Environmental Microbiology (2008) **10**(11), 2931–2941 doi:10.1111/j.1462-2920.2008.01775.x

Minireview

Relative contributions of archaea and bacteria to aerobic ammonia oxidation in the environment

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Summary

Traditionally, organisms responsible for major biogeochemical cycling processes have been determined by physiological characterization of environmental isolates in laboratory culture. Molecular techniques have, however, confirmed the widespread occurrence of abundant bacterial and archaeal groups with no cultivated representative, making it difficult to determine their ecosystem function. Until recently, ammonia oxidation, the first step in the globally important process of nitrification, was thought to be performed almost exclusively by bacteria. Metagenome studies, followed by laboratory isolation, then demonstrated the potential for significant ammonia oxidation by mesophilic crenarchaea, whose ecosystem function was previously unknown. Re-assessment of the role of bacteria in ammonia oxidation is now required and this article reviews the current evidence for the relative importance of bacteria and archaea. Much of this evidence is based on metagenomic analysis and molecular techniques for estimation of gene and gene transcript abundance, changes in ammonia oxidizer community structure during active nitrification and phylogeny of natural communities. These studies have been complemented by physiological characterization of a laboratory isolate and by incorporation of labelled substrates. Data from these studies provide increasingly convincing evidence for the importance of archaeal ammonia oxidizers in the global nitrogen cycle. They also highlight the need to re-assess the importance of ammonia-oxidizing bacteria, the requirement and limitations of molecular techniques in linking specific microbial groups to ecosystem function and the limitations of reliance on laboratory cultures.

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Introduction

The accumulation of evidence for the existence and importance of mesophilic archaea in ammonia oxidation has had a significant impact on the contemporary view of nitrification (Francis *et al*., 2007). Since the isolation of autotrophic prokaryotes in the late 19th century, it has been assumed that all autotrophic ammonia oxidizers are bacteria. Early isolates are presumed to have been bacterial and, until recently, all cultivated, aerobic, autotrophic ammonia oxidizers were betaproteobacteria or gammaproteobacteria (Koops *et al*., 2003). This article assesses the current evidence for the role of mesophilic archaeal ammonia oxidizers in terrestrial, marine and wastewater treatment environments, in comparison with that of bacteria. This includes evidence derived from molecular data, but the validity and reliability of these data in assessing ecosystem function should not be considered unique to ammonia oxidizers. They are relevant to molecular analysis of many other functional groups, including those that are well characterized in laboratory culture.

Discovery of putative archaeal ammonia oxidation through metagenomics

The discovery of genes encoding putative ammonia monooxygenase subunits associated with mesophilic crenarchaea exemplifies the power of environmental metagenomics in determining functional characteristics of uncultivated microorganisms. The first indications of crenarchaeal involvement in nitrogen transformations of global ecological significance arose from two different metagenomic approaches involving the sequencing of targeted soil-derived fosmids and high-throughput shotgun sequencing of marine water samples (Venter *et al*., 2004; Schleper *et al*., 2005). Treusch and colleagues (2005) analysed a fosmid clone (54d9) prepared from high-molecular-weight DNA from a sandy loam soil. This

43.3 kb contiguous fragment contained 45 open reading frames (43 coding genes and 16S and 23S rRNA genes). Phylogenetic analysis of the 16S rRNA gene sequence placed the organism from which the fragment was derived within the Group 1.1b 'soil' lineage of the crenarchaea. The coding genes included homologues to *amoA* and *amoB* subunits of the bacterial AMO/PMO family (encoding ammonia and particulate methane monooxygenases respectively) and a homologue of the prokaryotic *nirK* gene family (encoding nitrite reductase). Both gene families are present in bacterial ammonia oxidizers and AMO is responsible for the first step in ammonia oxidation.

The metagenome data set described by Venter and colleagues (2004) also contains *amo* gene homologues in a shotgun library from the Sargasso Sea, which could be assembled with other genes in a contig that suggested that they belonged to an archaeon. However, the absence of an rRNA gene on the scaffold prevented unequivocal support for a mesophilic crenarchaeal association. Unlike the soil fosmid, all three subunits (A, B and C) could be constructed in a *B-C-hypothetical-A* arrangement. Further insight was provided by assembly of the genome of *Cenarchaeum symbiosum*, an uncultivated symbiont of the marine sponge *Axinella mexicana*, following sequencing of fosmid clones from highly enriched communities (Hallam *et al*., 2006). The genome contains homologues of genes required for carbon dioxide fixation by the 3-hydroxypropionate cycle and most of the genes required for the TCA cycle, suggesting the possibility of heterotrophic or mixotrophic growth. Homologues were also identified to genes involved in ammonia oxidation (*amoA*, *B* and *C*), denitrification (*nirK*) and urea transport and metabolism. Further analysis of *C. symbiosum* suggests a more distant relationship to the cultivated hyperthermophilic crenarchaea than previously recognized (Brochier-Armanet *et al*., 2008), and it has been suggested that the mesophilic lineages belong to a distinct, third archaeal domain, the '*Thaumarchaeota*'. It will be fascinating to see whether these observations hold true when genome sequences of other mesophilic crenarchaea are analysed.

