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Cultivation-based and molecular approaches to characterisation of terrestrial and aquatic nitrifiers

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Abstract

Increased awareness of the metabolic diversity within autotrophic nitrifying bacteria has led to a re-evaluation of their role in the cycling of nitrogen in terrestrial and aquatic ecosystems. This has been accompanied by improvements in our ability to characterise natural populations of autotrophic ammonia oxidising bacteria through the application of molecular techniques. Molecular approaches indicate considerable diversity within natural populations and the association of different groups of ammonia oxidisers with different environments and changes in populations in response to environmental factors. To some extent, results from molecular approaches are consistent with those adopting laboratory enrichment and isolation strategies. Physiological studies on the latter demonstrate links between phylogenetic groups and possession of characteristics of relevance to ecological studies. Understanding of the significance of ammonia oxidiser species and functional diversity for global cycling of nitrogen require greater links between molecular analyses, physiological studies and measurements of nitrogen cycling processes. However, there is increasing evidence for physiological properties driving the environmental distribution of particular groups of ammonia oxidisers and for associations between nitrification process rates and ammonia oxidiser community structure.

Introduction

Nitrification, the oxidation of reduced forms of nitrogen, is essential for global cycling of nitrogen. In its most common form, it involves the oxidation of ammonia to nitrate, via nitrite, by two groups of autotrophic bacteria, ammonia oxidisers and nitrite oxidisers. Autotrophic nitrifiers are therefore responsible for mobilisation and leaching of N in terrestrial environments, leading to significant losses of ammonia-based fertiliser and nitrate pollution of groundwater and run-off waters. In aquatic environments ammonia oxidisers may compete with primary producers for ammonia and they also play a crucial role in removal of ammonia in wastewater treatment processes. Metabolic diversity within autotrophic nitrifiers is, however, high. Ammonia oxidisers can hydrolyse urea and oxidise carbon monoxide, methane and a range of recalcitrant organic compounds, while many nitrite oxidisers grow mixotrophically or heterotrophically on organic substrates. Both groups are also able to reduce oxidised forms of inorganic nitrogen, leading to production of NO, N_2O and N_2, particularly at low O_2 concentrations. They are therefore involved in production of greenhouse gases in both aquatic and terrestrial environments. The traditional, simplistic view of nitrification is further complicated by the existence of heterotrophic nitrification, which is the production of nitrate directly from organic N substrates as a by-product of the secondary metabolism of certain fungi and bacteria (Killham 1986). In addition, anaerobic nitrification has been demonstrated in wastewater treatment systems (Jetten et al. 1998) and is discussed elsewhere in this publication.

Realisation of this metabolic diversity has led to a re-evaluation of the processes associated with conversion of reduced N in natural environments, as a result of both process-based studies and physiological characterisation of the organisms responsible. The availability of molecular techniques has led to

a parallel revolution in our understanding of the diversity of the microbial groups involved in nitrification. The molecular approach has proved particularly useful in its application to autotrophic ammonia oxidisers, which are believed to be the major contributors to nitrification in natural environments. Molecular studies have now developed beyond characterisation of natural populations to assessment of the influence of environmental factors on natural populations. An understanding of the influence of such factors on ammonia oxidiser diversity, and their consequent impact on nitrification processes, requires consideration of physiological characteristics of different groups which, in turn, requires analysis of representative cultured strains. Different aspects of nitrifier ecology have been the subject of a number of reviews (Belser 1979; Prosser 1989; Laanbroek & Woldendorp 1995; Ward 1996; Head et al. 1998; Bothe et al. 2000a,b; Koops & Pommerening-Röser 2001; Kowalchuk & Stephen 2001; Zehr & Ward 2002). This article draws comparisons between the nature of enriched and pure cultures from natural environments and those detected using molecular techniques, and assesses progress in linking phylogenetic diversity and functional diversity to rates of processes carried out by this important group of microorganisms.

Cultivation-based techniques

Autotrophic ammonia oxidising bacteria from environmental samples are obtained by enrichment in inorganic medium containing ammonium (Allison & Prosser 1992). Omission of organic carbon discourages, but does not eliminate heterotrophs, which grow on organic by-products of ammonia oxidiser growth and on volatile organic compounds or contaminants of media and culture vessels. Pure cultures are obtained from liquid culture enrichments by end-point dilution and from solid medium enrichments by colony picking and subculture into liquid medium. In both cases, purification is made difficult by the slow growth and low yield of ammonia oxidisers on laboratory media. For example, incubation on solid medium for several weeks is required for the production of microscopic colonies. In some studies, end-point dilution has been combined with enumeration by the most probable number method, with isolation and identification of organisms from cultures at the highest dilutions exhibiting growth (Belser & Schmidt 1978). This allows characterisation of the most numerous cultivable organisms. Pure cultures also provide the opportunity to determine physiological characteristics of environmental relevance. Again, difficulties in cultivation of ammonia oxidisers and restricted availability of pure cultures have limited this approach, but published data are available for a number of relevant properties, including salt tolerance, ammonia sensitivity, urease activity and K_s values for ammonia and oxygen (Prosser 1989; Koops & Pommerening-Röser 2001)

Cultivation-based approaches to studies of natural populations suffer from well-accepted problems resulting from the selective nature of laboratory media and incubation conditions, competition from other organisms during incubation and difficulties in creating media and growth conditions that faithfully reproduce those in natural environments. These are exacerbated by the slow growth of ammonia oxidisers in liquid culture and on solid media and by the fact that pure cultures, once obtained, are difficult to keep free of contamination and often die out with repeated subculture. Despite these disadvantages, successful enrichment of a particular strain is necessary for unequivocal evidence of its presence and viability in an environment. In addition, laboratory cultures provide the potential for development of antibodies specific to particular groups, again allowing direct detection of target organisms in environmental samples.

Characterisation of pure cultures led to the traditional classification of autotrophic ammonia oxidisers into five genera, based on cell morphology and a limited number of additional phenotypic characters. Confirmation of taxonomic groupings, including species descriptions, has been achieved using DNA:DNA hybridisation of pure cultures of these organisms but this approach is necessarily limited by the difficulties in obtaining pure cultures of ammonia oxidisers. Identification of cultures using the available phenotypic characters is also difficult and these problems have restricted studies of natural diversity of autotrophic ammonia oxidisers to those utilising immunological techniques (e.g., Belser & Schmidt 1978; Ward & Carlucci 1985) and long-term isolation programmes.

