Stable isotope probing analysis of the influence of liming on root exudate utilization by soil microorganisms

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Summary
Rhizosphere microorganisms play an important role in soil carbon flow, through turnover of root exudates, but there is little information on which organisms are actively involved or on the influence of environmental conditions on active communities. In this study, a 13C pulse labelling field experiment was performed in an upland grassland soil, followed by RNA-stable isotope probing (SIP) analysis, to determine the effect of liming on the structure of the rhizosphere microbial community metabolizing root exudates. The lower limit of detection for SIP was determined in soil samples inoculated with a range of concentrations of 13C-labelled Pseudomonas fluorescens and was found to lie between 105 and 106 cells per gram of soil. The technique was capable of detecting microbial communities actively assimilating root exudates derived from recent photo-assimilate in the field. Denaturing gradient gel electrophoresis (DGGE) profiles of bacteria, archaea and fungi derived from fractions obtained from caesium trifluoroacetate (CsTFA) density gradient ultracentrifugation indicated that active communities in limed soils were more complex than those in unlimed soils and were more active in utilization of recently exuded 13C compounds. In limed soils, the majority of the community detected by standard RNA-DGGE analysis appeared to be utilizing root exudates. In unlimed soils, DGGE profiles from 12C and 13C RNA fractions differed, suggesting that a proportion of the active community was utilizing other sources of organic carbon. These differences may reflect differences in the amount of root exudation under the different conditions.

Introduction
The rhizosphere plays a fundamental role in the formation, maintenance and turnover of soil organic matter (SOM), influencing flow, storage and the sink–source balance of C. Despite this, understanding of the role of rhizosphere microbial communities in C flow is limited and little is known of roles of different members of the community in assimilating root exudate. C in SOM is derived mainly from rhizosphere C flow and decomposed shoots, roots and litter (Kuzyakov and Domanski, 2002). Most of the compounds mediating rhizosphere C flow are low-molecular weight and polymeric compounds, including carbohydrates, amino acids and organic acids, which are either easily assimilated by soil microorganisms or require production of extracellular enzymes before uptake (Killham and Yeomans, 2001). The C pool is fundamental as a driving force in soil microbial processes (Lugtenberg and Dekkers, 1999) and, consequently, in the interactions within rhizosphere communities and their physiological characteristics. The role of microbial communities is also an important factor when considering applied aspects, such as control of pathogens, bioremediation processes and enhancement of plant nutrient supply.

One direct approach to determination of C flow in plant–soil systems is the use of 13C and 14C isotopes pulsed into the systems as labelled CO2 (Meharg, 1994; Kuzyakov, 2002). This approach has been restricted to laboratory systems (Kuzyakov and Domanski, 2000; Kuzyakov et al., 2001), with the exception of a limited number of pulse...
labelling field experiments (Johnson et al., 2002; Staddon et al., 2003; Olsrud and Christensen, 2003; Rangel-Castro et al., 2004, 2005). Rangel-Castro and colleagues (2005) demonstrated that the flux of recently photosynthesised $^{13}$C to soil microbial biomass occurs within hours (i.e. less than 24 h) of incorporation of $^{13}$CO$_2$ into shoot biomass. Calculation of exponential decay kinetics for $^{13}$C incorporated indicated that the half-life of the isotope in the microbial biomass was 4.7 days. These data, coupled with results of Ostle and colleagues (2003), who found maximum incorporation of $^{13}$C into microbial RNA within 4–8 days of pulse labelling, demonstrate rapid incorporation and turnover of $^{13}$C by the soil microbial biomass.

Tracking $^{13}$C in cellular components (e.g. lipids and nucleic acids) (stable isotope probing) has recently been used to determine which functional groups actively assimilate $^{13}$C-labelled substrates (Boschker et al., 1998; Radazewski et al., 2000; Manefield et al., 2002a; Butler et al., 2003; Lueders et al., 2004a,b; Treonis et al., 2004). Stable isotope probing of DNA and RNA has been used in microcosm systems to detect active groups in assimilation of a range of organic compounds (Padmanabhan et al., 2003), phenol degradation (Manefield et al., 2002a), and methane and methanol assimilation (Radazewski et al., 2000; Lueders et al., 2004b). However, a recent attempt to identify soil microbes active in assimilation of rhizosphere C flow, in pots planted with grassland turfs, was unsuccessful (Griffiths et al., 2004). $^{13}$C dilution in nucleic acid samples was suggested as a factor preventing adequate separation of labelled and unlabelled RNA after centrifugation.

