Tracing the effect of nutrient and carbon supply on the biosynthesis and composition of lipids from marine microbes

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Abstract

In recent years the application of more sensitive biogeochemical and microbiological techniques has significantly increased our knowledge on the complex microbial community that inhabits the marine realm, ranging from the surface of the oceans to deeply buried sediments. Regardless of the recent advancements, the marine water column and the subsurface sediments remain among the least understood ecosystems on this planet. Particularly, microbial driven processes in marine environment, including the extent of microbial activity, their food-web strategies and their ecological function within the marine realm are poorly understood. The aim of the presented research was to expand our knowledge on the microbial community structure and their lipid-based adaption mechanisms to geochemical changes in the marine realm. To this end pure culture of a model organism, water column and sediment samples from different regions of the world were investigated using state-of-the-art analytical techniques. The composition of the microbial community was determined by optimized analysis of intact polar lipids (IPLs) and microbiological techniques, such as cell counting and quantitative polymerase chain reaction. Moreover, a robust analytical technique for lipid specific radio isotope probing (lipid-RIP) experiments was established.

The influence of substrate limitation on the lipid biosynthesis of marine ammonia oxidizing archaea, which are considered as one of the most abundant microorganism groups on Earth, was investigated by a lipid-RIP experiment performed with the role model organism *Nitrosopumilus maritimus* (chapter III). The experiments showed that *N. maritimus* preferentially produce polycyclic glycerol dibiphytanyl glycerol tetraether lipids (GDGTs) under nutrient limiting conditions, whereas an excess in ammonium results in a predominant synthesis of the acyclic GDGT. These findings suggest that nutrient availability has a strong impact on the biosynthesis of microbial lipids. Furthermore, the obtained results may have important implications for the interpretation of the TEX_{86} paleo sea surface temperature (SST) proxy, which is solely based on the varying degree of cyclisation of GDGTs. Besides, the performed lipid-RIP experiments suggest that archaeol (AR) is an intermediate during the synthesis of GDGTs in *N. maritimus*. So far this hypothesized lipid synthetic pathway was only observed in anaerobic *Euryarchaeota* before.

Euryarchaeota together with *Thaumarchaeota* are major organisms in the aerobic water column, but due to the lack of cultivable planktonic *Euryarchaeota*, their lipid composition is poorly understood. Hence, a lipid-RIP incubation experiment was performed with water column samples collected off Svalbard (Norway) applying two different ^{14}C-

labeled substrates (chapter IV): leucine tracing heterotrophic carbon assimilation and bicarbonate to investigate autotrophy. Biomarkers indicative for marine *Thaumarchaeota* were predominantly produced in the autotrophic experiments, while in the leucine incubations polyunsaturated ARs prevailed. Given that marine planktonic *Euryarchaeota* are heterotrophs, this suggests that polyunsaturated ARs are suitable biomarkers to track this phylum in the marine water column.

Based on the analysis of IPL- and DNA there is an ongoing debate whether Archaea or Bacteria are the dominant microbes in subsurface sediments, since both techniques provided contrasting results. Here, the microbial community was investigated in sediments from the Benguela upwelling system by optimized protocols for IPL and quantitative polymerase chain reaction analysis (chapter V). Both methodological approaches showed a good correlation and exhibited that Bacteria are highly abundant in marine subsurface sediments. Contrary to earlier IPL-based studies, these results indicate that Bacteria may outnumber Archaea in marine sediments, particularly within organic lean settings. The pronounced dominance of bacterial phospholipids with a diether glycerol-core, instead of the typical diester glycerol-core, identified this specialized core lipid as a way of coping with chronic energy stress in the investigated subsurface sediments.

Yet, methanogenesis, which is a major process in subsurface sediments, is exclusively performed by Archaea. To study the carbon assimilation pathway of this specialized methane producing community and the fate of their lipid biomarkers, a lipid-RIP incubation experiment stimulating methanogenesis was implemented (chapter VI). The results indicate that methanogens in marine sediments are predominantly autotrophs. A predominant production of AR within the IPL-fraction suggests that these lipids are suitable biomarkers to trace the active methanogens. Higher label incorporation into archaeal core lipids rather than into their IPLs was observed. Considering the short period of incubation (21 days), the obtained results imply that core lipids are actively synthesized by methanogenic archaea and are not exclusively formed by the degradation of their intact analogs as previously thought.

The here applied techniques provide important information on the biosynthesis of microbial lipids under energy limiting conditions, showing that Archaea and Bacteria modify their lipid composition according to changing nutrient levels. Moreover, the detailed investigation of the proxy potential of selected microbial lipids, provided important information for future biomarker-based studies. Therefore, this dissertation increased the current knowledge of the microbial community and the physiologic mechanisms of microbes in the marine realm.

Zusammenfassung

Die Weiterentwicklung von geomikrobiologischen und biogeochemischen Analysemethoden in den letzten Jahren, führte zu einer deutlichen Erweiterung des Wissens über die Mikroorganismen in marinen Systemen. Die Ergebnisse offenbarten einen großen Artenreichtum und hochkomplexe Gemeinschaften, die von der Wasseroberfläche der Ozeane bis hin zu der tiefen Sedimentbiosphäre reicht. Trotz dieses Erkenntnisgewinns bleiben die Wassersäule und die darunterliegenden Sedimente eines der am wenigsten untersuchten Ökosysteme der Erde. Insbesondere über die Funktion der Mikroben in den Stoffkreisläufen von marinen Systemen, ihre Nahrungskette und das Ausmaß der mikrobiellen Aktivität ist nur wenig bekannt. Ziel dieser Dissertation ist es unser gegenwärtiges Wissen über die mikrobiellen Gemeinschaften und den Einfluss von veränderten geochemischen Bedingen auf ihre mikrobielle Lipidbiosynthese in marinen Systemen zu erweitern. Im Verlauf dieser Dissertation wurden Untersuchungen an einer Reinkultur, sowie Proben aus der Wassersäule und marinen Sedimenten aus verschiedenen Regionen dieser Welt, mit der Hilfe modernster analytischer Methoden durgeführt. Die mikrobielle Gemeinschaft wurde mit Hilfe von optimierter biogeochemischer Analyse von intakten polaren Membranlipiden (IPL) und mikrobiologischen Methoden (Zellzählugen und quantitative Polymerase-Kettenreaktion) analysiert. Darüber hinaus wurde eine Methode entwickelt, die eine lipidspezifische Detektion des Einbaus von ^{14}C markierten Substraten während Inkubationsexperimenten erlaubt (Lipid-RIP).

Der Einfluss von Nährstofflimitation auf Ammoniak-oxidierende Archaeen, eine der am häufigsten in der Wassersäule vorkommende Organismengruppe, wurde mit Hilfe eines Lipid-RIP Experimentes an dem Beispielorganismus *Nitrosopumilus maritimus* untersucht. Die Ergebnisse haben gezeigt, dass *N. maritimus* in Folge von Nährstoffarmut bevorzugt zyklische Glycerol Dibiphytanyl Glycerol Tetraether (GDGTs) synthetisieren, während eine hohe Nährstoffverfügbarkeit hingegen zu einer erhöhten Produktion von azyklischem GDGT führt. Dieses deutet darauf hin, dass die Nährstoffverfügbarkeit einen großen Einfluss auf die Lipidbiosynthese von marinen AOA hat. Diese Beobachtungen könnten eine wichtige Rolle bei der Interpretation des TEX_{86}, einem auf der Zyklisierung von GDGTs beruhenden Oberflächenwasserthermometer, spielen. Des Weiteren deutet die schnelle Synthese von Archaeol zu Beginn des Experimentes daraufhin, dass Archaeol ein Zwischenprodukt bei der Bildung von GDGTs in *N. maritimus* ist. Dieser Lipidbiosyntheseweg konnte bisher nur in anaeroben *Euryarchaeota* gezeigt werden.

Euryarchaeota und *Thaumarchaeota* machen einen großen Teil der mikrobiellen Gesellschaft in der aeroben Wassersäule aus. Allerdings ist über die

Lipidzusammensetzung von planktonischen *Euryarchaeota* nur wenig bekannt, da es bis zum heutigen Tage keine Reinkulturen dieser Organismen gibt. Daher wurde ein Lipid-RIP Experiment an Wasserproben, entnommen westlichen von Spitzbergen (Norwegen), mit zwei Unterschiedlichen ^{14}C-Substraten durchgeführt: Leucin um heterotrophe Kohlenstoffassimilation zu untersuchen und Bikarbonat für autotrophe planktonische Archaeen (Kapitel IV). In den Inkubationen mit Bikarbonat wurden zum Großteil Biomarkerlipide gebildet, die mit *Thaumarchaeota* in Verbindung stehen, während in den Inkubationen mit Leucin hauptsächlich ungesättigte Archaeole gebildet wurden. Vorherige Studien haben gezeigt, dass planktonische *Euryarchaeota* organischen Kohlenstoff assimilieren. Daher deuten die Ergebnisse daraufhin, dass ungesättigte Archaeole ein guter Biomarker für diese Organismen darstellt.

Die Frage ob Archaeen oder Bakterien die dominierenden Organismen in der Tiefenbiosphäre sind, ist bis zum heutigen Tage nicht geklärt, da IPL und DNA Techniken sehr unterschiedlich Ergebnisse erbracht haben. Aus diesem Grund wurde die mikrobielle Gemeinschaft in Sedimentproben aus dem Benguela Auftriebsgebiet vor Namibia, mit Hilfe optimierter IPL Methoden und modifizierter quantitativer Polymerase-Kettenreaktion, untersucht (Kapitel V). Die beiden angewandten Techniken haben übereinstimmend gezeigt, dass Bakterien in Sedimenten weit verbreitet sind. Im Gegensatz zu früheren IPL-basierten Studien, deuten die Ergebnisse daraufhin, dass Bakterien möglicherweise gegenüber Archaeen den größeren Anteil an Mikroorganismen in nährstoffarmen Sedimenten ausmachen. Die gemessen Bakterienlipide waren hauptsächlich aus Phospholipiden mit einer Diether-struktur aufgebaut. Da diese Lipide bisher selten in Bakterienkulturen detektiert wurden, könnte dieses auf eine Anpassung der bakteriellen Zellmembran an die dauerhaft nährstoffarmen Bedingungen im Sediment hindeuten.

Methangenese ist einer der Hauptprozesse in marinen Sedimenten, der ausschließlich von Archaeen durchgeführt wird. Um die Lipidbiosynthese und Kohlenstoffassimilierung von Methan produzierenden Archaeen zu untersuchen, wurde ein lipid-RIP Experiment mit Sedimenten aus dem Rhone Delta durchgeführt. Während dieses Experimentes wurden Methan produzierende archaeen mit der Zugabe von Nährstoffen stimuliert. Die Ergebnisse lassen den Schluss zu, dass Methan produzierende archaeen im Rhone Delta zum Großteil autotrophe Organismen sind. Des Weiteren hat eine hohe Produktion von Archaeol in der IPL-fraktion gezeigt, dass dieses Lipid ein guter Biomarker für Methan produzierende archaeen darstellt. Die unpolaren Lipide haben am meisten ^{14}C, in den 21 Tagen des Experimentverlaufs, aufgenommen. Aufgrund der kurzen Inkubationszeit und der geringen Abbaurate von IPLs in marinen Sedimenten, erscheint eine mikrobielle Produktion von unpolaren Lipiden als wahrscheinlichste Erklärung. Daher ist anzunehmen,

dass unpolare Lipide von Methan produzierende archaeen gebildet werden und nicht ausschließlich durch den Abbau von IPLs entstehen, wie bisher angenommen.

Die hier angewandten Methoden erweitern unser Wissen, in Bezug auf die Lipidbiosynthese von Mikroben die nährstoffarmen Bedingungen ausgesetzt sind. In der Dissertation wurde gezeigt, dass sowohl Archaeen als auch Bakterien ihre Lipidzusammensetzung an veränderte Umweltbedingungen anpassen. Des Weiteren konnten einige Lipide mit bestimmten Prozessen oder Organismen in Verbindung gebracht werden, was insbesondere für zukünftige Lipidbiomarker basierte Studien von Bedeutung ist. Aus diesem Grund hat diese Dissertation einen wichtigen Beitrag geleistet um die mikrobielle Gemeinschaft und ihre physiologischen Strategien in marinen Systemen besser zu verstehen.

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List of abbreviations

1G	Monoglycosidic
2G	Diglycosidic
3G	Triglycosidic
AOA/AOB	Ammonia oxidizing archaea/bacteria
ACL	Average chain length
ACT	Acetate
AEG	Acylether glycerol
APCI	Atmospheric pressure chemical ionization
ANME	Anaerobic methane oxidizing archaea
AOM	Anaerobic oxidation of methane
AR	Archaeol
AR/T-ratio	Archaeol to tetraether ratio
BEL	Bacterial and eukaryal lipids
BDGT	Butanetriol dibiphytanyl glycerol tetraether
BL	Betaine lipid
Br-GDGT	Branched glycerol dibiphytanyl glycerol tetraether
CLs	Core-lipids
CPM/CPS	Counts per minute/counts per second
Cren	Crenarchaeol
DAG	Diacyl glycerol
DBI	Double bond index
DCM	Dichloromethane
DEG	Diether glycerol
DIC	Dissolved inorganic carbon
(D)OM	(Dissolved) organic matter
DPM	Disintegrations per minute
ESI-MS	Electrospray ionization mass spectrometry
Eq	Equation
FID	Flame ionization detector
GC	Gas chromatography
GDD	Glycerol dialkanol diether
GDGT 0-4	Glycerol dibiphytanyl glycerol tetraether with 0 to 4 pentacyclic rings
GTGT	Glycerol trialkyl glycerol tetraether
$g_{Sed\ d}$	Sediment dry weight in gram

HCl	Hydrochloric acid
HPH	Hexose-phosphohexose
HPLC	High performance liquid chromatography
IPL	Intact polar lipid
LEU	Leucine
Lipid-RIP	Lipid specific radioisotope probing
LSC	Liquid scintillation counting
Mbsf	Meter below the seafloor
Mbsl	Meter below the sea level
MCG	Miscellaneous crenarchaeotal group
McrA	methyl coenzyme M reductase
MeO-AR	Methoxy archaeol
MeO-AR/D-ratio	Methoxy archaeol to diether ratio
Methanol	MeOH
MG I-IV	Marine group I-IV
MRM	multiple reaction monitoring
MZ	Methanogenic zone
NP	Normal phase
nsDNA	Non-soluble deoxyribonucleic acid
OH-group	Hydroxyl-group
PC	Phosphatidylcholine
PDGT	Pentanetriol dibiphytanyl glycerol tetraether
PD	Phosphatidylethanolamine
PDME	Phosphatidyl–(N,N)-dimethylethanolamine
PG	Phosphatidylglycerol
PHG	Polar head group
PI	Phosphatidylinositol
PL	Phospholipids
PME	Phosphatidyl–(N)-methylethanolamine
PP	Primary production
RI	Ring index
(r)RNA	(ribosomal) Ribonucleic acid
RP	Reverse phase
sDNA	Soluble deoxyribonucleic acid
SIP	Stable isotope probing
SMTZ	Sulfate methane transition zone

SPM	Suspended particulate matter
SRB	Sulfate reducing bacteria
SRZ	Sulfate reduction zone
SST	Sea surface temperature
TEX_{86}	Tetraether index of tetraethers consisting of 86 carbon atoms
TLC	Thin layer chromatography
TLE	Total lipid extract
Uns	Unsaturated
Q-PCR	Quantitative polymerase chain reaction
Q-ToF-MS	Quadrupole-time-of-flight-mass spectrometry
Yr	Year

Chapter I

Introduction

I.1. The biogeochemical cycles in the marine realm - how microbes shape the environment

The marine ecosystem, covering more than 70% of the earth, plays an important role in the global carbon and nutrient cycle. Especially photoautotrophic phytoplankton is one of the major producers of oxygen (O_2) in the atmosphere, which is fundamental for life on Earth (reviewed by Falkowski, 2012). Simultaneously, organisms that inhabit the ocean and the underlying sediment fix vast amounts of carbon and therefore represent one of the major carbon sinks on Earth (reviewed by Falkowski et al., 1998). Moreover marine and benthic microbes are involved in the consumption of greenhouse gases, such as carbon dioxide (CO_2) and methane (CH_4), therefore playing an important role for the regulation of the global climate (reviewed by Falkowski et al., 1998). In addition, the marine ecosystem is a key player in the global nutrient cycle, which is particularly important to face anthropogenic eutrophication (reviewed by Arrigo, 2005). This chapter will introduce microbial processes in the ocean and underlying sediments and how the microbial community drives these biogeochemical cycles in the marine realm. Additionally, the introduction will include an overview of the techniques that can be applied to study the microbial communities in the marine ecosystem and how microbial processes can be traced in the environment. The last part will explain the pathway from the sample to the data point.

I.1.1. Microbial processes in the water column

As mentioned before, the world's oceans cover more than 70% of the Earth's surface, with a water column ranging from shallow shelf seas to deep sea ocean trenches, such as the Mariana trench reaching deeper than 11,000 meters below the sea level (mbsl). Thus, the marine environment represents the largest continuous habitat on this planet (Hügler and Sievert, 2011). The water column can be divided into different zones based on microbial

processes and available energy sources. In the sunlit surface layer (Fig. I.1; epipelagic zone) the majority of microorganisms have a sun-dependent phototrophic lifestyle (Bach et al., 2006). In the photic zone 40-50 petagram of carbon are produced per year mainly by photosynthetic phytoplankton accounting for ca. 50% of the global primary production (PP) (e.g. Longhurst et al., 1995; Antoine et al., 1996; Falkowski et al., 1998). The phytoplankton in the epipelagic zone is either consumed by zooplankton and fish or becomes dissolved organic matter (DOM) which is remineralized by heterotrophic microbes (Fig. I.1; Azam and Malfatti, 2007). The zooplankton and heterotrophic microbes in the epipelagic zone break down the dead biomass into smaller organic and inorganic molecules, such as dissolved inorganic carbon (DIC) and ammonium (NH_4^+; Fig. I.1; Middelburg, 2011). The inorganic products of the DOM remineralization are important nutrients for the photosynthetic phytoplankton (Azam and Malfatti; 2007). In addition, DIC and NH_4^+ are considered as the major substrates for chemolithoautotrophic microbes in the water column, which oxidize NH_4^+ in a two-step pathway to nitrate (NO_3^-; Fig. I.1, Middelburg, 2011). It is estimated that up to 1% of the PP in the epipelagic zone is produced by chemolithoautotrophic microbes (reviewed by Middelburg; 2011).

Approximately 20% of refractory DOM (see review by Carlson et al., 2011) escapes the remineralization in the epipelagic layer and is transported to the mesopelagic and the bathypelagic layer. Since no light is available in the latter two layers, the present microbes utilize either DOM or inorganic substances (Fig. I.1). Traditionally, heterotrophic microbes were considered to dominate the deep-water layers, which consume exported refractory DOM from the euphotic zone or *in situ* produced DOM. However, more recent studies showed that autotrophic planktonic archaea represent up to 20% of the picoplankton in the deeper water column (Karner et al., 2001; Schattenhofer et al., 2009). In addition, experiments with marine water column samples from the mesopelagic layer showed an active utilization of DIC as carbon substrate for marine planktonic archaea (e.g. Herndl et al., 2005; Reinthaler et al., 2010; Yakimov et al., 2011). For instance, Herndl et al. (2005) suggested that up to 84% of the autochthonously formed carbon in the mesopelagic zone of the North Atlantic is produced via autotrophy. Nevertheless, the knowledge about the microorganisms in the meso- and bathypelagic ocean is still very limited (Arístegui et al., 2009; Nagata et al., 2010) and further research has to be performed to unravel the activity of the microbes and their role in biogeochemical cycles in the meso- and bathypelagic ocean.

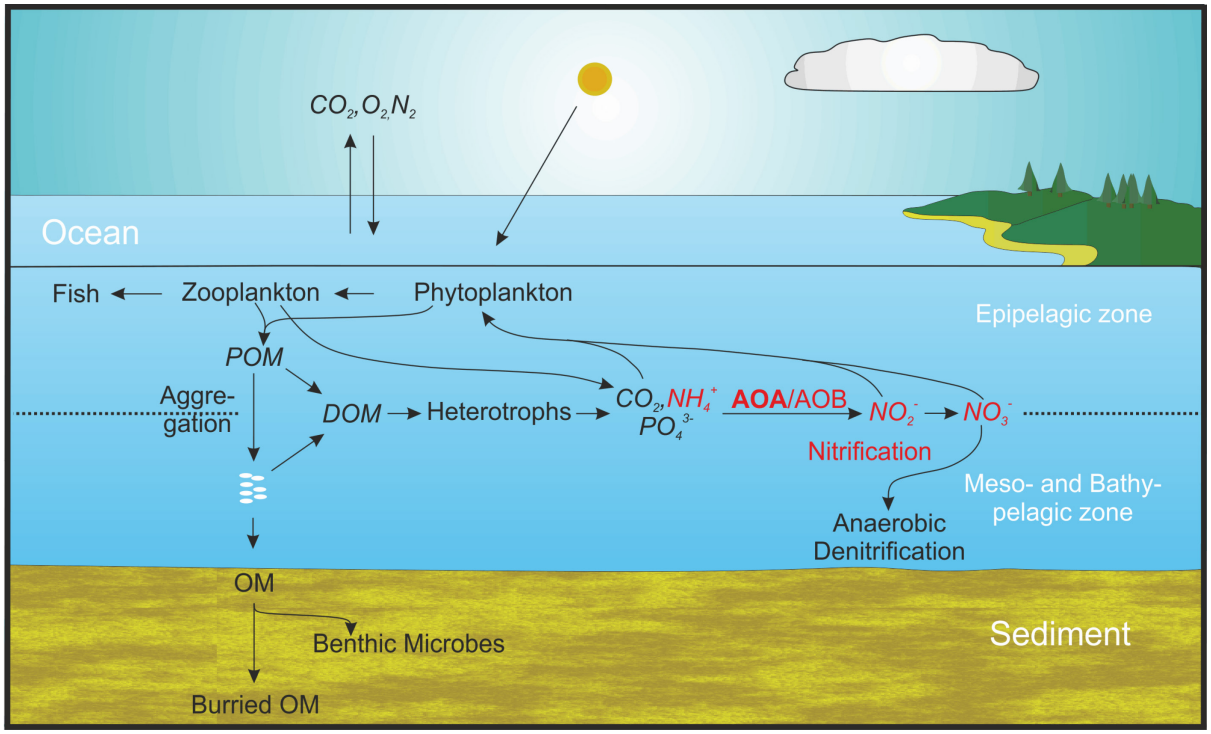

Fig. I.1. The major microbial processes in the water column. The diagram illustrates the different marine habitats and the major microbial processes in the water column. The figure displays the pathway from primary production of biomass by phytoplankton, to the deposition in the underlying sediments as organic matter (OM). Furthermore, the figure shows the nitrification pathway (highlighted in red) as an important process in the nitrogen cycle. The two microbial groups that perform the first and rate limiting processes during nitrification are displayed as AOA (ammonia oxidizing archaea) and AOB (ammonia oxidizing bacteria). Components or chemicals that are produced from or by organisms are written in italic. The figure was modified from Azam and Malfatti (2007) and Francis et al. (2007).

I.1.1.1. Marine Thaumarchaeota mediate a key process in the marine nitrogen cycle

Nitrogen is one of the most essential elements for life on earth and a major constituent in DNA, amino acids and proteins. Only a few microbial groups can fix the gaseous N_2 from the atmosphere, therefore N_2 is one of most limiting factors for microbial life in the water column. In the epipelagic zone phototrophic cyanobacteria are considered as the predominant N_2-fixing microbes (e.g. Karl et al., 1997; Capone et al., 2005), which is converted into bioavailable N-compounds such as NH_4^+ or NO_3^- (Fig. I.1). During nitrification, the NH_4^+ is first oxidized to nitrite (NO_2^-) and then to NO_3^-, providing important nutrients for the marine phytoplankton population. In addition, anaerobic microbes further convert NO_3^- to N_2, a process known as denitrification, in anoxic parts of the marine water column, such as oxygen minimum zones often found in upwelling regimes (e.g. Lam et al., 2009).

Until recently, ammonia oxidizing bacteria (AOB) were considered as the only microbes that perform the first and rate limiting step of nitrification (e.g. Koops et al., 2006). However, there is substantial evidence that the *Thaumarchaeota*, belonging to the marine planktonic archaea, are also involved in this processes (e.g. Könneke et al., 2005). The so

called ammonia oxidizing archaea (AOA) are ubiquitously found in the marine water column (e.g. Francis et al., 2005; Sintes et al., 2016; Smith et al., 2016) and recent studies suggest that AOA may be the main driver of NH_4^+-oxidation in the marine water column (e.g. Beman et al., 2008; Santoro et al., 2010; Beman et al., 2012; Smith et al., 2014; Smith et al., 2016). One explanation for the key role of the *Thaumarchaeota* in this pathway, is their ability to grow under extreme substrate limited conditions (Martens-Habbena et al., 2009). The constant low substrate threshold allows *Thaumarchaeota* to outcompete AOB in many NH_4^+-limited environments, such as the vast marine water column with NH_4^+-concentrations in the in the nM-range (e.g. Beman et al., 2012; Smith et al., 2014; Kim et al., 2016a). In order to study the physiology of *Thaumarchaeota*, several AOA-strains were cultured from the marine environment and soils in the last decade (Könneke et al., 2005; Tourna et al., 2011; Qin et al., 2014; Elling et al., 2015). Despite the increasing number of available cultures, the carbon assimilation pathway of AOA is not fully resolved yet. First results suggested that *Thaumarchaeota* utilize DIC as carbon source (e.g. Könneke et al., 2005; Park et al., 2010). However, some AOA strains are stimulated by additional organic substances, such as pyruvate and α-ketoglutaric acid, suggesting that these strains are obligate mixotrophs (Tourna et al., 2011; Qin et al., 2014). The high abundance of AOA in water column, their unique metabolism and key role in the nitrogen cycle, make *Thaumarchaeota* one of the most interesting microorganisms in the marine realm.

I.1.2. The biogeochemical cycles in marine sediments

Approximately 1% of the organic matter that is produced in the epipelagic zone escapes remineralization in the water column and reaches the sediments (Hedges and Keil, 1995). The organic matter (OM) that arrives in the sediments is heavily degraded by the microbes during the long residence times from the surface to the bottom of the ocean (reviewed by Arndt et al., 2013), thus producing extremely challenging conditions for the microbes that inhabit the marine sediments. Nevertheless, marine sediments, particularly in the upper surface layer, are known to host a highly diverse and active microbial community. The OM in the sediments is remineralized by chemolithoheterotrophs that utilize a range of different terminal electron acceptors (TEA; Fig. I.2; Froelich et al., 1979). Since oxygen respiration is the most energy efficient pathway for the microbes in the sediments, OM is first degraded with O_2, which diffuses from the water column into the upper layer of the sediment. In active sediments, such as the Wadden Sea, this processes is limited to the first few mm of the water-sediment interface (Schulz and Zabel, 2006). However, in less active deep-sea sediments, oxic respiration was detected up to several meters below the seafloor (mbsf) as shown in a study from the North Pacific Gyre (Røy et al., 2012). When O_2 is consumed, the

OM is further remineralized by anaerobic processes using inorganic TEAs. Depending on the energy yield of the different metabolic pathways, the OM is degraded by denitrification and manganese reduction followed by iron and sulfate reduction (Fig. I.2; Froelich et al., 1979). In active coastal settings the sulfate reduction is considered as the most important anaerobic OM remineralization pathway in sediments (e.g. Canfield, 1989).

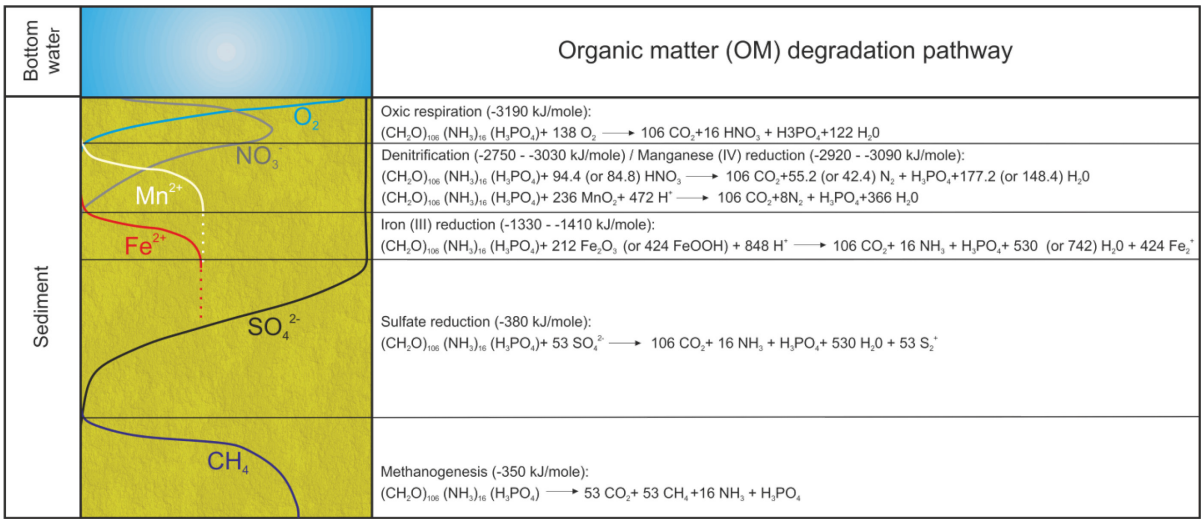

Fig. I.2. The degradation pathway of organic matter (OM) in marine sediments. On the left: A schematic pore water profile illustrating microbial degradation of OM by different terminal electron acceptors (TEAs; modified after Froelich et al., 1979; Schulz and Zabel, 2006). The sediment depth and concentrations of TEAs are shown with arbitrary units. On the right: The different microbial processes and reactions to degrade the OM (modified after Froelich et al., 1979; Schulz and Zabel, 2006). The numbers in brackets represent the energy yield under standard conditions (ΔG^0) of the different reactions in kilojoules per mole of glucose respired (Froelich et al., 1979).

The final step of OM remineralization, after consumption of the inorganic TEAs, is fermentation (Fig. I.3). This process yields the lowest energy during OM degradation. Fermentative microbes break down OM into smaller molecules, such as propionate, butyrate, lactate, aromatics, CO_2, hydrogen (H_2), acetate (ACT), alcohols and other molecules (Konhauser, 2007). Due to advection processes the produced molecules are transported to the surface sediments to fuel the above-mentioned metabolic pathways (Fig. I.3). For instance, H_2 and ACT are widely used by sulfate reducing bacteria (SRB) in the sulfate reduction zone (SRZ). Moreover, ACT, CO_2 and H_2 are utilized by methanogens as energy substrates to perform the final metabolic pathway, the microbial production of methane (Fig. I.3).

I.1.2.1. The deep biosphere - microbes under severe energy limitation

Before the 1950's, deeply buried sub-seafloor sediments were thought to be a flat uniform lens without any microbial activity (reviewed by Jørgensen and Boetius, 2007). A new dimension in this research field was gained in the middle of the 1990´s when Parkes et

al. (1994) showed that microbial cells are detected sediment depths of up to 500 mbsf. A more recent study suggests that the deep biosphere may actually extend to a depth of 2500 mbsf (Inagaki et al., 2015).

The organisms that inhabit deeply buried sediments must face tremendous challenges to survive under the harsh conditions in subsurface sediments. For instance the pressure, generated by the overlying water column and sediment layers, leads to a reduction of the pore space which has a profound impact on the microbial life in sediments (Fredrickson et al., 1997; reviewed by Rebata-Landa and Santamarina, 2006). Another widespread limit of life is suggested in depths between 2000 and 4000 mbsf (Parkes et al., 2000), where the geothermal gradient approaches the known biological limit of life at 122 °C (Takai et al., 2008). In addition, microbes must face extreme energy limitation in deeply buried subsurface sediments. The vast majority of the OM has been degraded in the water column and the upper surface sediment layers, therefore the remaining OM represents an accumulation of degradation resistant macromolecules that are unfavorable to microbes (Tegelaar et al., 1989; see review by Zonneveld et al., 2010). Consequently, very slow microbial community turn-over times of up to several thousands of years were calculated in the deep biosphere (Lipp and Hinrichs, 2009; Lomstein et al., 2012).

A more recent study suggested that community turn-over times are potentially up to two orders of magnitude shorter than previously thought (Braun et al., 2017). Moreover, it is estimated that up to 90% of the microbial activity in marine sediments is taking part in the deep biosphere (reviewed by Parkes et al., 2014). Both studies demonstrate that the microbes present in subsurface sediments are important participants in global biogeochemical cycles. Previous studies showed that subsurface sediments are predominantly inhabited by uncultured Archaea and Bacteria (e.g. Hug et al., 2016; Starnawski et al., 2017), that are considered to consume OM as an energy source (Biddle et al., 2006; Lloyd et al., 2013a; Meng et al., 2014; Seitz et al., 2016; Lazar et al., 2017). However, the different techniques that were applied to investigate the abundance Archaea and Bacteria in the deep biosphere provided contrasting results. With studies suggesting that Archaea dominate in subsurface sediments (Lipp et al., 2008), while others observed a bacterial dominance (e.g. Schippers et al., 2005; Nunoura et al., 2016; Buongiorno et al., 2017) and studies which showed an equal abundance of both domains (e.g. Breuker et al., 2013; Lloyd et al., 2013b). Despite the contradictory results of the previous mentioned studies, there are some groups of Archaea and Bacteria that are commonly referred as subsurface Prokaryotes. *Chloroflexi*, *Gammaproteobacteria*, *Planctomycetes* and *Atribacteria* (formerly JS1) are typical Bacteria detected in subsurface sediments (review by Parkes et al., 2014). Moreover, there are several uncultivated linages of Archaea which are ubiquitously found in subsurface sediments such as *Lokiarchaeota*, *Bathyarchaeota*, *Thermoplasmata*,

Thorachaeota, *Hadesarchaeota* and *Thaumarchaeota* (e.g. Baker et al., 2016; Lauer et al., 2016; Lever, 2016; Seitz et al., 2016). The investigation of life under extreme conditions and various open questions regarding the phylogenetic composition of the microbial community, make the deep biosphere one of the most interesting ecosystems on Earth.

I.1.2.2. Methanogenesis a key process in marine subsurface sediments

Marine sediments harbor vast amounts of CH_4 and up to 25% of the global annual production of this greenhouse gas is taking place in marine sediments (Reeburgh, 2007). The overwhelming majority of this CH_4 is produced by microbes. So far, all cultured methanogens belong to the *Euryarchaeota* and are distributed among seven taxonomic classes (Becker et al., 2016). The methanogenic archaea utilize low molecular substrates to produce CH_4, which are provided by fermentative bacteria. There are three different methanogenesis pathways: the acetoclastic, the hydrogenotrophic and the methylotrophic methanogenesis (Fig. I.3; e.g. Whiticar, 1999). During the acetoclastic methanogenesis, ACT is broken down into CO_2 and CH_4. Hydrogenotrophic methanogens reduce CO_2 with H_2 to produce CH_4. These two processes are severely curtailed in the SRZ, because the energetically more efficient SRB are able to outcompete methanogens for the substrates ACT and H_2 (Fig. I.3; Oremland and Polcin, 1982; Whiticar, 1999). The methylotrophic methanogens, on the other hand, utilize non-competitive substrates such as methanol, methylamines and methyl sulfides. Due to the consumption of substrates that are not favorable to other organisms, this group of methanogens can also thrive in zones where sulfate is still present. It is estimated that hydrogenotrophic methanogenesis is the most predominant pathway in sulfate free marine sediments (Whiticar, 1999). This can be explained by the fact that CO_2 accumulates in marine sediments, while ACT can be depleted due to the rapid consumption by SRB. Little is known about the contribution of methylotrophic methanogenesis, because this process cannot be distinguished from hydrogenotrophic methanogenesis based on isotope measurements (Whiticar, 1999; Conrad, 2005). Therefore, this pathway possibly remains the least explored microbial CH_4 production process in marine sediments.

Although, large quantities of methane are produced by methanogens in deeply buried sediments, previous studies showed that methane producers represent only a small fraction (<1%) of the microbes in subsurface sediments (reviewed by Lever, 2016). Instead, microbes that utilize OM as an energy source are considered to prevail in deeply buried sediments as described above. The mismatch between methane production and the abundance of methanogens demonstrates that methanogenesis in marine sediments is not fully understood yet and future research must be performed to relate methanogenic communities to this globally important process. For instance, a recent study suggested that the abundance of methanogens was underestimated in earlier studies and that *Bathyarchaeota* may also be able to perform methanogenesis (Evans et al., 2015).

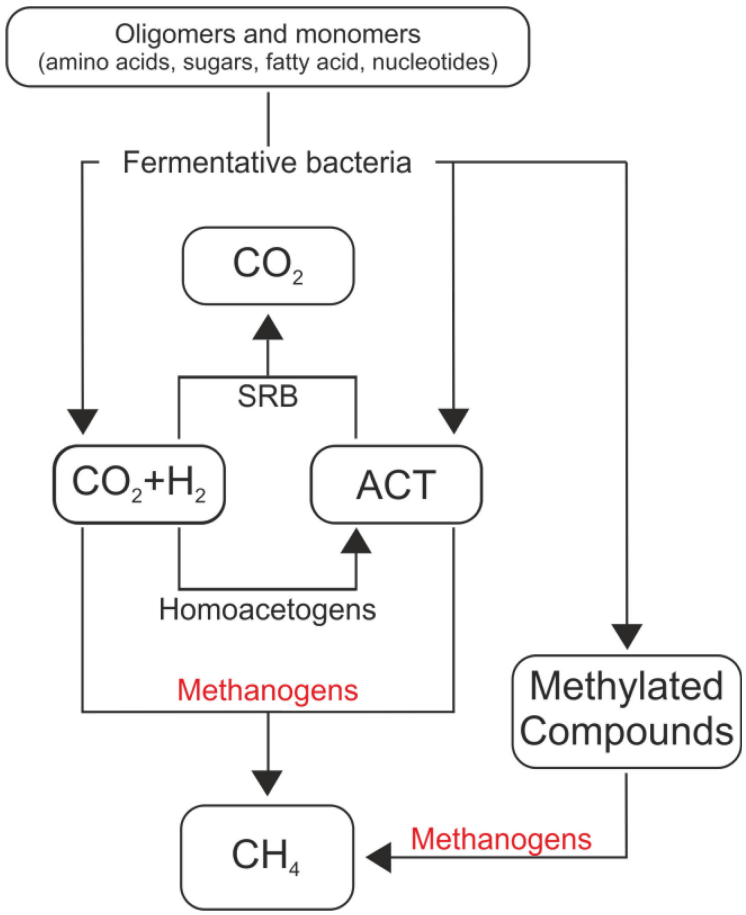

Fig. I. 3. The methane production pathways in marine sediments. Fermentative bacteria produce H_2 and acetate (ACT), which is utilized by sulfate reducing bacteria (SRB) utilize. In sulfate depleted sediments methanogens (shown in red) may produce methane either via hydrogenotrophic (H_2+CO_2) and acetoclastic (disproportion of ACT) methanogenesis. The consumption of methylated compounds by methylotrophic methanogens can be also performed in sulfate rich sediments. The figure was modified from Konhauser (2007)

I.2. How to study microbial life in the marine environment - an overview of the different techniques

The lack of cultivable representatives in the marine environment, particularly in the energy limited deep biosphere, led to the development of various culture independent techniques to study the size and the phylogenetic relationship of the microbial communities in marine environments (Fig. I.4). In this chapter an overview will be presented, to explain the advantages and disadvantages of the three major techniques that were applied in this thesis. A special focus will be set on the analysis of microbial lipids, because this was the predominantly applied technique in this dissertation.

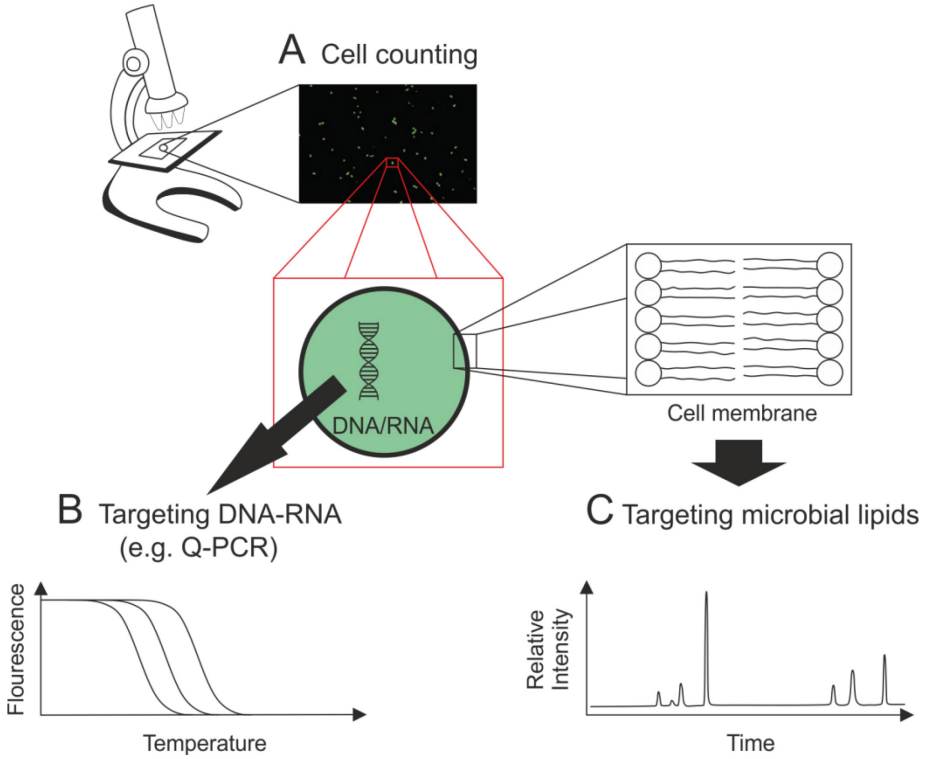

Fig. I.4. A scheme of the three different techniques that were applied to investigate the microbial community structure in this dissertation. Panel A shows results from heat sterilized sediments mixed with *Escherichia coli* (Morono et al., 2009). The red box magnifies into one single cell, with DNA (double helix) in the cytoplasm and a surrounding cell membrane (black layer). Panel B displays a typical Q-PCR (quantitative polymerase chain reaction) melting curve that enables the quantification of DNA. Panel C shows the cell membrane composition of a microbe that is built up of a lipid bilayer. The circles represent the hydrophilic outer part of the membrane (also known as head groups); whereas the thin black lines represent the hydrophobic center of the microbial cell membrane. The chromatogram was derived from a lipid analysis of *Methanothermobacter thermoautotrophicus*.

Historically, the classical approach for the quantification of the microbial biomass is cell counting. Thereby, cells are stained by a nucleic acid-binding dye (e.g., SYBR green or DAPI) and counted with an epifluorescence microscope. In turn, the cells can be identified by their fluorescence, while other particles such as minerals do not show this characteristic behavior. Cell counting was applied in numerous studies from the water column (e.g. Morris et al., 2002) and the subsurface biosphere (e.g. Parkes et al., 1994; Inagaki et al., 2015) to determine and quantify the size of microbial community. One of the major advantages of this technique is that cells can be quickly counted and, for example, calculated on board during a scientific expedition. However, cell counting provides only little information about the composition of the microbial community and within sediment samples cell counting can be difficult. For example, the presence of sediment particles can lead to high background fluorescence as well as, the number of cells may be underestimated since some cells may be hidden behind sediment particles. Nevertheless, cell counting is a powerful tool to gain a first impression of the present microbial community size.

The analysis of DNA and RNA molecules in environmental samples is probably the most frequently used technique to characterize the abundance and composition of the microbial community in the marine realm. The analysis of 16 S-rDNA allows to quantify the number of different microbes (e.g., Archaea vs. Bacteria) using a technique called quantitative polymerase chain reaction (Q-PCR). During Q-PCR primer-pairs are added to the extracted DNA that define which part of the DNA will be amplified. The DNA molecules are subsequently quantified by a fluorescent stain (e.g., SYBR green). The added primers are specific to detect certain microbial groups, e.g. Archaea, Bacteria, Eukarya (e.g. Breuker et al., 2013; Starnawski et al., 2017) or even microbial processes, such as methanogenesis, sulfate reduction or the oxidation of ammonia (e.g. Santoro et al., 2010; Schippers et al., 2012). Moreover, the phylogenetic relationship of the microbial community in environmental samples can be investigated by next generation sequencing technology of 16S rRNA (e.g. Lazar et al., 2017; Starnawski et al., 2017). Therefore, DNA-analysis has a large potential to establish detailed descriptions of the present microbial community in marine environmental samples, but there are also disadvantages. One major problem in subsurface sediments is the stability of the DNA. It was thought that DNA, particularly RNA, are labile and should be degraded directly after cell death (Schippers et al., 2005). However, other studies showed that up to 90% of the DNA in anoxic environments might originate from dead biomass (Dell'Anno and Danovaro, 2005; reviewed by Torti et al., 2015). Moreover, the applied primer-sets, which are usually developed from well characterized cultures, are often not able to cover the diverse uncultured microbial community in the water column and particularly in marine sediments (e.g. Lipp et al., 2008; Teske and Sørensen, 2008; Lever and Teske, 2015). Thus, the mismatch of primers can lead to an underestimation of certain microbial groups in the environment. This was, for instance, demonstrated by Teske and Sørensen (2008) who showed that earlier DNA-based techniques have strongly underestimated the abundance of Archaea in subsurface sediments.

The analysis of microbial lipids offers a technique that allows an unspecific investigation of the microbial community in environmental systems. Hence, microbial lipids from eukaryotic and prokaryotic cells may provide important information on the biogeochemistry, microbial ecology and environmental conditions during growth of the organisms in recent and ancient ecosystems. Lipid analysis has been used to describe the microbial community in a wide range of samples, such as cultures, the water column and sediment samples (e.g. Rossel et al., 2008; Lipp et al., 2008; Rossel et al., 2011; Elling et al., 2014; Zhu et al., 2016). Next, the application of lipids and their advantages and disadvantages as biomarker for microbes will be discussed in more detail.

I.2.1 The lipid biomarker concept - Tracing microbial communities and processes in the marine realm

The cell membrane constitutes a mechanical and physiochemical barrier between the inner cell and the external environment. Lipids are the main building blocks of the cell membrane that surround cells and ensure their integrity. The semipermeable layer of the cell membrane controls and maintains fluxes and gradients of ions nutrients and metabolic products between the cell's interior and exterior. Lipid membranes are thought to constitute between 5 and 10% of the cell dry weight (Cario et al., 2015). Microbial lipids in the cell membrane are composed of a hydrophilic head group, a glycerol backbone and a hydrophobic tail, altogether representing an intact polar lipid (IPL). The IPLs can be set-up as bilayer or monolayer in the cell membrane (Fig. I.5). There are various types of hydrophilic head groups in microbes such as phosphatidic, glycosidic, amine-based, sulfate-based head groups and combinations of them. IPLs bear chemotaxonomic information, which can be applied to differentiate between the microbial groups of Archaea, Bacteria and Eukarya.

I.2.1.1. Archaeal lipids

The archaeal lipid structure is distinctly different compared to bacterial and eukaryal lipids (Fig. I.5A and Fig. I.5B). The inner core structure is composed of a series of isoprenoid units resulting in a highly branched hydrophobic chain. The chain is connected to the glycerol backbone via ether bonds in a *sn*-2 and *sn*-3 position. The head group can either be glycosidic and/or phosphatidic (Elling et al., 2014; Yoshinaga et al., 2015a). However, in environmental samples archaeal lipids with a glycosidic head group prevailed in previous studies (e.g. Lipp and Hinrichs, 2009; Xie et al., 2014). The archaeal membrane can be arranged as a bilayer and a monolayer structure (Fig. I.5A)

The bilayer in Archaea is formed by two diether lipids (Fig. I.5A). The diether lipids (archaeol (AR)) consist of two C_{20} isoprenoidal phytanyl chains (biphytanyl chain), connected to the glycerol backbone. AR predominantly occurs in *Euryarchaeota*, but is also found in the lipid membrane of *Cren*- and *Thaumarchaeota* (reviewed by Schouten et al., 2013). AR can contain hydroxyl-(OH)-groups and unsaturations (uns) in the core structure, which are particularly identified in methanogenic archaea (Koga and Morii, 2005). Another variation is the extended AR, with an additional isoprenoid unit, found in various Archaea (e.g. *Methanomassiliicoccus luminyensis* (Becker et al., 2016)) and the macrocyclic AR as observed in *Methanococcus jannaschii* (Sprott et al., 1991). Moreover, short AR, lacking of one isoprenoid unit, is synthesized by *Methanothermobacter thermoautotrophicus* (Yoshinaga et al., 2015a).

