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Present and past contribution of anaerobic ammonium oxidation to nitrogen cycling as revealed by ladderane lipids

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Present and past contribution of anaerobic ammonium oxidation to nitrogen cycling as revealed by ladderane lipids

Ladderaanlipiden verschaffen inzicht in de huidige en vroegere bijdrage van anaerobe ammonium oxidatie aan de de stikstofkringloop

(met een samenvatting in het Nederlands)

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Contents

Chapter 1

Introduction

1.1 The marine nitrogen cycle

Nitrogen is often a limiting element for biological productivity, and therefore occupies a central role in ocean biogeochemistry. It also has a significant influence on cycles of many other elements, in particular carbon and phosphorous (Gruber, 2004). The marine nitrogen cycle (Figure 1) is a complex elemental cycle and chemical transformation of the different nitrogen species that exhibit a large range of oxidation states in the marine environment, from +5 (NO_3^-) to –3 (NH_4^+), are predominantly mediated by microbes as part of their metabolism, to synthesize cell material or to gain energy for growth. While most chemical forms of nitrogen in the ocean are biologically available (i.e. "fixed nitrogen"), the most abundant chemical form, dissolved nitrogen gas, N_2, is generally not.

The most important source for fixed nitrogen in the ocean is biological N_2 fixation, which refers to the conversion of N_2 into organic nitrogen, mainly performed by photoautotrophic organisms (i.e. cyanobacteria). Once the nitrogen fixers die and the cell remnants degrade, the nitrogen is subsequently released as ammonium during the process of remineralization. Under oxic conditions the ammonium formed can rapidly be taken up as a source for organic nitrogen by planktonic organisms thriving in the upper ocean (Suttle et al., 1990).

Fixed nitrogen in the form of ammonium is aerobically oxidized stepwise to nitrate and nitrite during the process of nitrification. The two steps of nitrification, the oxidation of ammonium to nitrite and the oxidation of nitrite to nitrate, are thought to be mediated by distinct chemo-autotrophic bacteria belonging to the group *Nitrosomonas, Nitrospira* and *Nitrosococcus* (Zehr et al., 2002; Purkhold et al., 2000). However, marine *Crenarchaeota*, which are among the most abundant micro-organisms in the ocean, have recently also been shown to oxidize ammonium under oxic conditions (Könneke et al., 2005; Wuchter et al., 2006). During nitrification, nitrous oxide (N_2O), which is a strong greenhouse gas, may be formed as an intermediate product. Most of the N_2O escapes to the atmosphere, thereby having a potential impact on the Earth's temperature and climate (Fluckiger et al., 2004).

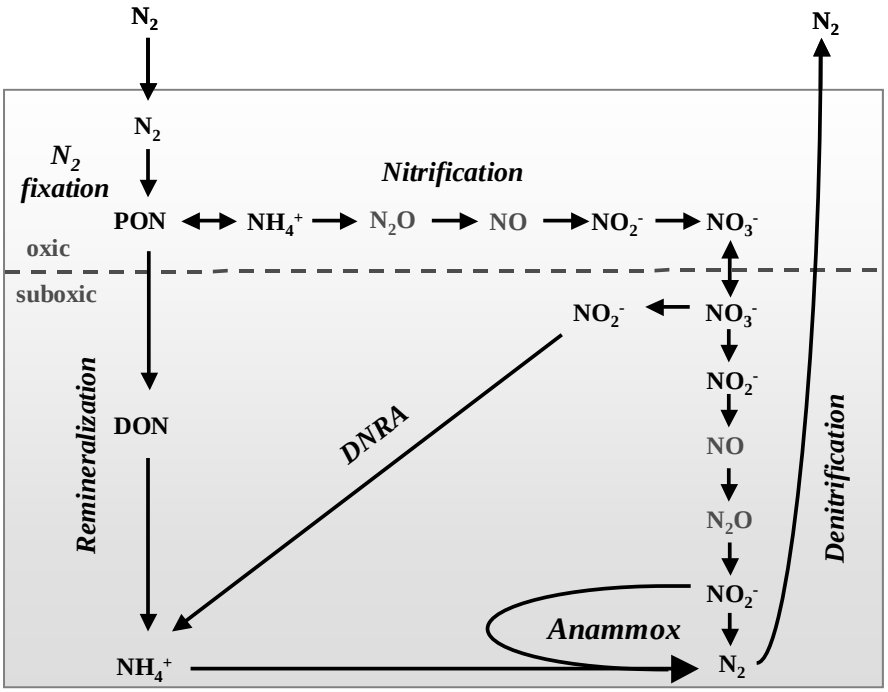

Figure 1. Simplified version of the marine nitrogen cycle including anammox (after Francis et al., 2007). DNRA = dissimilatory nitrate reduction to ammonium.

Denitrification is a process performed by heterotrophic bacteria that permanently remove fixed inorganic nitrogen from the ocean. Under anaerobic conditions denitrifying bacteria use nitrate instead of oxygen as an electron acceptor for the oxidation of organic matter:

$$5CH_2O + 4NO_3^- + 4H^+ = 2N_2 + 5CO_2 + 7H_2O \qquad (1)$$

N_2O is also an intermediate product formed during the reduction of nitrate to N_2. If some of this N_2O escapes further reduction to N_2, denitrification can act as a source of N_2O (Suntharalingam et al., 2000b). The majority of the N_2O formed in the ocean, however, appears to be formed in association with nitrification (Suntharalingam et al., 2000a).

The massive use of nitrogen-based fertilizers on agricultural soils is not only changing terrestrial ecosystems and freshwater systems, but increasingly also vulnerable

coastal systems by fueling phytoplankton blooms (Galloway et al., 2004; Beman et al., 2005). In addition, increased formation of reactive nitrogen compounds (mostly NO_x) during burning of fossil fuels over the past few decades causing acid rain and eutrophication in aquatic ecosystems (Galloway et al., 2002). The true impact of these man-made perturbations leading to an immense acceleration of the global nitrogen cycle is, however, poorly understood.

1.2 Anammox

Already decades ago, there were several indications that ammonium could also be oxidized anaerobically in nature. In 1941, the anaerobic oxidation of ammonium coupled to nitrite reduction was suggested to be a possible source of N_2 in the ocean (Hamm and Thompson, 1941). In a later study from an anoxic fjord Richards (1965) observed an unexplainable loss of ammonium under anoxic conditions, and it was suggested that this 'missing' ammonium was oxidized with nitrate to free N_2. In 1977, the possibility of ammonium oxidation with either nitrate or nitrite performed by chemosynthetic bacteria was proposed by Broda (1977) based on thermodynamical grounds. The first evidence for the existence of anaerobic ammonium oxidation (anammox) came about two decades later from a waste water treatment plant in Delft, the Netherlands (Mulder et al., 1995), and the anammox reaction was subsequently defined as the oxidation of ammonium under anoxic conditions with nitrite as the electron acceptor resulting in the release of N_2 (Van de Graaf et al., 1995).

$$NO_2^- + NH_4^+ = N_2 + 2H_2O \qquad (2)$$

The organisms performing the anammox reaction were successfully enriched from wastewater and identified as chemolithotrophic bacteria belonging to the clade *Planctomycetes* (Strous et al., 1998; Strous et al., 1999). Four genera of anammox bacteria have been enriched and classified, "*Candidatus* Kuenenia", "*Candidatus* Brocadia", "*Candidatus* Anammoxoglobus", and "*Candidatus* Scalindua" (Kartal et al., 2007; Schmid et al., 2003; Schmid et al., 2007; Strous et al., 1999) (Figure 2). The species "*Candidatus* Kuenenia stuttgartiensis", "*Candidatus* Brocadia fulgida", "*Candidatus* Brocadia anammoxidans", "*Candidatus* Anammoxoglobus propionicus", "*Candidatus* Scalindua wagneri", and "*Candidatus* Scalindua brodae" have been

identified in wastewater treatment systems. A close relative of the "*Candidatus* Scalindua spp", "*Candidatus* Scalindua sorokinii", is so far the only genus identified also in the marine environment and has been found to contribute significantly to the loss of fixed nitrogen from the ocean (see below) (Hamersley et al., 2007; Kuypers et al., 2003; Kuypers et al., 2005). Based on phylogenetic and genomic analysis, anammox bacteria seem to have a common ancestor, despite the large evolutionary difference between the four genera with on average only 85% 16S rRNA gene sequence similarity between environmental and wastewater anammox bacteria.

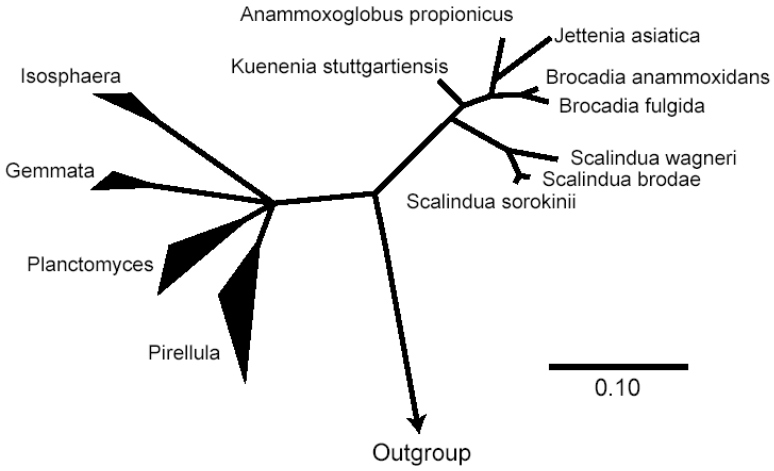

Figure 2. Phylogenetic tree of the different anammox genera, based on 16S rRNA (B. Kartal, PhD thesis).

Anammox bacteria are slow growers with doubling times of around 11-20 days (Strous et al., 1999). Their metabolism is reversibly inhibited by very low levels (<1 µM) of oxygen. All known anammox bacteria have a membrane-bound intracytoplasmic compartment, named anammoxosome (Figure 3), where anammox catabolism was shown to take place (van Niftrik et al., 2008; Lindsay et al., 2001; Van Niftrik et al., 2004). The membrane of this organelle-like compartment consists of unusual ladderane lipids containing either three or five linearly fused cyclobutane rings (Sinninghe Damsté et al., 2002) (Figure 3).

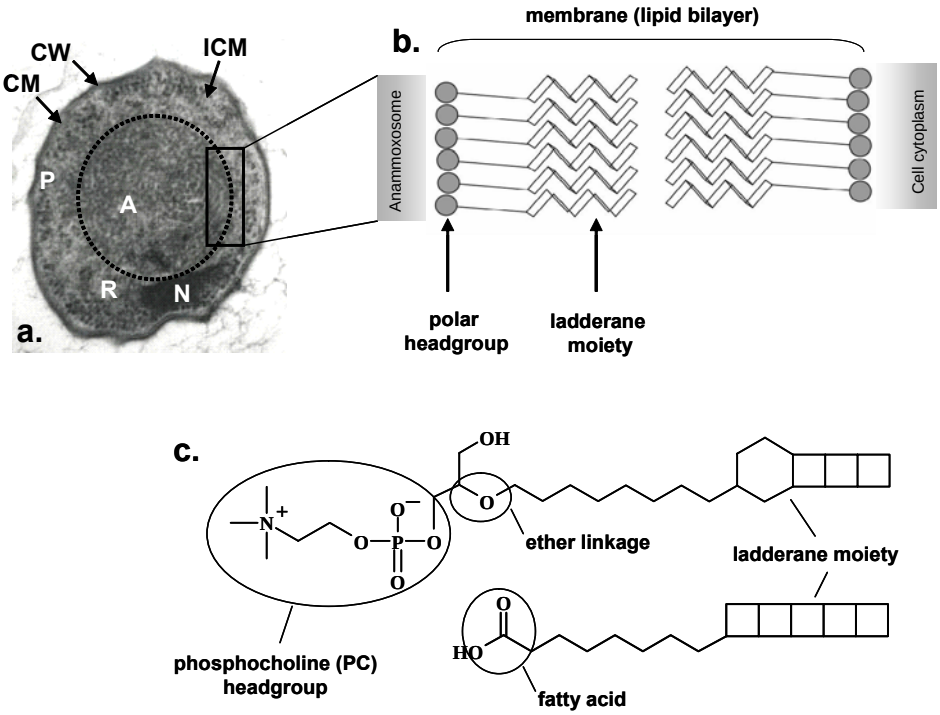

Figure 3a.) Transmission electron micrograph (TEM) of *Candidatus* "Brocadia anammoxidans" (photo by John Fuerst/Rick Webb). (A) anammoxosome, (N) nucleoid, (R) riboplasm, (P) paryphoplasm, (ICM) intracytoplasmic membrane, (CM) cytoplasmic membrane, (CW) cell wall. **b.)** scheme of the ladderane lipid membrane surrounding the anammoxosome (after Sinninghe Damsté et al., 2002). **c.)** chemical structures of different ladderane lipids.

Ladderane lipids occur in a variety of different forms, either ester- or ether bound to a glycerol backbone. Major polar headgroups of ladderane lipids are phosphocholine (PC), phosphoetanolamine (PE) and phosphoglycerol (PG) (Boumann et al., 2006; Rattray et al., 2008). Ladderane lipids provide an unusually dense membrane compared to conventional membranes (~1.5 kg dm^{-3} vs. ~1.0 kg dm^{-3}). Such a dense membrane was shown to be less resistant to diffusion as it is impermeable to the toxic intermediate of the anammox reaction, hydrazine, and might be a specific adaptation to the unusual anammox metabolism (Sinninghe Damsté et al., 2002). The biosynthetic pathway of ladderane lipids is so far unknown. Because ladderane lipids are unique to anammox bacteria, they provide excellent biomarkers for the detection of anammox in the natural environment.

The recent discovery of the anammox pathway (Figure 1) in the marine environment has led to a reevaluation of the mechanisms of nitrogen cycling in the ocean. So far, denitrification was regarded as the sole process removing fixed inorganic nitrogen from the ocean. First direct evidence for anaerobic ammonium oxidation with nitrite in marine sediments was based on incubation experiments with ^{15}N-labelled nitrogen species (Dalsgaard et al., 2002). In a combined approach using ^{15}N labeling techniques, microbial molecular techniques and ladderane biomarker lipids, Kuypers et al. (2003) found anammox to be a key process for nitrogen removal in the suboxic zone of the Black Sea, the world's largest anoxic basin. Since then, anammox has been found to be a ubiquitous process in many different natural settings ranging from marine and estuarine sediments (Engström et al., 2005; Rysgaard et al., 2004b; Trimmer et al., 2003), anoxic water columns (Dalsgaard et al., 2003), and even sea ice (Rysgaard et al., 2004a). Recent studies from the oxygen minimum zones (OMZs) off Namibia, Peru and Chile (Hamersley et al., 2007; Kuypers et al., 2005; Thamdrup et al., 2006) indicated that anammox was the dominant process responsible for the loss of fixed inorganic nitrogen, while denitrification was minor or absent. These findings have possible consequences for the interpretation of sedimentary $\delta^{15}N$ records as anammox might leave an imprint on the $\delta^{15}N$ signal as well which was so far attributed to denitrification only.

1.3 The past marine nitrogen cycle

Earth's climate has experienced many large changes, to which the global nitrogen cycle has sensitively responded (Gruber et al., 2008). This has been reconstructed by analysis of N_2O entrapped in ice cores as well as nitrogen stable isotope budgeting ($\delta^{15}N$) on marine sediment cores. The $\delta^{15}N$ of organic matter (OM) which settles from the surface water is a function of the $\delta^{15}N$ of the nitrate source and fractionation occurring during uptake of nitrate by phytoplankton (Altabet, 1988). Denitrification occurs under suboxic conditions in the water column resulting in a strong fractionation of about 27‰ (Brandes et al., 1998), leaving the residual nitrate enriched in ^{15}N. Upwelling of this ^{15}N-enriched nitrate also influences the isotopic composition of newly produced OM in the surface water by phytoplankton leading to high $\delta^{15}N$ values in particulate and sedimentary OM. Both $\delta^{15}N$ of marine OM, being indicative for the intensity of oceanic denitrification, and atmospheric N_2O

concentrations, which is determined by the magnitude of nitrification and denitrification, have undergone large and relatively rapid changes which are generally synchronous with climate variations: Cold periods are generally corresponding to low N_2O and $\delta^{15}N$ values, and *vice versa* (Fluckiger et al., 2004; Altabet et al., 1999). Furthermore, the marine nitrogen cycle was suggested to have an influence on atmospheric CO_2 concentrations and, therefore, on the global carbon cycle (Altabet et al., 2002), although changes in marine nitrification and denitrification as a primary trigger for changes in atmospheric CO_2 have been questioned (Gruber, 2004).

Due to its recent discovery, relatively little is yet known about the ubiquity and importance of anammox in the present-day marine nitrogen cycle. In addition, virtually nothing is known about the ubiquity of anammox in past marine settings, and its possible impact on the past oceanic nitrogen cycle remains unclear. The molecular phylogeny of the 16S rRNA genes suggest that anammox is a relatively ancient process and is hypothesized to impact the marine nitrogen cycle for hundreds of million of years, for example during periods in Earth history when parts of the oceans were predominantly anoxic (i.e. the so-called oceanic anoxic events) (e.g., Kuypers et al., 2004). Ladderane biomarkers could be a way to establish this but because of the thermal lability of their cyclobutane moieties (Sinninghe Damsté et al., 2005) these components are probably degraded during the burial of organic matter in the subsurface.

1.4 Diagenesis and catagenesis of sedimentary organic matter

Organic matter formed in the photic zone of the upper ocean and deposited on the sea floor is subjected to a range of biological, chemical and physical reactions (Figure 4). A large fraction (>90%) of the sedimentary organic matter is remineralized during transport in the water column and near the sediment-water interface during early diagenesis (Wakeham et al., 2002; Wakeham et al., 1989).

Microbial processes and consumption and reworking of sediment organic matter by macrofaunal activity are factors controlling the redox chemistry of sedimentary systems as well as the degree to which compounds are preserved in the sedimentary record. In addition, organic matter sorption to mineral surfaces or into organic matrices provides a mechanism by which organic matter is preserved (Keil et al., 1994; Burdige, 2007). A small fraction (typically ~0.5%, but this fraction is much

larger under anoxic conditions), however, may escape the biosphere and become part of the geosphere (Burdige, 2007).

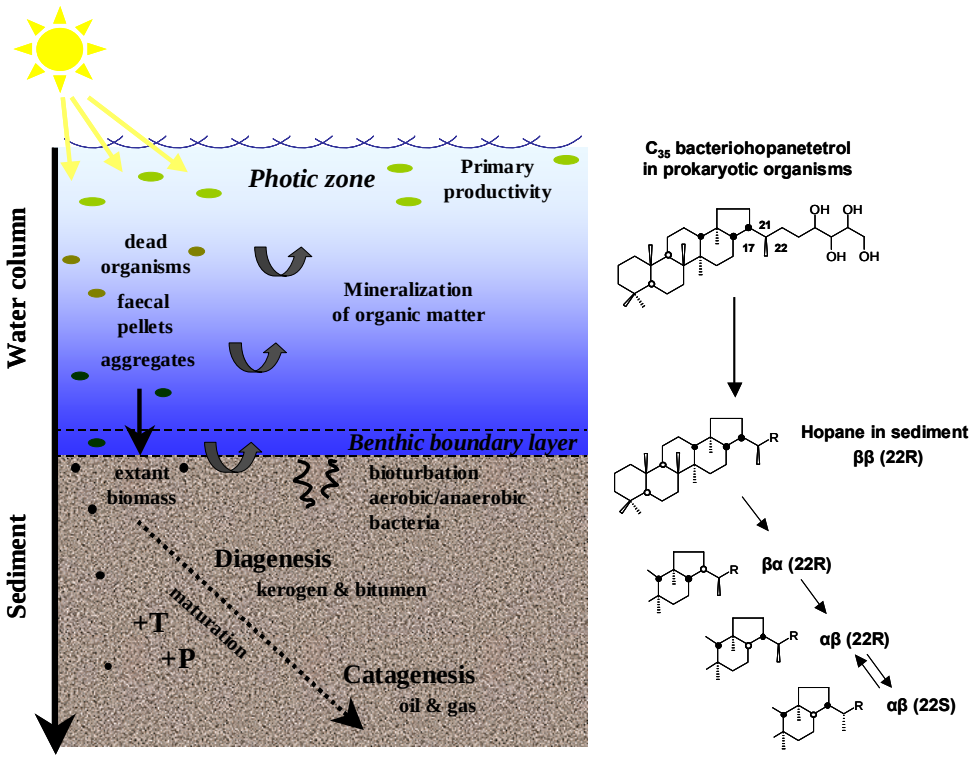

Figure 4. Schematic representation of the processes involved in the transformation of biogenic organic matter and hopanoid biomarkers during water column transport and subsequent sediment burial. Hopane isomerization occurs with increasing maturity resulting in mixtures of isomers, the ratio of which reflect their relative thermodynamic stabilities.

During geological time, the organic matter is buried and subjected to increasing temperatures causing initially mild chemical transformations known as diagenesis (Figure 4). Biomarkers, or molecular fossils, undergo only minor structural changes during processes associated with sedimentation and diagenesis and therefore provide useful information on the organic matter in sediments as well as environmental conditions during their deposition. With increasing burial depth, temperature and pressure increases leading to more severe reactions such as C-C bond cleavage (cracking) of organic matter. This process leading to the formation of petroleum is known as catagenesis and requires under typical burial conditions millions of years.

During catagenesis, biomarkers undergo structural changes, i.e., saturation, aromatization and isomerization reactions, that can be used to assess the thermal maturity of organic matter (Peters et al., 2005). For example, in C_{31}-C_{35} hopanes that are derived from bacteriohopanetetrol and related bacteriohopanes, the biologically conferred 22R configuration preserved during initial stages of diagenesis, undergoes isomerization via hydrogen exchange during catagenesis forming more 'geological' 22S epimers. At the C-17 and C-21 positions the configurations are initially mainly 17β,21β(H), which is the biological but thermodynamically unstable configuration. With increasing temperature the more stable 17β,21α(H), and 17α,21β(H) isomers are formed (Peters et al., 1991; Ensminger et al., 1974) (Figure 4).

The dia- and catagenetic fate of organic matter is ideally studied in the natural environment. This requires the occurrence of organic-rich rock formations that extend over a depth interval representing a range of thermal maturity levels from immature into the oil window, which are very scarce. Hydrous pyrolysis is a laboratory technique developed to simulate the natural maturation process, and basically involves heating of organic-rich sedimentary rock in a closed reactor in the presence of water at subcritical temperatures (<374°C) for a certain time (Lewan et al., 1979; Lewan, 1993). Geological processes, that would normally take millions of years, can then be mimicked in the laboratory within a few days or weeks. Previous studies have shown that reactions taking place with biomarkers during hydrous pyrolysis experiments resemble those occurring during natural maturation (e.g. Koopmans et al., 1996; Van Duin et al., 1997).

1.5 Scope and framework of this thesis

The aim of this thesis was to investigate the occurrence and importance of the anammox process in the present and past marine nitrogen cycle using the unique ladderane membrane lipid molecules of anammox bacteria. The applicability of ladderane lipids as a marker for anammox was tested in different recent environmental settings, and processes of transport, preservation and early diagenesis were examined. Another objective was to investigate the thermal stability of ladderane lipids during progressive dia- and catagenesis. To this end, the natural maturation process was simulated in the laboratory by hydrous pyrolysis of anammox bacterial cell material. This specific degradation approach provides detailed quantitative and qualitative

information on the fate of ladderane lipids and the suitability of ladderane lipid degradation products as markers for past anammox processes.

Chapter 2 describes the discovery of anammox in various hot springs from California and Nevada, USA. Ladderane biomarker lipids and 16S rRNA gene-based phylogeny revealed that anammox bacteria can thrive at thermophilic temperatures and, therefore, might form an important and as yet undiscovered link in the nitrogen cycle of hot spring environments.

Chapter 3 provides evidence for anammox in the oxygen minimum zone (OMZ) of the Arabian Sea, the world's most important area for denitrification. Lipid analysis of suspended particulate matter (SPM) and sediment trap material from locations offshore Oman are presented. The data show that ladderane biomarker lipids are present throughout the water column and most abundant in the upper part of the OMZ indicating the presence of anammox bacteria. Fluxes of ladderane lipids revealed a strong seasonal pattern, with highest fluxes during the southwest monsoon indicating enhanced anammox activity and sedimentation of ladderanes during the monsoon-driven high-productivity, high-vertical flux period. Anammox, besides denitrification, might therefore be an important sink for fixed inorganic nitrogen in the Arabian Sea OMZ.

In **Chapter 4 and 5** the anammox process is investigated in sediments of different oceanic regimes, the Irish Sea, and the continental shelf and slope in the Celtic Sea and offshore northwest Africa using different techniques, i.e. quantitative polymerase chain reaction (qPCR), ^{15}N labeling experiments and specific ladderane biomarker lipids. The combined data showed that anammox was ubiquitously present at all investigated sites. The ladderane monoether lipid with a PC headgroup was shown to be a suitable marker for living anammox cells as it degraded rapidly below the zone of anammox activity in the sediment, while the ladderane core lipids revealed a much higher preservation potential and became fossilized. Anammox, besides traditional denitrification, might constitute an important process removing nitrogen from marine sediments deposited on continental margins and should therefore be considered within further investigations of the marine sedimentary nitrogen budget.

Chapter 6 provides experimental insight in the degradation processes of ladderane lipids occurring at the sediment-water interface and subsurface during subsequent sediment burial. Slurries of anammox cell material, Wadden Sea sediment and water were heated at temperatures between 20 and 100°C for three days under fully

oxic conditions. Microbial activity, which was highest at temperatures between 40 and 60°C, causes degradation of the ladderane lipids by cleaving the alkyl chain attached to the ladderane moiety. A new high performance liquid chromatography (HPLC) method was developed for the detection of these specific ladderane degradation products and applied to various marine sediments.

Chapter 7 deals with the thermal stability of ladderane lipids. Anammox cell material was artificially matured by hydrous pyrolysis at constant temperatures ranging from 120 to 365°C for 72 hours to study the stability of ladderane lipids during progressive dia- and catagenesis. Ladderane lipids undergo structural modification due to breakdown of the condensed cyclobutane rings already at low temperatures, forming thermally more stable compounds. These products are still present at a low level of thermal maturity as determined by hopane isomerization ratios, and constitute suitable biomarkers for the detection of past anammox activity in immature ancient sediments and sedimentary rocks.

Chapter 8 reports on the detection of fossil ladderane lipids in a sediment core from the northern Arabian Sea providing for the first time evidence for past anammox activity. Concurrent variations in ladderane lipid abundances, $\delta^{15}N$ and TOC reflect changes in anammox activity in concert with OMZ intensity and surface productivity over the last glacial cycle. Furthermore, the data indicate that anammox might also cause strong isotopic fractionation, leaving an imprint on the $\delta^{15}N$ signal which is crucial for the understanding of the marine nitrogen cycle.

To summarize, the experimental and field work studies presented in this thesis contribute to a greater understanding of the distribution of anammox specific ladderane lipids in natural environments, their applicability as a marker for anammox bacteria in recent settings, and it provides first insights in their potential as a marker for the anammox process in past settings.

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Chapter 2

16S rRNA and lipid biomarker evidence for anaerobic ammonium-oxidizing bacteria (anammox) in California and Nevada hot springs

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Abstract

Anammox, the oxidation of ammonium with nitrite to dinitrogen gas under anoxic conditions, is an important process in mesophilic environments such as waste waters, oceans and freshwater systems, but little is known of this process at elevated temperatures. In this study, we investigated anammox in microbial mats and sediments obtained from several hot springs in California and Nevada (USA), using geochemical and molecular microbiological methods. Anammox bacteria-specific ladderane core lipids with concentrations ranging between 0.3 and 52 ng g^{-1} sediment were detected in five hot springs analyzed with temperatures up to 65°C. In addition, 16S rRNA gene analysis showed the presence of genes phylogenetically related to the known anammox bacteria *Candidatus* 'Brocadia fulgida', *Candidatus* 'Brocadia anammoxidans' and *Candidatus* 'Kuenenia stuttgartiensis' (96.5-99.8% sequence identity) in three hot springs with temperatures up to 52°C. Our data indicate that anammox bacteria may be able to thrive at thermophilic temperatures and thus may play a significant role in the nitrogen cycle of hot spring environments.

2.1 Introduction

Terrestrial hot springs provide suitable habitats for a variety of photoautotrophic and chemolithoautotrophic microorganisms (Castenholz, 1984; Ward *et al.*, 1998). Temperature and hydrogen sulfide content amongst others seem to be important factors, which affect the distribution and diversity of these organisms in hot springs (Purcell *et al.*, 2007; Ward *et al.*, 1998). These environments can contain dense microbial mats with complex communities which exhibit a high biodiversity including phototrophic organisms, mostly cyanobacteria and filamentous green nonsulfur bacteria (Ward *et al.*, 1987), thermophilic denitrifying bacteria (Hollocher *et al.*, 1992) and archaea (Barns *et al.*, 1996; Huang *et al.*, 2007; Kvist *et al.*, 2005; Meyer-Dombard *et al.*, 2005). Recently, thermophilic autotrophic crenarchaeal nitrifiers were enriched from terrestrial hot springs (de la Torre *et al.*, 2008; Hatzenpichler *et al.*, 2008) and were found to play an active role in the nitrogen cycle of these hot ecosystems (Reigstad *et al.*, 2008; Zhang *et al.*, 2008). Inspired by these findings, we started the present survey of various hot spring ecosystems in order to find indications of other microorganisms that may play a role in the conversion of nitrogen compounds in these thermophilic environments with a special focus on anaerobic ammonium oxidizing bacteria.

Anammox, the anaerobic ammonium oxidation to dinitrogen gas with nitrite as electron acceptor, is a key process in the global nitrogen cycle (see Arrigo, 2005; Brandes *et al.*, 2007; Francis *et al.*, 2007 for recent reviews), which constitutes a novel route to convert fixed inorganic nitrogen to gaseous N_2. The anammox process is linked to one group of organisms forming a distinct phylogenetic group related to the *Planctomycetes* (Schmid *et al.*, 2007; Strous *et al.*, 1999a). Anammox bacteria contain a special intracellular compartment called the anammoxosome, where anammox catabolism is assumed to take place (Sinninghe Damsté *et al.*, 2002; van Niftrik *et al.*, 2004, 2008). The membrane of this “organelle” consists of unusual ladderane lipids forming a dense barrier which reduces the permeability of the membrane to small molecules, e.g. protons or the toxic intermediate hydrazine of the anammox reaction, which can easily permeate less dense bacterial membranes (Sinninghe Damsté *et al.*, 2002). The ladderane core lipid contains three or five linearly concatenated cyclobutane rings either ester or ether bound to the glycerol backbone, which is unprecedented in nature. Anammox has been found in a range of environments, i.e. anoxic water columns (Dalsgaard *et al.*, 2003; Kuypers *et al.*, 2003), marine and estuarine sediments

(Dalsgaard *et al.*, 2002; Schmid *et al.*, 2007; Trimmer *et al.*, 2003), freshwater lakes (Schubert *et al.*, 2006) and also polar marine sediments and sea ice (Rysgaard *et al.*, 2004a; 2004b). The group of anammox bacteria is currently associated to at least five genera, *Candidatus* 'Brocadia', *Candidatus* 'Kuenenia', *Candidatus* 'Anammoxoglobus', *Candidatus* 'Jettenia' and *Candidatus* 'Scalindua' (Kartal *et al.*, 2007; Quan *et al.*, 2008; Schmid *et al.*, 2007; Strous *et al.*, 1999a). So far, available 16S rRNA gene sequences from marine environments were found to be closely related to *Candidatus* 'Scalindua sp.'(Kuypers *et al.*, 2003, 2005; Schmid *et al.*, 2007; Schubert *et al.*, 2006; Woebken *et al.*, 2008). Although the anammox process is well known in mesophilic environments, little is known about its importance in thermophilic and hyperthermophilic environments. Recently, Byrne *et al.* (2008), however, found indications for anammox activity at 60-85°C in deep-sea hydrothermal vent area's where temperatures as high as 153°C are observed.

To investigate the occurrence of anammox bacteria in high temperature environments, we studied microbial mats and sediments in five hot springs from California and Nevada (USA) with temperatures between 36.1 and 65.2°C where previously, in similar hot springs, also archaeal amoA genes and 16S rRNA genes related to crenarchaeaota were detected (Huang *et al.*, 2007; Zhang *et al.*, 2008). The samples were analysed using different molecular techniques for the detection of anammox bacteria, i.e., ladderane biomarker lipid analysis and 16S rRNA gene-based phylogeny.

2.2 Material and Methods

2.2.1 Sampling

Samples were collected from five locations in the Great Basin hot springs of Northern California and Western Nevada (USA) in February 2007, at the source and further downstream of the spring (Fig. 1). The hot springs were selected to represent a range of temperatures and chemistries (Table 1). At each site, temperature, pH and oxygen were measured *in situ* in the overlying water prior to collection of bacterial mats and sediments. Temperature was determined with an Ama-Digit ad 20 th digital thermometer.

The oxygen concentration was measured using WTW Oxi 315i digital meter. The pH was determined with a WTW pH 315i digital meter. Water samples for nutrient

analysis were poisoned with $HgCl_2$, transported cooled to the NIOZ and measured spectrophotometrically using an autoanalyzer system (Bran and Luebbe TRAACS 800+). Mat and sediment samples were stored frozen at -20°C until used for further analysis.

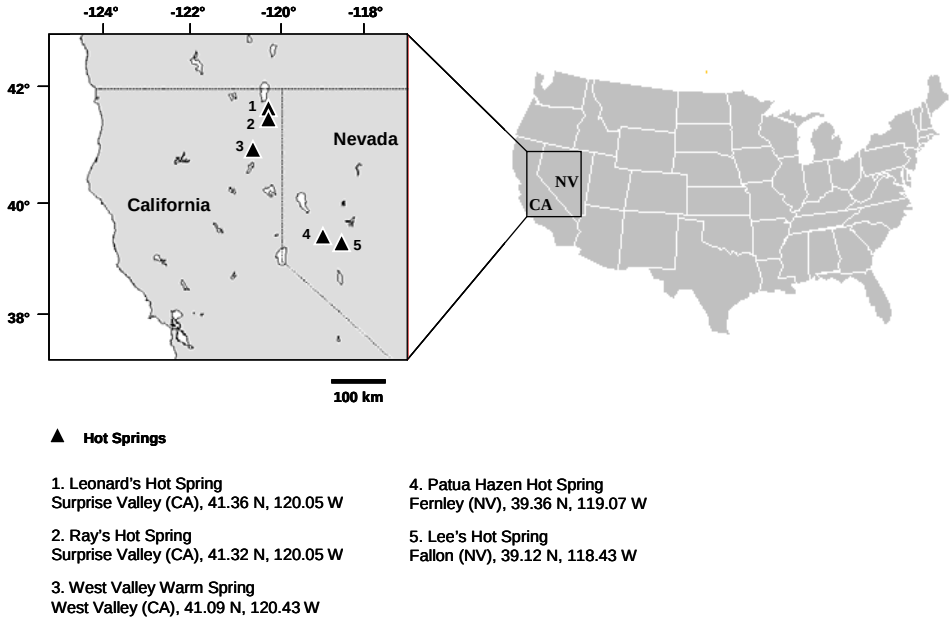

Figure 1. Map of hot spring locations in California and Nevada (USA).

2.2.2 Ladderane lipid analysis

Samples of about 50 mg to 13 g of freeze-dried and homogenized material were ultrasonically extracted 5x using a dichloromethane (DCM)-methanol mixture (2:1 by volume). The extracts were combined and the bulk of the solvent was removed by rotary evaporation under vacuum, and the extract was further dried over a Na_2SO_4 column. An aliquot of the lipid extract was saponified with aqueous 1 N KOH in methanol for 2 h at 100°C. Non-saponifiable lipids (neutral lipids) were extracted out of the basic solution (pH >13) using DCM. Fatty acids were obtained by acidifying the residue to pH 3 and subsequent extraction with DCM. The fatty acid fraction was methylated by adding diazomethane (CH_2N_2) to convert fatty acids into their corresponding methyl esters (FAMEs). The excess CH_2N_2 was removed by evaporation. To remove very polar components, aliquots of the FAMEs were eluted with ethyl

acetate over a small column filled with silica. Polyunsaturated fatty acids (PUFAs) were removed by eluting the aliquots with ethyl acetate over a small $AgNO_3$ (5%) impregnated silica column, yielding a saturated fatty acid fraction. The fatty acid fractions were dissolved in acetone and then filtered through a 0.45 µm, 4 mm diameter PTFE filter.

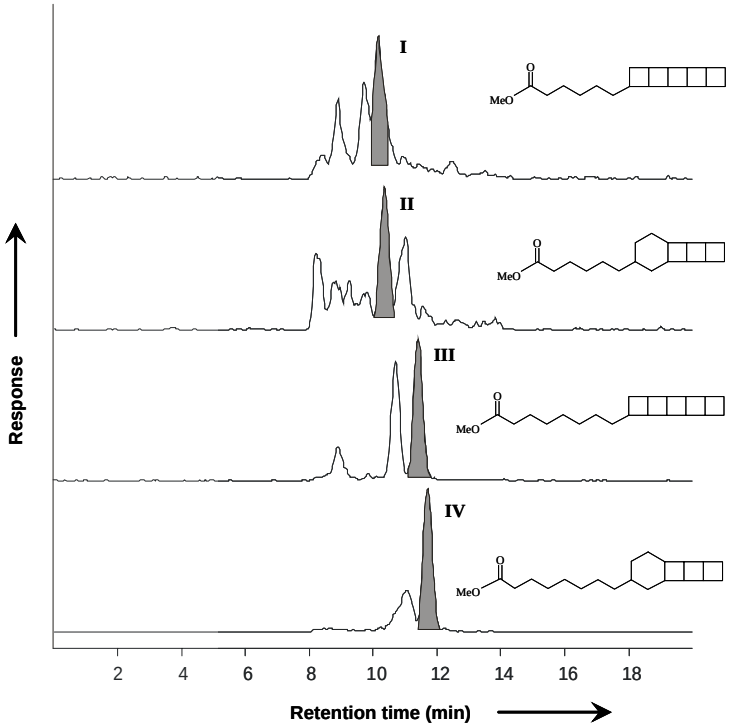

Figure 2. Partial selective reaction monitoring (SRM) traces of four ladderane core lipids obtained by HPLC/(APCI)-MS/MS analysis of a sediment sample from Leonard's Hot Spring, and their corresponding structures, (I) C_{18}-[5]-ladderane FAME, (II) C_{18}-[3]-ladderane FAME, (III) C_{20}-[5]-ladderane FAME, (IV) C_{20}-[3]-ladderane FAME. Fatty acids were analyzed as methyl esters (FAME).

These fractions were analysed by high performance liquid chromatography coupled to positive ion atmospheric pressure chemical ionization tandem mass spectrometry (HPLC/APCI-MS/MS) in selective reaction monitoring (SRM) mode as described Hopmans *et al.* (2006) with some modifications described by Rattray *et al.* (2008). Specifically, separation was achieved using a Zorbax Eclipse XDB-C_8 column

(3.0 x 250 mm, 5 µm; Agilent) and a flow rate of 0.18 ml/min MeOH. The source settings were: vaporizer temperature 475 °C, discharge current 2.5 µA, sheath gas (N_2) pressure 30 (arbitrary units), auxiliary gas (N_2) pressure 5 (arbitrary units), capillary temperature 350°C, source CID -10 V. Argon pressure was maintained at 1.5 mTorr in the second quadrupole. Quantification of ladderane lipids was done by using an external calibration curve using standards of isolated methylated ladderane fatty acids containing the [3]- and [5]- ladderane moieties (Fig. 2, structures I-IV) (Hopmans *et al.*, 2006; Rattray *et al.*, 2008; Sinninghe Damsté *et al.*, 2002). A detection limit (defined by a signal to noise ratio of 3) of 30-35 pg injected was achieved with this technique.

2.2.3 Molecular techniques and phylogenetic analysis

High molecular weight DNA isolation and hot-start polymerase chain reactions (PCR) were performed according to Schmid *et al.* (2000, 2005). The DNA from each site was used as a template in a PCR amplification with primer set Pla46F and 1390R. The products of these PCRs were used in a second nested PCR with primer sets AMX368F - AMX820R (codes with grey background, Fig. 3) or Pla46F - AMX820R (other codes). Products of the second PCR amplifications were subsequently cloned with the TOPO TA cloning kit (Invitrogen, Breda, The Netherlands) according to the manual of the manufacturer and sequenced. The 16S rRNA gene sequences of the clones were compared with their closest relatives in the GenBank database by BLASTN searches (http://www.ncbi.nih.nlm.edu/BLAST) and with the RDP classifier tool (http://rdp.cme.msu.edu/classifier/). Further phylogenetic and molecular evolutionary analyses were performed with the MEGA 4.1 program (Tamura *et al.*, 2007).

