Environmental Microbiology (2008) **10**(11), 2966–2978 doi:10.1111/j.1462-2920.2008.01701.x

The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria

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Summary

Autotrophic ammonia oxidation occurs in acid soils, even though laboratory cultures of isolated ammonia oxidizing bacteria fail to grow below neutral pH. To investigate whether archaea possessing ammonia monooxygenase genes were responsible for autotrophic nitrification in acid soils, the community structure and phylogeny of ammonia oxidizing bacteria and archaea were determined across a soil pH gradient (4.9–7.5) by amplifying 16S rRNA and *amoA* genes followed by denaturing gradient gel electrophoresis (DGGE) and sequence analysis. The structure of both communities changed with soil pH, with distinct populations in acid and neutral soils. Phylogenetic reconstructions of crenarchaeal 16S rRNA and *amo*A genes confirmed selection of distinct lineages within the pH gradient and high similarity in phylogenies indicated a high level of congruence between 16S rRNA and *amoA* genes. The abundance of archaeal and bacterial *amoA* gene copies and mRNA transcripts contrasted across the pH gradient. Archaeal *amoA* gene and transcript abundance decreased with increasing soil pH, while bacterial *amoA* gene abundance was generally lower and transcripts increased with increasing pH. Short-term activity was investigated by DGGE analysis of gene transcripts in microcosms containing acidic or neutral soil or mixed soil with pH readjusted to that of native soils. Although mixed soil microcosms contained identical archaeal ammonia oxidizer communities, those adapted to acidic or neutral pH ranges showed greater relative activity at their native soil pH. Findings indicate that different bacterial and archaeal ammonia oxidizer phylotypes are selected in soils of different pH and that these differences in community structure and abundances are reflected in different contributions to ammonia oxidizer activity. They also suggest that both groups of ammonia oxidizers have distinct physiological characteristics and ecological niches, with consequences for nitrification in acid soils.

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Introduction

Soil pH is known to have a considerable effect on the activities of microbial communities and the biogeochemical processes which they mediate. Soil pH will affect the chemical form, concentration and availability of substrates (Kemmitt *et al.*, 2006) and will influence cell growth and activity. There is also strong evidence that soil pH is an important determinant of bacterial diversity and community structure on a global scale (Fierer and Jackson, 2006). The mechanisms by which soil pH influences the growth and activity of some microbial functional groups have been determined through a combination of physiological and soil microcosm studies. For example, rates of nitrification and, in particular, ammonia oxidation in soil are significantly reduced in acid soils (de Boer and Kowalchuk, 2001), and significant batch growth of pure cultures of ammonia oxidizing bacteria in liquid growth media does not occur below pH 7 (de Boer and Laanbroek, 1989; Allison and Prosser, 1991; Jiang and Bakken, 1999). The generally accepted explanation for reduced growth and activity of ammonia oxidizers at low pH is the exponential reduction in NH_3 availability with decreasing pH, through ionization to NH_4^+ (Frijlink *et al.*, 1992), decreasing NH_3 diffusion and increasing the requirement for energy-dependent transport of NH_4^+. Although some acidophilic heterotrophs are capable of nitrification, autotrophic oxidation of inorganic

ammonia occurs in acid soils (Killham, 1990; de Boer *et al*., 1992), and laboratory studies have demonstrated mechanisms for low pH nitrification through biofilm formation (Allison and Prosser, 1991), continuous rather than batch growth (Allison and Prosser, 1993) and urease activity (de Boer and Laanbroek, 1989; Burton and Prosser, 2001; Pommerening-Röser and Koops, 2005).

Ammonia oxidation in acid soils may result from the selection for acidophilic ammonia oxidizers whose activity may reflect differences in, for example, propensities for biofilm formation and urease activity, or other unknown adaptive characteristics. For example, Stephen and colleagues (1996), Kowalchuk and colleagues (1998) and Nugroho and colleagues (2007) reported selection for *Nitrosospira* cluster 2 and 4 strains in acid and neutral soils, although this is not always found (Webster *et al*., 2002). These studies suggest selection and adaptation of particular phylogenetic lineages in low pH soil, but measure 'standing crops' of ammonia oxidizers rather than activities and potentially reflect effects on long-term differences in growth and colonization.

Recent studies suggest an important role for non-thermophilic crenarchaea in soil ammonia oxidation. This follows initial reports of crenarchaea-associated ammonia monooxygenase genes (*amoA* and *amoB* homologues) in soil metagenomic studies (Treusch *et al*., 2005), isolation of the first autotrophic ammonia oxidizing non-thermophilic crenarchaeon (Könneke *et al*., 2005) and subsequent quantification of bacterial and archaeal *amoA* genes in several soils (He *et al*., 2007; Leininger *et al*., 2006) that indicate a greater abundance of archaeal over bacterial ammonia oxidizers. Some studies (Treusch *et al*., 2005; Leininger *et al*., 2006; Tourna *et al*., 2008) also indicate a higher transcriptional activity of archaeal over bacterial ammonia oxidizers in soils.

The discovery of archaeal ammonia oxidation provides a further potential explanation for nitrification in acid soils, i.e. the activity of archaeal ammonia oxidizers and the potential for pH selection of acidophilic and neutrophilic archaeal ammonia oxidizers. The aim of this study was therefore to determine the influence of soil pH on archaeal ammonia oxidizer community structure and activity. To achieve this we revisited the long-term soil pH manipulation-field plots investigated by Stephen and colleagues (1996) and Kowalchuk and colleagues (1998), providing the additional opportunity to determine whether pH-related influences on bacterial ammonia oxidizer community structure persisted with time. Long-term changes in ammonia oxidizer communities were assessed by DNA-based analyses of 16S rRNA and *amoA* gene abundance and relative abundance. Transcriptional activity was assessed by targeting *amoA* gene transcript abundance and composition, in long-term field sites and in soil microcosms.

Results

Physicochemical analysis

Total carbon, nitrogen, ammonia, nitrite/nitrate, water content and pH were determined for triplicate field samples taken from a pH gradient in July 2006. The pH gradient consists of plots maintained at seven values in the range of 4.5–7.5 at 0.5 intervals since 1961. The maximum deviation from the target pH in any plot was 0.4 units with the measured pH values of each subplot being 4.9, 5.3, 5.9, 6.4, 6.9, 7.3 and 7.5 (SE < 0.1). With the exception of pH, measured soil physicochemical characteristics showed little variability between plots and no significant trends were observed with pH, as determined by analysis of variance. The average water, nitrogen and carbon contents across all plots on an oven dry weight basis were 23.6(0.4)%, 0.4(0.02)% and 7.0(0.4)%, respectively, and the average ammonia concentration was 1.5(0.1) mg NH_4^+-N g^{-1} soil. The only exception were concentrations of nitrite/nitrate in pH 4.9 and 5.3 soils (15.1 and 15.0 mg NO_2^-/NO_3^--N g^{-1} soil respectively) which were higher than those in soils of higher pH ($P = 0.02$) with the average concentration in all samples from pH 5.9–7.5 plots 10.2 (0.6) mg NO_2^-/NO_3^--N g^{-1} soil.

