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Microbial use of ^{15}N-labelled maize residues affected by winter temperature scenarios

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ABSTRACT

A 56-day incubation experiment was carried out to investigate decomposition and microbial use of ^{15}N-labelled maize (*Zea mays* L.) residues incubated under four winter temperature scenarios. The residues were mixed to mesocosms equivalent to 1.2 mg C and 42.5 μg N g^{-1} dry soil, after which the samples were incubated at a constant temperature of +4 °C, a constant −3 °C, and under multiple and single freeze–thaw conditions. A constant +4 °C was most favourable for microbial substrate use, with 4- and 6-fold higher total and maize-C mineralization, respectively, in comparison with constant frost. The cumulative maize mineralization was not determined by the frequency of freeze–thaw events, but regulated by the overall time of frost and thaw conditions. The decomposition of maize straw significantly increased soil organic C mineralization (in all scenarios) and incorporation into microbial biomass (in the freeze–thaw scenarios only). The positive priming effects observed were equivalent to an additional loss of total soil organic C of between about 0.2 (continuous frost) and 0.8% (single freeze–thaw). Microbial biomass was significantly increased after maize straw amendment, with constant frost and freeze–thaw scenarios not having any negative effect on microbial biomass C compared with constant +4 °C. Highest fungal biomass was found after constant frost without fresh substrates and also after extended frost followed by a warm period when fresh plant residues were present. On average, 50% of the added maize N were recovered in the soil total N after 56 days of constant 4 °C and in the freeze–thaw scenarios, with the strongest effect after single freezing and thawing.

1. Introduction

Although it is predicted that climate warming will be most pronounced at high latitudes (Houghton et al., 2001; IPCC, 2007), temperate soils may also be affected as they remain close to freezing point throughout the winter (Henry, 2008). In fact, trend analysis has shown an increase in mean annual temperature for Germany of about 0.8–1.1 °C from 1901 to 2000, with marked increases in winter precipitation (Schönwiese and Janoschitz, 2008). A positive trend for the mean annual temperature (increase of 1.3 °C from 1951 to 2005) and increased winter precipitation as well as a decrease in the number of days with minimum temperatures below 0 °C was also found by Haberlandt et al. (2010) for Lower Saxony, which is a large temperate area (357,000 km^2) in the north of Germany. Winter climate change may have stronger effects on microbial activity and C and N dynamics in fallow arable soils, due to a lack of protective plant cover in comparison with grassland or forest systems.

It is assumed that climate warming reduces the snow pack thickness, thus leading to lower soil temperatures or to higher frequencies of freezing and thawing, i.e. colder soils in a warmer world (Isard and Schaetzl, 1998; Groffman et al., 2001). Increased soil freezing can cause leaching losses of C and N (Groffman et al., 2001; Fitzhugh et al., 2001) as well as lower winter soil respiration (Monson et al., 2006). In addition, it has been suggested that soil freezing and thawing disrupts soil aggregates (Oztas and Fayetorbay, 2003; Six et al., 2004), plant material (Mellick and Seppelt, 1992; Harris and Safford, 1996) and microbial cells (Skogland et al., 1988; Yanai et al., 2004; Larsen et al., 2002). This enhances microbial activity upon thawing, due to increased availability of substrates or easily decomposable organic matter (Edwards and Cresser, 1992; Schimel and Clein, 1996; Lipson et al., 2000; Grogan et al., 2004; Sharma et al., 2006). Microorganisms are the main drivers of soil organic matter decomposition and nutrient

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cycles (Swift et al., 1979), with saprotrophic fungi being most important in decomposing plant residues in arable soils (Bowen and Harper, 1990; Cheshire et al., 1999). Contradictory impacts of freeze–thaw events on microorganisms have been reported, with either decreasing (Lipson et al., 1999; Pesaro et al., 2003) or no effects (Grogan et al., 2004; Sharma et al., 2006) on microbial biomass. If temperature drops below 0 °C, shifts in microbial substrate use occur (Schimel and Mikan, 2005), which may be accompanied by shifts in microbial community composition from bacteria towards fungi (Lipson et al., 2002; Schadt et al., 2003; Lipson and Schmidt, 2004; Sjursen et al., 2005).

