.9
5	Black Sea	GeoB 15105-2	3.98	4.4	38.9

[a] m below seafloor; [b] wet wt.; [c] Xie et al., 2013; [d] D'Hondt et al., 2003; [e] mixed with equal amount of sediment from each depth; [f] value for mixture of samples from different depths.

IV.3.2 Standard mixture

A PL standard mixture with equal amounts of di-C_{16}-PE, PE-AR, di-C_{16}-PC, di-C_{18}-PC, di-C_{21}-PC, di-O-C_{16}-PC, PC-AR, di-C_{16}-PS and di-C_{16}-PG was prepared for method development. To evaluate recovery after the cleanup protocols, 300 ng of each PL was split into three aliquots. One was directly analyzed as the reference using LC-ESI-MS, whereas the other two were processed with the HybridSPE cartridge or silica gel column before analysis. The amount injected for LC-ESI-MS was 10 ng of each PL for the reference or a volume fraction equivalent to 10 ng of originally added PL in the treated standard mixture. To obtain reliable results, each treatment was carried out in triplicate. The recovery of PLs was determined by normalizing the peak area of the treated samples against the reference (as % peak area of untreated sample).

IV.3.3. Sediment samples

Sediment samples (27.4-44.9 g wet wt.; Table IV.1) were extracted using a modified Bligh and Dyer method described by Sturt et al. (2004). Briefly, each sample was dispersed in a mixture of DCM/MeOH/phosphate buffer (pH 7.4; 1:2:0.8, v/v/v) and ultrasonically extracted twice for 10 min, followed by duplicate extraction with DCM/MeOH/CCl_3CO_2H (pH 2; 1:2:0.8, v/v/v). The mixture was centrifuged after each extraction step and the supernatants were combined in a separation funnel for phase separation. Combined with the DCM used for extraction of the aqueous phase, the organic phase was further washed with MilliQ water 3x and evaporated to dryness as the total lipid extract (TLE). The TLE was treated as follows with each treatment being carried out in triplicate.

(i) To compare PL recovery over the two cleanup protocols in the presence of sedimentary matrix, the TLE of Sample #1 (Table IV.1) was spiked with the PL standard mixture containing 300 ng of each PL and subjected to the same procedure outlined in Fig. IV.1a. The recovery of PLs was determined by normalizing the peak area of the treated samples against the reference.

(ii) TLEs of Samples #2 and #3 were selected as sedimentary background to examine the efficiency of the HybridSPE protocol for recovering different concentrations of PLs and/or in different types of matrices. The TLEs were split into three aliquots. Each was spiked with a different amount of PL standard mixture (0.4, 4 and 40 μg), respectively, and split into two sub-aliquots for

direct LC-ESI-MS measurements or analysis after the HybridSPE cartridge treatment (Fig. IV.1b). The mixture of PL standards was prepared with equal amounts of di-C_{16}-PE, PE-AR, di-C_{18}-PC, di-C_{21}-PC, di-O-C_{16}-PC and PC-AR. Since the experiment also aimed to compare quantification of PLs via both GC (as PLFAs) and LC analysis, di-C_{16}-PC, di-C_{16}-PS and di-C_{16}-PS, which contain the same FAs in the core structure as di-C_{16}-PE, were excluded from this standard mixture. Due to analytical difficulty related to low hydrolysis yield (i.e. independent of the procedure introduced here), PLFA data are not reported here. A proportion (1%) of each sub-aliquot (equivalent to 0.2, 2 and 20 ng of each PL, respectively) was used for LC-ESI-MS.

(iii) TLEs of Samples #4 and #5 were selected as study cases to demonstrate the capability of the HybridSPE protocol for detection of indigenous sedimentary PLs. Each TLE was split into two aliquots for direct LC-ESI-MS measurements as reference and cleanup with the HybridSPE cartridge prior to analysis.

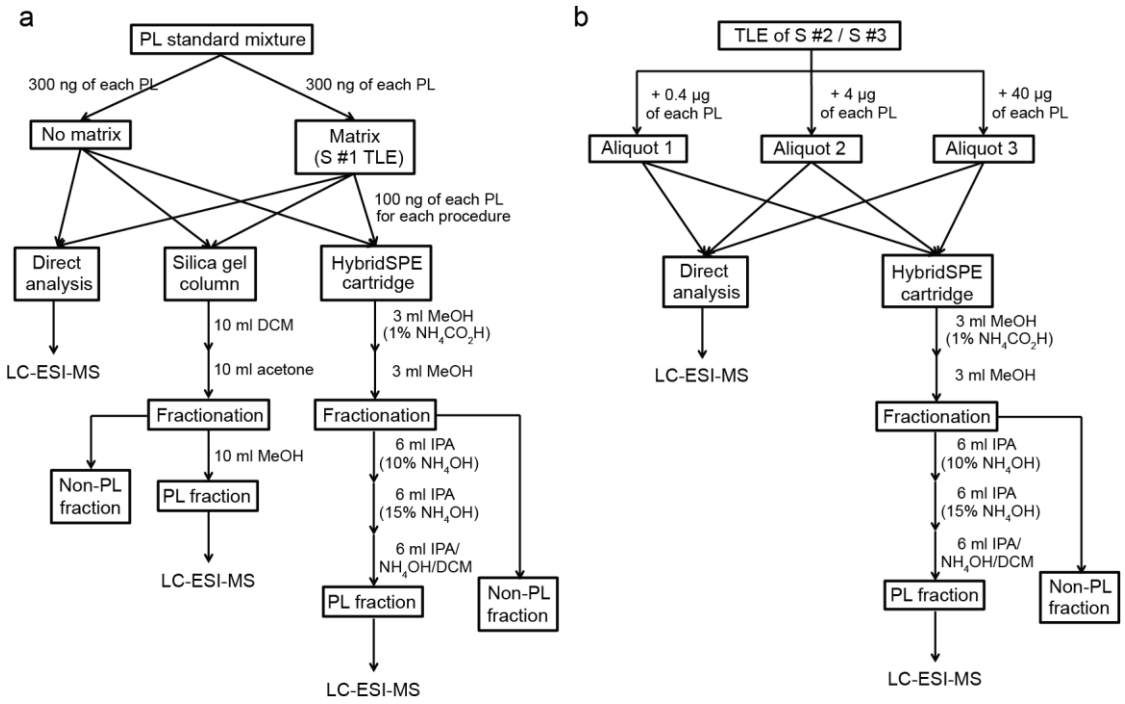


Fig. IV.1. Schematic overview of procedures for (a) comparison of HybridSPE®-Phospholipid cartridge and silica gel column for recovery of PLs with/without sedimentary matrix and (b) evaluation of HybridSPE protocol for recovering PLs at different concentrations and/or in different types of matrices. Each treatment was carried out in triplicate with each of the resulting solutions analyzed in duplicate using LC-ESI-MS.

For analysis of TOC, ca. 0.5 g of each sediment sample was decalcified with 10% HCl, washed with MilliQ water and freeze-dried. Aliquots were weighed and

measured with a Delta V elemental analyzer (Thermo Finnigan MAT GmbH, Bremen, Germany).

IV.3.4. Sample fractionation

The HybridSPE cartridge was rinsed 2x with 5 ml MeOH prior to use. Samples were dissolved in ca. 500 µl MeOH:HCO_2H (98%; 99:1, v/v), loaded onto the cartridge and eluted under vacuum. The cartridge was thereafter washed with 3 ml MeOH containing 1% NH_4CO_2H (wt.%) followed by 3 ml MeOH, both of which were collected and combined as the first fraction (F1). PLs were then eluted from the cartridge using three different eluents including 6 ml IPA containing 10% of NH_4OH solution (wt.%), followed by 6 ml IPA containing 15% of NH_4OH solution (wt.%) and finally 6 ml DCM:IPA:NH_4OH (5:9:6, v/v/v) under vacuum. All three eluents were collected and combined as the second fraction (F2, the PL fraction).

For comparison, the silica gel columns were also employed to fractionate TLEs using the method described by Mills et al. (2006) after slight modification. In brief, the columns were pre-conditioned with 10 ml DCM. Non-PL fractions were eluted with 10 ml DCM followed by 10 ml acetone, while PLs were subsequently collected with 10 ml MeOH. All fractions were evaporated to dryness under a stream of N_2 in a water bath at 37 °C and stored at -20 °C until analysis.

IV.3.5. Instrumentation

The LC-ESI-MS analysis was performed with a Dionex Ultimate 3000 ultra-high performance LC instrument coupled to a Bruker maXis ultra high resolution orthogonal acceleration quadrupole time-of-flight mass spectrometer, equipped with an ESI source and operated in positive mode (Bruker Daltonik, Bremen, Germany). Separation of PLs was achieved with a Waters Acquity UPLC BEH amide column (2.1 × 150 mm, 1.7 µm; Waters Corporation, Eschborn, Germany) based on Wörmer et al. (2013). To investigate the compatibility of the cleanup protocol for different LC methods, a Waters Acquity BEH C_{18} column (2.1 × 150 mm, 3 µm; Waters Corporation, Eschborn, Germany) was additionally employed (Wörmer et al., 2013). Samples were either dissolved in DCM/MeOH 9:1 for the former column or DCM/MeOH 1:9 for the latter. Compounds were assigned and integrated through

Data Analysis 4.0 software (Bruker Daltonik). Compound assignment was based on retention time, accurate mass (< 1 ppm) and diagnostic fragments (Sturt et al., 2004; Schubotz et al., 2009; Rossel et al., 2011). The extraction window of individual ion chromatograms was ± 0.01 *m/z* units. All samples were analyzed in duplicate with LC-ESI-MS.

IV.4. RESULTS AND DISCUSSION

IV.4.1. Recovery of PLs after cleanup

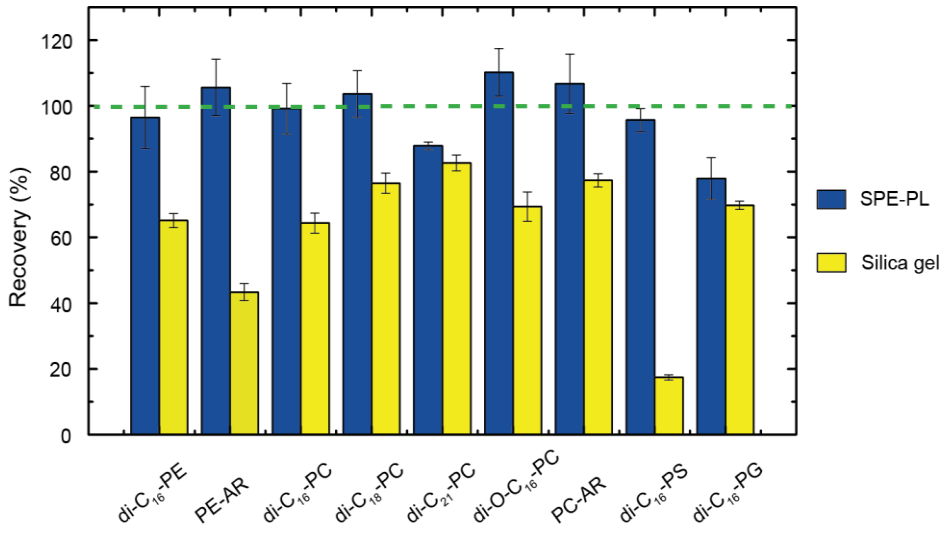


Fig. IV.2. Recovery of authentic PL standards with the HybridSPE®- Phospholipid cartridges (blue) and silica gel columns (yellow), analyzed using LC-ESI-MS and separated with a HILIC amide column. Recovery was calculated by normalizing the signals from the treated standards relative to the untreated standards, indicated by the dashed green line as 100%. Error bars represent standard error from triplicate cleanup experiments. Amount injected into LC-ESI-MS was 10 ng of each PL. (PE, phosphoethanolamine; PC, phosphocholine; PG, phosphatidylglycerol; PS, phosphoserine; AR, archaeol)

PLs were exclusively detected in F2, indicating the high affinity between zirconia-coated sorbents and phosphate-based head groups. The strength of the Lewis acid-base interaction was influenced by head group and side chain length; therefore, three eluents were applied to achieve maximum PL recovery. Our results showed that PL standards with different head groups and side chains were all well retrieved (78-110%), with an average recovery of 98% compared with the signals from untreated standards (Fig. IV.2). Insignificant discrimination among PLs of different types was found through the paired *t*-test (*P* 0.002; statistical analysis using SPSS 20). The ester-bound lipid di-C_{16}-PG showed the lowest recovery (78%), suggesting that

increased number of OH groups in the PG head group strengthened the retention of the PL on the HybridSPE cartridge. For comparison, the recovery of PL standards after the silica gel column ranged from 17% to 83% (avg. 63 ± 20%), i.e. substantially and systematically lower than that from the HybridSPE cartridge (*t*-test, *P* 0.950; Fig. IV.2). Such low recovery of PLs from the silica gel columns is consistent with the findings of Lengger et al. (2012).

We further examined the performance of the HybridSPE using the PL-spiked TLE from a surface sediment sample from the Wadden Sea (Sample #1). The results showed that signals from the spiked standards (Fig. IV.3a) and sedimentary PLs (Fig. IV.3b) were significantly improved in all cases after the HybridSPE cartridge, with a yield of 126-463% (avg. 240%) and 142-268% (avg. 205%), respectively, compared with the untreated aliquot. Noticeably, the greatest signal enhancement was for di-O-C_{16}-PC, PC-AR and PE-AR, followed by di-C_{16}-PC, di-C_{18}-PC and di-C_{16}-PE, whereas di-C_{16}-PS and di-C_{16}-PG showed only minor enhancement (Fig. IV3a). The difference in signal enhancement for individual PL standards between pure standards (Fig. IV.2) and standards in a TLE (Fig. IV.3a) is tentatively attributed to the varied efficiency of the HybridSPE cartridge in removing sedimentary matrices that co-elute with specific PLs during LC-ESI-MS analysis. The yield of indigenous sedimentary PLs approximated that of spiked PL standards with the same head groups except for $C_{32:1}$-PE (Fig. IV.3b), indicating no discrimination between spiked and sedimentary PLs.

Except for di-C_{21}-PC, the yield of spiked standards and sedimentary PLs ranged from 78% to 277% (avg. 162%) and 39% to 172% (avg. 101%), respectively, after fractionation with the silica gel column. The yield of PG-DAGs, e.g. di-C_{16}-PG, $C_{31:1}$-PG and $C_{34:4}$-PG, was similar to the yield with the HybridSPE cartridge, while others showed significantly lower recovery than the HybridSPE-PL fraction (Fig. IV.3). Unlike other PC-bearing lipids, di-C_{21}-PC displayed an unexpectedly high yield after the silica gel column, with the underlying mechanism remaining unclear. Taking di-C_{21}-PC as an exception, we found that the difference in PL recovery between two cleanup protocols was related to head group, with PE showing the greatest discrepancy. Moreover, when evaluated with average recovery, the HybridSPE protocol was a more effective method than silica gel chromatography, particularly in the presence of sedimentary matrices (cf. Figs. IV.2 and 3).

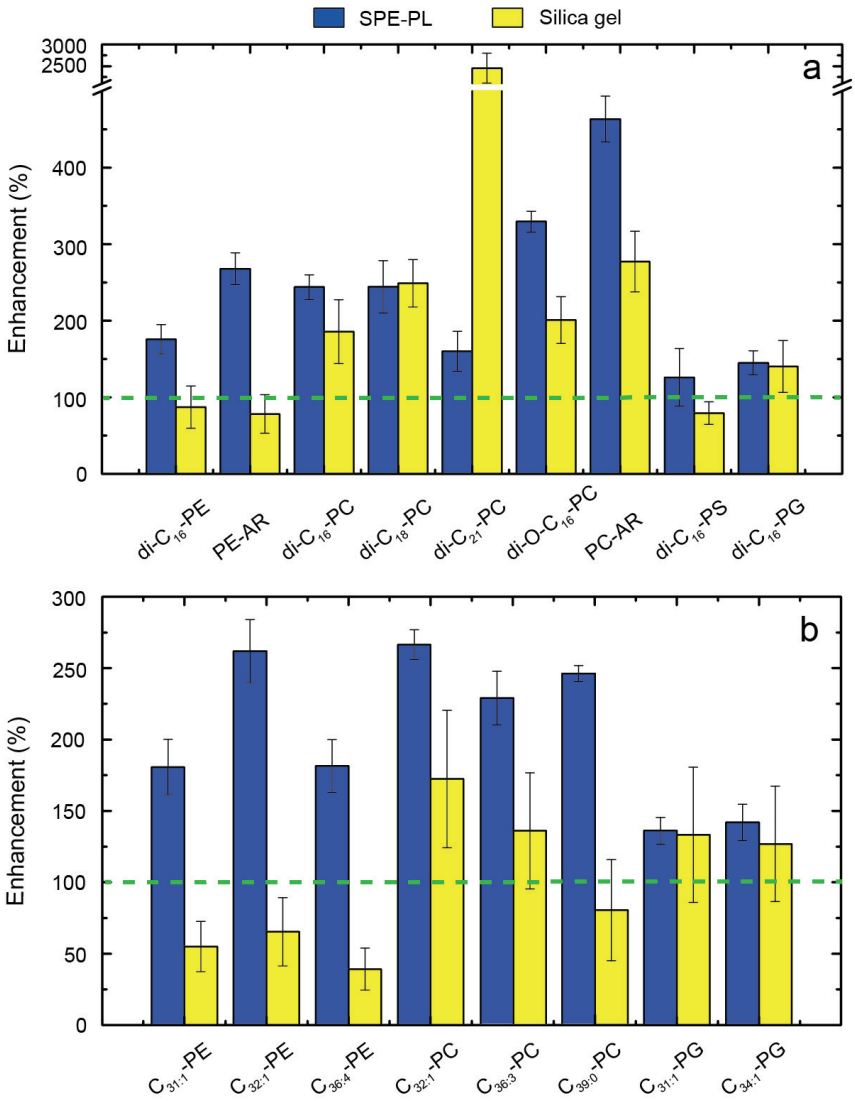


Fig. IV.3. Yield of (a) spiked PL standards (amount injected: 10 ng of each) and (b) indigenous sedimentary PLs from a TLE of a surface sediment from the Wadden Sea, Germany (Sample #1), after treatment with HybridSPE®- Phospholipid cartridges (blue) and silica gel columns (yellow). PLs were analyzed using LC-ESI-MS and separated with a HILIC amide column. Yield was calculated by normalizing the signals from the treated samples relative to the untreated samples, indicated by the dashed green line as 100%. Error bars represent standard error from triplicate cleanup experiments. Sedimentary PLs were indicated as total carbon number of both side chains followed by unsaturation.