2.3 Results and Discussion

2.3.1 Occurrence and distribution of ladderane lipids in California and Nevada hot springs

To search for the presence of anammox bacteria, we first analyzed ladderane biomarker lipids in microbial mats and sediments from several hot springs in California and Nevada, either from the source or further downstream (Table 1). We specifically targeted four ladderane core lipids (see structures in Fig. 2) that are present in nearly all currently known anammox genera (Rattray *et al.*, 2008).

Table 1. Physical and nutrient data for overlying waters and lipid data for microbial mats and sediments of California and Nevada hot springs

Site	Location	Sample description	Temp (°C)	pH	O_2 (%) *	NH_4^+ (µM)	NO_x (µM)	NO_2^- (µM)	C_{18} [5] (ng g^{-1} sed)	C_{18} [3] (ng g^{-1} sed)	C_{20} [5] (ng g^{-1} sed)	C_{20} [3] (ng g^{-1} sed)	Total (ng g^{-1}sed)	NL_5
1.1	Leonard's hot spring, CA	Mat (source)	52.4	7.7	50	0.7	0.6	0.2	1.9	0.4	5.6	4.9	12.8	0.75
1.3		Sediment (effluent)	36.1	8.4	83	0.0	0.9	0.1	0.3	0.05	0.4	0.2	1.0	0.57
2.1	Ray's hot spring, CA	Streamers (source)	89.1	8.2	n.a.	0.0	0.7	0.2	n.d.	n.d.	n.d.	n.d.	n.d.	-
2.4		Mat+Sed (effluent)	58.1	8.7	n.a.	2.3	6.9	6.0	n.d.	n.d.	n.d.	1.8	1.8	-
2.5		Mat+Sed (effluent)	37.1	8.9	n.a.	0.8	5.8	1.9	0.2	0.08	0.7	0.7	1.7	0.76
3	West Valley warm spring, CA	Sediment (source)	23.7	8.5	54	0.1	27.6	0.1	0.3	n.d.	n.d.	n.d.	0.3	-
4	Patua-Hazen hot spring, NV	Sediment (source)	51.3	7.9	n.a.	13.3	7.1	0.8	25.0	n.d.	19.8	7.3	52.0	0.44
5.1	Lee's hot spring, NV	Sediment (source)	96.6	8.2	45	2.3	0.6	0.5	n.d.	n.d.	n.d.	n.d.	n.d.	-
5.2		Sediment (effluent)	65.2	8.7	85	0.4	1.0	0.7	0.03	n.d.	0.2	0.15	0.4	0.88

n.d. not detected

n.a. not analyzed

% air saturation

Ladderane core lipids were detected in almost all hot spring samples with concentrations between 0.3 and 52 ng g^{-1} sediment (total concentration of all four ladderane lipids) and temperatures between 24 and 65°C (Table 1). Highest concentrations of ladderane lipids were detected at Patua-Hazen hot spring (52 ng g^{-1} sediment), followed by Leonard's hot spring source mat (12.8 ng g^{-1} sediment). At all other sites more distant from the hot spring source and at lower *in situ* temperatures only low concentrations between 0.3 and 1.8 ng g^{-1} sediment of ladderane lipids were found (Table 1). The overall low concentration (0-0.4 ng g^{-1} sediment) of the C_{18}-[3]-ladderane fatty acid can be explained by the fact that this specific lipid is only synthesized by marine anammox bacteria belonging to the 'Scalindua' genus (Rattray *et al.*, 2008). No ladderane lipids were detected in the sediment at the source of Lee's hot spring and in the streamers of Ray's hot spring where temperatures of 96.6°C and 89.1°C were recorded. These temperatures are probably too high for any anammox activity and only specific prokaryotes can thrive there. The high ladderane lipid concentration at Patua-Hazen hot spring coincides with substantially higher NH_4^+ and NO^-_x concentrations compared to other hot springs which may promote anammox activity (Table 1). Although oxygen concentrations in the waters overlying the mats were high (Table 1), the mats themselves are most likely anoxic as indicated by the occurrence of wax ester lipids specific for green nonsulfur-like bacteria (GNSLB) which are anoxygenic phototrophs (Schouten *et al.*, unpubl. results). Furthermore, Zhang *et al.* (2007) also found 16S rRNA gene sequences falling in the phylogenetic cluster of the GNSLB in hot spring mats from Nevada and California, indicating that these mats are anoxic or at least contain anaerobic microsites where anaerobic bacteria like anammox can thrive.

Further evidence for the *in situ* production of ladderane lipids in anammox bacteria was derived from the relative distribution of ladderane lipids. Anammox bacteria have recently been found to adapt to changing temperatures by modifying their membrane composition. The amount of shorter chain ladderane fatty acids increases relative to the amount of longer chain fatty acids at lower temperatures and *vice versa*. The iNdex of Ladderane lipids with 5 cyclobutane rings, termed NL_5, has been proposed to quantify this relative change (Rattray, 2008). The NL_5 of the ladderane lipids in the hot spring samples varied between 0.44 and 0.88 (Table 1), indicating relatively more production of the longer chain fatty acids at elevated temperatures (>25°C) at the investigated sites. Especially the high NL_5 value of 0.88 calculated for

Lee's hot spring (site 5.2) indicates adaptation of the membrane lipid composition to high temperatures, and further indicates that anammox bacteria might be thriving in these hot spring environments.

2.3.2 16S rRNA analysis of California and Nevada hot springs

Ladderane core lipids are derived from both living and fossil anammox bacterial biomass, and might not necessarily be indicators for active cells. We, therefore, also analyzed the 16S rRNA gene sequences from several of these environments. Amplification of 16S rRNA genes using a nested PCR approach with primers specific for anammox bacteria resulted in the detection of several 16S rRNA gene sequences from selected hot spring sites where ladderane core lipids were detected, i.e. Leonard's hot spring, Ray's hot spring, Patua-Hazen hot spring and Lee's hot spring, suggesting the presence of active anammox bacteria at these sites with temperatures ranging between 36.1 and 52.4°C. Phylogenetic analysis of the 16S ribosomal RNA gene sequences showed that some of them were related to known anammox bacteria (Fig. 3). At the source of Leonard's hot spring mat clones were closely related to *Candidatus* 'Kuenenia stuttgartiensis', while most downstream sequences were more closely related to both *Candidatus* 'Brocadia fulgida' and *Candidatus* 'Kuenenia stuttgartiensis'. At Ray's hot spring at 37.1°C sequences were found that are most closely related to *Candidatus* 'Brocadia anammoxidans'. At Patua Hazen hot spring, where highest concentrations of the ladderane core lipids were observed, clones were found which did not seem to fall in the known anammox clusters but seem to be positioned phylogenetically between *Planctomycetes* and anammox bacteria. This could mean that these organisms are also capable of anaerobic ammonium oxidation and also produce ladderane lipids like anammox bacteria, or the ladderane lipids detected at this site are not produced autochthonous, which seems rather unlikely in view of the high ladderane lipid concentrations.

Four clones closely related to *Dictyoglomus thermophilum* were found in sediments of Patua-Hazen and Lee's hot spring at temperatures of 51.3 and 65.5°C. *Dictyoglomus thermophilum* is an extremely thermophilic bacterium deeply rooted near the base of the order *Thermotogales* (Patel *et al.*, 1987).

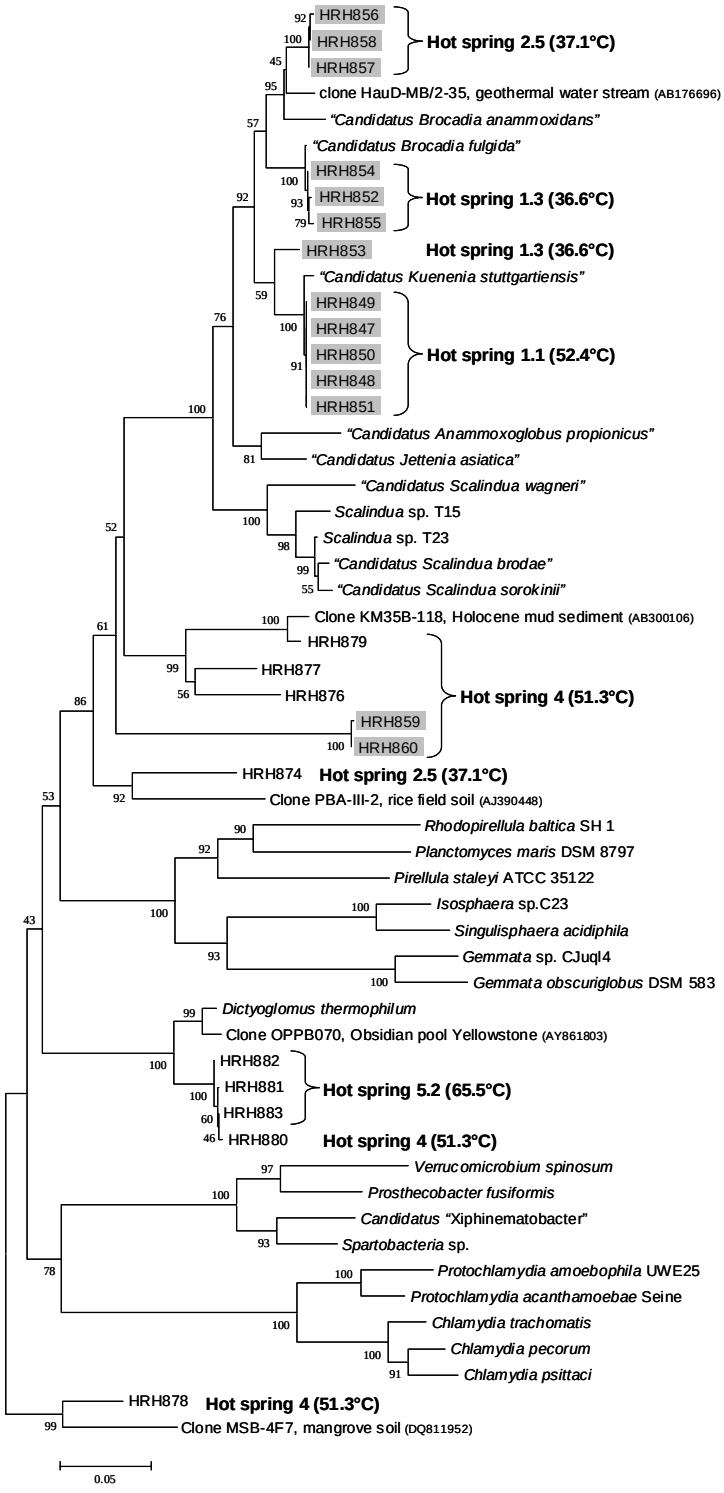
HRH856
HRH858
HRH857
Hot spring 2.5 (37.1°C)
clone HauD-MB/2-35, geothermal water stream (AB176696)
"Candidatus Brocadia anammoxidans"
"Candidatus Brocadia fulgida"
HRH854
HRH852
HRH855
Hot spring 1.3 (36.6°C)
HRH853
Hot spring 1.3 (36.6°C)
"Candidatus Kuenenia stuttgartiensis"
HRH849
HRH847
HRH850
HRH848
HRH851
Hot spring 1.1 (52.4°C)
"Candidatus Anammoxoglobus propionicus"
"Candidatus Jettenia asiatica"
"Candidatus Scalindua wagneri"
Scalindua sp. T15
Scalindua sp. T23
"Candidatus Scalindua brodae"
"Candidatus Scalindua sorokinii"
Clone KM35B-118, Holocene mud sediment (AB300106)
HRH879
HRH877
HRH876
Hot spring 4 (51.3°C)
HRH859
HRH860
HRH874
Hot spring 2.5 (37.1°C)
Clone PBA-III-2, rice field soil (AJ390448)
Rhodopirellula baltica SH 1
Planctomyces maris DSM 8797
Pirellula staleyi ATCC 35122
Isosphaera sp.C23
Singulisphaera acidiphila
Gemmata sp. CJuql4
Gemmata obscuriglobus DSM 583
Dictyoglomus thermophilum
Clone OPPB070, Obsidian pool Yellowstone (AY861803)
HRH882
HRH881
HRH883
Hot spring 5.2 (65.5°C)
HRH880
Hot spring 4 (51.3°C)
Verrucomicrobium spinosum
Prosthecobacter fusiformis
Candidatus "Xiphinematobacter"
Spartobacteria sp.
Protochlamydia amoebophila UWE25
Protochlamydia acanthamoebae Seine
Chlamydia trachomatis
Chlamydia pecorum
Chlamydia psittaci
HRH878
Hot spring 4 (51.3°C)
Clone MSB-4F7, mangrove soil (DQ811952)
0.05

Figure 3. Phylogenetic tree of 16S ribosomal RNA gene sequences determined by Neighbor-Joining method (Saitou *et al.*, 1987). The optimal tree with the sum of branch length = 2.69043569 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There were a total of 636 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007). Sequences were identified using primer-sets AMX368F-AMX820R (codes with grey background) or Pla46F-AMX820R (other codes).

Finally, some of the retrieved 16S rRNA gene sequences detected in Ray's hot spring (HRH874) and Patua Hazen hot spring (HRH876, 877, 879) were related to uncultivated bacteria falling in the cluster of Planctomycetes (Fig. 3).

2.3.3 Implications

Our combined results of ladderane lipids and 16S rRNA gene sequences suggest that anammox bacteria are present in hot springs at temperatures of at least up to 52°C. This is higher than the current temperatures at which anammox bacteria are found to be thriving. *Candidatus* Kuenenia and *Candidatus* Brocadia are genera present in wastewater treatment systems, and active at temperatures up to 43°C (Strous *et al.*, 1999b), while only members of the *Candidatus* Scalindua genus have so far been found in oxygen-limited marine environments at generally lower ambient temperatures (Schmid *et al.*, 2007; Woebken *et al.*, 2008). Thus, the fact that the sequences detected in the hot springs ranging in temperature between 36 and 52°C are more closely related to Kuenenia and Brocadia rather than Scalindua is in agreement with the generally higher optimal growth temperatures (ca. 35°C in bioreactors vs. in general much lower environmental temperatures of 12-15°C) of these species. Very recently, anammox activity was observed at 60-85°C in samples obtained from hydrothermal vent area's at the mid-Atlantic Ridge (Byrne *et al.*, 2008). In that study 16S rRNA gene sequences were retrieved that were also more closely related to Kuenenia sequences.

The results of the Patua-Hazen hot spring are intriguing, i.e. this spring contains abundant ladderane lipids but the 16S rRNA gene sequences detected form a cluster which does not fall into the currently known anammox bacteria but is more

related to deep-branching *Planctomycetes* as was recently also reported for a hot spring in Oklahoma, USA (Elshahed *et al.*, 2007). Future enrichment culture work may reveal whether these organisms are indeed capable of catalysing the anammox reaction. Nevertheless, our results indicate that anammox bacteria are present in hot springs, and may form an important and as yet undiscovered link in the nitrogen cycle of these hot spring environments.

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Chapter 3

The presence of ladderane lipids in the oxygen minimum zone of the Arabian Sea indicates nitrogen loss through anammox

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Abstract

Distributions of ladderane lipids that are characteristic of membranes of bacteria performing anaerobic ammonium oxidation (anammox) were determined in the northwestern Arabian Sea with respect to season, depth, and distance to the coast of Oman. Ladderane lipids were detected and quantified in suspended particulate matter (SPM) obtained from various water depths along a northwest-to-southeast transect during the Spring Intermonsoon period. Maximum concentrations of 5-8 pg L^{-1} generally occurred at 500 m in the upper part of the oxygen minimum zone (OMZ). Fluxes of ladderane lipids obtained from sediment trap material sampled at 500 m water depth ~350 km off the coast reveal a strong seasonal pattern apparently related to the annual monsoon cycle in the northern Arabian Sea, with highest fluxes of 125 ng $m^{-2}d^{-1}$ observed during the Southwest Monsoon. This 4-fold increase in flux during the SW Monsoon compared to the Spring Intermonsoon period may indicate higher anammox bacterial productivity or enhanced export of ladderanes during a period of high particulate matter flux or both. Anammox, in addition to denitrification, seems thus

responsible for a significant loss of nutrient nitrogen from OMZ waters in the Arabian Sea.

3.1 Introduction

Anammox, the anaerobic oxidation of ammonium, was first discovered in a wastewater treatment system about a decade ago (Mulder et al. 1995). The microorganisms performing anammox were identified as new members of the order *Planctomycetales*, one of the major and distinct divisions of the Bacteria (Strous et al. 1999). Anammox bacteria grow extremely slowly, dividing only once every two weeks (Strous et al. 1999). The anammox reaction involves the oxidation of ammonium under anoxic conditions with nitrite as the electron acceptor and dinitrogen gas as the product. Hydrazine (N_2H_2) and nitric oxide (NO) were found to be important intermediates in this process (Strous et al. 2006). Anammox bacteria have a specific intracytoplasmic compartment where the anammox reaction takes place, the anammoxosome. It is surrounded by a dense and highly impermeable lipid membrane, consisting of chemotaxonomically unique lipids. These so-called ladderane lipids are composed of up to five linearly concatenated cyclobutane rings (Sinninghe Damsté et al. 2002*c*). Such a dense membrane is thought to be required to maintain concentration gradients during the exceptionally slow anabolism of anammox bacteria and to protect the cell from the highly toxic intermediate hydrazine (Sinninghe Damsté et al. 2002*c*).

Evidence for anammox activity in the marine environment came from the Black Sea (Kuypers et al. 2003), the world's largest anoxic basin, where anammox was detected in the suboxic zone of the water column. Isotopic labelling studies have shown that anammox contributes substantially to N_2 production, i.e. up to 67 % in a continental shelf sediment (Thamdrup and Dalsgaard 2002), 19-35 % in the anoxic water column of Golfo Dulce, Costa Rica (Dalsgaard et al. 2003), and up to 19 % even in Arctic sea ice (Rysgaard and Glud 2004). Recently, anammox was also detected in the oxygen minimum zone (OMZ) of the Benguela upwelling system, one of the most productive regions of the world ocean, indicating that anammox not only takes place in anoxic environments but also in marine regions with low oxygen contents (Kuypers et al. 2005). It has thus been suggested that anammox might be a substantial sink for nitrogen in the OMZ waters off the Namibian coast (Kuypers et al. 2005). Until the discovery of anammox, the major (30-50%) sink of the global nutrient N removal occurring in OMZs

was mainly attributed to heterotrophic denitrification (Gruber and Sarmiento 1997; Brandes and Devol 2002).

The largest open-ocean OMZ is presently found in the Arabian Sea (Bange et al. 2000). The Arabian Sea is characterized by intense upwelling of nutrient-rich water during the Southwest and Northeast Monsoon periods leading to high primary production and enhanced downward particle flux (Honjo et al. 1999). Remineralization of sinking organic matter leads to a high oxygen demand in intermediate waters and causes an extensive OMZ between 150 and 1200 m depth. As much as ca. 20% of the major water column denitrification in the world ocean is estimated to take place in the Arabian Sea and thus this area has a significant influence on the global oceanic nitrogen budget (Codispoti 1989; Gruber and Sarmiento 1997). However, the contribution of anammox to loss of nitrogen in this area has yet to be established.

In this study we investigate whether anammox bacteria are present in the water column of the Arabian Sea and the extent to which anammox might be responsible for the removal of fixed inorganic nitrogen from oxygen depleted parts of the water column. We focus on the spatial and seasonal distribution of anammox-specific ladderane lipids in the northwestern Arabian Sea.

3.2 Material and methods

3.2.1 Suspended particulate matter and sediment traps

Suspended particulate matter (SPM) samples were collected at seven sites in the northwestern Arabian Sea (Fig. 1) during a U.S. Joint Global Ocean Flux Study (JGOFS) Arabian Sea Process Study cruise TN047 of the R/V *Thomas G. Thompson*. All stations (except station 13) form a transect perpendicular to the coast of Oman, with the most remote station being ca. 570 km off the coast. SPM samples used for this study were taken during the Spring Intermonsoon (SI) period in mid-May 1995, which is generally a period of low primary productivity. SPM samples were collected at specific depths of the water column (Table 1) by filtration of large volumes of water (~2,400 to 3,000 liters) through 292 mm diameter, precombusted glass fiber filters (nominal pore size 0.7 µm) with a Challenger Oceanic Mark II in situ pump. Filters were stored frozen (-20°C) until they were used for extraction.

Sinking particulate matter was collected in sediment traps (IRS-C traps; Peterson et al. 1993) deployed over an annual cycle along the southern U.S. JGOFS section from off the coast of Oman. The traps were deployed in November 1994 during cruise TN047 of R/V *Thomas G. Thompson* and recovered in January 1996 during cruise TN050. Samples used for this study derived from a ~ 500 m-deep sediment trap at site MS-3 (17°12'N, 59°36' E; Fig. 1) located ~ 350 km offshore in 3000 m of water (Honjo et al. 1999; Wakeham et al. 2002). Particulate matter was collected over variable time intervals, ranging from 9 to 34 days, with shorter intervals set for monsoon periods when high flux was expected, giving a total of 22 time-resolved samples for the study period of 408 days. Mercuric chloride was added as a biocide (Lee et al. 1992), and upon recovery, trap samples were sealed in their collection tubes and stored refrigerated until processing (Wakeham et al. 2002).

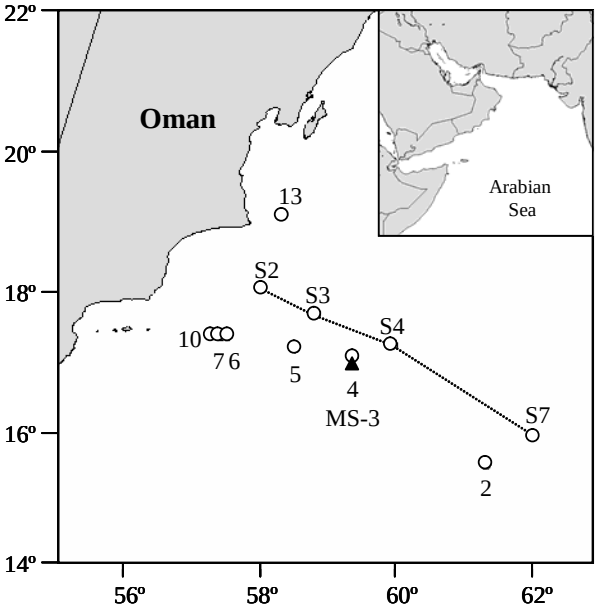

Figure 1. Site locations (open circles) of the 7 hydrographic stations where suspended particulate matter was obtained during the US JGOFS Arabian Sea Process Study cruise TTN047 in May 1995, and mooring site of IRS-C sediment trap MS-3 (filled triangle). The dotted line indicates part of the southern U.S. JGOFS Arabian Sea Process Study transect where nutrient data were collected in April 1995. The black dots indicate stations S2-S7.

3.2.2 Lipid analysis

Trap material was extracted with dichloromethane (DCM)-methanol (2:1 by volume; Wakeham et al. 2002). Lipid extracts were archived in solvent and stored at -20ºC since the time of the original studies until the ladderane lipid analyses made in this study. Filters were ultrasonically extracted with methanol, DCM-methanol (1:1, vol/vol), and DCM (three times). The bulk of the solvent was removed by rotary evaporation under vacuum, and the extract was further dried over a Na_2SO_4 column. An aliquot of the lipid extract was saponified under N_2 with aqueous 0.5 mol L^{-1} KOH in methanol for 2 h at 100°C. Non-saponifiable lipids (neutral lipids) were extracted out of the basic solution (pH>13) using hexane. Fatty acids were obtained by acidifying the residue to pH 2 and extraction with hexane thereafter. The fatty acid fraction was methylated by adding diazomethane (CH_2N_2) to convert fatty acids into their corresponding methyl esters (FAMEs). The excess CH_2N_2 was removed by evaporation. To remove very polar components, aliquots of the FAMEs were eluted with ethyl acetate over a small column filled with silica. Polyunsaturated fatty acids were removed by eluting the aliquots with ethyl acetate over a small $AgNO_3$ (5%) silica column yielding a saturated fatty acid fraction. These fractions were dissolved in acetone and then filtered through a 0.45 µm, 4 mm diameter PTFE filter.

An aliquot was analyzed by using high performance liquid chromatography coupled to positive ion atmospheric pressure chemical ionization tandem mass spectrometry (HPLC/APCI-MS/MS) under conditions described recently by Hopmans et al. (2006). This method was developed for the detection of three specific and most abundantly occurring ladderane fatty acids. These fatty acids (expressed as FAMEs) contain the biochemically unique [3]- and [5]- ladderane moieties composed of up to five linearly fused cyclobutane rings (see Fig. 2, structures I-III). Analysis of ladderane lipids were performed using an Agilent 1100 LC system, consisting of an inline membrane degassing unit, thermostatted auto injector and column compartment, coupled to a Quantum TSQ Ultra EM triple quadrupole mass spectrometer equipped with an Ion max source with atmospheric pressure chemical ionization (APCI) probe. Separation was achieved on two Zorbax Eclipse XDB-C_8 columns (4.6 x 150 mm, 5 µm, Agilent), coupled in series and maintained at 30 °C. Ladderane lipids were eluted with 0.4 ml min^{-1} methanol with a total run time of 20 min. Detection was achieved by positive ion APCI and selective reaction monitoring (SRM) of four specific fragments

for each ladderane lipid. The source settings were: vaporizer temperature 475 °C, discharge current 2.5 µA, sheath gas (N_2) pressure 50 (arbitrary units), auxiliary gas (N_2) pressure 5 (arbitrary units), capillary temperature 350 °C, source CID -10 V. Argon pressure was maintained at 1.5 mTorr in the second quadrupole. Quantification of ladderane lipids was done by using an external calibration curve using standards of isolated methylated ladderane fatty acids containing the [3]- and [5]- ladderane moieties (Sinninghe Damsté et al. 2002*c*; Hopmans et al. 2006). A detection limit (defined by a signal to noise ratio of 3) of 30-35 pg injected on-column was achieved with this technique.

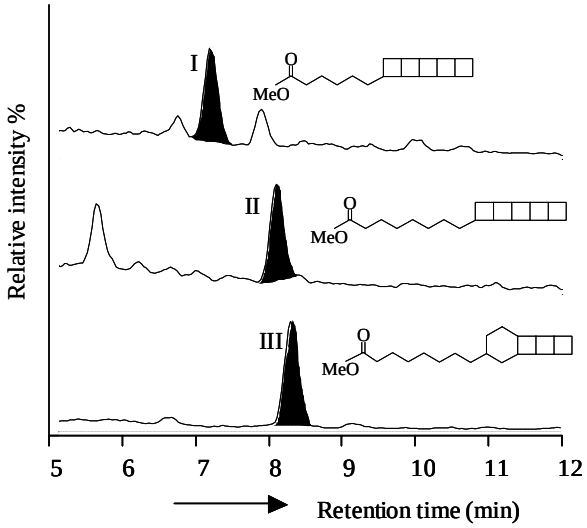

Figure 2. Partial SRM traces of three ladderane FAMEs obtained by HPLC/APCI-MS/MS analysis of suspended particulate matter at Station 5 (500 m water depth), and their corresponding structures. Trace I, II, and III show the pentyl-[5]-, the heptyl-[5]-, and the heptyl-[3]-ladderane FAMEs, respectively.

3.2.3 Statistical data treatment

Contour plots were generated using the Kriging interpolation technique in Surfer (version 7.04, 2001).

3.3 Results

We analysed a set of 18 SPM samples from different water depths along a northwest-to-southeast transect in the Arabian Sea off Oman, as well as a set of 22 trap samples from ~480 m water depth covering a whole monsoon cycle from November 1994 to January 1996. In nearly all samples ladderane fatty acids were detected in the saponified extracts of water filtrates and sediment trap material. Their relative

distribution was similar in all samples, i.e., it was dominated by the heptyl-[3]-ladderane FAME (Fig 2, III), followed by the pentyl-[5]- and heptyl-[5]-ladderane FAMEs (Fig. 2, I and II), both being 2 to 5 times lower in concentration. The concentrations of the three isomers were summed and are reported in Table 1.

3.3.1 Distribution of ladderane lipids in the water column

Figure 3 shows contour plots based on the concentrations of ladderane lipids (summed signal of the three ladderane fatty acids) in the water column offshore Oman during the Spring Intermonsoon, together with those based on nitrite, ammonium, and dissolved oxygen concentrations from nearby stations taken along the southern US JGOFS ASPS section during R/V *Thomas G. Thompson* cruise TN045 in early April 1995 (see http://usjgofs.whoi.edu), about 1 month earlier than the SPM sampling on cruise TN047.

Ladderane lipids were found in most of the samples from the oxygen depleted parts of the water column along the sample transect of the Arabian Sea (Table 1). Concentrations at ca. 100 m were below 1 pg L^{-1}, except at station 5 where concentrations of 3.4 pg L^{-1} were detected. Concentrations were highest at a water depth of around 500 m at all sites, varying from 5 to 8 pg L^{-1} (Fig. 3d). Concentrations at 1000 m water depth were approximately 1 pg L^{-1} or lower, and only near the coast (station 13) a higher concentration of 3 pg L^{-1} was found. Abundances of ladderanes at 1500 m water depth were <0.5 pg L^{-1} except station 5, where concentrations were around 6 pg L^{-1}.

3.3.2 Seasonal variations in ladderane lipid fluxes

Figure 4 shows a time-series of the ladderane lipid flux (summed fluxes of the three ladderane fatty acids) obtained from sinking particulate matter at station MS-3. A marked seasonality in ladderane lipid flux was observed, with maximum fluxes of 125 ng $m^{-2}d^{-1}$ during the beginning of the SW Monsoon. Strong winds from the southwest during the SW Monsoon induce upwelling of nutrient-rich bottom waters during the late spring and summer, leading to enhanced primary production in surface waters and enhanced particulate matter fluxes down the water column. During that SW Monsoon, ladderane lipid fluxes increased by a factor of ca. 3 within four weeks.

Table 1. Description of field stations, ladderane lipids, and nutrients

Ladderane lipids					Nutrients[a]					
Station	Location	Date	Depth (m)	FAME I-III ($pg\ L^{-1}$)	Station	Location	Date	$[O_2]$ ($mg\ L^{-1}$)	$[NO_2^-]$ ($\mu mol\ L^{-1}$)	$[NH_4^+]$ ($\mu mol\ L^{-1}$)
2	15°58'N 61°29'E	05-06 May 95	60	n.d.[b]	S7	15°99'N 61°98'E	31 March 95	4.20	0.81	0,00
			500	5,10				0.05	0.01	0,03
			1000	n.d.				0.16	0.00	0,00
			1500	n.d.				1.00	0.00	0,00
4	17°12'N 59°35'E	07-08 May 95	70	n.d.	S4	17°29'N 59°93'E	03 April 95	2.80	0.06	0,00
			1000	1,00				0.15	0.00	0,00
			1500	0,20				1.00	0.00	0,00
5	17°24'N 58°49'E	10-11 May 95	90	3,40	S3	17°73'N 58°80'E	05 April 95	~0.50	0.03	0,00
			500	8,10				0.18	0.00	0,00
			1500	6,00				0.89	0.00	0,00
6	17°41'N 57°50'E	13 May 95	500	5,50	S2	18°08'N 58°00'E	07 April 95	0.20	0.00	0,00
			1000	0,30				0.23	0.00	0,00
7	17°40'N 57°40'E	14 May 95	85	0,60	S2	18°08'N 58°00'E	07 April 95	3.20	0.06	0,00
10	17°44'N 57°29'E	16 May 95	80	n.d.	S2	18°08'N 58°00'E	07 April 95	3.20	0.06	0,00
			450	4,00				0.12	0.01	0,02
13	19°13'N 58°31'E	16-17 May 95	35	n.d.	NA	NA	NA	NA[c]	NA	NA
			500	6,60				NA	NA	NA
			1000	3,10				NA	NA	NA

[a] Data are from nearby stations S7 (for station 2), S4 (for station 4), S3 (for station 5), and S2 (for stations 6, 7, 10) of the U.S JGOFS Arabian Sea Process Study cruise 2 (see http://usjgofs.whoi.edu) in early April 1995

[b] n.d., not detected

[c] NA, not available

During the Spring and Fall Intermonsoon (SI and FI, respectively) periods, when wind strengths were relatively low, ladderane lipid fluxes were significantly reduced, varying between 3 and 20 ng m^{-2} d^{-1}. The NE Monsoon is characterized by moderate winds from the northeast during late fall and winter, and ladderane lipid fluxes of 65 ng m^{-2} d^{-1} were observed at the beginning of the NE Monsoon period, but then decreased to values as low as ~2 ng m^{-2} d^{-1} towards the end of the NE Monsoon. Overall, average ladderane lipid fluxes during the intermonsoon periods were 12.6 ng m^{-2} d^{-1} for the SI and 11.5 ng m^{-2} d^{-1} for the FI, whereas the average flux during the SW Monsoon increased about four times to 50 ng m^{-2} d^{-1}. The NW Monsoon period had an average flux of about 19 ng m^{-2} d^{-1}.

3.4 Discussion

3.4.1 Distribution of ladderane lipids in the water column

The presence of ladderane lipids in nearly all SPM samples strongly indicates that anammox bacteria occur throughout the water column of the northwestern Arabian Sea. Their distribution in the water column generally ranges from 85 m to 1000 m, and even to 1500 m water depth at station 5, which may reflect the dynamic and heterogeneous nature of the OMZ waters offshore Oman. Our data suggest that anammox bacteria are most abundant in the upper part of the OMZ at about 500 m water depth, but the low resolution of the SPM sampling (see grid points in Fig. 3d) precludes pinpointing the exact depth of maximum abundance. The observation that ladderane lipid and anammox bacteria abundances appear to be localized at 500 m rather than distributed throughout the OMZ (given our sampling resolution) is likely related to the distribution of nitrogen species in the OMZ. Both ammonium and nitrite are required for anammox. In the thermocline, where oxygen is still present, nitrite originates from phytoplankton reduction of nitrate, nitrification, or both (Codispoti and Christensen 1985), and a weak nitrite maximum is present in the thermocline (~100 m) along the whole transect (Fig. 3a). A quasi-permanent secondary nitrite maximum in the suboxic waters of the OMZ at ~200 to 350 m water depth with concentrations up to 3.4 µmol L^{-1} is associated with denitrification (Naqvi et al. 1991). This secondary nitrite maximum is reported to be strongest offshore the coast of Oman in the eastern and central Arabian Sea, whereas it is generally lacking along the northern and western boundaries due to re-oxygenation of subsurface waters (Naqvi et al. 1991). Ammonium

concentrations of maximum 0.17 µmol L^{-1} are found within the oxygenated thermocline, but a secondary ammonium maximum is present offshore at a water depth of around 500 m (Fig. 3b). Although the nutrient data reflect conditions of the water column during the Intermonsoon period, about four weeks earlier than the SPM sampling, temporal variability of suboxic conditions is thought to be minimal at these depths below the thermocline (Morrison et al. 1999).

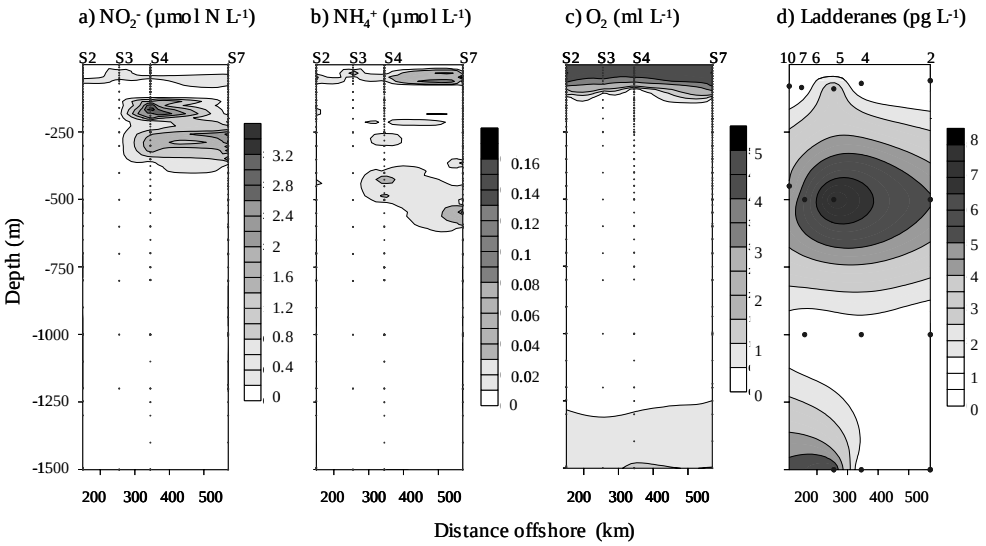

Figure 3. Contour plots of a) NO_2^-, b) NH_4^+, c) dissolved oxygen, and d) ladderane lipids at various depths in the water column on a northwest-to-southeast transect offshore Oman in the northern Arabian Sea. The black dots in panel d) indicate positions where samples were collected for ladderane lipid analysis (stations 2, 4, 5, 6, 7, and 10). NO_2^-, NH_4^+, and dissolved oxygen data are from nearby stations (S2, S3, S4, and S7) of the U.S. JGOFS Arabian Sea Process Study cruise 2 (see http://usjgofs.whoi.edu) in early April 1995. Grid points for the calculation of the contour plots are indicated.

The highest abundances of ladderane lipids were below the depth of the secondary nitrite maximum and close to the ammonium maximum, and this depth distribution strongly indicates active anammox at these depths. Anammox, in addition to denitrification, is therefore likely to contribute to the loss of nitrogen from the OMZ. Anammox might be coupled to reduction of nitrate to nitrite by denitrifying bacteria (Dalsgaard and Thamdrup 2002). The co-occurrence of suboxic conditions and nitrite

maxima suggests that denitrification is the dominant respiratory pathway (Morrison et al. 1998). However, Crenarchaeota have also been found to cope with extremely low oxygen levels, and concentrations of glycerol dibiphytanyl glycerol tetraethers (GDGTs) and crenarchaeol, the characteristic membrane lipid of Crenarchaeota (Sinninghe Damsté et al. 2002*b*) have been shown to be also abundant at 500 m depth in these Arabian Sea samples (Sinninghe Damsté et al. 2002*a*). As at least some of the Crenarchaeota are likely involved in nitrification (Könneke et al. 2005, Wuchter et al. 2006), they may, in addition to denitrifying bacteria, provide nitrite which could subsequently be used by anammox bacteria to transform ammonium to dinitrogen gas. However, further studies are needed to confirm this hypothesis.

Concentrations of ladderane lipids in the water column of the Arabian Sea are much lower than those previously reported from the Black Sea (1-4 ng L^{-1}; Kuypers et al. 2003) and the Benguela upwelling area (0.1-4 ng L^{-1}; Kuypers et al. 2005). This might be explained by the fact that the main zone where anammox potentially takes place, i.e., the zone of the secondary nitrite maximum at 200-400 m, was not sampled in this study. Furthermore, the main zone of denitrification (Morrison et al. 1998, Naqvi et al. 1991) where anammox might be more prevalent lies further offshore and to the southwest of the transect we sampled. Overall, the rate of water column denitrification in the Arabian Sea is generally believed to be globally significant and is thought to represent a major oceanic sink for reactive nitrogen (Codispoti 1989; Gruber and Sarmiento 1997). The imbalance between the nitrate deficit and the much higher excess of nitrogen gas probably from denitrification remains unexplained (Devol et al. 2006). One possible resolution could be that oxidation of ammonium by anammox bacteria is a significant potential source of excess N_2. Future resampling of the Arabian Sea at higher resolution and using different transects could resolve this important issue.

3.4.2 Seasonal variations in ladderane lipid fluxes

The sediment trap ladderane lipid flux data show a marked seasonal variability in the amount of ladderane lipids being transported vertically to the sediment trap at 500 m water depth. Ladderane fluxes generally show the same seasonal pattern as other biomarkers measured in sediment trap MS-3 (see Wakeham et al. 2002 for additional biomarker flux data), with highest fluxes during the SW Monsoon, low fluxes during the Intermonsoon periods, and moderate fluxes during the NE Monsoon, although the

timing of maximal flux varies among individual compounds. Ladderane lipid flux peaks near the start of the SW Monsoon (Fig. 4), which is about 2-4 weeks earlier than, for example, fluxes of alkenones or C_{30}-diols produced by photoautotrophic algae in surface waters (Wakeham et al. 2002). In contrast, the flux of organic carbon (OC, Fig. 4) is highest during the mid-to-late SW Monsoon period, presumably at the time of maximum export of phytoplankton-derived material following the high productivity SW Monsoon period. The flux of GDGTs (Wuchter et al. 2006, in press; Fig. 4), possibly derived from nitrifying Crenarchaeota, increased at the same time as the ladderane flux, but continued to increase towards the end of the SW Monsoon, parallel with OC.

