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Intact polar lipids of ammonia-oxidizing Archaea: Structural diversity and application in molecular ecology

Angela M. Pitcher

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Intact polar lipids of ammonia-oxidizing Archaea: Structural diversity and application in molecular ecology

Intacte polaire lipiden van ammoniak-oxiderende Archaea:
Structurele diversiteit en toepassing in moleculaire ecologie

(met een samenvatting in het Nederlands)

Proefschrift

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Angela Marie Pitcher
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Promotoren: Prof. dr. ir. J.S. Sinninghe Damsté

Prof. dr. ir. S. Schouten

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For Cecilia

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Chapter 1

General Introduction

The Discovery of Mesophilic Crenarchaeota

Until the 1970's, a dichotomy existed between small, single-celled microbes and multicellular higher-life forms. It was assumed that every living organism could be classified as one or the other, and hence the Tree of Life consisted of only two domains - the respective, Prokaryotes and Eukaryotes. However, once comparisons between the nucleic acid sequences of the genes coding for the broadly distributed ribosomal RNA complex began to reveal more detailed evolutionary relationships and taxonomic groupings within the Tree of Life, a third grouping emerged essentially dividing the Prokaryotes into two distinct domains, the Bacteria and the deeply branching "Archaebacteria" (Woese and Fox, 1977), now known as the Archaea. The Archaea contained two major phyla, the Crenarchaeota and Euryarchaeota (Woese et al., 1990) (Figure 1), and were generally associated with "extreme" environments thought to resemble the conditions of early Earth. It was because of this, and their deep-branching on the Tree of Life, that they were given the name "Archaea", which comes from the Greek language meaning "ancient things".

Not only are the Archaea genetically distinct from the other life domains but they are phenotypically unified by their unique cellular membrane lipids; the lipids of Archaea are based on ether linkages (deRosa et al., 1986; Woese and Fox, 1977), whereas those of Bacteria and Eukaryotes are based on ester linkages formed by the condensation of

alcohols and fatty acids (example structures are shown in Figure 1). Prior to the recognition of the Archaea as a coherent phylogenetic group, their ether-based membrane lipids were already seen as individual physiological adaptations, vital to their survival under harsh living conditions (Brock, 1978). Until the 1990s, Archaea were viewed as holistically

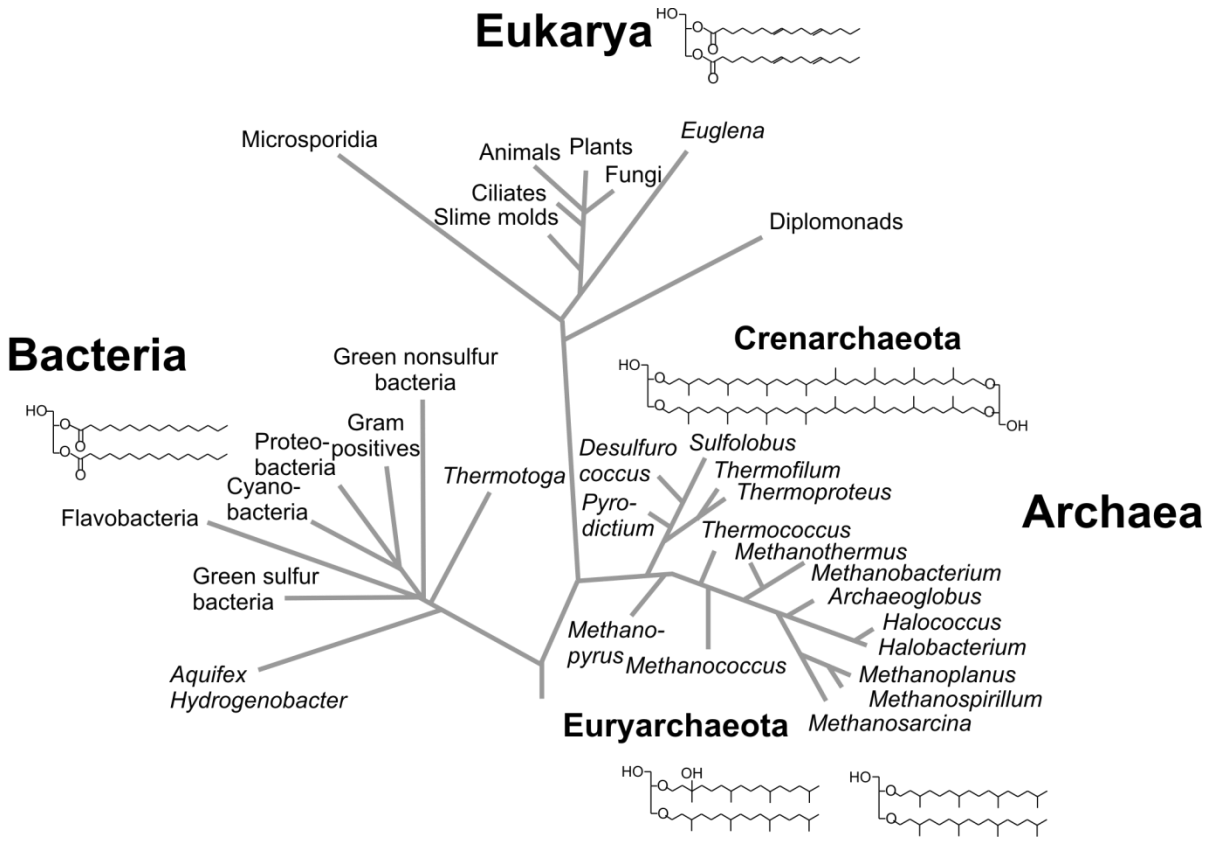


Figure 1. Tree of life showing the three Domains (Eukarya, Bacteria, and Archaea) and core membrane lipid structures typical of each. The Archaea is further divided into two major phyla: the Crenarchaeota which synthesize predominantly tetraether-based core membrane lipids, and the Euryarchaeota which more commonly synthesize diether-based core membrane lipids. Redrawn and modified from http://oceanexplorer.noaa.gov /explorations/06fire/background/microbiology/media/universal_tree2hotbugs.html; based on Woese et al. (1990).

extremophilic, living mainly in environments such as hypersaline lakes, hot springs, anoxic settings, and highly acidic and alkaline waters, representing three major phenotypes: methanogens, halophiles, and thermophiles. However, over the last decades, largely due to the development and application of the polymerase chain reaction (PCR) and the emergence of metagenomics (cultivation-independent analysis of DNA recovered from environmental samples), Archaea have been found to inhabit a broad range of cold and temperate habitats, including marshlands, soils, and marine waters.

The first evidence for the existence of non-extremophilic Crenarchaeota came from phylogenetic analysis of cloned 16S rRNA genes present in oxic coastal surface waters of the Santa Barbara Channel (DeLong, 1992) and epi- and mesopelagic depths in the Pacific Ocean (Fuhrman et al., 1992). Further work showed that Archaea make up a significant fraction of marine bacterioplankton assemblages (DeLong et al., 1993; Fuhrman et al., 1993). These mesophilic marine Archaea belonged to two main phylogenetic lineages, the "Group I" Crenarchaeota and "Group II" Euryarchaeota (cf. Figure 2). Recovery of abundant Group I Crenarchaeota from the deep sea of the Pacific and Atlantic suggested they are widespread and important in the bathypelagic ocean; based on the proportion of clones it was estimated that 10% of the deep-sea microbiota are comprised of planktonic Crenarchaeota (Fuhrman and Davis, 1997). A quantitative assessment using RNA-based fluorescent probes to determined depth distribution of marine Archaea and Bacteria by Karner et al. (2001), showed that Group I Crenarchaeota more accurately comprise approximately 20% of global marine picoplankton. Their work pointed out that while Bacteria always dominated the surface and epipelagic waters, Crenarchaeota were as abundant as Bacteria at meso- and bathypelagic depths over an entire year, and even exceeded Bacteria in abundance below 1000 m. Together, this suggested that mesophilic Crenarchaeota constitute a consistent and significant fraction of the marine microbiota, both in surface waters and in the deep-sea.

A wave of cultivation-independent studies on community DNA extracted from a broad range of marine and terrestrial environments led to the resolution of a much more detailed Archaeal phylogeny that included novel lineages within both the Crenarchaeota and Euryarchaota (Schleper et al., 2005) (Figure 2). The Group I Crenarchaeota was further

sub-divided to include the groupings I.1a and I.1b, representing sequences largely derived from the mesophilic marine and soil environments, respectively. Recent analysis of archaeal 16S rRNA gene sequences generated over the past several years, in addition to the genome of the marine crenarchaeon, *Cenarchaeum symbiosum*, has resulted in the proposal of a novel archaeal phylum, the "Thaumarchaeota", which contains all of the 16S rRNA sequences derived from mesophilic Crenarchaeota (Brochier-Armanet et al., 2008; Sprang et al., 2010)[1]. With the abundance, ubiquity and diversity of mesophilic Crenarchaeota well-established, a subsequent urgency to understand their biogeochemical significance, particularly in the marine realm, has materialized.

Role of mesophilic Crenarchaeaota in the marine nitrogen and carbon cycles

Initial evidence from the ^{13}C content of marine water column and sediment-derived archaeal lipids indicated that at least some marine Archaea are autotrophic, fixing bicarbonate as their main carbon source (Hoefs et al., 1997; Wuchter et al., 2003; Kuypers et al., 2001). The first successfully enriched mesophilic Archaeon, the Group I.1a crenarchaeote, *Nitrosopumilus maritimus* SCM1 (Könneke et al., 2005), also fixed bicarbonate and was shown to oxidize ammonia as its primary energy source. This provided important direct evidence of the potential role of marine Crenarchaeota in the biogeochemical cycling of nitrogen, a speculation which had been previously made based on field study correlations between Crenarchaeota abundances and nitrate concentrations (Murray et al., 1999; Sinninghe Damsté et al., 2002a).

Based on phylogenetic analysis of the gene encoding for alpha subunit of archaeal ammonia monooxygenase (*amoA*) - the enzyme responsible for the catalytic conversion of ammonia to hydroxylamine (NH_2OH) - ammonia-oxidizing Archaea (AOA) were found to be pervasive in regions of the oceanic water columns and sediments critical for nitrogen cycling (Francis et al., 2005; Venter et al., 2004). Although confirmation of ammonia oxidation by marine Crenarchaeota came from cultivation of *N. maritimus* (Könneke et al. 2005), it was isolated from a sea aquarium and not directly from a natural marine system.

[1] We recognize that current nomenclature for the ammonia-oxidizing 'Crenarchaeota' would more be more accurately described as ammonia-oxidizing 'Thaumarchaeota'. However, as this came about towards the completion of the thesis, we have maintained the (mesophilic) Crenarchaeota designation for consistency.

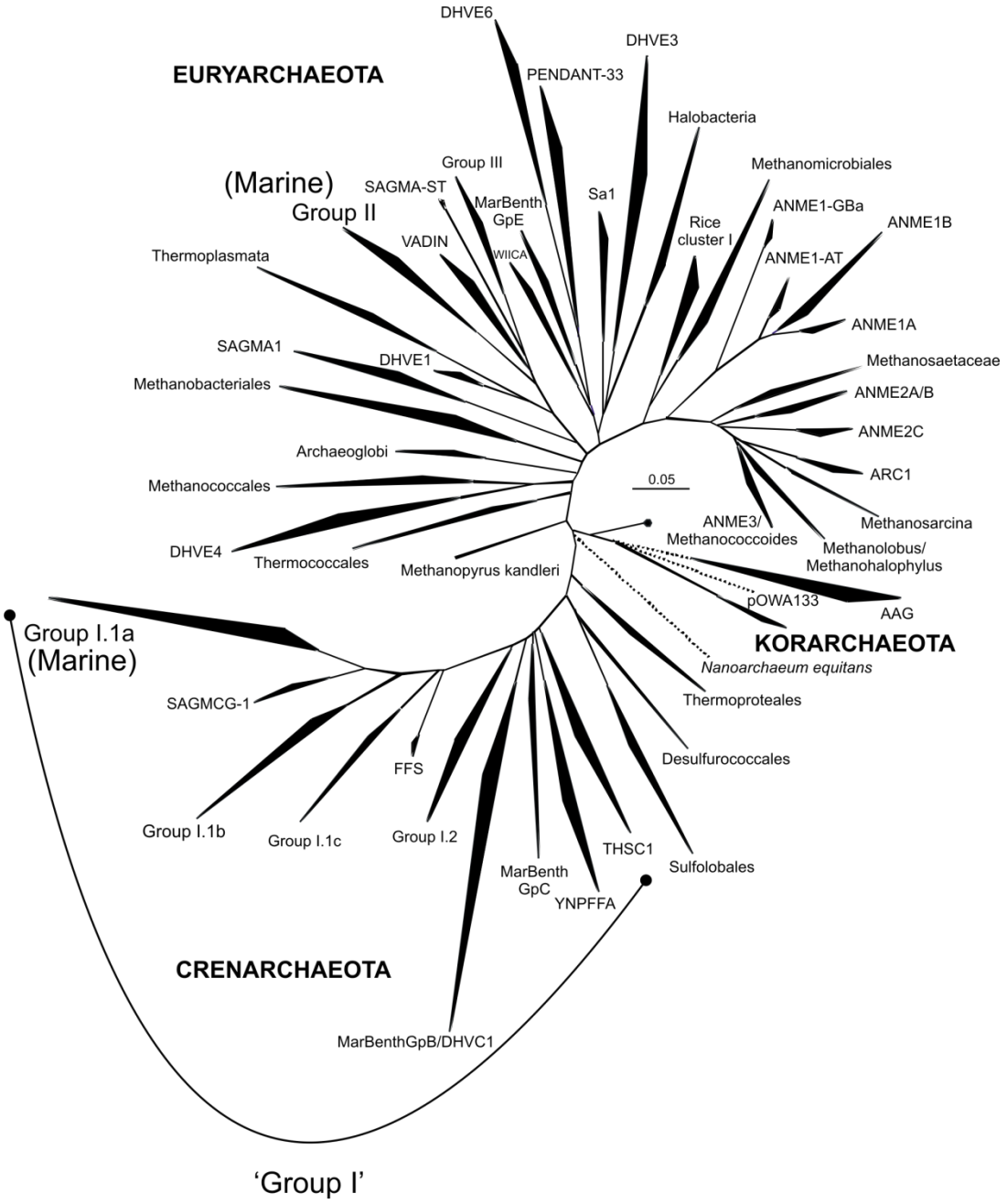


Figure 2. 16S rRNA sequence-based phylogeney of the Archaea, showing the diversity of the three phyla: Crenarchaeota, Euryarchaeota, and the Korarchaeota. Modified from Schleper et al. (2005). Dark colored triangles represent lineages with at least one cultivated representative; light colored triangles represent lineages defined purely from environmental clones.

Wuchter et al. (2006), however, directly connected ammonia oxidation in the coastal North Sea to an enriched mesophilic crenarchaeon closely related to *N. maritimus*. In addition, they showed that *amoA* genes of Archaea were much more abundant than those of Bacteria. Indeed, when compared directly to bacterial *amoA* in environmental samples, archaeal *amoA* is often present in much greater numbers (Leininger et al., 2006; Wuchter et al., 2006). Genomic analysis of the mesophilic Group I.1a crenarchaeote, *Cenarchaeum symbiosum*, provided further direct evidence for both carbon fixation and the use of reduced inorganic nitrogen as energy sources by these mesophilic Crenarchaeota (Hallam et al., 2006). This study also showed that *Cenarchaeum symbiosum* contains genes which could allow for heterotrophic carbon assimilation, thereby pointing to potential diverse capabilities of marine Crenarchaeota. This, together with evidence of amino acid uptake by marine Archaea (Herndl et al., 2005; Ouverney et al., 2000) suggests that the function of AOA in the marine carbon cycle is complex.

Ammonium oxidation is the first, and rate-limiting, step in the process of nitrification - the sequential oxidation of reduced inorganic nitrogen to more oxidized forms including nitrite and nitrate (cf. Figure 3). In its essence, nitrification connects nitrogen fixation and nitrogen loss: this microbially-mediated oxygenic process uses ammonium as an energy source, effectively converting the most bioavailable form of nitrogen to less bioavailable forms, while simultaneously forming substrates readily used in denitrification and anammox - two of the main metabolic processes linked to systemic nitrogen-loss (Codispoti et al., 2001; Kuypers et al., 2003). Traditionally, the *Proteobacteria* were viewed the sole actors in nitrification (Bothe et al., 2000), however, insights into the potential for Group I Crenarchaeota to oxidize ammonia have implicated them as potentially important actors in this process. While there is no doubt that they are very abundant, their actual importance (i.e. net effect on marine nitrification and influence on other nitrogen transformations) relative to their bacterial counterparts remains an unresolved issue. Unraveling their ecology and defining/constraining the niches in which AOA thrive will be imperative to our overall understanding of the marine nitrogen cycle.

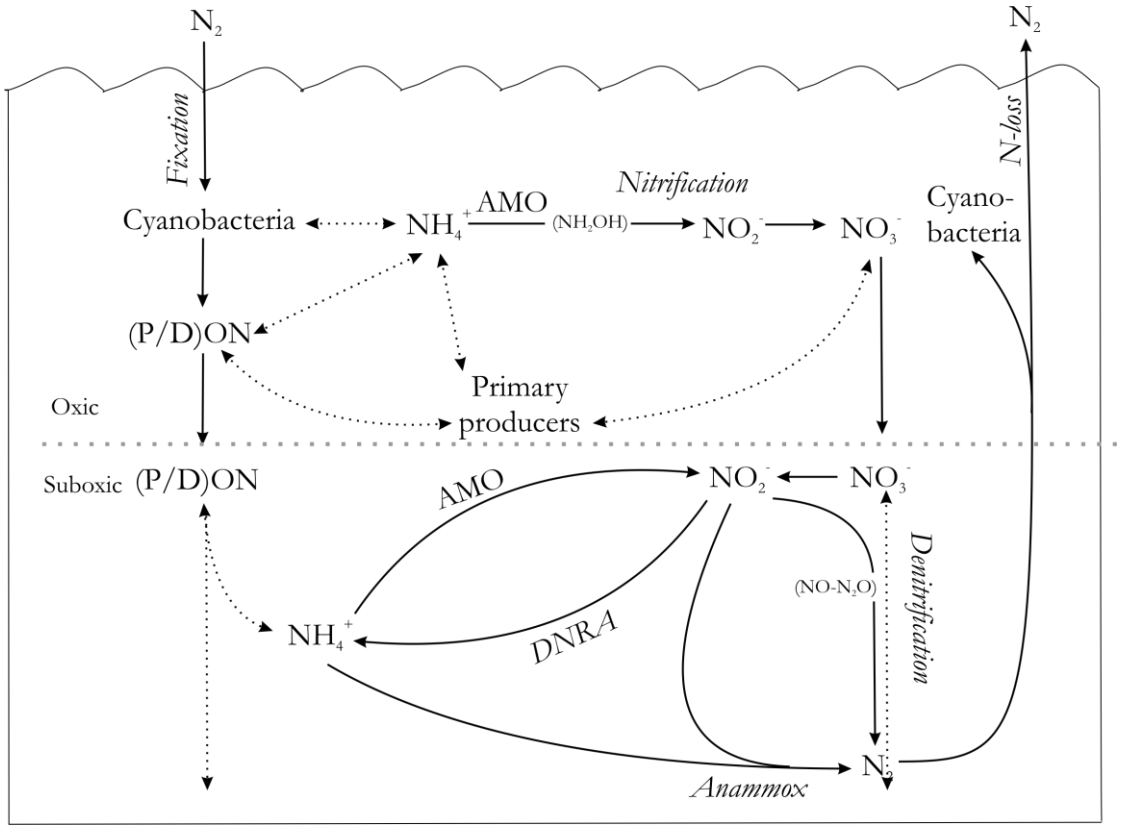


Figure 3. Conceptual marine nitrogen (N) cycle showing the general pathways of major processes occurring under oxic and suboxic conditions. Entry of N into the system mainly occurs through nitrogen gas (N_2)-fixation by diazotrophic cyanobacteria. In the upper, oxic water column direct release of ammonium (NH_4^+), and the process of remineralizeation of particulate organic nitrogen (PON) followed by subsequent ammonification of dissolved organic nitrogen (DON), yields free NH_4^+. This NH_4^+ can be oxidized to nitrite (NO_2^-) (catalyzed by the ammonia monooxygenase (AMO) enzyme), and further to nitrate (NO_3^-) (*nitrification*), or it can be assimilated directly into biomass. NO_2^- and NO_3^- in suboxic waters provide potential substrates for *denitrification* (sequential reduction of NO_3^- to N_2 gas, which is lost from the system and/or recaptured and fixed by diazotrophs), anaerobic oxidation of ammonium (*anammox*), and disassimilatory nitrite reduction to ammonium (*DNRA*). Black solid and dotted lines represent unidirectional and bidirectional flows, respectively.

Archaeal GDGTs

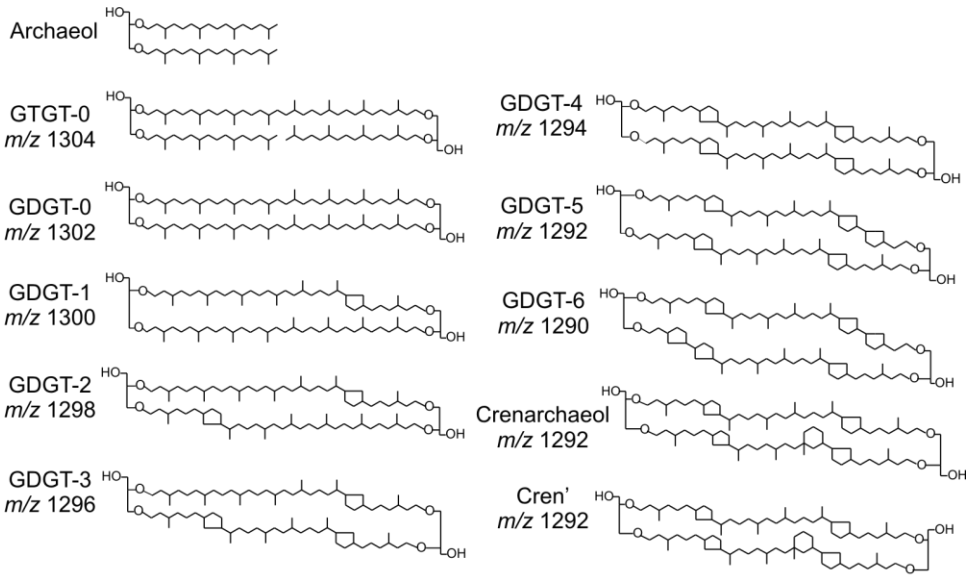
A major trait differentiating Archaea from the other life domains, Bacteria and Eukarya, is their cellular membrane lipid structure. The major characteristics distinguishing Archaeal lipids from Bacterial and Eukaryote lipids include: (i) the stereochemistry of glycerophosphate backbones (Archaea have *sn*-glycerol-1-phosphate (G-1-P) compared to (G-3-P) backbone of Bacteria and Eukaryotes), (ii) ether linkages between the glycerol moiety and hydrocarbon chains as opposed to ester-linkages, (iii) isoprenoid hydrocarbon chains as opposed to the mostly linear chains of fatty acids, and (iv) bipolar lipids that span the membrane forming a monolayer organization (cf. Koga and Morii, 2007 and references therein). Differences between the lipids of Archaea and Bacteria remain so definitive that it appears as though the advent of enantiomeric lipids was actually responsible for their evolutionary separation into distinct lines of descent (Koga et al., 1998).

The Crenarchaeota mainly produce glycerol dibiphytanyl glycerol tetraethers (GDGTs) (Figure 4), while other common ether-based lipids in the form of mono-, di- or triethers occur in abundance in Euryarchaeota. Archaeol, an acyclic diether (cf. Figures 1 and 4), is common among Euryarchaeota (particularly methanogens) and some Crenarchaeota (cf. Koga and Morii, 2005). The occurrence of GDGTs in (hyper)thermophilic Crenarchaeota was seen as a mechanism to provide enhanced stability in higher temperature, low pH, environments due to the stability of the ether bond and the monolayer properties of GDGTs. GDGTs may contain up to eight cyclopentane moieties, the number of which may be adjusted in response to temperature changes in order to keep the cell membrane in a liquid-crystalline state (deRosa and Gambcorta, 1988); increasing the number of cyclopentane rings increases the molecular density (Gabriel and Chong, 2000), thereby maintaining membrane integrity at high temperatures. GDGT-4 is a particularly abundant GDGT synthesized by hyperthermophilic Crenarchaeota.

Hoefs et al. (1997) found carbon skeletons derived from GDGTs with cyclopentane moeieties in the marine water column and sediments, linking these structures for the first time to mesophilic Crenarchaeota. This was confirmed by DeLong et al. (1998) who found similar biphytane carbon skeletons in picoplankton sampled from Antarctica and the Santa Monica Basin, as well as in *C. symbiosum*. Direct HPLC-MS analysis of intact core

GDGTs by Schouten et al. (Schouten et al., 2000) revealed abundant amounts of a unique GDGT (and lesser amounts of its regioisomer) containing a cyclohexane moiety in addition to four cyclopentane moieties in a number of mesophilic environments. NMR characterization of this GDGT extracted from Arabian Sea sediment confirmed its synthesis by the mesophilic marine crenarchaeon, and it was subsequently named "crenarchaeol". Membrane modeling showed its effect on the cell membrane to be opposite that of the hyperthermophiles - the introduction of the cyclohexane ring actually decreased membrane density (Sinninghe Damsté et al., 2002). Its absence in hyperthermophiles coupled to its abundance in mesophilic marine environments suggested that crenarchaeol may be specific to Group I.1a Crenarchaeota. However, subsequent studies have shown that crenarchaeol is ubiquitous in terrestrial environments (Weijers et al., 2006) as well as hot springs (Pearson et al., 2004). The lack of enrichment cultures has prevented any detailed studies of the occurrence of this specific GDGT within the Group I Crenarchaeota and its possible link, specifically, to AOA.

Core GDGTs, as described above, constitute the relatively apolar portion of a bipolar cell membrane and represent the archaeal lipid in the absence of a polar headgroup (Figure 5). Due to their stabile nature, a fraction of the GDGTs synthesized by Archaea in the water column and sediments tend to persist in the environment after cell death. In this sense, core GDGTs, when extracted as such from environmental samples, largely represent fossilized cell remnants. Since it is not possible to discern the age and provenance of GDGTs without the use of ^{14}C radiocarbon dating, their application to studies of modern microbial assemblages may provide limited useful information. In contrast, the propensity of GDGTs to fossilize and persist over geologic timescales has been paramount in the development of the TEX_{86} paleothermometer (Schouten et al., 2002) - a proxy which uses the relative abundance of a number of GDGTs (1-3) and the regioisomer of crenarchaeol to extrapolate past sea surface temperatures (Figure 5). The TEX_{86} proxy is based on the assumption that planktonic mesophilic Crenarchaeota living near the sea surface adjust their membrane lipid composition in accordance with temperature (analogous to their hyperthermophilic relatives), but have evolved the added incorporation of crenarchaeol and



$$TEX_{86} = \frac{[GDGT\text{-}2]+[GDGT\text{-}3]+[Cren']}{[GDGT\text{-}1]+[GDGT\text{-}2]+[GDGT\text{-}3]+[Cren']}$$

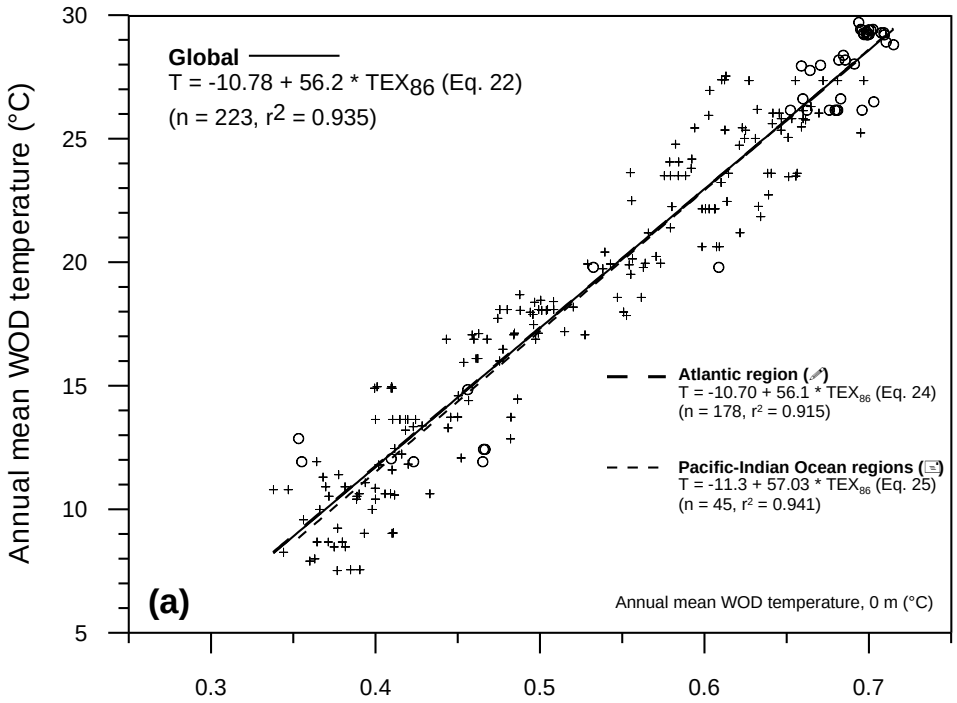


Figure 4. (Previous page) (Top) Structures of some core membrane lipids synthesized by Crenarchaeota, including the diether, archaeol, the glycerol trialkyl glycerol triether with no cyclopentane moieties (GTGT-0) and several glycerol dialkyl glycerol tetraethers with 0-6 cyclopentane moieties (GDGTs 0-6). Molecular weights are shown as *m/z* values. Crenarchaeol and the crenarchaeol regioisomer (cren') contain a unique cyclohexane moiety in addition to four cyclopentane moieties (Sinninghe Damsté et al., 2002). (Bottom) Formula for the calculation of the Tetraether Index of lipids with 86 carbon atoms (TEX_{86}) using concentrations of GDGTs depicted above (Schouten et al., 2002) and the TEX_{86} marine core-top global calibration used to calculate associated sea surface temperatures (Kim et al., 2008). Calibration temperatures were obtained from the World Ocean Database (WOD).

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its regioisomer to cope with the low temperatures of the marine water column. At present, although the empirical relationship between the TEX_{86} and temperature is statistically robust (cf. Figure 5), a complete understanding of why and how mesophilic Crenarchaeota adjust their membrane composition is lacking.

IPL GDGTs as Archaeal 'life' markers

Due to the stability of core lipids relative to their intact polar lipid (IPL) counterparts, their ease of extraction from environmental samples with common organic solvents, and the analytical capacity of conventional instrumentation, the study of biomarker lipids has traditionally focused on the analysis of core lipids. However, since core lipids are stable outside the cell membrane and can persist for long periods of time, studies of viable (or recently viable) cells are increasingly focusing on the analysis of IPLs as they occur in an intact cell membrane. Upon cell senescence, IPLs rapidly lose their polar headgroups via enzymatic hydrolysis (Harvey et al., 1986; White et al., 1979) and are therefore viewed as diagnostic for (recently) living microbes. IPLs can be extracted from organic matter using a modified organic solvent extraction scheme which employs the use of an aqueous phosphate buffer solution to prevent headgroup loss during sonication/sample work-up and enables partitioning of IPLs into the organic phase of the solution (Bligh and Dyer, 1959). These IPLs can subsequently be separated using column chromatography or thin layer chromatography into different of classes polar lipids (e.g.

glycolipids, phospholipids) (Guckert et al., 1985). These fractions can then be treated with chemical degradation procedures such as acid/base hydrolysis and/or ether bond cleavage with $HI/LiAlH_4$ (Hoefs et al., 1997) to remove polar headgroups and cleave ether bonds, to render GDGTs and/or GDGT-derived biphytanes amenable to analyses with HPLC-MS, or GC-MS, respectively. Only in recent years, have Archaeal IPLs been analyzed in their complete intact form using HPLC-ESI/MS (Rutters et al., 2002; Sturt et al., 2004) with improved structural identifications of their headgroups. Archaeal IPLs are represented by a large number of unique chemical structures (see structures reviewed in Koga et al., 1993; Koga and Morii, 2005; Sturt et al., 2004) that share some degree of

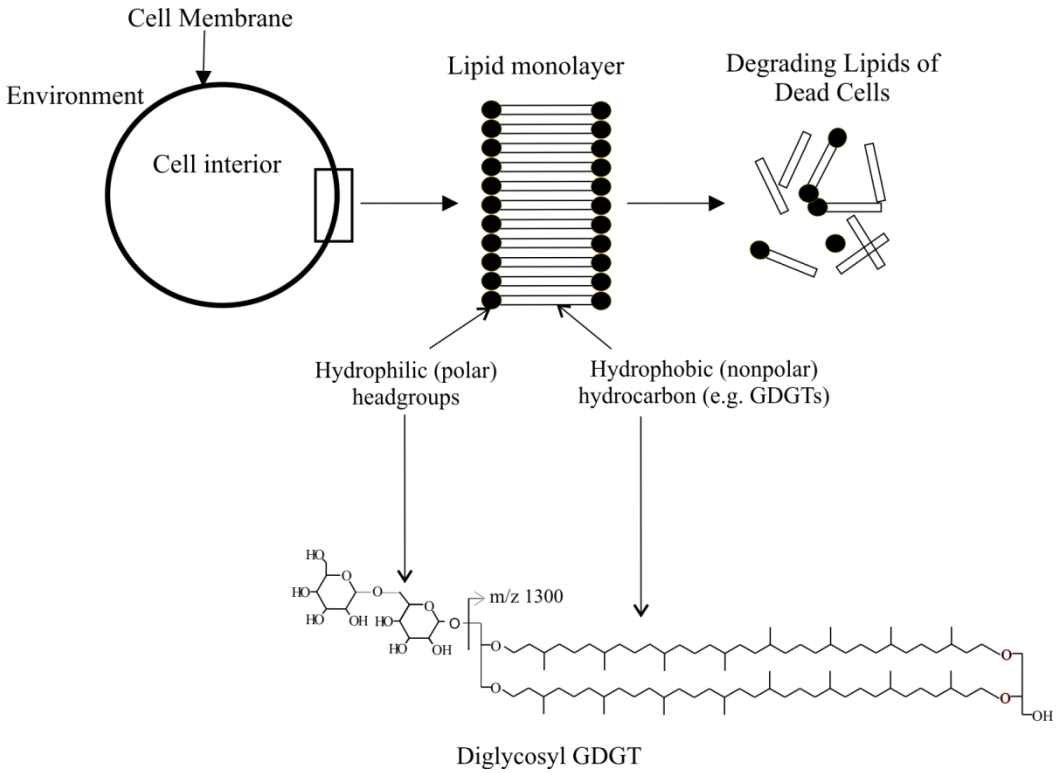

Figure 5. Schematic of a cell membrane and lipid monolayer (typical of Crenarchaeota) comprised of GDGTs with polar headgroups. The example intact polar lipid (IPL) shown is a diglycosyl GDGT-0 containing two hexose sugar moieties, commonly synthesized by a number of Crenarchaeota, including the marine Group I.1a crenarchaeote, *Nitrosopumilus maritimus* SCM1 (Schouten et al., 2008).

taxonomic specificity. It is assumed that the "composition of lipid component parts is widely shared by Archaea species belonging to the same family" (Koga and Morii, 2005) indicating that as chemotaxonomic markers, IPLs are more informative than their core lipid counterparts. Much of this insight has come from cultivated archaeal isolates, which however, almost exclusively represent the aforementioned extremophiles. By comparison, very little is known about the structures of IPLs synthesized by the widespread mesophilic Crenarchaeota, simply due to the lack of cultivated representatives. *Nitrosopumilus maritimus* SCM1 is the only mesophilic Crenarchaeon analyzed for its IPLs, however, it was originally isolated from a tropical marine aquarium. Based on the apparent phylogenetic diversity of mesophilic Crenarchaeota, it is clear that more cultivated representatives enriched directly from natural environments are needed to provide a representative and environmentally-relevant overview of the IPLs synthesized by this archaeal group.

Scope and framework of this thesis

The ubiquity of mesophilic Crenarchaeota has been well documented over the last two decades using advancing techniques in lipid analysis and (meta)genomics. First evidence from the mesophilic crenarchaeotes, *Nitrosopumilus maritimus* and *Cenarchaeum symbiosum*, and environmental studies suggest that the majority of mesophilic Crenarchaeota may be ammonia-oxidizers. However, the importance and prevalence of AOA is still poorly constrained and the biomarker specificity of GDGTs - particularly crenarchaeol to AOA - is also not yet clear. Only recently have ecological studies begun to integrate GDGT-based IPLs, which are supposedly better makers for living archaeal cells, into their molecular analyses (e.g. Biddle et al., 2006). The actual structures of IPL headgroups and associated GDGT composition of AOA are largely unknown, mainly because of difficulties in cultivating these recalcitrant microbes.

The aims of this thesis were to develop techniques for IPL-GDGT analysis, to investigate their phylogenetic distribution, and develop the application of GDGT-based IPLs to ecological studies of AOA. To achieve this, a major focus was on the development of analytical methods for the analysis of IPL GDGTs, which involved determining the

structures of GDGT-based IPLs synthesized by four novel enrichment cultures of AOA. Subsequent application of those results to a number of environmental studies was aimed at linking the distributions of AOA to their membrane lipid biomarkers.

Chapter 2 investigates the elution behavior of core and intact polar GDGTs over activated silica gel columns to obatin the optimal separation and enrichment of each GDGT class. It was found that elution schemes for column chromatography of bacterial core lipid and IPLs are inadequate for analogous separation of core and IPL GDGTs, which can have implications for distinguishing between living and fossil archaeal biomass. Using authentic GDGT standards, it was found that elution with hexane:ethyl acetate (3:1, v/v), ethyl acetate, followed by methanol, was the best scheme to obtain fractions enriched in core, glyco-, and phospho- GDGTs, respectively.

Chapter 3 provides the first demonstration of the specificity of IPL-GDGTs to living Archaea by combining the results described in Chapter 2 to obtain separate fossil and living GDGT fractions with quantitative PCR analysis of AOA genes extracted from two California hot springs. The results of this work reveal the occurrence of *in-situ* production of crenarchaeol by hot spring AOA, a previous speculation based solely on the analysis of core GDGTs.

Chapter 4 reports the unique GDGT distribution and IPL headgroups synthesized by a thermophilic soil Group I.1b ammonia-oxidizing Archaeon. "*Ca*. Nitrososphaera gargensis", an AOA enriched from a Russian hot spring (Hatzenpichler et al., 2008). "*Ca*. N. gargensis" synthesized similar IPL headgroups to those synthesized by *N. maritimus* SCM1, the only other AOA analyzed for its IPL composition. Confirmation of crenarchaeol synthesis by a third lineage of AOA (in addition to the marine Group I.1a and Thermophilic AOA Crenarchaeota) supports its hypothesized specificity to ammonia-oxidation and widespread distribution among the ammonia-oxidizing Archaea.

Chapter 5 describes the core and IPL composition of three additional cultures of AOA enriched directly from marine and estuarine sediments. The few studies that have been done on the detailed lipid structures synthesized by AOA in (enrichment) culture are based on AOA enriched from non-marine environments, i.e. a hot spring, an aquarium and a sponge. Investigation of the GDGT distributions confirmed crenarchaeol synthesis by

these enrichments of sedimentary AOA. IPL analysis indicated that crenarchaeol bound to hexose-phosphohexose headgroups is the best biomarker for AOA, as it is the only IPL common to all five AOA analyzed to date.

Chapter 6 shows the utility of IPLs as proxies for living microbes by analyzing the distribution of AOA and Anammox Bacteria throughout the Arabian Sea water column. Using the findings described in Chapter 4, a selected reaction monitoring (SRM) HPLC-ESI-MS/MS method to sensitively detect a suite of crenarchaeol-based IPLs was developed and applied to collected suspended particulate matter. This, in addition to SRM detection of the anammox-specific C_{20}-[3]-ladderane monoether PC, and quantitative PCR of corresponding 16S rRNA and metabolic genes, shows that IPLs track (recently) living organisms. The results depict the separate niches of AOA and Anammox Bacteria in the oxygen minimum zone possibly suggesting that, in contrast to other environments, their roles in the nitrogen cycle are not directly coupled.

Chapter 7 provides a comparison of core, IPL-derived, and directly analyzed IPL-GDGTs through the Arabian Sea oxygen minimum zone within the context of their application as biomarker lipids for marine Crenarchaeota. Differences between IPL profiles with different headgroups suggest a potential for fossilization of IPL-GDGTs with sugar-based headgroups which could ultimately affect the fossil core GDGT distribution. A lack of correlation between TEX_{86}-derived and *in situ* temperatures was noted, and suggests that deep-water Archaea do not adapt their membranes according to temperature.

Chapter 8 describes the seasonal distribution of AOA in the coastal North Sea over a 4-year interrupted time series using a combination of qPCR of AOA genes and core and IPL-GDGT analysis. This work expands on results obtained by Wuchter et al. (2006) and confirms the recurrence of AOA blooms in the coastal North Sea during periods of winter nitrification. ^{13}C label incorporation into crenarchaeol indicates that during periods of elevated abundance during the winter, these AOA are actively fixing bicarbonate.

In summary, the data presented in this thesis reveal that intact polar lipids of ammonia-oxidizing Archaea are robust tools for tracking both their abundance in natural environments. As biomarker lipids become important in modern microbial ecology studies, developing and adapting the use of IPLs, particularly in the case of AOA, will be

imperative to their application. The results presented here show that AOA synthesize crenarchaeol and other GDGTs bound to only a few major headgroups and that these IPLs can be used as specific markers to study their natural ecology.

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Chapter 2

Separation of core and intact polar archaeal tetraether lipids using silica columns: Insights into living and fossil biomass contributions

Angela Pitcher, Ellen C. Hopmans, Stefan Schouten, Jaap S. Sinninghe Damsté

NIOZ Royal Netherlands Institute for Sea Research, Department of Marine Organic Biogeochemistry, P.O. Box 59, 1790 AB Den Burg, The Netherlands

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Abstract

Intact polar lipids (IPLs) are frequently used as biomarkers for living microbial cells and can be separated from core lipids (i.e. lipids without polar headgroups), which are mainly derived from fossil (i.e. dead) cell material, using column chromatography. We have compared the effect of various silica column conditions on the separation and recovery of archaeal glycerol dialkyl glycerol tetraether (GDGT) core lipids, glycolipids and phosphoglycolipids using authentic standards and direct analysis with various high performance liquid chromatography-mass spectrometry (HPLC/MS) techniques. The commonly used procedure to separate these compound classes using dichloromethane, acetone and methanol as eluents, respectively, did not separate core GDGTs from glyco-

and phosphoglyco-GDGTs. In contrast, a recently described procedure using hexane: ethyl acetate (3:1, v/v), ethyl acetate and methanol achieved both high recovery and successful separation of core GDGTs from the other IPLs. Application of this method to a geothermally heated soil and suspended particulate matter from the North Sea showed that considerable qualitative and quantitative differences can occur between core and IPL-GDGTs. We conclude that this method is thus appropriate for the separation of intact archaeal IPLs and their fossil analogues.

Introduction

Intact polar lipids (IPLs) form the main constituents of all cellular membranes, and comprise a broad range of molecules. Among others, they include ester-linked phosphoglycerides (e.g. phosphatidylethanolamine, phosphatidylcholine, phosphatidyl-serine, and phosphatidyl-glycerol) which are generally abundant in Bacteria and Eukaryotes, and a variety of phospho- and glycolipids composed of glycerol units containing di-, or tetraether-linked isoprenoidal chains which are typical of archaeal membranes. Upon cell lysis, the covalently-bound polar headgroups of IPLs are quickly (within days) lost (White et al., 1970; Harvey et al., 1986) presumably via enzymatic hydrolysis, whereas the resulting core lipids are less readily degraded and may remain in the environment as part of the fossilized (i.e. 'dead') pool of cellular material. IPLs are, therefore, generally considered to be good biomarkers for living, viable organisms (e.g. Petsch et al., 2001; Biddle et al., 2006).

Analysis of IPLs typically starts with a modified Bligh and Dyer extraction (e.g. White et al., 1979), where the extract (BDE) contains both the IPLs and core lipids present in the original sample. To obtain separate fractions containing various lipid classes, the BDE can be separated over an activated silica gel column with a series of increasingly polar solvents. According to Guckert et al. (1985), fractionation over activated silica gel with dichloromethane (DCM) or chloroform, acetone, and then methanol (MeOH), yields a neutral fraction containing core lipids (CLs), a glycolipid (GL) fraction and a phospholipid

(PL) fraction, respectively. The GL and PL fractions can then be analysed intact directly, or indirectly after chemical degradation, thereby providing information about living microbial cells in the original samples.

Group I Crenarchaeota, a newly proposed phylum within the domain Archaea (Brochier-Armanet et al., 2008), are of great interest due to their recent emergence as major actors in the biogeochemical cycling of nitrogen and inorganic carbon (Könneke et al., 2005; Wuchter et al., 2006). Their cellular membranes differ from those of Bacteria and Eukaryotes in that they can contain glycerol dialkyl glycerol tetraether (GDGT)-based lipids. Archaeal GDGTs can contain 0-8 cyclopentane moieties and one particular GDGT, crenarchaeol, has so far only been found in Group I Crenarchaeota and contains 4 cyclopentane moieties and 1 cyclohexane moiety (Sinninghe Damsté et al., 2002b; Schouten et al., 2008). Distributions and concentrations of archaeal GDGTs have been determined by high performance liquid chromatography coupled to mass spectrometry (HPLC/MS) (Hopmans et al., 2000; Schouten et al., 2007) as core lipids, i.e. without a functional headgroup, in the marine water column (e.g. Sinninghe Damsté et al., 2002a; Coolen et al., 2007; Wakeham et al., 2007), marine sediments (e.g. Pancost et al., 2001), soils (e.g. Leininger et al., 2006 ; Weijers et al., 2006), and hot springs (e.g. Reigstad et al., 2008), in part to examine the ecological distributions of the Archaea living there and their importance in local biogeochemical process. Analysis of GDGT-derived biphytane carbon skeletons using gas chromatography coupled to mass spectrometry (GC-MS), has also been achieved by chemical ether bond cleavage of core GDGTs with HI followed by reduction with $LiAlH_4$ (e.g. Hoefs et al., 1997; DeLong et al., 1998; Pearson et al, 2001; Wakeham et al., 2003), a technique which has also proved useful in observing microbial activity, for example via GC-isotope ratio monitoring (IRM) measurements of ^{13}C incorporated into the tricyclic biphytanyl moiety of crenarchaeol (Wuchter et al., 2003).

The previously mentioned studies have analysed core lipid (CL) GDGTs in extracts obtained using traditional organic solvent extraction methods, designed to extract core lipids from environmental samples. While these extracts may indeed contain CL-GDGTs derived from recently living Archaea (i.e. core lipids that have lost their polar headgroups either recently in the environment or during sample work-up), it is likely that a substantial

proportion of the total GDGTs extracted using these methods were derived from fossil cell material. In contrast, other studies have specifically focussed on IPL-derived GDGTs by instead performing Bligh and Dyer extractions and subsequent acid- or base-catalyzed cleavage of polar headgroups prior to GDGT analysis via HPLC/atmospheric pressure chemical ionization (APCI)-MS and/or HI/$LiAlH_4$ followed by GC-MS (Summit et al., 2000; Pearson et al., 2004; Zhang et al., 2006; Ingalls et al., 2007; Pearson et al., 2008). This approach, however, may still include analysis of an unknown fossil component as these extracts can potentially also contain CL-GDGTs.

This problem can be overcome in two ways: (1) by direct analysis of the fully intact molecules (i.e. with polar headgroups still attached) via HPLC/electrospray ionization (ESI)-MS (e.g. Sturt et al., 2004; Biddle et al., 2006; Rossel et al., 2008), or (2) by removal of the fossil CL-GDGT background from IPL-GDGTs using column chromatography. Although HPLC/ESI-MS analyses the intact lipid as such (thus negating the need for fossil GDGT removal), quantification of the different compounds are difficult due to the large difference in ionization efficiencies (Mangelsdorf et al., 2005; Zink et al., 2008). Column separation of fossil and IPL-GDGTs followed by quantitative analysis of the core GDGTs after acid hydrolysis of the IPL fraction is a good alternative, as the GDGTs are structurally more similar and a quantitation method using an internal standard is available (Huguet et al., 2006). Traditional chromatography methods for achieving this have proved ineffective, however, as CL-GDGTs have been noted to elute in the GL fraction rather than the neutral fraction (e.g. Pearson et al., 2004; Wakeham et al., 2007). To address this, Oba et al. (2006) have proposed a modified chromatographic procedure to achieve full separation of CL-GDGTs from other IPL-GDGTs. They did not, however, directly analyse the intact GDGTs in their column fractions nor did they provide a quantitative assessment of their separation scheme.

In this work, we have evaluated the elution behavior of archaeal CL-, GL- and PL-GDGTs on silica gel columns and determined their recovery using authentic standards derived from the main phosphoglycolipid of *Thermoplasma acidophilum*, various chromatography schemes and HPLC/MS analysis. Furthermore, we have applied the alternative chromatographic scheme of Oba et al. (2006) to separate, quantify, and examine

the respective distributions of CL- and IPL-GDGTs in a geothermally heated soil from California and suspended particulate matter (SPM) from the North Sea.

Materials and methods

GDGT core and IPL standards

A phosphoglycolipid (PGL)-GDGT mixture (> 95% pure; Fig. 1) derived from *Thermoplasma acidophilum* was obtained from Matreya, LLC (Pleasant Gap, PA, USA). An aliquot (ca. 0.5 mg) was subjected to base hydrolysis to cleave the phosphoglycerol head group, generating a GL-GDGT standard mixture. Briefly, 2 ml of 1N KOH in methanol (MeOH) (96%) was added to 0.5 mg of the PGL-GDGT and the mixture refluxed for 1 h. The pH of the cooled solution was adjusted to pH 5 with 2N HCl:MeOH (1:1, v/v). Bidistilled water was added to give a final ratio of H_2O:MeOH (1:1, v/v) and the mixture was washed (x 3) with dichloromethane (DCM). The DCM fractions were combined and evaporated to dryness under rotary vacuum. The contents were transferred to a glass vial using DCM and stored at -20ºC until further analysis. A CL-GDGT standard mixture was prepared by acid catalyzed hydrolysis of both headgroups of the PGL-GDGT mixture. This was achieved by adding 2 ml of 5% HCl in MeOH to 0.5 mg of the GDGT PGL-GDGT mixture and refluxing for 3 h. The cooled solution was adjusted to pH 5 with 1N KOH in MeOH (96%) and processed as described above for the GL-GDGT mixture.

The production and purity of both the GL- and CL-GDGT standard mixtures were verified using HPLC/MS.

Separation of IPL and core GDGTs by column chromatography

We performed four different chromatography experiments with the GDGT standard mixtures (Table 1). Fractions were collected, dried under rotary vacuum and transferred directly without filtering to HPLC vials for analysis. This was done to avoid losses of IPL GDGTs due to adsorption to glassware or filters. The experiments were:

Experiment I: An aliquot of the CL-GDGT mixture was separated over an activated silica column [4.0 g silica gel (60 Mesh), activated at 130ºC for 3 h, cooled to room temperature in a desiccator and immediately transferred to a 0.8 cm diameter glass column]. The column was flushed with 25 ml DCM, followed by 25 ml acetone and 40 ml MeOH.

Experiment II: Aliquots of the CL-GDGT and GL-GDGT standard mixtures were combined to give a HPLC/MS response ratio of ca. 1:1. An aliquot of the resulting standard mixture ('CL:GL 1:1 mixture') was chromatographed over activated silica gel according to Experiment I, but with an additional elution step of 25 ml of 5% acetone in DCM following the elution step with DCM.

Experiment III: An aliquot of the CL:GL (1:1) mixture was separated according to Experiment I, but using 5% deactivated silica gel. For deactivation, 10 g activated silica was placed in a pre-combusted Erlenmeyer flask prior to immediate addition of 5% (by wt) bidistilled water. The mixture was sealed and shaken gently for 12 h prior to use.

Experiment IV: An aliquot of the CL:GL (1:1) mixture was chromatographed over 0.6 g activated silica gel using 3 ml hexane:ethyl acetate (3:1 v/v), 3 ml ethyl acetate, and 10 ml MeOH, according to Oba et al. (2006). In addition, an aliquot of the original PGL-GDGT standard mixture was eluted following the same protocol. Each of these column separations was repeated (x 3).

All CL:GL column fractions were analysed using HPLC/APCI-MS and the PGL-GDGT mixture fractions were analysed using HPLC/ESI-MS.

HPLC/APCI-MS

CL- and GL-GDGT mixtures were analysed using a procedure modified from that of Hopmans et al. (2000) and Schouten et al. (2007). Analysis was performed using an HP (Palo-Alto, CA, USA) 1100 series LC/MSD equipped with an autoinjector and Chemstation chromatography manager software. Separation was achieved with a Prevail Cyano column (2.1 x 150 mm, 3 µm; Alltech, Deerfield, IL, USA) maintained at 30 °C. The mobile phase gradient was extended compared to that of Schouten et al. (2007) to allow determination of both CL- and GL-GDGTs in a single analysis. For the first 5 min,

elution was isocratic with 99% A (hexane) and 1% B (iso-propanol), followed by a gradient to 1.8% B in 45 min and by a gradient to 10% B in 20 min. Total run time was 90 min. Flow rate was 0.2 ml/min. Detection was achieved with positive ion APCI under the following conditions: nebulizer pressure (N_2) 60 psi, vapourizer temperature 400 °C, drying gas (N_2) flow 6 l/min, and temperature 200 °C, corona current 5 µA, capillary voltage -3kV. A mass range of *m/z* 1250-1700 was scanned.

