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Soil nitrogen availability alters rhizodeposition carbon flux into the soil microbial community

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Abstract
Purpose Soil microorganisms are important in the cycling of plant nutrients. Soil microbial biomass, community structure, and activity are mainly affected by carbon substrate and nutrient availability. The objective was to test if both the overall soil microbial community structure and the community-utilizing plant-derived carbon entering the soil as rhizodeposition were affected by soil carbon (C) and nitrogen (N) availability.
Materials and methods A ^{13}C-CO_2 steady-state labeling experiment was conducted in a ryegrass system. Four soil treatments were established: control, amendment with carboxymethyl cellulose (CMC), amendment with ammonium nitrate (NF), combined CMC and NF. Soil phospholipid fatty acid (PLFA) and ^{13}C labeling PLFA were extracted and detected by isotope ratio mass spectrometer.
Results and discussion The combined CMC and NF treatment with appropriate C/N ratio (20) significantly enhanced soil microbial biomass C and N, but resulted in lower soil inorganic N concentrations. There was no significant difference in soil PLFA profile pattern between different treatments. In contrast, most of the ^{13}C was distributed into PLFAs 18:2ω6,9c, 18:1ω7c, and 18:1ω9c, indicative of fungi and gram-negative bacteria. The inorganic-only treatment was distinct in ^{13}C PLFA pattern from the other treatments in the first period of labeling. Factor loadings of individual PLFAs confirmed that gram-positive bacteria had relatively greater plant-derived C contents in the inorganic-only treatment, but fungi were more enriched in the other treatments.
Conclusions Amendments with CMC can improve N transformation processes, and the ryegrass rhizodeposition carbon flux into the soil microbial community is strongly modified by soil N availability.

Keywords ^{13}C · N availability · PLFA · Rhizodeposition carbon · Steady state labeling

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1 Introduction

Soil microorganisms are both a labile nutrient pool and an agent of the transformation and cycling of organic matter and plant nutrients in soils (Fierer et al. 2012). Soil C and N availability are key factors which modify the microbial community and thus affect organic matter decomposition and nutrient cycling (Chen et al. 2014). Changes in microbial population size, community structure, and activity are usually observed after amendment with organic matter or N fertilizer (Marschner et al. 2003; Bengtsson et al. 2003). Since soil microbial turnover rate and activity are highest when substrate input corresponds to stoichiometric C and N ratios (Hessen et al. 2004), combined inorganic N fertilizer with organic C treatment is usually used to increase soil nutrient availability and has been proven to be an effective method to reduce N losses and improve crop productivity (Palm et al. 1997; Zhang et al. 2012).

Root exudates are implicated as a key determinant of rhizosphere microbial biomass and activity, and can modify the biochemical interactions between plants and soil microorganisms (Prosser et al. 2006). Based on a meta-analysis of the available data, rhizodeposition is estimated to account for approximately 17 % of photoassimilate (Nguyen 2003). Rhizodeposition carbon compounds can strongly influence nutrient availability and microbial community structure due to their preferential use as substrates by microorganisms involved in nutrient transformations (Phillips et al. 2011). Soil C and N cycling are often coupled. The coupling of rhizodeposition C input to the functioning of microbial community can affect microbial growth and reproduction and be beneficial for soil N availability and plant N uptake (Paterson 2003). N fertilizer application reduces the competition for N between roots and soil microbes, and the amount of rhizodeposition C is decreased by increased N availability (Henry et al. 2005; Liljeroth et al. 1994).

The isotope labeling technique has been effectively used to track carbon flow and study the relative importance of rhizodeposition C and other C sources in determining soil microbial community structure and activity (West et al. 2006). Soil microbial communities can utilize both labile and recalcitrant carbon, including root exudates, soil organic matter, and plant litter. Phospholipid fatty acid (PLFA) analysis combined with stable isotope probing (SIP) can quantify the ^{13}C assimilated into microbial populations and determine the sources of C used by soil microbes (Yao et al. 2015). PLFA-SIP has been applied to study the decomposition of plant residues and the assimilation of organic matter (Williams et al. 2006; Murase et al. 2006) as well as the incorporation of photosynthates into microbial communities (Lu et al. 2004; Yao et al. 2012).