In order to follow microbial C and N dynamics around the freezing point, we combined the natural $\delta^{13}C$ value of maize straw, which is usually different from soil organic matter (Ryan and Aravena, 1994; Rochette et al., 1999; Potthoff et al., 2005), and an artificial enrichment in $\delta^{15}N$. To our knowledge, this is the first attempt to simultaneously follow microbial respiration and C and N sequestration into different fractions, such as microbial biomass, CO_2, particulate organic matter, extractable C and N, and soil total C and N, during the decomposition of a complex organic substrate at low temperatures. This incubation study addressed the following questions: (1) Do freeze–thaw scenarios accelerate the decomposition of straw-derived C and N in comparison with constant temperatures around 0 °C? (2) Is the microbial use, i.e. mineralization and immobilization, of straw-derived organic matter regulated by the frequency of freeze–thaw cycles?

2. Material and methods

2.1. Soil and plant material

The arable soil used for the experiment was taken from the upper 10 cm of an experimental site in Neu-Eichenberg near Witzenhausen (51°23′ N, 9°55′ E, Northern Hessia, Germany) in September 2009. The site is located at 240 m above sea level with a mean annual precipitation and temperature of 670 mm and 8.7 °C. The soil is classified as a Haplic Luvisol (FAO-WRB, 2006) with the following characteristics: 3.3% sand, 83.4% silt, 13.3% clay, a water holding capacity of 55%, a pH ($CaCl_2$) of 6.3, 1.4% total C, a $\delta^{13}C$ value of $-26.4 \pm 0.1‰$, 0.14% N and a $\delta^{15}N$ value of 7.8 ± 0.3. Plant tissue, insects and stones were removed by hand and the soil was sieved (<2 mm). The soil was incubated at 3 °C for 4 weeks before the experiment started. For ^{15}N labelling, maize was grown for six months in the greenhouse and fertilised once with 278 ml fertilizer solution containing 160 mg l^{-1} NH_4NO_3 enriched with 10 atom-% ^{15}N. Maize leaf residues were harvested in October 2009, air dried, chopped into pieces of 0.5 cm × 1 cm and stored in a paper bag at room temperature. The maize residues contained 43.7% (±0.3) C with a $\delta^{13}C$ value of −12. ‰, 1.8% (±0.01) N with a $\delta^{15}N$ value of 594.8‰ and had a C/N ratio of 24.7.

2.2. Incubation procedure

The incubation experiment was carried out in 2 L glass jars with 5 replicates per treatment, each containing soil with a water content of 20% on a dry weight basis (corresponding to 36% of the water holding capacity), equivalent to 300 g dry soil. After equilibration at 3 °C, maize residues were mixed thoroughly into half of the soil samples at amounts of 1.2 mg C and 42.5 μg N g^{-1} dry soil, the remaining samples serving as non-amended controls. All samples were then incubated simultaneously by temperature treatment in two climate cabinets (MV 600, LinTek, Germany) for 56 days. To simulate winter climate regimes, four different temperature treatments were applied: (1) a constant +4 °C ($+4_{CON}$), (2) a constant −3 °C (-3_{CON}), (3) multiple freeze–thaw cycles of 48 h at +4 and −3 °C, respectively ($+4/-3_{MULTIPLE}$), and (4) a single freeze–thaw cycle of four weeks at −3 °C between two warm periods of two weeks each at +4 °C ($+4/-3_{SINGLE}$).

Air samples for CO_2 measurement were taken on days 1, 4, 8, 10, 16, 18, 22, 36, 43, 46, 50, 52, 54 and 56 after the incubation period started. For this purpose, two evacuated gas containers (50 ml) were connected to one of two ports on the PVC lid of each glass jar, which was attached with two rubber bands to achieve an airtight seal. The second port was used to connect a 50 L gas bottle with CO_2-free synthetic air (synthetic air 5.0, ≥99.999 vol% purity, Air Liquide, Germany, at 20.5 ± 0.5% O_2 in 79.5 ± 0.5% N_2). Each glass jar and the connections of the attached gas containers were flushed with synthetic air for about 2 min to remove the CO_2 before the first sampling. The headspace volume of each jar was homogenized by a fan affixed to the inside of the PVC lid. To take the temperature differences between the synthetic air and the refrigerators/freezers into account, the synthetic air was first cooled in a styrofoam box by passing it through a 10-m flexible silicon tube covered with ice. After the flushing procedure, the first air sample (A_{T0}) was taken. The second air sample (A_{T1}) was taken after an accumulation period of 24 h. CO_2 concentrations were measured using an automated gas chromatograph with an electron capture detector according to Loftfield et al. (1997). The volume of CO_2 in the headspace-volume of each jar under standard conditions for temperature and pressure was calculated as:

