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MINI-REVIEW

New processes and players in the nitrogen cycle: the microbial ecology of anaerobic and archaeal ammonia oxidation

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Microbial activities drive the global nitrogen cycle, and in the past few years, our understanding of nitrogen cycling processes and the micro-organisms that mediate them has changed dramatically. During this time, the processes of anaerobic ammonium oxidation (anammox), and ammonia oxidation within the domain *Archaea*, have been recognized as two new links in the global nitrogen cycle. All available evidence indicates that these processes and organisms are critically important in the environment, and particularly in the ocean. Here we review what is currently known about the microbial ecology of anaerobic and archaeal ammonia oxidation, highlight relevant unknowns and discuss the implications of these discoveries for the global nitrogen and carbon cycles.
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Introduction

The element nitrogen (N) is an essential nutrient for all organisms, and as a critical component of proteins, N is fundamental to the structures and biochemical processes that define life. N is of such centrality that it has been suggested to be perhaps the best bio-signature for life on other planets (Capone *et al.*, 2006), yet our understanding of how this element is cycled on Earth has changed drastically in just the last few years. Here N exists in multiple oxidation states and chemical forms, and is rapidly converted by micro-organisms on land and in the sea. Until recently, the global N cycle was thought of as essentially 'linear' from the atmosphere and back again. The largest reservoir of N on Earth is triple-bonded N_2 gas (78% of the atmosphere) and must be fixed by microorganisms before it is readily useable by other organisms. N exists in its most reduced state within organisms, but is rapidly nitrified to nitrate (aerobically) when released following cell death and lysis. Nitrate is in turn denitrified to N_2 gas under suboxic to anoxic conditions, completing the cycle (Figure 1).

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Along this flowpath, micro-organisms directly catalyze the processes of nitrification and denitrification, but these two functional groups are a study in contrasts. In the conventional view of nitrification ($NH_3 \rightarrow NO_2^- \rightarrow NO_3^-$), the metabolic labour is divided between two distinct groups of organisms, ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). Ammonia oxidation is typically thought to be an obligatory aerobic, chemoautotrophic process restricted to just a few groups within the Proteobacteria (Kowalchuk and Stephen, 2001). In contrast, denitrification is primarily heterotrophic, facultative, occurs under low-oxygen conditions and is widespread among over 50 different genera (Zumft, 1997), including members of the *Bacteria, Archaea*, and – based on the surprising discovery of complete denitrification in a benthic foraminifer (Risgaard-Petersen *et al.*, 2006) – *Eukarya*. Known denitrifying bacteria and archaea posses several clusters of genes involved in denitrification (Philippot, 2002), and most are therefore capable of performing the multi-step process in its entirety ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$).

Much is now known about these processes and many of the micro-organisms involved, yet our understanding of the N cycle has been upended twice in the past few years, first by the discovery of *anaerobic* ammonium oxidation in natural systems, and more recently by the discovery of *aerobic* ammonia oxidation within the domain *Archaea*. Aerobic oxidation of ammonia by bacteria was first

