Ladderanes as tracers for present and past anaerobic ammonium oxidation

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Ladderanes as tracers for present and past anaerobic ammonium oxidation

Ladderanen als indicatoren voor het voorkomen van anaerobe ammonium oxidatie in het heden en verleden

met een samenvatting in het Nederlands

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So we beat on, boats against the current,
borne back ceaselessly into the past.

F. Scott Fitzgerald

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Summary

Ladderane lipids are membrane lipids produced exclusively by anaerobic ammonium oxidising (anammox) bacteria. Anammox bacteria are key players in the marine nitrogen cycle, as they perform the anammox reaction, converting equal parts of ammonium and nitrite into dinitrogen gas. This process is responsible for a significant loss of bio-available nitrogen from the oceanic system. In a living anammox cell, ladderane lipids are present in the form of intact polar lipids (IPLs). Upon cell death, these IPLs are degraded into more stable ladderane fatty acids or ladderane mono-ethers. Traditionally, four ladderane fatty acids and one ladderane mono-ether have been used as biomarkers to trace modern and past anammox activity in marine water columns and sediments. These lipids contain either three or five linearly concatenated cyclobutane moieties, and have a total of eighteen or twenty carbon atoms. In addition, the ratio of C_{18} and C_{20} ladderane fatty acids with five cyclobutane rings is used as a proxy (NL_5) for the temperature at which the anammox bacteria were living. NL_5 derived temperature can indicate whether anammox bacteria were proliferating in the water column or in the sediment. However, the fate of ladderane lipids as they undergo structural alterations during diagenesis and catagenesis is unknown, and the influence of these processes must be constrained before confidently applying these biomarkers to infer past anammox activity. This thesis is thus divided into two parts: the first concerns the use of ladderane lipids in modern marine and terrestrial environments to trace anammox bacteria, and the second deals with two distinct types of transformation products of anammox biomarkers, and their applicability in past marine settings.

To investigate the occurrence of anammox in soils, a laboratory enrichment culture was obtained from a Dutch peat soil and shown to contain two anammox bacterial clusters. These bacteria represented 50% of the population detectable by fluorescent in situ hybridisation (FISH) microscopy. The ladderane lipid profile of these novel bacteria showed that they contained all four ladderane fatty acids seen in previous anammox bacteria, indicating that ladderane lipids can also be used as biomarkers for terrestrial anammox bacteria. This may become essential as anthropogenic influence in soils continues to increase, and anammox activity in terrestrial environments becomes more important in agricultural settings.

Analysis of suspended particulate matter from several depths in the Eastern Tropical North Pacific (ETNP) indicated that anammox bacteria were living in the ETNP oxygen minimum zone (OMZ), and that anammox is also an important nitrogen process in this environment. The NL_5 derived temperature values in the ETNP were much lower than the in situ measured values. This was due to the fact that in the ETNP concentrations of C_{18} ladderane fatty acids were at least 10 fold the concentrations of C_{20} ladderane fatty acids. It was concluded that care must be taken when applying the NL_5 index as it is not universally applicable.

Ladderane fatty acid distributions in the euxinic Cariaco Basin, Venezuela, revealed that the presence of anammox bacteria is restricted to a specific water depth (235 – 350 m) in the chemocline, which was confirmed by anammox cell counts using FISH microscopy. This depth delimitation is most likely caused by the need for a low oxygen environment where there are also sufficient nitrite and ammonium substrates. Based on the presence of glyceryl dialkyl glycerol tetraethers at the same depth, part of the nitrite required by anammox bacteria living in the Cariaco Basin is probably provided by ammonia oxidising archaea.

To investigate the effect of oxic degradation on ladderane lipids, a laboratory aerobic degradation experiment was performed in which anammox cell material was subjected to oxic conditions. This led to the transformation of C_{18} and C_{20} ladderane fatty acids into short-chain ladderane fatty acids. One short-chain product was isolated by preparative Liquid

Chromatography (LC), and identified by Nuclear Magnetic Resonance (NMR) experiments to be a C_{14}-[3]-ladderane fatty acid. The identifications of the other two oxic products were inferred from this first identification and from mass spectra to be short-chain C_{14}-[5]-, and C_{16}-[3]-ladderane fatty acids. This transformation was likely microbially mediated, as the optimal production of short-chain ladderane fatty acids occurred at 40 °C, and a sterile control experiment resulted in no biodegraded products. Based on the structures of these biodegraded ladderane fatty acids, a β-oxidation, which removes two carbon atoms from the alkyl-side chain, was proposed as the degradation pathway. This process stopped at the ladderane ring structure.

The two C_{14} short-chain biodegraded ladderane fatty acids generated in the laboratory experiment were subsequently used in a new LC coupled to tandem Mass Spectrometry (LC/MS/MS) Selective Reaction Monitoring (SRM) method. This method was applied on marine suspended particulate matter and sediments in order to determine their natural occurrence. Short-chain ladderane fatty acids were shown to be abundant in settings where lipids from anammox bacteria had been exposed to oxic conditions after cell death (in OMZs and, to some extent, oxic sediments), but not in settings where ladderane lipids were deposited in an euxinic water column. Using degradation kinetics, it was determined that short-chain ladderane fatty acids are present in marine sediments for a longer period of time (10^7 years) than the original ladderane fatty acids, which had been used previously as tracers for past anammox activity. They, therefore, may be more suitable for detecting past anammox activity in ancient marine sediments. However, the fact that these short-chain ladderane lipids are thermally degraded during Gas Chromatography (GC) analyses indicates that they, like their longer-chained counterparts, are sensitive to thermal instability during the early stages of sediment maturation.

The fate of ladderane lipids during thermal maturation was investigated via hydrous pyrolysis experiments mimicking the natural catagenetic processes that take place over millions of years. Anammox cell material was thermally matured at temperatures ranging between 120 and 365 °C for three days. At experimental temperatures >200 °C, oil was generated. Two novel lipid classes were identified in the saturated aliphatic fractions of these oils. Representatives from both classes were isolated using preparative GC, and identified according to their mass spectra and 1D- and 2D-NMR experiments. The first class was shown to contain C_{19} and C_{20} ladderane-like hydrocarbons with tricyclododecane moieties, believed to be the result of rearrangement in the ladderane moieties accompanied by decarboxylation and ether cleavage of C_{20} ladderane fatty acids and mono-ethers, respectively. The second class contained branched long-chain ($C_{28} - C_{30}$) alkanes. However, the transformation pathway to the formation of these alkanes is ambiguous as their chain length exceeds those of ladderane lipids encountered in anammox cell material. Using compound specific $\delta^{13}C$ isotopic values, both lipid classes were confirmed to be the result of the thermal maturation of anammox lipids. These lipids were not detected in marine sediments, which may be due to either the low selectivity of the GC/MS method used, the fact that the screened sediments were too immature for the thermally mature lipids to have been generated, or that these lipids do not accurately represent the natural thermal maturation of anammox biomass.

The data presented in this thesis show that ladderane lipids can be used to trace anammox bacteria in marine as well as terrestrial settings. In relation to marine environments, anammox was shown to occur in two previously unexplored oxygen depleted zones, highlighting the ubiquitous nature of these micro-organisms. Transformation products of ladderane lipids, demonstrated using laboratory-based experiments simulating natural processes, revealed both oxidation and catagenetic products of anammox lipids, both of which are new biomarkers for past anammox activity.

Samenvatting

Ladderanen zijn de karakteristieke membraanlipiden van bacteriën die in staat zijn om anaëroob ammonium te oxideren (anammox). Anammox bacteriën spelen een belangrijke rol in de mariene stikstofcyclus omdat zij ammonium en nitriet omzetten in stikstofgas. Deze omzetting leidt tot een substantiële afname van biologisch beschikbaar stikstof in mariene ecosystemen. In levende anammox bacteriën zijn de ladderaan lipiden aanwezig als intacte polaire lipiden (IPLs). Na sterfte worden deze IPLs omgezet in meer stabielere ladderaan vetzuren of ladderaan mono-ethers door verlies van de polaire groep. Deze ladderanen worden gebruikt als 'biomarker' om de anammox activiteit in huidige en vroegere mariene milieu's te achterhalen. Ladderanen bevatten drie of vijf lineair aaneengeschakelde cyclobutaan groepen met totaal achttien of twintig koolstofatomen. De distributie van [5]-ladderaan vetzuren wordt gebruikt als index (NL_5) voor de omgevingstemperatuur waarin de anammox bacteriën leefden. Uit de NL_5 index van fossiele ladderanen kan worden afgeleid of de anammox bacteriën zich in de waterkolom of in het sediment bevonden.

Ondanks deze toepassingen is het gebruik van ladderaan lipiden voor reconstructie van anammox in het verleden nog beperkt. Dit is vooral het gevolg van de relatieve instabiliteit van ladderanen door de aanwezigheid van cyclobutaangroepen. Het lot van ladderaan lipiden gedurende zowel vroeg diagenetische omzetting in de eerste meters van begraving van het sediment als gedurende thermische degradatie tijdens diepere begraving is nog onbekend. Deze processen dienen beter begrepen te worden alvorens deze 'biomarkers' algemeen kunnen worden toegepast om de anammox activiteit in het verleden te reconstrueren. Dit proefschrift is in twee delen opgesplitst; het eerste deel betreft het gebruik van ladderaan lipiden om anammox bacteriën te traceren in huidige mariene en terrestrische milieu's, terwijl het tweede deel in gaat op twee specifieke vormen van omzetting van ladderanen en hun toepassing in het fossiele mariene milieu.

Om de aanwezigheid van anammox bacteriën in bodems te onderzoeken is er een bacteriële verrijkingscultuur verkregen uit een Nederlands veen. Twee clusters van anammox bacteriën vertegenwoordigden 50% van de populatie waarneembaar met de 'fluorescent in situ hybridisation' (FISH) techniek. Deze nieuwe anammox bacteriën bevatten de vier karakteristieke ladderaan vetzuren, hetgeen betekent dat ladderaan lipiden ook gebruikt kunnen worden als 'biomarkers' for terrestrische anammox bacteriën. Dit is belangrijk omdat de anthropogene invloed op bodems blijft toenemen, met name door agrarische activiteit, en daardoor anammox activiteit in terrestrische milieu's steeds belangrijker wordt.

Analyse van ladderaan lipiden in gesuspendeerd particulair materiaal op verschillende diepten in de noordoostelijke tropische Stille Oceaan (NTSO) liet zien dat anammox bacteriën aanwezig zijn in de zuurstofminimumzone (ZMZ) van de NTSO en dat ook hier anammox een belangrijk proces in de stikstofcyclus is. De met de NL_5 index gereconstrueerde temperaturen van de ZMZ van de NTSO waren veel lager dan de ter plekke gemeten temperaturen. Dit komt omdat de concentraties van C_{18} ladderaan vetzuren tien maal zo hoog is als de C_{20} ladderaan vetzuren, een distributie die niet eerder zo gevonden is. Hieruit moet geconcludeerd worden dat de NL_5 index niet universeel toepasbaar is.

Analyse van concentraties van ladderaan vetzuren in gesuspendeerd particulair materiaal op verschillende diepten van het gestratificeerde, euxinische Cariaco bekken laat zien dat de aanwezigheid van anammox bacteriën zich beperkt tot een water diepte van 235 tot 350 m. Dit werd bevestigd door het tellen van anammox cellen met FISH microscopie. Deze specifieke niche komt voort uit de behoefte van anammox bacteriën aan een omgeving met een lage zuurstofconcentratie met tegelijkertijd de aanwezigheid van voldoende ammonium

en nitriet. De piek in de concentratie van isoprenoïde glyceryl diakyl glycerol tetraethers (o.a. crenarchaeol) op dezelfde diepte toont aan dat een gedeelte van het nitriet noodzakelijk voor de anammox reactie waarschijnlijk afkomstig is van ammonium-oxiderende thaumarchaeota.

De invloed van aërobe afbraak op ladderaan lipiden werd onderzocht met een laboratorium experiment waarin celmateriaal van anammox bacteriën gemengd werd met slib uit de Waddenzee in een zuurstofrijke omgeving. Dit zorgde voor de omzetting van de C_{18} en C_{20} ladderaan vetzuren naar verkorte ladderaan vetzuren. Met vloeistofchromatografie werd één van deze verkorte ladderaan vetzuren geïsoleerd en met nucleaire magnetische resonantie (NMR) experimenten geïdentificeerd als een C_{14}-[3]-ladderaan vetzuur. Met behulp van massaspectrometrie werden de twee andere oxische producten geïdentificeerd als C_{14}-[5]-, en C_{16}-[3]-ladderaan vetzuren. Deze omzetting vond waarschijnlijk plaats onder de invloed van bacteriën aangezien de grootste hoeveelheid verkorte ladderaan vetzuren werd gevonden bij 40 °C en een steriel controle experiment geen omzettingsproducten opleverde. De structuur van de verkorte ladderaan vetzuren laat zien dat zij door microbiologische β-oxidatie gevormd zijn, een proces dat per stap twee koolstofatomen verwijderd van de alkyl zijketen. Dit proces stopt bij het bereiken van de ringstructuur van de ladderanen.

De twee verkorte C_{14} ladderaan vetzuren gevormd tijdens dit laboratoriumexperiment werden vervolgens gebruikt om een nieuwe gevoelige en specifieke analysemethode (LC/MS/MS) te ontwikkelen die vloeistofchromatografie koppelt aan massaspectrometrie, waarbij specifieke reacties in de ionenbron kunnen worden waargenomen ('selective reaction monitoring'). Deze methode werd vervolgens toegepast op gesuspendeerd materiaal in de oceaan en op mariene sedimenten om het natuurlijk voorkomen van verkorte ladderaan vetzuren te onderzoeken. Er werd aangetoond dat verkorte ladderaan vetzuren gevormd worden indien ladderanen afkomstig van de anammox bacteriën na sterfte worden blootgesteld aan zuurstofrijke condities (zoals in ZMZs en, tot op zekere hoogte, zuurstofrijke oppervlaktesedimenten), maar niet wanneer de ladderaan lipiden werden afgezet in anoxische bekkens zoals het Cariaco bekken, waar blootstelling aan zuurstof niet optreedt. Verkorte ladderaan vetzuren komen langer (tot wel 10^7 jaar) in mariene sedimenten voor dan de originele ladderaan vetzuren, die voorheen gebruikt werden om anammox activiteit in het verleden op te sporen, en zijn dus wellicht stabieler. Daarom zijn verkorte ladderaan lipiden wellicht beter om anammox activiteit in oude sedimenten te achterhalen. Ook deze korte ladderaan lipiden, net als hun tegenhangers met langere alkyl zijketens, zijn echter thermische instabiel en zullen daarom waarschijnlijk tijdens de vroege catagenese worden omgezet.

Het lot van ladderaan lipiden tijdens de vroege catagenese is onderzocht met behulp pyrolyse experimenten in de aanwezigheid van water die de natuurlijke processen die plaatsvinden tijdens de begraving van sediment gedurende miljoenen jaren goed kunnen nabootsen. Celmateriaal van anammox bacteriën werd gedurende drie dagen op een temperatuur variërend van 120 tot 365 °C verhit, waardoor natuurlijke catagenesereacties gesimuleerd werden. Bij temperaturen >200 °C werd olie gevormd. Twee nieuwe groepen van lipiden werden aangetroffen in de verzadigde alifatische fracties van deze oliën en twee vertegenwoordigers van deze lipiden werden geïsoleerd met preparatieve gaschromatografie en geïdentificeerd met behulp van massaspectrometrie en 1D- en 2D-NMR experimenten. De eerste klasse omvat C_{19} en C_{20} op ladderaan-gelijkende koolwaterstoffen met tricyclododecaan groepen. Zij worden waarschijnlijk gevormd door C-C breuk van de oorspronkelijke ladderaan groepen in combinatie met decarboxylatie of etherspliting van C_{20} ladderaan vetzuren en mono-ethers. De tweede klasse omvat lange (C_{28} – C_{30}) vertakte alkanen met twee ethyl of butyl groepen op specifieke

posities. De herkomst van deze alkanen is onzeker aangezien zij meer koolstofatomen bevatten dan de ladderanen. Verbindingsspecifieke $\delta^{13}C$ waarden bevestigen echter dat beide klassen van lipiden gevormd zijn door thermische afbraak van celmateriaal van anammox bacteriën. Beide soorten koolwaterstoffen konden niet worden aangetoond in oude mariene sedimenten waar anammox activiteit vermoed werd. Dit kan veroorzaakt worden doordat de gebruikte GC/MS methode niet selectief genoeg is, doordat de geanalyseerde sedimenten te weinig thermische degradatie hebben ondergaan om deze lipiden te produceren, of doordat deze lipiden toch niet representatief zijn voor het natuurlijke proces van thermische degradatie van ladderanen.

De in dit proefschrift gepresenteerde resultaten tonen aan dat ladderaan lipiden gebruikt kunnen worden om anammox bacteriën op te sporen in mariene en terrestrische milieus. Er is aangetoond dat anammox voorkomt in twee, nog niet eerder onderzochte, zuurstofarme zones in het mariene milieu. Dit illustreert het alomtegenwoordige karakter van de anammox reactie. De omzettingsproducten van ladderaan lipiden, aangetoond met laboratorium experimenten die de natuurlijke diagenese en catagenese processen nabootsen, laten zien dat zowel oxidatie en thermische degradatie van anammox lipiden leidt tot producten die gebruikt kunnen worden als nieuwe 'biomarkers' om anammox activiteit in het verleden op te sporen.

Chapter 1

Introduction

1.1 THE MARINE NITROGEN CYCLE

The marine nitrogen cycle is one of the more complex biogeochemical cycles. Reduction-oxidation (redox) reactions transform the most oxidised nitrogen species in the ocean, nitrate (NO_3^-), into the most reduced species, ammonium (NH_4^+), and vice versa (Fig. 1).

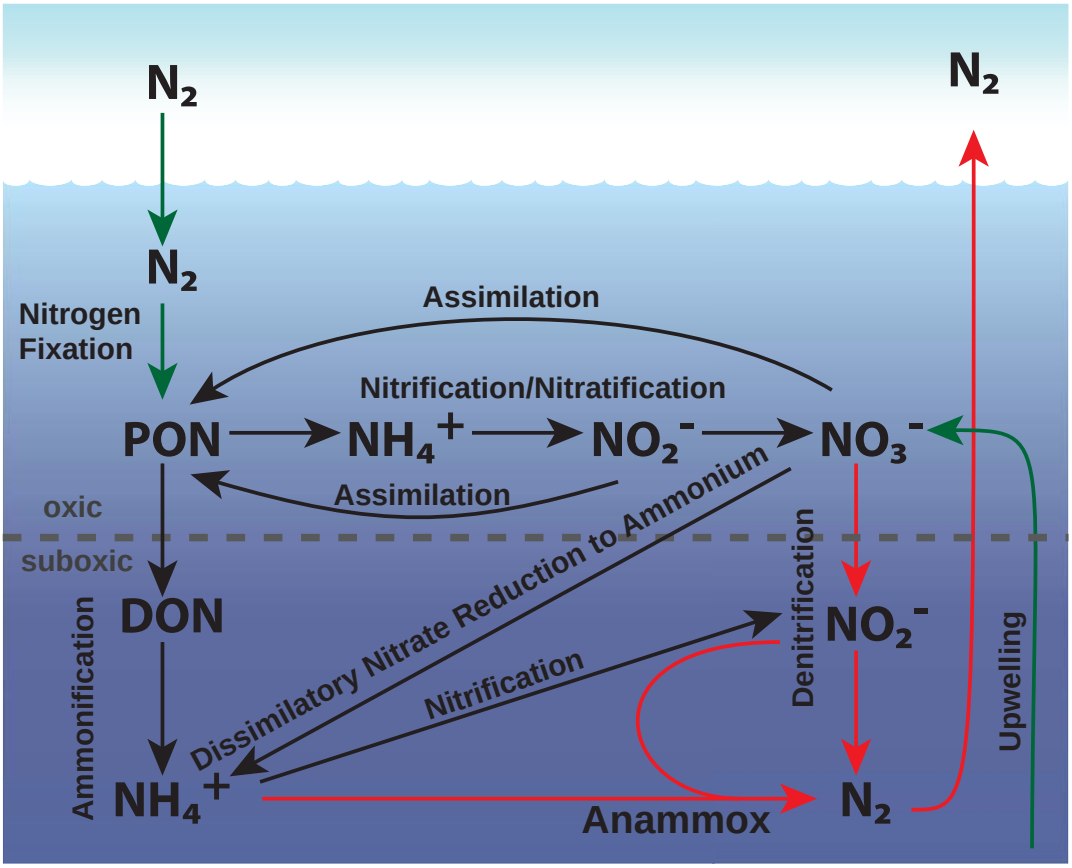

Figure 1: The marine biogeochemical nitrogen cycle, including simplified redox reactions. Losses of nitrogen from the ocean are indicated by red arrows, and gains of nitrogen by green. Modified from Arrigo (2005).

These transformations are regulated by microorganisms living in the water column and marine sediments (Arrigo, 2005; Brandes et al., 2007; Wright et al., 2012). Though dinitrogen gas (N_2) makes up 78% of the atmospheric mass, it is an inert gas. Apart from a few photoautotrophic microorganisms (belonging primarily to the genus cyanobacteria) using the enzyme nitrogenase to break its triple bond, N_2 is not used biologically in the marine environment (Gallon, 2001). As only specific organisms are able to fix and use the N_2 dissolved into the ocean from the atmosphere, the rest must obtain it in the forms of NO_3^-, NH_4^+, nitrite (NO_2^-), particulate organic nitrogen (PON), and dissolved organic nitrogen (DON).

The nitrogen cycle is intricately linked to the carbon cycle as nitrogen is essential in the biosynthesis of amino acids, nucleic acids, and some membrane lipids, and therefore, required for primary productivity in the ocean. However, bio-available nitrogen is not always abundant in the marine environment, and nitrogen often plays a limiting role to autotrophic organisms

fixing carbon dioxide (CO_2). This means the nitrogen cycle could influence atmospheric carbon sequestration in the deep ocean by regulating global primary productivity (Gruber and Galloway, 2008). Since the industrial revolution, there has been a marked anthropogenic effect on the global nitrogen cycle; however, the nitrogen budget in the ocean is still poorly constrained (Arrigo, 2005; Brandes et al., 2007). Thus, it is critical to understand the organisms responsible for nitrogen transformations in order to fully decipher the marine nitrogen cycle, as well as its impact on marine biota and the marine carbon sink.

The redox state of the environment determines which redox reactions are favoured, and which microorganisms proliferate. In the presence of oxygen, organisms assimilate nitrogen as NO_2^- or NO_3^- to form PON. After death, this PON can be remineralised to NH_4^+, which then may be oxidised to NO_2^- and further to NO_3^- by archaea and bacteria performing nitrification. This constant recycling provides nitrogen to primary producers. Yet, nitrogen is not conserved entirely in the oxic waters as some particulate organic matter sinks out of the water column, taking available nitrogen along with it. In anoxic environments, the system shifts towards organisms using reaction pathways, i.e., anaerobic ammonium oxidation and denitrification, that result in nitrogen loss, e.g., N_2 and nitrous oxide (N_2O) production (Mulder et al., 1995; Zumft, 1997).

These nitrogen cycle interactions occur in many natural oceanic settings, in particular within oxygen minimum zones (OMZs). OMZs occur when restricted ocean circulation and high oxygen consumption result in a thick vertical band of water with low oxygen concentration, bound by oxygenated water above and below. OMZs host an array of microorganisms performing nitrogen transformations as they take advantage of the varying redox environments (Bange et al., 2005; Wright et al., 2012).

1.2 **ANAMMOX**

Ammonium was long thought to be unreactive in anoxic conditions. However, in 1977, Broda published a paper based on thermodynamic principles in which he suggested that there was a redox reaction absent in nature: the oxidation of ammonium with nitrate or nitrite, which was potentially being performed by a missing lithotroph. Nearly 20 years later the "missing lithotroph" and its redox reaction were described (Mulder et al., 1995), e.g., anaerobic ammonium oxidation, or anammox, which is the oxidation of NH_4^+ by NO_2^- to produce N_2.

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$$

This reaction is performed by anammox bacteria. The production of N_2 through anammox is a sink of fixed nitrogen from the oceanic system (Dalsgaard and Thamdrup, 2002; Kuypers et al., 2005; Thamdrup et al., 2006; Hamersley et al., 2007).

1.2.1 ANAMMOX BACTERIA

Anammox bacteria are slow-growing, chemolithoautotrophic members in the order Brocadiales of the Planctomycetes phylum (Strous et al., 1999; Jetten et al., 2010). Before anammox was discovered in a Delft wastewater treatment plant (Mulder et al., 1995), denitrification was believed to be the sole producer of dinitrogen gas in the ocean. However, following anammox detection in the natural marine environment (first, in marine sediment incubations in 2002 by Thamdrup and Dalsgaard, followed by incubation and lipid biomarker

studies of two anoxic water columns, Dalsgaard et al., 2003; Kuypers et al., 2003), global estimates have anammox and denitrification sharing the production of N_2 50-50 (Arrigo, 2005; Brandes et al., 2007; Francis et al., 2007).

Though several species of anammox bacteria exist, thus far only species belonging to the genera *Scalindua* have been found in marine systems (Kuypers et al., 2005; Hamersley et al., 2007; Schmid et al., 2007). Anammox bacteria have been shown to play a crucial role in the present marine nitrogen cycle, especially in OMZs (Kuypers et al., 2005; Hamersley et al., 2007; Jaeschke et al., 2007; Pitcher et al., 2011). In our current climate setting of increasing ocean temperature and decreasing oceanic ventilation, OMZs are expanding (Wright et al., 2012); habitable environments for anammox bacteria are growing in size. It is therefore important to better describe the distribution of these bacteria, and to understand how they affect nutrient availability. Gene-based analyses (i.e., Kuypers et al., 2003; Hamersley et al., 2007; Pitcher et al., 2011) and isotope rate measurements (Thamdrup and Dalsgaard, 2002; Kuypers et al., 2005) have been used to study the modern distribution of anammox activity. Using the presence of specific biomarker lipids has become an increasingly more popular alternative. This method provide a potential tool to reconstruct past anammox activity, as lipids are better preserved in the sediment record than DNA and RNA (Hofreiter et al., 2001; Boere et al., 2011).

1.2.2 Ladderane lipids

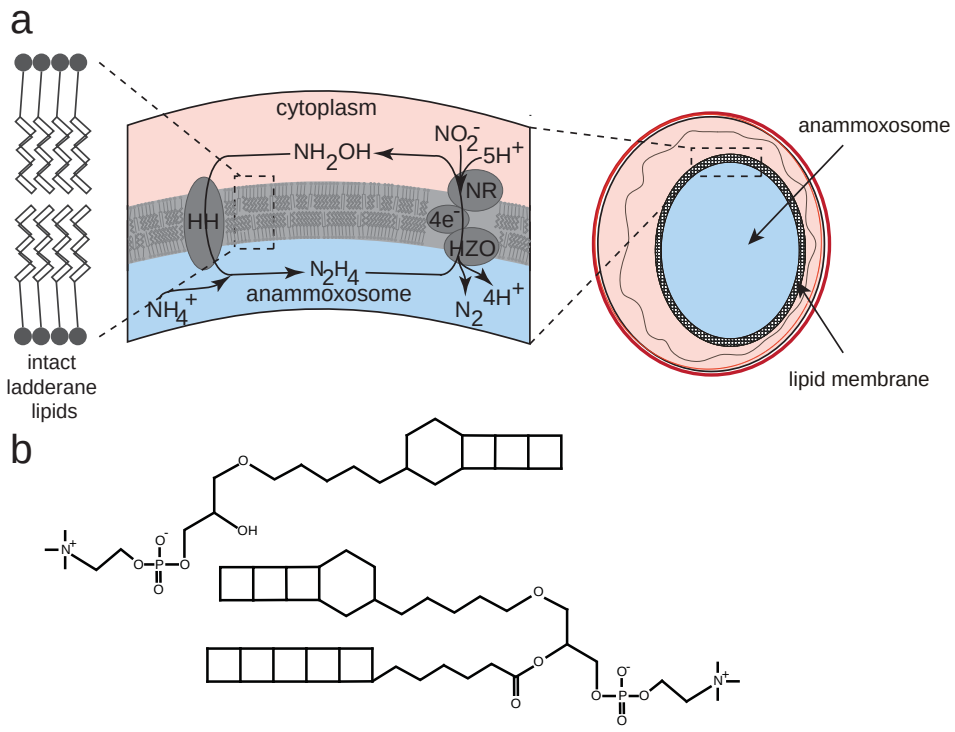

Figure 2: (a) Anammox cell structure, proposed model for the anammox process, and stacking of ladderane polar membrane lipids. HH, hydrazine (N_2H_4) hydrolase; HZO, hydrazine oxidizing enzyme; NR, nitrite reducing enzyme. (b) Examples of chemical structures of ladderane intact polar lipids (IPLs): ladderane phosphocholine (PC) monoalkylether (above), PC IPL with ether and ester linked ladderane moieties (below). Figure modified from Sinninghe Damsté et al. (2002a) and Kuypers et al (2003).

Anammox bacteria a synthesise unique set of lipids, i.e., ladderane lipids, so named because the stereochemical arrangement of their concatenated cyclobutane moieties resembles the form of a stepladder (Fig. 2a; Sinninghe Damsté et al., 2002a). The anammox reaction takes place inside an organelle-like structure within the cell, called the anammoxosome (Fig. 2a). It is believed that anammox bacteria dispense the energy to synthesise the strained cyclobutane bonds because these ladderane lipids form a dense anammoxosome membrane. This decreases membrane permeability, preventing the toxic intermediate of the anammox reaction (hydrazine) from diffusing into the cytoplasm and disrupting other cell functions (Sinninghe Damsté et al., 2002a). Thus, these lipids can be used as biomarkers for the presence of anammox bacteria, and are valuable to the detection of the anammox reaction in the natural environment.

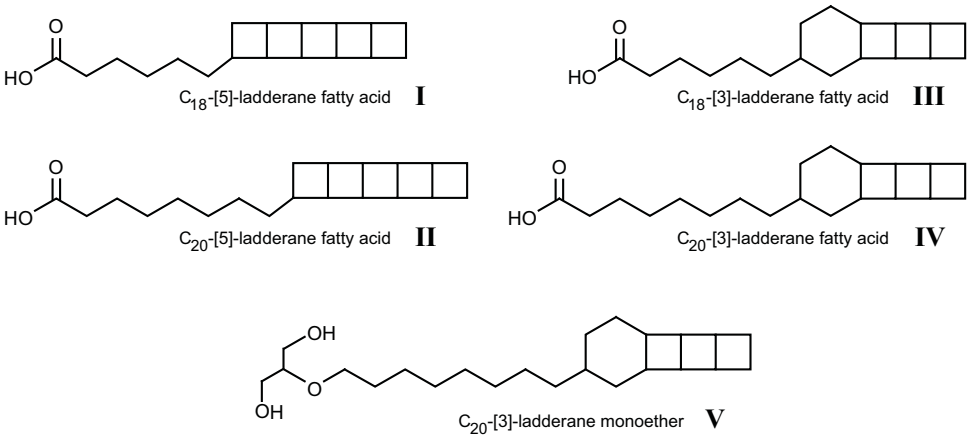

Figure 3: Chemical structures of the ladderane fatty acid lipids used as biomarkers for anammox bacteria. (I) C_{18}-[5]-ladderane fatty acid, (II) C_{20}-[5]-ladderane fatty acid, (III) C_{18}-[3]-ladderane fatty acid, (IV) C_{20}-[3]-ladderane fatty acid, (V) C_{20}-[3]-ladderane monoether.

Ladderane lipids are present in several forms (Sinninghe Damsté et al., 2002a; Sinninghe Damsté et al., 2005; Boumann et al., 2006). Within the cell, ladderane moieties are attached to polar lipid headgroups forming Intact Polar Lipids (IPLs) (Fig. 2b). Upon cell death, IPLs have been shown to degrade relatively quickly (Harvey et al., 1986; White et al., 1979), while core lipids remain more resistant. This also appears to be true for anammox lipids. Jaeschke et al. (2009a) showed that the concentration of a phosphocholine (PC) ladderane IPL decreased rapidly in the first centimeters of marine sediments, whereas ladderane core lipids, which were detected in deeper sediments, had a higher preservation potential. Traditionally, four ladderane fatty acids and a ladderane monoether core lipid are used as biomarkers for anammox activity (Fig. 3; Kuypers et al., 2005; Hamersley et al., 2007 Jaeschke et al., 2007; Jaeschke et al., 2009a; Jaeschke et al., 2010). These lipids contain a total of 18 or 20 carbon atoms, with either 3 or 5 cyclobutane rings.

The distribution of ladderane lipids has been show to vary with tempertature (Rattray et al., 2010). It was found that the chain length of ladderane fatty acids with five cyclobutane rings was dependent on the temperature at which the anammox bacteria were living. The tendency for chain length to increase with increasing temperature is not unique to ladderane fatty acids. This has been seen before in other bacterial membranes (Sinensky, 1974; Russell, 1984) that adapt

chain length to maintain membrane fluidity. Using the ratio of chain length of [5]-ladderanes, a proxy for in situ temperature was created: the iNdex of Ladderane lipids with 5 cyclobutane rings (NL_5; Equation 1). A global dataset of culture and marine samples was used to calibrate the relationship between temperature and chain length, which is described by Equation 2 and graphically represented by a 4th order sigmoidal relationship (Fig. 4). However, NL_5 is a new proxy and has not been widely applied. Relatively little is known about its limitations, nor about how broadly it can be used.

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

$$NL_5 = 0.2 + \frac{0.7}{1 + e^{-\left(\frac{\text{Temperature} - 16.3}{1.5}\right)}} \qquad \text{Equation 2}$$

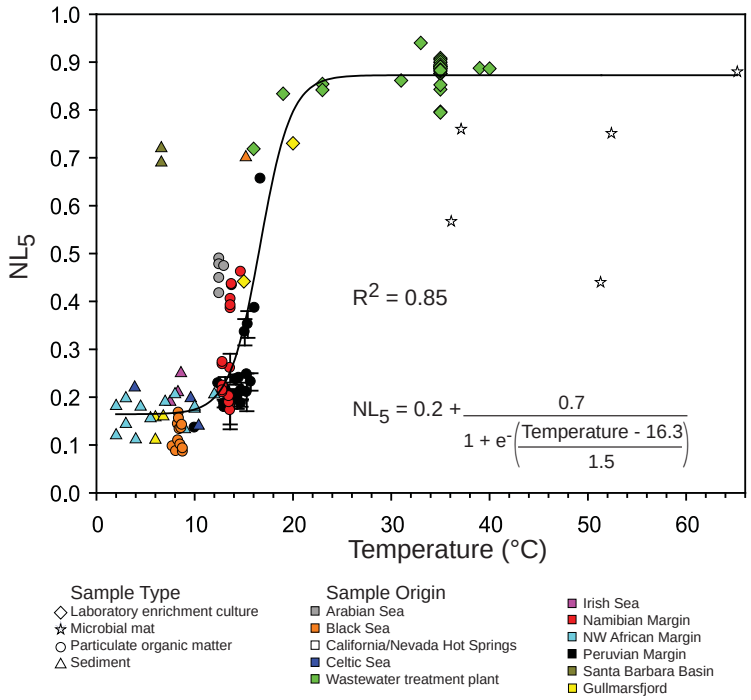

Figure 4: The index of ladderane lipids with 5 cyclobutane rings (NL_5) calculated using ladderane fatty acid distributions from sequencing batch reactor enrichment cultures, particulate organic matter, marine sediments, and microbial mats plotted against temperature. The equation relates to an empirical fourth-order sigmoidal regression. Figure from Rattray et al. (2010).

Ladderane lipids are unique tracers for the anammox process, and may also contain information on past temperatures. However, the application of ladderanes has been relatively limited, and only a fraction of the natural environment has been screened for the presence of anammox bacteria using these lipids. Many areas where ammonium and nitrite are available in the absence of oxygen, and where anammox is probably occurring, have yet to be studied.

1.3 LADDERANES AS POTENTIAL BIOMARKERS FOR PAST ANAMMOX

The past marine nitrogen cycle has been even more difficult to quantify than the modern one. While anammox is a relatively recent addition to our understanding of the nitrogen cycle, the process itself has probably been around for millions of years since anammox bacteria form a deep branching phylogenetic group (Strous et al., 2006). Indeed, it has been hypothesised that anammox was responsible for a decrease in nitrogen availability in the marine system during Cretaceous Ocean Anoxic Events (OAEs) (Kuypers et al., 2004). However, the detection of anammox bacteria using ladderane fatty acids has been limited to sediments <140,000 years (Jaeschke et al., 2009b), and laboratory experiments have shown that ladderane lipids disappear quickly when anammox biomass is artificially thermally matured (Fig. 5; Jaeschke et al., 2008). Therefore, though ladderanes are essential in tracing anammox presence and activity in modern marine settings, they are, as with any biomarker, useful only to the point where we know the effects of degradation on their distributions (Canuel and Martens, 1996). Thus, it is imperative to understand how these lipids react when exposed to common forms of dia- and catagenesis. We must first constraint the effects of these processes before ladderanes can be applied as tracers for past anammox activity.

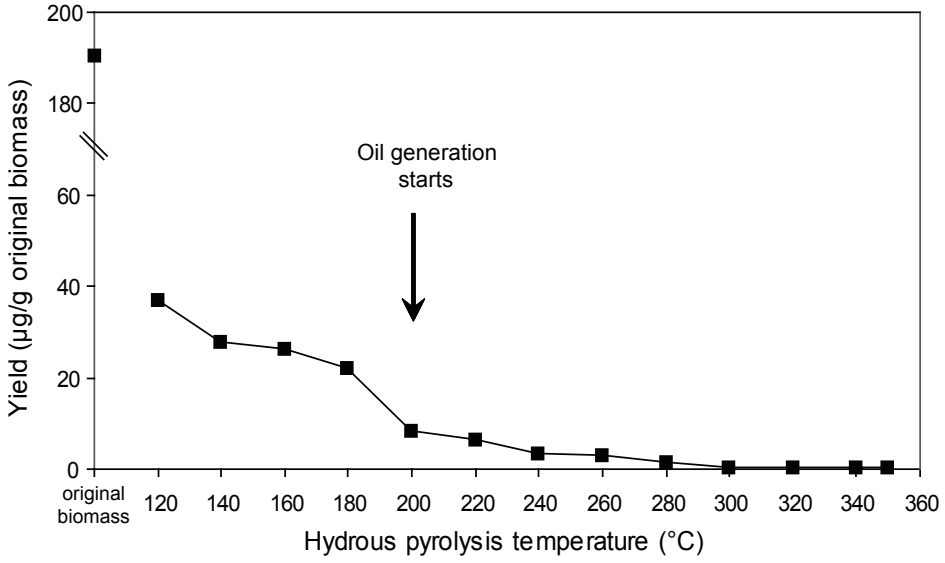

Figure 5: Concentration of ladderane lipids as a function of artificial maturation (hydrous pyrolysis) temperature (as anaylsed by gas chromatography) compared to the concentration of ladderane lipids in original, untreated anammox biomass. Note the break in the concentration axis. Figure modified from Jaeschke et al. (2008).

1.3.1 DIAGENESIS

It is estimated that less than 0.1 % of the organic matter produced in the photic zone is preserved in deep-water sediments (Henrichs, 1992; Wakeham et al., 2002). Much is remineralised, in the water column and during early diagenesis near the sediment-water interface, before eventual preservation (Hedges and Keil, 1995). Lipids like ladderanes provide valuable

information as biomarkers for specific biosynthetic pathways in source organisms (Peters et al., 2005; Volkman, 2005), which is fortuitous as, due to their aliphatic nature, lipids make up a significant portion of the organic matter preserved in the sediment record. However, lipids have been shown to undergo structural alterations by microbial and chemical processes already in the water column and later, once incorporated into sediments (Johnson and Calder, 1973; Sun and Wakeham, 1994; Hoefs et al., 2002; Rontani et al., 2006). Thus, in order to be able to reconstruct past biogeochemical cycles using molecular biogeochemistry, it is important to identify the diagenetic products of biomarker lipids such as ladderanes (Peters et al., 2005). The degree of lipid degradation is greatly determined by exposure time to oxygen, in the water column and in sediment pore water. In general, oxic conditions favour increased remineralisation of organic matter (Burdige, 2007). Currently, however, nothing is known of the effect of oxic degradation on ladderane lipid distributions.

