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Minireview

Lipids as paleomarkers to constrain the marine nitrogen cycle

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Summary

Global climate is, in part, regulated by the effect of microbial processes on biogeochemical cycling. The nitrogen cycle, in particular, is driven by microorganisms responsible for the fixation and loss of nitrogen, and the reduction-oxidation transformations of bio-available nitrogen. Within marine systems, nitrogen availability is often the limiting factor in the growth of autotrophic organisms, intrinsically linking the nitrogen and carbon cycles. In order to elucidate the state of these cycles in the past, and help envisage present and future variability, it is essential to understand the specific microbial processes responsible for transforming bio-available nitrogen species. As most microorganisms are soft-bodied and seldom leave behind physical fossils in the sedimentary record, recalcitrant lipid biomarkers are used to unravel microbial processes in the geological past. This review emphasises the recent advances in marine nitrogen cycle lipid biomarkers, underlines the missing links still needed to fully elucidate past shifts in this biogeochemically-important cycle, and provides examples of biomarker applications in the geological past.

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Biomarker lipids and the marine nitrogen cycle

Nitrogen is vital to the building blocks of life (e.g. nitrogen is a key element in amino acids, nucleic acids, and membrane lipids). The largest marine pool of nitrogen is dissolved dinitrogen (N_2). However, few organisms in the ocean are capable of utilising N_2; non-diazotrophic microbes must depend on the less substantial pools of bio-available nitrogen [i.e. nitrate (NO_3^-); nitrite (NO_2^-); ammonium (NH_4^+)] to sustain their activity (Fig. 1). The concentration of these different forms of nitrogen varies spatially and temporally in the ocean. In general, the highest concentrations of NO_2^- and NH_4^+ are found in the surface waters where they are assimilated by phytoplankton to form a pool of organic nitrogen. The concentrations of these two species decrease significantly at the base of the euphotic zone, as they are converted into NO_3^- by nitrifying microorganisms. NO_3^- on the other hand accumulates with water depth and is usually present an order of magnitude higher than NO_2^- and NH_4^+ (Gruber, 2008). The fate of nitrogen in deeper waters can either be towards burial as particulate matter, or complete loss as it is converted back to N_2 in anoxic conditions. Nevertheless, it must be emphasised that the presence of these bio-available forms of nitrogen in the ocean is inconsistent, and their concentrations in the water column are habitually low.

Nitrogen is often the limiting nutrient for primary productivity in marine environments, and our understanding of the marine carbon pump inherently depends on what we know of the nitrogen cycle. Since the global inception of the Haber-Bosch process in the early 20th century, anthropogenic activity has significantly influenced the marine nitrogen cycle. Run-off from fertilisers in coastal areas causes eutrophication of the water column, leading to oceanic dead zones (Altieri and Gedan, 2015), and changes in the extent and vertical structure of oxygen minimum zones (OMZs) (Paulmier and Ruiz-Pino, 2009; Wright *et al.*, 2012). Besides the obvious repercussions on ecology and climate, anthropogenic nitrogen input also greatly affects local economies by decreasing the available stock of commercial fish (Lavin *et al.*, 2006; Rabotyagov *et al.*, 2014).

