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Microbial interactions affect sources of priming induced by cellulose

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ABSTRACT

The recently developed 3-source-partitioning approach: addition of ^{14}C labeled organics to soil after C3–C4 vegetation changes, was used to distinguish C sources in three compartments, namely CO_2, microbial biomass and dissolved organic C (DOC) during decomposition of labeled cellulose. Microbial community structure (based on PLFA composition) and functions (based on enzyme activities and on microbial growth parameters) revealed mechanisms and drivers of priming effects (PE) induced by cellulose addition.

^{14}C-cellulose input caused negative PE within the first week and was accompanied by fast consumption of unlabelled DOC and its incorporation into microbial biomass. Microbial activation however, was not confirmed by substrate-induced respiration, nor by hydrolytic enzymes activity or by PLFA changes. A remarkable exception was a 2-fold increase in protozoan PLFA. Such an increase indicates that microorganisms feeding on cellulose and on DOC were quickly grazed by protozoans acting as a driver of microbial succession. This experimentally demonstrates the functioning of the microbial interactions: protozoan grazers provided for rapid recycling of nutrients and facilitated the succession of cellulose-degrading microorganisms during the second week of cellulose decomposition. An increase in the activity of cellulolytic enzymes caused short-term real PE accompanied by increase in abundance of slow-growing fungi and G(−) bacteria. Long-term real PE observed between 14 and 60 days after cellulose input was due to decomposition of SOM-originated hemicelluloses by fungi and G(+) bacteria. The CO_2 released by primed soil organic matter (SOM) decomposition was originated mainly from C younger than 12 years (63%) and only 37% were older than 12 years despite the recent and old C contributed almost equally (51 and 49%, respectively) to SOM under *Miscanthus giganteus*. This indicates that the SOM pools are involved in PE according to their availability. Despite 71% of the applied cellulose-C was sequestered in the soil, the net soil C-gain amounted only for 28% of the applied cellulose-C after factoring in the C losses by the PE. Our study emphasizes the role of food webs in the PE dynamics: cellulose input served as a driver activating the food chain through the microbial loop.

1. Introduction

The stability of global terrestrial C stocks such as soil organic matter (SOM) is mainly under environmental and biological control (Schmidt et al., 2011). It is also often affected by pulse inputs of plant-originated C (plant residues and rhizodeposits), which induce the cascade of reactions between living and dead pools of SOM (West and Six, 2007; Kuzyakov, 2010; Xu et al., 2011). This cascade comprises increase in respiratory activity and in enzyme production, changes in amounts of easily-available C and of soluble C. This, in turn, alters the size and structure of the soil microbial community (Paterson, 2009; Kuzyakov, 2011) and triggers priming effects (PE), i.e. accelerated SOM decomposition. It remains unclear which drivers are responsible for the decomposition of heterogeneous SOM pools co-existing in soil but differing in age, turnover rates and availability (Bengtson and Bengtsson, 2007; von Lützow and Kögel-Knabner, 2009; Salome et al., 2010). A number of PE mechanisms

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are currently being discussed from the perspective of competitive interactions between a broad range of prokaryotic and eukaryotic organisms in the soil. They are present in interconnected networks and food webs that are highly responsive to local environmental conditions (Coleman et al., 2004; Heaton et al., 2012).

One of the suggested mechanisms is that the decomposition of recalcitrant SOM involves succession and competition between fast- and slow-growing microorganisms, i.e. r- and K-strategists, respectively (Fontaine and Barot, 2005). Both microbial groups are activated by the input of available substrate; after its exhaustion, however, r-strategists lose the competition, while K-strategists produce specific enzymes that can decompose recalcitrant SOM compounds. It has also been hypothesized that the input of plant residues stimulates production of microbial enzymes that decompose recalcitrant SOM (Schimel and Weintraub, 2003; Allison et al., 2010; Schimel and Schaeffer, 2012). There is a lack of information on changes in microbial functions such as growth rates and enzyme activity during priming effects in the soil. Thus, neither hypothesis (microbial succession or enzymatic activity induction) has yet been experimentally proven. Enzymes that degrade cellulose (a major component of plant residues) can also decompose cellulose-like SOM compounds (Fontaine et al., 2004b). Structural changes in both bacterial and fungal communities were also detected during PEs induced either by cellulose (Fontaine et al., 2011) or by plant residues (Bernard et al., 2007; Nottingham et al., 2009). Nonetheless, it remains unclear how these changes in microbial community composition altered microbial functions, e.g., enzyme activity and microbial growth rates. Furthermore, to understand the underlying processes, consideration should be given to microbial loops which is a mechanism of bacterial and fungal interactions with microfauna. According to the microbial loop concept (Azam et al., 1983; Clarholm, 1985; Coleman, 1994), the concentration of plant-originated labile C in soil is very low; moreover, this C source is usually unavailable for soil microfauna, whereas it can be taken up by bacteria. C incorporated in bacterial cells represents a more concentrated C pool than DOC, and this pool is available for protozoans and other animals (Panikov, 2010). Thus, the microbial loop is considered as a kind of food web strongly influencing the decomposition of organics. How the soil PEs are affected by such microbial loops needs to be studied to shed light on PE mechanisms.

Even though tiny amounts of available substrate can induce PE, such effects are mainly apparent i.e. caused by accelerated microbial metabolism which is not followed by SOM decomposition (Blagodatskaya et al., 2007). Amounts of easily available C exceeding microbial biomass, however, caused real priming of both old and recent SOM (Blagodatskaya et al., 2011b). It remains unclear, at what extent old and recent SOM are subjected to real priming induced by low available substrate. The direct confirmation of real PE is an increase of SOM-originated C not only in CO_2, but also in microbial biomass (Blagodatskaya et al., 2011b). Thus, the partitioning of C sources in microbial biomass is required to distinguish the pathways of old and recent C involved in real PE. This necessitates the coupling of at least two isotopic approaches that enable 1) partitioning of primer C and SOM C, and 2) partitioning of old and recent C from SOM in pools and fluxes traced for priming: DOC, CO_2 and microbial biomass. Most previous studies on partitioning of C sources were conducted for the PEs induced by easily available organic substances of low molecular weight such as sugars and amino acids (Kuzyakov and Bol, 2004, 2006; Blagodatskaya et al., 2011a, see Blagodatskaya and Kuzyakov, 2008 for review). Only few experiments have distinguished the C sources in the PE occurring during decomposition of high molecular substrates that are not immediately available for microorganisms (Subke et al., 2004; Nottingham et al., 2009). Following Schimel and Weintraub (2003), we assume that the hydrolytic and oxidative enzymes produced to decompose natural polymers contained in plant residues such as cellulose are also involved in SOM decomposition. Thus, we hypothesized larger contribution of relatively recent SOM originated from partly decomposed plant residues to the PE induced by cellulose. Therefore, in contrast to most previous experiments of our and other groups, we applied ^{14}C cellulose as a primer substance to soil originating from a C3–C4 vegetation change in which the old (>12 years) and recent SOM have a different $\delta^{13}C$ signature. Accordingly, we combined artificial ^{14}C labeling with the partitioning based on ^{13}C natural abundance. This enabled distinguishing the relative and absolute contribution of three C sources: substrate added (^{14}C), recent (enriched ^{13}C) and old (depleted ^{13}C) SOM to the three C pools: CO_2, microbial biomass and DOC.

Our study was designed 1) to estimate the contribution of recent and old C in the PE induced by cellulose; and 2) to relate the PE observed with functional (microbial growth, enzymes activity) and structural (PLFA) changes in the microbial community. Protozoan PLFA (White et al., 1996; Fierer et al., 2003) were included in the analysis to reveal the presence and intensity of the microbial loop. Beyond examining the enzymes decomposing plant residue components cellulose (β-glucosidase and cellobiohydrolase) and hemicelluloses (xylanase) – we determined the activity of chitinase as an indicator of fungal biomass. This enabled us to relate the composition and functioning of the microbial community to their role in priming effects during cellulose decomposition.

