The influence of synthetic sheep urine on ammonia oxidizing bacterial communities in grassland soil

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Abstract
In grazed, grassland soils, sheep urine generates heterogeneity in ammonia concentrations, with potential impact on ammonia oxidizer community structure and soil N cycling. The influence of different levels of synthetic sheep urine on ammonia oxidizers was studied in grassland soil microcosms. ‘Total’ and active ammonia oxidizers were distinguished by comparing denaturing gradient gel electrophoresis (DGGE) profiles following PCR and RT-PCR amplification of 16S rRNA gene fragments, targeting DNA and RNA, respectively. The RNA-based approach indicated earlier, more reproducible and finer scale qualitative shifts in ammonia oxidizing communities than DNA-based analysis, but led to amplification of a small number of nonammonia oxidizer sequences. Qualitative changes in RNA-derived DGGE profiles were related to changes in nitrate accumulation. Sequence analysis of excised DGGE bands revealed that ammonia oxidizing communities in synthetic sheep urine-treated soils consisted mainly of Nitrosospira clusters 2, 3 and 4. Nitrosospira cluster 2 increased in relative abundance in microcosms treated with all levels of synthetic sheep urine. Low levels additionally led to increased relative abundance of Nitrosospira cluster 4 and medium and high levels increased relative abundance of cluster 3. Synthetic sheep urine is therefore likely to influence the spatial distribution and composition of ammonia oxidizer communities, with consequent effects on nitrate accumulation.

Introduction
Microbial communities are responsible for sustainable recycling of nutrients in aquatic and terrestrial ecosystems and for the biodegradation of waste material and toxic pollutants. Environmental change, such as atmospheric deposition of pollutants, elevated CO2 and temperature, intensive fertilization and management for improved productivity, is likely to impact on the composition of the soil microbial community (Pennanen et al., 1998; McCaig et al., 2001; Kent & Triplett, 2002; DeForest et al., 2004; Girvan et al., 2004). It may also result in a change in microbial diversity, with potential effects on nutrient recycling processes and ecosystem sustainability. The development of molecular techniques has enabled cultivation-independent analysis of natural microbial communities (Dahllof, 2002; Prosser, 2002; Torvik & Øvreås, 2002). Application of these techniques has, in some studies, demonstrated an influence of environmental change on microbial community structure, for example soil fertilization (Girvan et al., 2004), pesticide application (Engelen et al., 1998; Mahmood et al., 2005), temperature (Avrahami et al., 2003), soil management (Sun et al., 2004) and soil contamination with bimetallic sludge or heavy metals (Sandaas et al., 2001). However, detection of changes can be difficult when using DNA-targeted approaches, which assess ‘total’ (dormant and active) communities, as these may mask changes occurring in active organisms. In addition, the potential for mechanistic understanding of the impact of environmental change may be increased by analysis of functional groups that may be impacted by specific environmental factors.

The environmental change investigated in this study was amendment of a grassland soil with synthetic sheep urine. In grassland ecosystems, herbivore grazing contributes to sustainable N recycling but results in locally high concentrations of N in urine patches and potentially introduces spatial heterogeneity in microbial communities. The application of synthetic sheep urine increases soil pH and concentrations of dissolved organic C and organic and inorganic forms of N and P (Shand et al., 2000, 2002). Emissions of nitrous oxide (N2O) from grazed grassland are generally higher than from ungrazed grassland, and emissions from grasslands are...
typically greater than from arable fields or natural ecosystems (Smith et al., 1998). Few studies have investigated the effects of sheep urine on below-ground microbial communities. Williams et al. (2000) amended upland grassland soils with synthetic sheep urine and reported a dramatic but short-lived change in the structure and activity of the cultivable soil microbial community, increased bacterial numbers and increased microbial utilization of sugars, amino acids and amides. Nicol et al. (2004), using cultivation-independent, molecular methods, demonstrated a distinct and reproducible effect on bacterial communities in synthetic sheep urine-amended soil microcosms, but no detectable influence on the crenarchaeal community. Ammonia oxidizing bacteria are the functional group likely to be impacted most by such changes. These organisms oxidize ammonia to nitrite, the first stage of the nitrification process, and thereby play a major role in terrestrial N cycling. Application of inorganic N fertilizer to grassland soils can affect ammonia oxidizer community structure (Bruns et al., 1999; Phillips et al., 2000; Clegg et al., 2003), and complexity of ammonia oxidizer communities has been found to be greater in unimproved than in improved grassland soils (Webster et al., 2002). Recently Patra et al. (2005) reported a profound effect of grazing on the community structure of ammonia oxidizers, with differences in dominant sequences between intensively and lightly grazed grassland soils.