Most ^{13}C labeling PLFA studies in soil-plant systems have been based on the pulse-labeling technique (Butler et al. 2003; Treonis et al. 2004) but this technique does not label all plant C pools to the same degree, emphasizing current photosynthate (De Visser et al. 1997). The steady-state ^{13}C labeling approach can determine the relative turnover activities of individual PLFAs and show a gradual shift in one direction during the labeling period (Paterson et al. 2011). In this study, we conducted a ^{13}C-CO_2 steady-state labeling experiment in a ryegrass system with different amendments. The objective was to test if both the overall soil microbial community structure and the community-utilizing plant-derived carbon entering the soil as rhizodeposition were affected by soil C and N availability.

2 Materials and methods

2.1 Soil and ^{13}C steady-state labeling

A typical red clay soil (Ultisols) was collected from the plow layer (0–20 cm) in Longyou county (28°58′ N and 118°53′ E), Zhejiang Province, China, in September 2013. The air-dried soil was sieved through a 2-mm mesh. Some chemical and physical properties of the soil were as follows: pH (soil, water = 1: 2.5) 4.51, soil organic carbon 10.81-mg g^{-1} soil, total nitrogen 1.22-mg g^{-1} soil.

Ryegrass (*Lolium perenne* L.; cultivar ORE-TET) was grown in 250-ml containers, filled with 160-g air-dried soil, and rewetted to 50 % water holding capacity. In order to investigate the interactive effects of organic C and N fertilizer, four soil treatments were established: (1) control, CK; (2) amendment with carboxymethyl cellulose at the rate of 2000 mg C kg^{-1} soil, CMC; (3) amendment with ammonium nitrate (NH_4NO_3) at the rate of 100 mg total N kg^{-1} soil, NF; and (4) combined CMC and NF, CMC + NF. All the soils were amended with potassium dihydrogen phosphate (KH_2PO_4) at the rate of 50-mg P_2O_5 kg^{-1} soil. Seeds of ryegrass were germinated in Petri dishes containing deionized water at 30 °C in the dark, and, following germination, 50 seedlings were sown in each pot. Four days after sowing, 30 healthy seedlings were retained in each pot. The pots were placed in a growth chamber (day/night temperature, 30/25 °C; photoperiod, 12 h light; relative humidity of 80–90 %).

After 2 weeks' growth, steady-state labeling of $^{13}CO_2$ at 2.0 atom % excess and at ambient CO_2 concentrations was introduced. All the pots were arranged in a random block structure in a Perspex labeling chamber, housed within a controlled environment room. The chamber had ports at each end as inlets/outlets for gas flows and two access doors that maintained air-tight seals when closed (Yao et al. 2012). The steady-state $^{13}CO_2$ enrichment of the labeling chamber continuously aerated at a rate of 1.8×10^4 cm^3 min^{-1} was achieved by routing the compressed air supply via a self-regenerating pressure swing adsorption CO_2 scrubber unit. Soil water status was maintained by additions of deionized water every day during the 15 d labeling experiment; this was performed during the dark period in order to avoid affecting the ^{13}C- signature of CO_2 fixed by the plants.

Destructive harvests of plants and soils were taken after labeling for 0, 5, 10, and 15 days. Each treatment included three replicates. Soil was separated from roots and divided into two portions. The first portion was stored at 4 °C up to 7 days before microbial biomass C and N analysis, except that inorganic N (NH_4^+–N and NO_3^-–N) was immediately analyzed. The other portion was freeze-dried for PLFA analysis. Plant roots and shoots were separated and dried at 105 °C for 24 h, then weighed. The inorganic N was extracted from soil subsamples by shaking for 1 h in 1-M KCl (soil: solution, 1:10), filtering using a filter paper (Quantitative 203 grade filter paper, ϕ = 7 cm) and then detected colorimetrically using a micro-plate reader (Spectramax M5, Molecular Devices, USA) (Shand et al. 2008). Soil microbial biomass C and N were determined using the chloroform fumigation extraction method (Brookes et al. 1985; Vance et al. 1987; Wu et al. 1990). Extraction coefficients 0.45 and 0.54 were used for

calculating microbial biomass C and N, respectively. The N content in plants was determined by macro elemental analyzer (Vario Max, Elementar, Germany).