The monolayer membrane cell wall is built up by glycerol dibiphytanyl glycerol tetraethers (GDGTs), which are composed of two biphytanyl chains embedded between two

glycerol backbones (Fig. I.5A). The biosynthesis pathway of GDGTs in Archaea is, to this point, not fully resolved (reviewed by Pearson, 2014). Some studies suggested that GDGTs are formed by a head-to-head condensation of two intact AR-molecules, thus implying that ARs are intermediates in the tetraether synthesis pathway (see review by Koga and Morii, 2007). On the contrary, other studies came to the conclusion that the GDGTs are formed by highly unsaturated diethers, which are also intermediates during the AR synthesis (Poulter et al., 1988; Eguchi et al., 2003). Moreover, theories exist, suggesting that diethers are not involved in the synthesis of tetraether lipids (Villanueva et al., 2014). Despite the yet unresolved biosynthesis pathway, GDGTs are found in almost all Archaea (reviewed by Schouten et al., 2013). The inner core of the tetraether structure may be composed of a large variety of different structures. The most typical variation is the addition of one or multiple cyclopentane rings (Fig. I.5A). So far, up to eight cyclopentane rings have been observed in S*ulfolobus acidocaldarius* (De Rosa and Gambacorta, 1988)). A unique feature is the formation of a GDGT with four cyclopentane rings and one cyclohexane ring (crenarchaeol; Fig. I.5A), which is exclusively found in the phylum of *Thaumarchaeota* (Sinninghe Damsté et al., 2002; Schouten et al., 2008; Elling et al., 2014). Other modifications are the addition of OH-groups in methanogens and *Thaumarchaeota* (Liu et al., 2012; Elling et al., 2014), methylations (e.g. *M. thermoautotrophicus*; Knappy et al., 2009) and H-shaped GDGTs (e.g. *Methanothermus fervidus;* Morii et al., 1998). Moreover, lipids with a butanetriol (BDGT) and pentanetriol (PDGT) instead of a glycerol backbone were recently detected in environmental samples as well as isolates of the methylotrophic methanogen *Methanomassiliicoccus luminyensis* (Zhu et al., 2014; Becker et al., 2016).

I.2.1.2. Bacterial and eukaryal lipids

In contrast to Archaea, the hydrophobic chain of bacterial lipids is build-up by two alkyl chains connected in the *sn*-1 and *sn*-2 position of the glycerol backbone (Fig. I.5B). The hydrocarbon chains of Bacteria and Eukarya can vary in chain length and the degree of unsaturation. The majority of studies, that investigated the lipidome of Bacteria and Eukarya reported an ester bound connection between the backbone and inner core structure (reviewed by Valentine, 2007). These diacyl glycerol lipids (DAGs) are frequently detected in the water column and surface sediments (e.g. Lipp and Hinrichs, 2009; Schubotz et al., 2009). The bacterial DAG structure is predominantly composed of C_{14} to C_{20} fatty acids with one unsaturation (reviewed by Zhang and Rock, 2008), while polyunsaturated C_{20} or C_{22} fatty acids are typical constituents of the lipid membrane in marine Eukarya (Brett and Müller-Navarra, 1997). Moreover, diether (DEG) and acylether (AEG) bound lipids (Fig. I.5B) are found in SRB (Rütters et al., 2001; Sturt et al., 2004; Grossi et al., 2015; Vinçon-Laugier et al., 2016). In addition, DAG and AEG occur in selected members of *Planctomycetes* (Sinninghe Damsté et al., 2005) and thermophilic bacteria (e.g. Huber et al., 1996). Other

types of lipids that are identified in Eukarya are the betaine lipids (BL, e.g. in marine algae, Kato et al., 1996)), the nitrogen bearing sphingolipids in Eukarya and Bacteria (reviewed by Olsen and Jantzen, 2001) and the ornithine lipids that are exclusively found in Bacteria (reviewed Geiger et al., 2010). Moreover, branched GDGTs (br-GDGTs) of presumably bacterial origin have been detected in terrestrial peats and soils (e.g. Hopmans et al., 2004). The microbial source of these membrane spanning lipids are to this point not fully elucidated, but structural analogs occur in Bacteria from the order *Thermotogales* (Sinninghe Damsté et al., 2007) and in *Acidobacteria* (Sinninghe Damsté et al., 2011).

I.2.1.3. IPLs as biomarkers for microbial life in the marine realm

IPLs are ubiquitously found in marine environments and early experiments with marine sediments showed that they quickly degrade after cell death, particularly under oxic conditions (White et al., 1979; Harvey et al., 1986). Accordingly, it was proposed that IPLs serve as biomarkers for intact cells. However, Harvey et al. (1986) also observed differential degradation rates of lipids with a phosphatidic head group compared to glycolipids, suggesting a preferential preservation of glycolipids. Moreover, a study from North Sea sediments revealed that ether lipids are more stable than ester lipids (Logemann et al., 2011). Complementary to the previous studies, an incubation experiment showed extraordinary low degradation rates for archaeal glycolipids in subsurface sediments, resulting in turn-over times of several hundred thousand years (Xie et al., 2013a). Consequently, in low activity environments the connection between microbial life and IPLs must be interpreted with caution and ester bound phosphatidic lipids are presumably the best biomarkers for microbial life in the marine realm.

I.2.1.4. Variations of the membrane lipid structure and composition as a response of environmental changes

The lipid membrane of Archaea, Bacteria and Eukarya represents the barrier that protects the inner cell from the surrounding environment. However, in a wide range of environmental conditions the cell membrane of microorganisms must be capable to modify its structure and composition to allow cell integrity and metabolic activity of the organism with respect to different environments but also changes of its surrounding environment, such as variations of temperature, pH and/or nutrient availability. The ether bound lipids in Archaea are considered to reduce proton permeability compared to ester bonds predominantly found in Bacteria and Eukarya (Fig. I.5; Van De Vossenberg et al., 1998; Mathai et al., 2001; Konings et al., 2002). This allows to minimize the lateral ion flow-through the membrane and therefore lowers the energy cost for cell maintenance in energy starved environments (Valentine, 2007). Moreover, membrane spanning GDGTs dramatically decrease the permeability of ions at higher temperatures (e.g. Van De Vossenberg et al., 1995; Mathai et

al., 2001; Konings et al., 2002). Accordingly, Archaea are considered to be well adapted to energy limiting environments (Valentine, 2007).

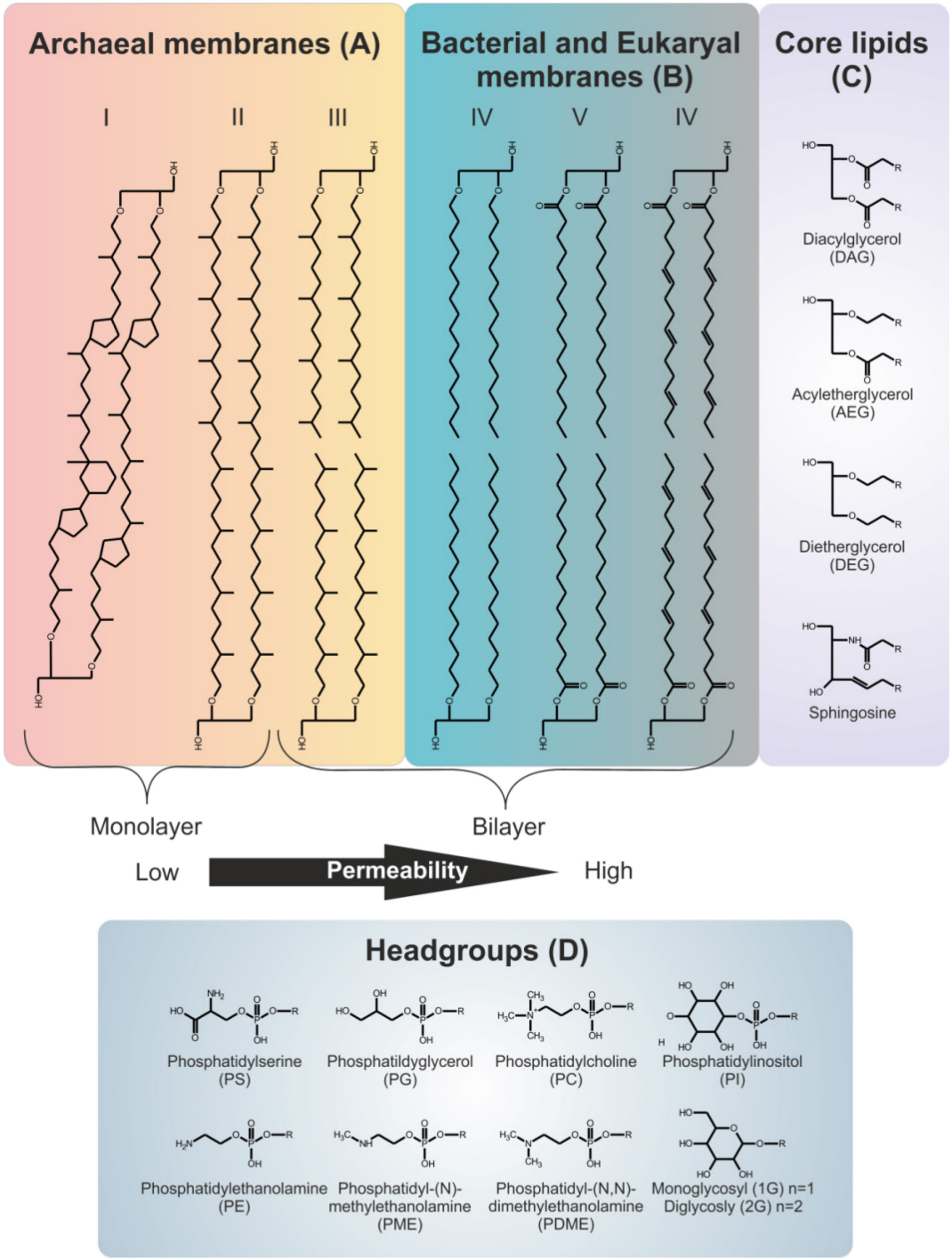

Fig. I.5. Detailed chemical structures of microbial lipids Panel A shows the typical structure of archaeal membranes and panel B structures of lipids from Bacteria and Eukarya. Archaeal lipids: crenarchaeol with cyclopentane rings and one cyclohexane ring (I), acyclic GDGT (II) and archaeol (III). Bacterial lipids with a diether glycerol backbone (DEG; IV) and diacyl glycerol backbone (DAG; V and VI). Panel C displays glycerol backbones that connect the hydrophobic tail with the polar head group. A variety of phosphatidic and glycosidic polar head groups are illustrated in panel D. The arrow shows the effect of the lipid structure on the cross lateral diffusion of ions and water through the lipid membrane (Valentine, 2007). The figure was modified after Valentine, (2007).

One of the best studied lipid adaptation mechanism among Archaea is the increasing number of cyclopentane rings in GDGTs with increasing growth temperature, which has been observed in thermophilic archaea (e.g. De Rosa et al., 1980; Uda et al., 2001) and mesophilic *Thaumarchaeota* (Elling et al., 2015; Qin et al., 2015). The exact effect of the cyclization in GDGTs on the membrane properties is not fully elucidated yet; however modeling experiments suggest that a higher number of rings lead to a tighter packing of the lipids and so stabilizes and at the same time reduces the permeability of the cell membrane (Fig. I.5; Gabriel and Chong, 2000; reviewed by Chong et al., 2012). Interestingly, the mesophilic *Thaumarchaeota* are usually characterized by a higher degree of cyclization than the thermophilic archaea (Elling et al., 2014; Qin et al., 2015). This suggests that an additional factor, apart from temperature, leads to the introduction of cyclopentane rings in the GDGTs of *Thaumarchaeota*. Hurley et al. (2016) hypothesized that this may be an adaptation of cell membrane to the severe NH_4^+ limitation of marine environments. Furthermore, decreasing pH-values result in an increasing cyclization of GDGTs in thermophilic archaea (Boyd et al., 2011), thus protecting the inner cell against the acidic conditions. Archaeal core lipid modifications are also implemented to increase the fluidity as a response to cold temperatures, as shown in the psychrophilic methanogen *Methanococcoides burtonii* (Nichols et al., 2004). This Archaeon synthesizes unsaturated archaeols with decreasing growth temperature to keep the lipid membrane in a liquid crystalline state even at low temperatures.

Bacteria and Eukarya, on the other hand, are capable to adapt their membrane corresponding to nutrient limitation and environmental stressors. For instance, *Listeria monocytogenes* and *Saccharomyces cerevisiae*, increase their fatty acid chain length with increasing growth temperature (Suutari et al., 1990; Russell et al., 1995). Moreover, variations in the degree of unsaturations were observed in other microbes as a response to changes in temperature, pH and salinity (Suutari et al., 1990; Gombos et al., 1994; Yuk and Marshall, 2004; Chihib et al., 2005). Both the increase of chain length and the decreasing number of fatty acid unsaturation reduces the water and ion permeability (De Gier et al., 1968; Paula et al., 1996). Furthermore, increasing abundances of ether bound lipids are identified in thermophilic and acidophilic bacteria (e.g. Goldfine and Langworthy, 1988; Huber et al., 1996; Sinninghe Damsté et al., 2007; Sinninghe Damsté et al., 2011), suggesting that these lipids are produced to form a more rigid lipid structure (Valentine, 2007). Indeed, experiments by Jansen and Blume (1995) showed that the bacterial ether bound lipids significantly decreased the membrane permeability compared to their ester bound counterparts.

In addition, alterations of the head group composition as a response to nutrient limitation are documented in Archaea, Bacteria and Eukarya. The Archaeon *M.*

thermoautotrophicus, for instance, preferentially synthesizes glycosidic head groups during energy limiting conditions (Yoshinaga et al., 2015a). Moreover, *Rhodobacter sphaeroides* as well as phytoplankton substitute phosphatidic head groups under phosphate-limiting conditions with glycolipids, BLs and sulphate bearing head groups (Benning et al., 1995; Van Mooy et al., 2009).

I.2.1.5. Labeling experiments - a powerful tool to investigate active microbial processes in the environment

As pointed out earlier the marine realm is inhabited by a great variety of organisms, thus representing a vast ecosystem for microbes that may use heterotrophic and/or autotrophic strategies to thrive in their specific environment. To describe the abundance of a certain microbe and their phylogenetic relationship, various culture independent techniques such as the analysis of DNA and IPLs have been successfully applied. However, both latter techniques do not provide information on the occurring microbial processes, the microbial activity and the utilized energy sources of the responsible organisms. In order to tackle this question labeling experiments provide a powerful tool (reviewed by Boschker and Middelburg, 2002; Radajewski et al., 2003; Wegener et al., 2016).

In order to study the autotrophic life style of *Thaumarchaeota* and methane oxidizing archaea, ^{13}C-bicarbonate (DIC) has been applied in previous studies (e.g. Park et al., 2010; Kellermann et al., 2012). Moreover, the microbial degradation of certain OM-pools was investigated by the consumption of labeled IPLs (e.g. Harvey et al., 1986; Xie et al., 2013a) or algal biomass (Kristensen et al., 1995; Graue et al., 2012). Labeling techniques were further applied to investigate specific microbially mediated processes like methanotrophy (e.g. Bodelier et al., 2000) or methanogenesis (Harvey et al., 1989). In addition, labeling experiments were used to investigate the importance of methylotrophic methanogenesis in marine sediments (e.g. Zhuang et al., 2016) or the biological degradation of substances hazardous to the environment (e.g. Roslev et al., 1998a). Apart from the reconstruction of biogeochemical processes in samples from marine systems, labeling experiments were further applied to investigate biosynthetic pathways, such as lipid synthesis, in pure or enrichment cultures (e.g. Nishihara et al., 1989; Nemoto et al., 2003; Kellermann et al., 2016a). The carbon assimilation of the microbes is traced by the incorporation of the labeled substrate in microbial lipids (Fig. I.6), DNA or other biomolecules. However, the analysis of label uptake into lipids is considered to be more sensitive than into DNA, since high label uptake is needed to detect the substrate incorporation into DNA (Wegener et al., 2012). This results in long incubation times, leading to a widespread distribution of the labeled substrate over the entire food-web (reviewed by Radajewski et al., 2003) and therefore complicates the investigation/interpretation of individual microbial processes.

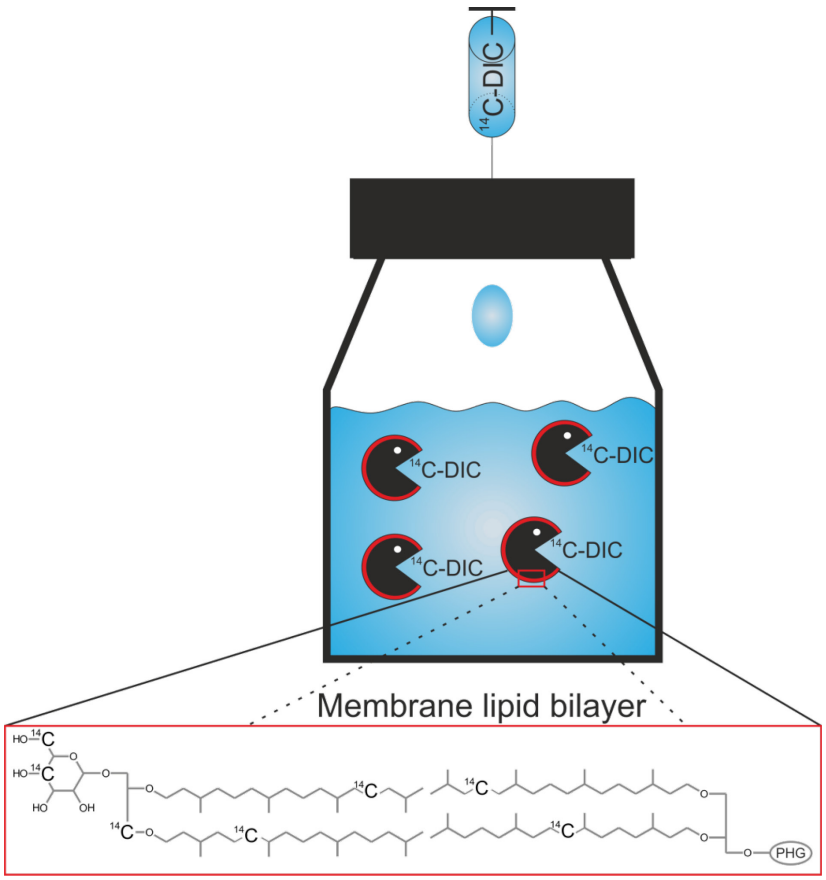

Fig. I.6. A schematic illustration of an incubation experiment using ^{14}C labeled bicarbonate (^{14}C-DIC) as a tracer to investigate carbon assimilation. The substrate is added by a syringe, to investigate the incorporation of DIC into microbial lipids. The cells of the organisms are displayed as black dots surrounded by the lipid membrane (red layer). The red panel shows a magnified view on membrane lipid bilayer. The ^{14}C in the chemical structure depicts the incorporation of radiotracer in the lipid membranes. Please note that the distribution of ^{14}C within the head group, the glycerol moiety and the isoprenoidal units is randomly arranged and such high ^{14}C incorporation per molecule are usually not reached (PHG=polar head group).

Two different types of labeling techniques are applied to investigate microbial processes via substrate incorporation into lipid molecules. The classical approach is the addition of a radioactive food source (e.g. ^{14}C-DIC; Fig. I.6) to an environmental or culture sample (e.g. Gaskell et al., 1976; White et al., 1977). The application of radioisotopes as a labeling substrate and their incorporation into lipids is also called lipid radio isotope probing (lipid-RIP). In lipid-RIP experiments, β-emitters like ^{14}C and ^{32}P are applied to study their incorporation into the biomass (e.g. White et al., 1977; Nishihara et al., 1989). Analytical developments in the late 1990's led to the development of stable isotope probing (SIP) with ^{13}C-labeled substrates (Boschker et al., 1998; reviewed by Boschker and Middelburg, 2002). Due to the easier handling of the substrates, SIP has become popular. However, previous studies that applied SIP in marine subsurface sediments to investigate the activity and carbon assimilation of Archaea with various substrates showed only minor or no significant

label incorporation into the lipid molecules, even after incubation periods exceeding more than one year (e.g. Lin et al., 2013; Lengger et al., 2014a).

In order to improve the sensitivity of this approach dual-SIP was developed, where deuterated water ($^{2}H_2O$) and ^{13}C-DIC are added to the sample (Wegener et al., 2012). This technique allows a sensitive measurement of the microbial activity via ^{2}H incorporation and an investigation of the food source of microbes by ^{13}C-DIC uptake. According to Wegener et al. (2012), the ratio of ^{2}H to ^{13}C-DIC incorporation displays whether the microbes are autotrophic (ratio close to 1) or heterotrophic (ratio close to 0.3). In their case study with microbially active sediments from the Baltic Sea, no significant incorporation of ^{13}C-DIC was detected into archaeal lipids. Therefore, Wegener et al. (2012) suggested that Archaea in subsurface sediments may likely be predominantly heterotrophs. On the other hand, the observations could also be explained by the low sensitivity of the ^{13}C method. When comparing lipid-RIP with dual-SIP, the ^{14}C method is considered to be more sensitive, because of the extremely low natural abundance of ^{14}C ($<10^{-9}$%) compared to the natural abundance of ^{2}H (0.015%) and ^{13}C (1.106%). Consequently, less ^{14}C must be incorporated into the biomass to generate an unequivocal signal by RIP compared to the dual-SIP method. Another problem generated by the lower sensitivity of SIP is that, due to the low incorporation rates, high quantities of label have to added to the sample (e.g. up to 25% ^{13}C-DIC; Wegener et al., 2012). High additions of DIC may have a low impact on the microbial community. However, large volumes of artificial organic substrates, which are normally scarce in energy starved environments, may alter the sample composition by triggering a certain group of microbes. Hence, lipid-RIP is considered as a valuable tool to trace the activity of microbes, particularly Archaea, in low-activity environmental samples.

I.3. The analysis of microbial lipids and RIP experiments - from the sample collection to the final data point

In this dissertation the microbial lipid composition and their lipid biosynthesis (via lipid-RIP) were analyzed in marine sediments and marine water column samples as well as pure cultures. The lipids were extracted by the application of two different protocols. (1) The modified Bligh and Dyer method (e.g. Sturt et al., 2004) was used to obtain archaeal, bacterial and eukaryal IPLs of water column and sediment samples. Thereby, a mixture of methanol (MeOH), dichloromethane (DCM) and an aqueous buffer in a ratio of 2:1:0.8 (v:v:v) was used to obtain the total lipid extract. (2) For culture experiments the cells were directly hydrolyzed by the addition of 1M hydrochloric acid (HCl) in MeOH for 16 hours at 70 °C as described by Becker et al. (2016). Compared to the Bligh and Dyer protocol, direct cell hydrolysis has the disadvantage that particularly phosphatidic polar head groups are cleaved

during this procedure and therefore some taxonomic information of the IPLs is lost (Fig. I.7). However, tests during this thesis with a culture of *M. thermoautotrophicus* (Fig. I.7 B and Fig. I.7 C) and published culture experiments showed that the acidic hydrolysis significantly improves the extraction efficiency of archaeal and bacterial tetraethers (Sinninghe Damsté et al., 2011; Cario et al., 2015; Becker et al., 2016). Subsequently, an aliquot of the extract was analyzed by high performance liquid chromatography coupled to an electrospray ionization mass spectrometer (HPLC/ESI-MS) to identify the microbial lipids and obtain their structural information by MS and MS/MS^2 measurements (e.g. Sturt et al., 2004). When derived from lipid-RIP incubation experiments, the extracts were further processed to investigate incorporation of ^{14}C from different substrates into individual lipids or lipid classes. In the following sections the analytical methods will be described in more detail (Fig. I.8).

I.3.1. HPLC-MS analysis

In the early days of lipid analysis of environmental samples, gas chromatography (GC) was widely applied to study microbial communities in marine ecosystems (e.g. Clark and Blumer, 1967; Boon et al., 1975). Up to this day, this technique does only allow to investigate apolar lipids (Lengger et al., 2017). Given that IPLs bear important chemotaxonomical information of the microbial communities, scientists developed techniques that allow an investigation of these lipids (e.g. Rütters et al., 2002; Sturt et al., 2004).

The chromatographic separation of the compounds of interest is an important step to allow an optimal identification and quantification of the lipids extracted from environmental samples. In principle two types of chromatographic separation exist: normal phase (NP) and the reverse phase (RP). The NP separates the compounds according to their polarity of the head group (e.g. phosphatidic vs. glycosidic head groups), whereas the RP allows separation according to the chain length of microbial lipids (e.g. AR vs. GDGT). Problematic during HPLC analysis may be the co-elution of compounds from the complex organic matrix in environmental samples that may hinder an optimal detection of the lipid composition (Mallet et al., 2004). In the last years, HPLC separation techniques have steadily evolved in order to minimize the co-elution (e.g. Wörmer et al., 2013; Zhu et al., 2013a), resulting in the identification of novel biomarkers in environmental samples, such as the BDGTs and PDGTs (Zhu et al., 2014).

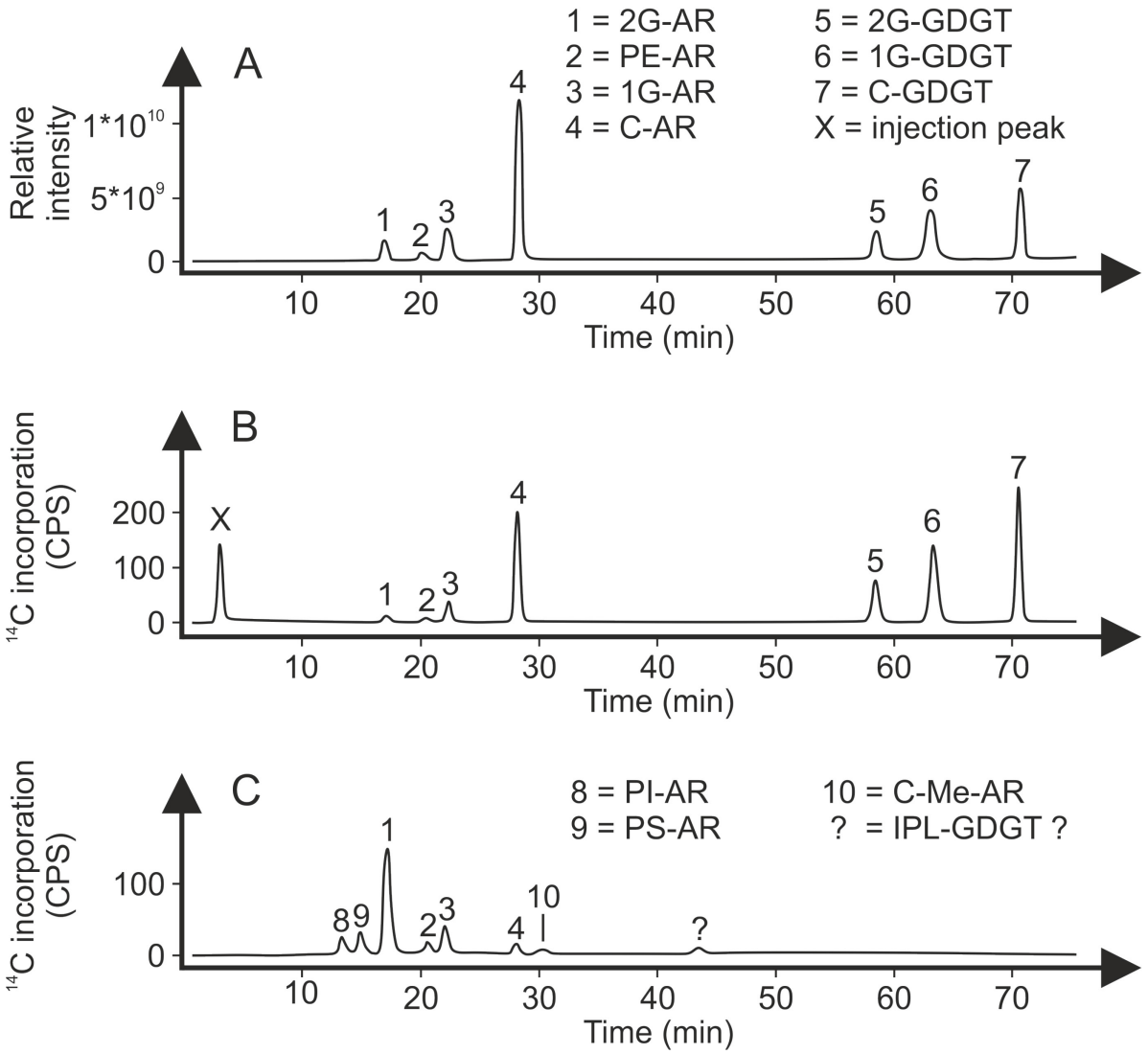

Fig. I.7. The different extraction efficiencies of the modified Bligh and Dyer protocol and acidic cell hydrolysis. The lipids were separated by reverse phase-HPLC on an analytical ACE3 C_{18} column as described by Evans et al. (2017). All three chromatograms are based on a ^{14}C incubation experiment with a *M. thermoautotrophicus* culture. In Panel A and B the culture was extracted by acidic cell hydrolysis and C displays the extraction with the modified Bligh and Dyer protocol (Sturt et al., 2004). Panel A shows the base peak chromatogram of the hydrolyzed *M. thermoautotrophicus* culture measured by HPLC-MS. In B and C a radio chromatogram of the ^{14}C incorporation into the lipids is displayed. The results in panel B and C were obtained by flow-through scintillation counting and are displayed as counts per second (CPS). The low incorporation into tetraethers in panel C compared to B shows the different extraction efficiencies of both applied protocols in culture samples. The injection peak (X) likely originates from cleaved head groups, which cannot be detected by the applied HPLC-MS system (A) due to their low mass. Different lipid structures were detected: archaeol (AR), methylated archaeol (Me-AR) and glycerol dibiphytanyl glycerol tetraethers (GDGTs). The associated head groups were: mono-glycosidic (1G), di-glycosidic (2G), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS).

The separated lipids are subsequently analyzed by MS, where the compounds of interest are first ionized in the ion-source and later detected according to their specific mass. For further structural information the lipids can be fragmented (MS^2). The combination of the

lipid mass and the fragmentation pattern provides a valuable tool to identify microbial lipids (Yoshinaga et al., 2011). Two different types of ion-sources are commonly used: the atmospheric pressure chemical ionization (APCI; e.g. Becker et al., 2015) and the electron spray ionization (ESI; e.g. Sturt et al., 2004). However, IPLs cannot be detected by APCI, for that reason lipid identification was predominantly carried out with the ESI ion-source in this dissertation.

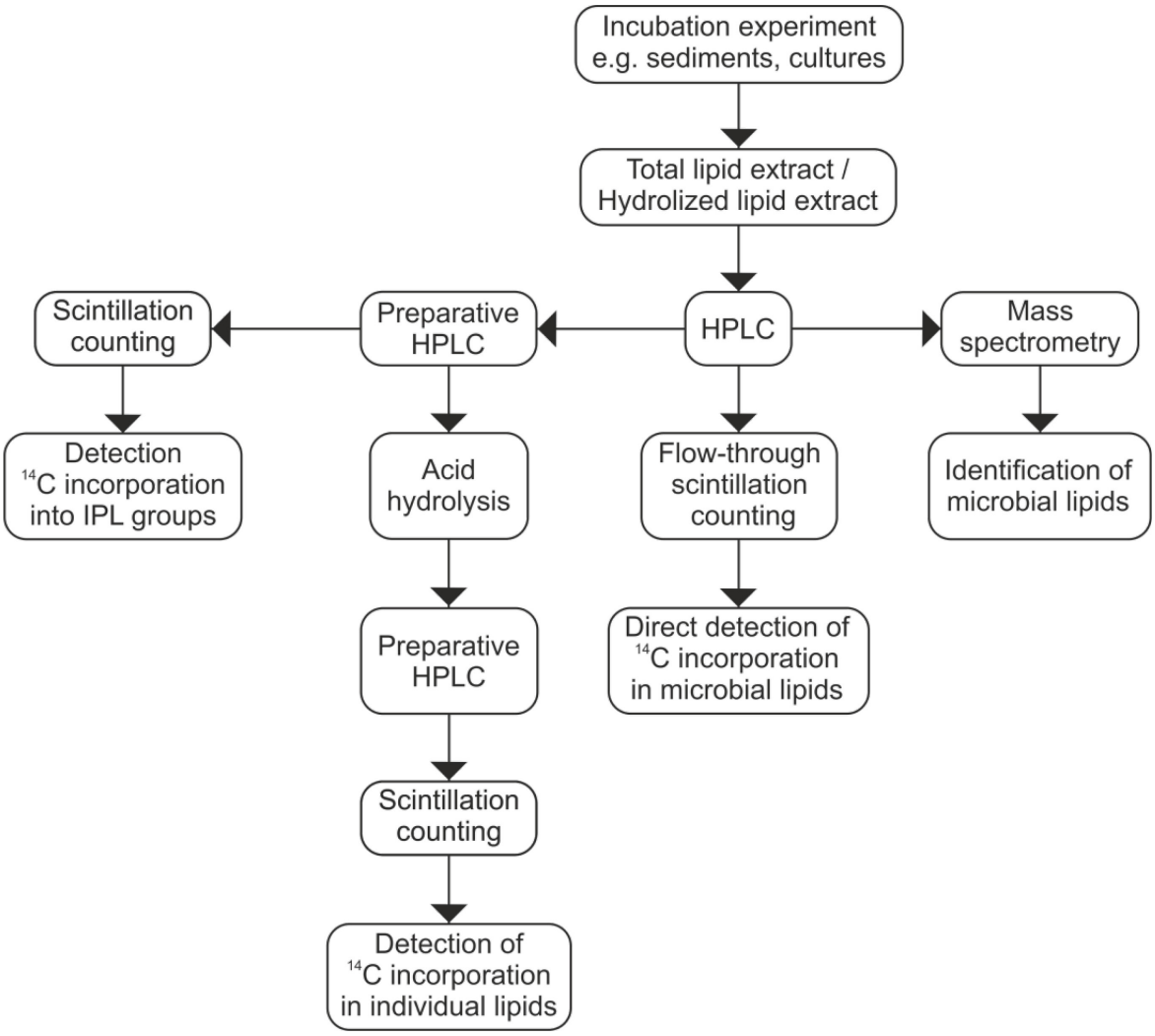

Fig. I.8. A detailed work flow diagram showing the major analytical steps performed in this PhD-thesis. Extracted lipids were separated by high performance liquid chromatography and analyzed by mass spectrometry. ^{14}C incorporation into lipids was either detected by flow-through scintillation counting coupled to a HPLC-system or the lipids were separated into individual fractions by preparative HPLC and measured by liquid scintillation counting (LSC).

I.3.2. Lipid specific RIP (lipid-RIP)

The most frequently applied technique to detect radio isotopic incorporation into microbial biomass is the liquid scintillation counting (LSC). To detect the incorporation of radioisotopes into membrane lipids by LSC, a scintillation solvent is added to the lipid extract

prior to the measurement. After addition, the emitting β-decay of the radioisotope excites the aromatic ingredients of the scintillation solvent. The energy is transported along the aromatic compounds to a scintillator that emits ultra-violet light. The strength and abundance of the ultra violet light flashes is proportional to the radioactivity of the analyte. Photomultipliers in the LSC convert this signal to an electronic pulse which is detected and recorded. The results are given as counts per minute/second (CPM/CPS) or disintegrations per minute (DPM). DPM-values have the advantage that the results were corrected for an internal standard in the LSC and therefore the results can be directly converted into a radioactive unit, such as Becquerel (Bq). Since this method is basically an optical method to determine the radioactivity, this method can be hindered by colorful (dark) samples (color quenching). Given that the lipid extracts of organic rich environmental samples can be dark, color quenching may strongly suppress the radioactive signal in studies conducted with marine sediments. Modern LSC systems are equipped with correction systems, but quenching must be considered when comparing extracts with different colors.

In order to study the incorporation of radioactive substrates into lipids some studies have measured the TLE to investigate the microbial activity in environmental samples (White et al., 1977; McKinley and Vestal, 1984; Phelps et al., 1989; Phelps et al., 1994). However, the direct measurement of the TLE does not allow to draw a relationship between the microbial community, their activity and the biosynthesis of lipids. For lipid specific RIP, the different microbial lipids are separated into individual lipids that are characteristic for certain microbes. Different approaches have been used to achieve this goal, all based on chromatographic separation. One option, which has been particularly used in earlier studies, is the application of thin layer chromatography (TLC; e.g. Brown and Johnston, 1962; Langworthy, 1977; Tornabene et al., 1980). Thereby, lipids are separated by TLC, dissolved from the stationary phase by organic solvents and then individual fractions are measured on the LSC. Due to analytical advantages of GC and liquid chromatography, these techniques have been widely applied in order to separate microbial compounds for RIP and SIP experiments (e.g. Bodelier et al., 2000; Takano et al., 2010; Kellermann et al., 2016a). After the separation by preparative GC or HPLC, the individual lipids or lipid groups are collected by a fraction collector and measured. The advantage of this technique compared to TLC-separation is the higher resolving power that provides a finer separation of the individual fractions compared to the TLC-separation. However, even GC or HPLC separation is limited regarding the purity of the obtained fractions. Therefore, several chromatographic steps have may be necessary to reach the requested purity. Particularly, the separation to the individual lipid level (e.g. single GDGT species) can require time consuming sample work-up and result in preferential loss of certain compounds. Accordingly only a few studies that applied HPLC and GC were able to investigate label incorporation into specific lipids (e.g. Takano et al.,

2010) and the majority separated on a compound class level, such as a separation between different head groups of GDGTs (Kellermann et al., 2016a) or the carbon number of fatty acids (Bodelier et al., 2000).

A third technique, which is widely applied in the pharmaceutical- and chemical-industry, is the flow-through scintillation counting coupled to a HPLC- or GC-system (Fig. I.8). Thereby, radio isotopic quantification is achieved by an on-line measurement of the flow-through scintillation counting-system that mixes the scintillation solvent with separated compounds from the eluent downstream of the HPLC (Fig. I.7 B and Fig. I.7 C). The mixture of solvent and radioactive marked lipids is measured within the counting cell by paired photomultiplier tubes. Due to small volumes of the counting cell (e.g. 250 µL) the flow-through scintillation counting provides reliable counts of the radioactively marked compounds over short time scales (Pinckney et al., 1996). Therefore, this system has the advantage over other techniques of a real-time detection of radiolabel incorporation of individual compounds. On the other hand, flow-through scintillation counting is less sensitive than the LSC approach coupled with preparative HPLC, due to shorter residence times in the measuring cell. In this PhD project, flow-through scintillation counting as well as preparative HPLC-based techniques were applied to study the biosynthesis of lipids, the microbial activity and the microbial processes taking place in environmental and culture samples.

Chapter II

Structure and objectives of this dissertation

In the last decades numerous studies have investigated the microbial communities that inhabit the marine environment, ranging from the surface of the water column to deeply buried marine sediments. However, these studies often provided diverging results and were limited in their attempt to assess activity and food-web strategies of the microbes that inhabit the marine realm.

The overall goal of this thesis was to better define the microbial communities that inhabit marine environments and to investigate their lipid adaptation mechanisms to changing biogeochemical conditions (Fig. II.1). To this aim sophisticated techniques were developed that allow the specific detection of substrate incorporation into lipid biomarkers. Coupled with the identification and quantification of IPLs by HPLC-MS and the analysis of prokaryotic 16S rRNA genes, this polyphasic approach provides a comprehensive overview on microbial communities that inhabit the marine environment and their metabolic and physiologic strategies to thrive under energy limiting conditions. This thesis can be subdivided into two parts: In the first part (chapter III and chapter IV) the effect of different environmental conditions and carbon sources on the lipid biosynthesis of marine planktonic archaea was investigated. The second part of this thesis concentrated on the benthic microbial community to investigate archaeal and bacterial membrane lipid adaptions to energy deficient conditions in subsurface sediments (chapter V and VI).

Scientific question in Chapter III:

- How does the NH_4^+-supply and growth stage affect the biosynthesis and cyclization of GDTGs in marine *Thaumarchaeota*?

Chapter III focused on the influence of nutrient supply on the biosynthesis of microbial lipids of marine *Thaumarchaeota*, which are ubiquitous in the water column. To this end, a pure isolate of this phylum (*Nitrosopumilus maritimus*) was grown in the presence of ^{14}C-bicarbonate (DIC) to trace the newly synthesized lipids incubated with excess and limited supply of NH_4^+. The ^{14}C incorporation was detected by a flow-through scintillation counting

coupled to an HPLC-system. This set-up, which has been used for the first time to investigate ^{14}C incorporation in microbial lipids in environmental research, enables a direct and straight-forward detection of label incorporation on an individual lipid basis (e.g. individual GDGT-species).

Scientific question in chapter V

- How do different water masses influence the planktonic archaeal community/physiology and do heterotrophic archaea produce different lipids than autotrophic archaea in the dark ocean?

In **chapter IV** the food-web sources of planktonic archaea were investigated in two sites with a contrasting oceanographic setting off Svalbard (Norway). The metabolic strategies of the Archaea were examined by lipid-RIP experiments with two different substrates (DIC and leucine (LEU)). DIC was applied to trace the production of autotrophic archaea and LEU incubations were performed to track lipid synthesis of the heterotrophic archaea. The incubations were performed for less than 5 days in the absence of light at 4°C.

Scientific question in Chapter V:

- How does the abundance of Archaea and Bacteria change along the transect from sediments rich in OM to organic lean sediments and do the microbes have physiologic strategies to cope with the chronic energy stress in subsurface sediments?

In **Chapter V** the microbial community present in subsurface sediments in the Benguela upwelling area was analyzed by applying improved protocols for the analysis of IPLs and 16S rRNA-genes. The utilization of these modified protocols allowed us to give a more realistic view on the microbial community that inhabits subsurface sediments off the Namibian coast. The unique depositional system in this region enables studying variations of the microbial community under contrasting conditions, ranging from the organic rich sediments below the upwelling cell to the organic lean sediments in the south Atlantic gyre.

Scientific question in chapter VI:

- Are benthic methanogens autotrophs or heterotrophs and how do varying energy sources affect the activity and the lipid biosynthesis of this community?

In **chapter VI** the metabolic strategies of benthic methanogenic archaea in sediments from three different geochemical zones was investigated in a 21 days lipid-RIP experiment. Methanogenesis was stimulated by a H_2/CO_2-headspace (hydrogenotrophic-set) and additional MeOH (methylotrophic-set) and compared to background community without

stimulation (background-set). The addition of ^{14}C-DIC and ^{14}C-ACT allowed to trace the autotrophic and heterotrophic carbon assimilation by methanogens and the background community. The labeled lipids were purified by preparative HPLC to analyze the ^{14}C incorporation in specific archaeal lipid groups.

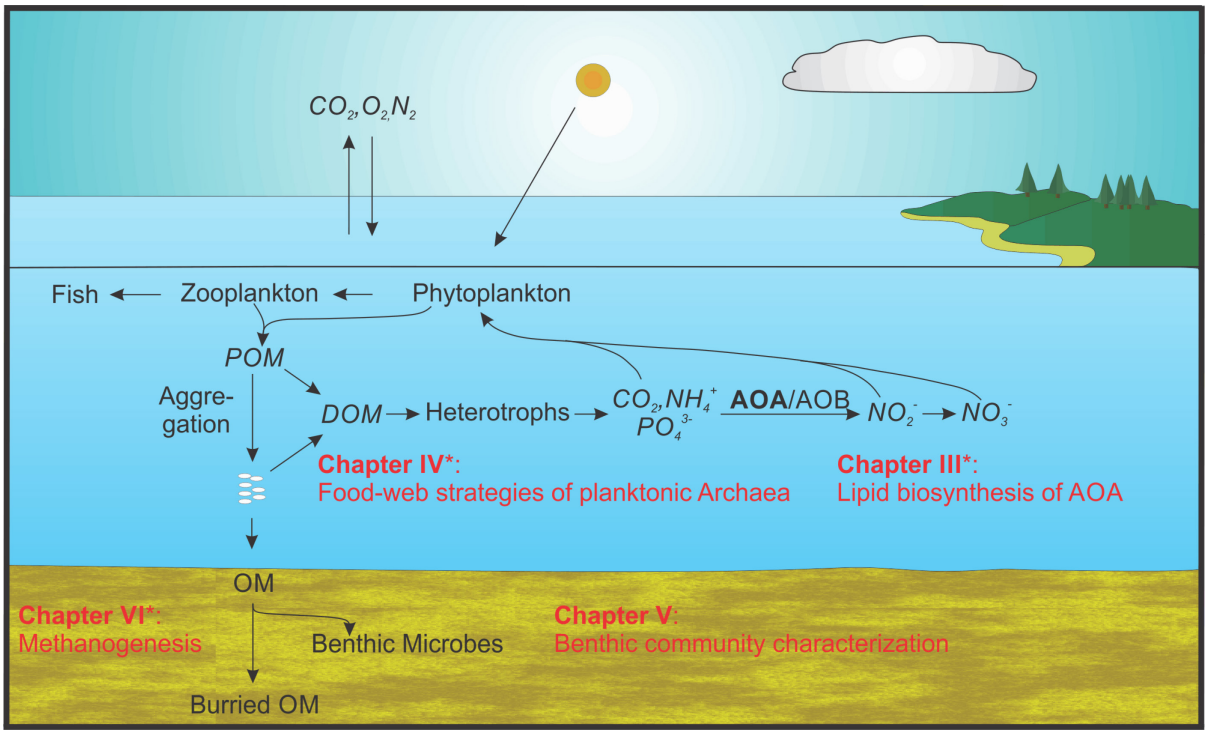

Fig. II. 1 A vertical scheme of the water column and underlying sediments, showing the four studies that were performed during this dissertation. In red: the four research chapters of this thesis. Investigations by applying a ^{14}C incorporation experiment are marked with a star*. In grey: the major microbial processes and the nitrification pathway. Modified from Azam and Malfatti (2007) and Francis et al. (2007).

II.1. Contributions to publications

Chapter III:

Lipid biosynthesis of *Nitrosopumilus maritimus* dissected by lipid specific radio isotope probing (Lipid-RIP) under contrasting ammonium supply

Thomas W. Evans, Martin Könneke, Julius S. Lipp, Rishi R. Adhikari, Heidi Taubner, Marcus Elvert, Kai-Uwe Hinrichs

Submitted at *Geochimica et Cosmochimica Acta*

T.W.E., M.K. and K.-U.H designed research; T.W.E. performed lab work; T.W.E. analyzed data with the help of M.K.; T.W.E wrote the manuscript with contributions from all co-authors.

Chapter IV:

The lipid biosynthesis of auto- and heterotrophic planktonic archaea examined by the incorporation of radiolabeled carbon substrates

Thomas W. Evans*, Nadine I. Goldenstein*, Marcus Elvert, Susan Mau, Ingeborg Bussmann and Kai-Uwe Hinrichs

*equal contribution

In preparation for *Organic Geochemistry*

N.I.G., T.W.E. and K.-U.H. designed project; T.W.E. and N.I.G. performed lab work; T.W.E. and N.I.G. analyzed the data; T.W.E. and N.I.G. wrote manuscript with contributions from all co-authors

Chapter V

Size and composition of subseafloor microbial community in the Benguela upwelling area examined from intact membrane lipid and DNA analysis

Thomas W. Evans, Lars Wörmer, Mark A. Lever, Julius S. Lipp , Lorenzo Lagostina, Yu-Shih Lin, Bo Barker Jørgensen and Kai-Uwe Hinrichs

Published in *Organic Geochemistry*, 2017, Vol. 111, pages 86-100
doi: 10.1016/j.orggeochem.2017.06.008

T.W.E., L.W., M.A.L. and K.-U.H designed project; T.W.E and L.L. performed lab work; T.W.E. analyzed data with the help of L.W. and M.A.L..; T.W.E, wrote the manuscript with contributions from all co-authors.