2. Materials and methods

2.1. Soil sampling and preparation

Soil – a loamy Gleyic Cambisol (WRB, 1998) was sampled from the upper layer (0–10 cm) of a field under *Miscanthus giganteus* ($C_{org} = 2.4 \pm 0.1\%$; $N_{total} = 0.20 \pm 0.01\%$, pH ($CaCl_2$) 5.1) and under an adjacent grassland ($C_{org} = 2.1 \pm 0.2\%$; $N_{total} = 0.21 \pm 0.01\%$, pH ($CaCl_2$) 5.1) at the experimental station of the University of Hohenheim, Baden-Württemberg, Germany (48°43′N, 9°13′E). The C_4 plant *M. giganteus* (Greef et Deu) ($\delta^{13}C = -11.8 \pm 0.21‰$) was grown for 12 years after grassland, causing a shift in the $\delta^{13}C$ of SOM from $-27 \pm 0.29‰$ to $-19 \pm 0.28‰$, which was most pronounced in upper soil layer (Schneckenberger and Kuzyakov, 2007). This $\delta^{13}C$ shift was used to distinguish between SOM older and younger than 12 years. We use the term 'recent' C for the C_4–C originated from *M. giganteus* because it is not older than 12 years. In contrast, for the C originated from previous C3 vegetation, which older than 12 years, we use the term 'old' C.

Prior to the experiment, the soil was sieved (<2.5 mm) and large roots and other plant debris were carefully removed. Fifteen gram (dry weight) sub-samples were pre-conditioned in 150-ml jars at 50% of the water holding capacity (WHC) according to Blagodatskaya et al. (2011b).

2.2. Experiment design and substrate application

Soil from both plots (C3–C4 soil under *M. giganteus* and C_3 soil under grassland used as C3 reference) was amended either with distilled water (control) or with cellulose. Uniformly labeled ^{14}C-cellulose, BIOTREND Chemicals, Köln, Germany was added to the unlabeled microcrystalline cellulose and thoroughly mixed in ball mill before being added to the soil at rate of 400 μg C g^{-1} soil (specific ^{14}C activity 17 kBq mg^{-1}) which corresponded to the rate of cellulose amendment in previous studies on priming effect (Fontaine et al., 2004b; Fontaine et al., 2011). Cellulose was applied to soil as a powder and was thoroughly mixed by spatula; then soil

was moistened with distilled water to reach a final moisture content of 60% of WHC. The C3 soil (with distilled water or with cellulose) was used as reference to estimate the $\delta^{13}C$ shifts between the pools caused by ^{13}C isotopic fractionation (Werth and Kuzyakov, 2010; Blagodatskaya et al., 2011b). The C3–C4 soil under the *M. giganteus* plot treated solely with water was used as control to estimate the changes in $\delta^{13}C$ caused by preferential utilization of ^{13}C-enriched recent C (Blagodatskaya et al., 2011a). Accordingly, the experimental design included 4 treatments: 2 soils with or without addition of cellulose. Thirty jars for each treatment of the respective soil were incubated and were further used for destructive samplings (in total 30•4 treatments = 120 jars, see below).

2.3. Incubation and sampling

After adding distilled water to the soil, small vials with 1.5 mL of 1 M NaOH were placed in the incubation vessels to trap CO_2. The jars were immediately closed air-tight and incubated for 103 days at 22 °C at 60% WHC. Periodically, the jars were opened for aeration, the vials with NaOH were removed and replaced by vials with a new 1.5 mL aliquot of 1 M NaOH. Aliquots of sampled NaOH from the three randomly chosen replicate jars for each treatment were used to measure the ^{14}C activity and total amount of trapped CO_2. These measurements were done at 1, 4, 7, 9, 12, 14, 19, 23, 27, 33, 48, 61, 71, 90, 103 days, (15 samplings in total). Another three jars for each treatment were used for the sampling of $^{13}CO_2$ at 7, 14, 60 and 103 days of the incubation. We made 3 destructive samplings (at 7, 14 and 60 days) and used 8 jars for each (15 g * 8 = 120 g of soil); in total 24 jars were used in each treatment to determine microbial biomass, DOC and $\delta^{13}C$ analyses for these two pools. Simultaneously microbial growth parameters, PLFA content and enzyme activity were measured in the same soil samples, namely during intensive (7 and 14 days) and slow (60 days) phases of cellulose decomposition (see below) when the largest changes in the PE were expected. The rest 6 jars in each treatment were used at the end of experiment to determine microbial biomass only.

2.4. Microbial biomass and DOC

Soil microbial biomass was determined by the chloroform fumigation extraction method (modified after Vance et al., 1987). After destructive sampling the soil was carefully mixed and moist soil (containing the equivalent of 10 g of oven-dry soil) was extracted with 40 ml of 0.05 M K_2SO_4. Another 10 g of soil (dry weight basis) were firstly fumigated with chloroform for 24 h and then extracted in the same way. The extracts were frozen until analyses for total C concentrations using a TOC/TIC analyzer (Multi N/C 2100, Analytik Jena, Germany).

The total amount of extractable microbial C (C_{MB}) was determined by the difference between K_2SO_4-extractable C in fumigated and non-fumigated soil using k_{ec} factor 0.45 (Wu et al., 1990). The cellulose-derived microbial C (C_{MB_C}) was calculated based on ^{14}C activity in microbial biomass divided by the ^{14}C-specific activity ($^{14}C/C$) of the added cellulose.

2.5. Chemical and isotopic analyses

CO_2 trapped in NaOH solution was precipitated with 0.5 M $BaCl_2$ solution. The total amount of CO_2 trapped in the vials was measured by titration of the NaOH excess with 0.1 M HCl using the phenolphthalein indicator (Zibilske, 1994).

For ^{13}C analyses the CO_2 trapped as Na_2CO_3 in 1.5 ml of NaOH was precipitated with 2 ml of 0.5 M $SrCl_2$ aqueous solution, washed and dried (see details in Blagodatskaya et al., 2011a, b). The $SrCO_3$ was analyzed for $\delta^{13}C$ values on the IRMS (Delta Plus XL IRMS, Thermo Finnigan MAT, Bremen, Germany). For the K_2SO_4-soluble C and for the microbial biomass C, an aliquot of the K_2SO_4 samples was pipetted directly into tin capsules and dried at 60 °C prior to IRMS analyses (according to Brant et al., 2006).

The ^{14}C activity of NaOH with trapped CO_2 was measured in the scintillation cocktail Rotiszint Eco Plus (Carl Roth, Germany) after decay of the chemiluminescence using a 1450 LSC & Luminescence Counter MicroBeta TriLux (Perkin Elmer Inc., USA). The ^{14}C counting efficiency was 87% and the ^{14}C activity measurement error did not exceed 2%.

2.6. Calculations to partition the three C sources

Calculations based on the three-source-partitioning were similar to those described in detail in Blagodatskaya et al. (2011b). Briefly, to partition three sources of C in CO_2, microbial biomass and DOC, we used the following steps. Initially, *the amount of cellulose-originated C in each pool* ($C_{Cel\text{-}derived}$, μg) was calculated based on the current ^{14}C radioactivity of the corresponding pool ($^{14}C_{curr}$, DPM), the amount of added cellulose (C_{Cel}, μg), and the radioactivity of the applied cellulose ($^{14}C_{Cel}$, DPM):

$$C_{Cel-derived} = {}^{14}C_{Curr} \cdot C_{Cel} / {}^{14}C_{Cel}. \quad (1)$$

Then, the amount of SOM-derived C was calculated as:

$$C_{SOM-derived} = C_{total} - C_{Cel-derived}, \quad (2)$$

where C_{total} is the total amount of C in the corresponding pool (CO_2, DOC, microbial biomass).

