Intact membrane lipids as tracers for microbial life in the marine deep biosphere

Dissertation
zur Erlangung des Doktorgrades
der Naturwissenschaften
- Dr. rer. Nat. –

Am Fachbereich Geowissenschaften

der Universität Bremen

vorgelegt von

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Bremen

Mai 2008

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Science may set limits to knowledge, but should not set limits to imagination.

Bertrand Russel, 1872-1970.

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THESIS ABSTRACT

The main objective of this thesis was to elucidate the structure and carbon metabolism of microbial communities living in deeply buried sediments by using intact polar lipids (IPL) as markers for active microbial cells. A globally distributed sample set obtained during ODP Legs 201, 204, and 207, IODP Expeditions 301 and 311, and cruises Sonne SO147, Karei KY04-11, and Professor Logatchev TTR15 was analyzed to constrain the composition and quantity of deep marine subseafloor life on a global scale. Surface sediments contained abundant bacterial phospholipids having phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylcholine as head groups with C16 and C18 acyl side chains, whereas the deeper sediment layers were dominated by archaeal glycolipids of tetraether and diether type with diglycosidic head groups. The transition from bacterial to archaeal lipids occurred within the top 0.1 mbsf of the sediment column. These results contrast previous studies based on microbiological methods which have identified a dominance of viable bacteria in deeply buried sediments. Supporting evidence comes from (*i*) "traditional" analysis of phospholipid-derived fatty acids which confirmed the low contribution of bacteria to the total population, and (*ii*) improved microbiological methods which detected a higher proportion of archaeal cells compared to previous studies that possibly discriminated against archaeal phylogenetic lineages.

Furthermore a decrease in IPL concentrations with depth was observed, following a similar relationship as previously seen for directly counted cells. IPL concentration was found to be dependent on concentration of sedimentary organic carbon, reflecting the inherent heterotrophic nature of this ecosystem. Modeling of organic carbon concentrations allowed to estimate the magnitude of the deep biosphere as 90 Pg of cellular C-units. This estimate is independent of microscopic cell counts and underlines the global importance of the deep biosphere. Carbon isotopic analysis indicated that the majority of the archaeal biomass is indeed heterotrophic and utilizes carbon derived from degradation of sedimentary organic matter to synthesize biomass. It could also be shown that methane oxidation in deeply buried sulfate-methane transition zones is mediated differently compared to seep-sites in surface sediments. Calculation of community turnover rates supported previous values on the order of hundreds to thousands of years. Such low rates point to maintenance energy requirements much lower than known from laboratory cultures and challenge our understanding of life.

KURZFASSUNG

Der Schwerpunkt dieser Arbeit basiert auf der Aufklärung der Struktur und dem Stoffwechselhaushalt von Mikrobengemeinschaften, die in tief versenkten Sedimenten leben. Diese Fragestellung soll mittels der Analyse von intakten polaren Lipiden (IPL) als Indikator für aktive mikrobielle Zellen angegangen werden. Ein weltweiter Probensatz an Sedimentkernen wurde auf folgenden Expeditionen geteucht: ODP 201, 204 und 207, IODP 301 und 311, und Sonne SO147, Karei KY04-11 und Professor Logatchev TTR15. Die Proben wurden in Hinblick auf Zusammensetzung und Ausmaß mikrobiellen Lebens unter dem Meeresboden analysiert. Oberflächensedimente enthielten hauptsächlich bakterielle Phospholipide mit Phophatidylethanolamin, Phosphatidylglycerol und Phosphatidylcholin als polarer Kopfgruppe und Fettsäureketten mit 16 bzw. 18 Kohlenstoffatomen. Tiefere Sedimentschichten waren dominiert durch Tetraether- und Dietherlipide von Archaeen stammend mit verschiedenen Zuckern als Kopfgruppe. Der Übergang von Bakterien- zu Archaeenlipiden fand in den ersten 10 cm des Sediments statt. Diese Ergebnisse stehen im Widerspruch zu vorherigen Studien welche mithilfe von mikrobiologischen Methoden gewonnen wurden, diese fanden Bakterien als vorherrschend mikrobielle Spezies in tief versenkten Sedimenten. Unterstützung für die Ergebnisse der IPL Analyse kommt von (*i*) der „traditionellen“ Analyse von Phospholipidfettsäuren, welche ebenfalls einen geringen Anteil von Bakterien an der Gesamtpopulation zeigten und (*ii*) verbesserten mikrobiologischen Methoden welche einen höheren Anteil von Archaeenzellen nachwiesen als vorherige Studien, die möglicherweise einige Archaeengruppen unterdrückten.

Weiterhin konnte ein Abfall von IPL Konzentrationen mit der Tiefe beobachtet werden, der vergleichbar mit den direkt gezählten Zellen ist. Die Konzentrationen der intakten polaren Lipide waren abhängig von sedimentären organischen Kohlenstoffkonzentrationen und spiegelten folglich den heterotrophen Charakter dieses Ökosystems wider. Modellierung der Konzentrationen des organischen Kohlenstoffs erlaubte eine Abschätzung der Größe der tiefen Biosphäre auf ca. 90 Pg von zellulärem Kohlenstoff. Diese Abschätzung ist unabhängig von mikroskopischen Zellzählungen und unterstreicht die globale Bedeutung der tiefen Biosphäre. Analyse der Isotopenzusammensetzung zeigte, dass der Hauptanteil der Archaeenbiomasse in der Tat heterotroph ist, d.h. Kohlenstoff aus degradiertem organischem Material in die Biomasse einbaut. Es konnte außerdem gezeigt werden, dass die anaerobe Oxidation von Methan in tief versenkten Sulfat-Methan Übergangszonen sich anders verhält als bekannt war von oberflächennahen Austrittsstellen von Methan. Die Berechnung von Umsatzraten ergab Werte im Bereich von Hunderten bis Tausenden von Jahren, welche

durchaus im Rahmen von bisherigen Abschätzungen liegen. Solch niedrigen Raten zeigen dass die Energie die zur Instandhaltung der Zelle nötig ist viel niedriger sein muss als man bisher aus Laborversuchen annahm. Dieser Befund stellt eine Herausforderung an unser bisheriges Verständnis von Leben dar.

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LIST OF ABBREVIATIONS

16S rRNA	Small subunit of ribosomal ribonucleic acid with a sedimentary unit of 16
ΔG_0	Standard free Gibbs energy
ΔG_R	Gibbs free energy changes
ANME	Anaerobic methanotrophic archaea
AODC	Acridine orange direct counts
AOM	Anaerobic oxidation of methane
APCI	Atmospheric pressure chemical ionization
AR	Archaeol
ATP	Adenosine triphosphate
CARD-FISH	Catalyzed reporter deposition fluorescent *in-situ* hybridization
CDP-DAG	Cytidindiphosphate diacylglycerol
Cds	Cytidindiphosphate-diacylglycerol synthase
Cfa	Cyclopropane fatty acid synthase
Cls	Cardiolipin synthase
CMP	Cytidinmonosphosphate
CoA	Coenzyme A
Cti	Phospholipid cis-trans isomerase
CTP	Cytidintriphosphate
Da	Dalton
DAG	Diacylglycerol
DCM	Dichloromethane
DEG	Dietherglycerol
DesA	Phospolipid acyl-chain desaturase
DGD	Dialkylglyceroldiether
DGGE	Denaturing gradient gel electrophoresis
DIC	Dissolved inorganic carbon
DNA	Desoxyribonucleic acid
DPG	Diphosphatidylglycerol
DSAG	Deep Sea Archaeal Group
DSDP	Deep Sea Drilling Project
EA-MS	Elemental analyzer mass spectrometer
ELSD	Evaporative light scattering detector
ESI	Electrospray ionization
FA	Fatty acids
FAME	Fatty acid methyl esters
FISH	Fluorescent *in-situ* hybridization
FISH-SIMS	Fluorescence *in-situ* hybridization selective ion mass spectrometry

GC-MS	Gas chromatography mass spectrometry
GDGT	Glyceroldialkylglyceroltetraether
GDNT	Glyceroldialkynonitoltetraether
G-1P	Glycerol-1-phosphate
G-3P	Glycerol-3 phosphate
Gly	Glycosyl
Gly-DG	Monoglycosyldiacylglycerol
GNS	Green non-sulfur bacteria
HPLC-MS	High performance liquid chromatography mass spectrometry
ID	Internal diameter
IMF	Instrumental Mass Fractionation
IODP	Integrated Ocean Drilling Program
IPL	Intact polar lipids
IT-MS	Ion-trap mass spectrometer
JS1	Japan Sea 1
LOD	Level of detection
m/z	Mass to charge ratio
MBG-B	Marine Benthic Group B
MBG-D	Marine Benthic Group D
MC	Multicorer
MCG	Miscellaneous Crenarchaeotic Group
MDA	Multiple displacement amplification
MeOH	Methanol
MG 1	Marine Group 1
MS1	Primary order mass spectrometry stage
MS2	Secondary order daughter ion mass spectra
MS^n	Higher order daughter ion mass spectra
MSD	Mass selective detector
MSWD	Mean squared weighted deviations
NMR	Nuclear magnetic resonance
NSF	National Science Foundation
OC	Organic carbon
ODP	Ocean Drilling Project
OM	Organic matter
OMZ	Oxygen minimum zone
PA	Phosphatidic acid
PAF	Platelet activation factor (1-*O*-hexadecyl-2-acetoyl-*sn*-glycero-3-phosphocholine)

PC	Phosphatidylcholine
PCR	Polymerase chain reaction
Pcs	Phosphatidylcholine synthase
PDME	Phosphatidyl-(N,N)-dimethylethanolamine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
Pgs	Phosphatidylglycerol synthase
PI	Phosphatidylinositol
P_i	Inorganic phosphate
Pis	Phosphatidylinositol synthase
PLFA	Polar lipid fatty acid
PME	Phosphatidyl-(N)-methylethanolamine
Pmt	Phospholipid-(N)-methyl-transferase
POC	Particulate organic carbon
PS	Phosphatidylserine
Psd	Phosphatidylserine decarboxylase
Pss	Phosphatidylserine synthase
Q-PCR	Quantitative polymerase chain reaction
rDNA	Ribosomal desoxyribonucleic acid
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT-PCR	Real time polymerase chain reaction
SAGMEG	South African Goldmine Euryarchaeotal Group
SAHC	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SBH	Slot-blot hybridization
SMTZ	Sulfate methane transition zone
SNR	Signal to noise ratio
SQ-DG	Sulfoquinovosyldiacylglycerol
SRB	Sulfate reducing bacteria
SST	Sea surface temperature
TCA-cycle	Tricarboxylic acid cycle
TLE	Total lipid extract
TOC	Total organic carbon
ToF-SIMS	Time of flight mass spectrometry
TSQ-MS	Triple stage quadrupole mass spectrometer
VFA	Volatile fatty acid
w.b.a.	Weighted by area

Chapter I

Introduction

GENERAL INTRODUCTION

This introductory section provides an overview on deep biosphere research and sets the frame for the following chapters. The role of marine sediments in the global carbon cycle is evaluated, followed by a short description of the direct and indirect evidence for living microorganisms below the seafloor. An overview of microbial diversity and quantities determined by microbiological methods follows. Some considerations about their metabolic capabilities and energy sources are presented.

I.1. THE DEEP BIOSPHERE – SETTING THE STAGE

Almost 70% of Earth's surface is covered with water. The oceans represent a vast habitat for primary producing organisms: 40% of the carbon that is fixed every year is mediated by phytoplankton living in the ocean (Figure I.1; Killops and Killops, 2005). A schematic of the global carbon cycle is shown in Figure I.1. The remains of organisms living in the water column and their faecal pellets comprise a particulate organic carbon (POC) pool of 30 Pg. A flux of 2 Pg yr^{1} of organic carbon escapes degradation in the water column, reaches the seafloor and accumulates as sediment. Most of this flux - 1.7 Pg yr^{-1} - is remineralized and

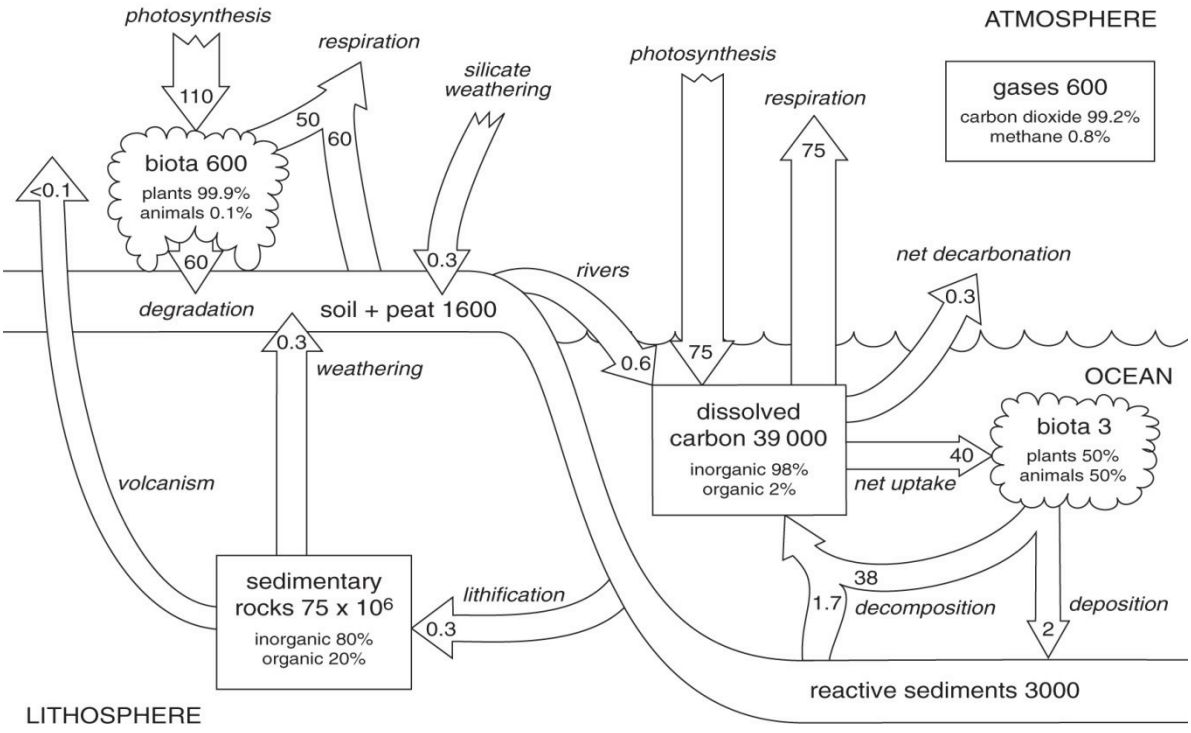

Figure I.1: Simplified scheme of the carbon cycle with reservoir sizes, annual fluxes and processes (taken from Killops and Killops (2005). Reservoir sizes and annual fluxes are given in Pg carbon (10^{15} g). This figure represents the preindustrial global carbon cycle showing the three main reservoirs: lithosphere, atmosphere, and ocean.

leached out of upper sediment layers. It is estimated that 3000 Pg of organic carbon are buried in those reactive sediments and are available for processes that interact with the water column. Finally, 0.3 Pg yr^{-1} of organic carbon is buried with increasing sedimentation as the sediments consolidate and lithify. The pool of sedimentary rocks is by far the largest carbon reservoir on Earth with a total size of 75 x 10^6 Pg of which 20% is organic carbon and the remaining 80% is comprised of inorganic carbon (Killops and Killops, 2005). The thickness of the sediment varies from (almost) non-existing in oceanic deserts like the South Pacific Gyre (Rea *et al.*, 2006) to more than 10 km on the eastern Indian continental margin (Figure I.2, Fowler 1990). This results from low primary productivity in the open ocean and higher productivity along the continental margins (Wollast 1998), discharge of terrestrial sediments from rivers (e.g. Lisitzin, 1996), and the tectonic movement of the plates. The latter includes the production of young oceanic crust at mid oceanic ridges and its transportation towards the continents where the oceanic plate subducts and sediments are "scraped off" and pile up. Considering the important role of marine sediments in the global carbon cycle, it is of great importance to better constrain mechanistic details of biogeochemical processes taking place in the oceanic subsurface sediments.

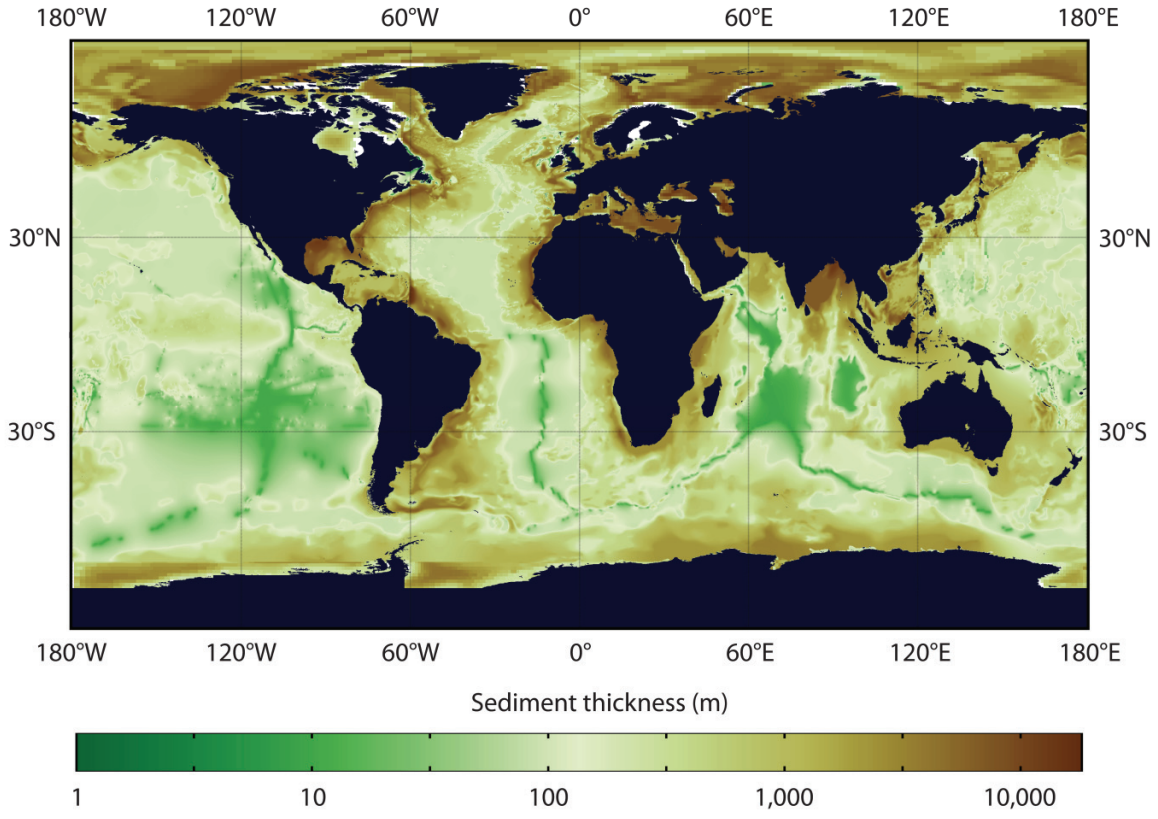

Figure I.2: Map showing the thickness of sediment in the oceans. Data from Laske and Masters (1997) (1° resolution) and the NGDC Sediment thickness database (Divins, retrieved 2008) (5' resolution). White color designates area where no sediment coverage information is available.

The three domains of life. Based on evolutionary relationships, all organisms can be grouped into the three domains of life: (1) eukarya, (2) bacteria, and (3) archaea (Madigan *et al.*, 2003). The measure for relatedness of organisms is based on similarity of highly conserved genetic sequences which are present in every cell: the small subunit ribosomal RNA (Figure I.3, Pace, 1997). Archaea and bacteria are grouped together as prokaryotes due to the absence of a cell nucleus, which distinguishes them from eukaryotic cells containing a nucleus. For a long time archaea have been thought to be a subgroup of bacteria and were called archaebacteria, until Woese and Fox (1977) recognized them as the third domain of life. Eukaryotes have a unicellular or multicellular organization, but are restricted to solely oxygen as electron acceptor. In contrary, prokaryotes are unicellular organisms, but are more diverse in their metabolic capabilities, e.g. they can utilize several electron acceptors and are therefore found in a wide range of habitats. Typically, archaea are thought to be confined to extreme environments, due to their tolerance towards high temperatures, acidity, or salinity. Just recently they were found to be ubiquitous in the oceans and not restricted to environmental niches (Karner *et al.*, 2001). Due to their more suitable physiology, it is expected that prokaryotes are responsible for the major portion of biogeochemical cycling of carbon in marine sediments.

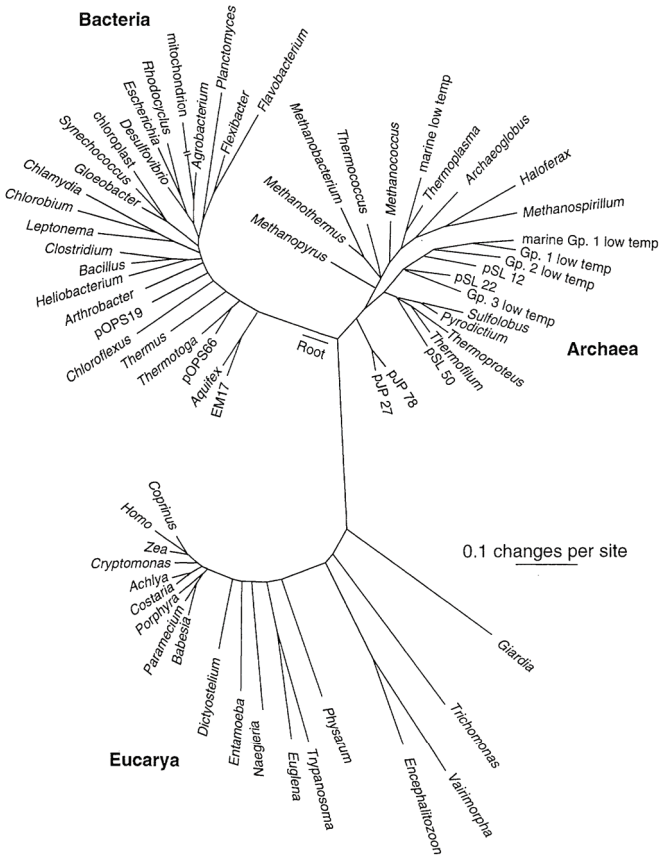

Figure I.3: Universal phylogenetic tree based on small subunit ribosomal RNA sequences. The scale bar corresponds to 0.1 changes per nucleotide. Figure taken from Pace (1997).

Microbially mediated processes. Marine sediments are host to a variety of processes dealing with organic matter degradation which over geological time scales profoundly affect the chemical composition of ocean and atmosphere on Earth (Holland, 1984, Kennett *et al.*, 2000). Microbial processes are known for their fundamental role in surface sediments (Jørgensen, 1983; Lochte and Turley, 1988) and for a long time it has been thought that microbial processes are restricted to the top tens of meters and that deeper sediment layers are devoid of life. Morita and Zobell (1955) defined the base of the marine biosphere at 7.47 meters below seafloor (mbsf) as they were not able to culture microorganisms from deeper sediments. It was thought that extreme conditions like low temperature, high pressure, low availability of energy sources, and decreasing porosity set the boundary for life. A few decades later with the implementation of the Deep Sea Drilling Project (DSDP) scientists were not any longer confined to the upper few meters available by gravity coring and it was possible to recover deeper sediments. Despite the lack of direct evidence for microbial life,

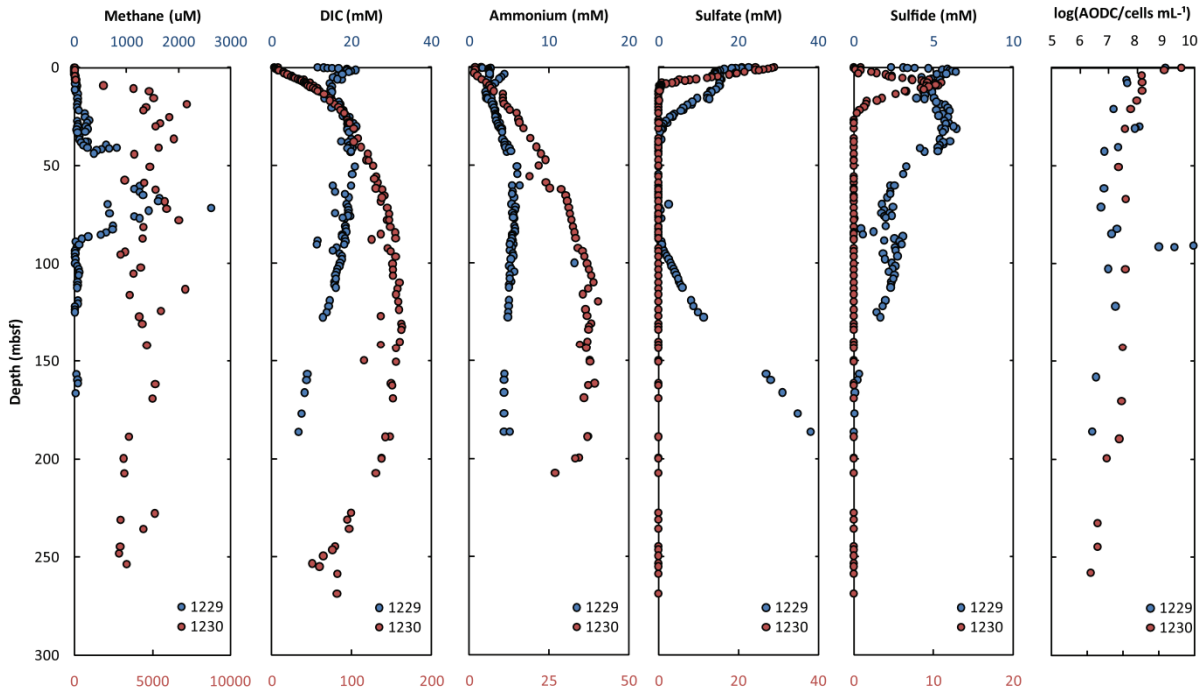

Figure I.4: Pore water profiles of ODP Leg 201 sediments drilled off Peru. Site 1229 is located on the Peru shelf in a water depth of 150 m. This site is influenced by a deep brine incursion reflected in the pore water profiles. Site 1230 is located on the lower slope of the Peru Trench in ~5100 m water depth. This site is methane-hydrate bearing. Methane concentrations are high in the methanogenic zone and decrease as they are oxidized by anaerobic methane oxidation coupled to sulfate reduction in the sulfate-methane transition zone (SMTZ). Sulfate diffuses into the sediments from the sediment-water interface and the deep brine incursion. Sulfide concentration is high in the horizon of intense sulfate reduction. Dissolved inorganic carbon (DIC) concentrations increase in zones of intense methane production. The ammonium profile reflects four main processes: organic matter degradation, microbial uptake, diffusion, and adsorption onto clays. Acridine orange direct counts (AODC) indicate the abundance of microbial cells and increase in the SMTZ. Data from ODP Initial Reports Volume 201 (D'Hondt *et al.*, 2003).

conspicuous porewater profiles were found which indirectly indicated the presence of microbially mediated processes (Figure I.4; Oremland *et al.*, 1982; Krumbein, 1983). Several other observations hinted at biological processes: production of methane from organic matter, biomarkers from bacteria, changes of stable isotope composition, and the enormous amounts of at least partially biologically produced methane hydrate in marine sediments, comprising four to eight times the amount of Earth's surface biomass and terrestrial soils combined (Kvenvolden, 1993). Although bacteria could be cultured from those sediments, it was not clear if they are thriving in the sediments *in situ* or if they are sea-water contaminants. Direct observations such as measurements of microbial activity, viable counts, genetic analysis, and proof for microbial adaptation to the conditions prevailing in deeply buried sediments, were lacking. Also, systematic studies covering the oceans horizontally and vertically had not been conducted. With the launch of the Ocean Drilling Program (ODP) in 1983 microbiologists tackled those questions actively and began to study bacterial distributions and activity. This resulted in evidence for *in situ* microbial life: high molecular DNA (desoxyribonucleic acid) was amplified (Rochelle *et al.*, 1994), rapidly growing enrichment cultures were prepared (Getliff *et al.*, 1992), bacteria physically adapted to the deep sea environment were identified (Bale *et al.*, 1997), bacterial activity was measured with radiotracers (Cragg *et al.*, 1990 and 1992), and growth rates were determined by thymidine incorporation (Parkes *et al.*, 2000). Rigorous contamination tests by addition of perfluorocarbon tracers and fluorescent microspheres to the drilling fluid have shown that the recovered samples were mostly uncontaminated. Such tests are now being routinely done on every ODP and IODP (Integrated Ocean Drilling Program) expedition dealing with microbiological studies (Smith *et al.*, 2000a and 2000b; House *et al.*, 2003; Lever *et al.*, 2006).

Detection of intact cells. Cell concentrations from globally distributed locations have been compiled by Parkes *et al.* (1994 and 2000) and show an exponential decline with depth (Figure I.5). Surprisingly, even sediments as deep as 800 mbsf - corresponding to an age of several tens of millions of years - still harbor millions of microbial cells per milliliter on average. Depending on oceanographical regime, cell concentrations are elevated (shallow water, high productivity, e.g. Peru Margin upwelling) or decreased (deep water, low productivity, e.g. open ocean sites) in relation to the average regression line. This indicates that the majority of cells counted in this fashion are intact and not dead. Based on the cell concentration regression line, Parkes *et al.* (1994) estimated the marine subsurface to harbor 10% of living carbon on Earth. In a similar way Whitman *et al.* (1998) estimated that marine

prokaryotic biomass constitutes "the hidden majority" with one-half to five-sixth of the total microbial biomass on Earth. However, the underlying sampling locations are somewhat skewed towards continental margin with higher cell concentrations and the numbers are likely overestimating the population.

Limiting factors for life. There is no clear definition regarding the upper boundary of the deep biosphere. Some studies exclude the upper 10 cm of sediment to avoid effects by animal mixing and precipitation (Whitman *et al.*, 1998), other definitions start at 1 mbsf (Jørgensen and Boetius, 2007) or 1.5 mbsf (D'Hondt *et al.*, 2002a). Some microbial ecologists use a more flexible definition as the boundary is locally variable and define the deep subsurface as "the sediment horizon where water column bacterial and archaeal communities are fading out, and solely sediment-typical bacterial and archaeal communities are remaining" (Teske and Sørensen, 2008). The deep biosphere is thought to continue to depths of several kilometers throughout the sediment column before microbial life is limited by hostile conditions. Several studies even suggest an extension into the upper layers of the oceanic crust (Fisk *et al.*, 1998;

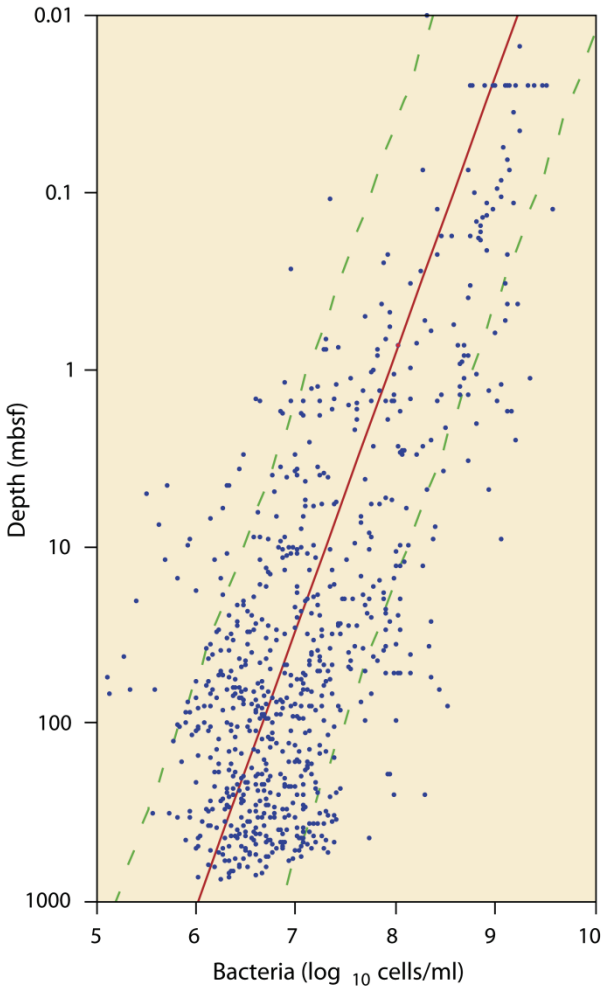

Figure I.5: Direct counts of microbial cells in ODP sediment samples. The detection limit is 10^4 cells mL^{-1}. Data sources are listed in Parkes *et al.* (2000). Figure adapted from D'Hondt *et al.* (2002b).

Cowen, 2003; Ehrhardt *et al.*, 2007; Huber *et al.*, 2006; Nakagawa *et al.*, 2006; Summit and Baross, 2001; Lysnes *et al.*, 2004a and 2004b; Thorseth *et al.*, 2001). Microbial cells were found to be relatively motile (Parkes *et al.*, 2000) and small enough to exploit the available pore space. However, low porosity might pose a limit to microbial life when grain size is low and burial depth is high (Rebata-Landa and Santamarina, 2006). Another limiting factor is temperature. Up to date, the highest temperature that microorganisms can endure and still be viable is 121°C (Kashefi and Lovley, 2003). In a typical marine environment with a geothermal gradient of 30°-50° km^{-1} this temperature is reached in a depth of 2-4 km. Until now, there are no sediment cores available for depths greater than 1 km. Hydrothermal sediments are the only means to study hyperthermophilic microorganisms in their natural sedimentary environment. Fouquet *et al.* (1997) analyzed samples from the Juan de Fuca Ridge and found geochemical evidence for continuous biological activity in sediments that were heated to more than 100°C. The detection of cells at even higher temperatures of more than 200°C (Cragg *et al.*, 2000) has raised the question if those microorganisms are living in the hot sediment *in situ* or are transported from shallower, cooler sediments by seawater recharge and lateral fluid flow (Membrillohernandez *et al.*, 1995).

I.2. MICROBIAL COMMUNITIES IN THE DEEP SUBSURFACE

Porewater profiles allow indirect conclusions about metabolic diversity of the responsible microbial populations, but not much is known about microbial diversity and quantities in the deep subsurface. There are two routes microbiologists can take: culture-dependent and culture-independent methods. Both methods and the results of their application are described below.

Culture dependent methods. Cultures represent an elegant way of studying the metabolic capabilities and genetic potential of microorganisms in detail (e.g. Giovannoni *et al.*, 2007). However, the preparation of cultures of deep biosphere representatives is no easy task (e.g. Amann *et al.*, 1995; Kaeberlein *et al.*, 2002). Culturing efforts have to reproduce the complex conditions prevailing in the natural habitat of the target organisms, for the deep subsurface this means absence of oxygen, low nutrient supply, low temperature, high pressure, and the presence of sediment particles for cell attachment. Owing to the slow *in-situ* growth rates and often the necessity of a syntrophic partner, only a small fraction of total cells are accessible by cultivation (0.001%-0.1% cultivability; Amann *et al.*, 1995; Kobayashi *et al.*, 2008 and references therein). This helps to explain why there are only few reports of successful

isolation of pure cultures from the deep subsurface (Bale *et al.*, 1997; Barnes *et al.*, 1998; Inagaki *et al.*, 2003; Mikucki *et al.*, 2003; Toffin *et al.*, 2004; D'Hondt *et al.*, 2004; Biddle *et al.*, 2005; Lee *et al.*, 2005; Batzke *et al.*, 2007).

Many of the cultured microorganisms represent novel lineages, with up to 14% difference of 16S rRNA gene sequences to their closest relative (D'Hondt *et al.*, 2004). The most commonly cultured genera are Firmicutes (related to *bacillus firmus*) and Proteobacteria (related to *rhizobium radiobacter*) (D'Hondt *et al.*, 2004; Batzke *et al.*, 2007). Many isolates are spore formers and might have been dormant for millions of years before they were supplied with substrates for cultivation. Their role in the biogeochemical processes in the subsurface is therefore questionable. Some cultivated lineages have cosmopolitan distribution, which is not surprising since the available substrates are largely similar in all sediments. However, others seem to correlate with geochemical parameters of their host environment, e.g. Firmicutes and Rhizobiaceae are often recovered from ocean margin sediments with abundant sulfate and low concentrations of methane. Another connection was found to oceanographic regime, e.g. typical ocean margin environments with high concentrations of organic carbon, high cell concentrations, and high metabolic rates favored cultivation of γ-Proteobacteria, while sites in the open ocean where those parameters are low, promoted growth of Actinobacteria. Physiologically the cultured representatives were characterized as generalists with the majority being facultatively anaerobic (Batzke *et al.*, 2007). Only three obligatory anaerobic isolates were successfully cultured (Bale *et al.*, 1997; Mikucki *et al.*, 2003; Kendall *et al.*, 2006), among them a methanogenic archaeon, *methanoculleus submarinus*.

Culture independent methods – 16S rRNA-based. Direct analysis of gene sequences from the environment is the only means to gain information on the majority of prokaryotes thriving in the ecosystems and avoid bias from selective growth of cultures (Amann *et al.*, 1995; Schloss and Handelsmann, 2004). Uncultured and novel archaeal and bacterial 16S rRNA gene sequences dominate clone libraries from the deep biosphere. Organisms known from seep environments like sulfate-reducing bacteria (SRB) or methanogenic and methane-oxidizing archaea do not seem to play an important role. This surprising observation can be explained by (a) novel phylogenetic lineages responsible for the observed geochemical profiles, (b) inefficient DNA extraction and primer bias for detection of certain lineages (Teske and Sørensen, 2008), and (c) very low population densities that are still high enough to account for the observed processes. For example, it was calculated for SMTZ in the Peru

Margin that a population of SRB according to 0.002-0.02% of total microbial population would be sufficient to explain the observed sulfate reduction rates (Parkes *et al.*, 2005). Such a low proportion is very unlikely to appear in clone libraries. Bacterial clone libraries are often dominated by *chloroflexi* (also termed green non-sulfur bacteria, GNS; Garrity *et al.*, 2002) or representatives of the Japan Sea 1 candidate division (JS1; Webster *et al.*, 2004). Archaeal clone libraries are usually dominated by members of the uncultured Miscellaneous Crenarchaeotic Group (MCG; Takai *et al.*, 2001; Inagaki *et al.*, 2003; Sørensen *et al.*, 2004; Teske, 2006), Marine Benthic Group B (MBG-B; Vetriani *et al.*, 1999; alternatively classified as DSAG by Inagaki *et al.*, 2003), Marine Group I (MG I; DeLong, 1992; Fuhrman *et al.*, 1992), or South African Goldmine Euryarchaeotal Group (SAGMEG; Takai *et al.*, 2001). Inagaki *et al.* (2006) analyzed the distribution and diversity of prokaryotes in sediments of the Peru and Cascadia Margins using clone libraries (Figure I.6) and found a strong correlation with methane hydrate content. Clone libraries constructed from hydrate-bearing sediments were consistently dominated by members of the JS1 candidate division (bacteria) and MBG-B (archaea), while hydrate-free sediments displayed GNS bacteria and archaea of the MCG.

Gene-based microbiological methods rely on efficient extraction of nucleic acids from sediments and subsequent primer-based amplification. There is ample evidence that the choice of primers for identification of the microbial community selectively suppresses the detection of some subsurface lineages (Teske and Sørensen, 2008). In addition, studies have suggested that DNA is resistant to degradation on timescales of thousands to millions of years (Coolen and Overmann, 1998; Inagaki *et al.*, 2005), thus making the detection of the *in-situ* population even more difficult. DNA can be adsorbed to sediment particles and as such is physically protected from degradation (Romanowski *et al.*, 1991; Demanèche *et al.*, 2001). This explains why it is possible to extract and sequence eukaryotic 18S rRNA gene sequences from Peru Margin surface sediments although eukaryotes could not directly be identified (Schippers and Neretin, 2006). Another method targeting only the active *in-situ* population is based on 16S rRNA. Ribonucleic acids (RNA) are less stable than their DNA-counterpart and are degraded rapidly after cell death (Fritsche, 1999). Archaeal RNA has been successfully extracted from subsurface sediments, reverse transcribed to DNA, amplified, and sequenced (Sørensen and Teske, 2006). The diversity in clone libraries constructed from 16S rDNA and 16S rRNA is largely similar with the exception of Marine Group I organisms which were not found in the rRNA clone libraries. Clonal frequencies of the 16S rRNA-based clone libraries reflect the diversity of the metabolically active population in contrast to the more temporally integrated gene-based clone libraries.

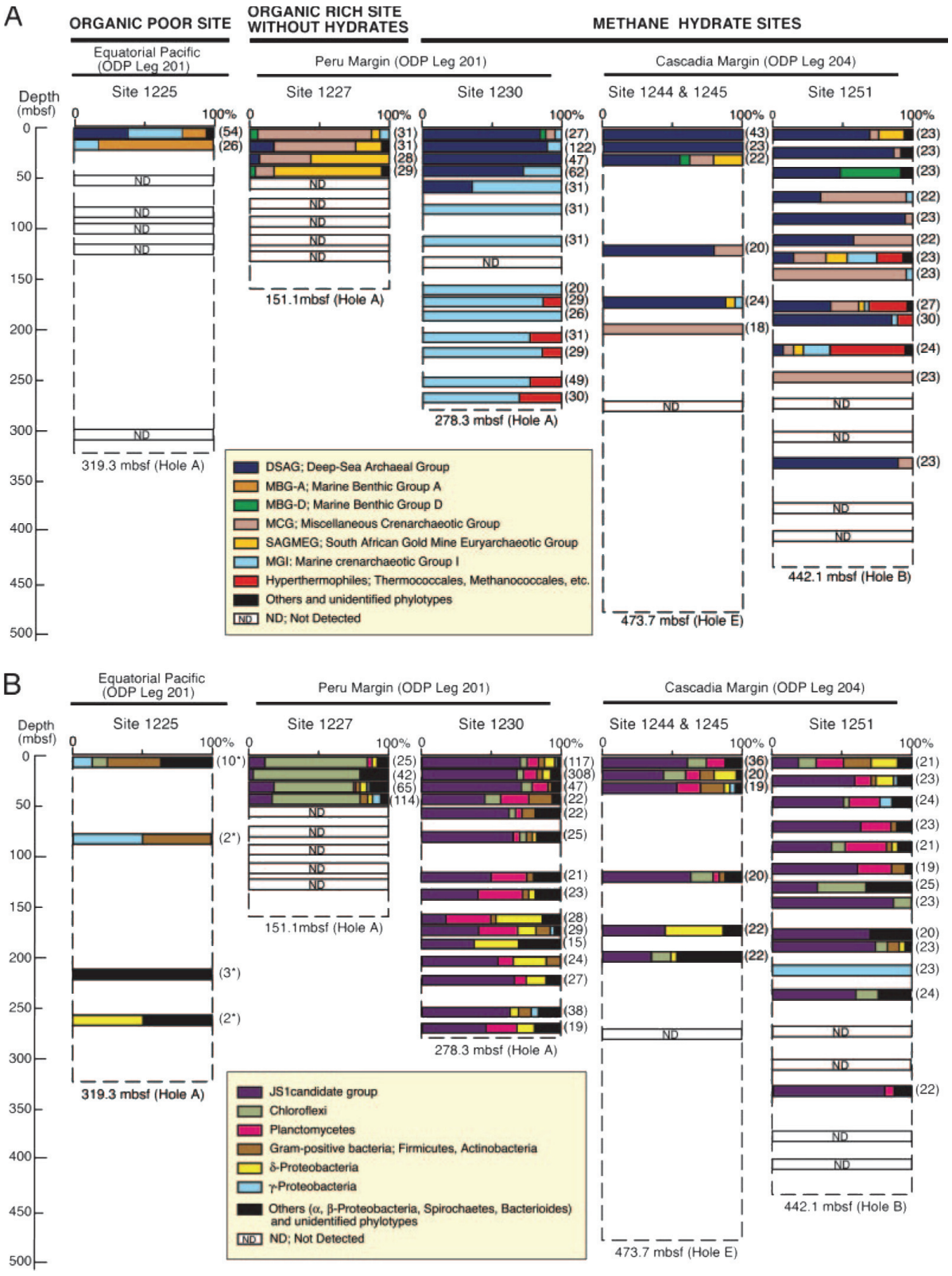

Figure I.6: Phylogenetic community structures based on 16S rRNA gene clone libraries of domains Archaea (**A**) and Bacteria (**B**) from ODP sediment core samples. Numbers of clones examined at each depth are indicated in parentheses. The phylogenetic affiliation of each clone sequence was determined by similarity analysis of 400-500 bp of 16S rRNA gene sequences. In each column diagram the relative abundances of clones classified with the (sub)phylum level are shown. Bacterial community structures at Site 1225 were evaluated by sequencing 200 bp of PCR fragments obtained by denaturing gradient gel electrophoresis (DGGE) analysis, and the number of major bands is indicated in parentheses with asterisk. Figure and caption reproduced from Inagaki *et al.* (2006).