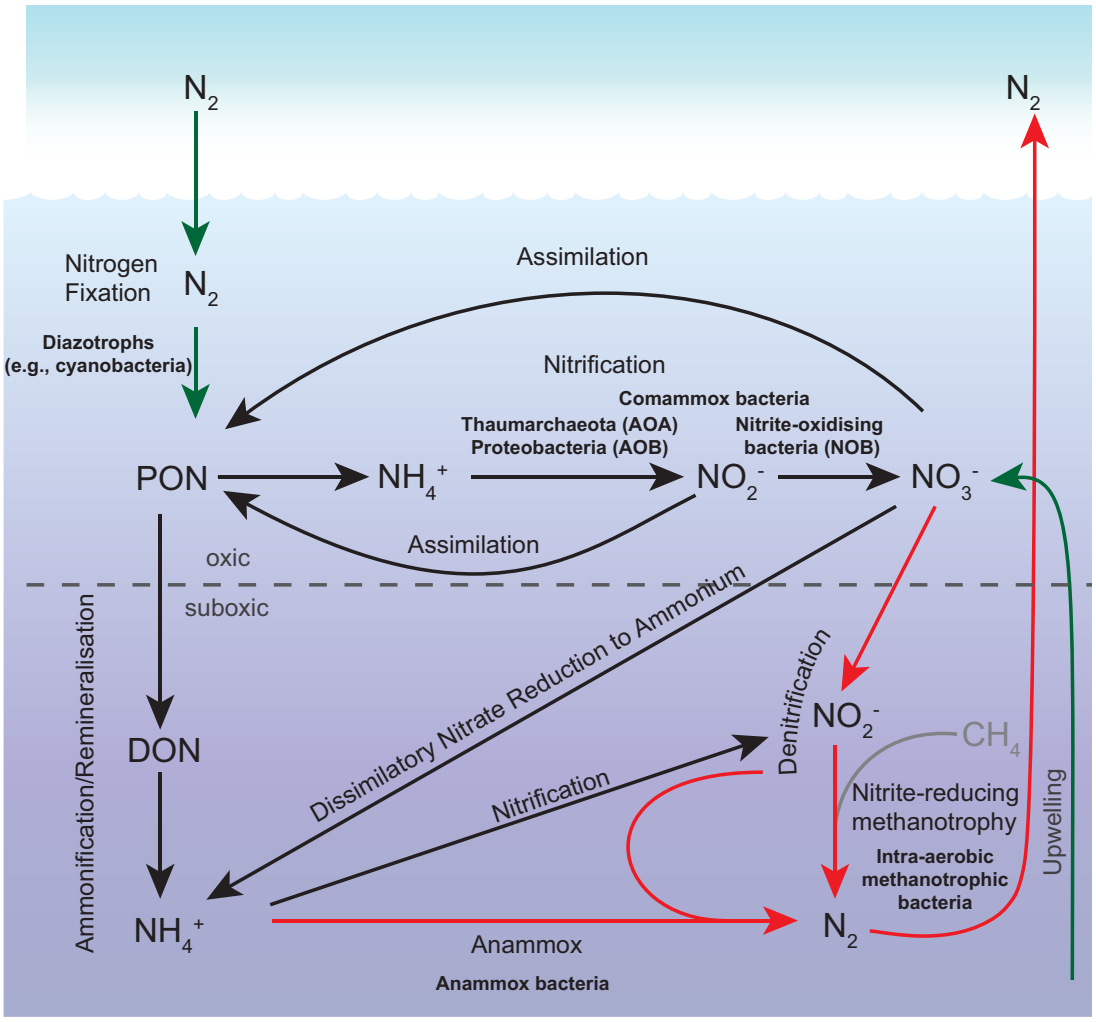

Fig. 1. The marine nitrogen cycle. Microorganisms with known biomarkers are indicated next to their transformation pathways. Losses of nitrogen are indicated by red arrows, sources in green. N_2: dinitrogen gas; PON: particulate organic nitrogen; DON: dissolved organic nitrogen; NH_4^+: ammonium; NO_2^-: nitrite; NO_3^-: nitrate. Figure adapted from Arrigo, 2005.

The transformations between chemical species of nitrogen are primarily regulated by microbial activity, with specialised organisms performing each process (Fig. 1). Nitrogen cycle microorganisms are often taxonomically divided, and many have structurally unique lipids, known as biomarkers (Peters *et al.*, 2005). By employing biomarker lipids, we can determine the contributions of different nitrogen cycle processes through Earth's history. Biomarkers are especially useful in reconstructing paleo-environments where genetic information has been degraded (cf. Hofreiter *et al.*, 2001). Subsequently, this biomarker information can be used to better predict modern and future nitrogen and carbon cycle variations by constraining biogeochemical models of the past [e.g. during the Cenomanian-Turonian Ocean Anoxic Event (OAE 2); Monteiro *et al.*, 2012].

Biomarkers exist for many of the key nitrogen biogeochemical processes. However, there are some glaring gaps, notably on the anoxic side of the nitrogen cycle. OMZs are found in the marine water column underlying productive photic zones, and are hotbeds for anaerobic nitrogen cycling processes, most of which result in net marine nitrogen loss through nitrogenous gas production. It has been estimated that half of the nitrogen loss in the ocean occurs within these OMZs (Codispoti *et al.*, 2001; Lam *et al.*, 2009). In our current dynamic ocean climate of increasing temperature and decreasing ventilation, OMZs are expanding (Wright *et al.*, 2012). It is imperative that we be able to predict how past perturbations in ocean biogeochemistry, especially within OMZs, affected the sensitivity of the system.

Several review papers have highlighted the marine nitrogen cycle (Gruber, 2004; 2008; Arrigo, 2005;

Capone, 2008; Lam and Kuypers, 2011; Zehr and Kudela, 2011; Voss *et al*., 2013; Ward and Jensen, 2014), but none have focused on nitrogen cycle biomarkers. Here, we describe the biomarker lipids known for key processes in the marine nitrogen cycle, how they can be applied to cycling in the geological past, and draw attention to the processes within the nitrogen cycle that still need resolved biomarkers.

Bringing nitrogen in: Diazotrophic organisms in the marine system

N_2 accounts for 94% (10^7 Tg N) of the nitrogen in the ocean (Gruber, 2008). Diazotrophy, or nitrogen-fixation, is essential to the fertilisation of ocean surface waters in oligotrophic regions, where there are no other significant inputs of nitrogen (Capone, 2001; Mahaffey *et al*., 2005). N_2-fixation has been estimated to contribute 36–50% of the nitrogen used by microbes in the Pacific and Atlantic gyres (Carpenter *et al*., 1999; Dore *et al*., 2002). The most important marine diazotrophs belong to the phylum Cyanobacteria (Carpenter and Romans, 1991; Capone *et al*., 1997; Karl *et al*., 1997; Gallon, 2001), and by far the most well-studied species are the filamentous *Trichodesmium* spp. However, unicellular cyanobacteria (Martínez-Pérez *et al*., 2016) and non-cyanobacteria diazotrophs (Farnelid *et al*., 2011) may also play an important role in N_2-fixation in marine systems. Diazotrophic organisms use the enzyme nitrogenase to break the triple bonds between the N atoms of N_2. Nitrogenase is inactive in the presence of oxygen, a fact that is problematic to the surface dwelling, primarily photoautotrophic, microorganisms using nitrogenase. Diazotrophs compensate for this in different ways. Some use the light/dark cycle to spatially separate these processes. Others use physical separation by compartmentalising diazotrophy into specialised cells. Below, we discuss two groups of diazotrophy biomarkers.

Bacteriohopanepolyols of cyanobacteria

Cyanobacteria are considered to be the most environmentally significant source of bacteriohopanepolyol lipids (BHPs) (Farrimond *et al*., 1998; Talbot and Farrimond, 2007). BHPs are the precursors to the most ubiquitous lipids found in geological materials: geohopanoids (e.g. Ourisson and Albrecht, 1992). BHPs are pentacyclic C_{30} triterpenoids linked to a polyfunctionalised side chain with e.g. hydroxyl, amine, or cyclic groups (Fig. 2). Although not all cyanobacteria synthesise BHPs, and many synthesise non-source specific BHPs, [e.g. bacteriohopanetetrol (BHT) and aminobacteriohopanetriol (aminotriol)], some marine N_2-fixing species of cyanobacteria have been shown to synthesise more characteristic BHPs. C-2 methylated BHPs (Fig. 2) were found in *Trichodesmium* and *Crocosphaera*, both ubiquitous marine species (Talbot *et al*., 2008). C-2 methylated hopanes (the diagenetic product of BHPs) were originally thought to be a biomarker for cyanobacteria, and have been used previously to indicate the rise of oxygenic photosynthesis (Summons *et al*., 1999) as well as the importance of N_2-fixation at times of stratified oceans (e.g. Cretaceous black shales; Kuypers *et al*., 2004). However, other bacteria have also been found to synthesise C-2 methylated BHPs [i.e. anoxygenic phototroph *Rhodopseudomonas palustris* TIE-1 (Rashby *et al*., 2007), *Methylobacterium* spp. (Bisseret *et al*., 1985; Knani *et al*., 1994) and *Bradyrhizobium* spp. (Bravo *et al*., 2001)]. Furthermore, the gene coding for the enzyme responsible for C-2 methylation in BHPs was found to occur across the bacterial domain (Welander *et al*., 2010). Thus, using the C-2 methylation in hopanoids as a biomarker for cyanobacteria input and the onset of atmospheric oxygen is complicated.

