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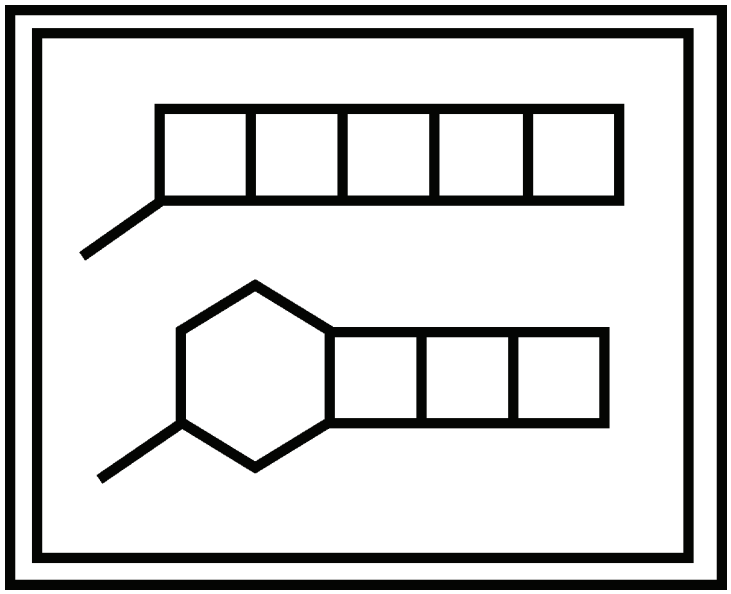
# Ladderane Lipids in Anammox Bacteria: Occurrence, Biosynthesis and Application as Environmental Markers



Jayne E. Rattray

# Ladderane Lipids in Anammox Bacteria: Occurrence, Biosynthesis and Application as Environmental Markers

Ladderaan Lipiden in Anammoxbacteriën: Voorkomen, Biosynthese en Toepassing als Tracers in het Milieu
(met een samenvatting in het Nederlands)

## Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
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op dinsdag 20 mei 2008 des middags te 4.15 uur

door

**Jayne Elizabeth Rattray**

geboren op 10 november 1975 te Dundee (Schotland)

| | |
|---|---|
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The Predator

*Small you may be and unseen by the eye,*
*You feed on fear but you cannot fly.*
*You don't stab, you don't run, or gnaw.*
*The end result could it be, the death of us a'!*

JR the older November 2006

To a mouse

*The best laid schemes o' mice an' men,*
*Gang aft agley.*
*An' lea'e us nought but grief an' pain,*
*For promis'd joy!*

Robert Burns, on turning her up her nest with the plough,
November, 1785

*aan Mark en Ukkie*
*to mum and dad*

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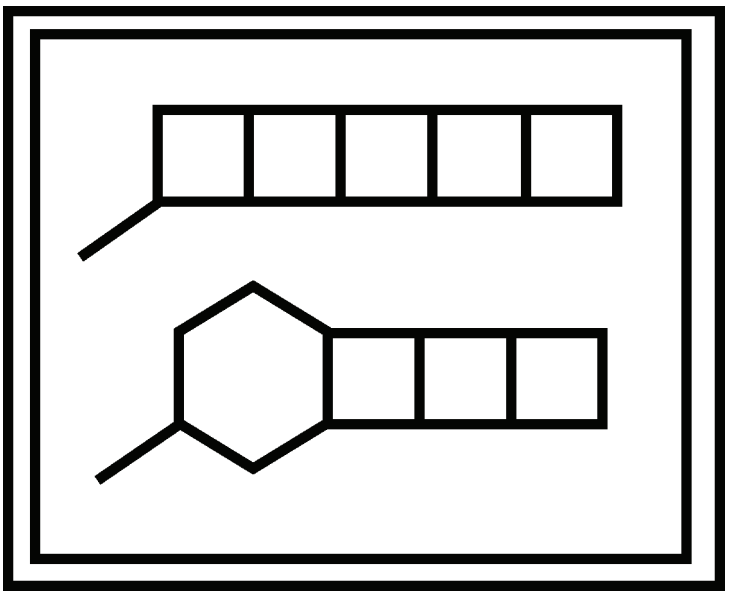
# table of contents



# chapter 1

## General introduction and thesis outline

### 1.1 Marine microbes

The world's oceans are teeming with complex assortments of microorganisms. Despite their small size, they are responsible for regulating the global biochemical cycling of several key elements such as carbon (C), nitrogen (N) and sulphur (S). Different types of cellular organisms can be classified into the three domains of life, based on their ribosomal RNA gene sequence, a gene common to all living organisms. These domains are the eukaryota, which include humans, animals and plants and are defined by having a membrane bound nucleus and other organelles. The other two domains of life, the archaea and bacteria, are both prokaryotic (lacking a cell nucleus) but are different from each other based on their genetic and biochemical make-up. Currently, classification into domains and species is primarily based on the rRNA gene (16S or 18S rRNA) phylogenies, where changes in the composition of rRNA over long periods of time generally correspond to changes in evolution (Woese and Fox, 1977). These three domains are however not static and it is known that viruses, which are not cellular, can affect oceanic biogeochemical cycling by influencing the mortality rates of cellular organisms (Suttle, 2007). In addition, gene loss, recombination, duplication and gene creation also cause exchange of information to occur between different species and even the different domains. Therefore, over time a large diversity of microbial species have evolved in the worlds oceans, most of which (and their corresponding roles in biogeochemical cycling) still remain to be discovered (Pedros-Alio, 2007).

Several molecular techniques are available for the identification and enumeration of *in situ* organisms. A commonly used technique is fluorescence *in situ* hybridization (FISH) where cells are hybridized with fluorescently labelled oligonucleotide probes which target the 16S rRNA, subsequently, cells can be viewed using epifluorescence microscopy (Amann et al., 1995). Quantitative (or real-time) PCR is a technique used to quantify the amount of DNA or RNA copies from microbes in different environments (Bassler et al., 1995). For both FISH and qPCR techniques, the use of species-specific primers and probes means these techniques can be used to discriminate between different microbial species. Alternatively, if a particular

(putative) gene is identified to encode an enzyme performing a specific process, in a specific organism, this can be used as a tracer for both the organism and process (Könneke et al., 2005). Metagenomic studies of ocean environments have also been successful in identifying genes and organisms responsible for particular biogeochemical processes (Venter et al., 2004).

A different type of technique commonly used to trace microorganisms in a particular environment is by the analysis of their membrane lipids. There are a wide range of membrane lipids, many of which are common to many organisms, but occasionally, lipids are found which are unique to only a limited number of species or genera, these are termed as 'biomarker lipids'. Although they are relatively rare, lipid biomarkers can be used to trace individual organisms in complex natural environments, e.g. sediments. Lipid membranes encapsulate the cell contents, and form a semi-permeable barrier, which allows the entry of essential nutrients and beneficial chemicals, and the exit of waste or toxic substances. Cell membranes must also therefore be able to adapt to variables in the surrounding environment e.g. temperature or salinity. The relationship between membrane lipids and temperature has been successfully applied in paleo-environmental studies as a method of reconstructing past sea surface and continental temperatures (Brassell et al., 1986; Schouten et al., 2002; Weijers et al., 2007).

Marine microorganisms are often difficult to isolate which makes it difficult to study their physiology. Therefore, the metabolic functioning of microorganisms in a particular environment needs to be studied *in situ*. Metabolic activity can be partly elucidated using isotope-labelling experiments. The addition of labelled substrates to a microbial population can often allow us to determine the rates of turnover of a particular element in that system. For example, in the nitrogen cycle, labelled substrates of inorganic nitrogen ($NO_2^-$, $NH_4^+$ or $NO_3^-$) are added to the system, a sample is taken, the microbial activity is then terminated and the amount of labelled gas produced is measured to give an estimate of the rate of nitrogen cycling in the system (Dalsgaard and Thamdrup, 2002; Nielsen, 1992; Seitzinger, 1988). Alternatively, insight into the large 'uncultivated majority' of microbial species in a particular population can be gained using stable isotope probing of DNA (Radajewski et al., 2000), RNA (Manefield et al., 2002a; Manefield et al., 2002b) or phospholipids

(Boschker et al., 1998; Wuchter et al., 2003). Further information about specific metabolic pathways (e.g. carbon or nitrogen acquisition) used by an organism can also be gained by analysing the natural stable isotopic composition of their lipids in relation to the total biomass and substrates (Guy et al., 1993; Madigan et al., 1989; Schouten et al., 2004).

## 1.2 The marine nitrogen cycle

### *1.2.1 Nitrogen fixation*

The marine nitrogen cycle is an example of an elemental cycle, which is predominantly mediated by microbes. The most abundant natural source of nitrogen available to the marine environment is the atmosphere (Fig. 1).

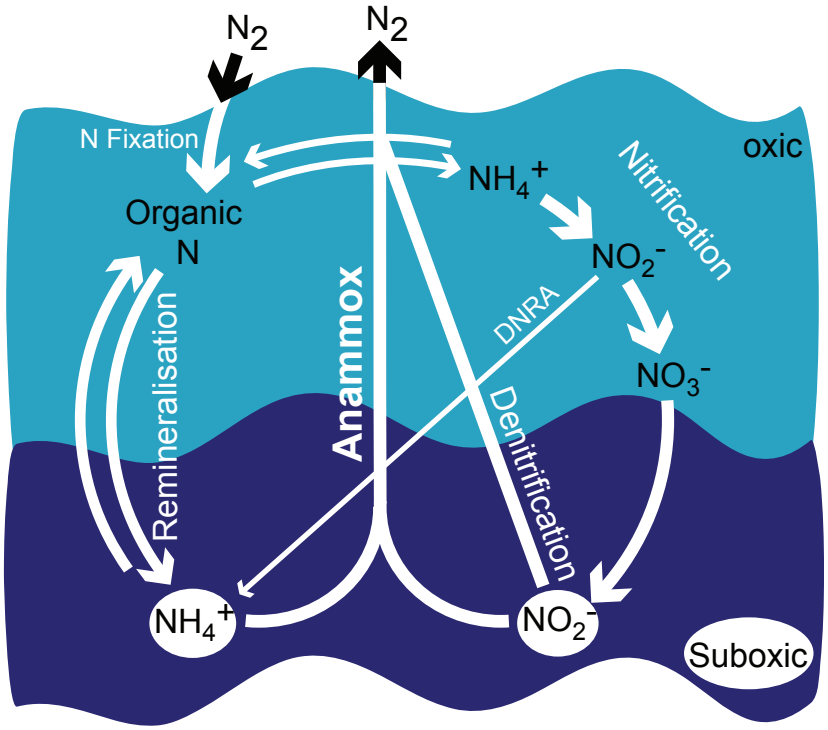



**Figure 1.** Simplified version of the marine nitrogen cycle following the discovery of the anammox reaction. DNRA – disimilatory nitrite reduction to ammonium.

Biologically available nitrogen is a major factor limiting primary production because most organisms cannot use molecular nitrogen gas ($N_2$). Atmospheric $N_2$ enters the water phase through the process of dissolution, where it can then be taken up and reduced by cyanobacteria residing in the photic zone. The cyanobacteria use this essential fixed nitrogen for the synthesis of amino acids and proteins. Stable isotope

measurements of cyanobacterial production have verified that roughly half of the organic nitrogen in the surface ocean originates from atmospheric $N_2$ (Capone et al., 2005). A major factor regulating nitrogen fixation is thought to be the ratio of nitrogen to phosphorus in seawater (Deutsch et al., 2007). Subsequently, when the $N_2$ fixers die and the cell remnants degrade to release inorganic ammonium, during the process of remineralisation. Under oxic conditions, this ammonium can be rapidly taken up and recycled in the upper ocean by planktonic communities who in turn degrade to re-release inorganic nitrogen compounds (Suttle et al., 1990).

### *1.2.2 Anthropogenic nitrogen inputs*

Anthropogenic sources of nitrogen to the ocean environment also occur, namely in the form of run-off from nitrate fertilizers used in agriculture and ammonium from sewers and untreated wastewater. In addition, the atmospheric deposition of fossil fuel derived nitrates to open oceans and coastal regions may result in elevated primary production, and indirectly lead to the elevated acidification of coastal regions (Doney et al., 2007). The true impact of anthropogenic inputs of nitrogen on the oceanic nitrogen cycle is yet to be properly understood.

### *1.2.3 Nitrification*

Fixed nitrogen in the form of ammonium can also be converted via aerobic oxidation to nitrite and nitrate, during the process of nitrification. Until recently, nitrification was thought to be carried out by members of the bacterial genera *Nitrosomonas*, *Nitrosospira* and *Nitrosococcus* (Purkhold et al., 2000). However, marine Crenarchaeota, which are some of the most abundant organisms in the world's oceans, have also been shown to aerobically oxidize ammonium (Könneke et al., 2007; Wuchter et al., 2006). In the natural environment, crenarchaeota are commonly identified by determining the number of copies of the gene archaeal *amoA*, which encode for the archaeal putative ammonia mono-oxygenase alfa subunit (Könneke et al., 2005), or by their characteristic tetraether membrane lipids (Schouten et al., 2000).

### *1.2.4 Denitrification*

Permanent removal of fixed inorganic nitrogen from marine systems occurs via the process of denitrification, which can operate under both aerobic and anaerobic

conditions. Before the discovery of the anammox reaction (see below), denitrification was thought to be the most important removal pathway of fixed inorganic nitrogen from the oceans (Codispoti and Christensen, 1985). Denitrification is carried out by a whole range of organisms including heterotrophic and lithotrophic bacteria, fungi, and archaea, some exclusively performing, and many facultatively performing, denitrification. Recently, benthic foraminifera have also been found to perform complete denitrification (Risgaard-Petersen et al., 2006). Under anaerobic conditions the metabolism of denitrifying organisms use nitrate instead of oxygen as an electron acceptor for the production of energy (equation 1).

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

If denitrification is incompletely carried out, significant amounts of the intermediates nitrite and nitrous oxide may be formed. For example, the oxygen minimum zone of the Arabian Sea has been estimated to contribute up to 18% of the total oceanic nitrous oxide emission (Law and Owens, 1990; Naqvi and Noronha, 1991). Nitrous oxide is a powerful greenhouse gas, therefore the removal of oceanic fixed nitrogen via incomplete denitrification, can impact both the ocean and atmosphere (Stein and Yung, 2003).

## 1.3 Anammox

In 1977, an Austrian physicist proposed the existence of two undiscovered types of lithotrophic bacteria, based fully on thermodynamical grounds (Broda, 1977). One of the bacteria was described to be a photosynthetic anaerobic ammonia oxidizer, analogous to coloured sulphur bacteria, the second was a chemosynthetic bacteria that could oxidize ammonia to $N_2$ using nitrate or oxygen as the electron acceptor. The latter of which (using nitrate or nitrite as the electron acceptor) has come to be known as the *an*aerobic *amm*onium *o*xidation (anammox) reaction. In a study on a highly stratified anoxic fjord, Richards et al. (1965) observed an unexplainable loss of ammonium. Unfortunately this study lacked sufficient evidence to prove the biological nature of the reaction and at the time, the general consensus was that ammonium was an inert substance under anoxic conditions and could not undergo oxidation. However, three decades later, Mulder et al. (1995) observed the anaerobic loss of ammonium from a denitrifying bioreactor and attributed it to

anammox. The anammox reaction was subsequently defined (van de Graaf et al., 1995) as involving the combination of ammonium and nitrite (the electron acceptor) resulting in the release of $N_2$ (equation 2).

$$1\ NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ = 1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}\ N_{0.15} + 2.03\ H_2O$$

(equation 2)

More recently however, anammox bacteria have been found to be versatile substrate users, with the ability to respire different energy sources with a range of electron acceptors (Guven et al., 2004; Kartal et al., 2007; Strous et al., 2006).

### *1.3.1 Classification of anammox bacteria*

Bacteria performing the anammox reaction were successfully enriched from wastewater and have been classified as chemolithotrophic bacteria of the clade *Planctomycetes* (Strous et al., 1998; Strous et al., 1999). Like all other bacteria classified as *Planctomyetes*, anammox contain special compartments in their cell physiology (Fig. 2). Anammox bacteria are also typified by their unusually slow metabolism, and cell division occurs roughly once a fortnight (Strous et al., 1999).

### *1.3.2 The anammoxosome*

Unlike their closest relatives, anammox bacteria contain a separated membrane bound intracytoplasmic compartment called the anammoxosome, the postulated site of anammox catabolism (Lindsay et al., 2001; van Niftrik et al., 2007; van Niftrik et al., 2004). Since its identification, this organelle has become one of the trademark characteristics used to classify anammox cells, with its identification as the site of the anammox reaction based on confined immunogold hydroxylamine oxidoreductase labelling (Lindsay et al., 2001). During the anammox reaction, a hydroxylamine oxidoreductase-like enzyme has been shown to oxidize the reaction intermediate hydrazine into $N_2$ (Schalk et al., 2000; Strous and Jetten, 2004; van de Graaf et al., 1997).

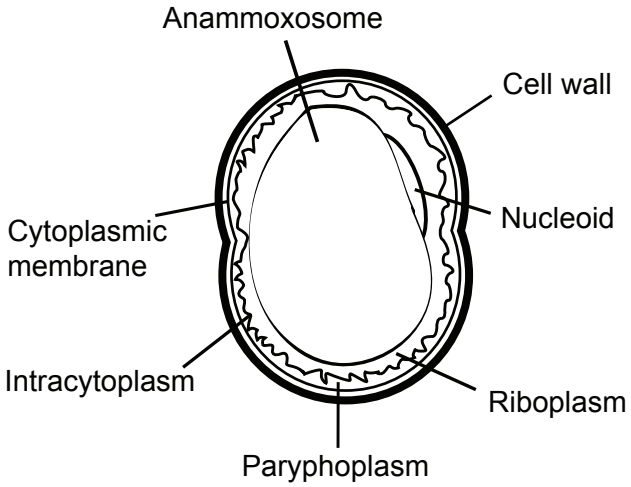



**Figure 2.** Schematic drawing of the anammox cell

### *1.3.3 Anammox cell biochemistry*

In 2002, anammox bacteria were found to contain unusual membrane lipids with cyclobutane rings (Sinninghe Damsté et al., 2002). These 'ladderane lipids' contained either three or five cyclobutane rings linearly fused with *cis* ring junctions (Fig. 3).

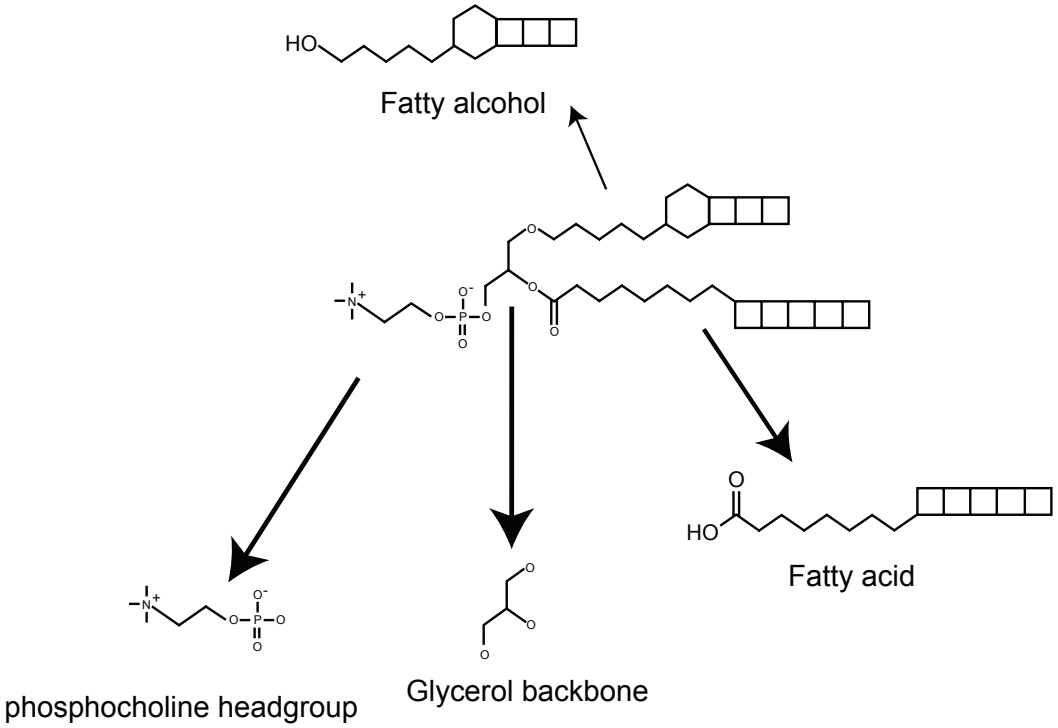



**Figure 3.** Intact ladderane phospholipid lipid with its different structural components

Since these lipids are, so far, unique to anammox bacteria they can be used as biomarkers for anammox cells and the anammox reaction. Ladderane lipids occur in a number of forms, i.e. fatty acids or fatty alcohols, which are ester or ether, bound, respectively, to a glycerol backbone. The occurrence of ether lipids in anammox is unusual, but they have been identified in a few other types of bacteria. However, the question then arises to the functional significance of ladderane lipids. Molecular modelling experiments have shown that these lipids provide an unusually dense membrane (up to 1.6 kg $dm^{-3}$), in comparison to conventional membranes (1.0 kg $dm^{-3}$) (Sinninghe Damsté et al., 2002). In addition, anammoxosomes have been shown to be impermeable to fluorophore treatments, indicating that the anammoxosome membrane was unusually dense (Lindsay et al., 2001; van Niftrik et al., 2007; 2004). The advantage of a biomembrane resistant to diffusion, and thus the evolution of ladderane lipids, could be an adaptation to the slow anammox metabolism, since diffusion of protons and the reaction intermediate hydrazine, is problematic at low rates of enzymatic activity (Sinninghe Damsté et al., 2002). Since linearly concatenated cyclobutane containing ladderane lipids are, currently, unique in nature, the pathway of biosynthesis is completely unknown.

### *1.3.4 Anammox ecology*

Since their initial taxonomic identification in 1999, four genera of anammox bacteria have been successfully enriched and classified; "*Candidatus* Brocadia", "*Candidatus* Kuenenia", "*Candidatus* Anammoxoglobus" and "*Candidatus* Scalindua" (Kartal et al., 2007; Schmid et al., 2007; Schmid et al., 2003; Schmid et al., 2000; Strous et al., 1999) (Fig. 4). The species "*Candidatus* Brocadia anammoxidans", "*Candidatus* Brocadia fulgida", "*Candidatus* Kuenenia stuttgartiensis", "*Candidatus* Anammoxoglobus propionicus", "*Candidatus* Scalindua brodae" and "*Candidatus* Scalindua wagneri" have all been identified in, or enriched from, wastewater treatment sludge or effluent, while "*Candidatus* Scalindua sorokinii" was identified in water column particulate matter in the Black Sea (see below). Based on phylogenetic and genomic analysis, anammox bacteria appear to have a common ancestor, despite the fact that there is a large evolutionary difference between the anammox genera, <85% 16S rRNA gene sequence identity between environmental and wastewater anammox (Jetten et al., 2005).

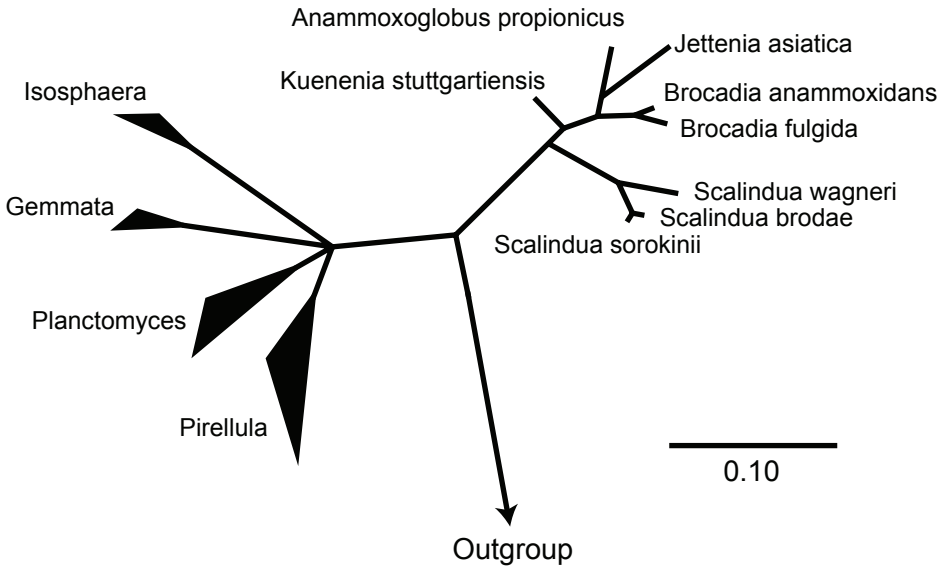



**Figure 4.** Phylogenetic tree of the different anammox genera, based on 16S rRNA (Created by Dr B. Kartal, PhD thesis)

### *1.3.5 Anammox and the marine nitrogen cycle*

Until recently, it was understood that the most important process responsible for the removal of nitrogen from the marine environment was denitrification (see above). However, the discovery of the anammox reaction has changed the way we view the removal of nitrogen under oceanic anoxic conditions. Dalsgaard and Thamdrup (2002) first showed using isotopic labelling techniques that anaerobic $NH_4^+$ oxidation with $NO_2^-$ occurred in sediments via the anammox pathway. In a study on the Black sea, the worlds largest anoxic basin, a combined approach of $^{15}N$ labelling techniques, molecular ecological, and ladderane lipid biomarkers was applied and it was found that the anammox reaction played a key role in water column nitrogen cycling (Kuypers et al., 2003). A subsequent study of anammox in the Benguela upwelling indicated that anammox (and not denitrification) was the dominant process responsible for permanent nitrogen removal from the oxygen minimum zone (Kuypers et al., 2005). Since the discovery of anammox in the natural environment, many studies have reported the occurrence of both the anammox process and the bacteria responsible. Environments investigated range from sediments to water columns (fresh water and saline conditions) and sea ice, covering a wide range of geographical positions as diverse as Antarctica to tropical Africa (Schmid et al., 2007; Schubert et al., 2006). However, it is interesting to note that despite the

apparent global prevalence of anammox bacteria, only members of the "*Candidatus* Scalindua" genus have been, so far, detected in anoxic marine ecosystems (Schmid et al., 2007).

The discovery of the anammox reaction is relatively recent, thus there are still many questions to be answered, regarding the global importance of anammox and its relationship to other processes in the oceanic nitrogen cycle. A recent study using a multi-technique approach has shown how anammox is coupled with both crenarcheal and bacterial nitrification in the Black sea (Lam et al., 2007), which has implications for the way we view the source of nitrogen in the anammox reaction. It has also recently been shown that there is link between nitrogen fixation and nitrogen removal processes, i.e. denitrification in the oceans (Capone et al., 2005; Deutsch et al., 2007; Capone and Knapp, 2007), but it is unknown how the anammox process contributes to this relationship. In addition to the larger ecological questions, techniques used for the detection of anammox bacteria, are still being developed and optimised, and currently nothing is known about the behaviour, biosynthesis or degradation of their unique biomarker ladderane lipids.

What is certain is that the anammox process has caused a major paradigm shift in our understanding of the previously 'well understood' nitrogen cycle, and in turn, its interaction with other biogeochemical cycles. However, due to increasing anthropogenic nutrient release and the onset of climate change, it becomes ever more important that we fully understand the processes and organisms operating in the nitrogen cycle.

## 1.4 Scope and framework of this thesis

The aim of this thesis is to provide information on the distribution of anammox bacteria and, in particular, the behaviour and biosynthetic pathway of their ladderane lipids. This thesis has therefore been divided into two sections, the first examining the distribution and biosynthetic pathway of ladderane lipids in cultured anammox bacteria and in the second part the abundance and distribution of ladderane lipids in different environments.

**Part I Ladderane lipids in cultured anammox bacteria**

**Chapter 2** describes the first successful laboratory cultivation of the marine anammox bacteria, "*Candidatus* Scalindua *spp*". Inoculum sediment from a Swedish fjord was cultured in a sequencing batch reactor and the enriched bacteria were found to metabolise hydrazine, contain a compartmentalised cellular ultrastructure, and synthesise ladderane lipids. In addition, "*Candidatus* Scalindua *spp*" was shown to have a versatile metabolism by using various carbon sources, and reducing nitrate, Fe (III) and Mn (IV). Substrate versatility could be a factor determining the global distribution of the Scalindua genera in marine anoxic ecosystems.

**Chapter 3** outlines the development of a new high performance liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry (HPLC/APCI-MS/MS) technique for the analysis of ladderane lipids in complex matrixes. Thermal degradation of ladderane lipids during gas chromatographic analysis, coupled with low concentrations in environmental samples, e.g. sediments, makes ladderane lipids difficult to identify and quantify. The new HPCL/APCI-MS/MS technique has been demonstrated to be highly sensitive and is thus expected to further aid investigation into ladderane lipids in natural environments.

**Chapter 4** reports on the ladderane core lipid and phospholipid composition of four species of anammox bacteria, each representing one of the four known genera. A range of ladderane phospholipids were found within the anammox bacteria, i.e. different combinations of hydrophobic tail types attached to the *sn*-1 position of the glycerol backbone, in combination with different types of polar headgroup; phosphocholine, phosphoethanolamine or phosphoglycerol (the latter identified for the first time in anammox lipids). In addition, two novel longer chain ladderane lipids were identified. The high abundance of intact ladderane phospholipids in extracts of *K. stuttgartiensis* suggested that ladderane lipids are not solely restricted to the anammoxosome membrane and must also be incorporated in to other membranes.

In **chapter 5** the genome of *K. stuttgartiensis* was examined using bioinformatics techniques with the aim of providing further clues to the pathway of ladderane lipid

biosynthesis. A comparative/ functional genomics analysis of a wide range of genomes showed that genes previously proposed to be involved in the biosynthesis of ladderane lipids may encode a new pathway for the anaerobic biosynthesis of polyunsaturated hydrocarbons (PUHCs). It is further suggested that PUHCs could be processed into ladderane lipids.

**Chapter 6** provides the first experimental insight into the pathway of ladderane lipid biosynthesis. 2-$^{13}$C labelled acetate was fed to an anammox enrichment culture of "*Candidatus* Brocadia fulgida". Labelling patterns of lipids obtained via $^{13}$C-NMR spectroscopic analysis indicated that "*Candidatus* Brocadia fulgida" synthesises $C_{16:0}$ and *iso*$C_{16:0}$ fatty acids according to known pathways of fatty acid biosynthesis. The labelling pattern in the $C_8$ *n*-alkyl chain of the ladderane dialkylmonoether also indicated use of the type II fatty acid biosynthetic route. However, the labelling pattern of carbon atoms in the cyclobutane and cyclohexane moieties did not correspond to known patterns of fatty acid synthesis, suggesting that a completely novel route of biosynthesis was used for ladderane synthesis.

**Part II Environmental application of ladderane lipids as indicators for anammox**

**Chapter 7** describes the discovery of anammox in the Peruvian oxygen minimum zone (OMZ). A range of techniques, i.e. $^{15}$N labelling techniques, fluorescent *in situ* hybridisation (FISH), quantitative polymerase chain reaction (qPCR) and specific biomarker 'ladderane' lipids, were used to determine the amount of anammox bacteria present, the species type, the rates of $N_2$ gas production and the distribution of biomarker ladderane lipids. The combined data set showed that the anammox reaction and anammox cells were ubiquitously present, and that the anammox reaction appeared to be solely responsible for the anaerobic removal of $N_2$ from the water column of the Peruvian OMZ.

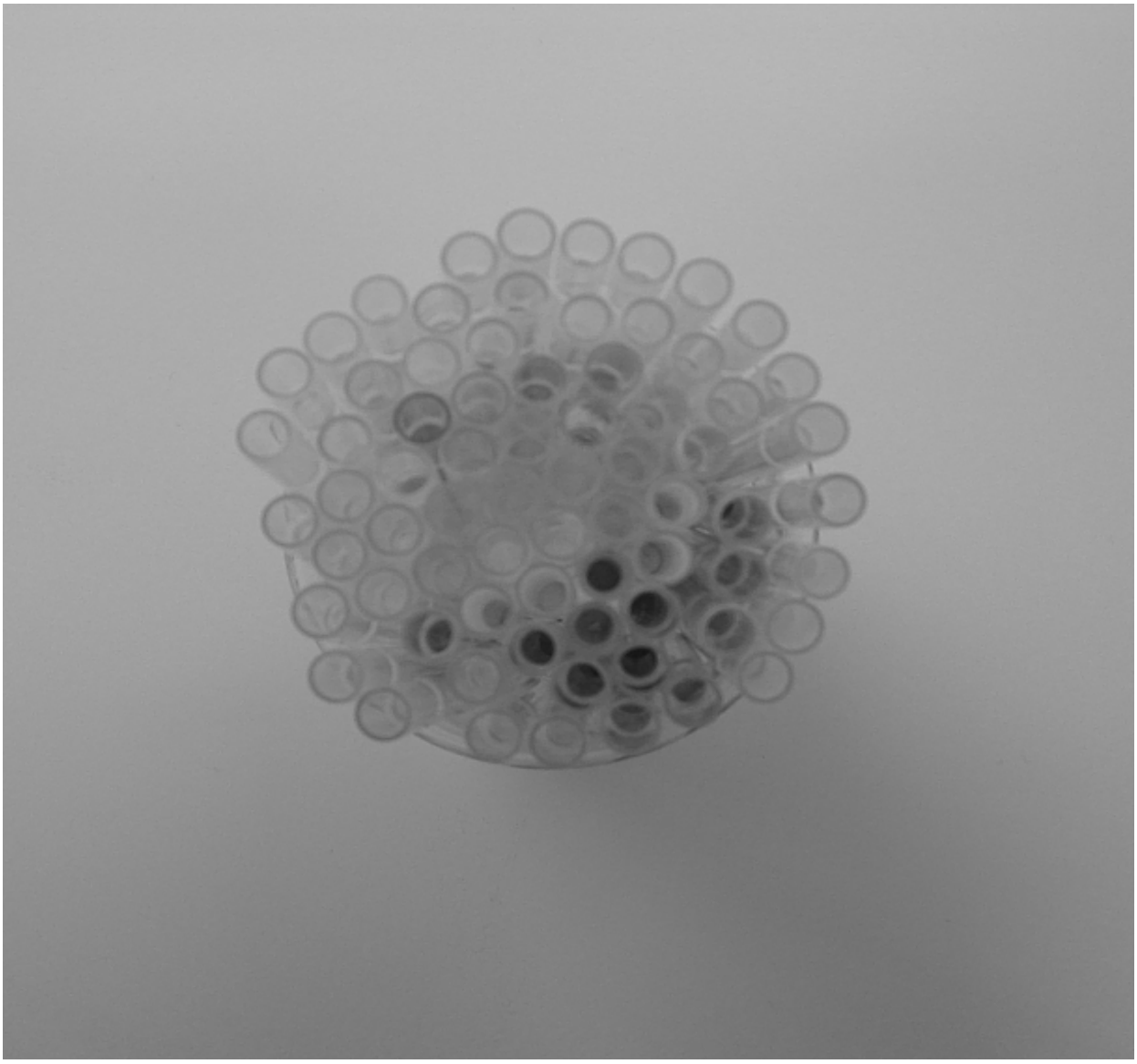
In **chapter 8** we statistically compared different techniques used to detect and quantify the distribution of anammox bacteria and the anammox reaction in the Peruvian and Namibian OMZ. Results from the Namibian OMZ showed that the higher the rates of $^{29}N_2$ production per cell, the more ladderane lipids each cell contained. Which could indicate that the expense of making extra ladderane lipids was saved for times of optimal growth conditions. In the Peruvian OMZ, qPCR copy

numbers and rates of $^{29}N_2$ production and rates of $^{29}N_2$ production and ladderane lipid concentrations were non-parametrically correlated. Observed disparities are probably due to differences in the physiological state or growth phase of the anammox communities at the different sites.

**Chapter 9** combines laboratory and environmental data to provide the first insight into the physiological alteration of ladderane lipids in response to temperature. Analysis of anammox biomass (including representatives of the four genera) grown in enrichment cultures showed that more shorter chained ladderane lipids were produced under colder conditions, and *vice versa*. This trend was also observed in particulate organic matter and surface sediments from a range of marine environments. To quantify the relationship between the $C_{18}$ and $C_{20}$ ladderane lipids containing 5 cyclobutane rings, the index of ladderane lipids with 5 cyclobutane rings ($NL_5$) was calculated. When plotting the $NL_5$ against *in situ* temperature a significant relationship was observed, best approximated by a 4$^{th}$ order sigmoidal curve ($R^2$= 0.92, p=<0.0001, *n*=158). The adaptation of chain length with temperature is similar to the regulation of common fatty acid distribution reported occurring in other bacteria. The $NL_5$ can be used to discriminate between the origins of ladderane lipids in marine surface sediments and may have promise as a paleo-temperature proxy.

To summarise, the work presented in this thesis contributes to a greater understanding of the distribution and synthesis of ladderane lipids in anammox bacteria and of the anammox reaction in natural environments. It has been established that the anammox reaction is of major influence in nitrogen cycling in the Peruvian OMZ, further supporting the idea that the anammox reaction plays a major role in the global cycling of nitrogen. On the molecular scale, it was found that ladderane lipids are not exclusive membrane lipids of the anammoxosome, and therefore must also be present in other membranes of the anammox cell. In addition, we have shown that anammox bacteria physiologically adapt the chain length of their ladderane lipids in response to *in situ* temperature, which may prove to be useful in future paleo-environmental studies. Finally, it was shown that ladderane lipids are not, as previously thought, biosynthesised via the known pathways of fatty acid biosynthesis.

# Part I
# ladderane lipids in cultured anammox bacteria



# chapter 2

## Enrichment and characterization of marine anammox bacteria associated with global nitrogen gas production

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- *Environmental Microbiology in press*

**Abstract**

Microbiological investigation of anaerobic ammonium oxidizing (anammox) bacteria has until now, been restricted to wastewater species. The present study describes the enrichment and characterization of two marine *Scalindua* species, the anammox genus that dominates almost all natural habitats investigated so far. The species were enriched from a marine sediment in the Gullmar Fjord (Sweden) using a medium based on Red Sea salt. Anammox cells comprised about 90% of the enrichment culture after 10 months. The enriched *Scalindua* bacteria displayed all typical features known for anammox bacteria, including turnover of hydrazine, the presence of ladderane lipids, and a compartmentalized cellular ultrastructure. The *Scalindua* species also showed a nitrate-dependent use of formate, acetate, and propionate, and performed a formate-dependent reduction of nitrate, Fe(III) and Mn(IV). This versatile metabolism may be the basis for the global distribution and substantial contribution of the marine *Scalindua* anammox bacteria to the nitrogen loss from oxygen-limited marine ecosystems.

## 2.1 Introduction

Decades ago, it was already observed that ammonium was consumed anaerobically in anoxic fjords (for an overview, see Dalsgaard et al., 2005). Although the anaerobic oxidation of ammonium was thermodynamically possible (Broda 1977), such observations were considered anomalous because ammonium was believed to be inert in the absence of oxygen. In 1995 the first biological evidence was presented for the anaerobic oxidation of ammonium with nitrite as electron acceptor (anammox) in a wastewater treatment bioreactor (Van de Graaf et al., 1995). Since then, it has become widely established that anammox is a significant sink for fixed nitrogen in the oceans (Thamdrup and Dalsgaard, 2002, Kuypers et al., 2005; Thamdrup et al., 2006; Jaeschke et al., 2007). It is currently estimated that anammox may be responsible for at least 50% of nitrogen removal from marine ecosystems (Devol, 2003; Arrigo, 2005; Brandes et al., 2007; Capone and Knapp, 2007; Hamersley et al., 2007).

So far, at least four genera of anammox bacteria have been isolated and enriched, and they form a monophyletic clade deeply branching in the phylum *Planctomycetes*. Of the four genera described, only one "*Candidatus* Scalindua" dominates in all marine environments investigated, as shown by FISH or PCR amplification of 16S rRNA genes (Kuypers et al., 2003; Kuypers et al., 2005; Penton et al., 2006; Schmid et al., 2007; Nakajima et al., 2008).

Anammox bacteria have not yet been obtained in pure culture, but they are routinely grown in enrichment cultures with efficient biomass retention where they constitute about 70-90% of the bacterial population (Strous et al., 1998; 2002 Tsushima et al., 2007). Using biomass from such enrichment cultures, it was demonstrated that the majority of membrane lipids of anammox bacteria have three or five linearly concatenated cyclobutane rings, and have become known as ladderane lipids (Sinninghe Damsté et al., 2002; Sinninghe Damsté et al., 2005). Intact polar glycerol-ladderane lipids contain phosphocholine or phosphoethanolamine as head groups (Boumann et al., 2006). So far, ladderane lipids have only been found in anammox bacteria and therefore they are considered to be unique biomarkers for the process of anaerobic ammonium oxidation. However, presently all knowledge on anammox bacteria is derived from

wastewater species, and for the interpretation of the oceanographic and molecular data we depend on extrapolation from wastewater studies.

In a recent paper, we reported the activity and identity of anammox bacteria in sediment of a Swedish fjord (Schmid et al., 2007). From the same site, we have used surface sediment (i.e. the upper 2 cm) as inoculum for anoxic laboratory bioreactors fed with a mineral medium based on Red Sea salt containing ammonium and nitrite. After 10 months of enrichment, anammox bacteria of the genus *Scalindua*, comprised about 90% of all bacterial cells. The marine *Scalindua* species were further characterized with respect to substrate use, lipid content, cellular ultrastructure and temperature response.

## 2.2 Materials and Methods

### *2.2.1 Sampling and enrichment*

All samples were taken from the deepest part of the Gullmar Fjord (Alsbäck, 58°15.5' N, 11°13.5' E, water depth 116 m). This site was previously shown to harbor appreciable amounts of anammox cells and activity (Schmid et al., 2007). The bottom water temperature, oxygen and nitrate concentration was 6°C, 160 µM and 15 µM, respectively. The sediment density was 1.18 g $ml^{-1}$. Sediment was collected with an Olausen box-corer and Plexiglas tubes (Ø 10 cm) were inserted into the sediment. The tubes were sealed, removed from the box-corer and transported to a temperature-controlled room. The upper 2 cm of the sediment was removed and sieved through a 1 mm mesh to exclude macrofauna and larger debris. Several samples were pooled, resulting in a final volume of about 2 liters. The sieved sediment was transported in a polypropylene bottle on ice and used to inoculate two sequencing batch bioreactors (SBR), one initially at 20°C (liquid volume 3 l) and one at 15°C (liquid volume 1.5 l). These were inoculated with 1 l and 0.5 l sieved and pooled sediment, respectively, and used for enrichment and cultivation of anammox bacteria (Strous et al., 1998). Each SBR cycle of 24 h consisted of 23h 30m of filling, 10 min of settling of the biomass (no stirring) and 20 min of pumping off liquid above the settled cells. During each filling period, one third of the volume was replaced per day, with demineralized water containing 33 g $l^{-1}$ sea salt (Red Sea Salt Fish Pharm Ltd., The Seahorse, Arnhem, NL), supplemented with $FeSO_4$, $KH_2PO_4$, ammonium, nitrite and nitrate as described in the results section. To maintain anoxic

conditions, the bioreactors and medium vessels were flushed continuously with $Ar/CO_2$ (95/5%, 10 ml $min^{-1}$). The 20-23°C SBR was stirred at 350 rpm with a turbine stirrer, while the 15°C reactor was operated at 250 rpm. The pH was controlled at 7.4 with 150 g $l^{-1}$ $NaHCO_3$.

### 2.2.2 *Activity assays*

Biomass samples were washed 3-5 times with sea salt medium until nitrite, nitrate and ammonium in the sample were less than 20 µM. The biomass, consisting of small aggregates that were resized by passing through an injection needle, was then transferred to 30 ml serum bottles. The bottles were sealed with 5 mm butyl rubber stoppers and made anoxic by alternately applying under-pressure and Argon gas five times. An overpressure of 1 bar was maintained in the bottles. The soluble substrates were added to the bottles from 100 mM anoxic stock solutions. To measure anaerobic ammonium oxidation activity, final concentrations of 3 mM $NO_2^-$ and $NH_4^+$ were used. In order to measure nitrate reduction activity, final concentrations of 3 mM $NO_3^-$ and 12.5 mM formate, 3 mM acetate or 2 mM propionate were used. To measure the intermediate accumulation of hydrazine, final concentrations of 5 mM $NH_2OH$ and 5 mM $NH_4^+$ were used. The bottles were incubated at room temperature (20-22°C) and were shaken continuously at 150 rpm for 6 h.

The reduction rates of $Fe^{3+}$ and $Mn^{4+}$ were measured by incubation of biofilm aggregates with δ-$MnO_2$ and amorphous FeOOH, respectively (Lovley and Phillips, 1986; Villalobos et al., 2003; Strous et al., 2006). Formate was added as electron donor.

### 2.2.3 *Analytical methods*

Nitrate, nitrite, propionate, acetate and formate were measured with high performance liquid chromatography (HPLC). Water samples from the batch incubations and the SBR were centrifuged and the supernatant was filtered through a 0.45 µm Spartan filter (Schleicher & Schuell). From the samples, 10 µl was analyzed using a Hewlett Packard 1050 series autosampler. A sodium hydroxide solution was used as the liquid phase at a flow rate of 1.5 ml $min^{-1}$. The anions were separated using a hydroxide gradient elution from 1 mM to 15 mM in 20 min. Separations were

performed on a 4x250mm Ionpac AS11-HC (Dionex, UK) column at 30°C. Anions were detected using a CD25 conductivity detector (Dionex UK). Ammonium was measured colorimetrically at 420 nm after a 30 min reaction of 40 µl sample containing 0.5 – 5 mM ammonium with 760 µl 0.54 % ortho-phthalaldehyde, 0.05 % β-mercaptoethanol and 10% ethanol in 400 mM potassium phosphate buffer (pH 7.3) (modified from Taylor et al., 1974). In addition, nitrite, hydroxylamine, hydrazine and protein were determined as described before (Strous et al., 1998; Kartal et al., 2006).

### *2.2.4 Electron Microscopy*

Small aggregates of anammox bacteria or percoll purified single cells (Kartal et al. 2007) were transferred into a 100 µm cavity of a planchette (3 mm diameter, 0.1/0.2 mm depth, Engineering Office M. Wohlwend GmbH, CH-9466 Sennwald, Switzerland) containing 1-hexadecene (Studer et al., 1989), closed with the flat side of a lecithin-coated planchette (3 mm, 0.3 mm depth) and cryofixed by high-pressure freezing (Leica EMHPF, Leica Microsystems, Vienna, Austria). Freeze-substitution was performed in acetone containing 2% osmium tetroxide, 0.2% uranyl acetate and 1% $H_2O$ (Walther and Ziegler, 2002). Samples were kept at -90°C for 47 hours, brought to -60°C at 2°C/hour, kept at -60°C for eight hours, brought to -30°C at 2°C/hour and kept at -30°C for eight hours in a freeze-substitution unit (AFS, Leica Microsystems, Vienna, Austria). Samples were washed four times for 30 minutes in the AFS at -30°C and once for 60 minutes on ice with acetone containing 2% osmium tetroxide and 1% $H_2O$ to remove the uranyl acetate. Osmium tetroxide and $H_2O$ were removed by washing two times for 30 minutes on ice with anhydrous acetone. Samples were gradually infiltrated with Epon resin (Mollenhauer, 1964). Epon was polymerized for 72 hours at 60°C.

All sections were cut using a Reichert Ultracut E Microtome (Leica Microsystems, Vienna, Austria). Ultra-thin (ca. 70 nm) sections were collected on formvar-carbon-coated copper square 50 mesh grids, post-stained with 20% (w/v) uranyl acetate in 70% (v/v) methanol/water for four minutes and Reynolds' lead citrate staining for two minutes (Reynolds, 1963). Cells were investigated at 80-120 kV in a transmission electron microscope (Tecnai10 or Tecnai12, FEI Company, Eindhoven, The Netherlands). Images were recorded using a CCD camera (MegaView II, AnalySis) as described by van Nitrik et al. (2007).

### 2.2.5 DNA extraction and 16S rRNA sequence comparison

Sediment material was suspended in 10 ml of DNA extraction buffer (100 mM Tris/HCl, pH 8.0; 100 mM sodium EDTA, pH 8.0; 100 mM sodium phosphate, pH 8.0; 1.5 M NaCl; 1% CTAB). Total genomic DNA was extracted as described previously (Schmid et al., 2007). The preferential PCR amplification of 16S rRNA genes of members of the Planctomycetales was performed with Pla46F (*E. coli* positions 46–63; Schmid et al., 2005) forward primer in combination with the universal reverse primer (*E. coli* positions 1529–1545). PCR was performed with a Tgradient cycler (Biometra, Goettingen, Germany). Negative controls (no DNA added) and positive controls (DNA from a "*Candidatus* Brocadia anammoxidans" enrichment culture) were included in all sets of amplifications. The optimal annealing temperature for the primer set Pla46F/630R regarding yield and specificity was 60 °C. The presence and size of amplification products were determined by agarose (1%) gel electrophoresis of 5 µl aliquots of the PCR products. The biofilm-derived 16S rRNA gene amplificates were cloned directly by using the TOPO TA Cloning kit following the instructions of the manufacturer (Invitrogen, Groningen, The Netherlands). Plasmid-DNA was isolated with the FlexiPrep Kit (Amersham Pharmacia Biotech Inc, Piscataway, NJ). Plasmids with an insert of the expected size were identified by agarose (1.0%) gel electrophoresis after EcoRI digestion (5 U, Eco RI-buffer for 3 h at 37 °C). Sequencing was done with the BigDye Terminator Cycle Sequencing v2.0 kit (Applied Biosystems, Foster City, CA). The reaction mixtures were analyzed with a 3700 DNA Analyzer (Applied Biosystems, Foster City, CA). The complete sequences of the 16S rRNA gene fragments were determined using M13 forward and reverse primers targeting vector sequences adjacent to the multiple cloning site as well as universal Eubacterial primers 609F (*E. coli* positions 785–806; 5'-TTA GAT ACC CCD GTA GT-3') and 699R (*E. coli* positions 1099–1114; 5'-AGG GTT GCG CTC GTT GC-3') targeting the 16S rRNA gene. The fragments that belonged to organisms from the anammox cluster, were also sequenced with the anammox specific internal primer Amx368 (*E. coli* position 368-386; 5'-TTC GCA ATG CCC GAA AGG-3'). The 16S rRNA gene sequences retrieved in this study were added to the 16S rRNA gene sequence database of the ARB program package (Ludwig et al., 2004). 16S rRNA sequences were aligned automatically using the respective tool of the ARB package. Subsequently, the alignments were corrected by visual inspection. Phylogenetic analysis of 16S rRNA

sequences were performed by applying neighbor-joining, ARB parsimony and maximum likelihood analysis (fastDNAml) to different data sets.

### *2.2.6 Fluorescence in situ Hybridization (FISH)*

Sediment (0.5 g) was fixed in 4% paraformaldehyde. In the last step of the fixation procedure we suspended the sample in 1 ml 50% ethanol in phosphate buffered saline pH 7.4. We used a mixture of anammox specific probes S-G-Sca-1309-a-A-21 (Sca1309), S-*-Scabr-1114-a-A-22 (Scabr1114), S-*-BS-820-a-A-22 (BS820), S-*-AMX-820-a-A-22 (AMX820), S-*-AMX-0368-a-A-18 (Amx368), a Planctomycetes specific probe S-P-Planc-0046-a-A-18 (Pla46) , and a mixture of S-D-Bact-0338-a-A-18, S-D-Bact-0338-b-A-18, S-D-Bact-0338-c-A-18 (EUBMix) (Loy et al., 2003). We designed an extra probe S-*-Scama-820-a-A-22 5'- TAA TTT CCT CTA CTT AGT GCC C-3' (ScaMa820), that specifically reacted with one of the organisms (represented by EU142947) found in this study. Probe Scama820 is specific for EU142947, but has 2 mismatches with EU142948. Probe AMX820 has one internal mismatch plus one at the 5' end to EU142948, and 2 internal mismatches to EU142947. Both probes target the same position on the 16S rRNA molecule (i.e. position 820 of the *E. coli* reference). Even though the second probe did not match exactly to either sequence, the probe combination Scama820 and AMX820 appeared to distinguish better between both species in a competitive hybridization experiment than any other probe combination. Further details about the probes and conditions used in this study can be found in Schmid et al. (2005). All probes were purchased as Cy3, Cy5 and 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS) labeled derivatives (Interactiva, Ulm, Germany). Hybridizations were performed as described previously (Schmid et al., 2003). Simultaneous hybridization with probes requiring different stringency was realized by a successive-hybridization procedure. Optimal hybridization conditions for hybridization probes were taken from Schmid et al. (2005). After hybridization and air-drying, slides were embedded in Vectashield (Vector Laboratories Inc., Burlingame, CA) with 4',6-diamidino-2-phenylindole (DAPI) for total cell counts. For image acquisitions we used a Zeiss axioplan 2 microscope (Zeiss, Jena, Germany). Every month, samples were routinely collected from the enrichment cultures and fixed. These samples were used to analyze the change in anammox population composition.

### 2.2.7 Lipid identification and $\delta^{13}C$

Cultured biomass was freeze-dried and ultrasonically extracted three times using methanol (MeOH), three times using a MeOH/dichloromethane (DCM) mixture (1:1, v/v) and three times using DCM. The extracts were combined, dried and subsequently methylated using $BF_3$/MeOH (with a known carbon isotopic composition of -20.5‰ vs Vienna PeeDee Belemnite (VPDB). The sample was initially eluted over a small silica column to remove highly polar material and subsequently treated with N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) (with a known isotopic composition of -40‰ vs VPDB) in pyridine at 60°C for 20 min. After the addition of ethyl acetate, the total lipid extract was analyzed on a Hewlett Packard HP6890 Series GC System fitted with on column injection and a flame ionization detector (FID). Identification of lipids was carried out on a Finnigan Trace GC Ultra, Thermoelectron Corporation, interfaced with a Finnigan Trace DSQ mass spectrometer. Ladderane lipids were identified using previously identified GC/MS mass spectra (Sinninghe Damsté et al., 2005). A ThermoFinnigan Delta Plus XL isotope ratio monitoring irm-GC/MS was used to analyze lipid stable carbon isotopes as described in detail elsewhere (Schouten et al., 2004). The isotopic compositions of fatty acids and alcohols were adjusted for the addition of extra carbon incorporated during sample derivatization.

Culture medium was analyzed for the $\delta^{13}C$ values of the total dissolved inorganic carbon (DIC), using a ThermoFinnigan Gas Bench II coupled to a Delta Plus irmMS. Headspace analysis was performed after 1 ml of culture supernatant (treated with $HgCl_2$) reacted with $H_3PO_4$ for at least 1 h at room temperature. An analytical standard deviation of 0.46‰ was achieved by performing the analysis 10 times. Stable isotope ratios were determined relative to the laboratory standard ($NaCO_3$, -0.57‰ vs VPDB) which was calibrated against NBS-18 carbonate (International Atomic Energy Agency (IAEA).

## 2.3 Results

### *2.3.1 Enrichment*

Compared to the conditions in wastewater treatment systems, the marine environment has different chemistry (more salt and less nutrients) and lower temperatures. Therefore we initially used artificial sea water to enrich anammox bacteria from the sediment of the Gullmar Fjord, but after several months of trial without technical problems, no anammox activity or cells could be detected. Finally, a medium based on Red Sea salt was applied, and to further increase the chances of successful enrichment, the Gullmar Fjord sediment was incubated in two parallel bioreactors, one at 15 °C and one at 20 °C. Apart from the temperatures and the volumes (1.5 and 3 L respectively) the bioreactor setups and operation was identical. At the start-up of the two cultures, the influent medium contained 0.5 mM nitrite, 1.0 mM ammonium and 1.5 mM nitrate. Although nitrate is a product of anammox and not a substrate, it was added to the medium to prevent the occurrence of sulfate reduction. After three months, both enrichment cultures consumed all nitrite supplied in the medium. From then on, nitrite and ammonium concentrations were increased whenever nitrite was completely consumed (Fig. 1). As soon as the nitrite concentration of the influent became 15 mM and higher, nitrate was not added to the influent anymore. At that moment, sufficient nitrate was produced by anammox to prevent sulfate reduction.

After 9 months of enrichment, the nitrite concentration reached 30 mM, but it could not be increased further without nitrite accumulating to toxic levels of several millimolars. On the contrary, anammox activity in both enrichment cultures slowly started to decline (Fig. 1). The activity could not be increased by raising the temperature from 20 to 23°C, but growth and activity was restored by adding 9 µM $FeSO_4$ and 0.2 mM phosphate to the influent medium (final concentrations). After 14 months, the cultures consumed approximately 30 mmol nitrite $l^{-1}$ $day^{-1}$ (at influent nitrite and ammonium concentration of 45 mM), and the medium composition and temperature (15 and 23 °C) were not changed further. From then on the actual concentrations in the cultures were <0.5 mM nitrite, 8 mM ammonium, 8 mM nitrate, indicating that all nitrite was consumed and converted at the expected anammox stoichiometry (see below). After the two years of enrichment, the appearance of the two cultures was strikingly different. The 23°C culture contained orange, cotton

wool-shaped flocks, whereas the 15°C enrichment culture contained brownish, granular aggregates.

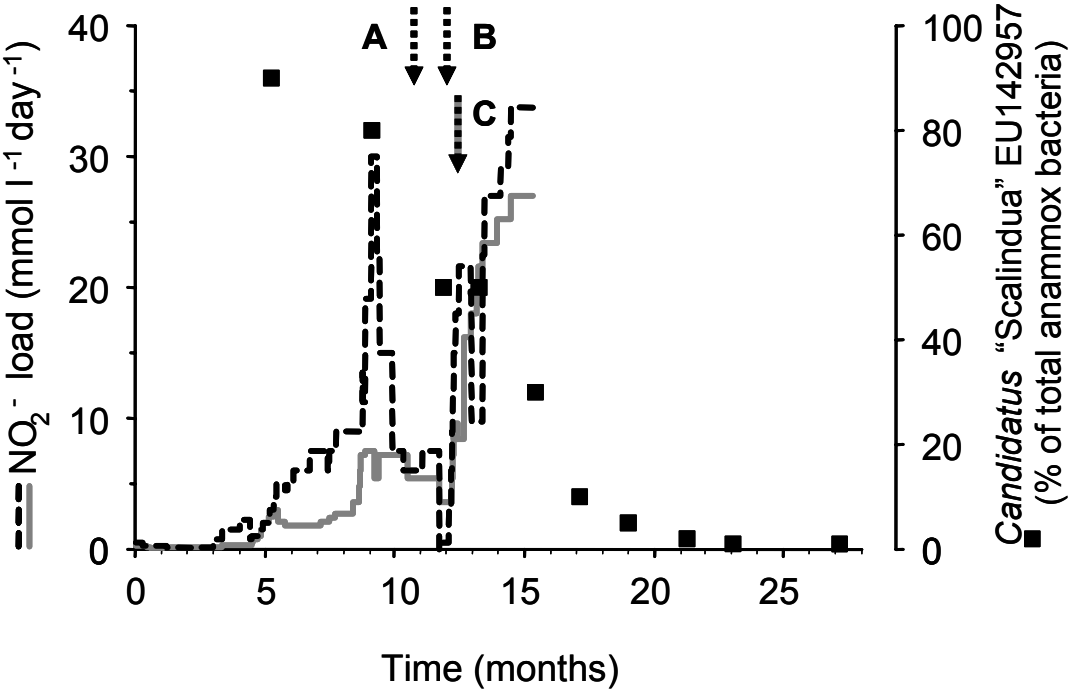


**Figure 1.** Enrichment of marine anammox bacteria from Gullmar Fjord, Sweden, in 2 bioreactors. Dashed black line: 20-23°C enrichment culture; Solid grey: 15°C enrichment culture. Black squares: FISH: percentage of Scalindua EU142957 positive cells of total anammox counts in the 20-23°C enrichment culture. In the 15°C enrichment culture, the population consisted of 40-60% of both "C*andidatus* Scalindua" species over the whole period. In both enrichments the final amount of anammox cells was 90% of all bacterial cells counted by EUBmix (Loy et al., 2003). Events: A: temperature rise from 20 to 23°C; B: Oxygen leakage problem; C: start of Phosphate and Iron supplementation. After around 15 months, a concentration of 45 mM of nitrite and ammonium was achieved in the influent medium of both bioreactors, and all nitrite was consumed, 8nM of ammonium was left, and about 8Mm nitrate was produced in agreement with the anammox stoichiometry (Strous et al., 1998). It was decided not to increase the nitrite load any further to avoid the risk of lethal concentrations of nitrite at the influent inlet.