Cultivation studies

Metagenomic data strongly suggest the potential for ammonia oxidation by mesophilic crenarchaea but confirmation requires demonstration in laboratory culture. This was reported by Könneke and colleagues (2005), who isolated *Nitrosopumilus maritimus* from a marine aquarium. *Nitrosopumilus maritimus* grows autotrophically with ammonia as the sole energy source and converts ammonia to nitrite with concomitant increase in cell number. It has a maximum specific growth rate of 0.033 h^{-1}, equivalent to a generation time of 21 h, and yielded 1.4×10^7 cells ml^{-1} on medium containing 500 μM ammonium. The highest reported specific growth rate for bacterial ammonia oxidizers is 0.088 h^{-1}, for *Nitrosomonas europaea*, but different studies of this strain report values in the range 0.017–0.088 h^{-1} (Prosser, 1989). Cells visualized by electron microscopy were straight rods, 0.17–0.22 mm in diameter × 0.5–0.9 mm in length, similar in size to crenarchaea detected in environmental samples, but smaller than cultivated bacterial ammonia oxidizers.

The potential for crenarchaeal ammonia oxidation has also been confirmed by enrichment (Hatzenpichler *et al*., 2008) and isolation (de la Torre *et al*., 2008) of ammonia-oxidizing archaea from terrestrial hot springs. Before the discovery of archaeal ammonia oxdizers, thermophiles associated with lineages thought to be representative of mesophilic crenarchaea had been reported following analysis of 16S rRNA gene sequences (Kvist *et al*., 2005) and Group 1-associated, glycerol dibiphytanyl glycerol tetraether (GDGT) lipids (including crenarchaeol) (Pearson *et al*., 2004). Enrichment of *Nitrososphaera gargensis* in cultures incubated at 46°C (Hatzenpichler *et al*., 2008) confirmed that some members of the Group 1.1b 'soil' lineage were at least moderately thermophilic and could perform autotrophic ammonia oxidation. However, this organism contrasts with the isolate *Nitrosocaldus yellowstonii* (de la Torre *et al*., 2008) which can grow up to 74°C with stoichiometric conversion of ammonia to nitrite, as found for *N. maritimus*. In addition, both organisms are placed in two relatively divergent lineages (Fig. 1A and B), indicating that adaptation out of (or into) thermophilic environments occurred multiple times.

Although enrichment of archaeal ammonia oxidizers from mesophilic aquatic (but not terrestrial) environments appears to be relatively easy, maintenance of enrichments and isolation of pure cultures are difficult. The reasons for this may become clear as cultivation conditions are investigated and optimized, and additional cultures are required to investigate, and compare, biochemistry and physiology of archaeal ammonia oxidizers. Nevertheless, characterization of *N. maritimus* provides non-circumstantial, direct evidence that (at least some) mesophilic crenarchaea are capable of autotrophic growth on ammonia. The similarity of the *amoA* gene sequences in *N. maritimus*, crenarchaeal metagenome fragments and environmental surveys (see below) suggests the potential for ammonia oxidation by a broad range of mesophilic crenarchaea in natural environments.

The extent to which isolation of *N. maritimus* informs our understanding of the relative importance of bacteria and archaea in nitrification highlights an issue of general importance in microbial ecology: identification of links between the presence of organisms and their ecosystem