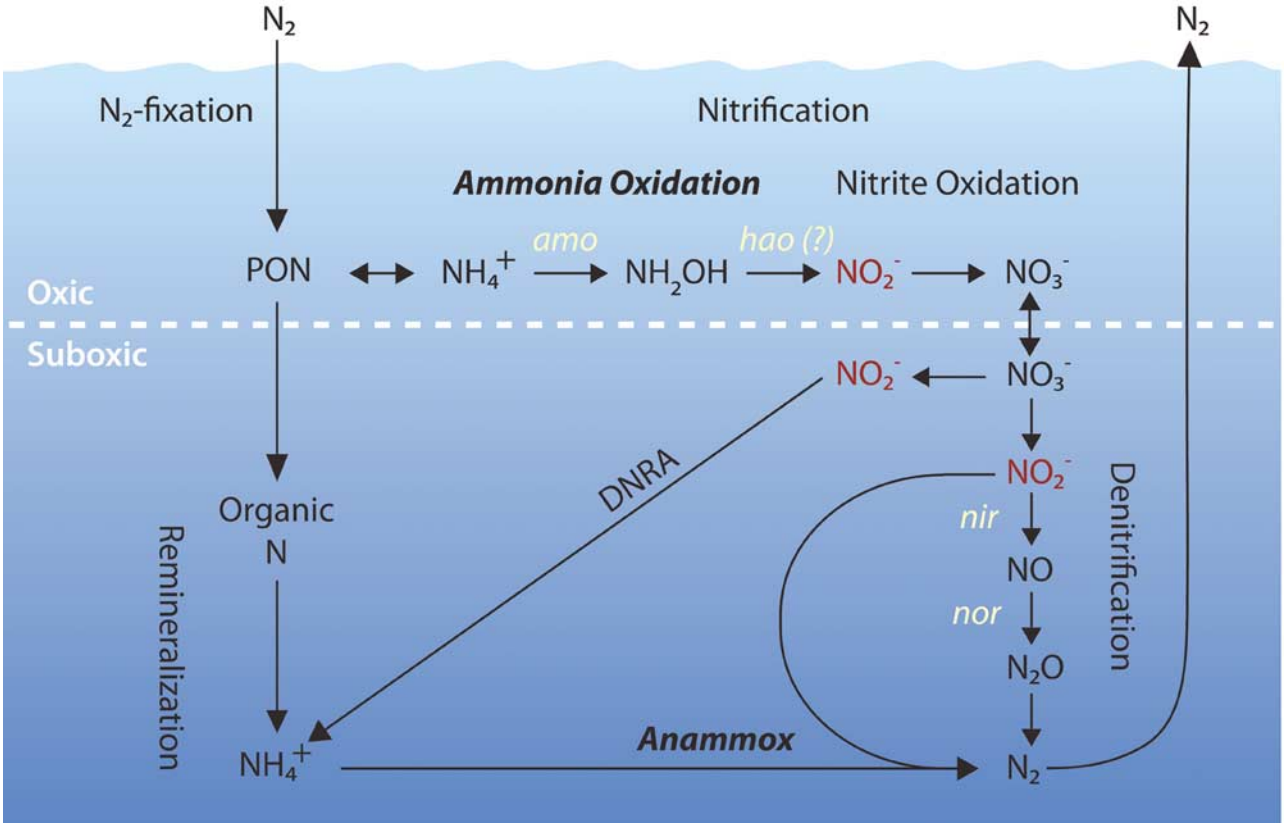

Figure 1 Microbial nitrogen transformations above, below and across an oxic/anoxic interface in the marine environment (based in part on Arrigo, 2005). Nitrite is highlighted in red to emphasize the central role of this metabolic intermediate/product within and between N-cycling pathways. Key functional genes discussed in the text are shown in yellow: *amo*, ammonia mono-oxygenase; *hao*, bacterial hydroxylamine oxidoreductase (? = unknown gene/enzyme in AOA); *nir*, nitrite reductase; and *nor*, nitric oxide reductase. For clarity, other functional genes and the process of nitrate/nitrite assimilation are not shown.

discovered over 100 years ago (Winogradsky, 1890), underlining just how rapidly these two major discoveries have taken place. In this review, we focus on recent developments related to the microbial ecology of anaerobic and archaeal ammonia oxidation. We expand upon existing reviews that cover various aspects of the microbial N cycle (Kowalchuk and Stephen, 2001; Zehr and Ward, 2002; Strous and Jetten, 2004; Arrigo, 2005; Kuypers *et al.*, 2006; Nicol and Schleper, 2006; Revsbech *et al.*, 2006), and focus particularly on archaeal ammonia oxidation, because this very recent discovery is an area of remarkably active research.

Anammox

Since the mid-1960s, oceanographers have recognized pervasive ammonium deficits in anoxic basins that hinted at the possible removal of ammonium by anaerobic microbial activity (Richards, 1965). Nevertheless, for the remainder of the century, heterotrophic denitrification was considered the only sink for fixed nitrogen under anoxic conditions in natural systems. The first evidence for anaerobic ammonium oxidation (anammox) to N_2 gas was obtained from anoxic (denitrifying) bioreactors of wastewater treatment plants (WWTPs) (Mulder *et al.*, 1995), where it was eventually determined that novel organisms related to Planctomycetales were capable of oxidizing ammonium using nitrite (rather than O_2) as the electron acceptor (Strous *et al.*, 1999). Befitting micro-organisms capable of such a novel metabolism, these 'anammox' bacteria have a number of truly unique features, including the use of hydrazine (N_2H_4, i.e., rocket fuel) as a free catabolic intermediate, the biosynthesis of ladderane lipids and the presence of an anammoxosome (intracytoplasmic compartment). All four currently recognized genera of anammox bacteria – *Candidatus* 'Brocadia', 'Kuenenia', 'Scalindula', and 'Anammoxoglobus' – share these unique physiological and morphological features.