IV.4.2. Application of the HybridSPE cartridge to marine sediments

In order to assess the impact of different PL abundances and matrices on cleanup efficiency with the HybridSPE protocol, sediment samples from two sites (Sample #2 and #3) were extracted and spiked with different amounts of PL standards as described above. Sample #2 from the Peru Margin represents a marine organic-rich sediment (5.5% TOC), whereas the sapropelic layers from the Eastern Mediterranean

Sea (Sample #3) offer a moderately elevated TOC content (1.5%; Table IV.1). In accordance with the results described above, the signals of all PL standards were consistently increased after cleanup with the HybridSPE cartridge at all concentration levels (Fig. IV.4). Greater enhancement was observed for Sample #3 than Sample #2, with yields of 151-266% relative to the untreated TLEs for the former and 110-189% for the latter. Moreover, with 0.2 ng of each spiked PL standard, some PLs were detectable only after cleanup (e.g. di-C_{16}-PE, PE-AR; Fig. IV.4), demonstrating the improved detectability of PLs after the HybridSPE treatment.

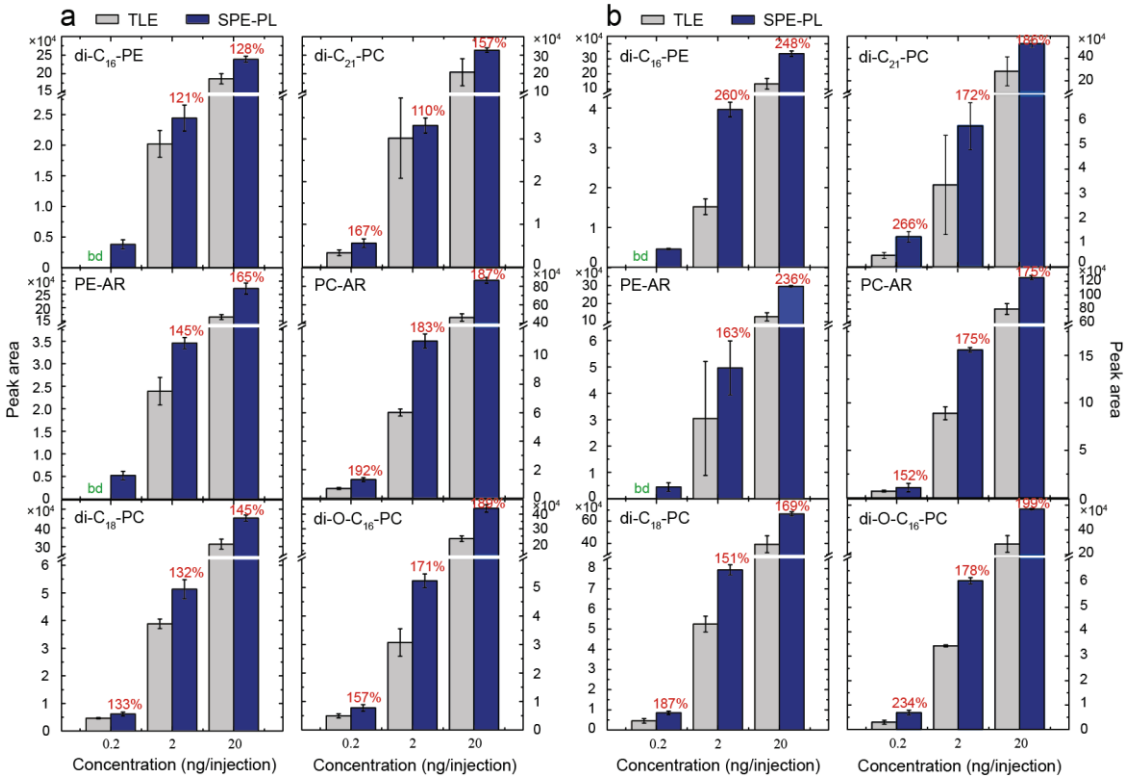


Fig. IV.4. Peak area of PL standards spiked in TLEs of (a) Sample #2 and (b) Sample #3 in different amount (equivalent to 0.2, 2, and 20 ng of each for LC-ESI-MS analysis) before (labeled as TLE) and after (labeled as SPE-PL) cleanup with the HybridSPE®-Phospholipid cartridges. Enhanced signals were normalized to peak area of untreated samples and labeled as % above each HybridSPE-PL fraction. Concentration of spiked standards covered a typical range encountered during analysis of sediment samples. Error bars represent standard error from triplicate cleanup experiments and analyzed with a HILIC amide column on LC-ESI-MS. (bd, below detection limit).

In view of significantly enhanced signals of spiked PLs, we additionally applied the HybridSPE protocol to two organic-rich samples (Sample #4, 4.0% TOC and Sample #5, 4.4% TOC; Table IV.1) for detection of indigenous PLs. The majority of organic compounds were not retained on the cartridge in both cases, resulting in a relatively matrix-free PL fraction that could be concentrated to a desired level for

trace PL analysis. We noted that glycolipids (GLs) were barely detectable in the HybridSPE-PL fraction, suggesting that the HybridSPE protocol can successfully separate PLs from GLs. However, we observed that relatively polar GLs (e.g., diglycosidic compounds) were partly retained on the HybridSPE sorbent (data not shown) and therefore recommend that a dedicated glycolipid analysis should be performed with another aliquot of the TLE. In Sample #4, signals of DEGs with PC, PE and PME head groups in the purified PL fraction were 124-135% compared to the untreated TLE (Fig. IV.5a). Likewise, signal enhancement of up to 250% relative to the TLE for PE-DEG and PME-DEG was found for Sample #5 (Fig. IV.5b). Different degrees of improvement after the proposed cleanup protocol may result from various sample matrices that co-eluted with specific PLs. Nevertheless, significantly enhanced PL signals in all tested samples suggest that the HybridSPE cartridge is highly advantageous for cleanup of a wide range of sediment samples.

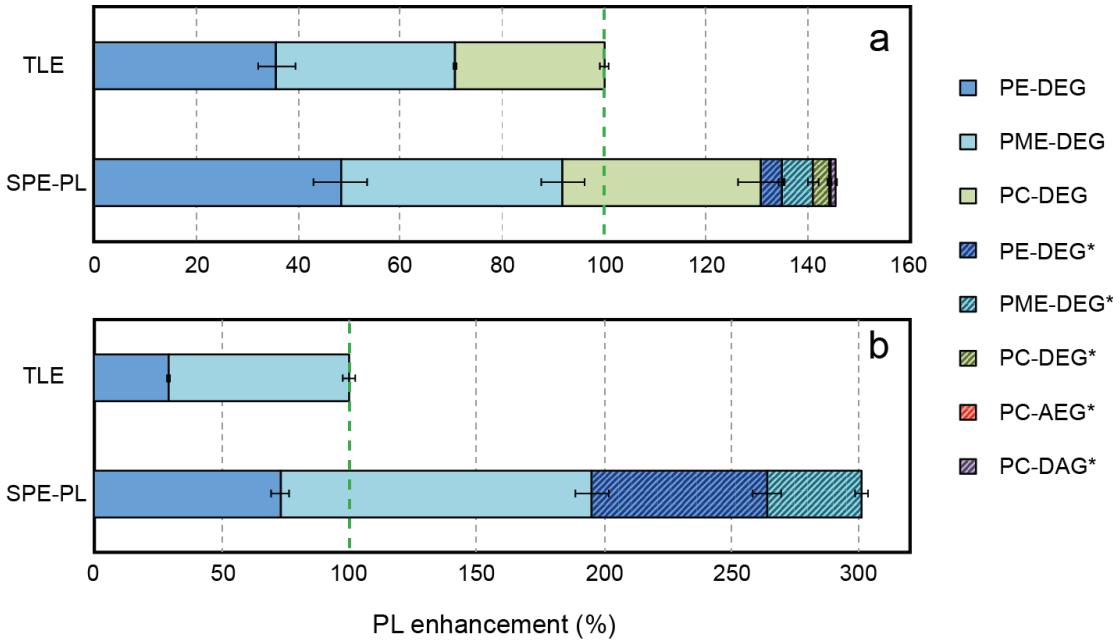


Fig. IV.5. Distributions of PLs in marine sediments from (a) White Oak River Basin (Sample #4) and (b) Black Sea (Sample #5) before (labeled as TLE) and after (labeled as SPE-PL) cleanup with the HybridSPE protocol. Samples were separated with a HILIC amide column on LC-ESI-MS. Total PL yield determined from the TLE was defined as 100%, whilst PL yield from the HybridSPE-PL fraction are reported relative to TLE yield for comparison. Filled bars represent PLs identified in both aliquots, while diagonal bars represent PLs exclusively identified after cleanup with the HybridSPE cartridge. Error bars represent standard deviation from triplicate cleanup processes. (* PL detected only after cleanup)

Notably, the high sensitivity resulting from the HybridSPE treatment was also associated with the observation of a greater diversity of PLs. Trace PLs, which were

not detectable in the reference aliquot, could be identified after the HybridSPE protocol (e.g. Fig. IV5; PL compounds labeled with *). In Sample #4, PC-DAGs and PC-AEGs were exclusively found in the HybridSPE-PL fraction (Fig. IV5a). Together with trace PC-DEGs, PE-DEGs and PME-DEGs (e.g. PE-DEG $C_{34:1}$, PME-DEG $C_{33:1}$), they accounted for 14% of the total PLs identified in the untreated sample. More remarkably, PE-DEGs and PME-DEGs, which were only identified in the HybridSPE-PL fraction, constituted 106% of the total PLs in Sample #5, i.e. an absolute amount exceeding the total PLs quantified in the untreated TLE. Such significant improvement in PL detection resulted in highly different lipid patterns after cleanup and should provide more accurate and comprehensive information on sedimentary PLs. It also suggested that previously published work might underestimate the population and diversity of sedimentary PLs.

IV.4.3. HybridSPE protocol in combination with different LC-MS methods

The results presented above are based on measurements using a HILIC amide column, which separates PLs according to head group polarity (Wörmer et al., 2013). Reversed phase (RP) LC provides an alternative approach for lipid fingerprinting by ESI-MS via separation based on chain length and hydrophobicity (Wörmer et al., 2013; Zhu et al., 2013) and would potentially benefit from an effective cleanup protocol. We therefore also analyzed the PL-spiked Samples #2 and #3 using a Waters Acquity BEH C_{18} column. The results showed that peak area of each PL in the post-cartridge fractions was improved by 3.3-7.6 and 7.6-16.4 times, respectively, compared to the untreated TLEs of Samples #2 and #3. Enhanced detection was also reflected in significantly improved signal/noise ratios for the spiked PL standards (Fig. IV.6). Noticeably, none of PL standards was detected in the non-treated samples when 0.2 ng of each PL was injected on column, which is 2-40 times higher than the reported detection limit of the LC method in matrix-free solvents (Wörmer et al., 2013). This indicates a greater sensitivity of the BEH C_{18} column to matrix-related ion suppression than of the HILIC amide column. It also implies that the cleanup procedure is beneficial to RP-MS-based analysis of PLs at low concentration. In addition, the PLs quantified in the F2 had up to ca. 90% of the intensity of authentic PL standards in pure solvent at the same concentration, suggesting that matrix effects were significantly reduced by the cartridge. In this regard, it is promising to combine

the HybridSPE protocol with RP separation on LC-ESI-MS to more comprehensively analyze distributional patterns within a lipid class.

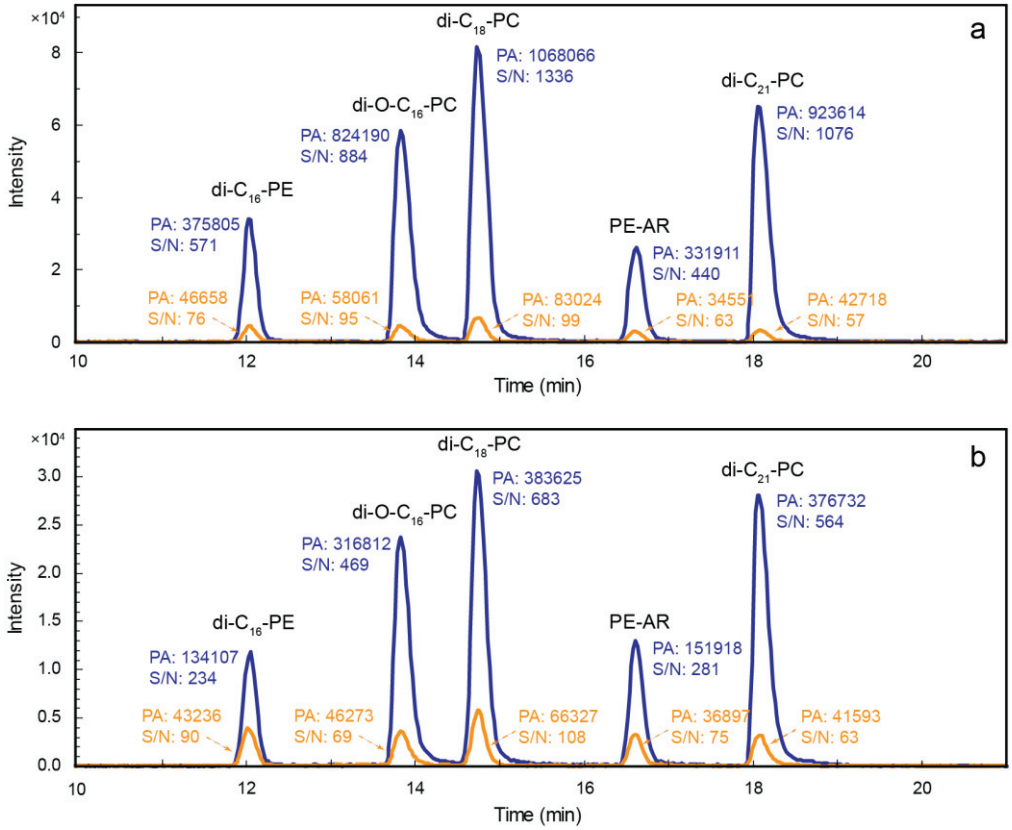


Fig. IV.6. Composite mass chromatograms of PL standards spiked into marine sediment samples from (a) Peru Margin (Sample #2) and (b) Eastern Mediterranean Sea (Sample #3) before (orange) and after (blue) purification with the proposed method and separated with a Waters Acquity BEH C_{18} column. Injection amount was equivalent to 20 ng of each PL standard. Selected ions were *m/z* 692.5225, 714.5044 (di-C_{16}-PE), 706.6109, 728.5928 (di-O-C_{16}-PC), 790.6320, 812.6140 (di-C_{18}-PC), 776.6892, 798.6711 (PE-AR) and 874.7259, 896.7079 (di-C_{21}-PC). Extraction window of the ion chromatograms was ± 0.01 *m/z* units.

IV.5. CONCLUSIONS

A cleanup protocol based on HybridSPE®-Phospholipid cartridge for diverse PLs was adapted to environmental samples prior to LC-ESI-MS analysis. The resulting new protocol is characterized by (i) strongly improved detectability of PLs, (ii) insignificant discrimination among various PL types, and (iii) negligible PL loss. Removal of extract matrix by the HybridSPE cartridge clearly enhances PL ionization, resulting in significantly lowered detection limits of PLs compared to the untreated aliquots. With sediment samples, some indigenous trace PLs were only detectable after cleanup with the HybridSPE cartridge, providing additional clues for tracking

the microbial community. With these advantages, the method offers more accurate and comprehensive PL fingerprints for the study of microbial PLs in marine sediments.

IV.6. ACKNOWLEDGEMENTS

Samples used in this research were taken during two field trips to the estuary of White Oak River Basin (North Carolina, USA) and a tidal flat of Wadden Sea (North Sea, Germany), respectively, and during RV Meteor cruise M84/1 (DARCSEAS), and Leg 201 of the Ocean Drilling Program (ODP), which is sponsored by the US National Science Foundation and participating countries under management of Joint Oceanographic Institutions (JOI), Inc. We thank the participating scientists and ship crews for sample recovery; J. Lipp for technical and scientific guidance, and two anonymous reviewers for constructive comments. The study was supported by the Deutsche Forschungsgemeinschaft through Grant Inst 144/300-1 (LC-qTOF system) and the Gottfried Wilhelm Leibniz Price awarded to K.-U.H., and by the European Research Council under the European Union's Seventh Framework Programme - "Ideas" Specific Programme, ERC Grant Agreement No. 247153 (to K.-U.H.). R.Z. is sponsored by the Chinese scholarship Council (CSC) and University of Bremen.

IV.7. REFERENCES

Bobbie, R., White, D.C., 1980. Characterization of benthic microbial community structure by High-Resolution Gas Chromatography of fatty acid methyl esters. Applied and Environmental Microbiology 39, 1212-1222.

DeLong, E.F., Yayanos, A.A., 1986. Biochemical function and ecological significance of novel bacterial lipids in deep-sea procaryotes. Applied and Environmental Microbiology 51, 730-737.

D'Hondt, S.L., Jørgensen, B.B., Miller, D.J. and Shipboard Scientific Party, 2003. Proceedings of the Ocean Drilling Program, Initial Reports, vol. 201. Ocean Drilling Program, Texas A & M University, College Station, TX. doi:10.2973/odp.proc.ir.201.2003.

Elvert, M., Boetius, A., Knittel, K., Jørgensen, B.B., 2003. Characterization of specific membrane fatty acids as chemotaxonomic markers for sulfate-reducing bacteria involved in anaerobic oxidation of methane. Geomicrobiology Journal 20, 403-419.

Fang, J., Barcelona, M.J., 1998. Structural determination and quantitative analysis of bacterial phospholipids using liquid chromatography/electrospray ionization/mass spectrometry. Journal of Microbiological Methods 33, 23-35.

Fang, J., Barcelona, M.J., Semrau, J.D., 2000. Characterization of methanotrophic bacteria on the basis of intact phospholipid profiles. FEMS Microbiology Letters 189, 67-72.