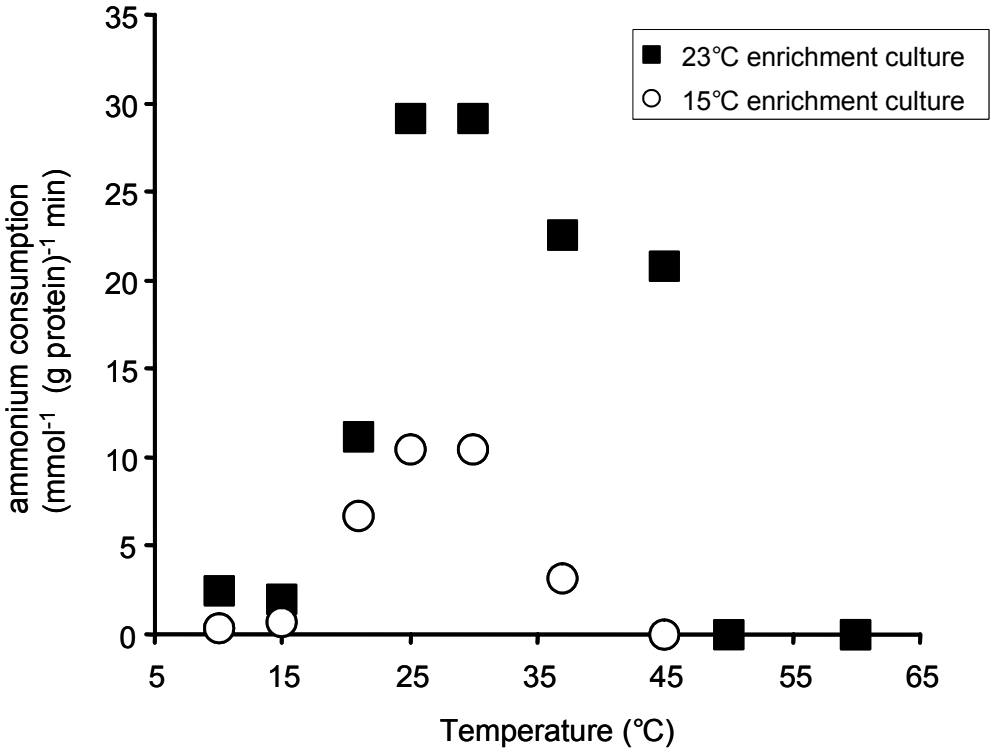


**Figure 2**. Temperature profiles of the two anammox enrichment cultures after two years of operation. The optimum anammox activity temperatures are not significantly different (n=2). However, at 45 ˚C, no activity was observed for a sample from the 15 ˚C enrichment culture, where clear activity was observed for the 23 ˚C culture. This observation was confirmed in a separate test. The standard deviation was no more than 5% in 2 separate experiments.

### *2.3.2 FISH and 16S rRNA gene analysis*

After one year of enrichment, 16S rRNA genes from both cultures were amplified with Planctomycetes specific primer Pla46F and universal bacterial primer 630R, and two clone libraries were constructed. Sequencing of 24 complete 16S rRNA clones indicated that both libraries of the 15 °C and 23 °C enrichment culture contained two anammox 16S rRNA gene sequences (EU142947 and EU142948) in about equal amounts.

Phylogenetic trees of the two 16S rRNA gene sequences (Fig. 3) indicated that both marine anammox strains were closely related to "*Candidatus* Scalindua sorokinii/brodae", the anammox species that predominates in the marine environment (Schmid et al., 2007; Wöebken et al., 2008). The tree topology was

supported by neighbor joining, maximum parsimony and maximum likelihood. A distance matrix for the two enriched anammox strains indicated that the Scalindua phylotype EU142948 was most closely related (16S rRNA gene 99.5% identical) to "*Candidatus* Scalindua brodae" and "*Candidatus* Scalindua sorokinii" (16S rRNA gene 98.5 % identical). Scalindua phylotype EU142947 was somewhat less related to these organisms with 96.9% 16S rRNA gene identity to "*Candidatus* Scalindua brodae" and 96.8% to "*Candidatus* Scalindua sorokinii", but was most similar (98.0%) to the 16S rRNA gene sequences retrieved from the Gulmar Fjord sediment (Schmid et al., 2007). A FISH probe, S-*-Scama-820-a-A-22 (Scama820), was constructed to match specifically with the 16S rRNA gene sequence of the latter (EU142947) Scalindua strain. The Scama820 probe had two mismatches with the EU142948 sequence.

During enrichment, samples for FISH were collected every month, fixed and stored at -20 °C. FISH analysis showed that upon inoculation, the amount of anammox cells was the same (between $10^7$ and $10^8$ anammox cells per ml or about 2% of the population) as previously reported for the Gullmar Fjord sediment (Schmid et al., 2007). After 5 months, the anammox cells made up around 20 % of the total bacterial cells, and this percentage increased after further enrichment under the same conditions. After 10 months of enrichment about 90% of all bacterial cells were anammox as determined with FISH analysis with anammox and eubacteria specific probes.

By competitive hybridization with probes Scama820 (specific for EU142947) and Amx820 (targeting EU142948), that both hybridize with the (*E. coli* reference) 820 position on the 16S rRNA gene, the two Scalindua populations could clearly be distinguished. Interestingly, these experiments showed that during the second year of enrichment EU142947-positive cells gradually disappeared from the 23 °C culture, which was ultimately completely dominated by anammox cells that hybridized to probe EU142948. The total amount of anammox cells in the culture did not change and stayed around 90% of all bacterial cells. This population shift did not occur in the 15 °C culture, in which both Scalindua populations remained approximately equally abundant. Apparently, the higher temperature was selective for EU142948-positive Scalindua cells.

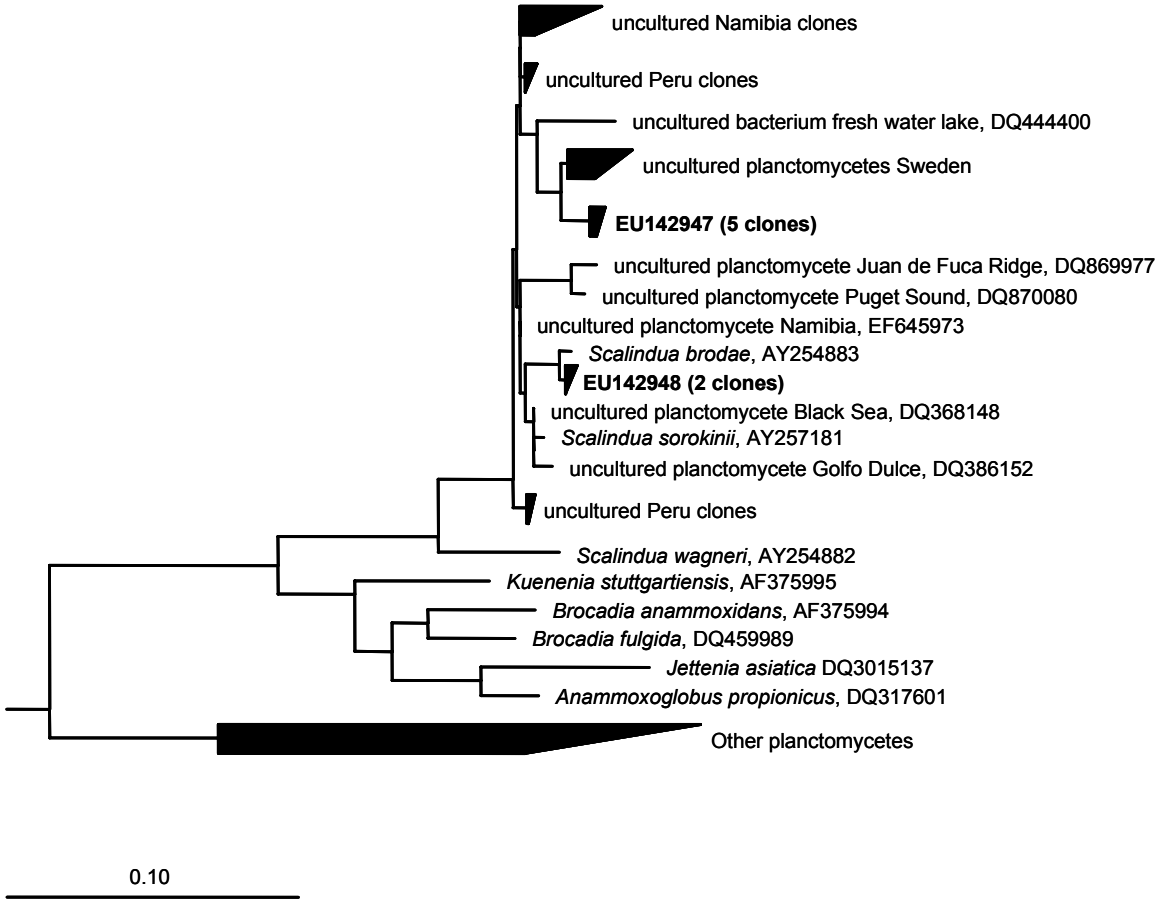


**Figure 3.** Maximum Likelihood phylogenetic relation of the 16S rRNA anammox bacterial partial sequences found in the enrichment cultures, compared to previously described "*Candidatus* Scalindua" species, environmental "*Candidatus* Scalindua" clones (Penton et al., 2006; Hamersley et al., 2007; Schmid et al., 2007) and other anammox species that have been enriched (Strous et al., 1998; Strous et al., 2006; Kartal et al., 2007;2008). The 15°C enrichment culture contained 2 closely related *Scalindua* species EU142957 and EU142958 in equal amounts. The 23°C reactor contained only EU142958 after 2 years of enrichment (see Fig. 1). Bar indicates 10% divergence.

No cells hybridized with probes specific for any of the wastewater anammox species "*Candidatus* Kuenenia", "*Candidatus* Brocadia", and "*Candidatus* Anammoxoglobus". Combined hybridization with Pla46, a probe targeting most planctomycete bacteria and the probes specific for marine *Scalindua* indicated that no other anammox bacteria were present in significant numbers.

### 2.3.3 Anammox Activity and Substrate Use

Fourteen months after inoculation, the anammox activity of the aggregates from both enrichment cultures was 10 (±5, n=4) μmol $NH_4^+$ (g protein)$^{-1}$ min$^{-1}$ when tested at 22°C. The suspended anammox cells collected from the effluent were more active, with activities of 23 (±3, n=4) μmol $NH_4^+$ (g protein)$^{-1}$ min$^{-1}$. In all cases, for each mol of ammonium 1.2 to 1.3 mol of nitrite was converted, similar to previously reported values (Strous et al., 1998). For each mol of nitrite about 0.1-0.2 moles of nitrate were formed. It has been hypothesized that this nitrite oxidation might supply electrons via reversed electron transport to replenish the electrons used for CO2 fixation (Strous et al., 2006). When the biomass was incubated with hydroxylamine, a transient accumulation of 0.2-0.4 mM hydrazine was observed. Production of hydrazine in the presence of hydroxylamine is diagnostic of anammox bacteria (van de Graaf et al 1997).

Both enrichment cultures consumed organic acids with nitrate as the electron acceptor, as observed in wastewater anammox bacteria (Kartal et al., 2007; Kartal et al. 2008). The rates for nitrate-dependent formate, acetate and propionate conversion were 7, 0.7 and 0.3 μmol (g protein)$^{-1}$ min$^{-1}$. The $Fe^{3+}$ and $Mn^{4+}$ reduction rates were measured with δ-MnO2 and amorphous FeOOH, with and without and formate as electron donor using aggregated biomass. In the presence of formate, both metal oxides were reduced at 0.06 and 0.03 μmol (g protein)$^{-1}$ min$^{-1}$, respectively. In the absence of formate, these rates were halved. The rates of formate-dependent metal reduction measured with aggregates of the marine *Scalindua* were comparable to rates observed in parallel experiments conducted with aggregates of "C*andidatus* Kuenenia" as positive controls. Both rates were somewhat lower than the Mn(IV) and Fe(III) reduction rates by single cells of "C*andidatus* Kuenenia" (Strous et al., 2006), that may experience less diffusion limitation.

When suspended biomass from the two enrichments was tested at different temperatures (Fig. 2), it appeared that the cells from the 15 °C culture had a maximum activity of 10 μmol (g protein)$^{-1}$ min$^{-1}$ at 30 °C, while the cells from the 23 °C reactor showed high anammox activity (up to 20 μmol (g protein)$^{-1}$ min$^{-1}$ until 45 °C.

### *2.3.4 Lipids*

After 15 months of enrichment, lipids were extracted from cells from both enrichment cultures and identified using gas chromatography-mass spectrometry (Fig. 4). The most abundant lipids in the sample were the ladderane fatty acids (**a-d)** which are specific for anammox bacteria (Sinninghe Damsté et al., 2002, 2005).

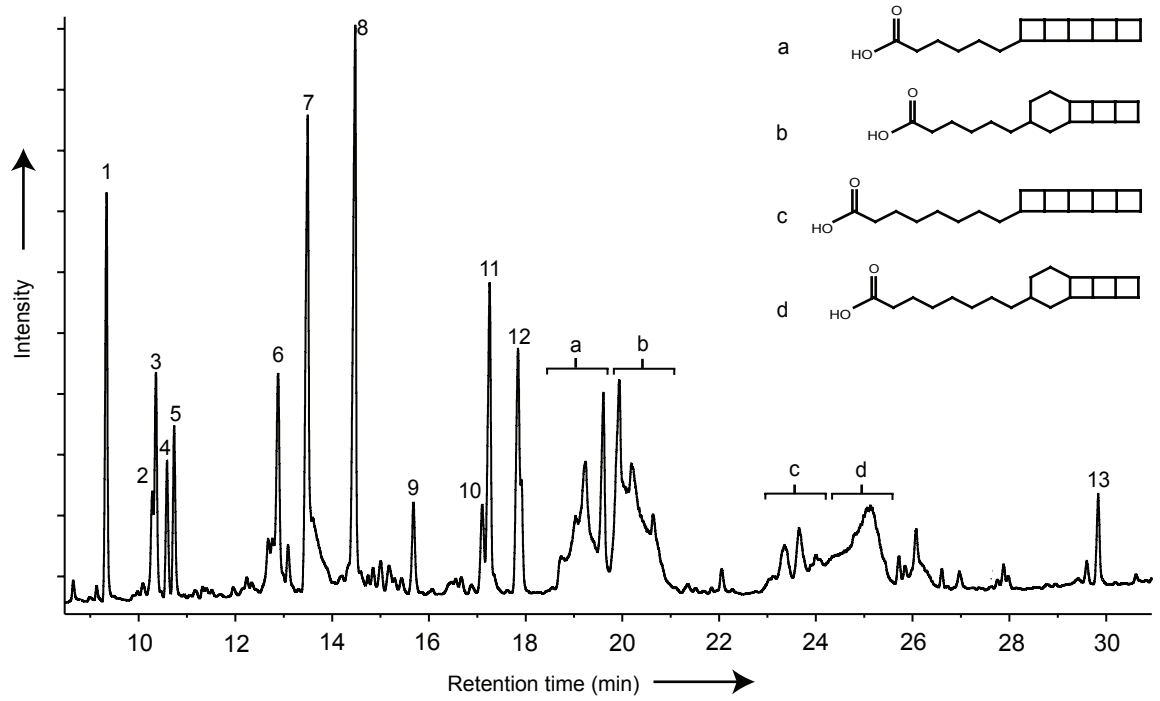


**Figure 4.** Gas chromatogram of the total lipid fraction of 15°C enrichment culture biomass. Key: (1) $C_{14}$ fatty acid, (2) 9-methylpentadecanoic acid, (3) 10-methylpentadecanoic acid, (4) *i*-$C_{15:0}$ fatty acid, (5) *ai*-$C_{15:0}$ fatty acid (6) $C_{16:1}$ fatty acid, (7) $C_{16:0}$ fatty acid, (8) 10-methylhexadecanoic acid, (9) $C_{17:0}$ fatty acid, (10) *i*-$C_{18:0}$ fatty acid, (11) $C_{18:1}$ fatty acid, (12) $C_{18:0}$ fatty acid, (13) squalene. Ladderane lipids are; (**a)** $C_{18}$ fatty acid with 5 cyclobutane rings, (**b)** $C_{18}$ fatty acid with 3 cyclobutane rings and one cyclohexane ring, **(c)** $C_{20}$ fatty acid with 5 cyclobutane rings, (**d)** $C_{20}$ fatty acid with 3 cyclobutane rings and one cyclohexane ring.

The core lipid ladderane fatty acid (**b**) was not observed previously in anammox enrichment cultures, but has been reported in water column particulate matter of the Black Sea and the Benguela upwelling system (Kuypers et al., 2003; Kuypers et al., 2005). No ladderane monoether was identified in the cultures, even though this compound was detected in the inoculum (Hopmans et al., 2006).

**Table 1.** Comparison of anammox specific fatty acids in the two enrichment cultures 15 months after inoculation. At that time, both reactors contained equal amounts of the same two "*Candidatus* Scalindua" species. The major causes for differences in lipid composition could be temperature and reactor geometry. Ladderane a-d relate to the structures in Fig. 2.

| | **% of total lipid extract** | |
|---|---|---|
| **Fatty acid** | **Sweden 15°C** | **Sweden 23°C** |
| **10Me $C_{16}$** | 9 | 14 |
| **Ladderane a** | 11 | 3 |
| **Ladderane b** | 15 | 4 |
| **Ladderane c** | 4 | 6 |
| **Ladderane d** | 5 | 9 |

The stable carbon isotopic composition of dissolved inorganic carbon (DIC) was -23.6 ± 0.5‰ and -24.8 ± 0.3‰ in the supernatants of the enrichment cultures grown at 15 and 23°C, respectively. Anammox specific lipids were depleted in $^{13}C$ relative to the DIC of the medium by about, 58‰ and 55‰, respectively (Table 1). This is consistent with isotope fractionation by wastewater anammox species and observed in natural waters (Schouten et al., 2004; Kuypers et al., 2003).

### *2.3.5 Ultrastructure*

After 18 months, Transmission Electron Microscopy was performed on thin sections of high-pressure frozen, freeze-substituted and Epon-embedded samples from both enrichment cultures. Anammox bacteria contain a membrane-bound intracytoplasmic compartment, known as the anammoxosome. The dominant organisms in the sample clearly showed this typical ultrastructural feature of anammox bacteria. Consistent with the molecular data at the time of sampling, it appeared that the enrichment culture at 15 °C contained two different anammox morphotypes (Fig. 5). One of these types was characterized by the presence of pili-like appendages that have a length of around 130 nm. (Van Niftrik et al., 2008).

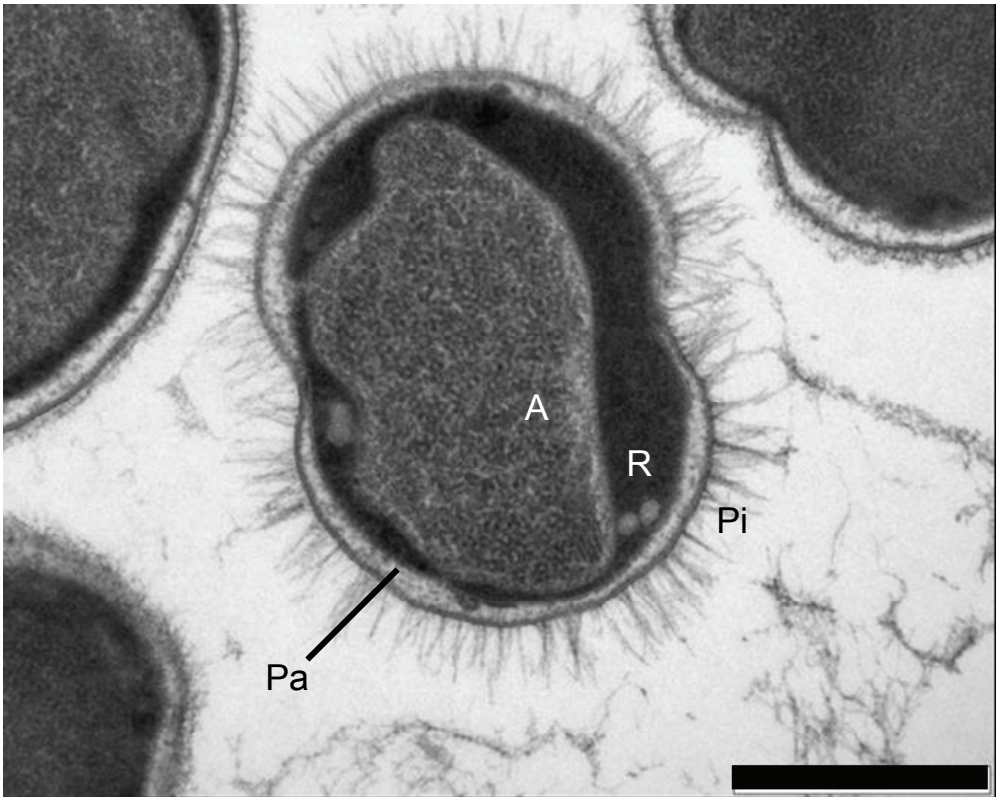


**Figure 5.** Transmission electron micrograph of "*Candidatus* Scalindua". Visible are anammoxosome (A), riboplasm (R), paryphoplasm (Pa). Pili-like appendages (Pi) have not been observed in anaerobic ammonium oxidizing bacteria before. Bar indicates 500 nm.

## 2.4 Discussion

Anaerobic ammonium oxidation (anammox) is a major sink of fixed nitrogen in the oceans. Molecular ecological surveys performed so far indicate that anammox biodiversity in this environment is limited to several closely related species of the genus "*Candidatus* Scalindua" (Nakajima et al., 2008; Penton et al., 2006; Schmid et al., 2007; Woebken et al., 2008). Since these marine anammox species were not yet available in enrichment culture, the interpretation of *in situ* data was based on extrapolations from experiments with anammox bacteria enriched from wastewater and co-cultures with other anammox bacteria (Kartal et al, 2006). Recently, "*Candidatus* Scalindua wagneri" was reported to be enriched from anammox bacteria collected on fabric from the sea floor, but with low population density and low conversion rates (70 mg N $l^{-1}$ $day^{-1}$ after 16 months, *i.e.* 5 mmol N $l^{-1}$ $day^{-1}$) and without supporting physiological evidence other than $^{15}N$ labeling tests (Nakajima et al., 2008).

Here we report the enrichment of marine anammox bacteria of the Scalindua branch to high cell densities, using a modified medium based on Red Sea salt. Compared to the standard anammox medium, this medium contained low concentrations of bicarbonate, magnesium, calcium, phosphate (< 0.2 µM), iron (< 0.03 nM) and other trace elements, but may contain micronutirents that were absent in the other media used in the first unsuccessful trials. Iron and/or phosphorous became the growth-limiting nutrients when the nitrite concentration in the influent was increased beyond 20 mM. Based on the growth yield of wastewater anammox species, the consumption of 20 mM nitrite corresponded to the fixation of 1 mmol $CO_2$ $l^{-1}$ influent medium. Based on general bacterial phosphate and iron requirements, it was very likely that the concentration of these compounds in the influent was too low to support the fixation of more than 0.2 mmol $CO_2$ $l^{-1}$ influent. Because of the risk of loosing an enrichment culture of a species that had never been enriched before, and due to the slow growing nature of the anammox bacteria, we did not test which of the two elements were most limiting at the moment that the growth of the culture deteriorated. Apparently, the culture depended on the iron and phosphate that was present in the inoculum or Red Sea salt. Subsequent addition of iron and phosphorous to the influent medium did restore growth and activity.

The 'standard' anammox medium described by Van de Graaf et al. (1996) was either, not successful for the enrichment of marine anammox bacteria, or resulted in the growth of wastewater species at the expense of marine species. Addition of salt to the 'standard' medium did not overcome this problem (Kartal et al., 2006). Previous enrichments (data not shown) with Namibian oxygen minimum zone water and Gullmar Fjord sediment using artificial seawater were also not successful. Recently, the use of deep sea water supplemented with 3 mM nitrite and ammonium was applied successfully for the partial (up to 5 %) enrichment of an anammox bacterium (related to *Candidatus* "Scalindua wagneri") from a marine sample (Nakajima et al. 2008).

It is unknown what factors contributed to the enrichment of marine anammox species in seawater. As indicated, a high salt concentration and lower temperatures were not sufficiently selective. Low bicarbonate, and microelement concentrations, the presence of unknown growth factors and the absence of high, potentially toxic concentrations of trace elements were other possible factors potentially contributing to successful enrichment.

It is interesting that the temperature difference between the two cultures appeared to be a selective condition. Application of 15 °C resulted in the coexistence two species of marine *Scalindua*, whereas 23 °C was (ultimately) selective for a single species corresponding to the 16S rRNA gene sequence EU142958. In the future, we will incubate the 15 °C culture at lower temperature to investigate whether lower temperatures are selective for Scalindua phylotype EU142957.

The two species of marine Scalindua enriched in this study are typical anammox bacteria, having the same compartmentalized cellular ultrastrucutre (van Niftrik et al., 2008). Like other anammox bacteria they are metabolically versatile using different electron donors and acceptors in addition to ammonium and nitrite. Interestingly, they displayed relatively high anammox rates, even though the temperature of the enrichment procedure was much lower than for the anammox species in wastewater. The observed high rates, up to 25 µmol (g protein)$^{-1}$ min$^{-1}$(at 22 ˚C) were consistent with cell counts and activity measurements in natural habitats (Kuypers et al., 2005; Schmid et al., 2007).

Formate and acetate oxidation rates were comparable to formate and acetate oxidation rates in other anammox species (Kartal et al., 2007; 2008). Propionate was oxidized at a much lower rate, consistent with the previous conclusion that propionate oxidation may be a specific trait of "*Candidatus* Anammoxglobus propionicus" (Kartal et al 2007). Reduction rates of manganese and iron were stimulated by the presence of formate as electron donor as previously shown for "*Candidatus* Kuenenia" (Strous et al., 2006). The *in situ* relevance of this trait needs to be investigated further (Hulth et al., 2005), but may be the basis for their global distribution in marine ecosystems.

The core lipid ladderane fatty acid b (Fig. 3), a C18 fatty acid with 3 cyclobutane rings and one cyclohexane ring, has not previously been observed in anammox enrichment cultures obtained from wastewater, but was previously reported in water column particulate matter of the Black Sea and Benguela upwelling systems (Kuypers et al., 2003; Kuypers et al., 2005). This indicates that ladderane fatty acid b may be specific to (marine) Scalindua species. Ladderane lipids were depleted in $^{13}C$ relative to the DIC of the medium, the carbon source for growth by up to 55-58‰. This strong $^{13}C$ depletion of ladderane lipids was also found for wastewater anammox bacteria. Experimental and genomic investigations have shown that the acetyl-CoA pathway (known to fractionate strongly against $^{13}C$) is used by the wastewater anammox bacterium *Kuenenia stuttgartiensis* (Schouten et al., 2004; Strous et al., 2006; Kartal et al., 2007).

## 2.5 Conclusion

Two species of marine *Scalindua* anammox were enriched from a marine sediment. Microbiological characterization of these enrichments showed that marine anammox bacteria conform to the typical anammox phenotype, currently based on species enriched from wastewater. The marine species also produced hydrazine in the presence of hydroxylamine, synthesized ladderane lipids, and possessed an intracytoplasmic compartment, known as the anammoxosome. They consumed the same substrates as the wastewater anammox bacteria, at similar rates but at lower temperatures. Their enrichment was dependent on the use of a Red Sea salt based medium medium. The enrichment of these species makes future genomic,

proteomic and physiological studies possible. Such data is necessary to advance the investigation and understanding of anammox marine ecology.

**Acknowledgements**

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Me OH
1:1
40
20

# chapter 3

## Improved analysis of ladderane lipids in biomass and sediments using high performance liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry

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**Abstract**

Ladderane lipids, containing 3 or 5 linearly concatenated cyclobutane moieties, are considered to be unique biomarkers for the process of anaerobic ammonium oxidation, an important link in the oceanic nitrogen cycle. Due to the thermal lability of the strained cyclobutane moieties, the ladderane lipids are difficult to analyze by gas chromatography, giving poor chromatography and high detection limits. A method combining high performance liquid chromatography coupled to positive ion atmospheric pressure chemical ionization-tandem mass spectrometry (HPLC/APCI-MS/MS), was developed for analysis of the most abundantly occurring ladderane lipids, occurring as fatty acids and ether-bound to glycerol. Detection was achieved by selective reaction monitoring of four specific fragmentations per ladderane lipid. Detection limits of 30-35 pg injected on column and a linear response ($r^2 > 0.99$) over nearly 3 orders of magnitude were achieved for all compounds. Using this method, these unique ladderane lipids were for the first time identified in a surface sediment from the Gullmarsfjorden, in concentrations ranging from 1.1 to 5.5 ng/g for the ladderane fatty acids and 0.7 ng/g for the monoether. It is foreseen that this method will allow the investigation of the occurrence of anaerobic ammonium oxidation in natural settings in much more detail than before.

## 3.1 Introduction

Recently, organisms capable of anaerobic ammonium oxidation ('anammox') in waste waters have been discovered (Strous et al., 1999). These bacteria of the order Planctomycetales combine ammonium and nitrite directly into $N_2$. The anammox process takes place in a special compartment ('anammoxosome') of the cell surrounded by a membrane consisting of unique linearly concatenated cyclobutane (ladderane) lipids (Sinninghe Damsté et al., 2002) (Fig. 1).

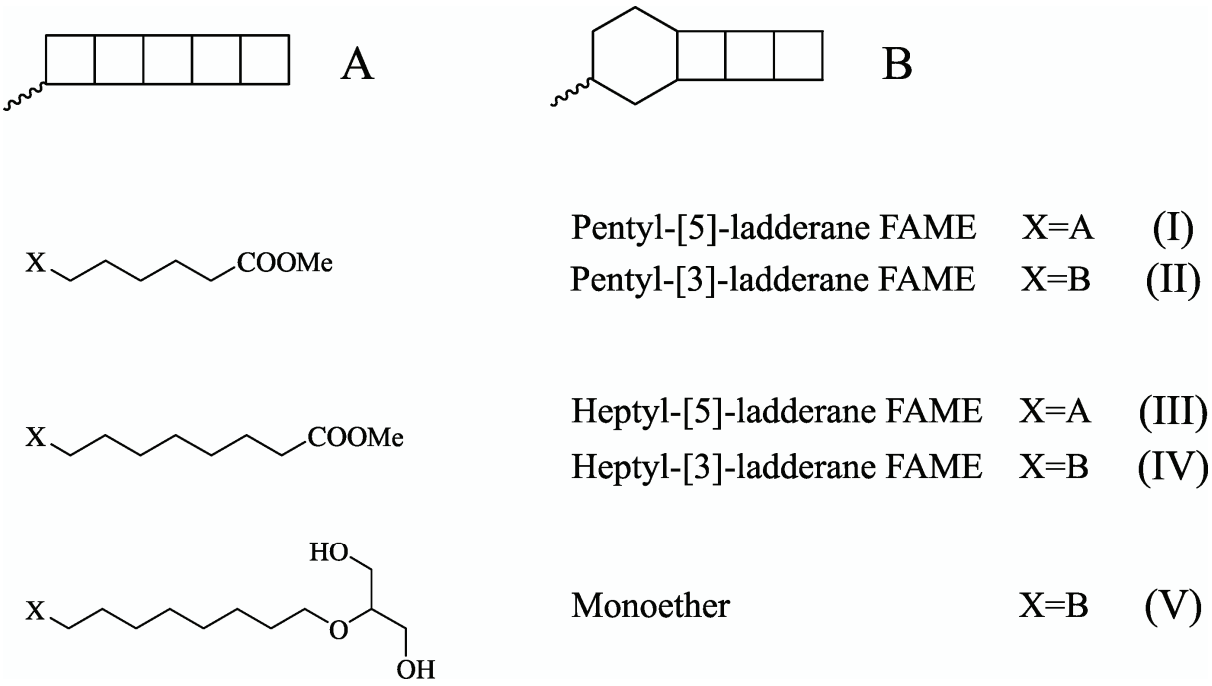


**Figure 1.** Structure of ladderane lipids

These lipids form an extremely dense membrane, which functions as a tight barrier against diffusion. This is required to maintain concentration gradients during the exceptionally slow anammox metabolism and to protect the remainder of the cell from toxic anammox intermediates such as hydrazine. Lipids containing the biochemically unique [5]- and [3]-ladderane moieties (Figure 1, structures A and B, respectively) exist in a variety of different forms. In two strains of *Candidatus* 'Brocadia anammoxidans' they occurred, in order of relative abundance, as fatty-acids (shown as fatty acid methyl esters (FAMEs) in Figure 1, structures I-IV), an *sn*-2-

alkyl glycerol monoether (Fig. 1, structure V), alcohols, *sn*-1,2-dialkyl glycerol diethers, and *sn*-2-*O*-alkyl, *sn*-1-acyl glycerol.

Although initial studies have focussed on anammox bacteria from waste water treatment plants, recent findings have indicated that anammox also occurs in the marine environment and may represent a quantitatively important process in the oceanic nitrogen cycle (Dalsgaard et al., 2003; Dalsgaard and Thamdrup, 2002; Kuypers et al., 2003). Kuypers et al. (2003) were the first to identify ladderane lipids in a natural seawater sample using gas chromatography (GC) and GC/mass spectrometry (GC/MS) analysis. Using a combination of molecular biological techniques, $^{15}N$ labelling studies and the ladderane lipids as unique biomarkers, they identified the anammox process in the water column of the Black Sea. Both the pentyl-[3]- and heptyl-[3]-ladderane fatty acids (II and IV), as well as the monoether (V) were detected (as FAMEs) in concentrations up to 4 ng/l in the suboxic water zone where anammox takes place. Recently, the anammox process was also shown to be important in the Benguela upwelling system (Kuypers et al., 2005). Up to 4 ng/l of both the pentyl-[3]- and heptyl-[3]-ladderane fatty acids was detected (as FAMEs) in the oxygen-minimum zone. Although active anammox processes have been shown to occur in sediments using $^{15}N$ labelling studies (Dalsgaard et al., 2003; Dalsgaard and Thamdrup, 2002; Engström et al., 2005), no reports of ladderanes in sediments have been made as of yet.

Ladderane lipids are difficult to analyze by GC/MS, giving poor chromatography and high detection limits (Sinninghe Damsté et al., 2005). This is due to the thermal lability of the strained cyclobutane moieties making up the [3]- and [5]-ladderane moieties. During GC analysis these components transform into thermally more stable degradation products, which are partially resolved from each other, resulting in the broad peaks observed. To circumvent these problems, a novel method for analysis of the most abundantly occurring ladderane lipids, the fatty acids (as FAMEs) and the monoether, using high performance liquid chromatography coupled to positive ion atmospheric pressure chemical ionization tandem mass spectrometry (HPLC/APCI-MS/MS), was developed. Examples will be shown of ladderane lipid analyses in biomass and sediments.

## 3.2 Experimental

### *3.2.1 Standards*

A qualitative standard mixture containing the heptyl-[3]- and –[5]-, and pentyl-[5]-ladderane FAMEs (described in Sinninghe Damsté et al., 2002) was available for method optimization. Quantitative standards of the fatty acid methyl esters of the heptyl-[3]- and heptyl–[5]-ladderanes, as well as the monoether were prepared from a large batch of anammox bacterial biomass (Sinninghe Damsté et al., 2002). Specifically, freeze dried culture material was ultrasonically extracted three times each with methanol (MeOH), MeOH/dichloromethane (DCM) (1:1, v/v) and DCM. The combined extracts were dried by rotary evaporation. The resulting extract was methylated with $BF_3$/methanol (20% solution) and the reaction mixture partitioned using bidistilled water and DCM. The DCM layer was dried and the resulting residue was separated over an aluminium oxide column with 3 column volumes each of hexane/DCM (4:1, v/v), (3:2, v/v), (1:1, v/v) and MeOH:DCM (2:1, v/v). Ladderane FAMEs eluted in the hexane:DCM (4:1) and (3:2) fractions, while the monoether eluted in the MeOH:DCM (2:1) fraction. Aliquots of the resulting lipid fractions were further fractionated with reversed phase HPLC using an Agilent 1100 LC system (Palo Alto, CA, USA). After injection onto two Zorbax Eclipse XDB-$C_8$ columns (4.6 x 150 mm, 5 µm, Agilent), coupled in series and maintained at 30 °C, the ladderane lipids were eluted with 0.4 mL/min methanol while collecting the eluents in 15 sec fractions. Purity of the resulting isolates was assessed by GC and GC/MS according to Sinninghe Damsté et al. (2002) and was at least 90%.

A standard mixture of poly-unsaturated FAMEs (PUFA-2 mix, Matreya, Inc., Pleasant Gap, PA, USA), consisting mainly of C16:0, C18:0, C18:1, C18:2, C18:3, C20:1, C20:2, C20:3 and C22:4 was used to identify interfering peaks in natural samples.

### *3.2.2 Samples*

Biomass from an anammox reactor, containing both *Candidatus* "Scalindua wagneri" and *Candidatus* "Kuenenia stuttgartiensis", was a kind gift from B. Kartal (Department of Microbiology, Radboud University Nijmegen). A composite sample of surface sediments (0-0.5 cm) was obtained from Gullmarsfjorden, Sweden, where Engström et al. (2005) previously showed an active anammox process in the sediment.

*3.2.3 Sample preparation*

Biomass material, as well as sediments, were freeze-dried, homogenized and ultrasonically extracted with MeOH/DCM (2:1, v/v; 5x). The combined extracts were dried by rotary evaporation and $Na_2SO_4$. In case of culture cell material, the extract was methylated with $BF_3$/MeOH (20% solution) and the reaction mixture partitioned using bidistilled water and DCM. The DCM layer was dried and this total lipid fraction was analyzed for ladderanes.

For sediments, the extract was dissolved in DCM and stirred overnight with copper powder, activated with 1 N HCl, to remove elemental sulfur. The desulfurized extracts were saponified with 1 N KOH (in MeOH 96%) by refluxing for 1 h. After adding 2 mL bidistilled water, the reaction mixture was partitioned 3x with DCM to obtain a neutral fraction. A fatty acid fraction was obtained by acidifying the residue with 2 N HCl/MeOH (1:1, v/v) and partitioning 3x with DCM. Both the neutral and fatty acid fraction were dried over $Na_2SO_4$. The fatty acid fractions were methylated with diazomethane ($CH_2N_2$) to convert fatty acids into their corresponding methyl esters. To remove very polar components, aliquots were eluted with ethyl acetate over a small column filled with silica. Polyunsaturated fatty acids were removed by eluting the aliquots with DCM over a small column packed with silica impregnated with $AgNO_3$.

Appropriate sample fractions (a total lipid extract, fatty acid fraction, or neutral fraction) were dissolved in acetone, filtered through a 0.45 µm, 4 mm diameter PTFE filter, and analyzed by HPLC/APCI-MS/MS as described below.

*3.2.4 Analytical HPLC/APCI-MS/MS*

Analyses were performed using an Agilent 1100 LC system, consisting of an inline membrane degassing unit, thermostated auto injector and column compartment, coupled to a Quantum TSQ Ultra EM triple quadrupole mass spectrometer (Thermo Inc, San Jose, CA, USA) 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. Injection volumes varied from 1 to 20 µL. Ladderanes were eluted with 0.4 mL/min methanol with a total run time of 20 min. Detection was achieved using

positive ion APCI and Selective Reaction Monitoring (SRM). The following optimal source settings were determined using loop injections of a qualitative mixture of the heptyl-[3]- and –[5]- and pentyl-[5]-ladderane FAMEs, and monoether[8]: 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. Optimal collision energies varied per monitored reaction and were determined by automatic tuning.

The heptyl-[3]- and –[5]-ladderane FAME as well as the monoether were quantified by comparing the response in the appropriate SRM trace to the response of known amounts of standards (amounts injected on column were 30 pg to 14.5 ng, 35 pg to 17.7 ng, and 10 pg to 10.6 ng for the heptyl-[3]-ladderane FAME, heptyl–[5]-ladderane FAME, and the monoether respectively). No quantitative standard was available for the pentyl-[5]-ladderane FAME and this ladderane was quantified by comparing its response to that of the heptyl-[5]-ladderane FAME.

## 3.3 Results and discussion

### *3.3.1 Development of MS/MS method*

In order to determine which reactions should be monitored in the SRM protocol, a series of MS/MS experiments at various collision energies were performed for each ladderane lipid. APCI-MS/MS spectra of the protonated molecules of the heptyl-[5]- and the heptyl-[3]-ladderane FAMEs are shown in Figures 2(a) and (b), respectively. For the heptyl-[5]-ladderane FAME, a protonated molecule is obtained at *m/z* 317. Loss of the methoxy and aldehyde functionalities results in a fragment at *m/z* 267. Loss of the alkyl chain results in a characteristic fragment for the [5]-ladderane moiety at *m/z* 161. It is this ladderane moiety then fragments in a series of secondary and tertiary reactions to produce clusters of fragments at *m/z* 145/147, 133, 119, 105, 91, and 79/81. As expected, the relative abundance of the larger fragments (i.e. *m/z* 269, 163, 133/135) decreased with increasing collision energy, while the abundance of the smaller fragments (i.e. 107/109, 93/95, 79/81, 67) increased. The APCI MS/MS spectrum of the protonated molecule of the pentyl-[5]-ladderane FAME (*m/z* 289) showed the same fragmentation pattern as the heptyl-[5]-ladderane FAME, as they both contain the [5]-ladderane moiety. The APCI-MS/MS spectrum of

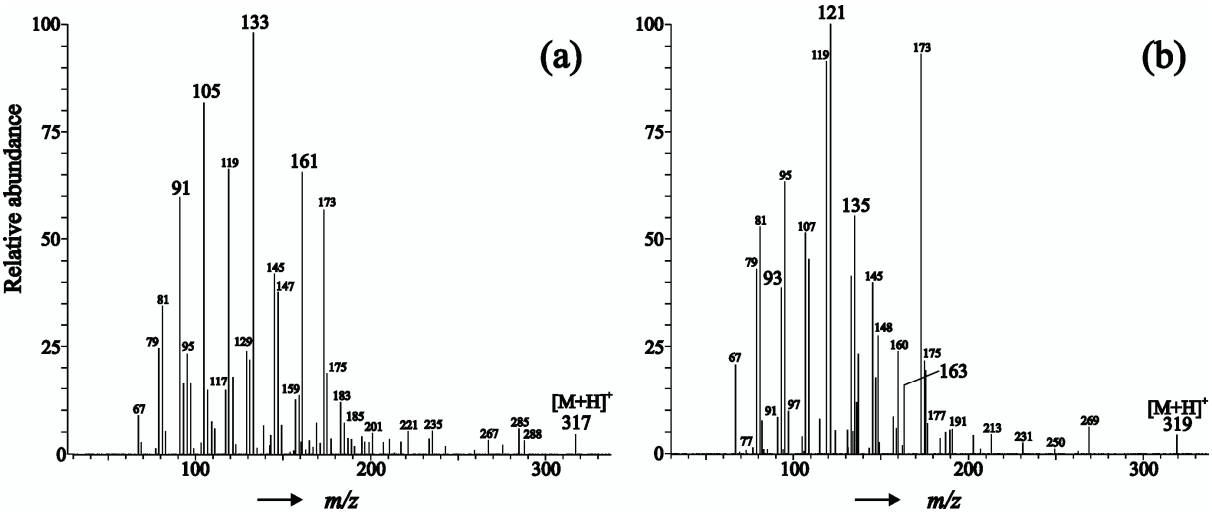


**Figure 2.** APCI-MS/MS spectra (1.5 mTorr argon, CE -25 V) of: (**a**) protonated molecule (*m*/z 317) of heptyl-[5]-ladderane FAME, and (**b**) protonated molecule (*m*/z 319) of heptyl-[3]-ladderane FAME. Indicated in larger type face are the product ions used for SRM.

the heptyl-[3]-ladderane FAME, with a protonated molecule at *m*/z 319, shows similar fragmentations to the heptyl-[5]-ladderane FAME. However, most fragment clusters are offset by 2 Da compared to their equivalent fragments in the mass spectrum of the heptyl-[5]-ladderane FAME, due to substitution of a hexyl instead of two concatenated cyclobutyls in this molecule. The mass spectra obtained by APCI-MS/MS for both the heptyl-[3]- and heptyl-[5]-ladderane FAMEs show similar fragments compared to spectra generated by GC/MS (Sinninghe Damsté et al., 2005).

Final 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 abundance. Table 1 lists the protonated molecule and selected product ions and respective collision energy for maximal abundance for each monitored ladderane lipid. Identical product ions are monitored for both the pentyl- and heptyl-[5]-ladderane FAMEs as they possess identical ladderane moieties. Likewise, 3 out of 4 product ions are identical for the heptyl-[3]-ladderane FAME and the monoether as they share the [3]-ladderane moiety. The monoether product ion of *m*/z 273 represents the loss of the glycerol moiety.

Table 1. Protonated molecule, selected product ions, and respective collision energy for maximal abundance for each monitored ladderane lipid.

| Ladderane lipid | Parent $[M+H]^+$ *m/z* | Daughter *m/z* | Collision Energy (V) |
|---|---|---|---|
| pentyl-[5]-ladderane FAME (I) | 289.3 | 91.1 | 37 |
| | | 105.1 | 33 |
| | | 133.1 | 24 |
| | | 161.1 | 18 |
| heptyl-[5]-ladderane FAME (III) | 317.3 | 91.1 | 37 |
| | | 105.1 | 35 |
| | | 133.1 | 24 |
| | | 161.1 | 19 |
| heptyl-[3]-ladderane FAME (IV) | 319.3 | 93.1 | 37 |
| | | 121.1 | 24 |
| | | 135.1 | 19 |
| | | 163.1 | 16 |
| Monoether (V) | 365.3 | 93.1 | 43 |
| | | 121.1 | 29 |
| | | 135.1 | 27 |
| | | 273.2 | 22 |

External standard curves were prepared for both the heptyl–[3]- and heptyl-[5]-ladderane FAMEs, as well as the monoether. Detection limits, defined at a signal to noise ratio of 3, were 30, 35, and 30 pg injected on column for the heptyl–[3]-ladderane FAME, heptyl-[5]-ladderane FAME and the monoether, respectively. The response was linear ($r^2 > 0.99$) over nearly 3 orders of magnitude for all three compounds. This shows that the developed method is capable of quantitatively and sensitively analyzing ladderane lipids. In comparison to previously used GC and GC/MS methods this method has the benefit of separating these lipids at low column temperatures preventing their thermal degradation.

### *3.3.2 Application of HPLC/MS/MS method*

To test the method we first applied it to cell material of anammox bacteria where the presence of ladderane lipids had already been established by GC/MS. Figure 3(a) shows the SRM traces of ladderane lipids as determined in a total extract of reactor biomass containing *Candidatus* "Scalindua wagneri" and *Candidatus* "Kuenenia stuttgartiensis". The heptyl-[5]-ladderane FAME was most abundant, followed by the heptyl-[3]-ladderane FAME. Concentrations of the pentyl-[5]-

ladderane FAME and monoether were approximately an order of magnitude less. No major interferences were observed in the SRM traces from other components.

The method was then tested on a more complex matrix, i.e. a surface sediment (0-0.5 cm) from the Gullmarsfjorden, Sweden, where anammox activity was detected previously using $^{15}N$ labelling (Engström et al., 2005). Screening of the fatty acid fraction by GC/MS revealed a large range of saturated and unsaturated FAMEs but ladderane FAMEs could not be detected. Figure 3(b) shows the SRM traces of the ladderane lipids in this same sediment. The FAMEs were determined in the fatty acid fraction, while the monoether was determined in the neutral fraction. After initial analysis of the fatty acid fraction large interfering peaks, eluting just before and co-eluting with the target compounds, were observed in all FAME SRM traces.

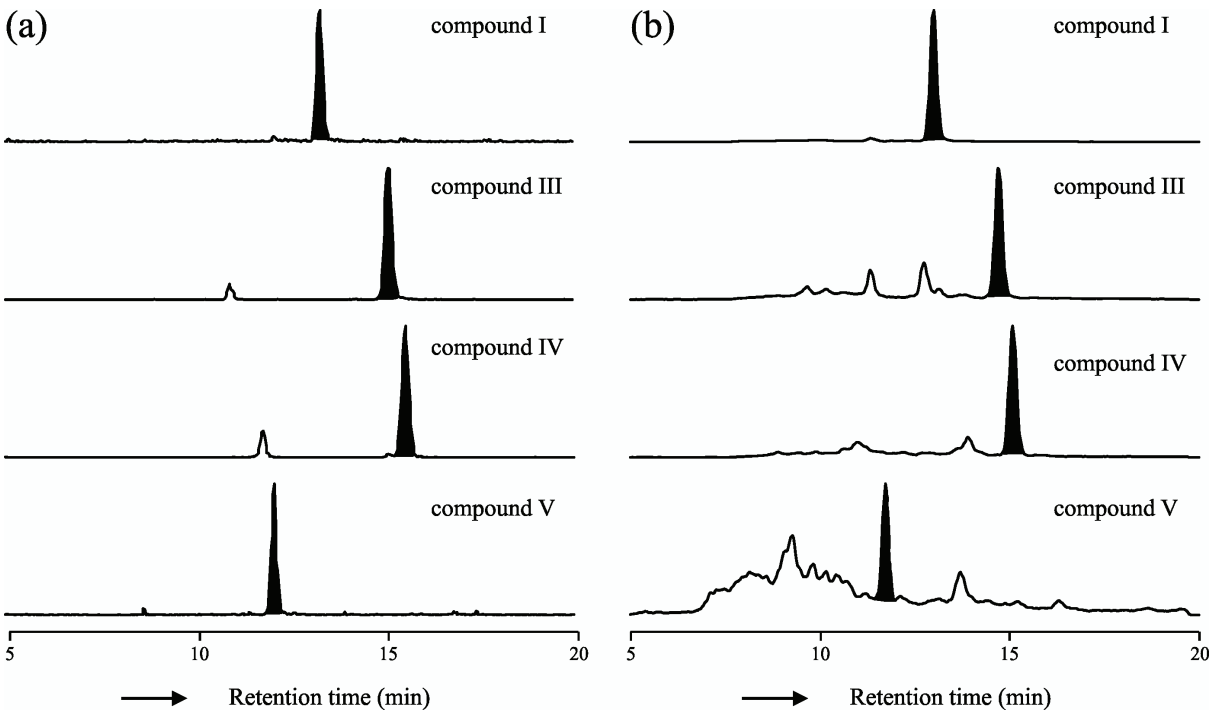


**Figure 3.** SRM traces of ladderane lipids in: (a) reactor biomass containing both *Candidatus* "Scalindua wagneri" and *Candidatus* "Kuenenia stuttgartiensis", and (b) surface sediment (0-0.5 cm) from Gullmarsfjorden, Sweden. Traces show the pentyl-[5]-, the heptyl-[5]-, the heptyl-[3]-ladderane FAMEs, and the monoether (compound I, III, IV, and V), respectively. For the reactor biomass all traces originate from an analysis of a total lipid extract. For the Gullmarsfjorden, the ladderane FAMEs (compound I, III and IV) were determined in the methylated fatty acid fraction, while the monoether (compound V) was determined in the neutral fraction.

Analysis of a standard mixture of poly-unsaturated FAMEs (PUFAs) showed that these interfering peaks were most likely due to the presence of high concentrations of PUFAs in the fatty acid fraction. Although the recorded APCI MS/MS spectra of PUFAs are distinctly different from those of the ladderane FAMEs (data not shown), the product ions selected for monitoring the ladderane lipids also occur in minor amounts in the mass spectra of PUFAs. However, to remove these interferences the PUFAs can easily be separated from ladderane FAMEs by column chromatography using silica impregnated with $AgNO_3$. PUFAs were effectively removed, while the recoveries of the ladderane FAMEs were over 90% as tested with standard mixtures. Measured concentrations were 5.5, 1.1 and 1.5 ng/g sediment for the pentyl-[5]-, heptyl-[5]-, and heptyl-[3]-ladderane FAMEs, respectively, and 0.7 ng/g for the monoether. This is the first report of the unique ladderane lipids in sediments. It is foreseen that this method will allow the investigation of the occurrence of anaerobic ammonium oxidation in natural settings in much more detail than before.

**Acknowledgements**

We gratefully acknowledge B. Kartal and Prof. M.S.M. Jetten of the Department of Micobiology, Radboud University Nijmegen, for providing biomass from an anammox reactor for this study.

# chapter 4

## Ladderane lipid distribution in four genera of anammox bacteria

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**Abstract**

Intact ladderane phospholipids and core lipids were studied in four species of anaerobic ammonium oxidizing (anammox) bacteria, each representing one of the four known genera. Each species of anammox bacteria contained $C_{18}$ and $C_{20}$ ladderane fatty acids with either 3 or 5 linearly condensed cyclobutane rings and a ladderane monoether containing a $C_{20}$ alkyl moiety with 3 cyclobutane rings. The presence of ladderane lipids in all four anammox species is consistent with their putative physiological role to provide a dense membrane around the anammoxosome, the postulated site of anammox catabolism. In contrast to the core lipids, large variations were observed in the distribution of ladderane phospholipids, i.e. different combinations of hydrophobic tail (ladderane, straight chain and methyl branched fatty acid) types attached to the glycerol backbone *sn*-1 position, in combination with different types of polar headgroup (phosphocholine, phosphoethanolamine or phosphoglycerol) attached to the *sn*-3 position. Intact ladderane lipids made up a high percentage of the lipid content in the cells of "*Candidatus* Kuenenia stuttgartiensis", suggesting that ladderane lipids are also present in membranes other than the anammoxosome. Finally, all four investigated species contained a $C_{27}$ hopanoid ketone and bacteriohopanetetrol, which, indicates that hopanoids are anaerobically synthesised by anammox bacteria.

## 4.1 Introduction

Bacteria capable of oxidizing ammonia under anoxic conditions (anammox bacteria) have been shown to be key organisms, both in the oceanic nitrogen cycle (Kuypers et al., 2003; Kuypers et al., 2005; Hamersley et al., 2007) and anaerobic wastewater treatment systems (van Dongen et al., 2001; Op den Camp et al., 2006; van der Star et al., 2007). They perform the anammox reaction by combining 1 mole of ammonia with 1.32 mole of nitrite to produce dinitrogen gas (van de Graaf et al., 1997). Anammox bacteria form a distinct phylogenetic group within the *Planctomycetes* (Strous et al., 1999; Schmid et al., 2007). The group of anammox bacteria is currently made up of four genera, "*Candidatus* Brocadia", "*Candidatus* Kuenenia", "*Candidatus* Anammoxoglobus" and "*Candidatus* Scalindua" (Strous et al., 1999; Schmid et al., 2000; Schmid et al., 2003; Kartal et al., 2007; Schmid et al., 2007). The species "*Candidatus* Brocadia anammoxidans", "*Candidatus* Brocadia fulgida", "*Candidatus* Kuenenia stuttgartiensis", "*Candidatus* Anammoxoglobus propionicus", "*Candidatus* Scalindua brodae" and "*Candidatus* Scalindua wagneri" have all been identified in or enriched from wastewater treatment sludge or effluent. In contrast, "*Candidatus* Scalindua sorokinii" was identified in water column particulate matter in the Black Sea (Kuypers et al., 2003; Schmid et al., 2003). Since then, other close relatives of "*Candidatus* Scalindua" have been discovered in the oceanic upwelling waters of the Namibian shelf and the Peruvian coast, all containing more than 97% 16S ribosomal RNA gene sequence similarity to "*Candidatus* S. sorokinii" and "*Candidatus* S. brodae" (Kuypers et al., 2005; Hamersley et al., 2007; Woebken et al., 2007). Anammox bacteria have also been detected in estuarine sediments (Risgaard-Petersen et al., 2004; Zhang et al., 2007; Rich et al., 2007) and lakes (Schubert et al., 2006), and the 16S rRNA gene sequences were also found to be closely related to those of "*Candidatus* Scalindua". Therefore, only close relatives (>95% sequence similarity) of the "*Candidatus* Scalindua" cluster have so far been reported to inhabit natural environments (Schmid et al., 2007).

Based on phylogenetic and genomic analysis, anammox bacteria appear to have a common ancestor, despite the fact that there is a large evolutionary difference between the four genera (on average 85% 16S rRNA gene sequence similarity between environmental and wastewater anammox bacteria). Furthermore, all

species share similar physiological traits (Jetten et al., 2003; Kartal et al., 2007). All successfully enriched anammox bacteria have been shown to contain a specialised compartment, a membrane bound 'organelle' called the anammoxosome (Strous et al., 1999; Sinninghe Damsté et al., 2002; Schmid et al., 2003; Kartal et al., 2007). On the basis of confined immunogold hydroxylamine oxidoreductase labelling, the anammoxosome is postulated to be the site of the anammox reaction (Lindsay et al., 2001; van Niftrik et al., 2007; van Niftrik et al., 2008). The membrane of the anammoxosome has been shown to consist mainly of unique ladderane lipids containing linearly concatenated cyclobutane rings (**a**-**d**, letters in bold relate to the lipid codes found in Fig. 1; Table 2) (Sinninghe Damsté et al., 2002). These lipids have been shown to form an unusually dense barrier, which reduces the permeability of the lipid membrane to fluorophores and therefore other small molecules (e.g. the anammox intermediate, hydrazine) that can easily permeate less dense bacterial membranes (Sinninghe Damsté et al., 2002). It is also thought that an electrochemical proton gradient may exist across the anammoxosome membrane which is involved in ATP synthesis, thereby the anammoxosome and thus ladderane lipids, have been assumed to play a role in energy conservation during the anammox reaction (van Niftrik et al., 2004). Recently, intact ladderane membrane phospholipids have been identified in an enrichment culture of "*Candidatus* K. stuttgartiensis" and shown to contain phosphocholine (PC) or phosphoethanolamine (PE) polar headgroups (Fig. 1., **II-VII**) (Boumann et al., 2006), but the headgroup composition of intact ladderane lipids in other anammox species is presently unknown.

Although for a number of anammox bacteria the core lipid composition has already been reported (Sinninghe Damsté et al., 2005; Kartal et al., 2007), a detailed analysis and comparison of the lipid composition in the different anammox genera has been lacking. Therefore, it is unknown if the composition and function of these unique lipids varies in the different genera. In this study we have analysed both the core lipid and phospholipid composition of enrichment cultures of four anammox species, each representing one of the currently recognised four anammox genera. The lipid compositions were compared to each other and interpreted in view of anammox phylogeny and culture conditions in the employed sequencing batch reactors.

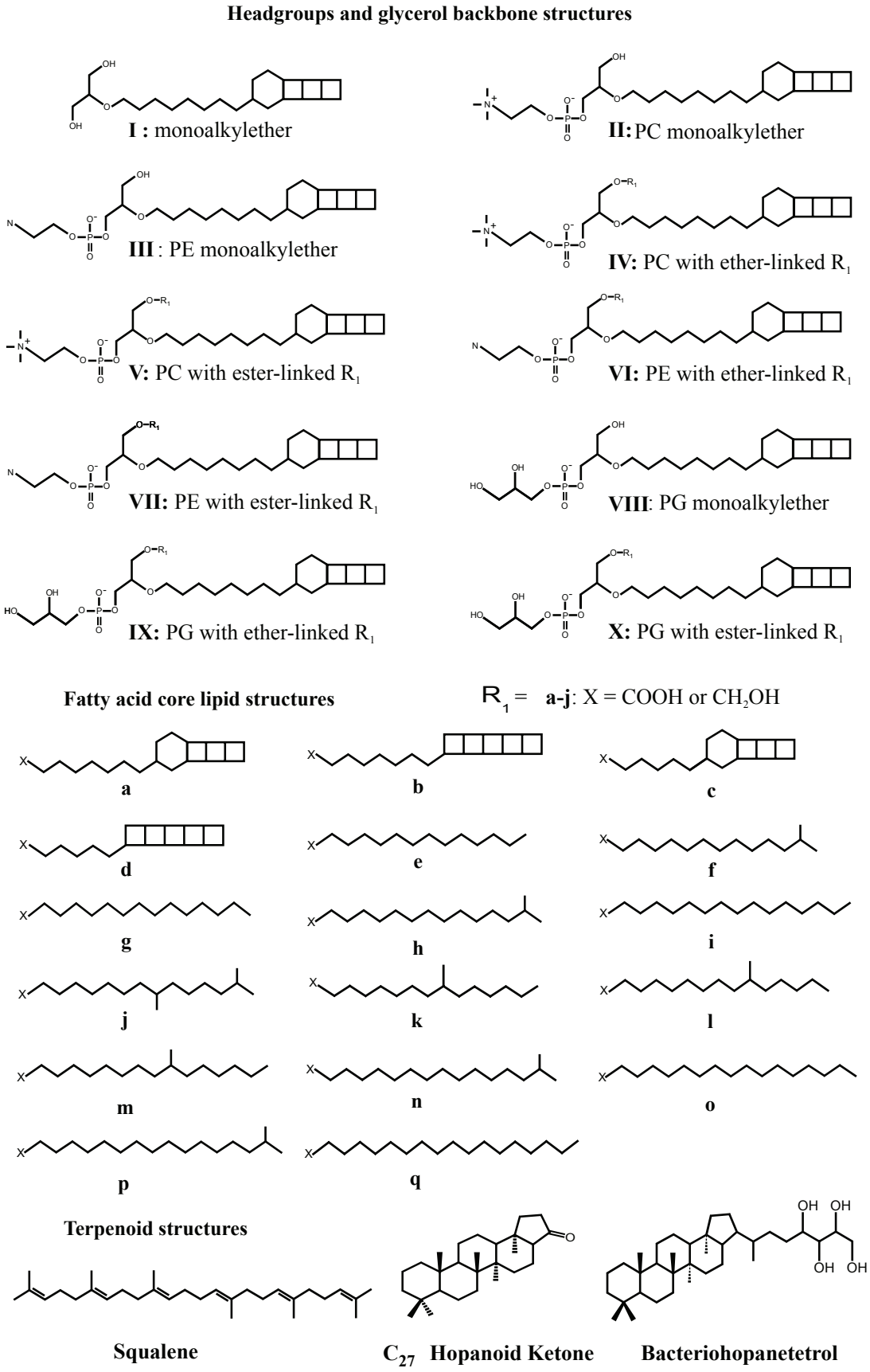


**Figure 1.** Types of core lipids and intact phospholipids lipids found in the anammox enrichment cultures

## 4.2 Materials and methods

### *4.2.1 Enrichment cultures*

Anammox cultures were grown over 5 months in sequencing batch reactors (SBRs), as described previously (Strous et al., 1998; Kartal et al., 2006; Kartal et al., 2007). Table 1 shows the SBR conditions and the percentage of anammox bacteria in the enrichment cultures. The amount of anammox cells was determined using fluorescence *in situ* hybridization (FISH). To prepare cells for FISH analysis, 1 ml of biomass sample was obtained from the reactor, fixed in paraformaldehyde and stored at -20$^{o}$C until analysis. Hybridization with fluorescently labelled probes was performed according to Schmid et al. (2000) and (2005). Probes were Cy3, Cy5 and 5(6)-carboxyflourescein-N-hydroxysiccinimide ester (FLUOS) labelled derivatives (Thermo Electron Corporation, Ulm, Germany). Subsequently slides were air dried and embedded in Vectashield (Vector Laboratories Inc., Burlingame, CA). A Zeiss axioplan 2 microscope (Zeiss, Jena, Germany) was used to inspect and estimate the number of anammox and other prokaryotic cells using fluorescence microscopy. Samples were analysed on triplicate slides from which triplicate pictures were taken and the percentage of anammox cells in the microbial community was counted on each slide and the average percentages have been reported. The three dimensional property of the aggregates means that the percentage cell counts are estimates of the actual anammox population.