In the second step, we calculated *the $\delta^{13}C$ value of SOM-originated C* in each pool ($\delta^{13}C_{SOM\text{-}derived}$) based on a mass balance equation (Balesdent and Mariotti, 1996). The $\delta^{13}C$ signature of cellulose-derived C (see below) was subtracted from the $\delta^{13}C$ signature of each pool, considering the contribution of the amount of cellulose-originated C estimated in the first step based on ^{14}C:

$$\delta^{13}C_{SOM-derived} = \left(\delta^{13}C_{total} \cdot C_{total} - \delta^{13}C_{Cel-derived} \cdot C_{Cel-derived}\right) / \left(C_{total} - C_{Cel-derived}\right) \quad (3)$$

where $\delta^{13}C_{total}$ and $\delta^{13}C_{Cel\text{-}derived}$ are the $\delta^{13}C$ values of the total and cellulose-originated C. The former value was measured experimentally as described in Section 2.5.

In the third step, *the contribution of recent and old C* in each pool in the C_3–C_4 soil was calculated based on the cellulose-corrected $\delta^{13}C$ signature of each pool (Eq. (3)) considering isotopic fractionation, which was assumed to be equal in reference and in *Miscanthus* soil. The amount of recent C_4-originated C in each pool is:

$$C_{C4-derived} = C_{SOM-derived} \cdot \left(\delta^{13}C_{SOM-derived} - \delta^{13}C_{C3\_ref}\right) / \left(\delta^{13}C_{C4\_plant} - \delta^{13}C_{C3\_plant}\right) \quad (4)$$

where $\delta^{13}C_{C3\_ref}$ is the $\delta^{13}C$ value of the corresponding pool in the reference C_3 soil at the corresponding sampling date calculated according Eq. (3). The denominator of Eq. (4) is equal to 16.2‰, i.e. to the difference between $\delta^{13}C$ signatures of C_4 and C_3 vegetation for our experiment corrected for isotopic fractionation during humification according to Schneckenberger and Kuzyakov (2007). Isotopic fractionation during SOM mineralization in the course of the incubation experiment was assumed to be equal for the soil under Miscanthus and for the reference soil with solely C3

vegetation (Werth and Kuzyakov, 2010). The corrections for $\delta^{13}C$ signatures of CO_2, microbial biomass, and DOC were made according to the data for isotopic fractionation in the reference soil under C3 vegetation. C_3-originated C in each pool was then calculated by subtracting the C_4-originated C (Eq. (4)) from the total amount of C in the corresponding pool.

In the last step, *the PE and the contributions of old and recent C to the PE* were calculated based on the changes in the $\delta^{13}C$ signature and the amount of extra-C in the three pools after cellulose addition (compared with the treatment without cellulose).

Cumulative PE, expressed in μg CO_2–C per g soil, was calculated based on experimental data as:

$$PE = {}^{12}CO_2^{amended} - CO_2^0 - CO_2^{Cel\text{-}derived}, \quad (5)$$

where ${}^{12}CO_2^{amended}$, $CO_2^{Cel\text{-}derived}$ and CO_2^0 is unlabeled ^{12}C–CO_2 evolved from soil amended with ^{14}C-cellulose, CO_2 originated from cellulose and CO_2 originated from soil without substrate addition, respectively.

The changes in $\delta^{13}C$ signature caused by preferential substrate utilization – which is not isotopic fractionation *per se* but is considered as preference for easily available and ^{13}C-enriched recent C (compared with ^{13}C depleted old C) – were considered for correct assessment of PE in all pools. The dynamic changes in $\delta^{13}C$ caused by preferential utilization were estimated in control C_3–C_4 soil treated solely with H_2O. For each pool, the PE was calculated separately for C_3 and C_4 carbon sources (C_{3_PE} and C_{4_PE}, respectively) considering the changes in contribution of old and recent C in control soil for each sampling date:

$$\begin{aligned} C_{3\_PE} &= C_{3\_CO_2}^{amended} - C_{3\_CO_2}^{control} \\ C_{4\_PE} &= C_{4\_CO_2}^{amended} - C_{4\_CO_2}^{control} \end{aligned} \quad (6)$$

The following equation was used to determine *the* $\delta^{13}C$ *of total microbial biomass* ($\delta^{13}C_{MB}$):

$$\delta^{13}C_{MB} = \left(\delta^{13}C_f \cdot C_f - \delta^{13}C_{nf} \cdot C_{nf}\right) / \left(C_f - C_{nf}\right) \quad (7)$$

where $\delta^{13}C_f$ and $\delta^{13}C_{nf}$ are the $\delta^{13}C$ values of the fumigated and non-fumigated samples, respectively, and C_f and C_{nf} are the amounts of C in the fumigated and non-fumigated K_2SO_4 samples, respectively.

2.7. Kinetics of substrate-induced respiration and calculation of microbial growth parameters and biomass

The dynamics of the CO_2 emission from soil amended with glucose and nutrients was used for estimation of carbon content in microbial biomass (C_{mic}) and the kinetic parameters of microbial growth (Blagodatsky et al., 2000). Soil sample (12.0–12.5 g wet weight, equivalent to 10 g dry soil) was amended with a powder mixture containing glucose (10 mg g^{-1}), talcum (20 mg g^{-1}), and mineral salts: $(NH_4)_2SO_4$ 1.9 mg g^{-1}, K_2HPO_4 2.25 mg g^{-1}, and $MgSO_4$ * $7H_2O$ 3.8 mg g^{-1}. Substrate concentrations sufficient for unlimited exponential growth of microorganisms were estimated in preliminary experiments in which increasing amounts of glucose were added. The amount of mineral salts was selected so that the substrate changed the pH of soil to <0.1 (Blagodatskaya et al., 2007).

After addition of the glucose–talcum mixture and mixing with a handheld kitchen blender, the soil samples were immediately placed into 24 plastic 50-mL tubes and the rate of CO_2 production was measured. Each sample was continuously aerated (100 mL min^{-1}) at 22 °C and the evolved CO_2 was measured every 1.5 h using an infrared detector (Gas Exchange Measurement System 2250, ADC, UK) connected to the gas handling unit with a flowmeter (ADC).

Microbial respiration in glucose amended soil (Substrate Induced Growth Response, SIGR) was used to calculate the following kinetic parameters: the specific growth rate of microorganisms (μ), the microbial biomass capable for immediate growth on glucose (AMB) – we accept that this part corresponds to active microbial biomass, physiological state index of microbial biomass before substrate addition (r_0), the total microbial biomass (TMB) responding by respiration to glucose addition, and the lag period (t_{lag}). This method was suggested by Panikov and Sizova (1996). The details of experimental procedure, calculations, fitting, and statistics are presented in Wutzler et al. (2012).

2.8. Phospholipid fatty acid analysis

A standard procedure of phospholipid fatty acid (PLFA) extraction with chloroform:methanol:citrate buffer (1 : 2 : 0.8; pH 4) followed by acid methylation to fatty acid methyl esters (FAME) and derivatization (Frostegård et al., 1991) was applied. The internal standard was 15 μg PLFA 19:0 (Biotrend, 50933 Cologne, Germany), and recovery standard 10 μL 13:0 FAME (SigmaeAdrich, 82024 Taufkirchen, Germany). Separated FAMEs were analyzed by capillary gas chromatography equipped with a flame ionization detector (GC-FID). For further details see Schmitt and Glaser (2011). Terminal-branched saturated PLFA (a15:0, i15:0, i16:0, i17:0, a17:0) were considered as markers for Gram-positive bacteria (G+); monounsaturated fatty acids (16:1ω7c, 18:1ω7c) and cyclopropyl saturated PLFA (cy17:0; cy19:0) were associated with Gram-negative bacteria (G-) (Frostegård et al., 1993); midchain branched saturated PLFA (10Me16:0, 10Me17:0, 10Me18:0) were associated with actinomycetes (Zelles, 1999). Typical markers for fungal PLFA (18:2ω6,9, 18:1ω9c) were used to indicate fungal biomass (Frostegård and Bååth, 1996), while the protozoans were identified by PLFA 20:4ω6,c (Moore-Kucera and Dick, 2008; Esperschütz et al., 2009). Short- or odd-chain saturated PLFA (14:0, 15:0, 16:0, 17:0) were considered as non-specific makers present in all microorganisms (Zelles, 1999).

