Links between ammonia oxidizer species composition, functional diversity and nitrification kinetics in grassland soils

Gordon Webster,[1][†] T. Martin Embley,[2][‡]
Thomas E. Freitag,[1] Zena Smith,[1] and
James I. Prosser[1]*

[1]*Department of Molecular and Cell Biology, University of
Aberdeen, Institute of Medical Sciences, Foresterhill,
Aberdeen AB25 2ZD, UK.*
[2]*Department of Zoology, Natural History Museum, London
SW7 5BD, UK*

Summary

**Molecular approaches have revealed considerable
diversity and uncultured novelty in natural prokary-
otic populations, but not direct links between the new
genotypes detected and ecosystem processes. Here
we describe the influence of the structure of commu-
nities of ammonia-oxidizing bacteria on nitrogen
cycling in microcosms containing natural and man-
aged grasslands and amended with artificial sheep
urine, a major factor determining local ammonia con-
centrations in these environments. Nitrification kinet-
ics were assessed by analysis of changes in urea,
ammonia, nitrite and nitrate concentrations and
ammonia oxidizer communities were characterized by
analysis of 16S rRNA genes amplified from extracted
DNA using ammonia oxidizer-specific primers. In nat-
ural soils, ammonia oxidizer community structure
determined the delay preceding nitrification, which
depended on the relative abundance of two *Nitrosos-
pira* clusters, termed 3a and 3b. In batch cultures,
pure culture and enrichment culture representatives
of *Nitrosospira* 3a were sensitive to high ammonia
concentration, while *Nitrosospira* cluster 3b represen-
tatives and *Nitrosomonas europaea* were tolerant.
Delays in nitrification occurred in natural soils domi-
nated by *Nitrosospira* cluster 3a and resulted from the
time required for growth of low concentrations of
Nitrosospira cluster 3b. In microcosms dominated by**
**_Nitrosospira_ cluster 3b and _Nitrosomonas_, no sub-
stantial delays were observed. In managed soils, no
delays in nitrification were detected, regardless of
initial ammonia oxidizer community structure, most
probably resulting from higher ammonia oxidizer cell
concentrations. The data therefore demonstrate a
direct link between bacterial community structure,
physiological diversity and ecosystem function.**

Introduction

Establishment of links between species diversity, func-
tional diversity and ecosystem processes is central to our
understanding of ecosystem function (Tilman *et al.*, 1997;
Hulot *et al.*, 2000; Loreau and Hector, 2001; Bradford
et al., 2002; Cardinale *et al.*, 2002; Worm *et al.*, 2002).
Analysis of these links has focused on communities of
higher organisms, in part through technical limitations
associated with analysis of prokaryotic communities.
However, the influence of prokaryotes on global bio-
geochemical processes is considerably greater than that
of eukaryotes (Whitman *et al.*, 1998) and there is consid-
erable scientific and public interest in the influence of
anthropogenic activities on biodiversity. Realization and
acceptance of the importance of prokaryotes in global
biogeochemical cycling (Whitman *et al.*, 1998) has coin-
cided with the development of molecular techniques that
have demonstrated considerable prokaryotic diversity and
the prevalence of uncultured groups (Pace, 1997; Hugen-
holtz *et al.*, 1998; Harris *et al.*, 2004; Webster *et al.*,
2004). To understand the importance of maintaining diver-
sity, it is therefore essential that the influence of environ-
mental factors on the diversity of bacterial communities
be understood, and that consequent effects on bio-
geochemical processes can be determined.

   Ammonia oxidation is central to global nitrogen cycling
and in terrestrial environments is carried out by
autotrophic ammonia-oxidizing bacteria that form a mono-
phyletic group within the *beta-proteobacteria*. Sequences
of 16S rRNA genes amplified from soil DNA and RNA
using ammonia oxidizer-specific primers show consider-
able diversity and may be grouped into at least seven
lineages, one corresponding to the *Nitrosospira* genus and
six lineages within the *Nitrosomonas* genus (Koops *et al.*,

2003). Analysis of sequences from pure cultures, enrichment cultures and environmental 16S rRNA gene sequences also provides evidence of subgroups within the *Nitrosospira* lineage (Stephen *et al.*, 1996). Differences in relative abundance of 16S rRNA gene sequences are frequently associated with differences in soil characteristics (Stephen *et al.*, 1998; Bruns *et al.*, 1999; Kowalchuk *et al.*, 2000a,b; Phillips *et al.*, 2000) but no link has been demonstrated with soil ammonia oxidation rates. In addition, although there appear to be links between phylogenetic groups, environmental origin and the limited number of physiological characteristics available (Koops and Pommerening-Röser, 2001), changes in community structure have not been explained in terms of the physiological attributes of different groups of ammonia oxidizers.

Ammonia concentration is likely to play an important role in determining ammonia oxidizer community structure, through potential differences in substrate affinities between different groups but also because of the inhibition of some ammonia oxidizers by high concentrations of ammonia (Koops *et al.*, 2003). In terrestrial environments, high ammonia concentration will result from the application of ammonia-based fertilizer and, locally, through excretion of waste material from grazing animals and decomposing organic matter. The aim of this study was to determine whether community structure was influenced by land management regimes leading to differences in ammonia concentration, whether influences could be explained in terms of physiological and functional diversity and whether these changes resulted in differences in nitrification kinetics. The study was carried out on natural upland grassland soils from the Sourhope Research Station, Scotland which typify upland grassland soils that provide the mainstay for grazing in the UK and are the subject of a major study into soil biodiversity. An understanding of nitrogen cycling in such sites is of particular importance following changes in land use strategies associated with extensification and reduced fertilizer applications. In unmanaged, natural grasslands, grazing sheep are important in providing high local nitrogen concentrations while managed soils are additionally subjected to regular inorganic N fertilization.