### *4.2.2 Total organic carbon analysis*

Anammox biomass was weighed into tin cups and analysed for organic carbon content using a Thermo Flash EA1112 Series Analyser, following the method of Verardo et al., (1990).

### *4.2.3 Core lipid analysis*

#### 4.2.3.1 Core lipid extraction

Samples of enrichment culture biomass and medium were obtained from the SBRs and the cells were precipitated using centrifugation (three samples each from the "*Candidatus* A. propionicus", "*Candidatus* B. fulgida" and "*Candidatus* K. stuttgartiensis" SBRs, grown over a period of 4 months, and one sample from the "*Candidatus* Scalindua" SBR). The supernatant was removed and discarded and the cell material was freeze dried. Approximately 20 mg dry weight biomass was

**Table 1**. Anammox sequencing batch reactor conditions

| Genera (*Candidatus*) | Species | FISH probes used | % Enriched Anammox | Reactor Temperature (°C) | Reactor pH |
|---|---|---|---|---|---|
| "Anammoxoglobus | propionicus" | EUB mix; EUB 338 (Amann et al., 1990)+ EUB (II) + EUB (III) (Daims et al., 1999)<br>Pla46 (Neef et al., 1998)<br>AMX 368 (Schmid et al., 2003) | ± 75 | 35 | 7.4±0.1 |
| "Brocadia | fulgida" | EUB Mix<br>Ban Mix (Schmid et al., 2001)<br>AMX 820 (Schmid et al., 2000) | ± 75 | 35 | 7.4* |
| "Kuenenia | stuttgartiensis" | EUB Mix<br>AMX 820<br>KST 1273 (Schmid et al., 2000) | ± 90 | 35 | 7.4* |
| "Scalindua" | | EUB Mix<br>AMX 368<br>BS820 (Schmid et al., 2005)<br>Scabr1114 (Schmid et al., 2005) | ± 75 | 20 | 7.5±0.1 |

* single measurement

extracted five times using a dichloromethane/methanol mixture (2:1 v/v). Combined extracts were dried using rotary evaporation yielding the total lipid extract. One aliquot of the total lipid extract was methylated using boron trifluoride in methanol and eluted with ethyl acetate over a small silica column and silylated using BSTFA (N,O-bis-(trimetylsilyl)trifluoroacetamide) in pyridine at 60°C for 15 min. For quantification purposes a known amount (3 µg) of the internal standard 6,6-$d_2$-3-methyleicosane (*ante iso* $C_{22}$) in ethyl acetate was added after silylation, and the derivatized extract was analysed using gas chromatography (GC) and gas chromatography mass spectrometry (GC/MS). Another aliquot of the total lipid extract was methylated as above and further purified by eluting (in ethyl acetate) over a small column containing $SiO_2$ impregnated with $AgNO_3$ (5%). The sample was subsequently dissolved in acetone, filtered and analysed using high performance liquid chromatography coupled to positive ion atmospheric pressure chemical ionization-tandem mass spectrometry (HPLC/APCI-MS/MS).

4.2.3.2 GC and GC/MS analysis

GC analysis was performed on a Hewlett Packard HP6890 Series GC System, fitted with on column injection and a flame ionisation detector. A helium carrier gas was used on a fused silica capillary column (25 m long, 0.32 mm Ø), coated with CP Sil5

(film thickness 0.12 µm). Samples were analysed using a GC where the samples were injected at 70°C, with the temperature rising to 130°C at a rate of 20°C per minute and then to 320°C at a rate of 4°C per minute, where the temperature was held constant for 10 minutes. 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 for GC in this section. Ladderane fatty acids and diethers were identified according to characteristic molecular weights and fragment ions in mass spectra published by Sinninghe Damsté et al. (2004, 2005).

4.2.3.3 HPLC/APCI-MS/MS analysis

Core ladderane lipids were analysed according to Hopmans et al. (2006) with some modifications. Briefly, samples dissolved in acetone were injected onto a Zorbax Eclipse XDB $C_8$ column (3.0 x 250 mm, 5 µm, Agilent) kept at 30°C. Ladderane core lipids were eluted using 0.18 ml $min^{-1}$ methanol and detected with a Quantum TSQ Ultra EM triple quadrupole mass spectrometer (Thermo Inc., San Jose, CA, USA), equipped with an ion max source with an APCI probe, in selective reaction monitoring (SRM) mode. In addition to analysing using a method containing the previously described SRM transitions to detect the $C_{20}$ [3], $C_{20}$ [5] and $C_{18}$ [5] ladderane fatty acids and the $C_{20}$ [3] ladderane monoether (Fig. 1, **a**, **b**, **d** & **I**) respectively, a new method was created which quantifies the $C_{18}$ [3] ladderane fatty acid (Fig. 1, **c**) and the 3 previously mentioned fatty acids. For this, transitions from *m*/z 291.3 ($[M+H]^+$) to *m*/z 93.1,121.1,135.1, and 163.1 at collision energies (CE) of 37, 24, 19, and 16 V, respectively, were added and the SRM transitions to detect the $C_{20}$ [3] ladderane monoether were removed.

Ladderane lipids were quantified using external standard curves of the $C_{20}$ [3] ladderane fatty acid (**a)** and the $C_{20}$ [5] ladderane fatty acid (**b**) and the $C_{20}$ [3] ladderane monoether (**I**). No quantitative standards were available for the $C_{18}$ [3] ladderane fatty acid (**c)** and the $C_{18}$ [5] ladderane fatty acid (**d**). Therefore these ladderanes have been quantified by comparing their responses to the responses of the $C_{20}$ [3] ladderane fatty acid (**a)** and $C_{20}$ [5] ladderane fatty acid (**b**), respectively.

*4.2.4 Phospholipid analysis*

4.2.4.1 Phospholipid extraction

The analysis of intact ladderane phospholipids using HPLC electrospray ionization MS/MS (HPLC/ESI-MS/MS) was performed as previously described (Boumann et al., 2006). Briefly, freeze dried biomass (one sample per species anammox) was extracted using a modified Bligh and Dyer (1959) method. A volume ratio of 2:1:0.8 (methanol: DCM: phosphate buffer, pH 7.4) was added and sonicated for 10 min. Subsequently a volume ratio of 1:0.1 (DCM : phosphate buffer, pH 7.4) was added and the solution was centrifuged. The methanol/ phosphate buffer phase was discarded and the DCM lipid phase was kept. The biomass was re-extracted using the same procedure. DCM fractions were combined, eluted over $NaSO_4$ (to remove any remaining water) and dried using $N_2$. The resulting Bligh Dyer extract was then dissolved into a hexane/isopropanol mixture (4:1, v/v) and analysed using HPLC/ESI-MS/MS.

4.2.4.2 HPLC/ESI-MS/MS

Bligh Dyer extracts were analysed using an Agilent 1100 series LC (Agilent, San Jose, CA) coupled to a Thermo TSQ Quantum ultra EM triple quadruple mass spectrometer with an Ion Max source with ESI probe (Thermo Electron Corporation, Waltham, MA) as described by Boumann et al. (2006). Separation was achieved on a LiChrosphere diol column 100A (250 mm x 2.1 mm, 5 mm particles; Alltech Associates Inc., Deerfield, IL) maintained at 30°C. A combination of two mobile phase solutions was used (A) hexane/2-propanol/formic acid/14.8M $NH_3$ ratios 79 : 20 :0.12 : 0.04 (volume in volume in volume in volume, v/v/v/v) and (B) 2-propanol/water/formic acid/14.8M $NH_3$ ratios 88 : 10 : 0.12 : 0.04 (v/v/v/v). Phospholipids were eluted with a flow rate of 0.2 ml $min^{-1}$ using the following elution program: 100% A to 35% A: 65% B over 45 min, maintained for 20 min, then back to 100% A for 20 min to re-equilibrate the column (Boumann et al., 2006). LC-MS/MS was performed in data-dependant mode with 2 scan events, where a positive ion scan (*m/z* 250–1000) was followed by product ion scan of the base peak of the mass spectrum of the first scan event (CE 20V, collision gas (argon) 0.8 mTorr). Searching the data for parent ions generating a product ion at m/z 184.1, representing the PC headgroup, identified PC lipid species.

The total lipid extracts were also analysed using a MS/MS routine with 2 scan events specifically targeting PE and PG lipid species. The first scan event was a neutral loss scan (*m/z* 300-1000, CE 30 V, 0.8 mTorr argon) for a loss of 141.0 Da, representing the PE headgroup. The second event was a neutral loss scan (*m/z* 300-1000, CE 30 V, 0.8 mTorr argon) for loss of 189.0 Da, representing the ammoniated PG headgroup. Under the chromatographic conditions used, PG species preferably form (80%) the ammoniated molecule ($[M+NH_4]^+$).

## 4.3. Results

### *4.3.1 Composition of anammox enrichment cultures*

The enrichment cultures used for lipid analysis were initially analysed using FISH to estimate the relative abundance of anammox bacteria. Results showed that about 75% of the bacterial population in the "*Candidatus* A. propionicus" enrichment culture hybridized with the newly developed oligonucleotide probe Apr-820 (Kartal et al., 2007) and did not hybridize with probes specific to any other anammox genera. In the enrichment culture of "*Candidatus* B. fulgida", approximately 75% of the bacterial population hybridised with both the probes Ban-mix and Amx-820. Reactor effluent samples containing "*Candidatus* K. stuttgartiensis" single cells consisted of about 90% "*Candidatus* K. stuttgartiensis" when hybridised with the probe KST-1273. Finally, the anammox oligonucleotide probe Scabr-1114 (Schmid et al., 2005) hybridised with about 75% of the bacterial population in the "*Candidatus* Scalindua" enrichment culture. Since there was only one detectable anammox genus per enrichment culture and this genus dominated each of the enrichment cultures, it is likely that most of the lipids analysed in this study were derived from the identified anammox bacterial genera and that lipids from other anammox bacteria were below the limit of detection.

### *4.3.2 Core lipid composition of anammox enrichment cultures*

Analysis of the total lipid extracts of the anammox enrichment cultures using GC/MS revealed that the dominant lipids in the cultures were ladderane lipids, straight-chain fatty acids, branched fatty acids, monounsaturated fatty acids and triterpenoids (Fig. 2). Calculation of the weight percentages showed that ladderane fatty acids constituted the largest lipid fraction in the enrichment cultures of "*Candidatus* B. fulgida" (63%) and "*Candidatus* K. stuttgartiensis" (45%) and that

unsaturated fatty acids did not make up a significant fraction in these cultures (Fig. 3). In contrast, in "*Candidatus* A. propionicus" and "*Candidatus* Scalindua" enrichment cultures, the relative weight percentages of ladderane fatty acids (24% and 22%, respectively) were similar to those of the unsaturated fatty acids and straight chain lipids. Branched fatty acids were the most abundant lipid type (29% branched fatty acids) in the "*Candidatus* Scalindua" enrichment culture. All anammox biomass investigated contained minor amounts of triterpenoids (≤ 6% total lipids). The distribution of the different lipids within each lipid class will be discussed in detail below.

4.3.2.1 Non-ladderane lipids

All anammox bacterial enrichment cultures contained straight chain fatty acids ranging from $C_{14}$ to $C_{18}$. The most dominant non-ladderane fatty acid in all the enrichment cultures was hexadecanoic acid (Fig. 2; Table 2). Unsaturated fatty acids in the anammox enrichment cultures ranged from $C_{15}$ to $C_{19}$, and only monounsaturated fatty acids were identified (Fig. 2; Table 2). The $C_{16:1}$ fatty acid was most abundant in cell material of "*Candidatus* A. propionicus" and "*Candidatus* Scalindua", whereas in cell material of "*Candidatus* K. stuttgartiensis" only the $C_{18:1}$ fatty acid was present. No unsaturated lipids were detectable in the "*Candidatus* B. fulgida" enrichment culture.

All enrichment cultures contained branched fatty acids, including the generic $C_{15}$-$C_{18}$ *iso* branched fatty acids (Fig. 2; Table 2). Less commonly occurring branched fatty acids with a 9,14-dimethylpentadecane (**j**), 9-methylpentadecane (**k**), 10-methylpentadecane (**l**) and 10-methylhexadecane (**m**) carbon skeleton were identified in the enrichment culture of "*Candidatus* Scalindua". "*Candidatus* A. propionicus" contained 9,14-dimethylpentadecane (**j**) and 10-methylhexadecane (**m**) and "*Candidatus* B. fulgida" contained 9,14-dimethylpentadecane (**j**), 9-methylpentadecane (**k**), and 10-methylpentadecane (**l**). Only 10-methylhexadecanoic acid (**m**) was detected in the "*Candidatus* K. stuttgartiensis" enrichment culture. Finally, triterpenoid lipids, in the form of a $C_{27}$ hopanoid ketone and bacteriohopanetetrol occurred in all enrichment cultures. Only the enrichment culture of "*Candidatus* A. propionicus" also contained squalene in detectable amounts.

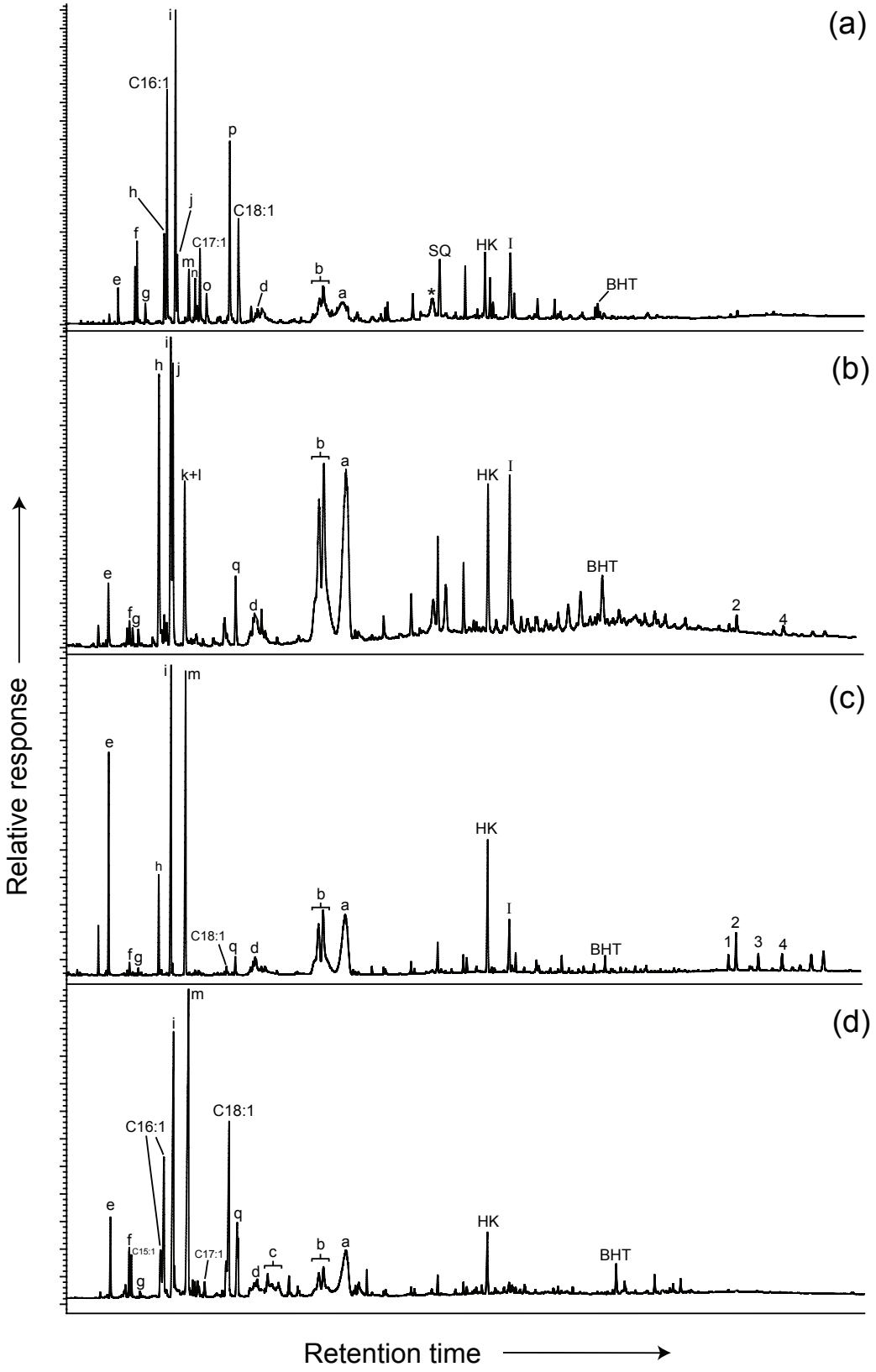


**Figure 2.** GC chromatograms of the total lipid extracts of anammox enrichment cultures. (a) "*Candidatus* Anammoxoglobus propionicus", (b) "*Candidatus* Brocadia fulgida", (c) "*Candidatus* Kuenenia stuttgartiensis", (d) "*Candidatus* Scalindua". Compounds; (Fig. 1 **a**-**q**, **l**), SQ = Squalene, HK = $C_{27}$ hopanoid ketone, BHT =bacteriohopanetetrol. 1 = **l** + ether linked *iso* $C_{14}$ alkyl chain, 2 = **l** + ether linked alkyl chain **e**, 3 = **l** + ether linked alkyl chain **h**, 4 = l + ether linked alkyl chain **o**.

**Table 2.** Lipid concentrations (µg $g^{-1}$ dry weight biomass) in anammox enrichments, analysed using GC and GC/MS. Values are the mean and standard deviations for three independently taken samples of an enrichment culture. ND, not detected

| | Anammoxoglobus propionicus | Brocadia fulgida | Kuenenia stuttgartiensis | Scalindua (a)(*) |
|---|---|---|---|---|
| **Ladderane fatty acids** | | | | |
| C20[3] fatty acid (**a**) | 130 ± 68 | 1000 ± 210 | 1000 ± 86 | 42 |
| C20[5] fatty acid (**b**) | 150 ± 77 | 1200 ± 260 | 980 ± 170 | 27 |
| C18[3] fatty acid (**c**) | ND | ND | ND | 17 |
| C18[5] fatty acid (**d**) | 16 ± 7 | 65 ± 8 | 28 ± 12 | 0.5 |
| **Ladderane diethers (*sn*-2, *sn*-1 positions)** | | | | |
| C20[3] , C16 | ND | ND | 47 ± 27 | ND |
| C20[3] , C17 | ND | 9 ± 2 | 51 ± 40 | ND |
| C20[3] , C18[5] | ND | ND | 9 ± 5 | ND |
| C20[3] , C20[5] | ND | ND | 9.5a | ND |
| C20[3] , *iso*-C14 | ND | ND | 58 ± 49 | ND |
| C20[3] , C14 | ND | 13 ± 5 | 63 ± 59 | ND |
| C20[3] , C15 | ND | ND | 20a | ND |
| C20[3] , *iso*-C16 | ND | ND | 37 ± 29 | ND |
| **Ladderane monoether** | | | | |
| C20[3] monoether (**I**) | 82 ± 26 | 130 ± 55 | 160a | ND |
| **Straight chain fatty acids** | | | | |
| *n*-C14 (**e**) | 15 ± 10 | 41 ± 29 | 340 ± 160 | 8.5 |
| *n*-C15 (**g**) | 32 ± 27 | 19 ± 10 | 12 ± 7 | 1 |
| *n*-C16 (**i**) | 180 ± 130 | 370 ± 94 | 650 ± 260 | 56 |
| *n*-C17 (**o**) | 22 ± 12 | ND | ND | ND |
| *n*-C18 (**q**) | ND | 100 ± 44 | 45 ± 6 | 14 |
| **Unsaturated fatty acids** | | | | |
| C15:1 | ND | ND | ND | 6 |
| C16:1 | 100 ± 93 | ND | ND | 24 |
| C17:1 | 43 ± 32 | ND | ND | 2 |
| C18:1 | 80 ± 76 | ND | 13 ± 9 | 34 |
| C19:1 | 76 ± 71 | ND | ND | ND |
| **Branched fatty acids** | | | | |
| *iso*-15 (**f**) | 22 ± 19 | 7 ± 7 | 17 ± 11 | 7 |
| *iso*-16 (**h**) | 37 ± 35 | 190 ± 48 | 170 ± 73 | ND |
| *iso*-17 (**n**) | 16 ± 7 | ND | ND | ND |
| *iso*-18 (**p**) | 62 ± 59 | ND | ND | ND |
| 9,14-dimethylpentadecane (**j**) | 27 ± 27 | 220 ± 10 | ND | 2 |
| 9-methylhexadecane and 10-methylpentadecane (**k+l**) | ND | 190 ± 16 | ND | 2 |
| 10-methylhexadecane (**m**) | 41 ± 24 | ND | 680 ± 240 | 75 |
| **Triterpenoids** | | | | |
| Squalene | 82 ± 26 | ND | ND | ND |
| C27 Hopanoid Ketone | 4 ± 1 | 0.5 ± 0.5 | 290 ± 150 | 2 |
| Bacteriohopanetetrol | 3.5 | 50 ± 50 | 5a | 1 |

(a) Single measurement
(*) The dry weight consisted for ca. 90% of salt, actual concentrations based on dry weight biomass are therefore estimated to be 10 fold higher.

4.3.2.2 Ladderane lipids

When analysing with GC/MS, the dominant ladderane fatty acid in the enrichment culture of "*Candidatus* A. propionicus" and "*Candidatus* B. fulgida" was the $C_{20}$ [5] ladderane fatty acid (**b**) whilst in enrichment cultures of "*Candidatus* K. stuttgartiensis" and "*Candidatus* Scalindua", the $C_{20}$ [3] ladderane fatty acid (**a**) was most dominant. In all genera, the $C_{18}$ [5] ladderane fatty acid (**d**) was present in the lowest concentration. Only the culture of "*Candidatus* Scalindua" contained a $C_{18}$ [3] ladderane fatty acid (**c**).

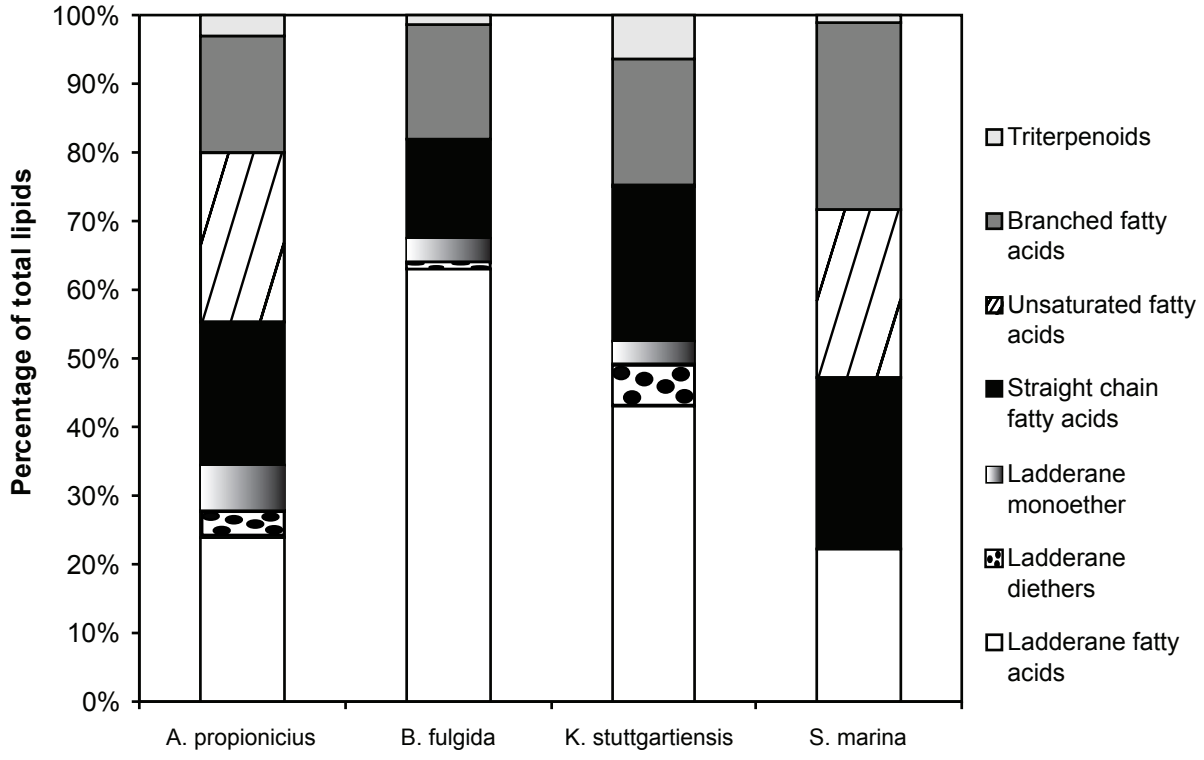


**Figure 3.** Weight percentage composition of lipid types in the total lipid extract (GC/MS analysed) of anammox SBR enrichment cultures.

Concentrations of ladderane fatty acids differed substantially between enrichment cultures and varied up to 50-fold in concentration (Table 2). The enrichment cultures of "*Candidatus* K. stuttgartiensis" and "*Candidatus* B. fulgida" contained ladderane diethers, which were absent in the cultures of "*Candidatus* Scalindua" or "*Candidatus* A. propionicus". The ladderane monoether (**I**) was abundant in enrichment cultures of "*Candidatus* A. propionicus" and "*Candidatus* B. fulgida",

but only present in one of the three analysed "*Candidatus* K. stuttgartiensis" biomass samples and was either below the limit of detection or not present in the enrichment culture of "*Candidatus* Scalindua".

In the "*Candidatus* A. propionicus" enrichment culture two new $C_{22}$ ladderane fatty acids were tentatively identified based on mass spectrometry (Fig. 4). These ladderanes had molecular ions 28 daltons higher than previously identified ladderane fatty acids (molecular ions at 344 and 346 dalton instead of 316 and 318 dalton, respectively; Fig. 4) and eluted later, which is in agreement with the attachment of a longer alkyl chain to the ladderane moieties.

Ladderane core lipids were additionally analysed using HPLC/APCI-MS/MS (Table 3). This separation technique avoids thermal alteration of ladderanes upon gas chromatographic analysis, since ladderane moieties are heat labile (Sinninghe Damsté et al., 2005) and HPLC/APCI-MS/MS has a much higher sensitivity than GC/MS (Hopmans et al., 2006). When analysing with HPLC/APCI-MS/MS the most dominant ladderane fatty acid in the enrichment culture of "*Candidatus* A. propionicus" was the $C_{20}$ [5] ladderane fatty acid (**b**). In "*Candidatus* B. fulgida" and "*Candidatus* K. stuttgartiensis" the $C_{20}$ [3] ladderane fatty acid (**a**) and the $C_{20}$[5] ladderane fatty acid (**b**) were the most dominant. In "*Candidatus* Scalindua" the $C_{20}$ [3] ladderane fatty acid (**a**) was the most dominant. All enrichment cultures contained the $C_{18}$ [3] ladderane fatty acid.

Differences were observed in the concentrations of the ladderane core lipids per g dry weight biomass when using either GC/MS or HPLC-MS/MS. Plotting the concentrations of ladderane fatty acids determined using these techniques resulted in a linear correlation ($R^2$ = 0.70). This relatively low coefficient of determination was due to poor correlation between ladderane lipids at concentrations above 600 µg $g^{-1}$. The reason for the difference between the two analytical techniques is perhaps due to thermal degradation of ladderane lipids created during GC analysis, resulting in broad peaks (Sinninghe Damsté et al., 2005) which are problematic to integrate and can mask other lipids eluting at the same retention times. Despite the disparity between the concentrations of ladderane lipids calculated from the two methods of analysis, the relative composition of the different ladderane fatty acids was similar

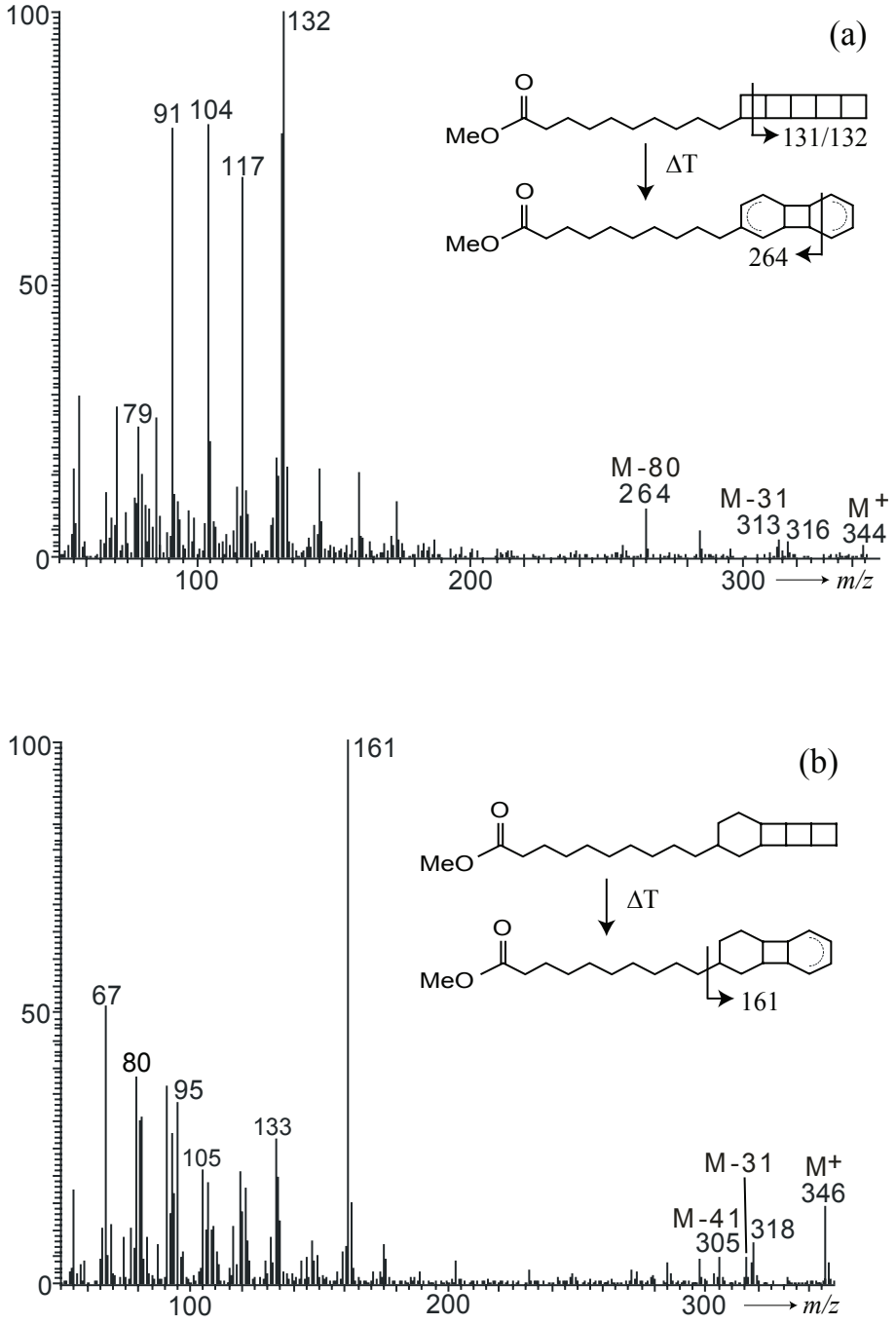


**Figure 4.** GC/MS mass spectrum of tentatively identified lipids in "*Candidatus* A. propionicus" samples. (a) Methyl ester of a $C_{22}$ ladderane fatty acid lipid containing five linearly concatenated cyclobutane rings, (b) Methyl ester of a $C_{22}$ ladderane fatty acid lipid containing one cyclohexane and three condensed cyclobutane moieties. The structures of the original lipids are indicated in the spectra but it should be noted that the mass spectra reflect their thermal degradation products formed during GC analysis as discussed by Sinninghe Damsté et al. (2005).

when applying both techniques. In the enrichment culture of "*Candidatus* Scalindua", the total concentrations of ladderane lipids (and other lipids) were

substantially lower than observed in the other enrichment cultures (Table 2). This is probably due to the presence of salt added to the reactor medium of "*Candidatus* Scalindua" to mimic 'marine' conditions. TOC analysis showed that the biomass of "*Candidatus* B. fulgida", "*Candidatus* A. propionicus" and "*Candidatus* K. stuttgartiensis" mainly consisted of organic carbon with TOC values ranging between 19 and 30%. In contrast "*Candidatus* Scalindua" biomass had only 3% TOC, due to the much higher amounts of inorganic material. When concentrations are calculated on the basis of organic carbon content rather than dry weight biomass, ladderane lipid concentrations of "*Candidatus* Scalindua" are in a similar order of magnitude as the other anammox bacteria.

**Table 3.** Ladderane lipid concentrations (µg $g^{-1}$ dry weight biomass) in anammox enrichments, analysed using HPLC/APCI-MS/MS. Values are the mean and standard deviations for three independently taken samples of an enrichment culture.

| | Anammoxoglobus propionicus | Brocadia fulgida | Kuenenia stuttgartiensis | Scalindua (a)(*) |
|---|---|---|---|---|
| **Ladderane fatty acids** | | | | |
| C20[3] fatty acid **a** | 290 ± 58 | 580 ± 280 | 1400 ± 340 | 92 |
| C20[5] fatty acid **b** | 450 ± 140 | 880 ± 400 | 1700 ± 310 | 75 |
| C18[3] fatty acid **c** | 100 ± 120 | 22 ± 9 | 650 ± 340 | 59 |
| C18[5] fatty acid **d** | 48 ± 18 | 93 ± 41 | 230 ± 36 | 27 |
| **Ladderane monoether** | | | | |
| C20[3] monoether **I** | 4 ± 5 | 540 ± 480 | 190 ± 95 | 1.5 |

(a) single measurement
(*) Dry weight consisted of additional salt, actual values dry weight biomass is circa 10 fold higher.

*4.3.3. Intact phospholipid composition*

All four anammox enrichment cultures contained ladderane polar lipids with PC, PE and PG (phosphocholine, phosphoethanolamine or phosphoglycerol) headgroups (Figs. 5, 6 and 7). Large differences in the ionization efficiencies of the different types of polar headgroups make it complicated to quantify the relative amount of these lipids and compare them between species. Therefore, the results for each species will be described in qualitative terms only.

4.3.3.1 "*Candidatus* A. propionicus"

The enrichment culture of "*Candidatus* A. propionicus" contained the ladderane PC polar lipids **IVa** – **IVn** and **Va** – **Vn** with **II** and **Vb** as the most dominant types, and non- ladderane PC polar lipids made up a minor component of the base peak chromatogram (Fig. 5; Table 4). Nine types of ladderane PE polar lipids were identified in this enrichment culture, with the most abundant of these being **VIIa** (Fig. 6; Table 4). Non-ladderane PE diester lipids were also tentatively identified from mass ions in the averaged mass spectra of the PE lipid cluster. They contained fatty acids with a combined carbon number between 28 and 32 with 0 to 2 double bonds, and were abundant in the PE base peak chromatogram. In addition, "*Candidatus* A. propionicus" contained nine types of ladderane PG polar lipids and **Xd** was the most abundant (Fig. 7; Table 4). Non-ladderane PG diester lipids were present in low abundances only.

4.3.3.2 "*Candidatus* B. fulgida"

The "*Candidatus* B. fulgida" enrichment culture contained the same PC polar lipids as identified in "*Candidatus* A. propionicus", but in this case **II, Vb** and **Va** were the most dominant types (Fig. 5; Table 4). Non ladderane PC polar lipids constituted a minor component of this enrichment culture. The "*Candidatus* B. fulgida" enrichment culture contained seven types of ladderane PE polar lipids and in addition a PE diether with a $C_{22}$ [5] ladderane alcohol, all of which were present in low abundances (Fig. 6; Table 4).

In the "*Candidatus* B. fulgida" enrichment culture nine ladderane PG lipids were detected (Fig. 7; Table 4), of which two types were dominant (**IXm** or **IXj** or **IXn** or **Xi** or **Xh** and **Xm** or **Xj** or **Xn**). Non-ladderane PG diester lipids were especially relatively abundant in the enrichment cultures of "*Candidatus* B. fulgida" and contained fatty acids with a combined carbon number between 28 and 32 with 0 or 1 double bond.

4.3.3.3 "*Candidatus* K. stuttgartiensis"

In contrast to the other enrichment cultures, **IVa, Va** and **II** were the most dominant PC lipids in the "*Candidatus* K. stuttgartiensis" enrichment culture while **Vd** and **Vc** could not be detected (Fig. 5; Table 4). The PE lipids **VIIm**/**VIIj**/**VIIn** and **VIIa** were the most abundant and mass ions of protonated ladderane lipids in the PE cluster

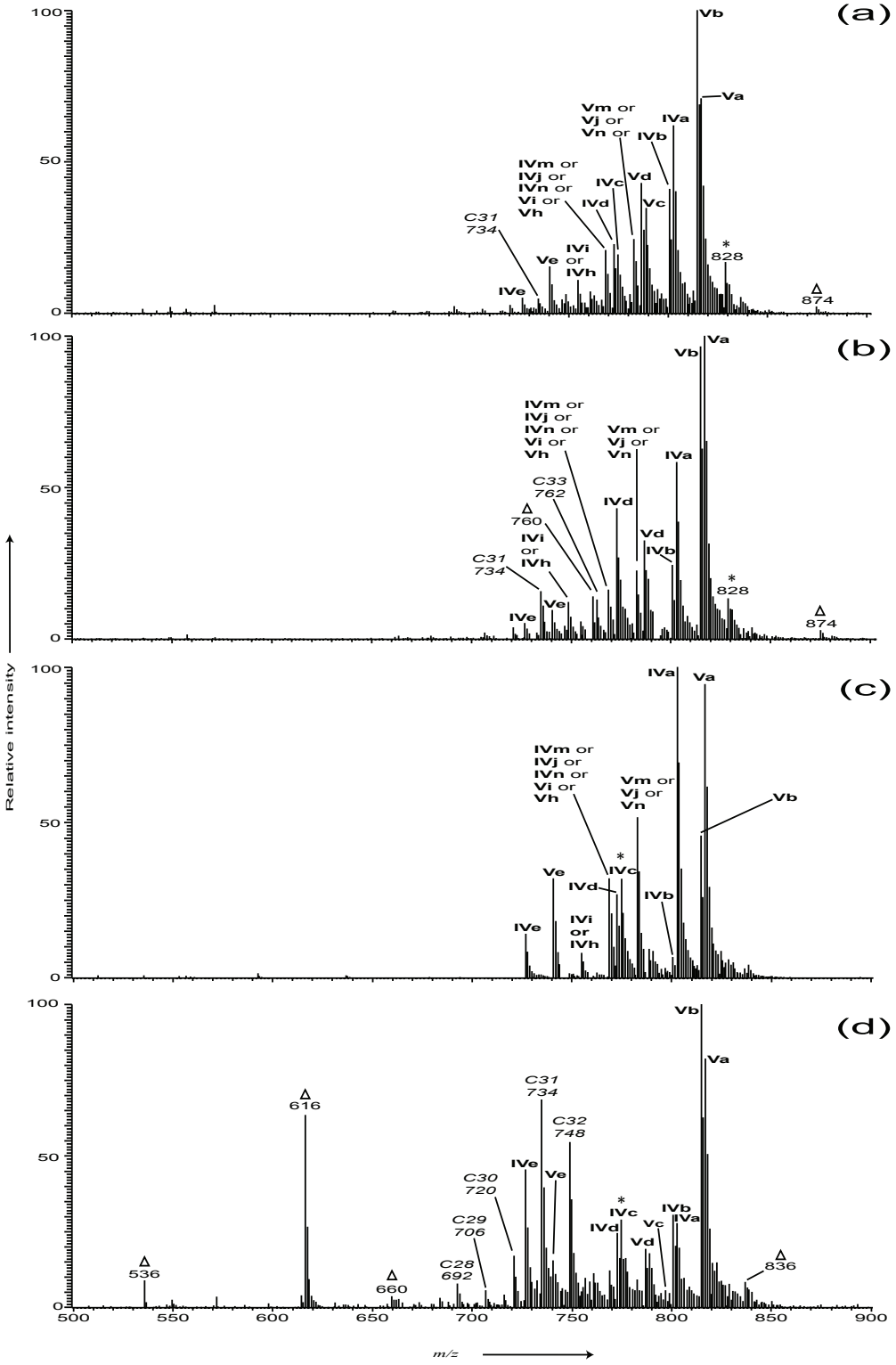


**Figure 5.** HPLC/ESI-MS/MS averaged mass spectra of polar lipids with phosphocholine (PC) headgroups, as identified by generation of a product ion at *m/z* 184.1 upon fragmentation of the protonated molecule, in total Bligh Dyer extracts. (a) "*Candidatus* Anammoxoglobus propionicus", (b) "*Candidatus* Brocadia fulgida", (c) "*Candidatus* Kuenenia stuttgartiensis", (d) "*Candidatus* Scalindua". Ladderane lipids are indicated in bold and non-ladderane diester lipids in italics, where the total carbon number is given. Triangles indicate unidentified compounds. Asterisks relate to ladderane lipids identified in this study. Mass spectra for the PC $C_{20}$ [3] ladderane monoalkylether (**II**) are not shown.

**Table 4.** Composition of ladderane phospholipids in anammox enrichment cultures, analysed using HPLC/ESI-MS/MS. Percentage relative intensity of *m/z* ion in total mass spectrum: *** = High (>66%), ** = Medium (33-66%), * = Low (<33%), - = not detected.

| Head group type | *m/z* | Anammoxoglobus propionicus | Brocadia fulgida | Kuenenia stuttgartiensis | Scalindua |
|---|---|---|---|---|---|
| **PC Headgroup** | | | | | |
| **II** | 530 | *** | *** | *** | - |
| **IVe** | 726 | * | * | * | ** |
| **Ve** | 740 | * | * | * | * |
| **IVi or IVh** | 754 | * | * | * | - |
| **IVm or IVj or IVn or Vi or Vh** | 768 | * | * | * | - |
| **IVd** | 772 | * | ** | * | * |
| **IVc** | 774 | * | - | * | * |
| **Vm or Vj or Vn** | 782 | * | * | ** | - |
| **Vd** | 786 | ** | * | - | * |
| **Vc** | 788 | ** | * | - | * |
| **IVb** | 800 | ** | - | * | * |
| **IVa** | 802 | ** | ** | *** | * |
| **Vb** | 814 | *** | *** | ** | *** |
| **Va** | 816 | ** | *** | *** | *** |
| **PE Headgroup** | | | | | |
| **III** | 488 | - | - | - | - |
| **VIe** | 684 | - | - | - | * |
| **VIIe** | 698 | * | - | ** | * |
| **VIi or VIh** | 712 | * | - | ** | - |
| **VIm or VIj or VIn or VIIi or VIIh** | 726 | * | * | ** | * |
| **VId** | 730 | * | * | * | - |
| **VIc** | 732 | ** | - | * | ** |
| **VIIm or VIIj or VIIn** | 740 | - | * | *** | * |
| **VIId** | 744 | * | * | ** | * |
| **VIIc** | 746 | - | - | ** | * |
| **VIb** | 758 | ** | * | ** | * |
| **VIa** | 760 | - | - | - | * |
| **VIIb** | 772 | ** | * | ** | - |
| **VIIa** | 774 | *** | * | *** | - |
| **PG Headgroup** | | | | | |
| **VIII** | 536 | - | - | - | - |
| **IXe** | 732 | - | - | - | - |
| **Xe** | 746 | * | * | * | * |
| **IXi or IXh** | 760 | * | * | * | * |
| **IXm or IXj or IXn or Xi or Xh** | 774 | * | *** | ** | * |
| **IXd** | 778 | * | - | - | * |
| **IXc** | 780 | ** | * | - | * |
| **Xm or Xj or Xn** | 788 | * | *** | *** | * |
| **Xd** | 792 | *** | * | - | * |
| **Xc** | 794 | - | * | - | * |
| **IXb** | 806 | ** | - | * | * |
| **IXa** | 808 | - | - | - | - |
| **Xb** | 820 | * | * | * | * |
| **Xa** | 822 | - | * | * | - |

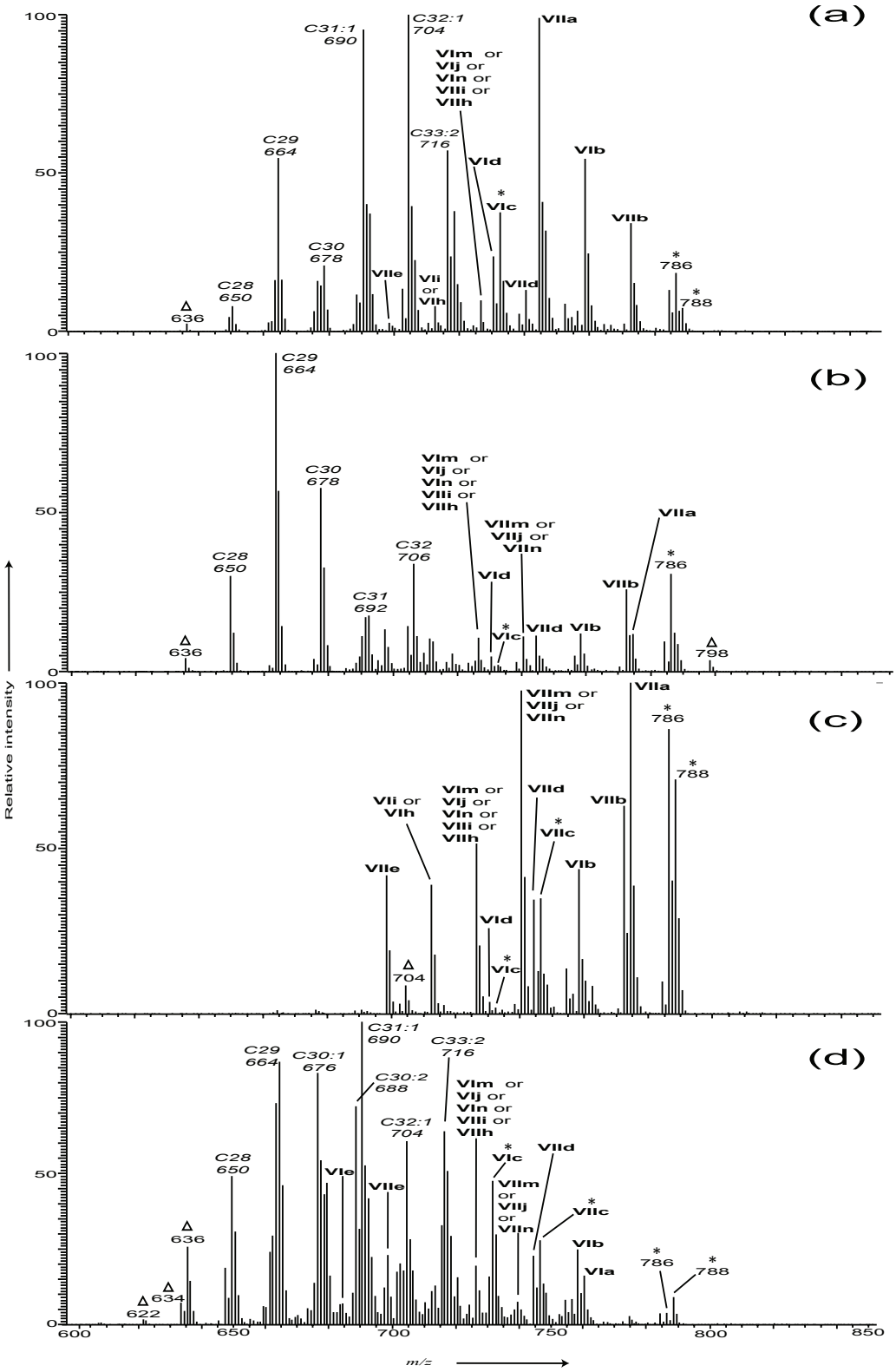


**Figure 6.** HPLC/ESI-MS/MS averaged mass spectra of neutral loss scans for loss of 141.0 Da, specifically targeting phosphoethanolamine (PE) headgroup containing polar lipids in total Bligh Dyer extracts. (a) "*Candidatus* Anammoxoglobus propionicus", (b) "*Candidatus* Brocadia fulgida", (c) "*Candidatus* Kuenenia stuttgartiensis", (d) "*Candidatus* Scalindua". Ladderane lipids are indicated in bold and non-ladderane diester lipids in italics, where the total carbon number is given. Triangles indicate unidentified compounds. Asterisks relate to ladderane lipids identified in this study. Mass spectra for the PE $C_{20}$ [3] ladderane monoalkylether (**III**) are not shown.

dominated the mass spectrum (Fig. 6; Table 4). The "*Candidatus* K. stuttgartiensis" enrichment culture contained seven different types of ladderane PG lipids, of which **Xm** or **Xj** or **Xn** was the most dominant (Fig. 7; Table 4). A unique feature of the "*Candidatus* K. stuttgartiensis" enrichment culture was that it also contained PG diethers with both the $C_{22}$ [3] ladderane and the $C_{22}$ [5] ladderane fatty alcohol. Unlike the other cultures analysed, non-ladderane PC, PE and PG lipids could not be identified in this enrichment culture.

4.3.3.4 "*Candidatus* Scalindua"

The "*Candidatus* Scalindua" enrichment culture contained predominantly **Vb** and **Va** as PC ladderane lipids and, unlike in the other cultures, **IVi/IVh**, **IVm/IVj/IVn/Vi/Vh**, **Vm/Vj/Vn** were not detected (Fig. 5; Table 4). In addition, the PC $C_{20}$ [3] ladderane monoether was also not present in the "*Candidatus* Scalindua" enrichment culture. In contrast to the other cultures, non-ladderane diester lipids formed the most dominant ions in the mass spectrum of the PC lipids of "*Candidatus* Scalindua". Non-ladderane diester lipids contained fatty acids with a combined carbon number ranging from 28 to 33 with no double bonds. Nine types of ladderane PE lipids were present in low abundance in "*Candidatus* Scalindua" with **VIc** being the most dominant. Ten types of ladderane PG lipids were also present in low abundance (Fig. 7; Table 4).

## 4.4 Discussion

### *4.4.1 Ladderane lipids*

All analysed anammox bacteria contained large amounts of ladderane fatty acid core lipids in similar proportions. The fact that all of these anammox bacteria biosynthesise such large amounts of ladderane lipids, despite their large genetic differences, and that these lipids have not been detected in any other bacterial species (including other *Planctomycetes*), indicates that this is a unique feature inherited from the earliest ancestor of anammox bacteria and is essential for the anammox way of life. This reinforces the idea that ladderane lipids are excellent biomarkers for anammox bacteria and the anammox process. Ladderane fatty acid concentrations were relatively constant over time within one genus. However, it was observed that the $C_{20}$ [3] ladderane monoether (**I**) was present in all samples (at varying concentrations) of the "*Candidatus* B. fulgida" and "*Candidatus* A.

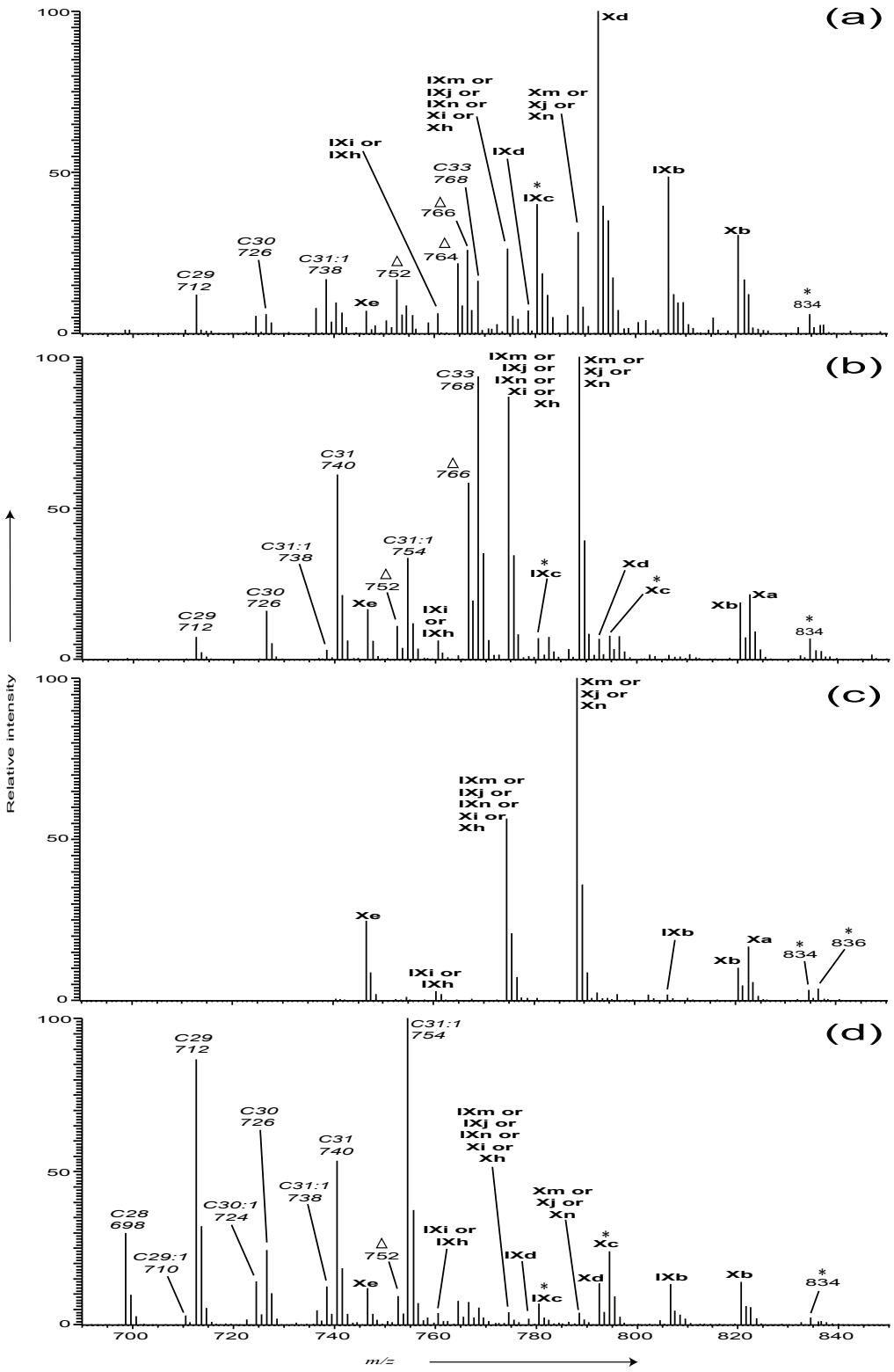


**Figure 7.** HPLC/ESI-MS/MS averaged mass spectra of neutral loss scans for losses of 189.0 Da, specifically targeting phosphoglycerol (PG) headgroup containing polar lipids in total Bligh Dyer extracts. (a) "*Candidatus* Anammoxoglobus propionicus", (b) "*Candidatus* Brocadia fulgida", (c) "*Candidatus* Kuenenia stuttgartiensis", (d) "*Candidatus* Scalindua". Ladderane lipids are indicated in bold and non-ladderane diester lipids in italics, where the total carbon number is given. Triangles indicate unidentified compounds. Asterisks relate to ladderane lipids identified in this study. Mass spectra for the PG $C_{20}$ [3] ladderane monoalkylether (**VIII**) are not shown.

propionicus" biomass, while in the biomass of "*Candidatus* K. stuttgartiensis" it was present in only one sample and in the biomass of "*Candidatus* Scalindua", the monoether was never detected, when analysing with GC/MS. However, when analysing using HPLC/APCI-MS/MS the monoether was observed in all samples, but in different relative amounts. A similar scenario was observed when analysing the $C_{18}$ [3] ladderane fatty acid using HPLC/APCI-MS/MS, highlighting the sensitivity of this technique in comparison to GC/MS. From the HPLC/APCI-MS/MS analysis we observed that the $C_{20}$ [3] ladderane monoether (**I**) varied, both within and between species, and in that respect behaves differently to most of the ladderane fatty acids investigated. The ladderane monoether, and its phospholipid counterpart, could perhaps be a by-product of cell lysis, formed during work up of the biomass, an intermediate of lipid biosynthesis or is present in this form in the cells of anammox bacteria. Previous studies on bacterial cells have shown that the formation of various lysis products are caused by the enzyme phospholipases which selectively cleave *sn*-1, *sn*-2 or *sn*-3 attached alkyl chains. This allows the organism to keep the composition of phospholipid types in the membrane bi-layer in balance with the surrounding environment (Paltauf, 1994).

All of the phospholipid types previously identified in "*Candidatus* K. stuttgartiensis" (Boumann et al., 2006) have been detected in the four anammox genera used in this study. In addition, we have also detected intact ladderane lipids containing PG headgroups and containing $C_{18}$ [3] ladderane fatty acid, $C_{22}$ [3] and [5] ladderane fatty acids. When comparing the "*Candidatus* K. stuttgartiensis" intact phospholipid profile published by Boumann et al. (2006) with the "*Candidatus* K. stuttgartiensis" intact phospholipid profile obtained in this study, we observed that the composition of the PC intact ladderane phospholipids was different. The profile of Boumann et al. (2006) showed a higher abundance of PC ether linked lipids and a much lower abundance of ladderane **Va**. The largest differences were observed with the PE headgroups where only ladderane lipid **VIId** dominated the chromatogram and the rest of the lipids were much less abundant, which is in sharp contrast to the PE lipid distribution presented in our study. This suggests that the culture conditions may influence the relative composition of phospholipids as these differed between our study and that of Boumann et al. (2006), with respect to reactor size (laboratory vs.

industrial scale), temperature (35°C vs. 30°C) and reactor ecology (i.e. different community composition).

*4.4.2 Non-ladderane lipids*

The largest differences between the four enrichment cultures were observed in the distributions of the non-ladderane fatty acids. However, considering that other organisms also inhabit the anammox SBRs, differences in these lipids could not be directly attributed to differences in the lipid composition of only anammox bacteria. Monounsaturated fatty acids were present in the core lipid profiles of all anammox enrichment cultures (Figure 2; Table 2). However, polar lipids containing monounsaturated fatty acids attached to the *sn-1* position of intact ladderane phospholipids have not been found in this study, or the study of Boumann et al. (2006), suggesting that monounsaturated fatty acids may not be derived from anammox bacteria.

Branched fatty acids made up between 16 and 28% of total core lipids in the anammox enrichment cultures. Some of these branched fatty acids (Table 2) are likely to be present in ladderane phospholipids and given the dominance (>75%) of anammox bacteria and the high abundance of unusual branched lipids, the majority of these lipids can be attributed to anammox bacteria. Previous studies using Percoll-purified anammox cells (>99% purity) also indicate that methyl branched fatty acids are derived from anammox biomass (Sinninghe Damsté et al., 2002; Sinninghe Damsté et al., 2005). The biomass of "*Candidatus* K. stuttgartiensis" and "*Candidatus* Scalindua" contain significant quantities of 10-methylhexadecanoic acid. Although this lipid is not unique to *Planctomycetes* (Dowling et al., 1986; Londry et al., 2004), it has been used as an indicator for the presence of anammox bacteria (Schubert et al., 2006). The branched fatty acids 9,14-dimethylpentadecanoic acid, 9-methylhexadecanoic acid and 10-methylpentadecanoic acid can be used to discriminate between the different anammox genera, with "*Candidatus* A. propionicus" and "*Candidatus* B. fulgida" synthesising 9,14-dimethylpentadecanoic acid, 9-methylhexadecanoic acid and 10-methylpentadecanoic acid and no (or below the detection limit) 10-methylhexadecanoic acid. In contrast, "*Candidatus* K. stuttgartiensis" synthesises relatively high amounts of 10-methylhexadecanoic acid with 9,14-

dimethylpentadecanoic acid, 9/10-methylhexadecanoic acid or 10-methylpentadecanoic acid below the detection limit. "*Candidatus* Scalindua" synthesises all of these branched fatty acids, but predominantly the 10-methylhexadecanoic acid.

Triterpenoids like squalene, bacteriohopanetetrol and a $C_{27}$ hopanoid have previously been identified from enrichment cultures of "*Candidatus* B. anammoxidans", "*Candidatus* K. stuttgartiensis", "*Candidatus* Scalindua wagneri" and "*Candidatus* Scalindua brodae" (Sinninghe Damsté et al., 2004; Sinninghe Damsté et al., 2005). Our results also suggest that all the known genera of anammox bacteria biosynthesise hopanoids. The recent assembly of the genome of "*Candidatus* K. stuttgartiensis" (Strous et al., 2006) showed that gene sequences encoding putative squalene hopane cyclases are present. This gives strong evidence that anammox bacteria are the source of hopanoids observed in the enrichment cultures and potentially in the natural environment.

*4.4.3 Ladderane lipids and the anammoxosome hypothesis*

Phospholipids (PC, PE and PG) in the 90% enriched "*Candidatus* K. stuttgartiensis" biomass contained almost exclusively lipids with ladderane moieties and a very minor fraction of non-ladderane lipids. Considering that the anammoxosome compartment in "*Candidatus* K. stuttgartiensis" makes up about 60% of the cell volume, taking into account membrane folding (van Niftrik et al., 2007, van Niftrik et al., 2008), the abundance of non-ladderane diester lipids is surprisingly low. This high abundance of ladderane lipids therefore suggests that ladderane lipids are not only restricted to the anammoxosome and are likely to be used in the construction of other cellular membranes, such as those surrounding the various cytoplasmic and intracytoplasmic compartments (van Niftrik et al., 2008).