Owing to their distinct metabolism and physiology, anammox bacteria received considerable attention in engineered systems, but were assumed to be minor players in the N cycle within natural ecosystems. However, in 2002, Thamdrup and Dalsgaard found anammox to be responsible for 24–67% of N loss in marine sediments (Thamdrup and Dalsgaard, 2002), and in 2003, two parallel studies demonstrated that anammox was directly

responsible for a substantial fraction of N loss in the ocean (Dalsgaard *et al.*, 2003; Kuypers *et al.*, 2003). In fact, 20–40% of N loss could be attributed to anammox in the suboxic water columns of the Black Sea and Gulfo Dulce (Dalsgaard *et al.*, 2003; Kuypers *et al.*, 2003), and more recent studies indicate that, if anything, anammox is responsible for an even greater percentage of marine N loss (Kuypers *et al.*, 2005; Hamersley *et al.*, 2007). The now well-established significance of anammox in the marine environment has emerged from a combination of ^{15}N-based tracer studies, analysis of ladderane lipid biomarkers, fluorescent *in situ* hybridization and phylogenetic and quantitative PCR analysis of 16S rRNA sequences (Schmid *et al.*, 2005). To date, anammox has been documented in marine, coastal and estuarine sediments (Thamdrup and Dalsgaard, 2002; Trimmer *et al.*, 2003; Risgaard-Petersen *et al.*, 2004; Rysgaard *et al.*, 2004; Engstrom *et al.*, 2005), anoxic basins (Dalsgaard *et al.*, 2003; Kuypers *et al.*, 2003), oxygen minimum zones (OMZs) off of West Africa, Chile and Peru (Kuypers *et al.*, 2005; Thamdrup *et al.*, 2006; Hamersley *et al.*, 2007), mangroves (Meyer *et al.*, 2005), sea-ice (Rysgaard and Glud, 2004) and freshwater lakes – including Lake Tanganyika, the second largest lake in the world (Schubert *et al.*, 2006). A recent PCR-based study using newly designed primers detected 'Scalindula'-like 16S rRNA genes in a variety of freshwater and marine sediments, as well as permafrost soil (Penton *et al.*, 2006). Although anammox activity has yet to be measured in soils, the occurrence of anammox in this broad array of aquatic environments suggests that this process is truly ubiquitous, and it seems likely that anammox will be found in virtually any N-containing ecosystem with a pronounced suboxic zone or chemocline.

In addition to their widespread distribution, anammox bacteria appear to be far more metabolically versatile than previously thought: these organisms have the capacity to couple the oxidation of various organic acids to the reduction of nitrate (Guven *et al.*, 2005); *Candidatus* 'Anammoxoglobus propionicus' outcompeted other anammox bacteria and heterotrophic denitrifiers for the oxidation of propionate in the presence of ammonium, nitrite and nitrate (Kartal *et al.*, 2007b); the capacity to respire iron and manganese oxides was suggested by the large number of encoded *c*-type cytochromes in the *Kuenenia stuttgartiensis* genome (by analogy to the metal-respiring bacteria *Geobacter* and *Shewanella*), and this ability was confirmed experimentally (Strous *et al.*, 2006). In addition, *K. stuttgartiensis* is capable of dissimilatory nitrate reduction to ammonium (DNRA), reducing NO_3^- to NH_4^+ even in the presence of 10 mM NH_4^+ (Kartal *et al.*, 2007a). Regardless of whether DNRA is performed by anammox or other (facultatively) anaerobic micro-organisms *in situ*, DNRA could provide NH_4^+ for anammox, and because this NH_4^+ is eventually lost as N_2 gas, the whole process is effectively concealed as denitrification – in other words, an even larger fraction of N loss from anoxic systems may be driven by anammox organisms. In fact, evidence for DNRA has been detected in the Benguela upwelling system (Kartal *et al.*, 2007a), where anammox bacteria actively remove massive amounts of N (Kuypers *et al.*, 2005).