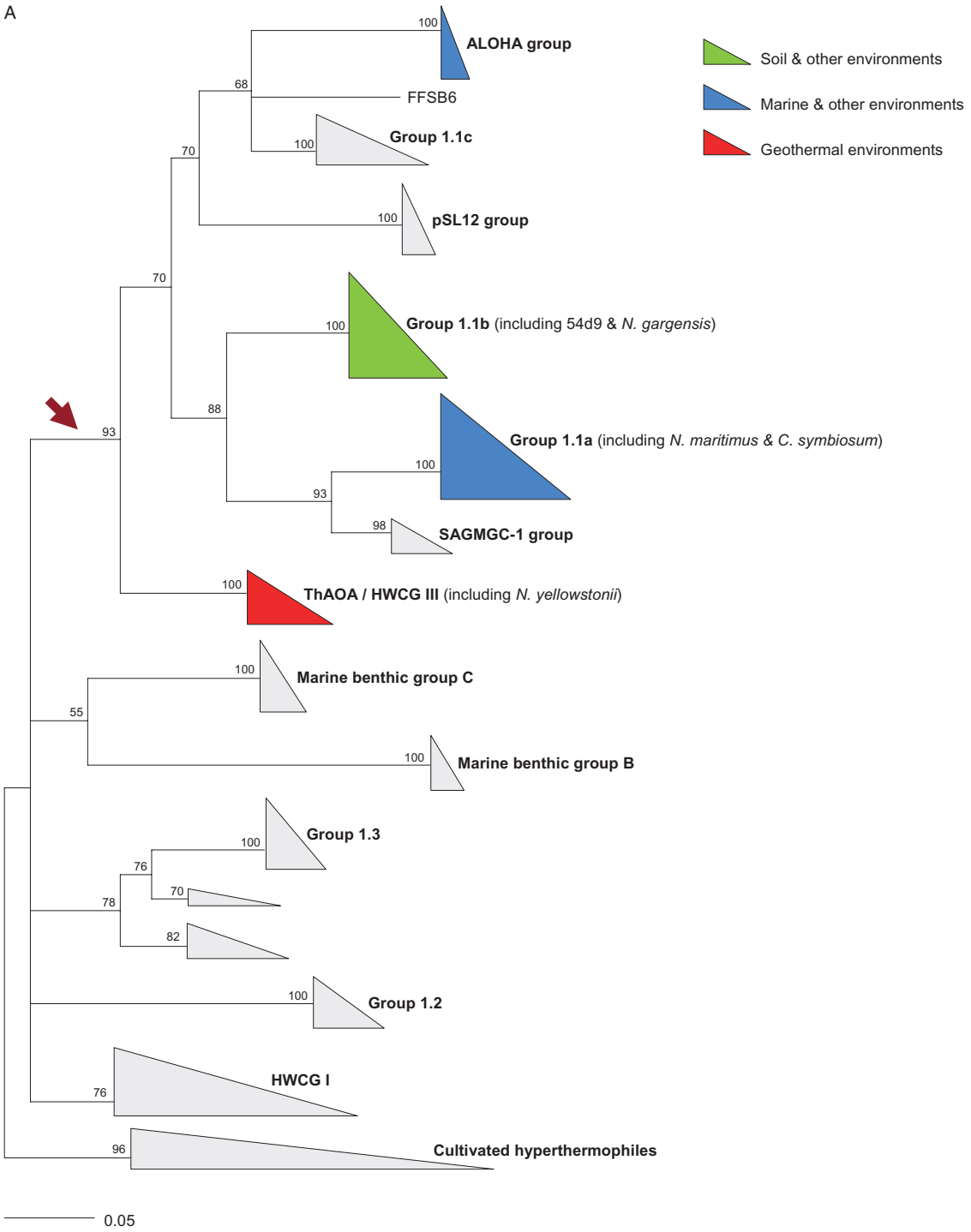

Fig. 1. Phylogenetic trees describing (A) major 16S rRNA gene- and (B) *amoA* gene-defined lineages within the kingdom *Crenarchaeota*. The height and length of triangular blocks are proportional to the number of sequences and maximum branch length within lineages respectively. Lineages with representatives that have been demonstrated or inferred to contain crenarchaeal *amo* genes are highlighted with the red arrow indicating the potential position of a monophyletic archaeal ammonia oxidizer lineage. Pair-wise distances with LogDet–Paralinear correction of unambiguously aligned positions were calculated with site-to-site variation (invariable sites plus eight variable rate categories estimated from the data). Bootstrap support was calculated using maximum likelihood, distance and parsimony methods (100, 1000 and 1000 replicates respectively) with values at major nodes representing the most conservative value from all three methods (expressed as a percentage). Multifurcation indicates where the relative branching order of major lineages could not be determined in the majority of bootstrap replicates with all methods. The scale bars represent an estimated 0.05 changes per nucleotide position.

