Medium-term response of microbial community to rhizodeposits of white clover and ryegrass and tracing of active processes induced by $^{13}$C and $^{15}$N labelled exudates

Gedrime Kusliene a, b, *, Jim Rasmussen a, Yakov Kuzyakov b, c, Jørgen Eriksen a

a Department of Agroecology, Faculty of Science and Technology, Aarhus University, Post Box 50, 8830 Tjele, Denmark
b Dept. of Soil Science of Temperate Ecosystems, University of Göttingen, Germany
c Dept. of Agricultural Soil Science, University of Göttingen, Germany

Article info
Article history:
Received 12 December 2013
Received in revised form 30 April 2014
Accepted 3 May 2014
Available online 16 May 2014

Keywords:
Root exudates
Rhizosphere processes
Plant--microbial interactions
Labelling approaches
Rhizodeposition

Abstract
Rhizodeposition affects the microbial community in the rhizosphere, and microbial composition and activity may therefore differ in soil depending on plant species. We hypothesised that these differences increase over the plant growth period because roots occupy larger soil volumes and release more rhizodeposits. We tested how such medium-term responses of the microbial community can be explained by the short-term utilisation of root exudates. To test this we analysed $^{15}$N incorporation into microbial biomass, phospholipid fatty acid (PLFA) composition and $^{13}$C incorporation into the PLFAs of specific microbial groups in soil under white clover (Trifolium repens L.) and ryegrass (Lolium perenne L.) following leaf-labelling with $^{13}$C-bicarbonate and $^{15}$N-urea. In this way microbial N and $^{15}$N and the composition of PLFAs reflect the medium-term (two months) response of microorganisms to rhizodeposits, whereas the $^{13}$C-label of the PLFAs reflects the short-term (one week) utilisation of root exudates following labelling of shoots. In the medium term, microbial biomass N and $^{15}$N were greater under the ryegrass, whereas total PLFA was higher under white clover. The relative abundance of fungi and actinomycetes was unaffected by plant species, but pool of Gram-negative and Gram-positive bacteria was greater under white clover at the 10 percent significance level. In the short term, microorganisms more actively utilised fresh exudates ($^{13}$C-labelled) of ryegrass than of white clover. We expected ryegrass exudates initially to be incorporated into bacterial PLFA and into fungi over time, but surprisingly fungi had the highest utilisation of ryegrass-derived C over the week. At 0–5 cm soil depth, white clover exudates were utilised only by bacteria, whereas fungi dominated at 5–15 cm. This reflects differences in the quality of white clover exudates or differences in the microbial community composition at the two depths. We conclude that despite clear short-term differences in microbial response to the exudates of white clover and ryegrass, this is only to a limited extent transferred into medium-term defects on the composition of the microbial communities under the two plant species. Hence, our study showed that different short-term C utilisation patterns may lead to similar medium-term responses of the microbial community.

1. Introduction

Improving the use efficiency of legume-derived N is a means of improving the sustainability of agricultural production. The ability of legumes to biologically fix atmospheric N and hence improve soil fertility via rhizodeposition (Høgh-Jensen and Schjoerring, 2000) is widely recognised. Of the forage legumes, white clover is one of the most commonly used in temperate grasslands. However, the knowledge of root--microbial interactions in white-clover-based grasslands is particularly limited.

The main input of N and C to the rhizosphere is via functional (excretions, secretions) and non-functional (diffusates, root debris) rhizodeposition in the form of carbohydrates, amino acids, fatty acids, enzymes, proteins, etc. (Badri et al., 2009). Root effects on the microbial community may differ in the short and medium term since rhizodeposits may alter bacterial and fungal communities (Jones et al., 1998; Zak et al., 2000) as well as change their activities (Blagodatskaya et al., 2009). Microbial communities play a key role in nutrient transformation and storage in the rhizosphere (Petersen et al., 2002) and in the mobilisation and/or mineralisation of soil...
organic matter (SOM) (Churchland et al., 2012). Short-term shifts in the activity of fungi and bacteria in the rhizosphere compared to the root-free soil are related to the quality (C/N ratio, lignin content, recalcitrance) (Grayston et al., 2001; Bai et al., 2012), diversity (Paterson et al., 2008) and quantity (De Vries et al., 2006) of exudates. Bacteria actively respond to low molecular weight compounds with a low C/N ratio (Ding et al., 2011) and show fast response to fresh exudates (Bell et al., 2003; Dungait et al., 2011). Fungi have the ability to decompose both easily-degradable substrates (De Graaff et al., 2010) and more recalcitrant compounds (Poll et al., 2008; Bai et al., 2012). However, high inputs of mineral N (Bardgett et al., 1996) at low levels of soil organic carbon (Petersen et al., 2002) negatively affect the fungal community. Previous studies show that the composition and activity of the microbial biomass vary depending on management (Clegg et al., 2003; Haubert et al., 2009; Bird et al., 2011), grassland plant species composition (Bardgett et al., 2003; Butler et al., 2003), grazing intensity (Hafner et al., 2012), water regime (Tian et al., 2013), root density (Helal and Sauerbeck, 1986), and soil depth (Petersen et al., 2002; Zhang et al., 2012). However, it is not clear whether short-term (hours to days) effects on microbial activities persist over time and structure the microbial community in the medium term (hours to days). Therefore, we investigated, how the utilisation of root exudates over the short term corresponds to the medium-term composition of the microbial community.