Results and discussion

Preliminary microcosm experiments

Preliminary studies were carried out in microcosms containing natural, unimproved grassland soil and managed soil, which had been subjected to long-term inorganic fertilizer treatment. Microcosms were amended with synthetic sheep urine and nitrification was measured in conjunction with molecular analysis of ammonia oxidizer communities. Urea was rapidly converted to ammonia, as

a result of ureolytic heterotrophic, nitrifying bacteria and free urease activity, and urea-N concentrations decreased from 1000 to <20 µg urea-N per gram within 10 h of amendment. Rates of urea hydrolysis were greater in improved soils (89.4 µg NH_4^+-N per gram dry weight of soil, SD = 30.6) than in unimproved soils (71.6 µg NH_4^+-N per gram dry weight of soil, SD = 56.8). These rates of urea hydrolysis are typical of grassland soils (Haynes and Williams, 1993) and result in localized increases in soil pH. Subsequent conversion of ammonia to nitrite and nitrate occurred during incubation for 84 days but rates of conversion and lag phases varied between microcosm experiments. In addition, molecular characterization by denaturing gradient gel electrophoresis (DGGE) analysis of polymerase chain reaction (PCR)-amplified 16S rRNA gene fragments indicated heterogeneity in the initial soil ammonia oxidizer communities. Denaturing gradient gel electrophoresis profiles at t = 0 contained several bands representative of different ammonia oxidizer clusters, differing in presence/absence and relative abundance (see Fig. 1 for example). Dominant DGGE bands often co-migrated with cluster controls, *Nitrosospira* cluster 3 (Fig. 1), *Nitrosomonas* cluster 7 and, rarely, *Nitrosospira* cluster 4 and a novel DGGE band was also observed. Similar heterogeneity in ammonia oxidizer communities between soil samples has been observed in a previous study of this site (Webster *et al.*, 2002) and other grassland soils (Bruns *et al.*, 1999; Kowalchuk *et al.*, 2000a,b) and there is evidence of an association between small scale spatial heterogeneity and that of soil physicochemical characteristics, particularly in unimproved grassland soils (Webster *et al.*, 2002). However, DGGE migration pattern alone could not be relied on for definitive identification of environmental ammonia oxidizer populations. Co-migration of certain bands from different clusters (*Nitrosospira* clusters 2 and 3) and the presence of multiple bands resulting from the degeneracy of the reverse PCR primer and possibly the forward primer (Kowalchuk *et al.*, 1997; McCaig *et al.*, 1999) prevents unambiguous identification and all bands of interest were excised and sequenced to confirm identity. Slight differences in the migration pattern of some cluster controls from those reported by Webster and colleagues (2002) were also observed and were thought to result from different gradient conditions between gels.

Identification and phylogenetic analysis of selected strains

Despite initial variability and heterogeneity at time zero, incubation with synthetic urine frequently led to dominance of DGGE profiles by a single band indicating a change from the initial community composition and selection for some members (Fig. 1). This novel band (arrowed) occurred close to but below the *Nitrosospira* cluster 4

Cluster controls   **Replicates**

1   2   3   4   5   6   7   8   9   10 11 12

0 days
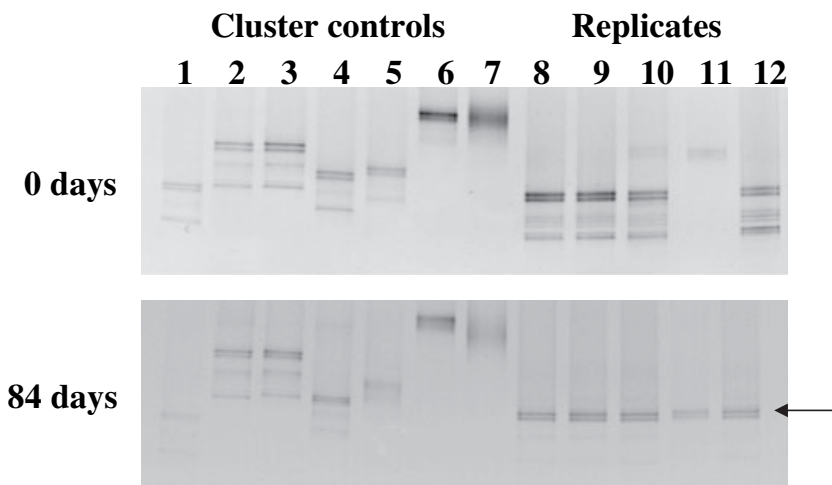
84 days

Fig. 1. Denaturing gradient gel electrophoresis (DGGE) gel of ammonia oxidizer 16S rRNA gene fragments amplified from replicate microcosms sampled immediately after establishment and after incubation for 84 days. Lanes 1–7 are ammonia oxidizer cluster controls as described in the text. Lanes 8–12 are profiles derived from unimproved, U4a soil samples from five independent replicate soil microcosms. Bands marked with an arrow represent novel DGGE band *Nitrosospira* cluster 3b.

control on DGGE gels, although sequences representative of this banding pattern had not been found at this site in the previous study (Webster *et al.*, 2002). The band was excised and sequenced and comparison with database ammonia oxidizer sequences placed it within *Nitrosospira* cluster 3, related to *Nitrosospira briensis*, although it did not migrate to the position of the *Nitrosospira* cluster 3 control. Cultured organisms representative of this band, and of other bands on DGGE profiles, were obtained by inoculation of inorganic salts medium containing ammonium with soil from unimproved and improved soils, with and without previous amendment with synthetic urine. The identity of ammonia oxidizers dominating these enrichment cultures was determined by PCR amplification of 16S rRNA gene fragments (Kowalchuk *et al.*, 1997), sequencing and DGGE analysis. Enrichment cultures dominated by representatives of *Nitrosospira* cluster 3 and migrating with the cluster 3 control, or with the novel band, were subcultured at least three times and representatives of each were sequenced for almost the entire length of their 16S rRNA genes. Detailed phylogenetic analysis indicated two subgroups within *Nitrosospira* cluster 3, termed here cluster 3a and cluster 3b (Fig. 2). One enrichment culture (En284) fell within cluster 3a, which also contained the pure culture isolate *Nitrosospira* NpAV. The novel sequence, which migrated below *Nitrosospira* cluster 4 on DGGE gels, fell within cluster 3b, which contained *N. briensis* and two enrichment cultures derived from the urine treated soils (EnU4a and EnMG6) that were closely related to the novel sequence (Fig. 2).