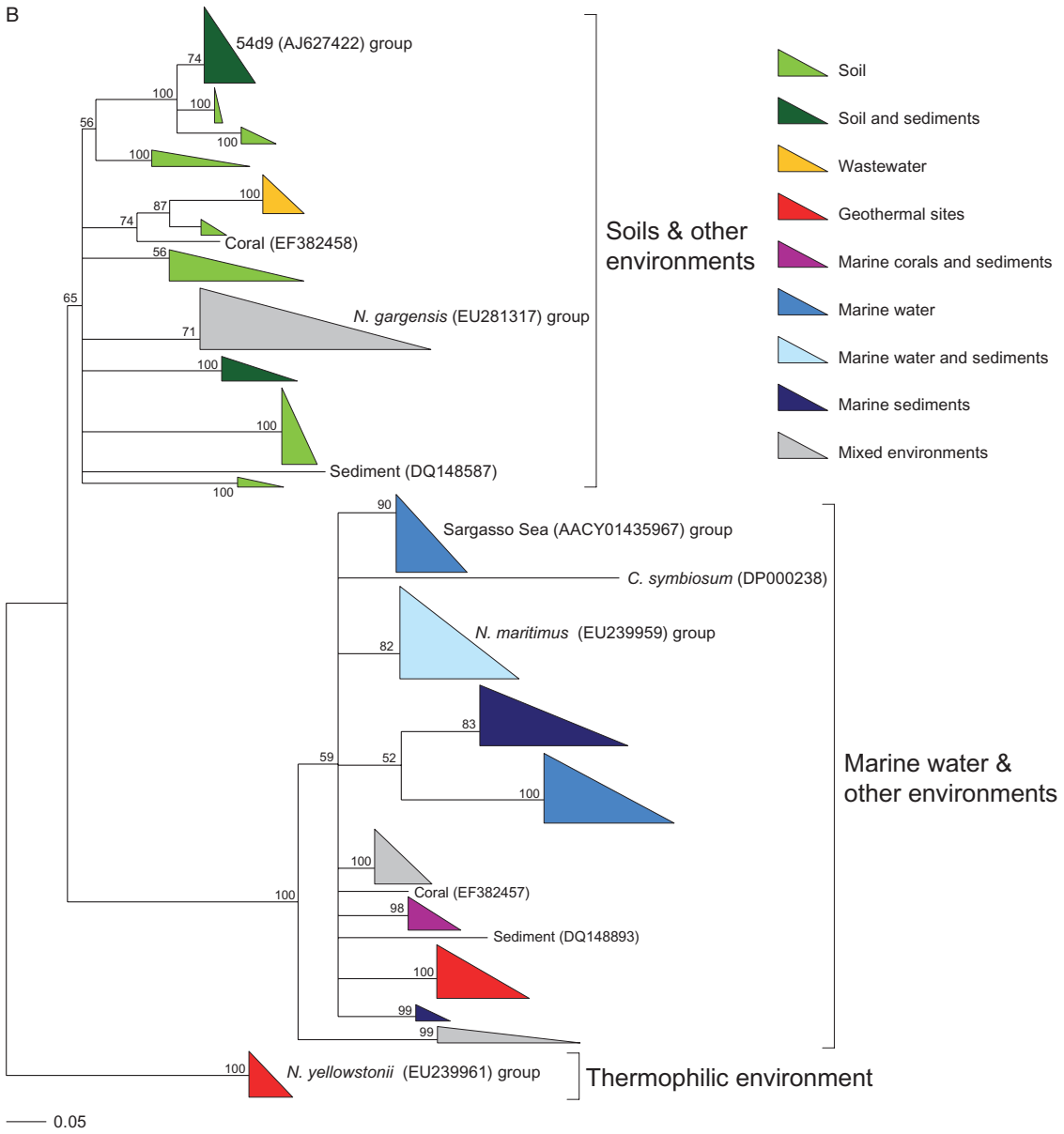

Fig. 1. *cont.*

functions. There is currently no reliable inhibitor that discriminates archaeal and bacterial ammonia oxidizers in environmental samples. We are therefore reliant on molecular techniques to assess whether even ammonia-oxidizing bacteria are actively oxidizing ammonia in the environment. Mere isolation of organisms from an environment does not demonstrate their activity and the fact that there are more bacterial than archaeal ammonia oxidizers in culture may be irrelevant. Isolation of *N. maritimus* therefore demands that we demonstrate that bacteria, and not just archaea, are responsible for oxidizing significant proportions of ammonia in the oceans and the soil. In this respect, both should be considered as putative ammonia oxidizers when studying natural environments. The following sections assess molecular data on the distribution, abundance and activity of archaeal ammonia oxidizers that attempt to achieve this.

Widespread distribution of archaeal *amo* genes (16S rRNA and *amoA* phylogeny)

Recovery of *amo* genes from soil (Treusch *et al*., 2005) and marine (Venter *et al*., 2004) environments from two distinct major clades of mesophilic crenarchaea (Group 1.1a and 1.1b respectively) allowed the design of primers that were used for environmental surveys of crenarchaeal

amoA genes. These showed that archaeal *amoA* genes, like crenarchaeal 16S rRNA genes (DeLong, 1998), were ubiquitous. For example, Francis and colleagues (2005) performed a comprehensive survey of marine and terrestrial ecosystems, finding a variety of lineages associated with specific habitats. Subsequent studies have revealed a global distribution of crenarchaeal *amoA* sequences in, for example, marine waters (Wuchter *et al*., 2006), estuarine sediments (Beman and Francis, 2006), corals (Beman *et al*., 2007), sponges (Steger *et al*., 2008), soil (Leininger *et al*., 2006), wastewater bioreactors (Park *et al*., 2006) and terrestrial hot springs (Weidler *et al*., 2007). Individual ecosystem studies (Nicol *et al*., 2008) and a wider comparison of diversity from all environments (Fig. 1A and B) indicate substantial congruence in the phylogeny of crenarchaeal ribosomal and *amo* genes. Within the context of relatively recent evolutionary history, phylogenetic analysis indicates little horizontal transfer of bacterial and archaeal *amo* genes. This contrasts with other genes involved in nitrogen transformations, for example, *nirK* (Cantera and Stein, 2007). Metagenomic and cultivation data indicate the presence of *amoA* in organisms belonging to marine and soil Group 1.1a (Venter *et al*., 2004; Könneke *et al*., 2005; Hallam *et al*., 2006) and 1.1b (Treusch *et al*., 2005; Hatzenpichler *et al*., 2008) crenarchaeal lineages respectively. There is also evidence for an association of *amoA* in a 16S rRNA gene-defined lineage found in ocean waters ('ALOHA' lineage, Fig. 1a) related to thermophilic pSL12-like sequences and Group 1.1c crenarchaea (Mincer *et al*., 2007). A distinct thermophilic crenarchaeal lineage (de la Torre *et al*., 2008) has also been discovered. However, despite the global distribution of archaeal *amoA* genes, 16S rRNA-defined diversity (including that represented by sequences from exclusively uncultivated organisms) is significantly greater than that associated with ammonia-oxidizing activity (Fig. 1A).

Both bacterial and crenarchaeal phylogenies demonstrate a significant level of ecological differentiation, with each lineage dominated by sequences from a particular habitat range. For example, most soil bacterial ammonia oxidizers belong to *Nitrosospira* clusters 2, 3 and 4, whereas sequences placed within cluster 1 are recovered from marine-influenced habitats (Purkhold *et al*., 2000). For mesophilic crenarchaea, most soils are dominated by sequences within the 1.1b lineage, while organisms belonging to the 1.1a lineage have been estimated to represent approximately 20% of all prokaryotes in the World's oceans (Karner *et al*., 2001). Similar patterns are emerging for archaeal *amoA* gene sequences. For example, most (though not all) soil-derived sequences fall in one major clade, and a large number of divergent, marine-dominated lineages form a well-supported clade distinct from the soil lineage. As with 16S rRNA genes, *amoA* sequences are retrieved from a wide variety of other environments placed within lineages generally considered to be 'marine' or 'soil', possibly reflecting bias in commonly studied environments. For example, sequences from terrestrial hot springs are closely related to a large number of soil-derived *amoA* and 'mesophilic' crenarchaeal 16S rRNA sequences.

Abundance of organisms possessing *amo* genes

Leininger and colleagues (2006) were the first to determine and compare abundances of bacterial and archaeal ammonia oxidizers, by qPCR amplification of *amoA* genes. GDGT lipid abundance and archaeal *amoA* gene abundance were correlated, indicating that most soil crenarchaea in these soils were ammonia oxidizers, and archaeal *amoA* genes were more abundant than those of bacteria in 12 soils with a range of cultivation and management histories. In some soils, archaeal *amoA* genes were more than two orders of magnitude greater, suggesting greater ecosystem function (Fig. 2). In others the differences were smaller and potentially within the range of experimental variability. Higher abundance of archaeal *amoA* genes has since been reported in two Chinese soils subjected to different fertilizer treatments and with pH values ranging from 3.7 to 6 (He *et al*., 2007) and from 8.3 to 8.7 (Shen *et al*., 2008) and in Scottish agricultural plots (pH 4.9–7.5; Nicol *et al*., 2008).