Biomarker techniques are powerful tools to investigate microbial groups and hence get insight into root–microbial interactions (Ding et al., 2011). Since phospholipid fatty acids (PLFAs) are components of living cells and rapidly decompose after cell death, they are useful biomarkers of living microbial groups (Fry, 2006). PLFA biomarkers reflect the composition of microbial groups including fungi, Gram-positive and Gram-negative bacteria, and actinomycetes (Abraham et al., 1998; Fang et al., 2001; Treonis et al., 2004).

Stable dual 13C/15N labelling in combination with analysis of biomarkers of soil microorganisms enables estimations of the contribution of various types of root exudates to C and N dynamics in soil. The 13C analyses of PLFAs have been used to investigate: 1) linkages between plants and microorganisms (Dungait et al., 2011) and 2) the effects of individual substances on microorganisms (Dungait et al., 2011; Apostle et al., 2013). However, previous investigations with, for example, sieved soil (harming the fungal hyphae) may not fully reflect in-situ conditions. Therefore, we investigated in our study the plant–microbial interactions under undisturbed field conditions.

The objective of the study was to examine the incorporation of 15N in the microbial community and the 13C uptake by microbial groups through analysis of PLFA composition in soil under white clover and ryegrass leaf-labelled with 15N and 13C. As root–microbial interactions are highly dynamic (Bardgett et al., 2005), we investigated N and C dynamics over a timescale of days to weeks.

This study examined the medium-term (two months) effects of rhizodeposits of white clover and ryegrass leaf-labelled with 15N and 13C. As root–microbial interactions are highly dynamic, we investigated N and C dynamics over a timescale of days to weeks.

This study examined the medium-term (two months) effects of rhizodeposits of white clover and ryegrass leaf-labelled with 15N and 13C. As root–microbial interactions are highly dynamic, we investigated N and C dynamics over a timescale of days to weeks.

2. Materials and methods

2.1. Experimental site and conditions

The experiment was conducted on a sandy loam at Foulumgård Experimental Station, Viborg, Denmark (55°28′N, 09°07′E). Since 1987 the site has been under intensive dairy farming with grassland-arable crop rotations (Eriksen et al., 1999, 2014). The soil is classified as a typic hapladult with 6.4% clay, 8.5% silt, 44% fine sand and 39% coarse sand. Soil contained 1.8% C at 0–5 cm and 1.6% C at 5–15 cm, and the N content was 0.18% at 0–5 cm and 0.15% at 5–15 cm. The field experiment was established in the beginning of April 2012 by installing 8 cm diameter and 20 cm high PVC cylinders (90 in total) in 2nd year perennial ryegrass (Lolium perenne L., 28 kg ha–1) and white clover (Trifolium repens L., 6 kg ha–1) pure stands in a randomised block design with four replicates of each plant type for each sampling date. The rainfall was 28 mm and 84 mm in May and June 2012, respectively, and the mean temperature was 12 °C. The mean annual temperature was 8 °C and precipitation 738 mm in 2012.

2.2. Leaf 15N/13C labelling

Leaf labelling with 15N-urea (99.6 atom%15N, 0.5% w/v) (Høgh-Jensen and Schjoerring, 2000) and Na215CO3 (99.9%, 0.01 M) (Rasmussen et al., 2013a) was conducted with five tubes per cylinder. White clover or ryegrass leaves were inserted into a 2-ml tube filled with 1 ml of the labelling solution and sealed with an inert plastic material (UNIGUM Sanitary putty, Unipak A/S, Gøhlen, Denmark) to avoid 15N/13C losses (Høgh-Jensen and Schjoerring, 2000). Labelling was started in the morning on May 1st and continued for 48 h. After the labelling was terminated, labelled leaves were wiped off with a paper towel to avoid soil contamination with 15N/13C after the tubes were removed. We assumed that more than 90% of the labelling solution was taken up by plants (Rasmussen et al., 2007).

2.3. Sampling times and initial sample preparation

Sampling of the soil-plant system was done 2 days before the labelling and 0 h, 1 d, 2 d, 4 d, 7 d, 14 d, 28 d and 2 months after labelling was terminated. At sampling, cylinders were excavated and placed in a cooling box and immediately transported to the lab and placed in a room at 2 °C. Samples were processed within 4 h after the excavation and within half an hour of the removal from the cylinder. First, plant shoots were cut at the soil surface and the soil removed from the cylinder. The soil core was then sliced at two depths to give 0–5 cm and 5–15 layers. The soil/root sample was taken and divided into four subsamples for the following analyses: water content (oven-dried at 105 °C), 15N/13C bulk analyses (oven-dried at 60 °C), 13C-PLFA (frozen at –18 °C)
and $^{15}$N microbial biomass (MB) (chloroform fumigation-extraction).