Culture independent methods – whole genome-based. Culture-dependent genomic protocols have unraveled whole genome sequences of several ecologically relevant marine microorganisms (see DeLong, 2005 and references therein). This provided important aspects on microbial evolution, biochemistry, physiology, and diversity. Recent improvements of sequencing techniques enable scientists to predict biological functions and identify the physiological and metabolic potential of microorganisms in marine environments without the necessity of cultivation (e.g. Schleper *et al.*, 2005 and references therein). Sogin *et al.* (2006) used a massively parallel DNA sequencing strategy on samples from North Atlantic deep water and diffuse hydrothermal vent fluids and found that the complexity of the bacterial communities was two orders of magnitude higher than previously reported for any other microbial environment. The samples were dominated by a few different populations, but the majority of the diversity was found in thousands of low-abundance populations, constituting the so called "rare biosphere". The rare biosphere is typically overlooked by conventional approaches that target only the most dominant species, but nevertheless provides an invaluable, potentially inexhaustible reservoir of genetic potential. When environmental conditions change and the prevailing microorganisms become extinct, just one cell of rare biosphere microbes able to cope with the changed conditions is enough to multiply and become dominant. Rare microorganisms also have a higher chance of survival. Main reasons for microbial death are infestation with viruses that lyse the cells or predation by protists; chances for both are less likely for rare microorganisms (Sogin *et al.*, 2006; Pedros-Alio, 2007).

Quantification of the active population. Clonal frequencies in clone libraries do not necessarily represent the quantities of the indigenous microorganisms. One approach for quantification is based on extraction of DNA and detection by quantitative polymerase chain reaction (Q-PCR). This technique has been successfully applied in marine sediments and revealed an active microbial population dominated by bacteria (Inagaki *et al.*, 2003 and 2006; Schippers *et al.*, 2005). However, a certain bias against archaea is likely. First, extraction of DNA from archaeal cells is more difficult due to high resistance to enzymatic, chemical and mechanical treatment for cell wall permeabilization (Teira *et al.*, 2004) and the DNA-yield is low (Newberry *et al.*, 2004) . Second, depending on the choice of primers, the detection of some archaeal lineages can be selectively suppressed (e.g. Teske and Sørensen, 2008). This can be avoided by using multiple displacement amplification (MDA; see Binga *et al.* (2008)

for a review) which is a recently developed, powerful technique for amplification of whole genomes from low-biomass communities that does not rely on primers.

In addition to selective favoring of some lineages, the presence of fossilized DNA blurs the signal of the active population (Coolen and Overmann, 1998, Inagaki *et al.*, 2005). This problem is avoided by targeting rRNA which is only present in metabolically active cells (Fritsche, 1999). The content of rRNA is proportional to metabolic activity and nutritional state of the cell (Molin and Givskov, 1999). Fluorescent *in-situ* hybridization (FISH) uses an oligonucleotide probe that hybridizes to specific regions in rRNA and can be visualized by fluorescent microscopy. A variant of FISH, catalyzed reporter deposition FISH (CARD-FISH), enhances the fluorescent signal with the enzyme horseradish peroxidase and provides an increased sensitivity to lower rRNA content (Pernthaler *et al.*, 2002). CARD-FISH is especially suitable for habitats like the deep subsurface with small, slow-growing or starving microorganisms. Studies of sediments from the Peru Margin obtained during ODP Leg 201 provided controversial results regarding community composition. Some studies identified significant proportions of archaea (Mauclaire *et al.*, 2004), while others have shown that bacteria make up the dominant fraction of active cells (Schippers *et al.*, 2005; Figure I.7). A study of sediments recovered during IODP Expedition 301 found no dominance of one domain over the other but could only detect a small fraction of 1% of total stainable cells (Engelen *et al.*, 2008). This demonstrates that even seemingly simple questions concerning the nature of the community structure in deep biosphere sediments are still open, e.g. if archaea or bacteria dominate.

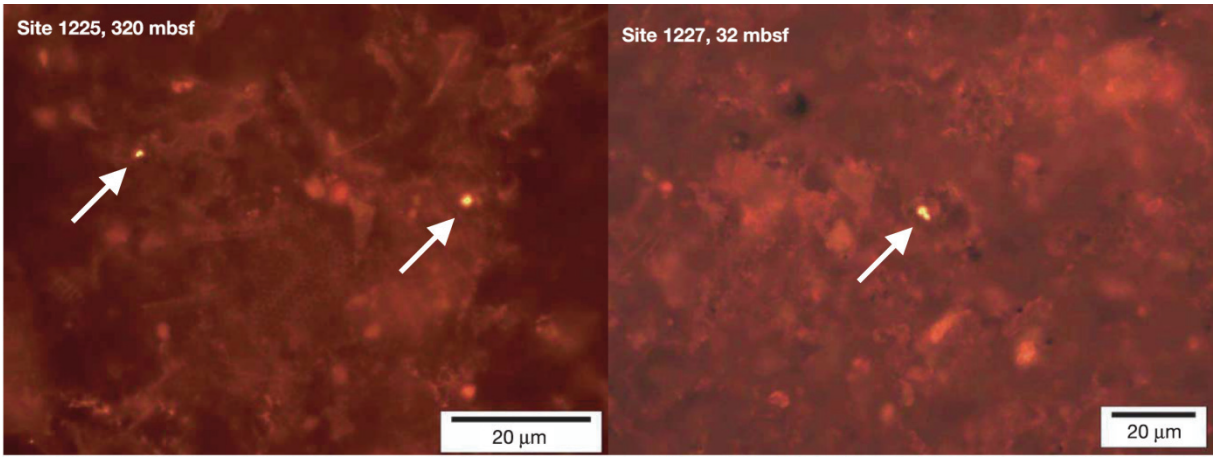

Figure I.7: Bacterial cells detected by CARD-FISH in sediments recovered from Peru Margin during ODP Leg 201. Arrows point to bacterial cells that have hybridized to the FISH probe. Picture taken from Schippers *et al.* (2005).

I.3. BIOGEOCHEMICAL PROCESSES – ENERGETIC CONSTRAINTS

The deep biosphere is "an enormous bioreactor that processes vast amounts of organic and inorganic materials" (DeLong, 2007a). Most of the energy fueling microbial life is derived from degradation of organic matter produced in the photic zone of the water column and transported rapidly to the seafloor. Despite this indirect dependence on photosynthesis deep biosphere microorganisms can also utilize geofuels that are produced in the oceanic crust (Bach and Edwards, 2003). Hydrogen is produced when seawater reacts with iron-bearing igneous rocks, a process which is important in the fresh basalts of mid-oceanic ridges (Schulte *et al.*, 2006). Another possibility of hydrogen formation deep within Earth's crust is radiolytic cleavage of water (Lin *et al.*, 2005). However, compared to organic matter degradation it is unlikely that those more regionally confined processes provide enough energy to sustain the majority of microbial life.

The degradation of organic matter is an oxidation process that is tightly coupled to the reduction of electron acceptors like oxygen, nitrate, manganese and iron cations, or sulfate which diffuse into sediments from the seawater. These are utilized in order of decreasing energy production per mole of organic carbon oxidized to carbon dioxide (Table I.1, Froelich *et al.*, 1979). Oxygen is depleted first, followed by nitrate, manganese and iron cations, and sulfate. When inorganic electron acceptors are depleted, organic matter is fermented:

Table I.1: Standard free Gibbs energies (ΔG_0) for oxidation reactions of sedimentary organic matter with Redfield ratio composition. Data taken from Froelich *et al.*, 1979.

Electron acceptor	Reaction	ΔG_0 (kJ mol^{-1} glucose)
Oxygen	$(CH_2O)_{106}(NH_3)_{16}(H_3PO_4) + 138\ O_2 \rightarrow 106\ CO_2 + 16\ HNO_3 + H_3PO_4 + 122\ H_2O$	-3190
Manganese	$(CH_2O)_{106}(NH_3)_{16}(H_3PO_4) + 236\ MnO_2 + 472\ H^+ \rightarrow 236\ Mn^{2+} + 106\ CO_2 + 8\ N_2 + H_3PO_4 + 366\ H_2O$	-3090 to -2920
Nitrate $\rightarrow N_2$	$(CH_2O)_{106}(NH_3)_{16}(H_3PO_4) + 94.4\ HNO_3 \rightarrow 106\ CO_2 + 55.2\ N_2 + H_3PO_4 + 177.2\ H_2O$	-3030
Nitrate $\rightarrow N_2 + NH_3$	$(CH_2O)_{106}(NH_3)_{16}(H_3PO_4) + 84.8\ HNO_3 \rightarrow 106\ CO_2 + 42.4\ N_2 + 16\ NH_3 + H_3PO_4 + 148.4\ H_2O$	-2750
Iron	$(CH_2O)_{106}(NH_3)_{16}(H_3PO_4) + 212\ Fe_2O_3$ (or $424\ FeOOH$) $+ 848\ H^+ \rightarrow 424\ Fe^{2+} + 106\ CO_2 + 16\ NH_3 + H_3PO_4 + 530\ H_2O$ (or $742\ H_2O$)	-1410 / -1330
Sulfate	$(CH_2O)_{106}(NH_3)_{16}(H_3PO_4) + 53\ SO_4^{2-} \rightarrow 106\ CO_2 + 16\ NH_3 + 53\ S^{2-} + H_3PO_4 + 106\ H_2O$	-380
Fermentation	$(CH_2O)_{106}(NH_3)_{16}(H_3PO_4) \rightarrow 53\ CO_2 + 53\ CH_4 + 16\ NH_3 + H_3PO_4$	-350

every mole of carbon that is oxidized to carbon dioxide needs another mole of carbon which is reduced to methane (Fenchel and Finlay, 1995). In case of deep brine incursion at the sediment base an additional redox zonation develops, where this order is turned upside-down (e.g. ODP Leg 201 site 1229, Figure I.4).

Microbial activity is influenced by the concentration of total organic carbon (TOC), e.g. zones of elevated TOC content like Mediterranean sapropels (Parkes *et al.*, 2000) or cretaceous black shales (Coolen *et al.*, 2002; Arndt *et al.*, 2006) lead to faster turnover processes. However, turnover in the deep subsurface was found to be orders of magnitude slower than in surface sediments (D'Hondt *et al.*, 2002a). Turnover times have been estimated to be in the range of hundreds to thousands of years (Whitman *et al.*, 1998; D'Hondt *et al.*, 2003) and sometimes reaching hundreds of thousands of years (Parkes *et al.*, 2000). Such long turnover times challenge the conventional way of thinking about life and raise the question of how cells can prevent the unavoidable damage to macromolecules that make up DNA, enzymes or the cell membrane. Metabolic rates per cells can be grouped into three categories: (a) active growth, (b) maintenance of functions but low enough nutrient level to prevent growth, and (c) survival mode where damage to essential macromolecules is repaired but no other functions are performed. The scaling of the three groups has been found to be 10^6:10^3:1 (Price and Sowers, 2004). In marine sediments, most of the cells fall into the category of survival mode which needs extremely little energy. A common survival strategy of bacteria is formation of endospores. Recently, a method for quantification has been developed and been applied to wadden sea sediments (Fichtel *et al.*, 2007). First results show that increasing depth leads to an increasing proportion of bacterial endospores to total prokaryotic biomass and it was estimated that endospores might represent a major part of global biomass. However, during their dormant state they do not contribute to the major biogeochemical processes.

In order to satisfy their energy demand, microorganisms have to develop novel metabolic strategies. One example is the metabolic cooperation of two or more species to thrive on low-energy carbon sources that neither partner could utilize on its own, e.g. the anaerobic bacterium *Synthrophus aciditrophicus* can efficiently degrade fatty acids or benzoate when a syntrophic partner consumes its metabolic waste products (DeLong, 2007b, McInerney *et al.*, 2007). Other examples of novel microbially mediated processes are ethanogenesis and propanogenesis (Hinrichs *et al.*, 2006), which were previously thought to be solely associated with thermal degradation of organic matter.

Geochemical interfaces such as the sulfate-methane transition zone (SMTZ) greatly stimulate microbial activity and lead to elevated cell concentrations (e.g. ODP Leg 201 site 1229, Figure I.4). Methane which is produced by fermentation in the methanogenic zone or thermally during cracking of organic matter diffuses upwards and is oxidized while sulfate diffusing into the sediment from the water column is reduced. This process is known from methane seeps where high methane and sulfate concentrations lead to high activity of a specialized microbial consortium consisting of anaerobic methane oxidizers (ANME) and sulfate reducing bacteria (Boetius *et al.*, 2000). It is unclear, if this process is mediated by the same microorganisms in the deep biosphere.

I.4. OBJECTIVES OF THIS THESIS

The overarching goals of studies concerning subsurface life are described and specific open questions addressed in this thesis are highlighted.

Open questions addressed in this thesis. The ultimate, overarching goal of studies on deep subseafloor life is to gain deeper understanding of the abundance of subsurface prokaryotic communities and their carbon metabolism, and to constrain their fundamental role in Earth's biogeochemical cycles. The approach used in this thesis focuses on deciphering the information encoded in structural and isotopic properties of intact membrane molecules (intact polar lipids, IPL). IPL analysis is a rapid and non-selective procedure which - in contrast to gene-based techniques – does not need prior knowledge of the community structure. This powerful tool provides (*i*) general taxonomic information on the active sedimentary community, (*ii*) constraints on the carbon fixation pathways and carbon substrates utilized by prokaryotes *in-situ*, and (*iii*) estimates on the population density.

When I started my PhD thesis in December 2003, the application of IPL analysis to environmental samples from marine sediments was still in its infancy. Rütters *et al.* (2002a and 2002b) were the first to use IPL analysis as tool to study microbial communities in intertidal flat sediments and were able to detect bacterial phospholipids throughout a 55 cm long sediment core. One year later, Zink *et al.* (2003) applied IPL analysis to surface sediments from Hydrate Ridge and deeply buried sediments from Nankai Trough. They extended the maximum depth of IPL presence to 745 mbsf and still detected a variety of IPL structures. However, those early studies only focused on phospholipids derived from bacteria and eukarya and neglected the presence of archaeal IPLs. This shortcoming was addressed in the study of Sturt *et al.* (2004), where the analytical window was extended to cover

bacterial/eukaryotal phospholipids as well as archaeal glycolipids. This study also provided first results of IPL concentration and species in sediments that were sampled during ODP Leg 201, which is until today the only ODP/IODP undertaking dedicated solely to the study of the deep biosphere. The analyses pointed to a major contribution of archaeal IPLs to the total inventory. Several archaeal IPL species have been identified, but the link to the producing organisms has not been unambiguously established. Although those studies provided first insight into diversity and abundance of intact polar lipids in subsurface sediments, the main research objectives "who is there?", "how many are there?", and "what are they doing?" were still unanswered.

In this thesis I want to address the following specific questions regarding deep subsurface life:

(1) What information on the taxonomic composition of microbial communities can be gleaned from intact membrane analysis?
Recent gene-based studies have revealed controversial results regarding dominance of archaea or bacteria.

(2) What is the quantitative extent of the deep marine biosphere?
Previous estimates were based on direct cell counts and the sample-set was skewed towards continental margin settings, potentially overestimating microbial biomass.

(3) Does structural lipid diversity reflect differences in the microbial community in surface and deeply buried sediments?
IPL can provide taxonomic affiliation of major microbial players, 16S rRNA-based clone libraries cannot provide quantitative information.

(4) Are the microbial communities and processes in deeply buried sulfate-methane transition zones similar to those observed in surface environments where methane - an important greenhouse gas - is oxidized anaerobically (e.g. at methane seeps)?
Most of our knowledge about anaerobic oxidation of methane is based on studies of surficial methane seeps und it is unclear if this process is accomplished by the same microorganisms.

These research questions were addressed in three first-author manuscripts which are presented in chapters II-IV. Chapter II presents a study of the carbon metabolism in deeply buried sulfate-methane transition zones of the Peru Margin using an investigative approach combining molecular-isotopic and rRNA-based techniques directly targeting research question 4. Chapter III reports on the composition and magnitude of the deep biosphere using IPLs as markers for the active prokaryotic community, addressing research questions 1 and 2. These results are based on analysis of a globally distributed sample-set spanning from surface to deeply buried sediments and are supported by improved molecular-biological protocols. Finally, chapter IV provides a study of the structural diversity and quantities of IPLs in marine sediments, in respect to research question 3. A map showing the locations studied in this thesis color-coded in red is provided in Figure I.8.

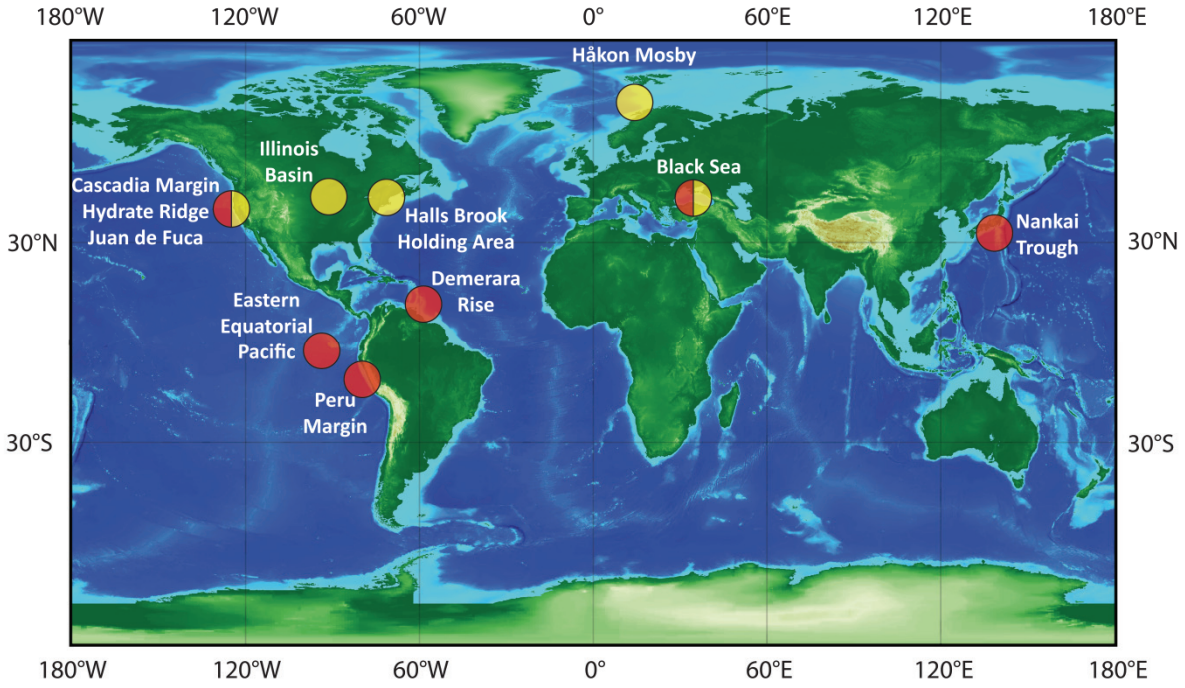

Figure I.8: Map showing the locations of samples analyzed in the chapters of this thesis. Locations are color-coded according to my contribution to the manuscripts: red color denotes sample locations analyzed on studies that yielded (joint) first author manuscripts and yellow color marks sample locations of manuscripts where I contributed as co-author.

Other opportunities to study microbial life with intact polar lipids. In addition to the three first-author manuscripts, I was able to contribute to four other projects where I am listed as co-author. Abstracts of those other studies are listed in chapter V. Chapter Va presents a study of vertical changes in microbial lipid composition and functional genes in a highly contaminated, meromictic lake. Through lipid-based techniques and compound-specific isotope analysis it was possible to detect marked differences in cell concentrations and

metabolic strategies in the different layers. Chapter Vb reports results of a study similar to chapter II, where carbon metabolism and microbial diversity in sediments drilled at Hydrate Ridge were studied. The focus of chapter Vc is on the application of IPLs as chemotaxonomic markers of archaeal and bacterial species involved in the anaerobic oxidation of methane and evaluates their relative concentrations in microbial mat samples dominated by three major community types. Chapter Vd presents a study characterizing the microbial community responsible for coal bed methane formation in the eastern Illinois basin using molecular biological and geochemical techniques. A methanogenic enrichment culture was isolated from coal bed drainage waters and characterized for its IPL-fingerprint which could serve as template for identification and quantification of the methanogenic contribution of this organism in the environment. The study areas of co-authorship manuscripts are color-coded in yellow in Figure I.8.

I.5. INTACT POLAR LIPIDS AS TOOL IN BIOGEOCHEMISTRY

Lipids are the building blocks of every cell membrane and are essential for multiple functions of the cell (Figure I.9). They provide a physical barrier to separate the cytoplasma from the environment and serve as "anchor" for trans-membrane proteins allowing substrate uptake and product release. The lipid synthetic pathway also provides precursors for protein modification and for the synthesis of other molecules (Cronan, 2003). The specialization of microorganisms to the wide range of processes in which membrane lipids are involved has led to the formation of a large diversity (Dowhan, 1997). The majority of membrane lipids are glycerol-based with two hydrophobic chains connected to the glycerol backbone via ester or ether bonds and a polar head group.

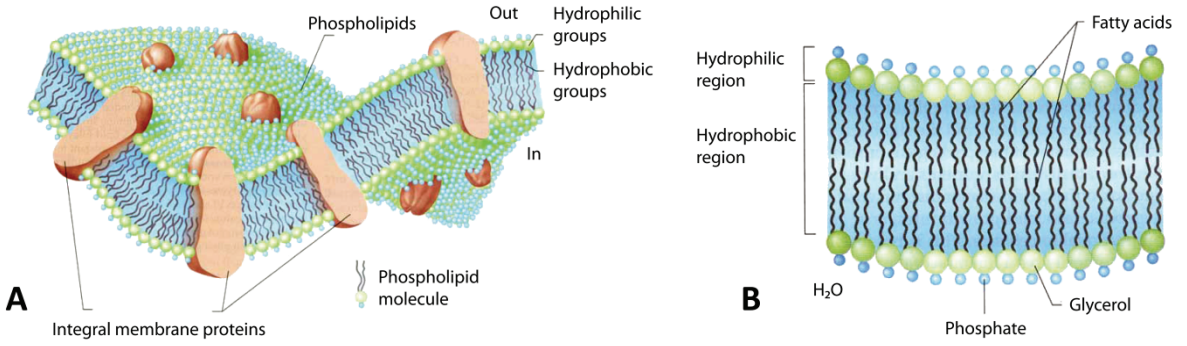

Figure I.9: Schematic structure of a bacterial phospholipid bilayer membrane. (**A**) Cytoplasmic membrane showing the lipid molecules and transmembrane proteins. The inner surface faces the cytoplasm and the outer part faces the environment. (**B**) Detailed view of the lipid bilayer. The polar headgroups are hydrophilic and face outwards to the aqueous environment, while the apolar fatty acids are hydrophobic and make up the interior of the membrane. Figure adopted from Madigan *et al.* (2003).

The composition of the membrane changes in response to environmental parameters like temperature, osmolarity, salinity, pH, or pressure to minimize energy expenditure and optimize cell growth (Cronan and Gelmann, 1975). This adaptation process results from *de-novo* production of lipids or alteration of existing phospholipid fatty acids by introduction of unsaturations or isopropyl rings or cis-trans isomerization of unsaturations (Zhang *et al.*, 2008; Figure I.10). For example, psychrophilic and piezophilic bacteria produce fatty acids with more unsaturations to maintain membrane fluidity (Fulco, 1983, DeLong and Yayanos, 1985 and 1986; Gounot, 1991; Kaneda, 1991; Nichols *et al.*, 1997; Yano *et al.*, 1997 and 1998; Russel and Nichols, 1999; Fang *et al.*, 2000a and 2003, Valentine and Valentine, 2004). Besides adaptation in the hydrophobic part of the molecule, the polar head group composition can also change with growth temperature (e.g. Hasegawa, 1980) and has a profound impact on phase transition temperature (Pluschke and Overath, 1981). This mechanism also supports the adaptation to changing environmental conditions.

The use of IPLs and their derivates in the marine environment as chemotaxonomic markers is reviewed below. A short description of stable-isotope analysis for unraveling the physiologies of the microorganisms is given followed by a brief introduction of the analytical procedure used for IPL analysis.

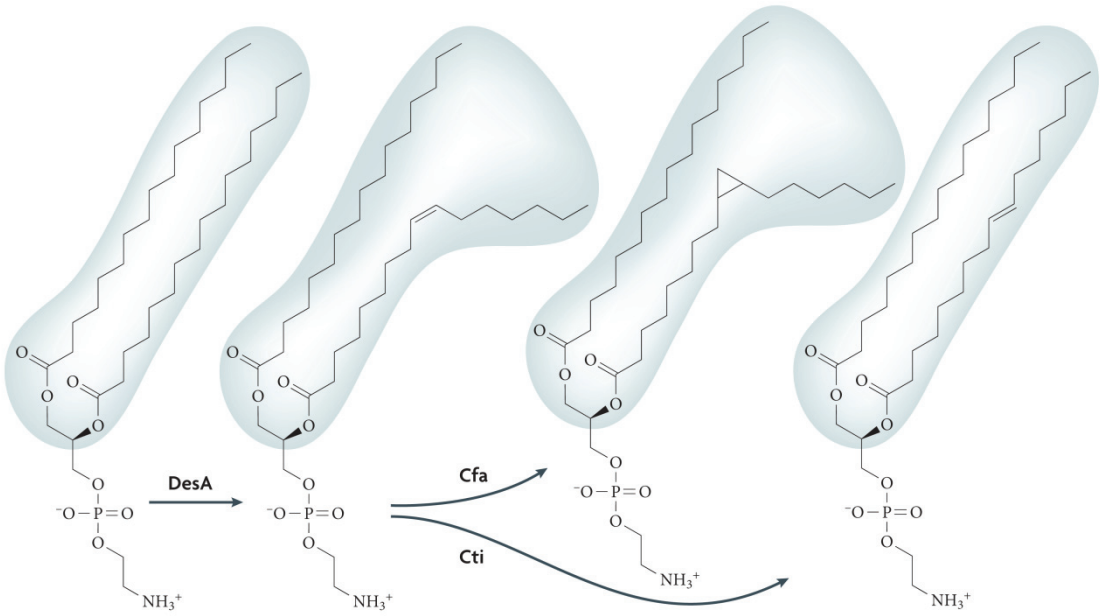

Figure I.10: Modification of existing phospholipid structures in bacteria shown at the example of a diacyl-phosphatidylethanolamine lipid. Phospholipid acyl-chain desaturase (DesA) introduces a double bond, which increases membrane fluidity due to greater molecular volume and lower package density in the bilayer. Cyclopropane fatty acid synthase (Cfa) modifies a double bond to from a cyclopropane ring, thereby increasing the stability towards acid stress. In some bacteria, phospholipid cis-trans isomerase (Cti) can replace a *cis* double bond with a *trans* double bond, which increases the membrane transition temperature. Figure adopted from Zhang *et al.* (2008).

Phospholipid-derived fatty acids (PLFA). In environmental samples typically the apolar derivates of membrane lipids were analyzed, either as breakdown products which were produced in the environment or as products prepared during sample preparation. PLFA are prepared by alkaline hydrolysis from membrane lipids after sample extraction. They are thought to select only for live bacteria, since the "parent" phospholipid is degraded rapidly by hydrolytic cleavage of the polar head group bond within days to weeks after cell death (White *et al.*, 1979, Harvey *et al.*, 1986). This allows the wide-spread application to study *in-situ* microbial community structures (e.g. Guezennec and Fiala-Medioni, 1996). The fatty acid chain structure holds information on the involved species and can be used as chemotaxonomic marker (Asselineau and Asselineau, 1990; Green and Scow, 2000; Pinkart *et al.*, 2002), whereas PLFA abundance has been used to determine cell concentrations (e.g. White and Findlay, 1988; Haack *et al.*, 1994). Many PLFA-based studies focused on marine subsurface sediments and studied the *in-situ* population (e.g. Summit *et al.*, 2000; Cardace *et al.*, 2006; Mills *et al.*, 2006). However, the assignment of PLFAs to organisms is difficult at times as the fatty acids in surface sediments represent a complex mixture of eukaryotic and prokaryotic fatty acids, often including contamination by wax esters from higher plant material (Volkman and Johns, 1977; Eglinton and Hamilton, 1967) which sometimes are difficult to exclude from analysis even in deeper samples (Aries *et al.*, 2001).

Intact polar membrane lipids (IPL). Recent advances in analytical chemistry allow analyzing the membrane lipids in their intact form as IPLs. Using high-performance liquid chromatography coupled to mass spectrometry (HPLC-MS) we can now explore the full potential of the lipid inventory, including the valuable taxonomic information of the polar head group (Imhoff, 1991; Fang and Barcelona, 1998; Ivanova *et al.*, 1999; Rütters *et al.*, 2001, 2002a, and 2002b). Direct comparison of PLFA and IPL analysis has shown that the chemotaxonomic potential is greater for analysis of the intact molecules (Fang *et al.*, 2000b, Rütters *et al.*, 2002b). IPL analysis hence allows direct characterization of the *in-situ* community. So far, studies of marine sediments have revealed bacterial IPLs with a variety of polar head groups and fatty acids (Fang *et al.*, 2000b; Rütters *et al.*, 2002a and 2002b; Zink *et al.*, 2003; Sturt *et al.*, 2004; Zink and Mangelsdorf, 2004; Fredricks and Hinrichs, 2007; Zink *et al.*, 2008). Typical lipids that were found are diacyl-based with C_{16} and C_{18} fatty acids with polar head groups phosphatidylethanolamine (PE), phosphatidyl-(N,N)-dimethylethanolamine

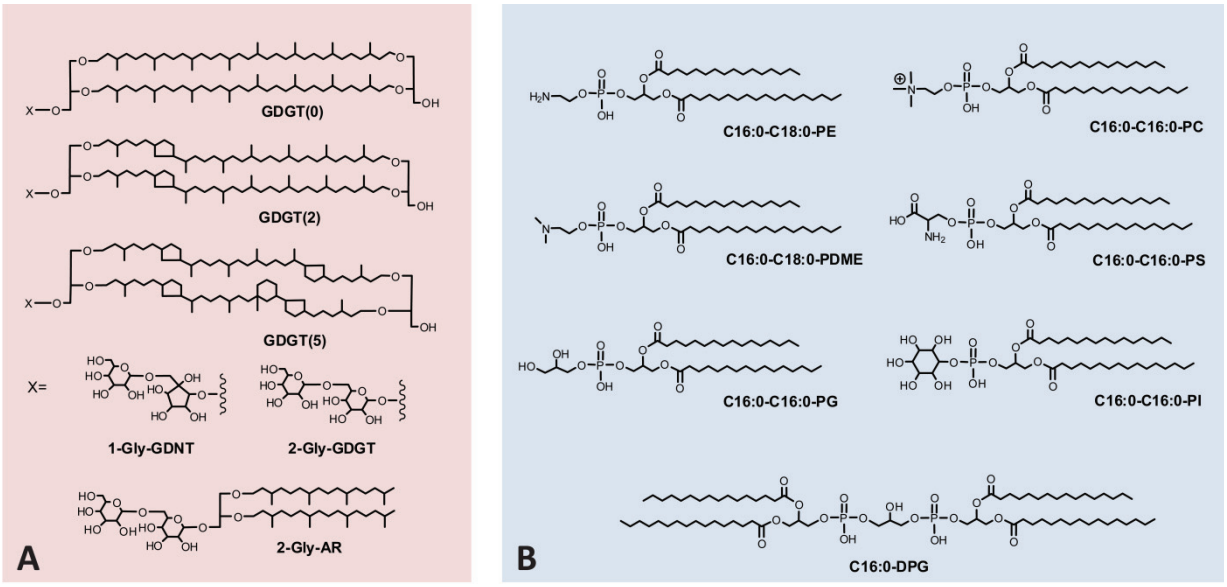

Figure I.11: Structures of IPLs found in marine sediments. (**A**) Archaeal glyceroldialkylglyceroltetraether (GDGT) and glyceroldiacyldiether (archaeol, AR) lipids. The number in brackets denotes the number of rings in the GDGT core lipid. Note the hexacyclic ring in the structure of GDGT(5) which is called crenarchaeol. GDNT tetraether lipids are based on a mixed nonitol/glycerol backbone. Typically glycosidic polar head groups with one or two hexoses are found. (**B**) Bacterial diacylglycerol (DAG) lipids. Fatty acids can vary. Shown are $C_{16:0}$ and $C_{18:0}$ with different polar headgroups. Two PG-DAG lipids can condensate to form DPG (cardiolipin). See text for full names of lipids.

(PDME), phosphatidylglycerol (PG), phosphatidylcholine (PC), diphosphatidylglycerol (DPG, cardiolipin), phosphatidylserine (PS) and phosphatidylinositol (PI) (see Figure I.11B for structures).

The biosynthetic network for synthesis of the various polar head groups is depicted in Figure I.12 (modified after Dowhan (1997), Cronan (2003) and Sohlenkamp *et al.* (2003). The synthesis starts with glycerol-3-phosphate (G-3P) which is available through reaction of glycerol with adenosine-triphosphate (ATP) in a reaction catalyzed by glycerol kinase. Two fatty acid chains are transferred to G-3P producing phosphatidic acid-diacylglycerol (PA-DAG). This step is activated by transfer of cytidintriphosphate (CTP) and the resulting CDP-DAG serves as starting point for synthesis of several lipid classes. The polar head groups PS, PC, PG, and PI are introduced by transfer of serine, choline, G-3P and myo-inositol, respectively, in reactions releasing cytidinmonophosphate (CMP). DPG is formed by reaction of two PG molecules, and PE is produced indirectly via decarboxylation of PS. PE is reactant for a second route to formation of PC via methylation in three steps with the intermediates phosphatidyl-(N)-methylethanolamine (PME) and PDME (for details see Dowhan, 1997; Cronan, 2003; Sohlenkamp *et al.*, 2003).

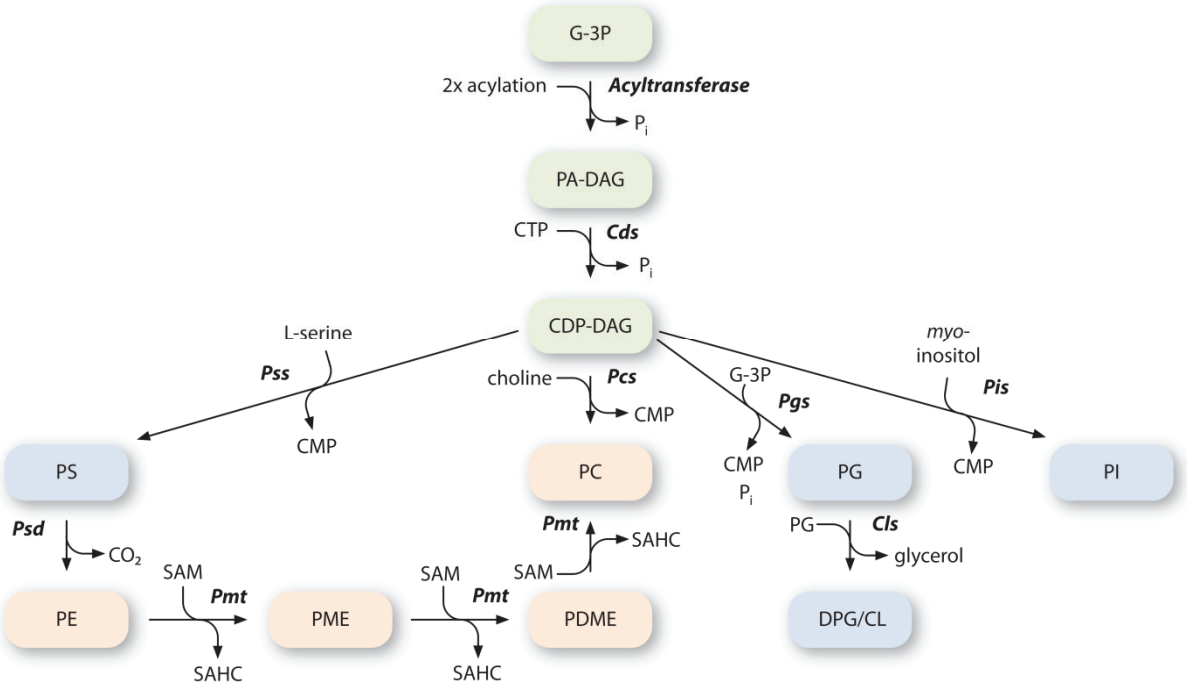

Figure I.12: Biosynthetic pathway for IPL synthesis in prokaryotes. Synthesis starts with glycerol-3-phosphate (G-3P), which is acylated twice to phosphatidic acid-diacylglycerol (PA-DAG) in a condensation reaction catalyzed by *acyltransferases*. PA-DAG then reacts with cytidintriphosphate (CTP) to cytidindiphosphate-diacylglycerol (CDP-DAG) in a reaction catalyzed by CDP-diacylglycerol synthase (*Cds*) which is releasing inorganic phosphate (P_i). CDP-DAG is the starting point for the synthesis of phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylglycerol (PG), and phosphatidylinositol (PI) in reactions catalyzed by phosphatidylserine synthase (*Pss*), phosphatidylcholine synthase (*Pcs*), phosphatidylglycerol synthase (*Pgs*), and phosphatidylinositol synthase (*Pis*), respectively, which all release cytidinmonophosphate (CMP). Two molecules of PG can be fused together to form cardiolipin (CL; also called diphosphatidylglycerol, DPG) with the enzyme cardiolipin synthase (*Cls*), thereby releasing glycerol. PS can be decarboxylated in a reaction catalyzed by phosphatidylserine decarboxylase (*Psd*) to form phosphatidylethanolamine (PE). In a series of methylation reactions catalyzed by phospholipid N-methyl-transferase (*Pmt*), PE can be converted to PC via phosphatidyl-(N)-methylethanolamine (PME) and phosphatidyl-(N,N)-dimethylethanolamine (PDME) using S-adenosylmethionine (SAM) as methyl-donor and releasing S-adenosylhomocysteine (SAHC) in each methylation step. Educts for IPL production are shown in green color, zwitterionic IPLs are shaded in red color, and anionic IPLs are colored in blue. Figure modified after Dowhan (1997), Cronan (2003) and Sohlenkamp *et al.* (2003).

Some IPLs have zwitterionic structures at physiological pH such as PE, PME, PDME,and PC, while others like PG, DPG, PS, and PI are anionic. The ionic characteristics of the membrane lipids define physiological and physical membrane properties. The zwitterionic IPLs comprise the majority of membrane phospholipids in eukaryotic cells, gram-negative bacteria (like *E. coli*) and many gram-positive bacteria (e.g. *Bacilli*, de Mendoz, 1993). PE is the most abundant IPL in bacteria (c.f. Dowhan, 1997), while PC has been identified as the major membrane forming lipid in eukaryotes (e.g. Raetz, 1986). It has been estimated that more than 10% of all bacteria posses PC (Sohlenkamp *et al.*, 2003). PME and PDME occur as intermediates in the synthesis of PC but depending on enzymatic activity the synthesis can slow down or even stop, allowing detection of methylated products in significant quantities.

PE and PC have traditionally been treated as interchangeable membrane lipids because both posses a dipole moment at the head group, but more recent studies have shown significant and important differences in chemistry and properties between those two lipid classes (Dowhan, 1997). The size of the polar head group increases from PE to PC, a property that has profound impact on the structure of the membrane. If small head groups are combined with large hydrophobic chains it is likely that the membrane forms nonbilayer structures due to the wedge-shape of the individual molecules. Relatively large head groups like PC form stable bilayer membranes and are only prone to produce nonbilayer structures under extreme conditions (Dowhan, 1997). Every biological membrane system has at least one nonbilayer-forming lipid component (Rilfors, 1984; Lindblom and Rilfors, 1989). The potential to form nonbilayer structures is important for cell division processes like membrane fusion and membrane vesicle formation as it influences the radius of membrane curvature (De Kruijff, 1997). It also has impact on the potential for movement of macromolecules through the membrane or lateral to it. Besides its structural role, PC is involved in a set of diverse cellular processes. In eukaryotes it can be a source of secondary messengers for signal transduction like diacylglycerol or phosphatidic acid (Sohlenkamp *et al.*, 2003).

The anionic membrane lipids serve a different, but equally important purpose. It has been speculated, that they form "islands of anionic membrane lipids" near the positively charged domains of peripheral membrane lipids (Dowhan, 1997). The electrostatic attraction thus promotes the membrane association of cytoplasmic proteins (Dowhan, 1997), an important function for synthesis of membrane-related products which have to be produced in close proximity. This effect can also help with activation of membrane-related enzymes which change their conformation when they get close to the anionic membrane. Furthermore, anionic membrane lipids play multiple and specific roles in the complex process of protein translocation in prokaryotic cells. PG is typically associated to photosynthetic organisms where it occurs in the thylakoid membranes and has an essential function in the photosynthetic machinery (Sakurai *et al.*, 2003; Hagio *et al.*, 2000; Wada and Murata, 2007), nevertheless, it has also been identified as the second most abundant lipid in bacterial membranes (Dowhan, 1997) including cultured representatives of the deep biosphere (Schubotz, 2005; Hinrichs *et al.*, unpublished data). PS is rarely found in higher concentrations in prokaryotes as it is an intermediate in PE biosynthesis. PI is an essential phospholipid involved in enzyme regulation of many key cellular functions in eukaryotes but is rarely found in bacteria.

The analytical window of IPL analysis is not only limited to analysis of bacterial and eukaryotic lipids but can be extended to include the third domain of life, the Archaea. Glycerol-based phospho- and glycolipids are building blocks of the archaeal cell membrane and comprise 2-6% of dry weight of the cell (Langworthy *et al.*, 1983; Ferrante *et al.*, 1990). Archaeal lipids are distinct from their bacterial and eukaryotal counterparts. In contrast to bacteria where the starting point for lipid synthesis is G-3P, archaeal lipid synthesis starts with glycerol-1-phosphate (G-1P) leading to stereochemically distinct membrane lipids of archaea and bacteria. Additionally, they use ether bonds to connect the glycerol backbone to isoprenoidal hydrocarbon chains in order to strengthen the cell membrane against extreme conditions (De Rosa *et al.*, 1989). Archaeal isoprenoidal lipids exist as diethers with two chains and also as membrane-spanning tetraethers with four ether bonds and varying number of rings in the two biphytane chains. The degree of cyclization and the diether to tetraether-ratio depends on growth temperature (DeRosa and Gambacorta, 1988; Gliozzi *et al.*, 1983; Uda *et al.*, 2001; Lai *et al.*, 2008). Polar head groups of archaeal IPLs are either purely hexose- and phosphate-based or a mixture of both (Koga *et al.*, 1993, Sturt *et al.*, 2004; Schouten *et al.*, 2008; see Figure I.11A for structures). The nature of the polar head group in combination with its core lipid allows the use of archaeal IPLs as chemotaxonomic marker. Analysis of lipid component parts in cultures of methanogenic archaea showed the specificy of the lipid composition up to the genus-level (Koga *et al.*, 1993; Koga *et al.*, 1998; Whitman *et al.*, 2001). Further support for the concept of chemotaxonomy comes from a broader study of cultures of environmentally relevant cren- and euryarchaea such as *Archaeoglobales*, *Methanobacteriales*, *Methanococcales* and *Thermoproteales*. It was observed that crenarchaea produce mostly glycosidic head groups attached to tetraether core lipids with a high degree of cyclization, whereas euryarchaea abundantly produce mixed phospho/glyco head groups, diether lipids and tetraether IPLs with a low number of rings in the core (Hinrichs *et al.*, unpublished data).

Analytical procedure for IPL analysis. IPLs can be extracted from pure biomass of cultures or sediments with a modified Bligh and Dyer procedure (White and Ringelberg, 1998). After extraction in several steps of ultrasonication with a mixture of methanol (MeOH), dichloromethane (DCM), and buffer the total lipid extract (TLE) is evaporated to dryness. An aliquot of the TLE is re-dissolved in a mixture of DCM/MeOH for injection to the chromatographic system. It has been found that a ratio of DCM:MeOH of 5:1 gives minimal disturbance of the chromatographic separation and is still polar enough to dissolve all

compounds of a typical IPL mixture (Buchs and Schubotz, unpublished results). From an analytical viewpoint, IPLs are complex molecules of high polarity and low stability. The best way to separate a mixture is by high performance liquid chromatography (HPLC) with a normal-phase eluent system and stationary phase. Two types of column packing material have been described in the literature: pure silica-gel based (Lutzke and Braughler, 1990) and silica gel chemically bonded to hydroxyl groups (Olsson *et al.*, 1996). The polar head group is the most polar part of IPL molecules, and chromatographic separation according to polarity separates the IPL mixture into lipid classes like PG, PE, or PC (see Figure I.13 for chromatogram and density map).

The detection of the analyte molecules can either be done with an evaporative light scattering detector (ELSD) or a mass selective detector (MSD). While the ELSD has the benefit of a linear response over a wide range of concentrations with equal detection efficiency, problems like co-elution of IPL classes, high noise background due to buffer in the eluent-system and detection of non-IPL compounds hinder the application in environmental samples. This is effectively circumvented when the MSD is used, because it offers another selection criterion: the mass-to-charge ratio. The transfer of ions from the liquid analyte to gas phase ions which can be manipulated in the MSD is done via electrospray ionization (ESI) probe. ESI is a soft ionization technique which is ideally suited for polar molecules like IPLs. However, not all classes of IPLs are ionized with the same efficiency. This calls for calibration curves with IPL standards that cover the range of IPLs expected in the sample to correct for a different response. In addition, it is useful to spike the sample with a recovery standard before it is extracted. This way it is also possible to correct for losses during extraction or adsorption to active glass surfaces while transferring the TLE (e.g. Pettitt *et al.*, 2006).