Land management strategies aimed at improving plant production, including application of lime, are known to affect microbial activities such as C and N transformations (Hopkins, 1997; Neale et al., 1997). In a previous study, liming was found to affect C flow in plant shoots and roots of upland grassland plots (Rangel-Castro et al., 2004) and turnover of C in microbial biomass of limed soil plots was faster than in unlimed plots (Rangel-Castro et al., 2005). This could result from differences in active rhizosphere microbial communities in limed and unlimed soils. The main objective of this study was therefore to use RNA-SIP to determine if differences in C turnover in limed and unlimed soils were due to differences in the active microbial community structure. In addition, lower limits for detection of $^{13}$C-labelled cells in soil, using SIP, were determined.

**Results**

SIP optimization

Preliminary experiments were carried out using $^{13}$C-labelled *Escherichia coli* and *Pseudomonas fluorescens* cells to determine the location of $^{13}$C and $^{12}$C RNA following centrifugation and to determine lower detection limits of $^{13}$C-labelled cells. Results were similar for both organisms and only *P. fluorescens* analyses are presented. Figure 1 illustrates DGGE profiles from fractions derived from soil containing $^{13}$C and $^{12}$C RNA from a $^{13}$C-labelled *Pseudomonas putida* phenol-degrading strain. Therefore, fraction 7 was not considered for further analyses and fractions 8–10 were analysed as representative of $^{13}$C RNA fractions.

The $^{13}$C community showed the complex DGGE banding patterns typical of soil bacterial communities, with one band corresponding also to *P. fluorescens*, although this was not expected because of its likely low abundance in comparison with a total cell concentration of approximately 10$^9$ cells per gram of soil. Denaturing gradient gel electrophoresis profiles derived from $^{13}$C RNA (fractions 4–6) were dominated by the *P. fluo"rescens* band, although sequences representative of the total soil community were detected as faint bands in some fractions. This indicates that some $^{13}$C sequences could have migrated to the $^{13}$C zone during centrifugation and were amplified during the polymerase chain reaction (PCR). Denaturing gradient gel electrophoresis profiles derived from $^{12}$C extracted from soil containing $^{13}$C cells per gram showed similar complexity and a very faint band, corresponding to *P. fluorescens*, was observed. Products representing *P. fluorescens* were not detected in the $^{13}$C fractions, indicating that the detection limit lies within the range of 10$^5$–10$^6$ cells per gram for this organism. Again, faint bands representative of sequences from the soil bacterial community were observed in fractions 5 and 6, but none was detected in fraction 4.

**Microbial incorporation of root exudates**

To confirm that density gradient centrifugation led to fractionation of $^{13}$C and $^{12}$C RNA, and that $^{13}$C root exudates had been assimilated by soil microorganisms, fractions along the gradient were analysed for $^{13}$C. The data, illustrated in Fig. 2, show $\delta$C values significantly greater than natural abundance values (see Table 1) in fractions 4–6, as expected, but label was also detected in other fractions, indicating that separation was not completely efficient. However, the proportion of $^{13}$C RNA in fractions 8–10 will be negligible, in comparison with $^{12}$C, and will not have compromised analysis of these fractions. High levels of $^{13}$C were also detected in fraction 3 but amplification of sequences was less reliable and consistent, and occa-
sionally represented only high GC content sequences. Nevertheless, future studies should examine this fraction, which may contain sequences that are not present in fractions 4–6 but which potentially are highly $^{13}$C-labelled.

Table 1 shows $\delta^{13}$C values of RNA extracted from soils collected 3 h and 5 days after completion of pulse labelling. $^{13}$C enrichment was highest in samples collected after 3 h of labelling and were greater than natural abundance values. Denaturing gradient gel electrophoresis profiles generated by reverse transcription polymerase chain reaction (RT-PCR) amplification, using bacterial primers, of fractions sampled along the $^{12}$C–$^{13}$C gradient are illustrated in Fig. 3. For limed soils, no difference could be detected by visual examination of profiles in different fractions. Denaturing gradient gel electrophoresis profiles reflect relative abundance of different sequence types but provide no information on absolute numbers or biomass, or abundance of sequence types. Similar amounts of RT-PCR product were added to each lane, leading to similarities in total intensity within lanes for each fraction, rather than differences in levels in soil samples of $^{12}$C and $^{13}$C RNA. Similarities in DGGE profiles in each lane therefore indicate similarities in relative abundance of different sequence types in $^{12}$C- and $^{13}$C RNA. Denaturing gradient gel electrophoresis profiles from $^{12}$C RNA fractions are equivalent to those from standard RNA-DGGE analyses, which are considered to represent active members of the community. Similarity between these profiles and those from $^{13}$C RNA DGGE therefore indicates that these organisms were actively incorporating root exudates. In contrast, DGGE profiles derived from $^{12}$C and $^{13}$C fractions differed for unlimed soils. For example, bands marked I, II and III increased in relative intensity in $^{13}$C fractions, while those marked A, B and C decreased in relative intensity. Other bands showed no detectable difference in