Fredricks, H.F., Hinrichs, K.-U., 2007. Data report: intact membrane lipids as indicators of subsurface life in cretaceous and paleogene sediments from sites 1257 and 1258. Proceedings of the Ocean Drilling Program, Science Research 207. doi:10.2973/odp.proc.sr.207.112.2007

Frostegård, Å., Tunlid, A., Bååth, E., 1993. Phospholipid fatty acid composition, biomass, and activity of microbial communities from two soil types experimentally exposed to different heavy metals. Applied and Environmental Microbiology 59, 3605-3617.

Guckert, J.B., Hood, M.A., White, D.C., 1986. Phospholipid ester-linked fatty acid profile changes during nutrient deprivation of *Vibrio cholerae*: Incerases in the *trans/cis* ratio and proportions of cyclopropyl fatty acids. Applied and Environmental Microbiology 52, 794-801.

Harvey, H.R., Fallon, R.D., Patton, J.S., 1986. The effect of organic matter and oxygen on the degradation of bacterial membrane lipids in marine sediments. Geochimica et Cosmochimica Acta 50, 795-804.

Jiang, H., Zhang, Y., Ida, M., LaFayette, A., Fast, D.M., 2011. Determination of carboplatin in human plasma using HybridSPE-precipitation along with liquid chromatography-tandem mass spectrometry. Journal of Chromatography B 879, 2162-2170.

Koga, Y., Morii, H., 2005. Recent advances in structural research on ether lipids from archaea including comparative and physiological aspects. Bioscience, Biotechnology, and Biochemistry 69, 2019-2034.

Lengger, S.K., Hopmans, E.C., Sinninghe Damsté, J.S., Schouten, S., 2012. Comparison of extraction and work up techniques for analysis of core and intact polar tetraether lipids from sedimentary environments. Organic Geochemistry 47, 34-40.

Lipp, J.S., Morono, Y., Inagaki, F., Hinrichs, K.-U., 2008. Significant contribution of Archaea to extant biomass in marine subsurface sediments. Nature 454, 991-994.

Liu, X., Lipp, J.S., Hinrichs, K.-U., 2011. Distribution of intact and core GDGTs in marine sediments. Organic Geochemistry 42, 368-375.

Logemann, J., Graue, J., Köster, J., Engelen, B., Rullkötter, J., Cypionka, H., 2011. A laboratory experiment of intact polar lipid degradation in sandy sediments. Biogeosciences 8, 2547-2560.

Mills, C.T., Dias, R.F., Graham, D., Mandernack, K.W., 2006. Determination of phospholipid fatty acid structures and stable carbon isotope compositions of deep-sea sediments of the Northwest Pacific, ODP site 1179. Marine Chemistry 98, 197-209.

Moriarty, M., Lee, A., O'Connell, B., Lehane, M., Keeley, H., Furey, A., 2012. The application and validation of HybridSPE-Precipitation cartridge technology for the rapid clean-up of serum matrices (from phospholipids) for the clinical analysis of serotonin, dopamine and melatonin. Chromatographia 75, 1257-1269.

Peterse, F., Hopmans, E.C., Schouten, S., Mets, A., Rijpstra, I.C., Sinninghe Damsté, J.S., 2011. Identification and distribution of intact polar branched tetraether lipids in peat and soil. Organic Geochemistry 42, 1007-1015.

Pitcher A., Hopmans, E.C., Schouten S., Sinninghe Damsté, J.S., 2009. Separation of core and intact polar archaeal tetraether lipids using silica columns: Insights into living and fossil biomass contributions. Organic Geochemistry 40, 12-19.

Pucci, V., Di Palma, S., Alfieri, A., Bonelli, F., Monteagudo, E., 2009. A novel strategy for reducing phospholipids-based matrix effect in LC-ESI-MS bioanalysis by means of HybridSPE. Journal of Pharmaceutical and Biomedical Analysis 50, 867-871.

Rossel, P.E., Elvert, M., Ramette, A., Boetius, A., Hinrichs, K.-U., 2011. Factors controlling the distribution of anaerobic methanotrophic communities in marine environments: evidence from intact polar membrane lipids. Geochimica et Cosmochimica Acta 75, 164-184.

Rütters, H., Sass, H., Cypionka, H., Rullkötter, J., 2002. Phospholipid analysis as a tool to study complex microbial communities in marine sediments. Journal of Microbiological Methods 48, 149-160.

Schubotz, F., Wakeham, S.G., Lipp, J.S., Fredricks, H.F., Hinrichs, K.-U., 2009. Detection of microbial biomass by intact polar membrane lipid analysis in the water column and surface sediments of the Black Sea. Environmental Microbiology 11, 2720-2734.

Sturt, H.F., Summons, R.E., Smith, K., Elvert, M., Hinrichs, K.-U., 2004. Intact polar membrane lipids in prokaryotes and sediments deciphered by high-performance liquid chromatography/electrospray ionization multistage mass spectrometry--new biomarkers for biogeochemistry and microbial ecology. Rapid Communications in Mass Spectrometry 18, 617-628.

Sundh, I., Nilsson, M., Borga, P., 1997. Variation in microbial community structure in two boreal peatlands as determined by analysis of phospholipid fatty acid profiles. Applied and Environmental Microbiology 63, 1476-1482.

Vestal, J.R., White, D.C., 1989. Lipid analysis in microbial ecology. Bioscience 39, 535-541.

White, D., Davis, W., Nickels, J., King, J., Bobbie, R., 1979. Determination of the sedimentary microbial biomass by extractible lipid phosphate. Oecologia 40, 51-62.

Wörmer, L., Lipp, J.S., Schröder, J.M., Hinrichs, K.-U., 2013. Application of two new LC-ESI-MS methods for improved detection of intact polar lipids (IPLs) in environmental samples. Organic Geochemistry 59, 10-21.

Xie, S., Lipp, J.S., Wegener, G., Ferdelman, T.G., Hinrichs, K.-U., 2013. Turnover of microbial lipids in the deep biosphere and growth of benthic archaeal populations. Proceedings of the National Academy of Sciences, U.S.A. 110, 6010-6014.

Zelles, L., Bai, Q.Y., Beck, T., Beese, F., 1992. Signature fatty acids in phospholipids and lipopolysaccharides as indicators of microbial biomass and community structure in agricultural soils. Soil Biology and Biochemistry 24, 317-323.

Zhu, C., Lipp, J.S., Wörmer, L., Becjer, K.W., Schröder, J., Hinrichs, K.-U., 2003. Comprehensive glycerol ether lipid fingerprints through a novel reverse-phase liquid chromatography-mass spectrometry protocol. Organic Geochemistry 65, 53-62.

Zink, K.G., Wilkes, H., Disko, U., Elvert, M., Horsfield, B., 2003. Intact phospholipids-microbial ‘‘life markers’’ in marine deep subsurface sediments. Organic Geochemistry 34, 755-769.

Zink, K.G., Mangelsdorf, K., 2004. Efficient and rapid method for extraction of intact phospholipids from sediments combined with molecular structure elucidation using LC-ESI-MS-MS analysis. Analytical and Bioanalytical Chemistry 380, 798-812.

Chapter V

Distributions of phospholipids in oil-bearing hydrothermal field of the Guaymas Basin

Rong Zhu[1], Yu-Shih Lin[1, 2], Kai-Uwe Hinrichs[1]

Data Report

[1] Organic Geochemistry Group, MARUM-Center for Marine Environmental Sciences & Department of Geosciences, University of Bremen, 28359 Bremen, Germany

[2] Department of Oceanography, National Sun Yat-Sen University, No.70 Lienhai Rd, Kaohsiung 804, Taiwan

V.1. ABSTRACT

The hydrothermal field of the Guaymas Basin hosts a variety of microbial biomass that includes all three domains of life. Because phospholipids (PLs) can be indicative of active microorganisms and bear taxonomic information, we investigated the distributions of the intact PLs to compare the microbial communities at a hydrothermal site (Dive 4568-1) and an adjacent non-hydrothermal site (Dive 4567-6). PLs were observed at the hydrothermal site, suggesting the presence of living microbial biomass in sediments with a steep temperature gradient, and were still detectable up to ~90°C. Compared to the non-hydrothermal site, distributions of PLs showed less diversity at the hydrothermal site, indicating a comparatively simple but specialized thermophilic microbial community.

V.2. INTRODUCTION

The hydrothermal environment of the Guaymas Basin receives high sediment input from the water column of the Gulf of California, Mexico, and is covered by a thick, organic-rich sediment layer with the total organic carbon (TOC) content up to 12% near the surface sediment (De la Lanza-Espino and Soto, 1999). Magmatic heating of the sediment from the Guaymas Basin leads to thermogenic decay of the organic matter and drives the production of petroleum (Gieskes et al., 1988; Simoneit et al., 1992). The vent fluids and sediments of the Guaymas Basin host unusually rich microbial diversity, including a large variety of bacteria, archaea and eukaryotes (Teske et al., 2002). For instance, bacterial mats found at the sediment-water interface can be observed visually and are dominated by the filamentous sulfur bacteria *Beggiatoa* spp. (Nelson et al., 1989; Weber and Jørgensen, 2002); previous gene-based studies have also shown the predominance of hydrothermophilic methanogens *Methanomicrobiales* and *Methanosarcinales* in surface sediments from the Guaymas Basin (Teske et al., 2002; Dhillon et al., 2003).

Analysis of microbial biomarkers provided insights into the taxonomic composition of the microbial community and microbial metabolisms. The distributions and stable carbon isotopic compositions of lipids from the hydrothermally active areas in the Guaymas Basin, which exhibit a steep sedimentary temperature gradient, indicated the presence of anaerobic methanotrophic archaea

(Teske et al., 2002; Schouten et al., 2003). By employing stable-isotope-probing experiments, Kellermann et al. (2012) suggested that the anaerobic methane-oxidizing archaea oxidized methane but assimilated inorganic carbon and should be classified as methane-oxidizing chemoorganoautotrophs.

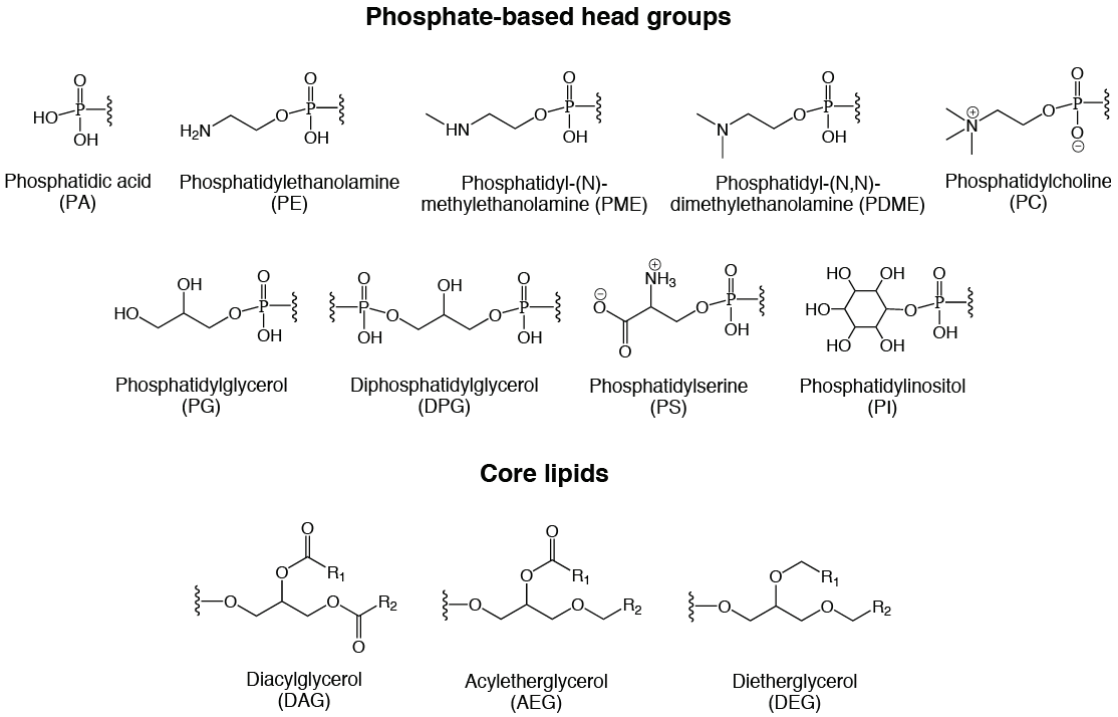


Fig. V.1. Structures of phosphate-based head groups and core lipids.

Previous studies have demonstrated that phospholipids (PLs) bear chemotaxonomic information both on the polar head groups and acyl and alkyl side chains (Fig. V.1; Fang et al., 2000; Zink et al., 2003; Sturt et al., 2004; Schubotz et al., 2009) and decompose rapidly after cell lysis (White et al., 1979; Harvey et al., 1986; Logemann et al., 2011). The intact PLs therefore can indicate the presence of vegetative cells and have been widely targeted as a culture- and gene-independent approach for decoding microbial communities (Zelles et al., 1992; Lipp et al., 2008; Schubotz et al., 2009; Peterse et al., 2011). In combination with a recently adapted solid phase extraction (SPE)-based cleanup protocol (Zhu et al., 2013), comprehensive microbial PL fingerprints can be assigned and quantified, with minimum interference from co-eluting compounds in the sedimentary matrix, using liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS; Wörmer et al., 2013). Here, we studied the distributions of PLs in oil-bearing sediments from the hydrothermal field of the Guaymas Basin. PLs in an adjacent

non-hydrothermal site were examined for comparison. The results suggested the presence of living microbial biomass up to maximum sedimentary temperatures of ~90°C. Compared to the non-hydrothermal site, the hydrothermal site exhibited less variable PL distribution, indicating that a comparatively specialized thermophilic microbial community.

V.3. MATERIAL AND METHODS

V.3.1. Material

Sediment samples were retrieved during two Alvin dives 4567-6 (27°00.476 N, 111°24.433 W) and 4568-1 (27° 00.449 N, 111° 24.347 W) during the RV Atlantis cruise to the Guaymas Basin, Gulf of California, Mexico (November 22 to December 6, 2009). Dive 4568-1 was located in the oil-bearing hydrothermally active area, where sedimentary temperature steeply increased from ~3°C to ~100°C within 35 cm (Fig. V.2.d). The location of this core was near or within the *Beggiatoa* mats. Dive 4567-6 was located in an adjacent non-hydrothermal area, with sedimentary temperature fairly constant at 3.6 ± 0.4°C in the upper 50 cm below the seafloor (cmbsf; Fig. V.2.a).

The PL standard, 2-dihenarachidoyl-*sn*-glycero-3-phosphocholine (di-C_{21}-PC), was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA) and used as an internal standard for quantification of PLs. The HybridSPE®-Phospholipid cartridges (500 mg; 6 ml) were obtained from Supelco Chemical Co. (Munich, Germany). All the solvents and chemicals used in this study were provided by Sigma-Aldrich Chemie GmbH (Munich, Germany) or Merck KGaA (Darmstadt, Germany).

V.3.2. Sample preparation

Sediment samples (15-20 g, wet weight) were spiked with the internal standard di-C_{21}-PC and extracted using a modified Bligh and Dyer method (Sturt et al., 2004). For analysis of PLs, aliquots of the resulting TLEs were treated with the HybridSPE®-Phospholipid cartridges according to the method described by Zhu et al. (2013). The purified PL fractions were evaporated to dryness under a stream of N_2 and stored at -20 °C until analysis. For analysis of TOC content, ca. 0.5 g of each

sediment sample was decalcified with 10% HCl, washed with MilliQ water and freeze-dried. Aliquots were weighed and measured with a Delta V elemental analyzer (Thermo Finnigan MAT GmbH, Bremen, Germany).

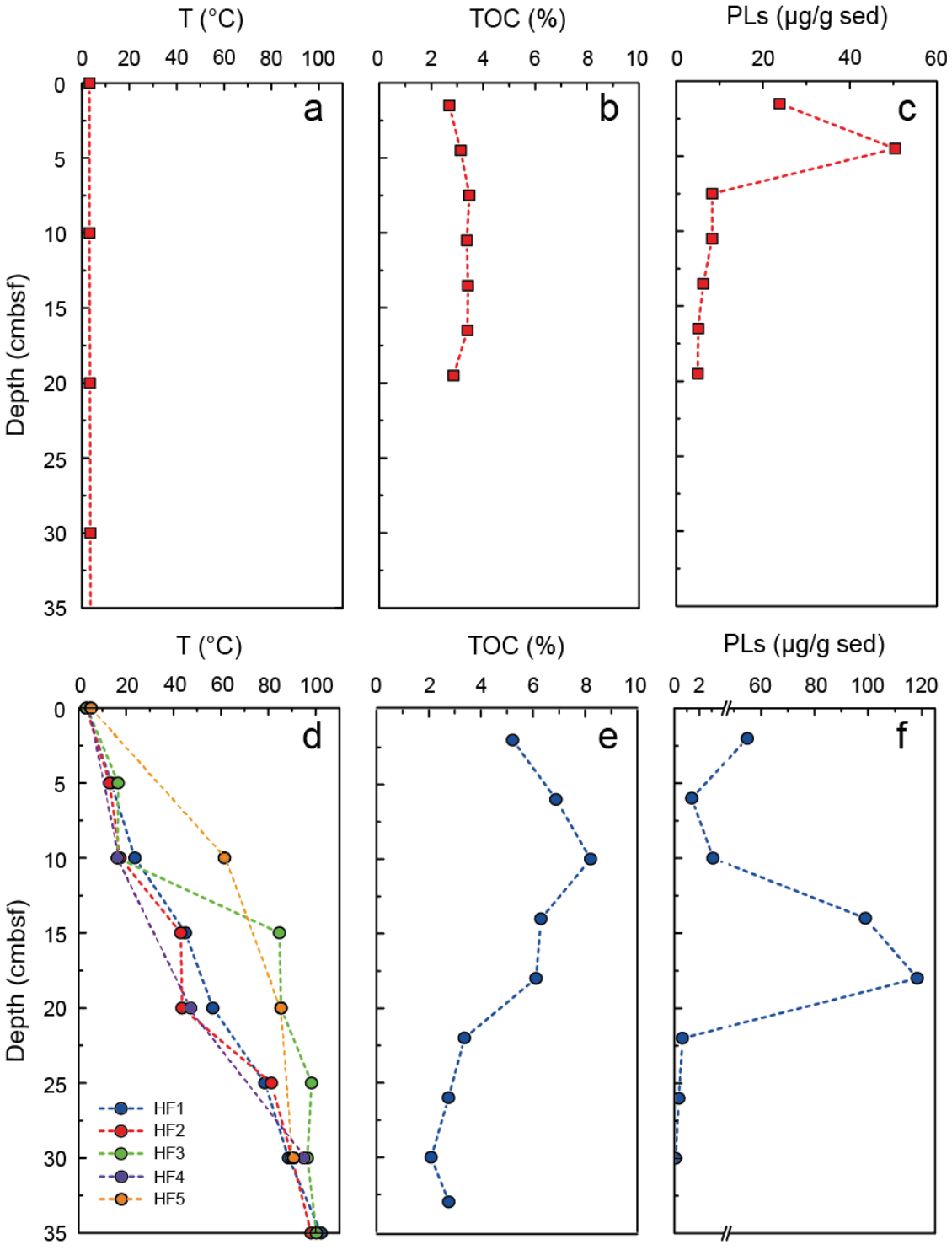


Fig. V.2. Depth profiles of sedimentary temperature (a, d), total organic carbon (TOC) content (b, e), and phospholipid (PL) concentration (c, f) in Dive 4567-6 (a, b, c) and Dive 4568-1 (d, e, f). HF1-HF5 indicate sedimentary temperature profiles from five independent measurements.