Another effect which has to be taken into account while quantifying IPLs is ion-suppression (e.g. Mallet *et al.*, 2004). Ion-suppression happens during the ionization step in the ion source when charge-transfer to the analyte is suppressed due to co-eluting substances. The reverse effect - ion enhancement - is also possible when the co-elution effectively helps to ionize the analyte. Ion-suppression and -enhancement effects are especially common when analyzing samples with complex sediment matrices. Two categories of co-eluting substances occur: molecules with similar polarity (and retention time) as IPLs that do not produce ions in ESI, or high concentrations of multiple IPLs that elute at the same retention time and compete for charge. The presence of ion-suppression and -enhancement can be checked for by constant infusion of an IPL standard downstream to the column while analyzing a sample

(e.g. Mallet *et al.*, 2004). When mass-spectrometrically "invisible" compounds elute from the column, the signal of the IPL standard is enhanced or suppressed at the corresponding retention time. In case of co-elution of multiple IPLs with high concentrations, usually the analysis of a more diluted sample fixes the problem. In general, the cleaner a sample is, the better is the quantification. This can be achieved by performing a chromatographic sample-cleanup step prior to analysis. However, in this case care has to be taken that no compounds are retained on the column.

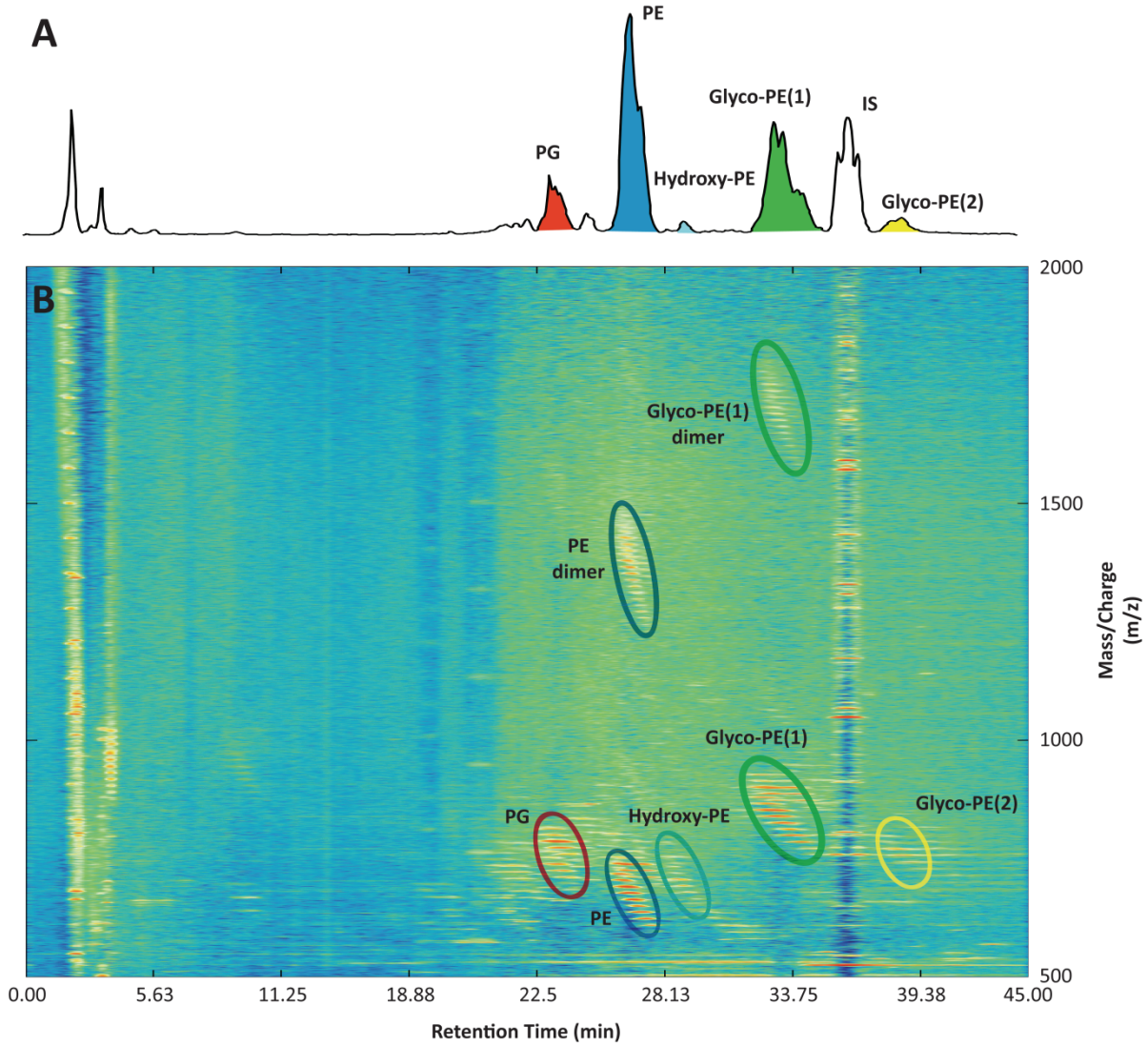

Figure I.13: HPLC-MS chromatogram and density map obtained from analysis of a cultured bacterial representative of the deep biosphere, *Shewanella sp.* (data from Schubotz, 2005). (**A**) Base peak plot chromatogram showing IPL classes in different colors. Peak shape is influenced by co-elution of IPL species with varying fatty acid side chains. (**B**) Density map adding the extra dimension of mass to charge ratio. Intensity is color-coded with warmer color representing higher intensity. Lipids belonging to the same class are encircled and labeled. Varying fatty acid chain length and unsaturation give rise to the formation of a series in the density map: IPLs with longer fatty acids and higher mass decrease the polarity and elute at earlier retention time and vice versa. The density map was digitally enhanced and filtered with a Matlab script (The MathWorks) for better illustration.

The use of special MSDs can provide additional structural information on the target analytes. Two types of MSD are useful for this task: triple stage quadrupole mass spectrometers (TSQ-MS) or ion-trap mass spectrometers (IT-MS). TSQ-MS are more sensitive but do not offer the wealth of structural information that IT-MS can provide. IT-MS can be programmed to automatically trap ions of interest and produce secondary and higher order daughter ion mass spectra (MS^n). For IPL structure elucidation it is especially useful to use ionization modes with positive and negative polarity. Positively charged IPL ions often loose the polar head group as neutral molecule in the MS2 step thus providing information on the lipid class. When the sample is analyzed in negative ionization mode, information about the individual fatty acid chains is gained (see Figure I.14). The combination of both polarity modes enables complete structural elucidation of IPL molecules.

Complex mixtures of IPLs are now being routinely analyzed with the HPLC-MS technique. Using a specialized variant of ESI - nano-spray ionization - it is possible to increase the sensitivity to the subpicomolar level. Such low amounts of IPLs equal the quantities found in 1000 cells or less (Brügger *et al.*, 1997). Until now, this sensitive technique has not been applied to the analysis of extracts from marine subsurface sediments. This environment is analytically very challenging due to low biomass concentrations and the complex matrix of sedimentary organic material.

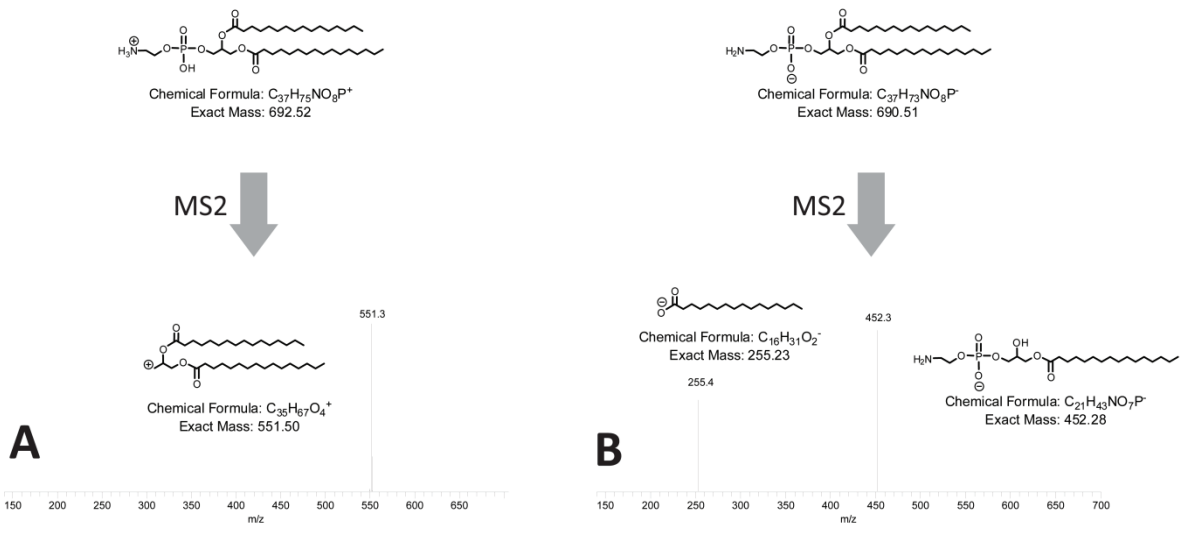

Figure I.14: Mass spectra and corresponding structures of fragments of dipalmitoyl-phosphatidylethanolamine (C16:0-C16:0-PE) obtained in positive and negative ionization mode. PE readily forms positively and negatively charged ions due to its zwitterionic structure. (**A**) Positive ionization mode. The MS2 daughter ion spectrum shows loss of the PE head group with a mass of 141 Da. The core fragment mass of 551 Da allows to calculate the sum of carbon atoms in both fatty acid chains (C32:0) but no information about the individual chain length is obtained. (**B**) Negative ionization mode. Two MS2 daughter ions are formed, one belonging to the fatty acid (m/z 255) and the other to a lyso fragment (m/z 542). The information of both fragments allows assigning individual fatty acid chain length to the IPL molecule. Note that asymmetric fatty acid chains produce two fatty acid and two lyso fragments.

I.6. STABLE CARBON ISOTOPES AS INDICATOR FOR METABOLIC STRATEGIES

Elements are composed of one or more isotopes that have nuclei with the same number of protons but different number of neutrons. The varying number of elementary particles in the nucleus leads to different molecular mass. This has consequences during chemical reactions, where the light isotopes are partially separated from the heavier isotopes in a process called isotope fractionation (see Hayes (1982) for a review). Typically, the lighter isotope reactants react faster and as a result the products are isotopically depleted. The change in isotopic composition is usually very low. For practical reasons scientists have developed the δ-notation which describes the deviation of an isotopic ratio to a reference standard of known isotopic composition in ‰ (equation I.1).

$$\delta X = \left[\left(R_{sample} - R_{reference}\right) - 1\right] \cdot 1000 \qquad \text{(I.1)}$$

In this equation X is the less-abundant isotope (e.g. ^{13}C), and R is the isotopic ratio measured for sample and reference (e.g. $^{13}C/^{12}C$). For the five principal light elements of interest the reference standards are:

(a) H, O: Standard Mean Ocean Water (SMOW)
(b) C, O: Vienna Pee Dee Belemnite (VPDB)
(c) N: Atmospheric Air
(d) S: Canyon Diablo Meteorite

Some elements have unstable (radioactive) isotopes, either because their decay is so slow that significant amounts still exist since their formation, or because they are continually produced by radiation processes. Of the 94 elements occurring naturally on Earth, only 80 elements have stable isotopes. One of them is carbon, which occurs in nature as mixture of the stable isotopes ^{12}C (98.9%) and ^{13}C (1.1%) (Killops and Killops, 2005). Stable carbon isotope analysis is widely used to track the carbon fixation pathway in microorganisms. For this purpose, the isotopic composition of all carbon pools has to be known, in particular total organic carbon, dissolved inorganic carbon, and hydrocarbon gases like methane. Figure I.15 shows the carbon isotopic values of common carbon reservoirs found in nature (Trumbore and Druffel, 1995).

Useful tools for tracing substrates from the source to metabolic waste-product or biomass are ^{13}C-labeled substances like bicarbonate, methane, glucose, or acetate (see review by Boschker and Middelburg, 2002). The uptake of substrate can be monitored by isotopic analysis of biomass over time and carbon assimilation rates can be determined. Furthermore, the basic mode of carbon metabolism can be identified. In contrast to unstable isotopic

tracers which can be readily detected by radioactive radiation even in low concentrations, stable isotopic tracers have to be added in significant quantities to be detectable. However, addition of strongly labeled substrates to a system alters concentrations and kinetic parameters and might render the results useless (cf. Hayes, 2006).

A better approach is based on analysis of the natural abundance of isotopes in substrates and products. Enzyme driven biochemical carbon fixation shifts the carbon isotopic composition of the products towards more depleted values depending on the process and pathway (Table I.2). The residual carbon substrate pool becomes therefore increasingly

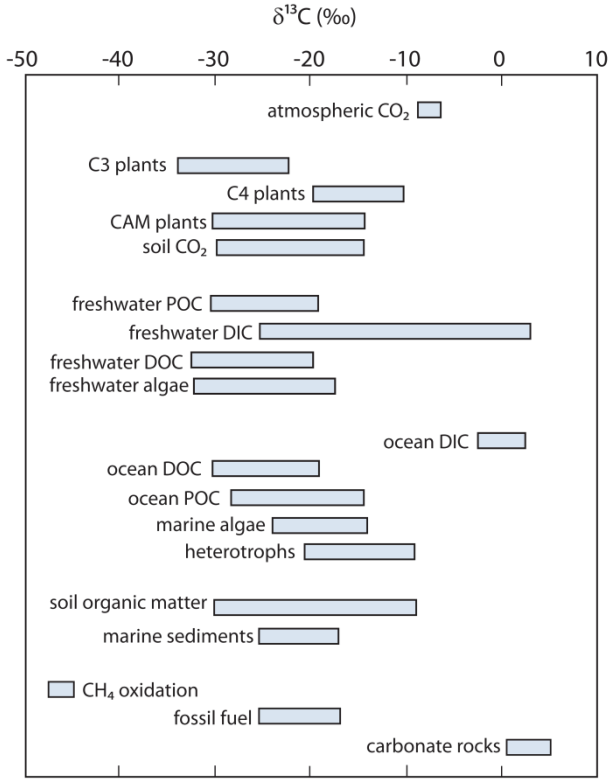

Figure I.15: Different forms of carbon are characterized by widely varying $\delta^{13}C$ values as a result of fractionation processes. Shown are the ranges of $\delta^{13}C$ values for common carbon reservoirs. Figure adopted from Trumbore and Druffel (1995).

enriched in ^{13}C over time. For example, assimilation of carbon during anaerobic oxidation of methane (AOM) yields carbon isotopic values of the methane-oxidizers (measured as archaeol lipids) which are 15-50‰ depleted relative to their carbon substrate, methane (Hinrichs and Boetius, 2002). Methane which is biologically produced is already significantly depleted in ^{13}C (Figure I.15) and further isotopic fractionation results in extraordinarily depleted values of -100 to -120‰ for some lipids (cf. Hinrichs *et al.*, 1999). The exceptional position of methane carbon isotopes therefore greatly simplifies the identification of methane-carbon related metabolic processes in the environment.

The isotopic analysis of metabolic intermediates like volatile fatty acids (VFA, Heuer *et al.*, 2006) represents a novel and powerful tool for the reconstruction of carbon flow. Acetate

is the central intermediate in anaerobic metabolism like organic matter fermentation or autotrophic acetogenesis and an important substrate for a variety of microorganisms like sulfate-reducing bacteria and methanogenic archaea (Cappenberg and Prins, 1974; Sørensen *et al.*, 1981; Winfrey and Ward, 1983).

Table I.2: Isotope fractionation effects associated to different modes of carbon metabolism.

Pathway/process	Fractionation (biomass – substrate)	Reference
	Autotrophic growth	
C3 plants, Rubisco	10-22‰	Hayes (2001)
C4 plants, Rubisco	2-15‰	Hayes (2001)
Acetyl-CoA	15-36‰	Hayes (2001)
Reductive or reverse TCA Cycle	4-13‰	Hayes (2001)
3-Hydroxypropionate Cycle	0‰	Hayes (2001)
	Heterotrophic growth	
Heterotrophy	2‰	Coffin *et al.* (1989)
	Methane assimilation	
Anaerobic methane oxidation	15-50‰	Hinrichs and Boetius (2002)
Aerobic methane oxidation	10-20‰	Barker and Fritz (1981)

I.7. CONTRIBUTIONS TO PUBLICATIONS

This thesis includes the complete versions of three manuscripts for publication in international journals (chapters II-IV). Chapters Va to Vd include abstracts of manuscripts that are also part of this thesis but are not included in full for reasons of length. Two manuscripts are already published (chapter II and Vd), one is in press (chapter Vc), three are in the revision stage (chapters III, Va, and Vb) and one is a draft version close to submission (chapter IV).

Chapter II – full manuscript

Heterotrophic Archaea dominate sedimentary subsurface ecosystems off Peru
Jennifer F. Biddle, Julius S. Lipp, Mark A. Lever, Karen G. Lloyd, Ketil B. Sørensen, Rika Anderson, Helen F. Fredricks, Marcus Elvert, Timothy J. Kelly, Daniel P. Schrag, Mitchell L. Sogin, Jean E. Brenchley, Andreas Teske, Christopher H. House, and Kai-Uwe Hinrichs

Jennifer F. Biddle and Julius S. Lipp contributed equally to this work and are listed alphabetically as joint first authors. Julius S. Lipp extracted membrane lipids from sediment samples, developed a new analytical protocol for selective isotopic analysis and determination of relative concentrations, and performed geochemical modeling and thermodynamic calculations of energy fluxes and maintenance energies. Rika Anderson helped with lipid analysis during her summer student fellowship. Marcus Elvert and Helen F. Fredricks provided expertise on biomarker analysis. Daniel P. Schrag provided isotopic data of DIC; Mitchell L. Sogin contributed new reagents and analytical tools; Mark A. Lever, Karen G. Lloyd, Ketil B. Sørensen, and Andreas Teske provided clone library data; Jennifer F. Biddle, Timothy J. Kelly, Christopher H. House, and Jean E. Brenchley performed FISH-SIMS analysis. Jennifer F. Biddle, Julius S. Lipp and Kai-Uwe Hinrichs wrote the paper jointly with editorial input from all co-authors. Published in *Proceedings of the National Academy of Sciences of the USA*, vol. 103, no. 10, page 3846-3851, doi:10.1073/pnas.0600035103

Chapter III – full manuscript

Significant contribution of Archaea to extant biomass in marine subsurface sediments
Julius S. Lipp, Yuki Morono, Fumio Inagaki, and Kai-Uwe Hinrichs

Julius S. Lipp developed and validated the analytical protocol for lipid extraction and quantification, performed lipid analysis and data handling, and wrote the Matlab scripts for

geochemical and sedimentological modeling. Ulrike Proske helped with lipid extraction and sample preparation in the laboratory. Yuki Morono and Fumio Inagaki provided results from DNA extraction and molecular analyses, Kai-Uwe Hinrichs helped with geochemical modeling. Julius S. Lipp and Kai-Uwe Hinrichs wrote the paper with input from all co-authors. The manuscript has been reviewed by *Nature;* minor revisions have been requested by the editor.

Chapter IV – full manuscript

Structural diversity of intact polar lipids in marine sediments

Julius S. Lipp and Kai-Uwe Hinrichs

Julius S. Lipp developed and validated the analytical protocol for lipid extraction, separation of polar and apolar GDGTs, mass spectrometric characterization of unknown compounds, and performed box-modeling for lipid-stability calculations using Matlab scripts and Excel with input from Kai-Uwe Hinrichs. Ulrike Proske and Nadine Buchs assisted with lipid extraction and sample preparation. Julius S. Lipp wrote the paper with input from Kai-Uwe Hinrichs. The manuscript is prepared for submission to *Geochimica and Cosmochimica Acta*.

Chapter Va – abstract only

Vertical distribution of microbial lipids and functional genes in chemically distinct layers of a highly polluted meromictic lake

Tobias F. Ertefai, Meredith C. Fisher, Helen F. Fredricks, Julius S. Lipp, Ann Pearson, Daniel Birgel, Kai M. Udert, Coleen M. Cavanaugh, Philip M. Gschwend, and Kai-Uwe Hinrichs

Tobias F. Ertefai analyzed, identified and quantified intact polar lipids with input from Julius S. Lipp and Helen F. Fredricks. Tobias F. Ertefai and Daniel Birgel analyzed Bacteriohopanepolyols with analytical expertise by Julius S. Lipp. Meredith C. Fisher, Ann Pearson, Kai M. Udert, Coleen M. Cavanaugh, and Philip M. Gschwend provided molecular biological data. Tobias F. Ertefai wrote the paper with input from all co-authors. The manuscript is in revision at *Organic Geochemistry*.

Chapter Vb – abstract only

Microbial community composition and potential activity in hydrate bearing deep subsurface sediments of Hydrate Ridge (Cascadia Margin, ODP Leg 204)

Beth! N. Orcutt, Julius S. Lipp, Katrin Knittel, Kai-Uwe Hinrichs, Samantha B. Joye, and Antje Boetius

Beth! N. Orcutt and Julius S. Lipp performed method development and validation for lipid analysis and analyzed intact polar lipid concentrations and prepared fractions for isotopic analysis. Julius S. Lipp prepared fractions for analysis of apolar lipids and analyzed them isotopically and performed ether cleavage reactions and isotopic analysis of derived apolar compounds. Beth! N. Orcutt, Katrin Knittel, Samantha B. Joye, and Antje Boetius performed molecular biological analyses. Beth! N. Orcutt wrote the paper with input from all co-authors. The manuscript is in the revision stage at *Environmental Microbiology*.

Chapter Vc – abstract only

Intact polar lipids of anaerobic methanotrophic archaea and associated bacteria

Pamela E. Rossel, Julius S. Lipp, Helen F. Fredricks, Julia Arnds, Antje Boetius, Marcus Elvert, and Kai-Uwe Hinrichs

Pamela E. Rossel, Julius S. Lipp, and Helen F. Fredricks analyzed intact polar lipids with assistance from Marcus Elvert. Julius S. Lipp helped with identification and quantification of compounds and provided expertise for mathematical combination of separate analytical runs. Julia Arnds and Antje Boetius provided molecular biological data. Pamela E. Rossel and Kai-Uwe Hinrichs wrote the paper with input from all co-authors. The manuscript is in press at *Organic Geochemistry*. doi:10.1016/j.orggeochem.2008.02.021

Chapter Vd – abstract only

Methane-Producing Microbial Community in a Coal Bed of the Illinois Basin

Dariusz Strapoc, Flynn W. Picardal, Courtney Turich, Irene Schaperdoth, Jennifer M. Macalady, Julius S. Lipp, Yu-Shih Lin, Tobias F. Ertefai, Florence Schubotz, Kai-Uwe Hinrichs, Maria Mastalerz, and Arndt Schimmelmann

Julius S. Lipp analyzed intact polar lipids in enrichment cultures with assistance from Tobias F. Ertefai. Julius S. Lipp designed mass spectrometric experiments targeting unknown lipid constituents for characterization of all membrane lipids and performed quantification. Yu-Shih Lin and Kai-Uwe Hinrichs performed thermodynamic calculations; Florence Schubotz provided acetate concentrations; Dariusz Strapoc, Flynn W. Picardal, Courtney Turich, Irene Schaperdoth, Jennifer L. Macalady, Maria Mastalerz, and Arndt Schimmelmann provided microbiological and microscopical data. Dariusz Strapoc wrote the manuscript with input from all co-authors. Published in *Applied and Environmental Microbiology*, vol. 74, no. 8, page 2424-2432, doi:10.1128/AEM.02341-07 © American Society for Microbiology, April 2008.

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Chapter II

Heterotrophic Archaea dominate sedimentary subsurface ecosystems off Peru

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Published in *Proceedings of the National Academy of Sciences of the USA*, vol. 103, no. 10, page 3846-3851, doi:10.1073/pnas.0600035103

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II.1. PRINTED MANUSCRIPT

ABSTRACT

Studies of deeply buried, sedimentary microbial communities and associated biogeochemical processes during Ocean Drilling Program Leg 201 showed elevated prokaryotic cell numbers in sediment layers where methane is consumed anaerobically at the expense of sulfate. Here, we show that extractable archaeal rRNA, selecting only for active community members in these ecosystems, is dominated by sequences of uncultivated Archaea affiliated with the Marine Benthic Group B and the Miscellaneous Crenarchaeotal Group, whereas known methanotrophic Archaea are not detectable. Carbon flow reconstructions based on stable isotopic compositions of whole archaeal cells, intact archaeal membrane lipids, and other sedimentary carbon pools indicate that these Archaea assimilate sedimentary organic compounds other than methane even though methanotrophy accounts for a major fraction of carbon cycled in these ecosystems. Oxidation of methane by members of Marine Benthic Group B and the Miscellaneous Crenarchaeotal Group without assimilation of methane–carbon provides a plausible explanation. Maintenance energies of these subsurface communities appear to be orders of magnitude lower than minimum values known from laboratory observations, and ecosystem-level carbon budgets suggest that community turnover times are on the order of 100–2,000 years. Our study provides clues about the metabolic functionality of two cosmopolitan groups of uncultured Archaea.

INTRODUCTION

The microbial ecosystem in deeply buried marine sediments may comprise one-tenth of Earth's living biomass (Parkes *et al.*, 2000; Whitman *et al.*, 1998), but little is known about the organisms, their physiologies, and their influence on surface environments. Geochemical modeling suggests that microbial respiration in marine subsurface sediments is largely dominated by sulfate reduction coupled to the anaerobic oxidation of methane (AOM) (D'Hondt *et al.*, 2002). In deeply buried sediments, this process is typically observed in a sediment horizon into which both methane and sulfate diffuse, hereafter termed ''sulfate-methane transition zone'' (SMTZ), and where both compounds are consumed in equimolar amounts (e.g., Iversen and Jørgensen, 1985). Our knowledge about the microorganisms mediating AOM is largely based on studies of methane seep environments, where high concentrations of methane and sulfate promote high activities of syntrophic consortia of archaea and bacteria (e.g., Hinrichs and Boetius, 2002). Little is known about the microorganisms mediating AOM in diffusive settings with low fluxes of methane and sulfate such as deeply buried SMTZs.

During Ocean Drilling Program (ODP) Leg 201, which targeted sediments underlying highly productive surface waters off Peru, strikingly elevated concentrations of cells were detected by the acridine orange staining technique in three deeply buried SMTZs (D'Hondt *et al.*, 2003; D'Hondt *et al.*, 2004) (see Supplementary Figure II.4). The SMTZs are located at sediment depths of ≈10 m (ODP Site 1230), ≈30 m (Site 1229, top SMTZ), ≈40 m (Site 1227), and ≈88 m (Site 1229, bottom SMTZ) and may have a vertical extension of up to a few meters as indicated by various geochemical parameters (D'Hondt *et al.*, 2003). Microbial activity in these sediments is indicated by a large variety of geochemical parameters (D'Hondt *et al.*, 2003; D'Hondt *et al.*, 2004), the presence of cultivable Bacteria (D'Hondt *et al.*, 2004), intact cells, and intact DNA of both Archaea and Bacteria (Inagaki *et al.*, 2006; Mauclaire *et al.*, 2004; Parkes *et al.*, 2005; Schippers *et al.*, 2005). These studies suggest that (*i*) a significant fraction of the cells detected with acridine orange is active (Schippers *et al.*, 2005), and (*ii*) a high genetic diversity exists and is somehow linked to geochemical conditions (Inagaki *et al.*, 2006, Parkes *et al.*, 2005), while (*iii*) views of the composition of microbial communities derived from different approaches remain controversial (Inagaki *et al.*, 2006; Mauclaire *et al.*, 2004; Parkes *et al.*, 2005; Schippers *et al.*, 2005).

Here, we constrain community composition and processes in deeply buried sediments in and outside SMTZs using a previously undescribed combination of molecular and isotopic

techniques that provide insight into the ecosystem's carbon flow regime while selecting for only truly active community members.

RESULTS AND DISCUSSION

Archaeal communities in SMTZs. Extractable archaeal 16S rRNA is an indicator of active subsurface Archaea while excluding inactive cells and fossil DNA (Inagaki *et al.*, 2005). In the four SMTZs sampled during ODP Leg 201, 16S rRNA is dominated by sequences belonging to the uncultured Marine Benthic Group B and Miscellaneous Crenarchaeotal Group (Figure II.1; see also Supplementary Figure II.5). Both previously undescribed groups also have been detected by analysis of DNA from the investigated sites (Inagaki *et al.*, 2006, Parkes *et al.*, 2005) and, in fact, are widely distributed throughout marine subsurface environments (Inagaki *et al.*, 2003, Coolen *et al.*, 2002) (A.T., M.A.L., K.G.L., and K.B.S., unpublished data), but their physiologies are unknown. Notably, sequences from methanotrophic Archaea that inhabit surface sediments (Orphan *et al.*, 2001a) were not detected in rRNA and also have been absent in DNA libraries (Inagaki *et al.*, 2006; Parkes *et al.*, 2005).

Similar to rRNA, intact polar lipids (IPLs) also are presumed to represent living rather than fossil biomass (Sturt *et al.*, 2004). The IPLs from these sediments are dominated by archaeal glyceroldialkylglyceroltetraethers (GDGT), followed by glyceroldialkyldiether, both types attached to diglycosidic polar headgroups (Figure II.2; see also Supplementary Table II.3). The presence of calditol-based IPLs (Sturt *et al.*, 2004) and crenarchaeol-derived alkyl chains (Damste *et al.*, 2002) (Figure II.2) are indicators of crenarchaeotal community members, in agreement with rRNA results. Counts of cells hybridized to a domain-specific archaeal fluorescent *in situ* hybridization (FISH) probe (Figure II.2) offer independent evidence in support of a sizeable and active archaeal community. As with the extraction of rRNA and IPLs, the FISH protocols applied in our study are considered to select for active members of the population.

Both counts of FISH-labeled Bacteria and Archaea and relative distributions of IPLs (Table II.1) suggest that the active portion of the microbial community is dominated by Archaea. At all sites, the highest relative abundance of Archaea is found within SMTZs. The situation is not much different outside SMTZs; only two samples from 0.7 m below seafloor at Sites 1227 and 1229 showed slightly higher bacterial than archaeal counts (Table II.1). Similarly, bacterial IPLs were not identified in any of the analyzed samples. Unidentified compounds present in some samples do not show any of the mass spectrometric properties

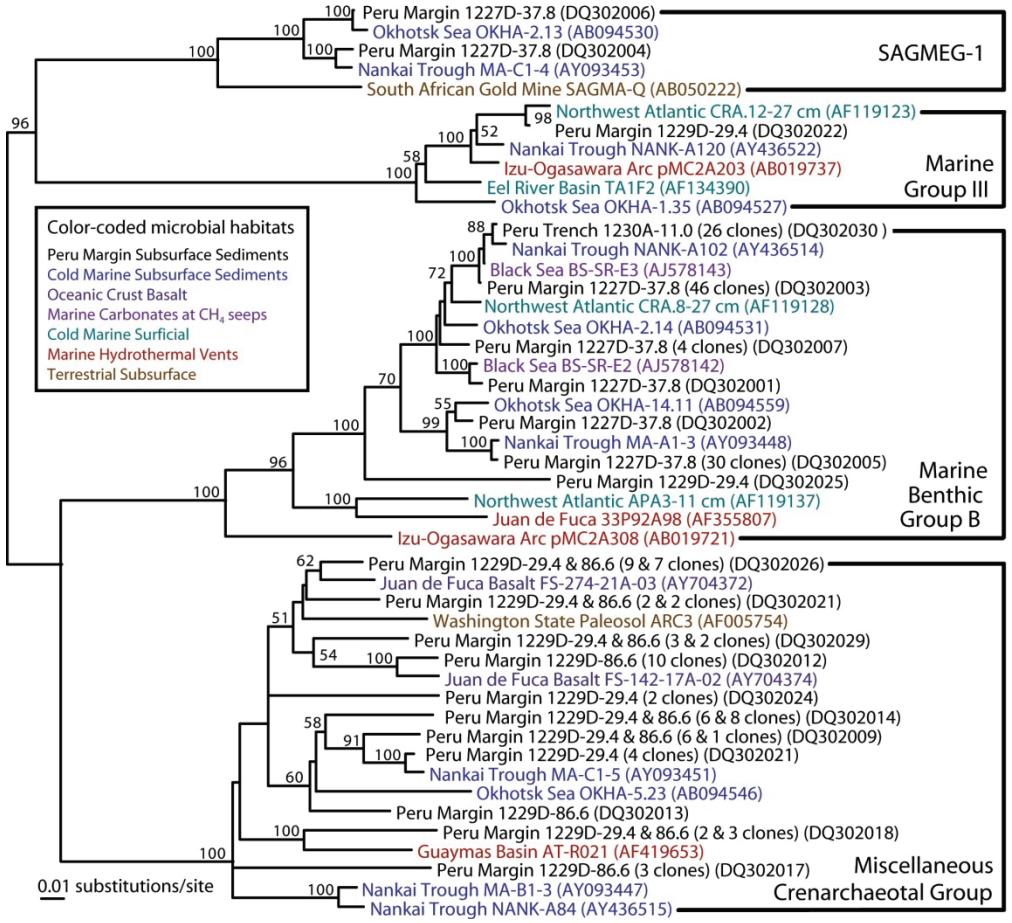

Figure II.1: A 16S rRNA archaeal phylogenetic tree based on maximum likelihood distances of ~900 nucleotide positions (16S rRNA positions 23–914). Bootstrap numbers are based on 200 resamplings. Sequences were obtained from four sediment samples from each of the four SMTZs encountered during ODP Leg 201: 1227D-37.8 (ODP Hole-depth in hole in meters below seafloor), 1229D-29.4, 1229D-86.8, and 1230A-11.0. Closely related sequence clusters are represented by single sequences, annotated with the number of near-identical 16S rRNA clones that they represent and their Gen-Bank accession numbers. Sequences are color coded by habitat to illustrate the diverse environmental range of these uncultured archaeal lineages.

observed during analysis of a large variety of bacterial IPLs in both environmental samples and cultures of environmentally relevant bacteria (K.-U.H., H.F.F., J.S.L., F. Schubotz, and T. Mohr, unpublished data) and are considered unlikely to be of bacterial origin. In SMTZs at Sites 1227 and 1229, archaeal cells account for at least 80% of total hybridized Archaea and Bacteria, whereas at Site 1230 the archaeal proportion is at ≈60%, somewhat lower. The highest concentrations of archaeal cells, up to $6x10^6$ cells per cm^{-3} sediment, were found at the two SMTZs at Site 1229, followed by Site 1230 (Table II.1). The relative abundance of archaeal lipids also peaks at the two SMTZs of Site 1229 (Table II.1). Notably, relative yields of archaeal rRNA determined throughout the sediment column at Site 1227 were ≈2 orders of magnitude higher in the SMTZ than outside, suggestive of a drastic increase of active archaea (K.B.S. and A.T., unpublished data).

Table II.1: Quantification of active archaeal cells hybridized with archaeal FISH Probe Arch915-Cy3, percentage archaea of total prokaryotes, and relative quantities of archaeal IPLs

Sample core-depth, mbsf	Archaeal cells 10^6 cm^{-3} sed.	Archaea %	Archaeal IPLs relative, %
1227D-0.7	9.7 ± 0.11	35	
1227D-34.5	1.3 ± 0.15	96	
1227A-37.6*			48 ± 6
1227D-38.0*	1.5 ± 0.01	98	
1227D-38.2*			39 ± 4
1227A-40.5*	1.7 ± 0.08	98	
1227A-40.9*			31 ± 5
1229D-0.7	2.8 ± 0.40	45	
1229D-1.4			10
1229D-4.3	5.4 ± 0.20	68	
1229D-12.9	9.2 ± 1.61	69	
1229D-29.7*			80 ± 6
1229D-29.8*	6.3 ± 1.13	80	
1229D-30.4*	5.9 ± 0.98	86	
1229D-32.4*			22
1229D-49.8	1 ± 0.17	81	
1229D-49.9			20
1229D-54.3	3.2 ± 0.31	67	
1229D-86.8*	3.2 ± 0.35	94	
1229D-87.1*			100 ± 9
1229D-89.1*	6.4 ± 1.59	98	
1229A-121.1			14
1229A-121.4	1.5 ± 0.37	63	
1230A-0.8			4
1230A-1.3	1.7 ± 0.44	54	
1230C-9.1*	4.3 ± 2.0	63	
1230B-9.7*			17 ± 1
1230B-10.1*			14 ± 2
1230C-10.3*	3.1 ± 0.1	61	
1230C-10.7*	2.9 ± 0.36	56	
Average	2.9	82	34

Percentage archaea of total prokaryotes is based on cells hybridized with probes Arch915-Cy3 and EUB338-FITC. Relative quantities of archaeal IPLs were normalized to the sample with maximum concentration, 1229D-87.1. Values are ±SD of three replicates. mbsf, m below seafloor; sed, sediment. *Samples from SMTZs.

Support for an important role of Archaea in subsurface sediments off Peru comes from a study of Site 1229 by Mauclaire *et al.* (2004), who counted archaeal cells directly with catalyzed reporter deposition (CARD)-FISH, a technique shown to increase the sensitivity for detecting moderately active cells (Amann *et al.*, 1992). Conversely, Schippers *et al.* (2005) inferred from quantitative PCR that DNA extracts from sediments at Sites 1227 and 1230, including those from SMTZs, are dominated by Bacteria, whereas archaeal cell estimates are 10^5 ml^{-1} and lower. In the same study, CARD-FISH did not detect Archaea in amounts sufficient for direct counts (Schippers *et al.*, 2005). These conflicting data stress that distinguishing between presence and activity of subsurface prokaryotes remains a major problem.

Isotopic Constraints on the Carbon Metabolism of Subsurface Archaeal Communities. The profiles of $\delta^{13}C$ of dissolved inorganic carbon (DIC) exhibit minima in SMTZs (profiles not shown) and suggest production of DIC by AOM. $\delta^{13}C$ of archaeal biomass depends on the $\delta^{13}C$ of the substrates used for cell growth and/or the carbon fixation pathway (e.g. Hayes, 2001). Because of strong ^{13}C-depletions commonly observed in methane, the biomass of methane-oxidizing Archaea is typically strongly depleted in ^{13}C (e.g. Hinrichs *et al.*, 1999). $\delta^{13}C$of deeply buried Archaea was determined by two independent techniques in adjacent samples, i.e., on whole cells by FISH coupled to secondary ion mass spectrometry (SIMS) (cf. Orphan *et al.*, 2001b) and by compound-specific analyses of IPLs. The $\delta^{13}C$ values of archaeal cells and IPLs in SMTZs are uniform and average at -20‰ (n = 8 samples; 98 cells total) and -24‰ (n = 8 samples; expressed as weighted average of δ values obtained for hydrocarbon derivatives prepared from IPLs), respectively, and are not significantly different from values obtained for archaeal biomass in sediments above the SMTZ (Figure II.3).

We draw the following conclusions from the isotopic relationships between archaeal biomass and other sedimentary carbon pools (Figure II.3). (*i*) Given the consistently high ^{13}C-depletion in methane, the δ values of archaeal biomass indicate that only a small fraction,

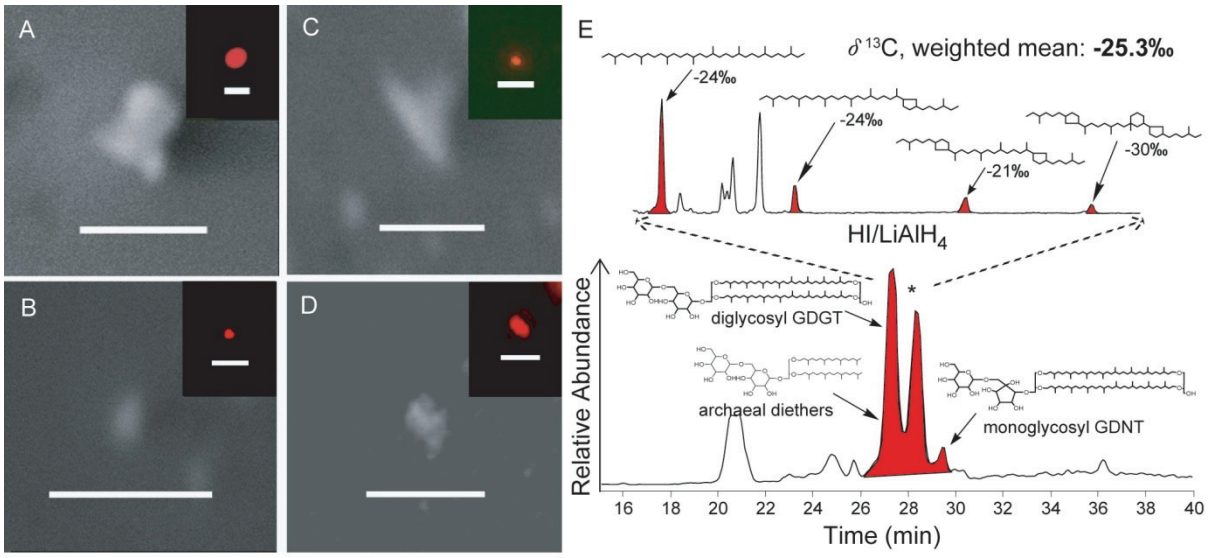

Figure II.2: Microscopic images of archaeal cells (A–D) and chromatograms of gas and liquid chromatographic analyses of archaeal lipids (E). **(A–D)** Environmental scanning electron microscopy (ESEM) images of individual cell targets in the following samples: 1227D-34.4 (**A**) (ODP Hole-depth in hole in meters below seafloor), 1229D-29.8 (**B**), 1229D-86.8 (**C**), and 1230C-9.1 (**D**). (Scale bars: 1 µm.) *Insets* show FISH images of the same cells. All ESEM analyses showed small cells, most <1 µm in diameter. All images have been subjected to postexposure software enhancement to increase brightness and contrast. (**E Lower**) Positive ion base-peak HPLC-MS chromatogram of a fraction resulting from consecutive trapping of archaeal IPLs by preparative HPLC-MS of sample 1229D-87.1. Red labeled peaks designate archaeal glyceroldialkyl-glyceroltetraethers (GDGTs) with smaller quantities of coeluting archaeal glyceroldialkylethers (structures shown; *, tentatively identified as GDGT derivative). (**E Upper**) Total ion current GC-MS chromatogram of isoprenoid hydrocarbons released from IPLs by ether cleavage and subsequent reduction ($\delta^{13}C$ of individual compounds is reported in Supplementary Table II.3).

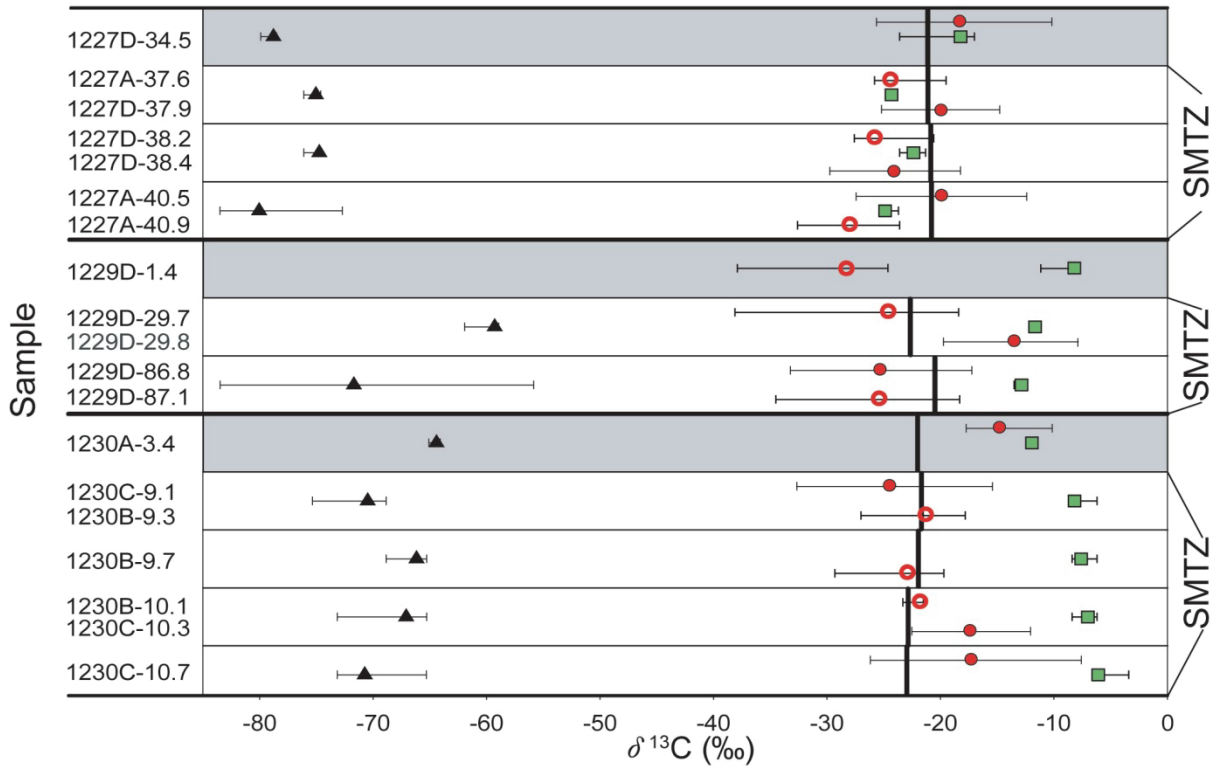

Figure II.3: $\delta^{13}C$ values of sedimentary methane (filled triangles; linearly interpolated from sample depth used for gas analysis to depth of samples used for FISH and IPL analysis; endpoints of bars indicate actual δ values of methane), DIC (green squares; linearly interpolated, as above), archaeal cells determined by FISH–secondary ion MS (filled red circles; weighted mean of all samples for each site; bars show the error on that mean; mean squares weighted deviation ranges were 0.1–1.5), and archaeal IPLs (open red circles; circle shows the weighted mean $\delta^{13}C$ of all IPL-derived archaeal hydrocarbons; line shows the range of individual compounds). Brown bars designate $\delta^{13}C$ of TOC. Gray-shaded samples stem from sediments above the SMTZ. Sample names: ODP Hole-depth in hole in meters below seafloor.

if any, of the archaeal populations relies on methane as carbon source. (*ii*) The relatively small ^{13}C-depletion of archaeal biomass relative to DIC indicates that autotrophic processes such as methanogenesis via CO_2 reduction are not fueling the bulk of the archaeal communities (House *et al.*, 2003). (*iii*) The small ^{13}C-depletion of lipids relative to bulk biomass eliminates an important role of methylotrophic methanogens (Summons *et al.*, 1998). (*iv*) Instead, the similarity of isotopic compositions of archaeal biomass and total organic carbon (TOC) is striking and suggests that the bulk archaeal community assimilates organic compounds derived from fossil organic matter (OM). The variability of isotopic compositions of both hydrocarbon derivatives prepared from IPLs (Supplementary Table II.3) and individual archaeal cells (see Supplementary Table II.4) is consistent with some versatility of metabolic reactions and/or carbon sources used by community members, but no single isotopic value points strongly toward assimilation of methane-derived carbon.