<table>
<thead>
<tr>
<th>Time after completion of pulse labelling</th>
<th>Sample</th>
<th>$\delta^{13}$C</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 h</td>
<td>Unlimed</td>
<td>−14.34</td>
</tr>
<tr>
<td></td>
<td>Limed</td>
<td>−13.85</td>
</tr>
<tr>
<td>5 days</td>
<td>Unlimed</td>
<td>−20.00</td>
</tr>
<tr>
<td></td>
<td>Limed</td>
<td>−24.06</td>
</tr>
<tr>
<td>Natural abundance</td>
<td>Unlimed</td>
<td>−23.90</td>
</tr>
<tr>
<td></td>
<td>Limed</td>
<td>−22.80</td>
</tr>
</tbody>
</table>
Stable isotope probing of soil microbial communities

relative intensity along the gradient. These data indicate that a proportion of the active community, as determined by 12C RNA DGGE profiles, was active through utilization of root exudates but that other members were active through utilization of other forms of soil carbon. Those sequence types derived from organisms utilizing root exudates will have reduced relative band intensity in the 12C fractions, while those utilizing other forms of soil carbon will show greater or similar relative band intensity.

Archaeal DGGE profiles (Fig. 4) showed a similar pattern in limed soils, with no detectable change in relative intensity of bands in DGGE profiles in fractions taken along the 12C–13C gradient. Again, this indicates that the activity of archaea detected by targeting RNA was due to utilization and incorporation of root exudates. In unlimed soils, however, no band was detected in 13C fractions, indicating that any archaea in these soils utilizing root exudates were below the limit of detection of the technique and/or that activity was due to utilization of other forms of organic carbon. Archaeal communities have been characterized previously at Sourhope and are dominated by group 1.1b and 1.1c crenarchaea (Nicol et al., 2003). The large difference in the percentage of GC content of 16S rRNA genes of these two lineages results in group 1.1c sequences typically migrating further than group 1.1b sequences in DGGE analysis. Comparison with the marker lane containing sequences representative of different crenarchaeal lineages including group 1.1b and 1.1c indicates that both these lineages may be present and utilize root exudates (Fig. 4).

Denaturing gradient gel electrophoresis profiles generated using fungal 18S rRNA gene primers for amplification of RNA from limed and unlimed soils showed similar behaviour to bacterial profiles (Fig. 5). In limed soils, DGGE profiles generated from 12C and 13C fractions were similar, while differences were observed for unlimed soils. This provides further evidence for use of alternative organic carbon substrates in unlimed soils, while the majority of the active fungal community in limed soils were utilizing root exudates. In addition, visual observation of DGGE profiles indicates that the communities in limed soils were more complex than in unlimed soils.

Sequencing of clones unique to 13C fractions in unlimed soils

Sequences were determined for three clones of bacterial origin and two of fungal origin containing 16S rRNA and 18S rRNA gene inserts similar to those which increased in relative abundance in 13C fractions derived from

Fig. 3. Denaturing gradient gel electrophoresis banding pattern of bacterial SSU RNA partial sequences of fractions 4–6 (13C) and 8–10 (12C) obtained by ultracentrifugation of CsTFA density gradients of RNA extractions from limed and unlimed grassland soils labelled in the field with 13CO2. M represents markers consisting of ammonia oxidizer clones amplified with the same bacterial primers.