V.3.3. Instrumentation

Each sample was analyzed on a Dionex Ultimate 3000 ultra-high performance LC instrument coupled to a Bruker maXis ultra high resolution orthogonal acceleration quadrupole time-of-flight mass spectrometer, equipped with an ESI source in positive mode (Bruker Daltonik, Bremen, Germany), and separated with a Waters Acquity UPLC BEH amide column (2.1 × 150 mm, 1.7 µm; Waters Corporation, Eschborn, Germany; Wörmer et al., 2013). Each lipid was assigned and integrated using Data Analysis 4.1 software (Bruker Daltonik). Compound assignment was based on retention time, accurate mass (< 1 ppm) and diagnostic mass fragments (Sturt et al., 2004; Schubotz et al., 2009; Rossel et al., 2011). The extraction window of individual ion chromatograms was ± 0.01 *m/z* units. PLs were assigned according to the total carbon number of both side chains. Concentrations of PLs were calculated from peak areas of the extracted ion chromatograms relative to that of internal standard di-C_{21}-PC.

V.4. RESULTS AND DISCUSSION

V.4.1. Quantitative distribution of PLs

At the non-hydrothermal site (Dive 4567-6), TOC was fairly constant throughout the core, averaging (± SD) 4.9 ± 2.0% (Fig. V.2.b). Total intact PLs ranged from 5-50.4 µg/g sediment. The highest PL concentration was observed at 4.5 cmbsf and steeply decreased, by an order of magnitude, towards the bottom of the core at 19.5 cmbsf (Fig. V.2.c). Sediments from the hydrothermal site (Dive 4568-1) were rich in organic matter (average TOC = 4.9 ± 2.0%), with a maximum TOC of 8.2% at 10 cmbsf (Fig. V.2.e). In this hydrothermal setting, total intact PLs ranged between 0.09 and 118.3 µg/g sediment (Fig. V.2.f). In contrast to the non-hydrothermal site, the PL concentration in the surface sediment (2 cmbsf) was 55 µg/g sediment, which was near or within the *Beggiatoa* mat, consistent with previous reported bacterial PL concentrations determined in the microbial mats at the Chapopote Knoll (Schubotz et al., 2011). The total PL concentration steeply decreased thereafter by up to fortyfold, followed by a subsurface maximum at 14-18 cmbsf, where the sedimentary temperature was around 45-60°C (Fig. V.2.d). Such high PL concentrations (i.e. 118.3 µg/g sediment) are rare. High sulfate reduction rates have been observed in Guaymas

Basin sediments, with the maximum values occurring in samples at 50-60°C (Jørgensen et al., 1990; Elsgaard et al., 1994). Therefore, the remarkably increased PL concentrations in the hydrothermal site could have been predominantly derived from thermophilic sulfate-reducing bacteria. PLs were still detectable at 30 cmbsf, suggesting the presence of living microbial biomass up to a sedimentary temperature of ~90°C.

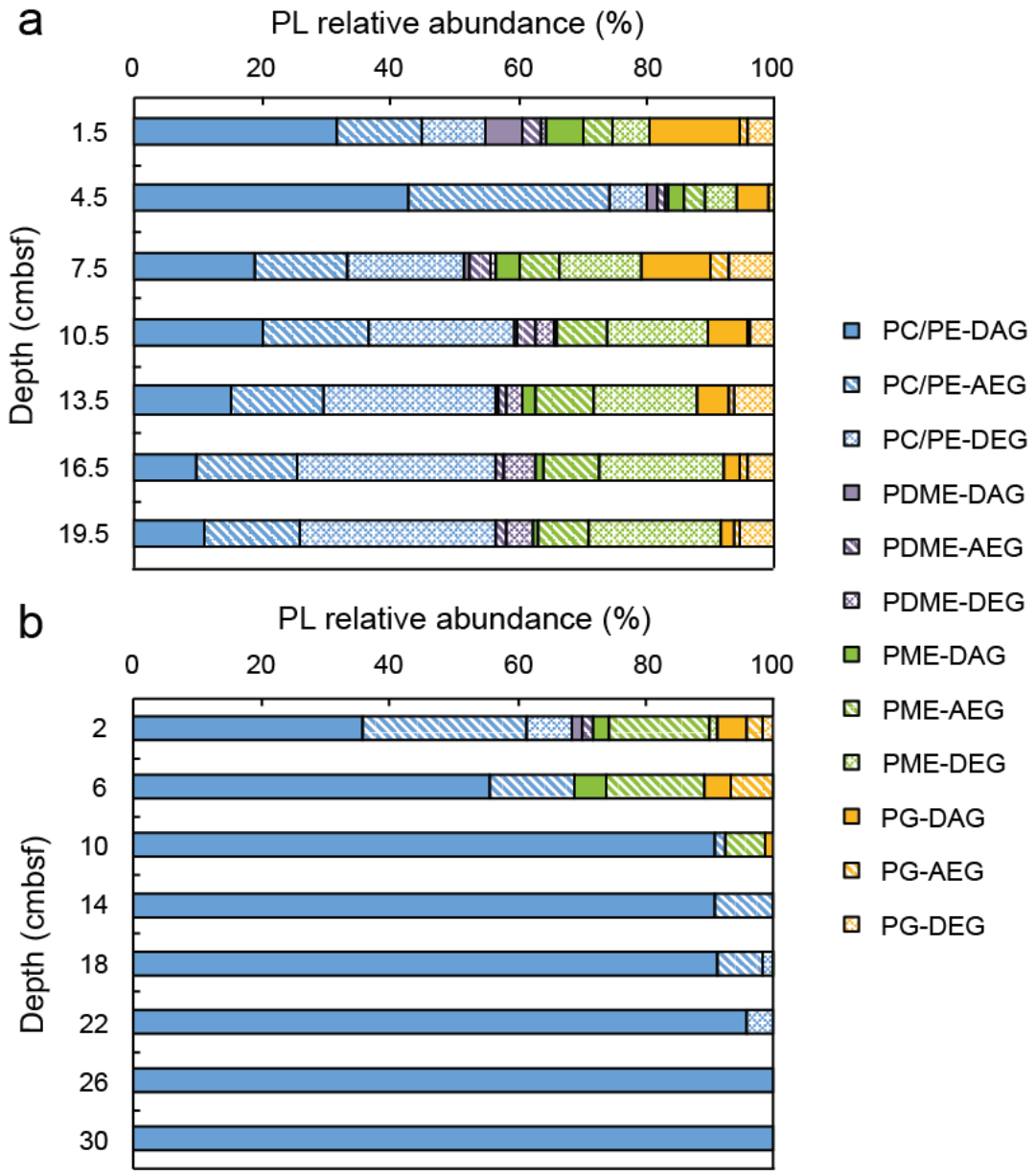


Fig. V.3. Relative distribution of PLs in non-hydrothermal (a) and hydrothermal (b) sediments in the Guyamas Basin. Abbreviations: phosphocholine, PC; phosphatidyl-(N, N)-dimethylethanolamines, PDME; phosphoethanolamine, PE; phosphatidylglycerol, PG; phosphatidyl-(N)-methylethanolamines, PME; diacylglycerol, DAG; acyletherglycerol, AEG; dietherglycerol, DEG.

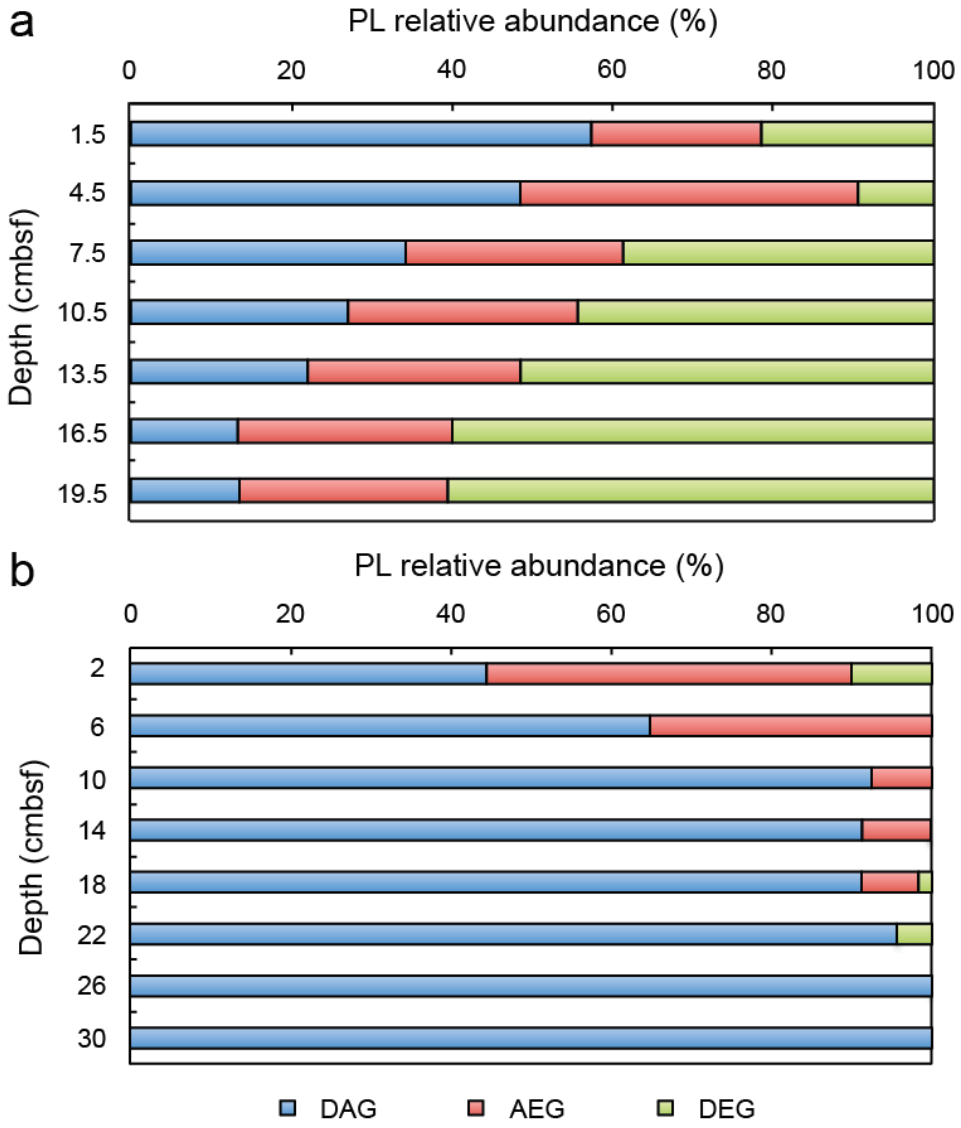


Fig. V.4. Relative abundances of PL core structures in non-hydrothermal (a) and hydrothermal (b) sediments in the Guaymas Basin.

V.4.2. Compositional distribution of PLs

PLs with phosphocholine (PC) or phosphoethanolamine (PE) head groups were the most abundant PLs at both sites, followed by PLs consisting of a phosphatidyl-(N)-methylethanolamine (PME) head group (Fig. V.3.). PLs with phosphatidyl-(N, N)-dimethylethanolamine (PDME) or phosphatidylglycerol (PG) head groups were also present at both sites, but were usually minor constituents. In the non-hydrothermal site, the proportions of PC and PE lipids accounted for 52-85% of total PLs, reaching a maximum at 4.5 cmbsf (Fig. V.3.a), where the PL concentration was highest. PLs with PDME, PME or PG head groups made up 6 ± 2%, 22 ± 8% and 12 ± 6% of total PLs, respectively. In the hydrothermal site, the

combined proportion of PC and PE lipids accounted for > 69% of the total PLs in upper sediments, and was completely dominant below 14 cmbsf (Fig. V.3.b). PLs with PDME, PME or PG head groups constituted ~3%, < 20% and < 11% of the total PLs, respectively, with the PDME lipids only detectable in the uppermost sample. The PL composition in the uppermost sample was similar to the reference site, suggesting the presence of eukaryotes and other bacteria in addition to *Beggiatoa*. The less variable PL distributions in the deeper samples of the hydrothermal site indicated a comparatively simple but specialized thermophilic microbial community compared to the reference site.

The vertical distributions of the PL core structures are summarized in Fig. V.4. PLs with core structures of diacylglycerol (DAG), acyletherglycerol (AEG) and dietherglycerol (DEG) were present at both sites. In the non-hydrothermal site, DAGs accounted for 57% of the total PLs in the uppermost sample and gradually decreased with depth until 13% at 19.5 cmbsf (Fig. V.4.a). The proportions of AEGs was fairly constant throughout the core, with a slight increase at 4.5 cmbsf. The relative abundance of DEGs decreased at a depth of 4.5 cmbsf and increased with depth thereafter, reaching 61% at 19.5 cmbsf. The distributions of the PL core structures were significantly different in the hydrothermal site. DAGs accounted for 44% of the total PLs in the uppermost sample, increased with depth, and completely dominated the PL pool below 26 cmbsf (Fig. V.4.b). AEGs made up ~40% of the total PLs above 6 cmbsf, then steeply decreased by a factor of ~5 in the deeper samples. Unexpectedly, DEGs were only observed at depths of 2, 14, 18, and 22 cmbsf, representing a very minor PL component. Previous studies have reported that PE/PME-DEGs could be derived from sulfate-reducing bacteria (Rütter et al., 2001; Sturt et al., 2004; Schubotz et al., 2009) or methanotrophic bacteria (Fang et al., 2000; Rossel et al., 2011). Because lipids containing an ester-bond are presumed to be more labile than those with an ether-bond (Schouten et al., 2010; Logemann et al., 2011), DEGs are expected to be observed in the deeper samples from both sites. However, DEGs were predominant only in the non-hydrothermal site (Fig. V.3.a) and were barely detected in the samples between 14-18 cmbsf from the hydrothermal site (Fig. V.3.b), where were suspected to host sulfate-reducing bacteria (Jørgensen et al., 1990; Elsgaard et al., 1994). Our results thus suggest that the thermophilic sulfate-reducing bacteria in these horizons produced PC/PE-DAGs (Rütter et al., 2001; Sturt et al., 2004;

Schubotz et al, 2009).

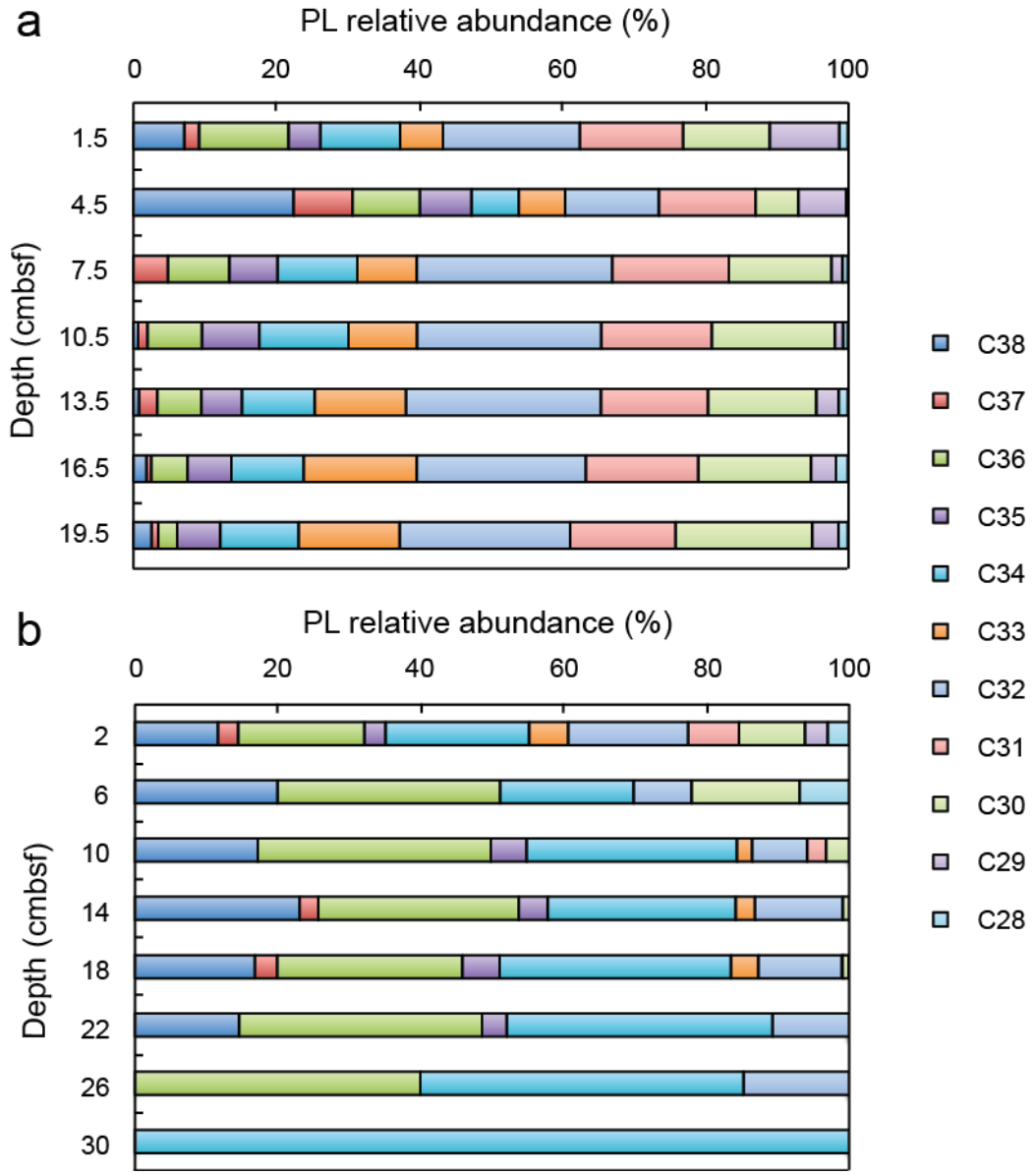


Fig. V.5. Relative distribution of PL side chains in non-hydrothermal (a) and hydrothermal (b) sediments in the Guyamas Basin. PLs are indicated as total carbon number of both side chains. Note that only side chains containing 28 to 38 carbon atoms are considered.