Molecular-based techniques

Analysis of 16S rRNA gene sequences provides the basis for phylogenetic classification of microorganisms and was first applied to a limited number of ammonia oxidisers by Woese et al. (1984, 1985), with

subsequent analysis of sequences from 11 pure cultures by Head et al. (1993). These analyses placed *Nitrosococcus oceanus* in the γ-proteobacteria and other strains in two genera, *Nitrosomonas* and *Nitrosospira*, within the β-proteobacteria, *Nitrosospira* incorporating the genera *Nitrosovibrio* and *Nitrosolobus*. These sequence data were used to construct primers (McCaig et al. 1994) for the specific amplification of ammonia oxidiser 16S rRNA genes from DNA extracted from environmental samples, thereby providing a means of characterising natural populations of ammonia oxidisers. This approach involves analysis of amplified 16S rRNA genes by cloning, followed by sequencing and phylogenetic analysis of members of the clone library, or by fingerprinting techniques such as denaturing and temperature gradient gel electrophoresis (DGGE, TGGE; Muyzer et al. 1993; Muyzer & Smalla 1998). Alternatively, sequence data can be used to design ammonia oxidiser specific oligonucleotide probes for *in situ* detection by fluorescence *in situ* hybridisation (FISH, DeLong et al. 1989). Molecular analysis has also been extended by the development and application of primers for genes encoding ammonia monooxygenase, in particular *amoA*, with subsequent analysis by cloning, sequencing, DGGE (Webster et al. 2002) or T-RFLP (see Bothe et al. 2000a,b).

This molecular approach has significant advantages for analysis of ammonia oxidiser communities. Most importantly, analysis can be carried out without enrichment and isolation of pure cultures, thereby removing the major problems discussed above. In addition, acquisition of sequence data is more rapid than isolation processes and characterisation on the basis of molecular data is more informative than by analysis of phenotypic characteristics. Furthermore, *in situ* detection using 16S rRNA probing has advantages over traditional immunological techniques in that it does not require pure cultures for generation of antibodies, again eliminating the need for prior cultivation of target organisms. *In situ* analysis of ammonia oxidisers has been achieved in samples from wastewater treatment processes, where populations are high, and has demonstrated their existence in discrete clusters (e.g., Schramm et al. 1999). In aquatic environments, the low cell concentrations of ammonia oxidisers reduce the applicability of this approach, while analysis of soil samples is further complicated by background fluorescence and colonisation of particulate material. Here discussion will focus on characterisation of ammonia oxidiser populations in natural terrestrial and aquatic environments.

Characterisation of natural diversity

PCR amplification of 16S rRNA genes from environmental samples has been carried out using a range of primer sets with varying degrees of specificity (see below). Phylogenetic analysis of these environmental sequences, and of those obtained from pure and enrichment cultures, presents a relatively consistent picture of the diversity of ammonia oxidisers in natural environments. Ammonia oxidisers within the γ-proteobacteria have only been detected in marine environments and pure culture representatives of this group have only been isolated from marine environments. They appear to be rare in comparison to β-proteobacterial ammonia oxidisers, although few molecular studies have targeted this group.

Within the β-proteobacteria, there is significant diversity within sequences from enrichment cultures and environmental clones. Sampling of clone libraries rarely reveals identical sequences, although differences in sequence are not always great and, in some cases, may be due to sequencing and other errors. Most environmental studies, however, analyse relatively small gene fragments, reducing the ability to discriminate between strains. Analysis of environmental sequences provided further evidence for the subdivision of β-proteobacterial ammonia oxidisers into two genera, and also indicated the existence of at least seven subclusters, four within *Nitrosospira* and three within *Nitrosomonas* (Stephen et al. 1996). In addition to characterising diversity, these data enabled refinement of primers and probes for ammonia oxidisers and for the proposed subclusters. A number of subsequent studies of full-length sequences of 16S rRNA gene sequences from pure culture representatives of β-proteobacterial ammonia oxidisers (Teske et al. 1994; Pommeringening-Röser et al. 1996; Utåker et al. 1996b; Suwa et al. 1997; Purkhold et al. 2000) provide further support for these clusters. The most extensive study (Purkhold et al. 2000) compared phylogenetic analysis of 16S rRNA genes with that of a key functional gene, *amoA*, encoding ammonia monooxygenase. The two phylogenies indicate similar evolutionary relationships and suggest additional subgroups/clusters to those defined by Stephen et al. (1996) within the *Nitrosomonas* genus. Their study focused on *Nitrosomonas* and clusters within the *Ni-*

trosospira genus are less stable when subjected to different forms of phylogenetic analysis. Molecular techniques potentially involve a number of biases, including cell lysis techniques, DNA extraction efficiency, DNA purification methods, PCR bias, formation of chimeras, sequencing errors, multiple gene copies, biofilm formation and cloning errors. These must be considered when interpreting molecular data but little is known of their relative contributions and significance, and it is often possible to design protocols and controls to determine and minimise their influence. However, a particularly important consideration is the specificity of PCR primers and probes. Purkhold et al. (2000) extended the analysis carried out by Utåker & Nes (1998) and demonstrated that a single primer set, designed by McCaig et al. (1994), was capable of amplifying all known β-proteobacterial ammonia oxidiser sequences. This primer set does, however, also amplify non-ammonia oxidiser sequences in some environments and the use of more specific but non-inclusive primers and probes, either alone or in a nested PCR, may provide advantages in some applications. Their study also illustrates that our ignorance of natural populations necessitates continual review and modification of primers and probes as additional information becomes available.

The development and availability of primers and probes for ammonia oxidisers and for the proposed subclusters enabled the construction of hypotheses regarding the links between the presence, absence and relative abundance of particular phylogenetic groups and their ecology and habitats. The molecular approach, in itself, gives little information on the mechanisms controlling the distribution of particular groups of ammonia oxidisers. However, although relatively few physiological characteristics have been studied in a limited number of strains, there is evidence for links between species diversity and functional diversity (Koops & Pommerening-Röser 2001). Particular environments tend to yield similar groups of organisms in enrichment and purification procedures and these links between the molecular and laboratory based studies will be discussed below.

Molecular analysis of soil samples commonly yields sequences representative of *Nitrosospira* clusters 2, 3 and 4 and pure and enrichment cultures representative of these clusters have been obtained only from terrestrial environments. In contrast, *Nitrosomonas* cluster 6a clones and cultures are restricted to marine environments while *Nitrosomonas* cluster 7 sequences are obtained from a range of environments, including marine and non-marine environments. These results suggest differences in physiological characteristics between clusters and one of the exciting areas for future research is discovery of the links between metabolic diversity and genetic diversity indicated by 16S rRNA and *amoA* sequences. This will require extension of both the range of metabolic properties and of laboratory cultures and will be driven most efficiently by hypotheses arising from molecular studies.