1.3.2 Catagenesis

Over the course of millions of years, organic matter buried in marine sediment is exposed to increasing temperature and pressure. At temperatures of 60 – 100 °C, thermal alterations begin to crack chemical bonds in a process termed catagenesis. However, it is often problematic to study catagenetic transformations of specific biomarkers in natural settings, as this would require that identical sedimentary organic matter be subjected to varying degrees of thermal maturation. For the study of catagenesis of ladderane lipids, the difficulty is compounded by the fact that is problematic to find environments where organisms like anammox bacteria are the dominant source of organic matter. Studying the effects of catagenesis on specific biomarkers is however possible, in a laboratory setting, by simulating catagenesis using an artificial maturation technique such as hydrous pyrolysis (Lewan et al., 1979; Lewan, 1993). Hydrous pyrolysis is the heating of a rock or biomass sample in a closed reactor to subcritical temperatures in the presence of water over the course of several days. Essentially, fair representations of natural processes that would take place over millions of years are mimicked quickly.

In 2008, Jaeschke et al. reported hydrous pyrolysis experiments performed on anammox biomass, and found that ladderane lipids undergo structural modification already at low temperatures. Their cyclobutane rings were shown to open, with concomitant formation of double bonds, a process which has been observed previously on ladderane lipids during gas chromatography (Sinninghe Damsté et al., 2005). However, no thermally stable aliphatic products of anammox lipids, which would be most resistant in the sedimentary record, were reported.

1.4 Scope of this thesis

The aim of this thesis was to explore the applicability of ladderane lipids for anammox in natural environments as well as the impact of oxic degradation and catagenesis on ladderane lipids. The framework of this thesis is divided into two parts. The first part explores the importance of anammox in modern settings, both in marine and terrestrial environments. The second part of this thesis explores the fate of anammox lipids through their transformations by diagenetic and catagenetic processes.

1.4.1 PART I: LADDERANES IN ANAMMOX BACTERIA AND IN PRESENT-DAY OCEAN SYSTEMS

In **Chapter 2**, novel terrestrial anammox bacteria are described. This is the first time that a lab enrichment of soil anammox has been performed. The culture was obtained from an area where local agricultural runoff influenced the soil to become nitrogen-enriched (Hierdense Beek, Staverden, Netherlands). After 8 months, the culture contained 40 – 50% anammox bacteria, representing two separate clusters, neither having been described previously. Ladderane fatty acids were detected in this enrichment culture, with the C_{20}-[5]-ladderane being the most abundant, similar to the ladderane distribution of *Scalindua* species. This suggests ladderane fatty acids can be used to trace terrestrial anammox bacteria, as they have been used already in marine environments, which might lead to better understanding of the contribution of anammox bacteria to the soil nitrogen cycle.

Chapter 3 reports the presence of anammox in the Eastern Tropical North Pacific (ETNP). The ETNP contains a shallow OMZ, starting at a water depth <100 m. Four water column depth profiles of ladderane fatty acids were used to describe anammox activity in the ETNP. Ladderanes were detected in highest concentrations at the depth of nitrite maxima, suggesting that nitrite was the limiting substrate controlling anammox activity in the ETNP. C_{18} ladderanes dominated over C_{20} ladderanes in the ETNP, which is dissimilar to previous ladderane distributions in cultures and in the marine environment. This affected the NL_5 ratio inferred temperatures, highlighting that caution must be taken to apply this proxy only in settings where there is no bias between C_{18} and C_{20} ladderanes.

The importance of anammox in the chemocline of the Cariaco Basin is explored in **Chapter 4**. A temperature gradient controls the stratification and maintains anoxia in the Cariaco Basin, off the coast of Venezuela. Chemolithoautotrophy is an important process in the Cariaco Basin and accounts for a significant portion of the flux of OM below the chemocline. Ladderane fatty acid concentrations show that anammox is confined to a ~115 m thick section of the core chemocline (235 – ~350 m). Anammox cell numbers determined by FISH counts confirmed the presence of anaerobic ammonium oxidising bacteria. The presence of thaumarchaeal lipids at the same water depth suggests that marine archaea are providing at least a part of the nitrite for anammox activity.

1.4.2 PART II: DIAGENESIS AND CATAGENESIS OF LADDERANE BIOMARKERS

Chapter 5 describes degradation experiments where anammox biomass was mixed into slurries with Wadden Sea sediment and water, under oxic conditions. The experimental set-ups were heated at temperatures between 20 and 100 °C for 72 h. Degradation of ladderane fatty acids resulted in the production of short chain ladderane fatty acids, identified by nuclear magnetic resonance (NMR) and mass spectral analyses. The highest concentration of degradation products occurred at 40 °C, indicating that microbial activity was likely shortening the alkyl side chain of ladderane fatty acids two carbon atoms at a time, using a β-oxidation pathway. A new high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) method was developed to detect these degradation products in the natural environment.

This method was used in **Chapter 6** to describe the natural occurrence and distribution of two of the short chain ladderane oxic degradation products. In the water column of the anoxic Cariaco Basin, no oxidation products were identified, though original ladderane fatty acids were present. This suggests that the β-oxidation pathway is limited by the availability of

oxygen to the degrading microorganisms. In the Arabian Sea OMZ water column, short chain ladderane fatty acids were detected, indicating that the oxygen concentration required for this pathway is in fact quite low (<3 μM). In the sediments below the OMZ, short chain ladderane fatty acids were abundant and were shown to degrade at a slower rate than the original ladderane fatty acids. Finally, in deep sediments from under the Peru upwelling, short chain ladderane fatty acids were much more abundant than the original ladderane fatty acids. However, short chain ladderanes were not detected below sediment from the late Pleistocene, suggesting that they too are degraded, but might make an alternative biomarker for past anammox activity in OMZ settings.

Chapter 7 investigates the production of potential thermally stable anammox biomarker lipids. The aliphatic fractions of oil generated during the hydrous pyrolysis of anammox biomass showed that two unique component classes were produced at relatively high temperatures. Using NMR and mass spectral identifications, these classes were found to be $C_{28} - C_{30}$ branched alkanes and ladderane hydrocarbons with C_{19} and C_{20} homologues. However, neither of these classes was detected in samples from geological periods where anammox is thought to have taken place. This could be due to the low detection method used, the fact that the sediments screened were too immature for these lipids to have been generated, or it may be that these components are not naturally occurring catagenetic products of anammox lipids.

To summarise, the results presented in this thesis emphasise that anammox is an important process not only in the marine environment, but also in soils. However, the ladderane lipids that are currently used to trace anammox activity undergo relatively quick transformations in the water column and in sediments. These diagenetic products are often more abundant than the original ladderanes, and have the potential to be used to trace past anammox activity and the importance of anammox in the nitrogen cycle.

Part I: Ladderanes in anammox bacteria and in present-day ocean systems

Chapter 2

New anaerobic, ammonium-oxidising community enriched from peat soil

This chapter is based on
Hu, B.-I., Rush, D., van der Biezen, E., Zheng, P., van Mullekom, M., Schouten, S., Sinninghe Damsté, J.S., Smolders, A.J.P., Jetten, M.S.M., and Kartal, B. (2011) *Applied Environmental Microbiology* 77, 966-971.

Anaerobic ammonium-oxidising (anammox) bacteria have been recognised as an important sink for fixed nitrogen and are detected in many natural environments. However, their presence in terrestrial ecosystems has long been overlooked and their contribution to the nitrogen cycling in natural and agricultural soils is currently unknown. Here we describe the enrichment and characterisation of anammox bacteria from a nitrogen-loaded peat soil. After 8 months of incubation with the natural surface water of the sampling site and increasing ammonium and nitrite concentrations, anammox cells comprised 40 – 50% of the enrichment culture. The two dominant anammox phylotypes were affiliated to '*Candidatus* Jettenia asiatica' and '*Candidatus* Brocadia fulgida'. The enrichment culture converted ammonium and nitrite to dinitrogen gas with the previously reported stoichiometry (1:1.27) and had a maximum specific anaerobic ammonium oxidation rate of 0.94 mmol $NH_4^+ \cdot g$ (dry weight)$^{-1} \cdot h^{-1}$ at pH 7.1 and 32°C. The diagnostic anammox-specific lipids were detected at a concentration 650 ng·g (dry weight)$^{-1}$, and C_{20}-[3]-ladderane was the most abundant ladderane lipid.

2.1 INTRODUCTION

Anaerobic ammonium-oxidising (anmmox) bacteria form a deep-branching, monophyletic group within the Planctomycetes and anaerobically oxidise ammonium (NH_4^+) to dinitrogen gas (N_2) with nitrite (NO_2^-) as an electron acceptor (Jetten et al., 2010). They are active at redox transition zones, particularly in oceanic oxygen minimum zones (Dalsgaard et al., 2003; Kuypers et al., 2005; Hamersley et al., 2007; Lam et al., 2009) and in other marine ecosystems (Kuypers et al., 2003; Byrne et al., 2009; Engstrom et al., 2009; Glud et al., 2009). The discovery of anammox bacteria led to the realisation that a substantial part of the nitrogen loss that is observed in the marine environment — up to 50% of the total nitrogen turnover — was due to the activity of these bacteria (Arrigo, 2005). There are also several studies reporting the presence of anammox bacteria in many freshwater and marine sediments (Meyer et al., 2005; Penton et al., 2006; Rich et al., 2008; Dale et al., 2009; Jaeschke et al., 2009c). However, little is known to date about the distribution, diversity and activity of anaerobic ammonium oxidation in terrestrial ecosystems. Only this year anammox bacteria were detected in permafrost and agricultural soils by a 16S rRNA based molecular approach (Humbert et al., 2010). Nevertheless, anammox bacteria originating from terrestrial ecosystems have yet to be enriched in the laboratory under controlled conditions, and the physiology of these bacteria is still unknown.

Since anammox bacteria depend on the concomitant presence of nitrate/nitrite (NO_x) and NH_4^+ under oxygen-limited conditions, oxic-anoxic interfaces in terrestrial ecosystems should provide a suitable habitat for anammox bacteria. It is believed that in the marine environment, the source of NH_4^+ is mineralisation and nitrifiers or dissimilatory nitrate reduction provides the necessary NO_2^- and NH_4^+ (Kartal et al., 2007a; Lam et al., 2007; Lam et al., 2009). In many heavily farmed soil ecosystems agricultural run-off contributes significantly to the flux of inorganic nitrogen in the form of NH_4^+ and nitrate (NO_3^-).

Since the 1960's anthropogenic nitrogen deposition has led to such an increased inorganic nitrogen load in the Netherlands (Smolders et al., 2010). At the sampling site in Hierdense Beek valley, a swampy peat soil is fed by NO_3^--enriched local groundwater which infiltrates the adjacent pine forest. In the pine forest atmospheric ammonia, originating from nearby agricultural activities is intercepted by the canopy and nitrified in the forest soil. As a result, local ground water is acidified and strongly enriched in NO_3^-. This NO_3^--enriched ground water flows underneath the peat towards the 'Hierdense Beek' (Smolders et al., 2010). The NH_4^+-rich peat soil comes in contact with NO_3^--rich groundwater and oxygen is rapidly depleted in these organic soils creating a perfect environment for anammox bacteria.

The aim of the present study was to cultivate bacteria from peat soil and describe these soil-derived anammox bacteria. In order to enrich microorganisms indigenous to the peaty soil, the in situ niche should be mimicked as closely as possible in the laboratory (Kartal and Strous, 2008). To this end, we used a sequencing batch reactor (SBR) that provides the biomass retention that is essential for the cultivation of slow growing bacteria (Strous et al., 1998). The SBR system, combined with a "standardised" medium (van de Graaf et al., 1996) rich in inorganic nutrients, was used in the previous studies describing enrichment cultures of fresh water anammox bacteria (Strous et al., 1998; Kartal et al., 2007b; Kartal et al., 2008). However, the attempts to grow marine anammox species were only successful when not only the salinity but also the micronutrient concentrations of the marine environment were simulated by the

inclusion of Red Sea salt (van de Vossenberg et al., 2008). Here, with a similar approach, we used the in situ surface water to recreate the field-like conditions that would most suit the anammox bacteria from the soil ecosystem.

After six months of enrichment, the culture consisted of 40 to 50% anammox bacteria affiliated with two previously unknown species, contained the diagnostic ladderane lipids and could oxidise NH_4^+ at a rate of 0.94 mmol $NH_4^+ \cdot g$ (dry weight)$^{-1} \cdot h^{-1}$. The enrichment culture described here, presents the first insights to the physiology of anammox bacteria detected in terrestrial environments.

2.2 **MATERIALS AND METHODS**

2.2.1 SAMPLING SITE

The sampling site is located in the Hierdense Beek, Landgoed Staverden, the Netherlands (52°16' N, 5°44' E). Samples were taken at 0.1 m, 1 m and 2 m with a corer. The NO_3^- and NH_4^+ concentrations, pH and organic content of the samples were measured as described previously (Tomassen et al., 2004).

2.2 OPERATION OF THE BIOREACTOR

An SBR (working volume 5 L) was used for the enrichment and cultivation of anammox bacteria from soil (Strous et al., 1998). In order to prepare the influent medium, surface water was sterilised by hemofiltration (Fresenius Medical Care, Bad Homburg, Germany). Each SBR cycle consisted of 11 h of filling, 45 min of biomass settling and 15 min of drawing of the liquid. During each filling period, 0.5 L of the surface water originating from the sampling site containing NO_2^-, NH_4^+ and NO_3^- (concentrations specified in Results) was added continuously to the reactor at a flow rate of 0.5 mL$\cdot$min^{-1}. To maintain anoxic conditions, the reactor and the medium vessel were flushed continuously with Ar-CO_2 (95:5, 10 mL$\cdot$min^{-1}). The SBR was stirred at 200 rpm and was operated at room temperature (20 °C). The pH in the SBR was kept at 7.0 via the CO_2 present in the supplied gas and an automated pH control unit supplying $KHCO_3$.

2.2.3 ANAMMOX ACTIVITY ASSAYS

Biomass (40 mL) from the SBR was transferred to 60 mL serum bottles. The biomass was not washed, because a washing step would have reduced the activity of the anammox bacteria considerably. Bottles were sealed with 5-mm-radius butyl rubber stoppers and were made anoxic by alternately applying underpressure and Ar-CO_2 gas at least 7 times. An overpressure of 10^5 Pa was maintained in the bottles. Soluble substrates were added to the bottles from 10 mM anoxic stock solutions. To measure anaerobic ammonium oxidation activity, final concentrations of 250 µM, 500 µM or 1000 µM NO_2^- were used. Ammonium was not added to the incubations because it was in excess in the reactor. Bottles were incubated at pH 7, 20 °C and were shaken continuously at 200 rpm for 1 day. The activity assays with ^{15}N-labeled nitrogen compounds were conducted as described above except $^{15}NH_4^+$ and $^{15}NO_2^-$ were used at concentrations of 250 µM, 500 µM or 1000 µM (99% pure; purchased as chloride and sodium salts, respectively, from Cambridge Isotope Laboratories Inc., Andover, MA, U.S.A.).

2.2.4 Optimum temperature and pH assays

For the optimum temperature analysis, a range of 15 to 35 °C with 5 °C increments was used. For the optimum pH determination, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer in combination with HCl or NaOH was used to adjust the pH to a range of 6.5 to 8.5 (with 0.5 pH unit increments). Final concentrations of 250 μM NH_4^+ and NO_2^- were used in all tests, and the incubations were conducted as described above. The pH of each sample was measured again at the end of the incubation to determine any changes. A highly enriched culture of "*Candidatus* Brocadia fulgida" was used as a positive control in the assays (Kartal et al., 2010).

2.2.5 Analytical methods

In the activity tests, NO_2^- and NH_4^+ were measured as described by Kartal et al. (2006). The isotopic compositions of dinitrogen gas and nitrous oxide were determined with a gas chromatograph (Agilent 6890) coupled to a quadruple inert mass spectrometer (Agilent 5975c). The total solids (TS) were quantified by drying at 105 °C and weighing after cooling in a desiccator.

2.2.6 DNA extraction and PCR

The original soil sample (1 g) or the biomass from the SBR (1.5 mL) was centrifuged. DNA was extracted with a PowerSoil DNA isolation kit (Mobio, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. DNA was then dissolved in 50 μL ultrapure water, and kept at 4 °C until further analysis.

The primer combination Amx368F-Amx820R was used for the preferential amplification of the 16S rRNA genes of anammox bacteria (Schmid et al., 2003). PCR fragments were cloned directly using the pGEM-T Easy cloning kit (Promega, Leiden, the Netherlands) according to the manufacturer's instructions. Plasmid DNA was isolated and purified with the GeneJET plasmid miniprep kit (Fermentas, Burlington, Canada). Plasmids were digested with 5 U EcoRI enzyme in EcoRI buffer for 1.5 h at 37 °C. The digestion products were examined for an insert with the expected size by agarose gel (1%) electrophoresis.

2.2.7 Sequencing and phylogenetic analysis

The sequences of the 16S rRNA gene fragments were determined by using the M13 forward primer, the M13 reverse primer, and an internal primer targeting vector sequences adjacent to the multiple cloning sites. Phylogenetic analyses were performed with the neighbour-joining method using the Tamura-3 algorithm and pairwise deletion substitution model (Tamura et al., 2007), and were tested by bootstrap analysis with 3,000 replications.

2.2.8 Fluorescence in situ hybridisation (FISH)

Biomass (1.5 mL) was harvested from the enrichment culture and was fixed in paraformaldehyde. Hybridisations with fluorescent probes were performed as described previously (Schmid, et al., 2003). All probes were purchased as Cy3-, Cy5- and 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (Fluos)-labeled derivatives from Thermo Electron Corporation (Ulm, Germany). The following probes were used to monitor the enrichment of the anammox population as described in Schmid et al. (2003): Amx 368 (S-*-Amx-0368-a-A-18, specific for all known anammox genera; Schmid et al., 2003), Amx 820 (S-*-

Amx-0820-a-A-22, specific for "*Candidatus* Kuenenia stuttgartiensis" and "*Candidatus* Brocadia anammoxidans; Schmid et al., 2000), EUB 338 (S-D-Bact-0338-a- A-18; Amann et al., 1990), EUB 338 II (S-D-Bact-0338-b-A-18), EUB 338 III (S-D-Bact-0338-c-A-18), together with EUB and EUBII, specific for most bacteria; (Daims et al., 1999), and Pla46 (S-P-Planc-0046-a-A-18), specific for Planctomycetales (Neef et al., 1998).

2.2.9 Lipid Analysis

Biomass (50 mL) was harvested from the SBR and freeze-dried. It was extracted with a modified Bligh-Dyer method according to Rattray et al. (2008). The sample was ultrasonically extracted for 15 min using methanol: dichloromethane (DCM): phosphate buffer, pH 7.4 at a volume ratio of 2:1:0.8 (v/v/v). The supernatant was collected, and the residue was re-extracted ultrasonically twice. The solvent ratio of the combined supernatants was adjusted to 1:1:0.9 (v/v/v), and the supernatants were centrifuged. The bottom DCM layer was collected and the remaining solvent re-extracted twice with DCM. The DCM layers were combined and dried under a rotary evaporator. The extract was then eluted over Na_2SO_4 and dried under N_2.

An aliquot of the Bligh-Dyer extract was separated over a small column of activated silica (60 mesh; activated for 3 h at 130 °C and cooled in a desiccator to room temperature). The aliquot was eluted 3 times with 2:1 (v/v, ethyl acetate: hexane) then 3 times with methanol in order to separate the anammox ladderane lipids (methanol fraction) from soil material (ethyl acetate: hexane fraction). The methanol fraction was saponified by refluxing with aqueous KOH (in 96% methanol) for 1 h. Fatty acids were obtained by acidifying the sample to a pH of 3 with 1N HCl in methanol, followed by extraction using DCM. The fatty acids were converted to their corresponding fatty acid methyl esters (FAMEs) by methylation with diazomethane (CH_2N_2). Excess CH_2N_2 was removed by evaporation under N_2. Polyunsaturated fatty acids (PUFAs) were removed by elution of the sample over a small $AgNO_3$ (5%)-impregnated silica column with DCM.

The fatty acid fraction was dissolved in acetone and filtered through a 0.45-μm-pore size, 4-mm-diameter polytetrafluoroethylene (PTFE) filter and analysed by high performance liquid chromatography coupled to positive-ion atmospheric pressure chemical ionisation tandem mass spectrometry (HPLC/APCI-MS/MS) in selective reaction monitoring (SRM) mode, as described in Hopmans et al. (2006) and modified in Rattray et al. (2008). Ladderane lipids were quantified using an external calibration curve of standards of isolated methylated ladderane fatty acids containing the [3]- and [5]- ladderane moieties (Sinninghe Damsté et al., 2002a; Hopmans et al., 2006; Rattray et al., 2008). A detection limit of 30 – 35 pg injected was achieved with this technique.

2.2.10 Nucleotide sequence accession numbers

The sequences determined at the time of inoculation have been deposited in GenBank under accession numbers HQ637487 (cluster 1), HQ637488 (cluster 2), and HQ637489 (cluster 3). Those determined after 8 months of incubation have been deposited in GenBank under accession numbers HQ610833 (cluster 1) and HQ610834 (cluster 2).

2.3 RESULTS

2.3.1 SAMPLING SITE

The NO_3^- and NH_4^+ profiles of the peat soil at the Hierdense beek valley showed that these nitrogen species co-occurred between 1 and 2 m of the depth profile. The measurements also suggested that there was a significant consumption of NO_3^- and NH_4^+ at these sampling points (Fig 1). Throughout the depth profile, O_2 concentration was below the detection limit (15 μM).

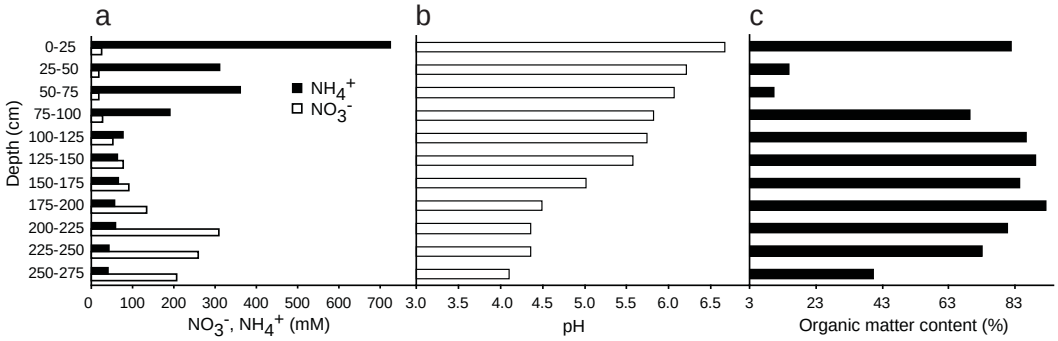

Figure 1. (a) Nitrate and ammonium concentration, (b) pH, and (c) organic matter content of the sampling site.

2.3.2 SCREENING OF INOCULUM

Five samples were taken from Hierdense Beek peat soil at two locations and three depths (Table 1). Samples HB1, HB4 and HB5 were taken from the peat soil and samples HB2 and HB3 from the adjacent grassland. PCR amplification with a primer combination of Pla46F and universal reverse primer 630R (Planctomycete specific) did not result in any sequences affiliated with anaerobic anammox bacteria. When the primer set 368F-820R (specific for anammox bacteria) was used, three sequence clusters affiliated with anammox bacteria could be detected in samples HB1, HB4 and HB5. All three clusters were detected in sample HB5; thus, it was decided that sample HB5 would be suitable for inoculating a bioreactor for the enrichment of soil anammox bacteria.

2.3.3 ENRICHMENT OF ANAMMOX BACTERIA

A SBR with a working volume of 5 L was inoculated with 1 kg (wet weight) of soil homogenised with surface water from Hierdense Beek (soil:surface water; 1:4). The influent medium was prepared by filtering surface water from Hierdense Beek with a hemolfilter (Fresenius Medical Care, Bad Homburg, Germany). Initially, the medium was supplemented with 0.5 mM NO_2^- and 1 mM NH_4^+ and NO_3^-. The substrate concentrations were gradually increased to 4mM. The effluent concentration of NO_2^- was always below detection limit (10 μM). The population diversity of the enrichment culture and the abundance of anammox bacteria were monitored by FISH and 16S rRNA gene clone libraries.

2.3.4 ACTIVITY TESTS

The anammox activity of the enrichment culture was tested every 2 months during the operation of the reactor. Anammox activity was detected in the batch incubations for the first time after 4 months. This activity increased with a doubling time of 27 days in the following 4

months, and reached a pseudo steady-state. The highest specific activity for the enrichment culture was 0.94 (± 0.04) mmol $NH_4^+ \cdot h^{-1} \cdot g$ (dry weight)$^{-1}$ with a stoichiometry of 1:1.27 (NO_2^-:NH_4^+). The optimal temperature and pH of the culture were 32 °C and 7.1, respectively. These parameters were different from the control culture (~80% enriched "*Candidatus* Brocadia fulgida"; Kartal et al., 2010), which had maximum specific activity at 30 °C and pH 7.8 with a rate of 9 mmol $NH_4^+ \cdot h^{-1} \cdot g$ (dry weight)$^{-1}$.

Table 1. Nitrate and ammonium concentrations, depth, and pH of the samples.

Location	Sample site	Concentration (μM)		Depth (m)	pH
		Nitrate	Ammonium		
HB1	Peat soil	13	740	0.1	6.8
HB2	Dry grassland	14	8	2	4.5
HB3	Dry grassland	9	7.5	1	5.8
HB4	Peat soil	220	1.4	2	4.6
HB5	Peat soil	603	43	1	5.8

2.3.5 16S rRNA analysis and FISH microscopy

In order to determine the species of anammox present in the enrichment culture, we applied our anammox-specific 16S rRNA sequences approach. DNA extraction, PCR amplification (with the Amx368F-820R primer set), cloning, and sequencing were conducted at the time of the inoculation and after 8 months. Twenty clones were randomly selected for sequencing at each sampling point. Phylogenetic analysis of the clones at the time of the inoculation revealed that there were three detectable genera in the Hierdense Beek valley, in contrast to marine sediments, where only one genus has been detected until now (Sup. Fig. 1). At the end of 8 months, when a pseudo steady-state was reached, two anammox phylotypes, each represented with 9 out of 19 sequences, could be detected in the clone libraries. These clusters (clusters 1 and 2) were unique groups with only 96% sequence similarity to the closest known anammox species, "*Candidatus* Jettenia asiatica" and "*Candidatus* Brocadia fulgida", respectively (Fig. 2).

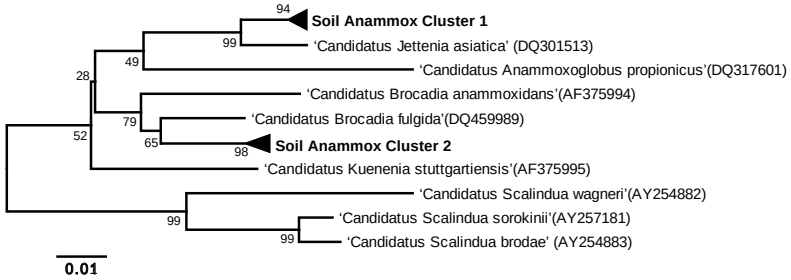

Figure 2. Phylogenetic tree depicting the 16S rRNA gene sequence diversity of the anammox species in the soil enrichment culture after 8 months, and the relation of these sequences to known anammox bacteria.

The enriched anammox bacteria could also be visualised by FISH microscopy. Anammox clusters were identified after 6 months of enrichment with the Amx820 probe, specific for "*Candidatus* Brocadia"-, "*Candidatus* Jettenia"-, and "*Candidatus* Kuenenia"- like bacteria (Fig. 3).

2.3.6 Lipid Analysis

Four ladderane fatty acids (as FAMEs) were analysed by HPLC/APCI-MS/MS. All scanned ladderane fatty acids were detected in the enrichment culture (Fig. 4, b-e). The dominant ladderane fatty acid in the culture was the C_{20}-[3]-ladderane (Fig. 4e) and constituted 46% of the total ladderane lipid concentration. The three remaining ladderanes, C_{18}-[5]-, C_{18}-[3]-, and C_{20}-[5]-ladderane fatty acids, contributed 14%, 11%, and 29%, respectively. The combined total concentration of ladderane fatty acids in the soil culture was 650 ng·g (dry weight)$^{-1}$.

2.4 DISCUSSION

The surroundings of the Hierdense Beek valley, the Netherlands, have been strongly enriched in anthropogenic nitrogen for more than 40 years due to agricultural activity. Since the 1980's part of the grasslands in the valley has become progressively wetter, resulting in the development of a swampy peat soil. This can be attributed to the anaerobic decomposition of the deeper peat layer, most likely because the groundwater is enriched in NO_3^-, which serves as an alternative electron acceptor (Smolders et al., 2010).

For the sampling site, anammox bacteria were detected by PCR cloning in three of the five samples (HB1, HB4 and HB5) taken from different depths, and showed a high diversity (3 distinct species) for such a limited set of samples. It is most likely that separate microniches existed in the heterogeneous soil samples, suggesting that the terrestrial ecosystems harboured different anammox species, unlike the ubiquitous (and almost exclusive) presence of "*Candidatus* Scalindua" genus in the oceans (Engstrom et al., 2005; Penton et al., 2006; Schmid et al., 2007; Lam et al., 2009). The two samples (HB2 and HB3) in which anammox bacteria could not be detected were from a dry grassland in the sampling site, where NO_x concentrations in the ground water were much lower (<10 μM). Nevertheless, the possibility that primer bias may account for the negative result cannot be ruled out.

Our past studies have shown that the inorganic medium described by van de Graaf et al. (1996) was suitable for the enrichment of wastewater anammox species, but was most

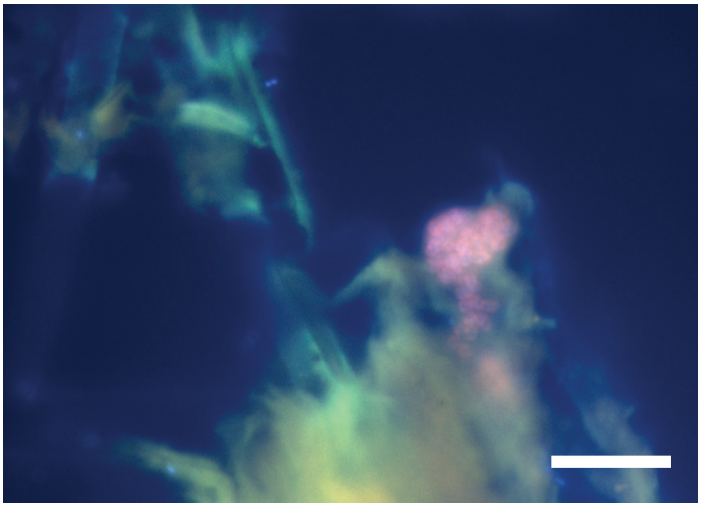

Figure 3. A fluorescence in situ hybridisation micrograph of the anammox enrichment culture after 6 months. The anammox bacteria are hybridised with Amx820 (Cy3; red) and EUB I,II,III probes (Fluos; green) probes, and with the DNA stain DAPI (pink). The scale bar is 10 μm.

probably inhibitory to the species originating from natural ecosystems that were adapted for survival under nutrient limitation (van de Graaf et al., 1996; van de Vossenberg et al., 2008). In order to enrich the indigenous anammox bacteria, the in situ surface water (hemofilter sterilised) was used as influent medium with low initial NO_2^- and NH_4^+ concentrations (0.5 mM). This resulted in a slow, but steady increase in the consumption of the substrates in the reactor. The first detection of anammox activity in batch incubations was followed by an increase in the anaerobic ammonium oxidation rate, and the responsible bacteria could be visualised with FISH microscopy.

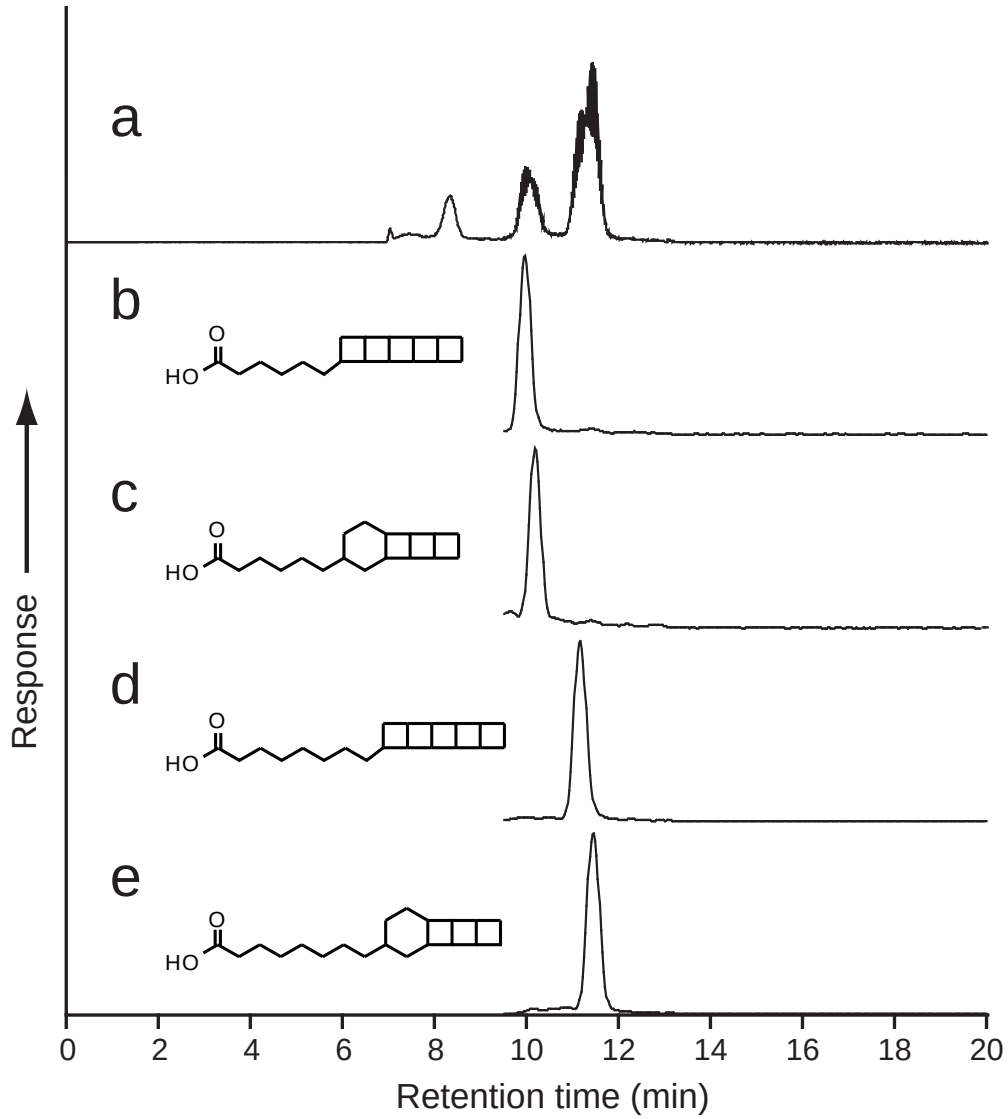

Figure 4. (a) Base peak chromatogram, and (b,c,d, and e) SRM trace of 4 ladderane FAMEs obtained HPLC/APCI-MS/MS analysis of freeze-dried soil biomass. Traces b, c, d, and e show the C_{18}-[5]-, C_{18}-[3]-, C_{20}-[5]-, and C_{20}-[3]-ladderane FAMEs, respectively.

The first anammox cells that could be detected by FISH were loosely attached cells (data not shown). After six months of enrichment, as observed for previous enrichment cultures (Schmid et al., 2003; Kartal et al., 2007a; van den Heuvel et al., 2010), the anammox bacteria were present in tightly packed clusters and constituted about 40 to 50% of the population detectable with FISH microscopy and counterstaining with DNA dye, 4',6-diamidino-2-phenylindole (DAPI; Fig 3). The detected cells seemed to be attached to soil particles which could facilitate better settling, necessary for growth in a SBR (Fig. 3).

In contrast to the previous reports that showed one anammox strain per enrichment culture, there were two clusters of anammox bacteria in the enrichment culture after 8 months (Fig. 2). Neither of these clusters corresponded to previously described anammox species. Phylogenetic analysis based on the 16S rRNA gene showed that these clusters had the highest similarities (96%) to "*Candidatus* Jettenia asiatica" and "*Candidatus* Brocadia fulgida". The niche differentiation of anammox bacteria could be defined by micronutrient concentrations, or the inclusion of an alternative energy source (Kartal et al., 2007a; Kartal et al., 2007b; van de Vossenberg et al., 2008). Compared to the almost exclusively used activated-sludge inoculum, seeding material from the sediment environment most likely contained more difficult-to-degrade and slow released organic compounds (e.g. humic acids). It has been reported that the endogenous electron donors in soils could as long as several months to be completely degraded anaerobically (van den Heuvel et al., 2010). In the current enrichment culture, there were probably still enough soil-derived compounds to allow the presence of two groups of anammox bacteria (van den Heuvel et al., 2010). Because the enrichment culture was fed with in situ surface water, the compound(s) that affect the selection of a certain species might be constantly supplied to the enrichment culture leading to the enrichment of two different species.

The biomass from the enrichment culture had a substrate (NO_2^- and NH_4^+) conversion stoichiometry of 1:1.27, which was almost identical to the previously reported value, 1:1.3 (van de Graaf et al., 1996). The highest anaerobic ammonium-oxidising activity was at 32 °C and pH 7. The optimum pH for the activity reflected the operational conditions of the bioreactor. The maximum specific activity of the biomass from the reactor was 0.94 (± 0.04) mmol $NH_4^+ \cdot h^{-1} \cdot g$ (dry weight)$^{-1}$. This lower specific activity (approximately 10% of the control) could be an underrepresentation due to the large amount of non-biological particles present in the enrichment culture as a result of the very long solids retention times (SRT) required for the enrichment of anammox bacteria.

The long SRT also led to a high abundance of soil- and plant-derived lipids in the fatty acid fraction isolated from the bioreactor. Once these lipids were removed, the ladderane lipids, so far a unique biomarker for anammox bacteria (Sinninghe Damsté et al., 2002a), could be detected in relatively high abundances in the enrichment culture. The concentrations of ladderane lipids were lower than those previously reported in highly enriched cultures (75 to 90%, Rattray et al., 2008), which is most likely due to the contribution of non-biological particles to the dry weight measurements. C_{20} ladderane fatty acids dominated over shorter-chained C_{18} fatty acids, a lipid distribution comparable to that found in other anammox enrichment cultures. The most abundant lipid type was the C_{20} fatty acid with 3 cyclobutane rings and one cyclohexane ring, as in the previously described enrichment culture of "*Candidatus* Scalindua"-like bacteria.

In this study, two previously unknown anammox species were enriched from a soil deposited with anthropogenic nitrogen. The physiological parameters and substrate conversion stoichiometry conformed to those for the previously described species. The soil enrichment culture also contained the diagnostic ladderane lipids unique to anammox bacteria, in particular the C_{20}-[3]-ladderane fatty acid. The enrichment of an anammox species from the terrestrial ecosystem is a first step towards better understanding the contribution of anammox bacteria to the nitrogen cycling in natural and agricultural soils.