Fig. 4. Denaturing gradient gel electrophoresis banding pattern of archaeal SSU RNA partial sequences of fractions 4–6 (13C) and 8–10 (12C) obtained by ultracentrifugation of CsTFA density gradients of RNA extractions from limed and unlimed grassland soils labelled in the field with 13CO2. M represents a marker consisting of cloned 16S rRNA gene sequences representative of different group 1 Crenarchaeota lineages including 1.1b and 1.1c.
unlimed soil samples. Sequences of archaeal bands were not determined, as no archaeal band was preferentially enriched in $^{13}$C RNA. To confirm reliability of sequences, three different colonies with the same insert were sequenced. Final analyses of the sequences were based on sequences of approximately 600 bp. Sequences were submitted to BLAST search and closest matches with sequences from GenBank are listed in Table 2. Bacterial sequences were most closely related to *Sphingomonas* and *Mycobacterium* isolates and an isolate from rhizosphere soil from a grazed pasture (Joseph *et al*., 2003). The two fungal sequences were most closely related to *Sistotrema eximum* and *Rhodotorula glutinis*.

### Discussion

Previous studies employing SIP have employed laboratory systems in which incorporation is optimized by application of relatively high levels of label, repeated applications and minimization of losses of labelled substrates. Field application of label reduces the sensitivity of the technique and a seeding experiment, with $^{13}$C-labelled *P. fluorescens*, was therefore carried out to determine the minimum level of detection of the method, which was found to lie between $10^5$ and $10^6$ cells per gram of soil. Sensitivity will be affected by a number of factors, including the efficiency of cell lysis, RNA extraction and PCR amplification, all of which may vary with target organism, soil type, primers and protocols used. In addition, inoculated cells were labelled by growth on fully labelled glucose, maximizing labelling of RNA, and the detection limit will be higher for partially labelled cells. This experiment also allowed assessment of the efficiency of separation of $^{13}$C- and $^{13}$C-labelled RNA and of potential generation of random PCR products at low levels of target DNA, as observed in other studies. Results indicate successful separation of $^{13}$C and $^{12}$C RNA but with low level cross-contamination of $^{12}$C and $^{13}$C RNA. For example, a number of bands derived from the indigenous (unlabelled) soil community were detected in fractions 5 and 6, suggesting some migration of $^{12}$C RNA to the $^{13}$C fractions during centrifugation, while the *P. fluorescens* band was detectable in $^{12}$C fractions. However, there was no significant carry-over of $^{12}$C to fraction 4. Lueders and colleagues (2004a) found that caesium trifluoroacetate (CSTFA) separation of labelled RNA was most efficient in gradients containing RNA from a single bacterial species, with reduced efficiency in gradients derived from two or more species. Lueders and colleagues (2004a) and Manefield and colleagues (2002a) considered reduced efficiency to be related to the presence of rRNA from species with similar buoyant densities, and is the most likely explanation for cross-contamination in the complex rhizosphere community investigated here.

### Table 2. Closest database matches of sequences corresponding to bacterial and fungal bands with increased relative abundance in $^{13}$C RNA fractions from unlimed soils.

<table>
<thead>
<tr>
<th>Band number and origin</th>
<th>Accession number</th>
<th>Closest match</th>
<th>% Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Bacteria</td>
<td>AY705870</td>
<td><em>Sphingomonas</em> sp. CSSB-2 AB167382</td>
<td>99</td>
</tr>
<tr>
<td>II Bacteria</td>
<td>AY705871</td>
<td>Bacterium Ellin5080* AY234497</td>
<td>97</td>
</tr>
<tr>
<td>III Bacteria</td>
<td>AY705872</td>
<td><em>Mycobacterium</em> sp. M175 AY004157</td>
<td>99</td>
</tr>
<tr>
<td>I Fungi</td>
<td>AY705873</td>
<td><em>Sistotrema eximum</em> AF334935</td>
<td>98</td>
</tr>
<tr>
<td>II Fungi</td>
<td>AY705874</td>
<td><em>Rhodotorula glutinis</em> AB018403</td>
<td>99</td>
</tr>
</tbody>
</table>

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* a. Strain isolated from a rotationally grazed pasture (Joseph *et al*., 2003).