$$CO_{2_{Headspace}}(ml) = V_{Net} \times \Delta CO_2 \times \frac{p_n \times T_n}{p_n \times (T_n + IT)} \quad (1)$$

where V_{Net} is the headspace volume (ml), ΔCO_2 is the difference between the CO_2 concentrations A_{T1} and A_{T0} (expressed as %), p_n is the standard atmospheric pressure (hPa), T_n is the standard temperature (K) and *IT* is the temperature during the incubation (°C). Soil respiration expressed as CO_2–C was then calculated by the following equation:

$$CO_2 - C\left(\mu g\ g^{-1} soil\ d^{-1}\right) = \frac{CO_{2_{Headspace}} \times M_{CO_2}}{V_{m0}} \times 0.2729 \times \frac{1000}{ds} \times \frac{24}{\Delta t} \quad (2)$$

where $CO_{2\ Headspace}$ is the volume of CO_2 (ml) under normal conditions related to the respective incubation temperature, M_{CO_2} is the molar mass of CO_2 (mg), V_{m0} is the volume of one mol of a gas under normal conditions (ml), 0.2729 is the mass fraction of C in CO_2, ds is the dry weight of the incubated soil sample (g) and Δt is the time between A_{T0} and A_{T1} (*h*). Air samples for $\delta^{13}C$ analysis of evolved CO_2 were taken with a syringe (35 ml) directly after A_{T1} and stored in 12 ml Labco Exetainer vials (Labco Limited, UK). CO_2–$\delta^{13}C$ analyses were performed on a Delta plus IRMS (Thermo Scientific, Bremen, Germany). Soil sub-samples for analysis were taken at the end of the incubation on day 56 after all visible particles of maize straw residues had been removed from the amended soil samples by sieving (2 mm).

2.3. Analytical procedures

Microbial biomass C and N were estimated by fumigation extraction (Brookes et al., 1985; Vance et al., 1987). A sub-sample of 20 g moist soil was taken and separated into two portions of 10 g. One portion was fumigated at 25 °C with ethanol-free $CHCl_3$, which was removed after 24 h. Fumigated and non-fumigated samples were extracted for 30 min with 40 ml of 0.05 M K_2SO_4 (Potthoff et al., 2003) by horizontal shaking at 200 rev min^{-1} and filtered (hw3, Sartorius Stedim Biotech, Göttingen, Germany). Organic C

and total N in the extracts were measured after combustion at 850 °C using a Dimatoc 100 automatic analyser (Dimatec, Essen, Germany). Microbial biomass C was calculated as E_C/k_{EC}, where E_C = (organic C extracted from fumigated soil) − (organic C extracted from non-fumigated soil) and $k_{EC} = 0.45$ (Wu et al., 1990; Joergensen, 1996). Microbial biomass N was calculated as E_N/k_{EN}, where E_N = (total N extracted from fumigated soil) − (total N extracted from non-fumigated soil) and $k_{EN} = 0.54$ (Brookes et al., 1985; Joergensen and Mueller, 1996). The conversion values k_{EC} and k_{EN} have been repeatedly used in freeze–thaw cycle experiments (Larsen et al., 2002; Sjursen et al., 2005; Fan et al., 2012). For the determination of ^{13}C and ^{15}N-isotope composition of microbial biomass, 20 ml aliquots of 0.05 M K_2SO_4 extracts of fumigated and non-fumigated samples were freeze dried for about 3 days. The freeze–dried K_2SO_4 extracts were analysed for ^{13}C and ^{15}N-isotope composition by isotope-ratio mass spectrometry (Delta plus, Finnigan, Bremen).

The fungal cell-membrane component ergosterol was extracted from 2 g moist soil with 100 ml ethanol (96%) according to Djajakirana et al. (1996). Quantitative determination of ergosterol was then performed by reversed-phase HPLC analysis with 100% methanol as the mobile phase and detected at a wavelength of 282 nm (Dionex UVD 170 L).