The objective of this study was to determine whether amendment of grassland soil with synthetic sheep urine, at different levels, influenced the composition of total and active ammonia oxidizer communities, using two cultivation-independent 16S rRNA gene-based approaches. The first involved PCR-amplification of gene fragments from DNA extracted directly from soil, and provides information on both active and dormant ammonia oxidizers. The second approach involved reverse-transcription-PCR (RT-PCR) amplification of the same sequences from extracted RNA and is believed to provide a better indication of active members of the community, which will contain higher cellular ribosome abundance (Kerkhof & Kemp, 1999). In both approaches, PCR and RT-PCR amplifications were performed using ammonia oxidizer specific primers and the resultant PCR products were analysed by denaturing gradient gel electrophoresis (DGGE).

Materials and methods

Soil microcosms

Laboratory microcosms were established containing grassland soil collected in May 2001 from an unimproved plot at the Sourhope Research Station located in the Borders region of Scotland (map reference NT 850 205). Vegetation cover was dominated by Agrostis capillaris, Festuca ovina and Galium saxatile and further details of the site can be found in McCaig et al. (1999). After sampling, the soil was passed through a 3.35 mm sieve to remove roots and stones and stored at 4°C. Microcosms consisted of 10 g soil in 28-mL Universal glass bottles with screw caps (Fisher Scientific, Loughborough, UK) amended with 1 mL of either deionized water (control, C) or one of three levels of synthetic sheep urine, giving final concentrations of 100 (low N, LN), 500 (medium N, MN) and 1000 (high N, HN) μg urea-N g⁻¹ soil. The HN treatment was equivalent to soil N levels following typical sheep urination and is equivalent to 500 kg ha⁻¹ (Haynes & Williams, 1993) and moisture content was 40% (w/v). The composition of synthetic sheep urine (per litre ddH₂O) was: KHCO₃, 23.3 g; KBr, 6.7 g; KCl, 4.20 g; K₂SO₄, 2.30 g; glycine, 4.84 g; and urea, 21.43 g (Clough et al., 1996) and MN and LN consisted of HN diluted in deionized water. Triplicate microcosms for each treatment were incubated in the dark at 15°C and were harvested and sampled destructively after incubation for 0, 2, 4, 7, 14, 21, 28, 42 and 56 days. At each harvest, soil samples were stored at −80°C until extraction of nucleic acids and determination of ammonia and nitrite + nitrate concentrations.

Nucleic acid extraction

Nucleic acids were extracted from 0.5 g soil samples according to Griffiths et al. (2000). In brief, cells were lysed for two cycles of 20 s with a Ribolyser cell disruptor (Hybaid Ltd., Ashford, UK) at 4 m s⁻¹ in 2 mL Blue Matrix Ribolyser tubes (Hybaid Ltd.) containing 0.5 mL hexadecyltrimethylammonium bromide extraction buffer, 0.5 mL phenol-chloroform-isooamyl alcohol (25:24:1 volume in volume (v/v), pH 8.0) and ceramic and silica beads. Further extraction and precipitation of nucleic acids involved removal of phenol with an equal volume of chloroform-isooamyl alcohol (24:1) followed by precipitation from the aqueous layer with two volumes of 30% (weight in volume, w/v) polyethylene glycol 6000–1.6 M NaCl. Pelleted nucleic acids were washed in ice-cold 70% (v/v) ethanol, air-dried and resuspended in 50 μL of RNase-free sterile water.

PCR amplification of ammonia oxidizer 16S rRNA genes

Ammonia oxidizer 16S rRNA genes were amplified from extracted soil DNA using a nested approach, with primary PCR amplification with CTO189f–CTO654r primers (Kowalchuk et al., 1997), specific for the majority of soil autotrophic betaproteobacterial ammonia oxidizers, and secondary amplification with universal bacterial 357f–GC–518r primers (Muyzer et al., 1993). PCR amplifications were carried out in 50 μL reaction volumes with a PCR Express thermal cycler (Hybaid). The reaction mixture contained 20 ng of template DNA, 0.2 M of each primer,
1 × PCR buffer (Bioline, London, UK), 250 μM each deoxynucleoside triphosphate, 1.5 mM MgCl₂, 20 ng bovine serum albumin (for primary PCR amplification only) and 1 U of Biotaq™ DNA polymerase (Bioline). The thermocycling conditions for both primer-sets have been described by Freitag & Prosser (2003). The CTO189f–CTO654r and 357f-GC–518r primers amplified 465 and 161 bp fragments, respectively. PCR amplification products were analysed by electrophoresis on 1% (w/v) agarose gels and detected by staining with ethidium bromide (Sambrook et al., 1989).