Despite low levels of cross-contamination, the seeding experiment and differences in $^{12}$C and $^{13}$C profiles derived from unlimed field samples provided confidence that presence of bands in the $^{13}$C fractions only was due to migration of $^{12}$C to the $^{13}$C fractions, but due to labelling of RNA. The differences in $^{12}$C and $^{13}$C DGGE profiles are similar to the changes observed by Lueders and colleagues (2004b) in the terminal restriction fragment length polymorphism (T-RFLP) fingerprints of ‘heavy’ and ‘light’ fractions of methylotrophic bacteria of rice soils labelled with $^{13}$C-methanol. In addition, similar DGGE banding patterns throughout the $^{12}$C–$^{13}$C gradient observed in limed soils indicate that there was no amplification of spurious sequences. An alternative explanation for the DGGE banding pattern observed in limed soils is that the greater community complexity may have increased contamination of $^{13}$C fractions by $^{12}$C, masking $^{13}$C-labelled sequences. However, the high similarity throughout the fractions (in terms of complexity and band intensity) and the differences in $\delta^{13}$C (Fig. 2) indicate that this was not the case. Density gradient centrifugation was employed to separate $^{13}$C and $^{12}$C RNA but can also lead to fractionation through differences in GC content. This approach has been employed to examine the diversity of communities differing in GC content (Holben et al., 2004) and SIP is potentially susceptible to bias towards GC-rich organisms. However, this bias will be reduced in $^{13}$C-labelled nucleic acids and may also be lower in RNA, for which GC content may be less variable, than in DNA. The close similarity of communities across the $^{12}$C and $^{13}$C gradients in limed soils indicates that any such bias was not significant.

Griffiths and colleagues (2004) were unable to separate $^{13}$C and $^{12}$C RNA using SIP from grassland turfs planted in pots and labelled with $^{13}$CO$_2$ and bacterial DGGE analyses of fractions from soil samples showed less complex banding patterns than those reported here. Although the density gradient protocols applied in both studies were similar, minor modifications have improved separation efficiency. For example, ultracentrifugation was carried out with a vertical rotor, considered most efficient for isopycnic separation (Rickwood et al., 1994), and RT-PCR amplification targeting large amplicons, followed by nested amplification using primers yielding shorter sequences, may have improved DGGE analysis. Improvements in separation efficiency would, however, be valuable and a second centrifugation of $^{13}$C fractions would enhance separation, although potentially reducing $^{13}$C signals. Sensitivity could also be improved for investigation of utilization of root exudates by addition of $^{13}$C-compounds directly to soil but this will eliminate effects of spatial interactions between plant roots and the rhizosphere microbial community, which are critical in competition for C flux from plants to soil.

Rangel-Castro and colleagues (2005) demonstrated rapid transport of photosynthate through soil biomass to bulk soil C, with maximal incorporation within 24 h of labelling, and Padmanabhan and colleagues (2003) reported rapid incorporation of $^{13}$C-labelled compounds added directly to soil. Nucleic acids were therefore extracted in this study 3 h after labelling, to maximize recovery of $^{13}$C and detection of primary utilizers of root exudates. Using this strategy, RNA-SIP combined with DGGE analysis of small subunit (SSU) rRNA gene fragments, enabled characterization of bacterial and fungal communities utilizing root exudates in limed and unlimed soils and archaea in limed soils within this time period. The seeding experiment indicates that the signal detected will have resulted from activity of biomass equivalent to at least $10^6$ bacterial cells per gram of soil. Total culturable bacterial cell concentrations at this site are reported in the range $10^3$–$10^4$ cells per gram of soil (Griffiths et al., 2004) but total cell concentrations are likely to be 1–2 orders of magnitude greater. The seeding inoculum therefore represents approximately 0.1–0.01% of the total bacterial community, although it is unlikely that all active cells will have been fully labelled. Similar comparisons for fungal biomass are difficult but archaeal communities comprise approximately 1% of the total prokaryote RNA or fluorescently labelled cells (Buckley et al., 1998; Sandaa et al., 1999). The lack of signal from $^{13}$C rRNA amplified using archaeal primers provides further evidence for a lower detection limit in the range $10^5$–$10^7$ cells per gram of soil.