### 2.4. Analyses

#### 2.4.1. Bulk $^{15}$N/$^{13}$C soil and plant analyses

Plant shoots (labelled leaves included), roots, and soil sub-samples were ground to a fine powder in a ball-mill for total C and N content and $^{15}$N/$^{13}$C analysis by a PDZ Europa ANCA-GSL elemental analyser interfaced to a PDZ Europe 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) at the UC Davis Stable Isotope Facility (UC Davis, CA, USA).

#### 2.4.2. Microbial biomass

Chloroform fumigation extraction according to Brookes et al., (1985) was performed to assess soil microbial biomass. Fumigated and non-fumigated samples were extracted with ultrapure water instead of K$_2$SO$_4$ in order to avoid salty residues that interfere with stable isotope analyses (Nordin et al., 2004) and analysed for total dissolved N (TDN) and $^{15}$N enrichment. Briefly, analyses for TDN were performed by adding 7.5 ml persulfate reagent and autoclaving at 121°C for 30 min in order to oxidise organic N to inorganic N. To this was added 7.4 g of KCl, 0.2 g MgO and 0.4 g Devardas reagent. A fibreglass filter trap was acidified by adding 20 μl of 1.5 M H$_2$SO$_4$ packed in Teflon paper and placed in a bottle with the sample (Solorzano and Sharp, 1980; Sørensen and Jensen, 1991). The diffusion was processed for five days to ensure complete reduction of NO$_3$ to NH$_4$. The glass filter traps were subsequently dried and packed into tin capsules for $^{15}$N and N measurement. Nitrogen content and $^{15}$N enrichment were determined as described above.

#### 2.4.3. PLFA extraction and analysis

PLFA content was determined for soil sampled on days 0, 1, 2, and 7 after labelling. PLFA extraction followed the modified procedure by Petersen and Klug (1994). Briefly, 2 g freeze-dried soil sample was extracted for 2 h with a phosphate:methanol:chloroform (0.8:2:1) buffer. This was followed by phase separation by adding chloroform and phosphate buffer (1:1 v:v), shaking, and leaving the solution overnight. Following centrifugation (15 min, 1500 × g), the organic phase was separated and evaporated under N$_2$ at 37°C. PLFA was eluted with methanol by solid-phase extraction columns (Bond Elut 1CC LRC-Si, Agilent Tech, USA) and evaporated under N$_2$ at 37°C. Nonadecanoic acid methyl ester (19:0) and tridecanoic acid methyl ester (13:0) were used as internal standards. PLFA extracts were diluted in 200 μL of hexane and analysed via gas-chromatography-combustion-isotopic ratio mass spectrometry (GC-c-IRMS) using a GC (Trace GC Ultra, Thermo Scientific, Bremen, Germany) containing a fused silica column (Agilent HP-5MS, 0.25 mm id × 60 m, film thickness 0.25 μm) with helium as carrier gas (1.5 mL/min). The GC was coupled via a GC combustion interface (GC-Isolink, Thermo Scientific, Bremen, Germany) in continuous flow mode to a Thermo Scientific Delta V Advantage isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany) at Department of Biology, Lund University, Sweden.

#### 2.5. Calculations and statistics

##### 2.5.1. Microbial biomass

Microbial biomass N content (MB-N) was MB-N/$\Delta$N, where MB-N = (N extracted from fumigated samples) – (N extracted from non-fumigated samples) and $\Delta$N = 0.45 (Nordin et al., 2004). Microbial biomass $^{15}$N content was calculated as the difference in the $^{15}$N content between fumigated and non-fumigated samples:

$$^{15}$N$_{\text{MB}} = \left( \frac{^{15}N_{\text{fum}} \times N_{\text{fum}} - ^{15}N_{\text{unfum}} \times N_{\text{unfum}}}{K_{\Delta N}} \right) - \left( \frac{^{15}N_{\text{fum}} \times N_{\text{fum}} - ^{15}N_{\text{unfum}} \times N_{\text{unfum}}}{K_{\Delta N}} \right)$$

where $^{15}$N$_{\text{MB}}$ denotes microbial biomass $^{15}$N content, $^{15}N_{\text{fum}}$ and $^{15}N_{\text{unfum}}$ are the $^{15}$N enrichment (at%) of, respectively, fumigated labelled and unlabelled (control) samples, $^{15}N_{\text{fum}}$ and $^{15}N_{\text{unfum}}$ are the $^{15}$N enrichment (at%) of, respectively, unfumigated labelled and unlabelled (control) samples and $K_{\Delta N} = 0.45$ (Nordin et al., 2004).

##### 2.5.2. PLFA

The PLFAs detected at a concentration of less than 0.4 mol% were excluded from the data set. $^{31}$C values were expressed against Vienna PeeDee Belemnite (VPDB). FAMEs were identified by relative retention time comparing samples with an FAME standard mix (BAME mix supelco, 47885-U, Sigma Aldrich, Stockholm, Sweden). In order to avoid the risk of co-elution, PLFAs that were included in our calculations had baseline separated peaks. PLFA concentrations were calculated relative to the concentration of the internal standard C19:0. Raw $^{31}$C values were corrected by calibration curves from external standards with known $^{31}$C values: acetylilide, caffeine, C16:0, C18:0, C20:0. All $^{31}$C values were corrected for the additional C by derivatisation:

$$^{31}$C = \left( \frac{^{31}$C_{\text{FAME}}}{^{31}$C_{\text{MeOH}}} \right)$$

where $^{31}$C$_{\text{FAME}}$ is the measured $^{31}$C value of the FAME after methylation and $^{31}$C$_{\text{MeOH}}$ is the $^{31}$C value for the methanol used for derivatisation (−37.7$_{\text{meas}}$ ± 3.23$_{\text{meas}}$). $^{31}$C atom% excess in individual PLFAs was calculated by subtracting the $^{13}$C excess of unlabelled PLFA. PLFA nomenclature was used as described earlier (Petersen et al., 2002). Table 1 lists the PLFAs that were detected and used for further analyses.