RT-PCR amplification of ammonia oxidizer 16S rRNA genes

DNA was removed from crude nucleic acid extracts by treatment with DNase for 1 h at 37°C according to the manufacturer’s recommendations (Promega, Madison, WI). Reverse transcription of RNA to complementary DNA (cDNA) was performed according to Griffiths et al. (2004) with modifications. The RNA secondary structure was melted by incubating RNA samples with the reverse 16S rRNA gene primer, CTO654r, at 70°C for 10 min. Samples of annealed primer-template were then chilled on ice and 8 μL of RT reaction mixture (SuperScript RNase H-Reverse Transcriptase, 5× First-Strand Buffer and 0.1 M dithiothreitol) was added as specified by the manufacturer (Invitrogen Ltd., Paisley, UK). Control reactions (without RT) were run to check for DNA contamination of RNA samples. Reverse transcription was carried out at 42°C for 50 min and the enzyme was subsequently heat-inactivated for 10 min at 70°C. PCR amplification of cDNA template and subsequent analysis were performed in a 50 μL reaction volume with 1 μL of cDNA template, as described for PCR-amplification products.

DGGE analysis

Denaturing gradient gel electrophoresis (DGGE) analysis of PCR products was carried out using the D-Code Universal Mutation Detection System (Bio-Rad, Hercules, CA). Briefly, 8% polyacrylamide gels containing a gradient of 30–60% denaturant were prepared using a gradient maker (Fisher Scientific, Loughborough, UK), with 100% denaturing solution defined as 7 M urea and 40% formamide (McCaig et al., 2001). Wells were loaded with equal amounts of PCR products and electrophoresis was carried out for 16 h at 75 V in 1× TAE buffer at a constant temperature of 60°C. Following electrophoresis, gels were stained for 30 min in 1× TAE buffer with ethidium bromide (final concentration 1.0 mg L⁻¹) and destained in ddH₂O for 10 min. Gels were scanned using a Gel Doc 2000 UV transilluminator (BioRad) and selected gels were silver stained as described in McCaig et al. (2001) for a greater resolution of banding profiles.

Recovery and purification of DNA template from DGGE bands

Fifteen bands were excised for sequencing from both DNA- and RNA-DGGE gels, including most major bands common or specific to a particular synthetic sheep urine treatment and minor or less intense bands. Sequences were obtained from several replicate bands with identical migratory positions in different lanes to ascertain whether selected bands migrating to similar positions had identical sequences. Bands were excised with a sterile razor blade and DNA was eluted by incubation of the crushed band in 25 μL sterile ddH₂O at 65°C for 30 min, followed by centrifugation at 5000 × g for 1 min. The eluted DNA (1 μL) was used as template for PCR amplification using universal bacterial 357f-GC–518r primers (Muyzer et al., 1993) under the conditions described above and the resultant amplified products were analysed by agarose gel electrophoresis. The purity of amplification products was checked by DGGE analysis. In most cases, products yielded several bands, in addition to the band of interest, necessitating further (typically two to three) rounds of excision, PCR amplification and DGGE analysis to attain purity. The melting behaviour and correct migration of PCR products of purified bands were confirmed by DGGE analysis of these products and environmental PCR products on the same gel. Purified PCR products were subjected to a final cleaning step as described by Boyle & Law (1995).

Sequencing and sequence comparisons

Purified PCR products were sequenced with 357f–518r primers (Muyzer et al., 1993) using the BigDye Terminator cycle-sequencing kit (PE Biosystems, North Warrington, UK) and sequences were analysed using an ABI377 automated sequencer (PE Biosystems). The BLASTN search tool (Altschul et al., 1990) was used to find sequence homology and to determine the most similar sequences in the GenBank database. All sequences from this study were deposited to the GenBank under the accession numbers DQ017527–DQ017556.

Ammonia, nitrite and nitrate analyses

Ammonium, nitrite and nitrate were extracted from 4 g soil in 15 mL of 1 M KCl after shaking for 30 min on a rotary shaker, as described in Allen (1989). Nitrite concentrations were negligible in all samples. The extracts were analysed on a Tecator 5010 Flow Injection Analyzer (Foss UK Ltd.) for ammonium and nitrite + nitrate and pH was measured in water.
Results