2.9. Enzyme assays

Extracellular enzyme activities in soil were measured using fluorogenically labeled substrates according to Sanaullah et al. (2011). Four fluorogenic enzyme substrates based on 4-methylumbelliferone (MUF) were used for the assessment of enzyme activities: MUF-β-D-xylopyranoside (MUF-X; EC 3.2.1) for xylanase, MUF-β-D-cellobioside (MUF-C; EC 3.2.1) for β-cellobiosidase, MUF-β-D -glucopyranoside (MUF-G; EC 3.2.1.21) for β-glucosidase and MUF-N-acetyl-β-D-glucosaminide dehydrate (MUF-NAG; EC 3.2.1.14) for chitinase.

The MUF-substrates were dissolved in 2 ml of 2-methoxyethanol (Hoppe, 1983). Pre-dissolved MUF-substrates were further diluted with sterile distilled water to obtain the desired concentrations (see below). All chemicals were purchased from Fluka (Germany).

The fresh soil samples (equivalent to 1 g soil dry weight) were suspended in water (25 ml) and shaken on an overhead shaker for 30 min at room temperature and at maximum speed (500 rpm) to ensure thorough mixing. A sub-sample of the soil suspension (0.5 ml) was added to 1.5 ml of each substrate solution (containing 200 μmol MUF), already pipetted in Deep Well Plates (24-wells × 10 ml, HJ-Bioanalytik GmbH, Germany). Saturation concentrations of fluorogenic substrates were determined in preliminary experiments. The microplates were incubated at 22 °C for 1 h for enzymes releasing monomers (β-glucosidase, and chitinase)

and 3 h for enzymes releasing oligomers (β-cellobiosidase, xylanase). The calibration solutions were prepared using soil suspension (0.5 ml) and MUF of different concentrations (0–100 μmol, 1.5 ml). Deep Well Plates with the soil-substrates and soil-calibration solutions were centrifuged (3000 rpm for 10 min). Thereafter, 1 ml of supernatant was transferred to 24-well microplates (Becton Dickinson, USA). Inhibition of the reaction and maximization of the fluorescence intensity through alkalinization of the medium was found unnecessary because of the high sensitivity and fast processing power of the analytical equipment used to measure fluorescence (Marx et al., 2001). Fluorescence was measured in microplates within 2–3 min after pipetting at an excitation wavelength of 355 nm and an emission wavelength of 460 nm, slit width of 25 nm, with a Victor^3 1420-050 Multilabel Counter (PerkinElmer, USA).

Calibration curves as well as the controls for the autofluorescence of the substrate and for quenching effect (Marx et al., 2001; Pritsch et al., 2004) were included in every series of enzyme measurements. Enzyme activities were expressed as MUF release in μmol per g soil and hour (μmol g^{-1} h^{-1}).

2.10. Statistical analyses

The means of three replicates with standard errors (SE) for CO_2, microbial biomass and DOC values are presented in the figures. The significance of temporal differences between means for each C source in CO_2, microbial biomass or DOC is shown as LSD (5%) estimated by one-way ANOVA. Standard deviations (SD) of treatment-to-control ratios for enzymes activity and PLFA content were calculated considering the mean values and the SDs for corresponding parameters in control and cellulose amended soil.

3. Results

3.1. CO_2 dynamics and priming effects during cellulose decomposition

Three phases of cellulose decomposition (intensive, slow and retardation of decomposition) were revealed by the $^{14}CO_2$ efflux rate (Fig. 1A). Cellulose applied to both *Miscanthus* (C3–C4) and to reference (C3) soils was decomposed to CO_2 most intensively between day 4 and 14 after addition. Applied cellulose was mineralized to CO_2 on day 14 for 12.3 ± 0.4% (Fig. 1A). A similar amount of ^{14}C (12.8 ± 0.5%) was slowly mineralized during next 46 days, while only 4.2 ± 0.4% of cellulose was evolved as $^{14}CO_2$–C within 60–103 days after cellulose addition, indicating retardation of decomposition (Fig. 1A). In total, 29.4 ± 0.3% of cellulose C was mineralized to CO_2 in the course of the 103-day incubation. Despite intensive cellulose decomposition, no differences in the total amount of CO_2 were observed until day 12 of the experiment between control and cellulose-treated soil (Fig. 1B). This indicated a negative priming effect during the first two weeks after cellulose addition (Fig. 1B) due to a shift in the microbial community from SOM to cellulose decomposition. Primed CO_2 increased already at day 9 after cellulose addition; nonetheless, cumulative PE became positive only at day 19 (Fig. 1B). At the end of incubation the PE caused by cellulose amounted to 25.1% of the CO_2–C evolved from the control and 42.3% of the cellulose-C added to the soil.

3.2. Sources of carbon pools

The C sources were partitioned at the dates of destructive sampling, i.e. for the time intervals 0–7, 7–14, 14–60 and 60–103 days after cellulose addition.

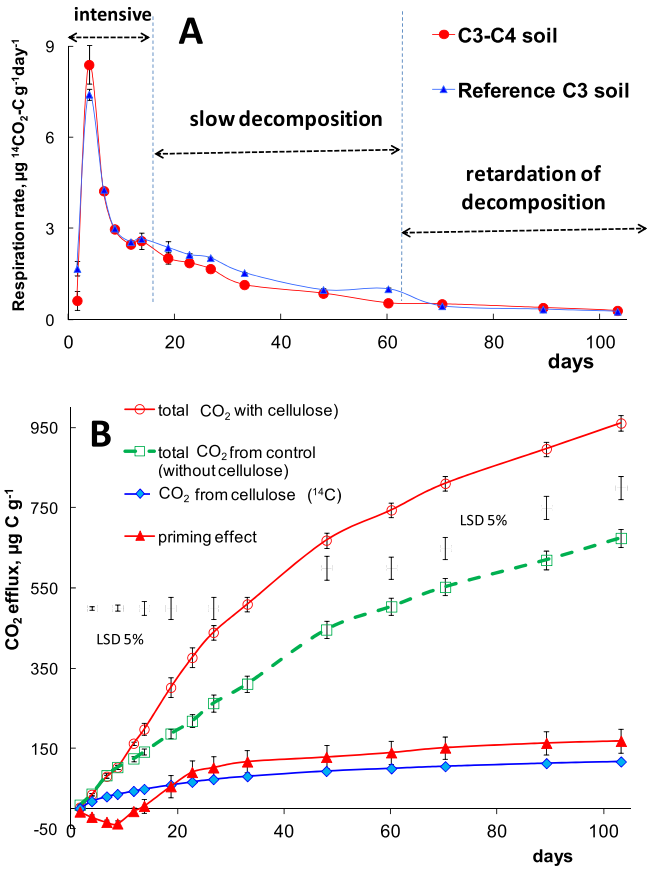

Fig. 1. Dynamics of the cellulose-originated ^{14}C–CO_2 efflux rate from reference (C3) and *Miscanthus* (C3–C4) soils amended with cellulose (A), and cumulative CO_2 fluxes from control and cellulose amended (C3–C4) soils, from cellulose (^{14}C) and calculated cumulative priming effect (B). Bars indicate standard errors of the means (n = 3). The significance of differences between means (C sources) for each sampling is shown as LSD (5%) on panel B.

3.2.1. DOC

An almost 35% decrease in DOC was induced during the first week by cellulose addition (Fig. 2A). This decrease was due to 31% and 43% reduction of recent (C4) and old (C3) C, respectively. The fraction of cellulose-originated C in DOC decreased from 8.2% (first week) to 4.1% (60 days) (Fig. 2A). Despite a gradual increase in the total amount of DOC from the second week after cellulose addition, the final DOC content was still significantly lower at 103 day than before cellulose addition. The portion of old C in total DOC increased from 62 to 77% during cellulose decomposition, while the contribution of recent C decreased from 36 to 13%.