The vertical distributions of PL side chain length are summarized in Fig. V.5. PLs consisting of 30-32 carbons in the side chains were predominant in the non-hydrothermal site, whereas PLs in the hydrothermal site mostly contained 34, 36 and 38 carbons in the side chains, clearly indicating community diversities between the two sites. Because PLs were measured by LC-ESI-MS in positive mode, it was difficult to distinguish bacterial and eukaryotic PLs that differed in the length of individual fatty acid (FA) side chains and degree of unsaturation. Based on currently

available data, bacterial PLs likely accounted for less proportions in the hydrothermal site compared to the reference site. Additional experiments, such as analysis of the intact PLs by LC-ESC-MS in negative mode and/or analysis of the FA side chains on gas chromatography (GC)-MS would provide additional information on the microbial communities in the study area.

V.5. CONCLUSION AND OUTLOOK

Distributions of the intact PLs in oil-bearing sediments from the hydrothermal field of the Guaymas Basin were investigated using a recently adapted SPE-based cleanup protocol for removing majority of the sedimentary matrices. At the hydrothermal site, PLs were detectable in sediments with temperatures up to ~90°C, suggesting the presence of living microbial biomass in the thermally heated sediments. Moreover, the PLs exhibited less diversity in the hydrothermal site, indicating a comparatively simple but specialized thermophilic microbial community. Ongoing experiments, including microbial cell counts, performed by colleagues from the University of North Carolina, will provide additional information regarding the microbial population in the Guaymas sediments. Future analysis of PLs by LC-ESC-MS in negative mode and analysis of the PL-derived FA (PLFA) on GC-MS will provide even more comprehensive PL fingerprints of microbial communities. Analysis of the stable carbon isotopic composition of PLFAs will allow for an in situ assessment of the microbial metabolisms in Guaymas sediments.

V.6. ACKNOWLEDGEMENTS

Samples used in this study were taken during RV Atlantis cruise to the Guaymas Basin, Gulf of California. We thank the participating scientists and ship crews for sample recovery; J. S. Lipp for technical and scientific guidance, M. Y. Yoshinaga for precious advice. This study was supported by the Deutsche Forschungsgemeinschaft through Grant Inst 144/300-1 (LC-qTOF system) and the Gottfried Wilhelm Leibniz Price awarded to K.-U.H., and by the European Research Council under the European Union's Seventh Framework Programme - “Ideas” Specific Programme, ERC Grant Agreement No. 247153 (to K.-U.H.). Sample

acquisition was supported by Grant NSF OCE-0647633.

V.7. REFERENCES

De la Lanza-Espino, G., Soto, L.A., 1999. Sedimentary geochemistry of hydrothermal vents in Guaymas Basin, Gulf of California, Mexico. Applied geochemistry 14, 499-510.

Dhillon, A., Lever, M., Lloyd, K.G., Albert, D.B., Sogin, M.L., Teske, A., 2005. Methanogen diversity evidenced by molecular characterization of methyl coenzyme M reductase A (mcrA) genes in hydrothermal sediments of the Guaymas Basin. Applied and environmental microbiology 71, 4592-4601.

Elsgaard, L., Isaksen, M.F., Jørgensen, B.B., Alayse, A.-M., Jannasch, H.W., 1994. Microbial sulfate reduction in deep-sea sediments at the Guaymas Basin hydrothermal vent area: Influence of temperature and substrates. Geochimica et Cosmochimica Acta 58, 3335-3343.

Fang, J., Barcelona, M.J., Semrau, J.D., 2000. Characterization of methanotrophic bacteria on the basis of intact phospholipid profiles. FEMS Microbiology Letters 189, 67-72.

Gieskes, J.M., Simoneit, B.R., Brown, T., Shaw, T., Wang, Y.-C., Magenheim, A., 1988. Hydrothermal fluids and petroleum in surface sediments of Guaymas Basin, Gulf of California; a case study. The Canadian Mineralogist 26, 589-602.

Harvey, H.R., Fallon, R.D., Patton, J.S., 1986. The effect of organic matter and oxygen on the degradation of bacterial membrane lipids in marine sediments. Geochimica et Cosmochimica Acta 50, 795-804.

Jørgensen, B.B., Zawacki, L.X., Jannasch, H.W., 1990. Thermophilic bacterial sulfate reduction in deep-sea sediments at the Guaymas Basin hydrothermal vent site (Gulf of California). Deep Sea Research Part A. Oceanographic Research Papers 37, 695-710.

Kellermann, M.Y., Wegener, G., Elvert, M., Yoshinaga, M.Y., Lin, Y.S., Holler, T., Mollar, X.P., Knittel, K., Hinrichs, K.-U., 2012. Autotrophy as a predominant mode of carbon fixation in anaerobic methane-oxidizing microbial communities. Proceedings of the National Academy of Sciences of the United States of America 109, 19321-19326.

Lipp, J.S., Morono, Y., Inagaki, F., Hinrichs, K.-U., 2008. Significant contribution of Archaea to extant biomass in marine subsurface sediments. Nature 454, 991-994.

Logemann, J., Graue, J., Köster, J., Engelen, B., Rullkötter, J., Cypionka, H., 2011. A laboratory experiment of intact polar lipid degradation in sandy sediments. Biogeosciences 8, 2547-2560.

Nelson, D.C., Wirsen, C.O., Jannasch, H.W., 1989. Characterization of large, autotrophic Beggiatoa spp. abundant at hydrothermal vents of the Guaymas Basin. Applied and Environmental Microbiology 55, 2909-2917.

Peterse, F., Hopmans, E.C., Schouten, S., Mets, A., Rijpstra, W.I.C., Sinninghe Damsté, J.S., 2011. Identification and distribution of intact polar branched

tetraether lipids in peat and soil. Organic Geochemistry 42, 1007-1015.

Rossel, P.E., Elvert, M., Ramette, A., Boetius, A., Hinrichs, K.-U., 2011. Factors controlling the distribution of anaerobic methanotrophic communities in marine environments: evidence from intact polar membrane lipids. Geochimica et Cosmochimica Acta 75, 164-184.

Rütters, H., Sass, H., Cypionka, H., Rullkötter, J., 2001. Monoalkylether phospholipids in the sulfate-reducing bacteria Desulfosarcina variabilis and Desulforhabdus amnigenus. Archives of Microbiology 176, 435-442.

Schouten, S., Wakeham, S.G., Hopmans, E.C., Sinninghe Damsté, J.S., 2003. Biogeochemical evidence that thermophilic archaea mediate the anaerobic oxidation of methane. Applied and environmental microbiology 69, 1680-1686.

Schouten, S., Middelburg, J.J., Hopmans, E.C., Sinninghe Damsté, J.S., 2010. Fossilization and degradation of intact polar lipids in deep subsurface sediments: a theoretical approach. Geochimica et Cosmochimica Acta 74, 3806-3814.

Schubotz, F., Wakeham, S.G., Lipp, J.S., Fredricks, H.F., Hinrichs, K.-U., 2009. Detection of microbial biomass by intact polar membrane lipid analysis in the water column and surface sediments of the Black Sea. Environmental Microbiology 11, 2720-2734.

Schubotz, F., Lipp, J.S., Elvert, M., Kasten, S., Mollar, X.P., Zabel, M., Bohrmann, G., Hinrichs, K.-U., 2011. Petroleum degradation and associated microbial signatures at the Chapopote asphalt volcano, Southern Gulf of Mexico. Geochimica et Cosmochimica Acta 75, 4377-4398.

Simoneit, B., Leif, R., Sturz, A., Sturdivant, A., Gieskes, J., 1992. Geochemistry of shallow sediments in Guaymas Basin, Gulf of California: hydrothermal gas and oil migration and effects of mineralogy. Organic geochemistry 18, 765-784.

Sturt, H.F., Summons, R.E., Smith, K., Elvert, M., Hinrichs, K.-U., 2004. Intact polar membrane lipids in prokaryotes and sediments deciphered by high-performance liquid chromatography/electrospray ionization multistage mass spectrometry--new biomarkers for biogeochemistry and microbial ecology. Rapid Communications in Mass Spectrometry 18, 617-628.

Teske, A., Hinrichs, K.-U., Edgcomb, V., de Vera Gomez, A., Kysela, D., Sylva, S.P., Sogin, M.L., Jannasch, H.W., 2002. Microbial diversity of hydrothermal sediments in the Guaymas Basin: evidence for anaerobic methanotrophic communities. Applied and Environmental Microbiology 68, 1994-2007.

Weber, A., Jørgensen, B.B., 2002. Bacterial sulfate reduction in hydrothermal sediments of the Guaymas Basin, Gulf of California, Mexico. Deep Sea Research Part I: Oceanographic Research Papers 49, 827-841.

White, D., Davis, W., Nickels, J., King, J., Bobbie, R., 1979. Determination of the sedimentary microbial biomass by extractible lipid phosphate. Oecologia 40, 51-62.

Wörmer, L., Lipp, J.S., Schröder, J.M., Hinrichs, K.-U., 2013. Application of two new LC-ESI-MS methods for improved detection of intact polar lipids (IPLs) in environmental samples. Organic Geochemistry 59, 10-21.

Zelles, L., Bai, Q., Beck, T., Beese, F., 1992. Signature fatty acids in phospholipids

and lipopolysaccharides as indicators of microbial biomass and community structure in agricultural soils. Soil Biology and Biochemistry 24, 317-323.

Zhu, R., Evans, T.W., Wörmer, L., Lin, Y.-S., Zhu, C., Hinrichs, K.-U., 2013. Improved sensitivity of sedimentary phospholipid analysis resulting from a novel extract cleanup strategy. Organic Geochemistry 65, 46-52.

Zink, K.G., Wilkes, H., Disko, U., Elvert, M., Horsfield, B., 2003. Intact phospholipids—microbial ‘‘life markers’’ in marine deep subsurface sediments. Organic Geochemistry 34, 755-769.

Chapter VI

Detection of microbial biomass in subseafloor sediment by pyrolysis-GC/MS

Rong Zhu*, Gerard J. M. Versteegh, Kai-Uwe Hinrichs

In preparation for *Journal of Analytical and Applied Pyrolysis*

Organic Geochemistry Group, MARUM Center for Marine Environmental Sciences & Department of Geosciences, University of Bremen, Leobener Str., 28359 Bremen, Germany

* Corresponding author. Email address: rongzhu1018@gmail.com (R. Zhu)

VI.1. ABSTRACT

The conventional approaches for qualitative and quantitative assessment of microbial biomass in sediments, e.g. via quantitative polymerase chain reaction or intact polar lipids analysis, are rather tedious and time-consuming. Therefore, we exploited the applicability of pyrolysis-gas chromatography/mass spectrometry for this purpose. Analysis of microbial cultures of different bacterial and archaeal lineages as well as reference substances and model compounds provided a molecular fingerprint for tracking microbial biomass. The diagnostic fingerprint consists of benzyl nitrile (derived from DNA and protein), 2-furanmethanol (from DNA and peptidoglycan), indole (from peptidoglycan and protein), phenol (from DNA, peptidoglycan and protein) and pyrrole (from peptidoglycan). Detection of microbial signals through this molecular fingerprint required a cell density of at least 10^6 cells/g, whereas at least 10^7 cells/g was necessary for quantification. The pyrolysis method was applied to marine sediments from different depths (until ~400 m below the seafloor at the Canterbury Basin) and with different organic carbon contents (0.11-6.99%). This approach enables rapidly screening of sediment samples for the presence of microbial signals and quantification bulk populations.

VI.2. INTRODUCTION

The conventional approaches for qualitative and quantitative assessment of microbial biomass in sediments, e.g. via cell counting, quantitative polymerase chain reaction, or intact polar lipids analysis, are rather tedious and time-consuming. Pyrolysis-gas chromatography/mass spectrometry (py-GC/MS) may provide an alternative route. Py-GC/MS is a useful technique for the characterization of non-volatile organic macromolecules in complex matrices. Potential advantages of a pyrolysis route for determination of biomass include minimal sample preparation and sample size (submilligram), rapid analysis and low cost (Meuzelaar et al., 1974; Stankiewicz et al., 1997a; Barshick et al., 1999).

During the past decades, py-GC/MS has been successfully employed to investigate a wide range of biological substances, such as DNA (Jarman, 1980; Poinar and Stankiewicz, 1999), proteins (Tsuge and Matsubara, 1985; Langhammer et al., 1986; Knicker et al., 2001), chitin (Lal and Hayes, 1984; van der Kaaden et al, 1984;

Sato et al., 1998), sporopollenin, algaenan and dinosporin (see review de Leeuw et al., 2006). Additionally, different strains or gram-type of microorganisms can be identified and differentiated through ratios of diagnostic fragments and multivariate statistics (e.g. principal component analysis, linear discriminant analysis; Smith et al, 1987; Barshick et al., 1999; Miketova et al., 2003; Snyder et al., 2004). In this way, the pyrolysis product 2-picolinamide has been identified in gram-positive (G_+) bacteria with a concentration approximately ten times higher than in gram-negative (G_-) bacteria and employed to assign bacterial gram-type (Dworzanski et al., 2005).

Tetramethylammonium hydroxide (TMAH) has been widely applied for online methylation, enabling assessment of the highly polar structural fragments of macromolecules (e.g. Schulten and Sorge, 1995; Fabbri and Helleur, 1999; Gallois et al., 2007; Grasset et al., 2009; Versteegh et al., 2012). As a strong base, TMAH is not solely involved in pyrolytic reactions but also participates in high temperature saponification or transesterification (Hatcher and Clifford, 1994). However, the methyl derivatives are formed not only through hydrolysis with TMAH but also through methanolysis, with the latter reaction resulting from methanol serving as solvent of TMAH (Ishida et al., 1995; Challinor, 2001). The use of a polar GC column enables separation and quantification of the released polar compounds (e.g. alcohols) without derivatization, which provides a direct way for assessing the released polar compounds and enables further investigation of pyrolytic mechanisms.

The aim of this study is to explore the application of py-GC/MS as an assay for rapid detection and quantification of microbial biomass in sediments, using a polar column and without derivatization.

VI.3. MATERIAL AND METHODS

VI.3.1. Material

To obtain pyrolysis fingerprints for detection of microbial biomass, materials selected for analysis were (i) amino sugars (e.g. *N*-Acetyl-D-glucosamine, GlcNAc); (ii) biopolymers (e.g. chitin from crab shells, peptidoglycan from *Staphylococcus aureus*, peptidoglycan from *Micrococcus luteus*, pBR322 DNA isolated from *Escherichia coli*, Bovine Serum Albumin) and (iii) microbial cultures (4 bacteria and 13 archaea; Table VI.1). The following low-molecular-weight compounds were used as proxy for the quantification of microbial biomass: benzyl nitrile, 2-furanmethanol,

indole, phenol and pyrrole. Naphthalene-D8 was employed as an internal standard and added before analysis. All the standards, biological material and the other chemicals were obtained from Sigma-Aldrich (Munich, Germany) and MBI Fermentas GmbH (St. Leon-Rot, Germany). The microbial cultures were either provided by Prof. Michael Thomm (University of Regensburg, Germany) or from our lab collections. Sediment samples from three locations, RV Sonne cruise 147 Station ST2, ODP Leg 201 Site 1229 and IODP Expedition 317 Site U1352, were used during method development and validation (Table VI.2).

Table VI.1. Microbial cultures analyzed.

Domains	Microorganism*
Bacteria	*Acetobacterium woodii* (G_+)
	Desulfotomaculum geothermicum (G_-)
	Desulfovibrio gigas (G_-)
	Thermotoga maritima (G_-)
Archaea	*Archaeoglobus fulgidus*
	Archaeoglobus profundus
	Methanobacterium thermoautotrophicum
	Methanococcus jannschii
	Methanococcus maripaludis
	Methanococcus thermolithotrophicus
	Methanosarcina barkeri
	Methanotorris igneus
	Pyrobaculum islandicum
	Pyrobaculum oguniense
	Pyrolcaculum calidifontis
	Pyrococcus furious
	Sulfolobus acidocaldarius

* Bacterial gram-types in parentheses; G_+, gram-positive; G_-, gram-negative.

VI.3.2. Sample preparation

Each sample was lyophilized over night, milled and homogenized using a CryoMill (Retsch, Haan, Germany; milling conditions: frequency of rod vibration, 20

s^{-1}; milling duration, 2 min; under liquid nitrogen) and loaded into a quartz tube (CDS Analytical Inc., Kamp Lintfort, Germany) prior to analysis.

The detection limit of the microbial signals was determined by diluting freeze-dried cultures of *Thermotoga maritima* and *Methanobacterium thermoautotrophicum* with de-activated silica gel in a concentration series of 10^4-10^{12} cells/g using one order of magnitude intervals; the respective py-GC/MS analysis was conducted in the order from low to high cell concentration. Silica gel was pre-combusted at 450°C overnight for de-activation in order to impede any adsorption of pyrolysis fragments, and analyzed by py-GC/MS for blank check.

Table VI.2. Marine sediment samples analyzed.