Changes in soil NH$_4$$^+$, NO$_3^-$ and pH

Synthetic sheep urine application led to an immediate (first sample point) increase in soil NH$_4$$^+$ concentration, due to hydrolysis of urea. This was followed by a further increase in NH$_4$$^+$ concentration, which reached a maximum at 7 days. For MN and HN treatments, these maxima were significantly greater than levels of N applied and presumably resulted from the release of bound ammonium. NH$_4$$^+$ concentration thereafter decreased gradually until the end of the incubation period (day 56) (Fig. 1a). NH$_4$$^+$ concentration in control microcosms increased continuously during incubation, reaching a maximum value of 42 ± 3.0 µg N mL$^{-1}$. Soil NO$_3^-$ concentrations increased after lag phases of 2, 7 and 7 days in LN, MN and HN treatments, respectively. During the lag phases, NO$_3^-$ concentrations were in the ranges 25–23, 24–13 and 24–3 µg N mL$^{-1}$ for LN, MN and HN treatments, respectively. NO$_3^-$ concentrations increased throughout the experiment in HN-treated microcosms but reached maxima in other treatments and in the control (Fig. 1b). The increase in ammonium concentration immediately after synthetic sheep urine addition was accompanied by an increase in soil pH (H$_2$O), which reached maxima of 5.0, 5.4, 6.7 and 7.4 for control, LN, MN and HN treatments, respectively. Soil pH then decreased, as ammonium was converted to nitrite and nitrate, reaching values in the range 4.9–5.1 by day 56 in all treatments (Fig. 1c).

DNA-targeted analysis of ammonia oxidizer communities

DNA-derived DGGE profiles of abundant bacterial communities in control and treated microcosms at day 0 showed no differences, as determined by visual examination (data not presented). Small treatment-associated changes in DNA-DGGE profiles were detectable by day 2 and further, significant changes occurred until day 28, after which DGGE profiles remained the same (Fig. 2). Fifteen bands could be discriminated in DGGE profiles (Fig. 3), and changes in their relative abundance are indicated by arrows in Fig. 2. The relative intensity of several of these bands changed during incubation of control microcosms and small differences between profiles of control and synthetic sheep urine-treated microcosms were apparent after incubation for 2 days. These differences increased as incubation proceeded. The relative intensity of bands D4–D6, D9 and D10 increased in profiles derived from LN-treated microcosms, with differences increasing, in comparison with other treatments, until day 28, after which they were maintained until termination of the experiment (Figs 2, 3). Bands D11 and D15 increased in relative intensity in HN treatments, showing the greatest treatment differences on day 14. In contrast, bands D4–D6, D9 and D10 decreased in relative intensity in MN-treated microcosms and to a greater extent in HN microcosms. Bands D11–D15 increased in relative intensity in MN and HN treatments, reaching the highest relative intensities in the latter. Band D13 showed a higher intensity in MN microcosms than in other treatments (Fig. 2).

Bands showing the highest relative intensity and greatest changes during incubation were excised and sequenced. Sequences of most of the bands that originated from DNA-derived DGGE profiles (bands D1–D15) had homologies to 16S rRNA sequences that fell within the betaproteobacterial
Fig. 2. DNA- and RNA-derived denaturing gradient gel electrophoresis (DGGE) banding profiles of ammonia oxidizer communities following application of three levels of synthetic sheep urine: 100 (low N, LN), 500 (medium N, MN) and 1000 (high N, HN) µg urea-N g⁻¹ soil sampled at several points during incubation for 56 days. Lanes 1–3 relate to samples from triplicate microcosms of control (C) or synthetic sheep urine treatments that were harvested destructively on each sampling occasion. Bands marked with arrows have been assigned numbers (R1–15 or D1–15) (see Fig. 3) and were excised and sequenced (for identities see Table 1). Grey and black arrowheads indicate bands that appeared exclusively in DNA- and RNA-derived DGGE banding profiles, respectively. White arrowheads indicate bands that either responded to synthetic sheep urine treatments or, in most cases, were common to DNA- and RNA-derived DGGE profiles. Markers in lane M consisted of controls for ammonia oxidizer clusters I–VII (Stephen et al., 1996): I, EnvB1-8 (Nitrosospira); II, pH4.2A/27 (Nitrosospira); III, pH4.2A/4 (Nitrosospira); IV, pH7B/C3 (Nitrosospira); V, EnvA1-21 (Nitrosomonas); VI, EnvC1-19 (Nitrosomonas); VII, Nitrosomonas europaea.
ammonia oxidizer clade (Table 1) and demonstrated that the ammonia oxidizing bacterial community was composed of *Nitrosospira* clusters 1, 2, 3, 4 and *Nitrosomonas* cluster 5. *Nitrosospira* cluster 1 band D9 and cluster 4 bands D4, D5, D7 and D8 had the highest intensities in LN treatments; clusters 2 bands D6, D10 and D11, D12 were generally more abundant in LN and MN/HN treatments respectively; and cluster 3 bands D13–15 had greater intensities in MN and HN treatments (Fig. 3). *Nitrosospira* cluster 5 bands D1 and D2 appeared in samples collected after incubation for 28 days. The intensities of these bands were greatest in control samples, but decreased in the LN treatment and were faint in profiles from MN and HN treatments (Fig. 3). *Nitrosospira* cluster 5 bands D1 and D2 appeared in samples collected after incubation for 28 days. The intensities of these bands were greatest in control samples, but decreased in the LN treatment and were faint in profiles from MN and HN treatments (Fig. 3). *Nitrosospira* cluster 5 bands D1 and D2 appeared in samples collected after incubation for 28 days. The intensities of these bands were greatest in control samples, but decreased in the LN treatment and were faint in profiles from MN and HN treatments (Fig. 3). *Nitrosospira* cluster 5 bands D1 and D2 appeared in samples collected after incubation for 28 days. The intensities of these bands were greatest in control samples, but decreased in the LN treatment and were faint in profiles from MN and HN treatments (Fig. 3). *Nitrosospira* cluster 5 bands D1 and D2 appeared in samples collected after incubation for 28 days. The intensities of these bands were greatest in control samples, but decreased in the LN treatment and were faint in profiles from MN and HN treatments (Fig. 3).