A more appropriate BHP biomarker for marine N_2-fixation may be BHT cyclitol ether (BHT-CE; Fig. 2) with an unsaturation at the C-6 or C-11 position. Unsaturated BHT-CE is synthesised by *Trichodesmium* sp. (Talbot *et al*., 2008), one of the most prolific and biogeochemically-important marine cyanobacteria. However, this lipid has also been found in acetic acid bacterial cultures (Simonin *et al*., 1994; Herrmann *et al*., 1996; Talbot *et al*., 2007), and several strains of *Burkholderia cepacia* isolated from acetic grasslands (Cvejic *et al*., 2000). Thus, care must be taken when applying unsaturated BHT-CE as a biomarker for *Trichodesmium*. As it is unlikely that either of these other types of bacteria proliferate in open marine settings, unsaturated BHT-CE was successfully applied as a biomarker for *Trichodesmium* in sediments from the Congo deep sea-fan to interpret the past marine nitrogen cycle (Handley *et al*., 2010). Unsaturated BHT-CE was present in unprecedented abundance at a specific sedimentary horizon (480–610 ka). Here, the abundance of unsaturated BHT-CE was interpreted to be caused by a decrease in nutrient supply, which resulted in increased N_2-fixation by diazotrophic cyanobacteria.

Heterocyst glycolipids

A large group of diazotrophic cyanobacteria contain heterocysts, specialised cells where diazotrophy occurs under induced low oxygen conditions. To maintain anoxia, heterocystous cyanobacteria surround these cells with a thick enveloppe limiting the diffusion of gases (Walsby, 1985; Murry and Wolk, 1989), which is made up of glycolipids known as heterocyst glycolipids (Nichols and Wood, 1968; Bryce *et al*., 1972; Lambein and Wolk, 1973). Heterocyst glycolipids consist of sugar

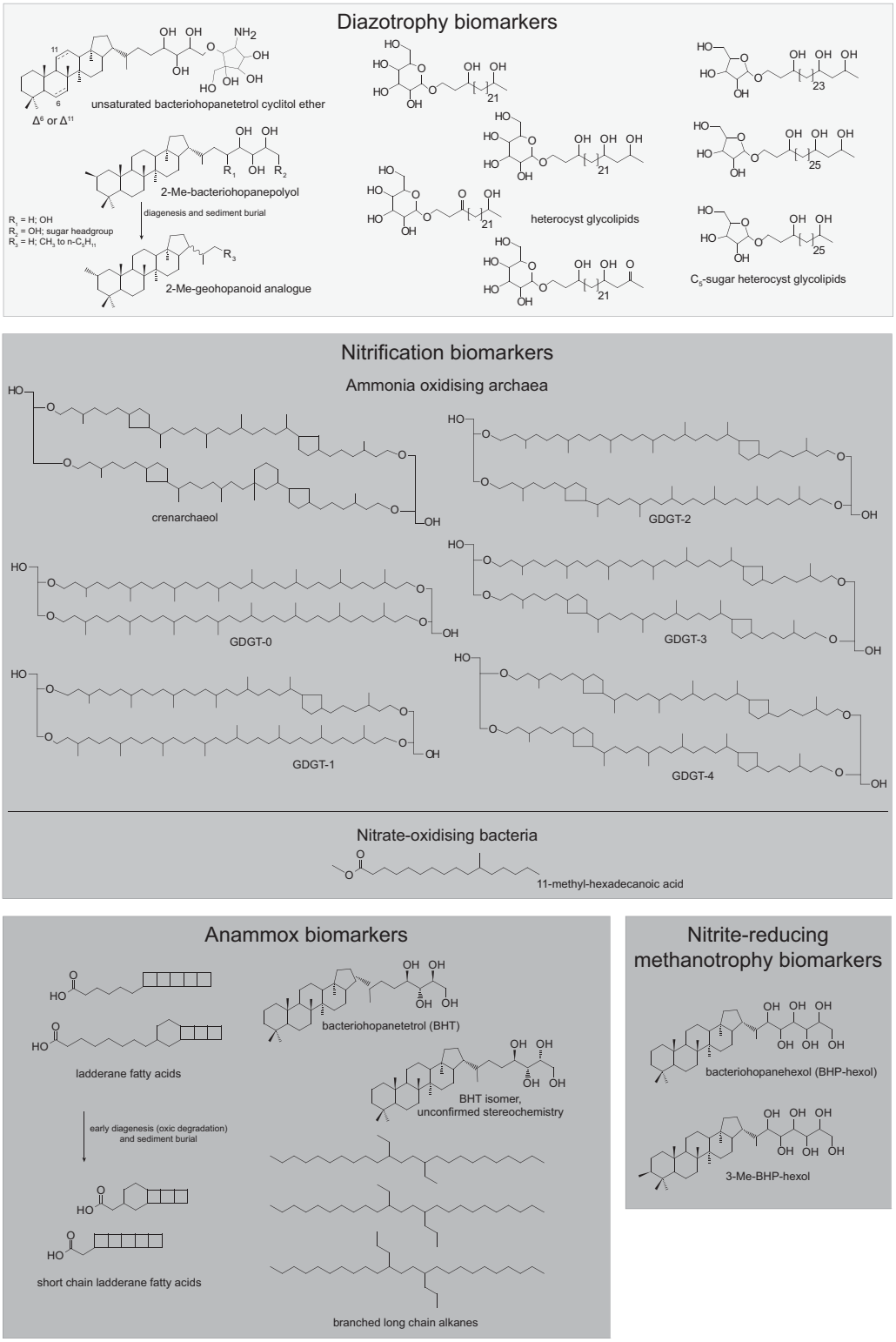

Fig. 2. Biomarkers of microbial processes in the marine nitrogen cycle. Diazotrophy biomarkers: unsaturated bacteriohopanetetrol cyclitol ether; 2-methyl-bacteriohopanepoyols are diagenetically altered in sediment burial to 2-methyl-geohopanoids; heterocyst glycolipids with C_6 and C_5 sugar head groups. Nitrification biomarkers: crenarchaeol and GDGT-0-4; 11-methyl-hexadecanoic acid. Anammox biomarkers: ladderane fatty acids undergo oxic degradation into short chain ladderane fatty acids; bacteriohopanetetrol stereoisomer; anammox bacterial biomass is thermally altered to produce branched long chain alkanes. Nitrite-reducing methanotrophy biomarkers: bacteriohopanehexol; 3-methyl-bacteriohopanehexol.