Long-term influence of pH on ammonia oxidizer communities

Long-term changes in crenarchaeal and ammonia oxidizer communities were investigated by molecular analysis of soil samples from plots over an actual pH range of 4.9–7.5. Bacterial ammonia oxidizer communities were characterized by denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA gene fragments (Fig. 1A). DGGE profiles varied with soil pH, indicating changes in the relative abundances of bacterial ammonia oxidizer phylotypes. Soil at pH 4.9 was dominated by two bands that decreased in relative intensity as soil pH increased and were not detectable in high soil pH plots. Comparison with sequences in a marker lane run alongside the environmental samples showed that one of these bands belonged to *Nitrosospira* cluster 2, as found previously to dominate in the lowest pH soils (Stephen *et al*., 1998). In contrast, several bands were present in pH 7.5 soil which decreased in relative intensity as soil pH decreased and were not detectable in pH 4.9 soil. These included *Nitrosospira* cluster 3 sequences, as found previously (Stephen *et al*., 1998). Successful and reproducible amplification of bacterial *amoA* genes was only obtained using primers amoA-1-f and amoA-2-r (Rotthauwe *et al*., 1997) without a GC clamp which reduced PCR efficiency, and therefore DGGE analysis of *amoA*-defined AOB communities was not performed. In addition,

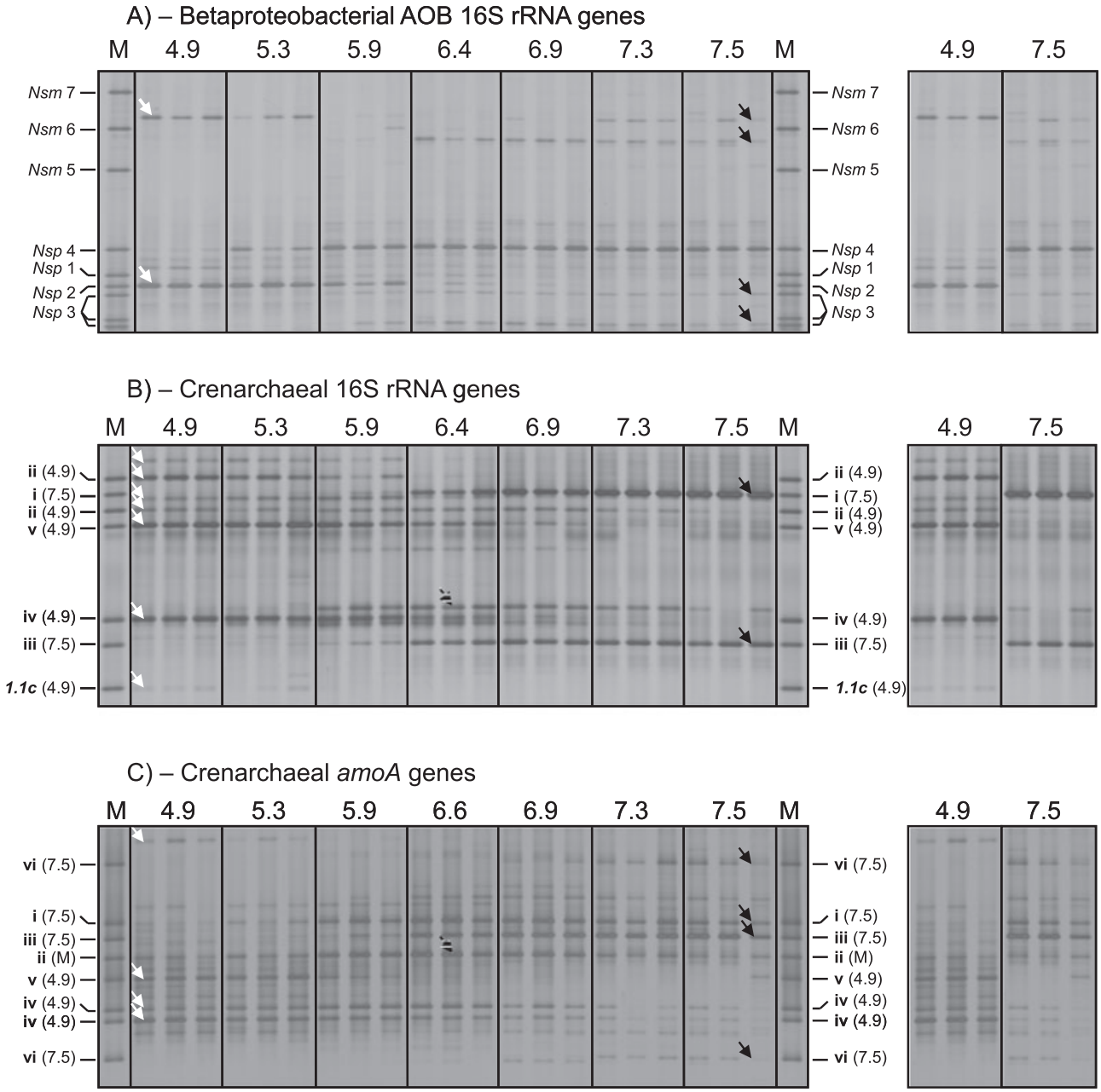

Fig. 1. Denaturing gradient gel electrophoresis (DGGE) profiles of bacterial ammonia oxidizer, crenarchaea and archaeal ammonia oxidizers in soils maintained for 46 years at pH values in the range 4.5–7.5. Each lane represents a profile derived from an individual soil sample. Band positions highlighted by white and black arrows are those considered to decrease and increase, respectively, with increasing soil pH, and those that show greatest relative intensity in the middle of the pH gradient (~6.0) are highlighted with a striped arrow.
A. Betaproteobacterial ammonia oxidizing bacteria 16S rRNA genes. M denotes a marker lane composed of nine different 16S rRNA gene PCR products derived from sequences representative of seven different bacterial ammonia oxidizer clusters.
B. Crenarchaeal 16S rRNA genes. M denotes a marker lane composed of 771f/957r PCR products of seven clones (in descending order CS16S-25-4.9, CS16S-55-7.5, CS16S-23-4.9, CS16S-2-4.9, CS16S-9-4.9, CS16S-72-7.5 and CS16S-1-4.9) representative of major clades of sequences (i–vi) identified in clone libraries (see Fig. 3A) dominated either by sequences from soil of pH 4.9 or 7.5.
C. Crenarchaeal *amoA* genes. M denotes a marker lane composed of CrenamA23f/CrenamoA616r PCR products of eight clones (in descending order CSamoA-71-7.5, CSamoA-54-7.5, CSamoA-50-7.5, CSamoA-47-7.5, CSamoA-31-4.9, CSamoA-22-4.9, CSamoA-25-4.9 and CSamoA-72-7.5) representative of major clades of sequences (i–vi) identified in clone libraries (see Fig. 3B). Clades were either dominated by sequences from soil of pH 4.9, 7.5 or a mixture of both (M).

betaproteobacterial ammonia oxidizers are known to be adequately profiled using the 16S rRNA-based approach used in this study (e.g. Freitag and Prosser, 2004; Freitag *et al.*, 2006).