### RNA-derived ammonia oxidizer communities

RNA-derived profiles also showed no difference between untreated controls and samples harvested immediately after synthetic sheep urine applications and, as for DNA-DGGE analysis, treatment-associated differences were evident in profiles obtained after incubation for 2 days (Fig. 2). DGGE profiles of control soils exhibited rather persistent banding patterns with little or no change in band intensities during incubation of microcosms. Synthetic sheep urine treatment led to a decrease in the relative intensity of bands R1–R4, which were prominent in the control profiles, and an increase in the relative intensity of bands R11–R15, except in the LN treatment. The changes in relative intensity of these bands increased in magnitude as incubation proceeded and, by day 28, indicated a distinct shift in ammonia oxidizing bacterial community resulting from synthetic sheep urine treatment. DGGE profiles from microcosms sampled after day 28 showed little change, suggesting no further change in ammonia oxidizer community structure (Fig. 2).

The nature of the change in DGGE profile varied with synthetic sheep urine concentration. In general, increasing synthetic sheep urine concentration was associated with an increase in the relative intensity of bands with higher GC content. For example, the relative intensities of bands R5–R7, R9 and R10 were greater in the LN treatment than in control, MN or HN treatments, indicating that this level of synthetic sheep urine enhanced the activity of this particular ammonia oxidizing bacterial community (Fig. 2). Bands R11–R15 increased in relative intensity in the MN-treated microcosms, whereas bands R12 and R13 decreased in relative intensity in the HN treatment and bands R14 and R15 maintained high intensities (Fig. 2).

Denaturing gradient gel electrophoresis bands showing the highest relative intensity and the greatest changes in RNA-derived profiles during incubation were excised and sequenced. Bands R1–R4 and R8, which appeared exclusively in RNA-derived DGGE profiles (Fig. 3), showed sequence homologies to uncultured *Gammaproteobacteria* and have no phylogenetic relation to known ammonia oxidizers (Table 1). However, all of the sequenced bands that were either prominent or corresponded to DNA-
derived bands (with identical migratory positions) were of known betaproteobacterial ammonia oxidizers with established phylogenies (Table 1). The RNA-derived ammonia oxidizing community was also dominated by *Nitrosospira* clusters 2, 3 and 4, and their distribution with different treatments corresponded with that for DNA-DGGE sequences. Thus, *Nitrosospira* cluster 4 bands R5, R6 and R9 had the highest intensities in LN treatments, clusters 2 bands R7, R10 and R11, R12 were generally more intense in LN and MN/HN treatments, respectively, and cluster 3 bands R13–15 had higher intensities in MN and HN treatments (Fig. 3). Ten of the sequenced betaproteobacterial ammonia oxidizer bands were common to both DNA- and RNA-derived DGGE profiles (Table 1). However, the five bands that were specific to DNA-derived DGGE profiles were derived from ammonia oxidizing bacteria. The five bands that were specific to RNA-derived DGGE profiles were of *Gammaproteobacteria* (presumably nonammonia oxidizers) (Table 1).