functionalities glycosidically bound to long-chain (C_{26}–C_{32}) diols, triols, keto-ols, or keto-diols (Fig. 2). The distribution of heterocyst glycolipids in cyanobacteria was screened using high-performance liquid chromatography coupled to electrospray ionisation tandem mass spectrometry, and was found to vary according to taxonomic groups (Bauersachs *et al*., 2009a,b). For example, species belonging to the family *Nostocaceae* synthesise the glycolipids 1-(O-hexose)−3,25-hexacosanediol and 1-(O-hexose)−3-keto-25-hexacosanol, whereas members of the *Calothrix* genus primarily synthesise 1-(O-hexose)−3,25,27-octacosanetriol and 1-(O-hexose)−3-keto-25,27-octacosanediol.

In marine systems, heterocystous cyanobacteria are also commonly found in symbiosis with diatoms (Villareal, 1992). Schouten *et al*. (2013a) found that these endosymbiotic marine cyanobacteria species synthesise heterocyst glycolipids with distinct C-5 sugar moieties (Fig. 2). These have been found in suspended particulate matter and surface sediments from the marine Amazon plume region, and proposed to be specific biomarkers for marine endosymbiotic heterocystous cyanobacteria (Bale *et al*., 2015).

Converting waste into nutrients: Nitrification

Most fixed organic nitrogen is remineralised back into NH_4^+ by heterotrophic bacteria. This pool of reduced nitrogen is then oxidised by nitrifying microorganisms (Fig. 1). Nitrification is the primary source of oxidised nitrogen species in the ocean, converting reduced NH_4^+ to oxidised NO_2^- and NO_3^-. Traditionally, nitrification is divided into two distinct steps, performed by different groups of microorganisms: (1) ammonia oxidation to nitrite [initially thought to be performed by ammonia-oxidising members of Proteobacteria (AOB), and since expanded to include ammonia-oxidising archaea (AOA)], and (2) nitrite oxidation to nitrate (nitrite-oxidising bacteria; NOB) (Ward, 2013a). Recently, specific species of the bacterial genus *Nitrospira* have been identified performing both of the nitrification steps completely [complete ammonia oxidation (comammox); Daims *et al*., 2015; van Kessel *et al*., 2015; Pinto *et al*., 2016].

Lipids of ammonium-oxidising bacteria (AOB)

The most common AOBs in natural environments belong to the genus *Nitrosomonas*. However, the lipids of *Nitrosomonas* spp. are undiagnostic, including generic BHPs (aminobacteriohopanetriol, diploptene) and fatty acids (unsaturated-, *cis*-9- and *trans*−9-hexadecanoic acids) (Sakata *et al*., 2008).

Glycerol dibiphytanyl glycerol tetraethers (GDGTs) of AOA

The discovery that archaea, which dominate the marine mesopelagic zone (Karner *et al*., 2001), were responsible for a significant amount of ammonia oxidation in the ocean was revolutionary (Francis *et al*., 2005; Könneke *et al*., 2005; Wuchter *et al*., 2006). To date, the phylum Thaumarchaeota contains three orders of autotrophic ammonia oxidisers, all of which synthesise a suite of archaeal membrane lipids known as glycerol dibiphytanyl glycerol tetraethers (GDGTs). The stereochemical structure of crenarchaeol, the one abundant GDGT found so far exclusively in Thaumarchaeota was first described by Sinninghe Damsté *et al*. (2002a). Crenarchaeol was named for the 'Creanarchaeota' phylum, the mesopholic branch of which has since been reclassified as 'Thaumarchaeota' (Brochier-Armanet *et al*., 2008; Spang *et al*., 2010). Crenarchaeol contains two cyclopentyl moieties in one of its isoprenoid chains, and one cyclohexyl moiety and two cyclopentyl moieties in its other chain (Fig. 2). AOA also synthesise common and thus non-source-specific GDGT-0 and GDGT-1-4 (Fig. 2). The application of crenarchaeol as a biomarker for AOA has been challenged by the suggestion that Marine Group II Euryarchaeota may synthesise this lipid (Lincoln *et al*., 2014). However, there are no cultivated relatives of Marine Group II. Currently, both phylogenomic analysis of genes responsible for archaeal lipid biosynthesis (Villanueva *et al*., 2017) and environmental data (cf. Schouten *et al*., 2014) point to Thaumarchaeota being the sole source of crenarchaeol. It should be noted that some Thaumarchaeota are not obligate chemolithoautotrophs (Mußmann *et al*., 2011), and the presence of thaumarchaeal GDGTs does not necessarily mean ammonia oxidation has been occurring in the environment.