2.2 PLFA analysis

Lipids were extracted from soil and PLFA, analyses followed the method of Bligh and Dyer (1959) as modified by Frostegård et al. (1993). From the freeze dried soil sub-samples, 2.0 g was extracted twice using a total of 22.8 ml of a chloroform-methanol-citrate buffer mixture (1:2:0.8 v/v/v), and the phospholipids were separated from neutral and glycolipids on a silicic acid column. The phospholipids were derivatized into fatty acid methyl esters (FAMES). Methylnonadecanoate fatty acid (19:0) was added as the internal standard. The concentration and ^{13}C label of the FAMES were carried out by using a GC Trace Ultra with combustion column attached via a GC Combustion III to a Delta V Advantage isotope ratio mass spectrometer (Thermo Fisher Scientific, USA). Samples (1–2 μl) were injected in splitless mode (Column HP-5, 50-m length, 0.2-mm i.d., film thickness of 0.33 μm; Agilent Technologies Inc., Santa Clara, USA), and the running conditions were as described by Thornton et al. (2011).

The PLFA concentrations were determined based on the combined area of the peaks for all ions (m/z 44, 45 and 46) relative to the internal standard. The C isotope ratios were calculated from a CO_2 reference gas (Thornton et al. 2011). The ^{13}C value of the C added to each PLFA molecule during methylation to their corresponding FAMES was accounted for using a mass balance approach (Paterson et al. 2008). Nomenclature of fatty acids follows that used by Tunlid and White (1992). Briefly, the suffix c for cis refer to the geometric isomer. The prefixes i- and a- refer to iso- and anteiso-branched fatty acids. The suffix -10Me indicates a methyl group on the tenth carbon atom from the carboxyl end of the molecule. A cyclopropyl ring is indicated as cy-, and "OH" indicates a hydroxyl group. In total, 29 PLFAs were identified in these samples; however, only a subset of 22 PLFAs, comprising of about 95 % of the total PLFA concentration, were present in sufficient concentration for accurate isotope analysis. We therefore defined ^{13}C incorporation into the soil microbial biomass as the total ^{13}C incorporation into this subset of 22 PLFAs. The proportion of plant-derived labeled C in each PLFA was determined using a mass balance approach (Yao et al. 2012).

2.3 Statistical analysis

Soil chemical properties and PLFA data were compared by a one-way ANOVA. The significance of any difference was defined according to statistical convention at $p<0.05$. The percentage distribution of ^{13}C among PLFAs was calculated and used for principal component analysis, after generating a correction matrix to transform the data to unit variance. All statistical analyses were made using GenStat 16th Edition (VSN International, Oxford, UK).

3 Results

After 2 weeks' growth before labeling, there was no significant difference in ryegrass biomass except for CK (Fig. 1). After labeling for 15 days, the CMC + NF treatment had the highest growth rate, and the dry weight was 0.37 g pot^{-1}, which was significantly higher than the CK (0.24 g pot^{-1}) and CMC (0.27 g pot^{-1}) treatments. Compared to the NF treatment, the combined organic C with N fertilizer treatment improved plant biomass. We found that the NF treatment had the highest soil inorganic N content in the first 10 days of labeling. However, no significant differences in soil inorganic N concentration were observed among all the treatments at the end of the labeling period (Fig. 2). Interestingly, soil inorganic N concentration in the CMC + NF treatment was much lower than the inorganic-only fertilizer treatment in the first 10 days of labeling, and there was no significant difference in CK, CMC, and CMC + NF treatments during the whole labeling period.

Final microbial biomass C in the soils ranged from 144 to 228 μg C g^{-1} (Fig. 3). Application of N fertilizer significantly increased soil microbial biomass N. The combination of organic C with N fertilizer further increased soil microbial biomass such that the CMC + NF treatment had the highest value. Microbial biomass N in the CMC + NF treatment was 34.1 μg N g^{-1}, which was much higher than the CK (21.1 μg N g^{-1}), CMC (23.0 μg N g^{-1}), and NF (28.9 μg N g^{-1}) treatments.