DGGE profiles of crenarchaeal 16S rRNA genes were similar to those of the bacterial community. Community structure was highly reproducible within replicate soil samples at each pH and distinct differences in profiles were observed across the pH gradient (Fig. 1B). Seven bands dominated pH 4.9 soil, and decreased in relative intensity as pH increased, while two bands showed the reverse behaviour. One band position showed greatest relative intensity in the middle of the gradient (pH ~6.0). DGGE profiles of crenarchaeal *amoA* genes followed the same pattern (Fig. 1C), with most band positions showing greatest relative intensity at the lowest or highest pH, and one at pH ~6.4. For all three assays, there was no overlap in bands dominating profiles from pH 4.9 and 7.5 plots, suggesting distinct dominating crenarchaeal and bacterial ammonia oxidizers.

Direct comparison of abundances of archaeal and bacterial ammonia oxidizers was assessed by qPCR amplification of *amoA* genes, using primers specific for crenarchaeal *amoA* and betaproteobacterial *amoA* genes (Fig. 2A). Abundance of archaeal *amoA* genes decreased significantly from approximately $9.9 ¥ 10^6$-$2.1 ¥ 10^6$ copies g^{-1} soil as soil pH increased from 4.9 to 7.5 (ANOVA, log-transformed data, $P = 1.2 ¥ 10^{-5}$). Abundance of bacterial *amoA* genes was 0.8–3.1% of archaeal *amoA* genes across all soils. Bacterial *amoA* gene abundance also varied significantly with pH (ANOVA, log-transformed data, $P = 0.01$), but differences were less than those for archaeal genes, and no obvious trend was evident.

Transcriptional activities of archaeal and bacterial ammonia oxidizers were compared by quantification of *amoA* gene transcripts (Fig. 2B). Archaeal *amoA* transcript abundance decreased with increasing soil pH, while that of bacterial *amoA* transcripts increased. Archaeal *amoA* gene transcripts were more abundant than bacterial transcripts at all sites, the latter comprising 0.03–8.9% of archaeal transcripts.

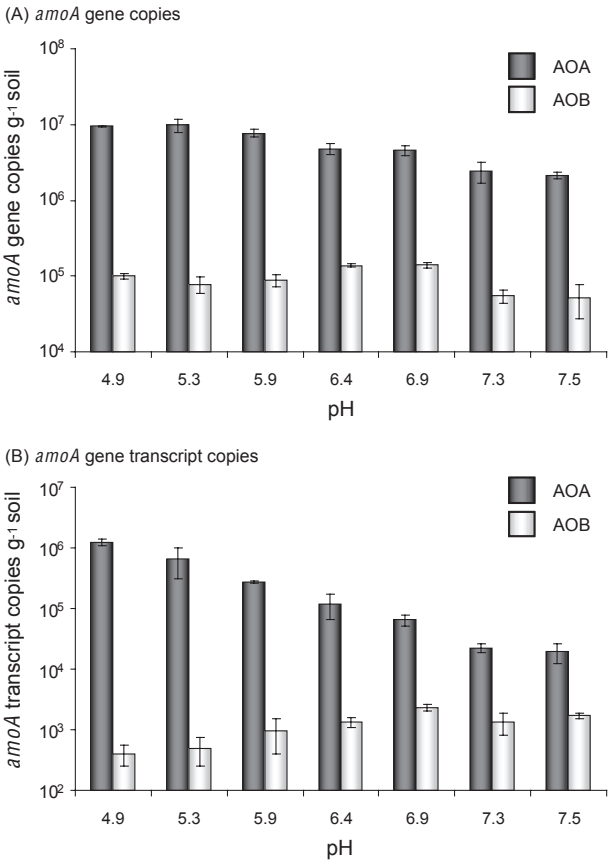

Fig. 2. Quantification of crenarchaeal and bacterial *amoA* gene (A) and transcript copies (B) in soils with pH values in the range 4.5–7.5. Three replicate field samples were analysed for each soil pH. Error bars represent standard errors.

Phylogenetic analysis

DGGE profiles in Fig. 1 indicated that specific phylotypes were selected by soils of different pH. To determine whether these different phylotypes represented distinct phylogenetic groups, six clone libraries of crenarchaeal 16S rRNA and *amoA* genes were constructed from PCR products of approximately 1400 and 624 bp, respectively, derived from the three 4.9 and three 7.5 field replicates. Twelve clones were selected from each library for sequencing (72 16S rRNA and 72 *amoA* sequences in total). Sequences from short or failed reads or of apparent chimeric origin were excluded from analysis, leaving 135 of 144 selected clones for phylogenetic analysis. Sequences were aligned with reference taxa and subjected to distance, parsimony and maximum likelihood phylogenetic analyses for both 16S rRNA gene and translated *amoA* sequences (Fig. 3). A number of distinct clades, representing diverse phylogenetic lineages and containing exclusively sequences from low (4.9) or high (7.5) pH sites were observed for both genes. There was no evidence of bias associated with individual clone libraries as each distinct clade (containing three or more sequences) was represented by sequences from more than one clone library.