For determination of particulate organic matter (POM), the soil samples (250 g moist soil) were dispersed in 400 ml of saturated sodium chloride, shaken by hand and allowed to stand for 45 min (Magid and Kjærgaard, 2001; Muhammad et al., 2006). The samples were poured gradually onto two sieves of 0.4 mm and 0.063 mm mesh size and washed with tap water. The aggregates were destroyed by pushing the soil through the sieve during the washing procedure until the water passing through the sieve became clear. The material retained on the 0.4 mm sieve (POM 0.4–2 mm) as well as the washed POM > 2 mm fraction were then transferred into a crucible, dried at 60 °C, weighed and milled for further analysis (total C, total N, $\delta^{13}C$, $\delta^{15}N$).

2.4. Calculations and statistical analysis

Isotope values are expressed in delta notation relative to VPDB and air for ^{13}C and ^{15}N, respectively. The $\delta^{13}C$ value of the microbial biomass ($\delta^{13}C_{MB}$) was calculated by the following equation (Ryan and Aravena, 1994; Potthoff et al., 2003):

$$\delta^{13}C_{MB}(‰) = \frac{\left(\delta^{13}C_{fum} \times C_{fum}\right) - \left(\delta^{13}C_{nfum} \times C_{nfum}\right)}{\left(C_{fum} - C_{nfum}\right)} \quad (3)$$

where C_{fum} and C_{nfum} represent the mass of C (μg g^{-1}) extracted from the fumigated and non-fumigated samples, respectively, and $\delta^{13}C_{fum}$ and $\delta^{13}C_{nfum}$ represent the corresponding $\delta^{13}C$ values. Accordingly, the $\delta^{15}N$ value of the microbial biomass ($\delta^{15}N_{MB}$) was calculated as follows (Dijkstra et al., 2006; Zareitalabad et al., 2010):

$$\delta^{15}N_{MB}(‰) = \frac{\left(\delta^{15}N_{fum} \times N_{fum}\right) - \left(\delta^{15}N_{nfum} \times N_{nfum}\right)}{\left(N_{fum} - N_{nfum}\right)} \quad (4)$$

The fraction of maize-derived C ($f_{maize\text{-}C}$) was calculated for each individual replicate of all treatments from the $\delta^{13}C$ data according to a two pool-mixing model with the following equation:

$$f_{maize-C}(\%) = \frac{\left(\delta^{13}C_{sample} - \delta^{13}C_{control}\right)}{\left(\delta^{13}C_{maize} - \delta^{13}C_{control}\right)} \times 100 \quad (5)$$

where $\delta^{13}C_{sample}$ represents the $\delta^{13}C$ value of SOC, POM-C (0.4–2 mm), microbial biomass C at day 56, and CO_2-C at each measurement day; $\delta^{13}C_{control}$ is the average $\delta^{13}C$ value of the non-amended control samples within each temperature treatment and $\delta^{13}C_{maize}$ is the $\delta^{13}C$ of the maize residues. Accordingly, the fraction of maize-derived N ($f_{maize\text{-}N}$) was calculated by the following equation:

$$f_{maize-N}(\%) = \frac{\left(\delta^{15}N_{sample} - \delta^{15}N_{control}\right)}{\left(\delta^{15}N_{maize} - \delta^{15}N_{control}\right)} \times 100 \quad (6)$$

where $\delta^{15}N_{sample}$ represents the $\delta^{15}N$ value of soil total N, POM-N (0.4–2 mm), microbial biomass N at day 56; $\delta^{15}N_{control}$ is the average $\delta^{15}N$ value of the non-amended control samples within each temperature treatment and $\delta^{15}N_{maize}$ is the $\delta^{15}N$ of the maize residues.