Coupled DNRA–anammox is indistinguishable from denitrification based on most conventional isotope tracer experiments, such that more sophisticated tracer methods and/or gene marker studies are needed to assess the importance of this process in the environment. Although a calcium-dependent cytochrome *c* protein with a high rate of nitrite reduction to ammonium was partially purified from *K. stuttgartiensis*, and candidate genes for this enzyme were putatively identified in the *K. stuttgartiensis* genome (Kartal *et al.*, 2007a), there is currently no established functional gene marker for analyzing anammox bacteria in the environment. This is in clear contrast to other N-cycling microorganisms. For example, functional genes encoding nitrite reductase (*nirK* and *nirS*) and ammonia mono-oxygenase (*amoA*) are commonly used molecular markers for studying communities of denitrifiers and aerobic ammonia oxidizers, whereas only 16S rRNA genes have been used to detect anammox bacteria. However, functional genes encoding the most defining metabolic feature of anammox – hydrazine metabolism – are beginning to be identified: candidate hydrazine hydrolase and hydrazine dehydrogenase genes have been identified in the *K. stuttgartiensis* genome (Strous *et al.*, 2006), and a hydrazine-oxidizing enzyme (HZO) and the underlying genes have recently been identified in a related organism (Shimamura *et al.*, 2007). Once these functional genes are definitively and specifically linked to anammox, analysis of anammox functional gene abundance and expression in the environment becomes a real possibility.

At the global scale, the overall contribution of anammox to global N loss is still unclear. On land, few if any measurements of anammox activity exist, but the widespread occurrence of anammox in marine sediments and suboxic water columns indicates that this process could be responsible for a substantial proportion of fixed N loss from the ocean. In marine sediments, the proportion of N loss that can be attributed to anammox appears to increase with water depth (Thamdrup and Dalsgaard, 2002; Trimmer *et al.*, 2003; Risgaard-Petersen *et al.*, 2004; Rysgaard *et al.*, 2004; Engstrom *et al.*, 2005); this suggests anammox may be important in slope and hemipelagic sediments, where modeling results indicate that the majority of benthic N losses take place (Middelburg *et al.*, 1996). A substantial portion of marine N loss occurs in oceanic water columns, specifically in spatially-constrained OMZs over a relatively limited depth range. To the best of our knowledge, there is so far little published evidence from ^{15}N-labelling experiments that nitrate

is directly converted into N_2 by heterotrophic denitrifiers in these regions, whereas anammox appears to dominate N_2 production in all published experiments to date (Kuypers *et al.*, 2005; Thamdrup *et al.*, 2006; Hamersley *et al.*, 2007). Altogether, these studies indicate that anammox is probably responsible for 30–50% of all marine N loss (Kuypers *et al.*, 2006).

Ammonia-oxidizing archaea

As anammox continues to be explored as a 'new' process in the N cycle, new 'players' in the N cycle have also been identified, and they are among the most widely distributed and abundant groups of micro-organisms on the planet – the mesophilic *Crenarchaeota*. Although archaea were previously characterized as extremophiles, mesophilic archaea are now recognized to be an ubiquitous component of marine plankton (DeLong, 1992; Fuhrman *et al.*, 1992), with the marine 'group 1' clade of *Crenarchaeota* alone comprising over 20% of picoplankton in the world ocean (Karner *et al.*, 2001). These organisms are estimated to number a staggering 10^{28} cells in total; however, because of our inability to cultivate them, for the last 15 years our understanding of their physiology and biogeochemical function remained almost entirely speculative. Remarkably, two complementary metagenomic studies of seawater (Venter *et al.*, 2004) and soil (Treusch *et al.*, 2005) revealed putative ammonia mono-oxygenase (*amoA*) genes derived from uncultivated *Crenarchaeota*, suggesting the genetic capacity for ammonia oxidation. More specifically, Venter *et al.* identified an *amoA*-like gene on an archaeal-associated scaffold, whereas Treusch *et al.* found a similar gene on the same 43-kb soil DNA fragment as a 16S rRNA gene derived from a member of the group 1.1b *Crenarchaeota* – the most widespread crenarchaeal group in soils (Ochsenreiter *et al.*, 2003).

