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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

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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

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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 (weeks to months). 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 $^{13}C/^{15}N$ 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 ^{13}C 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; Apostel 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 ^{15}N in the microbial community and the ^{13}C uptake by microbial groups through analysis of PLFA composition in soil under white clover and ryegrass leaf-labelled with ^{15}N and ^{13}C. 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 on the microbial community in the rhizosphere by examining the PLFA composition and ^{15}N incorporation in the microbial community, and the short-term (seven days) utilisation of ^{13}C-enriched exudates by microbial groups. We hypothesised:

- a greater incorporation of root-released N into the microbial community under ryegrass compared to white clover because of stronger N limitation under ryegrass and because of its higher release of energy-rich root-derived C stimulating microbial growth and turnover in the rhizosphere;
- a greater incorporation of released exudates into microorganisms in the uppermost layer (0–5 cm) compared to the deeper layer due to higher root density resulting in more available substrate for microbial growth;
- a lower incorporation of root-released C into fungal PLFA under white clover than under ryegrass because legumes exudate inorganic (NH_4^+) and organic N compounds with a low C/N ratio (de Neergaard et al., 2002), while fungi are more C-dependent;
- finally, we expected that bacteria incorporate C immediately after its release into the rhizosphere with a temporal shift of C to fungi in later stages.

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 $^{15}N/^{13}C$ labelling

Leaf labelling with ^{15}N-urea (99.6 atom% ^{15}N, 0.5% w/v) (Høgh-Jensen and Schjoerring, 2000) and $Na_2^{13}CO_3$ (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, Galten, Denmark) to avoid $^{15}N/^{13}C$ 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 $^{15}N/^{13}C$ 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 shaken carefully to separate roots from the soil that was passed through a 2-mm sieve. Plant leaves and washed plant roots were oven-dried at 60°°C to constant weight. The soil sample was thoroughly mixed and divided into four subsamples for the following analyses: water content (oven-dried at 110°°C), $^{15}N/^{13}C$ bulk analyses (oven-dried at 60°°C), ^{13}C-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 subsamples were ground to a fine powder in a ball-mill for total C and N content and $\delta^{15}N/^{13}C$ 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_2SO_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_2SO_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/K_{en}, where MB-N = (N extracted from fumigated samples) − (N extracted from non-fumigated samples) and $K_{en} = 0.45$ (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:

$$^{15}N_{SMB} = \left(\left(^{15}Nat\%_{fum} - {}^{15}Nat\%_{fumcontrol}\right) \times N_{fum} - \left(^{15}Nat\%_{unfum} - {}^{15}Nat\%_{unfumcontrol}\right) \times N_{unfum}\right) \times K_{en}$$

where $^{15}N_{SMB}$ denotes microbial biomass ^{15}N content, $^{15}Nat\%_{fum}$ and $^{15}Nat\%_{fumcontrol}$ are the ^{15}N enrichment (at%) of, respectively, fumigated labelled and unlabelled (control) samples, $^{15}Nat\%_{unfum}$ and $^{15}Nat\%_{unfumcontrol}$ are the ^{15}N enrichment (at%) of, respectively, unfumigated labelled and unlabelled (control) samples and $K_{en} = 0.45$ (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. $\delta^{13}C$ 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 $\delta^{13}C$ values were corrected by calibration curves from external standards with known $\delta^{13}C$ values: acetanilide, caffeine, C16:0, C18:0, C20:0. All $\delta^{13}C$ values were corrected for the additional C by derivatisation:

$$\delta^{13}C = \frac{\left[(n_{FA} + 1)\,\delta^{13}C_{FAME} - \delta^{13}C_{MeOH}\right]}{n_{FA}}$$

where n_{FA} refers to the number of C-atoms of the fatty acid (FA) component, $\delta^{13}C_{FAME}$ is the measured $\delta^{13}C$ value of the FAME after methylation and $\delta^{13}C_{MeOH}$ is the $\delta^{13}C$ value for the methanol used for derivatisation (−37.7‰ ± 3.23‰). ^{13}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.

Statistical analyses were performed using the R statistical software. The effect of plant species, depth and time after labelling on the ^{15}N and ^{13}C enrichment of bulk plant and soil, the ^{15}N enrichment of microbial biomass and the relative abundance of individual PLFAs and ^{13}C-PLFAs was evaluated by factorial analysis of variance (ANOVA) and Tukey's HSD post-hoc test.

3. Results

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

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

Table 1
List of PLFA used in data analysis.