To determine priming effects, the amount of soil-derived C in the respective fractions of maize amended samples (C_3–C_{sample}) was obtained by subtracting the maize-derived C from the total amount of C. If the difference between C_3–C_{sample} and C_3–$C_{control}$ ($p \leq 0.05$, *t*-test) was significant, priming effects were calculated using the following equation:

$$PE(\%) = \frac{\left(C_3 - C_{sample} - C_3 - C_{control}\right)}{C_3 - C_{control}} \times 100 \quad (7)$$

where C_3–$C_{control}$ is the amount of SOC in each fraction of the non-amended control samples. Isotopic fractionation during microbial decomposition processes is still a controversial issue and often suggested to be negligible and of little importance (Ehleringer et al., 2000; Ekblad et al., 2002). Also, Collins et al. (2000) found no evidence of isotopic discrimination after incubating maize residues for 50 days. We therefore assume that no fractionation occurred during the incubation process (see also Rochette et al., 1999)

The data presented in tables and figures are arithmetic means expressed on an oven-dry basis (about 24 h at 105 °C), standard deviations are given in brackets. The Kolmogorov–Smirnoff test was used to check for normal distribution. Significance of treatment effects was tested by a one-way analysis of variance (ANOVA) using post hoc Tukey HSD analysis, and significant differences were determined at $P \leq 0.05$. All statistical calculations were performed using SPSS Statistics 17.0 (SPSS Inc., Chicago, USA).

3. Results

3.1. Maize-derived C and N fractions

Application of maize residues increased ^{13}C in SOC on average by 0.46‰ and in K_2SO_4 extractable C on average by 0.50‰ (Fig. 1a, Table 1). However, this enrichment was only significant for the $+4/-3_{SINGLE}$ treatment. For microbial biomass C, the $\delta^{13}C$ values of the maize amended samples varied around −22.5‰ and were thus significantly enriched in ^{13}C by on average 3.35‰, with highest values in the $+4/-3_{SINGLE}$ treatment. In the POM 0.4–2 mm fraction, maize amendment increased ^{13}C by a mean of 4.7‰ in comparison with the control samples. The highest enrichment in this fraction was again found in the $+4/-3_{SINGLE}$ treatment. The application of maize residues also significantly increased ^{15}N in all compartments analysed (Table 1). The ^{15}N in soil total N, microbial biomass N and K_2SO_4 extractable N was increased by 9.8‰, 77‰ and 184‰, respectively (Fig. 1b, Table 1). Here, the highest values were found in the $+4/-3_{SINGLE}$ treatment, while samples under constant frost showed the lowest increases.