#### 3. Results

##### 3.1. Plant biomass and $^{15}$N and $^{13}$C enrichment

Rye grass had higher ($P < 0.001$) root biomass than white clover (Table 2). Rye grass and white clover shoot biomass yields were similar but higher than generally reported at this site (Eriksen et al., 2012).

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Name</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>Gram positive</td>
<td>i15:0, i15:0, i16:0, i17:0, i17:0</td>
</tr>
<tr>
<td>Gram negative</td>
<td>16:i0&lt;7c, 18:i0&lt;7c, 18:i0&lt;7c, cy19</td>
<td>Stromberger et al., 2012</td>
</tr>
<tr>
<td>Non-specific</td>
<td>14:0, 15:0, 16:i0&lt;5c, 16:0</td>
<td>Bird et al., 2011</td>
</tr>
<tr>
<td>Actinomycetes</td>
<td>10Me16:0, 10Me18:0</td>
<td>Frostegård and Bååth, 1996</td>
</tr>
<tr>
<td>Fungi</td>
<td>18:2&lt;69</td>
<td>Stromberger et al., 2012</td>
</tr>
</tbody>
</table>
because stubble was included. Nitrogen accumulation was significantly higher \((P < 0.001)\) in white clover, whereas C yields were similar in both plants, resulting in a higher C/N ratio in ryegrass. The atmospheric nitrogen fixation by white clover pure stands was determined using the \(15\text{N}\) dilution method (Høgh-Jensen and Schjoerring, 2000) in adjacent plots. The nitrogen derived from the atmosphere (Ndfa) amounted 80% of N in white clover.

Leaf \(15\text{N}/^{13}\text{C}\) dual labelling led to the expected \(15\text{N}\) and \(^{13}\text{C}\) enrichment of shoots and roots (Fig. 1). Ryegrass shoots and roots at 5–15 cm had a higher \((P < 0.001)\) \(15\text{N}\) enrichment than white clover, whereas no significant difference was observed between the roots of both species at 0–5 cm. The \(15\text{N}\) enrichment of white clover roots decreased with soil depth which was the opposite to ryegrass. White clover shoots and roots had significantly higher \((P < 0.001)\) \(^{13}\text{C}\) enrichment than ryegrass. The \(^{13}\text{C}\) enrichment of white clover roots was the same at both depths, whereas ryegrass roots at 5–15 cm had higher \(^{13}\text{C}\) enrichment than at 0–5 cm \((P < 0.001)\).

### 3.2. Soil \(^{15}\text{N}\) and \(^{13}\text{C}\) enrichment

Although \(^{15}\text{N}\) and \(^{13}\text{C}\) enrichment in the soil was low, differences were observed between white clover and ryegrass (Fig. 2). Under ryegrass at 0–5 cm depth, soil \(^{15}\text{N}\) enrichment was highest on day 7 and slightly decreased thereafter, whereas under white clover it increased until day 56. Soil \(^{13}\text{C}\) enrichment at 5–15 cm depth was similar for both species. Soil \(^{13}\text{C}\) enrichment was significantly higher \((P < 0.001)\) under ryegrass than white clover at 0–5 cm depth. The \(^{13}\text{C}\) enrichment at 5–15 cm was higher in white clover soil in the first few days of the experiment, whereas it was highest under ryegrass later on.

### 3.3. Medium-term effect of sward type on the microbial biomass and functional groups

Medium-term effects of sward type were evaluated from the differences developed during the lifetime of the one-year-old

<table>
<thead>
<tr>
<th>Plant characteristics.a</th>
<th>White clover</th>
<th>Ryegrass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot Biomass [kg/m²]</td>
<td>1.95 ± 0.09</td>
<td>1.54 ± 0.04</td>
</tr>
<tr>
<td>Shoot N Yield [g/m²]</td>
<td>63 ± 4</td>
<td>44 ± 2</td>
</tr>
<tr>
<td>Shoot C/N</td>
<td>12 ± 1</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>Shoot 15N/13C enrich.</td>
<td>2.1 ± 0.13</td>
<td>1.5 ± 0.12</td>
</tr>
<tr>
<td>Shoot 15N/13C enrich.</td>
<td>33 ± 2</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>Shoot 15N/13C enrich.</td>
<td>26 ± 2</td>
<td>41 ± 2</td>
</tr>
<tr>
<td>Root 0–5 cm Biomass [kg/m²]</td>
<td>1.35 ± 0.01</td>
<td>1.35 ± 0.01</td>
</tr>
<tr>
<td>Root 0–5 cm N Yield [g/m²]</td>
<td>39 ± 2</td>
<td>39 ± 2</td>
</tr>
<tr>
<td>Root 0–5 cm C/N</td>
<td>13 ± 1</td>
<td>13 ± 1</td>
</tr>
<tr>
<td>Root 0–5 cm 15N/13C enrich.</td>
<td>1.46 ± 0.03</td>
<td>1.46 ± 0.03</td>
</tr>
<tr>
<td>Root 0–5 cm 15N/13C enrich.</td>
<td>17 ± 1</td>
<td>17 ± 1</td>
</tr>
<tr>
<td>Root 0–5 cm 15N/13C enrich.</td>
<td>34 ± 3</td>
<td>34 ± 3</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SE (standard error) from 32 replications.