Molecular analysis targeting RNA, rather than DNA, is considered to provide an indication of the active community, based on the assumption that faster-growing and more active cells will have higher levels of cellular RNA (Moller et al., 1995). While this has been demonstrated for several organisms (Klappenbach et al., 2000), RNA-based analysis of activity may be complicated by slow turnover of RNA following starvation (Flardh et al., 1992). The similarity of bacterial, archaeal and fungal RNA-DGGE profiles from $^{12}$C- and $^{13}$C-labelled RNA extracted from limed suggests that RNA-DGGE provides a good indication of active communities under these conditions. Differences in $^{12}$C and $^{13}$C-profiles from unlimed soils, however, suggest utilization of other sources of organic carbon. The major effect of timing will be to increase soil pH, which has been shown to influence microbial phospholipid fatty acid patterns and concentrations in rhizosphere and bulk soils (Bååth and Anderson, 2003; Söderberg et al., 2004). Microbial communities in the grassland soils examined in this study are also affected by pH (Nicol et al., 2004). Denaturing gradient gel electrophoresis banding patterns were more complex in limed than in unlimed soils, consistent with similar findings for chitinolytic (Metcalfe et al., 2002) and ammonia oxidizer (Gray et al., 2003) communities in grassland soils. This
complexity could result in faster turnover of C (Rangel-Castro et al., 2005), possibly through the combined activities of several groups within the microbial community. Stimulation of a more diverse community is likely under limed conditions as a wider range of microbes will compete for rhizosphere C flow, and the flux of readily utilisable C from grass roots is greater, with reduced soil acidity (Meharg and Killn, 1990). Sequences were determined for bacterial and fungal DGGE bands increasing in relative intensity in $^{13}$C fractions from unlimed soils. The small number of sequences obtained, and limited information on function from SSU RNA phylogeny, make interpretation of data difficult. *Myxobacterium* and *Sphingomonas* spp. can degrade polycyclic aromatic hydrocarbons, and the results may support assimilation of root exudation by organisms capable of degrading complex organic compounds (Lynch and Whipp, 1990; Bremer and Kuikman, 1994). The two fungal sequences obtained were most closely related to *Sistotrema eximum*, a resupinate homobasidiomycete belonging to the general group of wood-decaying fungi, and *R. glutinis*, heterobasidiomycete yeast. However, sequence similarities were not sufficiently high for reliable identification and prediction of ecosystem function is not possible. Although few bands were sequenced, it was surprising that no mycorrhizal fungal sequence was detected in $^{13}$C RNA, as most grassland plants are colonized by arbuscular mycorrhizal fungi (Read et al., 1976) and mycorrhizal associations are fundamental to C cycling in grasslands (Johnson et al., 2002). This may represent primer bias and future studies should employ a wider range of primer sequences for investigation of specific microbial groups.

In conclusion, this study demonstrates the applicability of SIP for analysis of assimilation of root exudates in the field and indicates the sensitivity of the currently available technology. The results demonstrate significant differences in utilization of root exudates between limed and unlimed grassland soils and provide the basis for future investigations into the role of specific microbial groups in C flow within the rhizosphere.

### Experimental procedures

#### Field site and pulse labelling

The $^{13}$CO$_2$ pulse labelling experiment was carried out at the Sourhope Field Experimental Site (NT855196, 320 a.s.l.) located in the Scottish Borders region. General information about this seminatural upland grassland can be obtained from the Soil Biodiversity Programme web site (http://soilbio.nerc.ac.uk/Sourhope.htm). The experimental site is divided into five replicate blocks, each subdivided into six plots to which different treatments have been applied. Four blocks were used as replicates and two plots corresponding to unlimed and limed (144 kg CaCO$_3$ per plot) treatments were selected from each block. On 6 June 2003 acrylic labeling chambers, diameter 400 mm, were used to pulse $^{13}$CO$_2$ (up to 99% atom% $^{13}$C) to vegetation patches in each block for a period of 6 h, as described previously (Ostle et al., 2000; Rangel-Castro et al., 2004). CO$_2$ concentrations inside the chambers were maintained at 360–400 p.p.m. during the pulse labelling period.

#### Sampling and sample processing

Soil cores (5 cm diameter × 15 cm depth) were removed from each plot immediately after pulse labelling and after a further 3, 6, 12, 24 h and 5 days. Samples were immediately frozen, transported to the laboratory and stored at −80 °C until further processing.

#### $^{13}$C analysis of extracted nucleic acids

Approximately 5 g of soil was carefully separated from roots from each soil core and nucleic acids were extracted according to Griffiths and colleagues (2000). RNA and DNA were separated and cleaned using the Qiagen RNA/DNA kit (QIAGEN, West Sussex UK) following the manufacturer's protocol. Concentrations and purity of RNA extractions were determined spectrophotometrically (Eppendorf Biophotometer, Cambridge UK) and 1 μg of RNA was cut with 61.6 μg of glucose, freeze dried in 6 × 4 mm tin cups (Elemental Microanalysis, Okehampton UK). $^{13}$C was analysed (Manefield et al., 2002b) by isotope ratio mass spectrometry (IRMS) at the Scottish Crop Research Institute (SCRI) using the equation:

\[
\delta^{13}C \text{(in } \%e) = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000,
\]

where $R = ^{13}C/^{12}C$, the isotopic ratio for the material (i.e. RNA sample and standard material). The $R_{\text{standard}}$ was Pee Dee Belemnite (Wang and Hsieh, 2002).