3.2.2. Microbial biomass

Initially, the microbial pool consisted mainly of recent C4–C (80.7%) and only to 19.3% of old C3–C (Fig. 2B). This shows that microorganisms reutilized mainly organics that recently entered the soil. Adding cellulose resulted in a 51% increase in the microbial biomass on day 7. As confirmed by ^{14}C in microbial biomass, this increase was mainly due to cellulose (27%) and increase in old C3-originated SOM (14.5%) (Fig. 2B). However, a further 25% increase in microbial C during the second week of incubation originated exclusively from old C3 (Fig. 2B). The strong decrease in microbial biomass below the initial level during the slow phase (60–103 days) of cellulose decomposition was mainly due to a 2.2-fold reduction in the amount of recent MBC. As a result, the relative contribution of C4-SOM to total biomass decreased from 67 to 56% at the end of incubation.

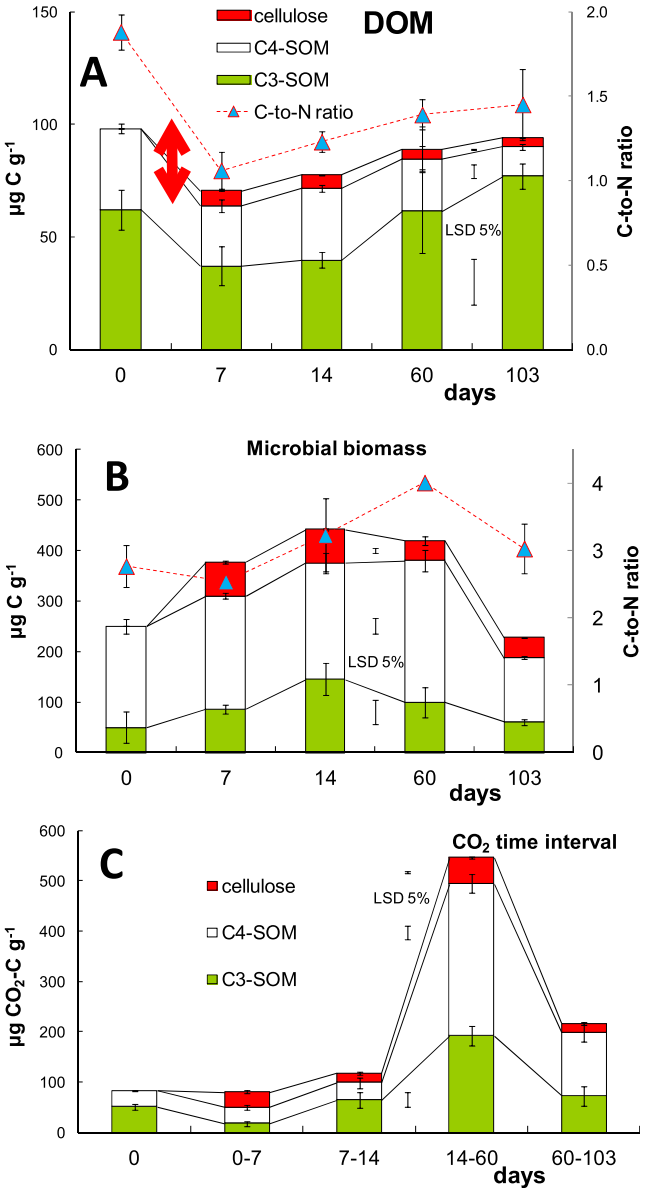

Fig. 2. Contribution of three C sources to dissolved organic matter (A), microbial biomass (B), and to cumulative CO_2 efflux (C), in soil after cellulose addition. The three sources include: 1) SOM-C older than 12 years (C_3-SOM), 2) SOM-C younger than 12 years (C_4-SOM), and 3) C of added cellulose (C-cellulose). C-to-N ratios in DOM and microbial biomass (flush after fumigation) are plotted against right *Y* axes. Error bars indicate standard errors of the means ($n = 3$). The significance of temporal differences between means for each of C sources is shown as LSD (5%).

3.2.3. *CO_2 efflux*

The cumulative CO_2 amount did not differ between the untreated control and soil with cellulose during the first week after addition (Fig. 2C). Nonetheless, about 38% of the respired CO_2 originated from cellulose, indicating the switch of soil microorganisms to cellulose decomposition. The portion of cellulose-C in CO_2 continuously decreased down to 12% of cumulative CO_2 efflux at the end of incubation, while the contribution of recent C increased up to 52%. Old C dominated in the CO_2 efflux during the intensive stage of cellulose decomposition between day 7 and 14 after cellulose treatment, comprising 55% of the CO_2 emission.

3.3. *Sources of priming effects induced by cellulose*

The reduction in DOC content caused by cellulose addition resulted in a strong negative priming effect, mainly due to the decrease of old soil C in the soluble fraction (Fig. 3A). The negative PE for old C, however, was much smaller at day 14 versus day 7 after cellulose amendment. Moreover, the PE for recent C tended to be positive during the intensive stage of cellulose decomposition, indicating accelerated decomposition of plant-originated recent C induced by cellulose. Recent and old C contributed almost equally to the positive PE observed in the DOC pool at day 60 of cellulose decomposition, while the relatively small PE at day 103 was mainly originated from old C.

Both the old and recent carbon fractions consumed from the dissolved organic pool were revealed as being primed C in microbial biomass during the first two weeks after cellulose addition (Fig. 3B). The contribution of old C in the PE strongly increased during intensive cellulose mineralization and comprised 82% of total PE in the microbial biomass at day 14. The decrease in microbial biomass during the slow phase of cellulose decomposition

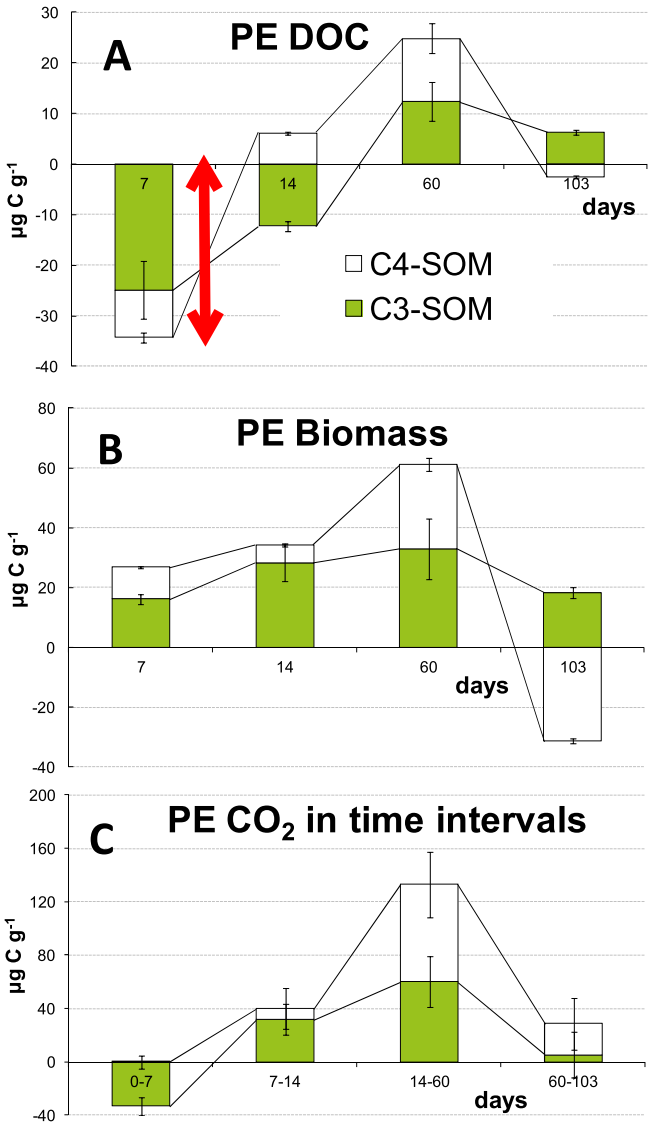

Fig. 3. Contribution of recent (C_4) and old (C_3) SOM to the priming effect in DOC (A), microbial biomass (B), and in cumulative CO_2 efflux (C), in soil after cellulose addition.

was mainly due to recent C (Fig. 2B), and old C was the sole source of primed C in MBC (Fig. 3B).