HPLC/ESI-MS

PGL-GDGTs were analysed according to the procedure described by Boumann et al. (2006). Briefly, analysis was performed using an Agilent (Palo-Alto, CA, US) 1100 series LC equipped with an autoinjector and coupled to a Thermo TSQ Quantum EM triple quadrupole mass spectrometer equipped with an Ion Max source with ESI probe. Separation was achieved with an Inertsil diol column (2.1 x 250 mm, 5 µm; Alltech, Deerfield, IL, USA), maintained at 30 °C. The following linear gradient was used, with a flow rate of 0.2 ml/min: 100% A to 35% A:65% B in 45 min, maintained for 20 min, then back to 100% A for 20 min to re-equilibrate the column, where A = hexane/2-propanol/formic acid/14.8 M NH_{3aq} (79:20:0.12:0.04 v/v/v/v) and B = 2-propanol/water/formic acid/14.8 M NH_{3aq} (88:10:0.12:0.04 v/v/v/v). Detection was achieved using positive ion ESI/MS by scanning a range of *m/z* 1000-2000.

Recovery of GDGT standards

The recovery of CL-, GL- and PGL-GDGTs from each column fraction was estimated by comparing the integrated peak areas of CL- and GL-GDGTs in the base peak chromatograms with the corresponding peak areas integrated from the base peak chromatogram obtained from an equivalent amount of the starting CL:GL (1:1) mixture injected on column.

Analysis of core and IPL GDGTs of a soil and SPM

A soil sampled near Leonard's Hot Spring, California, USA (41˚36.086 N, 120˚05.135 W) in January 2007 and a suspended particulate matter (SPM) sample obtained

from the North Sea in December 2007 were freeze-dried and extracted using a modified Bligh and Dyer technique. A single-phase solvent mixture of MeOH:DCM:phosphate buffer(2:1:0.8, v/v/v) was added to the sample in a centrifuge tube and placed in an ultrasonic bath for ten minutes. DCM and phosphate buffer was added to give a new volume ratio (1:1:0.9, v/v/v). The extract and residue was separated by centrifuging at 2500 rpm for five minutes. The MeOH/phosphate buffer phase was removed and discarded and the DCM phase was collected in a round-bottom flask. The combined DCM phases were reduced under rotary vacuum and dried over a Na_2SO_4 column.

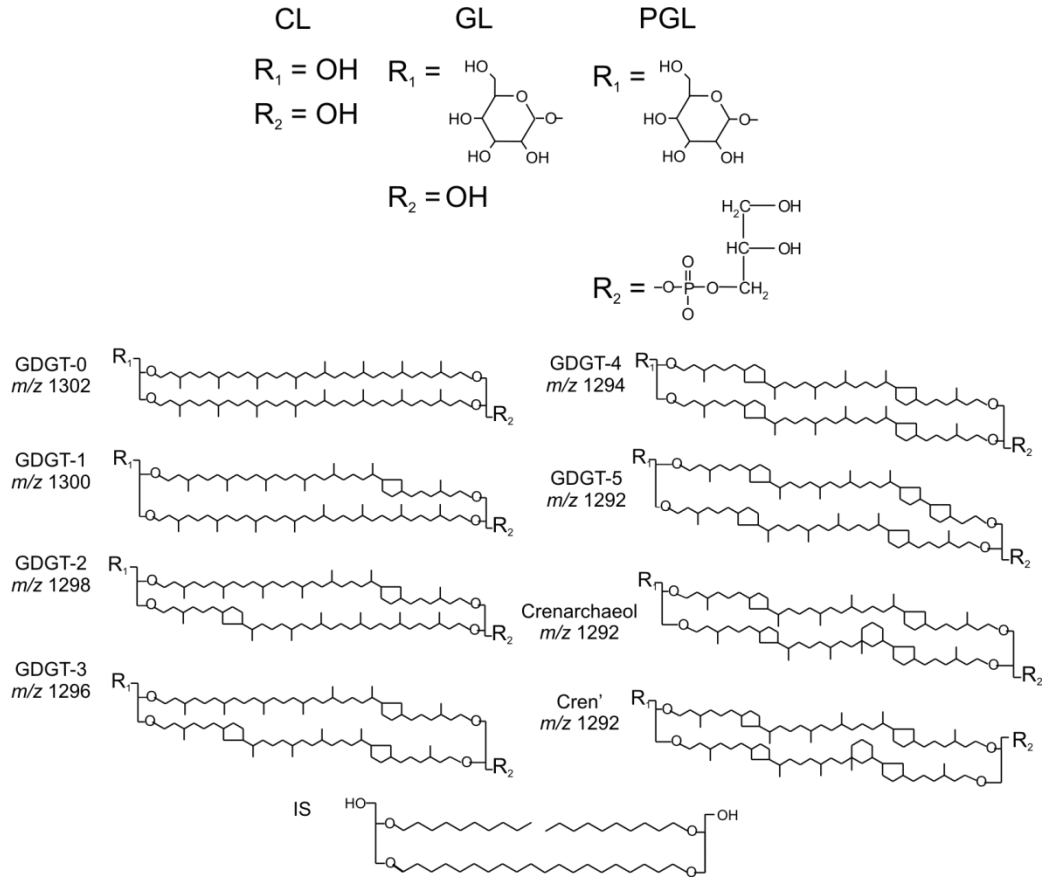

Figure 1. Structures of GDGT standard mixtures used. Core lipid (CL), glycolipid (GL), and phosphoglycolipid (PGL) GDGT standards. IS = C_{46} internal standard added to samples for quantification of GDGTs with HPLC/APCI-MS.

An aliquot (80%) of the BDE was fractionated over pre-activated silica according to column experiment IV. The IPL-GDGT fractions were pooled and 99 ng of a C_{46} internal standard (Figure 1, 'IS') was added to both the CL- and IPL-GDGT column fractions (Huguet et al., 2006). An aliquot of the IPL-GDGT fraction was directly analysed for CL-GDGTs by HPLC/APCI-MS to quantify any fossil carry-over into this fraction. The bulk of the material was subjected to acid-catalyzed polar head group cleavage by refluxing in 2 ml of 5% HCl as described above. CL-GDGTs were analysed using an HPLC/APCI-MS-single ion monitoring (SIM) method described previously (Hopmans et al., 2000, Schouten et al., 2007) and GDGT concentrations were calculated according to Huguet et al. (2006). Finally, 20% of the BDE was directly analysed using the modified APCI method for the analysis of core GDGTs and monohexose-GDGTs.

Results and discussion

Detection and identification of PGL-, CL- and GL-GDGT standard mixtures

Before carrying out any column separations, we determined the composition of the commercially available PGL-standard mixture using HPLC/ESI-MS (Figure 2). This showed that, besides the expected PGL-GDGTs, the mixture contained minor amounts of GL-GDGTs. The various GL- and PGL-GDGTs (structures in Fig. 1) were assigned on the basisof the presence of three characteristic ions, i.e. $[M+H]^+$, $[M+NH_4]^+$ and $[M+Na]^+$ (see insets in Figure 2). The dominant PGL-GDGTs contained two and three cyclopentane moieties (core GDGT-3 and 4). To produce a CL-GDGT standard mixture, we performed acid hydrolysis of the PGL-GDGT standard mixture to cleave both phosphate bonds as well as glycoside bonds. The successful production of the CL-GDGT standard mixture was verified using HPLC/APCI-MS (Figure 3a). The CL-GDGTs were readily assigned via the presence of characteristic $[M+H]^+$ ions and established retention times (Hopmans et al., 2000) and shown to be composed of GDGTs 0 to 5, with GDGT-3 dominating.

To produce a GL-GDGT standard mixture we performed base hydrolysis of the PGL-GDGT standard mixture to cleave the phosphate ester bonds, but leaving the

glycoside bond intact. For the analysis of the GL-GDGT mixture, we developed a new HPLC/APCI-MS procedure, based on those from Hopmans et al. (2000) and Schouten et al. (2007), to allow for the simultaneous analysis of CL-GDGTs and GL-GDGTs.

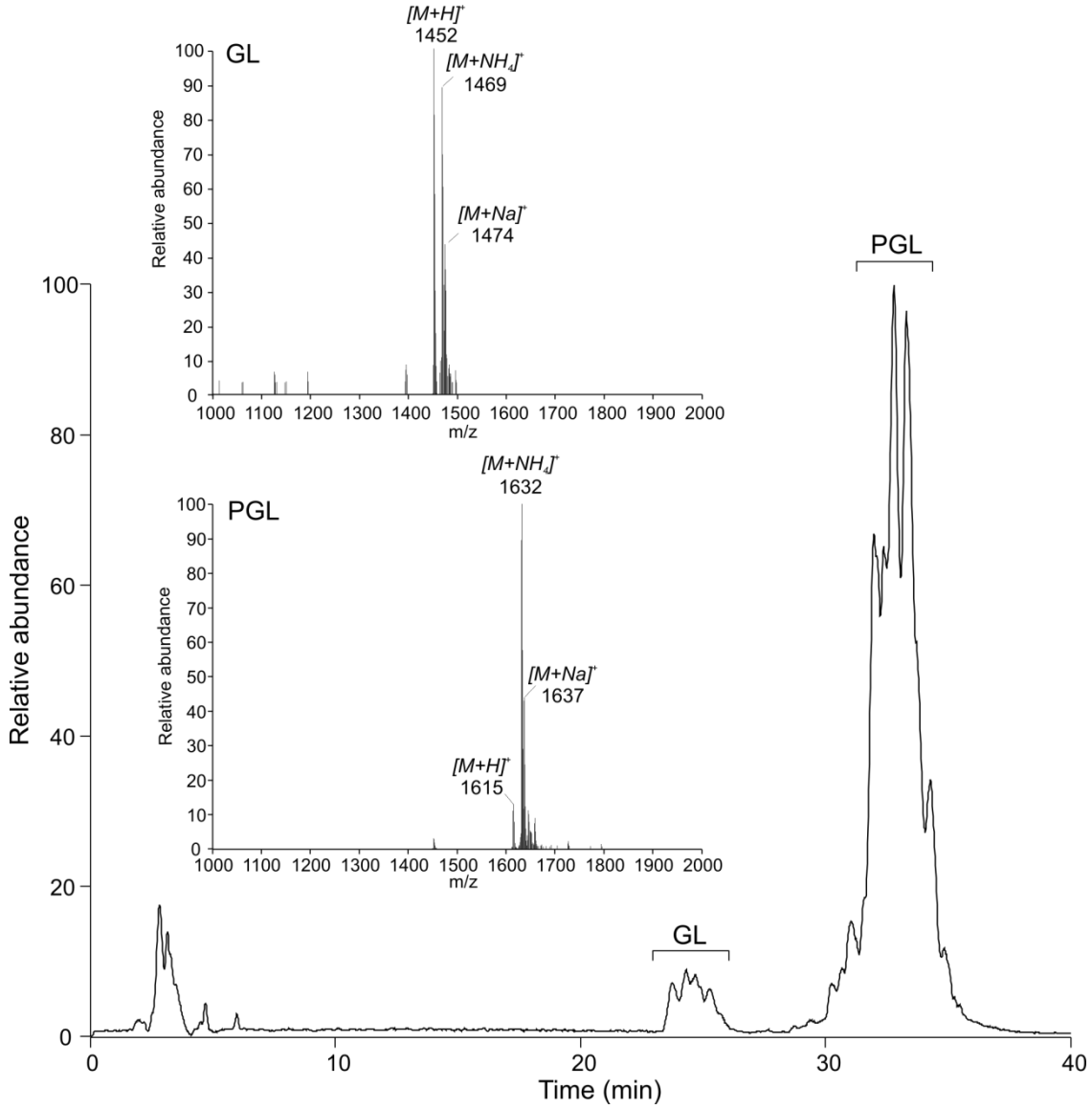

Figure 2. HPLC/ESI base peak chromatogram of phosphoglycolipid standard (PGL). Minor amounts of glycolipid GDGTs (GL) were also present in the standard mixture. The top inset shows spectra for the dominant GL-GDGT-3, containing two cyclopentane moieties. The spectra of the dominant PGL in the mixture, also containing core GDGT-3, is shown in the bottom inset.

Individual GL-GDGTs peaks were less well-resolved, but all GL-GDGT components could be assigned and only minor amounts of CL-GDGTs were detected in the base hydrolysis fraction (Figure 3b). The mass spectra of the GL-GDGT mixture showed only minor peaks representing the $[M+H]^+$ and $[M+H]^+$+1, and dominant fragment ions corresponding to the loss of the sugar moiety (Figure 3b), similar to what is observed for GL-GDGTs when analysed by HPLC/ESI-MS (Sturt et al., 2004; Schouten et al., 2008). Likely, in-source fragmentation during APCI resulted in the loss of the hexose head group from the GL-GDGTs. Nevertheless, the clear separation in retention time between CL-GDGT and GL-GDGTs allows simultaneous analysis of these compounds classes, something not achieved previously.

Recovery of CL-, GL-, and PGL-GDGTs from silica gel columns

Experiments I-III

Experiment I was designed to test the commonly used silica chromatography method for isolation of CL-, GL- and PGL-GDGT fractions using DCM, acetone and MeOH, respectively (Guckert et al., 1985). Column chromatography with the CL-GDGT mixture showed that almost all (> 99%) GDGTs were recovered with acetone. No GDGTs were observed in either of the DCM or MeOH fractions (Table 1). These results show that, although CL-GDGTs lack polar headgroups and are thus comparatively 'non-polar' relative to their IPL analogues, they are still sufficiently polar such that DCM will not elute them from a silica gel column along with other non-polar compounds.

Based on these results, we attempted to separate CL-GDGT from IPL-GDGTs, first using slight modifications from Guckert et al. (1985). The most critical step was to separate CL-GDGTs from the monohexose GL-GDGTs, since the latter would be among the IPL-GDGTs closest in polarity to their CL-GDGT analogues. For experiment II, an aliquot of the CL:GL (1:1) mixture was chromatographed over an activated silica column according to experiment I, but with an additional elution step of 5% acetone in DCM between the DCM and acetone steps. We suspected that this slight increase in solvent polarity might be sufficient to successfully elute the CL-GDGT components from the column. This was not

the case, however, as only 16% of the original CL-GDGTs eluted with 5% acetone in DCM and the majority of recovered CL-GDGTs (73%) still eluted with pure acetone (Table 1). Surprisingly, acetone did not appear to effectively elute the GL-GDGTs. Only 29% of the original GL-GDGTs were recovered from the column, 2% of which were observed in the acetone fraction, with the remaining 27% in the MeOH fraction.

In experiment III, we used 5% deactivated silica (with water) to decrease the polarity of the stationary phase such that the CL-GDGTs would elute more readily with DCM. This was not the case, however, as once again all of the recovered CL-GDGTs (64%) were observed in the acetone fraction. This time, however, all of the recovered GL-

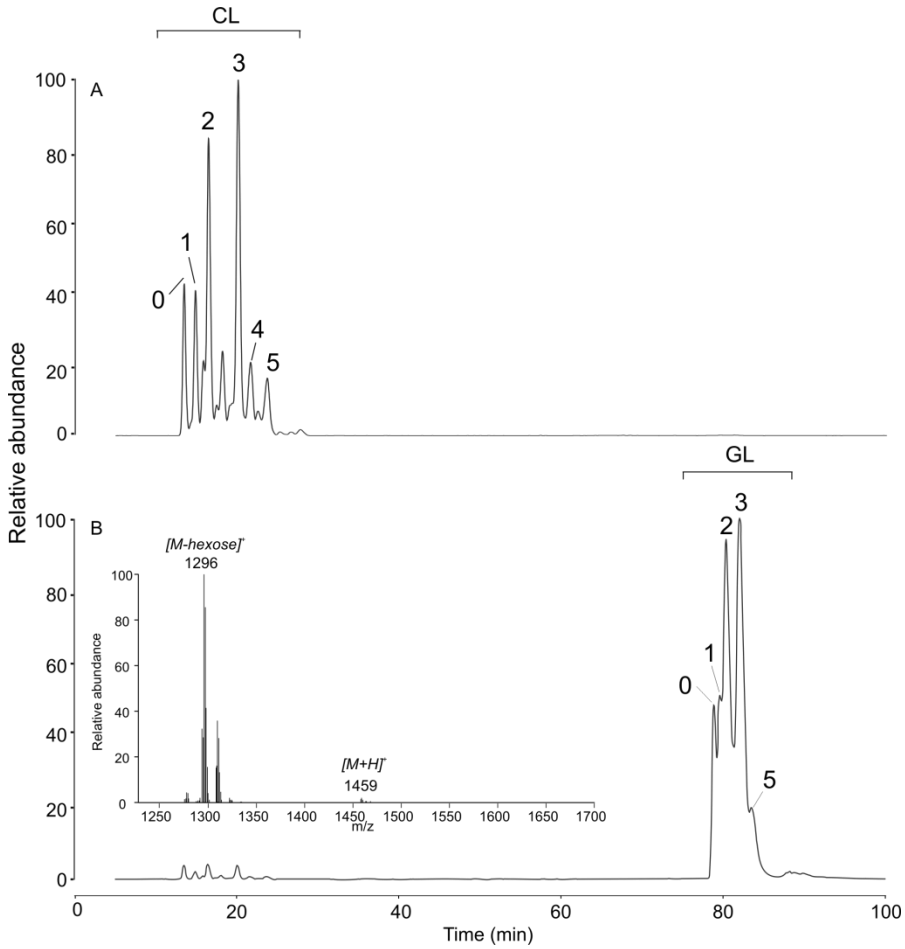

Figure 3. HPLC/APCI base peak chromatograms of core (top) and glycolipid (bottom) GDGT mixtures derived from acid and base hydrolysis, respectively, of the PGL-GDGT standard. Peak labels correspond to GDGT-0 to GDGT 5.

GDGTs (27%) eluted with acetone, suggesting that column deactivation did have an effect on the retention of GL-GDGTs even though CL-GDGT retention appeared unaffected. These findings show that the commonly applied silica chromatography method is unsuitable for separating CL-GDGTs from IPL-GDGTs and that the recovery of GDGTs after column separation is highly variable.

A remarkable result of column experiments I-III is the low recovery of the GL-GDGTs compared to CL-GDGTs (Table 1). Ultrasonic extraction of the silica after each experiment with DCM:MeOH did not, however, afford additional GL-GDGTs, indicating that the GL-GDGTs unaccounted for were not adsorbed to the silica after complete elution and that all lipids originally adsorbed to the silica had been successfully eluted from the column. Rinsing of the glass vials used during the column experiments with MeOH did, however, recover an additional ca. 20-30% of the GL-GDGTs, indicating that a large proportion readily adsorbs to active spots at the surface of the glassware. The importance of this phenomenon has recently been noted for IPL analysis of phosphoinositides in biological samples (Pettitt et al., 2006). Although this artifact effect resulted in a low recovery of GL-GDGTs from columns in experiments I-III, it did not affect the chromatographic separation of the CL- and GL-GDGTs once adsorbed to the silica, which was our primary concern in this study. Furthermore, in the analysis of environmental samples, the problem may be substantially lessened due to adsorption of extract matrix to the glassware, thereby allowing for higher recovery of IPLs.

To improve GL-GDGT recovery in experiment IV, we minimized, and eliminated wherever possible, steps that involved contact of the lipids with glassware. Sample material was transferred to the column using plastic pipette tips, directly placed on to the centre of the silica adsorbent to avoid contact with the glass column, and glass vials containing IPLs were repeatedly rinsed and subjected to sonication in an ultrasonic bath with each consecutive eluent which was also then also added to the column.

Table 1. Recovery of core- (CL), glyco- (GL) and phosphoglyco- (PGL) GDGTs from silica gel column experiments I-IV. (n.a. = not analysed; n.d. = not detected; standard deviations shown for experiment IV, n =3)

Experiment	Eluent	Volume (ml)	Silica	% GDGT Recovery		
				CL	GL	PGL
I	DCM	25	4.0 g, 130ºC, 3h	n.d.	n.a.	n.a.
	acetone	25		99	n.a.	n.a.
	MeOH	40		n.d.	n.a.	n.a.
II	DCM	25	4.0 g, 130ºC, 3h	n.d.	n.d.	n.a.
	acetone-DCM, 5% vol	25		16	n.d.	n.a.
	acetone	25		73	3	n.a.
	MeOH	40		n.d.	26	n.a.
III	DCM	25	4.0 g, 5% deactivated 130ºC, 3h	n.d.	n.d.	n.a.
	acetone	25		64	26	n.a.
	MeOH	40		n.d.	n.d.	n.a.
IV	hexane:ethyl acetate 3:1 v/v	3	0.6 g, 130ºC, overnight	60 ± 26	n.d.	n.d.
	ethyl acetate	3		n.d.	62 ± 24	n.d.
	MeOH	10		n.d.	33 ± 5	64 ± 13

Experiment IV

A modified elution scheme recently proposed by Oba et al. (2006) suggests that separation of GDGTs over activated silica can be achieved by eluting with hexane:ethyl acetate (3:1 v/v) which should elute CL-GDGTs, and ethyl acetate followed by MeOH to elute GDGT-containing IPLs. To test this, we separated an aliquot of the CL:GL (1:1) mixture over a small column containing 0.6 g activated silica gel with the solvents in the prescribed volumes and analysed the three fractions using HPLC/APCI-MS. Recovered CL-GDGTs (60 ± 26%) were only observed in the hexane:ethyl acetate (3:1 v/v) fraction and no GL-GDGTs were found in this fraction. This solvent mixture is therefore indeed suitable for complete separation of CL-GDGTs from other IPL-GDGTs. The majority of recovered GL-GDGTs (62 ± 24%) was observed in the ethyl acetate fraction, although a large proportion (33 ± 5%) was also observed in the MeOH fraction. Remarkably, our improvements in handling glassware resulted in a substantially increased recovery of GL-

GDGTs (> 95%) emphasizing the significance of IPL-glassware interaction. From an aliquot of the original PGL-GDGT standard mixture eluted according to the same column protocol, 64 ± 13% was recovered in the MeOH fraction, as determined from HPLC/ESI-MS (Table 1).

The Oba et al. (2006) separation scheme thus gives complete separation and high yields of CL- and GL-GDGTs, although complete separation of GL- and PL-GDGTs is likely not achieved. In any case, combining the GL- and PL-GDGT fractions will still give a mixture containing predominantly IPL-GDGTs from living Archaea, where prior separation of CL-GDGTs from these will minimize concomitant analysis of fossil GDGTs derived from non-living cellular material.

CL- and IPL-GDGTs in California soil and North Sea SPM

We applied our modified Oba et al. (2006) column separation scheme to test the separation of CL- and IPL-GDGTs on Bligh and Dyer extracts of a geothermally heated soil from California and marine SPM from the North Sea. To test if we successfully separated the CL-GDGTs from the IPL-GDGTs we also analysed the absolute amounts of CL-GDGTs in the IPL-GDGT fraction (Table 2). The results show that there are still some CL-GDGTs in the IPL-GDGT fraction. For the soils this was on average 6% of the total GDGTs quantified from the IPL fraction after acid hydrolysis, while for the SPM this was on average 11%. This means that the column fraction boundaries were not as sharp for our environmental samples as those observed for the GDGT standards, an effect that can likely be attributed to the impact of sample matrices. The GDGT distributions in the CL- and acid-hydrolyzed IPL-fractions of the soil were dramatically different (Figure 4a, Table 2). The CL-GDGT fraction was dominated by crenarchaeol and GDGT-0, whereas there were substantially larger amounts of GDGT-1 and GDGT-2 in the acid-hydrolyzed IPL fraction. These qualitative differences between the CL- and IPL-GDGT fractions were also reflected in the different distributions of CL-GDGTs and the mono-hexose GDGTs using our new HPLC/APCI-MS method (Figure 4b). The total CL- and IPL-GDGTs measured approximately 13 and 54 ng/g dry soil, respectively, indicating a potential fossil contribution to the total GDGT pool in this soil of about 19% (Table 2). If we correct for

the presence of CL-GDGTs in the IPL-GDGT fraction, then the fossil contribution increased to 23%. In contrast, the CL- and IPL-GDGT distributions in North Sea SPM were similar with GDGT-0 and crenarchaeol dominating in both fractions. In this case, the fossil contribution to the total GDGT pool was substantially higher than for the soil, at approximately 64% (Table 2).

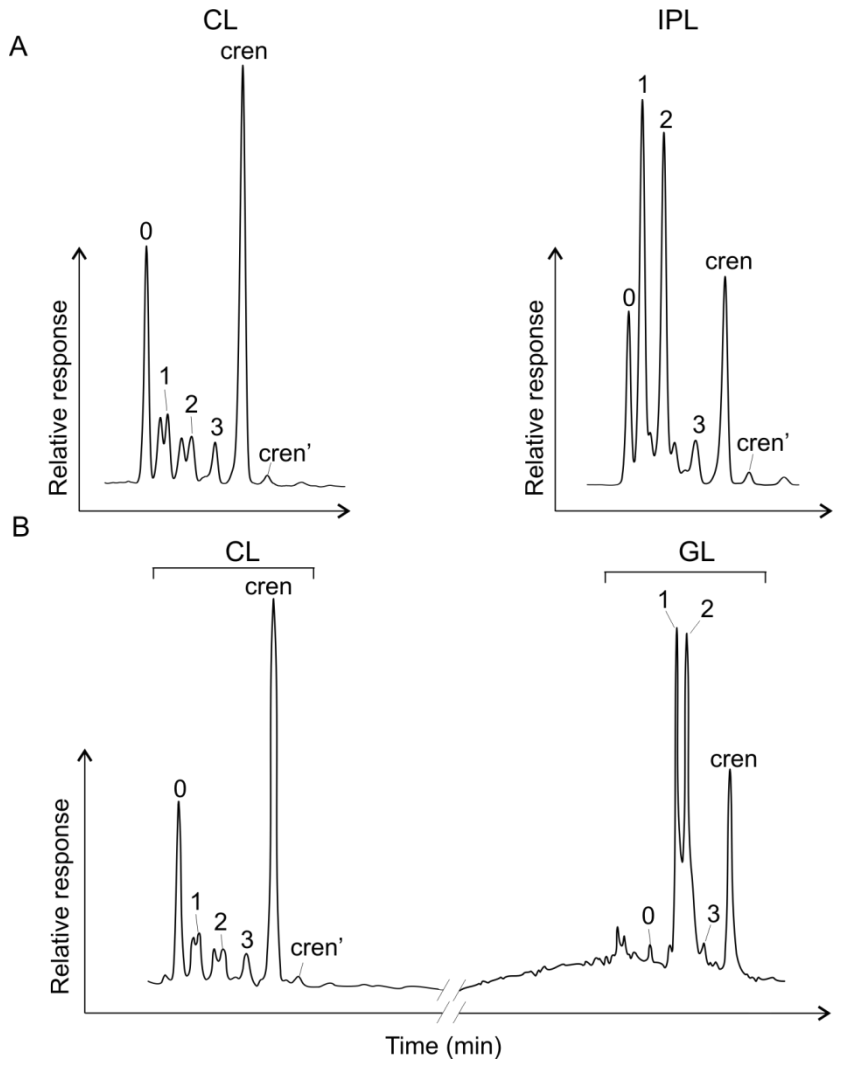

Figure 4. (A) HPLC/APCI-MS base peak chromatograms of GDGTs recovered from a Bligh and Dyer extract of a geothermally heated soil in a CL-GDGT column fraction eluted with hexane:ethyl acetate (3:1 v/v) (left) and in the IPL-GDGT fraction after acid-catalyzed cleavage of polar headgroups (right). (B) HPLC/APCI-MS base peak chromatogram of the total BDE with an extended gradient to detect both CL- and monohexose-GDGTs. Peak labels correspond to GDGT-0 to GDGT 5.

Table 2. GDGT concentrations measured from column fractions of Bligh and Dyer extracted soil and suspended particulate matter obtained using the Oba et al (2006) separation scheme. 'CL's = core GDGTs, 'CL in IPL' = core GDGTs in IPL fraction prior to acid hydrolysis, 'IPL' = GDGTs in the IPL fraction after acid hydrolysis (corrected for the presence of CL-GDGTs by subtracting 'CL in IPL' values from the total IPL-GDGTs).

Sample	Column fraction	GDGT concentration (ng per g dry soil[a]/liter seawater[b])						
		0 (*m/z* 1302)	1 (m*/z* 1300)	2 (*m/z* 1298)	3 (*m/z* 1296)	cren (*m/z* 1292)	cren' (*m/z* 1292)	total
Soil[a]	CL	4.4	0.9	0.8	0.7	5.8	0.2	12.8
	CL in IPL	0.7	0.2	0.2	0.2	2.3	0.0	3.6
	IPL	9.0	17.7	16.5	1.7	8.2	0.6	53.7
SPM[b]	CL	61.1	5.7	2.3	1.0	49.2	0.4	119.3
	CL in IPL	3.8	0.4	0.2	0.1	4.2	0.0	8.7
	IPL	43.3	3.6	2.0	1.0	22.2	0.3	72.3

[a]Soil sampled near Leonard's hot spring, California, USA (January 2007)

[b]Suspended particulate matter filtered from North Sea water, Texel, The Netherlands (December 2007)

Implications

A number of studies have used the traditional DCM, acetone, MeOH elution sequence to separate Bacterial IPLs, but also used the same method to separate archaeal CL-, GL- and PL-GDGTs. Our results, together with those of Oba et al. (2006), show that archaeal GDGTs in an eluted acetone fraction from a Bligh and Dyer extract will contain a large proportion of CL-GDGTs and only minor amounts of GL-GDGTs. Thus, previous studies which have not directly analysed the IPL-GDGTs, but CL-GDGTs derived from column fractions from Bligh Dyer extracts, may have analysed GDGTs derived from fossil material in addition to those from living microbes. Our direct assessment using GDGT standard mixtures confirms that complete separation of CL-GDGTs from other IPL-GDGTs can be achieved using a hexane:ethyl acetate (3:1 v/v) mixture over pre-activated silica, a method previously advocated by Oba et al. (2006). However, environmental sample matrices may impact the sharpness of fraction boundaries.

We did not check this column protocol with bacterial or eukaryotic IPLs, which may show different chromatographic retention and recoveries. Original silica column chromatography protocols were developed for the separation of bacterial and eukaryotic phospho di-ester lipids and may thus be more suitable for such analyses. Since archaeal, bacterial, and eukaryotic IPLs will elute with different solvents, multiple column procedures on aliquots of a partitioned total lipid extract may be necessary when the analysis of IPLs among the various domains of life are the goal**.**

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Chapter 3

In situ production of crenarchaeol in two California hot springs

Angela Pitcher, Stefan Schouten and Jaap S. Sinninghe Damsté

NIOZ Royal Netherlands Institute for Sea Research, Department of Marine Organic Biogeochemistry, P.O. Box 59, 1790 AB Den Burg, The Netherlands

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Abstract

Crenarchaeol - a membrane-spanning glycerol dialkyl glycerol tetraether (GDGT) containing a cyclohexane moiety in addition to four cyclopentane moieties – was originally hypothesized to be synthesized exclusively by mesophilic Crenarchaeota. Recent studies reporting the occurrence of crenarchaeol in hot springs and as a membrane constituent of the recently isolated thermophilic Crenarchaeote, "*Candidatus* Nitrosocaldus yellowstonii", however, have raised questions regarding its taxonomic distribution and function. To determine whether crenarchaeol in hot springs is indeed synthesized by Archaea *in situ* or is of allochthonous origin, we quantified crenarchaeol present in the form of both intact polar lipids (IPLs) and core lipids (CLs) in sediments of two California hot springs and in nearby soils. IPL-derived crenarchaeol was found in both hot springs and soils, suggesting *in situ* production of this GDGT over a wide temperature range (12-89°C). Quantification of archaeal *amoA* gene abundance by quantitative PCR showed a good correspondence

with IPL-crenarchaeol, suggesting that it was, indeed, derived from living cells and that crenarchaeol-synthesizing Archaea in our samples may also be ammonia oxidizers.

Introduction

Numerous groups of Archaea synthesize isoprenoid glycerol dialkyl glycerol tetraethers (GDGTs) as a major component of their core membrane lipids, which can contain up to eight cyclopentane moieties (e.g., reference 7; Figure 1). Increasing the number of cyclopentane moieties results in denser packing of membrane lipids, allowing for the maintenance of both cellular membrane integrity at high temperatures and stable proton gradients at low pH conditions (Gabriel and Chong, 2000). This biophysical characteristic is hypothesized to be among those traits essential for the survival and persistence of Archaea in the 'extreme' environments in which they are commonly found (Valentine, 2007). GDGTs are synthesized by a large number of cultivated Archaea (see overviews in Macalady et al., 2004 and Schouten et al., 2007) and in nature they are abundant in hot springs (Pearson et al., 2004; Zhang et al., 2006; Schouten et al., 2007; Pearson et al., 2008), for example, where Archaea are known to thrive at high temperatures and over a wide pH range (Madigan et al., 2003; Chaban et al., 2006).

Crenarchaeol is unique among the GDGTs in that it contains a cyclohexane moiety in addition to four cyclopentane moieties (Figure 1). It was first reported in large abundances from Holocene and ancient sediments collected from various marine settings as supporting evidence for the widespread distribution of low-temperature relatives of hyperthermophilic Archaea (Schouten et al., 2000). It was later proposed that crenarchaeol was synthesized exclusively by marine Group I Crenarchaeota (Sinninghe Damsté et al., 2002) – a hypothesis further supported by core lipid analysis of the mesophilic marine group I.1a Crenarchaeotes, "*Candidatus* Cenarchaeum symbiosum" (Sinninghe Damsté et al., 2002) and *Nitrosopumulis maritimus* SCM1 (Schouten et al., 2008), which showed that both of these organisms synthesize crenarchaeol at moderate temperatures. In addition to this, the apparent absence of crenarchaeol in cultures of (hyper)thermophilic Archaea

(overviews in Macalady et al., 2004 and Schouten et al., 2007) and molecular modeling (Gabriel and Chong, 2000; Sinninghe Damsté et al., 2002), led to the hypothesis that crenarchaeol decreases lipid density, effectively allowing archaeal membranes composed of membrane-spanning GDGTs to function at mesophilic temperatures (Sinninghe Damsté et al., 2002). Hence, crenarchaeol synthesis was thought to be instrumental in the evolution and radiation of mesophilic Crenarchaeota from thermophilic habitats (Kuypers et al., 2001).

Recent studies, however, have reported the occurrence of crenarchaeol in hot springs with temperatures of up to 86.5°C (Pearson et al., 2004; Zhang et al., 2006; Schouten et al., 2007; Pearson et al., 2008). This work has been debated to some extent as there exists the potential for allochtonous input of fossilized lipid material from weathering of nearby soils where mesophilic Crenarchaeota may thrive: Schouten et al. (2007) found

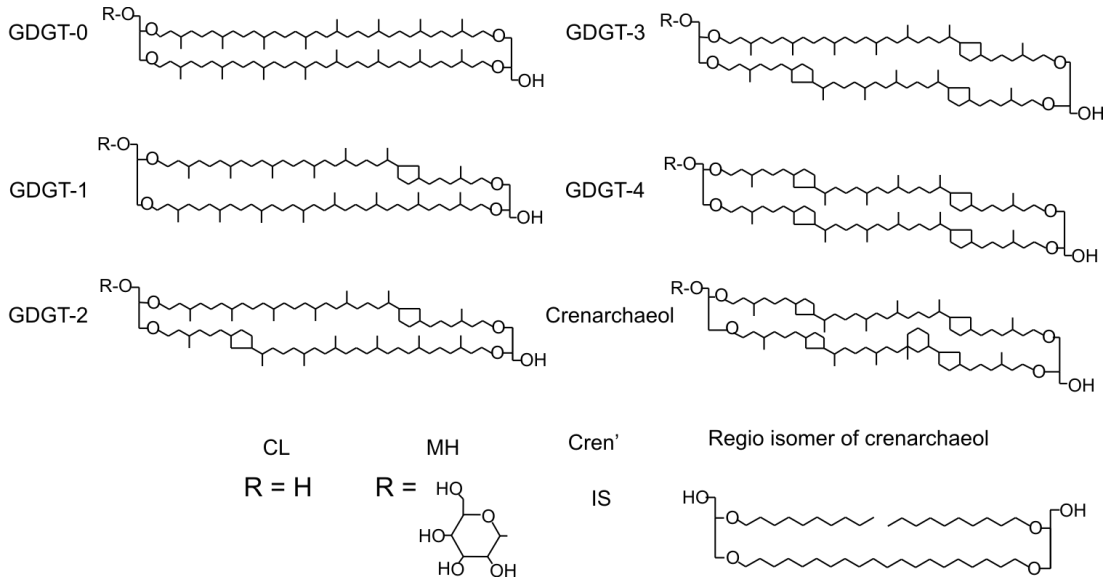

Figure 1. Structures of glycerol dialkyl glycerol tetraethers (GDGTs) referred to in the text. "CL"core lipid**; "**MH" = mono-hexose; "IS" = C_{46} internal standard.

large relative amounts of specific soil bacteria biomarkers in tandem with crenarchaeol in Yellowstone hot springs. In contrast, Reigstad et al. (2008) reported the occurrence of crenarchaeol in the absence of soil specific biomarkers in Icelandic hot springs. Furthermore, the recently isolated thermophilic crenarchaeote, "*Candidatus* Nitrosocaldus yellowstonii", was shown to synthesize crenarchaeol at a growth temperature of 72°C (de la Torre et al., 2008).

Core lipids (CL) that occur in biological membranes generally contain polar head groups such as sugars and phosphates, which are rapidly cleaved upon cell senescence (White et al., 1979; Harvey et al., 1986). The loss of head groups from the intact polar lipids (IPL) leaves relatively recalcitrant CLs to accumulate in the environment over time as fossil biomarkers. Therefore, depending on the extraction and/or analytical protocols, CLs present in environmental lipid extracts may be derived from both living cells and fossil biomass, including a mixture of both CL and IPL-GDGTs. Most studies on the presence of crenarchaeol in hot springs to date have analyzed directly extracted CL-crenarchaeol or CL-crenarchaeol released by acid hydrolysis of Bligh and Dyer IPL lipid extracts, i.e. without prior separation of CL-GDGTs from IPL-GDGTs (Pearson et al., 2004; Zhang et al., 2006; Schouten et al., 2007; Pearson et al., 2008; Reigstad et al., 2008). In these cases the reported GDGT distributions represent an integrated signal of both 'living' and fossilized material, rendering it impossible to distinguish what proportion (if any) of the observed crenarchaeol was derived from local living archaeal communities. Thus, the *in situ* production of crenarchaeol in hot springs and its importance relative to the *in situ* production of other archaeal GDGTs remains uncertain.

Here we have used a recently described chromatographic method (Oba et al., 2006; Pitcher et al., 2009) to separately quantify the potential contribution of both *in situ*-produced, and fossilized, crenarchaeol (as well as other archaeal GDGTs) in two Californian hot springs and their surrounding soils. In addition, we have quantified the amount of the archaeal *amoA* and archaeal 16S rRNA gene copies from one site to make quantitative comparisons between gene abundance and IPL-GDGT concentrations.

Materials and Methods

Sampling and Study Area

Material from two hot springs, Leonard's hot spring (41˚36.086N, 120˚05.135W) and "Ray's" hot spring (41˚31.855N, 120˚04.966W), both located in Surprise Valley in northwestern California, USA, were sampled as well as soils in a perpendicular transect extending from the hot springs in January 2007. Hot spring material consisted of surface mat and sediment (Leonard's hot spring) and surface sediment material ("Ray's" hot spring) taken with a metal spoon. From the edge of Leonard's and "Ray's" hot spring, soil samples (upper 10 cm) were taken along a perpendicular transect at distances of 10, 30, 60, and 150 cm, and 10, 20, 40, 80, and 250 cm, respectively. Sample material to be analyzed for lipids was stored at -20˚C until further analysis, and samples from which DNA was to be extracted were stored at -80˚C. The *in situ* soil temperature at 10 cm depth and the hot spring water temperature of each site were measured with an Ama-Digit ad 20 th digital thermometer and pH of the soils was determined with freeze dried samples in distilled water (10:25 w/v), after vigorous shaking of the mixture for 1 min and allowing the particles settle for 30 min.

GDGT extraction, column fractionation, and acid hydrolysis

The mat/sediment and soil samples were freeze-dried and extracted three times using a modified Bligh and Dyer technique (Bligh and Dyer, 1959) to obtain CL- and IPL-GDGTs. A single-phase solvent mixture of methanol:dichloromethane (DCM):phosphate buffer (2:1:0.8, v/v/v) was added to the sample in a centrifuge tube and placed in an ultrasonic bath for ten minutes. DCM and phosphate buffer was added to give a new volume ratio (1:1:0.9, v/v/v). The extract and residue was separated by centrifuging at 2500 rpm for five minutes. The methanol/phosphate buffer phase was removed and the DCM phase was collected in a round-bottom flask. We did not substitute with a 5% tirchloric acetic acid solution for the aqueous phase in the last two extractions as previously suggested (Sturt et al., 2004) because we were unsure how this would affect the stability of IPL headgroups and potentially artificially create CLs. Thus, IPL concentrations may be

potential underestimates based on our extraction procedure. The combined DCM phases were reduced under rotary vacuum and dried over a Na_2SO_4 column. The total Bligh Dyer extract (BDE) was fractionated over pre-activated silica gel (60 mesh) with 3 column volumes of hexane:ethyl acetate (3:1, v/v) to obtain the core lipid GDGT (CL-GDGT) fraction and then with 3 column volumes of ethyl acetate followed by 3 column volumes methanol to obtain an intact polar lipid (IPL)-GDGT fraction according to Oba et al. (2006) and Pitcher et al. (2009).

The IPL-GDGT fraction was subjected to acid hydrolysis to cleave polar head groups by refluxing in 2 ml of 5% HCl in MeOH (96%) for 3 h. The pH of the cooled solution was adjusted to pH 5 with 2N KOH:MeOH (1:1, v/v). Bidistilled water was added to a final ratio of H_2O:MeOH (1:1, v/v) and this mixture was washed 3x with DCM. The DCM fractions were collected and evaporated to dryness under rotary vacuum. A known amount of C_{46} internal GDGT standard (14) was added to both the IPL-GDGT fraction (before acid hydrolysis) and the CL-GDGT fraction, which were subsequently analyzed by HPLC/MS.

GDGT analysis

Archaeal GDGTs were analyzed using a modified procedure from Hopmans et al. (2000) and Schouten et al. (2007). Analyses were performed using an HP (Palo-Alto, CA, US) 1100 series LC/MSD equipped with an auto-injector and Chemstation chromatography manager software. For the first 5 min elution was isocratic with 99% A (hexane) and 1% B (iso-propanol), followed by a gradient to 1.8% B in 45 min. Separation was achieved on a Prevail Cyano column (2.1 x 150 mm, 3 µm; Alltech, Deerfield, IL, USA), maintained at 30°C. Total run time was 40 minutes with a flow rate of 0.2 ml min^{-1}. After each analysis the column was cleaned by back flushing hexane/propanol (9:1, v:v) at 0.2 ml min^{-1} for 10 min. Detection was achieved by positive ion APCI with the following conditions: nebulizer pressure (N_2) 60 psi, vaporizer temperature 400°C, drying gas (N_2) flow 6 L min^{-1} and temperature 200°C, corona current 5 µA, capillary voltage -3kV. Archaeal GDGTs were detected with single ion monitoring of their protonated molecules $[M + H]^+$. SIM parameters were set to detect protonated molecules of six common isoprenoid GDGTs (*m/z*

1302, 1300, 1298, 1296, 1294, 1292) as well as the protonated molecule of the C_{46} GDGT internal standard (*m/z* 744), with a dwell time of 237 ms per ion.

For the simultaneous analysis of CL and monohexose-GDGTs in the total Bligh-Dyer extract the mobile phase gradient described above was extended as described previously (26). For the first 5 min elution was isocratic with 99% A (hexane) and 1% B (iso-propanol), followed by a gradient to 1.8% B in 45 min. This was followed by a gradient to 10% B in 20 min. Total run time for this method was 90 min. Detection was achieved according to the MS-method described above.

Quantification of GDGTs was achieved by adding a known amount of C_{46} GDGT internal standard to each fraction. To check the performance of our column separations of the environmental samples, we quantified CL-GDGTs present in the IPL-GDGT fraction prior to acid hydrolysis. This showed that on average only 8.7 ± 5.4% of the IPL-GDGTs were present as CL-GDGTs prior to acid hydrolysis. However, since a proportion of these could have resulted from degradation of intact GDGTs prior to and during analysis, we did not correct for them in our final concentration measurements. Thus, the IPL-GDGT concentrations presented here may be slight overestimations. Because crenarchaeol and GDGT-4 co-elute, the concentrations of each were corrected according to Weijers et al. (2006).

DNA extraction and real-time PCR amplification of archaeal genes

DNA extraction of 0.2-0.4 g of sample material from Leonard's hot spring and adjacent soils was carried out using an Ultra Clean Soil Extraction Kit (MoBio Labs, USA) following the manufacturers' protocol. Archaeal 16S rRNA and archaeal *amoA* gene copy numbers were determined using the Parch519f/Arc915r (Coolen et al., 2004) and Arch-*amoA*-for/Arch-*amoA*-rev (Coolen et al., 2007) primer pairs. Real time quantification was performed using an iCycler (Bio-Rad, Hercules, CA) with all reactions proceeding with an initial denaturing for 5 min at 94ºC, followed by 38 cycles of denaturing for 30 s at 94ºC, 40 s of primer annealing at 64ºC and 58.5ºC for 16S rRNA and *amoA* genes, respectively, and primer extension for 40 s at 72ºC. Real-time increase in fluorescence by SYBRGreen (Molecular Probes) indicated accumulation of double-stranded amplicons. Calibration of

gene copy numbers was performed using real-time standard curves generated with known copy numbers ranging from 10^2 and 10^7 of an enriched Marine Group I Crenarchaeote from the North Sea (Wuchter et al., 2006), which were generated during the same PCR reactions and with the same primers used to detect the environmental genes.

Results

Distribution and abundance of CL- and IPL-GDGTs

HPLC-MS analysis of the CL and IPL-GDGT fractions revealed differences in GDGT distribution between both hot springs (Figure 2a-d). The IPL-GDGT distribution in Leonard's spring was dominated by crenarchaeol, with substantial amounts of GDGTs 0 and 2, and minor amounts of GDGTs 1 and 3 and the crenarchaeol regioisomer (Figure 2a). In "Ray's" hot spring IPL-crenarchaeol was also present, however, in contrast to Leonard's hot spring, the relative amounts were low compared to the other GDGTs. IPLs from "Ray's" spring were dominated by GDGT-4 but also contained substantial amounts of GDGTs 0-3 (Figure 2b). The CL-GDGT distributions in the hot springs were generally similar to those of the corresponding IPL-GDGTs (Figure 2c-d). Notable differences include a larger relative contribution of GDGT-0 and a smaller relative contribution of crenarchaeol in both springs. The distributions of GDGTs 1-3 were similar in both the IPL and CL fractions. GDGT distributions from the soils differed substantially from the corresponding hot spring sediments at each site (Figure 2e-h). Crenarchaeol was still relatively abundant among the IPL-GDGTs in the soil sampled 10 cm from Leonard's hot spring, however this soil was dominated by two GDGTs, which appear to be early eluting isomers of GDGTs 1 and 2 (Figure 2e). IPL-GDGTs obtained from soil sampled 10 cm away from the edge of "Ray's" hot spring were dominated by crenarchaeol, with substantially lower amounts of the other GDGTs (Figure 2f).

The CL-GDGT distribution in the 10 cm soil sampled near Leonard's hot spring was quite different from the corresponding IPL distribution (cf. Figs. 2e and 2g): GDGTs 1 and 2 were in comparatively low abundance, while crenarchaeol dominated along with a

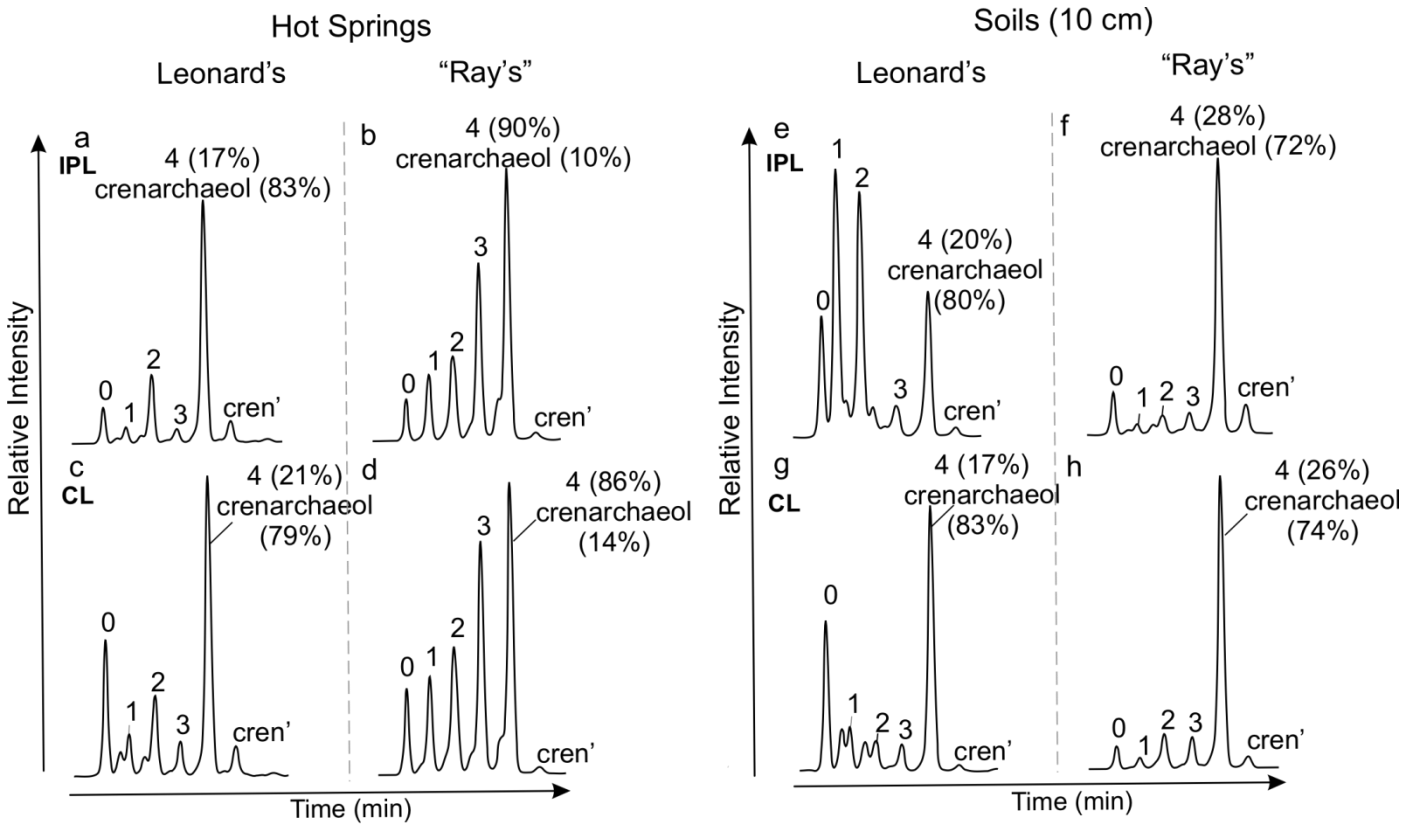

Figure 2. HPLC-APCI-MS base peak chromatograms showing the relative abundance of IPL- and CL-derived GDGTs from Leonard's and "Ray's" hot springs (a-d) and in soils sampled 10 cm from each spring (e-h). Peak numbers and labels correspond to GDGT structures shown in Figure 1 (e.g. "0" = GDGT-0). Numbers in brackets next to co-eluting GDGT-4 and crenarchaeol indicate their relative contribution to the peak area.

substantial amount of GDGT-0. The early eluting isomers of GDGTs 1 and 2, which were in high abundance in the IPL fraction, occurred in much lower relative abundance here, albeit still present in near equal amounts relative to GDGTs 1 and 2. The CL-GDGT distribution obtained from the soil near "Ray's" hot spring was similar to that of the corresponding IPLs, with only slightly less GDGT-0 and slightly more GDGTs 2 and 3 (Figure 2h).

Quantification of the IPL and CL-GDGT fractions showed that the total IPL-GDGT concentration was always higher than the total CL-GDGT concentration, which ranged from 1.4 - 540 ng g^{-1} and 0.4 - 44 ng g^{-1}, respectively (Table 1). "Ray's" hot spring contained by far the highest concentration of IPL-GDGTs, with concentrations dropping significantly along the soil transect to ca. 1.4 ng g^{-1} (Table 1, Figure 3a). Much lower total concentrations of IPL-GDGTs were observed in Leonard's hot spring (11 ng g^{-1}), but higher concentrations of IPLs were observed in the surrounding soils (Table 1, Figure 3b).

IPL-crenarchaeol concentrations varied from 0.8 - 22 ng g^{-1} and contributed between 4 - 61% to the total IPL-GDGT concentrations (Table 1). The highest concentration of IPL-crenarchaeol was actually observed in "Ray's" hot spring, at 89°C, although it contributed only ca. 4% to the total archaeal IPL-GDGT concentration (Figure 3c). IPL-crenarchaeol concentration decreased slightly in the 10 cm soil to 20 ng g^{-1}, and then decreased considerably in soils sampled further away from the hot spring. In contrast, Leonard's hot spring contained lower absolute amounts of IPL-crenarchaeol (5.8 ng g^{-1}), however, here it represented ca. 63% of the total IPL-GDGTs quantified (Figure 3d). In soils sampled away from Leonard's spring, crenarchaeol concentration increased to ca.15 ng g^{-1} (at 30 cm) and then decreased again. CL-crenarchaeol ranged from 0.0 - 16 ng g^{-1} and was always present in lower amounts than IPL-crenarchaeol in the same sample.