It is unlikely that only ladderane lipids are present in the outer membrane of anammox bacteria since, in comparison to conventional cell membranes, ladderane lipids create an unusually dense membrane, which would prevent permeability to essential small molecules. Therefore, it is more feasible that a combination of ladderane and non-ladderane lipids (e.g. straight chain and unusual methyl branched fatty acids) are used to ensure that other cell membranes are

more permeable than the anammoxosome membrane. In addition, hopanoids have been found to be abundant in all anammox genera. Hopanoids are believed to function as membrane rigidifiers, playing a similar role like sterols in eukaryotes (Kannenberg and Poralla, 1999). Therefore the potential combination of both hopanoids and ladderane lipids in the outer cell membranes of anammox bacteria raises the question why anammox bacteria would additionally synthesise hopanoids to rigidify their outer membranes, when the membrane already contains a substantial amount of dense and rigid ladderane lipids. Further research is needed to clarify the biophysical role of ladderanes and hopanoids in the membranes of anammox bacteria.

**Conclusions**

Despite a large difference in molecular phylogeny all the anammox genera studied produce the same types of ladderane core lipids in similar compositions, with the exception of "*Candidatus* A. propionicus", from which two new $C_{22}$ ladderane fatty acid lipids were identified. Ladderane core lipids can be combined into many different ladderane phospholipid combinations, containing a variety of different *sn*-1 tails and PC, PE or PG head groups. Ladderane phospholipid compositions varied considerably in each anammox species and the variations in composition are probably a species related effect or due to differences in the culturing conditions. This study highlights that ladderane fatty acid biosynthesis in anammox bacteria is relatively unchanged since the divergence of the different genera occurred, which further supports the applicability of ladderane lipids as biomarkers for all species of anammox and the anammox process. In contrast, combinations of several unusual methyl branched fatty acids appear to be specific to the different anammox genera. Finally, hopanoids have been identified in all genera of anammox bacteria, but further investigation is required to understand the biological function of ladderanes and hopanoids in anammox cell membranes.

**Acknowledgments**

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# chapter 5

## A comparative genomics study of gene products potentially encoding ladderane lipid biosynthesis

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- *in preparation*

**Abstract**

The membrane fatty acids of anaerobic ammonium oxidizing (anammox) bacteria contain linearly concatenated cyclobutane moieties, so far unique to biology. These moieties are under high ring strain and are synthesised by a presently unknown biosynthetic pathway. Gene clusters encoding enzymes of fatty acid biosynthesis in the anammox bacteria *Kuenenia stuttgartiensis* and 137 other organisms were analysed and compared *in silico* to gain further insight into the pathway of (ladderane) fatty acid biosynthesis. Four large gene clusters encode fatty acid biosynthesis in K. stuttgartiensis. Next to the regular enzyme complex needed for fatty acid biosynthesis (FASII), the presence of four putative S-adenosyl-methionine (SAM) radical enzymes, two enzymes similar to phytoene desaturases and many divergent paralogues of β-ketoacyl-ACP synthase was unusual. Surprisingly, extensive synteny was observed with FASII gene clusters in the deltaproteobacterium *Desulfotalea psychrophila*. No ladderane lipids were detected in lipid extracts of this organism but we did find unusual polyunsaturated hydrocarbons (PUHC), not detected in *K. stuttgartiensis*. We suggest that the unusual gene clusters of *K. stuttgartiensis* and *D. psychrophila* encode a novel pathway for anaerobic PUFA biosynthesis and that *K. stuttgartiensis* further processes PUFA into ladderane lipids, in similar fashion to the previously proposed route of ladderane lipid biosynthesis.

## 5.1 Introduction

Anammox (anaerobic ammonium oxidizing) bacteria are unusual specimens of the phylum *Planctomycetales* (Strous et al., 1999a). They play an important role in the oceanic nitrogen cycle (Dalsgaard and Thamdrup, 2002; Hamersley et al., 2007; Kuypers et al., 2005) and are implemented at industrial scale to remove ammonium from wastewater (Op den Camp et al., 2006; van der Star et al., 2007). Anammox catabolism uses one 1 of ammonia and 1.32 moles of nitrite to produce dinitrogen gas in the absence of oxygen (van de Graaf et al., 1997). Exploitation of this energy source under anaerobic conditions is thought to have resulted in the evolution of unique cellular architecture (Strous et al., 1999b; Strous et al., 1999a; van Niftrik et al., 2007). For example, the cell membranes of anammox bacteria are comprised of linearly concatenated cyclobutane moieties, aptly named 'ladderane' lipids (Sinninghe Damsté et al., 2002). Ladderane lipids consist of two different cyclobutane moieties, which are synthesised into a variety of lipid structures; fatty acids (Fig. 1), alcohols, mono-ethers, di-ethers and mixed ether-ester phospholipids (Boumann et al., 2006; Sinninghe Damsté et al., 2005).

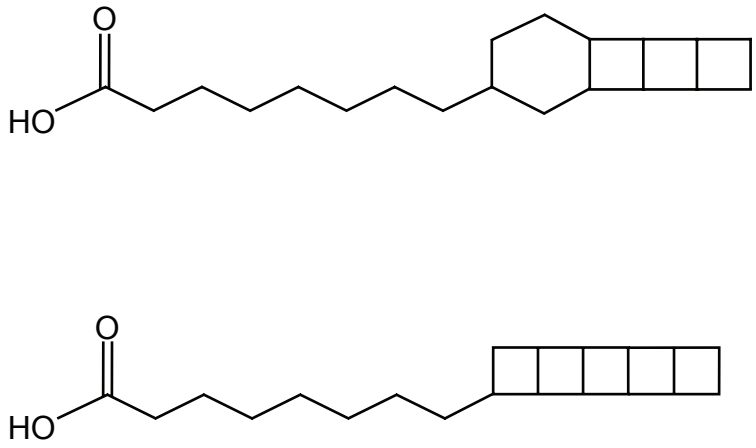


**Figure 1.** Ladderane fatty acids containing 3 or 5 linearly concatenated cyclobutane moieties.

The first hypothesis for ladderane lipid biosynthesis, as proposed by Sinninghe Damsté et al., (2005), involves ring closure of a $C_{20}$ polyunsaturated fatty acid at $C_9$ and $C_{20}$ ($C_{12}$ macrocycle formation) and subsequent carbon-carbon bonding, to create the

cyclobutane rings. Due to the structural similarity of the ladderane lipid moieties, the number of cyclization steps could then be increased or reduced to synthesise the 3 or 5 cyclobutane ring containing moieties. Mascitti and Corey (2006) suggest that ladderane biosynthesis could occur via a cascade type polycyclization, using a substrate like the allenic $C_{20}$ fatty acid 9,10,12,16,18,19-docosahexaenoic acid. Despite the fact that allenic fatty acids are rare in the natural environment and to the best of our knowledge have only been reported to occur higher plants (Aitzetmuller et al., 1997; Bagby et al., 1965; Jie et al., 2003), anaerobic bacteria including other *Planctomycetes* have been reported to have the ability of synthesising lipids containing multiple double bonds (Knoblauch et al., 1999; Kulichevskaya et al., 2007a; Kulichevskaya et al., 2007b).

Fatty acid synthesis in bacteria and plants usually occurs via type II fatty acid synthesis (FASII), recently reviewed by White et al. (2005). In *Escherichia coli*, FASII is made up of seven separate soluble proteins, each encoded by a discrete gene. Fig. 2 displays the sequence of events during FASII biosynthesis, with additional information given in Table 1. A crucial enzyme in this pathway is the acyl carrier protein (ACP), which transports lipid intermediates (as ACP thioesters) between different enzymes in the FASII pathway. In the initiation module, ACP is activated, condensed with malonyl CoA (using Malonyl-CoA:ACP transacylase, FabD) and undergoes the first condensation reaction via β-Ketoacyl-ACP Synthase III (FabH). After leaving the initiation cycle the acetoacetyl-ACP intermediate enters the elongation cycle (via FabB or FabF) where four enzymatic reactions catalyse the growth of the lipid chain by two carbons per pathway cycle. Due to the similarity of the gene paralogues *fabB* and *fabF* (and the enzymes they encode), they will be further discussed as *fabBF*. The FabBF isozymes condense the growing acyl-ACP with malonyl-ACP to extend the chain by two carbon atoms, and can differentiate between the entrance of a growing lipid chain or a new lipid chain.

After this, the lipid intermediate undergoes reduction of the carbonyl group by the NADPH dependant β-ketoacyl-ACP reductase (FabG), producing the intermediate β-ketoacyl-ACP. The isozymes FabA and FabZ (β-hydroxyacyl-ACP dehydratases) perform the third step in the elongation cycle, the initiation of unsaturated fatty acid biosynthesis in the growing lipid chain. β-hydroxyacyl-ACP is initially dehydrated to

yield a double bond in the $C_2$-$C_3$ region, creating *trans*-2-butenoyl-ACP. This product can then proceed in the cycle to have the double bond reduced by FabI (enoyl-ACP reductase), or alternatively, FabA can isomerize e.g. *trans*-2-decenoyl-ACP to *cis*-3-decenoyl-ACP, bypassing FabI to be used by FabB and initiating the first cycle of unsaturated fatty acid biosynthesis (Heath and Rock, 1996). At the last step of the cycle, FabI and the isozyme FabK reduce the double bond ($C_2$-$C_3$) in *trans*-2-decenoyl-ACP to form butyryl-ACP, using NADPH as the electron donor. The fatty acid then resides in the elongation cycle until the required acyl chain length is achieved, at which point, the fatty acid is released from the cycle to undergo further processing into intact membrane lipids. In aerobic bacteria, unsaturated fatty acids can be biosynthesised by modifying the saturated end products of the FASII pathway. In anaerobic bacteria, monounsaturated lipids are produced in the FASII pathway itself, by inserting a double bond during chain elongation (FabAZ produces a *cis*-3-decenoyl-ACP substrate to bypass FabI). FASII is also versatile pathway, it can be used for synthesising various lengths of normal straight chain fatty acids, hydroxy, *iso* and *ante-iso* branched fatty acids and non-lipid cellular metabolites including molecules for quorum sensing and lipoic acid (White et al., 2005).

Initial analysis of the *K. stuttgartiensis* genome has provided several clues to the potential pathway of ladderane lipid biosynthesis (Strous et al., 2006). In *K. stuttgartiensis*, four gene clusters putatively encode fatty acid biosynthesis. Apart from encoding typical FASII enzymes, two clusters additionally contain genes encoding unusual S-adenosylmethionine (SAM) radical enzymes. The ability of these enzymes to catalyse diverse and unusual reactions (Cheek and Broderick, 2001; Jarrett, 2003; Sofia et al., 2001) was the basis to propose that SAM radical enzymes perform a key role in the biosynthesis of ladderane lipids (Strous et al., 2006). In addition to SAM radical enzymes, other genes not normally associated with fatty acid biosynthesis, were also detected. In this study we aimed to provide further insight into ladderane biosynthesis in anammox bacteria, by applying detailed analysis and functional prediction of *K. stuttgartiensis* gene products and comparing these with fatty acid gene clusters from a diverse range of bacterial and archaeal genomes.

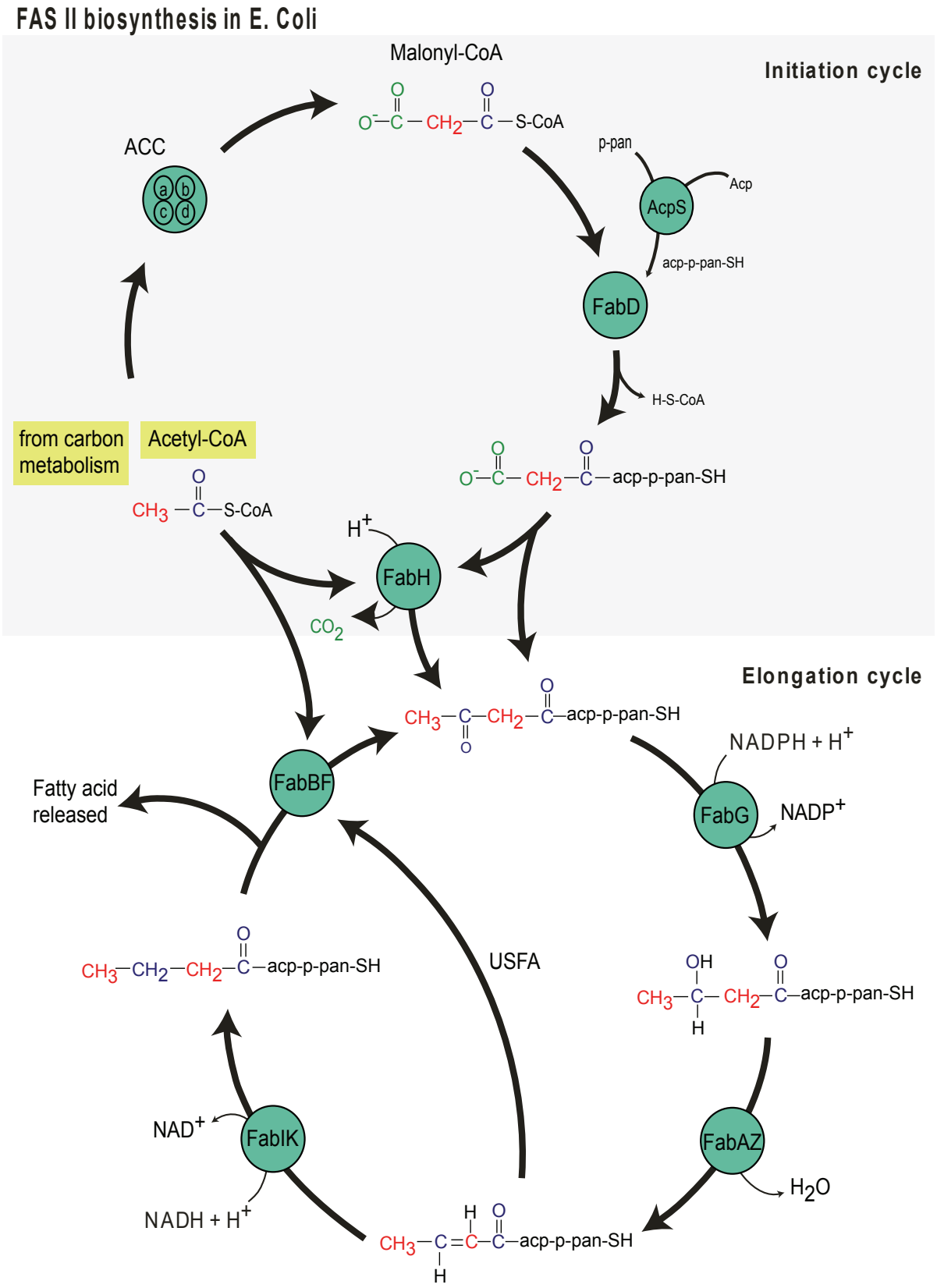


**Figure 2.** Schematic diagram of the flow of carbon during type II fatty acid biosynthesis in *Escherichia Coli*, adapted from White et al. (2005). Table 1 contains enzyme name abbreviations. USFA, pathway of unsaturated fatty acid biosynthesis, which bypasses enzyme FabIK.

**Table 1.** Gene products and enzymes encoding fatty acid synthesis in *E. coli* (White et al., 2005).

| Enzyme | Full name enzyme | Gene Product |
|---|---|---|
| ACP | Acyl carrier protein | *acpP* |
| AacpS | ACP synthase | *acpS* |
| AccABCD | Acetyl-CoA carboxylase (ACC) | *accABCD* |
| FabD | Malonyl-CoA ACP transacylase | *fabD* |
| FabH | *β*-Ketoacyl-ACP synthase III | *fabH* |
| FabB | *β*-Ketoacyl-ACP synthase I | *fabB* |
| FabF | *β*-Ketoacyl-ACP synthase II | *fabF* |
| FabG | *β*-Ketoacyl-ACP reductase | *fabG* |
| FabA | *β*-Hydroxydecanoyl-ACP dehydratase | *fabA* |
| FabZ | *β*-Hydroxyacyl-ACP dehydratase | *fabZ* |
| FabI | Enoyl-ACP reductase I | *fabI* |
| FabK | Enoyl-ACP reductase II | *fabK* |
| FabR/ FadR | Transcriptional activator/ repressor | *fabR/ fadR* |

## 5.2 Experimental procedure

### 5.2.1 *Database retrieval and data assessment*

Genomic information from the *K. stuttgartiensis* genome was extracted from PEDANT and NCBI databases. Amino acid sequences encoding lipid biosynthesis in other organisms were retrieved from a selection of online databases (http://www.genome.ad.jp/kegg,http://www.tigr.org, http://www.ncbi.nlm.nih.gov). BLAST (Basic logical alignment search tool) searches were performed using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) and the online National Centre for Biotechnology and Information (NCBI, Bethesda, MD) database (http:www.ncbi.nlm.nih.gov/BLAST). Amino acid sequences were analysed using MEGA 3.1 (Kumar et al., 2004) and aligned using ClustalW with a Gonnet protein weight matrix. Protein active sites were identified by comparison to predetermined protein crystal structures available on the Research Collaboratory for Structural Bioinformatics (RCSB) protein data bank. For phylogenetic analysis, amino acid sequences were aligned using ClustalW and manually refined. Minimum evolution phylogeny with bootstrapping (500 re-samplings) was performed using MEGA 3.1 (Kumar et al., 2004). Protein active sites were determined using CDART (Conserved Domain Architecture Retrieval Tool, NCBI) and the conserved domain database (CDD, NCBI). Alignment of sequences using this database, are related to the percentage similarity of residue conservation patterns from other members of that particular protein family. Protein sequences were also blasted in NCBI using CD-

Search (Conserved Domain Search) where the proteins are compared using similarities in architecture rather than sequence alignment (Kumar et al., 2004).

### *5.2.2 Comparative genomics*

Lipid gene clusters from the genomes of a representative selection of 137 different organisms publicly available in the databases in June 2006 were compared with the four gene clusters of *K. stuttgartiensis*. The genomes were screened for gene clusters, which contained at least 2 of the normal fatty acid biosynthesis genes and 2 of the accessory genes present in the gene clusters of *K. stuttgartiensis.* Genes were considered homologous when they shared at least 20% amino acid identity over at least 70% of the length. The screening was performed with a custom script that used, as the input, reciprocal BLAST searches of the *K. stuttgartiensis* genes against the 137 genomes.

### *5.2.3 Biomass culturing, extraction and analysis of lipids*

Biomass of *Desulfotalea psychrophila* was cultured at DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH), and grown according to DSMZ protocols (www.dsmz.de). Biomass was frozen, freeze-dried, ultrasonically extracted, saponified and analysed for lipids as described previously (Sinninghe Damsté et al., 2005). The total lipid extract was analysed using gas chromatography (GC) and gas chromatography mass spectrometry (GC/MS) as described by Kartal et al., (2007). For identification of molecular ions of selected compounds the lipid extract was analysed by GC/Chemical Ionization (CI)/MS. These were performed using a HP 6890 Series GC System (Hewlett Packard) equipped with a 25 m X 0.32 mm X 0.12 µm CPSil 5CB (Chrompack) silica column coupled to an HP 5973 Mass Selective Detector. Helium was used as a carrier gas and $CH_4$ as the reaction gas. Ladderane lipid analysis was performed using the high performance liquid chromatography (HPLC)-MS/MS procedure of Hopmans et al. (Hopmans et al., 2006). Stable carbon isotope analysis of individual lipids was performed as described in detail elsewhere (Schouten et al., 2004). Using derivatizing agents with known isotopic compositions, and subsequently adjusting the isotopic compositions of the fatty acids and alcohols, determined the isotopic composition of the extra carbon added during sample derivatization. $\delta^{13}C$ values of the sodium lactate crystals used as the carbon

source for anammox bacteria were determined using Elemental analysis isotope ratio monitoring (EA-IRM)/MS as described previously (Kartal et al., 2007).

## 5.3 Results

### *5.3.1 Comparative genomics analysis*.

*K. stuttgartiensis* contained the complete set of genes encoding FASII biosynthesis (Table 1; Figure 2). Most genes were present as a single copy but multiple paralogues of acyl carrier protein (*acp*, 3 copies) and β-ketoacyl-ACP synthase (*fabB/F,* 7 copies) were present in the lipid gene clusters. Multiple copies of *acp* were also present in *Mycobacteria* and in organisms employing polyketide biosynthesis (e.g. *Shewanella* sp.). A distant relative to *K. stuttgartiensis*, the planctomycete *Rhodopirellula baltica* and the unrelated deltaproteobacterium *Desulfotalea psychrophila* both contained 5 *fabB/F* paralogues.

We studied in detail the active site residues essential for the functioning of *fabB/F*, since deviation in active site residues could point to the use of alternative substrates by different paralogues. The *K. stuttgartiensis fabB/F* paralogues Kuste3606, Kuste2805 and Kuste3348 display significant differences in the 'active' site residues (Table 2), whereas the primary structure of the active site of the other four *fabB/F* sequences were highly conserved. As seen in Table 2, the *R. baltica fabB/F* paralogues RB 3714 (NP_865682) and RB4527 (NP_866153) also miss the active site cysteine (Cys163), which is substituted for a negative hydrophilic amino acid, Asp and Glu in the respective samples. Deviations in the sequences of these two paralogues of *R. baltica* are very similar to those observed in *K. stuttgartiensis*, which is consistent with the grouping of these paralogues together in the phylogenetic tree in Fig. 3.

The upper section of the minimum evolution phylogenetic tree shows the 'standard' *fabB/F* gene sequences from a selection of bacteria, and the lower section shows the grouping of unusual *fabB/F* paralogues, which contain distinct active sites. The only other *fabB/F* gene product analysed which displayed deviation in the active site residues was in *G. sulfurreducens* (NP_951518). This sequence was shorter than all other *fabB/F* sequences (341 amino acids) and as a result missed the last hydrophobic residue.

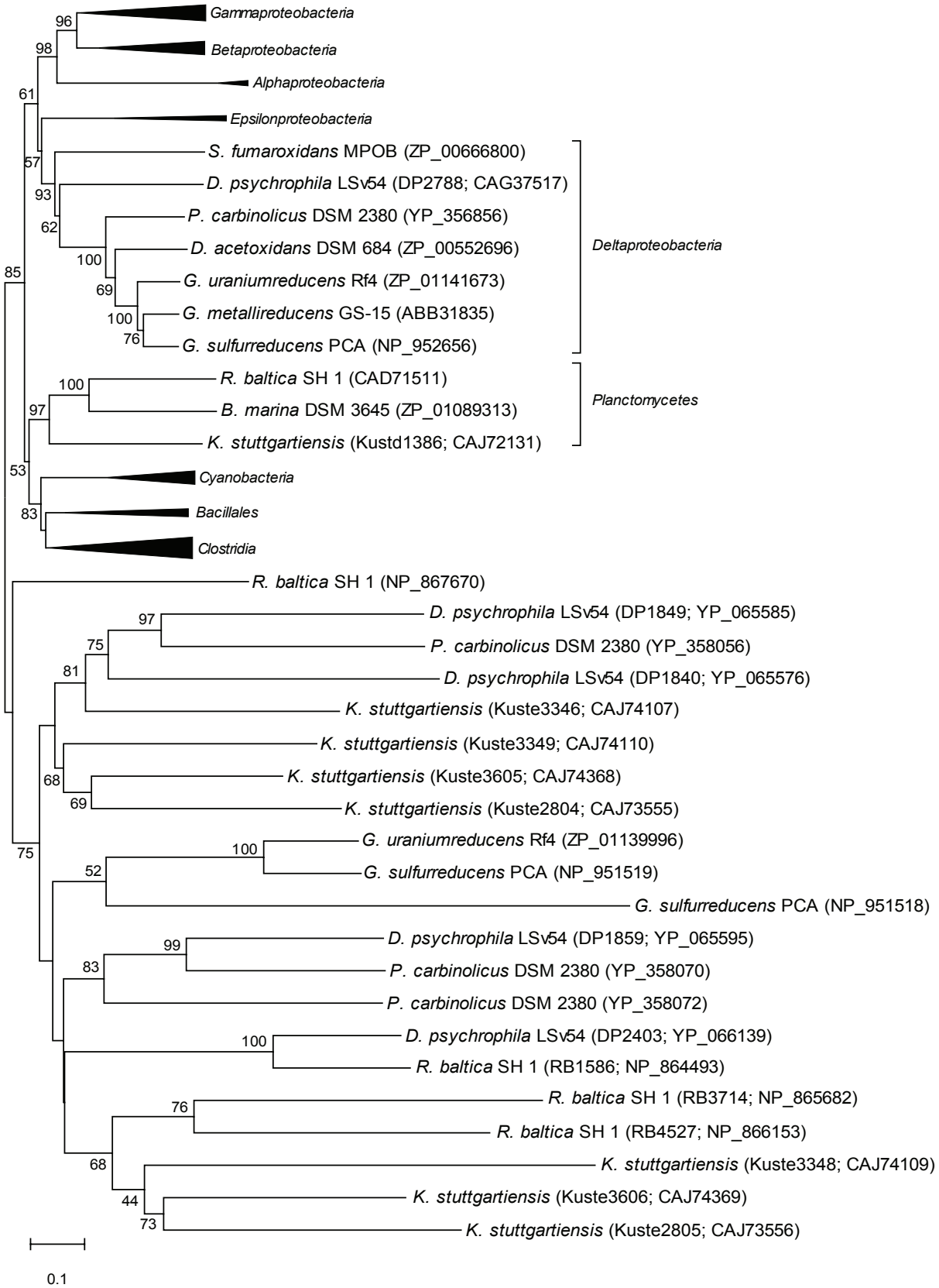


**Figure 3.** A minimum evolution phylogenetic tree for *fabB* and *fabF* displaying distribution of the protein paralogues. Bootstrap values correspond to the consensus of 500 replications, final tree was based on bootstrap consensus. Missing gaps were treated using pairwise deletion. Accession numbers of all proteins and locus tags of gene products mentioned in text are shown.

**Table 2.** Deviations in enzyme active sites in *fabB/F* paralogues in *K. stuttgartiensis*, *R. baltica*, *G. sulfurreducens* in comparison to 'typical' *fabB/F* sequences in e.g. Kustd1386 or *E. coli* (YP_540234). Functional role of amino acids taken from White et al. (2005). Key; *Italics* = *hydrophilic (polar amino acids)*, Bold = hydrophobic (non-polar amino acids), (*) = Basic, (‡) = Acidic or amide, (s) = Sulphur containing, (a) = Aromatic, (i) = Imino acid.

| **Gene sequence** | **Active site amino acids from *fabB/F* sequence** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Typical *fabB/F* sequence and function | Active site | Decarboxylation | Unknown | Unknown | Electronic charge on histidine ring | Decarboxylation | Electronic charge on histidine ring | Guides acyl chain entry into binding pocket |
| | *Cys163*s | *His298** | *Thr300* | *Thr302* | *Lys328** | *His333** | *Glu342*‡ | **Phe392**a |
| Kuste3606 *CAJ74369* | **Val163** | *Asn298*‡ | *Tyr300*a | **Gly302** | *Lys328** | *Glu333*‡ | *Gln342*‡ | *Arg392** |
| Kuste2805 *CAJ73556* | **Phe163**a | *Asn298*‡ | *Asn300*‡ | *Cys302*s | *Lys328** | *Glu333*‡ | *Gln342*‡ | **Phe392**a |
| Kuste3348 *CAJ4109* | *Ser163* | *Ser298* | **Pro300**i | **Phe302**a | *Asp328*‡ | *Asn333*‡ | *Asn342*‡ | *Ser392* |
| *R. baltica* NP 865682 | *Asp163*‡ | *His298** | **Met300**s | *Asp302*‡ | **Ile328** | *His333** | **Gly342** | *Ser392* |
| *R. baltica* NP 866153 | *Glu163*‡ | *His298** | **Leu300** | *Asp302*‡ | *Lys328** | *Asn333*‡ | *Glu342*‡ | **Pro392**i |
| *G. sulfurreducens* NP 951518 | **Phe163**a | **Pro298**i | **Ala300** | **Ala302** | **Gly328** | *Arg333** | **Ala342** | - |

This sequence consisted of mainly hydrophobic residues instead of the hydrophilic amino acids found in typical *fabB/F* sequences. Information in Table 2 and Figure 3 indicate that Kuste3348 is unique to *K. stuttgartiensis*. The rest of the recognised FASII gene products were analysed and (in comparison to other organisms) were not found to be atypical, with respect to amino acid sequence and the number of paralogues.

*K. stuttgartiensis* contains FASII gene clusters with six genes encoding putative S-adenosylmethionine (SAM) radicals or methylase enzymes and two genes with some homology to phytoene dehydrogenases. To determine just how unusual this combination is, we searched databases for the occurrence of similar gene clusters in other organisms and found that such clusters were rare but not unique. Some deltaproteobacteria (*Desulfotalea psychrophila*, members of the genus *Geobacter* and *Pelobacter carbinolicus*) contained similar gene clusters (Fig. 4, a). The synteny with *D. psychrophila* was striking, apart from the presence of similar SAM and 'phytoene dehydrogenase' genes, a gene encoding a new membrane protein with unknown function and a 'phenylacetyl-CoA ligase' were also in synteny, the pairwise identity of these 'unusual' enzymes is shown in Fig. 4 (b).

A small number of open reading frames in the *K. stuttgartiensis* gene clusters were not in synteny with gene clusters in *D. psychrophila* and had little sequence homology with open reading frames in any other organisms. Proteins with no assigned function were Kuste3352 (an unknown protein), Kuste3351 (a polysaccharide deacetylase), Kuste3347 (a methyl transferase), Kuste2803 (a SAM radical enzyme with an additional B12 binding domain) (Figure 4, a). In particular, Kuste3347 was unlike other currently defined methyl transferases. Therefore we constructed a minimum evolution phylogenetic tree of closest relatives of Kuste3347, obtained using closest matches in BLAST (Fig. 5).
For comparative purposes methyl transferases with other putative functions have been shown, including cyclopropane mycolic acid synthases, which are involved in cyclic fatty acid production. The closest relative to Kuste3347 was a gene product encoding a methyltransferase type 11 in the deltaproteobacterium *Geobacter bemidjiensis*.

Apart from the FASII gene clusters we also investigated if there were clusters encoding lipid synthesis via the polyketide or isoprenoid pathways. No gene products were located in the *K. stuttgartiensis* (or the *D. psychrophila*) genome, which resemble polyketide synthases known to synthesise PUFA in marine organisms (Metz et al., 2001), or indeed any other types of polyketide like enzymes. In *K. stuttgartiensis*, gene sequences encoding enzymes for isoprenoid synthesis were found for the 2-C-methyl-D-erythritol 4-phosphate (MEP or non-mevalonate) pathway (containing dxs, dxr, ygbP, ychB, ygbB, gcpE and lytB). Key genes encoding enzymes in the mevalonate pathway were absent (i.e. hmgs and hmgr). Common to sequences encoding isoprenoid enzymes, gene products for the production of isoprenoids were scattered around the genome and did not occur in one specific gene cluster. *K. stuttgartiensis* also contained gene products for the production of terpenoid lipids.

### *5.3.2. Lipid analysis*

The striking synteny of gene products encoding fatty acid biosynthesis in *K. stuttgartiensis* and *D. psychrophila* prompted us to investigate the lipid composition of *D. psychrophila*. Core lipid fractions of *D. psychrophila* were analysed for ladderane lipids using GC/MS and HPLC-MS/MS. Ladderane lipids were not identified in the *D. psychrophila* biomass. However, unusual polyunsaturated hydrocarbons (PUHC) $C_{31:8}$ and $C_{31:9}$, with molecular masses of 418 and 420 respectively, were identified using CI/MS. The PUHCs formed 5% of the total lipid fraction (Table 3). The $\delta^{13}C$ values of PUHCs and fatty acids were determined and their isotopic composition relative to the carbon source lactate was calculated, PUHCs, the most abundant fatty acids and cholesterol are shown in Table 3. The PUHC are similar in $^{13}C$ relative to the average $\delta^{13}C$ of the monounsaturated fatty acids.

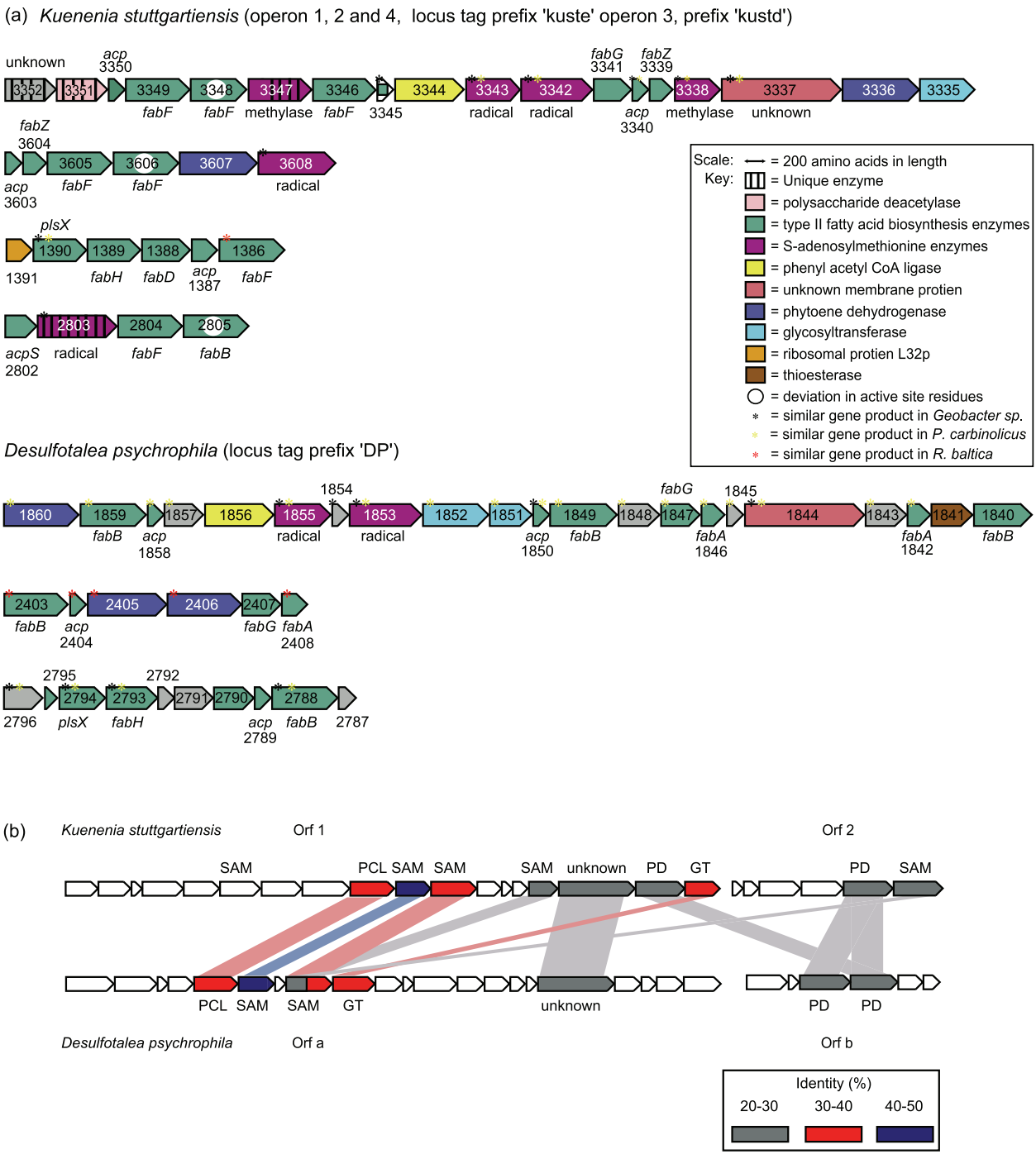


**Figure 4.** (a) Functional assignment of enzymes in open reading frames (Orf) encoding fatty acid biosynthesis in *K. stuttgartiensis* and *D. psychrophila*. (b) Pairwise comparison (identities) of unusual enzymes found in (a). Pairwise comparisons were conducted using BLAST with a BLOSUM62 matrix file and gap penalties of 11/1 (existence/ extension). SAM, S-adenosylmethionine; PCL, phenylacetyl CoA ligase; OX, oxidoreductase; GT, glycosyltransferase; PD, phytoene dehydrogenase.

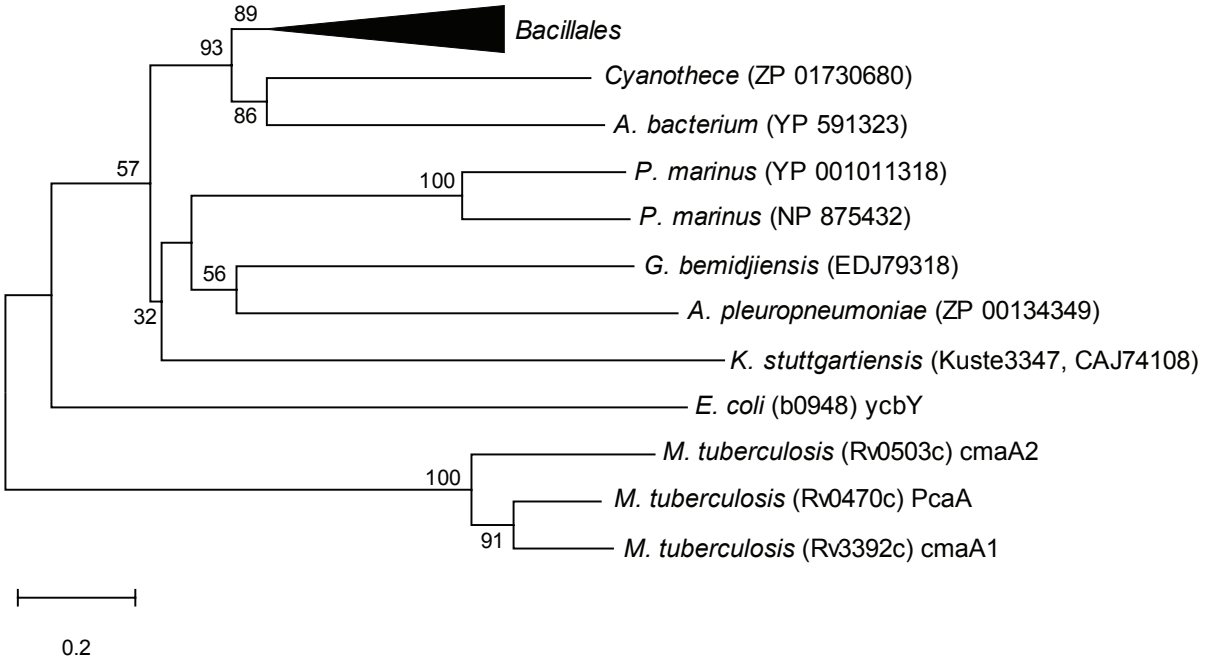


**Figure 5.** A minimum evolution phylogenetic tree of closest relatives of Kuste3347, an unusual methyl transferase (obtained using closest matches in BLAST). Bootstrap values correspond to the consensus of 500 replications, final tree was based on bootstrap consensus. Missing gaps were treated using pairwise deletion. Accession numbers of all proteins and the locus tag of the *K. stuttgartiensis* gene product are shown. For comparative purposes methyl transferases with other putative functions are shown; ycbY, a putative N-6 adenine-specific DNA methylase; PcaA, mycolic acid synthase; cmaA1, cyclopropane mycolic acid synthase; 1 cmaA2, cyclopropane mycolic acid synthase 2.

**Table 3.** Carbon fractionation of major lipids in *D. psychrophila* calculated relative to the carbon source, lactate (-28.9‰). PUHC; polyunsaturated hydrocarbon. Mean values of duplicate measurements of one culture are given, replicate values deviated by a maximum of ± 1 ‰.

| Lipid / Substrate | % abundance in total lipid extract | $\Delta\ \delta^{13}C$‰ relative to lactate |
|---|---|---|
| C31:8 (PUHC) | 4 | -20.1 |
| C31:9 (PUHC) | 1 | -20.4 |
| C14:0 | 2 | -8.0 |
| C16:0 | 12 | -11.0 |
| C16:1 | (39, 15) | (-16.1, -20.8[a]) |
| C17:1 | 1 | -13.5 |
| C18:1 | (2, 2[a]) | (-28.5, -22.8[a]) |
| Cholesterol | 1 | 1.9 |

[a] two isomers, separated by retention time.

## 5.4 Discussion

Different versions of *acp* in *K. stuttgartiensis* are probably employed to transport structurally different biosynthetic intermediates. It is possible that in the case of ladderane fatty acids, the lipid intermediates are too varied in structure to perform biosynthesis using a single *acp*. Structural differences could be explained by a varying degree of carbon chain rigidity and the presence of intermediate functional groups. In addition, multiple paralogues of *fabB/F* were also identified. *fabB/F* catalyses the addition of a new monomer to a growing lipid chain. The presence of seven *fabB/F* paralogues could therefore indicate to the addition of different monomers to the chain (e.g. unsaturated or branched fatty acids) or could be required to accommodate a chain that undergoes major backbone rearrangements during elongation. At this stage we can not exclude the synthesis of ladderane lipids via a shared synergistic pathway, it is therefore possible that some of the *fabB/F* paralogues function with intermediates from outside FASII, e.g. from the isoprenoid pathway. Numerous copies of *acp* would support this hypothesis since the diffusible properties of the acp protein would allow it to act as a transporter of external ladderane intermediates into FASII. As found with *acp*, multiple paralogues of *fabB/F* were not unique to *K. stuttgartiensis*. Its distant relative, the *Planctomycete Rhodopirellula baltica* and the unrelated deltaproteobacterium *Desulfotalea psychrophila* both contained *5 fabB/F* paralogues. To investigate further, we have studied in detail the active site residues, which are essential for performing the functions of *fabB/F*, since deviation in active site residues could point to the use of alternative substrates by different paralogues. The comparative genomic analysis of the FASII gene products of *K. stuttgartiensis* has indicated that this particular FASII pathway could accommodate a carbon backbone that undergoes major structural rearrangement. It may also facilitate the extension of the backbone with compounds other than acyl-CoA. Surprisingly, most of this extra functionality seems to be shared with a small number of other organisms, most notably the related *R. baltica* and the unrelated *D. psychrophila*.

*K. stuttgartiensis* contains FASII gene clusters encoding a number of genes with no recognised function in lipid biosynthesis. The presence of SAM radical or methyl enzymes in gene clusters encoding fatty acid biosynthesis in many of the analysed organisms was unexpected and the synteny between these unusual gene clusters in

*K. stuttgartiensis* and *D. psychrophila* was particularly unusual (Figure 4, b). Although *D. psychrophila* did not contain ladderane lipids, it did contain unusual $C_{31:8}$ and $C_{31:9}$ polyene hydrocarbons, which have been previously identified in psychrophillic bacteria and in members of the *Planctomycetes* (Kulichevskaya et al., 2007b; Kulichevskaya et al., 2007a; Nichols et al., 1995). Saturated polyene hydrocarbons are known to be produced via decarboxylation of (e.g. $C_{32}$) fatty acids (Finnerty, 1989). Our results showed that the $\delta^{13}C$ of the PUHCs in *D. psychrophila* were similar to the $\delta^{13}C$ of the monounsaturated fatty acids, which suggests that these lipids have undergone similar fractionation processes and implies that they have been synthesised via the same biosynthetic pathway. Although polyunsaturated hydrocarbons have not been identified in *K. stuttgartiensis*, the biosynthesis of ladderane lipids could, as previously suggested by Sinninghe Damsté et al., (2005), begin with a polyunsaturated fatty acid. Therefore, the synteny between *K. stuttgartiensis* and *D. psychrophila* may be explained by shared genes, as part of a shared pathway for the production of polyunsaturated fatty acids via a novel anaerobic mechanism that proceeds from FASII gene products or operates on the *acp*-bound intermediates of the FASII pathway.

There are two classes of oxidative enzymes encoded in the FASII gene clusters of *K. stuttgartiensis* and *D. psychrophila* that could perform the insertion of double bonds into a growing lipid chain. In *K. stuttgartiensis* there are two (Kust3336, Kuste3607) and in *D. psychrophila* there are three (DP1860, DP2405, DP2406) open reading frames, which encode for flavin containing amine-oxidoreductases, similar in architecture to phytoene dehydrogenases. Phytoene dehydrogenases introduce double bonds into isoprenoid chains. However, it should be noted that isoprenoids are constructed from IPP units, already containing double bonds and that it is a more difficult task to introduce the first double bond into a saturated lipid. Therefore, it is more probable that the phytoene dehydrogenase like enzymes, encoded by *K. stuttgartiensis* and *D. psychrophila*, introduce subsequent double bonds into an already unsaturated fatty acid. The second class of oxidative enzymes, the SAM radical enzymes, are however, more powerful. At the expense of adenosyl methionine they can abstract a hydrogen atom from a carbon, yielding a carbon radical (-CH-). In theory, these enzymes have the thermodynamic capability to introduce an initial double bond into a saturated fatty acid. Pairwise comparison

between the unusual gene products in *K. stuttgartiensis* and *D. psychrophila* (Figure 4, b) indicates that many of the unusual *D. psychrophila* open reading frames are homologous to those in *K. stuttgartiensis* and therefore could drive a similar pathway. Remaining synteny between the *K. stuttgartiensis* and *D. psychrophila* FASII gene clusters could well be related to the post-processing of the FASII products. The acyl-CoA ligase encoded in both organisms (annotated as phenylacetyl-CoA ligase and F390 synthetase, respectively) could well perform the reaction for the first step of glycerol attachment and the unknown membrane protein (containing a putative lipid transport domain) may be involved in the transport and insertion of unusual lipids into the membrane, as hypothesised with similar Mmp gene sequences found *in Mycobacterium tuberculosis* (Tekaia et al., 1999).

Some open reading frames in the *K. stuttgartiensis* gene clusters were not in synteny with gene clusters in other organisms. It is tempting to speculate that the proteins encoded by these open reading frames catalyse the most spectacular part of the pathway, the oxidative cyclisation of PUFAs into ladderanes. Although the analysis of alternative pathways of lipid biosynthesis (isoprenoid and polyketide) supplied no additional clues to the pathway of ladderane biosynthesis, they could still provide additional ladderane building blocks in FASII. For example an IPP building block could also undergo oxidative cyclisation and ring closure via SAM radicals. The latter possibility is consistent with the fact that all of the synteny between *K. stuttgartiensis* and *D. psychrophila* is restricted to one of the four gene clusters. This cluster could in fact encode a pathway of PUFA biosynthesis, which is parallel and unrelated to ladderane biosynthesis.

Large differences in the amino acid sequences of *fabB/F* paralogues in *K. stuttgartiensis*, have lead us to suggest that these enzymes are capable of bringing unusual intermediates (possible precursors for backbone rearrangement) into the FASII pathway. Furthermore, gene clusters encoding fatty acid biosynthesis in *K. stuttgartiensis*, contain an unusual combination of genes with remarkable synteny to fatty acid encoding gene clusters in the *D. psychrophila* genome. We have established that although *D. psychrophila* does not produce ladderane lipids under the growth conditions investigated, it does produce unusual polyunsaturated hydrocarbons. Both isotope fractionation values and the gene cluster content have

shown consistency with the biosynthesis of these polyunsaturated molecules via the pathway of fatty acid biosynthesis. Therefore we propose that two SAM radical enzymes and a number of homologous 'phytoene desaturase' like enzymes are part of a new pathway of PUFA biosynthesis in anaerobic bacteria. In addition, the presence of these genes in *K. stuttgartiensis* is consistent with the suggestion that ladderane lipids could be synthesised from a polyunsaturated precursor (Mascitti and Corey, 2006; Nouri and Tantillo, 2006; Sinninghe Damsté et al., 2005).

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# chapter 6

## Constraints on the biosynthesis of ladderane lipids in anammox bacteria using carbon isotopic labelling

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- *in preparation*

**Abstract**

Ladderane lipids are unusual membrane lipids of bacteria, which anaerobically oxidise ammonium to dinitrogen gas (anammox). Ladderane lipids contain linearly concatenated cyclobutane rings for which the pathway of biosynthesis is currently unknown. To constrain the possible biosynthetic routes of these lipids, 2-$^{13}$C labelled acetate was added to a culture of the anammox bacterium "*Candidatus* Brocadia fulgida". Labelling patterns obtained by high field $^{13}$C-NMR spectroscopy of isolated lipids indicated that "*Candidatus* Brocadia fulgida" synthesises $C_{16:0}$ and *i*$C_{16:0}$ fatty acids according to the known pathway of type II fatty acid biosynthesis. The $^{13}$C-labelling pattern of the $C_8$ alkyl chain of the $C_{20}$[3] ladderane monoether also indicated use of the type II synthetic route. However, carbon atoms in the cyclobutane rings and cyclohexane ring were non-specifically labelled, and did not correspond to known patterns of fatty acid synthesis. Taken together, this indicates that it is unlikely that ladderane lipids are formed from the cyclisation of polyunsaturated fatty acids as previously hypothesised, and suggests an alternative, although as yet unknown, pathway of biosynthesis.

## 6.1 Introduction

Anaerobic ammonium oxidising (anammox) bacteria are important in nitrogen cycling in marine (Hamersley et al., 2007; Kuypers et al., 2003; Kuypers et al., 2005), lake (Schubert et al., 2006) and wastewater (Op den Camp et al., 2006; van der Star et al., 2007) ecosystems, by anaerobically catabolising $NH_4^+$ and $NO_2^-$ to form $N_2$ (van de Graaf et al., 1997). Anammox bacteria have an unusual cell biology (Strous et al., 1999) and lipid chemistry (Sinninghe Damsté et al., 2002). They contain an anammoxosome, the intracytoplasmic compartment where anammox catabolism is thought to take place (Lindsay et al., 2001; van Niftrik et al., 2004, in press a). The lipid membrane of the anammoxosome contains unique linearly concatenated cyclobutane (3 or 5 ring) containing lipids, termed as "ladderane lipids" (Sinninghe Damsté et al., 2002). Lipid modelling and fluorophore permeation experiments using anammox cells have shown that ladderane lipids form a dense and rigid membrane, providing a tight barrier against diffusion (Sinninghe Damsté et al., 2002). Ladderane moieties can be incorporated into a variety of lipid structures, i.e. as fatty acids which can be ester bound to the glycerol backbone or as alkyl moieties ether bound to glycerol (Sinninghe Damsté et al., 2005). Ladderane phospholipids contain either phosphocholine, phosphoethanolamine or phosphoglycerol head group attachments (Boumann et al., 2006; Rattray et al., *in press*).

Naturally occurring cyclobutane structures are rare in nature and the linearly concatenated cyclobutane rings produced by anammox bacteria are so far unique. As a consequence, the pathway of ladderane lipid biosynthesis is unknown. Sinninghe Damste et al. (2005) have hypothesised that ladderane synthesis involves the folding of a $C_{20}$ polyunsaturated fatty acid with ring closure at the $C_9$ and $C_{20}$ position, forming a $C_{12}$ macrocycle, followed by internal cyclisation within the macrocycle with formation of the [3] ladderane moiety (where the 3 or 5 in square brackets indicates the number of cyclobutane rings). Formation of the [5] ladderane moiety would then require one more cyclisation step. Mascitti and Corey (2006) suggested that ladderane biosynthesis could occur via a cascade type polycyclisation, using a $C_{20}$ allenic fatty acid as a substrate. In addition, a variety of other possible scenarios for polycyclisation and/or folding of highly unsaturated acyclic lipid precursors have been suggested for ladderane lipid biosynthesis, by Nouri and Tantillo (2006). In a comparative bioinformatics study of the genome of

*Kuenenia stuttgartiensis*, Rattray et al. (*in prep*) postulated that some of the more unusual open reading frames, located in regions encoding enzymes for fatty acid biosynthesis, probably encode a new pathway for the synthesis of unsaturated fatty acids. The final ladderane moiety could then be folded from the unsaturated fatty acid to create linearly concatenated cyclobutane structures. Thus, regardless of the different mechanisms postulated, all of the hypothetical routes of ladderane biosynthesis make use of a polyunsaturated fatty acid as the precursor molecule from which ladderane lipids are formed.

A commonly used technique to elucidate lipid biosynthetic pathways is application of position specific $^{13}C$ labelled substrates (e.g. acetate, pyruvate, glucose or amino acids) as tracer molecules (Bloch, 1965; Massé et al., 2004; Ponticorvo et al., 1949; Rohmer et al., 1993). The initial route of fatty acid biosynthesis in prokaryotes was first elucidated for *Escherichia coli*, which has since become the model pathway for type II fatty acid biosynthesis (White et al., 2005). In short, fatty acids are biosynthesised by the condensation of acetyl-ACP (acyl carrier protein) and malonyl-ACP, in a repeated cycle where at the end of each revolution, two carbon units are added. Rittenberg and Bloch (1945) showed, using isotopic labelling experiments, that the condensation units were derived from acetic acid. When 1-$^{13}C$ acetate (carbonyl position labelled) is used as substrate, the odd numbered carbon positions of fatty acids will be labelled, while use of 2-$^{13}C$ acetate (methyl position labelled) will label the even numbered carbon positions. Since most of the postulated synthetic routes for ladderane synthesis assume that unsaturated fatty acids form the parent molecule, we performed labelling experiments with 2-$^{13}C$ acetate on cultures of the anammox bacteria "*Candidatus* Brocadia fulgida". Using this method, we investigated the hypothesis that the carbon atoms in ladderane lipids are synthesised via the type II fatty acid biosynthetic pathway. Although anammox are facultative chemolithoautotrophs (Schouten et al., 2004; Strous et al., 1999), deriving most carbon from $CO_2$ fixed via the acetyl CoA pathway, acetate is an intermediate of this pathway and its incorporation would be expected. In addition, the conversion of small organic acids (i.e. formate, acetate, and propionate) by anammox bacteria has been recently documented (Kartal et al, 2007; 2008). Furthermore, it has been previously demonstrated that photoautotrophs can incorporate sufficient $^{13}C$ labelled acetate into pathways of lipid synthesis, to

reveal details of the biosynthetic route used (Massé et al., 2004; Schwender et al., 1996). Therefore, with the use of $^{13}C$ labelled acetate tracer experiments, we aimed to provide experimental data on the pathway of ladderane lipid biosynthesis.

## 6.2 Experimental Procedures

### *6.2.1 Anammox enrichment cultures*

"*Candidatus* Brocadia fulgida" was cultivated in a sequencing batch reactor (SBR), as described previously (Kartal et al., 2007; 2008; Strous et al., 1998). In addition, the culture was supplied with 100 ml of 8 mM 2-$^{13}C$ acetic acid (99% purity, Cambridge Isotope Laboratories) per day (in the medium), for a total of 40 days. The biomass was harvested at the end of the experiment. To study the fate of acetate in the continuous culture, a duplicate experiment was set up using the bacterium "*Candidatus* Brocadia fulgida", where the SBR was supplied with 8 mM 2-$^{13}C$ acetic acid (in the medium), per day for a total of 18 days. DIC (dissolved inorganic carbon) samples were taken at regular intervals during the experiment.

### *6.2.2 Isolation of lipids*

Biomass for lipid extraction was harvested at the end of the experiment, freeze dried (yielding ca. 9 g dry weight biomass) and ultrasonically extracted five times using a mixture of 2:1 (v:v) dichloromethane (DCM):methanol (MeOH). Biomass residues were base hydrolysed using 0.5 mol $l^{-1}$ KOH in methanol for 1 h at 100°C and the extracts were combined. Total extracts were evaporated to dryness using rotary evaporation. The total extract was methylated using $BF_3$ in methanol (known isotopic composition -43‰ vs. VPDB). Lipids were separated using column chromatography as previously described by Hopmans et al. (2006). The $C_{20}$[3] ladderane glycerol monoether (Fig. 1, c) was obtained in pure form from the 2:1 (v:v) DCM:MeOH fraction. The $C_{16:0}$ and *iso*$C_{16:0}$ fatty acid methyl esters (Fig. 1, a and b, respectively) were isolated from the 4:1 (v:v) hexane:DCM fraction by high performance liquid chromatography (HPLC) (Hopmans et al., 2006).

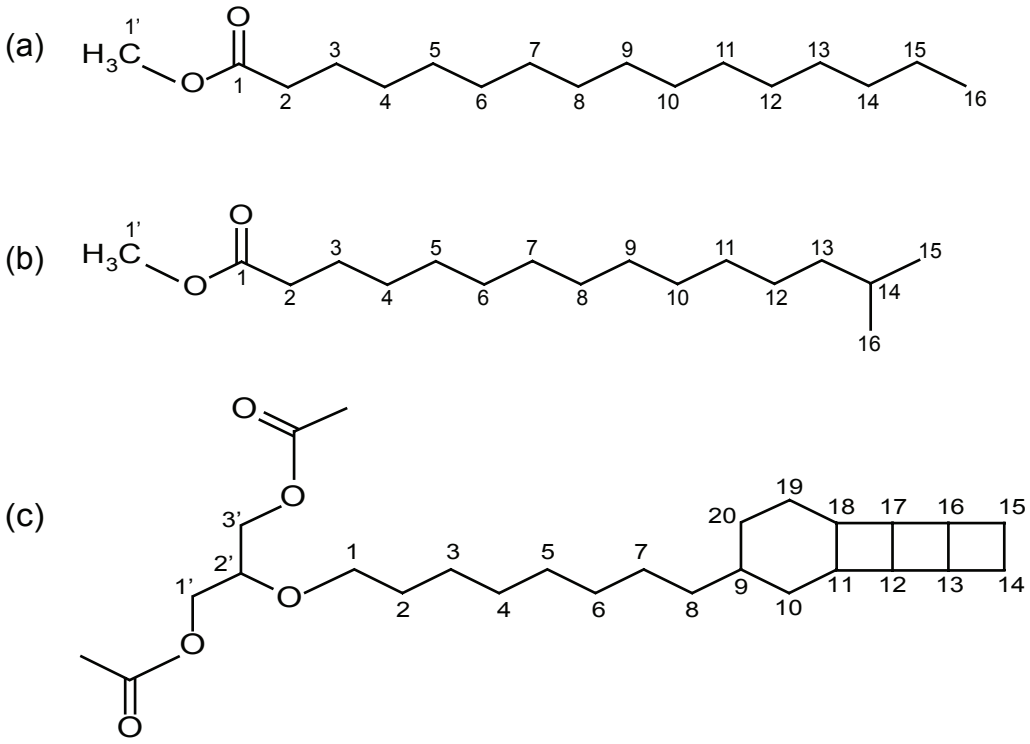


**Figure 1.** Structures of anammox lipids analysed using NMR, illustrating carbon numbering system. (a) $C_{16:0}$ fatty acid (b) *i* $C_{16:0}$ fatty acid (c) $C_{20}$[3] ladderane monoether.

Prior to nuclear magnetic resonance (NMR) spectroscopy the purified $C_{20}$[3] ladderane monoether was acetylated using 2 ml acetic anhydride and 30µl pyridine and heating at 55°C for 1 h. The acetylated mixture was then combined with distilled water and hexane, extracted using a separatory funnel and dried. A non-labelled enrichment culture of "*Candidatus* Brocadia anammoxidans" was previously cultured and the $C_{20}$[3] ladderane monoether extracted and prepared as described by Sinninghe Damsté (2002). Additionally, the non-labelled $C_{16:0}$ and *iso*$C_{16:0}$ fatty acid methyl esters were isolated by HPLC as described above. The purity of the isolates was analysed using gas chromatography (GC) and GC/mass spectrometry (MS), as previously described by Sinninghe Damsté et al. (2005). Table 1 displays the weights and purities of the individual lipid fractions.

**Table 1.** Weights and purities of lipids used for NMR analysis

| | Labelled biomass | | Non-labelled biomass | |
|---|---|---|---|---|
| | Weight (mg) | Purity (%) | Weight (mg) | Purity (%) |
| **$C_{16:0}$** | 0.4 | 83 | 0.9 | 92 |
| ***i*$C_{16:0}$** | 1.8 | 80 | 0.5 | 89 |
| **$C_{20}$[3] ladderane monoether** | 20.4 | 93 | 2.0* | 98* |

* reported by Sinninghe Damsté et al. (2002).

### 6.2.3 *Lipid and stable isotope analysis*

Biomass of "*Candidatus* Brocadia fulgida" taken at 0 and 60 days of the labelling experiment was freeze dried and extracted as described previously (Sinninghe Damsté et al., 2005). Aliquots of the total lipid extract were silylated using BSTFA (N,O-bis-(trimetylsilyl)trifluoroacetamide) in pyridine at 60°C for 15 min and dissolved in ethyl acetate. Lipid stable carbon isotopes were measured using a ThermoFinnigan Delta C, GC/isotope ratio monitoring (IRM)-MS system as previously described (Schouten et al., 1998). The isotopic compositions of fatty acids and alcohols were corrected for additional carbon from sample derivatization.

The stable carbon isotopic composition of the total DIC in the reactor was measured in the duplicate experiment (see above) to investigate if there was breakdown of the labelled acetate into $CO_2$ in the culture medium. Headspace analysis was performed on the reactor supernatant and analysed on a gas bench IRM system as described by Schouten et al. (2004). Stable isotope ratios were determined relative to a laboratory standard ($NaCO_3$, -0.57‰ vs. VPDB, Vienna Pee-Dee Belemnite) which was calibrated against NBS-18 carbonate (International Atomic Energy Agency (IAEA). The delta symbol (δ) in relation to stable isotope analysis, relates to per mille.