Chapter VI

Carbon assimilation and fate of methanogenic lipid biomarkers in marine sediments tracked by lipid radio isotope probing

Thomas W. Evans*, Sarah Coffinet *, Martin Könneke, Kevin W. Becker, Julius S. Lipp, Marcus Elvert and Kai-Uwe Hinrichs
*equal contribution

In preparation for *Geochimica et Cosmochimica Acta*

T.W.E., S.C., M.K. and K.-U.H designed project; T.W.E. and S.C. performed lab work; T.W.E. and S.C. analyzed the data; K.W.B. analyzed intact polar lipids in sediments; T.W.E. wrote the manuscript with contributions from the co-authors.

Chapter III

Lipid biosynthesis of *Nitrosopumilus maritimus* dissected by lipid specific radio isotope probing (Lipid-RIP) under contrasting ammonium supply

Thomas W. Evans[a,#], Martin Könneke[a,b], Julius S. Lipp[a], Rishi R. Adhikari[a], Heidi Taubner[a], Marcus Elvert[a], Kai-Uwe Hinrichs[a]

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III.1. Abstract

Ammonia oxidizing archaea (AOA) are among the most abundant microbial organisms in the world's oceans and are one of the major sources of glycerol dibiphytanyl glycerol tetraethers (GDGTs) in the water column and underlying sediments. However, little is known about the critical steps during biosynthesis of GDGTs, which form the basis of the TEX_{86} paleothermometer. Recent results showed that, apart from temperature, physiological factors (e.g. growth stage and variations of the ammonium oxidation rate) may affect the TEX_{86} temperature proxy. A 72-hour ^{14}C bicarbonate incubation experiment was performed to trace the effect of NH_4^+ supply and limitation on the production of individual membrane lipids by the AOA model organism *Nitrosopumilus maritimus*. The ^{14}C incorporation was monitored at five-time intervals by liquid chromatography coupled to flow-through scintillation counting of the hydrolyzed extract, allowing a straight forward and sensitive on-line detection of the degree and incremental changes of ^{14}C incorporation into TEX_{86}-related and other archaeal lipids on time scales lower than a single cell cycle. The experiments showed that NH_4^+ limitation results in a higher cyclization of GDGTs with a preferential synthesis of crenarchaeol, whereas excess supply led to predominant production of GDGT-0. Consequently, the culture with excess NH_4^+ resulted in up to 10 °C lower reconstructed incubation temperatures than the NH_4^+ limited culture using the TEX_{86}^L calibration. The TEX_{86}^L reconstructed temperatures in the limited culture approached the incubation temperature when NH_4^+ was exhausted, which may provide support for GDGT based sea surface temperature application in oligotrophic paleoenvironments. Interestingly, a high relative production of archaeol was observed at the beginning of both experiments (ca. 25%); likewise, the degree of cyclization was initially lowest indicating delayed production of cycloalkylated derivatives. This pattern is consistent with *N. maritimus* synthesizing GDGTs involving head-to-head reaction of two archaeol molecules and subsequent cyclization of the acyclic tetraether. This study provides robust information on the biosynthesis of GDGTs in *N. maritimus* and thereby advances our understanding of the influence of NH_4^+ supply on the TEX_{86} proxy.

Keywords: Ammonia oxidizing archaea, ^{14}C radioisotope probing, GDGTs synthesis, ammonium supply, TEX_{86}, CCaT, ring index, flow-through scintillation counting

III.2. Introduction

Ammonia oxidizing archaea (AOA) affiliated with the phylum *Thaumarchaeota* are among the most abundant microbes in the world's oceans, representing ca. 20% of marine picoplankton (Karner et al., 2001; Schattenhofer et al., 2009). In the marine environment, AOA are thought to play a key role in the nitrogen cycle since they are involved in the oxidation of ammonium (NH_4^+) to nitrite (NO_2^-), which is the primary and rate-limiting step of nitrification (Könneke et al., 2005; Beman et al., 2008; Martens-Habbena et al., 2009).

The cell membrane envelope of marine AOA is largely comprised of glycerol dibiphytanyl glycerol tetraethers (GDGTs) and to a lesser extent of glycerol diphytanyl glycerol diether lipids (archaeols; ARs; Schouten et al., 2008; Elling et al., 2014). AOA are thought to be one of the major sources of GDGTs in the water column (reviewed by Pearson and Ingalls, 2013, and Schouten et al., 2013). GDGTs of marine *Thaumarchaeota* contain zero to four cyclopentane rings, and crenarchaeol contains four cyclopentane and one cyclohexane ring (Sinninghe Damsté et al., 2002; Schouten et al., 2008; Elling et al., 2014). Crenarchaeol was proposed as a specific biomarker for *Thaumarchaeota*, since it has only been identified in this phylum (Sinninghe Damsté et al., 2002; de la Torre et al., 2008; Pitcher et al., 2010; Elling et al., 2014; Elling et al., 2017).

The degree of cyclization of GDGTs forms the basis of the TEX_{86} proxy for reconstruction of past sea surface temperatures (SST; Schouten et al., 2002) being justified by the good correlation between the calculated SST in core top sediments and the actual SST (Schouten et al., 2002; Rueda et al., 2009; Kim et al., 2010; Wei et al., 2011; Zhu et al., 2011). However, TEX_{86} calculated temperatures in suspended particulate material (SPM) in the water column showed large variations compared to *in situ* temperatures, (e.g. Wuchter et al., 2005; Turich et al., 2007; Basse et al., 2014; Xie et al., 2014; Kim et al., 2015; Kim et al., 2016a), suggesting that the degree of cyclization not solely depends on temperature (reviewed by Pearson and Ingalls, 2013). For instance, thermophilic archaea were shown to produce a higher degree of cyclization with decreasing pH (reviewed by Oger and Cario, 2013). More recently, Qin et al. (2015) and Hurley et al. (2016) showed that oxygen limitation and reduced NH_4^+ oxidation rate increase the TEX_{86} and accordingly the corresponding SST.

Recent studies investigating the lipid composition of marine AOA (e.g., *N. maritimus*) in response to varying environmental conditions are based on mass spectrometric identification and quantification of archaeal lipids (Elling et al., 2014; Elling et al., 2015; Qin et al., 2015; Hurley et al., 2016). However, this approach is limited into tracing short-term variations of lipid biosynthesis due to integration of lipid signals from various microbial generations, growth stages and environmental conditions. To overcome these analytical limitations, labeling experiments were previously performed to investigate the biosynthesis

pathway of archaeal lipids. For example, studies based on (i) radio isotope probing (RIP; e.g. Nishihara et al., 1989; Nemoto et al., 2003) and (ii) stable isotope probing (SIP; Kellermann et al., 2016a) suggested that AR is the intermediate during GDGT synthesis. However, this hypothesis remains controversial (reviewed by Pearson, 2014), resulting in the proposal of an alternative diether independent GDGT biosynthesis pathway (Villanueva et al., 2014).

Labeling experiments with thaumarchaeal isolates are scarce and have applied SIP to trace their carbon source (Jung et al., 2014; Kim et al., 2016b), whereas RIP-experiments were not performed yet. RIP incubation experiments with other cultured isolates or environmental samples have isolated individual lipids by thin layer or liquid chromatography (e.g. Nishihara et al., 1989; Roslev et al., 1998b) followed by off-line ^{14}C-detection. Chromatographic separation coupled with fraction collection may, however, result in inaccuracies and therefore several chromatographic steps must be performed to attain the required lipid purity. Flow-through scintillation counting allows an on-line compound-specific detection of the ^{14}C incorporation in organic molecules (Pinckney et al., 1996), but this technique has rarely been used in environmental science (Pinckney et al., 1996; Yakimov et al., 2011) and to our knowledge has not yet been applied to trace the incorporation of ^{14}C into individual lipids.

In this study, a 72-hour (h) experiment was performed with ^{14}C-bicarbonate to trace individual lipid production in the marine AOA model organism *N. maritimus*. *N. maritimus* was grown with both excess and limited NH_4^+ supply. The approach of lipid specific radio isotope probing (lipid-RIP) enabled us to investigate the effect of nutrition on the cyclization of GDGTs and the potential production of intermediates during tetraether synthesis. The ^{14}C incorporation into lipids was followed by coupled HPLC flow-through scintillation counting after cell hydrolysis of the total lipid extract, allowing specific resolution and accurate detection of individual TEX_{86}-related and other archaeal lipids.

III.3. Material and Methods

III.3.1. Cultivation

A 3-L pre-culture of *N. maritimus* was grown aerobically at 28 °C with 0.8 mM NH_4Cl in a modified SCM-1 medium (Könneke et al., 2005; Martens-Habbena et al., 2009). The pre-culture was inoculated with 200 mL of a *N. maritimus* culture, which was in exponential growth phase. The growth of the pre-culture was monitored via nitrite formation and purity was routinely checked by phase-contrast microscopy. After consumption of 0.5 mM NH_4^+, the pre-culture split for two experiments (1.25 L each). In experiment 1 (“non-limited”) 1 mM NH_4Cl and 2 MBq $Na^{14}CO_3$ (ARC; specific activity: 1.85-2.22 GBq $mmol^{-1}$) were added to the

culture. The culture in experiment 2 ("limited") was treated with 2 MBq $Na^{14}CO_3$, but no additional NH_4Cl was added. Both cultures were further grown at 28 °C for 72 h. The growth was verified by nitrite measurement, ^{14}C incorporation into cells, and direct count of SYBR-Green I stained cells (Lunau et al., 2005). Cells were harvested by centrifugation (30 minutes followed by 20 minutes at 3200x g) at five-time intervals after label addition (12, 24, 36, 48 and 72 h). Each experiment was performed with a single incubation. The reproducibility of results is supported by the internal consistency of the results obtained for the five time points. The cell pellets were stored frozen at -20 °C upon extraction. Due to increasing cellular concentrations over the course of the experiment, 400 mL of culture was harvested at 12 h and 24 h, while only 150 mL was harvested at 36 h, 48 h and 72 h.

III.3.2. ^{14}C incorporation into cells

^{14}C incorporation into cells was measured in duplicates on a filter with a nominal pore size of 0.1 µm (Merck Millipore Ltd.). 1 mL of the culture was mixed with 5 mL of a phosphate-buffer, filtered and washed two times with 10 mL phosphate buffer. The filter and 3 mL of scintillation solvent (Ultima Gold, PerkinElmer) were added to a scintillation vial and the radioactivity was measured for 100 min on a Liquid Scintillation Analyzer Tri-Carb® 2810 (Perkin Elmer). ^{14}C in the cells was measured from a starting time set as the addition of ^{14}C to the culture. The results presented were corrected for the time zero.

III.3.3. Extraction of lipids

Cell pellets were directly hydrolyzed by addition of 1M hydrochloric acid in methanol (MeOH) after addition of combusted sand to the extraction vial in order to facilitate cell rupture (Becker et al., 2016). The mixture was sonicated twice for 20 min in the ultrasonic bath and then hydrolyzed for 16 h at 70 °C. Following acid hydrolysis, the extract was sonicated for 20 min and assembled in a separatory funnel. The sand residue was re-extracted three times with dichloromethane (DCM):MeOH 5:1 (v:v) for 20 min by sonication. Deionized water was added to the extract, and following phase separation the organic phase, was drawn off. The aqueous phase was washed once with DCM and the organic phase three times with deionized water. The extract was dried under a stream of N_2 and stored at -20 °C until measurement.

III.3.4. ^{14}C incorporation into individual lipids

Lipid separation was carried out with an Agilent 1200 HPLC-system (Agilent Technologies, Wilmington, DE, USA) by an ACE3 C_{18} column (2.1x150 mm, 3 µm particle

size, Advanced Chromatography Technologies, Aberdeen, Scotland), following Evans et al. (2017). The column effluent was mixed with a liquid scintillation solvent (Insta-Flour, Perkin Elmer) supplied by an external pump (Raytest, Straubenhardt, Germany) at a flow rate of 1 mL min^{-1}. ^{14}C signals of individual lipids were detected with a RAMONA Quattro (Raytest) scintillation counter, equipped with a 4 x 220 µL flow cell and 4 pairs of 2 x 1 1/8" photomultipliers. Previously incubated *N. maritimus* culture showed a standard deviation of 8% of the total ^{14}C incorporation into lipids in the replicates (appendix A Table III.S1). The standard deviation of the ring index (RI) and the TEX_{86} is less than 2% in the replicates (appendix A Table III.S1).

III.3.5. Identification of microbial lipids

Archaeal lipids were identified with an LCQ Deca XP ion trap mass spectrometer (MS; Thermo Scientific, San Jose, CA, USA) under electro spray ionization (ESI) mode coupled to an Agilent 1200 HPLC-system. The separation of the lipids was carried out using the same column setting as described above. Lipids were identified by their retention time and characteristic MS^2 fragmentation. Integration was performed based on the extracted ion trace of the major ions [M+H+] and [M+NH_4+]. The quantification of lipids was achieved using C_{46}-GTGT (glycerol trialkyl glycerol tetraether; Huguet et al., 2006) added as an injection standard to the samples prior to the analysis (appendix A Fig. III.S1 and Table III.S2)

III.3.6. Calculations

Temperature reconstruction was based on the TEX_{86}^{L} (GDGT index-1) and the corresponding core top calibration as described by Kim et al. (2010):

$$TEX_{86}^{L}(GDGT\ index-1) = log\frac{(GDGT\ 2)}{(GDGT\ 1)+(GDGT\ 2)+(GDGT\ 3)} \qquad Eq.1$$

$$T[°C] = 67.5 \times TEX_{86}^{L} + 46.9 \qquad Eq.2$$

Additionally, temperature reconstruction was performed by using the CCaT and the LC based core top calibration described by Wörmer et al. (2014):

$$CCaT = \frac{Cren + Cren'}{GDGT\ 0 + Cren + Cren'} \qquad Eq.3$$

$$T[°C] = -18.758 + 64.774 \times CCaT$$

$Eq. 4$

To evaluate the cyclization of GDGTs, the RI was calculated following Eq. 5 (Pearson et al., 2004), where GDGT 0-4 and crenarchaeol (cren) is the fractional abundance of individual GDGTs relative to all GDGTs, including GDGT-0:

$$RI = \frac{1 \times (GDGT\ 1) + 2 \times (GDGT\ 2) + 3 \times (GDGT\ 3) + 4 \times (GDGT\ 4) + 5 \times (Cren + Cren')}{(GDGT\ 0) + (GDGT\ 1) + (GDGT\ 2) + (GDGT\ 3) + (GDGT\ 4) + (Cren + Cren')}$$

$Eq. 5$

The ratio of archaeol to tetraether (AR/T) in mole percent (mole%) and the ratio of methoxy archaeol to diethers (MeO-AR/D) were calculated by the following equations:

$$AR/T = \frac{AR}{Tetraethers + AR}$$

$Eq. 6$

$$MeO\ AR/D\ = \frac{MeO\ AR}{Diethers\ (AR + MeO\ AR)}$$

$Eq. 7$

III.4. Results

III.4.1. Growth of *N. maritimus* at different ammonium supply

After a period of 112 h of growth (exponential growth phase), when 0.5 M NH_4^+ were consumed, a pre-culture of *N. maritimus* was split for a non-limited (with additional NH_4^+) and a limited experiment. Setting this point to time zero, growth of *N. maritimus* was monitored by nitrite formation (Fig. III.1A), cell counts (Fig. III.1B) and ^{14}C incorporation into cells (Fig. III.1C). The non-limited culture remained in the growth phase, as indicated by an increasing concentration of nitrite and pronounced ^{14}C incorporation into biomass. The increased NH_4^+ respiration rate resulted in an elevated biomass production as determined by cell concentration. In the non-limited *N. maritimus* culture cells numbers doubled within 45 h. The limited experiment entered the transition to stationary phase after 12 h. The stationary phase was reached after 36 h when NH_4^+ was completely consumed as observed by a cessation of nitrite formation, incorporation of ^{14}C into biomass and constant cell counts.

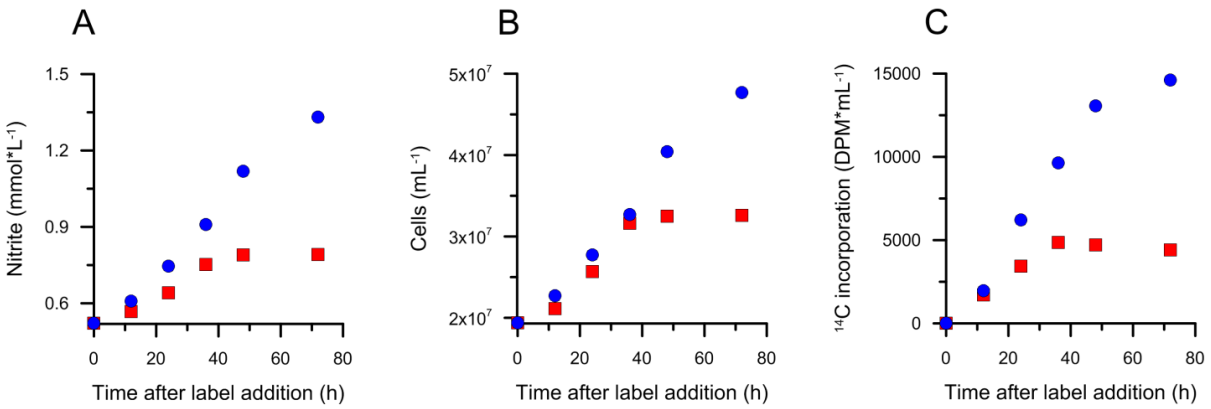

Fig. III.1 Growth of *N. maritimus* in both batch cultures, the NH_4^+ is shown as blue circles and the NH_4^+ limited culture as red squares. (A) nitrite production, (B) cell counts and (C) ^{14}C incorporation into cells.

III.4.2. ^{14}C incorporation into individual lipids and their relative distribution

Cells were extracted by acid hydrolysis. Nevertheless, ^{14}C incorporation showed that between 7% and 38% of the total radioactivity found in lipids was detected in monoglycosidic GDGTs (1G-GDGTs; Fig. III.2). Therefore, the concentration of 1G- and core-GDGTs (c-GDGTs) was summed, to take this non-hydrolyzed pool of intact GDGTs into account. The comparison of the calculated RI and TEX_{86}^L derived SST based on the c-GDGT vs. 1G and c-GDGTs is shown in Table III.S3 (appendix A) and differed by less than 0.1 and 2 °C, respectively.

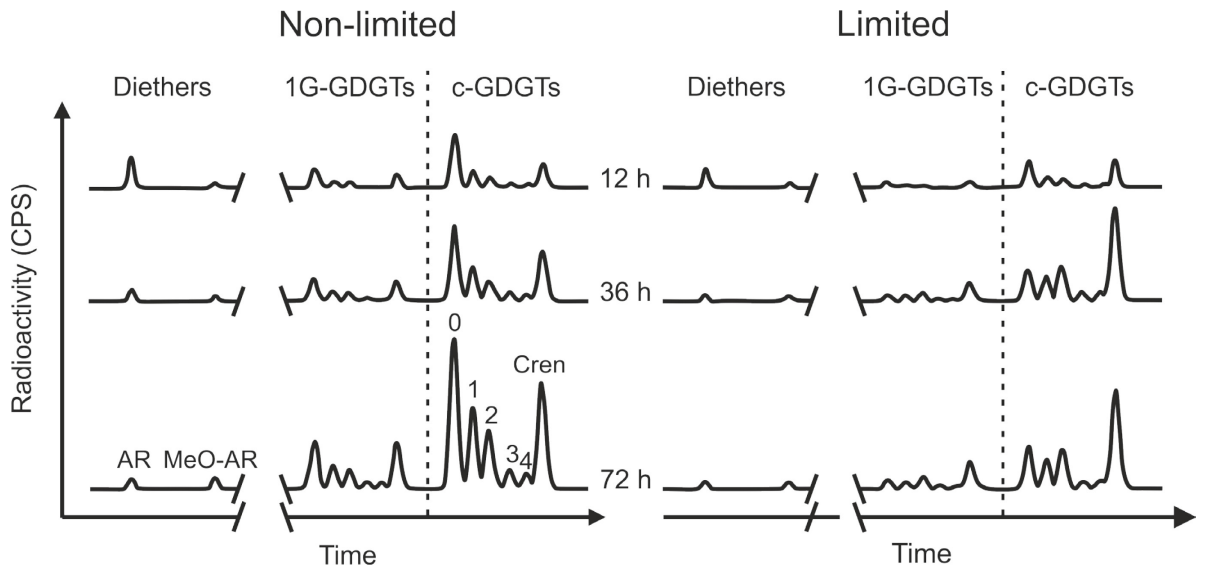

Fig. III.2. Radio chromatograms of the ^{14}C incorporation into individual diethers and glycerol dibiphytanyl glycerol tetraethers (GDGTs) over time in the non-limited and limited experiment. The radioactivity is shown as counts per second (CPS). The numbers 0 to 4 identify the number of rings of the corresponding GDGTs and crenarchaeol (cren). Note: At different harvesting time points the amount of extracted culture varied. (12 h = 400 mL, 24 h 400 mL, 36 h = 150 mL; 72 h = 150 mL).

The ^{14}C incorporation experiment exhibited strong differences between the non-limited and the limited culture (Fig. III.2). About three times more lipids were synthesized in the non-limited (261 ng ^{14}C L^{-1}) compared to the limited experiment (85 ng ^{14}C L^{-1}). The non-limited culture showed a nearly constant incorporation of ^{14}C into tetraethers and methoxy archaeol (MeO-AR) during growth (Fig. III.3). On the contrary, AR was only produced during the early growth stage and remained approximately constant further on. At the end of the non-limited experiment the highest label uptake was observed for GDGT-0 followed by crenarchaeol, GDGT-1 and GDGT-2. The other GDGTs showed only minor ^{14}C incorporation. The relative distribution of the ^{14}C incorporation into the lipids in the non-limited experiment exhibited a decrease of the AR/T-ratio from 23% to 3% during the experiment (Fig. III.4A), while the MeO-AR/D-ratio increased with time (Fig. III.4B). The relative ^{14}C incorporation into individual GDGTs revealed a decrease of the dominant GDGT-0 by 11%, while the relative ^{14}C incorporation into the other GDGTs slightly increased (Fig. III.5A).

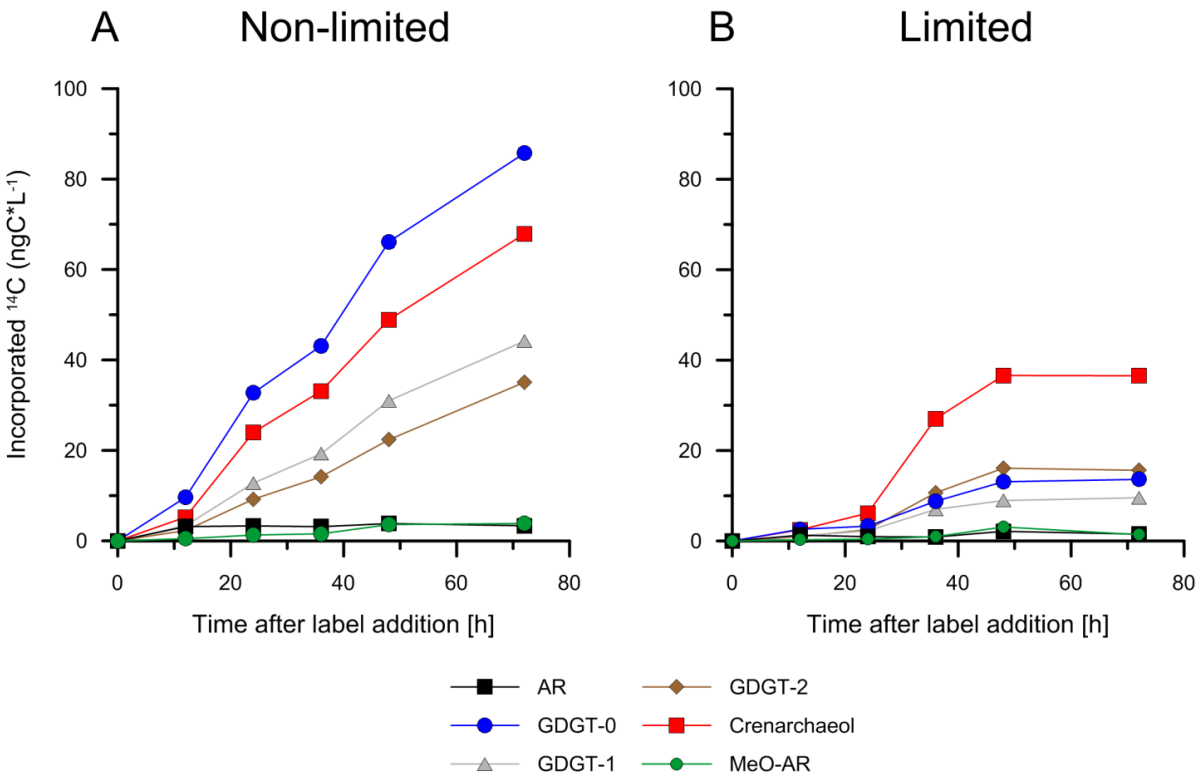

Fig. III.3. ^{14}C incorporation into individual GDGTs and diethers. The non-limited experiment is shown in panel (A) and the limited in panel (B). GDGT synthesis is calculated based on the sum of 1G-GDGTs and the equivalent c-GDGTs (compare with Fig. III.2).

The limited culture exhibited higher variations of the GDGT production than the non-limited culture (Fig. III.3). During growth, the GDGT-0 and crenarchaeol showed almost similar ^{14}C incorporation, followed by GDGT-1 and GDGT-2. A predominant production of crenarchaeol and GDGT-2 compared to the other GDGTs was observed at the transition to

the stationary phase. The AR/T-ratio and the MeO-AR/D-ratio showed a similar pattern in the limited experiment as observed in the non-limited experiment (Fig. III.4A and Fig. III.4B). The relative distribution of the ^{14}C incorporation into the GDGTs exhibited a decrease of GDGT-0 by 19% at the transition to the stationary phase (Fig. III.5B). The relative ^{14}C incorporation into GDGT-2 and crenarchaeol, on the contrary, showed an increase by 9 and 12%, respectively. During the stationary phase little variation was observed.

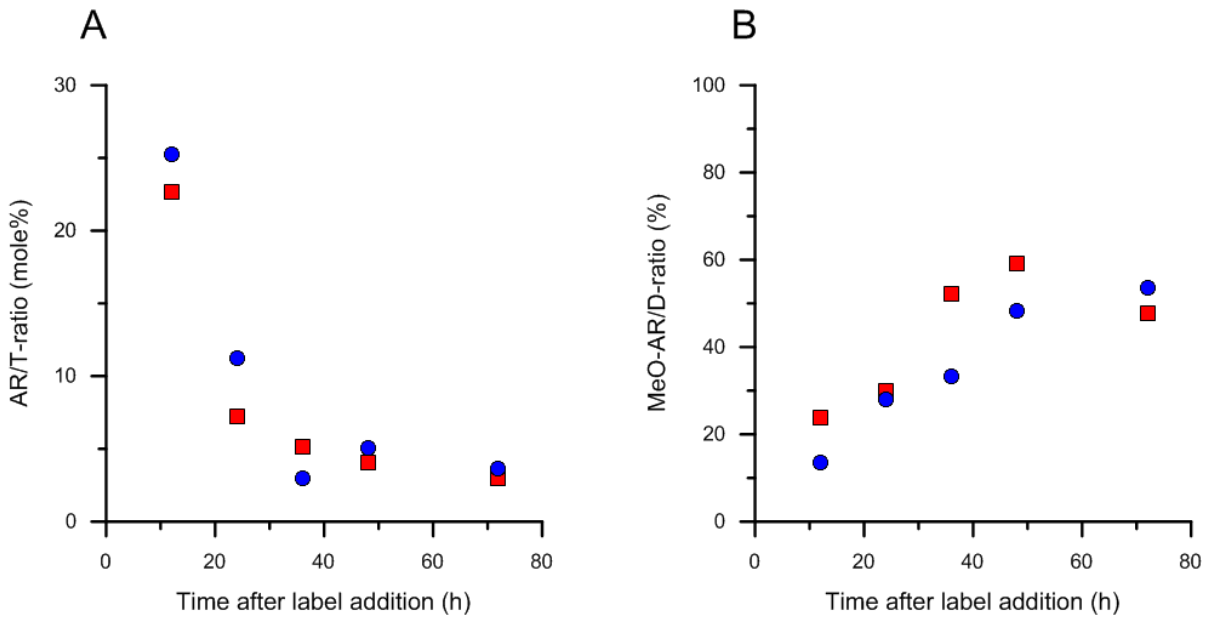

Fig. III.4 The AR to tetraether-ratio (AR/T -ratio) of the experiment with NH_4^+ excess (blue circles) and limited experiment (red squares) calculated in mole percent (mole%) over time (A). Panel B shows the ratio of methoxy archaeol to sum of the diethers (MeO-AR/D-ratio) during the experiment. The ^{14}C incorporation into tetraethers is calculated based on the sum of 1G-GDGTs and the equivalent c-GDGTs (compare with Fig. III.2).

Complementary to ^{14}C scintillation, the total microbial lipids were analyzed by MS. Results showed a low AR/T-ratio (< 0.1%; appendix A Table III.S2). The relative abundance of GDGT-0/-1 in the non-limited experiment increased at the expense of GDGT-2 and crenarchaeol during growth (appendix A Fig. III.S1A). The limited experiment showed, on the contrary, an increase of GDGT-2 and crenarchaeol (appendix A Fig. III.S1B).

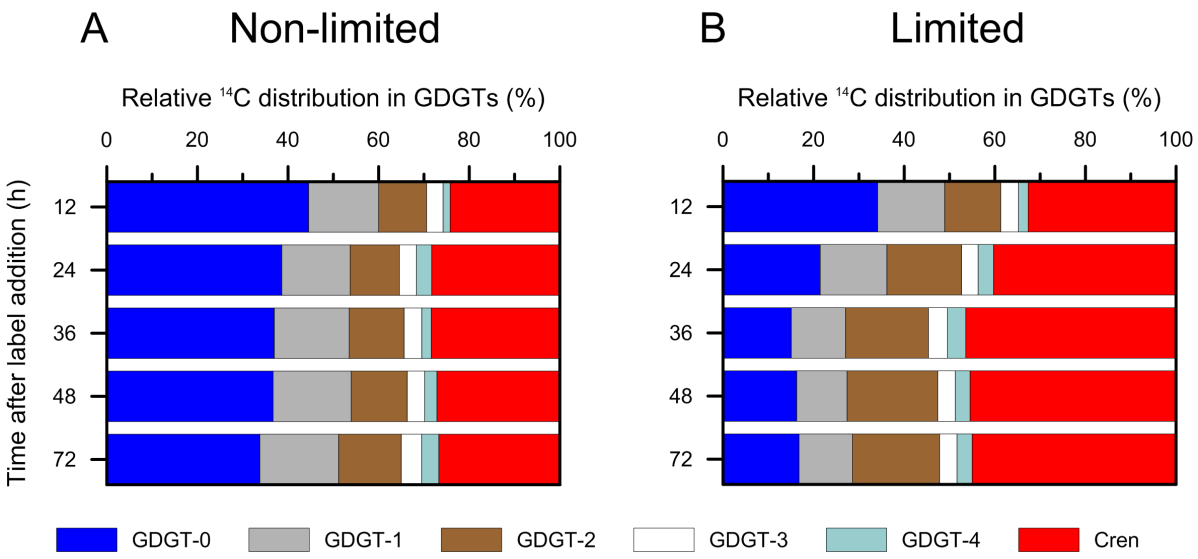

Fig. III.5. Relative ring distribution of produced lipids of the NH_4^+ non-limited (A) and the limited (B) incubation during the experiments. The distributions are calculated based on the sum of 1G-GDGTs and the equivalent c-GDGTs (compare with Fig. III.2).

III.4.3. Effect of ammonium supply on the cyclization of GDGTs

The good separation of individual ^{14}C-GDGTs signals by flow-through scintillation counting allowed us to calculate the weighted cyclization of the synthesized GDGTs (RI; Pearson et al., 2004). However, due to the absence of a defined peak of the regioisomer of crenarchaeol we were not able to estimate the SST using the classical TEX_{86} calibration by (Schouten et al., 2002). Therefore, the TEX_{86}^L (GDGT index-1; Kim et al., 2010) was computed, a derivative of the TEX_{86} not including the crenarchaeol regioisomer. Likewise, the calculation of the CCat as defined by Wörmer et al. (2014) did not involve the crenarchaeol regioisomer.

The RI in the non-limited culture increased during the experiment from 1.8 to 2.1 (Fig. III.6A). The reconstructed water temperature based on the TEX_{86}^L ranged between 17 and 19 °C in the non-limited culture and was approximately 10 °C lower than the incubation temperature (Fig. III.6B). The temperature calculated from the CCaT increased from 4 °C after 12 h to 10 °C at the end of the incubation and was more than 20 °C “colder” than the incubation temperature.

The limited experiment exhibited a pronounced increase of the RI and the temperature at the transition from the growth phase to the stationary phase and remained rather constant thereafter. The RI was up to one unit higher than in the non-limited experiment. Moreover, the TEX_{86}^L derived temperature was more than 10 °C and the CCaT based SST more than 20 °C higher compared to the culture incubated with excess NH_4^+,

respectively. The water temperature reconstructed from both proxies reached the incubation temperature in stationary phase in the limited experiment.

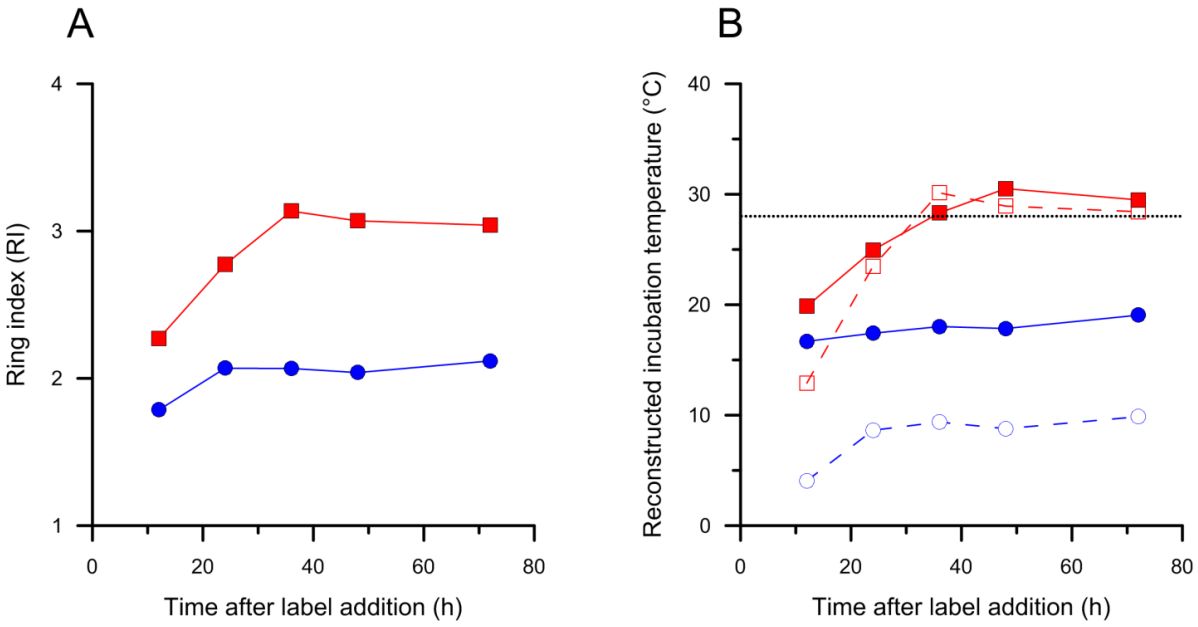

Fig. III.6. The calculated ring index based on the ^{14}C incorporation into GDGTs (A) of the non-limited (blue circles) and limited experiment (red squares). Panel B: The corresponding incubation temperature was reconstructed based on the TEX_{86}^{L} after Kim et al. (2010) and CCaT (Wörmer et al., 2014). Temperatures calculated from the TEX_{86}^{L} are displayed as filled symbols (circles = non-limited experiment, squares = limited incubation), while CCaT based results are shown as open symbols (circles non-limited experiment, squares = limited incubation). The dotted line shows the incubation temperature at 28°C. RI and incubation temperature are based on the sum of 1G-GDGTs and the equivalent c-GDGTs (compare with Fig. III.2).

III.5. Discussion

III.5.1. Methodological considerations

A 72 h ^{14}C bicarbonate incubation experiment with *N. maritimus* was performed to investigate the biosynthesis of diethers and GDGTs at different growth states with respect to the NH_4^+ supply. Multiple sampling points within such a short period allow monitoring lipid biosynthetic responses to changing nutrient supply on the time scale of one cell cycle (45 h in the experiment with NH_4^+ excess) and less. Longer batch incubations result in an accumulation of metabolic products and therefore overprint the lipid synthesis signal.

To the best of our knowledge this is the first lipid-RIP study in environmental geosciences that has applied flow-through scintillation counting to quantify the incorporation of a ^{14}C-labeled substrate into chromatographically well-resolved individual lipids (Fig. III.2)

and thus accurately determines biosynthesis of individual proxy-relevant compounds. By contrast, previous studies targeting archaeal lipids using SIP protocols employed labor-intensive ether cleavage techniques (e.g. Wuchter et al., 2003; Park et al., 2010; Lin et al., 2013; Kellermann et al., 2016a) to obtain GDGTs amenable for gas chromatography-based compound specific isotope analysis, while at the same time accepting a loss of information due to the non-specificity of the resulting biphytanes with respect to their GDGT precursors.

The co-elution of ^{14}C labeled lipids in flow-through scintillation counting may complicate the quantification of lipid-specific ^{14}C. To minimize the risk of co-elution during the measurement, the cells were hydrolyzed to convert intact polar into core lipids. While resulting in a loss of information, the acid hydrolysis of cells may drastically increase the extraction efficiency of archaeal and bacterial tetraethers compared to commonly applied extraction techniques (e.g. Sinninghe Damsté et al., 2011; Cario et al., 2015; Weber et al., 2017). Nevertheless, the measurements revealed that up to 38% of the total radioactivity found in lipids, was detected in 1G-GDGTs. Incomplete hydrolysis, possibly combined with proportionally higher ^{14}C assimilation into the glycosidic head group, could account for this observation. Indeed, a < 5% level of incomplete hydrolysis has been observed in experiments with the total lipid extract of *N. maritimus* (Elling et al., 2014), while preferential incorporation of ^{13}C into glycosidic head groups was identified in SIP experiments sedimentary archaeal populations (Lin et al., 2013).

Previous studies investigating the effect of physiological conditions or growth stage on the lipid distribution of marine AOA were predominantly based on MS analysis with cell harvesting after 6 to 40 days (Elling et al., 2014; Elling et al., 2015; Qin et al., 2015). The experimental approach applied in this study has the advantage that lipid production in *N. maritimus* can be directly traced via ^{14}C incorporation, whereas MS-based results, particularly in short-term experiments, are limited in their accuracy by the existing lipid pool of the culture at the beginning of the experiment. The differences between the two approaches are documented by example of the relative distributions of GDGTs and diethers in this study. Based on the ^{14}C incorporation, the non-limited (RI: 1.8-2.1) and the limited culture (RI: 2.3-3.0) showed a lower cyclization of the GDGTs (Fig. III.6A) than the total archaeal lipids obtained after MS quantification (RI: 3.6-4.2; appendix A Table III.S2). Previously published RIs for GDGTs in *N. maritimus* ranged between 3.1 and 3.3 during growth (Elling et al., 2014). The difference between the MS-based RI in our experiment compared to the study by Elling and colleagues (2014) possibly results from the application of two different mass spectrometers for quantification (see appendix A Table III.S4). Additionally, the ^{14}C incubation experiments revealed high label incorporation into diethers (3-25%) whereas MS-based results showed little contribution (< 0.1%; appendix A Table III.S2). Previous studies resulted in a heterogeneous picture with Elling et al. (2014) reporting diether abundances of

up to 13%, while another study that applied direct cell hydrolysis reported only “little” amounts of diethers (Schouten et al., 2008).

III.5.2. Biosynthesis of diethers and tetraethers as a function of cell cycle stage

In both the NH_4^+ non-limited and limited culture, the AR/T-ratio showed the same tendency with time (Fig. III.4A). One possible explanation for these observations is a head-to-head condensation of two AR-molecules forming GDGTs. This hypothesis was proposed from previous labeling experiments, which exhibited similar variations in the labeling pattern between AR and GDGTs in *Euryarchaeota* over time (Nishihara et al., 1989; Kellermann et al., 2016a). This mechanism was further investigated by a pulse-chase labeling experiment with terbinafine, an inhibitor for the formation of GDGTs, which showed that intact labeled ARs were converted to GDGTs after the removal of the inhibitor (Nemoto et al., 2003). Our results agree well with the hypothesis of AR being a precursor of GDGTs in *N. maritimus*. Furthermore, the constant AR/T-ratio in both experiments after 24 h suggests that the GDGT production is in equilibrium with the formation of AR during ongoing growth (Fig. III.4A). We further substantiated this hypothesis by prolonged a 140 h ^{14}C bicarbonate incubation experiment using the Euryarchaeon *Methanothermobacter thermoautotrophicus* that indicated an analogous behavior (appendix A Fig. III.S2). Specifically, we observed a high AR/T-ratio at the beginning of the experiment, a decrease within the first 14 h and relatively constant values after ~24 h, again suggesting equilibrium between AR formation and conversion to GDGTs.

The distinctly lower cyclization of GDGTs (Fig. III.6) in the non-limited and the limited ^{14}C experiment with *N. maritimus* compared to the MS measurements of this (appendix A Table III.S2) and previous studies (Schouten et al., 2008; Elling et al., 2014) further indicates a preferential synthesis of tetraethers with less cyclopentane/hexane rings during short-term incubation. This may suggest that the cyclization of GDGTs is initiated from the acyclic GDGT precursor and increases gradually to form multicyclic GDGTs (De Rosa et al., 1986). Therefore, our results challenge the pathway proposed for archaeal GDGT synthesis by Villanueva et al. (2014) based on the examination of amino acid sequences of central biosynthetic enzymes. According to their study, biphytanyl derivatives, including cycloalkylated ones, are being formed from two phytanyl derivatives (both acyclic and cycloalkylated) prior to etherbond linkage to glycerol. While our results do not categorically rule out this possibility, the apparent intermediate role of AR (Fig. III.4A) and the lower degree of cyclization in the exponential phase (Fig. III.6A) are rather supportive of the “conventional” pathway described by Koga and Morii (2007) involving initial synthesis of acyclic di- and tetraethers prior to cycloalkylation.

During the experiment an increase of the MeO-AR/D-ratio was observed in both incubations (Fig. III.4B). This suggests that MeO-AR, the second diether identified in this study, accumulates in the cell membrane and is not converted to GDGTs as proposed for AR. Although the biological function of the apolar MeO-AR remains unknown it was proposed as a specific biomarker for the phylum *Thaumarchaeota* (Elling et al., 2014; Elling et al., 2017). A potential function could be energy storage to overcome phases of dormancy, as observed for apolar triacyl glycerols in marine bacteria (reviewed by Alvarez and Steinbüchel, 2002). Elling et al. (2015) proposed that MeO-AR might be involved in the increase of membrane fluidity in marine AOA by separating the polar lipids, given that the abundance of this lipid inversely correlated with growth temperature in the isolate NAOA-2. A similar function was suggested for squalene in halophilic archaea (Lanyi et al., 1974).

III.5.3. Influence of the NH_4^+ respiration rate on the cyclization of GDGTs

Experiments with isolates of marine AOA have shown that the cyclization of GDGTs broadly correlates with growth temperature, resulting in an increase of RI with increasing temperature (e.g. Elling et al., 2015). However, recent results suggest that factors other than temperature might also affect the cyclization of GDTGs in marine *Thaumarchaeota* (Qin et al., 2015; Hurley et al., 2016).

The experiments performed here showed a strong effect of the NH_4^+ supply on the cyclization of the GDGTs. The NH_4^+ limited experiment revealed an up to one-unit higher RI than the non-limited culture grown with excess NH_4^+. This agrees well with recent findings giving evidence of a strong correlation between the cyclization of the total extracted lipids of *N. maritimus* with respect to variations in NH_4^+ respiration rates (Hurley et al., 2016). The study by Hurley et al. (2016) applied a different incubation strategy by the use of a chemostat system, while in our study *N. maritimus* was grown in batch cultures. The chemostat system allows direct manipulation of the NH_4^+-supply and other physical and chemical parameters. In batch cultures, on the other hand, the control of biological, physical and chemical variables during the course of incubation is difficult as nutrients are consumed and metabolic products accumulate; however, this effect is deemed negligible given the short duration of 72 h of our experiments. The good agreement between chemostat and the current batch results strongly suggests that the cyclization of GDGTs observed in our experiments is primarily influenced by NH_4^+-supply rather than by other parameters.

The effect of the cyclization of GDGTs on the membrane properties of Archaea is not fully constrained, but several studies of thermophilic archaea observed that cyclization increases with temperature and decreasing pH (see review by Oger and Cario, 2013). Accordingly, it was argued that cyclopentane/hexane rings in GDGTs stabilize the

membrane, and modeling experiments suggest a tighter lipid packing with an increasing number of rings in GDGTs (Gabriel and Chong, 2000). Marine AOA are well adapted for oligotrophic growth in NH_4^+ depleted environments (Martens-Habbena et al., 2009). Therefore, the variations of the newly synthesized GDGTs observed in *N. maritimus* suggest a response of the lipid membrane that allows energy conservation under NH_4^+ limited conditions due to a lowered permeability, which provide an energetic advantage for AOA under NH_4^+ limiting conditions as hypothesized by Hurley et al. (2016).

III.5.4. Implications for the TEX_{86} paleothermometer and environmental SST reconstructions

This strong relationship between the number of cyclopentane rings in surface sediments and the SST led to the development of the TEX_{86} paleothermometer (Schouten et al., 2002). The correlation between temperature and cyclization of GDGTs was confirmed in incubations with marine water samples (e.g. Schouten et al., 2007; Wuchter et al., 2004) and isolated marine *Thaumarchaeota* (Elling et al., 2014; Elling et al., 2015; Qin et al., 2015). However, the detailed mechanisms controlling the TEX_{86} are still not fully constrained. Thus, we monitored variations of the TEX_{86}^L during the course of the experiment, which was established for the reconstruction of the SST in particularly cold regions, but is also deemed appropriate for temperatures of up to 30 °C (Kim et al., 2010).

The ^{14}C-based results of the experiment with excess NH_4^+ led to TEX_{86}^L reconstructed incubation temperatures that were up to 10 °C lower than the limited experiment and the incubation temperature, while the calculated incubation temperature in the NH_4^+ limited experiment reached the incubation temperature in stationary phase (Fig. III.6B). This demonstrates the impact of the NH_4^+ supply on the TEX_{86} paleothermometer proxy. Excess NH_4^+ supply results in preferential production of GDGTs leading to underestimation of the calculated temperature. This cooling effect is even more pronounced for the CCaT, an index factoring in the relative abundances of GDGT-0 and crenarchaeol and its regioisomer and introduced as SST sensitive proxy for mass spectrometry imaging of marine sediment cores (Wörmer et al., 2014). CCaT-based reconstructed temperature estimates in the non-limited experiment are 4 °C during initial growth and then stabilize around 10 °C after 24 h (Fig. III.6). Further research is required to validate to what degree these lipid patterns are associated with excess NH_4^+ supply and whether underestimation of SST in oceanic and other environments may ultimately inform us about past periods of enhanced nutrient supply.