ACKNOWLEDGEMENTS

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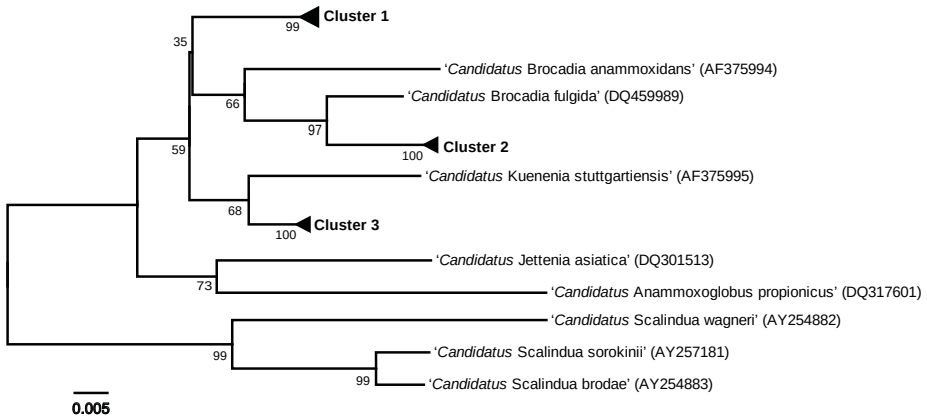

Supplementary Figure 1. Phylogenetic tree depicting the 16S rRNA gene sequence diversity of the anammox species in samples HB1, HB4, and HB5. Cluster 1 contains sequences from samples HB4 and HB5, Cluster 2 contains sequences from samples HB1 and HB5 and Cluster 3 contains sequences from all samples.

Chapter 3

Biomarker evidence for anammox in the oxygen minimum zone of the Eastern Tropical North Pacific

This chapter is based on
Rush, D., Wakeham, S.G., Hopmans, E.C., Schouten, S., Sinninghe Damsté, J.S. (2012) *Organic Geochemisty*, in press.

Anaerobic oxidation of ammonium (anammox) is an important process in the marine nitrogen cycle. It has been estimated to contribute up to 50% of nitrogen loss from the ocean, and is especially prevalent within oxygen minimum zones (OMZs). Here we studied the presence and distribution of anammox in the extended OMZ of the Eastern Tropical North Pacific (ETNP) using ladderane fatty acids (FAs), specific biomarkers for anammox bacteria. The validity of ladderane FAs as proxies for anammox bacteria was demonstrated by their excellent correspondence with anammox 16S rRNA functional gene abundances, and their expression and intact polar ladderane lipid concentrations in suspended particulate matter (SPM) from the Arabian Sea. In the ETNP, SPM was collected from various water depths at four stations along a northwest to southeast cruise transect, and ladderane FAs were analysed at each station. In all SPM samples where ladderane lipids were detected, C_{18} ladderane FAs were on average 5 fold more abundant than C_{20} ladderane FAs. Maximum concentrations in ladderane FAs (1.1 – 2.3 ng L^{-1}) were recorded at 400 – 600 m water depth, often corresponding to the depth of the secondary nitrite maximum. In one of the four stations, a second maximum in the ladderane FA concentration was noted at a shallower depth (i.e. at 85 m), coinciding with higher nitrite availability at this water depth. This suggests that nitrite, along with oxygen, may be a limiting factor for anammox activity in the ETNP. Anammox lipids were abundant within the OMZ at all stations, and concentrations were comparable to those in other OMZs, suggesting that anammox may be responsible for a significant loss of nitrogen in the OMZ of the ETNP.

3.1 INTRODUCTION

Anaerobic ammonium oxidation (anammox) is the conversion of equal amounts of ammonium (NH_4^+) and nitrite (NO_2^-) to dinitrogen gas (N_2) (van de Graaf et al., 1995). This reaction is performed by anammox bacteria, a deep branching monophyletic group within the phylum Planctomycetes. The anammox reaction takes place within an organelle-like structure, the anammoxosome (Lindsay et al., 2001; van Niftrik et al., 2004) of the cell. The densely packed lipids surrounding the anammoxosome, called ladderane lipids, prevent the toxic intermediates from diffusing into the rest of the cell membrane (Sinninghe Damsté et al., 2002a). Ladderane lipids contain 3 to 5 concatenated cyclobutane moieties and are unique biomarker lipids for anammox bacteria. The presence of ladderane lipids in suspended particulate matter (SPM) in the water column is indicative of the presence of anammox bacteria and thus the occurrence of the anammox reaction (Hamersley et al., 2007; Jaeschke et al., 2007). Complimentary studies comparing genetic, isotopic and lipid techniques (Kuypers et al., 2003; Kuypers et al., 2005; Wakeham et al., 2007; Rattray, 2008; Jaeschke et al., 2010; Pitcher et al., 2011; Brandsma et al., 2011) have shown that ladderane lipids, both as fatty acids (FAs) as well as intact polar lipids, reflect well the extent to which anammox bacteria occur in the environment.

Production of N_2 through anammox represents an important loss of nitrogen from the oceanic system, affecting the marine nitrogen cycle (Devol, 2003; Arrigo, 2005). Anammox occurs in low oxygen (O_2) environments where NH_4^+ and NO_2^- are freely available to the bacteria, and is active ubiquitously in the O_2 depleted ocean, both in the water column of oxygen minimum zones (OMZs) and within anoxic sediments (Thamdrup and Dalsgaard, 2002; Kuypers et al., 2003; Dalsgaard et al., 2003; Kuypers et al., 2005; Hamersley et al., 2007). Anammox has been shown to be quantitatively important for the nitrogen cycle in most OMZs, however, in some OMZs, such as the Arabian Sea, its prominence is debated (cf. Ward et al., 2009; Jensen et al., 2011). Recently, Pitcher et al. (2011) found anammox bacteria, through lipid- and gene-based analyses, to be abundant within the core of the Arabian Sea OMZ, showing that at least in a certain niche, anammox may be an important process in the Arabian Sea OMZ.

One of the most prominent OMZs in the present day ocean is the Eastern Tropical North Pacific (ETNP). It has been extensively studied (Lavin et al., 2006), partly due to the economic significance it has for the fishing industry of the region. Coastal and open-ocean upwelling in the ETNP is caused by Ekman pumping. The O_2 concentrations in the ETNP OMZ are lower than most other low oxygen regions in the ocean (Fiedler and Talley, 2006). The OMZ is quite shallow, starting at <100 m below the surface, and is maintained by high productivity, a permanent picnocline that hinders ventilation, and slow circulation that prevents penetration of oxygen into the old, oxygen-depleted deep waters (Sarmiento et al., 1988). The intermediate waters in the Gulf of Tehuantepec, off southeastern Mexico, are sourced from the west by the Equatorial Undercurrent. Once this current reaches the ETNP, its waters are depleted in O_2 after having traveled across the Pacific (Hendy and Pedersen, 2006). The Costa Rica Dome, located to the east of the Gulf of Tehuantepec, is another upwelling feature of the ETNP that has consequences on the biogeochemistry of the waters. Seasonal changes in coastal water movement, the Intertropical Convergence Zone (ITCZ), and geostrophic currents at 10°N (Kessler, 2006) cause high primary productivity in the region. The strong temperature and density gradients in the ETNP also affect nutrient concentrations and a strong nutricline has already been observed (Chavez et al., 1996). A secondary nitrite maximum is evident in

some parts of the ETNP (Brandhorst, 1959; Cline and Richards, 1972).

The ETNP OMZ would therefore seem an ideal niche for anammox activity, but it is unclear whether anammox occurs there or not. Here, we analysed SPM for ladderane FAs along a cruise transect in the ETNP to investigate the areal and depth distribution of anammox bacteria.

3.2 **Materials and Methods**

3.2.1 Study sites and sampling methods

Samples were collected during the Eastern Tropical Pacific cruise aboard the R/V *Seward Johnson* (Autumn 2007). Station 1 was located within the "Tehuantepec Bowl" (Kessler, 2006), west of the Gulf of Tehuantepec. The cruise followed a south-east transect towards the Costa Rica Dome, where Station 8 was located (Fig. 1). SPM was collected on ashed glass fiber filters (GFF; 142 mm diameter; 0.7 μm nominal pore size) at four stations (Table 1) using McLane WTS-LV in situ filtration systems that were mounted to a CTD, which determined the depths of the pumps. Two GFFs were used in sequence to minimise the loss of small particles passing through a single filter. O_2 was measured using a Seabird 43 oxygen sensor, calibrated against dissolved O_2 determined by Winkler micro-oxygen titration following instructions of the manufacturer. Water samples for nutrient profiles were collected at each station in 10 L Niskin bottles mounted on a rosette to the CTD. The detection limits for O_2, NO_2^-, and NH_4^+ were 0.1 μM, 0.01 μM and 0.38 μM, respectively (Podlaska et al., 2012).

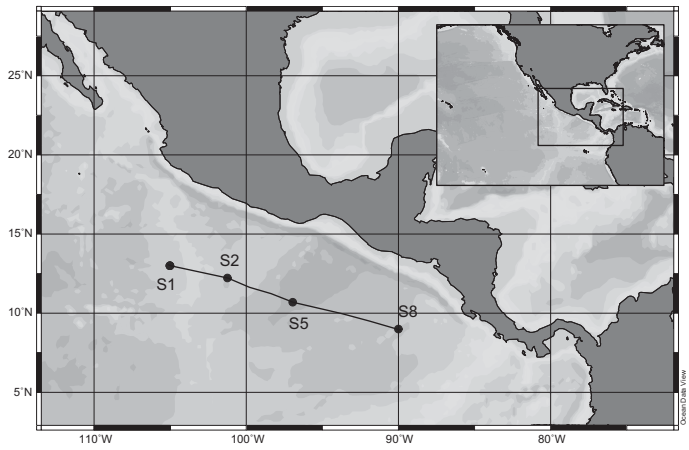

Figure 1: Location of cruise transect in the Eastern Tropical North Pacific (aboard the R/V *Seward Johnson* from 18 October to 17 November, 2007). SPM was sampled by in situ pumping at various water depths at Stations 1, 2, 5, and 8 (Table 1).

3.2.2 Ladderane fatty acid extraction

SPM filters were Soxhlet extracted using dichloromethane (DCM): methanol (MeOH), 9:1 v/v for 8 h. Aliquots of the extracts were saponified by refluxing with aqueous KOH (in 96% MeOH) at 100°C for 1h. FAs were obtained by acidifying the samples with 1N HCl in MeOH to a pH of 3 and extracted using DCM. FAs were converted to FA methyl esters (FAMEs) by methylation with diazomethane (CH_2N_2). Excess CH_2N_2 was removed by evaporation under N_2. Polyunsaturated FAs (PUFAs) were removed by separation over a small $AgNO_3$ (5%) impregnated silica column by elution with DCM. The saturated FA fraction was analysed (as FAMEs) by high performance liquid chromatography – positive ion atmospheric pressure chemical ionisation tandem mass spectrometry (HPLC/APCI-MS/MS).

3.2.3 Arabian Sea SPM ladderane analysis

SPM filters from a depth profile in the Arabian Sea (21° 55' 36 N, 63° 10' 36 E) were collected aboard the R/V *Pelagia* during the Northeast winter monsoon (January 2009; see Pitcher et al., 2011 for details). Particulate organic matter was extracted as described by Pitcher et al. (2011). Freeze-dried filters were extracted using a modified Bligh-Dyer method (Bligh and Dyer, 1959; Rattray et al., 2008a). The Bligh-Dyer lipid extract was saponified and ladderane FAs were extracted as described above.

3.2.4 HPLC/APCI-MS/MS ladderane lipid analysis

The saturated FAME fraction was dissolved in acetone and filtered through a 0.45 μm, 4 mm diameter PTFE filter and analysed by HPLC/APCI-MS/MS in selective reaction monitoring (SRM) mode described in Hopmans et al. (2006), modified in Rattray et al. (2008a). Four ladderane FAs (Fig. 2) were quantified using external calibration curves of two isolated methylated ladderane FA standards (C_{20}-[3]-ladderane FA, and C_{20}-[5]-ladderane FA) (Hopmans et al., 2006; Rattray et al., 2008a). A detection limit of 30 – 35 pg injected was achieved with this technique.

NL_5 (iNdex of Ladderane lipids with 5 cyclobutane rings) is based on the observation that the relative length of the ladderane alkyl side chain adapts to variations in the in situ temperature of several anammox bacteria grown in enrichment cultures (Rattray et al., 2010). NL_5 values were calculated according to the equation of Rattray et al. (2010), where

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

and

$$NL_5 = 0.2 + \frac{0.7}{1 + e^{-\left(\frac{\text{Temperature} - 16.3}{1.5}\right)}} \qquad \text{Equation 2}$$

NL_5 values <0.2 were not transformed into temperature as NL_5 does not change with temperature below this value (Rattray et al., 2010).

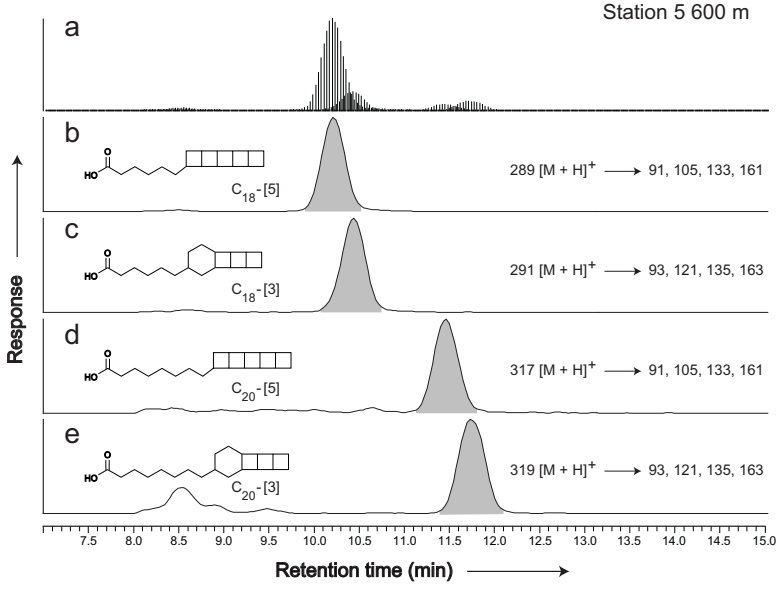

Figure 2: Base peak chromatogram (a) and Selective Reaction Monitoring (SRM) traces (b,c,d, and e) of 4 ladderane fatty acids (as their methyl esters) obtained by HPLC/APCI-MS/MS analysis of saponified lipid extract of SPM at Station 5, 600 m water depth. Traces b, c, d, and e show the C_{18}-[5]-ladderane FAME, C_{18}-[3]-ladderane FAME, C_{20}-[5]-ladderane FAME, and C_{20}-[3]-ladderane FAME, respectively.

Table 1. Location of particulate matter samples in the Eastern Tropical North Pacific, ladderane concentrations, NL_5, and nutrients taken aboard the R/V *Seward Johnson* (18 October – 17 November 2007); n.d., not detected; N/A, data not available.

Station	Depth (m)	Temperature CTD (°C)	Water volume filtered (L)	C_{18} Ladderane FAMEs (ng/L)	C_{20} Ladderane FAMEs (ng/L)	Total Ladderane FAMEs (ng/L)	NL_5	O_2 (μmol/kg^{-1})[a]	NO_2^- (μM)[a]	NH_4^+ (μM)[a]
1	3	27.5	575	n.d.	n.d.	n.d.	-[b]	200	0.01	0.57
13°00' N	25	25.8	193	n.d.	n.d.	n.d.	-	166	0.51	0.95
105°00' W	35	21.0	1636	n.d.	n.d.	n.d.	-	67	0.48	0.66
	75	14.1	1013	0.03	0.00	0.03	-	3	0.00	0.56
	120	12.5	1459	0.01	0.00	0.01	-	6	0.00	0.56
	200	N/A	1647	0.04	<0.01	0.05	-	N/A	N/A	N/A
	300	10.6	800	0.38	0.09	0.47	0.0	2	0.00	0.86
	400	9.5	1544	1.17	0.27	1.44	0.0	2	0.70	0.62
	600[c]	8.0	1337	0.79	0.18	0.97	0.0	2	0.70	0.54
	725[d]	6.0	1616	0.04	<0.01	0.04	0.0	2	0.00	0.86
	830	5.6	1389	0.00	0.00	<0.01	-	5	0.00	0.45
	1250	3.8	1507	0.00	0.00	<0.01	-	33	0.52	0.95
2	3[e]	27.8	1071	n.d.	n.d.	n.d.	-	198	0.00	0.48
12°14' N	26	27.7	1166	n.d.	n.d.	n.d.	-	182	0.52	1.10
101°13' W	55[f]	14.9	1647	0.07	0.00	0.07	-	3	1.52	0.73
	85	13.4	1435	1.15	0.24	1.39	0.0	2	1.73	0.44
	115[g]	13.3	1517	0.88	0.20	1.08	0.0	2	2.19	0.55
	200	11.4	181	0.46	0.06	0.52	0.0	2	1.52	0.72
	400	N/A	1645	1.16	0.30	1.46	0.1	N/A	N/A	N/A
	600[d]	7.6	1476	0.15	0.03	0.18	0.0	2	0.79	0.61
	830	5.3	1514	n.d.	n.d.	n.d.	-	3	0.30	1.03
5	3[e]	25.5	223	n.d.	n.d.	n.d.	-	211	0.13	0.98
10°41' N	25[h]	25.5	130	n.d.	n.d.	n.d.	-	211	0.13	1.07
96°56' W	50	21.5	1647	n.d.	n.d.	n.d.	-	76	0.24	0.83
	75[i]	14.3	1331	0.15	0.01	0.16	0.0	5	0.01	0.78
	125[j]	13.5	1420	n.d.	n.d.	n.d.	-	6	0.01	0.79
	250	11.3	1328	n.d.	n.d.	n.d.	-	15	0.01	0.80
	400	9.6	1023	0.96	0.19	1.15	0.0	2	0.90	0.80
	600[c]	8.2	995	2.00	0.28	2.28	0.0	2	0.57	0.88
	830[d]	5.9	1709	0.06	0.01	0.08	0.0	3	0.01	0.80
8	3[e]	27.1	387	n.d.	n.d.	n.d.	-	197	0.06	0.98
9°00' N	10	27.1	336	n.d.	n.d.	n.d.	-	197	0.06	0.98
90°00' W	25[h]	26.4	1647	n.d.	n.d.	n.d.	-	119	0.08	0.73
	50	14.1	554	n.d.	n.d.	n.d.	-	42	0.05	0.57
	125[j]	13.5	1457	n.d.	n.d.	n.d.	-	23	0.00	0.65
	200	12.2	1640	0.01	0.00	0.01	-	10	0.00	0.66
	350[k]	10.3	1603	0.85	0.21	1.07	0.0	2	1.13	0.70
	450	8.6	1543	0.10	0.02	0.11	0.0	2	1.08	0.72
	550[c]	7.9	1891	0.37	0.03	0.41	0.0	2	0.20	0.71
	650[l]	6.3	1733	0.01	<0.01	0.01	0.0	3	0.00	0.69
	750[m]	6.1	1506	n.d.	n.d.	n.d.	-	8	0.00	0.69
	1000	4.5	1561	n.d.	n.d.	n.d.	-	26	0.00	0.69
	1250	3.8	1366	n.d.	n.d.	n.d.	-	46	0.00	0.70

Nutrient concentration of water sample taken close to depth of SPM sample[c–m].

[a] From Podlaska et al. (2012).

[b] C_{20} or C_{18} ladderane FA concentration were below the limit of detection, so NL_5 not calculated.

[c] 500 m.

[d] 750 m.

[e] 2m.

[f] 60 m.

[g] 90 m.

[h] 20 m.

[i] 80 m

[j] 100 m.

[k] 340 m.

[l] 660 m.

[m] 700 m.

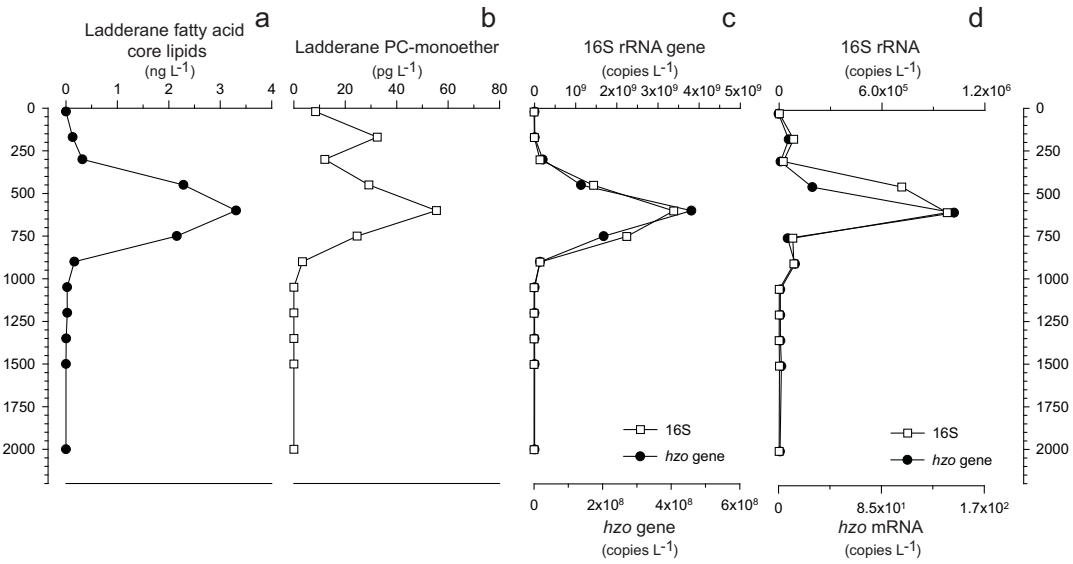

Fig 3: Depth profile of distributions of biomarker lipids, copy numbers of specific genes, and gene expression in the Arabian Sea water column: (a) ladderane fatty acid core lipids, (b) ladderane PC-monoether concentration, (c) anammox bacterial 16S rRNA and hydrazine oxydoreductase (*hzo*) gene abundances, and (d) anammox bacterial 16S rRNA and hydrazine *hzo* mRNA abundances. Data in b-d are modified from Pitcher et al., 2011.

3.3 Results and Discussion

We examined ladderane FA core lipids as markers for anammox bacteria. Since FAs have a relatively low degradation rate, compared to e.g., intact polar lipids or genes, the question may arise as to whether these FAs are mainly representing fossil anammox (cf. Jaeschke et al., 2009b; Brandsma et al., 2011), or also living anammox bacteria. To validate the use of ladderane FA core lipids as biomarkers for active anammox bacteria in the ETNP OMZ, we analysed these compounds in SPM from the Arabian Sea (Fig. 3a). At this station, Pitcher et al. (2011) previously reported intact polar ladderane lipid concentrations, anammox 16S rRNA, as well as hydrazine oxydoreductase (*hzo;* the functional gene for an important step in the anammox reaction) gene abundances and their expressions (Fig. 3, b–d). The distribution of ladderane FA core lipids (Fig. 3a) followed the same trend with depth as both the intact polar ladderane lipid (Fig. 3b) as well as the abundances of the 16S rDNA and *hzo* genes and their expressions (Fig. 3, c and d), suggesting that ladderane FA core lipids are representative of anammox bacteria living in the OMZ water column. Indeed, Kuypers et al. (2003) showed that the distributions of two ladderane FAs corresponded well to the distribution of anammox activity measured by ^{15}N tracer experiments. Thus, ladderane FAs are likely a suitable tool to study the presence and distribution of anammox bacteria in an OMZ water column setting when genetic, isotopic, and intact polar lipid studies are not possible.

Figure 4 shows the depth distribution plots of O_2, NO_2^-, NH_4^+ and ladderane FAs at all four stations along the cruise transect in the ETNP. O_2 concentrations (Table 1; Fig. 4) dropped to minimum values (<5 μmol kg^{-1}) in the first 100 m of all stations. Ammonium concentrations varied between 0.44, near detection limit, and 2.51 μM. The primary NO_2^-

maximum occurred within the euphotic zone (<100 m), likely resulting from the excretion of nutrients by zooplankton coupled to the nitrification of NH_4^+ (Dore and Karl, 1996; Fiedler and Talley, 2006). In the ETNP, a deeper, secondary NO_2^- maximum (Fig. 4, 400 – 600 m) with consistently higher concentrations than the primary NO_2^- maximum occurred within the OMZ and is likely caused by bacteria reducing nitrate to NO_2^- (Brandhorst, 1959; Cline and Richards, 1972). At Station 2 (Fig. 4b), the OMZ shoaled which resulted in the secondary NO_2^- maximum occurring at the upper limit of the OMZ. Nitrate reduction is believed to be a source of NO_2^- for anammox in the Peruvian OMZ (Lam et al., 2009), and likely plays a similar role in the ETNP.

Table 2. Location of particulate matter samples, ladderane concentrations, NL_5 values, NL_5 derived temperature, CTD measured temperature, and nutrients data taken in the Arabian Sea aboard the R/V *Pelagia* (January 2009); n.d., not detected; N/A, data not available.

Depth (m)	C_{18} Ladderane FAMEs (ng/L)	C_{20} Ladderane FAMEs (ng/L)	Total Ladderane FAMEs (ng/L)	NL_5	Temperature NL_5 derived (°C)	Temperature CTD (°C)	Nutrients		
							O_2 (μM)	NO_2^- (μM)	NH_4^+ (μM)
20	n.d.	n.d.	n.d.	-[a]	N/A	24.8	192	0.04	0.09
170	0.08	0.06	0.13	0.27	13.0	19.0	5	0.62	0.14
300	0.17	0.15	0.31	0.29	13.4	15.6	6	0.03	0.01
450	1.17	1.12	2.28	0.31	13.8	13.6	4	0.02	0.03
600	1.75	1.56	3.31	0.32	13.9	12.0	3	0.5	0.06
750	1.18	0.97	2.15	0.29	13.5	11.0	3	0.02	0.05
900	0.10	0.07	0.16	0.26	12.9	9.9	4	0.02	0.10
1050	0.02	<0.01	0.02	-	N/A	8.5	5	0.02	0.10
1200	<0.01	0.02	0.03	0.46	15.5	7.5	10	0.02	0.08
1350	<0.01	<0.01	<0.01	-	N/A	6.5	19	0.02	0.07
1500	n.d.	n.d.	n.d.	-	N/A	5.5	29	0.01	>0.01
2000	n.d.	n.d.	n.d.	-	N/A	3.2	65	0.03	0.04

[a] C_{18} or C_{20} ladderane FA concentration were below the limit of detection, so NL_5 not calculated.

Ladderane FAs were detected at every station in the ETNP in selected depth intervals. They were dominated by the C_{18} ladderane FAs which were on average 5× more abundant than C_{20} ladderane FAs (Fig. 2a; Table 1). This is a stark difference from what has been seen previously in a study of ladderane FAs in four different genera of anammox bacteria (Rattray et al., 2008a), where C_{20} ladderane FAs were found to dominate over C_{18} ladderane FAs. In the Arabian Sea, concentrations of C_{18} ladderane FAs were at the same order of magnitude as C_{20}-ladderane FAs (Table 2). However, ladderane FA distributions in the suboxic zone of the Cariaco Basin also showed C_{18} ladderane FAs to be 5× more abundant than C_{20} ladderane FAs (Wakeham et al., 2012). Ladderane distributions in the Peruvian and Namibian upwelling areas (Rattray, 2008b) also showed a dominance of C_{18} ladderane FAs, although the difference between C_{18} and C_{20} ladderane FAs in these settings were not as large as in the ETNP, or the Cariaco Basin. The substantial difference in ladderane FA distributions might be caused by a different genus of anammox living in these OMZs than what has been seen previously in cultures and other natural environments. Another possible explanation for such a variation in distribution is differences in environmental factors. For example, Rattray et al. (2010) found ladderane chain lengths increased with increasing temperature in enrichment cultures as well as in SPM from different environments. However, due to the dominance of C_{18} ladderane FAs, NL_5 values were <0.2 in the ETNP, values at which the NL_5 no longer changes with temperature (Rattray et al., 2010). Indeed, ETNP calculated temperatures did not correlate

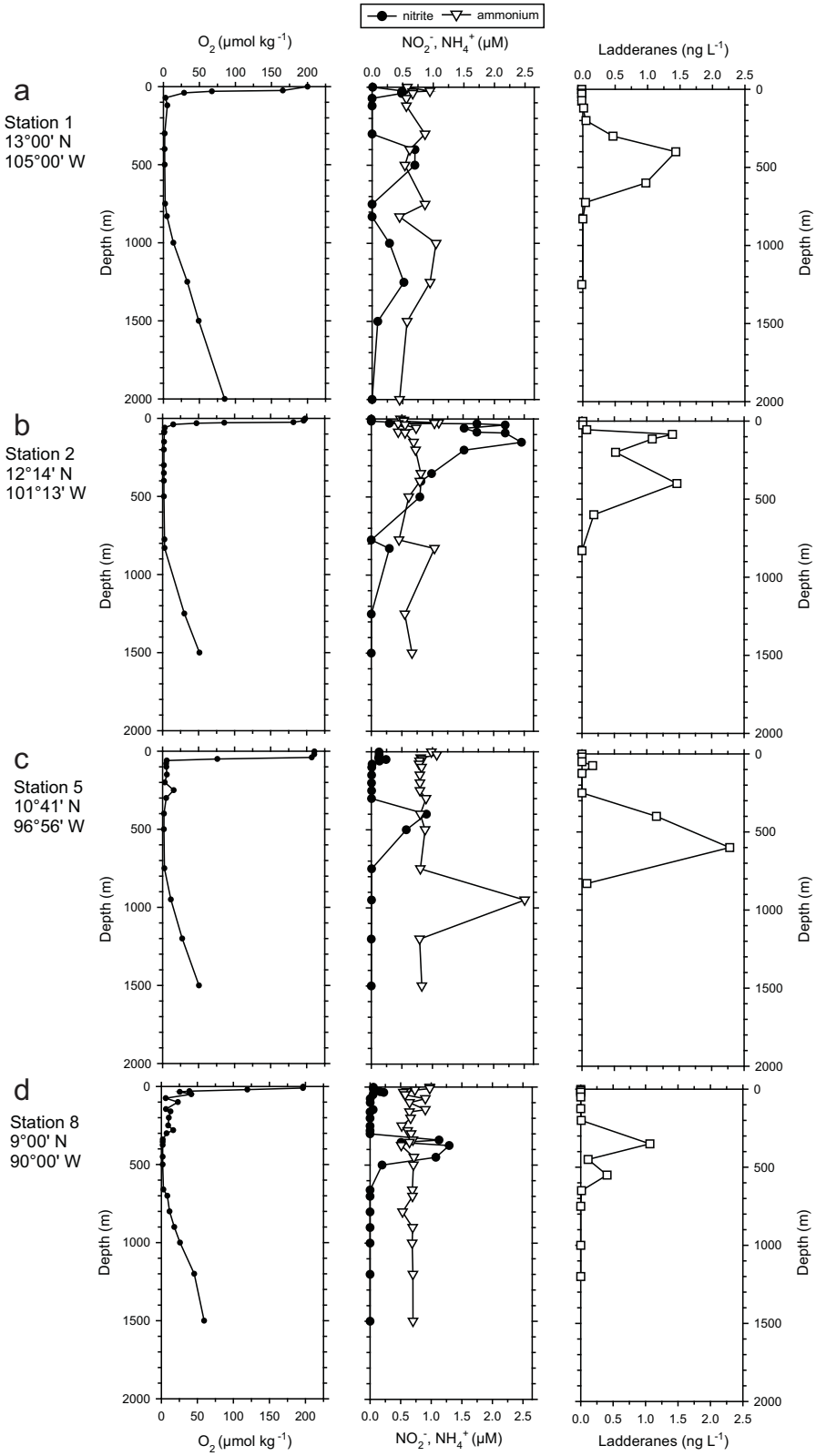
nitrite
ammonium
O2 (µmol kg-1)
NO2-, NH4+ (µM)
Ladderanes (ng L-1)
a
Station 1
13°00' N
105°00' W
b
Station 2
12°14' N
101°13' W
c
Station 5
10°41' N
96°56' W
d
Station 8
9°00' N
90°00' W
Depth (m)
0
500
1000
1500
2000
0 50 100 150 200
0.0 0.5 1.0 1.5 2.0 2.5

Figure 4 (previous page): Depth profile plots of oxygen, nutrients, and ladderane lipids in the water column at (a) Station 1, (b) Station 2, (c) Station 5, and (d) Station 8 in the Eastern Tropical North Pacific. The first column shows the oxygen concentrations, the second column shows concentrations of nitrite (black circles) and ammonium (white triangles), and the third column shows the concentration of total ladderane lipids. Locations of stations are in Fig. 1. Oxygen, nitrite, and ammonium concentrations from Podlaska et al. (2012).

with in situ temperatures whereas the average NL_5 values in the Arabian Sea (13 ± 1°C; Table 2) correlated well with the average OMZ in situ temperatures measured by way of CTD (12 ± 3°C; Pitcher et al., 2011). Thus, it appears that the NL_5 index only works in some settings, and caution must be used when applying the index to OMZs, especially when there is a dominance of C_{18} ladderane FAs.

The detection of ladderane FAs at all stations unambiguously indicates that anammox occurs within the OMZ of the ETNP, and, on the basis of our results from the Arabian Sea OMZ (Fig. 3), likely indicates the presence of living anammox bacteria. The peaks in the concentration of ladderane FAs corresponded to the secondary nitrite maximum at all stations. At Station 1 (Fig. 4a), ladderane concentrations were maximum at 400 m. The highest

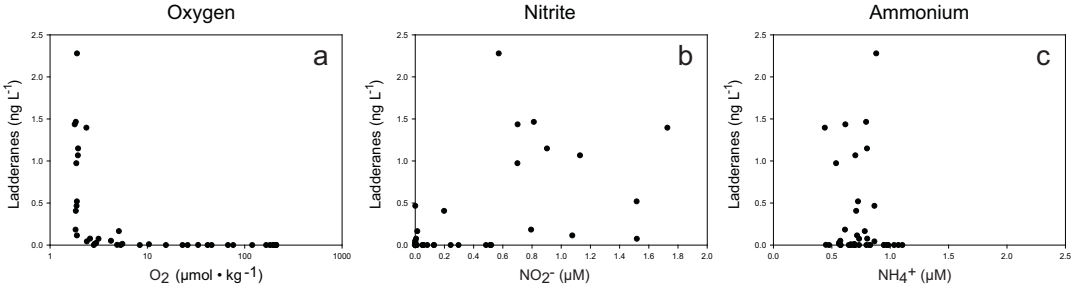

Figure 5: Plots of possible controls on anammox activity: Abundances of ladderane lipids are plotted versus the concentrations of (a) oxygen, (b) nitrite, and (c) ammonium. Note log scale of oxygen concentration in (a). Oxygen, nitrite, and ammonium concentrations from Podlaska et al. (2012).

concentrations of ladderane FAs occurred at Station 5 at 600 m (Fig. 4c; 2.3 ng L^{-1}). The highest abundance of ladderane FAs at Station 8 was between 350 and 550 m (Fig. 4d). At Station 2 (Fig. 4b), concentrations of ladderane FAs occurred at this depth interval but a secondary maximum also occurred at a much shallower depth (85 – 115 m) than at the other stations. This was due to the secondary nitrite maximum being at the upper limit of the OMZ (Fig. 4b, 50 – 200 m). The depth at which ladderane lipids occured in the ETNP were primarily within the lower part of the OMZ.

In other OMZ environments, either NO_2^- (Jaeschke et al., 2007) or NH_4^+ (Hamersley et al., 2007) has been suggested to be the limiting factor controlling anammox activity. To examine potential controls on anammox activity in the ETNP, we compared concentration of ladderane FAs with several environmental parameters (Fig. 5). Ladderane FAs have been shown to reasonably indicate the presence of living anammox bacteria in OMZs (Kuypers et al., 2003; this study); however, they might also represent a fossil pool. Therefore, caution must be employed when using ladderane FAs to interpret the controls on anammox activity. Nonetheless, in the ETNP, ladderane FAs were only detected at O_2 concentrations below a threshold of 10 µmol kg^{-1} (approx. 11 µM, Fig. 5a), which is comparable to the Benguela Upwelling where ladderanes

were detected only in waters with <9 μM of oxygen (Kuypers et al., 2005). This is explained by the fact that anammox bacteria are anaerobic, and can only tolerate low concentrations of O_2 (Strous et al., 1997). A substantial concentration of ladderanes (>0.41 ng L^{-1}) were only found when NO_2^- concentrations were >0.2 μM (Fig. 5b), with the exception of Station 1, at 300 m, where ladderanes were detected while no NO_2^- was detected. However, the NO_2^- concentrations measured in situ might not be representative of the nitrite turnover or flux in the system. In the Arabian Sea OMZ (Pitcher et al., 2011), anammox was found to be thriving at depths where NO_2^- concentrations were below 0.5 μM, and high NO_2^- turnover was speculated to be the cause of the low NO_2^- concentrations observed. Alternatively, the ladderane FAs present at 300 m at Station 1 in the ETNP could have been mainly of fossil origin. High ladderane concentrations were found at both low and high NH_4^+ concentrations, suggesting that ammonium was not a limiting factor. The highest abundances of ladderane FAs occurred within the lower OMZ and corresponded to the highest concentrations in NO_2^- (Fig. 4), which suggests that both NO_2^- and O_2 are likely the limiting factors controlling anammox activity in the ETNP.

Ladderane concentrations reported in this study of the ETNP are comparable to those previously reported in other OMZs of the ocean (0-58 ng L^{-1} in the Peru upwelling, Hamersley et al., 2007; 0.1-3.3 ng L^{-1} in the Arabian Sea, this study). This suggests that concentrations of anammox bacteria cells are similar to those in other OMZ where they have been shown to contribute substantially to the loss of nitrogen, and likely indicates that anammox plays an important role in the nitrogen cycle of the ETNP.

3.4 Conclusions

We found ladderane lipid fatty acids, tracers for the anammox reaction, throughout the Eastern Tropical North Pacific oxygen minimum zone, indicating that anammox bacteria are thriving in these oxygen depleted waters. Comparison of the distribution of ladderanes at each station with those of nutrients tentatively suggests that anammox activity is limited by nitrite and oxygen availability. Concentrations of ladderanes at all stations were comparable to those reported in other OMZs. Anammox in the ETNP thus probably plays an important role in the nitrogen cycle in this region, as it does in other OMZs.

Acknowledgements

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Chapter 4

Anammox in the chemocline of the Cariaco Basin

These data have been published in an extended form as
Biomarkers, chemistry and microbiology show chemoautotrophy in a multilayer chemocline in the Cariaco Basin.

Wakeham, S.G., Turich, C., Schubotz, F., Podlaska, A., Li, X.N., Valera, R., Astor, Y., Sáenz, J.P., Rush, D., Sinninghe Damsté, J.S., Summons, R.E., Scranton, M.I., Taylor, G.T., and Hinrichs, K.U. (2012) *Deep-Sea Research Part I* 63, 133–156.

Anaerobic ammonium oxidation (anammox) is an important process in the marine nitrogen cycle. We studied the distribution of ladderane membrane lipids, unique biomarkers for anammox bacteria, in the Cariaco Basin. The Cariaco Basin is the world's largest marine anoxic basin. A comprehensive investigation of the water column (42 – 750 m) bracketing the redox boundary (a 250-m thick "chemocline") of the Cariaco Basin was conducted to evaluate linkages between ladderane fatty acid concentrations, dissolved chemical species, and the distribution of the associated redox process, anammox. The detection of ladderane fatty acids was reserved to a specific depth of the chemocline (between ~ 235 and 350 m), where both of the substrates, ammonium and nitrite, required for the anammox reaction were available, and where oxygen levels were sufficiently low. Peak ladderane concentration (6.5 ng L^{-1}) occurred at 245 m, at the same depth that anammox cell counts, determined by FISH, peaked. Nitrification by thaumarchaeota is most likely the source of nitrite for anammox, as shown by the distribution of intact polar and core lipid glyceryl dialkyl glycerol tetraethers. This is the first study of anammox in the Cariaco Basin, and shows that anammox is responsible for a loss of nitrogen from this anoxic basin.