### 6.2.5 *$^{1}$H-and $^{13}$C-NMR spectroscopy*

The isolated $C_{20}$[3] ladderane monoethers, $C_{16:0}$ and *i*$C_{16:0}$ fatty acids were dissolved in DCM (x 2) and $CHCl_3$ (x3) and $CDCl_3$ (x2) and evaporated to dryness. Finally, the compounds were transferred into NMR tubes dissolved in 0.75 ml $CDCl_3$. Isolated lipids were studied by one dimensional high-field $^{1}$H-and $^{13}$C-NMR spectroscopy. $^{13}$C NMR spectra were taken at 25°C on a Varian Unity Inova 500 MHz spectrometer

operating at 125,684 MHz, equipped with a 5mm pulsed field gradient $^{13}C$ ($^{31}P/^{1}H$) probe ($^{13}C$ observe, $^{1}H$ decoupling). The conditions used for the carbon spectra were a sweep width of 37825 Hz, relaxation delay of 30 seconds and 1.3 second acquisition time, with a pulse width of 4.8125 us corresponding with a 45 degree pulse, where a 90 flip angle corresponded with 9.625 us at 54 dB. Proton decoupling was performed with WALTZ-16 modulation during acquisition at 499.79 MHz. Lipids were analysed consecutively with their non-labelled counterparts, isolated previously (Sinninghe Damsté et al., 2002), to enable comparison of signal heights under identical conditions and relaxation times. $^{13}C$ peak assignments were made based on published data (Gunstone et al., 1976; Gunstone, 1993; Sinninghe Damsté et al., 2002). It should be noted that the delta symbol ($\delta$) in relation to NMR analysis, relates to ppm.

### 6.3 Results & Discussion

Comparison of the $\delta^{13}C$ values of lipids at 0 days with values at 40 days showed that all lipids incorporated small amounts of the $^{13}C$ label to some degree, with most incorporation occurring in the non-ladderane fatty acids including the $C_{16:0}$ and *i*$C_{16:0}$ fatty acids (Table 2).

**Table 2.** $^{13}C$ label incorporation into specific compounds as determined by stable isotope analysis. Where $\delta$ relates to per mille.

| | **$^{13}C$ per atom (%)** | | **$\Delta\delta^{13}C$ per atom (%)** |
|---|---|---|---|
| | Start experiment | End experiment | |
| ***i*$C_{16:0}$** | 1.03 | 1.51 | 0.48 |
| **$C_{16:0}$** | 1.04 | 2.50 | 1.46 |
| **$C_{20}$[3] ladderane fatty acid** | 1.03 | 1.38 | 0.35 |
| **$C_{20}$[5] ladderane fatty acid** | 1.03 | 1.34 | 0.31 |
| **$C_{20}$[3] ladderane monoether** | 1.03 | 1.28 | 0.25 |

Lipids at the start of the experiment were, as expected, highly depleted in $^{13}C$, due to the large fractionation of $^{13}C$ through carbon fixation via the acetyl-CoA pathway (Schouten et al., 2004; Strous et al., 2006). Based on the GC/IRM-MS $\delta^{13}C$

measurements, it was found that the $C_{20}$[3] ladderane monoether contained approximately 1.3% $^{13}C$ label, the $C_{16:0}$, 2.5% $^{13}C$ label and the *i*$C_{16:0}$ fatty acid 1.5 % $^{13}C$ label, compared to the natural abundance of $^{13}C$ (~1.1%). The ladderane fatty acids were found to be more enriched in $^{13}C$ label, in comparison to the $C_{20}$[3] ladderane monoether.

High field $^{13}C$ NMR was then used to determine the positioning of the label incorporation in the $C_{16:0}$ and *i*$C_{16:0}$ fatty acid and the $C_{20}$[3] ladderane monoether. Label incorporation into the $C_{16:0}$ and *i*$C_{16:0}$ fatty acids was determined to give an indication of the success of acetate incorporation into the pathway of fatty acid biosynthesis. To compare the peak heights of individual carbon atoms from the separate NMR experiments on the non-labelled and labelled compounds, the peak height of the individual carbon atoms in each compound were first normalised on the peak height of the carbon atom of the derivatising agent (i.e. methyl ester in case of fatty acids, methyl carbon of acetate group in case of the $C_{20}$[3] ladderane monoether). Subsequently, the relative degree of labelling of each carbon atom was estimated by dividing the normalised peak height of the labelled compound by the normalised peak height of the non-labelled compound, to give (equation 1) the 'relative enhancement of NMR peak height' (Massé et al., 2004).

$$Relative\ enhancement\ NMR\ peak\ height = \frac{\left(\frac{I_n}{I_d}\right)_{labelled}}{\left(\frac{I_n}{I_d}\right)_{non-labelled}}$$

(equation 1)

Where $I_n$= peak height of carbon atom n and $I_d$=peak height of carbon atom derived from the derivatizing reagents (Figure 1; C1' for a and b and the methyl carbon of the $COCH_3$ group for c). This calculation showed that NMR peak heights of a number of carbon atoms were enhanced indicating position specific $^{13}C$ labelling (Tables 3, 4 and 6). Furthermore, the mean relative enhancement of NMR peak height for the $C_{16:0}$ fatty acid gave the highest value, followed by the *i*$C_{16:0}$ fatty acid and the $C_{20}$[3] ladderane monoether (Tables 3, 4 and 6), which is in full agreement with results from our compound specific carbon isotope analysis. Below we discuss the specific labelling patterns of the different lipids.

### *6.3.1 $^{13}C$ label incorporation into the $C_{16:0}$ fatty acid*

The generic $C_{16:0}$ fatty acid constituted 2% of the total lipid extract in the enrichment culture (75+5% dominated by "*Candidatus* Brocadia fulgida"). Anammox bacteria have been reported to incorporate the generic $C_{16:0}$ fatty acid into its intact phospholipids (Boumann et al., 2006; Rattray et al., *submitted*) and also in the present study, the high depletion of $C_{16:0}$ indicated that it was a biosynthesised by "*Candidatus* Brocadia fulgida". The labelling pattern of the $C_{16:0}$ fatty acid is therefore used as a guide to the success of the incorporation of 2-$^{13}C$ acetate into the pathway of fatty acid biosynthesis.

**Table 3.** Relative enhancement of $^{13}C$ in NMR analysis of the $C_{16:0}$, isolated from the "*Candidatus* Brocadia fulgida" enrichment culture. Carbon atoms expected to be enriched according to type II straight chain fatty acid biosynthesis are highlighted in bold (even carbons labelled in $^{13}C$ and carbons at odd numbers not labelled).

| | **$C_{16:0}$ fatty acid** | |
|---|---|---|
| Carbon No. | δ (ppm) | Relative enhancement of NMR peak height |
| 1' | 51.3 | 1.0 |
| 1 | 174.3 | 1.5 |
| **2** | **34.1** | 11.2 |
| 3 | 25.0 | 1.6 |
| **4** | **29.2** | 10.7 |
| 5 | 29.2 | 1.7 |
| **6** | **29.4** | 8.3 |
| 13 | 29.3 | 0.9 |
| **14** | **32.0** | 10.4 |
| 15 | 22.8 | 1.4 |
| **16** | **13.9** | 12.3 |
| **Average** | | 6.0 |

The relative enhancement of NMR peak intensity for the $C_{16:0}$ showed that all carbon atoms were enriched in $^{13}C$, but carbon positions at even numbered positions from $C_1$ to $C_6$ and $C_{13}$ and $C_{16}$, contained about 10 fold more label (Table 3). The labelling pattern between $C_7$ and $C_{12}$ has been omitted from Table 3 because overlapping peaks in the NMR spectra, precluded reliable allocation of the correct peak height to the different carbon atoms in the $C_{16:0}$ fatty acid (Gunstone et al., 1976; Gunstone, 1993). The strong labelling of the even numbered positions from $C_1$ to $C_6$ and $C_{13}$ and $C_{16}$, fully agrees with biosynthesis via the pathway of type II fatty acid

biosynthesis (Table 3). Previous work assembling and investigating the genome of the related anammox bacterium *Kuenenia stuttgartiensis* showed that this genome contains the full set of gene products necessary for type II fatty acid biosynthesis (Strous et al., 2006; Rattray et al., *in prep*). Therefore, assuming that the related bacterium "*Candidatus* Brocadia fulgida" also performs type II fatty acid biosynthesis via the regular pathway, these results suggest that 2-$^{13}$C acetate was successfully incorporated into the pathway of type II fatty acid biosynthesis of the anammox bacterium.

*6.3.2. $^{13}$C label incorporation into the iC16:0 fatty acid*

The less generic *i*$C_{16:0}$ fatty acid has been previously reported in all published fatty acid lipid profiles from anammox bacteria (Boumann et al., 2006; Kartal et al., 2007; Kartal et al., 2008; Rattray et al., *submitted*; Sinninghe Damsté et al., 2005) and thus it is likely that it is fully derived from "*Candidatus* Brocadia fulgida". For carbon atoms $C_7$ to $C_{12}$ we could not accurately determine the relative peak enhancement similar to the $C_{16:0}$, because of overlapping peaks in the NMR spectra (Gunstone, 1993). The $^{13}$C labelling pattern of the remaining carbon atoms of the *i*C16:0 fatty acid showed that all carbon atoms were enriched in $^{13}$C about 2 fold, including those not thought to be derived from acetate, i.e. carbon atoms $C_{13}$-$C_{16}$ (Table 4). Carbon atoms at these positions are most likely incorporated via pyruvate, L-valine and isobutyryl-CoA, brought into the pathway of fatty acid biosynthesis for the production of even numbered *iso* fatty acids (Willecke and Pardee, 1971). It is therefore likely that the $^{13}$C label was partly scrambled as acetate was broken down into $CO_2$ in the bioreactor and was subsequently utilised by the anammox bacteria as a carbon source for all biosynthetic pathways. To investigate this hypothesis we performed a separate experiment in which we added labelled acetate to a sequencing batch culture of "*Candidatus* Brocadia fulgida" and monitored the isotopic composition of the dissolved inorganic carbon (DIC). Indeed, it was found that the delta $^{13}$C values of DIC became substantially enriched in $^{13}$C, relative to the standard VPDB (Table 5). During the first seven days of the experiment, the delta $^{13}$C values of the DIC increased by ca. 200%, and further increased in delta $^{13}$C value but by lower amounts, in the subsequent weeks. This $CO_2$ could then be 're-fixed' by "*Candidatus* Brocadia fulgida", as previously reported for this anammox bacterium (Kartal et al., 2008), and thus $^{13}$C label could be incorporated into other biosynthetic pathways

**Table 4.** Relative enhancement of $^{13}C$ in NMR analysis of the $iC_{16:0}$, isolated from the "*Candidatus* Brocadia fulgida" enrichment culture. Carbon atoms expected to be enriched according to the pathway of type II fatty acid biosynthesis are highlighted in bold.

| | | ***i*C$_{16:0}$ fatty acid** | |
|---|---|---|---|
| Carbon No. | δ (ppm) | Relative enhancement of NMR peak height | $CO_2$ Scrambling correction |
| 1' | 51.43 | 1.0 | - |
| 1 | 174.35 | 1.9 | 0.5 |
| **2** | **34.13** | 3.4 | 1.9 |
| 3 | 24.97 | 1.5 | 0.0 |
| **4** | **29.16** | 3.2 | 1.7 |
| 5 | 29.255 | 1.7 | 0.3 |
| **6** | **29.45** | 2.5 | 1.0 |
| 13 | 39.06 | 1.6 | 0.1 |
| 14 | 27.96 | 1.4 | - |
| 15-16 | 22.66 | 1.4 | - |
| **Average** | | 2.1 | |

and cellular components. This finding indicates that some of the $^{13}C$ label in the $C_{16:0}$ also probably derives from $^{13}C$ labelled $CO_2$, however, the amount is not possible to quantify since there are no parts of the molecule which do not derive from the pathway of fatty acid biosynthesis.

**Table 5.** Delta $^{13}C$ (permille) of the dissolved organic carbon (DIC) in the sequencing batch reactor supernatant over time.

| **Day of experiment** | **DIC $\delta^{13}C$ (‰ vs VPDB)** |
|---|---|
| 0 | -25.2 |
| 7 | +206 |
| 10 | +331 |
| 14 | +392 |
| 18 | +352 |

To correct for the scrambling effect by the incorporation of $^{13}C$ labelled $CO_2$, we calculated the mean enhancement of the $C_{13}$-$C_{16}$ NMR peak intensity and

subtracted this value from the enhancement of NMR peak intensity of carbon atoms $C_1$-$C_6$ (Table 4, heading '$CO_2$ scrambling correction'). The resulting corrected relative peak enhancement indicated that $^{13}C$ label derived from the $C_2$ carbon atom of acetate was incorporated at carbon positions with an even number. Only a low amount or no label was incorporated at the odd numbered carbon atoms. This pattern of $^{13}C$ incorporation is in agreement with the labelling pattern expected for an *i*$C_{16:0}$ lipid produced via type II fatty acid biosynthesis. Therefore, our results suggest that $^{13}C$ labelled acetate has been directly incorporated by "*Candidatus* Brocadia fulgida" into $C_{16:0}$ and *i*$C_{16:0}$ fatty acids and yielded $^{13}C$ labelling patterns in accordance to the expected pathways of type II fatty acid biosynthesis.

### *6.3.3. $^{13}C$ label incorporation into the $C_{20}$[3] ladderane monoether*

As observed with the *i*$C_{16:0}$ fatty acid, all carbon atoms show a relative peak enhancement in the $C_{20}$[3] ladderane monoether, suggesting that some $^{13}C$ label scrambling had occurred (Table 6). To correct for the $CO_2$ scrambling, the mean relative peak enhancement of the glycerol head group ($C_{1'}$, $C_{2'}$, $C_{3'}$) was calculated, since carbon atoms in the glycerol backbone are derived from glycerol and not from acetate (Hsu and Fox, 1970). We then subtracted this value from the relative peak enhancement of the $C_1$-$C_{20}$ carbon atoms in the ladderane moiety (Table 6, heading '$CO_2$ scrambling correction'). In table 6 some peaks have been omitted due to partially overlapping peaks.

The resulting corrected relative enhancement of peak intensity in the ladderane monoether indicated that carbon atoms $C_{10}$, $C_{12}$ to $C_{14}$, $C_{16}$ to $C_{18}$ and $C_{20}$ were labelled with $^{13}C$ (Table 6). The absence of labelling at positions $C_1$, $C_3$, $C_7$, $C_9$ and $C_{11}$ would be in agreement with the labelling pattern expected from the pathway of type II fatty acid biosynthesis. However, the absence of label in $C_8$ does not fit this expected pattern. Assigned peaks $C_{10}$, $C_{12}$ to $C_{14}$, $C_{16}$ to $C_{18}$ and $C_{20}$ of the $C_{20}$[3] ladderane monoether moiety were all more or less uniformly labelled. However, it was striking that these carbon atoms all contained a much lower enhancement of NMR peak height in comparison to carbon atoms in either the $C_{16:0}$ or the sections of the *i*$C_{16:0}$ synthesised via type II fatty acid biosynthesis. Instead, the cyclobutane rings of the ladderane lipid give similar values of NMR peak height enhancement to the

**Table 6.** Relative enhancement of $^{13}C$ in NMR analysis and the scrambling correction for the $C_{20}$[3] ladderane monoether. For the scrambling correction >0 = $^{13}C$ incorporation at position, <0 = no $^{13}C$ incorporation.

| | | **$C_{20}$[3] ladderane monoether** | |
|---|---|---|---|
| Carbon No. | δ (ppm) | Relative enhancement of NMR peak height | $CO_2$ Scrambling correction |
| 1 | 70.70 | 1.4 | -0.2 |
| 2 | 29.90 | n.a | - |
| 3 | 25.97 | 1.3 | -0.3 |
| 4 | 29.92 | n.a. | - |
| 5 | 29.64 | n.a. | - |
| 6 | 26.87 | n.a. | - |
| 7 | 29.42 | 1.5 | -0.1 |
| 8 | 38.14 | 1.5 | -0.1 |
| 9 | 32.48 | 1.4 | -0.2 |
| 10 | 34.22 | 1.7 | 0.1 |
| 11 | 37.89 | 1.6 | -0.1 |
| 12 | 49.39 | 1.9 | 0.3 |
| 13 | 41.54 | 1.8 | 0.2 |
| 14 | 26.17 | 1.8 | 0.1 |
| 15 | 25.49 | n.a. | - |
| 16 | 42.25 | 2.0 | 0.3 |
| 17 | 47.33 | 2.0 | 0.4 |
| 18 | 37.67 | 2.1 | 0.4 |
| 19 | 25.50 | n.a. | - |
| 20 | 28.19 | 1.8 | 0.1 |
| 1' | 35.35 | 1.6 | - |
| 2' | 75.03 | 1.6 | - |
| 3' | 35.35 | 1.6 | - |
| C=O | 170.77 | 0.9 | - |
| COCH3 | 20.83 | 1.0 | - |
| **Mean** | | 1.7 | |

n.a. not applicable due to overlapping peak areas.

*iso* group of the $iC_{16:0}$ and therefore appear to be synthesised via a pathway of biosynthesis which is not directly incorporating acetate. Unfortunately, the rather non-specific labelling of the cyclohexane and cyclobutane rings does not allow us to speculate on what the exact biosynthetic route of ladderane lipids is. The pattern of non-labelling in the acyclic part of the ladderane moiety and the non-specific labelling in the ring structures suggest that they originated as two separate units which were perhaps synthesised in different biosynthetic pathways and assembled together at a later stage.

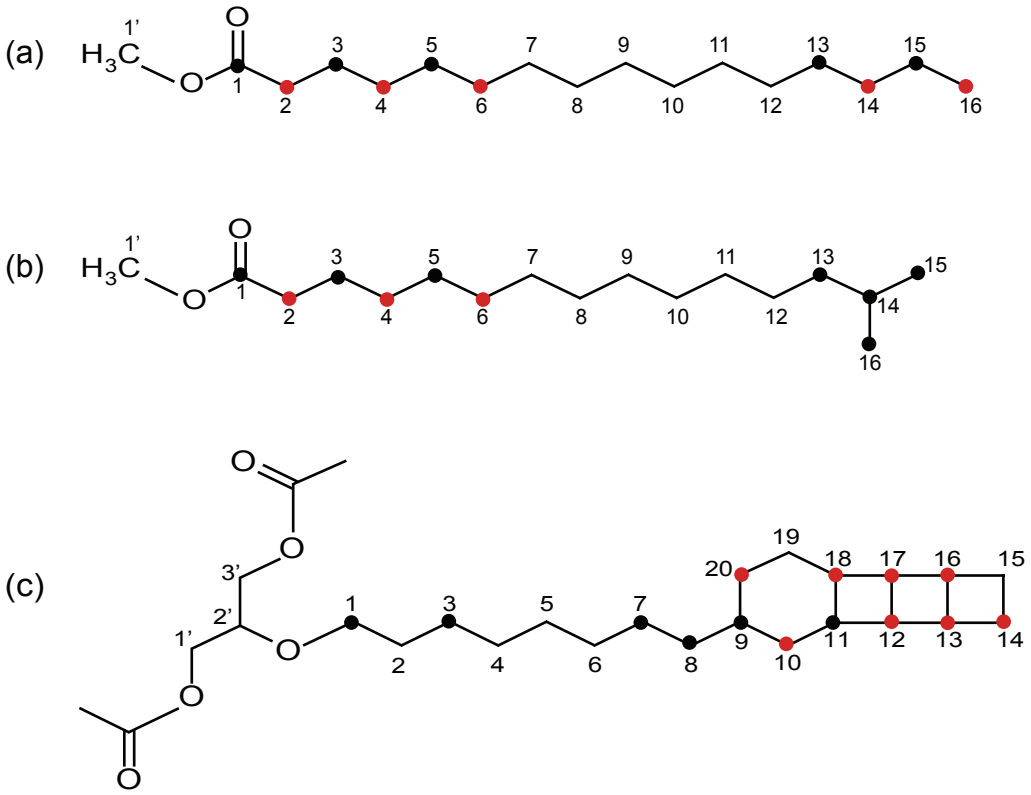


**Figure 2.** Pattern of [2-$^{13}$C] incorporation in the (a) $C_{16:0}$ fatty acid (b) $iC_{16:0}$ fatty acid (after correction for $CO_2$ scrambling) and the (c) $C_{20}$[3] ladderane monoether (after correction for $CO_2$ scrambling). Red dots indicate label has been incorporated at that position and black dots indicate no incorporation. No dot indicates that peaks overlapped on the NMR spectra and incorporation could not be assigned to a specific carbon atom.

Therefore, it does not seem that the original precursor for the ladderane moiety was a polyunsaturated fatty acid. However, it has been observed that the genome of the related anammox bacterium *Kuenenia stuttgartiensis* contained gene products capable of encoding multiple, unusual paralogues of the enzyme FabB/F, which is capable of bringing compounds into the elongation cycle of fatty acid biosynthesis (Rattray et al. in prep). Therefore, it is possible that this anammox bacterium can feed an unusual and unknown compound into the pathway of type II fatty acid biosynthesis. However, more experimental research using other types of labelled substrates is now needed, to resolve the biosynthesis of the ladderane moiety itself.

**Acknowledgments**

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# Part II
# environmental application of ladderane lipids as indicators for anammox bacteria

# chapter 7

## Anaerobic ammonium oxidation in the Peruvian oxygen minimum zone

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**Abstract**

We investigated the microbial pathways of nitrogen (N) loss in an April 2005 transect through the Peruvian oxygen minimum zone (OMZ) at 12° S latitude using short anaerobic incubations with $^{15}N$-labeled substrates, and molecular-ecological and lipid-biomarker studies. In incubations with $^{15}NH_4^+$, immediate production of $^{14}N^{15}N$, but not $^{15}N^{15}N$, indicated that $N_2$ was produced by the pairing of labeled $^{15}NH_4^+$ with *in situ* $^{14}NO_2^-$ via anaerobic ammonium oxidation (anammox). Supporting this finding, we also found anammox-related 16S ribosomal ribonucleic acid (rRNA) gene sequences similar to those previously known from other marine water columns where anammox activity was measured. We identified and enumerated anammox bacteria via fluorescence *in situ* hybridization (FISH) and quantitative polymerase chain reaction (qPCR), and found ladderane membrane lipids specific to anammox bacteria, wherever anammox activity was measured by our isotope tracer method. However, in incubations with $^{15}NO_3^-$ or $^{15}NO_2^-$, where denitrification would have been expected to produce $^{15}N^{15}N$ by pairing of oxidized $^{15}N$ ions, $^{15}N^{15}N$ production was not detected before 24 h, showing that denitrification of fixed N to $N_2$ was not taking

place in our samples. At the time and locality of our study, anammox, rather than denitrification, was responsible for $N_2$ production in the Peruvian OMZ waters.

## 7.1 Introduction

The Peruvian upwelling system in the eastern tropical South Pacific Ocean (ETSP) is an area of high productivity that generates one of the ocean's largest masses of suboxic water. Longshore geostrophic winds cause the Ekman transport of the Subtropical Surface Water (ca. >25 m deep) westward, bringing subsurface waters to the euphotic zone (Friederich and Codispoti 1987; Copin-Montégut and Raimbault 1994). The upwelled water has its source in the Equatorial Undercurrent, a poleward current of hypoxic, nutrient-enriched Equatorial Subsurface Water. The upwelling of this water into the euphotic zone supports high primary productivity, and decomposition of this phytoplankton biomass further depletes dissolved oxygen, resulting in the development of an OMZ that extends from the thermocline – as little as 20 m below the surface – to the shelf sediments, and westward over the continental slope and abyssal plain (Fig. 1A).

Decomposing organic matter should release dissolved nutrients in the same proportion as that of the originating material. A deficit in the ratio of regenerated inorganic nitrogen ($NH_4^+$, $NO_3^-$, and $NO_2^-$) to phosphate relative to the N:P ratio of mixed-layer organic matter (the 'N-deficit') has been attributed to a loss of fixed nitrogen as $N_2$ within this and other oceanic OMZs (Codispoti and Packard 1980; Deutsch et al. 2001). Such calculations have indicated that 30 to 50% of the total oceanic nitrogen loss may occur in OMZs (Codispoti et al. 2001). Until recently, the only known process that could explain this loss was denitrification, the reduction of nitrate to $N_2$ gas via $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$ by heterotrophic bacteria (Codispoti and Christensen 1985). However, direct measurements of the reduction of $NO_3^-$ to $N_2$ in oceanic water columns have been lacking. Denitrification has been inferred from the distribution of water-column nitrite, thought to accumulate as a result of incomplete nitrate reduction (Codispoti and Packard, 1980; Codispoti and Christensen 1985), and from the $^{15}N$ enrichment of residual nitrate by the isotopic discrimination associated with nitrate reduction (Brandes et al. 1998).

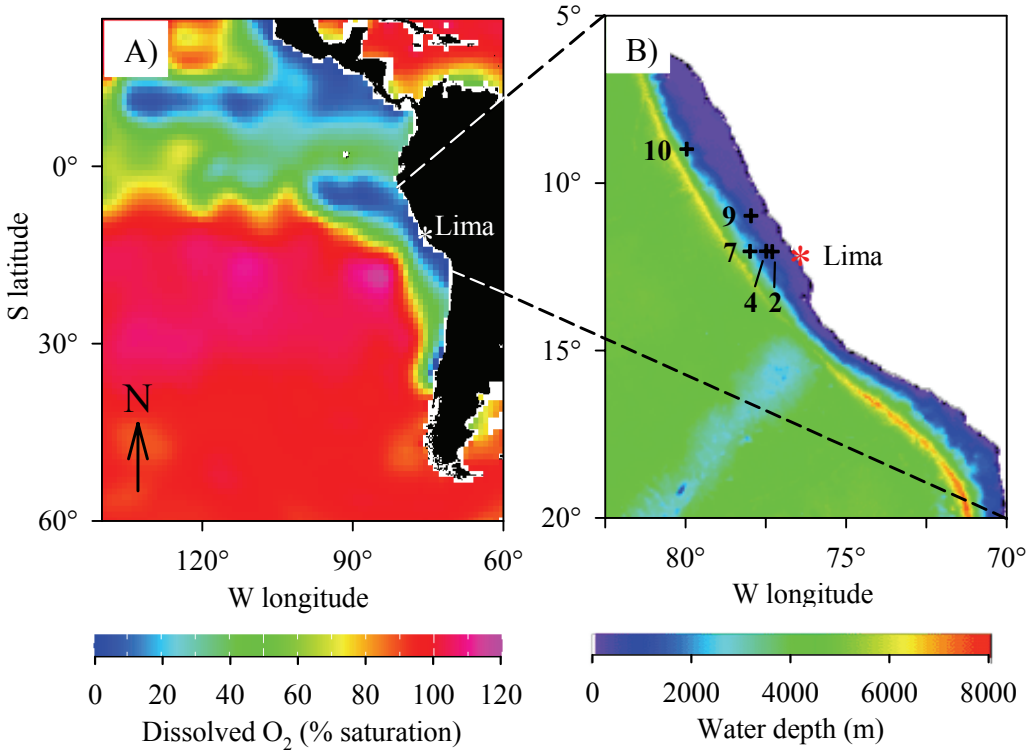


**Figure 1.** (A) Dissolved oxygen concentrations (% saturation) in the equatorial Pacific at 100 m depth (image source: http://iridl.ldeo.columbia.edu/SOURCES/.NOAA/.NODC/.WOA01/). (B).Bathymetry of Peruvian shelf and location of sampling stations. See Table 1 for station reference data. Sta. 2, 4, and 7 of transect at 12° S are shown; Sta. 1, 3, 5, and 6 (not sampled for anammox) are not shown (image source: http://topex.ucsd.edu/marine_topo/).

The activity of the electron transport system (ETS) in cell-free extracts, a measure of the reductive potential of the water column cellular machinery, has been assumed (in suboxic waters) to indicate denitrification potential, and denitrification rates have been estimated from ETS activity using theoretical and empirical correction factors (Codispoti and Packard 1980). Denitrification has also been inferred from the spatial gradients in N-deficits, with advection and diffusion calculated from reference and literature values (Codispoti and Packard 1980), or from chlorofluorocarbon-derived water-mass ages (Deutsch et al. 2001). Other, incubation-based, measures of nitrogen metabolism have not directly addressed $N_2$ production. Measurements of $N_2O$ production under acetylene amendment have given contradictory results and their interpretation may be uncertain because of the multiple pathways of $N_2O$ production and consumption and the artifacts associated with the acetylene technique. Other measures have included nitrite production from nitrate in cell-free

extracts (Packard et al. 1978), and isotopic tracer measurements of the reduction of nitrate to nitrite (Lipschultz et al. 1990). However, none of these approaches have directly measured the loss of fixed inorganic nitrogen as $N_2$ gas, nor confirmed the mechanism of $N_2$ production with metabolic or microbiological evidence.

Recently, a novel microbial process known as anammox (anaerobic ammonium oxidation) has been show to be a significant contributor to nitrogen losses in marine and estuarine sediments (Thamdrup and Dalsgaard 2002; Trimmer et al. 2003; Meyer et al. 2005), in sea ice (Rysgaard and Glud 2004), and in the anoxic waters of basins isolated from oxygenated deep circulation (Dalsgaard et al. 2003; Kuypers et al. 2003). Anammox is a chemolithotrophic process whereby one mole of ammonium is oxidized with one mole of nitrite in the absence of oxygen to produce $N_2$ gas (Srous et al. 1999):

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \qquad (1)$$

The potential for such a pathway in the ocean was postulated by Richards et al. (1965), but was first observed in wastewater (Mulder et al. 1995), and shown to be performed by hitherto-unknown members of the bacterial Order *Planctomycetales* (Strous et al. 1999). $N_2$ generated through anammox can be distinguished from that generated by denitrification by the pattern of $N_2$-labeling observed in anaerobic incubations with $^{15}NH_4^+$, $^{15}NO_2^-$, or $^{15}NO_3^-$. The technique of isotope pairing can distinguish anammox from denitrification through the measurement of the relative production of the differently-labeled $N_2$ isotopologues ($^{14}N^{14}N$, $^{14}N^{15}N$, and $^{15}N^{15}N$) (Nielsen 1992; Thamdrup and Dalsgaard 2002; Risgaard-Petersen et al. 2003). Since this discovery, anammox has been shown to play a significant role in many marine environments (reviewed in: Dalsgaard et al. 2005; Kuypers et al. 2006). Further, anammox has been shown to be responsible for large N losses from the OMZ waters of the dynamic Benguela upwelling system off the coast of Namibia, whereas no evidence for heterotrophic denitrification was found (Kuypers et al. 2005). This discovery led to the hypothesis that anammox might be important in other OMZs such as the Peruvian. The aim of this study was to investigate the role of these two microbial $N_2$ loss processes – denitrification and/or anammox – in nitrogen loss from Peruvian OMZ waters.

## 7.2. Methods

### *7.2.1 Study site*

Water column profiles of physico-chemical parameters and water samples for nutrient analyses, $^{15}N$ incubations, and anammox indicators, were collected during a cruise of the IMARPE R/V José Olaya off the coast of Peru in April 2005. Sampling stations were located along a transect at 12° S latitude near Callao, ranging from nearshore waters (47 m water depth) to the continental slope (ca. 2400 m depth) (Table 1, Fig. 1B). In addition, two shelf stations were sampled farther north (Sta. 9 and 10 (respectively, 11° and 9° S latitude: Ocean Drilling Project Sta. 1228 and 1229); Fig. 1B). All the investigated stations are within the region of intense seasonal upwelling, primary production, and water column anoxia that form the extensive OMZ off the coast of Peru and northern Chile (Fig. 1A; Codispoti and Packard 1980).

**Table 1.** Characteristics of study stations within the Peruvian upwelling system (see Fig. 1B).

| Station | Latitude ° S | Longitude ° W | Water depth (m) | Sampling date(s) |
|---|---|---|---|---|
| 2 | 12.05 | 77.30 | 96 | 15 Apr 2005<br>21 Apr 2005 |
| 4 | 12.03 | 77.49 | 141 | 17 Apr 2005 |
| 7 | 12.04 | 77.99 | 2400 | 22 Apr 2005 |
| 9 | 10.96 | 77.96 | 152 | 20 Apr 2005 |
| 10 | 8.99 | 79.96 | 442 | 19 Apr 2005 |

### *7.2.2 Water column profiles*

We obtained profiles of salinity, temperature, oxygen, and chlorophyll *a* (Chl *a*) fluorescence with a conductivity, temperature, and depth (CTD) system equipped with an oxygen electrode and a fluorometer (Sea-Bird Electronics, Bellevue, USA). Water samples for high-resolution nutrient analyses and $^{15}N$ incubations were obtained by a pump-CTD (Kuypers et al. 2003) or by GoFlow bottles (Hydrobios, Kiel-Holtenau, Germany). Nitrite was measured immediately on board (detection limit 0.03 µmol $L^{-1}$), and water samples were frozen (-20° C) for later analysis of nitrate, ammonium, and phosphate by autoanalysis (TRAACS 800, Bran & Lubbe, Hamburg, Germany; detection limits 0.1, 0.3, and 0.1 µmol $L^{-1}$, respectively) (Grasshoff 1999). The fixed N deficit was determined by the formula:

$$N\ deficit = 12.6 \times [PO_4^{3-}] - ([NO_3^-] + [NO_2^-] + [NH_4^+]) \quad (2)$$

where the constant 12.6 is the empirically-determined N:P ratio of organic matter produced in these waters (Codispoti and Packard 1980).

### 7.2.3 *Long-term temperature and $O_2$ monitoring*

A time-series (1996 – 2005) of vertical profiles of temperature and oxygen at Sta. 2 has been constructed to show interannual variability (Gutiérrez et al. in press). Water samples were collected monthly with Niskin bottles. Temperature was measured by inversion thermometer through 2001 and by CTD from 2002 onwards. Dissolved oxygen was determined by the Winkler method (Grasshoff 1999).

### 7.2.4 *$^{15}N$ incubations and analysis*

$^{15}N$ labeling experiments were performed at Sta. 2, 4, 7, 9, and 10 (Fig. 1B). Water was collected across the suboxic zone at 4 to 7 depths per station, with one further sample collected from within the oxycline (ca. 25 to 50 µmol $L^{-1}$ $O_2$) as a control. The $^{15}N$ incubations were begun immediately after sampling. The experimental method was slightly modified from that previously published by Dalsgaard et al. (2003). Briefly, 250 mL of Peruvian OMZ waters collected by pump-CTD were flushed with He for 15 min and amended with $^{15}NH_4^+$, $^{15}NH_4^+$ + $^{14}NO_2^-$, or $^{15}NO_2^-$ (to 5 µmol $L^{-1}$ each), or with $^{15}NO_3^-$ (20 µmol $L^{-1}$) (isotopes: Campro Scientific, Berlin, Germany). Water from above the suboxic zone was equilibrated with 20% v/v air to produce a final $O_2$ concentration of ca. 50 µmol $L^{-1}$ (similar to in situ values). Labeled seawater was transferred into 12-mL Exetainers (Labco, High Wycombe, U.K.) and incubated for up to 24 h in the dark at in situ temperatures. The Exetainers were destructively sampled at 0, 6, 12, and 24 h with $HgCl_2$ to stop biological activity. $^{14}N^{15}N$:$^{14}N^{14}N$ and $^{15}N^{15}N$:$^{14}N^{14}N$ ratios of He-equilibrated headspaces were determined by gas chromatography/isotope ratio mass spectrometry (Fisons VG Optima, UK).

### 7.2.5 *Lipid biomarkers*

Evidence for the presence of anammox bacteria was sought using anammox-specific ladderane membrane lipids (Sinninghe Damsté et al. 2002). Particulate matter was collected using in situ pumps (McLane WTS-LV Sampler, East Falmouth, USA) to filter large volumes of water (ca. 500 L per sample) through precombusted glass-fiber filters (GF/F, nominal pore size: 0.7 µm, Whatman, Maidstone, UK). The filters were freeze-dried and extracted with 3× methanol, 3×

methanol:dichloromethane (1:1 v/v), and 3× dichloromethane to obtain total lipid extracts. Aliquots of the lipid extracts were saponified and subsequently methylated with diazomethane. Samples were analyzed by high-performance liquid chromatography/mass spectrometry as described elsewhere (Hopmans et al. 2006). Lipid quantification was performed using external authentic ladderane standards, isolated from anammox enrichment cultures. The reproducibility of the HPLC/MS ladderane concentration measurements was >95% (determined by triplicate analysis). Since GF/F filters, with a pore size of 0.7 µm, may undersample anammox cells, the calculated ladderane-lipid concentrations represent minimum values.

### *7.2.6 DNA extraction and phylogenetic analysis*

For deoxyribonucleic acid (DNA) extraction, seawater samples (200 to 400 mL) were filtered onto polycarbonate GTTP membrane filters (47 mm diam., 0.22 µm pore size, Millipore, Eschborn, Germany) and stored at -20° C. DNA extraction and PCR amplification targeting Planctomycete 16S rRNA genes were as described in Schmid et al. (2000), except that 1392R (Stahl et al. 1988) was used as the reverse primer. The anammox-related 16S rRNA sequences obtained in this study were deposited in GenBank (accession numbers DQ534720-DQ534744).

### *7.2.7 FISH and total microbial abundance*

Water samples were fixed with particle-free paraformaldehyde solution (final concentration, 1% v/v) for 1 h at room temperature, or overnight at 4° C. Fixed samples were filtered onto white polycarbonate membrane filters (described above) and stored at -20° C until analysis. Filter material was hybridized (Glöckner et al. 1996) with fluorescently-labeled oligonucleotide probes (biomers.net, Ulm, Germany) specific for anammox bacteria. For the detection of the entire Candidatus *Scalindua wagneri* and *Scalindua sorokinii* clade a mixture of two probes were used: probe BS-820 (5'-TAA TTC CCT CTA CTT AGT GCC C-3') (Kuypers et al. 2003), and a newly-developed probe BS-820-C (5'-TAA TCC CCT CTA CTT AGT GCC C-3'). We used the unlabeled probe AMX-820 (5'-AAA ACC CCT CTA CTT AGT GCC C-3') (Schmid et al. 2000) as a competitor. Filters were also stained non-specifically for DNA with 4,6-diamidino-2-phenylindole (DAPI), and the relative abundance of anammox cells was determined by epifluorescence microscopy.

Total microbial abundance was determined by flow cytometry calibrated with manual microscopic counting of DAPI-stained cells.

*7.2.8 Quantitative PCR (qPCR)*

Anammox bacteria were also quantified with the TaqMan fluorogenic PCR method (Livak et al. 1995), using DNA extracted from polycarbonate membrane filters (described above). A primer and fluorogenic probe set was designed for all known anammox or anammox-like bacteria using the Primer Express v.2.0 software (Applied Biosystems, Darmstadt, Germany), based on sequences retrieved from the GenBank and from our own environmental sequence databases. The resulting primer set: AMX-808-F (5′-ARC YGT AAA CGA TGG GCA CTA A-3′) and AMX-1040-R (5′-CAG CCA TGC AAC ACC TGT RAT A-3′), and the probe AMX-931 (5′-TCG CAC AAG CGG TGG AGC ATG TGG CTT A-3′) had melting temperatures (58°, 57°, and 67° C respectively) within the recommended range for the TaqMan system, and the specificity of their sequences was verified with the ARB software package, BLAST (Altschul et al. 1990), and the Probe Match tool from the Ribosomal Database Project II (Cole et al. 2005). qPCR was performed on a iQ5 Real-Time PCR System (Bio-Rad Laboratories, Hercules, USA) for 2 min at 50° C and 10 min at 95° C, followed by 50 cycles of 15 s each at 95° C and 2 min at 60° C. DNAs extracted from various anammox enrichment cultures were used as positive controls and standards. All samples and standards were analyzed in triplicate. The resulting anammox cell numbers were calibrated for absolute quantity against FISH counts (qPCR = 0.0307 (FISH) +2100; $r^2 = 0.7$, $p < 0.01$).

## 7.3 Results

*7.3.1 Site characterization*

The Peruvian shelf waters along our vertical transect at 12° S latitude were thermally stratified in mid-April 2005 with a pronounced OMZ extending from ca. 30 m water depth to the shelf sediments (Figs. 2A, 3). Within the OMZ, $O_2$ concentrations were typically less than 10 µmol $L^{-1}$ (Fig. 2A). N deficits in the OMZ waters were largest near the coast and in shelf bottom waters, ranging from <5 µmol N $L^{-1}$ over the slope to >15 µmol N $L^{-1}$ near the shore (Fig. 2B), while Chl *a* concentrations in the mixed layer of the euphotic zone ranged up to 1.0 µg $L^{-1}$ (Fig. 2C). Long-term (10 yr) temperature and $O_2$ monitoring of the water column at Sta. 2 shows that thermal stratification

and subsurface hypoxia are common conditions, and the oxycline depth typically fluctuates between 10 and 50 m (Fig. 3). Instances of complete water column mixing and oxygenation were related to El Niño-Southern Oscillation (ENSO) temperature anomalies, as during the El Niño of 1997 to 1998 (red bars on Fig. 3 indicate ENSO warm periods), which otherwise were generally accompanied by a deepening of the oxycline. Cold-temperature anomalies, as during the La Niña of 1998 to 1999 (blue bars on Fig. 3 indicate ENSO cold periods) were generally associated with shoaling of the oxycline and a water column dominated by suboxic conditions.

### *7.3.2 Physico-chemical structure of the water column*

During our cruise over the Peruvian shelf in April 2005, nitrate concentrations within the OMZ waters ranged from ca. 10 to 30 µmol $L^{-1}$, and nitrite concentrations ranged from ca. 0.2 to 5.0 µmol $L^{-1}$ (Fig. 4, first column). Nitrate and nitrite concentrations were much lower in waters of the mixed layer. Two nitrite maxima were observed at all stations, one just below the oxycline, and a second near the sediments, or centered at ca. 300 m over the slope at Sta. 7. Ammonium concentrations were typically <1 µmol $L^{-1}$, though maxima (up to 3 µmol $L^{-1}$) were found at the base of the oxycline at all stations. Nutrient, oxygen, and temperature-compensated density ($\sigma_T$) profiles taken 6 d apart at Sta. 2 showed the dynamic physico-chemical structure of the water column, which is also apparent from the long-term monitoring record (Fig. 3).

By 21 April, Sta. 2 waters had stratified, forming a defined and deeper mixed layer nearly 30 m thick (Fig. 4B), possibly following reduced wind stress. The $O_2$ concentrations in the surface waters at this station on both dates were low (<50% saturation), indicating the upwelling of low-oxygen waters and high respiration rates. Although surface water $O_2$ concentrations at Sta. 4 and 7 – farther offshore – were near saturation, the oxycline depth was similar to that at Sta. 2, with steep oxygen gradients near the surface, and extensive water-column suboxia. Steep density gradients of up to 0.033 kg $m^{-4}$ maintained the oxyclines at Sta. 4 and 7 (Fig. 4, second column).

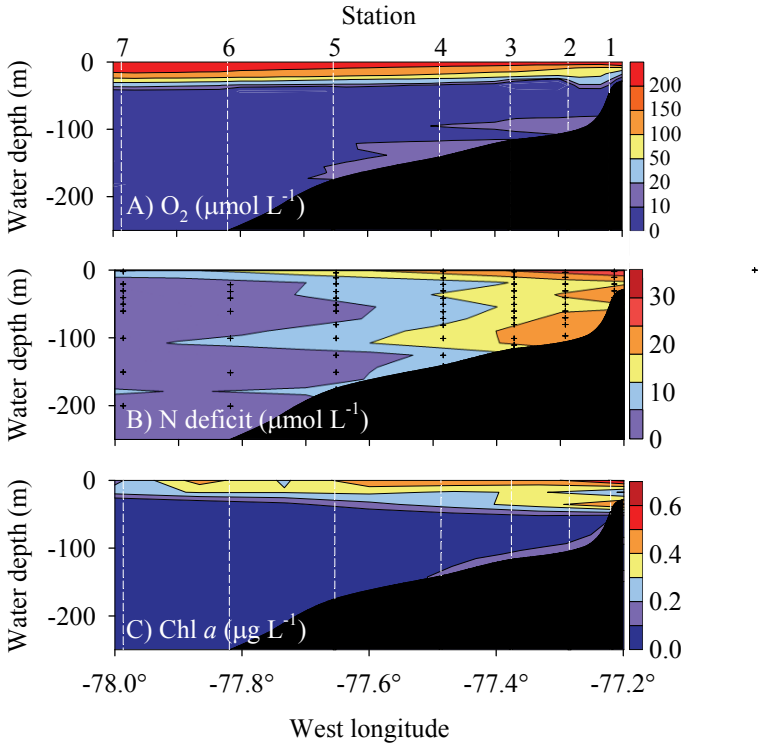


**Figure 2.** Depth profiles along 12° S. Station numbers are those of Table 1 and Fig. 1B, with 4 additional stations interposed along the same latitude. Dashed lines in panels A and C represent locations of continuous profiles. Crosses in panel B represent locations of GoFlow bottle sample collection. (A) Dissolved $O_2$. (B) N deficit = 12.6 x $[PO_4^{3-}]$ – $[NH_4^+ + NO_2^- + NO_3^-]$. (C) Chlorophyll *a* (fluorescence).

### *7.3.3 $^{15}$N-labeling incubations*

Significant $^{14}N^{15}N$ production was detected in 48 out of 60 OMZ water-sample incubations, i.e. with $^{15}NH_4^+$, $^{15}NH_4^+$ + $^{14}NO_2^-$, $^{15}NO_2^-$, or $^{15}NO_3^-$ amendments (average detection limit (including sample handling) for $^{14}N^{15}N$ = 1.1 nmol $L^{-1}$) (Fig. 4, third column). $^{14}N^{15}N$ was produced at every location and depth within the OMZ from at least one of the isotope treatments. The production of $^{14}N^{15}N$ in these incubations was linear with time ($r^2 \geq 0.7$, e.g. Fig. 5), although with $^{15}NO_3^-$, it was sometimes delayed (e.g. Fig. 5D). Of the incubations where $^{14}N^{15}N$ production was not detected, half (6 out of 12) were incubations with $^{15}NO_3^-$. $^{14}N^{15}N$ production in anaerobic incubations with $^{15}NH_4^+$ ranged from 1.5 ± 0.1 nmol $L^{-1}$ $d^{-1}$ at 60 m at Sta. 2 (21 April; Fig. 4B) to a maximum of 384 ± 11 nmol $L^{-1}$ $d^{-1}$ at Sta. 2 (15 April; Fig. 4A). At two stations (Sta. 2 (15 April) and 7), $^{14}N^{15}N$ production rates with $^{15}NH_4^+$ were significantly enhanced by the addition of $^{14}NO_2^-$. $^{14}N^{15}N$ production from $^{15}NO_2^-$

ranged from 0 nmol $L^{-1}$ $d^{-1}$ at two depths at Sta. 7 to 48 ± 4 nmol $L^{-1}$ $d^{-1}$ at Sta. 4. Significantly lower rates of $^{14}N^{15}N$ production (maximum 27 nmol $L^{-1}$ $d^{-1}$) were measured in incubations with $^{15}NO_3^-$. In contrast to $^{14}N^{15}N$, the production of $^{15}N^{15}N$ was undetectable in all 6-h and 12-h incubations (average detection limit (including sample handling) = 0.9 nmol $L^{-1}$). $^{15}N^{15}N$ was detected only in 6 out of 60 of the 24-h incubations, and only from the $^{15}NO_2^-$ or $^{15}NO_3^-$ substrate. Both $^{14}N^{15}N$ and $^{15}N^{15}N$ production were undetectable in aerobic incubations with water from the mixed layer.

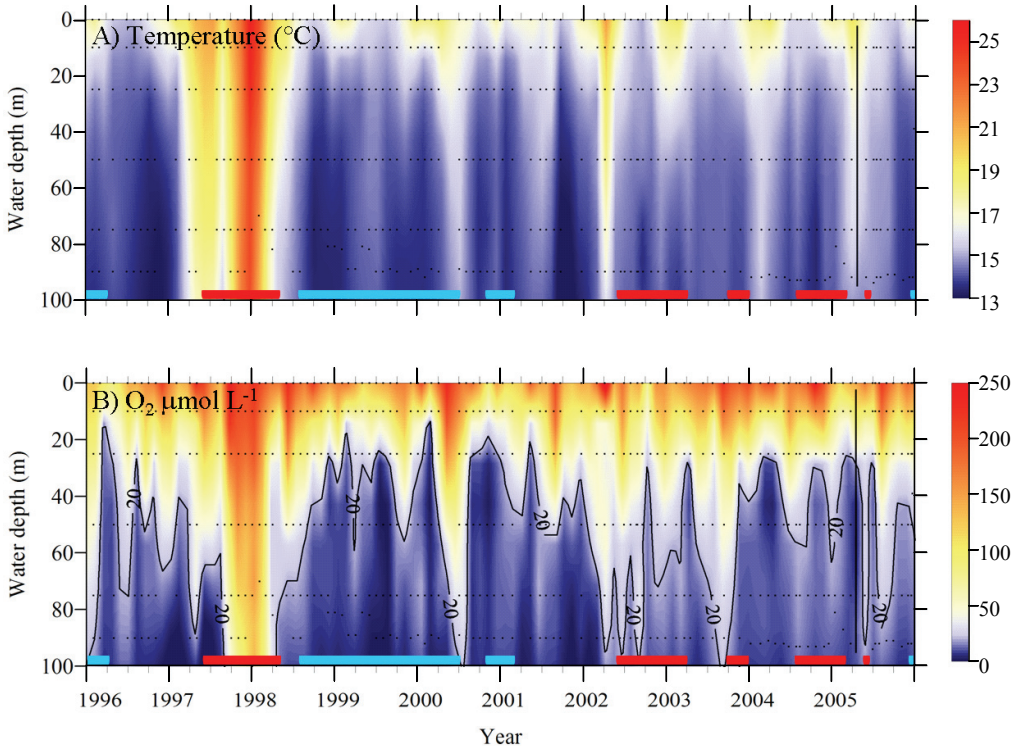


**Figure 3.** Time-series of (A) temperature (° C) and (B) $O_2$ (µmol $L^{-1}$) profiles at Sta. 2 (Fig. 1B) (Gutiérrez et al. in press). Vertical line on (A) represents our study date (Apr 2005). Contour line on (B) indicates the 20 µmol $O_2$ $L^{-1}$ isopleth. Temperature anomalies are the Oceanic Niño Index, and are shown as bars near the x-axis in red (>0.5° C: El Niño/Southern Oscillation (ENSO) warm periods) and light blue (<-0.5° C: ENSO cold periods). Note correspondence between ENSO events and water column oxygenation.
(http://www.cpc.ncep.noaa.gov/products/analysis_monitoring/ensostuff/ensoyears.shtml)

*7.3.4 Abundance, phylogeny and lipid biomarkers of anammox bacteria*

Anammox bacteria were enumerated by FISH with anammox-specific 16S rRNA oligonucleotide probes and by qPCR. Anammox bacteria were found at all depths where $^{14}N^{15}N$ production was observed (Fig. 4). Their abundance in the OMZ ranged up to a maximum of 13 $\times 10^4$ to 15 $\times 10^4$ $mL^{-1}$ at Sta. 2 (15 April). Anammox cells represented on average 2.2 to 3.1% of the total microbial abundance at Sta. 2, but only 0.8% at Sta. 4 (Table 2). Phylogenetic analysis of the Planctomycete-specific 16S rDNA clone library detected sequences with 98% sequence idenitity as the known anammox bacteria Candidatus "*Scalindua sorokinii*" found in the Black Sea and the Benguela upwelling (Kuypers et al. 2003; 2005).

Particulate matter collected from the OMZ waters was also examined for the presence of ladderanes: membrane lipids specific to anammox bacteria (Table 2, Fig. 4, fourth column). No ladderanes were found in the mixed layer, but they were found at all depths where $^{14}N^{15}N$ production was observed, at concentrations ranging up to 58 ng $L^{-1}$. The differing depth distributions of anammox bacteria numbers and ladderane lipid concentrations at some stations, may have resulted from the >12-h difference in sampling times, but when averaged over the whole water column, mean anammox bacteria numbers at each site were highly correlated with mean ladderane lipid concentrations ($r$ = 0.96).

**Table 2.** Anammox indicators and activities in the Peruvian upwelling OMZ (station locations: Fig. 1B). All values are arithmetic means of OMZ measurements except the depth-integrated potential anammox rate [a]. The number of observations for each mean is as in Fig. 4. Errors are standard errors. nd = no data.

| Station | Anammox cell numbers [b] ($\times 10^4$ mL$^{-1}$) | Anammox cells (% of total cells) | Ladderane lipids (ng L$^{-1}$) | Depth-integrated anammox rate [a] (mmol N m$^{-2}$ d$^{-1}$) | Cell-specific anammox rate (fmol cell$^{-1}$ d$^{-1}$) |
|---|---|---|---|---|---|
| 2 (15 April) | 10.1 ± 1.5 | 3.1 ± 0.1 | 36 ± 11 | 11.5 | 1.7 |
| 2 (21 April) | 5.1 ± 1.4 | 2.2 ± 0.4 | 45 (at 92 m) | 1.3 | 0.4 |
| 4 | 2.2 ± 0.8 | 0.8 ± 0.2 | 12 ± 4 | 6.2 | 2.4 |
| 7 | 3.9 ± 1.4 | nd | 8 ± 4 | 34.0 | 1.9 |
| 9 | nd | nd | 16 ± 14 | 8.8 | nd |
| 10 | 0.21 ± 0.05 | nd | 4 ± 2 | 1.5 | 1.9 |

[a] This is a potential rate calculated from the non-substrate-limited $^{15}NH_4^+ + {}^{14}NO_2^-$ treatment (Fig. 4). Rate measurements were depth-integrated by summing the product of the averages of two adjacent rate measurements and the depth interval between them. At Sta. 7, where anammox activity was only determined in the upper 400 m of the 2400 m water column, potential anammox rates were arbitrarily set to decrease to 0 at 600 m.

[b] Anammox cell numbers for Sta. 2 and 4 determined by FISH; Sta. 7 and 10 determined by qPCR.

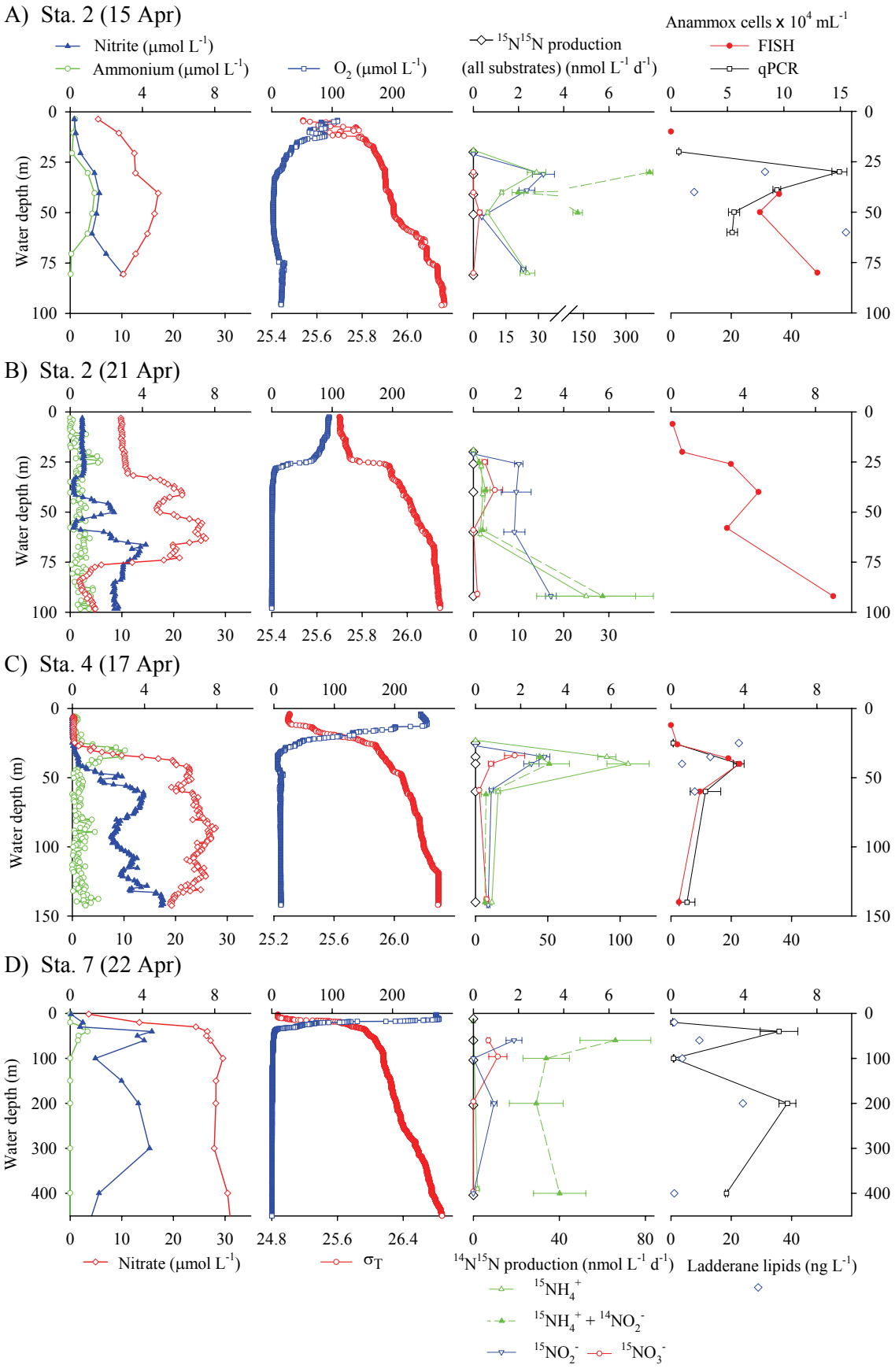
A) Sta. 2 (15 Apr)
B) Sta. 2 (21 Apr)
C) Sta. 4 (17 Apr)
D) Sta. 7 (22 Apr)
Nitrite (µmol L-1)
Ammonium (µmol L-1)
O2 (µmol L-1)
15N15N production (all substrates) (nmol L-1 d-1)
Anammox cells x 10^4 mL-1
FISH
qPCR
Water depth (m)
Nitrate (µmol L-1)
σT
14N15N production (nmol L-1 d-1)
15NH4+
15NH4+ + 14NO2-
15NO2- 15NO3-
Ladderane lipids (ng L-1)

**Figure 4.** Chemical zonation and distribution of anammox indicators (see Fig. 1B for station locations). (A) Sta. 2 (15 Apr 2005). (B) Sta. 2 (21 Apr 2005). (C) Sta. 4. (D) Sta. 7. The first column of figures shows the depth distribution of ammonium and nitrite (top axis), and nitrate (bottom nitrate), with water samples collected either by high-resolution pumpcast (B and C) or by GoFlow bottle water collection (A and D). The second column shows profiles of dissolved $O_2$ (top axis) and $\sigma_T$ (bottom axis - note different y-axis scales). The third column shows $^{15}N^{15}N$ (top axis) and $^{14}N^{15}N$ (bottom axis - note different y-axis scales) production in incubations with four combinations of isotopically-enriched DIN species. The fourth column shows the abundance of anammox cells as determined by FISH and qPCR (top axis - note different y-axis scales), and the concentration of anammox-specific ladderane membrane lipid biomarkers (bottom axis).

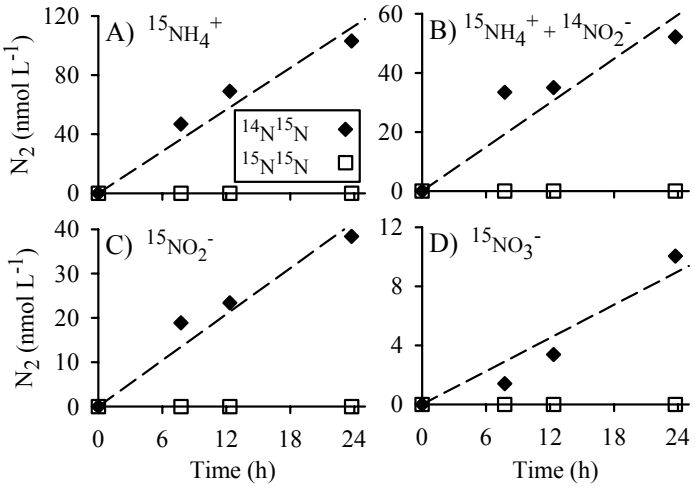


**Figure 5.** Production of $^{15}N$-labeled $N_2$ from $^{15}N$-labeled substrates in anaerobic incubations of water from 40 m depth at Sta. 4. Dashed line is least-squares fit to $^{14}N^{15}N$ data. (A) Production of $^{14}N^{15}N$ from $^{15}NH_4^+$ indicates anammox activity. (Rate: 106 nmol $^{14}N^{15}N$ $L^{-1}$ $d^{-1}$, $r^2$ = 0.96). (B) Unlabeled $^{14}NO_2^-$ added along with $^{15}NH_4^+$ to test for nitrite limitation of anammox. (Rate: 51 nmol $^{14}N^{15}N$ $L^{-1}$ $d^{-1}$, $r^2$ = 0.87). (C) Production of $^{14}N^{15}N$ from $^{15}NO_2^-$ without $^{15}N^{15}N$ production indicates anammox, rather than denitrification, since the in situ $NO_2^-$ pool was highly (>50%) $^{15}N$ labeled. (Rate: 39 nmol $^{14}N^{15}N$ $L^{-1}$ $d^{-1}$, $r^2$ = 0.96). (D) Production of $^{14}N^{15}N$ from $^{15}NO_3^-$ without $^{15}N^{15}N$ production indicates nitrate reduction to nitrite coupled to anammox, rather than denitrification, since the situ $NO_3^-$ pool was highly (>50%) $^{15}N$ labeled. (Rate: 11 nmol $^{14}N^{15}N$ $L^{-1}$ $d^{-1}$, $r^2$ = 0.94)

## 7.4 Discussion

### *7.4.1 Denitrification*

We incubated water column samples from the Peruvian OMZ with $^{15}NO_2^-$ or $^{15}NO_3^-$ to elucidate the microbial process responsible for the loss of fixed nitrogen from the Peruvian OMZ. Since denitrification pairs two nitrite and/or nitrate ions to produce $N_2$, and since the pools of these N species in our incubations were labeled >50% with $^{15}N$, denitrification would be expected to produce significant amounts of $^{15}N^{15}N$ through random pairing (Nielsen 1992). The theoretical ratio of the production of $^{15}N^{15}N$ to $^{14}N^{15}N$ by denitrification is:

$$\frac{^{15}N^{15}N}{^{14}N^{15}N} = \frac{F}{2(1-F)} \quad (3)$$

where *F* is the fractional $^{15}N$ composition of the nitrate or nitrite pools (Nielsen 1992; Kuypers et al. 2006). Isotopic fractionation would not have an appreciable effect with this degree of labeling. Since the value of *F* in our experiments was no less than 0.5, $N_2$ production via denitrification would have yielded at least 0.5 mole of $^{15}N^{15}N$ for every mole of $^{14}N^{15}N$ produced. Although $^{14}N^{15}N$ was produced linearly beginning at 0 − 6 h in 20 of the 32 experiments with $^{15}NO_2^-$ or $^{15}NO_3^-$ ($r^2$ ≥0.7), no $^{15}N^{15}N$ production was detected during any of the experiments before 24 h. Thus, our results did not provide evidence for in situ denitrification of nitrate or nitrite to $N_2$. However, $^{15}N^{15}N$ was detected at 24 h in 6 of 32 experiments. Similar results were obtained in both the Benguela upwelling system and in the Golfo Dulce, where $^{15}N^{15}N$ production was not detected until 48 h (Dalsgaard et al. 2003; Kuypers et al. 2005). The potential to respire nitrate is widespread among bacteria and archaea (Zumft 1997). The production of $^{15}N^{15}N$ in 6 of the 32 24-h incubations indicates that facultatively anaerobic heterotrophs that can use nitrate in the absence of oxygen are present in the Peruvian OMZ waters but does not indicate that nitrate was directly converted to $N_2$ by denitrifying microorganisms in situ in the Peruvian OMZ waters.