Two clusters, *Nitrosospira* cluster 1 and *Nitrosomonas* cluster 5, contain only sequences from marine environments and are of particular interest in that no pure culture representative of these clusters has yet been obtained. Unequivocal evidence of the presence, if not role, of these organisms in the environment requires their isolation and demonstration of ammonia oxidising activity. This has not yet been achieved, although enrichment cultures have been obtained but did not persist (McCaig, Embley & Prosser unpublished observations). The absence of laboratory cultures also means that we can only speculate on their physiological properties and role in marine nitrification. Indeed, confirmation that all sequences within the phylogenetic grouping containing β-proteobacterial ammonia oxidisers actually carry out ammonia oxidation requires additional techniques. However, confidence is provided by the lack of non-ammonia oxidisers with sequences that fall within this grouping and the fact that all pure and enrichment cultures performing autotrophic ammonia oxidation fall within this group, with the exception of γ-proteobacterial ammonia oxidisers. Of greater concern is the apparent lack of *Nitrosomonas* strains found in environmental samples and, in particular, of those related closely to the model organism *Nitrosomonas europaea* (Stephen et al. 1996). This has been reported by a number of other workers. For example, Hiorns et al. (1995) could detect *Nitrosospira* sequences, but not *Nitrosomonas* sequences, in environmental DNA from soil, activated sludge, lake water and sediment samples. Both genera were, however, represented in enrichments obtained from these environments. In some cases the inability to detect *Nitrosomonas* in these environments may reflect deficiencies in the primers and probes used. However, much of our knowledge of the biochemistry and physiology of ammonia oxidising bacteria is derived from laboratory studies of *N. europaea* and the relevance of this information to ammonia oxidisers operating in natural environments must be questioned. Certainly, greater emphasis must

Table 1. Numbers and (in parentheses) percentages of partial 16S rDNA sequences from terrestrial and marine environmental clones and enrichment cultures between β-proteobacterial ammonia oxidiser clusters defined by Stephen et al. (1996). From Smith et al. (2001), with permission.

	Terrestrial samples		Marine samples	
	Enrichment cultures	Environmental clones	Enrichment cultures	Environmental clones
Nitrosospira cluster 1			3 (17)	31 (78)
Nitrosospira cluster 2	4 (14)	21 (43)		
Nitrosospira cluster 3	19 (66)	10 (20)		
Nitrosospira cluster 4	5 (17)	15 (31)		
Nitrosomonas cluster 5			2 (11)	6 (15)
Nitrosomonas cluster 6		3 (6)	11 (61)	3 (8)
Nitrosomonas cluster 7	1 (3)		1 (6)	
Other[a]	2 (6)	1 (2)	1 (6)	
Total	31	50	18	40

[a] One clone and two enrichments belonged to the *Nitrosospira* clade but could be placed within any clusters previously defined in Stephen et al. (1996) using the available data. One marine sediment enrichment, while belonging to the ammonia oxidiser clade, was not associated strongly with either genus. These sequences were not used in calculating percentages.

be placed on *Nitrosospira* in future ecophysiological studies.

Sequence analysis of laboratory enrichments

A major limitation of traditional approaches to the study of microbial diversity is the selection, in the laboratory, of organisms that are not representative of the natural community. This may be tested by comparison of sequences amplified from environmental DNA with those from enrichment cultures obtained from the same environmental samples. The traditional method for enumeration of ammonia oxidisers, the most probable number method, also provides a source of sequences from culturable organisms.

Smith et al. (2001) compared sequences from environmental DNA and enrichment cultures from both soil and marine sediment samples. Many of the enrichment culture sequences were identical, over the ~300 base-pair region analysed, suggesting selection for particular strains in laboratory culture. The possibility exists, however, that more detailed analysis, e.g., of longer sequences or of functional genes, may reveal additional diversity within these cultures. Identical clone sequences were obtained only rarely and only a small proportion of clone sequences were identical to those in enrichment cultures. Thus, 16 and 2.5% of enrichment sequences were identical to those in clone libraries sampled from terrestrial and marine environments, respectively. This suggests that selective pressures imposed by laboratory cultivation may be greater for the marine ammonia oxidiser populations investigated. However, for both samples, the percentage of 'culturable' organisms was greater than the 1% value frequently quoted for bacterial populations (Torsvik et al. 1996). In addition, the relative abundance of sequences representative of different clusters differed significantly between clone libraries and enrichment cultures. For soil samples, there was further evidence of laboratory selection. Environmental clones contained representatives of *Nitrosospira* clusters 2, 3 and 4, with a small percentage of *Nitrosomonas* cluster 6. The majority (66%) of enrichment cultures contained *Nitrosospira* cluster 3, with lower percentages of *Nitrosospira* clusters 2 and 4 and *Nitrosomonas* cluster 7 (Table 1). Enrichment cultures from marine samples were dominated (61%) by *Nitrosomonas* cluster 6, with fewer numbers of *Nitrosospira* cluster 1 and *Nitrosomonas* clusters 5 and 7. In contrast, the majority of clones examined contained sequences representative of *Nitrosomonas* cluster 1, with lower representation by *Nitrosomonas* clusters 5 and 6. The community structures indicated by molecular analyses of enrichment cultures and of environmental DNA from the same environmental samples therefore differ significantly, while analysis of sequence data also indicates laboratory selection of particular strains. This may be the major source of differences between the two approaches, and molecular analysis may provide a better indication of the structure of indigenous communit-

ies. It must be remembered, however, that molecular techniques are also subject to bias and there is currently little information on the nature and extent of such biases.

Phillips et al. (2000) also compared sequences derived from environmental DNA and from laboratory cultures from the same soil samples, the laboratory cultures being obtained from MPN counts, using media containing a range of ammonia concentrations. Sequences obtained from bands excised randomly from DGGE gels derived from a wide range of soil treatments were representative of *Nitrosospira* cluster 3. MPN cultures contained *Nitrosospira* cluster 3 sequences, but also representatives of *Nitrosospira* cluster 4 and *Nitrosomonas* clusters 6 and 7, which were not detected in environmental DNA. Interestingly, two of the *Nitrosomonas* sequences were closely related to *N. europaea*. Selection for *Nitrosomonas* was greatest in MPN counts employing media with 1000 μg NH_4^+-N ml^{-1}. The results therefore suggested selective enrichment in laboratory medium, particularly at high ammonium concentrations, of strains that were not representative of the natural population.