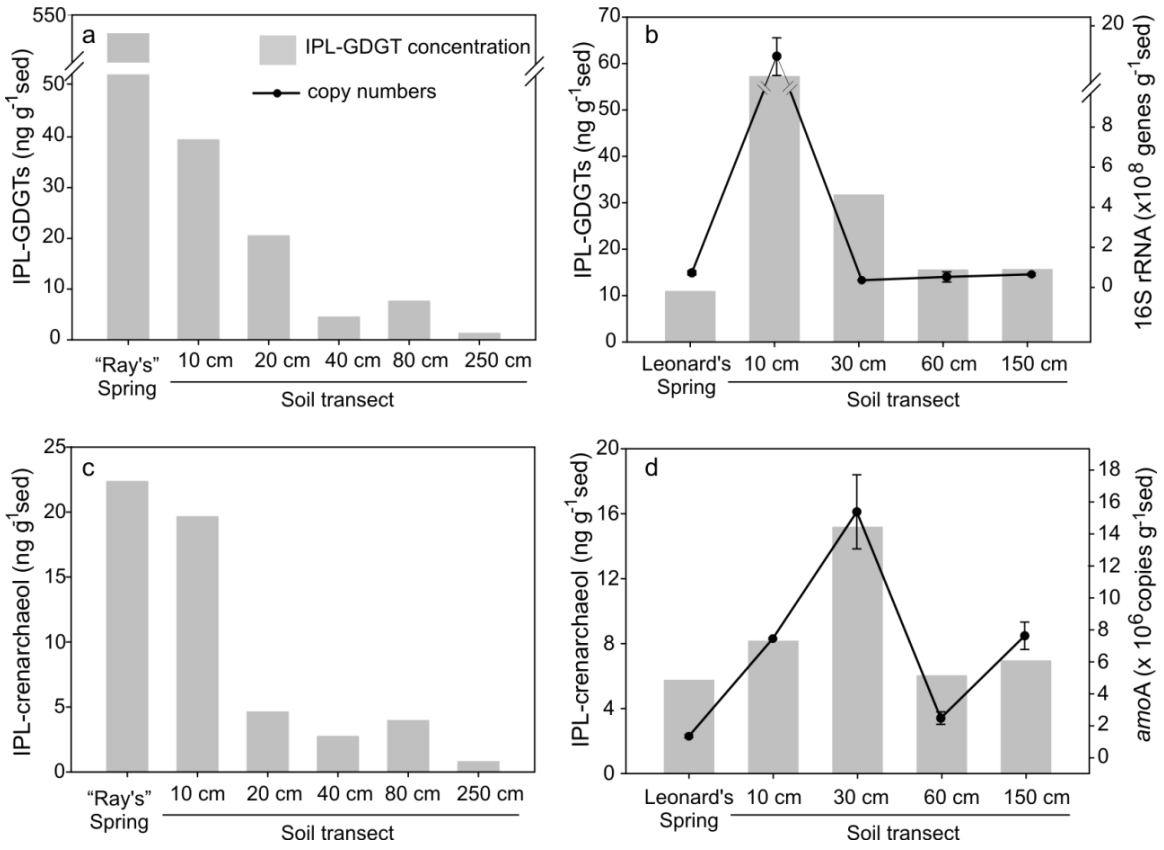

Figure 3. Total archaeal IPL-derived GDGTs (a, b) and IPL-derived crenarchaeol (c, d) from "Ray's" hot spring and Leonard's hot spring as well as the corresponding soil transects are shown as bars. Archaeal 16S rRNA gene (b) and archaeal *amoA* (d) copy numbers measured by qPCR from Leonard's site are shown as marked lines, with error bars (in some cases smaller than the symbol size) representing the standard deviation of copy numbers obtained from triplicate qPCR reactions

Table 1. Absolute concentrations of IPL- and CL-derived GDGTs for all hot spring and soil samples, IPL-estimated archaeal cell densities, and archaeal 16S rRNA and *amoA* gene copy numbers (for Leonard's spring only). "na" = not analyzed; "cren" = crenarchaeol. Numbers correspond to GDGT structures shown in Figure 1 (e.g. "0" = GDGT-0).

site	sample	distance from spring (cm)	Temp (°C)	pH	abundance of GDGTs (ng/g dry sample)															Estimated Archaeal cells/g	Archaeal 16S copies/g sed	*amoA* copies/g sed
					IPL								CL									
					0	1	2	3	4	cren	cren'	total	0	1	2	3	4	cren	cren'	total			
Leonard's	Hot spring	0	58	8.4	1.1	0.4	1.6	0.4	1.1	5.8	0.6	11	0.1	0.0	0.0	0.0	0.0	0.2	0.0	0.4	1.1E+07	7.3E+07	5.3E+05
	soil	10	36	8.6	9.7	18*	17*	1.9	2.1	8.2	0.6	22	4.4	0.9	0.8	0.7	1.2	5.8	0.2	14	5.7E+07	1.8E+09	7.1E+06
	soil	30	28	8.4	1.3	6.6	4.4	0.8	na	15	3.4	32	0.1	0.1	0.1	0.0	nd	0.0	0.0	0.5	3.2E+07	3.6E+07	1.6E+07
	soil	60	20	8.6	0.8	4.0	2.3	0.4	na	6.0	2.1	16	0.2	0.2	0.1	0.1	nd	2.9	0.3	3.9	1.6E+07	5.2E+07	1.8E+06
	soil	150	14	8.6	2.8	1.8	1.2	0.6	na	7.0	2.4	16	1.4	0.1	0.1	0.1	nd	1.3	0.3	3.2	1.6E+07	6.5E+07	7.3E+06
"Ray's"	hot spring	0	89	8.2	42	48	75	130	210	22	7.5	540	5.4	4.6	6.8	11	13	2.1	0.4	44	5.4E+08	na	na
	soil	10	42	7.0	4.8	0.9	1.8	1.9	7.6	20	2.9	40	2.0	0.8	2.7	2.2	5.8	16	0.9	31	4.0E+07	na	na
	soil	20	33	6.1	2.2	2.5	3.9	6.5	na	4.7	0.8	21	0.2	0.1	0.3	0.2	nd	3.5	0.3	4.5	2.1E+07	na	na
	Soil	40	25	6.9	0.4	0.2	0.4	0.3	na	2.8	0.4	4.6	0.2	0.1	0.2	0.1	nd	0.8	0.1	1.4	4.6E+06	na	na
	soil	80	20	7.0	1.6	1.1	0.7	0.3	na	4.0	0.0	7.7	0.2	0.1	0.1	0.1	nd	1.3	0.2	1.9	7.7E+06	na	na
	soil	250	12	7.7	0.2	0.0	0.1	0.1	na	0.8	0.1	1.4	0.1	0.0	0.0	0.0	nd	0.2	0.0	0.5	1.4E+06	na	na

pH of streamers near source sediment samples

na = not analyzed

* GDGTs 1 and 2 in Leonard's 10-cm soil are leading regioisomers (see Fig. 2e,g)

Distribution of monohexose-GDGTs

Analysis of the total Bligh and Dyer extracts with HPLC-APCI-MS using a newly described method (Pitcher et al., 2009) allowed for the detection of both the CL-GDGT and monohexose (MH)-derived GDGTs (Figure 1) in a single HPLC/MS run (Figure 4). The base peak chromatograms of each hot spring showed relatively low amounts of CL-GDGTs compared to MH-GDGTs which is in agreement with the higher concentrations of IPL-GDGTs compared to CL-GDGTs quantified after hydrolysis (Table 1). Crenarchaeol dominated both the CL and MH-GDGTs in Leonard's hot spring, whereas crenarchaeol represented a minor component of the CL and MH-GDGTs in "Ray's" hot spring. We did not quantify the MH-GDGTs directly due to lack of an authentic MH-GDGT standard and, therefore, cannot conclude what proportion of the IPL-GDGTs in our samples quantified after acid hydrolysis was comprised of IPLs with a MH moiety.

Archaeal 16S rRNA and *amoA* gene abundance at Leonard's hot spring

Archaeal 16S rRNA gene abundance in Leonard's hot spring was 7.3×10^7 copies g^{-1}. Similar gene abundances were found in the adjacent soils with the exception of soil at 10 cm distance, which had 1.8×10^9 copies g^{-1} (Table 1, Figure 3b). Archaeal *amoA* gene copy numbers ranged from 5.3×10^5 to 1.6×10^7 copies g^{-1}, with the lowest abundance in the hot spring, and the highest abundance observed in soil sampled 30 cm from the spring (Figure 3d). The *amoA* gene abundance decreased at 60 cm distance from the hot spring and increased again at 150 cm.

Discussion

Sources of GDGTs in hot springs and soils

The occurrence of GDGTs we observed in the hot springs is consistent with the known prevalence of thermophilic Archaea in these environments (Madigan et al., 2003; Chaban et al., 2006). They are likely sourced by a mixed archaeal community, as GDGTs 0 to 4 are synthesized in various relative abundances by many cultured representatives of

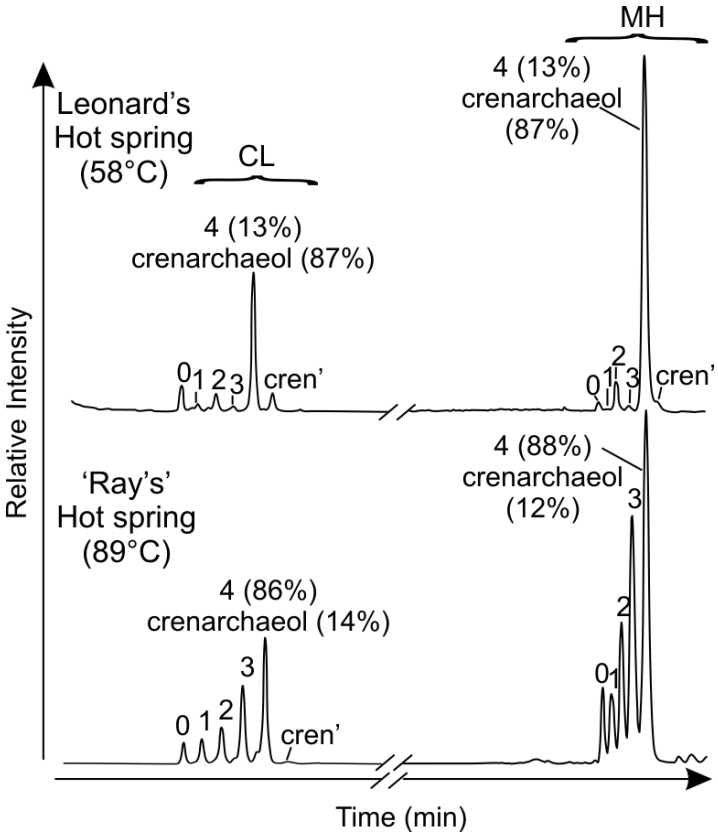

Figure 4. HPLC-APCI-MS base peak chromatograms of total Bligh and Dyer extracts showing the relative abundance of core lipid (CL)- and monohexose (MH)-derived GDGTs in each hot spring. Peak numbers and labels correspond to GDGT structures shown in Figure 1 (e.g. "0" = GDGT-0). Numbers in brackets next to GDGT-4 and crenarchaeol indicate the percent composition each in the labeled peak where these GDGTs co-elute.

(hyper) thermophilic Euryarchaeota and Crenarchaeota (Macalady et al., 2004; Schouten et al., 2007). For example, most cultured Euryarchaeota, including members of the orders Thermococcales (Sugai et al., 2004), Archaeoglobales (Thurl and Schafer, 1988), Methanosarcinales (Hoefs et al., 1997), Methanobacteriales (Langworthy and Pond, 1986), Methanococcales (Thurl and Schafer, 1988) and Methanomicrobiales (Sprott et al., 1994), produce only GDGT-0. Other Euryarchaeotes, however, such as Methanopyrales (Gattinger et al., 2002; van der Meer et al., unpublished results), and members of the ANME-1 (Blumemburg et al., 2004) and DHVE2 (Reysenbach et al., 2006) clusters, produce various amounts of GDGTs 0-4, while some members of Thermoplasmatales (Macalady et al., 2004) produce GDGTs with up to eight cyclopentane rings. Most cultured (hyper)thermophilic Crenarchaeota synthesize at least GDGTs 0-4 and some can also synthesize GDGTs with up to 8 cyclopentane moieties (c.f. Schouten et al., 2007). In contrast, cultivated representatives of the group I.1a Crenarchaeota, *N. maritimus* SCM1

(Könneke et al., 2005) and "*Candidatus* Cenarchaeum symbiosum" (Preston et al., 1996), group I.1b Crenarchaeota, "*Candidatus* Nitrosophaera gargensis" (Pitcher et al., unpublished results), and from the ThAOA/HWCG III cluster of the Crenarchaeota, "*Candidatus* Nitrosocaldus yellowstonii" (de la Torre et al., 2008), all synthesize substantial amounts of GDGT-0 and/or crenarchaeol with minor amounts of GDGTs 1-3 (Sinninghe Damsté et al., 2002; de la Torre et al., 2008; Schouten et al., 2008). It is therefore likely that GDGT 0-4 measured in this study have multiple sources and that crenarchaeol is derived predominantly from Crenarchaeota falling in the group I.1a, I.1b and ThAOA/HWCG III clusters of the Crenarchaeota.

The contrasting IPL-GDGT distributions in Leonard's and "Ray's" hot spring indicate significant differences in the resident archaeal communities. The IPL-GDGT distribution in Leonard's hot spring is similar to others observed for isolates of the group I.1a Crenarchaeota, whereas the IPL-GDGT distribution in "Rays" hot spring is similar to those observed in (hyper)thermophilic Crenarchaeota and some Euryarchaeota (e.g. Schouten et al., 2007; Pearson et al., 2008). The high temperature of "Ray's" (89°C) versus that of Leonard's hot springs (58°C) may be a substantial controlling factor in governing the differences in archaeal community composition, as Leonard's and "Ray's" hot springs were similar in pH and geographically very close.

For soils, the IPL-GDGT distributions are similar to those commonly observed for isolates of the group I.1a Crenarchaeota, except for the soil closest to Leonard's hot spring which shows unusually abundant early eluting isomers of GDGTs 1-2. GDGTs 1-2 are abundant components in ANME-1 Archaea (Pancost et al., 2001; Schouten et al., 2003; Blumenburg et al., 2004). Indeed, in contrast to the other soils, this soil was composed mainly of fine, clay-like material and had a sulfidic smell, indicating anoxic conditions which could support such a community (Table 1). However, no ANME-Archaea have been reported in a soil environment and the existence of such a community in this soil sample has yet to be identified.

Contributions of fossil and IPL GDGTs

The underlying presupposition to our work is that the IPL-GDGTs were derived from living biomass and thus produced *in situ*. This relies mainly on two assumptions: *(1)* that GDGTs in the hydrolyzed IPL fraction were not significantly contaminated by CL-GDGTs and *(2)* that GDGTs with intact polar head groups are only derived from living biomass. The first assumption is supported by the direct analysis of the MH-GDGTs, which confirmed the presence of GDGTs with polar head groups in our samples and that they are likely more abundant than those of CL-GDGTs (Figure 4). It should also be noted that MH-GDGTs represent only a part of the total IPL-GDGT pool, as Archaea are capable of synthesizing a variety of other head groups including dihexoses and phosphohexoses (Sturt et al., 2004; Koga and Morii, 2005; Schouten et al., 2008).

Support for our second assumption may come by estimating archaeal cell numbers from IPL-GDGT concentrations. If we assume that the average GDGT-synthesizing Archaeon in our samples is approximately the size of the thermophilic group I.1b crenarchaeote, "*Candidatus* Nitrosophaera gargensis" (0.9 ± 0.3 μm diameter cocci; ref. 11), and that there is ca. 1.5×10^7 GDGT molecules μm^{-2} in the archaeal membrane (Gabriel and Chong, 2000), the IPL-estimated archaeal cell concentrations for Leonard's site (Table 1) fall within an order of magnitude of archaeal 16S rRNA gene copies g^{-1} except the 10-cm soil sample (see below). We did not quantify all GDGTs that are known to be produced by Archaea, e.g., GDGTs with 5-8 cyclopentane rings, which could account for some of the observed offset between gene abundance and IPL-GDGT based cell estimates. Nevertheless, the good correspondence between IPL-GDGT based cell estimates and gene abundance supports our assumption that the IPL-GDGTs we quantified were indeed derived mostly from living Archaea.

In the 10 cm soil of Leonard's hot spring, the IPL-GDGT based cell estimate was ca. 31 times lower than the corresponding number of 16S rRNA gene copies (Table 1). Here, the highest abundance of both archaeal 16S rRNA genes and IPL-GDGTs were measured, however, this maximum represented an increase in archaeal 16S rRNA copy numbers from the spring to the 10 cm soil of ca. 25 fold compared to the corresponding increase in IPL-GDGT concentration of ca. 5 fold only. This discrepancy could be due to

the presence of a dominant microbial community consisting primarily, for example, of methanogens, which predominantly produce diglyceride dialkyl ether lipids (DGDs) such as archaeol and hydroxy-archaeol as their main membrane core lipids (Koga and Morii, 2005), which we did not measure. Indeed, this anomaly occurred in the soil with an IPL-GDGT distribution typical for ANME-1 Archaea (Figure 2e) and which appeared anoxic.

Absolute quantification of GDGTs from both CL and IPL-GDGT fractions (Table 1) shows that the contribution of fossilized GDGTs to the total GDGT pool (i.e. summed total of IPL- and CL-GDGTs) are as high as 40%. It is also important to note that this percentage varied widely among individual GDGTs (Figure 5). For both hot springs, the contribution of fossil GDGTs to the total pool was fairly low (ca. 3 – 8%) and relatively even among individual GDGTs. However, fossil GDGT contribution in the surrounding soils was substantially higher (up to 44%), and was more variable among individual GDGTs. For example, ca. 2% of the total crenarchaeol measured in Leonard's hot spring was derived from fossil material, whereas crenarchaeol extracted from the soil located 10 cm away from the hot spring contained over 40% fossilized material. Similarly in "Ray's" hot spring, ca. 9% of crenarchaeol was of fossil origin, whereas fossil crenarchaeol contributed 45% to the total crenarchaeol extracted from the nearest soil. Such a large variation in the fossil contribution to the GDGT distribution in soils and hot springs implies that, in general, analysis of GDGT distributions obtained from directly extracted CL- GDGTs or from CL-GDGTs present in acid hydrolyzed Bligh and Dyer IPL extracts where background CL-GDGTs have not first been removed, may be influenced by a substantial fossil signal and potentially skew the GDGT distribution of the living archaeal community, although the data show this effect appears to be expressed more strongly in soils than in the hot springs.

In situ production of crenarchaeol in hot springs and soils

Separate analysis of CL and IPL-GDGT fractions allowed us to evaluate whether or not the crenarchaeol found in our hot springs was produced *in situ*. In both Leonard's and "Ray's" hot spring we did find IPL-crenarchaeol (Figure 3c, d) and comparatively minor amounts of fossil crenarchaeol (Table 1), suggesting that crenarchaeol is indeed being synthesized *in situ* by (hyper)thermophilic Crenarchaeota and that allochtonous input is not

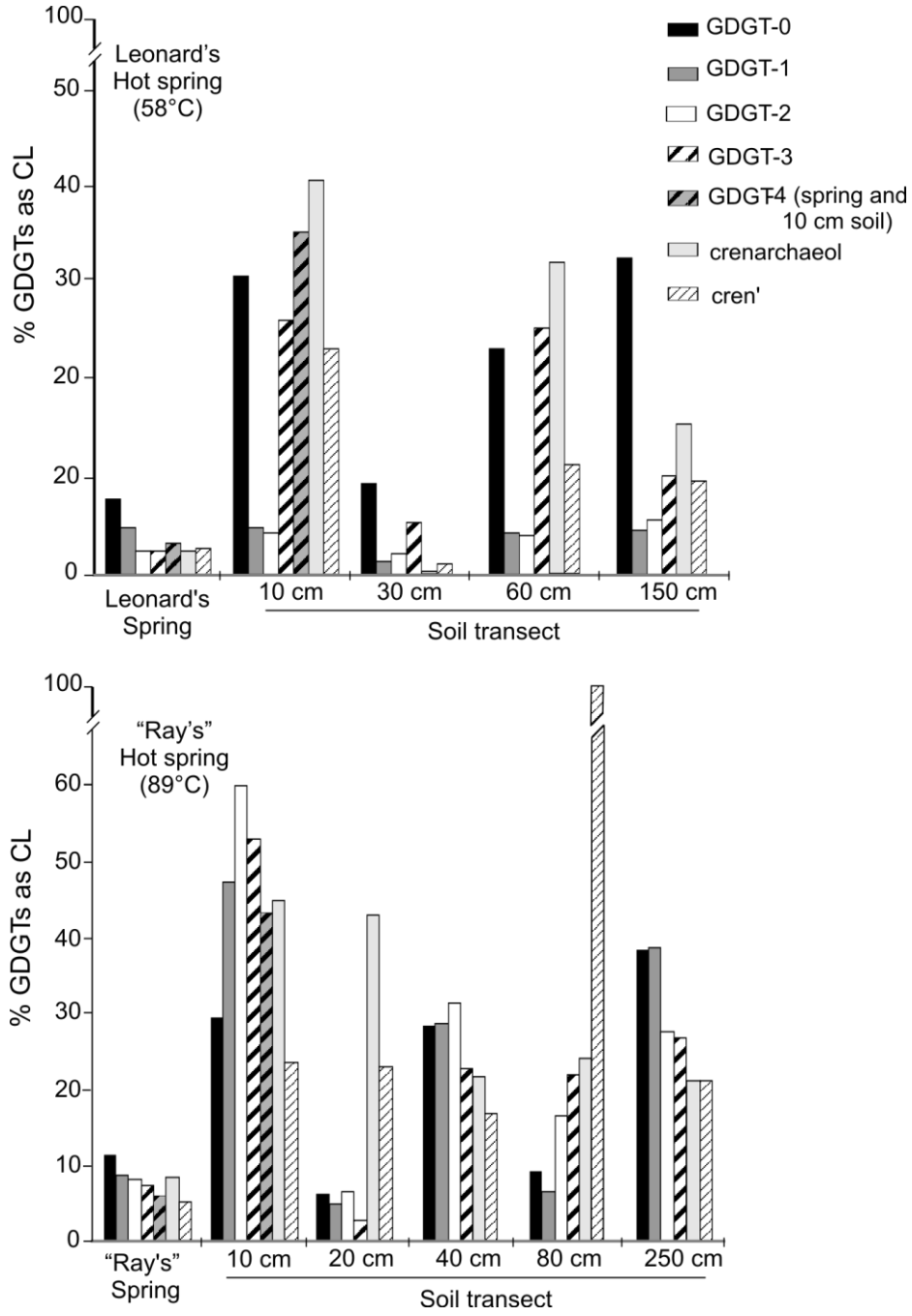

Figure 5. Contribution of fossilized GDGTs to the total pool in each hot spring and soil transect. Each shaded/patterned bar represents an individual GDGT isomer as indicated in the legend. GDGT-4 was only quantified for the hot spring and the 10-cm soil samples as it was nearly absent in the other soils.

important. This is supported by the direct analysis of our total Bligh and Dyer aliquots which confirmed the presence of MH-crenarchaeol (Figure 4). A monohexose moiety is a common head group synthesized by Archaea and has been identified as one of the forms of IPL-crenarchaeol in the cell membrane of *N. maritimus* SCM1 (Schouten et al., 2008). There are some differences between the relative distributions of MH-GDGTs and acid-hydrolyzed IPL-GDGTs (Figure 2), likely indicating the presence of IPL-crenarchaeol with multiple head groups. Indeed, *N. maritimus* SCM1 contains IPL-GDGTs with two hexose moieties and a phosphohexose moiety in addition to MH-GDGTs (Schouten et al., 2008).

The presence of IPL-crenarchaeol shows that crenarchaeol synthesis occurs in hot springs and soils over a wide temperature range (12 to 89ºC). Our findings corroborate those of others who have found crenarchaeol in hot springs (Pearson et al., 2004; Zhang et al., 2006; Schouten et al., 2007; Pearson et al., 2008; Reigstad et al., 2008) and soils (Leininger et al., 2006; Weijers et al., 2006), and are supported by work showing crenarchaeol is synthesized by enrichment cultures of ammonia-oxidizing (hyper)thermophiles (de la Torre et al., 2008, Pitcher et al., unpublished results). Further confirmation of *in situ* production of crenarchaeol comes from the analysis of archaeal *amoA*, the gene coding for a subunit of ammonia monooxygenase, which is the membrane-bound protein that catalyzes the rate limiting step in the biochemical process of ammonia oxidation. In general, we find that *amoA* copy numbers and IPL-crenarchaeol concentration follow similar patterns at Leonard's site (Figure 3d). This also further corroborates the hypothesis that crenarchaeol is predominantly derived from Crenarchaeota involved in ammonia oxidation (de la Torre et al., 2006).

Interestingly, there is not a significant relationship between the relative abundance of IPL-crenarchaeol and temperature, as might have been expected based on the results of previous studies in hot springs (Zhang et al., 2006) or in marine environments (Schouten et al., 2002). This is likely because the other GDGTs are sourced from a variety of Archaea and not only from specific groups of Crenarchaeota. Shifts in the relative abundance of GDGTs in such terrestrial environments are more likely to reflect changes in community structure than in the marine environment, where archaeal diversity is lower and environmental conditions are comparatively stable. There is, however, some

correspondence between IPL-crenarchaeol abundance and pH, i.e. the pH of "Ray's" hot spring is much more alkaline than the corresponding soil transect and there is a substantial decrease in IPL-crenarchaeol concentration between the spring and soils (Table 1). In contrast, the pH of Leonard's spring is quite similar to the surrounding soils, and IPL-crenarchaeol concentrations are quite similar as well. Our data set is too small to derive statistically significant relationships, however, these results compare well with those of Weijers et al. (2006) and Pearson et al. (2008), who also found a positive relationship between crenarchaeol and pH.

Our results, together with those reported previously (Pearson et al., 2004; Zhang et al., 2006; Schouten et al., 2007; Pearson et al., 2008), raise an intriguing question: Why is crenarchaeol specifically synthesized by selected groups of Crenarchaeota? The fact that all of the crenarchaeol-synthesizing Archaea cultured to date are also ammonia oxidizers suggests that crenarchaeol may be diagnostic for the ammonia-oxidizing Crenarchaeota. This is supported by the correspondence between archaeal *amoA* gene copy abundance and IPL-crenarchaeol concentrations (Figure 3d) implying that the crenarchaeol-synthesizing Archaea at our study sites may also be predominantly ammonia oxidizers. Nevertheless, we cannot yet exclude the possibility that crenarchaeol synthesis was an evolutionary trait that allowed (hyper)thermophilic Archaea to expand into temperate environments (Kuypers et al., 2002). It is possible that a group of mesophillic Crenarchaeota subsequently evolved back to hot environments and utilized alternative mechanisms for maintenance of cellular membrane integrity, while still producing crenarchaeol. Further studies on the biophysical properties of crenarchaeol are needed to elucidate its functional utility in the cell membranes of Crenarchaeota.

Conclusions

IPL-crenarchaeol derived from living Archaea was found in two hot Californian springs, indicating that crenarchaeol is synthesized *in situ* at high temperatures. The correspondence between IPL-crenarchaeol and archaeal *amoA* in Leonard's hot spring and surrounding soils further implies that the crenarchaeol-synthesizing Crenarchaeota were

predominantly ammonia-oxidizers. Quantification of both CL- and IPL-GDGTs showed that long-term accumulation of fossilized lipids may contribute substantially to the total lipid pool, especially in soils (up to 40%). These fossilized lipids may potentially mask relationships between biomarker distribution and microbial community structure.

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Chapter 4

Crenarchaeol dominates the membrane lipids of "*Candidatus* Nitrososphaera gargensis", a thermophilic Group I.1b Archaeon

Angela Pitcher[1], Nicolas Rychlik[2], Ellen C. Hopmans[1], Eva Spieck[2], W. Irene C. Rijpstra[1], Jort Ossebaar[1], Stefan Schouten[1], Michael Wagner[3], and Jaap S. Sinninghe Damsté[1]

[1] NIOZ Royal Netherlands Institute for Sea Research, Department of Marine Organic Biogeochemistry, P.O. Box 59, 1790 AB, Den Burg, The Netherlands

[2] University of Hamburg, Biocenter Klein Flottbek, Microbiology & Biotechnology, Ohnhorststrasse 18, D-22609 Hamburg, Germany

[3] University of Vienna, Department of Microbial Ecology, Althanstraße 14, A-1090 Wien, Austria.

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Abstract

Analyses of archaeal membrane lipids are increasingly being included in ecological studies as a comparatively unbiased complement to gene-based microbiological approaches. For example, crenarchaeol, a glycerol dialkyl glycerol tetraether (GDGT) with a unique cyclohexane moiety, has been postulated as biomarker for ammonia-oxidizing Archaea (AOA). Crenarchaeol has been detected in *Nitrosopumilus maritimus* and "*Candidatus*

Nitrosocaldus yellowstonii" representing two of the three lineages within the Crenarchaeota containing described AOA. Here, we present the membrane GDGT composition of "*Candidatus* Nitrososphaera gargensis", a moderately thermophilic AOA, and the only cultivated Group I.1b Crenarchaeon. At a cultivation temperature of 46°C, GDGTs of this organism consisted primarily of crenarchaeol, its regioisomer, and a novel GDGT. Intriguingly, "*Ca*. N. gargensis" is the first cultivated archaeon to synthesize substantial amounts of the crenarchaeol regioisomer, a compound found in large relative abundances in tropical ocean water and some soils, and an important component of the TEX_{86} paleothermometer. Intact polar lipid (IPL) analysis revealed that "*Ca*. N. gargensis" synthesizes IPLs similar to those reported for the Goup I.1a AOA, *Nitrosopumilus maritimus SCMI*, in addition to IPLs containing uncharacterized headgroups. Overall, the unique GDGT composition of "*Ca*. N. gargensis" extends the known taxonomic distribution of crenarchaeol synthesis to the Group I.1b Crenarchaeota, implicating this clade as a potentially important source of crenarchaeol in soils and moderately high temperature environments. Moreover, this work supports the hypothesis that crenarchaeol is specific to all AOA and highlights specific lipids which may prove useful as biomarkers for "*Ca*. N. gargensis"-like AOA.

Introduction

The importance of Group I Crenarchaeota (i.e. those not belonging to the well known class of Thermoprotei) in the biogeochemical cycling of nitrogen and carbon is becoming increasingly evident, as culture-independent studies reveal the ubiquity and potential activity of these organisms in nature (e.g. Francis et al., 2007; Beman et al., 2008; Prosser and Nicol, 2008 and references therein). Environmental analyses demonstrating the presence of abundant and diverse putative Group I Crenarchaeota-associated genes coding for 16S rRNA and the alpha subunit of ammonia monooxygenase (*amoA*) in the marine water column (Francis et al., 2005), estuarine sediments (Beman and Francis,2006), sponges (Steger et al., 2008), soils (Leininger et al., 2006), and hot springs (Zhang et al.,

2008), indicate that these prevalent Archaea are also predominantly ammonia oxidizers. Archaeal *amoA* copy numbers often exceed those of Bacteria, suggesting that Archaea may even dominate Bacteria in ammonia oxidation under certain conditions (e.g. Leininger et al., 2006; Wuchter et al., 2006; Mincer et al., 2007; Martens-Habbena et al., 2009). Despite their apparent ubiquity and potential ecological importance, culturing efforts in concert with these discoveries have resulted in the enrichment and characterization of only a few ammonia-oxidizing Archaea (AOA) to date, together representing three phylogenetic lineages (Könneke et al., 2005; de la Torre et al., 2008; Hatzenpichler et al., 2008). The recent enrichment of "*Candidatus* Nitrososphaera gargensis" (Hatzenpichler et al., 2008) has demonstrated the capacity for ammonia oxidation by moderately-thermophilic Archaea and extended this capability to the Group I.1b Crenarchaeota, a clade comprised predominantly of 16S rRNA gene sequences recovered from soils.

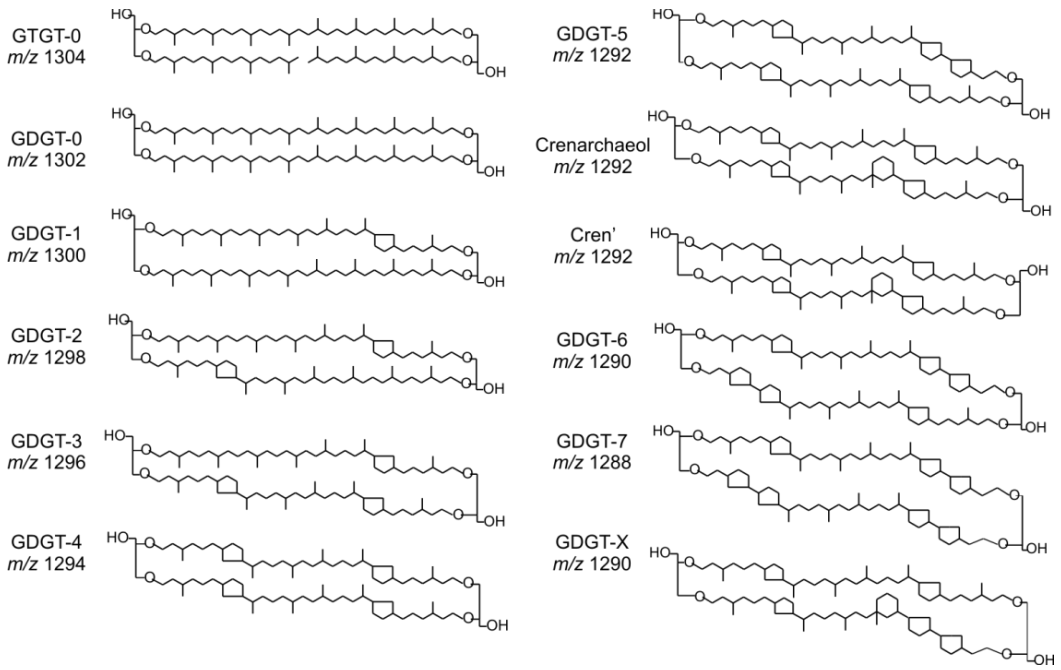

Figure 1. Structures analyzed in this study: glycerol trialkyl tetraether (GTGT)-0, glycerol dialkyl glycerol tetraethers (GDGTs) 0-7 including crenarchaeol, the crenarchaeol regioisomer (cren'), and the novel GDGT-X.

The core (i.e. apolar) component of crenarchaeal cellular membrane lipids, in general, is dominated by glycerol dialkyl glycerol tetraethers (GDGTs) (e.g. Figure 1), which may contain multiple cyclopentane moieties (see overviews in Macalady et al., 2004; Schouten et al., 2007). For a time, a unique GDGT, crenarchaeol – which contains a cyclohexane moiety in addition to its four cyclopentane moieties (Figure 1) – was observed in natural samples but not in crenarchaeal cultures until its formal characterization from GDGTs extracted from a co-culture of *Cenarchaeum symbiosum* and the marine sponge *Axinella mexicana* (Sinninghe Damsté et al., 2002). Consequently, it was proposed that the crenarchaeol found so ubiquitously in nature originated from mesophilic Crenarchaeota, a hypothesis supported by confirmation of crenarchaeol synthesis by the marine Group I.1a Crenarchaeon, *Nitrosopumilus maritimus* SCM1 (Schouten et al., 2008).

Since then, however, crenarchaeol recovery from hot springs (Pearson et al., 2004; Zhang et al., 2006; Pitcher et al., 2009) and confirmation of crenarchaeol synthesis by the ammonia-oxidizing thermophile (growth temperature of 72°C), "*Ca.* N. yellowstonii" (ThAOA/HWCG III) (de la Torre et al., 2008), has shown that crenarchaeol is also synthesized by non-mesophilic Crenarchaeota, effectively negating the original hypothesis. Instead, a number of studies have linked the presence of putative AOA to the occurrence of crenarchaeol (Wuchter et al., 2004; Coolen et al., 2007; Schouten et al., 2007). The significant correlation between GDGT concentrations (including crenarchaeol) and Crenarchaeota Group I.1b *amoA* copy numbers in soils, further indicated that Group I.1b AOA specifically may also synthesize crenarchaeol (Leininger et al., 2006). Taken together, these findings suggest that crenarchaeol is a specific biomarker for AOA (de la Torre et al., 2008).

In addition to crenarchaeol, the regioisomer of crenarchaeol (cf. Sinninghe Damsté et al., 2002) is also abundant in certain settings, particularly in tropical marine environments (Schouten et al., 2002; Kim et al., 2008). However, none of the previously enriched or isolated Crenarchaea produce substantial amounts of the crenarchaeol regioisomer, despite producing crenarchaeol (Sinninghe Damsté et al., 2002; Wuchter et al., 2005; Schouten et al., 2007; de la Torre et al., 2008; Schouten et al., 2008). This discrepancy is also important to investigate as the regioisomer of crenarchaeol is used,

together with several other GDGTs, in the TEX_{86} paleothermometer (Schouten et al., 2002) (Figure 1). The TEX_{86} has been shown to correlate well with *in situ* temperatures in sea water enrichment cultures (Wuchter et al., 2004; Schouten et al., 2007), as well as the marine water column (Wuchter et al., 2005). However, a calibration of the TEX_{86} by cultivation efforts has not been possible due to the near-lack of the regioisomer in cultures compared to relative abundances observed in natural environments at similar temperatures (cf. Schouten et al., 2007). Through analysis of the core GDGT and intact polar lipid (IPL) GDGT composition of "*Ca.* N. gargensis", we have been able to further investigate the phylogenetic distribution of crenarchaeol and its regioisomer in AOA. In addition, we have analyzed lipids of "*Ca.* N. gargensis" cultivated at three growth temperatures to determine whether slight temperature differences result in modification of its GDGT distribution.

Materials and Methods

Culture conditions

The ammonia-oxidizing enrichment culture of "*Candidatus* Nitrososphaera gargensis" (Hatzenpichler et al., 2008) was grown in mineral medium modified from Krümmel and Harms containing KH_2PO_4 (0.4 mM), KCl (1 mM), $MgSO_4$ (0.2 mM), $CaCl_2$ (1 mM) and NaCl (10 mM). One ml trace element solution (Ehrich et al., 1995) and a small amount of cresol red were added to the final medium. In contrast to the previous protocol (Hatzenpichler et al., 2008), the substrate concentration was reduced to 0.5 mM NH_4Cl from 2.0 mM. 4.5 l of medium was prepared in 5 l bottles and inoculated with 50 ml of an active preculture. Incubation was performed in the dark at 46°C for 3 weeks with moderate stirring (150 rpm). The pH was adjusted to 7.8 and kept constant by daily titration using 15% (w/v) $NaHCO_3$. The consumption of ammonia was regularly measured by test sticks (Merck) followed by replenishing the substrate with a 5 mM sterile stock solution. In total, 3-4 mM NH_4Cl were oxidized before cells were harvested by centrifugation (14000 g) and washed in 0.9% NaCl. Several 5-l bottles were collected, and the pellet was stored at -20°C until final analysis. In the enrichments used in this study, a single archaeal OTU was present, which showed 98-99% 16S RNA gene sequence

similarity to "*Ca*. N. gargensis" (Hatzenpichler et al., 2008). In addition, the enrichment also contained a betaproteobacterium and possibly several other Bacteria (Hatzenpichler et al., 2008). Cells for the temperature experiment were grown in 3-l flasks with 1.5 l medium without stirring.

Core lipid analysis

Acid hydrolysis was performed on freeze-dried biomass of cultivated "*Ca.* Nitrososphaera gargensis" to cleave polar headgroups. Biomass was refluxed in 2 ml of 5% HCl in MeOH for 3 h. The cooled solution was adjusted to pH 5 with 2N KOH:MeOH (1:1, v/v). Bidistilled water was added to a final ratio of H_2O:MeOH (1:1, v/v) and this mixture was washed 3 times with DCM. The DCM fractions were collected and dried over Na_2SO_4. The extract was dissolved in hexane:propanol (99:1 v/v) filtered over a 0.4 µm PTFE filter before analysis by high performance liquid chromatography atmospheric pressure chemical ionization mass spectrometry (HPLC-APCI/MS).

HPLC-APCI/MS analysis

Archaeal GDGTs were analyzed using a modified procedure from Hopmans et al. (2000) and Schouten et al. (2007). Archaeal GDGTs were detected with full scan analysis from *m/z* 900-1400 and the relative abundance determined by integration of the peak areas of their $[M+H]^+$ and $[M+H+1]^+$ ions.

Identification of a novel GDGT-'X'

Individual GDGTs of "*Ca.* N. gargensis" were isolated by semi-preparative HPLC according to the procedure described by Smittenberg et al. (2002). The fraction enriched in GDGT-X was subjected to ether bond cleavage as described by Hoefs et al. (1997) and analyzed by gas chromatography/mass spectrometry (GC/MS) for biphytanes. GC/MS analysis was performed on a Thermofinnigan TRACE gas chromatograph equipped with a fused silica capillary column (25 m × 0.32 mm) coated with CP Sil-5 (film thickness 0.12 µm) and helium as the carrier gas. Sample was dissolved in hexane and injected at 70°C. Subsequently the oven was programmed to increase to 130°C at 20°C min^{-1} and then to

320°C at 4°C min^{-1}, where it was held for 10 min. The gas chromatograph was coupled with a Thermofinnigan DSQ quadrupole mass spectrometer with an ionization energy of 70 eV scanning a mass range of *m/z* 50-800 at three scans s^{-1}.

Intact Polar Lipid analysis

Intact polar lipids were extracted from freeze-dried biomass using a modified Bligh and Dyer technique (Bligh and Dyer,1959). A known volume of single-phase solvent mixture of methanol (MeOH):dichloromethane (DCM):phosphate buffer (2:1:0.8, v/v/v) was added to the sample in a centrifuge tube and placed in an ultrasonic bath for ten min. The extract and residue were separated by centrifuging at 2500 rpm for 5 min and the solvent mixture collected in a separate flask (3 times). The DCM and phosphate buffer were added to the single-phase extract to give a new ratio of MeOH:DCM:phosphate buffer (1:1:0.9, v/v/v), and to induce phase separation. The extract was centrifuged at 2500 rpm for 5 min. DCM phase was collected in a round-bottom flask and the methanol:phosphate buffer phase was washed two additional times with DCM. The combined DCM phases were reduced under rotary vacuum and evaporated to dryness under a stream of N_2. Residual biomass was re-extracted using 5% trichloroacetic acid instead of phosphate buffer according to Sturt et al. (2004), and analyzed separately. This extract contained the same IPLs identified in the first extraction but in much lower amounts.

HPLC-Electrospray Ionization (ESI)/MS

IPL-GDGTs were analyzed according to conditions described previously (Schouten et al., 2008) as modified from Sturt et al. (2004). For the analysis an Agilent (Palo-Alto, CA, US) 1100 series LC equipped with a thermostatted auto-injector was coupled to a Thermo TSQ Quantum EM triple quadrupole mass spectrometer equipped with an Ion Max source with ESI probe. Detection was achieved using positive ion ESI/MS by scanning mass range *m/z* 1000-2000.

Results

Core lipids

HPLC-APCI/MS analysis of the GDGTs released from acid-hydrolyzed whole-cell biomass from "*Ca.* Nitrososphaera gargensis", grown at its optimal growth temperature of 46°C, revealed that it produces predominantly crenarchaeol and the crenarchaeol regioisomer (Figure 2b). GDGTs 0-4 were also present, but in very low abundances relative to crenarchaeol and its regioisomer (Table 1). In addition, a GDGT with a $[M+H]^+$ of *m/z* 1290 (i.e., six rings or double bonds) was present, which eluted earlier than GDGT-6 known to occur in (hyper)thermophilic Archaea, suggesting that it represented a novel core membrane lipid (henceforth referred to as 'GDGT-X').

Tentative identification of GDGT-X was performed after isolation with semi-preparative HPLC and treatment with $HI/LiAlH_4$ to release the carbon skeletons of the GDGT. GC/MS analysis revealed the presence of two C_{40} isoprenoidal hydrocarbons present in approximately equal abundance, indicating that together they comprised the intact GDGT-X. The first eluting isoprenoid had a mass spectrum and retention time identical to that of a biphytane with two cyclopentane moieties (de Rosa and Gambacorta,1988; Hoefs et al., 1997; Schouten et al., 1998), with characteristic fragment ions at *m/z* 97, 125, 165, and 194, and an M^+ ion of *m/z* 558 (Figure 3a). The second eluting compound had a mass spectrum with fragment ions at *m/z* 95, 151, 163, and 261 and a fragment of *m/z* 539, likely corresponding to an M^+-15 ion (Figure 3b). The latter would be in agreement with a biphytane carbon skeleton containing 4 cyclic moieties; the mass spectrum of this compound is quite similar to that of the biphytane containing 2 cyclopentane moieties and a cyclohexane moiety present in crenarchaeol (Schouten et al., 1998), except that the fragment ions 165 and 263 are now two Daltons lower. These fragments would be consistent with the same biphytane carbon skeleton present in crenarchaeol but containing an additional cyclopentane moiety at the end of the alkyl chain. Indeed, it is well known that hyperthermophilic Crenarchaeota make cyclopentane moieties at this position of the biphytane carbon skeleton when grown at high temperatures (de Rosa and Gambacorta et al., 1988). Based on this, the structure of GDGT-X was tentatively

identified as being nearly identical to that of crenarchaeol but with an additional cyclopentane moiety (Figure 1).

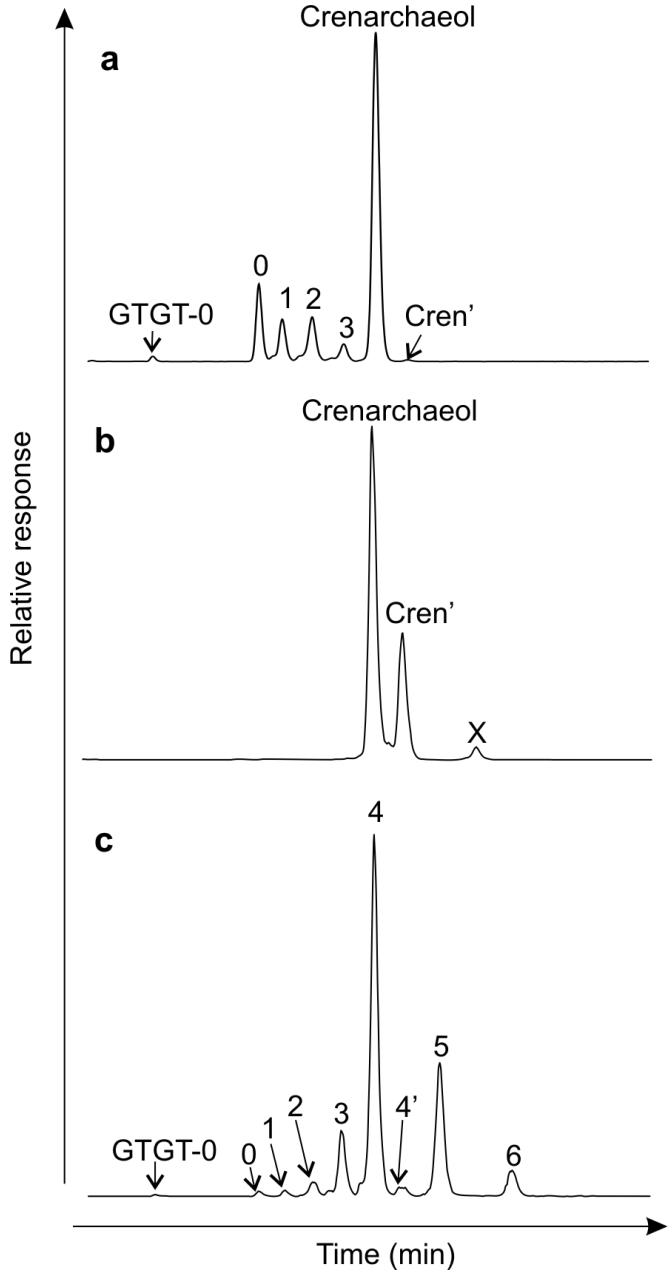

Figure 2. HPLC-APCI base peak chromatograms showing GDGTs derived from acid-hydrolyzed biomass of (a) *Nitrosopumulis maritimus* SCM1 (Schouten et al., 2008), (b) "*Ca*. Nitrososphaera gargensis" grown at 46°C, and (c) *Sulfolobus solfataricus* P2 strain DSM1617 (Ellen et al., 2009). Peak labels: GTGT-0 is labeled as such; other peaks follow GDGT designations according to Fig. 1 (e.g. "1" = GDGT-1).

To test whether "*Ca.* N. gargensis" would adjust its GDGT composition when grown at different temperatures, media was inoculated with the original enrichment culture and incubated at 42°C, 46°C, and 50°C for three weeks. Acid hydrolysis of biomass harvested during the end of logarithmic phase growth revealed that at all three temperatures, crenarchaeol and its regioisomer remained the most abundant GDGTs, with significant amounts of GDGT-X. There were also notable amounts of GDGT-4 and an unknown isomer of GDGT-4 in the 46°C culture, which were less abundant in the original enrichment culture. Furthermore, at all growth temperatures, "*Ca.* N. gargensis" continued to produce only minor amounts of GDGTs 0-3, each comprising ≤ 2% of total GDGTs, and barely detectable amounts of GTGT-1 and GDGT-6 (data not shown). The relative abundances of GDGTs 0-3 were slightly higher at 42°C than at 46° and 50°C. In contrast, the relative abundances of GDGTs 4 and X, were highest at 46°C (Table 1).

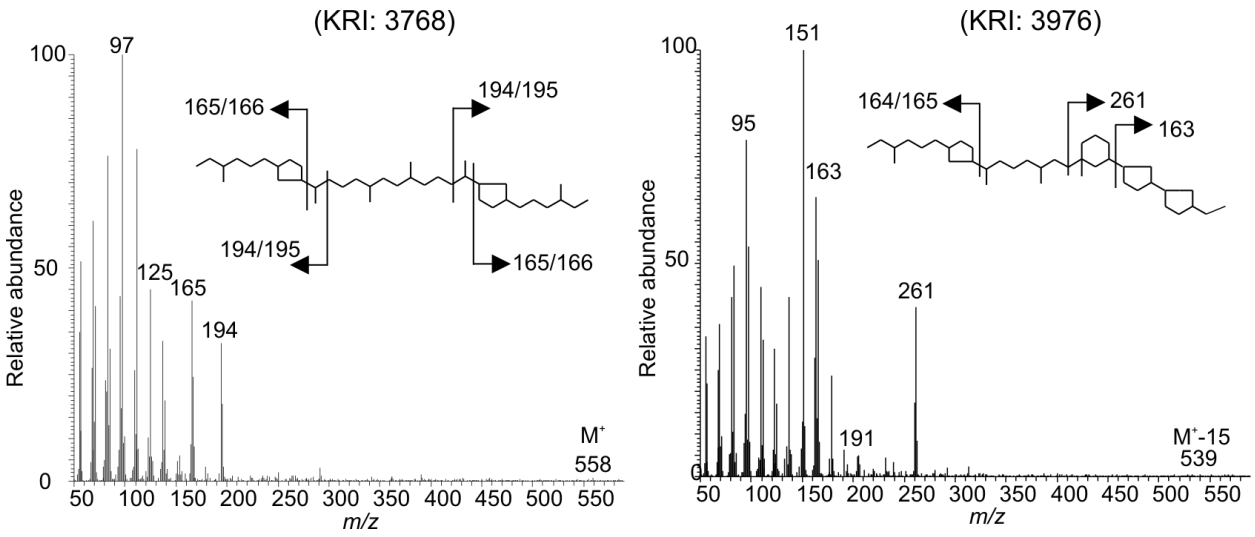

Figure 3. Mass spectra corresponding to biphytanes released from a prepared GDGT fraction enriched in GDGT-X after treatment with HI/$LiAlH_4$. Kirchman Retention Indices (KRI) are indicated in brackets.

Table 1 Relative abundance of GDGTs recovered from acid-hydrolyzed biomass of the original enrichment culture material, and "*Ca* Nitrososphaera gargensis" grown at 42°C, 46°C, and 50°C. GDGT numbers correspond to structures shown in Figure 1.

growth temperature (C°)	GDGT relative abundance									TEX_{86}	TEX_{86}-temp (C°)
	0	1	2	3	4	4'	cren	cren'	X		
46[1]	0.10	0.12	0.15	0.32	2.2	6.2	65	21	4.5	0.994	45.1
42	0.32	0.38	0.47	1.0	1.1	2.1	69	24	1.6	0.985	44.6
46	0.12	0.14	0.17	0.38	2.0	8.3	65	19	4.2	0.993	45.1
50	0.10	0.13	0.17	0.38	1.5	4.1	70	21	2.3	0.994	45.1

Abbreviations: GDGT, glycerol dialkyl glycerol tetraether; TEX_{86}, TetraEther Index of tetraethers consisting of 86 carbon atoms; cren, crenarchaeol; cren', crenarchaeol regioisomer. [1] original enrichment culture used to innoculate media incubated at 42°C, 46°C, and 50°C (below).

Intact Polar Lipids

HPLC-ESI/MS analysis of the Bligh and Dyer extract of "*Ca.* N. gargensis" showed four peaks with single molecular ions and two major peak clusters with IPLs that were not baseline separated (Figure 4). Based on their mass spectra, resulting from in-source fragmentation, six IPLs were tentatively identified (Table 2, Figure 4).

The first peak (1) was identified as crenarchaeol with a glycosidically bound hexose headgroup (Figure 4). The dominant ions included 1454, 1471, and 1476, representing the $[M+H]^+$ of the intact polar lipid, ammonium ($[M+NH_4]^+$), and sodium ($[M+Na]^+$) adducts, respectively. An additional dominant ion at *m/z* 1292 indicated that crenarchaeol or the crenarchaeol regioisomer was the core GDGT of this polar lipid.