---

![Fig. 1. \(^{15}\text{N}\) excess (at.%) and \(^{13}\text{C}\) of plant shoots and roots as a function of length of time after labelling (mean ± SE, \(n = 4\)).](image-url)
swards of white clover and ryegrass. The size of the microbial N pool (MB-N) was larger ($P < 0.05$) in ryegrass soil than under white clover at both depths (Fig. 3). Furthermore, it was significantly larger ($P < 0.001$) in the uppermost layer under both plant species (Fig. 3) compared to deeper down. Plant-derived N was incorporated into microbial biomass under both swards. MB-$^{15}$N in soil under ryegrass was at both depths higher ($P < 0.01$) than in soil under white clover (Fig. 3).

![Fig. 2. $^{15}$N excess and $^{13}$C in bulk soil under ryegrass and clover pure stands (mean ± SE, n = 4).](image)

![Fig. 3. Microbial biomass N (average of eight samplings) (mean ± SE, n = 32) and $^{15}$N content under ryegrass and clover pure stands at 0–5 cm and 5–15 cm depth (mean ± SE, n = 4).](image)
Soil samples taken on days 0, 1, 2 and 7 after labelling were analysed for individual PLFA contents. The concentration of total PLFA (sum of 17 PLFAs) varied from 30 to 50 nmol g⁻¹ soil and was significantly higher in the 0–5 cm than the 5–15 cm layer (P < 0.001) for both species (Fig. 4). Furthermore, the total PLFA content was higher (P = 0.09) under white clover than under ryegrass. PLFAs of non-specific (16:0) and Gram-positive bacteria (i15:0, a15:0) were the most abundant in soil under ryegrass and white clover at both depths (Table 3). The PLFAs were dominated by bacteria (approx. 90%), with Gram-negative bacteria being most abundant, while fungi and actinomycetes accounted for around 10% under both plants. The relative abundance of bacteria, fungi and actinomycetes was unaffected by species, but Gram-negative bacterial PLFA (P = 0.07) and Gram-positive bacterial PLFA (P = 0.09) were higher under white clover than under ryegrass and for both species were significantly higher at 0–5 cm than at 5–15 cm (P < 0.001 and P < 0.01, respectively, Fig. 4). Thus, in the medium-term, the microbial biomass under ryegrass had a larger incorporation of N and ¹⁵N than under white clover, whereas total PLFA was higher under white clover than under ryegrass due to a greater bacterial PLFA.

3.4. Utilisation of recent assimilates by microbial groups

Plant-derived ¹³C was incorporated into 17 individual PLFAs (Table 4). In ryegrass soil at 0–5 cm depth, the highest ¹³C enrichment (more than 10 mol% on mean values) was observed in the PLFAs of non-specific bacteria (16:0) and fungi (18:2ω6:9), while PLFAs of Gram-positive bacteria (i15:0, a15:0) showed the highest ¹³C enrichment at 5–15 cm. White-clover-derived ¹³C was mostly incorporated into PLFAs of Gram-positive bacteria (a15:0, non-specific bacteria (16:0) and actinomycetes (10Me16:0) at 0–5 cm depth, while PLFAs of actinomycetes (10Me16:0), fungi (18:2ω6:9) and cyclopropyl bacteria (cy19:0) had the highest ¹³C enrichment at 5–15 cm depth.

The ¹³C-labelled exudates under ryegrass and white clover were incorporated into different groups of microorganisms (Fig. 5). The ¹³C incorporation into Gram-positive and Gram-negative bacterial and fungal PLFAs was affected by length of time since labelling (P < 0.001), species and depth (P < 0.01). The incorporation into actinomycetes was found to be unaffected by any of those factors. The specific activity of fungi in the uptake of ¹³C exudates was higher under ryegrass than white clover (Fig. 6). Bacteria (Gram-positive and Gram-negative) and actinomycetes were less active and showed increasing ¹³C incorporation over time. White clover exudates at 0–5 cm were primarily incorporated into Gram-positive and Gram-negative bacterial PLFA, while no ¹³C enrichment of fungal PLFA was detected under white clover. Overall, the greater short-term activity of fungi under ryegrass than white clover did not translate into medium-term differences in fungal PLFA under the two species. In contrast, the difference in short-term bacterial activity under white clover compared to ryegrass did result in a greater medium-term bacterial biomass under white clover than under ryegrass.