Group	Name	Reference
Bacteria		
Gram positive	*i*15:0, *a*15:0, *i*16:0, *i*17:0, *a*17:0	Frostegård and Bååth, 1996
Gram negative	16:1ω7c, 18:1ω7c, 18:1ω9c, cy:19	Stromberger et al., 2012,
Non-specific	14:0, 15:0, 16:1ω5c, 16:0	Frostegård and Bååth, 1996
Actinomycetes	10Me16:0, 10Me18:0	Bird et al., 2011
Fungi	18:2ω6,9	Stromberger et al., 2012

Table 2
Plant characteristics.[a]

		White clover			Ryegrass		
		Biomass [kg/m^2]	N Yield [g/m^2]	C/N	Biomass [kg/m^2]	N Yield [g/m^2]	C/N
Shoot		1.95 ± 0.09	63 ± 4	12 ± 1	2.1 ± 0.13	33 ± 2	26 ± 2
Root	0–5 cm	1.49 ± 0.04	44 ± 2	14 ± 1	2.1 ± 0.12	19 ± 1	41 ± 2
	5–15 cm	1.35 ± 0.01	39 ± 2	13 ± 1	1.46 ± 0.03	17 ± 1	34 ± 3

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

2014) 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}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}N/^{13}C dual labelling led to the expected ^{15}N and ^{13}C enrichment of shoots and roots (Fig. 1). Ryegrass shoots and roots at 5–15 cm had a higher ($P < 0.001$) ^{15}N enrichment than white clover, whereas no significant difference was observed between the roots of both species at 0–5 cm. The ^{15}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}C enrichment than ryegrass. The ^{13}C enrichment of white clover roots was the same at both depths, whereas ryegrass roots at 5–15 cm had higher ^{13}C enrichment than at 0–5 cm ($P < 0.001$).

3.2. Soil ^{15}N and ^{13}C enrichment

Although ^{15}N and ^{13}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}N enrichment was highest on day 7 and slightly decreased thereafter, whereas under white clover it increased until day 56. Soil ^{15}N enrichment at 5–15 cm depth was similar for both species. Soil ^{13}C enrichment was significantly higher ($P < 0.001$) under ryegrass than white clover at 0–5 cm depth. The ^{13}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