4.1 INTRODUCTION

Anammox, the anaerobic oxidation of ammonium by nitrite to produce dinitrogen gas (N_2), was first identified in the natural environment in the early 2000's (Thamdrup and Dalsgaard, 2002; Dalsgaard et al., 2003; Kuypers et al., 2003). Since then, it has been proposed that anammox is responsible for up to 50% of N_2 production in marine environments (Arrigo, 2005; Brandes et al., 2007; Francis et al., 2007). Anammox is performed by a phylogenetic group of Brocadiales related to the order Planctomycetales, a distinct division of bacteria which is phylogenetically as distant from proteobacteria as from archaea (Strous et al., 1999; Chistoserdova et al., 2004). Ladderane fatty acids, containing hydrocarbon chains of linearly condensed cyclobutane rings, are components of the membrane of the specialised organelle, the anammoxosome, in which the anammox process takes place (Sinninghe Damsté et al., 2002a; Sinninghe Damsté et al., 2005; Rattray et al., 2008). Ladderane lipids are biomarkers for the anammox bacteria, and the identification of ladderane fatty acids in suspended particulate matter (SPM) in the water column has previously been used to indicate the presence of anammox bacteria and thus the occurrence of the anammox reaction (Kuypers et al., 2003; Hamersley et al., 2007; Jaeschke et al., 2007).

Anammox has been shown to occur in environments where the oxygen concentration is low, and where ammonium and nitrite are available. Anammox is active ubiquitously in the oxygen depleted regions of the ocean, i.e., in the water column of oxygen minimum zones (OMZs) as well as within anoxic sediments (Thamdrup and Dalsgaard, 2002; Dalsgaard et al., 2003; Kuypers et al., 2003; Kuypers et al., 2005; Hamersley et al., 2007). Anammox has previously been identified in the Black Sea anoxic basin (Kuypers et al., 2003), which has limited mixing with the Mediterranean Sea. However, to fully understand the influence of anammox in marine environments, it is important to investigate other anoxic basins, and the world's largest marine anoxic basin, the Cariaco Basin, has yet to be investigated.

This study uses a high resolution sampling scheme to generate data on the hydrography (temperature, fluorescence, transmissivity and dissolved oxygen), nutrient chemistry (NO_3^-, NO_2^-, NH_4^+, H_2S), and lipid biomarkers (ladderane fatty acids). These data are used to define linkages between ladderane biomarkers and distributions of major dissolved chemical species.

4.2 SITE DESCRIPTION

The Cariaco Basin is located on the continental shelf of northern Venezuela (Fig. 1). A shallow sill (<150 m) isolates it from the Caribbean Sea, and a second, deeper saddle (~1000 m) restricts circulation between the two deep 1400 m sub-basins. Upwelling, regional rainfall, primary productivity, and particle export are all driven by the seasonal migration of the Inter-Tropical Convergence Zone (ITCZ). In winter, the ITCZ is below the equator promoting sustained easterly Trade Winds, strong upwelling and export of autochthonous organic matter (OM). During summer the ITCZ is farther north, bringing high precipitation and increased sedimentation of allochthonous lithogenic material from the Venezuelan uplands (Muller-Karger et al., 2004; Goñi et al., 2009). The lithogenic and OM records preserved in Cariaco sediments suggest significant variation in the location and strength of the ITCZ over regional climate cycles.

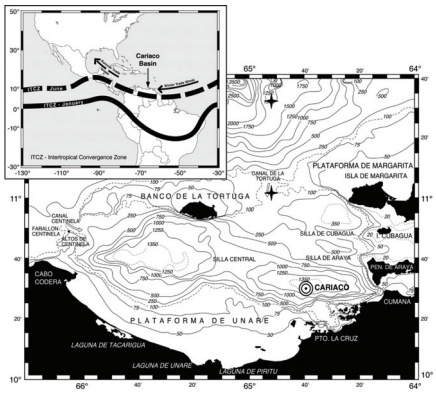

Figure 1. Map showing the location of the Cariaco Basin, and the effect of the ITCZ position on seasonal upwelling (boreal winter) during November sampling, and high precipitation and run-off (summer). Suspended particles were collected in November, 2007 (CAR-139) at the CARIACO time-series site in the Eastern Basin.

In the Cariaco Basin, the US-Venezuelan CARIACO Ocean Time Series Program (Carbon Retention in a Colored Ocean; http://www.imars.usf.edu/CAR) has collected more than 15 years of monthly measurements of physical, chemical and biological properties, complemented by SeaWIFS satellite observations and biannual process-study cruises (Thunell et al., 2000; Muller-Karger et al., 2004; Thunell et al., 2007; Goñi et al., 2009, for background). High seasonal coastal surface productivity and restricted water exchange between the Caribbean Sea and the deep Cariaco Basin maintain a sulfidic ("euxinic") zone from ~260 m to the seafloor at ~1400 m (Astor et al., 2003; Scranton et al., 2006). Between oxygenated surface waters and sulfidic bottom waters lies a well-developed ~250-m thick zone of strong chemical gradients and associated microbiological community, hereafter termed "the chemocline".

Biogeochemical redox processes within the chemocline support a diverse microbial community (Madrid et al., 2001; Stoeck et al., 2003; Lin et al., 2006, Taylor et al., 2006; Lin et al., 2007; Lin et al., 2008) that produces diagnostic lipid biomarkers (Wakeham, 1990; Freeman et al., 1994; Wakeham et al., 2004). Various oxidants (O_2, MnO_2, Fe_2O_3, NO_3^- $S_2O_3^{2-}$, SO_3^{2-}, S^0) and reductants (H_2S, NH_4^+, CH_4, Fe^{2+}, Mn^{2+}) could support chemoautotrophy (Scranton et al., 1988; Scranton et al., 2001; Taylor et al., 2001; Ho et al., 2002; Ho et al., 2004; Hayes et al., 2006; Li et al., 2008; Percy et al., 2008; Li et al., 2012). Denitrifying, metal-reducing, and ammonium and sulfide oxidising microbial populations are all active within different depth intervals. Chemoautotrophy in the Cariaco Basin equates to 10-130% of contemporaneous primary production and responds to interannual and decadal changes in surface productivity and deep-water ventilation rather than short-term surface processes of seasonal upwelling and blooms (Taylor et al., 2001). As a result, the flux of organic carbon (OC) captured in sediment traps below the chemocline frequently exceeds the flux exported from above, further implicating mid-water chemoautotrophic production as an important secondary source of OC in the water column and to the sediments in addition to surface water photoautotophy (Taylor et al. 2001).

Anoxic and laminated sediments of the Cariaco Basin preserve a record of Holocene climate change, including changes in upwelling intensity, planktonic community structure and regional rainfall resulting from the shifting position of the ITCZ (Goñi et al., 2009, and references cited). Recognising the significance of mid-water chemoautotrophy from a lipid biomarker perspective aids in identifying water column sources of biomarkers as proxies for biological processes and climate change, especially for past oceans characterised by euxinic conditions and extensive OC deposition. The underlying causes of oceanic anoxic events

(OAEs) that may lead to organic-rich sediments deposited under oxygen-deficient water column conditions are the subject of ongoing investigations (e.g., Koopmans et al., 1996a; Kuypers et al., 2001; Hinrichs et al., 2003; Brocks and Pearson, 2005, Jaeschke et al., 2009a) given recent suggestions that ocean warming and increased stratification caused by global climate change lead to declining dissolved oxygen in the interior of the ocean (Keeling et al., 2010).

4.3 METHODS

4.3.1 SAMPLING

All samples were collected during the CAR-139 cruise in late November, 2007, at the CARIACO time-series site (10°30'N, 64°40'W; Fig. 1). SPM for biomarker analyses was collected using two McLane WTS-LV in situ filtration systems deployed in tandem on 21-22 November using B/O *Paraguachoa* operated by Estación de Investigaciones Marinas de Margarita (EDIMAR). Ten SPM samples were collected between nominal depths of 205 – 346 m (205, 220, 236, 245, 256, 270, 276, 296, 324, 346), along with a sample at the fluorescence maximum (42 m). Bad weather forced the *Paraguachoa* back to port. On 28 November, which was the previously scheduled sediment trap turn-around cruise, a deep anoxic zone sample was collected at 750 m using EDIMAR's B/O *Hermano Gines*. A Seabird CTD on the hydrowire directly beneath the pumps provided real-time depth (used to estimate the depths of the pumps), temperature, salinity, fluorescence (WetLab ECO-AFL), beam attenuation (WetLab C-star transmissometer), and dissolved oxygen (SBE 43 oxygen probe). Between 200 – 850 L were filtered during ~2-h pump deployments. Two particle sizes were collected using a 53 μm Nitex prefilter and two stacked 142 mm Whatman glass fiber filters (GFF; nominally 0.7 μm; ashed at 500ºC for 8 h). Only the GFFs, representing 0.7 - 53 μm material, were analysed in this investigation. Filters were frozen immediately, and returned to the laboratory frozen. Because GFF may undersample the microbial comunity (Lee et al., 1995; Gasol and Moran, 1999), we stacked two GFF together to try to minimise any discrimination while still filtering the large volumes needed for biomarker analyses.

4.3.2 SEAWATER CHEMISTRY

Seawater samples for water column chemistry were collected with Teflon-lined Niskin bottles on a rosette. Niskin bottles were pressurised with nitrogen and subsamples for nutrients were filtered through 0.7 μm GF/Fs. Nitrate, nitrite and ammonium were analysed as described by Scranton et al. (2001). Sulfide was analysed following Li et al. (2008).

4.3.3 LADDERANE FATTY ACIDS ANALYSIS

Lipids were Soxhlet-extracted using dichloromethane:methanol (DCM:MeOH; 9:1 v/v) for 8 h. Extracted lipids were partitioned into DCM against 5% NaCl solution and dried over Na_2SO_4. Splits of extracts were saponified with aqueous KOH (96% MeOH) for 1 h, and fatty acids were collected (3×) with DCM after acidifying with 1 N HCl in MeOH (pH 3). Fatty acids were then methylated with diazomethane, and cleaned up over a silica nitrate column. The saturated fatty acid (as methyl esters) fraction was dissolved in acetone and filtered through a 0.45μm, 4 mm diameter PTFE filter for analysis of core ladderane fatty acids (Fig. 2, **I – IV**), analysed as C_{18}-[5]-, C_{18}–[3]-, C_{20}-[5]- and C_{20}-[3]-ladderane methyl esters by high performance liquid chromatography coupled to positive ion atmospheric pressure chemical ionisation tandem with

mass spectrometry (HPLC-APCI-MS/MS) with a ThermoScientific Quantum TSQ Ultra EM triple quadrupole mass spectrometer (Hopmans et al., 2006; modified by Rattray et al., 2008). Detection was achieved by selective reaction ion monitoring (SRM) of fragments specific to each of the four core ladderane fatty acids. An external calibration curve of two isolated methylated ladderane fatty acids standards (C_{20}-[3]-ladderane fatty acid, and C_{20}-[5]-ladderane fatty acid; Hopmans et al., 2006; Rattray et al., 2008) was used to quantify the ladderane fatty acids. A detection limit of 30 – 35 pg injected was achieved with this technique.

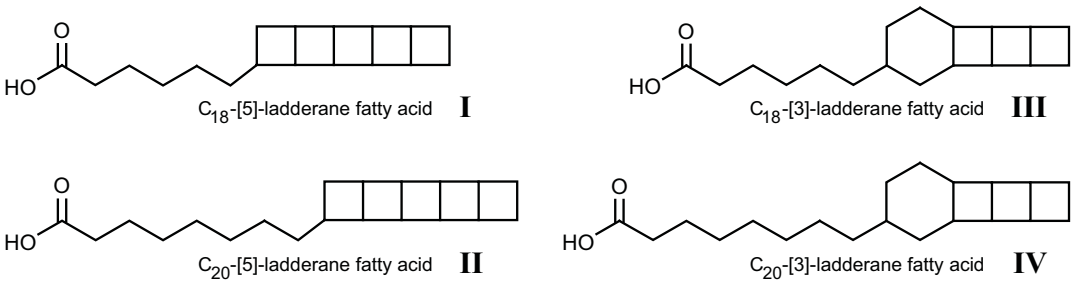

Figure 2. Chemical structures of the ladderane fatty acid lipids used as biomarkers for anammox bacteria. (IV) C18-[5]-ladderane fatty acid, (V) C20-[5]-ladderane fatty acid, (VI) C18-[3]-ladderane fatty acid, and (VII) C20-[3]-ladderane fatty acid.

4.4 **RESULTS**

4.4.1 WATER COLUMN CONDITIONS AND CHEMISTRY DURING CAR-139

Hydrographic (Fig. 3) and chemical data (Fig. 4, a and b) obtained during for CAR-139 provide multiple lines of evidence for a strong redoxcline in the Cariaco Basin. Stratification and maintenance of anoxia in this system are due to the temperature gradient (Fig. 3a), in contrast to the Black Sea where the salinity contrast controls the density structure (Scranton et al., 2001; Astor et al., 2003).

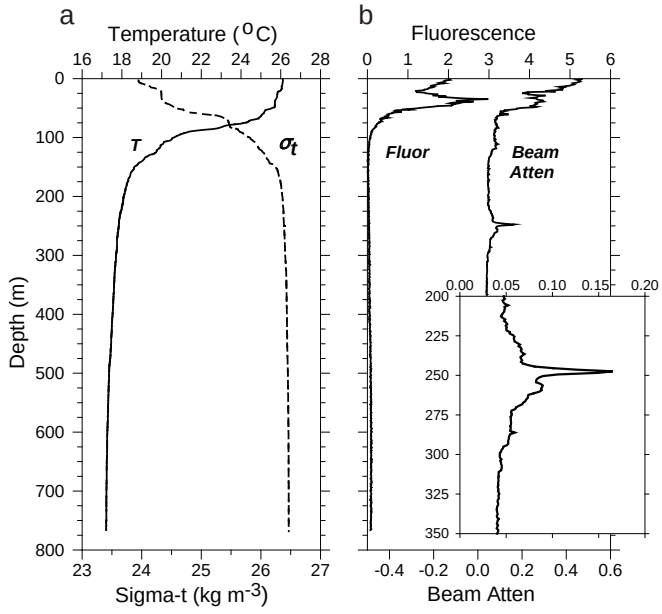

Figure 3. Hydrography for CAR-139 (0-800 m). (a) Temperature and potential density; (b) fluorescence and beam attenuation. The inset shows the fluorescence between 200 and 350 m.

The chemocline is characterised by opposing gradients of oxygen, nitrate, and sulfide (Fig. 4). Remineralisation of exported particulate OM from the euphotic zone consumes dissolved oxygen so that below ~230 m, dissolved O_2 was below its detection limit of 2 – 5 μM (Fig. 4a). Nitrate released by OM remineralisation had a broad concentration peak centered at ~150 m (~13 μM, Fig. 4b), then decreased, but showed a small secondary peak at 260 m (4.4 μM). Nitrite had a weak peak at 150 m (0.05 μM) but a stronger peak at 260 m (0.6 μM). Sulfide was first detected (~1 μM) at 260 m (Fig. 4a). Ammonium (Fig. 4b) was first detected at 250 m, increasing in concentration into the euxinic deeper waters.

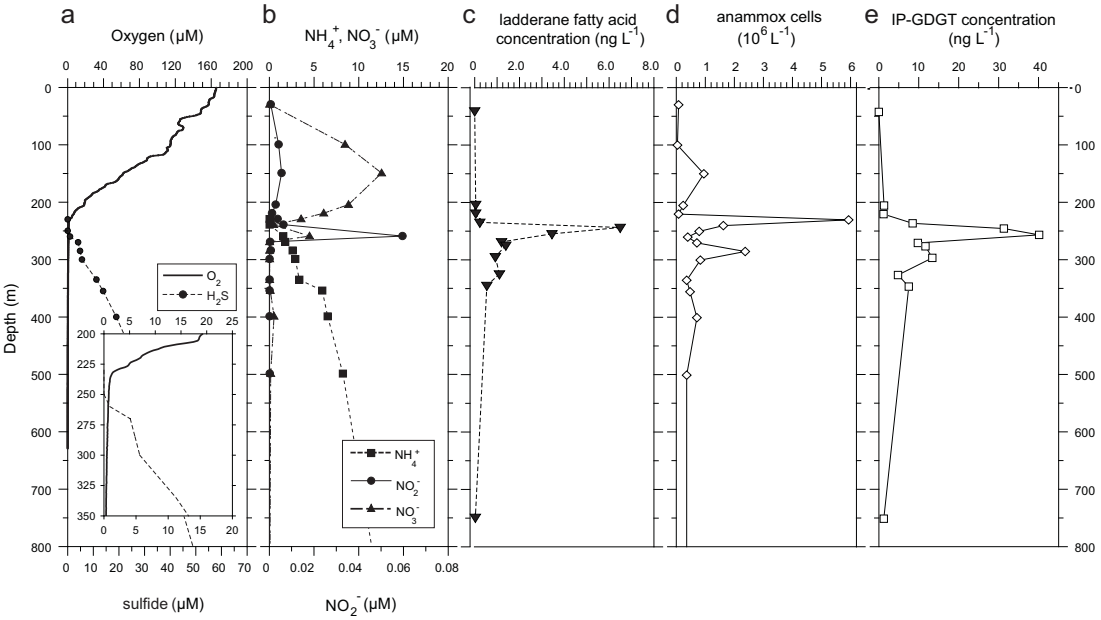

Figure 4. Depth profile of chemistry, nutrients, anammox cell numbers, and lipid biomarkers for CAR-139 (0 – 800 m). (a) Dissolved oxygen (solid line), H_2S (solid circles, dashed line); (b) nitrogen species: NH_4^+ (squares), NO_3^- (triangles), and NO_2^- (circles) concentrations obtained during CTD pumping casts. (c) Concentration of ladderane fatty acids, obtained from in situ pump filters. (d) cell numbers for ammonium oxidizing bacteria (determined by fluorescent in situ hybridisation using rRNA oligonucleotide probe AMX368; Schmid et al., 2003). Note that these samples were filtered on 0.2 μm Millipore GTTP membranes. (e) concentration of intact polar glyceryl dialkyl glycerol tetraethers (IP-GDGT) from in situ pump filters. Anammox cell numbers and IP-GDGT data from Wakeham et al., (2012).

During the CAR-139 sampling campaign, the fluorescence maximum was located at ~40 m (Fig. 3b). A second "lens" of light scattering particles at 240-250 m was seen as a peak in beam attenuation (Fig. 3b), and became the target depth for SPM sampling since we surmised this to be a microbe-enriched suboxic layer. Consequently, 10 SPM samples were obtained at depths between 205 and 346 m.

Table 1: Distribution of ladderane fatty acids in the Cariaco Basin water column. n.d., not detected.

Water column depth (m)	Ladderane fatty acid concentration (ng L^{-1})			
	C_{18}-[5]-ladderane	C_{18}-[3]-ladderane	C_{20}-[5]-ladderane	C_{20}-[3]-ladderane
42	n.d.	n.d.	n.d.	n.d.
205	0.0	0.0	n.d.	0.01
220	0.0	0.0	0.0	0.0
236	0.1	0.1	0.0	0.0
245	4.1	1.6	0.2	0.6
256	2.1	0.8	0.1	0.4
270	0.8	0.3	0.1	0.1
276	0.8	0.3	0.1	0.2
296	0.5	0.2	0.1	0.1
326	0.7	0.2	0.1	0.1
346	0.3	0.1	0.0	0.1
750	0.0	0.0	n.d.	0.0

4.4.2 LADDERANE FATTY ACIDS

Ladderane fatty acids were abundant in the core chemocline (Fig. 4c), ranging from near detection limit at 236 m and below 346 m, to a maximum of 6.5 ng L^{-1} at 245 m depth. They consisted of C_{18} and C_{20} fatty acids, containing either 3- or 5- linearly concatenated cyclobutane rings (Sinninghe Damsté et al., 2002a), i.e., C_{18}-[5]-, C_{20}-[5]-, C_{18}-[3]-, and C_{20}-[3]-fatty acids (**I – IV**). Despite significant concentration changes down the water column for total ladderanes, there was little variation in the relative distributions of the four core ladderanes (Table 1). The C_{18}-compounds together constituted up to ~85% of total ladderanes (of which the C_{18}-[5]-ladderane fatty acid and C_{18}-[3]-ladderane fatty acid were ~60% and ~20% of total ladderanes, respectively), whereas the remaining ~20% were the C_{20}-compounds (with C_{20}-[3]-ladderane fatty acid the more abundant).

4.5 **DISCUSSION**

A sharp peak in four ladderane fatty acids occurs at 245 m water depth (Fig. 4c), indicating the presence of anammox bacteria within the core chemocline of the Cariaco Basin. This is confirmed by a co-occurence of nitrite and ammonium (Fig. 4b), as well as by a peak in anammox bacteria (AMX368-positive cells; Schmid et al., 2003), determined by fluorescent in situ hybridisation (FISH) (Fig. 4d; data from Wakeham et al., 2012). There is, however, a slight offset in the peak depths of ladderane concentration (245 m), NO_2^- concentration (256 m), and anammox bacterial cell counts (230 m). It is uncertain whether this is a real spatial separation or an artifact of the week's time between the lipid sampling (21-22 November) and the microbial and nutrient samplings (30 November).

The fact that anammox is most prevalent in the suboxic zone of the Cariaco Basin (Fig. 4a) fits with anammox bacteria being able to function in the presence oxygen, albeit at low levels. In culture, anammox can be inhibited at concentrations as low as 1 µM O_2 (Strous et al., 1997), but anammox bacteria have been recovered from suboxic waters of the Benguela Current OMZ with 9 µM O_2 (Kuypers et al., 2005). Sulfide apparently also inhibits anammox

(Jensen et al., 2008); anammox activity in the Cariaco Basin is therefore probably restricted to the depth interval above which H_2S was first detected (Fig. 4a; 260 m).

Diverse nitrogen cycling processes by microbes other than anammox occur in the core chemocline (245 – 270 m) of the Cariaco Basin (Wakeham et al., 2012). Nitrate, nitrite and ammonium all approach zero at the bottom of the suboxic zone (~270 m), suggesting competition between nitrification (oxidation of ammonium to NO_3^- via NO_2^-), denitrification (reduction of NO_3^- to N_2 via NO_2^- at the expense of OM), and anammox (anaerobic oxidation of ammonium with NO_2^-). In suboxic environments, ammonia oxidising archaea (AOA) and ammonia oxidising bacteria (AOB) may coexist by competing for available ammonium diffusing into the suboxic zone from the underlying anoxic zone (Lam and Kuypers, 2011). AOA may out-compete AOB in environments where sulfur-oxidizing bacteria are present (Park et al., 2010). Enrichment studies also show preferential growth of AOA over AOB at low oxygen tensions (Park et al., 2010).

Cutltivated marine Thaumarchaeota are up to now all light-independent chemoautotrophic nitrifiers, oxidising ammonium to nitrite (Könneke et al., 2005; Wuchter et al., 2006; Martens-Habbena et al., 2009). Glycerol dialkyl glycerol tetraethers (GDGTs) are specific lipids found in Euryarchaeota, Crenarchaeota (see compilation in Schouten et al., 2007a), and Thaumarchaeota (DeLong et al., 1998; Sinninghe Damsté et al., 2002b; Schouten et al., 2008). Crenarchaeol has been proposed as a specific biomarker for AOA (de la Torre et al., 2008; Pitcher et al., 2010). Therefore, the peak in intact polar (IP-)GDGT and the abundance of crenarchaeol among both IP- and core GDGT at 256 m (Fig. 4e; data from Wakeham et al., 2012) indicate the co-existence of AOA with anammox bacteria in a narrow confine (~25 m) of the suboxic core of the Carico Basin chemocline.

A similar co-occurrence apparently also occurs in the thin suboxic zone of the Black Sea (Lam et al., 2007; Wakeham et al., 2007), where thaumarchaeota has been suggested to supply nitrite for anammox (Coolen et al., 2007). In contrast, anammox and AOA were segregated by ~400 m in the OMZ of the Arabian Sea (Pitcher et al., 2011), most likely because the O_2 gradient in the Arabian Sea is much less steep. AOA in the Arabian Sea occured in the oxycline at ~170 m depth, where dissolved O_2 concentration was ~5 μM, whereas anammox peaked in the core of the OMZ at ~600 m and ~1 μM O_2. Nevertheless, in the Cariaco Basin it appears that at least a part of the nitrite required by anammox bacteria for the anaerobic oxidation of ammonium is provided by ammonium oxidation by AOA.

4.6 Conclusions

Within the core of the Cariaco Basin chemocline (235 – ~350 m), the transition into anoxic waters together with the availability of nitrite and ammonium provide anammox bacteria a suitable depth in which to be active. Peak concentration in anammox ladderane biomarkers co-occurred with a peak in anammox cell abundance. Nitrfying thaumarchaeota may be a source of nitrite for anammox, as indicated by the co-occurrence of crenarchaeol and ladderane lipids. Chemoautotrophy in marine water columns produces diagnostic biomarkers that provide valuable information about the biogeochemical processes at work. Many of these same biomarkers, including ladderane lipids, are used as molecular fossils to infer ancient oceanic environments, in particular during OAEs. Such hind-casting interpretations require a sound understanding of biogeochemical processes in the contemporary ocean. Further research

in the Cariaco Basin and similar environments will be needed to fully confirm relationships between water column chemoautotrophy and its manifestation in the sediment record.

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Part II: Diagenesis and catagenesis of ladderane biomarkers

Chapter 5

Short-chain ladderanes: oxic biodegradation products of anammox lipids

This chapter is based on
Rush, D., Jaeschke, A., Hopmans, E.C., Geenevasen, J.A.J., Schouten, S., Sinninghe Damsté, J.S. (2011) *Geochimica et Cosmochimica Acta* 75, 1662-1671.

Anammox, the microbial anaerobic oxidation of ammonium by nitrite to produce dinitrogen gas, has been recognised as a key process in both the marine and freshwater nitrogen cycles, and found to be a major sink for fixed inorganic nitrogen in the oceans. Ladderane lipids are unique anammox bacterial membrane lipids that have been used as biomarkers for anammox bacteria in recent and past environmental settings. However, the fate of ladderane lipids during diagenesis is as of yet unknown. In this study, we performed oxic degradation experiments (at 20 – 100 °C) with anammox bacterial biomass to simulate early diagenetic processes occurring in the water column and at the sediment-water interface. Abundances of C_{18} and C_{20} ladderane lipids decreased with increasing temperatures, testifying to their labile nature. The most abundant products formed were ladderane lipids with a shorter alkyl side-chain (C_{14} and C_{16} ladderane fatty acids), which was unambiguously established using two-dimensional NMR techniques on an isolated C_{14}-[3]-ladderane fatty acid. The most pronounced production of these short-chain lipids was at 40 °C, suggesting that degradation of ladderane lipids was microbially mediated, likely through a β-oxidation pathway. An HPLC-MS/MS method was developed for the detection of these ladderane alteration products in environmental samples and positively tested on various sediments. This showed that the ladderanes formed during degradation experiments also naturally occur in the marine environment. Thus, short-chain ladderane lipids may complement the original longer-chain ladderane lipids as suitable biomarkers for the detection of anammox processes in past depositional environments.

5.1 INTRODUCTION

Lipids provide much information as biomarkers for specific biosynthetic pathways in source organisms (Peters et al., 2005; Volkman, 2005). However, during settling in the water column and at the sediment water interface, lipids can undergo structural alterations by biotic and abiotic processes. Depending on structure, protection by a mineral or organic matrix, and oxygen levels, different alterations of individual biomarker lipids takes place, e.g., preferential degradation of shorter-chain *n*-alkanes (Johnson and Calder, 1973), incorporation of sulfur into functionalised lipids (Sinninghe Damsté et al., 1989), and autoxidation of alkenones (Rontani et al., 2006). Unsaturated and cyclic components are generally considered more susceptible to degradation than saturated components (Sun and Wakeham, 1994; Hoefs et al., 1998; Hoefs et al., 2002; Sinninghe Damsté et al., 2002c). Identifying the diagenetic products of biomarker lipids is an important tool in molecular biogeochemistry as it enables the reconstruction of past biomarker presence (Peters et al., 2005).

Ladderane lipids (Sinninghe Damsté et al., 2002a) are membrane lipids specific for anammox bacteria, belonging to the Planctomycetes, which perform anaerobic ammonium oxidation to dinitrogen gas with nitrite as the electron acceptor (Van de Graaf et al., 1995; Strous et al., 1999). Ladderane lipids consist of either three or five linearly concatenated cyclobutane rings (Fig. 1). These lipids form a dense and highly impermeable membrane surrounding the intracellular compartment where the anammox reaction takes place: the anammoxosome (Lindsay et al., 2001; Van Niftrik et al., 2004). Such a dense membrane is thought to be required to maintain concentration gradients during the exceptionally slow metabolism of anammox bacteria, and would also protect the remainder of the cell from the highly toxic intermediate hydrazine (Sinninghe Damsté et al., 2002a; van Niftrik et al., 2010). Since its discovery in a wastewater treatment system (Mulder et al., 1995), many studies have shown that anammox bacteria are ubiquitous in the marine environment and that the anammox process constitutes a substantial sink for fixed inorganic nitrogen in the oceans (Dalsgaard and Thamdrup, 2002; Kuypers et al., 2005; Thamdrup et al., 2006; Hamersley et al., 2007), which previously was solely attributed to heterotrophic denitrification. Ladderane lipids have been detected in oxygen minimum zones (OMZs; Hamersley et al., 2007; Jaeschke et al., 2007) and found to be exported through the water column (Jaeschke et al., 2007). However, the effect of (oxic) degradation on ladderane lipids occurring during transport through the water column and after deposition on the seafloor is presently unknown.

In this study, we investigated the effect of oxic degradation by incubating anammox cell material with Wadden Sea sediment and water, under oxic conditions. We monitored the degradation of ladderane lipids and analysed the products formed during these experiments in order to evaluate potential early diagenetic processes of ladderanes which might occur in the natural environment.

5.2 MATERIAL AND METHODS

5.2.1 EXPERIMENTAL SETUP

Two sources of anammox cell material were used in this experiment: (1) An anammox enrichment culture grown over 5 months in sequencing batch reactors (SBRs) as described by Strous et al. (1998), which contained two species of anammox bacteria, "*Candidatus* Kuenenia

stuttgartiensis" and "*Candidatus* Brocadia fulgida"; and (2) biomass from the Dokhaven wastewater treatment plant, shown to contain 70% bacterial dominance of "*Candidatus* Kuenenia stuttgartiensis" (Schmid et al., 2000). All cell material was freeze-dried and stored at -20 °C until further use.

For each degradation experiment approximately 20 – 30 mg of the SBRs anammox cell material was thoroughly mixed with 1.5 – 2 g of freeze-dried sandy sediment derived from the Wadden Sea, a tidal flat area situated close to NIOZ, and placed in open tubes. Ca. 1 – 2 mL of bi-distilled water was added to obtain a thick slurry, so that the cell material could be well dispersed throughout the experiment. These samples were then incubated for 72 h at temperatures of 20, 40, 50, 60, 70, 80 and 100 °C, respectively, and gently stirred to maintain aeration during the experiments. Afterwards, the supernatant was removed and the residue was freeze-dried again. A control experiment was performed at 40 °C for 72 h, using SBRs anammox cell material mixed with sterilised sediment (autoclaved for 20 min at 120 °C).

In order to isolate degradation products, a large batch degradation experiment was also performed. In an open flask, 1.5 g of anammox cell material from the Paques wastewater treatment plant was thoroughly mixed with 100 g of freeze-dried sediment. The mixture was gently stirred at 40 °C with 100 mL of bi-distilled water, which was refilled throughout the course of the week-long experiment to replace water lost due to evaporation. After 7 days, the experiment was stopped, the supernatant was removed, and the residue was freeze-dried.

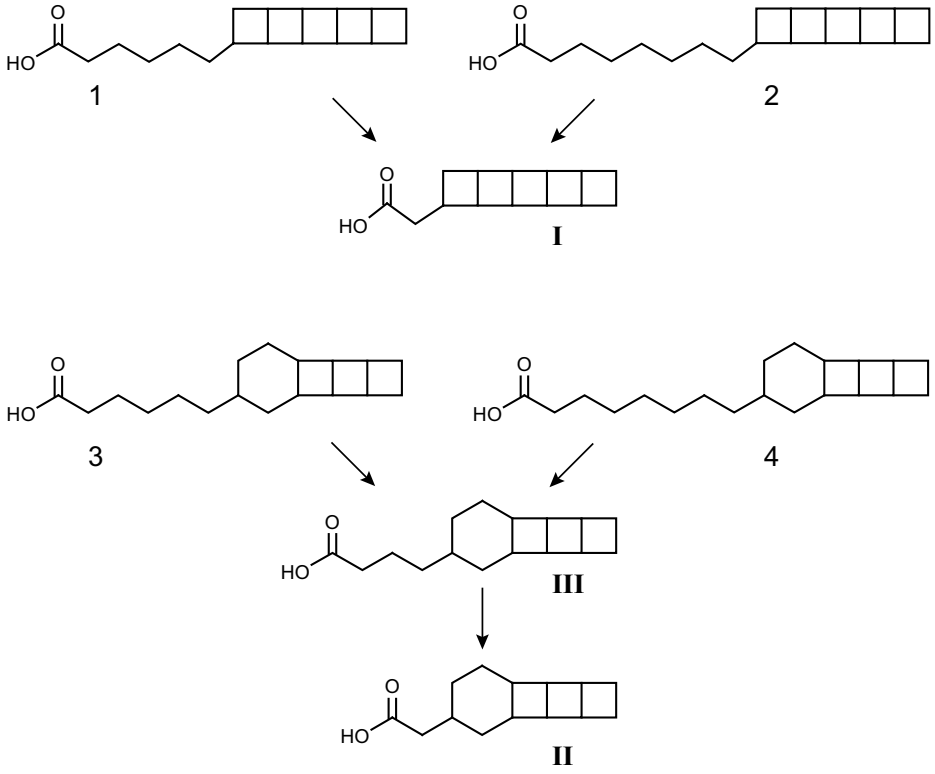


Figure 1. Structures of ladderane lipids derived from anammox bacteria and their transformation products derived from microbial oxic degradation. (1) C_{18}-[5]-ladderane FA, (2) C_{20}-[5]-ladderane FA, (3) C_{18}-[3]-ladderane FA, (4) C_{20}-[3]-ladderane FA, (**I**) C_{14}-[5]-ladderane FA, (**II**) C_{14}-[3]-ladderane FA, and (**III**) C_{16}-[3]-ladderane FA. FA, fatty acid.

5.2.2 EXTRACTION AND FRACTIONATION

Samples from the degradation experiments were ultrasonically extracted five times using a dichloromethane (DCM):methanol mixture (2:1 v/v). Combined extracts were dried using rotary evaporation, yielding the total lipid extract (TLE).

Aliquots of the TLEs from the temperature experiments were saponified with aqueous 1 N KOH in methanol for 2 h. Fatty acids were obtained by acidifying the solution to pH 3 and extracting with DCM. The fatty acid fraction was methylated by adding diazomethane to convert fatty acids into their corresponding fatty acid methyl esters (FAMEs). To remove very polar components, aliquots of the FAMEs were eluted with ethyl acetate over a small column filled with silica. Extracts derived from sediments were further eluted with DCM over a small silver nitrate-impregnated (5%) silica column to remove polyunsaturated fatty acids, yielding a saturated fatty acid fraction. For quantification, a known amount of internal standard (*ante iso* C_{22} alkane) in ethyl acetate was added prior to gas chromatography (GC) and GC/mass spectrometry (MS) analyses.

For high performance liquid chromatography tandem mass spectrometry (HPLC–MS/MS) analysis, an aliquot of the methylated TLE was dissolved in acetone and filtered through a 0.45 μm, 4 mm diameter PTFE filter.

5.2.3 ISOLATION OF SHORT-CHAIN LADDERANE FATTY ACIDS

Freeze-dried residue of the large batch degradation experiment was ultrasonically extracted five times using a DCM:methanol mixture (2:1 v/v). Combined extracts were dried using rotary evaporation, yielding the TLE (1.1 g). The total extract was methylated using BF_3 in methanol. Lipids were separated over an activated Al_2O_3 large column (75 × 250 mm) using four solvent mixtures: hexane:DCM (9:1), hexane:DCM (1:1), DCM, and DCM:methanol (1:1) (v/v). The short-chain ladderane fatty acids eluted in the hexane:DCM (1:1) fraction. Two short-chain components (**I** and **II**; see Figure 1 for structures) were further isolated by repetitive semi-preparative HPLC (high performance liquid chromatography) following conditions described by Hopmans et al. (2006). Briefly, aliquots of the hexane:DCM (1:1) fraction were injected onto two Zorbax Eclipse XDB-C_8 columns (4.6 × 150 mm, 5 μm, Agilent, coupled in series and maintained at 30 °C). Ladderane lipids were eluted using 0.4 mL/min methanol and fractions of 15 s were collected. Purity of the resulting fractions was assessed by GC and GC/MS. The purity of the isolated methyl ester C_{14}-[3]-ladderane fatty acid (**II**) was found to be 97%. The structure of this component was established using 2D-nuclear magnetic resonance (NMR) spectroscopy. The second short-chain fatty acid, C_{14}-[5]-ladderane fatty acid (**I**), was sufficiently enriched during the preparative HPLC to be used in the development of a HPLC coupled to positive ion atmospheric pressure chemical ionisation tandem mass spectrometry (HPLC/APCI-MS/MS) method, but the total amount of **I** was insufficient for 2D NMR studies.

5.2.4 GC AND GC/MS ANALYSIS

GC analysis was performed using a Hewlett-Packard 6890 instrument equipped with an on-column injector and a flame ionisation detector (FID). A fused silica capillary column (25 m × 0.32 mm) coated with CP Sil-5 (film thickness 0.12 μm) was used with helium as carrier gas. The samples were injected at 70 °C. The GC oven temperature was subsequently raised to 130 °C at a rate of 20 °C/min, and then at 4 °C/min to 320 °C, which was held for 15 min. GC/MS analysis was carried out using a Finnigan Trace GC Ultra, Thermo Electron

Corporation, interfaced with a Finnigan Trace DSQ mass spectrometer, with a mass range of *m/z* 40–800. GC conditions for GC/MS were the same as those described above for GC. The components formed during thermal destruction of the ladderane lipids during GC analysis were identified according to retention times and mass spectra described by Sinninghe Damsté et al. (2005).

5.2.5 HPLC/APCI-MS/MS ANALYSIS

The HPLC/APCI-MS/MS method developed by Hopmans et al. (2006) and modified by Rattray et al. (2008) was expanded to include Selective Reaction Monitoring (SRM) transitions diagnostic for the newly detected short-chain components with concatenated cyclobutane moieties (Table 1). First, an aliquot from the 40 °C degradation experiment was analysed using chromatographic and source conditions identical to those described by Rattray et al. (2008). Detection was achieved by MS/MS in data dependent scan mode where a positive ion scan (*m/z* 200–400) was followed by a product ion scan (collision energy 25 V, 0.8 mTorr argon, collision gas) of the base peak of the generated mass spectrum. Based on the results of this experiment, diagnostic fragments for each of the short-chain ladderane lipids (**I**, **II**, and **III**) were identified. Optimal conditions for each monitored SRM transition were determined by an automatic tuning procedure, while directly infusing the isolated C_{14}-[3]-ladderane fatty acid (**II**) and the fraction enriched in C_{14}-[5]-ladderane fatty acid (**I**) (Table 1). Collision gas pressure was maintained at 1.5 mTorr for these experiments. No standard was available for component **III** and thus the collision energies for its reactions were set to 25 V (Table 1).

5.2.6 ^{1}H AND ^{13}C NMR SPECTROSCOPY

The C_{14}-[3]-ladderane fatty acid methyl ester isolated using semi-preparative HPLC was dissolved and dried in (2×) DCM, (2×) $CHCl_3$, (2×) tetrachloroethane, and (2×) $CDCl_3$, then transferred to an NMR tube using 0.75 mL $CDCl_3$. 1D and 2D ^{1}H and ^{13}C NMR analyses were performed on a Bruker DMX-600 spectrometer equipped with a TCI CryoProbe at 298 K, as previously reported by Sinninghe Damsté et al. (2005).

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

Ladderane lipid alteration product	$[M+H]^+$ *m/z*	Product *m/z*	Optimum Collision Energy (V)
I	233.2	117.0	37
		131.0	32
		158.9	24
		172.9	23
II	235.2	119.2	34
		133.2	32
		161.2	24
		175.2	22
III	263.2	93.5	25[a]
		121.3	25[a]
		135.2	25[a]
		171.1	25[a]

[a]Collision energy was set at 25 V for all transitions monitored for component **III** as no standard was available for optimisation.