The negative PE in CO_2 efflux observed during the first week originated solely from C3–C (Fig. 3C), revealing the switch of old-SOM decomposers to degradation of cellulose. During the second week of cellulose decomposition, however, the contribution of old C to positive PE in CO_2 efflux was nearly 80%. In the course of further incubation, the portion of old CO_2–C in the PE decreased down to 18% at the late stage of the experiment. In total, the PE in CO_2 efflux consisted to 37% of old C3 and to 63% of recent C4. This demonstrates a small shift compared with SOM, where the recent and old C contributed almost equally (51 and 49%, respectively).

3.4. Microbial growth parameters

During the intensive phase of cellulose decomposition an 11–24% decrease in μ_m-values was observed in the cellulose treatment compared with control soil (Fig. 4A). Despite slower specific growth rates, a 3–4-fold increase in active MB and a 2–4 h shorter lag-period was observed in the cellulose-decomposing community versus the control soil. Surprisingly, the SIGR and fumigation approaches demonstrated opposite responses of total biomass to cellulose addition. Despite the strong increase in microbial biomass confirmed by fumigation (Fig. 2B), the SIGR approach showed an almost 20% reduction in total biomass of microorganisms responding to glucose one week after cellulose addition to soil (Fig. 4A). Such a discrepancy between the two methods indicated an occurrence of factors restricting the respiratory activity of cellulose decomposers. During the second week of incubation, SIGR-biomass in cellulose-treated soil increased and exceeded control values by 21%. The 2.7-fold increase in active and the 15% increase in total MB still remained at day 60, while the μ_m recovered almost to the control values.

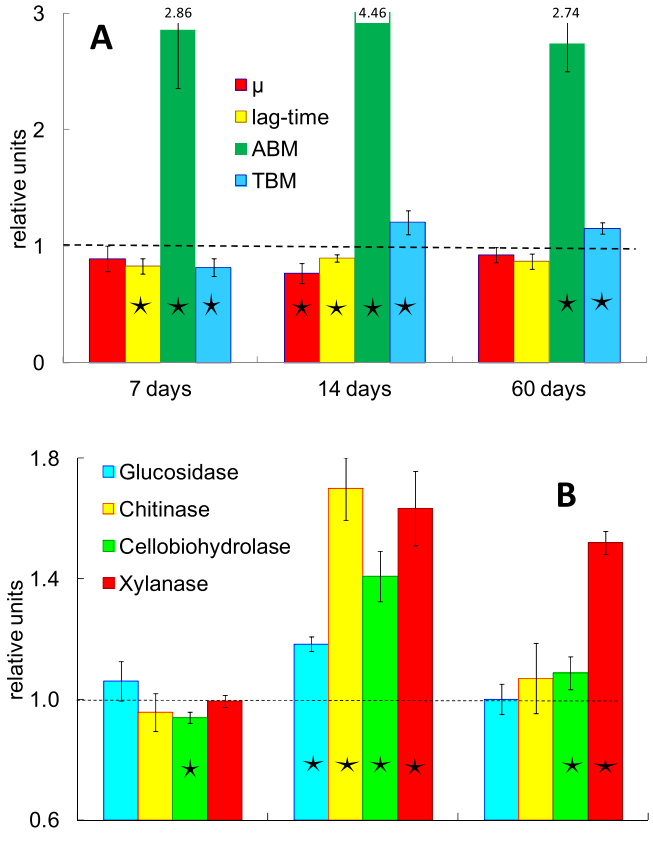

Fig. 4. Relative changes in microbial growth parameters (A), and in enzyme activities in soil treated with cellulose as compared with untreated control at three sampling dates (B). The dashed line (=1) indicates no changes compared to control without cellulose addition. The bars labeled by asterisks indicate significant difference from control.

3.5. Enzymatic activity

Despite the clear indication of cellulose mineralization by $^{14}CO_2$ (Fig. 2) and its incorporation in microbial biomass (Fig. 2B), there were no acceleration in hydrolytic enzyme activity in soils with cellulose versus untreated control until day 7 (Fig. 4B). Furthermore, even a slight (6%) decrease in cellobiohydrolase activity was detected one week after cellulose addition. After two weeks, however, hydrolytic enzyme activity significantly increased compared to the untreated soil (Fig. 4B). The largest increase (70%) was observed for chitinase activity, whereas the activity of xylanase, cellobiohydrolase and β-glucosidase increased by 63, 41 and 18%, respectively. The activity of xylanase and cellobiohydrolase was maintained at high levels exceeding those in control soil by 52 and 9%, respectively, even 2 months after cellulose addition.

3.6. PLFA content

One week after cellulose addition, no significant changes in specific bacterial and fungal PLFA content were observed, except the doubling of several common and protozoan (20:4ω6) PLFAs (Fig. 5A).

Two weeks of cellulose decomposition (Fig. 5B) resulted in a 13% decrease in the two G(+) PLFAs (i15:0; a15:0) while the content of three G(−) PLFAs: 16:1ω7c, 18:1ω7c and cy19:0 increased for 21, 15 and 18%, respectively. The largest increase, about 50%, was detected for the fungal PLFA 18:2ω6,9; however, the content of the second fungal PLFA analyzed (18:1ω9c) increased by only 12%. The content of protozoan PLFA decreased to the control level.

Reverse changes were observed for G(+) and G(−) PLFAs in the two months after cellulose addition (Fig. 5C). The content of both G(+) PLFAs increased by about 80%, while the content of two G(−) PLFAs: 18:1ω7c and cy19:0 decreased, respectively, by 41 and 66% compared with control soil. The higher content of both fungal PLFAs (18:2ω6,9 – by 62% and 18:1ω9c – by 44%) remained in cellulose-treated versus untreated soil until 60 days of the experiment. The content of protozoan PLFA continued to decrease and was 23% lower than in the control soil.

4. Discussion

4.1. The size and nature of the priming effect

Added cellulose C amounted to 160% of microbial biomass C and initiated a priming effect of 25% of CO_2 efflux from control soil. This priming fits well to the relationship between the input of plant residues and primed CO_2–C as presented in the literature (Fig. 3 in Blagodatskaya and Kuzyakov, 2008). The absolute amount of primed C released as CO_2 was similar to that triggered by cellulose addition in the study of Fontaine et al. (2004b) considering the same decomposition period (70 days: 152 and 140 μg C g^{-1}, respectively).

The positive PE in the CO_2 flux (40 μg CO_2–C g^{-1}) during the first two weeks after cellulose addition was comparable with the amount of C primed in microbial biomass (34.3 μg C g^{-1}). Therefore, this PE was mainly real. The PE induced by maize leaf-litter (Nottingham et al., 2009) was *ca.* 2-fold higher, while the PE caused by wheat straw (Guenet et al., 2010) was *ca.* 2-fold lower than the cellulose-induced priming (Fontaine et al., 2004b, current study). Thus, the amount of SOM-C losses during decomposition of plant residues is mediated by substrate quality and composition.