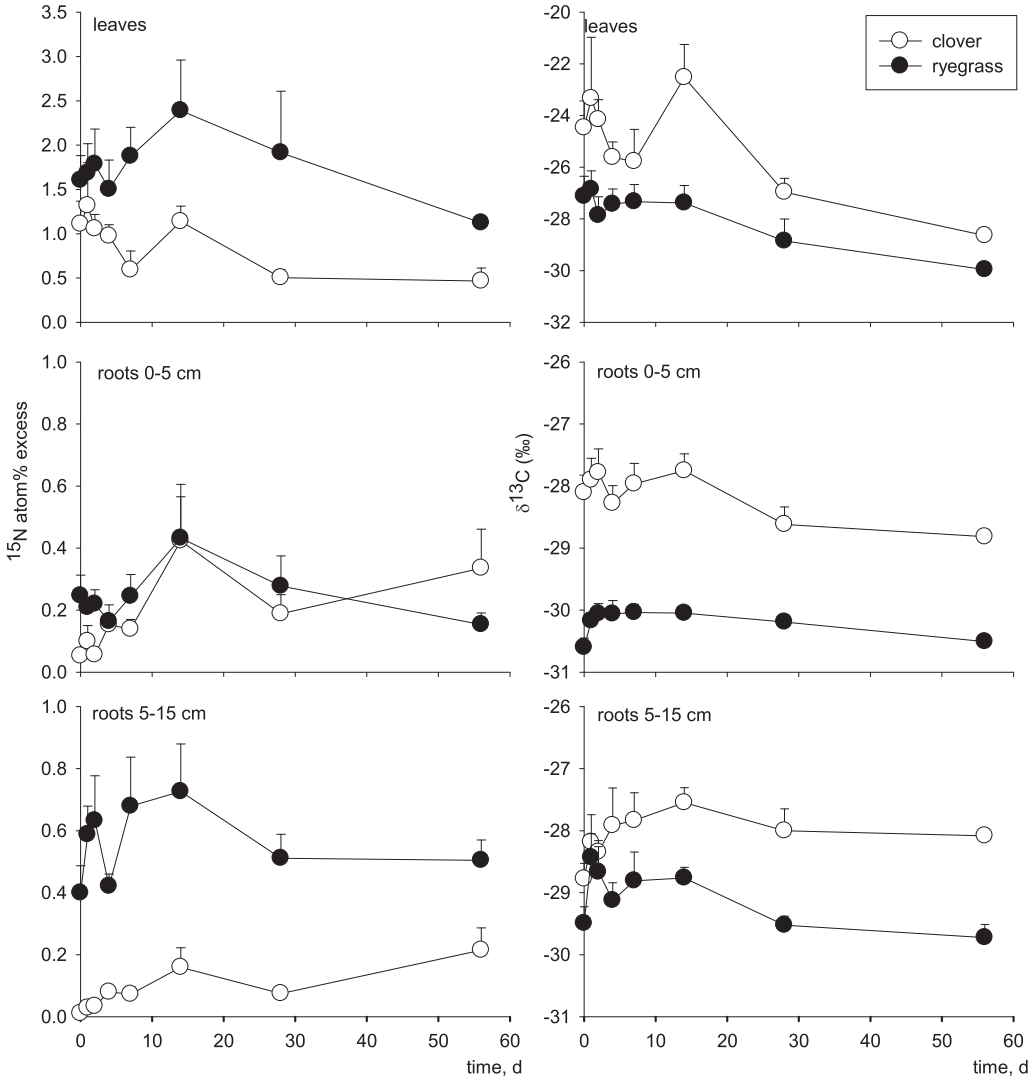

Fig. 1. ^{15}N excess (at.%) and δ^{13}C of plant shoots and roots as a function of length of time after labelling (mean ± SE, $n = 4$).

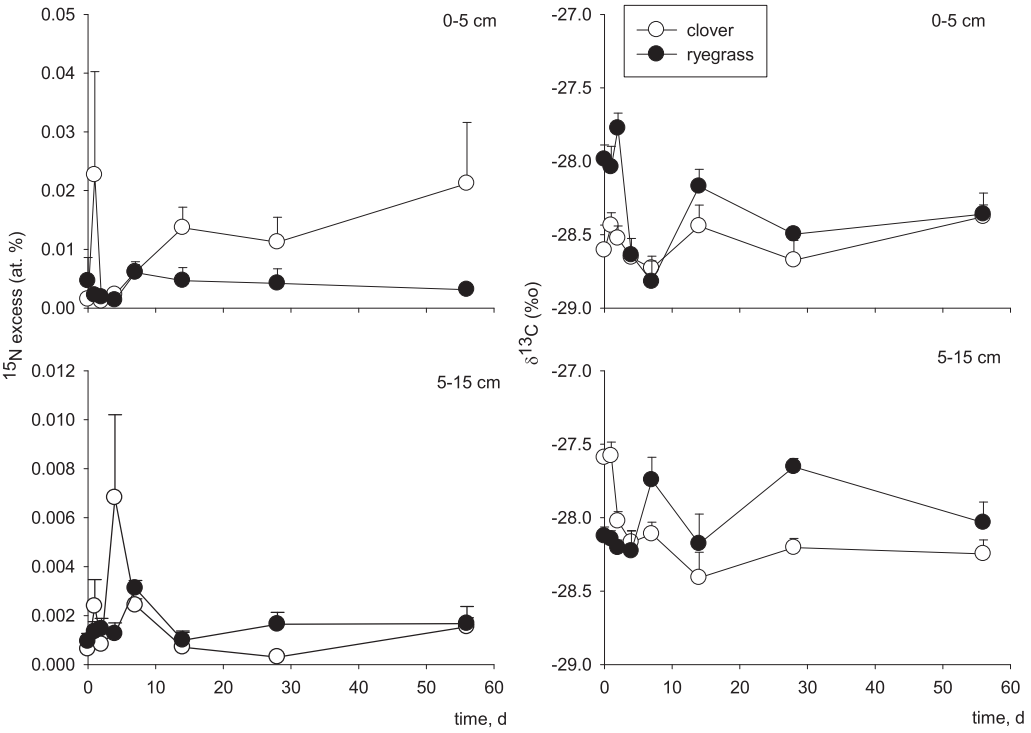

Fig. 2. ^{15}N excess and δ^{13}C in bulk soil under ryegrass and clover pure stands (mean ± SE, $n = 4$).

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).