4. Discussion

To our knowledge, this is the first in-situ study where the ¹⁵N/¹³C leaf-labelling technique has been followed by compound-specific ¹³C-PLFA analysis to investigate microbial utilisation of root exudates under white clover and ryegrass. The ¹³C leaf-labelling with bicarbonate (Rasmussen et al., 2013a) resulted in plants (shoots and roots) and bulk soil being ¹³C enriched already after two days, similar to other studies with ¹³CO₂ or ¹⁴CO₂-labelling (Dilkes et al., 2004). This indicates that the ¹³C introduced to plants via bicarbonate leaf-labelling is allocated similarly to the ¹³C fixed via photosynthesis (¹³CO₂-labelling). With the leaf-labelling technique there is a risk of ¹⁵N/¹³C entering the soil from leaf decay (Dahlin

![Fig. 4](image_url). The content of total PLFA (average of four samplings) (mean ± SE, n = 16) and functional groups (Gram-positive and Gram-negative bacteria, actinomycetes and fungi) under ryegrass and white clover pure stands at 0–5 and 5–15 cm depth (mean ± SE, n = 4).
Table 3
Mole percentage distribution of PLFAs under the ryegrass and white clover at 0
and 5 cm depth (mol%).

<table>
<thead>
<tr>
<th>Species</th>
<th>Depth 14:0</th>
<th>i15:0</th>
<th>a15:0</th>
<th>15:0</th>
<th>i16:0</th>
<th>16:1 uc</th>
<th>16:1 uc</th>
<th>16:0</th>
<th>10Me16:0</th>
<th>i17:0</th>
<th>a17:0</th>
<th>18:2 6,9</th>
<th>18:1 uc</th>
<th>18:1 uc</th>
<th>10Me18:0</th>
<th>cy19:0</th>
<th>20:0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ryegrass 0 cm</td>
<td>1.35</td>
<td>11</td>
<td>8</td>
<td>0.90</td>
<td>4</td>
<td>4</td>
<td>8</td>
<td>18</td>
<td>2.73b</td>
<td>1.95</td>
<td>1.09</td>
<td>6</td>
<td>8</td>
<td>8</td>
<td>4</td>
<td>9b</td>
<td>3</td>
</tr>
<tr>
<td>Ryegrass 5 cm</td>
<td>1.35</td>
<td>11</td>
<td>8</td>
<td>0.90</td>
<td>4</td>
<td>4</td>
<td>8</td>
<td>20</td>
<td>3.02a</td>
<td>2</td>
<td>1.13</td>
<td>7</td>
<td>7</td>
<td>4</td>
<td>4</td>
<td>10a</td>
<td>3</td>
</tr>
<tr>
<td>Clover 0 cm</td>
<td>1.48</td>
<td>10</td>
<td>9</td>
<td>1.72</td>
<td>4</td>
<td>4</td>
<td>8</td>
<td>19</td>
<td>2b</td>
<td>1.97</td>
<td>1.22</td>
<td>4</td>
<td>10</td>
<td>9</td>
<td>4</td>
<td>7b</td>
<td>2</td>
</tr>
<tr>
<td>Clover 5 cm</td>
<td>1.35</td>
<td>11</td>
<td>9</td>
<td>0.75</td>
<td>4</td>
<td>4</td>
<td>7</td>
<td>18</td>
<td>4a</td>
<td>1.91</td>
<td>1.24</td>
<td>6</td>
<td>8</td>
<td>7</td>
<td>4</td>
<td>11a</td>
<td>3</td>
</tr>
</tbody>
</table>

Species ns ns ns ns ns ns ns ns ns ns ns ns ns ns ns ns ns ns
Depth ns ns ns ns ns ns ns ns ns * ns ns ns ns ns ** ns

Letters behind mean values indicate significant differences within depth of each species. Data are presented as mean SE (standard error) from four replications.

Microbial N and 15N enrichment in soil was higher under ryegrass than under white clover, which confirmed our first hypothesis of a larger microbial community under ryegrass. This indicates better conditions for microbial activity and utilisation of exudates under ryegrass. Higher root density and carbon-rich ryegrass exudates (Domanski et al., 2001) probably activated microbial organisms, resulting in higher N immobilisation (Schimel et al., 1989). In white clover, a higher availability of N in the rhizosphere due to the low C/N ratio of exudates (Høgh-Jensen and Schjoerring, 2001) probably resulted in a faster turnover of fresh exudates.