The second peak (2) appeared to consist of a single IPL with a protonated molecule at *m/z* 1629 Daltons ($[M+NH_4]^+$ and $[M+Na]^+$ at *m/z* 1647 and 1652, respectively) and fragments at *m/z* 1292 and 1454 corresponding to crenarchaeol and crenarchaeol with a monohexose group. This suggests that this IPL is a monohexose crenarchaeol with an additional but unknown headgroup of 176 Daltons, however it was not possible to determine the structure of this additional headgroup based on mass spectrometry. Similar intact polar GDGTs with a headgroup of 176 Daltons have, to the best of our knowledge, not yet been reported elsewhere. The molecular weight of the headgroup is 14 Daltons higher than that of a hexose moiety, suggesting that it is possibly a methylated hexose moiety. However, further identification by isolation and NMR techniques is needed to confirm this hypothesis.

Most peaks in the cluster containing peak 3 (indicated by stars, Figure 4) contained ions with *m/z* ratios below 1200 in their mass spectra, suggesting that they did not contain GDGTs as core lipids. This was not surprising since the culture was an enrichment containing multiple species of Bacteria in addition to "*Ca*. N. gargensis". The GDGT-based IPL eluting in Peak 3 itself gave a mass spectrum identical to that described previously for diglycosidic GDGTs (Schouten et al, 2008), with $[M+H]^+$, $[M+NH_4]^+$ and $[M+Na]^+$ ions corresponding to *m/z* values of 1616, 1633, and 1638, respectively. Most peaks in the next eluting cluster (Figure 4) consisted mainly of unknown compounds, again unrelated to GDGTs as they had fragment ions all below 1200. The IPL eluting in peak 4, with an

$[M+H]^+$ at *m/z* 1792, had a mass spectrum suggestive of diglycosidic crenarchaeol with an additional unknown headgroup of 176 Daltons. Likely, this unknown headgroup is the same as that found for the IPL represented by peak 2.

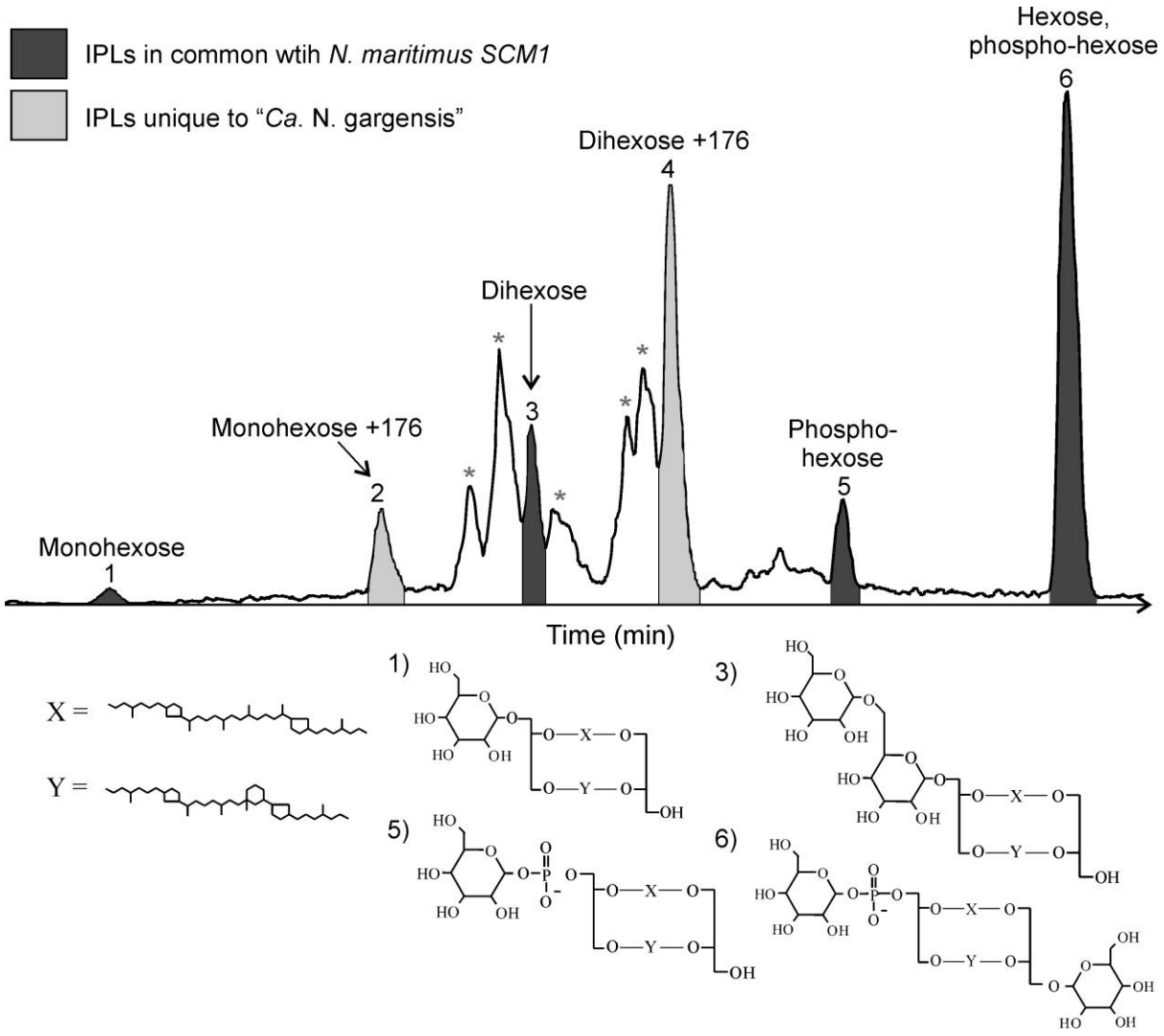

Figure 4. HPLC-ESI/MS base peak chromatogram of intact polar lipids extracted from "*Ca*. Nitrososphaera gargensis". Numbered peaks correspond to structures described in Table 2 and tentative structures drawn below the chromatogram (with the exception of IPLs eluting in peaks 2 and 4, which were not drawn due to the unknown nature of the 176-

Dalton moiety). Stars indicate unknown compounds with fragment ions below *m/z* 1200 (i.e. not GDGT-based).

The mass spectrum of the IPL eluting in peak 5 (Figure 4) is in agreement with that of phospho-hexose crenarchaeol based on the *m/z* values of 1534, 1556, and 1578, which corresponded to the $[M+H]^+$, $[M+NH_4]^+$ and $[M+Na]^+$ ions, respectively (Table 2). This compound has been reported in *N. maritimus* SCM1 . Finally, the IPL in the last eluting peak (6) (Figure 4) had a mass spectrum suggestive of crenarchaeol with a glycosidically bound hexose headgroup and a phospho-hexose headgroup with *m/z* values of 1696, 1713, and 1718 corresponding to the $[M+H]^+$, $[M+NH_4]^+$ and $[M+Na]^+$ ions, respectively (Figure 3, Table 2). This IPL has also been described previously for *N. maritimus* SCM1 (Schouten et al., 2008).

Table 2. Summary of the main ions in the mass spectra of the intact polar lipids of "*Ca* . Nitrososphaera gargensis" shown in Figure 4.

peak	fragments	$[M+H]^+$	$[M+NH_4]^+$	$[M+Na]^+$	headgroup
1	1292	1454	1471	1476	monohexose
2	1292, 1454	1630	1647	1652	monohexose+176
3	1292	1616	1633	1638	dihexose
4	1292, 1454	1792	1809	1814	dihexose+176
5	none	1534	1556	1578	phospho-hexose
6	1534	1696	1713	1718	hexose, phospho-hexose

Discussion

Phylogenetic occurrence of crenarchaeol

Our results show that "*Candidatus* Nitrososphaera gargensis", a moderately thermophilic, ammonia-oxidizing Group I.1b Crenarchaeon, synthesizes crenarchaeol as its

main core membrane GDGT. This expands the phylogenetic distribution of crenarchaeol synthesis to include, in addition to the marine Group I.1a Crenarchaeota (Sinninghe Damsté et al., 2002; Schouten et al., 2008) and the ThAOA/HWCG III clade (de la Torre et al., 2008), the soil Group I.1b Crenarchaeota, which all belong to the monophyletic lineage of AOA recently described by Prosser and Nicol (2008) (cf. Figure 5). In addition, our findings confirm previous circumstantial evidence for crenarchaeol synthesis by Group I.1b Crenarchaeota based on the ubiquitous presence of crenarchaeol in soils (Weijers et al., 2006), and the significant correlation linking ammonia-oxidizing soil Archaea to crenarchaeol synthesis (Leininger et al., 2006). Our results signify that all cultivated representatives of Group I Crenarchaeota to date synthesize crenarchaeol, thus providing support for the suggested specificity of crenarchaeol to Archaea involved in ammonia oxidation (de la Torre et al., 2008). Crenarchaeol has also been detected at sites of archaeal ammonia oxidation in the marine water column of the coastal North Sea (Wuchter et al., 2006), the Black Sea (Coolen et al., 2007), Icelandic hot springs (Reigstad et al., 2008), Nevada and California hot springs (Pearson et al., 2004; Zhang et al., 2006; Pitcher et al., 2009) and agricultural soils (Leininger et al., 2006). Although Wuchter et al. (2006) and Leininger et al. (2006) found a strong correlation between crenarchaeol abundance and *amoA* copy numbers, due to a lack of cultured representatives from Group I Crenarchaeota falling outside of the recognized AOA it cannot yet be unambiguously concluded that crenarchaeol synthesis is actually restricted to AOA.

Our results differ from those of *N. maritimus* and other AOA enrichment cultures in that the GDGT composition of "*Candidatus* Nitrososphaera gargensis" is dominated by crenarchaeol and the crenarchaeol regioisomer (cf. Figure 2a, b). All previously analyzed AOA contained substantial amounts of other GDGTs in addition to crenarchaeol, most notably GDGT-0 in *N. maritimus* SCM1 (Schouten et al., 2008) and *C. symbiosium* (Sinninghe Damsté et al., 2002), and large amounts of glycerol trialkyl glycerol tetraethers (GTGTs) synthesized by "*Ca*. N. yellowstonii" (de la Torre et al., 2008). These large differences in tetraether distribution between the different organisms may be due to the different optimal growth temperatures of individual isolates/enrichment cultures, which range from 28°C for *N. maritimus* SCM1 to 72°C for "*Ca*. N. yellowstonii". Indeed,

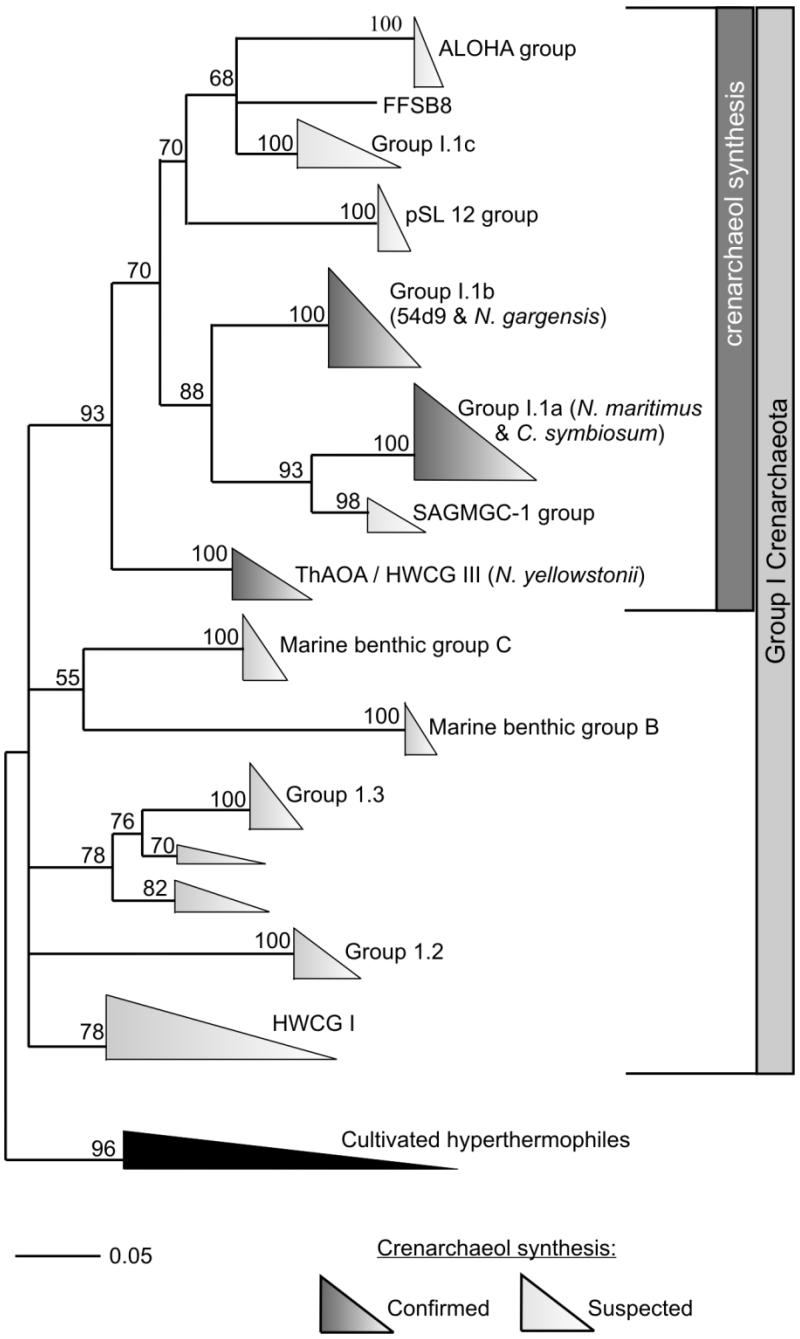

Figure 5. 16S rRNA gene-based phylogeny of the Crenarchaeota redrawn from Prosser and Nicol (2008), highlighting confirmed and suspected crenarchaeol synthesizers within the 'Group I' Crenarchaeota, based on the potential widespread distribution of archaeal ammonia-oxidization within this monophyletic clade and its hypothesized link to crenarchaeol synthesis.

cultivation of "*Ca*. N. gargensis" at temperatures lower than its optimal growth temperature resulted in a slight increase in GDGT-0. Alternatively, these differences in composition may reflect genetic differences affecting membrane lipid biosynthesis between the phylogenetic clusters I.1a, I.1b and ThAOA/HWCG III. The later hypothesis implies that differences in the Crenarchaeota community composition between environmental samples may have a notable effect on the composition of environmental GDGT assemblages.

Specific GDGTs for phylogenetic clusters in Group I Crenarchaeota

The core lipids of "*Ca*. N. gargensis" contain a unique GDGT-X (Figure 1, Figure 2). The addition of a cyclopentane moiety is homologous to thermophilic temperature adaptations observed in other cultivated hyperthermophiles (e.g. *Sulfolobus*) which increase the number of cyclopentane rings in their GDGTs as temperature increases. Indeed, this GDGT is not found in the mesophilic *N. maritimus* SCM1, but it was also not reported to be present in the thermophilic "*Ca*. N. yellowstonii" by de la Torre et al. (2008). The relative amount of GDGT-X also did not increase when "*Ca*. N. gargensis" was grown at higher temperatures. Thus, GDGT-X may be unique to (thermophilic) Group I.1b Crenarchaeota rather than an adaptation to elevated growth temperature.

"*Ca.* N. gargensis" also synthesizes two IPL headgroups containing moieties corresponding to 176 Daltons (Figure 4, peaks 2 and 4). To the best of our knowledge, such a moiety has not been reported yet for cultures or in natural environments. Schouten et al. (2008) reported IPLs with an unknown headgroup of 180 Daltons in *N. maritimus* SCM1, which is not present in "*Ca*. N. gargensis". On the other hand, "*Ca.* N. gargensis" also synthesizes some IPLs with headgroups identical to those synthesized by *N. maritimus* SCM1 (Schouten et al., 2008); IPLs with a monohexose, dihexose and hexose-phosphohexose moieties (Figure 4) are common to both species, indicating that these IPLs may become useful biomarkers for broad AOA screens. Direct detection of some crenarchaeal IPLs (i.e. containing crenarchaeol) has been achieved in deep sea sediments using HPLC-ESI/MS (Schouten et al., 2008). They found IPLs containing crenarchaeol with hexose and dihexose moieties but not with a phospo-hexose moiety that is present in

both *N. maritimus* SCM1 and "*Ca.* N. gargensis". Interestingly, they also found GDGTs with an unknown headgroup of 180 Daltons, similar to that synthesized by *N. maritimus* SCM1. This might indicate a specific contribution of Group I.1a Crenarchaeota in deep sea sediments, although they did not recover any Group I.1a Crenarchaeal 16S rRNA gene sequences from their clone libraries. An alternative explanation is that archaeal members derived from, for example, Marine Benthic Group B, which were well-represented in those recovered sequences, synthesize similar IPL headgroups and possibly crenarchaeol. Again, cultivated representatives of sedimentary Archaea are needed to confirm this.

Distribution and occurrence of the crenarchaeol regioisomer

The crenarchaeol regioisomer has, until now, been reported in very low (i.e. <5 % of total GDGTs) amounts in cultured/enriched Group I Crenarchaeota representatives. Neither *N. maritimus* nor *C. symbiosum* synthesize substantial amounts of the regioisomer in culture (Sinninghe Damsté et al., 2002; Schouten et al., 2008), and only trace amounts were observed in "*Ca.* Nitrosocaldus yellowstonii" when grown at 72°C (de la Torre et al., 2008). Similarly, only small amounts of the regioisomer were found in marine Group I.1a archaeal enrichments obtained from the North Sea (Wuchter et al., 2004) and the Indian Ocean (Schouten et al., 2007) when cultivated over a range of temperatures (5-35°C and 25-40°C, respectively). Trace amounts of crenarchaeol regioisomer detected in these cultures and enrichments show that these Group I Crenarchaeota are indeed capable of synthesizing the crenarchaeol regioisomer, however only appear to do so to a small degree.

Despite its limited production in cultured Crenarchaeota until now, the crenarchaeol regioisomer is abundant in some natural environments, particularly in tropical marine waters and sediments as well as in lake sediments, though its abundance rarely exceeds ca. 15% of the corresponding crenarchaeol concentration (Schouten et al., 2002; Wuchter et al., 2005; Kim et al., 2008. In some tropical soils, however, the relative abundance of the crenarchaeol isomer is much higher and approaches ca. 40% of the local crenarchaeol concentration (Sinninghe Damsté et al., unpublished results; Weijers et al., 2006). Members of the soil Group I.1b crenarchaeota with a GDGT composition similar to that of "*Ca.* N. gargensis" are likely responsible for this unusual environmental GDGT pattern. A

potential contribution of Group I.1b Crenarchaeota to the increased crenarchaeol regioisomer abundances in tropical marine waters and sediments appears less parsimonious, as sequences affiliated with Group I.1b are not often reported in open marine environments. For example, Herfort et al. (2007) and Coolen et al. (2007) only found 16S rRNA gene sequences related to marine Group I Crenarchaeota in the North Sea and the Black Sea water columns, respectively. It should be noted, however, that Group I.1b sequences have indeed been recovered from some marine sediments (e.g. Park et al., 2008; Sahan and Muyzer, 2008) and corals (Beman et al., 2007). Thus, three plausible explanations exist for the high regioisomer abundance in tropical marine waters: (i) culture conditions used to enrich or cultivate marine Group I.1a Crenarchaeota up to now have simply not been effective at stimulating production of the crenarchaeol regioisomer, (ii) previously overlooked Group I.1b AOA are responsible for the regioisomer production in tropical marine waters, or (iii) large differences in crenarchaeol regioisomer production exist among individual Group I.1a species. The latter two hypotheses are supported by Shah et al. (2008), who present evidence supporting alternative biological or geographical origins of crenarchaeol regioisomer which was isolated from a mixed sedimentary GDGT assemblage.

Our results also have implications for TEX_{86} paleothermometry, as paleoreconstructions of sea surface temperatures using TEX_{86} rely on the inclusion of the crenarchaeol regioisomer in the calculated TEX_{86} ratio. If regioisomer synthesis is not only controlled by temperature, but also by the occurrence of specific phylogenetic clusters of Crenarchaeota producing enhanced amounts of the regioisomer, then this may complicate the use of the TEX_{86}. Such a scenario may explain part of the scatter observed in the TEX_{86}-temperature calibration (Kim et al., 2008). At this point, however, it is impossible to evaluate which potential shifts in TEX_{86}-temperature estimates will be caused by shifts in crenarchaeal populations in the natural environment since such community shifts are potentially influenced by a wide variety of factors including optimal growth temperatures, substrate availability, presence of competitors and predators, and species biogeography.

Our results with "*Ca*. N. gargensis" may be used to test the TEX_{86} at high temperatures, although we note that "*Ca*. N. gargensis" is a moderately thermophilic

organism isolated from a non-marine environment. A TEX_{86} value of 1 would (linearly) extrapolate to a temperature of 45.4°C using the Kim et al. (2008) calibration. This occurs when the crenarchaeol regioisomer is highly dominating over GDGTs 1-3, such as what is observed for "*Ca*. N. gargensis"; the TEX_{86}-derived temperature calculated from our biomass grown at 46°C was 45.1°C (cf. Table 1). There was no notable difference in the relative GDGT abundances when "*Ca*. N. gargensis" was grown at 46 °C or 50°C, but at 42°C the increase in the relative abundance of GDGTs 1-3 resulted in a slightly lower, but analytically robust, TEX_{86} (Table 1). The TEX_{86} empirical relationship predicts a more pronounced increase in GDGTs 1-3 than we observed - i.e. shifting from 46°C to 42°C should correspond to a TEX_{86} decrease of 0.050 (i.e. from 0.99 to 0.94). The measured TEX_{86} decrease of only 0.008 signifies that factors in addition to temperature influence the membrane GDGT distribution at high (and likely low) temperatures, rendering the GDGT-temperature relationship non-linear in these regions. Nevertheless, our results indicate that temperature does indeed exert some influence over the relative abundance of these GDGTs among individual species within the Group I.1b, similar to phenomena observed by incubation experiments with Group I.1a Crenarchaeota (Wuchter et al., 2004; Schouten et al., 2007).

Ultimately, the confirmation of crenarchaeol synthesis within the soil Group I.1b Crenarchaeota further supports the hypothesis that crenarchaeol is synthesized by all Archaea performing ammonia oxidation. However, since no cultivated representatives outside the recognized AOA lineages of the Group I Crenarchaeota are available at this moment, it cannot yet be evaluated how widespread crenarchaeol synthesis is among this group. "*Ca.* N. gargensis" is the first AOA for which the production of large amounts of the crenarchaeol regioisomer has been demonstrated, suggesting that Group I1.b Crenarchaeota are a likely source of the documented elevated regioisomer abundances in some natural environments. Furthermore, the unique lipids (a novel GDGT and IPL with novel headgroups) synthesized by "*Ca*. N. gargensis" may prove useful in future studies as biomarkers for Group I.1b Crenarchaeota or a sub-clade of this group. Screening more crenarchaeal enrichment cultures as they become available for their IPLs will determine if indeed this is the case. In contrast, environmental screening for IPLs common to *N.*

maritimus and "*Ca*. N. gargensis" (e.g. crenarchaeol with a hexose and phospho-hexose headgroup) may prove useful for the general detection of AOA.

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Chapter 5

Core and intact polar glycerol dibiphytanyl glycerol tetraether lipids of ammonia-oxidizing Archaea enriched from marine and estuarine sediments

Angela Pitcher[1], Ellen C. Hopmans[1], Annika C. Mosier[2], Soo-Je Park[3], Sung-Keun Rhee[3], Christopher A. Francis[2], Stefan Schouten[1] and Jaap S. Sinninghe Damsté[1]

[1] NIOZ Royal Netherlands Institute for Sea Research, Department of Marine Organic Biogeochemistry, P.O. Box 59, 1790 AB, Den Burg, The Netherlands
[2] Stanford University, Department of Environmental Earth System Science, Stanford, CA 94305, USA.
[3] Chungbuk National University, Department of Microbiology, 12 Gaeshin-dong, Heungduk-gu, Cheongju 361-763, South Korea

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Abstract

Glycerol dibiphytanyl glycerol tetraether (GDGT)-based intact membrane lipids are increasingly being used as compliments to conventional molecular methods in ecological studies of ammonia-oxidizing Archaea (AOA) in the marine environment. However, the few studies that have been done on the detailed lipid structures synthesized by AOA in

(enrichment) culture are based on species enriched from non-marine environments, i.e. a hot spring, an aquarium filter and a sponge. Here we have analyzed core and intact polar lipid (IPL)-GDGTs synthesized by three newly available AOA enriched directly from marine sediments taken from the San Francisco Bay Estuary ("*Ca*. Nitrosoarchaeum limnia"), and coastal marine sediments from Svalbard and South Korea. Like previously screened AOA, the sedimentary AOA all synthesize crenarchaeol (a GDGT containing a cyclohexane moiety and four cyclopentane moieties) as a major core GDGT, thereby supporting the hypothesis that crenarchaeol is a biomarker lipid for AOA. The IPL headgroups synthesized by sedimentary AOA comprised mainly monohexose, dihexose, phosphohexose, and hexose-phosphohexose moieties. The hexose-phosphohexose headgroup bound to crenarchaeol was common to all enrichments and, in fact, the only IPL common to every AOA enrichment analyzed to date. This apparent specificity, in combination with its inferred lability, suggests that it may be the most suitable biomarker lipid to trace living AOA. GDGTs bound to headgroups with a mass of 180 Daltons of unknown structure appears to be specific to the marine group I.1a AOA: they were synthesized by all three sedimentary AOA and "*Ca.* N. maritimus", however, they were absent in the Group I.1b AOA, "*Ca*. N. gargensis".

Introduction

The discovery of ammonia-oxidizing Archaea (AOA) and their apparent importance in the biogeochemical cycling of nitrogen (Francis et al., 2005; Könneke et al., 2005; Wuchter et al., 2006), has encouraged the ongoing study of their ecology and efforts to enrich and cultivate individual species. AOA perform the aerobic oxidation of ammonia to nitrite - the first and rate-limiting step in the process of nitrification - and are abundant in many natural environments, often outnumbering their ammonia-oxidizing bacterial counterparts (based on quantitative comparisons of 16S rRNA and *amoA* genes, and *in situ* hybridization techniques) (Leininger et al., 2006; Wuchter et al., 2006; Park et al., 2008). To date, cultivation efforts have resulted in the enrichment and characterization of three

AOA, representing three phylogenetic lineages within the Group I AOA: "*Ca.* Nitrosopumilus maritimus SCM1" (Könneke et al., 2005), "*Ca*. Nitrosocaldus yellowstonii" (de la Torre et al. 2008), and "*Ca*. Nitrososphaera gargensis" (Hatzenpichler et al., 2008). In addition, "*Ca*. Cenarchaeum symbiosum", a Group I AOA living in symbiosis with the marine sponge, *Axinella mexicana*, has not been cultivated but has been well characterized (Preston et al., 1996; Hallam et al., 2006a; Hallam et al., 2006b).

It has been recently shown that all enriched AOA synthesize the membrane lipid crenarchaeol (Sinninghe Damsté et al., 2002b; de la Torre et al., 2008; Schouten et al., 2008; Pitcher et al., 2010b) – a unique glycerol dibiphytanyl glycerol tetraether (GDGT) including four cyclopentane moieties and a cyclohexane moiety (Figure 1) – which has not been encountered in any cultivated (hyper)thermophilic Crenarchaeota or Euryarchaeota until now (Sinninghe Damsté et al., 2002b). These include the three cultivated species mentioned above and "*Ca.* C. symbiosum" (Sinninghe Damsté et al., 2002b), in addition to uncharacterized AOA enriched from the North Sea and the Indian Ocean (Wuchter et al., 2004; Schouten et al., 2007a). A close association between crenarchaeol and putative AOA has been previously noted in diverse environments (e.g. Leininger et al., 2006; Coolen et al., 2007; Pitcher et al., 2009) and this, coupled to its occurrence in cultivated representatives, suggests that it may in fact be specific to AOA (de la Torre et al., 2008; Pitcher et al., 2010b).

Aside from crenarchaeol, AOA also synthesize GDGTs with 0-3 cyclopentane moieties (Figure 1). Generally, GDGT-0 and crenarchaeol predominate with lower relative abundances of GDGTs 1-3, and similar GDGT distributions have been recovered from marine suspended particulate matter and sediments (Schouten et al., 2000 and references therein; Schouten et al., 2002). The occurrence of GDGT-4 has also been noted in "*Ca*. N. maritimus", "*Ca*. N. gargensis" and "*Ca*. N. yellowstonii", but it is typically present in small relative amounts (de la Torre et al., 2008; Schouten et al., 2008; Pitcher et al., 2010b). GDGT analysis has been used to provide DNA-independent information on the structure and function of archaeal communities in numerous marine environments, and has proved useful in substantiating or complimenting other molecular evidence (Biddle et al., 2006; Coolen et al., 2007). Incorporation of ^{13}C-labelled bicarbonate into the GDGTs of a

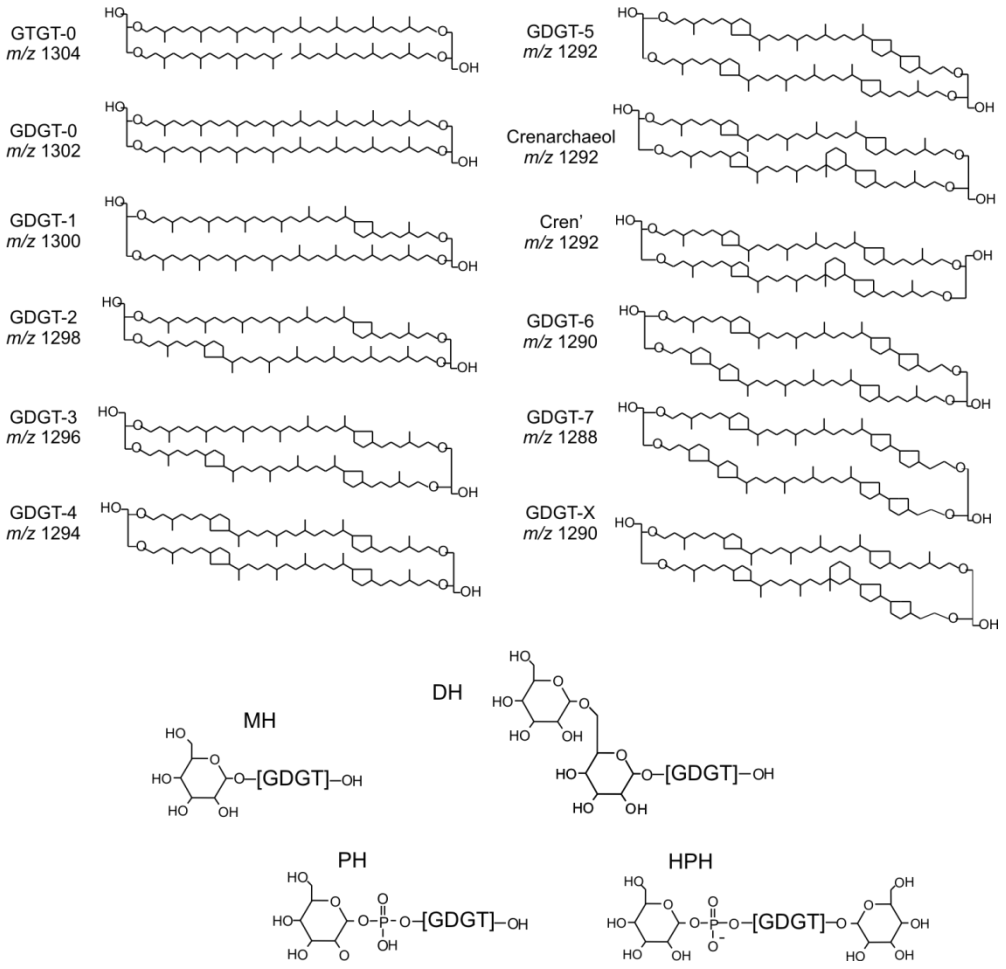

Figure 1. Structures of diglycerol di(tri)alkyl tetraethers GD(T)GTs commonly synthesized by Archaea and molecular weights shown as *m/z* values. GDGT-X was recently identified as a major core GDGT in the soil AOA, "*Ca*. Nitrososphaera gargensis", however it has not been observed in other cultivated Archaea or environmental GDGT distributions thus far. Below, IPLs identified in this work (and also in "*Ca*. N. maritimus" and "*Ca*. N. gargensis"); polar headgroups are shown in full structure: monohexose (MH), dihexose (DH), phosphohexose (PH), and hexose + phosphohexose (HPH). It should be noted that the exact positions of the polar head groups are unknown.

marine crenarchaeote enriched from the North Sea provided the first direct evidence for an autotrophic lifestyle of planktonic mesophilic AOA (Wuchter et al., 2003), and determination of the radiocarbon content of GDGTs in the marine water column suggested that autotrophy accounts for approximately 83% of the carbon assimilated by mesopelagic AOA, the rest coming from heterotrophic consumption of organic carbon (Ingalls et al., 2006).

Compared to core GDGTs (as described above), their intact polar lipid (IPL) counterparts (i.e. GDGTs bound to a polar headgroup(s)) may give more specific information on putative microbial community structure. IPLs are thought to derive from living (or recently living) organisms, as the covalently bound polar headgroups are relatively rapidly lost upon cell senescence (White et al., 1979; Harvey et al., 1986). GDGT-based IPLs have been used previously as tracers of AOA in marine sediments and water columns (Biddle et al., 2006; Schubotz et al., 2009), to determine the viability of microbes where conventional molecular approaches may show a relative bias (e.g. such as in the deep biosphere) (Lipp et al., 2008), and in quantitatively distinguishing between living and dead cell material (Pitcher et al., 2009; Huguet et al., 2010). The additional structural information obtained from polar headgroup analysis of IPLs, as opposed to core lipid analysis only, may also be potentially useful for inferring the presence of certain phylogenetic lineages. Overall, IPLs give more relevant information regarding the local viable organisms compared to the analysis of core lipids, which often largely represent fossilized organic matter.

Of the four AOA cultures that have been profiled for their GDGT composition, only "*Ca*. N. maritimus", a Group I.1a AOA isolated from an aquarium, and "*Ca*. N. gargensis", a Group I.1b AOA enriched from a hot spring, have been profiled for their GDGT-based IPLs (Schouten et al., 2008; Pitcher et al., 2010b). "*Ca*. N. gargensis" has an unusual GDGT profile and is phylogenetically more closely related to soil AOA than marine AOA. Although "*Ca*. N. maritimus" is closely related to sequences of marine AOA, it was enriched from sea aquarium gravel, not a natural marine environment. Further development and progress in the application of IPL analysis to marine AOA ecology

Table 1. Overview of AOA analyzed for GDGTs

Enrichment/Isolate	Location	Source	Temperature (°C)		NH_4^+ (μM)	pH	Reference
			In situ	Culture			
"*Ca* . Nitrosoarchaeum limnia"	San Fransisco Bay Estuary	estuarine sediment	21.6	22	500	7.0-7.2	Blainey et al., 2010; This work
SJ'	Donhae, South Korea	coastal marine sediment	0.5	25	100	8.2	Park et al., 2010; This work
AR'	Svalbard, Norway	coastal marine sediment	-0.5	25	100	8.2	Park et al., 2010; This work
"Ca. Nitrosopumilus maritimus"	tropical aquarium	gravel	21-23	28	500	7.0-7.2	Könneke et al., 2005; Schouten et al., 2008
"*Ca* . Nitrososphaera gargensis"	Siberia Garga Hot Spring	microbial mat	<60	46	500	7.8	Lebedeva et al., 2005; Pitcher et al., 2010
"*Ca* . Nitrosocaldus yellowstonii"*	Yellowstone Heart Lake hot spring	sediment	70-80	72	95	8.3	de la Toore et al., 2008
" Ca. Cenarchaeum symbiosum*"*	Santa Barbara, California	marine sponge *A. mexicana*	8-18	10	*n.s.*	*n.s.*	Sinninghe Damsté et al., 2002; Preston et al., 1996

* analyzed for core lipids, no IPLs

n.s. = not specified

necessitates the study of GDGTs and their associated IPLs synthesized by AOA actually enriched from marine environments similar to those commonly studied.

Here we have analyzed three new cultures of Group I.1a AOA enriched from marine sediments off the coast of Svalbard, Norway ('AR') and South Korea ('SJ') (Park et al., 2010), as well as sediments from the San Francisco Bay estuary ("*Ca*. Nitrosoarchaeum limnia") (Blainey et al., 2010), for their IPL and core GDGTs. Although Group I AOA were first identified in the marine water column (Delong, 1992; Fuhrman, 1992), a large abundance and diversity of sedimentary AOA has been demonstrated (Francis et al., 2005). Recovery of IPL-GDGTs from marine sediments has also led to questions of whether or not they are produced *in situ* or are derived from planktonic AOA living in the water column (Lipp and Hinrichs, 2009). Analysis of three sedimentary AOA enrichment cultures from contrasting geographies provides the opportunity to address this issue. Moreover, comparisons among our findings and with those of previously studied AOA contribute additional information on the diversity of marine AOA GDGTs and IPLs, and give insight into lipid biomarkers specific to this phylogenetic lineage of Archaea, the Group I Crenarchaeota.

Materials and Methods

Cultivation of archaeal ammonia-oxidizers

Enrichment cultures of "*Candidatus* Nitrosoarchaeum limnia" SFB1 were grown as previously described (Blainey et al., 2010). Briefly, surface sediments collected in northern part of the San Francisco Bay estuary (site SU001S; 38°5'55.75"N, 122° 2'47.40"W) were inoculated into a modified low-salinity version of Synthetic Crenarchaeota Medium (Könneke et al., 2005) as described by Mosier and Francis (2008). At the time of sampling, nutrient concentrations in the bottom water just above the sediments were 2 µM ammonia, 14 µM nitrate, and 0.9 µM nitrite. Salinity was 7.9 psu, temperature was 21.6 C, and sediment C:N ratio was 15.8 (data courtesy of the San Francisco Bay Regional Monitoring Program). For lipid analysis, 585 ml of culture was filtered over one 0.22 µm

Duopore filter and 1710 ml of culture was filtered over two 0.7 µm GF/F (47 mm diameter) filters.

Marine sediments (ca. 100 g of sediment and 1L of sea water) were collected from the Donghae (E 128º35', N 38º20'; depth 650 m) and Svalbard (Arctic region, E 016°28', N 78°21'; depth 78 m) in sterilized glass bottles and mixed in the field by stirring with a sterile spatula. Approximately 10 ml of the each sediments slurry was transferred to a sterile conical tube and transported back to the laboratory at 4°C. Cultures were grown aerobically in natural seawater media, which contains autoclaved coastal seawater from Donghae (South Korea) supplemented with ammonium chloride (1 mM), sodium thiosulfate (0.1 mM), sodium bicarbonate (2.5 mM), potassium phosphate (0.1mM), trace element mixture (1×) (Widdel and Bak 1992) and vitamin solution (1×) (Widdel and Bak, 1992). The pH was adjusted to 8.2 using 1 N NaOH or HCl. The cultures from Donghae, South Korea (SJ), and the Arctic sea of Svalbard (AR) were incubated at a static condition with daily intermittent inverting instead of continuous shaking. After oxidation of ammonia (typically after two weeks), 5% of the total culture volume were routinely transferred to new seawater media at 25°C in dark condition. After batch culture, the pH of this medium was not significantly changed (8.0 to 8.2). The cells used for lipid work was harvested by centrifugation at 8,000 rpm (5,200 g) for 1 h.

Intact Polar Lipid analysis

IPLs of "*Ca*. N. limnia" were extracted separately from the 0.2 µm Duopore and 0.7 um GF/F filters. Whole cell pellets of 'AR' and 'SJ' biomass were extracted as such. IPLs were extracted using a modified Bligh and Dyer technique (Bligh and Dyer, 1959): A known volume of single-phase solvent mixture of methanol (MeOH):dichloromethane (DCM):phosphate buffer (2:1:0.8, v/v/v) was added to the sample in a centrifuge tube and placed in an ultrasonic bath for ten min. The extract and residue were separated by centrifuging at 2500 rpm for 5 min and the solvent mixture collected in a separate flask (3 times). DCM and phosphate buffer were added to the single-phase extract to give a new ratio of MeOH:DCM:phosphate buffer (1:1:0.9, v/v/v), and to induce phase separation. The extract was centrifuged at 2500 rpm for 5 min. The DCM phase was collected in a round-

bottom flask and the MeOH:phosphate buffer phase was washed two additional times with DCM. The combined DCM phases were reduced under rotary vacuum and evaporated to dryness under a stream of N_2.

Residual biomass of "*Ca*. N. limnia" left on both Duopore and GF/F filters was re-extracted substituting 5% trichloroacetic acid (TCA) for the phosphate buffer according to Sturt et al. (2004), and analyzed separately to determine the effectiveness and efficiency of the regular Bligh Dyer extraction. After Bligh and Dyer extraction of 'AR' and 'SJ' biomass, there was not enough residual biomass to perform a TCA extraction.

HPLC-Electrospray Ionization (ESI)/MS

IPL-GDGTs were analyzed according to conditions described previously (Schouten et al., 2008) as modified from Sturt et al. (2004). For the analysis an Agilent (Palo-Alto, CA, US) 1100 series LC equipped with a thermostat auto-injector was coupled to a Thermo TSQ Quantum EM triple quadrupole mass spectrometer equipped with an Ion Max source with ESI probe. Detection was achieved using positive ion ESI/MS by scanning mass range *m/z* 1000-2000.

Core lipid analysis

Acid hydrolysis was performed on aliquots of Bligh and Dyer extracts obtained from filtered "*Ca*. N. limnia" biomass and on whole cells of 'SJ' and 'AR', to cleave polar headgroups and release core GDGTs. Biomass was refluxed in 2 ml of 5% HCl in MeOH for 3 h. The cooled solution was adjusted to pH 5 with 2N KOH:MeOH (1:1, v/v). Bidistilled water was added to a final ratio of H_2O:MeOH (1:1, v/v) and this mixture was washed 3 times with DCM. The DCM fractions were collected and dried over Na_2SO_4. The extract was dissolved in hexane:propanol (99:1 v/v) filtered over a 0.4 µm PTFE filter before analysis by high performance liquid chromatography atmospheric pressure chemical ionization mass spectrometry (HPLC-APCI/MS).

HPLC-APCI/MS

Core GDGTs were analyzed using a modified procedure from Hopmans et al. (2000) and Schouten et al. (2007b). GDGTs were detected with full-scan analysis from *m/z* 900-1400 and were quantified using single ion monitoring (SIM) of glycerol trialkyl glycerol tetraethers (GTGTs) with *m/z* 1304, and GDGTs with *m/z* 1302, 1300, 1298, 1296, 1294, 1292, 1290, and 1288. The relative abundance was determined by integration of the extracted peak areas of their $[M+H]^+$. Because crenarchaeol and GDGT-4 co-elute and naturally occurring isotopes of crenarchaeol contribute to the GDGT-4 signal, the integrated peak area was corrected by subtracting a response equal to 45% of the crenarchaeol peak from the 1294 peak, according to theoretical isotope distributions described in Hopmans et al. (2000).

Results

Core GDGT distributions

HPLC-APCI-MS analysis of acid-hydrolyzed enrichment culture material revealed the presence of GDGTs 0-4, crenarchaeol and the regioisomer of crenarchaeol – in all three cultures (Figure 2). Although SIM parameters were set to detect GDGTs 6, 7 and X (Figure 1), none of these GDGTs were detected. Trace amounts of the tribiphytanyl glycerol tetraether, GTGT-0, (Figure 1) were detected in enrichment cultures AR and "*Ca*. N. limnia" while it was not detected in the SJ enrichment (Table 2). GDGTs of all AOA enrichments were dominated by GDGT-0 and crenarchaeol but the relative amounts of the other GDGTs were quite different. The Southern Korea enrichment (SJ) showed a large contribution of GDGT-4 (34% of the peak area representing co-eluting GDGT-4 and crenarchaeol) while the enrichments from San Francisco Bay and coastal Svalbard sediments (AR) contained much less (10-11% of total peak area) (Figure 2, Table 2). "*Ca*. N. limnia" also showed a distinctly higher relative abundance of GDGT-1 (Figure 2a) compared to SJ and AR, which showed a somewhat similar relative contribution of GDGTs 1-3 (Figures 2b and c). In addition, the SJ and AR enrichments contained notable

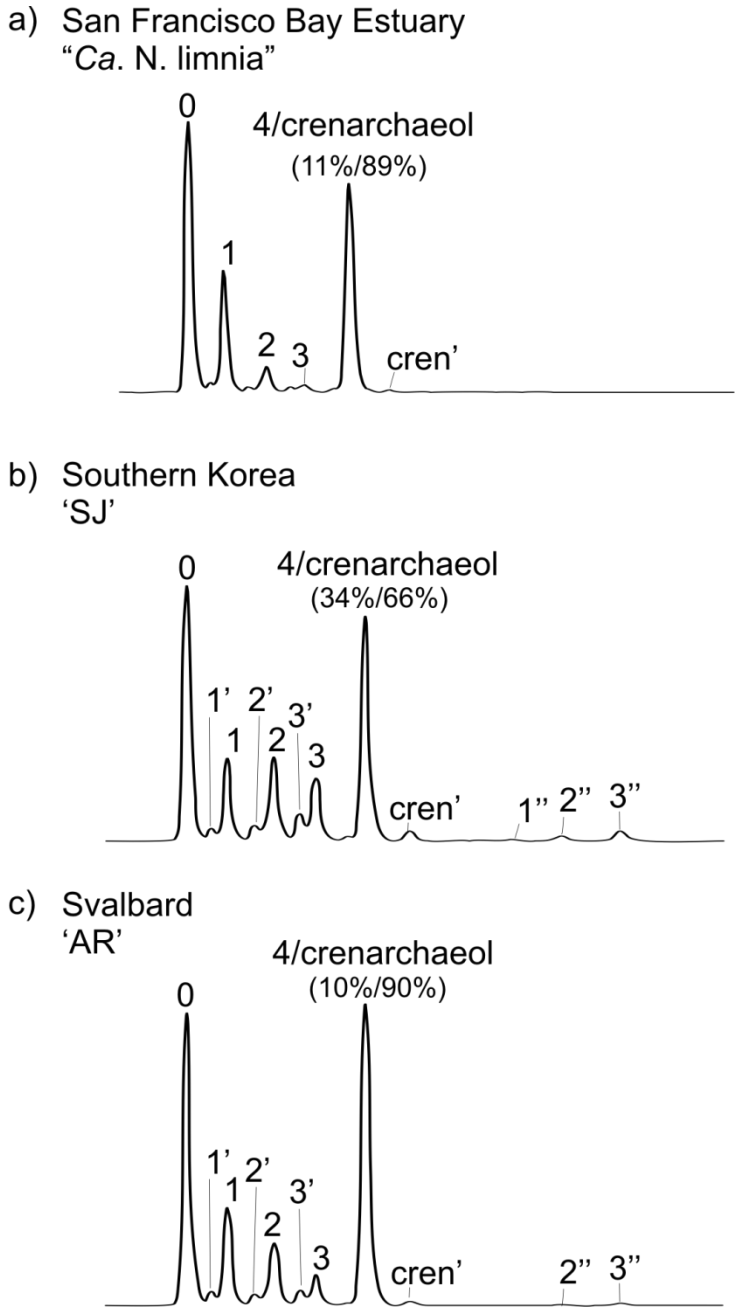

Figure 2. Base peak chromatogram of core GDGT profiles of a) San Francisco Bay enrichment "*Ca*. N. limnia", b) coastal South Korea enrichment "SJ", and c) coastal Svalbard, Norway enrichment "AR". Numbers over peaks correspond to GDGT structures shown in Figure 1. Numbers with ', or '', represent unknown structures with the same *m/z* as the corresponding GDGT. Percentages listed below peak '4/crenarchaeol' indicate the respective contribution of each GDGT to the area of that peak, as corrected according to Hopmans et al. (2000).

Table 2. Fractional abundance of core GDGTs (and GTGT-0) synthesized by the sedimentary AOA analyzed in this work.

Enrichment	GTGT-0	GDGT Relative Abundance [a]													TEX_{86} [b]	TEX- temp (°C) [c]
		0	1	1'	1"	2	2'	2"	3	3'	3"	4	4'	cren	cren'	
"*Ca*. N. limnia"	0.001	0.35	0.17	0.011	nd	0.046	0.005	nd	0.013	0.006	nd	0.041	0.005	0.34	0.004	0.27	0.6
SJ	0.00	0.26	0.084	0.013	0.004	0.11	0.021	0.012	0.076	0.033	0.026	0.12	nd	0.24	0.012	0.70	28.0
AR	0.001	0.31	0.102	0.012	0.002	0.088	0.013	0.003	0.037	0.018	0.008	0.040	nd	0.35	0.006	0.56	21.4

[a] structures shown in Figure 1.

[b] TEX_{86} values were calculated according to Schouten et al. (2002)

[c] temperature calculated according to Kim et al. (2010) using the TEX_{86}^{H} calibration for SJ and AR and the TEX_{86}^{L} calibration for "*Ca.* N. limnia"

nd = not detected

contributions of early eluting isomers of GDGTs 1-3 (i.e. 1', 2', and 3'), which were present, although in lower relative abundance in "*Ca*. N. limnia" (Table 2). Interestingly, relatively low but notable amounts of GDGTs with similar molecular masses as GDGTs 1-3, but with much later elution times, were present in SJ and AR (labeled as 1'', 2'', and 3'' in Figures 2b and c), suggesting the presence of novel GDGTs and/or isomers which have not previously been reported.

IPL-GDGT distributions

HPLC-ESI/MS analysis of the Bligh and Dyer extracts of the three enrichment cultures revealed the presence two major clusters of peaks and a number of minor peaks (Figure 3). GDGT-based IPLs were tentatively indentified via mass spectral analysis, and showed that all three enriched AOA synthesized similar IPLs containing both glyco and phospho headgroups, although the relative abundances of these IPL classes differed somewhat between cultures. Identification of the major core GDGTs associated with the different IPLs was based on molecular weight and diagnostic fragments of core GDGTs in the mass spectra. This has been previously shown to reveal the same distributions as GDGTs analyzed after acid-hydrolysis of prepared HPLC IPL peak fractions (Schouten et al., 2008).

All of the peaks in the first cluster of polar lipids (represented by peaks 1-6; Figure 3) were identified as GDGT-based glycolipids (GL). Peaks 1-4 represent dihexose (DH) GDGTs with each peak associated with a different major core lipid (cf. Table 3). The main ions in the mass spectra of each IPL corresponded to the ammoniated (NH_4^+), $[M+18]^+$, and sodiated (Na), $[M+23]^+$, adducts of the IPL, and minor ions represented the core GDGTs. Mass spectra of peak 5 were consistent with a monohexose (MH) IPL with an additional headgroup of 180 Daltons ('MH+180'), with GDGTs 1-3 as the major core lipids, in all three cultures. This IPL, containing the 180 Dalton headgroup, has been identified previously in "*Ca.* N. maritimus SCM1" and has also been recovered from marine sediments (Lipp and Hinrichs, 2008; Schouten et al., 2008), however, the precise structure of this headgroup remains unknown. In SJ, this IPL was in present in much higher relative abundances than in "*Ca*. N. limnia" and AR, dominating the GL cluster of the base

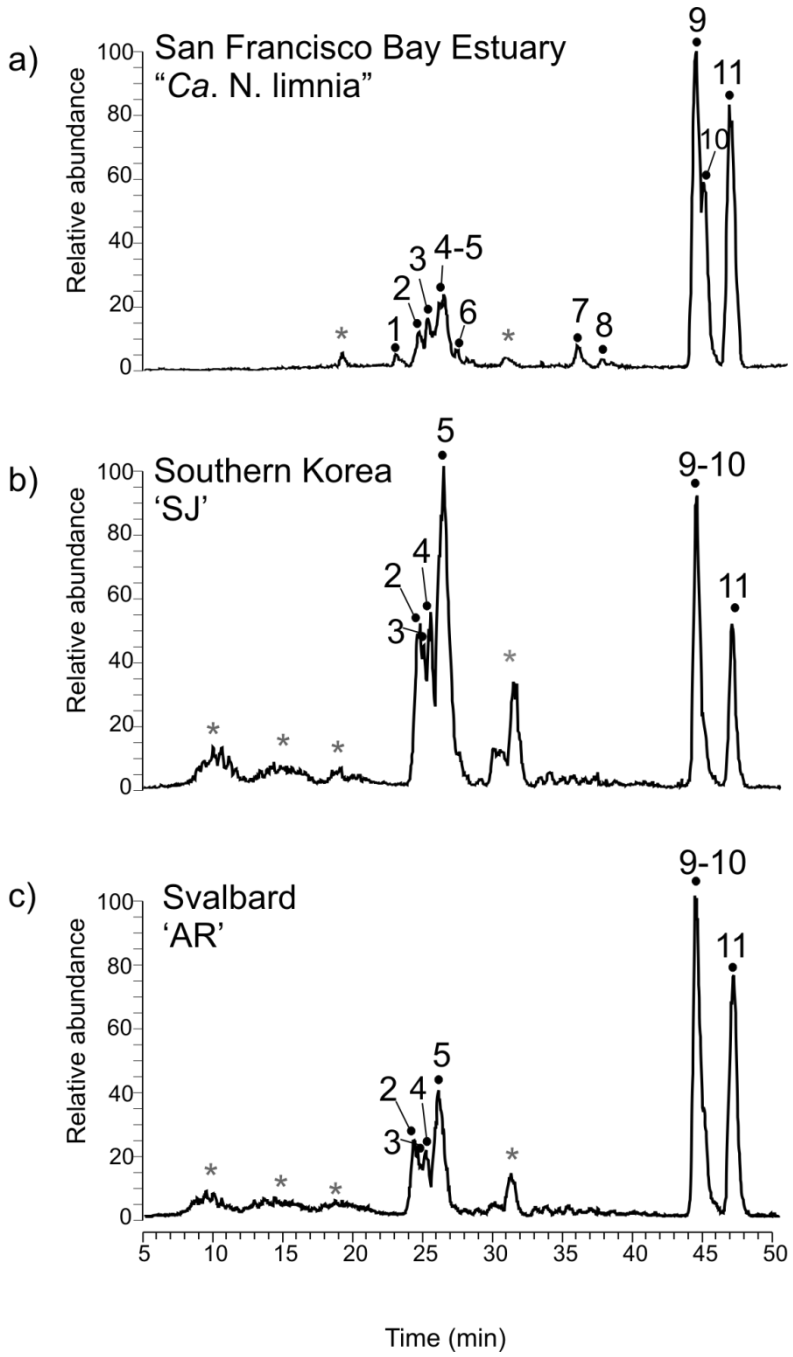

Figure 3. Base peak chromatogram of GDGT-based IPL profiles (*m/z* 1000-2000) of a) San Francisco Bay enrichment "*Ca*. N. limnia", b) coastal South Korea enrichment (SJ), and c) coastal Svalbard, Norway enrichment (AR). Peak numbers correspond to IPL structures described in Table 3. Stars indicate non-identified peaks.