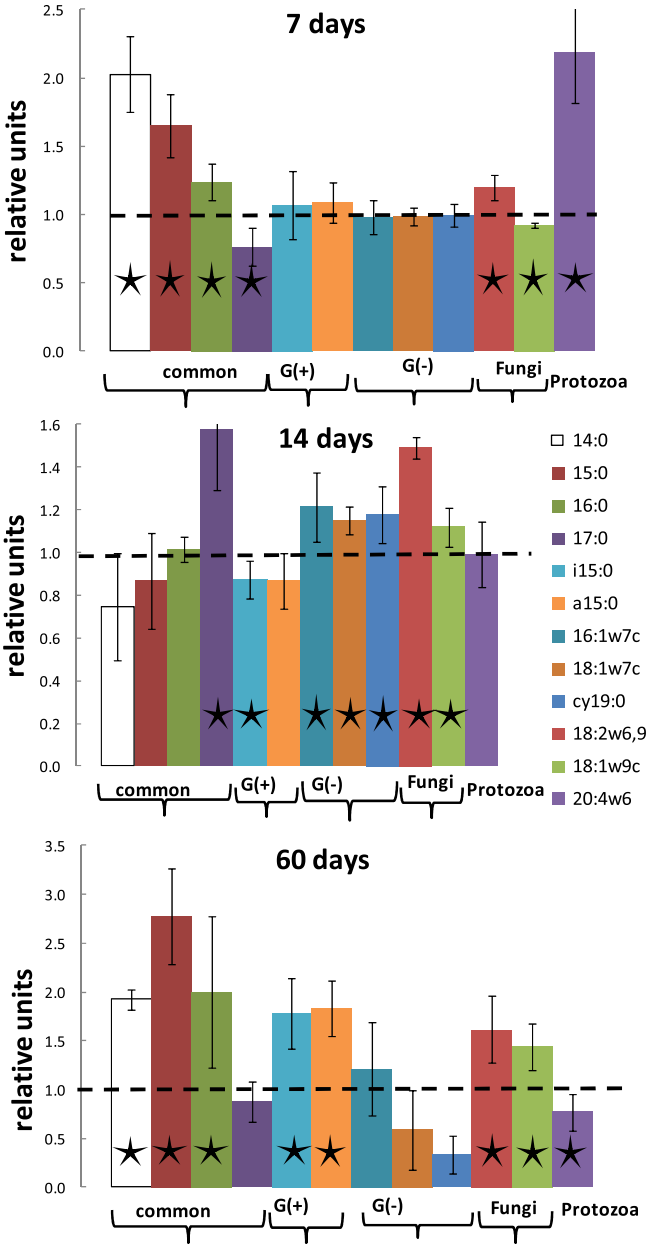

Fig. 5. Relative changes in PLFA content in soil treated with cellulose as compared with untreated control at sampling dates. The dashed line (=1) indicates no changes compared to control without cellulose addition. The bars labeled by asterisks indicate significant difference from control.

The PE in the CO_2 efflux during the first week after cellulose addition was negative and was accompanied by a reduction in DOC. This could be due to increased utilization of dissolved C, or its reduced production (e.g. via reduced production of SOM-acting enzymes). The former process, likely, dominated in our study as an accelerated uptake of soil organics was already detected both in DOC (as decrease) and in MBC (as increase). Thus, the application of either readily (glucose, Blagodatskaya et al., 2011b) or less available (cellulose, this study) substrates induced a rapid DOC reduction accompanied by a C increase in microbial biomass. Accordingly, the pool of labile dissolved C with fast turnover (Bengtson and Bengtsson, 2007) was initially subjected to PE. This negative PE in DOC at the early stage of substrate decomposition apparently initiates the microbial loop mechanism when dispersed molecules of soluble C are concentrated in microbial cells (Clarholm, 1985; Panikov, 2010).

4.2. Carbon budget in the presence of a priming effect

If the priming effect is not considered, the amount of cellulose originated C remaining in soil at the end of the experiment (103 days) comprised 71% of the cellulose-C input. Considering the C losses due to the PE, however, only 28% of C input from cellulose ultimately contributed to C sequestration in soil. In contrast to Fontaine et al. (2004a), who found that cellulose input resulted in C sequestration only when accompanied by high nutrient enrichment, our study revealed positive soil C balance after solely cellulose application. This may reflect the lower C:N ratio of SOM 10 in our study vs. 17 in the study of Fontaine et al. (2004a).

The cumulative contribution of old C to the total PE in the CO_2 flux induced by cellulose was 37% (Fig. 6). This indicates that old SOM is an important C source for soil microorganisms even in the presence of more readily available plant-derived carbon (Paterson, 2009). The contribution of recent C (63%), however, was 10% greater than in the PE induced by a similar glucose amount in the same soil (Blagodatskaya et al., 2011b). The glucose input may cause a catabolic repression of cellulolytic enzymes synthesis and reduce decomposition of plant residues. The cellulose input, on the contrary, activated cellulases enzyme complex (Fig. 4B).

4.3. Composition and functioning of the microbial community during priming

Cellulose input to the soil resulted in rapid DOC-C decrease within the first week. A subsequent increase in C was detected by fumigation of microbial biomass, but no such increase (and even

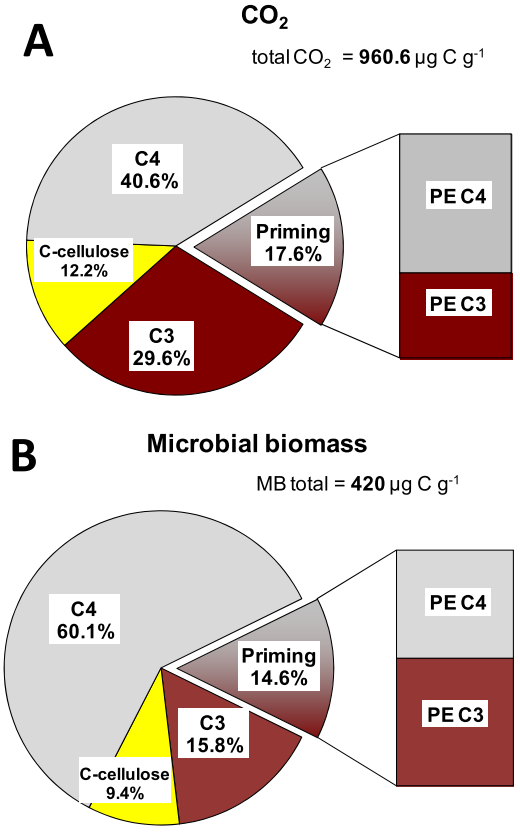

Fig. 6. Contribution of three C sources to cumulative CO_2 efflux (A) and microbial biomass (B) after cellulose addition. The priming effect is presented as the right segment of the pie-plot and contribution of the old and recent C to the primed C is shown as stacked columns. The three sources include: 1) SOM-C older than 12 years (C3), 2) SOM-C younger than 12 years (C4), and 3) C of added cellulose (C-cellulose).

slight decrease) was observed either in SIGR-biomass or in enzyme activities. Specific PLFA biomarkers also showed no significant changes, with the remarkable exception of the two-fold increase in protozoan PLFAs. We therefore assume that cellulose input served as a driver activating the food chain through the microbial loop: during the first days after cellulose input, the soluble C was consumed by bacteria, which were later grazed on by protozoans. This corresponds well to the typical microbial succession after substrate input or even after soil moistening: maximal bacterial activity at 2–3 days is substituted by raised activity of protozoans at days 4–5 (Clarholm, 1985, 1994; Saetre and Stark, 2005; Christensen et al., 2007), which then rapidly decreases, enabling rapid nutrient recycling by protozoan grazers (Pomeroy, 1974). As other microorganisms, the protozoans are sensitive to fumigation and their contribution to total heterotrophic respiration induced by glucose is minor (Kuikman et al., 1990). Thus, the protozoan biomass containing the cells of grazed bacteria contributed to the total microbial biomass determined by fumigation while SIGR-biomass decreased due to bacterial grazing by protozoans.