The total PLFAs (± standard deviation) in the CK, CMC, NF, and CMC + NF treatments were 27.0 ± 4.7, 30.3 ± 1.9, 34.2 ± 4.0, and 38.9 ± 4.8 nmol g^{-1} soil, respectively, with no significant change during the labeling period. The total

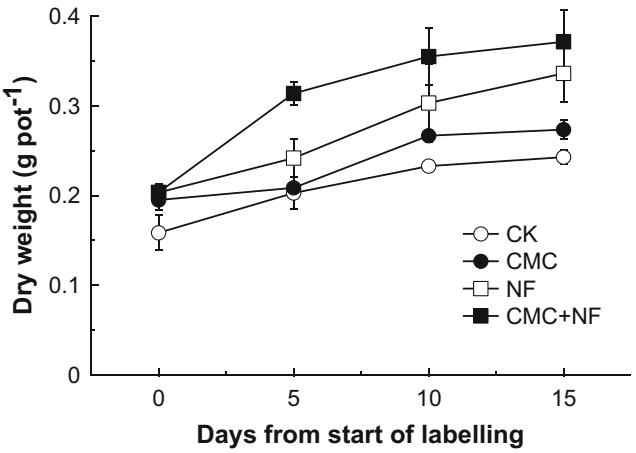

Fig. 1 Effect of organic C and N fertilizer on plant biomass during a 15-day $^{13}CO_2$ labeling experiment. *CK* control, *CMC* organic carbon alone, *NF* N fertilizer alone, *CMC + NF* combined organic carbon and N fertilizer. Values are means ± s.d. ($n = 3$)

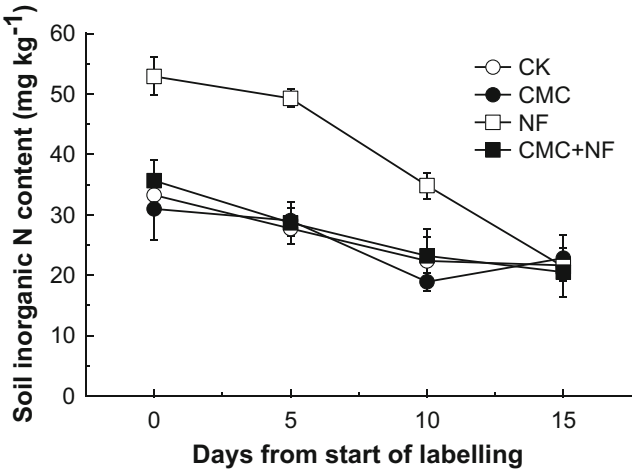

Fig. 2 Total inorganic N in the soil during a 15-day $^{13}CO_2$ labeling experiment. *CK* control, *CMC* organic carbon alone, *NF* N fertilizer alone, *CMC + NF* combined organic carbon and N fertilizer. Values are means ± s.d. ($n = 3$)

amount of PLFAs was significantly correlated with soil microbial biomass obtained from the fumigation-extraction method. Twenty-nine PLFAs with chain lengths from C14 to C19 were identified which varied significantly in their relative abundance (Fig. 4a). There was no difference in PLFA profile pattern based on principal component analysis (PCA) between different treatments and different labeling days.

At the end of labeling, the total ^{13}C incorporation into the PLFA pools in the CK, CMC, NF, and CMC + NF treatments were 1633, 1855, 2488, and 2699 ng g^{-1} soil, respectively. The ^{13}C was not evenly distributed among PLFAs. The highest relative abundances were observed for PLFAs 18:2ω6,9c and 16:0, followed by 18:1ω7c, and 18:1ω9c (Fig. 4b). PCA analysis of ^{13}C PLFA data showed that the NF treatment was distinct from the other treatments in the first 5 and 10 days of labeling, but no real significant difference was found at the end of labeling (Fig. 5). In the first period of labeling (Fig. 5a), the NF treatment significantly separated along PC1, accounting for 59.5 % of the variation. Factor loadings of individual PLFAs on PC1 confirmed that i16:0 (score 0.154) and i17:0 (score 0.136) had relatively greater plant-derived C contents in the NF treatment. The loadings also confirmed that 18:2ω6,9c (score −0.835) was more enriched in the other treatments (Table S1, Electronic Supplementary Material).