Greater abundance of archaeal *amoA* genes has been seen as evidence of a greater role for archaea in nitrification. However, interpretation of abundance data must be cautious. The presence or high abundance of a functional gene does not mean that the function is operating. The gene might only be expressed under rare combinations of environmental conditions and the *amoA* gene product may give alternative ecosystem functions to ammonia oxidation (in both bacteria and archaea). Bacteria and archaea are phylogenetically distant, with significant differences in cell physiology and structure, and archaeal ammonia oxidizers appear to be smaller than bacterial ammonia oxidizers. Activities per cell may therefore differ significantly. Bacterial AMO is a multifunctional enzyme, oxidizing methane, carbon monoxide and a range of organic compounds, although these do not provide growth substrates in laboratory culture. Little is currently known of the alternative substrates for archaeal AMO but the significantly different nucleotide and predicted amino acid sequences may have consequences for functional diversity. The potential alternative metabolism and growth strategies for mesophilic crenarchaea must also be considered. Their abundance may result not from ammonia oxidation but, for example, from mixotrophic or heterotrophic growth. This would mirror the situation for nitrite oxidizers, whose higher abundance than bacterial

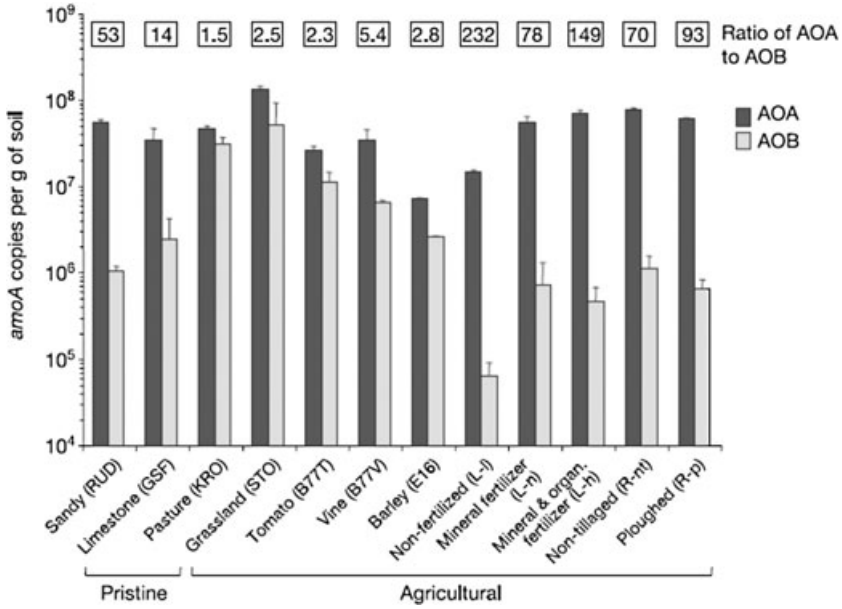

Fig. 2. Abundance and abundance ratios (in boxes) of archaeal (AOA) and bacterial (AOB) *amoA* genes in 12 soils with a range of soil management and fertilization histories. (From Leininger *et al*., 2006, with permission.)

ammonia oxidizers has been explained by mixotrophic growth. Higher crenarchaeal *amoA* gene abundance may therefore be unrelated to ammonia oxidation.

Methodological issues must also be considered. Bacterial and archaeal *amoA* genes fall in distinct and distant clusters and gene primers differ significantly. There is therefore the potential for primer bias (favouring detection of either bacteria or archaea). Differences in archaeal and bacterial cell structure accentuate the usual concerns about biases associated with cell lysis, DNA extraction efficiency, DNA stability and others. These are rarely tested and difficult to test, but Leininger and colleagues (2006) did observe differences in archaeal and bacterial cell lysis and DNA extraction efficiency when applying bead-beating for different periods. Finally, quantification of gene abundance in environmental samples by qPCR is at a relatively early stage of development and significant care must be taken in accepting it as a reliable measure of absolute gene abundance (Smith *et al*., 2006).

Changes in relative abundance

Although functional gene abundance does not necessarily demonstrate functional activity, and qPCR may not accurately measure absolute gene abundance, changes in *amoA* gene abundance associated with active nitrification in environmental samples provide more convincing evidence of relative roles of bacteria and archaea in ammonia oxidation. These have been reported in marine mesocosms and in long-term field soil studies.

Wuchter and colleagues (2006) provided the strongest current evidence for archaeal ammonia oxidation in marine environments. In an initial study, they investigated the effect of warming on communities in a mesocosm containing 850 l of North Sea water. Crenarchaeal abundance increased and correlated with a decrease in ammonia concentration. An increase in crenarchaeal abundance was also correlated with a decrease in ammonia and increases in nitrite and nitrate in mesocosms containing 20 l of this 'enriched' water amended with ammonium. Estimated doubling times and cell activities required for observed conversion of ammonia agreed with those reported for *N. maritimus* (Könneke *et al*., 2005). Archaeal 16S rRNA and archaeal *amoA* gene abundances were similar and sequences of both genes showed high homology and identity, respectively, to corresponding genes in *N. maritimus*. *amoA* gene sequences were also similar to marine archaeal *amoA* gene sequences found in marine metagenomic (Venter *et al*., 2004) and environmental (e.g. Francis *et al*., 2005) studies. Bacterial ammonia oxidizer abundances did not increase and were significantly lower than those of crenarchaea during nitrification. Similar correlations were found during regular monitoring of North Sea coastal waters for 12 months, and the archaeal community was dominated by an organism with sequence type homologous to that in the mesocosms. Rates of increase in archaeal abundance were comparable to those of *N. maritimus* cultures but estimated cell activities were higher, suggesting a possible contribution from bacteria. However, bacterial ammonia oxidizers were one to two orders of magnitude less abundant during the highest rates of nitrification. The study also showed good correlations between abundances determined by qPCR of 16S rRNA and *amoA* genes and direct enumeration using CARD-FISH.