The definitive link between these novel *amoA* genes and archaeal ammonia oxidation was recently and convincingly established by cultivation of an ammonia-oxidizing crenarchaeon – designated *Nitrosopumilus maritimus* – from a saltwater aquarium (Könneke *et al.*, 2005). *N. maritimus* grows chemoautotrophically to cell densities of 10^7 ml^{-1} via the near-stoichiometric conversion of ammonia into nitrite, and with bicarbonate as a sole carbon source (organic carbon actually inhibited growth) (Könneke *et al.*, 2005). 16S rRNA phylogeny places this organism firmly in the group 1.1a *Crenarchaeota* – the first cultivated representative from this exceptionally abundant archaeal group – and it contains putative ammonia mono-oxygenase genes *amoA*, *amoB* and *amoC*.

Hot on the heels of this discovery came the first molecular evidence demonstrating the archaeal *amoA* gene to be pervasive in areas of the ocean that are critical for the global nitrogen cycle – including the base of the euphotic zone, suboxic water columns and coastal/estuarine sediments (Francis *et al.*, 2005). For the first time, these data indicated that many marine *Crenarchaeota* might be capable of ammonia oxidation, and these organisms were identified as putative ammonia-oxidizing archaea (AOA) (Francis *et al.*, 2005). In addition, phylogenetic analysis of several hundred archaeal *amoA* sequences revealed diverse and distinct AOA communities associated with different habitats and sampling sites, with little overlap between water columns and sediments. Considering that only a few genera of Bacteria (*Nitrosomonas*, *Nitrosospira* and *Nitrosococcus*) were thought to be involved in ammonia oxidation (Purkhold *et al.*, 2000), this diversity among AOA is all the more remarkable.

Following these initial insights, our picture of AOA in the environment has become more complete and more compelling. For example, AOA appear to be active in natural samples, based on quantification of *amoA* gene expression in soil microcosms, specifically increased *amoA* expression in the presence of elevated ammonia concentrations (Treusch *et al.*, 2005). Metagenomic analysis of the only other established species within the marine group 1.1a *Crenarchaeota*, the uncultivated sponge symbiont *Cenarchaeum symbiosum*, identified putative ammonia mono-oxygenase genes (*amoA, amoB, amoC*), as well as homologs of ammonia permease, urease, a urea transport system, putative nitrite reductase and nitric oxide reductase accessory protein – all potentially associated with chemoautotrophic ammonia oxidation (Hallam *et al.*, 2006a, b). Surprisingly, homologs for critical components of the second enzymatic step of bacterial ammonia oxidation – hydroxylamine oxidoreductase and cytochromes c_{554} and c_{552} – were not identified (Hallam *et al.*, 2006b), indicating that if *C. symbiosum* is in fact capable of ammonia oxidation, it apparently employs a different mechanism than known AOB for catalyzing this key reaction (Hallam *et al.*, 2006a). This has been interpreted by some as evidence that archaeal ammonia oxidation evolved fairly late by incorporating an AMO-like biochemical function into an ammonia-independent metabolism (Klotz *et al.*, 2006).

Multiple components of a modified 3-hydroxypropionate cycle (for autotrophic carbon assimilation) – as well as genes predicted to encode a nearly complete oxidative tricarboxylic acid cycle (consistent with organic carbon consumption) – were also present in *C. symbiosum*, suggesting that this organism has the potential to function either as a strict autotroph, or as a mixotroph utilizing both CO_2 and organic material as carbon sources (Hallam *et al.*, 2006a, b). In fact, organic geochemical evidence indicate that either or both of these metabolic lifestyles are real possibilities. Previous studies have demonstrated uptake of organic compounds by

archaea in the form of amino acids (Ouverney and Fuhrman, 2000; Herndl *et al.*, 2005; Teira *et al.*, 2006), whereas signatures of chemoautotrophy have been identified among the archaea based on the carbon isotopic composition of archaeal membrane lipids and remains (Kuypers *et al.*, 2001; Pearson *et al.*, 2001), and the direct incorporation of ^{13}C-labeled bicarbonate into crenarchaeal lipids in the absence of light (Wuchter *et al.*, 2003). Most recently, Ingalls *et al.* used the natural distribution of radiocarbon in archaeal membrane lipids to quantify the bulk carbon metabolism of archaeal communities at two depths in the subtropical North Pacific gyre. In surface waters, archaea incorporated modern carbon into their membrane lipids, whereas archaea at 670 m incorporated carbon that was isotopically enriched relative to inorganic carbon at the same depth (Ingalls *et al.*, 2006). On the basis of an isotopic mass balance model, 83% of *in situ* archaeal production at 670 m was estimated to be chemoautotrophic, and the balance heterotrophic. It remains to be determined whether this is indicative of archaeal mixotrophy, or a mixed community of autotrophic and heterotrophic archaea dominated by autotrophs.