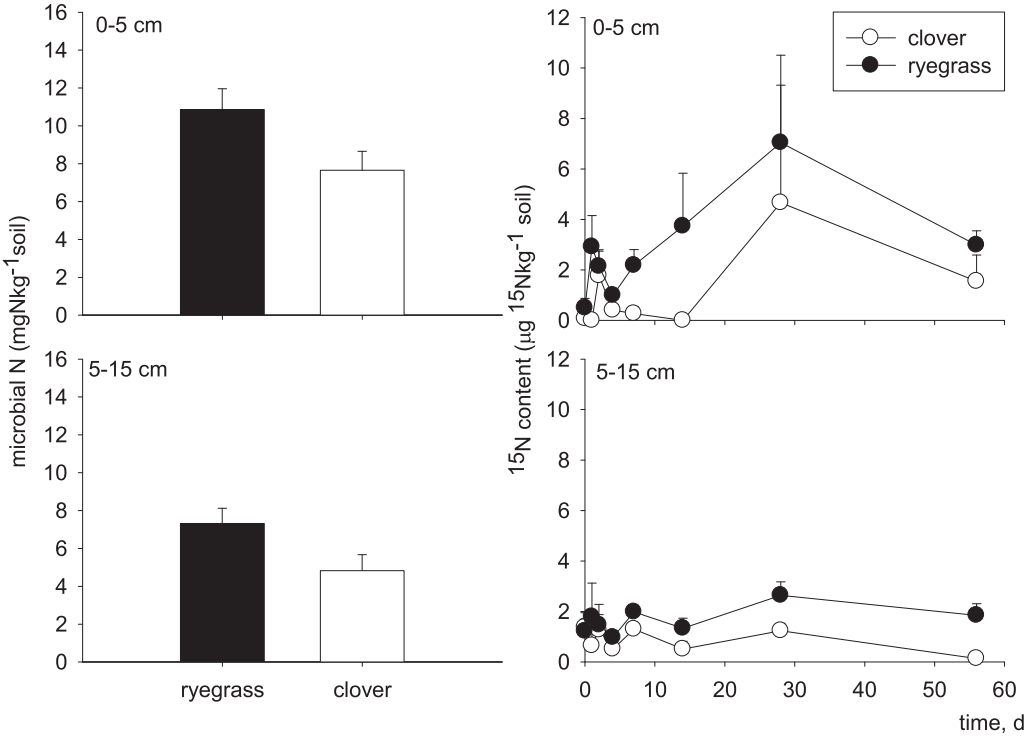

Fig. 3. Microbial biomass N (average of eight samplings) (mean ± SE, $n = 32$) and ^{15}N content under ryegrass and clover pure stands at 0–5 cm and 5–15 cm depth (mean ± SE, $n = 4$).

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 (*i*15:0, *a*15:0) 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 ^{15}N 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 ^{13}C was incorporated into 17 individual PLFAs (Table 4). In ryegrass soil at 0–5 cm depth, the highest ^{13}C 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 (*i*15:0), actinomycetes (10Me16:0) and fungi (18:2ω6,9) showed the highest ^{13}C enrichment at 5–15 cm. White-clover-derived ^{13}C 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 ^{13}C enrichment at 5–15 cm depth.

The ^{13}C-labelled exudates under ryegrass and white clover were incorporated into different groups of microorganisms (Fig. 5). The ^{13}C 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 ^{13}C exudates was higher under ryegrass than white clover (Fig. 6). Bacteria (Gram-positive and Gram-negative) and actinomycetes were less active and showed increasing ^{13}C incorporation over time. White clover exudates at 0–5 cm were primarily incorporated into Gram-positive and Gram-negative bacterial PLFA, while no ^{13}C 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 $^{15}N/^{13}C$ leaf-labelling technique has been followed by compound-specific ^{13}C-PLFA analysis to investigate microbial utilisation of root exudates under white clover and ryegrass. The ^{13}C leaf-labelling with bicarbonate (Rasmussen et al., 2013a) resulted in plants (shoots and roots) and bulk soil being ^{13}C enriched already after two days, similar to other studies with $^{13}CO_2$ or $^{14}CO_2$-labelling (Dilkes et al., 2004). This indicates that the ^{13}C introduced to plants via bicarbonate leaf-labelling is allocated similarly to the ^{13}C fixed via photosynthesis ($^{13}CO_2$-labelling). With the leaf-labelling technique there is a risk of $^{15}N/^{13}C$ entering the soil from leaf decay (Dahlin