Remaining material was stored at −80 °C for no longer than 2 weeks before isopycnic ultracentrifugation.

#### Density gradient separation and fractionation of labelled and non-labelled RNA

Density gradient solutions of CsTFA (Amersham Biosciences) were used to separate $^{13}$C-labelled and non-labelled RNA from unlimed and limed soil samples by isopycnic ultracentrifugation using a modified version of the method described by Manefield and colleagues (2002b). Aliquots containing 500 μg of RNA were transferred to Polyallomer Bell-top Quick-Seal centrifuge tubes (11 × 32 mm; Beckman Coulter, Fullerton, CA) and centrifuged (Beckman TL-100 Ultracentrifuge) in a vertical rotor (Beckman TLV100) at 150 000 g for 40 h at 20 °C. Tubes were fractionated using a Fraction Recovery System (Beckman) by displacement with water and 12 fractions were collected in sterile Eppendorf tubes (0.5 ml) each containing approximately 85 μl of the density gradient. RNA was precipitated by washing each fraction twice with 100 μl of ice-cold isopropanol. Recovered RNA samples were re-suspended in 20 μl of RNase free water and stored at −80 °C. Analysis of $\delta^{13}C$ by IRMS (as
Reverse transcription reactions were performed by addition of reverse transcriptase (Invitrogen, Paisley, UK) using reverse primers 1492r (Lane, 1991) and EF3 (Smit et al., 1999) to target fungal 18S rRNA genes. Reverse transcription reactions were performed by addition of 4 µl 20 pmol reverse primer and 0.4 µl 25 mM dNTP to 10 µl of RNA template. Samples were first incubated at 65°C for 5 min and then transferred to ice for 2 min. After addition of 4 µl of 5× First-Strand Buffer and 2 µl 0.1 M dithiothreitol (DTT), samples were incubated at 52°C for 2 min. One microlitre of the reverse transcriptase was then added and tubes were incubated at 52°C for 50 min. Reverse transcriptase activity was inactivated by heating at 70°C for 15 min. Two negative controls were performed with all reactions [no template (water only) and template but no RT enzyme]. The resulting cDNA was amplified using a nested PCR approach with subsequent analysis by DGGE. Polymerase chain reaction amplifications consisted of 50 µl of reactions with 2 mM MgCl₂, 1 mM dNTPs and 0.4 µM each primer. The first PCR amplification step was performed using the forward and reverse primers 27f (Lane, 1991) and 1492r (Muyzer et al., 1988) targeting bacteria, rSAf (Nicol et al., 1987) targeting fungi (White et al., 1990; Smit et al., 1999) targeting archaea (Nicol et al., 2004) and SSU 0817 and NS4 targeting fungi (White et al., 1990; Smit et al., 1999). Polymerase chain reaction amplification conditions are presented in Table 3.

<table>
<thead>
<tr>
<th>Domain</th>
<th>PCR round</th>
<th>PCR amplification conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>1</td>
<td>95°C at 5 min 94°C at 30 s, 55°C at 30 s, 72°C at 1 min for 35 cycles 72°C at 10 min</td>
</tr>
<tr>
<td>Fungi</td>
<td>2</td>
<td>95°C at 5 min 94°C at 30 s, 55°C at 30 s, 72°C at 1 min for 35 cycles 72°C at 10 min</td>
</tr>
<tr>
<td>Archaea</td>
<td>1</td>
<td>95°C at 5 min 94°C at 30 s, 55°C at 30 s, 72°C at 1 min for five cycles 92°C at 30 s, 55°C at 30 s, 72°C at 1 min for 30 cycles 72°C at 10 min</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>95°C at 5 min; followed by five cycles of 94°C at 30 s, 63°C at 30 s, 72°C at 1 min; followed by 30 cycles of 92°C at 30 s, 63°C at 30 s, 72°C at 1 min; followed by 72°C at 10 min</td>
</tr>
</tbody>
</table>

DGGE analysis

Denaturing gradient gel electrophoresis analyses of PCR products from each fraction were performed using a DCode universal mutation detection system (Bio-Rad Laboratories, Hertfordshire UK). Polyacrylamide gels with 30–60%, 45–70% and 10–50% denaturing gradients were used to separate 13C- and 12C-enriched SSU sequences for bacteria, archaea and fungi respectively. Gels were run for 16 h and 75 V at 60°C and were silver-stained (McCaig et al., 2001) for visual comparison of banding patterns among the fractions from each single CsTFA gradient density.