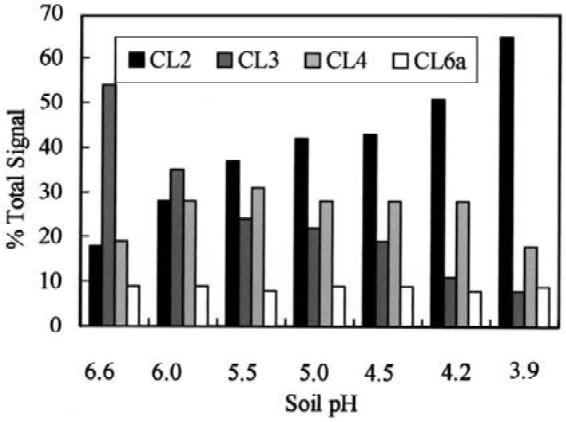

Figure 1. Quantification of hybridisation signals from DGGE gels of 16S rRNA genes amplified from DNA extracted from soil maintained at a range of pH values, using ammonia oxidiser specific primers. Quantification was achieved by phosphoimaging and profile analysis of each lane. The percentage total signal is the hybridisation signal for each sample, expressed as a normalised percentage of the signal for the cluster standard. Least significant difference (LSD) values for clusters 2, 3, 4 and 6a were 2.13, 2.36, 1.50 and 1.15 respectively. From Stephen et al. (1998), with permission.

Influence of soil characteristics on ammonia oxidiser communities

Soil pH

The growth of pure cultures of ammonia oxidising bacteria in liquid batch culture does not occur at pH values below 6.5 (Allison & Prosser 1991), because of increased ionisation of ammonia, the substrate for ammonia monooxygenase. Despite this, autotrophic nitrification has been reported in acid soils at pH values as low as 3.5 (de Boer et al. 1988; de Boer & Kowalchuk 2000). Explanations for autotrophic nitrification in acid soils include growth and/or activity on surfaces (Allison & Prosser 1993) or in aggregates (de Boer et al. 1991) and ureolytic activity (de Boer & Laanbroek 1989; Allison & Prosser 1991), as urea hydrolysis occurs at low pH values, enabling oxidation of ammonia produced intracellularly (Burton & Prosser 2001). A further explanation, the existence of strains adapted to low pH environments, could not be tested using traditional, cultivation-based techniques because of the possible lack of culturability of such strains. Molecular analysis removes this limitation and was used by Stephen et al. (1996, 1998) to test the hypothesis that pH influenced the ammonia oxidiser community structure in soils maintained over a long period at pH values ranging from 3.9 to 7.2. Sequence analysis of members of clone libraries constructed from 16S rRNA genes amplified from soils of pH 4.5 and 7.2 indicated an influence of pH on the relative abundance of different ammonia oxidiser clusters. *Nitrosospira* cluster 2 appeared to represent a greater proportion of the population at pH 4.5 than did *Nitrosospira* cluster 3, with the situation reversed at pH 7.2. No such difference in the relative abundance of the other clusters detected, *Nitrosospira* cluster 4 and *Nitrosomonas* cluster 6, was observed (Stephen et al. 1996). A more comprehensive analysis of soils with pH values ranging from 3.9 to 6.6 was then carried out using a different set of PCR primers and DGGE, combined with oligonucleotide probing, to distinguish clusters with similar banding patterns (Stephen et al. 1998). The relative abundance of different ammonia oxidiser clusters was estimated by quantification of band intensities normalised against a general probe. This provided further support for selection by soils of different pH. An increase in the relative abundance of *Nitrosospira* cluster 3 was observed with increasing pH and a corresponding decrease in *Nitrosospira* cluster 2 (Figure 1). There were also indications of a peak in the relative abundance of *Nitrosospira* cluster 4 at pH 5.5 but little detectable variation in *Nitrosomonas* cluster 6 with pH.

These results suggest the existence of groups of ammonia oxidisers that are adapted to growth at different soil pH values. This adaptation could be related to the proposed mechanisms for nitrification at low pH. For example, the greater relative abundance of *Nitrosospira* cluster 2 in acid soils could be due to a greater prevalence of ureolytic capability within this group or to factors that enhance growth on surfaces or in aggregates, all of which have been shown to facilitate growth at low pH. Alternatively, other mechanisms may be responsible. Unfortunately, little is known of the relationships between phylogeny and physiology within *Nitrosospira*. Koops & Pommerening-Röser (2001) report more frequent isolation of strains classified previously as *Nitrosolobus* (now considered within *Nitrosospira*) from soils of neutral pH, but ureolytic activity in these strains does not appear to increase abundance in acid soils. In contrast, terrestrial isolates of *Nitrosospira* and *Nitrosovibrio* strains were obtained from acid soils and their ureolytic activity could explain growth at low pH. It is currently not clear whether these strains fall within clusters 2 and 3, nor whether the distinction between these clusters is sufficiently robust to expect significant differences in physiology.

It must also be remembered that relatively few environments have been studied. Kowalchuk et al. (2000a) carried out molecular analysis of ammonia oxidisers in grassland soils and also reported selection for *Nitrosospira* clusters 2 and 4 at low pH values and Hastings et al. (2000) detected *Nitrosospira* in acid forest soils (pH 4.1), with lower abundance of *Nitrosomonas*. Changes in pH in a sand dune system indicated some pH-associated changes in *Nitrosospira* communities, while *Nitrosomonas* was detected only in the most seaward location examined (Kowalchuk et al. 1997). Laverman et al. (2001) found domination by a group of closely related *Nitrosospira* cluster 2 sequences and were unable to detect any temporal or spatial changes in community structure. However, other acid soils subjected to similar analyses (Bruns et al. 1999; Phillips et al. 2000; Webster et al. 2002) did not detect high relative abundance of *Nitrosospira* cluster 2 and populations were instead dominated by *Nitrosospira* cluster 3. It is therefore important to avoid generalisations regarding links between species diversity and environmental factors from a limited number of environments, particularly when so little is known of the physiological characteristics, and other mechanisms, that may drive microbial diversity and community structure.

Nitrogen fertilisation

It is generally accepted that long-term treatment with ammonium-based fertilisers increases populations of ammonia oxidisers but little is known of the effects of such treatments on community structure and diversity. Similarly, liming provides more favourable growth conditions, but may also alter the relative abundance of different groups, as indicated above. The ability to detect changes in community structure following nitrogen fertilisation, and other soil management procedures, depends both on the extent of shifts in ammonia oxidiser populations and on the techniques used to detect changes. The former will depend on the relative growth and death rates of organisms influenced by the changes imposed by treatments. Unless the influence of treatments is substantial and effected immediately, the low growth rates and survival capabilities of ammonia oxidisers are likely to make detection of changes difficult. Small and subtle changes are unlikely to be detected by fingerprinting techniques, such as DGGE and probing, which distinguish broad groupings. Analysis of RNA, rather than DNA, may increase sensitivity but fine scale analysis requires sequencing of clone libraries or bands excised from DGGE gels.