Degradation of sedimentary OM probably provides a substantial fraction of carbon for the archaeal community. Likely intermediates are low-molecular-weight organic compounds

such as acetate and formate, which are present in all Leg 201 sediments and exhibit elevated concentrations at the SMTZs of Site 1229 (D'Hondt *et al.*, 2003).

Energy Fluxes and Maintenance Energies. Conceptually, we consider AOM and slow degradation of refractory sedimentary OM as the two principal sources of energy for communities in SMTZs. Electron donors that are independent of photosynthetically produced OM and relevant in other subsurface settings are not important in the Peru Margin subsurface, e.g., H_2 andO_2 from radiolysis of water (Lin *et al.*, 2005; Blair *et al.*, 2005) or H_2 from rock–water reactions (Stevens and McKinley, 1995). Anaerobic degradation of sedimentary OM is typically accomplished by complex syntrophic associations and involves fermentative breakdown of large organic molecules and subsequent reaction of products to CH_4 and CO_2 or, when coupled to sulfate reduction, only to CO_2. Methane fluxes, determined from methane concentration profiles, account for 8% (1229, top SMTZ), 33% (Site 1230), 43% (1229, bottom SMTZ), and 114% (Site 1227) of sulfate fluxes (Table II.2). Although loss of methane gas during sample retrieval from high pressure environments could lead to underestimation of fluxes of methane relative to those of sulfate, alternative sinks of sulfate such as organotrophic sulfate reduction could explain the differences of fluxes of methane and sulfate at the SMTZs at Sites 1229 and 1230. If organotrophic sulfate reduction were performed by archaea and their growth efficiencies were not vastly higher than those of presumed archaeal methanotrophs, the excess in sulfate fluxes over those of methane is not large enough to wipe out an isotopic signature of methane assimilation in all except the upper SMTZ at Site 1229.

The rates of the biologically mediated ''basal'' degradation of sedimentary OM in SMTZs were estimated on the basis of a diagenetic model (Middelburg, 1989) that describes the decrease in reactivity with age of the OM (Table II.2). The resulting rates are lower than (Sites 1227 and 1230) to approximately equal to (Site 1229) modeled rates of methane turnover in SMTZs (Table II.2). Gibbs free-energy changes (ΔG_R) in SMTZs were estimated from turnover rates of both methane and sedimentary organic carbon, and energies to maintain the observed microbial populations were computed by using an empirical model that simulates minimum values known from laboratory cultures (Tijhuis *et al.*, 1993). The results show that the ΔG_R estimates can only account for a small fraction of the energy that would be required for maintaining the observed communities (Table II.2). Two principal conclusions can be drawn from the energetic considerations: (*i*) Real maintenance energies in subsurface environments must be much lower than what has been experimentally determined in

Table II.2: Average values of total hybridized cells, fluxes, rates of AOM and OC degradation, and related ΔG_R energies required to maintain the observed populations, and community turnover times in SMTZs.

Site; SMTZ	Hybr. cells[1]	CH_4 flux into SMTZ[2]	SO_4^{2-} flux into SMTZ[2]	AOM rate[3]	OC-degr. rate[4]	ΔG_R, AOM[5]	ΔG_R, OC degr.[6]	Maint. energy[7]	Turn-over time[8]
1227	1.6	0.16	0.14	1.6	0.1	27	2.8/4.7	15,000	360/2150
1229T	7.4	0.08	0.95	0.8	2.9	14	67/114	71,000	70/410
1229B	5.0	0.13	0.30	1.3	1.1	15	27/46	55,000	120/690
1230	6.7	0.88	2.58	8.8	1.7	114	39/66	20,000	110/640

Additional information on the calculations is provided in *Supplementary Text*.

[1]Total hybridized cells with probes Arch915-Cy3 and EUB338-FITC (Table II.1). Values are x 10^6 cm^{-3}.

[2] Fluxes were calculated from pore water concentrations and physical property data (D'Hondt *et al.*, 2003) according to Schulz (2000). Only the linear parts of methane and sulfate gradients into SMTZs were considered. Values are x 10^{-6} mol cm^{-2} yr^{-1}.

[3] Estimated from fluxes of methane, assuming a vertical extension of the reactive zone of the SMTZ of 100 cm. Values are x 10^{-9} mol cm^{-3} yr^{-1}.

[4] Based on the diagenetic model by Middelburg (1989). Values are x 10^{-9} mol cm^{-3} yr^{-1}.

[5] Using a ΔG_R for AOM of -17.2 kJ mol^{-1} (1227), -16.9 kJ mol^{-1} (1229T), -11.9 kJ mol^{-1} (1229B), and -12.9 kJ mol^{-1} (1230). Values are x 10^{-6} J cm^{-3} yr^{-1}.

[6] Calculated using hexadecane as model compound for sedimentary OM (Orphan *et al.*, 2001a); assumes DG_R of syntrophic breakdown under methanogenic (-23.4 kJ mol^{-1} C, lower value) and sulfate-reducing conditions (-40 kJ mol^{-1} C, upper value) (Zengler *et al.*, 1999). Values are x 10^{-6} J cm^{-3} yr^{-1}.

[7] Based on a calculation of temperature-dependent, species-independent maintenance energies (Tijhuis *et al.*, 1993). The cellular maintenance energies resulting from this model are close to the lowest known values determined experimentally for anaerobic bacteria (*Acetobacterium woodii* grown on lactate; Scholten and Conrad, 2000). Values are x 10^{-6} J cm^{-3} yr^{-1}.

[8] Based on total amount of carbon in the population of hybridized cells, and OC degradation rate in combination with growth efficiencies of 0.01 (Del Giorgio and Cole, 1998) (higher value; planktonic heterotrophic bacteria in highly oligotrophic ocean) to 0.06 (Scholten and Conrad, 2000) (lower value; maximum value found for syntrophic propionate-degrading community).

laboratory cultures (e.g. Tijhuis *et al.*, 1993; Scholten and Conrad, 2000), and (*ii*) AOM is a principal source of metabolic energy in SMTZs. Explanations follow.

(*i*) Multiple lines of evidence suggest the presence of ≈10^6 active prokaryotic cells per cm^3 of sediment (Table II.1), but sedimentary biogeochemical processes can only account for 0.1–2% of the population if conventional maintenance energy requirements are applicable. Conceptually, cellular maintenance energies are probably lower in dormant cells (Harder, 1997), which may be abundant in the deep subsurface. In fact, a dormant fraction of subsurface cells provides one possible explanation for our estimates of long turnover times of subsurface populations (Table II.2). Isotopic data indicate that the bulk of the carbon assimilated by subsurface communities is derived from sedimentary OM. With estimated degradation rates in SMTZs ranging from 0.1 to 2.9 x 10^{-9} mol C cm^{-3} yr^{-1}, and a range of growth efficiencies of 0.01–0.06 (Scholten and Conrad, 2000; Del Giorgio and Cole, 1998), we derive a range of turnover times for the population of 70–2,150 years (Table II.2). Turnover of microbial communities in SMTZs is likely faster than outside where lower total ΔG_R related to the absence of AOM could conceivably result in lower growth efficiencies (cf. Del Giorgio and Cole, 1998). Such a vertical distribution of turnover times could provide

an explanation for the great excess of cells in SMTZs that are observed by acridine orange staining (cf. Supplementary Figure II.4) but do not hybridize to FISH probes, i.e., ‘‘recently’’ active cells that have not been degraded yet.

(*ii*) The high fraction of total ΔG_R in SMTZs accounted for by AOM (Table II.2), combined with the lack of ^{13}C-depletion in archaeal biomass (Figure II.3), suggests that members of the Marine Benthic Group B and/or Miscellaneous Crenarchaeotal Group Archaea oxidize methane but do not assimilate its carbon. Such ‘‘dissimilatory’’ methane-oxidizing process could account for our observations and would resemble metabolic strategies found in other Archaea. For example, several CO_2-reducing methanogens use auxiliary carbon substrates and complex organic nutrients for synthesis of cell material and growth, while CO_2 is converted into methane (Boone *et al.*, 2001a and 2001b).

Marine Benthic Group B and Miscellaneous Crenarchaeotal Group Archaea, presumably dominant members in subsurface ecosystems, have cosmopolitan distributions in a wide range of biogeochemically distinct sedimentary settings (Figure II.1 and Supplementary Figure II.5), suggestive of a considerable ecophysiological flexibility. More widespread and improved quantifications may show that these subsurface Archaea, in analogy to planktonic Marine Group I Archaea in the ocean (Karner *et al.*, 2001), constitute a significant fraction of the prokaryotic biomass in Earth’s subsurface. Our insights into carbon sources of these uncultured Archaea may form essential building blocks toward understanding the microbial carbon cycle in deep marine sediments.

MATERIALS AND METHODS

Sample Collection. All samples were collected on ODP Leg 201 at Sites 1227, 1229, and 1230 (D’Hondt *et al.*, 2003). Whole round cores frozen at -80°C were used for lipid and RNA studies. Fixed sediment was used for FISH studies (D’Hondt *et al.*, 2003).

FISH Analysis. Fixed samples kept at -20°C were diluted in 1xPBS and spotted on 1-inch glass rounds and 10-welled slides. Dried samples were subjected to traditional FISH (Orphan *et al.*, 2001b) by using the standard probes ARCH915-CY3 (Amann *et al.*, 1990) and EUB338-FITC (Stahl and Amann, 1991). Slides were dehydrated in a series of ethanol baths (55%, 85%, 95%; 3 min each), then covered with 10 µl of hybridization buffer (House *et al.*, 2003) (20% formamide, pH 8.0/40 ng of each probe). Slides were incubated in a premoistened hybridization chamber for 90 min at 46°C, then washed for 15 min at 48°C (Orphan *et al.*, 2001b) and viewed with a Nikon E800 microscope. Because both archaeal and bacterial probes were used at the same time in experiments, the digital overlay of the two

fluorochrome images served to identify nonspecific binding of probes, and only cells illuminated by one fluorochrome were counted. Exposure times of 0.5–5 s were needed to capture cell fluorescence, and only single cells were observed. Counts are based on a total of 400 cells counted per horizon, viewed in random fields. Error is reported as the standard deviation of three replicate counts on each sample. Separately, both CY3 and FITC NON probes were used; however, the amount of nonspecific binding was not significant compared with the estimated error of the counts. The reversal of probe fluorochromes produced similar results (data not shown). Additional controls were run by using pure cultures of microbes with and without added sediment. Samples on 1-inch glass rounds had the location of cells recorded and documented with a series of phase contrast and epifluorescent images using 10x, 40x, 60x, and 100x dry objectives on a Nikon E800. PHOTOSHOP software (Adobe Systems, San Jose, CA) was used to enhance and organize these images into digital maps of each slide. Targets were then located on a FEI Quanta 200 Environmental Scanning Electron Microscope (Penn State, Materials Resource Institute) to ensure that individual cells were separated from other material. Rounds were subsequently gold coated and used for microprobe analysis.

Isotopic Composition of Whole Cells by Secondary Ion MS Analysis. The carbon isotopic composition of individual target cells was analyzed on the IMS 1270 (Cameca, Paris) at the Ion Microprobe Facility at the University of California, Los Angeles. Secondary C_2^- ions were sputtered by a 10-µm-diameter Cs^+ beam from the target biomass and analyzed in monocollection mode by using the electron multiplier with a field aperture of 5 μm^2. Instrumental mass fractionation was calibrated by the comparison of *Escherichia coli* cells measured by ion microprobe and by elemental analyzer-MS, and data were appropriately corrected (data not shown). Data of individual cells, resulting weighted means of all cells, the errors on that mean, and the mean squared weighted deviations are reported in Supplementary Table II.4.

Extraction and Separation of Lipids. Fifty grams of dry sediment were spiked with an internal standard (1-*O*-hexadecyl-2-acetoyl-*sn*-glycero-3-phosphocholine; PAF) and extracted by a modified Bligh and Dyer method in four steps (Sturt *et al.*, 2004) followed by 10-min centrifugation at 800 x *g*. The combined supernatants were washed with water and evaporated to dryness. The total lipid extract was separated chromatographically in two fractions on a glass column (4 g of silica gel, 60 Mesh; apolar fraction, 20 ml of dichloromethane; polar fraction containing glycolipids and phospholipids, 20 ml of acetone followed by 40 ml of methanol).

HPLC-MS Analysis of IPLs. HPLC-MS analysis was performed at the University of Bremen. Details are provided in Sturt *et al.* (2004). Relative concentrations of IPLs for intersample comparison were calculated based on MS response of molecular ions relative to that of known amounts of the internal standard. Lack of authentic standards prevented determination of absolute concentrations because response factors of different IPLs vary significantly. IPL concentrations (roughly estimated in the lower ng/g sediment-range) were too low for detection with an evaporative-light-scattering detector.

Preparation of IPL Derivatives for Isotopic Analysis. Stable carbon isotopic compositions, $\delta^{13}C$, were determined on isopranyl derivatives prepared from archaeal IPLs by ether cleavage with HI and subsequent reduction with $LiAlH_4$ (e.g. DeLong *et al.*, 1998). To avoid incorporation of ether-bound alkyl moieties that may be present in polar, high-molecular-weight organic material, we collected archaeal IPLs during 10 repeated injections on a wide-bore preparative HPLC column (LiChrosphere Si60, 5 µm, 250 x 10 mm; Alltech Associates) with a fraction collector. The flow rate was 1.5 ml/min, and the solvent gradient was 100% A to 100% B in 120 min, hold for 30 min followed by 90 min re-equilibration with 100% A; eluents were as in Sturt *et al.* (2004).

MS and Isotopic Analysis of IPL Derivatives. Products released from archaeal IPLs were identified and quantified on a Trace GC-MS (ThermoFinnigan, San Jose, CA). The GC was operated at 310°C in split/splitless mode and equipped with a Varian VF5-ms capillary column (L=30 m; ID = 0.25 mm; 0.25 µm film thickness; He as carrier gas; flow = 1 ml/min). The column temperature was programmed from 60°C (1 min) at 10°C/min to 150°C followed by 4°C/min to 310°C (25 min). Compound-specific isotope analyses of IPL derivatives were performed on a Hewlett–Packard 5890 GC equipped with an on column injector and an IVA OV-1 capillary column (L = 60 m; ID = 0.32 mm; 0.25-µm film thickness). The column temperature was programmed from 60°C (1 min) at 10°C/min to 150°C followed by 4°C/min to 310°C (30 min). The GC was coupled via a combustion interface (set at 940°C) to a MAT252 isotope-ratio-monitoring mass spectrometer (ThermoFinnigan).

Isotopic Analysis of Methane, TOC, and DIC. $\delta^{13}C$ of methane, TOC, and DIC was determined by using standard protocols. Details are provided in *Supplementary Text*, which is published as supplementary information on the PNAS web site.

Ribosomal RNA Extraction, Reverse Transcription, Cloning, and Sequencing. RNA was extracted from the center of whole-round core samples 1227D-37.8, 1229D-29.7, 1229D-86.8, and 1230A-11.1. Following a modification of published protocols (MacGregor

et al., 1997; Stahl *et al.*, 1988), a volume of 4 ml sediment was mixed with 5 ml of phenol (pH 5), 5 ml of 5x extraction buffer (250mM NaAc/50mM EDTA, pH 5), and 0.5 ml of 20% SDS, distributed over 2-ml bead beating tubes with 0.5 g of 0.1-mm zirconium beads (Biospec Products, Bartlesville, OK), homogenized for 30 s at level 6.5 on a FastPrep FP120 homogenizer (Qbiogene, Carlsbad, CA), and centrifuged (5 min; 16,000 x *g*; 4°C). Aqueous phases were removed and saved. The remaining phenol and sediment pellets were homogenized and extracted once more using 300 µl of 1x extraction buffer. The combined aqueous phases were extracted in phenol, 1:1 phenol/chloroform, and chloroform by vortexing and centrifugation. RNA was precipitated for 2 h at -20°C in 0.5 vol of 7.5 M ammonium acetate and 1 vol of isopropanol. Precipitates were washed in 70% ethanol, air dried, and resuspended in 90 µl of water. Each extraction product was incubated with 10 µl of DNAse buffer and 4 µl of DNAse (Fisher) for 30 min at 37°C and purified (RNeasy Mini Kit; Qiagen, Valencia, CA). Negative controls were run as parallel extractions without sediment.

The RNA was reverse transcribed and amplified with primers 8f (Teske *et al.*, 2002) and 915r (DeLong, 1992) by using the One-Step RT-PCR kit (Qiagen) and the following PCR protocol: (*i*) 30-min reverse transcription at 50°C, (*ii*) 15-min *Taq* polymerase activation at 95°C, (*iii*) 40 cycles of 30-45 s denaturation at 92–94°C, 30–45 s annealing at 58°C, 4 min amplification at 72°C, and (*iv*) a final amplification step of 6–10 min. RT-PCR products were checked by gel electrophoresis on 1.5% agarose gels. PCR assays without the reverse transcription step showed the absence of DNA contamination.

RT-PCR products were cloned with the TOPO XL PCR cloning kit (Invitrogen) according to the manufacturer's instructions. Plasmid extraction, purification and cycle sequencing was performed at the Marine Biological Laboratory (Woods Hole, MA). Sequences were BLAST analyzed against GenBank (www.ncbi.nlm.nih.gov/blast) and screened for chimeras with the CHECK_CHIMERA application (Ribosomal Database Project, http://geta.life.uiuc.edu/RDP/commands/chimera.html) and by constructing phylogenetic trees from sequence fragments (base pairs 1–250, 251–500, 501-end). Sequences were aligned in ARB (www.arb-home.de), and alignments were edited in SEQPUP (Version 0.6, http://iubio.bio.indiana.edu/soft/molbio/seqpup/java/seqpup-doc.html). Phylogenetic trees (maximum likelihood distance) and bootstrap analyses (200 replicates) were performed in PAUP4.0* (Sinauer Associates, Sunderland, MA).

ACKNOWLEDGEMENTS

We thank the ODP Leg 201 Shipboard Scientific Party for sample recovery and data; C. G. Johnson, H. Buschhoff, and M. Segl for technical support during isotopic analysis of methane and TOC; and J. M. Hayes, A. Schippers, and two anonymous reviewers for constructive comments that improved this manuscript. Samples for this research were provided by ODP [sponsored by the National Science Foundation (NSF) and participating countries under the Joint Oceanographic Institutions]. This work was supported by Deutsche Forschungsgemeinschaft (to J.S.L., R.A., M.E., and K.-U.H. at Research Center for Ocean Margins and Grant Hi 616-4 to K.U.-H.); National Aeronautics and Space Administration Astrobiology Institute Grants NNA04CC06A (to J.E.B. and C.H.H. at Pennsylvania State University), NCC 2-1275 (to M.A.L., K.G.L., K.B.S., H.F.F., A.T., and K.-U.H. at the University of Rhode Island), and NCC 2-1054 (to M.L.S. and A.T. at the Marine Biological Laboratory); the G. Unger Vetlesen Foundation; U.S. Department of Energy Grant DE-FG02-93ER20117; and NSF Grant MCB03-48492. J.F.B. was supported by NSF Integrative Graduate Education and Research Traineeship Program Grant DGE-9972759 and a Schlanger fellowship from the Joint Oceanographic Institutions (JOI). M.A.L. was supported in part by post cruise support from JOI. The University of California, Los Angeles ion microprobe is supported by the W. M. Keck Foundation and NSF Grant EAR 01-13563. This work is Research Center for Ocean Margins Publication No. 359.

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II.1. SUPPLEMENTARY ONLINE MATERIAL

(*Online at http://www.pnas.org/cgi/content/full/0600035103/DC1*)

Supplementary text

(I) Isotopic Analysis of Methane, Total Organic Carbon (TOC), Dissolved Organic Carbon (DIC). $\delta^{13}C$ of methane was determined at Woods Hole Oceanographic Institution in headspace gas samples (D'Hondt *et al.*, 2003) using a 1000-µl gastight syringe. 50 to 100 µl were injected into a Gerstel CIS-4 injector on an HP6890 gas chromatograph that was operated in split mode with a split ratio of 0.01. Gaseous hydrocarbons were separated on a 30 m x 0.3 mm ID Alltech AT-Q column with helium carrier gas flow of 3.0 ml/min. The column temperature was held isothermal at 50°C. An integral fused silica combustion system at 950°C converted all organic components to CO_2. Isotopic data were acquired and processed on a Finnigan MAT Delta Plus isotope-ratio-monitoring mass spectrometer using the Isodat NT data package. $\delta^{13}C$ of TOC was measured at Bremen from 10 mg of decalcified dry sediment filled to silver capsules. Samples were measured in duplicate on an elemental analyzer (ThermoQuest EA/NA 1110) coupled to a ThermoFinnigan Delta Plus isotope ratio mass spectrometer via a ConFlow II interface. Average values are reported after correction with a known standard. The error is ±0.1‰. $\delta^{13}C$ of DIC was measured on filtered pore water samples that were poisoned with $HgCl_2$ after squeezing. CO_2 was extracted from pore waters by acidification with phosphoric acid, and isotope measurements were performed at Harvard University. The error, based on replicate measurements and analysis of laboratory standards, is ±0.1‰.

(II) Calculations (Table II.2). Organic carbon degradation rates v_{OM} were calculated on the basis of an empirical, diagenetic model by Middelburg (1989) (Eq. II.1-II.3). For Site 1227, 1229, and 1230, linear sedimentation rates of 1, 11, and 4 cm kyr^{-1}, respectively, were used to calculate the ages of sediments in the SMTZs. Initial ages *a* are an expression of the organic matter's initial reactivity; we applied 500 yrs for the shallow-water Sites 1227 and 1229 and 1000 yrs for the deep-water Site 1230. Organic carbon contents assumed for surface sediments, $c_{TOC(t0)}$, are 13% (mean value for five sites in the region of Sites 1227 and 1229; PANGAEA database (www.pangaea.de)) and 4.8% (Site 1230, average of Peru continental margin (Seiter *et al.*, 2004)), ρ_{sed} is the mean density of sediment in SMTZ and m_C is 12.011 g C mol^{-1}.

$$k(t) = 0.16 \cdot (a + t)^{-0.95} \qquad \text{(II.1)}$$

$$c_{TOC}(t) = c_{TOC(t_0)} \cdot e^{3.2 \cdot (a^{0.05} - (a+t)^{0.05})} \quad \text{(II.2)}$$

$$\upsilon_{OM} = \frac{c_{TOC}(t) \cdot k(t) \cdot \rho_{sed}}{m_C} \quad \text{(II.3)}$$

Maintenance energies for populations of hybridized cells $E_{m,pop}$ were calculated using a temperature-dependent, species-independent model (Harder, 1997) (Eq. II.4) with N_{cells} being the number of hybridized cells and using a cellular carbon content m_{cell} of 19 fg C cell^{-1} (Morita, 1997), a constant for anaerobic bacteria A of 4.99 x 10^{12} kJ (g dry wt.)$^{-1}$ d^{-1}, an activation energy E_A of 69.4 kJ mol^{-1}, the universal gas constant R (8.314 J mol^{-1} K^{-1}), and *in situ* temperatures T in individual SMTZs (D'Hondt *et al.*, 2003).

$$E_{m,pop} = N_{cells} \cdot m_{cell} \cdot A \cdot e^{-E_A / RT} \quad \text{(II.4)}$$

The turnover time of the population $t_{turnover}$ was calculated according to Eq. II.5 on the basis of the total quantity of carbon in the population of hybridized cells and OC degradation rate in combination with growth efficiencies e_g of 0.01 (Del Giorgio and Cole, 1998) and 0.06 (Scholten and Conrad, 2000).

$$t_{turnover} = \frac{N_{cells} \cdot m_{cell}}{\upsilon_{OM} \cdot e_g \cdot m_C} \quad \text{(II.5)}$$

The ΔG_R for the AOM reaction (e.g., Hinrichs and Boetius, 2002) was calculated using Eq. II.6 for *in situ* temperatures and activities using tabulated activity coefficients (Millero and Pierrot, 1998) and a standard Gibbs free energy, DG_0, of -31 kJ mol^{-1} (with methane as aqueous species).

$$\Delta G_R = \Delta G_0 + RT \ln\left(\frac{a\left(HCO_3^-(aq)\right) \cdot a\left(HS^-(aq)\right)}{a(CH_4(aq)) \cdot a(SO_4^{2-}(aq))}\right) \quad \text{(II.6)}$$

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Supplementary Tables

Supplementary Table II.3: $\delta^{13}C$ values (in ‰) of hydrocarbon derivatives chemically released from intact lipids and percentages.

Sample	Diether derivatives		Tetraether derivatives Biphytanes				
	Phytane	2,6,10,14,18-PMI	0 rings	1 ring	2 rings	3 rings, *cren*	3 rings
1227A-37.6	-25.5 (25%)	n. d.	-25.0 (38%)	-21.3 (13%)	-21.2 (11%)	-25.4 (13%)	n. d.
1227D-38.2	-25.8 (46%)	-27.2 (22%)	-26.7 (19%)	-21.3 (7%)	-24.2 (4%)	-21.2 (1%)	-17.1 (1%)
1227A-40.9	-26.7 (20%)	n. d.	-29.4 (39%)	-25.0 (6%)	-26.5 (12%)	-28.2 (23%)	n. d.
1229D-1.4	-24.7 (59%)	n. d.	-34.7 (24%)	-32.0 (8%)	-27.5 (6%)	-37.0 (3%)	n. d.
1229D-29.7	-37.6 (18%)	-24.4 (4%)	-23.5 (41%)	-19.4 (25%)	-21.3 (9%)	-20.3 (2%)	-19.9 (1%)
1229D-87.1	-34.0 (12%)	n. d.	-24.0 (54%)	-24.4 (16%)	-20.7 (12%)	-29.8 (6%)	n. d.
1230B-9.3	-26.5 (7%)	n. d.	-21.2 (35%)	-18.5 (13%)	-20.3 (19%)	-22.1 (26%)	n. d.
1230B-9.7	-25.6 (9%)	n. d.	-22.3 (46%)	-20.7 (14%)	-21.7 (15%)	-25.6 (16%)	n. d.
1230B-10.1	n. d.	n. d.	-23.0 (40%)	-21.5 (24%)	-21.9 (18%)	-22.5 (16%)	-23.1 (2%)
Average	-28.3	-25.8	-25.5	-22.7	-22.8	-25.8	-20.0

Supplementary Table II.4: Individual $\delta^{13}C$ cell values as determined by FISH-SIMS

Cell number	$\delta^{13}C$	error
1227D 4H6, 34.5 mbsf		
Cell 1	-19.0	12.5
Cell 2	-8.4	13.9
Cell 3	-28.4	12.1
Cell 4	-14.3	12.6
Cell 5	-21.9	27.0
weighted mean	**error on mean**	**MSWD**
-18.4	8.0	0.4
1227D 5H2, 37.9 mbsf		
Cell 1	-14.8	6.4
Cell 2	-29.4	7.5
Cell 3	-22.0	19.3
Cell 4	-14.7	10.1
Cell 5	-23.0	10.4
Cell 6	-15.0	14.1
Cell 7	-12.4	11.5
Cell 8	-15.0	30.0
Cell 9	-16.8	18.3
Cell 10	-22.5	18.2
Cell 11	-19.2	22.7
Cell 12	-10.9	9.8
Cell 13	-12.7	10.4
Cell 14	-16.2	29.5
Cell 15	-17.5	4.8
Cell 16	-31.2	17.9
Cell 17	-15.2	20.9
Cell 18	-13.2	6.7
Cell 19	-23.4	5.1
Cell 20	-21.8	5.3
Cell 21	-27.0	8.9
Cell 22	-21.2	10.3
Cell 23	-17.2	6.7
Cell 24	-16.0	8.6
Cell 25	-18.3	9.2
Cell 26	-31.4	15.2
Cell 27	-27.7	5.4
Cell 28	-20.3	7.4
weighted mean	**error on mean**	**MSWD**
-20.0	5.4	0.4
1227D 5H2, 38.4 mbsf		
Cell 1	-25.3	7.3
Cell 2	-15.6	7.2
Cell 3	-19.6	10.4
Cell 4	-30.3	8.2
Cell 5	-20.5	8.2
Cell 6	-35.8	12.8
Cell 7	-29.2	12.2
Cell 8	-17.2	12.7
Cell 9	-33.1	10.6
Cell 10	-17.4	17.7
Cell 11	-25.3	7.3
Cell 12	-13.5	52.9
Cell 13	-21.1	12.1
Cell 14	-10.7	13.3
Cell 15	-29.6	17.1
Cell 16	-18.0	53.7
Cell 17	-30.8	10.9
Cell 18	-24.4	7.1
Cell 19	-20.7	7.7
Cell 20	-27.2	8.7
weighted mean	**error on mean**	**MSWD**
-23.9	5.8	0.4
1227A 5H5, 40.5 mbsf		
Cell 1	-4.6	32.1
Cell 2	-17.2	5.8
Cell 3	-23.9	11.5
Cell 4	-41.6	19.7
weighted mean	**error on mean**	**MSWD**
-19.7	7.6	0.6

Cell number	$\delta^{13}C$	error
1229D 4H3, 29.8 mbsf		
Cell 1	-20.9	4.5
Cell 2	-14.1	5.9
Cell 3	-12.7	4.5
Cell 4	-17.3	10.3
Cell 5	-6.1	5.3
Cell 6	-14.4	12.2
Cell 7	-20.4	13.3
Cell 8	-8.8	4.5
Cell 9	-22.0	7.3
weighted mean	**error on mean**	**MSWD**
-13.8	5.8	0.9
1229D 12H3, 87.1 mbsf		
Cell 1	-29.7	9.5
Cell 2	7.1	48.7
Cell 3	-67.7	72.1
Cell 4	-20.1	75.4
Cell 5	-1.3	69.1
Cell 6	-25.8	52.0
Cell 7	-18.7	22.2
Cell 8	-22.1	13.3
Cell 9	-30.1	113.0
Cell 10	-13.2	128.0
weighted mean	**error on mean**	**MSWD**
-25.6	8.4	0.1

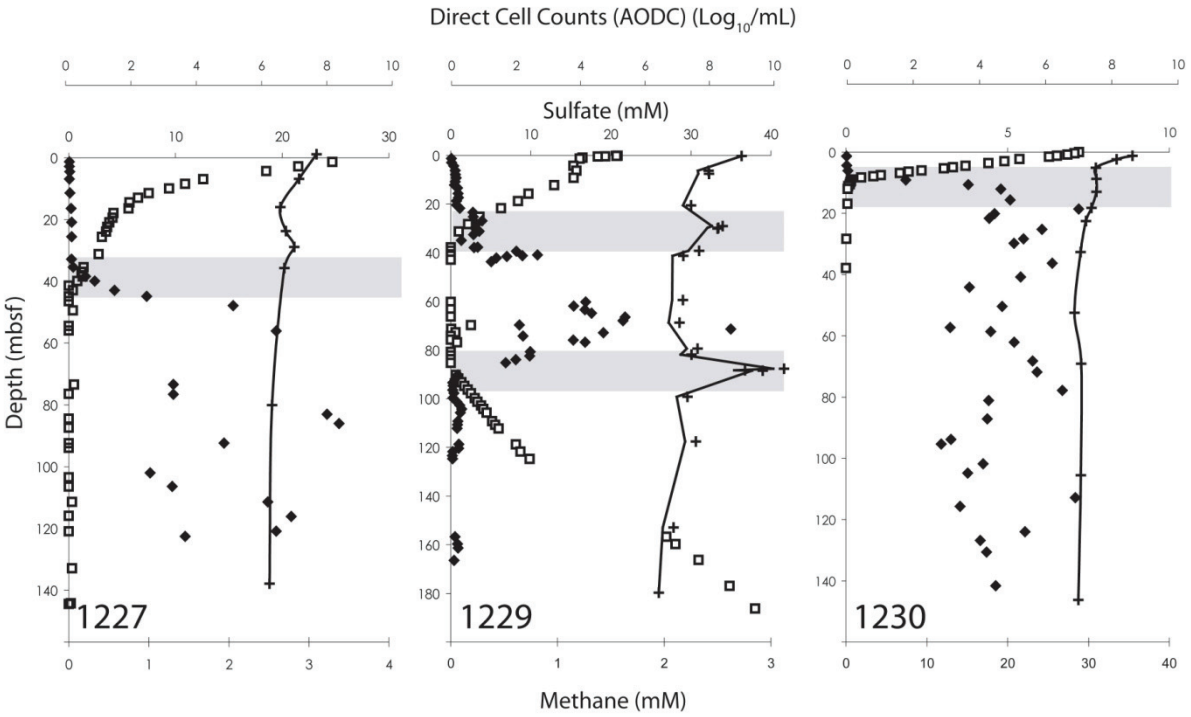
Cell number	$\delta^{13}C$	error
1230A 1H3, 3.4 mbsf		
Cell 1	-12.7	9.1
Cell 2	-10.5	18.4
Cell 3	-20.9	12.7
Cell 4	-13.2	8.5
weighted mean	**error on mean**	**MSWD**
-14.2	3.7	0.1
1230C 2H4, 9.1 mbsf		
Cell 1	-25.0	6.9
Cell 2	-28.0	6.2
Cell 3	-11.9	7.8
Cell 4	-20.1	9.8
Cell 5	-31.8	10.0
Cell 6	-22.6	9.2
Cell 7	-33.9	6.8
Cell 8	-1.8	35.5
Cell 9	-28.4	8.0
Cell 10	-18.0	17.5
Cell 11	-6.1	11.0
weighted mean	**error on mean**	**MSWD**
-24.3	8.3	0.9
1230C 2H4, 10.3 mbsf		
Cell 1	-16.6	6.2
Cell 2	-25.8	9.6
Cell 3	-16.6	7.8
Cell 4	-16.5	6.3
Cell 5	-9.4	9.5
Cell 6	-8.6	8.6
Cell 7	-21.4	7.2
Cell 8	-20.2	8.1
Cell 9	-24.7	11.5
Cell 10	-20.3	14.9
weighted mean	**error on mean**	**MSWD**
-17.5	5.1	0.4
1230C 2H5, 10.7 mbsf		
Cell 1	-20.9	6.6
Cell 2	-3.7	8.0
Cell 3	-8.0	8.0
Cell 4	-27.7	6.2
Cell 5	-14.6	9.0
Cell 6	-19.4	12.0
weighted mean	**error on mean**	**MSWD**
-17.1	9.5	1.5

Supplementary Table II.4: Individual $\delta^{13}C$ cell values as determined by FISH-SIMS. Single cell results shown have been corrected for an instrumental mass fractionation (IMF). The uncertainty on each cell measurement (1σ) (shown in error column) was calculated by adding in quadrature the precision of the individual cell measurement with the uncertainty of IMF. In most of these cases (with error > 5‰), the uncertainty was dictated predominately by the lack of precision on the individual cell analysis, due to unfavorable counting statistics arising from the small amount of carbon in each cell. IMF was determined each day by comparing a series of measured $\delta^{13}C$ values of *E. coli* cells to the $\delta^{13}C$ for the bulk *E. coli* culture as determined by EA-MS. The weighted means and mean squared weighted deviations (MSWD) for each horizon were calculated from the corrected values and calculated uncertainty (shown in the table).

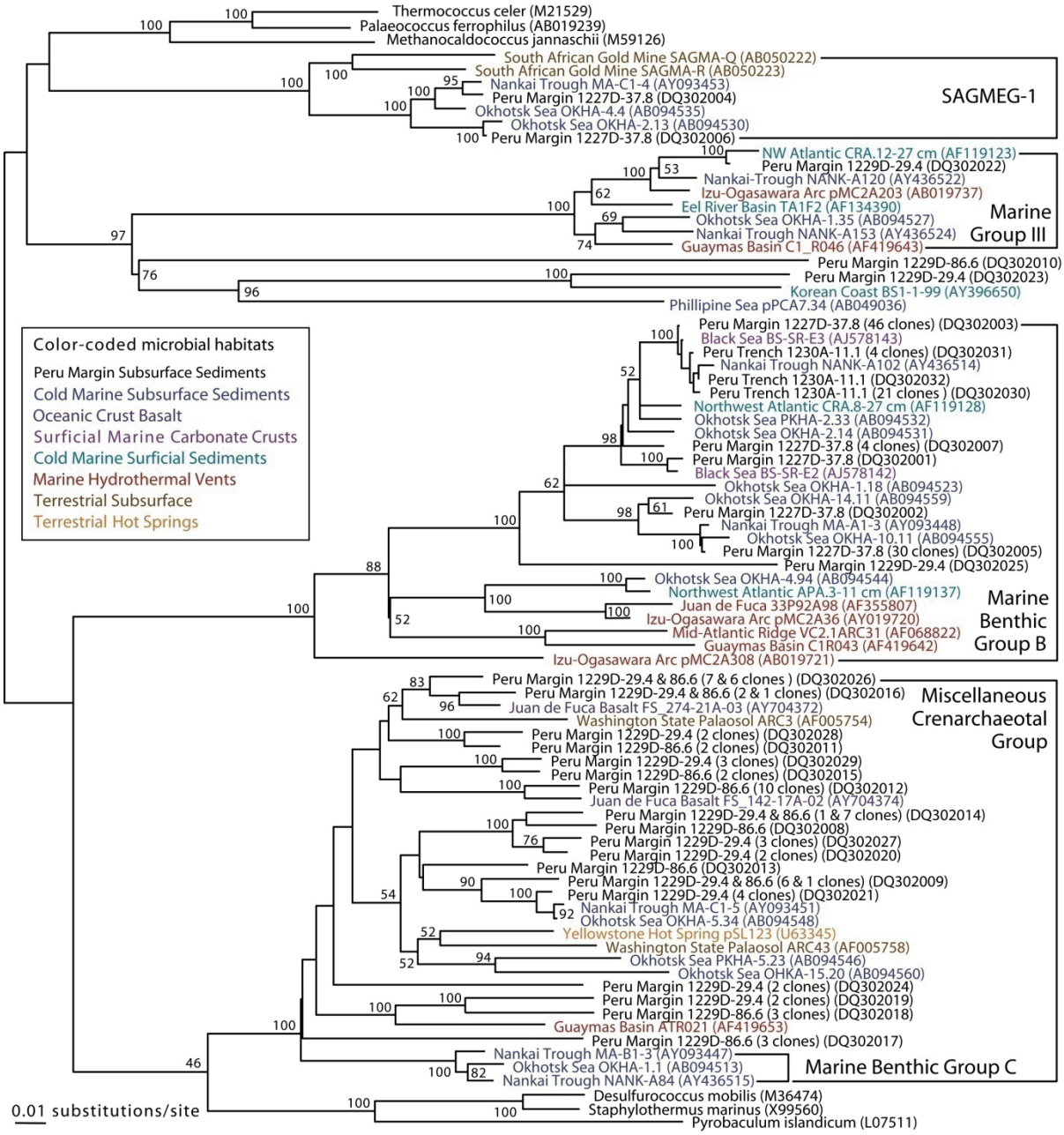
$$MSWD = \sum_i \left(\left[X_{wm} - X_i \right] \cdot 2 / \sigma_i^{\,2} \right) / (n-1)$$

where X_{wm} = weighted mean of the X_i, σ_i = 1 standard error in measurement of X_i, and n = # of measurements on each horizon.

Supplementary Figures



Supplementary Figure II.4: Concentrations of sulfate (open squares), methane (diamonds), and counted cells after staining with acridine orange (AODC) (crosses) (D'Hondt *et al.*, 2003). SMTZs are marked by gray bars.



Supplementary Figure II.5: 16S rRNA archaeal phylogenetic tree based on maximum likelihood distances of ca. 900 nucleotide positions (16S rRNA positions 23 - 914). Compared to Figure II.1, this tree includes a greater variety of reference sequences from different habitats, and shows groups of closely related Peru Margin subsurface sequences in finer resolution. As previously, bootstrap numbers are based on 200 resamplings. The sequences were obtained from four sediment samples from each of the four SMTZ encountered during ODP Leg 201: 1227D-37.8 (ODP Hole-depth in hole in meters below seafloor), 1229D-29.4, 1229D-86.8, and 1230A-11.0. Closely related sequence clusters are represented by single sequences in the tree, annotated with the number of near-identical 16S rRNA clones that they represent, and their GenBank accession numbers. Sequence names consist of sample name and GenBank accession numbers. Sequences are color coded by habitat to illustrate the diverse environmental range of these uncultured archaeal lineages.

Chapter III

Significant contribution of Archaea to extant biomass in marine subsurface sediments

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In revision at *Nature*

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III.1. PRINTED MANUSCRIPT

ABSTRACT

Deep drilling into the marine seafloor has uncovered a vast sedimentary ecosystem of microbial cells (Parkes *et al.*, 1994; D'Hondt *et al.*, 2004). Extrapolation of direct counts of stained microbial cells to the total volume of habitable marine subsurface sediments suggests that 56 Pg (Parkes *et al., 1994*) and 303 Pg (Whitman *et al.*, 1998) of cellular carbon could be stored in this largely unexplored habitat. From recent studies employing various culture-independent techniques no clear picture has yet emerged as to whether Archaea or Bacteria are more abundant in this extensive ecosystem (Mauclaire *et al.*, 2004; Schippers *et al.*, 2005; Inagaki *et al.*, 2006; Biddle *et al.*, 2006). Here we show that in subsurface sediments buried deeper than 1 meter in a wide range of oceanographic settings, at least 87% of intact polar membrane lipids, biomarkers for the presence of live cells (Sturt *et al.*, 2004; Biddle *et al.*, 2006), are attributable to archaeal membranes, suggesting that Archaea constitute a major fraction of the biomass. Results obtained from modified quantitative PCR and slot-blot hybridisation protocols support the lipid-based evidence and indicate that these techniques have previously underestimated archaeal biomass. The concentrations of intact lipids are proportional to those of total organic carbon. This relationship was used to derive an estimate independent of cell counts for the size of the global marine subsurface biosphere, with inherent uncertainties being comparable to those of previous estimates. Our estimate of 90 Pg of C is consistent within an order of magnitude to other estimates of deeply buried biomass (56 - 303 Pg) and underscores the importance of marine subsurface habitats for global biomass budgets.

The discovery of a subsurface biosphere in deeply buried marine sediments has fundamentally influenced our view of life on Earth. Microbial life has been found down to at least 800 meters below seafloor (mbsf) in sediments that were deposited tens of millions of years ago (Parkes *et al.*, 1994). Recent work resulting from dedicated drilling expeditions suggests a remarkable diversity of both Archaea and Bacteria and revealed some systematic links between abundance and structure of microbial communities and sediment geochemistry (Parkes *et al.*, 2005; Biddle *et al.*, 2006; Inagaki *et al.*, 2003 and 2006). However, in intensively studied subsurface sediments at the Peru Margin, culture-independent studies could not resolve whether Archaea or Bacteria are more abundant and whether they are being detected with equal efficiency (Mauclaire *et al*., 2004; Biddle *et al.*, 2006; Inagaki *et al.*, 2003 and 2006; Schippers *et al.*, 2005).