Given the sheer numbers of *Crenarchaeota* in the ocean and evidence that many are AOA, it is not surprising that AOA appear to be much more abundant than AOB (Leininger *et al.*, 2006; Wuchter *et al.*, 2006; Mincer *et al.*, in press), which typically comprise $<0.1\%$ of microbial communities (Ward, 2000). In the North Sea and North Atlantic, archaeal *amoA* gene copy numbers were 10–1000 times those of betaproteobacterial *amoA* and correlated with cell counts of *Crenarchaeota* (Wuchter *et al.*, 2006). In a study of 12 pristine and agricultural soils spanning three climate zones, archaeal *amoA* gene copy numbers were up to 3000 times those of the betaproteobacteria and correlated with *Crenarchaeota*-specific lipids, including crenarchaeol (Leininger *et al.*, 2006). Reverse transcription quantitative PCR studies and complementary DNA analysis demonstrated the expressional activity of AOA *in situ*, and supported the numerical dominance of AOA over AOB. Together, these findings suggest that most mesophilic *Crenarchaeota* are AOA, and that these organisms are the numerically dominant ammonia oxidizers in the ocean and in soils.

Adding to this emerging paradigm of AOA ubiquity, AOA have also recently been detected in nitrifying wastewater treatment bioreactors (Park *et al.*, 2006), where 50 of 75 archaeal *amoA* sequences recovered in activated sludge from Oregon, Wisconsin, Pennsylvania and New Jersey were virtually identical. In estuarine sediments from Bahía del Tóbari, Mexico, AOA communities from the interior of the estuary were phylogenetically distinct from those found at the mouths of the estuary, and the distribution of these two archaeal *amoA* 'ecotypes' was consistent with *amoA* genes being widespread within both group 1.1a and 1.1b *Crenarchaeota* (Beman and Francis, 2006). Closely related archaeal *amoA* sequences were recently recovered from an Austrian radioactive thermal spring (Weidler *et al.*, 2007) and a Colorado geothermal mine adit (Spear, Barton, Robertson, Francis and Pace, unpublished results), which suggests AOA may be important in subsurface/cave ecosystems, and could be indicative of crenarchaeal ammonia oxidation at higher temperatures (for example, 45–50°C). Although these subsurface environments are not truly 'hyperthermophilic', non-marine crenarchaeol has been detected in Nevada hot springs with temperatures from 40°C to 84°C (Pearson *et al.*, 2004; Zhang *et al.*, 2006), and archaeal *amoA* genes also seem to occur widely in terrestrial hot springs up to 86°C (Zhang *et al.*, unpublished results). Reports of N-fixation in 53.5-63.4°C hot spring cyanobacterial mats (Steunou *et al.*, 2006), and at 92°C in a deep-sea hydrothermal vent methanogen (Mehta and Baross, 2006), may be indicative of active N cycling in high-temperature environments. At this stage, it is unclear whether *amoA* genes are also associated with some thermophilic *Crenarchaeaota* lineages, or whether the thermotolerance and ecological niche of 'mesophilic' *Crenarchaeota* is more extensive than currently thought; both are certain to be areas of active research.

Recent results from the North Pacific Ocean add at least one piece to this puzzle. Here Mincer *et al.*, (in press) found discrepancies between archaeal *amoA* gene copy numbers and crenarchaeal 16S rRNA gene copy numbers, where *amoA* was several orders of magnitude more abundant than 16S rRNA at certain depths. However, when 16S rRNA genes corresponding to the deeply branching pSL12 clade (originally discovered in a Yellowstone hot spring; Barns *et al.*, 1996) were specifically quantified, crenarchaeal *amoA* and 16S rRNA gene copy numbers were more comparable. This intriguing finding suggests that the pSL12 clade – which has only been reported a few times previously in the marine environment (Vetriani *et al.*, 1999; van der Wielen *et al.*, 2005; Zaballos *et al.*, 2006) – may be widespread and at times abundant in the ocean. These data can also be interpreted as evidence for *amoA* within the pSL12 clade, potentially adding to the list of crenarchaeal groups known to be capable of ammonia oxidation (Mincer *et al.*, in press). Finally, corroborating a growing number of studies, direct comparison of AOA and AOB abundance in the North Pacific Gyre and Monterey Bay showed AOA to be up to two orders of magnitude more abundant than their often undetectable betaproteobacterial counterparts (Mincer *et al.*, in press).