On the other hand, in the NH_4^+ limited experiment the reconstructed temperatures based on both the TEX_{86}^L and the CCaT are in good agreement with the incubation temperature from 36 hours onward, suggesting that at low NH_4^+ concentrations tetraether

based SST estimates may faithfully record actual ambient temperatures in ocean waters. Given that NH_4^+ concentrations in modern oceans are usually in the nM-range (e.g. Beman et al., 2012; Smith et al., 2014; Kim et al., 2016a), our results provide support for GDGT based SST applications in oligotrophic paleoenvironments

The observed relationship between NH_4^+ respiration and TEX_{86}^L in both experiments may help to explain the discrepancies of the SST calculated from SPM and *in situ* water column temperature of the in the epi- and meso/bathypelagic ocean. GDGT-2 and crenarchaeol are predominantly produced in the experiment with NH_4^+ limitation. Previous studies, that measured the relative abundance of GDGTs in the water column, commonly showed an increase of GDGT-2 and the crenarchaeol-isomer in the oligotrophic meso/bathypelagic compared to the epipelagic layer (Basse et al., 2014; Kim et al., 2015; Kim et al., 2016a). The meso/bathypelagic layer is characterized by reduced NH_4^+ oxidation rates compared to the epipelagic ocean (Beman et al., 2008; Santoro et al., 2010; Smith et al., 2016). The variations of the oxidation rates result in a clustering of marine AOA with depth into a more active high NH_4^+ “surface” cluster and less active low NH_4^+ “subsurface” cluster (Santoro et al., 2010; Beman et al., 2012; Sintes et al., 2013; Smith et al., 2014; Smith et al., 2016). The combination of extremely low NH_4^+ respiration rates and changes in the predominant marine AOA species with depth potentially affects the TEX_{86} paleothermometer and results in reconstructed temperatures warmer than the *in situ* temperature.

In polar to temperate coastal regions a cold-biased TEX_{86} signal is commonly observed in core top sediments, resulting in colder SSTs than the *in situ* temperature (e.g. Herfort et al., 2006; Leider et al., 2010; Ho et al., 2014; Wang et al., 2015). Different hypotheses were offered to explain the too cold TEX_{86}-derived temperatures, such as an alteration of the marine AOA GDGT-signature by tetraether lipid producing planktonic *Euryarchaeota* (Wang et al., 2015) or a preferential growth of marine AOA during winter (Herfort et al., 2006). However, our experiments suggest that an alternative mechanism should be considered, i.e., in coastal waters marine AOA are stimulated by terrestrial input of NH_4^+ resulting in enhanced cyclization of GDGTs and thus cold TEX_{86}-derived SSTs. In oligotrophic open ocean settings, on the contrary, where less NH_4^+ is available than in coastal waters, marine AOA produce a GDGT pattern that leads to warmer TEX_{86} based SSTs, as observed by Ho et al. (2014) and Leider et al. (2010). Therefore, we suggest that GDGT distributions should be further examined for their proxy potential by tracking changes in NH_4^+ supply.

III.6. Conclusion

This study used lipid-RIP employing HPLC separation and flow-through scintillation counting to investigate rapid biosynthetic responses of the membrane lipid composition of the marine AOA *N. maritimus* to NH_4^+ supply and limitation. The sensitivity of the technique enabled accurate determination of freshly produced lipids at multiple time points within a time span of single cell cycle (45 hours). In comparison to previous studies with AOA cultures that harvested lipids after ~5 to 10 cycles, we were able to solidify hypothetical precursor-product relationships between AR and GDGTs unaffected by accumulation of compounds from several cell cycles and contrasting batch cultivation conditions. AR was preferentially produced at the beginning of the experiment, before reaching an equilibrium state with an AR/T ratio of 4%. Excess NH_4^+-supply resulted in distinct GDGT production patterns with a reduced cyclization of GDGTs and correspondingly lower TEX_{86}^L and CCaT based temperature estimates compared to incubation temperatures. The calculated temperature in the limited experiment approached incubation temperature when NH_4^+ was exhausted, indicating that temperature proxies might reflect the SST in oligotrophic paleoenvironments.

III.7. Acknowledgments

We thank F. J. Elling for providing *N. maritimus* biomass. This study was supported by the Deutsche Forschungsgemeinschaft through the Gottfried Wilhelm Leibniz Price to KUH (HI-616/14-1) and the Heisenberg fellowship awarded to MK (KO 3651/3-1).

III.8. Appendix A: supporting information

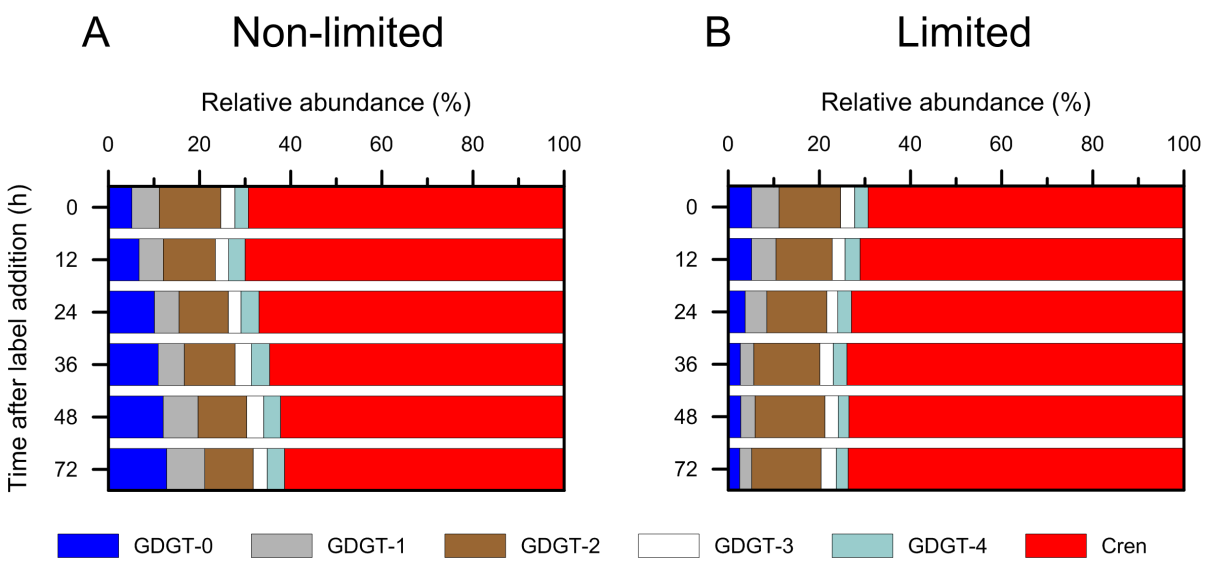

Fig. III.S1. Relative abundance of glycerol dibiphytanyl glycerol tetraether (GDGTs) measured by mass spectrometry in the experiment with NH_4^+ excess (A) and NH_4^+ limitation (B) during the experiment.

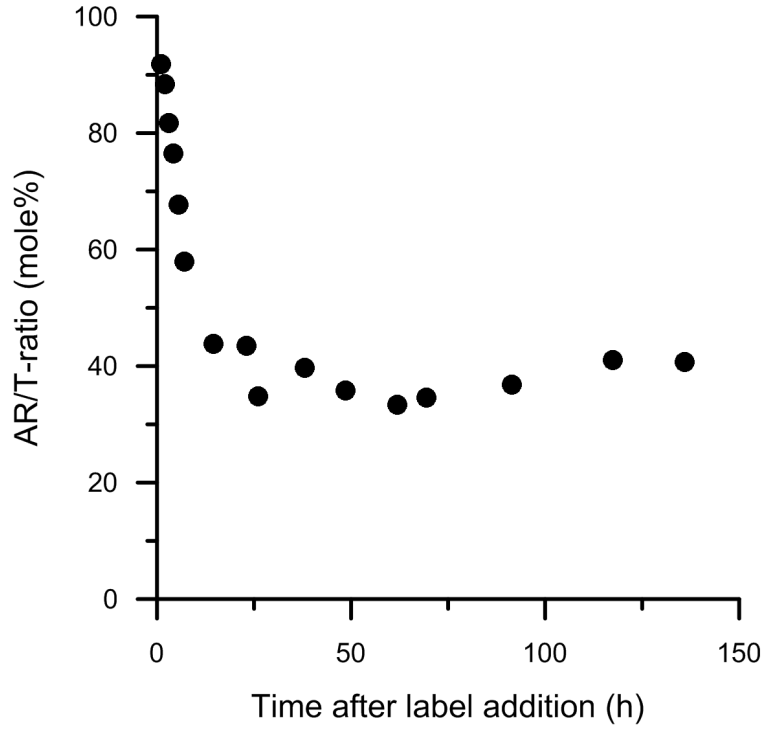

Fig. III.S2. Archaeol to tetraether ratio (AR/T-ratio) of a 140 hour ^{14}C-bicarbonate incubation experiment performed with *Methanothermobacter thermoautotrophicus* calculated in mole percent (mole%). Incorporation of ^{14}C into individual lipids was measured by flow-through scintillation counting after acidic cell hydrolysis.

Table III.S1: A triplicate ^{14}C measurement of a *Nitrosopumilus maritimus* culture by flow-through scintillation counting. The table displays the radioactivity in counts per second (cps) detected in the major glycerol dibiphytanyl glycerol tetraethers (GDGT-0, -1, -2, cren) and the total ^{14}C in GDGTs with the corresponding ring index (RI; Pearson et al., 2004) and the reconstructed incubation temperature (Temp). The culture was harvested in late stationary phase after 20 days of incubation and extracted by acid hydrolysis. [1]GDGTs represent the ^{14}C incorporation into all tetraethers including the minor GDGT-3 and GDGT-4 that are not displayed in the table. [2]Temp calculated by TEX_{86}^{L} (Kim et al., 2010). [3]Temp based on CCaT (Wörmer et al., 2014).

#	GDGT-0 (cps)	GDGT-1 (cps)	GDGT-2 (cps)	Cren (cps)	[1]GDGTs (cps)	RI	[2]Temp (°C)	[3]Temp (°C)
1	701	618	2211	5120	9224	3.5	34.3	38.2
2	601	468	1821	4451	7721	3.6	35.7	38.3
3	761	599	2302	4764	8915	3.4	35.2	37.0
Average	687	561	2111	4763	8621	3.5	35.0	37.9
Standard deviation	66	67	208	274	648	0.1	0.6	0.6

Table III.S2. Variation of the archaeol to tetraether ratio (AR/T-ratio), methoxy archaeol to diether ratio (MeO-AR/D-ratio), the ring index (RI) and the incubated calculated temperature (Temp) based on the total lipids measured by mass spectrometry in the experiment with excess NH_4^+ and the limited experiment. The SST was calculated by the TEX_{86}^{L} (GDGT index-1; Kim et al., 2010) and the RI according to Pearson et al. (2004).

	Non-limited experiment				Limited experiment			
Time (h)	AR/T-ratio (%)	MeO-AR/D-ratio (%)	RI	Temp (°C)	AR/T-ratio (%)	MeO-AR/D-ratio (%)	RI	Temp (°C)
0	0.03	33.8	4.0	36	0.03	33.8	4.0	36
12	0.03	25.7	4.0	34	0.02	35.8	.1	36
24	0.06	28.9	3.9	28	0.01	41.0	4.2	39
36	0.10	34.4	3.8	27	0.01	48.8	4.2	41
48	0.03	35.3	3.7	25	0.02	66.8	4.2	41
72	0.01	49.0	3.6	24	0.02	56.4	4.2	41

Table III.S3. Ring index (RI, Pearson et al., 2004) and the reconstructed growth temperature derived from the ^{14}C incorporation into core glycerol dibiphytanyl glycerol tetraether distribution (c-GDGT) and into total GDGTs. The SST was calculated based on the TEX_{86}^{L} (GDGT-1 index) following Kim et al. (2010) and the CCaT as described by Wörmer et al. (2014).

	RI		TEX_{86}^{L} temperature (°C)		CCaT temperature (°C)	
Time (h)	c-GDGTs	Total-GDGTs	c-GDGTs	Total-GDGTs	c-GDGTs	Total-GDGTs
Limited experiment						
12	2.20	2.27	19.7	19.9	11.8	12.9
24	2.72	2.77	24.2	25.0	22.9	23.5
36	3.10	3.14	28.5	28.3	30.0	30.1
48	3.07	3.07	30.3	30.5	29.6	28.9
72	2.97	3.04	29.7	29.5	27.8	28.4
Non-limited experiment						
12	1.71	1.79	16.1	16.7	2.9	4.1
24	1.98	2.07	17.2	17.4	7.6	8.6
36	2.02	2.07	17.7	18.0	8.9	9.4
48	2.01	2.04	17.4	17.8	8.6	8.8
72	2.03	2.12	19.4	19.1	8.9	9.9

Table III.S4. Relative glycerol dibiphytanyl glycerol tetraether distribution (GDGT), ring index (RI; Pearson et al., 2004) and the TEX_{86}^{L} (GDGT index-1) derived incubation temperature – comparing measurements by the LCQ mass spectrometer (this study) and Q-ToF (e.g. Elling et al., 2014). The incubation temperature was calculated based on the TEX_{86}^{L} as described by Kim et al. (2010). The results show the mean values of triplicate (Q-ToF) and fivefold (LCQ) measurements of a *Nitrosopumilus maritimus* culture, extracted by acid hydrolysis. The *N. maritimus* culture was harvested in exponential growth phase.

MS	GDGT-0 (%)	GDGT-1 (%)	GDGT-2 (%)	GDGT-3 (%)	GDGT-4 (%)	Cren (%)	RI	Temp (°C)
LCQ	5.1	5.3	12.0	2.4	1.2	73.5	4.1	32
Q-ToF	12.7	10.4	19.2	4.1	3.8	49.7	3.2	30

Chapter IV

The lipid biosynthesis of auto- and heterotrophic planktonic archaea examined by the incorporation of radiolabeled carbon substrates

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IV.1. Abstract

Planktonic archaea occur ubiquitously in the marine water column and are considered to be important participants in global nutrient cycles. They can be subdivided into two phyla: *Thaumarchaeota* (formerly classified as *Crenarchaeota*) and *Euryarchaeota*. The lipid biosynthesis of planktonic archaea is still poorly understood. To this end, mesocosm experiments were performed with water samples collected in different water depths from two oceanographically contrasting sites, from the shelf and a fjord, offshore Svalbard, Norway. The activity and lipid biosynthesis of planktonic archaea was investigated by radioisotope probing experiments with two ^{14}C labeled substrates. To trace heterotrophic growth, 2-^{14}C-leucine was applied, while ^{14}C-bicarbonate was used to constrain autotrophic carbon assimilation by marine planktonic archaea. For samples from both sites, the experiments with leucine showed a high incorporation of ^{14}C into unsaturated archaeol derivatives, representing 65 to 70% of the total labeled archaeal lipids. This finding substantiates previous suggestions, based on indirect evidence, of unsaturated archaeols being suitable biomarkers for marine *Euryarchaeota*. Additionally, the incorporation of leucine into methoxy archaeol, a specific biomarker for *Thaumarchaeota*, suggests, that these organisms are capable of a mixotrophic lifestyle. The mesocosm experiments with bicarbonate showed a predominant production of glycerol dibiphytanyl glycerol tetraethers (GDGTs) and methoxy archaeol. A higher incorporation of ^{14}C-DIC into lipids associated with planktonic *Euryarchaeota* in the fjord compared to the shelf further suggests that these organisms are mixotrophs and that *Euryarchaeota* are more active in coastal (fjord) compared to open ocean settings (shelf). Hence, this study provides new insights on preferential heterotrophic and autotrophic carbon assimilation of planktonic archaeal communities from specific niches in polar waters and provides evidence for specific euryarchaeal biomarker lipids.

Keywords: Radioisotope probing, planktonic archaea, autotrophy, heterotrophy, *Euryarchaeota*, *Thaumarchaeota*, diethers, tetraethers

IV.2. Introduction

Planktonic marine archaea can be subdivided into two major phyla. One of them is *Thaumarchaeota*, belonging to the Marine Group I (MG I; formerly classified as *Crenarchaeota*; Brochier-Armanet et al., 2008). It is estimated that MG I compromises 20% of the marine prokaryotes in the open oceans (Karner et al., 2001; Schattenhofer et al., 2009). Experiments with pure cultures (e.g. Könneke et al., 2005) and environmental gene assays (e.g. Francis et al., 2005; Santoro et al., 2010) suggest that marine *Thaumarchaeota* obtain energy via oxidation of ammonia to nitrite, which is the primary and rate-limiting step of nitrification. Therefore, *Thaumarchaeota* are considered to be key players in the nitrogen cycle (e.g. Beman et al., 2008, 2012; Santoro et al., 2010; Smith et al., 2016). *Thaumarchaeota* are thought to be predominantly autotrophs that utilize bicarbonate (DIC) as a carbon source to produce biomass (e.g. Wuchter et al., 2003; Könneke et al., 2005). However, based on environmental and pure culture studies, mixotrophic behavior has also been indicated (Ingalls et al., 2006; Tourna et al., 2011; Alonso-Sáez et al., 2012; Qin et al., 2014). The Marine Groups II to IV (MG II to IV) belong to the phylum *Euryarchaeota* (DeLong, 1992; DeLong, 1998; López-García et al., 2001). Among these, MG II is considered to be the most abundant group in pelagic environments (DeLong, 1992; Karner et al., 2001). Due to the lack of cultivated representatives of marine planktonic *Euryarchaeota*, identification of their preferential carbon fixation pathway remains elusive. However, application of microautoradiography to environmental samples (Herndl et al., 2005; Alderkamp et al., 2006) as well as metagenomics data suggest that *Euryarchaeota* are heterotrophs, utilizing amino acids and higher molecular organic carbon sources (Iverson et al., 2012; Deschamps et al., 2014; Martin-Cuadrado et al., 2015; Orsi et al., 2015). Previous studies showed that MG II *Euryarchaeota* prevail in the photic layer, while marine *Thaumarchaeota* (MG I) and MG III are predominantly identified in the dark ocean (e.g. Massana et al., 2000; Karner et al., 2001; Herndl et al., 2005; Kirchman et al., 2007).

Since DNA-based techniques do not provide information on microbial response to changing environmental regimes, the investigation of microbial membrane lipids provides a valuable tool to study these mechanisms. Based on the presence of biomarker lipids, information on the environmental distribution of marine planktonic archaea is often gained via *in situ* pump filtration of pelagic biomass (e.g. Basse et al., 2014; Lincoln et al., 2014; Xie et al., 2014; Zhu et al., 2016). Earlier studies focused on the investigation of glycerol dibiphytanyl glycerol tetraethers (GDGTs) which are ubiquitously detected in the marine water column (Wuchter et al., 2005; Schouten et al., 2012). It is suggested that GDGTs, containing zero to four cyclopentane rings, and crenarchaeol, containing four cyclopentane and one cyclohexane ring, are produced by *Thaumarchaeota* (e.g. Sinninghe Damsté et al.,

2002; de la Torre et al., 2008; Schouten et al., 2008; Pitcher et al., 2010; Elling et al., 2014). On the contrary, planktonic *Euryarchaeota* are considered to predominantly construct their lipid membrane from diphytanyl glycerol diether (archaeol; AR; Ingalls et al., 2006). However, the lipid-based chemotaxonomic differentiation of the two marine planktonic archaeal phyla is not exclusive, as indicated by recent studies, that suggested crenarchaeol as a building block of the euryarchaeal lipid membrane (Lincoln et al., 2014) and showed that *Thaumarchaeota* contain diether lipids (Elling et al., 2017). Therefore, more specific biomarkers were proposed, with methoxy archaeol (MeO-AR) being a marker for *Thaumarchaeot*a (Elling et al., 2014; Elling et al., 2015). Even though the lipidome of marine *Euryarchaeota* is less constrained, unsaturated (uns)-AR and uns-GDGT were empirically identified as potential biomarkers for these organisms (Zhu et al., 2016).

DNA- and lipid-based techniques have been successfully applied to environmental samples to investigate the abundance of microbial communities and their phylogeny. However, these techniques provide limited information on microbial activity, biomass formation and food-web strategies of prokaryotes in the marine environment. Isotope labeling experiments have been shown to be a valuable tool for the investigation of microbial processes in environmental samples (reviewed by Boschker and Middelburg, 2002; Radajewski et al., 2003; Wegener et al., 2016). Isotopic tracers, for instance, were successfully applied to evaluate the utilization of different carbon sources by marine pelagic archaea and bacteria (e.g. Herndl et al., 2005; Kirchman et al., 2007; Alonso-Sáez et al., 2012). Those results showed that autotrophic planktonic archaea are responsible for up to 84% of the total prokaryotic production in the North Atlantic (Herndl et al., 2005). However, most environmental studies concentrated on the incorporation of labeled substrates into DNA or cells, whereas experiments that targeted microbial lipid biomarkers are scarce (Wuchter et al., 2003). Accordingly, the lipid biosynthetic pathways of heterotrophic and autotrophic planktonic archaea are poorly understood. Unraveling the lipid synthesis of marine archaea is crucial to understand their physiology and adaptation to environmental changes. Moreover, labeling studies with archaeal lipids, which are ubiquitously detected in the marine water column, will provide important information to evaluate their biomarker potential and to assign the lipids to their biological source.

To investigate the activity of planktonic archaea and the associated biosynthesis of specific lipid biomarkers, ^{14}C-labeling experiments were performed in this study. The microbial communities were investigated in marine water column samples from two sites offshore Svalbard. The ^{14}C-labeling experiments were performed with two different substrates: ^{14}C-leucine (LEU) and ^{14}C-DIC. ^{14}C-LEU incorporation is applied to asses heterotrophic carbon assimilation (Kirchman et al., 1985; Kirchman, 2001), but has not been combined with lipid radioisotope probing (lipid-RIP). Inorganic carbon is the main/only carbon

source for chemolithoautotrophic organisms, which fixate carbon either via the Calvin-Benson-Bassham cycle (reviewed by Hügler and Sievert, 2011) or, in the case of Archaea, via the 3-hydroxypropionate/4-hydroxybutyrate cycle (Berg et al., 2007). Hence, ^{14}C-LEU and ^{14}C-DIC represent characteristic sources for heterotrophic and autotrophic carbon assimilation, respectively and are thus suitable tracers for tracking the production of lipid biomarkers by these groups of planktonic archaea.

IV.3. Material and Method

IV.3.1. Study site and sample collection

Samples were collected during cruise HE449 with RV Heincke in August 2015 in proximity to the Svalbard archipelago. To study the activity of planktonic archaea in contrasting oceanic habitats, two sites were chosen for the incubation experiments: an open ocean setting located in a depression on the Hornsund Shelf (Hornsund Basin; GeoB20110), and an enclosed fjord with little exchange of water masses (Van Mijenfjorden; GeoB20129; Fig. IV.1).

The water masses west of Spitsbergen are characterized by the South Cape and the West Spitzbergen Current. The South Cape Current transports cold and fresh Arctic water from the Barents Sea (Saloranta and Haugan, 2001), while the West Spitzbergen Current carries warmer and more saline water from the Atlantic (Aagaard et al., 1987). The two water masses are separated by the Arctic front. Near the ocean surface (0-50 meters below the sea level; mbsl) the Arctic front has a strong density gradient that prevents the water masses from mixing, while the subsurface front (50- 150 mbsl) appears only as a temperature-salinity boundary (Saloranta and Svendsen, 2001). The depths sampled at Hornsund Basin (Fig. IV.1) hence represent water masses transported with the South Cape Current, mixing with Atlantic water at greater depth. The South Cape Current prevents Atlantic water from entering the fjords along the west coast of Spitsbergen. Moreover, the highly sheltered Akseløya island at the entrance of the Van Mijenfjorden acts as a barrier and limits exchange with waters from the shelf. During summer, the water column in the fjords at the west coast of Svalbard develops a three layer structure, with fresh surface water at the top, derived from sea ice melting and river/glacial input (Cottier et al., 2005). The intermediate layer (Svendsen et al., 2002) evolves via mixing between fresh surface water and the underlying saline water mass, which is generated during sea ice formation and preserved over time behind the sill (Cottier et al., 2005).

The sampling and hydrographic measurements were carried out using the ship-based Sea Bird SBE911 plus CTD (conductivity, temperature, depth). The water samples were

collected by 12 Niskin bottles (5 L each) attached to the rosette. At each site three different water depths were investigated. At the station located on the Hornsund Shelf samples were taken at 5, 30 and 80 mbsl, while in the Van Mijenfjorden sampling depths were 5, 20 and 65 mbsl, to collect samples from the different water masses. For each incubation experiment 10 L of the collected water was immediately transferred to sterile 20 L Schott glass bottles and incubated on board with ^{14}C labeled substrates.

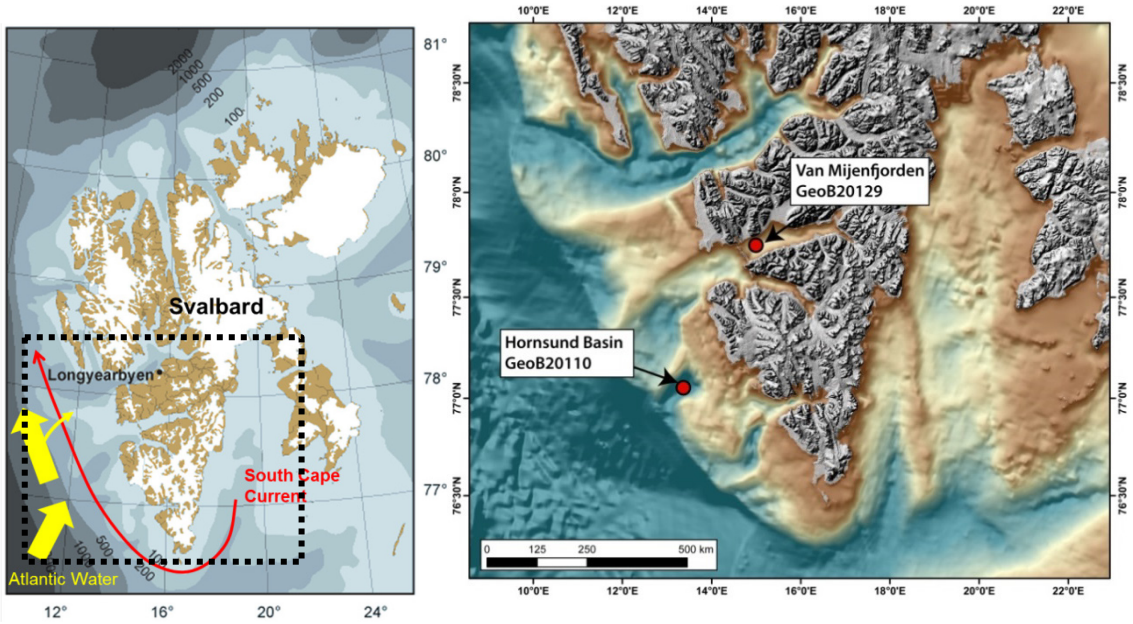

Fig. IV.1. Map of the Svalbard archipelago and the main currents along its western coast (left). Detailed map of the sampling sites investigated during the cruise HE449 (right).

IV.3.2. The *in situ* composition of archaeal lipids

The two study sites are characterized by depth-dependent differences of the *in situ* archaeal lipid composition (Goldenstein et al., in preparation), indicating distinct archaeal communities within the analyzed water masses. Uns-ARs are the predominant archaeal lipids in the two upper samples at each site samples, representing between 50% (Van Mijenfjorden) and 65% (Hornsund Basin) of the total archaeal lipids (appendix A Fig. IV.S1). The IPL-GDGTs become the major fraction of the archaeal lipid pool in the water column at 65 mbsl in the Van Mijenfjorden and at 80 mbsl at the Hornsund Basin (appendix A Fig. IV.S1). The IPL-GDGTs are characterized by a high fraction of hydroxylated GDGTs, particularly in the Van Mijenfjorden samples (ca. 50% of the total IPL-GDGTs in the fjord).

IV.3.3. Incubation experiments

For each site and sampling depth, three incubation experiments were performed with 10 L of seawater in 20 L Schott bottles (Fig. IV.2). One bottle per site and depth was treated with 0.03 MBq 2-^{14}C-L-LEU (specific activity: 1.85-2.22 GBq*mmol^{-1}; American Radioactive Chemicals) to monitor heterotrophic productivity. A second bottle was treated with 0.12 MBq ^{14}C-DIC (specific activity: 1.85-2.22 GBq*mmol^{-1}; American Radioactive Chemicals) to investigate autotrophic carbon fixation. The third bottle was incubated as a control without ^{14}C-labeled substrate. The three experiments were incubated at ambient pressure, in the dark, and at 4°C to simulate *in situ* conditions (*in situ* temperature range 0-8 °C). The shipboard movement and daily stirring kept the incubations agitated. Growth of organisms was monitored by daily measurements of ^{14}C incorporation into total cells in the ^{14}C amended samples. On-board measurement of ^{14}C incorporation of radioactive substrates into biomass was conducted by scintillation counting of biomass in 10 mL subsamples filtered by 25 mm diameter filters with a nominal pore size of 0.1 µm (Merck Millipore Ltd). Filters were rinsed with 10 mL of a mixture of phosphate buffer saline and Milli-Q water (v:v; 10:90) to remove extracellular radioactive material and transferred to a scintillation vial. 3 mL of the liquid scintillation counting solvent were added to the sample (Ultima Gold, Perkin Elmer) and the samples were counted for 15 minutes on a portable scintillation counter (Hidex). The initial ^{14}C-incorporation into cells was recorded at the beginning of the experiment. The incorporation of ^{14}C into cells at later growth stages was corrected for the value recorded at the beginning of the experiment. The results of the onboard ^{14}C measurement are shown as counts per minute (CPM). The mesocosms were stopped when the stationary growth phase was reached after 96 hours (h; Hornsund Basin) or 120 h (Van Mijenfjorden) of incubation. Samples were harvested via filtration of incubated biomass on polyethersulfone membrane filters (Sartorius Stedim Biotech; pore size 0.1 µm), using pre-filters with 3 µm pore size for exclusion of multicellular organisms. Filters were immediately stored frozen at -80 °C for shore-based analyses of ^{14}C incorporation into lipid biomarkers.

IV.3.4. Lipid extraction

Lipids were extracted from frozen filter samples using a modified Bligh and Dyer protocol following Sturt et al. (2004). Samples were treated with a mixture of dichloromethane:methanol:aqueous buffer (DCM:MeOH:buffer; 1:2:0.8; v:v:v) and placed in an ultrasonic bath for 10 minutes. This procedure was performed in four steps, using phosphate buffer (K_2HPO_4 − 50 mM at pH 7.4) for the first two steps, and trichloroacetic acid buffer (50 g/L, pH 2) for the last two steps. After sonication, the mixture was centrifuged (10 minutes at 2000 revolutions per minute) and the supernatant was collected. DCM and

deionized Milli-Q water (1:1; v/v) were added to the combined supernatants to allow phase separation. The organic phase was separated and the remaining aqueous phase was washed three times with DCM. The collected organic phase was subsequently washed three times with Milli-Q water and dried under a stream of nitrogen at room temperature. The TLE was stored at -20°C for further analysis.

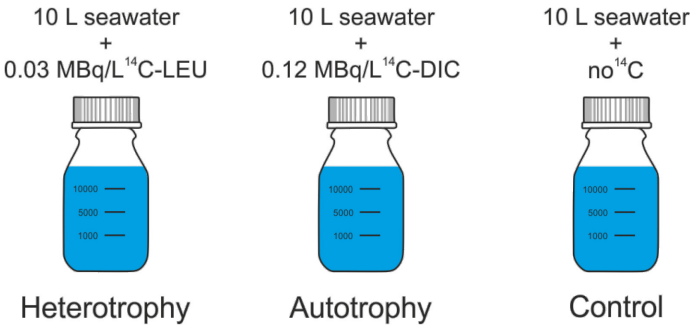

Fig. IV.2. Experimental setup for mesocosm experiments performed at the Hornsund Basin and the Van Mijenfjorden. For each site and each depth, three bottles were filled with 10 L seawater. Bottle one and two were amended with ^{14}C-leucine (LEU) or ^{14}C-bicarbonate (DIC), respectively. Bottle three was incubated without additional ^{14}C as a control.

IV.3.5. Analysis of ^{14}C assimilation

The extracted lipids were separated using an Agilent 1200 high-pressure liquid chromatography (HPLC) equipped with a ACE3 C_{18} column using reversed phase separation following Evans et al. (2017). The HPLC was either coupled to a fraction collector (Gilson) for preparative LC-based fractionation, or to a mass spectrometer (MS; LCQ Deca XP plus Thermo Fisher) for lipid identification. The time-based fraction collection method was established based on retention times of compounds of interest present in a representative master sample. The representative sample was obtained by combining lipid extracts derived from the planktonic thaumarchaeon *N. maritimus* (Elling et al., 2014) and the euryarchaeon *Cuniculiplasma divulgatum* (Golyshina et al., 2016). The lipid fractions contained eukaryal and bacterial lipids in F1, archaeal unsaturated archaeols (uns-AR) in F2, core archaeol (c-AR) in F3, methoxy archaeol (MeO-AR) in F4, head group containing intact polar (IPL)-GDGT, hydroxylated (OH) and unsaturated intact and core GDGT in F5 and core GDGT (c-GDGT) in F6 (Fig. IV.3). The F2 also contained quinones as minor compounds, which were neglected in the interpretation of the signal, since these compounds were more than two orders of magnitude less abundant than uns-AR. The capillary connection from the HPLC to the MS and from the HPLC to the fraction collector had the same length and diameter, guaranteeing similar separation and elution properties in MS and the fraction collection mode. Extracted lipids of all incubations were fractionated by four individual injections of 10 µL to avoid column overload and to maximize recovery. Potential shifts of retention times

were assessed by running the representative sample in MS mode after every fourth preparative HPLC run (minimum once per day).

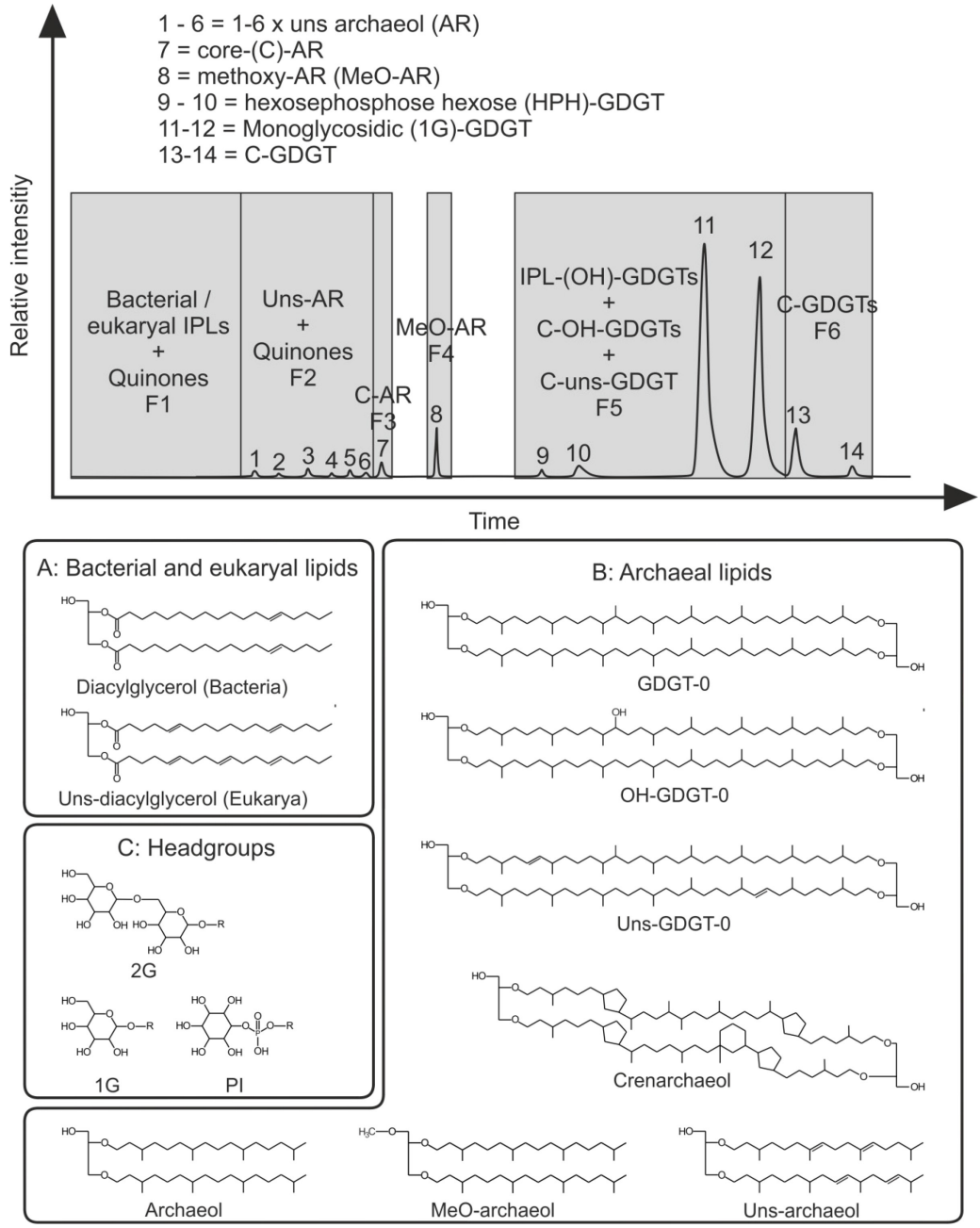

Fig. IV.3. The lipid separation strategy of this study. A schematic chromatogram of fractions obtained from liquid chromatography of a representative sample, obtained by combining lipid extracts from planktonic Thaumarchaeon *N. maritimus* and the Euryarchaeon *C. divulgatum*, measured on the mass spectrometer (top). Structures of exemplary compounds for the fractions are shown below the chromatogram (bottom). Panel A shows two diacyl glycerol lipids that are typically identified in Bacteria and Eukarya. In Panel B archaeal lipids that were investigated in this study: glycerol dibiphytanyl glycerol tetraether without rings (GDGT-0), GDGT-0 with an additional hydroxyl-group (OH) or unsaturations (uns), crenarchaeol, archaeol, methoxy archaeol (MeO-AR) and unsaturated archaeol (uns-AR) with four unsaturations. Different head groups typically identified in planktonic archaea are displayed in panel C, including the diglycosidic (2G), monoglycosidic (1G) and a phosphatidic (phosphatidylinositol, PI) head group.

Identical fractions and samples of the four preparative LC-runs were recombined for scintillation counting. Each sample was dissolved in 500 µL DCM:MeOH (5:1; v:v), transferred to a scintillation vial, and treated with 3 mL of a liquid scintillation counting solvent (Ultima Gold, Perkin Elmer). The fractions were counted for 100 minutes each by a TriCarb® 2810 TR scintillation counter (PerkinElmer). Content of radioactively labeled ^{14}C in lipid structures was measured as disintegrations per minute (DPM). The DPM in the individual fraction was corrected by the DPM measured in the control incubations. DPM values were utilized to calculate relative distribution and potential rates of ^{14}C incorporation into archaeal lipids. Following studies by Herndl et al. (2005) and Yakimov et al. (2011), DPM calculations were based on average specific activities (2.035 GBq/mmol) for the two substrates, resulting in $9.919*10^{-8}$ mmol ^{14}C/DPM.

IV.4. Results and Discussion

IV.4.1. Rates of ^{14}C-based total community activities in mesocosm experiments over time

The activity of the total microbial community within the batch incubation experiments was monitored daily via label incorporation into total cells (Fig. IV. 4). The ^{14}C-LEU-treated biomass showed a 100-fold higher ^{14}C incorporation than the ^{14}C-DIC amended incubations (Fig. IV.4). The large difference observed for the two substrates can be explained by the size of the natural pools of DIC and LEU. The natural DIC concentrations in the Arctic Ocean range around 2 mM (Anderson et al., 2010), whereas LEU is usually present at concentrations lower than 1 µM (e.g. Dittmar et al., 2001). Accordingly, the high natural DIC concentrations result in a dilution of the ^{14}C-DIC and lower labeling strength of this substrate compared to ^{14}C-LEU. However, since natural concentrations of DIC and LEU were not measured in this study, no conversion factor for the dilution effect of the ^{14}C-DIC was included. Consequently, the potential rates of the ^{14}C assimilation of DIC and LEU into cells and lipids are not directly compared in this chapter.

The extensive incorporation of LEU at the Hornsund Basin into total microbial cells shows that the heterotrophic microbes were highly active in this site (Fig. IV.4A). On the shelf, the shallower two samples (5 + 30 mbsl) showed an exponential LEU based cell growth within the first 72 h, while no cell growth was observed thereafter. This suggests a direct response of the shallow communities to the ^{14}C-LEU addition, probably followed by a decrease in activity due to the exhaustion of nutrients. The Hornsund Basin sample from 30 mbsl showed the highest incorporation (ca. 7000 CPM) of ^{14}C-LEU followed by the experiments from 5 mbsl (ca. 5000 CPM; Fig. IV.4A). More enhanced activities at 30 mbsl

could be related to mixing of nutrient rich Arctic water (Rich et al., 1997) with underlying Atlantic water, resulting in a warmer and more saline intermediate water mass with a similar nutrient content as in the surface. A previous study showed that an increasing temperature results in a higher metabolic rate of the microbial community (Price and Sowers, 2004). Therefore, the marine microbes might preferentially inhabit the mixing zone at 30 mbsl with warmer conditions and high nutrient supply from the Arctic waters, which allows faster growth of the heterotrophic community. An alternative explanation for the lower activity of the heterotrophic community in the samples from 5 mbsf could be that the incubations were performed in the dark, thus phototrophic microbes, which are more active in the sunlit surface of the water column, were not traced in this study.

The deepest samples from Hornsund Basin (80 mbsl) exhibited the lowest ^{14}C-LEU incorporation (Fig. IV.4A). This indicates that the activity of the microbial community is lower in the deeper part compared to the shallower depths of the water column. However, in contrast to the other two samples from this site, a continuous growth without an exhaustion of nutrients was observed during the experiment. The lower activity of the heterotrophic microbial community in 80 m water depth, may suggest that Atlantic waters are nutrient poor compared to the Arctic water. In addition, most of the favorable OM might have been consumed in the uppers parts of the water column, resulting in lower growth rates in the sample from 80 mbsl compared to the shallower incubations.

The surface samples from the Van Mijenfjorden also showed an exponential LEU incorporation, while no further growth was observed after 72 h. At this site the sample from 5 mbsl showed the highest incorporation of ^{14}C-LEU (Fig. IV.4B). This maximum in activity in the surface waters of the fjord may be related to nutrient enrichments and input of labile bioavailable organic matter, from intense terrestrial runoff by glacial melting, stimulating the heterotrophic community (Hood et al., 2009). Similar to the Hornsund Basin the deepest sample showed the lowest activity but continues growth during the course of the experiment. The clear zonation of decreasing community activities from surface to depth in the Van Mijenfjorden is considered to be related to a development of nutrient-poor local bottom waters due to reduced exchange of water masses (Cottier et al., 2005).

Incubations treated with ^{14}C-DIC exhibited little ^{14}C incorporation (below 30 CPM/10 mL), due to the dilution of the radiolabeled substrate in the natural DIC pool (Fig. IV.4C and Fig. IV.4D). The assimilation of ^{14}C-DIC was relatively constant over all depths in both sites, indicating similar utilization of DIC by the total autotrophic community. This could be due to generally homogenous distributions of DIC concentrations in the Arctic Ocean (Anderson et al., 2010). Moreover, incubation in the dark excluded ^{14}C-DIC incorporation by photoautotrophic phytoplankton, which are particularly active in the summer in the proximity of the Svalbard archipelago (Hop et al., 2002; Iversen and Seuthe, 2011).

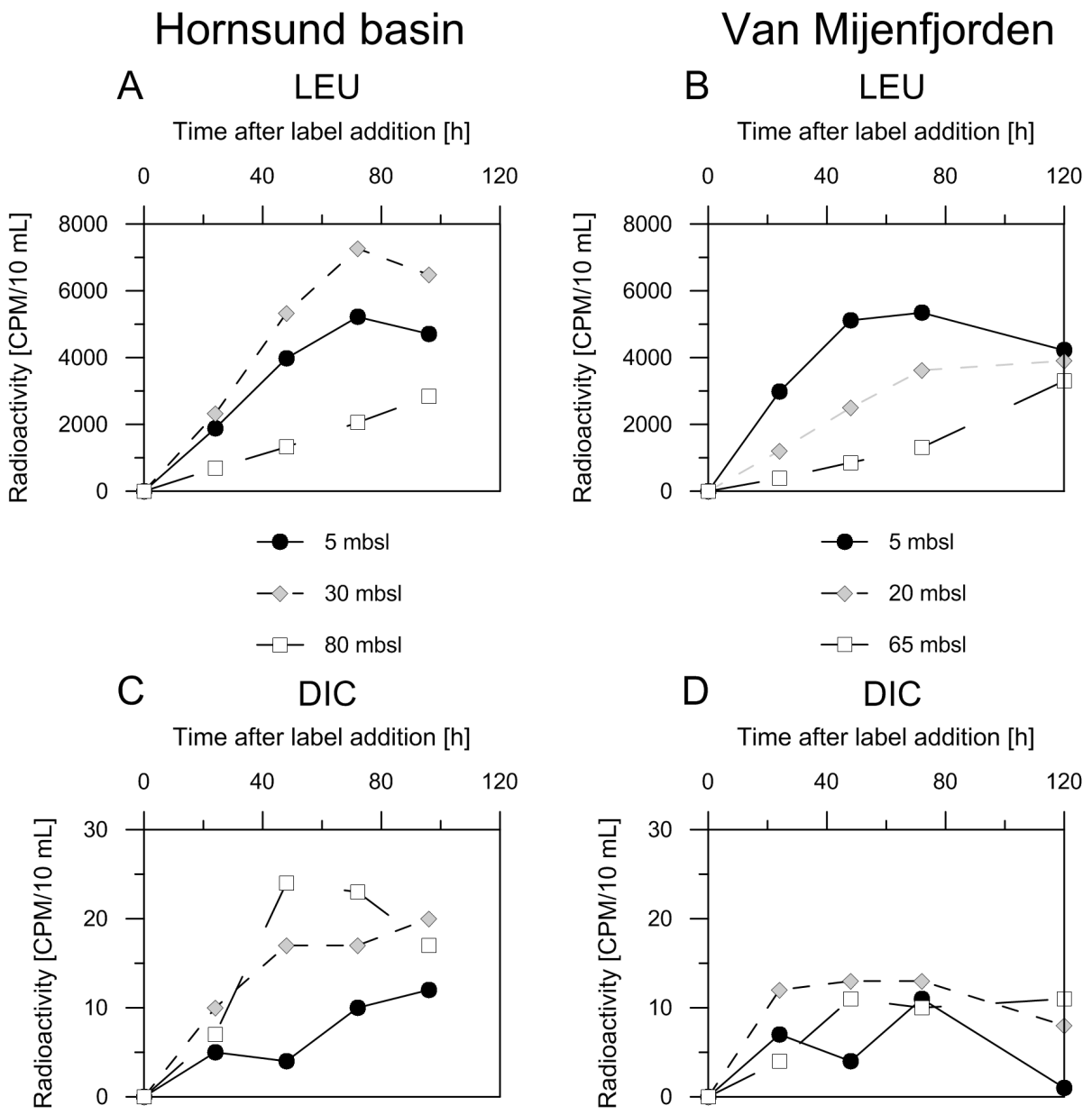

Fig. IV.4. Growth curves of batch incubation experiments based on incorporation of ^{14}C labeled carbon sources into total cells: leucine (LEU; A and B) and bicarbonate (DIC; C and D). Cells for ^{14}C measurement were collected by filtration. The filters had a nominal pore size of 0.1 µm and ^{14}C in the cells was measured for 15 minutes by scintillation counting. The results are given as counts per minute (CPM) per 10 mL of measured seawater. Experiments A and C were conducted with water samples acquired from the Hornsund Basin, while experiments B and D were performed with seawater from the Van Mijenfjorden. The measured values are corrected for the t_0 after label addition.