These issues are exemplified by studies on Long-Term Ecological Research (LTER) sites subjected to a number of different fertilisation and cultivation conditions for many years. Bruns et al. (1999) adopted a cloning and sequencing approach and, although analysing a limited number of clones, detected differences in ammonia oxidiser communities between different treatments. In soils that had been tilled, *Nitrosospira* cluster 3 sequences only were detected, while sequences representative of *Nitrosospira* clusters 3 and 4 and *Nitrosomonas* cluster 6 were detected in never-tilled soils. Sequence analysis also indicated an increase in relative abundance of *Nitrosospira* cluster 3 in fertilised soils. Phillips et al. (2000) studied similar plots (see legend to Figure 2) and found differences in nitrification potential associated with fertilisation. To determine whether these differences were due to increases in ammonia oxidiser populations, enumeration was carried out using the MPN techniques and competitive PCR (cPCR) of ammonia oxidiser 16S rRNA genes. MPN counts were not correlated with fertilisation and estimated numbers were significantly lower in medium containing higher concentrations of ammonium (Figure 2). This suggests underestimation of population size due to selective growth on labor-

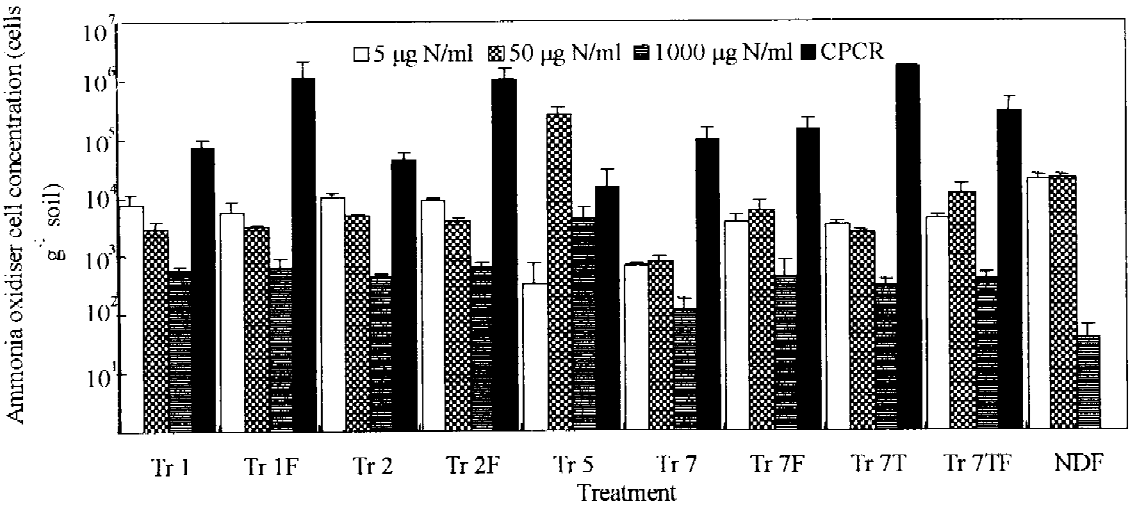

Figure 2. Ammonia oxidiser cell concentrations in LTER soils, as determined by the MPN method with medium containing 5, 50, or 1000 mg of NH_4^+-N ml^{-1} and by cPCR. Treatment (Tr) 1 is conventional tilling, treatment 2 is no tilling, treatment 5 has a *Populus* perennial cover crop, and treatment 7 was historically tilled (now in 7-year successional grassland). Error bars represent the standard error for six replicate samples of each treatment (for the NDF samples, $n = 3$). Suffixes T and F indicate tillage and fertilization, respectively, such that 7TF represents treatment 7, successional grassland, tilled and fertilized. Counts for treatments 1, 2, and 7 and for NDF were determined with samples collected in October 1996, and those for treatment 5 were determined with samples collected in March 1997. From Phillips et al. (1999), with permission.

atory media. Further evidence for this was provided by cPCR counts, which were 1–2 orders of magnitude greater than MPN counts. In addition, the variability associated with cPCR counts was lower and estimated numbers were correlated with fertiliser treatments. Phillips et al. (2000) measured community changes using DGGE analysis of 16S rDNA and did not detect any correlation between nitrification rates and ammonia oxidiser DGGE banding patterns in soils from different treatments, even when bands were sequenced. In contrast, Kowalchuk et al. (2000b) detected a decrease in the relative abundance of *Nitrosospira* cluster 3 sequences, in comparison to *Nitrosospira* cluster 4, with increasing time since fertilisation in chalk grassland soils. This is consistent with the findings of Bruns et al. (1999) that an increase in *Nitrosospira* cluster 3 sequences was associated with fertilisation. Again, MPN counts were carried out and sequence analysis of cultures obtained suggested that ammonia oxidiser community structure was determined by ammonium supply.

The importance of the time period over which changes are monitored was demonstrated by investigation of the effect of ammonium nitrate on ammonia oxidiser populations (Mendum et al. 1999). Enumeration by cPCR of both 16S rRNA and *amo*A genes did not detect any change in populations within 3 days of fertiliser addition, despite increases in the rate of nitrification. Higher numbers were found, however, at the next sampling point (6 weeks), by which time rates of nitrification had declined.

Soil heterogeneity

A common finding in studies of the diversity of ammonia oxidisers, and of other microbial groups, is considerable variation and heterogeneity in populations. This may result from heterogeneity in the soil environment, particularly in properties that are likely to influence growth, activity and survival of particular populations. For ammonia oxidisers, factors of potential importance include ammonium concentration and pH, which are likely to vary at scales influencing bacterial populations. To test this hypothesis, Webster et al. (2002) determined the heterogeneity in ammonia oxidiser populations by analysis of 16S rRNA genes and *amoA* genes, using a combination of DGGE and oligonucleotide probing. Two grassland soils were studied, managed soils that had been subject to regular addition of inorganic nitrogen fertiliser, and unimproved soils for which the major nitrogen input was sheep urination, although both sites were grazed by sheep. In this study, soil management did not result in detectable changes in the relative abundance of different ammonia oxidiser clusters, but the DGGE patterns from unimproved soils were more heterogeneous than

those from improved soils, with representatives of a greater number of ammonia oxidiser clusters than improved soils. Small-scale heterogeneity was investigated in replicate 0.5-g subsamples of soil from unimproved and improved soils. DGGE banding patterns from the former varied significantly between replicates samples, in terms of both the type and number of clusters represented. In contrast, banding patterns from replicate 0.5-g samples of improved soil were more homogeneous. The differences in heterogeneity in ammonia oxidiser populations were reflected in similar differences in heterogeneity in soil pH and ammonium concentration, with greater variability in replicate samples of unimproved soil.