Similar patterns in the distribution of these clades with respect to reference taxa would indicate a high level of congruence between phylogenies derived from 16S rRNA and *amoA* genes. The majority of sequences fell within major lineages previously characterized as being dominated by sequences from soil environments [labelled '1.1b (soil)' and 'soil/sediment' in 16S rRNA and *amoA* gene trees respectively]. Within the soil lineage for both

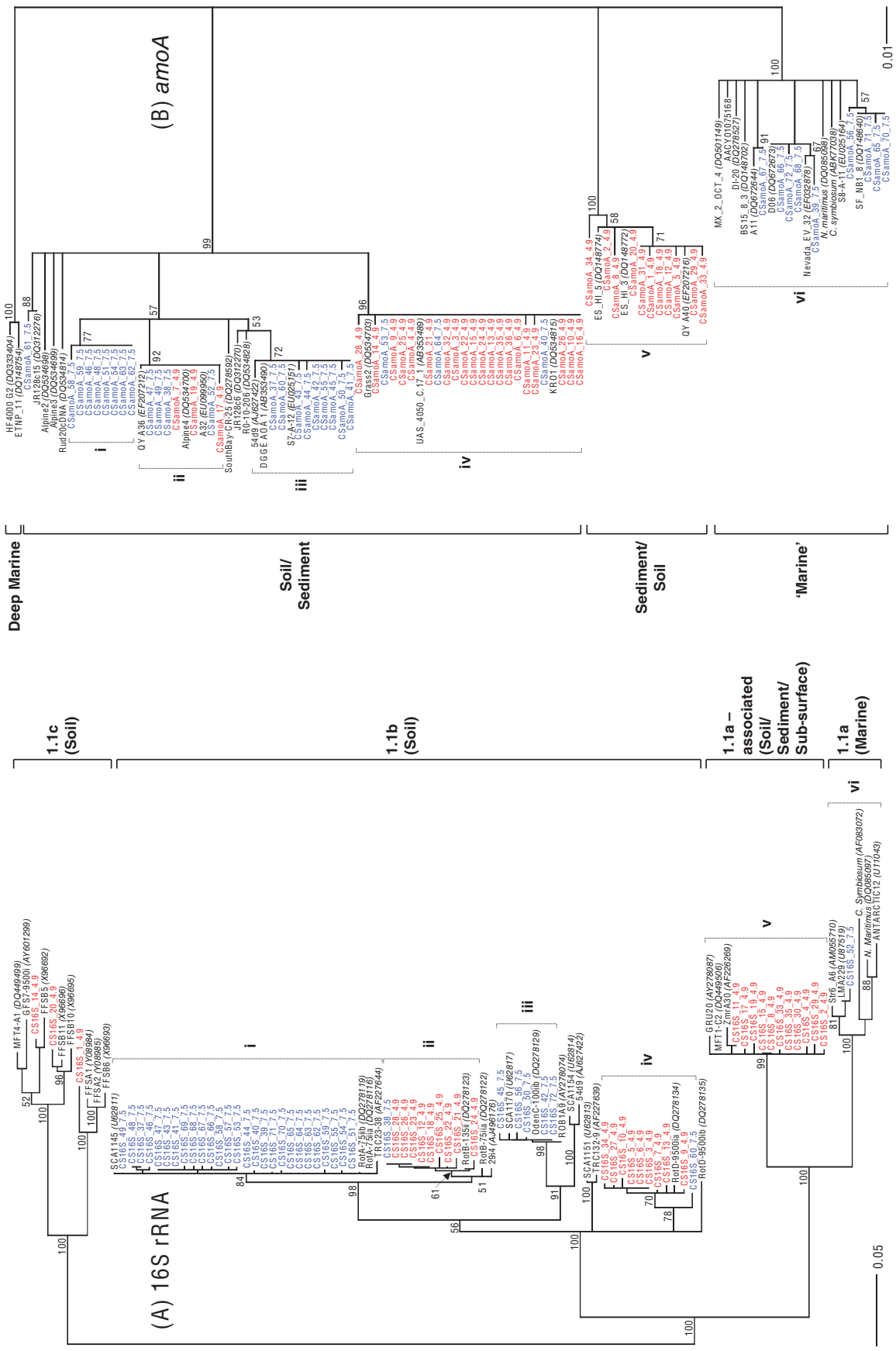

Fig. 3. Phylogenetic analysis of cloned crenarchaeal 16S rRNA and *amoA* gene PCR products. Six clone libraries were produced for each gene with numbered sequences (in red) 1–12, 13–24 and 25–36 derived from three pH 4.9 soil samples, and numbered sequences (in blue) 37–48, 49–60 and 61–72 derived from three pH 7.5 soil samples. Reference sequences are described as 'Name (*accession number*)'. Bootstrap support at major nodes represent the most conservative value from distance, parsimony and ML analyses (expressed as a percentage). Multifurcation indicates where the relative branching order could not be determined in the majority of bootstrap replicates using all three methods. Clades highlighted i–vi indicate potential groups of 16S rRNA and *amoA* gene sequences potentially derived from the same group of organisms.
A. 16S rRNA gene sequences. LogDet/Paralinear pairwise distances of unambiguously aligned positions were calculated using variable sites only (338 positions) estimated from a maximum-likelihood model. Clades labelled 1.1a (Marine), 1.1b (Soil) and 1.1c (Soil) are Group 1 16S rRNA-defined lineages dominated by sequences retrieved from marine and soil environments. The tree was rooted with 1.1c lineage sequences.
B. *amoA* genes sequences. Pairwise distances of 168 aligned amino acid positions were calculated using the JTT substitution model with site variation (invariable sites and eight variable gamma rates). Clades are labelled according to most frequently represented environment. The tree was rooted with two sequences of a deep marine water clade (DM).

genes, four major clades were observed. Three clades formed one monophyletic cluster with modest bootstrap support, within which two clades (i and iii) were represented exclusively by sequences from the high pH plots. The third clade (ii) contained a mixture of low and high pH soil-derived sequences. A fourth clade (iv), that was distinct from the monophyletic group of three clades (i–iii) and contained a mixture of sequences, but dominated by pH 4.9 sequences, was also present in both phylogenies. For both genes, only high pH sequences were placed in a clade (vi) with marine-like sequences including *C. symbiosum* and *N. maritimus* (although only one sequence was associated in the 16S rRNA gene analysis). In addition, for both genes, a cluster of 11, exclusively low pH sequences (labelled v) was observed that was distinct from the soil and marine clusters containing 54d9, *C. symbiosum* and *N. maritimus*. In summary, a remarkable congruence was observed between the phylogenetic calculations for both the 16S rRNA and *amo*A genes of crenarchaea.

Three 16S rRNA gene sequences were retrieved from the 1.1c lineage from low pH soils. This group was relatively distant from the monophyletic, *amoA*-associated, 1.1a/b lineage and no indication that this lineage possesses ammonia monooxygenase can be found in these results or from other studies.

To identify the phylogenetic origin of bands profiled by DGGE analysis, marker lanes composed of representative clones from the major phylogenetic lineages identified in the clone libraries were constructed from both the 16S rRNA and *amoA* libraries (Fig. 1B and C) and run alongside the environmental profiles. Clones were screened by DGGE analysis and in all cases, individual band positions represented by multiple clones were found to be represented by one unique phylotype. As indicated by the phylogenetic analyses, clones belonging to those clades identified as exclusively low or high pH co-migrated with bands found only in the low or high pH profiles for both crenarchaeal 16S rRNA and *amo*A assays.