*Links between delays in nitrification activity and ammonia oxidizer community structure*

A more detailed comparison of the effects of synthetic urine on nitrification and ammonia oxidizer populations was carried out in duplicate microcosms using soil from both natural (unimproved, U4a) and managed (improved, MG6) grassland soils and confirmed the heterogeneity observed in preliminary experiments. Two extremes in nitrification kinetics (differences in the nitrification lag period) were observed and are illustrated in Fig. 3. Apparent lag periods before detectable nitrate production ranged from approximately 7 days (data not shown) to 35 days (Fig. 3A) for the unimproved soil microcosms and were approximately 5 days for the improved soil microcosms (Fig. 3B). Molecular analysis of ammonia oxidizer communities during incubation demonstrated a strong relationship between the relative abundances of *Nitrosospira* clusters 3a and 3b in the original community and the length of the lag period. Dominance by *Nitrosospira* cluster 3a sequences was associated with a significant delay in detectable nitrate production (Fig. 3A). The detectable presence of *Nitrosospira* cluster 3b sequences in initial communities was associated with a much shorter lag or an absence of a detectable lag in nitrate production (Fig. 3B). In all microcosms incubated for 84 days, only *Nitrosospira* cluster 3b sequences were detected in the final population, regardless of initial community structure. However, links between relative abundance of strains and relative intensities of bands on DGGE gels can be compromised by DNA extraction efficiency and PCR biases, but in this study these factors will have applied equally to all analyses, and changes in profiles are therefore likely to provide a good indication of changes in relative abundance.

To increase replication and assess the influence of land management regimes on nitrifier community responses, microcosm incubations were repeated, for a shorter period of 28 days, with 10 replicates of both natural and managed soils. Nitrification delays were observed in all natural soil microcosms (frequently greater than 20 days) that initially contained *Nitrosospira* cluster 3a sequences (Fig. 4). In all cases, these sequences decreased in rela-
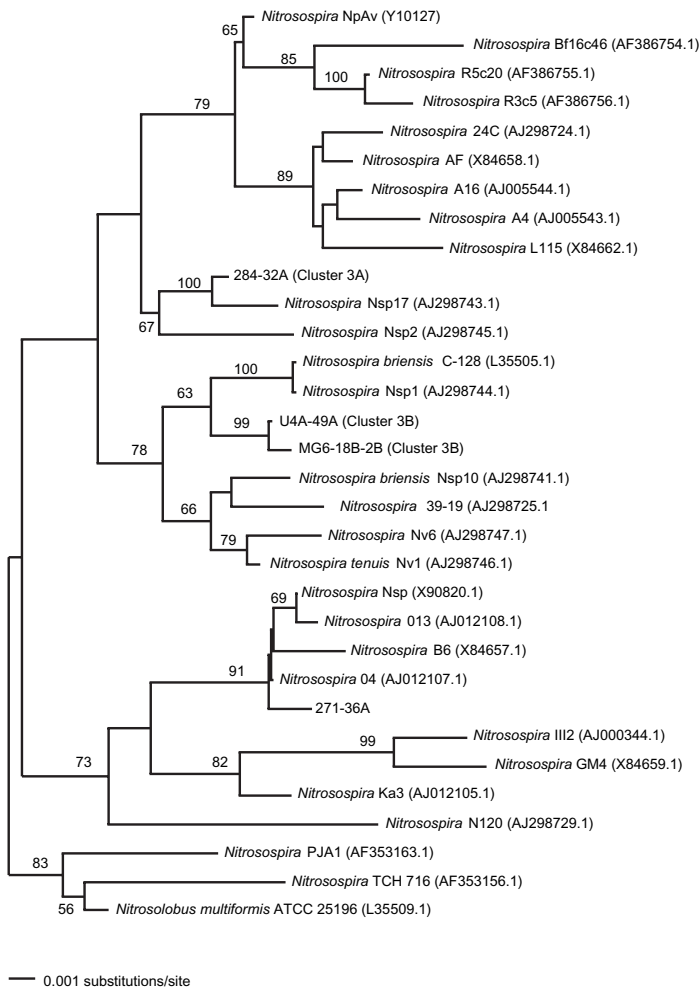
Fig. 2. Relationships between full-length 16S rRNA gene sequences from enrichment cultures and from reference pure cultures of ammonia oxidizers. Bootstrap values derived by LogDet/Paralinear distances of variable sites are presented at nodes when they exceed 50% of replicates. Scale equals 0.1% estimated substitutions calculated using the Jukes and Cantor correction (Jukes and Cantor, 1969). Sequences 284-23A, U4A-49A and MG6-18B-2B were cloned from enrichment cultures En284, EnU4a and EnMG6 respectively.
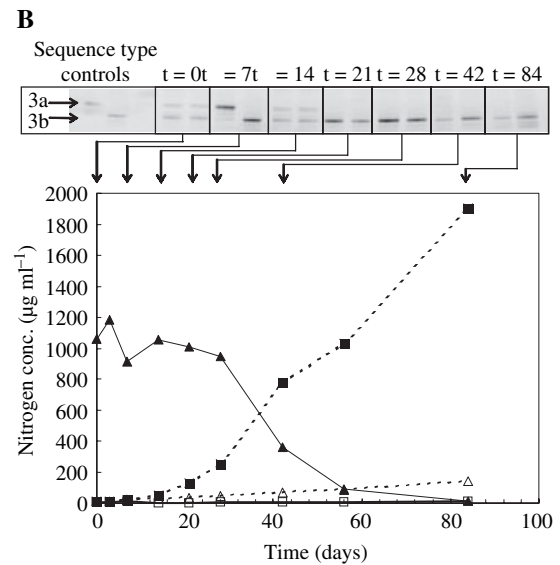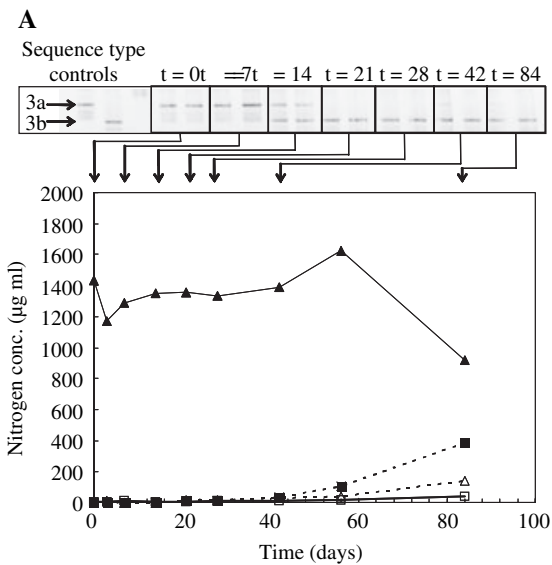
Fig. 3. Changes in ammonium (▲, △) and nitrite + nitrate (■, □) concentrations and ammonia oxidizer sequence types in control microcosms (□, △) and microcosms amended with synthetic sheep urine (■, ▲). (A) and (B) are DGGE profiles of ammonia oxidizer communities from microcosms established with unimproved (A) and improved (B) soil and amended with urea at t = 0. Triplicate soil microcosms were sampled destructively at each time point and mean values plotted. Standard errors were less than the plotted symbol size.