5.3 RESULTS AND DISCUSSION

5.3.1 DEGRADATION EXPERIMENTS

The effect of oxygen and temperature on the preservation of ladderane lipids was investigated using aliquots of biomass from an anammox enrichment culture, which contained "*Candidatus* Kuenenia stuttgartiensis" and "*Candidatus* Brocadia fulgida", mixed with surface sediment derived from the Wadden Sea. These experiments were incubated in open tubes at temperatures ranging from 20 to 100 °C for three days. The original C_{18} and C_{20} ladderane fatty acids (**1–4**; see Fig. 1 for structures), as analysed with GC, substantially decreased in concentration with increasing experimental temperature (Figs. 2 and 3a). Compared to the original unheated anammox biomass, ladderane lipids incubated at 20 °C were 2 – 4 times lower in concentration, and then slowly decreased further with increasing temperature. At 100 °C, these lipids were still detectable but had decreased substantially in concentrations, leaving <10% of the original lipids.

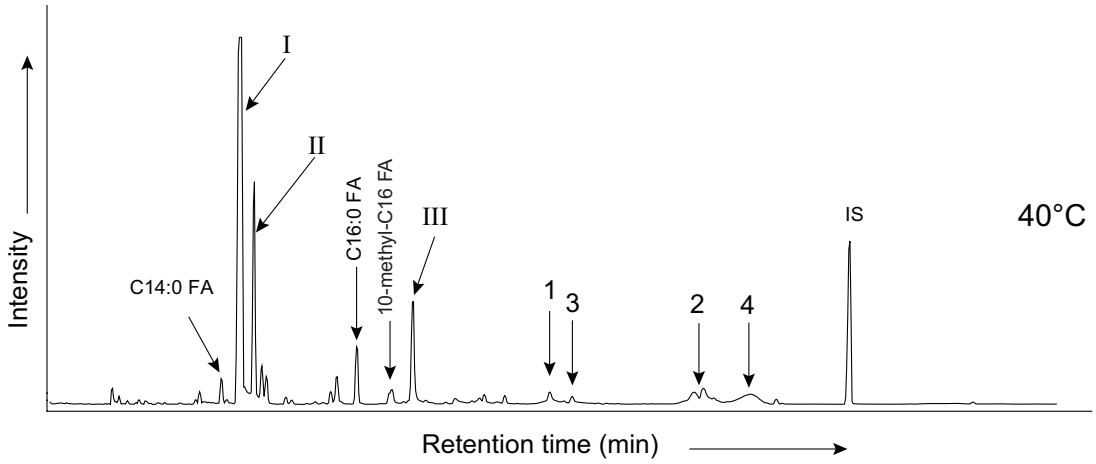


Figure 2. Gas chromatogram of the fatty acid fraction of anammox biomass incubated for three days at 40 °C. Numbers correspond to structures shown in Figure 1. IS denotes internal standard (*ante iso* C_{22} alkane).

Several new components were produced during the incubation experiments and were detected with GC/MS (Fig. 2). Components **I** and **II** were present at incubation temperatures between 20 and 70 °C, while **III** was only detected in the 40 °C experiment (Fig. 3b). At temperatures of 80 °C and above, these new components were not detected (Fig. 3b). The highest concentrations of the three components were found at a temperature of 40 °C (Fig. 3b). GC/MS analysis revealed that the mass spectra for **I–III** were similar to those of known C_{18} and C_{20} ladderane fatty acids (Sinninghe Damsté et al., 2005), i.e., loss of the alkyl chain resulting in fragments m/z 131, 132, and 160, 161, but with different molecular ions, i.e., m/z 232, m/z 234, and m/z 262 for **I**, **II**, and **III**, respectively (Fig. 4, a-c). The molecular ion of **I** is 84 Da less than that of the C_{20}-[5]-ladderane fatty acid (**2**), or 56 Da less than that of the C_{18}-[5]-ladderane fatty acid (**1**), which, together with similar fragmentation patterns, suggests that **I**

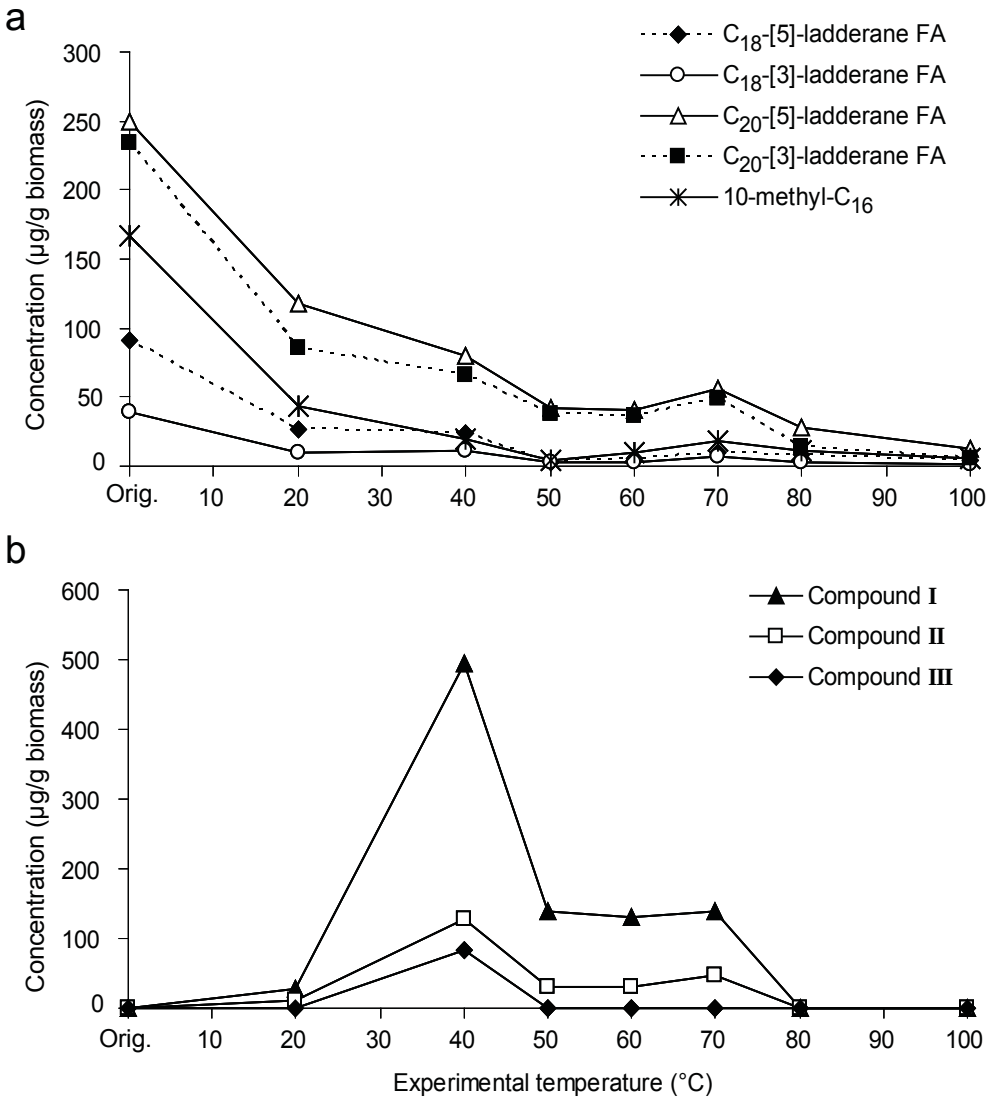


Figure 3. Concentration of (a) known ladderane lipids and 10-methylhexadecanoic acid, and (b) short-chain ladderane transformation products as a function of temperature in the fatty acid fractions after base hydrolysis and analysed by GC.

is a C_{14}-[5]-ladderane fatty acid. The molecular ion of **II** is 84 Da less than that of the C_{20}-[3]-ladderane fatty acid (**4**), or 56 Da less than that of the C_{18}-[3]-ladderane fatty acid (**3**), suggesting component **II** is a C_{14}-[3]-ladderane fatty acid. Finally, the molecular ion of **III** is 56 Da less than that of the C_{20}-[3]-ladderane fatty acid (**4**), or 28 Da less than that of the C_{18}-[3]-ladderane fatty acid (**3**), suggesting that **III** is a C_{16}-[3]-ladderane fatty acid. The ladderane moiety, which has been found to be heat labile (Sinninghe Damsté et al., 2005) and form thermally more stable products at hydrous pyrolysis temperatures of 120 °C and above (Jaeschke et al., 2008), was still intact at 100 °C.

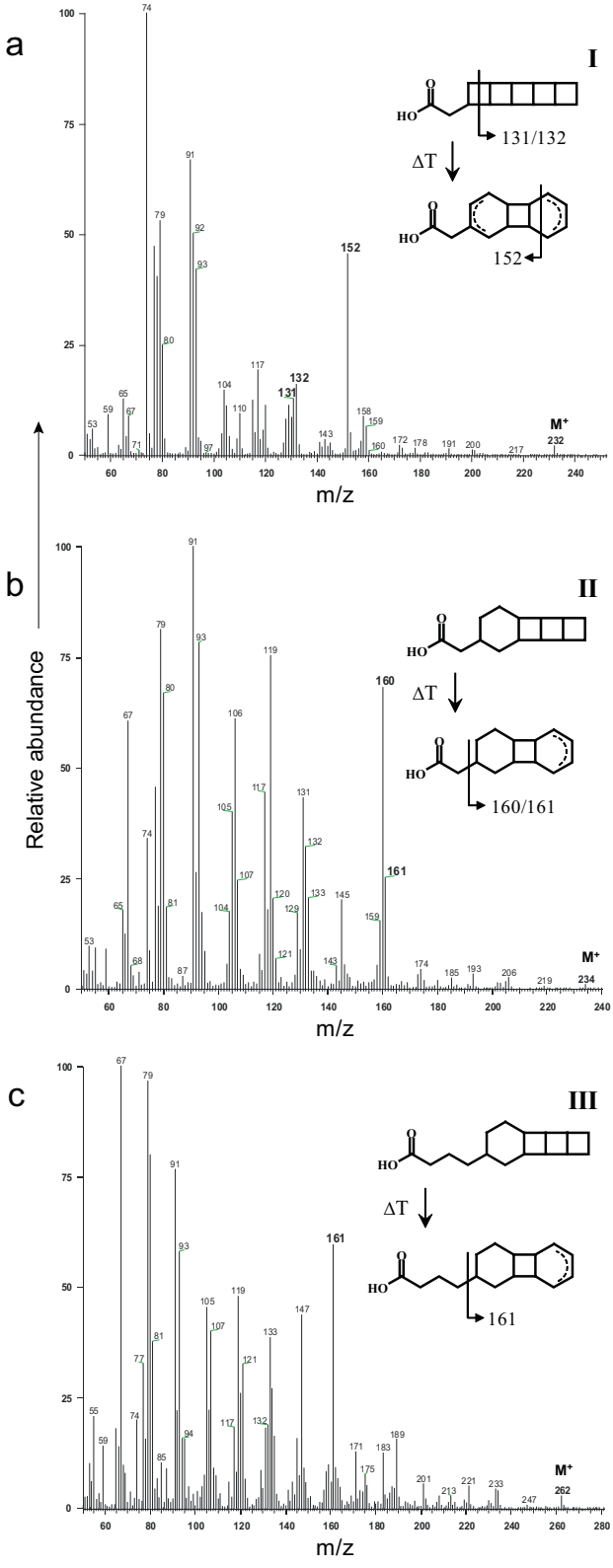
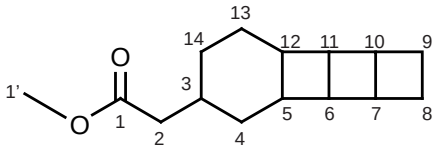


Figure 4. GC/MS spectra of the short-chain ladderane fatty acids **I**, **II**, and **III** analysed as their methyl ester derivatives. (a) C_{14}-[5]-ladderane fatty acid, (b) C_{14}-[3]-ladderane fatty acid, and (c) C_{16}-[3]-ladderane fatty acid. It should be noted that the mass spectra reflect the thermal degradation products formed during GC analysis as discussed by Sinninghe Damsté et al. (2005).

To unambiguously establish the structure of the short-chain ladderane analysis, a large scale experiment was performed, which enabled the isolation of 1.6 mg of the most abundant component (**II**) for structural identification by high-resolution NMR spectroscopy (Table 2). ^{1}H NMR revealed no olefinic protons as well as shifts (ppm) in aliphatic protons that were akin to ladderane cyclobutane rings. ^{13}C NMR revealed 15 carbon atoms (1 carbonyl carbon, 1 primary carbon in the terminal position of an ester, 6 secondary carbons, and 7 tertiary carbons) which is consistent with a C_{14}-[3]-ladderane FAME. The identification of the ladderane moiety was established by the application of 2D-NMR techniques (HSQC, COSY, TOSCY). These studies also confirmed that the conformational properties of component **II** were identical to those of the first ladderanes identified (Sinninghe Damsté et al., 2002a; Mascitti and Corey, 2004). These structural identifications imply that the new fatty acids are formed by the oxidation of the alkyl chain on the original ladderane fatty acids.

Table 2. Proton and Carbon NMR data for component **II** (C_{14}-[3]-ladderane fatty acid methyl ester), formed during the oxic degradation of ladderane biomass.



C-number	Stereo-chemistry	Proton Shift (ppm)	Carbon shift (ppm)[a]				2D NMR correlations	
			Primary	Secondary	Tertiary	Quaternary	COSY	TOSCY
1		—				173.62	—	—
2		2.24 (d, 2H)		42.32			H3	H3, H4α, H4β, H5, H13, H14α, H14β
3		1.76 (m, 1H)			29.96		H2, H4β	H2
4	α	1.80 (m, 1H)		33.93			H4β, H5	H4β, H14α
	β	1.11 (m, 1H)					H3, H4α, H5	H5, H6, H13, H14α
5		2.27 (m, 1H)			37.43		H4α, H4β, H6, H12	H4α, H4β, H13
6		2.31 (m, 1H)			49.18		H5	H4α, H4β, H12
7		2.75 (m, 1H)			41.38		H8α, H10	H8α, H8β, H9α, H9β, H10, H11, H12
8	α	2.50 (m, 1H)		26.15			H7, H8β, H9α, H9β	H7, H8β, H9β, H10
	β	1.84 (m, 1H)					H8α, H9α, H9β	H7, H8α, H9α, H10
9	α	2.42 (m, 1H)		25.39			H8α, H8β, H9β, H10	H7, H8β, H9β, H10
	β	1.95 (m, 1H)					H8α, H8β, H9α, H10	H7, H8α, H9α, H10
10		2.63 (m, 1H)			42.18		H7, H9α, H9β, H11, H12	H7, H8α, H8β, H9α, H9β, H11, H12
11		2.45 (m, 1H)			47.07		H10, H12	H7, H10, H12
12		2.31 (m, 1H)			37.03		H5, H10, H11, H13	H4α, H4β, H5, H6, H10, H11, H13, H14α, H14β
13		1.54 (m, 2H)		25.23			H12, H14α, H14β	H4α, H4β, H5, H12, H14β
14	α	1.60 (m, 1H)		27.83			H13, H14β	H2, H4α, H4β, H12, H13, H14β
	β	1.18 (m, 1H)					H13, H14α	H2, H4α, H4β, H12, H13, H14α
1'		3.68 (s, 3H)	51.38				—	—

[a] connection was established by an HSQC experiment

The question arises as to whether or not these short-chain fatty acids are formed abiotically, or through microbial activity. The fact that ladderane lipids with shorter alkyl chains occur only at temperatures between 20 and 70 °C, and have maximum concentrations at 40 °C, strongly suggested microbial degradation. To confirm this, a control experiment was performed with anammox biomass and sterilised sediment under the same conditions (40 °C, 72 h). No C_{14} or C_{16} ladderane fatty acids were detected, demonstrating that their formation was microbially mediated.

The oxidation of alkyl side chains of cyclic components has been shown previously during microbial degradation experiments. For example, several microorganisms are able to degrade *n*-alkylcyclohexanes by an alkyl side-chain oxidation pathway, the so-called β-oxidation pathway (Beam and Perry, 1974; Dutta and Harayama, 2001; Koma et al., 2003). This pathway

includes four reactions that occur in repeated cycles. In each cycle, as the alkyl chain is oxidised, it is progressively shortened by two carbon atoms. Depending on whether there is an odd or even number of carbon atoms in the alkyl side-chain, carboxylic or acetic acid derivatives are formed, respectively. Typically, acetic acid derivatives are metabolically recalcitrant; they cannot be further oxidised by β-oxidation and usually accumulate (Beam and Perry, 1974). Therefore, we suggest a similar degradation pathway is likely for the ladderane lipids, as their chemical structure comprises of an even number of carbons in the alkyl chain and a cyclic group, which is comparable to that of *n*-alkylcyclohexanes with even-numbered side-chains. The C_{14} and C_{16} ladderane fatty acids (**I, II,** and **III**) detected in the degradation experiments are thus derived from C_{18} and C_{20} ladderane fatty acids (**1**-**4**) via the β-oxidation route (Fig. 1) (cf. Dutta and Harayama, 2001). Component **II** could be derived from **III** by further oxidation of the alkyl side-chain by two carbon atoms during ongoing degradation. It should be noted that the C_{16}-[3]-ladderane fatty acid with ($[M+H]^+$) of m/z 263 was reported previously by Rattray et al. (2010) in an anammox enrichment culture, suggesting that this shorter-chain fatty acid may be synthesised as an adaptation to lower cultivation temperatures. Since this lipid was not present in the starting biomass, our results, however, indicate that, in this case, the shorter-chain ladderane fatty acids were products of microbial oxidation.

5.3.2 Environmental occurrence of short-chain ladderane fatty acids

To investigate whether the products formed in our degradation experiments are also formed in natural environments, we analysed a number of sediments for the presence of short-chain ladderane fatty acids. We thus aimed to expand the existing HPLC/APCI-MS/MS SRM method developed for the original ladderane lipids in the natural environment by Hopmans et al. (2006), and modified by Rattray et al. (2008), to include the short-chain ladderane fatty acids (Table 1).

We first analysed an aliquot from the 40 °C experiment, in which the biodegraded ladderane products were initially detected, by MS/MS in data dependent scan mode (Fig. 5b). The results showed two additional peaks, besides those of the original ladderane fatty acids which were found in the biomass before the degradation experiment (Fig. 5a) and in the 80 °C experiment (Fig. 5c). At 40 °C, the peak with a retention time of ca. 8 min. actually consisted of two co-eluting components with protonated molecules ($[M+H]^+$) of m/z 233 and 235, respectively. The peak with a retention time of ca. 9 min represented a component with a protonated molecule of m/z 263. The APCI-MS/MS spectra of the protonated molecules of these biodegraded ladderanes showed similar product ions to those reported to be representative of the ladderane moieties in the original ladderane fatty acids (Hopmans et al., 2006) (Fig. 6a-c, showing mass spectra for products **I–III**, respectively). Therefore, these peaks likely represented the C_{14} and C_{16} shorter-chain ladderane fatty acids. Confirmation came from the HPLC/MS/MS analyses of the isolated C_{14}-[3]-ladderane fatty acid and the enriched fraction of C_{14}-[5]-ladderane fatty acid, which showed the same retention times and MS/MS mass spectra as those identified in the 40 °C experiment. The selection of product ions for SRM was based on their diagnostic value for the concatenated cyclobutane moieties of the ladderane molecules as well as on their high relative abundances in the mass spectra. Parent ions, selected product ions, and respective collision energies for maximal abundance of each monitored short-chain fatty acid are listed in Table 1.

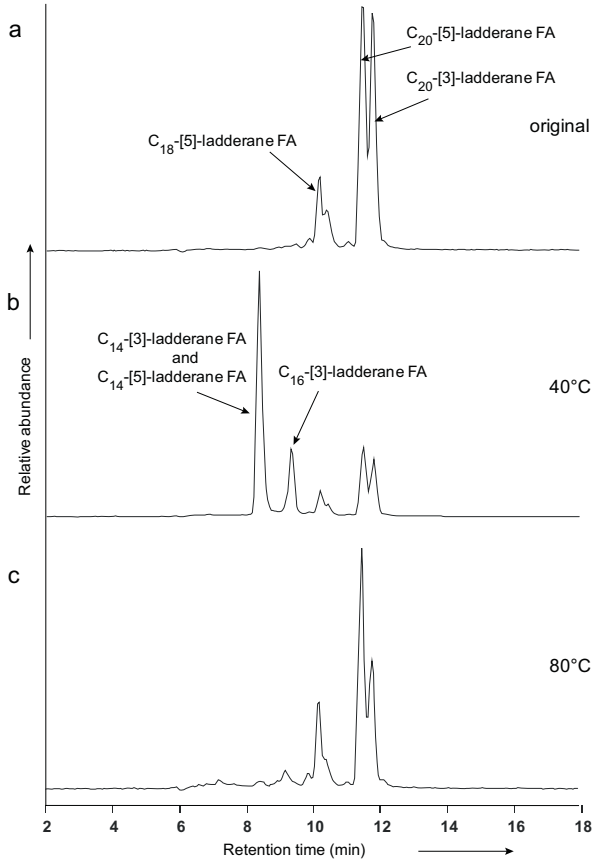


Figure 5. HPLC base peak chromatograms of the fatty acid fraction (analysed as their methyl ester derivatives) showing the distribution of ladderane fatty acids in anammox degradation experiments: original (a), heated for three days at 40 °C (b), and heated for 3 days at 80 °C (c).

To test this modified method, it was applied on an aliquot of the methylated TLE from the 40 °C experiment (Fig. 7a). C_{14}-[5]-ladderane fatty acid (**I**) was found to be most abundant, followed by C_{14}-[3]-ladderane fatty acid (**II**), which was one order of magnitude lower in abundance. C_{16}-[3]-ladderane fatty acid (**III**) was approximately 1.5 times lower in abundance than component **II**. The method was then used to analyse sediments derived from various locations where C_{18} and C_{20} anammox lipids have previously been detected, i.e., off northwest Africa (08°54.0'N, 14°56.1W; 1–2 cmbsf), the Arabian Sea (22°32.9'N, 64°02.8'E; 193 cmbsf), and the Irish Sea (53°53.0'N, 5°35.6'W; 1–2 cmbsf) (Jaeschke et al., 2007; Jaeschke et al., 2009a; Jaeschke et al., 2010). As shown in the partial SRM traces in Fig. 7b-d, the ladderane lipid degradation products with shorter-chain lengths could indeed be detected with the modified HPLC/APCI-MS/MS method in all three sediments analysed. In these sediments, the C_{14}-[3]-ladderane fatty acid (**II**) was the most abundant, followed by C_{14}-[5]-ladderane fatty acid (**I**), at 1.5–2 times lower abundance. C_{16}-[3]-ladderane fatty acid (**III**) was about two orders of magnitude lower in abundance.

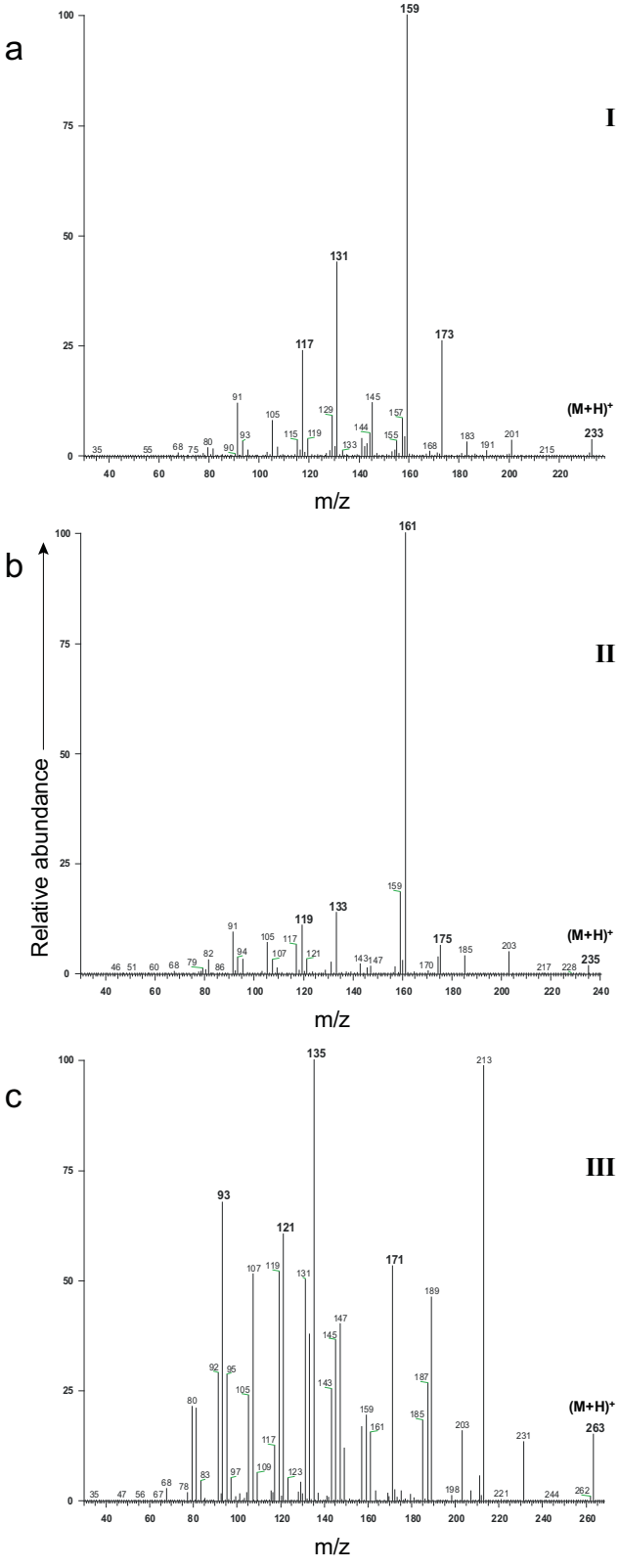


Figure 6. APCI-MS/MS spectra of the protonated molecular ions of three different ladderane lipid transformation products: (a) component **I** (*m/z* 233), (b) component **II** (*m/z* 235), and (c) component **III** (*m/z* 263). Structures of the original lipids and the alteration products are depicted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

The occurrence of these short-chain ladderane alteration products in marine sediments suggests that ladderane lipids are degraded in the natural environment in a way similar to our simulated degradation experiments, i.e., via a microbially mediated β-oxidation pathway. Thus, the short-chain ladderane fatty acids **I – III** may be useful biomarkers for anammox bacteria, especially in sediments underlying oxygen minimum zones, where ladderane lipids produced in the OMZ have been re-exposed to oxygen during settling through the water column and at the sediment–water interface. Future investigations of short-chain ladderanes in natural

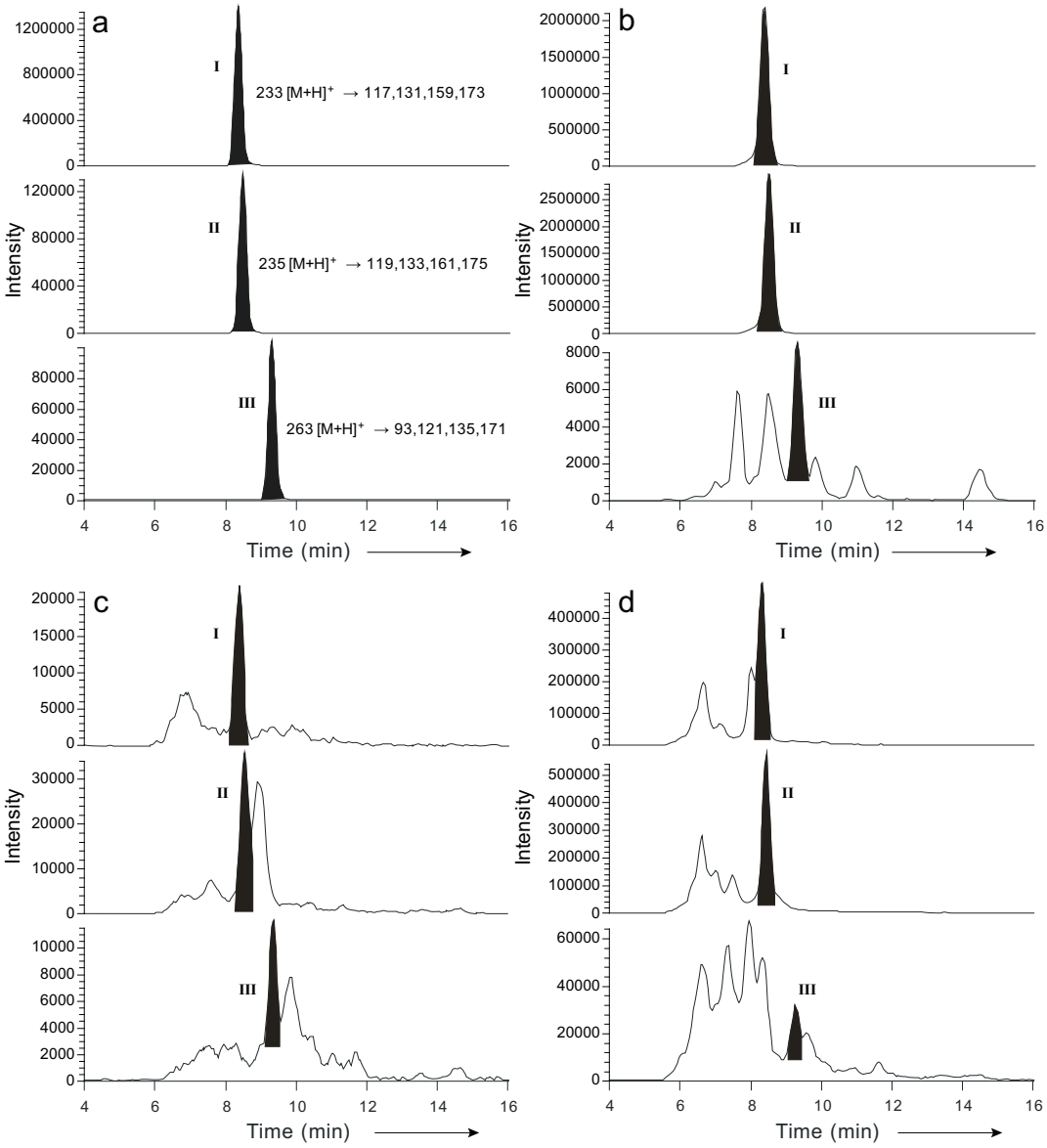


Figure 7. SRM traces of short-chain ladderane fatty acids (analysed as their methyl ester derivatives) in (a) anammox biomass heated at 40 °C for three days, and in sediments from (b) the Arabian Sea, (c) the Irish Sea, and (d) offshore northwest Africa.

settings should reveal the quantitative importance of these compounds as biomarkers for oxic ladderane degradation.

5.4 CONCLUSIONS

The results of our laboratory studies strongly suggest that under oxic conditions ladderane lipids can be microbially degraded via the β-oxidation pathway of the alkyl side-chain, resulting in shorter-chain ladderane lipids. The detection of these biodegraded ladderane lipids in marine sediments using a modified HPLC/APCI–MS/MS method indicates that these components are also produced in the natural environment. These short-chain ladderane lipids may be a useful biomarker for past OMZ anammox processes in immature sediments.

ACKNOWLEDGEMENTS

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Chapter 6

Occurrence and distribution of ladderane oxidation products in different oceanic regimes

This chapter is based on
Rush, D., Hopmans, E.C., Wakeham, S.G., Schouten, S., Sinninghe Damsté, J.S. (2012) *Biogeosciences* 9, 2407-2418.

Ladderane fatty acids are commonly used as biomarkers for bacteria involved in anaerobic ammonium oxidation (anammox). These lipids have been experimentally shown to undergo aerobic microbial degradation to form short-chain ladderane fatty acids. However, nothing is known of the production or the distribution of these oxic biodegradation products in the natural environment. In this study, we analysed marine water column particulate matter and sediment from three different oceanic regimes for the presence of ladderane oxidation products (C_{14} ladderane fatty acids) and of original ladderane fatty acids (C_{18} and C_{20} ladderane fatty acids). We found that ladderane oxidation products, i.e., C_{14} ladderane fatty acids, are already produced within the water column of the Arabian Sea oxygen minimum zone (OMZ) and thus only low amounts of oxygen (< 3 μM) are needed for the β-oxidation of original ladderane fatty acids to proceed. However, no short-chain ladderane fatty acids were detected in the Cariaco Basin water column, where oxygen concentrations were below detection limit, suggesting that the β-oxidation pathway is inhibited by the absence of molecular oxygen, or that the microbes performing the degradation are not proliferating under these conditions. Comparison of distributions of ladderane fatty acids indicates that short-chain ladderane fatty acids are mostly produced in the water column and at the sediment surface, before being preserved deeper in the sediments. Short-chain ladderane fatty acids were abundant in Arabian Sea and Peru Margin sediments (ODP Leg 201), often in higher concentrations than the original ladderane fatty acids. In a sediment core taken from within the Arabian Sea OMZ, short-chain ladderanes made up more than 90% of the total ladderanes at depths greater than 5 cm below sea floor. We also found short-chain ladderanes in higher concentrations in hydrolysed sediment residues compared to those freely occurring in lipid extracts, suggesting that they had become bound to the sediment matrix. Furthermore, these matrix-bound short-chain ladderanes were found at greater sediment depths than short-chain ladderanes in the lipid extract, suggesting that binding to the sediment matrix aids the preservation of these lipids. Though sedimentary degradation of short-chain ladderane fatty acids did occur, it appeared to be at a slower rate than that of the original ladderane fatty acids, and short-chain ladderane fatty acids were found in sediments from the late Pleistocene (~100 kyr). Together these results suggest that the oxic degradation products of ladderane fatty acids may be suitable biomarkers for past anammox activity in OMZs.

6.1 INTRODUCTION

Anaerobic ammonium oxidation (anammox) is the conversion of ammonium through nitrite reduction to dinitrogen gas, performed by select Planctomycetes bacteria. Since its discovery in a waste water treatment plant (Mulder et al., 1995), evidence of anammox activity has been found in anoxic marine and estuarine sediments (Thamdrup and Dalsgaard, 2002; Trimmer et al., 2003; Jaeschke et al., 2009a;), as well as in the water columns of oxygen minimum zones (OMZs; Kuypers et al., 2005; Thamdrup et al., 2006; Hamersley et al., 2007; Pitcher et al., 2011) and euxinic basins (Kuypers et al., 2003; Wakeham et al., 2012). Anammox has been shown to be responsible for a significant loss of nitrogen from the oceanic system, especially in OMZs (Jaeschke et al., 2007; Jensen et al., 2011), although the relative importance of anammox versus denitrification is unresolved (Ward et al., 2009; Lam et al., 2011).

Anammox bacterial cells contain characteristic biomarker lipids, called ladderane lipids, which are comprised of concatenated cyclobutane moieties (Sinninghe Damsté et al., 2002a). Ladderane lipids form the outer membrane of the anammoxosome, the organelle-like structure within the bacterial cell where the anammox reaction is believed to take place. Traditionally, C_{18} and C_{20} ladderane fatty acids with either 3 or 5 cyclobutane moieties (Fig. 1, **IV** – **VII**) have been used to trace anammox (Kuypers et al., 2003; Kuypers et al., 2005; Hopmans et al., 2006; Jaeschke et al., 2009a), and the preservation of ladderane lipids in sediments may indicate past anammox activity (Jaeschke et al., 2009b). Still, relatively little is known about their fate in the water column or in the sediment.

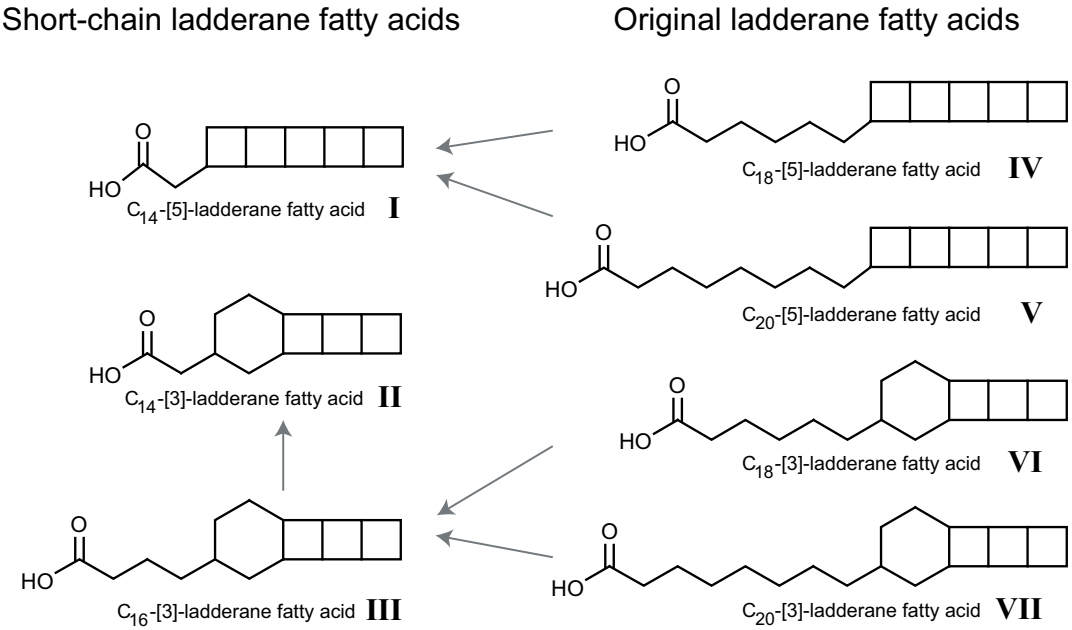

Figure 1: Chemical structures of the ladderane lipids analysed in this study. Short-chain ladderane fatty acids: (**I**) C_{14}-[5]-ladderane fatty acid, (**II**) C_{14}-[3]-ladderane fatty acid, and (**III**) C_{16}-[3]-ladderane fatty acid. Original ladderane fatty acids: (**IV**) C_{18}-[5]-ladderane fatty acid, (**V**) C_{20}-[5]-ladderane fatty acid, (**VI**) C_{18}-[3]-ladderane fatty acid, and (**VII**) C_{20}-[3]-ladderane fatty acid. Two short-chain ladderane fatty acids (I, II) were chosen as oxic degradation products of original ladderane fatty acids. Grey arrows indicate presumed degradation pathways.

Recently, we reported biodegradation products of C_{18} and C_{20} ladderane fatty acids that were produced during an oxic degradation experiment (Rush et al., 2011). Aerobic degradation of anammox biomass resulted in the production of short-chain (C_{14} and C_{16}) ladderane fatty acids (Fig. 1, **I – III**) that were mostly likely formed via a β-oxidation pathway. This pathway has been observed previously in degradation experiments on *n*-alkyl substituted cycloalkanes (Beam and Perry, 1974; Dutta and Harayama, 2001). A preliminary investigation of a few marine sediments in this earlier study showed that biodegraded ladderane products are present in nature as well. It is unclear, however, how important they are in general, under which conditions they are formed, and if they are preserved in sediments.

Here, we investigated the occurrence and distribution of the short-chain ladderane fatty acid oxidation products in suspended particulate matter (SPM) and marine sediments from different oceanic regimes where anammox activity has been shown to occur, i.e., the OMZs of the Arabian Sea (Jaeschke et al., 2007; Jensen et al., 2011) and the Peru Margin (Hamersley et al., 2007), and the euxinic Cariaco Basin (Wakeham et al., 2012). We compared the presence of the original ladderane fatty acids to that of the short-chain ladderane fatty acids to determine the extent of biodegradation and the possible application of these new lipid biomarkers as tracers for past anammox activity.