Effect of pH on short-term crenarchaeal ammonia oxidizer activity

Analysis of long-term, pH-manipulated plots indicated that crenarchaeal ammonia oxidizer phylotypes had different pH preferences. To explore this further, short-term activity of different communities was determined in soil microcosm experiments in which nitrification, community changes and relative transcriptional activity were determined. Soil was sampled from high and low pH soils of the gradient (actual pH values of 6.9 and 4.3), sieved and homogenized, and 500 g of each soil was mixed. The mixed soil was then split into two 500 g batches with the pH of one re-adjusted to approximately 4.3 and the other to 6.9 using aluminium sulfate and calcium hydroxide, respectively. Microcosms were then established in triplicate for destructive sampling for each of the two original soils and for the high and low pH mixed soils, amended with ammonium (20 μg g^{-1} soil) and incubated for 24 days. Nitrification kinetics (ammonia disappearance) are presented in Fig. 4. Ammonia decreased to below 1 μg NH_4^+-N g^{-1} soil within 2 days in native pH 6.9 soil and within 4 days in the mixed soil adjusted to pH 6.9. Nitrification was slower in the two pH 4.3 soil microcosms, as expected, but again the rate of ammonia decrease was greater in the native soil, which oxidized ammonia within 8 days, while ammonia depletion in the mixed soil adjusted to pH 4.3 occurred after 12 days. For each of the four soils there was no significant difference in the pH between each set of destructively sampled microcosms throughout the course of the experiment. These results indicate that the acid- or neutral-specific ammonia oxidizer communities were 'diluted' in the mixed soils by communities adapted to native pH values, leading to a reduction in total nitrification rate.

Total nucleic acids were extracted from microcosms sampled on day 4 (during high nitrification activity), day 12 (after ammonia concentrations had decreased below 1 mg NH_4^+-N g^{-1} soil in all microcosms) and day 24. The

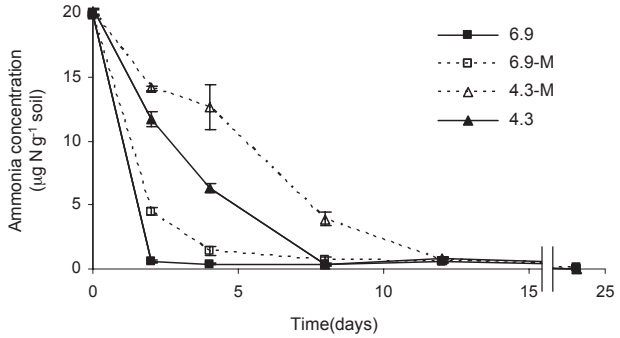

Fig. 4. Changes in soil ammonia concentration during incubation for 24 days of microcosms containing soils supplemented with 20 mg $NH4^+$-N g^{-1} soil. Triplicate microcosms were destructively sampled at each time point for two native soils from plots maintained at pH 4.3 and 6.9 and mixtures of these two soil following adjustment of soil pH to 4.3–6.9. Error bars represent standard error of the mean of triplicate microcosms.

transcriptionally active crenarchaeal ammonia oxidizing community was assessed by DGGE analysis of *amoA* gene transcripts. RT-PCR products were obtained from all samples and DGGE profiles were compared by principal component analysis (PCA) (Fig. 5). Crenarchaeal communities in the native soils had contrasting *amoA* transcript profiles, with bands in high and low pH samples co-migrating with sequences in the DGGE marker typical of high and low pH soils. At day 4, the high and low pH mixed soils resembled each other and the native pH 4.3 soil. This is indicated by PCA analysis, with little separation along PC1, which explained 83.1% of the variability. At days 12 and 24, bands considered representative of low and high pH soils (in comparison to the clone marker lane) showed increased differential relative expression at low and high pH respectively; PCA analyses showed greater separation of the two sets of mixed soils, and the pH 4.3 mixed soil communities became highly similar to the native 4.3 soil. These results therefore indicate that the different crenarchaeal populations were only active in particular pH ranges. The greatest differences in expression profiles were observed in day 24 profiles when nitrification activity was presumably much lower than that at day 4, and indicates that differential activity occurred in soil with low ammonia concentrations.

Discussion

Long-term pH selection within bacterial and crenarchaeal ammonia oxidizers

Autotrophic nitrification occurs in acid soils, despite the inability of cultivated bacterial ammonia oxidizers to grow in liquid batch culture at pH values below 7. Previous studies provided evidence for selection of different ammonia oxidizer phylotypes in soils maintained in the pH range 4.5–7.5 since 1961 (Stephen *et al*., 1996; Kowalchuk *et al*., 1998). This study aimed to determine whether similar selection occurred for ammonia oxidizing archaea in these soils and whether bacterial ammonia oxidizer selection persisted since the previous study performed 10 years earlier. DGGE analysis of 16S rRNA gene fragments, amplified using primers targeting the breadth of betaproteobacterial ammonia oxidizer diversity, enabled comparison of pH selection in soil plots and showed pH selection of the same bacterial ammonia oxidizer phylotypes as observed by Stephen and colleagues (1998). Although these two studies used different primer sets for profiling the bacterial ammonia oxidizer communities, comparison with sequences in the marker lane revealed selection by *Nitrosospira* clusters 2 and 3 in acid and neutral soils respectively. These communities therefore appear to be stable over more than a decade. Crenarchaeal community structure analyses, using both 16S rRNA and *amoA* genes, revealed similar, long-term selection in these soils for distinct phylotypes at low and high pH and no detectable overlap in community structure.

pH selection of bacterial and archaeal ammonia oxidizers and their activity

Quantification of *amoA* genes by qPCR showed contrasting pH effects, with bacterial and archaeal *amoA* abundance, respectively, decreasing and increasing as soil acidity increased. Gene abundances were similar to those observed by Leininger and colleagues (2006), who also found greater abundance of archaeal than bacterial *amoA* genes, as observed here in all soil pH plots. Gene abundance provides information on potential ammonia oxidizing activity but transcriptional activity is a measure of protein (i.e. ammonia monooxygenase subunits) production potential in soil. Archaeal and bacterial *amoA* gene transcript abundance showed contrasting patterns with soil pH. Archaeal transcript abundance decreased significantly with increasing soil pH, whereas bacterial transcripts increased with increasing pH to a maximum at 6.9, before decreasing slightly at 7.3 and 7.5. Interestingly, this correlates with measurements of autotrophic (i.e. acetylene-blocked) nitrification in these plots by Killham (1990) where maximum rates of nitrification occurred in the pH 6.5 plots. Bacterial *amoA* gene expression was much lower than that of archaea at low pH but increased with increasing pH. These contrasting trends in specific gene expression may reflect different preferences of archaeal and bacterial ammonia oxidizers for available ammonia concentration or fundamental differences in physiology and metabolism. In this respect, it is interesting that overall transcript abundance was not proportional