4 Discussion

The combined impact of inorganic and organic fertilizers on soil organic matter content and crop yield has been stressed by many studies (e.g. Pan et al. 2009; Zhang et al. 2012). Compared to inorganic-only fertilizer treatment, the combination of organic carbon and inorganic N fertilizer treatment decreased soil inorganic N, but improved soil microbial biomass and plant growth (Fig. 1 and Fig. 3). Soil microorganisms determine N availability for plant uptake or loss mainly through the transformation processes of mineralization, nitrification, and immobilization. The balance between these processes can regulate the pool size of soil inorganic N and accordingly plant uptake (Shi et al. 2006; Herencia et al. 2008; Dinesh et al. 2010). Soil inorganic N was much lower in the CMC + NF treatment than in the NF treatment, indicating more inorganic N was incorporated into microorganisms (Cheng et al. 2014). Soil microbial biomass C and N increased with the amendment of CMC (Fig. 3), indicating that microbial N turnover rates were promoted and that inorganic N fertilizer can be more easily incorporated into soil microbial biomass. Soil C and N cycling are tightly coupled in soils. Barrett and Buke (2000) showed that soil carbon content alone can explain more than 60 % of the variation in potential N immobilization. Organic amendments typically increased soil microbial biomass through the supply of available C as a food source to the generally C-limited microbes in arable soils (Diacono and Montemurro 2010; Lazcano et al. 2013). The input of readily available organic compounds can change the predominant soil microbial type (Meidute et al. 2008) and N transformation processes (Bengtsson et al. 2003), and microbial activity is the highest when soil available C and N ratio matches microbial demands (Chen et al. 2014). In this study,

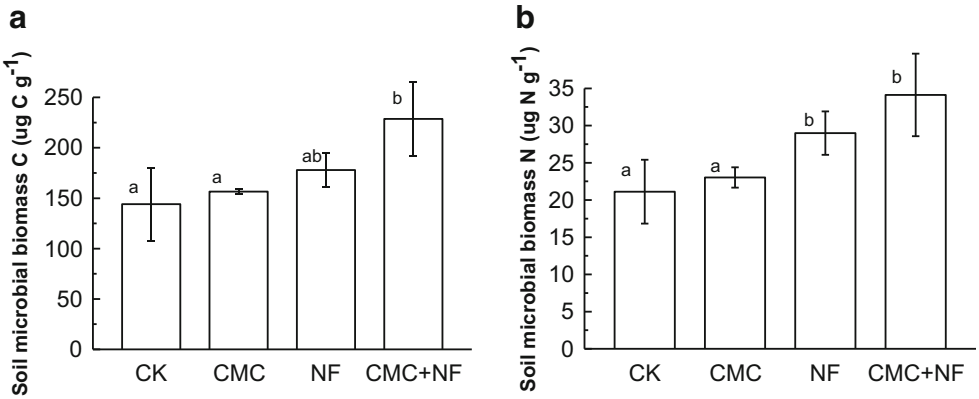

Fig. 3 Microbial biomass C (**a**) and N (**b**) at the end of labeling. *CK* control, *CMC* organic carbon alone, *NF* N fertilizer alone, *CMC + NF* combined organic carbon and N fertilizer. Values are means ± s.d. ($n = 3$)