Phospholipid-based fatty acids (PLFA) as molecular building blocks of cell membranes were introduced and validated as measure for live bacterial biomass by White *et al.* (1979) because they are rapidly degraded after cell death. This concept was extended to the analysis of intact phospho- and glycolipids in order to include Archaea, which were discriminated against by the PLFA approach (Sturt *et al.*, 2004; Biddle *et al.*, 2006). These intact polar lipids (IPLs) are suitable for quantifying live biomass and broadly constraining the phylogenetic affiliation of the major contributors to the pool of biomass in natural ecosystems (Rütters *et al.*, 2002; Zink *et al.*, 2003; Sturt *et al.*, 2004; Biddle *et al.*, 2006; Fredricks and Hinrichs, 2007). Hence IPLs should provide a basis for a robust estimate of biomass.

By combining lipid- and DNA-based approaches that were specifically designed and modified to meet the analytical requirements of the deep marine subsurface, we have obtained evidence suggesting that Archaea contribute substantially to the microbial biomass residing in this habitat (Figure III.1). In subsurface sediment samples spanning a depth range from 0.01 to 367 meters below seafloor (mbsf) from sites in the Black Sea, Nankai Trough, Peru Margin, Hydrate Ridge, Cascadia Margin, Juan de Fuca, Demerara Rise, and Equatorial Pacific (see *Supplementary Table III.2*), concentrations of IPLs declined with depth, exhibiting a statistically significant ($p<0.0001$) linear log(depth)-log[IPL] relationship in analogy to a global data set of directly counted cells (Figure III.1A) (Parkes *et al.*, 1994). The observed IPL concentrations cover more than three orders of magnitude from 12 to 16000 ng mL^{-1} sediment. Using published conversion factors for lipid mass and cell numbers (Simon and Azam, 1989), these values correspond to an estimated range of cell concentrations of $9x10^6$ to $1x10^{10}$ mL^{-1} (assuming cells of 0.5 µm diameter, Biddle *et al.*, 2006; Inagaki *et al.*, unpublished data; *see Supplementary Methods)*. These ranges are

consistent with those resulting from direct cell counts in marine subsurface environments (D'Hondt *et al.*, 2004; Parkes *et al.*, 2005; Schippers *et al.*, 2005; Inagaki *et al.,* unpublished data).

Surface sediments are dominated by bacterial IPLs with possible admixtures of eukaryotic lipids (Figure III.1B). The major bacterial IPLs identified comprise phosphatidylglycerol, phosphatidylethanolamine, and phosphatidylcholine diacylglycerides with C_{16} and C_{18} acyl groups (see *Supplementary Figure III.4*). The major archaeal lipids, generally dominant in sediments deeper than 0.1 mbsf, are diglycosidic derivatives of archaeol and glyceroldialkylglyceroltetraether (GDGT) lipids (see *Supplementary Figure III.4*). Both the distribution of polar headgroups, the presence of

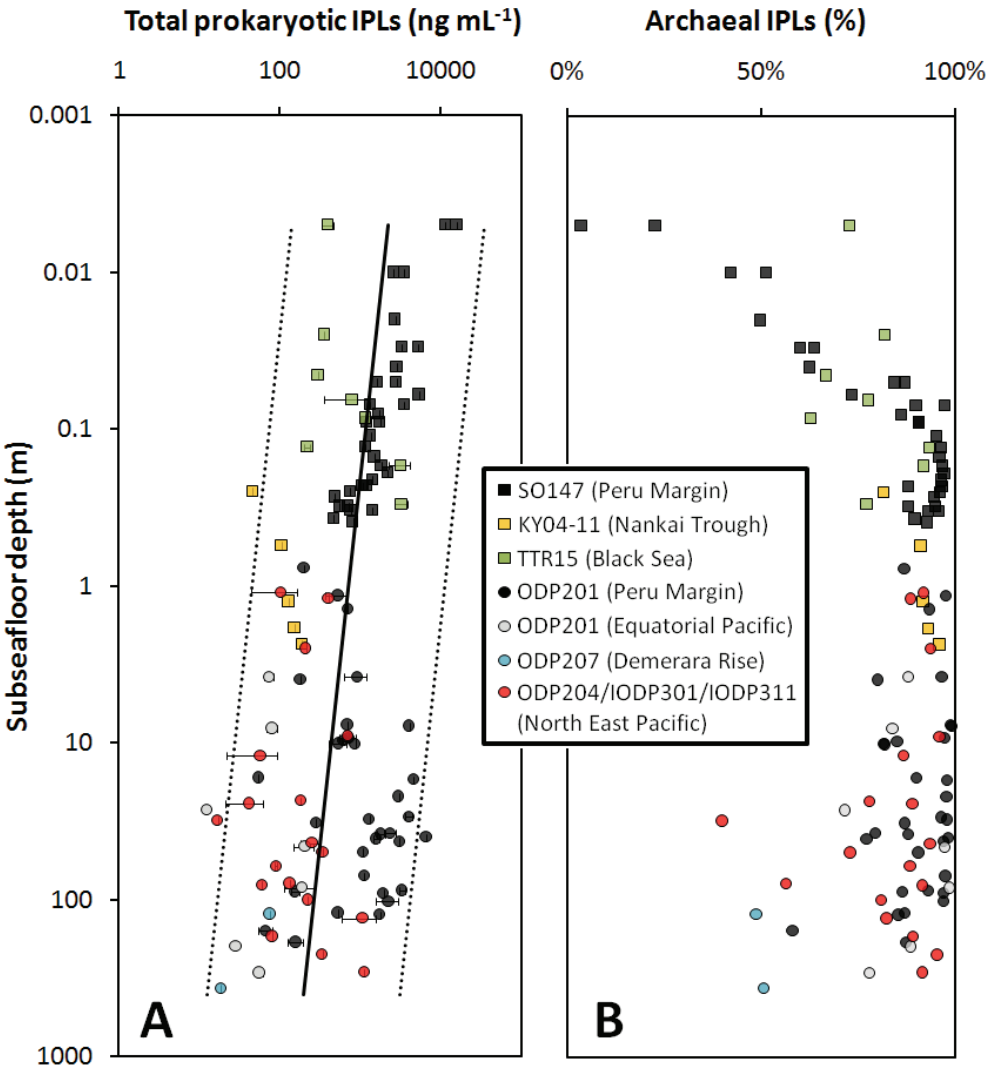

Figure III.1: (A) Depth profiles of IPLs in marine sediments. Error bars show standard deviation of repeated injection of total lipid extracts. Regression lines: total microbial IPLs (solid line, $\log [IPL]_{total} = -0.214 \times \log [depth] + 2.853$, N=104, R^2=0.20, p<0.0001)), and 95% prediction interval (dotted line, standard error of estimate: 0.60), both generated using least-squares analysis. **(B)** Relative contribution of archaeal IPLs to total microbial IPL contents. The relative archaeal contribution was corrected for instrumental limit of detection. If no Bacteria were detected, the limit of detection was assumed as bacterial proportion (*see Supplementary Methods*). Actual bacterial contribution may be lower.

glyceroldialkylnonitoltetraether (GDNT), and a relatively high abundance of GDGT core lipids with multiple rings is consistent with substantial contribution of crenarchaeota (Biddle *et al.*, 2006) and in line with RNA and DNA-based gene libraries in these environments, which indicate the widespread presence of phylogenetically diverse Archaea affiliated with the Marine Benthic Group-B (MBG-B, alternatively classified as Deep-Sea Archaeal Group [DSAG]), South African Gold Mine Euryarchaeotic Group (SAGMEG) and Miscellaneous Crenarchaeotal Group (MCG) (Parkes *et al.*, 2005; Biddle *et al.*, 2006; Inagaki *et al.*, 2003 and 2006; Teske, 2006). Concentrations of bacterial IPLs decline rapidly within the first 0.1 mbsf to levels significantly lower than those of their archaeal counterparts (Figure III.1B).

Does this apparent archaeal dominance result from selective preservation of fossil archaeal lipids and/or analytical discrimination of bacterial lipids, or, are archaeal cells much more abundant in deeply buried sediments? Several points argue against selective preservation and discrimination:

Improved protocols of DNA extraction and purification, and the quantification protocols of slot-blot hybridization (SBH) and Q-PCR (*see Supplementary Methods*) of samples from the Peru Margin (ODP Leg 201) and the Juan de Fuca Ridge Flank (IODP Expedition 301) indicate that previous analyses of similar and identical samples largely underestimated archaeal biomass (Schippers *et al.*, 2005; Inagaki *et al.*, 2006) (Figure III.2, *Supplementary Table III.3*). Similarly high abundances of Archaea were observed in at a methane hydrate-bearing site off Japan (CK06-06) (Figure III.2), where cells were generally only 200-600 nm in size (Inagaki *et al.*, unpublished data). The discrepancies between previous and current DNA-based results can be explained by four factors: (I) previous incomplete cell lysis during extraction of DNA from archaeal cells with mechanically rigid cell walls that are highly resistant to physical and enzymatic disruption (see *Supplementary Figure III.6*); (II) the implementation of quality control of template DNA after extraction and purification using multiple displacement amplification with phi29 polymerase (see *Supplementary Figure III.5*) to obtain highly pure, inhibitor-free DNA; (III) signal detection and quantification methods with lower number of oligonucleotides potentially discriminating against sequences from sedimentary Archaea previously led to overestimation of bacterial numbers (Teske and Sørensen, 2008), and (IV) lower 16S rRNA gene copy numbers in archaeal compared to bacterial genomes (Acinas *et al.*, 2004). Even though the DNA-based estimates of relative amounts of archaeal biomass in these environments increased by an order of magnitude relative to previous studies (Schippers *et al.*, 2005; Inagaki *et al.*, 2006), we note that the new data result in minimum estimates of the archaeal contribution. Nevertheless, our DNA-based

results clearly show that archaeal populations in the deep marine subsurface are larger than previous methods suggested.

Absence of discrimination against bacterial lipids is indicated by the consistency of results obtained from analysis of both PLFAs and IPLs in selected samples (see *Supplementary Table III.4*). Low PLFA concentrations are in agreement with generally low concentrations found in other studies in samples below 10 mbsf (Summit *et al.*, 2000; Cardace *et al.*, 2006); these are too low to account for the typical range of observed cell concentrations (see *Supplementary Table III.4*). There is no evidence that glycosidic lipids, i.e., those generally dominating the subsurface sediments, preferentially accumulate in sediments. For example, glycolipids produced by marine algae and cyanobacteria (Van Mooy *et al.*, 2006) were not detected in our sample set, not even in the uppermost sediment layers situated in the anoxic Black Sea and the oxygen minimum zone off Peru, thus requiring that they were degraded promptly even under anoxic conditions. This rapid removal is probably due to their

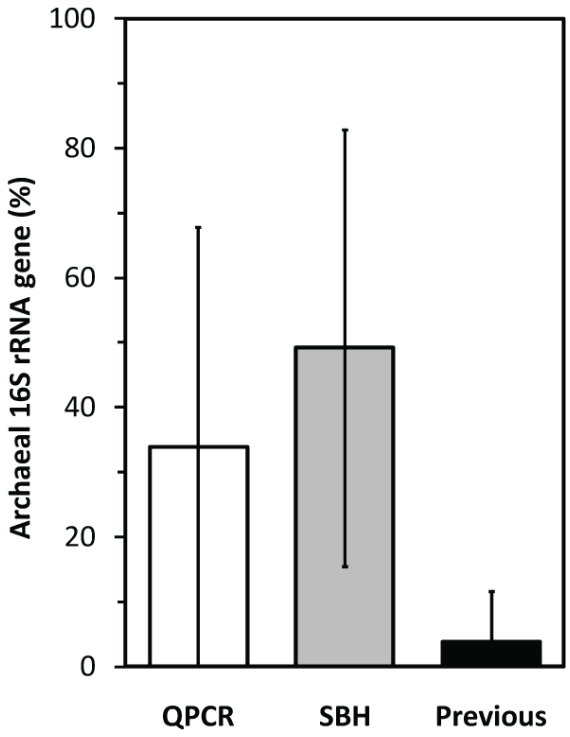

Figure III.2: Relative abundance of archaeal 16S rRNA genes to total microbial (Archaea + Bacteria) 16S rRNA genes in sediment cores evaluated by quantitative PCR (Q-PCR) and slot-blot hybridization (SBH) using genomic DNA amplified with phi29 polymerase as reaction template. The primer sets of Bac27F/EUB338R-I and ARC806F/ARC958R were used for Q-PCR amplification, and detection of 16S rRNA genes in SBH was done with EUB338-I, II, III and ARC915 probes labelled with digoxigenin (*see Supplementary Methods*). Average score of archaeal abundance evaluated by Q-PCR (white bar, n = 96) and SBH (gray bar, n= 115). Individual values were shown in *Supplementary Table III.3*. Note that the archaeal abundances have increased from previously reported data (black bar, average of *n* = 93 samples in previous studies is 3.7%; Schippers *et al.*, 2005; Inagaki *et al.*, 2006) by using improved DNA extraction and detection protocols. The archaeal proportion is likely still being underestimated due to sequence mismatch against some specific phylotypes and/or not-yet-detected archaeal components (e.g., the ARC915 probe has one- and two-base mismatches with sequences of the South African Gold Mine Euryarchaeotic Group [SAGMEG]) (Teske and Sørensen, 2008).

exposure to extracellular enzymes that are produced by sedimentary microorganisms for catalysing the initial hydrolytic steps of substrate degradation. These enzymes hydrolyse glycosidic bonds in polymeric carbohydrates rapidly on the order of days to weeks (Arnosti and Jørgensen, 2006) and are likely to catalyse the same reactions in glycosidic IPLs. Rapid turnover of glycosidic archaeal IPLs under natural conditions is also evident in results by Thiel *et al.* (2007) who analyzed anaerobic methanotrophic mats from the anoxic Black Sea and showed elegantly that certain areas of the mat are dominated by non-intact GDGT rather than intact, diglycosidic GDGT, consistent with hydrolysis of glycosidic bonds on timescales similar to cell turnover. Moreover, the distribution of archaeal core lipids in the pool of IPLs is clearly distinct from that of their non-intact, fossil counterparts (data not shown), suggesting that the extant archaeal population differs from the fossil population consisting of fossil planktonic and/or fossil sedimentary species deposited during earlier stages of the sediment's history. Jointly, these multiple lines of evidence argue against a fossil character of glycosidic archaeal IPLs.

What is the significance of an archaeal-dominated subsurface ecosystem? Based on empirical and theoretical consideration, Valentine (2007) recently argued that Archaea should be better adapted than Bacteria to extreme low-energy conditions such as those found in the deep biosphere while Bacteria are more successful in dynamic environments. Specifically, he argues that the lower membrane permeability resulting from the lipid structure lowers the demand for maintenance energy of an archaeal cell relative to a bacterial cell. Consequently, we suggest that even if the available free energy were to be evenly shared between Archaea and Bacteria, the lower energy requirement of Archaea for maintaining cell integrity under conditions of limited energy availability would result in longer life cycles, longer community-turnover times, and thus to larger standing stocks compared to Bacteria. Hence, although Archaea are more abundant than Bacteria, they do not necessarily rule the biogeochemical processes in the deep subsurface.

The sedimentary IPLs may be used as a proxy for the quantity of live microbial biomass in subsurface environments. We observed a statistically significant ($p<0.0001$) double logarithmic relationship between [IPL] and total organic carbon (TOC), extending over more than two and four orders of magnitude in TOC and IPL concentration (Figure III.3, $n = 101$, $R^2 = 0.56$), respectively, and testifying to the heterotrophic nature of the subsurface ecosystem. The relationship further indicates that the quantity of fossil organic matter is an important controlling factor for the amount of microbial biomass.

This relationship can be used to derive an estimate of the global inventory of biomass in marine subsurface sediments from well constrained concentrations of TOC. Although we currently do not have representation of the most oligotrophic open-ocean sites in our sample set, we consider this approach as less susceptible to biases in sample selection towards continental margins than previously used relationships based on cell counts vs. depth relationships (Parkes *et al.*, 1994; Whitman *et al.*, 1998). We estimate that prokaryotes inhabit a global marine sediment volume of 193 x 10^6 km^3 (see *Supplementary Methods* for details regarding calculations). Using a combination of regional distributions of TOC concentrations in surface sediments (Seiter *et al.*, 2004) and diagenetic models describing the degradation of TOC with time (Middelburg *et al.*, 1993), we derived an average TOC content in habitable subsurface sediments of 0.13% (see *Supplementary Tables 5-6*). This value is identical to a recent estimate of the average TOC content in subducting sediment (Hayes and Waldbauer, 2006). It results in a quantity of TOC in habitable subsurface sediments of 3.7 x 10^5 Pg, i.e., ~2.5% of the estimated TOC in the earth crust (Sigman and Boyle, 2000). For the amount of IPL in the respective sediment volume we obtain 7.1 Pg IPL (Table III.1, see *Supplementary Methods*), which can be converted into 90 Pg of microbial biomass-C (assuming spherical cells with a diameter of 0.5 µm, see *Supplementary Methods*

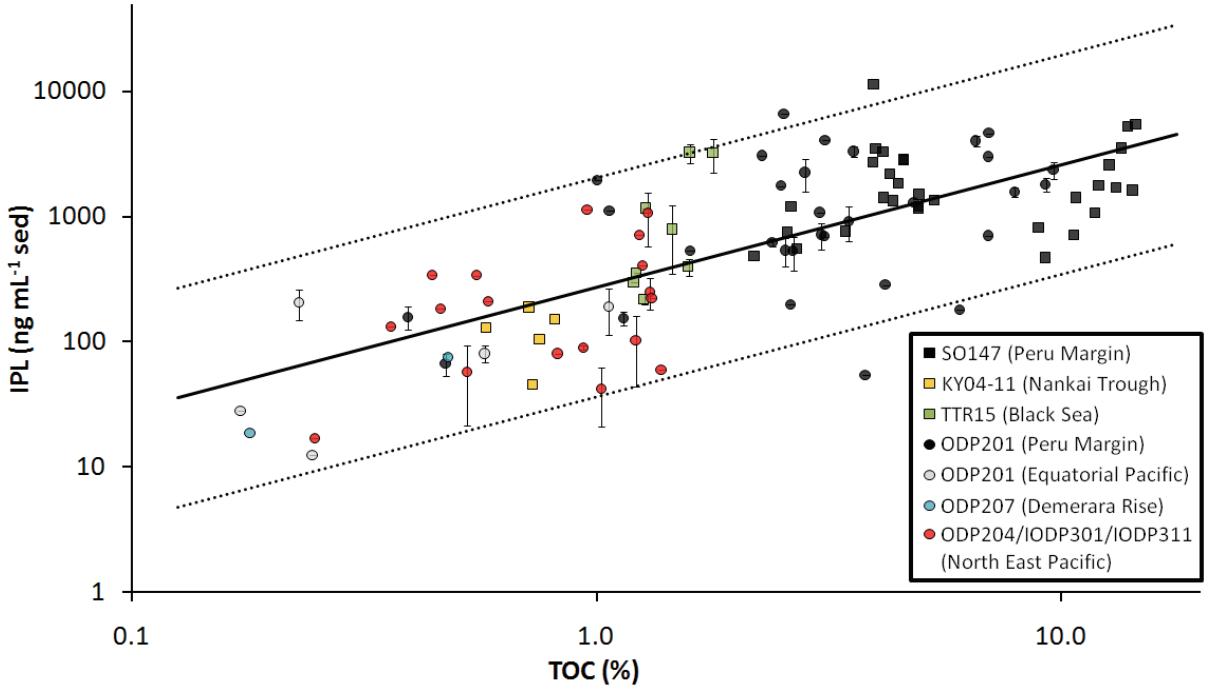

Figure III.3: Correlation of concentrations of IPLs and total organic carbon. Error bars show standard deviation of repeated injection of total lipid extracts. Regression lines: log $[IPL]_{total}$ vs. log [TOC] (solid line; log $[IPL]_{total}$ = 0.979 x log [TOC] + 2.436, N=101, R^2=0.56, p<0.0001, least squares analysis), and 95% prediction interval (dotted line, standard error of estimate: 0.44).

for information on the influence of cell size). The inherent uncertainties resulting from the data scatter of the 95% prediction intervals in the log[IPL]-log[TOC] relationship (± 0.9 orders of magnitude) are comparable to those encountered in log[cells]-log[depth] relationships previously used for estimating marine subsurface biomass (± 1 order of magnitude) (Parkes *et al.*, 1994; Whitman *et al.*, 1998).

We provide a refined and independent view on the quantity and composition of marine subsurface biomass. Our results suggest a vast ecosystem, in which Archaea contribute a major fraction to the standing stock of biomass. The environmental functions, strategies of survival and growth in extremely low-energy flux habitats, and physiologies of the key phylogenetic groups that presumably contribute largely to this biomass pool remain to be clarified. In combination with estimates of the relative contributions of the two taxonomic domains to water column biomass (Whitman *et al.*, 1998; Karner *et al.*, 2001), our data imply that in the marine realm, Archaea are more abundant than Bacteria.

Table III.1: Estimate of TOC concentrations in habitable marine sediment derived from diagenetic modelling (see *Supplementary Methods*) of concentrations at the sediment surface (Seiter *et al.*, 2004), resulting total inventories of TOC and IPL, and total inventories of microbial biomass-C for a hypothetical cell diameter of 500 nm.

	Open Ocean	Continental Margin	World Ocean
TOC average, range (%)	0.099; 0.07-0.56	0.204; 0.16-0.95	0.129
Amount of TOC (Pg)	2.0×10^5	1.7×10^5	3.7×10^5
Amount of IPL (Pg)	3.9	3.2	7.1
Biomass-C (Pg), 500-nm cells	50	40	90

METHODS SUMMARY

Extraction and analysis of lipids (IPL and PLFA). Frozen sediment was spiked with an internal intact polar lipid standard and extracted using a modified Bligh and Dyer protocol. The total lipid extract was analyzed on a HPLC system equipped with an ion-trap mass spectrometer (ThermoFinnigan LCQ Deca XP) following details published in Sturt *et al.* (2004). Response factors relative to the internal standard were determined offline for six commercially available IPL standards. Concentrations of individual IPLs in the sediment samples were calculated from mass chromatograms relative to the peak area of the known amount of internal standard and taking individual response factors into account (see *Supplementary Methods*). PLFAs were released from the polar fraction after fractionation of the total extract on a glass column via mild alkaline methanolysis. Analysis of the produced fatty acid methyl esters was done on a ThermoFinnigan Trace GC-MS system.

Extraction and amplification of DNA, and quantitative polymerase chain reaction (PCR) and slot-blot hybridization (SBH) analysis. Subseafloor sediment samples were collected from various cruises (see *Supplementary Table III.2*) and were immediately frozen and stored at -80°C until processing. DNA was extracted using a Beads Beating Kit (Nippon Gene, Tokyo, Japan) after crushing frozen sediments in a mill cooled by liquid nitrogen to maximize physical cell disruption. Cell disruption efficiency was monitored by epifluorescence microscopy with SYBR Green I (see *Supplementary Methods* and *Supplementary Figure III.6*). The DNA was purified and amplified by multiple displacement amplification using phi29 polymerase before analysis with quantitative polymerase chain reaction (Q-PCR) and slot-blot hybridization (see *Supplementary Methods*).

Quantitative biomass estimates. Estimates of the carbon sequestered in cellular carbon of marine subsurface prokaryotes are based on the correlation of [IPL] and [TOC] (Figure III.3). Global TOC quantities in marine sediments habitable by prokaryotes were estimated on the basis of modern concentrations of TOC in surface sediments (Seiter *et al.*, 2004), a diagenetic model describing the degradation of organic carbon with time (Middelburg *et al.*, 1993), and a global volume of habitable sediments resulting from the assumption that temperature is the major variable limiting microbial life at great depth (Whitman *et al.*, 1998). The resulting inventory of cellular lipids was transferred into a carbon inventory of marine subsurface prokaryotes using published conversion factors (Simon and Azam, 1989) (see *Supplementary Methods*).

ACKNOWLEDGEMENTS

Samples for this research were provided by the Integrated Ocean Drilling Program (IODP Expeditions 301 and 311), the Ocean Drilling Program (ODP Legs 201, 204, 207) (sponsored by the U.S. National Science Foundation and participating countries under the Joint Oceanographic Institutions, Inc.), cruises Sonne SO147, Karei KY04-11, Professor Logatchev TTR15, and Chikyu Shakedown Expedition CK06-06. We thank the participating crews and scientists of for sample recovery and data; U. Proske for assistance with lipid extraction and sample preparation; J. M. Hayes and A. Teske for useful comments on an earlier version of this manuscript; H. F. Fredricks, F. Schubotz for discussion of data; V. Heuer, A. Schippers, T. Toki for sample donation; B. Kockisch for TOC analysis; T. Terada and N. Masui for DNA extraction and analyses. This work was supported by Deutsche Forschungsgemeinschaft (through MARUM Center for Marine Environmental Sciences and grant Hi616-4 from the priority program IODP/ODP) and JAMSTEC Multidisciplinary Research Promotion Award 2007 (to Y.M. and F.I.). This is MARUM Publication No. ###.

Author Contributions. JSL, geochemical and lipid analysis, geochemical modelling, method development, paper writing; YM and FI, DNA extraction, molecular analyses; KUH, study design, paper writing, geochemical modelling. All authors participated in data analysis and interpretation and provided editorial comments on the manuscript.

Author Information. Raw data is available online in the Pangaea database (www.pangaea.de). Reprints and permissions information is available at http://npg.nature.com/reprintsandpermissions. The authors declare that they have no competing financial interests. Correspondence and requests for materials should be addressed to khinrichs@uni-bremen.de.

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III.2. SUPPORTING ONLINE MATERIAL

(I) Materials and Methods

Sample collection. Samples were collected during ODP Legs 201 (Peru Margin and Eastern Equatorial Pacific), 204 (Hydrate Ridge), 207 (Demerara Rise), IODP Expeditions 301 (Juan de Fuca Ridge Flank), 311 (Cascadia Margin), *DV Chikyu* Shakedown Expedition CK06-06 (offshore the Shimokita Peninsula, Japan), and cruises Sonne SO147 (Peru Margin), Professor Logatchev TTR15 (Black Sea), and Karei KY04-11 (Nankai Trough). The sediments were immediately frozen and stored at -80°C until extraction of lipids and DNA.

DNA extraction and multiple displacement amplification of subseafloor genomes. DNA was extracted from 6 g of wet sediment by using ISOIL for Beads Beating kit (Nippon Gene, Tokyo, Japan) according to the modified manufacturer's instructions as follows: sediments were crushed in a liquid nitrogen chamber with FREEZER/MILL 6850 (SPEX SamplePrep LLC, NJ) prior to bead-beating to maximize cell disruption, since very low cell disruption efficiencies were observed when DNA was extracted by previously used techniques without physical treatments (Inagaki *et al.*, 2006). Cell disruption efficiency was monitored by epifluorescence microscopy with SYBR Green I, indicating that approximately 80-90% of total cells were physically disrupted (*Supplementary Figure* III.5). However, the physical disruption using FREEZER/MILL was found to be unsuitable for the sandy sediments, likely due to serious damage to the nucleic acids by sand particles while crushing. The derived DNA solution after chemical and enzymatic lysis with SDS and lysozyme was further purified with magnet beads (MagExtractor^{TM} -PCR & Gel Clean up, TOYOBO, Tokyo, Japan). Purified DNA was amplified by multiple displacement amplification (MDA) using phi29 polymerase of the Illustra GenomiPhi V2 Kit (GE Healthcare Bioscience, Tokyo, Japan). Reaction mixtures were prepared in a lamina-flow clean bench to avoid potential contamination of DNA from the air. Genome amplifications including negative controls without samples were monitored by real-time PCR with SYBR Green I (Stephanauska and Sieracki, 2007), and no amplification of negative controls was observed during the amplification step (see *Supplementary Figure III.6*). The amplified genomes were further purified with Montague PCR (Millipore, Bedford, MA) by removing random primers and unreacted dNTPs.

Quantitative PCR and slot-blot hybridization analysis. To estimate copy numbers of archaeal and bacterial 16S rRNA genes, quantitative PCR (Q-PCR) was performed with a Power SYBR Green PCR Master Mix by ABI 7300 real-time PCR system according to the manufacturer's instructions (Applied Biosystems, Foster city, CA). For bacterial and archaeal 16S rRNA gene amplifications, the primer sets of Bac27F (Lane, 1985) and EUB338R-I (Amann *et al.*, 1990a), ARC806F (Takai *et al.*, 2000) and ARC958R (DeLong, 1992) were used, respectively. The standard curves for bacterial and archaeal 16S rRNA genes were obtained from genome DNA of *Escherichia coli* and *Pyrococcus horikoshii*, respectively (R^2 = 0.998 for Bacteria and 0.995 for Archaea). To minimize potential bias by sequence mismatch for molecular detection, we performed slot-blot hybridization (SBH) using highly conserved probes and purified MDA-genomes blotted on nylon membranes (Roche Diagnostics, Tokyo, Japan). For detection of bacterial and archaeal 16S rRNA genes, EUB338-I, II, III (Amann *et al.*, 1990a, Daims *et al.*, 1999) and ARC915 (Amann *et al.*, 1990b) probes labelled with digoxigenin were used, respectively. The sequence similarity of the ARC915 probe to 3,978 archaeal 16S rRNA gene sequences including almost all deep marine subsurface clones in our ARB software database showed that the probe is highly conserved among archaeal 16S rRNA genes: i.e. perfect match: 3,539/3,978 sequences (88.96%), one-base mismatch: 3,904/3,978 sequences (98.14%). The probe match analysis revealed that the sequences with the South African Gold Mine Euryarchaeotic Group (SAGMEG) have on- or two-base mismatches against ARC915, indicating a potential bias for signal detection by SBH. The membranes were hybridized in Ultrahyb Oligo buffer (Ambion, Austin, TX) at 42°C and washed twice with 0.2 x SSC (1x SSC: 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.1% SDS at 42°C and twice with 0.5x SSC containing 0.1% SDS at 46°C. Signals were detected by immunological and chemiluminescent detection with the chemi-luminescent substrate CSPD® using the DIG luminescent detection kit (Roche Diagnostics, Tokyo, Japan). Genome DNA of *Escherichia coli* and *Pyrococcus horikoshii* were used as standards for bacterial and archaeal 16S rRNA gene detection. False-positive detection of archaeal/bacterial 16S rRNA gene by cross-hybridization of archaeal/bacterial probes was checked and no signal was detected on *E. coli* and *P. horikoshii* genome blots by ARC915 and EUB338-I, II, III probes respectively.

Extraction of intact polar lipids. Frozen material (10 - 50 g of wet sediment for ODP/IODP samples; 1-10 g of freeze-dried sediment for SO147, TTR15, KY04-11 samples) was spiked with an internal standard (1-O-hexadecyl-2-acetoyl-*sn*-glycero-3-phosphocholine; PAF) and extracted using a modified Bligh and Dyer protocol in four steps (Sturt *et al.*, 2004). After

centrifugation at 800 x g the supernatants were combined, washed with water and carefully evaporated to dryness under a stream of nitrogen at 35°C and stored at -20°C until analysis.

HPLC-MS analysis and quantification of IPLs. The total lipid extract was dissolved in 0.1 – 1 ml of Methanol:DCM (1:1, v/v) and separated on a ThermoFinnigan Surveyor HPLC System equipped with a diol column coupled to a ThermoFinnigan LCQ Deca XP Plus ion trap mass spectrometer using an electrospray ionization (ESI) ion source. Details of chromatography and mass spectrometry are provided in Sturt *et al.* (2004).

Individual Response factors relative to our internal standard PAF were determined by injection of a series of standard solutions (di-C16-PC, di-C16-PG, di-C16-PE, 1-Gly-DG, GDGT; Matreya, USA; Avanti Polar Lipids, USA) in amounts ranging from 1 ng to 500 ng. Absolute IPL concentrations were calculated from areas of molecular ions in mass chromatograms relative to that of PAF, taking the response factors of archaeal and bacterial IPLs in relation to the internal standard into account. Multiple extractions of different sediment aliquots from 10-cm subcores and repeated injection of the total lipid extract provided standard deviations (typically ~20%) of the absolute IPL concentrations for a subset of 31 samples. For samples in which exclusively archaeal IPLs were detected, the proportion of bacterial lipids was assumed to be the level of detection (LOD). The LOD was calculated in a similar manner to the absolute IPL concentrations from the minimum peak area that could be identified as a signal in the respective mass chromatograms (typically 10^8 peak area units). It was then corrected with an averaged response factor for the two standards di-C16-PG and di-C16-PE representing the most common bacterial IPL types and multiplied by a factor of two assuming that the diversity of bacterial IPLs is higher than the diversity of archaeal lipids due to the larger variation in carbon atom numbers of ester-bound fatty acids (i.e., in a typical sediment sample containing both archaeal and bacterial IPLs, typically twice as many individual compounds are identifiable). A signal-to-noise ratio (SNR) of higher than three was maintained for all analyses. All absolute IPL concentrations were normalized to the amount of extracted sediment and the bulk sediment density value was used to calculate concentrations from volume of sediment.

Preparation and analysis of phospholipid derived fatty acids (PLFA). Total lipid extracts were fractionated on a glass column packed with 500 mg silica (Silica Gel 60, 60 - 200 µm, Roth, Germany) following the procedure of Mills *et al.* (2006). The apolar fraction was eluted with 5 ml dichloromethane and the polar fraction with 10 ml acetone followed by 5 ml methanol. After evaporation to dryness, the polar fraction was dissolved in 1 ml of toluene/methanol (1:1, v/v) and subjected to mild alkaline methanolysis (White *et al.*, 1979;

White and Ringelberg, 1998) to produce fatty acid methyl esters (FAMEs). FAMEs were dissolved in hexane and cholestane was added as injection standard. The identification and quantification was done on a Trace GC-MS system (ThermoFinnigan, San Jose, CA). The GC injector temperature was 310°C in split/splitless mode and separation was achieved on a Restek Rxi-5ms capillary column (L=30 m; ID=0.25 mm; 0.25 µm film thickness; carrier gas: He at 1 ml/min flow rate). The GC was programmed to 60°C (hold for 1 min), followed by heating at 10°C/min to 150°C, then 4°C/min to 320°C (hold for 60 min). Concentrations of FAMEs were normalized to volume of sediment.

Analysis of total organic carbon (TOC) concentration. Contents of total organic carbon were determined on decalcified sediments after acidification of ~50 mg of freeze dried sediment with 3 N HCl. Residues were analyzed on a Leco CS200 analyzer.

(II) Calculations of inventories of TOC, prokaryotic lipids, and cellular carbon in marine sediments

Horizontal and vertical distribution of TOC, IPLs and biomass-C in marine sediments. Regional provinces based on organic carbon content in surface sediments have been defined by Seiter *et al.* (2004). Sediment coverage and area of the provinces were calculated with MATLAB using a grid resolution of 5' from the NGDC sediment thickness database (Divins, 2008) for all cells where both TOC and sediment thickness were available (*Supplementary Table* III.6). The maximum sediment thickness was limited to 3000 m to account for the temperature limit which prevents life in very deep sediments (cf. Whitman *et al.*, 1998). Several of the provinces were combined to two major environmental types: open-ocean and continental margin sediments (see *Supplementary Table III.6*) with different geological and geochemical parameters (see *Supplementary Table III.5*). Down-core concentrations of TOC were calculated on the basis of an empirical diagenetic model (Eq. III.1) (Middelburg *et al.*, 1993). Equation III.3 describes *[TOC](t)* as a function of sediment age *t* and initial age *a,* and is obtained after substitution of Eq. III.1 into first-order kinetics of organic carbon degradation (Eq. III.2) followed by integration. Initial ages *a* are an expression of the organic matter's initial reactivity and were chosen by reverse fitting of the calculated TOC concentration profile to measured values in the deeper layers. Numerical integration of *[TOC](t)* over time from present to the age of the sediment basement or, at continental margins, the base of the habitable zone, and division by the covered time span (t_{base}) results in the average organic carbon concentration $[TOC]_{av}$ in the environment (Eq. III.4). Using the empirical relationship

of Eq. III.5, the average concentration of IPLs $[IPL]_{av}$ can be calculated from $[TOC]_{av}$. The total amount of IPLs ($m_{IPL,sed}$) is calculated according to Eq. III.6 with the volume of the sediment V_{sed} taken from *Supplementary Table III.5* and assuming an average sediment density ρ_{sed} of 1.5 g/cm^3. The calculation of biomass-C units ($m_{C,sed}$) is based on Eq. III.7 and is shown for a cell diameter of 500 nm (Table III.1).

$$k(t) = 0.21 \cdot (a + t)^{-0.99} \quad \text{(III.1)}$$

$$\frac{d[TOC]}{dt} = -k(t) \cdot [TOC] \quad \text{(III.2)}$$

$$[TOC](t) = [TOC](0) \cdot e^{21 \cdot (\sqrt[100]{a} - \sqrt[100]{a+t})} \quad \text{(III.3)}$$

$$[TOC]_{av} = \int_0^{t_{base}} [TOC](t)\, dt \cdot \frac{1}{t_{base}} \quad \text{(III.4)}$$

$$log[IPL]_{av} = 0.979 \cdot \log[TOC]_{av} + 2.436 \quad \text{(III.5)}$$

$$m_{IPL,sed} = [IPL]_{av} \cdot V_{sed} \cdot \rho_{sed} \quad \text{(III.6)}$$

$$m_{C,sed} = \frac{m_{C,cell}}{m_{IPL,cell}} \cdot m_{IPL,sed} \quad \text{(III.7)}$$

Amounts of IPL and carbon in cells. Published values for cellular amounts of lipids and total carbon vary widely (Bratbak, 1985; Balkwill *et al.*, 1988; Simon *et al.*, 1989; Morita, 1997; Madigan *et al.*, 2000; Summit *et al.*, 2000; Inagaki *et al.*, unpublished data). Simon and Azam (1989) published well established values for the macromolecular composition of an average spherical marine bacterium in a cell diameter range of 370-910 nm. Their results show that the ratio of cellular carbon to cell membrane lipids does not vary much with cell volume (ratio = 11-15) due to decreasing water content in smaller cells. We transferred their model to archaea by accounting for a lower membrane thickness of 5-6 nm (Bakowsky *et al.*, 2000) and a lower protein content of 60% (DeRosa *et al.*, 1986) and found conversion factors of archaeal lipid/cell that for spherical cells with diameters of 400 to 700 nm, these are on average only 10% higher than the values reported for bacteria by Simon and Azam (1989). The amount of IPL/cell ($m_{IPL,cell}$), carbon/cell ($m_{C,cell}$), and the conversion factor cellular carbon/IPL calculated using the power functions for a cell diameter of 500 nm are 1.4 fg, 18 fg and 13.

(III) References

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SUPPLEMENTARY TABLES

Supplementary Table III.2: Sampling site characteristics and number of samples analyzed for IPLs and molecular biological based methods.

Cruise	Station/Site	Position	Water depth	# of samples
SO147	2MC	11°35.0'S 77°3.1'W	86 m	19
SO147	47MC	9°44.4'S 78°45.1'W	155 m	13
KY04-11	PC08	33°7.3'N 136°28.8'E	2400 m	5
TTR15	BS269M	41°57.5'N 41°17.6'E	850 m	8
ODP201	1226	3°5.7'S 90°49.1'W	3297 m	7
ODP201	1227	8°59.5'S 90°57.4'W	427 m	8
ODP201	1229	10°58.6'S 77°57.5'W	152 m	18
ODP201	1230	9°6.8'S 80°35.0'W	5086 m	6
ODP204	1250	44°34.1'N 125°9.0'W	796 m	7
ODP207	1257	9°27.2'N 54°20.5'W	2951 m	1
ODP207	1258	9°26.0'N 54°44.0'W	3192 m	1
IODP301	1301	47°45.2'N 127°45.8'W	2656m	4
IODP311	1326	48°37.6'N 127°3.0'W	1828 m	2
IODP311	1327	48°41.9'N 126°51.9'W	1304 m	3
IODP311	1329	48°47.3'N 126°40.7'W	946 m	2
CK06-06	C9001	41°10.6'N 142°12.1'E	1180 m	21

Supplementary Table III.3: Relative abundance of archaeal 16S rRNA genes in sediment cores evaluated by quantitative PCR (Q-PCR) and slot-blot hybridization (SBH).

Site	Depth (mbsf)	Archaeal 16S rRNA gene copy number ratio (%)[a]	
		Q-PCR (N = 3)	SBH (N ≥ 2)
1226E	3.2	50.1 ± 9.1	67.7 ± 38.4
1226B	46.7	25.9 ± 6.2	>12
1227D	0.3	0.08 ± 0.05	82.8 ± 20.4
1227A	16.6	0.06 ± 0.03	11.2 ± 5.11
1227D	42.0	21.7 ± 4.9	40.3 ± 38.0
1227A	75.1	72.6 ± 8.2	59.7 ± 36.8
1230A	0.3	15.1 ± 7.0	49.9 ± 40.9
1230A	6.3	3.1 ± 4.4	8.5 ± 2.2
1230A	29.8	n.d.[c]	43.8 ± 47.9
1230A	73.8	86.9 ± 2.7	66.7 ± 25.3
1230A	209.3	n.d.[c]	17.4 ± 14.1
1301C	2.5	82.7 ± 8.4	56.4 ± 40.8
1301C	51.2	10.1 ± 4.0	46.7 ± 40.8
1301C	90.8	84.3 ± 2.3	36.4 ± 9.8
1301D	122.5	n.d.[c]	n.d.[c]
1301C	259.5	n.d.[c]	53.9 ± 32.4
CK06-06	1.0	39.9 ± 5.7	58.4 ± 32.4
CK06-06	5.2	50.1 ± 1.1	67.2 ± 34.5
CK06-06	8.1	18.1 ± 0.3	58.4 ± 30.3
CK06-06	13.5	97.6 ± 0.8	44.6 ± 30.1
CK06-06	21.7	1.8 ± 0.2	61.2 ± 12.1
CK06-06	31.0	1.5 ± 0.2	52.0 ± 20.9
CK06-06	59.5	0.4 ± 0.2	23.0 (n=1)
CK06-06	78.5	37.6 ± 3.6	4.3 ± 0.2
CK06-06	97.6	6.6 ± 5.5	n.d.[c]
CK06-06	116.6	98.1 ± 0.3	61.8 ± 17.9
CK06-06	134.5	56.5 ± 10.8	54.3 ± 45.7
CK06-06	154.5	0.02 ± 0.03	87.0 (n=1)
CK06-06	171.8	>0.1[b]	>18.9 (n=1)[b]
CK06-06	191.5	14.3 ± 5.2	15.5 ± 16.9
CK06-06	208.3	0.7 ± 0.1	n.d.[c]
CK06-06	216.8	23.2 ± 3.0	30.3 ± 19.0
CK06-06	228.6	88.6 ± 2.6	>11.9 (n=1)[b]
CK06-06	264.9	>97.8[b]	20.2 ± 14.0
CK06-06	346.3	30.0 ± 4.5	60.9 ± 48.2
CK06-06	358.6	34.3 ± 19	46.3 ± 31.0

[a]Archaeal 16S rRNA gene copy number ratio = archaeal 16S rRNA genes / (archaeal + bacterial 16S rRNA genes).
[b]The value indicates the minimum archaeal 16S rRNA ratio based on the detection limit of bacterial 16S rRNA gene copy number when no bacterial signals were detected.
[c]n.d.: not detected

Supplementary Table III.4: PLFA and IPL concentrations (in ng mL^{-1}) of selected sediment cores. When no bacterial IPLs were detected the LOD was assumed as bacterial IPL concentration (see *Supplementary Methods*). The higher PLFA relative to IPL concentrations in sample SO147-2MC might be partially due to inclusion of fatty acids from undefined polar precursors (Aries *et al.*, 2001) as evidenced by the presence of land-plant derived fatty acids (C20-C26).