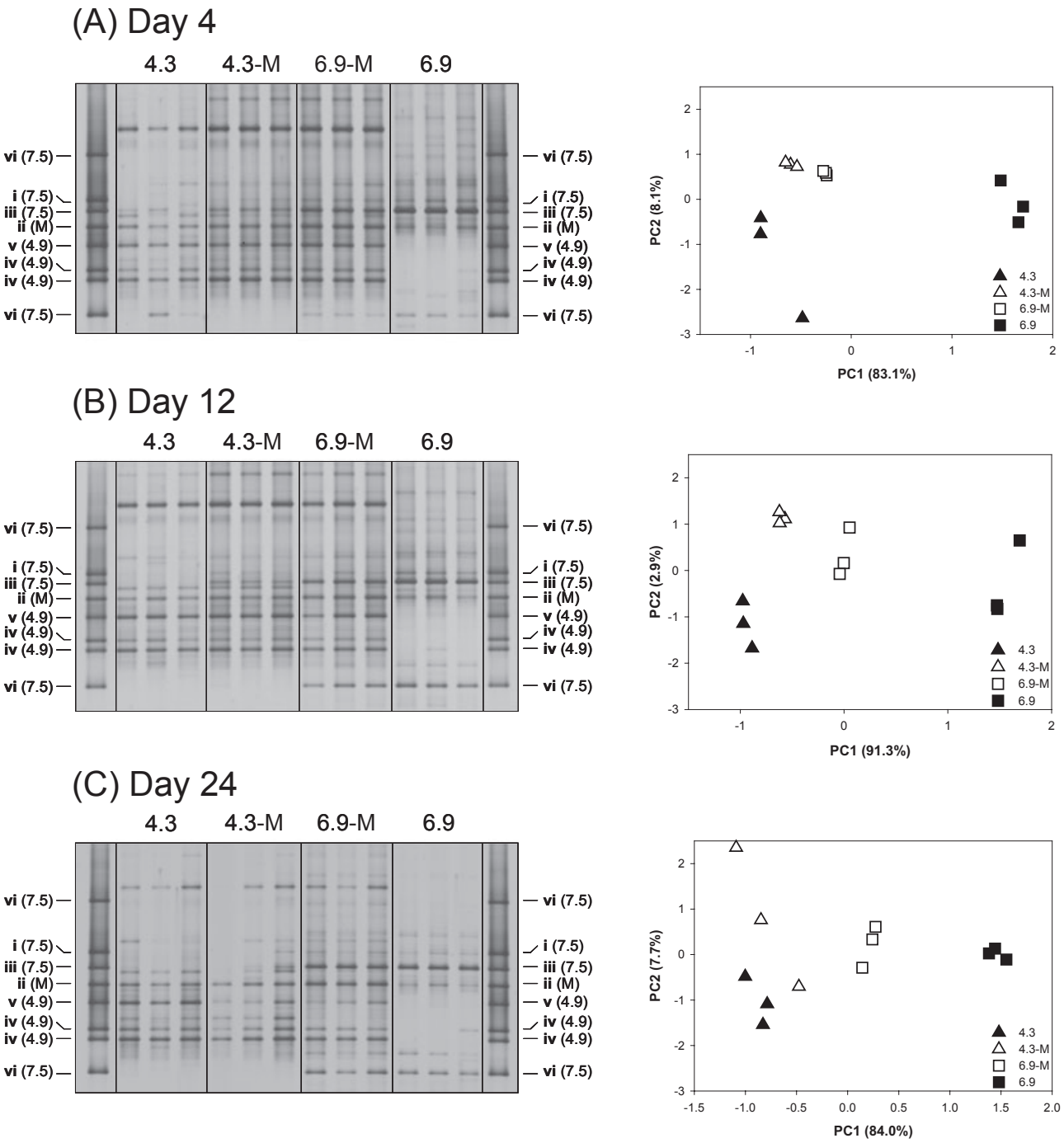

Fig. 5. Short-term, *amoA* transcriptional activity of crenarchaeal communities in native and mixed soils at acidic and neutral pH. Samples were run alongside a DGGE marker composed of PCR products derived from eight *amoA* clones representative of major clades of sequences identified in clone libraries (see Fig. 1). DGGE and principal component analysis of crenarchaeal *amoA* transcripts from microcosms after incubation for 4 days (A), 12 days (B) and 24 days (C).

to nitrification, being greatest at low pH, where nitrification is lowest in these soils (Killham, 1990), and may hint at additional functional activities. However, it should be noted that changes in the mRNA transcript abundance may not directly reflect protein production and activity. For example, we do not know the half-life of *amo*A mRNA, which may even vary depending on the growth conditions of the organisms. In addition, transcript numbers were lower than gene copies in all samples. Although this may reflect some loss of mRNA and/or conversion of only a

proportion of mRNA into cDNA during reverse transcription, these data probably indicate that a large proportion of the organisms were inactive or the amounts measured reflect steady-state levels. The results therefore highlight that caution is required when relating molecular indicators of activity (gene expression, protein levels) to cellular and process activity, particularly where community structure and environmental factors are changing significantly. However, the presence of distinct phylotypes and the highest ratio of archaeal versus bacterial transcriptional activity occurring in the lowest pH soils indicate that autotrophic ammonia oxidation in acidic soils may be largely attributable to archaea. If the dominant ammonia oxidizers in the soils were indeed AOA, it is tempting to estimate cell activity rates from these data. For example, in the lowest pH soil where AOA *amo*A gene copies were 100¥ more abundant than their bacterial counterparts, the rate of ammonia oxidation was approximately 40 mmol-day^{-1} (as measured over the first four days of the transcription experiment). If the dominant community of AOA cells were present at 10^7 cells g^{-1} soil (assuming one *amo*A gene copy equated to one cell), they would have an average activity of around 40 fmol NH_3 AOA cell^{-1} day^{-1} which is within the range of that found for AOB (Prosser, 1989). This is also comparable to rates of approximately 7 and 40 fmol NH_3 cell^{-1} day^{-1} that can be calculated for the cultivated AOA *Nitrosopumilus maritimus* (Könneke *et al*., 2005) and *Nitrosocaldus yellowstonii* (de la Torre *et al*., 2008) respectively.

Short-term, transcriptional activity was determined in native pH 4.3 and 6.9 soils and in mixed soils adjusted to these two pH values. It was not possible to determine transcriptional activity of ammonia oxidizing bacteria as no product was obtained, but DGGE profiles of archaeal *amoA* gene transcripts were similar to *amoA* gene profiles. Thus, the abundant archaeal ammonia oxidizer phylotypes at the different pH values are also likely to be the most active. Although the transcription profiles of the mixed soils were not identical to those in native soils with similar pH values, those band positions that were reproducibly different in relative intensities between the two sets of mixed soils were those that were associated specifically with the same pH in the native soils. DGGE analysis of the DNA-defined community structure showed no differences between the two mixed soils (data not shown). This therefore indicates that crenarchaea do not constitutively express *amoA*, but do so specifically in response to particular pH ranges.