### Table 1. Closest matches between 16S rRNA gene sequences of bands excised from DNA- and RNA-derived denaturing gradient gel electrophoresis (DGGE) gels and sequences from the GenBank databases obtained using the BLASTN search tool

<table>
<thead>
<tr>
<th>Band no.</th>
<th>GenBank accession no.</th>
<th>Percentage similarity</th>
<th>Total entries with similar score</th>
<th>Closest relative in GenBank[^1] [name, (accession number)]</th>
<th>AOB cluster[^2]</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>DQ017527</td>
<td>99</td>
<td>2</td>
<td><em>Betaproteobacterium</em> sp. (clone EnvA1-21) (Z69091)</td>
<td>5</td>
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<tr>
<td>D2</td>
<td>DQ017528</td>
<td>100</td>
<td>2</td>
<td><em>Betaproteobacterium</em> sp. (clone EnvA1-21) (Z69091)</td>
<td>5</td>
</tr>
<tr>
<td>D3</td>
<td>DQ017529</td>
<td>98</td>
<td>2</td>
<td><em>Betaproteobacterium</em> sp. (clone EnvA1-21) (Z69091)</td>
<td>5</td>
</tr>
<tr>
<td>D7</td>
<td>DQ017533</td>
<td>100</td>
<td>44</td>
<td>Uncultured <em>Nitrososphaera</em> sp. clone SBMPs11 (AY293113)</td>
<td>4</td>
</tr>
<tr>
<td>D9</td>
<td>DQ017535</td>
<td>100</td>
<td>21</td>
<td>Uncultured <em>Nitrososphaera</em> sp. isolate DGGE gel band ORC3 (AY773203)</td>
<td>1</td>
</tr>
<tr>
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<td>2</td>
<td>Uncultured bacterium clone DUN1.27-1495+_B02 (AY724026)</td>
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<tr>
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<td>100</td>
<td>2</td>
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<td>96</td>
<td>13</td>
<td>Grassland soil clone saf1.107 (AF078179)</td>
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</tr>
<tr>
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<td>DQ017549</td>
<td>100</td>
<td>1</td>
<td>Uncultured soil bacterium clone 829-2 (AY326574)</td>
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<tr>
<td>D4/R5[^3]</td>
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</tr>
<tr>
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<tr>
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<td>35</td>
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<tr>
<td>D8/R9</td>
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<td>Uncultured <em>Nitrososphaera</em> sp. SSCP band S3 (AY274172)</td>
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<td>35</td>
<td>Uncultured <em>Nitrososphaera</em> sp. clone SBMPs10 (AY293112)</td>
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<td>100</td>
<td>34</td>
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<td>2</td>
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<tr>
<td>D13/R13</td>
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<td>23</td>
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<td>3</td>
</tr>
<tr>
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<td>1</td>
<td>Uncultured <em>Betaproteobacterium</em> E2.pH4/50/FL (AF178087)</td>
<td>3</td>
</tr>
</tbody>
</table>

[^1]: Percentage similarity between the sequence of a band excised from DGGE gel and the closest match in GenBank.
[^2]: Identities are based on closest NCBI sequences of phylogenetically related bacteria.
[^3]: Relatedness to AOB cluster number, indicating that the closest relative of the sequences is a nonammonia oxidizer.
[^4]: Dx/Rx indicates that DNA- and RNA-derived sequences are identical.

[^5]: The prominent bands in ammonia oxidizer DGGE profiles that showed a response to sheep urine addition to soils.
In summary, the relative intensity of RNA-DGGE bands R11 and R12 increased in MN microcosms after incubation for 7 days, whereas corresponding bands in DNA-DGGE gels, D11 and D12, showed no treatment effect at this stage. Similarly, RNA-DGGE bands R14 and R15 increased in relative intensity in MN and HN microcosms after incubation for 14 days, whereas a similar change in DNA-DGGE bands D14 and D15 was not observed until 28 days.

Relationships between changes in DGGE profiles and in nitrate accumulation

Temporal patterns in DGGE profiles and nitrification dynamics were compared to assess links between changes in community structure and process rates. There were no clear trends or changes in RNA-derived DGGE profiles from control microcosms, except for slight changes in intensity of bands R6 and R10 after incubation for 28 days. This corresponded with maximum nitrate concentrations in control microcosms, which decreased with further incubation (Fig. 1b), with no further visible change in DGGE profiles. In the LN treatment, the increase in relative intensities of bands R5–R7, R9 and R10, between days 2 and 28 of incubation, was associated with NO$_3^-$ production that commenced after a short lag phase of 2 days and continued until day 42, by which time nitrate production was almost complete. In the MN treatment, the increase in relative intensities of bands R11–R15 at day 7 coincided with NO$_3^-$ production. This continued throughout the incubation period, although changes in community structure were not evident after 28 days. The HN treatment also showed a lag phase of 7 days prior to NO$_3^-$ production, but in this case several bands (R11, R14 and R15) had already increased in relative intensity.