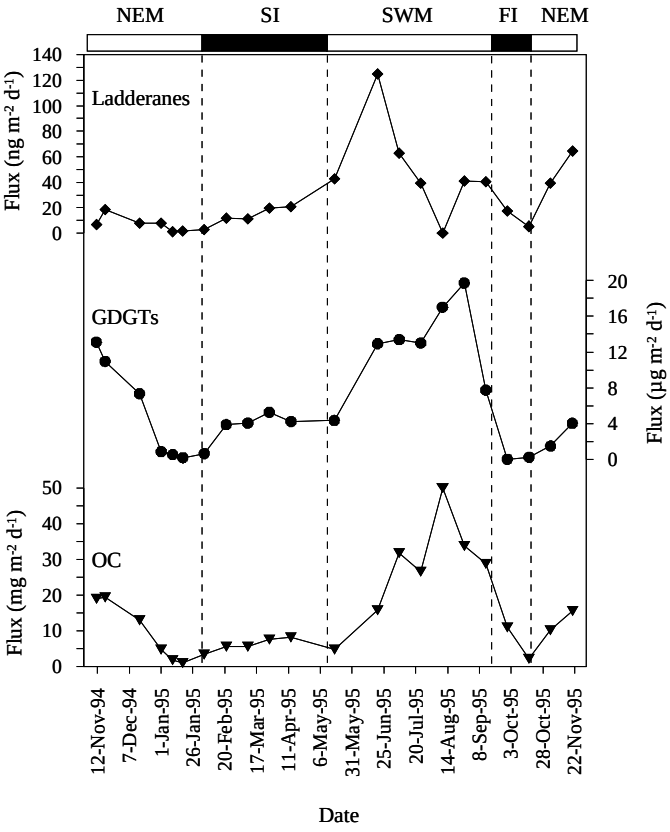

Figure 4. Summed ladderane lipid, GDGT (Wuchter et al. 2006, in press), and organic carbon (OC) (Wakeham et al., 2002) fluxes for IRS-C trap MS-3 deployed at ~ 500 m water depth. Data points represent the centers of collection intervals. Bars at the top of the plot and dotted vertical lines indicate the NE Monsoon (NEM), Spring Intermonsoon (SI), SW Monsoon (SWM), and Fall Intermonsoon (FI) periods of 1994/1995 after Weller et al. (1998).

Anammox bacterial cells are probably too small to settle themselves and other mechanisms (e.g. marine snow aggregation and faecal pellet formation) have to be invoked to explain the presence of ladderane lipids in descending particles. Increased primary productivity during the SW Monsoon leads to enhanced particle export through the water column (Honjo et al. 1999) due to a shift in enhanced food web dynamics and, consequently, in an increased marine snow aggregation and faecal pellet formation. Vertical migration by zooplankton in the Arabian Sea, even into the OMZ (Smith et al. 1998; Wishner et al. 1998) could also provide an active-transport mechanism by which ladderanes are injected directly as faecal pellets into the trap during times of high productivity. In these cases the maximum ladderane flux would be expected to coincide with the maximum in the OC flux, which is clearly not the case (Fig. 3). One of the factors complicating a comparison of lipid and OC fluxes is that ladderanes are likely produced at much greater depths than other biomarkers and the bulk of the OC, leading to differences in settling times and likely temporal offsets. Furthermore, there may also be changes in anammox bacterial productivity due to changing nutrient concentrations. Clearly, more work involving temporal studies of the water column are needed to investigate the seasonality of anammox activity. Regardless, the fact that ladderane lipids were detected in settling particles eventually reaching the sea-floor makes them potential biomarkers to assess anammox activity also in the geological past. For this, however, further research on the stability of ladderane lipids upon sediment burial is required.

Ladderane lipids derived from membranes of anammox bacteria have been identified in suspended particulate matter in the water column of the Arabian Sea during the Spring Intermonsoon period, with maximum concentrations in the upper part of the OMZ. Fluxes of ladderane lipids obtained by sediment trapping over an annual cycle revealed a strong seasonal pattern, with highest fluxes in the beginning of the SW Monsoon period. Enhanced vertical flux during the SW Monsoon may be related to a combination of increased ladderane lipid biosynthesis due to enhanced anammox activity and enhanced sedimentation of ladderanes during the monsoon-driven high productivity/high vertical flux period. The occurrence of ladderane lipids in the oxygen deficient part of the water column throughout the whole year indicates that, in addition

to denitrification, anammox may be an important sink for fixed inorganic nitrogen in the OMZ of the northern Arabian Sea.

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Chapter 4

Comparison of ladderane phospholipid and core lipids as indicators for anaerobic ammonium oxidation (anammox) in marine sediments

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Abstract

We investigated anaerobic ammonium oxidation (anammox) in continental shelf and slope sediments of the Irish and Celtic Seas by using anammox specific ladderane biomarker lipids. We used the presence of an intact ladderane phospholipid as a direct indicator for living anammox bacteria, and compared it with the abundance of ladderane core lipids derived from both living and dead bacterial biomass. All investigated sediments contained ladderane core lipids as well as the intact ladderane phospholipid, in agreement with ^{15}N-labeling experiments which revealed anammox activity at all sites. Ladderane core lipid and intact ladderane phospholipid concentrations were significantly correlated (R^2=0.957 and 0.464, respectively) with anammox activity over the transect of the continental shelf and slope sediments. In the Irish Sea (50-100 m water depth) highest abundances of the intact ladderane phospholipid were found in the upper 2 cm of the sediment, indicating a zone of active anammox. A sharp decline further down-core suggested a strong decrease in anammox biomass and rapid degradation of the intact lipids. In comparison, ladderane core lipids were 1-2 orders of magnitude higher in concentration than the intact ladderane phospholipid and accumulated as fossil remnants with depth. In the slope sediments of

the Celtic Sea both ladderane core lipids and the intact ladderane phospholipid were found in sediments at water depths ranging from 500 to 2000 m. Here, anammox seemed to be active at greater depths of the sediment (>2 cm). Mean abundances of both intact and core ladderane lipids in whole sediment cores increased downslope, indicating an increasing importance of anammox in deeper slope sediments.

4.1 Introduction

Anammox is the anaerobic oxidation of ammonium coupled to nitrite reduction with dinitrogen as the end product. The microorganisms performing anammox were identified as deep-branching members of the *Planctomycetales* (Strous et al., 1999). Since the discovery of anammox in a wastewater treatment system about a decade ago (Mulder et al., 1995), the process has been found to be significant for nitrogen cycling in the marine environment, and constitutes a novel route to remove fixed nitrogen from oxygen deficient zones of the water column (Dalsgaard et al., 2003; Jaeschke et al., 2007; Kuypers et al., 2003), anoxic sediments (Dalsgaard and Thamdrup, 2002; Trimmer et al., 2003), and even in sea ice (Rysgaard and Glud, 2004). In the oxygen minimum zones (OMZ) of Namibia (Kuypers et al., 2005), Chile (Thamdrup et al., 2006) and Peru (Hamersley et al., 2007) anammox was shown to be the dominant N_2 production pathway. Before, denitrification was considered to be the major process responsible for removing nitrogen from the oceans, and especially important in organic-rich sediments deposited along continental margins (Codispoti et al., 2001). Identification of the anammox process in marine and estuarine sediments was achieved mainly through patterns of isotope pairing and accounted for <2% to ~70% of the total N_2 production (Dalsgaard and Thamdrup, 2002; Thamdrup and Dalsgaard, 2002; Trimmer et al., 2003). The relative importance of anammox relative to denitrification was mainly attributed to changes in organic matter reactivity and the availability of reductants in sediments (Engstrom et al., 2005; Thamdrup and Dalsgaard, 2002). Quantification of anammox rates were all based on sediment-water slurries or closed jar incubations of surface sediments. However, information on anammox activity with sediment depth and the anammox bacterial communities in marine sediments are relatively scarce.

Ladderane lipids are specific membrane lipids unique to anammox bacteria (Sinninghe Damsté et al., 2002). The ladderane core lipid consists of three or five

linearly fused cyclobutane rings either ester- or ether-linked to the glycerol backbone via an alkyl chain. These ladderanes form a dense and highly impermeable membrane surrounding the anammoxosome, an intracytoplasmic compartment, and protecting the remainder of the cell from the toxic catabolic intermediate hydrazine (N_2H_4). Ladderane core lipids have so far been successfully applied as biomarkers for anammox bacteria in the suboxic water column of the Black Sea (Kuypers et al., 2003), oxygen minimum zones (OMZ) off Namibia (Kuypers et al., 2005), Peru (Hamersley et al., 2007) as well as in the Arabian Sea (Jaeschke et al., 2007). However, no distinction between living and dead anammox cells could be made as these core lipids do not occur as such in anammox cell membranes but are derived from intact ladderane membrane lipids. Previously, Boumann et al. (2006) and Rattray et al. (2008) showed that intact ladderane phospholipids comprise either phosphocholine (PC), phosphoethanolamine (PE), or phosphoglycerol (PG) as the major polar headgroup attached to the glycerol backbone. Intact phospholipids are due to their ionic nature more soluble to water and less resistant to enzymatic cleavage, therefore being rapidly decomposed after cell death, i.e., by loosing their polar headgroup (Harvey et al., 1986; White et al., 1979). As such, they are thought to be suitable biomarkers for the study of living microbial communities (Sturt et al., 2004). Thus, intact ladderane phospholipids might be more appropriate indicators for active anammox bacteria than ladderane core lipids.

In this study we applied anammox specific ladderane lipids in sediments of different oceanic regimes, the Irish Sea (50-100 m water depth) and along the slope of the Celtic Sea (500-2000 m water depth) as markers for the detection of anammox bacteria. To distinguish between living anammox cells and their dead remnants, we compared abundances of an intact C_{20}-[3]-ladderane monoether-PC lipid versus ladderane core lipids with sediment depth using newly developed high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) techniques.

4.2 Material and methods

4.2.1 Study sites and sampling

During a cruise with R/V *Pelagia* in March 2006 the occurrence and spatial distribution of anammox was investigated at six stations in the Irish and Celtic Sea, both side arms of the Atlantic Ocean (Fig. 1, Table 1). The sampling sites in the Irish Sea, located between Ireland and Great Britain were at 50 to 100 m water depth. The

sediments were composed of dark mud, mainly clay. Total organic carbon (TOC) values of surface sediments were 0.8-1.4 %. In the Celtic Sea, which is expanding south of Ireland, three stations were located along a steep shelf break at 500, 1000, and 2000 m water depth, respectively. The sediments at 500 m depth consisted mainly of shell-bearing sands, at greater depths mostly of clay-bearing fine sands, and TOC values were 0.04-0.5 %. Oxygen penetration depths measured with a Clark-type O_2 microsensor, ranged from 0.7 to 1.6 cm at the investigated sites in the Irish Sea, and from 1.2 to 2.0 cm in the Celtic Sea sediments. Sediment was collected by a box-corer, and only undisturbed cores with clear overlying water were used. Sediment cores for nutrient analysis were sliced into 0.5 cm pieces directly after core retrieval. For FISH analysis, sediment was sliced at 3 mm intervals to a depth of 36 mm. The sediment (~ 1g x 5 replicates) from each section was fixed (4 % v/v paraformaldehyde in PBS solution) and allowed to stand at room temperature for 2 h. Each sample was then washed twice (PBS) and re-suspended in PBS-ethanol solution (50 % v/v). All samples were subsequently stored at -20ºC until required. Subcores for ladderane lipid analysis were collected in Plexiglas tubes of 12 cm diameter. Sediment samples were cut into 1-cm sections and stored at -20°C until used for analysis.

4.2.2 NO_2^-, NO_3^-, NH_4^+ porewater profiles

Sediment samples were centrifugated, and the pore water was immediately analyzed on board for NO_2^-, NO_3^-, and NH_4^+ using a segmented flow auto analyzer (Skalar, The Netherlands) and standard colorimetric techniques.

4.2.3 Fluorescence *in situ* hybridisation (FISH)

The FISH analyses were conducted based on the methods described by Schmid et al. (2000; 2003). The anammox specific probes S-*-Amx 0368-a-A-18 (detecting all anammox organisms) (Schmid et al., 2003) and S-*-BS-820-a-A-22 (targeting *Scalindua sorokinii* and *Scalindua wagneri*) (Kuypers et al., 2003) were used in combination with S-P-Planc-0046-a-A-18 (identifying the *Planctomycetales*) (Neef et al., 1998). To maximise probe binding, the hybridization buffer was set at a stringency of 25% formamide. Following hybridisation, sediment samples were counterstained with DAPI (fluorochrome 4'6-diamino-2-phenylindole dihydrochloride) mounted in Vectashield (Vector Laboratories, Peterborough, UK) and viewed using a Leica DM RA2 epiflouresecent microscope (Leica microsystems, UK). Images were captured from

two visual fields using Openlab (Improvision, Coventry, UK) and processed with Adobe PHOTOSHOP (Adobe Systems, Edinburgh, UK). Adjustments to colour contrast and brightness were uniform across all images. Five FISH experiments were performed at each depth interval using labelled oligonucleotide probes purchased as Cy3, Cy5 and 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (Fluos) labelled derivatives from MWG-Biotech (London, UK) and Thermo Electron (Ulm, Germany).

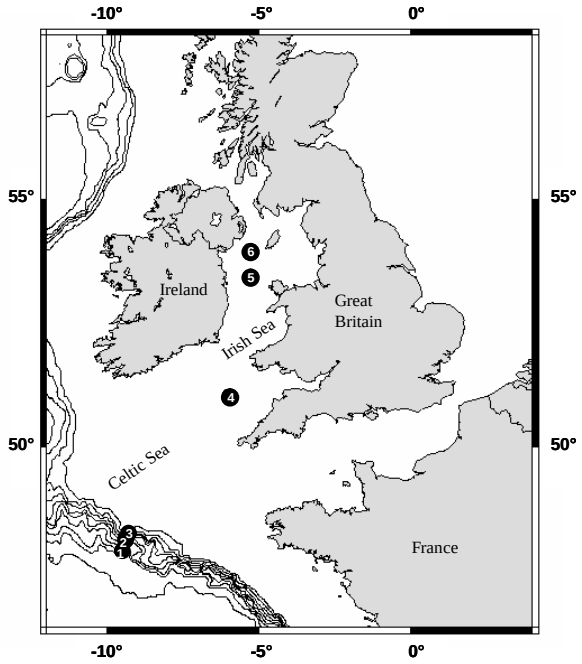


Figure 1. Locations of the six stations in the Irish Sea and the Celtic Sea shelfbreak, respectively.

4.2.4 ^{15}N incubations and analysis

Sediment samples (oxic and suboxic layers 0-2 cm) were collected from the box-corer and slurries (30 ml, 50 % v/v) were prepared anaerobically in serum bottles (37 ml) as previously described (Trimmer et al., 2003). After preincubation overnight the slurries were enriched from concentrated stocks of $^{15}NH_4Cl$ (120 mM $^{15}NH_4Cl$ [99.3 ^{15}N atom %] Sigma-Aldrich, Poole, United Kingdom) to give a final concentration of 500 µM $^{15}NH_4^+$ and $Na^{14}NO_3^-$ (93 mM, respectively, VWR International Ltd, Lutterworth, United Kingdom) to a final concentration of 100 µM or 0 µM in the case of controls. Production of $^{29}N_2$ after overnight incubation was taken as proof-positive of the anammox reaction (Risgaard-Petersen et al., 2004; Thamdrup and Dalsgaard, 2002;

Trimmer et al., 2003) and was quantified in subsamples (50 µL) of the headspace in each slurry using continuous flow isotope ratio mass spectrometry (Delta Mat Plus, Thermo-Finnigan, Bremen, Germany) (Trimmer et al., 2005).

Table 1. Site characteristics

Station	Location	Date	Latitude	Longitude	water depth (m)	O_2 penetration depth (mm)	TOC (%)
1	Celtic Sea	16-Mar-06	48.03.69 N	9.51.11 W	2006	11.7	0.49
2	Celtic Sea	29-Mar-06	48.10.70 N	9.42.40 W	1035	11.8	0.17
3	Celtic Sea	1-Apr-06	48.16.02 N	9.42.54 W	497	19.9	0.04
4	Irish Sea	20-Mar-06	51.13.10 N	6.05.96 W	106	8	0.82
5	Irish Sea	23-Mar-06	53.52.95 N	5.35.56 W	104	16.4	1.36
6	Irish Sea	26-Mar-06	54.07.14 N	5.35.05 W	54	7.2	1.25

4.2.5 Ladderane core lipid analysis

Samples of ca. 6-20 g of freeze-dried and homogenized sediment were ultrasonically extracted 5x using dichloromethane (DCM)-methanol (2:1 by volume). The extracts were combined and the bulk of the solvent was removed by rotary evaporation under vacuum, and the extract was dried over a Na_2SO_4 column. An aliquot of the lipid extract was saponified with aqueous 1 N KOH in methanol for 2 h. Non-saponifiable lipids (neutral lipids) were extracted out of the basic solution (pH>13) using DCM. Fatty acids were obtained by acidifying the residue to pH 3 and subsequent extraction with DCM. The fatty acid fraction was methylated by adding diazomethane (CH_2N_2) to convert fatty acids into their corresponding methyl esters (FAMEs). The excess CH_2N_2 was removed by evaporation. To remove very polar components, aliquots of the FAMEs were eluted with ethyl acetate over a small column filled with silica. Polyunsaturated fatty acids (PUFAs) were removed by eluting the aliquots with ethyl acetate over a small $AgNO_3$ (5%) impregnated silica column, yielding a saturated fatty acid fraction. An aliquot of the neutral fraction was eluted with DCM/methanol (1:1; v/v) over a small column filled with activated aluminium oxide. Both fatty acid and neutral fractions were dissolved in acetone and then filtered through a 0.45 µm, 4 mm diameter PTFE filter.

These fractions were analyzed by high performance liquid chromatography coupled to positive ion atmospheric pressure chemical ionization tandem mass spectrometry (HPLC/APCI-MS/MS) as described by Hopmans et al. (2006) with some modifications. Specifically, separation was achieved using a Zorbax Eclipse XDB-C_8

column (3.0 x 250 mm, 5 µm; Agilent) and a flow rate of 0.18 ml/min MeOH. The source settings were: vaporizer temperature 475 °C, discharge current 2.5 µA, sheath gas (N_2) pressure 30 (arbitrary units), auxiliary gas (N_2) pressure 5 (arbitrary units), capillary temperature 350°C, source CID -10 V. Argon pressure was maintained at 1.5 mTorr in the second quadrupole. Quantification of ladderane lipids was done by using an external calibration curve using standards of isolated methylated ladderane fatty acids containing the [3]- and [5]- ladderane moieties (Fig. 2, structures I-III) and a monoether containing the [3]-ladderane moiety (Fig. 2, structure IV) (Hopmans et al., 2006; Sinninghe Damsté et al., 2002). A detection limit (defined by a signal to noise ratio of 3) of 30-35 pg injected was achieved with this technique. The analytical reproducibility of ladderane core lipid concentrations based on duplicate analysis was better than 8%.

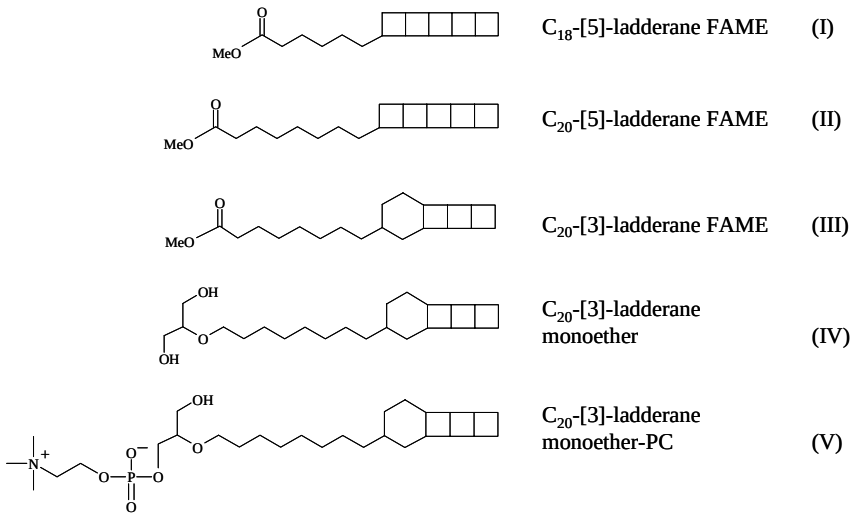


Figure 2. Chemical structures of the ladderane lipids analyzed in this study; (I) C_{18}-[5]-ladderane FAME, (II) C_{20}-[5]-ladderane FAME, (III) C_{20}-[3]-ladderane FAME, (IV) C_{20}-[3]-ladderane monoether, (V) C_{20}-[3]-ladderane monoether-PC.

4.2.6 C_{20}-[3]-ladderane monoether-PC lipid analysis

Sediment samples (6-20 g dry weight) were ultrasonically extracted according to the method of Bligh and Dyer (Bligh and Dyer, 1959) with some modifications using a mixture of methanol, DCM and phosphate buffer at pH 7.4 (2:1:0.8 v/v/v). After sonication for 10 min, further DCM and buffer were added to the mixture to achieve a

final methanol/DCM/buffer ratio of 1:1:0.9 (v/v/v). The phases were separated by centrifugation and the extraction repeated three more times. The extracts were combined and the bulk of the solvent was removed by rotary evaporation under vacuum. An aliquot of the extract was dissolved in a hexane/2-propanol mixture (79:21 v/v) and filtered through a 0.45 µm, 4 mm diameter RC filter.

The C_{20}-[3]-monoether lipid containing a PC headgroup (Fig. 2, structure V) was analyzed by HPLC/electrospray ionization (ESI)-MS/MS based on Boumann et al. (2006) with some modifications. Separation was achieved on a LiChrospher diol column (250 mm x 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}: 90% A:10 % B to 70% A: 30% B over 10 min, maintained for 20 min, then to 35% A:65% B in 15 min, maintained for 15 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} ratios 79:20:0.12:0.04 (v/v/v/v) and B = 2-propanol/water/formic acid/14.8 M NH_{3aq} ratios 88:10:0.12:0.04 (v/v/v/v). Detection of the C_{20}-[3]-monoalkylether-PC was achieved by selective reaction monitoring (SRM) of the transition from *m/z* 530 $[M+H]^+$ to *m/z* 184 (corresponding to the PC-headgroup), with 1.5 mTorr Argon as collision gas and 20V collision energy. Quantification of the intact ladderane monoetherlipid was done by an external calibration curve of an isolated C_{20}-[3]-ladderane monoether-PC standard (43% purity; see below). A detection limit of 10 pg injected was achieved with this technique. The analytical reproducibility of the C_{20}-[3]-ladderane monoether-PC lipid concentration was better than 12%.

For quantification, a C_{20}-[3]-ladderane monoether-PC standard was isolated from anammox cell material from a wastewater treatment plant of Paques B.V., Balk, The Netherlands. The freeze-dried anammox cell material was extracted using a modified Bligh and Dyer method (Bligh and Dyer, 1959) as described above. The C_{20}-[3]-ladderane monoether-PC was isolated by semi-preparative HPLC using an HP 1100 LC system (Hewlett Packard, Palo Alto, CA, USA) equipped with thermostatted autoinjector and column oven, coupled to an Isco (Lincoln, NE, USA) Foxy Jr. Fraction collector. After injection of the filtered extract onto a LiChrospher DIOL 100 Å (250 x 10 mm, 5 µm; Alltech) column, maintained at 25 °C, the C_{20}-[3]-ladderane monoether-PC was eluted using the identical gradient and mobile phase composition as described above for the LC/ESI-MS/MS analysis, but with a flow rate of 3 ml/min. The eluent was collected in 1 min fractions. Small aliquots of the collected fractions were analyzed by

flow injection analysis (FIA) using the same HP HPLC system as described above, coupled to an HP 1100 MSD mass spectrometer using ESI. Conditions for ESI-MS were as follows: nebulizer pressure 20 psi N_2, drying gas (N_2) 10 l/min, gas temperature 200 °C, capillary voltage 3500 V. Injections were made at 1 min intervals into a stream of 60%A:40%B (see above for composition of A and B) at a flow rate of 0.2 ml/min. Positive ion spectra were generated by scanning *m/z* 300-1000. Fractions containing the C_{20}-[3]-ladderane monoether-PC were pooled and dried under a stream of nitrogen. The purity of the isolated C_{20}-[3]-ladderane monoether-PC was determined by weighing the pooled fractions and comparing the calculated phosphorus content to the measured lipid phosphorus content as determined by the colorimetric method described by Fiske and Subbarow (1925).

4.3 Results

4.3.1 NO_2^-, NO_3^-, NH_4^+ porewater profiles

Nitrite concentrations at all sites were typically low (<2 µM) besides site 1 where concentrations reached 17 µM in the surface sediment (Fig. 3). Ammonium, diffusing upwards from deeper sediment depths and reaching concentrations up to 120-130 µM, is re-oxidized in the oxygenated part of the sediments (Fig. 3). Nitrate concentrations reached maxima of 8-12 µM in the upper 2 cm due to nitrification (sites 2, 4, 5, 6), while maxima of 42 and 33 µM were observed at site 1 and 3, respectively.

4.3.2 ^{15}N-labeling incubations and FISH analysis

To confirm the presence of anammox bacteria, we determined the $^{29}N_2$ production after incubation with $^{15}NH_4$ and $^{14}NO_3$, and performed FISH analyses. Significant production of $^{29}N_2$ (P<0.001) was measured in anaerobic sediment samples at all sites (Table 2). The production of $^{29}N_2$ was fairly consistent across sites 1 to 4 but was markedly greater at sites 5 and 6, though the screening exercise employed here using the top 2 cm of sediment uniformly across each site may not truly reflect the real potential of anammox to N_2 production at each site.

FISH analysis with anammox-specific 16S rRNA probes confirmed the presence of anammox bacteria at all sites (Fig. 4). Prokaryotes affiliated with *Candidatus* S. sorokinii/ *Candidatus* S. wagneri were the only detectable anammox bacteria and no other Planctomycetes were detected.

Table 2. Production of $^{29}N_2$ from incubation with $^{15}NH_4^+$ and $^{14}NO_3^-$ in anaerobic surface sediment (ca 0-2 cm) slurries from selected sites.

	Production of $^{29}N_2$ (nmol ml^{-1} wet sediment)					
Site	1	2	3	4	5	6
Mean	**1.3**	**2.8**	**2.0**	**2.1**	**25.7**	**6.3**
se, *n* = 4	**0.2**	**0.1**	**0.4**	**0.1**	**1.1**	**0.4**

4.3.3 Ladderane core lipids

At all six stations in the Celtic and Irish Sea, ladderane fatty acids and the ladderane monoether were detected (Fig. 3). Their distribution was similar in all samples, i.e., it was dominated by the C_{20}-[3]-ladderane monoether, followed by the C_{18}-[5]-ladderane FAME in 2-3 times lower concentration, and the C_{20}-[3]- and C_{20}-[5]-ladderane FAME both in 6-10 times lower concentrations.

At station 1 (2000 m water depth) in the Celtic Sea the summed concentration of all four ladderane lipids were below 1 ng/g sediment in the first two cm, then increased downcore up to 33 ng/g sediment at 8 cm depth (Fig. 3). At station 2 (1000 m water depth) ladderane lipid concentrations were overall low, ranging between 0.6 and 2.7 ng/g sediment. At station 3 (500 m water depth) the concentration of ladderanes were <1 ng/g sediment down to 4 cm, then increased to 8 ng/g sediment at 6 cm, and subsequently decreased to 2 ng/g sediment. In the Irish Sea, ladderane lipid concentration at station 4 (100 m water depth) showed little variation with values ranging between 7.5 and 10.5 ng/g sediment. At station 5 (100 m water depth) ladderane concentrations in the upper two cm were ~42 ng/g sediment, increased to 53 ng/g sediment between 3 and 5 cm, and then decreased to values of ~47 ng/g sediment. At station 6 (50 m water depth) highest concentration of ladderane lipids of 27 ng/g sediment were found in the upper two cm, then decreasing to 19 ng/g sediment at 4 cm, and ranging between 23 and 19 ng/g sediment further downcore.

4.3.4 C_{20}-[3]-ladderane monoether-PC lipid

Since anammox bacteria are generally in low abundance in the environment (Kuypers et al., 2005; Hamersley et al., 2007; Schmid et al. 2007), and thus likely also their phospholipids, we developed a new HPLC/ESI/SRM-MS method to selectively and quantatively analyse the C_{20}-[3]-ladderane monoether containing a PC headgroup. Using this technique, we detected this compound at all six stations in the Irish and Celtic Sea (Fig. 3). At station 1 (2000 m) in the Celtic Sea the concentrations varied in

the upper 3.5 cm between 10 and 14 pg/g sediment (minimum of 3 pg/g sediment at 2.5 cm depth), then increased downcore to ca. 60 pg/g sediment. At station 2 (1000 m) concentrations were overall low, showing a maximum at 2.5 cm depth of ca. 20 pg/g sediment. Below 5 cm no C_{20}-[3]-ladderane monoether-PC lipid was detected. Lowest concentrations were detected at station 3 (500 m) with values ranging from <1 to 3 pg/g sediment. At station 4 (100 m) in the Irish Sea concentrations decreased rapidly from ca. 20 pg/g sediment at the surface to 1-1.5 pg/g sediment below 2 cm depth. At station 5 (100 m) concentrations decreased constantly downcore from ca. 20 pg/g sediment in the upper part to ca 5 pg/g sediment at 7.5 cm. At station 6 (50 m) concentrations decrease quickly within the upper two cm from ca. 40 pg/g sediment at the surface to 1-4 pg/g sediment below 2 cm.

4.4 Discussion

4.4.1 Ladderane core lipids versus intact phospholipids in marine sediments

The presence of ladderane membrane lipids that are unique to anammox bacteria, FISH analysis and ^{15}N incubation experiments provide strong evidence for the occurrence of anaerobic ammonium oxidation in sediments along a transect of different oceanic regimes i.e., shelf sediments of the Irish Sea (50-100 m water depth) and slope sediments of the Celtic Sea (between 500 and 2000 m water depth). However, apparent differences in the intact C_{20}-[3]-ladderane monoether-PC lipid versus core lipids can be seen in terms of total abundances and sediment depth in the Irish and Celtic Sea (Fig. 3, columns 2 and 3). In the Irish Sea, depth profiles show high contents of both ladderane core lipids and the C_{20}-[3]-ladderane monoether-PC lipid in surface sediments (Fig. 3d-f), although concentrations of the C_{20}-[3]-ladderane monoether-PC lipid are about two orders of magnitude lower than the corresponding ladderane monoether core lipid. The sharp decrease in concentration of the C_{20}-[3]-ladderane monoether-PC lipid is most distinct at station 4 and 6 (Fig. 3d and f) while the concentration of the ladderane core lipids remain more or less constant over the depth range. This difference can be readily explained by inferring that the C_{20}-[3]-ladderane monoether-PC lipid indicates the zone of anammox activity and below this zone it degrades rapidly with depth. In contrast, the ladderane core lipids seem to possess a much higher preservation potential and accumulate as dead cell remnants with depth.

Celtic Sea (slope)

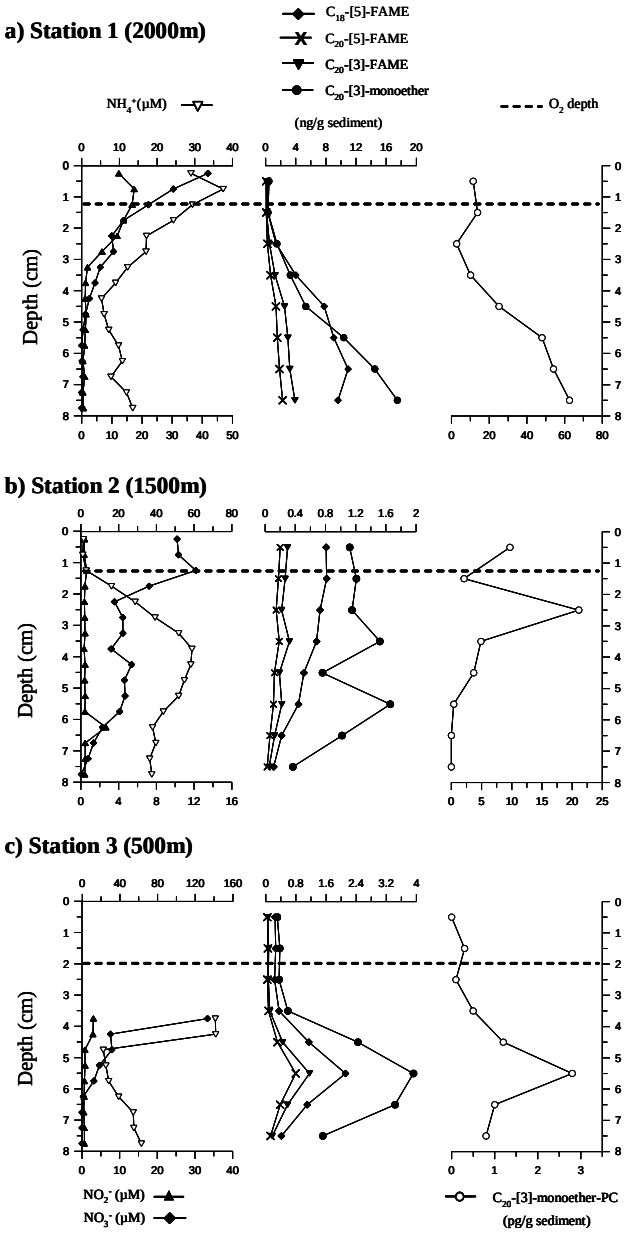
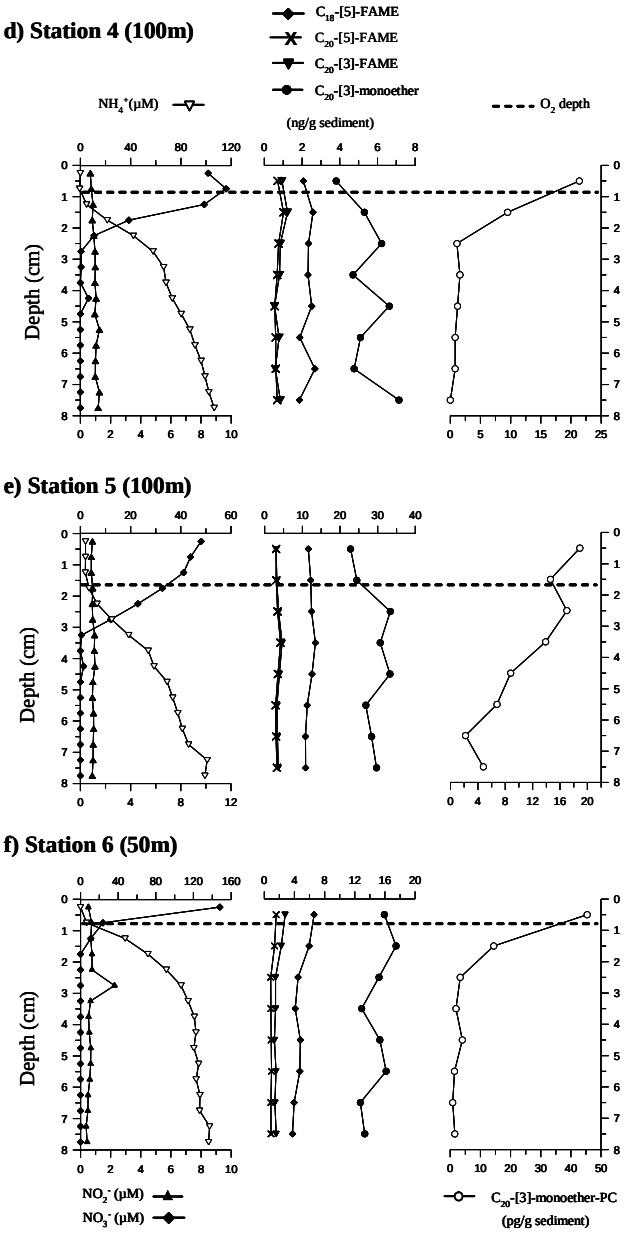


Figure 3. Nutrient porewater profiles and distribution of anammox specific ladderane membrane lipids from the Celtic Sea shelfbreak (a-c) and the Irish Sea (d-f). The first column shows the profiles of NH_4^+ (top axis), and NO_3^- and NO_2^- (bottom axis). The second column shows the concentration of four different ladderane core lipids, C_{18}-[5]-ladderane FAME (filled diamond),

Irish Sea (shelf)



C_{20}-[5]-ladderane FAME (cross), C_{20}-[3]-ladderane FAME (filled triangle), and C_{20}-[3]-ladderane monoether (filled circle) (top axis). The third column shows the concentration of C_{20}-[3]-ladderane monoether-PC (open circle) (bottom axis). Note different x-axis scales for all columns. The dashed line indicates the oxygen penetration depth in the sediment.

At station 5 (Fig. 3e) the decrease in concentration of the C_{20}-[3]-ladderane monoether-PC lipid was not as sharp as observed at the other two sites in the Irish Sea. Here, high benthic (i.e., nephrop) activity was observed in the muddy, almost liquid sediments probably resulting in a more mixed signal, and oxygen penetration was much deeper in comparison to station 4 and 6 (Fig. 3d and f). In sediments of the Celtic Sea shelf break, concentrations of ladderane core lipids at station 2 and 3 (Fig. 3b and c) remain low over the whole depth range while they increase below the oxic sediments at station 1 (Fig. 3a). The concentration profile of the C_{20}-[3]-ladderane monoether-PC lipid reveals the same trend as the core lipids, although being 2-3 orders of magnitude lower in concentration than the core lipids. Here, ladderane core lipids run more parallel with the zone of indicated anammox activity. However, at station 2 (Fig. 3b) a distinct peak in the intact C_{20}-[3]-ladderane monoether-PC lipid is observed which is not mirrored in the core lipid profile. If mean values of the ladderane lipid concentrations calculated for each site over the whole depth range it is obvious that both ladderane core lipids and the C_{20}-[3]-ladderane monoether-PC lipid increase with increasing water depth at the three slope sites 1, 2 and 3, respectively (Fig. 5). Mean ladderane core lipid concentrations are highest at site 5 in the Irish Sea which is consistent with highest anammox activities from sediment slurries (Table 2), while mean C_{20}-[3]-ladderane monoether-PC lipid concentrations are relatively lower. Anammox activity was found to be different in spring and autumn in the Irish Sea with in general higher activities during autumn, while the difference was less at the slope sites in the Celtic Sea (Trimmer and Nicholls, 2009). Therefore, in the Irish Sea there might be a higher contribution of dead biomass to the ladderane lipid pool than in the Celtic Sea.

The fast degradation with depth and in general low concentrations of the intact C_{20}-[3]-ladderane monoether-PC lipid which is 10-100 times lower than the corresponding core lipid in sediments of the Irish and Celtic Sea demonstrates its labile character. Once the PC headgroup is hydrolyzed after cell death, the core ladderane monoether is left and accumulates with sediment depth (Fig. 3). The much lower concentration of the C_{20}-[3]-ladderane monoether-PC lipid versus its corresponding ladderane monoether core lipid may also be partly due to the fact that we are currently only able to detect and quantify the monoether with a PC headgroup using our new HPLC/ESI/SRM-MS technique, whilst it is known that the intact polar ladderane phospholipids also contain other headgroups like phosphoethanolamine and phosphoglycerol, although in much lower abundance (Boumann et al., 2006; Rattray et

al., 2008). This might have led to an underestimation of the actual intact ladderane phospholipid concentration. The ladderane monoether core lipids analyzed after base hydrolysis include therefore the sum of all intact ladderane monoethers with different headgroups (PC, PG and PE). With respect to mean ladderane lipid concentrations for each site, the C_{20}-[3]-ladderane monoether-PC lipid correlates with the C_{20}-[3]-ladderane monoether core lipid in the Irish and Celtic Sea, although with different slopes (Fig. 6). Although revealing different patterns with sediment depth, both the C_{20}-[3]-ladderane monoether-PC lipid and the corresponding C_{20}-[3]-ladderane monoether core lipid show the same trend in terms of mean concentrations per site, indicating the amount of anammox cells in the sediment.