Unimproved, U4a grassland

Improved, MG6 grassland
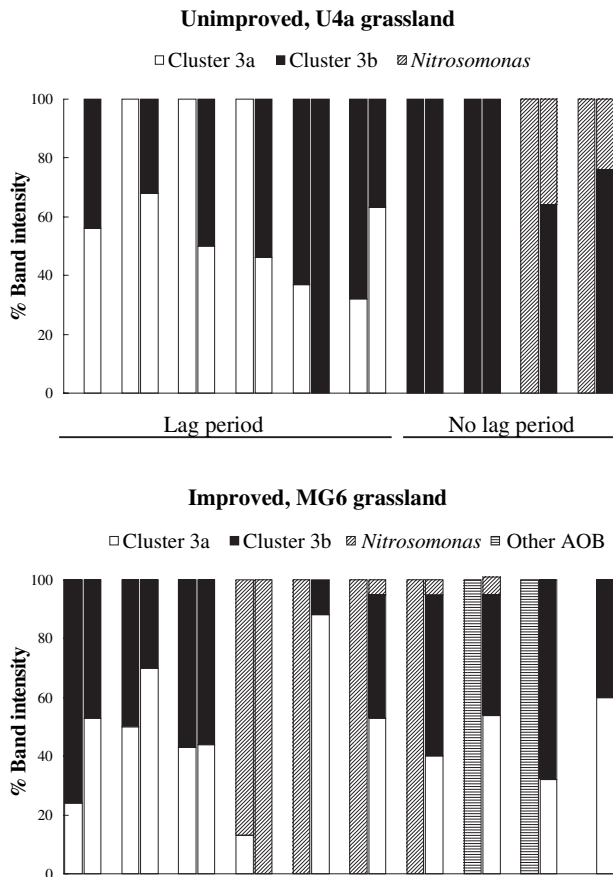
Fig. 4. Relative abundance of ammonia oxidizer sequence types in 10 replicate microcosms containing unimproved (U4a) soil and 10 containing improved (MG6) grassland soil amended with synthetic sheep urine and incubated for 28 days. Paired stacked bars represent relative abundance initially and after incubation for 28 days. Polymerase chain reaction products were not obtained from U4a soil replicate 1 or MG6 replicate 10 at t = 0. Microcosms in which a lag period was detected, or not detected, before nitrite production are indicated.

tive abundance, or became undetectable during incubation, resulting from increases in relative abundance of *Nitrosospira* cluster 3b. No delay in nitrification activity was observed in natural soil microcosms dominated initially by *Nitrosospira* cluster 3b or by *Nitrosomonas* sequences and, again, *Nitrosospira* cluster 3b sequences increased in relative abundance during incubation, even in microcosms in which *Nitrosomonas* was initially detected (Fig. 4). These data therefore confirm the early findings but also demonstrate that *Nitrosospira* cluster 3b can also, apparently, outcompete *Nitrosomonas*, which is traditionally considered to grow faster than *Nitrosospira* strains at high ammonium concentrations.

In the majority of managed soil microcosms, nitrification activity was detected without delay, regardless of initial community structure. Changes in relative abundance of different ammonia oxidizer groups caused by the addition

of sheep urine were more variable and less predictable than in natural soils. There was greater variability in the initial communities which contained *Nitrosomonas* species and *Nitrosospira* clusters 3a and 3b, but also some additional ammonia oxidizers clusters. In some cases relative abundance of *Nitrosospira* cluster 3a increased at the expense of cluster 3b, in contrast to previous results, and in one microcosm *Nitrosomonas* remained the dominant cluster. However, in the majority of microcosms containing managed soil, *Nitrosospira* cluster 3a or 3b sequences dominated communities at 28 days, regardless of initial ammonia oxidizer community structure and in which these sequences were often undetectable or at low relative abundance.