Location	Site	Depth (mbsf*)	TOC (%)
Peru Margin	R/V Sonne cruise 147 Station ST2	0.005	3.96
	ODP Leg 201 Site 1229	1.4	6.99
		102.2	2.28
Canterbury Basin	IODP Exp. 317 Site U1352	1.1	0.48
		11.6	0.13
		40.1	0.11
		137.3	0.18
		231.4	0.18
		403.0	0.20
		633.4	0.26
		816.3	0.15
		1113.4	0.15
		1164.2	0.30

* m below the seafloor

VI.3.3. Instrumentation

Py-GC/MS was performed using a CDS Pyroprobe 5000 series system (CDS Analytical Inc.) coupled to an Agilent 7890 GC/5975C VL MSD in electron ionization mode (Agilent Technologies Deutschland GmbH, Böblingen, Germany)

equipped with an Optima Wax column (25 m × 0.25 mm, 0.25 µm film thickness; Macherey-Nagel GmbH & Co. KG, Düren, Germany). The pyrolysis program was initiated at 200 °C and held for 1 s, then increased to 500 °C at 20 °C/min and held for 1 min. The GC temperature program was initiated at 50 °C and held for 0.5 min, increased to 200 °C at 4 °C/min and held for 5 min, then raised to 250 °C at 4 °C/min and held for 5 min, and finally decreased to 50 °C at 100 °C/min and held for 1 min. The MS was programmed in full scan (50-600 m/z) for characterization of the pyrolysis fragments and in selective ion monitoring mode for quantification. The pyrolysis fragments derived from biopolymers for tracking biomass and the corresponding ions monitored are listed in Table VI.3.

VI.4. RESULTS

VI.4.1. Pyrolysis fragments for tracking microbial biomass

A number of characteristic pyrolysis fragments have been identified from different biological sources (Table VI.3). The pyrolysis fragments were usually generated from multiple biopolymers, except for acetamidoacetaldehyde (only detected from GlcNAc), 2-picolinamide (only from peptidoglycan; Fig. VI.1), methyl-phenol, 3-methyl-butanenitrile and 4-methyl-pentanenitrile (from protein only). Most pyrolysis fragments were also detectable in the microbial cultures, with their relative abundances differed significantly (e.g. Fig. VI.2a, b, c). Levoglucosenone and acetamidoacetaldehyde were absent in the pyrolysis products of all microbes, whist 2-picolinamide was detectable in all cultures except in the two archaeal strains *Pyrobaculum oguniense* and *Sulfolobus acidocaldarius*.

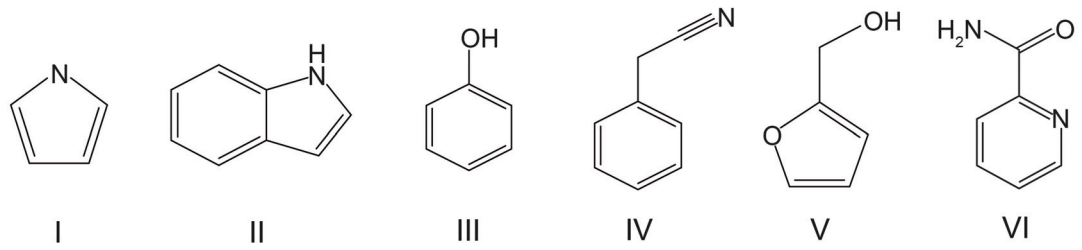


Fig. VI.1. Structures of the selected pyrolysis fragments from microbial biomass: (I) pyrrole, (II) indole, (III) phenol, (IV) benzyl nitrile, (V) 2-furanmethanol and (VI) 2-picolinamide.

Table VI.3. Selected pyrolysis fragments identified from the standards and microbial biomass in this study.

Formula	Pyrolysis fragment	Ions (m/z)	Major source[a]				
			Protein	Peptidoglycan	DNA	Chitin	GlcNAc[b]
C_8H_8	styrene	78, 104	1, 2, 8	14			
C_6H_6O	phenol[c]	66, 94	1, 6, 7, 8, 12, 14	14	14		
C_7H_8O	methyl-phenol	77, 90, 107	1, 6, 7, 8, 12				
$C_2H_4O_2$	acetic acid	43, 60	14	14	14	5, 14	
$C_3H_6O_2$	1-hydroxy-2-propanone	43, 74	14	14		5	
$C_5H_6O_2$	2-furanmethanol[c]	98		12, 14	12, 14		
$C_6H_6O_3$	levoglucosenone	53, 98		14		5, 14	
C_5H_9N	3-methyl-butanenitrile	43, 68	1				
$C_6H_{11}N$	4-methyl-pentanenitrile	43, 55, 82	1				
C_8H_7N	benzyl nitrile[c]	90, 117	1, 2, 12, 14		1		
C_5H_5N	pyridine	52, 79	1, 11, 12		12	5, 10, 14	9
C_6H_7N	methyl-pyridine	66, 78, 93	1				9
C_4H_5N	pyrrole[c]	67	1, 8, 14			10	
C_5H_7N	methyl-pyrrole	80, 81	1, 11			10	
C_8H_7N	indole[c]	90, 117	1, 2, 6, 7, 8, 11	14			
C_2H_5NO	acetamide	44, 59	14	3, 11, 14	14	5, 10, 14	
$C_4H_7NO_2$	acetamidoacetaldehyde	43, 72, 73					4, 14
$C_6H_6N_2O$	2-picolinamide	79, 122		13, 14			

Table VI.3. (continued)

[a] Numbers refer to studies reported sources of the corresponding pyrolysis fragments: 1. Simmonds, 1970; 2. Smith et al., 1974; 3. Hudson et al., 1982; 4. Franich et al., 1984; 5. van der Kaaden et al., 1984; 6. Tsuge and Matsubara, 1985; 7. Chiavari and Galletti, 1992; 8. Stankiewicz et al., 1997b; 9. Chen and Ho, 1998; 10. Sato et al., 1998; 11. Chefetz et al., 2002; 12. Snyder et al., 2004; 13. Dworzanski et al., 2005; 14. this study.

[b] GlcNAc refers to N-Acetylglucosamine.

[c] Compounds selected for identification and quantification of microbial biomass.

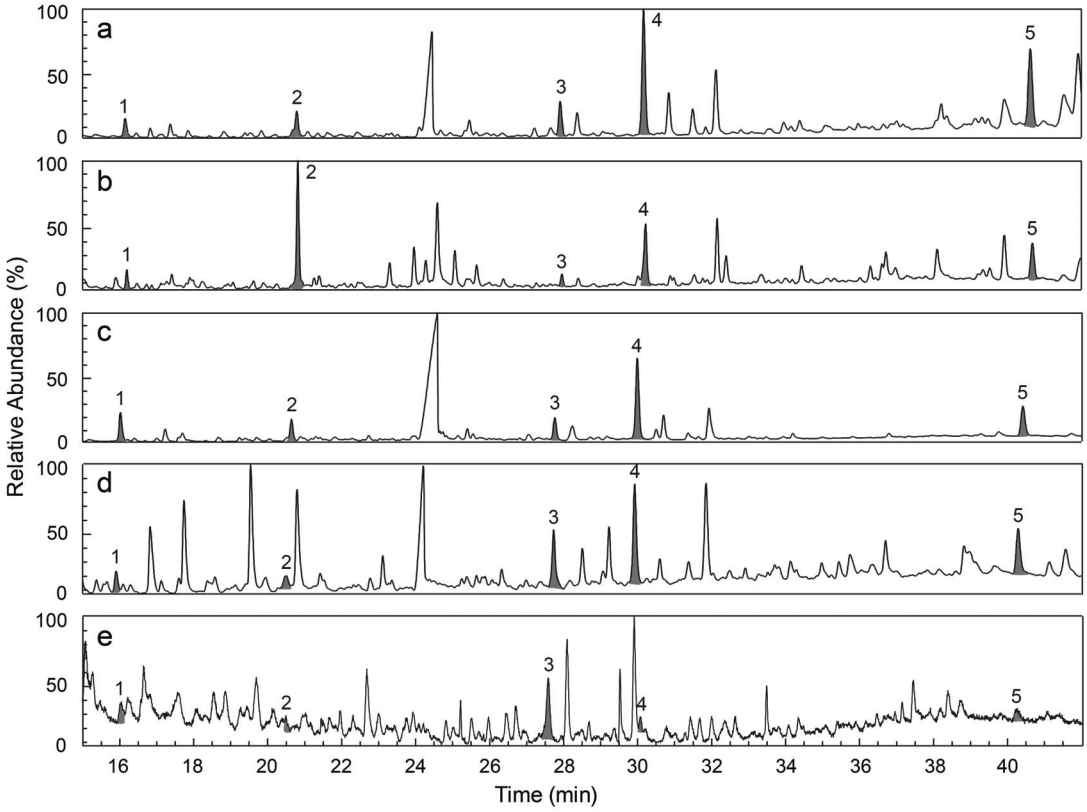


Fig. VI.2. Partial py-GC/MS chromatograms of (a) gram-positive bacterium *Acetobacterium woodii*, (b) gram-negative bacterium *Thermotoga maritima*, (c) archaeon *Methanosarcina barkeri*, (d) a surface sediment sample from Peru Margin (ST2 0-1, 0.005 mbsf) and (e) a subsurface sediment sample from Canterbury Basin (Site U1352B, 48X3, 402.9 mbsf). The filled peaks indicate the selected pyrolysis fragments for tracking microbial signals (1: pyrrole; 2: 2-furanmethanol; 3: benzyl nitrile; 4: phenol; 5: indole).

Clear differences in pyrolysate relative compositions were observed among microorganisms. However, with multivariate statistic analysis, these differences did not show a diagnostic pattern that can be used for distinguishing different lineages of microbial biomass (data not shown). Five pyrolysis fragments, i.e. benzyl nitrile (from DNA and protein), 2-furanmethanol (from DNA and peptidoglycan), indole (from peptidoglycan and protein), phenol (from DNA, peptidoglycan and protein) and pyrrole (from peptidoglycan; Fig. VI.1; Table VI.3), occurred consistently in all

pyrolysates and were selected as indicators for microbial biomass in sediments. Pyrolysis of these compounds exhibited highly linear relationships in a concentration range of 1-200 ng/µl for each standard and respective peak area (relative to the internal standard naphthalene-D8; Fig. VI.3).

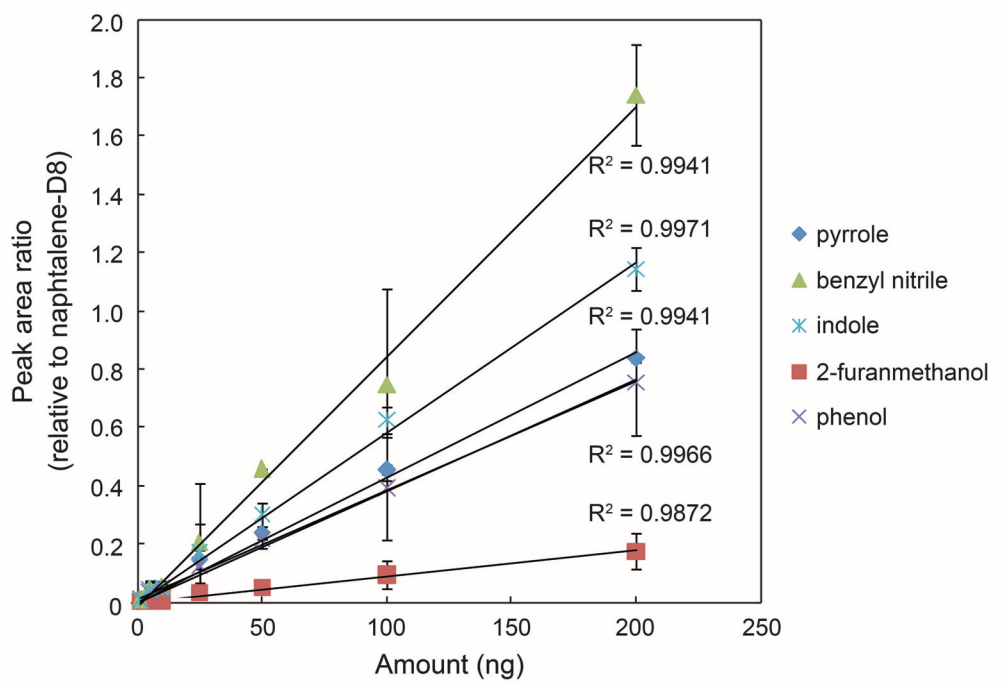


Fig. VI.3. Calibration curves of selected pyrolysis fragments between concentrations and corresponding peak area ratios (relative to internal standard naphthalene-D8). Error bars represent standard errors from triplicate measurements.

VI.4.2. Detection of microbial biomass in marine sediment

Analysis of microbial biomass *Thermotoga maritima* and *Methanobacterium thermoautotrophicum* in a dilution series demonstrated that the detection limit in terms of the selected pyrolysis fragments was equivalent to a cell density of 10^6 cells/g (e.g. *Thermotoga maritima*; Fig. VI.4). Concentrations of the selected pyrolysis fingerprints were nonlinearly correlated with microbial biomass for cell densities between 10^6-10^9 cells/g, whereas they increased linearly with cell densities above 10^9 cells/g. Relationship between the responses of all selected pyrolysis fingerprints and microbial cell density exhibited a similar trend compared to individual compounds, but the former started to increase at a cell density of 10^7 cells/g. Pyrolysis of marine sediments from different depths and different total organic carbon (TOC) contents (Table VI.2) showed that the selected pyrolysis

products were detectable until a sample depth of ~400 mbsf, with strongly different distributions of pyrolysis fingerprints among the samples (e.g. Fig. VI.2d, e).

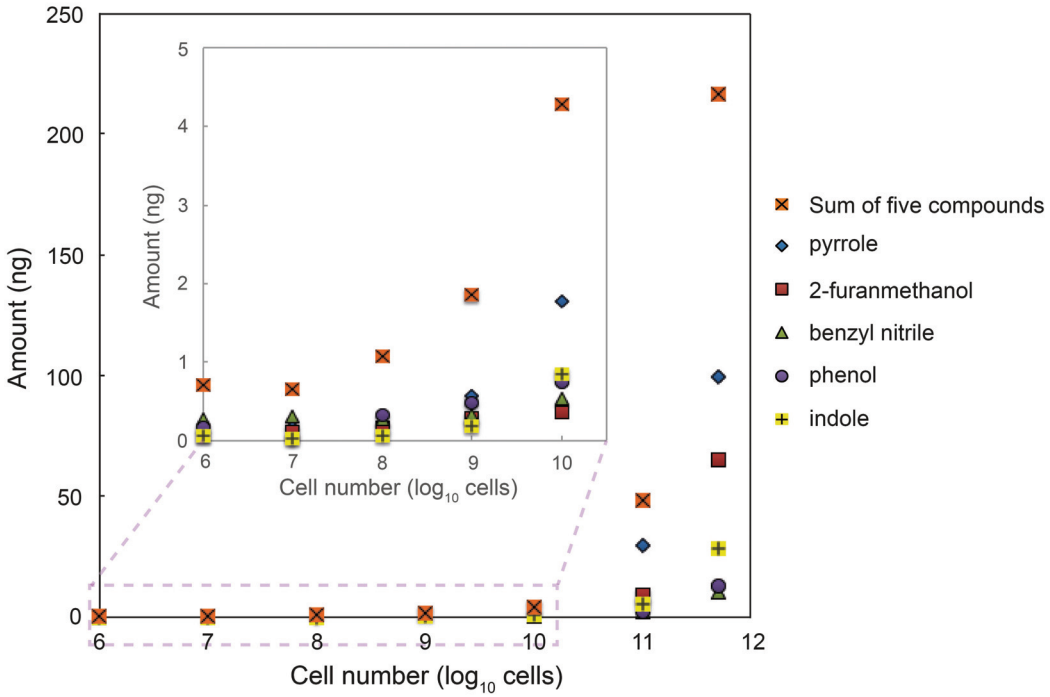


Fig. VI.4. Yields of the selected pyrolysis fragments upon pyrolysis of different numbers of *Thermotoga maritima* cells.

VI.5. DISCUSSION

The pyrolysis products generated from standards and biopolymers are generally identical to those reported previously in studies of isolated biopolymers (Table VI.3). We note that most of these products are also produced by pyrolysis of microbial biomass. The absense of levoglucosenone and acetamidoacetaldehyde in microbial biomass is ascribed to the low proportions of their precursors in combination with the limited sample sizes employed for pyrolysis. Peptidoglycan, for example, only accounts for < 2% of the dry cell weight for *Escherichia coli* (Mengin-Lecreulx and van Heijenoort, 1985). Although the proportion of peptidoglycan can be up to eight-fold higher in gram-positive bacteria (Schleifer and Kandler, 1972; Madigan and Martinko, 2005), levoglucosenone is still barely detectable due to the intense adjacent peaks. In contrast, 2-picolinamide is more abundant than leveglucosenone in the pyrolysis products of peptidoglycan, and consequently is detectable in the majority of investigated microbial biomass samples. Since archaea lack of peptidoglycan (Schleifer and Kandler, 1972; Madigan and Martinko, 2005), the presence of 2-

picolinamide both in bacteria and in most archaea suggests that 2-picolinamide orginates from rearrangement of peptide-derived amino acid-residues, because the peptides are significant components in bacterial peptidoglycan and most archaeal cell walls (Kandler and König, 1998).

Our selection of the pyrolysis fingerprints, i.e. benzyl nitrile, 2-furanmethanol, indole, phenol and pyrrole, for identification and quantification of microbial biomass, is based on the detectability of the compounds under our current analysis conditions. With the methodology applied, the minimum cell density required for tracking microbial signals, i.e. 10^6 cells/g, is consistent with the low range of cellular concentrations previously reported in marine continental margin sediment (Parkes et al., 2000), whereas the total responses of the selected pyrolysis products suggest that the threshold for quantification of microorganisms requires a cell density of > 10^7 cells/g. Among a cell density range of 10^6-10^9 cells/g, the nonlinear relationship between the cell numbers and the yields of the target compounds presumably due to a concentration dependent kinetic mechanism involved in formation of these compounds by py-GC/MS. It also can be attributed to limitation of the dilution technique, i.e. the calculated cell densities may not be accurate after 10^7-fold dilution. Previous studies have reported microbial populations in surface sediments in the orders of 10^8-10^9 cells (Parkes et al, 2000; Kallmeyer et al., 2012), which falls in the range for reliable quantification of microbial biomass by the current py-GC/MS approach. However, implementation of the approach for accurate determination of microbial population sizes in deep marine sediments, where cell densities tend to be lower, will be a challenge. Unexpectedly, the selected biomass fingerprint is detectable in deeply buried marine sediment until ~400 mbsf (Fig. VI.2e), where cell density is less than 10^5 cells/g (Ciobanu et al., in review). This finding suggests that a proportion of microbial debris or microbe-related compounds are preserved in marine sediments and the total determined pyrolysis fingerprint includes both active cells and necromass.