	SO147-2MC-0.05	1226B-45.87	1301C-49.90	1229A-185.88
Σ C14-C18	4667.9 (95%)	4.8 (95%)	6.8 (90%)	1.5 (52%)
Σ C20-C26	252.9 (5%)	0.2 (5%)	0.8 (10%)	1.4 (48%)
Total PLFA	4920.8	5.0	7.6	2.9
Bacterial IPLs	1035.5	5.4 (LOD)	125.6 (LOD)	22.7 (LOD)

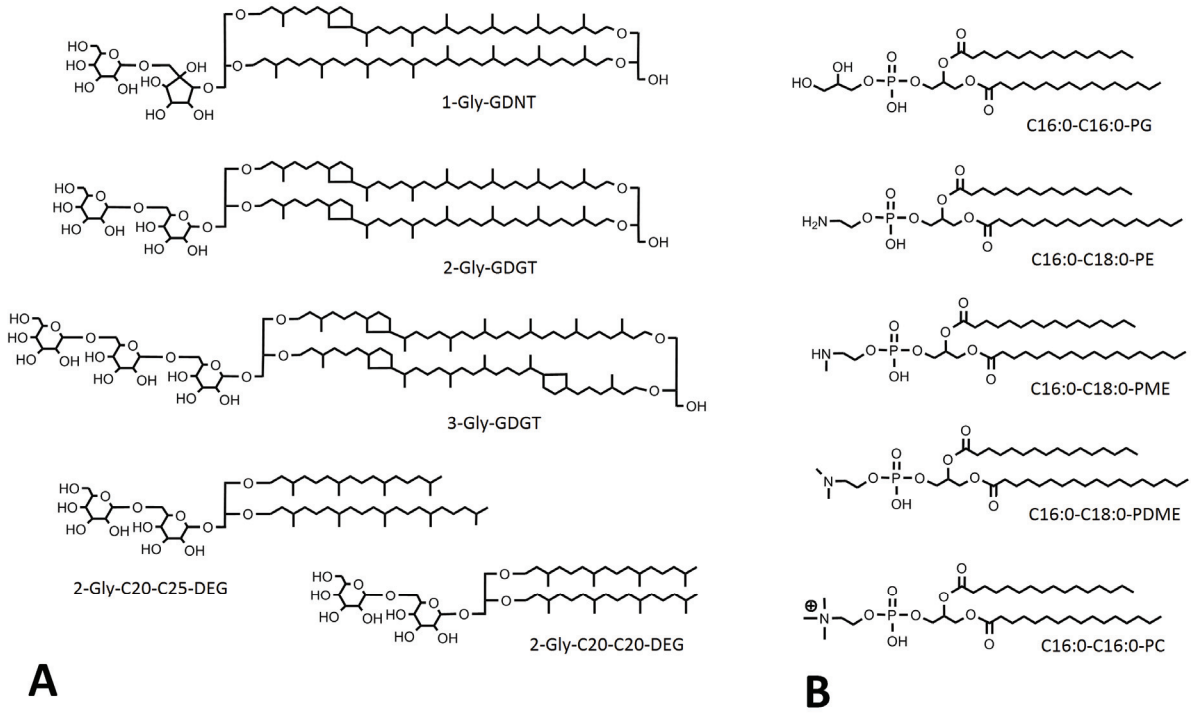
Supplementary Table III.5: Parameters and assumptions for modelling of TOC distribution in marine environment types. Estimates in open ocean and continental margin sediments are based on average sediment coverage of 416 m and 1952 m, respectively. A total area of marine sediments of 360 x 10^6 km^2 (Lisitzin, 1996*)* was used to calculate sediment volumes. Note that this value is higher than the sum of open ocean and continental margin area in *Supplementary Table III.6* of 312 x 10^6 km^2. The relative proportions of 92% open ocean and 8% continental margin are adopted in the area not covered by the NGDC database (Divins, 2008) and/or the study of Seiter *et al.* (2004).

	Open Ocean	Continental Margin, habitable zone
Percent of ocean area (%)	92.1	7.9
Sediment coverage (m)	416	1952
Volume of sediment (10^6 km^3)	137.9	55.4
TOC in surface sediment (%)	0.56	0.95
Sedimentation rate (cm kyr^{-1})	0.33	10
Age of sediment base (Myr)	124.9	19.5
Initial Age (yr)	3 x 10^4	1 x 10^4

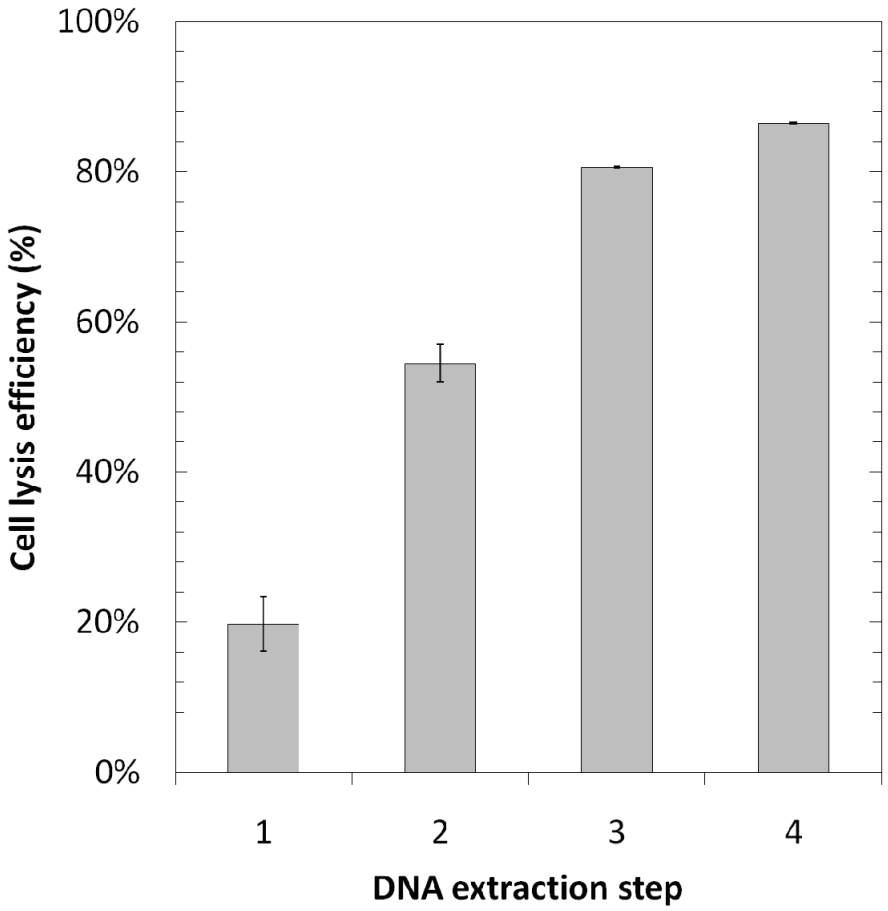
Supplementary Table III.6: Area and TOC contents in marine sediment provinces defined by Seiter *et al.* (2004). Provinces of open ocean and continental margin settings were combined to two major environment types and the average TOC content was weighted by area (w.b.a.).

Province	Area (10^6 km^2)	Percent of total area (%)	TOC in surface sediment (top 5 cmbsf) (%)	Sediment coverage in habitable sediments (m)
NEPAC	25.98	8.3	0.4	160
NWPAC	18.87	6.0	0.6	347
SEPAC	54.37	17.4	0.5	132
SWPAC	9.27	3.0	0.8	636
TROPAC2	12.32	3.9	0.8	399
TROPAC	30.86	9.9	1.2	197
NOATL	29.35	9.4	0.4	704
SOATL	35.31	11.3	0.4	476
ETROPAT	3.11	1.0	0.7	585
IND	58.87	18.9	0.4	571
EARAB	0.97	0.3	1.2	1343
WARAB	2.20	0.7	1.5	1167
GROE	1.69	0.5	0.7	816
ANT	4.50	1.4	0.3	1522
Σ Open Ocean	**287.68**	**92.1**	**0.56 (w.b.a.)**	**416 (w.b.a.)**
EUR1	0.295	0.09	0.8	1632
EUR2	1.47	0.47	0.3	1959
NWAMCO	1.77	0.57	1.7	538
NEAMCO	5.92	1.90	0.9	2227
SEAFCO	2.60	0.83	0.5	1834
WAFCO	1.29	0.41	0.6	2402
TANZACO	0.921	0.30	1.0	2155
SOMALICO	0.295	0.09	0.7	1197
GUI	0.353	0.11	1.1	2366
CANAR	0.486	0.16	0.6	2327
GUBRACO	1.58	0.50	0.4	2569
ARGCO	1.53	0.49	0.3	1643
BRAZCO	1.40	0.45	0.5	2194
RIOPLATA	0.813	0.26	0.8	2558
EICO	0.409	0.13	1.0	3000
SWACO	1.92	0.62	1.5	2331
NAMBCO	0.543	0.17	2.7	1618
PERCO	0.542	0.17	4.8	292
CHICO	0.479	0.15	1.5	646
Σ Continental Margin	**24.63**	**7.9**	**0.95 (w.b.a.)**	**1952 (w.b.a.)**

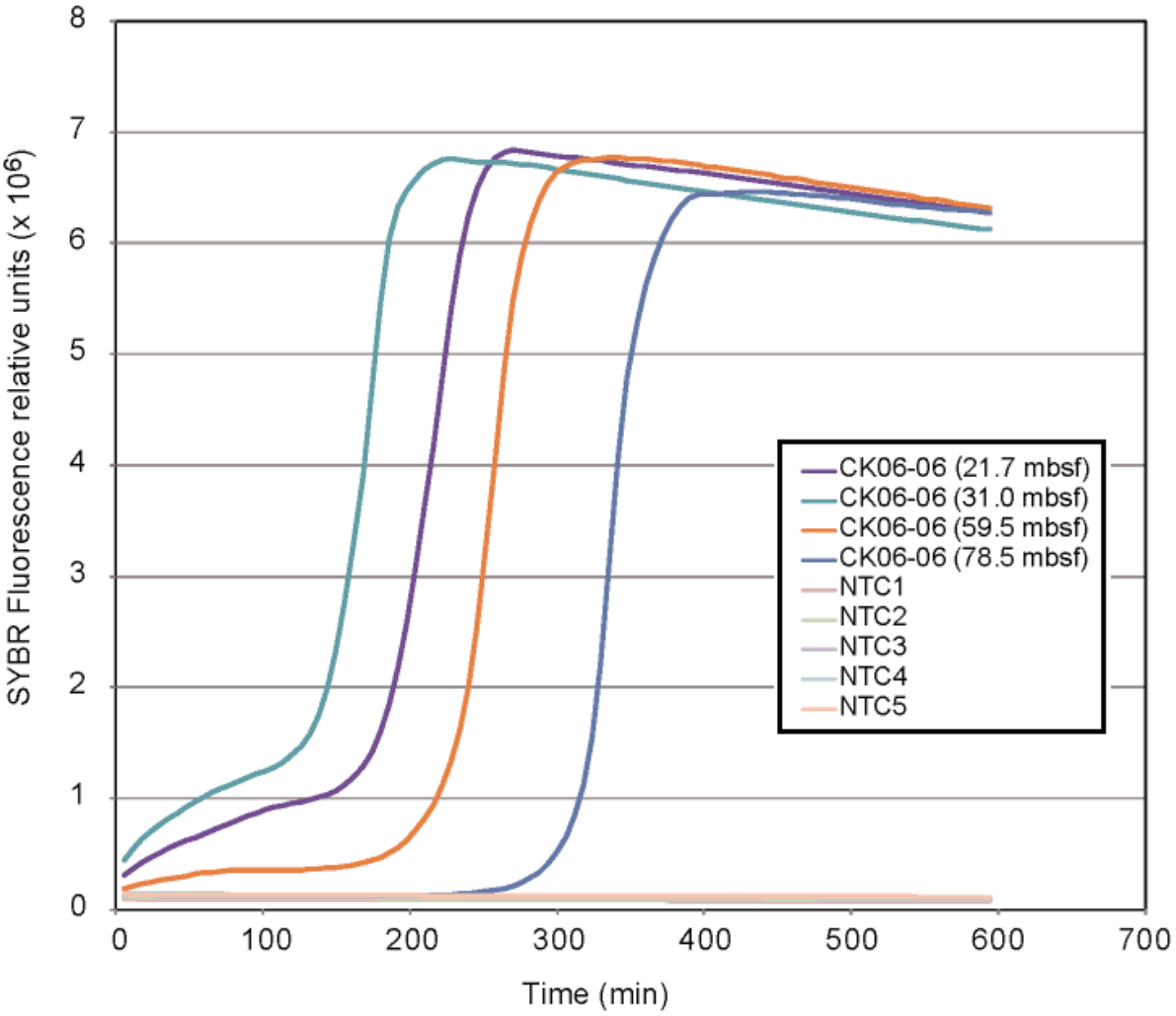
SUPPLEMENTARY FIGURES



Supplementary Figure III.4: Structures of microbial IPLs detected in marine sediments. **(A)** Archaeal tetraethers with 0-5 rings were found, examples of the core lipid structures with 1-3 rings are shown. **(B)** The most abundant bacterial phospholipid fatty acids were C16:0, C16:1, C18:0, and C18:1. Headgroups: 1-Gly: monoglycosyl, 2-Gly: diglycosyl, 3-Gly: triglycosyl, PG: phosphatidylglycerol, PE: phosphatidylethanolamine, PME: phosphatidyl-(N)-methylethanolamine, PDME: phosphatidyl-(N,N)-dimethylethanolamine. Core lipids: GDGT: glyceroldialkylglyceroltetraether, GDNT: glyceroldialkylnonitoltetraether, DGD: dialkylglyceroldiether.



Supplementary Figure III.5: Cell lysis efficiency after chemical, enzymatic, and physical disruption of sub-seafloor sediments. The number of SYBR Green I-stained cells were evaluated under fluorescent microscopy after the following DNA extraction steps: 1) SDS and enzymatic (lysozyme and proteinase K) treatment, 2) beads beating treatment and step (1), 3) freeze mill at beating rate 8 and step (1), and 4) freeze mill at beating rate 15 and step (1). The experiments were carried out using the same core sediment sample (Code: CK06-06-2T-3). Error bars indicate the standard deviation (n = 5).



Supplementary Figure III.6: An example of quality control monitoring of multiple displacement amplification evaluated by quantitative real-time PCR with SYBR Green I. The deep marine subsurface genomes amplified by the reaction of multiple displacement amplification with phi29 polymerase were obtained without amplification of negative control samples. NTC: no-template control.

Chapter IV

Structural diversity of intact polar lipids in marine sediments

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Prepared for submission to

Geochimica et Cosmochimica Acta

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ABSTRACT

Marine sediments harbor an enormous quantity of microorganisms including a multitude of novel species. The inhabitable zone of the marine sediment column begins at the sediment-water interface and is thought to reach to depths of several thousands of meters. Studies of the microbial diversity in this extant ecosystem have mostly relied on molecular biological techniques. We used a complementary method - intact polar membrane lipid analysis - to characterize the *in-situ* microbial community in sediments covering a wide range of environmental conditions. A variety of bacterial and eukaryotal phospholipids were detected in surface sediments from the Peru Margin. Deeply buried sediments were dominated by archaeal diether and tetraether lipids with various polar head group and core lipid structures. The comparison of ring distributions in intact and non-intact tetraether lipids revealed a distinct pattern, suggesting that the *in-situ* archaeal population is different from any fossil contribution deposited earlier. This affects paleotemperatures reconstructed using the TEX86 paleotemperature proxy, where temperatures were found to be systematically higher when calculated from intact lipids. We explored the effect of varying cell and lipid stability on the sedimentary pool by box-modeling. Results show that (a) constant supply of freshly synthesized lipid is necessary, and (b) lipid input rates decrease with burial depth. We present several arguments for rapid turnover of lipids under a variety of naturally occurring conditions. Our results underline the usefulness of intact polar membrane lipids as readily available markers targeting live microbial communities in marine sediments.

IV.1. INTRODUCTION

Drilling into marine sediments has revealed the presence of a deeply buried vast microbial ecosystem. The magnitude of this largely unexplored habitat has been estimated to about 56 - 303 Pg of cellular carbon or 8 - 35x10^{29} prokaryotic cells, representing 55-85% of total prokaryotes in marine sediments or 10-30% of total biomass on Earth (Whitman *et al.*, 1998, Parkes *et al.*, 1994). A more recent estimate found a more conservative amount of 90 Pg of cellular carbon (Lipp *et al.*, in revision). The activity of these cells was found to be orders of magnitude lower than in surface sediments (D'Hondt *et al.*, 2002) with long turnover times in the order of hundreds to thousands of years (Whitman *et al.*, 1998; D'Hondt *et al.*, 2003; Biddle *et al.*, 2006) and even up to 100 kyr in Mediterranean Sapropels (Parkes *et al.*, 2000). The activity is stimulated at geochemical interfaces like the sulfate-methane transition zone in the Peru Margin where the activity rates were even higher than in surface sediments (Parkes *et al.*, 2005). Little is known about the organisms and the processes they are mediating. Recent studies using culture–independent molecular biological techniques have revealed the presence of unique microbial communities in the marine subsurface (Vetriani *et al.*, 1999, Sørensen *et al.*, 2004, Biddle *et al.*, 2006; Inagaki *et al.*, 2003 and 2006; Sørensen and Teske, 2006; Teske, 2006; Webster *et al.*, 2006). However, even seemingly simple questions concerning the nature of the community structure remain controversial, e.g. if archaea or bacteria dominate (Mauclaire *et al.*, 2004; Schippers *et al.*, 2005; Biddle *et al.*, 2006; Inagaki *et al.*, 2006; Schippers and Neretin, 2006; Lipp *et al.*, in revision). The overall low biomass in the deep subsurface and complex matrix of sedimentary organic material makes this environment analytically very challenging. Gene-based microbiological methods rely on efficient extraction of DNA or RNA and subsequent primer-based amplification (e.g. quantitative polymerase chain reaction, Q-PCR) or direct hybridization with genetic probes (e.g. fluorescent *in-situ* hybridization, FISH; catalyzed reporter deposition FISH, CARD-FISH). There is ample evidence that the choice of primers and probes for quantification and identification of the microbial community selectively suppresses the detection of some subsurface archaeal lineages (Lipp *et al.*, in revision; Teske and Sørensen, 2008). In addition, studies have suggested that DNA is resistant to degradation on timescales of thousands to millions of years (Coolen and Overmann, 1998; Inagaki *et al.*, 2005), making the detection of the *in-situ* population even more difficult.

A promising method for detection and quantification of the *in-situ* community is based on membrane lipids. The analysis is a rapid procedure and non-selective since no prior knowledge of the community structure is necessary. In addition, lipids can be used to unravel

the physiologies of the organisms by analysis of stable-isotope abundance. Lipids are the building blocks of every cell membrane and are essential for multiple functions of the cell. They provide a physical barrier to separate the cytoplasma from the environment and serve as "anchor" for trans-membrane proteins allowing substrate uptake and product release. It is expected that the wide range of processes in which membrane lipids are involved has led to the formation of a large diversity (Dowhan, 1997). The majority of bacterial membrane lipids are glycerol-based with two hydrophobic chains connected to the glycerol backbone via ester bonds and a phosphate-based polar head group (e.g. Figure IV.1B). The composition of the membrane changes in response to environmental parameters like temperature, osmolarity, salinity, pH, or pressure to minimize energy expenditure and optimize cell growth (Cronan and Gelmann, 1975). This adaptation process results from *de-novo* production of lipids or alteration of existing phospholipid fatty acids by introduction of unsaturations or isopropyl rings or cis-trans isomerization of unsaturations (Zhang *et al.*, 2008). For example, psychrophilic and piezophilic bacteria produce fatty acids with more unsaturations to maintain membrane fluidity (Fulco, 1983, DeLong and Yayanos, 1985 and 1986, Yano *et al.*, 1997, Kaneda, 1991, Fang *et al.*, 2000a; Fang *et al.*, 2003).

In environmental samples typically the apolar derivates of membrane lipids were analyzed, either as breakdown products which were produced in the environment or as products prepared during sample preparation (e.g. phospholipid-derived fatty acids, PLFA). PLFA have been validated as markers for live bacteria, since the "parent" phospholipid is degraded by hydrolytic cleavage of the polar head group bond within days to weeks after cell death (White *et al.*, 1979a, Harvey *et al.*, 1986). This allows the wide-spread analysis of *in-situ* microbial community structures (e.g. Guezennec and Fiala-Medioni, 1996). The fatty acid chain structure holds information about the involved species and can be used as chemotaxonomic marker (Asselineau and Asselineau, 1990), whereas PLFA abundance has been used to infer cell concentrations (e.g. White and Findlay, 1988; Haack *et al.*, 1994). Many PLFA-based studies focused on marine sediments and studied the *in-situ* population (Summit *et al.*, 2000; Cardace *et al.*, 2006; Mills *et al.*, 2006). However, the assignment of PLFAs to organisms is difficult at times as the fatty acids in surface sediments represent a complex mixture of eukaryotic and prokaryotic fatty acids, often including contamination by wax esters from higher plant material (Volkman and Johns, 1977; Eglinton and Hamilton, 1967) which sometimes are difficult to exclude from analysis even in deeper samples (Aries *et al.*, 2001).

Recent advances in analytical chemistry allow the analysis of the membrane lipids in their intact form as intact polar lipids (IPL). Using high-performance liquid chromatography coupled to mass spectrometry (HPLC-MS) we can now explore the full potential of the lipid inventory, including valuable information on the polar head group (Fang and Barcelona, 1998; Rütters *et al.*, 2001, 2002a, and 2002b). Direct comparison of PLFA and IPL analysis has shown that the chemotaxonomic potential is greater for analysis of the intact molecules (Fang *et al.*, 2000b). IPL analysis allows direct characterization of the *in-situ* community. Studies of marine sediments have revealed bacterial IPLs with a variety of polar head groups and fatty acids (Fang *et al.*, 2000b; Rütters *et al.*, 2002a and 2002b; Zink *et al.*, 2003; Sturt *et al.*, 2004; Zink and Mangelsdorf, 2004; Fredricks and Hinrichs, 2007; Zink *et al.*, 2008; Lipp *et al.*, in revision).

The analytical window of IPL analysis is not only limited to analysis of bacterial and eukaryotic lipids but can be extended to include the third domain of life, the Archaea. Glycerol-based phospho- and glycolipids are building blocks of the archaeal cell membrane and comprise 2-6% of dry weight of the cell (Langworthy *et al.*, 1983; Ferrante *et al.*, 1990). Archaeal lipid hydrophobic chains are distinct from their bacterial and eukaryotal counterparts. Archaea use ether bonds to connect the glycerol backbone to isoprenoidal hydrocarbon chains in order to strengthen the cell membrane against extreme conditions (De Rosa *et al.*, 1989). In addition, they exist as diethers with two chains and also as membrane-spanning tetraethers with four ether bonds and varying number of rings in the two

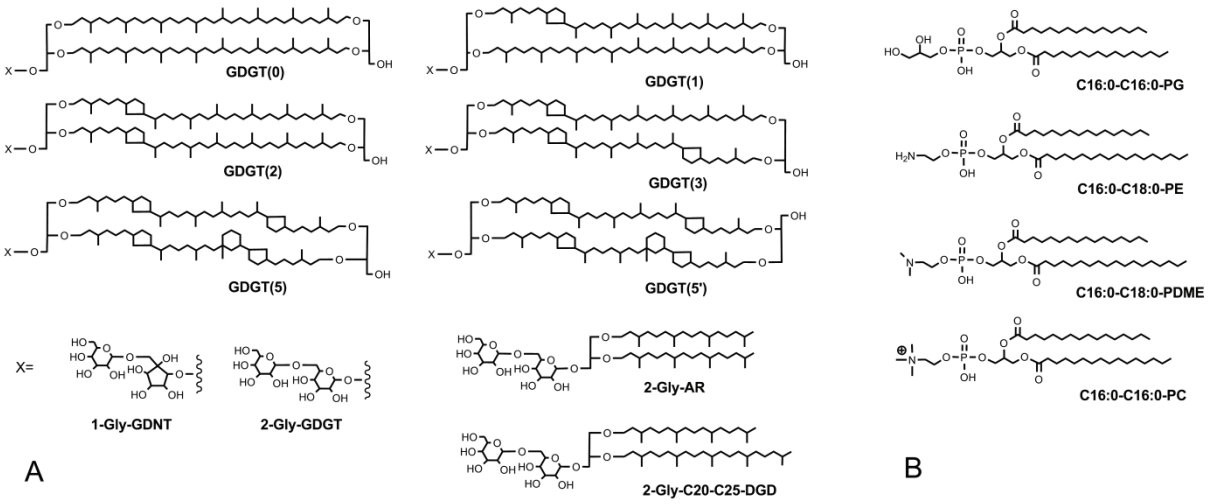

Figure IV.1: Structures of prokaryotic IPLs detected in marine sediments. (**A**) Archaeal tetraether- and diether lipids with different polar head groups and core lipid structure were found. (**B**) Bacterial phospholipids with various polar head groups and the fatty acids C16:0, C16:1, C18:0, and C18:1 were identified. Head groups: 1-Gly: monoglycosyl, 2-Gly: diglycosyl, PG: phosphatidylglycerol, PE: phosphatidylethanolamine, PDME: phosphatidyl-(N,N)-dimethylethanolamine, PC: phosphatidylcholine. Core lipids: GDGT: glyceroldialkylglyceroltetraether, GDNT: glyceroldialkylnonitoltetraether, AR: archaeol, C20-C25-DGD: dialkylglyceroldiether with mixed isoprenoidal chains of phytane (C20) and PMI (C25).

biphytane chains. The degree of cyclization depends on growth temperature (DeRosa and Gambacorta, 1988; Gliozzi *et al.*, 1983; Uda *et al.*, 2001). Polar head groups of archaeal IPLs are either purely hexose- and phosphate-based or a mixture of both (Koga *et al.*, 1993, Sturt *et al.*, 2004; Rossel *et al.*, in press; Schouten *et al.*, 2008; Strapoc *et al.*, 2008). The nature of the polar head group and core lipid allows the use of archaeal IPLs as chemotaxonomic marker. Analysis of lipid component parts in cultures of methanogenic archaea showed that the lipid composition is specific up to the genus-level (Koga *et al.*, 1993; Koga *et al.*, 1998; Whitman *et al.*, 2001). Further support for the concept of chemotaxonomy comes from a broader study of cultures of environmentally relevant cren- and euryarchaea such as *Archaeoglobales*, *Methanobacteriales*, *Methanococcales* and *Thermoproteales*. It was found that crenarchaea produce mostly glycosidic head groups attached to tetraether core lipids with a high degree of cyclization, whereas euryarchaea abundantly produce mixed phospho/glyco head groups, diether lipids and tetraether IPLs with a low number of rings in the core (Hinrichs *et al.*, unpublished data).

Few studies targeting both archaeal and bacterial IPLs in marine sediments exist, and these analyses point to a major contribution of archaeal IPLs to the total inventory (Sturt *et al.*, 2004; Biddle *et al.*, 2006; Fredricks and Hinrichs, 2007; Lipp *et al.*, in revision). Several unknown archaeal IPL species have been found but the link to the producing organism has not been unambiguously established. The dominance of archaeal IPLs in deep sediments has raised questions about lipid stability and possible selective preservation.

We want to explore the structural diversity and abundance of IPLs in marine sediments using a combination of PLFA, IPL and apolar core lipid analysis to assess the lipid diversity in deeply buried sediments and find out if it matches the variety found in surface-near sediments. Furthermore, we will use box-modeling to simulate IPL concentration profiles with to varying lipid stability and re-evaluate the possibility of preservation.

IV.2. MATERIALS AND METHODS

Sample collection. Sediment surface samples were collected from Peru Margin (Sonne SO147, stations 2MC and 47MC; Kudrass *et al.*, 2000), Black Sea (Professor Logatchev TTR15, station BS269M; Akhmetzhanov *et al.*, 2007) and Nankai Trough (Karei KY04-11, station PC08; JAMSTEC Japan), subsurface samples were collected from Hydrate Ridge (ODP204, Sites 1249 and 1250; Tréhu *et al.*, 2003), Juan de Fuca Ridge (IODP301, Site 1301; Shipboard Scientific Party, 2004), Cascadia Margin (IODP311, Sites 1326, 1327, and 1329; Shipboard Scientific Party, 2005), Peru Margin and Eastern Equatorial Pacific

(ODP201, Sites 1226, 1227, 1229, and 1230; D'Hondt *et al.*, 2003), and Demerara Rise (ODP207, Sites 1257 and 1258; Erbacher *et al.*, 2004). All samples were immediately frozen and stored at -80°C until extraction and analysis.

Extraction of intact polar lipids (IPL). Frozen sediment was spiked with an internal standard (1-O-hexadecyl-2-acetoyl-*sn*-glycero-3-phosphocholine; PAF) and extracted using a modified Bligh and Dyer protocol as described previously (Sturt *et al.*, 2004). Total lipids were extracted in four steps with a solvent mixture of dichloromethane/methanol/buffer [1:2:0.8; v/v] using 4 ml solvent per gram of sediment and extraction step. The first two steps used phosphate buffer (8.7 g/L KH_2PO_4, pH 7.4) and the final two steps trichloroacetic acid buffer (50 g/L CCl_3COOH). After sonication for 10 min and centrifugation at 800 x g for 10 min the supernatants were combined in a separatory funnel, washed with water, and carefully evaporated to dryness under a nitrogen stream at 40°C. The total lipid extract (TLE) was stored at -20°C until analysis.

Analysis of intact polar lipids. Intact polar lipids were analyzed on a ThermoFinnigan Surveyor HPLC system coupled to a ThermoFinnigan LCQ Deca XP Plus ion trap mass spectrometer equipped with electrospray ionization source (ESI) under conditions described previously (Sturt *et al.*, 2004). The total lipid extract was dissolved in 0.1-1 ml of methanol/dichloromethane [1:1, v/v] and 10 µl were injected to a LiChrosphere Diol-100 column (150x2.1 mm, 5 µm, Alltech, Germany) equipped with a guard column of the same packing material. Lipid components in the polar fraction were separated according to head group polarity. Details of chromatography and mass spectrometry are provided in Sturt *et al.* (2004). IPLs were identified based on their characteristic fragmentation patterns in MS2 spectra (cf. Sturt *et al.*, 2004). Details of IPL quantification and calculation of level of detection (LOD) are described in Lipp *et al.* (in revision). In brief, concentrations were calculated from peak areas in mass chromatograms relative to the internal standard and corrected with individual response factors determined offline from IPL standards. The LOD was calculated in a similar way from the smallest peak that could be identified as signal in the mass chromatogram, maintaining a signal-to-noise ratio of three. In order to better represent the proportion of bacteria, the LOD concentration was multiplied by a factor of two assuming that the diversity of bacterial lipids is higher than the diversity of archaeal lipids.

Preparation of enriched IPL fractions. A subset of samples was subjected to preparative HPLC for preconcentration of archaeal IPLs (glyceroldialkylglyceroltetraethers, GDGT; dialkylglyceroldiethers, DGD). This step is necessary to prepare clean fractions that can be

injected to the HPLC at higher concentration and allows identification and quantification of all archaeal IPLs, including minor components. Fractions were prepared using a preparative LiChrosphere Si60 column (250x10 mm, 5 µm, Alltech, Germany) with a fraction collector following established parameters (Biddle *et al.*, 2006). The fraction containing archaeal IPLs (45-50 min retention time) was evaporated to dryness, re-dissolved in 0.2 ml dichloromethane/methanol [1:1, v:v] and analyzed using the analytical conditions described above.

Preparation and analysis of GDGT core lipids. The distribution of rings in GDGTs was determined for polar GDGTs (intact form with polar head group) and apolar GDGTs (without polar head group). The TLE corresponding to 30 g of sediment was separated on a glass column packed with 1.5 g silica gel (Silica Gel 60, 60 – 200 µm, Roth, Germany) and preconditioned with dichloromethane. Three fractions were prepared: F1 containing apolar lipids (10 ml dichloromethane), F2 containing apolar GDGT core lipids (10 ml dichloromethane/acetone [3:1, v/v]), and F3 containing polar GDGTs (10 ml dichloromethane/methanol [3:1, v/v] followed by 10 ml dichloromethane/methanol [1:1, v/v] and 40 ml methanol). Analysis of fractions F2 and F3 showed nearly complete separation of polar and apolar GDGTs. Polar GDGTs in F3 were converted to GDGT core lipids by hydrolysis of the glycosidic bond to the sugar head group. Fraction F3 was dissolved in 1 ml of a reaction mixture of 6 M HCl/methanol/dichloromethane [1:9:1, v/v] and heated to 70°C for 24 h. The cleaved fraction F3 and untreated F2 were evaporated and re-dissolved in 300 µl of hexane/isopropanol [99:1 v/v] and analyzed following slightly modified conditions described by Hopmans *et al.* (2000). GDGTs were separated on an Econosphere NH_2 column (4.5 x 250 mm, Alltech, Germany) heated to 30°C in a ThermoFinnigan Surveyor HPLC system. The following gradient was used at 1 ml/min flow rate: hold isocratically 99:1 (hexane/isopropanol, [v/v]) for 5 min, then ramp to 98.2:1.8 (hexane/isopropanol, [v/v]) at 45 min, followed by flushing with 95:5 (hexane/isopropanol, [v/v]) for 10 min and equilibrating with 99:1 (hexane/isopropanol, [v/v]) for 10 min to prepare the system for the next injection. Mass spectrometric identification and quantification was achieved on a ThermoFinnigan LCQ Deca XP Plus ion trap mass spectrometer coupled to the HPLC by an atmospheric pressure chemical ionization (APCI) interface. APCI settings were as follows: capillary temperature 200°C, source heater temperature 400°C, sheath gas flow 30 arbitrary units, source current 5 µA, while other parameters were optimized by manual tuning during infusion of a hydrolyzed commercially available intact GDGT standard (Matreya, USA). Quantification of core lipids and calculation of relative ring distribution was done from mass

chromatograms of the M^+ ions. Quantification of GDGT(4) was not done due to co-elution of m/z 1294 (M^+) with the $[M+2]^+$ isotope peak of GDGT(5) and the lack of end member samples consisting of only GDGT(5) and GDGT(0) to allow for correction following Zhang *et al.* (2006).

Preparation and analysis of phospholipid derived fatty acids (PLFA). Total lipid extracts were separated on a glass column packed with 0.5 g silica gel (Silica Gel 60, 60 – 200 µm, Roth, Germany) following the procedure described by Mills *et al.* (2006). Two fractions were eluted: apolar F1 (5 ml dichloromethane) and polar F2 (10 ml acetone followed by 5 ml methanol). After evaporation to dryness the polar fraction was dissolved in 1 ml of toluene/methanol [1:1, v/v] and fatty acid methyl esters (FAME) were produced by mild alkaline methanolysis (White *et al.*, 1979b; White and Ringelberg, 1998). FAMEs were dissolved in hexane and cholestane was added as injection standard. The identification and quantification was done on a ThermoFinnigan Trace GC-MS system (ThermoFinnigan, San Jose, USA) using analytical conditions described earlier (Lipp *et al.*, in revision).

Modeling of IPL concentration and distribution in marine sediments. The first kinetic model of the process of organic matter degradation in marine sediments was proposed by Berner (1964). This model assumes a constant first-order degradation rate for the organic matter as a whole. A more refined model considers that organic matter is composed of several components with different reactivity and proposes first order decay for every component (Jørgensen, 1979). Among the components that can be explained with first-order kinetics are live cells, DNA and RNA, and apolar and polar lipids. We used a box-model with first-order kinetics to describe the decay of IPLs. The simulated box is the equivalent to 1 mL of sediment and the amount of IPL it contains ($n_{IPL,total}$) is simulated over time in discrete time steps of duration t_{step}. We used constant values for IPL half-life covering three orders of magnitude (1 kyr, 10 kyr and 100 kyr) to model the concentration of lipids over time. The depth interval spans from 0.01 to 1000 mbsf, which is the equivalent to 100 yr to 10 Myr when using a sedimentation rate of 10 cm kyr^{-1}.

Simple model of IPL decay with input of IPLs in surface sediment only. In order to simulate conditions where input of IPLs only occurs in the surface sediment and no IPLs are produced *in-situ* the input flux was set to zero for the whole sediment column. The total amount $IPL_{IPL,total}(0)$ starts with a value of 1862 ng as predicted by the regression line of Lipp *et al.* (in revision) for the shallowest simulated depth (0.01 mbsf). The amount of IPL that is removed in each discrete modeling step *n* from the previous step (*n-1*) is calculated using

first-order kinetics with half-life $T_{1/2,}$ a step size $t_{step,}$ and the total amount of IPL in the previous step as described in equation IV.1.

$$n_{IPL,removed}(n) = \left[1 - \left(\frac{1}{2}\right)^{\frac{t_{step}}{T_{1/2}}}\right] \cdot n_{IPL,total}(n-1) \quad \text{(IV.1)}$$

Finally, the total amount of IPL in step *n* was calculated using equation IV.2 by subtraction of the amount of IPL that is removed from the total amount of IPL in the previous step.

$$n_{IPL,total}(n) = n_{IPL,total}(n-1) - n_{IPL,removed}(n) \quad \text{(IV.2)}$$

Simple model of IPL decay allowing input of IPLs in subsurface sediments. In order to explore how the profile changes when sedimentary input of IPL is allowed, we extended the box model to account for a constant input flux (equation IV.3). Half-life and input flux was selected to match the modeled profile to the observed IPL regression line (Lipp *et al.*, in revision). The input flux $F_{IPL,input}$ was multiplied with the duration of a time step to yield the amount of IPL that has to be added in each step (equation IV.4). The remaining model conditions are similar to the model without sedimentary IPL input.

$$n_{IPL,total}(n) = n_{IPL,total}(n-1) - n_{IPL,removed}(n) + n_{IPL,input} \quad \text{(IV.3)}$$

$$n_{IPL,input} = F_{IPL,input} \cdot t_{step} \quad \text{(IV.4)}$$

We also performed "reverse" modeling to calculate the IPL input flux as a function of depth that is necessary to match the model result as close as possible to the IPL regression line. The amount of IPL input was calculated as deviation of the model result to the amount calculated according to the regression line according to equation IV.5. Negative values for the input term were set to zero. Input flux was calculated according to equation IV.6 with step size t_{step}.

$$n_{IPL,input}(n) = n_{IPL,removed}(n) - \left(n_{IPL,total}(n-1) - n_{IPL,regress}(n)\right) \quad \text{(IV.5)}$$

$$F_{IPL,input}(n) = \frac{n_{IPL,input}(n)}{t_{step}} \quad \text{(IV.6)}$$

IV.3. RESULTS AND DISCUSSION

We have analyzed 104 samples covering various sites in the world, including the Peru Margin, Black Sea, Nankai Trough, Eastern Equatorial Pacific, Hydrate Ridge, Juan de Fuca Ridge, Cascadia Margin, and Demerara Rise (Lipp *et al.*, in revision). Lipid concentrations have been reported in Lipp *et al.* (in revision). All samples contain abundant archaeal glycolipids and only 12 out of 104 samples show signs of phospholipids with concentrations high enough for unambiguous identification.

Distribution of bacterial lipids in marine sediments

Bacterial phospholipids were detected in surface samples from the Peru Margin (Table IV.1) where two sites were sampled by multicoring (47MC and 2MC). In both cores diacylglyceride lipids with the polar head groups phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) were found (see Figure IV.1B for structures). The fatty acid chains connected to the glycerol backbone of the intact lipids were identified in negative ionization mode and found to be C_{16} and C_{18} with up to one unsaturation (Lipp *et al.*, in revision). PE is the most abundant phospholipid in both cores with relative contributions of 43% to 100%, while the abundance of PG and PC varies (Table IV.1). Site MC47 contains minor amounts of PC in the top sediment sample and the proportion of PG decreases sharply with depth from 43% to levels below the detection limit at 5 cmbsf. Concentrations range from 15000 ng mL^{-1} sediment at the sediment-water interface to 1150 ng mL^{-1} at 7 cmbsf. Site 2MC displays more diversity in IPLs: PE is the dominating lipid except in the very top sample with relative contributions increasing with depth from

Table IV.1: Relative distribution of polar head groups in bacterial IPL and observed concentrations.

Sample	Concentration (ng mL^{-1} sediment)	PG (%)	PE (%)	PC (%)
47MC-0.005	15236	43	51	7
47MC-0.01	1332	19	81	n.d.
47MC-0.03	1932	23	77	n.d.
47MC-0.05	95	n.d.	100	n.d.
47MC-0.06	1152	n.d.	100	n.d.
2MC-0.005	8763	9	43	48
2MC-0.01	1676	18	47	35
2MC-0.02	1345	12	62	26
2MC-0.03	1155	n.d.	83	17
2MC-0.05	1036	19	65	16
2MC-0.07	193	n.d.	67	33
2MC-0.09	80	n.d.	100	n.d.

n.d. not detected

43-100% and PC becomes second most abundant with 48% at the sediment top and decreasing to levels below the detection limit at 9 cmbsf. The relative contribution of PG is scattered and spans from 9% to 19%. Overall the concentration of total bacterial IPLs decreases from 8700 ng mL^{-1} to 80 ng mL^{-1} at 9 cmbsf. PE is the most abundant phospholipid in bacteria (cf. Dowhan *et al.*, 1997), in sediments it has often been attributed to sulfate reducing bacteria (SRB) where it usually occurs esterified to glycerol-based diacyl- (DAG), diether- (DGD) and mixed acylether-chains (Rütters *et al.*, 2001; Sturt *et al.*, 2004; Rossel *et al.*, in press; Hinrichs *et al.*, unpublished data). We could only detect DAG-PE, but its occurrence can be linked with great confidence to the presence of SRB based on previous analyses that detected maximum sulfate reduction rates in the upper 5 cm of the cores (Böning *et al.*, 2004) and also described highest amounts of DNA from SRB within the top 10 cmbsf (Schippers and Neretin, 2006). Although it has been suggested that the microbial community structure is largely similar according to DNA analysis (Schippers and Neretin, 2006) there are some profound differences in the lipid profiles of PG and PC. PG is typically associated to photosynthetic organisms where it occurs in the thylakoid membranes (Sakurai *et al.*, 2003; Hagio *et al.*, 2000; Wada and Murata, 2007), nevertheless, it has also been identified as the second most abundant lipid in bacterial membranes (Dowhan, 1997) including cultured representatives of the deep biosphere (Schubotz, 2005; Hinrichs *et al.*, unpublished data). PC has been identified as the major membrane forming lipid in eukaryotes (e.g. Raetz, 1986) and it is estimated that more than 10% of all bacteria posses PC (Sohlenkamp *et al.*, 2003). The higher concentration of PC in sediments from 2MC therefore suggests a larger eukaryotic contribution, most likely through accumulation of sinking particles from the upper water column. Another explanation could be a difference in bacterial community structure. Although there is no microbiological evidence for a community difference, it is not unlikely that the different geochemistry of the two cores, e.g. different concentrations of total organic carbon (TOC), affects the microbial community. The higher concentrations of bacterial IPLs in samples from station MC47 are generally consistent with higher concentrations of sedimentary organic carbon which is used as substrate (Lipp *et al.*, in revision).

The detection of bacterial phospholipids in the remaining samples is severely hampered by the level of detection (LOD) of the analytical procedure which suffers from overall low concentrations (Lipp *et al.*, in revision) and strong matrix effects due to ion suppression (e.g. Mallet *et al.*, 2004). In order to get a more complete picture of the bacterial lipid diversity we prepared phospholipid derived fatty acids (PLFA) from polar extracts of five samples representing different environments (Table IV.2). Branched and unbranched fatty acids (FA)

with a chain length of C_{14} to C_{26} and up to one double bond were detected. The highest concentrations were found for FAs $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, and $C_{18:1}$ which is consistent with the results of analysis of the intact lipids and previous studies of PLFA in deeply buried sediments (Summit *et al.*, 2000; Cardace *et al.*, 2006; Mills *et al.*, 2006). Notably, FAs with very long acyl-chains of 20 to 26 carbon atoms have been found in all samples with relative amounts of up to 50% of total PLFA in samples 1249F-54.3 and 1229A-185.9. It has been argued that very long chain fatty acids could be a signature of oligotrophic lifestyle or thermal protection of bacterial membranes (Summit *et al.*, 2000), however, another explanation is that they are derived from land-plants and were included in undefined polar precursors that could not be separated during PLFA preparation (Aries *et al.*, 2001). Consequently, this leads to the conclusion that the concentrations of FAs C_{14}-C_{18}, which are typically attributed to bacteria, might also be partially influenced by fossil FAs and thus *in situ* bacterial abundance might be overestimated. This becomes apparent when comparing the concentrations of total PLFA with total bacterial IPL in sample 2MC-0.05: the concentrations of PLFA are higher than IPL by a factor of five. The other four samples have lower concentrations of PLFA suggesting only a minor contribution from land plant derived fatty acids. Concentrations of bacterial IPLs in those samples have been estimated from the LOD (Lipp *et al.*, in revision) and are up to one order of magnitude higher than actual measured total PLFA concentration.

Table IV.2: Concentrations (in ng mL^{-1}) and relative proportions of PLFAs and bacterial IPLs.

	2MC-0.05 *Peru Margin*	1226B-45.9 *Equatorial Pacific*	1301C-49.9 *Juan de Fuca Ridge*	1249F-54.3 *Hydrate Ridge*	1229A-185.9 *Peru Margin*
C14:0	94 (2%)	n.d.	n.d.	0.5 (3%)	n.d.
C15:0	37 (1%)	n.d.	n.d.	0.9 (6%)	n.d.
aiC15:0	251 (5%)	n.d.	n.d.	n.d.	n.d.
iC15:0	112 (2%)	n.d.	n.d.	n.d.	n.d.
C16:0	1566 (32%)	2.3 (45%)	2.8 (37%)	3.3 (22%)	0.9 (31%)
C16:1	698 (14%)	n.d.	0.6 (7%)	0.8 (5%)	n.d.
iC16:0	91 (2%)	n.d.	n.d.	n.d.	n.d.
C18:0	256 (5%)	0.5 (10%)	1.1 (15%)	1.3 (8%)	0.6 (21%)
C18:1	1563 (32%)	2.0 (40%)	2.3 (31%)	1.5 (10%)	n.d.
Σ C14 - C18	4668 (95%)	4.8 (95%)	6.8 (90%)	8.1 (54%)	1.5 (52%)
C20:0	107 (2%)	0.2 (5%)	0.8 (10%)	0.7 (4%)	0.4 (13%)
C22:0	53 (1%)	n.d.	n.d.	2.0 (13%)	0.5 (19%)
C24:0	83 (2%)	n.d.	n.d.	3.1 (21%)	0.5 (16%)
C26:0	10 (0.2%)	n.d.	n.d.	1.1 (8%)	n.d.
Σ C20 - C26	253 (5%)	0.2 (5%)	0.8 (10%)	6.9 (46%)	1.4 (48%)
Σ PLFA	4921	5	7.5	15	2.9
bacterial IPL	1036	5.4[a]	126[a]	28[a]	23[a]

n.d. - not detected; [a] – level of detection assumed as bacterial IPL concentration

Distribution of archaeal lipids in marine sediments

All analyzed samples contain abundant archaeal glycolipids with either diether or tetraether core structure. We identified glyceroldialkylglyceroltetraether (GDGT), glyceroldialkylnonitoltetraether (GDNT) and dialkylglyceroldiethers (DGD) with different isopranyl hydrocarbon chain combinations: C20-C20-DGD (archaeol) and C20-C25-DGD (mixed phytane/pentamethylicosane) (see Figure IV.1A for structures). The polar head groups are of glycosidic origin and either one or two hexoses are bound to the core lipid. One GDGT species (termed "GDGT + m/z 342") contains an unknown head group of 18 Da larger mass than regular diglycosidic GDGT (see section below for detailed description). GDGT and GDNT core lipids contained 0-5 rings in their biphytane chains as revealed by detailed mass spectrometric analysis.