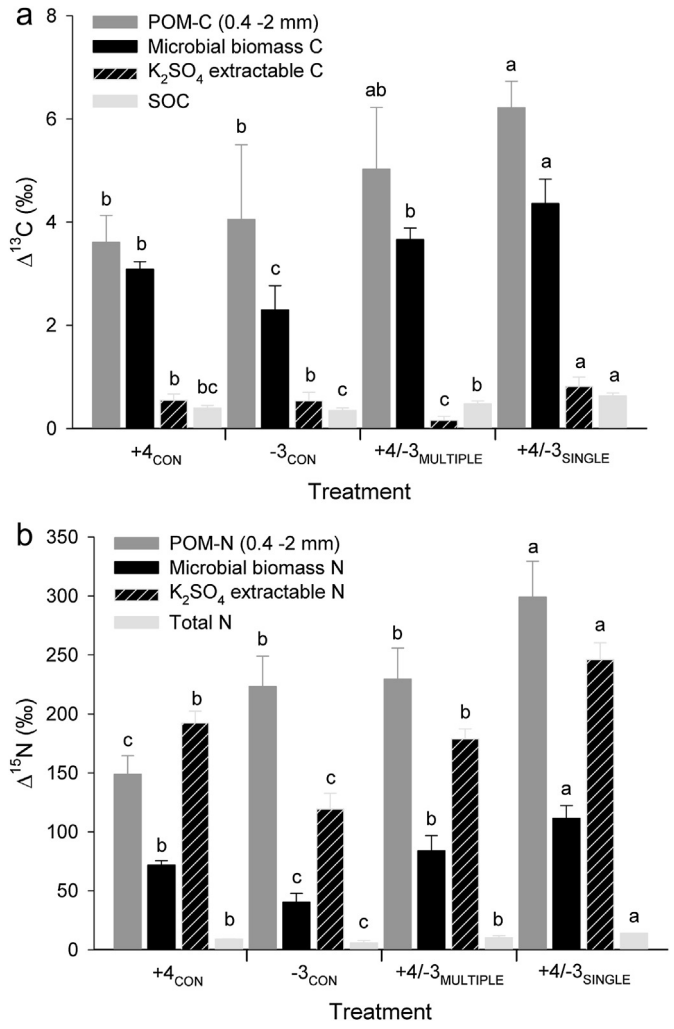

Fig. 1. a+b. Isotopic enrichment in (a) POM-C (0.4–2 mm), microbial biomass C, K_2SO_4 extractable C, soil organic C (SOC) and (b) POM-N (0.4–2 mm), microbial biomass N, K_2SO_4 extractable N and soil total N (total N) in maize-amended samples compared with non amended controls at the end of the 56-day incubation; error bars show ± one standard deviation ($n = 5$); different letters above the columns indicate a significant differences ($P < 0.05$).

On average, 99% of the maize C and 96% of maize N added were recovered in the different compartments (Fig. 2a+b, Table 2). About 36% of the maize C and 61% of the maize N were transferred to SOC and soil total N in the $+4/-3_{SINGLE}$ treatment. In the other treatments, only 25% of the maize C and 41% of the maize N were found on average in these compartments. Also, nearly 6% of the maize C and about 10% of the maize N were transferred to the POM 0.4–2 mm fraction in the $+4/-3_{SINGLE}$ treatment, whereas in the other treatments the recovery for this fraction was around 2% for maize C and nearly 4% for the added maize N.

3.2. C mineralization

Microbial activity was very low in all temperature scenarios without substrate addition. Lowest CO_2 emission was measured in the constant frost scenario (-3_{CON}), with a mean respiration rate of 0.6 μg CO_2–C g^{-1} soil d^{-1} (Fig. 3a). Mean heterotrophic respiration in the $+4_{CON}$ treatment was about 45% higher. Multiple freeze–thaw cycles increased the C mineralization over that of the $+4_{CON}$ and -3_{CON} treatments and averaged 1.0 μg CO_2–C g^{-1} soil d^{-1} (Fig. 3b). Here, mean respiration rates at +4 and −3 °C were about 21% and 40% higher than those of the $+4_{CON}$ and -3_{CON} samples, respectively. Mean respiration rate of the $+4/-3_{SINGLE}$ treated samples at −3 °C four days after the temperature change was 0.6 μg CO_2–C g^{-1} soil d^{-1} and not significantly different to that of the -3_{CON} samples. Four days after the samples were thawed again, respiration rates doubled and decreased thereafter to the level of the first 14 days (Fig. 3b).

The addition of maize residues led to an immediate increase in CO_2 evolution, which was less pronounced under the constant frost scenario. Microbial respiration in the $+4_{CON}$ treatment peaked on day 4 and decreased continuously thereafter (Fig. 3c). Mean respiration rate was 6.6 μg CO_2–C g^{-1} soil d^{-1}. Lowest C mineralization was again observed in the -3_{CON} treatment and averaged 1.9 μg CO_2–C g^{-1} soil d^{-1}. Until the end of the experiment, no clear pattern of microbial substrate utilization was observed. Samples that were exposed to multiple freeze–thaw cycles had an average respiration rate of 5.2 μg CO_2–C g^{-1} soil d^{-1} (Fig. 3d). When measured at +4 °C the samples had 9.4% higher C mineralization rates than samples of the $+4_{CON}$ treatment on the same days. However, these were only minor and not significant. On the other hand, at −3 °C the respiration rates were about 81% higher than mineralization rates of the -3_{CON} samples on the same days. In the $+4/-3_{SINGLE}$ treatment, microbial respiration also peaked four days after application of maize residues, with a mean rate of 13.3 μg CO_2–C g^{-1} soil d^{-1}, and decreased sharply thereafter, especially when the incubation temperature was set to −3 °C (Fig. 3d). CO_2 evolution at −3 °C equilibrated four days after temperature change and was on average 16% higher than respiration rates of -3_{CON} samples on the same days. Thawing of the samples led to a 4.5 fold increase in respiration four days after the incubation temperature was set back to 4 °C. Thereafter, microbial respiration decreased again but was on average still 1.6 times higher than respiration rates of the $+4_{CON}$ samples at that time.