New paradigms

Our understanding of these new processes and players in the microbial N cycle has evolved in

opposite directions for anammox and the AOA. In the case of anammox, initial observations based on biogeochemistry led to the discovery of these organisms in the environment, and we are only now beginning to determine the biochemical pathways and genes involved in anammox. AOA were first identified via functional gene sequences recovered directly from the environment, and we still do not know what their full contributions are to N biogeochemistry.

The lesson to be learned from this is that neither of these processes can be fully characterized without employing multiple, complimentary approaches – including molecular approaches, metagenomics, cultivation and (bio)geochemistry – to address a growing number of questions. For example, how do the diversity, abundance and activity of these organisms compare with their presumed competitors, the denitrifiers and AOB? Anammox is the dominant N loss process in several OMZs, but is this true for all? Are there times and places in the ocean where conventional denitrification dominates N loss? And why does denitrification appear to be of greater significance in sediments and lakes? Similarly, AOA are far more abundant than AOB in a number of environments – are there exceptions? Is this true with regard to their activity? Are these organisms truly 'functionally equivalent'? Do AOA, AOB and anammox bacteria and their respective 'ecotypes' respond differently to environmental perturbations and gradients (for example N, oxygen, light, salinity)?

Ultimately, a new N cycle paradigm will only emerge through considering all of these processes and microbial groups together, particularly in their use of common substrates and in environments where they coexist. For example, AOA, AOB, anammox bacteria and denitrifiers all appear to possess nitrite reductase (*nirK/S*) genes (Casciotti and Ward, 2001; Treusch *et al.*, 2005; Strous *et al.*, 2006; Hallam *et al.*, 2006b; Cantera and Stein, 2007). Although in some cases these genes may only be involved in nitrite detoxification, this parallel suggests that nitrite could act as an important substrate for all of these organisms. More broadly, if most of the 10^{28} *Crenarchaeota* cells in the ocean are capable of ammonia oxidation (as all available evidence indicates), and do so even at fairly low rates, the fact that nitrite only rarely accumulates in the ocean necessitates a large and active pool of nitrite-oxidizing organisms. In direct support of this idea, correlation between the quantitative depth distributions of planktonic crenarchaea and 16S rRNA sequences similar to those of known nitrite-oxidizing *Nitrospina* species suggests metabolic coupling between AOA and NOB (via nitrite) in the ocean (Mincer *et al.*, in press).

The distribution of the radiatively active trace gas nitrous oxide (N_2O) may also be strongly influenced by these organisms: AOB (Casciotti and Ward, 2005), denitrifiers, and now AOA (Hallam *et al.*, 2006b)