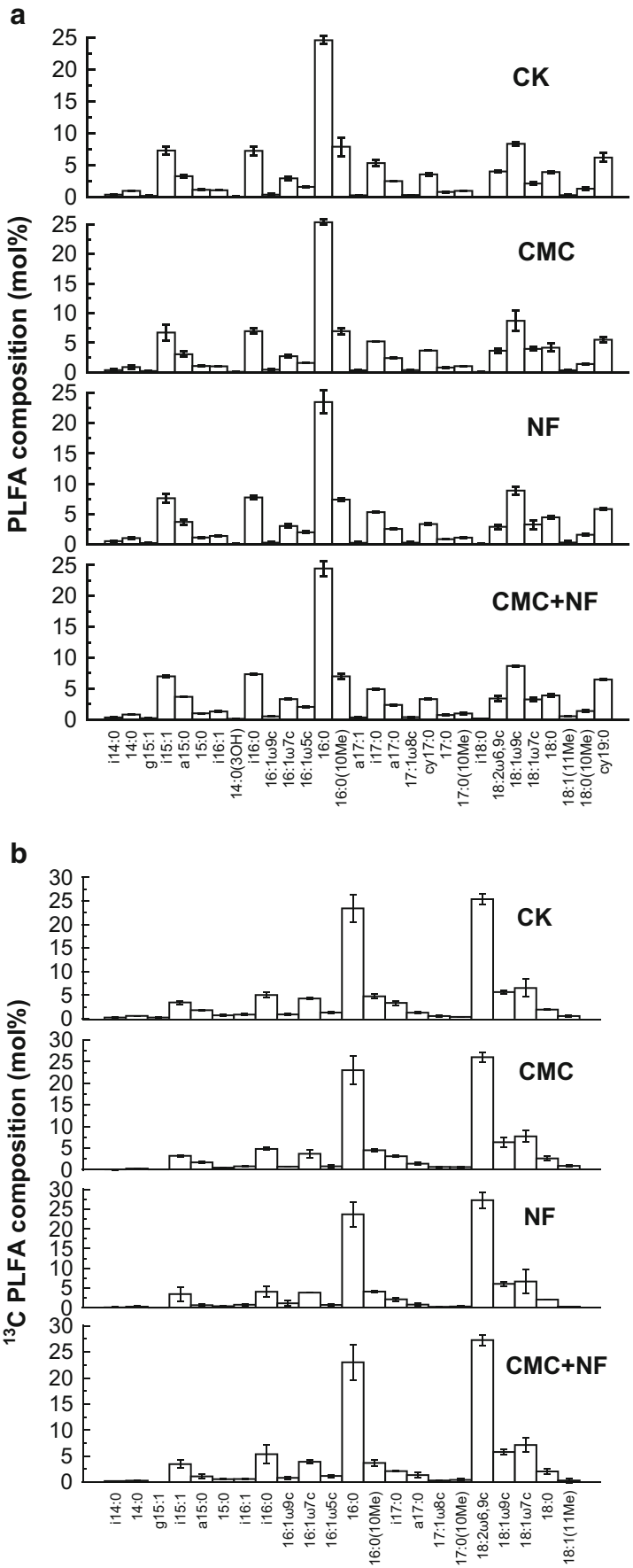

Fig. 4 Relative abundance (mol %) of total PLFAs (**a**) and ^{13}C-PLFAs (**b**) at the end of labeling. *CK* control, *CMC* organic carbon alone, *NF* N fertilizer alone, *CMC + NF* combined organic carbon and N fertilizer. Values are means ± s.d. ($n = 3$)