Cumulative CO_2 production (Fig. 4) in the control samples averaged 48 μg C g^{-1} soil and increased in the order $-3_{CON} < +4/-3_{SINGLE} < +4_{CON} < +4/-3_{MULTIPLE}$. Total CO_2 production after application of maize residues was highest in the $+4_{CON}$ treatment, with around 396 μg C g^{-1} soil, which was almost four times higher than in the -3_{CON} treatment. 21% of respired CO_2–C originated from the added maize C in the $+4_{CON}$ treatment, compared with only 3% in the -3_{CON} treatment. No significant difference in total C mineralization was found between the $+4/-3_{MULTIPLE}$ and the $+4/-3_{SINGLE}$ treatments, at roughly 300 μg C g^{-1} soil. In both treatments, 11.5% of the evolved CO_2–C originated from the added maize C.

The application of maize residues always significantly increased the mineralization of soil-derived C in comparison with the control samples (Fig. 4). The strongest increase in extra soil-derived CO_2–C production was found in the $+4_{CON}$ treatment as well as the freeze–thaw cycle treated samples. In the $+4_{CON}$ and -3_{CON} treatments, maize amendment led to an additional cumulative mineralization of 96.6 and 31.2 μg SOC g^{-1} soil, according to an overall PE of 180% and 85% (equivalent to 0.7 and 0.2% of total SOC). Maize amended samples in the $+4/-3_{MULTIPLE}$ and $+4/-3_{SINGLE}$ treatments respired around 111 μg g^{-1} soil of additional soil C, equivalent to 0.8% of total SOC and an overall PE of 170% and 270%, respectively.

3.3. Soil microbial biomass and extractable C and N

The contents of microbial biomass C in the samples without application of maize residues at the end of the incubation period were very similar between the four treatments, at about 210 μg g^{-1} soil (Fig. 5a, Table 3). No significant effects of the temperature treatments were observed. Mixing of maize residues into

Table 1
$\delta^{13}C$ values in soil organic C, K_2SO_4 extractable C, microbial biomass C, POM-C 0.4–2 mm and >2 mm at the end of the 56-day incubation; $\delta^{15}N$ values in soil total N, K_2SO_4 extractable N, microbial biomass N, POM-N 0.4–2 mm and >2 mm at the end of the 56-day incubation in control and maize amended samples of four temperature treatments.

Treatment	SOC	K_2SO_4 extractable C	Microbial biomass C	POM-C 0.4–2 mm	POM-C >2 mm
	$\delta^{13}C$ (‰)				
Maize					
$+4_{CON}$	−26.4 abc	−24.4 ab	−22.7 bc	−25.4 b	−14.9 a
-3_{CON}	−26.5 abc	−24.3 ab	−23.3 c	−24.8 b	−15.3 a
$+4/-3_{MULTIPLE}$	−26.3 ab	−24.6 b	−22.2 ab	−23.6 ab	−15.5 a
$+4/-3_{SINGLE}$	−26.2 a	−23.7 a	−21.6 a	−22.6 a	−15.6 a
Control					
$+4_{CON}$	−26.8 bc	−24.8 b	−25.8 d	−29.0 c	–
-3_{CON}	−26.9 c	−24.9 b	−25.6 d	−28.7 c	–
$+4/-3_{MULTIPLE}$	−26.8 bc	−24.7 b	−25.9 d	−28.9 c	–
$+4/-3_{SINGLE}$	−26.9 c	−24.9 b	−26.0 d	−28.7 c	–
CV (±%)	0.8	1.2	1.2	1.9	3.6
Treatment	Total N	K_2SO_4 extractable N	Microbial biomass N	POM-N 0.4–2 mm	POM-N > 2 mm
	$\delta^{15}N$ (‰)				
Maize					
$+4_{CON}$	16.9 b	197 b	78 b	156.c	483 c
-3_{CON}	12.9 c	124 c	45 c	231 b	581 a
$+4/-3_{MULTIPLE}$	17.2 b	184 b	88 b	237 b	522 bc
$+4/-3_{SINGLE}$	22.0 a	251 a	116 a	307 a	537 ab
Control					
$+4_{CON}$	7.8 d	5.0 d	5.6 d	7.4 d	–
-3_{CON}	7.7 d	4.9 d	5.0 de	7.6 d	–
$+4/-3_{MULTIPLE}$	7.8 d	5.0 d	4.5 e	7.8 d	–
$+4/-3_{SINGLE}$	7.9 d	5.0 d	4.3 e	7.9 d	–
CV (±%)	3.0	6.1	11.2	8.0	4.8