Discussion

Nitrification dynamics

Urea in the applied synthetic sheep urine was converted rapidly to NH$_4^+$ by the indigenous ureolytic microbial community, leading to an increase in soil pH and NH$_4^+$ concentration. The subsequent increase in NH$_4^+$ concentration in all treatments, but particularly in MN and HN treatments, was most likely due to the increase in soil pH, leading to mobilization of adsorbed ammonium and to a general increase in microbial activity. The latter could also have been stimulated by organic carbon in synthetic sheep urine, leading to enhanced mineralization of organic matter. As a result, the observed changes in community structure cannot be attributed to increased ammonium concentration alone, but may have been associated with changes in soil pH. Similar changes in soil pH and NH$_4^+$ and NO$_3^-$ concentrations have been observed in larger-scale studies of the effects of natural and synthetic sheep urine addition to field sites and sward boxes (Shand et al., 2002). As nitrification proceeded, nitrate production and ammonia removal resulted in a decrease in pH, eventually to pH values of 4.9–5.1, which inhibited nitrification and prevented complete conversion of ammonium.

Nitrification rates in grassland soils, measured in the field or in soil microcosms, frequently exhibit significant variability. In many cases, this variability can be attributed to variation in the lag phase prior to nitrification (Webster et al., 2005). Variability is greatest in soils with no history of inorganic fertilization (Webster et al., 2002) and is associated with the relative abundance of groups of ammonia oxidizers with different sensitivities to high ammonia concentration (Webster et al., 2005). In fertilized soils, variability appears to be less due to the higher abundance of ammonia oxidizers. In this study, which employed unfertilized grassland soil, a lag phase of 2 days, prior to detectable nitrate production, was observed in the LN treatment, while MN and HN treatments exhibited lag periods of approximately 7 days. The most likely explanation for this longer lag period is sensitivity of at least a proportion of the ammonia oxidizer community to high ammonia concentrations. The lag period would then reflect time required for ammonia tolerant strains within the community to grow. This is evidenced by selection of fewer bands and an increase in relative intensities of bands D11–D15 or R11–R15 at day 14 to day 56 gels (Figs 2, 3). In addition, DNA- and RNA-derived DGGE profiles of HN treatments contained few bands, indicating a decrease in richness of active and growing ammonia oxidizers through ammonia-inhibition of some members of the community, suggested by a decrease in relative intensity of bands D1–D10 and R1–R10 (Fig. 3). Alternative explanations for lag phases include changes in the balance between nitrate assimilation and denitrification, during changes in organic carbon availability, and inability to measure small increases in nitrate concentration.

Changes in ammonia oxidizer community structure

One aim of this study was to assess differences between DNA- and RNA-targeted approaches, and the potential of the latter approach to detect active, rather than total, ammonia oxidizer communities and provide an earlier and more sensitive indication of community changes. Although both approaches demonstrated similar changes in community structure, RNA-targeted analysis indicated finer-scale qualitative shifts in ammonia oxidizing communities in response to synthetic sheep urine addition to soils. This is most evident in changes in relative intensity of bands 11 and 12 in MN microcosms and of bands 14 and 15 in MN and HN microcosms. Thus, in most cases, RNA-DGGE provided an earlier indication of community changes and also
demonstrated greater consistency between replicates. Despite this, temporal and treatment differences indicated by DNA-DGGE and RNA-DGGE were similar. This has been observed in other studies (Freitag & Prosser, 2003; Mahmood et al., 2005) and indicates that active (RNA-targeted) communities were also the most abundance members of the total (DNA-targeted) community.

 Sequencing and phylogenetic analysis of DGGE bands provided evidence for selection of Nitrosospira cluster 4 in LN treatments, Nitrosospira cluster 2 in LN, MN and HN treatments, and Nitrosospira cluster 3 in MN and HN treatments. Previous studies (Webster et al., 2005) have demonstrated links between nitrification dynamics and relative abundance of Nitrosospira clusters 3a and 3b in synthetic sheep urine-treated soils, which were explained on the basis of greater ammonia sensitivity in cluster 3a. In the present study, Nitrosospira cluster 3 bands had the highest relative intensities in MN and HN treatments, but it was not possible to discriminate between clusters 3a and 3b, due to the shortness of the sequence (161 bp) retrieved from the excised bands. Other studies have demonstrated changes in ammonia oxidizer community structure associated with fertilization. For example, Mendum & Hirsch (2002) observed dominance by Nitrosospira cluster 3 in fertilized arable plots, whereas unfertilized plots were dominated by Nitrosospira cluster 4, supporting our findings. Webster et al. (2002) found that unfertilized grassland soils were dominated by Nitrosospira clusters 1 and 3 and Nitrosomonas cluster 7, but detected only Nitrosospira clusters 1 and 3 in fertilized soil. However, selection is not always observed. For example, Jordan et al. (2005) reported abundance of Nitrosospira clusters 2, 3 and 4 in a US forest soil subject to atmospheric N deposition, but found no clear association between community composition, soil N content and pH. Similarly Avrahami et al. (2002) observed no effect of ammonium concentration (6.5–395 μg NH₄⁺-N g⁻¹ dry weight soil) on ammonia oxidizer communities dominated by Nitrosospira clusters 3, 4 and 7. These studies employed lower ammonium concentrations than are present in synthetic sheep urine, and ammonia toxicity will have been less. However, using the same soil but two levels of a slow-release N-fertilizer (resulting in an increase up to 800 μg NH₄⁺-N g⁻¹ dry weight soil) and a longer incubation period (6.5–20 weeks) Avrahami et al. (2003) were able to demonstrate a shift in the community structure of ammonia oxidizing bacteria. In the present study Nitrosomonas cluster 5 sequences (bands D1–D3) did not persist throughout the incubation period (56 days), and their reduction in profiles from MN and HN treatments suggests a sensitivity of these strains to increased levels of N. Nitrosomonas cluster 5 sequences have been considered to be specific to the marine environment, yet there is evidence of their presence in soil (Radajewski et al., 2002). However, the lack of cultured representatives of Nitrosomonas cluster 5 prevents reliable assessment of their role in soil nitrification.