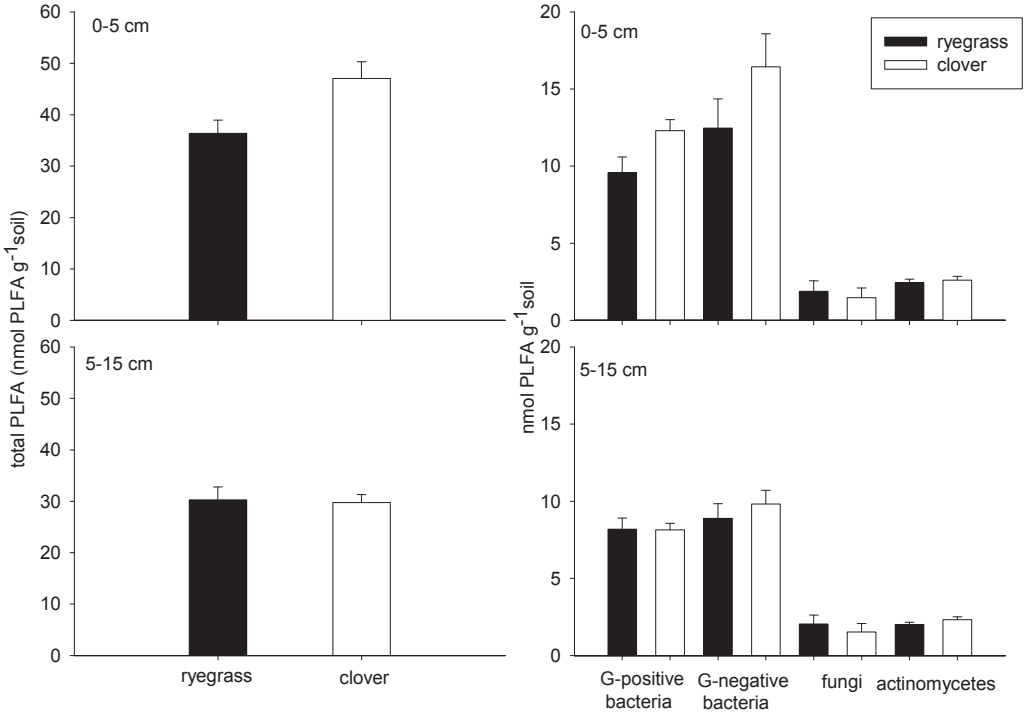

Fig. 4. The content of total PLFA (average of four samplings) (mean ± SE, $n = 16$) and functional groups (Gram-positive and Gram-negative bacteria, actinomycetes and fungi) under ryegrass and white clover pure stands at 0–5 and 5–15 cm depth (mean ± SE, $n = 4$).

Table 3
Mole percentage distribution of PLFAs under the ryegrass and white clover at 0–5 and 5–15 cm depth (mol%).

Species	Depth	14:0	i15:0	a15:0	15:0	i16:0	16:1ω7c	16:1ω5c	16:0	10Me16:0	i17:0	a17:0	18:2ω6,9	18:1ω9c	18:1ω7c	10Me18:0	cy19:0	20:0
Ryegrass	0–5 cm	1.35	11	8	0.90	4	4	8	18	2.73b	1.95	1.09	6	8	8	4	9b	3
	5–15 cm	1.35	11	8	0.77	4	4	8	20	3.02a	2	1.13	7	7	4	4	10a	3
Clover	0–5 cm	1.48	10	9	1.72	4	4	8	19	2b	1.97	1.22	4	10	9	4	7b	2
	5–15 cm	1.35	11	9	0.75	4	4	7	18	4a	1.91	1.24	6	8	7	4	11a	3
Species		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
Species × depth		ns	ns	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.
ns – $P > 0.05$, * – $P < 0.05$, ** – $P < 0.01$, *** – $P < 0.001$.

and Stenberg, 2010). However, since the experiment was conducted in the early part of the growing season and for a short time period, it is very unlikely that leaf decay significantly affected the results, especially that of PLFA analyses.

White clover shoots and roots were less ^{15}N enriched than ryegrass due to dilution caused by clover N_2 fixation, while ryegrass was less ^{13}C enriched than white clover. This is consistent with Rasmussen et al. (2013a) who found higher ^{14}C incorporation in white clover compared to ryegrass both for atmospheric $^{14}CO_2$-labelling and ^{14}C-bicarbonate leaf-labelling. Soil under ryegrass was more ^{13}C enriched than under white clover as found in other studies (de Neergaard and Gorissen, 2004).