IV.4.2. Rates of microbial radiocarbon uptake into archaeal lipids

At the Hornsund Basin a higher relative incorporation of ^{14}C-LEU into bacterial/eukaryal lipids (> 90%) compared to archaeal lipids was detected (appendix A Fig. IV.S2). The relative incorporation of ^{14}C-DIC into Bacteria compared to Archaea was lower, representing 40 to 60% at the Hornsund Basin (appendix A Fig. IV.S2). The higher

incorporation of leucine-derived ^{14}C into bacterial biomass is commonly observed (Kirchman et al., 2007; Yokokawa et al., 2012). Therefore, LEU is generally applied as a tracer for bacterial (heterotrophic) production, in comparison to ^{14}C-DIC-based measurements used to quantify primary productivity (Viviani and Church, 2017). The present study confirms the previous results that bacterial productivity is predominantly linked to heterotrophic carbon assimilation in the water column (Kirchman et al., 2007; Yokokawa et al., 2012). The ^{14}C incorporation into archaeal lipids at the Hornsund Basin revealed only little variation of label incorporation as a function of depth for both applied ^{14}C substrates (Fig. IV.5A and Fig. IV.5C). This may suggest a similar response of the archaeal community in the different water depths at the shelf, regardless of their preferred carbon source.

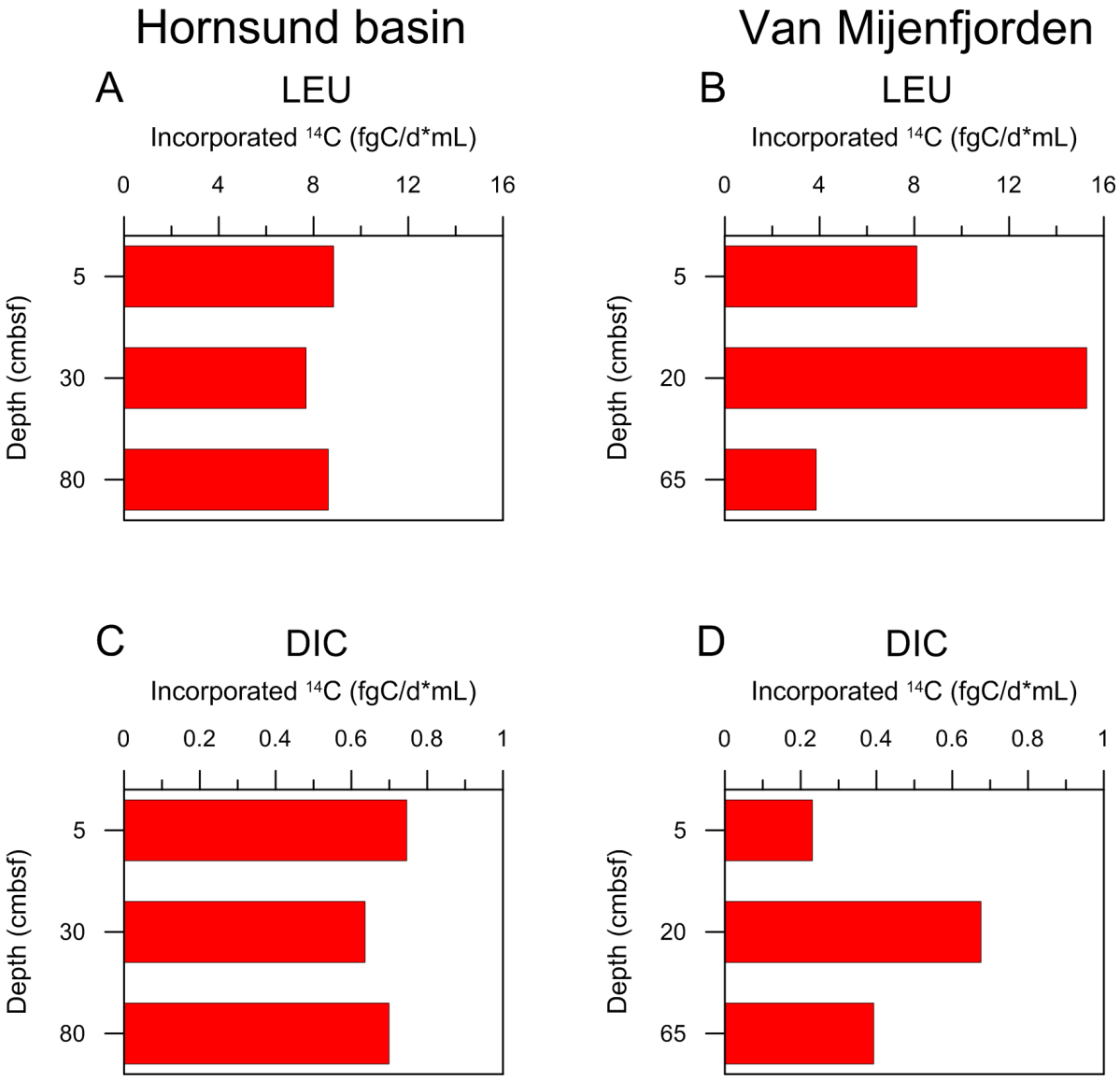

Fig. IV.5. ^{14}C assimilation rates in fg carbon per day and mL (fgC/d*mL) in the seawater incubations acquired from the Hornsund Basin (A + C) and the Van Mijenfjorden (B + D) determined from incorporation of ^{14}C-leucine (LEU, A and B) and ^{14}C-bicarbonate (DIC, C and D) into archaeal lipids.

Similar to the Hornsund Basin, the ^{14}C-LEU experiments in the Van Mijenfjorden showed a predominant incorporation into bacterial/eukaryal lipids (> 90%; appendix A Fig. IV.S2). The ^{14}C assimilation of both applied substrates into archaeal lipids revealed a strong stratification in the different sampling depths in the fjord, which was not observed in the other station (Fig. IV.5). The labeling strength of both applied carbon substrates in the sample collected at 20 mbsl was approximately twice than those of the mesocosms from shallower and deeper water masses (Fig. IV.5B and Fig. IV.5D). The fjords west of Svalbard show a three-layered stratification during the summer, with a nutrient rich surface, an intermediate mixing zone and a nutrient depleted deeper layer. The nutrient enriched surface shows highest microbial activities (Fig. IV.4B), dominated by energetically efficient and fast growing Bacteria and Eukarya (Iversen and Seuthe, 2011). It may be hypothesized that the heterotrophic archaeal ^{14}C-LEU consumers and ^{14}C-DIC assimilating autotrophic archaea are outcompeted by the community that inhabits the nutrient rich surface layer and are forced to evade to intermediate waters in 20 mbsl, resulting in the observed high archaeal activities found in the mixing zone (20 mbsl). Moreover, the ^{14}C-DIC incubations showed that general less DIC is incorporated into archaeal lipids in the Van Mijenfjorden compared to the incubations at the Hornsund Basin, indicating that autotrophy is a less important process for the Archaea in the fjord than at the shelf (Fig. IV.5). This may be explained by the high OM input via glacial melting in the Van Mijenfjorden, providing carbon sources for heterotrophic archaea. The OM input could preferentially stimulate heterotrophic archaea in the fjord, while autotrophic archaea might be outcompeted due to their slow growth and reproduction rates (Pelve et al., 2011). Alternatively, the autotrophic archaea might be outcompeted by the highly active autotrophic phytoplankton found in the fjords west of Svalbard (Iversen and Seuthe, 2011). Both hypotheses may result in a low abundance of autotrophic archaea in the Van Mijenfjorden and, hence, lower ^{14}C-DIC assimilation rate compared to the shelf.

IV.4.3. Substrate-specific ^{14}C-based composition of de-novo synthesized archaeal lipid biomarkers

To correlate the synthesis of archaeal biomarker lipids with heterotrophic/autotrophic carbon assimilation of their respective producers, ^{14}C-labeled lipids were separated into groups by preparative HPLC. The archaeal lipids were fractionated into: uns-AR (F2), AR (F3), MeO-AR (F4), IPL (including IPL-, OH- and uns-c-GDGT, F5) and c-GDGT (F6) (Fig. IV.3). The relative distribution of ^{14}C in each archaeal lipid fraction is displayed in figure IV.6.

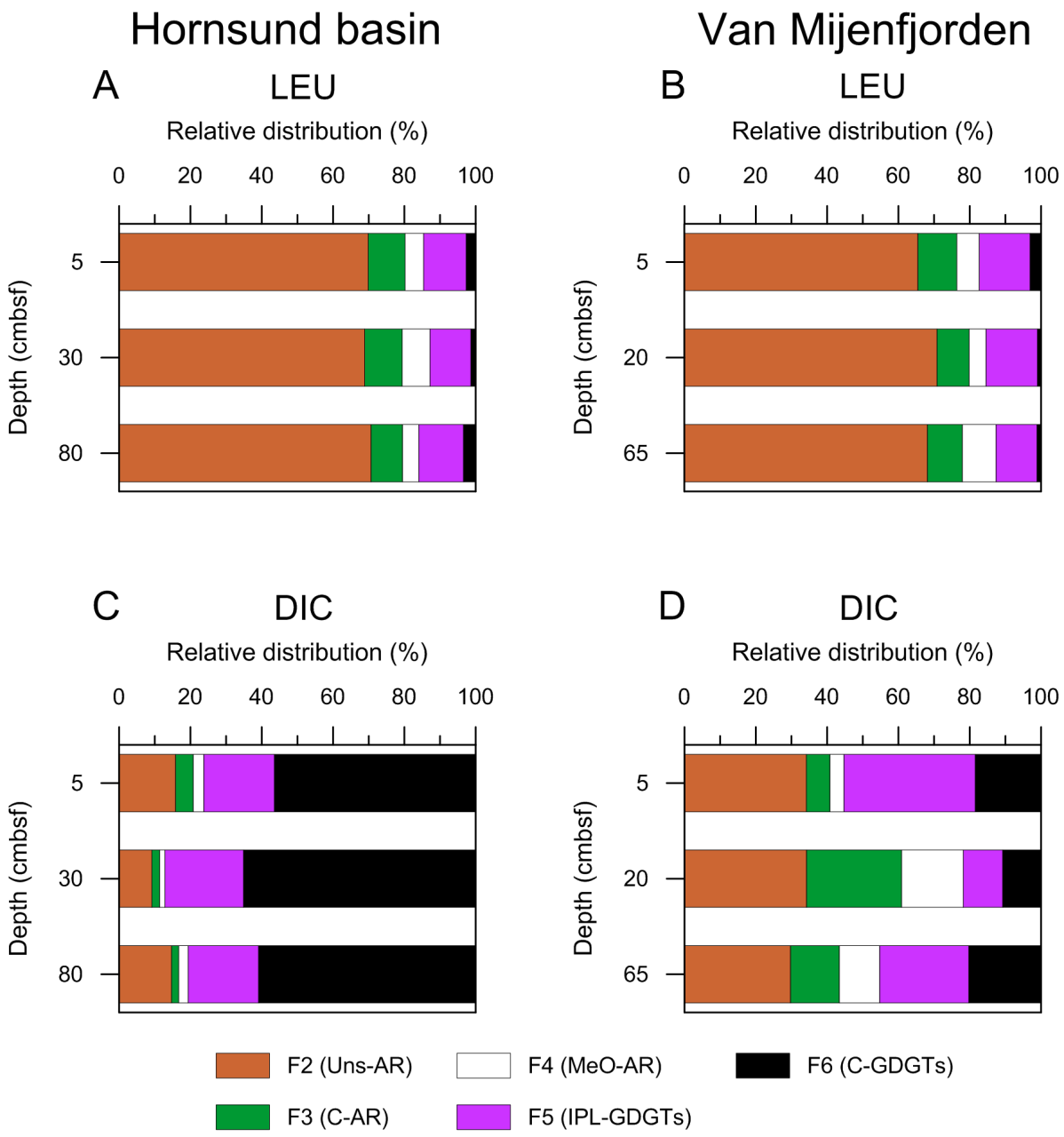

Fig. IV.6. Relative distribution of ^{14}C incorporation into archaeal lipid groups from the mesocosm experiments performed at Hornsund Basin (A + C) and Van Mijenfjorden (B + D). Lipid groups are based on preparative LC fractions as visualized in Figure IV.3: unsaturated archaeol (uns-AR), methoxy archaeol (MeO-AR), core archaeol (c-AR), intact polar lipid glycerol dibiphytanyl glycerol tetraethers (IPL-GDGTs and core-GDGTs (c-GDGTs). Panel A and B shows the incubation with ^{14}C-leucine (LEU) and Panel C and D with ^{14}C-bicarbonate (DIC).

The ^{14}C-based relative abundance of archaeal lipids from the incubations with ^{14}C-LEU exhibited similar distributions of lipid groups for samples from both sites and depths (Fig. IV.6A and Fig. IV.6B). About 80% of the label was incorporated into diethers, such as uns-AR (>60%) and AR (ca. 10%), while archaeal tetraether lipids made up less than 20% of the relative abundance (Fig. IV.6A and Fig. IV.6B). Based on CARD-FISH coupled with microautoradiography, earlier studies showed a preferential incorporation of ^{14}C-LEU into

euryarchaeal cells rather than into *Thaumarchaeota* (Herndl et al., 2005; Alderkamp et al., 2006). These findings were further supported by phylogenetic studies, which identified planktonic *Euryarchaeota* as heterotrophic organisms (e.g. Iverson et al., 2012; Baker et al., 2013; Deschamps et al., 2014; Martin-Cuadrado et al., 2015; Orsi et al., 2015). Hence, the results indicate that heterotrophic *Euryarchaeota* predominantly synthesize diether lipids rather than tetraethers and agree with the assumption that the cell membrane of planktonic *Euryarchaeota* is mainly composed of diethers (Ingalls et al., 2006). Moreover, the predominant production of uns-AR indicates that this lipid type is a suitable biomarker for future studies to investigate the abundance of planktonic *Euryarchaeota* in the water column. This hypothesis is further sustained by the low abundance of uns-AR (< 0.01%) found in the cell membrane of cultured *Thaumarchaeota* (Elling et al., 2014, Elling et al., 2017). Hence, the results support the empirical findings by Zhu et al. (2016) that marine *Euryarchaeota* are the principal producers of uns-AR in the marine water column.

Since incubation experiments were exclusively conducted in the dark, no phototrophic energy metabolism mechanism, as described earlier in euryarchaeal genetic studies (Frigaard et al., 2006; Iverson et al., 2012; Martin-Cuadrado et al., 2015), can be expected. This supports the absence of *proteorhodopsin*, which are light-dependent proton pumps, from certain euryarchaeal clades described in previous studies (e.g. Deschamps et al., 2014). The incorporation of ^{14}C-LEU into MeO-AR (Fig. IV.6A and Fig. IV.6B), a specific marker for *Thaumarchaeota* (Elling et al., 2014, Elling et al., 2015), supports previous results suggesting a mixotrophic metabolism of these Archaea in pure cultures (e.g. Tourna et al., 2011; Qin et al., 2014) and from environmental assessments (e.g. Agogué et al., 2008; Alonso-Sáez et al., 2012).

The ^{14}C-DIC enrichments at the Hornsund Basin showed a similar labeling pattern in the three investigated depths (Fig. IV.6C). ^{14}C-DIC was preferentially incorporated into tetraethers (~80%) and to a lower extent into diethers (20%; Fig. IV.6C). Marine *Thaumarchaeota* are considered to predominantly utilize DIC as a carbon substrate (Wuchter et al., 2003; Könneke et al., 2005), thus indicating that *Thaumarchaeota* at this site produce GDGTs. This is in agreement with culture studies (Schouten et al., 2008; Elling et al., 2014, Elling et al., 2017) and environmental studies (e.g. Schouten et al., 2002; Sinninghe Damsté et al., 2002), that showed GDGTs to be the major building blocks of the cell membrane of *Thaumarchaeota*. Unexpectedly, ^{14}C-DIC was primarily incorporated into c-GDGTs representing up to 60% of the labeled archaeal lipids, whereas IPL-GDGTs accounted for 20%. Assuming that IPL-GDGTs are markers for living archaeal cells and c-GDGTs represent dead archaeal biomass (e.g. Lipp et al., 2008; Lipp and Hinrichs, 2009), this distribution could indicate high quantities of labeled dead archaeal lipid material in the incubation. Lipid turnover times in the water column have not been investigated yet, but

experiments performed in aerobic sediments observed turnover times of several hundred days (Harvey et al., 1986). Although it may be assumed that turnover times are shorter in the water column than in sediments, the short incubation period of 96 hours in this site suggests that c-GDGTs are actively synthesized by autotrophic archaea and not exclusively produced via the degradation of their head group containing analogs. A high production of c-GDGTs was further observed in ^{14}C experiments tracing the lipid biosynthesis of methanogens in subsurface sediments from the Rhone delta (chapter VI). Moreover, a recent study, tracing the production of bacterial branched GDGTs of incubated peat samples, also reported a high production of bacterial c-GDGTs compared to the corresponding IPL-GDGTs within one to two months of incubation (Huguet et al., 2017). The observations from the other two studies support the hypothesis that environmental archaea and bacteria also synthesize c-GDGTs. Huguet et al. (2017) suggested that c-GDGTs may have a biological functional within the cell membrane, as suggested for the MeO-AR in *N. maritimus* (Elling et al., 2015). In addition, c-GDGTs could represent an intermediate stage during the formation of IPL-GDGTs in environmental archaea, as supported by labeling experiments with benthic archaea that showed exclusive carbon uptake into the glycerol backbone (Takano et al., 2010). These authors explained their observations by *de novo* synthesis of the glycerol backbone combined with recycling of biphytane chains from the environment. However, a preferential loss of IPL-GDGTs during lipid extraction by the modified Bligh and Dyer protocol (e.g. Weber et al., 2017) and/or separation (Lengger et al., 2012a) cannot be excluded.

The samples from the Van Mijenfjorden showed a higher relative ^{14}C-DIC incorporation into diethers, particularly into uns-AR, compared to the Hornsund Basin (Fig. IV.6C and Fig. IV.6D). This may suggest a change of the autotrophic thaumarchaeal community structure in the Van Mijenfjorden compared to the Hornsund Basin, or an adaption of their membrane lipid composition to the different environmental conditions in the fjord and on the shelf. However, since low concentrations of uns-AR were observed in cultured *Thaumarchaeota* (Elling et al., 2014, Elling et al., 2017), it is unlikely that uns-AR are produced by this phylum in the Van Mijenfjorden. The results rather indicate that planktonic *Euryarchaeota*, considered as the producers of that uns-AR (this study; Zhu et al., 2016), are mixotrophs that are capable of assimilating amino acids and as well as DIC. This hypothesis is supported by experiments with water column samples collected in the North Atlantic, which showed that planktonic *Euryarchaeota* predominantly incorporate amino acids but at the same time utilize ^{14}C-DIC as a carbon substrate (Herndl et al., 2005). Alternatively, the high production of lipids associated with planktonic *Euryarchaeota* may be explained by the ability of heterotrophs to fixate up to 30% of their carbon budget from inorganic carbon sources (Sorokin, 1966; Roslev et al., 2004; Wegener et al., 2012). The high relative ^{14}C-DIC incorporation into diethers in the samples from the Van Mijenfjorden further suggests that

Euryarchaeota are more active in coastal regions, compared to the open ocean setting at the Hornsund Basin. These findings are consistent with a study from the Beaufort Sea, which showed a predominance of *Euryarchaeota* in riverine and coastal samples, while *Thaumarchaeota* dominated in the marine setting on the shelf (Galand et al., 2008). Nevertheless, biomarkers indicative for *Thaumarchaeota* (MeO-AR and GDGTs) prevailed in the samples from 5 mbsf and 65 mbsf in the Van Mijenfjorden (Fig. IV.6D). The depth related changes of the ^{14}C-DIC labeling pattern in the Van Mijenfjorden (Fig. IV.6) further indicate that the autotrophic community sensitively responds to the stratification and the resulting variations of nutrient supply in the different water masses of the fjord.

In contrast to the Hornsund Basin, the relative ^{14}C-DIC incorporation into archaeal lipids from Van Mijenfjorden did not show enhanced synthesis of c-GDGT (20% compared to 38% IPL-GDGT; Fig. IV.6D). However, *in situ* lipid characterization by Goldenstein et al. (in preparation) showed unusually high quantities of intact and core mono- and di-hydroxylated GDGT in the Van Mijenfjorden samples. The hydroxylated c-GDGT co-elute within the IPL-GDGT-fraction (cf. Fig. IV.3). Thus, the high relative radioactivity in the IPL-GDGT-fraction may result from both, ^{14}C incorporation into IPL-GDGTs and c-GDGTs with hydroxyl-groups. Therefore, the results from this site further support the hypothesis that core and hydroxylated c-GDGTs are not exclusively produced by the degradation of IPL-GDGTs.

IV.5. Conclusions and summary

In situ lipid distributions derived from suspended particulate matter represent a valuable tool to take ‘snap-shots’ of the status of the prevailing archaeal community. However, information on metabolic processes and response of lipid biosynthetic activity to changing environmental conditions are not captured with this approach. Therefore, conclusions on archaeal response to nutrients and community dynamics cannot be inferred. To obtain this information, a ^{14}C labeling experiment was performed. This approach was applied to a shelf site and a fjord site in the Arctic Ocean off Svalbard, to investigate the biosynthesis of lipid biomarkers by the heterotrophic and autotrophic planktonic archaeal community. The lipid-specific analysis of the ^{14}C-LEU incorporation showed a preferential incorporation into uns-AR at both sites, indicating that these lipids are a suitable marker for heterotrophic planktonic *Euryarchaeota*. Furthermore, the synthesis of MeO-AR exclusively found in *Thaumarchaeota* in the ^{14}C-LEU experiments supports their mixotrophic metabolism. In the ^{14}C-DIC incubation experiments from the Hornsund Basin the label was predominantly incorporated into biomarkers that are indicative for *Thaumarchaeota*. The high incorporation of ^{14}C-DIC into c-GDGTs in combination with the short incubation period of less than five days, suggests that apolar lipids are actively synthesized by planktonic archaea. Thus, the

results imply that c-GDGTs in the water column are not exclusively produced by the degradation of their intact analogs. The incorporation of ^{14}C-DIC into uns-AR and AR in the Van Mijenfjorden may indicate that planktonic *Euryarchaeota* are mixotrophs. This study is the first approach to provide an identification of the lipid biosynthesis of heterotrophic and autotrophic planktonic archaea. Hence, the methodology here establishes a link between metabolic mechanisms and the production of specific lipid-biomarker molecules.

IV.6. Acknowledgements

We thank the crew and scientist from R/V Heincke cruise HE449 for sample recovery. The study was funded by the Deutsche Forschungsgemeinschaft through the Gottfried Wilhelm Leibniz Price (HI 616/14-1).

IV.7. Appendix A: supporting information

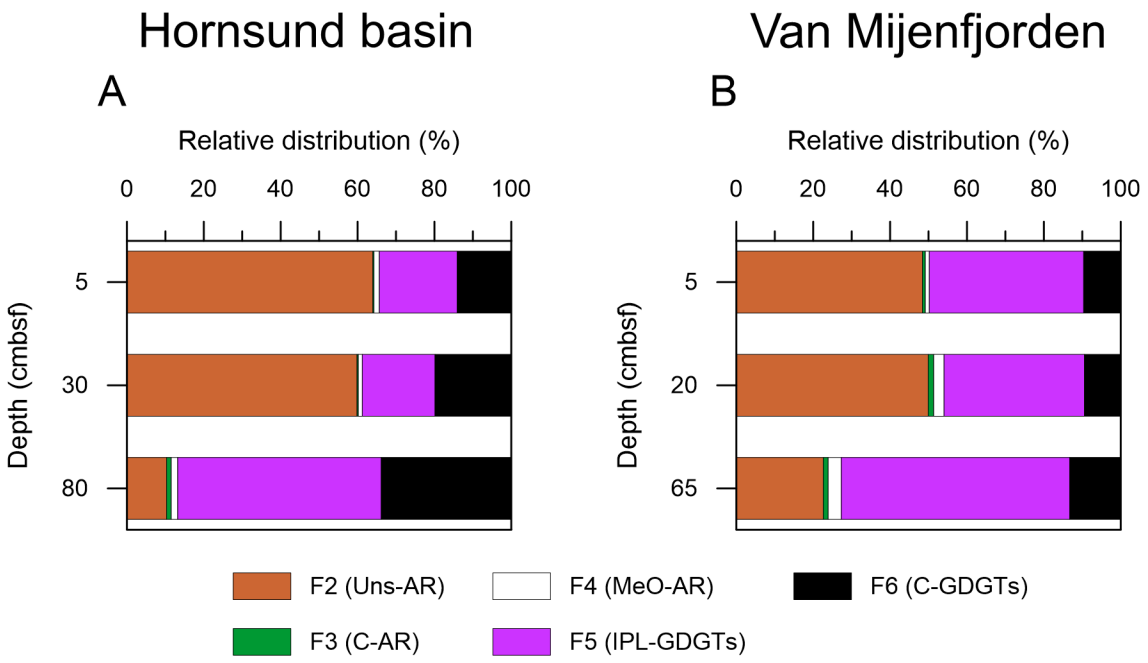

Fig. IV.S1. The relative archaeal IPL-distribution in the samples from the Hornsund Basin (A) and Van Mijenfjorden (B) before the incubation experiment started (Goldenstein et al., in preparation). The samples were collected by filtration with a nominal pore size of 0.3, 0.7 and 2.7 µm. The IPL distribution represents a mean value of the three analyzed pore size fractions.

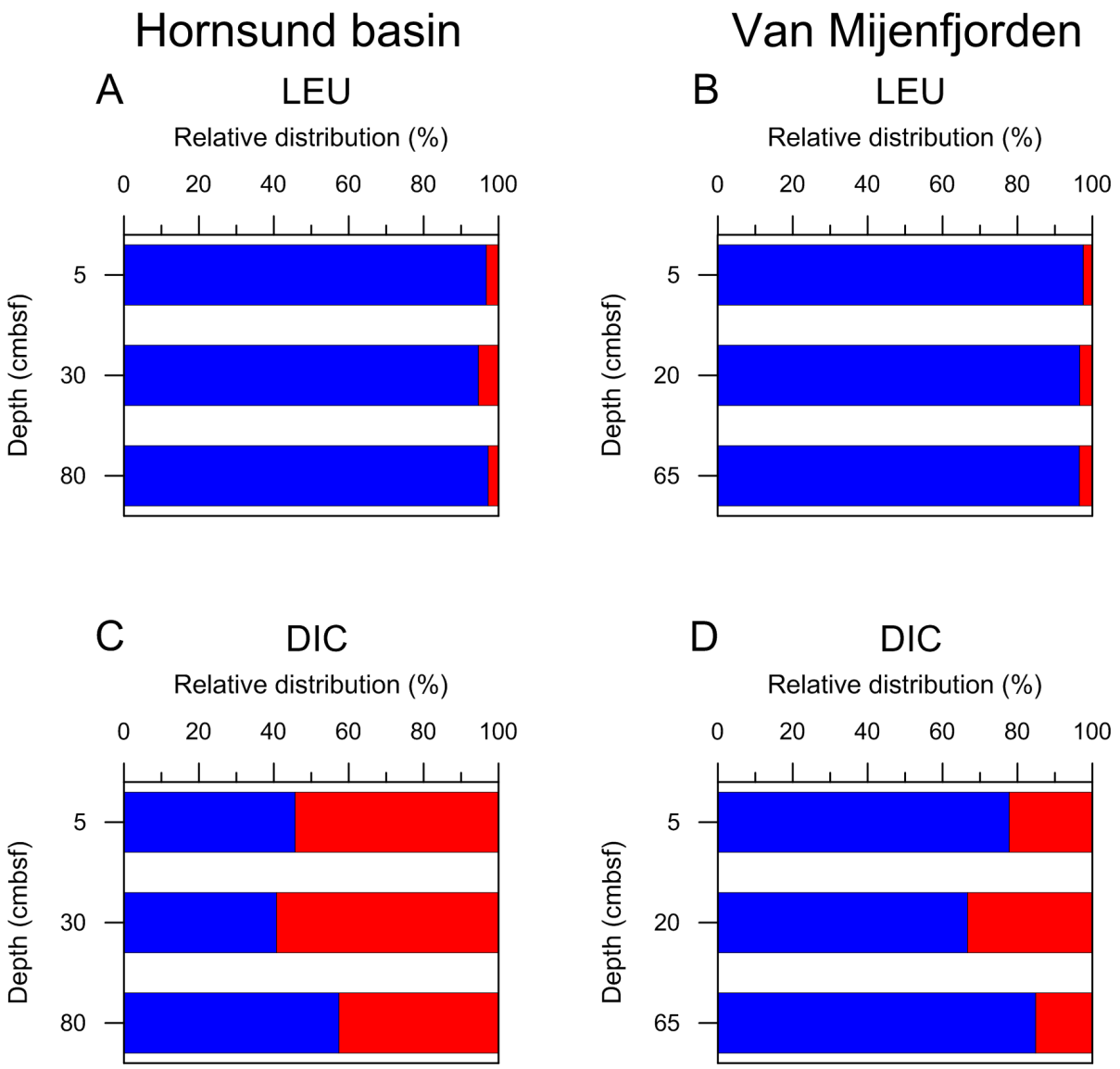

Fig. IV.S2. Relative distribution of ^{14}C incorporation into bacterial/eukaryal lipids (blue bars) and archaeal lipids (red bars) from the mesocosm experiments performed at Hornsund Basin (A and C) and Van Mijenfjorden (V and D). Panel A and B shows the incubation with ^{14}C-leucine (LEU) and Panel C and D with ^{14}C-bicarbonate (DIC).

Chapter V

Size and composition of subseafloor microbial community in the Benguela upwelling area examined from intact membrane lipid and DNA analysis

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V.1. Abstract

Subsurface sediments are among the largest, but also among the least understood ecosystems on Earth. However, novel analytical methods are contributing to constrain uncertainties regarding this unique environment. Here, the microbial community in subsurface sediments was investigated along a transect through the Benguela upwelling system from the organic-rich shelf to the organic-lean bottom of the slope. For this, we combined examination of intact polar lipids (IPLs), purified using a selective clean-up protocol, with analysis of non-soluble DNA (nsDNA) using a quantitative polymerase chain reaction. Both methods aimed at overcoming potential bias introduced by fossil fractions of either lipids or DNA. Our data revealed an abundant microbial community, which decreased with sediment depth and distance from the shore. Interestingly, both methods revealed similar trends in the microbial abundance for three of the four sample locations and the ratio of Archaea to Bacteria showed consistency for the two techniques in samples from the deeper sediment column (> 50 cm). Both techniques suggested that Bacteria dominated the surface samples. The deeper subsurface sediment exhibited an increasing relative abundance of Archaea, yet only in the organic-rich inner shelf, while Bacteria remained dominant in sediments on the slope. With increasing subsurface depth, glycerol diether lipids became the dominant bacterial lipid type, suggesting changes in the bacterial community structure. This may implicate a membrane lipid adaptation to cope with severe energy limitation and thereby allow Bacteria to outcompete Archaea.

Keywords: Sediments; Benguela upwelling system; subseafloor life; IPLs; non-soluble DNA; quantitative polymerase chain reaction.

V.2. Introduction

Since the first findings of microbial cells in subseafloor sediments down to several hundred m below the seafloor in the mid-1990s (Parkes et al., 1994), research on this ecosystem has intensified. During recent years, estimates of the size of the microbial community varied strongly, ranging from 30% of the total biomass on Earth (Whitman et al., 1998) to 1% (Kallmeyer et al., 2012). Biogeochemical relevance, community composition and adaptive strategies of this deep biosphere remain among the major scientific issues to be elucidated, and are targeted by an increasing number of cell count-, biomarker-, and DNA-based studies (e.g. Schippers et al., 2005; Inagaki et al., 2006; Lipp et al., 2008; Morono et al., 2009; Webster et al., 2009; Lomstein et al., 2012; Lloyd et al., 2013b; Breuker et al., 2013; Inagaki et al., 2015).

DNA provides valuable information on the subsurface community, e.g. via sequencing of 16S rRNA genes by next generation sequencing technology. Studies using these techniques showed that the subsurface communities are inhabited largely by uncharacterized groups of Archaea and Bacteria (e.g. Biddle et al., 2006; Inagaki et al., 2006; Hug et al., 2016; Lever, 2016). DNA-based results using quantitative polymerase chain reactions on 16S rRNA genes and/or fluorescent in situ hybridization suggested a strong dominance of Bacteria in deeply buried sediments of the Peru Margin (Schippers et al., 2005; Inagaki et al., 2006). At other sites the abundances of Archaea and Bacteria were close to a 1/1-ratio (e.g. Engelen et al., 2008; Webster et al., 2009; Schippers et al., 2012; Breuker et al., 2013; Lloyd et al., 2013b). Another method for the characterization of subsurface life is based on the analysis of intact polar lipids (IPLs), which usually consist of a polar head group and a glycerol backbone linked to alkyl chain(s) via ester/ether bond(s). The polar head is usually made up of a phosphatidic IPL and/or glycosidic IPL head group and bears taxonomic information (e.g. Fang et al., 2000; Rütters et al., 2001; Sturt et al., 2004; Koga and Morii, 2005). Moreover, studies have stated that microorganisms are capable of adapting their membrane properties with respect to variation in growth temperature (e.g. Hasegawa et al., 1980; Van De Vossenberg et al., 1995), salt tolerance (e.g. Yamauchi et al., 1992; Kellermann et al., 2016b) and severe energy limitation (Valentine, 2007). In systems with high microbial activity, in which biomass is turned over rather quickly, IPLs are useful microbial biomass proxies since it was shown that their polar head group is rapidly degraded after cell death (White et al., 1979; Harvey et al., 1986). In contrast to the DNA based results, IPLs suggested a strong dominance of Archaea in subsurface sediments from the Peru margin (Lipp et al., 2008; Lipp and Hinrichs, 2009).

These conflicting results led to discussions as to whether IPL- or DNA-based analyses overestimate or underestimate the abundance of Archaea and Bacteria due to

systematic biases (cf. Pearson, 2008). In the case of IPLs, an analytical challenge is related to mass spectrometry (MS) ion suppression due to the complex organic matrix in environmental samples (Mallet et al., 2004). This results in high background signals and a lowered detectability of IPLs, in particular with a phosphatidic head group, due to inefficient ionization (Wörmer et al., 2013). A further challenge that biases this method is the preservation efficiency of IPLs in subsurface sediments. Harvey et al. (1986) observed a higher degradation rate for phosphatidic IPLs than for their glycosidic counterparts, and Logemann et al. (2011) showed that ether-based lipids, independent of their polar head group type, degrade more slowly than their ester analogues. Recently, radiotracer experiments also confirmed that archaeal glycolipids degrade more slowly than bacterial ester-based lipids (Xie et al., 2013a). These structural features, associated prominently with archaeal IPLs, may have resulted in an overestimation of living Archaea in previous studies (cf. Lipp and Hinrichs, 2009). On the other hand, DNA-based techniques have also been shown to be impacted by systematic effects such as inefficient extraction (Lipp et al., 2008; Lever et al., 2015) and primer bias (Teske and Sørensen, 2008; Lever and Teske, 2015). A further limitation of the microbial community analysis from DNA is related to potential bias through the pool of extracellular DNA, which has been suggested to account for a significant fraction of total sedimentary DNA (Dell'Anno and Danovaro, 2005, Corinaldesi et al., 2011, Lever et al., 2015). The fact that a significant fraction of DNA is not associated with living organisms, instead being from the remains of deceased organisms, was neglected in most studies.

To overcome these methodological difficulties, modified methods were applied here to subsurface sediment samples. For IPLs, a clean-up procedure, based on solid phase extraction of lipids with a phosphatidic head group, was introduced by Zhu et al., (2013b). This results in improved detection of phosphatidic IPLs in environmental samples due to a reduction of the hindering matrix. On the DNA-side, a novel method was used that removes water-soluble DNA (sDNA), most likely adsorbed in the native sediment matrix, by washing with a mild alkaline phosphate buffer prior to cell lysis (Lever et al., 2015). As cell integrity is preserved, the pool of non-soluble DNA (nsDNA) left behind includes intact cells with their intracellular DNA. Despite the initial removal of sDNA, which accounts for 10-90% of total DNA, nsDNA extracted with this protocol affords higher archaeal and bacterial gene copy numbers than widely used commercial extraction kits that target total DNA (Lever et al., 2015).

Both improved techniques were applied here to surface and subsurface sediment samples from the Benguela upwelling system. The system is located at the southwest African continental margin off Namibia and is formed by a combination of steady southeastern trade winds, the shelf topography and the Benguela Current. The resulting onshore transport of

cold nutrient-rich Eastern South Atlantic Central Water to the euphotic zone (Emeis et al., 2009) creates one of the most productive upwelling areas in the ocean (Carr, 2002). The result is a range of different depositional settings, including organic-rich sediments below the upwelling cell and organic-lean sediments in the South Atlantic Gyre (cf. Lin et al., 2012). The region of the Namibian continental margin therefore provides an excellent environmental system for studying the microbial community in subsurface sediments under different depositional conditions. The depositional and biogeochemical processes in these sediments have been widely studied (e.g. Giraudeau et al., 2000; Borchers et al., 2005; Inthorn et al., 2006; Mollenhauer et al., 2007; Goldhammer et al., 2011), but research focusing on the microbial community in the sediments is scarce, with only one study available, which shows rather equal abundances of Archaea and Bacteria (Schippers et al., 2012).

We aimed at providing a detailed estimation of archaeal and bacterial abundances through combined IPL and nsDNA analysis. Particular focus was on the IPL composition, since recent analytical improvements (Wörmer et al., 2013; Zhu et al., 2013a) resulted in protocols that provide a comprehensive view of the lipidome, and thus of microbial life, in subsurface sediments (e.g. Zhu et al., 2014; Meador et al., 2015). The approach should aid in identifying the factors controlling the domain-level distribution and microbial membrane composition, which might reflect physiological adaptation of microorganisms to energy limitation in this heterogeneous environment.

V.3. Material and Methods

V.3.1. Sampling and study site

Samples were investigated from four stations (GeoB12810, GeoB12802, GeoB12811, GeoB12808) along a transect through the upwelling system representing water depths between 120 and 3800 m (Table V.1). The samples were collected in Spring 2008 during RV Meteor cruise M76/1 (Zabel and Ferdelman, 2008). At each site, a combination of multi-cores and gravity cores was collected. The multi-cores were sampled from 0-30 cm below the seafloor (cmbsf) and the gravity cores from 50-560 cmbsf. The samples were stored in sterile polyethylene bags (Whirl-Pak, Nasco), transported on dry ice and preserved at -80 °C.

Station GeoB12810 (Fig. V.1) is in the up to 15 m thick organic rich Namibian mud belt (Bremner and Willis, 1993) underneath the main upwelling cell. The mud belt covers an area of 18,000 km² (Emeis et al., 2004) and consists of a diatomaceous ooze with large fractions of foraminifera and shell fragments (Bremner and Willis, 1993). These sediments are characterized by a high deposition rate of modern (fresh) organic matter (OM) (Mollenhauer et al., 2007), due to (i) high vertical particle flux from the phytoplankton-rich

surface water, (ii) shallow water depth, and (iii) periodically anoxic bottom water (Brüchert et al., 2003; Borchers et al., 2005; Inthorn et al., 2006). Published pore water profiles of the station show intensive SO_4^{2-} reduction in the upper 250 cmbsf (Goldhammer et al., 2011). Sediments deeper than 270 cmbsf showed increasing CH_4 concentration, suggesting methanogenesis (Supplementary material, Table V.S1).

Station GeoB12802 on the upper slope of the continental margin has sediments with high OM content (Inthorn et al., 2006). At this depocenter the lateral exceeds the vertical input of OM, causing deposition of suspended pre-aged OM transported from the shelf sediment (Giraudeau et al., 2000; Inthorn et al., 2006; Mollenhauer et al., 2007). Pore water data show Fe reduction in the upper 10 cm, followed by the SO_4^{2-} reducing zone ranging from 10 to 260 cmbsf (Goldhammer et al., 2011; Lin et al., 2012). The methanogenic zone was observed below a depth of 260 cmbsf (Lin et al., 2012).

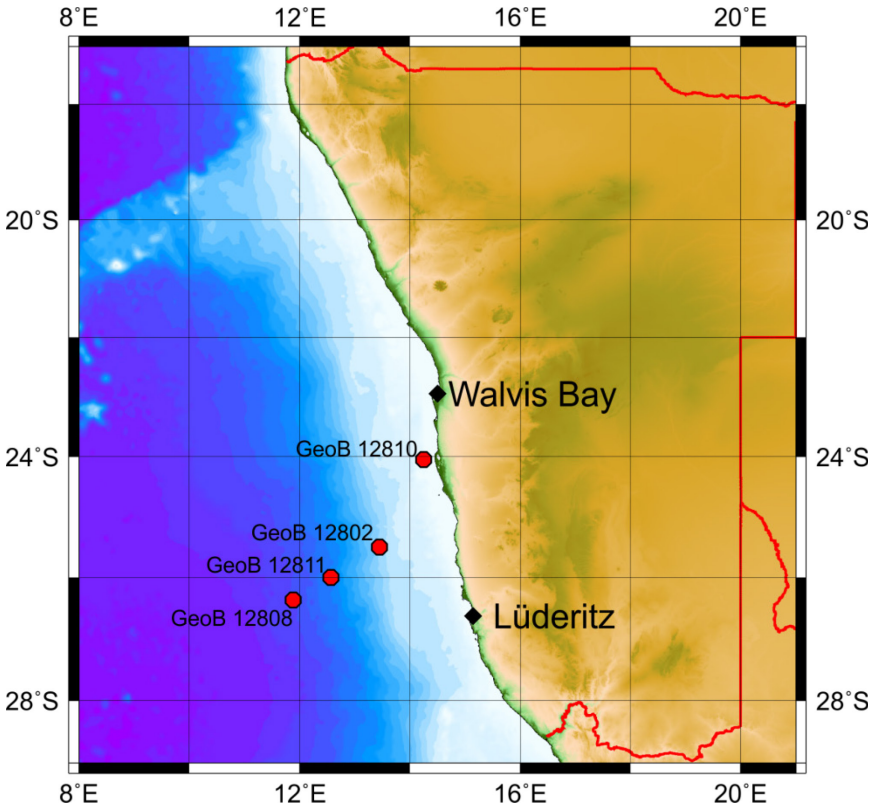

Fig. V.1. Sample stations for transect through the Benguela upwelling system of cruise M 76/1.

Stations GeoB12811 and GeoB12808 are further offshore, on the middle and lower slope, respectively. The sediments here are enriched in carbonate, while sand and organic carbon contents are lower than at the shallower sites (Bremner and Willis, 1993; Lin et al., 2012). The surface sediments are characterized by a slightly younger OM than at the depocenter (Inthorn et al., 2006; Mollenhauer et al., 2007). At the station from the middle slope (Geo 12811) Fe reduction (3-10 cmsbf) and SO_4^{2-} reduction (>10 cmbsf) were also observed (Goldhammer et al., 2011; Lin et al., 2012). Further offshore Fe concentration close

to the detection limit and rather constant SO_4^{2-} concentration throughout the core indicate a ceasing importance of both processes (Lin et al., 2012). The methanogenic zone was not reached during coring at Stations GeoB12811 and GeoB12808.

Table V.1. Sample stations for transect though the Benguela upwelling area. Average TOC content calculated from Lin et al. (2012). Sediment deposition rate values for mud belt and middle to lower slope sediments adopted from Mollenhauer et al. 2002 (all except depocenter) and Inthorn et al., (2006; depocenter).

Station number	Regional classification	Water depth (m)	TOC (%)	Sedimentation rate (SR) (cm/ky)
GeoB12810	Inner shelf (mud belt)	120	8.08	50-60
GeoB12802	Upper slope (depocenter)	791	7.35	112
GeoB12811	Middle slope	2980	4.27	5-17
GeoB12808	Lower slope	3796	1.24	5-17

V.3.2. DNA analysis

The nsDNA was extracted from 1 g frozen sediment as described by Lever et al. (2015). After initial removal of sDNA using carbonate dissolution solution by washing with a solution of 0.43 M NaOAc, 0.43 M $MeCO_2H$, 10 mM ethylenediaminetetraacetic acid (EDTA), 100 mM Na hexametaphosphate, 3% NaCl and Tris-EDTA buffer (300 mM Tris Hydrochloride, 10mM EDTA, 3% NaCl; pH 10.0), nsDNA was extracted using lysis protocol III (Lever et al., 2015). The resulting DNA extract was washed 2x with cold $CHCl_3$/isoamyl alcohol (24:1), precipitated with 2.5 volumes EtOH and 20 µg linear polyacrylamide/ml DNA extract, and underwent final purification using the CleanAll RNA/DNA Clean-up and Concentration Kit (Norgen, Biotek).

16S rRNA gene copy numbers were amplified via a quantitative polymerase chain reaction (PCR) on a LightCycler 480 (Roche). Each PCR reaction contained 10 µl FastStart Universal SYBR Green I Master Mix (Roche), 2 µl bovine serum albumin (10 mg/ml), 1 µl each of forward and reverse primer (10 pmol/µl), 4 µl molecular grade water and 2 µl DNA extract. The 16S rRNA gene copies of the nsDNA extract were amplified in duplicate measurements (Appendix; Fig. V.S1). The nsDNA gene copy numbers shown are the mean value of both measurements. For the quantification of archaeal 16S rRNA genes, Arch806F (Takai and Horikoshi, 2000) and Arch958R (DeLong, 1992) were used as primer pair. Bacteria were quantified using the primers Bac8F (Turner et al., 1999) and Bac338Rabc (Daims et al., 1999).

The 16S rRNA copy numbers were determined using the following thermal cycler protocol: 95 ºC for enzyme activation for 5 min followed by 50 PCR cycles; each PCR cycle

consisted of 30 s denaturation at 95 ºC, 30 s annealing at 55 ºC, 15 s elongation at 72 ºC and 5 s data acquisition at 80 ºC. Standards for 16S rRNA quantification were made from 10x serial dilution of plasmids (Promega), containing amplified fragments of the relevant gene. Bacterial standards were made using a sequence related to *Sphingomonadales* and standards for Archaea were made from a sequence affiliated with *Methanosarcina*.

V.3.3. IPL analysis

Frozen wet sediment samples were extracted in four steps after a modified Bligh and Dyer protocol (Sturt et al., 2004). The samples were extracted with 4 ml solvent/g sediment mixture of MeOH, dichloromethane (DCM) and aqueous buffer (2:1:0.8;v:v:v) and sonicated for 10 min using ultrasonic sticks with a pulse of 70% and a duty cycle of 60% (Bandelin Sonorex). A monopotassium phosphate buffer (8.7 g/l, pH 7.4) was used for the first two steps and a CCl_3CO_2H acid buffer (50 g/l, pH 2) for the final two steps. The sonicated sediment was centrifuged, the supernatants combined in a separatory funnel and DCM and water added in order to allow phase separation. Pooled organic layers were washed 3x with deionized Milli-Q water. The solvent was removed from the final extract under N_2 flow and the extract stored at -20 °C. Afterwards the extract was washed with Milli-Q water, the solvent removed at 36 °C under a stream of N_2 and the extract stored at -20 °C.

The total lipid extract was split into two aliquots, of which one was directly measured to detect the glycosidic lipids while the other cleaned up by HybridSPE-PPT cartridges (Supelco, 6 mg). The cartridge has the advantage over silica gel columns that it combines a high recovery of an average 98% (78 – 110%) with no significant discrimination among the different tested phospholipids (PLs). Moreover, the clean-up-procedure results in 1.2 - 2.7-fold more intense signal compared to a untreated sample (Zhu et al., 2013b).

According to the method described by Zhu et al. (2013b), the sample was loaded onto the cartridge with MeOH and HCO_2H (99:1), followed by the removal of phosphate-free lipids with MeOH and ammonium formate. The phospholipids (PL) were eluted with isopropanol and DCM in the presence of NH_4OH.

Detection and identification of IPLs were carried out using a maXis ultra-high resolution quadrupole time-of-flight mass spectrometer (Q-ToF-MS instrument; Bruker) with an electron spray ionization (ESI) source, coupled to a Dionex Ultimate 3000RS ultra high pressure liquid chromatography (UHPLC) instrument. The bulk sample was separated on a Waters Acquity BEH C_{18} column (150 x 2.1 mm; 1.7 µm particle size), while the PL fraction was separated with a Waters Acquity amide column (150 x 2.1 mm; 1.7 µm particle size) used in hydrophilic interaction liquid chromatography mode as described by Wörmer et al. (2013). Lipids were detected in positive ionization mode scanning from *m/z* 150 to 2,000.