6.2 Materials and Methods

6.2.1 Study sites and sampling methods

Three areas (Fig. 2; Table 1) were studied, i.e., the Arabian Sea (AS), the Peru Margin (PM), and the Cariaco Basin (CB). In the AS, a series of 10 surface sediment cores down a depth profile along the Murray Ridge as well as SPM samples at one station were taken in the Arabian Sea on R/V *Pelagia* cruise 64PE301 during the Northeast winter monsoon (January 2009; see Pitcher et al., 2011; Lengger et al., 2012). This ridge protrudes into the AS OMZ; surface sediment oxygen concentrations varied from minimum (3 μM) on the top of the ridge to higher values (86 μM) at the lower sites. Surface sediments (0 – 0.5 cm below sea floor; cmbsf) were sampled at ten sites. Whole sediment cores, varying in length from 20 to 32 cmbsf, were analysed at three stations (Station 1: 885 m water depth, within the OMZ; Station 4, 1306 m water depth, directly below the OMZ; and Station 10, 3003 m water depth, below the OMZ). On the PM, a sediment core (185.6 mbsf) from the Peru shelf was sampled at 151 meters water depth, under the major marine Peru upwelling area, during ODP Leg 201 (Site 1229; D'Hondt et al., 2003). This core was located in the Salaverry Basin, which is completely filled with hemipelagic sedimentation. Past shifts in the intensity of productivity has caused the upper boundary of the OMZ to reach the seafloor in this area (Suess et al., 1988). Finally, water column SPM samples from the CB were collected at the 'Cariaco Basin time series' site (continental shelf, northern Venezuela; 10˚N30', 64˚W40') in November 2007 during CARIACO cruise 139 (CAR-139; Wakeham et al., 2012). At water depths between 230 and 260 m, both O_2 and H_2S were below detection limits (~2 μM and ~1 μM, respectively). Sulfide was first detected at 260 m (see also Ho et al., 2004, for comparison). Euxinic deep waters in the CB are caused by the degradation of sinking organic matter produced by high primary productivity, and the limited reoxygenation of subsurface waters due to the restricted water circulation within the basin (Muller-Karger et al., 2001; Muller-Karger et al., 2004).

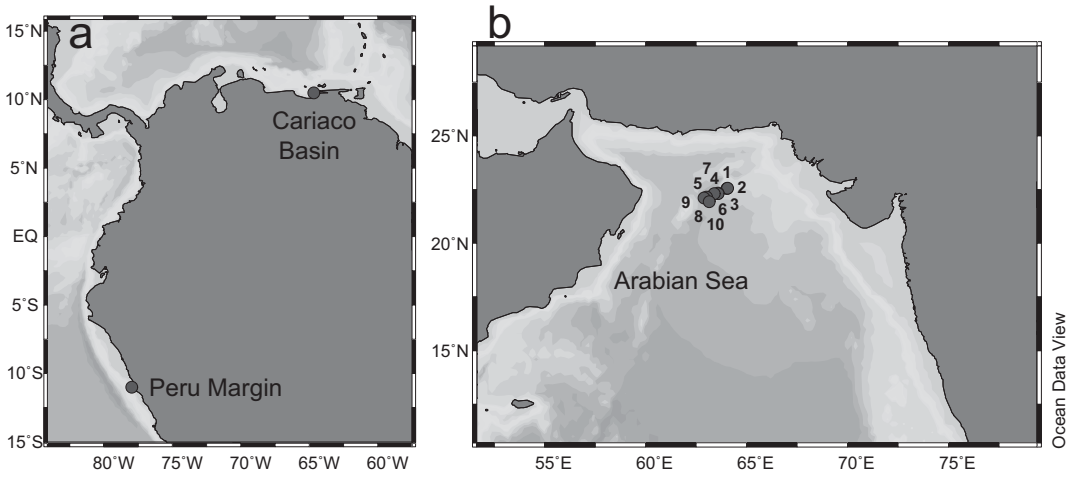

Figure 2: Location of sampling sites. Circles correspond to a deep sediment core (185.6 mbsf) in the Peru Margin (ODP Leg 201 - Site 1229), a water column particulate matter depth profile in the Cariaco Basin (aboard the R/V *Paraguachoa*) and a series of surface sediments in the Arabian Sea (aboard R/V *Pelagia* cruise 64PE301). The Arabian particulate water column site is Station 10. Surface sediments were taken at stations 1 through 10, located along Murray Ridge, while sediment cores of up to 32 cmbsf depth were taken at Stations 1, 4, and 10.

6.2.2 Extraction

6.2.2.1 Cariaco Basin SPM

CB water column SPM samples, collected by large-volume in situ filtration, were Soxhlet extracted wet with dichloromentane:methanol (DCM:MeOH) as described by Wakeham et al. (1997; 2012). The total lipid extract (TLE) was dried to near-dryness using a rotary evaporator and was kept frozen at -20 °C.

6.2.2.2 Arabian Sea SPM

AS SPM was obtained by large-volume in situ filtration and extracted as described by Pitcher et al. (2011). Briefly, freeze-dried filters were extracted using a modified Bligh-Dyer method (Bligh and Dyer, 1959; Rattray et al., 2008). The sample was ultrasonically extracted for 15 min using a volume ratio of 2:1:0.8 (v/v/v; MeOH: DCM: phosphate buffer, pH 7.4). The supernatant was collected and the residue was re-extracted ultrasonically two times. The solvent ratio of the combined supernatants was adjusted to 1:1:0.9 (v/v/v; MeOH: DCM: phosphate buffer) and centrifuged. The bottom DCM layer was collected and the remaining solvent re-extracted twice with DCM. The DCM layers were combined and dried to near-dryness under rotary evaporator. The TLE was then kept frozen at -20 °C.

6.2.2.3 Arabian Sea sediments

Slices of the first 0 – 0.5 cm of AS cores were taken on board the ship and immediately stored at -80 °C and transported at -20 °C (Lengger et al., 2012). Deeper core analyses were done on 0.5 cm slice intervals to 2 cmbsf and then on 1 cm slice intervals to 20 or 32 cmbsf, of cores at stations 1, 4, and 10 (Lengger et al., 2012). Freeze-dried sediments were extracted 4 times for 5 min by an accelerated solvent extractor (ASE 200, DIONEX) using a solvent

mixture of DCM:MeOH (3:1). The effect of ASE extraction on ladderane recovery was tested using different temperatures and pressures. An extraction at relatively low temperature (40 °C) and high pressure (6.9 MPa) was determined to effectively extract ladderane lipids without loss due to thermal degradation. The obtained TLEs were dried down using a Turbo Vap LV (Caliper Life Sciences) and stored at 4 °C.

Table 1. Location, type, and water depth of samples used in this study.

Site	Location (latitude, longitude)	Station	Sample Type	Water depth sampled (m)	Sediment depth sampled
Cariaco Basin (CB)	10° 30' 00" N, 64° 40' 01" W		SPM[a]	42, 205, 220, 236, 245, 256, 270, 276, 296, 326, 346, 750	
Arabian Sea (AS)	21° 55' 36" N, 63° 10' 36" E		SPM [a]	20, 170, 300, 450, 600, 750, 900, 1050, 1200, 1350, 1500, 2000	
	22° 32' 53" N, 64° 02' 23" E	1	sediment core (32 cmbsf[b])	885	0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 24, 28, 32 cmbsf[b]
	22° 33' 55" N, 64° 03' 46" E	2	sediment core	1013	surface[d]
	22° 19' 55" N, 63° 36' 00" E	3	sediment core	1172	surface[d]
	22° 18' 00" N, 63° 36' 00" E	4	sediment core (20 cmbsf[b])	1306	0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20 cmbsf[b]
	22° 09' 18" N, 63° 12' 45" E	5	sediment core	1379	surface[d]
	22° 04' 42" N, 63° 04' 30" E	6	sediment core	1495	surface[d]
	22° 18' 30" N, 63° 24' 30" E	7	sediment core	1786	surface[d]
	22° 08' 41" N, 63° 01' 07" E	8	sediment core	1970	surface [d]
	22° 06' 17" N, 62° 53' 42" E	9	sediment core	2470	surface[d]
	21° 55' 44" N, 63° 09' 30" E	10	sediment core (20 cmbsf[b])	3003	0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20 cmbsf[b]
Peru Margin (PM)	10° 59' 01" S, 77° 57' 47" W		sediment core (185.6 mbsf[c])	151	0.2, 0.8, 6.2, 6.5, 7.8, 11.4, 25.5, 30.6, 39.5, 41.1, 42.4, 54.7, 65.4, 81.5, 85.6, 87.1, 89.0, 102.1, 157.6, 185.6 mbsf[c]

[a]SPM: suspended particulate matter
[b]cmbsf: centimeters below sea floor
[c]mbsf: meters below sea floor
[d]surface sediment: 0 – 0.5 cmbsf

6.2.2.4 Peru Margin sediments

Slices of the deep core from the PM (ODP Leg 201 – Site 1229) were taken at depths of 0.2, 0.8, 6.2, 6.5, 7.8, 11.4, 25.5, 30.6, 39.5, 41.1, 42.4, 54.7, 65.4, 81.5, 85.6, 87.1, 89.0, 102.1, 157.6, and 185.6 mbsf, and extracted using the modified Bligh-Dyer method as described for the AS SPM. TLEs were stored at -20 °C.

6.2.3 Ladderane fatty acid work-up

Aliquots of the prepared TLEs and the PM sediment residues were saponified by refluxing with aqueous KOH (in 96% MeOH) for 1 h. Fatty acids were obtained by acidifying the saponified samples to a pH of 3 with 1N HCl in MeOH and extracted using DCM. The fatty acids were converted to their corresponding fatty acid methyl esters (FAMEs) by methylation with diazomethane (CH_2N_2). The standard practice of removing excess CH_2N_2 by evaporation under a stream of N_2 was found to contribute to a significant loss of the volatile short-chain ladderane fatty acids. Thus, N_2 was not used to aid evaporation. Polyunsaturated fatty acids (PUFAs) were removed by eluting the sample over a small $AgNO_3$ (5%) impregnated silica column with DCM. Fatty acid fractions were stored at 4 °C until analysis.

6.2.4 HPLC/APCI-MS/MS ladderane lipid analysis

The fatty acid fractions were dissolved in acetone, filtered through 0.45 μm, 4 mm diameter PTFE filters, and analysed by high performance liquid chromatography coupled to positive ion atmospheric pressure chemical ionisation tandem mass spectrometry (HPLC/APCI-MS/MS) in selective reaction monitoring (SRM) mode as originally described in Hopmans et al. (2006), and modified and expanded to include two of the short-chain oxic degradation products (Rush et al., 2011). Four ladderane fatty acids (Fig. 1, **IV – VII**) were used in this study as indicators of the original anammox ladderane lipids from biomass. As the C_{16} ladderane fatty acid with 3 cyclobutane rings (C_{16}-[3]-ladderane fatty acid; Fig. 1, **III**) had been detected previously in minor amounts in an anammox enrichment culture (Rattray et al., 2010), and thus can be produced as such, it was decided to exclude the C_{16}-[3]-ladderane fatty acid SRM transition and include only the C_{14} ladderane fatty acid with 5 cyclobutane rings (C_{14}-[5]-ladderane fatty acid; Fig. 1, **I**) and the C_{14}-[3]-ladderane fatty acid (Fig. 1, **II**) in the SRM method. We assume here that de novo production of C_{14} ladderane fatty acids in anammox bacteria does not occur, or only in minor amounts, as observed in enrichment cultures (Rattray et al., 2010). Ladderane lipids were quantified using external calibration curves of three standards of isolated methylated ladderane fatty acids (C_{14}-[3]-ladderane fatty acid, C_{20}-[3]-ladderane fatty acid, and C_{20}-[5]-ladderane fatty acid) (Rush et al., 2011; Hopmans et al., 2006; Rattray et al., 2008). A detection limit of 30 – 35 pg injected was achieved with this technique. Ladderane fatty acid concentrations in sediment samples were expressed per gram of dry weight sediment (g^{-1}).

6.2.5 NL_5

The NL_5 (i<u>N</u>dex of <u>L</u>adderane lipids with <u>5</u> cyclobutane rings) is based on the observation that the relative length of the ladderane alkyl side-chain adapts to variations in growth temperature of the anammox bacteria in cultures, as well as in the natural environment (Rattray et al., 2010). NL_5 values and corresponding temperatures were calculated according to

the equations of Rattray et al. (2010):

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

and

$$NL_5 = 0.2 + \frac{0.7}{1 + e^{-\left(\frac{\text{Temperature} - 16.3}{1.5}\right)}} \qquad \text{Equation 2}$$

6.3 **RESULTS**

6.3.1 CARIACO BASIN WATER COLUMN

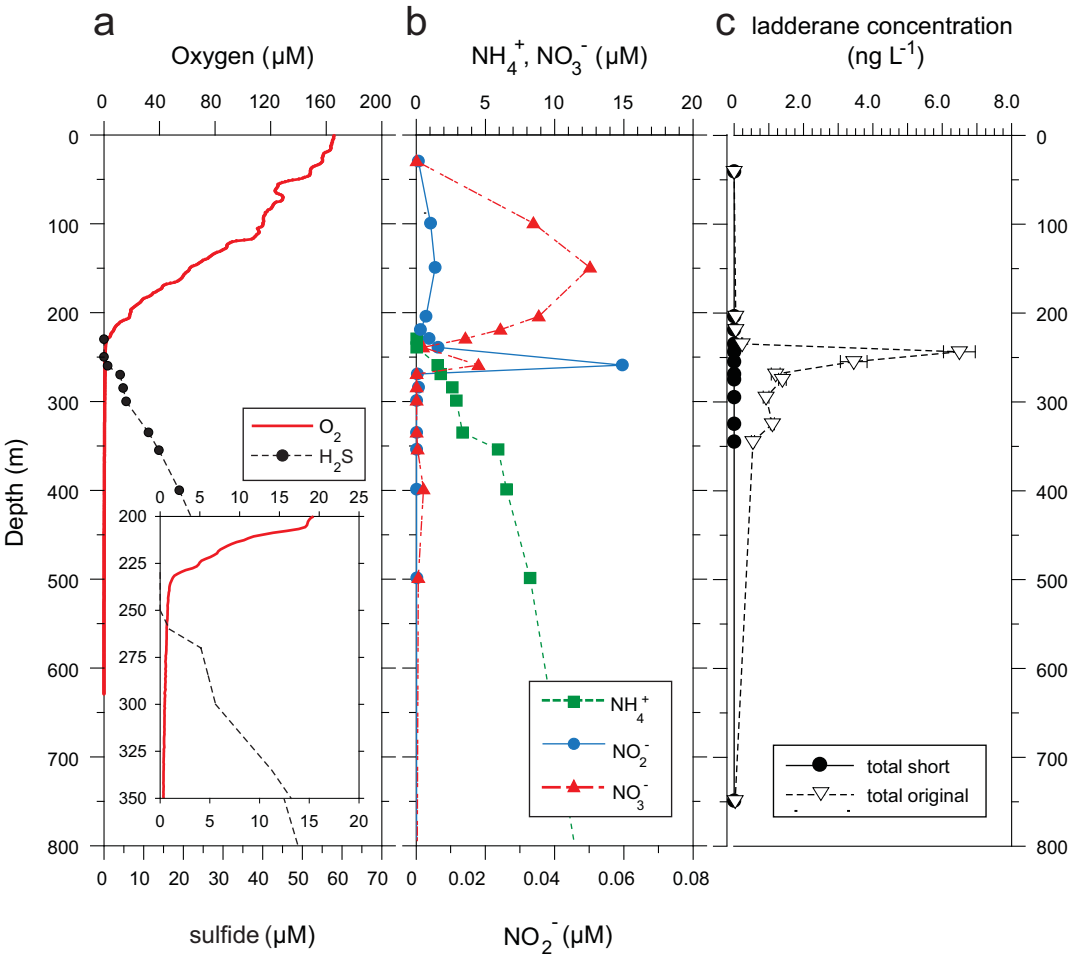

Figure 3: (a) Oxygen (red line), H_2S (black circles), (b) NH_4^+ (green squares), NO_2^- (blue circles), and NO_3^- (red triangles) concentrations obtained during CTD pumping casts at the 'Cariaco Basin time series' site during cruise 139 (CAR139). (c) Concentrations of original (open triangles) and short-chain (filled circles) ladderane fatty acids were obtained from in situ pump filters. Data, except those of short-chain ladderane fatty acids, from Wakeham et al. (2012).

As previous reported by Wakeham et al. (2012), the original C_{18} and C_{20} ladderane fatty acids were not detected within the oxic CB waters, and were only detected in SPM at 205 m water depth (Fig. 3a; dissolved O_2 = 18 μM), and in relatively low concentrations (0.07 ng L^{-1}; Fig. 3c). Concentrations of the unaltered, original ladderanes showed a sharp peak to 8.1 ng L^{-1} at 245 m (dissolved O_2 below detection limit of ~2 μM), which corresponded to a peak in available nutrients (Fig. 3b). Our analyses showed that short-chain ladderane fatty acids were not detected in any CB SPM.

6.3.2 Arabian Sea water column

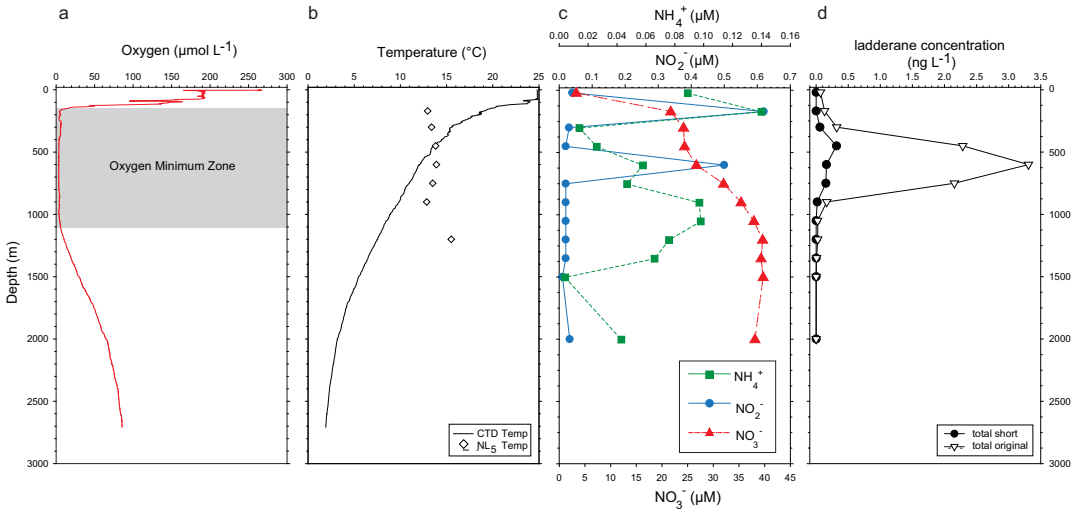

Figure 4: Arabian Sea water column depth profiles of (a) dissolved oxygen concentrations (b) CTD measured temperature and NL_5 temperature (open lozenges) derived from water column particulate matter, (c) NH_4^+ (green squares), NO_2^- (blue circles), and NO_3^- (red triangles) concentrations, and (d) concentrations of ladderane fatty acids (original: open triangles; short-chain: filled circles). Samples were taken during R/V *Pelagia* cruise 64PE301. Oxygen and temperature CTD data are from Pitcher et al., (2011), and original ladderane fatty acid data are from Rush et al. (2012a).

Ladderane lipid analyses of AS SPM (Fig. 4, c and d) showed that the highest concentrations of original ladderane fatty acids (3.3 ng L^{-1}) occurred in the middle of the OMZ, at 600 m water depth (dissolved O_2 = 3 μM; Fig. 4a) (Rush et al., 2012a). Substantial amounts of short-chain ladderane fatty acids were detected at all depths between 300 and 900 m, with a maximum (0.3 ng L^{-1}) occurring at 450 m. They accounted for up to 20% of total ladderane fatty acids within the OMZ (Fig. 4d). NL_5-derived temperature estimates (14 ± 1 °C; Fig. 4b) (Rush et al., 2012a) corresponded to the average temperature measured by CTD (12 ± 3 °C; Fig. 4b) of the OMZ (Pitcher et al., 2011).

6.3.3 ARABIAN SEA SEDIMENTS

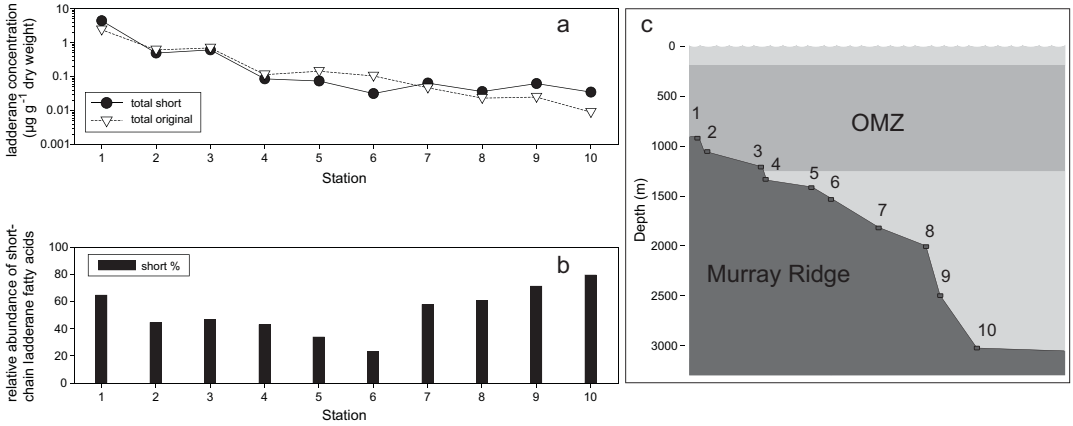

Figure 5: (a) The concentrations (original: open triangles; short-chain: filled circles) of ladderane fatty acids and (b) proportion of short chain ladderane fatty acids in surface sediment along the Murray Ridge (c), stations 1 – 10 in the Arabian Sea collected during R/V *Pelagia* cruise 64PE301. Note the log scale in concentrations.

Analyses of AS surface sediments (0 – 0.5 cmbsf) along the Murray Ridge (Fig. 5a and b) showed that the highest concentrations of both original and short-chain ladderane fatty acids were detected at Station 1 (885 m water depth) at the top of the ridge, within the OMZ. Concentrations of total original ladderane fatty acids were 2.4 µg g^{-1}. Concentrations of original ladderanes fatty acids were one order of magnitude lower at Stations 2 and 3, which are still within the OMZ, but near its base. Concentrations were another order of magnitude lower just below the OMZ at Stations 4 to 6 (1306 to 1495 m water depth), and another order of magnitude lower at Stations 7 to 10 (Fig. 5a and b). Below the AS OMZ, original ladderane fatty acid concentrations varied from 110 to 9 ng g^{-1} for Stations 4 and 10 (3003 m water depth), respectively. Concentrations of short-chain ladderane fatty acids were 4.4 µg g^{-1} at shallowest Station 1 and decreased down the ridge with increasing water depth, with a concentration of 0.04 ng g^{-1} at the deepest station. Beneath the OMZ, the relative proportion of short-chain ladderane fatty acids increased with increasing water depth (Fig. 5b). At the deepest site sampled (Station 10) short-chain ladderanes made up 80% of the total ladderane fatty acid concentration.

Three AS sediment cores along Murray Ridge were also analysed for ladderane fatty acids (Fig. 6). At Station 1, within the OMZ, we observed the highest concentrations of ladderane fatty acids (Fig. 6a). The concentration of original ladderanes at the top (0.5 cmbsf) of this core was 4.9 µg g^{-1} and decreased with depth down to 0.1 µg g^{-1} at 32 cmbsf. Short-chain ladderane concentration at the top of the core was 8.0 µg g^{-1} and fluctuated between a minimum of 4.4 µg g^{-1}, at 6 cmbsf, and a maximum of 10.4 µg g^{-1}, at 9 cmbsf. The short-chain ladderane fatty acids were the most abundant ladderane fatty acids (Fig. 6b), accounting for more than 60% of total ladderanes from 0.5 to 5 cmbsf and >90% at deeper depths. NL_5-derived temperature estimates (Fig. 6c) were constant (14 – 15 °C) down the core.

At Station 4 (Fig. 6, d-f), concentrations of the original ladderane fatty acids peaked at 2 cmbsf (0.58 µg g^{-1}) and decreased steadily there below. Concentrations at 20 cmbsf were 0.02 µg g^{-1}. Total short-chain ladderane fatty acid concentrations decreased from 0.36 µg g^{-1} at

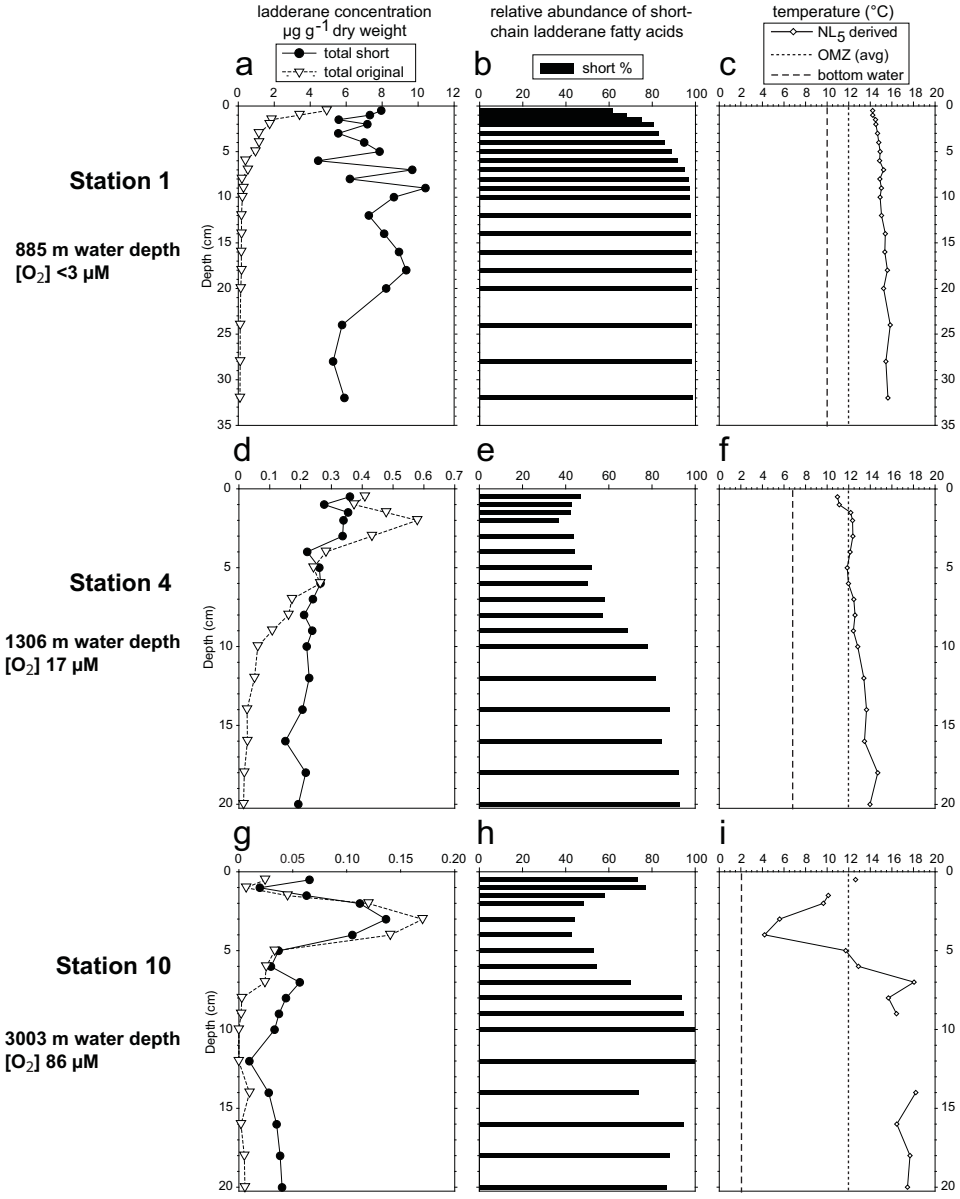

Figure 6: Vertical distributions of concentrations of ladderane fatty acids (original: open triangles; short-chain: filled circles), proportion of short-chain ladderane fatty acids, and temperatures (open lozenges: NL_5 derived temperature; dotted line: oxygen minimum zone average temperature; dashed line: bottom water temperature at station) in Arabian Sea sediment cores. Station 1 (a, b, and c), Station 4 (d, e, and f) and Station 10 (g, h, and i). Samples were taken during R/V *Pelagia* cruise 64PE301.

0.5 cmbsf to 0.19 µg g^{-1} at 20 cmbsf. Below 5 cmbsf, the short-chain ladderane lipids made up more than 50% of total ladderane fatty acids. Temperature estimates from NL_5 at Station 4 increased gradually from 11 °C at the top of the core to 14 °C at 20 cmbsf.

The concentrations of both original ladderane and short-chain ladderane fatty acids at Station 10 showed a peak of 0.17 and 0.14 µg g^{-1}, respectively, at 3 cmbsf (Fig. 6g). No original ladderane fatty acids were detected at 10 or 12 cmbsf. Proportionally, the short-chain

ladderane fatty acids were more abundant below 5 cmbsf (Fig. 6h). NL_5-derived temperatures at this depth horizon dropped from 12.5 °C at 0.5 cmbsf to 4.2 °C at 4 cmbsf (Fig. 6i). The NL_5-derived temperature at 1 cmbsf could not be calculated as no C_{20}-[5]-ladderane fatty acid was detected.

6.3.4 PERU MARGIN SEDIMENTS

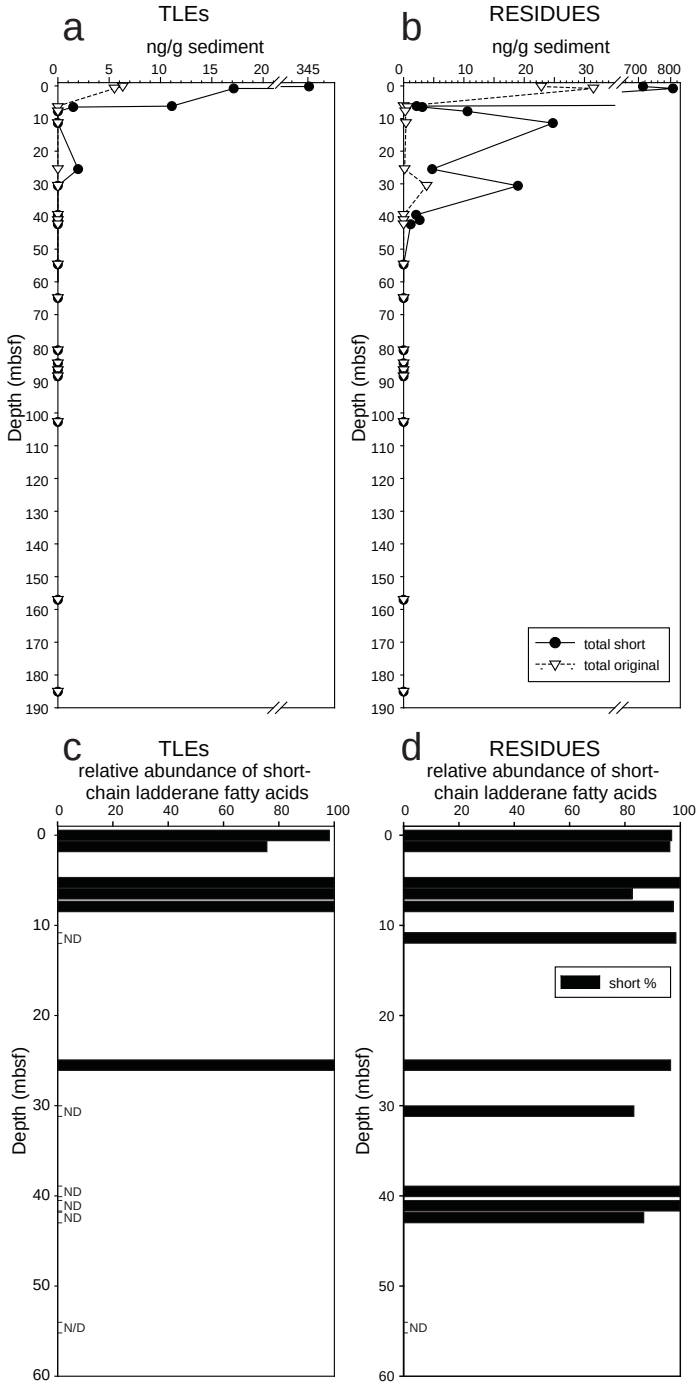

Figure 7: Peru Margin (ODP Leg 201 - Site 1229) depth profile of deep sediment core. Freely extractable ladderane fatty acids (total lipid extracts –TLEs) and matrix-bound ladderane fatty acids (RESIDUES) were analysed. Concentrations of ladderane fatty acids in the TLE (a) and residue (b) fractions (original: open triangles; short-chain: filled circles). No ladderane fatty acids were detected below 42.2 m in either the TLE or the residue fraction. Note breaks in concentration scales for both TLE and residue fractions. Proportion of short-chain ladderane fatty acids in the TLE (c) and residue (d) fractions. ND: Not Detected.

Twenty 1 cm thick sediment slices down to 185.6 meters below sea floor (mbsf) on the PM were analysed, but ladderane fatty acids were only detected down to 25.5 mbsf in the TLEs. Ladderane concentrations showed a maximum (Fig. 7a) at the shallowest sediment (0.2 mbsf), with 6 ng g^{-1} for original ladderane fatty acids and 350 ng g^{-1} for short-chain ladderane fatty acids. Concentrations dropped substantially in the first 5 m, and the deepest sediments in which ladderane fatty acids were detected in the TLEs were 0.8 m and 25.5 mbsf for original ladderanes and short-chain ladderanes, respectively.

Hydrolysis of PM sediment residue left after Bligh-Dyer extraction released substantial amounts of ladderane fatty acids. Matrix-bound ladderane fatty acid concentrations (Fig. 7b; residues) were higher by at least two-fold than freely extractable ladderane fatty acids (Fig. 7a; TLEs). Matrix-bound ladderane fatty acids had maximum concentrations at 0.8 mbsf (original ladderanes: 30 ng g^{-1}; short-chain ladderanes: 800 ng g^{-1}) and were detected down to 42.4 mbsf. At all depths where ladderanes were detected, the short-chain ladderane fatty acids were the most abundant (>75%).

6.4 Discussion

6.4.1 Production of short chain ladderane fatty acids

The production of short-chain ladderane fatty acids is thought to proceed via a microbially mediated β-oxidation pathway (Rush et al., 2011). If so, then the production of these biodegraded compounds in principle requires the presence of molecular oxygen (Beam and Perry, 1974; Dutta and Harayama, 2001; Koma et al., 2003). Indeed, the absence of short-chain ladderane fatty acids in SPM within the anoxic and sulfidic water of the Cariaco Basin supports this. However, short-chain ladderane fatty acids were detected, albeit in low relative amounts (<20%, Fig. 4d), within the oxygen depleted waters in the Arabian Sea (Fig. 4b). This suggests that only low levels of oxygen (<3 μM; Pitcher et al., 2011) are required for the biodegradation pathway to proceed, that the degradation of ladderane fatty acids happens relatively quickly, i.e., already in the water column, but that degradation apparently does not occur in waters devoid of oxygen.

Short-chain ladderane fatty acids occur in all AS surface and deeper sediments in substantial amounts (40 – 100% of total ladderane fatty acids, Figs. 4 and 5). These short-chain ladderane fatty acids could be derived from the sedimentation of those produced in the water column (Fig. 4), from the sedimentary β-oxidation of original ladderane fatty acids produced in the water column but deposited undegraded in the sediments, or of original ladderane fatty acids that were produced in the sediment itself (cf. Jaeschke et al., 2009a; Jaeschke et al., 2010), as anammox has been shown to occur within anoxic sediments (Thamdrup and Dalsgaard, 2002; Brandsma et al., 2011). NL_5 temperatures can be indicative of the temperature at which the anammox bacteria were living when their ladderane lipids were produced (Rattray et al., 2010), although caution must be used when applying this index as it has shown to be not generally applicable (Rush et al., 2012a). Here we use NL_5 only to highlight the differences in the origin of original ladderane fatty acids, i.e., between water column and sedimentary production, rather than apply it for accurate temperature reconstructions. NL_5 derived temperatures from the AS sediment cores (16 ± 2 °C; Figs. 6c, 6f, and 6i) mostly correspond to temperatures within the OMZ water column (average CTD measured temperature of 12 ± 3 °C; Fig. 4b; Pitcher et al., 2011), but not the temperatures of the surface sediments (i.e. AS bottom waters 2 – 10 °C; Fig.

4b). This suggests that most of the original ladderane lipids within the sediment were produced in the water column.

Large differences in ladderane fatty acid concentrations are observed among the AS sediment surface samples (Fig. 5a), with stations below the OMZ having much lower concentrations than those within. This is likely due to the prolonged exposure of original ladderane fatty acids to biodegrading conditions during transport through the oxygenated water column and at the sediment surface. The proportion of short-chain ladderanes increases from no more than 20% in the AS water column (Fig. 4d) to up to 80% at the sediment surface (Fig. 5b). However, the degradation of original ladderanes did not translate into an accumulation of short-chain ladderane fatty acids.

Though most of the original ladderane fatty acids were produced in the water column, indications of benthic production of both original and short-chain ladderane fatty acids are observed at Station 10 (Fig. 6g): a peak in short-chain ladderane fatty acids occurs at the same depth horizon (2 – 5 cmbsf) as a peak in original ladderane fatty acids. As anammox bacteria live in anoxic or low oxygen environments (Thamdrup and Dalsgaard, 2002; Kuypers et al., 2003; Jaeschke et al., 2007; Brandsma et al., 2011), conditions for an active anammox bacterial community are ideal near the oxycline in the sediment. Indeed, the temperatures of ladderane production calculated by NL_5 at this depth horizon (Fig. 6i) decrease substantially down to 4 °C, which correspond to bottom water temperatures of Station 10, indicating in situ produced original ladderanes. The oxygen penetration depth of this core is 2 cmbsf (Lengger et al., 2012), which indicates that oxygen may be available for the β-oxidation pathway near the depth at which ladderane fatty acids are synthesised. This suggests that the short-chain ladderane concentration peak in this core is predominantly the result of oxidation of the in situ produced ladderane fatty acids, as opposed to the burial of short-chain ladderanes degraded in the water column and at the sediment surface. However, the benthic production of ladderanes at 2 – 5 cmbsf did not affect the NL_5 derived temperatures at depths below this interval, possibly because this in situ produced material (both original and short-chain ladderane fatty acids) is more readily degraded than the more resistant, older material that settled from the water column (Middelburg, 1989; Cowie et al., 1992; Keil et al., 1994).

In situ production of original ladderane fatty acids in sediments also appears to have occurred at Station 4, as we observe a peak of original ladderane fatty acids at 2 cmbsf (Fig. 6d). In contrast to Station 10, this peak was not accompanied by a substantial increase in short-chain ladderane fatty acids. In this case, oxygen possibly did not penetrate sufficiently into the sediment core (the oxygen penetration depth is 0.4 cm; Lengger et al., 2012) for the in situ produced original ladderane fatty acids to degrade to short-chain ladderane fatty acids via the β-oxidation pathway. Alternatively, the increase in concentration of original ladderane fatty acids is the result of past fluctuations in the flux of anammox lipids to the sea floor (i.e. resulting from an expansion or an intensification of the OMZ), as no change in NL_5 derived temperatures was observed at this station. This indicates that the original ladderane fatty acids were not produced in the sediment, but rather primarily in the OMZ. Thus, only specific redox conditions, i.e., low enough concentrations of oxygen for anammox to occur and high enough concentrations of oxygen for microbial degradation of ladderane fatty acids to proceed, allow for the biodegradation of in situ produced original ladderane fatty in the sediment. Therefore, short-chain ladderane fatty acids in anoxic sediments are likely fossil.

6.4.2 Burial and preservation of short chain ladderane fatty acids

One of the aims of this study was to determine whether short-chain ladderane fatty acids are better preserved in the sediment record than the original ladderane fatty acids that have been used previously as indicators of past anammox activity (Jaeschke et al., 2009b). In all cores investigated, short-chain ladderane fatty acids made up >75% of total ladderane lipids in sediments deeper than 10 cmbsf (Figs. 6b, 6e, and 6h; Figs. 7c and 7d), which suggests that short-chain ladderane fatty acids are better preserved in sediment than the original ladderane fatty acids. The degradation of original ladderane fatty acids did not, however, result in the accumulation of short-chain ladderane fatty acids. Short-chain ladderane fatty acid concentrations also decreased with depth in all cores, but generally at a much slower rate than the original ladderanes (Figs. 8c and 8d). This indicates that short-chain ladderane lipids are likely further anaerobically transformed though at a slower rate than the long chain ladderane fatty acids. The products of this degradation pathway are unknown, and thus, it is not possible to trace anammox with biomarkers any further.