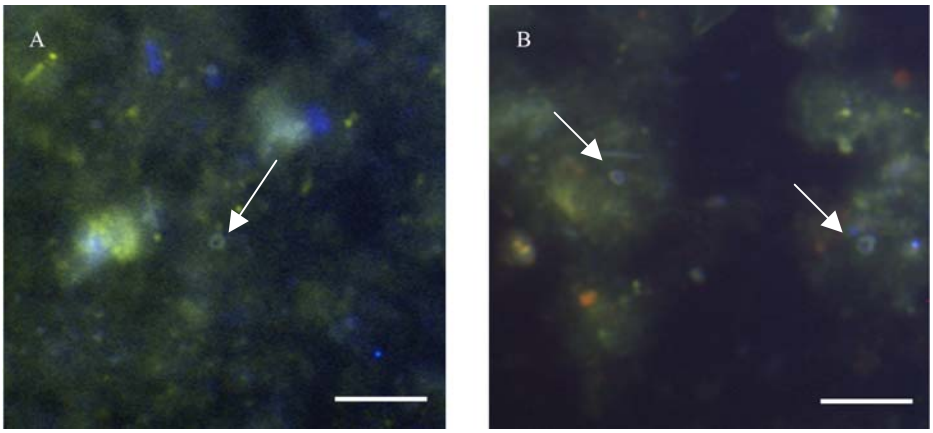


Figure 4. Detection of anammox organisms with fluorescence *in situ* hybridisation from (a) site 1, -36mm (b) and site 5, -9mm; arrows indicate the presence of 'white' anammox cells. This is the result of triple hybridisation of probes Amx 0368 (CY3), Pla 46 (CY5) and BS 820 (fluos) in combination with DAPI (staining all DNA blue). The scale bar represents 5µm.

4.4.2 Implications for anaerobic ammonium oxidation in marine sediments

Our results for the lipid analysis compare well with our ^{15}N tracer experiments performed on sediment slurries (Table 2). Low production of ^{29}N in surface sediments (ca. 0-2 cm) of the Celtic Sea slope sites 1-3 is consistent with very low abundances of ladderane lipids, both core and the C_{20}-[3]-ladderane monoether-PC lipid, at these sites (Fig. 3a-c). Highest concentrations of the C_{20}-[3]-ladderane monoether-PC lipid were found in the muds of the Irish Sea, indicating the zone of active anammox in the upper two cm of the sediment (Fig. 3d-f). Station 6 (Fig. 3f) reveals highest concentrations of

45 pg/g sediment in the upper first cm, which is two times higher than concentrations observed at station 4 and 5, respectively (Fig. 3d and e), while ^{29}N production was by far the highest at station 5 (Table 2). This can be explained by the fact that at station 5 the concentration of the C_{20}-[3]-ladderane monoether-PC lipid remained more or less constant over the upper 2-3 cm, while at station 6 it decreased rapidly in concentration within the upper two cm. A moderate organic carbon content (0.8-1.4%) in sediments of the Irish Sea, together with high concentrations of NH_4^+ (50-120 µmol) in the pore water diffusing upwards may stimulate the anammox process near the sediment-water interface where it might be coupled to nitrate reduction (Dalsgaard and Thamdrup, 2002).

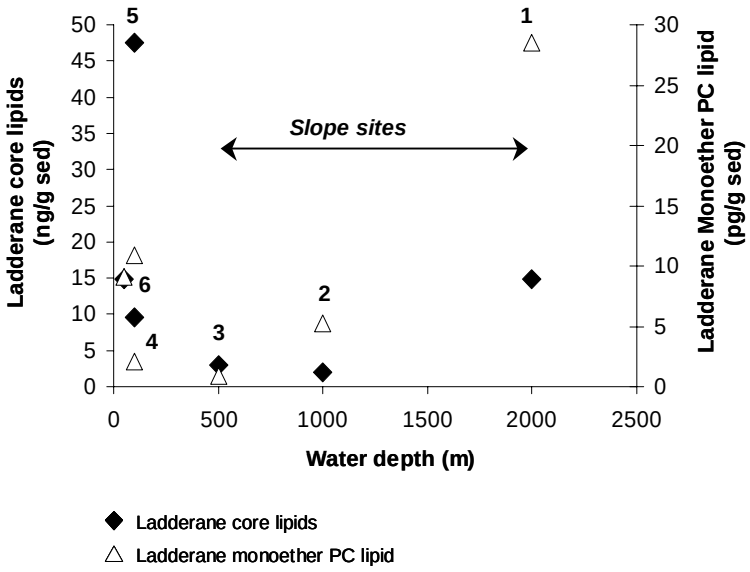


Figure 5. Mean concentrations of ladderane core lipids (filled diamond) and the C_{20}-[3]-ladderane monoether-PC lipid (open triangle) in whole sediment cores at six sites along a transect from the shelf (Irish Sea) and slope (Celtic Sea) in relation to water depth.

High NO_3^- concentrations in the pore water could be essential for maintaining anammox as already suggested by Rysgaard et al. (2004), Risgaard-Petersen et al. (2005) and Trimmer et al. (2005). In the Celtic Sea, concentrations of the C_{20}-[3]-ladderane monoether-PC lipid in the upper few cm of the sediments that consist mainly of shell-bearing sands are ca 2-15 times lower than in the Irish Sea (Fig. 3a-c). Here, deep

oxygen penetration into the sediment, low organic carbon (0.04-0.5%) and nutrient availability are probably less favourable for anammox. With increasing sediment depth though, the concentrations of the C_{20}-[3]-ladderane monoether-PC lipid at stations 1-3 increased below the oxygenated part of the sediment. At station 3, a maximum in C_{20}-[3]-ladderane monoether-PC lipid, although in low absolute amounts, is observed directly below a peak in ammonium, nitrate and nitrite. At station 2, the C_{20}-[3]-ladderane monoether-PC lipid concentration peaks just below the oxygenated part of the sediment where ammonium and nitrate concentrations are decreasing. Here, C_{20}-[3]-ladderane monoether-PC lipid concentrations of 21 pg/g sediment are comparable to those observed in surface sediments of station 4 and 5. As indicated by the concentration profile of the C_{20}-[3]-ladderane monoether-PC lipid at station 1, anammox bacteria seem to be active much deeper in the sediment, and concentrations of ca. 60 pg/g sediment at 7.5 cm depth are even the highest from all stations investigated. As shown in Fig. 5, mean ladderane lipid concentrations of both core lipids and the C_{20}-[3]-ladderane monoether-PC lipid increased at greater water depth, indicating higher abundances of anammox bacteria in deeper slope sediments.

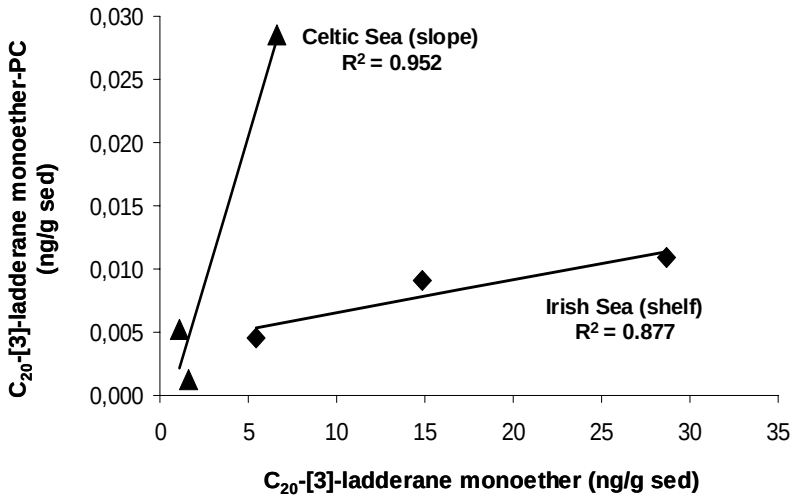


Figure 6. Mean concentrations of the C_{20}-[3]-ladderane monoether-PC lipid in relation to the C_{20}-[3]-ladderane monoether concentration in whole sediment cores at six sites along a transect from the shelf (Irish Sea) and slope (Celtic Sea).

Pore water NH_4^+ concentrations decreased with increasing water depth, indicating less organic matter remineralization, which favours anammox over denitrification (Dalsgaard and Thamdrup, 2002; Engström et al., 2005).

The low concentrations of the ladderane lipids (ranging from ng to pg/g sediment) is in agreement with in general low abundances of anammox bacteria in the natural environment, where they make only a small fraction of the total bacterial community, ranging from <1 to 3% in oxygen minimum zones of Namibia and Peru (Kuypers et al., 2005; Hamersley et al., 2007) and about 2 to 8% in different marine sediments (Schmid et al., 2007). However, despite their low abundance they have been reported to contribute significantly to N_2 production in these environments.

Anammox bacteria are known to be sensitive to oxygen and their metabolism is inhibited by oxygen concentrations as low as 1 µM (Strous et al., 1997). However, Kuypers et al. (2005) found anammox activity in waters of the Namibian OMZ where oxygen concentrations were up to 9 µM. In our study, the C_{20}-[3]-ladderane monoether-PC lipid was detected in sediments where oxygen was still at 50% of air-saturation. In the Irish Sea (Fig. 3d-f) highest concentrations of the C_{20}-[3]-ladderane monoether-PC lipid were found within the upper oxygenated cm of the sediment. Possible explanations could be that anammox bacteria present at higher oxygen levels are in a dormant state, or thriving in anaerobic microniches within the sediment attached to aggregates as suggested by Kuypers et al. (2005) and Woebken et al. (2007). Furthermore, macrofaunal activity (bioturbation) or physical mixing (reworking) of sediments can lead to alternating oxic and anoxic conditions in the sediment, especially in coastal and continental margin sediments (Aller et al., 2004). In general, anammox in slope sediments of the Celtic Sea seems to be active at deeper sediment depths compared to the Irish Sea, where the intact C_{20}-[3]-ladderane monoether-PC lipid was most abundant in the upper two cm of the sediment. In terms of mean ladderane lipid abundances calculated per site the data indicate that the importance of anammox increases in deeper slope sediments (Fig. 5). The mean anammox N_2 gas production rates measured in intact sediment cores from the same sites were highest in the Irish Sea shelf sediments but contributed only 33% the total N_2 gas production (Trimmer and Nicholls, 2009). In the slope sites of the Celtic Sea, however, total N_2 production was in general lower compared to the Irish Sea but the contribution of anammox increased to values up to 65% of total N_2 production. Interestingly, we find a significant correlation (R^2=0.957) between ladderane core lipids and anammox activity, as reported by Trimmer and

Nicholls (2009) for intact sediment cores from the same sites, over the whole transect from shelf to slope sediments, while for the C_{20}-[3]-ladderane monoether-PC lipid the correlation is less significant (R^2=0.464) (Fig. 7). These data strongly support the results of our lipid concentration data and indicate that ladderane lipids are suitable markers for anammox activity in sediments.

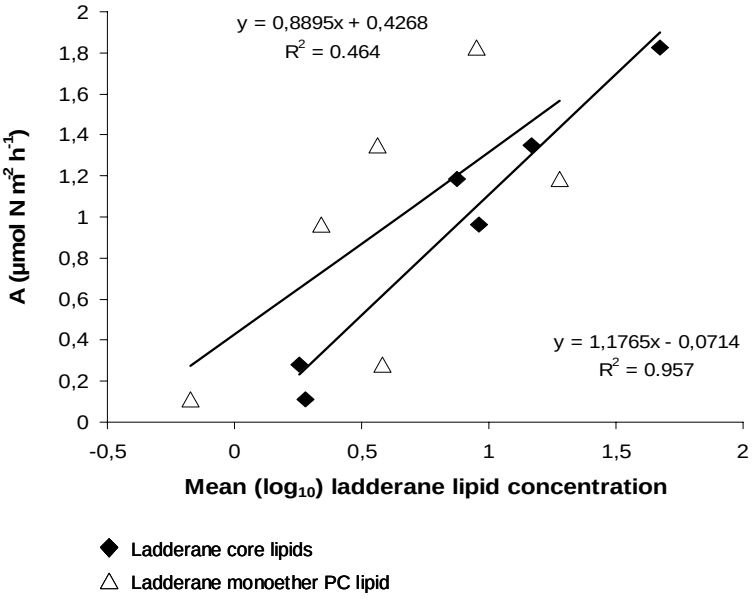


Figure 7. Relation between anammox activity A (from Trimmer and Nicholls, 2009) and mean concentrations of ladderane core lipids (filled diamond) and the C_{20}-[3]-ladderane monoether-PC lipid (open triangle) in whole sediment cores at six sites along a transect from the shelf (Irish Sea) and slope (Celtic Sea).

Most studies on anammox activity so far focused on surface sediment slurries of relatively shallow shelf sediments (Dalsgaard and Thamdrup, 2002; Engström et al., 2005). Our data show that more detailed down-core studies are needed to better assess the location of anammox activity in marine sediments, especially in offshore areas where anammox bacteria are active at greater sediment depths. Our study suggests that anammox, besides traditional denitrification, is also an important process removing nitrogen from marine sediments deposited on continental margins and especially on deeper slope sediments, and should be considered in further investigations of the marine sedimentary nitrogen budget.

4.5 Conclusions

Ladderane lipids have been applied for the detection of anammox bacteria in sediments along a transect of the continental shelf (Irish Sea) and slope (Celtic Sea). The relatively fast degradation of the C_{20}-[3]-ladderane monoether-PC lipid with sediment depth, and the good agreement with ^{15}N tracer experiments indicates its applicability as a marker for living anammox cells or at least "fresh" membrane remnants. Ladderane core lipids are derived from both living and dead anammox cells and reveal a generally higher preservation potential in the sediment. The mean ladderane lipid abundances calculated per site significantly correlate with anammox activity, corroborating their applicability as suitable markers for anammox.

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Chapter 5

Anaerobic ammonium oxidation (anammox) in continental shelf and slope sediments off northwest Africa

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Abstract

Anaerobic ammonium oxidation (anammox) has been found to be an important process in the marine and freshwater nitrogen cycle converting fixed inorganic nitrogen into gaseous N_2. In this study the presence and abundance of anammox bacteria were investigated in continental shelf and slope sediments (ca. 300-3000 m water depth) off northwest Africa in a combined approach applying quantitative polymerase chain reaction (q-PCR) analysis of anammox specific 16S rRNA genes and anammox specific ladderane biomarker lipids. We used the presence of an intact ladderane phospholipid as a direct indicator for living anammox bacteria and compared it with the abundance of ladderane core lipids derived from both living and dead bacterial biomass. All investigated sediments contained ladderane lipids, both intact and core lipids, in agreement with the presence of anammox-specific 16S rRNA gene copies, indicating that anammox is present at all sites. Concentrations of ladderane core lipids in core top sediments varied between 0.3 and 97 ng/g sediment with highest concentrations detected at the sites located on the shelf at shallower water depths between 300 and 500 m. In contrast, the C_{20}-[3]-ladderane monoether-PC lipid was most abundant in a core top sediment from 1500 m water depth. Both anammox specific 16S rRNA gene copy numbers and the concentration of the C_{20}-[3]-ladderane monoether-PC lipid increased

downcore in sediments located at greater water depths showing highest concentrations of $1.2*10^8$ copies/g sediment and 30 pg/g sediment, respectively, at the deepest station of 3000 m water depth. This suggests that the abundance of anammox bacteria is higher in sediments at intermediate to deep water depths were carbon mineralisation rates are lower.

5.1 Introduction

Recent studies have indicated that anammox, the anaerobic ammonium oxidation to dinitrogen gas with nitrite as electron acceptor, is a key process in the global marine nitrogen cycle (Arrigo, 2005; Hulth et al., 2005) constituting a novel route to remove fixed inorganic nitrogen which was so far attributed to heterotrophic denitrification. This process has been detected in various environments, i.e. anoxic water columns (Dalsgaard et al., 2003; Kuypers et al., 2003), marine and estuarine sediments (Dalsgaard et al., 2002; Trimmer et al., 2003), freshwater lakes (Schubert et al., 2006), terrestrial hot springs (Jaeschke et al., 2008 in press) and also polar marine sediments and sea ice (Rysgaard et al., 2004a; Rysgaard et al., 2004b). In the oxygen minimum zones (OMZ) off Namibia (Kuypers et al., 2005), Chile (Thamdrup et al., 2006) and Peru (Hamersley et al., 2007) anammox was shown to be the dominant N_2 production pathway, while denitrification was minor or even absent. In marine sediments the relative contribution of anammox to total N_2 production measured by ^{15}N isotope pairing, ranged from a few percent to ca. 70%, and was originally suggested to be correlated with sediment remineralization rate and water depth (Dalsgaard et al., 2002; Engström et al., 2005; Thamdrup et al., 2002). Schmid et al. (2007) and Penton et al. (2006) found evidence for anammox in a wide range of anoxic ecosystems covering arctic, temperate and tropical shelf sediments. Little, however, is known about the occurrence and distribution of anammox with sediment depth or along continental slopes or even in the deep sea.

The anammox process is so far linked to one group of organisms forming a distinct phylogenetic group within the *Planctomycetes* (Schmid et al., 2007; Strous et al., 1999). The group of anammox bacteria is currently associated to at least four genera, *Candidatus* 'Brocadia', *Candidatus* 'Kuenenia', *Candidatus* 'Anammoxoglobus' and *Candidatus* 'Scalindua' (Kartal et al., 2007; Schmid et al., 2007; Strous et al., 1999). So far, available 16S rRNA gene sequences from marine environments were found to be

closely related to *Candidatus* 'Scalindua sp.' (Kuypers et al., 2003; Kuypers et al., 2005; Schmid et al., 2007; Schubert et al., 2006), while in terrestrial hot springs they were closely related to *Candidatus* 'Brocadia' and *Candidatus* 'Kuenenia' (Jaeschke et al., 2008 in press). Anammox bacteria contain a separated intracytoplasmic compartment called the anammoxosome, where anammox catabolism was shown to take place (Lindsay et al., 2001; van Niftrik et al., 2008; Van Niftrik et al., 2004). The membrane of this "organelle" consists of unusual ladderane lipids forming a dense barrier which reduces the permeability of the membrane to small molecules, e.g. the toxic intermediate of the anammox reaction, hydrazine, which can easily permeate less dense bacterial membranes (Sinninghe Damsté et al., 2002). The ladderane core lipid contains three or five linearly concatenated cyclobutane rings either ester or ether bound to the glycerol backbone, which is unprecedented in nature.

Ladderane core lipids have been applied as biomarkers for anammox activity in anoxic waters of the Black Sea (Kuypers et al., 2003) as well as in oxygen minimum zones (OMZ) off Namibia (Kuypers et al., 2005), Peru (Hamersley et al., 2007) and the Arabian Sea (Jaeschke et al., 2007). However, these lipids may not necessarily indicate the presence of active anammox bacteria but may also be derived from fossil biomass because ladderane core lipids do not occur as such in anammox cell membranes but are derived from intact ladderane lipids containing polar headgroups. Recently, Boumann et al. (2006) and Rattray et al. (2008) have shown that that intact ladderane lipids comprise phosphocholine (PC), phosphoethanolamine (PE) or phosphoglycerol (PG) as the major headgroups attached to the glycerol backbone. Intact phospholipids are due to their ionic nature more soluble to water and less resistant to enzymatic cleavage, therefore being rapidly decomposed after cell death, i.e. by loosing their polar headgroup (Harvey et al., 1986; White et al., 1979). Intact phospholipids are thought to be suitable biomarkers for the study of living microbial communities (Sturt et al., 2004). Indeed, Jaeschke et al. (unpubl. results) showed that a specific ladderane C_{20}-[3]-monoether lipid with a PC-headgroup is a potentially suitable indicator for active anammox bacteria in marine sediments. However, comparison of this new marker for the detection of anammox bacteria with other molecular markers like real-time PCR (qPCR) or fluorescence in situ hybridization (FISH) was missing.

In this study we investigated anammox in marine sediments off northwest Africa derived from a large range of water depths varying between ca. 300 and 3000 m

using different molecular techniques. To distinguish between living anammox cells and their dead remnants, we compared abundances of an intact C_{20}-[3]-ladderane monoether-PC lipid versus ladderane core lipids with sediment depth using different high performance liquid chromatography (HPLC)-tandem mass spectrometry (MS/MS) techniques. Furthermore, q-PCR was used to quantify the amount 16S rRNA gene copies of anammox bacteria in different environments (Hamersley et al., 2007; Schmid et al., 2005) and compared with the lipid results.

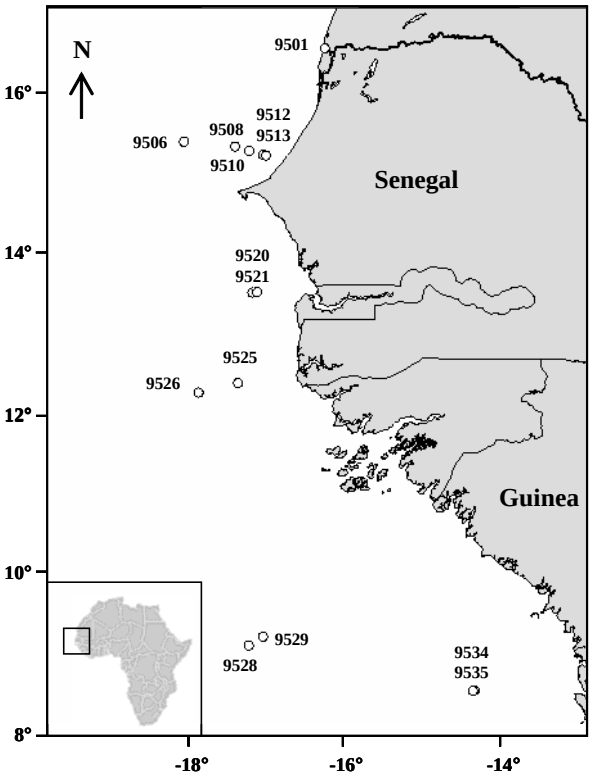

Figure 1. Location of sediment cores investigated off northwest Africa.

5.2 Material and Methods

5.2.1 Study sites and sampling

The sediment cores analysed in this study were collected during R/V *Meteor* cruise M65/1 from the continental shelf and slope off northwest Africa in June 2005 (Mulitza et al., 2006) which is characterized by coastal upwelling of nutrient-rich deep waters to the euphotic zone resulting in high primary productivity. Elevated oxygen

consumption related to high organic matter decomposition is leading to hypoxic conditions at intermediate water depths between 300 and 700 m (Stramma et al., 2008). In total 14 sediment cores were retrieved off Senegal and Guinea (16.5ºN-8.5ºN) from water depths ranging between 300 and 3000 m (Table 1, Fig. 1). The sediments consisted mainly of homogenous greenish muds. All sediment cores were obtained by multicorers. Cores for pore water analysis were transferred to the cooling lab immediately after recovery and processed at a temperature of about 4°C. Cores for lipid and DNA analysis were sectioned into 1 cm slices immediately after recovery and stored deep-frozen until used for analysis.

Table 1. Characteristics of study sites off northwest Africa

Station GeoB	Date 2005	Latitude (°N)	Longitude (°W)	WD (m)	TOC (%)
9501	12.6	16°50'38	16°43'96	330	2,6
9506	14.6	15°36'50	18°20'99	2964	1,2
9508	15.6	15°29'97	17°56'82	2385	2,0
9510	15.6	15°25'00	17°39'23	1567	2,4
9512	16.6	15°20'21	17°21'99	787	2,2
9513	16.6	15°19'10	17°17'70	498	1,3
9520	19.6	13°49'77	17°35'45	1102	1,8
9521	19.6	13°50'87	17°29'43	522	2,3
9525	21.6	12°38'40	17°52'75	2648	2,8
9526	21.6	12°26'12	18°03'37	3223	2,6
9528	23.6	09°09'96	17°39'84	3062	0,9
9529	24.6	09°21'18	17°22'13	1234	1,1
9534	26.6	08°54'04	14°56'14	493	4,2
9535	26.6	08°52'53	14°57'64	666	4,3

TOC was analyzed on sediment core top samples
WD: water depth

5.2.2 Ladderane lipid analysis

5.2.2.1 Ladderane core lipids

Typically ca. 6-20 g of freeze-dried and homogenized sediment were ultrasonically extracted 5x using a dichloromethane (DCM)-methanol mixture (2:1 by volume). The extracts were combined and the bulk of the solvent was removed by rotary evaporation under vacuum, and the extract was further dried over a Na_2SO_4 column. An aliquot of the lipid extract was saponified with aqueous 1 N KOH in methanol for 2 h at 100°C. Non-saponifiable lipids (neutral lipids) were extracted out of the basic solution (pH>13) using DCM. Fatty acids were obtained by acidifying the residue to pH 3 and subsequent extraction with DCM. The fatty acid fraction was methylated by adding diazomethane (CH_2N_2) to convert fatty acids into their corresponding fatty acid methyl esters (FAMEs). The excess CH_2N_2 was removed by evaporation. To remove very polar components, aliquots of the FAMEs were eluted with ethyl acetate over a small column filled with silica. Polyunsaturated fatty acids (PUFAs) were removed by eluting the aliquots with ethyl acetate over a small $AgNO_3$ (5%) impregnated silica column, yielding a saturated fatty acid fraction. An aliquot of the neutral fraction was eluted with DCM/methanol (1:1; v/v) over a small column filled with activated aluminium oxide. Both fatty acid and neutral fractions were dissolved in acetone and then filtered through a 0.45 µm, 4 mm diameter PTFE filter.

These fractions were analyzed by high performance liquid chromatography coupled to positive ion atmospheric pressure chemical ionization tandem mass spectrometry (HPLC/APCI-MS/MS) as described by Hopmans et al. (2006) with some modifications. Specifically, separation was achieved using a Zorbax Eclipse XDB-C_8 column (3.0 x 250 mm, 5 µm; Agilent) and a flow rate of 0.18 ml/min MeOH. The source settings were: vaporizer temperature 475 °C, discharge current 2.5 µA, sheath gas (N_2) pressure 30 (arbitrary units), auxiliary gas (N_2) pressure 5 (arbitrary units), capillary temperature 350°C, source CID -10 V. Argon pressure was maintained at 1.5 mTorr in the second quadrupole. Quantification of ladderane lipids was done by using an external calibration curve using standards of isolated methylated ladderane fatty acids containing the [3]- and [5]- ladderane moieties (Fig.2, structures I-III) and a monoether containing the [3]-ladderane moiety (Fig.2, structure IV) (Sinninghe Damsté et al., 2002; Hopmans et al., 2006). A detection limit (defined by a signal to noise ratio

of 3) of 30-35 pg injected was achieved with this technique. The reproducibility of the concentrations based on duplicate measurements was between 3 and 8%.

The iNdex of Ladderane lipids with 5 cyclobutane rings, an index for the relative chain length of ladderane lipids, was calculated according to Rattray (2008):

$$NL_5 = \frac{C_{20}[5]\,\text{fatty acid}}{(C_{18}[5]\,\text{fatty acid} + C_{20}[5]\,\text{fatty acid}}$$

The NL_5 was then converted to a temperature estimate using the equation of Rattray (2008):

$$NL_5 = 0.2 + \frac{0.7}{1 + e^{-\left(\frac{\text{Temp}-16.0}{1.6}\right)}}$$

The reproducibility of the NL_5 based on duplicate measurements was better than 0.005.

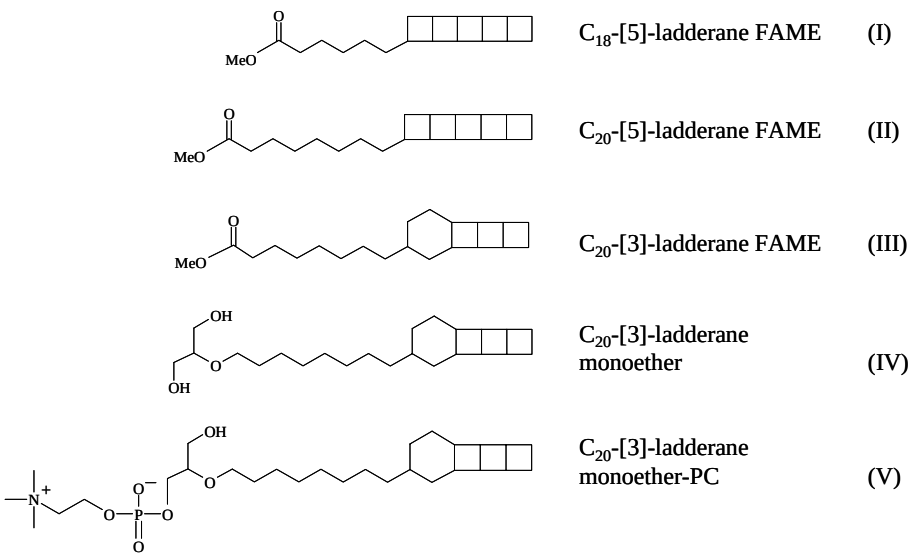

Figure 2. Chemical structures of the ladderane lipids analyzed in this study; (I) C_{18}-[5]-ladderane FAME, (II) C_{20}-[5]-ladderane FAME, (III) C_{20}-[3]-ladderane FAME, (IV) C_{20}-[3]-ladderane monoether, (V) C_{20}-[3]-ladderane monoether-PC. FAME = fatty acid methyl ester.

5.2.2.2 C_{20}-[3]-ladderane monoether-PC lipid analysis

Sediment samples (6-20 g dry weight) were ultrasonically extracted according to the method of Bligh and Dyer (1959) with some modifications using a mixture of methanol, DCM and phosphate buffer at pH 7.4 (2:1:0.8 v/v/v). After sonication for 10 min, further DCM and buffer were added to the mixture to achieve a final methanol/DCM/buffer ratio of 1:1:0.9 (v/v/v). The phases were separated by centrifugation and the extraction repeated three more times. The extracts were combined and the bulk of the solvent was removed by rotary evaporation under vacuum. An aliquot of the extract was dissolved in a DCM/methanol mixture (9:1, v/v) and filtered through a 0.45 µm, 4 mm diameter RC filter.

The C_{20}-[3]-monoether lipid containing a PC headgroup (Fig.2, structure V) was analyzed by HPLC/electrospray ionization (ESI)-MS/MS based on the method described by Boumann et al. (2006) with some modifications. Separation was achieved on a LiChrospher diol column (250 mm x 2.1 mm, 5 µm particles) maintained at 30°C. The following linear gradient was used with a flow rate of 0.2 mL min^{-1}: 90% A:10% B to 70% A: 30% B over 10 min, maintained for 20 min, then to 35% A:65% B in 15 min, maintained for 15 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} ratios 79:20:0.12:0.04 (v/v/v/v) and B = 2-propanol/water/formic acid/14.8 M NH_{3aq} ratios 88:10:0.12:0.04 (v/v/v/v). Detection of the C_{20}-[3]-monoalkylether-PC was achieved by SRM of the transition from *m/z* 530 $[M+H]^+$ to *m/z* 184 (corresponding to the PC-headgroup), with 1.5 mTorr Argon as collision gas and 20V collision energy. Quantification of the intact ladderane monoetherlipid was done by an external calibration curve of an isolated C_{20}-[3]-ladderane monoether-PC standard (43% purity). A detection limit of 10 pg injected was achieved with this technique. The reproducibility based on duplicate measurements was better than 12%.

5.2.3 DNA extraction and Q-PCR

Total DNA from sediment cores GeoB 9501, 9506, 9510 and 9513 was extracted from ca. 0.25-0.33 g sediment sample using the PowerSoil™ DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, USA). An aliquot of the total DNA extract was subjected to agarose gel electrophoresis to determine DNA quality and quantity. All

extracts contained mainly higher molecular weight DNA (~20 kbp fragments) at a concentration of >10 ng/µl.

Undiluted, as well as 5 and 25 times diluted (in order to check for inhibition) DNA extracts were subjected to quantitative PCR (q-PCR) analysis. As inhibition was not observed, the data from the undiluted samples were used for q-PCR.

Realtime PCR amplification was performed using the 16S rRNA gene specific primer set Brod541F (5'-GAGCACGTAGGTGGGTTTGT-3') -Brod1260R (5'-GGATTCGCTTCACCTCTCGG-3') for detecting anammox bacteria in sediments according to Penton et al. (2006). Reaction mixtures (20 µl) contained 1 unit of Picomaxx^{TM} high-fidelity DNA polymerase, 2 µl of 10x Picomaxx PCR buffer (both Stratagene), 0.25 mM of each dNTP, 8 µg of BSA, 0.25 µM of each primer, 50,000 times diluted SYBRgreen (Molecular Probes), a final concentration of 10 nM fluorescein, 3 mM of $MgCl_2$ and ultra pure sterile water (Sigma). All reactions involved initial denaturing (4 min at 95°C), followed by 35-40 cycles including denaturing (30 s at 94°C), 40 s of primer annealing of 62°C, a fotomoment at 80°C for 25 s and primer extension (60 s at 72°C). Real time PCR was performed in triplet measurements per sample using an iCycler (Biorad, Hercules, California). Calibration of the samples was performed by using a dilution series of the 719 bp purified PCR product, ranging from 10^7 to 10^1 copies/ reaction, of a culture of *Candidatus* Scalindua wagneri were used as qPCR standards. Realtime PCR was followed by meltcurve analysis and gel electrophoresis to check for the presence of a-specific products such as primer dimers, which were both not detected.

5.2.4 Pore water and sediment chemistry

Multicores were processed in a glove box under argon atmosphere at about 4°C. The sampling resolution was 0.25-1 cm. Pore water was extracted by pressure filtration using Teflon squeezers that were operated with argon at a pressure gradually increasing up to 4 bars. The pore water was retrieved through 0.2 µm cellulose acetate membrane filters. Ammonium and nitrate concentrations were determined on board immediately after sampling using a segmented flow auto analyzer and standard colorimetric techniques. Nitrite was not analyzed, therefore, nitrate was used instead.

Freeze-dried and powdered surface sediment (0-1 cm) samples were decalcified with dilute (2N) HCl, rinsed with demineralized water to remove $CaCl_2$ and freeze-dried again. Total organic carbon (TOC) was measured against a benzoic acid laboratory standard (C = 68.80%) on a Carlo Erba Flash elemental analyzer coupled to a Thermofinnigan Delta^{plus} mass spectrometer. The reproducibility based on duplicate measurements was better than 0.2%.

5.3 Results

5.3.1 Ladderane core lipid analysis

A set of 14 sediment core tops derived from different water depths (ca. 300-3000 m) off northwest Africa was analyzed for ladderane core lipids (Fig. 1). In all samples ladderane core lipids were detected (Fig. 3a). Their relative distribution was similar in all samples, i.e. it was dominated by the C_{18}-[5]-ladderane FAME (I), followed by the C_{20}-[3]-ladderane monoether (IV), being 1.5-3.5 times lower in concentration, and the C_{20}-[3]- and C_{20}-[5]-ladderane FAMEs (II and III), both 2-8 times lower in concentration. Total ladderane lipid abundances varied between 0.3 and 97 ng/g sediment (Fig. 3a).

In addition to the surface sediments, four sediment cores from the Mauritania mudbelt and along the continental slope off Senegal were analyzed to study the ladderane core lipid distribution with sediment depth (Table 2, Fig. 4). Highest concentrations of the ladderane core lipids up to 139 ng/g sediment were detected at the shallowest station GeoB9501 (water depth 330 m). At station GeoB9513 (water depth 500 m) ladderane lipid concentrations are 70 ng/g sediment at the surface, then decreased further downcore to values of 48 ng/g sediment at 5 cm depth (Fig. 4b). At station GeoB9510 (water depth 1500 m) concentrations of ladderanes increased from 49 ng/g sediment in the surface to 70 ng/g at 2 cm depth, and then decreased further downcore to values of 21 ng/g sediment at 5 cm depth (Fig. 4c). At the deepest station GeoB9506 (water depth 3000 m) ladderane concentrations increased from about 4 ng/g sediment in the surface to 39 ng/g sediment at 4 cm depth (Fig. 4d).

Anammox bacteria have been found to alter their membrane composition in response to temperature changes, i.e. an increase in the amount of shorter chain ladderane fatty acids relative to the amount of longer chain fatty acids at lower temperatures and *vice versa* (Rattray, 2008). The NL_5 ratio (see Material and Methods

section) has been proposed as a means to quantify this relative change. The NL_5 of the ladderane lipids in the surface sediments and the sediment cores was generally low varying between 0.11 and 0.22 in surface sediments, suggesting they were synthesized at temperatures below 10°C (Rattray , 2008). Hardly any variation in the NL_5 was observed with sediment depth (Table2, Fig. 4).

Table 2. Field stations, ladderane lipids, 16S rRNA copy numbers and nutrients

		Ladderane core lipids (ng/g sediment)								Nutrients	
Station	depth	C18-[5]	C20-[5]	C20-[3]	C20-[3]	sum	ladderane	PC-mono	16S rRNA	NH4+	NO3-
GeoB	(cm)	FA	FA	FA	mono		index	(pg/g sed)	(copies/g sed)	µmol/l	µmol/l
9501	0,5	37,2	10,4	5,7	41,7	95,0	0,218	4,0	1,20E+07	12,6	15,6
	1,5	42,6	14,5	14,4	67,2	138,7	0,254	1,1	1,08E+07	38,9	5,5
	2,5	32,6	10,7	6,4	52,3	101,9	0,247	0,9	8,88E+06	54,2	6,7
	3,5	28,1	8,1	4,6	25,9	66,6	0,223	0,2	1,20E+07	58,2	1,7
	4,5	27,4	8,4	5,1	54,9	95,8	0,235	0,0	6,73E+06	58,3	1,1
9513	0,5	41,7	9,0	7,3	12,4	70,4	0,178	7,6	8,34E+06	22,5	25,8
	1,5	32,2	6,7	5,3	33,9	78,1	0,172	3,3	4,05E+07	37,5	2,0
	2,5	21,7	4,9	3,7	30,5	60,8	0,184	1,5	3,58E+07	19,5	0,0
	3,5	8,0	1,8	1,4	36,9	48,1	0,187	3,1	9,22E+06	19,8	0,8
	4,5	14,5	3,6	2,7	27,1	47,9	0,197	3,7	2,47E+07	26,3	0,6
9510	0,5	19,2	2,5	3,5	5,4	30,6	0,116	19,5	6,25E+07	3,7	25,9
	1,5	27,3	4,3	6,3	32,1	70,0	0,136	12,9	6,96E+07	8,4	4,1
	2,5	26,2	3,4	4,5	30,8	65,0	0,116	4,1	8,36E+07	25,4	0,5
	3,5	12,6	2,1	2,9	15,6	33,2	0,144	1,8	8,21E+07	29,0	0,3
	4,5	7,5	1,1	1,5	11,1	21,2	0,129	0,0	2,25E+07	42,9	0,0
9506	0,5	1,5	0,2	0,3	2,3	4,4	0,138	6,5	1,21E+07	8,0	33,6
	1,5	5,3	0,8	1,1	5,6	12,8	0,128	10,4	2,13E+07	7,7	35,7
	2,5	9,1	1,6	2,5	12,4	25,6	0,146	29,5	1,03E+08	7,4	18,3
	3,5	16,8	2,6	4,4	15,4	39,2	0,133	3,8	1,23E+08	7,2	5,3
	4,5	12,6	1,4	2,2	17,0	33,2	0,101	2,9	7,50E+07	7,0	0,9

FA: fatty acid

5.3.2 C_{20}-[3]-ladderane monoether-PC lipid analysis

Eight core tops (Table 1) were analyzed for the C_{20}-[3]-ladderane monoether-PC lipid. The compound was detected in all core tops and its concentration varied between 4 and 19 pg/g sediment (Fig. 3b). The concentration of the ladderane C_{20}-[3]-ladderane monoether-PC lipid was also determined in four sediment cores derived from the Mauritania mudbelt and along the continental slope off Senegal (Table 2, Fig. 4). Concentrations were ranging between <1 and and 30 pg/g sediment. Lowest

concentrations of <10 pg/g sediment were detected at stations GeoB9501 and GeoB9513 located at 330 and 500 m water depth (Table 2, Fig. 4a, b). The two deeper sites GeoB9510 and GeoB9506 located at 1500 and 3000 m water depth along the slope revealed higher concentrations of the C_{20}-[3]-ladderane monoether-PC lipid with maximum values of 20 and 30 pg/g sediment (Table2, Fig. 4b, c).