Table 3. Main mass spectra ions, polar headgroups and major core GDGTs associated with each IPL peak (Figure 3). DH = dihexose; MH = monohexose; PH = phosphohexose; HPH = hexose-phosphohexose; '180' refers to an additional moiety of 180 Daltons.

Figure 3 Peak	Main Ions (*m/z*)	IPL headgroup	Main Core GDGTs
1	1302-1304, 1643-1645, 1648-1650	DH	GTGT-0, GDGT-0
2	1298-1300, 1639-1641, 1644-1646	DH	GDGT 2
3	1294-1296, 1635-1637, 1640-1642	DH	GDGT 3
4	1294-1296, 1635-1637, 1640-1642	DH	GDGT 4
5	1296-1300, 1638-1642, 1655-1659, 1660-1664	MH+180	GDGTs 1-3
6	1298, 1658, 1675, 1680	2 x 180	GDGT 2
7	1534, 1544, 1561, 1566	PH	GDGT-0
8	1534, 1551, 1556	PH	Crenarchaeol
9	1544, 1706, 1723, 1728	HPH	GDGT-0
10	1542, 1704, 1721, 1726	HPH	GDGT-1
11	1534, 1696, 1713, 1718	HPH	Crenarchaeol

peak chromatogram. In general the GL cluster in SJ represented a much higher proportion of the GDGT-based IPLs than the other two AOA. Peak 6 was very small, and co-eluted with the tail of peak 5 observed in "*Ca*. N. limnia", and was not identified in SJ or AR. The dominant ions were consistent with an IPL containing two of the unknown 180 Dalton headgroups coupled to a GDGT-2 core lipid (Figure 4). In this case, the $[M+H]^+$ ion was quite apparent (*m/z* 1658), along with the $[M+NH_4]^+$ and $[M+Na]^+$ ions. GDGT-2 appeared to be the dominant core GDGT as revealed by the *m/z* 1298 in the mass spectrum. To the best of our knowledge, this particular IPL has not yet been previously reported.

Peaks 7 and 8 indicated IPLs with a phosphohexose (PH) headgroup coupled to GDGT-0 and crenarchaeol, respectively. These peaks were unique to "*Ca*. N. limnia", present in apparently low relative amounts in comparison to the other IPLs. Peaks 9-11 are striking in their dominance in the IPL profiles of all three AOA cultures, representing IPLs with a hexose headgroup in addition to a phosphohexose headgroup (HPH) coupled to GDGT-0, GDGT-1, and crenarchaeol, respectively. The distribution of these three HPH-

IPLs was nearly identical for the three cultures, despite variable distributions of the GLs. Furthermore, the distributions of the HPH IPLs share similarities with the total core GDGT distributions (Figure 2), which were dominated by GDGTs 0 and crenarchaeol, with lower amounts of GDGT-1. In general it appears that GDGT-0 and crenarchaeol are more closely associated with the HPH headgroups, rather than with the DH and '180' headgroups, which were more commonly associated with the minor GDGTs 2-4. A similar trend was noticed in "*Ca.* N. maritimus SCM1" (Schouten et al., 2008).

Comparison of filter pore size and BDE and TCA extraction on IPLs of "*Ca.* N. limnia" culture

Archaeal lipids in the natural environment are typically studied by filtering water through GF/F filters (Sinninghe Damsté et al., 2002a; Coolen et al., 2007). To test the effect of filter material and pore size on the collection and extraction of IPLs, suspended "*Ca*. N. limnia" culture was filtered onto both Duopore (DUO) and GF/F filters, with nominal pore sizes of 0.22 µm and 0.7 µm, respectively. The results from equivalent amounts of injected lipid extract from the four separate extracts are summarized in Table 4. The sequential BDE and TCA extracts of the DUO filter and the BDE extract of the GF/F-filter showed almost identical IPL profiles, while only two IPL-GDGT peaks were detected in the TCA extract of the GF/F-filtered cells (possibly because of the low amounts recovered)(Table 4). The overall extraction yield (BDE+TCA) from GF/Fs (based on summed IPL response per ml culture; Table 4) was ca. eight times lower than that of the DUO filters, indicating that significantly more cells per ml of culture filtered were captured on the DUO filters. In addition, TCA extraction of the DUO filters yielded much more additional GDGTs (41% of total IPL-GDGTs) than the TCA-extracted GF/F filter (6% of total IPL-GDGTs)

Table 4. Yields from sequential Bligh and Dyer (BDE) and trichloroacetic acid (TCA) extractions of "*Ca*. N. limnia" culture filtered over duopore and GF/F filters

"*Ca* . N. limnia" peak (Figure 3a)	0.22 µm Duopore		0.7 µm GF/F	
	BDE	TCA	BDE	TCA
Distribution (% summed peak areas 1-11)				
1-4	9.5	9.7	9.4	0.0
5	11	10	11	0.0
6	1.1	1.0	1.2	0.0
7	1.7	1.7	1.9	0.0
8	0.62	0.34	nd	0.0
9-10	47	44	42	39
11	29	33	35	61
Yield (% total BDE+TCA signal)				
1-4	58	42	100	0
5	60	40	100	0
6	62	38	100	0
7	59	41	100	0
8	72	28	nd	nd
9-10	61	39	93	7.0
11	56	44	89	11
Total yield (%)	59	41	94	6.0
Total response per ml culture ($x10^5$)[a]	8.4	5.9	1.6	.11

[a] sum of integrated peak areas; nd = not detected

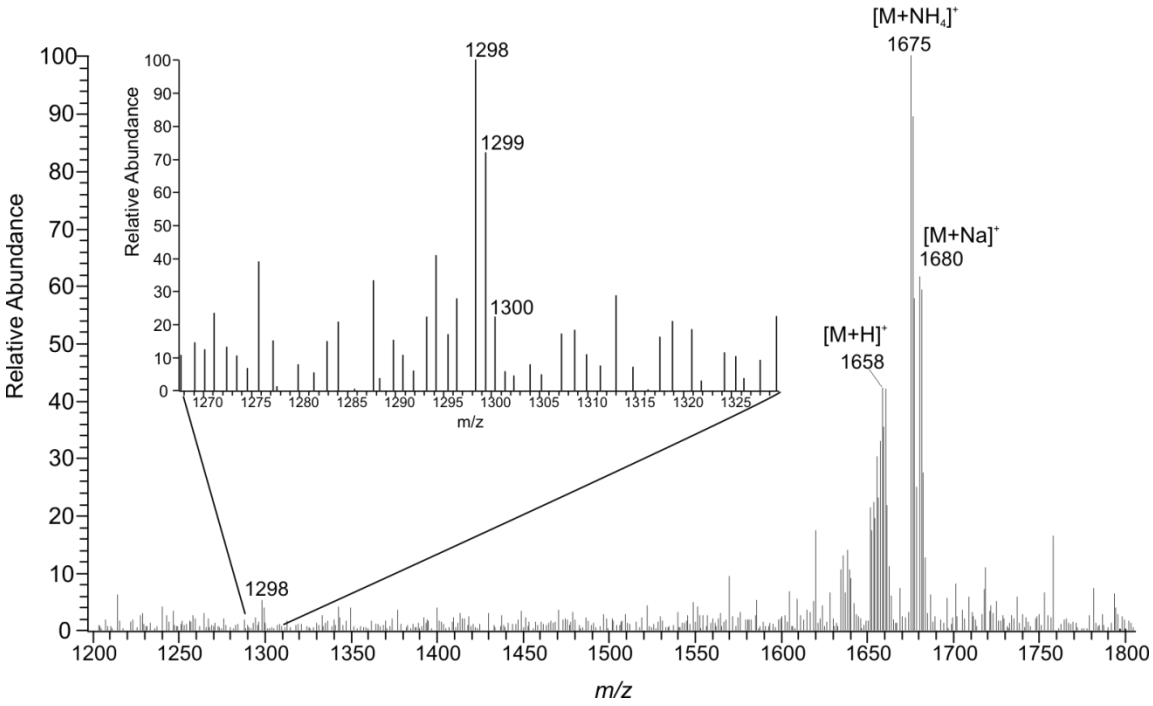

Figure 4. Mass spectrum of peak 6 in the base peak chromatogram of the HPLC/MS analysis of the extract of "*Ca*. N. limnia" showing ions consistent with an IPL containing two 180 Dalton headgroups and GDGT-2 as its core lipid.

Discussion

Core GDGTs

Research over the past few years has highlighted the omnipresence of crenarchaeol in the marine environment, as simultaneous studies have called attention to the ubiquity and potential importance of AOA in these environments using molecular ecological approaches. While a few empirical studies have pointed to a link between AOA and the occurrence of crenarchaeol in marine water columns, marine sponges, and soils (Sinninghe Damsté et al., 2002b; Wuchter et al., 2004; Leininger et al., 2006; Coolen et al., 2007),

crenarchaeol synthesis by an AOA was only unambiguously confirmed by its discovery in "*Ca.* N. maritimus SCM1" (Schouten et al., 2008), an AOA isolated from tropical aquarium gravel (Könneke et al., 2005). Subsequent confirmation of crenarchaeol synthesis by two other AOA, "*Ca*. Nitrosocaldus yellowstonii" (de la Torre et al., 2008) and "*Ca*. Nitrososphaera gargensis" (Pitcher et al., 2010b) led to the proposal that crenarchaeol is synthesized exclusively by AOA and is therefore a specific biomarker for this crenarchaeal lineage (de la Torre et al., 2008; Pitcher et al., 2010b). The weight of this hypothesis, however, has relied mainly on analysis of the aforementioned AOA, which were enriched from non-marine sources (cf. Table 1).

A significant amount of work on AOA has centered on the marine realm where AOA are ubiquitously abundant, however, GDGT data derived from lipid analysis of AOA enriched directly from marine suspended particulate matter and sediments has been lacking until now. Confirmation of crenarchaeol and crenarchaeol regioisomer synthesis by "*Ca*. N. limnia", SJ and AR (enriched directly from marine sediments) further substantiates the possible specificity of crenarchaeol to AOA and provides the strongest evidence thus far that crenarchaeol recovered from marine environments derives from AOA living there. Furthermore, our findings confirm that *in situ* production by sedimentary AOA may contribute to the GDGTs recovered from marine sediments.

The phylogeny based on the 16S rRNA gene of all of the AOA analyzed for GDGTs to date is shown in Figure 5: crenarchaeol synthesis in three Group I lineages indicates that it is widely distributed. Crenarchaeol synthesis by multiple representatives from the Group I.1a further implies the likelihood that crenarchaeol synthesis is widely occurring throughout this group, regardless of species and/or habitat (e.g. planktonic or sedimentary). All of the Group I enrichments thus far are AOA, implying the possibility that crenarchaeol synthesis may actually be exclusive to Archaea involved in ammonia oxidation. Verification of this would require lipid analysis of enriched, non-ammonia oxidizing representatives of the Group I Crenarchaeota, however, no such cultures exist at this time.

In addition to crenarchaeol, all AOA that have been screened for core GDGTs also synthesize GDGTs 0-4 and, with the exception of "*Ca*. N. gargensis", produce GDGT-0 in

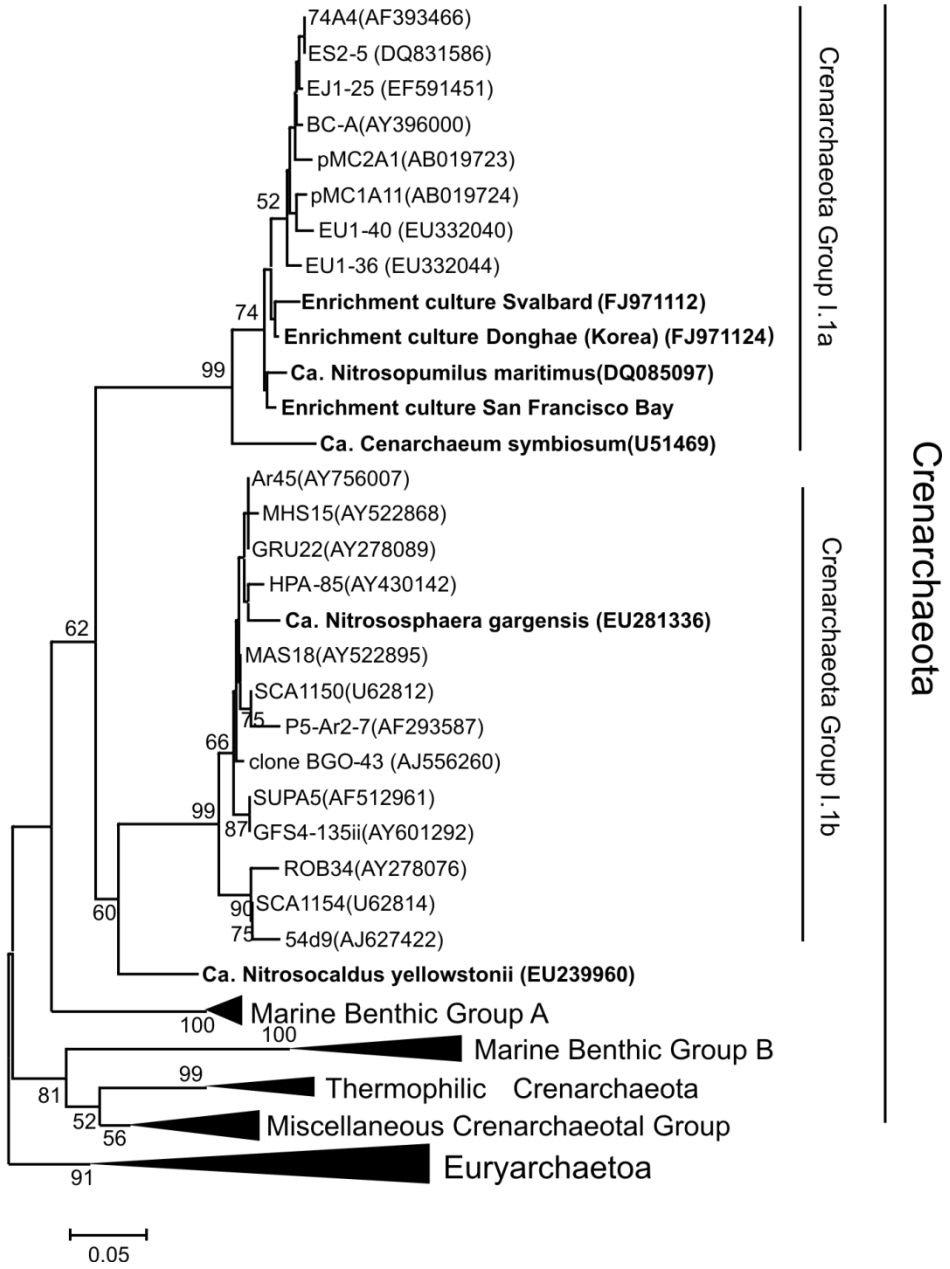

Figure 5. Summarized archaeal phylogeny based on the 16S rRNA gene showing detailed groupings with previously cultivated representatives (bold type) in addition to those analyzed in this work. For phylogenetic analysis, the gene sequences of related taxa were obtained from the GenBank database, and multiple alignments were performed using the Clustal X program (Thompson et al., 1997). The phylogenetic trees were constructed by using the MEGA 3 computer software program (Kumar et al., 2004).

high relative abundances, often comparable to crenarchaeol. GDGTs 1-4 are usually produced in lower amounts than GDGT-0 and crenarchaeol; their comparative abundances vary significantly between cultures. Similarly, our data yielded different distributions of GDGTs 1-4 for all three enrichment cultures, (Figure 3, Table 2). The high abundance of GDGT-4, particularly in SJ (34% of the combined crenarchaeol+GDGT-4 peak; cf. Figure 2b) is intriguing, as it has not been observed in such high abundances in any other AOA. GDGT-4 is often the most abundant GDGT in (hyper)thermophilic Archaea, presumably because its structure (containing four cyclopentane moieties; Figure 1) promotes dense packing of lipids which helps maintain the integrity of the cell membrane at high temperatures where it would otherwise be compromised. Of the three AOA studied here, AR and SJ were enriched from among the lowest *in situ* temperatures (-0.5 - 0.5°C), and in comparison with other AOA isolated and cultivated at much higher temperatures (i.e. "*Ca*. N. gargensis and "*Ca*. N. yellowstonii"; Table 1), the relative abundance of GDGT-4 in especially SJ is anomalously high. The occurrence of such relatively large amounts of GDGT-4 in an archaeon enriched from such a low temperature is unusual and difficult to explain at present.

Precisely why GDGT profiles of Group I Crenarchaeota enrichments differ from each other is still largely unknown, and may have to do with a number of physical conditions. Temperature, specifically, has been shown to exhibit control over GDGT composition in both (hyper)thermophilic Crenarchaeota and Euryarchaeota (Uda et al., 2001) and mesophilic Group I Crenarchaeota (Wuchter et al., 2004; Schouten et al., 2007a). An empirical correlation between marine sea surface temperature (SST) and sedimentary GDGT distribution comprised of GDGTs 1-3 and the crenarchaeol regioisomer, has led to the development of the TEX_{86} temperature proxy (Schouten et al., 2002), which is based on the premise that planktonic marine Group I Crenarchaeota adapt their GDGT distribution according to temperature and this signal is recorded in fossil GDGTs that reach the sediments. The cultures of "*Ca*. N. limnia", SJ, and AR, represent relevant AOA upon which to test whether sedimentary AOA adapt their membrane in a similar way.

Despite similar cultivation temperatures (22-25°C), the TEX_{86} values of "*Ca*. N. limnia", SJ, and AR were quite different (Table 2). The TEX_{86}-derived temperatures for SJ and AR (28°C and 21°C; Table 2) are around the cultivation temperatures (25°C). These sedimentary AOA do follow the marine TEX_{86} calibration recently presented in Kim et al. 2010), consistent with findings reported for "*Ca.* N. maritimus" and "*Ca*. N. gargensis", a Group I.1b crenarchaeote enriched from a hot spring (Pitcher et al., 2010b). In contrast, the TEX_{86}-derived temperature of "*Ca*. N. limnia" is ca. 0°C, far below the cultivation and *in situ* temperatures of 22°C and 21.6°C, respectively. The reason for this is presently unclear but may have to do with the fact that it was cultivated at a lower pH than the SJ and AR (Table 1). Shimada et al. (2008) reported that *Thermoplasma acidophilum* cultivated in the pH range 1.2-3.0 decreased the number of cyclopentane moieties in the membrane GDGTs at lower pH, the same response as is observed here for the sedimentary AOA. Cultivation at a lower salinity (i.e. "*Ca*. N. limnia" was grown at ~8‰ salinity) is an unlikely explanation for the low TEX_{86} value of "*Ca*. N. limnia" since Wuchter et al. (2004) already demonstrated for a marine Group I crenarchaeotal enrichment that salinity has no effect on TEX_{86}. This is further corroborated by the identical marine and lacustrine TEX_{86}-temperature calibrations (Powers et al., 2010).

Lipids with the same molecular weight as GDGTs 1-3 but with slightly earlier elution times (usually leading the main GDGT peak) have been recovered from the environment (e.g. Pitcher et al., 2009), and appear similar to those observed in the sedimentary AOA enrichments (Figure 2b-c, GDGTs 1', 2', and 3'). More commonly reported is a lipid similar to GDGT-4 (Pearson et al., 2008; Sinninghe Damsté et al., 2002b), however GDGT-4' was not detected in SJ and AR, and present in trace amounts in "*Ca*. N. limnia" (Table 2). Unambiguous identification of these compounds has yet to be performed, however, MS-MS data show that mass spectral wise they are identical to the later-eluting GDGTs, and therefore represent structural isomers (Hopmans et al., unpublished data). In some soils and hot spring sediments, these isomers can be as abundant as the known GDGTs (Pitcher et al., 2009), however, they are not abundant in marine suspended particulate matter and surface sediments. Their abundance in the AOA studied here compared to other AOA, points to sedimentary AOA as primary producers of

these unknown lipids in marine environments, however their sources in terrestrial environments remains unknown. In addition, some other late-eluting lipids were present in the SJ and AR (Figure 2 b-c, GDGTs 1'', 2'', and 3''). At the moment, however, their structures remain enigmatic. A late-eluting lipid related to crenarchaeol with an additional cyclopentane moiety (*m/z* 1290, GDGT X; Figure 1) was recently identified in the moderate thermophile "*Ca*. N. gargensis", that falls into the soil Group I.1b AOA (Pitcher et al., 2010b). We did not identify this GDGT in either of the AOA enrichments analyzed in this study.

IPL-GDGTs

IPL headgroups synthesized in notable amounts by all three cultures include DH, 'MH+180', and HPH moieties, suggesting they are important in the cell membranes of marine AOA. Trace MH and only small apparent amounts of PH headgroup moieties were detected in "*Ca*. N. limnia", which were completely absent in SJ and AR, signifying that these headgroups may not be widely synthesized by marine AOA, despite apparently larger relative abundances of these IPLs in "*Ca*. N. maritimus" (Schouten et al., 2008). Comparison of the IPL composition of "*Ca*. N. limnia", SJ, and AR AOA with those identified previously for "*Ca*. N. maritimus" and "*Ca*. N. gargensis" showed that they all make IPL-GDGTs with DH and HPH headgroups (Table 4). Further comparison of the GDGTs associated with each headgroup indicated that HPH bound to crenarchaeol represents the only IPL common to all five species, consequently rendering HPH-crenarchaeol the best biomarker lipid for the detection of living AOA. In addition to its uniqueness to AOA, PLs in general are superior to GLs as indicators of living organisms due to their lower stability outside the cell membrane (i.e. upon cell senescence) (Harvey et al., 1986; Schouten et al., 2010).

Lipp and Hinrichs (2009) found differences between core and GL-derived GDGT distributions, concluding that they must derive from different source communities (e.g. fossil planktonic versus *in situ* production by sedimentary Archaea). Although our results show that sedimentary Group I Crenarchaeota do indeed synthesize a suite of GDGTs (including crenarchaeol and its regioisomer), GDGTs associated with particular headgroups

seem to occur in a similar way as those in "*Ca*. N. maritimus": GDGTs 0, 1 and crenarchaeol are largely associated with PH and HPH headgroups, while minor GDGTs 2-4 are usually associated with DH and 'MH+180' moieties (cf. Table 3). Thus, synthesis of IPL-GDGTs by sedimentary AOA is not necessarily different than that of pelagic AOA. Indeed, our results suggest that different distributions in sedimentary core and IPL-GDGT pools, as observed by Lipp and Hinrichs (2009) may be due to differences in the lability of PLs compared to GLs (i.e. free PLs degrade much more rapidly than GLs): degradation of PLs will release GDGTs with a different composition than those of GLs. If so, then these differences do not reflect the variable contributions of planktonic versus sedimentary Group I Crenarchaetoa. The presence of GL-GDGTs in sediments does not unambiguously implicate the presence of living sedimentary AOA, as a proportion of the GLs synthesized in the water column have the potential to reach seafloor sediments (Schouten et al., 2010). In cases where sediments do not contain any GDGT-based PLs (i.e. with a PH or HPH headgroup), particularly HPH-crenarchaeol, the presence of a substantial and viable sedimentary AOA community cannot be unambiguously established using lipid analysis alone.

The implications drawn from differences between glycosidic and phosphate-based IPLs raise additional interest in other structurally enigmatic headgroup moieties. Common among the AOA is a 'MH+180' Dalton headgroup (Table 3), which was originally identified in marine sediments (Sturt et al., 2004; Biddle et al., 2006) prior to confirmation of its biosynthesis by "*Ca*. N. maritimus" (Schouten et al., 2008). With the exception of "*Ca*. N. gargensis", all four other AOA synthesize IPL-GDGTs containing this headgroup, signifying that it may be a commonly synthesized moiety among Group I.1a AOA. IPL analysis of additional AOA from other Group I lineages, however, is needed to confirm this. Nevertheless, MH+180 bound to crenarchaeol has recently been detected in water column SPM (Pitcher et al., 2010a). Interestingly, "*Ca*. N. limnia" synthesizes what we have tentatively identified as a GDGT-based IPL with a double 180 Dalton moiety (Figure 3, Table 4), which has not been previously reported as far as we are aware.

Effects of filter type and TCA extraction on IPLs of "*Ca.* N. limnia"

In contrast to sediments, which are typically freeze-dried and extracted as such, GDGTs present in the marine water column are often recovered by filtration of SPM over GF/F filters, however, the reported size of marine Crenarchaeota (e.g. 0.5x0.15 µm for "*Ca.* N. maritimus"; (Könneke et al., 2005) is often smaller than the GF/F nominal pore size (0.7 µm) typically used for particulate matter filtration intended for lipid analysis. The low recovery of suspended culture of "*Ca.* N. limnia" on GF/Fs compared to the DUO filters (which have 0.7 µm and 0.22 µm pore sizes, respectively) (Table 4), suggests that when planktonic Group I Crenarchaeota are not particle-associated (e.g. as they often are in more turbid waters, such as the North Sea (Wuchter et al., 2006)), a large proportion of cells present will not be caught on those filters. However, since the effective pore size will decrease with an increasing load on the filter, field experiments filtering turbid waters should result in much higher recovery than we observed here (Herfort et al., 2006). While in our study, nominal pore size had no major observable effect on the IPL distribution (which was not surprising as the culture was uni-archaeal), it could result in quantitative biases in GDGT/IPL distributions recovered from the environment where differences could exist in the cell size and/or shape, and propensity of local Group I Crenarchaeota to associate with particles.

Sequential extraction of the DUO filters with TCA resulted in a substantial additional recovery of IPLs (i.e. 41% of the total recovered IPLs), with the same distribution as those extracted using Bligh and Dyer solvents, in contrast to the GFF filters, where TCA extraction did not yield substantial additional amounts. Previously, it was noted that sequential extraction of whole cell biomass of "*Ca.* N. gargensis" with TCA did not result in significant increase in IPL yields (Pitcher et al., 2010b), thereby suggesting that IPL profiles of SJ and AR are representative and that BDE alone is sufficient for a qualitative assessment of IPLs in culture material, particulate matter and sediments. However, for quantitative analysis, filtration of water over filters with smaller pore size and additional extraction with TCA may need to be performed.

Conclusions

We report the core and IPL-GDGTs synthesized by three sedimentary marine Group I.1a AOA, enriched from the marine sediments from the San Francisco Bay Estuary ("*Ca*. N. limnia"), the South Korean coast (SJ) and Svalbard, Norway (AR). These cultures represent the first AOA enriched directly from the marine environment and therefore provide the most robust comparison to sedimentary GDGT distributions. They all synthesize crenarchaeol and minor amounts of the crenarchaeol regioisomer, confirming crenarchaeol as a widespread biomarker for AOA in the marine environment and a potential *in situ* contribution of local Group I Crenarchaeota to sedimentary GDGT pools. Major GDGT-based IPLs synthesized by "*Ca*. N. limnia", SJ, and AR, contained HPH-headgroups attached primarily to crenarchaeol and GDGT-0, in addition to a variety of sugar-based headgroups bound mainly to minor GDGTs 2-4. The occurrence of HPH-crenarchaeol in all AOA analyzed for IPLs thus far points to its utility as a general biomarker for viable AOA. Systematic differences in GDGT-headgroup associations may have implications for our understanding of environmental GDGT distributions, particularly when trying to dissociate 'dead' from 'living' archaeal lipid signals using core and GL-GDGT distributions, respectively.

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Chapter 6

Niche partitioning of ammonia-oxidizing Archaea and anammox Bacteria in the Arabian Sea oxygen minimum zone as determined by a combined intact polar lipid and gene-based approach

Angela Pitcher[1], Laura Villanueva[1], Ellen C. Hopmans[1], Stefan Schouten[1,2], Gert-Jan Reichart[2] and Jaap S. Sinninghe Damsté[1,2]

[1] NIOZ Royal Netherlands Institute for Sea Research, Departments of Marine Organic Biogeochemistry and Biological Oceanography, PO Box 59, 179AB Den Burg, The Netherlands

[2] Department of Earth Sciences, Faculty of Geosciences Utrecht University, P.O. Box 80.021, 3508 TA Utrecht, The Netherlands

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Abstract

Ammonia-oxidizing Archaea (AOA) and anaerobic ammonium oxidizing (anammox) Bacteria have emerged as significant actors in the marine nitrogen cycle. In oxygen-limited settings particularly, they affect fixed-nitrogen loss through the oxidation of ammonium to nitrite and dinitrogen gas, respectively. Potential for interaction between these groups

exists, however, their distributions are rarely determined in tandem. Here we have examined the vertical distribution of AOA and anammox Bacteria through the Arabian Sea oxygen minimum zone (OMZ), the most intense and vertically-exaggerate OMZ in the global ocean, using a unique combination of intact polar lipid (IPL) and gene-based analyses. To screen for AOA-specific IPLs, we developed an HPLC/MS/MS method targeting hexose-phosphohexose (HPH) crenarchaeol, a common IPL of cultivated AOA. HPH-crenarchaeol showed highest abundances in the upper and lower OMZ transition zones at oxygen concentrations of ca. 5 µM, coincident with peaks in both Marine Group I Crenarchaeota 16S rRNA and crenarchaeal *amoA* gene abundances. In contrast, concentrations of anammox-specific IPLs peaked within the core of the OMZ at 600 m where oxygen reached the lowest measured levels, and coincided with peak anammox 16S rRNA gene abundances. Together, the data reveal a unique depth distribution of abundant putative AOA and anammox Bacteria. Partitioning of their respective niches by ≥ 400 m suggests that direct coupling of their metabolisms does not occur in the Arabian Sea OMZ.

Introduction

The oxygen minimum zone (OMZ) of the Arabian Sea represents a globally important site for oceanic nitrogen (N) loss (Bange et al., 2000; Bange et al., 2005 and references therein). Its vertical distribution extends from approximately 150 to 1000 meters below sea level with oxygen concentrations at times as low as 0.1µM (Morrison et al., 1999; Paulmier and Ruiz-Pino, 2009), rendering it the most expansive and intense OMZ in the global ocean. N-cycling in the Arabian Sea is so important in fact, that it may impact the earth's climate due to (OMZ intensity-related) fluctuations in N-loss via heterotrophic denitrification (e.g. Suthhof et al., 2001)**.** This process simultaneously liberates the potent greenhouse gas, nitrous oxide (N_2O), as a metabolic intermediate while circumventing the biological pump via respiratory emancipation of carbon dioxide (CO_2).

Anaerobic ammonium oxidation (anammox) also occurs to a great extent in oxygen-limited waters where canonical denitrification was conventionally assumed to

dominate N-loss (c.f. Arrigo, 2005; Brandes et al., 2007 and references therein). This autotrophic metabolism combines ammonium (NH_4^+) with nitrite (NO_2^-) to form dinitrogen gas (N_2) (Strous et al., 1999), most of which is then lost from the system. Anammox may be responsible for up to 40% of N-loss in some anoxic marine environments (Dalsgaard et al., 2003; Kuypers et al., 2003; Kuypers et al., 2005), although recent evidence suggests that its contribution to N-loss in the Arabian Sea OMZ may be considerably less (Ward et al., 2009). As their role in oceanic N-cycling is gradually becoming more clear (Lam et al., 2009; Voss and Montoya, 2009), the questions of when, where and under what conditions anammox Bacteria thrive is receiving ever-increasing attention.

Ammonia-oxidizing Archaea (AOA), also use NH_4^+ as an electron donor which is oxidized aerobically to NO_2^-. The ubiquity and abundance of the marine Group I Crenarchaeota (MCGI) and their associated genes coding for the alpha subunit of ammonia monooxygenase enzyme (*amoA*) in marine waters have suggested their potential importance in oceanic nitrification (Wuchter et al., 2006; Francis et al., 2007), subsequently stimulating a need to define the ecological niches in which they are most likely to persist (Prosser and Nicol, 2008; Erguder et al., 2009). AOA have been previously recovered from multiple oceanic oxygen-limited settings where they appear best adapted to depths below the photic zone at or near the onset of suboxia (Coolen et al., 2007; Lam et al., 2007; Beman et al., 2008; Lam et al., 2009; Molina et al., 2010), but their effect on marine nitrification is still unclear.

The distribution of AOA in the Arabian Sea remains largely unstudied; their contribution to nitrification within this OMZ remains unresolved. The same holds for the annamox bacteria, although recent work has demonstrated their activity in the upper depths of the Arabian Sea (Ward et al., 2009). A secondary NO_2^- maximum (SNM) is often observed between depths of ca. 200-500 m (Naqvi, 1991; Naqvi, 1994), which is assumed to be the primary result of denitrification (specifically NO_2^- derived from NO_3^- reduction) and the most likely site for anammox activity (Ward et al., 2009; Bulow et al., 2010). The depth relation between anammox Bacteria and AOA in the Arabian Sea has yet to be determined, and could provide valuable information regarding their niche preferences.

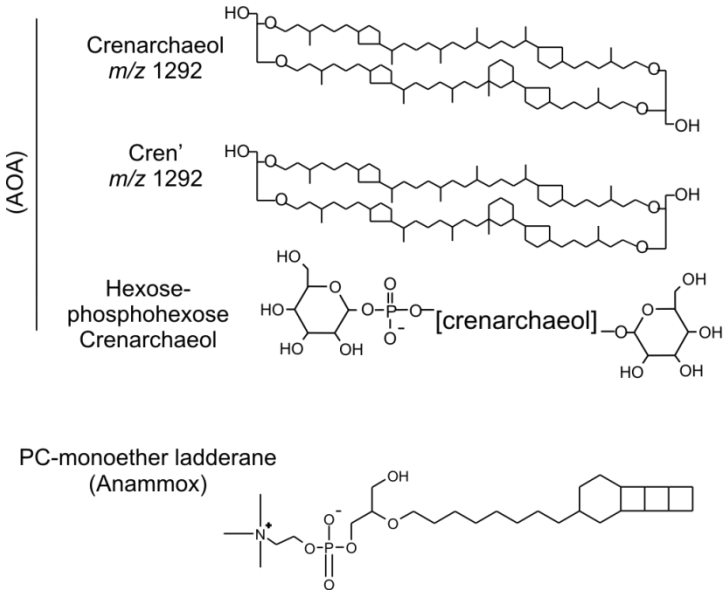

Figure 1. Structures of lipids specific to ammonia-oxidizing Archaea (AOA): crenarchaeol, the crenarchaeol regioisomer (Cren'), and crenarchaeol with hexose and phosphohexose headgroups, and Anammox Bacteria: C_{20}-[3]-monoether ladderane lipid containing a PC headgroup.

The first studies to implicate the presence of anammox Bacteria and AOA in the Arabian Sea quantified core (i.e. fossil) membrane lipids recovered from suspended particulate matter (SPM) ranging in depth from 35 to 1500 m (Sinninghe Damsté et al., 2002a; Jaeschke et al., 2007). Crenarchaeol, a glycerol dialkyl glycerol tetraether (GDGT) with four cyclopentane moieties and one cyclohexane moiety (Sinninghe Damsté et al., 2002b) (Figure 1) which is thought to be specific to AOA (de la Torre et al., 2008; Pitcher et al., 2010b), was recovered from SPM at four depths in the water column with highest concentrations located just below the photic zone (Sinninghe Damsté et al., 2002a). From these same samples, anammox-specific ladderane core lipids were also recovered and found to be most abundant within the core of the OMZ at depths of ca. 500 m (Jaeschke et al., 2007). The core lipid data suggests that AOA and anammox Bacteria may not occupy proximal water column depths. Such a suggestion is highly speculative, however, since (i)

core lipid quantification is unlikely to reflect active *in situ* lipid production as fossilized lipids can, in some cases, represent the majority of lipids recovered (e.g. Pitcher et al., 2009b) and (ii) the resolution of SPM sampling was relatively low. This first caveat can be readily addressed by the analysis of intact polar lipids (IPLs), which consist of the core membrane lipids still covalently bound to polar headgroups. These molecules are relatively labile (i.e. unstable outside of the intact cell) (White et al., 1979), thus IPL distributions may more accurately represent lipids synthesized by living, or recently living, cells.

So far, two representatives of Group I AOA, and four genera of anammox Bacteria, have been screened for their IPLs (Boumann et al., 2006; Schouten et al., 2008; Rattray et al., 2008; Pitcher et al., 2010b). Selected reaction monitoring (SRM) of the C_{20}-[3]-monoether ladderane lipid containing a PC headgroup (henceforth referred to as 'PC-monoether ladderane') (Figure 1) has allowed for highly specific detection of this lipid to detect living anammox Bacteria in a number of sedimentary environments (Jaeschke et al., 2009). Until now, SRM has not been employed for AOA IPL measurements - which have consequently relied on indirect methods (Pitcher et al., 2009a; Huguet et al., 2010) or less sensitive full-scan analysis (Sturt et al., 2004; e.g. Biddle et al., 2006; Schubotz et al., 2009). In this study, we developed a novel SRM method to target AOA-specific IPLs. In addition, we have applied the PC-monoether ladderane SRM of Jaeschke et al. (2009) to marine SPM for the first time. The aim of this work was to employ both SRM methods on marine SPM from the Arabian Sea water column and combine these results with conventional quantitative gene-based approaches to delineate the respective distributions of both AOA and anammox Bacteria through the Arabian Sea water column in a higher resolution than the previous lipid studies.

Materials and Methods

Physical properties of the water column

A free-falling conductivity-temperature-depth (CTD) system equipped with attached oxygen, turbidity, and fluorescence sensors, was deployed to record the physical

properties of the water column at a depth profile station in the Northern Arabian Sea (lat 21°55.6', long 63°10.6') (Figure 2).

SPM Sampling

A depth profile of 12 SPM samples was collected at our sampling station by large-volume (ca. 200-1700 L) *in situ* pump filtration onto pre-washed 0.7 µm GF/F filters (Pall Corporation). A total of six deployments of two McLane WTS-LV *in situ* pumps (McLane Laboratories Inc., Falmouth) were carried out between 14 and 20th of January 2009. Upon retrieval of the pumps, GF/F filters containing SPM were removed and immediately frozen at -80°C. A rosette sampler containing 24×12-L Niskin bottles was attached to the CTD deployed during each pump cast to collect water from for inorganic nutrient analysis and onboard filtration of DNA. For nutrients, ca. 5-mL samples were filtered over 0.45-µm 25-mm Acrodisc HT Tuffryn Membrane syringe filters (Pall Corporation) into pre-rinsed pony vials. DNA was filtered onboard from water decanted from the Niskin bottles via clean Teflon tubing into pre-rinsed 20-L Nalgene bottles in a dark climate chamber maintained at 7°C over 142-mm, 0.2-µm PC filters (Millipore, Isopore) and immediately stored at -80°C.

Extraction and Analysis of Intact Polar Lipids

Intact polar lipids were extracted from freeze-dried biomass using a modified Bligh and Dyer technique (Bligh and Dyer, 1959). A known volume of single-phase solvent mixture of methanol (MeOH):dichloromethane (DCM):phosphate buffer (2:1:0.8, v/v/v) was added to the sample in a glass centrifuge tube and placed in an ultrasonic bath for 10 min. The extract and residue were separated by centrifuging at 2500 rpm for 5 min and the solvent mixture collected in a separate flask (repeated 3 times). The DCM and phosphate buffer were added to the single-phase extract to give a new ratio of MeOH:DCM:phosphate buffer (1:1:0.9, v/v/v), and to induce phase separation. The extract was centrifuged at 2500 rpm for 5 min. The DCM phase was collected in a round-bottom flask and the MeOH:phosphate buffer phase was washed 2 additional times with DCM. The combined DCM phases were reduced under rotary vacuum and evaporated to dryness under a stream of N_2.

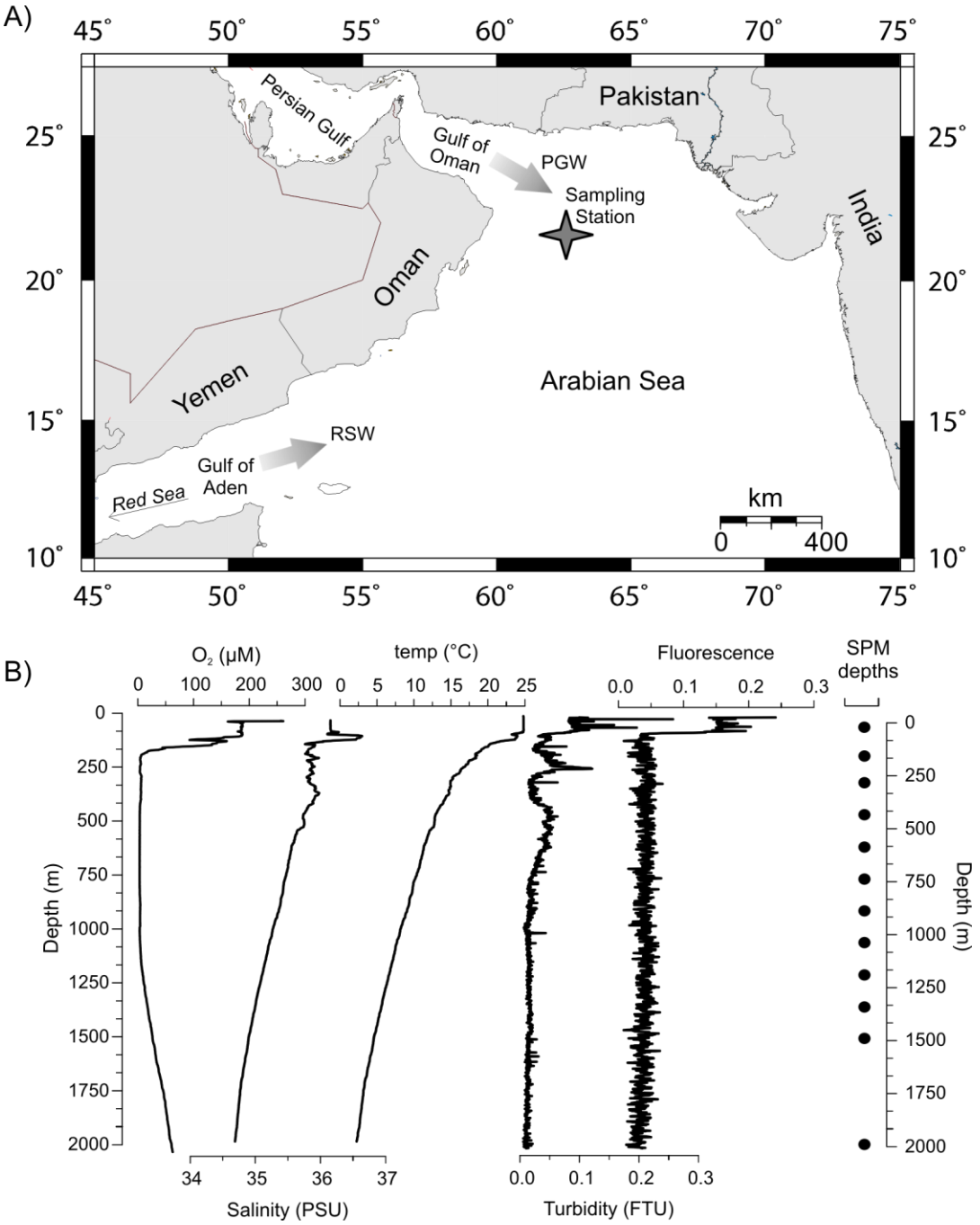

Figure 2. (A) Location of Arabian Sea sampling station (star; lat 21°55', long 63°10'). Persian Gulf Water (PGW) and to a much lesser extent, Red Sea Water (RSW), influence the oxygen minimum zone (OMZ) at our study site. (B) Hydrographic characteristics of the water column at our sampling site as recorded from the conductivity-temperature-depth (CTD) sensors, and suspended particulate matter (SPM) sampling depths.

Selected Reaction Monitoring of Specific IPLs

Detection of crenarchaeol-based IPLs was achieved by HPLC/electrospray ionization (ESI)–MS/MS using chromatographic conditions and source settings as described by Schouten et al. (2008). In order to detect the specific crenarchaeol-based IPLs in high sensitivity we developed a selected reaction monitoring (SRM) method using IPLs extracted from "*Ca*. Nitrososphaera gargensis" (Hatzenpichler et al., 2008) biomass (Pitcher et al., 2010b). Crenarchaeol-hexose, crenarchaeol-dihexose, crenarchaeol-hexose–phosphohexose, crenarchaeol-hexose-'176', and crenarchaeol-dihexose-'176' ('176' represents an unknown headgroup with a mass of 176 Dalton) were targeted for SRM. Transitions were based on published mass spectra and optimized by direct infusion of an IPL extract of "*Ca*. N. gargensis" resulting in the conditions listed in Table 1, with an Argon collision gas pressure of 0.8 mTorr. An additional SRM transition was created for crenarchaeol with a hexose and an additional unknown headgroup with a mass of 180 Daltons, which is a major IPL in *Nitrosopumilus maritimus* SCM1 (Schouten et al., 2008). Since there was not sufficient IPL extract of *Nitrosopumilus maritimus* available to optimize the SRM transition for this compound, the SRM conditions were based on those for crenarchaeol-hexose-'176'.

There was insufficient "*Ca*. N. gargensis" biomass to prepare purified standards for quantification of crenarchaeol-based IPLs. Therefore, IPLs were quantified as the integrated IPL peak area response L^{-1}. This approach reveals depth-related distribution patterns in individual IPLs but does not allow the comparison of different IPL abundances. Long-term instrument response was monitored by injection of equivalent amounts of "*Ca*. N. gargensis" Bligh and Dyer extract, every 6 to 10 runs.

The C_{20}-[3]-monoether ladderane lipid containing a PC headgroup (PC-monoether ladderane) (Figure 1) was analyzed by HPLC/ESI-MS/MS using an SRM method described previously (Jaeschke et al., 2009).

Total DNA extraction

DNA was extracted from SPM filtered onto 142-mm, 0.2-µm polycarbonate filters. Filters were cut into ca. 0.5 × 0.5 cm squares with sterile scissors prior to extraction. Cells

were lysed by bead-beating with 1.5 g of sterile 0.1-mm zirconium beads (Biospec Products, Inc.) in an extraction buffer containing 10 mM Tris-HCl pH 8, 25 mM Na_2EDTA pH 8, 1% (v/v) sodium dodecyl sulfate (SDS), 100 mM NaCl, and molecular biology grade water. Samples were incubated at 70°C for 30 minutes and then extracted with phenol-chloroform (Sambrook et al., 1989). After extraction, DNA was precipitated using ice-cold ethanol, dried, and re-dissolved in 100 µl of 10 mM Tris-HCl, pH 8. Total nucleic acid concentrations were quantified spectrophotometrically (Nanodrop, Thermo Scientific, Wilmington, DE, USA) and checked by agarose gel electrophoresis for quality. Extracts were kept frozen at -80°C.

Preparation of the Q-PCR standards

The Archaeal 16S rRNA gene for the standard was amplified by using the archaeal-specific 16S rRNA gene primer set Ar8F (5'-TCC GGT TGA TCC TGC C-3'; (Teske et al., 2002) and Ar958r (5'-YCC GGC GTT GAM TCC AAT T-3'; Lane, 1991; Tm = 54 °C) and genomic DNA of *Nitrosopumilus maritimus.* The archaeal *amoA* gene of the standard was amplified from DNA of *N. maritimus* with primers Nitrosop_*amoA*F (5'-GCT AAG ACG ATG TAC ACA CTA-5', this study) and Cren*AmoA*ModR (5'-AAG CGG CCA TCC ATC TGT A-3'; Mincer et al. (2007); Tm = 51 °C). 16S rRNA fragment of anammox Bacteria was amplified from a sample of a wastewater treatment plant described in Jaeschke et al. (2008) with primers An7F (5'-GGC ATG CAA GTC GAA CGA GG-3') and An1388R (5'-GCT TGA CGG GCG GTG TG-3'; Penton et al. (2006); Tm = 58 °C). PCR reaction mixture was the following (final concentration): Q-solution 1x; PCR buffer 1x; BSA (200 µg ml^{-1}); dNTPs (20 µM); primers (0.2 pmol $µl^{-1}$); $MgCl_2$ (1.5 mM); 1.25 U Taq polymerase (Qiagen, Valencia, CA, USA). PCR conditions for these amplifications were the following: 95°C, 5 min; 35× [95°C, 1 min; Tm, 1 min; 72°C, 1 min]; final extension 72°C, 5 min. PCR products were gel purified (QIAquick gel purification kit, Qiagen) and cloned in the TOPO-TA cloning® kit from Invitrogen (Carlsbad, CA, USA) and transformed in *E. coli* TOP10 cells following the manufacturer's recommendations. Recombinant clones were screened by sequencing using M13F (-20) (5'-GTA AAA CGA CGG CCA G-3') and M13R (5'-CAG GAA ACA GCT ATG AC-3') primers with

BigDye® v1.1 sequencing kit on a ABI PRISM® 310 Genetic analyzer (Applied Biosystems, Foster city, CA, USA). Recombinant plasmids were purified by Qiagen Miniprep kit, linearized by restriction digestion and quantified by Nanodrop before using as standard for quantification. Marine Crenarchaeota Group I (MCGI) 16S rRNA gene fragment for Q-PCR standard was obtained as described previously (Coolen et al., 2007). Copy numbers of the genes were calculated directly from the concentration of the purified DNA. Ten-fold serial dilutions ranging from 10^7 to 10^0 copies of a known copy number of the standard were used per Q-PCR reaction in triplicate to generate an external quantification standard.

Q-PCR analysis

All Q-PCR analyses were performed on a Biorad CFX96™ Real-Time System/C1000 Thermal cycler equipped with CFX Manager™ Software. The copy numbers of archaeal 16S rRNA genes were estimated by using the 16S specific primers Parch519F and ARC915R (Coolen et al., 2004); MCGI 16S rRNA genes with MCGI-391F and MCGI-554R as described by Coolen et al., 2007; archaeal *amoA* with primers Cren*AmoA*Q-F (5'-GCA RGT MGG WAA RTT CTA YAA-3') and Cren*AmoA*ModR (Mincer et al., 2007); 16S rRNA genes of anammox with Brod541F (5'-GAG CAC GTA GGT GGG TTT GT-3') and Amx820R (5'-AAA ACC CCT CTA CTT AGT GCC C-3') as described by (Li et al., 2010). Gene copies were determined in triplicate on diluted DNA extract (30×). The reaction mixture (25 µl) contained 1 unit of Pico Maxx high fidelity DNA polymerase (Stratagene, Agilent Technologies, Santa Clara, CA, USA) 2.5 µl of 10x Pico Maxx PCR buffer, 2.5 µl 2.5 mM of each dNTP, 0.5 µl BSA (20 mg ml^{-1}), 0.02 pmol μl^{-1} of primers, 10 000 times diluted SYBR Green® (Invitrogen) (optimized concentration), 0.5 µl of 50 mM $MgCl_2$ in ultra pure sterile water. All reactions were performed in iCycler iQ™ 96-well plates (Bio-Rad, Hercules CA, US) with optical tape (Bio-Rad). One µL of diluted or non-diluted environmental DNA was added to 24 µL of mix in each well. Specificity of the reaction was tested with a gradient melting temperature assay. The cycling conditions for the Q-PCR reaction were the following: 95°C, 4 min; 40–45× [95°C, 30 s; Tm, 40 s; 72°C, 30 s]; final extension 80°C, 25 s. Specificity for Q-PCR reaction was

tested on agarose gel electrophoresis and with a melting curve analysis (50°C–95°C; with a read every 0.5°C held for 1 s between each read) in order to identify unspecific PCR products such as primer dimers or fragments with unexpected fragment lengths. Melting temperature, PCR efficiencies and correlation coefficients for standard curves were as follows: for the Archaeal 16S rRNA gene assay, Tm = 62°C, 105.8% and R^2 = 0.997; MCGI 16S rRNA gene assay, Tm = 61°C, 80.2% and R^2 = 0.989; for the archaeal *amoA* assay, Tm = 55°C, 72% and R^2 = 0.998; for the 16S rRNA gene anammox, Tm = 59°C, 87.7% and R^2 = 0.99.

Results

Hydrographic setting and water column chemistry

A depth profile of twelve SPM samples was taken at the southeast slope of the Murray Ridge where the water depth reaches 3010 m (Figure 2). Dissolved oxygen concentration as measured by the CTD oxygen sensor, decreased from fully saturated at the surface to ca 2.5 µM (i.e. near detection limit) within the core of the OMZ and increased again with depth starting at 1050 m (Figure 2B). Lack of measurable H_2S also indicated absence of euxinic conditions. Salinity showed increases at 95 m and 325-400 m, corresponding to the influx of Arabian Sea High Salinity Water (ASHSW) and the Persian Gulf Outflow (PGW) water masses, respectively (Shetye et al., 1994). Particulate matter, as indicated by turbidity, was concentrated towards the surface where fluorescence was also high, but also showed increased amounts between 170-300 m, and 450-750 m. Ammonium (NH_4^+) concentrations showed some variation with depth, peaking just above the OMZ at 0.14 µM. In contrast, nitrite (NO_2^-) peaked closer to the surface (0.53 µM) and showed elevated concentrations again at 600 m (0.18 µM) (Figure 4B).