VI.6. CONCLUSION AND OUTLOOK

By analyzing a wide range of biological material using py-GC/MS, five pyrolysis products, i.e. benzyl nitrile, 2-furanmethanol, indole, phenol and pyrrole,

appear suitable for rapid detection and quantification of microbial biomass in marine sediment. Within a concentration range of 10^4-10^9 microbial cells per sample (equivalent to cell densities of 10^7-10^{12} cells/g), the GC/MS response of the selected pyrolysis products reliably reflects different concentrations of the source biomass, which validates the analytical approach. The detection limit of the approach is equivalent to a cell density of at least 10^6 cells/g, but at least 10^7 cells/g are necessary for quantification of microbial biomass. Analyses of sediment samples from different depths and with different TOC contents show that microbial signals can be tracked until ~400 mbsf at the Canterbury Basin (TOC 0.11%), indicating assemblages of active cells and necromass in the deep sediment. Combination with additional sample preparation steps (e.g. demineralization, density separation), or using a more advanced technique (e.g. multiple pyrolyses of one sample with cryotrapping, a more sensitive MS) may reduce the detection limit and enable accurate determination of microbial biomass in deeply buried sediments.

VI.7. ACKNOWLEDGEMENTS

Samples used in this study were retrieved during RV Sonne cruise 147, ODP Leg 201 and IODP Expedition 317. ODP Leg 201 is sponsored by the U.S. National Science Foundation (NSF) and participating countries under management of Joint Oceanographic Institutions (JOI), Inc., while IODP Expedition 317 is sponsored by the U.S. National Science Foundation (NSF), Japan's Ministry of Education, Culture, Sports, Science and Technology, and other IODP members. We thank the participating crews and scientists for sample recovery; Prof. M. Thomm, University of Regensburg, and other collaborators for providing the cultures; J. Lipp for valuable advices; R. Himmelsbach and S. Reiners for technical assistance. This study is sponsored by the Deutsche Forschungsgemeinschaft (DFG) through Grant HI 616/11-1 (Cell Surf) to K.-U.H. and the DFG Heisenberg Grants VE-486/2 and VE-486/3 to G.J.M.V. R.Z. is sponsored by the China Scholarship Council (CSC) and the Gottfried-Wilhelm Leibniz Program of the DFG (through the Leibniz Price to K.-U.H.).

VI.8. REFERENCES

Barshick, S., Wolf, D., Vass, A., 1999. Differentiation of microorganisms based on pyrolysis-ion trap mass spectrometry using chemical ionization. Analytical Chemistry 71, 633-641.

Challinor, J.M., 2001. Review: the development and applications of thermally assisted hydrolysis and methylation reactions. Journal of Analytical and Applied Pyrolysis 61, 3-34.

Chefetz, B., Tarchitzky, J., Deshmukh, A.P., Hatcher, P.G., Chen, Y., 2002. Structural characterization of soil organic matter and humic acids in particle-size fractions of an agricultural soil. Soil Science Society of America Journal 66, 129-141.

Chen, J., Ho, C.-T., 1998. Volatile compounds formed from thermal degradation of glucosamine in a dry system. Journal of Agricultural and Food Chemistry 46, 1971-1974.

Chiavari, G., Galletti, G.C., 1992. Pyrolysis–gas chromatography/mass spectrometry of amino acids. Journal of Analytical and Applied Pyrolysis 24, 123-137.

de Leeuw, J.W., Versteegh, G.J.M., van Bergen, P.F., 2006. Biomacromolecules of plants and algae and their fossil analogues. Plant Ecology 189, 209-233.

Dworzanski, J.P., Tripathi, A., Snyder, A.P., Maswdeh, W.M., Wick, C.H., 2005. Novel biomarkers for Gram-type differentiation of bacteria by pyrolysis–gas chromatography–mass spectrometry. Journal of Analytical and Applied Pyrolysis 73, 29-38.

Fabbri, D., Helleur, R., 1999. Characterization of the tetramethylammonium hydroxide thermochemolysis products of carbohydrates. Journal of Analytical and Applied Pyrolysis 49, 277-293.

Franich, R.A., Goodin, S.J., Wilkins, A.L., 1984. Acetamidofurans, acetamidopyrones, and acetamidoacetaldehyde from pyrolysis of chitin and N-acetylglucosamine. Journal of Analytical and Applied Pyrolysis 7, 91-100.

Gallois, N., Templier, J., Derenne, S., 2007. Pyrolysis-gas chromatography–mass spectrometry of the 20 protein amino acids in the presence of TMAH. Journal of Analytical and Applied Pyrolysis 80, 216-230.

Grasset, L., Rovira, P., Amblès, A., 2009. TMAH-preparative thermochemolysis for the characterization of organic matter in densimetric fractions of a Mediterranean forest soil. Journal of Analytical and Applied Pyrolysis 85, 435-441.

Hatcher, P.G., Clifford, D.J., 1994. Flash pyrolysis and *in situ* methylation of humic acids from soil. Organic Geochemistry 21, 1081-1092.

Hudson, J.R., Morgan, S.L., Fox, A., 1982. Quantitative pyrolysis gas chromatography-mass spectrometry of bacterial cell walls. Analytical Biochemistry 120, 59-65.

Ishida, Y., Ohtani, H., Tsuge, S., 1995. Effects of solvents and inorganic salts on the reactive pyrolysis of aromatic polyester in the presence of tetramethylammonium hydroxide studied by pyrolysis-gas chromatography/mass spectrometry. Journal of Analytical and Applied Pyrolysis 33, 167-180.

Jarman, M., 1980. Pyrolysis of deoxyribonucleic acid: isolation and mass spectrometry of individual pyrolysis products. Journal of Analytical and Applied Pyrolysis 2, 217-223.

Kallmeyer, J., Pockalny, R., Adhikari, R.R., Smith, D.C., D'Hondt, S., 2012. Global distribution of microbial abundance and biomass in subseafloor sediment. Proceedings of the National Academy of Sciences of the United States of America 109, 16213-16216.

Kandler, O., König, H., 1998. Cell wall polymers in Archaea (Archaebacteria). Cellular and Molecular Life Sciences 54, 305-308.

Knicker, H., del Río, J.C., Hatcher, P.G., Minard, R.D., 2001. Identification of protein remnants in insoluble geopolymers using TMAH thermochemolysis/GC–MS. Organic Geochemistry 32, 397-409.

Lal, G., Hayes, E., 1984. Determination of the amine content of chitosan by pyrolysis-gas chromatography. Journal of Analytical and Applied Pyrolysis 6, 183-193.

Langhammer, M., Lüderwald, I., Simons, A., 1986. Analytical pyrolysis of proteins. Fresenius' Journal of Analytical Chemistry 324, 5-8.

Madigan, M.T., Martinko, J.M., 2005. Brock Biology of Microorganisms 11th edn. Prentice Hall.

Mengin-Lecreulx, D., van Heijenoort, J., 1985. Effect of growth conditions on peptidoglycan content and cytoplasmic steps of its biosynthesis in Escherichia coli. Journal of Bacteriology 163, 208-212.

Meuzelaar, H.L., Kistemaker, P.G., Posthumus, M.A., 1974. Recent advances in pyrolysis mass spectrometry of complex biological materials. Biological Mass Spectrometry 1, 312-319.

Miketova, P., Abbas-Hawks, C., Voorhees, K.J., Hadfield, T.L., 2003. Microorganism Gram-type differentiation of whole cells based on pyrolysis high-resolution mass spectrometry data. Journal of Analytical and Applied Pyrolysis 67, 109-122.

Parkes, R.J., Cragg, B.A., Wellsbury, P., 2000. Recent studies on bacterial populations and processes in subseafloor sediments: a review. Hydrogeology Journal 8, 11-28.

Poinar, H.N., Stankiewicz, B.A., 1999. Protein preservation and DNA retrieval from ancient tissues. Proceedings of the National Academy of Sciences of the United States of America 96, 8426-8431.

Sato, H., Mizutani, S., Tsuge, S., Ohtani, H., Aoi, K., Takasu, A., Okada, M., Kobayashi, S., Kiyosada, T., Shoda, S., 1998. Determination of the degree of acetylation of chitin/chitosan by pyrolysis-gas chromatography in the presence of oxalic acid. Analytical Chemistry 70, 7-12.

Schleifer, K.H., Kandler, O., 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriological Reviews 36, 407-477.

Schulten, H.R., Sorge, C., 1995. Pyrolysis methylation mass spectrometry of whole soils. European Journal of Soil Science 46, 567-579.

Simmonds, P., 1970. Whole microorganisms studied by pyrolysis-gas chromatography-mass spectrometry: significance for extraterrestrial life detection experiments. Applied and Environmental Microbiology 20, 567-572.

Smith, C.S., Morgan, S.L., Parks, C.D., Fox, A., Pritchard, D.G., 1987. Chemical marker for the differentiation of group A and group B streptococci by pyrolysis-gas chromatography-mass spectrometry. Analytical Chemistry 59, 1410-1413.

Smith, W.T., Harris, T.B., Patterson, J.M., 1974. Pyrolysis of soybean protein and an amino acid mixture having the same amino acid composition. Journal of Agricultural and Food Chemistry 22, 480-483.

Snyder, A.P., Dworzanski, J.P., Tripathi, A., Maswadeh, W.M., Wick, C.H., 2004. Correlation of mass spectrometry identified bacterial biomarkers from a fielded pyrolysis-gas chromatography-ion mobility spectrometry biodetector with the microbiological gram stain classification scheme. Analytical Chemistry 76, 6492-6499.

Stankiewicz, B.A., Briggs, D.E., Evershed, R.P., 1997a. Chemical composition of Paleozoic and Mesozoic fossil invertebrate cuticles as revealed by pyrolysis-gas chromatography/mass spectrometry. Energy & Fuels 11, 515-521.

Stankiewicz, B.A., Hutchins, J.C., Thomson, R., Briggs, D.E., Evershed, R.P., 1997b. Assessment of bog-body tissue preservation by pyrolysis-gas chromatography/mass spectrometry. Rapid Communications in Mass Spectrometry 11, 1884-1890.

Tsuge, S., Matsubara, H., 1985. High-resolution pyrolysis-gas chromatography of proteins and related materials. Journal of Analytical and Applied Pyrolysis 8, 49-64.

van der Kaaden, A., Boon, J., de Leeuw, J., de Lange, F., Schuyl, P., Schulten, H., Bahr, U., 1984. Comparison of analytical pyrolysis techniques in the characterization of chitin. Analytical Chemistry 56, 2160-2165.

Versteegh, G.J., Blokker, P., Bogus, K.A., Harding, I.C., Lewis, J., Oltmanns, S., Rochon, A., Zonneveld, K.A., 2012. Infra red spectroscopy, flash pyrolysis, thermally assisted hydrolysis and methylation (THM) in the presence of tetramethylammonium hydroxide (TMAH) of cultured and sediment-derived *Lingulodinium polyedrum* (Dinoflagellata) cyst walls. Organic Geochemistry 43, 92-102.

Chapter VII

Concluding remarks and perspectives

VII. 1. SUMMARY AND CONCLUSIONS

Gene- and cultivation-independent biomarker approaches have been widely employed for investigation of microbial communities in various marine environments (e.g. Lipp et al., 2008; Schubotz et al., 2009; Lomstein et al., 2012). However, the physiologies and roles of microorganisms in marine deep biosphere remain elusive, in most of the cases due to complex sample matrices and/or low concentrations of the respective biomarkers in marine sediments. Facing these obstacles, three independent methods have been developed targeting biomarkers with different biochemical functions, i.e. amino sugars, phospholipids (PLs) and low-molecular weight compounds produced during pyrolysis of microbial biomass. Each resulting method was subsequently applied to constrain microbial activity, community composition and metabolisms in a selected set of marine subseafloor sediments.

The first part of the thesis (Chapters II and III) focused on quantitative and stable carbon isotopic analysis of amino sugars. Amino sugars are significant sedimentary components largely derived from microorganisms and invertebrates, and have been widely applied as a molecular proxy to study microbially mediated transformation of organic matter (OM) and indicate microbial community compositions in soil science (e.g. Guggenberger et al., 1999; Zhang et al., 1999; Glaser et al., 2004). In order to establish a robust protocol for stable carbon isotopic analysis of amino sugars in marine sediments, we tested various preparation steps previously reported for analysis of amino sugar in soils with marine sediments, including acid hydrolysis, purification, and derivatization. The combination of the most effective steps resulted in a preparation protocol adapted for gas chromatography (GC)-based isotopic analysis of amino sugars at trace levels (limit of detection = 20 ng per amino sugar; Chapter II). The resulting protocol significantly reduced the limit of precise analysis of amino sugar isotopic composition by a factor of ten compared to the high performance liquid chromatography (HPLC)-based method (Bodé et al., 2009). Moreover, use of the protocol did not introduce significant isotopic fractionation during sample preparation, except for the derivatization step, which can be corrected for with a derivatization standard. The optimized protocol involves acid hydrolysis of the sediments with hot HCl (Zhang and Amelung, 1996), neutralization with KOH and desalting with a Supelclean^{TM} ENVI-Carb^{TM} Plus SPE cartridge, conversion of amino sugars into aldononitrile acetates (ANA; Guerrant and Moss, 1984) and purification of the ANA

derivatives with silica gel column (Lin et al., 2010). For samples that exhibit low signals of muramic acid (MurA) and/or mannosamine (ManN) due to the high background generated by sedimentary matrix or high abundance of the adjacent peak of galactosamine (GalN), amino sugar extracts are separated into two fractions, i.e. hexosamine fraction and MurA fraction, with preparative HPLC. Each resulting fractions can be further concentrated for GC-IRMS analysis.

By applying this protocol to sediments from two locations, ODP Leg 201 Site 1229 at the Peru Margin and IODP Expedition 317 Site U1352 at the Canterbury Basin, comprehensive information of the microbial community in subseafloor sediments was obtained (Chapter III). The total amino sugars accounted for minor to substantial proportions of the OM (e.g. 0.4-5.0% of the total organic carbon, TOC) at both sites. Downcore distributions and ratios of amino sugars are distinctive at the two sites, reflecting different OM type, i.e. organic-rich and predominant prokaryotic sources of the marine OM in Peru sediments (D'Hondt et al., 2003), and organic-lean and dominant terrestrial input of OM in the Canterbury Basin (Fulthorpe et al., 2011).

MurA is a diagnostic bacterial biomarker that is exclusively derived from bacterial cell walls (Schleifer and Kandler, 1972; Madigan and Martinko, 2005) and has been presumed to decompose in surface sediments on timescales of days (King and White, 1977; Moriarty, 1977). However, compiling the distributions of MurA (Niggemann and Schubert, 2006; Lomstein et al., 2009; Carstens and Schubert, 2012; Lomstein et al., 2012; this study) and microbial cell counts (Parkes et al., 2000; D'Hondt et al., 2003; Ciobanu et al., in review) in sediments of various types and from different sites, we estimated the intracellular MurA generally accounted for < 15% of the total MurA in sediments, i.e. the majority of MurA is not associated with active bacteria, which challenges the application of MurA as a strict biomarker for quantification of bacterial population in the marine deep biosphere. Even though increased concentrations of amino sugars were observed in the sulfate-methane transition zones (SMTZs) of both sites, which host elevated concentrations of microbial cells (D'Hondt et al., 2003; Ciobanu et al., in review), indicating increased contribution from microbial biomass in these horizons. Notably, significant ^{13}C-depletion of MurA and ManN was observed in the SMTZs of both sites, indicating the isotopic imprints of indigenous microorganisms that assimilate methane-derived carbon at these geochemical interfaces.

The second part of this thesis (Chapter IV and V) aims to decipher the composition of microbial communities in marine sediments by quantitative PL analysis with LC-electrospray ionization (ESI)-MS. PLs are principal constituents of microbial cytoplasmic membranes and bear chemotaxonomic information (e.g. Fang et al., 2000; Zink et al., 2003; Sturt et al., 2004; Schubotz et al., 2009). As they decompose rapidly upon cell lysis (White et al., 1979; Harvey et al., 1986; Logemann et al., 2011), analysis of PLs can provide direct clues on composition and population of the living microbial biomass in environmental samples. However, such analysis in the subseafloor sediments is usually constrained by very low PL concentrations and relatively high detection limit caused by strong ion suppression of sedimentary matrices during LC-ESI-MS analysis. In this context, a cleanup protocol using a HybridSPE®-Phospholipid cartridge for purification of PLs was adapted for analysis of PLs in sedimentary lipid extracts (Chapter IV). The HybridSPE cartridge is filled with zirconia-coated sorbent and exhibits high affinity for PLs due to the Lewis acid-base interaction between zirconia and phosphate moieties, whereas the retention of a wide range of basic, neutral and acidic compounds is minimal. The majority of sedimentary matrix components interfering with ESI-MS analysis therefore can be removed by this cleanup protocol, resulting in a relatively matrix-free PL fraction and consequently enhanced PL ionization efficiency and significantly lowered detection limits of PLs. Compared to the untreated total lipid extract (TLE) or cleanup with the conventional silica gel chromatography, the HybridSPE-based protocol significantly resulted in an up to threefold higher detectability of PLs. Discriminations among different PL types were negligible for the analysis of samples at different depths and with different TOC contents. Application of the cleanup protocol to sediment samples revealed trace quantities of some indigenous PLs that were only detectable after the cleanup. This implies that more comprehensive microbial PL fingerprints can be revealed after the HybridSPE cleanup protocol.