Arguably the most important application of AOA lipids is to paleo-thermometry. The number of rings in the GDGT structures is temperature-dependent, a fact that has been exploited to create the TEX_{86} paleothermometer (Schouten *et al*., 2002; Kim *et al*., 2010). The TEX_{86} proxy has been applied extensively to determine sea surface temperatures (SST) of paleoenvironments up until the Middle Jurassic (160 Ma) (Jenkyns *et al*., 2012; for full review, see Schouten *et al*., 2013b). Some research has contested whether TEX_{86} does in fact reflect SST (Shah *et al*., 2008; Ho and Laepple, 2016). The paradox of TEX_{86} is that though it seemingly records surface temperature, the Thaumarchaeota responsible for synthesising GDGTs reside in both the shallow and deep water column. Ammonia monoxygenase (amoA) gene sequences have established the division into 'shallow' and 'deep' water

clusters of AOA Thaumarchaota (e.g. Francis *et al*., 2005; Hallam *et al*., 2006; Hu *et al*., 2011). This trend was also observed recently in the production of GDGTs within the water column by evaluating a key gene involved in the lipid biosynthetic pathway coding for GDGT precursor geranylgeranylglyceryl phosphate (GGGP) synthase in Thaumarcheaota (Villanueva *et al*., 2015). These authors observed a positive correlation between an increase of GGGP synthase and the relative proportion of GDGT-2 with water depth, suggesting a niche differentiation in GDGT distributions. Interestingly, this depth speciation was not observed in 16S gene-based phylogeny (Schouten *et al*., 2012). Ammonia availability was suggested to be responsible for the niche partitioning of thaumarchaeota in the Arabian Sea (Villanueva *et al*., 2015). The 'deep' cluster of AOA on the Portuguese continental margin was found to contribute significantly to the GDGTs present in sediments at greater water depth, implying this non-surface population does have an influence on TEX_{86} (Kim *et al*., 2016). Hurley *et al*. (2016) recently suggested that the distribution of GDGTs of thaumarchaeota may be dependant on the nitrification rate. These studies merely highlight the further work required to understand the signal that TEX_{86} records.

Fatty acids of nitrite-oxidising bacteria (NOB)

Nitrospina spp. are the most abundant NOB in marine systems, and dominate microaerophilic zones of OMZs (Lücker *et al*., 2013). Fatty acids of four genera of NOB (*Nitrobacter*, *Nitrococcus*, *Nitrospina*, and *Nitrospira*) have been described, and each genus appears to have its own distinct profile (Auran and Schmidt, 1972; 1976; Lipski *et al*., 2001) (Fig. 2). Although fatty acids are typically not specific enough to be applied as biomarkers, these differences would enable differentiation between NOB bacteria in particular environments. A novel fatty acid, 11-methyl-hexadecanoic acid, was identified in *Nitrospira moscoviensis* (Lipski *et al*., 2001). Unfortunately, however, fatty acids are not recalcitrant enough to endure diagenesis in buried marine sediments, and hence, fatty acids can typically not be used in sediments older than ca. 1 Ma.

Given that the comammox bacteria described performing complete nitrification belong to the genus *Nitrospira*, it is possible that comammox species synthesise 11-methyl-hexadecanoic acid. However, non-comammox species of *Nitrospira* also synthesise 11-methyl-hexadecanoic acid (Lebedeva *et al*., 2011), showing that this fatty acid is not an appropriate biomarker for comammox. The *Nitrospira* genus is diverse. *Nitrospira* has at least six sub-lineages (Daims *et al*., 2001), and has been found in various natural environments (e.g. marine, Watson *et al*., 1986; fresh water, Daims *et al*., 2001; and terrestrial, Lebedeva *et al*., 2011; Pester *et al*., 2014). Lipski *et al*. (2001) found that the two species they studied belonging to *Nitrospira* already showed variations between their lipids. Thus, it is possible that comammox *Nitrospira* spp. have their own distinct fatty acid lipid profile, which has yet to be characterized.

Closing the circle: Denitrifying processes return nitrogen to the atmosphere

In anoxic environments, the system shifts towards favouring organisms that use reaction pathways resulting in net nitrogen loss through the production of N-containing gases, i.e. nitrous oxide (N_2O) and N_2 (Mulder *et al*., 1995; Zumft, 1997). There are two major pathways of nitrogen loss from the marine environment: denitrification and anaerobic ammonium oxidation (anammox) (Fig. 1). In modern marine environments, the production of N_2 by anammox and denitrification represents an important sink of bio-available nitrogen.

Before the discovery of anammox in a waste water treatment plant (Mulder *et al*., 1995), NH_4^+ was thought to be unreactive in anoxic systems. However, in the following decades the bacteria that anaerobically oxidise NH_4^+ using NO_2^- have been found to be prevalent in marine systems (Dalsgaard and Thamdrup, 2002; Kuypers *et al*., 2003; Capone, 2008; Gruber, 2008), especially within OMZs (Kuypers *et al*., 2005; Hamersley *et al*., 2007; Lam *et al*., 2009; Sollai *et al*., 2015). Anammox bacteria are slow-growing, taking weeks to divide (Strous *et al*., 1999), which might be to their advantage in certain systems as they may not have to respond to sudden changes in substrate concentration. For example, the anammox reaction was found consistently, though at low rates, within the Eastern Tropical South Pacific OMZ, whereas the presence of denitrification was sporadic (Dalsgaard *et al*., 2012). This could indicate that anammox represents a constant background loss of nitrogen from anoxic systems.

Ladderane and BHT lipids of anammox bacteria

One of the most challenging puzzles in membrane lipid composition of the last 15 years was the stereochemical structure of lipids found exclusively in anammox bacteria. Strained cyclobutyl moieties require such a high amount of energy to synthesise that they were not thought to exist in nature. However, isolated lipids from anammox bacterial enrichments proved to have three or five concatenated cyclobutyl moieties (Fig. 2; Sinninghe Damsté *et al*., 2002b), and were named 'ladderane lipids' for their 3-dimensional structure, which was later confirmed by total synthesis (Mascitti and Corey, 2004; Mercer *et al*., 2016). Ladderanes play an important role

within the anammox cells. These lipids create a dense membrane bilayer that prevents the diffusion of the toxic intermediate gas (hydrazine) of the anammox reaction into other cell functions. The anammox reaction is thus entirely contained within the organelle-like anammoxosome. Originally thought to be specific to the anammoxosome, ladderane lipids have subsequently been found in other anammox cell fractions (Neumann *et al*., 2014). These results seem to indicate that the anammox bacteria do not possess a mechanism to sort their lipids. Nevertheless, ladderanes are excellent biomarkers because they are uniquely synthesised by anammox bacteria.