The present study employed CTO189f–CTO654r primers (Kowalchuk et al., 1997) that specifically amplify ammonia oxidizing bacteria but can amplify sequences from nonammonia oxidizers when ammonia oxidizers are at low abundance. All sequences obtained from DNA-derived DGGE bands belonged to Betaproteobacteria and all were from ammonia oxidizing bacteria. However, RNA-derived DGGE profiles of control soils and early harvests of synthetic sheep urine applied soils contained several gammaproteobacterial sequences. Interestingly, in the DGGE profiles of synthetic sheep urine-treated soils, these bands gradually decreased in relative abundance and final DGGE profiles contained only ammonia oxidizers. Others have reported amplification of nonammonia oxidizing bacteria by CTO189f–CTO654r primers (Backman et al., 2003; Cebron et al., 2004; Mahmood et al., 2006). One explanation for their amplification when targeting RNA is their relatively high activity in control soils (and early harvests of synthetic sheep urine-treated soils) compared to ammonia oxidizing bacteria, resulting in relatively high copy numbers of RNA templates used for RT-PCR. Since the majority of RNA- and DNA-derived sequences that dominated the ammonia oxidizing bacterial community were common to both approaches, it appears that the relatively high copy numbers of RNA templates of nonammonia oxidizers had no competitive effect on the relative abundance of ammonia oxidizers in the RNA-based approach. In addition, there is the possibility of mismatches in CTO189f and CTO654r primer sites that might result in amplification of sequences of nonammonia oxidizing Gammaproteobacteria. For example, CTO189f and CTO654r primers have been found to exhibit one and seven mismatches, respectively, when aligned against clone sequences of the 16S rRNA gene from the genera Nitrosomonas and Nitrosospira. However, RNA-DGGE resulted in relatively high copy numbers of RNA templates used for RT-PCR. Since the majority of RNA- and DNA-derived sequences that dominated the ammonia oxidizing bacterial community were common to both approaches, it appears that the relatively high copy numbers of RNA templates of nonammonia oxidizers had no competitive effect on the relative abundance of ammonia oxidizers in the RNA-based approach. In addition, there is the possibility of mismatches in CTO189f and CTO654r primer sites that might result in amplification of sequences of nonammonia oxidizing Gammaproteobacteria. For example, CTO189f and CTO654r primers have been found to exhibit one and seven mismatches, respectively, when aligned against clone sequence DUN1_27-1495_+B02 (AY724026) (Gammaproteobacteria) that has 99% similarity to sequence of band R1 (Table 1).

The changes in community structure observed in this study may result from selection through difference in ammonia concentration preferences but other factors may also have influenced community structure. Differences in salt concentrations may have introduced different degrees of osmotic stress and soil pH, N and P, dissolved organic C and N will all have been influenced by amendment with synthetic sheep urine (Shand et al., 2000; 2002); all can influence soil microbial activity and community structure. For example, Nicol et al. (2004) studied the effect of amendment with synthetic sheep urine or ammonium nitrate and pH change of unimproved grassland soil and found a differential effect on bacterial, but not crenarchaeal community structure. Synthetic sheep urine has also been shown to influence soil respiration, microbial activity and bacterial abundance in two pastures in the Scottish uplands.
(Williams et al., 2000). This study clearly demonstrates that in response to synthetic sheep urine, DNA-and RNA-derived DGGE profiles of abundant/total and active ammonia oxidizing bacteria, respectively, exhibit similar changes in communities. However, the RNA-based approach depicts finer-scale qualitative changes in community structure earlier than DNA-derived DGGE profiles. Moreover, we have been able to establish links between relative changes in RNA-derived DGGE profiles and nitrate accumulation.

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References