*7.4.2. Anammox*

We found $^{15}N$ tracer, molecular, and lipid biomarker evidence for anaerobic ammonium oxidation throughout the Peruvian upwelling OMZ in April 2005. Water samples incubated anaerobically with $^{15}NH_4^+$ produced significant amounts of $^{14}N^{15}N$, but no detectable $^{15}N^{15}N$. The exclusive production of $^{14}N^{15}N$ without delay indicated that $^{15}NH_4^+$ was being oxidized by *in situ* $^{14}NO_2^-$ to $N_2$ via the anammox reaction (Eq. 1) and excluded the suggested (Hulth et al. 1999) alternative pathways of anaerobic oxidation of $NH_4^+$ to $NO_3^-$, $NO_2^-$,or $N_2$ with $MnO_2$ (Thamdrup and Dalsgaard 2002; Trimmer et al. 2003). $^{14}N^{15}N$ production in these incubations was linear ($r^2 \geq 0.7$), indicating that the anammox bacteria were active from time 0 and were therefore likely active *in situ* (Fig. 5A). Since $^{15}N^{15}N$ production was not detected in incubations with $^{15}NO_2^-$ or $^{15}NO_3^-$, even though the *in situ* $NO_2^-$ and $NO_3^-$ pools were highly labeled, all of the $^{14}N^{15}N$ produced must have been produced via the pairing of $^{15}N$ labeled $^{15}NO_2^-$ with *in situ* $^{14}NH_4^+$ via the anammox reaction. The production of $^{14}N^{15}N$ in incubations with $^{15}NO_3^-$ therefore suggests that $^{15}NO_3^-$ was first converted to $^{15}NO_2^-$ by dissimilatory nitrate reduction, and that this reaction was coupled to anammox. However, it is not yet known whether the reduction of nitrate to nitrite resulted from incomplete denitrification by classical heterotrophic denitrifiers or by other nitrate-respiring organisms. In contrast to these results from OMZ waters, no $^{15}N$-labled $N_2$ was produced in any of the incubations with water from the oxic mixed layer (Fig. 4). Further evidence for the importance of anammox bacteria in the Peruvian OMZ waters came from molecular identification of the anammox bacteria via FISH and qPCR (Fig. 4). Ladderane lipids specific for anammox bacteria were also found within the Peruvian OMZ waters we investigated. These anammox-specific molecular and lipid biomarkers were found at all stations and depths where anammox activity was measured with $^{15}N$ incubations, but were not found (lipids, FISH), or were found at greatly reduced concentrations (qPCR) above the oxycline.

Anammox activity measured by our $^{15}N$ incubations had maxima at the base of the oxycline at all stations, and near the sediments at Sta. 2 (Fig. 4). The depth-distributions of FISH and qPCR counts of anammox cells were highly correlated with $^{14}N^{15}N$ production (from $^{15}NH_4^+$ or $^{15}NO_2^-$) within stations ($r$ = 0.77 to 0.95). Cell-specific potential anammox rates in the Peruvian OMZ (0.4 to 2.4 fmol cell$^{-1}$ d$^{-1}$, Table 2) were

comparable to rates from the Benguela upwelling system and the Black Sea (4.5, and 3 to 4 fmol cell$^{-1}$ d$^{-1}$, respectively) (Kuypers et al. 2005). Although anammox metabolism in wastewater bioreactors is reversibly inhibited at $O_2$ concentrations as low as 1 µmol L$^{-1}$ (Strous et al. 1997), $^{15}$N-labeling experiments, FISH, qPCR, and ladderane biomarkers showed that anammox bacteria were abundant in waters with *in situ* oxygen concentrations of up to 20 µmol L$^{-1}$ (Fig. 4), and these cells were able to begin anammox metabolism immediately following establishment of anoxic incubation conditions. Similar activity patterns were also observed in oxygen-containing samples collected from the Benguela upwelling system (Kuypers et al. 2005). The anammox bacteria at this high oxygen concentration may be dormant (although they were able to produce $N_2$ from $NH_4^+$ immediately upon re-establishment of anoxia during our labeling experiments), or they may be metabolizing in anoxic microniches within marine snow particles (Ploug 2001). Anammox bacteria in waste water reactors may have doubling rates as short as 1.8 d, in contrast with early reports of an 11-d doubling period, suggesting that they may have considerable flexibility in growing to meet changing environmental conditions (Isaka et al. 2006). The large temporal, spatial, and vertical heterogeneity in anammox activity, cell numbers and activities in the Peruvian OMZ (Fig. 4, Table 2), and the presence of active populations in oxygen-containing strata, indicates that anammox bacteria have the physiological flexibility to persist in this dynamic environment.

Ammonium concentrations in OMZs are often very low, and it has been suggested that anammox could account for the "missing" ammonium which was predicted to have been regenerated by heterotrophic denitrification in the water column (Richards 1965; Devol 2003). An initial appraisal of the significance of anammox in the ocean suggested a scenario where anammox 'scavenging' of the ammonium regenerated during organic matter respiration by heterotrophic denitrification could at most be responsible for 28 to 48% of the total OMZ $N_2$ production, depending on the C:N ratio of the organic matter respired by denitrifiers (Devol 2003). However, if anammox can occur without denitrification in OMZ water columns, as suggested by our data and those of Kuypers et al. (2005), then another source of the ammonium to drive anammox must be found.

The peak in ammonium concentration and anammox activity that we observed just below the oxycline (also observed by Kuypers et al. (2005) in the Benguela upwelling) indicates that anammox may be driven in part by aerobically-regenerated ammonium diffusing into the suboxic zone. Earlier measures of Peruvian OMZ community metabolism (ETS activity and nitrate reduction) also showed intense activity just below the oxycline (Codispoti and Packard 1980; Lipschultz et al. 1990). Heterotrophic dissimilatory reduction of nitrate, suggested by our $^{15}NO_3^-$ incubations, could provide an additional source of remineralized ammonium at these depths. A second maximum in anammox activity seen near the sediments at Sta. 2 (Figs. 4A, 4B) and at Sta. 9 and 10 (data not shown) - as well as in the Benguela upwelling (Kuypers et al. 2005) - might be driven by benthic ammonium fluxes (Dalsgaard et al. 2003). These observations suggest that maximum $N_2$ loss may not take place in the core of the deep nitrite maximum as suggested earlier, but near the sediments and the oxycline, where ammonium is most available (Fig. 4).

*7.4.3 Contribution of anammox and denitrification to N deficits in the Peruvian OMZ*

Annual N losses in the Peruvian upwelling system previously have been estimated at 15 to 25 Tg N $yr^{-1}$ based on measurements of ETS activity from the "inner main secondary nitrite maximum zone" within 175 km of the Peruvian coast ($3.26 \times 10^{11}$ $m^2$; Codispoti and Packard 1980). Lipschultz et al. (1990) estimated 8.9 Tg N $yr^{-1}$ for the same area from the production of $^{15}NO_2^-$ in incubations with $^{15}NO_3^-$ tracer. The estimate of Codispoti and Packard (1980), derived from the diffusive and advective transport of N deficits through 16° S latitude and adjusted to the same area, was ca. 14 Tg N $yr^{-1}$, while the estimate of Deutsch et al. (2001), based on the change in N* (an N deficit measure) with chlorofluorocarbon (CFC)-derived ETSP water-mass ages, when adjusted to the same area, yields ca. 19 Tg N $yr^{-1}$.

The rates of $^{14}N^{15}N$ production attributable to anammox that we derived from our $^{15}N$ incubation experiments were made under conditions of excess substrate availability, and we did not derive *in situ* anammox rates from them. Further, we recognize that the temporal and spatial scale of our sampling cannot be considered representative of the full range variation within the Peruvian OMZ. However, our measurements represent the first direct measurements of $N_2$ production in the Peruvian OMZ waters, and we do not believe that they are necessarily less realistic

than previous estimates from denitrification proxies and similarly restricted datasets. To compare the magnitude of our results to previously published areal N-loss estimates, we calculated a depth-integrated potential anammox rate (using results from the non-substrate-limited $^{15}NH_4^+$ + $^{14}NO_2^-$ incubations) at our five Peruvian OMZ stations, to obtain an overall mean potential anammox rate of 11 mmol N $m^{-2}$ $d^{-1}$ (Table 2), equivalent to 18 Tg N $yr^{-1}$ when scaled to the same area as the above estimates from previous studies (3.26 x $10^{11}$ $m^2$). This estimate falls well within the range estimated in the previous studies (9 to 25 Tg N $yr^{-1}$), suggesting that the magnitude of the anammox N-loss rates we measured were not unreasonable, and could potentially account for a significant proportion of the total N-loss from the Peruvian upwelling system.

$N_2$ losses via anammox have been shown to be significant in many anoxic marine sediments and water columns investigated so far. However, the consequences of anammox activity for carbon and nitrogen cycling within the marine water column are still poorly understood. The significance of $N_2$ production via anammox or denitrification in OMZs lies not only in the identity of the responsible species, but in the metabolic differences between the two processes, which have differing consequences for the carbon cycle (chemoautotrophy versus heterotrophy) and for the cycling of other inorganic N species in the N cycle, particularly ammonium and nitrous oxide. Our results showing that the $N_2$ production in the Peruvian upwelling OMZ in April 2005 was via anammox, rather than denitrification, are surprising, given the long-held expectation that OMZ N deficits would result from heterotrophic denitrification. However, earlier studies assumed that OMZ N deficits resulted from heterotrophic denitrification because no other $N_2$-generating process was known, and the assays used in these studies could not differentiate denitrification from anammox. Our results from $^{15}N$-labeling experiments, supported by molecular and lipid biomarker data, are in agreement with prior results (Dalsgaard et al. 2003; Kuypers et al. 2005), and show that a hitherto unexpected process – anammox – can be the sole N-loss pathway in the OMZ waters of the ETSP.

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# chapter 8

## A comparison of analytical techniques used for the detection of anaerobic ammonium oxidation in the Namibian and Peruvian oxygen minimum zones

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- *in preparation*

**Abstract**

Methods of analysis used to indicate the presence and activity of anammox bacteria and the anammox process, i.e. $^{15}N$ labelling techniques, fluorescent *in situ* hybridisation (FISH), quantitative polymerase chain reaction (qPCR) and specific biomarker 'ladderane' lipids, all relate to different properties of anammox bacteria. We have assessed the comparability of these techniques using statistical analysis on two data sets obtained from water column samples in the Namibian and Peruvian oxygen minimum zones (OMZs). Analysis showed that rates of $^{29}N_2$ production obtained using $^{15}N$ labelling techniques significantly correlate with ladderane lipid concentrations in both upwelling systems and with anammox FISH counts in the Namibian upwelling. Ladderane lipid concentrations obtained by either high performance liquid chromatography mass spectrometry/ mass spectrometry (HPLC/APCI-MS/MS) or gas chromatography MS (GC/MS) were significantly correlated, and statistical testing indicated that the ladderane lipid concentrations analysed using HPLC/APCI-MS/MS were not statistically different to those analysed using GC/MS. Significant correlations between ladderane lipid concentrations and rates of $^{29}N_2$ production suggest that ladderane lipids are derived mainly from metabolically active cells. Results from the Namibian upwelling showed that the higher the rates of $^{29}N_2$ production per cell, the more ladderane lipids each cell

contains, suggesting that the anammoxosome contains more ladderane lipids and is at same time capable of producing more $N_2$. This could indicate that the expense of making extra ladderane lipids is saved for times when optimal growth conditions arise. In the Peruvian upwelling qPCR copy numbers and rates of $^{29}N_2$ production and rates of $^{29}N_2$ production and ladderane lipid concentrations were non-parametrically correlated. Observed disparities in this data set could be due to differences in the physiological state or growth phase of the anammox communities at the different sites.

## 8.1 Introduction

Anaerobic ammonium oxidizing (anammox) bacteria produce gaseous $N_2$ by catabolising one mole of nitrite with one mole of ammonium (van de Graaf et al., 1997). Since its discovery, the anammox process has become implemented for the large scale, low cost removal of ammonium from wastewater (van Dongen et al., 2001). Growing evidence indicates that anammox bacteria make a significant contribution to the global loss of nutrient N in the form of $N_2$ from anoxic marine water columns and sediments (Dalsgaard and Thamdrup 2002; Dalsgaard et al., 2003; Kuypers et al., 2003; Kuypers et al., 2005; Hamersley et al., 2007; Schmid et al., 2007). In addition, the anammox process also appears to play an important role in nitrogen cycling in tropical and temperate freshwaters and sediments (Jetten et al., 2003; Schubert et al., 2006; Trimmer et al., 2003). Currently, the anammox process can be detected using $^{15}N$ containing substrates, while the bacteria responsible for this process can be detected /quantified by fluorescent *in situ* hybridization (FISH) with specific 16S rRNA-targeted probes, quantitative polymerase chain reaction (qPCR or real-time PCR) using selective primer and probe sets and specific biomarker 'ladderane' lipids which are unique to anammox bacteria.

$^{15}N$ isotope pairing techniques involve the addition of labelled nitrogen compounds ($^{15}NH_4^+$, $^{15}NH_4^+ + {}^{14}NO_2^-$, $^{14}NH_4^+ + {}^{15}NO_2^-$ or $^{14}NH_4^+ + {}^{15}NO_3^-$) to anaerobic samples, which, if containing active anammox bacteria, results in the production of isotopically labelled $N_2$ with different masses (Dalsgaard et al., 2003). Terminating cell activity by adding $HgCl_2$ at different time intervals halts the production of $N_2$ over time. Isotopic ratios of $N_2$, i.e. $^{14}N^{15}N$ : $^{14}N^{14}N$ and $^{15}N^{15}N$ :$^{14}N^{14}N$, can then be determined using gas chromatography (GC)/ isotope ratio mass spectrometry

(IRMS). The $^{15}N$ labelling technique was initially developed to determine the rate of $N_2$ production from heterotrophic denitrification by incubating anaerobic samples with different amounts of $^{15}NO_3^-$ (Nielsen 1992). The relative abundance of $^{29}N_2$ ($^{15}NH_4^+$ + $^{14}NO_2^-$) is now used to determine anammox activity. Although this method gives information on the rate of $^{29}N_2$ production via the anammox reaction, it provides no further information on the abundance and type of organism responsible for the anaerobic oxidation of ammonium.

The presence and abundance of anammox bacteria in environments can be determined using fluorescent *in situ* hybridization (FISH). Water, sediment or biomass is fixed in paraformaldehyde, filtered and subsequently hybridized with fluorescently labelled oligonucleotide probes targeting the 16S rRNA of anammox bacteria (Schmid et al., 2003; Amann et al., 1995). The relative abundance of cells can then be enumerated using epifluorescence microscopy. The effectiveness of FISH is found when applying combinations of specific oligonucleotide probes, which can allow discrimination to be made between the different anammox species and the surrounding community structure (Schmid et al., 2005). Additionally, you can see which microbes are adjacent or even in direct contact with the anammox bacteria and potential interactions can be postulated (Woebken et al., 2007). However, living anammox cells that were not actively performing the anammox process can also be detected by FISH, which could lead to an overestimation of the metabolically active anammox cells (Schmid et al., 2007).

Quantitative (or real-time) PCR can be used to quantify the amount of DNA or RNA copies from anammox bacteria in different environments (Lam et al., 2007; Hamersley et al., 2007). This technique is based on the principle that the cycle number at which the amount of PCR products starts to exponentially increase is linearly correlated to the amount of starting targeted materials. Special primer and probe sets have been designed to target the 16S rRNA genes specific to anammox bacteria (Hamersley et al., 2007), and the anammox bacteria were quantified using the TaqMan fluorogenic PCR method, calibrated against known standards (Bassler et al., 1995; Livak et al., 1995). This technique is rapidly gaining popularity due to its relatively fast, less labour intensive (in comparison to cell enumeration with FISH) and reasonably accurate quantification of DNA and RNA levels in environmental

samples. Like FISH analysis, the use of species-specific primers and probes means that qPCR can be used to discriminate between the different species of anammox (Schmid et al., 2005).

The presence of anammox bacteria can also be indicated by detection of their unique membrane lipids called ladderanes (Sinninghe Damsté et al., 2002). These lipids are postulated to form the membrane bilayer of the anammoxosome, a cellular compartment peculiar to anammox bacteria (Lindsay et al., 2001). Ladderane lipids come in a variety of forms e.g. fatty acids, glycerol diethers, glycerol ether-esters and an *sn*-2 glycerol monoether (Sinninghe Damsté et al., 2005), and can be quantified using gas chromatography/mass spectrometry (GC/MS) (Kuypers et al., 2003; Sinninghe Damsté et al., 2002). However, application of this technique on environmental samples with low ladderane lipid concentrations in complex matrixes can be problematic due to the poor gas chromatographic behaviour of ladderane lipids and related high detection limits (Sinninghe Damsté et al., 2005). Recently, a high performance liquid chromatography/ atmospheric pressure chemical ionization (HPLC/APCI)-MS/MS method allowing determination of low levels of ladderane lipids in complex matrixes (e.g. sediments) has been developed (Hopmans et al., 2006). In comparison to GC/MS, the chromatography of ladderane lipids is superior when using HPLC/APCI-MS/MS and detection limits of ca. 35 pg can be achieved. Since ladderane lipids occur in all genera of anammox bacteria (Rattray et al, submitted) and have so far not been found in other organisms, this makes them excellent biomarkers. However, these lipids may also be present in non-living organic matter and thus may not necessarily indicate the presence of metabolically active anammox bacteria.

Although the four methods of detection described above are used to provide evidence of the anammox process and anammox bacteria, they relate to different aspects, i.e. rates of $N_2$ production, cellular abundance of anammox bacteria and the amount of membrane lipids. Different types of quantitative information are therefore obtained. In an attempt to evaluate the comparability of the aforementioned detection techniques with each other we have statistically analysed data sets obtained from two OMZs, a part of which have been previously reported by Kuypers et al. (2005) and Hamersley et al. (2007).

## 8.2 Materials and Procedures

### *8.2.1 Data set selection*

Data sets from the Namibian upwelling (Kuypers et al., 2005) and Peruvian upwelling (Hamersley et al., 2007) systems have been selected for analysis because they comprise the two most comprehensive multi-technique studies so far used to define the role of the anammox process in the cycling of oceanic nitrogen (see Fig. 1; Table 1 for sampling locations). These regions were originally selected for study because these oxygen minimum zones are generally believed to be responsible for 30-50% of total oceanic inorganic nitrogen loss. Both studies were conducted in the oxygen minimum zones during the spring season.

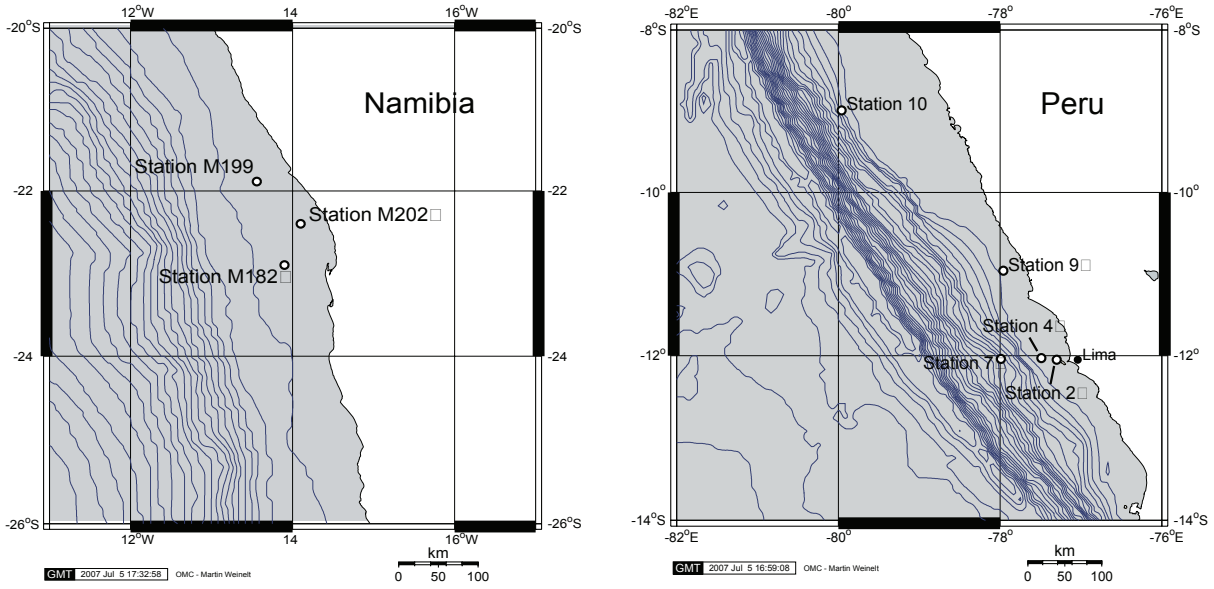


**Figure 1.** Maps of sampling sites in the Namibian and Peruvian upwelling systems. Contour lines are derived from the ETPO5 data set (U.S. National Geophysical Data Centre), where contour intervals are < 2 degrees: contour interval = 100 m, > 2 degrees: contour interval = 200 m. (Source: Online Map Creation at www.aquarius.geomar.de).

**Table 1.** Sampling sites and depths in the Peruvian and Namibian upwelling systems

| Upwelling system | Station | Location (°S, °W) | Site depth (m) | Date |
|---|---|---|---|---|
| Namibia | M182 | -22.90°S, 13.90°W | 132 | 21.03.03 |
| Namibia | M202 | -22.40°S, 14.10°W | 70 | 27.03.03 |
| Peru | 2 | -12.05°S, -77.30°W | 96 | 15.04.05 |
| Peru | 4 | -12.03°S, -77.49°W | 141 | 17.04.05 |
| Peru | 7 | -12.04°S, -77.99°W | 2,400 | 22.04.05 |
| Peru | 9 | -10.96°S, -77.96°W | 152 | 20.04.05 |
| Peru | 10 | -8.99°S, -79.96°W | 442 | 19.04.05 |

In addition to the data on the Namibian upwelling from Kuypers et al. (2005), we have re-analysed these samples (see Fig. 2. for ladderane lipids analysed) using HPLC/APCI-MS/MS as described by Hopmans et al., (2006). We also supplement the Peruvian upwelling data from Hamersley et al., (2007) by presenting individual (not the summed total) ladderane lipids in this study. Not only does this additional information contribute an extra comparison between the two methods of analyses, but it also allows us to see if individual ladderane lipids from different settings behave in a similar way.

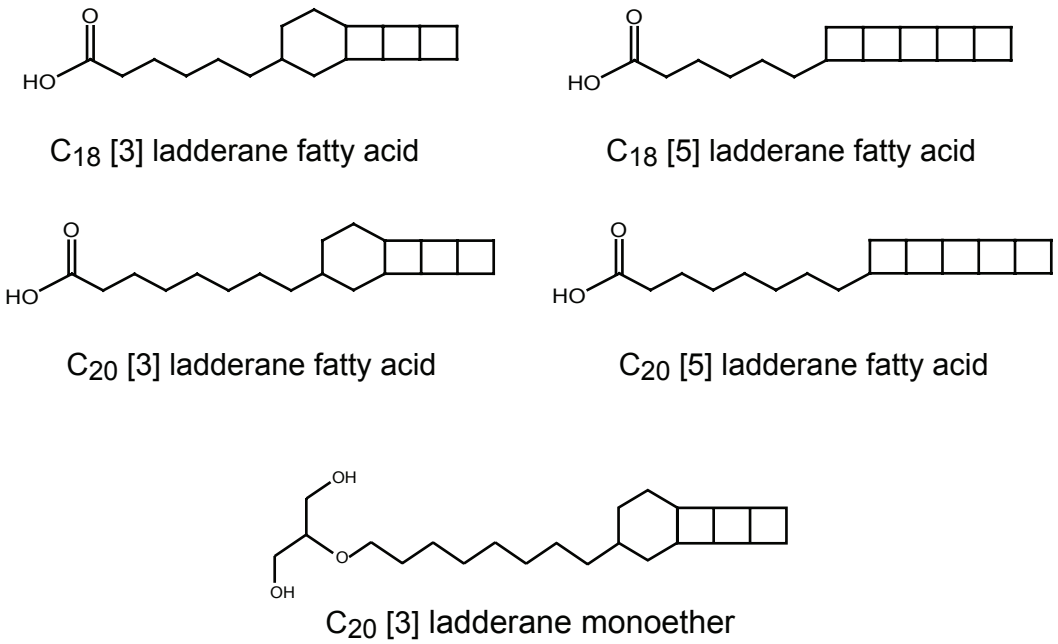


**Figure 2.** Structures of analysed ladderane fatty acids and ladderane monoether. Nomenclature of ladderane lipids; total carbon numbers followed by the number of cyclobutane rings in square brackets.

### *8.2.2 Methods used for additional ladderane lipid analysis*

Total lipid extracts from Namibian upwelling stations M182 and M202 were extracted as previously described by Kuypers et al. (2005). The total lipid extracts were methylated, dissolved in ethyl acetate and eluted over a small $AgNO_3$ impregnated silica column and finally analysed using HPLC/APCI-MS/MS as described by Hopmans et al. (2006). Quantification using HPLC/APCI-MS/MS was performed using external ladderane standards, isolated from anammox enrichment cultures. The reproducibility of the HPLC/APCI-MS/MS concentration measurements was ±5%.

## 8.3. Assessment

### *8.3.1 Statistical analysis of the Namibian and Peruvian data sets*

In order to evaluate the Namibian and Peruvian data sets we have applied statistical analysis to the (in part) previously published data sets. However, we have now used the original qPCR copy numbers of the Peruvian data set instead of the anammox cell counts (calculated by calibration to FISH counts) published by Hamersley et al., (2007). Furthermore, we have analysed the additional ladderane lipid concentration data for both sites in this study.

The results from all techniques have been statistically analysed using Sigma Stat version 3.1. Kolmogorov-Smirnov normality tests indicated that the combined data was not normally distributed and box plots were constructed to indicate outliers. Data was transformed using various techniques (logarithmic, square root, and arcsin) and the best fit was chosen by analysing and comparing quantile/quantile plots. In the Namibian upwelling data the FISH counts, the $C_{20}$ [3] ladderane fatty acid concentrations obtained by GC/MS, the total amount of ladderane lipids analysed by GC/MS and the $^{29}N_2$ rates were found to be normally distributed. All other variables were logarithmically transformed before analysis by calculating the parametric Pearson correlation coefficient (r). Results have been tabulated in a correlation matrix for the Namibia upwelling data set (Table 2). In the data from the Peruvian upwelling, the only normally distributed data set was from the anammox FISH counts. The remaining variables in the data set were logarithmically transformed, with exception of the $C_{18}$ [3] ladderane fatty acid (HPLC/APCI-MS/MS analysed) for which it was not possible to successfully transform into a normalised data set. Therefore in table 3, the Pearson product moment correlation coefficients

**Table 2.** Correlation matrix of Pearson product moment correlation coefficients indicating relationships between the methods of analysis used to indicate anammox bacteria and the anammox process in the Namibian upwelling. FA: fatty acid.

**Namibia Stations M182 and M202 Pearson product moment correlation coefficients**

| | FISH counts | C18[3] Ladderane FA GC/MS | C20 [3] Ladderane FA GC/MS | C20[3] Ladderane monoether GC/MS | Total ladderane lipids GC/MS | C18[3] Ladderane FA HPLC-MS/MS | C18 [5] Ladderane FA HPLC-MS/MS | C20 [3] Ladderane FA HPLC-MS/MS | C20 [5] Ladderane FA HPLC-MS/MS | C20[3] Ladderane monoether HPLC-MS/MS | Total ladderane lipids HPLC-MS/MS |
|---|---|---|---|---|---|---|---|---|---|---|---|
| C18 [3] Ladderane FA GC/MS | **0.84 n=10** | | | | | | | | | | |
| C20 [3] Ladderane FA GC/MS | **0.88 n=10** | **0.96 n=10** | | | | | | | | | |
| C20 [3] Ladderane monoether GC/MS | 0.46 n=9 | **0.81 n=9** | **0.76 n=9** | | | | | | | | |
| Total ladderane lipids GC/MS | 0.60 n=10 | **0.87 n=10** | **0.80 n=10** | **0.87 n=9** | | | | | | | |
| C18 [3] Ladderane FA HPLC-MS/MS | **0.91 n=10** | **0.93 n=10** | **0.93 n=10** | 0.65 n=9 | **0.79 n=10** | | | | | | |
| C18 [5] Ladderane FA HPLC-MS/MS | **0.91 n=10** | **0.94 n=10** | **0.94 n=10** | *0.67 n=9* | **0.80 n=10** | **1.00 n=10** | | | | | |
| C20 [3] Ladderane FA HPLC-MS/MS | **0.82 n=10** | **0.84 n=10** | **0.86 n=10** | 0.52 n=9 | *0.67 n=10* | **0.95 n=10** | **0.94 n=15** | | | | |
| C20 [5] Ladderane FA HPLC-MS/MS | **0.86 n=10** | **0.87 n=10** | **0.88 n=10** | 0.56 n=9 | *0.71 n=10* | **0.98 n=10** | **0.96 n=15** | **1.0 n=15** | | | |
| C20 [3] Ladderane monoether HPLC-MS/MS | 0.61 n=5 | 0.45 n=5 | 0.34 n=5 | -0.78 n=4 | -0.30 n=5 | 0.55 n=5 | 0.55 n=5 | 0.58 n=5 | 0.6 n=5 | | |
| Total ladderane lipids HPLC-MS/MS | **0.89 n=10** | **0.92 n=10** | **0.92 n=10** | 0.65 n=9 | **0.77 n=10** | **1.00 n=10** | **0.97 n=15** | **0.99 n=15** | **1.0 n=15** | 0.57 n=5 | |
| $^{29}N_2$ production rates | **0.88 n=10** | **0.86 n=10** | **0.90 n=10** | **0.90 n=10** | *0.75 n=10* | **0.85 n=10** | 0.51 n=15 | 0.19 n=15 | 0.31 n=15 | 0.34 n=5 | 0.35 n=15 |

**Pearson product moment correlation, significant at 0.01 (2 tailed)**
*Pearson product moment correlation significant at 0.05 (2 tailed)*
No relationship between variables p>0.05

**Table 3.** Correlation matrix of Pearson product moment or Spearman rank correlation coefficients indicating relationships between the methods of analysis used to indicate anammox bacteria and the anammox process in the Peruvian upwelling. FA: fatty acid.

**Peru all stations Pearson product moment or Spearman rank correlation coefficients**

| | FISH counts | qPCR Copy numbers | C18 [3] Ladderane FA HPLC-MS/MS | C18 [5] Ladderane FA HPLC-MS/MS | C20 [3] Ladderane FA HPLC-MS/MS | C20 [5] Ladderane FA HPLC-MS/MS | C20 [3] Ladderane monoether HPLC-MS/MS | Total ladderane lipids HPLC-MS/MS |
|---|---|---|---|---|---|---|---|---|
| qPCR Copy numbers | *0.80 n=6* | | | | | | | |
| C18 [3] Ladderane FA HPLC-MS/MS | *0.52 n=6* | 0.40 n=17 | | | | | | |
| C18 [5] Ladderane FA HPLC-MS/MS | 0.18 n=6 | 0.02 n=15 | **0.61 n=23** | | | | | |
| C20 [3] Ladderane FA HPLC-MS/MS | 0.14 n=6 | -0.04 n=15 | **0.57 n=23** | **0.97 n=21** | | | | |
| C20 [5] Ladderane FA HPLC-MS/MS | 0.10 n=6 | 0.03 n=15 | **0.54 n=23** | **0.95 n=20** | **0.97 n=20** | | | |
| C20 [3] Ladderane monoether HPLC-MS/MS | 0.23 n=6 | 0.01 n=15 | **0.57 n=23** | **0.94 n=21** | **0.91 n=21** | **0.89 n=20** | | |
| Total ladderane lipids HPLC-MS/MS | 0.26 n=6 | 0.07 n=15 | **0.65 n=23** | **0.96 n=21** | **0.96 n=22** | **0.93 n=20** | **0.96 n=21** | |
| $^{29}N_2$ production rates | 0.09 n=6 | *0.50 n=15* | 0.38 n=15 | **0.67 n=15** | *0.50 n=14* | *0.54 n=14* | *0.50 n=14* | *0.59 n=14* |

Spearman rank correlation (non-linear)

**Correlation, significant at 0.01 (2 tailed)**
*Correlation significant at 0.05 (2 tailed)*
No relationship between variables p>0.05

are displayed for logarithmically transformed data, and the Spearman rank correlation coefficients for the $C_{18}$ [3] ladderane fatty acid against the other variables.

Calculation of Spearman rank correlation coefficients ignores data missing from the set but being a rank correlation, requires the same amount of observations per analysis. The coefficient (R) can be interpreted in the same way as the parametric Pearson correlation coefficient (r) and the levels of significance are calculated from the ranked data. However it should be noted that calculation of the parametric Pearson correlation coefficients gives a more stringent test. Below we discuss the observed correlations and their implications.

### *8.3.2 Comparison of ladderane lipid concentrations*

Results from the Namibian upwelling station M182 showed that the ladderane $C_{18}$ [3] ladderane fatty acid was the most abundant ladderane lipid analysed using HPLC/APCI-MS/MS with concentrations ranging from 0.3 to 0.8 ng $l^{-1}$ (Fig. 3c). The $C_{18}$ [5] ladderane fatty acid displayed the same distribution, ranging in concentrations from 0.3 to 0.7 ng $l^{-1}$. Concentrations of the $C_{20}$ [3] ladderane fatty acid ranged from 0.2 to 0.7 ng $l^{-1}$ and a similar trend is observed with the $C_{20}$ [5] ladderane fatty acid (concentrations ranging from 0.2 to 0.6 ng $l^{-1}$). The $C_{20}$ [3] ladderane monoether was only present above the detection limit at 121 m depth (0.1 ng $l^{-1}$).

When analysing lipid extracts from Namibian upwelling station M202 using HPLC/APCI-MS/MS (Fig. 4c), the $C_{18}$ [3] ladderane fatty acid and the $C_{18}$ [5] ladderane fatty acid were found to be the most abundant lipids, with concentrations ranging from 2.5 to 13 ng $l^{-1}$ in both cases. The $C_{20}$ [3] ladderane fatty acid was the least abundant and concentrations ranged from 0.7 to 3.9 ng $l^{-1}$ and the $C_{20}$ [3] ladderane monoether concentrations from 0.2 to 0.6 ng $l^{-1}$. All lipids displayed an increase in concentration with depth.

Ladderane lipid extracts from Namibian upwelling station M199 were analysed using HPLC/APCI-MS/MS (Fig. 5a), the $C_{18}$ [5] ladderane fatty acid was found to be the most abundant lipid, and the concentrations ranged from 0.0 to 1.9 ng $l^{-1}$. The $C_{20}$[5] ladderane fatty acid was the least abundant and concentrations ranged from 0 to 0.4 ng $l^{-1}$. All lipids displayed an increase in concentration with depth.

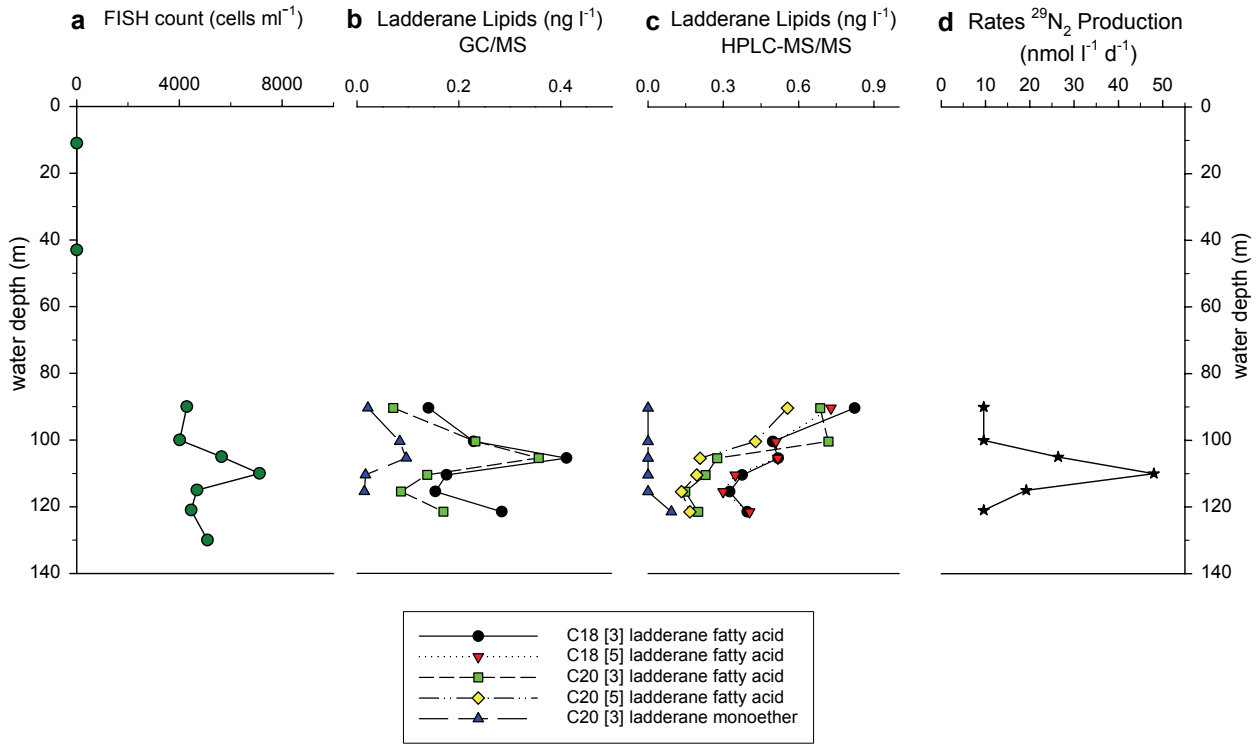


**Figure 3.** Namibian upwelling stations M182 (calculated using data from depths 105 m, 110 m, 115 m, 121 m) and M202, the concentration of total measured ladderane lipids per cell (using FISH cell counts) fg cell$^{-1}$ and the rate of $^{29}N_2$ production per cell in fmol cell$^{-1}$ d$^{-1}$.

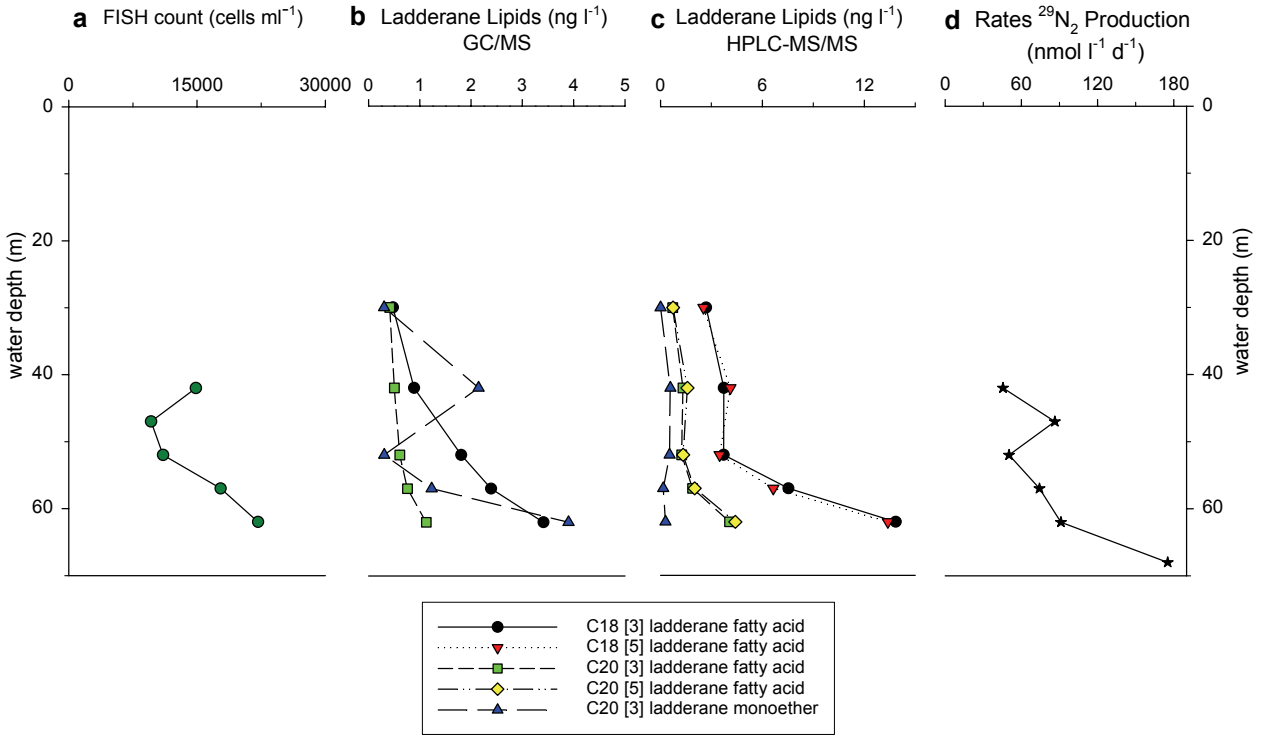


**Figure 4.** *In situ* data from site M182 in Namibia. (a) Anammox FISH count in cells per ml seawater. (b) GC/MS analysed ladderane lipids; $C_{18}$ [3] ladderane fatty acid, $C_{20}$ [3] ladderane fatty acid and $C_{20}$ [3] ladderane *sn*-2-glycerol monoether in nanograms per litre. (c) HPLC/APCI-MS/MS analysed ladderane lipids; $C_{18}$ [3] ladderane fatty acid, $C_{18}$ [5] ladderane fatty acid, $C_{20}$ [3] ladderane fatty acid, $C_{20}$ [5] ladderane fatty acid and $C_{20}$ [3] ladderane *sn*-2-glycerol monoether in nanograms per litre (d) Rate of $^{29}N_2$ production from the addition of $^{15}NH_4$ (Kuypers et al., 2005).

Ladderane lipid concentrations as determined by HPLC/APCI-MS/MS at the Peruvian station 2 (Fig. 6c) ranged from 0.7 to 25 ng $l^{-1}$ with the $C_{18}$ [5] ladderane fatty acid consistently displaying the highest concentration with depth and the $C_{20}$ [5] ladderane fatty acid displaying the lowest concentration. At 60 m the $C_{18}$ [5] ladderane fatty acid and the $C_{20}$ [3] ladderane monoether increase in concentration relative to the $C_{18}$ [3] ladderane fatty acid, $C_{20}$ [5] ladderane fatty acid and the $C_{20}$ [3] ladderane fatty acid. At station 4 (Fig. 7c) the lowest ladderane lipid concentration was displayed by the $C_{18}$ [3] ladderane fatty acid (0.3 ng $l^{-1}$) and this lipid was consistently in lowest concentration at 40 and 60 m. The highest ladderane lipid concentration was displayed by the $C_{20}$ [3] ladderane monoether (7.7 ng $l^{-1}$). However, the $C_{20}$ [3] ladderane monoether did not always display the highest concentration with depth, interchanging with the $C_{18}$ [5] ladderane fatty acid at 35 m and 60 m. Lowest concentrations at station 7 (Fig. 8b) were displayed by the $C_{20}$ [5] ladderane fatty acid (0.1 to 2.4 ng $l^{-1}$) and the $C_{18}$ [3] fatty acid (0 to 5.5 ng $l^{-1}$). The highest concentrations of ladderane lipids alternated with depth between the $C_{18}$ [5] ladderane fatty acid (9.4 ng $l^{-1}$ at 200 m) and the $C_{20}$ [3] ladderane monoether (4.4 ng $l^{-1}$ at 60 m). At station 9 (Fig. 9b) no $C_{20}$ [5] ladderane fatty acid or $C_{20}$ [3] ladderane monoether, and only trace amounts of other ladderane fatty acids were found at 25 m. At 145 m the lipid concentrations were highest ($C_{18}$ [5] ladderane fatty acid; 22 ng $l^{-1}$) and displayed a change in the lipid composition, which is also observed at higher lipid concentrations at stations 2 and 7. At station 10 (Fig. 10b), ladderane lipids were below the detection limit at 40 and 80 m. The highest concentrations of ladderane lipids were measured at 410 m, with the highest concentration held by the $C_{18}$ [5] ladderane fatty acid (6.3 ng $l^{-1}$).

At both Namibian stations we observed that the ladderane fatty acids followed a similar distribution, however the $C_{20}$ [3] ladderane monoether did not follow the same distribution with depth. In Peruvian upwelling waters the different ladderane lipid species appear to follow a more varied distribution, at higher concentrations. In some cases the distribution also changes with depth, but follows no observable pattern (e.g. nutrient concentrations or cell counts), suggesting that another factor may control the distribution of the different ladderane lipid species.

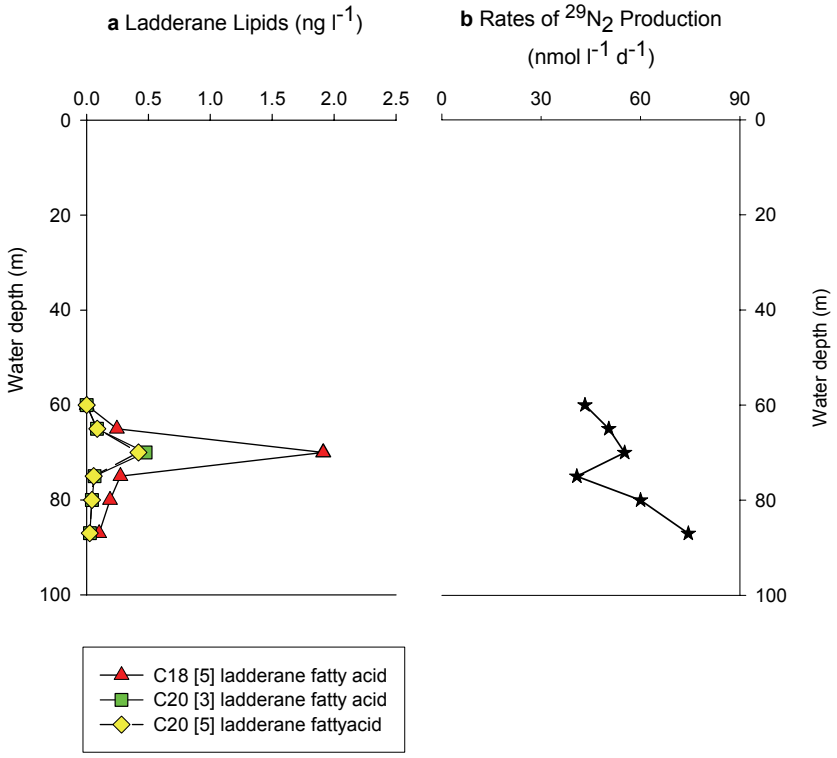


**Figure 5.** *In situ* data from site M199 in Namibia. (a) HPLC/APCI-MS/MS analysed ladderane lipids; $C_{18}$ [5] ladderane fatty acid, $C_{20}$ [3] ladderane fatty acid, $C_{20}$ [5] ladderane fatty acid in nanograms per litre (b) Rate of $^{29}N_2$ production from the addition of $^{15}NH_4$ (Kuypers et al., 2005).

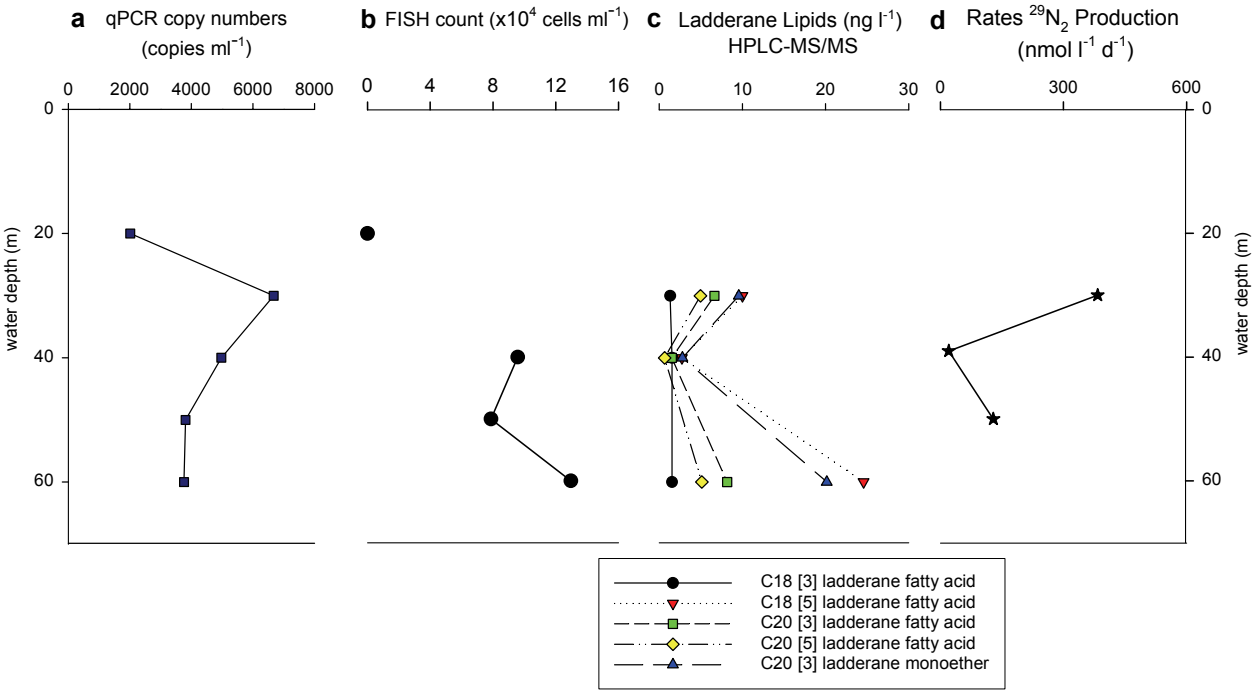


**Figure 6.** *In situ* data from Peru station 2. (a) qPCR copy numbers in gene copies per ml. (b) Anammox FISH count in cells per ml seawater. (c) HPLC/APCI-MS/MS analysed ladderane lipids; $C_{18}$ [3] ladderane fatty acid, $C_{18}$ [5] ladderane fatty acid, $C_{20}$ [3] ladderane fatty acid, $C_{20}$ [5] ladderane fatty acid and $C_{20}$ [3] ladderane *sn*-2-glycerol monoether in nanograms per litre. (d) Rate of $^{29}N_2$ production from the addition of $^{15}NH_4$ (Hamersley et al., 2007).

Comparison of the two different techniques used for quantifying ladderane lipid concentrations, HPLC/APCI-MS/MS and GC/MS (summed total lipids) as determined for the Namibian upwelling area (Table 2) showed there is a significant Pearson product moment correlation ($r = 0.77$, $p = <0.01$, $n=10$). In addition, we applied an unpaired t-test to the untransformed data set, which indicated that the two methods of measuring ladderane lipids did not give statistically significant different results ($p=0.096$). However, in nine out of ten cases, HPLC/APCI-MS/MS quantification yielded higher concentrations than quantification using GC/MS. The best correlations existed between individual ladderane lipids analysed using the same technique. For example, when using GC/MS the $C_{18}$ [3] ladderane fatty acid and $C_{20}$[3] ladderane fatty acid ($r = 0.96$, $p<0.01$, $n=10$) in the Namibian upwelling and when using HPLC/APCI-MS/MS the $C_{18}$ [5] ladderane fatty acid and the $C_{20}$ [3] monoether ($r = 0.94$, $p<0.01$, $n=21$) in the Peruvian upwelling. The lower correlation between the techniques, which could be due to the differences in quantifying ladderane lipids when using GC/MS, as reported by Hopmans et al. (2006). Higher concentrations obtained when using HPLC/APCI-MS/MS are probably due to the lack of co-eluting compounds and thermal degradation of cyclobutane rings encountered when analysing using GC/MS.

### *8.3.3 Comparison of anammox cell abundance, $^{29}N_2$ production rates and ladderane lipids*

To investigate if ladderane lipids are primarily derived from living or dead biomass, we compared ladderane lipid concentrations with FISH counts. In the Namibian upwelling we observed significant relationships between FISH and HPLC/APCI-MS/MS quantified total ladderane lipids ($r = 0.89$, $p<0.01$, $n=10$). The $C_{18}$ [3] ladderane fatty acid and $C_{20}$ [3] ladderane fatty acid (GC/MS analysed) were also significantly correlated to the FISH counts. However, even when using both techniques of ladderane lipid analysis, the $C_{20}$ [3] monoether was not strongly correlated to other variables. This and the lack of correlation to other ladderane fatty acids, indicates that the $C_{20}$ [3] monoether behaves in differently to other ladderane lipids, as previously reported by Rattray et al (*in press*).

The significant relationships found between anammox cell abundance and ladderane lipids suggest that ladderane lipids in the Namibian upwelling system are

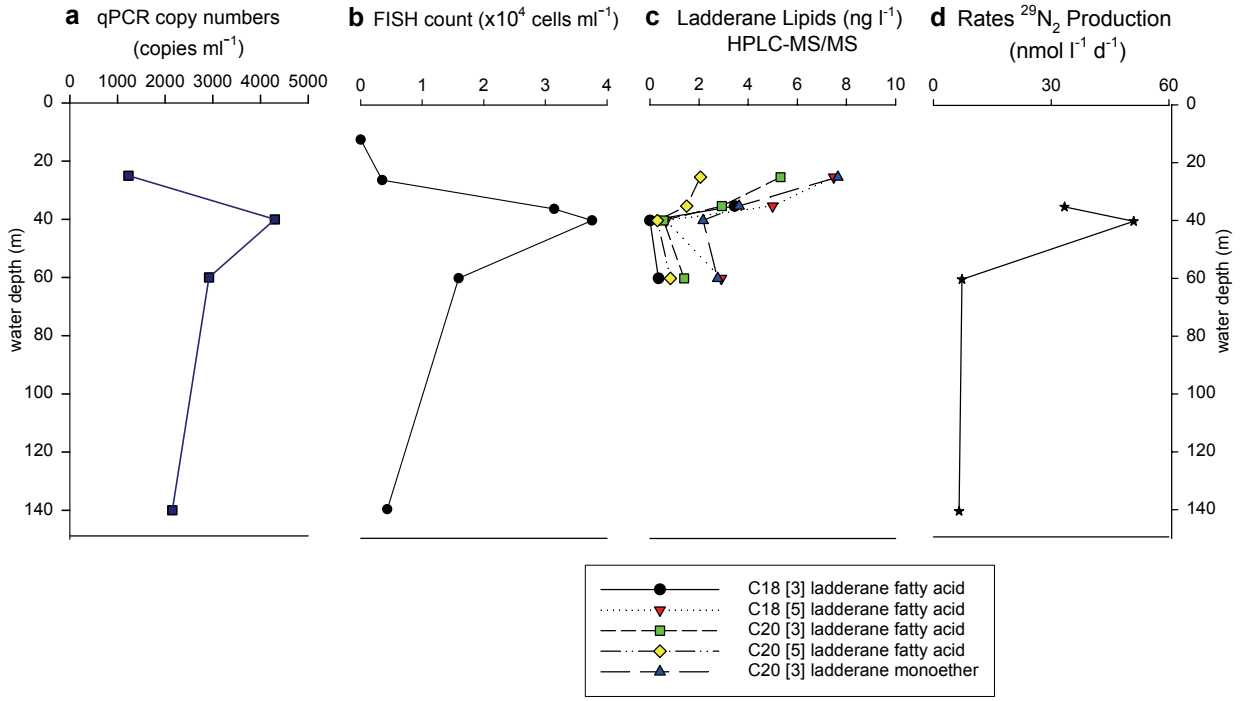


**Figure 7.** *In situ* data from site Peru station 4. (a) qPCR copy numbers in gene copies per ml. (b) Anammox FISH count in cells per ml seawater. (c) HPLC/APCI-MS/MS analysed ladderane lipids; $C_{18}$ [3] ladderane fatty acid, $C_{18}$ [5] ladderane fatty acid, $C_{20}$ [3] ladderane fatty acid, $C_{20}$ [5] ladderane fatty acid and $C_{20}$ [3] ladderane *sn*-2-glycerol monoether in nanograms per litre. (d) Rate of $^{29}N_2$ production from the addition of $^{15}NH_4$ (Hamersley et al., 2007).

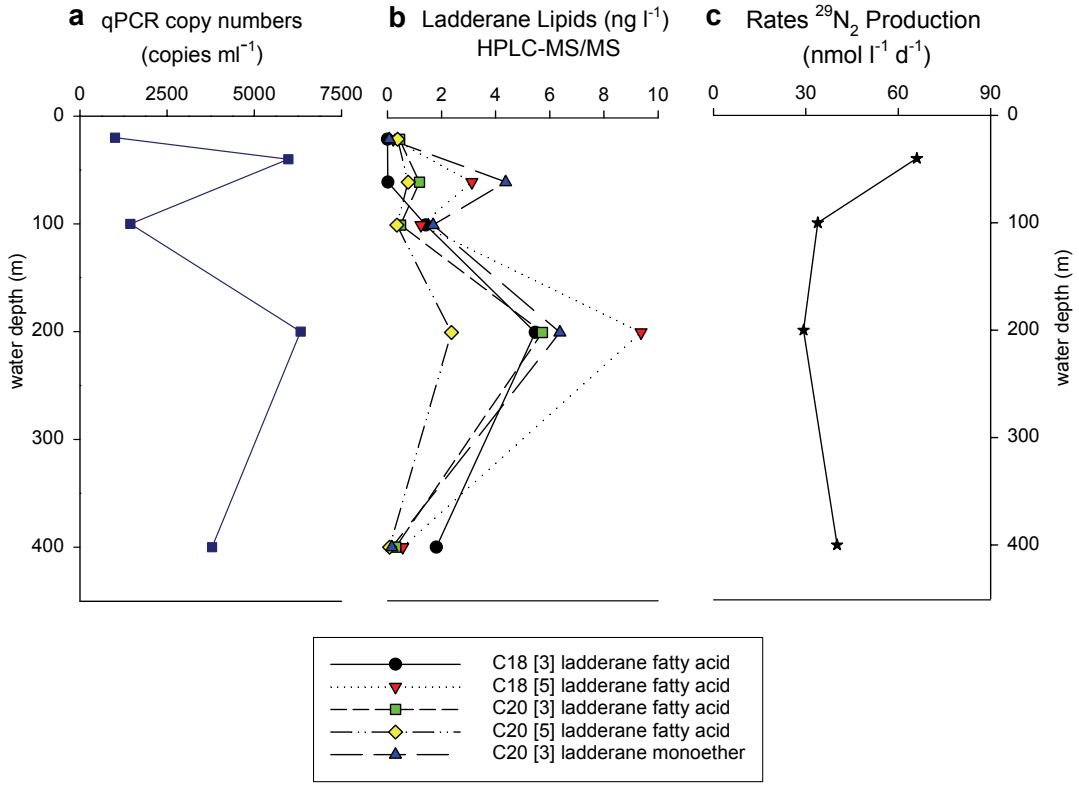


**Figure 8.** *In situ* data from Peru station 7. (a) qPCR copy numbers in gene copies per ml. (b) HPLC/APCI-MS/MS analysed ladderane lipids; $C_{18}$ [3] ladderane fatty acid, $C_{18}$ [5] ladderane fatty acid, $C_{20}$ [3] ladderane fatty acid, $C_{20}$ [5] ladderane fatty acid and $C_{20}$ [3] ladderane *sn*-2-glycerol monoether in nanograms per litre. (c) Rate of $^{29}N_2$ production from the addition of $^{15}NH_4$ (Hamersley et al., 2007).

mainly derived from living anammox cells. To investigate if the living anammox cells were actively performing anammox we compared cell abundance with $^{29}N_2$ production rates. FISH cell counts from Namibia gave a significant relationship where r = 0.88, p<0.01, n =10, indicating that the majority of anammox cells were actively catabolising via the anammox reaction.

In the Peruvian upwelling system, relationships appeared to exist between the ladderane lipid concentrations and anammox bacterial abundance (qPCR copy numbers) over depth, at individual stations (e.g. 2 and 7), however, there were insufficient data points to perform reliable statistical analysis. When combining the data for all Peruvian stations, the $C_{18}$ [3] ladderane fatty acid was non-parametrically correlated (R= 0.52, p<0.05, n=6) to the FISH counts. The lack of correlation with other ladderane lipids and anammox bacterial abundance suggests that ladderane lipids were either not related to the living anammox cell fraction, or that anammox cells contain different amounts of ladderane lipids. Anammox FISH counts and/ or qPCR copy numbers did appear to be significantly correlated with the rates of $^{29}N_2$ production at some individual stations (e.g. station 2 and 4), however, there were insufficient data points to reliably draw statistical conclusions. When combining the data for all sites, a non-parametric correlation between qPCR copy numbers and $^{29}N_2$ production rates was found (R= 0.50, p<0.05, n=15), but no significant correlation was observed between the combined data for the FISH counts and rates of $^{29}N_2$ production. However, significant Spearman rank correlations between ladderane lipids (individual and total) and the rate of $^{29}N_2$ production were found, which suggests that anammox metabolic activity and ladderane lipids are probably derived from living anammox cells.