MS^2 scans were obtained in data dependent mode. For each MS full scan up to three MS^2 experiments, targeting the most abundant ions, were performed. Lipid assignment was achieved by monitoring exact masses of possible parent ions (present as either H^+, NH_4^+ or Na^+ adducts) in combination with characteristic fragmentation patterns. Quantification was achieved by comparison of parent ion responses relative to a known amounts of an internal standard [1,2-dihenarachidoyl-sn-glycero-3-phosphocholine, C_{21}-PC-DAG, Avanti Lipids for phospholipids; C_{46}-GTGT (glycerol trialkyl glycerol tetraether) for glycolipids; Huguet et al. (2006)] and with the use of response factors. Response factors were determined from commercial or purified IPL standards for both applied HPLC columns on the Q-ToF-MS after triplicate measurements. For bacterial and eukaryal diether glycerols (DEGs) with a phosphatidylethanolamine (PE), phosphatidyl –(N)-methylethanolamine (PME) or phosphatidyl –(N,N)-dimethylethanolamine (PDME) head group the response factors were determined from a PE-archaeol (AR) due to the lack of standards for these lipid types. No phosphatidic-sphingolipid was available at the time of measurement so that a relative response factor of unity was chosen for these lipids. We acknowledge that previous studies have applied the commercially available glucocerebrosides to determine the response factor (e.g. Fulton et al., 2014). The results for the lipid measurements are based on single injections. However, triplicate measurements for response factor determination showed a standard deviation of different measured standards of between 3 and 17% (mean 7%) for the BEH C_{18} column and between 2 and 10% (mean 4%) for the amide column.

To detect minor archaeal IPLs in very low concentration, the samples were also analyzed using a Dionex Ultimate 3000RS UHPLC system (Thermo Scientific Dionex, Dreieich, Germany) coupled to an ABSCIEX QTRAP 4500 mass spectrometer (ABSCIEX, Darmstadt, Germany) by a TurboIonSpray ion source in ESI mode. The samples were chromatographically separated on an ACE3 C_{18} column (150 x 2.1 mm; particle size 3 µm) equipped with guard column using a method modified from Zhu et al. (2013a). Column temperature was 45 °C and eluent A consisted of MeOH, HCO_2H and 14.8 M NH_4OH (100:0.04:0.1; v:v:v) and eluent B was prepared from isopropanol, formic acid and 14.8 M NH_4OH (100:0.04:0.1; v:v:v). The flow rate was 200 µl/min and the gradient was programmed to 100% A for 10 min, followed by an increase to 24% B at 15 min and a further increase to 56% B at 60 min. Finally, the column was cleaned with 90% B for 15 min and re-equilibrated with 100% A from 76 to 90 min. Samples were dissolved in MeOH and a typical volume of 10 µl was injected.

Ion source parameters were optimized by way of flow injection analysis of standards into the HPLC eluent stream, leading to the following values: curtain gas 35 units, ion spray 5.5 kV, 300 °C, ion source gas 1 flow 30 units, ion source gas 2 flow 25 units. Detection was achieved in scheduled multiple reaction monitoring (MRM). MRM transitions monitored the

fragmentation of the polar head group of the ammoniated molecular ions (e.g. *m/z* 1723.3 → 1544.3 for HPH-GDGT-0, *m/z* 1425.4 → 1246.3 for 1G-GDD-0, *m/z* 1495.4 → 1316.3 for 1G-BDGT-0, *m/z* 1481.5 → 1302.3 for 1G-GDGT-0, *m/z* 1635.2 → 1456.1 for 1G-GDGT-0-PG and *m/z* 848.8 → 669.7 for 1G-OH-AR). MRM transition parameters (declustering potential, collision energy, collision cell exit potential) and retention times for scheduled MRM were optimized and determined via chromatographic runs of standards or extract containing the compounds of interest.

V.3.4. Quantification of archaeal and bacterial abundances

To quantify archaeal and bacterial abundance, the fraction of Archaea to the total microbes was calculated for both data sets. For the nsDNA-data the ratio was calculated from gene copy concentrations of Archaea vs. the total prokaryotic 16S rRNA gene copy numbers. IPL abundance data, for both total concentration and IPL-based archaeal abundance, were based solely on the Q-ToF data in order to avoid bias from combining measurements from two different machines. The archaeal lipids are represented by glycerol dialkyl glycerol tetraether (GDGT) and AR core structures built-up of isoprenoid units. Bacterial and eukaryal lipids (BELs) are defined by non-isoprenoidal core structures with glycerol backbones (Yoshinaga et al., 2011). Core lipid structures for Bacteria and Eukarya include diacyl glycerols (DAGs), acylether glycerols (AEGs) and diether glycerol (DEGs), but also betaine lipids (BLs) and sphingolipids. Bacterial and eukaryal IPLs can have multiple sources due to a highly similar lipid structure and associated head groups in both domains. Thus BELs can only be partly assigned to specific organisms and therefore these lipids were grouped together as non-archaeal lipids with a bacterial or eukaryal source.

V.3.5. Calculations

To evaluate the membrane properties provided by the IPLs the average chain length (ACL; Eq. 1) and the double bond index (DBI; Eq. 2) were calculated for individual IPL types. *Cx* and *Cy* stand for the abundance of a compound with *x* carbons or *y* unsaturations in the apolar chain.

$$\mathrm{ACL} = \frac{\sum \mathrm{x_i} * \mathrm{Cx_i}}{\sum \mathrm{Cx_i}} \qquad Eq.1$$

$$\mathrm{DBI} = \frac{\sum \mathrm{y_i} * \mathrm{Cy_i}}{\sum \mathrm{Cy_i}}$$

$Eq. 2$

V.4. Results and Discussion

V.4.1. DNA-based data

The total prokaryotic 16S rRNA gene copy concentrations depicted a high population density at the sediment-water interface, ranging from 10^8 (lower slope) to 10^9 (mud belt) copies/g wet sediment (Fig. V.2A). With increasing sediment depth the prokaryotic gene copy concentration generally decreased as observed previously (e.g. Schippers et al., 2012) particularly at the offshore sites. At the mid-slope station GeoB12811, a sharp drop in the prokaryotic gene copy concentration was observed within in the top 10 cm, followed by a strong increase at greater depth.

At the sediment-water interface, bacterial gene copy concentration values outnumbered archaeal genes at all stations (Fig. V.3). At the stations from the mud belt and the depocenter, the concentrations of archaeal gene copies increased in the upper 20 cm. In the mud belt archaeal gene copies further increased down core, whereas bacterial gene copy concentrations declined steadily in the subsurface sediments. In the depocenter, archaeal and bacterial gene copy numbers shared a similar trend. At the stations from the middle and lower slope the gene concentration sharply decreased in the top 20 cm by one to two orders of magnitude for bacterial genes and two to three orders for archaeal genes, respectively. At the middle slope the concentrations showed a second maximum at a depth of 248 cmbsf, while at the lower slope, gene copy concentrations of Archaea and Bacteria stayed rather constant below 20 cmbsf. A prevalence of archaeal genes was only observed in the deeper sediments of the mud belt, while bacterial genes dominated on the slope.

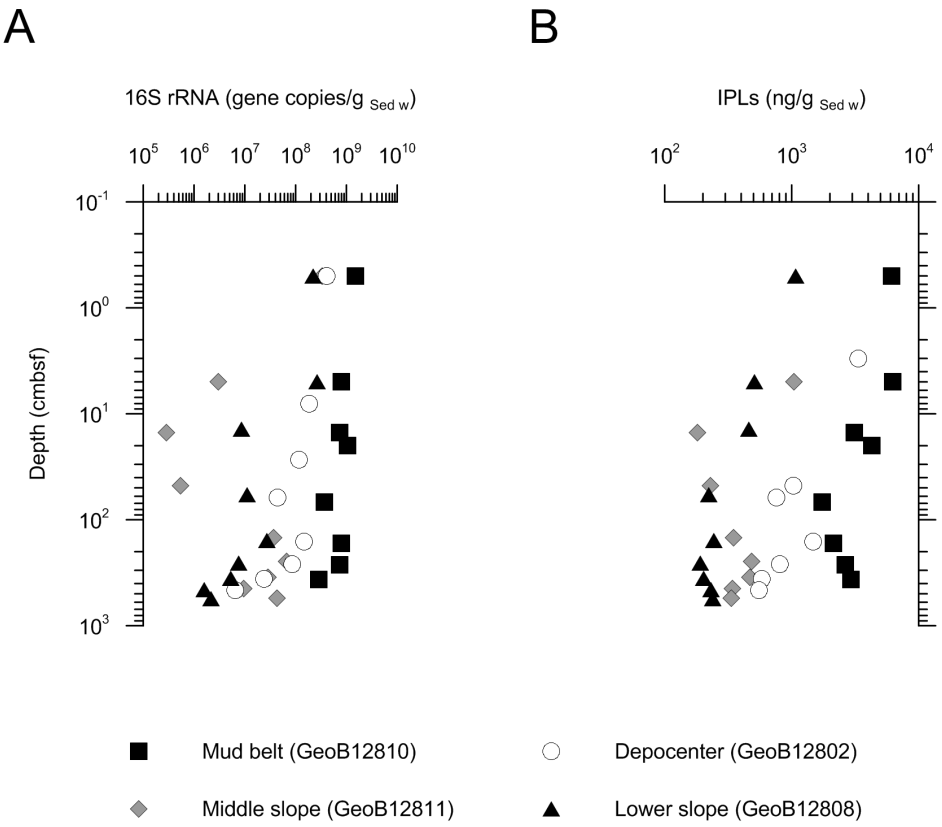

Fig. V.2. Depth profiles of total Prokaryotic (Archaea + Bacteria) 16S rRNA gene copy numbers/g wet sediment (A) and total IPL concentrations ng/g wet sediment (B). The total IPL concentrations was calculated from the Q-ToF data.

V.4.2. IPL classes and potential microbial sources

Like the nsDNA data, the total IPL concentration was generally highest at the sediment-water interface, ranging from 10^3 to 10^4 ng/g wet sediment (Fig. V.2B). With increasing distance from the shore and greater sediment depth the IPL abundance decreased. Moreover, the IPL concentration from the middle slope station exhibited a minimum at 15 cmbsf, as observed also for the nsDNA-data.

V.4.2.1. Major archaeal lipids

Archaeal glycosidic IPLs were detected in all samples (Fig. V.4). The fraction of archaeal IPLs in the mud belt increased with depth, peaking at 168 cmbsf. Such a subsurface maximum was also observed in the other sediment cores, yet at decreasing depth in the offshore direction (162, 10 and 14 cmbsf, respectively).

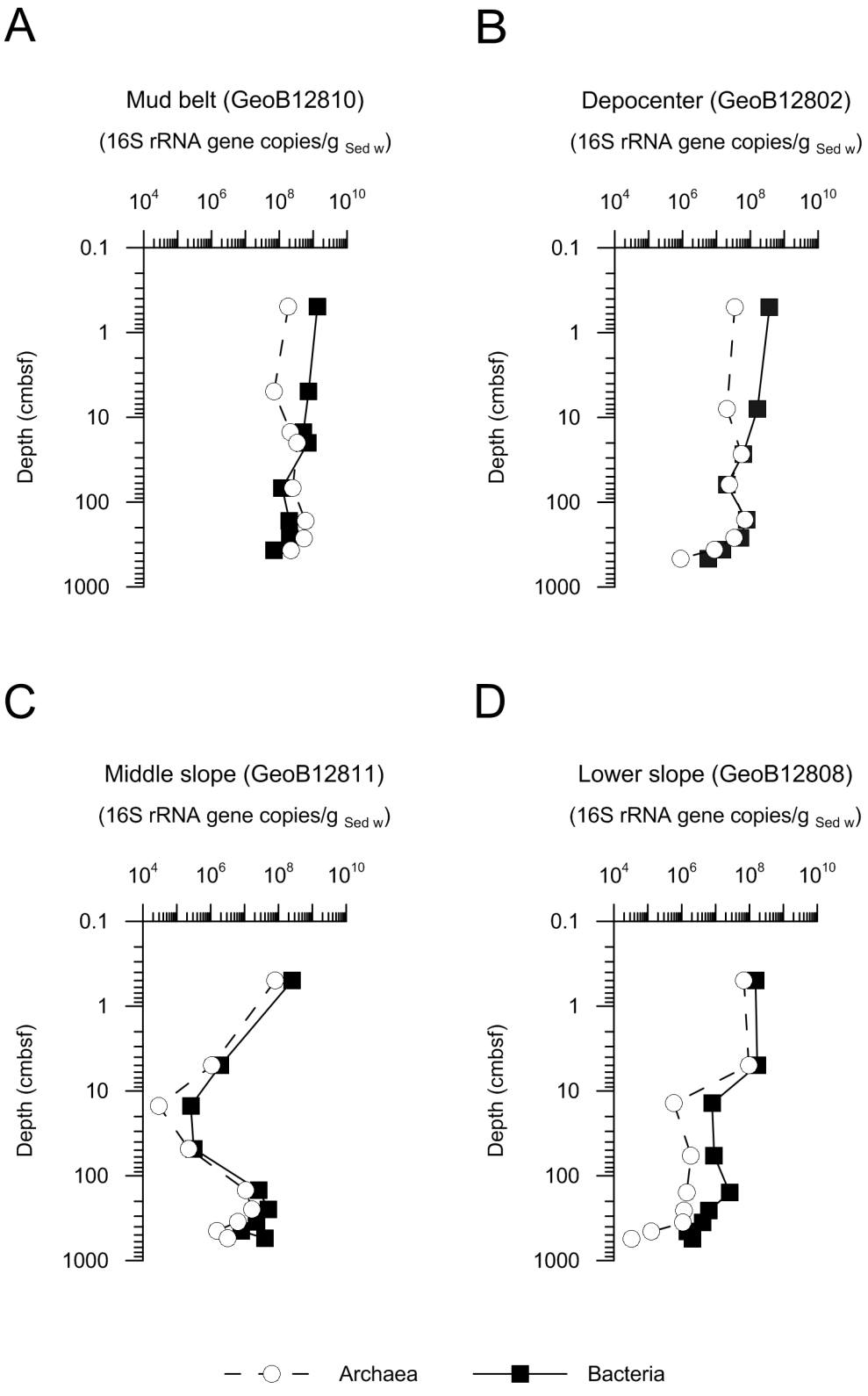

Fig. V.3. Depth profiles of the archaeal and bacterial 16S rRNA gene copy numbers/g wet sediment along the transect from mud belt (A) to lower slope (D).

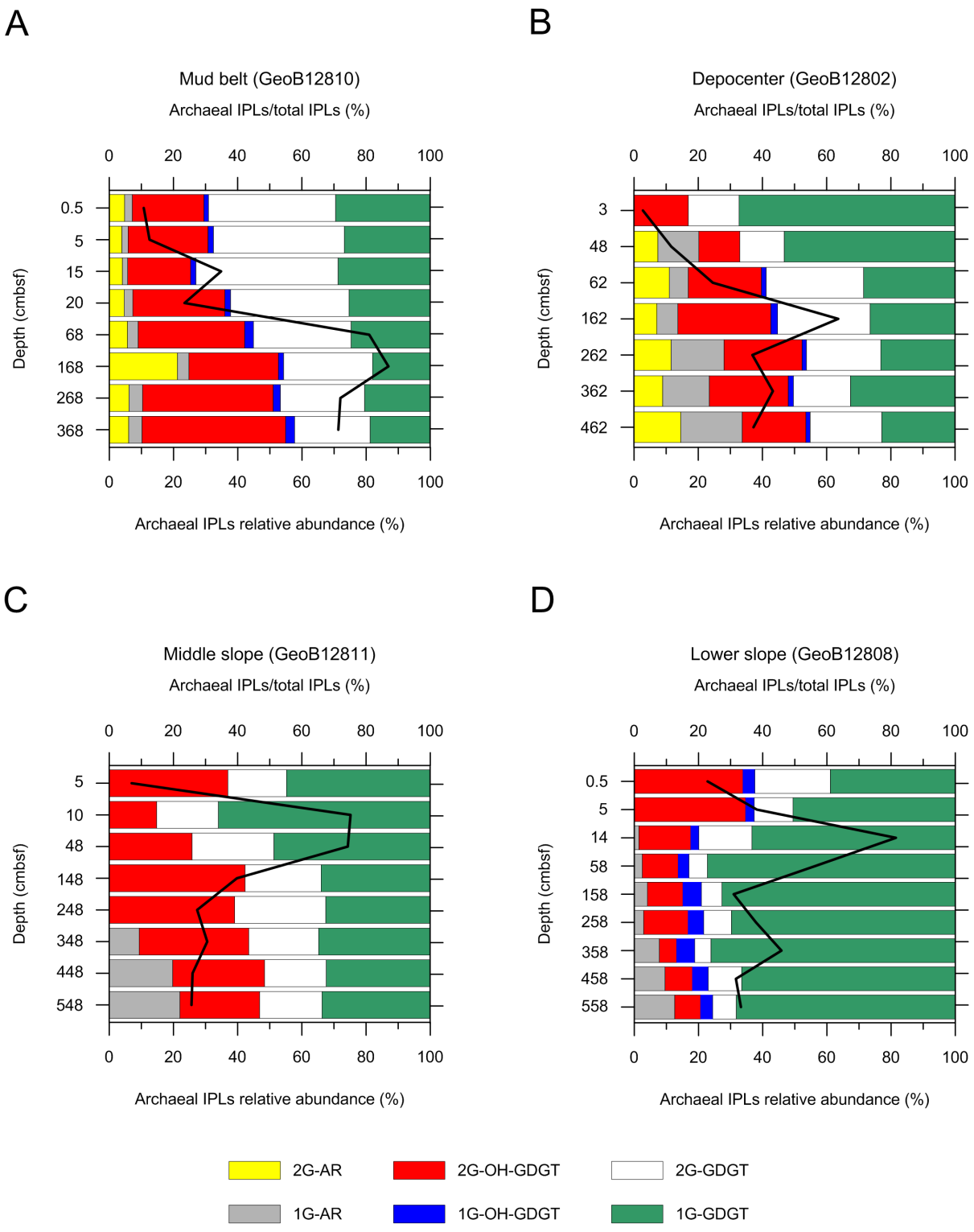

Fig. V.4. Distribution of major archaeal lipids, in four sediment cores along the transect across the Namibian shelf and slope. The archaeal lipids were quantified from Q-ToF and corrected with response factors. Two ratios were calculated: the relative contribution of major archaeal IPLs to total IPLs (solid line) and the relative abundance of individual lipid species (stacked bars).

The most abundant lipids were GDGTs with a glycosidic head group, which are common lipid constituents of archaeal membranes (Schouten et al., 2013). These compounds have been widely detected in the marine water column (e.g. Schubotz et al.,

2009; Xie et al., 2014) and marine sediments (e.g. Lipp et al., 2008; Lipp and Hinrichs, 2009; Rossel et al., 2011; Meador et al., 2015; Yoshinaga et al., 2015b).

Four different types of intact polar GDGTs were found: mono- and diglycosidic species (1G-GDGT and 2G-GDGT) and their monohydroxylated counterparts (1G-OH-GDGT and 2G-OH-GDGT). The relative abundance of 1G-GDGT increased in the offshore direction, coinciding with a clear down core increasing trend at the station from the lower slope (Fig. V.4). In contrast, 2G-GDGT and 2G-OH-GDGT were more abundant at the nearshore stations and 2G-OH-GDGT increased with depth in the mud belt. OH-GDGTs are widely distributed in environmental samples (Lipp and Hinrichs, 2009; Liu et al., 2012) and are major constituents in the marine ammonium oxidizing archaeon (AOA) *Nitrosopumilus maritimus* (Elling et al., 2014), while also being present in the methanogen *Methanothermococcus thermolithotrophicus* (Liu et al., 2012). 1G- AR was detected at all sites, while 2G-AR was only abundant in the samples from the mud belt and the depocenter, where the sum of both lipids represent up to 35% of the total archaeal lipids. ARs have been reported in subsurface sediments and are associated with the heterotrophic consumption of OM (Biddle et al., 2006; Lipp and Hinrichs, 2009). Glycosidic archaeol is widely distributed among members of the domain Archaea assigned to ANME-1/-2 (Rossel et al., 2008; Kellermann et al., 2016a), numerous methanogens (Koga et al., 1993) and *N. maritimus* (Elling et al., 2014). Stable isotope probing experiments indicate that intact ARs are potential biomarkers for actively growing Archaea (Kellermann et al., 2016a).

V.4.2.2. Minor archaeal lipids

More sensitive, targeted analysis using triple quadrupole MS revealed the presence of a number of additional archaeal lipids: two phosphatidic-GDGTs, 1G-GDGT-PG (phosphatidylglycerol) and hexose phosphohexose (HPH)-GDGT and several species of 1G and 2G lipids, including butanetriol DGTs (BDGTs), glycerol dibiphytanol diethers (GDDs) and monohydroxylated archaeol (OH-AR) (Fig. V.5). The phosphatidic GDGTs showed rapidly decreasing concentration within the uppermost cm, as reported for HPH-crenarchaeol (Lengger et al., 2012b). The minor archaeal glycosidic lipids that were identified in the sediments share a common down core pattern (Fig. V.5), similar to the pattern of 1G-GDGT.

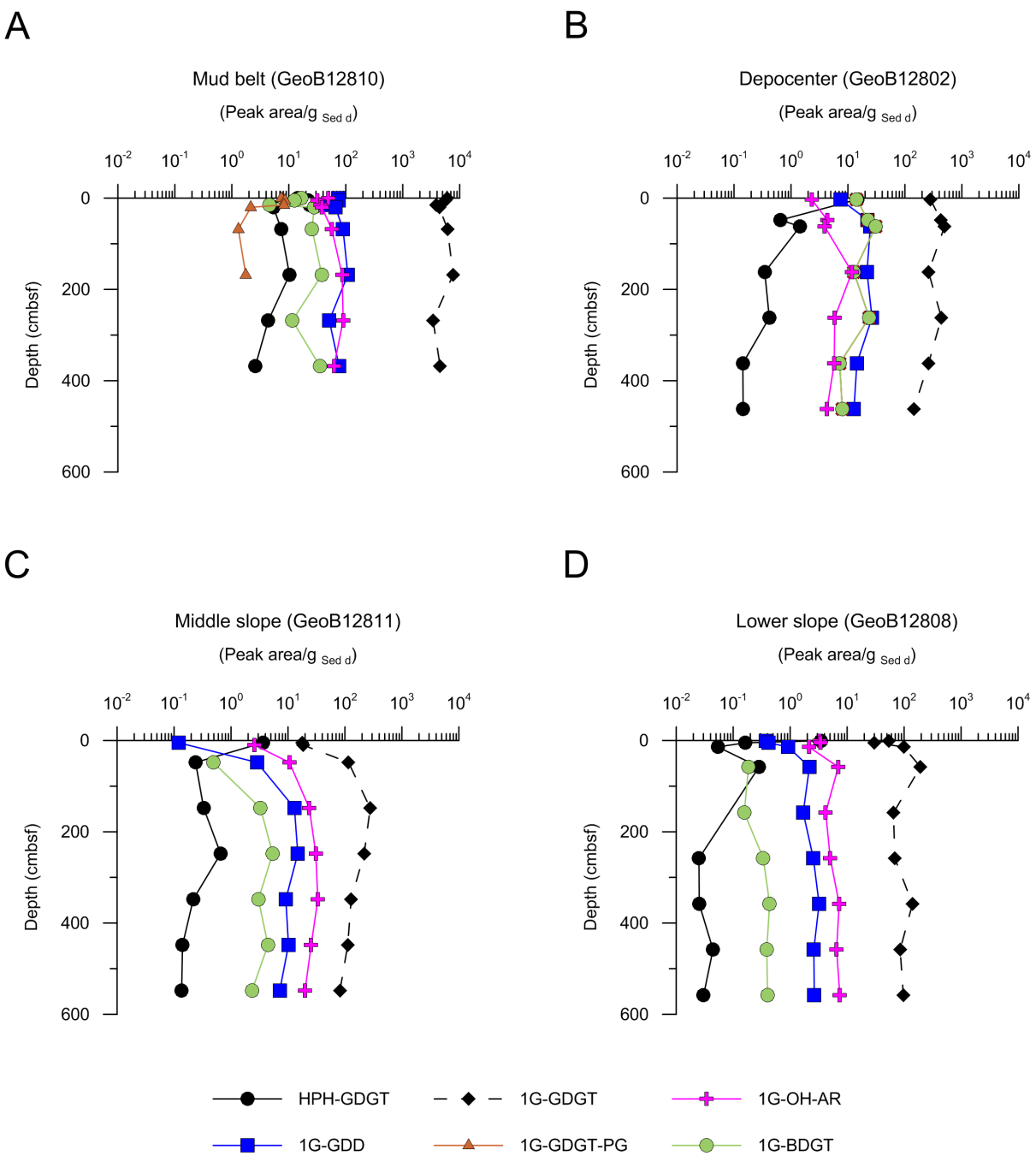

Fig. V.5. Down core profiles of abundances of minor archaeal IPLs and 1G-GDGT as reference along the transect from the mud belt (A) to the lower slope (D); data were acquired in MRM mode on a triple-stage quadrupole MS and given in unit-less peak area/g dry sediment, after normalization to the extraction standard (C_{46}-GTGT) without correcting for response factors.

HPH-crenarchaeol, a lipid produced by *Thaumarchaeota* (Schouten et al., 2008; Pitcher et al., 2010; Elling et al., 2014) has been detected up to 600 cmbsf at the Peru Margin (Lengger et al., 2014b), close to the depth reported here. During systematic examination of the thaumarchaeal lipidome, Elling et al. (2014) observed that HPH-GDGTs are especially abundant during early growth stages and suggested these lipids are linked to actively growing cells. 1G-GDGT-PG was only identified at the station from the mud belt

down to a depth of 168 cmbsf. It has been found in ANME-1 (Rossel et al., 2011) and Archaea of the order of *Thermoplasmatales* (Swain et al., 1997; Golyshina et al., 2016). Therefore, a possible biological source may be *Marine benthic group-D*, which is widely distributed in marine sediments (Teske and Sørensen, 2008) and is closely related to *Thermoplasmatales* (Vetriani et al., 1999). 1G-GDDs were identified in batch cultures of *N. maritimus* (Meador et al., 2014) suggesting a biosynthetic rather than diagenetic origin. OH-AR is most commonly associated with the anaerobic oxidation of methane (AOM; e.g. Hinrichs et al., 2000) and methanogens (Koga and Nakano, 2008). For 1G-BDGT a putative link to the *Miscellaneous Crenarchaeota Group* was established (Meador et al., 2015). However recent results have shown that *Methanomassiliicoccus luminyensis*, a methanogenic archaeon, is a source for BDGTs (Becker et al., 2016). The occurrence of lipids characteristic for methanogenic archaea within the SO_4^{2-} reduction zone suggests that methanogens utilizing non-competitive substrates such as MeOH and methylamines inhabit these sediments, as recently demonstrated for the Orca Basin (Zhuang et al., 2016). For example, *M. luminyensis* is associated with methylotrophy (Dridi et al., 2012), underlining that methanogenic organisms consuming non-competitive substrates are a potential biological source of these lipids.

V.4.2.3. Bacterial and eukaryal lipids

Different groups of intact BELs with a phosphatidic head group were present in the samples (Fig. V.6). To our knowledge, this study provides the most comprehensive account of their distribution in marine subsurface sediments to date. BELs with a glycosidic head group, which are typically identified in marine algae (Schubotz et al., 2009), were not detected in the sediments. This is consistent with the observation of Schubotz et al. (2009) who showed that these lipid disappeared below the top 1 cm in sediments from the Black Sea. A large fraction of BELs relative to total IPLs was observed at the sediment-water interface. The fraction decreased to a minimum between 168 cmbsf (mud belt) and 14 cmbsf (lower slope). The minimum was followed by a second maximum in the deeper sediments of the depocenter, middle and lower slope. Moreover, a lower diversity of BELs was observed in the deeper sediments.

Phosphatidic IPLs with a DEG core structure and a PE, PME and PDME head group, dominated the BEL pool in the sediments > 20 cmbsf at all stations. They were present in all samples, except the two uppermost ones from the lower slope where O_2 may penetrate to a depth of up to 20 cmbsf (Glud et al., 1994). This shows that PE, PME and PDME-DEGs are restricted to anoxic milieu, as suggested previously (Rossel et al., 2008; Rossel et al., 2011; Schubotz et al., 2009; Seidel et al., 2012). Based on their carbon stable isotopic composition, DEGs have been associated with the bacterial SO_4^{2-} reducing syntrophic partner (SRB) taking part in in AOM (Hinrichs et al., 2000). A further IPL study revealed a correlation

between PE-DEGs and ANME, reinforcing the idea of their potential as a proxy for SRB involved in AOM (Rossel et al., 2008). Apart from the AOM process, DEG lipids were recently found in mesophilic heterotrophic SRB (Grossi et al., 2015; Vinçon-Laugier et al., 2016). However, according to Kraft et al. (2013), these organisms represent only a minor part of the total microbial community on the Namibian shelf. Furthermore, rather constant SO_4^{2-}-concentrations throughout the sediment column at the lower slope and DEGs within the methanogenic zone at the stations from the mud belt and the depocenter predict that additional biological sources for these lipids cannot be excluded (Grossi et al., 2015). Accordingly, Schippers et al. (2012) showed a dominance of *Chloroflexi* and *JS1* (*Atribacteria*) over SRB in the subsurface sediments of the Benguela upwelling system. Moreover, PE-DEG and other ether bound lipids were also found in the soil bacterium *Myxococcus xanthus* during starvation-induced sporulation (Ring et al., 2006), thereby implying bacterial endospores to be a potential source.

BELs built of a DAG or an AEG core connected to either PC, PG, PE, PME or PDME head groups strongly dominated the surface sediments and were absent from sediments > 20 cmbsf apart from the mud belt. PE-DAGs are widely found in marine surface sediments (e.g Schubotz et al., 2009; Rossel et al., 2011) and their ubiquitous abundance in Bacteria hinders their assignment to a specific biological source, even though they are often attributed to SRB (cf. Makula and Finnerty, 1974; Rütters et al., 2001; Sturt et al., 2004). DAGs with a PG head group are highly abundant in phototrophic organisms (Asselineau and Trüper, 1982; Imhoff et al., 1982) and PC-DAGs are the most abundant lipids in Eukarya (Raetz, 1986), and are found in almost all eukaryotic algae (Kato et al., 1996; Harwood, 1998) and some diatoms (Berge et al., 1995). Hence a large fraction of these lipids may be transported from the euphotic zone to the surface sediments, as was also observed in the Black Sea (Schubotz et al., 2009). Indeed, the fatty acid distributions of the PC-DAGs at all stations showed a high ACL (35 to 40 carbon atoms) and DBI (3-9 unsaturations) vs. the other identified BELs. These polyunsaturated long chain fatty acids are typically identified in algae (e.g. Brett and Müller-Navarra, 1997). However, since up to 15% of all Bacteria build up their membrane with PC-DAGs (Geiger et al., 2013), benthic production cannot be excluded.

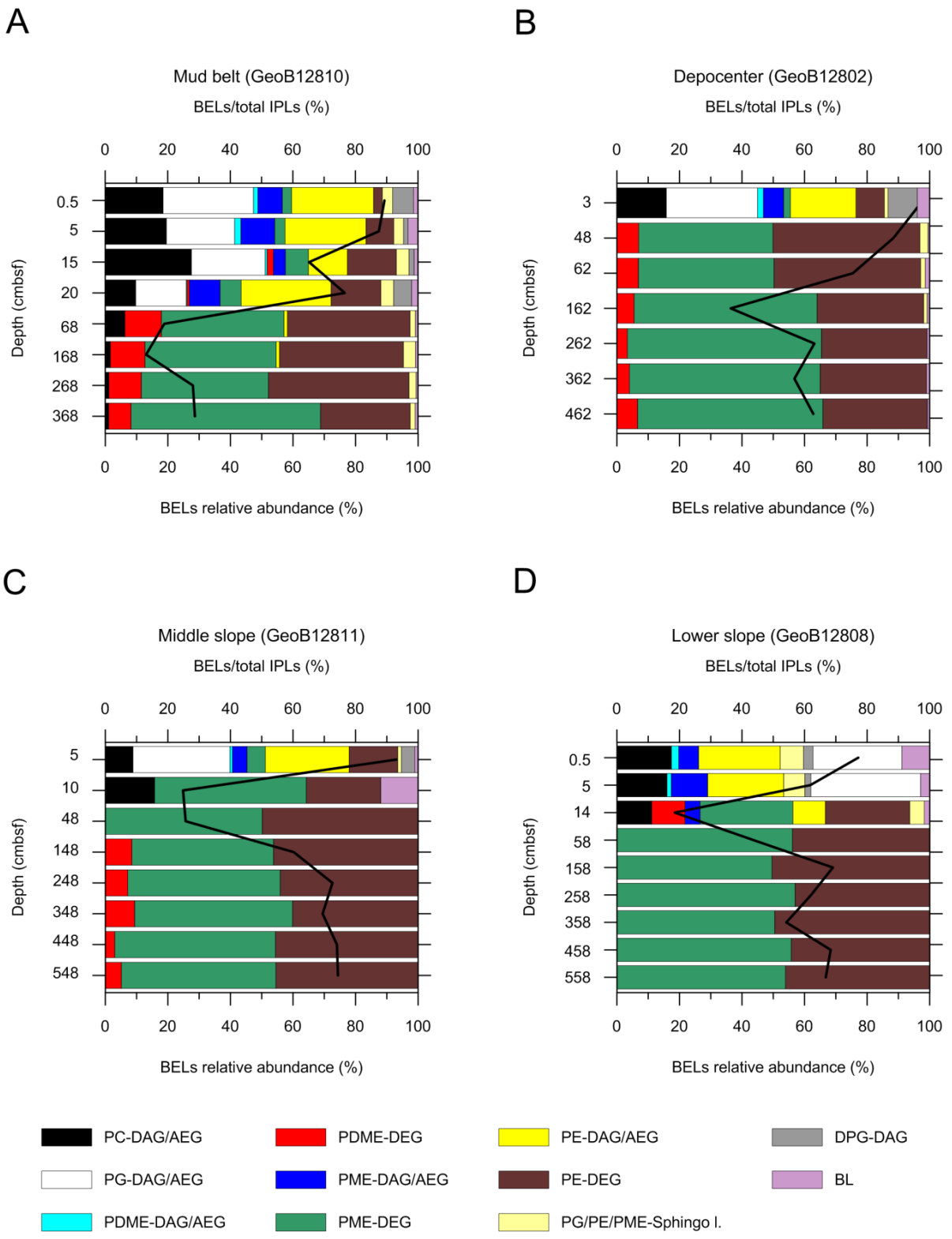

Fig. V.6. BELs along transect through the Benguela upwelling system from mud belt (A) to the lower slope (D). The results were corrected with response factors. A response factor of unity was chosen for sphingolipids because no standard was available. The solid line shows the relative contribution of BELs to total IPLs and the stacked bars depict the relative abundance of individual lipid species.

The relative abundance of sphingolipids and betaine lipids (BLs) stayed rather constant in the mud belt and the depocenter samples, while in the stations on the slope these

lipids were not identified at depths greater than 14 cmbsf (Fig. V.6.). Sphingolipids have been identified in mammals and Bacteria (Kato et al., 1995; Kato et al., 1996; Olsen and Jantzen, 2001). Additionally, sphingolipids with glycosidic head groups have been widely identified in viruses and virus-infected algae (e.g Fulton et al., 2014; Vardi et al., 2009). Since BLs are characteristic constituents of algae and lower plants (Eichenberger, 1982; Sato and Furuya, 1984) and their occurrence among Bacteria is limited (Geiger et al., 2010), BLs and Sphingolipids might also represent algal detritus exported from the water column to the sediment. However, in the mud belt and the depocenter, traces of BLs were detected at greater depths with varying core lipid structures (Fig. V.7A). Schubotz et al. (2009) also observed variations in the core structure of BLs between the oxic zone, dominated by even carbon numbered fatty acid chains, and the suboxic zone (odd carbon numbers) in the water column of the Black Sea. They suggested that unknown anaerobic bacteria produce the odd numbered BLs in the anoxic water column. Accordingly the observed variations of the core lipid structure in the sediments from the Benguela upwelling may also indicate an *in situ* production by anaerobic bacteria.

V.4.3. Potential membrane lipid adaptations signaled by glycerol ether lipid dominance in the subsurface sediments

The bacterial IPL distributions in subsurface sediments are dominated by complex assemblages of DEG compounds (Fig. V.6). This is an interesting observation given their rarity in mesophilic bacterial isolates. Apart from their scarcity in bacterial isolates, ether bound core lipids have been found in various hydrothermal (e.g. Jahnke et al., 2001; Pancost et al., 2006; Bradley et al., 2009) and mesophilic marine ecosystems hosting syntrophic AOM consortia (e.g. Hinrichs et al., 2000; Pancost et al., 2001; Orphan et al., 2001; Elvert et al., 2005). The known mesophilic bacterial isolates are limited to SRBs (Grossi et al., 2015; Vinçon-Laugier et al., 2016) and selected members of the *Planctomycetes* (Sinninghe Damsté et al., 2005).

The prevalence of DEGs in the subsurface sediments vs. surface sediments suggests a variation in the bacterial community with increasing depth. It has been shown that membrane lipids with ether linkages reduce the water and osmotic permeability of the membrane compared with their ester bound counterparts (Jansen and Blume, 1995). Experiments with archaeal ether bound lipids confirmed the lower proton permeability relative to bacterial ester-bonds (Van de Vossenberg et al., 1998; Mathai et al., 2001; Konings et al., 2002). This agrees with the hypothesis that strongly energy limiting environments may select for special membrane properties, such as low permeability, which are required for survival under these conditions (Valentine, 2007).

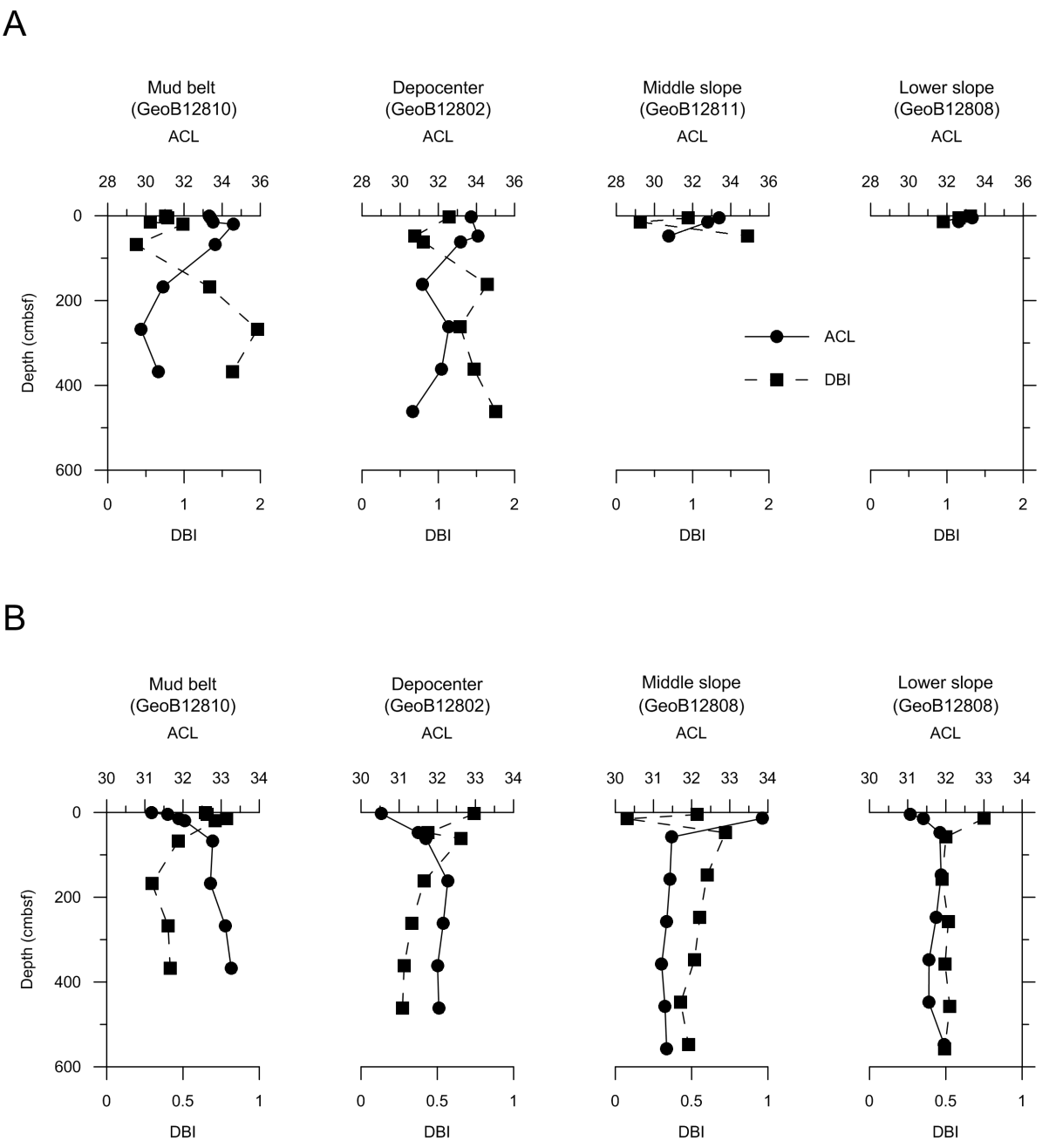

Fig. V.7. Depth variations of the average chain length (ACL) and degree of unsaturation of alkyl groups (DBI, double bond index) of BLs (A) and PE-DEG (B), expressed as mass weighted averages according to Eqs 1 and 2. NB different axis scales of A and B.

An indication that the prevalence of these DEGs is due to *in situ* production rather than preferential preservation due to their relatively recalcitrant nature (cf. Logemann et al., 2011), is the increase in chain lengths of up to two carbons accompanied by a decrease in unsaturation with greater depth (Fig. V.7B). De Gier et al. (1968) showed that increasing chain length and a lower number of unsaturations in the alkyl chain result in decreased permeability and therefore enhanced protection of the cell against energy loss. A positive correlation between protection efficiency, in particular against proton permeability, and chain length was also revealed in a study by Paula et al. (1996). Therefore, the reliance on

relatively stable glycerol ether-based membranes in combination with longer alkyl chain length and lower number of unsaturations may be characteristic for Bacteria under energy limiting environments.

V.4.4. Comparison of IPL- and DNA-datasets

The fraction of Archaea in the total microbial community was estimated from the IPL- and DNA-based methods (Fig. V.8) to gain insight into the subsurface microbial community of the upwelling system. The estimates reflect similar trends for both techniques in the samples from the mud belt, depocenter and middle slope. By contrast, the two techniques resulted in similar down core patterns for samples from the lower slope, but the archaeal fraction in the IPL dataset strongly exceeded the archaeal fraction from the nsDNA measurements. Nevertheless, the good agreement between lipid- and nsDNA-based methods for the quantitative assessment of community composition on the domain level is unprecedented, and a clear improvement compared to previous studies in marine sediments (e.g. Lipp et al., 2008; Briggs et al., 2012). This is manifested by the strong linear correlation ($R^2 = 0.72$, $p < 0.00001$; Fig. V.9) between the two sets of estimated archaeal fraction in the gravity core samples (> 50 cmbsf). This correlation is attributed to a common source contributing to the signals from both IPLs and nsDNA. Since sDNA was removed prior to extraction, the common source for both biomolecular pools in the deeply buried sediments is possibly intact microbial cells.

Two subsets of the data deviate most obviously from the good correlation between both data sets. The first subset, which does not show a good agreement between techniques, refers to an enhanced IPL based archaeal fraction compared with nsDNA and comprises the samples from the lower slope. The dominance of archaeal lipids at this site could be caused by a slow decomposition rate of relatively stable glycosidic ether lipids in this oligotrophic environment. Therefore, the archaeal ratio determined from IPLs at this site may be overestimated due to an increased contribution of fossil lipids. This agrees well with the dominance of 1G-GDGT in these samples, which was suggested to be more stable during post-depositional degradation than the diglycosidic species (Lengger et al., 2013).

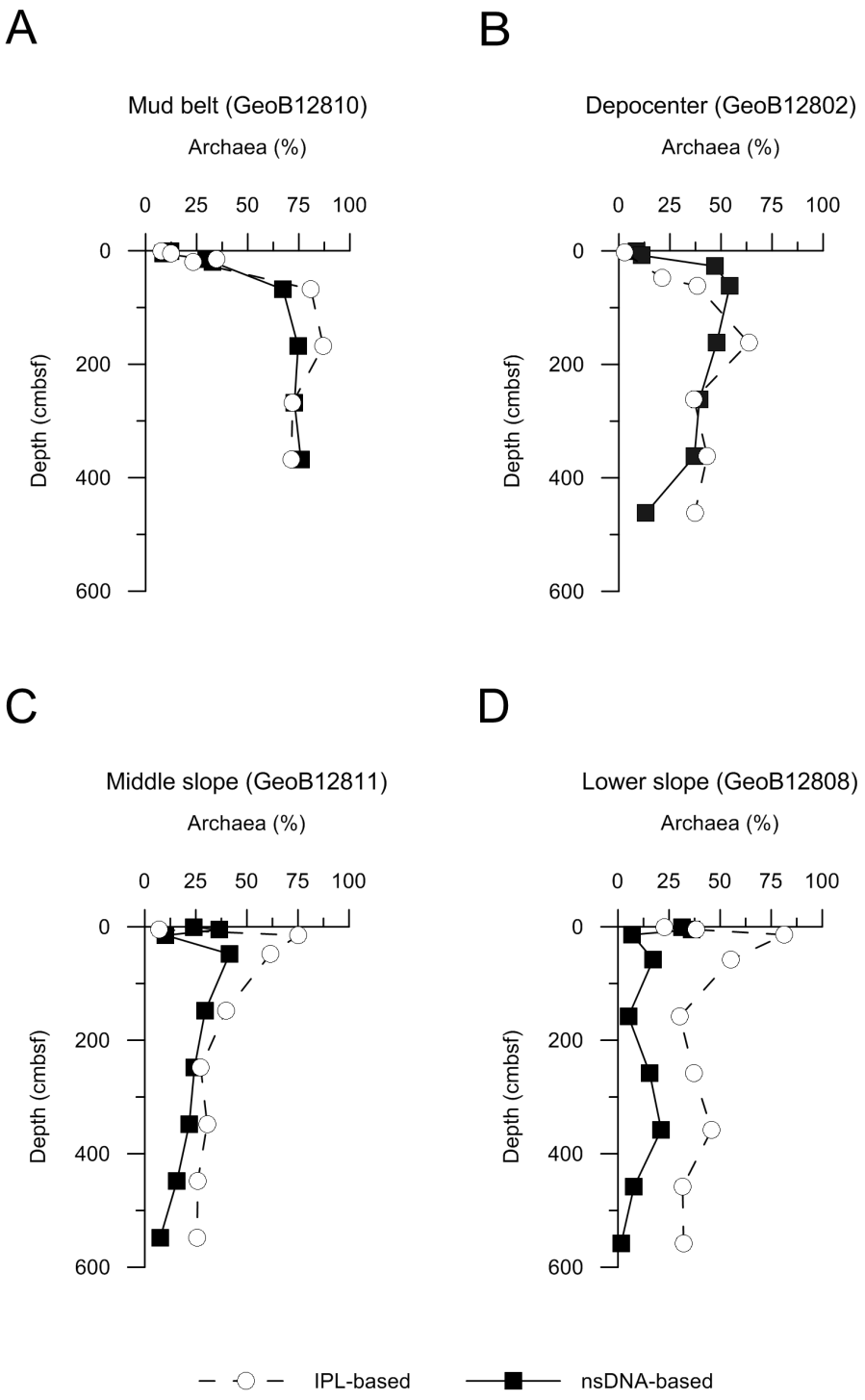

Fig. V.8. Proportion of Archaea relative to the total microbial population estimated from analysis of IPLs and 16S rRNA gene copy numbers, ranging from the mud belt (A) to the lower slope (D). The IPL data are from Q-ToF analysis and were corrected with response factors.

The second subset of samples shows a predominance of BELs in the lipid data set compared to the nsDNA-data in some surface samples, most notably on the middle slope (5 cmbsf). Given that in the surface sediments a certain proportion of BELs can be tentatively assigned to marine algae (e.g. BLs, PC-DAGs, PG-DAGs and sphingolipids) this suggests an

accumulation of lipids from algal detritus derived from the water column in the uppermost sediments.