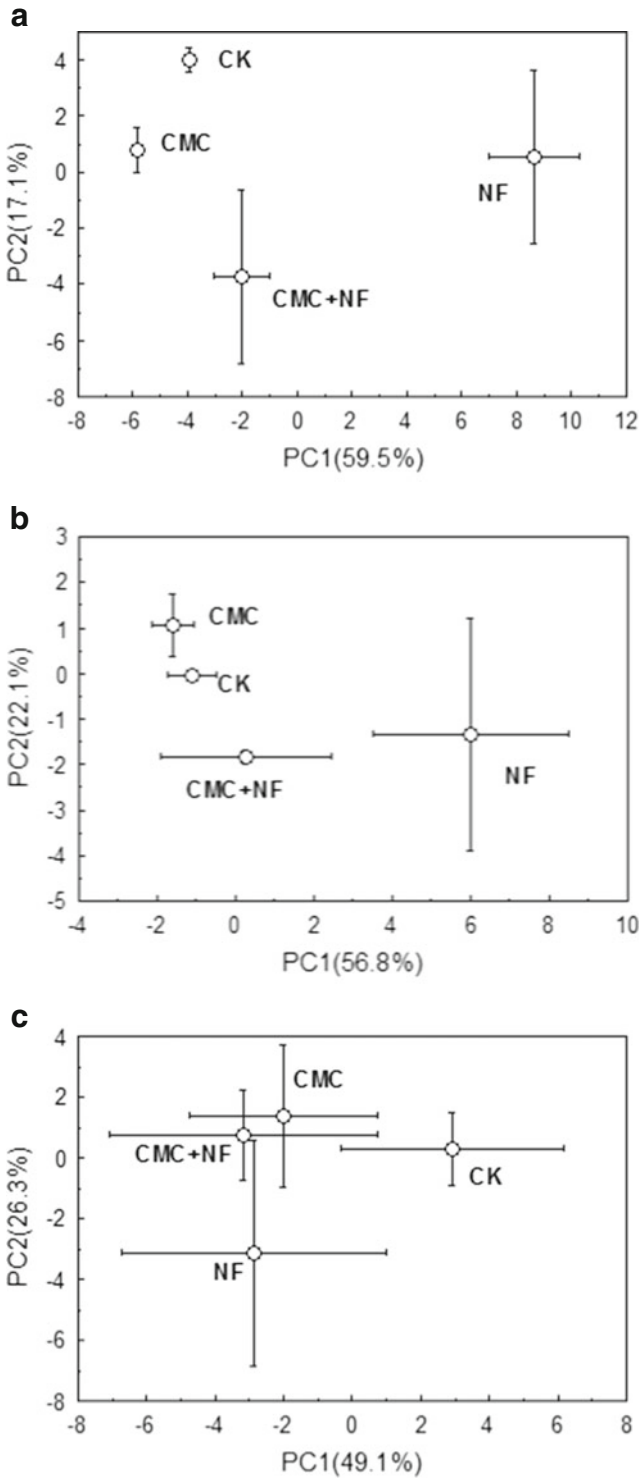

Fig. 5 Principal component analysis of the relative abundance (mol %) of ^{13}C PLFAs of individual soil samples after labeling 5 days (**a**), 10 days (**b**), and 15 days (**c**). *CK* control, *CMC* organic carbon alone, *NF* N fertilizer alone, *CMC + NF* combined organic carbon and N fertilizer. Values are means ± s.d. ($n = 3$)

the soil C/N ratio was quite low (8.9), and soil labile organic C was very limited for microbial N cycling. The enhanced microbial N turnover and plant productivity in the combination of CMC and inorganic N fertilizer treatment could be accounted for by the appropriate stoichiometric C and N ratios of the substrate input.

Similar to soil microbial biomass, application of inorganic N fertilizer or organic amendment (CMC) increased the amount of PLFA. The whole soil PLFA and ^{13}C-PLFA were significantly correlated with soil microbial biomass and plant yield. Available C and N are two key limiting factors for soil microbial growth (Flavel and Murphy 2006). Both inorganic N fertilizer and organic fertilizers can stimulate microbial processes, increase soil microbial biomass, and crop productivity by improved nutrient availability (Sarathchandra et al. 2001; Liu et al. 2010). Generally, different types of amendments differ in organic matter composition or C/N ratio, and this in turn, can affect the transformation rate and change microbial community structure (Marschner et al. 2003). Differences in microbial composition in soil with chemical and organic fertilization were identified based on the analysis of 16S rRNA gene libraries (Gu et al. 2009; Su et al. 2015). Interestingly, there was no significant change in soil microbial community structure with the addition of different amendments based on PLFA profile pattern. This result suggested that changes in microbial biomass and nutrient availability do not always involve a change in microbial community structure (Yao et al. 2000; Franco-Otero et al. 2012), since even the same species of microorganisms can carry out different functions and activities. Due to the complexity and heterogeneity of C sources in soil-plant ecosystems, including root exudates, soil organic matter, and plant litter, specific microbial groups can feed on different organic compounds, and the diversity in labile C sources is likely to support the similar microbial PLFA pattern observed in the present study. On the other hand, PLFA patterns do not provide high-resolution phylogenetic information or detailed species composition (Frostegård et al. 2011). The limitation of the low resolution in PLFA analysis may be another reason for no significant difference in PLFA patterns. Although different amendments had no significant effect on the soil PLFA pattern, some specific PLFA indicators changed in the different treatments. The ratio of cy17:0/16:ω7c, which is an indicator of environmental stress (Frostegård et al. 2011), was much higher in the inorganic-only fertilizer treatments (1.7) than in the other treatments (1.2–1.3). This result may suggest a high level of inorganic N has a stress effect in the C-limited soil.

The microbial PLFA profile derived from root exudates was distinct from the whole soil PLFA profile, which suggested that a subset of the microbial population assimilated ryegrass root derived C (Fig. 5). Most of the ^{13}C (>60 %) was distributed into four straight chain fatty acids, 18:2ω6,9c, 16:0, 18:1ω7c, and 18:1ω9c, in all the soil treatments. This suggested that microbial groups containing these PLFAs were relatively more active and competitive for the available substrates released from ryegrass roots than microbial groups indicated by total PLFA which received little ^{13}C. The observed

differences in the whole soil PLFA and ^{13}C-PLFA profiles are in agreement with Butler et al. (2003) who showed that most plant-derived C was incorporated into 18:2ω6,9c, followed by 16:0 and 18:1ω7c. Carbon substrate is the main reason to select for specific microbial groups and to change the composition of the soil microbial community (Ros et al. 2006; Zhong et al. 2010). The high heterogeneity of available C sources in soil may result in a more even distribution in whole soil PLFAs than in ^{13}C PLFAs. The most common PLFAs in eukaryotic soil microorganisms, predominantly fungi, include 18:2ω6,9c, 16:0 and 18:1ω9c. Nearly all PLFA-SIP experiments suggest that these PLFAs have a high incorporation of plant rhizodeposition C (Butler et al. 2003; Lu et al. 2004; Yao et al. 2012). However, since plant root usually contain the PLFAs 18:2ω6,9c, and 18:1ω9c (Lovell et al. 2001; Lu et al. 2004), some input of PLFAs derived from fine ryegrass roots cannot be completely excluded.