Soil pH and crenarchaeal phylogeny

The extent to which crenarchaeal community analysis reflects that of ammonia oxidizing crenarchaea is unknown. *AmoA* genes have been found in members of 16S rRNA-defined crenarchaeal groups 1.1a (*C. symbiosum* and *N. maritimus*), 1.1b (soil fosmid 54d9) and have been indirectly associated with organisms within the pSL12 clade (Mincer *et al*., 2007). However, it is not known whether other mesophilic crenarchaeal groups (e.g. 1.1c) also possess ammonia monooxygenase genes and analysis of crenarchaeal 16S rRNA genes may therefore not be diagnostic of crenarchaeal ammonia oxidizers. Nevertheless, most soil archaeal sequences fall within the 1.1b lineage (e.g. Buckley *et al*., 1998; Ochsenreiter *et al*., 2003), and most of those detected here may fall within this lineage. Therefore, 16S rRNA-based analysis may target largely *amoA*-possessing organisms if ammonia monooxygenase is possessed by organisms throughout the lineage.

A number of distinct clades contained sequences exclusively derived from high or low pH soils for both genes. However, low- or high-pH clades did not form monophyletic groups in either analysis, indicating several independent adaptations to a particular soil pH range. The inclusion of marine and soil reference taxa [including those sequences from soil fosmid clone 54d9 (Treusch *et al*., 2005), *C. symbiosum* (Hallam *et al*., 2006) and *N. maritimus* (Könneke *et al*., 2005)] provided anchors for inferring congruence. The majority of sequences were placed within the dominant 'soil' lineage with four major clusters of sequences. The relative branching order (i.e. those containing exclusively pH 7.5 sequences, or a mixture of 4.9 and 7.5 sequences, and the placement of 54d9) mirrored that of the *amoA* phylogeny in the 'soil' lineage. One discrepancy between 16S rRNA and *amoA* phylogenies was the relative number of sequences in 16S rRNA Group 1.1a and *amoA* marine groups, which contained one and nine pH 7.5 sequences respectively. However, both groups are linked with the inclusion of 16S rRNA and *amoA* sequences of *N. maritimus* and *C. symbiosum*. The high congruence in patterns of 16S rRNA- and *amoA*-sequence distribution in the phylogenetic analyses suggests coevolution of both genes and strongly indicates that the majority of organisms profiled by 16S rRNA gene analysis also possessed *amoA* genes.

pH-associated distribution of specific crenarchaeal 16S rRNA and *amoA*-defined lineages observed here is supported by findings of other studies. For example, Nicol and colleagues (2005; 2006) observed a highly structured succession of *Crenarchaea* in the foreland of a receding glacier. This chronosequence of soil development had a gradient of soil pH from 7.5 to 4.2 with phylogenetically distinct clades of sequences associated with soils of different pH ranges. Sequences placed within 16S rRNA 'clade i' in this study (Fig. 3) contained sequences from exclusively pH 7.5 plots and grouped with sequences associated with glacier foreland soils with a pH greater than 7 ('RotA' sequences). Similarly, 'clade iv' in this study,

was dominated by sequences from pH 4.5 plots and was closely related to sequences associated with glacier foreland soils with a pH less than 5 ('RotD' sequences). It is also interesting to note that archaeal ammonia oxidizer clades described by He and colleagues (2007) were related to different fertilizer regimens but also, to some extent, soil pH.

In conclusion, this study confirms that different archaeal and bacterial ammonia oxidizer phylotypes are selected in soils of different pH. Quantification of gene copies confirms this selection and quantification of gene transcripts indicates that these differences in community structure and abundance are reflected in different contributions to ammonia oxidizer activity. The findings suggest that archaeal and bacterial ammonia oxidizer subgroups have distinct physiological characteristics and ecological niches, with consequences for nitrification in acid soils. Although crenarchaeal cell numbers and transcripts dominated over their bacterial counterparts, the pattern of changing ammonia oxidizer bacterial gene copies and transcriptional levels correlated more closely with measured nitrification rates and the relevance of transcriptional activity to ecosystem function therefore requires further investigation.

Experimental procedures

Field site and sample collection

The study site was a section of an agricultural field (Scottish Agricultural College, Craibstone, Scotland, Grid reference NJ872104) containing a series of plots each of which have been maintained since 1961 with a gradient of pH values of approximately 4.5, 5.0, 5.5, 6.0, 6.6, 7.0 and 7.5 by addition of either lime or aluminium sulfate. Plots undergo an 8-year crop rotation cycle, and detailed soil characteristics are provided by Kemp and colleagues (1992). The plots are identical to those investigated by Stephen and colleagues (1996; 1998), which were sampled in August 2006 and had supported a crop of potatoes in the previous year. For long-term analysis of ammonia oxidizer communities, triplicate 200 g soils samples were removed at 1 m intervals from the surface 10 cm within each pH-controlled subplot and were homogenized by passing through a 3.35 mm mesh sieve. Approximately 50 g soil was archived at −20°C for molecular analysis and the remainder was stored at 5°C for physicochemical analysis. Larger masses (2 kg) of soil from pH 4.5 and pH 7 plots were collected and sieved for microcosm experiments.

Soil microcosms

Eighteen replicate microcosms containing 10 g soil were constructed in 30 ml Universal bottles for four different sets of soils (72 in total) for destructive sampling. Two sets contained sieved soil from either the pH 4.5 plot or the pH 7.0 plot, with actual pH values of 4.3 and 6.9, respectively, measured in deionized water [using a ratio of 1:2 soil : water (w/v), shaking for 15 min and settling for 30 min before measurement]. The remaining two sets contained 10 g of mixed pH 4.3 and pH 6.9 soil that had been thoroughly mixed before adjustment back to approximately pH 4.3 or 6.9 by addition of 8 or 2 mg g^{-1} soil of $Al_2(SO4)_3$ or $Ca(OH)_2$, respectively, and left for 24 h prior to establishing microcosms. Each microcosm was individually supplemented with 1 ml of 8 mM ammonium sulfate to give a final concentration of 20 mg NH_4^+-N g^{-1} soil. After incubation at room temperature for 0, 2, 4, 8, 12 and 24 days, 2 g soil was removed from each microcosm and immediately frozen at −80°C. The remaining 8 g soil was used for determination of pH and colorimetric determination of ammonia concentration by flow injection analysis (FIA star 5010 Analyzer, Tecator, USA; Allen, 1989).