Lipids adsorbed onto the clay sediment matrix have been shown to be protected from microbial degradation and mineralisation (;Keil et al., 1994; Hedges and Keil, 1995; Hedges et al., 2001; Ding and Henrichs, 2002; Hoefs et al., 2002). Therefore, we also investigated the presence of the freely extractable and of the matrix-bound (residue) ladderane fatty acids in a long core from the Peru Margin (Fig. 7). Indeed, matrix-bound ladderane fatty acids were detected down to 42.4 mbsf but freely extractable ladderane fatty acids only down to 25.5 mbsf (Figs. 7, a-b). This difference highlights the potential for a degree of protection by the sediment matrix, perhaps by making lipids less susceptible to microbial attack. Analyses of matrix-bound lipids could, therefore, extend the detection depth of ladderane fatty acids in the sediment record. However, more deeply buried sediments did not contain any matrix-bound ladderane fatty acids, suggesting that ultimately they are lost through diagenesis or that anammox activity was negligible at the time of deposition. Likely, fossil short-chain ladderane fatty acids are too labile to be used as markers for ancient anammox in sediments older than the late Quaternary, as we show below. Thermal degradation products of ladderane lipids (see Jaeschke et al., 2008) might be more useful biomarkers in older sediments.

Using age models available for the three AS cores (Lengger et al., 2012), we can plot the relative concentrations of original and short-chain ladderane fatty acids versus relative time (Figs. 8, a and b, respectively), in which relative time is the age of the sample relative to the age of maximum original ladderane fatty acid concentration (t_0) (Station 1: 0.04 ka, 0.5 cmbsf; Station 4: 0.53 ka, 2 cmbsf; Station 10: 0.36 ka, 3 cmbsf), and relative concentration is defined as the ladderane fatty acid concentration compared to that at t_0. A strong logarithmic decrease in concentration with increasing time is observed, especially for the original ladderane fatty acids. A weaker, but still significant, decrease in relative concentration was observed for the short-chain ladderane fatty acids at two stations (Stations 4 and 10; Fig. 8b). The decrease is not apparent for Station 1, where the concentration of short-chain ladderane fatty acids was variable throughout the core and no significant decrease with depth was observed.

Previous studies of organic matter (OM) degradation (Middelburg, 1989; Canuel and Martens, 1996), have shown that sedimentary degradation of OM does not have a constant reactivity, and the first-order degradation constant (k) decreases with time. OM is considered to have several refractory and more labile portions when using a first-order type degradation model (Middelburg, 1989). This can be visualised by calculating k according to Equation (3)

(Canuel and Martens, 1996),

$$k = \frac{-\ln\left(\frac{C_t}{C_{t0}}\right)}{t} \qquad \text{Equation 3}$$

where t is relative time, C_t is the concentration at time t and C_{t0} is the concentration at initial time t_0. If log k is plotted against log relative time, strong correlations in the original (Fig. 8c; $R^2 = 0.40 – 0.93$) as well as the short-chain (Fig. 8d; $R^2 = 0.60$ and 0.80) ladderane fatty acids are observed. This indicates that, as with sedimentary OM and other sedimentary organic compounds, the rate of degradation strongly decreases with time (Canuel and Martens, 1996; Middelburg, 1989; cf. Janssen, 1984). The slopes of the lines of best fit were in the same order of magnitude for the original and the short-chain ladderane fatty acids, indicating that the *decrease* in reactivity follows the same trend for both classes of ladderane fatty acids. However, the intercept is lower for the short-chain ladderane fatty acids (-0.46 and 0.14) compared to the original ladderane fatty acids (0 – 0.51), indicating that the latter are initially degraded faster than the short-chain ladderane fatty acids.

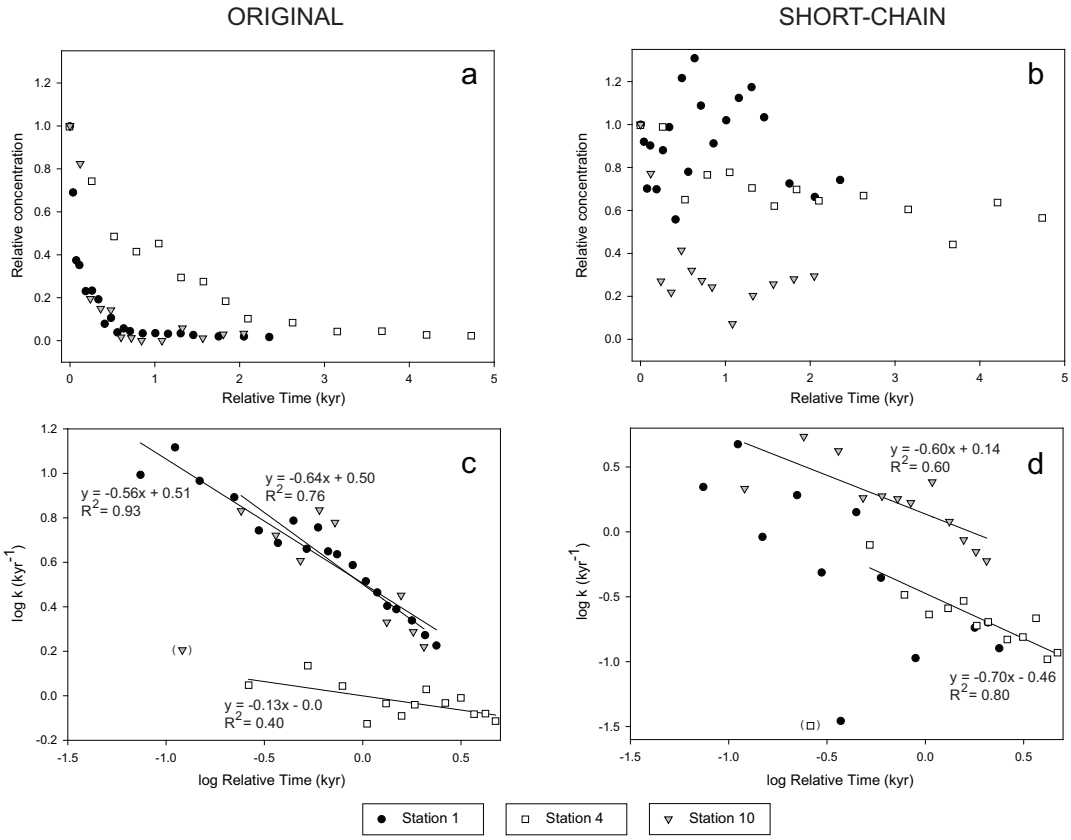

Figure 8: Plots demonstrating the degradation of original and short-chain ladderane fatty acids in three Arabian Sea sediment cores. Relative concentration (where $C_i = 1$) versus relative time for (a) original ladderane fatty acids and (b) short-chain ladderane fatty acids. Degradation was assumed to start from the depth of maximum original ladderane fatty acid concentrations. Correlation plot of log k versus log relative time for (c) original ladderane fatty acids and (d) short-chain ladderane fatty acids. Station 1: filled circles; Station 4: open squares; Station 10: grey triangles.

Our data can potentially be used to determine at what age original and short-chain ladderane fatty acid concentrations drop below detection limit (~200 pg g^{-1}) in the Arabian Sea sedimentary record, based on the correlation between log *k* vs. log time (Figs. 8c and 8d). For original ladderane fatty acids, the time to reach the detection limit ranged from 8 – 13 kyr, less than the results of Jaeschke et al. (2009b), who still detected original ladderane fatty acids in 140 ka old AS sediments. However, the time to reach the detection limit is dependent on the initial concentrations, and therefore, for other sediment cores the time when concentrations drop below detection limit may be different. Nevertheless, given that the surface concentrations of original ladderane fatty acids in the AS sediments are relatively high, 1 – 2 orders of magnitude higher than sediments from the African shelf (2 – 60 ng g^{-1}; Jaeschke et al., 2010) and the Irish Sea (0 – 20 ng g^{-1}; Jaeschke et al., 2009a), we can assume that for most sediment records, original ladderane fatty acids may rarely be detected in sediments >10 kyr. The detection limit is reached much later for the short-chain ladderane fatty acids (50 to 10^4 kyr), suggesting that they degrade much slower than the original ladderane fatty acids. Indeed, in the PM core we detected short chain ladderane fatty acids to be more abundant at greater depths (Fig. 7c and d). However, in the PM core at depths >42.4 mbsf, neither short chain nor original ladderane fatty acids could be detected. This depth corresponds to a time boundary in the late Pleistocene (Hart and Miller, 2006), and tentatively suggests that short-chain ladderane fatty acids will, like the original ladderane fatty acids, not be preserved beyond the Quaternary.

6.5 CONCLUSIONS

Short-chain ladderane fatty acids formed by β-oxidation of original ladderane fatty acids are ubiquitous in oxygen minimum zones. Short-chain ladderane fatty acids are dominantly formed in oxygen deficient water columns, but not anoxic waters, and are further oxidised while settling through the water column and later at the sediment surface. In the sediment record, short-chain biodegradation products are generally more abundant and degrade slower than the original four ladderane fatty acids used previously as biomarkers for past anammox activity. Matrix-bound short-chain ladderane fatty acids were detected at greater sediment depths than those that were freely extractable, and, though the biodegraded ladderanes, like the original ladderanes, are degraded in the sediment, the rate of degradation seems to be lower. In future work related to detecting past anammox activity, we propose that the residues containing matrix-bound ladderane fatty acids should also be analysed. We suggest that short-chain ladderane fatty acids be considered as an alternative biomarkers for past anammox activity in late Quaternary sediments from below oxygen minimum zones.

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Chapter 7

Generation of thermally stable aliphatic hydrocarbons by hydrous pyrolysis of anammox biomass and the evaluation of their biomarker potential

This chapter is based on
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Anammox, the microbial anaerobic oxidation of ammonium by nitrite to produce dinitrogen gas, has been recognised as a key process in the marine, freshwater and soil nitrogen cycles, and found to be a major sink for fixed inorganic nitrogen in the oceans. Ladderane lipids are unique anammox bacterial membrane lipids that have been used as biomarkers for anammox bacteria in recent and past environmental settings. However, the fate of ladderane lipids during diagenesis and early catagenesis is not yet well constrained. In this study, hydrous pyrolysis experiments were performed on anammox biomass, and aliphatic hydrocarbons, present in oils generated at temperatures 220 – 365 °C, were analysed. Two unique classes of lipids were detected, and a representative homologue of each class was isolated and rigorously identified using 2D-NMR. One class consisted of C_{19} and C_{20} alkyl branched tricyclododecanes. The C_{20} product was likely derived from ether cleavage and ring-opening of the C_{20}-[3]- or [5]-ladderane glycerol ether, whereas the C_{19} homologue is probably formed from the ring-opening and decarboxylation of C_{20} ladderane fatty acids. The other class consisted of C_{28} to C_{30} branched alkanes with two internal ethyl, or propyl groups. Both classes were generated at hydrous pyrolysis temperatures above 260 °C, with maximum generation occurring between 320 and 335 °C, indicating that they are thermally mature aliphatic products of anammox lipids. Stable carbon isotopic values of these products were depleted in ^{13}C, similar to carbon isotope values observed in the original unheated anammox lipids. A range of sediments from geological periods where anammox is believed to have been an important process were screened for the presence of these catagenetic products. However, these lipids were not detected. This is likely because the concentrations of these products were too low to be detected, as anammox biomass does not account for a significant proportion of microbial biomass in the environment. Alternatively, the sediments screened might have been too immature for these products to have been generated. Thirdly, the artificially produced thermally mature products of anammox lipids may not reflect the natural diagenetic and catagenetic products of ladderane lipids.

7.1 INTRODUCTION

Anaerobic ammonium oxidation (anammox) is an important process in the marine nitrogen cycle, contributing up to 50% of fixed nitrogen loss from marine environments (Kuypers et al., 2005; Hamersley et al., 2007). Anammox is the oxidation of ammonium with nitrite as the electron acceptor (Mulder et al., 1995; Strous et al., 1999; Jetten et al., 2009; Jetten et al., 2010). Anammox bacteria are responsible for this reaction, which takes place in a specialised oraganelle-like structure, the anammoxosome (van Niftrik et al., 2008a; van Niftrik et al., 2008b). This compartment is enclosed by specific membrane lipids made of linearly concatenated cyclobutane rings (Sinninghe Damsté et al., 2002a). These lipids are unique to the anammox bacteria and have been used previously as biomarkers for the presence of the anammox reaction in the natural environment (Fig. 1, **I**-**V**; Kuypers et al., 2003; Jaeschke et al., 2009a; Jaeschke et al., 2010; Wakeham et al., 2012). However, ladderane fatty acids are transformed relatively quickly during sediment burial as evident from sediment records (Jaeschke et al., 2009b; Rush et al., 2012b), as well as artificial maturation experiments (Jaeschke et al., 2008; Rush et al., 2011). This is probably due to their cyclobutane ring structures, which are highly strained, and are likely susceptible to structural rearrangements during diagenesis and catagenesis.

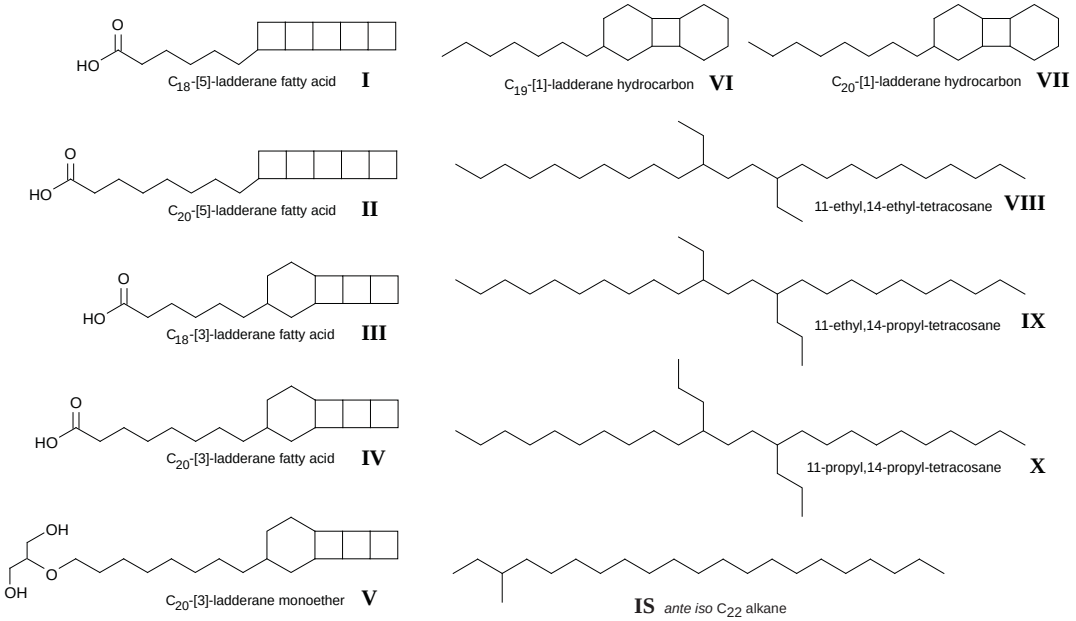


Figure 1. Chemical structures of anammox lipids and their supposed thermal maturation products. (I) C_{18}-[5]-ladderane fatty acid, (II) C_{20}-[5]-ladderane fatty acid, (III) C_{18}-[3]-ladderane fatty acid, (IV) C_{20}-[3]-ladderane fatty acid, (V) C_{20}-[3]-ladderane monoether, (VI) 4-heptyl-tricyclo[6.4.0.0.^{2,7}]dodecane, (VII) 4-octyl-tricyclo[6.4.0.0.^{2,7}]dodecane, (VIII) 11-ethyl,14-ethyl-tetracosane, (IX) 11-ethyl,14-propyl-tetracosane, and (X) 11-propyl,14-propyl-tetracosane. IS: internal standard, *ante iso* C_{22} alkane.

Lipids undergo different structural alterations during sedimentary processes (i.e. hydration, sulfurisation, oxidation, condensation; Gaskell and Eglinton, 1975; Sinninghe Damsté et al., 1989; Sinninghe Damsté et al., 2002c; de Leeuw et al., 2006). Artificial maturation

processes such as hydrous pyrolysis have been shown to simulate the generation of crude oil from organic-rich shales by heating them submerged in water in a closed reactor over a limited time period (up to one week) at temperatures in the range of 280 – 370 °C (Lewan et al., 1979; Lewan, 1993). In addition to simulating catagenesis experiments, hydrous pyrolysis has been used previously to study the maturation processes of organic biomarkers (e.g. Koopmans et al., 1996b; Schouten et al., 2004) using temperatures <280 °C. Jaeschke et al. (2008) used hydrous pyrolysis experiments performed on anammox biomass to show that ladderane lipids undergo structural modification during hydrous pyrolysis already at low temperatures (120 °C). The cyclobutane rings of ladderane fatty acids and a ladderane monoether were shown to open, with concomitant formation of double bonds, forming several thermally more stable components with condensed cyclohexenyl groups, a process that has been observed already on ladderane lipids during Gas Chromatography (GC; Sinninghe Damsté et al., 2005). Here, we investigate the potential production of additional thermally stable biomarkers of anammox bacteria, by examining the aliphatic hydrocarbons present in the oils generated during these hydrous pyrolysis experiments. These components would likely be more stable and comprise more suitable biomarkers for past anammox activity.

7.2 **MATERIALS AND METHODS**

7.2.1 HYDROUS PYROLYSIS EXPERIMENT

The conditions of the hydrous pyrolysis experiments were previously described in Jaeschke et al. (2008). Briefly, 75 g of anammox cell biomass (the anammox species "*Candidatus* Kuenenia stuttgartiensis" made up to 70% of the bacterial population from the sludge of a waste water treatment plant (Dokhaven, Rotterdam, the Netherlands); obtained from Paques B.V. (Balk, the Netherlands)), and 500 g of distilled water were filled into carburised 1 L Hastelloy-C276 reactors. Reactor contents were then artificially matured at temperatures which were kept constant between 120 and 365°C. After 72 h, the experiments were stopped and cooled to room temperature within 24 h. The oils which were generated at temperatures between 200 and 365 °C were recovered either from the water surface with a pipette, or from the reactor walls by rinsing with benzene.

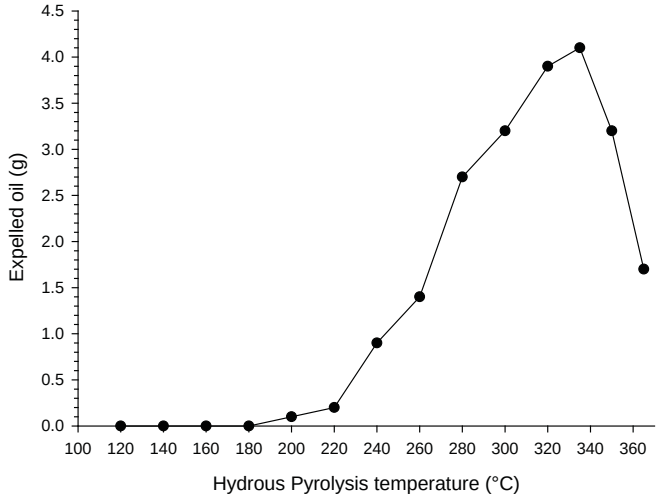


Figure 2. Amount of oil generated as a function of the hydrous pyrolysis temperature.

7.2.2 EXTRACTION AND FRACTIONATION

Aliquots (3 – 10 mg) of the expelled oils were separated over small Al_2O_3 columns with hexane, hexane:dichloromethane (DCM) (9:1; v/v), and DCM:methanol (1:1; v/v). Saturated components were isolated from the hexane fractions by eluting them over $AgNO_3$ impregnated silica columns using hexane as eluent. A known amount of internal standard (*ante iso* C_{22} alkane) was added to the hexane fractions for GC quantification, and the aliquots were air dried.

7.2.3 GC AND GC-IRMS ANALYSIS

GC analysis was performed by a Hewlett-Packard (HP) 6890 instrument equipped with an on-column injector and a flame ionisation detector (FID). Helium was used as the carrier gas through a fused silica capillary column (25 m × 0.32 mm) coated with CP Sil-5 (0.12 μm film thickness). Samples were injected at 70 °C and the GC oven temperature was subsequently raised to 130 °C at a rate of 20 °C min^{-1}, and then at 4 °C min^{-1} to 320 °C, which was held for 15 min. GC/MS analysis was carried out using a Finnigan Trace GC Ultra, Thermo Electron Corporation, interfaced with a Finnigan Trace DSQ mass spectrometer, with a mass range of m/z 40 – 800. GC conditions for GC/Mass Spectrometry (MS) were the same as those described above for GC. The molecular weight of a branched long-chain alkane was determined by Chemical Ionisation (CI)-MS on a HP 5973 mass spectrometer.

Compound-specific carbon isotope ($\delta^{13}C$) analyses were carried out using a ThermoFinnigan DELTA-V irm-GC/MS system. The column, carrier gas, and temperature program conditions were the same as described above for GC analyses. The $\delta^{13}C$ values for individual components are reported in the standard delta per mil (‰) notation against the Vienna Pee Dee Belemnite standard (Table 2).

7.2.4 ISOLATION OF ALKYL-TRICYCLODODECANE AND BRANCHED LONG-CHAIN ALKANE

Two components were isolated using preparative GC (prep-GC), in order to rigorously identify them using two-dimensional nuclear magnetic resonance (NMR). First, large aliquots (2 – 3 g) of expelled oils from the 280 and 300 °C hydrous pyrolysis experiments were fractionated over large (25 cm × 2 cm) Al_2O_3 columns with hexane, hexane:dichloromethane (DCM) (9:1; v/v), and DCM:methanol (1:1; v/v). The hexane fractions were further cleaned up over large $AgNO_3$-impregnated silica columns. The obtained saturated hydrocarbon fractions were combined and used for the isolation of these two components, performed on an HP 6890 GC coupled to a Gerstel Preparative Collector system. Five μL of the purified hexane fractions was injected at 70 °C by a HP 7683 series autoinjector, onto a CP-Sil 5 column (25 m × 32 mm, 0.52 μm film thickness). The GC temperature program was run to 130 °C at 20 °C min^{-1}, followed to 320 °C at 4 °C min^{-1}, which was held for 10 min. A small fraction of the effluent was diverted to a flame ionisation detector (FID), while approximately 99% of the effluent was sent to the preparative system. The contents of the fraction traps, cooled to 10 °C, were collected using dichloromethane. Over a thousand injections were performed to trap sufficient material. Sample purity was determined by GC-FID.

7.2.5 ^{1}H AND ^{13}C NMR SPECTROSCOPY

The two components isolated using prep-GC were dissolved and dried in (2×) DCM, (2×) $CHCl_3$, (2×) tetrachloroethane, and (2×) $CDCl_3$, then transferred to NMR tubes using 0.75

mL $CDCl_3$. ^1H and ^{13}C NMR analyses were performed on a Bruker DMX-600 spectrometer equipped with TCI cryoProbe and on a Bruker AV-750 with a 5 mm triple TXI-zGRAD probe, both at 298 K, as previously reported by Sinninghe Damsté et al. (2005).

7.3 **Results and Discussion**

Anammox biomass was artificially matured using hydrous pyrolysis in order to examine the effects of diagenesis and catagenesis on anammox biomarker lipids (Jaeschke et al., 2008). Oils were generated at maturation temperatures from 200 to 365 °C (Fig. 2, Table 1), which were then analysed for aliphatic hydrocarbons, potentially thermally stable biomarker lipids of anammox bacteria. GC analyses of the aliphatic fractions of these oils (e.g. Fig. 3) showed that they contained C_{29} – C_{35} hopanes and hopenes, as well as branched- and straight-chain C_{15} – C_{17} alkanes, which might be the catagenetic products of 9-methylhexadecanoic acid, 10-methylhexadecanoic acid, and 9,14-dimethylkexadecanoic acid acids present in anammox biomass (Sinninghe Damsté et al., 2005).

Table 1. Absolute amount of recovered oils, and relative amounts of selected components in the artificially matured anammox biomass. **VI** – **XII** correspond to structures in Figure 1.

		Hydrous Pyrolysis Temperature (°C)									
		200	220	240	260	280	300	320	335	350	365
Weight expelled oil (g)		0.1	0.2	0.9	1.4	2.7	3.2	3.9	4.1	3.2	1.7
Lipid yield (µg component/ gram original biomass)											
Ladderane	**VI**	-[a]	-	-	-	-	6	13	16	14	8
hydrocarbons	**VII**	-	<1	-	<1	4	25	49	48	21	10
branched	**VIII**	-	-	-	-	78	29	54	45	21	9
long-chain	**IX**	-	-	-	<1	24	93	165	134	66	25
alkanes	**X**	-	-	-	-	5	16	27	20	9	3

[a]Not detected

At temperatures >260 °C, two clusters of component classes with unknown mass spectra were evident, one eluting between the C_{19} and C_{21} *n*-alkanes and another between the C_{26} and C_{28} *n*-alkanes (Fig. 4; Table 1). Neither of these lipid classes was detected in the original anammox biomass (Jaeschke et al., 2008), indicating that they are products formed upon thermal maturation. Furthermore, isotopic analysis show that they are ^{13}C depleted (-40.0 – -49.4‰; Table 2), similar to the original unheated, anammox lipids (~-48‰; Table 2) suggesting they are derived from anammox lipids. The most abundant homologue from each class was isolated using prep-GC in order to fully elucidate their structures by NMR and aid in identifying their homologues using mass spectral interpretation. An earlier-eluting class, containing components with mass fragments indicative of ladderane origin (m/z 161), eluted between the C_{15} and C_{17} *n*-alkanes, but these components were too low in abundance to be able to identify them definitively with MS or isolate them for structural elucidation by NMR experiments.

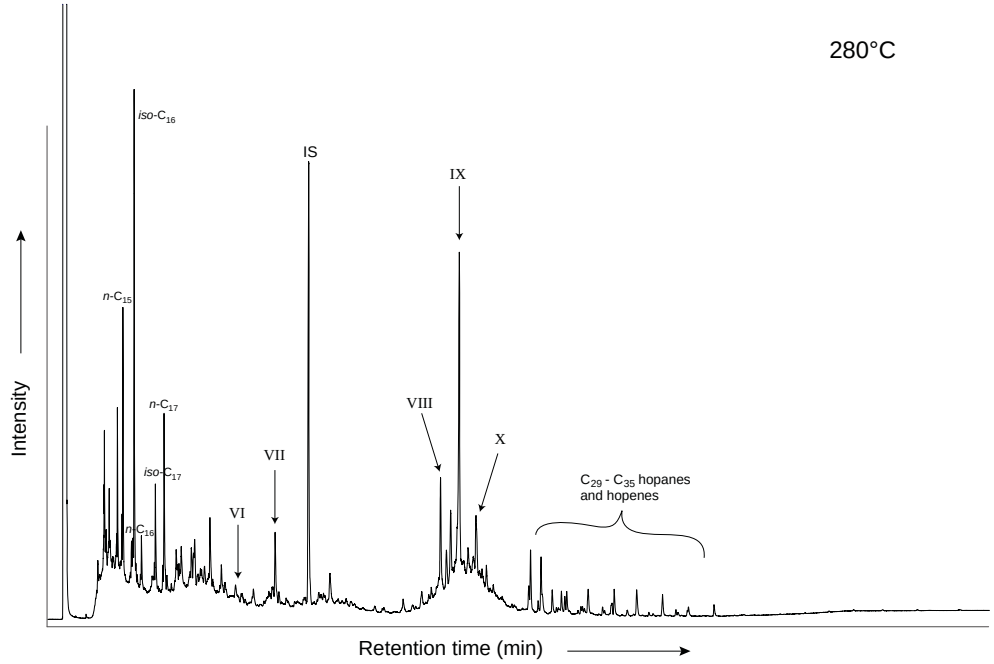


Figure 3. Gas chromatogram of the aliphatic (hexane) fraction of the oil expelled during the 280 °C hydrous pyrolysis experiment. Numbers correspond to structures in Figure 1. IS denotes internal standard (*ante iso* C_{22} alkane).

7.3.1 IDENTIFICATION OF ALKYL-BRANCHED TRICYCLODODECANES

7.3.1.1 THE ISOLATED TRICYCLODODECANE

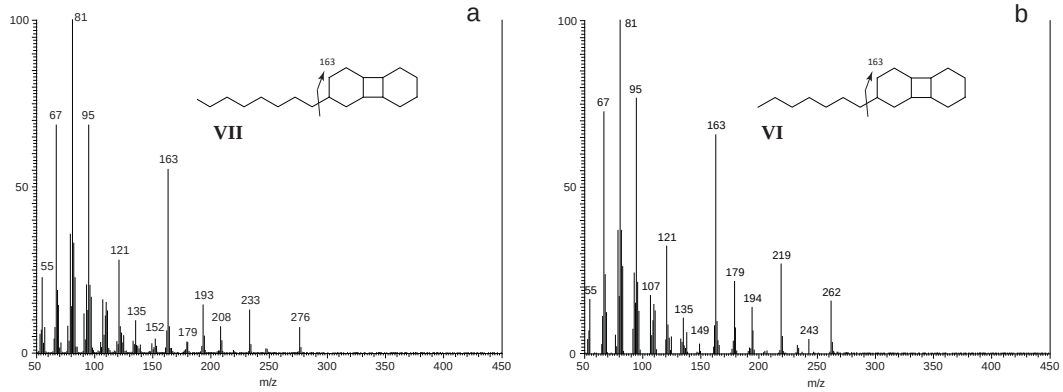


Figure 4. Mass spectra (corrected for background) of (a) **VII**, and (b) **VI** with tentative corresponding structures. Mass spectrum of (a) is from the isolated C_{20}-[1]-ladderane hydrocarbon. The spectrum for **VI** is from the saturated aliphatic fraction of the oil generated at 320 °C.

The most abundant homologue of the first cluster of hydrocarbons showed a major fragment ion at m/z 163 in its mass spectrum (**VII**; Fig. 4a), reminiscent of mass spectra of thermal degradation products of ladderane fatty acids (Sinninghe Damsté et al., 2005). The Kováts Retention Index (RI; Kováts, 1961) of this component was 2071. The molecular ion of this hydrocarbon was m/z 276, suggesting it is a C_{20} alkane with three double bond equivalents ($C_{20}H_{36}$). Since this component was present in the saturated hydrocarbon fraction isolated

by chromatography on a $AgNO_3$ impregnated silica column, it most likely does not contain double bonds. This component was isolated using more than 1000 injections on a prep-GC, which resulted in 0.6 mg of **VII** (84% pure, as determined by GC).

Table 2. Stable carbon isotopic composition and retention indices of selected lipids in the original anammox biomass and in the oils generated from the hydrous pyrolysis of anammox biomass (saturated aliphatic fraction of the 280°C hydrous pyrolysis experiment). Unheated and 200 °C anammox lipid data from Jaeschke et al., (2008). Roman numerals correspond to structures in Figure 1.

Lipid	$\delta^{13}C$ (‰ vs VPDB)			Retention Index (Kováts Factor)
	Unheated	200 °C	280 °C	
***iso*-C_{16}**	-50.9	-49.4		
C_{16}:0	-41.7	-42.0		
10-methyl-hexadecanoic acid	-44.8	-43.3		
IV	-48.9	-45.1		
V	-48.7	-45.4		
VII			-49.4 ± 0.3	2071
VIII			-47.1 ± 0.1	2600
IX			-41.8 ± 0.2	2665
X			-40.0 ± 0.3	2731

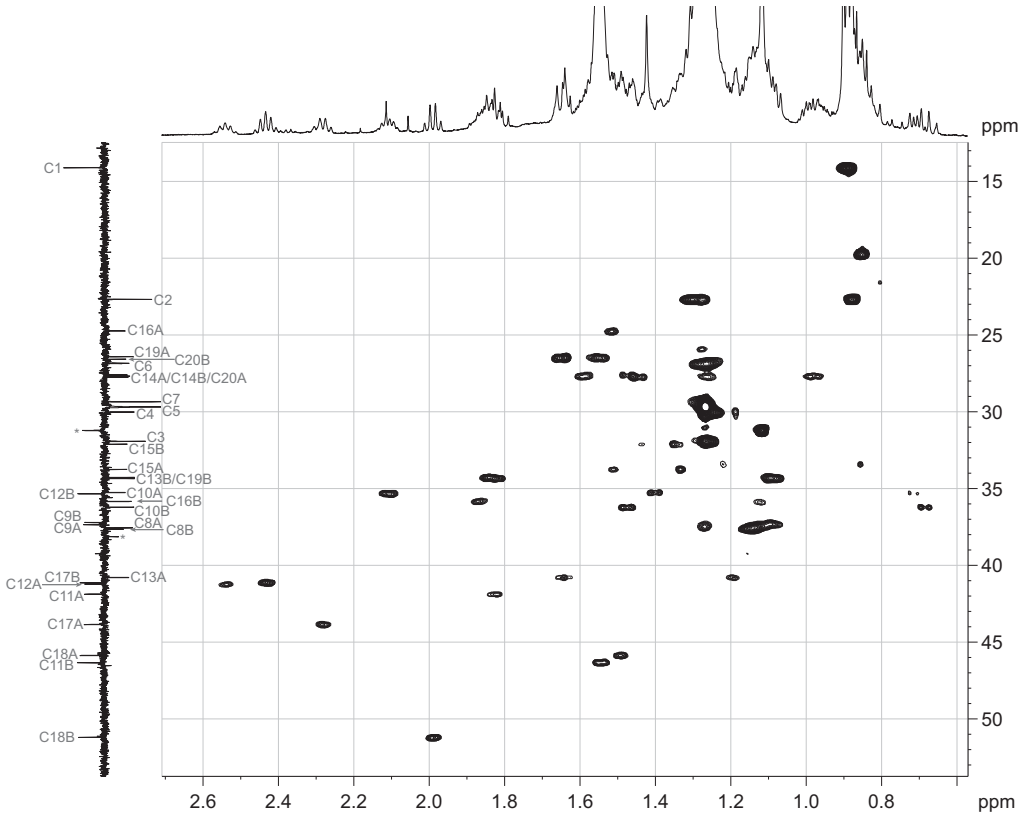
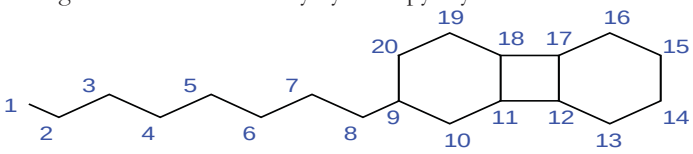


Figure 5. Heteronuclear single-quantum correlation spectroscopy (HSQC) spectrum for isolated component **VII**. Carbon atoms are numbered according to the assignments in Table 3. * denotes impurity.

Table 3. Proton and Carbon NMR data for the two isomers (A and B) of components **VII** formed during artificial maturation by hydrous pyrolysis.



C-number[a]	Tentative Stereo-chemistry	Proton Shift (ppm)	Carbon shift (ppm)[a]			2D NMR correlations	
			Primary	Secondary	Tertiary	COSY	HMBC
Alkyl chain							
1		0.86	14.1			H2	H2, H3
2		1.28		22.7		H1	H3
3		1.25		31.9			H1, H2
4		1.17		30.0			
5		1.24		29.7			
6		1.27		26.9			
7		1.24		29.4			
8A		1.28		37.6			
8B		1.16		37.7			
Ring A							
9		1.10			37.4	H10αβ	H10
10	α	1.40		35.3		H9, H10β, H11	H9, H11, H13α, H18(w)
	β	0.72 (dd)				H9, H10α, H11, H19	H11, H18(w)
11		1.82 (d)			41.9	H10αβ, H18	H10αβ, H13αβ, H17, H19
12		2.55 (quint)			41.2	H13αβ, H16, H17	H13αβ, H16
13	α	1.64 (t)		40.8		H12, H13β, H14(w)	H10, H11, H12, H17
	β	1.20				H12, H13	H11, H12
14		1.46		27.8		H15α	H15
15	α	1.51		33.8		H14, H15β	H14
	β	1.34				H15α	
16		1.51		24.7		H12	H12, H17, H18
17		2.30 (dd)			43.8	H12, H18	H11, H13α, H16
18		1.49			45.9	H11, H17	H16, H10αβ (w)
19		1.56		26.4		H10β	H11, H20α
20	α	1.57		27.7			H19
	β	1.23					
Ring B							
9		1.10			37.2	H10β	H10α (w)
10	α	1.47 (dd, J = 12.5Hz)		36.2		H10β, H11	H9(w), H11, H12, H18
	β	0.67(dd)				H9, H10α, H11	H11
11		1.54			46.4	H10αβ	H10αβ, H12
12		2.11			35.4	H13αβ, H14(w), H16(w), H19α(w)	H10α, H11, H13α(w), H14, H17, H18

Table 3. continued.

C-number[a]	Tentative Stereo-chemistry	Proton Shift (ppm)	Carbon shift (ppm)[a]			2D NMR correlations	
			Primary	Secondary	Tertiary	COSY	HMBC
Ring B (con't)							
13	α	1.83 (d)		34.2		H12(w), H13β	H18(w)
	β	1.10				H12, H13α, H14	H17, H18(w)
14		1.57		27.6		H12, H13β	H12, H17
15	α	1.45		32.1		H15β	
	β	1.36				H15α	
16	α	1.85		35.9		H16β, H17	H13α, H18(w), H19
	β	1.13				H12(w), H16α, H17	H18(w), H19
17		2.45 (quint)			41.1	H16αβ, H18	H12, H13αβ, H14, H19
18		1.98 (quart)			51.2	H17, H19αβ	H10α, H12, H13αβ(w), H16αβ(w)
19	α	1.84		34.3		H12(w), H18, H19β	H16, H17
	β	1.12				H18, H19α	
20	α	1.66		26.6			
	β	1.55					

[a]carbon-proton connections were established by an HSQC experiment.

The ^{1}H-NMR spectrum of **VII** was complex, but showed distinct signals in the 2.0 – 2.6 ppm range, probably representing the protons of a cyclobutane system (cf. Sinninghe Damsté et al., 2002a). In addition, the spectrum revealed two high-field double doublets at 0.67 and 0.72 ppm (Table 3), possibly representing two axial protons in cyclohexane ring structures.

None of the shifts observed in the APT (attached proton test) ^{13}C-NMR spectrum were indicative of double bond carbons, suggesting the absence of double bonds in **VII**. The APT spectrum revealed 32 distinct carbon shifts (Table 3), one primary carbon, ten tertiary, and twenty-one secondary carbons. The molecular composition of this component (see above) indicated a C_{20} molecule, which seems to be inconsistent with the number of carbons in the ^{13}C NMR data. However, the peak intensities of the primary carbon at 14.1 ppm, and some of the secondary signals, were substantially enhanced relative to all other carbon signals. Most of these secondary carbons could be assigned as carbons in an aliphatic chain (Table 3). This tentatively suggests that the isolate contained two co-eluting isomers with C_8 *n*-alkyl side-chains showing identical carbon shifts, attached to non-equivalent C_{12} ring moieties, which would in total make up for thirty-two distinct carbon shifts.