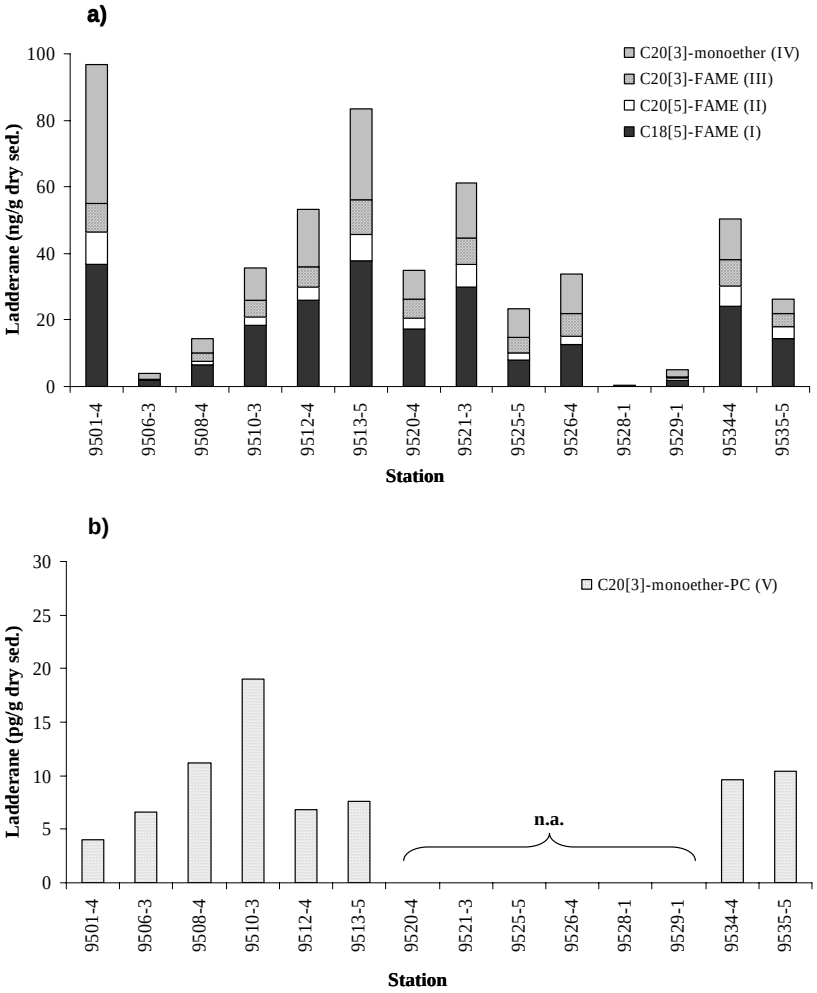

Figure 3. a) concentrations and distribution of ladderane core lipids in surface sediments from different water depths offshore Senegal and Guinea, b) concentration of the C_{20}-[3]-ladderane monoether-PC in selected surface sediments from different water depths offshore Senegal and Guinea.

5.3.3 Q-PCR analysis

Q-PCR analysis using a primer set selective for anammox bacteria was performed for the four sediment cores which were also analyzed for ladderane lipids. 16S rRNA gene copy numbers ranged between $6.7*10^6$ to $1.2*10^8$ (Table2). As observed with the C_{20}-[3]-ladderane monoether-PC lipid concentrations, lowest 16S rRNA gene copy numbers at the two shallow sites GeoB9501 and GeoB9513 (Table 2, Fig. 4a, b), while the two sites located at deeper water depths revealed 16S rRNA gene copy numbers that were about 1-1.5 orders of magnitudes higher (Table 2, Fig. 4c, d).

5.3.4 Pore water and sediment chemistry

Pore water ammonium and nitrate concentrations were analyzed from four sediment cores derived from the Mauritania mudbelt and along the continental slope off Senegal (Table 2, Fig. 4). Nutrient concentrations in the pore water ranged between 4 and 58 µmol/l for ammonium and between 1 and 36 µmol/l for nitrate. Ammonium concentrations were highest at the shallowest station GeoB9501 (58 µmol/l), and decreased at the sites further downslope with increasing water depth where concentrations were only 7-8 µmol/l at the deepest site, while nitrate concentrations of 36 µmol/l were highest at the deepest station GeoB9506 (Table 2, Fig. 4).

5.4 Discussion

5.4.1 Spatial distribution and origin of ladderane lipids

The detection of ladderane core lipids in all surface sediments indicate that anammox is occurring in shelf and also slope sediments offshore northwest Africa up to water depths of ca. 3000 m. The distribution of ladderane core lipids reveals a distinct pattern with, in general, higher abundances in sediments located at shallower water depths and closer distance to the coast (Fig. 3a, Fig. 5a). In contrast, the concentrations of the C_{20}-[3]-ladderane monoether-PC lipid, which is a marker for living anammox biomass, is highest in the surface sediment at a water depth of 1500 m while it is lower at deeper and shallower water depths (Fig. 5c). The distribution of ladderane core lipids and the intact C_{20}-[3]-ladderane monoether-PC lipid in core top sediments thus show different patterns (Fig. 3). Especially at station GeoB 9501, where highest concentrations of the core lipids were detected, the C_{20}-[3]-ladderane monoether-PC lipid is relatively low in concentration.

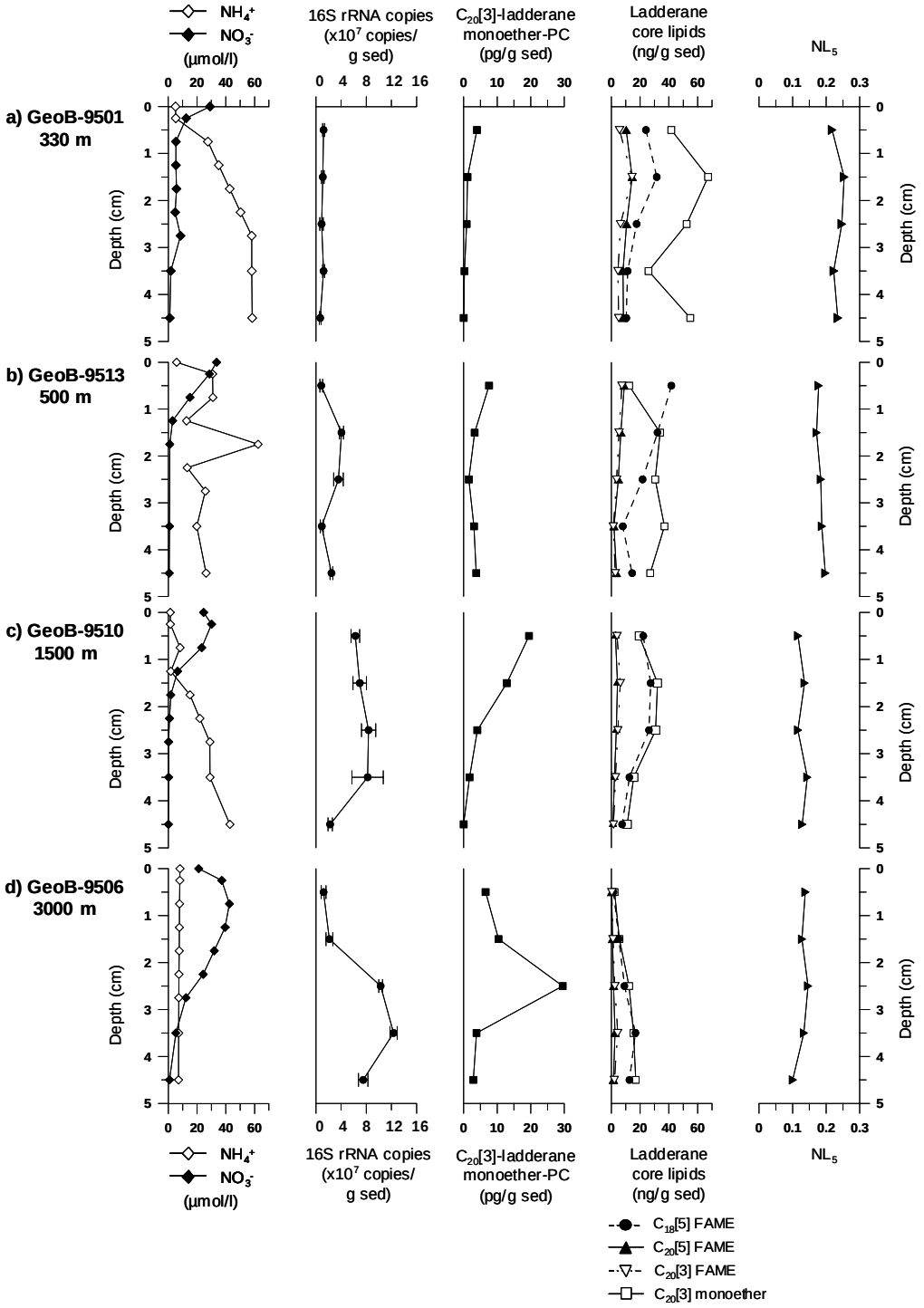
a) GeoB-9501
330 m
b) GeoB-9513
500 m
c) GeoB-9510
1500 m
d) GeoB-9506
3000 m
Depth (cm)
NH4+
NO3-
(µmol/l)
16S rRNA copies
(x10^7 copies/
g sed)
C20[3]-ladderane
monoether-PC
(pg/g sed)
Ladderane
core lipids
(ng/g sed)
NL5
C18[5] FAME
C20[5] FAME
C20[3] FAME
C20[3] monoether

Figure 4. Nutrient pore water profiles, 16S rRNA copy numbers and distribution of anammox specific ladderane membrane lipids in sediments from the mudbelt off Mauritania at 330 m water depth (a), and along the continental slope off Senegal at water depths of 500, 1500 and 3000 m, respectively (b-d). The first column shows pore water concentration profiles of NH_4^+ and NO_3^-. The second column shows the number of 16S rRNA gene copies of anammox bacteria. The third column shows the concentration of the C_{20}-[3]-ladderane monoether-PC. The fourth column shows the concentration of four different ladderane core lipids. The fifth column shows the NL_5, a temperature index based on the ratio of ladderane lipids with different chain length (see text). FAME = fatty acid methyl ester.

This could indicate that some of the ladderane core lipids detected in the sediments might actually have originated from dead anammox bacterial biomass settling from the overlying OMZ. Ladderane lipids are known to be produced in OMZ offshore Peru, in the Arabian Sea and in the Benguela upwelling area (Hamersley et al., 2007; Jaeschke et al., 2007; Kuypers et al., 2005), and ladderane core lipids have been found in settling particles in the Arabian Sea (Jaeschke et al., 2007). The OMZ off northwest Africa is less pronounced (hypoxic conditions) than these OMZs although its intensity has increased over the past 50 years (Stramma et al., 2008).

One way of evaluating the origin of ladderane lipids is to examine their relative distribution. Anammox bacteria have been found to adjust their membrane composition with temperature, i.e. an increase of the chain length of the ladderane fatty acids (C_{20}-[5]-ladderane FAME compared to the C_{18}-[5]-ladderane FAME) with higher growth temperature has been noted (Rattray, 2008). The NL_5 index, which quantifies these changes, has low values ranging from 0.11 to 0.22 in the surface sediments with values slightly decreasing with increasing water depth (Fig. 6). These values suggest that the sedimentary ladderanes were synthesized at temperatures below 10°C (Rattray, 2008). In contrast, ladderane lipids produced only in the overlying OMZ with temperatures of 14-15°C would possess slightly higher NL_5 values of ca. 0.3-0.4 as indicated by the dashed line in Fig. 6. CTD derived bottom water temperatures were between 2.5 and 12°C. The NL_5 thus indicates that anammox is most probably an *in situ* process in the sediment. However, this does not exclude that part of the ladderane lipids are derived from anammox bacteria in the OMZ nor that anammox may not be an important process in the OMZ. Part of the settling ladderane lipids might have been degraded during transport through the oxic water column, especially at greater water depths. Analysis of

suspended particulate matter or sediment trap material is needed to provide further insights in the occurrence of anammox in the water column.

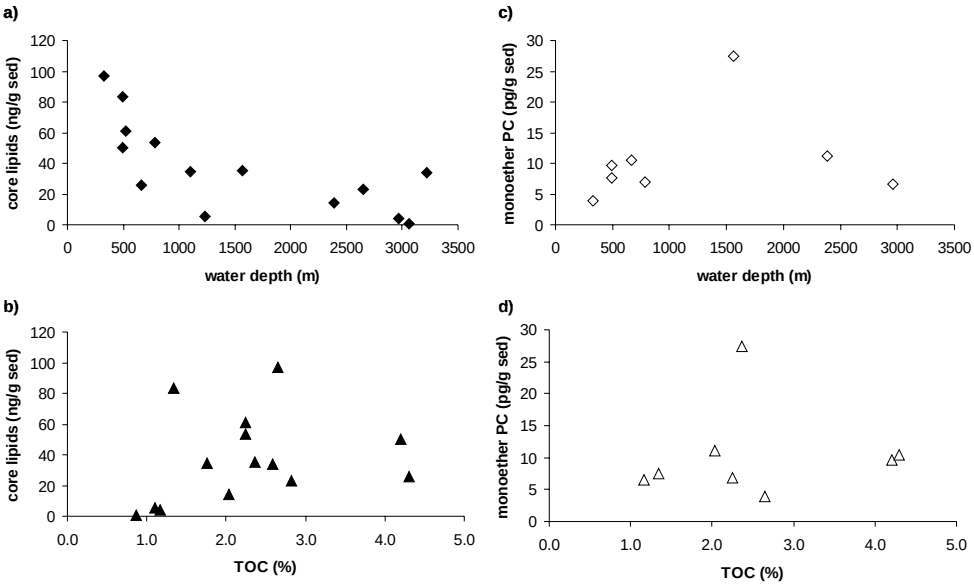

Figure 5. Summed ladderane core lipid and C_{20}-[3]-ladderane monoether-PC concentrations in surface sediments in relation to water column depth (a and c, respectively) and TOC (b and d, respectively).

Factors controlling the abundance of ladderane lipids in sediments, and thus anammox productivity, might be the organic matter flux coming from the overlying high productivity waters and reaching these sediments, the availability of nitrate, nitrite and ammonium in the sediment and bottom water, the depth of the water column, and the oxygen content of overlying waters. Water depth and sediment mineralization are generally related because with a deeper water column a larger fraction of the organic matter is remineralized during transport to the sediment resulting in a lower organic load of the sediment. The TOC content of the sediments, however, seems not to be related to the ladderane lipid concentration and highest ladderane concentrations were detected at intermediate TOC concentrations between 2% and 3% (Fig. 5b). Similarly, the highest concentration of the C_{20}-[3]-ladderane monoether-PC lipid is found at an

intermediate TOC content of ca. 2.5% while in general the concentrations are not related to TOC contents which vary between 1 and 4% (Fig. 5d).

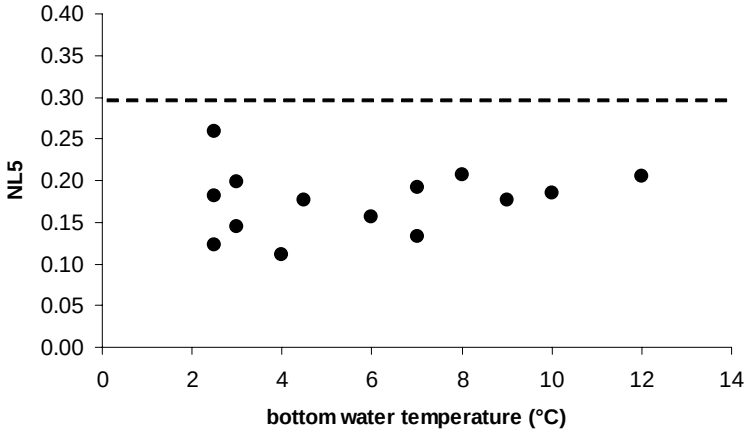

Figure 6. NL_5 calculated for surface sediments in relation to bottom water temperatures. The dotted line indicates NL_5 values expected from oxygen minimum zones derived ladderane lipids.

5.4.2 Distribution of anammox with sediment depth

The depth profiles investigated at the three stations along the continental slope off Senegal show decreasing ladderane core lipid concentrations with increasing water depth (Figs. 4b-d). While the stations GeoB9501 and GeoB9513 do not show much variation in ladderane concentration with sediment depth (Figs. 4a-b), it decreases at station GeoB9510 below 4 cm depth (Fig. 4c), and at station GeoB9506 it increases continuously with sediment depth (Fig. 4d). Apparent differences, however, can be seen in the distribution of the core lipid versus the intact lipid in terms of total abundances and depth. The concentration of the intact C_{20}-[3]-monoether lipid containing a PC headgroup, is in general 3-4 magnitudes lower than the corresponding monoether core lipid. This large difference can be explained by inferring that the intact ladderane PC monoether may represent the actual zone of anammox activity and rapidly degrades with sediment depth. Moreover, the core ladderane monoether comprises also, although less abundant, phosphoglycerol (PG) and phosphoethanolamine (PE) headgroups, (Boumann et al., 2006; Rattray et al., 2008) which we were not able to quantify with our current method. Strikingly, the concentration of the intact ladderane monoether increases with increasing water depth, showing the opposite trend to the ladderane core lipids. The different patterns of ladderane core lipids compared to the intact C_{20}-[3]-

ladderane monoether can be explained by the fact that ladderane core lipids are derived from both living and dead anammox cells in the sediment and in the overlying OMZ. Ladderane core lipids have a higher preservation potential, resulting in much higher concentrations compared to intact ladderanes, and therefore accumulate in the sediment.

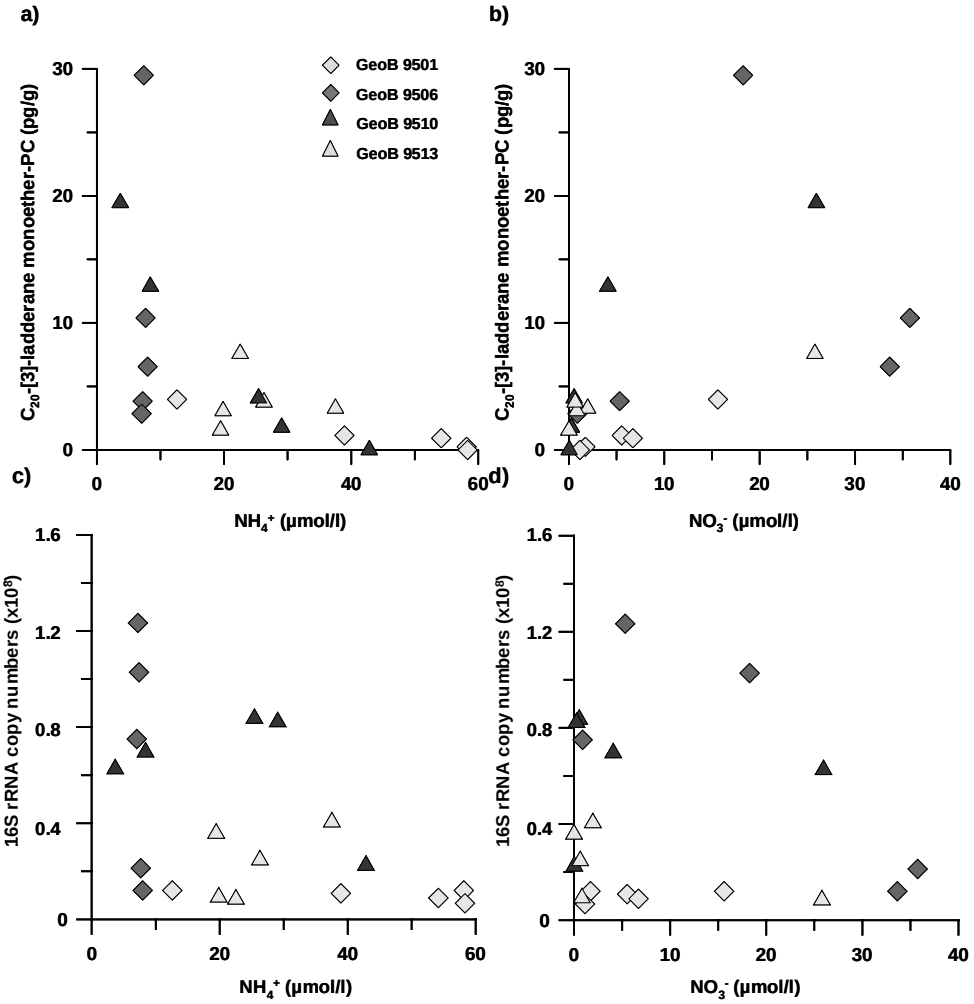

Figure 7. C_{20}-[3]-ladderane monoether-PC concentrations and anammox specific 16S rRNA copy numbers in relation to pore water NH_4^+ (a and c, respectively) and NO_3^- concentrations (b and d, respectively) from sediment cores GeoB9501, 9506, 9510, and 9513, respectively.

The 16S rRNA gene copy numbers of anammox bacteria in the sediment also increase with increasing water depth, and also show an increase with sediment depth at the stations located at deeper water depths, similar to what is observed with the intact C_{20}-

[3]-ladderane monoether lipid. Both are used as markers for the detection of anammox bacteria. Differences, however, can be seen in the depth distribution of these two markers (Fig. 4). From the 16S rRNA gene copy numbers and the intact C_{20}-[3]-ladderane monoether lipid data it is apparent that with increasing water depth the zone of living anammox bacteria moves deeper into the sediment. This increase in 16S rRNA gene copy numbers of anammox bacteria and intact C_{20}-[3]-ladderane monoether lipid with sediment depth at the stations located at deeper water depths, correlates with the fact that the penetration depth of nitrate, and probably oxygen as well, also increases continuously with increasing water depth, most distinct at the deepest station GeoB9506 (Fig. 4d). Earlier studies by Dalsgaard and Thamdrup (2002) and Engström et al. (2005) showed the relative importance of anammox (compared to denitrification) to be independent of pore water nitrate concentrations, but was mainly influenced by carbon mineralization rates in the sediment. Ammonium, initially released to pore water during early diagenesis, seems to be non-linearly related to the concentration of the intact C_{20}-[3]-ladderane monoether-PC lipid (Fig. 7a) and also, although less distinctive, to 16S rRNA gene copy numbers of anammox bacteria (Fig. 7c) at stations GeoB 9501, 9506, 9610, and 9513. Engström et al. (2005) reported a similar relation between the relative contribution of anammox to total N_2 production and pore water ammonium, where the relative importance of anammox was found to be highest at the least reactive site. Both, the concentration of the intact C_{20}-[3]-ladderane monoether-PC lipid and the 16S rRNA gene copy numbers of anammox bacteria seem not to be related to pore water nitrate concentrations (Fig. 7b, d). Our data indicate that the importance of anammox in removing nitrogen from sediments seems to be highest at greater water depths where organic matter mineralization is lower, and is in agreement with what was found earlier by Thamdrup and Dalsgaard (2002). This becomes even more obvious regarding increasing C_{20}-[3]-ladderane monoether-PC lipid concentrations and 16S rRNA copy numbers in whole sediments cores along the depth transects, further indicating the increasing importance of anammox at greater water depths (Fig. 8a, b). Ladderane core lipid concentrations along the same transect, however, reveal the opposite pattern, strongly indicating a higher contribution of water column derived ladderane lipids at shallower water depths and closer to the coast (Fig. 8c).

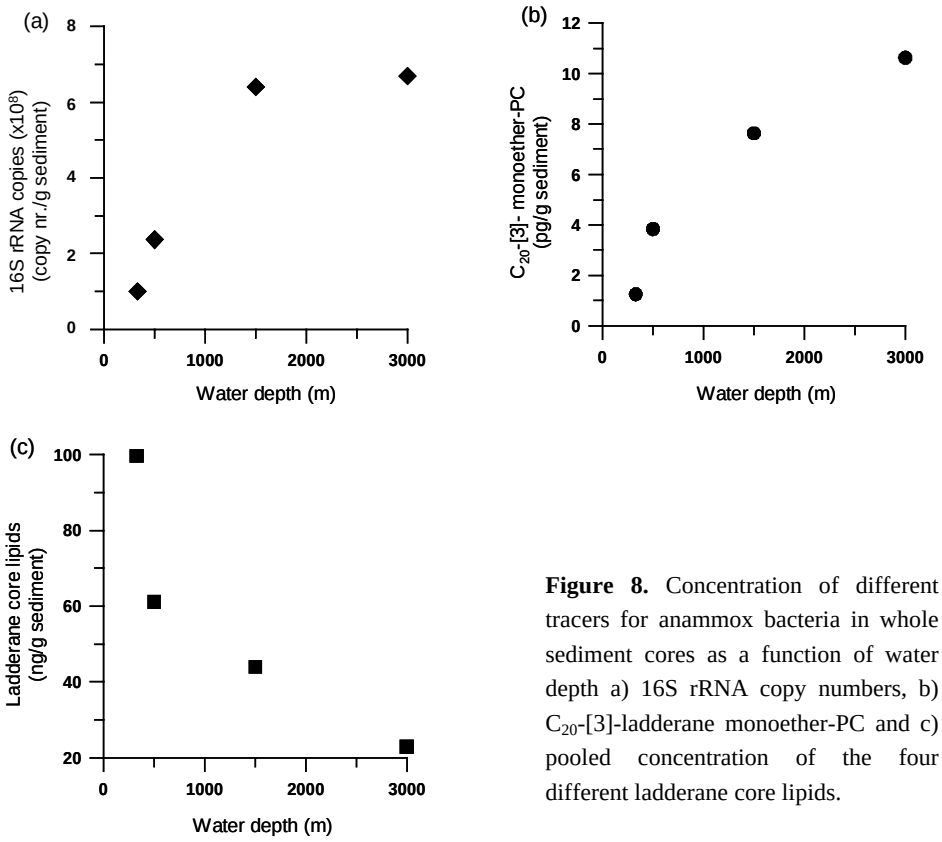

Figure 8. Concentration of different tracers for anammox bacteria in whole sediment cores as a function of water depth a) 16S rRNA copy numbers, b) C_{20}-[3]-ladderane monoether-PC and c) pooled concentration of the four different ladderane core lipids.

5.5 Conclusions

Our combined approach using molecular microbial and geochemical methods showed that anammox is ubiquitous in continental shelf and slope sediments off Senegal and Guinea. Although anammox in the OMZ off northwest Africa might have contributed to some part of the ladderane core lipid pool in the sediment, especially at shallower water depths and closer to the coast, the distribution of ladderane lipids (as reflected by the NL_5 index) indicates that they predominantly are derived from sedimentary anammox bacteria. Both, anammox specific 16S rRNA gene copy numbers and the intact C_{20}-[3]-ladderane monoether lipid indicate that anammox bacteria are more abundant in sediments located at greater water depths and also at greater sediment depths where carbon mineralisation rates are generally lower.

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Chapter 6

An experimental study on the biodegradation of ladderane lipids

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Abstract

Anammox, the microbial anaerobic oxidation of ammonium with nitrite to dinitrogen gas, has been recognized as a main process both in the marine and freshwater nitrogen cycle, and found to be a major sink for fixed inorganic nitrogen in the oceans. Ladderane lipids are unique anammox bacterial membrane lipids that have been used as a marker for anammox bacteria in different recent environmental settings. However, the fate of ladderane lipids during diagenesis is as yet unknown. In this study, we performed oxic degradation experiments with anammox bacterial biomass at temperatures ranging from 20 to 100°C to simulate early diagenetic processes occurring at the sediment-water interface as well as thermal degradation during subsequent sediment burial. Abundances of ladderane lipids decreased with increasing experimental temperatures leaving <10% of the original lipids at 100°C compared to the starting material, testifying to the labile nature of ladderane lipids. The most abundant products formed were ladderane lipids with a shorter alkyl chain (C_{14} to C_{16} ladderane fatty acids), most pronounced at 40°C. This suggests that, at relatively low temperatures, ladderane lipids are microbially degraded via a beta-oxidation pathway. An HPLC-MS/MS method was developed for the detection of these ladderane alteration products in environmental samples and tested on various sediments. This showed that these specific ladderane products formed during degradation experiments are also present in the marine environment. Thus, short-chain ladderane lipids may, in addition to the

regular longer chain ladderane lipids, be suitable biomarkers for the detection of the anammox process in past depositional environments.

6.1 Introduction

Organic matter (OM) settling from the surface of the ocean through the water column to the sediment as well as OM produced *in situ* in the sediment is subjected to a range of biological, physical and chemical processes. More than 90% of OM is remineralized already in the water column and during early diagenesis near the sediment-water interface leaving typically only <0.1% of the OM produced in the photic zone to be preserved in sediments (Henrichs, 1992; Wakeham et al., 2002). The oxygen exposure time and the oxygen concentration in the water column and in the sediment pore water plays a crucial role in the extent of OM degradation (Hedges and Keil, 1995; Burdige, 2007). The preservation of organic compounds in sediments depends both on their intrinsic reactivity toward enzymatic degradation and the degree to which the molecules are physically protected by association with a resistant mineral or organic matrix (Canuel and Martens, 1996; Burdige, 2007). Once incorporated into the sediments, lipids can undergo further structural alteration by biotic and abiotic processes. Depending on structure, packaging and oxygen levels, alteration of individual biomarker lipids takes place at different rates, e.g., preferential degradation of shorter chain n-alkanes (Johnson and Calder, 1973), autoxidation of alkenones (Rontani et al., 2006), and unsaturated compounds are generally considered more susceptible to degradation than saturated compounds (Sun and Wakeham, 1994; Hoefs et al., 1998; 2002; Sinninghe Damsté et al., 2002a).

Lipids provide much information as markers for specific source organisms as well as tracers for diagenetic processes. Ladderane lipids (Sinninghe Damsté et al., 2002b) are membrane lipids specific for a group of organisms belonging to the *Planctomycetes* that perform anaerobic ammonium oxidation to dinitrogen gas with nitrite as the electron acceptor (anammox) (Van de Graaf et al., 1995; Strous et al., 1999). Ladderane lipids consist of either three or five linearly concatenated cyclobutane rings (Fig. 1) and form a dense and highly impermeable membrane surrounding the anammoxosome, an intracellular compartment where the anammox reaction takes place (Lindsay et al., 2001; Van Niftrik et al., 2004). Such a dense membrane is thought to be required to maintain concentration gradients during the exceptionally slow anabolism of

anammox bacteria and to protect the remainder of the cell from the highly toxic intermediate hydrazine (Sinninghe Damsté et al., 2002b). Since its discovery about a decade ago in a wastewater treatment system (Mulder et al., 1995), many studies have shown that anammox bacteria are ubiquitous in the marine environment and that the process constitutes a substantial sink for fixed inorganic nitrogen in the oceans (Dalsgaard and Thamdrup, 2002; Kuypers et al., 2005; Thamdrup et al., 2006; Hamersley et al., 2007), which before was solely attributed to heterotrophic denitrification. Ladderane lipids have been detected in the environment (Kuypers et al., 2005; Hamersley et al., 2007) and found to be exported through the water column (Jaeschke et al., 2007). However, the effect of oxic degradation on ladderane lipids occurring during transport through the water column and after deposition on the seafloor is presently unknown.

In this study, we investigated the effect of oxic and thermal degradation by incubating anammox cell material with sediment from a tidal flat area and water at temperatures between 20 and 100°C for 72 hours under oxic conditions. We monitored the degradation of ladderane lipids and analyzed the products formed during these experiments in order to evaluate potential early diagenetic processes of ladderanes which may occur in the natural environment.

6.2 Material and methods

6.2.1 Experimental setup

An anammox enrichment culture grown over 5 months in sequencing batch reactors (SBRs) as described by Strous et al. (1998), contained two species of anammox bacteria: *Candidatus* Kuenenia stuttgartiensis and *Candidatus* Brocadia fulgida. The suspension of enrichment culture biomass and medium obtained from the SBRs was centrifuged and the supernatant was removed. The cell material was freeze-dried and stored at -20°C until further use. For each degradation experiment approximately 20-30 mg of anammox cell material was thoroughly mixed with 1.5-2 g freeze-dried sediment derived from the Wadden Sea, a tidal flat area situated close to NIOZ, and placed in open flasks. Ca. 1-2 ml of bi-distilled water was added to obtain a thick slurry, so that the cell material could be well dispersed throughout the experiments. Samples were incubated for 72 h at temperatures of 20, 40, 50, 60, 70, 80 and 100°C, respectively, and gently stirred to maintain aeration during the experiments. Afterwards, the supernatant

was removed and the residue was freeze-dried again. Another experiment was performed with Anammox biomass at 40°C for 72 h using sediment sterilized (autoclaved for 20 min at 121°C) prior to use.

6.2.2 Extraction and fractionation

Samples from the degradation experiments were ultrasonically extracted five times using a dichloromethane (DCM)/methanol mixture (2:1 v/v). Combined extracts were dried using rotary evaporation yielding the total lipid extract (TLE). An aliquot of the lipid extract was saponified with aqueous 1 N KOH in methanol for 2 h at 100°C. Non-saponifiable lipids (neutral lipids) were extracted out of the basic solution (pH>13) using DCM. Fatty acids were obtained by acidifying the residue to pH 3 and subsequent extraction with DCM. The fatty acid fraction was methylated by adding diazomethane (CH_2N_2) to convert fatty acids into their corresponding fatty acid methyl esters (FAMEs). To remove very polar components, aliquots of the FAMEs were eluted with ethyl acetate over a small column filled with silica. Extracts derived from sediments were further eluted with DCM over a small silver nitrate ($AgNO_3$)-impregnated (5%) silica column to remove polyunsaturated fatty acids, yielding a saturated fatty acid fraction. For high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) analysis, an aliquot of the methylated TLE was dissolved in acetone and filtered through a 0.45 µm, 4 mm diameter PTFE filter. For quantification, a known amount of the internal standard (*ante iso* C_{22} alkane) in ethyl acetate was added prior to GC analysis.

6.2.3 GC and GC/MS analysis

GC analysis was performed using a Hewlett-Packard 6890 instrument equipped with an on-column injector and a flame ionization detector (FID). A fused silica capillary column (25 m x 0.32 mm) coated with CP Sil-5 (film thickness 0.12 µm) was used with helium as carrier gas. The samples were injected at 70°C. The GC oven temperature was subsequently raised to 130°C at a rate of 20°C/min, and then at 4°C/min to 320°C, which was held for 15 min. GC/MS analysis was carried out using a Finnigan Trace GC Ultra, Thermo Electron Corporation, interfaced with a Finnigan Trace DSQ mass spectrometer, using a mass range of *m/z* 40-800. GC conditions for GC/MS were the same as those described above for GC. The compounds formed during

thermal destruction of the ladderane lipids during GC analysis were identified according to retention times and mass spectra described by Sinninghe Damsté et al. (2005).

6.2.4 HPLC/APCI-MS/MS analysis

An aliquot of the methylated TLE was analyzed for ladderanes by high performance liquid chromatography coupled to positive ion atmospheric pressure chemical ionization tandem mass spectrometry (HPLC/APCI-MS/MS) in selective reaction monitoring (SRM) mode as described by Hopmans et al. (2006) with some modifications described by Rattray et al. (2008) and Jaeschke et al. (2008). Specifically, separation was achieved using a Zorbax Eclipse XDB-C_8 column (3.0 x 250 mm, 5 µm; Agilent) and a flow rate of 0.18 ml/min MeOH. The source settings were: vaporizer temperature 475 °C, discharge current 2.5 µA, sheath gas (N_2) pressure 30 (arbitrary units), auxiliary gas (N_2) pressure 5 (arbitrary units), capillary temperature 350°C, source CID -10 V. Argon pressure was maintained at 1.5 mTorr in the second quadrupole.

Table 1. Protonated molecules and selected product ions for the SRM detection of short-chain ladderane fatty acids.

Ladderane lipid alteration product	$[M+H]^+$ *m/z*	Product* *m/z*
Compound I	233.2	117.1
		131.1
		159.1
		173.1
Compound II	235.2	119.1
		133.1
		161.1
		175.1
Compound III	263.2	93.5
		121.3
		135.2
		171.1

*Collision energy was set at 25 V for all transitions monitored.

In order to detect ladderane fatty acid methyl esters with shorter chain lengths as produced during the degradation experiments (Fig. 1), the enrichment culture biomass extract from the 40°C experiment was analysed by MS^2 in data-dependant mode with three scan events, where a positive ion scan (*m/z* 200-400) was followed by a product ion scan of the base peak of the mass spectrum of the first scan event and the second most abundant ion (collision energy 25 V, collision gas (argon), 0.8 mTorr). Based on the results of this experiment, the method published by Hopmans et al. (2006) was modified to include SRM transitions diagnostic for the concatenated cyclobutane moieties (Table 1). For these experiments chromatographic and APCI source conditions were as described above.

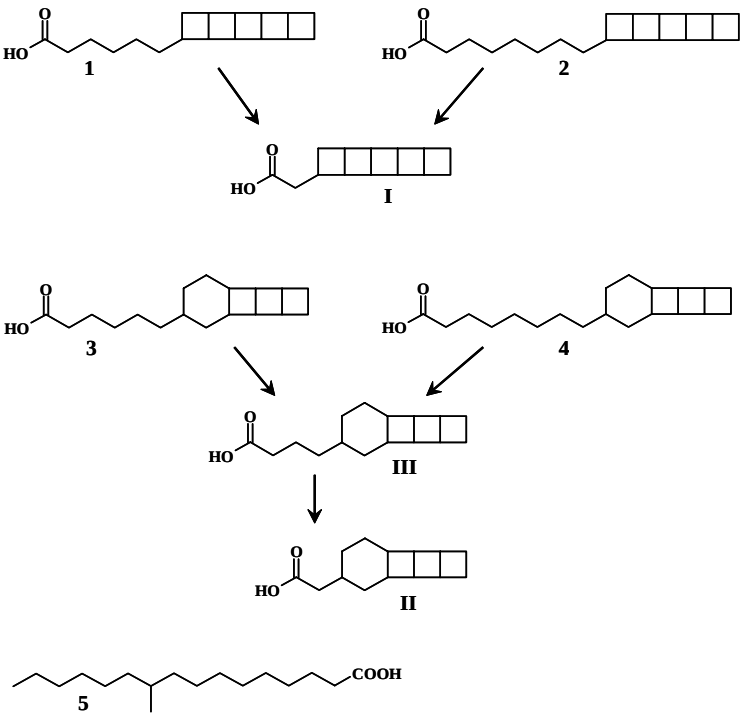

Figure 1. Structures of ladderane lipids derived from anammox enrichment cultures and their transformation products derived from microbial oxic degradation. 1) C_{18}-[5]-ladderane FA, 2) C_{20}-[5]-ladderane FA, 3) C_{18}-[3]-ladderane FA, 4) C_{20}-[3]-ladderane FA, I) C_{14}-[5]-ladderane FA, II) C_{14}-[3]-ladderane FA, III) C_{16}-[3]-ladderane FA. 5) 10-methylhexadecanoic acid. Key: FA=fatty acid.

6.3 Results and discussion

6.3.1 Degradation experiments

To investigate the effect of oxygen and temperature on the preservation of ladderane lipids, aliquots of biomass of an anammox enrichment culture containing *Candidatus* Kuenenia stuttgartiensis and *Candidatus* Brocadia fulgida were mixed with surface sediment derived from a tidal flat area and incubated in open flasks at temperatures ranging from 20 to 100°C for three days. Ladderane lipids (as analyzed with GC) decreased in concentration with increasing experimental temperature (Fig. 2, Fig. 3a). Compared to the original unheated sample, ladderane lipids incubated at 20°C were already 2-4 times lower in concentration, and then slowly further decreased with increasing temperatures. At 100°C ladderane lipids were still detectable but decreased further in concentration leaving only <10% of the original lipids. The ladderane moiety which has been found to be heat labile (Sinninghe Damsté et al., 2005) and forming thermally more stable products at hydrous pyrolysis temperatures of 120°C onwards (Jaeschke et al., 2008), however, was still intact at 100°C.

Several new components were detected with GC-MS (Fig. 2b, Fig. 3b). I and II (see Fig. 1 for structures) were present at temperatures between 20 and 70°C, while III was only detected in the 40°C experiment. The highest concentrations of the three components were found at a temperature of 40°C (Fig. 3b). GC-MS analysis revealed mass spectra for I-III similar to those of known C_{18}-C_{20} ladderane fatty acids (Sinninghe Damsté et al., 2005), i.e. loss of the alkyl chain resulting in fragments m/z 131, 132, 152 and 160, 161, but with different molecular ions, i.e., *m/z* 232, *m/z* 234, and *m/z* 262 for I, II, and III, respectively (Fig. 4). The molecular ion of I is 84 Da less than that of the C_{20}-[5]-ladderane fatty acid (**2**) or 56 Da less than that of the C_{18}-[5]-ladderane fatty acid (**1**), suggesting I is a C_{14}-[5]-ladderane fatty acid. The molecular ion of II is 84 Da less than that of the C_{20}-[3]-ladderane fatty acid (**4**) or 56 Da less than that of the C_{18}-[3]-ladderane fatty acid (**3**), suggesting compound II is a C_{14}-[3]-ladderane fatty acid. Finally, the molecular ion of III is 56 Da less than that of the C_{20}-[3]-ladderane fatty acid (**4**) or 28 Da less than that of the C_{18}-[3]-ladderane fatty acid (**3**), suggesting that III is a C_{16}-[3]-ladderane fatty acid. These structural identifications suggest that these fatty acids are formed by oxidation of the alkyl chain of the original ladderane fatty acids.