Influence of high urea/ammonia concentration

Measurements of nitrification and potential nitrification rates in environmental samples, particularly of unfertilised soils, often show high levels of variability. The reasons for this are not always clear, but may result from differences in ammonia oxidiser communities, which may themselves vary through spatial heterogeneity in ammonium supply. This is likely to be greater in unimproved soils, where the major N input is sheep urination, than in soil that is fertilised and ploughed regularly. Webster et al. (submitted) studied the effects of high concentrations of nitrogen addition (1 mg urea-N g^{-1} soil) in soil microcosms. Incubations resulted in dominance of a single 16S rDNA sequence which was frequently a representative of *Nitrosospira* cluster 3. Detailed phylogenetic analysis of 16S rRNA genes from pure cultures, enrichment cultures and environmental clones resulted in its subdivision into clusters 3a and 3b. Sequences representative of cluster 3b were selected in microcosms treated with high levels of nitrogen. Nitrification kinetics varied significantly between microcosms, due to variation in apparent lag periods, and the length of the lag period was correlated with the initial relative abundance of clusters 3a, 3b and *Nitrosomonas* sequences. Soils dominated by *Nitrosospira* cluster 3a exhibited significant apparent lags, while other soils nitrified more rapidly. In addition, prolonged incubation led to selection for *Nitrosomonas* or *Nitrosospira* cluster 3b, in preference to cluster 3a. Batch culture experiments on pure and enrichment culture representatives of these clusters demonstrated the differences in kinetics to be due to inhibition of *Nitrosospira* cluster 3a by high ammonium concentrations, and the lag periods to be required for growth of ammonium tolerant *Nitrosospira* cluster 3b or *Nitrosomonas* strains from low population levels. Little information is available on ammonia sensitivity from studies of pure cultures of *Nitrosospira*, but the species originally classified as *Nitrosolobus* may belong to cluster 3a, while *Nitrosospira* and *Nitrosovibrio* may tentatively be placed in cluster 3b. The differences in distribution of isolates of these species referred to above may therefore be associated with phylogenetic and physiological distinctions. However, the most significant finding is demonstration of an important link between nitrification kinetics, community structure and differences in physiological characteristics of ammonia oxidisers.

Hastings et al. (1997) and Ceccherini et al. (1998) investigated the effects of the application of swine manure, containing high concentrations of ammonium, on soil ammonia oxidisers. *Nitrosospira* 16S rRNA genes were detected in both untreated and amended soils, but *Nitrosomonas* was only detected in soils that had been treated with slurry. In addition, *Nitrosomonas*-specific *amoA* genes were only detected in amended soils. Although quantitative methods were not used, the strength of signals following hybridisation with oligonucleotide probes was used to assess changes in relative abundance of the two genera after treatment for one and three years. The data indicated an increase in the relative abundance of *Nitrosomonas* within the ammonia oxidiser population and are consistent with the traditional belief that *Nitrosomonas* is better adapted to growth at high ammonium concentrations that *Nitrosospira*. The studies described above, however, indicate that the situation may be more complex and that some *Nitrosospira* strains may be capable of growth in high ammonium environments.

Ammonia oxidisers in freshwater environments

Hastings et al. (1998) characterised ammonia oxidiser populations in the water column and in littoral and profundal sediments of a eutrophic freshwater lake by MPN enumeration and molecular analysis of ammonia oxidiser 16S rRNA genes in environmental DNA and in positive MPN cultures, and by analysis of amplified *amoA* genes. MPN counts were greater in sediments than in the water column, with higher numbers and higher nitrification potentials in littoral, rather than profundal sediments. MPN counts were carried out using medium containing high and low concentrations of ammonium and results provided evidence of differences in sensitivity and tolerance to high concentrations of different members of the am-

monia oxidiser population, as found by Bruns et al. (1999) and Phillips *et al*. (2000) for soil populations. In general, samples from the water column gave positive enrichments at low ammonium concentrations, with enrichment at high concentrations from sediment samples. Moreover, enrichments from sediment samples contained *Nitrosomonas* and/or *Nitrosospira* (as detected by specific probes) but low-ammonia enrichments from lake water samples contained *Nitrosomonas*, while high-ammonia enrichments contained *Nitrosospira*. This therefore challenges the general belief, derived from studies with *N. europaea*, that *Nitrosomonas* is most frequently isolated because it prefers growth at high ammonium concentration and demonstrates the existence of ammonia-tolerant strains of *Nitrosospira*. *Nitrosomonas* sequences were not detected following amplification of 16S rDNA from environmental DNA, but *Nitrosomonas*-like *amoA* sequences were detected. In contrast, *Nitrosospira* 16S rDNA was detected in all samples, supporting earlier findings that this group are more numerous and potentially more important in nitrification in these environments. These data do, however, contrast with the detection of *Nitrosomonas* sequences, related to *Nitrosomonas ureae*, in a range of freshwater environments (Speksnijder et al. 1998). Kowalchuk et al. (1998) also reported *N. ureae*-like sequences in MPN cultures from lake sediment but only *Nitrosospira* clusters 3 and 4 were detected by amplification of 16S rDNA from environmental DNA. No relationship could be discerned between seasonal changes in nitrification rates and ammonia oxidiser communities.

A study of a temperate oligotrophic lake (Whitby et al. 1999) may help explain the inability to detect *Nitrosomonas* by amplification of 16S rDNA from environmental DNA, but its presence in enrichment cultures. Again, *Nitrosospira* was detected in both lake water and sediment samples, at all times of the year. *Nitrosomonas* was also detected, but only during the summer months when ammonium concentration increased. Sequences related to *N. europaea* and *N. eutropha* were detected in littoral and profundal sediment samples, respectively. The appearance of *Nitrosomonas* may, therefore, be due to more rapid growth at higher ammonium concentrations, as in enrichment cultures, or to faster recovery from ammonia starvation. This does, however, conflict with the data above, indicating that *Nitrosospira* can prefer high ammonium concentrations in enrichments. The distribution of *N. europaea* and *N. eutropha* between different locations also suggests subtle differences in their physiology. More pertinent, however, is the demonstration in this study of the inability to provide consistent explanations for all observed apparent links between ammonia oxidiser community structure and physiological characteristics of populations distinguished on the basis of 16S rDNA sequences. This may be due, in part, to analysis of relatively small numbers of samples and insufficient replication.