Ten tertiary carbon atoms were apparent; two of these could be assigned to the tertiary carbon in a cyclohexane moiety bearing the C_8 alkyl side-chain (Table 3), as previously assigned in various ladderane lipid structures (cf. Sinninghe Damsté et al., 2002a; 2005). The heteronuclear single quantum coherence spectroscopy (HSQC) experiment (Fig. 5) revealed correlations of the remaining eight tertiary carbon atoms at 35.4 – 51.2 ppm, with proton shifts at 1.49 to 2.55 ppm (six of these were above 1.8 ppm; Table 3), probably representing the carbon shifts of the carbon atoms of two distinct cyclobutane moieties. These assignments were confirmed by connectivities observed in 2D-NMR (correlation spectroscopy (COSY) and heteronuclear multiple-bond correlation spectroscopy (HMBC)) experiments (Table 3). Thus, the configuration of the tricyclic moieties must be such that the cyclobutanes are condensed

to two cyclohexyl moieties, resulting in a 4-octyl-tricyclo[6.4.0.0.^{2,7}]dodecane, or, briefly, a C_{20}-[1]-ladderane hydrocarbon (**VII**; Fig. 1). This assignment is consistent with the ^{13}C shifts of the remaining carbon atoms, and the NMR data of their attached protons (Table 3). The differences in the NMR data of the isomer tricyclododecane moieties are probably due to their different stereochemistries. It is known that the stereoisomers of this type of ring system (tricyclo[6.4.0.0.^{2,7}]dodecane) result in distinctly different shifts in their carbon atoms (Salomon et al., 1974). It, however, it remains enigmatic as to why these two stereoisomers elute as a single GC peak.

7.3.1.2 Mass spectral characteristics of tricyclododecane homologues

A related homologue **(VI**; Fig. 4b; $M^+ = m/z$ 262) eluted earlier (RI = 1943) than the partially NMR-identified C_{20}-[1]-ladderane hydrocarbon, and shared similar fragment ions (m/z 81, 95, and 163), which represented the fragmentations of the tricyclododecane group. It was, therefore, identified as 4-heptyl-tricyclo[6.4.0.0.^{2,7}]dodecane, or briefly a C_{19}-[1]-ladderane hydrocarbon (**VI**; Fig. 1). C_{18} homologues of these lipids might have been present in the hydrous pyrolysis experiment performed at 320 and 335 °C, but their abundances were too low to establish conclusive identifications.

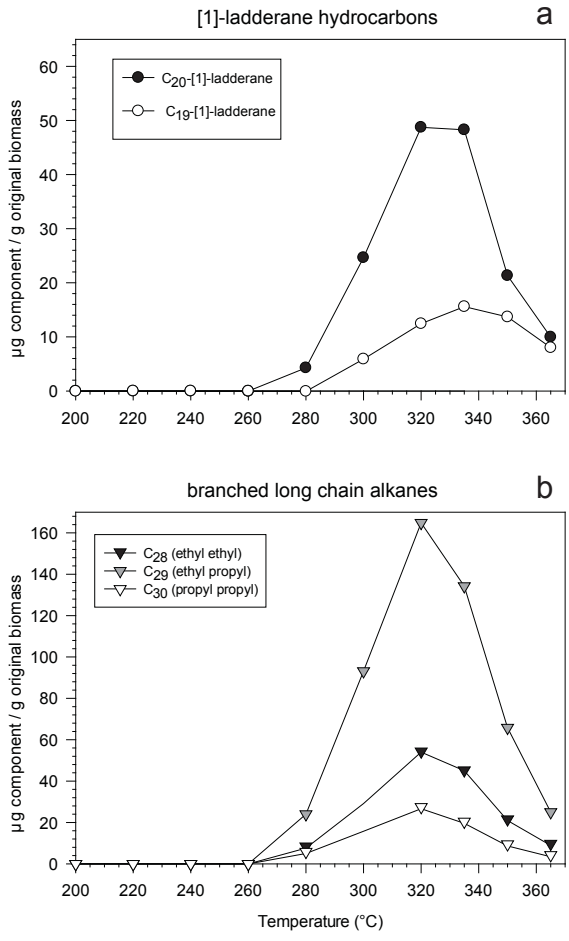


Figure 6. Concentration (µg component · g (original anammox biomass)$^{-1}$) of the components found in the generated oils as a function of hydrous pyrolysis temperature. (a) [1]-ladderane hydrocarbons, (b) branched long-chain alkanes.

7.3.1.3 GENERATION AND FORMATION PATHWAY OF ALKYL-BRANCHED TRICYCLODODECANES

These results demonstrate that hydrous pyrolysis of anammox biomass generates C_{19} and C_{20} alkyl-branched tricyclododecane homologues. The [1]-ladderane hydrocarbons (**VI** and **VII**) were generated in significant amounts at temperatures of 280 – 365 °C, with the highest yields between 320 and 335 °C (Fig. 6a).

The formation of [1]-ladderane components (**VI** and **VII**; Fig. 1) from original ladderane lipids likely goes through the breakdown of one or two bonds of the ladderane cyclobutane rings (Fig. 7), as already observed during thermal analyses by GC (Sinninghe Damsté et al., 2005). This same mechanism of rearrangement has been proposed to be responsible for other ladderane hydrous pyrolysis products (e.g. after ring breakdown, internal proton shifts form olefinic rings; Jaeschke et al., 2008). These olefinic rings would then be hydrogenated to form saturated hydrocarbon rings. The number of carbons in the C_{20} tricyclododecane (**VII**) is in agreement with the cleavage of the ether bond in C_{20} ladderane ethers (Fig. 7a). The C_{19} tricyclododecane, in contrast, is probably formed via the decarboxylation of ladderane fatty acids (Fig. 7b).

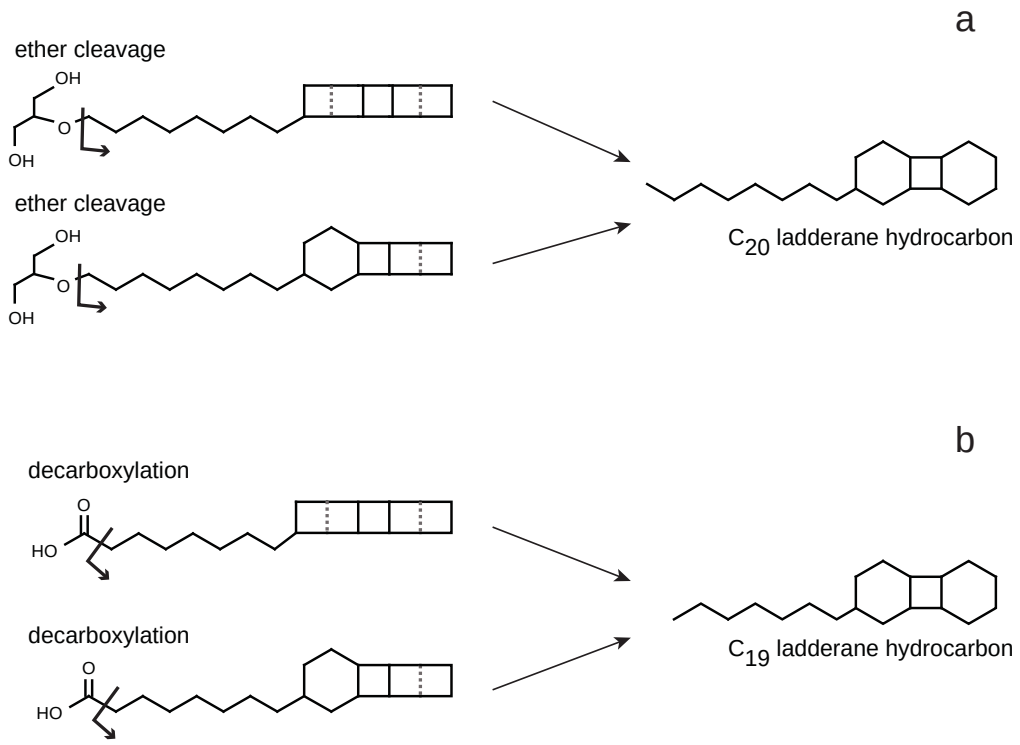


Figure 7. Possible transformation pathways for [1]-ladderane hydrocarbons. (a) C_{20} fatty acids become C_{19} tricyclododecane, whereas (b) C_{20} ethers become C_{20} tricyclododecane. Stippled grey lines indicate bonds where ring cleavage probably occurs.

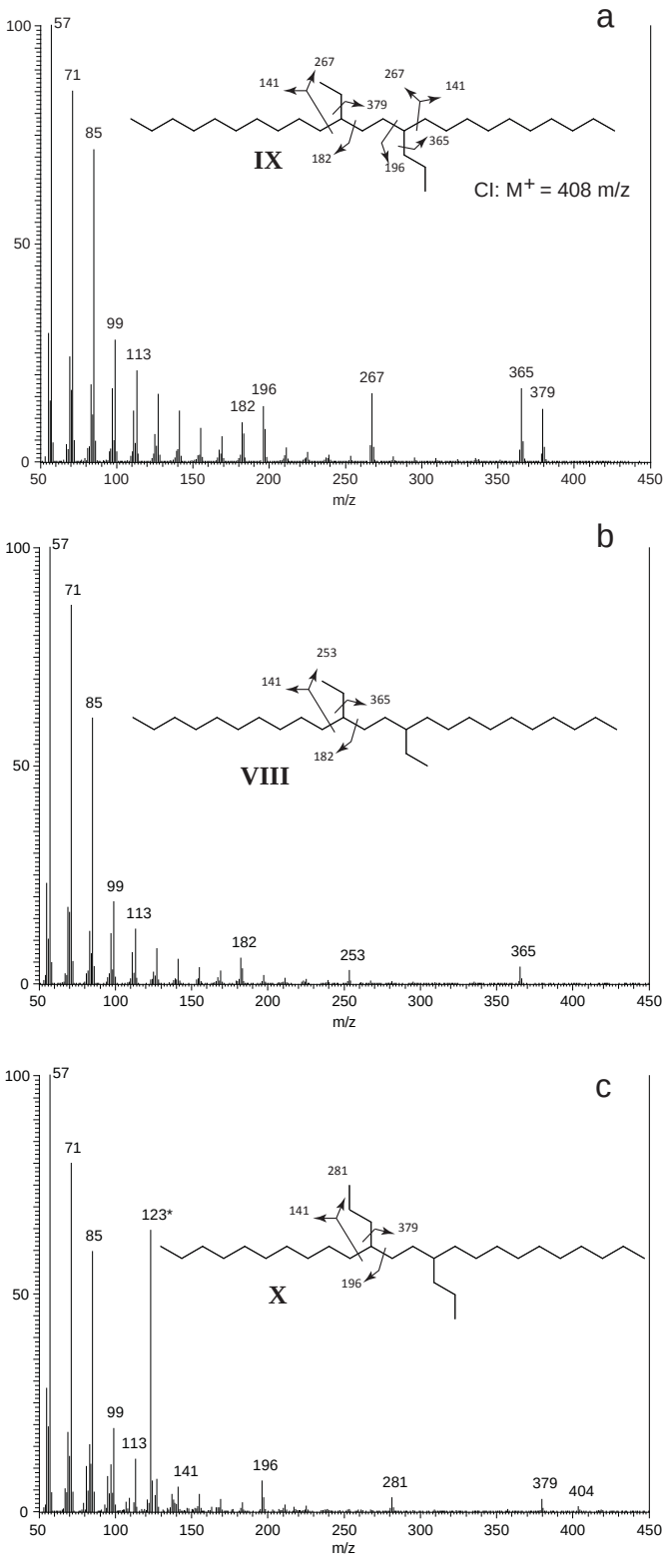


Figure 8. Mass spectra (corrected for background) of (a) **IX**, (b) **VIII**, and (c) **X**, with corresponding structures (tentatively identified for (b) and (c)). Mass spectra of (a) is from the isolated C_{29} branched long chain alkane. Molecular ion (M^+) for **IX** was determined by Chemical Ionisation (CI). Other spectra are from the saturated aliphatic fraction of the oil generated at 320 °C. *denotes impurity in the mass spectra.

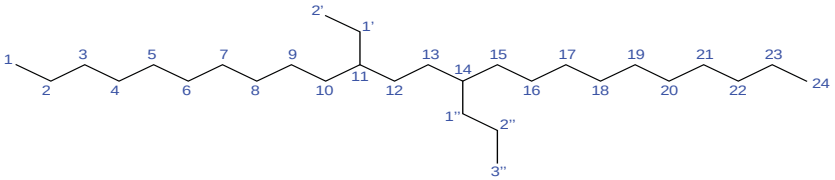
7.3.2 IDENTIFICATION OF BRANCHED LONG-CHAIN ALKANES

The second unknown lipid class is composed of three components (Fig. 3) which elute before the C_{29} – C_{35} hopanes and hopenes. Their mass spectra are characterised by major mass fragment ions representative of alkanes (i.e. m/z 57, 71, 85, 99, etc.), as well as even fragment ions (m/z 182 and 196) and fragment ions in the high m/z range at 253, 267, 281, 365, 379, suggesting that these components be branched alkanes. To fully elucidate the structure of this series of components, the most abundant component was isolated by prep-GC, with a yield of 1.1 mg and a GC purity of 96%.

7.3.2.1 THE MOST ABUNDANT HOMOLOGUE

The mass spectrum of the isolated homologue (**IX**; Fig. 8a) revealed characteristic elevated fragment ions at m/z 182, 196, 267, 365, and 379. The molecular ion of this component was not observed in the electron impact mass spectrum, but was determined using GC-CI-MS, and found to be m/z 408, which indicates a $C_{29}H_{60}$ branched alkane.

Table 4. Proton and Carbon NMR data for component **IX** (11-ethyl,14-propyl-tetracosane), formed during artificial maturation by hydrous pyrolysis.



C-number[a]	Proton Shift (ppm)	Carbon shift (ppm)[a]			2D-NMR correlations		Predicted carbon shift (ppm)[b]
		Primary	Secondary	Tertiary	COSY	HMBC	
1	0.89 (t, 3H)	14.1			H2, H23	H2, H23, H3, H22	14.1
2	1.27-1.30		22.7		H1, H24		20.7
3	1.26		31.9				30.1
4 – 10	~1.3		~29.5				
11	1.17			39.2			38.2
12	1.22		33.2				32.8
13	1.22		33.7				33.2
14	1.25			37.5			35.8
15 – 21	~1.3		~29.5				
22	1.26		31.9				30.1
23	1.27-1.30		22.7		H1, H24		20.7
24	0.89 (t, 3H)	14.1			H2, H23	H2, H23,H3, H22	14.1
1'	1.27		25.9		H2'		28.0
2'	0.84 (t, 3H)	10.9			H1'	H1', H11	12.0
1''	1.21		36.1		H2''		37.4
2''	1.28		19.9		H3''		18.6
3''	0.88 (t, 3H)	14.6			H2''	H2'',H1''	14.5

[a]carbon-proton connections were established by an HSQC experiment
[b]calculated according to the additivity principle (Friebolin, 1991)

The APT ^{13}C-NMR carbon spectrum (Table 4) revealed four primary carbons: two signals at 10.9 and 14.6 ppm, and a higher intensity signal at 14.1, probably representing two primary carbon atoms. This was confirmed by the HSQC experiment (Fig. 9), which showed that the shift of the carbon atoms at 14.1 was correlated with a triplet at 0.89 ppm in the ^{1}H NMR spectrum, representing six attached protons, which was twice the intensity of the two other observed triplets at 0.84 and 0.88 ppm (Table 4). Furthermore, the APT ^{13}C NMR spectrum, in addition to a substantial number of secondary carbon atoms, showed two tertiary carbon atoms (37.5 and 39.2 ppm). The intensity of the carbon signals at 14.1, 22.7, 31.9 ppm were twice the strength of most other signals, and were characteristic of carbons at the end of an *n*-alkyl chain. This, in in combination with the presence of two additional methyl groups and two tertiary carbon atoms (Table 4), indicates that this component is a dialkyl substituted *n*-alkane. Since the additional methyl groups are represented in the ^{1}H NMR spectrum as triplets, they must each be connected to secondary carbon atoms. The HMBC experiment indicated that the triplet at 0.84 ppm showed connectivities with a secondary carbon atom at 25.93 ppm and a tertiary carbon atom at 39.24 ppm, establishing the presence of an ethyl group. Additionally, the triplet at 0.88 ppm showed connectivities with two secondary carbon atoms (at 19.9 and 36.1 ppm), representing a propyl, or longer, alkyl moiety. However, the observed mass spectral fragmentation (Fig. 8a) clearly reveals the loss of 29 Da (*m/z* 379; loss of ethyl) as well as 43 Da (*m/z* 365; loss of propyl), establishing both side chains. The position of the substituents (i.e. at C-11 and C-14; Fig. 8a) is also clearly indicated by the mass spectral fragment ions *m/z* 182, 196, and 257, establishing the identity of this component as 11-ethyl,14-propyl-tetracosane (**IX**; Fig. 1). Using the additivity principle (Friebolin, 1991), the chemical shifts of the carbon atoms were calculated, and they match the observed shifts for an 11-ethyl,14-propyl-tetracosane. The RI of this component was 2665, which is in agreement with the calculated RI value (2680) for this internally branched C_{29} alkane, according to the equation of Kissin and Feulmer (1986).

7.3.2.2 Mass spectral identification of branched long-chain alkane homologues

In addition to **IX**, two other components were detected with similar mass spectra (**VIII** and **X**; Fig. 8, b and c, respectively). The mass spectral fragments *m/z* 183 and 253 (Fig. 8b) of **VIII** suggest internal branching points at C-11 and C-14, likely with two ethyl substitutions. The branching points for two propyl chains in component **X** are probably also at C-11 and C-14 based on the *m/z* 196 and 281 fragment ions (Fig. 8c). The RI for **VIII** and **X** were 2600 and 2725, and are in agreement with calculated retention indices (2610 and 2750, respectively). Thus, these components are tentatively identified as C_{28} 11-ethyl,14-ethyl-tetracosane (**VIII**), and C_{30} 11-propyl,14-propyl-tetracosane (**X**), structures closely related to that of the unambiguously identified **IX**.

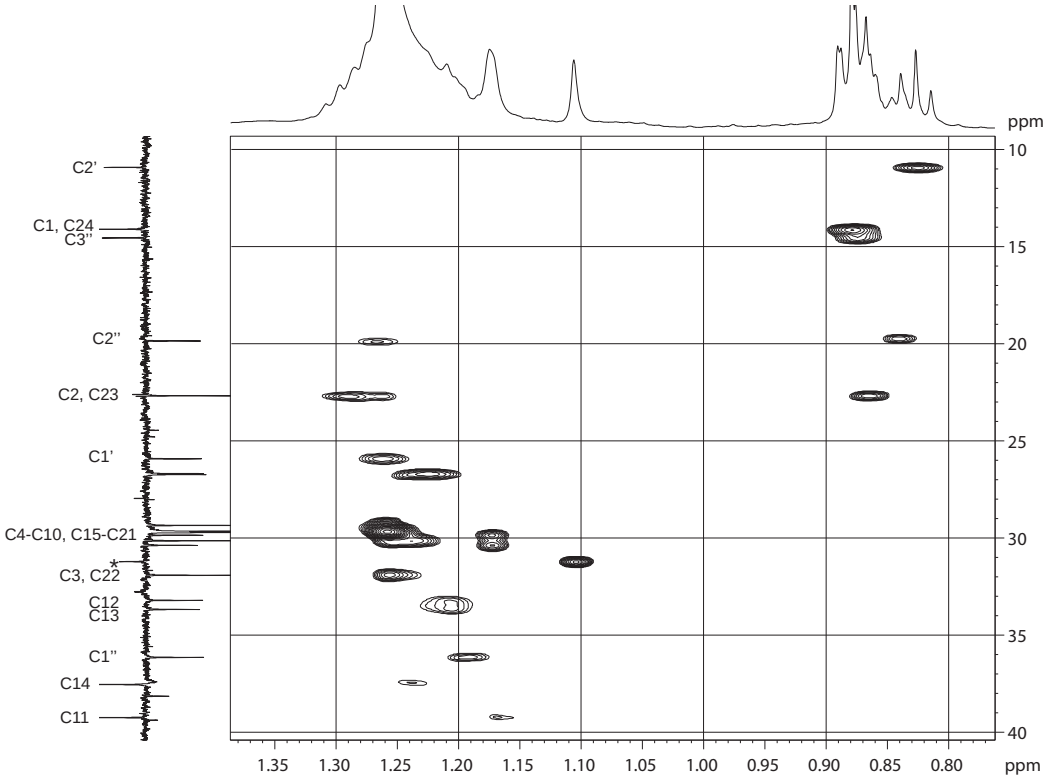


Figure 9. Heteronuclear single-quantum correlation spectroscopy (HSQC) for isolated component **IX**. Carbon atoms are numbered according to the assignments in Table 4. * denotes impurity.

7.3.2.3 Generation and formation pathway of branched long-chain alkanes

Branched long-chain alkanes were generated at temperatures >260 °C, with maximal abundances all occurring at 320 °C (Fig. 5c). These components were the most dominant aliphatic hydrocarbons (Fig. 3) in the hydrous pyrolysis oils, which, along with their depletions in $\delta^{13}C$ (Table 2), seems to indicate catagenetic products of anammox biomass. However, branched long-chain alkanes have not been reported previously in anammox biomass. Furthermore, their chain lengths are substantially longer than those of the major ladderanes identified in anammox bacteria, i.e., $C_{16} – C_{24}$ (Rattray et al., 2010). A very speculative pathway of formation could possibly involve the modification of $C_{28} – C_{30}$ ladderane lipids, or ladderane ethers with two aliphatic chains bound to one ladderane moiety (Fig. 10), which would not have been detected in earlier GC screening of anammox lipids. The ether bonds of these ladderane lipids would be cleaved during hydrous pyrolysis, and the ladderane moieties opened up to form a long aliphatic chain. Clearly, there is need for a reinvestigation of biosynthesised lipids of anammox bacteria.

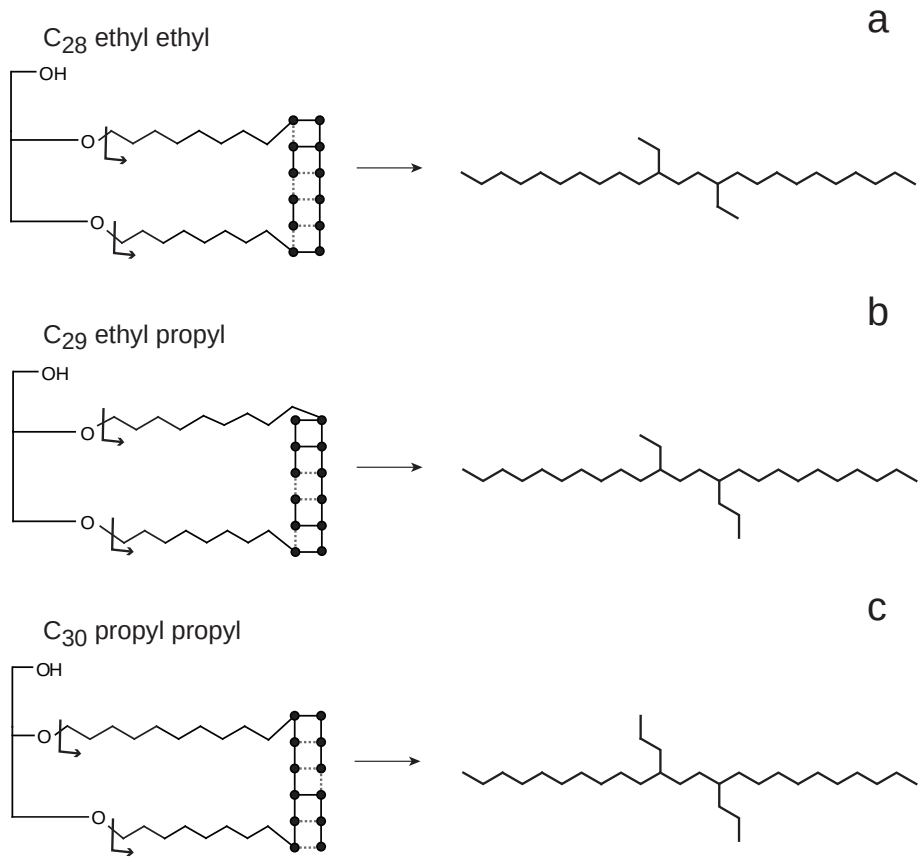


Figure 10. Suggested transformation pathways for the (a) C_{28}, (b) C_{29}, and (c) C_{30} branched long-chain alkanes. These speculative pathways involve original ladderane lipids that have not previously been detected in anammox biomass. Stippled grey lines indicate bonds where ring cleavage possibly occurs.

7.3.3 Environmental occurrence

Sediments deposited during geological periods where anammox activity is believed to have been high, i.e., stratified anoxic basins, were screened for the presence of these thermally stable component classes (Table 5). However, none of the thermally stable catagenetic products of anammox lipids produced during hydrous pyrolysis could be detected. This is possibly due to the low abundance of ladderanes in environmental lipid mixtures. Anammox bacteria are slow growing Planctomycetes (Strous et al., 1999), and the abundance of their lipids in the natural environment is low. The detection limit of ladderanes in modern sediments is in the ng range (<1 – 580 ng/g dry sediment; Jaeschke et al., 2009a; Jaeschke et al., 2009b; Jaeschke et al., 2010; Rush et al., 2012b), with a maximum of 4.9 μg/g sediment reported in an Arabian Sea core top (Rush et al., 2012b). It is likely that the amount of their thermally stable components would be even lower in ancient sediments. Here, we used single quadrupole MS, whereas to detect ladderanes in modern sediment samples we used a selective reaction monitoring triple quadrupole MS method (Hopmans et al. 2006). Therefore, our method is likely not sensitive enough, and more sensitive methods such as 2D-GC-Time of Flight-MS should be tested.

Table 5. Sources of samples screened for thermally mature products of anammox biomass in this study.

Origin of sample	Number of samples screened	Geological age	Reference
Sapropel cores, Mediteranean	32	Pliocene	(Menzel et al., 2005)
Lomonosov Ridge, Arctic	30	Upper Eocene/Lower Paleocene	(Schouten et al., 2007b)
Alpha Ridge, Arctic	5	Maastrichtian/ Campanian	(Jenkyns et al., 2004)
Denmark	23	PETM	Schoon et al., unpublished
Walvis Ridge, South Atlantic	26	Turonian/Cenomanian	(Forster et al., 2008)
Umbria, Italy	12	Cenomanian	(Tsikos et al., 2004a)
Furlo, Italy	4	Cenomanian	Forster et al., unpublished
North West Greece	6	Cenomanian/ Albian	(Tsikos et al., 2004b)
Demerara Rise, Tropical West Atlantic	6	Albian	(Forster et al., 2004)
Mid Pacific Mountains	32	Aptian	van Breugel et al., unpublished
Cismon Valley, Italy	35	Aptian	(van Breugel et al., 2007)
Paris Basin, France	4	Toarcian	(van Breugel et al., 2006)

A second explanation for the non-detection of these ladderane catagenetic products could be that the screened sediments were too immature. The 22S/(22S+22R) ratio and the ββ/(αβ+βα+ββ) ratio of C_{31} homohopanes are often used as biomarker maturity indices (Seifert and Moldowan, 1980; van Duin et al., 1997), and the ratios produced during these hydrous pyrolysis experiments are reported in Jaeschke et al., (2008). The maximum generation of both ladderane component classes was at high hydrous pyrolysis temperatures (320 – 335 °C; Fig. 6). At these temperatures, the ratio of ββ/(αβ+βα+ββ) was 0, indicating that these products are thermally mature. This is supported by the 22S/(22S+22R) ratio, which was ~0.4. Thus, these screened sediments might not have been exposed to the catagenetic processes needed for the generation of these products.

Alternatively, these detected ladderane thermal transformation products could not reflect the natural degradation of anammox biomass. There are some discrepancies between natural petroleum formation and hydrous pyrolysis simulations (Lewan, 1985). At high temperatures, differences in the oil generated by anhydrous pyrolysis and hydrous pyrolysis showed that hydrous pyrolysis resulted in an increased hydrocarbon yield and might not mimic natural oil generation (Comet et al., 1986). Therefore, perhaps the hydrocarbons generated by the hydrous pyrolysis of anammox biomass are not naturally generated catagenetic products of anammox lipids.

7.4 Conclusions

Hydrous pyrolysis experiments of anammox biomass results in the generation of two distinct lipid classes: branched long-chain alkanes and ladderane hydrocarbons. However, these thermally stable lipids were not detected in the environment. They are either not naturally occurring products, the sediment samples screened were not mature enough for these products to have been generated, or the method used was not sensitive enough to be able to detect them. To test this, further study using a more selective, multidimensional GC/MS technique on mature sediments is likely needed.

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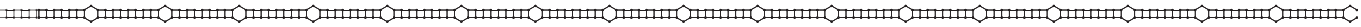

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Besides work, there was the social stuff, the two often overlapping. Which was greatly thanks to my girls. The surprise Efteling weekend you threw pretty much sums it up: you are wonderful! I couldn't have asked for a better group of friends. I'm so thankful to have you in my life, to share the highs, (sometimes the lows), and the wine… especially the wine. Sabine, before you started at the NIOZ, all I can remember is grey. You put a lot of colour into the place. You're a fantastic friend who has constantly made me laugh (whether you intended to, or not). I'm fortunate that you arrived on Texel when you did. I remember coming back from my holiday in Canada and the first thing "someone" told me about you was, "I hear she's pretty hot." But you're not just that. You're fun, and a brilliant friend. And I hope that, despite the fact that you say you're bad at keeping in touch, we remain as close as we are. Julie, there is no one else I would have rather spent the days on the beach with in Cape Verde while Sabine busied herself on the water. I truly enjoyed getting to relax with you. When Craig was determinedly scouring the NIOZ for friends for you, I was a little worried we were getting a weirdo (which let's face it, would have been an accurate description for any of us anyway), but you have turned out to be a great friend who never judges and just listens. I'm proud to be able to call you a friend. I look forward to spending more time with you and your family, and getting to see Lauren grow up into as beautiful of a person (inside and out) as you are. Nicole, I am so grateful that you came into my life that I'm not sure I can express it properly (oh boy, that's already the setup for the gushy love note that's to follow). From our first outing to Alkmaar, I knew that I was so lucky to have found someone I could share everything with (from the serious to the downright silly). It's going to be hard not having you right around the corner, getting to cycle with you to work in the morning, or coming over to sit in your gorgeous back yard. Thanks for being an excellent friend and confidant. I know it will stay a great friendship despite the distance. I love you.

Julie and Nicole, thank you in advance for all the tedious tasks you'll be doing as my paranimfen. I don't think I could have picked a better (or more organised) duet of ladies.

Of course there were always others who made the party as well: Allert and Roos, thank you for being so much fun to talk to. I enjoyed having you over after Cees' defense for a very emotional 1 a.m. chat. Amrit and Felix, the NIOZ was so much livelier while you two were in town. Arnaud you were always fun to talk to and I'm so impressed that you lived with – and survived – Sabine). Eric, thank you for leaving on my desk a paper entitled "A non-Darcy model for recirculating flow through a fluid-sediment interface in a cylindrical container" with a note chastising the authors for even imagining a world like that. It brightened my day. The GEO crew: Furu, Rik, Thomas, Wim, and company, meeting up for Friday beers, or just a coffee chat was always a pleasure! Ingmar, thanks for having such a positive attitude. You made buying a kasssoufflé fun. Jenny, thanks for having such an infectiously cheer. Jeremy and Silvia, thank you for being such a nice couple of people, and thanks for the surprise pecan pies. They were always so good. I wish you lots of happiness in the US as well as wherever your travels take you. Lennart, thank you for always being so happy, and so well supplied with gin! Lukas, thanks for being so fun (and FULL of energy). Niamh (whoop whoop), it was wonderful having you on Texel for those (3?) months. You're a class A lady. Thanks for hosting us in Dublin. Richard, you were truly a great person to have around. So positive and funny. Thanks. Santi, it was always entertaining (at the coffee table, or elsewhere). Guaranteed interesting conversation. Tristan, thanks for sharing my last train from Texel (as a bewoner) to the airport. And for the much appreciated drink at Schiphol. Ulrike, I'll never forget your "girl spirit" when it came to going up against the boys in indoor football. Thank you for being such a great person.

Jesse, I'm glad we met. Thank you for showing me what it's like in the "real world". I'm happy that it is just as complex as science. (Though maybe it's you making it more complex?)... Xofo!

Juliane, thank you for convincing me to get on a bike, even when I didn't want to. The ice cream was always worth it. I hope you continue to enjoy the cycling on the island, and have an ice cream or three for me! P.S., have you started your introduction yet?!

Paul, our conversations always meant so much to me. I had a fabulous time with you, whether one-on-one at dinner, or in a big party drinking ginger beer and rum/chocolate vodka. You're a grand guy so I'll go on the record: it probably wasn't the mung beans. Eva, thanks for getting down on the floor with Tess whenever I brought her over, giving her a bowl of water, and especially for shrugging it off when she dribbled it all over your house!

Yvo, I'm so lucky to have gotten to know you over the years. You are a true friend. Thank you for the numerous things you've helped me out with over the years. It was so nice to have you (and Nicole, too) over in BC for a visit. You are a wonderful couple, and I'm so happy to see how happy you make each other. I hope that you come over to Newcastle soon! One last thing, I've never met a man who was more willing to go the extra mile for a costume. Whether it was a Ghostbuster, a Polar Bear, or the Mad Hatter, you were always impressively dressed. (So maybe come over on Halloween?!)

To the NIOZ football team (Allert, Bert, Craig, Cees, Dave, Eli, Ingmar, Jan Dirk, Leon, Luke, Maarten, Matthijs, Pedro, Roeland, Santiago, Yvo, and others), honestly I have to say that watching you play was often painful. But that's okay. By the end, I had accepted that, and the beer and chat afterwards always made for a great Thursday evening. Sorry I was a bad WAG in the last year.

The British girls: Amanda, Claire, Jenni, Kate, Lesley, Libby, Nicole, Nikki, Rachel, Sharyn, and Thalia (wow, there really were a lot of you). You all gave me a great show of what it would be like in the UK (though, a bit more insight into the amount of alcohol consumed would have been appreciated). Thank you for being such sweet, lovely people. Lesley, I'll never forget coming home to the house on Buytengors and you being trapped inside by spiders which had "taken over all the exits". I think the following people who lived in that house must have been confused by all the spider guts on the walls. Thanks for being a great housemate and friend. I hope to come visit you in the (I'm sure) exciting place life takes you next! Jenni, thank you for being a fun dance partner and for organising rounders on the beach. Kate (and Jez), thank you for being such great fun to have around. Libby and Nikki, thanks for being a pleasure to hang out with in Wales. I truly enjoyed getting to spend time with you two while having a (few) drinks at the Balcken. Rachel, it was so nice having you as a (practically) neighbour. You're an incredibly kind person, and it was always lovely having tea and a chat with you. I hope to come and visit you and Pascal and Morgan in Liverpool. Thalia, it was so great to have you on Texel. You're so sweet. I hope you enjoy the house on Westergeest for years to come.

I'd like to thank the families Bale and Witte for making me feel at home in Plymouth and on Texel. Yvo, you have a fantastic set of siblings and a truly terrific mum. Nicole, spending the holidays with your amazing family was wonderful. And whenever they were over on Texel was always sure to be a blast. New Year's with you, Sabine, and Helen at Bonnie's family home in

Falmouth was so gezellig. Thanks to all you ladies!

All the dog walkers: Marloes and Lisa, Simone, Suzanne, Inge, and Eveline, as well as interim care from the Stuuts, Joost and Esther, Nicole and Yvo, Sabine, Rachel and Meinard, Craig, and a very-pregnant-Julie who had to sit on a disobedient, adolescent dog in the centre of Den Helder (I wish I could have seen that). Thank you!!! Though having Tess was a lot of work for a scientist who had to go here and there, you all made it so much easier.

Craig, thank you for feeding me beers (or wine) on Friday afternoons (and any afternoon, really). Thanks for being a great friend, and a genuinely nice guy. Also, I think she probably would have managed on her own, but I'll give you the credit, Julie and I are a pretty good match. Take care of those two beautiful redheads!

Jan-Berend, you're lucky to have so many fabulous women surrounding you. Meta: Thank you for being such a friendly face in Den Burg, and for always yelling out a hello as you all cycled past to school in the morning. Mathilde and Britte, thanks for making Tess realise how much she loves kids. And Nynke, ik ben trots op je. You went from being scared of a little puppy to hugging a 40 kg dog. Goed gedaan, meisje.

Family van der Land, Kor, Géa, Vroni, Joost, Bas and Esther, thank you all for welcoming me with open arms. Seeing Cees have such a supportive and close family has really made me happy.

Meinard, thanks for being a great housemate. And despite the loss of onions, apples, bananas (peel and all), lasagne, hot cross buns, numerous ballpoint pens, sandwiches, bread and various others, thank you for still putting up with Tess and giving her an enthusiastic pat once in a while. Rachel, it was so nice having you at home for a few weeks in the last half year of my thesis. Not only because it meant that Tess had company during the day, but also because it gave me someone to come home to and have a chat with about things unrelated to science. I hope you and Meinard enjoy the Kogerstraat for years to come!

Mélanie, ma belle, merci pour ces apéros chez toi à Montpellier, à Vauvenargues, à Marseille, et à Aix, et pendant notre vacance ensemble en Nouvelle-Zélande. Les vacances passées avec toi étaient toujours du fun. Mais surtout, je te remercie d'avoir été une très bonne amie (de proche aussi bien que de loin). And thank you for a very special birthday in Manapouri that involved delicious wine, but not-so-great canned dinner. Je t'aime. Micha, Cristina et Saskia, qui m'ont rendu visite sur cette belle ile paumée, merci. Kasia, thanks for putting me up in Marseille. I'm so happy you're in such a great place, surrounded by two lovely men. Jesteś miłością mojego życia!

Ali, Caroline, Erin, Kendra, Kyle, Liseanne, Malika, Matt, and Mike, I miss you guys, and occasionally feel bad about not being around so much, but when I'm home, you constantly reassure me that coming home is like I have never left (does that mean I have the maturity of an 18-year old?). I'm so lucky that I can just fall back into these fantastic friendships that I treasure so much.

Mum and Dad, your support over the years has really been unfaltering. Thank you so much for

always being so positive, and for coming over to visit so often (so that I never had the chance to get homesick). I love you.

Eric, thanks for being such a thoughtful little brother. I am really quite proud of the man you've become. The week you spent over on Texel was really fun. Hopefully you can come visit in Newcastle.

To those who will be leaving shortly after I did, I want to thank you for making the last 4 years what they were. And to those I leave behind, I hope to come back often and visit the beautiful island of Texel. Finally, to the new wave of NIOZers, I hope you have as great a time on Texel as I did. Savour it (wind, rain, and all), it goes by quicker than you can imagine.

And to the new set of people who I've gotten to know in Newcastle, thank you for making the transition so easy. I have to feeling that the next three years are going to be great!

This has turned into a sort of Thank You Manifesto, but I hope I have managed to convey how much I appreciated my time on Texel, and the people who were part of my life during my stay. Before I finish, there is one last person I really want to thank. Cees, you are an exceptional human being. The respect I have for you cannot be measured. Thank you for sharing the laughs (lots and lots of them) over the years, for being a best friend, for being an understanding person, and most importantly thank you for being there for me every step of the way. Also, thanks for being supportive of the decision to get a dumb, slobbering dog (even if you were sceptical at first), and for sharing the burden (and joy) of looking after her. I'm lucky to have you in my life. A lot!

…ladderaf

Darci Joan Rush was born on March 6th, 1984 at the base of the Rocky Mountains. When she was five, her family settled in Maple Ridge, B.C., which was to become her home for the following thirteen years. Very early on, she expressed interest in science, having won her first science competition in kindergarten by building a thermal container that kept an ice cube from melting the longest. Thanks to the fabulous 80's TV program *Danger Bay*, Darci quickly became captivated by the marine world. After graduating from Maple Ridge Secondary School in 2002, she moved to Canada's capital to begin studying Biochemistry and Biotechnology. However, she grew impatient with a prospective career away from the natural environment, and decided to pack up and move to Marseille, France in 2004. She undertook a Bachelor's (and subsequent Master's) degree in Oceanography and Marine Biogeochemistry at le Centre d'Océanologie de Marseille. She completed a Master's research project investigating the silica cycle in the Southern Ocean (to this day, diatoms are still her favourite little beast). After graduating in 2007, Darci returned to Canada for a short time before being tempted back to Europe, this time the Netherlands, to begin a PhD at the Royal NIOZ in August, 2008. There, she worked on what culminated into this body of work. She left Texel in August, 2012 to start a postdoctoral position investigating bacteriohopanepolyols as biomarkers for aerobic methane oxidation at Newcastle University.