Sediments from hydrothermal field of the Guaymas Basin host a large variety of microorganisms in all three domains of life (Teske et al., 2002; Weber and Jørgensen, 2002; Dhillon et al., 2005). However, analysis of microbial biomarkers in the untreated TLE using LC-ESI-MS suffers strong interferences of the oily matrices. With our HybridSPE-based cleanup protocol, indigenous PLs can be extracted from the oily matrices, resulting in improved detection of PLs on LC-ESI-MS and

comprehensive PL fingerprints for decoding microbial communities in the Guaymas sediments (Chapter V). In the hydrothermal site, where sedimentary temperature steeply increased from ~3°C to ~100°C within 35 cm, PLs were still detectable up to a maximum sedimentary temperature of ~90°C, indicating the presence of thermophilic microorganisms in these heated sediments. Distributions of PLs exhibited remarkable structural differences between the hydrothermal and non-hydrothermal sites. For example, PLs with phosphocholine (PC) or phosphoethanolamine (PE) head groups accounted for > 69% of the total PLs in surface sediments of the hydrothermal site and increased to 100% below 14 cmbsf, whereas PC/PE accounted for 52-85% of the total PLs in the non-hydrothermal site with a maximum proportion observed at 4.5 cmbsf. The lower PL diversity in the hydrothermal site suggested the presence of a relatively simple but specialized thermophilic microbial community compared to the non-hydrothermal site.

In the third part of the thesis (Chapter VI), a pyrolysis (py)-GC/MS based protocol was introduced for rapid determination of microbial biomass in marine sediments. Py-GC/MS is an efficient technique to characterize nonvolatile organic macromolecules with minimal sample pretreatment. Analysis of different bacterial and archaeal cultures as well as reference biological material using py-GC/MS provided pyrolysis fingerprints for tracking microbial biomass. The diagnostic fingerprints consists of benzyl nitrile (from DNA and protein), 2-furanmethanol (from DNA and peptidoglycan), indole (from peptidoglycan and protein), phenol (from DNA, peptidoglycan and protein) and pyrrole (from peptidoglycan) were selected for tracking microbial signals in several mg-sized samples of marine sediments. Analysis of microbial biomass in a dilution series showed that the detection limit in terms of the selected pyrolysis fragments was equivalent to a cell density of 10^6 cells/g, whereas quantification of active microbial biomass required a cell density of at least 10^7 cells/g. With the py-GC/MS approach, microbial signals in the form of the pyrolysis fingerprints were detectable in marine sediments at different subseafloor depths (until ~400 m below the seafloor at the Canterbury Basin) and with different organic carbon contents (0.11-6.99%). In the deeply buried sediments of the Canterbury Basin, microbial signals were still detectable at depth where living microbial cells were at least one order of magnitude lower than the minimal cell density required by the py-GC/MS protocol (Ciobanu et al., in press), suggesting that

the assessed signals were assemblages of active cells and necromass and presumed predominantly from fossil microbial biomass.

To summarize, the three independent methods were developed for investigation of microbial communities through analysis of different groups of biomarkers. Each method has its advantages as well as limitations. The py-GC/MS approach provides a promising perspective for rapid detection of microbial signals with minimal sample preparation and sample size, but the current approach quantifies the microbial population as assemblages of active cells and necromass in marine sediments. Analysis of amino sugars can reveal more information on microbial contributions to marine OM, microbial community compositions and their metabolisms. Although a large proportion of amino sugars are associated with microbial necromass, stable carbon isotopic compositions of amino sugars, in particular for MurA and ManN, exhibit isotopic imprints of indigenous microorganisms in sediment horizons of enhanced microbial activities (e.g. in SMTZ or cold seep sample). By contrast, analysis of PLs only targets active microbial biomass and can provide additional clues for assessing microbial community population and composition. However, such analysis is preferable for investigation of bacteria and eukaryotes, as archaeal-derived PLs are barely detectable in subseafloor sediments. In this regard, the combination of analyses of different biomarkers will result in more comprehensive information for deciphering microbial communities in the deep biosphere.

VII. 2. PERSPECTIVES

Methods developed in this thesis for different groups of microbial biomarkers, i.e. amino sugars, PLs and pyrolysis products of microbial biomass and selected biopolymers extend the toolbox for the investigation of microbial communities in environmental samples. The application of theses method provides new information on microbial communities in the deep biosphere. Of particular interests for the future work is detection of bacterial signals in the deeply buried subseafloor sediments via intact PLs analysis. By applying the developed cleanup protocol, large quantity of sedimentary extract can be concentrated to a desired level for LC analysis with minimal background interference, thus can provide substantial clues on the abundance, composition and metabolism of the vegetative bacterial communities. In combination

with microbiological molecular techniques will result in better illumination of microbial communities and their biogeochemical processes in the deep biosphere.

However, I also note that each biomarker study in this thesis has unsolved questions and/or need to be further improved.

- Recent studies showed the endospore population was on the order of 10^7 cells/ml sediments in sediments from the Aarhua Bay and the Peru Margin (Langerhuus et al., 2012; Lomstein et al., 2012). Analysis of dipicolinic acid in sediments from different regions will provide a global distribution of endospores. It also helps to figure out the proportions of endospore-derived MurA in the MurA pool. If substantial abundance of endospores do present in the deep biosphere as Lomstein et al. (2012) suggested, we should expect very minor fractions of extracellular MurA in the subseafloor sediments. Therefore, MurA could be an useful biomarker targeting both bacteria and endospores in the deep biosphere.
- Analysis of intact PLs in oil-bearing sediments of Guaymas Basin revealed the presence of living microbial biomass and their diversity in the hydrothermal site. Further phylogenetic studies will provide additional information of the microbial community composition. Future works involving analysis of PL-derived FA (PLFA) on GC-MS will reveal more comprehensive PL fingerprints of microbial communities and may also illuminate specific microbial groups. In addition, analysis of stable carbon isotopic composition of PLFA will provide new information on microbial metabolisms in the Guaymas sediments.
- Compared to the conventional biomarker approaches, the py-GC/MS technique provides an alternative way for rapid determination of microbial biomass in sediments. However, due to limited sample size for pyrolysis, detection limit of the current py-GC/MS approach is not satisfying for samples consisting a cell density below 10^7 cells/g. In combination with additional sample preparation steps (e.g. demineralization, density separation), or using a more advanced technique (e.g. multiple pyrolyses of one sample with cryotrapping, a more sensitive MS) may reduce the detection limit. During demineralization or density separation, extracellular fragments can be separated from the living microbial cells (Kallmeyer et al., 2008), thus enabling qualitative and quantitative assessment of living microbial biomass in marine sediments. In addition, this

technique could be further developed into sensitive detection of biomolecules in astrobiological studies.

VII. 3. REFERENCES

Amelung, W., Cheshire, M.V., Guggenberger, G., 1996. Determination of neutral and acidic sugars in soil by capillary gas-liquid chromatography after trifluoroacetic acid hydrolysis. Soil Biology and Biochemistry, 28, 1631-1639.

Carstens, D., Schubert, C.J., 2012. Amino acid and amino sugar transformation during sedimentation in lacustrine systems. Organic Geochemistry 50, 26-35.

Cowie, G.L., Hedges, J.I., 1984. Determination of neutral sugars in plankton, sediments, and wood by capillary gas chromatography of equilibrated isomeric mixtures. Analytical Chemistry, 56, 497-504.

Dhillon, A., Lever, M., Lloyd, K.G., Albert, D.B., Sogin, M.L., Teske, A., 2005. Methanogen diversity evidenced by molecular characterization of methyl coenzyme M reductase A (mcrA) genes in hydrothermal sediments of the Guaymas Basin. Applied and environmental microbiology 71, 4592-4601.

D'Hondt, S.L., Jørgensen, B.B., Miller, D.J. and Shipboard Scientific Party, 2003. Proc. ODP Init. Repts. 201. Ocean Drilling Program, Texas A & M University, College Station, TX. doi:10.2973/odp.proc.ir.201.2003.

Fang, J., Barcelona, M.J., Semrau, J.D., 2000. Characterization of methanotrophic bacteria on the basis of intact phospholipid profiles. FEMS Microbiology Letters 189, 67-72.

Fulthorpe, C.S., Hoyanagi, K., Blum, P., and the Expedition 317 Scientists, 2011. Proc. IODP 317: Tokyo (Integrated Ocean Drilling Program Management International, Inc.). doi:10.2204/iodp.proc.317.2011.

Glaser, B., Turrión, M., Alef, K., 2004. Amino sugars and muramic acid--biomarkers for soil microbial community structure analysis. Soil Biology and Biochemistry 36, 399-407.

Guerrant, G., Moss, C., 1984. Determination of monosaccharides as aldononitrile, O-methyloxime, alditol, and cyclitol acetate derivatives by gas chromatography. Analytical Chemistry 56, 633-638.

Guggenberger, G., Frey, S., Six, J., Paustian, K., Elliott, E., 1999. Bacterial and fungal cell-wall residues in conventional and no-tillage agroecosystems. Soil Science Society of America Journal 63, 1188-1198.

Harvey, H.R., Fallon, R.D., Patton, J.S., 1986. The effect of organic matter and oxygen on the degradation of bacterial membrane lipids in marine sediments. Geochimica et Cosmochimica Acta 50, 795-804.

Kallmeyer, J., Smith, D.C., Spivack, A.J., D'Hondt, S., 2008. New cell extraction procedure applied to deep subsurface sediments. Limnology and Oceanography: Methods 6, 236-245.

King, J.D., White, D.C., 1977. Muramic acid as a measure of microbial biomass in

estuarine and marine samples. Applied and Environmental Microbiology 33, 777-783.

Langerhuus, A.T., Røy, H., Lever, M.A., Morono, Y., Inagaki, F., Jørgensen, B.B., Lomstein B.A., 2012. Endospore Abundance and d:l-Amino Acid Modeling of Bacterial Turnover in Holocene Marine Sediment (Aarhus Bay). Geochimica et Cosmochimica Acta 99, 87-99.

Lin, Y.-S., Lipp, J.S., Yoshinaga, M.Y., Lin, S.H., Elvert, M., Hinrichs, K.-U., 2010. Intramolecular stable carbon isotopic analysis of archaeal glycosyl tetraether lipids. Rapid Communications in Mass Spectrometry 24, 2817-2826.

Lipp, J.S., Morono, Y., Inagaki, F., Hinrichs, K.-U., 2008. Significant contribution of Archaea to extant biomass in marine subsurface sediments. Nature 454, 991-994.

Logemann, J., Graue, J., Köster, J., Engelen, B., Rullkötter, J., Cypionka, H., 2011. A laboratory experiment of intact polar lipid degradation in sandy sediments. Biogeosciences 8, 2547-2560.

Lomstein, B., Niggemann, J., Jørgensen, B., Langerhuus, A., 2009. Accumulation of prokaryotic remains during organic matter diagenesis in surface sediments off Peru. Limnology and Oceanography 54, 1139-1151.

Lomstein, B.A., Langerhuus, A.T., D'Hondt, S., Jørgensen, B.B., Spivack, A.J., 2012. Endospore abundance, microbial growth and necromass turnover in deep sub-seafloor sediment. Nature 484, 101-104.

Madigan, M.T., Martinko J.M., 2005. Brock Biology of Microorganisms 11th edn. Prentice Hall, Upper Saddle River, USA.

Moriarty, D.J.W., 1977. Improved method using muramic acid to estimate biomass of bacteria in sediments. Oecologia 26, 317-323.

Niggemann, J., Schubert, C., 2006. Sources and fate of amino sugars in coastal Peruvian sediments. Geochimica et Cosmochimica Acta 70, 2229-2237.

Parkes, R.J., Cragg, B.A., Wellsbury, P., 2000. Recent studies on bacterial populations and processes in subseafloor sediments: a review. Hydrogeology Journal 8, 11-28.

Schleifer, K., Kandler, O., 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriological Reviews 36, 407-477.

Schubotz, F., Wakeham, S.G., Lipp, J.S., Fredricks, H.F., Hinrichs, K.-U., 2009. Detection of microbial biomass by intact polar membrane lipid analysis in the water column and surface sediments of the Black Sea. Environmental Microbiology 11, 2720-2734.

Sturt, H.F., Summons, R.E., Smith, K., Elvert, M., Hinrichs, K.-U., 2004. Intact polar membrane lipids in prokaryotes and sediments deciphered by high-performance liquid chromatography/electrospray ionization multistage mass spectrometry--new biomarkers for biogeochemistry and microbial ecology. Rapid Communications in Mass Spectrometry 18, 617-628.

Teske, A., Hinrichs, K.-U., Edgcomb, V., de Vera Gomez, A., Kysela, D., Sylva, S.P., Sogin, M.L., Jannasch, H.W., 2002. Microbial diversity of hydrothermal sediments in the Guaymas Basin: evidence for anaerobic methanotrophic communities. Applied and Environmental Microbiology 68, 1994-2007.

Weber, A., Jørgensen, B.B., 2002. Bacterial sulfate reduction in hydrothermal sediments of the Guaymas Basin, Gulf of California, Mexico. Deep Sea Research Part I: Oceanographic Research Papers 49, 827-841.

White, D., Davis, W., Nickels, J., King, J., Bobbie, R., 1979. Determination of the sedimentary microbial biomass by extractible lipid phosphate. Oecologia 40, 51-62.

Zhang, X., Amelung, W., 1996. Gas chromatographic determination of muramic acid, glucosamine, mannosamine, and galactosamine in soils. Soil Biology and Biochemistry 28, 1201-1206.

Zhang, X., Amelung, W., Yuan, Y., Samson-Liebig, S., Brown, L., Zech, W., 1999. Land-use effects on amino sugars in particle size fractions of an Argiudoll. Applied Soil Ecology 11, 271-275.

Zink, K.G., Wilkes, H., Disko, U., Elvert, M., Horsfield, B., 2003. Intact phospholipids—microbial ‘‘life markers’’ in marine deep subsurface sediments. Organic Geochemistry 34, 755-769.

ACKNOWLEDGEMENTS

It is a long journey to get here. I feel very luck and grateful to have so many people help me and encourage me during these years. Without their continuous help, I cannot get here.

The deepest thanks certainly go to my supervisor, Prof. Dr. Kai-Uwe Hinrichs. I thank him for accepting me as his PhD student, giving me the opportunity to work under his supervision and leading me to the fascinating world of the deep biosphere. He continued to guide me by inspiring ideas and fruitful discussion. I especially appreciate his trust, encouragement, and continuous support throughout the years. Moreover, I would not be able to finish my research without the generous financial supports from Kai through the Gottfried-Wilhelm Leibniz Price. I also thank Prof. Dr. Wolfgang Bach, who agreed to be my second supervisor on short notice.

All of my work would not have been possible without the assistance of Dr. Julius Lipp and Dr. Yu-Shih Lin. I always had a lot of questions and troubles bothering you. Your patience, encouragement and valuable suggestions always inspired me.

I enjoyed very much and benefited a lot from our group members. Dr. Gerard Versteegh, thank your for joining the pyrolysis project. I gained a lot from your guidance on designing the project and fruitful discussion. Dr. Travis Meador, thanks for your constructive suggestions and comments on the amino sugar project, as well as for helping me revise the introduction and data report chapters during your Christmas holidays. Dr. Marcos Yoshinaga, thank you for your comments and constructive ideas on interpretation of amino sugar and PL data. Dr. Lars Wörmer, Thomas Evans and Dr. Chun Zhu, thanks for your contributions during development of the PL cleanup method. Dr. Marcus Elvert, thank your for teaching me to interpret the GC-MS data and discussing about the PLFA analysis. Dr. Verena Heuer, thank you for showing the HPLC-IRMS system to me when I was fresh here and completely knew nothing about that machine. Felix Elling, thanks a lot for helping me to translate the abstract during your new year's holiday. Kevin Becker, Jan Schröder and Dr. Xiao-Lei Liu, thank you for your help with the q-TOF, discussion and other things. Dr. Sitan Xie, thank you for picking up me when I arrived in Bremen for the first time and showing me the detailed steps of your IPL-based model. Many thanks to Guangchao, who encouraged me and helped me all the time.

I would like to thank Xavier Prieto-Mollar, Jenny Wendt, Jessica Arndt, Raike Himmelsbach and Benrard Viehweger, who always helped me with the instruments, ordering lab staff and all the other support in the lab. Many thanks to our secretary, Birgit Schmincke, who is always very patient and helped me with all the administrative stuff; Dr. Heidi Taubner and Evert Kramer, who take well charge of our samples and computer system, respectively. My gratitude is also to Florences, Matthias, Arne, Shuchai, Huangmin, Nadine, Martin, Xiaoxia, Frauke, Weichao, Richi, Marshall and all my colleagues. The working atmosphere has been so wonderful and enjoyable.

I also thank Dr. Mark Lever and Prof. Bo Jørgensen from University of Aarhus, who organized and supported me for the cruise to the Aarhus Bay.

I want to thank my Chinese friends in Bremen, Xin, Yancheng, Enqing, Li, Xu, Wenwen, Yi, Mingming, Kai, Gao, Zhuo, Hang, Tingting, Jianping, and other friends who have spent great time chatting with me and helped me. Because of you, my life in Bremen is enjoyable and full of wonderful memories. I need to thank Ursula for teaching me German, although I was not a good student.

A special thanks to Bin, who accompanied me walking through the last year of my PhD. It was you to make the darkest time of my PhD full of sunshine. Thank you for taking good care of me, sharing so much joy with me and continuously encouraging me. Most importantly, I am very grateful that you walk into my life and always give me numerous surprises, which makes my life delightful and colorful.

感谢毕彩丰教授，范玉华教授多年来对我的培养，关心以及对我出国留学的支持。感谢国内的小伙伴们，你们的支持和关心，一路陪伴着我；特别感谢磊，总在我最需要的时候帮助我、鼓励我，你给我的最贴心的温暖，给了我克服所有困难的勇气。

感谢我的爸爸妈妈。四年前你们克服了无比的担心和不舍，支持我出国读书的决定。你们对我无尽的支持和鼓励，让我即使远在他乡也能时时刻刻都感受着你们的爱。感谢我的小姨、小姨夫，谢谢你们多年来对我学习生活上的极大的关

心和帮助。谢谢三姨、三姨夫、姑姑、姥爷以及其他亲人们对我的关心和爱护，谢谢你们！

感谢国家留学基金委（CSC）为我提供三年奖学金资助。

衷心感谢所有关心、帮助过我的人。

N a m e : ..... Datum .....

Anschrift : .....

E r k l ä r u n g

Hiermit versichere ich, dass ich

1. die Arbeit ohne unerlaubte fremde Hilfe angefertigt habe,

2. keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe und

3. die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

_____, den .....

(Unterschrift)