Distribution and abundance of lipids and genes

We optimized a newly developed SRM method for five of the main crenarchaeol-based IPLs synthesized by "*Ca*. N. gargensis" with the following headgroups: hexose,

hexose+'176', dihexose, dihexose+'176' and hexose+phosphohexose (Figure 3A, Table 1). Of these IPLs, three were detected at high levels in the Arabian Sea SPM: the monohexose (MH), dihexose (DH), and hexose-phosphohexose (HPH)-crenarchaeol, each of which were present at all depths but in variable relative abundances (example SPM trace shown in Figure 3B). Trace amounts of the hexose+'180' crenarchaeol IPL found in *N. maritimus* SCM1 were also sometimes observed (data not shown). For comparison with Archaeal genes we focused on HPH-crenarchaeol as opposed to the hexose-based IPLs, it is an abundant compound in all screened AOA thus far (Schouten et al., 2008; Pitcher et al., 2010a; Pitcher et al., 2010b) and is likely to be a superior biomarker for putative AOA due to the labile nature of the phosphate-ester bond compared to the glycosidic ether bond (Harvey et al., 1986; Schouten et al., 2010).

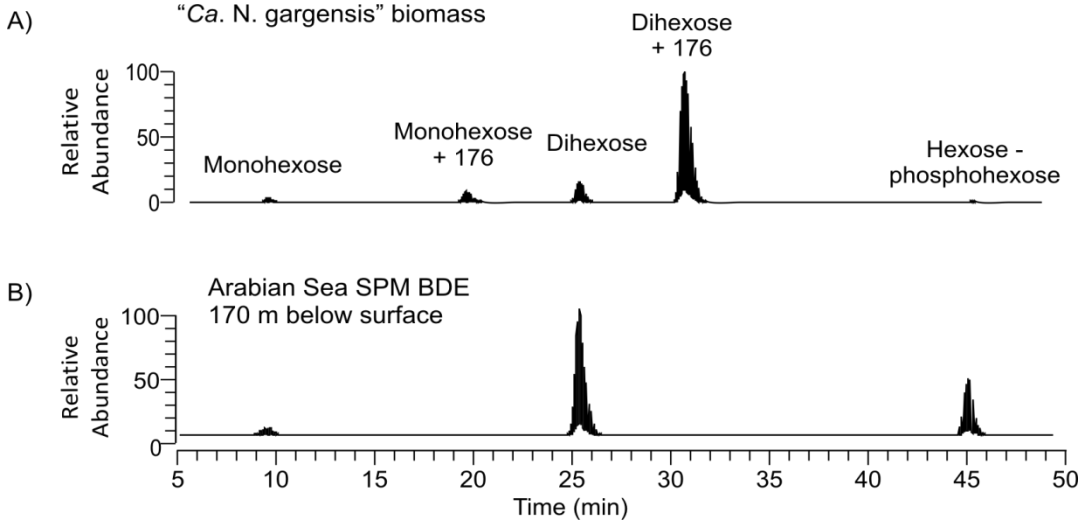

Figure 3. Selective reaction monitoring (SRM) of crenarchaeol-based IPLs with headgroups (labeled peaks; see Table 1 for specific monitored transitions). Total ion current of the SRM traces of (A) "*Ca*. N. gargensis" biomass and (B) total ion current of the SRM trace of suspended particulate matter collected at 170 m from the Arabian Sea.

Table 1. Parameters used for selected reaction monitoring of specific crenarchaeol-based IPLs

headgroup	fragments	Parent $[M+NH_4]^+$	Product *m/z*	Collision Energy (V)
monohexose	1292	1471.2	1292.3	-24
monohexose+176	1292, 1454	1647.4	1292.3	-35
dihexose	1292	1633.5	1292.3	-28
dihexose+176	1292, 1454	1809.0	1292.3	-35
hexose, phospho-hexose	1534	1713.4	1534.4	-35
monohexose+180*	1292, 1454	1651.4	1292.3	-35

* Used the same conditions as monohexose + 176. This is a common IPL synthesized by the marine species, *Nitrosopumulis maritimus* (Schouten et al. 2008) but is not synthesized by "*Ca* . N. gargensis" (Pitcher et al. 2010).

HPH-crenarchaeol showed a substantial increase from the surface waters to 170 m, where its relative abundance reached the highest detected levels and then decreased rapidly to 450 m (Figure 4C). HPH-crenarchaeol abundance was high again between 600 and 1200 m, peaking a second time at 1050 m. Copy numbers of marine Group I Crenarchaeota (MCGI) 16S and crenarchaeal *amoA* genes followed IPL distributions, also peaking at 170 and 1050 m (Figure 4D). Total Archaeal 16S rRNA gene copy numbers peaked at these depths, but also at 600 m. Archaeal 16S copies were more abundant than MCGI 16S genes at all depths, but particularly in the upper half of the water column where the fraction of Archaea represented by MCGI ranged from only 3-30% (Figure 4E).

Anammox Bacteria markers, PC-monoether ladderane and 16S rRNA genes, co-varied well throughout the water column (Figure 4F and G). In contrast to AOA, maximum abundances were observed in the core of the OMZ between 450 and 750 m. Both PC-monoether ladderanes and anammox 16S genes were low outside this range, with the exception of the PC-monoether ladderane concentration measured at 170 m which was notably elevated compared to gene copies. Full scan MS analysis showed that additional lipids in the water column (e.g. PC-lysolipids) contributed to the SRM signal here in contrast to other SPM samples and therefore this data point is omitted from further discussion.

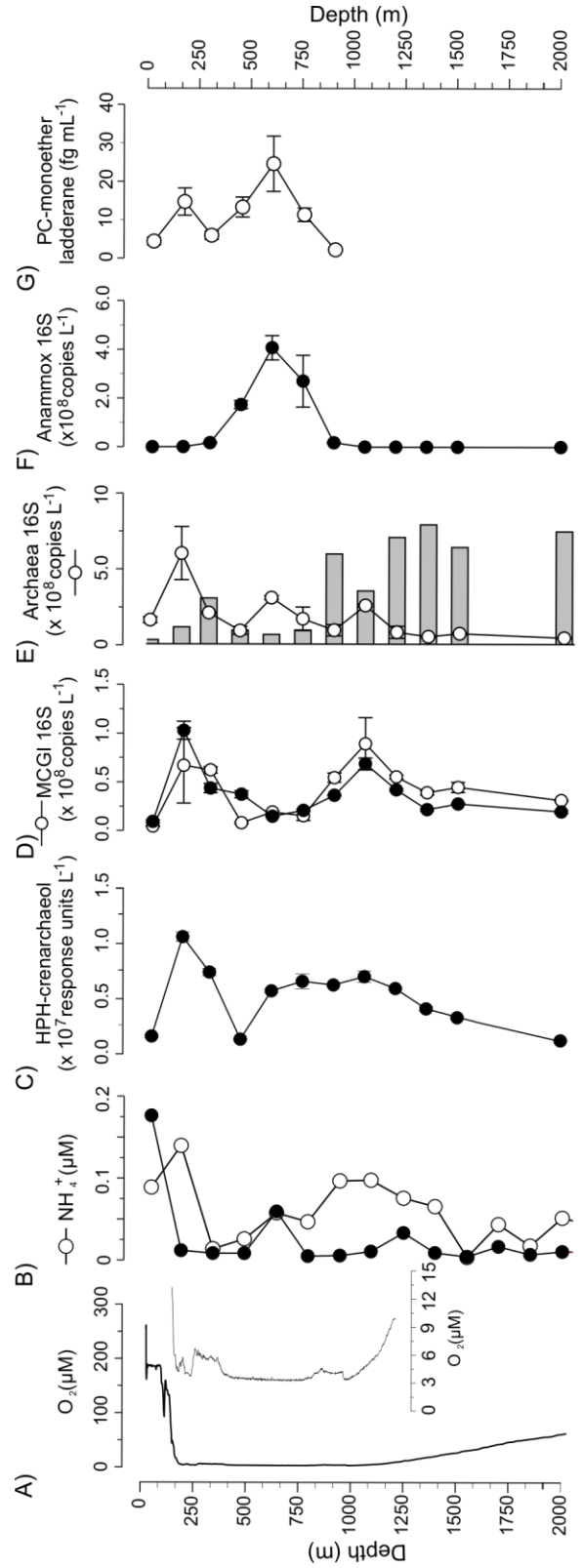

Figure 4. Depth profile at sampling station: (A) Oxygen (O_2); (B) ammonium (NH_4^+) and nitrite (NO_2^-); (C) HPH-crenarchaeol; (D) MCGI 16S rRNA and crenarchaeal *amoA* gene abundances; (E) Archaea 16S rRNA gene abundance and percent of total Archaea represented by MCGI (bars); (F) anammox Bacteria 16S rRNA gene abundance; and (G) PC-monoether ladderane abundance.

Discussion

Depth distributions of AOA

The results of our IPL and gene-based analyses show that AOA are abundant in the Arabian Sea and confirm previous evidence for the occurrence of planktonic Crenarchaeota in the Arabian Sea based on the recovery of fossil crenarchaeol (Sinninghe Damsté et al., 2002a). The specificity of crenarchaeol to AOA (de la Torre et al., 2008; Pitcher et al., 2010b) coupled to the occurrence of HPH-crenarchaeol in all screened AOA to date (Schouten et al., 2008; Pitcher et al. 2010a; Pitcher et al., 2010b), render HPH-crenarchaeol as the most fitting biomarker lipid for tracking AOA. Similarities between AOA genes and IPL profiles (Figure 4) indicate that our AOA-specific crenarchaeol-based IPL SRM is a suitable tool for tracking AOA occurrence in the marine water column.

AmoA and MCGI 16S rRNA gene abundances were positively correlated, suggesting that the majority of local MCGI are ammonia oxidizers. However, the correlation was somewhat weak (R^2 = 0.52) and the archaeal *amoA*:16S ratio was high (average of 3.8) compared to other environmental studies (Wuchter et al., 2006; Mincer et al., 2007; e.g. Beman et al., 2008), indicating that other ammonia-oxidizing organisms may be present (e.g. such as *amoA*-containing pSL12 Archaea - a deeply branching crenarchaeal group related to a hotspring clade) (Mincer et al., 2007). Indeed, not all of the Archaea in our samples hybridized with our MCGI Crenarchaeota primer (Figure 4E).

The presence of a putative AOA community deep in the Arabian Sea is in agreement with recent studies implicating AOA in deep sea nitrification (Konstantinidis et al., 2009; Church et al., 2010; Santoro et al., 2010). We note that this contrasts studies suggesting that deep-dwelling Crenarchaeota do not oxidize ammonia, based on low crenarchaeal *amoA*:16S ratios at depth (Agogue et al., 2008; Kalanetra et al., 2009). Recent work, however, has demonstrated primer mismatches which do not allow for the detection of *amoA* 'Cluster B' sequences, which are commonly recovered from deeper waters (Mincer et al., 2007; Beman et al., 2008), and may be responsible for that discrepancy (Konstantinidis et al., 2009).

The coincidence of peak HPH-crenarchaeol and AOA 16S rRNA and *amoA* gene abundances at 170 and 1050 m points to the OMZ transition zones as possible preferred niches for Arabian Sea Crenarchaeota and indicates that they are adapted to cope with low oxygen concentrations since at both depths, oxygen was ca. 5 µM (Figure 4). Supporting our finding are other water column studies that have revealed similar subsurface peaks in putative AOA abundance at or near the onset of low oxygen concentrations (Coolen et al., 2007; Lam et al., 2007; Beman et al., 2008; Lam et al., 2009; Molina et al., 2010). Although, AOA have been recovered in high abundance from oxygenated marine waters (Wuchter et al., 2006; e.g. Herfort et al., 2007) including the 20 m SPM sample at our station, and have been grown successfully under fully-oxic culture conditions (Könneke et al., 2005; Hatzenpichler et al., 2008; de la Torre et al., 2008), their abundance was much higher at 170 and 1050 m relative to the fully-oxygenated surface water and well-ventilated bottom waters (Figure 4). This indicates that AOA in the Arabian Sea are specifically apt to cope with low-oxygen levels. This is in agreement with recent enrichment studies that showed preferential growth of two AOA at low oxygen concentrations (Park et al., 2010).

Within the OMZ, AOA abundance decreases towards the mid-OMZ depths yielding the lowest abundances between 600-750 m, where oxygen concentrations reach minimal values (i.e. at the detection limit of the CTD oxygen sensor). This could be an indication that oxygen levels are too low within the core of the OMZ to support aerobic AOA activity. If so, then the AOA in the Arabian Sea exhibit an especially narrow range of preferred oxygen conditions (i.e. 5 µM > O_2 > 2.5 µM). Low oxygen has been shown to inhibit growth of *N. maritimus* SCM1 in culture (Km = 3.91 µM), suggesting a limited capacity for survival under very low oxygen/anoxic conditions in nature (Martens-Habbena et al., 2009). In contrast, two recent sedimentary AOA enrichments were shown to grow best under oxygen-limitation (Park et al., 2010). Their presence in the mid-OMZ may be further restricted by competition for NH_4^+ (and/or O_2) with other microbes, such as anammox Bacteria. NH_4^+ concentrations were lower than Km values reported for *N. maritimus* SCM1 (Martens-Habbena et al., 2009), indicating that AOA could be limited by NH_4^+ concentrations at mid-OMZ depths (assuming environmental AOA are also adapted to low substrate conditions). The pointed decrease in AOA abundance towards the mid-OMZ

occurred despite an increase in *in situ* NH_4^+ concentrations, suggesting that oxygen was a more influential factor in determining their depth distribution at this time and location.

Depth distributions of anammox Bacteria

Anammox 16S rRNA gene abundance and PC-monoether ladderane profiles co-vary well (Figure 4F and G) and maximum abundance of anammox genes and IPLs occurred between 450 and 750 m, evidencing a prominent community of putative anammox Bacteria at this depth range. Substantially lower abundance of genes and IPLs at other depths suggests that mid-OMZ depths are optimal for anammox bacteria at our station and time of sampling, despite contrasting evidence that they are present and active at much shallower depths at other stations in the Arabian Sea (Ward et al., 2009; Bulow et al., 2010). Anammox Bacteria have been closely associated with NO_2^- maxima previously in the Black Sea (Kuypers et al., 2003; Lam et al., 2007) and in the Arabian Sea (Ward et al., 2009; Bulow et al., 2010). Therefore, we did not expect to find such high abundances of anammox bacteria in the absence of a strong SNM at our sampling station; within the OMZ NO_2^- was only slightly elevated (0.18 µM) at a single depth (600 m) (Figure 4B). This observation suggests that significant anammox communities may also exist elsewhere where NO_2^- is in lower concentrations, however we note that *in situ* concentration may not be representative of actual flux or turnover. Interestingly, previous core ladderane analyses from Arabian Sea SPM also showed maximum lipid abundances below the SNM at ca. 600 m, at multiple stations off the Omani coast (Jaeschke et al., 2007). Although core lipids were used in this case (as opposed to IPLs) and the sampling resolution was much lower, this nevertheless substantiates our findings and the likelihood of anammox communities existing outside/below the SNM of the Arabian Sea (and potentially other low-NO_2^- marine environments).

The anammox 16S rRNA gene abundances we observed between 450 and 750 m (1.7-4.1 x 10^8) are comparable to those observed by Ward et al. (2009) at shallower depths (80-250 m) where NO_2^- was ca. 5-10 µM. Since Ward et al. (2009) did not analyze samples deeper than the SNM for genes and anammox activity it is not possible to say whether a significant community of anammox Bacteria was present from 450-700 m at their stations.

Nevertheless, our findings indicate that anammox Bacteria may be important in removing nitrogen from core OMZ depths in the Arabian Sea in addition to any denitrification which may also be occurring.

The Arabian Sea SNM has been attributed to denitrification activity in the Arabian Sea (i.e. accumulated NO_2^- from NO_3^- reduction). The absence of a strong SNM at our site suggests the possibility that denitrification may not have been intense, resulting in only scant amounts of denitrification-derived NO_2^- as a substrate for anammox. Alternatively, depending on the activity level of local anammox bacteria, rapid conversion of NO_2^- could mask intense high denitrification. Additional NO_2^- could derive non-locally, namely from the Persian Gulf outflow where high salinity surface waters flow into the Gulf of Oman through the Strait of Hormuz, typically to a mean outflow depth of 275 m in the northern Arabian Sea (Shetye et al., 1994). Water between 250 and 400 m at our sampling station did show an increase in salinity characteristic of PGW, however from 450-750 m no such salinity increase was observed (Figure 2). From the present data is not possible to determine whether the PGW and/or local denitrification was influencing the anammox community at our site. Nevertheless, the identification of abundant biomarker lipids and genes pointing to a substantial community of anammox Bacteria in the Arabian Sea at the core of the OMZ in the absence of a prominent SNM, is an intriguing observation which could imply a large potential contribution of anammox Bacteria to N-loss here.

Implications for potential metabolic coupling of archaeal ammonia-oxidation and anammox

AOA could theoretically provide substrates for anammox in the form of NO_2^-. NH_4^+, however, is required by both and therefore direct coupling of these metabolisms would require competition, and/or alternative sources of this substrate. Despite the fact that NH_4^+ oxidation requires oxygen and anammox Bacteria are inhibited by as little as 1 µM of oxygen in culture (Strous et al., 1997), anammox bacteria have been recovered from water with oxygen of 9 µM (Kuypers et al., 2005), and putative AOA are commonly recovered from near-suboxic waters. Recent work suggested that indeed AOA may contribute up to 40% of the NO_2^- required by anammox in the Black Sea (Lam et al., 2007). Although AOA

and anammox Bacteria could theoretically occupy similar depths in the Arabian Sea, our results suggest that this is not the case, evidenced mainly by the large vertical segregation (> 400 m) of their respective niches (cf. Figure 4). This is in line with suggestions made by Ward et al. (2009), who also hypothesized a non-existent relationship between aerobic ammonium oxidation and anammox at shallower depths. However, if AOA at the time of their sampling campaign would have exhibited the same distribution as we observed, it is possible that the anammox observed at 200 m by Ward et al. (2009) could have indeed been coupled to archaeal ammonia-oxidation. More work is needed to resolve these questions.

Conclusions

Analysis of IPLs, 16S rRNA and metabolic genes recovered from the Arabian Sea water column, showed the presence of substantial communities of putative AOA and anammox Bacteria at the OMZ boundaries, and at core OMZ depths, respectively. Oxygen concentration seems to be a major factor controlling the vertical distribution, and thus niche partitioning of both groups. Whether this is a special case at our site, or a prominent feature of the entire Arabian Sea remains to be determined. Nevertheless, our data illustrate a detailed depth-related community distribution of AOA and anammox together, for the first time in the Arabian Sea which could have implications for our understanding of N-cycling in the Arabian Sea (particularly when and where SNM are less intense or absent) and its overall importance in global marine N-loss. The use of SRM to detect specific IPLs of MCGI and anammox Bacteria appears to be a robust approach for tracking these microbes in the marine water column.

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Chapter 7

Distribution of core and intact polar glycerol dibiphytanyl glycerol tetraether lipids in the Arabian Sea oxygen minimum zone: Implications for their use as biomarker lipids and the TEX_{86} paleothermometer

Stefan Schouten, Angela Pitcher, Ellen C. Hopmans, Laura Villanueva, Judith van Bleijswijk and Jaap S. Sinninghe Damsté

Departments of Marine Organic Biogeochemistry and Biological Oceanography, Royal Netherlands Institute for Sea Research, P.O. Box 59, 1790 AB, Den Burg, The Netherlands

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Abstract

Glycerol dibipytanyl glycerol tetraether lipids (GDGTs) have proven to be important biomarker lipids for groups of Archaea and their distribution is used for paleoenvironmental proxies, particularly paleotemperature. In this study, we analyzed GDGTs in the form of core lipids and as intact polar lipids in suspended particles in the water column of the Arabian Sea which contains a large oxygen minimum zone (OMZ). GDGTs, both as core lipid and as intact polar lipid (IPL), were detected throughout the water column but were most abundant at the upper part of the OMZ. The depth profiles of

IPLs with a crenarchaeol core lipid match previously reported profiles of genes specific for ammonia-oxidizing Archaea (AOA), such as 16S rRNA and archaeal *amoA* genes. Some differences were noted between the different IPL concentration profiles, however, i.e. those with a phosphohexose head group matched the AOA-gene profile better than those with a hexose head group. These differences suggest that the hexose IPL pool contained a fossil component or that part of them were synthesized by other Archaea. IPL-derived GDGTs, presumably derived from living Archaea, were generally less abundant than core lipid GDGTs, derived from fossil organic matter. Their concentration profile also somewhat matched AOA gene abundances, but a second peak in abundance within the OMZ was not found in the AOA gene concentration profile. This represents additional evidence for a fossil contribution to the IPL pool or could point to non-AOA Archaea as additional GDGT sources within the core of the OMZ. TEX_{86} values, calculated from both of fossil and IPL-derived GDGTs, increased from surface waters to the core of the OMZ, below which they decreased again, not correlating with *in situ* temperature. This suggests that either (*i*) the majority of core lipids and IPLs in the deep water column are of fossil origin, (*ii*) that changes in GDGT distributions related to archaeal community shifts overprint the temperature signal contained in GDGT distributions or (*iii*) that deep water Archaea do not adjust their GDGT composition to temperature.

Introduction

Molecular biological studies have revealed that Archaea are ubiquitous and one of the dominant groups of picoplankton in today's ocean (e.g. DeLong et al., 1992; Fuhrman et al., 1992; Karner et al., 2001; Herndl et al., 2005; Auguet et al., 2010). Within the marine realm, Marine Crenarchaeota Group I (MCGI) is one of the most important groups of Archaea and genomic, labeling, and cultivation studies have shown that they possess both heterotrophic and autotrophic metabolism (Hoefs et al., 1997; Wuchter et al., 2003; Herndl et al., 2005; Könneke et al., 2005; Hallam et al., 2006: Ingalls et al., 2006). Particularly, members of the MCGI have shown to be predominantly chemoautotrophic ammonia-

oxidizers (Könneke et al., 2005; Wuchter et al., 2006b). These ammonia-oxidizing Archaea (AOA) are often more abundant than their ammonia-oxidizing Bacteria counterparts (e.g. Wuchter et al., 2006b) and, in this way, form a potentially important component of the global carbon and nitrogen cycles.

Environmental and cultivation studies have shown that members of the MCGI make GDGTs with 0-4 cyclopentane moieties (GDGTs 0-3; Figure 1), including an unusual GDGT, crenarchaeol which also contains a cyclohexane moiety in addition to four cyclopentane moieties (Schouten et al., 2000; Sinninghe Damsté et al., 2002b). Recent studies suggest that crenarchaeol may be synthesized exclusively by AOA within the Group I Crenarchaeota (de la Torre et al., 2008; Schouten et al., 2008; Pitcher et al., 2010a), rendering it a potentially suitable marker to trace living AOA. Furthermore, since this GDGT is preserved in sediments up to millions of years old it can be used to trace the past occurrence of AOA (e.g. Schouten et al., 2000 and 2003; Kuypers et al., 2001; Carillio-Hernandez et al., 2003).

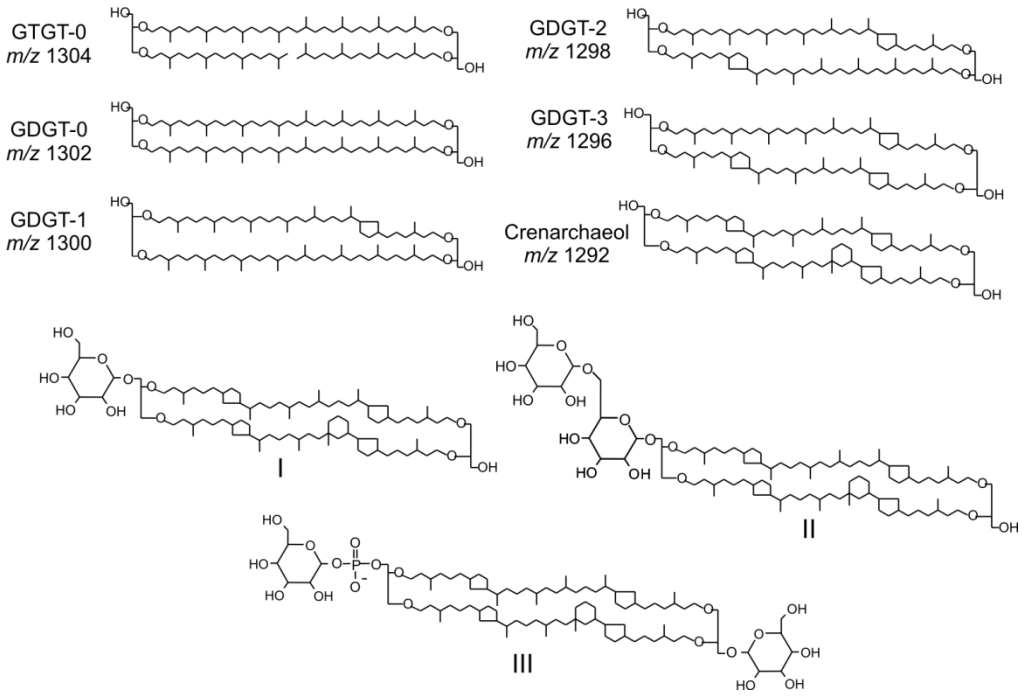

Figure 1. Structures of GDGTs and crenarchaeol IPLs discussed in the text.

In addition to their utility as biomarker lipids, the distribution of crenarchaeotal GDGTs in the marine environment has also been shown to strongly correlate to temperature, i.e. with increasing temperature an increase in the relative abundance GDGT with cyclopentane moieties is observed (Schouten et al., 2002; Wuchter et al., 2004). Based on this premise, the TEX_{86} paleotemperature proxy was developed and has been calibrated through a number of marine surface sediment studies (e.g. Schouten et al., 2002; Kim et al., 2008 and 2010). Additional supporting evidence for the empirical nature of the TEX_{86} calibrations has come from mesocosm enrichment studies (e.g. Wuchter et al., 2004; Schouten et al., 2007b), water column studies (e.g. Wuchter et al., 2005) and sediment trap studies (e.g. Wuchter et al., 2006a). One of the most intriguing aspects is that TEX_{86} seems to reflect surface temperature conditions despite the fact that MCGI are abundant throughout the water column though abundances tend to maximize in the upper 200 m of the water column (e.g. Karner et al., 2001; Herndl et al., 2005; Beman et al., 2008; Santoro et al., 2010). This apparent contradiction has been explained by the suggestion that vertical transport of archaeal lipids synthesized in the (near)surface waters to the sediment is facilitated by their incorporation into fecal pellets within the photic zone (c.f. Wakeham et al., 2003; Huguet et al., 2006b). So far, however, water column studies have not shed much light on this matter, but have revealed another intriguing observation: TEX_{86} values of surface waters correlated with *in situ* temperature but those of deeper water did not (Wuchter et al., 2005; Turich et al., 2007). This was, in part, explained by the hypothesis that the GDGTs analyzed were core lipids mostly derived from fossil cell material, particularly as the TEX_{86} values of GDGTs in meso- and bathypelagic water did show some correlation with surface water temperature suggesting the deep water GDGTs were derived from the upper part of the water column (Wuchter et al., 2005).

In contrast to core lipids, intact polar lipid GDGTs, i.e. with polar head groups attached to the core GDGT lipids (e.g. structures **I-III**, Figure 1), may provide more suitable tracers for living Archaea (Sturt et al., 2004; Biddle et al., 2006; Lipp et al., 2008; Schubotz et al., 2009) as they are thought to be degraded relatively quickly (i.e. within days) after cell death. AOA enrichment cultures generally containe IPL-GDGTs with a mono-hexose (MH) moiety (**I;** Figure 1), a dihexose (DH) moiety (**II**) or hexose and

phosphohexose (HPH) moieties (**III**) (Schouten et al., 2008; Pitcher et al., 2010a and 2010c). In addition, the only axenic isolate of Group I Crenarchaeota, *Nitrosopumilus maritimus* SCM1, and AOA enrichments from marine sediments, contained GDGTs with a hexose moiety and an unknown head group of 180 Daltons (Schouten et al., 2008, Pitcher et al., 2010c). Analysis of these specific IPL-GDGTs, as opposed to core lipid GDGTs, may be more suitable to trace *in situ*-produced GDGTs in the marine water column. It should be noted, however, that the application of IPL-GDGTs as markers for living cells has recently been questioned (Schouten et al., 2010; Bauersachs et al., 2010; Huguet et al., 2010a) and it has been suggested that most of the GDGTs, including IPL-GDGTs, in environmental samples are not from growing cells (Huguet et al., 2010b).

Here we analyzed GDGTs in the form of core lipids and intact polar lipids in the Arabian Sea water column to address their suitability as biomarker lipids for AOA and evaluate the effect of temperature on their distribution in the marine water column. The Arabian Sea contains the largest oxygen minimum zone (OMZ) of the present day oceans. An earlier study of GDGT core lipids in the Arabian Sea showed that they were present throughout the water column but particularly abundant within the OMZ (Sinninghe Damsté et al., 2002a). In a separate publication, we show that AOA genes are abundant within the Arabian Sea OMZ and that their concentration profile matches that of a specific IPL of AOA, crenarchaeol with a HPH head group (Pitcher et al., 2010b). In this study, we report the general distribution of GDGTs as IPLs and as core lipids (CL), either present as such or derived from IPLs. The results shed light on sources and fate of GDGTs in the marine water column and the relation of GDGT distribution with temperature.

Materials and Methods

Suspended particulate matter sampling and extraction

A depth profile of 12 suspended particulate matter (SPM) samples was taken at lat 21°55.6', long 63°10.6', at the southeast slope of the Murray Ridge where the water depth reaches ca. 3000 m (Figure 2). Dissolved oxygen decreased from fully oxic at the surface

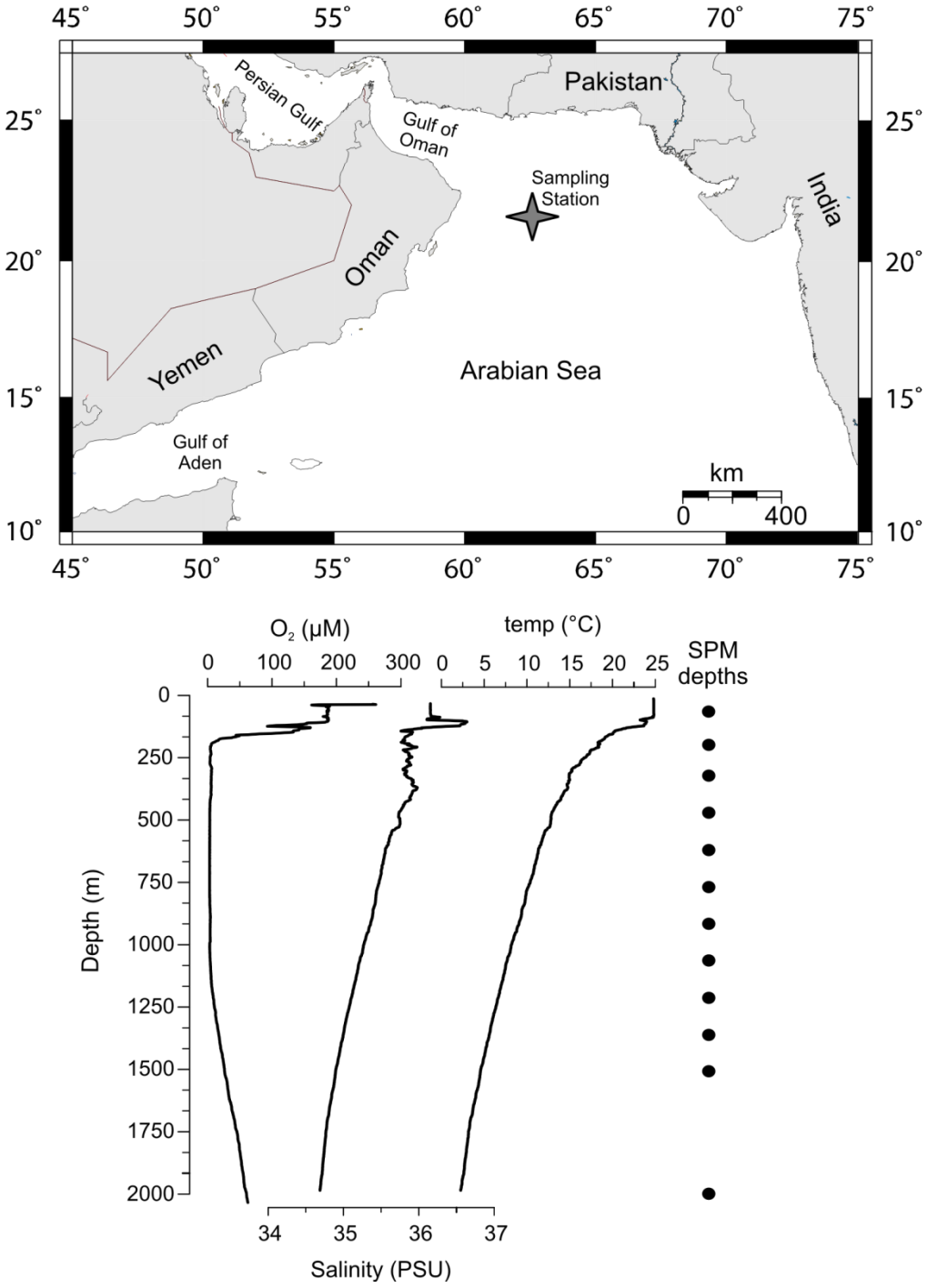

Figure 2. Location map and continuous CTD profile of oxygen, salinity and temperature (from Pitcher et al. (2010b)).

to 2.5 µM within the core of the OMZ and increased again with depth starting at ca.1050 m (Figure 2b). Salinity showed increases at 95 m and 325-400 m, corresponding to the Arabian Sea High Salinity Water and the Persian Gulf Outflow water masses, respectively (Shetye et al., 1994). Details on the suspended particulate matter (SPM) sampling have been described previously by Pitcher et al. (2010b). Briefly, SPM was collected by large-volume (ca.1200-1700 L) in situ pump filtration using McLane pumps onto pre-ashed 0.7 µm GF/F filters during a cruise of the R/V Pelagia in December 2008/January 2009. Upon retrieval of the pumps the GF/F filters were removed and immediately frozen at -80°C.

Intact polar lipids were extracted from freeze-dried filters using a modified Bligh and Dyer technique (Bligh and Dyer, 1959). A known volume of single-phase solvent mixture of methanol (MeOH):dichloromethane (DCM):phosphate buffer (2:1:0.8, v/v/v) was added to the sample in a centrifuge tube and placed in an ultrasonic bath for ten min. The extract and residue were separated by centrifuging at 2500 rpm for 5 min and the solvent mixture collected in a separate flask (3 times). The DCM and phosphate buffer were added to the single-phase extract to give a new ratio of MeOH:DCM:phosphate buffer (1:1:0.9, v/v/v), and to induce phase separation. The extract was centrifuged at 2500 rpm for 5 min. The DCM phase was collected in a round-bottom flask and the methanol:phosphate buffer phase was washed two additional times with DCM. The combined DCM phases were reduced under rotary vacuum and evaporated to dryness under a stream of N_2. A recent study evaluating the different extraction procedures used for GDGT analysis found widely different extraction yields and suggested that the commonly used Bligh Dyer technique is underestimating GDGT concentrations (Huguet et al., 2010b). Though we recognize the uncertainty this introduces in our absolute GDGT concentrations (see also discussion below) the main goal of our study was to compare depth trends and relative distributions within the Arabian Sea water column and it also allowed us to compare it with previously reported results.

One aliquot of the Bligh-Dyer extract was analyzed as such on a high performance liquid chromatography (HPLC)-Electrospray Ionisation (EI)-Mass Spectrometer (MS)-MS. Another aliquot was separated into a IPL and CL fractions according to Oba et al. (2006) and Pitcher et al. (2009), except that a hexane:ethyl acetate (EtOAc) (1:1, v/v) mixture was

used to obtain the CL fractions, and that IPL fractions were retrieved by flushing the silica column with MeOH. A C_{46} GDGT internal standard was added to both CL and IPL fractions according to Huguet et al (2006). The IPL fractions were subjected to acid hydrolysis (3 h reflux with 1N HCl/MeOH solution) to cleave the head groups, thereby releasing the GDGTs as core lipids (IPL-derived GDGTs). The IPL-derived and CL fractions were dissolved in hexane:isopropanol (99:1, v/v), filtered over a 0.45 µm PTFE filter, and concentrated to about 3 mg mL^{-1} prior to analysis using HPLC/ atmospheric pressure chemical ionization (APCI)–MS.

HPLC/APCI-MS analysis

Core lipid GDGTs were analyzed on an Agilent 1100 series LC/MSD SL according to Schouten et al. (2007). The injection volume was 10 µl for all samples, and selective ion monitoring of the $[M+H]^+$ was used to detect and quantify the different GDGTs. Absolute quantification was done according to Huguet et al. (2006a). The TEX_{86} was calculated as follows (Schouten et al., 2002):

TEX_{86} = (GDGT-2+GDGT-3+Crenisomer/(GDGT-1+GDGT-2+GDGT-3+Crenisomer) [Eq. 1]

The TEX_{86} values were related to temperature according to the following empirical relationship based on globally distributed core top sediments with annual mean SST [r^2=0.87; n=255, p<0.0001] (Kim et al., 2010):

$SST = +38.6 + 68.4 * (\log TEX_{86})$ [Eq. 2]

This calibration introduces a non-linear term, TEX^H_{86} (defined as the logarithmic function of TEX_{86}), improving accuracy in the estimation of warm temperatures (Kim et al., 2010).

HPLC/ESI-MS^2 analysis

IPLs in the Bligh Dyer extracts were analyzed with HPLC/ESI–MS/MS using either a ThermoScience Quantum triple quadrupole MS or a ThermoScience LTQ Ion Trap MS.

Ion trap MS analysis

For general detection of IPLs, Bligh Dyer extracts were analyzed using HPLC-ESI-Ion trap MS. Lipid extracts were dissolved in hexane:2-propanol:water (72:27:1, v/v/v) at a concentration of 2 mg mL^{-1} and filtered through a 0.45 µm regenerated cellulose (RC) filter (Alltech Associates Inc., Deerfield, IL) prior to injection. Intact polar lipids (IPLs) were analyzed according to Sturt et al. (2004) with some modifications. An Agilent 1200 series LC (Agilent, San Jose, CA), equipped with thermostatted auto-injector and column oven, coupled to a Thermo LTQ XL linear ion trap with Ion Max source with ESI probe (Thermo Fisher Scientific), was used. Separation was achieved on an Lichrosphere diol column (250 x 2.1 µm, 5 um particles; Alltech Associates Inc., Deerfield, IL) maintained at 30°C. The following elution program was used with a flow rate of 0.2 mL min^{-1}: 100% A for 1 min, followed by a linear gradient to 66% A: 34% B in 17 min, maintained for 12 min, followed by a linear gradient to 35% A: 65% B in 15 min, where A = hexane/2-propanol/formic acid/14.8 M NH_{3aq} (79:20:0.12:0.04, v/v/v/v) and B = 2-propanol/water/formic acid/ 14.8 M NH_{3aq} (88:10:0.12:0.04, v/v/v/v). Total run time was 60 min with a re-equilibration period of 20 min in between runs. For MS detection source parameters were optimized using loop injections of standard IPLs (1,2-dipalmitoyl-sn-glycero-3-phosphocholine, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine, 1,2-dipalmitoyl-sn-glycero-3-phospho-L-serine, 1,2-dipalmitoyl-sn-glycero-3-phospho-rac-glycerol, 1,2-dipalmitoyl-sn-glycero-3-phosphate, and soya L-a-phosphatidylinositol (Avanti Polar Lipids, Alabaster, AL) and 1,2-distearoyl-3-O-β-D-galactosyl-sn-glycerol,1,2-distearoyl-3-O-(α-D-galactosyl1-6)-β-D-galactosyl-sn-glycerol, and main phospholipid (MPL) of *Thermoplasma acidophilum* (Matreya LCC, Pleasant Gap PA)) into a stream of 0.2 mL min^{-1} of 60% A: 40% B. ESI settings were as follows: capillary temperature 275 °C, sheath gas (N_2) pressure 25 (arbitrary units), auxiliary gas (N_2) pressure 15 (arbitrary units), sweep gas (N_2) pressure 20 (arbitrary units), spray voltage 4.5 kV (positive ion ESI). The lipid extract was analyzed by

an MS routine where a positive ion scan (m/z 400-2000) was followed by data dependant MS^2 experiments where the 4 most abundant ions of the mass spectrum were fragmented (normalize collision energy (NCE) 25, isolation width (IW) 5.0, activation Q 0.175).

Triple quadrupole analysis

In order to detect the specific crenarchaeol IPLs in high sensitivity we used the Selected Reaction Monitoring (SRM) method of Pitcher et al. (2010b) using the HPLC-ESI-triple quadrupole MS. This method allows the detection of IPLs with crenarchaeol as a core lipid and five different head groups, i.e. a monohexose (**I**), a dihexose (**II**), a hexose and an unknown head group with a mass of 180 Daltons ('180'), a hexose and an unknown head group with a mass of 176 Daltons ('176') and HPH head groups (**III**) (Pitcher et al., 2010b). Prior to analysis by HPLC/ESI-MS/MS, extracts were dissolved in DCM/MeOH (9:1, v/v), ultrasonicated, and filtered using a regenerated cellulose 0.45µm filter (Alltech, Deerfield, IL). Separation was achieved on a LiChrospher diol column (250 × 2.1 mm, 5 µm particles, Alltech) maintained at 30°C. The following linear gradient was used with a flow rate of 0.2 mL min^{-1}: 100% A:0% B to 35% A: 65% B over 45 min, maintained for 20 min, then back to 100% A for 20 min to re-equilibrate the column. Detection of crenarchaeol-based IPLs was achieved by using conditions, and monitoring the transitions as described by Pitcher et al. (2010b). Since we did not have purified standards for quantification of crenarchaeol-based IPLs, we quantified these IPLs as the peak area response L^{-1} that revealed depth-related distribution patterns in individual IPLs but did not allow for absolute comparison of different IPL abundances. Fractions were analyzed in triplicate and long-term instrument response was monitored by injection of equivalent amounts of the Bligh and Dyer extract of "*Ca.* N. gargensis" biomass, every 6 to 10 runs.

Phylogeny

DNA was extracted as described by Pitcher et al. (2010b), followed by DGGE analysis of PCR-amplied archaeal 16S rRNA genes using conditions described by Wuchter et al. (2006). The presence and absence of similar DGGE bands in samples from other depths (lanes) was scored with GelCompar II software (Applied Maths).

The phylogenetic affiliation of the partial archaeal 16S rRNA gene sequences recovered from the DGGE bands were compared to release 102 of the Silva SSU Ref database (Pruesse et al., 2007) and most recent submissions from NCBI database (http://www.ncbi.nlm.nih.gov/) using the ARB software package (Ludwig et al., 2004). Using the ARB Parsimony tool, we first added our partial sequences to a reference tree supplied with the Silva database. Subsequently, with a subset of the reference database, including the most relevant sequences and closest relatives, we performed Maximum Likelyhood analyses using the RaxML program (Stamatakis et al., 2005 and 2008) implemented in ARB. We used GTRMix model settings with rapid bootstrap analyses (100 runs) with and without applying a filter for positional variability in Archaea. The overall topologies of the resulting trees and the clustering of our new sequences were similar with all methods.

Results

Intact polar lipids

Analysis of Bligh Dyer extracts of the SPM collected from the Arabian Sea using HPLC-ESI-Ion trap MS showed a large diversity of IPLs. The IPLs which could be identified were acyl glycerols with betaine (DGTS/DGTA), phosphocholine, phosphoethanolamines, phosphoethanol(di)methylamines and sulfoquinovosyl head groups (data not shown). IPLs with GDGT core lipids, however, could hardly be identified from the complex IPL mixtures, even using 3D ion density maps of retention time, mass and intensity (cf. Lipp and Hinrichs, 2009), suggesting that these IPLs formed either a minor part of the total IPL pool or that their ionization efficiency was much lower than that of the other IPLs which were detected. In order to increase the specificity of analysis for IPL-GDGTs, we re-analyzed the SPM extracts with a limited mass range of 1000-2000 Daltons, thereby excluding most of the IPLs present which had molecular weights <1000. Detection of IPL-GDGTs was still difficult but GDGTs with a monohexose (MH), a dihexose (DH) and a hexose-phosphohexose (HPH) head groups could be identified. Within the SPM

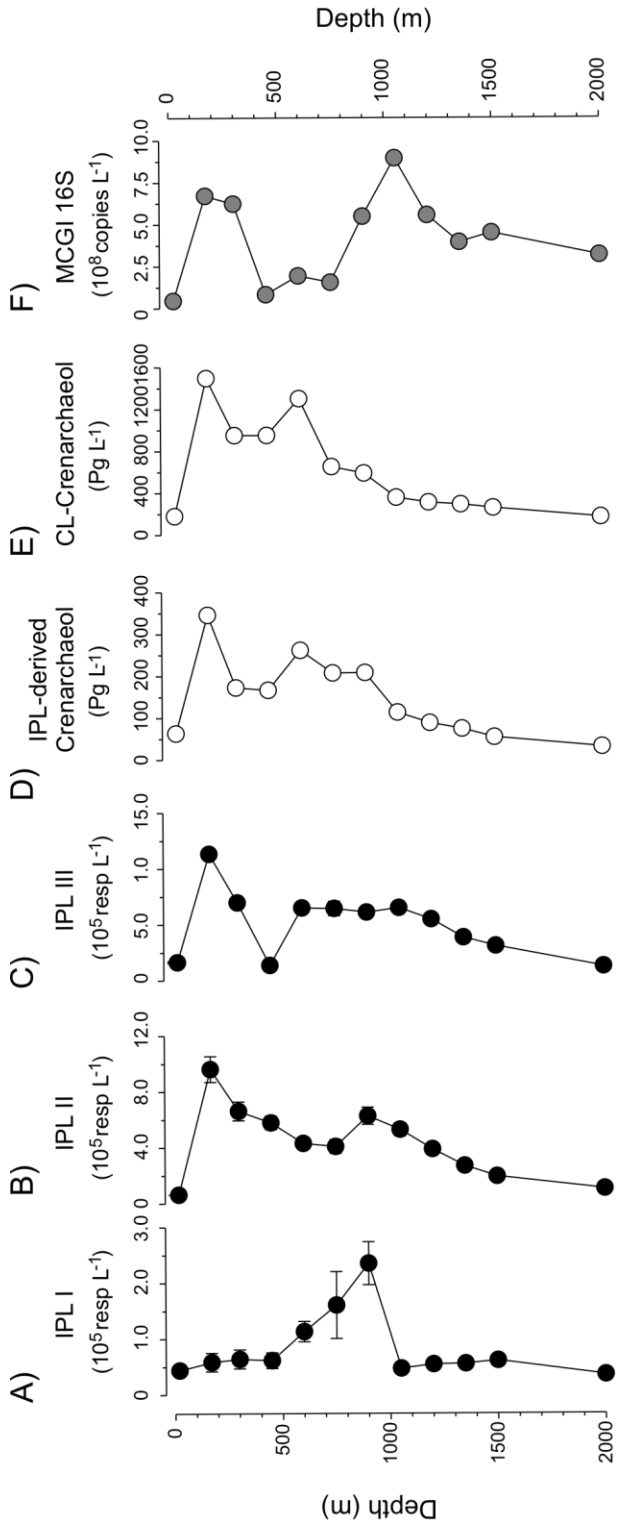

Figure 3. Depth distribution of concentrations of (A) mono-hexose crenarchaeol (**I**), (B) di-hexose crenarchaeol (**II**), (C) hexose, phospho-hexose crenarchaeol (**III**) (from Pitcher et al., 2010b), (D) IPL-derived crenarchaeol, (E) core crenarchaeol, and (F) MCGI 16S rRNA gene copy numbers. Note that the concentrations of the crenarchaeol IPLs are given in peak area response per liter due to a lack of standard. For structures of GDGTs see Figure 2.

originating from the OMZ, MS-MS spectra indicated that these IPL-GDGTs predominantly contained crenarchaeol as core lipid with trace amounts of GDGT-0 as core lipid, while the latter core lipid was more predominant in SPM below the OMZ. However, signals were low and IPLs peak areas could not be reliably integrated.

To further enhance the detection of IPL-GDGTs, we used a specific SRM method targeted at the detection of five IPLs with crenarchaeol as a core lipid (Pitcher et al., 2010b). With the exception of crenarchaeol containing the '176' head group, all four other crenarchaeol-based IPLs were detected in the SPM extracts. Crenarchaeol with MH (**I**), DH (**II**) and HPH head groups (**III**) gave abundant signals in all SPM samples while the crenarchaeol with the '180' head group was near detection limit in most samples and its peak area could not be accurately quantified. The depth distributions of the crenarchaeol IPLs were quite different: HPH crenarchaeol (**III**) peaked at 170 m depth (near the onset of OMZ), below which it generally decreased in abundance, while the MH crenarchaeol (**I**) gradually increased in abundance, reaching its maximum at 900 m depth, in the lower part of the OMZ (Figure 3a-c). Maximum abundance of the DH crenarchaeol (**II**) was observed at 170 m depth, but it peaked again at 900 m.

CL and IPL-derived GDGTs

To quantify the abundance and distribution of GDGTs present as core lipids or as IPLs, CL-GDGTs were separated from IPL-GDGTs using column chromatography (Oba et al., 2006; Pitcher et al., 2009), after which the IPL-GDGTs were acid hydrolyzed to yield CL-GDGTs. Quantification of both GDGT pools showed that CL-GDGTs were in substantially higher abundances (summed total ranging from CL-GDGT 300-2400 pg L^{-1}) than IPL-derived GDGTs (summed total ranging from 90-600 pg L^{-1}) (Figure 4; Table 1). In general, CL-GDGTs showed a similar depth profile compared to each other with maximum abundance at 170 m depth, below which they steadily decreased in concentration. The exception was CL-crenarchaeol and the CL-crenarchaeol isomer, which peaked at 600 m to abundances almost as high as values observed at 170 m. IPL-derived GDGTs also showed a relatively similar depth profile compared to each other but different

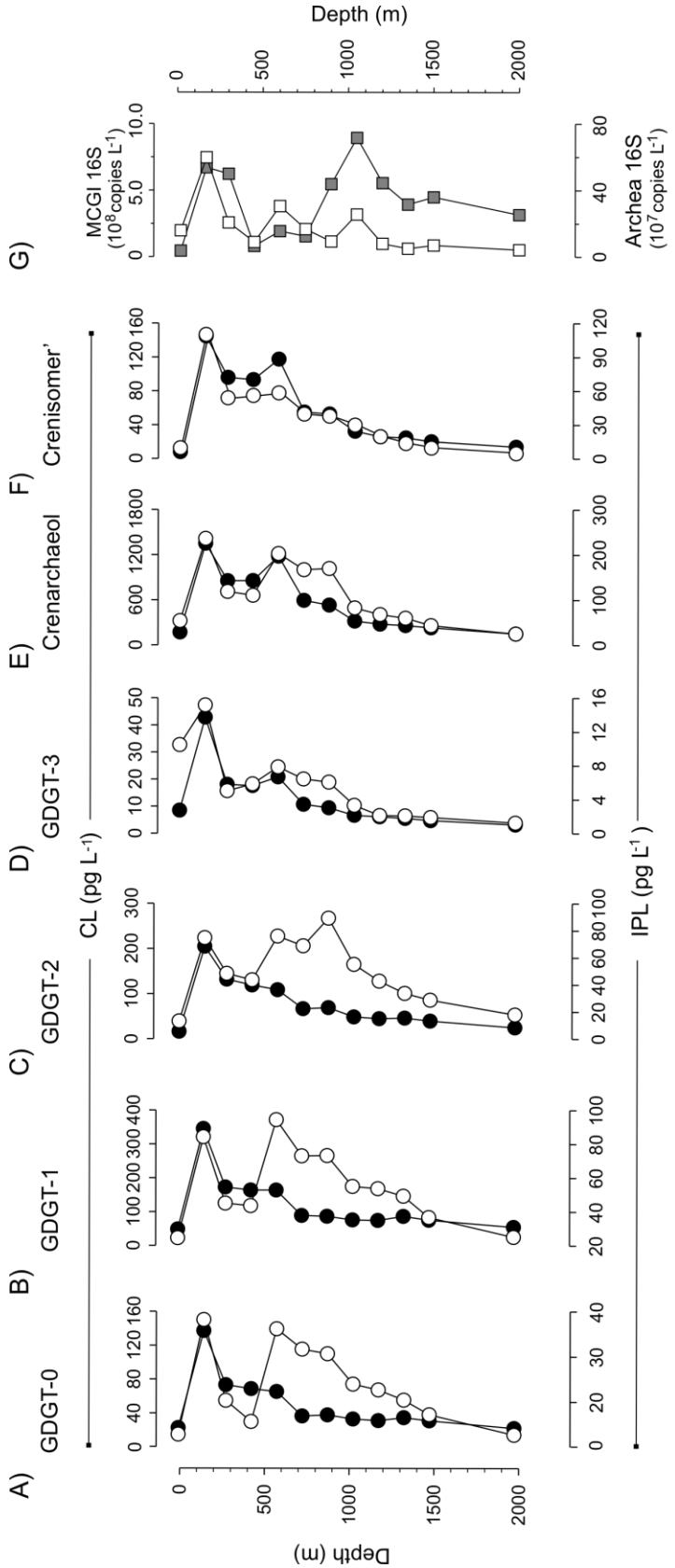

Figure 4. Depth distribution of concentrations of core lipid (CL) and intact polar lipid (IPL) derived GDGTs. (A) GDGT-0, (B) GDGT-1, (C) GDGT-2, (D) GDGT-3, (E) crenarchaeol, and (F) crenarchaeol regio-isomer; (G) MCGI and total Archaea 16S rRNA gene copy numbers. Note the separate scales of CL and IPL-derived GDGTs. For structures of GDGTs see Figure 2.