Unfortunately, both the strain on the cyclobutyl moieties in addition to the carboxyl functionality of ladderane fatty acids means that these biomarkers do not tolerate diagenesis to a significant extent, and have not been found in mature marine sediments. Recently, a relatively rare stereoisomer of BHT (BHT isomer) was found to be synthesised in high abundance by marine anammox bacteria (Fig. 2; Rush *et al*., 2014a). This BHP was also found in sediments from an anoxic marine fjord. Considering BHPs have been found in sediments of 56 Ma old (Talbot *et al*., 2016), BHT isomer shows potential to fill the gap as a biomarker for anammox in sediments that are not yet thermally mature, but which are older than the limit of diagenetic alteration for ladderanes.

Nitrite-reducing methanotrophy biomarkers

Methylomirabilis oxyfera is an exceptional methanotroph that produces its own oxygen from NO_2^- via the production of NO by nitrite reductase (Ettwig *et al*., 2010). This O_2 is subsequently used to oxidise methane. *M. oxyfera* was shown to synthesise characteristic BHP biomarkers: bacteriohopanehexol (BHP-hexol) and 3-Me-BHP-hexol (Fig. 2; Kool *et al*., 2014). Although the significance of this process within the marine nitrogen cycle is currently unknown, intra-aerobic methanotrophy has recently been detected in marine OMZs, suggesting it may play a role in the nitrogen cycle of these environments (Padilla *et al*., 2016). Using 3-Me-BHP-hexol, we may be able to understand past NO_2^- availability in dynamic OMZ systems, where up to 50% of marine bioavailable nitrogen loss occurs (Codispoti *et al*., 2001; Lam *et al*., 2009).

Anaerobic nitrogen cycle processes without specific biomarker lipids

The importance of dissimilatory nitrate reduction to ammonium (DNRA; Fig. 1) in marine systems is unresolved. DNRA was proposed to be an important source of NH_4^+ in the Peruvian OMZ (Lam *et al*., 2009), but was not detected within the Chilean OMZ (De Brabandere *et al*., 2014).There are currently no biomarker lipids available to trace past shifts of DNRA.

Perhaps the most versatile anaerobic nitrogen cycle process is denitrification, where NO_3^- is reduced via NO_2^-, NO, and N_2O to N_2 (Fig. 1). However, this process is not related to a specific taxonomic group (e.g. autotrophic and heterotrophic bacteria, archaea, and foraminifera can all perform denitrification; Zumft, 1997; Risgaard-Petersen *et al*., 2006), which severely complicates finding biomarkers that are specific to denitrification. It is thus more likely that if we are going to trace denitrification using lipids, it will be with a suite of biomarkers related to individual denitrifiers. Another possible avenue is using anammox biomarkers to infer denitrification. Ward (2013b) proposed that the proportion of anammox is thermodynamically linked to denitrification, where the latter accounts for 70% of N loss from the ocean. If this proves to hold true over nitrogen cycle history, we could reconstruct total nitrogen loss due to anammox and denitrification solely using anammox biomarkers.

Application of biomarkers to interpret nitrogen cycling in the geological past

Nitrogen cycle biomarkers can be applied to the modern ocean, complementing molecular, genetic, and isotope-labeling approaches (e.g. Kuypers *et al*., 2003; 2005; Hamersley *et al*., 2007; Pitcher *et al*., 2011). However, such biomarkers are especially beneficial for the geological past, where these other techniques can no longer be used. Below, we discuss four examples of nitrogen cycle biomarker application.

Ladderane lipids: The importance of anammox in the past arabian sea OMZ

The abundance of ladderane fatty acids was investigated in sediments underlying the Arabian Sea OMZ, spanning the last 140 ka (Fig. 3a; Jaeschke *et al*., 2009). During this time period, the maxima in ladderane lipid concentration correlated with events of high total organic carbon (TOC) content and elevated $\delta^{15}N$. This would appear to indicate enhanced anammox activity coincides with periods of increased intensity in the Arabian Sea OMZ and large-scale changes in the nitrogen cycle (i.e. preferential removal of the lighter ^{14}N isotope). Unfortunately, it has so far not been possible to assess anammox further back in time using ladderane lipids as they are not resistant to the degradative stresses of sediment burial. Ladderane fatty acids undergo β-oxidation in the presence of even very low oxygen concentrations to form short chain ladderane fatty acids, which have been found in sediments underlying OMZs (Rush *et al*., 2011; 2012). However, these too appear to undergo further