The negative PE during the first week indicated that protozoan grazing prevented accelerated decomposition of SOM (Fig. 7). Bacterial grazing followed by protozoan growth usually results in a strong increase in N availability (Clarholm, 1985) which as observed in our study by the strong decrease in the C:N ratio in the dissolved organic matter pool (Fig. 2, 7 days). The decrease in the content of protozoan PLFAs to control (14th day) or even lower (60th day) levels supported the assumption of decreased protozoan activity (due to partial death or switch to resting cystic forms) during the second week after cellulose addition. Nutrients and growth factors released by protozoans (Bonkowski et al., 2000, Fig. 7) facilitated the next wave of microbial succession that degraded cellulose (Blackwood and Paul, 2003; Zelenev et al., 2005, 2006). This was revealed as an increase in G(−) and in fungal PLFAs. The stimulation of the G(−) bacteria during the intensive phase of cellulose decomposition is consistent with previous studies that identified the dominance of G(−) proteobacteria in the decomposition of cellulose or plant residues (Bernard et al., 2007; Haichar et al., 2007) and the increase in G(−) PLFAs at the similar stage of food web development (Esperschütz et al., 2009). The increase in fungal PLFA on day 14 of intensive cellulose decomposition was supported by the increase in fungal ergosterol and in PLFA content, which peaked at between 15 and 20 days of plant residues decomposition (Rousk and Bååth, 2007a; Marschner et al., 2011).

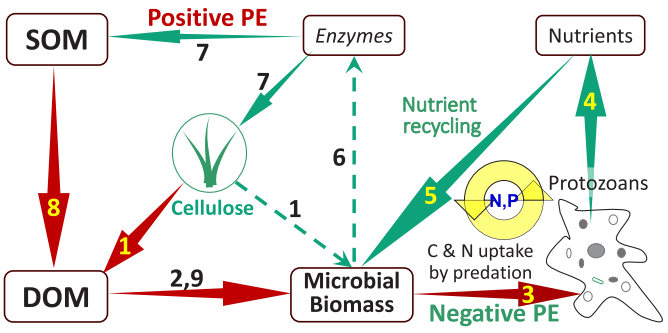

Fig. 7. Conceptual scheme of microbial loop driving the PEs in soil after cellulose addition. 1 – microbial uptake of cellulose degraded by existing enzymes leads to microbial biomass increase; 2 – DOC uptake by increased microbial biomass; 3 – microbial biomass decrease due to grazing by protozoa and observed negative priming effect; 4 – protozoan turnover and die-off lead to nutrient release; 5 – nutrients (N) uptake by microorganisms; 6 – accelerated enzyme production by microorganisms supplied with C and nutrients; 7 – increase in cellulose degradation rate and positive priming effect (SOM decomposition) by increased enzyme pool; 8 – increased DOC supply due to increasing decomposition rates of SOM, and 9 – closing the loop with microbial biomass increase.

A significant (24%) decrease in the microbial growth rate was observed during intensive cellulose decomposition, and this decrease corresponded to a 4-fold increase in active biomass. Slower growth of the cellulose-decomposing community can indicate a shift to microorganisms with K-strategy, which benefit from the low-degradable substrates due to a higher affinity of their enzyme systems to complex long-chain molecules (Blagodatskaya et al., 2009). Considering the simultaneous 60% increase in fungal PLFAs, we conclude that the lower growth rates coincided with an increased contribution of fungi to total microbial community because, on average, fungi decomposing polymeric compounds grow slower than bacteria dominating on soluble substrates (Rousk and Bååth, 2007b). The strong increase in active microbial biomass during the intensive phase of cellulose decomposition coincided with higher activity of the enzymes decomposing chitin, cellulose and hemicelluloses (Fig. 4B). Extracellular enzymes produced during intensive cellulose decomposition also participated in SOM decomposition causing positive PE (Fontaine et al., 2004b). Thus, a possible mechanism of the PE occurring during the intensive phase of cellulose degradation is non-targeted hydrolysis of SOM-components by cellulolytic enzymes produced by fungi and G(−) bacteria. Increase in bacterial PLFA, however, can also indicate that the monomers released as a result of fungal enzymatic activity were commensally used by G(−) bacteria in course of a further food web development (Esperschütz et al., 2009). Note, however, that our experimental design simulated only one of the most simple situations, e.g. occasional C input to bulk or non-rhizosphere soil. The whole predator chain above protozoa (which were possibly lacking during soil sieving) and plant–microbial interactions might well change the functioning of the microbial loop.

The slow phase of cellulose degradation was accompanied by an increase in fungal and G(+) PLFAs and in xylanase activity (Figs. 4B and 5). The increase in fungal PLFAs supported the observations based on PLFA and DNA markers that fungi have a strong competitive advantage in the long-term utilization of cellulose (Fontaine et al., 2011). The increase in G(+) PLFAs is in accordance with the 3-fold increase in G(+) PLFA after 49 days of decomposition of the recalcitrant fraction of plant residues (Paterson et al., 2008). The increase in xylanase activity implied that the slow phase of cellulose degradation was accompanied by the decomposition of hemicellulose compounds of SOM.

We conclude that the partitioning of C sources in total and in primed C pools during cellulose decomposition, combined with the results of PLFA and enzyme activity analyses, demonstrated the functioning of the microbial loop as a particular type of food chain in terrestrial ecosystems (Clarholm, 1985; Coleman, 1994; Panikov, 2010). C input in the form of cellulose activated the microbial loop mechanism (Fig. 7). This was evident as an accelerated uptake of soluble C by cellulose-degrading bacteria (stages 1 and 2 in Fig. 7). These were then rapidly grazed by protozoa, causing a drop in enzyme activity and negative PE (stage 3 in Fig. 7). N released by protozoans after bacterial consumption (Fig. 2; stages 4 and 5 in Fig. 7) activated intensive cellulose decomposition by fungi and G(−) bacteria followed by slow cellulose decomposition by fungi and G(+) bacteria (stage 6 in Fig. 7). This corresponded to increased activity of hydrolytic enzymes degrading cellulose (stage 7 in Fig. 7). Extracellular cellulolytic enzymes commensally decomposed cellulose-like compounds of SOM causing real PE (stage 8 in Fig. 7) resulting in increase of DOC content (stage 9 in Fig. 7).

5. Conclusions

The combination of ^{14}C labeling and ^{13}C natural abundance enabled us to evaluate *three* C sources: 1) C of the added cellulose

(^{14}C), 2) SOM C older than 12 years, and 3) SOM C younger than 12 years. These 3 sources were evaluated in three pools and fluxes: 1) DOC, 2) microbial biomass, and 3) CO_2 evolved from soil. This combination of trace approaches revealed the sources of primed C.

The 35% decrease in the dissolved organic C pool after cellulose addition was the first indication of the PE. This decrease was mainly due to C older than 12 years. Soil C taken up from the DOC pool was incorporated in microbial biomass. At least a one-week delay occurred between the PE detected in the microbial C pool and in the CO_2 efflux, which also mainly originated from the old C during the second week of incubation. This clearly illustrates that decomposition of soil organics is accomplished through the soluble pool and is microbially mediated. The decrease in DOC and simultaneous increase in protozoan PLFA indicated that the microbial loop functions as a driver of microbial community succession. Intensive cellulose decomposition caused short-term real PE (days 7–14) of cellulose-like SOM compounds by slow-growing fungi and G(−) bacteria. Long-term PE (days 14–103) was due to the decomposition of SOM-originated hemicelluloses by fungi and G(+) bacteria. Despite 71% of the C input in the form of cellulose was sequestered in the soil the net soil C-balance amounted only for 28% after considering the C losses by the PE. The sources of carbon lost from soil and substituted by cellulose C were old (37%) and recent (63%) SOM indicating that both C sources are subjected to soil priming (Fig. 6). At that, the partly decomposed plant residues were the main source of the PE induced by cellulose input.

We conclude that 3-source-partitioning based on ^{14}C labeling and ^{13}C natural abundance, in combination with monitoring the structure and functioning of the microbial community by PLFA, is a very powerful tool for source identification and evaluation of mechanisms and drivers of priming effects.

Acknowledgments

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