4.1. Medium-term effects of sward type on the microbial biomass and functional groups

Microbial N and ^{15}N 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 microorganisms, 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 (18:2ω6,9) 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 ^{13}C-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%).

Species	Depth	Time, d	14:0	i15:0	a15:0	15:0	i16:0	16:1ω7c	16:1ω5c	16:0	10Me16:0	i17:0	a17:0	18:2ω6,9	18:1ω9c	18:1ω7c	10Me18:0	cy19:0	20:0
Ryegrass	0–5 cm	0	3ab	10a	14a	0a	9a	0a	0a	17a	6ab	0.6a	0.3a	15a	6a	4ab	5a	3 ab	8a
		1	23a	0b	0b	7a	7a	0a	0a	0b	18a	1.2a	20a	22a	0a	0b	0a†	0b	1.3a†
		2	1.8b	9a	10a	0a	7a	5a	0.7a	19a	0.3b	0a	0a†	15a†	3a	18a	0.1a	7a	5a
		7	0.6b	8a	9ab	0a	6a	0a	0a	9ab	1.2b	0a	0a	41a	1.0a	10ab	2a	6 ab	6a
	5–15 cm	0	1.1a	23a	12a	0.3a	8a	9a	6a	21a	0.3b	0a	0a	13a	0a	2b†	0a	5a	0a
		1	1.3a	0b	0b	10a	6a	0a	0b	0b	44a	4a	6a	29a	0a	0b	0a†	0.3a	0a
		2	0.5a	13a	8a	0a	6a	2a	5a	8ab	0.1b	0.6a	0a†	36a	0a†	8ab	1.7a	7a	4a
		7	0.1a	13a	8a	0.5a	4a	6a	5a	8ab	0.8b	0a†	0a	32a	0a†	12a	3a	5a	2a
White clover	0–5 cm	0	5a	13a	21a	0.2a	0.1a	4a	7a	35a	3b	0a	0b†	0a†	7a	0a	1.3a	1.0a	1.3a
		1	1.3a	0b	0c	21a	0.0a	0a	0a	0b	29a	20a	28a	0a	0a	0a	0a†	0a	ND
		2	3a	16a	23a	0.6a	0.7a	0a	10a	39a	4b	0a	0b	ND	0a	0a	0a	2a	0a
		7	2a	8ab	11b	9a	1.7a	21a	4a	37a	1.9b	0a†	0b	0a	1.5a	0a	0a	2a	1.5a
	5–15 cm	0	2a	1.3a	0a	0a	9a	20a	9a	7a	29a	0a	0a	ND	0.5a	0a	1.5a	20a	0b
		1	0b	0a	0a	0a†	0b	26a	6a	0a	31a	1.0a	4a	9a	12a	0a	0a	11a	0b†
		2	0.2b	6a	0a	0a	9a	15a	12a	1.2a	4a	0a	0a	21b	0a	0a	1.5a	19a	12a
		7	0b	0a	0a	0.1a	3ab	8a	4a	3a	1.3a	0a	0a	67b	0a	0a	3a	9a	2b
Species			ns	***	ns	ns	***	**	***	*	ns	ns	ns	*	ns	***	ns	ns	ns
Time			*	***	***	*	ns	ns	*	***	***	*	***	**	ns	***	ns	ns	*
Depth			**	ns	***	ns	ns	*	*	***	ns	ns	*	**	ns	ns	ns	**	ns
Species × depth			**	***	***	ns	**	ns	ns	***	ns	ns	ns	*	ns	ns	ns	**	**
Species × time			ns	**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	***	ns	ns	ns
Species × time × depth			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 over time. Data are presented as mean ± SE (standard error) from four replications.
ns $-P > 0.05$,* $-P < 0.05$,** $-P < 0.01$,*** $-P < 0.001$.
† indicate 1 replicate.
ND, not determined.