β-Proteobacterial ammonia oxidiser communities were studied further by Whitby et al. (2001a,b) in the eutrophic and oligotrophic basins of a large temperate lake. Again *Nitrosospira* sequences were amplified from all lake water and sediment samples, but *Nitrosomonas* was only detected in the oligotrophic basin. Lake water enrichments were obtained at low or medium ammonium concentrations, while sediment enrichments were also obtained in medium containing high ammonium concentration. This provides further evidence that relative abundance of different groups of ammonia oxidisers may be related to physiological differences associated with sensitivity and tolerance to high ammonium concentrations. Sensitivity to salt concentration may lead to differences in populations in estuarine ecosystems. De Bie et al. (2001) observed dominance by *Nitrosomonas* cluster 6a-like in freshwater regions of the Schelde estuary and by a novel *Nitrosomonas* sequence group in brackish regions. Differences in relative abundance of different groups were related to gradients in salt, oxygen and ammonia concentrations and in most environments it is unlikely that a single environmental factor will explain the sequence diversity observed within and between different sites.

Ammonia oxidisers in marine environments

Marine isolates belong to the γ-proteobacteria and to the β-proteobacterial groups *Nitrosomonas* cluster 6b (*Nitrosomonas marina*) and *Nitrosomonas* cluster 7 (*N. europaea*) (Koops & Pommerening-Röser 2001). Laboratory studies indicate all to be halophilic or halotolerant (Pommerening-Röser et al. 1996) while urease activity is found in those groups isolated from oligotrophic environments, where low ammonia concentrations may increase the importance of urea as a nitrogen source.

Sequences representative of ammonia oxidisers have been detected by PCR amplification of 16S rRNA genes from marine environments by a number of workers. For example, Stephen et al. (1996) charac-

terised ammonia oxidisers from a number of Scottish marine sites and detected sequences within *Nitrosomonas* clusters 6 and 7. In addition, they detected sequences representative of *Nitrosomonas* clusters 5 and *Nitrosospira* cluster 1. This was the first report of marine nitrosospiras, but subsequent studies have shown them to be common in marine environments. No cultured representatives are available for *Nitrosospira* cluster 1 or *Nitrosomonas* cluster 5. Voytek & Ward (1995) detected β-proteobacterial ammonia oxidisers in the South Californian Bight and Lake Bonney, a hypersaline lake in Antarctica. More detailed analysis of ammonia oxidiser 16S rDNA from another hypersaline lake in California (Ward *et al*. 2000) revealed sequences closely related to *N. europaea* and *N. eutropha*, correlating with physiological studies that indicate organisms within this cluster to be haloterant (Koops & Pommerening-Röser 2001). Primers were also constructed that were specific for ammonia oxidisers belonging to the γ-proteobacteria, but these did not generate detectable amplification products. Nold et al. (2000) also failed to detect γ-proteobacterial ammonia oxidisers in amplification products obtained using primers specific for ammonia monooxygenase and methane monooxygenase, their results suggesting that methane oxidation in these environments is carried out predominantly by methanotrophs, rather than ammonia oxidisers, and that ammonia oxidisers are dominated by those within the β-proteobacterial group. Interestingly, the *amoA* sequences obtained clustered with sequences obtained from pure cultures of *Nitrosomonas* or *Nitrosospira* but formed a distinct branch. One possibility, suggested by the authors, is that these sequences may be derived from *Nitrosospira* cluster 1 or *Nitrosomonas* cluster 5 discussed above.

A number of studies have attempted to establish links between marine ammonia oxidiser community structure and environmental factors and niches. McCaig et al. (1999a,b) characterised ammonia oxidiser populations in marine sediments surrounding a fish farm. Immediately beneath the fish cage and at 20 m, both nitrification and denitrification were inhibited but at 40 m sediment appearance and process rates were similar to those in pristine waters. DGGE analysis of ammonia oxidiser 16S rRNA genes, amplified from extracted DNA, indicated associated changes in community structure. The relative abundance of *Nitrosomonas* cluster 5 sequences was greatest in polluted sediment, immediately beneath the fish cages. Relative abundance was lower at 20 m and these cluster 5 sequences were undetectable at 40 m (Figure 3). In contrast, *Nitrosospira* cluster 1 and *Nitrosomonas* cluster 6 sequences increased in relative abundance with increasing distance from the fish cage. The results therefore suggest that *Nitrosomonas* cluster 5 was selected for within polluted fish-farm sediments and that its relative abundance was influenced by the extent of pollution. The absence of cultured representatives of this group, and consequent ignorance of distinguishing physiological characteristics, prevent speculation regarding the reasons for such selection.

In marine systems, particulate material, produced mainly in surface waters, provides an environment that potentially favours growth of ammonia oxidisers. Decomposition of organic material in particles leads to production of ammonia at concentrations significantly greater than those in surrounding water that may result in differences between particle-associated and planktonic populations. For example, conditions within particles might favour growth of *Nitrosomonas*, which is believed to prefer high ammonia concentrations, while the low ammonia concentrations in bulk seawater might favour *Nitrosospira*. To test this hypothesis, Phillips et al. (1999) analysed ammonia oxidiser 16S rDNA sequences from planktonic and particle-associated samples from the North Western Mediterranean Sea. Clone libraries were sampled and sequenced (partial and full length) and were also analysed by probing with oligonucleotide probes, specific to different groups of ammonia-oxidisers, to estimate the relative abundance of sequence types. Both approaches demonstrated dominance of aggregates by sequences related to *N. eutropha*, while planktonic samples were dominated by sequences related to *Nitrosospira* cluster 1. Depth-associated differences in communities were also assessed by probing of clones and both planktonic and particle-associated libraries showed depth-related changes in ammonia oxidiser populations. Differencesbetween planktonic and particle-associated populations were greatest at 400 m, with 4% of the clones in the planktonic library identified as *Nitrosomonas* and 96% related to *Nitrosospira* cluster 1. In contrast, all ammonia-oxidiser-positive clones sampled from the particle-associated library were members of the *Nitrosomonas* group. This indicates different niches for *Nitrosomonas* and *Nitrosospira* in this environment that may be due to differences in *in situ* ammonium concentrations. It must be remembered, however, that many other factors potentially influence colonisation of particulate material and controlled laboratory experiments are required to determine the mechanisms responsible for such dif-