Changes in bacterial and archaeal ammonia oxidizer soil communities have been observed in long-term field studies designed to investigate the influence of soil pH (in the range 4.9–7.5) over a period of 46 years (Nicol *et al*., 2008). Both communities changed significantly with soil pH, with different phylotypes selected at low, intermediate and alkaline pH. Long-term nitrogen, phosphorus and potassium fertilization regimes led to contrasting behaviour in two Chinese soils. In acid soils, abundance of both archaeal and bacterial *amoA* genes responded to fertilizer regime, but only archaeal community structure was affected (He *et al*., 2007). In an alkaline soil, archaeal *amoA* genes were more abundant, but only bacterial ammonia oxidizer community structure responded to fertilizer regime (Shen *et al*., 2008). Together, these studies suggest links between pH and the relative contributions of bacteria and archaea to soil nitrification.

Transcriptional analysis

As discussed above, the presence of a functional gene, or functional gene homologue, is not necessarily evidence of the associated ecosystem function. Stronger evidence comes from expression of that gene and concomitant measurement of the function. Expression of archaeal *amoA* genes was first reported, in soil, by Treusch and colleagues (2005) and Leininger and colleagues (2006) subsequently compared bacterial and archaeal gene transcription by quantification of *amoA* gene transcripts in three soils. Archaeal *amoA* transcripts were detected in all soils and ratios of archaeal : bacterial *amoA* transcript abundance were slightly lower than gene abundance ratios, but followed similar patterns and supported a significant archaeal contribution to ammonia oxidation. Further evidence was provided by the first high-throughput sequencing, metatranscriptomic analysis of an environmental sample (Leininger *et al*., 2006). A cDNA library of RNA extracted from a soil with similar archaeal and bacterial *amoA* gene abundances was sequenced by pyrosequencing, thereby avoiding cloning bias associated with traditional clone library analysis of gene abundance. Archaeal gene relative abundance within the cDNA reads was similar to that indicated by qPCR of *amoA* genes, suggesting that the majority of archaea possessed *amoA*, and the ratio of full-length bacterial : archaeal 16S rRNA genes was similar to that found with qPCR. The transcript sequences were dominated by rRNA transcripts, but two *amoA* genes were detected, both of which were archaeal, while no bacterial *amoA* gene transcript was detected. The data therefore indicate that archaeal ammonia oxidizers have greater transcriptional activity in addition to greater *amoA* gene abundance. Although this provides supporting evidence that archaea play a greater role than bacteria in soil nitrification, analyses were not replicated, numbers of *amoA* transcripts were low and differences between bacterial and archaeal *amoA* transcripts could have arisen through stochastic effects. Nevertheless, the consistency of abundance and relative abundance values for genes and transcripts obtained with independent methods increases confidence in the methods used.

Soil microcosm studies have also shown changes in transcriptionally active archaeal, but not bacterial communities during active nitrification. Tourna and colleagues (2008) investigated the influence of temperature on nitrification and soil ammonia oxidizer communities. Transcriptionally active ammonia oxidizers were characterized by denaturing gradient gel electrophoresis (DGGE) analysis of bacterial ammonia oxidizer or crenarchaeal 16S rRNA genes and bacterial and archaeal *amoA* genes. All were analysed following RT-PCR amplification of extracted soil RNA and 16S rRNA gene transcripts of both bacterial ammonia oxidizers and crenarchaea were obtained. Bacterial ammonia oxidizer 16S rRNA gene transcript DGGE profiles showed little change during nitrification but, in contrast, both crenarchaeal 16S rRNA and archaeal *amoA* transcript profiles showed marked changes (Fig. 3), suggesting different responses of different phylogenetic groups during nitrification. These changes increased with both time and temperature. The ability to detect archaeal *amoA* gene expression during nitrification is evidence of their involvement in soil ammonia oxidation, and changes in transcript relative abundance are highly suggestive of differential responses of different members of the archaeal community to different conditions.

Similar observations have been made on the influence of soil pH on ammonia oxidizers, with additional information on transcript abundance (Nicol *et al*., 2008). Figure 4 illustrates archaeal and bacterial gene and transcript abundances in soil adjusted to different pH values. Archaeal *amoA* gene abundance decreased slightly with increasing pH, while bacterial *amoA* gene abundance showed little variation (Fig. 4a). Archaeal *amoA* gene transcript abundance also decreased with increasing pH, but to a much greater extent, while bacterial transcript abundance increased. These pH-associated differences are best illustrated in changes in the ratio of gene transcript : gene abundance (Fig. 4b). At all soil pH values, archaeal genes and gene transcripts were more abundant than those of bacteria, implying greater potential activity and greater transcriptional activity. Transcriptional activity of archaeal *amoA* sequence types associated with different pH soils was also determined in a microcosm experiment in which pH 4.3 and 6.9 soils were mixed, re-adjusted to either pH 4.3 or pH 6.9 and then incubated. Nitrification occurred in both mixtures and in the original soils. DGGE profiles of archaeal *amoA* genes and gene