Links between ammonia oxidizer genotype and phenotype

To assess the relationship between genotype and phenotype, the tolerance of different ammonia oxidizer groups to high ammonia concentrations was determined during batch growth of pure cultures of *Nitrosomonas europaea*, *Nitrosospira* NpAV (cluster 3a) and *N. briensis* C-128 (cluster 3b) and enrichment cultures in which representatives of *Nitrosospira* cluster 3a (En284) and 3b (EnU4A, EnMG6) were the only ammonia oxidizer genotypes detected. All representatives of *Nitrosospira* sequence cluster 3b and *N. europaea* grew at all ammonia concentrations but representatives of *Nitrosospira* sequence cluster 3a were inhibited at the highest concentration (Fig. 5). A limited number of strains were tested, and it is possible that this pattern of tolerance and sensitivity to high ammonia concentration may not be followed by all members of clusters 3a and 3b, but enrichments from the study site showed similar tolerance characteristics to representative pure cultures. This inhibitory concentration was equivalent to initial concentrations in synthetic urine-amended microcosms and to concentrations in soil following sheep urination.

Significance of community structure for ecosystem processes

This study involved use of laboratory soil microcosms to study specific aspects considered to be important in the field, but other factors, in particular plant species composition, may have an influence on ammonia oxidizer communities and nitrification rates. Nevertheless, the experimental data provide an explanation for the heterogeneity in nitrification rates, frequently observed in natural soils, through differences in the relative abundances of different components of the ammonia oxidizer community. Communities with high relative abundance of *Nitrosospira* sequence cluster 3b are able to respond rapidly to input
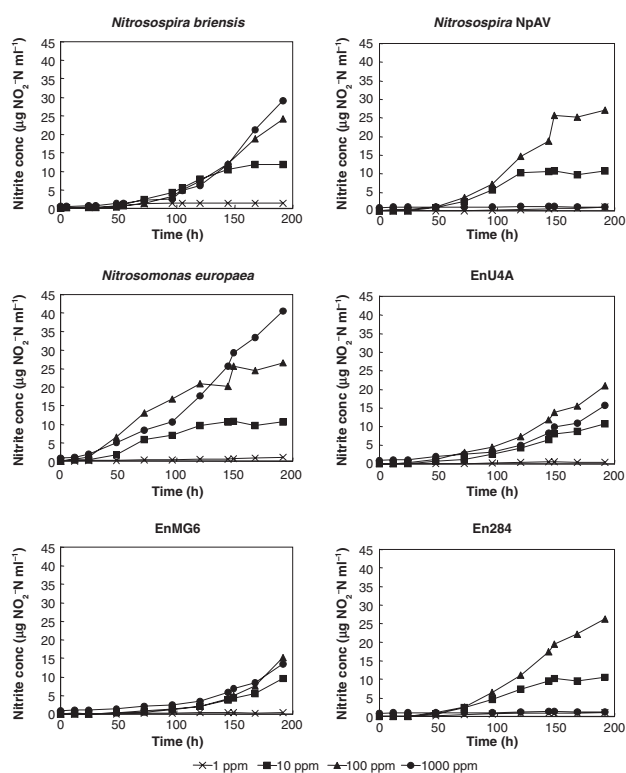
Fig. 5. The influence of initial ammonia concentration on growth of representatives of different phylogenetic groups of ammonia oxidizers: pure cultures of *Nitrosospira briensis* (cluster 3b), *Nitrosospira* NpAV (cluster 3a) and *Nitrosomonas europaea* (cluster 6) and isolates EnU4a (cluster 3b), EnMG6 (cluster 3b) and En284 (cluster 3a). Strains were grown in liquid batch culture with initial ammonia concentrations of 1, 10, 100 or 1000 μg ml^{-1} NH_4^+-N. Triplicate cultures were set up for each strain at each initial ammonia concentration. Replicates for each strain showed the same response to ammonia concentration and results of single replicate cultures only are presented.

of high concentrations of ammonia, which is converted without delay. In communities initially dominated by representatives of *Nitrosospira* sequence cluster 3a, nitrification is delayed until the growth and emergence of *Nitrosospira* cluster 3b strains. Analysis of pure cultures and of cultures enriched from the same soil samples shows the differences in nitrification kinetics to result from inhibition, by high ammonia, of *Nitrosospira* cluster 3a, but not cluster 3b strains. The data therefore demonstrate a direct link between community structure and physiological and functional diversity of an important component of the soil bacterial community and their impact on an ecosystem process. This impact is of considerable significance for nitrogen dynamics in grassland communities. The observed delays in nitrification, and consequent persistence of ammonia, will benefit plant growth, while rapid nitrification will result in losses through denitrification, increasing N_2O production, or leaching, leading to pollution of receiving waters. The apparent lag periods before

detectable nitrification activity may reflect the time required for activation of dormant populations, which in pure cultures increased with starvation (Batchelor *et al.*, 1997). However, an alternative, and more likely explanation is that *Nitrosospira* cluster 3b populations were present at low abundance and that nitrate production would not be detectable until growth to cell concentrations of approximately 10^6 cells per gram had occurred. Minimum doubling times of cultivated ammonia oxidizers are typically in the range 12 h to several days (Prosser, 1989). A population present at 10^3 cells per gram, growing exponentially with a doubling time of 2 days, would require approximately 20 days to reach 10^6 cells per gram.