The lack of correlation between the combined Peruvian data set could be due to differences in the hydrographic conditions of the region, and in addition, the abundance of anammox may be influenced different factors, i.e. anammox rates are much lower at the deep-water stations 9 and 10. Upwelling systems are prone to being highly dynamic, meaning that changes in temporal or spatial sampling will result in inadvertently sampling a different microbial community. In addition, spatial differences can also account for these differences, for example the roll of the ship can easily cause a 5 m difference in sampling depth (e.g. Namibia station M182

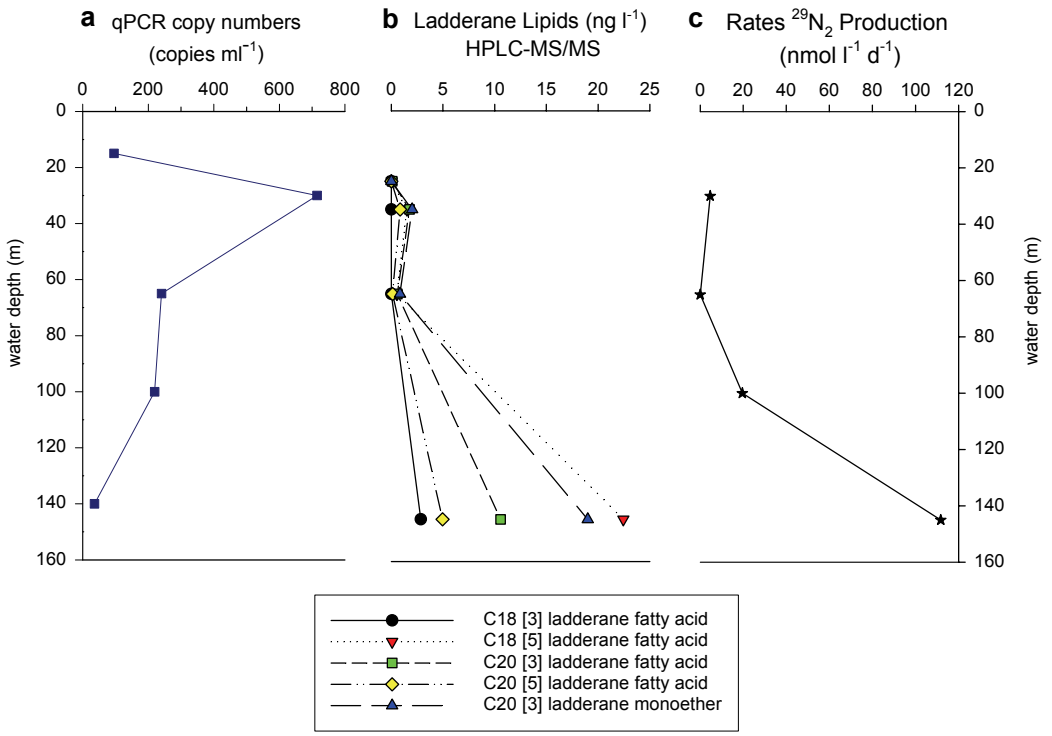


**Figure 9.** *In situ* data from Peru station 9. (a)-(c) as for Figure 8

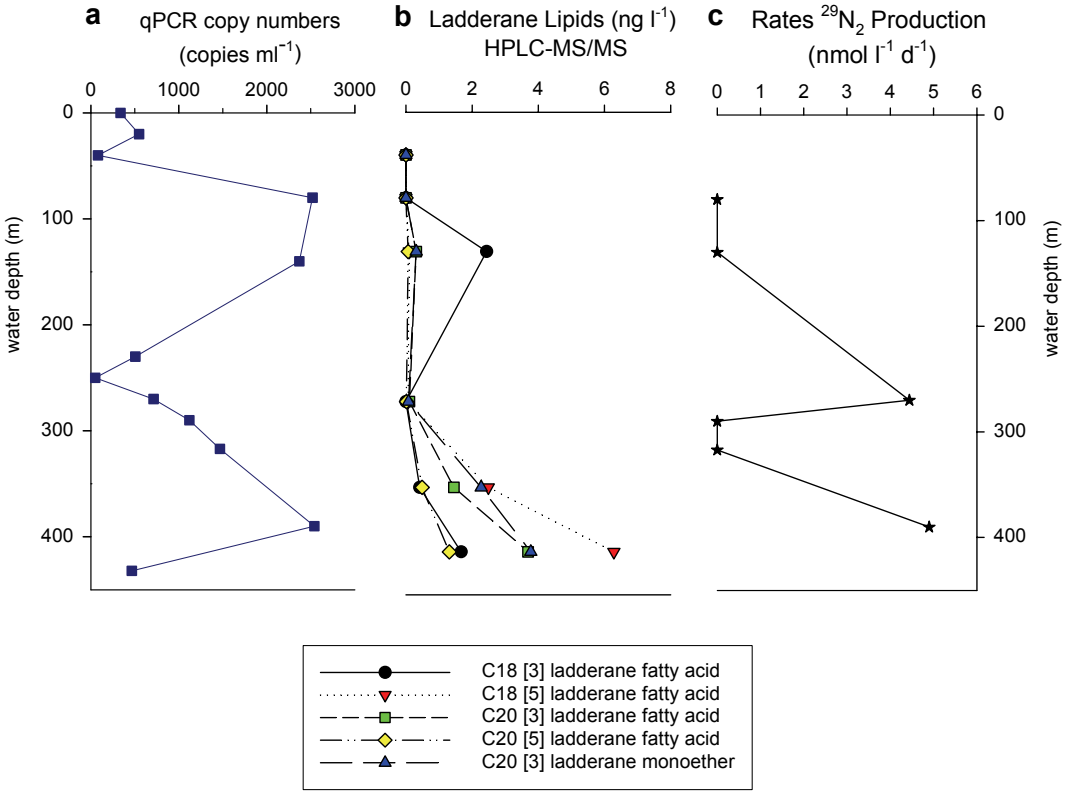


**Figure 10.** *In situ* data from Peru station 10. (a)-(c) as for Figure 8.

and M199, ladderane lipid concentrations and $^{29}N_2$ production). Therefore to summarise, it appears that ladderane lipids in the Namibian upwelling system are mainly derived from living and metabolically active anammox cells. However, the relationship between ladderane lipids and metabolically active anammox cells in the Peruvian upwelling is less well defined, which is perhaps due to anammox bacteria adapting to deal with different hydrographic conditions of this particular upwelling system.

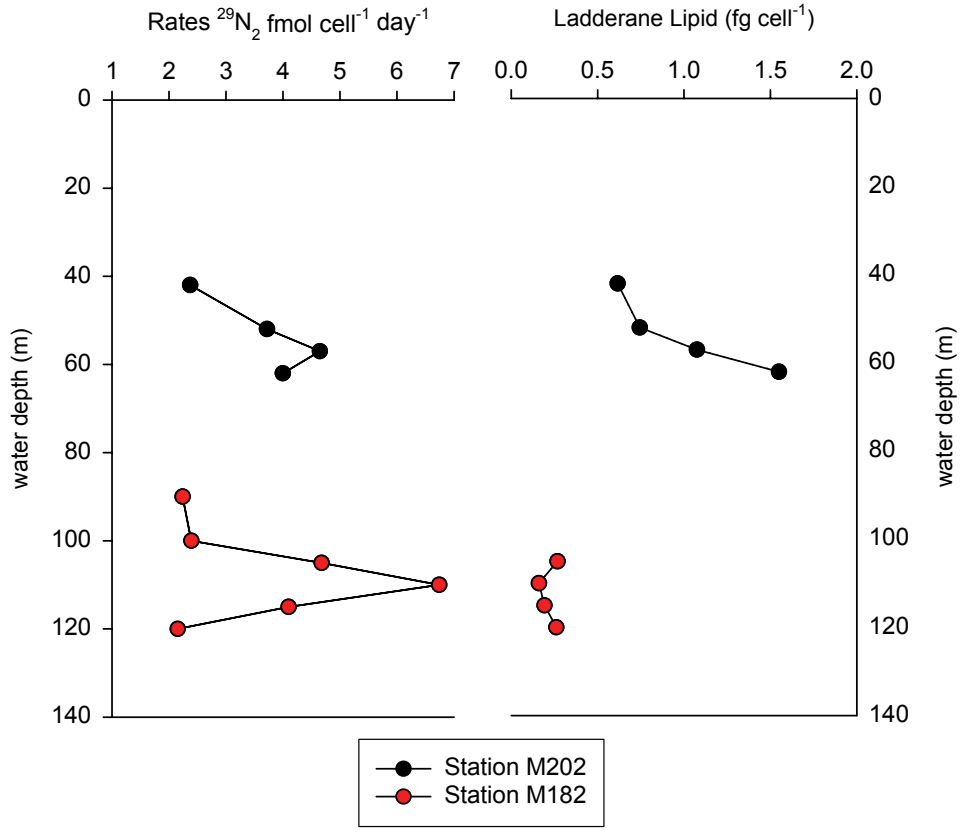


**Figure 11.** Namibian upwelling stations M182 (calculated using data from depths 105 m, 110m, 115 m, 121 m) and M202, the concentration of total measured ladderane lipids per cell (using FISH cell counts) fg cell$^{-1}$ and the rate of $^{29}N_2$ production per cell in fmol cell$^{-1}$ d$^{-1}$.

## 8.4 Discussion

Our statistical analysis has established for the Namibian upwelling that most of the anammox cells are metabolically active and that ladderane lipids are predominantly derived from these living cells. This relationship has allowed calculation of the total ladderane lipid concentrations per cell, per depth, using the *in situ* FISH cell counts and ladderane lipid concentrations and rates of $^{29}N_2$ production per cell, using the *in situ* rates of $^{29}N_2$ production and ladderane lipid concentrations (Fig. 11). Cell specific ladderane lipid concentrations at station M182 ranged from 0.2 to 0.3 fg cell$^{-1}$, and rates of $^{29}N_2$ production per cell ranged from 2.2 to 6.7 fmol cell$^{-1}$ day$^{-1}$. At station M202, cell specific ladderane concentrations ranged from 0.6 to 1.5 fg cell$^{-1}$. The rates of $^{29}N_2$ production previously published by Kuypers et al. (2005) ranged from 2.4 to 4.6 fmol cell$^{-1}$ day$^{-1}$, were in a similar range to the rates (3-4 fmol cell$^{-1}$ day$^{-1}$) calculated for the Black Sea water column (Kuypers et al., 2003). The cell specific rate for anammox growing in sequencing batch reactors has been calculated to range from 2-20 fmol cell$^{-1}$ day$^{-1}$ (Strous et al., 1999) and the cell specific ladderane concentration has been calculated to range from 0.6. to 8.0 fg cell$^{-1}$ (J. Rattray, unpublished data). Results from the Namibian upwelling suggest that the higher the rates of $^{29}N_2$ production per cell, the more ladderane lipids each cell contains. Since the cyclobutane rings in the ladderane lipids are highly strained (Sinninghe Damsté et al., 2002), they likely require a substantial amount of energy for biosynthesis. Therefore, it seems reasonable to assume that anammox bacteria only produce ladderane lipids at times of optimal growth conditions and increased metabolic activity. It has been proposed that the lipid membrane of the anammoxosome is constructed of ladderane lipids (Sinninghe Damsté et al., 2002), which encapsulate the site of the anammox reaction (Lindsay et al., 2001). An increase in the production of ladderane lipids may therefore also relate to an increase in the size of the anammoxosome and a related increase in the surface area of the site of anammox catabolism.

A lack of correlation in the Peruvian upwelling data set, specifically between the anammox cell counts (FISH and qPCR) and ladderane lipid concentrations and rates of $^{29}N_2$ production warrant it unsuitable to calculate cell specific $^{29}N_2$ rates or ladderane concentrations. The strong correlation between the FISH counts and qPCR copy numbers mostly reflects the fact that these samples originate from the

same water mass and measure the same variable. Poor correlation between the rates of $^{29}N_2$ production and the cell counts suggests that anammox bacteria in the natural environment alter their rate of anammox catabolism. The $^{15}N$ labelling incubations also showed that anammox catabolism in anammox bacteria from the Peruvian upwelling system responded immediately to addition of substrates under anaerobic conditions. We also observed that ladderane lipid concentrations were not directly correlated with the FISH or qPCR copy numbers, which was probably a reflection of the sometimes ≥12 hr time difference in sampling at some stations (Hamersley et al., 2006). However, as reported by Hamersley et al. (2006) the mean anammox bacterial numbers were highly correlated ($r$ = 0.96) with the mean ladderane lipid concentrations. Therefore, correlations between ladderane lipids and other variables in the Peruvian upwelling could be a direct reflection of this highly fluctuating and dynamic upwelling system.

In this study it has been observed that anammox cell numbers in both upwelling systems are in a similar order of magnitude, while rates of $^{29}N_2$ and ladderane lipid concentrations were significantly higher in the Peruvian upwelling system. Ladderane lipids in both upwelling systems appear to be related mainly to living anammox cells and correlation between FISH cell counts and $^{29}N_2$ rates indicated that the majority of anammox cells were metabolically active in the Namibian upwelling. Results suggest that energy is channelled into ladderane lipid biosynthesis when the optimal conditions for cell growth arise.

**8.5 Comments and Recommendations**

Despite the flaws of the methods we have discussed, it is clear that using a combination of techniques to define anammox bacteria and the anammox process in the natural environment provides the most complete picture. Since it is rarely possible to work on a state of the art research vessel in stable water masses, it is perhaps most realistic to develop existing sampling techniques and equipment. For example, when taking samples for lipid analysis, ca. 500 litres water needs to be filtered *in situ* which can take up to 3 h. The large volumes of water were required for achieving detection of ladderane lipids when using GC/MS. When using HPLC/APCI-MS/MS to analyse the ladderane lipids, the detection limit is considerably lower and interference from co-eluting compounds are removed. Our estimates are that the

volume of filtered water can be reduced to ca. 50 litres for expected areas of high anammox density (comparable to the Peru OMZ shelf regions) or at less densely populated sites ca. 250 litres (comparable to the Peru deep water stations). This in turn will reduce the sampling time to between 40 min and 1.5 h, thereby improving the comparability with other samples (provided they are taken around the same time). Perhaps equipping *in situ* pumps with Niskin bottles, or a CTD system with an *in situ* pump, could also give a more synchronised and thus more actual impression of the anammox population and process in dynamic upwelling environments.


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# chapter 9

## Temperature control of ladderane lipid chain length in anammox bacteria

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- *Environmental Microbiology Submitted*

**Abstract**

Anaerobic ammonium oxidizing (anammox) bacteria possess the unique ability to synthesise fatty acids containing linearly concatenated cyclobutane rings, termed 'ladderane lipids'. In this study we have investigated the effect of temperature on the ladderane lipid composition of anammox enrichment cultures, including all four genera of anammox bacteria, marine particulate organic matter and surface sediments. The results show a significant ($R^2$= 0.92, p=<0.0001, *n*=158) positive sigmoidal relationship between ladderane lipid chain length and *in situ* temperature, i.e. there is an increase in the relative abundance of ladderane lipids with shorter alkyl chains at lower temperatures and *vice versa*, particularly between 12°C and 20°C. This adaptation of chain length with temperature is similar to the regulation of common fatty acid compositions found in other bacteria, and is thought to be the result of maintaining constant membrane fluidity under different temperature regimes (homeoviscous adaptation). Our results can be used to discriminate between the origins of ladderane lipids in marine sediments, i.e. if ladderanes are produced *in situ* in relatively cold surface sediments or if they are fossil remnants originating from the warmer upper water column.

## 9.1 Introduction

Anammox bacteria possess the unique capability to oxidize $NH_4^+$ with $NO_2^-$ to $N_2$ under anoxic conditions (van de Graaf et al., 1997). Since the discovery of the anammox process in a wastewater treatment plant in the Netherlands (Mulder et al., 1995), it appears to be omnipresent in suboxic environments around the globe. Anammox therefore forms an important link in both the oceanic (Dalsgaard et al., 2003; Hamersley et al., 2007; Kuypers et al., 2003; Kuypers et al., 2005; Schmid et al., 2007) and freshwater nitrogen cycles (Jetten et al., 2003; Schubert et al., 2006). Anammox bacteria, like all *Planctomycetes*, contain intracellular compartments, but unlike other *Planctomycetes*, they contain a unique 'organelle' called the anammoxosome (Lindsay et al., 2001; Sinninghe Damsté et al., 2002; van Niftrik et al., 2004). The membrane of this compartment is constructed from unusual 'ladderane' lipids (Sinninghe Damsté et al., 2002). The core ladderane lipids consist of $C_{18}$ and $C_{20}$ fatty acids containing either 3 or 5 linearly concatenated cyclobutane rings, which are ether and/or ester bound to a glycerol backbone (Sinninghe Damsté et al., 2005). In addition, the intact polar lipids possessing these core lipid structures contain different types of polar head groups, i.e. phosphatidyl choline (PC), phosphatidyl ethanolamine (PE) or phosphatidyl glycerol (PG) (Boumann et al., 2006; Rattray et al., submitted). Modelling experiments have indicated that ladderane lipids provide a denser cell membrane than conventional membrane lipids and since the anammoxosome appears impenetrable to fluorophores, the ladderane membrane could well function in the role of cell energy conservation (Sinninghe Damsté et al., 2002; van Niftrik et al., 2004).

Experimental evidence has shown that enrichment cultures of anammox bacteria isolated from wastewater treatment reactors grow in enrichment cultures over a wide temperature range i.e. 20-43°C with an optimum temperature of ca. 35°C (Strous et al., 1999). In the natural environment the anammox process has been reported to occur at temperatures as low as -2.5°C (Dalsgaard and Thamdrup, 2002; Rysgaard and Glud, 2004). Given that anammox bacteria appear to proliferate in such a wide temperature range, the cell must be able to adapt to the surrounding environment. In other bacteria containing common fatty acids, temperature adaptation can be achieved by modifying the composition of the membrane bilayers to deal with changes in membrane viscosity due to alterations in

temperature. This process has been well documented and is termed as "homeoviscous adaptation", i.e. the fatty acid composition is changed to maintain membrane fluidity (Russell, 1984; Sandercock and Russell, 1980; Sinensky, 1974). However, it is not known how anammox bacteria, with their highly unusual ladderane lipids, react to changes in temperature, specifically with regards to lipid composition. To investigate this we cultured and analysed anammox bacteria grown at different temperatures in sequencing batch reactors (SBR) and obtained samples from different natural environments, which covered a wide range of temperatures and analysed the ladderane lipid composition.

## 9.2. Materials and Methods

### *9.2.1 Cultivation of anammox bacteria*

Three sequencing batch reactors (SBR), 2 litres in volume, were used for the cultivation of an anammox enrichment culture containing about 75% "*Candidatus* Brocadia fulgida" (Strous et al., 1998). "*Candidatus* Brocadia fulgida" inoculum biomass was initially cultured at 35°C, then split between three sequencing batch reactors set at temperatures of 16°C, 25°C and 35°C, with the biomass cultured at 35°C remaining in the already operational "*Candidatus* Brocadia fulgida" SBR. The enrichment cultures were grown on mineral medium containing $NO_2^-$, $NH_4^+$ and $NO_3^-$ prepared as described by van de Graaf et al. (1996). Anoxic conditions were maintained by continuously flushing the SBR with $Ar/CO_2$ (95/5%, 10 ml $min^{-1}$) and mixing of the SBR contents was performed using a turbine stirrer (ca. 250 rpm). The temperature of the three reactors was held constant using water jackets. The reactors were operated for 85 days and 20 ml biomass / reactor medium samples were taken weekly. The biomass suspension was centrifuged, the supernatant removed and the supernatant pH measured. The biomass pellet frozen at –20°C until further analysis.

The anammox bacteria in the 16°C and 25°C reactors were not cultured at their optimum temperature (35°C) and as a consequence were less active. As a result of this the nutrient loading to the SBR was gradually modified to prevent excess nutrients from poisoning the culture. $NO_2^-$ and $NO_3^-$ levels were monitored daily using test strips and when concentrations were elevated, the pump system was stopped

until the excess nutrients were consumed, or alternatively the pump system was set to a lower speed.

In addition, samples were taken from full-grown cultures of other anammox species grown at a variety of temperatures i.e. "*Candidatus* Brocadia anammoxidans" (35°C), "*Candidatus* Kuenenia stuttgartiensis" (35°C; aggregate cells, 23°C; single cells), "*Candidatus* Anammoxoglobus propionicus" (33°C) and "*Candidatus* Scalindua spp." (15°C, 20°C). Enrichment cultures were grown in sequencing batch reactors (SBR), as described previously (Kartal et al., 2006; Kartal et al., 2007; Strous et al., 1998).

### 9.2.2 *Environmental samples*

Particulate organic matter (POM) and surface sediments were obtained from a variety of sources (Table 1). POM samples were collected from the Namibian, Peruvian and Arabian Sea oxygen minimum zones and the Black Sea as described previously (Hamersley et al., 2007; Jaeschke et al., 2007; Wakeham et al., 2007; Kuypers et al., 2005). Surface sediment and sediment trap samples from the Santa Barbara basin was collected as described previously by Huguet et al. (2007). Surface sediments were also collected from the Gullmarsfjord (Sweden) in 2005, from the NW-African continental shelf and slope on a cruise with the R/V *Meteor* (leg M65/1) in summer 2005 (Mulitza et al., 2006), from the Irish and Celtic Sea on a cruise with the R/V *Pelagia* in spring 2006 (Jaeschke et al., in prep), and from the Black Sea on a cruise with the R/V *Knorr* (KN172/8) in May 2003 (Wakeham et al., 2007).

### 9.2.3 *Ladderane core lipid analysis*

Approximately 20 mg dry weight enrichment culture biomass was ultrasonically extracted using three times methanol, three times methanol / dichloromethane (DCM) and three times DCM. Combined extracts were dried using rotary evaporation and methylated using $BF_3$ in methanol, heating for 15 min at 60°C. An aliquot of the methylated total lipid extract was eluted in ethyl acetate over a small silica column containing (5%) $AgNO_3^-$ to remove polyunsaturated fatty acids. Fractions were subsequently dissolved in acetone and then filtered through a 0.45 µm, 4 mm diameter polytetrafluorethylene (PTFE) filter and analysed using high

performance liquid chromatography (HPLC)/atmospheric pressure chemical ionization (APCI) tandem mass spectrometry (MS)/MS.

**Table 1.** Source of samples investigated for ladderane lipids in this study

| Geographic area | n | Environment | Anammox species | Ladderanes previously reported |
|---|---|---|---|---|
| Namibian upwelling | 14 | Water column | "*Candidatus* Scalindua sorokinii'' and "*Candidatus* Scalindua brodae'' | Kuypers et al. (2005) |
| Peruvian upwelling | 22 | Water column | "*Candidatus* Scalindua sorokinii'' | Hamersley et al. (2007) |
| Gullmarsfjord (Sweden) | 3 | Surface sediment | "*Candidatus* Scalindua" | Hopmans et al. (2006) van de Vossenberg et al. (*in press*) |
| NW Africa | 13 | Surface sediment | "*Candidatus* Scalindua brodae" | - |
| Black sea | 15 | Sediment traps, surface sediment water column | "Candidatus Scalindua *sp.*" | Wakeham et al., 2007, *in press* |
| Santa Barbara Basin (California) | 3 | Sediment traps and surface sediment | na. | - |
| Irish Sea | 3 | Surface sediment | na. | Jaeschke et al. *in prep* |
| Celtic Sea | 3 | Surface sediment | na. | Jaeschke et al. *in prep* |
| Arabian Sea | 5 | Water column | na. | Jaeschke et al. (2007) |
| Not relevant | 11 | Laboratory SBR | "*Candidatus* Brocadia anammoxidans", "*Candidatus* Brocadia fulgida", "*Candidatus* Kuenenia stuttgartiensis", "*Candidatus* Anammoxoglobus propionicus", "*Candidatus* Scalindua *spp.*" | - |

na. = not available

The POM total lipid extracts from Namibian upwelling stations M182 and M202 were extracted as previously described by Kuypers et al. (2005) and from the Peruvian upwelling system as previously described by Hamersley et al. (2007). The total lipid extracts were methylated, dissolved in ethyl acetate and eluted over a small $AgNO_3$ (5%) impregnated silica column, subsequently dissolved in acetone and filtered as described above and finally analysed using HPLC/APCI-MS/MS.

POM from the Black Sea and Arabian Sea were extracted as described by Wakeham et al. (2007) and Jaeschke et al. (2007), respectively. Surface sediments from the Celtic and Irish Seas were ground and extracted as described by Jaeschke et al. (in preparation).

#### 9.2.3.1. HPLC/APCI-MS/MS analysis

Ladderane lipids in enrichment culture biomass, POM and sediments were analysed for $C_{18}$ [5], $C_{20}$ [5] and [3] ladderane fatty acids by HPLC/APCI-MS/MS according to Hopmans et al. (2006), with some modifications. Briefly, samples dissolved in acetone were injected onto a Zorbax Eclipse XDB $C_8$ column (3.0 x 250 mm, 5 µm, Agilent) kept at 30°C. Ladderane fatty acids were eluted using 0.18 ml $min^{-1}$ methanol and detected with a Quantum TSQ Ultra EM triple quadrupole mass spectrometer (Thermo Inc., San Jose, CA, USA), equipped with an ion max source with APCI probe, in selective reaction monitoring (SRM) mode.

In order to identify ladderane fatty acid methyl esters with shorter and/or longer chain lengths than previously reported, two enrichment culture biomass extracts (representing cold, 15°C, and warm, 35°C, cultured temperatures) were analysed using MS/MS in product ion scan mode (collision energy 25V, collision gas 1.5 mTorr Argon; chromatographic and source conditions were as described above) with parent masses set to match the predicted protonated molecules ($[M+H]^+$) of all possible $C_{16}$-$C_{24}$ ladderane fatty acid methyl esters, i.e. *m*/z 261.2, 289.3, 317.3, 345.3 373.3 and *m*/z 263.2, 291.3, 319.3, 347.3, 375.3 for the even numbered $C_{16}$ to $C_{24}$, [5] and [3] ladderane fatty acids, respectively. Based on the results of this experiment, the method published by Hopmans et al. (2006) was modified to include SRM transitions to quantify the $C_{16}$ to $C_{24}$, [5] and [3] ladderane fatty acids (Figs. 1&2; a, b, d, g, h, i and j) in addition to the $C_{18}$ [5] ladderane fatty acid and $C_{20}$ [5] and [3] ladderane fatty acids (Figs. 1&2, c, e, f).

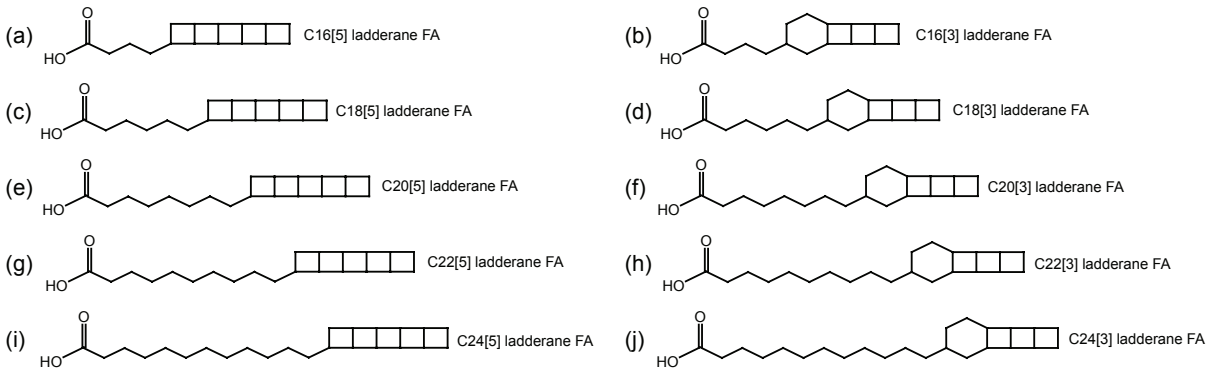


**Figure 1.** Homologous series of ladderane fatty acid core lipid structures. FA: fatty acid.

The SRM transitions used to detect the monoether were removed. For the ladderane fatty acids containing 5 concatenated cyclobutane moieties, transitions from their respective protonated molecules ($[M+H]^+$) to *m/z* 91.1,105.1,133.1, and 161.1 at collision energies (CE) of 37, 35, 24, and 19 V, respectively, were monitored. For the ladderane fatty acid containing 3 concatenated cyclobutanes, transitions from the respective protonated molecules ($[M+H]^+$) to *m/z* 93.1,121.1,135.1, and 163.1 at collision energies (CE) of 37, 24, 19, and 16 V, respectively, were monitored. Quantitative standards are only available for the $C_{20}$ [5] and [3] ladderane fatty acids (Fig. 1, e & f). Therefore, all ladderane fatty acids containing 5 concatenated cyclobutanes were quantified using an external standard curve of the $C_{20}$ [5] ladderane fatty acid, while the ladderane fatty acids containing 3 cyclobutanes were quantified using an external standard curve of the $C_{20}$ [3] ladderane fatty acid. All samples, with exception of the sediment samples, were re-analysed for $C_{16}$-$C_{24}$, [5] and [3] ladderane fatty acid methyl esters using this modified method.

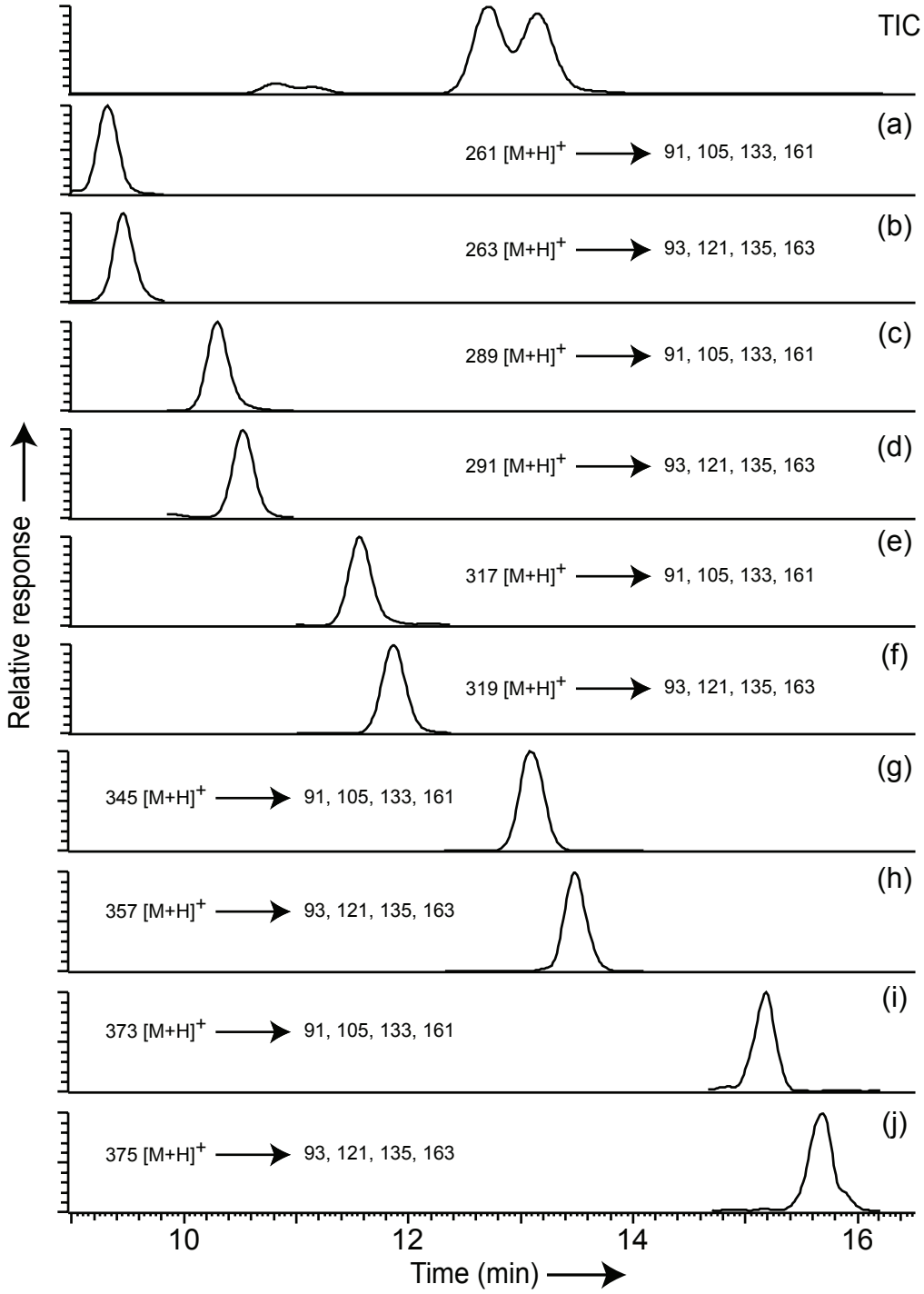


**Figure 2.** HPLC/APCI-MS/MS chromatogram illustrating previously unreported protonated molecules and selected product ions for shorter $C_{16}$ and longer $C_{24}$ ladderane lipids and previously reported $C_{18}$-$C_{22}$ ladderane lipids. (a)-(j) corresponds to structures in figure 1.

9.2.3.2 Ladderane phospholipid analysis

Freeze dried biomass was extracted using the modified Bligh and Dyer (1959) method of Boumann et al. (2006) and analysed using HPLC/Electron Spray Ionisation (ESI)-MS/MS. The Bligh Dyer extracts were analysed using an Agilent 1100 series LC (Agilent, San Jose, CA) coupled to a Thermo TSQ Quantum ultra EM triple quadruple mass spectrometer with an Ion Max source with ESI probe as described by Boumann et al. (2006), with minor modifications. Scanning for parent ions generating a product ion at m/z 184.1 represented the PC headgroup and identified the PC lipid species. Peak areas of individual PC lipids were integrated from peak areas in the first quadrupole MS and ratios were subsequently calculated.

## 9.3. Results and discussion

### *9.3.1 Anammox enrichment cultures*

Aliquots of an inoculum of "*Candidatus* Brocadia fulgida", initially cultured at 35°C were grown in three SBRs at 16, 25 and 35°C. Except for a brief period between 14 and 36 days, the distribution of the ladderane fatty acids in the 35°C reactor remained similar to the distribution found in the inoculum culture (Fig. 3). However, a major decrease in the relative amount of the $C_{20}$ [5] ladderane fatty acid was observed in the biomass cultured at lower temperatures. To quantify the change in the relative amount of the $C_{20}$ [5] ladderane fatty acid, its abundance was normalised relative to the other ladderane fatty acids. Since fatty acid chain length has been shown to change with temperature for generic fatty acids in other bacteria (Russell, 1990; Russell, 1984; Russell, 1997; Sandercock and Russell, 1980; Sinensky, 1974), we normalised it on the fatty acids with equivalent amounts of cyclobutane rings, but with different chain lengths. Therefore, we have quantified the trends in ladderane chain length using the iNdex of Ladderane lipids with 5 cyclobutane rings ($NL_5$):

$$NL_5 = \frac{C_{20}[5]\text{fatty acid}}{(C_{18}[5]\text{fatty acid} + C_{20}[5]\text{fatty acid})} \qquad [1]$$

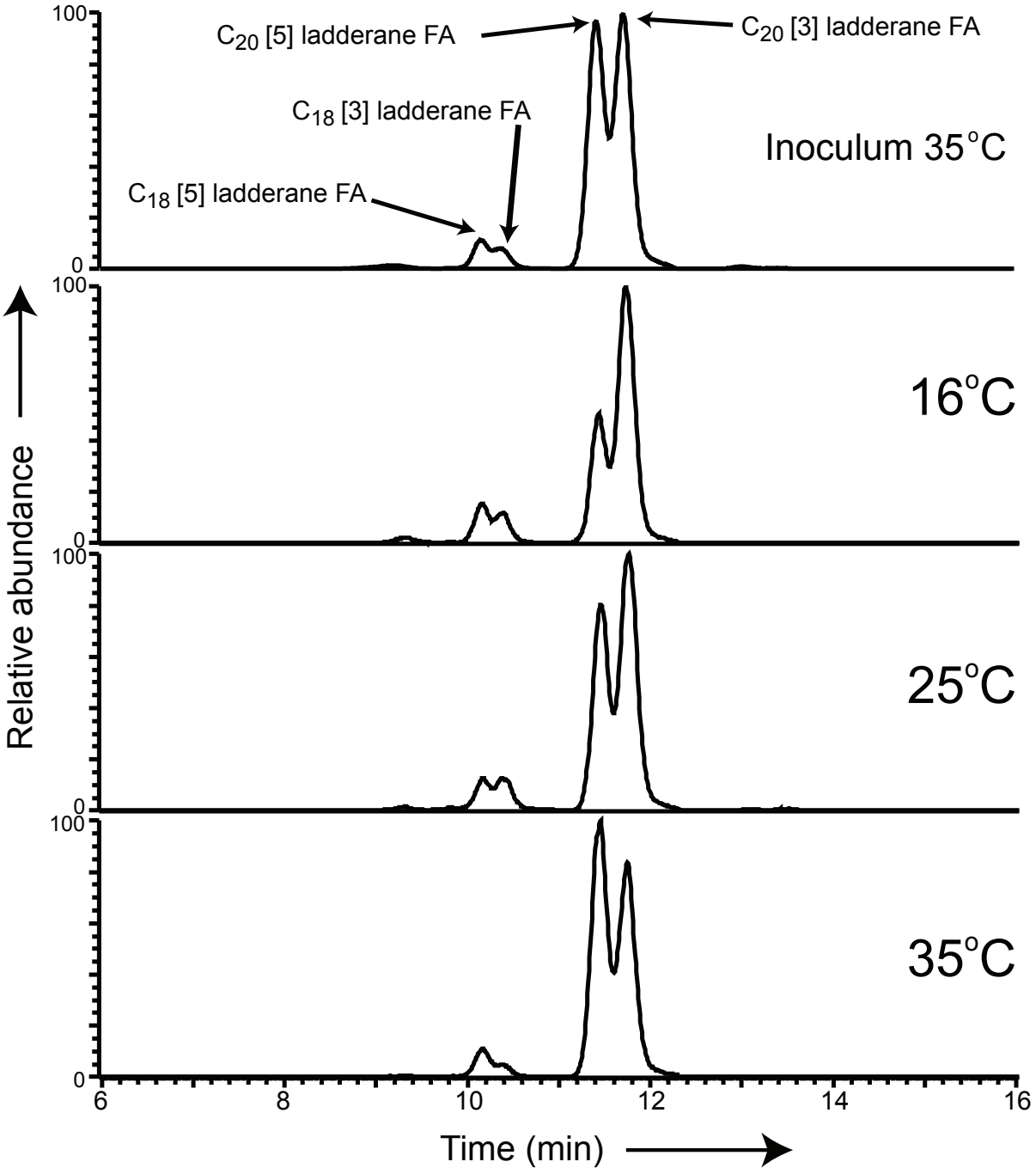


**Figure 3.** HPLC base peak chromatograms showing the distribution of ladderane fatty acids in SBR enrichment cultures of "*Candidatus* Brocadia fulgida" grown at 16, 25 and 35°C, on the 75th day of culturing. FA: fatty acid. The largest variation with temperature is observed with the $C_{20}$ [3] ladderane fatty acid and the $C_{20}$ [5] ladderane fatty acid.

The $NL_5$ appeared to function in response to the *in situ* temperature, with the $NL_5$ from the 35°C cultured biomass remaining around an index value of 0.9 (Fig. 4), except for a change in the fatty acid composition after 14 days, which coincided with the addition of a supplementary batch of "*Candidatus* Brocadia fulgida" kept at relatively low (8°C) temperature. In contrast, the $NL_5$ in anammox biomass cultured at 25°C showed a gradual decrease in the index value from 0.89 to 0.84 and the $NL_5$ in anammox biomass cultured at 16°C displayed a sharp decrease in value from 0.89 to between 0.65 and 0.75 (Fig. 4). The adjustment of the membrane fatty acid composition in the 16°C and 25°C reactors took 50 and 26 days, respectively. Since the typical doubling time of anammox bacteria is around 14 days, it appears that "*Candidatus* Brocadia fulgida" regulates its membrane lipids quickly to adapt to the new temperature regime, especially considering that some of the new signal will be diluted by the divided cells, which already contain membranes adapted to the *in situ* temperature of the inoculum.

We also analysed the anammox biomass for intact ladderane lipids containing PC head groups to investigate if the adaptation of chain length with temperature would be visible in the intact polar lipids (data not shown). At the start of the experiment, lipids containing PC head groups were nearly identical in composition at all temperatures, with a high abundance of $C_{20}$ [3] ladderane fatty acids ether or ester bound to the *sn*-1 position of the glycerol backbone. Samples taken at the end of the experiment showed a distinct difference in composition, i.e. the base peak chromatogram of intact ladderane lipids from the 16°C and 25°C reactors showed an increased abundance of ladderane lipids with shorter chain $C_{18}$ [5] ladderane moieties ether/ester bound to the glycerol *sn*-1 position. In contrast, the base peak chromatogram of intact ladderane lipids from the 35°C reactor was almost identical to the chromatograms obtained at the start of the experiment. This suggests that "*Candidatus* Brocadia fulgida" alters its polar ether/ester ladderane lipid composition in response to the *in situ* temperature.

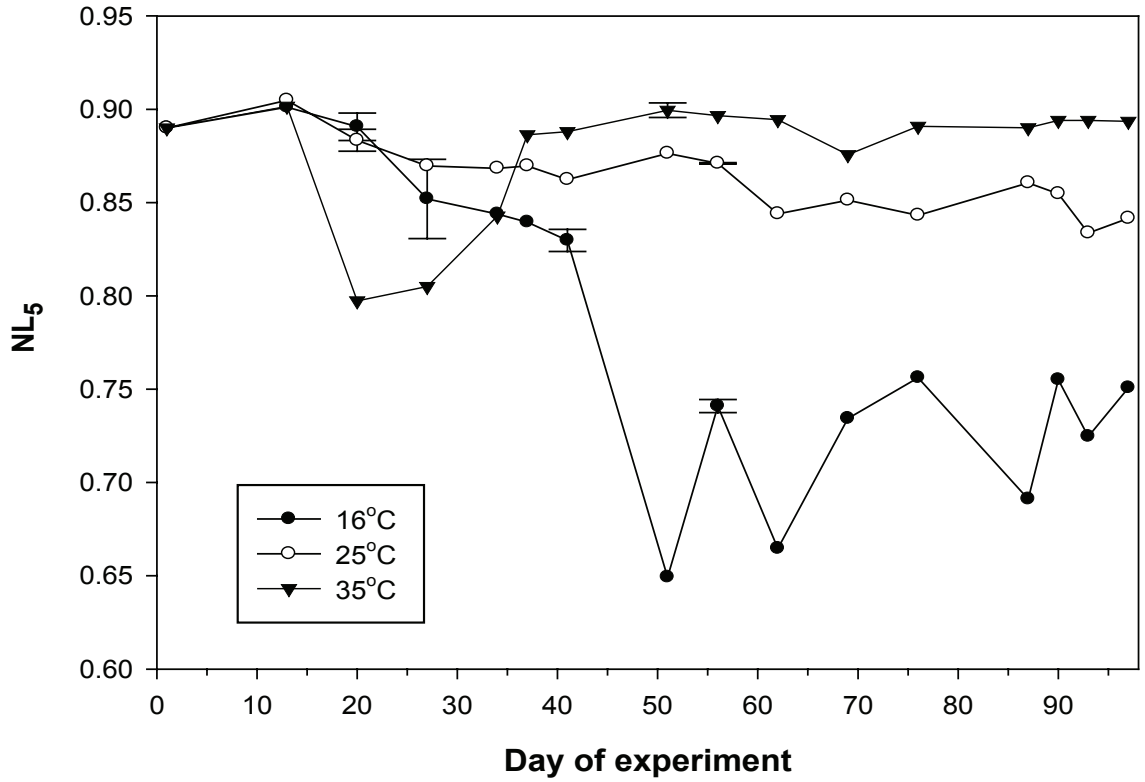


**Figure 4**. Ratio between ladderane lipids containing 5 cyclobutane rings and hydrocarbon chain lengths of 18 and 20 carbon atoms plotted over time for anammox biomass cultured in 16, 25 and 35°C reactors. The control reactor was kept at 35°C whereas biomass in the 16°C and 25°C reactors was originally cultured at 35°C. Error bars relate to the standard deviation of the ratio calculated from duplicate or triplicate samples worked up and analysed separately.

In order to investigate if other species of anammox bacteria showed the same behaviour of adjusting their ladderane lipid chain length with temperature, we analysed ladderane lipids in SBR enrichment cultures of three other species of anammox bacteria, "*Candidatus* Anammoxoglobus propionicus", "*Candidatus* Scalindua *spp.*" and "*Candidatus* Kuenenia stuttgartiensis" (Table 2). Indeed, species cultured at lower temperatures contained higher amounts of the $C_{18}$ [5] ladderane fatty acid and consequently lower percentages of the $C_{20}$ [5] ladderane fatty acid, when compared to biomass grown at higher temperatures. Therefore, in all anammox species a lower $NL_5$ was calculated from biomass cultured at lower temperatures and *vice versa* (Table 2).

**Table 2.** Relative percentages of ladderane fatty acids in a selection of enrichment cultures and environmental samples. FA=fatty acid.

| | | | Relative Amount Ladderane (%) | | | |
|---|---|---|---|---|---|---|
| **Culture** | **Location** | **Temperature °C** | **$C_{18}$[5]FA** | **$C_{20}$[3]FA** | **$C_{20}$[5]FA** | **$NL_5$** |
| *"Candidatus* A. propionicus*"* | Radboud University Nijmegen | 35 | 6 | 26 | 68 | 0.92 |
| *"Candidatus* A. propionicus*"* | idem | 23 | 10 | 37 | 53 | 0.87 |
| *"Candidatus* B. fulgida*"* | idem | 35 | 8 | 28 | 64 | 0.90 |
| *"Candidatus* B. fulgida*"* | idem | 35 | 8 | 27 | 65 | 0.90 |
| *"Candidatus* B. fulgida*"* | idem | 35 | 8 | 27 | 65 | 0.93 |
| *"Candidatus* K. stuttgartiensis*"* | idem | 35 | 7 | 27 | 66 | 0.90 |
| *"Candidatus* K. stuttgartiensis*"* | idem | 35 | 8 | 27 | 65 | 0.90 |
| *"Candidatus* K. stuttgartiensis*"* | idem | 35 | 8 | 27 | 65 | 0.90 |
| *"Candidatus* K. stuttgartiensis*"* | idem | 35 | 7 | 27 | 66 | 0.90 |
| *"Candidatus* Scalindua*"* | idem | 15 | 38 | 31 | 31 | 0.44 |
| *"Candidatus* Scalindua*"* | idem | 20 | 16 | 42 | 42 | 0.73 |
| **POM** | **(Location)** | | | | | |
| Peru Upwelling (30m) | 12°05'S, 77°30'W | 15.4 | 50 | 23 | 27 | 0.35 |
| Peru Upwelling (40m) | 12°05'S, 77°30'W | 15.3 | 60 | 23 | 17 | 0.22 |
| Peru Upwelling (60m) | 12°05'S, 77°30'W | 14.7 | 68 | 16 | 16 | 0.19 |
| Peru Upwelling (92m) | 12°05'S, 77°30'W | 14.2 | 61 | 24 | 15 | 0.20 |
| Peru Upwelling (200m) | 12°04'S, 77°99'W | 13.1 | 63 | 17 | 20 | 0.22 |
| Peru Upwelling (400m) | 12°04'S, 77°99'W | 10.0 | 59 | 25 | 16 | 0.15 |
| Peru Upwelling (35m) | 10°96'S, 77°96'W | 16.1 | 33 | 45 | 22 | 0.42 |
| Peru Upwelling (130m) | 8°99'S, 79°96'W | 13.8 | 25 | 52 | 23 | 0.48 |
| Peru Upwelling (270m) | 8°99'S, 79°96'W | 12.4 | 40 | 44 | 16 | 0.28 |
| Namibian Upwelling (60m) | -22°00'S, 13°75'E | 13.6 | 60 | 21 | 19 | 0.24 |
| Namibian Upwelling (65m) | -22°00'S, 13°75'E | 13.6 | 61 | 18 | 21 | 0.25 |
| Namibian Upwelling (70m) | -22°00'S, 13°75'E | 13.6 | 64 | 15 | 21 | 0.25 |
| Namibian Upwelling (75m) | -22°00'S, 13°75'E | 13.5 | 57 | 19 | 24 | 0.25 |
| Namibian Upwelling (80m) | -22°00'S, 13°75'E | 13.5* | 59 | 17 | 24 | 0.29 |
| Arabian Sea (500m) | 15°58'N, 61°29'E | 12.5 | 15 | 72 | 13 | 0.45 |
| Arabian Sea (500m) | 17°24'N, 58°49'E | 12.5 | 19 | 63 | 18 | 0.49 |
| Arabian Sea (500m) | 17°41'N, 57°50'E | 12.5 | 20 | 62 | 18 | 0.48 |
| Arabian Sea (450m) | 17°44'N, 57°29'E | 13.0 | 18 | 66 | 16 | 0.47 |
| Arabian Sea (500m) | 19°13'N, 58°31'E | 12.5 | 23 | 61 | 16 | 0.42 |
| Black Sea (62m) | 43°33'N, 34°00'W | 7.8 | 61 | 32 | 7 | 0.10 |
| Black Sea (85m) | 43°33'N, 34°00'W | 8.3 | 70 | 21 | 9 | 0.11 |
| Black Sea (100m) | 43°33'N, 34°00'W | 8.4 | 46 | 45 | 9 | 0.16 |
| Black Sea (200m) | 43°33'N, 34°00'W | 8.7 | 63 | 27 | 10 | 0.14 |
| Black Sea (300m) | 43°33'N, 34°00'W | 8.8 | 68 | 26 | 6 | 0.10 |
| Black Sea (400m) | 43°33'N, 34°00'W | 8.8 | 48 | 47 | 5 | 0.09 |
| **Surface sediment** | | | | | | |
| Irish Sea (104 m) | 53°52'N, 5°35'W | 8 | 56 | 25 | 19 | 0.19 |
| Celtic Sea (2006 m) | 48°03'N, 9°51'W | 3.8 | 58 | 25 | 16 | 0.22 |
| Celtic Sea (497 m) | 48°16'N, 9°42'W | 10.5 | 72 | 16 | 12 | 0.14 |
| N.W. Africa (2964 m) | 15°36'N, 18°20'W | 2* | 74 | 15 | 10 | 0.12 |
| N.W. Africa (498 m) | 13°50'N, 17°29'W | 10 | 70 | 14 | 16 | 0.18 |
| N.W. Africa (1234 m) | 09°21'N, 17°22'W | 4.5 | 70 | 15 | 15 | 0.18 |
| N.W. Africa (666 m) | 08°52'N, 14°57'W | 7 | 68 | 16 | 16 | 0.19 |
| Santa Barbara Basin (590 m) | 34°14'N, 120°02'W | 6.0[1], 15.1[2] | 26 | 18 | 56 | 0.72 |

[1]Surface sediment temperature
[2]Upper water column temperature
*Used in figure 5

### 9.3.2 *Particulate organic matter and sediments*

To investigate whether the ladderane fatty acid composition also varied with temperature in natural settings, we analysed POM and surface sediments where anammox activity had been previously documented. Fig. 5 shows three base peak chromatograms from POM and surface sediment at *in situ* temperatures from the 'extreme temperature ends' of the environmental data set (Table 2). The chromatogram clearly shows that the abundance of the $C_{20}$ [5] ladderane fatty acid is lower in the surface sediment of the N.W. African continental slope (at ca.

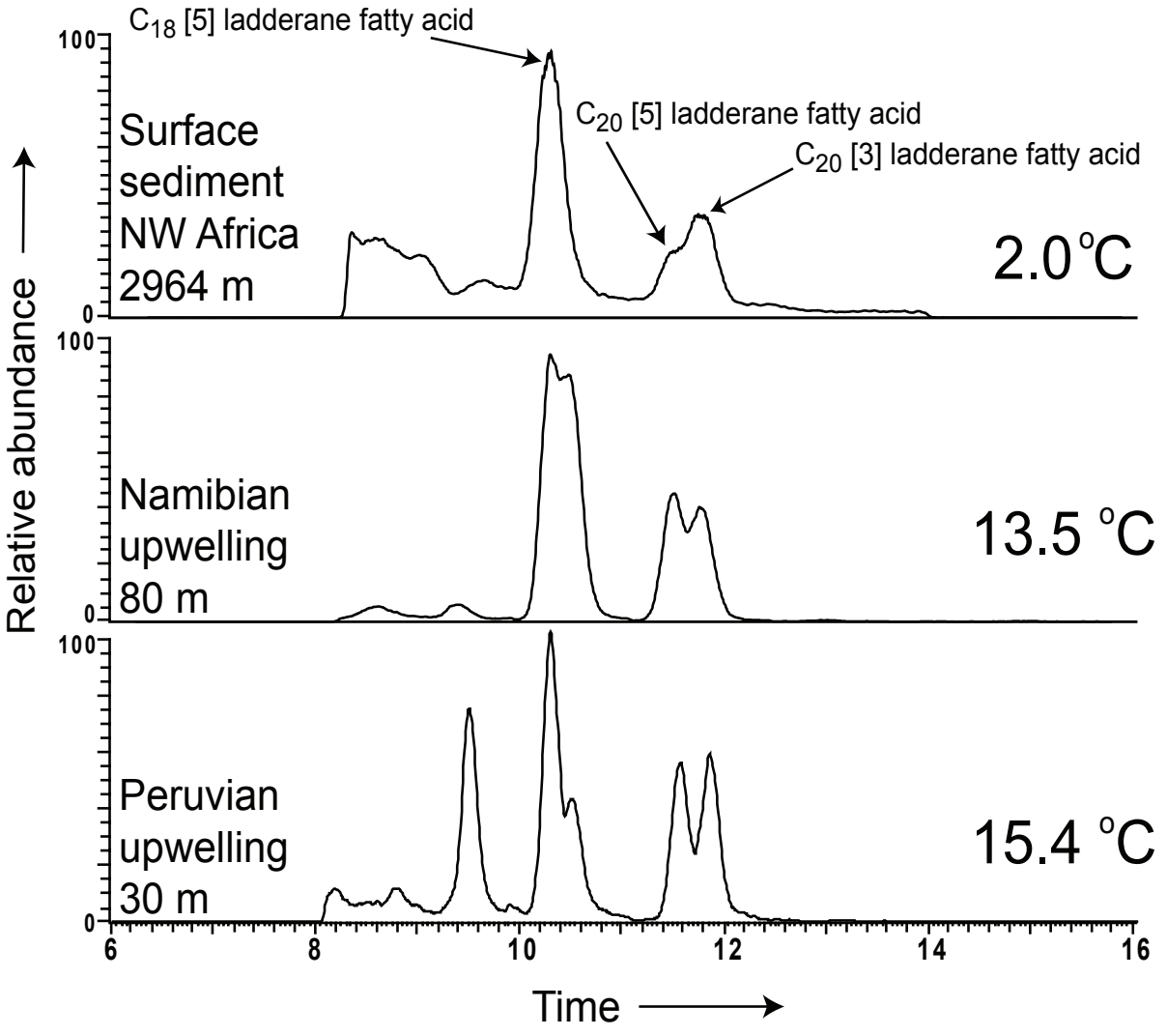


**Figure 5.** HPLC base peak chromatograms showing the differences in the distribution of ladderane fatty acids produced at *in situ* temperatures in surface sediment from the N.W. African continental slope and POM from the Namibian and Peruvian upwelling systems.

3,000 m water depth, where the temperature was 2°C), in comparison to the higher abundance of the $C_{20}$ [5] ladderane fatty acid in the Peru POM sample (15.4°C). Since the ladderane fatty acids in POM and sediment showed a similar response to temperature as observed in the anammox enrichment cultures, we calculated $NL_5$ for the environmental samples and plotted this together with the enrichment culture data against the *in situ* temperature. This resulted in a significant 4th order sigmoidal relationship ($R^2$= 0.92 p=<0.0001, *n*=157) between the $NL_5$ and temperature (Fig. 6). The largest amount of change in ladderane lipid chain length takes place between 12°C and 20°C, while there is no significant change in chain length at temperatures <12°C and >20°C. This trend strongly suggests that all anammox bacteria change their ladderane fatty acid chain length to adapt to the *in situ* temperature over the range of 12 to 20°C.

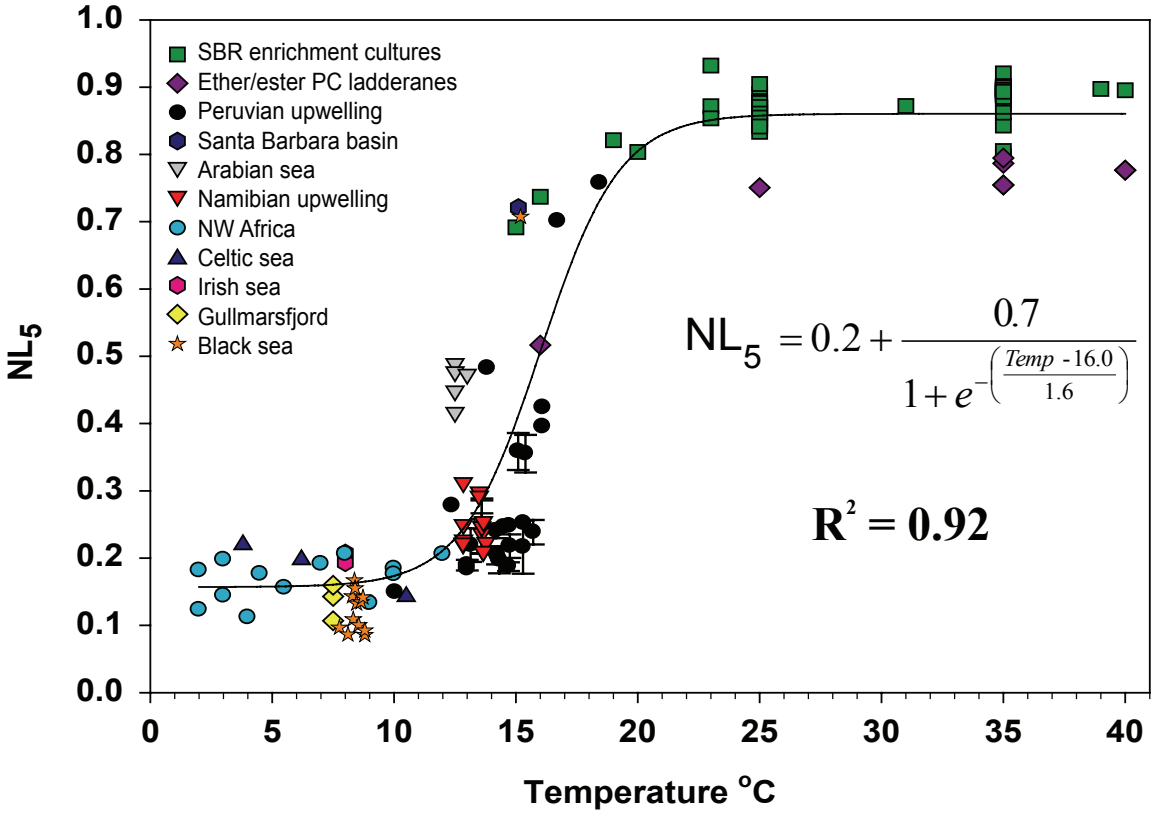


**Figure 6**. The $NL_5$ from a range of marine environments and sequencing batch reactor enrichment cultures plotted against temperature. The equation relates to an empirical 4th order sigmoidal regression. Error bars related to the standard deviation of samples independently worked up and analysed, ranged from 0.01 to 0.04.

*9.3.3 Longer and shorter ladderane fatty acids*

The large change in the chain length of ladderane fatty acids, between 12 and 20°C and apparently constant chain lengths at temperatures <12°C and >20°C raises the question if ladderane fatty acids with acyl chain lengths <5 and >9 carbon atoms can also be found in anammox bacteria in addition to the previously identified $C_{18}$, $C_{20}$ and $C_{22}$ ladderane fatty acids (Sinninghe Damsté et al., 2005; Rattray et al., *in press*).

Based on these results, the SRM method (Hopmans et al., 2006) was modified to include SRM transitions specific for $C_{16,}$ $C_{22}$ and $C_{24}$ [5] and [3] ladderane fatty acids. This showed that the $C_{18}$ and $C_{20}$ [5] and [3] ladderane fatty acids were always dominant, and that the $C_{16}$, $C_{22}$ and $C_{24}$ [5] and [3] ladderane fatty acids constituted only a minor fraction of the ladderane fatty acids. In fact, the latter fatty acids never comprised more than 2% of the total ladderane fatty acids extracted from the anammox bacterial biomass grown at 16°C, 25°C and 35°C. These lipids were also observed to form a minor fraction of the total ladderane lipid composition of the POM and sediment samples. Since the $C_{16}$, $C_{22}$ and $C_{24}$ [5] and [3] ladderane fatty acid components constituted a minor part of the total ladderane fatty acids, we assume here that they do not play a major role in the biochemical functioning of membranes containing ladderane lipids.