The structural diversity of archaeal lipids of the deep biosphere has so far not been investigated in great detail nor put into context to their environmental setting (Sturt *et al.*, 2004; Biddle *et al.*, 2006; Fredricks and Hinrichs; 2007; Lipp *et al.*, in revision). We have looked in detail at eight samples from the Peru Margin that were enriched in archaeal IPLs by preparative HPLC (Biddle *et al.*, 2006). Seven of the samples are from sediment layers where methane is oxidized at the expense of sulfate (sulfate methane transition zones, SMTZ; cf. Biddle *et al.*, 2006) and one sample was selected from a shallower horizon (Table IV.3). These enriched fractions have high enough concentrations to provide high quality MS data and allow quantification of all archaeal compounds, even of the minor compounds. Five archaeal IPLs were identified in relative amounts of 0.3% to 79%: diglycosidic GDGT (2-Gly-GDGT), "GDGT + m/z 342" with unknown polar head group, monoglycosidic GDNT (1-Gly-GDNT), diglycosidic archaeol (2-Gly-DGD-A), and diglycosidic DGD with mixed isopranyl chains (2-Gly-DGD-B). All samples contained high amounts of 2-Gly-GDGT with relative proportions of 60-80% followed by 17-30% of "GDGT + m/z 342". The remaining 0.3-10% of archaeal IPLs were 1-Gly-GDNT and the two diether species. The number of rings in the tetraether core varied among the different species: 2-Gly-GDGT contains acyclic to pentacyclic core lipids, "GDGT + m/z 342" was identified with 2-4 rings, and the nonitol-based tetraether was found exclusively in the monocyclic form. Notably, the distribution of GDGTs in the surface sample is distinct from the more uniform samples of the SMTZ: concentrations of 2-Gly-GDGT are higher with almost 80% compared to 60-70% in the SMTZ samples and concentrations of "GDGT + m/z 342" are lower with 17% instead of 23-30%.

Geochemical and sedimentary properties like substrate availability and associated predominant metabolic processes influence the composition of the microbial community (e.g. Parkes *et al.*, 2005) which results in a variety of archaeal lipids in changing relative proportions dependent on microbial regime. However, it is challenging to connect the lipid distribution patterns with phylogenetic information of microbial community structure gained from 16S rRNA analysis. The frequency of clones in clone libraries does not necessarily reflect the abundance of the associated organisms. Recent studies of different primer sets selected for construction of clone libraries found inherent bias against certain archaeal lineages (Teske and Sørensen, 2008). This is further complicated by the fact that some studies focused on 16S rRNA genes with a potentially higher proportion of fossil DNA (Coolen and Overmann, 1998), while others have analyzed reverse-transcribed 16S rRNA which is thought to target only active cells. We compared the relative concentrations of the IPL species with the dominant phylotypes described by Biddle *et al.* (2006) and Sørensen and Teske (2006) who used the same primer set and focused on RNA (Table IV.3). There is no evident relationship of lipid distribution and phylogenetic archaeal community structure in the sample set. A comparison of IPLs and geochemical parameters also did not establish an obvious connection (data not shown). However, samples from ODP Leg 201 site 1230 did not show the presence of diether-based lipids, a fact that clearly distinguishes samples from this site from the rest of the sample set. Site 1230 is located at the Peru Trench in a water

Table IV.3: Archaeal IPL distribution in fractions enriched by preparative HPLC.

Sample	2-Gly-GDGT (%)	"GDGT + m/z 342" (%)	1-Gly-GDNT (%)	2-Gly-AR (%)	2-Gly-C25-AR (%)	Dominant phylotype[a]
Sulfate-methane transition zone sediments						
1227D-38.2	66.0	25.3	3.4	1.0	4.3	MBG-B
1227A-37.6	70.7	22.9	3.9	0.4	2.1	MBG-B
1227A-40.9	61.2	26.0	n.d.	3.5	9.2	MCG
1229D-29.7	69.5	27.8	1.9	0.3	0.5	MCG
1229D-87.1	64.7	28.5	3.8	1.8	1.2	MCG
1230B-9.3	69.2	29.7	1.1	n.d.	n.d.	MBG-B
1230B-9.7	73.6	26.4	n.d.	n.d.	n.d.	MBG-B
Surface near sediment						
1229D-1.4	78.9	17.0	1.4	1.7	n.d.	n.a.
GDGT rings	0-5	2-4	1	-	-	-

n.d. - not detected; n.a. – not analyzed
"GDGT + m/z 342" - tentatively identified GDGT with a 18 Da larger head group than 2-Gly-GDGT
AR - archaeol (C20-C20-DGD); C25-AR - C20-C25-DGD
MBG-B – Marine Benthic Group B; MCG – Miscellaneous Crenarchaeotic Group
[a] - as described in Biddle *et al.* (2006) and Sørensen and Teske (2006)

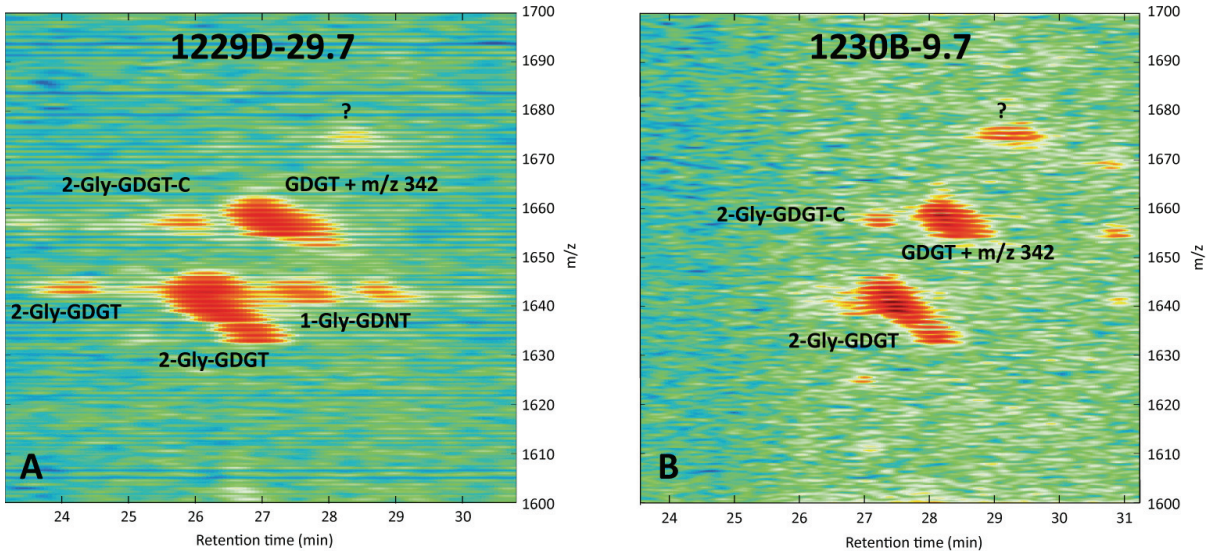

Figure IV.2: Density maps showing magnified peaks in the tetraether area. Samples were pre-concentrated tetraether IPLs using preparative HPLC to allow identification of trace compounds. (**A**) Density map of HPLC-MS analysis of sample 1229D-29.7 and identified archaeal tetraether lipids. Note the two species of 2-Gly-GDGT and 1-Gly-GDNT possibly derived from different sugars in the polar head group. (**B**) Density map of sample 1230B-9.7 showing only 2-Gly-GDGT-C, 2-Gly-GDGT, and "GDGT + m/z 342". "?" denotes unknown compound with m/z 1674 where no MS2 fragments could be observed. The density map was digitally enhanced and filtered with a Matlab (TheMathWorks) script for better demonstration.

depth of 5086 m and harbors hydrate-rich sediments. These conditions and the low *in-situ* temperature of 4°C compared to 10°C and 15°C for sites 1227 and 1229 could select for a different archaeal population and explain the absence of diether-based membrane lipids.

In addition to the composition of the archaeal membrane lipids regarding relative concentrations we analyzed differences in the "fine structure" of single lipid species. Figure IV.2 shows two density maps highlighting the tetraether region of the HPLC-MS chromatograms of the two samples 1229D-29.7 and 1230B-9.7. Sample 1229D-29.7 exhibits two separate peaks each for 2-Gly-GDGT and 1-Gly-GDNT with slightly different retention times, whereas the other sample suggests the presence of a single species only. A logical explanation of this difference in polarity while having similar mass spectrometric fragmentation patterns is the presence of different glycosidic head groups. Glycolipids with varying hexoses cannot be distinguished by their MS2 mass spectra but the different structure could lead to shifts in relative polarity and thus retention time of the intact lipids. Several sugars have been found in the glycosidic head group of archaea, including the hexoses glucose, galactose, mannose, and gulose and modified carbohydrates like (N)-acetylglucosamine and sulfated sugars (see Koga *et al.*, 2005 and references therein). Unfortunately, concentrations of the glycolipids have not been high enough to prove this hypothesis by GC-MS analysis of the carbohydrate components after acidic hydrolysis. Large scale extraction of sediments and enrichment of the glycolipids by preparative HPLC needs to

be done. The detection of the various sugars could then be done with either GC-MS (Guerrant and Moss, 1984; Koga *et al.*, 1993) or thin-layer chromatography and development of the plates with specific dyes.

Structures of archaeal tetraether lipids

To investigate the structures of the archaeal tetraethers found in all analyzed samples, we focused on sample ODP201 1229D-29.7, in which high concentrations of all species were observed. The extract was analyzed with specially designed mass spectrometric method in the mass range m/z 1600-1700 while applying a mass filter that prevents ions of less than m/z 1500 to enter the ion trap of the instrument. This guarantees low space-charge effects in the trap and ensures a high signal-to-noise (SNR) ratio and good yields of daughter ions in the MS2 and MS3 fragmentation stages to help identification. Four different tetraether species were identified and were designated as Peaks 1-4 in the chromatogram (Figure IV.3A).

Two of the peaks (Peak 2 and 4) occur in the mass range of m/z 1634 – 1644. Analysis of the fragmentation pattern in MS2 identified 2-Gly-GDGT (Peak 2) and 1-Gly-GDNT (Peak 4). Figure IV.3B shows the daughter ion mass spectra for the dicyclic and monocyclic tetraether structures. 2-Gly-GDGT (m/z 1640, ammonium adducted pseudomolecular ion with two cyclopentyl rings) looses the diglycosidic head group including the ammonium ion (two hexose units of 162 Da plus 18 Da of NH_4^+) in the MS2 stage and produces a characteristic core lipid ion at m/z 1298. 2-Gly-GDGT lipids have been identified in a variety of environmentally relevant cultivated cren- and euryarchaea such as *Archaeoglobales*, *Methanobacteriales*, *Methanococcales* and *Thermoproteales* (Hinrichs *et al.*, unpublished data), anaerobic methane oxidizers of the ANME-1 clade in Black Sea mats (Thiel *et al.*, 2007; Rossel *et al.*, in press), methanogen cultures (Koga *et al.*, 1993), marine group I crenarchaea (Schouten *et al.*, 2008) and extracts of subseafloor sediments (Sturt *et al.*, 2004; Biddle *et al.*, 2006; Lipp *et al.*, in revision). Peak 4 has a much lower abundance but could still be identified by the typical fragmentation pattern of GDNTs. The MS2 daughter ion spectrum shows a strong peak at m/z 1460 which is consistent with the loss of one hexose group of 162 Da plus an ammonium adduct from the pseudomolecular ion at m/z 1640. The difference in fragmentation is caused by the nonitol backbone structure which has a strong ether bond instead of an easily cleaved glycosidic bond. GDNT lipids have only been found in hyperthermophilic crenarchaea of the order sulfolobales (DeRosa *et al.*, 1980; Gambacorta, 2002; Sturt *et al.*, 2004) and in deeply buried sediments off Peru (Biddle *et al.*, 2006; Lipp *et al.*, in revision) where they have been connected to redox cycling of sulfur during anaerobic oxidation of methane (Sturt *et al.*, 2004).

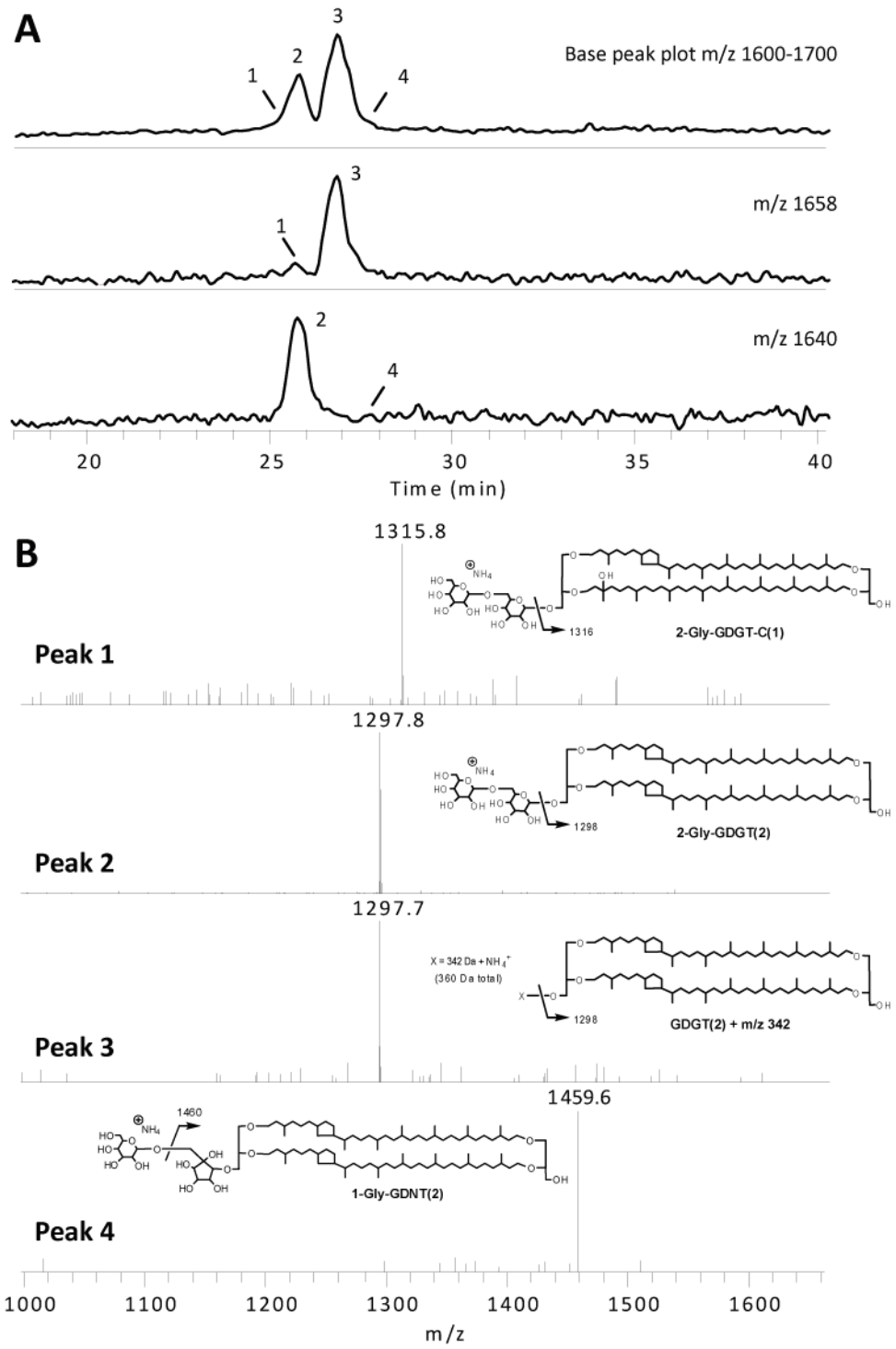

Figure IV.3: Chromatograms and mass spectra of GDGTs in TLE of sample 1229D-29.7. (**A**) Base peak chromatogram of m/z 1600-1700 and extracted ion mass chromatograms for m/z 1658 and m/z 1640. Peaks 1-4 were identified to have a m/z of 1658, 1640, 1658, and 1640 in MS1 mass spectrometry stage. (**B**) Daughter ion spectra of peaks 1-4 measured in MS2 stage and corresponding (tentatively) identified structures (see text for details).

The other two peaks illustrated in Figure IV.3A are found in the mass range of m/z 1652 - 1660. The first compound - eluting as Peak 1 - is present in traces only and produces a strong ion at m/z 1316. The loss of a neutral fragment of 342 Da is in line with a diglycosidic head group; however the ion at m/z 1316 represents an unknown tetraether core

structure. Interestingly, ions of such m/z 1316 have been observed during analysis of GDGT core lipids in an acid hydrolyzed extract of the same sediment sample (data not shown). A tentative structure is shown in Figure IV.3B consisting of a hydroxybiphytane chain in the GDGT core structure. Such an additional hydroxyl group would be lost during the harsh conditions of ether cleavage or strong acid treatment for preparation of GDGT core lipids as known from hydroxyarchaeol (Ferrante *et al.*, 1988; Koga *et al.*, 1993). The intrinsic lability together with low concentrations could explain why no evidence for unusual biphytane chains was found in ether cleaved hydrocarbon fractions (Biddle *et al.*, 2006; Orcutt *et al.*, submitted; Lin *et al.*, unpublished results). The last tetraether compound (Peak 3) produces a pseudomolecular ion of m/z 1658 in the MS1 full scan and a characteristic fragment ion of m/z 1298 which can be attributed to a GDGT core structure with two pentacyclic rings. This tetraether lipid, termed "GDGT + m/z 342" in this study, has been found abundantly in subseafloor sediments (Sturt *et al.*, 2004; Biddle *et al.*, 2006; Fredricks and Hinrichs, 2007) and a cultured representative of Marine Group I water column archaea (Schouten et al, 2008) but the structure is still unclear. It has been termed as "GDGT +14-diglycosyl" reflecting a core lipid 14 Da larger than the usual GDGT core and possibly including an additional CH_2 unit (Fredricks and Hinrichs, 2007), while other studies describe a mass difference in the polar head group, possibly derived from a modified hexose (Sturt *et al.*, 2004; Biddle *et al.*, 2006). Schouten *et al.* (2008) found evidence for a GDGT core structure with 3 rings and a polar head group consisting of one hexose and an unidentified additional group of 180 Da, however they also found an unidentified peak at m/z 1521 in acid hydrolyzed extract and suggest that this ion might represent the core GDGT. Our results suggest a difference in the polar head group of 18 Da which coincides with a shift towards later retention time from 26 min for the regular 2-Gly-GDGT to 27 min for "GDGT + m/z 342", indicating a head group that is more polar than two hexose units. A close structural similarity of "GDGT + m/z 342" and 2-Gly-GDGT is reflected by the similar behavior of adduct formation in negative ionization mode: 2-Gly-GDGT yields negatively charged adduct ions with formate of m/z 1668 and the unknown "GDGT + m/z 342" produces abundant ions with m/z 1686. The feature of additional 18 Da in the polar head group seems to be independent of adduct formation with NH_4^+ in positive ion formation or formate while analyzing in negative ionization mode. The detailed structural characterization of "GDGT + m/z 342" remains a challenge for the future. Preparative HPLC for purification and subsequent degradation reactions or direct structure elucidation by NMR techniques would be a potential route if enough material exists.

Our results suggest that the diversity of archaeal lipids in the deep subsurface is higher than previously thought. The IPL inventory most likely represents a mixture of lipids derived from cren- and euryarchaea. However, a direct link to the *in-situ* producing organisms cannot be established because cultures of the predominant sedimentary phylogenetic groups are not available.

Distribution of rings in core lipids of intact and free (fossil) GDGTs

The apparent dominance of intact archaeal lipids in subsurface marine sediments raises the question of how they influence the sedimentary pool of GDGTs without polar head group. GDGTs can contain up to eight rings in the core structure (DeRosa and Gambacorta, 1988) and the number of rings has been found to increase with growth temperature (DeRosa and Gambacorta, 1988; Gliozzi *et al.*, 1983; Uda *et al.*, 2001). This has led to the development of the TEX86 (TetraEther index of tetraethers consisting of 86 carbon atoms) proxy for reconstruction of past sea surface temperatures (SST) (Schouten *et al.*, 2002). The TEX86 is based on the distribution of rings in fossil GDGTs and is defined as:

$$TEX86 = \frac{GDGT(2)+GDGT(3)+GDGT(5')}{GDGT(1)+GDGT(2)+GDGT(3)+GDGT(5')} \quad \text{(IV.7)}$$

The correlation of TEX86 with SST was empirically found to be:

$$TEX86 = 0.015T + 0.28 \quad \text{(IV.8)}$$

The proxy is based on the premise that the lipids contributing to it are formed exclusively by planktonic crenarchaea in the water column and that they are transported efficiently to the sediments after cell death (Wuchter *et al.*, 2005; Wuchter *et al.*, 2006). However, sediments are not devoid of live crenarchaea which potentially produce similar lipids as their water column relatives. Phylogenetic relatives of water column MG I archaea have been found abundantly in deeply buried sediments of site 1230 (Inagaki *et al.*, 2006). They represent a specialized subclade that might have evolved independently from their water-column relatives (Sørensen *et al.*, 2004; Teske and Sørensen, 2008). Recently, it was proposed that planktonic crenarchaea might belong to a third archaeal phylum, the thaumarchaea (Brochier-Armanet *et al.*, 2008). It was suggested that this ancient archaeal lineage branches very basal in the tree of life and might even be more ancient than crenarchaea. Thus, it is possible that some of the traits of MG I water column archaea have been inherited to their sedimentary crenarchaeal descendents like the ubiquitous Marine Benthic Group B and the Miscellaneous Crenarchaeotic Group where only limited information regarding metabolism and lipid composition is available.

The impact of intact archaeal lipids produced *in-situ* by those dominant crenarchaea on the TEX86 proxy has been neglected and remains unclear. We analyzed the distribution of rings in the biphytane chains of intact GDGTs from eight samples of the Peru Margin and compared the observed pattern to the core lipids of non-intact (=fossil) GDGTs of the same samples. Four of the samples were from within the SMTZ, three samples were from near surface horizons of less than 1.5 mbsf depth and one sample was selected as reference between surface and the SMTZ (Table IV.4). Our results show the characteristic marine pattern for both the intact and the fossil lipids (Schouten *et al.*, 2000 and 2002; Damste *et al.*, 2002a; Wuchter *et al.*; 2005): abundant GDGT(0) and crenarchaeol GDGT(5) and minor amounts of GDGT(1), GDGT(2), GDGT(3), and crenarchaeol regioisomer GDGT(5') (see Figure IV.1 and Figure IV.4). Concentrations of GDGT(4) could not be determined due to co-elution with an isotopic species of crenarchaeol and the lack of pure standards for correction. However, concentrations of GDGT(4) in the intact form are likely very low as seen in HPLC-MS chromatograms. The density plots (Figure IV.2) show a gap in the series

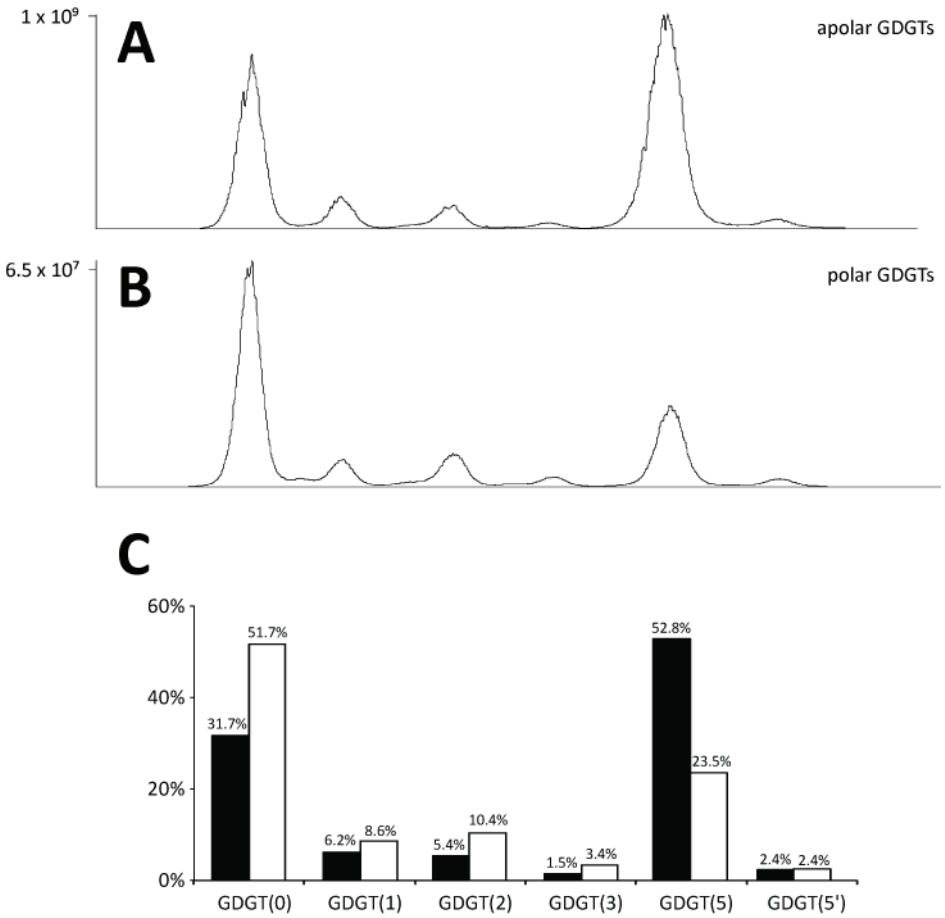

Figure IV.4: Ring distribution in GDGTs of sample 1229D-1.4. (**A**) Base peak chromatogram of the apolar fraction. (**B**) Base peak chromatogram showing ring distribution of the GDGT core lipids in the intact fraction. Note the different scale on the intensity axis. (**C**) Relative proportions of apolar (black bars) and polar (white bars) GDGTs after quantification of peak area in mass chromatograms.

of GDGTs where GDGT(4) would be seen. GDGT(5) has been unambiguously identified to be crenarchaeol in previous studies of the same sediments by ether cleavage and subsequent analysis by GC-MS (Biddle *et al.*, 2006).

Separation and analysis of the fossil GDGT showed crenarchaeol as dominating GDGT with 40-50%, followed by GDGT(0) with 30-40%. GDGTs with 1-3 rings and the crenarchaeol regioisomer were minor compounds. The average number of rings is relatively high in a range of 2.1-3.0. Two groups are similar: (a) SO147 station 2MC and ODP sites 1227 and 1229, and (b) ODP site 1230. Samples from the first group are underlying highly productive waters of the Peru upwelling system and are located in water depths of 86 m (2MC), 427 m (1227), and 152 m (1229) (Kudrass *et al.*, 2000; D'Hondt *et al.*, 2003). TOC contents are in the range of 3-7%. The calculated SST values span from 18-21°C and are similar to temperatures reconstructed from late quaternary sediments of the Nazca Ridge (ODP Leg 202, site 1237; Abe *et al.*, 2006; Prahl *et al.*, 2006; Wara and Ravelo, 2006) and present day values observed by satellite (Pathfinder Advanced Very High Resolution Radiometer; http://podaac.jpl.nasa.gov). The exception is sample 1229A-16.6, which has a lower TEX86 value and corresponding SST (16°C). This is due to a lower amount of monocyclic GDGT in this sample, which according to the definition of TEX86 has a strong effect on calculated SST. The two samples from site 1230 are distinct in a lower contribution of crenarchaeol and higher proportions of GDGT(1) and GDGT(2). This is reflected in lower values of average number of rings, TEX86 and corresponding SST of 15-17°C.

The analysis of the intact GDGTs revealed a different pattern: GDGT(0) is most abundant with 40-50% of total GDGTs, followed by GDGT(5) (crenarchaeol) with 16-30% and monocyclic and dicyclic GDGTs with average relative amounts of 10% each. GDGT(3) and the regioisomer of crenarchaeol are present in minor amounts of 2-6%. The average number of rings in the core lipids is in the range of 1.5-2.2. Comparison of biphytanes released after ether cleavage (Biddle *et al.*, 2006) and the pattern of biphytanes calculated from the observed core lipids assuming structures of Figure IV.1 shows good agreement (data not shown). Here, the eight samples can be grouped in four clusters with largely similar ring distribution: (1) the surface-near samples 2MC-0.33, 1227D-1.2, 1229D-1.4, and shallower SMTZ 1229D-29.7; (2) samples from the Peru Trench site 1230B-9.3 and 1230B-10.1; (3) sample 1229A-16.6; and (4) sample 1229D-87.1 from the deep SMTZ of site 1229. Group 1 has an average composition of 45-50% GDGT(0), 9-10% GDGT(1), 11-12% GDGT(2), 3-5% GDGT(3), 23-26% crenarchaeol, and 2-3% crenarchaeol regioisomer. The second group has a notably lower contribution of GDGTs with 1-3 rings and GDGT(5') at the expense of a

higher proportion of crenarchaeol. Sample 1229A-16.6 differs in lower relative concentration of GDGT(0) and higher amounts of GDGTs with 1-5 rings which is also reflected in the higher average number of rings. Sample 1229D-87.1 has a higher contribution from GDGTs with 1-3 rings and the crenarchaeol regioisomer and relatively low concentration of crenarchaeol.

The fact that core lipids of intact GDGTs are different than the fossil pool suggests a different origin and at least two distinct populations of archaeal lipids. The fossil GDGTs in the sediment represent a mixture of fossil GDGTs from water column archaea and decayed GDGTs from sedimentary archaea, whereas the intact GDGTs are solely derived from live archaea thriving in the sediment. This is supported by the presence of crenarchaeol with a diglycosidic head group in the fraction containing the intact lipids. Previously, crenarchaeol has been proposed as unique marker for planktonic archaea of Marine Group I in the water column (Damste *et al.*, 2002b) and is found abundantly in water samples and in the underlying sedimentary record. However, it has also been found in non-marine samples of hot-springs (Pearson *et al.*, 2004; Zhang *et al.*, 2006), which led to the isolation of a thermophilic archaeon from a hot spring in Yellowstone Park and direct evidence for crenarchaeol synthesis at elevated temperature (de la Torre *et al.*, 2008). The identification of intact crenarchaeol in marine sediments (Sturt *et al.*, 2004; Biddle *et al.*, 2006; Lipp *et al.*, in revision) has further broadened the range of sources. In marine sediments the crenarchaeol core lipid was always found in conjunction with a diglycosidic head group (2-Gly-GDGT), a combination which has not been identified in a cultured representative of non-thermophilic crenarchaea affiliated with MG I (Schouten *et al.*, 2008). It has been suggested that 2-Gly-GDGTs with crenarchaeol core are fossil remains of planktonic archaea (Schouten *et al.*, 2008). However, this scenario is unlikely as it would require selective degradation of the intact lipids with monoglycosidic or mixed sugar/phosphatidyl head groups which are predominantly found in the non-thermophilic archaea culture and are suspiciously lacking in marine sediments. The other possibility, fossil remains from uncultured water-column archaea producing 2-Gly-GDGT(5) warrants further exploration as more cultures become available in the future.

Degradation of intact GDGTs derived from archaea thriving in the sediment produces GDGTs without polar head group and contributes to the pool of fossil GDGTs. Any signal carried from the water column is therefore “diluted” and more and more influenced by the sedimentary signal with increasing burial depth. In order to assess the magnitude of this effect on the TEX86 temperature proxy we calculated SST from the ring distributions of

Table IV.4: Distribution of rings in GDGT core lipids determined directly for fossil GDGTs and after hydrolysis of the glycosidic head group for intact GDGTs. Relative proportions are average values of duplicate measurements and errors are calculated as maximum deviation from the mean. Values of TEX86 temperature proxy and corresponding sea surface temperatures (SST) are calculated using the equations in Schouten *et al.* (2002) with errors determined by error propagation. Relative proportions of intact and fossil GDGTs were determined as ratio of total GDGT peak areas of intact and fossil GDGTs injected from the same amount of solvent.

	2MC-0.33	1227D-1.2	1229D-1.4	1229A-16.6	1229D-29.7	1229D-87.1	1230B-9.3	1230B-10.1
				GDGT core (fossil)				
GDGT(0) (%)	37.2 ± 0.7	38.6 ± 2.0	31.7 ± 1.8	41.2 ± 0.4	34.5 ± 1.5	36.1 ± 0.6	40.3 ± 0.1	39.2 ± 0. 8
GDGT(1) (%)	5.7 ± 0.4	6.6 ± 0.5	6.2 ± 0.3	5.8 ± 0.04	7.4 ± 0.3	6.9 ± 0.5	8.5 ± 0.9	9.9 ± 1.6
GDGT(2) (%)	4.1 ± 0.2	5.1 ± 0.6	5.4 ± 0.3	3.7 ± 0.04	6.5 ± 0.2	6.0 ± 0.4	6.0 ± 0.4	7.8 ± 1.3
GDGT(3) (%)	1.2 ± 0.1	1.6 ± 0.2	1.5 ± 0.1	1.3 ± 0.02	1.9 ± 0.1	1.7 ± 0.1	0.8 ± 0.05	1.0 ± 0.2
GDGT(5) (%)	50.1 ± 1.5	46.2 ± 3.2	52.9 ± 2.2	46.8 ± 0.02	47.2 ± 0.7	47.2 ± 0.6	42.2 ± 1.3	39.8 ± 3.7
GDGT(5') (%)	1.7 ± 0.01	1.9 ± 0.1	2.4 ± 0.1	1.1 ± 0.5	2.6 ± 0.3	2.1 ± 0.1	2.1 ± 0.1	2.3 ± 0.2
average # of rings[a]	2.8 ± 0.1	2.6 ± 0.2	3.0 ± 0.1	2.6 ± 0.03	2.7 ± 0.1	2.7 ± 0.1	2.1 ± 0.1	2.4 ± 0.2
TEX86	0.55 ± 0.03	0.57 ± 0.05	0.60 ± 0.02	0.52 ± 0.02	0.60 ± 0.02	0.59 ± 0.03	0.51 ± 0.04	0.53 ± 0.08
SST (°C)	18.2 ± 2.1	19.1 ± 3.0	21.3 ± 1.5	15.7 ± 1.6	21.1 ± 1.4	20.3 ± 2.2	15.4 ± 2.8	16.6 ± 5.3
				GDGT core (intact)				
GDGT(0) (%)	45.0 ± 0.3	45.0 ± 0.1	51.7 ± 0.2	37.5 ± 0.3	49.7 ± 0.1	50.5 ± 0.1	45.8[b]	43.5[b]
GDGT(1) (%)	9.6 ± 0.3	9.8 ± 0.1	8.6 ± 0.03	9.8 ± 0.4	8.9 ± 0.001	10.1 ± 0.1	8.7[b]	10.2[b]
GDGT(2) (%)	11.6 ± 0.1	12.4 ± 0.1	10.4 ± 0.05	13.3 ± 0.06	10.3 ± 0.02	14.6 ± 0.03	9.6[b]	11.7[b]
GDGT(3) (%)	4.5 ± 0.02	4.7 ± 0.02	3.4 ± 0.02	5.8 ± 0.1	3.3 ± 0.03	5.4 ± 0.01	1.7[b]	2.7[b]
GDGT(5) (%)	26.4 ± 0.1	25.3 ± 0.01	23.5 ± 0.1	30.6 ± 0.9	25.6 ± 0.1	15.8 ± 0.02	31.9[b]	28.7[b]
GDGT(5') (%)	2.9 ± 0.02	2.7 ± 0.02	2.4 ± 0.1	3.0 ± 0.4	2.0 ± 0.03	3.5 ± 0.004	2.3[b]	3.2[b]
average # of rings[a]	1.9 ± 0.01	1.9 ± 0.01	1.7 ± 0.02	2.2 ± 0.07	1.8 ± 0.01	1.5 ± 0.01	2.0[b]	2.0[b]
TEX86	0.66 ± 0.01	0.67 ± 0.01	0.65 ± 0.01	0.69 ± 0.01	0.64 ± 0.01	0.70 ± 0.01	0.61[b]	0.63[b]
SST (°C)	25.6 ± 0.7	25.9 ± 0.7	24.8 ± 0.7	27.4 ± 0.9	23.8 ± 0.7	27.9 ± 0.7	22.0[b]	23.5[b]
Δ SST (°C)	7.3 ± 2.8	6.8 ± 3.7	3.5 ± 2.2	11.7 ± 2.5	2.7 ± 2.1	7.6 ± 2.9	6.6[b]	6.9[b]
intact/fossil (%)	16.2 ± 2.1	5.5 ± 0.1	4.9 ± 0.1	9.4 ± 0.3	7.3 ± 3.2	3.5 ± 1.6	2.0[b]	0.4[b]

[a] - calculated as weighted average; [b] – sample was analyzed only once (n=1)

intact GDGTs (Table IV.4). Temperatures are in a range of 22-27°C and the offset to the SST calculated for fossil GDGTs is between 3-12°C. As the TEX86 is strongly dependant on the

concentration of the monocyclic GDGT and the relative amount of this compound is higher for the intact lipid this shift towards higher calculated temperatures is easily explainable. The SST is relatively uniform for the shallower samples of stations 2MC, 1227, and 1229 in a range of 24-28°C and differs from site 1230 where lower temperatures of 22-24°C are calculated. Especially intriguing is the hypothesis of an adaptation of membrane composition to *in situ* temperatures of the much colder ODP site 1230. However, it is unlikely that the definition of the TEX86 ratio is relevant for sediment-based archaea as the involved species are different. Again, the differences between core lipid distributions and resulting TEX86 are strongly suggestive of different sources. The only way to explain this signal as fossil water column is under the assumption that the temperature/TEX86 relationship is entirely different. Our studies suggest that the constant flux of GDGT core lipids derived from decaying sedimentary archaea is not negligible and is likely to influence calculated SST. The intact GDGT pool has a size of up to 16% of the fossil components (Table IV.4). It is possible to roughly estimate the potential impact of intact GDGT decay on the fossil pool. Assuming an average pool size of the intact GDGTs of 5% of fossil GDGTs and a burial efficiency of 10%, every turnover of intact GDGTs will increase the pool of fossil GDGTs by the equivalent of 0.5%. When decay of fossil GDGTs is neglected, fossil GDGTs will accumulate with time and the imprint from sedimentary GDGTs on the fossil pool will increase with longer burial times. For example, 100 generations of intact GDGTs being turned with above mentioned relative proportions will increase the size of the fossil pool by 64% to a final composition of 60% water column and 40% sedimentary signal (data not shown). Similarly, 1000 generations (e.g. with a turnover time of 100 kyr and a water column signal from 100 Myr ago in the Cretaceous) will change the proportion to 99% sediment-derived. When degradation of fossil GDGTs is admitted, the signal from the water column will be wiped out even earlier. This shows the potentially large effect of sedimentary archaeal populations and their diverse intact GDGT inventory on paleotemperature reconstruction, and calls for further systematic studies.

Modeling IPL concentration and distribution in subsurface sediments

Considering the very high turnover times that have been estimated for all populations in the deep subsurface, it cannot be excluded that the extracted IPLs from the sediment represent a mixture of IPLs from live cells and sedimentary IPLs derived from dead cells which are not yet hydrolyzed. In a simple model the degradation of extractable IPL is described by first-order kinetics (e.g. Berner *et al.*, 1964; Jørgensen, 1979; Middelburg *et al.*, 1989) with an apparent half-life that corresponds to a combination of cell and IPL half-life. This

simplification is applicable because cell death is tightly coupled to sedimentary IPL input. We created a simple box-model to reconstruct extractable IPL concentration profiles for several possible scenarios. We note that, in principle, the models are also applicable to cells, DNA, RNA, and other cellular constituents.

Turnover of prokaryotic cells and lipids. Accumulation of IPLs to levels that exceed the cellular content of the extant population will only occur when the critical step of IPL degradation, i.e. hydrolysis of the glycosidic or phosphate-ester bond to the polar head group, proceeds more slowly than the mean community turnover time. As long as this criterion is fulfilled, the relative stability of archaeal vs. bacterial IPLs is irrelevant for their use as biomass tracers and IPL profiles will closely reflect live cell concentrations. Multiple lines of evidence support that archaeal glycolipids are similarly reactive as phospholipids and, more importantly, highly unlikely to persist on geological time scales.

Surface sediments from the anoxic Black Sea and the oxygen minimum zone (OMZ) off Peru are devoid of glycolipids typically produced by marine algae and cyanobacteria (cf. Lipp *et al.*, in revision). A similar lack of glycolipids derived from the photic zone was observed by Schubotz *et al.* (in preparation), who quantified concentrations of the lipids monoglycosyl-diacylglycerol (1-Gly-DG) and sulfoquinovosyldiacylglycerol (SQ-DG) in water column samples and the underlying sediments in the oligotrophic central Black Sea. They found that those lipids are abundant in the photic zone and are transported effectively through the water column so that they accumulate in a sediment-water suspension, termed as "fluff", directly above the sediment. However, they were not found in the top cm of the sedimentary record. Lipid components found in the ubiquitous planktonic Marine Group I archaea (Schouten *et al.*, 2008) were also undetectable in the study by Lipp *et al.* (in revision). Residence times of particles of fresh planktonic debris in the oxic water column were likely minimal in both studies, since it is well established that a major mechanism of export of fresh organic material into surface sediments is taking place via large, rapidly sinking particles (Fowler and Knauer, 1986), including fecal pellets. The impact of this mechanism on the export of labile organic compounds is supported by the presence of other labile compounds such as a complex series of carotenoid pigments in surface sediments from the Peruvian OMZ (e.g., Repeta and Gagosian, 1987). The observed lack of glycolipids in surface samples deposited under these conditions is consistent with these compounds being highly unstable even under anoxic conditions and unable to survive on geological timescales.

Fast turnover is also supported by laboratory incubation studies mimicking water column and sedimentary conditions. Harvey *et al.* (1986) used sensitive radiotracers to study

the relative stability of intact archaeal glycolipids and bacterial phospholipids in sediments and found slower relative degradation rates for glycolipids. However, overall degradation under anoxic conditions was very slow: after 80 days 80-90% of the glycolipid was still intact and only 5-15% of the bacterial phospholipid was respired. Unfortunately the experiment was not conducted long enough to calculate turnover times. Harvey *et al.* (1995) studied the water-column decomposition of phytoplankton in oxic and anoxic incubations in a flow-through system and determined turnover times of 44 to 160 days for diatom and cyanobacterial lipids. Sun *et al.* (2000) looked at the fate of algal lipids using stable isotope tracers and found first-order degradation constants of 0.01 to 0.2 day^{-1} which corresponds to a mean turnover time of 5-100 days. Finally, Emerson and Hedges (1988) found TOC turnover times of 4-256 days for the degradation of algae in the water column under various conditions.

Microorganisms in subsurface environments produce extracellular enzymes to digest organic substrates and make them available for cell uptake. Intact lipids from dead cells are exposed to these enzymes and their degradation is likely to be caused primarily by efficient extracellular hydrolases. Studies of organic carbon degradation in marine sediments with labeled polymeric carbohydrates as model substances (Arnosti, 2004; Arnosti and Jørgensen, 2006) demonstrated that polymeric carbohydrates are cycled on the order of days to weeks, and concluded that the enzymatic activity cleaving the glycosidic bonds is very high. When considering the relative stability of bacterial phospho-ester and archaeal glycosidic bonds and taking into account that both phosphate and sugar head groups are attractive substrates, any preservation on geologic time scales, selective or not, seems extremely unlikely. Also, without enzymes promoting the initial steps of organic matter degradation, microbial activity would stall. This is inconceivable given multiple lines of evidence such as cell counts, geochemical gradients, and the existence of deeply buried methane hydrate deposits consisting of substantial fractions of biologically produced methane.

Rapid turnover of diglycosidic archaeal IPLs, the major compound in the subsurface, was demonstrated recently by Thiel *et al.* (2007). They studied fresh microbial mats in anoxic waters of the Black Sea by ToF-SIMS and found that turnover of archaeal cell populations results in the presence of substantial amounts of apolar (non-intact) GDGT-based lipids. The ANME-1 archaea in these mats produce intact lipids dominated by diglycosidic GDGT (cf. Rossel *et al.*, in press), a lipid also found in many methanogens (e.g. Koga *et al.*, 1993) and structurally closely related to the major IPLs in subsurface sediments. ToF-SIMS analysis shows that apolar GDGT is spatially concentrated in small cell-like particles in some

areas of the mat while other areas contain largely intact diglycosidic GDGT, consistent with the presence of live cells. The detection pattern of the apolar GDGT lipids and the fact that they are still agglomerated in cell-like morphologies is strong evidence for hydrolysis of glycosidic bonds progressing on similar time scales as cell turnover.