Nucleic acid extraction, cDNA synthesis and standard PCR amplification

Nucleic acids were extracted from 0.5 g soil subsamples from microcosms and from field samples, as described by Griffiths and colleagues (2000) and as modified by Nicol and colleagues (2005). To generate cDNA, RNA was purified using a RNeasy kit (Qiagen, Crawley, UK) before a dilution of the extract was treated with RQ1 DNase (Promega, Southampton, UK). cDNA was produced using Superscript II reverse transcriptase (Invitrogen, Paisley, UK). Random hexamer primers (Invitrogen) were used at a concentration of 160 pmol per reaction. Two negative controls were performed with all reactions. The first control contained soil RNA template and all DNase/RT reagents except for the final addition of RT enzyme. A second control contained no template (water only) to ensure that all reagents were free of possible contaminants.

For DGGE analysis of AOB communities, 16S rRNA genes were amplified from extracted DNA using a nested PCR approach (Freitag *et al*., 2006). Primary amplification used CTO189f and CTO654r PCR primers (Kowalchuk *et al*., 1997) and amplicons were nested with P3 (357f-GC) and P2 (518r) (Muyzer *et al*., 1993). Primary amplification of archaeal 16S rRNA genes was performed using primers A109f (Großkopf *et al*., 1998) and a modified version of 1492r (Lane, 1991) to avoid mismatches with soil crenarchaeal sequences over this region (G.W. Nicol, unpubl. data) with the primer shortened and modified with an additional degeneracy (5′-GYYACCTTGTTACGACTT-3′). These products were used for cloning and also as template for secondary amplification for DGGE analysis using crenarchaeal-biased primers 771f and 957r (Ochsenreiter *et al*., 2003) with primer 957r containing additionally the GC clamp of primer P3 (Muyzer *et al*., 1993). Both AOB and crenarchaeal 16S rRNA gene PCR assays used two sets of 35 cycles. Ammonia oxidizing archaea were characterized by cloning and DGGE analysis of *amoA* genes amplified using primers CrenamA23f/CrenamoA616r (Tourna *et al*., 2008). Cycling conditions for amplifying all genes were 95°C for 5 min; followed by 10 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min; followed by 25 cycles of 92°C for 30 s, 55°C for 30 s, 72°C for 1 min; followed by 72°C at 10 min, except for primer set A109f/1492r, which used an extension time of 2 min.

DGGE analysis

DGGE was performed using a DCode Universal Mutation Detection System (Bio-Rad, Hertfordshire, UK) as described previously (Nicol *et al*., 2005). Gels contained 8% (w/v) polyacrylamide and a linear gradient of 15–55% denaturant for crenarchaeal *amoA* assays and 35–70% for both 16S rRNA assays. Gels were electrophoresed in 7 l of 1¥ TAE buffer at a constant temperature of 60°C for 900 min at 100 V and silver-stained as previously described (Nicol *et al*., 2005) before scanning using an Epson GT9600 scanner with transparency unit (Epson, Hemel Hempstead, UK). To compare community profiles using PCA, relative (within lane) band intensities were quantified by densitometry analysis of normalized DGGE profiles using Phoretix 1-D gel analysis software (Phoretix International, Newcastle-Upon-Tyne, UK) as previously described (Nicol *et al*., 2007) before performing PCA with PAST v1.75 (Hammer *et al*., 2001).

Quantitative PCR

Quantification of bacterial *amo*A genes was based on a protocol described previously (Leininger *et al*., 2006). Primers *amo*A-1F* (Stephen *et al*., 1999) and *amo*A-2R (Rotthauwe *et al*., 1997) were used, and a dilution series of a known amount of a linearized plasmid (pCR4-TOPO, Invitrogen) containing the *amo*A gene of *Nitrosospira multiformis* ATCC25196 was used as a standard with efficiencies ranging from 81% to 86%. For crenarchaeal *amoA*, qPCR was performed with CrenamoA23f and a degenerated version of CrenamoA616r (5′-GCCATCCABCKRTANGTCCA-3′) and a dilution series of the linearized soil fosmid clone 54d9 was used as a standard. A fragment of 624 bp was amplified in a SybrGreenI® approach with efficiencies of 86–93%. Each reaction was performed in a 20 ml volume containing 0.2 mg ml^{-1} BSA, 1.5 mM of each primer and 10 ml of QuantiTect SYBR Green PCR Master Mix (Qiagen). For DNA analyses, 5 ng of DNA was added to each reaction. For transcript analysis, the cDNA added was equivalent to 0.36% of the total RNA extracted g^{-1} soil. Amplification was performed in a DNA Engine Opticon 2 System (MJ Research). Melting curve analysis was performed at the end of all qPCR runs to indicate amplification of specific products only, before confirmation by standard agarose gel electrophoresis. No inhibitory effects of environmental DNA extracts on PCR amplification were detected when known amounts of standard were spiked with environmental DNA or using the method described by Horz and colleagues (2004). Standards ranged over seven orders of magnitude and the limit of detection was 5 copies g^{-1} soil. All qPCR data presented were from independent extractions from triplicate field samples and triplicate independent PCR amplifications.

Cloning and sequence analysis of 16S rRNA and amoA *gene sequences*

First-round 16S rRNA and *amoA* gene PCR products were cloned into pGEM-T Easy vector (Promega, Southampton, UK) and amplicons were selected from each library after successful amplification of colony inserts using vector primers M13f and M13r. For 16S rRNA gene sequences, LogDet/Paralinear distances and tree construction (neighbour-joining) were performed using PAUP v4.01 (Swofford, 1998) as described previously (Nicol *et al*., 2005). Bootstrap support was calculated using PAUP, PHYML (Guindon and Gascuel, 2003) and PHYLIP (Felsenstein, 2007) for maximum likelihood, distance and parsimony analyses (100, 1000 and 1000 replicates respectively. Distance analysis of derived crenarchaeal *amoA* protein sequences and bootstrap support (maximum likelihood, distance and parsimony analyses; 1000 replicates each) were calculated using the Jones, Taylor and Thornton (JTT) substitution model with site variation (invariable sites and eight variable gamma rates) using PHYML (Guindon and Gascuel, 2003) and PHYLIP (Felsenstein, 2007). Sequences of chimeric origin were checked by analysing alignments with Ballerophon (Huber *et al*., 2004) and partial treeing analysis.

Statistical analysis

Gene abundances, DGGE relative band intensities and nitrification process data from triplicate microcosms were analysed using a general linear model (GLM) of analysis of variance (ANOVA) using Minitab 14 (Minitab, PA, USA).

Accession numbers

All sequences have been deposited in the GenBank database with accession numbers EU856113–EU856247.

Acknowledgements

G.W.N. is funded by a NERC Advanced Fellowship (NE/D010195/1). The authors would like to thank Mr Lawrence Maurice and the SAC Craibstone Estate (Aberdeen) for access to the Woodlands Field pH plots and the anonymous reviewers who made useful suggestions which benefited this manuscript.

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