Continued tillage and fertilization lead to higher populations of ammonia oxidizers (Phillips *et al.*, 2000) and as a consequence higher measurable rates of nitrification in managed soils (Webster *et al.*, 2002). This is the probable explanation for the absence of detectable delays in nitrification in the majority of managed soil microcosms. It is likely that continued ammonia fertilization led to higher cell concentrations of all ammonia oxidizer groups, including those tolerant of high ammonia concentrations. This may also explain the unpredicted changes in relative abundance of different groups. At high population levels, the proportional change in cell concentration resulting from growth on ammonia supplied will be less and will be more difficult to detect using DGGE. There was evidence, however, that *Nitrosospira* strains were able to outcompete *Nitrosomonas* during incubations. These findings demonstrate the importance of considering abundance as well as diversity of microbial communities when assessing influence on process rates. Where populations are low, as in natural soils, the study also highlights our lack of knowledge of survival and death rates of nitrifiers and other bacteria in the soil. Thus, although the study demonstrated clear links between ammonia oxidizer community structure, functional diversity and nitrification rates, it was not possible to assess the stability of selected communities, the time required for reduction in cell concentrations if fertilizer application are discontinued nor the rates of reduction in relative abundance of ammonia-tolerant strains when ammonia concentrations decrease.

Experimental procedures

Microcosm studies

Natural (Unimproved – U4a) and managed (Improved – MG6) upland grassland (National Vegetation Classification) soil samples (0–10 cm depth) were collected from the Fasset Hill site (National Grid Reference NT 852207) at Sourhope Research Station, Scotland, UK on 5 October 1999 (duplicate samples of U4a and MG6), 21 November 1999 (duplicate samples of U4a and MG6) and 18 September 2000 (10×

samples, 2 m apart for each soil type) and stored overnight at 4°C. Bulked soil samples were mixed and sieved (3–4 mm mesh size) to remove stones and plant roots and were used immediately in soil microcosms. Soil microcosms were constructed consisting of 10 g of soil in 40 ml loosely fitting, screw-capped bottles mixed with 1 ml of either sterile distilled water or synthetic sheep urine (Williams *et al.*, 1999), giving a final concentration of 1 mg urea-N per gram of soil, which is typical of concentrations in soil immediately following sheep urination (Haynes and Williams, 1993). Microcosms were maintained at 35–40% soil water content at 10°C in the dark for up to 84 days and were sampled destructively with a minimum of three replicates at several time points for quantitative analysis of urea-N, ammonia-N, nitrite-N and nitrate-N, using an Alpkem RFA Autoanalyser (Alpkem Corporation, Clackamas, OR, USA), and for molecular analysis of ammonia oxidizer communities. Microcosms used in the increased replication experiment were incubated under the same conditions for only 28 days and individual microcosms for each soil replicate were sampled destructively for further analysis. All soil microcosm samples used in this study were stored at −20°C before chemical and molecular analysis. Urease activity was determined as described by Kandeler and Gerber (1988).

Molecular analysis of ammonia oxidizers

For analysis of ammonia oxidizer communities, DNA was extracted from 0.5 g of soil as described by Webster and colleagues (2002). Briefly, cells were lysed using a Hybaid Ribolyser™ Cell Disrupter (Hybaid, Ashford, UK) (speed 4, 10 s; two times) and DNA was extracted in Tris-buffered phenol and re-extracted in Tris-buffered phenol, followed by an equal volume of chloroform. DNA was concentrated and purified by dialysis using a Microcon YM-100 centrifugal filter device (Millipore Corporation, Bedford, MA, USA), resuspended in TE buffer and further purified by standard low melting point agarose (1.0%; w/v) gel electrophoresis. High-molecular-weight DNA (>10 kb) was removed, cleaned and purified using a Bio-Rad 'Freeze and Squeeze' spin column (Bio-Rad Laboratories, Hemel Hempstead, UK) before amplification of 16S rRNA gene fragments using a nested PCR approach. Primary amplification was carried out using the betaproteobacterial ammonia oxidizer selective βAMOf/βAMOr primer set (McCaig *et al.*, 1994), generating 1.1 kb products that were then amplified using the CTO189f/CTO654r primer pair (Kowalchuk *et al.*, 1997) that is specific for all known betaproteobacterial ammonia oxidizers, except some members of the *Nitrosomonas oligotropha* cluster and with several mismatches with the *Nitrosomonas communis* cluster. Details of primers and the PCR protocols are given by Webster and colleagues (2002). Polymerase chain reaction amplification was also carried out using DNA extracted from 16S rRNA gene clones or pure cultures representative of previously described *Nitrosomonas* and *Nitrosospira* 16S rRNA gene-based phylogenetic clusters (Stephen *et al.*, 1996), for use as reference sequences for analysis by DGGE, as follows: EnvB1-8 (*Nitrosospira* cluster 1), pH 4.2 A/27 (*Nitrosospira* cluster 2), pH 4.2 A/4 (*Nitrosospira* cluster 3a), pH 7B/C3 (*Nitrosospira* cluster 4), EnvA1-21 (*Nitrosomonas* cluster 5), EnvC1-19 (*Nitrosomonas* cluster 6), *N. europaea*

ATCC 25978 (*Nitrosomonas* cluster 7) and *N. briensis* C-128 (*Nitrosospira* cluster 3b).

Denaturing gradient gel electrophoresis analysis

Polymerase chain reaction products (465 bp) were analysed by DGGE as described previously (Webster *et al.*, 2002) using a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories) and a denaturant gradient of 35–50% [100% denaturant is defined as 7 M urea with 40% (v/v) formamide]. Denaturant gels were made with the aid of a 50 ml volume Gradient Mixer (Fisher Scientific, Loughborough, UK) and electrophoresed at 200 V for 5 h at 60°C. Gels were stained with ethidium bromide and gel images captured using a UVP ImageStore 5000 (Ultra Violet Products, San Gabriel, CA, USA). Gel images were analysed using the Phoretix one-dimensional gel analysis software (version 4.00), Phoretix International, UK and band intensity expressed as a percentage of each lane. Individual DGGE bands of interest were excised, reamplified, purified and sequenced (see Webster *et al.*, 2002 for details). Phylogenetic analysis was carried out as described below.