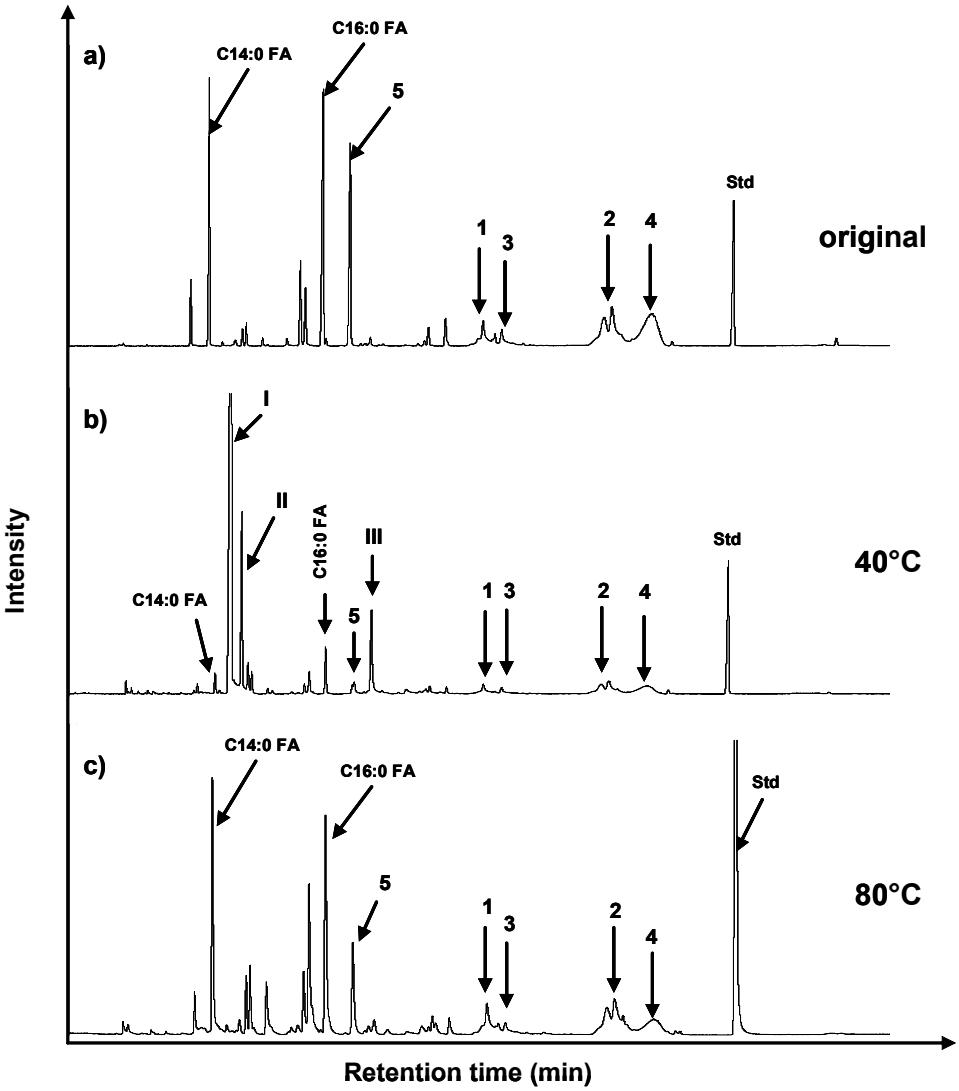

Figure 2. Gas chromatograms of the fatty acid fractions of a) anammox biomass and anammox biomass incubated for three days at b) 40°C, c) 80°C. Numbers correspond to structures shown in Fig. 1.

The question arises whether these compounds are formed abiotically or due to microbial activity. The fact that ladderane lipids with shorter alkyl chains occur only at temperatures between 20 and 70°C, and have maximum concentrations at 40°C, strongly suggested microbial degradation. In a control experiment performed with sterilized sediment and anammox biomass under the same conditions (40°C, 72h),

neither of the new ladderane fatty acids were detected. Indeed, the process of oxidation of the alkyl side chain of cyclic compounds has been shown before during microbial degradation.

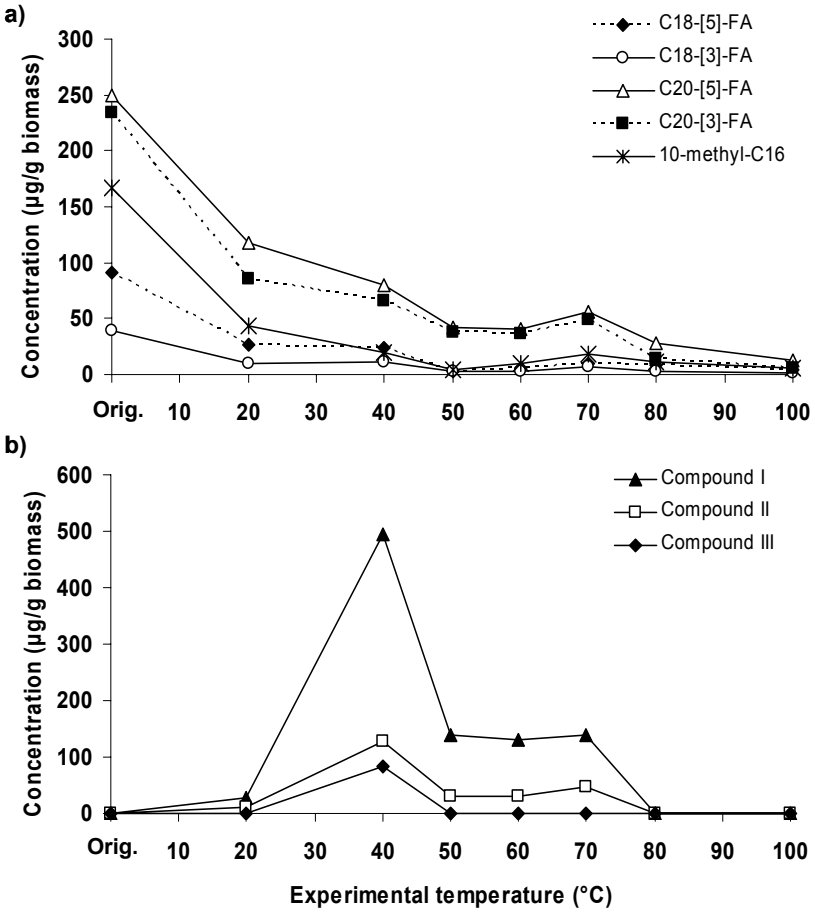

Figure 3. Concentration of a) known ladderane lipids and the 10-methylhexadecanoic acid, and b) short-chain ladderane transformation products as a function of temperature in the fatty acid fractions after base hydrolysis and analyzed by GC.

For example, several microorganisms are able to degrade *n*-alkylcyclohexanes by an alkyl side chain oxidation pathway, the so-called β-oxidation pathway (Beam and Perry, 1974; Dutta and Harayama, 2001; Koma et al., 2003). This pathway includes four reactions that occur in repeated cycles with each compound. In each cycle, the alkyl chain is progressively shortened by two carbon atoms as it is oxidized.

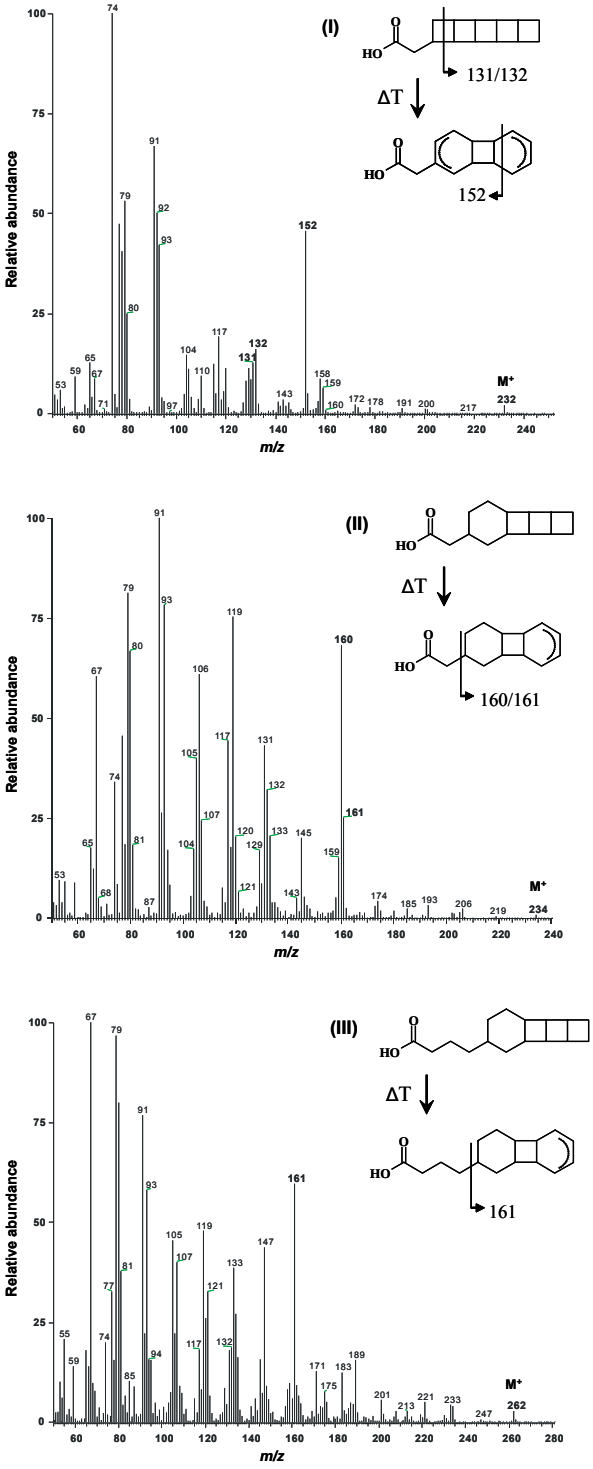

Figure 4. GC/MS spectra of the short-chain ladderane fatty acids I, II, and III. It should be noted that the mass spectra reflect the thermal degradation products formed during GC analysis as discussed by Sinninghe Damsté et al. (2005).

Depending on whether there is an odd or even number of carbon atoms in the alkyl side chain, carboxylic or acetic acid derivatives are formed, which cannot be further oxidized by β-oxidation and typically accumulate. A similar degradation pathway is likely for the ladderane lipids as their chemical structure also comprises a cyclic part and is, in that respect, comparable to that of *n*-alkylcyclohexanes. Therefore, we suggest that the novel ladderane fatty acids (I, II, and III) detected in the 20, 40, 50, 60, and 70°C experiments are derived from longer chain ladderane fatty acids (**1**-**4**) and formed via the β-oxidation route (Dutta and Harayama, 2001) as shown in Fig. 1. II could be derived from III by further oxidation of the alkyl side chain by two carbon atoms during ongoing degradation. The C_{16}-[3]-ladderane fatty acid with ($[M+H]^+$) of 263 Da was reported earlier by Rattray (2008), who found this compound in an anammox enrichment culture suggesting that this shorter-chain fatty acid may be synthesized as an adaptation to lower cultivation temperatures. Since this lipid was not present in the starting biomass, our results, however, indicate that the shorter chain ladderane fatty acids were products of microbial oxidation.

6.3.2 Environmental occurrence of short chain ladderane fatty acids

To investigate whether the products formed in our degradation experiments are also formed in natural environments, we aimed to analyze a number of sediments for the presence of short-chain ladderane fatty acids. However, the low abundances of ladderane lipids in environmental samples which are in the range of picograms to nanograms l^{-1} or g^{-1} sediment (Hopmans et al., 2006; Hamersley et al., 2007; Jaeschke et al., 2007) make them difficult to detect with GC/MS. To enhance the sensitivity of detection of these short-chain ladderane fatty acids, we modified the HPLC/APCI-MS/MS method recently developed for the detection of ladderane lipids in the natural environment by Hopmans et al. (2006) in order to identify the short-chain ladderane lipids formed during the degradation experiments. We first analyzed the ladderane products formed at 40°C with a data dependant scan experiment (Fig. 5b) which showed, besides the regular ladderane fatty acids, two additional major peaks The peak with a retention time of ca. 8 min. actually consists of two co-eluting compounds with protonated molecules ($[M+H]^+$) of 233 Th and 235 Th, respectively, while the peak with a retention time of ca. 9 min represents a compound with a protonated molecule at *m/z* 236. The APCI-MS/MS spectra of the protonated molecules of these ladderane lipid alteration products show similar product ions to those reported for the longer chain

ladderane fatty acids by Hopmans et al. (2006) (Fig. 6a-c). Thus, these peaks likely represent the C_{14} and C_{16} shorter chain ladderane fatty acids.

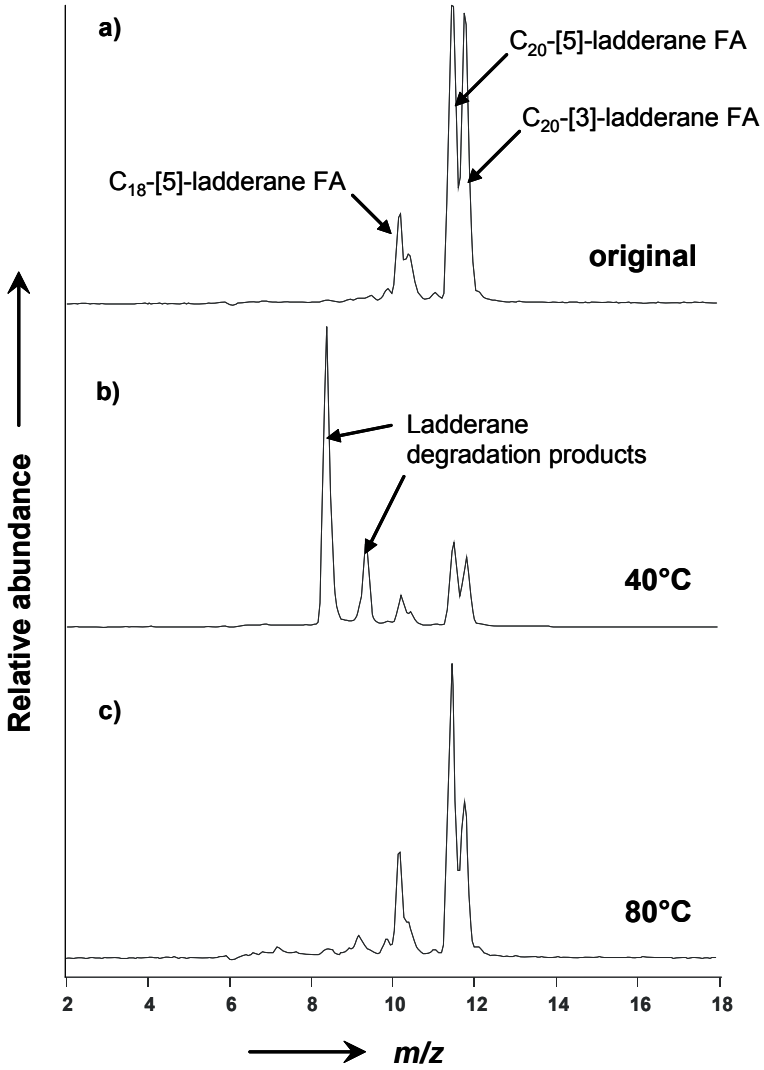

Figure 5. HPLC chromatogram of the fatty acid fraction (analyzed as their methyl ester derivatives) in anammox enrichment cultures a) original, and heated for three days at b) 40°C, and at c) 80°C.

To detect very small amounts of these compounds, we developed a selective reaction monitoring (SRM) method using four specific fragmentations per ladderane

lipid (cf. Hopmans et al., 2006). The selection of product ions for SRM was based on their diagnostic value for the concatenated cyclobutane moieties of the ladderane molecules as well as their high relative abundances in the mass spectra (Fig. 6, Table 1).

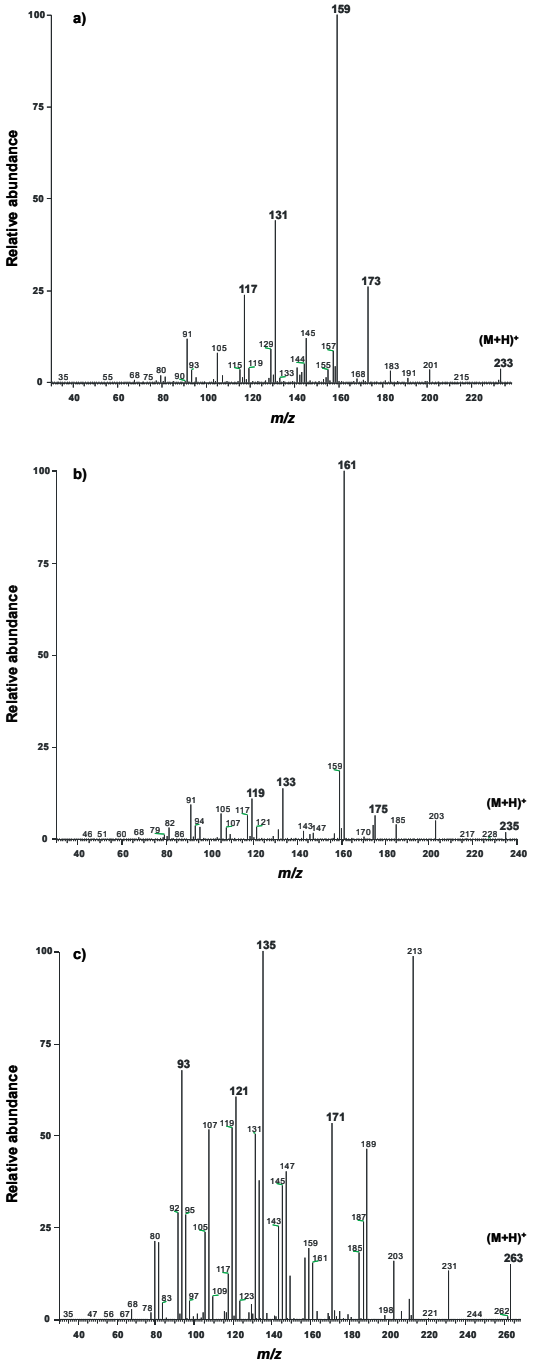

Figure 6. APCI-MS/MS spectra of the protonated molecular ions of three different ladderane lipid transformation products a) compound I (*m/z* 233), b) compound II (*m/z* 235), c) compound III (*m/z* 263). Structures of the original lipids and the alteration products are depicted in Fig. 1.

To test this modified method, we first applied it to the cell material of anammox bacteria, heated at 40°C, where the ladderane alteration compounds were initially detected (Fig. 7a).

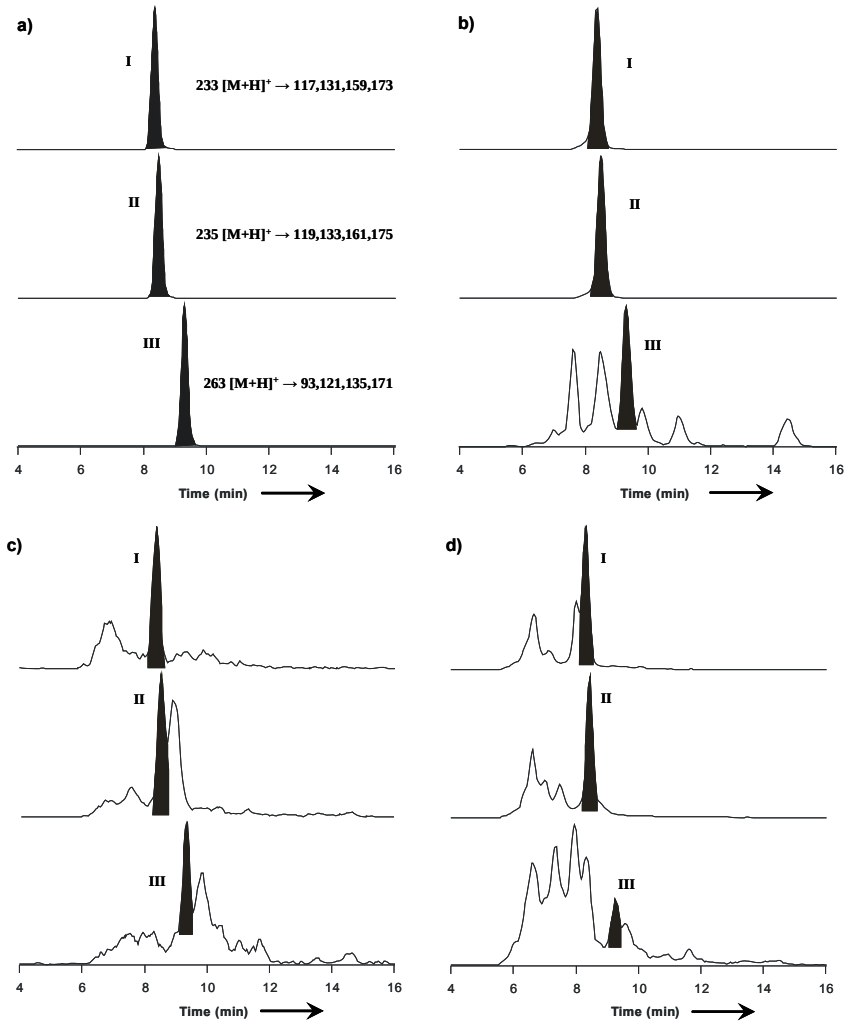

Figure 7. SRM traces of short-chain ladderane fatty acids in a) anammox biomass heated at 40°C for three days, and in sediments from b) the Arabian Sea, c) the Irish Sea and d) offshore northwest Africa.

I was most abundant, followed by II being one order of magnitude lower in abundance, while III, which was only detected in the sample heated at 40°C, was about 1.5 orders of magnitude lower in abundance. The method was then used to analyze sediments derived

from various locations, i.e., the Arabian Sea, Irish Sea and off northwest Africa where intact anammox lipids have previously been detected (Jaeschke et al., unpublished results). As shown in the partial SRM traces in Fig. 7b, c and d, the ladderane lipid degradation products with shorter chain lengths could indeed be detected with the modified HPLC/APCI-MS/MS method in all three sediments analyzed. In these sediments, II was the most abundant, followed by I in about 1.5-2 times lower abundance, while III was about two orders of magnitude lower in abundance. The occurrence of these short-chain ladderane alteration products in marine sediments suggests that ladderane lipids are degraded in the natural environment in a way similar to our simulated degradation experiments, i.e., via a microbially mediated β-oxidation pathway. Thus, the short-chain ladderane fatty acids I-III may be useful biomarkers for anammox bacteria, especially in sediments underlying oxygen minimum zones (OMZ) where ladderane lipids are re-exposed to oxygen after production in the OMZ and sedimentation.

6.4 Conclusions

The results of our laboratory studies suggest that under oxic conditions ladderane lipids are microbially degraded via the β-oxidation pathway where the alkyl side chain is oxidized resulting in shorter chain ladderane lipids. The detection of these biodegraded ladderane lipids in marine sediments using a modified HPLC/APCI-MS/MS method indicates that these compounds are also produced in the natural environment.

Acknowledgements

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Chapter 7

Thermal stability of ladderane lipids as determined by hydrous pyrolysis

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Abstract

Anaerobic ammonium oxidation (anammox) has been recognized as a major process resulting in loss of fixed inorganic nitrogen in the marine environment. Ladderane lipids, membrane lipids unique to anammox bacteria, have been used as markers for the detection of anammox in marine settings. However, the fate of ladderane lipids after sediment burial and maturation is unknown. In this study, anammox bacterial cell material was artificially matured by hydrous pyrolysis at constant temperatures ranging from 120 to 365 °C for 72 h to study the stability of ladderane lipids during progressive dia- and catagenesis. HPLC-MS/MS analysis revealed that structural alterations of ladderane lipids already occurred at 120 °C. At temperatures >140 °C, ladderane lipids were absent and only more thermally stable products could be detected, i.e., ladderane derivatives in which some of the cyclobutane rings were opened. These diagenetic products of ladderane lipids were still detectable up to temperatures of 260 °C using GC-MS. Thus, ladderane lipids are unlikely to occur in ancient sediments and sedimentary rocks, but specific diagenetic products of ladderane lipids will likely be present in sediments and sedimentary rocks of relatively low maturity (i.e., C_{31} hopane 22S/(22S+22R) ratio <0.2 or ββ/(αβ+βα+ββ) ratio of >0.5.

7.1 Introduction

Anammox, the anaerobic oxidation of ammonium coupled to nitrite reduction with N_2 as the end product, is performed by chemolithoautotrophic bacteria identified as a distinct phylogenetic group within the *Planctomycetes* (Strous et al., 1999; Schmid et al., 2007). The anammox process was first detected in a wastewater treatment plant in 1995 (Mulder et al., 1995; Van de Graaf et al., 1995), and later it was also observed to be ubiquitous in the marine environment where it contributed significantly to the loss of inorganic nitrogen from the ocean (Devol, 2003; Kuypers et al., 2005; Hamersley et al., 2007). Anammox bacteria contain a separated intracytoplasmic compartment named the anammoxosome, where anammox catabolism was shown to take place (Sinninghe Damsté et al., 2002; Van Niftrik et al., 2004). The membrane of this "organelle" consists of unusual ladderane lipids forming a dense barrier which is thought to reduce the permeability of the membrane to small molecules, e.g. the toxic intermediate of the anammox reaction, hydrazine, which can easily permeate less dense bacterial membranes (Sinninghe Damsté et al., 2002). Ladderane lipids occur in a variety of different forms either ester or ether bound to the glycerol backbone, and contain three or five linearly fused cyclobutane rings (Fig. 1, structures I-IV), which is unprecedented in nature. Ladderanes have been applied as biomarkers for anammox activity in anoxic waters of the Black Sea (Kuypers et al., 2003), oxygen depleted zones of the ocean (Kuypers et al., 2003; Hamersley et al., 2007; Jaeschke et al., 2007) and in marine sediments (Hopmans et al., 2006).

During periods in the geological past when the world's oceans experienced water column anoxia, so-called oceanic anoxic events (OAEs), anammox has been proposed to be an important process in the marine nitrogen cycle, as a sink for fixed nitrogen (Kuypers et al., 2004). However, this hypothesis has remained untested so far. Fossil remnants of ladderane lipids have recently been found in a sediment core from the Arabian Sea, suggesting that anammox activity occurred over the last glacial cycle (Jaeschke et al., unpublished results). However, it is not known if ladderane lipids can be found in more ancient mature sediments and sedimentary rocks. Due to the highly strained conformation of the cyclobutane moieties, the structures of the ladderanes are probably altered during diagenesis and maturation. Indeed, the ladderane moiety has been found to be thermally labile, and conversion of ladderane lipids into thermally

more stable degradation products has been observed during gas chromatographic analysis (Sinninghe Damsté et al., 2005).

Hydrous pyrolysis is a laboratory technique developed to simulate the natural maturation process by heating organic rich sedimentary rock in a closed reactor in the presence of liquid water at subcritical temperatures (<374 °C) for several days (Lewan et al., 1979; Lewan, 1993). Previous studies have shown that reactions involving biomarkers during hydrous pyrolysis experiments mimic those occurring during natural maturation (e.g., Koopmans et al., 1996). In this study, anammox biomass was artificially matured using hydrous pyrolysis to investigate the thermal stability of ladderane lipids and their possible degradation products, which can potentially be applied as molecular indicators for anammox activity in the geological past.

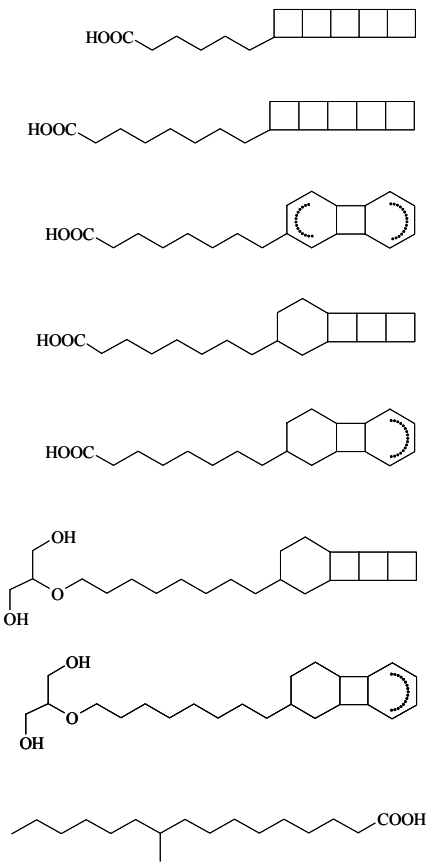

Figure 1. Structures of selected ladderane and other core lipids analyzed in this study. (I) C_{18}-[5]-ladderane fatty acid; (II) C_{20}-[5]-ladderane fatty acid; (II') thermal degradation product of II; (III) C_{20}-[3]-ladderane fatty acid; (III') thermal degradation product of III'; (IV) C_{20}-[3]-ladderane monoether; (IV') thermal degradation product of IV; (V) 10-methyl-hexadecanoic acid.

7.2 Material and methods

7.2.1 Sample description

Anammox cell material was obtained from a wastewater treatment plant of Paques B.V. (Balk, the Netherlands) located at the Waterboard Hollandse Delta (WHSD) in Rotterdam, the Netherlands. Coupled to partial nitrification, anammox bacteria convert ammonium and nitrite to dinitrogen gas in a cost effective and sustainable way. The material was filtered through a 0.5 mm sieve, freeze dried and homogenized. Fluorescence *in-situ* hybridization (FISH) analysis confirmed the dominance of the anammox bacterium *Kuenenia stuttgartiensis* in the reactor (ca. 70% of the bacterial population) (Boumann et al., 2006).

7.2.2 Hydrous pyrolysis

A detailed description of hydrous pyrolysis experiments is given by Lewan (1993). In short, carburized 1 litre Hastelloy-C276 reactors were filled each with 75 g of anammox cell material and 500 g of distilled water. A 1 mm Cr-Ni screen was placed on top of the sample to prevent cell material from floating on the water surface during the loading of the experiment and product collection at the end of the experiment. Sample volume (based on an approximate sample density), reactor volume and amount of added water were calculated to ensure that the sample remains submerged in a liquid water phase throughout the experiment. After leak check, the remaining reactor volume was filled with helium at a pressure of 1.38 bars. Artificial maturation was accomplished by heating aliquots isothermally for 72 h at temperatures from 120 to 365°C (Table 1). The temperatures were continuously monitored during the experiments at 30-s intervals with type J thermocouples. Standard deviations varied between 0.2 and 0.6 °C for the experiments, except for the experiments at 120 and 140 °C, which had standard deviations of 1.2 and 1.3 °C. After the experiments were completed and cooled to room temperatures (18-20 °C) within 24 h, the gas pressure and temperature were recorded and gas samples were collected in 30-50 cm^3 stainless steel cylinders. The remaining gas was vented and the reactor was opened to quantitatively collect the reaction products. Experiments at temperatures ranging between 260 and 350 °C generated an expelled oil, which was recovered from the water surface with a pipette. The reactor walls, reactor head and thermowell were rinsed with benzene to recover any sorbed oil films, which occurred in experiments starting at 200 °C. The water recovered

from the reactor was filtered (0.45 μm) and an aliquot immediately analyzed for pH and Eh at room temperature. The remaining water was kept to analyze water soluble compounds. The remaining cell material (i.e., residue) was removed from the reactor and freeze dried. More than 95 % of the originally loaded material was recovered from all experiments as reacted water, expelled oil, generated gas and anammox residue. The product yields from the hydrous pyrolysis experiments are given in Table 1 with Eh and pH values for the recovered water. The deficit between the recovered products and the original 75 g of starting anammox biomass is attributed to the aqueous products dissolved in the recovered water (i.e., proteins, carbohydrates, calcium and phosphorous), which were not analysed.

Table 1. Analytical data for anammox biomass (75 g) artificially matured by hydrous pyrolysis at a range of different temperatures for 72 hours.

Temp (°C)	pH	Eh (eV)	gas (g)	exp oil (g)	residue (g)	$\delta^{13}C$ (‰)	TOC (wt %)
orig.	5.7	-135	0	0	75.0	-34.1	23.6
120	5.4	63	0.3	0	63.2	-34.9	19.7
140	5.6	-6	0.7	0	57.7	-35.3	15.3
160	6.3	-140	1.5	0	55.8	-35.4	14.5
180	6.9	-197	1.7	0	52.8	-36.1	15.4
200	7.5	-198	2.5	0.1	51.8	-36.0	14.0
220	7.3	-252	2.4	0.2	51.9	-36.3	14.1
240	7.6	-253	2.6	0.9	48.5	-36.3	11.5
260	7.7	-251	2.9	1.4	47.1	-36.4	11.5
280	7.8	-267	2.7	2.7	46.4	-35.8	8.9
300	7.9	-281	2.8	3.2	42.7	-35.4	6.0
320	8.0	-290	3.6	3.9	41.0	-34.8	4.9
335	7.9	-307	5.0	4.1	38.8	-34.5	4.0
350	7.7	-303	5.6	3.2	45.8	-34.0	3.4
365	7.9	-329	6.9	1.7	40.2	-33.7	4.3

7.2.3 Extraction and fractionation

Aliquots of the residue (2-3 g) were repeatedly ultrasonically extracted (5x) with a mixture of dichloromethane (DCM) and methanol (2:1 v:v). The bulk of the

solvent was removed by rotary evaporation under vacuum, and the extract was further dried over a Na_2SO_4 column to obtain the total lipid extract (TLE). The TLE was methylated with boron trifluoride (BF_3) in methanol to convert fatty acids into their corresponding methyl esters (FAMEs). To remove very polar components, the extract was subsequently eluted with ethyl acetate over a silica column. For HPLC-MS/MS analysis, an aliquot of the methylated TLE was dissolved in acetone and filtered through a 0.45-µm, 4-mm-diameter PTFE filter. For gas chromatography (GC), GC-mass spectrometry (MS) and isotope ratio monitoring (irm) GC-MS analyses, another aliquot of the extract was silylated with bis (trimethyl) trifluoroacetamide (BSTFA) in pyridine at 60 °C for 20 min to convert alcohols in trimethylsilyl (TMS) ether derivatives, and the extract was dissolved in ethyl acetate. An aliquot of the separately collected oil that formed during experiments at 200-365 °C, was fractionated by column chromatography with Al_2O_3 into apolar and polar fractions by elution with hexane:DCM (9:1 v:v) and DCM:methanol (1:1 v:v), respectively. For quantification, a known amount of the internal standard (*anteiso*-C_{22} alkane) in ethyl acetate was added after silylation prior to GC analysis.

7.2.4 HPLC/APCI-MS/MS analysis

An aliquot of the methylated TLE was analyzed for ladderanes by high performance liquid chromatography coupled to positive ion atmospheric pressure chemical ionization tandem mass spectrometry (HPLC/APCI-MS/MS) as described by Hopmans et al. (2006) with some modifications. Specifically, separation was achieved using a Zorbax Eclipse XDB-C_8 column (3.0 x 250 mm, 5 µm; Agilent) and a flow rate of 0.18 ml/min MeOH. The source settings were: vaporizer temperature 475 °C, discharge current 2.5 µA, sheath gas (N_2) pressure 30 (arbitrary units), auxiliary gas (N_2) pressure 5 (arbitrary units), capillary temperature 350 °C, and source CID -10 V. Argon pressure was maintained at 1.5 mTorr in the second quadrupole. Ladderane lipids were quantified using external standard curves of the C_{20}-[5]- and C_{20}-[3]-fatty acids (Fig. 1, structures II and III) and the C_{20}-[3]-monoether (Fig. 1, structure IV) (Sinninghe Damsté et al., 2002; Hopmans et al., 2006). A detection limit (defined by a signal-to-noise ratio of 3) of 30-35 pg injected was achieved with this technique.

7.2.5 GC and GC/MS analysis

GC analysis was performed using a Hewlett-Packard 6890 instrument equipped with an on-column injector and a flame ionization detector (FID). A fused silica capillary column (25 m x 0.32 mm) coated with CP Sil-5 (film thickness 0.12 µm) was used with helium as carrier gas. The samples were injected at 70 °C. The GC oven temperature was subsequently raised to 130 °C at a rate of 20 °C/min, and then at 4 °C/min to 320 °C, which was held for 15 min. GC/MS analysis was carried out using a Finnigan Trace GC Ultra, Thermo Electron Corporation, interfaced with a Finnigan Trace DSQ mass spectrometer, using a mass range of *m/z* 40-800. GC conditions for GC/MS were the same as those described above for GC. The compounds formed during thermal destruction of the ladderane lipids were identified according to retention times and mass spectra described by Sinninghe Damsté et al. (2005). The reproducibility of concentration measurements based on duplicate analysis of the samples was between 1 and 7 %.

7.2.6 $\delta^{13}C$ and TOC analysis

Total organic carbon (TOC) and carbon isotope ratios ($\delta^{13}C$) of the residual matter were measured against a benzoic acid laboratory standard (%C = 68.80, $\delta^{13}C$ = -28.1 ‰), calibrated against the NBS22 standard ($\delta^{13}C$ = -30.03 ‰), on a Carlo Erba Flash elemental analyzer coupled to a ThermoFinnigan Delta^{plus} mass spectrometer. All analytical results are reported in the usual δ notation, in per mil relative to the Vienna Pee Dee belemnite (VPDB) standard. The reproducibility of concentrations based on duplicate analysis of samples was better than 0.3 ‰ for $\delta^{13}C$ and better than 0.6 % for TOC.

7.2.7 Compound specific isotope analysis

The $\delta^{13}C$ values of the ladderane lipids were determined using a ThermoFinnigan DELTA-C irmGC-MS system. A fused silica capillary column (50 m x 0.32 mm) coated with CP-Sil 5 (film thickness 0.12 µm) was used with helium as carrier gas. The column conditions and temperature program were the same as described above for GC analyses. $\delta^{13}C$ values were corrected for the additional carbon added during the derivatization step (BF_3/methanol: $\delta^{13}C$ = -20.5 ‰; BSTFA: $\delta^{13}C$ = -36.8

‰). The $\delta^{13}C$ values for individual compounds are reported in the standard delta notation against VPDB standard.

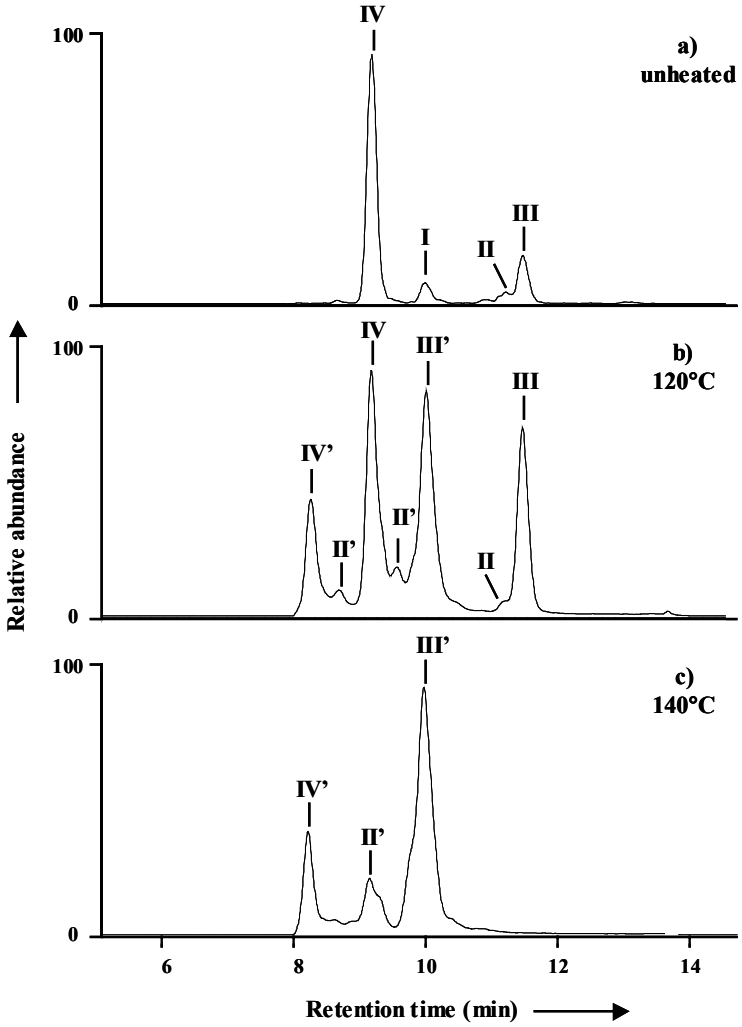

Figure 2. HPLC base peak chromatograms showing the distribution of ladderane lipids (analyzed as their methyl ester derivatives) in biomass (comprised of 70% of anammox bacteria) derived from a wastewater treatment plant. a) original, and artificially matured for 72 hours at b) 120 °C, c) 140 °C. I-IV corresponds to structures in Fig. 1. II'-IV' are possible thermal degradation products of ladderane lipids, II' and III' are those identified by Sinninghe Damsté et al. (2005).