Table 1. Concentrations and TEX_{86} values of GDGTs present as core lipids and derived from intact polar lipids. MCGI = copy numbers of Marine Crenarchaeotal Group I from Pitcher et al. (2010b). cren=crenarchaeol; creniso=crenarchaeol isomer

depth	Core GDGTs (pg L^{-1})								IPL GDGTs (pg L^{-1})								MCGI 16S (Copies L^{-1})	Theoretical IPLs
	GDGT-0	GDGT-1	GDGT-2	GDGT-3	cren	creniso	Total	TEX_{86}	GDGT-0	GDGT-1	GDGT-2	GDGT-3	cren	creniso	Total	TEX_{86}		
20	49	23	17	9	172	8	306	0.59	25	13	13	10	54	9	137	0.72	4.50E+06	5
170	346	137	205	43	1352	145	2441	0.74	84	38	75	15	236	110	597	0.84	6.70E+07	67
300	173	73	132	18	855	96	1476	0.77	45	20	48	5	119	54	312	0.84	6.20E+07	62
450	164	69	119	18	856	93	1439	0.77	44	16	44	6	110	56	292	0.87	8.20E+06	8
600	164	65	109	21	1182	118	1837	0.79	94	36	76	8	203	58	509	0.8	1.90E+07	19
750	89	37	67	11	595	55	939	0.78	73	32	69	6	167	40	412	0.78	1.60E+07	15
900	87	38	69	10	534	53	861	0.78	73	31	89	6	170	38	431	0.81	5.50E+07	55
1050	77	33	49	7	322	33	564	0.73	55	24	55	3	83	30	262	0.79	8.90E+07	90
1200	75	31	45	6	282	26	508	0.71	54	23	43	2	68	20	220	0.74	5.60E+07	56
1350	87	35	46	6	264	25	500	0.69	49	20	34	2	60	14	188	0.71	3.90E+07	39
1500	76	31	40	5	234	20	440	0.68	37	17	29	2	44	10	144	0.7	4.50E+07	45
2000	55	22	25	3	153	14	295	0.66	25	13	18	1	25	5	91	0.65	3.10E+07	32

notably from the CL-GDGTs. In addition to peaking at 170 m, they also presented a broader peak between 600 and 900 m (Figure 4).

TEX_{86} values for both CL-GDGTs and IPL-derived GDGTs showed a similar depth profile (Figure 5; Table 1). The TEX_{86} value of CL-GDGTs substantially increased from surface (0.59) to 600 m depth (0.79) after which it steadily decreased to a value of 0.66. IPL-derived GDGTs TEX_{86} values increased from 0.72 to 0.87 from surface to 450 m depth and subsequently decreased to a value of 0.65. In general, IPL-derived GDGT TEX_{86} values tended to be substantially higher than that of CL-GDGTs in SPM of the upper 600 m and only slightly higher in the deeper water SPM.

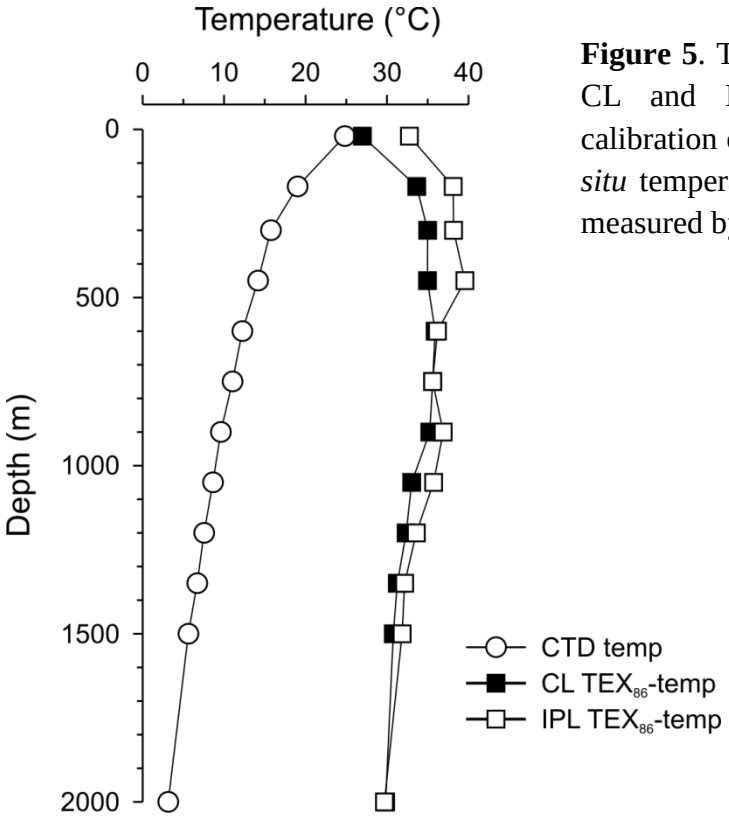

Figure 5. TEX_{86}-derived temperatures using CL and IPL-derived GDGTs with the calibration of Kim et al. (2010), as well as *in situ* temperatures at location of sampling as measured by CTD.

Archaeal phylogeny

To gain insights into the archaeal diversity of the Arabian Sea at the sampled depths we performed DGGE analysis and sequenced excised bands. The majority of sequences fall within the Group I Crenarchaeota and sequences falling within this group were detected at all depths (Figure 6). Sequences belonging to Group III Euryarchaeota were also detected at all depths between 20-2000 m while sequences of Group II Euryarchaeota were detected at 300 and 450 m depth only.

Discussion

IPL- and CL-crenarchaeol as tracers for Crenarchaeota

Crenarchaeol has been suggested to be a specific lipid for AOA within the Crenarchaeota (Sinninghe Damsté et al., 2002; de la Torre et al., 2008; Schouten et al., 2008; Pitcher et al., 2010a and 2010c) and thus may form a suitable tracer for AOA in the marine water column. Crenarchaeol IPLs in the Arabian Sea mainly occur with a (mono/di) hexose and phosphohexose head group. GDGTs with a MH and DH head groups have been previously reported in the water column of the Black Sea (Schubotz et al., 2009) and in marine subsurface sediments (Sturt et al., 2004; Biddle et al., 2006; Lipp et al., 2008; Lipp and Hinrichs, 2009) but GDGTs with a HPH head group have not been reported yet in environmental studies, despite its presence in all Group I Crenarchaeota enrichment cultures investigated until now (Schouten et al., 2008; Pitcher et al., 2010a and 2010c). The reason for its apparent absence in previous environmental IPL studies is not clear and might simply result from Archaea in these environments producing different IPLs. Alternatively, this may be due to methodological differences in sample work-up or method of analysis. GDGTs with an HPH head group could be identified both using ion trap MS as well as by SRM analysis though the latter was much more sensitive. The apparent absence of crenarchaeol with the ‘176’ head group may not be surprising, as this particular IPL has so far only been reported in the thermophilic Group I.1b Crenarchaeota “*Ca*. N. gargensis” (Pitcher et al., 2010a), which was isolated from a terrestrial hot spring (Hatzenpichler et al., 2008). The low abundance of crenarchaeol with the ‘180’ head group is perhaps also not

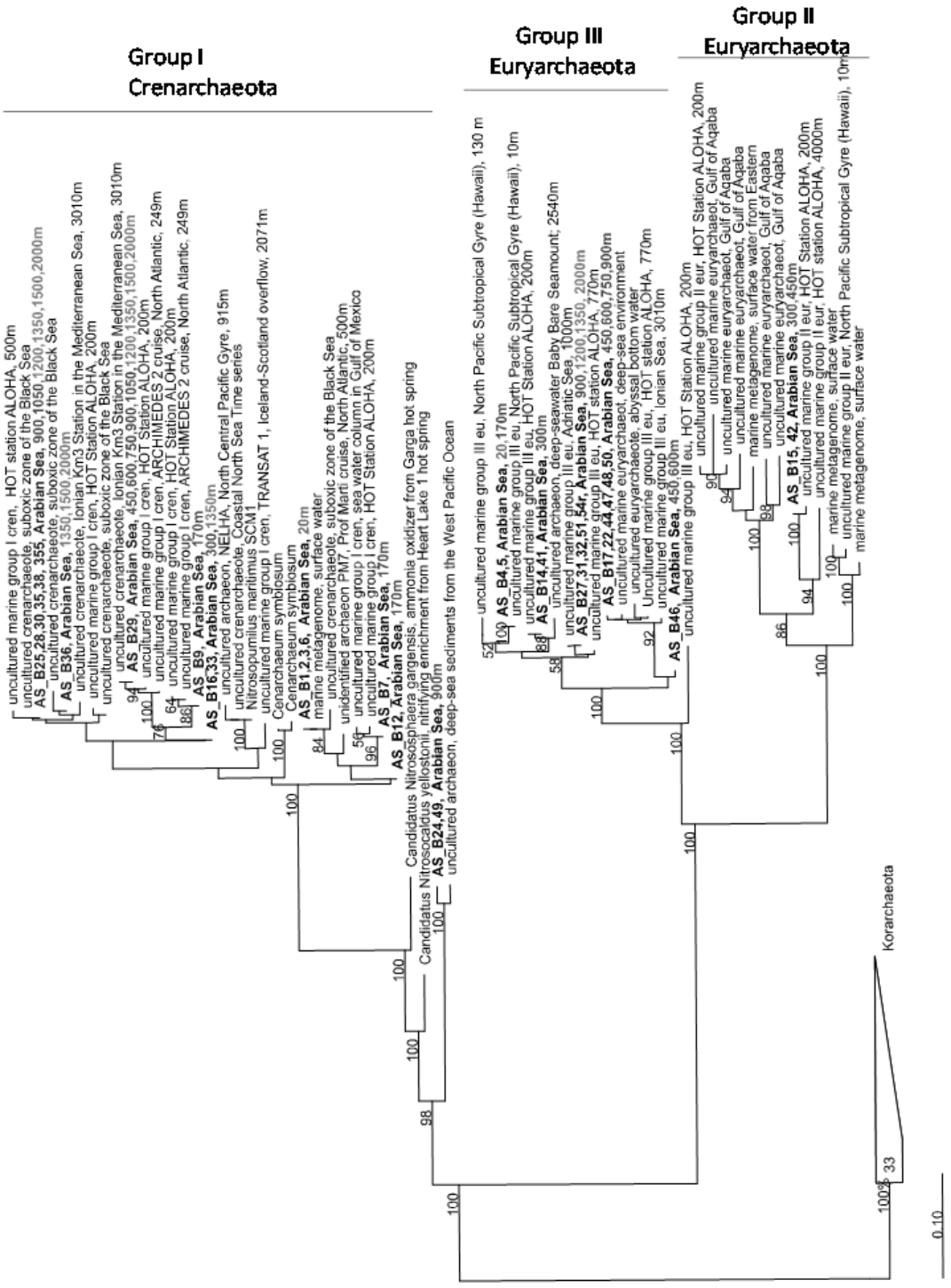
Group I
Crenarchaeota
Group III
Euryarchaeota
Group II
Euryarchaeota
uncultured marine group I cren, HOT station ALOHA, 500m
uncultured crenarchaeote, suboxic zone of the Black Sea
AS_B25,28,30,35,38, 355, Arabian Sea, 900,1050,1200,1350,1500,2000m
uncultured crenarchaeote, suboxic zone of the Black Sea
AS_B36, Arabian Sea, 1350,1500,2000m
uncultured crenarchaeote, Ionian Km3 Station in the Mediterranean Sea, 3010m
uncultured marine group I cren, HOT Station ALOHA, 200m
uncultured crenarchaeote, suboxic zone of the Black Sea
uncultured crenarchaeote, Ionian Km3 Station in the Mediterranean Sea, 3010m
AS_B29, Arabian Sea, 450,600,750,900,1050,1200,1350,1500,2000m
uncultured marine group I cren, HOT Station ALOHA, 200m
uncultured marine group I cren, ARCHIMEDES 2 cruise, North Atlantic, 249m
uncultured marine group I cren, HOT Station ALOHA, 200m
uncultured marine group I cren, ARCHIMEDES 2 cruise, North Atlantic, 249m
AS_B9, Arabian Sea, 170m
AS_B16,33, Arabian Sea, 300,1350m
uncultured archaeon, NELHA, North Central Pacific Gyre, 915m
uncultured crenarchaeote, Coastal North Sea Time series
Nitrosopumilus maritimus SCM1
uncultured marine group I cren, TRANSAT 1, Iceland-Scotland overflow, 2071m
Cenarchaeum symbiosum
Cenarchaeum symbiosum
AS_B1,2,3,6, Arabian Sea, 20m
marine metagenome, surface water
uncultured crenarchaeote, suboxic zone of the Black Sea
unidentified archaeon PM7, Prof Marti cruise, North Atlantic, 500m
uncultured marine group I cren, sea water column in Gulf of Mexico
uncultured marine group I cren, HOT Station ALOHA, 200m
AS_B7, Arabian Sea, 170m
AS_B12, Arabian Sea, 170m
Candidatus Nitrososphaera gargensis, ammonia oxidizer from Garga hot spring
Candidatus Nitrosocaldus yellostonii, nitrifying enrichment from Heart Lake 1 hot spring
AS_B24,49, Arabian Sea, 900m
uncultured archaeon, deep-sea sediments from the West Pacific Ocean
uncultured marine group III eu, North Pacific Subtropical Gyre (Hawaii), 130 m
AS_B4,5, Arabian Sea, 20,170m
uncultured marine group III eu, North Pacific Subtropical Gyre (Hawaii), 10m
uncultured marine group III eu, HOT Station ALOHA, 200m
AS_B14,41, Arabian Sea, 300m
uncultured archaeon, deep-seawater Baby Bare Seamount, 2540m
uncultured marine group III eu, Adriatic Sea, 1000m
AS_B27,31,32,51,54, Arabian Sea, 900,1200,1350, 2000m
uncultured marine group III eu, HOT station ALOHA, 770m
AS_B17,22,44,47,48,50, Arabian Sea, 450,600,750,900m
uncultured marine euryarchaeot, deep-sea environment
uncultured euryarchaeote, abyssal bottom water
Uncultured marine group III eu, HOT station ALOHA, 770m
uncultured marine group III eu, Ionian Sea, 3010m
AS_B46, Arabian Sea, 450,600m
uncultured marine group III eu, HOT Station ALOHA, 200m
uncultured marine group II eur, HOT Station ALOHA, 200m
uncultured marine euryarchaeot, Gulf of Aqaba
uncultured marine euryarchaeot, Gulf of Aqaba
uncultured marine euryarchaeot, Gulf of Aqaba
marine metagenome, surface water from Eastern
uncultured marine euryarchaeot, Gulf of Aqaba
uncultured marine euryarchaeot, Gulf of Aqaba
AS_B15, 42, Arabian Sea, 300,450m
uncultured marine group II eur, HOT Station ALOHA, 200m
uncultured marine group II eur, HOT station ALOHA, 4000m
marine metagenome, surface water
uncultured marine group II eur, North Pacific Subtropical Gyre (Hawaii), 10m
marine metagenome, surface water
Korarchaeota
0.10

Figure 6. (Previous page) Maximum likelihood tree with bootstrap analyses (100 runs) of partial 16S rRNA gene sequences retrieved by DGGE analysis from SPM of the Arabian Sea.

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surprising, as *N. maritimus* SCM1 synthesizes this IPL mainly with GDGTs 2 and 3, not crenarchaeol, as core lipids (Schouten et al., 2008). Indeed, Lipp and Hinrichs (2009) also found that in marine subsurface sediments IPLs with the '180' head group mainly contained GDGTs 2-3 as core lipids.

In order to evaluate the robustness of the different crenarchaeol-based IPLs in tracing living AOA, we compared them with the MCGI 16S rRNA gene abundance depth profile from Pitcher et al. (2010b; Figure 3), who proposed that most of the MCGI in the Arabian Sea were AOA and thus were likely to produce crenarchaeol. Since DNA outside the cell is relatively quickly degraded (e.g. Corinaldesi et al., 2008), the abundance of MCGI 16S rRNA is likely to reflect the abundance of (recently) living Crenarchaeota. The HPH crenarchaeol (**III**) matches the MCGI gene abundance (Pitcher et al., 2010b) quite well in the upper water column, although the deep peak in gene abundance at 1050 m depth is less apparent in the IPL profile (Figure 3). This suggests that this particular IPL might be suitable to trace the abundance of ammonia-oxidizing Crenarchaeota in the marine water column. The DH crenarchaeol (**II**) shows a similar profile although it has a second maximum at 900 m depth instead of 1050 m (Figure 3). The MH crenarchaeol (**I**) depth profile is rather different from both the MCGI gene abundance and the other IPLs, showing low abundances at 170 m depth, where Crenarchaeota and other IPLs are most abundant, and at 1050 m depth, where the second peak in MCGI abundance occurs. Instead, it gradually increases throughout the OMZ to reach its maximum abundance at 900 m depth (Figure 3). These differences may point to shifts in the relative abundance of the various IPLs in the cell membrane, e.g. MCGI at 170 m depth make less IPLs with a MH head group than those at 900 m. Alternatively, the MH IPL pool may contain a substantial fossil component, rendering its distribution unrepresentative of recently living Crenarchaeota. Indeed, Schouten et al. (2010) predicted, based on kinetic degradation models, that ether lipids with glycosidic head groups would be more persistent in the water column outside a

living cell membrane than those with phospho head groups. Thus, MH-GDGTs may be diagenetically formed from, for example, HPH-GDGTs and preferentially accumulate within the OMZ, where degradation rates of glycosidic ether lipids could be further diminished by low oxygen concentrations (Harvey et al., 1986; Schouten et al., 2010).

Another potentially suitable tracer for living AOA is the concentration of crenarchaeol released by acid hydrolysis from the IPL fraction of the Bligh Dyer extract. In principle, this IPL-derived crenarchaeol should represent the sum of all crenarchaeol-based IPLs present in the SPM, including those that may have escaped detection by SRM analysis (i.e. with head groups other than those we targeted and not visible in the ion trap MS analysis). The concentration profile of IPL-derived crenarchaeol also has a maximum at 170 m depth, consistent with the genes and directly measured IPLs, however, another broad peak in concentration was observed between 600 and 900 m and no peak at 1050 m depth is apparent (Figure 3). This suggests that the IPL-derived crenarchaeol in our samples may contain a fossil component from 600-900 m depth, which could conceivably derive from accumulated glycosidic ether lipids (such as MH-crenarchaeol). An apparent mismatch between IPL-derived GDGTs and archaeal 16S rRNA gene abundance was also found by Huguet et al. (2010a), i.e. they found highly variable amounts of IPL-derived GDGTs per 16S rRNA copy which were difficult to explain by variations in growth state and cell size only.

Finally, until now most SPM studies have used CL crenarchaeol as a tracer for marine Crenarchaeota (e.g. Sinninghe Damsté et al. 2002; Wakeham et al., 2003; Wuchter et al., 2005), and this is in fact the dominant form of crenarchaeol in the SPM we sampled; IPL-derived crenarchaeol represented only 13-32% of CL crenarchaeol (Table 1). Comparison of the CL crenarchaeol depth profile with that of the MCGI gene abundance showed the 170 m-maximum, but also high abundances at 600 m, and no peak at 1050 m depth (Figure 3). This suggests that CL crenarchaeol may not serve as a good general marker for viable AOA (cf. Huguet et al., 2010b), although it may be useful in locating depths of maximum MCGI abundances in similar settings. However, this is likely only to be the case if the depth of production of crenarchaeol has not shifted a substantial degree

over time, as CL-crenarchaeol likely represents an integrated signal of longer time periods (months to years).

To summarize the above points, of all the different lipids with a crenarchaeol moiety examined in this study, it seems that the best tracer for recently active Crenarchaeota is HPH-crenarchaeol, as it best matches MCGI gene abundances, is the most specific to AOA, and is the most labile molecule. Discrepancies between HPH-crenarchaeol and the 16S rRNA gene abundance depth profile, may be in part due to the accumulation of fossil IPLs within the OMZ (see above), however this phenomenon cannot explain the absence of a peak in IPL-derived crenarchaeol abundance at 1050 m depth, where MCGI genes were as high as at 170 m (Figure 4). There may be several explanations for this apparent mismatch with the MCGI 16S rRNA gene depth profile.

The MCGI population at 1050 m depth may be genetically different from those at 170 m and may produce significantly less crenarchaeol (Figure 4). Indeed, the MCGI sequence retrieved from 1050 m depth differed slightly from those retrieved from 170 m (Figure 6). However, Pitcher et al. (2010b) showed that archaeal genes coding for the alpha subunit of ammonia monooxygenase (*amoA*), the enzyme responsible for the first step of ammonia oxidation, also peaked at 1050 m depth, implying that the MCGI at this depth are also predominantly ammonia oxidizers. Since the archaeal 16S rRNA gene sequences at this depth were closely related to the AOA enrichment cultures *Cenarchaeum symbiosum* and *N. martimus* SCM1, both of which are known to produce crenarchaeol (Sinninghe Damsté et al., 2002a; Schouten et al., 2008), we would expect AOA at this depth to also synthesize crenarchaeol. Another explanation might be that the amount of IPL GDGTs per archaeal cell is highly variable and lower amounts of GDGTs are produced per cell in the deeper waters, thereby masking maxima in deeper water archaeal abundances (Huguet et al., 2010a). Finally, the fact should be considered that quantifications were done on SPM obtained by filtering with different pore size filters, i.e. 0.2 µm for DNA and 0.7 µm for lipids. Since the size of Group I Crenarchaeota is typically well below 1 µm (Könneke et al., 2005), a large proportion of the archaeal population which would have been captured by the 0.2 µm filter and subsequently used for DNA analysis, may have passed through the 0.7 µm filter. To investigate this we estimated the concentration of IPLs based on the copy

numbers of MCGI. Assuming one 16S rRNA gene per cell (Walker et al., 2009) and 1 fg of GDGTs per cell (as theoretically modeled by Sinninghe Damsté et al. (2002a); Lipp et al. (2008)), the amount of IPL-GDGTs which could potentially be present is ca. 2 orders of magnitude higher than the IPL-derived GDGT concentrations we measured (Table 2), representing up to 4% only of potentially present IPL-GDGTs (Figure 7). The major uncertainty in our estimates is the amount of GDGTs per cell. Huguet et al. (2010a) recently showed that in *N. maritimus* SCM1 biomass this amount may actually be much higher, making the observed discrepancy even larger, but in deeper waters (>50 m) can be as low as ~0.5 fg cell^{-1}. However, even assuming this lower cell concentration our recovery of IPL-GDGTs does not exceed 10%. Possibly the low recovery is due to the low extraction efficiency of the Bligh Dyer extractions but more likely these calculations suggest that the small-sized archaeal cells were not quantitatively captured by the larger pore-sized (0.7 µm) GF/F filters used for SPM filtration for lipid analysis. If the filtration efficiency changes with depth (e.g. due to the turbidity of the waters which are lower in the deeper Arabian Sea (cf. Pitcher et al., 2010b), this could potentially cause mismatches in archaeal lipid profiles with those of archaeal gene abundance. Indeed, Pitcher et al. (2010c) found that filtration of biomass of a crenarchaeotal enrichment culture resulted in only 12% recovery with a 0.7 µm filter compared to those of a 0.2 µm filter. It should be noted, however, that Herfort et al. (2006) analyzed GDGT concentrations in the North Sea with sequential filters of 0.7 and 0.2 µm pore size and only recovered <5% in the 0.2 µm filters. In this case, the filtration with 0.7 µm filters was likely more efficient due to the higher turbidity in this coastal sea, where particulate matter can rapidly clog filters, effectively reducing the nominal pore size. Therefore, it is likely in our samples that at least some GDGTs were lost through the large pore-size filters, however, exactly how much is impossible to say.

Sources for other IPL derived and CL-GDGTs

Our results also allow for evaluating which of the other GDGTs (which are more generally occurring in Archaea) can be attributed to MCGI. Nearly all GDGTs, both as IPL as well as CL, have a maximum in abundance at 170 m depth coinciding with the

maximum in MCGI gene abundance and in IPL-derived and CL crenarchaeol (Figure 4). This suggests that at this depth most of the GDGTs are derived from these Archaea. This correspondence holds for much of the depth profile, suggesting that most of the IPL-derived GDGTs are sourced by MCGI. However, IPL-derived GDGTs 0-2 tend to have a more pronounce peak in abundance at 600-900 m depth (Figure 4) than IPL-derived crenarchaeol, which may suggest that these GDGTs are produced *in situ* by Archaea not belonging to the MCGI, as the MCGI 16S rRNA gene abundance is not showing a pronounced maximum at this depth. Interestingly, total archaeal 16S rRNA gene abundance (from Pitcher et al., 2010b) shows a maximum at 600 m depth, which is not as pronounced as in the MCGI 16S rRNA gene abundance (Figure 4). Turich et al. (2007) speculated that Marine Group II Euryarchaeota synthesized GDGTs 0-2 in the epipelagic part of the marine water column. However, Wuchter (2006) did not find a correspondence between concentrations of GDGTs and those of Marine Group II Euryarchaeota in the

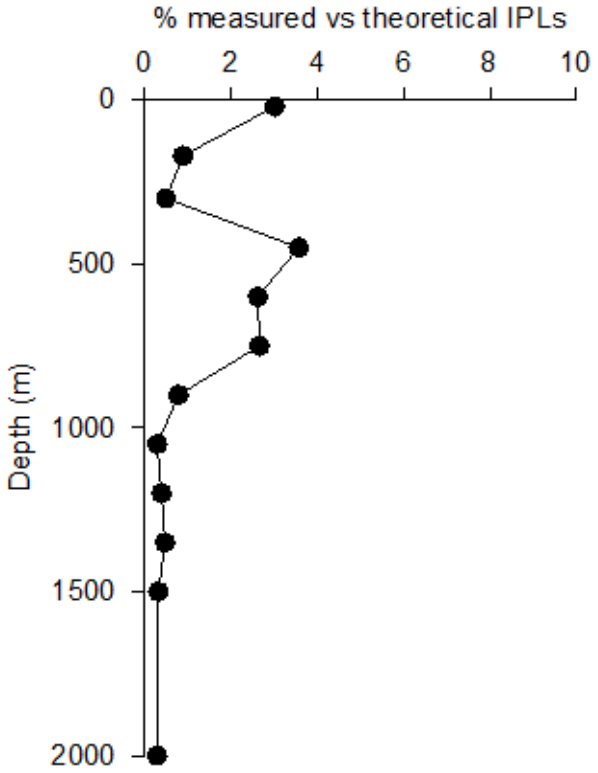

Figure 7. Relative percentage of total amount of measured intact polar lipid (IPL) derived GDGTs versus IPL-GDGTs estimated from the copy number concentration of MCGI 16S rRNA gene abundance.

coastal North Sea. In the Arabian Sea, sequences of Marine Group II Euryarchaeota were only detected at 300 and 450 m depth, making it unlikely that they contributed to the GDGT pool. However, sequences of Group III Euryarchaeota were detected at all depths (Figure 6), including a cluster falling within the DH148-W24 cluster (Galand et al., 2009). Previous studies have observed that Group III Euryarchaeota is rarely detected in marine ecosystems, in which MCGI Crenarchaeota and Group II Euryarchaeota are abundant (Massana et al., 2000). In addition, the depths from which Group III euryarchaeotal sequences have been retrieved suggest that this group is specific to the deep ocean. Sequences falling in this cluster were specifically detected in SPM from 600-900 m depth, where we also observe peaks in IPL-derived GDGTs 0-2. Thus, these Archaea may potentially contribute to some extent to the GDGT pool. Alternatively, at 900 m-depth sequences were detected which fall within the MCGI but are distantly related to sequences retrieved at other depths and also distantly related to known crenarchaeol synthesizers (Pitcher et al., 2010a and references cited therein). Thus, at this point it is unclear if Archaea other than Crenarchaeota have contributed substantially to the GDGT pool, in particular GDGTs 0-2. Interestingly, the 600-900 m depth maximum in IPL-derived GDGTs is not at all reflected in the CL-GDGTs depth profile: these GDGTs only show a maximum abundance at 170 m depth. This may mean that production of GDGTs at 600-900 m depth is only a relatively recent phenomenon and has not contributed yet substantially to the more "time-integrated" pool of CL-GDGTs.

GDGT distribution in relation to temperature

TEX_{86} values of GDGTs in the marine water column have, until now, mainly been analyzed using core lipids derived from fossil matter and therefore do not necessarily need to reflect *in situ* water temperatures (cf. Wuchter et al., 2005). Although the results discussed above show that IPL-derived GDGTs may still contain a fossil component, particularly at 600-900 m depth, they are more likely to reflect the composition of *in situ*-produced GDGTs compared to CL-GDGTs. Indeed, TEX_{86} values of IPL-derived GDGTs differ from CL-GDGTs, the latter being generally lower (Figure 5). However, the overall trend in TEX_{86} values through the water column is nearly identical, showing relatively low

values in surface waters and an increase between 170 and 450 m depth, below which they decrease reaching near-surface values. When converted to temperature estimates using the calibration of Kim et al. (2010) it becomes apparent that only the surface water TEX_{86} values correspond reasonably with *in situ* measured temperatures, while in the remaining part of the water column TEX_{86}-derived temperatures vastly overestimate *in situ* temperatures. This is in line with previous observations (Wuchter et al., 2005; Turich et al., 2007). There may be several explanations for this enigmatic result. Firstly, the IPL-derived GDGTs may contain a large contribution of fossil material derived from warmer waters, thereby masking the distribution of *in situ*-produced GDGTs (cf. Schouten et al., 2010; Huguet et al., 2010). If this would be the case, since deepwater TEX_{86} temperature estimates exceed those of the upper part of the water column (Figure 5), the fossil IPLs would have to be derived from allochtonous material transported from remote warm surface waters such as the Persian Gulf. Another explanation may be that Crenarchaeota living deep in the water column do not adjust their membrane GDGT composition according to temperature. However, adaptation of membrane lipids to temperature has been shown in enrichment studies of Group I Crenarchaeota (Wuchter et al., 2004; Schouten et al., 2007b). Furthermore, a sediment trap study in the Arabian Sea has shown that TEX_{86} varies over the seasonal cycle suggesting membrane lipid adaptation of upper water column Crenarchaeota does take place *in situ* (Wuchter et al., 2006). It is, therefore, unclear why deep water Crenarchaeota would have a similar GDGT lipid composition at temperatures as low as 4°C compared to those in the surface waters living at 25°C. Another explanation may be that temperature adaptation of membrane lipids may be blurred because of a genetic overprint, since the phylogenetic distribution of the marine Archaea changes with depth (Figure 6). However, Schouten et al. (2007b) also had genetically different populations in different mesocosms incubated with tropical surface waters but nevertheless found a good correspondence of TEX_{86} with incubation temperature. It remains therefore unclear why no relation between TEX_{86} and temperature is observed for IPL-derived GDGTs in marine water column SPM.

The question remains how the TEX_{86} values in the water column eventually determine that of what is settling to the sediment floor. The TEX_{86} value in the surface

sediment (0-0.5cm) at the site of water column sampling is 0.71 for both CL- and IPL-derived GDGTs (Lengger et al., in preparation). This is similar to, or slightly higher than, those of the CL- and IPL-derived GDGTs in the surface waters and the deeper oxic zone, but substantially lower than those of CL- and IPL-derived GDGTs in the oxygen minimum zone. The weighted average TEX_{86} value of CL-GDGTs, the largest pool of GDGTs in the water column, normalized on concentration and integrated over the entire water depth is 0.74, higher than that of the surface sediment. If the GDGT pool of the oxygen minimum zone is excluded, then a substantially lower value, 0.69 is obtained, relatively close to that of the surface sediment. A similar observation is made for the IPL-derived GDGTs: normalized on concentration and integrated over the entire water depth the TEX_{86} value of IPL-derived GDGTs is 0.79, substantially higher than in surface sediment; This value is 0.72 (close to that of the surface sediment) when excluding IPL-derived GDGTs from the oxygen minimum zone. Although these data do not give precise clues about the depth origin of CL- and IPL-derived GDGTs, they do suggest that the contribution of GDGTs synthesized in the oxygen minimum zone, where they are actually the most abundant, do not contribute substantially to the signal archived in the sediment floor. This is consistent with the idea that GDGTs in deeper, suboxic/anoxic parts of the water column do not constitute a large proportion of the GDGTs settling on the sediment floor (cf. Wakeham et al., 2003). However, to properly evaluate the origin of the TEX_{86} values of GDGTs in surface sediments of the Arabian Sea, analysis of GDGTs in sediment particles collected by a sediment trap is needed and is subject of future study.

Conclusions

Direct and indirect analysis of IPL-GDGTs in the water column of the Arabian Sea showed, in general, a reasonable correspondence of IPL crenarchaeol depth profile with a depth profile based on 16S rRNA gene copy number of Marine Group I Crenarchaeota, suggesting that they are one of the dominant sources of crenarchaeol and other GDGTs. However, there is likely also a fossil pool of IPL-derived GDGTs, which contribute to

different extents to the other different GDGTs as well. Enigmatically, IPL-derived TEX_{86} values do not vary with *in situ* temperature for reasons that are presently unclear. Comparison with surface sediment data suggests that fossil GDGTs in the surface sediment are unlikely to be derived from the oxygen minimum zone, where the largest concentrations of GDGTs are actually found, but from the overlying surface waters.

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Chapter 8

Crenarchaeol tracks winter blooms of planktonic ammonia-oxidizing Crenarchaeota in the coastal North Sea

Angela Pitcher[1], Cornelia Wuchter[1,2], Kathi Siedenberg[1,3], Stefan Schouten[1], and Jaap S. Sinninghe Damsté[1]

[1] Department of Marine Organic Biogeochemistry, Royal Netherlands Institute for Sea Research, P.O. Box 59, 1790 AB, Den Burg, The Netherlands

[2] Current address: Woods Hole Oceanographic Institution, 266 Woods Hole Rd., MS# 52, Woods Hole, Ma. 02543

[3] Department of Marine Geology MARUM University of Bremen Leobener Strasse D-28359 Bremen Germany

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Abstract

Ammonia-oxidizing Archaea (AOA) were previously recognized as important actors in the nitrogen cycle of the coastal North Sea during the winter of 2002-2003. We followed the abundance and distribution of AOA in the North Sea from April 2003-February 2005 by quantification of archaeal genes and core glycerol dibiphytanyl glycerol tetraether (GDGT) membrane lipids, to determine whether their abundance in the North Sea is seasonal.

GDGT and gene abundance patterns showed a repeated annual increase during winters and were nearly absent during the summer months. Crenarchaeol - a GDGT hypothesized to be specific to AOA - represented a major fraction of the GDGTs and varied in concert with AOA gene abundance, thereby pointing to AOA as the predominant source of crenarchaeol (in addition to the other GDGTs) in North Sea waters. The presence of crenarchaeol-based intact polar lipids (IPLs) from October 2007-March 2008 confirmed that the GDGTs recovered were derived from living AOA, as IPLs are rapidly degraded upon cell senescence and thus their occurrence represents (recently) living biomass more robustly than their fossil (i.e. core GDGT) counterparts. Dark incubations of North Sea water sampled during the 2007-2008 seasonal cycle with ^{13}C-labeled bicarbonate revealed incorporation of inorganic carbon into IPL-derived GDGTs, directly showing autotrophic production of Crenarchaeota biomass during the winter. Inhibition of ^{13}C uptake by nitrification inhibitors further confirmed that ammonia-oxidation was their main source of energy for carbon fixation. Together our results indicate that winter blooms of planktonic AOA in the coastal North Sea appear to be recurrent and predictable, occurring annually between November and February. These findings confirm previous preliminary observations and emphasize the potential importance of AOA in North Sea nitrogen cycling.

Introduction

The ubiquity of marine Archaea throughout the global seas and oceans has become well-established knowledge over the last two decades (e.g. DeLong, 1992; Venter et al., 2004). From this microbial domain, 16S rRNA gene sequences representative of two major phyla - the Crenarchaeota and Euryarchaeota - are now routinely recovered from temperate marine waters. A particular association has been made between the occurrence of marine Group I.1a Crenarchaeota (MCGI) and genes coding for the alpha subunit of archaeal ammonia monooxygenase (*amoA*) (the enzyme responsible for the oxidation of ammonia to hydrolylamine), suggesting that these Crenarchaeota are predominantly ammonia oxidizers

(Francis et al., 2005) and may play a significant role in marine nitrification (Wuchter et al., 2006). Thus far, four Group I Crenarchaeota enrichment cultures have been characterized. These include *Cenarchaeum symbiosum* (Hallam et al., 2006a), *Nitrosopumilus maritimus* (Könneke et al., 2005), "*Ca.* Nitrososphaera gargensis" (Hatzenpichler et al., 2008), and "*Ca.* Nitrosocaldus yellowstonii" (de la Torre et al., 2008), all which oxidize ammonia and fix bicarbonate. In contrast to the Crenarchaeota, metabolic functions of the marine Euryarchaeota remain more enigmatic, and with no currently cultivated representatives to study in physiological detail, their primary biogeochemical functions in the marine environment remain largely unknown.

The cell membrane lipids of Crenarchaeota consist mainly of glycerol dialkyl glycerol tetraethers (GDGTs) (Figure 1). Quantification of GDGTs is being increasingly used in addition to conventional molecular techniques in microbial ecology studies of Crenarchaeota (e.g. Sinninghe Damsté et al., 2002; Pitcher et al., 2009b). Crenarchaeol, a GDGT with a cyclohexane moiety in addition to four cyclopentane moieties, was recovered from the marine environment (Schouten et al., 2000) prior to its recovery from *Cenarchaeum symbiosum* (an AOA that lives in symbiosis with the marine sponge, *Axinella mexicana*; (Preston et al., 1996; Sinninghe Damsté et al., 2002). Since then, crenarchaeol has been found in environments where putative AOA exist (Leininger et al., 2006; Coolen et al., 2007; Pitcher et al., 2009a), and its synthesis by numerous AOA enrichments has been confirmed (Schouten et al., 2008; Pitcher et al., 2010a and 2010b). This, coupled to the apparent absence of crenarchaeol in non-Group I crenarchaeotes, suggests that crenarchaeol may be a specific biomarker for AOA.

GDGTs as they exist in a viable cell each contain a covalently bound polar headgroup (e.g. Figure 1). Upon cell senescence, these headgroups are thought to be rapidly lost leaving behind the comparatively recalcitrant core GDGTs, which largely represent the fossilized remains of formerly living cells. Intact polar lipids (IPLs) are so labile in comparison, that they likely represent the presence of (recently) living cells. AOA are known to synthesize GDGTs with both sugar and phosphate-based polar headgroups (Schouten et al., 2008; Pitcher et al., 2010b) and recently we developed a selected reaction monitoring (SRM) HPLC-ESI/MS method to screen for a number of these crenarchaeol-

based IPLs directly as a more sensitive method for determining the presence of viable AOA (Pitcher et al., 2010c).

The first direct evidence for autotrophy by marine Crenarchaeota came from incorporation of ^{13}C labeled bicarbonate into GDGTs during dark incubation of water sampled from the North Sea (Wuchter et al., 2003). Subsequent analyses of Crenarchaeota genes (both 16S rRNA and *amoA*) (Wuchter et al., 2006) and core GDGT abundances (Wuchter, 2006) showed dramatic increases over the winter of 2002-2003, suggesting a particular adaptation for growth during the winter months. Enrichment of a North Sea marine Group I.1a crenarchaeote confirmed that those Crenarchaeota were indeed AOA (Wuchter et al., 2006).

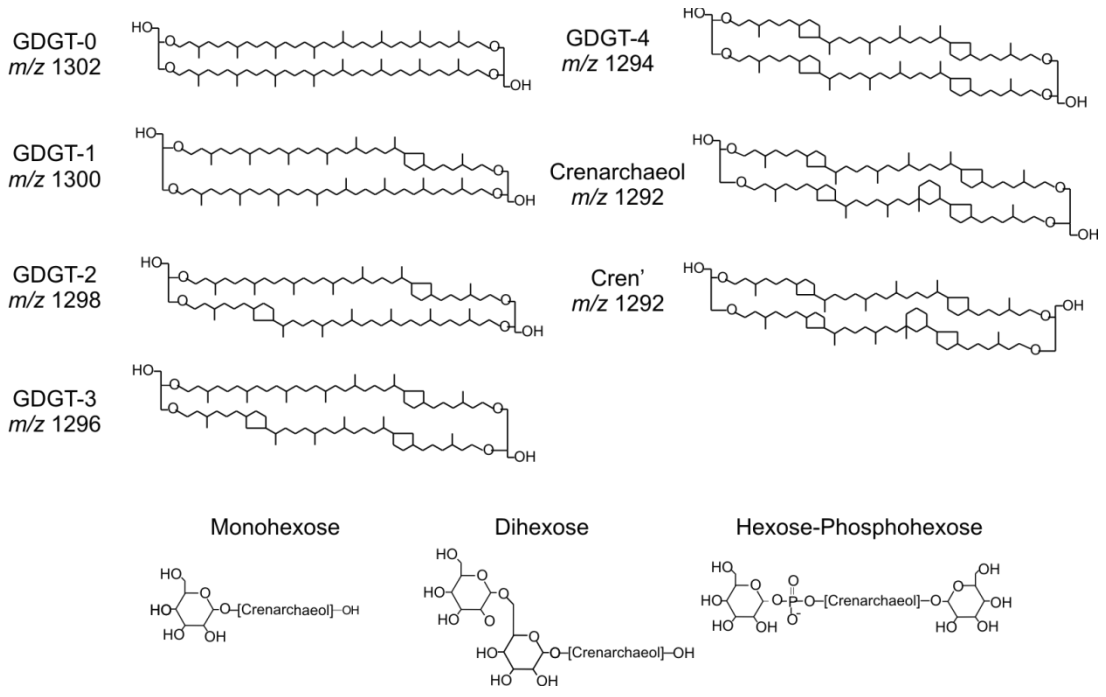

Figure 1. Structures of GDGTs analyzed in this study. Molecular weight of core GDGTs are shown as *m/z* values. Structures of crenarchaeol-based IPLs analyzed by selected reaction monitoring are shown, with the exception of a monohexose-crenarchaeol + 180 Dalton headgroup. The structure of the 180 Dalton moiety remains unknown. Cren' = crenarchaeol regioisomer.

Until now it has remained unclear if North Sea AOA exhibit strong seasonal patterns, and how productive they are during times of elevated abundance. Furthermore, since only core GDGTs were analyzed previously, it is unclear if they are all derived from living cells. Therefore, we have expanded upon the data set of Wuchter et al. (2006) by sampling suspended particulate matter (SPM) from the North Sea for an additional three years (2003-2005 and 2007-2008) to determine if and when 'blooms' of AOA occur, and to assess the utility of crenarchaeol as a tracer for active AOA in this dynamic coastal system. We have quantified the abundance of MCGI 16S rRNA genes and *amoA* genes, in addition to archaeal GDGTs including crenarchaeol. Core and IPL-derived GDGTs recovered from SPM sampled during October 2007 to April 2008 were quantified separately and compared with distribution of crenarchaeol-based IPLs analyzed directly via SRM. Finally, water sampled from the North Sea during 2007-2008 was incubated with ^{13}C-labeled bicarbonate, with and without addition of nitrification inhibitors, to determine the inorganic carbon-fixation activity of MCGI and its relation to nitrification.

Materials and Methods

Study site and sampling

The sampling site is situated at the western entrance of the North Sea into the Wadden Sea at the Island Texel (53°00'25"N, 4°78'27"E) (Figure 2). With each incoming-tide, water from the coastal North Sea moves as far as 25 km into the Wadden Sea (Potsma, 1954). At high tide, water collected at the NIOZ jetty represents Dutch coastal North Sea waters since the estuarine influence is minimal. Strong tidal currents assure that the water is vertically mixed. Therefore, surface water samples taken during high tide are fully oxygenated and representative of the entire water column.

Water samples were taken for DNA and lipid analyses from April 2003 to February 2005, and again from October 2007 to April 2008. For DNA analysis, measured volumes (ca. 1 L) of water were filtered through a 0.2 μm pore size polycarbonate filter (Schleicher

& Schuell were used during 2003-2005, and Millipore filters were used during 2007-2008), 142 mm filter diameter) and stored at -80ºC until extraction. For lipid analyses, a measured volume (ca. 20 L) of water was filtered sequentially through pre-ashed 3 µm, and 0.7 µm-pore-size, glass fiber filters (GF/F, Pall, 142 mm filter diameter). GF/F filters were stored at -20ºC until extraction. Temperature, salinity, and inorganic nutrients were measured weekly as part of a long-term monitoring program of the NIOZ.

Extraction and quantification of total DNA

Six ml of extraction buffer (10 mM Tris HCl, 25mM EDTA, 1vol% SDS, 100 mM NaCl) and 0.1 ml zirconium beads were added to the filters and total DNA was extracted with standard phenol, phenol/chloroform/isoamyl-alcohol and chloroform, and precipitated with ice-cold ethanol according to Sambrook et al. (1989). The DNA-pellet was re-dissolved in ultra-pure DNA and Dnase-free sterile water (Sigma, St. Louis, MO, USA). From each of the total DNA extracts a subsample was subjected to agarose gel electrophoresis to determine the quality of the extracted DNA.

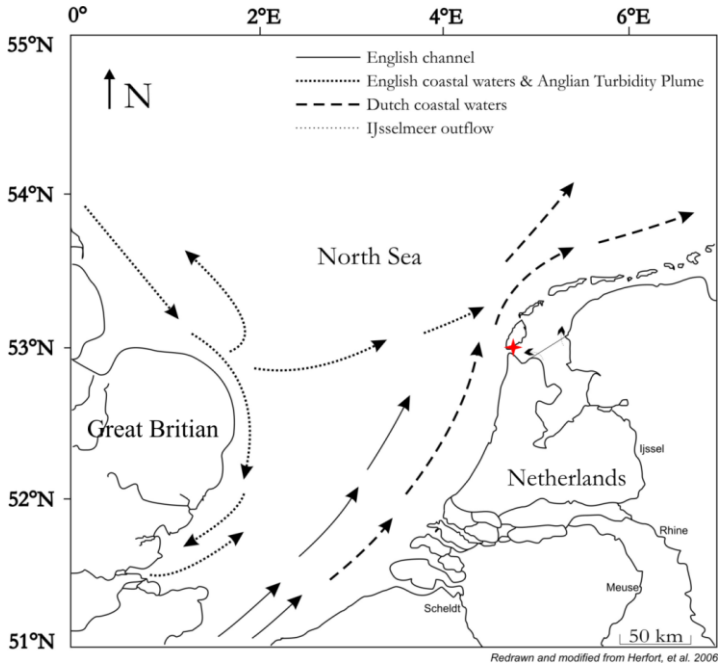

Figure 2. Map of the North Sea and sampling site (star).

Real-time quantitative PCR (Q-PCR)

Quantitative PCR was performed in an iCycler system (BioRad). To quantify the 16S rRNA and *amoA* gene copy numbers, PCR conditions and primers were used as described in Wuchter et al. (2006). Accumulation of amplified rRNA genes was followed online as the increase in fluorescence due to the binding of the fluorescent dye SYBRGreen (Molecular Probes). Reaction mixtures (20 µl) contained 1 unit of Picomaxx^{TM} High Fidelity DNA polymerase, 2 µl of 10× Picomaxx PCR buffer (both Stratagene), 200 µM of each dNTP, 20 µg of BSA, 0.2 µM of primers, 50,000 times diluted SYBRGreen, 3 mM of $MgCl_2$ and ultra-pure sterile water (Sigma). Known amounts of template DNA from each sample was added to each Q-PCR reaction. Quantitative calibration of the samples was achieved using a dilution series containing known copy numbers of purified standards derived from cloned genomic DNA of an AOA previously enriched from the North Sea (Wuchter et al., 2006) to create standard curves ranging from 1 to 10^8 copies per reaction.

Lipid extraction and analysis

Core GDGT extraction of SPM

The GF/F filters were freeze-dried and cut into small pieces with sterile scissors before being ultrasonically extracted four times using dichloromethane (DCM)/methanol (MeOH) (1:1, v/v). The total lipid extracts were eluted over an activated Al_2O_3 column by eluting with MeOH/DCM (1:1, v/v) to obtain a fraction enriched in core GDGTs. Solvent was removed from the eluent under a stream of nitrogen (N_2) and the residue was dissolved by sonication (5 min) in hexane:propanol (99:1, v/v). The resulting suspension was filtered through a 0.45-µm-pore-size, 4 mm diameter Teflon filter prior to injection. The GDGTs were analyzed by high performance liquid chromatography (HPLC)-APCI/MS using conditions modified from Hopmans et al. (2000). Analyses were performed using an HP (Palo-Alto, CA, USA) 1100 series LC-MS equipped with an auto-injector and Chemstation chromatography manager software. Separation was achieved on a Prevail Cyano column (2.1 x 150 mm, 3 µm; Alltech, Deerfield, IL, USA), maintained at 30°C. GDGTs were eluted isocratically with 99% A and 1% B for 5 min, followed by a linear gradient to 1.8% B in 45 min, where A = hexane and B = propanol. Flow rate was 0.2 ml/min. After each

analysis, the column was cleaned by back-flushing hexane/propanol (90:10, v/v) at 0.2 ml min^{-1} for 10 min. Detection was achieved using atmospheric pressure positive ion chemical ionization mass spectrometry (APCI-MS) of the eluent. Conditions for APCI-MS were as follows: nebulizer pressure 60 psi, vaporizer temperature 400°C, drying gas (N_2) flow 6 L min^{-1} and 200°C, capillary voltage -3 kV, corona 5 μA (~ 3.2 kV). GDGTs were detected by single ion monitoring of their $[M+H]^+$ ions and quantified by integration of the peak areas and comparison with a standard curve of a GDGT-0 standard.

Intact polar lipid extraction of SPM

Intact polar lipids (IPLs) were extracted from SPM sampled during 2007-2008 using a modified Bligh and Dyer technique (Bligh and Dyer, 1959). A known volume of single-phase solvent mixture of MeOH:DCM:phosphate buffer (2:1:0.8, v/v/v) was added to the sample in a glass centrifuge tube and placed in an ultrasonic bath for 10 minutes. The extract and residue were separated by centrifuging at 2500 rpm for 5 min and the solvent mixture collected in a separate flask (repeated 3 ×). The DCM and phosphate buffer were added to the single-phase extract to give a new ratio of MeOH:DCM:phosphate buffer (1:1:0.9, v/v/v), and to induce phase separation. The extract was centrifuged at 2500 rpm for 5 min. The DCM phase was collected in a round-bottom flask and the MeOH:phosphate buffer phase was washed 2 additional times with DCM. The combined DCM phases were reduced under rotary vacuum and evaporated to dryness under a stream of N_2.

An aliquot of each Bligh and Dyer extract was eluted over pre-activated silica gel (60 mesh) with three column volumes of hexane:ethyl acetate (3:1, v/v) and MeOH to obtain fractions enriched in core GDGTs and IPL-GDGTs, respectively (Pitcher et al., 2009b). To each fraction a known amount of a C_{46} internal standard was added (Huguet et al., 2006). Core GDGT fractions were evaporated under N_2 to dryness and then analyzed with HPLC-APCI/MS as core GDGTs using conditions described above. The IPL fraction was evaporated to dryness and then subjected to acid hydrolysis by reflux in 1M HCl for 1 hour to cleave any polar headgroups, following methods described in (Pitcher et al., 2009a). The resulting IPL-derived core GDGTs were analyzed with HPLC-APCI/MS using

conditions described above and quantification of GDGTs was achieved using the internal standard as described in Huguet et al. (2006).

Selected Reaction Monitoring of Specific IPLs

An aliquot of Bligh and Dyer extract was used for direct analysis of crenarchaeol-based IPLs shown in Figure 1 (Pitcher et al., 2010c). Detection was achieved by HPLC/electrospray ionization (ESI)–MS/MS. Separation was achieved on a LiChrospher diol column (250 × 2.1 mm, 5 µm particles, Alltech) maintained at 30°C. The following linear gradient was used with a flow rate of 0.2 mL min^{-1} 100% A:0% B to 35% A: 65% B over 45 min, maintained for 20 min, then back to 100% A for 20 min to re-equilibrate the column. Detection of crenarchaeol-based IPLs was achieved by using conditions, and monitoring the transitions, described in Pitcher et al. (2010c). Due to the lack of a purified standard for quantification, crenarchaeol-based IPLs were quantified as the integrated IPL peak area response L^{-1}. This approach reveals the relative distribution of individual IPLs over time, but does not allow the comparison of different IPL absolute abundances.

Stable isotope probing of AOA

Approximately every two weeks from October 2007 to April 2008, 4 × 20 L of North Sea water was sampled into clean Nalgene containers and incubated with ^{13}C-labeled bicarbonate in the dark at *in situ* temperatures for approximately 24 h to measure inorganic carbon uptake rates by marine Crenarchaeota. Four treatments were used: one container was left as a non-treated control, while bicarbonate (300 mg of 99% ^{13}C-labeled, equivalent to an addition of ca. 9% of the total bicarbonate) was added to each of the other three containers. Nitrification inhibitors were added to two of the three ^{13}C-labeled incubations to determine the effects on AOA carbon-fixation: 100 mg of N-serve (2-chloro-6-(trichloromethyl)pyridine; 5 mg mL^{-1}) was added to one container to inhibit ammonium oxidation (Bédard and Knowles, 1989; de Bie et al., 2002), and 21 g sodium chlorate ($NaClO_3$, 10 mM) was added to another container to inhibit nitrite oxidation (Belser and Mays, 1980). After each sampling, inhibitors were added immediately and the incubations were gently aerated (bubbled with air) and given ca. 2 h to equilibrate in a dark,

temperature-controlled, incubation chamber prior to the addition of ^{13}C-labeled substrates. After incubation for approximately 24 h the water was filtered over 0.7 µm GF/F filters and analyzed for GDGTs. The filters were freeze-dried and extracted using a modified Bligh and Dyer technique, and core GDGT and IPL-derived GDGT fractions were generated as described above.