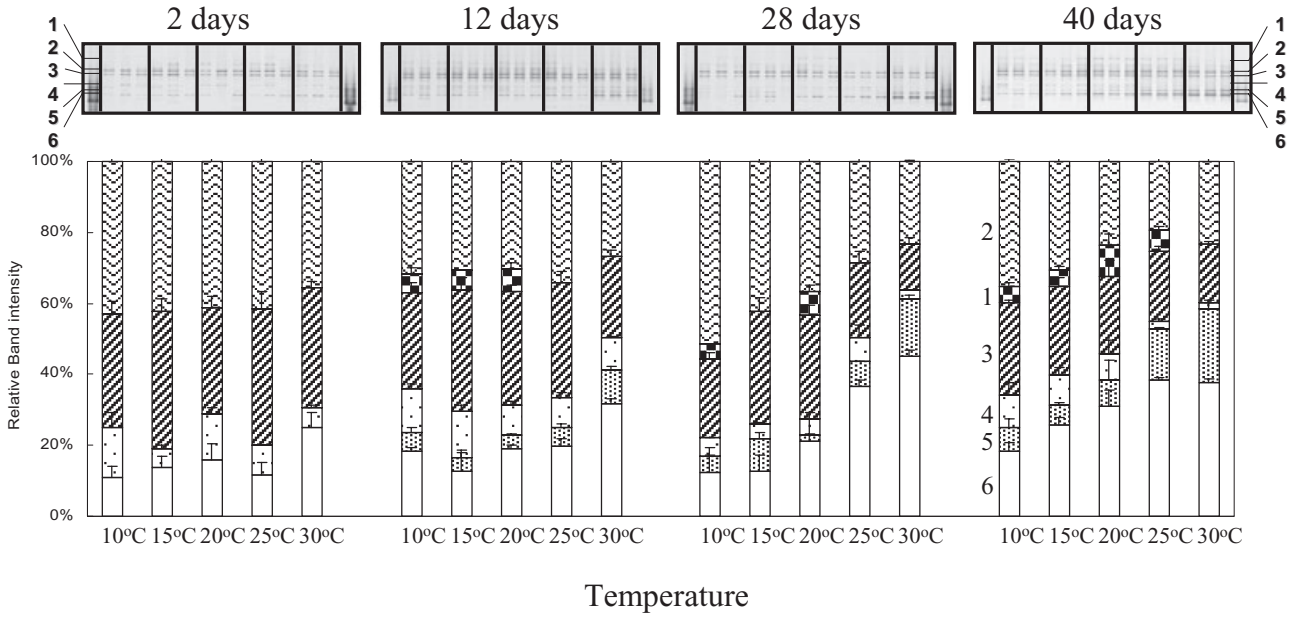

Fig. 3. Denaturing gradient gel electrophoresis of archaeal *amoA* gene transcripts in mRNA extracted from soil microcosms incubated at temperatures in the range 10–30°C and sampled at 2, 12, 26 and 40 days. Histograms represent relative intensity of bands within individual lanes from triplicate microcosms. Data are presented as means of triplicate values and standard errors. Numbers relate to bands that were sequenced. (From Tourna *et al.*, 2008, with permission.)

transcripts were similar, suggesting that dominant archaeal ammonia oxidizer phylotypes were most likely to dominate archaeal ammonia oxdizer activity. In addition, transcript sequence types associated with different pH soils showed changes in relative abundance in DGGE profiles. The data indicate short-term transcriptional responses to pH, rather than constitutive expression of *amoA* genes.

This study does, however, demonstrate some of the difficulties in analysing transcriptomic data. First, all of the caveats regarding qPCR and comparisons between archaeal and bacterial gene abundances discussed above apply here. Second, the extent to which functional gene expression reflects ecosystem function depends on a number of factors, including mRNA stability and turnover, protein turnover and cellular and environmental conditions that support physiological activity. Third, in all cases gene transcripts were less abundant than genes. This may indicate that a large proportion of the potential ammonia oxidizer community is inactive, with respect to ammonia oxidation, and/or that qPCR of genes is significantly more efficient than that of gene transcripts. Nevertheless, the ability to detect archaeal *amoA* gene expression at levels higher than those of bacteria during active soil nitrification, coupled with greater changes in archaeal transcript sequence type and relative abundance, provides strong evidence for their role in soil ammonia oxidation.

Isotope incorporation

Incorporation of specific substrates provides direct evidence of ecosystem function, using techniques such as microautoradiography (Wagner *et al.*, 2006) and stable isotope probing (SIP) (Dumont and Murrell, 2005). Direct demonstration of ammonia oxidation by such techniques is not possible, as nitrite, the product of ammonia oxidation, is not assimilated. However, autotrophic growth, accompanying ammonia oxidation, can be determined by incorporation of ^{14}C- or ^{13}C-labelled CO_2 or assessment of $^{13}C/^{12}C$ isotopic ratios in biomass.