7.3 Results and discussion

7.3.1 HPLC-MS/MS analysis

To investigate the effect of thermal maturity on ladderane lipids, anammox biomass was artificially matured for three days using hydrous pyrolysis (Lewan, 1993) at temperatures ranging from 120 to 365 °C. Ladderane lipids were analyzed with HPLC/APCI-MS/MS using the transition of the protonated molecules to selected product ions of four ladderane lipids. This highly sensitive technique avoids thermal alteration of ladderanes upon gas chromatographic analysis (Hopmans et al., 2006) and has been used to detect ladderanes in environmental samples (Hamersley et al., 2007; Jaeschke et al., 2007). In the unheated sample the C_{20}-[3]-monoether (IV) is the most abundant component, followed by the C_{20}-[3]-fatty acid (III) in about 10 times lower concentration, and minor amounts of the fatty acids with a [5]-ladderane moiety (I, II) (Fig. 2a).

In the sample from the 120 °C experiment additional peaks appeared (Fig. 2b, II', III', IV') which may represent degradation products of the ladderane lipids (see later) whereas the original ladderane lipids were already low in abundance when compared to the starting material, with a loss of about 99 % (I), 95 % (II), 77 % (III), and 78 % (IV), respectively. At a hydrous pyrolysis temperature of 140 °C none of the ladderane lipids in their original form could be detected, suggesting that these compounds were completely degraded (Fig. 2c). Analysis of the early eluting compounds formed at these temperatures (II', III', IV') by MS/MS in data-dependent mode revealed the same protonated molecular ion and almost identical fragmentation patterns as the intact ladderanes, signifying similar structures. The retention times of these components were earlier by ca. 1 min compared to the parent ladderane, indicating that these compounds are more polar. Collectively, this suggests that these newly formed compounds represent ladderanes in which one of the cyclobutane rings opened, resulting in the formation of a double bond within the ladderane moiety. This is similar to what has been observed for ladderane lipids during GC analysis, i.e., cleavage and internal proton shifts of bonds within the [5]-ladderane moiety leading to a moiety comprised of one cyclobutane ring with two condensed cyclohexenyl groups (Fig. 1, structure II')(cf. Sinninghe Damsté et al., 2005). Transformation of two cyclobutane rings into one cyclohexene ring was further suggested for the lipids containing the [3]-ladderane moiety (Fig. 1, structure III'). This is supported by HPLC-MS/MS analysis of

isolated standards of these products formed during GC analysis. Sinninghe Damsté et al. (2005) showed that these products have similar mass spectra and retention times as those formed in the 120 and 140 °C hydrous pyrolysis experiments. For the C_{20}-[3]-ladderane monoether (Fig. 1, structure IV) no isolated degradation products existed but almost identical fragmentation patterns of the two peaks detected with selective reaction monitoring (Fig. 2b, IV and IV') also suggests ring opening and formation of a thermally more stable product (Fig. 1, structure IV') in the same way as shown for the C_{20}-[3]-ladderane fatty acid.

These results indicate that the diagenetic products of ladderanes formed during hydrous pyrolysis experiments are the same as those formed during GC analysis. At 120 °C, already 87 % of the C_{20}-[5]-ladderane fatty acid is transformed into the thermally more stable form, whereas for the C_{20}-[3]-ladderane fatty acid and the C_{20}-[3]-ladderane monoether this is 49 and 47 %, respectively. Thus, ladderane lipids are transformed at the very early stages of maturation, which suggests that original ladderanes are unlikely to be present in mature sediments and sedimentary rocks.

7.3.2 GC and GC-MS analysis

Since HPLC-MS/MS analysis indicated that ladderane lipids were quickly transformed into diagenetic products similar to those formed during GC analysis, we also analysed lipids using GC and GC-MS. In the unheated sample the distribution of ladderane lipids was similar to that revealed by HPLC analysis, i.e., the thermally stable product of the C_{20}-[3]-ladderane monoether (Fig. 1, structure IV'; Fig. 3, peak 9) formed upon GC analysis was highest in concentration, followed by a broad peak representing the thermally stable products of the C_{20}-[3]-ladderane fatty acid (Fig. 1, structure III'; Fig. 3, peak 6), which is two times lower in concentration. Broad peaks representing the thermally stable products derived from the two fatty acids with the [5]-ladderane moieties are in the lowest concentrations (Fig. 1, structure II'; Fig. 3, peaks 4 and 5). At a hydrous pyrolysis temperature of 120 °C the concentration of these lipids already decreased by 70-100 % compared to the unheated sample (Fig. 4a). The products of the C_{18}-[5]-ladderane fatty acid (Fig. 1, structure I) were below detection limit using GC-MS at the lowest hydrous pyrolysis temperature, whereas the C_{20}-[5]-ladderane fatty acid products (Fig. 1, structure II) were detectable until 180 °C.

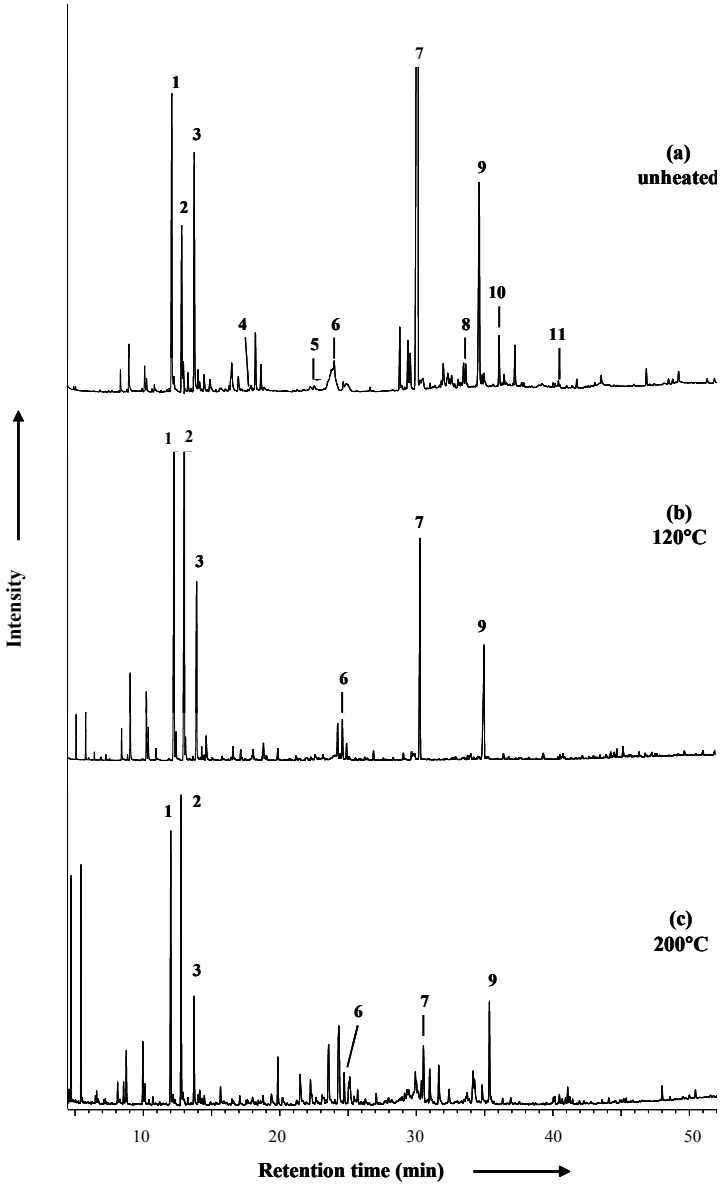

Figure 3. Gas chromatograms of the total lipid fractions (analyzed as their methyl ester and TMS derivatives) in anammox biomass derived from a wastewater treatment plant. (a) original biomass, and samples artificially matured for 72 hours at (b) 120 °C, (c) 200 °C. Compound numbers represent (1) *iso* hexadecanoic acid; (2) n-C_{16}; (3) 10-methyl-hexadecanoic acid; (4) C_{18}-[5]-ladderane fatty acid; (5) C_{20}-[5]-ladderane fatty acid; (6) C_{20}-[3]-ladderane fatty acid; (7) squalene; (8) C_{27} hopanoid ketone; (9) C_{20}-[3]-ladderane monoether; (10) diploptene; (11) diplopterol.

Remarkably, the broad peak shape of the C_{20}-[3]-ladderane fatty acid (Fig. 3a, Peak 6) evident in the original sample disappeared at 120 °C and instead a sharp peak was observed (Fig. 3b, peak 6) with the same mass spectrum. This suggests that opening of the cyclobutane rings and conversion into thermally more stable products had already occurred during hydrous pyrolysis, in good agreement with the HPLC-MS/MS results. With increasing artificial maturation temperatures the concentration of the diagenetic products derived from the [3]-ladderane moiety further decreased, and were below detection limit at 280 °C (Fig. 4a), indicating that all compounds were thermally degraded. Thus, GC and GC-MS analysis revealed that a high percentage (70-100 %) of the ladderane lipids already disappeared at low temperatures but that the thermally more stable ladderane degradation products were still detectable at 260 °C.

To study the effect of thermal maturity on ladderane fatty acids in comparison to normal fatty acids we analyzed the 10-methylhexadecanoic acid in the unheated and artificially matured anammox biomass. The 10-methylhexadecanoic acid (Fig. 1, structure V, Fig. 3, peak 3), although not unique to *Planctomycetes* (Londry et al., 2004), has been used as an indicator for the presence of anammox bacteria in a freshwater lake (Schubert et al., 2006). Compared to the starting material a 40 % decrease in concentration of the 10-methylhexadecanoic acid is observed at 120 °C (Fig. 4a). A further decrease is observed with increasing temperatures but low concentrations of the fatty acids are still detectable at 350 °C. Thus, compared to the conventional fatty acids, ladderane fatty acids have a much lower thermal stability that attests to the thermal instability of the structurally strained cyclobutane moieties.

7.3.3 Stable carbon isotopic composition and TOC

Chemolithoautotrophic anammox bacteria have been shown to possess a carbon fixation pathway (the acetyl-CoA pathway)(Strous et al., 2006) that strongly fractionates against ^{13}C, resulting in distinct isotopic signatures, i.e., ladderane lipids are depleted by as much as 47 ‰ relative to the carbon source (CO_2) of anammox bacteria (Schouten et al., 2004). The stable carbon isotopic composition of the C_{20}-[3]-ladderane fatty acid and the C_{20}-[3]-ladderane monoether analyzed in this study also revealed substantially depleted $\delta^{13}C$ values of -48.9 and -48.7 ‰, respectively, for the unheated sample (Table 2), whereas the bulk isotopic composition of the anammox starting

material has a more ^{13}C enriched $\delta^{13}C$ value of -34.1 ‰ (Table 1). The stable carbon isotopic composition of the 10-methylhexadecanoic acid with values of -44.8 to -43.4 ‰ (Table 2) is also substantially depleted in ^{13}C compared to other bacterial lipids ($\delta^{13}C$= ca -20 to -30 ‰) (Schmid et al., 2003), which strongly suggests that this lipid originated from anammox bacteria. With increasing hydrous pyrolysis temperatures the bulk biomass $\delta^{13}C$ values become isotopically lighter by about 2 ‰ which is probably due to the loss of thermally labile functional groups enriched in ^{13}C, i.e., proteins and carbohydrates, whereas $\delta^{13}C$ values of the ladderane lipids become slightly more enriched by about 2-4 ‰. With increasing temperatures (>260 °C) thermal cleavage of preferentially ^{12}C-^{12}C bonds during oil and gas generation leads to isotopically heavier $\delta^{13}C$ values of the biomass (-33.7 ‰ at 365 °C). However, the ^{13}C depleted signal is mainly preserved also at higher maturity, and this characteristic feature may be used in the assignments of diagenetic products of anammox lipids.

Table 2. Stable carbon isotopic ($\delta^{13}C$) data for selected lipids obtained from anammox biomass. All fatty acids were analyzed as methyl esters (FAME).

	$\delta^{13}C$ (‰ vs VPDB)		
lipid	unheated	120 °C	200 °C
iso-C16	-50.9	-49.2	-49.4
C16:0	-41.7	-42.9	-42.0
10-Me-C16	-44.8	-43.7	-43.3
C20-[3]-FA	-48.9	-48.0	-45.1
C20-[3]-monoether	-48.7	-45.6	-45.4

7.3.4 Implications for the occurrence of ladderane lipids in ancient sediments

These results can be used to make predictions of the occurrence of ladderane lipids in ancient sediments. The 22S/(22S+22R) ratio and the ββ/(αβ+βα+ββ) ratio for the C_{31} homohopanes, which are generated from bacteriohopanetetrol and possible other hopanepolyol derivatives present in anammox biomass (Sinninghe Damsté et al., 2004) during artificial maturation, are generally used biomarker maturity parameters with maximum values of ca. 0.6 and 0, respectively (Seifert and Moldowan, 1980; Van Duin et al., 1997). They can thus be used to examine the degree of thermal maturation of ladderane lipids in our hydrous pyrolysis experiments (Fig. 4b).

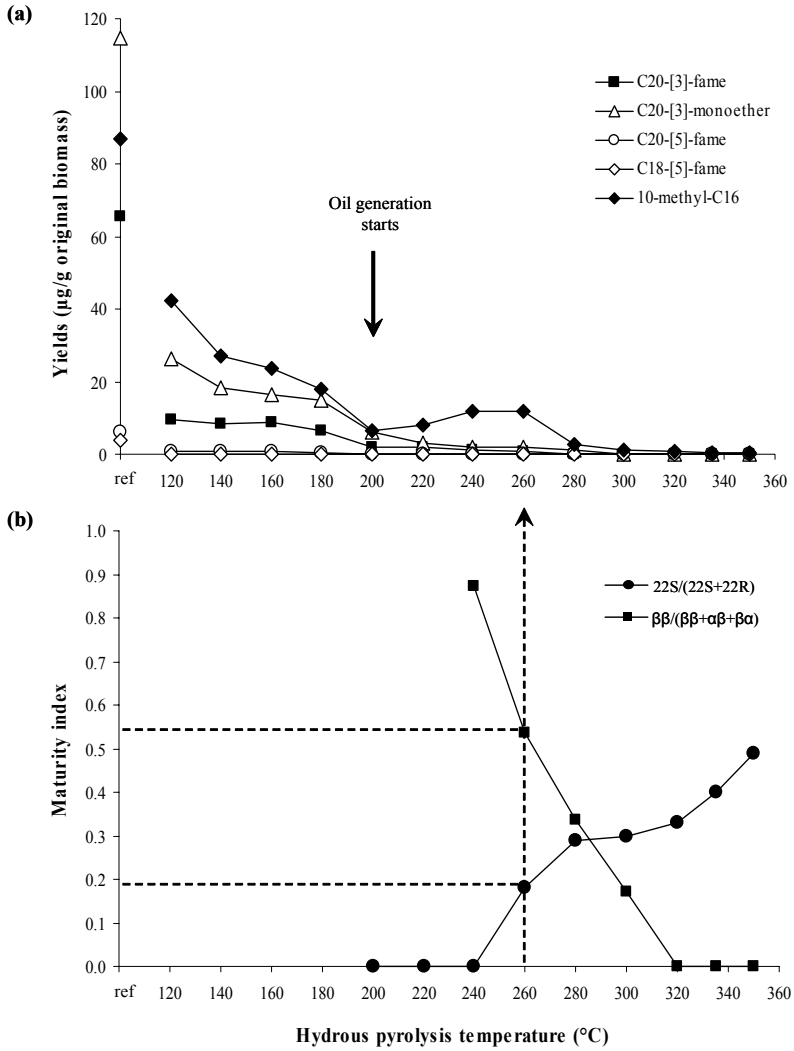

Figure 4. Concentration and ratios of compounds as a function of hydrous pyrolysis temperature. a) the most abundant ladderane lipids and the 10-methyl-hexadecanoic acid as analyzed by GC; b) diagram showing two hopane maturity parameters, the ββ/(ββ+αβ+βα) C_{31} homohopane ratio and the 22S/(22S+22R)-17α,21β(H) C_{31} homohopane ratio as a function of maturation temperature. The stippled line indicates the temperature and maturity index where diagenetic products of ladderane lipids were still detectable by GC-MS.

Ladderane lipids already disappear at 140 °C where no αβ or 22S hopanoid isomers have yet formed, and therefore, these lipids are not expected to be found even in relatively immature ancient sediments or sedimentary rocks. Thermal diagenetic

products of ladderane lipids containing the [3]-ladderane moiety were detected at hydrous pyrolysis temperatures of ≤ 260 °C, which corresponds to a 17α,21β(H)-hopane 22S/(22S+22R) ratio of ca. 0.2. This ratio corresponds to pre-oil generation maturities (Peters et al., 2005) and suggests that ladderane lipids are only expected to be present in sediments and sedimentary rocks with a relatively low level of thermal maturity. At 260 °C, the thermally unstable 17β,21β(H) isomer of the C_{31} hopane is still present (Fig. 4b), which is another indication of the relatively low level of thermal maturity of ladderane lipids revealed by our artificial maturation experiments. Therefore, reconstruction of past anammox activity based on diagenetic products of ladderane lipids is only feasible for relatively immature sediments and sedimentary rocks. Since ladderane lipids are likely to be present in low abundance in the depositional environment (e.g., 5 to <0.5 ng/g sediment; Jaeschke et al., unpublished results) and a large part (>70%) of them are lost during the earliest stages of maturation, new analytical methods targeting diagenetic products of ladderanes will need to be developed in order to trace their presence in ancient sediments.

7.4 Conclusions

Artificial maturation of anammox bacterial biomass by hydrous pyrolysis revealed that ladderane lipids rapidly undergo structural modification due to breakdown of the condensed cyclobutane rings at temperatures as low as 120 °C, forming thermally more stable compounds. Thermally stable products derived from ladderane lipids with a [3]-ladderane moiety were detectable by GC-MS from hydrous-pyrolysis experiments at temperatures as high as 260 °C, which corresponds to C_{31} 17α,21β(H)-homohopane 22S/(22S+22R) ratios of 0.2 or ββ/(αβ+βα+ββ) ratios of >0.5. These diagenetic products might, in combination with their highly ^{13}C depleted carbon isotopic signature, constitute suitable biomarkers for the detection of past anammox activity in immature ancient sediments and sedimentary rocks.

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Chapter 8

Molecular fossil evidence for anaerobic ammonium oxidation in the Arabian Sea over the last glacial cycle

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Abstract

Anaerobic ammonium oxidation (anammox) has been recognized as an important process converting fixed nitrogen to N_2 in many marine environments, thereby having a major impact on the present-day marine nitrogen cycle. However, essentially nothing is known about the importance of anammox in past marine nitrogen cycles. In this study, we analyzed the distribution of fossil ladderane lipids, derived from bacteria performing anammox, in a sediment core from the northern Arabian Sea. Concentrations of ladderane lipids varied between 0.3 and 5.3 ng/g sediment during the past 140 kyr, with high values observed during the Holocene, intervals during the last glacial as well as during the penultimate interglacial. Maxima in ladderane lipid abundances correlate with high TOC (4-6 %) and elevated $\delta^{15}N$ (>8 ‰) values. Anammox activity, therefore, seems enhanced during periods characterized by an intense oxygen minimum zone (OMZ). Low concentrations of ladderanes (<0.5 ng/g sediment), indicating low anammox activity, coincide with periods during which the OMZ was severely diminished. Since anammox activity co-varied with OMZ intensity it may play an important role in the loss of fixed inorganic nitrogen from the global

ocean on glacial interglacial time scales, which was so far attributed only to heterotrophic denitrification.

8.1 Introduction

The global oceanic nitrogen budget is presently not well established with respect to the balance of sources and sinks (Altabet, 2007; Brandes and Devol, 2002; Codispoti et al., 2001). Oceanic N_2 fixation constitutes the major source for nitrogen while sedimentary and pelagic denitrification is widely thought to be the predominant sink. Total sources and sinks are estimated to be between 200 and >400 Tg/yr (Codispoti et al., 2001; Gruber and Sarmiento, 1997). One way of establishing the nitrogen budget is to use the stable nitrogen isotope ratio ($\delta^{15}N$) of organic matter (OM). The $\delta^{15}N$ of OM which settles from the surface water is a function of the $\delta^{15}N$ of the nitrate source and fractionation occurring during uptake of nitrate by phytoplankton (Altabet, 1988). The average oceanic $\delta^{15}N$ of dissolved nitrate is around 5 ‰ (Altabet, 1988; Sigman et al., 1997). Denitrification occurs under suboxic conditions in the water column resulting in a strong fractionation of ~27 ‰ (Brandes et al., 1998), leaving the residual nitrate enriched in ^{15}N. Upwelling of this ^{15}N-enriched nitrate also influences the isotopic composition of newly produced OM in the surface water by phytoplankton leading to high $\delta^{15}N$ values in particulate and sedimentary OM. In contrast, although denitrification taking place within the sediment is changing the overall nitrogen inventory, it is believed to produce no significant change in $\delta^{15}N$ (Altabet, 2007; Brandes and Devol, 2002). Thus, OM $\delta^{15}N$ is mainly thought to reflect the rate of denitrification in the water column. Past changes in the fixed nitrogen budget can be deduced from changes in atmospheric N_2O concentrations (Fluckiger et al., 2004). Major changes in the extent of denitrification have furthermore been inferred from changes in $\delta^{15}N$ in sedimentary records on longer timescales (Altabet et al., 1999; Altabet et al., 2002; Ganeshram et al., 2000; Suthhof et al., 2001). Because these changes were parallel with atmospheric CO_2 concentrations, a link between the two has been suggested (Altabet et al., 2002).

Globally, about 30-50% of the total nitrogen loss takes place in oxygen minimum zones (OMZs) and was until recently exclusively attributed to heterotrophic denitrification (Codispoti et al., 2001; Gruber and Sarmiento, 1997). The Arabian Sea comprises one of the largest OMZs presently found in the oceans, and ~20 % of the

water column denitrification is estimated to take place here (Bange et al., 2000). The Arabian Sea, therefore, is believed to have a substantial influence on the global marine fixed nitrogen budget (Gruber and Sarmiento, 1997).

Already in the 1980's several studies suspected other processes than denitrification to contribute to N_2 production without corresponding decrease in the nitrate content (Codispoti and Christensen, 1985; Naqvi, 1987), and Devol and co-workers (2006) suggested anammox to be responsible for the observed mismatch between denitrification estimated from nitrate deficits and measured excess N_2 concentrations in the OMZ of the Arabian Sea. Recent studies indicate anammox to be the dominant N_2 production pathway in the OMZs off Namibia (Kuypers et al., 2005), Chile (Thamdrup et al., 2006) and Peru (Hamersley et al., 2007), while the contribution of denitrification was only minor or did not exist at all. Recently, we found evidence for anammox in the Arabian Sea by studying the spatial and seasonal distribution of ladderane lipids in OMZ waters off Oman (Jaeschke et al., 2007). Ladderane lipids are specific membrane lipids unique to anammox bacteria which mediate the anaerobic oxidation of ammonium (Sinninghe Damsté et al., 2002). The ladderane core lipid consists of three or five linearly concatenated cyclobutane rings either ester- or ether-linked to the glycerol backbone. These ladderanes form a dense and highly impermeable membrane surrounding the anammoxosome, an intracellular compartment, where anammox catabolism takes place. Ladderane lipids have so far been successfully applied as biomarkers for anammox bacteria in the suboxic water column of the Black Sea (Kuypers et al., 2003; Wakeham et al., 2007), OMZs off Namibia (Kuypers et al., 2005), Peru (Hamersley et al., 2007), and in the Arabian Sea (Jaeschke et al., 2007).

The dominance and role of anammox in comparison to denitrification in several present-day OMZs, raises the question how past changes in the intensity of anammox impacted the nitrogen budget. In this study we investigate past anammox activity using fossilized ladderane lipids in a sediment core from the northern Arabian Sea (Murray Ridge) spanning the last 140 kyr. In conjunction with bulk geochemical tracers (TOC, $\delta^{15}N$), the abundance of ladderane lipids is used to reconstruct changes in past anammox activity and to assess its potential impact on the oceanic nitrogen budget.

8.2 Material and methods

8.2.1 Oceanographic setting and core location

The Arabian Sea climate and hydrography is strongly influenced by the seasonal change of monsoonal winds driven by changes in the pressure gradient between the Tibetan plateau and the South Indian Ocean (Wyrtki, 1973). During summer, warm and humid southwest (SW) monsoonal winds induce coastal and open ocean upwelling of nutrient-rich deep waters to the euphotic zone resulting in primary productivity rates estimated to be 200 to 400 $gCm^{-2}yr^{-1}$ (Kabanova, 1968; Qasim, 1982). During winter, dry and relatively cold northeast (NE) monsoonal winds from the Asian landmass are prevailing. Productivity is then generally low as the NE monsoon suppresses upwelling, although deepening of the mixed layer and subsequent nutrient injection in the euphotic zone gives rise to a second, more modest productivity maximum (Sawant and Madhupratap, 1996). A stable and pronounced OMZ with O_2 values of less than 2 µmol/L persists at water depths between 150 and 1200 m (Wyrtki, 1973) as a result of the high biological productivity in combination with moderate ventilation of the thermocline waters.

Sediment core MD04-2879 (22°32.9'N, 64°02.8'E) was recovered with R/V *Marion Dufresne* in 2004 from the Murray Ridge in the northern Arabian Sea, from a water depth of 920 m (Fig. 1) well within the present-day OMZ. Oxygen concentration in the bottom waters is below 2 µmol/L (Van Bennekom et al., 1995). The sediment generally consists of homogeneous, dark-greenish to light greenish/grey hemipelagic muds.

8.2.2 Chronostratigraphy

It has been shown in several studies (Reichart et al., 2004; Reichart et al., 1998) that short-lived occurrences of deep-dwelling planktic foraminifera *Globorotalia truncatulinoides* and *Globorotalia crassaformis* match very well with cooling events in the North-Atlantic. These species are not present in the modern Arabian Sea as they rely for their reproductive cycle on deep overturning during intense winter mixing, which is associated with an enhanced NE monsoon during cold periods. Their high relative abundances during those short periods make them accurate biostratigraphic markers for Arabian Sea sediment records.

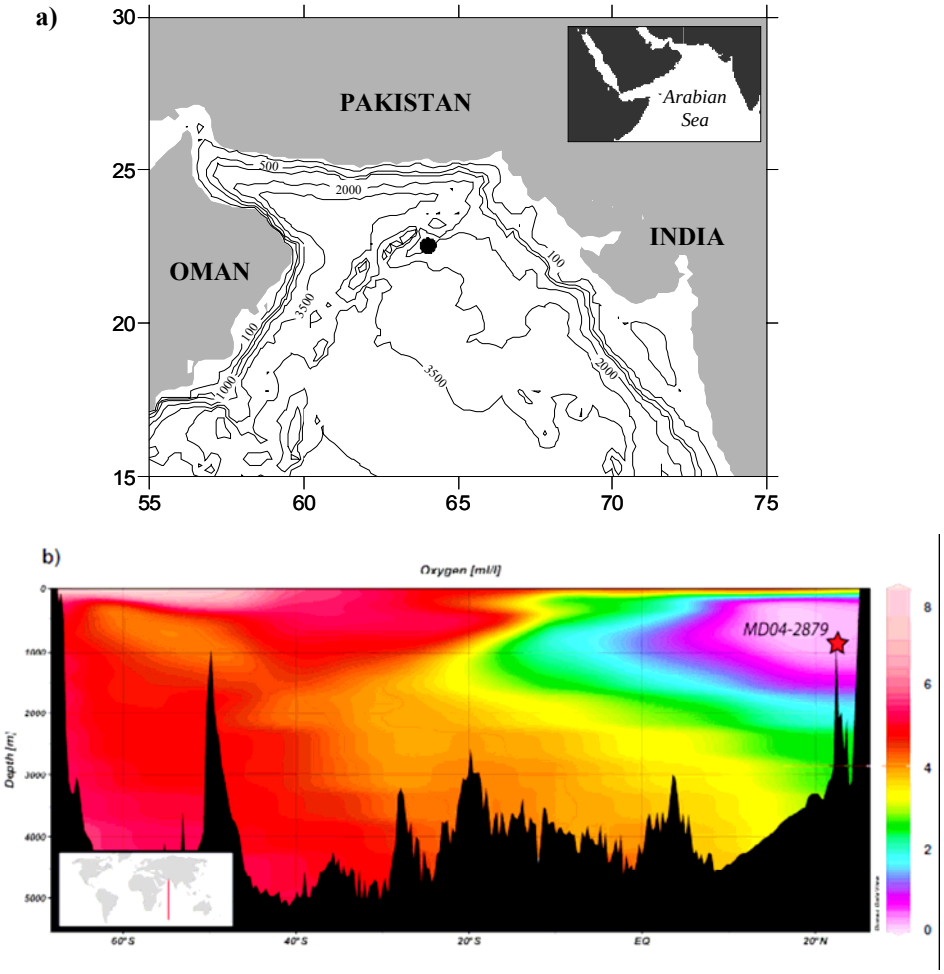

Figure 1. a) map showing the location of core MD04-2879 (22°32.9'N, 64°02.8'E, 920 mbss), b) column dissolved oxygen concentrations (ml/l shown in colour) and seafloor topography along a transect (indicated by red line in small map).

Species of *G. truncatulinoides* and *G. crassaformis* have been counted on split samples from the 150-600 μm size fraction and are expressed as specimen/g sediment (Fig. 2). The occurrence peaks in MD04-2879 have been tuned to extreme North Atlantic cooling events as recorded in an alkenone record from the Iberian margin (Martrat et al., 2007). The age model is based on 15 age control points (Fig. 2). Ages for each individual sample depth have been derived by linear interpolation between calibration points. A second order polynomial fit arrives at a correlation coefficient (r) of 0.989. Sedimentation rates vary between 2 and 30 cm/kyr. Sedimentation rates in the

Northern Arabian Sea co-vary in general with changes in calcium carbonate preservation, which is strongly influenced by supralysoclinal dissolution related to post-depositional organic matter degradation under dysoxic conditions (Den Dulk et al., 2000).

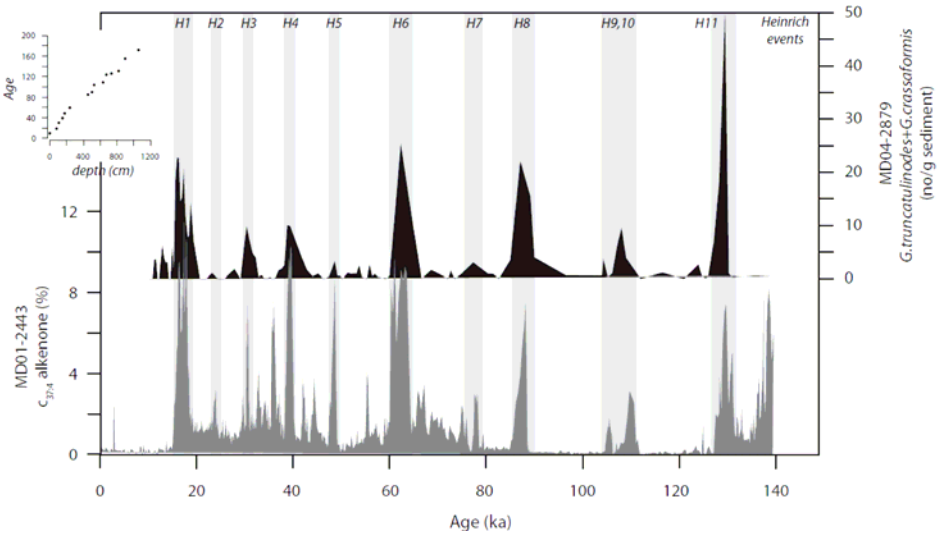

Figure 2. Age model for MD04-2879. Tuning of deep dwelling planktic foraminifera in MD04-2879 to North Atlantic cold events; *G. truncatulinodes* and *G. crassaformis* per g sediment in MD04-2879 (upper panel), relative proportion of tetraunsaturated C37 alkenone to total C37 alkenones (C37:4) in MD01-2443 (Martrat et al., 2007) (lower panel). Red dots are calibration points. Grey bars indicate timing of Heinrich events according to Rashid et al. (2003).

8.2.3 Total organic carbon (TOC) and $\delta^{15}N$ analysis

Freeze-dried and powdered sediment samples were decalcified with dilute (2N) HCl, rinsed with demineralised water to remove $CaCl_2$ and freeze-dried again afterwards. Total organic carbon (TOC) was measured against a benzoic acid laboratory standard (C = 68.80 %), $\delta^{15}N$ against the lab standards acetanilide ($\delta^{15}N$ = 1.3 ‰ vs. air) and glycine ($\delta^{15}N$ = 2.43 ‰ vs. air) on an elemental analyzer (Carlo Erba Flash) coupled online to a mass spectrometer (Thermofinnigan Delta^{plus}). Based on analysing standards and duplicate analysis of samples the analytical reproducibility for TOC was found to be better than 0.2 %. $\delta^{15}N$ values are expressed in per mil relative to atmospheric N_2, with reproducibility better than 0.3 ‰.

8.2.4 Lipid extraction and analysis

Samples of ca. 4 g of freeze-dried and homogenized sediment were repeatedly (5x) extracted with a dichloromethane (DCM)-methanol mixture (2:1 by volume). The extracts were combined and the bulk of the solvent was removed by rotary evaporation under vacuum, and the extract was dried over a Na_2SO_4 column. An aliquot of the lipid extract was saponified with aqueous 1 N KOH in methanol for 2 h at 100°C. Non-saponifiable lipids (neutral lipids) were extracted out of the basic solution (pH>13) using DCM. Fatty acids were obtained by acidifying the residue to pH 3 and subsequent extraction with DCM. The fatty acid fraction was methylated by adding diazomethane (CH_2N_2) to convert fatty acids into their corresponding methyl esters (FAMEs). Excess CH_2N_2 was removed by evaporation. To remove very polar components, aliquots of the FAMEs were eluted with ethyl acetate over a small column filled with silica. Polyunsaturated fatty acids were removed by eluting the aliquots with ethyl acetate over a small $AgNO_3$ (5 %) impregnated silica column, yielding a saturated fatty acid fraction. The fatty acid fractions were dissolved in acetone and then filtered through a 0.45 µm, 4 mm diameter PTFE filter.

These fractions were analyzed by high performance liquid chromatography coupled to positive ion atmospheric pressure chemical ionization tandem mass spectrometry (HPLC/APCI-MS/MS) as described by (Hopmans et al., 2006) with some modifications. Specifically, separation was achieved using a Zorbax Eclipse XDB-C_8 column (3.0 x 250 mm, 5 µm; Agilent) and a flow rate of 0.18 ml/min MeOH. The source settings were: vaporizer temperature 475°C, discharge current 2.5 µA, sheath gas (N_2) pressure 30 (arbitrary units), auxiliary gas (N_2) pressure 5 (arbitrary units), capillary temperature 350°C, source CID -10 V. Argon pressure was maintained at 1.5 mTorr in the second quadrupole. Quantification of ladderane lipids was done by using an external calibration curve using previously obtained standards (Hopmans et al., 2006; Sinninghe Damsté et al., 2002) of isolated methylated ladderane fatty acids containing the [3]- and [5]- ladderane moieties (Fig. 3, structures I-IV). A detection limit (defined by a signal to noise ratio of 3) of 30-35 pg injected was achieved with this technique. The reproducibility of ladderane lipid concentration based on duplicate analysis of the samples was better than 8%.

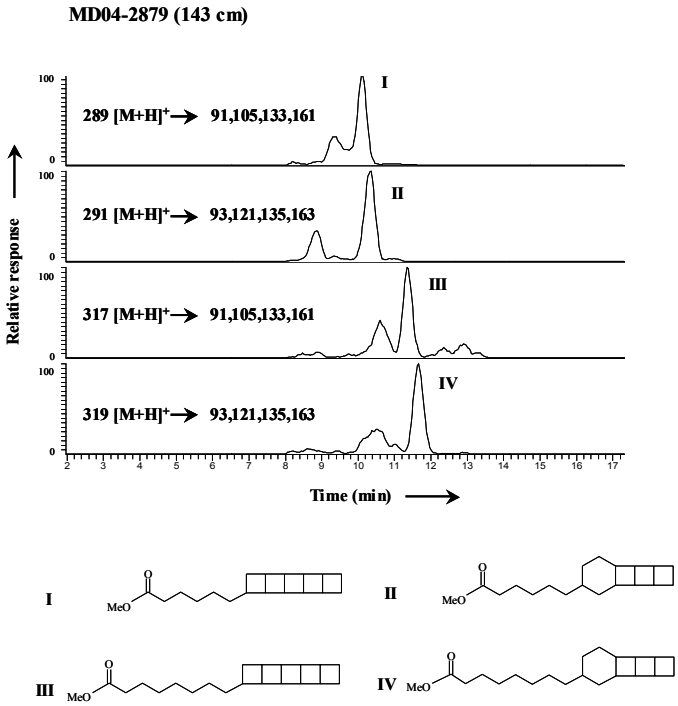

Figure 3. HPLC/APCI-MS/MS chromatograms generated using the transition of the protonated molecules to selected product ions for ladderane lipids analyzed in this study. (I)-(IV) correspond to structures of ladderane lipids shown below. (I) C_{18}-[5]-ladderane FAME, (II) C_{20}-[5]-ladderane FAME; (III) C_{18}-[3]-ladderane FAME, (IV) C_{20}-[3]-ladderane FAME.

The iNdex of Ladderane lipids with 5 cyclobutane rings, an index for the relative chain length of ladderane lipids, was calculated according to Rattray (2008):

$$NL_5 = \frac{C_{20}[5]\,\text{fatty acid}}{(C_{18}[5]\,\text{fatty acid} + C_{20}[5]\,\text{fatty acid}}$$

The NL_5, based on culture experiments with anammox bacteria, water column and surface sediment samples, was then converted to a temperature estimate using the equation of Rattray (2008):

$$NL_5 = 0.2 + \frac{0.7}{1 + e^{-\left(\frac{Temp - 16.0}{1.6}\right)}}$$

The reproducibility of the NL_5 based on duplicate measurements was better than 0.07.

8.3. Results

8.3.1 Organic carbon and ^{15}N content

The TOC record of core MD04-2879 shows changes between 0.6 and 5.9 % over the last 140 kyr (Fig. 4a). Highest TOC values were observed during the early Holocene (4.8 %), the penultimate interglacial (marine isotope stage 5e) (5.5 %) as well as during several warmer episodes within the last glacial (3.4-5.9 %). TOC values increased by about a factor of five over the last deglaciation. Overall, the TOC pattern is in line with those from previous studies from the Murray Ridge area (Reichart et al., 1997).

The $\delta^{15}N$ record exhibits values ranging from 4 to 9.6 ‰ during the last 140 kyr (Fig. 4b). Maxima in the $\delta^{15}N$ record characterize the early Holocene (>7.5 ‰), the penultimate interglacial (7.5-8.8 ‰) and several warmer episodes within the last glacial (>8 ‰). An increase in $\delta^{15}N$ of >2 ‰ from the Last Glacial Maximum (LGM) to the early Holocene is observed. These results are consistent with other studies from different parts of the Arabian Sea (Altabet et al., 1999; Altabet, 2007; Reichart et al., 1998).

8.3.2 Ladderane lipids

Ladderane lipids were detected in all sediment samples studied of core MD04-2879 (Fig. 4c). Ladderane lipid concentrations (total concentrations of the four components analyzed, structures I-IV in Fig. 2) ranged from 0.3 to 5.3 ng/g dry weight sediment. Maxima in the summed ladderane lipid concentrations are found during the early Holocene and the penultimate interglacial as well as during several intervals within the last glacial with values of 2.5 to 5.3 ng/g. Concentration minima coincide with the LGM and colder episodes within the last glacial with ladderane lipid concentrations generally below 1 ng/g. During the last deglaciation, ladderane lipid concentrations increased by a factor of 4.