Phylogenetic analysis of ammonia oxidizer 16S rRNA gene sequences

In order to obtain full-length representative sequences of *Nitrosospira* clusters 3a and 3b, DNA from enrichment cultures was used as template for PCR and amplified with eubacterial 16S rRNA gene primers (De Long, 1992). Amplification products were cloned using the vector pGEM-T Easy (Promega, Madison, WI, USA) and plasmid inserts were screened by DGGE to confirm that they corresponded to clusters 3a and 3b sequence types and sequenced on both strands. Reference sequences were recovered from GenBank, aligned using CLUSTAL W (Thompson *et al.*, 1994) and adjusted by eye. Regions of ambiguous alignment were removed leaving 1208 sites (88 variable sites) for phylogenetic analysis. Data were analysed using PAUP*4.0b 10 (PPC) written by Swofford (1998). The LogDet method (Lockhart *et al.*, 1994) was used as the primary tool in these analyses, but other methods including maximum likelihood gave similar tree topologies. The analysis was limited to variable positions estimated using maximum likelihood (proportion of invariable sites pinvar = 0.900). Bootstrapping (1000 replicates) was carried out to assess support for nodes.

Nucleotide sequence accession numbers

All nucleotide sequences obtained on this study have been submitted to GenBank under accession numbers AY727031–AY727034.

Growth and inhibition of pure and enriched cultures of ammonia oxidizers

Pure cultures of *N. briensis* C-128, *Nitrosospira* NpAV and *N. europaea* ATCC 25978 were obtained, respectively, from F. Valois, Woods Hole, E. Schmidt, University of Minnesota and

ATCC. Cultures were maintained as described by Keen and Prosser (1987) in Skinner and Walker (1961) medium, modified by Powell and Prosser (1986), containing 50 μg ml^{-1} NH$_4^+$-N as ammonia sulfate. Contamination by heterotrophs was detected by microscopic examination and by plating on Nutrient Agar (Oxoid) and incubation at 25°C for at least 21 days. Growth was assessed by spot tests for ammonia (Nessler's Reagent) and nitrite (Griess Ilosvay's Reagents 1 and 2) and reduction in pH. Enrichment cultures of ammonia oxidizers were obtained by inoculation of 150 ml of modified Skinner and Walker (1961) medium, containing 100 μg ml^{-1} NH$_4^+$-N, with 1.5 g of soil. Flasks were incubated at 28°C in the dark and pH was maintained at values in the range 7–8.5 by aseptic addition of sterile 5% (w/v) sodium carbonate solution. Growth was assessed by ammonium utilization and nitrite production. Stationary-phase cultures, in which ammonium had been completely utilized, were subcultured by 1% inoculation into 150 ml of the same medium and incubated as described above. Enrichment cultures used for growth experiments were subcultured at least three times.

Growth experiments were carried out in triplicate under the conditions described above but with initial ammonium concentrations of 1, 10, 100 or 1000 μg ml^{-1} NH$_4^+$-N. Flasks were inoculated with 2–7×10^5 cells per millilitre (collected as described by Jiang and Bakken, 1999) of an actively growing pure culture of *N. briensis* C-128 (related to *Nitrosospira* cluster 3b), *Nitrosospira* NpAV (related to *Nitrosospira* cluster 3a) and *N. europaea* and three enrichment cultures EnU4a (*Nitrosospira* cluster 3b), EnMG6 (*Nitrosospira* cluster 3b) and En284 (*Nitrosospira* cluster 3a). Growth was assessed by colorimetric quantitative analysis of 1 ml of samples for ammonia-N and nitrite-N concentrations by autoanalysis as above.

Acknowledgements

References

Batchelor, S.E., Cooper, M., Chhabra, S.R., Glover, L.A., Stewart, G.S.A.B., Williams, P., and Prosser, J.I. (1997) Cell-density regulated recovery of starved biofilm populations of ammonia oxidising bacteria. *Appl Environ Microbiol* **63:** 2281–2286.

Bruns, M.A., Stephen, J.R., Kowalchuk, G.A., Prosser, J.I., and Paul, E.A. (1999) Comparative diversity of ammonia oxidiser 16S rRNA gene sequences in native, tilled, and successional soils. *Appl Environ Microbiol* **65:** 2994–3000.

Bradford, M.A., Jones, T.H., Bardgett, R.D., Black, H., Boag, B., Bonkowski, M., *et al.* (2002) Impacts of soil faunal functional composition on model grassland ecosystems. *Science* **29:** 601–603.

Cardinale, B.J., Palmer, M.A., and Collins, S.L. (2002) Species diversity enhances ecosystem functioning through interspecific facilitation. *Nature* **415:** 426–429.

De Long, E.F. (1992) Archaea in coastal marine environments. *Proc Natl Acad Sci USA* **89:** 5685–5689.

Harris, J.K., Kelley, S.T., and Pace, N.R. (2004) New perspectives on uncultured bacterial phylogenetic division OP11. *Appl Environ Microbiol* **70:** 845–849.

Haynes, R.J., and Williams, P.H. (1993) Nutrient cycling and soil fertility in the grazed pasture ecosystem. In *Advances in Agronomy.* Sparks, D.L. (ed). San Diego, CA, USA: Academic Press, pp. 119–199.

Hugenholtz, P., Goebel, B.M., and Pace, N.R. (1998) Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J Bacteriol* **180:** 4765–4774.

Hulot, F.D., Lacroix, G., Lescher-Moutoue, F.O., and Loreau, M. (2000) Functional diversity governs ecosystem response to nutrient enrichment. *Nature* **405:** 340–344.

Jiang, Q.Q., and Bakken, L.R. (1999) Comparison of *Nitrosospira* strains isolated from terrestrial environments. *FEMS Microbiol Ecol* **30:** 171–186.

Jukes, T.H., and Cantor, C.R. (1969) Evolution of protein molecules. In *Mammalian Protein Metabolism*. Munro, H.N. (ed.). New York: Academic Press, pp. 21–132.

Kandeler, E., and Gerber, H. (1988) Short-term assay of soil urease activity using colorimetric determination of ammonium. *Biol Fert Soil* **6:** 68–72.