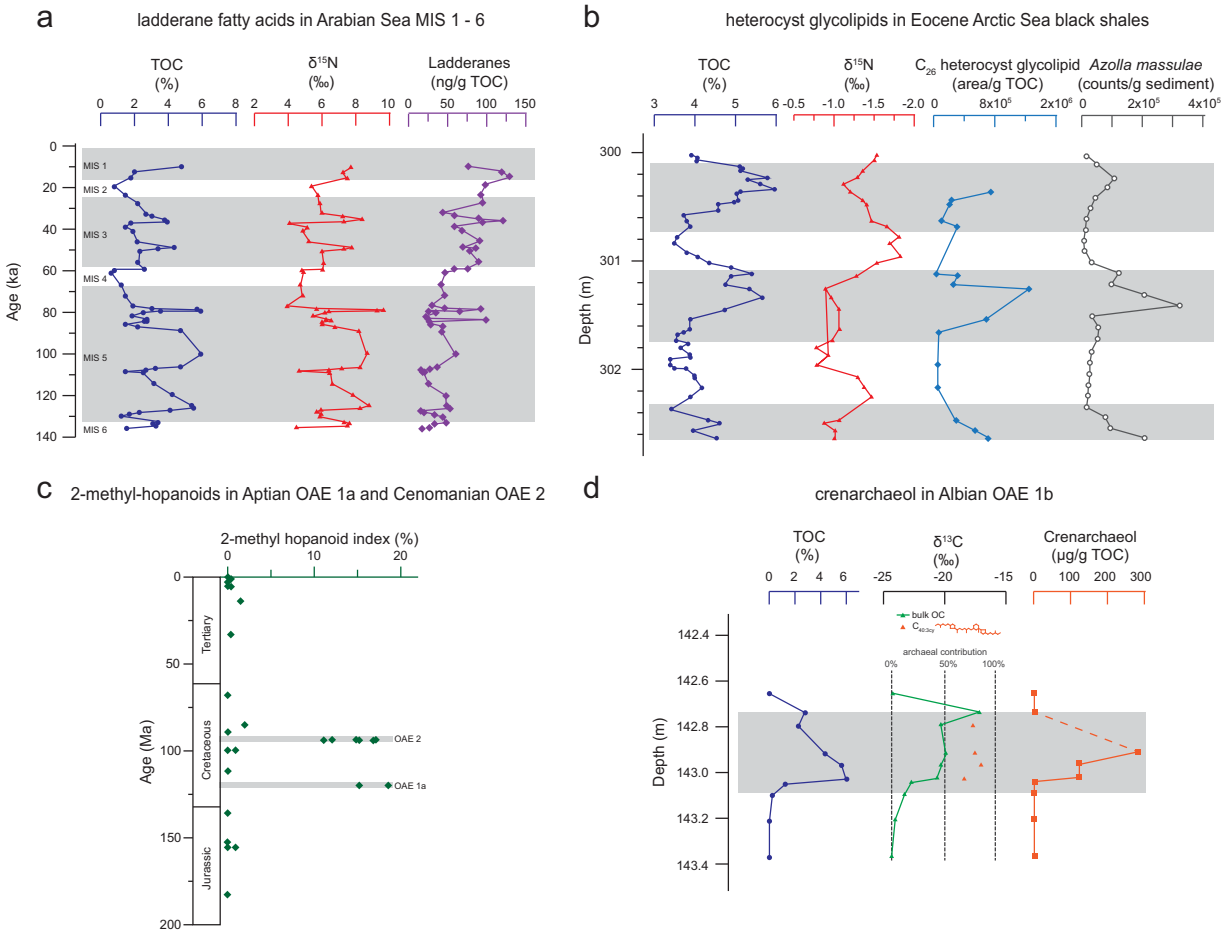

Fig. 3. Biomarker evidence for paleo-nitrogen cycling processes from (a) ladderane fatty acid abundances (Jaeschke *et al.*, 2009) of Arabian Sea sediments underlying the oxygen minimum zone (core MD04-2879) over the past 140 ka, spanning Marine Isotope Stages (MIS) 1 to 6; (b) heterocyst glycolipid abundances (Bauersachs *et al.*, 2010) and *Azolla massulae* counts (Speelman *et al.*, 2009) of Eocene Arctic Ocean sediments including black shale intervals (Integrated Ocean Drilling Project Expedition 302, ACEX); (c) 2-methyl-hopanoids indices (Kuypers *et al.*, 2004) of organic-rich sediment from the last 200 Ma, including the Cenomanian ocean anoxic event (OAE 2) (Deep Sea Drilling Project Sites 144, 367, and 603B, proto North Atlantic) and Aptian OAE 1a (Cismon apticore, Southern Alps, Italy, Aptian Thetys; Deep Sea Drilling Project Site 463, Pacific Ocean). Values represent averages of 1-24 samples.; (d) crenarchaeol abundances and carbon isotopic values of tricyclic biphytanes ($C_{40:3cy}$) derived from archaea (Kuypers *et al.*, 2001) of Albian OAE 1b (Ocean Drilling Project site 1049C). Warm MIS and anoxic events are indicated as grey shaded areas in the graphs. TOC: total organic carbon; $\delta^{15}N$: bulk sediment nitrogen isotope value (in per mil vs. N_{2atm}); 2-methyl-hopanoid index = weighted average of the dominant 2-methyl/2-desmethylhopanoid pairs; $\delta^{13}C$: carbon isotope values (in per mil vs. Vienna Pee Dee Belemnite) for bulk organic carbon (OC) and $C_{40:3cy}$.

degradation, and these Arabian Sea sediments are currently the oldest known detection of past anammox activity (Jaeschke *et al.*, 2009).

In order to potentially reveal degradation lipid products better-suited for past detection of the anammox reaction, conditions undergone during catagenesis of organic matter were simulated; anammox biomass was artificially thermally matured via hydrous pyrolysis (Jaeschke *et al.*, 2008). Ladderane lipids were found to be completely structurally altered at relatively low levels of thermal stress. However, thermally stable products in which some of the cyclobutane rings had opened, could still be detected at 260°C (Jaeschke *et al.*, 2008). Additionally, unusual branched long chain alkanes originating from anammox biomass were identified in the oils generated during these artificial maturation experiments (Fig. 2; Rush *et al.*, 2014b). Investigating these alkanes alongside BHT isomer might be an appropriate approach to detect anammox in thermally more mature sediments such as those deposited in the Cretaceous during so called oceanic anoxic events (OAEs).

Heterocyst glycolipids: Expansion of cyanobacteria during arctic black shale deposition

Like the modern-day Black Sea, the Arctic Ocean during the middle Eocene (ca. 49 Ma) was a warm, stratified basin with fresh surface waters. Such conditions induced

an intense bloom of the free-floating aquatic fern, *Azolla*. *Azolla* microspores occurred abundantly in the palynological record (Brinkhuis *et al*., 2006), and corresponded to increased TOC values. These *Azolla* ferns probably thrived in the fresh Arctic surface waters and high CO_2 atmosphere. The carbon fixed by *Azolla* subsequently represented a significant sink of CO_2 as their biomass was incorporated into buried sedimentary organic matter (Speelman *et al*., 2009). Bauersachs *et al*. (2010) found co-variance in the enhanced input of *Azolla* spores and the abundance of heterocyst glycolipids in these high TOC Eocene Arctic black shale sediments (Fig. 3b). Additionally, the averaged values of the bulk sedimentary nitrogen isotope ratio ($\delta^{15}N$) at the time were $-1‰$, which is consistent with a primarily diazotrophic signal. These results would seem to indicate that diazotrophs lived in symbiosis with *Azolla*, as seen in modern systems (Peters, 1991), and that they supplied fixed nitrogen to a phytoplankton community in the stratified Arctic Ocean. It is possible that N_2-fixation sustained photoautotrophic productivity that formed the black shales of the Eocene Arctic. These results demonstrate the excellent potential of heterocyst glycolipids as biomarkers for past diazotrophy.