The 17 PLFAs detected in our study were assigned to five groups: Gram-positive and Gram-negative bacteria, non-specific bacteria, actinomycetes and fungi (Fontaine et al., 2011). To test whether our results are comparable with other studies, we grouped PLFAs into groups of Gram-positive and Gram-negative bacteria, fungi and actinomycetes as they were used in different studies. The dynamics of fungi, Gram-positive and Gram-negative bacteria and actinomycetes followed the same pattern, even though different combinations of PLFA were used. Fungal PLFA (18:2 6,9) and non-specific bacterial PLFA (16:0) also occur in plant litters. However, as soil was passed through a 2-mm sieve and visible roots removed, we expect that plant roots did not significantly affect the PLFA results (Bardgett and McAlister, 1999; Butler et al., 2003). As expected, we found a significantly larger total PLFA pool at 0–5 cm depth than at 5–15 cm, which was probably due to the higher root density in the uppermost layer resulting in more readily available substrate for microbial growth. This is also supported by the larger MB-N pools in this layer. Comparing the two plant species, there was greater relative abundance of total PLFA and Gram-positive and Gram-negative bacterial groups under white clover than under ryegrass. This contradicts previous studies where the bacterial community was mainly defined by the quality and composition of SOM (Millard and Singh, 2010). It also seems to contradict the higher presence of MB-N under ryegrass. However, the PLFA data do not directly reflect the C microbial pool, because PLFA-C was not converted to microbial biomass C. The results revealed that plant species affect total PLFA-C and MB-N in their rhizospheres. The latter may explain why these two species, when grown together, have a strong positive impact on soil N fertility.

4.2. Short-term effects of exudates on the activity of fungi and bacteria within the microbial biomass

Soil samples taken on days 0, 1, 2 and 7 after labelling were analysed for individual PLFA contents. The total 13C-PLFA content showed that microorganisms more intensively utilised labelled ryegrass exudates than white clover exudates. Fast utilisation of
Table 4
Mole percentage distribution of $^{13}$C enriched microbial PLFA under the ryegrass and white clover at 0–5 and 5–15 cm depth (mol%).

<table>
<thead>
<tr>
<th>Species</th>
<th>Depth</th>
<th>Time, d</th>
<th>14:0</th>
<th>i15:0</th>
<th>a15:0</th>
<th>a16:0</th>
<th>16:0</th>
<th>10Me16:0</th>
<th>a17:0</th>
<th>18:2ω6,9</th>
<th>18:1ω7c</th>
<th>10Me18:0</th>
<th>cy19:0</th>
<th>20:0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ryegrass</td>
<td>0–5 cm</td>
<td>0</td>
<td>3ab</td>
<td>10a</td>
<td>14a</td>
<td>0a</td>
<td>9a</td>
<td>0a</td>
<td>17a</td>
<td>6ab</td>
<td>0.6a</td>
<td>0.3a</td>
<td>15a</td>
<td>6a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>23a</td>
<td>0b</td>
<td>0b</td>
<td>7a</td>
<td>7a</td>
<td>0a</td>
<td>0a</td>
<td>0b</td>
<td>18a</td>
<td>1.2a</td>
<td>20a</td>
<td>0a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>1.8b</td>
<td>9a</td>
<td>10a</td>
<td>0a</td>
<td>7a</td>
<td>5a</td>
<td>0.7a</td>
<td>19a</td>
<td>0.3b</td>
<td>0a</td>
<td>0a</td>
<td>15a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>0.6b</td>
<td>8a</td>
<td>9ab</td>
<td>0a</td>
<td>6a</td>
<td>0a</td>
<td>0a</td>
<td>9ab</td>
<td>1.2b</td>
<td>0a</td>
<td>0a</td>
<td>41a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0–15 cm</td>
<td>0.3a</td>
<td>8a</td>
<td>9a</td>
<td>6a</td>
<td>6a</td>
<td>0a</td>
<td>0a</td>
<td>21a</td>
<td>0.3b</td>
<td>0a</td>
<td>0a</td>
<td>13a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>1.3a</td>
<td>0b</td>
<td>0b</td>
<td>10a</td>
<td>6a</td>
<td>0a</td>
<td>0b</td>
<td>0b</td>
<td>44a</td>
<td>4a</td>
<td>6a</td>
<td>29a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0.5a</td>
<td>13a</td>
<td>8a</td>
<td>0a</td>
<td>6a</td>
<td>2a</td>
<td>5a</td>
<td>8ab</td>
<td>0.1b</td>
<td>0.6a</td>
<td>0a</td>
<td>36a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>0.1a</td>
<td>13a</td>
<td>8a</td>
<td>0.5a</td>
<td>4a</td>
<td>6a</td>
<td>5a</td>
<td>8ab</td>
<td>0.8b</td>
<td>0a</td>
<td>0a</td>
<td>32a</td>
</tr>
<tr>
<td>White clover</td>
<td>0–5 cm</td>
<td>0</td>
<td>5a</td>
<td>13a</td>
<td>21a</td>
<td>0.2a</td>
<td>0.1a</td>
<td>4a</td>
<td>7a</td>
<td>35a</td>
<td>3b</td>
<td>0a</td>
<td>0a</td>
<td>7a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>1.3a</td>
<td>0b</td>
<td>0c</td>
<td>21a</td>
<td>0.0a</td>
<td>0a</td>
<td>0a</td>
<td>0b</td>
<td>29a</td>
<td>20a</td>
<td>28a</td>
<td>0a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>3a</td>
<td>16a</td>
<td>23a</td>
<td>0.6a</td>
<td>0.7a</td>
<td>0a</td>
<td>10a</td>
<td>39a</td>
<td>0b</td>
<td>0a</td>
<td>0a</td>
<td>0a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>2a</td>
<td>8ab</td>
<td>11b</td>
<td>9a</td>
<td>1.7a</td>
<td>21a</td>
<td>4a</td>
<td>37a</td>
<td>0.1a</td>
<td>0a</td>
<td>0a</td>
<td>1.5a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5–15 cm</td>
<td>0.2a</td>
<td>6a</td>
<td>0a</td>
<td>0a</td>
<td>9a</td>
<td>15a</td>
<td>12a</td>
<td>1.2a</td>
<td>4a</td>
<td>0a</td>
<td>0a</td>
<td>21b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>0b</td>
<td>0a</td>
<td>0a</td>
<td>0b</td>
<td>26a</td>
<td>6a</td>
<td>0a</td>
<td>31a</td>
<td>1.0a</td>
<td>4a</td>
<td>9a</td>
<td>12a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0.2b</td>
<td>6a</td>
<td>0a</td>
<td>0a</td>
<td>9a</td>
<td>15a</td>
<td>12a</td>
<td>1.2a</td>
<td>4a</td>
<td>0a</td>
<td>0a</td>
<td>21b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>0b</td>
<td>0a</td>
<td>0a</td>
<td>0.1a</td>
<td>3ab</td>
<td>8a</td>
<td>4a</td>
<td>3a</td>
<td>1.3a</td>
<td>0a</td>
<td>0a</td>
<td>67b</td>
</tr>
</tbody>
</table>