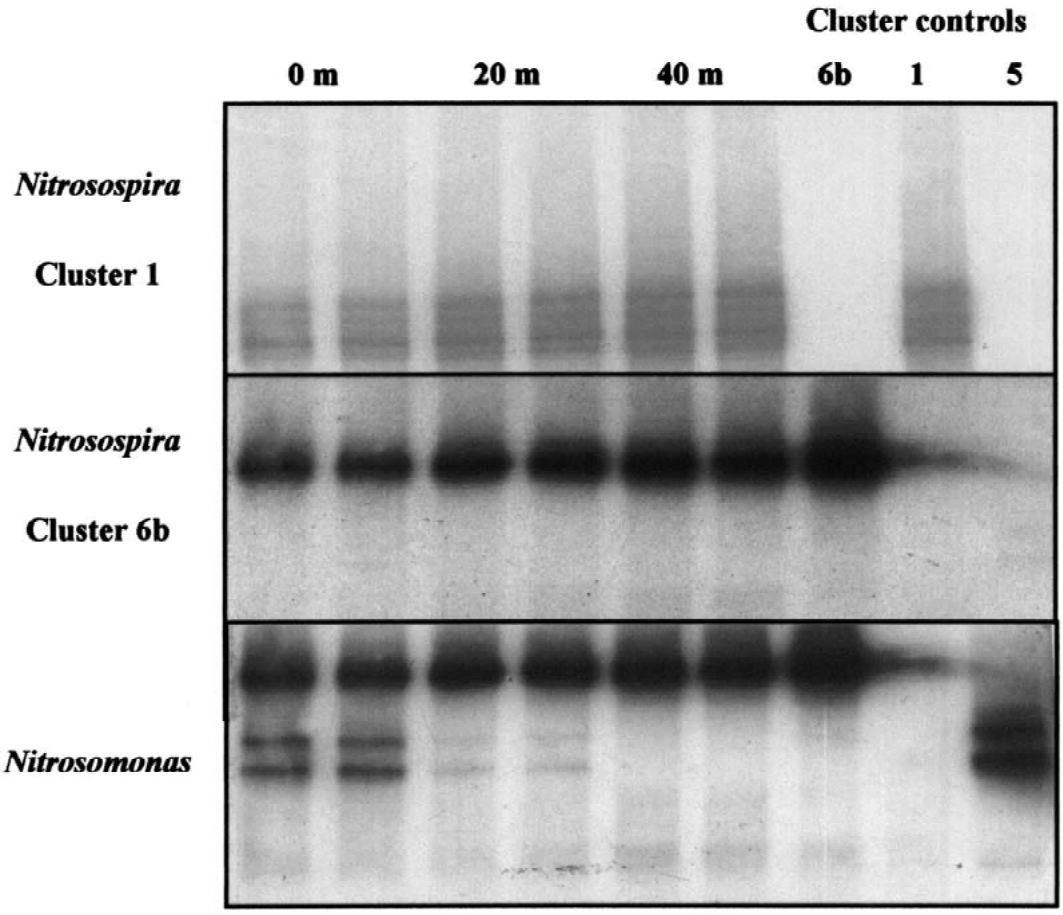

Figure 3. DGGE analysis and Southern oligonucleotide hybridisation following PCR amplification of β-subgroup ammonia-oxidising bacteria from 0- to 5-mm depth horizon sediment samples obtained directly beneath a fish cage (0 m) and at distances of 20 and 40 m. Lanes 1–6 represent duplicate samples from stations A (lanes 1 and 2), B (lanes 3 and 4), and C (lanes 5 and 6); lanes 7–9 represent cluster controls: lane 7, EnvC1-17 *(Nitrosomonas* cluster 6b); lane 8, EnvB2-11 *(Nitrosospira* cluster 1); lane 9, EnvA1-21 *(Nitrosomonas* cluster 5). From McCaig et al. (1999), with permission.

ferences in community structure. For example, pure and enrichment cultures exhibit different degrees of production of extracellular material which may encourage aggregate formation and are implicated in floc production in wastewater treatment processes (Stehr et al. 1995).

Ward, Voytek & Witzel (1997) determined the presence or absence of β-proteobacterial ammonia oxidisers at different depths in two lakes, using three sets of primers which enabled distinction between all β-proteobacterial ammonia oxidisers, *N. europaea* and a marine *Nitrosomonas* species. Differences in detectable sequences were found with depth in both lakes, but were greater in the lake that was stratified at the time of sampling. Voytek et al. (1999) detected both β-proteobacterial and γ-proteobacterial ammonia oxidisers in Antarctic lakes, the latter only in saline lakes. The depths at which ammonia oxidisers were detected correlated with estimates of nitrification rates and indicated a role for ammonia oxidisers in nitrous oxide production in these environments. Bano & Hollibaugh (2000) also examined the distribution with depth of ammonia oxidisers in the Arctic Ocean. Samples were obtained over several years at depths ranging from 5 to 235 m and the proportions of samples in which ammonia oxidisers could be detected was determined to provide an indication of cell concentrations. DGGE analysis indicated low diversity, with a single band dominating for most samples but with some showing additional bands. The dominant band was representative of *Nitrosospira* cluster 1, while others fell within *Nitrosomonas* cluster 5. There was also evidence,

based on percentages of samples in which ammonia oxidisers sequences were detected, for lower numbers in surface waters (where growth may be inhibited by light) and at greater depths (where concentrations of organic matter will be lower, reducing supply of ammonium through decomposition).

Conclusions

Molecular techniques have led to enormous advances in our ability to characterise the diversity of ammonia oxidising bacteria and their distribution in natural environments. Studies on a range of environments, by different researchers, have generated a number of common findings. For example, the importance of *Nitrosospira* in both terrestrial and aquatic environments is now recognised, the sensitivity and tolerance to high ammonium concentrations appears important and differences have been shown between communities assessed by cultivation-based and cultivation-independent approaches. The available information on physiological characteristics of pure cultures of ammonia oxidisers suggests links between phylogenetic groups and physiological characteristics of ecological relevance and, in some cases, can explain the distribution of ammonia oxidisers in natural environments and their response to changes in environmental conditions. The challenge for the future is to link molecular and traditional approaches more closely to investigate changes in communities that are associated with variation in environmental factors and physicochemical characteristics and, importantly, to establish the relationships between ammonia oxidiser community structure and nitrification and associated ecosystem processes, to determine the significance of the diversity observed in natural communities.

Studies on ammonia oxidisers provide information of generic importance. The majority of ammonia oxidisers that are believed to dominate natural populations are found within an apparently exclusive monophyletic group. This, coupled with the relative ease with which nitrification processes can be measured in natural environments, provides significant advantages in answering general questions regarding links between species, physiological and functional diversity and biogeochemical cycling. Studies addressing such questions can also take advantage of important techniques being developed in other areas. For example, stable isotope probing (Radajewski et al. 2000; Whitby et al. 2001; Radajewski *et al*. 2002) and combined 16S rRNA probing and microautoradiography (Lee et al. 1999; Ouverney & Fuhrman 1999) provide the potential for *in situ* analysis of metabolism of particular compounds by specific microbial groups, while greater replication and the development of more reliable quantitative molecular techniques are required. In addition, the sequencing of the genome of *N. europaea* (http://spider.jgi-psf.org/JGI_microbial/html/nitrosomonas_homepage.html) potentiates analysis of differences between ammonia oxidiser strains and more comprehensive analysis of the influence of environmental factors on metabolism, and its variability between different ammonia oxidiser clusters.

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