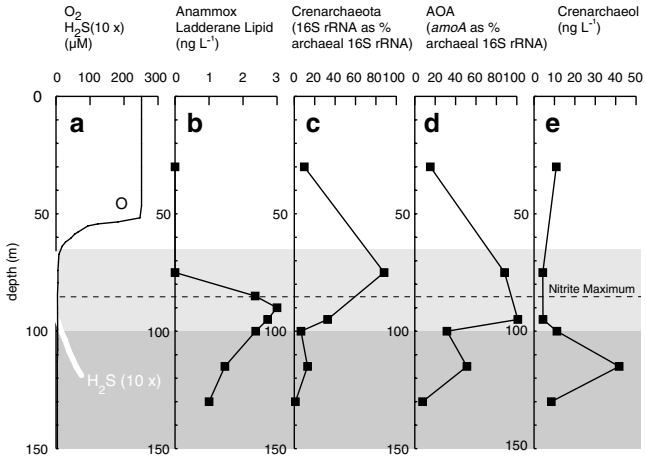

Figure 2 Depth profiles from the Black Sea of (**a**) oxygen (black line) and sulphide (white line); (**b**) the anammox ladderane lipid FAME 1; (**c**) *Crenarchaeota* (marine group 1.1a *Crenarchaeota* 16S rRNA genes as a percent of total archaeal 16S rRNA genes); (**d**) AOA (archaeal *amoA* genes as a percent of total archaeal 16S rRNA genes); and (**e**) the (cren)archaeal membrane lipid crenarchaeol. The suboxic zone ($O_2 < 3\,\mu M$) is indicated by light grey shading, and the sulfidic zone ($H_2S > 0\,\mu M$) by dark gray shading. Dashed line indicates depth of nitrite maximum ($NO_2^- > 0.3\,\mu M$). Similarities and differences in the distributions of anaerobic and archaeal ammonia oxidizers may or may not be indicative of direct metabolic coupling. Data replotted from Kuypers *et al.* (2003) and Coolen *et al.* (2007).

possess nitric oxide reductase (*nor*) genes that could be involved in N_2O production. Anammox bacteria are now known to produce N_2O as well (Kartal *et al.*, 2007a) – not as an intermediate of the anammox reaction but apparently as a result of NO detoxification. Even without AOA and anammox in the mix, the sources of N_2O are highly uncertain; with two new potential contributors, our understanding of how, where and why N_2O is produced may need rethinking.

Production of N_2O is likely to be of particular importance under suboxic conditions, and here our understanding of anammox and the AOA – and the N cycle in general – is at its most muddled. The fact that anammox appears to dominate N loss in OMZs seriously challenges our understanding of organic matter remineralization in these regions. Without remineralization of NH_4^+ from organic matter via denitrification, it is unclear how anammox could be sustained; one possibility is that microaerobic heterotrophs are responsible for regenerating N under low, but non-zero, oxygen conditions commonly found in OMZs. Although AOA are presumably aerobic ammonia oxidizers, AOA (Francis *et al.*, 2005; Coolen *et al.*, 2007) and the archaea generally (Kuypers *et al.*, 2001; Sinninghe Damste *et al.*, 2002), appear to be remarkably successful under low oxygen conditions. We know anammox bacteria and AOA are found at the same depths in the Black Sea (Kuypers *et al.*, 2003; Francis *et al.*, 2005; Kirkpatrick *et al.*, 2006; Coolen *et al.*, 2007), and we assume elsewhere; do they compete for

dissolved NH_4^+ under low oxygen, or are there potentially beneficial interactions among these organisms via the supply of critical substrates, such as nitrite? For example, in the CANON (Completely Autotrophic Nitrogen removal Over Nitrite) process (Third *et al.*, 2001), AOB provide nitrite and also create anoxic microenvironments for anammox bacteria (via O_2 consumption) – do AOA play a similar co-operative role in natural systems? Correlations between AOA and anammox bacteria in OMZs – similar to those observed between Archaea and NOB (Mincer *et al.*, in press) – would be good evidence of this and should be explored. For example, Coolen *et al.* (2007) found archaeal *amoA* gene copies to be highest at the same suboxic depth where anammox bacteria were also abundant in the Black Sea (Kuypers *et al.*, 2003) (see Figure 2). Surprisingly, crenarchaeol concentrations were highest in the sulfidic zone of the Black Sea. These lipids may be derived from crenarchaeal remains sinking through the water column, however archaeal communities recovered in this zone were very different from those found at other depths, suggestive of active growth (Coolen *et al.*, 2007). There are a number of possible interpretations of these data, but it does seem clear that marine *Crenarchaeota* have considerable metabolic and ecological flexibility, based on their presence under sulfidic conditions and evident success in suboxic environments.

Here, too, the interplay between N cycle and the carbon cycle becomes particularly salient. If N loss in OMZs is principally driven by the autotrophic process of anammox rather than heterotrophic denitrification, what happens to organic carbon, if it is not remineralized via denitrification? With regard to the archaea, their exceptional numbers mean that even if they perform chemoautrophic ammonia oxidation at relatively low rates, they still represent a substantial source of carbon in the deep ocean (Herndl *et al.*, 2005; Ingalls *et al.*, 2006) – is this truly the case? Although answers to some of these questions have already begun to emerge, it is clear that the microbial ecology of anammox bacteria and AOA will be an area of active research for years to come, and will be essential to our understanding of the global N and carbon cycles going forward.

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