8.4 Discussion

8.4.1 Evidence for past anammox in the Arabian Sea

The fossil ladderane lipid record from sediment core MD04-2879 provides the first evidence for past anammox activity. The record provides evidence that anammox occurred continuously over at least the last 140,000 years in the Arabian Sea (Fig. 4c). Variations in the concentration of ladderanes further suggest that significant changes in anammox activity took place over that period. Ladderane lipid concentration and TOC content reveal similar patterns, suggesting that both are controlled by the same mechanism. Reichart et al. (1998) showed that variations in TOC are mainly controlled by variations in primary productivity. Their evidence for a productivity signal is based on co-varying patterns of *G. bulloides*, an indicator for upwelling, Ba/Al ratios which are linked to surface water productivity driven by the summer monsoon, and similar TOC variations in sediment cores from different water depths. Furthermore, fluctuations in TOC and biomarker concentrations in the Arabian Sea sediment cores have also been interpreted as resulting primarily from changing productivity in surface waters (Schubert et al., 1998; Schulte et al., 1999). Based on stable nitrogen isotopes ($\delta^{15}N$), benthic foraminiferal assemblages and trace metals, OMZ intensity and thickness have been shown to fluctuate strongly in the past in line with changes in primary productivity, resulting in changes in bottom and pore water conditions (Altabet et al., 1999; Den Dulk et al., 2000; Reichart et al., 1998; Schulte et al., 1999; Suthhof et al., 2001; Van der Weijden et al., 2006). This suggests that anammox activity was enhanced during times of higher surface productivity and a strong OMZ. However, biomarker lipids have also been shown to have differential preservation in response to changes in the extent of the OMZ intensity (Sinninghe Damsté et al., 2002). Thus, although it seems likely that the observed variations in ladderane lipid concentration primarily reflect changes in anammox productivity, we can not exclude that changes in bottom water redox conditions affected the preservation of the primary ladderane lipid signal. However, when we normalize the ladderane lipid concentration on OC to account for this effect, the general pattern remains the same suggesting that production rather then preservation was the main control on the ladderane lipid distributions (Fig. 5).

8.4.2 Origin of the anammox signal

Important for the interpretation of the ladderane record is the location of the source of these lipids, i.e. anammox occurring in the water column (OMZ) or in the anoxic sediment. Evidence for anammox in the present-day OMZ comes from the presence of ladderane lipids in suspended particulate matter (SPM), and analysis of sediment trap material revealing that these ladderane lipids produced in the water column are transported down to the ocean floor (Jaeschke et al., 2007). However, a sedimentary source for ladderane lipids can not be excluded, as anammox is also a widespread process in organic-rich sediments of coastal and shelf areas (Dalsgaard and Thamdrup, 2002; Thamdrup and Dalsgaard, 2002). Since the core location presently is in the OMZ, high organic carbon contents and low bottom water oxygen could also result here in elevated anammox activity in the sediment. To discriminate between ladderane lipids derived from water column and sedimentary anammox, we applied the iNdex of Ladderane lipids with 5 cyclobutane rings, termed NL_5 (Rattray, 2008). Anammox bacteria have been found to alter their membrane composition in response to temperature changes, i.e., amount of shorter chain ladderane fatty acids increases relative to the amount of longer chain fatty acids at lower temperatures and *vice versa*. Most changes in ladderane lipid chain length take place between 12 and 20°C, while no significant change in chain length has been observed at temperatures <12°C and >20°C. The NL_5 index has been proposed to quantify this relative change (Rattray, 2008). The NL_5 index of the ladderane lipids at the Murray Ridge varied between 0.16 and 0.58 over the last 140 kyr (Fig. 4d). Converted to temperatures using the correlation between the NL_5 and temperature proposed by Rattray (2008) this suggests that ladderanes were synthesized at temperatures between <10 and 17°C. Analysis of SPM samples from a transect offshore Oman at water depths of 450-500 m (Jaeschke et al., 2007) showed NL_5 values between 0.45 and 0.49, which suggested that they were synthesized at temperatures of ca 15°C which is in good agreement with today's temperatures at these depths. The bottom water temperature at the core site (920m) is around 9°C, which would correspond to a NL_5 value below 0.2. Most NL_5 values in our record are above 0.2 (indicated by the dashed line in Fig. 4d), suggesting that the ladderane lipids are to a large extent derived from the water column. Furthermore, bottom water temperatures might have been even lower during the glacial and thus the NL_5 threshold value for ladderanes synthesized might have been even lower.

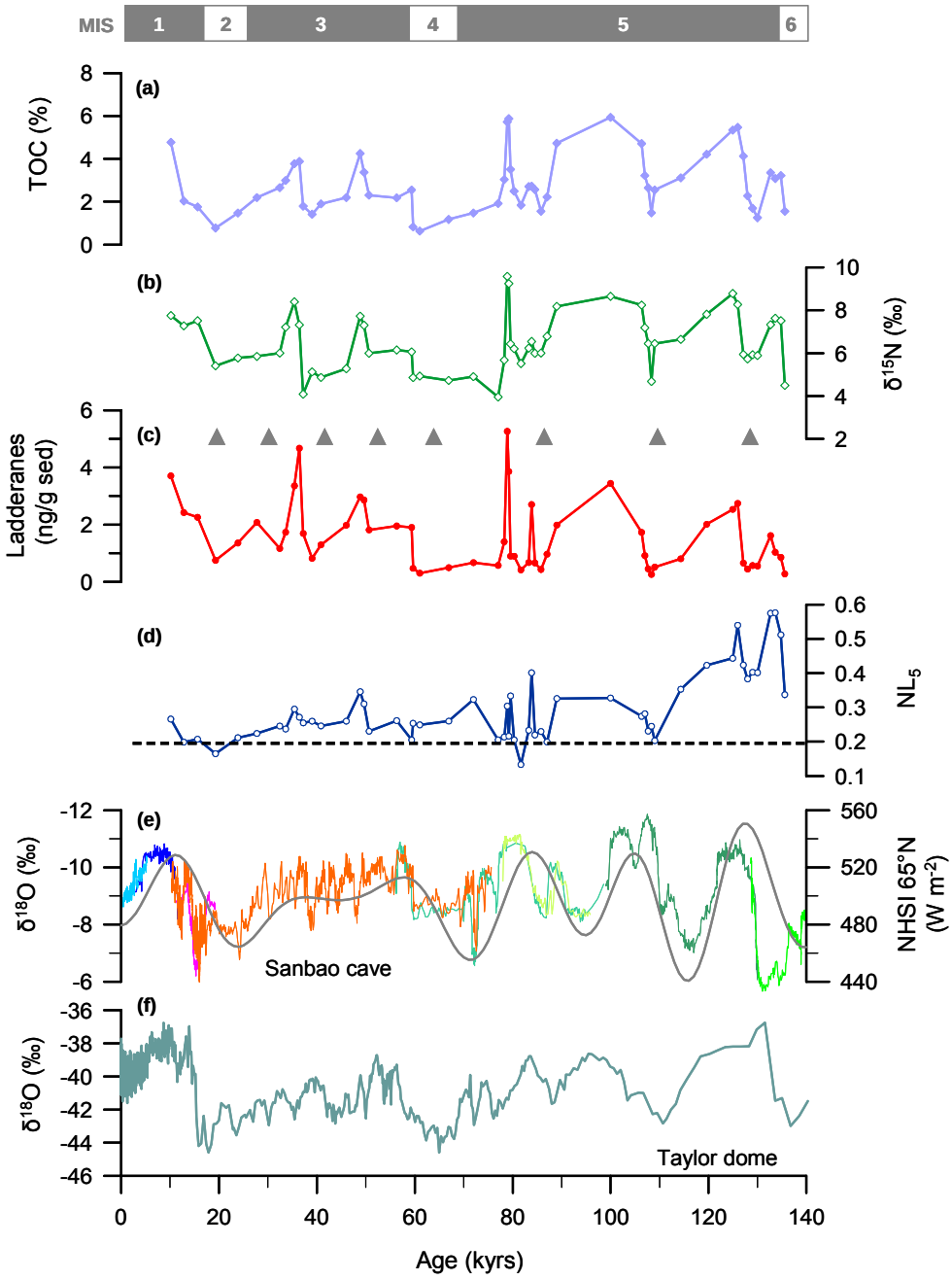
MIS
1
2
3
4
5
6
(a)
TOC (%)
(b)
δ15N (‰)
(c)
Ladderanes (ng/g sed)
(d)
NL5
(e)
δ18O (‰)
Sanbao cave
NHSI 65°N (W m-2)
(f)
δ18O (‰)
Taylor dome
Age (kyrs)

Figure 4. (a) weight percentage of total organic carbon (TOC), (b) stable nitrogen isotope ratio ($\delta^{15}N$) for sediment core MD04-2879, (c) total ladderane lipid concentrations (i.e., I-IV, Fig. 2) for core MD04-2879, (d) NL_5 as a temperature proxy, in this study used to locate the origin of the anammox signal, (e) $\delta^{18}O$ records from the Sanbao cave, China (Wang et al., 2008) (light blue stalagmite SB26; blue SB10; pink SB3; turquoise SB22; grey SB25-1); dark green SB23; light green SB11) and Hulu cave (Wang et al., 2001) (orange), and NHSI (Northern Hemisphere summer insolation, 21 July) at 65°N, (f) $\delta^{18}O$ from the Taylor dome ice core as a proxy for Antarctic air temperatures (Grootes et al., 2001). The dashed line represents a NL_5 value below expected for lipids produced only by sedimentary anammox bacteria. Grey triangles indicate the occurrence of deep-dwelling planktic foraminifera *G. truncatulinoides* and *G. crassaformis*. MIS=marine isotope stage.

During the penultimate interglacial NL_5 values increase to 0.4-0.6 which corresponds to water temperature estimates of 15-17°C. This increase in the NL_5 during this period follows the general global warming observed in ice cores from Antarctica (Fig. 4f) and is in line with global climate change. Finally, our ladderane lipid record follows the $\delta^{18}O$ records from Sanbao cave and Hulu cave speleothems (Fig. 4e) (Wang et al., 2001; Wang et al., 2008) which are indicators for monsoon intensity and vary in response to changes in Northern Hemisphere summer solar radiation on orbital scales (Fig. 4e, grey curve). Variations in monsoon intensity triggers upwelling and primary productivity, again causing changes in OMZ strength and likely also anammox activity (Fig. 4c). Taken together, our ladderane lipid record thus seems to primarily reflect past changes in anammox activity in the OMZ.

8.4.3 Implications for nitrogen cycling in the ocean

The down-core $\delta^{15}N$ record from the Murray Ridge (Fig. 4b) shows a similar range in $\delta^{15}N$ (4-9 ‰) as observed in other records from the Arabian Sea, indicating that these variations are caused by large-scale changes in nitrogen cycling in the Arabian Sea (Altabet et al., 1999; Suthhof et al., 2001). Strikingly, high $\delta^{15}N$ values (Fig. 4b) in our record correspond to high concentrations of ladderane lipids (Fig. 4c), suggesting that anammox may also leave an imprint on the $\delta^{15}N$ signature. This implies that previous assumptions on past changes in denitrification have to be reconsidered. Since the isotopic fractionation of anammox in the water column and in sediments is not known, it is not possible to assess the extent to which anammox contributes to the observed $\delta^{15}N$ signal, which is required for a better interpretation of sedimentary $\delta^{15}N$

records. Preliminary results, however, indicate that anammox also causes a strong nitrogen isotopic fractionation, similar to that observed with denitrification (G. Lavik, pers. comm.). Indeed, in areas where anammox rather than denitrification was found to be the dominant process (Hamersley et al., 2007; Kuypers et al., 2005; Thamdrup et al., 2006) the observed ^{15}N-fractionations are similar to those attributed to denitrification (Galbraith et al., 2004; Ganeshram et al., 2000). Therefore, it is likely that anammox leaves a similar nitrogen isotopic imprint as denitrification. The general coincidence of high ladderane lipid concentrations with enriched δ^{15}N values is thus indicative of enhanced anammox activity and/or denitrification during periods characterized by a pronounced OMZ. Strong SW monsoon driven upwelling of nutrient-rich waters leading to high surface productivity and subsequent remineralization of OM is resulting in strong oxygen deficiency at intermediate water depths which is driving the anammox process in the OMZ. During the coldest glacial episodes, the extent and intensity of the OMZ is thought to have been much reduced or even absent as is indicated by low δ^{15}N values approaching 4 ‰, which is slightly lower than average modern values for marine nitrate (Altabet et al., 1999). Reduced SW monsoonal winds and more intense cooling of the Asian continent during the last glacial period leading to stronger and colder NE monsoonal winds (Duplessy, 1982; Reichart et al., 1998) caused intensified winter mixing and a better ventilation of the OMZ. High water column oxygenation is also evidenced by decreased Sr/Ca ratios as well as the basinwide occurrence of *G. truncatulinoides* and *G. crassaformis*. The occurrence events of the deep-dwelling planktic foraminifera (Fig. 2) correlate with minima in δ^{15}N and ladderane lipid concentrations in our record derived from the same location (Fig. 4; grey triangles). Our results, however, suggest that there was still some minor anammox activity. However, during these phases, anammox activity in the sediment might have contributed to a larger extent to the ladderane lipids detected in our record as suggested by the low NL_5 values (Fig. 4d).

During several intervals δ^{15}N values up to 9.6 ‰ are observed which is 3-4 ‰ higher compared to average glacial values and which coincides with maxima in ladderane lipid concentrations >5 ng/g sediment, indicating conditions comparable to the Holocene. Maxima in δ^{15}N and ladderanes showed that anammox played a prominent role during the last glacial contributing to the net loss of fixed nitrogen from the ocean. This alternative mechanism for the removal of fixed inorganic nitrogen via the anammox reaction, suggested by the ladderane lipid record is in line with previous

studies indicating suboxic conditions and a well established OMZ in the Arabian Sea during interstadials (Altabet et al., 1999; Altabet et al., 2002; Suthhof et al., 2001). During the penultimate interglacial warm period, high TOC and $\delta^{15}N$ values indicate enhanced productivity and a strong OMZ although the abundance of ladderane lipids is somewhat lower compared to the Holocene and values associated with the warmer glacial episodes. However, we can not exclude that at these depths of the core (654-808 cm, 115-130 kyrs) ladderane lipids might have been partly lost through ongoing diagenesis resulting in lower ladderane lipid concentrations (Fig. 5).

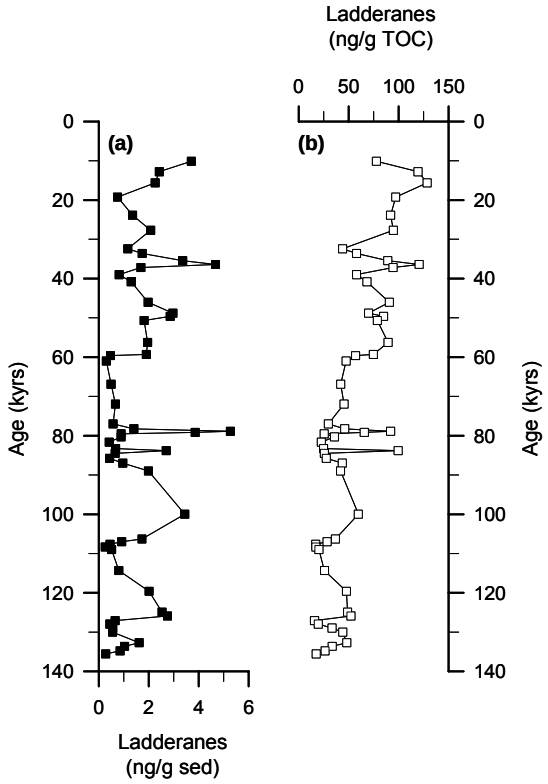

Figure 5. Concentration of ladderane lipids a) per g sediment, b) normalized to organic carbon (TOC) for sediment core MD04-2879.

Anammox and denitrification could, by changing the oceanic combined nitrogen inventory, influence marine productivity, thereby modulating atmospheric pCO_2 (Altabet et al., 2002). Changes in the marine nitrogen inventory controlling past changes in CO_2 levels is, however, thought to be unlikely (Gruber, 2004). On the other hand the greenhouse gas nitrous oxide (N_2O) is an intermediate in the oxidation of ammonium to nitrate (nitrification) and reduction of nitrate to N_2 (denitrification) in the

ocean, and could thus potentially also directly influence Earth's climate. N_2O has undergone large variations synchronous with rapid climatic changes (Fluckiger et al., 2004). Denitrification in the Arabian Sea was proposed to be one possible source contributing to the observed changes in atmospheric N_2O (Suthhof et al., 2001). The anammox reaction, however, does not involve the liberation of N_2O as an intermediate. A substantial contribution of anammox in converting fixed nitrogen into gaseous N_2 in the Arabian Sea in the past makes it thus unlikely that denitrification has contributed substantially to the changes in the atmospheric N_2O but rather that nitrification might be the dominant oceanic source for atmospheric N_2O.

8.5. Conclusions

The detection of fossil ladderane lipids in our record shows for the first time that anaerobic ammonium oxidation has been occurring in the Arabian Sea at least over the last 140 kyrs. Concurrent variations in $\delta^{15}N$ and abundance of ladderane lipids reflect changes in anammox intensity in the OMZ, leaving an imprint on the $\delta^{15}N$ signature. Anammox was, therefore, also responsible for losses of fixed nitrogen from the Arabian Sea, and might be responsible for the suggested imbalance in the local marine nitrogen budget. Further studies including better constraints on nitrogen isotopic fractionation during anammox are needed to assess the extent to which anammox contributes to the production of N_2 in the present-day OMZ, and thus to better understand past, present, and future changes in the ocean's nitrogen cycle.

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Synthesis

Since the first detection of anammox in the natural environment about five years ago, anammox has been found to be omnipresent in the ocean where oxygen concentrations are low, and a key player in the marine nitrogen cycle. There are various different markers for the detection of anammox, i.e. fluorescence in-situ hybridization (FISH), quantitative polymerase chain reaction (qPCR), nutrient profiles, ^{15}N-labelling experiments, and ladderane lipids. By combining these different techniques, a better understanding of the distribution, abundance, and activity of anammox bacteria in the environment has been obtained, although still many environments remain to be studied for the presence of anammox. Therefore, in this thesis a number of environments not previously investigated were examined for the presence and activity of anammox bacteria. This showed that there is a potential important role of anammox bacteria in the nitrogen cycle of hot spring environments. In fact, the results suggest that these organisms might actually also be thermophilic, which is supported by the recent detection of anammox in hydrothermal vents (Byrne et al., 2008). This, however, requires further research, for instance isolation and cultivation of the potential anammox bacterial species that was detected in a hot spring where no known anammox bacteria were detected but where ladderane lipids were found in relatively high abundance. Furthermore, ^{15}N-labelling experiments could give more detailed information about potential anammox activity and importance of anammox bacteria in the nitrogen cycle of these hot springs.

The detection of ladderane lipids in water and trap samples from the Arabian Sea for the first time indicated the presence of anammox bacteria in the oxygen minimum zone (OMZ) of the Arabian Sea. The OMZ of the Arabian Sea is one of the largest present-day OMZ and therefore believed to significantly impact the global nitrogen cycle. The results are in agreement with other studies showing the presence of anammox in different OMZs (Kuypers et al., 2005; Hamersley et al., 2007). Although the results show its presence, no information was obtained yet on its relative importance. Detailed investigation of the OMZ, for example using FISH, intact ladderane lipids, and ^{15}N-labelling experiments, are required to identify the organisms, locate the actual zone of anammox activity and to assess the actual importance of anammox as a sink for nutrient N in this area. Besides OMZs, sediments on the

continental slope and in the deep sea are assumed to be responsible for about 50% of the sediment N_2 production. Anammox so far has been found to be a sink for fixed nitrogen in a number of shelf sediments, contributing significantly (up to 70%) to the production of gaseous N_2 (Thamdrup and Dalsgaard, 2002). Evidence for anammox in deep slope sediments is provided in this thesis, and the apparent increase of anammox bacteria in sediments at greater water depths empasizes the need for further, more detailed mapping of anammox in the deep ocean. Hence, anammox may also be a ubiquitous process in sediments of the deep ocean.

The molecular phylogeny of the 16S rRNA genes suggest that *Planctomycetes*, to which anammox bacteria belong, are one of the earliest branches of the bacterial domain (Brochier and Philippe, 2002). Anammox, therefore, is believed to be a process that originated relatively early in the evolution of life on our planet. Therefore, anammox, probably affected the oceanic nitrogen cycle already for hundreds if not thousands of millions of years. For example, during several periods in Earth history, when parts of the oceans experienced anoxic conditions, so-called oceanic anoxic events during the Jurassic and Cretaceous, anammox could have been contributed to the loss of nutrient nitrogen from the ocean. Since anammox bacteria leave no inorganic fossil remains, molecular fossils are likely the only tool to trace their past occurrence. For tracing the past occurrence of anammox, ladderane lipids would be very suitable biomarkers. However, the fate of ladderane lipids after cell death was unknown and their peculiar chemical structure, i.e. the highly strained cyclobutane moieties, suggests that they are thermally labile and will not survive for substantial periods of time in the earth crust. One of the aims of this thesis was to better understand the behaviour of ladderane lipids upon degradation processes and to assess its suitability as a unique biomarker for anammox bacteria in present-day environments and ancient sediments. New, highly sensitive HPLC-MS/MS methods were developed and enabled us to detect ladderane lipids in environmental samples where they are presentin very low concentrations in the range of pg to ng g^{-1} sediment.

Thermal degradation experiments performed with anammox biomass revealed that ladderane lipids are indeed thermally labile, although less than initially thought. It also revealed that that more stable alteration products are formed that can theoretically be used as anammox markers as they are predicted to be present in ancient immature sediments. In addition, the 10-methyl hexadecanoic acid might constitute an alternative biomarker for anammox in past settings, as it is more abundant and less prone to

degradation. Although not unique only to anammox bacteria, its highly depleted carbon isotopic signature makes it a suitable marker for past anammox. Application of these potential markers in ancient immature sediments, however, will require new analytical methods for the detection of the thermal degradation products. The probably very low concentrations of these compounds in natural samples, caused by the fact that anammox divide very slowly, might hinder their detection.

Evidence that ladderane lipids are settling to the sea floor was obtained for the Arabian Sea, suggesting that they potentially may fossilize in the sediment. Indeed, fossil ladderane lipids were detected in a sediment core from the Arabian Sea. The ladderane lipid concentration varied in concert with nitrogen stable isotopic concentration ($\delta^{15}N$) which is believed to be an indicator for the intensity of denitrification, and thus OMZ intensity. If anammox bacteria cause a strong nitrogen isotopic fractionation in a similar way denitrifiers do, they could also influence the $\delta^{15}N$. This indicates that there is a need to resolve how anammox exactly fractionates nitrogen and to which extent it contributes to the natural $\delta^{15}N$ signal observed in marine sediments. This would be crucial for the interpretation of many $\delta^{15}N$ records currently used to reconstruct past intensity of OMZ and nutrient status. From our investigations it was also clear that degradation of ladderanes took place deeper in the core and investigations of other cores underneath OMZs or anoxic zones are needed to confirm that intact fossil ladderane lipids might be suitable biomarkers, at least for Quaternary sediments. Likely for more ancient sediments, thermal degradation products of ladderanes need to be used as tracers for past anammox.

This work here clearly showed that ladderane lipids have an excellent potential as biomarker lipids for anammox bacteria and that the anammox process is widespread. These lipids might also be useful as markers for past anammox in ancient immature sediments, and initial studies on the degradation products of ladderanes have provided clues for the search of diagenetic products of anammox lipids.

Summary

Anammox, the anaerobic oxidation of ammonium to dinitrogen gas with nitrite as the electron acceptor, constitutes a novel route to convert biologically available (fixed) nitrogen to gaseous N_2. This process is mediated by specific bacteria belonging to the Planctomycetes that were initially discovered in waste water systems. Within the nine years after their discovery, anammox bacteria have been identified as key players in the global nitrogen cycle. They have been found in different suboxic to anoxic environments, including oxygen minimum zones, marine and freshwater sediments, tropical lakes and even in sea ice. Anammox is now acknowledged as an important process for the removal of fixed inorganic nitrogen from the oceans, freshwater and wastewater treatment systems, which was so far solely attributed to heterotrophic denitrification. However, relatively little is known about anammox bacteria, specifically their unusual biology and the mechanisms regulating their occurrence in the natural environment. The potential importance of anammox in past settings is also an open question.

Anammox bacteria contain a specific intracellular compartment, the anammoxosome, in which anammox catabolism is thought to take place. The membrane of this organelle is composed of unique ladderane lipids containing either three or five linearly fused cyclobutane rings. Ladderane lipids provide an usually dense membrane which is thought to protect the remainder of the cell from the toxic intermediate of the anammox reaction, hydrazine. Their unusual chemical structure which is so far unprecedented in nature, make ladderane lipids ideal markers for the detection of anammox bacteria. This thesis aimed to get a better understanding of the presence and distribution of ladderane lipids as a marker for anammox in different present day environmental settings, to examine processes of transport, preservation and early diagenesis of ladderane lipids as well as to elucidate their potential suitability as a tracer for past anammox processes.

Anammox was up to now only documented in mesophilic environments such as waste waters, oceans, and freshwater systems. Analysis of ladderane lipids and 16S rRNA genes in terrestrial hot springs from California and Nevada (USA) revealed that anammox bacteria can also thrive at thermophilic temperatures. These anammox bacteria were phylogenetically related to the wastewater species Kuenenia and Brocadia

rather than Scalindua which is abundant in the marine environment. Anammox bacteria may form an important and as yet undiscovered link in the nitrogen cycle of hot spring environments.

The oxygen minimum zone (OMZ) in the Arabian Sea, with its thickness of 1,000 m and wide geographical extent, is one of largest OMZs presently found in the oceans. In this OMZ a substantial part of the global nitrogen loss takes and was so far fully attributed to denitrification. Analysis of suspended particulate matter (SPM) derived from a transect offshore Oman in the northwestern Arabian Sea revealed that ladderane lipids are present throughout the water column and most abundant in the upper part of the OMZ, indicating the presence of anammox bacteria. Fluxes of ladderane lipids, derived from ladderane lipid concentrations in sediment particles, revealed a strong seasonal pattern, with highest fluxes during the southwest monsoon. This indicated enhanced anammox activity during the monsoon-driven high-productivity, high-vertical flux period and showed that the anammox biomarker signal is transported to the sediment.

Ladderane lipids, along with a range of other techniques, were applied to investigate anammox in sediments along transects of the continental shelf (50-100 m) and the continental slope down to 3,000 m in the North Atlantic. The combined data showed that anammox bacteria were present at all sites. A specific ladderane monoether with a phosphocholine (PC) headgroup was for the first time applied as a marker for intact anammox cells in marine sediments. As the polar headgroup is rapidly being hydrolyzed after cell death, intact lipids are in general good indicators for living organisms. The abundances of the intact ladderane monoether-PC lipid and 16S rRNA gene copy numbers of anammox bacteria both increased in sediment cores located at greater water depth and were negatively correlated to pore water ammonium concentrations, indicating higher abundances of anammox bacteria in sediments with low carbon mineralization rates. In sediments underlying the OMZ off northwest Africa, increasing ladderane core lipid concentrations upslope indicate a contribution of fossil lipids derived from anammox bacteria in the oxygen depleted water column, especially at shallower water depths and closer to the coast. This is different from what has been observed in slope sediments further north in the Atlantic, where the water column is fully oxic. Anammox, besides traditional denitrification, constitutes an important process removing nitrogen from marine sediments deposited on continental margin, and especially in deeper slope sediments.

Organic matter produced in the water column and in the sediment, including lipids, is degraded to a large extent leaving typically only <1% to be preserved in the sediment and modifying the structures of lipids. To study the effect of early diagenesis on ladderane lipids, anammox bacterial biomass was incubated with marine sediments under oxic conditions and at elevated temperatures to simulate early diagenetic processes occurring at the sediment-water interface as well as thermal degradation during subsequent sediment burial. The most abundant products formed were ladderane lipids with a shorter alkyl chain, most pronounced at 40°C. These products were not formed when the sediments were sterilized, indicating that ladderane lipids were microbially degraded via a beta-oxidation pathway. A new high performance liquid chromatography (HPLC) method was developed for the detection of these specific short-chain ladderane degradation products. Detection of these biodegraded ladderane lipids in various marine sediments indicates that these compounds are also produced in the natural environment and can potentially be used as tracers for anammox.

Anammox cell material was artificially matured using hydrous pyrolysis at constant temperatures ranging from 120 to 365°C to study the thermal stability of ladderane lipids during simulated progressive diagenesis and catagenesis. Despite their fast destruction already at the lowest experimental temperature, ladderane lipids were found to form thermally more stable products that were still detectable at 260°C. Maturity levels, based on hopane isomerization ratios, were further determined to predict at which biomarker maturity ladderane products might still be present. Based on these experimental findings, ladderane alteration products might constitute suitable markers for past anammox in immature ancient sediments and sedimentary rocks.

Fossil, intact ladderane lipids were detected in a sediment core from the northern Arabian Sea spanning ca. 130,000 years. This provided evidence for past anammox activity for the first time. Calculations of the NL_5, a temperature related index based for the relative chain length of ladderane lipids, indicated that the source for ladderane lipids was mainly from the water column, although sedimentary anammox might have contributed to the ladderane pool, especially during cold periods with a severely diminished OMZ. Concurrent variations in ladderane lipid abundances, nitrogen stable isotopes ($\delta^{15}N$) and total organic carbon (TOC) suggested that changes in anammox productivity were in concert with OMZ intensity and surface productivity over the last glacial cycle. The correlation between ladderane lipids and $\delta^{15}N$ further indicates that anammox might also cause strong isotopic fractionation, influencing the

$\delta^{15}N$ signal in a similar way as denitrification. This is crucial for the interpretation of sedimentary $\delta^{15}N$ and for the understanding of past changes in the marine nitrogen cycle.

To conclude, the present study showed the general applicability of ladderane lipids as tracers for anammox bacteria in different present-day environmental settings. It also contributed to a better understanding of the behavior of ladderane lipids upon microbial degradation and thermal maturity and showed that diagenetic products of ladderane lipids are probably suitable biomarkers for the detection of anammox in ancient sediments, such as Cretaceous black shales. This, however, remains a challenge for future studies. The detection of intact core lipids in Arabian Sea sediments provided already insight in past anammox activity.

Samenvatting

Anammox, de anaërobe oxidatie van ammonium met nitriet als electronenacceptor, is een nieuwe route in de stikstofcyclus waarbij stikstof in een vorm beschikbaar voor de meeste organismen ('biologisch beschikbaar stikstof') omgezet wordt tot stikstofgas. Dit proces wordt uitgevoerd door bacteriën die tot de planctomyceten behoren. Deze bacteriën zijn ongeveer tien jaar geleden voor het eerst in afvalwaterzuiveringsinstallaties ontdekt. Sindsdien is vast komen te staan dat anammox bacteriën een zeer belangrijke rol spelen in de globale stikstofkringloop. Anammox bacteriën zijn aangetroffende in sub-oxische en anoxische milieu's, in de zuurstofminimumzones van oceanen, in mariene en lacustriene sedimenten, in tropische meren en zelfs in zeeijs. Anammox wordt momenteel gezien als een proces dat een belangrijke rol speelt bij de verwijdering van biologisch beschikbaar stikstof uit de oceaan, uit zoetwatersystemen en in afvalwaterzuiveringsinstallaties, daar waar tot voor kort heterotrofe denitrificatie gedacht werd een sleutelrol te spelen. Er is echter nog steeds maar weinig bekend over anammox bacteriën, met name met betrekking tot de bijzondere biologie van deze organismen en de mechanismen die het voorkomen van anammox bacteriën in de natuur reguleren. Of anammox ook in vroegere mileu's een rol gespeeld heeft, is tevens een open vraag.

Anammox bacteriën bevatten een specifiek intracellulair compartiment, het anammoxosoom genaamd, waar het anammox catabolisme zich afspeelt. Het membraan van dit organel bestaat uit unieke ladderaan lipiden die opgebouwd zijn uit 3 of 5 lineair gecondenseerde cyclobutaaneenheden. Deze ladderaan lipiden vormen een ongewoon dicht membraan dat mogelijk de rest van de cel beschermt tegen het toxische intermedair (hydrazine) van de anammox reactie. De unieke structuur van deze lipiden maken de ladderanen uitstekende 'markers' voor de detectie van anammox bacteriën. In dit proefschrift worden ladderaan lipiden gebruikt als tracers voor anammox bacteriën in verschillende milieu's. Tevens wordt het transport van ladderaan lipiden uit de waterkolom van de oceaan naar het sediment, en de preservatie en vroege diagenese van ladderaan lipiden bestudeerd om te kijken of fossiele ladderanen gebruikt zouden kunnen worden als tracers van activiteit van anammox bacteriën in het geologisch verleden.

Het voorkomen van anammox was tot op heden alleen bekend in mesofiele milieu's zoals afvalwater,en mariene en zoetwatersystemen. De analyse van ladderaan lipiden en 16S rRNA genen in heetwaterbronnen in Californië en Nevada (USA) lieten zien dat anammox bacteriën ook onder thermofiele condities kunnen bestaan. De gevonden anaammox bacteriën waren verwant aan de soorten *Kuenenia* en *Brocadia*, die in afvalwater voorkomen, maar niet aan de mariene soort *Scalindua*. Dit toont aan dat anammox bacteriën mogelijk een belangrijke rol spelen in de stikstofkringloop van heetwaterbronnen.

De zuurstofminimumzone (ZMZ) in de Arabische Zee is ongeveer 1000 m dik en beslaat een groot oppervlak en is daarmee één van de grootste ter wereld. In deze ZMZ wordt biologisch beschikbaar stikstof omgezet in stokstofgas door, naar men aannam, heterotrofe denitrificatie. Dit stikstofverlies in de Arabische Zee draagt significant bij aan het totale stikstofverlies in de oceanen. Analyse van ladderaan lipiden in gesuspendeerd particulair materiaal bemonsterd van een transect vanaf de kust van Oman in de noordwestelijke Arabische Zee liet zien dat anammox bacteriën overal voorkomen maar in hoogste concentraties in het bovenste gedeelte van de ZMZ. De flux van ladderaan lipiden, bepaald door analyse van zinkende deeltjes bemonsterd met een sedimentval, liet een sterk seizonaal patroon zien, met de hoogste fluxwaarden tijdens de zuidwestelijke moesson. Dit duidde op een hogere activiteit van anammox bacteriën gedurende de periode van opwelling van nutriënt-rijk bodemwater en de daarmee gepaard gaande toename in primaire productiviteit. De sedimentval data laten zien dat ladderaan lipiden kunnen sedimenteren en dus mogelijk gebruikt kunnen worden om het voorkomen van anammox in de vroegere oceaan vast te stellen.

Ladderaan lipiden, samen met tal van andere methoden, werden toegepast om het voorkomen van anammox in sedimenten van het continentale plat (50-100 m) en de continentale helling tot zo'n 3000 m diepte in de Noord Atlantische Oceaan te onderzoeken. Anammox bacteriën werden in alle onderzochte sedimenten aangetroffen. Een specifiek ladderaan lipide dat een fosfocholine groep bevatte werd voor het eerst als een marker voor intacte anammox cellen in mariene sedimenten gebruikt. Intacte membraanlipiden zijn goede markers voor "levende"cellen omdat de polaire kopgroepen snel verdwijnen door hydrolyse wanneer een cel afsterft. De concentratie aan intacte ladderaan lipiden en het aantal copiën van het 16S rRNA gen van

anammoxbacteriën namen beide toe met de diepte in sedimentkernen genomen op grotere diepte en waren negatief gecorreleerd met de ammoniumconcentratie in het poriewater. In de sedimenten beneden de ZMZ ten noordwesten van Afrika was in ondieper water nabij de kust een grotere bijdrage van fossiele ladderaan lipiden (dat wil zeggen zonder polaire kopgroep) waarschijnlijk afkomstig van anammox bacteriën uit de ZMZ. Dit was niet het geval in sediment van een transect meer naar het noorden in de Atlantische Oceaan waar de waterkolom volledig oxisch was. De data laten zien dat anammox, naast de traditionele denitrificatie, een belangrijk proces is voor de verwijdering van biologisch beschikbaar stikstof uit sedimenten van het continentale plat maar, vooral, de continentale helling.

Een groot gedeelte van het organisch materiaal, inclusief de lipiden, wordt afgebroken. In de meeste gevallen wordt slechts minder dan 1 % van het materiaal dat geproduceerd wordt gepreserveerd in mariene sedimenten. Daarnaast vinden tijdens de vroege diagenese ook vaak veranderingen in de structuur van lipiden plaats. Om het effect van vroege diagenese op ladderaan lipiden te onderzoeken werd anammox biomassa geïncubeerd met vers marien sediment uit de Waddenzee onder oxische condities bij verhoogde temperatuur. Hiermee werd zowel getracht vroeg diagenetisch processen in de eerste meters van het sediment als thermische degradatie tijdens de diepere begraving in de aardkorst te simuleren. De belangrijkste producten die gevormd werden, vooral bij 40°C, waren ladderaan lipiden met een verkorte alkyl zijketen. Deze producten werden niet gevormd wanneer het mariene sediment vaarafgaand aan de incubatie werd gesteriliseerd. Dit gaf aan dat zij door microbiologische β-oxidatie gevormd zijn. Een nieuwe HPLC-MS methode werd ontwikkeld om de gebiodegradeerde ladderaan lipiden te kunnen detecteren in complexe matrices. Toepassing van deze methode op mariene sedimenten toonde aan dat deze gebiodegradeerde ladderanen ook van nature voorkomen en mogelijk gebruikt kunnen worden als tracers voor anammox.

Anammox biomassa werd ook blootgesteld aan artificiële maturatie door middel van pyrolyse in de aanwezigheid van water bij constante temperatuur variërend van 120 tot 365°C gedurende drie dagen om de thermische stabiliteit van ladderaan lipiden tijdens dia- en catagenese te bestuderen. Hoewel ladderaan lipiden reeds bij de laagst toegepaste temperatuur in aanzienlijke mate werden afgebroken, werden er

thermisch stabiele omzettingsproducten gedetecteerd tot een temperatuur van 260°C. Met behulp van de isomerisatie van hopanoïden kon een maturatiegraad vastgesteld worden waar beneden de thermische producten van ladderanen nog steeds gedetecteerd kunnen worden. Op basis van deze experimentele bevindingen kan geconcludeerd worden dat ladderaan lipiden in potentie gebruikt kunnen worden in relatief thermisch onrijpe sedimenten om het voorkomen van anammox in het verleden te traceren.

Fossiele ladderaan lipiden werden gedetecteerd in tot 130.000 jaar oude sedimenten van een kern uit de noordelijke Arabische Zee en verschaften hiermee voor het eerst bewijs dat ook in het Aardse verleden anammox bacteriën actief geweest zijn. Deze fossiele ladderaan lipiden zijn zeer waarschijnlijk afkomstig van anammox bacteriën uit de ZMZ, hoewel een kleine bijdrage van anammox bacteriën uit het sediment niet uitgesloten kan worden vooral in tijden met een minder sterke ZMZ. Bewijs voor een afkomst uit de ZMZ werd geleverd door de bepaling van de NL_5, een temperatuur-gerelateerde index op basis van de laderaan lipiden distributie. Ladderaan concentraties correleerden positief met $\delta^{15}N$ en TOC waarden, hetgeen suggereert dat variaties in de intensiteit van de ZMZ, primaire productie en anammox activiteit over de laatste glaciale cyclus met elkaar samenhangen. Dit impliceert bovendien dat ook anammox leidt tot een sterke isotoopfractionering van stikstof, net als denitrificatie, hetgeen van groot belang is voor de interpretatie van het sedimentaire $\delta^{15}N$ signaal en de daaraan gekoppelde vroegere veranderingen in de stikstofkringloop.

Het werk beschreven in dit proefschrift heeft aangetoond dat ladderaan lipiden bruikbare tracers voor anammox activiteit in tal van veschillende milieu's zijn. Zowel microbiologische degradatie als thermische maturatie heeft invloed op de structuur van ladderaan lipiden maar desalniettemin kunnen de producten van deze processen nog steeds als anammox markers gebruikt worden. Detectie van fossiele anammox lipiden in sedimenten uit de Arabische Zee verschaftte informatie over vroegere anammox activiteit. Toekomstige studies, mede gebaseerd op de resultaten van dit proefschrift, zullen echter nog moeten aan tonen tot hoever we terug kunnen gaan in de geologische tijd. De zwarte schalies afgezet in het Krijt vormen daartoe een grote uitdaging.

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Curriculum Vitae

Andrea Jaeschke was born on the 12th of June 1972 in Bremen, Germany. After finishing high school, she attended technical school in Bremen and afterwards was working for five years as a laboratory technician in the departments of Biology and Glaciology of the Alfred-Wegener Institute (AWI) in Bremerhaven, Germany. She started to study Geology at University of Bremen in 1997 and obtained her masters in 2004. From August 2004 to October 2008 she completed her PhD project at the Royal Netherlands Institute for Sea Research (NIOZ) in the department of Marine Organic Biogeochemistry, under the supervision of Prof. Dr. Ir. Jaap Sinninghe Damsté and Dr. Ir. Stefan Schouten.

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