Letters behind mean values indicate significant differences within depth of each species over time. Data are presented as mean ± SE (standard error) from four replications.

- *P < 0.05,
- **P < 0.01,
- ***P < 0.001.

ND, not determined.
root exudates by microorganisms strongly depends on the quality and quantity of released C and N (Bais et al., 2006). White clover exudates are rich in N, and C is deposited to the soil mainly in amino acid form with very fast microbial decomposition (Jones et al., 2002; Dippold and Kuzyakov, 2013). Therefore, the low $^{13}$C content in the rhizosphere under white clover indicates a fast decomposition of N-rich white clover exudates and larger $^{13}$C losses due to respiration under this crop than under ryegrass.

Ryegrass and white clover exudates were differently utilised by bacteria (Gram-positive and Gram-negative), fungi and...
short-chain peptides (Farrell et al., 2013) probably caused the higher root biomass (Petersen et al., 2002; Butler et al., 2003) or soil organic C for fungal activity. Actinomycetal PLFA had a higher specific activity than bacterial PLFA under ryegrass at 0–5 cm layer than at 5–15 cm. As the root biomass of white clover at 5–15 cm was only slightly lower than at 0–5 cm, other explanations apart from differences in root density effect are needed. Previous studies have shown that nODULES and root tips of legumes are the main sites of microbial incorporation of fungal PLFA. Hence, the present study indicates that injection of specific easily-available substrates only to a limited extent reproduces the actual short-term processes in the rhizosphere.

The higher fungal PLFA 13C enrichment in soil under ryegrass was probably due to C-rich ryegrass exudates (Treonis et al., 2004; Hannula et al., 2012). Surprisingly, we found that the extent of 13C incorporation into fungal PLFA under ryegrass at both depths was at the same level, which contradicts previous studies reporting a higher abundance of fungal PLFA in the upper soil layers related to higher root biomass (Petersen et al., 2002; Butler et al., 2003) or soil organic C (Treonis et al., 2002). Therefore, our study showed that utilisation of fresh C exudates by microbial groups observed under ryegrass and white clover were related only to a limited extent in medium-term responses of the microbial communities. As an example, under white clover at 0–5 cm our medium-term results showed that fungi were abundant, but in the short term fungi did not utilise 13C from white clover, which implies that the fungi relied on alternative C sources to fresh white clover exudates. Hence, different short-term C utilisation patterns may lead to similar medium-term responses of the microbial communities under the different species. Further, our study showed that utilisation of fresh C exudates cannot well explain microbial N dynamics. Forage legumes, like white clover, are mainly grown in mixture with perennial grasses. Therefore, better knowledge about the processes in the rhizosphere under mixed plant communities is needed to predict processes controlling SOM decomposition, nutrient mobilisation/imobilisation and plant uptake.

5. Conclusions

Dual 13C/15N leaf labelling is a good tool for targeted tracing of C and N in mixed microbial communities. Plants showed very fast below-ground allocation of 13C and 15N and this labelling method can therefore be applied in short-term studies. Our results showed clear differences between the microbial communities under ryegrass and white clover in the short-term utilisation of root exudates. Under ryegrass, fungi more actively utilised fresh C, whereas bacteria more actively did so under white clover. These short-term differences, however, explained only to a limited extent the medium-term differences in the microbial communities under the two plant species. The results for ryegrass resembled studies where C tracer addition was done via CO2-labelling, whereas transformation of white clover root exudates were more in keeping with the findings of studies using injections of simple and specific organic compounds like amino acids and glucose into the soil.

Acknowledgement

The authors wish to acknowledge Karin Dyrberg for technical assistance and Margit Schacht for proof reading. The work was funded by the Danish Council for Independent Research (Technology and Production Sciences) as part of the project ‘Does white clover induce biennial bursts in N leaching from grassland?’ (Grant no. 10-082182).

References