Simple model of IPL decay without sedimentary input. In the first box-model we assumed that IPLs are only produced in surface-near sediments and no input occurs in the underlying sediments. This situation allows analyzing the effect of different degradation timescales on the modeled IPL concentration profile and comparison to the observed decline of IPL with depth (Lipp *et al.*, in revision). In our box-model the IPL concentration begins at 1860 ng mL^{-1} in the first step as predicted by the IPL regression line. The half-life is varied by several orders of magnitude representing reasonable estimates for turnover in the deep biosphere (Whitman *et al.*, 1998; D'Hondt *et al.*, 2003; Parkes *et al.*, 2000; Biddle *et al.*, 2006): 1 kyr, 10 kyr, and 100 kyr. Results of the model are shown in Figure IV.5A. The constructed IPL profile follows a different path compared to the regression line of log[IPL] vs. log(depth). In the first steps the modeled lipid concentrations stay high and are even higher than the regression line of the observed concentration; after several half-life/depth equivalents it drops below the observed concentration. The half-life influences the shape of the profile, however, the concentrations ultimately approach zero regardless of half-life. This is evidence for the necessity of continuous input of freshly synthesized IPL throughout the sediment column.

Simple model of IPL decay combined with sedimentary input. The second box-model involves continuous input of IPL in the sediment column. We modeled two reasonable situations: (a) fast IPL turnover with a half-life of 1 kyr and (b) slow turnover of IPLs using a half-life of 100 kyr (Figure IV.5B). The IPL production rates were selected by trial-and-error ensuring that the calculated IPL profiles are within the 95% prediction interval of log[IPL] vs. log(depth). Naturally, the fast turnover model requires a higher constant input flux of IPL (0.2 ng yr^{-1}) than the slower model (0.001 ng yr^{-1}). After a certain amount of time the input flux is balanced by an equal removal flux and the system reaches a steady-state. Overall compared to the no-production model, allowing input of fresh IPL shows better agreement with the observed IPL profile.

We used "reverse" modeling to match the modeled profile as close as possible to the observed lipid profile. In this approach the input and removal flux are the parameters that are calculated and half-life is held constant. The resulting rates are shown in Figure IV.5C.

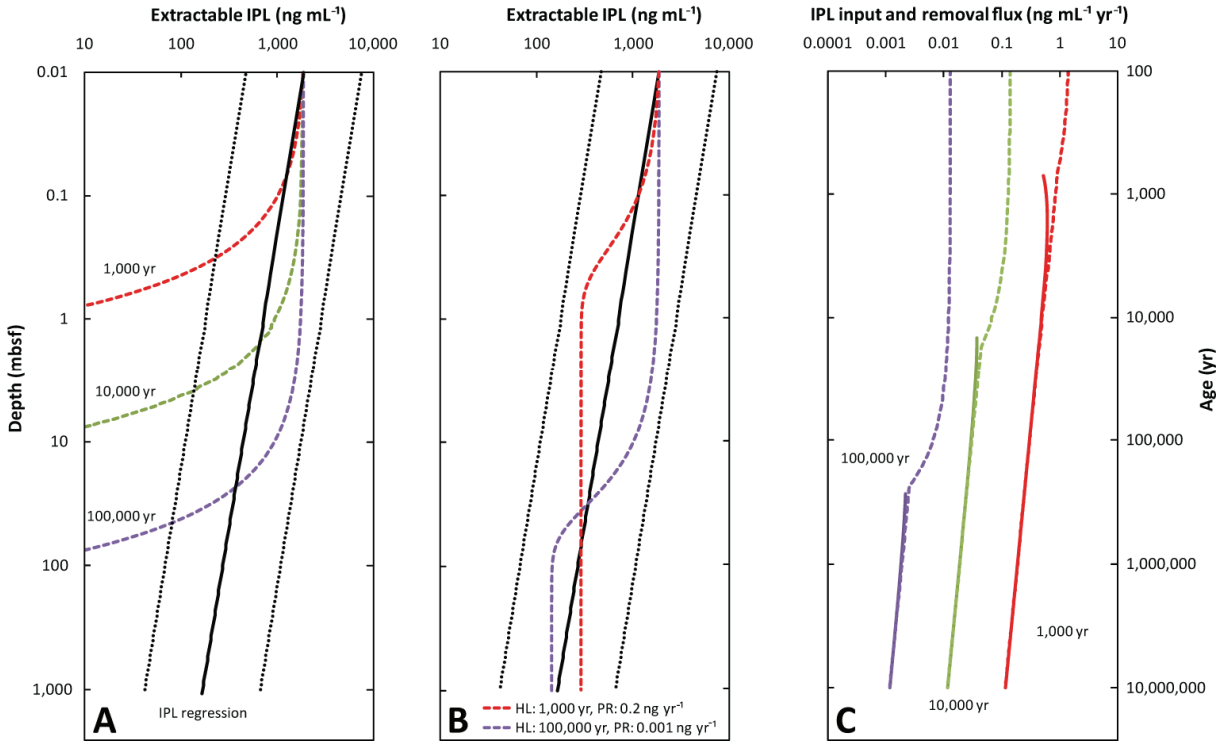

Figure IV.5: Box modeling results of extractable IPL concentration with depth. Note that the IPL concentration is the sum of lipids derived from live cells and free sedimentary species representing a fossil component. Sedimentation rate used in calculation was 10 cm kyr^{-1} (see Material and Methods for details of calculation). (**A**) Simple model of IPL decay over the period integrated by the sediment column with input of IPLs in surface sediments only (IPL is 100% sedimentary, no input from live cells). Shown is the modeled concentration applying first-order kinetics with varying values for half-life. Black solid line represents regression line and 95% prediction interval (dotted line) of log[IPL] vs. log(depth) of Lipp *et al.* (in revision) for comparison. (**B**) Model of IPL concentration allowing constant sedimentary input of IPL from dying cells for two scenarios with different activity: (a) "fast" system, half-life is 1 kyr with an IPL input flux of 0.2 ng IPL yr^{-1} and (b) "slow" system, half-life of 100 kyr with lower input flux of 0.001 ng IPL yr^{-1}. Input fluxes were selected so that the modeled profile is in the range of observed extractable IPL (black lines). (**C**) Reverse modeling of IPL input fluxes necessary to explain the observed IPL regression line in panel A for three different half-lives. Removal flux is calculated according to first-order kinetics and shown as dashed line. Input flux (solid line) is starting with zero and becomes positive with depth as the modeled IPL concentrations are lower than the regression line.

Several conclusions can be drawn: (a) input and removal flux decrease with depth with a similar slope as observed IPL concentrations; (b) in deeper sediment layers the flux of input and removal are almost balanced with the latter being slightly higher and reducing the extractable IPL reservoir with time; (c) the difference of IPL input and removal flux equals the first derivative of the modeled IPL concentration with time; and (d) the order of magnitude of the removal rate is determined by the used half-life.

IPL turnover times can be calculated by division of the reservoir size by IPL removal flux. The similar slope of reservoir size and removal flux decrease yields a constant turnover time in the deeper sediment layers. For IPL half-lives in the range of 1 to 100 kyr the model predicts turnover times τ ranging from 1.44 to 144 kyr. This is in line with values calculated according to the relationship between half-life and turnover time for exponential decay: $\tau = T_{1/2}/\ln(2)$. The modeled IPL input and removal fluxes are in the range of 0.001 to

0.1 ng mL^{-1} yr^{-1} for the deepest layers (Figure IV.5C); which can be converted to cell concentration fluxes of $7.1x10^2$ to $7.1x10^4$ cells mL^{-1} yr^{-1} (using a factor of 1.4 fg IPL $cell^{-1}$; Lipp *et al.*, in revision).. These values are similar to published growth rates obtained by [^{3}H]-thymidine incorporation measurements for deeper layer sediments from Blake Ridge (10^2 cells mL^{-1} day^{-1}) (Parkes *et al.*, 2000).

Cell production rates calculated from IPL flux for deeper sediment layers are assumed to be maximum values and real turnover rates might actually be lower because of simplifications in the modeling approach. The IPL removal process is assumed to follow first-order kinetics with a constant half-life and disregards the concentration and efficiency of the enzymes responsible for IPL degradation. However, the degradation process is likely to become slower as enzymatic activity ceases with depth and the organic carbon gets more recalcitrant. This is supported by the diagenetic model of Middelburg (1989) where the degradation constant of organic matter decay decreases increasing burial time. Such decreasing activity is compulsory to avoid exhaustion of the pool of total organic carbon. A typical marine sediment with a TOC content of 2% and density of 1 g mL^{-1} contains $2x10^7$ ng TOC. Assuming a growth increase of $7.1x10^4$ cells mL^{-1} yr^{-1}, a corresponding cellular carbon production of $1.3x10^6$ fg C mL^{-1} yr^{-1} (18 fg C $cell^{-1}$, Lipp *et al.*, in revision), and carbon assimilation efficiency of 1% this leads to 130 ng TOC mL^{-1} yr^{-1} that needs to be converted to sustain the observed cellular growth. The pool of organic carbon would thus be depleted within 160 kyr. When a lower production rate of $7.1x10^2$ cells mL^{-1} yr^{-1} is put through the same calculation, the TOC pool would last for 16 Myr.

The most likely scenario involves relatively fast turnover on the order of hundreds to thousands of years at the surface and increasingly slower turnover with increasing depth to values in the tens to hundreds of thousands of years. This resolves the apparent discrepancy of low IPL input rates in Figure IV.5C in the top of the sediment column and the observation of actively growing cells in the same horizons (Parkes *et al.*, 2000).

IV.4. SUMMARY AND CONCLUSIONS

IPL analysis is a useful tool for characterization of the *in-situ* microbial community in marine sediments. We explored the IPL inventory in sediments reaching from near-surface to deeply-buried. This study provides evidence for larger lipid diversity in deeply buried sediments than previously thought:

- Bacterial lipids were found in surface sediments from the Peru Margin. The distribution of polar head groups revealed a predominance of sulfate-reducing bacteria. Analysis of PLFA showed the presence of bacterial remains throughout the sediment column, however it was not entirely possible to separate between live and fossil biomass due to inclusion of fossil FA into polar macromolecules.
- Deeply buried sediments are dominated by archaeal IPLs of diether or tetraether type. The distribution of polar head groups was rather uniform in seven samples from sulfate-methane transition zones and distinct from on surface-near sediment sample, pointing to different microbial communities.
- We characterized four archaeal tetraether lipids by mass spectrometry and provide evidence for a novel core lipid with hydroxybiphytane.
- A detailed comparison of the ring distributions of polar and apolar GDGTs revealed distinct patterns, suggesting that the live sedimentary archaeal population is different from any fossil contribution that was deposited earlier.
- The effect of the sedimentary archaeal population on the TEX86 proxy for paleoreconstruction of sea surface temperature was evaluated and found to potentially have significant impact. Temperatures reconstructed from the core lipid distribution in intact lipids were systematically higher than from the fossil pool.
- Box-models were constructed to shed light on lipid stability and potential accumulation of fossil IPLs with increasing burial time. It became evident that constant supply of freshly synthesized archaeal IPL in the whole sediment column is mandatory to explain the observed IPL profile, regardless of IPL stability.
- The input flux was calculated for the scenarios of fast and slow IPL turnover and found to be in line with growth rates reported in the literature. The flux rates decrease with depth, possibly as a result of increasing recalcitrance of organic substrates and decreasing enzymatic degradation activity. Turnover times decrease with increasing depth from hundreds to thousands of years to tens to hundreds of thousands of years.

IV.5. ACKNOWLEDGEMENTS

Samples for this research were provided by the Integrated Ocean Drilling Program (IODP Expeditions 301 and 311), the Ocean Drilling Program (ODP Legs 201, 204, 207) (sponsored by the U.S. National Science Foundation and participating countries under the Joint Oceanographic Institutions, Inc.), cruises Sonne SO147, Karei KY04-11, and Professor Logachev TTR15. We thank the participating crews and scientists for sample recovery and data; U. Proske and N. Buchs for assistance with lipid extraction and sample preparation; D. Birgel for biomarker analytical expertise; H. F. Fredricks and F. Schubotz for discussion of data and useful comments on the manuscript; and V. Heuer and A. Schippers for sample donation. This work was supported by Deutsche Forschungsgemeinschaft (through MARUM Center for Marine Environmental Sciences and grant Hi616-4 from the priority program IODP/ODP). This is MARUM Publication No. ###.

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Chapter Va

Vertical distribution of microbial lipids and functional genes in chemically distinct layers of a highly polluted meromictic lake

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ABSTRACT

We examined vertical changes in microbial lipid composition and functional genes in the 4.5-m deep water column of a highly contaminated, meromictic lake in Eastern Massachusetts. Lipid-based techniques and compound-specific isotopic analysis indicated marked differences in community structure between the sulfate and CH_4-laden hypolimnion and the aerated epiliminon. The major intact polar lipids (IPLs) detected throughout the water column included diacyl-phosphatidylethanolamine, its methylated derivatives phosphatidyl-(N)-methylethanolamine and phosphatidyl-(N,N)-dimethylethanolamine, phosphatidyl-glycerol, diphosphatidylglycerol, phosphatidylcholine, and the glycolipids monoglycosyl-diacylglycerol and glycuronic acid-dialkylglycerol. These compounds were largely attributed to bacteria. The predominance of betaine lipids in the epilimnion was consistent with the dominant role of eukaryotic photoautotrophs in the oxic surface. Bacteriohopanepolyols and slight negative inflections of $\delta^{13}C$ values of bacterial phospholipid-bound fatty acids (PLFAs) at the metalimnion indicated a low contribution of methanotrophic bacteria to the pool of metalimnic bacteria. In the hypolimnion, dialkylglycerol-phosphatidylethanolamine, C_{17}-PLFAs and dsrAB gene sequence data suggested the presence of sulfate reducing bacteria (SRB). Five distinct groups of sulfate reducers and methanogens were detected in the hypolimnion. While SRB appeared to inhabit the hypolimnion, the methanogens most likely entered the lake through the inflow of CH_4-laden groundwater. We did not detect both methyl coenzyme M reductase genes and characteristic lipids related to known anaerobic CH_4-oxidizing Archaea although the hypolimnion exhibited conditions conducive for anaerobic methanotrophy. CH_4 was expected to represent an important carbon source. However, the large amount of dissolved organic carbon appeared to be the major pool of carbon for the lake's microbial biomass.

Chapter Vb

Microbial community composition and potential activity in hydrate-bearing deep subsurface sediments of Hydrate Ridge (Cascadia Margin, ODP Leg 204)

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ABSTRACT

One of the current challenges in microbial biogeochemistry is to understand the energy sources fueling life in the deep biosphere. The process of anaerobic oxidation of methane (AOM) has been inferred in deep sediment samples from geochemical profiles; however, the responsible microorganisms have not been identified. In near-surface sediments from the summit of southern Hydrate Ridge (ODP Leg 204), highly ^{13}C-depleted archaeal lipids indicated the incorporation of methane-derived carbon into microbial biomass and an important role of anaerobic methane oxidizing archaea (ANME). This signal was not found in sediment layers below 1 m. Instead, crenarchaeotal lipids and 16S rDNA sequences dominated a geochemically well-defined sulfate methane transition zone (SMTZ); however, isotopic evidence suggests that these microbes did not incorporate methane-derived carbon into their biomass. ANME-1 archaea were detected in deeply buried sediments within the hydrate stability zone (54 m depth) together with low potential rates of AOM. In these sediments, ANME-1 sequences dominated the archaeal 16S rRNA gene library and co-occurred with 16S rRNA gene sequences related to sulfate-reducing bacteria commonly found at cold seeps (Seep-SRB2 branch). Our results indicate that anaerobic methanotrophs may be present in deep biosphere sediments, but their contribution to energy flow and carbon assimilation of microbial subsurface communities remains unconstrained.

Chapter Vc

Intact polar lipids of anaerobic methanotrophic archaea and associated bacteria

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In press in *Organic Geochemistry*
doi:10.1016/j.orggeochem.2008.02.021

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ABSTRACT

Previous biomarker studies of microbes involved in anaerobic oxidation of methane (AOM) have targeted non-polar lipids. We have extended the biomarker approach to include intact polar lipids (IPLs) and show here that the major community types involved in AOM at marine methane seeps can be clearly distinguished by these compounds. The lipid profile of methanotrophic communities with dominant ANME-1 archaea mainly comprises diglycosidic GDGT derivatives. IPL distributions of microbial communities dominated by ANME-2 or ANME-3 archaea are consistent with their phylogenetic affiliation with the euryarchaeal order *Methanosarcinales*, i.e., the lipids are dominated by phosphate-based polar derivatives of archaeol and hydroxyarchaeol. IPLs of associated bacteria strongly differed among the three community types analyzed here; these differences testify to the diversity of bacteria in AOM environments. Generally, the bacterial members of methanotrophic communities are dominated by phosphatidylethanolamine and phosphatidyl-(N,N)-dimethylethanolamine species; polar dialkylglycerolether are dominant in the ANME-1 community while in ANME-2 and ANME-3 communities mixed acyl/ether glycerol derivatives are most abundant. The relative concentration of bacterial lipids associated with ANME-1 dominated communities appears significantly lower than in ANME-2 and ANME-3 dominated communities. Our results demonstrate that IPL analysis provides valuable molecular fingerprints of biomass composition in natural microbial communities and enables taxonomic differentiation at the rank of families to orders.

Chapter Vd

Methane-Producing Microbial Community in a Coal Bed of the Illinois Basin

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Published in *Applied and Environmental Microbiology*, vol. 74, no. 8, page 2424-2432, doi:10.1128/AEM.02341-07

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ABSTRACT

A series of molecular and geochemical studies were performed to study microbial, coal bed methane formation in the eastern Illinois Basin. Results suggest that organic matter is biodegraded to simple molecules, such as H_2 and CO_2, which fuel methanogenesis and the generation of large coal bed methane reserves. Small-subunit rRNA analysis of both the *in-situ* microbial community and highly purified, methanogenic enrichments indicated that *Methanocorpusculum* is the dominant genus. Additionally, we characterized this methanogenic microorganism using scanning electron microscopy and distribution of intact polar cell membrane lipids. Phylogenetic studies of coal water samples helped us develop a model of methanogenic biodegradation of macromolecular coal and coal-derived oil by a complex microbial community. Based on enrichments, phylogenetic analyses, and calculated free energies at in situ subsurface conditions for relevant metabolisms (H_2-utilizing methanogenesis, acetoclastic methanogenesis, and homoacetogenesis), H_2-utilizing methanogenesis appears to be the dominant terminal process of biodegradation of coal organic matter at this location.

Chapter VI

Concluding Remarks and Perspectives

VI.1. CONCLUSIONS

This thesis contributed to the deeper understanding of biogeochemical processes occurring in the deep marine subseafloor through the application of intact polar lipids (IPL) as markers for active microbial cells. In the course of my work I was able to address three important open questions in deep biosphere research pertaining to (*i*) broad taxonomic affiliation of subsurface microbes, (*ii)* microbial population density of the active sedimentary community, and (*iii*) their carbon metabolism.

IPLs analyzed in a globally distributed sample-set revealed a dominance of archaeal lipids in samples deeper than 10 cmbsf. The major archaeal IPLs were diglycosidic derivatives of archaeol and glyceroldialkylglyceroltetraethers (GDGT) lipids with concentrations ranging from 12 to 6600 ng mL^{-1}. Bacterial lipids were only detected in surface sediments and were mixed with IPLs from eukarya, most likely stemming from the water column. The major bacterial IPLs identified comprised phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and phosphatidylcholine (PC) diacylglycerides with C16 and C18 acyl groups in concentrations spanning from 470 to 15800 ng mL^{-1}. The transition between bacterial IPLs in the surface and archaeal IPLs in the deep sediment layers occurred in the top 10 cm of the sediment.

This observation was surprising at first, considering that 16S-gene-based microbiological methods pointed to minor archaeal contributions of only a few percent of total cells (Schippers *et al.*, 2005; Inagaki *et al.*, 2006). However, even among the microbiologists there is no consensus; some of them suggested that archaea make up a significant fraction (Mauclaire *et al.*, 2004) and results from both fluorescent *in-situ* hybridization (FISH) and 16S rRNA-extraction support this conclusion (Biddle *et al.*, 2006; see chapter II in this thesis). Although we did not find any sign of bacterial IPLs in deeply buried samples, it does not mean bacteria are not present. The level of detection (LOD) was variable between 3 and 600 ng IPL g^{-1} sediment, corresponding to cell concentrations of 1.8×10^6 to 4.3×10^8 cells mL^{-1} if we assume typical deep-biosphere cells with a diameter of 500 nm. The variability of LOD was most strongly dependent on matrix effects leading to ion suppression, which reduced the LOD relative to a solution of pure compounds by a factor of 2-10. The percentage of archaeal lipids of total lipids was therefore calculated conservatively with the LOD as bacterial proportion in a way likely overestimating the importance of bacteria. Application of the more sensitive analysis of polar lipid-derived fatty acids (PLFA) supported the conclusion of low bacterial abundance. In order to also capture the full

diversity of minor compounds, more sensitive measurements of IPLs will be required, possibly with extremely sensitive techniques like NanoSpray ionization.

The observation of significant proportions of archaea is further supported by newly developed microbiological methods: improved protocols of DNA extraction and purification and development of less biased microbiological quantification techniques based on slot-blot hybridization (SBH) and quantitative-polymerase chain reaction (Q-PCR) indicated that previous analyses had largely underestimated the archaeal population (Lipp *et al.*, in revision; see chapter III in this thesis). Furthermore, the dominance of archaea in the deep biosphere is consistent with a recent study by Valentine (2007) who argued that archaea are better adapted to extreme low-energy conditions due to their lower membrane permeability and special catabolic pathways. In energy- and nutrient-limited conditions such as the ones prevailing in the deep biosphere, this archaeal advantage could lead to longer life-cycles, longer community-turnover times, and a larger standing stock of archaeal biomass compared to bacteria.

The structural diversity of archaeal IPLs in deeply buried sediments is low and could potentially be explained by three factors: (1) a low phylogenetic diversity, (2) a special adaptation mechanism of the archaeal population to subsurface conditions, which is reflected uniform lipid composition, or (3) archaeal lipids are selectively preserved and the detected IPLs represent the most stable lipid classes.

Lipid distribution patterns could not directly be linked to the phylogenetic structure based on 16S rRNA clone libraries. This can be explained with different selectivity of the two techniques: while gene-based surveys of microbial diversity may also detect organisms that are present at very low abundance, the lipid-based technique will essentially detect the signals of those prokaryotes that contribute most substantially to the bulk microbial biomass. In that respect, IPL diversity does not reflect information on total diversity but it yields information on the structure of a microbial community which is not accessible by other techniques currently applied to study subsurface environments. There were some differences in relative abundance of archaeal lipids but none of them pointed to a specific distribution pattern for the major archaeal phyla found in the clone libraries. This contrasts results obtained in other settings where lipid distributions are more diverse and can be tracked to the phylogenetic affiliation of major community members on the level of families to orders (e.g. Ertefai *et al.*, in revision; Rossel *et al.*, in press,; see chapters Va and Vc in this thesis). The reason for this observation might be that the chemotaxonomic potential of IPLs cannot be explored in environments where microbial communities have to cope with nutrient and energy

limited conditions like the ones in the deep biosphere. The membrane composition of all major phylogenetic groups possibly represents the optimal adaptation minimizing energy-loss. This could potentially also explain the lack of phosphate-based archaeal lipids and could be evaluated with incubation studies to test if starving microbial communities in fact produce similar IPLs regardless of phylogenetic relationship.

In light of the slow pace of life and the very long community turnover times in the range of thousands to hundreds of thousands of years which were estimated for the deep subsurface, it cannot be excluded that the extracted IPLs from the sediment represent a mixture of IPLs from live cells and sedimentary IPLs derived from dead cells which are not yet degraded. The rate of IPL degradation under natural conditions in subsurface environments will ultimately affect the value of IPLs as markers for the active microbial population. Accumulation of IPLs to levels exceeding the cellular content of the living stock of biomass can only occur when the critical step of degradation proceeds more slowly than the mean community turnover time. We created simple box-models to constrain IPL turnover times from the observed declining concentration profile and tested two plausible scenarios. The first model assumed that IPLs are only produced in surface-near sediments and no input occurs in the underlying sediments, thus representing a truly fossil signal. The second box-model allowed continuous input of freshly synthesized IPL to the sediment column. A key result of these models is that continuous input of IPL throughout the sediment column is required in order to account for the observed concentration profiles. The most likely scenario involves fast turnover at the surface and decreasing rates with depth. This is supported by ceasing enzymatic activity with depth as observed from decreasing degradation rates of organic matter degradation (Middelburg *et al.*, 1989). Still, the calculated IPL degradation rates are in a similar range or faster as community turnover times and would therefore allow the use of IPLs as markers targeting only the active prokaryotic population.

The apparent dominance of archaeal IPLs in deeper horizons also raised the question of selective preservation of archaeal lipids. Phospholipids have been directly validated as measure for live biomass (White *et al.*, 1979), but similar evidence is still lacking for archaeal glycolipids. In addition to the conclusions gained from the box-models above which also include the observed archaeal lipids, there is ample experimental evidence supporting fast archaeal IPL turnover: (*i*) the distribution of archaeal core lipids of the live sedimentary archaeal population is clearly distinct from the fossil contribution of archaea which represents at least partially planktonic species, and secondly (*ii*) there is no evidence that glycosidic lipids like the ones found in archaea preferentially accumulate in sediments, thus indicating

rapid degradation even under anoxic conditions in the Black Sea and oxygen minimum zones off Peru. Future incubation studies under oxic and anoxic as well as biological and abiological conditions could shed more light on the relative timescales of archaeal and bacterial lipid degradation.

The production of tetraether-type archaeal lipids by the sedimentary microbial population and the fate of those lipids after cell death could have a profound impact on the paleotemperature proxy TEX86. The TEX86 is based on the ring distribution in GDGT lipids of planktonic archaea that are thought to be preserved in the sediment and reflects ancient sea surface temperatures. This proxy has been widely applied to reconstruct past sea surface temperatures, however, initial results point to consistently higher temperatures due to inclusion of sediment-derived GDGT core lipids in the calculations.

In our global data set we observed a decline in IPL concentrations with depth, following a log[IPL]-log(depth) relationship similar to the one observed for directly counted cells (Parkes *et al.*, 1994). IPL concentrations in sediments can be used as proxy for live microbial biomass in the same fashion as estimates which are based on direct counting of cells. However, a simple integration of concentration profiles over depth relies on a sample-set representative for all oceanographic regimes, including low productivity open ocean sites. Unfortunately so far most drilling expeditions have mainly obtained samples from continental margin areas and none of the samples in our set can be truly considered “open ocean”. Therefore we followed a different approach circumventing potential sample bias and used an observed relationship between IPL and total organic carbon (TOC) concentrations. Increased TOC concentrations were positively correlated to increased concentrations of lipids, reflecting the inherent heterotrophic nature of the subsurface biosphere ecosystem. TOC concentrations in marine subsurface sediments were modeled using a combination of regional distributions of TOC in surface sediments (Seiter *et al.*, 2004) and a diagenetic model of TOC decay (Middelburg *et al.*, 1993). An average TOC content of 0.13% was estimated for habitable sediments, resulting in a quantity of 3.7×10^5 Pg TOC and 7.1 Pg of IPL when converted via the TOC-IPL relationship. Using published values for cellular carbon content we derived a total amount of carbon in prokaryotic cells of 90 Pg with inherent uncertainties of roughly one order of magnitude – comparable to previous estimates (Parkes *et al.*, 1994; Whitman *et al.*, 1998). In contrast to the previous results this estimate is based on a direct chemical method and is independent of microscopic cell counting.

Another goal of this thesis was to shed light on the carbon metabolism of the microbial community. The majority of processes in the deep subsurface rely on the degradation of

organic carbon as energy yielding process and also as carbon source. However, it was unknown how the important process of anaerobic oxidation of methane (AOM) is mediated in deeply buried diffusive settings. Most of our knowledge on AOM is based on analysis of surface sediments in seep areas where high fluxes of methane and high concentrations of sulfate lead to highly active syntrophic microbial consortia of methane-oxidizing archaea and sulfate-reducing bacteria (cf. Hinrichs and Boetius, 2002). In order to clarify if AOM is mediated in a similar way in the sulfate-methane transition zones (SMTZ) of deeply buried sediments, we applied an interdisciplinary approach. $\delta^{13}C$ values of archaeal biomass were analyzed by two independent techniques which only select for the active population: FISH coupled to secondary ion mass spectrometry (FISH-SIMS) and compound-specific analysis of IPLs. A comparison of the isotopic composition of biomass and sedimentary carbon pools revealed that (*i*) only a small fraction of the archaeal community relies on methane as carbon source, (*ii*) a major contribution of autotrophic methanogenesis via CO_2 reduction can be excluded, (*iii*) an important role of methylotrophic methanogens is unlikely, and (*iv*) instead the isotopic similarity of total organic carbon (TOC) and archaeal biomass suggests that the bulk of the archaeal community assimilates organic compounds derived from fossil organic matter.

A possible explanation for the lack of an isotopic signature of depleted biogenic methane in archaeal biomass would be that the methane flux into the SMTZ is only small in comparison to organic carbon degradation. Methane fluxes derived from porewater profiles and organic carbon degradation rates calculated according to a diagenetic model (Middelburg, 1989) were largely similar and suggested that - assuming similar carbon assimilation efficiencies for methane oxidation and TOC degradation - both processes should be reflected equally in the isotopic composition of archaeal biomass. In this light, the lack of isotopic depletion in archaeal biomass suggests that the archaeal community dominating the SMTZ can utilize the extra energy derived from AOM but does not assimilate its carbon. Such dissimilatory processes are known from other archaea, e.g. some CO_2-reducing methanogens use auxiliary carbon substrates and complex organic substrates for synthesis of cell material and growth, while CO_2 is only converted to methane but not incorporated into the cell. A possible explanation is that use of C1-compounds for synthesis of biomass is more energy-intensive than the use of C2-compounds like acetate which is available from degradation of organic matter. The carbon metabolism can be further explored by incubation experiments of sediment samples with different isotopically labeled substrates.

Estimated population turnover times based on TOC degradation rate and the observed population ranged from 70 to 2150 years indicating the slow pace of microbial life in the deep subsurface and confirming previous calculations. Gibbs free-energy changes were calculated from turnover rates of both methane and sedimentary organic carbon and compared with calculated energies necessary to maintain the observed microbial populations. The results show that energy derived from sedimentary processes can only account for a small fraction of the observed communities when traditional maintenance energy values are applied. Real maintenance energies in the deep biosphere must be much lower than values determined in laboratory cultures. Such low energies would be in-line with the results from Price and Sowers (2004), who estimated metabolic rates of cells in survival mode, where only damage to essential macromolecules is repaired but no other functions are performed, to be a factor of 10^6 smaller than for actively growing cells.

The results obtained in the course of this thesis suggest a vast marine subsurface sedimentary ecosystem, in which archaea contribute a major fraction to the standing stock of biomass. The dominant phylogenetic groups were affiliated to the Marine Benthic Group B (MBG-B), Miscellaneous Crenarchaeotic Group (MCG), and South African Goldmine Euryarchaeotal Group (SAGMEG). Carbon flow reconstructions in geochemical transition zones, such as the sulfate methane transition zone indicate that these archaea assimilate sedimentary organic compounds instead of methane. Heterotrophic carbon uptake is further supported by the observed [TOC] - [IPL] correlation of a globally spanning sample set. Geochemical modeling could show that microbial turnover is extraordinarily slow with community turnover times in the order of thousands to hundreds of thousands of years, contesting our definition of life.

VI.2. PERSPECTIVES: INTACT POLAR LIPIDS IN SUBSURFACE BIOSPHERE RESEARCH

This thesis contributed significantly on constraining microbial structures and processes in deeply buried sediments; nevertheless, we are still at the beginning to fully comprehend the complexity of this ecosystem. On the one hand this is due to the difficult accessibility and on the other hand due to the analytical challenges and inherent extraordinarily slow pace of life. Several potential applications of IPL analysis and incubation studies with isotopically labeled substrates which can strongly contribute to this research field are listed below:

(a) Degradation studies with archaeal and bacterial IPLs under simulated sedimentary conditions for validation of IPLs as unbiased marker for the active population

(b) Analysis of more samples, possibly covering open ocean sediments, to obtain a more representative sample-set and verify the log[TOC]-log[IPL] relationship used for the biomass estimate

(c) ^{13}C labeling experiments with heterotrophic substrates to prove the heterotrophic character of archaea in the deep biosphere

(d) Structure elucidation of archaeal tetraether lipids, especially "GDGT + m/z 342" which was found in marine sediments as well as a culture of planktonic MG I-crenarchaea

(e) Culturing experiments under energy- and nutrient-starved conditions to simulate conditions prevailing in the deep biosphere and unravel potential membrane adaptation processes

Degradation studies of archaeal and bacterial IPLs. PLFA analysis has been validated as measure for live bacterial biomass by White *et al.* (1979) due to the rapid degradation of intact phospholipids after cell death. Conceptually, archaeal lipids degrade in a similar manner by cleavage of the glycosidic bond to the polar head group. Knowledge of the timescale on which IPL degradation proceeds is compulsory for their application as marker for the active prokaryotic population. If several species of lipids are involved, they should ideally be degraded with similar reaction rates in order to obtain unbiased results. Harvey *et al.* (1986) used sensitive radiotracers to study the relative stability of intact archaeal glycolipids and bacterial phospholipids in sediments under varying environmental conditions. However, since degradation progressed slowly under anoxic conditions and the experiment was only conducted for 80 days, no turnover times could be calculated and it remained unresolved if archaeal and bacterial lipids degrade similarly fast. Despite indirect evidence

for turnover proceeding more rapidly than geological timescales, it is of utmost importance to estimate the order of magnitude. I therefore suggest to conduct an experiment comparing archaeal and bacterial IPL degradation under biological and abiological conditions for a time span long enough to allow calculation of turnover times and gain at least a rough estimate on whether degradation happens within months, years or centuries. Such an experiment was started one year ago and initial results indicate that abiological degradation of archaeal IPLs proceeds on timescales of years (Rossel *et al.*, unpublished results).

Analysis of open ocean sediment samples. The sample-sets analyzed in the studies of Parkes *et al.* (1994 and 2000) and in chapter III of this thesis are all somewhat skewed towards continental margins. In the course of DSDP, ODP, and IODP drilling, the majority of sites were located at continental margins. However, according to the study by Seiter *et al.* (2004) and the results presented in chapter III of this thesis, more than 90% of the marine area can be considered as "open ocean". It is unclear, if conclusions regarding taxonomic affiliation, quantitative extent and links to geochemical parameters obtained in continental margin settings hold true in open ocean sediments. For example, it is not certain if the log[IPL]-log[TOC] relationship described in chapter III which is used to estimate biomass on a global scale truly applies to open-ocean sediments. While some samples of this oceanographic environment type were obtained during ODP Leg 201 (e.g. site 1225), the level of detection (LOD) of the mass spectrometer used in our laboratory prevents detection of IPLs due to very low concentrations. Specialized analytical techniques such as NanoSpray ionization (NSI) coupled to more advanced, sensitive mass spectrometers allow the analysis of trace IPL amounts equivalent to 1000 cells (Brügger *et al.*, 1997). I therefore propose to apply this technique to sediments already available in our laboratory and to follow-up expeditions that will be undertaken in the next years. NSI analysis can also be applied on sediment extracts that were already analyzed in the study presented in chapter III and showed abundant archaeal lipid concentrations but lacked bacterial lipids due to sensitivity issues. The estimate of 87% archaeal lipids of total lipids could therefore be confirmed or revised to higher values. This would provide a significant step towards estimates of the importance of bacteria in deeply buried sediments.

Stable carbon isotope labeling experiments. The study presented in chapter II provided evidence that the methane cycling in deeply buried sulfate-methane transition zones (SMTZ) is mediated differently than in surface-near seep environments. It was shown, that archaea

belonging to the ubiquitous major groups Marine Benthic Group B (MBG-B) and Miscellaneous Crenarchaeotic Group (MCG) are heterotrophic and take up carbon derived from sedimentary organic carbon. In addition, it was suggested that they can utilize energy gained from methane-oxidation in the SMTZ but do not incorporate methane-derived carbon into their biomass. I propose to perform incubation studies with stable carbon-labeled substrates to shed more light on the carbon metabolism of those major archaeal groups and to provide direct evidence for heterotrophic carbon uptake. Such an incubation experiment was started 9 months ago using sediment from the SMTZ of samples obtained from Hydrate Ridge during ODP Leg 204. To test different carbon uptake pathways, the ^{13}C-labeled substrates acetate (acetoclastic processes), bicarbonate (autotrophy), methane (methanotrophy), and biomass from cyanobacteria (heterotrophy) were added. Initial results show carbon assimilation into crenarchaeal biomass solely via heterotrophy, although turnover rates were very low (Lin *et al.*, unpublished results).

Structure elucidation and determination of the source of novel archaeal lipids. The diversity of archaeal lipids found in deeply buried marine sediments was found to be high (see chapter IV). Several lipid classes were identified but the link to the producing organism has not been established unequivocally. In order to distinguish more specifically between archaeal groups like MBG-B or MCG it is necessary to connect certain archaeal lipids more closely to those major taxa. Two archaeal tetraether lipids were characterized mass spectrometrically in the study presented in chapter IV: one was previously unknown and the other was often found in extracts from marine sediments and in a cultured representative of planktonic crenarchaea of the Marine Group I. It has been speculated, that the presence of the latter compound in deeply buried sediments is due to fossil preservation of remains of planktonic archaea (Schouten *et al.*, 2008). However, this is unlikely since other components found in the culture are not found in the sediments (see chapter IV). I propose to study the distribution of those uncharacterized tetraether lipids in a variety of sediments in concert with studies of phylogenetic affiliation to major archaeal groups. This would unravel the linkage between lipid types and the producing organisms.

Culturing experiments under limiting conditions. Most of our knowledge about membrane composition of environmentally relevant microorganisms comes from laboratory cultures. However, the growth conditions have profound impact on the lipid-structures that are produced, e.g. more unsaturated fatty acids at lower growth temperatures in bacteria or more

rings in the tetraether core lipids of archaea at higher temperature. It was surprising to see that deep biosphere-archaea produce solely sugar-based lipids. Production of glyco- instead of phospholipids is a strategy that microorganisms use if they become limited in phosphorus (Van Mooy *et al.*, 2006). However, this is very unlikely in the deep biosphere where concentrations of phosphate reach up to several hundred micromolar. Instead, production of glycolipids could be a strategy to survive conditions of limiting energy and nutrients. I therefore propose to conduct culturing experiments under energy- and nutrient-starving conditions and monitor the effect on the membrane composition to get more realistic molecular fingerprints.

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Acknowledgements

I would like to thank Kai-Uwe Hinrichs for accepting me as his first PhD-student and introducing me to the exciting world of IPLs and the deep biosphere. I especially appreciated the trust, freedom and continuous support through the years. I also would like to thank Jörn Peckmann for agreeing to be second supervisor on short notice. Many thanks also to Wolfgang Bach and Stuart Wakeham for joining my PhD committee.

There are many people that supported me during my studies, I would like to start by thanking Laura and Daniel for the fun year in the office back in the days when we were still confined to the TAB building. Thanks to Laura also for persistently correcting my English. I am indebted to Helen Fredricks for teaching me everything about LCMS and keeping up a good mood especially when something is wrong with the instrument….again.

I enjoyed very much collaborating with all the guests that came to our lab over the years: Rika Anderson, Kara Bogus, Mea Cook, Helen Fredricks, Roberta Hansman, Laura Hmelo, Fumio Inagaki, CarriAyne Jones, Nele Meckler, Yuki Morono, Marianne Nuzzo, Thomas Oldenburg, Beth! Orcutt, John Pohlman, Tracy Quan, James Saenz, Dariusz Strapoc, Roger Summons, Sean Sylva, Courtney Turich, Stuart Wakeham and Gunter Wegener.

I greatly valued the input and discussion on manuscripts from John Hayes, Axel Schippers and Andreas Teske. With fond memories I look back at my stay in Chapel Hill, where Mark Lever, Karen Lloyd and Ketil Sørensen took good care of me (Bingo!!). Although never having met her until two years after publication, I greatly enjoyed working on the PNAS paper together with Jennifer Biddle.

Thanks to Fumio Inagaki who over Mensa lunch invited me to the Natsushima cruise, it was an unforgettable experience. Ko-ichi Nakamura is also thanked for excellent post-cruise trip organization, I had a lot of fun!

A lot of the work done in our lab would not have been possible without the help of Wolfgang Bevern. Without him I would never have dared to completely disassemble the mass spectrometer.

My deepest thanks also go to my HiWi-assistants: Nadine Buchs, Ulrike Proske and Felix Elling. Thank you for enduring hundreds of boring Bligh and Dyer extractions.

During my thesis I co-supervised several thesis projects and I also would like to thank the students: Corinna Harms, Henning Meyer, Matthias Kellermann, and Florence Schubotz.

I was lucky to have done my PhD work in such a great working group. Thanks for providing everyday support and always a nice chat during a cup of coffee (or a bucket). Special thanks to Verena and Solveig for entertaining me in the office. Marcus and Xavi always kept the lab running and Birgit organized the administrative stuff.

I would like to thank Verena, Solveig, Marcus, Tobi E., Matthias, Yu-Shih, Arne, Xiaolei, Xavi, Pamela, Frauke, Birgit, Florence, Julio, Marcos, Daniel, Henning, Boonraksa, Steffi L., Steffi T., Monika, Esther, Tobi H., Benni, Lars, Simone, Jan, Marlene, Sebastian, Nadine, Florian, Kai and Jörn for creating a wonderful working atmosphere.

I am very glad that I was here at the right time in the right place to supervise Florence's master thesis and later "intensify" our relationship. Thank you for your love and support!

My deepest thanks go to my family, especially Masi, Mechthild and Anna. Thank you for always being there for me!

Oral presentations

September 2007	International Meeting on Organic Geochemistry (IMOG), Torquay, England "The Deep Biosphere: quantitative and taxonomic constraints through microbial lipids"
August 2007	Goldschmidt Conference 2007, Cologne, Germany „The Deep Biosphere: quantitative and taxonomic constraints through microbial lipids"
June 2007	4th Northern Germany Organic Geochemistry Meeting, Bremen, Germany „The Deep Biosphere: quantitative and taxonomic constraints through microbial lipids"
March 2007	IODP-ICDP Kolloquium 2007, Potsdam, Germany „The Deep Biosphere: quantitative and taxonomic constraints through microbial lipids"
January 2006	Seminar series "Aktuelle Forschungsarbeiten in der mikrobiellen Ökologie und Ökophysiologie", Oldenburg, Bremen "Molecular-isotopic constraints on biogeochemical processes in the deep marine subsurface"
September 2005	International Meeting on Organic Geochemistry (IMOG), Seville, Spain "Carbon isotopic constraints on the metabolism of archaea inhabiting deeply buried sulfate/methane transition zones"
June 2005	1st Northern Germany Organic Geochemistry Meeting, Bremen, Germany "Molecular-isotopic constraints on biogeochemical processes in the deep marine subsurface"

Poster presentations

March 2006	IODP/ICDP Kolloqium, Greifswald, Germany "Novel heterotrophic Archaea dominate deeply buried sulfate-methane transition zones in sediments off Peru"
March 2005	IODP/ICDP Kolloquium, Potsdam, Germany "Active biomass in the marine subsurface tracked by intact polar lipids"
October 2004	Dark Energy workshop, WHOI, USA "Isotopic Composition of Living Archaeal Biomass in Deeply Buried Sediments"

Cruises

October – November 2005	Cruise NT05-8 with the Japanese R/V NATSUSHIMA of the Japan Agency for Marine-Earth Science and Technology (JAMSTEC). Exploration of hydrothermal venting sites in the Mariana arc.

(Co-)Supervised theses

April – September 2008 (planned defense date)	M.Sc. thesis of Nadine Buchs (Title: "Biogeochemical analysis of microbial biomass using HPLC/ESI-IT-MS^n")
July – December 2007	M.Sc. thesis of Matthias Kellermann (Title: "Lipid Biomarkers of Thiotrophic and Methanotrophic Symbionts in different Bathymodiolus mussel species: Chemical and Isotopic Analysis")
January – September 2005	M.Sc. thesis of Florence Schubotz (Title: "Investigation of Intact Polar Lipids of Bacteria Isolated from the Deep Marine Subsurface"), received RCOM award for best master thesis 2006.
May – June 2005	B.Sc. thesis of Henning Meyer (Title: "Method development and Application: Lipid based quantification of active microbial biomass in marine sediments")

<u>Erklärung</u> gemäß § 6 Abs. 5 der Promotionsordnung der Universität Bremen

für die mathematischen, natur- und ingenieurwissenschaftlichen Fachbereiche

Hiermit versichere ich, dass ich die vorliegende Arbeit

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Bremen, 13. Mai 2008

Julius Lipp