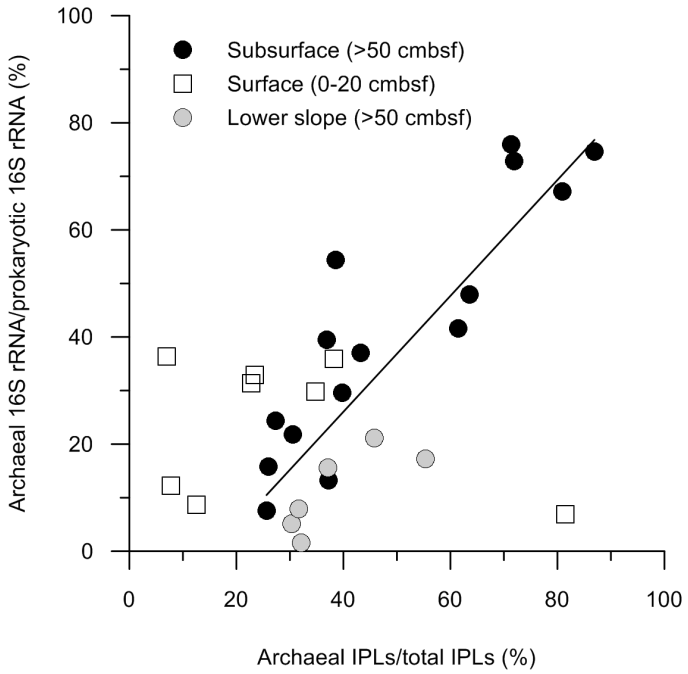

Fig. V.9. Plotted archaeal fraction vs. total prokaryotic community detected with nsDNA vs. archaeal fraction of the total IPLs measured with Q-ToF. Only samples where both methods were applied are shown (see Table V.S2). The line shows the regression line for the gravity cores of the four analyzed stations.

V.4.5. Relationship between domain composition and environmental conditions

The mud belt station hosts the largest microbial community, with a strong bacterial dominance in the topmost centimeters (Fig. V.8). This matches studies showing that the combination of high total organic carbon content and fresh OM results in the most active microbial community (e.g. Bühring et al., 2006). In particular, the input of fresh OM has been shown to control the activity of microorganisms (Aspetsberger et al., 2007). Moreover, the results suggest that this community is driven mainly by bacterial heterotrophs that outcompete Archaea in the uppermost centimeters, where facile terminal electron acceptors are available. At the stations further offshore, the size of the microbial community at the sediment-water interface is smaller, coinciding with the input of more refractory OM.

Microbial cell concentration has been shown to be strongly influenced by deposition rate (Kallmeyer et al., 2012), as fast sediment burial will result in shorter exposure time of labile compounds to O_2, thereby aiding transport of favorable substrates to greater depth. Moreover, a high sedimentation rate results in preferential burial of less degraded OM. Consequently, several studies have shown good correlation between sedimentation rate and

OM burial (Müller and Suess, 1979; Canfield, 1994). In our area the sediment deposition rate varies strongly across the transect, with a high rate in the mud belt and the depocenter and moderate to low rate further offshore (Table V.1). Similar to the study by Kallmeyer et al. (2012) a decrease in microbial population size with decreasing deposition rate was observed.

With increasing sediment depth, the size of the microbial community gradually decreased, apart from the pronounced minimum between 3 and 168 cmbsf at the station from the middle slope (Fig. V.2.), corresponding with the growing age of the OM at greater depth (Mollenhauer et al., 2007). Moreover, a distinct change in the domain composition along the profiles was observed. The sediments from the mud belt are characterized by a sharp increase in archaeal abundance in the upper 1 m, reaching up to 75% of the total prokaryotic community (Fig. V.8A). Down core this abundance is relatively constant in both data sets from the mud belt station, showing that the redox zonation had only little effect on the relative abundance of Archaea and Bacteria in the sediments, as observed for sediments from the Peru margin (Biddle et al., 2006). With increasing distance to the shore, the archaeal fraction peaks at shallower depths and becomes less pronounced, representing < 25% at the in the deeper sediments from the lower slope for the nsDNA based data (Fig. V.8D.). This dominance of Bacteria offshore is accompanied by decreasing OM content in the sediment column (Table V.1). Furthermore, a low organic carbon mineralization rate at the stations from the middle and lower slope compared with the mud belt and depocenter is reflected by low concentrations of dissolved inorganic carbon and NH_4^+ in the pore water (Goldhammer et al., 2011; Lin et al., 2012; Lagostina et al., 2015). These parameters, coupled with the lower SO_4^{2-} reduction rate further offshore, indicate lower microbial activity in the middle and lower slope stations vs. the station in the mud belt. Measurement of oxygen isotopes in inorganic PO_4^{3-} and its concentration confirmed the higher microbial activity coupled with a highly efficient microbial turnover of inorganic PO_4^{3-} in the mud belt, whereas samples taken from the slope showed lower microbial activity (Goldhammer et al., 2011).

The combination of low OM burial in the sediment, input of more refractory OM from the water column and the low microbial activity indicated from geochemical data at the stations from the middle and lower slope suggests that Bacteria dominate at the energetically less favorable sites vs. Archaea. These findings contradict with the hypothesis that Archaea outcompete Bacteria due to structural and metabolic advantages in energy limited subsurface sediments (Valentine, 2007). However, the unique composition of bacterial lipids detected in the deeper sediments seems to be ideally suited for low energy systems and might partially compensate for the theoretical disadvantages of Bacteria vs. Archaea.

V.5. Acknowledgments

We thank the crew and scientists from R/V Meteor cruise M76/1 for sample recovery and two anonymous reviewers for useful comments. The study was funded by the European Research Council under the European Union's Seventh Framework Program – ''Ideas'' Specific Program, ERC grant agreement # 247153 (Advanced Grant DARCLIFE; PI K.-U.H.), ERC grant agreement # 294200 (Advanced Grant MICROENERGY; PI B.B.J) and the Deutsche Forschungsgemeinschaft through the Gottfried Wilhelm Leibniz Price (HI 616-14). The Center for Geomicrobiology is funded by the Danish National Research Foundation.

V.6. Appendix A: supporting information

Table V.S1. The measured methane concentrations of the station from the mud belt (GeoB12810). Methane concentrations of this site were measured at same time and by the identical method as the other stations that were reported by Lin et al., (2012)

Sample depth [cmbsf]	CH_4 concentration [mM/l]
5	0.002
10	0.005
15	0.006
20	0.007
25	0.011
30	0.011
35	0.013
68	0.019
80	0.016
130	0.019
168	0.033
180	0.039
212	0.039
230	0.049
255	0.056
268	0.65
280	1.58
300	1.71
315	3.96
350	4,67
368	6.09

Table V.S2. The investigated depths and TOC-values of the sediment samples analyzed in this study. TOC-values were taken from Lin et al. (2012).

Sediment core	Sample depth [cmbsf]	Method	TOC [%]
GeoB12810	0.5	nsDNA,IPL	-
GeoB12810	5	nsDNA,IPL	7.43

Table V.S2. (continued) The investigated depths and TOC-values of the sediment samples analyzed in this study. TOC-values were taken from Lin et al. (2012).

Sediment core	Sample depth [cmbsf]	Method	TOC [%]
GeoB12810	15	nsDNA,IPL	5.53
GeoB12810	20	nsDNA,IPL	8.6
GeoB12810	68	nsDNA,IPL	4.2
GeoB12810	168	nsDNA,IPL	9.11
GeoB12810	268	nsDNA,IPL	7.28
GeoB12810	368	nsDNA,IPL	11.25
GeoB12802	0.5	nsDNA	-
GeoB12802	3	IPL	-
GeoB12802	8	nsDNA	-
GeoB12802	27	nsDNA	-
GeoB12802	48	IPL	-
GeoB12802	62	nsDNA,IPL	7.41
GeoB12802	162	nsDNA,IPL	5.25
GeoB12802	262	nsDNA,IPL	6.86
GeoB12802	362	nsDNA,IPL	5.99
GeoB12802	462	nsDNA,IPL	7.79
GeoB12811	0.5	nsDNA	-
GeoB12811	5	nsDNA,IPL	-
GeoB12811	10	IPL	-
GeoB12811	15	nsDNA	-
GeoB12811	48	nsDNA,IPL	1.66
GeoB12811	148	nsDNA,IPL	4.85
GeoB12811	248	nsDNA,IPL	4.64
GeoB12811	348	nsDNA,IPL	5.33
GeoB12811	448	nsDNA,IPL	5.13
GeoB12811	548	nsDNA,IPL	4.01
GeoB12808	0.5	nsDNA,IPL	-
GeoB12808	5	nsDNA,IPL	-
GeoB12808	14	nsDNA,IPL	-
GeoB12808	58	nsDNA,IPL	1.80
GeoB12808	158	nsDNA,IPL	1.04

Table V.S2. (continued) The investigated depths and TOC-values of the sediment samples analyzed in this study. TOC-values were taken from Lin et al. (2012).

Sediment core	Sample depth [cmbsf]	Method	TOC [%]
GeoB12808	258	nsDNA,IPL	1.09
GeoB12808	358	nsDNA,IPL	1.12
GeoB12808	458	nsDNA,IPL	1.36
GeoB12808	558	nsDNA,IPL	1.03

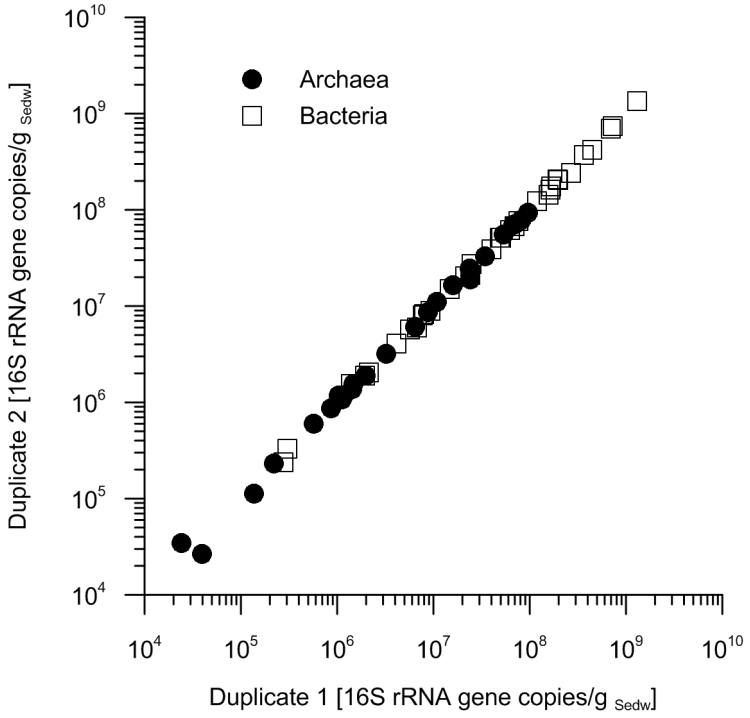

Fig. V.S1. The duplicate measurements of the archaeal and bacterial 16S rRNA gene copy numbers per gram wet sediment in this study.

Chapter VI

Carbon assimilation and fate of methanogenic lipid biomarkers in marine sediments tracked by lipid radio isotope probing

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VI.1. Abstract

Methanogenesis is an important terminal step of organic matter remineralization and is exclusively performed by anaerobic archaea. In anoxic marine sediments, methanogenesis represents one key process, particularly in layers that are depleted in electron acceptors like oxygen, nitrate or sulfate. So far, seven taxonomic groups of methanogenic archaea that utilize either hydrogen/carbon dioxide (hydrogenotrophic), acetate (acetoclastic) or methylated compounds (methylotrophic methanogenesis) as energy source have been cultured. However, the activity, mechanisms of carbon assimilation and lipid biosynthesis of methanogenic archaea in marine sediments are largely unconstrained. To this aim a ^{14}C lipid radioisotope probing incubation experiment, using either ^{14}C-bicarbonate or ^{14}C-acetate as carbon source, was performed in marine sediments of the Rhone delta (Mediterranean Sea). Methanogenesis in anaerobic sediment slurries was stimulated by a hydrogen/carbon dioxide headspace (hydrogenotrophic-set) and additional methanol (methylotrophic-set) as energy source and compared to the not-stimulated background community. The incubations were conducted at 30 °C for 21 days with sediments from three geochemical zones (sulfate reduction zone, sulfate methane transition zone and methanogenic zone). At all depths, the experiments with ^{14}C-bicarbonate showed a higher carbon assimilation rate compared to ^{14}C-acetate, indicating that the methanogenic communities are predominantly autotrophs. Unexpectedly, the apolar core lipids showed a higher incorporation of ^{14}C than the intact polar lipids in almost all incubated slurries. The intact polar lipids were characterized by a predominant ^{14}C incorporation into archaeols in the stimulated samples and the control-set, while the apolar core lipid-fraction consisted predominantly of tetraethers over archaeols in most of the slurries. This indicates that archaeols are good biomarkers for benthic archaea in active estuarine sediments, regardless of the nutrient supply or archaeal community. The different labeling pattern of core and intact lipids and the short incubation period implies that apolar lipids are actively synthesized during the course of the experiments. Although the exact biological function of core lipids remains to be addressed, the results suggest that apolar lipids in marine sediments are not exclusively formed by the degradation of intact polar lipids.

Keywords: ^{14}C incubation experiments, Archaea, methanogenesis, diether, tetraether, lipid biosynthesis, autotrophy, heterotrophy

VI.2. Introduction

Methane (CH_4) is one of the most important greenhouse gases on this planet and is produced in various environments, such as soils, rice fields, wetlands and marine sediments, as well as in many animal digestion tracts. Marine sediments host one of the largest reservoirs for CH_4 and account for 7 to 25% of the global production of CH_4 (Reeburgh, 2007). The vast majority of this CH_4 is produced biotically by microbes. Methanogenesis is a terminal step during organic matter (OM) degradation in anoxic sediments (Froelich et al., 1979) and is mediated by strictly anaerobic methanogenic archaea (reviewed by Garcia et al., 2000). With regard to their energy source, three distinct pathways are performed by Archaea: (i) the hydrogenotrophic, (ii) the acetoclastic and (iii) the methylotrophic methanogenesis (e.g. Whiticar, 1999). During hydrogenotrophic methanogenesis carbon dioxide (CO_2) is respired with hydrogen (H_2) resulting in CH_4 and water. Acetoclastic methanogens utilize acetate (ACT) as energy source, which is cleaved into CO_2 and CH_4. Since sulfate respiration with H_2 and ACT is thermodynamically favored, hydrogenotrophic and acetoclastic methanogenesis are outcompeted by sulfate-reducing bacteria (SRB) (Whiticar, 1999). Therefore, hydrogenotrophic and acetoclastic methanogenesis predominantly occur in sulfate depleted sediments (Oremland and Polcin, 1982; Whiticar, 1999). Methylotrophic archaea consume methylated substrates, such as methanol (MeOH), dimethyl sulfide and methylamines, which cannot be utilized by SRB. By using those non-competitive substrates, methylotrophic can also be abundant in the sulfate reducing zone (SRZ, Oremland and Polcin, 1982).

So far, all cultured methanogens belong to the *Euryarchaeota* and are distributed among seven taxonomic orders: *Methanopyrales*, *Methanococcales*, *Methanobacteriales*, *Methanomicrobiales, Methanocellales, Methanosarcinales* and *Methanomassiliicoccales*. The majority of the cultivated methanogens perform hydrogenotrophic methanogenesis, while *Methanosarcinales* are the only cultivated representative for acetoclastic methanogenesis so far (reviewed by Offre et al., 2013). In addition, *Methanosarcinales* (Weimer and Zeikus, 1978) and the recently described *Methanomassiliicoccales* can utilize methylated compounds as energy sources (e.g. Dridi et al., 2012; Iino et al., 2013). The carbon assimilation pathways in the different methanogenic classes are diverse and depend on the individual organism and growth conditions (Weimer and Zeikus, 1978). However, approximately 50% of the methanogens are considered to utilize CO_2 (autotrophy) as carbon source, whereas the other half assimilate organic matter (OM, heterotrophy), such as ACT and more complex organic molecules (Whitman et al., 2006).

Methyl coenzyme M reductase (mcrA) is a common molecular biomarker to investigate phylogenetic relationships of methanogens in environmental samples (Springer et

al., 1995; Hallam et al., 2003). However, despite the widespread production of CH_4 in subsurface sediments, genomic studies revealed that methanogens represent only 1% of the archaeal community (reviewed by Lever, 2016). The detection of methanogenic communities by DNA based techniques depends on the specificity of the applied primer sets. In this sense, it was shown that the application of general mcrA primer pairs does not cover the phylogenetic diverse methanogenic community in the sediments from the Guaymas Basin and resulted in an underestimation of CH_4 producing archaea in marine sediments (Lever and Teske, 2015).

The analysis of the concentration and distribution of the archaeal membrane lipids is a technique that potentially allows a less biased investigation of the archaeal community in marine sediments. The investigation of cultures revealed a distinctly different lipid distribution in methanogens compared to marine archaea (reviewed by Schouten et al., 2013). The lipidome of methanogens is usually characterized by a predominance of archaeol (AR) over acyclic glycerol dibiphytanyl glycerol tetraethers (GDGTs), while the introduction of pentacyclic moieties is rare among methanogens (see review by Koga and Nakano, 2008). Moreover, the natural isotopic signatures of the individual lipids can be measured, which provides insights into the metabolic pathways of the microbial community (reviewed by Pancost and Sinninghe Damsté, 2003). The analysis of the lipid distribution in combination with the natural isotopic composition of the lipids allows to estimate the contribution of methanogenic community in environmental samples, as demonstrated in various environmental case studies (e.g. Pancost and Sinninghe Damsté, 2003; Schubotz et al., 2011; Zhuang et al., 2016).

While DNA and lipid-based fingerprinting can be successfully applied to determine the phylogenic distribution of methanogens in marine sediments, neither of them, allows to track the metabolic activity, biomass synthesis and carbon assimilation pathways in environmental samples. In order to trace these processes, incubation experiments using labeled substrates are a valuable tool (see reviews by Boschker and Middelburg, 2002 and Wegener et al., 2016). Particularly, labeling experiments aiming at microbial lipids allow a highly sensitive investigation of microbial processes, because less cell reproduction cycles and shorter incubation periods are needed compared to experiments with DNA or RNA (Wegener et al., 2012). However, previous studies that targeted archaeal lipids with stable isotope probing (SIP) in marine sediments showed only minor or no significant uptake of different ^{13}C-substrates (e.g. Lin et al., 2013; Lengger et al., 2014a). To improve the sensitivity of stable isotope probing, the dual-SIP-method has been developed using a combination of deuterated water (^2H_2O) as a marker for microbial activity and ^{13}C-bicarbonate (^{13}C-DIC) as a marker of metabolic processes (Wegener et al., 2012). In their case study with sediments from the Baltic Sea, Wegener and colleagues were able to detect ^2H incorporation, but showed no

significant consumption of ^{13}C-DIC. Here we propose the utilization of radio isotopes to trace substrate uptake into lipids (Lipid-RIP) as a more sensitive method due to the low natural abundance of ^{14}C compared to ^{13}C (< 10^{-9}% vs. 1.06%).

To investigate the activity, the carbon fixation pathways and the lipid biosynthetic pathways of methanogenic archaea in marine sediments a lipid-RIP experiment was performed. Methanogens were stimulated by the addition of H_2/CO_2 and H_2/CO_2 with additional methanol (MeOH) in the sediment incubations and compared with the background community without stimulation. Two ^{14}C-labeled substrates were added to the slurries to trace autotrophic (^{14}C-DIC) and heterotrophic (^{14}C-ACT) carbon fixation pathways. To investigate the ^{14}C incorporation into individual archaeal lipids, the extracted labeled lipids were chromatographically separated into three different archaeal lipid pools that are ubiquitously found in environmental samples (e.g. Lipp and Hinrichs, 2009): apolar core (CLs), monoglycosidic (1GLs) and di-/triglycosidic lipids (2G+3GLs). Subsequently each of the three pools where chromatographically divided into lipid structures that are typically detected in methanogens (AR and GDGTs). This experimental set-up provides a unique view into lipid biosynthesis of methanogenic archaea.

VI.3. Material and Methods

VI.3.1. Sampling and study site

The incubation experiments and lipid analysis were performed with sediments from the prodelta of the Rhone River (GeoB17306; Fig. VI.1) in the Gulf of Lions (Mediterranean Sea) collected in Spring 2013 during the DARCSEAS II cruise (POS 450, Heuer et al., 2013).

The Rhone River is one of the major freshwater sources (1700 m³/s) to the Mediterranean Sea (Sempéré et al., 2000) and transports considerable quantities of fine grained sand particles, terrestrial organic matter (OM) and nutrients that are deposited at the river mouth (Sempéré et al., 2000; Rabouille et al., 2008). The input of terrestrial sediments from the Rhone River coupled with remobilization results in high sedimentation rates of up to 20-50 cm yr^{-1} (Marion et al., 2010). The enhanced input of nutrients from the river coupled with wind-driven upwelling leads to a high primary production in the water column, making the Gulf of Lions one of the most productive regions in the oligotrophic Mediterranean Sea (Ludwig et al., 2009). This results in high sulfate reduction rates in the surface sediments of the investigated station (Zhuang, 2014). The sulfate methane transition zone (SMTZ) is located below the sulfate reduction zone (SRZ), at a depth between 80 and 100 cm below the seafloor (cmbsf). The deeper sediments are characterized by high CH_4 concentrations indicative for the methanogenic zone (MZ; Zhuang, 2014).

The samples at site GeoB17306 (43°18.96'N, 4°52.17'E) were collected by a combination of multicorer and gravity corer at 30 m below the sea level. Immediately after core retrieval, samples for incubation experiments were transferred to pre-sterilized 250 mL Schott bottles and sealed with sterile gas tight butyl stoppers. The headspace was then exchanged with N_2 to ensure anoxic conditions and samples were stored at 4 °C. Additionally, sediments from the same core were sampled for intact polar lipid (IPLs) analysis. The lipid samples were transferred to 150 mL vials and kept frozen at -20 °C until analysis.

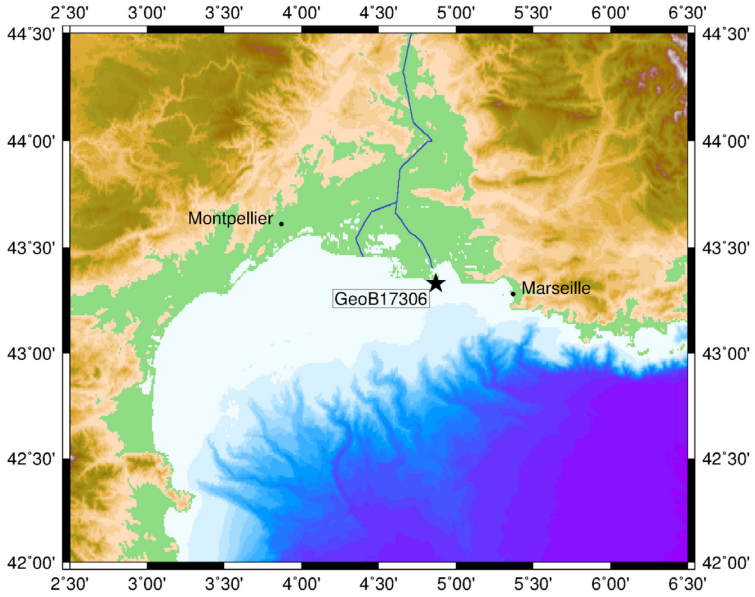

Fig. VI.1. Location of the investigated sediment samples collected at GeoB17306 (43°18.96'N, 4°52.17'E) in the Mediterranean Sea.

VI.3.2. Incubation set-up

Incubation experiments were performed with sediments from three depths: the SRZ (0-12 cmbsf), the SMTZ (80-85 cmbsf) and the MZ (133-140 cmbsf). Slurries were prepared under anoxic conditions with sediment and anoxic artificial seawater medium. Each incubation experiment was prepared from a 60 ml slurry aliquot (20 mL sediment + 40 mL medium) in 100 ml sterilized bottles sealed with pre-cooked and sterilized butyl rubber stoppers. At each depth, three different experimental set-ups were applied during cell growth (Table VI.1). Methanogenesis was either stimulated by a H_2/CO_2-headspace (80/20, v/v; called H_2/CO_2-set; Table VI.1) or a H_2/CO_2-headspace with additional 0.4 mL/L unlabeled MeOH (H_2/MeOH-set; Table VI.1). The third set was conducted with a N_2-headspace (control) to trace the sediment background activity. Each of the different set-ups and the control were incubated either with ^{14}C-DIC (1 MBq/sample; specific activity 1850-2200

MBq/mmol, American Radiolabeled Chemicals) or 2-^{14}C-ACT (1 MBq/sample; specific activity 1850-2200 MBq/mmol, American Radiolabeled Chemicals) as carbon source (Table VI.1). Slurries incubated with 2-^{14}C-ACT were further amended with 0.04 g/L unlabeled ACT (Table VI.1). Incubations were run in duplicates for the SRZ (0-12 cmbsf) and MZ (133-140 cmbsf) except for the control. No control and no duplicates were performed for the SMTZ depth due to sample material limitation. The samples were incubated at 30 °C and harvested after 21 days by freezing at -20 °C. They were stored frozen upon lipid extraction.

The anoxic sulfate-free artificial seawater like medium was prepared according to Xie et al. (2013b), but without additional DIC in the medium. Furthermore, 0.002g/L KH_2PO_4 and 0.1g/L NH_4^+, trace elements and vitamins (Widdel and Bak, 1992), were added to the medium. Reducing conditions were obtained by the addition of 0.5 g/L NaS_2*9 H_2O to the medium. Bacterial growth was inhibited by streptomycin (Schatz et al., 1944).

Table VI.1. The lipid-RIP incubation set-up of this study.

Incubation set	**Control-set[1]**		**H_2/CO_2-set[2]**		**H_2/MeOH-set[2]**	
Headspace	N_2		H_2/CO_2		H_2/CO_2	
Methanol-addition (unlabeled)	No		No		Yes	
Acetate-addition (unlabeled)	No	Yes	No	Yes	No	Yes
^{14}C substrate	^{14}C-DIC	^{14}C-ACT	^{14}C-DIC	^{14}C-ACT	^{14}C-DIC	^{14}C-ACT

[1]No control experiment was performed at the SMTZ depth (80-85 cmbsf) due to sample limitation. [2]Duplicates were performed for the samples at the SRZ depth (0-12 cmbsf) and MZ (133-140 cmbsf).

VI.3.3. CH_4 analysis

CH_4 production during incubation was monitored weekly for all samples by gas chromatography flame ionization detection (GC-FID) according to Ertefai et al. (2010). In brief, 250 µL of the headspace were collected by a gas tight syringe, immediately injected into a GC (HP 5890 Series II, Hewlett-Packard) equipped with a CARBOXEN fused silica-column (30m x 0.32mm) and detected by a flame ionization detector (FID). Quantification was achieved by external calibration of a reference gas (100 PPM, Air Liquide).

VI.3.4. Lipid analysis

Lipids were extracted from incubated slurries and from the original untreated sediment samples. The slurry of the incubation experiments were pre-extracted by the addition of MeOH and dichloromethane (DCM) to the slurry reaching a final ratio of 2:1:1 (MeOH: DCM: slurry; v:v:v) in an ultrasonic bath for 20 minutes (Bandelin Sonorex). The rest of the procedure was similar for the two types of sediments (incubated and untreated) and followed a modified Bligh and Dyer protocol (Sturt et al., 2004). Samples were extracted in four steps using a mixture of MeOH, DCM and an aqueous buffer (2:1:0.8; v:v:v). The first two extractions were performed with a phosphate buffer (8.7 g/L KH_2PO_4, pH 7.4) followed by two times with a trichloroacetic acid buffer (50 g/L, pH 2). The sonicated sediment was centrifuged at 1250 revolutions per minute for 10 minutes and supernatants were collected in a separatory funnel. After extraction, deionized water and DCM were added for phase separation and the organic phase was drawn off. This step was repeated three times. The pooled organic phase was then three times washed with deionized water. The total lipid extract (TLE) was gently dried under a stream of N_2 and stored at – 20 °C before lipid distribution analysis (untreated sediment) or further treatment (incubated sediment).

VI.3.4.1. Lipid fractionation by preparative liquid chromatography

^{14}C labeled lipids from the incubations were purified by a combination of two different preparative HPLC separation steps by a 1200-Series HPLC-system (Agilent). During the first step the extracted TLEs were chromatographically separated into three fractions: the first (0-9 min) containing apolar CLs, the second (12-21 min) containing 1GLs and the third (24-30 min) containing the 2G+3GLs. The separation was achieved over a LiChrosphere Diol-100 column (250 x 10 mm, 5 µm particle size, Alltech), maintained at 30 °C. Eluent A consisted of n-hexane and isopropanol (IPA; 90:10; v:v) and Eluent B of 100% IPA. The run started with 0% B, ramped to 24% at 15 min and then further increased to 100% of B at 20 min. The 100% B was hold for 10 min, followed by a 10 min equilibration with 100% A. the flow rate was set to 3 mL/min for the first 15 min and the equilibration phase, but lowered to 2 mL/min between 15-31 min. The TLEs were dissolved in 200 µL of 5:1 DCM:MeOH and injected twice (100 µL each).

After the first separation step the fractions that contained IPLs (1GL- and 2G+3GL-fractions) were hydrolyzed to release the polar head groups. The acid hydrolysis was carried out according to Elling et al. (2014). In brief, the 1GL- and 2G+3GL-fraction were dissolved in 1 mL of the hydrolyzing solution (1 M hydrochloric acid in MeOH), hydrolyzed for 3 hours at 70 °C and then evaporated.

The hydrolyzed IPL-fraction and CL-fraction were subsequently dissolved in 50 µL n-hexane and separated according to their lipid structures by a protocol modified from Meador

et al. (2015). The lipids were separated into two individual fractions: the AR-fraction (7.5 and 9 min), and the GDGT-fraction (16-22 min). Preparative HPLC was performed on a PerfectSil CN-3 column (5 µm, 250 x 10 mm 5 µm particle size, MZ-Analysentechnik). Eluent A consisted of n-hexane and IPA (99:1; v:v) and eluent B of hexane and IPA (90:10; v:v). The column was operated at 30 °C with a flow rate of 2.5 mL/min. The elution gradient was set for the first 5 min with 0% B, then increased to 10% B (12 min) and reached 100% B after 30 min. 100% B was held for 10 min. The column was equilibrated with 100% A for 10 min before the next run.

Time-based fractionation was established empirically by the measurement of a representative master sample which contained the lipids of interest for this study on a 1200-Series HPLC-system (Agilent) coupled to an LCQ Deca XP Ion Trap MS (Thermo Finnigan). Based on these measurements, a conservative time-based fraction collection of the lipids was established. The fraction collection of the incubated samples was carried out by the same HPLC-system coupled with a fraction collector (Gilson). The capillary connection from the HPLC to the MS and HPLC to the fraction collector had the same length and diameter, guaranteeing a similar separation and elution of the lipids measured by the MS and the fraction collection. To verify the purity of the individual, the representative master sample was measured daily.

VI.3.4.2. Analysis of ^{14}C incorporation into lipids

For the detection of the radiolabel incorporation into the lipids, the purified fractions were dissolved in 500 µL hexane, transferred to the scintillation vial and mixed with 3 mL of scintillation solvent (Ultima Gold, PerkinElmer). ^{14}C content was counted on a TriCarb 2810 TR (PerkinElmer) for 10 min. The DPM values (disintegration per minute) were corrected according to a blank sample.

VI.3.4.3. Lipid quantification from the sediment samples

Detection and quantification of IPLs in the original sediment samples was carried out on a maXis ultra-high resolution quadrupole-time-of-flight mass spectrometer (Bruker), coupled to an Ultimate 3000RS ultra high pressure liquid chromatography instrument (Dionex). The IPLs were chromatographically separated by an ACE3 C_{18} column (150 x 2.1 mm; particle size 3 µm) as described by Zhu et al. (2013a).

The lipids were detected in positive ionization mode scanning a mass range of 150 – 2000 Da. Structural information on the lipids was obtained by MS^2-scans, with up to three MS^2 experiments per MS full scan. The MS^2-experiments targeted the most abundant ions. Lipids were identified by their characteristic fragmentation pattern, the exact mass and retention time of the parent ions. The IPLs were quantified by the comparison of the parent ions with a C_{46}-Glycerol trialkyl glycerol tetraether (GTGT; Huguet et al., 2006) added as

injection standard. The concentrations of the IPLs were corrected to response factors determined by purified and commercially available standards following the procedure described by Becker et al. (2016).

VI.3.5. Calculations

After preparation of the slurries, the incubations with ^{14}C-DIC contained 8.33 mM of DIC, while the ones with ^{14}C-ACT contained 0.33 mM of ACT. According to the specific activity and the substrate concentration, the labeling strength of the ^{14}C-DIC experiments (0.01% ^{14}C DIC to total DIC) was 25 times lower than the labeling strength in ^{14}C-ACT incubations (2.39% ^{14}C ACT to total ACT). To correct for this difference in labeling strength the measured radioactivity in the ^{14}C-ACT samples were divided by a factor of 25 to allow a direct comparison with the ^{14}C-DIC results. Additionally, the lipid production rates per year ($prod_{lipid}$) were calculated based on the radioactivity in the individual lipids (DPM_{lipids}), the initial radioactivity added at the beginning of the incubation ($DPM_{initial}$), the concentration of DIC and ACT in the incubation ($C_{initial}$), the dry weight of the sample ($M_{Sed\ d}$) and the incubation time (T_{end}, Eq. 1).

$$prod_{lipid}\ (ng\ x\ y^{-1}\ x\ g_{Sed\ d}^{-1}) = \frac{DPM_{lipids}\ x\ C_{initial}}{DPM_{initial}\ x\ M_{Sed\ d}\ x\ T_{end}} \qquad Eq.1$$

VI.4. Results

VI.4.1. Lipid distribution in sediments

The initial lipid distribution was analyzed in core GeoB17306 at four depths: 4.5, 32.5, 70 and 255 cmbsf. IPL analysis exhibited a dominance of diethers over the tetraethers at the surface of the sediment (4.5 cmbsf), whereas GDGTs were predominant in the deeper sediments (Fig. VI.2A). The intact diethers consisted of 1G-AR and 2G-AR and both lipid types showed similar relative abundances. GDGTs with one to three sugar head groups were detected in the sediments. The IPL-GDGT distribution was dominated by 1G-GDGT followed by 2G- and 3G-GDGT. In addition, 1G-and 2G-GDGTs with a hydroxyl-group (OH-GDGT) (<2.5% of the total IPLs), butanetriol dibiphytanyl glycerol tetraethers (BDGTs) and pentanetriol dibiphytanyl glycerol tetraethers (PDGTs; BDGTs + PDGTs: 3.5 - 11%) were detected in the samples.

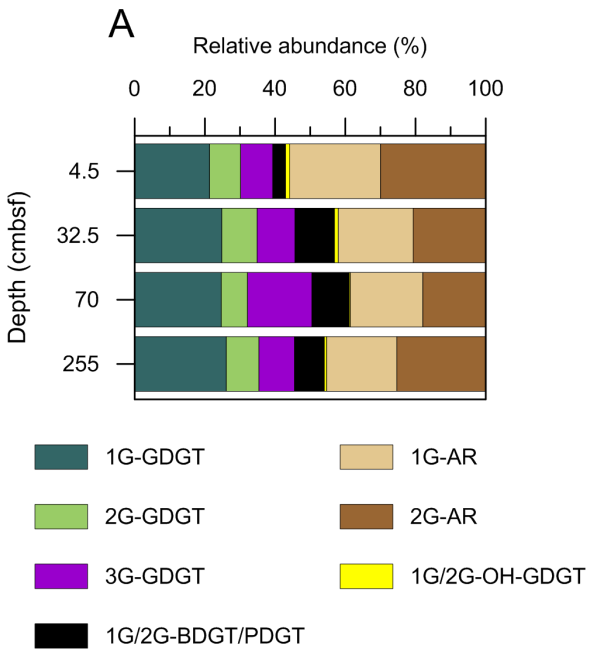

Fig. VI.2. Relative abundance of IPLs over depth (cmbsf) in the core GeoB17306.

VI.4.2. CH_4 production during ^{14}C incubation experiments

CH_4 measurements in the H_2/CO_2-set and H_2/MeOH-set showed that CH_4 production was several orders of magnitude higher than in the control samples (N_2) after 21 days of incubation (Fig. VI.3). Furthermore, the experiments with MeOH addition yielded higher production of CH_4 than the samples without MeOH for every depth. The samples from the SRZ (0-12 cmbsf) and MZ (133 to 140 cmbsf) produced approximately the same quantities of CH_4, whereas CH_4 formation in the SMTZ was about three times lower than in the incubations from the SRZ and MZ. No consistent differences between the ^{14}C-DIC and ^{14}C-ACT incubations could be observed, with values being close to each other in all but two cases: CH_4 production in the ^{14}C-DIC incubation was lower in the H_2/CO_2-set of the SRZ and in the H_2/MeOH set of the SMTZ than in the corresponding ^{14}C-ACT incubations.

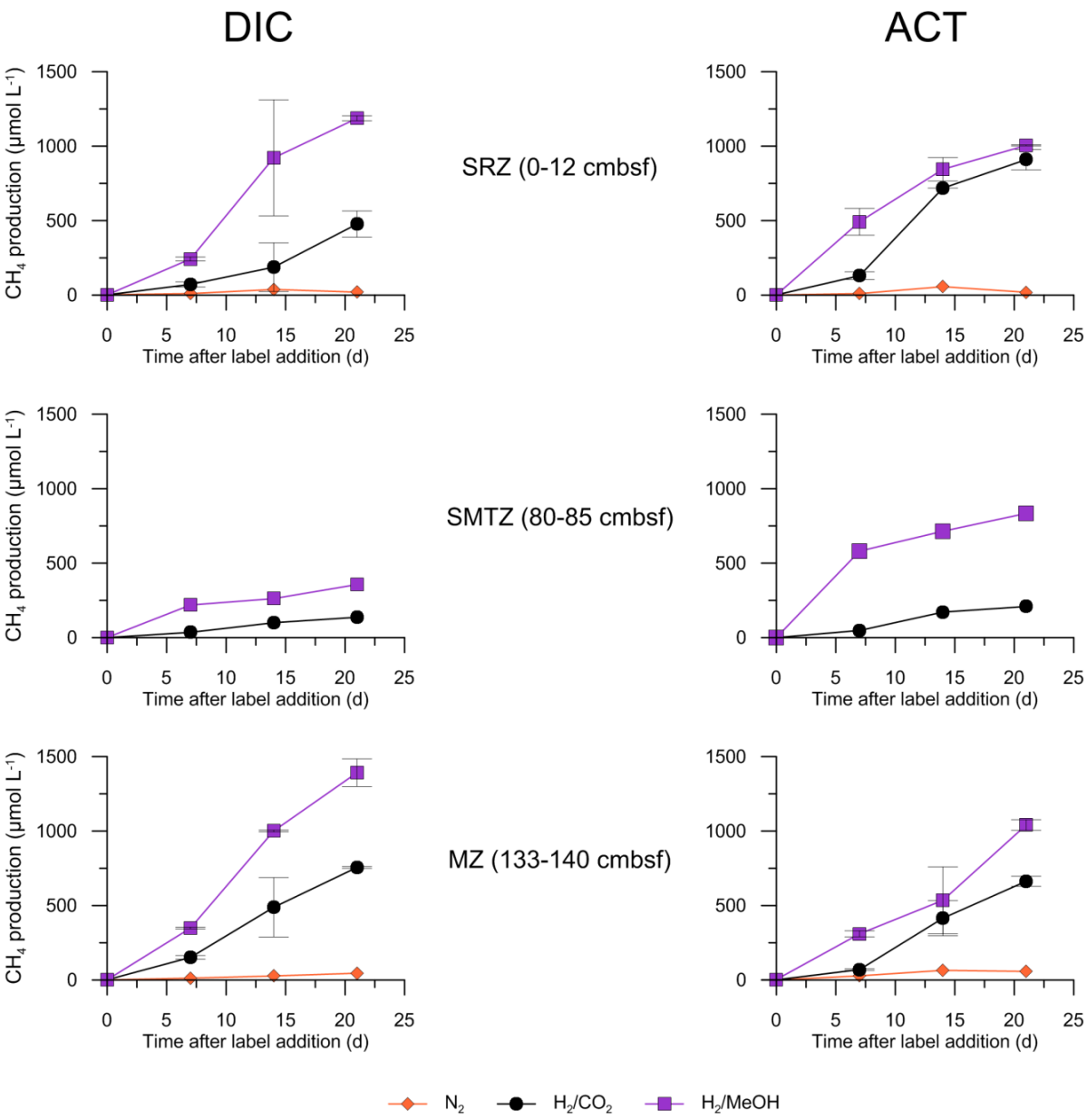

Fig. VI.3. CH_4 production in the different experimental set-ups (N_2, H_2/CO_2, H_2/MeOH) with ^{14}C-bicarbonate (DIC) addition on the left side and ^{14}C-acetate (ACT) addition on the right side. The results are shown for the three different investigated depths: the sulfate reduction zone (SRZ), the sulfate CH_4 transition zone (SMTZ) and the methanogenic zone (MZ). Error bars correspond to the individual values of the duplicates. The ACE-results were devided by 25 to correct for different labeling strengths in the DIC and ACE experiments (see material methods), respectively.

VI.4.3. ^{14}C incorporation in the total archaeal lipid extract of the different experimental set-ups

The ^{14}C incorporation in the stimulated experiments was 2 to 20 times higher than in the control incubations and higher in the ^{14}C-DIC incubations than in the ^{14}C-ACT samples (Fig. VI.4). The highest incorporation of ^{14}C was observed in the samples from the SRZ,

followed by the MZ. In the SRZ a 3.5 times higher incorporation of ^{14}C was observed in the H_2/MeOH-set compared to the H_2/CO_2-incubations for both carbon substrates. On the contrary, the SMTZ was characterized by a higher ^{14}C-value in the H_2/CO_2-sets than in the H_2/MeOH-set. A variable pattern was observed in the samples from the MZ: the ^{14}C-DIC incubations showed a predominant ^{14}C incorporation into the H_2/MeOH-set, while the ^{14}C-ACT uptake was highest in the H_2/CO_2-experiments.

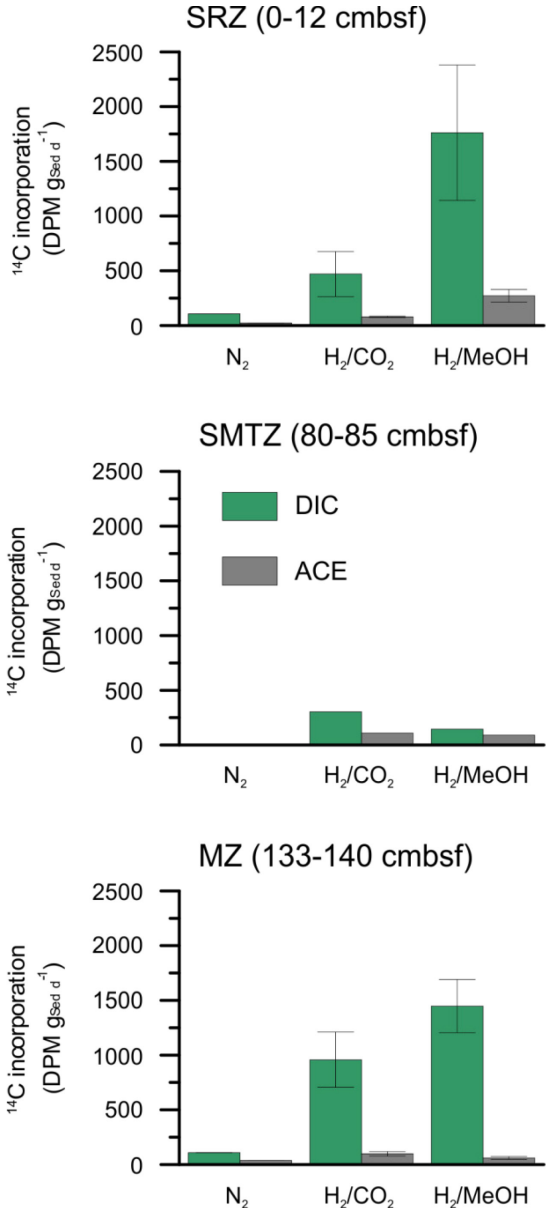

Fig. VI.4. Total ^{14}C-incorporation into the detected fractions in desintigrations per minute (DPM) per gram dry sediments for the two carbon sources after 21 days of incubation: bicarbonate (DIC) and acetate (ACT). The results are shown for the three investigated depths (sulfate reduction zone (SRZ); sulfate methane transition zone (SMTZ); methanogenic zone (MZ)). Error bars correspond to the individual values of the duplicates. The ACE-results were devided by 25 to correct for different labeling strengths in the DIC and ACE experiments (see material methods), respectively.

VI.4.4. ^{14}C incorporation into specific archaeal lipids:

The TLE of the different experimental set-ups was separated into three different lipid pools that are typically detected in environmental samples (CLs, 1GLs and 2G+3GLs). The relative ^{14}C incorporation into these three lipid pools is shown in Fig. VI.5.

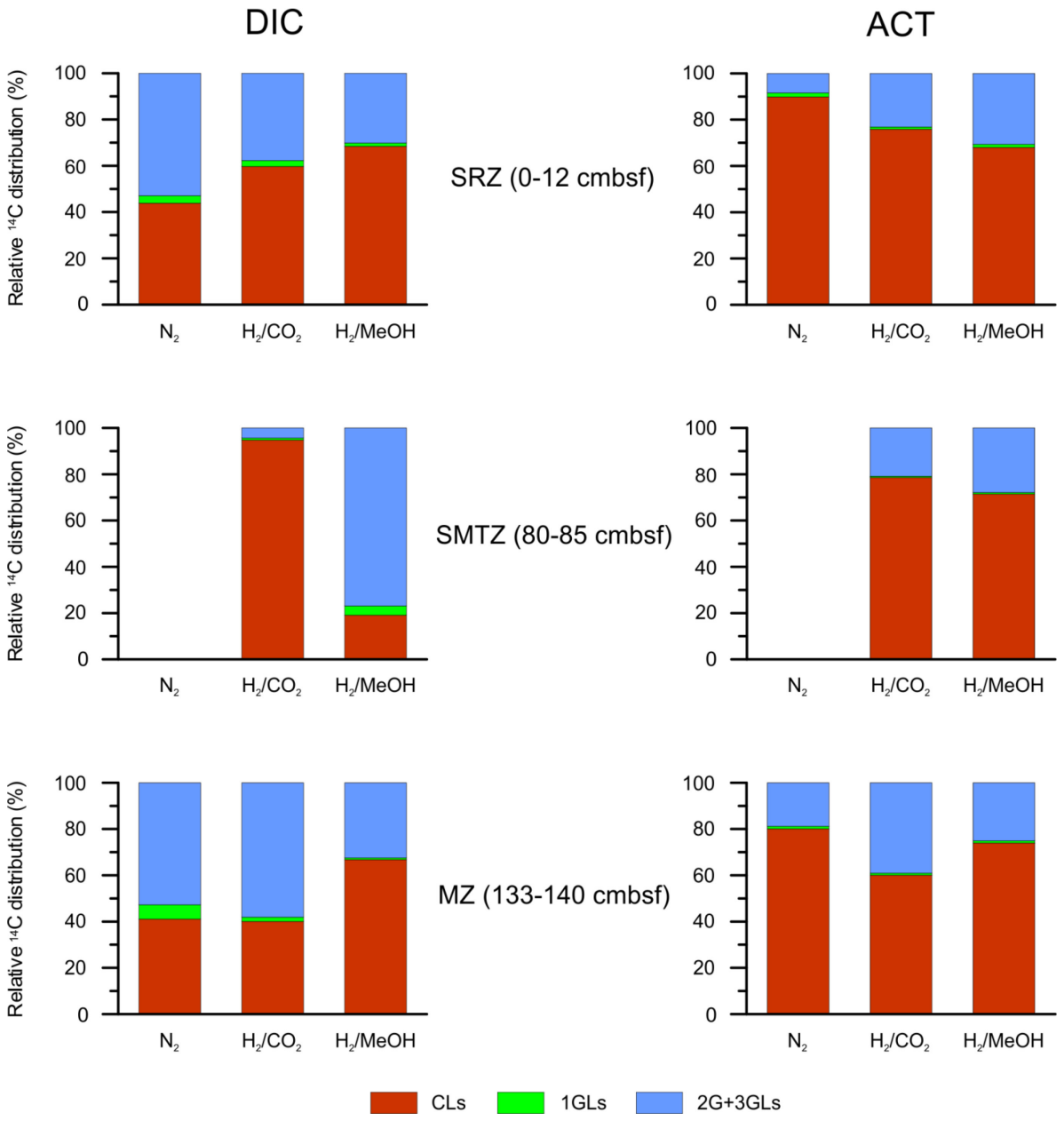

Fig. VI.5. Relative ^{14}C distribution in the core (CLs), monoglycosidic (1GLs) and di- and tri-glycosidic (2G+3GLs) lipids of the different experimental set-ups in the three investigated depths (sulfate reduction zone (SRZ), sulfate methane transition zone (SMTZ) and methanogenic zone (MZ)). The figure shows on the left the incubations with ^{14}C-DIC (bicarbonate) and on the right with ^{14}C-ACT (acetate).