## 9.4 Implications

Our results clearly show that anammox bacteria adjust their membrane composition according to temperature, i.e. longer chain lengths with higher temperatures and *vice versa*. Adjusting the production of fatty acids with different chain lengths as a response to temperature has previously been reported in a number of bacteria. For example, when *Micrococcus cryophilus* was cultured over a temperature range of –4 to 25°C, a four fold variation in the ratio of the $C_{18:1}$ versus the $C_{16}$ fatty acid (and no change in the type of unsaturation) was observed (Sandercock and Russell, 1980). In the gammaproteobacterium *Escherichia coli* the membrane lipid properties are also dependent on the temperature at which the cells are grown (Haest et al., 1969). In this case, adaptation was achieved by increasing the relative abundance of saturated long chained fatty acids, as a response to an increase in the growth temperature (Sinensky, 1974). The response of membrane lipids in *M. cryophilus* and *E. coli* to temperature, is similar to what was observed occurring with

the ladderane lipid chain length in anammox bacteria, thus this behaviour is likely to be due to homeoviscous adaptation. Shortening the acyl chain length has the effect of disrupting acyl chain packing and thereby lowering the liquid-crystaline transition gel phase transition temperature, in comparison to packing with only longer chain lipids (Russell, 1984). The alteration of membrane lipids in turn allows the organism to modulate the activity of intrinsic proteins and maintain the rate of reactions for functions like electron transport, ion pumping and nutrient uptake (McElhaney, 1982; Russell, 1990; Sandermann and Strominger, 1972). It is therefore possible that the same mechanisms are work within the ladderane lipid containing membranes of anammox bacteria.

Since our results suggest that different species of anammox bacteria in different environments, modify ladderane lipid fatty acid chain length in a similar way to temperature, one possible application of our results could be to discriminate between the origins of ladderane lipids in marine sediments, i.e. if ladderane lipids are produced *in situ* in cold surface sediments or if they originate from the warmer upper water column. The origin of these lipids can be especially problematic in cases where there is an anoxic or oxygen minimum water column overlying the anoxic sediment as in both the water column and/or sedimentary environment anammox activity can be expected. This approach can be illustrated by analysis of a surface sediment from the Santa Barbara basin, which has an overlying anoxic water column. Analysis of the sedimentary ladderane composition gave an $NL_5$ value of 0.72. This would correspond, using our sigmoidal correlation curve, to temperatures of about 15°C, which is substantially higher than the surface sediment temperature of the Santa Barbara basin surface sediment (6°C). Thus, the $NL_5$ value may indicate that these ladderane lipids are fossil remnants, which predominantly originated from the warmer upper water column rather than the colder surface sediments. This approach could be especially helpful in studies where fossil ladderane lipids are used to reconstruct past anammox activity.

## 9.5 Conclusions

Our study has shown that different species of anammox bacteria, indigenous to either wastewater or marine environments, display the same mechanism of ladderane lipid composition adaptation in response to temperature, i.e. the amount of ladderane lipids containing shorter acyl chains increase at lower temperatures and *vice versa*, particularly between 12°C and 20°C. This variation in ladderane chain length in anammox bacteria, which is likely to be a form of homeoviscous adaptation, has been quantified in the $NL_5$, which strongly correlates with *in situ* temperature. Using the $NL_5$ it seems possible to determine the origin of ladderane lipids in sediments, i.e. from *in situ* production in the relatively cold sediment or from transport and preservation of lipids derived from the warmer upper water column.

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## references


Aitzetmuller,K., Tsevegsuren,N., and Vosmann,K. (1997) A new allenic fatty acid in Phlomis (Lamiaceae) seed oil. *Fett-Lipid* **99:** 74-78.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**: 403-410.

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA (1990) Combination of 16S Ribosomal-RNA-Targeted Oligonucleotide Probes with Flow-Cytometry for Analyzing Mixed Microbial-Populations. *Appl Environ Microbiol* **56**:1919-1925

Amann,R.I., Ludwig,W., and Schleifer,K.H. (1995) Phylogenetic Identification and In Situ Detection of Individual Microbial-Cells Without Cultivation. *Microbiol Rev* **59:** 143-169.

Arrigo, K.R. (2005) Marine microorganisms and global nutrient cycles. *Nature* **437**: 349-355.

Boumann, H.A., Hopmans, E.C., van de Leemput, I., Op den Camp, H.J.M., van de Vossenberg, J., Strous, M. et al. (2006) Ladderane phospholipids in anammox bacteria comprise phosphocholine and phosphoethanolamine headgroups. *FEMS Microbiology Letters* **258**: 297-304.

Bassler,H.A., Flood,S.J.A., Livak,K.J., Marmaro,J., Knorr,R., and Batt,C.A. (1995) Use of A Fluorogenic Probe in A PCR-Based Assay for the Detection of Listeria-Monocytogenes. *Appl Environ Microbiol* **61:** 3724-3728.

Bagby,M.O., Smith,C.R., and Wolff,I.A. (1965) Laballenic Acid . A New Allenic Acid from Leonotis Nepetaefolia Seed Oil. *J Org Chem* **30:** 4227-&.

Bligh, E.G., Dyer, W.J. (1959) A Rapid Method of Total Lipid Extraction and Purification. *Can J Biochem Physiol* **37**:911-917

Bloch,K. (1965) Biological Synthesis of Cholesterol. *Science* **150:** 19-28.

Boschker,H.T.S., Nold,S.C., Wellsbury,P., Bos,D., de Graaf,W., Pel,R. et al. (1998) Direct linking of microbial populations to specific biogeochemical processes by C-13-labelling of biomarkers. *Nature* **392:** 801-805.

Boumann, H.A., Hopmans, E.C., van de Leemput, I., Op den Camp, H.J.M., van de Vossenberg, J., Strous, M., Jetten, M.S.M., Sinninghe Damsté, J.S., Schouten, S., (2006) Ladderane phospholipids in anammox bacteria comprise phosphocholine and phosphoethanolamine headgroups. *FEMS Microbiol Lett* **258**:297-304

Brandes, J. A., A. H. Devol, T. Yoshinari, D. A. Jayakumar, and Naqvi, S. W. A. (1998). Isotopic composition of nitrate in the central Arabian Sea and eastern tropical North Pacific: A tracer for mixing and nitrogen cycles. *Limnol Oceanogr* **43:** 1680-1689.

Brandes, J.A., Devol, A.H., and Deutsch, C. (2007) New developments in the marine nitrogen cycle. *Chem Revs* **107**: 577-589.

Brassell,S.C., Eglinton,G., Marlowe,I.T., Pflaumann,U., and Sarnthein,M. (1986) Molecular stratigraphy: a new tool for climatic assessment. *Nature* **320:** 129-133.

Broda,E. (1977) Two kinds of lithotrophs missing in nature. *Z allg Mikrobiol* **17:** 491-493.

Cheek,J. and Broderick,J.B. (2001) Adenosylmethionine-dependent iron-sulfur enzymes: versatile clusters in a radical new role. *Journal of Biological Inorganic Chemistry* **6:** 209-226.

Capone,D.G., Burns,J.A., Montoya,J.P., Subramaniam,A., Mahaffey,C., Gunderson,T. et al. (2005) Nitrogen fixation by Trichodesmium spp.: An important source of new nitrogen to the tropical and subtropical North Atlantic Ocean. *Global Biogeochem Cy* **19**.

Capone,D.G. and Knapp,A.N. (2007) Oceanography - A marine nitrogen cycle fix? *Nature* **445:** 159-160.

Codispoti, L. A., and Packard, T. T. (1980). Denitrification rates in the eastern tropical South Pacific. *J Mar Res* **38:** 453-477.

Codispoti,L.A. and Christensen,J.P. (1985) Nitrification, Denitrification and Nitrous-Oxide Cycling in the Eastern Tropical South-Pacific Ocean. *Mar Chem* **16:** 277-300.

Codispoti, L. A., J. A. Brandes, J. P. Christensen, A. H. Devol, S. W. A. Naqvi, H. W. Paerl, and Yoshinari, T. (2001). The oceanic fixed nitrogen and nitrous oxide budgets: Moving targets as we enter the Anthropocene? *Sci Mar* **65:** 85-105.

Cole J. R., B. Chai, R. J. Farris, Q. Wang, S. A. Kulam, D. M. McGarrell, G. M. Garrity, and Tiedje, J. M. (2005). The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Res* 2005 **1** 33(Database Issue):D294-D296.

Copin-Montégut, C., and Raimbault, P. (1994). The Peruvian upwelling near 15S in August 1986. Results of continuous measurements of physical and chemical properties between 0 and 200 m depth. *Deep-Sea Res I* **41:** 439-467.

Daims H, Bruhl A, Amann R, Schleifer KH, Wagner M (1999) The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. *System Appl Microbiol* **22**:434-444

Dalsgaard,T. and Thamdrup,B. (2002) Factors controlling anaerobic ammonium oxidation with nitrite in marine sediments. *Appl Environ Microbiol* **68:** 3802-3808.

Dalsgaard,T., Canfield,D.E., Petersen,J., Thamdrup,B., and Acuña-Gonzàlez,J. (2003) $N_2$ production by the anammox reaction in the anoxic water column of Golfo Dulce, Costa Rica. *Nature* **422:** 606-608.

Dalsgaard, T., Thamdrup, B., and Canfield, D.E. (2005) Anaerobic ammonium oxidation (anammox) in the marine environment. *Research in Microbiology* **156**: 457-464.

Deutsch, C., N. Gruber, R. M. Key, and Sarmiento, J. L. (2001). Denitrification and $N_2$ fixation in the Pacific Ocean. *Global Biogeochem Cycles* **15**: 483-506.

Deutsch,C., Sarmiento,J.L., Sigman,D.M., Gruber,N., and Dunne,J.P. (2007) Spatial coupling of nitrogen inputs and losses in the ocean. *Nature* **445:** 163-167.

Devol, A.H. (2003) Nitrogen cycle - Solution to a marine mystery. *Nature* **422**: 575-576.

Doney,S.C., Mahowald,N., Lima,I., Feely,R.A., MacKenzie,F.T., Lamarque,J.F., and Rasch,P.J. (2007) Impact of anthropogenic atmospheric nitrogen and sulfur deposition on ocean acidification and the inorganic carbon system. *Proc Natl Acd Sci USA* **104:** 14580-14585.

Dowling,N.J.E., Widdel,F., and White,D.C. (1986) Phospholipid Ester-Linked Fatty-Acid Biomarkers of Acetate-Oxidizing Sulfate-Reducers and Other Sulfide-Forming Bacteria. *J Gen Microbiol* **132:** 1815-1825.

Finnerty,W.R. (1989) Microbial lipid metabolism. In *Microbial Lipids Volume 2*. London: Academic Press Limited, pp. 525-566.

Glöckner, F. O., R. Amann, A. Alfreider, J. Pernthaler, R. Psenner, K. Trebesius, and K. H. Schleifer (1996). An in situ hybridization protocol for detection and identification of planktonic bacteria. *System. Appl. Microbiol*. **19**: 403-406.

Grasshoff, K., M. Ehrdardt, K. Kremling, and Anderson, L. G. (1999). *Methods of Seawater Analysis.* Wiley.

Gunstone,F.D., Pollard,M.R., Scrimgeour,C.M., Gilman,N.W., and Holland,B.C. (1976) Fatty Acids. Part 48. $^{13}$C Nuclear Magnetic Resonance Studies of Acetylenic Fatty Acids. *Chem Phys Lipids* **17:** 1-13.

Gunstone,F.D. (1993) High Resolution $^{13}$C NMR Study of Synthetic Branched-Chain Acids and of Wool Wax Acids and Isostearic Acid. *Chem Phys Lipids* **65:** 155-163.

Gutiérrez, D., E. Enríquez, S. Purca, J. Pasapera, L. Quipúzcoa, G. Flores, and Graco, M. In press. Remotely-driven temporal variations of the subsurface dissolved oxygen content and responses of the continental shelf benthic subsystem off Central Peru. *In* Extended Abstracts Book. *International Conference on the Humboldt Current System*, 27 Nov – 2 Dec, Lima, Peru.

Guven,D., van de Pas-Schoonen,K., Schmid,M.C., Strous,M., Jetten,M.S.M., Sozen,S. et al. (2004) Implementation of the anammox process for improved nitrogen removal. *Journal of Environmental Science and Health Part A-Toxic/Hazardous Substances & Environmental Engineering* **39:** 1729-1738.

Guy,R.D., Fogel,M.L., and Berry,J.A. (1993) Photosynthetic Fractionation of the Stable Isotopes of Oxygen and Carbon. *Plant Physiol* **101:** 37-47.

Haest,C.W.M., Degier,J., and Deenen,L.L.M. (1969) Changes in Chemical and Barrier Properties of Membrane Lipids of E-Coli by Variation of Temperature of Growth. *Chem Phys Lipids* **3:** 413-417.

Hamersley, M.R., Lavik, G., Woebken, D., Rattray, J.E., Lam, P., Hopmans, E.C. et al. (2007) Anaerobic ammonium oxidation in the Peruvian oxygen minimum zone. *Limnology and Oceanography* **52**: 921-931.

Heath,R.J. and Rock,C.O. (1996) Roles of the FabA and FabZ beta-hydroxyacyl-acyl carrier protein dehydratases in Escherichia coli fatty acid biosynthesis. *Journal of Biological Chemistry* **271:** 27795-27801.

Hopmans, E.C., Kienhuis, M.V.M., Rattray, J.E., Jaeschke, A., Schouten, S., and Sinninghe Damsté, J.S. (2006) Improved analysis of ladderane lipids in biomass and sediments using high-performance liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry. *Rapid Communications in Mass Spectrometry* **20**: 2099-2103.

Huguet,C., Schimmelmann,A., Thunell,R., Lourens,L.J., Damste,J.S.S., and Schouten,S. (2007) A study of the TEX86 paleothermometer in the water column and sediments of the Santa Barbara Basin, California. *Paleoceanography* **22**.

Hulth, S., R. C. Aller, and Gilbert, F. (1999). Coupled anoxic nitrification/manganese reduction in marine sediments. *Geochim Cosmochim Acta* **63**: 49-66.

Hulth, S., Aller, R.C., Canfield, D.E., Dalsgaard, T., Engström, P., Gilbert, F. et al. (2005) Nitrogen removal in marine environments: recent findings and future research challenges. *Marine Chemistry* **94**: 125-145.

Hsu,C.C. and Fox,C.F. (1970) Induction of Lactose Transport System in A Lipid-Synthesis-Defective Mutant of *Escherichia Coli*. *J Bacteriol* **103:** 410-416.

Isaka, K., Y. Date, T. Sumino, S. Yoshie, and Tsuneda, S. (2006). Growth characteristic of anaerobic ammonium-oxidizing bacteria in an anaerobic biological filtrated reactor. *Appl Microbiol Biotechnol* **70**: 47-52.

Jaeschke,A., Hopmans,E.C., Wakeham,S.G., Schouten,S., and Damste,J.S.S. (2007) The presence of ladderane lipids in the oxygen minimum zone of the Arabian Sea indicates nitrogen loss through anammox. *Limnol Oceanogr* **52:** 780-786.

Jarrett,J.T. (2003) The generation of 5 '-deoxyadenosyl radicals by adenosylmethionine-dependent radical enzymes. *Current Opinion in Chemical Biology* **7:** 174-182.

Jie,M.S.F.L., Lau,M.M.L., Lam,C.N.W., Alam,M.S., Metzger,J.O., and Biermann,U. (2003) Novel halo-oxo-allenic fatty ester derivatives from epoxidized methyl santalbate (methyl trans-11-octadecen-9-ynoate). *Chem Phys Lipids* **125:** 93-101.

Jetten,M.S.M., Sliekers,O., Kuypers,M., Dalsgaard,T., van Niftrik,L., Cirpus,I. et al. (2003) Anaerobic ammonium oxidation by marine and freshwater planctomycete-like bacteria. *Appl Microbiol Biotechnol* **63:** 107-114.

Kannenberg,E. and Poralla,K. (1999) Hopanoid Biosynthesis and Function in Bacteria. *Naturwissenschaften* **86:** 168-176.

Kartal, B., Koleva, M., Arsov, R., van der Star, W., Jetten, M.S.M., and Strous, M. (2006) Adaptation of a freshwater anammox population to high salinity wastewater. *Journal of Biotechnology* **126**: 546-553.

Kartal,B., Rattray,J., van Niftrik,L.A., van der Star,W.R., van de Vossenberg,J., Schmid,M.C. et al. (2007) Candidatus "Anammoxoglobus propionicus" a new

propionate oxidizing species of anaerobic ammonium oxidizing bacteria. *Syst Appl Microbiol* **30:** 39-49.

Kartal, B., van Niftrik, L., Rattray, J., van de Vossenberg, J.L., Schmid, M.C., Sinninghe Damsté, J.S., Jetten, M.S.M., and Strous M (2008) *Candidatus* 'Brocadia fulgida': an autofluorescent anaerobic ammonium oxidizing bacterium. FEMS Microbiol Ecol. **63(1)**: 46-55.

Knoblauch,C., Sahm,K., and Jorgensen,B.B. (1999) Psychrophilic sulfate-reducing bacteria isolated from permanently cold Arctic marine sediments: description of Desulfofrigrus oceanense gen. nov., sp nov., Desulfofrigus fragile sp nov., Desulfofaba gelida gen. nov., sp nov., Desulfotalea psychrophila gen. nov., sp nov and Desulfotalea arctica sp nov. *Int J Syst Bacteriol* **49:** 1631-1643.

Konneke,M., Bernhard,A.E., de la Torre,J.R., Walker,C.B., Waterbury,J.B., and Stahl,D.A. (2005) Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* **437:** 543-546.

Kulichevskaya,I.S., Ivanova,A.O., Baulina,O.I., Bodelier,P.L.E., Sinninghe Damsté,J.S., and Dedysh,S.N. Singulisphaera acidiphila gen. nov., sp. nov., a Non-Filamentous, Isosphaera-like Panctomycete from Acidic Northern Wetlands. *In Press.*

Kulichevskaya,I.S., Ivanova,A.O., Belova,S.E., Baulina,O.I., Bodelier,P.L.E., Rijpstra,W.I.C. et al. Schlesneria paludicola gen. nov., sp. nov., the First Acidophilic Member of the Order *Planctomycetales* from *Sphagnum*-dominated Boreal Weatlands. *In Press.*

Kumar,S., Tamura,K., and Nei,M. (2004) MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics* **5:** 150-163.

Kuypers,M.M.M., Sliekers,A.O., Lavik,G., Schmid,M., Jørgensen,B.B., Kuenen,J.G. et al. (2003) Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. *Nature* **422:** 608-611.

Kuypers,M.M.M., Lavik,G., Woebken,D., Schmid,M., Fuchs,B.M., Amann,R. et al. (2005) Massive nitrogen loss from the Benguela upwelling system through anaerobic ammonium oxidation. *Proc Natl Acd Sci USA* **102:** 6478-6483.

Kuypers, M. M. M., G. Lavik, and Thamdrup, B. (2006). Anaerobic ammonium oxidation in the marine environment, p. 311-335. *In* L. N. Neretin [ed.], *Past and present water column anoxia*. Springer.

Lam,P., Jensen,M.M., Lavik,G., McGinnis,D.F., Muller,B., Schubert,C.J. et al. (2007) Linking crenarchaeal and bacterial nitrification to anammox in the Black Sea. *Proc Natl Acd Sci USA* **104:** 7104-7109.

Law,C.S. and Owens,N.J.P. (1990) Significant Flux of Atmospheric Nitrous-Oxide from the Northwest Indian-Ocean. *Nature* **346:** 826-828.

Lindsay,M.R., Webb,R.I., Strous,M., Jetten,M.S.M., Butler,M.K., Forde,R.J., and Fuerst,J.A. (2001) Cell compartmentalisation in planctomycetes: novel types of structural organisation for the bacterial cell. *Arch Microbiol* **175:** 413-429.

Lipschultz, F., S. C. Wofsy, B. B. Ward, L. A. Codispoti, G. Friedrich, and Elkins, J. W. (1990). Bacterial transformations of inorganic nitrogen in the oxygen-deficient waters of the eastern tropical South Pacific Ocean. *Deep-Sea Res* **37:** 1513-1541.

Livak, K. J., S. J. A. Flood, J. Marmaro, W. Giusti, and K. Deetz (1995). Oligonucleotides with Fluorescent Dyes at Opposite Ends Provide A Quenched Probe System Useful for Detecting PCR Product and Nucleic-Acid Hybridization. *PCR-Methods and Applications* **4**: 357-362.

Lovley, D.R., and Phillips, E.J.P. (1986) Organic matter mineralization with reduction of ferric iron in anaerobic sediments. *Applied and Environmental Microbiology* **51**: 683-689.

Loy, A., Horn, M., and Wagner, M. (2003) probeBase: an online resource for rRNA-targeted oligonucleotide probes. *Nucleic Acids Research* **31**: 514-516.

Londry,K.L., Jahnke,L.L., and Marais,D.J.D. (2004) Stable carbon isotope ratios of lipid biomarkers of sulfate-reducing bacteria. *Appl Environ Microbiol* **70:** 745-751.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar et al. (2004) ARB: a software environment for sequence data. *Nucleic Acids Research* **32**: 1363-1371.

Madigan,M.T., Takigiku,R., Lee,R.G., Gest,H., and Hayes,J.M. (1989) Carbon Isotope Fractionation by Thermophilic Phototrophic Sulfur Bacteria - Evidence for Autotrophic Growth in Natural-Populations. *Appl Environ Microbiol* **55:** 639-644.

Manefield,M., Whiteley,A.S., Griffiths,R.I., and Bailey,M.J. (2002a) RNA stable isotope probing, a novel means of linking microbial community function to Phylogeny. *Appl Environ Microbiol* **68:** 5367-5373.

Manefield,M., Whiteley,A.S., Ostle,N., Ineson,P., and Bailey,M.J. (2002b) Technical considerations for RNA-based stable isotope probing: an approach to associating microbial diversity with microbial community function. *Rapid Commun Mass Spectrom* **16:** 2179-2183.

Mascitti,V. and Corey,E.J. (2006) Enantioselective synthesis of pentacycloanammoxic acid. *J Am Chem Soc* **128:** 3118-3119.

Massé,G., Belt,S.T., Rowland,S.J., and Rohmer,M. (2004) Isoprenoid biosynthesis in the diatoms *Rhizosolenia setigera* (Brightwell) and *Haslea ostrearia* (Simonsen). *Proc Natl Acd Sci USA* **101:** 4413-4418.

McElhaney,R.N. (1982) Effects of Membrane-Lipids on Transport and Enzymic Activities. *Curr Top Membr Trans* **17:** 317-380.

Metz,J.G., Roessler,P., Facciotti,D., Levering,C., Dittrich,F., Lassner,M. et al. (2001) Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes. *Science* **293:** 290-293.

Mincer,T.J., Church,M.J., Taylor,L.T., Preston,C., Kar,D.M., and DeLong,E.F. (2007) Quantitative distribution of presumptive archaeal and bacterial nitrifiers in Monterey Bay and the North Pacific Subtropical Gyre. *Environ Microbiol* **9:** 1162-1175.

Mollenhauer, H.H. (1964) Plastic embedding mixtures for use in electron microscopy. *Stain Technology* **39**: 111-114.

Mulder,A., van de Graaf,A.A., Robertson,L.A., and Kuenen,J.G. (1995) Anaerobic ammonium oxidation discovered in a denitrifying fluidized-bed reactor. *FEMS Microbiol Ecol* **16:** 177-183.

Mulitza, S., and cruise participants (2006). Report and preliminary results of METEOR cruise 65/1, Dakar-Dakar, 11.06.-1.07.2005, 151 pages, Bremen.

Myer, R. L., N. Risgaard-Petersen, and Allen, D. E. (2005). Correlation between anammox activity and microscale distribution of nitrite in a subtropical mangrove sediment. *Appl Environ Microbiol* **71**: 6142-6149.

Nakajima J., Sakka M., Kimura T., Furukawa K. and Sakka K. (2008) Enrichment of anammox bacteria from marine environment for the construction of a bioremediation reactor. *Appl Microbio. Biotechnol* **77**(5):1159-1166.

Naqvi,S.W.A. and Noronha,R.J. (1991) Nitrous-Oxide in the Arabian Sea. *Deep-Sea Research Part A-Oceanographic Research Papers* **38:** 871-890.

Neef A, Amann R, Schlesner H, Schleifer KH (1998) Monitoring a widespread bacterial group: i*n situ* detection of planctomycetes with 16S rRNA-targeted probes. *Microbiol -UK* **144**: 3257-3266

Nichols,D.S., Nichols,P.D., and McMeekin,T.A. (1995) A New n-$C_{31:9}$ Polyene Hydrocarbon from Antarctic Bacteria. *FEMS Microbiol Lett* **125:** 281-285.

Nielsen,L.P. (1992) Denitrification in Sediment Determined from Nitrogen Isotope Pairing. *FEMS Microbiol Ecol* **86:** 357-362.

Nouri,D.H. and Tantillo,D.J. (2006) They came from the deep: Syntheses, applications, and biology of ladderanes. *Current Organic Chemistry* **10:** 2055-2074.

Op den Camp,H.J.M., Kartal,B., Guven,D., van Niftrik,L.A., Haaijer,S.C.M., van der Star,W.R. et al. (2006) Global impact and application of the anaerobic ammonium-oxidizing (anammox) bacteria. *Biochem Soc Trans* **34:** 174-178.

Packard, T. T., Dugdale, R. C., Goering, J. J., and Barber, R. T. (1978). Nitrate reductase activity in the subsurface waters of the Peru Current. J. Mar. Res. **36:** 59-76.
Ploug, H. 2001. Small-scale oxygen fluxes and remineralization in sinking aggregates. *Limnol Oceanogr* **46**: 1624-1631.

Paltauf,F. (1994) Ether lipids in biomembranes. *Chem Phys Lipids* **74:** 101-139.

Pedros-Alio,C. (2007) Dipping into the rare biosphere. *Science* **315:** 192-193.

Penton, C.R., Devol, A.H., and Tiedje, J.M. (2006) Molecular evidence for the broad distribution of anaerobic ammonium-oxidizing bacteria in freshwater and marine sediments. *Appl Environ Microbiol* **72**: 6829-6832.

Ponticorvo,L., Rittenberg,D., and Bloch,K. (1949) The Utilization of Acetate for the Synthesis of Fatty Acids, Cholesterol, and Protoporphyrin. *Journal of Biological Chemistry* **179:** 839-842.

Purkhold,U., Pommerening-Roser,A., Juretschko,S., Schmid,M.C., Koops,H.P., and Wagner,M. (2000) Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: Implications for molecular diversity surveys. *Appl Environ Microbiol* **66:** 5368-5382.

Radajewski,S., Ineson,P., Parekh,N.R., and Murrell,J.C. (2000) Stable-isotope probing as a tool in microbial ecology. *Nature* **403:** 646-649.

Reynolds, E.S. (1963) Use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology* **17**: 208-212.

Richards,F.A., Cline,J.D., Broenkow,W.W., and Atkinson,L.P. (1965) Some Consequences of the Decomposition of Organic Matter in Lake Nitinat, an Anoxic Fjord. *Limnol Oceanogr* **10 (Suppl):** 185-201.

Risgaard-Petersen, N. L. P. Nielsen, S. Rysgaard, T. Dalsgaard, and Meyer, R. L. (2003). Application of the isotope pairing technique in sediments where anammox and denitrification coexist. *Limnol Oceanogr Methods* **1**: 63-73.

Risgaard-Petersen,N., Meyer,R.L., Schmid,M., Jetten,M.S.M., Enrich-Prast,A., Rysgaard,S., and Revsbech,N.P. (2004) Anaerobic ammonium oxidation in an estuarine sediment. *Aquat Microb Ecol* **36:** 293-304.

Risgaard-Petersen,N., Langezaal,A.M., Ingvardsen,S., Schmid,M.C., Jetten,M.S.M., Op den Camp,H.J.M. et al. (2006) Evidence for complete denitrification in a benthic foraminifer. *Nature* **443:** 93-96.

Rittenberg,D. and Bloch,K. (1945) The Utilization of Acetic Acid for the Synthesis of Fatty Acids. *Journal of Biological Chemistry* **160:** 417-424.

Rohmer,M., Knani,M., Simonin,P., Sutter,B., and Sahm,H. (1993) Isoprenoid Biosynthesis in Bacteria - A Novel Pathway for the Early Steps Leading to Isopentenyl Diphosphate. *Biochem J* **295:** 517-524.

Russell,N.J. (1984) Mechanisms of Thermal Adaptation in Bacteria - Blueprints for Survival. *Trends in Biochemical Sciences* **9:** 108-112.

Russell,N.J. (1990) Cold Adaptation of Microorganisms. *Philos T Roy Soc B* **326:** 595-611.

Rysgaard,S. and Glud,R.N. (2004) Anaerobic $N_2$ production in Arctic sea ice. *Limnol Oceanogr* **49:** 86-94.

Sandercock,S.P. and Russell,N.J. (1980) The Elongation of Exogenous Fatty-Acids and the Control of Phospholipid Acyl Chain-Length in Micrococcus-Cryophilus. *Biochem J* **188:** 585-592.

Sandermann,H. and Strominger,J.L. (1972) Purification and Properties of C55-Isoprenoid Alcohol Phosphokinase from Staphylococcus-Aureus. *Journal of Biological Chemistry* **247:** 5123-5131.

Schalk,J., de Vries,S., Kuenen,J.G., and Jetten,M.S.M. (2000) Involvement of a novel hydroxylamine oxidoreductase in anaerobic ammonium oxidation. *Biochem* **39:** 5405-5412.

Schmid,M., Twachtmann,U., Klein,M., Strous,M., Juretschko,S., Jetten,M. et al. (2000) Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation. *System Appl Microbiol* **23:** 93-106.

Schmid M, Schmitz-Esser S, Jetten M, Wagner M (2001) 16S-23S rDNA intergenic spacer and 23S rDNA of anaerobic ammonium-oxidizing bacteria: implications for phylogeny and *in situ* detection. *Environ Microbiol* **3**: 450-459

Schmid,M., Walsh,K., Webb,R., Rijpstra,W.I.C., van de Pas-Schoonen,K., Verbruggen,M.J. et al. (2003) *Candidatus* "Scalindua brodae", sp nov., *Candidatus* "Scalindua wagneri", sp nov.,Two new species of anaerobic ammonium oxidizing bacteria. *System Appl Microbiol* **26:** 529-538.

Schmid,M.C., Maas,B., Dapena,A., van de Pas-Schoonen,K., van de Vossenberg,J., Kartal,B. et al. (2005) Biomarkers for In Situ Detection of Anaerobic Ammonium-Oxidizing (Anammox) Bacteria. *Appl Environ Microbiol* **71:** 1677-1684.

Schmid,M.C., Risgaard-Petersen,N., van de Vossenberg,J., Kuypers,M.M.M., Lavik,G., Petersen,J. et al. (2007) Anaerobic ammonium-oxidizing bacteria in marine environments:widespread occurrence but low diversity. *Environ Microbiol* **9:** 1476-1484.

Schouten,S., Breteler,W.C.M.K., Blokker,P., Schogt,N., Rijpstra,W.I.C., Grice,K. et al. (1998) Biosynthetic effects on the stable carbon isotopic compositions of algal lipids: Implications for deciphering the carbon isotopic biomarker record. *Geochim Cosmochim Acta* **62:** 1397-1406.

Schouten,S., Hopmans,E., Pancost,R.D., and Sinninghe Damsté,J.S. (2000) Widespread occurrence of structurally diverse tetraether membrane lipids: Evidence for the ubiquitous presence of low-temperature relatives of hyperthermophiles. *PNAS* **97:** 14421-14426.

Schouten,S., Hopmans,E.C., Schefuss,E., and Damste,J.S.S. (2002) Distributional variations in marine crenarchaeotal membrane lipids: a new tool for reconstructing ancient sea water temperatures? *Earth Planet Sci Lett* **204:** 265-274.

Schouten,S., Strous,M., Kuypers,M.M.M., Rijpstra,W.I.C., Baas,M., Schubert,C.J. et al. (2004) Stable carbon isotopic fractionations associated with inorganic carbon fixation by anaerobic ammonium-oxidizing bacteria. *Appl Environ Microbiol* **70:** 3785-3788.

Schubert,C.J., Durisch-Kaiser,E., Wehrli,B., Thamdrup,B., Lam,P., and Kuypers,M.M.M. (2006) Anaerobic ammonium oxidation in a tropical freshwater system (Lake Tanganyika). *Environ Microbiol* **8:** 1857-1863.

Schwender,J., Seemann,M., Lichtenthaler,H.K., and Rohmer,M. (1996) Biosynthesis of isoprenoids (carotenoids, sterols, prenyl side-chains of chlorophylls and plastoquinone) via a novel pyruvate/glyceraldehyde 3-phosphate non-mevalonate pathway in the green alga Scenedesmus obliquus. *Biochem J* **316:** 73-80.

Seitzinger,S.P. (1988) Denitrification in Fresh-Water and Coastal Marine Ecosystems - Ecological and Geochemical Significance. *Limnol Oceanogr* **33:** 702-724.

Sinensky,M. (1974) Homeoviscous Adaptation - Homeostatic Process That Regulates Viscosity of Membrane Lipids in Escherichia-Coli. *Proc Natl Acd Sci USA* **71:** 522-525.

Sinninghe Damsté,J.S., Rijpstra,W.I.C., Schouten,S., Fuerst,J.A., Jetten,M.S.M., and Strous,M. (2004) The occurrence of hopanoids in planctomycetes: implications for the sedimentary biomarker record. *Org Geochem* **35:** 561-566.

Sinninghe Damsté,J.S., Strous,M., Rijpstra,W.I.C., Hopmans,E.C., Geenevasen,J.A.J., van Duin,A.C.T. et al. (2002) Linearly concatenated cyclobutane lipids from a dense bacterial membrane. *Nature* **419:** 708-712.

Sinninghe Damsté, J.S., Rijpstra, W.I.C., Geenevasen, J.A.J., Strous, M., and Jetten, M.S.M. (2005) Structural identification of ladderane and other membrane lipids of planctomycetes capable of anaerobic ammonium oxidation (anammox). *FEBS Journal* **272**: 4270-4283.

Sofia,H.J., Chen,G., Hetzler,B.G., Reyes-Spindola,J.F., and Miller,N.E. (2001) Radical SAM, a novel protein superfamily linking unresolved steps in familiar biosynthetic pathways with radical mechanisms: functional characterization using new analysis and information visualization methods. *Nucleic Acids Res* **29:** 1097-1106.

Stahl, D. A., B. Flesher, H. R. Mansfield, and Montgomery, L. (1988). Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. *Appl Environ Microbiol* **54**: 1079-1084.

Stein,L.Y. and Yung,Y.L. (2003) Production, isotopic composition, and atmospheric fate of biologically produced nitrous oxide. *Annu Rev Earth Planet Sci* **31:** 329-356.

Strous,M., Heijnen,J.J., Kuenen,J.G., and Jetten,M.S.M. (1998) The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms. *Appl Microbiol Biotechnol* **50:** 589-596.

Strous,M., Fuerst,J.A., Kramer,E.H.M., Logemann,S., Muyzer,G., van de Pas-Schoonen,K.T. et al. (1999) Missing lithotroph identified as new planctomycete. *Nature* **400:** 446-449.

Strous,M., Kuenen,J.G., and Jetten,M.S.M. (1999b) Key physiology of anaerobic ammonium oxidation. *Appl Environ Microbiol* **65:** 3248-3250.

Strous,M., Pelletier,E., Mangenot,S., Rattei,T., Lehner,A., Taylor,M.W. et al. (2006) Deciphering the evolution and metabolism of an anammox bacterium from a community genome. *Nature* **440:** 790-794.

Strous,M. and Jetten,M.S.M. (2004) Anaerobic oxidation of methane and ammonium. *Annu Rev Microbiol* **58:** 99-117.

Studer, D., Michel, M., and Müller, M. (1989) High-pressure freezing comes of age. *Scanning Microscopy Supplement* **3**: 253-269.

Suttle,C.A. (2007) Marine viruses - major players in the global ecosystem. *Nature Reviews Microbiology* **5:** 801-812.

Suttle,C.A., Fuhrman,J.A., and Capone,D.G. (1990) Rapid Ammonium Cycling and Concentration-Dependent Partitioning of Ammonium and Phosphate - Implications for Carbon Transfer in Planktonic Communities. *Limnol Oceanogr* **35:** 424-433.

Taylor, S., Ninjoor, V., Dowd, D.M., and Tappel, A.L. (1974) Cathepsin B2 measurement by sensitive fluorometric ammonia analysis. *Analytical Biochemistry* **60**: 153-162.

Thamdrup, B., Dalsgaard, T., Jensen, M.M., Ulloa, O., Farías, L., and Escribano, R. (2006) Anaerobic ammonium oxidation in the oxygen-deficient waters off northern Chile. *Limnol Oceanogr* **51**: 2145-2156.

Tekaia,F., Gordon,S.V., Garnier,T., Brosch,R., Barrell,B.G., and Cole,S.T. (1999) Analysis of the proteome of *Mycobacterium tuberculosis in silico*. *Tubercle and Lung Disease* **79:** 329-342.

Trimmer, M., J. C. Nicholls, and B. Deflandre (2003). Anaerobic ammonium oxidation measured in sediments along the Thames estuary, United Kingdom. *Appl Environ Microbiol* **69**: 6447-6454.

Tsushima I., Ogasawara Y., Shimokawa M., Kindaichi T., and Okabe S. (2007) Development of a super high-rate Anammox reactor and in situ analysis of biofilm structure and function. *Water Science Technology* **55**(8-9): 9-17.

van de Graaf,A.A., de Bruin,P., Robertson,L.A., Jetten,M.S.M., and Kuenen,J.G. (1997) Metabolic pathway of anaerobic ammonium oxidation on basis of $^{15}$N-studies in a Fluidized bed reactor. *Microbiology-UK* **143:** 2415-2421.

van de Graaf,A.A., Mulder,A., De Bruijn,P., Jetten,M.S.M., Robertson,L.A., and Kuenen,J.G. (1995) Anaerobic oxidation of ammonium is a biologically mediated process. *Appl Environ Microbiol* **61:** 1246-1251.

van Dongen, U., M. S. M. Jetten, and M. C. M. van Loosdrecht (2001). The SHARON((R))-Anammox((R)) process for treatment of ammonium rich wastewater. *Water Sci Technol* **44**: 153-160.

van der Star,W.R.L., Abma,W.R., Bloomers,D., Mulder,J.W., Tokutomi,T., Strous,M. et al. (2007) Startup of reactors for anoxic ammonium oxidation: Experiences from the first full-scale anammox reactor in Rotterdam. *Wat Res* **41:** 4149-4163.

van de Vossenberg, J., Thamdrup, B., and Dalsgaard, T. (2002) Production of N2 through anaerobic ammonium oxidation coupled to nitrate reduction in marine sediments. *Appl Envi Microbiol* 68: 1312-1318.

van Niftrik,L.A., Fuerst,J.A., Sinninghe Damsté,J.S., Kuenen,J.G., Jetten,M.S.M., and Strous,M. (2004) The anammoxosome: an intracytoplasmic compartment in anammox bacteria. *FEMS Microbiol Lett* **233:** 7-13.

van Niftrik, L., Geerts, W.J., van Donselaar, E.G., Humbel, B.M., Yakushevska, A., Verkleij, A.J., Jetten, M.S.M., and Strous, M. (2007) Combined structural and chemical analysis of the anammoxosome: A membrane-bounded intracytoplasmic compartment in anammox bacteria. *J Struct Biol*. DOI:10.1016/j.jsb.2007.05.005

van Niftrik,L., Geerts,W.J.C., van Donselaar,E.G., Humbel,B.M., Webb,R.I., Fuerst,J.A. et al. (2008) Linking ultrastructure & function in four genera of anaerobic ammonium-oxidizing bacteria: cell plan, glycogen storage and localization of cytochrome *c* protiens. *J Bacteriol*. **190**(2): 708-717.

Venter,J.C., Remington,K., Heidelberg,J.F., Halpern,A.L., Rusch,D., Eisen,J.A. et al. (2004) Environmental genome shotgun sequencing of the Sargasso Sea. *Science* **304:** 66-74.

Verardo DJ, Froelich PN, McIntyre A (1990) Determination of organic-carbon and nitrogen in marine-sediments using the Carlo-Erba-NA-1500 analyzer. Deep-Sea Research Part A-Oceanographic Research Papers 37:157-165

Villalobos, M., Toner, B., Bargar, J., and Sposito, G. (2003) Characterization of the manganese oxide produced by *Pseudomonas putida* strain MnB1. *Geochimica et Cosmochimica Acta* **67**: 2649-2662.

Walther, P., and Ziegler, A. (2002) Freeze substitution of high-pressure frozen samples: the visibility of biological membranes is improved when the substitution medium contains water. *Journal of Microscopy (Oxford)* **208**: 3-10.

Weijers,J.W.H., Schouten,S., and Damste,J.S.S. (2007) Bacterial tetraether membrane lipids in soils and their application in palaeoenvironmental studies. *Geochim Cosmochim Acta* **71:** A1098.

White,S.W., Zheng,J., Zhang,Y.M., and Rock,C.O. (2005) The structural biology of type II fatty acid biosynthesis. *Annu Rev Biochem* **74:** 791-831.

Willecke,K. and Pardee,A.B. (1971) Fatty Acid-Requiring Mutant of Bacillus-Subtilis Defective in Branched Chain Alpha-Keto Acid Dehydrogenase. *Journal of Biological Chemistry* **246:** 5264-5272.

Woebken,D., Fuchs,B.A., Kuypers,M.A.A., and Amann,R. (2007) Potential interactions of particle-associated anammox bacteria with bacterial and archaeal partners in the Namibian upwelling system. *Appl Environ Microbiol* **73:** 4648-4657.

Woebken D., Lam P., Kuypers M., Wajih S., Naqvi A., Kartal B., Strous M., Jetten M.S.M., Fuchs, B.M., and Amann, R.I. (2007) A microdiversity study of anammox

bacteria reveals novel Candidatus *Scalindua* phylotype in marine oxygen minimum zones. *Environ. Microbiol in press.*

Woese,C.R. and Fox,G.E. (1977) Phylogenetic Structure of Prokaryotic Domain - Primary Kingdoms. *Proc Natl Acd Sci USA* **74:** 5088-5090.

Wuchter,C., Abbas,B., Coolen,M.J.L., Herfort,L., van Bleijswijk,J., Timmers,P. et al. (2006) Archaeal nitrification in the ocean. *Proc Natl Acd Sci USA* **103:** 12317-12322.

Wuchter,C., Schouten,S., Boschker,H.T.S., and Damste,J.S.S. (2003) Bicarbonate uptake by marine Crenarchaeota. *FEMS Microbiol Lett* **219:** 203-207.

Zhang,Y., Ruan,X.H., den Camp,H.J.M.O., Smits,T.J.M., Jetten,M.S.M., and Schmid,M.C. (2007) Diversity and abundance of aerobic and anaerobic ammonium-oxidizing bacteria in freshwater sediments of the Xinyi River (China). *Environ Microbiol* **9:** 2375-2382.

Zumft, W. G. (1997). Cell biology and molecular basis of denitrification. *Microbiol Mol Biol Rev* **61**: 533-616.

## summary

Ladderane lipids are unusual membrane lipids of anammox bacteria. They can contain either three or five cyclobutane rings, and are so far unique in nature. This means that the lipids can be used as a tracer for anammox bacteria. Anammox bacteria are recently identified members of the nitrogen cycle, with the ability to combine ammonium with nitrite and form dinitrogen gas, in waters or sediments containing little or no oxygen. The anammox process is now being used commercially to remove ammonium during wastewater treatment, as a sustainable alternative to denitrification. In the natural environment anammox bacteria have been found in many different oxygen-limited environments, including marine oxygen minimum zones, marine and freshwater sediments, tropical lakes and even sea ice. Despite their apparent omnipresence, we are only just starting to understand anammox bacteria, their unusual biology and functioning in the natural environment. This thesis describes research conducted into the occurrence and biosynthesis of ladderane lipids in anammox bacteria, and their application as environmental markers for anammox bacteria.

For the first time, an anammox bacterium from the natural environment was successfully enriched using sediment from a Swedish fjord as inoculum. Prior to this, anammox bacteria had only been cultured from wastewater. Cells of the enrichment culture of "*Candidatus* Scalindua *spp.*" were shown to contain key features of anammox bacteria, including hydrazine production, a compartmentalised cellular ultrastructure and ladderane lipids. "*Candidatus* Scalindua *spp.*" was found to use various carbon sources, and reduce nitrate, Fe (III) and Mn (IV). The versatile metabolism of these anammox bacteria is thought to be one of the factors determining the widespread global distribution of the Scalindua genera in marine anoxic ecosystems.

To improve the analysis of ladderane lipids in complex matrixes a novel high performance liquid chromatography (HPLC) atmospheric pressure chemical ionization (APCI) tandem mass spectrometry (MS/MS) technique was developed which allowed us to investigate and quantify these unusual membrane lipids with more accuracy than previously achievable. Using this and other HPLC/MS

techniques, the ladderane core lipid and phospholipid composition of four different species of anammox bacteria, each representing one of the four known genera, was analysed and results showed that all anammox bacteria contained a wide range of ladderane phospholipids. These were made up of different combinations of hydrophobic tail types attached to the *sn*-1 position of the glycerol backbone, in combination with different types of polar headgroup; phosphocholine, phosphoethanolamine or phosphoglycerol were identified. It was also found that ladderane lipids are not solely exclusive membrane lipids of the anammoxosome, and must be incorporated into other membranes in the anammox cell.

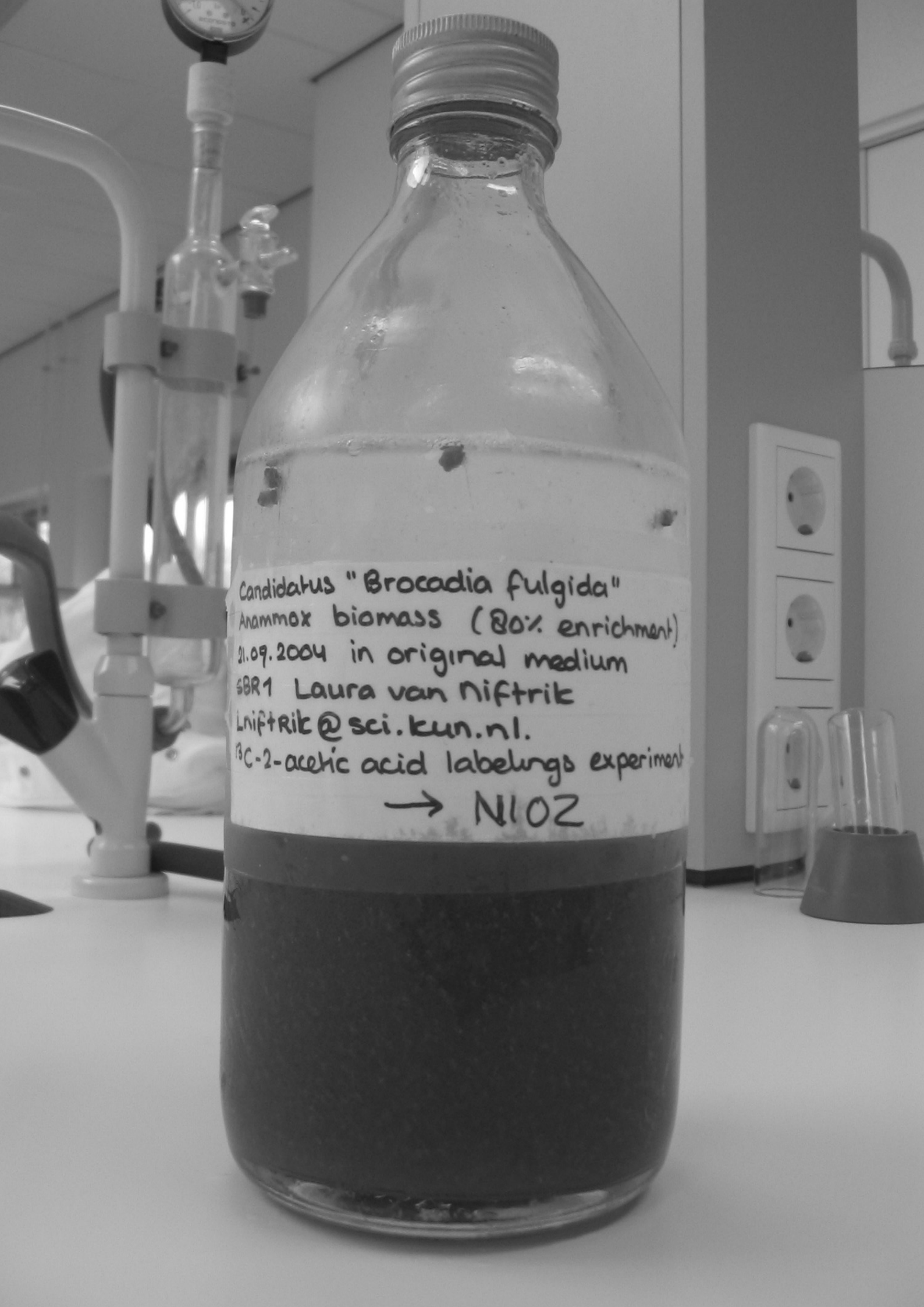
To investigate the biosynthetic route of ladderane lipids, a comparative/ functional genomics analysis of *K. stuttgartiensis* and a wide range of other bacterial and archaeal genomes was performed which indicated that genes previously proposed to be involved in the biosynthesis of ladderane lipids, may in fact encode a new pathway for the anaerobic biosynthesis of polyunsaturated hydrocarbons (PUHCs). PUHCs could then be further rearranged into ladderane lipids. However, subsequent results from a $^{13}$C-labelling study indicated that biosynthesis is probably carried out via a different pathway. During 2-$^{13}$C acetate labelling experiments, labelling patterns of lipids obtained via $^{13}$C-NMR spectroscopic analysis indicated that "*Candidatus* Brocadia fulgida" synthesises $C_{16:0}$ and *iso*$C_{16:0}$ fatty acids according to known pathways of fatty acid biosynthesis. The labelling pattern of the *n*-octyl chain of the ladderane monoether also indicated use of the type II fatty acid biosynthetic route. However, the labelling pattern of carbon atoms in the cyclobutane and cyclohexane moieties of the ladderane group did not correspond to known patterns of fatty acid synthesis, suggesting that a novel but as yet unknown route of biosynthesis is used for ladderane synthesis.

Ladderane lipids, along with a range of other techniques, were applied to establish that anammox bacteria are present and that the anammox reaction is a major process in nitrogen cycling in the Peruvian oxygen minimum zone (OMZ), supporting the idea that the anammox reaction plays a major role in the global cycling of nitrogen. No evidence was found for the permanent removal of nitrogen via denitrification from this system. Statistical analysis of the data obtained from the Peruvian OMZ, in combination with data from the Namibian OMZ was also carried

out. Results from the Namibian OMZ showed that the higher the rates of $^{29}N_2$ production per cell, the more ladderane lipids each cell contained. This may indicate that biosynthesis of the energetically expensive ladderane lipids was mainly done during times of optimal growth conditions. In the Peruvian OMZ, qPCR copy numbers and rates of $^{29}N_2$ production and ladderane lipid concentrations were non-parametrically correlated. Observed disparities were probably due to differences in the physiological state or growth phase of the anammox communities at the different sites.

The effect of temperature on the distribution of ladderane lipids in anammox bacteria was also investigated. Analysis of anammox biomass (including representatives of the four genera) grown in enrichment cultures indicated that more shorter chained ladderane lipids were produced under colder conditions, and *vice versa*. The same trend of chain length adaptation also occurred in ladderane lipids from particulate organic matter and in surface sediments from a wide range of marine environments. To quantify the relationship between the $C_{18}$ and $C_{20}$ ladderane lipids containing 5 cyclobutane rings, the index of ladderane lipids with 5 cyclobutane rings ($NL_5$) was calculated. When plotting the $NL_5$ against *in situ* temperature a significant relationship was observed, best approximated by a 4$^{th}$ order sigmoidal curve ($R^2$= 0.92, p=<0.0001, *n*=158). The adaptation of chain length with temperature was similar to the regulation of common fatty acid distribution reported occurring in other bacteria. It is proposed that the $NL_5$ can be used to discriminate between the origins of ladderane lipids in marine surface sediments and may have future application as a paleo-temperature proxy.

To conclude, work contained in this thesis has supported the claim that ladderane lipids can be successfully applied tracers for all species of anammox bacteria, both in the laboratory and in the natural environment. We have demonstrated that anammox bacteria alter the chain length of their ladderane lipids to adjust to *in situ* temperature, with the possibility to be further used in paleo-temperature research. Work unravelling the biosynthesis of ladderane lipids has shown that they are not synthesised via known patterns of fatty acid synthesis, and that a novel, but as yet unknown, route of biosynthesis must be used.


Candidatus "Brocadia fulgida"
Anammox biomass (80% enrichment)
21.09.2004 in original medium
SBR1 Laura van Niftrik
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13C-2-acetic acid labelings experiment
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## samenvatting

Bacteriën, die in staat zijn anaeroob ammonium te oxideren (anammox), zijn recentelijk geïdentificeerd als nieuwe leden van de biogeochemische stikstofcyclus; in zuurstofarm water en sedimenten zetten zij ammonium samen met nitriet direct om in stikstofgas. Het anammox proces wordt op dit moment commercieel toegepast bij het verwijderen van ammonium uit afvalwater, als duurzaam alternatief voor denitrificatie. In de natuur is het anammox proces in tal van zuurstofarme milieu's aangetoond, in de zuurstofloze Zwarte Zee, in de zuurstofminimum zones in de oceaan, in zee- en zoetwater sedimenten, in tropische meren en zelfs in zee-ijs. Ladderaanlipiden zijn de karakteristieke membraanlipiden van anammoxbacteriën. Zij bevatten drie of vijf cyclobutaanringen en zijn zijn unieke membraanlipiden. Hierdoor is het mogelijk deze lipiden te gebruiken als tracer voor anammoxbacteriën. We staan pas aan het begin om het functioneren van anammoxbacteriën in de natuurlijke omgeving en hun opmerkelijke biochemie te begrijpen. Dit proefschrift beschrijft onderzoek gedaan naar de aanwezigheid en biosynthese van ladderaanlipiden in anammoxbacteriën en hun toepassing als tracers.

Voor het eerst is een anammoxbacterie uit een natuurlijk milieu, succesvol opgehoopt, startend met een sediment uit een Zweeds fjord, als inoculum. Voorheen zijn anammoxbacteriën alleen verrijkt vanuit afvalwaterslib. In de verrijkte cultuur van "*Candidatus* Scalindua *spp*." zijn belangrijke kenmerken van annamox bacteriën aangetoond, zoals hydrazine productie, een organelachtige celstructuur en de aanwezigheid van ladderaanlipiden. "*Candidatus* Scalindua *spp*." is in staat te zijn diverse koolstofbronnen te gebruiken, en nitraat, Fe (III) en Mn (IV) te reduceren. Het veelzijdige metabolisme van deze anammoxbacterie kan een mogelijke verklaring zijn voor de wereldwijde verspreiding van het Scalindua genus in zuurstofarme mariene ecosystemen.

Om de analyse van ladderaanlipiden aanwezig in complexe matrices te verbeteren is een nieuwe hogedrukvloeistofchromatografie (HPLC)/atmosferische druk chemische ionisatie (APCI)/tandem mass spectrometrie (MS/MS) techniek ontwikkeld. Deze techniek maakt het mogelijk deze bijzondere, naar thermolabiele, membraanlipiden te identificeren en veel nauwkeuriger te kwantificeren. Door gebruikmaking van deze en andere HPLC/MS technieken zijn de ladderaanlipiden

en de ladderaanfosfolipiden van vier verschillende soorten anammoxbacteriën, die elk één van de op dit moment bekende genera representeren, geanalyseerd. Alle vier anammoxbacteriën bevatten een breed spectrum aan ladderaanfosfolipiden, welke zijn opgebouwd uit verschillende combinaties van hydrofobe ladderanen gebonden op de *sn*-1 positie van de glycerol groep met verschillende typen van polair hoofdgroepen; fosfocholine, fosfoethanolamine of fosfoglycerol. Ook is vastgesteld dat ladderaanlipiden niet alleen lipiden van het membraan van het anammoxosoom zijn, maar ook deel uit makenvan de celmembraan van de anammox cel.

Om de biosynthesische route van ladderaanlipiden te onderzoeken is een vergelijkende en functionele genoomanalyse verricht aan *K. stuttgartiensis* en een breed spectrum van andere bacteriën en archaea. Hieruit bleek dat eerder voorgestelde genomen welke betrokken zijn bij de biosynthese van ladderaanlipiden mogelijk een nieuwe synthese route aangeven voor de anaerobe biosynthese van poly-onverzadigde koolwaterstoffen (POKs). POKs zouden vervolgens verder omgezet kunnen worden tot ladderaanlipiden. De resultaten van een $^{13}$C-labeling studie geven echter aan dat de biosynthese via een ander route moet worden uitgevoerd. Door middel van 2-$^{13}$C acetaat label experimenten zijn labelingspatronen van lipiden verkregen middels $^{13}$C-NMR analyse, welke laten zien dat "*Candidatus* Brocadia fulgida" $C_{16:0}$ en *iso*$C_{16:0}$ vetzuren synthetiseren volgens bekende biosynthese routes. Het labelingspatroon van de *n*-octyl keten van de ladderaanmonoether suggereert ook het gebruik van het type II route van de biosynthese van vetzuren. Het labelelingspatroon van koolstofatomen in de cyclobutane en cyclohexane delen van de ladderaan groep correspondeerdt echter niet met het te verwachten labelingspatroon van de biosynthese van vetzuren. Dit suggereert dat er een andere, maar tot dusver onbekende, route voor de biosynthese wordt gebruikt voor de synthese van ladderaanlipiden.

Samen met andere technieken zijn ladderaanlipiden gebruikt om de aanwezigheid en de activiteit van anammox in de zuurstofminimum zone (ZMZ) voor de kust van Peru aan te tonen. De resultaten ondersteunen de gedachte dat het anammoxproces een grote rol speelt in de stikstofcyclus in dit soort systemen en waarschijnlijk op een globale schaal. Er zijn geen bewijzen gevonden voor een permanente verwijdering van stikstof door middel van denitrificatie. Tevens is er een statistische analyse uitgevoerd van de data verkregen uit de ZMZs voor de kust van

Peru en de kust van Namibië. De analyse van de data van Namibië laat zien dat wanneer er sprake is van een grotere productie $^{29}N_2$ per anammox cel (bij incubatie met $^{15}NH_4^+$), er per cel meer ladderaanlipiden aanwezig zijn. Dit kan erop wijzen dat de biosynthese van de hoog energiebehoeftige ladderaan lipiden vooral heeft plaatsgevonden tijdens optimale groeicondities. In de OMZ voor de kust van Peru zijn het aantal 16S rRNA kopien, de hoeveelheid geproduceerde $^{29}N_2$, en de concentratie aan ladderaan lipiden zijn niet-parametrisch gecorreleerd. De waargenomen verschillen zijn waarschijnlijk te verklaren door verschillen in fysiologische toestand of groeifase van de anammoxcellen op de diverse locaties.

Het effect van temperatuur op de distributie van ladderaanlipiden in anammoxbacteriën is ook onderzocht. Analyse van anammox biomassa van alle vier verschillende genera, gekweekt in verrijkte culturen, laat zien dat er meer ladderaanlipiden met kortere ketens worden geproduceerd onder koudere omstandigheden, en *vice versa*. Dezelfde trend ten aanzien van adaptatie van ketenlengte deed zich voor binnen ladderaanlipiden afkomstig van gesuspendeerd organisch materiaal en van oppervlakte sedimenten van een breed spectrum mariene milieus. Om de relatie tussen $C_{18}$ en $C_{20}$ ladderaanlipiden welke 5 cyclobutane ringen bevatten te kwantificeren, is een index van ladderaanlipiden met 5 cylcobutane ringen ($NL_5$) berekend. De $NL_5$ index laat ten opzichte van *in situ* temperaturen een significante relatie zien; de beste benadering werd verkregen met een 4$^{de}$ orde sigmoidale curve ($R^2$=0.92, p=<0.0001, n=158). De aanpassing van ketenlengte van ladderaan lipiden aan groeitemperatuur is vergelijkbaar met de regulatie van de vetzuurdistributie zoals gevonden bij andere bacteriën. De $NL_5$ kan gebruikt worden bij het onderscheiden van ladderaanlipiden afkomstig van oppervlakte sedimenten die afkomstig uit de waterkolom en zijn toepassing vindt als een paleotemperatuur proxy.

In conclusie, het werk in dit proefschrift ondersteund de stelling dat ladderaan lipiden met succes toegepast kunnen worden als tracers voor anammoxbacteriën. Verder is aangetoond dat anammoxbacteriën de ketenlengte van hun ladderaanlipiden veranderen om zich aan te passen aan de omgevingstemperatuur, een eigenschap die mogelijk gebruikt kan worden voor paleo-temperatuuronderzoek. Tenslotte laten studies van de biosynthese van ladderaanlipiden zien dat zij niet gesynthetiseerd worden via de bekende maar een andere, tot dusver onbekende, biochemische syntheseroute.


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## curriculum vitae

Jayne Elizabeth Rattray was born on the 10th of November 1975 in Dundee, Scotland and grew up close to the village of Eastriggs. After attending Annan Academy and then working full time for three years, she completed an HND in Environmental Protection and Management at the Scottish Agricultural Collage in Ayr in 1998. Subsequently she was awarded a BSc Honours in Environmental Science at the University of Plymouth in 2000, after which she moved to the Netherlands and worked in an unrelated field. After raising enough funds Jayne embarked on a Master of Science degree in Environmental Science at Wageningen University in 2001 and obtained her masters in 2003. Her studies focused mainly on aquatic ecology and water quality management, with specialisation in the nitrogen cycle and denitrification.

In January 2004 Jayne started a PhD 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. This PhD project was part of an international collaboration between the NIOZ, the Nutrient Group of the Max Plank Institute in Bremen (under supervision of Dr. Ir. Marcel Kuypers) and the Department of Microbiology of the Radboud University, Nijmegen (under supervision of Prof. Dr. Ir. Mike Jetten en Dr. Ir. Marc Strous) and has resulted in the production of this thesis.

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