In PLFA-SIP analysis, one important advantage is to be able to determine the sources of C used by soil microorganisms based on ^{13}C-PLFA turnover. Most ^{13}C-labelled rhizodeposition C was distributed into characteristically fungal (18:2ω6,9c) and gram-negative bacterial (18:1ω7c) PLFAs, and their rates of increase in δ^{13}C were very high (>4‰ d^{-1}), suggesting that these PLFAs are derived from microbes mainly reliant on rhizodeposition. In contrast, the relative abundance of i15:1, a17:0 and i17:0 (characteristic of gram-positive bacteria) was higher in whole soil PLFA than that in ^{13}C-PLFA (Fig. 4). The rates of δ^{13}C increase were relatively slow (<2‰ d^{-1}), suggesting that these PLFAs were mainly derived from microbes more reliant on soil organic matter. The high ^{13}C enrichment and turnover rate of 18:2ω6,9c and 18:1ω7c confirmed that it is fungi and gram-negative bacteria that preferentially utilize plant-derived labile C sources. Similar clear distinctions have been observed between communities degrading soil- and litter- derived C, with proportionally more fungi and general microorganisms utilizing litter derived C and more gram-positive bacteria utilizing soil-derived C (Creamer et al. 2015).

The δ^{13}C values of soil PLFA increased during the steady-state labeling indicating that root-derived C is an important source of readily available C for soil microorganisms. Both the total ^{13}C incorporation into PLFAs and plant biomass were the highest in the CMC + NF treatment. The results suggested that C substrate availability is the most important factor for microbial growth in this soil (Nguyen 2003). The importance of root exudation in regulating the size and composition of the microbial community is well known (Broeckling et al. 2008). In response to exudates, changes in microbial population and activity may stimulate a microbial demand for other nutrients, which can be met by increasing enzyme synthesis and promoting N transformations (Mergel et al. 1998; Dijkstra et al. 2009). Yin et al. (2013) demonstrated that root exudation can influence soil organic matter decomposition and microbial N cycling. Principal component analysis on the relative abundance of ^{13}C-PLFAs showed that inorganic N fertilizer had a significant effect on the incorporation of ^{13}C-labeled rhizodeposition C into soil microbial communities, implying an influence of plant root andmineral N interactions on soil microbial communities. Our current results provide knowledge of how different amendments affect the utilization of ryegrass rhizodeposition by soil microorganisms. The PLFAs i16:0 and i17:0, which are indicators of gram-positive bacteria (Butler et al. 2003), showed the highest ^{13}C enrichment in the NF treatment, while 18:2ω6,9c, the indicator of fungal PLFA (Frostegård et al. 2011), was more enriched in the other treatments. The lower ^{13}C enrichment of PLFA18:2ω6,9c in the NF treatment suggested that fungi generally exhibit a lower utilization of rhizodeposition C than soil-derived C with high inorganic N content. The previous studies found that inorganic fertilizers can suppress the utilization of rhizodeposition C by Actinobacteria but increase the utilization by Fibrobacteres (Ai et al. 2015). The changes in ^{13}C-PLFA pattern implied that the increase in soil N availability can decrease the dependence of rhizosphere microorganisms on plant-derived C sources. The effect of soil available N on the soil microbial community assimilating rhizodeposition C may be due either to the altered quality and quantity of root exudates (Kuzyakov et al. 2002) or to a change in microbial C use efficiency (Shi et al. 2006; Ai et al. 2015).

5 Conclusions

The results of this study have demonstrated that the application of ammonium nitrate combined with CMC significantly enhanced soil microbial biomass and N transformation processes, which are mainly achieved by the appropriate C and N ratio of substrate input. On the other hand, the impact of ryegrass roots on the microbial community structure is strongly modified by soil N availability. The increase in N availability can reduce the dependence of soil microorganisms on rhizodeposition C. Further work is needed to elucidate the changes in detailed species composition and phylogenetic structure using high-resolution nucleic acid-based techniques.

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