Cloning, screening and sequencing of clones

Products from first-round PCR amplification of lower fractions representing 13C-labelled regions of the CsTFA gradient densities were cloned using the pGEM®-T Easy Vector System I (Promega, Southampton, UK) in E. coli XL1-Blue MRF™ Kan supercompetent cells (Stratagene, La Jolla, CA). Transformed colonies with the correct insert were subjected to PCR amplification using the group-specific primers and amplification products were screened using DGGE as described above. Three clones containing inserts of bacterial and fungal origin, corresponding to DGGE bands with migration patterns identical to bands from environmental samples, were PCR-amplified using the M13f and M13r primers. The M13 products were then sequenced in both directions using the corresponding bacterial and fungal primers and the BigDye terminator sequencing kit (PE Biosystems, Foster City, CA). Sequences were analysed using an ABI 377 automated sequencer (PE Biosystems, Foster City, CA). The partial SSU rRNA gene sequences of clones that gave the same banding pattern were aligned using the program CLUSTAL W (http://www.ebi.ac.uk/clustalw/) and closest related sequences were identified by a BLASTN search (Altschul et al., 1990) of the GenBank database.

Table 3. Polymerase chain reaction conditions for first and second round amplifications targeting SSU RNA of bacteria, fungi and archaea.
Optimization and testing of stable isotope probing (SIP) procedure

Stable isotope probing analysis was carried using RNA extracted from \( ^{13}\text{C}- \)labelled \( E.\ coli \) (strain K12 10218 NCIMB) and \( P.\ fluorescens \) (strain 8146 NCIMB) cells to determine the location of \( ^{12}\text{C} \) and \( ^{13}\text{C} \) RNA following centrifugation and to determine the lower limit for detection of \( ^{13}\text{C}- \)labelled cells in the system used. Cells of both strains were grown in liquid Minimal Medium 9 (Maniatis et al., 1982) containing \( ^{13}\text{C} \)-labelled glucose, fully labelled (CK Gas Products, Hampshire UK) as the sole carbon source, such that the majority of cells were \( ^{13}\text{C} \)-labelled. Cells were incubated overnight at 37°C, harvested by centrifugation at 9300 g for 10 min and total cell concentration was determined using a Thoma chamber. Labelled cells were then inoculated into soil to give final cell concentrations in the range \( 10^3 \)–\( 10^9 \) cells per gram of soil. Nucleic acids were extracted within 30 min of inoculation and \( ^{12}\text{C} \)- and \( ^{13}\text{C} \)-labelled RNA were separated and centrifuged as described above. 16S rRNA genes were RT-PCR amplified using bacteria-specific primers from samples taken from fractions along the \( ^{12}\text{C} \)–\( ^{13}\text{C} \) gradient and amplification products were analysed by DGGE. The location of \( ^{12}\text{C} \) and \( ^{13}\text{C} \) RNA after centrifugation in these samples could be clearly detected by ethidium bromide staining of agarose gels and silver staining of DGGE. However, the lower levels of \( ^{13}\text{C} \) RNA extracted from field samples necessitated analysis of fractions along the expected \( ^{12}\text{C} \)–\( ^{13}\text{C} \) gradient to assess both the success of the procedure and to provide greater confidence in discriminating communities incorporating the label. A total of 14 fractions were obtained but extreme fractions gave spuruous results with random and inconsistent PCR amplification, presumably of low-level contaminants in these fractions. For instance, DGGE patterns of the lowest fractions, 1 and 2, sometimes contained bands that corresponded to sequences rich in GC content, rather than \( ^{13}\text{C} \)-labelled bands. In contrast, fractions from the top of the gradient (fractions 11 and 12) presented the same banding patterns as \( ^{12}\text{C} \) RNA sequences, occasionally with reduced band intensity.

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References