Keen, G.A., and Prosser, J.I. (1987) Steady state and transient growth of autotrophic nitrifying bacteria. *Arch Microbiol* **147:** 73–79.

Koops, H.-P., and Pommerening-Röser, A. (2001) Distribution and ecophysiology of the nitrifying bacteria emphasizing cultured species. *FEMS Microbiol Ecol* **37:** 1–9.

Koops, H.-P., Purkhold, U., Pommerening-Röser, A., Timmermann, G., and Wagner, M. (2003) The lithoautotrophic ammonia-oxidising bacteria. In *The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community*, 3rd edn, release 3.13. Dworkin, D., Falkow, S., Rosenberg, E., Schleifer, K.-H., and Stackebrandt, E. (eds). NY, USA: Springer-Verlag [WWW document]. URL http://link.springer-ny.com/link/service/books/10125/

Kowalchuk, G.A., Stephen, J.R., De Boer, W., Prosser, J.I., Embley, T.M., and Woldendorp, J.W. (1997) Analysis of ammonia-oxidising bacteria of the beta subdivision of the class Proteobacteria in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. *Appl Environ Microbiol* **63:** 1489–1497.

Kowalchuk, G.A., Stienstra, A.W., Helig, G.H.J., Stephen, J.R., and Woldendorp, J.W. (2000a) Molecular analysis of ammonia oxidising bacteria in soil of successional grasslands of the Drentsche A (The Netherlands). *FEMS Microbiol Ecol* **31:** 207–215.

Kowalchuk, G.A., Stienstra, A.W., Helig, G.H.J., Stephen, J.R., and Woldendorp, J.W. (2000b) Changes in the community structure of ammonia oxidizing bacteria during secondary succession of calcareous grasslands. *Environ Microbiol* **2:** 99–110.

Lockhart, P.J., Steel, M.A., Hendy, M.D., and Penny, D. (1994) Recovering evolutionary trees under a more realistic model of sequence evolution. *Mol Biol Evol* **11:** 605–612.

Loreau, M., and Hector, A. (2001) Partitioning selection and complementarity in biodiversity experiments. *Nature* **412:** 72–76.

McCaig, A.E., Embley, T.M., and Prosser, J.I. (1994) Molecular analysis of enrichment cultures of marine ammonia oxidisers. *FEMS Microbiol Lett* **120:** 363–368.

McCaig, A.E., Phillips, C.J., Stephen, J.R., Kowalchuk, G.A., Harvey, S.M., Herbert, R.A., *et al.* (1999) Nitrogen cycling and community structure of proteobacterial β-subgroup ammonia-oxidizing bacteria within polluted marine fish farm sediments. *Appl Environ Microbiol* **65:** 213–220.

Pace, N.R. (1997) A molecular view of microbial diversity and the biosphere. *Science* **276:** 734–740.

Phillips, C.J., Harris, D., Dollhopf, S.L., Gross, K.L., Prosser, J.I., and Paul, E.A. (2000) Effects of agronomic treatments on the structure and function of ammonia oxidising communities. *Appl Environ Microbiol* **66:** 5410–5418.

Powell, S.J., and Prosser, J.I. (1986) Inhibition of ammonium oxidation by nitrapyrin in soil and liquid culture. *Appl Environ Microbiol* **52:** 782–787.

Prosser, J.I. (1989) Autotrophic nitrification in bacteria. *Adv Microbial Physiol* **30:** 125–181.

Skinner, F.A., and Walker, N. (1961) Growth of *Nitrosomonas europaea* in batch and continuous culture. *Arch Microbiol* **38:** 339–349.

Stephen, J.R., McCaig, A.E., Smith, Z., Prosser, J.I., and Embley, T.M. (1996) Molecular diversity of soil and marine 16S rDNA sequences related to β-subgroup ammonia oxidising bacteria. *Appl Environ Microbiol* **62:** 4147–4154.

Stephen, J.R., Kowalchuk, G.A., Bruns, M.A., McCaig, A.E., Phillips, C.J., Embley, T.M., and Prosser, J.I. (1998) Analysis of β-subgroup ammonia oxidiser populations in soil by DGGE analysis and hierarchical phylogenetic probing. *Appl Environ Microbiol* **64:** 2958–2965.

Swofford, D.L. (1998) *paup*. Phylogenetic Analysis Using Parsimony (*and Other Methods).* Sunderland, MA, USA: Sinauer Associates.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994) CLUSTAL_W: improving the sensitivity of progressive multiple alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22:** 4673–4680.

Tilman, D., Knops, J., Wedin, D., Reich, P., Ritchie, M., and Siemann, E. (1997) The influence of functional diversity and composition on ecosystem processes. *Science* **277:** 1300–1302.

Webster, G., Embley, T.M., and Prosser, J.I. (2002) Grassland management regimes reduce small-scale heterogeneity and species diversity of β-proteobacterial ammonia oxidiser populations. *Appl Environ Microbiol* **68:** 20–30.

Webster, G., Parkes, R.J., Fry, J.C., and Weightman, A.J. (2004) Widespread occurrence of a novel division of Bacteria identified by 16S rRNA gene sequences originally found in deep marine sediments. *Appl Environ Microbiol* **70:** 5708–5713.

Whitman, W.B., Coleman, D.C., and Wiebe, W.J. (1998) Prokaryotes: the unseen majority. *Proc Natl Acad Sci USA* **95:** 6578–6583.

Williams, B.L., Shand, C.A., Sellers, S., and Young, M.E. (1999) Impact of synthetic sheep urine on N and P in two pastures in the Scottish uplands. *Plant Soil* **214:** 93–103.

Worm, B., Lotze, H.K., Hillebrand, H., and Sommer, U. (2002) Consumer versus resource control of species diversity and ecosystem functioning. *Nature* **417:** 848–851.