2-Methyl-hopanoids: Cyanobacteria sustained primary production during cretaceous OAEs

Past expansions of OMZs have been hypothesised to trigger OAEs (Jenkyns, 2010). OAEs represent one of the most abrupt perturbations of major global biogeochemical cycles of the last 200 Ma, and were accompanied by a rise of the redoxcline to such an extent that sulphide-containing waters occurred in the photic zone, providing a niche for photosynthetic anoxygenic green sulfur bacteria (Sinninghe Damsté and Köster, 1998). This resulted in widespread deposition of organic rich black shales (Arthur and Sageman, 1994). The extreme scenario of an OAE would involve a total reorganisation of the nitrogen cycle.

It is probable that like the Eocene Arctic, surface waters of Cretaceous OAEs 1a and 2 were characterized by increased cyanobacterial activity. The extraordinary abundance of, albeit less source-specific, 2-Me-hopanoids during these OAEs (Fig. 3c) indicates a shift towards diazotrophy as the primary source of nitrogen (Kuypers *et al*., 2004). Increased N_2-fixation could have been induced by limited vertical mixing of deep-water nitrogen species due to a stratification of the water column. It has been proposed that increased denitrification and anammox activity in the expanded anoxic waters of OAEs would have led to a substantial decrease in nitrogen availability (Kuypers *et al*., 2004). In order sustain oceanic primary productivity, this loss of nitrogen would have needed to be compensated by increased cyanobacterial N_2-fixation. This is seemingly apparent by the increased levels of 2-Me hopanoids (Fig. 3c). However, this nitrogen loss hypothesis should still be tested by investigating BHT isomer and branched long chain alkanes in these OAE sediments.

Crenarchaeol: Albian OAE bloom of thaumarchaeota

In disagreement with lipid evidence from OAEs 1a and 2, biomarkers for diazotrophs were not detected in Albian OAE 1b sediments from the proto North Atlantic and Tethys Ocean (Kuypers *et al*., 2001; Tsikos *et al*., 2004). In contrast, OAE 1b sediments contained an unprecedented contribution of crenarchaeol and organic material derived from nitrifying archaea (e.g. Fig. 3d; proto North Atlantic; Kuypers *et al*., 2001). This was evident from the $\delta^{13}C$ isotopic values of TOC, which was substantially enriched ($\sim -20‰$) during OAE 1b, compared with non-OAE conditions ($\sim -25‰$) (Fig. 3d). Neither plant (-28 to $-29‰$; leaf-waxes), algal (-27 to $-29‰$; steroids), nor bacterial (-24 to $-27‰$; hopanes) lipids showed the large change in $\delta^{13}C$ observed for TOC, suggesting a temporary source of ^{13}C-enriched organic matter during OAE 1b (Kuypers *et al*., 2002). Extant Thaumarchaeota use a modified hydroxypropionate-hydroxybutyrate cycle carbon fixation pathway (Berg *et al*., 2010; Könneke *et al*., 2014), which results in ^{13}C-enriched AOA lipids in sediment of the present-day ocean (Schouten *et al*., 2013b). In agreement with this, the tricyclic biphytane ($C_{40:3cy}$), a derived ether-cleaved component of crenarchaeol, is ^{13}C-enriched during OAE 1b (-17 to $-18‰$; Fig. 3). The same holds for AOA-derived macromolecular matter (Kuypers *et al*., 2002). Thus, the ^{13}C enrichment of bulk TOC in OAE 1b was likely due to enhanced archaeal contribution to the bulk organic carbon pool. These results would seem to indicate that AOA played a significant role in the onset and maintenance of OAE 1b.

The reason for the abundance of AOA during OAE 1b remains enigmatic. It seems that only a trigger independent of primary production (the source of organic nitrogen and ultimately ammonium in the present-day ocean) could effect this change. The mid-Cretaceous was a period of increased oceanic crust formation through enhanced volcanism (Larson, 1991). This volcanic activity would have introduced vast amounts of NH_4^+ to the marine water column. As some nitrifiers can function at very low oxygen concentrations, they can thrive at the oxic-anoxic water boundary and provide oxidised nitrogen species to denitrifying organisms in anaerobic niches (Lam and Kuypers, 2011; Füssel *et al*., 2012). During OAE 1b, it is possible that AOA were oxidising volcanic NH_4^+ to NO_2^-, which was provided to denitrifying

organisms. The consequent production of N_2 before this bio-available nitrogen could be exploited by photoautotrophs may have helped maintain water column anoxia during the Albian OAE. In comparison, injection of NH_4^+ from a modern submarine arc-volcano has been shown to support a nitrifying archaea community (closely related to *Nitrosopumilus maritimus*) in Fe-rich microbial mats (Kilias *et al*., 2013). Here, AOA is potentially supplying oxidised nitrogen species to be used to chemical oxidise hydrothermal Fe^{2+} into ferrihydrites.

These results show that there was no universal mechanism for OAE formation and black shale deposition in the Cretaceous. This further underscores the need to better constrain the marine nitrogen cycle during diverse past biogeochemical events in order to predict future change.

Conclusions

Biomarker lipids have been used effectively to constrain past nitrogen cycling. However, available biomarkers are still limited, especially for anaerobic nitrogen cycling processes. Without biomarker observations in paleoenvironments, we cannot predict how the current expansion of marine oxygen minimum and anoxic zones will affect the removal of bio-available nitrogen, nor the feedback mechanisms that will take place within the nitrogen cycle. Accordingly, more robust lipids must be developed as biomarkers for (anaerobic) nitrogen cycle processes.

In order for a biomarker to be used appropriately it must first be rigorously identified. Regrettably, this is often the most time consuming step in the pathway to introducing a novel biomarker. Advances in instrumentation (e.g. two-dimensional nuclear magnetic resonance, X-ray crystallography, scanning probe microscopy, gas chromatography, liquid chromatography, mass spectrometry) have primarily shaped the direction and limitation of biomarker identification/application. Often the biomarker community must wait for the development of improved methods in order to move forward with biomarker innovation. However, it is clearly essential to the advancement of novel biomarker for marine nitrogen cycle processes to invest the time in undertaking these types of investigations.

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