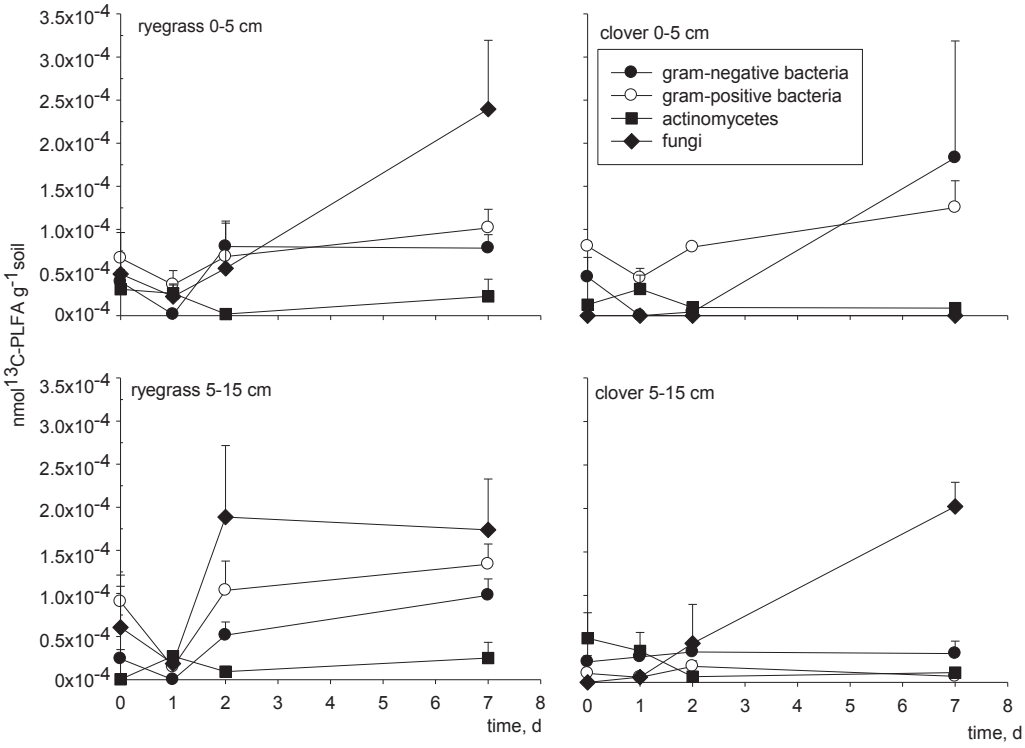

Fig. 5. Microbial (Gram-positive and Gram-negative bacteria, actinomycetes and fungi) ^{13}C-PLFA content under ryegrass and white clover pure stands at 0–5 and 5–15 cm depth (mean ± SE, $n = 4$).

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

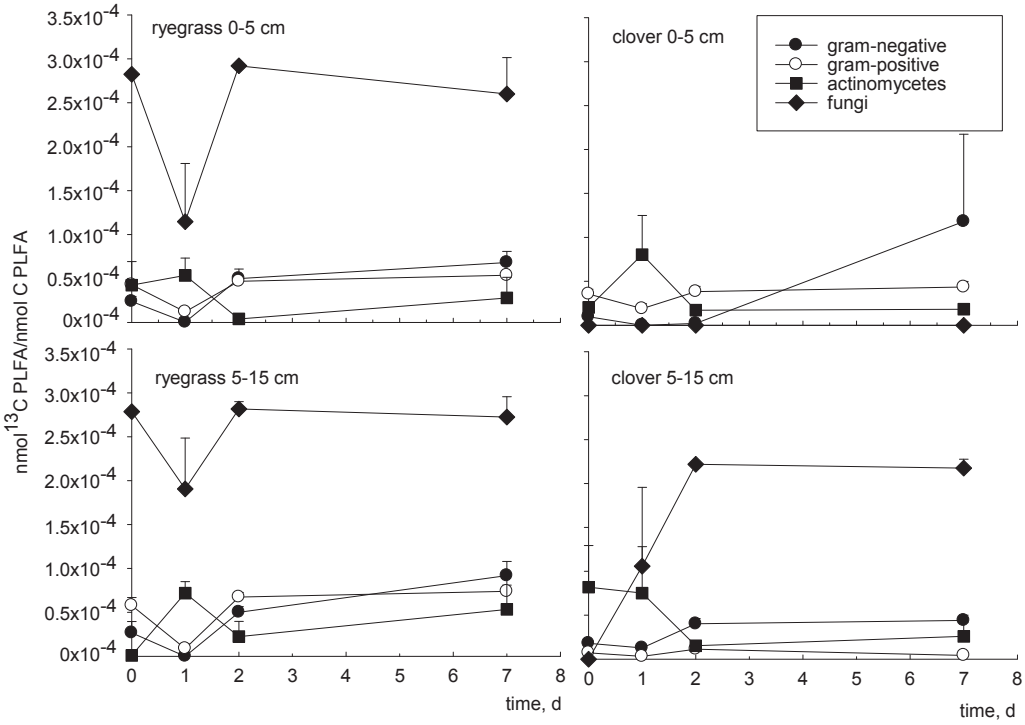

Fig. 6. Specific activity of microorganisms (Gram-positive and Gram-negative bacteria, actinomycetes and fungi) under ryegrass and white clover pure stands at 0–5 and 5–15 cm depth (mean ± SE, $n = 4$).