Each lipid pool was further separated in the two main archaeal lipid structures that are encountered in methanogenic archaea: AR and GDGTs. The relative ^{14}C-DIC and ^{14}C-ACT

incorporation into AR and GDGTs for each investigated lipid pool are displayed in Figs. VI.6 and VI.7, respectively.

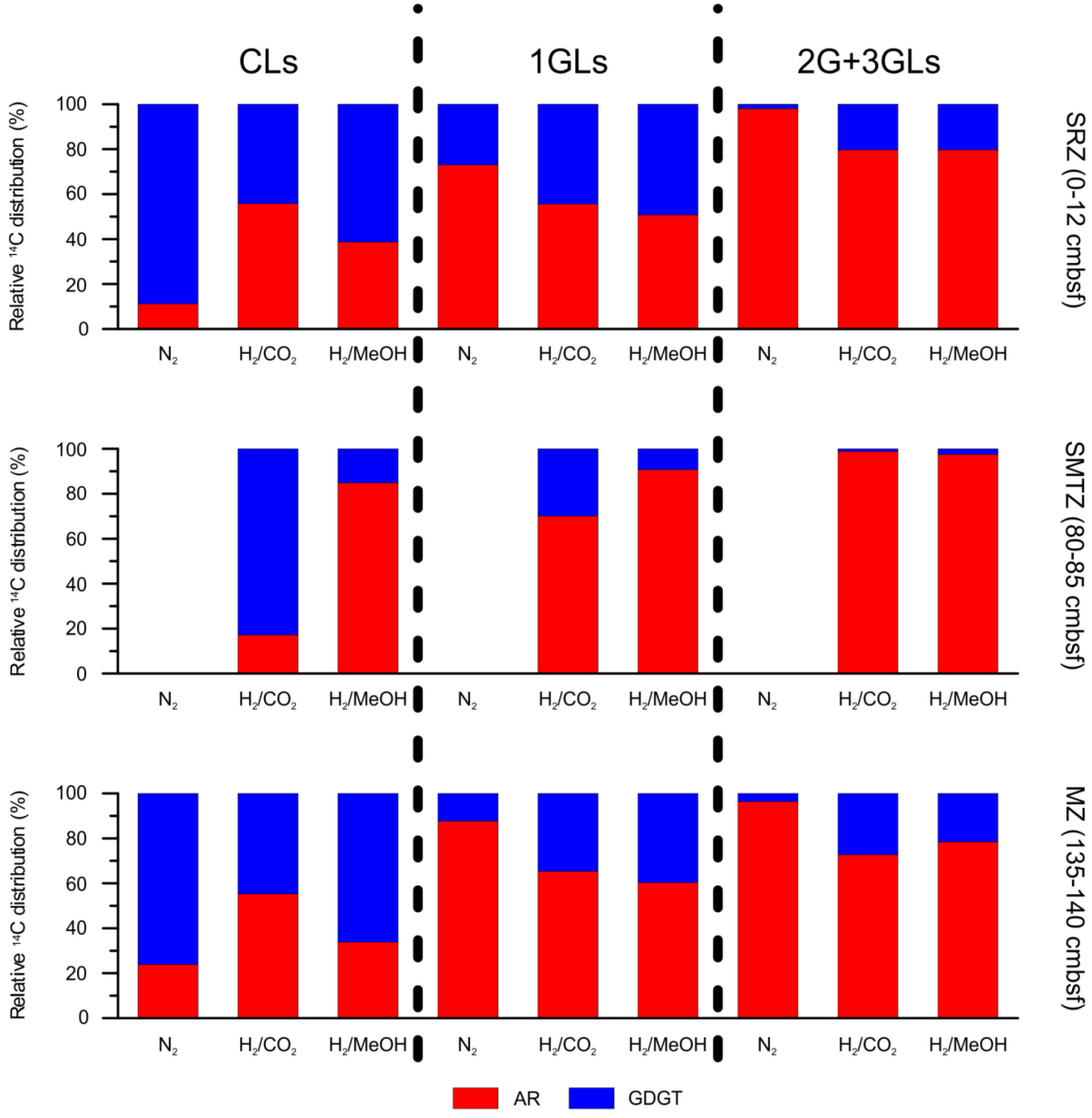

Fig. VI.6. Relative ^{14}C-DIC (bicarbonate) incorporation into archaeol (AR) and glycerol dialkyl glycerol tetraethers (GDGTs) according to the lipid pool (CLs = core lipids, 1GLs = monoglycosidic lipids and 2G+3GLs = di-/tri-glycosidic lipids). The results are shown for the three different incubation depths (sulfate reduction zone (SRZ), sulfate methane transition zone (SMTZ) and methanogenic zone (MZ)).

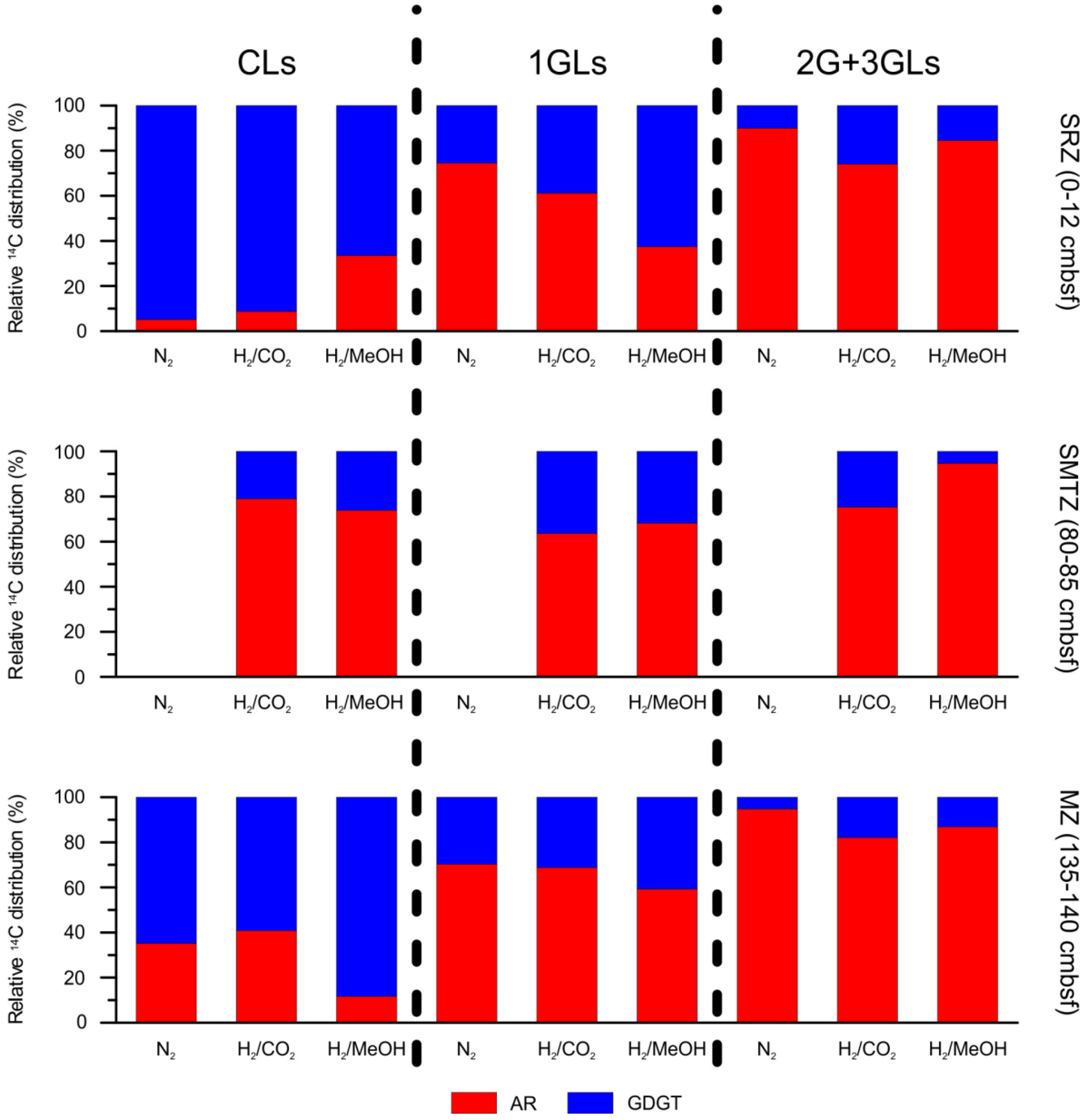

Fig. VI.7. Relative ^{14}C-ACT (acetate) incorporation into archaeol (AR) and glycerol dialkyl glycerol tetraethers (GDGTs) according to the lipid pool (CLs = core lipids, 1GLs = monoglycosidic lipids and 2G+3GLs = di-/tri-glycosidic lipids). The results are shown for the three different incubation depths. (sulfate reduction zone (SRZ), sulfate methane transition zone (SMTZ) and methanogenic zone (MZ)).

The relative ^{14}C incorporation into the different investigated lipid pools (Fig. VI.5) exhibited a high label in the CL-fraction representing more than 40%, except one sample from the SMTZ. The second highest incorporation of ^{14}C into most of the samples was observed in the 2G+3GL-fraction. The 1GL-fraction represents less than 5% of ^{14}C incorporation in the incubations for all depths and energy set-ups.

The relative ^{14}C incorporation into the individual lipid structures (AR and GDGTs) for each lipid pool is displayed in Fig. VI.6 (^{14}C-DIC) and VI.7 (^{14}C-ACT), respectively. At the SRZ and the MZ, the CL-pool revealed a high proportion of ^{14}C labeled GDGTs (44 - 95%), while the 2G+3GL-pool exhibited a strong predominance of ^{14}C labeled AR (73 - 98%). The

proportion of AR and GDGTs in the 1GLs pool was less contrasted (37 - 88% labeled AR) which corresponds to an intermediate lipid distribution between the CL- and 2G+3GL-pools. The samples from the SMTZ showed a different lipid distribution. Apart from one sample, the labeled AR were predominant in every pool (CLs, 1GLs, 2G+3GLs) for every energy set-up (64 - 99% labeled AR).

VI.5. Discussion

VI.5.1. Autotrophy as the major pathway for benthic methanogenic communities

In this study, incubation experiments with two different ^{14}C labeled substrates (DIC and ACT) were performed to trace the lipid biosynthesis of the methanogenic and the background community in marine sediments from the Rhone delta. In each incubation flask, methanogenesis was traced by CH_4 production (Fig. VI.3). Moreover, microbial activity and utilization of the different carbon substrates were tracked by measuring the ^{14}C incorporation into archaeal lipids (Fig. VI.4).

The CH_4 production and the ^{14}C incorporation into archaeal lipids in the incubations are strongly enhanced in the H_2/CO_2 and the H_2/MeOH experiments compared to the control incubations (Fig. VI.3 and Fig. VI.4), indicating that the added energy sources strongly stimulated the methanogenic community. The high CH_4 production and ^{14}C incorporation in the methanol amended set (H_2/MeOH-set) suggests that methylotrophy is the major methanogenic energy pathway in the SRZ (Fig. VI.3 and Fig. VI.4). This is in good agreement with a previous study that calculated rates for the different methanogenic pathways with untreated sediment incubations from the same core in the Rhone delta and observed a predominant utilization of methylated compounds to perform methanogenesis (Zhuang, 2014). Moreover, the measurements revealed that hydrogenotrophic methanogens (H_2/CO_2-set) are active in the SRZ (Fig. VI.3 and Fig. VI.4). Previous, studies showed that competitive methanogenesis is severely curtailed in sulfate-rich marine sediments (reviewed by Whiticar, 1999). However, the addition of streptomycin inhibited the bacterial community and therefore the hydrogenotrophic methanogenic community in the incubated slurries was not outcompeted for their substrates by SRB. Similar observations have been made in earlier studies where the SRB were inhibited, resulting in an increased production of CH_4 in sulfate enriched sediments (e.g. Abram and Nedwell, 1978). Moreover, the study with untreated sediments from the Rhone delta by Zhuang (2014) revealed that hydrogenotrophic methanogens contribute up to 50% of the CH_4 production in the SRZ. This suggests that hydrogenotrophic methanogenesis may also be important in sulfate rich marine sediments and may represent a source of atmospheric CH_4.

In the SMTZ, the CH_4 production (Fig. VI.3) and ^{14}C incorporation (Fig. VI.4) into microbial lipids is significantly lower than in the other investigated geochemical zones. On the contrary, Zhuang (2014) observed the highest MeOH and H_2/CO_2 utilization rates in the SMTZ. The SMTZ is characterized by a highly complex community of anaerobic methanotrophic archaea (ANME) that thrive in symbiosis with SRB and potentially other bacterial groups (reviewed by Knittel and Boetius, 2009). The addition of streptomycin in the present study inhibited the bacterial symbionts of the ANME community and therefore likely reduced the activity of the ANME community in our incubation, while Zhuang (2014) did not use any bacterial inhibitors. ANME are closely related to *Methanosarcinales* and *Methanococcoides* (Lloyd et al., 2011) and there are several lines of evidence that anaerobic methanotrophs are capable to perform methanogenesis, particularly at high CH_4 concentrations in marine sediments (e.g. Lloyd et al., 2011; Bertram et al., 2013). Accordingly, the inactivation of the ANME community in our experiments could explain the low CH_4 production and ^{14}C incorporation and the mismatch with the study from Zhuang (2014).

The slurries amended with MeOH in the MZ showed highest CH_4 (Fig. VI.3). This is unexpected, given that earlier studies observed that the methylotrophic pathway accounts for less than 10% of the total CH_4 production in sulfate depleted sediments (Lovley and Klug, 1983; Zhuang, 2014). However, the addition of 10mM MeOH, which is several orders of magnitude higher than the natural concentration in the sediments from the Rhone delta (<2 µM; Zhuang, 2014) could have stimulated the methylotrophic energy pathway. Higher ^{14}C-incorporation rates in the H_2/CO_2-set from MZ compared to the SRZ indicate that the relative importance of hydrogenotrophic methanogenesis increases in this geochemical zone, as also observed by Zhuang, 2014.

McrA based clone libraries of site GeoB17306 suggested that three different orders of methanogens are inhabiting the sediments of the Rhone delta: *Methanomicrobiales*, *Methanosarcinales* and *Methanomassiliicoccales* which are only found in the SMTZ (Zhuang, 2014). Moreover, the methanotrophs ANME-2 and ANME-3 were found in site GeoB17306. The utilization of MeOH as an energy source to perform methanogenesis has so far been described in cultivated representatives of *Methanosarcinales* (Weimer and Zeikus, 1978 reviewed by Offre et al., 2013) and *Methanomassiliicoccales* (e.g. Borrel et al., 2012; Dridi et al., 2012; Paul et al., 2012; Iino et al., 2013). Based on genomic data, a recent study proposed that *Methanomassiliicoccales* assimilate ACT as carbon source (Söllinger et al., 2016), whereas *Methanosarcinales* are capable of utilizing various carbon substrates (e.g. DIC, ACT, methylated compounds; reviewed by Whitman et al., 2006). Given the six (SRZ) to 22 (MZ, Fig. VI.4) times higher incorporation of ^{14}C-DIC into the microbial lipids compared to ^{14}C-ACT in the H_2/MeOH-sets, the results suggest that *Methanosarcinales* are the

predominantly active methylotrophic methanogens in the experiments. Moreover, incubation experiments with ANME dominated sediments showed a high CH_4 production after the addition of MeOH (Bertram et al., 2013), which utilize DIC rather than ACE as a carbon source (Kellermann et al., 2012; Bertram et al., 2013). Therefore, ANME may also have contributed to methanogenesis in the H_2/MeOH-sets. The H_2/CO_2-sets from the different depths also showed a predominant incorporation of ^{14}C-DIC over ^{14}C-ACT. There is a wide range of different methanogens that perform hydrogenotrophic methanogenesis as an energy pathway (see review by Offre et al., 2013), but according to the clone library results of Zhuang (2014), *Methanosarcinales*, *Methanomicrobiales* and ANMEs are candidates to perform this methanogenic pathway in this study. Many species of *Methanomicrobiales* are known to require ACT as a carbon source, while strict autotrophy is rare among this group of methanogens (reviewed by Garcia et al., 2006). Accordingly, this suggests that *Methanosarcinales* and potentially ANMEs are also active in the H_2/CO_2-set slurries during this study.

The results showed that autotrophy is the predominant carbon assimilation pathway in all three investigated depths geochemical environments, independent of substrates added (Fig. VI.4). It must be considered that the incubations performed during this study may have overestimated the relevance of autotrophy, due to the incorporation of ^{14}C-DIC by heterotrophic archaea. For instance, up to 33% of the assimilated carbon in heterotrophic bacteria may be derived from DIC (Sorokin, 1966; Roslev et al., 2004; Wegener et al., 2012). Nevertheless, approximately half of the cultured methanogens utilize DIC as carbon substrate, while others consume ACT and more complex OM (reviewd by Whitman et al., 2006). Therefore, the here obtained results are in a good agreement with cultivation-based studies. Ultimately, this result should also be completed and confirmed by the investigation of the incorporation of more complex OM such as yeast, peptones and butyrate, which are known carbon sources for several methanogenic groups (reviewed by Whitman et al., 2006).

VI.5.2. Lipid-RIP as a tool to track benthic microbial processes

One of the major advantages of labeling experiments is that lipid production rates can be calculated, providing a quantitative estimation of carbon fixation pathways and metabolic rates. The production of archaeal lipids during the incubation experiment was calculated based on the total ^{14}C incorporation into the archaeal lipids (Fig. VI.8). The lowest lipid production rates were observed in the control samples ranging from 245 to 250 ng $g_{Sed\ d}^{-1}$ y^{-1} for the ^{14}C-DIC and from 37 to 62 ng $g_{Sed\ d}^{-1}y^{-1}$ for the ^{14}C-ACT incubation. Apart from the SMTZ, the stimulated samples showed substantially higher incorporation rates with ^{14}C-DIC

(1083- 4057 ng $g_{Sed\ d}^{-1}$ y^{-1}) and with ^{14}C-ACT (100- 448 ng $g_{Sed\ d}^{-1}$ y^{-1}) than the control samples.

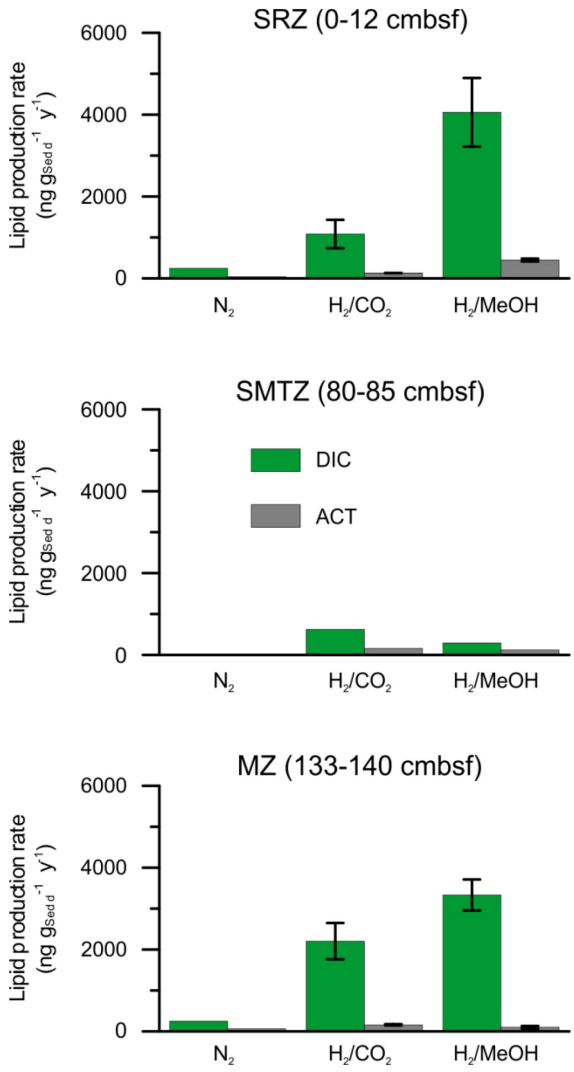

Fig. VI.8. The lipid production rates according to the ^{14}C incorporation of bicarbonate (DIC) and acetate (ACT) into archaeal lipids. The results are displayed for the three depths (sulfate reduction zone, SRZ; sulfate methane transition zone, SMTZ; methanogenic zone, MZ). Error bars represent the average deviation of the duplicates.

Previous studies have calculated lipid production rates from different sediment environments (Table VI.2). The comparison of the results from the present experiments with the previous studies is based on the control samples, because this set of samples was not stimulated by additional energy sources. Therefore, it is assumed that the control samples represent the activity of the background benthic community. The archaeal lipid and cell production rates in the control incubations were several orders of magnitude higher than the rates from the deeply buried Cascadia Margin sediments (Table VI.2). This can be explained by the high sediment age (35000 y, Lin et al., 2013) in deeply buried sediments of the Cascadia Margin, resulting in a limited supply of favorable nutrients and OM (reviewed by

Arndt et al., 2013). Accordingly, very low metabolic rates have been observed in deeply buried sediments with a high sediment age (Røy et al., 2012; see review by Hoehler and Jørgensen, 2013). In addition, archaeal lipid production rates, based on dual-SIP experiments, have been calculated in the Himmersfjärden (Table VI.2; Wegener et al., 2012). The sedimentation rates in the Himmersfjärden (0.8- 1.3 cm y^{-1}, Bianchi et al., 2002; Reuss et al., 2005) predict a sediment age that ranges from 0.8 y (shallow) to 90 y (deep), suggesting a more active setting than in the Cascadian Margin closer to the conditions encountered in the Rhone delta (sediment age 0.1-4 y Heuer et al., 2013). However, the archaeal lipid production rates in the samples from the Himmersfjärden were up to four orders of magnitude lower than the rates from the Rhone delta (Table VI.2).

Table VI.2. A comparison of lipid production rates observed in this study and other locations.

Study	Site	Additional information	Lipid production [ng $g_{sed\ d}^{-1}$ y^{-1}]
Present	Rhone delta	Control incubations	37-250
Present	Rhone delta	Stimulated incubations	100-4057
Lin et al. (2013)	Hydrate Ridge, Cascadia Margin	Subsurface sediments (800 cmbsf)	0.001-0.031
Wegener et al. (2012)	Fjord Himmersfjärden (Baltic Sea)	Surface sediments (1-72 cmbsf)	0.25-1.44
Kellermann et al. (2012)	Guaymas Basin	Hydrothermal vent, ANME-1 enriched Stimulated with CH_4	10000
Kellermann et al. (2016a)	ANME-1 enrichment culture	Stimulated with CH_4	3893

This suggests that additional factors led to the different archaeal lipid production rates in the two studies. Our experiments were performed at 30 °C, while Wegener et al. (2012) applied colder temperatures (4 °C). According to previous experiments, a more than 20 °C higher incubation temperature results in a two orders of magnitude increased metabolic activity of the microbial community (Price and Sowers, 2004). Therefore, the higher incubation temperatures presumably led to enhanced archaeal lipid production rates in the present study. Also, here, the label incorporation was detected into the archaeal core lipids, which consist of one or two glycerol backbone(s) and two apolar chains, whereas Wegener et al. (2012) measured the label incorporation in the apolar chains only. This could have considerably underestimated the archaeal production rates from Himmersfjärden. Indeed, incubation experiments with benthic archaea showed that labeled glucose and biomass was

incorporated into the glycerol backbone, rather than into the apolar chains (Takano et al., 2010; Lin et al., 2013). The dissimilar incubation temperatures and experimental procedures may explain the very different lipid production rates in the present study and in the one from the Himmersfjärden. Our results suggest that the archaeal lipid production rates in coastal-estuarine sediments are higher than previously thought.

The stimulated incubations showed a strongly enhanced archaeal activity compared to the control samples (Fig. VI.8). This suggests, that benthic methanogens are capable of high lipid production rates, when supported with a sufficient supply of energy sources. To the best of our knowledge no lipid production rates for methanogenic communities, from cultures or environmental settings have been reported, yet. However, the presented lipid production rates are in the range of stimulated ANME communities from the Guaymas Basin (Table VI.2; Kellermann et al., 2012; reviewed by Wegener et al., 2016). This suggests that growth of methanogens is high at high substrate availability and comparable to growth of anaerobic methanotrophs.

VI.5.3. Comparison of the labeling pattern in individual lipids: Implications for the accumulation and biosynthesis of archaeal lipids in subsurface sediments

The ^{14}C incorporation into the 2G+3GL-fraction was up to 45 times higher than the incorporation into the 1GL-fraction (Fig. VI.5). This pattern was observed for both substrates and in the three different set-ups. On the contrary, the IPL-distribution in the sediment measured before the incubation showed a one-to-one ratio of the 1GL- and the 2G+3GL-fraction (Fig. VI.2A). The mismatch between the low production of 1GLs in the incubation experiments and their high abundance in the sediments suggests that these lipids are preferentially accumulated in the marine sediments from the Rhone delta. The 1GLs in the sediments are either accumulated by (i) transport from the water column or soil and deposition in the sediments and/or (ii) degradation of 2G+3GLs to produce 1GLs. Previous studies showed, that 1G-GDGTs are highly abundant in the water column and dominate over the 2G-species (Lincoln et al., 2014; Xie et al., 2014). Additionally, the analysis of archaeal lipids in suspended particulate matter from the water column of the Rhone delta revealed a predominance of 1G-GDGTs (75-100% of the total archaeal IPLs (Goldenstein et al., 2013)). Accordingly, a contribution of 1GLs from the water column to the sediment pool seems likely. On the other hand, the IPL-pool in the water column contained no 1G-AR (Goldenstein et al., 2013), while it accounts for up to 30% of the IPLs in the sediments (Fig. VI.2A). Thus, a contribution from the water column cannot exclusively explain the accumulation of 1GLs in the marine sediments. Another hypothesis is the production of 1GLs by degradation of polyglycosidic lipids. A study from the Madeira Abyssal Plain showed that 2G-GDGTs are

more labile than the 1G-GDGTs (Lengger et al., 2013) and hypothesized that the later are produced by the degradation of the 2G-GDGTs. Additionally, the conditions during the incubation experiments may have not targeted the microbes that produce 1GLs (e.g. utilization of different substrates or incubation temperature). However, the most likely explanation is that a contribution of 1GLs from the water column and *in situ* degradation of 2G+3GLs resulted in the preferential accumulation of monoglycosidic lipids in the sediments, while benthic archaea seemed to predominantly produce IPLs with polyglycosidic head groups.

The ^{14}C-incorporation into AR and GDGTs in the IPL-pool (1GLs-and 2G+3GL-fraction) revealed a comparable distribution for both applied substrates in the stimulated (H_2/CO_2 and H_2/MeOH) and in the control set (Fig. VI.6 and VI.7). The incubations with DIC and ACT showed a preferential ^{14}C uptake into AR over GDGTs. Moreover, the ^{14}C incorporation into AR was higher in the 2G+3GL-fraction (mean for both substrates: 86% AR of the total ^{14}C incorporation in this pool) than in the 1GL-fraction (mean 66% AR). Previous studies that investigated the lipid distribution of mesophilic methanogen isolates showed that several taxonomic groups predominantly build-up their lipid membrane by AR rather than GDGTs (reviewed by Schouten et al., 2013). Particularly *Methanosarcinales*, which are the potentially stimulated methanogens in the present study (as discussed above), are characterized by a predominance of AR over GDGTs in their cell membrane (e.g. De Rosa and Gambacorta, 1988; Hoefs et al., 1997; Bauersachs et al., 2015). Additionally, labeling experiments with the hydrogenotrophic methanogen *Methanothermobacter thermoautotrophicus* (Nishihara et al., 1989) exhibited a preferential label incorporation into IPL-AR during short-term incubation. Therefore, the here obtained results suggest that 2G-AR is good marker for actively growing methanogens in marine sediments. Alternatively, IPL-AR may represent an intermediate lipid during the synthesis of IPL-GDGTs. Thus, several studies suggested that IPL-GDGTs are synthesized by a head-to-head reaction of two IPL-AR molecules (see review by Koga and Morii, 2007). This may suggest, that Archaea synthesize IPL-AR first, which, in later growth stages, is transformed to IPL-GDGTs. Hence, in short term incubation experiments (this study 21 days), the predominant production of the intermediate IPL-AR could be a common feature among a wide range of benthic archaea. In addition, this proposed pathway predicts a higher production of IPL-GDGTs at longer timescales, which may explain their predominance usually observed in subsurface sediments (e.g. Lipp et al., 2008; Lipp and Hinrichs, 2009; Meador et al., 2015; Evans et al., 2017).

The high ^{14}C incorporation into the apolar lipid pool is unexpected (Fig. VI.5), given that previous studies proposed that sedimentary CLs are fossil remnants from the water column mixed with lipids from decayed benthic archaea (e.g. Sturt et al., 2004; Biddle et al., 2006; Lipp et al., 2008; Lipp and Hinrichs, 2009). However, a recent study, that performed

dual-SIP incubation experiments in a French peatland, observed 20 times higher production rates of bacterial branched core GDGTs than the corresponding intact branched GDGTs (Huguet et al., 2017). Moreover, a study performing lipid-RIP experiments with planktonic archaea observed a high ^{14}C-DIC incorporation into c-GDGTs within less than 120 h of incubation (chapter IV). The degradation of ^{14}C-labeled IPLs, caused by the high microbial activity in the Rhone delta and in peat, may result in a accumulation of "fossil" ^{14}C-labeled CLs. Incubation experiments have revealed that glycosidic archaeal lipids are stable, with half-life times of several thousand years in active surface sediments from the Wadden Sea (Xie et al., 2013a). Consequently, a production of CLs from glycosidic archaeal lipids within the short incubation period in the present (21 days) and the peatland study (one to two months) is rather unlikely. Phosphatidic lipids are more labile resulting in shorter half-life times in the range of 100 days under aerobic conditions to 100 years in anoxic environments (White et al., 1979; Harvey et al., 1986; Logemann et al., 2011). However, since no archaeal lipids with a phosphatidic head group were detected in the Rhone delta (Fig. VI.2A) these can be excluded as source for the CLs. Furthermore, a distinctly different labeling pattern of AR vs. GDGTs in the CL-fraction (predominant ^{14}C incorporation into GDGTs) compared to the IPL-pool (predominant ^{14}C incorporation into AR) is observed in most of the samples in the present study (Fig. VI.6 and Fig. VI.7). Because AR and GDGTs are built up of an ether bound core structure, it may be assumed that IPL-AR and IPL-GDGTs have as similar degradation rate in marine sediments. Accordingly, a similar ratio of AR to GDGTs in the CL- and IPL-pool would be expected, if the apolar lipids were degradation products of IPLs. Thus, the degradation of IPLs can hardly explain the incorporation of label in the CLs and rather suggests an active synthesis of apolar lipids. The preferential incorporation of labeled carbon substrates in CLs has only been observed in environmental experiments (this study; chapter IV; Huguet et al., 2017), while labeling experiments with an ANME-1 enrichment culture showed less than 10% incorporation into CLs compared to IPLs (Kellermann et al., 2016a). This may suggest, that in the environment CLs fulfill a biological function in Archaea and Bacteria. The biological role of apolar lipids in organisms is still controversial (Elling et al., 2014; Meador et al., 2014; Cario et al., 2015), but CLs are considered to affect the stability of the cell membrane and/or may be involved in the storage of energy. In energy limited environments, such as marine subsurface sediments, cell stability and energy storage are crucial factors to allow cell maintenance (Valentine, 2007).

Another explanation for the high ^{14}C incorporation into the CL-fraction is, that apolar lipids could be biosynthetic intermediates of IPLs. Takano et al. (2010) hypothesized that benthic archaea synthesize *de novo* the glycerol backbone, while the fossil biphytanes are recycled from the sediment. In this sense the CLs could represent a biosynthetic transition state, before the polar head group is attached to the IPL, which eventually builds up the cell

membrane. Apolar AR and GDGTs might accumulate in this transition state resulting in a high ^{14}C label incorporation in the CL-pool. This hypothesis may suggest that benthic archaea are capable of an alternative IPL-GDGT formation pathway (via apolar GDGTs), probably due to energy limitation in the marine sediments, which does not involve the previously mentioned head-to-head reaction of two IPL-AR molecules, observed in culture experiments (Nishihara et al., 1989; Nemoto et al., 2003; Koga and Morii, 2007; Kellermann et al., 2016a). For instance, Villanueva et al. (2014) proposed an AR independent GDGT formation pathway, where GDGTs are directly synthesized from biphytanes. However, based on the results obtained here this pathway is speculative. Thus, the samples from the SMTZ showed a different labeling pattern of AR and GDGTs in the CL-pool in three of the four incubations (Fig. VI.6 and Fig. VI.7). Moreover, a preferential loss of IPLs during extraction (e.g. Huguet et al., 2010) or separation (Lengger et al., 2012a) may also explain the observed predominance of CLs. Therefore, future research is required to elucidate bio-physiological function and biosynthetic processes of apolar CLs in benthic microbes.

VI.6. Conclusion

The lipid-RIP technique applied here allows to investigate the lipid biosynthesis of autotrophic and heterotrophic archaea in marine sediments. Further this is one of the first studies able to trace carbon assimilation of methanogenic archaea in a detailed and comprehensive way in this environment. Consequently, this study provides important information on microbial mediated methanogenesis and their carbon assimilation pathways in marine sediments. The results showed that methylotrophy is the major methanogenesis pathway in the SRZ. Moreover, a predominant utilization of DIC as a carbon substrate indicates that the methanogens are autotrophs rather than heterotrophs. In combination with previously published genetic data, lipid-RIP suggests that member of the *Methanosarcinales* are the predominantly active Archaea in the here performed experiments. The lipid-based analysis demonstrates that active benthic archaea, regardless of the nutrient supply, predominantly produce 2G-AR. Thus, 2G-AR could be a suitable biomarker to trace microbial life in sediments. The high incorporation of ^{14}C-substrates into core lipids, coupled with the short incubation period, indicates that core lipids are not only derived from the degradation of IPLs, but can also be actively produced by benthic archaea. The results may suggest that benthic archaea are capable of an alternative GDGT-formation pathway with apolar GDGTs as intermediate lipids.

VI.7. Acknowledgements

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Chapter VII

Concluding remarks

VII.1. Summary and conclusion

This PhD-thesis focused on the investigation of some key actors of the marine microbiome, on their activity and their lipid biosynthesis under different conditions such as nutrient and energy limitation in pure cultures and environmental samples. The investigated environmental samples covered a wide range of marine settings from the surface of the water column to the subsurface sediments from different research areas around the world. Flow-through scintillation counting was established which, for the first time in environmental research, allowed an on-line detection of individual ^{14}C labeled lipids and thereby the investigation of lipid biosynthesis (chapter III). Additionally, separation techniques based on preparative HPLC (chapters IV and VI), were implemented together with radioisotope probing, which allow a highly sensitive detection of the ^{14}C incorporation into microbial lipids. In the first part of this thesis (chapters III and IV) the microbial activity, food-web strategies and biosynthesis of planktonic archaeal membrane lipids were tracked by lipid radioisotope probing (lipid-RIP). In the second part, the microbial community and their physiological strategies in subsurface sediments were successfully examined by the analysis of intact polar lipids (IPLs) and quantitative polymerase chain reaction (Q-PCR) by applying optimized protocols, which allow the clean-up of the lipid extract and an improved extraction of DNA (Zhu et al., 2013b; Lever et al., 2015). Moreover, the lipid biosynthesis of methanogenic archaea, performing one of the key processes in marine sediments, was investigated by lipid-RIP in sediments from the Rhone delta (chapter VI).

In **chapter III** a ^{14}C-DIC incubation experiment with *N. maritimus* was performed to investigate the effect of ammonium supply and growth phase on the biosynthesis of glycerol dibiphytanyl glycerol tetraethers (GDGT) and diethers in marine *Thaumarchaeota*. ^{14}C incorporation into lipids was measured by flow-through scintillation counting, which allowed a compound specific detection of the ^{14}C incorporation into individual lipids in a single

measurement. Incubations of *N. maritimus* with a limited NH_4^+ supply resulted in lower growth rates, compared to the culture with a sufficient supply. Moreover, crenarchaeol was predominantly synthesized in the limited culture, whereas the non-limited culture produced preferentially acyclic-GDGT during intense growth. These findings support the theory that Archaea modify their lipid membrane as a response to environmental changes and that cyclization is an indicator of growth-limiting conditions (e.g. nutrient limitation). NH_4^+-respiration rate thus can have a strong impact on the TEX_{86} paleotemperature proxy and this fact should be considered when evaluating reconstructed sea surface temperatures. The results infer that the ring distribution of GDGTs can be applied to trace the NH_4^+-supply of marine ammonium oxidizing archaea. Additionally, the production of archaeol (AR) and GDGTs markedly changed during the non-limited as well as the limited experiment, with a high synthesis of AR at the beginning and a predominant production of GDGTs during later growth. The observations of this experiment in combination with a second experiment conducted with the methanogen *M. thermoautotrophicus* suggest that GDGTs are formed by a head-to-head reaction of two AR-molecules. This agrees with previous experiments based on the cultivation of *Euryarchaeota* (see review by Koga and Morii, 2007).

In **chapter IV** the effect of varying environmental conditions on marine planktonic archaea and their food-web-strategies was further investigated in a lipid-RIP incubation experiment with water column samples. The samples were collected at two sites with a contrasting oceanographic setting: a shelf (Hornsund Basin) and a fjord (Van Mijenfjorden) in the proximity of Svalbard. Two labeled substrates were applied: ^{14}C-leucine (^{14}C-LEU) to investigate lipid biosynthesis of heterotrophic planktonic archaea and ^{14}C-bicarbonate (^{14}C-DIC) to study autotrophs. The predominant production of uns-AR in the ^{14}C-LEU incubations, suggests that uns-AR is a suitable biomarker to track planktonic *Euryarchaeota,* which are considered to be predominantly heterotrophs (e.g. Iverson et al., 2012; Orsi et al., 2015). The incorporation of LEU into MeO-AR, a specific biomarker indicative for *Thaumarchaeota*, demonstrates that these organisms are mixotrophs and capable of utilizing amino acids as a carbon source. The high incorporation of ^{14}C-DIC into core GDGTs within less than 120 hours of incubation indicates that apolar GDGTs are actively synthesized by autotrophic planktonic archaea. Therefore, the results may imply that the commonly observed predominance of core GDGTs in the water column is not exclusively derived from the degradation of IPLs (see also **chapter VI**).

In **chapter V**, the microbial community in subsurface sediments along a transect through the Benguela upwelling area was investigated by an optimized IPL- and Q-PCR-analysis, to avoid potential biases observed in previous studies such as ion suppression of phospholipids and inefficient DNA extraction. A selective clean-up protocol was applied, which significantly increases the detection of phospholipids in environmental samples (Zhu et

al., 2013b). Additionally, a recently developed method by Lever et al. (2015) was employed, that provides a high extraction yield of DNA in environmental samples and separates the analyzed water insoluble DNA, from the water-soluble DNA. Thus, allowing to remove most of the extracellular DNA derived from lysed cells in the water-soluble DNA. As opposed to earlier studies (e.g. Lipp et al., 2008; Briggs et al., 2012), the IPL- and Q-PCR based data are highly consistent. In fact, bacterial phospholipids were detected in the investigated sediment cores down to a depth of 560 cm below the seafloor (cmbsf), while previous studies were not able to detect bacterial lipids deeper than 10 cmbsf in sediments from the Peru upwelling area (Lipp et al., 2008; Lipp and Hinrichs, 2009). The results suggest, that the complex matrix in environmental samples hindered the identification of bacterial phospholipids in earlier studies. A dominance of Bacteria was observed in all surface sediments and in the organic lean, less active subsurface sites further offshore. Archaea prevailed in the subsurface sediments below the upwelling cell with high OM-burial and sedimentation-rates. The observation that Bacteria are predominantly present in the less active sites further offshore contradicts with theoretical considerations, which suggested that Archaea outcompete Bacteria in energy starved environments (Valentine, 2007) due to their metabolic strategies and cell membrane properties. However, with increasing depth bacterial phospholipids were predominantly built up by stable and less permeable diether glycerol-lipids (DEGs). The predominant production of stable DEGs may compensate the theoretical disadvantage of Bacteria and allow them to thrive in energy starved environments.

In **chapter VI**, a ^{14}C labeling experiment was performed in three different geochemical zones in sediments from the Rhone delta using ^{14}C-acteate (^{14}C-ACT) (heterotrophic) and ^{14}C-DIC (autotrophic) as carbon sources. The aim of this study was to investigate the utilization of the two different carbon substrates by methanogens and to trace the biosynthesis of their lipids compared to the lipid production by the background community. The methane production showed that hydrogenotrophic and methylotrophic methanogens are highly active when they are stimulated by the addition of a H_2/CO_2 headspace and methanol, respectively. Furthermore, it was demonstrated that methylotrophy is the major methanogenic pathway in the sulfate reduction zone. The incubation experiments further revealed that methanogens inhabiting the Rhone delta sediments are predominantly autotrophs. Combined with 16S sequence data the results suggest that *Methanosarcinales* are the predominantly active methanogens in the performed experiments. The predominant ^{14}C incorporation into the IPL-ARs in the stimulated experiments and the background community indicates that benthic archaea predominantly produce AR during cell-growth. Thus, the results suggest that IPL-AR is a suitable lipid biomarker to track active benthic archaea. The low incorporation of ^{14}C into IPL-GDGTs compared to IPL-AR observed in this experiment may suggest that previous studies, which predominantly identified IPL-

GDGTs in marine sediments (e.g. Lipp et al., 2008; Lipp and Hinrichs, 2009; Meador et al., 2015), have overestimated the archaeal abundance in subsurface sediments. The high ^{14}C incorporation into apolar lipids, particularly into core GDGTs, in combination with the short incubation period (21 days) indicate, that benthic archaea actively produce core lipids. Thus, apolar lipids may be synthesized as intermediates before the head group is attached to form the IPL. This hypothesis indicates that benthic archaea are capable of an alternative IPL-GDGT formation pathway (via apolar GDGT), that does not involve the head-to-head reaction of IPL-AR as observed in experiments with *N. maritimus* in chapter III. In addition, a biological function of apolar lipids, such as a stabilization of the membrane or energy storage, cannot be excluded. However, the observation that core lipids may not exclusively be produced by the degradation of IPLs, indicates that the role of apolar lipids in marine environments should be revised.

VII.2. Future perspective

This thesis applied novel state-of-the-art techniques to investigate microbial communities in different environments and has therefore contributed to extend the current knowledge of the microbial food-web and of microbial lipid biosynthesis strategies to survive under energy starved conditions. At the same time, the present results have raised further interesting questions and may lead to new hypothesis that require verification:

1) Nutrient availability drives GDGT cyclization in planktonic archaea:

In chapter III it was shown that the biosynthesis of GDGTs in the cell membrane of *N. maritimus* depends on the ammonium supply. The marine water column, with assemblages of different AOA and other archaeal communities, is a much more heterogenic system compared to a culture experiment. Although the here presented data is in a good agreement with observations in natural marine environments, it remains elusive whether the diverse AOA community in the water column shows a similar lipid biosynthetic response to changes of the ammonium supply. To close this gap of knowledge, comprehensive lipid-RIP experiments with marine water samples collected at coastal and open ocean sites and incubated with different nutrient supply may provide important information to understand the GDGT-biosynthesis of marine AOA. This may allow to elucidate the mechanisms that control the ring distribution and the TEX_{86}-paleotemperature proxy. Additionally, this comprehensive work could be applied to develop a novel proxy to estimate the ammonium supply of marine AOA based on the ring distribution of GDGTs.

2) DEG lipids are specific biomarkers for Bacteria in the deep biosphere and are synthesized to cope with energy starvation:

Phosphatidic bacterial lipids with a DEG core lipid structure were ubiquitously detected in the deeply buried sediments of the Namibian margin (chapter V), but have not been detected in other investigated marine subsurface sediments yet (Lipp et al., 2008; Lipp and Hinrichs, 2009). Accordingly, a biomarker analysis of sediment samples, ideally from diverse depositional systems, measured with improved techniques (e.g. Zhu et al., 2013b), could give important information whether these lipids are common among benthic bacteria in marine sediments or if the here obtained results are specific to the Namibian margin. Moreover, little is known about the source organisms of these lipids. Phosphatidic DEGs have been detected in mesophilic SRB (Grossi et al., 2015; Vinçon-Laugier et al., 2016), however, additional microbial sources are likely, given that these lipids were also identified in samples beneath the sulfate reduction zone on the Namibian margin. Accordingly, incubation and labeling experiments with subsurface sediments coupled with DNA sequencing techniques may improve our current knowledge on the source organisms of these lipids and allow investigating their metabolism by evaluating their carbon source.

3) Core lipids are actively synthesized as intermediates during IPL synthesis by planktonic and benthic archaea:

The experiments from Svalbard and the Rhone delta showed predominant label incorporation into apolar lipids. The shortness of the incubation period suggests that the core lipids are actively synthesized in the cell as intermediates lipids during the formation of IPLs. However, this IPL synthesis pathway has not been observed in culture experiments before and apolar lipids in the environment are usually considered as degradation products of IPLs in environmental samples. To ultimately validate the active synthesis of apolar lipids by environmental archaea, a specific short term ^{14}C incubation experiment should be implemented with samples from the water column and marine sediments. Moreover, frequent sampling to obtain a time-series experiment, as performed in chapter III, would allow to follow the lipid biosynthesis of apolar lipids and IPLs. Hence, this experiment could be applied to validate the here proposed lipid biosynthesis pathway that core lipids represent a transition state during the synthesis of IPLs in environmental archaea.

Chapter VIII

Contributions as co-author

Identification, formation and distribution of fatty acid-substituted glycerol dialkyl glycerol tetraethers in marine sediments

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VIII.1 Abstract

Archaeal isoprenoid glycerol dialkyl glycerol tetraethers (GDGTs) are abundant components of marine sedimentary organic matter and are used in paleoenvironmental reconstructions. During diagenesis, a significant fraction of free GDGTs can be incorporated

into macromolecules. However, the diagenetic reaction mechanisms involving GDGTs remain unresolved. We identified a potential macromolecule precursor, fatty acid-substituted GDGTs (FA-GDGTs), in a globally distributed set of sediment samples and the archaeal culture *Nitrosopumilus maritimus* by high performance liquid chromatography-tandem mass spectrometry (HPLC-MS^2). Instead of a biosynthetic origin, a diagenetic origin of these compounds is proposed. First, a stable isotope probing experiment with *N. maritimus* did not reveal label uptake into the fatty acids and second, FA-GDGTs in environmental samples appear to be in chemical equilibrium with GDGTs. Moreover, the fatty acids connected to FA-GDGTs are similar to the free fatty acids found in the investigated sediments. FA-GDGTs are likely produced by esterification of free fatty acids and GDGTs in sediments during early diagenesis and their formation seems to be dependent on reactant availability, hydrolytic conditions and water activity.

Keywords: GDGTs, fatty acids, diagenesis, fatty acid GDGTs, marine sediment

Chapter IX

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