Kuypers and colleagues (2001) reported that mesophilic crenarchaeal GDGTs, recovered from ancient oceanic shale deposits over 100 million years old, contained $^{13}C/^{12}C$ isotopic ratio signatures considered typical of chemoautotrophs. Studies had also revealed contemporaneous uptake of inorganic carbon. Pearson and colleagues (2001) studied the ^{14}C signatures in different layers of deep-sea sediments containing surface-derived carbon fixed by phytoplankton either before or after nuclear testing began in the 1950s, which increased the ^{14}C content in atmospheric carbon. Unlike many bacterial lipid groups, there was no evidence of increased ^{14}C in crenarchaeal lipids in recently formed sediments, indicating that the carbon source for crenarchaeal growth was dissolved inorganic carbon, not influenced by surface primary production. Stable isotope probing experiments

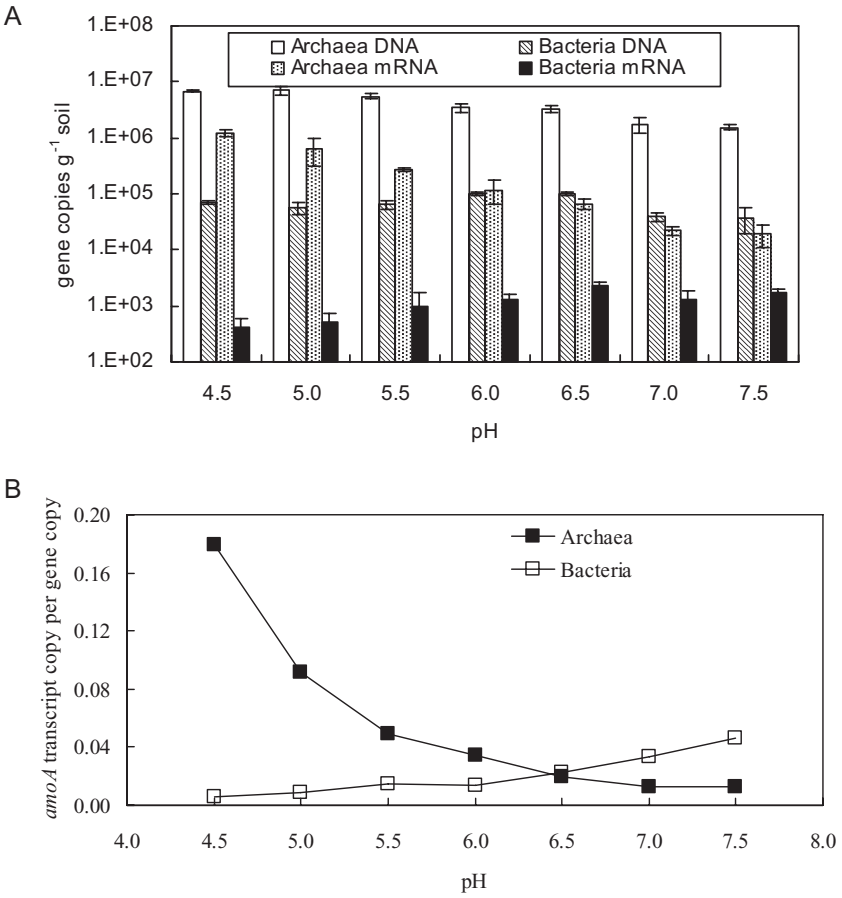

Fig. 4. Abundances of crenarchaeal and bacterial *amoA* gene and transcript copies (A) and ratios of gene transcript : gene abundance in soils maintained at pH values ranging from 4.5 to 7.5. Error bars represent standard errors of replicate field samples at each soil pH. (Based on data in Nicol *et al.*, 2008, with permission.)

have demonstrated autotrophy in marine ammonia oxidizers. In an *in situ* sodium bicarbonate experiment using marine surface water, Wuchter and colleagues (2003) demonstrated uptake of ^{13}C-CO_2, with the majority of all autotrophically incorporated lipid carbon found in crenarchaeal isoprenoids. However, Ingalls and colleagues (2006) found that at a depth of 670 m in the Pacific Ocean, the ^{14}C signature of archaeal GDGTs could not be explained by incorporation of dissolved inorganic carbon and calculated that 17% of the lipid carbon was derived from heterotrophic consumption of organic carbon. This would indicate that populations represented a mixture of different carbon metabolisms or perhaps mixotrophy. Indeed, Ouverney and Fuhrman (2000) demonstrated uptake of amino acids by planktonic archaea, indicating potential heterotrophy, but it was not determined whether these archaea were crenarchaea. DNA-SIP has been used to demonstrate CO_2 incorporation by nitrifiers in estuarine sediments, but no information was sought on archaea (Freitag *et al.*, 2006). Recently, Hatzenpichler and colleagues (2008) elegantly demonstrated the differential incorporation of bicarbonate in cultures of *Nitrososphaera gargensis* at the cellular level over a range of ammonium concentrations, using a combination of microautoradiography and catalyzed reporter deposition (CARD)-FISH.

Future

This article represents a snapshot in a rapidly developing story and new data will soon inform the bacterial–archaeal nitrification debate. Crucial to this will be analysis of the genome and the physiological characteristics of *N. maritimus* and other cultivated archaeal ammonia oxidizers, with extrapolation of findings to other crenarchaea. This will hopefully inform ecological studies and also enrichment and isolation strategies, as a wider range of cultures are required (including soil isolates). Such cultures will also facilitate the search for differential inhibitors of archaeal and bacterial nitrification, which will relieve some of the current reliance on molecular techniques for analysis of their relative importance. This will greatly increase our ability to determine, for example, whether archaeal ammonia oxidizers have a role in N_2O production, methane oxidation and heterotrophy. In assessing the ecosystem function of these organisms, we must be cautious in interpreting experimental data and critical of the techniques being used. However, when different, inde-

pendent techniques have been used to analyse the same samples, results have generally been consistent. No technique is without bias, but bias in, for example, molecular techniques applies equally to bacteria and archaea and, in the absence of evidence to the contrary, can apply in both directions. The accumulating data suggest strongly that archaeal ammonia oxidizers may have greater role than bacteria in some environments.

Acknowledgements

The authors would like to thank Dr Sven Leininger (University of Bergen) for supplying the alignment of *amo*A sequences used in Fig. 1B.

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