actinomycetes. From other studies we expected both Gram-negative and Gram-positive bacteria to be most active in decomposing fresh exudates (Bell et al., 2003) with this activity shifting to fungi and actinomycetes over time, but surprisingly, fungi were most active in utilising ryegrass exudates already from day 0 following labelling. Generally, in studies where ^{13}C-amino acids, sugars or labelled plant residues have been used (Bird et al., 2011; Dungait et al., 2011; Apostel et al., 2013; Sullivan and Hart, 2013; Lemanski and Scheu, 2014), the highest ^{13}C activity is in Gram-positive and Gram-negative bacterial PLFA, whereas studies using $^{13}CO_2$-labelling show the highest ^{13}C activity in fungal (Butler et al., 2003; Treonis et al., 2004) and Gram-negative bacterial PLFA (Tian et al., 2013). 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 ^{13}C 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 ^{13}C 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 carbon (Petersen et al., 2002). Therefore, exudate composition and availability were probably more important than root density or soil organic C for fungal activity. Actinomycetal PLFA had a higher specific activity than bacterial PLFA under ryegrass at 0–5 cm on days 0 and 1, showing that actinomycetes out-competed bacteria for ryegrass exudates. This finding differs from previous results that reported very low ^{13}C incorporation into actinomycetes (Dungait et al., 2011; Tian et al., 2013).

It was interesting that the exudates of white clover and ryegrass were utilised by different microbial groups. The ^{13}C enrichment of bacterial PLFA showed that Gram-positive and Gram-negative bacteria more actively utilised fresh white clover exudates in the 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 amino acid exudation (Lesuffleur and Cliquet, 2010). Therefore, higher nodule density and the competitive superiority of bacteria over fungi in utilising free amino acids and short-chain peptides (Farrell et al., 2013) probably caused the higher ^{13}C incorporation into bacterial PLFA in the 0–5 cm layer. Low ^{13}C enrichment in bacterial PLFA under white clover at 5–15 cm indicates that white clover exudated fewer C-rich compounds in this layer for bacteria to live on, probably due to lower root activity (Rasmussen et al., 2013b) or lower nodule abundance. Alternatively, the lower ^{13}C enrichment could be due to bacteria being in the dormant state (Blagodatskaya and Kuzyakov, 2013).

Fungal utilisation of exudates was also markedly different depending on whether it was under ryegrass or white clover. We expected lower exudate incorporation into fungi under white clover than under ryegrass due to a lower C/N ratio in legume exudates. Surprisingly, at 0–5 cm under white clover, fungi were not active, even though the relative abundance of fungi in the soil was similar to under ryegrass. Probably the fungi continuously utilised old, more recalcitrant, white clover rhizodeposits at 0–5 cm or stayed dormant (Blagodatskaya and Kuzyakov, 2013). The higher concentration of inorganic N and low C/N ratio of white clover exudates could have resulted in a deficiency of polymerised compounds (Fontaine et al., 2011), making fungi decompose more recalcitrant C compounds (Sullivan and Hart, 2013). Greater N availability enables fungi to use C sources that are not available to bacteria (Steer and Harris, 2000). Therefore, an increased ^{13}C enrichment of fungal PLFA over time at 5–15 cm indicated that fungi actually started decomposing more recalcitrant white clover deposits.

Our results were consistent with field studies where plants were ^{13}C-CO_2-labelled and thus more adequately reflecting exudation of a complex suite of organics (soluble and polymeric). Studies with soil injections of amino acids (Apostel et al., 2013) or glucose (Dungait et al., 2011; Garcia-Pausas and Paterson, 2011; Lemanski and Scheu, 2014) seem to mostly reflect processes that occur in the rhizosphere under legumes and less so under non-N-fixing plants.

Our study showed that the differences in short-term C utilisation of exudates by microbial groups observed under ryegrass and white clover were reflected 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 ^{13}C 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/immobilisation and plant uptake.

5. Conclusions

Dual $^{13}C/^{15}N$ 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 ^{13}C and ^{15}N 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 CO_2-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.

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