IPL-derived GDGTs were analyzed for ^{13}C-label incorporation by treatment with $HI/LiAlH_4$ to release the biphytanes (Hoefs et al., 1997). The $\delta^{13}C$ values of GDGT-derived biphytanes were measured by isotopic-ratio-monitoring (IRM)-gas chromatography/mass spectrometry (GC/MS). Compound-specific ^{13}C analyses were performed with an Agilent 6800 GC coupled to a Thermo Fisher Delta V isotope ratio monitoring mass spectrometer. Isotope values were measured against calibrated external reference gas and performance was checked by daily injections of two internal perdeuterated n-alkane standards. The ^{13}C values are reported in the standard delta notation against the Vienna Pee Dee Belemnite (VPDB) standard. Samples were run in at least duplicate. Incorporation of ^{13}C into the tricyclic biphytane derived from crenarchaeol (BIP-3) during incubations is expressed as $\Delta\delta^{13}C$ values (the difference between $\delta^{13}C$-labeled incubations and $\delta^{13}C$-control incubations).

Results

Seasonal dynamics in environmental parameters

Figure 3 summarizes chemical and physical data collected from coastal North Sea water sampled at high tides continuously from 2002 to 2008. Concentrations of ammonium (NH_4^+) were generally highest between November and January, ranging from ca. 10-13 µM during winter sampling periods (grey vertical bars) however, an anomalous spike in NH_4^+ occurred during Mid-May of 2004 which was not observed in other seasons. Nitrite (NO_2^-) concentrations were comparatively low throughout the year (0-3 µM), peaking during the winter as well. Annual declines in NH_4^+ and NO_2^- in the middle of the winter coincided with increases in nitrate (NO_3^-), suggesting periods of net nitrification (cf. Wuchter et al.,

2006). NO_3^- reached peak concentrations (ca. 60-90 µM) between January-February each year when the coastal North Sea reached its lowest temperatures (ca. 2-4°C). A subsequent steady decline in NO_3^- to ca. 0.2-0.5 µM was achieved by around August of each year presumably as a result of assimilation by spring and summer-blooming phytoplankton, while water temperatures rose to maxima of ca. 20-22°C. Annual oscillations in salinity were observed as well, with several sharp dips reaching as low as 20 permil during the winter, evidencing increased fresh water flux into the North Sea during these times.

Archaeal GDGTs

Core GDGT abundances

Figure 4 (A-C) shows the abundances of core GDGTs (grey circles), crenarchaeol, GDGT-0 and GDGTs 1-3 combined, measured from February 2002 to February 2005, and October 2007 to April 2008. Overall concentrations of all core GDGTs were low during the spring and summer months, and gradually increased starting around October-November to reach peak abundances during either January or February each year (Table 1). Subsequently, concentrations quickly decreased to near-autumn values by March-April each year. Crenarchaeol and GDGT-0 were dominant among the GDGTs, with maximum winter concentrations reaching 100-140 ng L^{-1} and 150-170 ng L^{-1}, respectively. GDGTs 1-3 represented a minor component of the total GDGT pool, their combined abundance reaching between 20-30 ng L^{-1} at winter maxima. In general, the winters in which sampling occurred were typified by analogous peaks of similar concentrations (cf. Figure 4 A-C). Overall, GDGT abundance correlated well with each other (R^2=0.83-0.97).

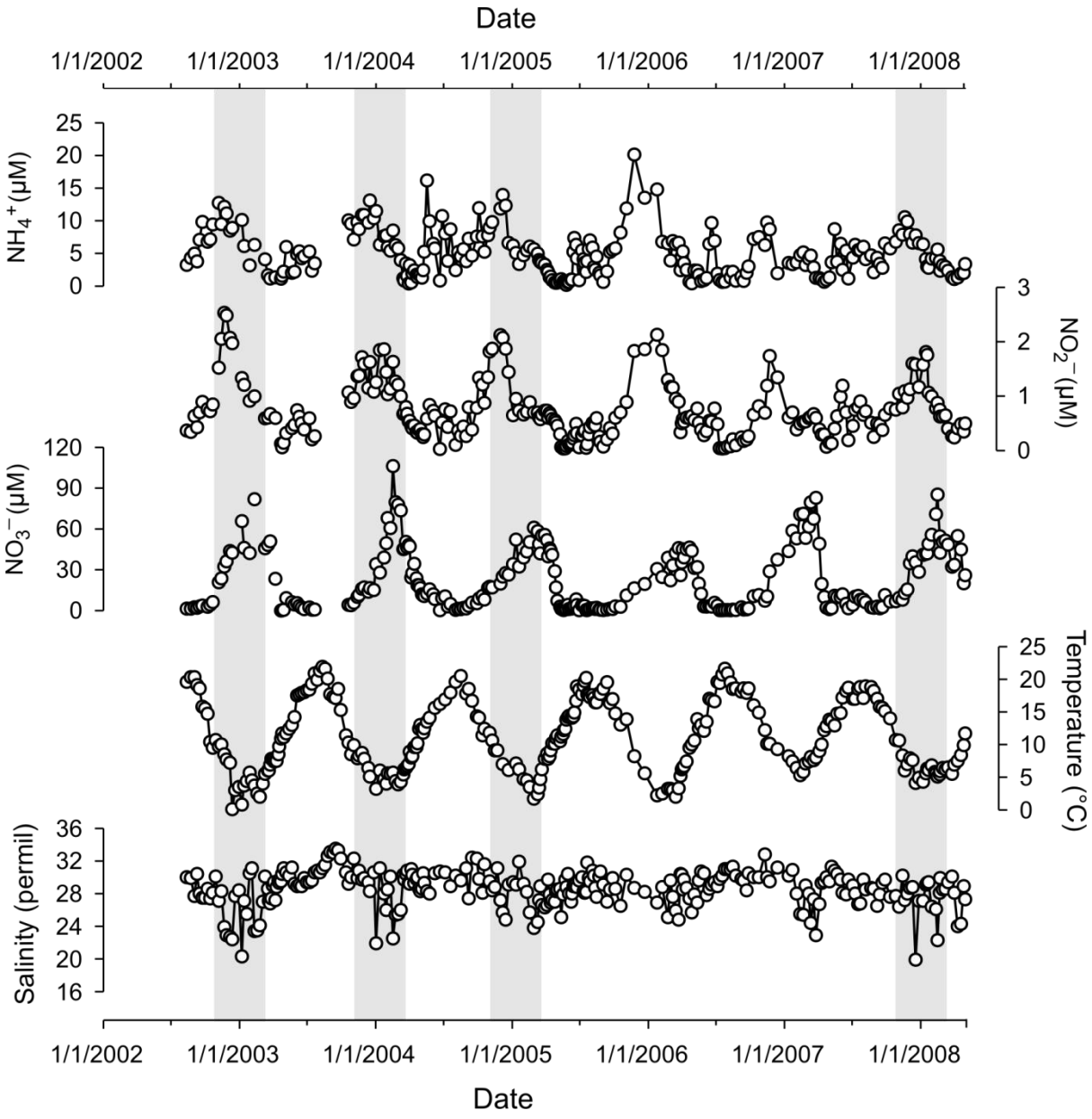

Figure 3. Inorganic nutrient concentrations (µM), ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-), temperature and salinity measured from 2002-2008. Grey bars indicate winter periods.

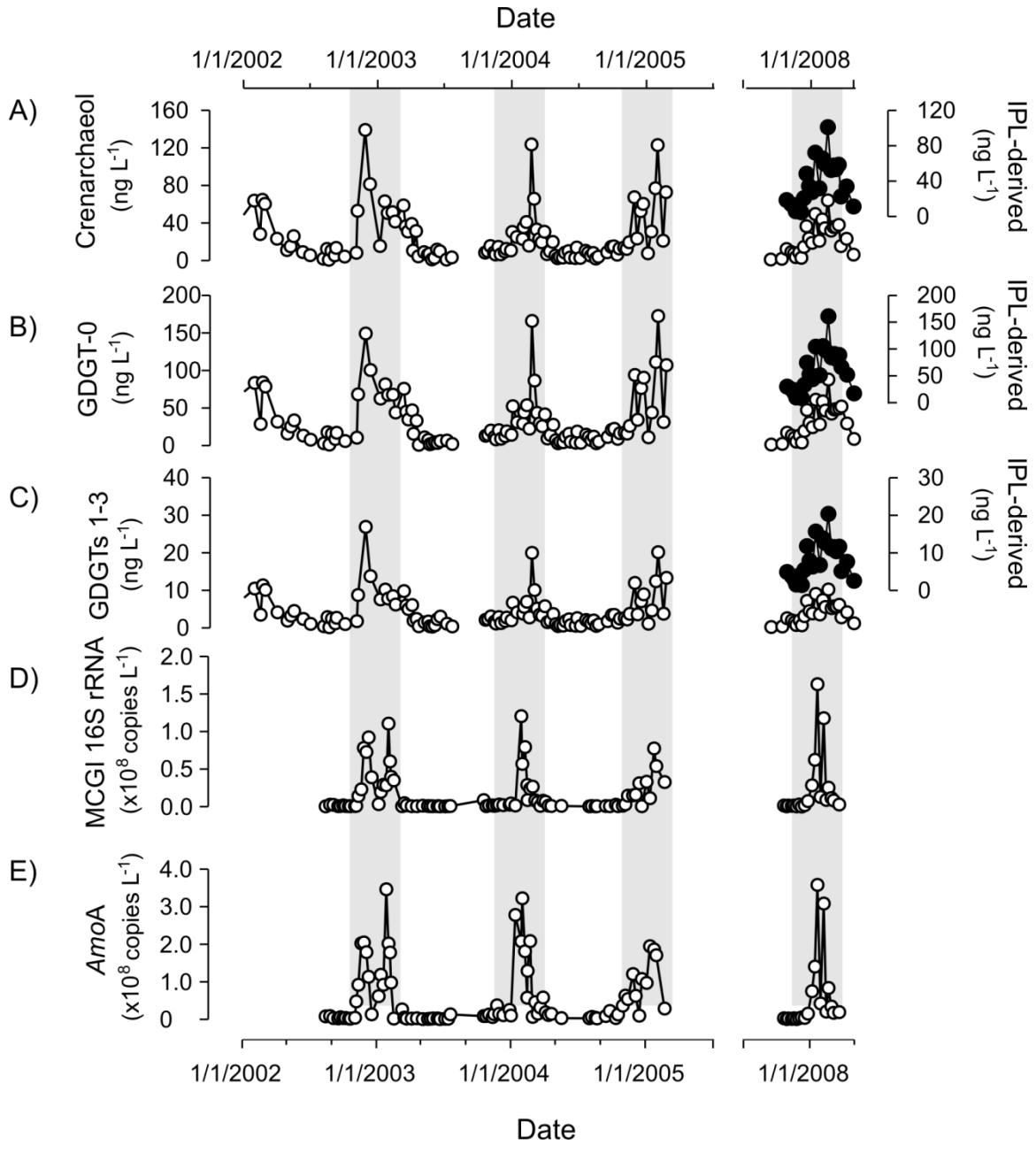

Figure 4. Concentrations of core (white circles) and IPL-derived (black circles) (A) crenarchaeol, (B) GDGT-0, and summed GDGTs 1-3 (C) recovered from North Sea suspended particulate matter from 2002-2005 and 2007-2008. Copy numbers of (D) 16S rRNA genes for MCGI and (E) archaeal *amoA* genes peaked during the winter months (grey bars), and matched fairly well to GDGT abundances.

Core and IPL-derived GDGT abundances 2007-2008

Over the winter of 2007-2008 core and IPL-derived GDGT abundances were quantified separately after Bligh and Dyer extraction of GFF filters, as opposed to conventional organic solvent methods used to extract GDGTs from SPM collected during 2002-2005. Total abundances of core and IPL-derived GDGTs over this time period were well-correlated with each other (R^2=0.89), with IPL-derived GDGTs representing an average of 41 ± 5% of the total GDGT pool (i.e. core and IPL-derived GDGTs combined). Core GDGTs measured during the 2007-2008 winter season were not as abundant as previous years, however when the abundances of core and IPL-derived GDGTs were combined (black circles, Figure 4 A-C), the GDGT concentrations were comparable from season to season. This suggests that a substantial proportion of the core GDGTs measured during 2002-2005 were derived from IPLs that were present in the SPM but lost their polar headgroups during the sample workup. Comparison of the relative GDGT distributions over the season showed that GDGT-0 is present in slightly higher amounts in the core fraction compared to the IPL fraction (56 ± 6% as core GDGT of total GDGTs), while for crenarchaeol the major part was present as core lipid (65 ± 6% of total GDGTs). Minor GDGTs 1-3 were roughly equally distributed over the core and IPL fractions (51 ± 6%).

Selected reaction monitoring (SRM) of crenarchaeol-based IPLs

Using a recently developed SRM method we were able to follow the relative abundances of various crenarchaeol-based IPLs (Figure 1) in the 2007-2008 SPM series (Figure 5 A-D). Crenarchaeol with monohexose (MH) and hexose-phosphohexose (HPH) headgroups were detected at every date sampled, in comparison to crenarchaeol with dihexose (DH) and monohexose +180 Dalton ('MH+180') moieties which were undetectable in some SPM samples taken near the beginning and end of the sampling period (cf. Figure 5B and C). IPL-GDGTs with 'MH+180' headgroup have been identified in a number of enriched marine Group I.1a AOA (Pitcher et al., 2010a; Schouten et al., 2008), however, the precise structure is still unknown. Individual and total IPL responses correlated well with quantified IPL-derived crenarchaeol (Figure 5E) (R^2=0.81-0.87), i.e.

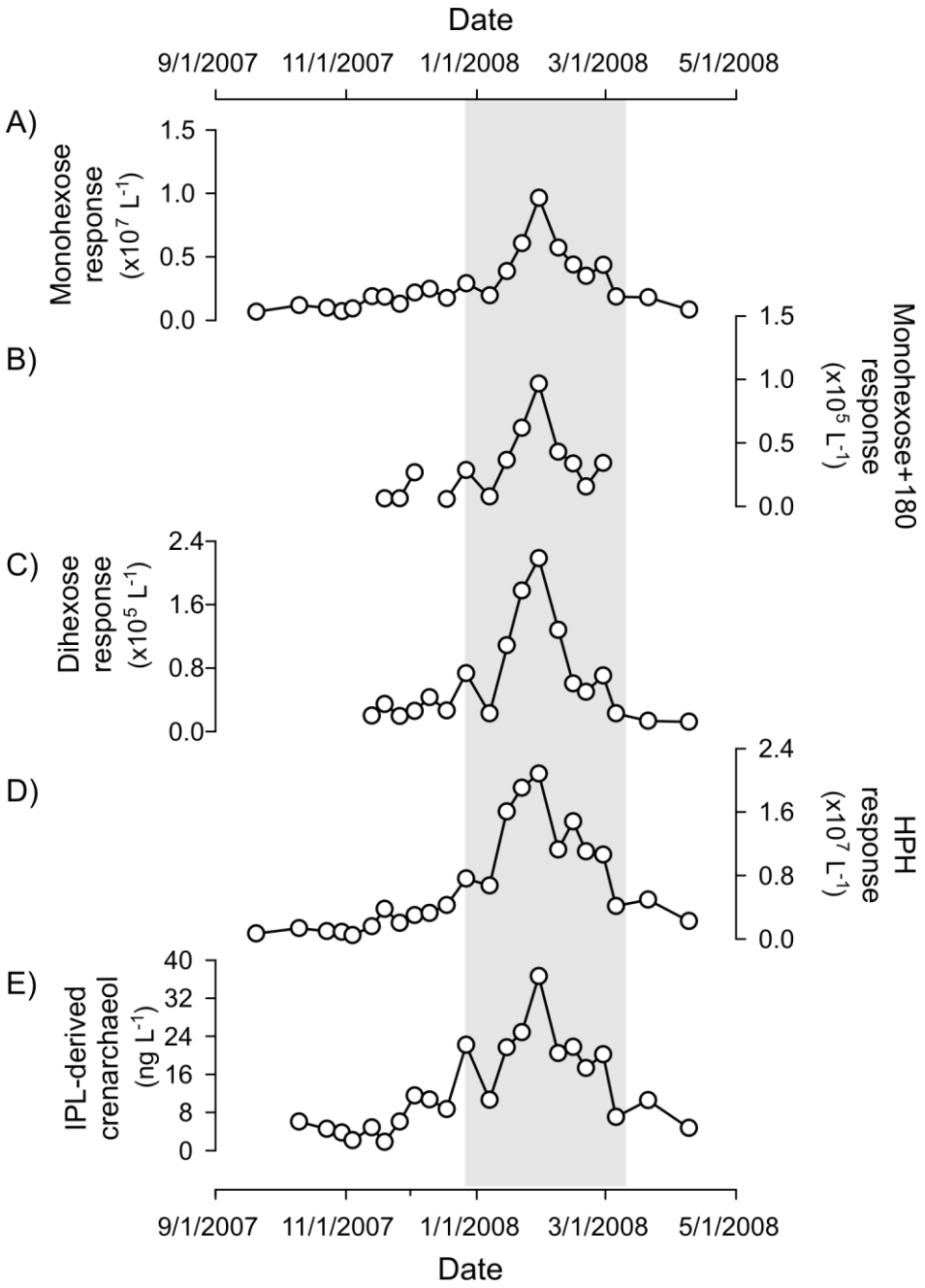

Figure 5. Seasonal profiles (2007-2008) showing the integrated instrument response for selected reaction monitoring of crenarchaeol-based IPLs with (A) monohexose (MH), (B) monohexose + 180 Dalton moiety (MH+180), (C) dihexose (DH), and (D) hexose-phosphohexose (HPH) headgroups, as well as (E) IPL-derived crenarchaeol concentrations.

there was no apparent offset between the occurrence of the various IPL-crenarchaeol and core crenarchaeol in the North Sea during this sampling period. This suggests that IPL-derived crenarchaeol is indeed mostly derived from IPLs.

Annual variation in Archaeal genes

Over the entire sample period, the copy numbers of MCGI 16S rRNA and *amoA* genes co-varied generally well (R^2=0.56). Notable increases in gene abundance occurred near just prior to winter each sampling season, with MCGI 16S rRNA and *amoA* gene abundances increasing from near detection limit to ca. 10^8 L^{-1} (Figure 4 D-E). Elevated gene abundances (>10^7 copies L^{-1}) were maintained throughout much of the winter and into the early spring, although the length of this period varied from year to year, ending between the end of March to mid-April (Table 1). In the first sampling season (2002-2003), Wuchter et al. (2006) revealed two notable peaks in gene abundance of approximately equal magnitude, however, the rest of the sampling seasons showed only a single peak (Figure 4). Despite annual variation in the duration of elevated gene abundances, annual maxima were achieved within a relatively narrow time-frame (between January 14 and February 2, with the exception of Wuchter et al. (2006) who noted an additional 'early' peak in November of 2002), where MCGI and *amoA* abundances increased to 7×10^7 - 2×10^8 and 2 - 4×10^8 copies L^{-1}, respectively (Table 1).

^{13}C incorporation into crenarchaeol

IRM-GC/MS analysis of HI/AlH_4-treated IPLs showed incorporation of ^{13}C into GDGT-derived biphytanes. Here we discuss only incorporation into the tricyclic biphytane derived from crenarchaeol (Bip-3), as this GDGT is the most specific to marine AOA (Sinninghe Damsté et al., 2002). Over the nine incubations performed during the winter of 2007-2008, $\Delta\delta^{13}$C values of Bip-3 ranged from 0-44‰ (Figure 6), peaking on January 30, when maximum IPL-GDGT abundances were also observed. In general, the trend in $\Delta\delta^{13}$ of Bip-3 followed the seasonal variation in the concentration of HPH-crenarchaeol. The

Table 1 . Summary of maximum MCGI 16S rRNA and *AmoA* genes and core GDGT abundances measured during bloom periods.

year	duration of 'bloom'		max genes			max GDGTs			
				abundance (copies L^{-1})			abundance (ng L^{-1})		
	start	end	date	MCGI	*amoA*	date	cren	GDGT-0	GDGTs 1-3
2002-3	7-Nov	12-Mar	21-Nov	7.79E+07	2.0E+08	28-Nov	139.0	148.9	26.8
			28-Jan	1.10E+08	3.5E+08	1-Jan	62.9	81.4	10.2
2003-4	3-Nov	21-Apr	2-Feb	7.90E+07	3.2E+08	23-Feb	123.5	165.7	19.9
2004-5	19-Oct	23-Feb	14-Jan	7.72E+07	1.9E+08	31-Jan	122.8	172.2	20.1
2007-8	27-Dec	21-Mar	22-Jan	1.63E+08	3.6E+08	30-Jan	100.8*	160.9*	20.4*

* GDGT abundances are the summed total of quantified core GDGTs and IPL-derived GDGTs

same trend in $\Delta\delta^{13}$ values of Bip-3 was visible in the incubations containing the inhibitors N-serve (which inhibits ammonia oxidation) and sodium chlorate (to inhibit nitrite oxidation; Belser and Mays, 1980) but the absolute values were much less (Figure 7), i.e. up to 14‰ for chlorate and up to 5‰ for N-serve.

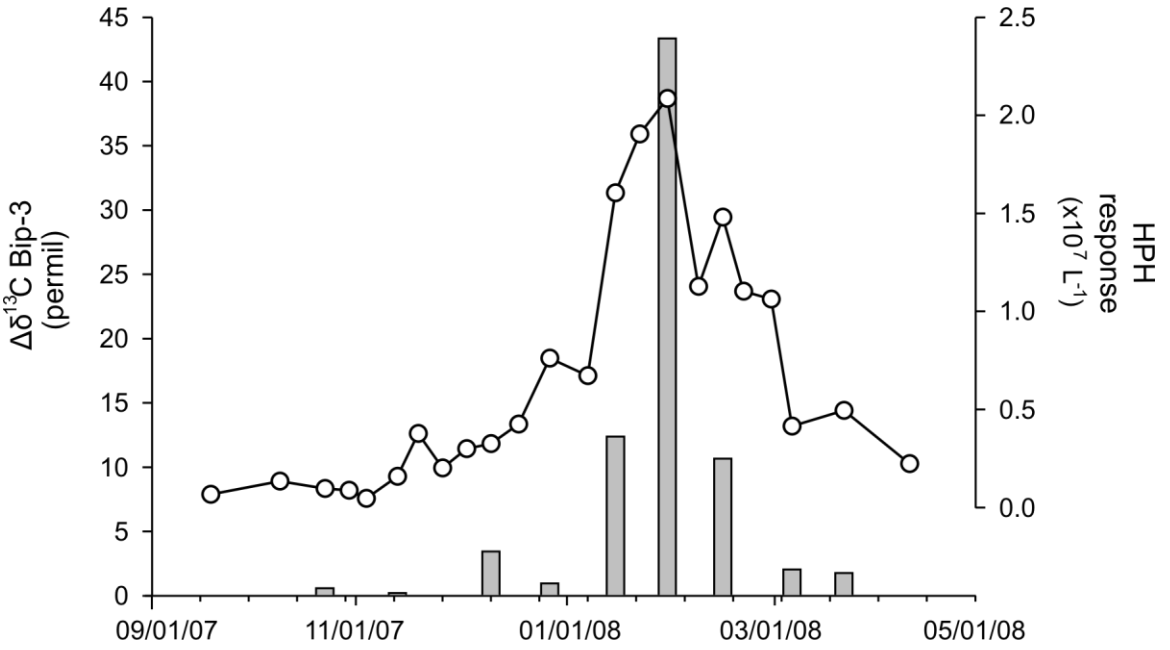

Figure 6. ^{13}C-incorporation into the crenarchaeol-derived biphytane with three cyclic moieties (Bip-3) after incubations performed during the 2007-2008 winter (grey bars). $\Delta\delta^{13}$C values represent the difference in ^{13}C content of Bip-3 from non-labeled (control) and ^{13}C-bicarbonate labeled incubations. White circles indicate the seasonal profile of HPH-crenarchaeol.

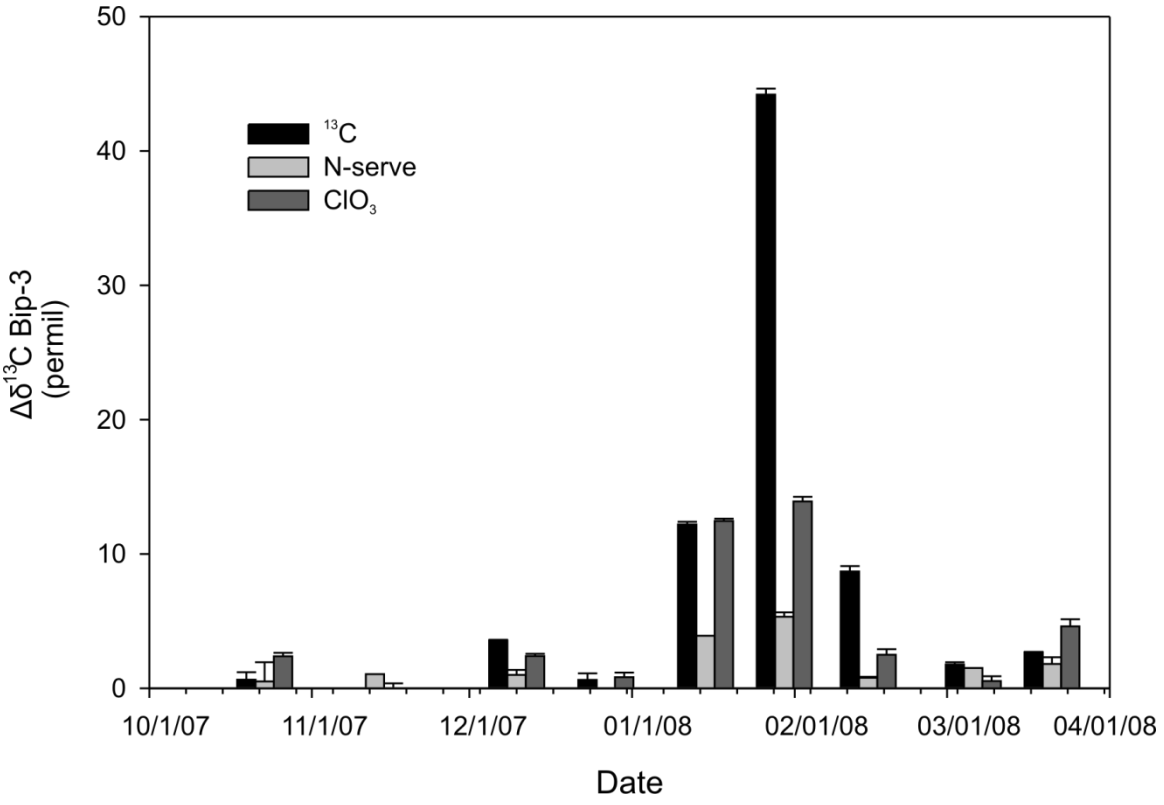

Figure 7. Δδ ^{13}C values of Bip-3, derived from crenarchaeol, after incubation with ^{13}C bicarbonate only, and with the inhibitors chlorate and N-serve over during the 2007-2008 winter. Error bars indicate standard deviations of replicate measurements.

Discussion

Crenarchaeol as a marker for ammonia-oxidizing Crenarchaeota in the North Sea

Comparison of annual increases in GDGT and gene abundances show that both occur during similar times in winter periods (Figure 4, shaded bars). There was an overall correspondence between crenarchaeol abundance and *amoA* and MCGI gene copy numbers, which showed distinct seasonal increases and decreases. This suggests that crenarchaeol is derived mainly from AOA living in the North Sea. AOA in the North Sea

are closely related to *N. maritimus* (Herfort et al., 2007; Wuchter et al., 2006), a marine AOA known to synthesize crenarchaeol (Schouten et al., 2008). Current evidence that crenarchaeol is specific to AOA comes mainly from culture studies (Sinninghe Damsté et al., 2002; de la Torre et al., 2008; Schouten et al., 2008; Pitcher et al., 2010a and 2010b) and mesocosm experiments (Wuchter et al., 2004; Schouten et al., 2007). Our field data now provide strong environmental evidence that crenarchaeol recovered from marine environments indeed predominately comes from AOA.

Additional support for the fact that core crenarchaeol (and other GDGTs) measured throughout the time series is derived from living AOA comes from the presence of crenarchaeol-based intact polar lipids (Figure 4 D-E, Figure 6), which are generally indicative of the presence of living cells (White et al., 1979; Harvey et al., 1986; Sturt et al., 2004). Correlated absolute abundances of individual GDGTs in the core and IPL-derived fractions (data not shown) suggest they are likely sourced from the same microbial population. If the IPLs and core GDGTs were derived from different sources, i.e. living versus fossil biomass, one could expect differences in concentration patterns as the controls on delivery of dead material through e.g. sediment transport is different from the growth of *in situ* microbial populations. In addition, the correspondence between HPH-crenarchaeol abundance, gene abundances and ^{13}C incorporation into IPL-derived GDGTs indicate that IPL-GDGT concentrations may not only indicate the abundance, but also productivity, of MCGI AOA.

Comparison of core and IPL-derived GDGT distributions showed that minor GDGTs were present in roughly equal abundance in the CL and IPL fractions. GDGT-0 was present in higher relative abundance in the IPL fraction in contrast to crenarchaeol, which was present in higher relative amounts in the CL fraction. While this could indicate slightly different sources for CL and IPL GDGTs, the comparative GDGT distributions and correlated abundances of core and IPL-derived GDGTs are similar enough to infer that they are derived from similar populations of AOA in the North Sea. The slight differences noted could be reflective of a number of factors related to the specific headgroups attached to given GDGTs. Differential degradation rates of phospho and glycolipids (Schouten et al., 2010) could feasibly contribute to slight differences in the core and IPL-derived GDGT

pools. Marine Group I.1a AOA synthesize GDGT-based IPLs with GDGTs 1-4 more closely associated with glycolipids and GDGTs 0 and crenarchaeol more closely associated with phospholipids (Schouten et al., 2008; Pitcher et al., 2010a and 2010b). Since the latter are more labile once free from the cell membrane (i.e. upon cell senescence), it stands to reason that if the majority of crenarchaeol synthesized by North Sea AOA is bound to a phosphate headgroup, relatively more may be recovered as a core GDGT. The fact that the relative abundance of GDGT-0 is higher in the IPL-derived GDGT pool may reflect that proportionally more GDGT-0 is bound predominantly to sugar-based headgroups, which are less labile.

While AOA recovered from marine water columns are usually attributed to planktonic species, we cannot exclude a contribution by sedimentary AOA in the North Sea, where high amounts of suspended material persist in the water column (ca. 50 m deep at maximum) throughout the year and the forceful tides combined with rough weather (especially in autumn and winter), undoubtedly result in sediment re-suspension from the seafloor. Indeed, three sedimentary marine AOA enrichments were recently shown to synthesize crenarchaeol and crenarchaeol-based IPLs analyzed in this study (Pitcher et al., 2010b).

Seasonal recurrence of AOA and their role in the North Sea N cycle

Incorporation of ^{13}C from bicarbonate into IPL-derived crenarchaeol unambiguously shows that during MCGI bloom periods at least a portion of the population is autotrophic (Figure 6). A strong reduction of crenarchaeal carbon fixation was observed after the addition of N-serve (an inhibitor of ammonium oxidation; de Bie et al., 2002), strongly indicating that the MCGI producing crenarchaeol are likely oxidizing ammonium as an energy source for autotrophic fixation (Figure 7). Inhibition by N-serve was not complete, suggesting that either 1) the concentration of N-serve was not high enough to completely inhibit ammonia oxidation, 2) that some of the crenarchaeol-synthesizing MCGI used alternative substrates for energy, while still fixing inorganic carbon or 3) that CO_2 was taking up through anaplerotic reactions. Genomic analysis of the MCGI crenarchaeote, *Cenarchaeum symbiosum*, and environmental samples showed that marine

Crenarchaeota can use other forms of reduced nitrogen as an energy source (Hallam et al., 2006a; Hallam et al., 2006b). Significant, albeit less than with N-serve, reduction of carbon fixation was observed with the addition of chlorate (which inhibits nitrite oxidation; Belser and Mays, 1980)(Figure 7). This indicates that NO_2^- accumulation during the incubation periods may have affected the ammonia-oxidation activity of AOA, thereby suggesting that nitrite-oxidizing bacteria are essential partners for AOA in the natural environment.

The question remains why the annual peak abundance of AOA always occurs during the winter. Similar observations (i.e. MCGI Crenarchaeota peak in abundance during winter times), have been made for the greater North Sea (Herfort et al., 2007), waters off the Antarctic Peninsula (Murray et al., 1998), the Santa Barbara Channel in California (Massana et al. 1997) and off the Mediterranean coast of Spain (Galand et al., 2010). The highly predictable seasonal pattern of the MCGI Crenarchaeota in the North Sea, and other regions, suggests that this group is highly optimized for a specific set of environmental conditions, such as light, temperature and nutrients, and reflect a low degree of functional redundancy (cf. Furhman et al., 2006). One obvious reason why this regular seasonal pattern occurs in these widely different regions would be competition for NH_4^+ which, with respect to phytoplankton, is more favorable during winter as light and temperature are unsuitable for phytoplankton growth. Experiments with *N. maritimus* SCM1 suggest a high affinity for ammonium, indicating that marine AOA have a particular adaptation to low ammonium concentrations (Martens-Habbena et al., 2009). Although *in situ* NH_4^+ concentrations are relatively high (3-20 μM) in the coastal North Sea during the winter compared to the Km value for *N. maritimus* SCM1, NH_4^+ concentrations are still low compared to the Km values reported for some common marine AOB (Martens-Habbena et al., 2009). It is therefore conceivable that AOB do not play an important role in North Sea ammonia-oxidation. Indeed Wuchter et al. (2006) noted much lower copy numbers of bacterial *amoA* than archaeal *amoA* in the North Sea.

Conclusions

Patterns in the abundance of AOA genes and both core and IPL-derived GDGTs in the coastal North Sea, demonstrate a pronounced seasonal effect on the ecology of AOA. Here we have noted a long-term association between crenarchaeol and AOA abundance. While it is still not exactly clear to what extent certain environmental factors influence AOA ecology in the North Sea, peak abundances during the winter months support previous hypotheses that light regime and competition with phytoplankton and other bacteria are important variables. ^{13}C incorporation into crenarchaeol, which substantially decreased when ammonia oxidation was inhibited, showed that North Sea AOA are active chemoautotrophs during the winter months, peaking in CO_2 uptake rates at the peak of their bloom. Overall, crenarchaeol appears to be an effective tool to track the occurrence of AOA in the North Sea and together, the data emphasize the potential importance of AOA in North Sea nitrification and carbon fixation during the winter.

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Summary

Non-extremophilic Crenarchaeota are ubiquitous, and comprise a major component of the microbial assemblages in many modern-day systems. Several studies have analyzed glycerol dialkyl glycerol tetraether (GDGT) membrane lipids synthesized by Crenarchaeota to interpret the presence, distribution, and activity of these microbes in various modern environments. The use of cellular membrane lipids in molecular ecology studies provides added value to conventional (meta)genomic approaches, partly in the form of independence from biases associated with the extraction and analysis of nucleic acids. However, disentangling biomarker lipid signals derived from living and dead cells has remained a challenge. This thesis describes investigations aimed at developing the use of intact polar lipids (IPLs) in ecological studies of ammonia-oxidizing Crenarchaeota (AOA), as crenarchaeotal IPLs containing polar head groups bound to the core GDGT are assumed to best represent living Crenarchaeota. To this end, improvements to both indirect and direct GDGT-based IPL analyses were made, with the latter based largely on information obtained from four novel enrichment cultures of ammonia-oxidizing Crenarchaeota. The findings of these studies were applied to three different environmental settings: two California hot springs, the Arabian Sea oxygen minimum zone (OMZ), and the coastal North Sea. Comparisons between IPL and DNA-based molecular data reveal a more complete picture of the distribution and abundance of ammonia-oxidizing Crenarchaeota at those sites, in addition to demonstrating the general robustness of IPL analyses in molecular ecology studies.

When IPL-GDGTs are analyzed (and quantified) after removal of the polar head group by hydrolysis, chromatographic fractionation of core and IPL GDGTs is first necessary in order to discern between dead and live GDGT signals. Conventional column fractionation schemes based on the separation of bacterial glyco- and phospholipids were found unsuitable for the separation of GDGT-based IPLs. Over activated silica, elution of GDGT-IPL standards with hexane:ethyl acetate (3:1, v/v), ethyl acetate, followed by methanol, was shown to yield fractions highly enriched in core, glyco-, and phospho-

GDGTs, respectively. The inadequate separation of GDGT classes using old separation schemes could result in significant qualitative and quantitative differences, and thus the modified solvent elution protocol presented in this study should be used in future work.

The ability to separate core and IPL-GDGTs allowed for the determination of the origin of crenarchaeol in terrestrial hot springs. The hypothesis that crenarchaeol was synthesized exclusively by mesophilic Crenarchaeota was called into question upon its discovery in terrestrial hot springs and its synthesis by the thermophilic AOA, "*Ca*. Nitrocaldus yellowstonii". Recovery of abundant crenarchaeol in the IPL-GDGT fractions extracted from two California hot springs confirmed that crenarchaeol is indeed synthesized *in situ.* In addition, a correspondence between *amoA* gene and IPL-derived crenarchaeol abundances suggested that the crenarchaeol recovered from the hot springs was synthesized by ammonia-oxidizing Crenarchaeota.

The characterization of ammonia-oxidizing Crenarchaeota has been hampered by difficulties in their enrichment, and therefore limited data exists on the IPL-GDGTs synthesized by these microbes in culture. Analysis of the recently enriched Group I.1b AOA, "*Ca*. Nitrososphaera gargensis", revealed a GDGT distribution consisting almost exclusively of crenarchaeol and the crenarchaeol regio-isomer. This finding extends the taxonomic distribution of crenarchaeol synthesis to a new phylogenetic lineage within the Group I Crenarchaeota, and implicates members of this group as important contributors to crenarchaeol recovered from soils. In addition, lower amounts of a tentatively identified GDGT containing a cyclohexane moieties in addition to five cyclopentane moieties were present. The GDGT-associated polar headgroups consisted of monohexose, dihexose, phosphohexose and hexose-phosphohexose moieties in addition to headgroups consisting of mono- and dihexose sugars with an additional moiety of176 Dalton. Together, these data contribute substantially to the current knowledge of IPLs synthesized by AOA and support the hypothesis that crenarchaeol is specific to ammonia-oxidizers. TEX_{86} (a GDGT-based geochemical proxy used to reconstruct past sea surface temperatures)-derived temperatures calculated using the GDGT distribution of "*Ca*. N. gargensis" matched its original cultivation temperature of 46°C, however they did not change according to short term cultivation at 42°C and 50°C. This indicates that individual species may not adjust their

membrane GDGTs dramatically according to temperature or that such a physiological adaptation would take much longer.

Additional support for the specificity of crenarchaeol to AOA comes from analysis of IPL-GDGTs synthesized by additional ammonia-oxidizing Crenarchaeota enriched from marine sediments. Three novel enrichment cultures all synthesized abundant crenarchaeol in addition to other GDGTs commonly recovered from marine suspended particulate matter (SPM), and polar headgroups similar to those synthesized by *Nitrosopumilus maritimus* SCM1 and "*Ca*. N. gargensis". A comparison of the GDGT distributions associated with each polar head group identified prior to, and including, this study revealed a commonality of hexose-phosphohexose crenarchaeol to all AOA, thereby pointing to this IPL as the ideal biomarker to track living ammonia-oxidizing Crenarchaeota in the environment.

A HPLC/ESI-MS^2 selected reaction monitoring method aimed at the detection of five different crenarchaeol-based IPLs was developed using extracts of biomass of "*Ca*. N. gargensis", to screen for the presence of viable AOA through the Arabian Sea oxygen minimum zone (OMZ). The vertical distribution of hexose-phosphohexose crenarchaeol was marked by a prominent peak at the oxycline in addition to a less-pronounced peak at the bottom of the OMZ which matched peaks in Crenarchaeota 16S and *amoA* gene abundances. The general correspondence between IPL and gene profiles in this study demonstrates the robustness of HPH-crenarchaeol as a marker for living ammonia-oxidizing Crenarchaeota. A comparison of the depth distribution of PC-monoether ladderane IPL derived from anaerobic ammonia oxidizing (anammox) Bacteria and 16S rRNA gene abundances, which peaked at mid-OMZ depths, suggest that despite theoretical potential, little opportunity may exist for metabolic coupling between these groups at this location due to ≥ 400 m vertical separation of their respective niches.

A more detailed comparison of directly-analyzed crenarchaeol-based IPLs, IPL-derived GDGTs, and core GDGTs was made through the Arabian Sea OMZ. The results suggest that a portion of IPLs may actually persist as molecular fossils, and support the idea of differential degradation of glycolipids and phospholipids. This is in contrast with the assumption that all IPLs degrade rapidly upon cell senescence which has conventionally justified their use as general 'life' markers. Despite a good correspondence

at the surface (ca. 20 m depth), TEX_{86}-calculated temperatures derived from core and IPL-derived GDGT distributions did not follow temperature changes with depth. A contribution of IPLs to the fossil GDGT pool could account for this.

Increases in AOA abundance were notable during the winter months between November and February of an interrupted time series spanning the years 2002-2008. GDGT-based IPLs were used to track the seasonal occurrence and carbon-fixation activity of marine AOA in the coastal North Sea from 2007-2008. During this time crenarchaeol-based IPLs showed the same temporal distribution, regardless of headgroup, indicating that in this dynamic system a fossil contribution of IPLs to the GDGT pool is less likely than in the Arabian Sea. Incubations of North Sea water with ^{13}C-bicarbonate resulted in label incorporation into the tricyclic biphytane derived from IPL-crenarchaeol, confirming that the Crenarchaeota in the North Sea surface waters actively fix bicarbonate during their winter blooms. Lower ^{13}C-incorporation was observed in incubations containing nitrification inhibitors (Nserve and chlorate) further indicating that these Crenarchaeota are predominantly ammonia-oxidizers.

To conclude, the present study demonstrates that intact polar GDGTs are excellent tools to study the ecology of Crenarchaeota in modern-day environments. Continued application of IPLs to molecular ecology studies will enhance our understanding of the role of AOA in both carbon and nitrogen cycling. In addition, constraining controls on the environmental distributions of GDGTs, including crenarchaeol and its regioisomer, will aid in a better understanding of their use in geochemical proxies, such as the TEX_{86} paleothermometer.

Sammenvatting

Niet-extremofiele Crenarchaeota zijn alomtegenwoordig en vormen een belangrijk onderdeel van microbiële levensgemeenschappen in vele hedendaagse ecosystemen. Deze microörganismen biosynthetiseren glycerol dialkyl glycerol tetraethers (GDGT) als membraanlipiden en worden gebruikt om de aanwezigheid en activiteit van deze microben in diverse moderne ecosystemen te duiden. Daarnaast worden de distributies van deze GDGTs gebruikt om zeewatertemperaturen uit een ver verleden te reconstrueren met behulp van de zogenaamde TEX_{86} palaeothermometer. Het gebruik van membraanlipiden in de moleculaire ecologie biedt toegevoegde waarde ten opzichte van (meta)genomische benaderingen omdat het een onafhankelijke methode is ten opzichte van die gebaseerd op nucleïnezuren (DNA of RNA) waarin de extractie tot een mogelijke bevoordeling van bepaalde groepen microörganismen kan leiden. Anderzijds is het niet altijd duidelijk of specifieke biomarker lipiden afkomstig zijn van levende of dode cellen. IPLs afkomstig van crenarchaeota bestaan uit een GDGT, die de kern vormt, waaraan één of meerdere polaire groepen gebonden zijn. Zij worden verondersteld markers te zijn voor levende Crenarchaeota. Dit proefschrift beschrijft onderzoek dat gericht is op de ontwikkeling van het gebruik van intacte polaire lipiden (IPLs) in ecologische studies van ammoniak-oxiderende Crenarchaeota (AOA). Om dit te bewerkstelligen werden verbeteringen aangebracht in zowel de directe IPL analysemethode als de indirecte methode dieGDGT afkomstig van IPLs analyseert. Informatie verkregen door analyse van vier nieuwe verrijkingsculturen van ammoniak-oxiderende Crenarchaeota speelde hierbij een belangrijke rol. De nieuw ontwikkelde analysemethoden werden toegepast op drie verschillende milieu's: heetwaterbronnen uit Californië, de Arabische Zee zuurstof minimum zone (OMZ), en het kustwater van de Noordzee. Vergelijkingen tussen IPL en op DNA-gebaseerde moleculaire gegevens tonen een completer beeld van de verspreiding en abundantie van ammoniak-oxiderende Crenarchaeota in deze ecosystemen. Daarnaast toont het de algemene robuustheid van IPL analyses in moleculaire ecologische studies aan.

Om onderscheid te kunnen maken tussen GDGTs afkomstig van levende organismen en die aanwezig in fossiel organisch materiaal is een chromatografische fractionering van de IPL GDGTs en die waar de polaire groep reeds van verwijderd is vereist. Vervolgens kan de IPL GDGT fractie onderworpen worden aan zure hydrolyse, waarna in beide fracties de GDGTs geanalyseerd kunnen worden. Conventionele kolomscheiding gebaseerd op de scheiding van bacteriële glyco-en fosfolipiden werden niet geschikt bevonden voor de scheiding van GDGT gebaseerde IPLs. Scheiding over geactiveerde silicagel, met elutie van achtereenvolgens hexaan: ethylacetaat (3:1, v/v), ethylacetaat, en methanol, leidde tot fracties sterk verrijkt in GDGTs zonder polaire groep, glyco- en fosfo-GDGTs, respectievelijk. Deze sterk verbeterde methode zal toegepast moeten worden in toekomstige studies.

De oorsprong van crenarchaeol (de meest karakteristieke GDGT membraanlipide van AOA) in terrestrische warmwaterbronnen werd bestudeerd met behulp van bovenstaande methode. De hypothese dat crenarchaeol uitsluitend werd gesynthetiseerd door mesofiele Crenarchaeota werd in twijfel getrokken nadat crenarchaeol in terrestrische warmwaterbronnen gevonden werd en aangetoond werd dat crenachaeol gebiosynthetiseerd wordt door de thermofiele AOA, "*Ca*. Nitrocaldus yellowstonii". Crenarchaeol werd in relatief grote hoeveelheden aangetroffen in de IPL-GDGT fracties afkomstig van heetwaterbronnen uit Californië. Dit bevestigde dat crenarchaeol inderdaad *in situ* gebiosynthetiseerd wordt bij hoge temperatuur door ammoniak-oxiderende Crenarchaeota. Dit blijkt tevens uit de goede overeenkomst in de concentraties van enerzijds het *amoA* gen en anderzijds van IPL-afkomstig crenarchaeol.

De karakterisering van AOA wordt belemmerd door de moeilijkheden in het verrijken en in cultuur brengen van AOA. Daarom zijn er slechts beperkte gegevens beschikbaar over de IPL-GDGTs gebiosynthetiseerd door deze microben. Analyse van de onlangs verrijkte Groep I.1b AOA, "Ca. Nitrososphaera gargensis", onthulde een GDGT distributie die bijna uitsluitend bestaat uit crenarchaeol en de crenarchaeol regio-isomeer. Deze bevinding breidt de taxonomische verdeling van crenarchaeol synthese tot een nieuwe fylogenetische lijn binnen de Groep I Crenarchaeota, en impliceert dat microben in deze groep een belangrijke bijdrage aan crenarchaeol in bodems leveren. De polaire

groepen van de GDGT omvatten monohexoses, dihexoses, fosfo-hexoses en hexose-fosfo-hexoses. Daarnaast komen GDGTs met mono- en dihexoses en een onbekende polaire groep met een massa van 176 Dalton voor. Deze gegevens vormen een wezenlijke bijdrage aan de huidige kennis van IPLs gebiosynthetiseerd door AOA en ondersteunen de hypothese dat crenarchaeol specifiek is voor AOA. De TEX_{86}-afgeleide temperatuur op basis van de GDGT distributie van "Ca. N. gargensis" komt goed overeen met de cultuurtemperatuur van 46 ° C, maar TEX_{86} waarden veranderen niet wanneer deze temperatuur voor een relatief korte termijn naar 42 of 50 °C gebracht wordt. Dit wijst er mogelijk op dat sommige soorten AOAs niet in staat zijn hun GDGT membraansamenstelling aan te passen aan de temperatuur of dat een dergelijke fysiologische adaptatie veel langer duurt dan de hier gebruikte kweektijd.

Aanvullende steun voor de specificiteit van crenarchaeol voor AOA komt uit analyse van IPL-afkomstige GDGTs gebiosynthetiseerd door nieuwe ophopingsculturen van AOA verrijkt uit mariene sedimenten. Drie nieuwe soorten biosynthetiseerde overvloedige crenarchaeol samen met andere GDGTs, die allen aangetroffen worden in mariene zwevende deeltjes. De samenstelling van de polaire groepen van de GDGTs is vergelijkbaar met die gebiosynthetiseerd door *Nitrosopumilus maritimus* SCM1 en "Ca. N. gargensis". In al deze AOAs vormt hexose-phosphohexose crenarchaeol een hoofdbestanddeel, waardoor deze IPL gezien kan worden als de ideale biomarker om AOAs in het milieu op te sporen.

Een HPLC/ESI-MS^2 methode gebaseerd op specifieke reacties in de ionenbron van de massapectrometer en gericht op de opsporing van vijf verschillende crenarchaeol gebaseerde IPLs werd ontwikkeld met behulp van het extract van biomassa van "Ca. N. gargensis ". Vervolgens werd deze methode toegepast om de aanwezigheid van levende AOA in de Arabische Zee zuurstof minimum zone te bepalen. De verticale verdeling van hexose-fosfo-hexose crenarchaeol werd gekenmerkt door een prominent concentratiemaximum in de oxycline. Daarnaast werd er een minder uitgesproken concentratiemaximum in het diepste gedeelte van de OMZ aangetroffen. Deze maxima kwamen overeen met die in 16S rRNA en *amoA* gen abundanties afkomstig van AOA. De goede overeenkomst in IPL- en gen- profielen in deze studie toont de robuustheid van

HPH-crenarchaeol als een marker voor levende ammoniak-oxiderende Crenarchaeota. Ook het concentratieprofiel van de PC-monoether ladderane IPL, afkomstig van anaërobe ammoniak-oxiderende (anammox) bacteriën, en dat van de 16S rRNA-gen afkomstig van deze bacteriën kwam goed overeen, met een maximum in het midden van de OMZ. Deze gegevens suggereren dat, ondanks de theoretische mogelijkheid, er geen metabolische koppeling tussen AOA en anammox bacteriën is op deze locatie vanwege de 400 m verticale scheiding van hun respectieve niches.

In deze monsterset werd ook een vergelijking gemaakt van direct geanalyseerde crenarchaeol-IPLs, van IPL-afkomstige GDGTs, en GDGTs zonder polaire groep. De resultaten suggereren dat een deel van de IPLs daadwerkelijk kan blijven bestaan als moleculaire fossielen en ondersteunt het idee van differentiële afbraaksnelheden voor glycolipiden en fosfolipiden. Dit contrasteert met de veronderstelling dat alle IPLs zeer snelle degradatie ondergaan na het doodgaan van de cel, de conventionele veronderstelling die het gebruik van IPLs als markers voor 'levende' materie rechtvaardigt. Ondanks een goede overeenkomst aan het wateroppervlak (ca. 20 m diepte), kwamen met TEX_{86}-berekende temperaturen afgeleid van GDGT met en zonder polaire groep niet overeen met *in-situ* temperaturen. Dit heeft mogelijk te maken met een preferentiele omzetting van bepaalde IPL-GDGTs.

GDGTs en AOA gen concentraties zijn gebruikt om de ecologie van AOA in de wateren van de Noordzee te bestuderen. Sterke verhoging van de abundantie van AOA waren waarneembaar in de wintermaanden tussen november en februari van een onderbroken tijdreeks over de jaren 2002-2008. IPL-GDGTs werden gebruikt om het seizoensgebonden voorkomen en de koolstoffixatie activiteit van mariene AOA in het kustwater van de Noordzee in 2007-2008 te bestuderen. Crenarchaeol-IPLs vertoonde dezelfde temporele distributieals die van AOA genen, aangevende dat ze levende AOA reflecteren. Incubaties van de Noordzee water met ^{13}C-bicarbonaat resulteerde in label-opname in de tricyclische biphytane afgeleid van IPL-crenarchaeol. Dit bevestigde dat de Crenarchaeota in de Noordzee oppervlaktewateren actief bicarbonaat opnemen tijdens de winter bloei. Veel lagere ^{13}C-opname werd waargenomen in incubaties met nitrificatie

remmers (Nserve en chloraat), hetgeen aangaf dat de autotrofe Crenarchaeota overwegend uit AOA bestaan.

Concluderend kan gesteld worden dat de huidige studie aantoont dat IPL-GDGTs uitstekende verbindingen zijn om de ecologie van Crenarchaeota in hedendaagse milieu's te bestuderen. Verdere toepassing van IPLs in de moleculaire ecologie studies zal ons begrip van de rol van de AOA in zowel de koolstof- als de stikstofcyclus verder vergroten. Dit zal tevens zorgen voor een verbeterd inzicht in de sturende factoren op de samenstelling van GDGTs, met inbegrip van crenarchaeol en haar regio-isomeer, in verschillende milieu's, op haar beurt leidende tot een beter begrip van het gebruik op GDGT gebaseerde geochemische proxies, zoals de TEX_{86} paleothermometer.

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Curriculum vitae

Angela Pitcher was born on the 18th of September 1980 in Corner Brook (Newfoundland) Canada. After finishing high school, she began her study of Biology. In 2006 she received her Bachelor of Science Honours in Biology, with a minor concentration in physical Geography, from Mount Allison University in Sackville (New Brunswick), Canada. During her undergraduate studies, Angela worked as a research assistant with the Department of Geography, collecting field data on Bay of Fundy salt marshes and studying the biogeochemistry of methanogenesis in wetland sediments under the supervision of Dr. Jeff Ollerhead and Dr. Doug Campbell. From 2006 to 2010 she worked as a PhD student at the Royal Netherlands Institute for Sea Research (NIOZ) in the Department of Marine Organic Biogeochemistry, under the supervision of Jaap S. Sinninghe Damsté and Stefan Schouten. Her PhD research focused on the application of membrane lipids to the study of ammonia-oxidizing Archaea. Angela currently lives in London, England.