Different letters within a column indicate a significant difference ($P < 0.05$; Tukey HSD, $n = 5$); CV = pooled coefficient of variation between replicate incubations ($n = 5$).

the soil significantly increased microbial biomass C to a mean of 330 μg g^{-1} soil at the end of the incubation. While the significantly highest amount of microbial biomass C was in the $+4/-3_{SINGLE}$ treated samples, there was no significant difference for the +4- and -3_{CON} treatments or for the $+4_{CON}$ and $+4/-3_{MULTIPLE}$ treated samples (Fig. 5a, Table 3). Microbial biomass N in the control samples was not significantly affected by the temperature treatment and averaged 20 μg g^{-1} soil (Fig. 5b, Table 3). Except for the -3_{CON} treatment, application of maize residues significantly increased microbial biomass N to a mean of 27 μg g^{-1} soil. Samples of the $+4/-3_{SINGLE}$ treatment showed significantly highest amounts of microbial biomass N. In contrast to microbial biomass C, $+4_{CON}$ treated samples had significantly higher amounts of microbial biomass N than the -3_{CON} treatment. The microbial biomass C to N ratio significantly increased in the maize amended samples, except for the $+4_{CON}$ treatment, with highest values in the freeze–thaw cycle treatments (Table 3).

Without application of maize residues, the contents of fungal ergosterol were about 0.4 μg g^{-1} soil, samples of the -3_{CON} treatment showing highest amounts (Table 3). Application of maize residues significantly increased the ergosterol content, regardless of the temperature treatment, to an average of 0.6 μg g^{-1} soil. As for microbial biomass C and N, the highest contents of ergosterol were found in the $+4/-3_{SINGLE}$ treated samples. There was no significant difference between the other temperature scenarios. In comparison with the control samples, application of maize residues significantly increased the ergosterol to microbial biomass C ratio in the $+4/-3_{MULTIPLE}$ treatment (Table 3).

Significant differences in the incorporation of maize-derived C into the microbial biomass were found between all temperature treatments (Fig. 5a, Table 2). From the added maize C, 6 and 4% were incorporated in the $+4_{CON}$ and -3_{CON} treatments, respectively. Multiple freeze–thaw cycles led to an incorporation of 7%, whereas the highest amount of maize-derived C was found in the $+4/-3_{SINGLE}$ treatment, at 10%. The highest amount of maize-derived N (12%) in the microbial biomass was also found in this treatment (Fig. 5b, Table 2). In the $+4_{CON}$ and the $+4/-3_{MULTIPLE}$ treatments, about 7% of the added maize N were incorporated, whereas only 2% of the added maize N were found in the -3_{CON} treatment.

After application of maize residues, soil-derived C and N were additionally incorporated into the microbial biomass in all temperature scenarios. This led to 20–40% increases in soil-derived microbial biomass C and N (corresponding to 0.3–0.7% of SOC and total soil N), being most significant for the $+4/-3_{SINGLE}$ treatments soil.

The contents of K_2SO_4 extractable C and N in the control samples were on average 14 and 4 μg g^{-1} soil, respectively (Table 3). Application of maize residues led to a general increase in K_2SO_4 extractable C ranging from 39% (-3_{CON}) to 64% ($+4/-3_{MULTIPLE}$), and to a 15% increase in K_2SO_4 extractable N only in the -3_{CON} and $+4/-3_{SINGLE}$ treatments.

4. Discussion

4.1. Effects on C mineralization

Regardless of the temperature treatment, the C mineralization significantly increased in the residue-amended samples, indicating a clear turnover of the added maize residues at temperatures near and below 0 °C. However, as a consequence of the permanent frost, maize residue decomposition and thus mineralization rates in the -3_{CON} scenario were in general significantly decreased during the incubation and remained constantly low throughout the experiment. A similar pattern of microbial substrate use under constantly frozen conditions was found by Drotz et al. (2010). They observed a lag-phase in microbial CO_2 production of about 60 days in glucose amended soil samples frozen at −4 °C, after which the respiration rates strongly increased. This was accompanied by the production of glycerol, which they first detected after 34 days. Glycerol is

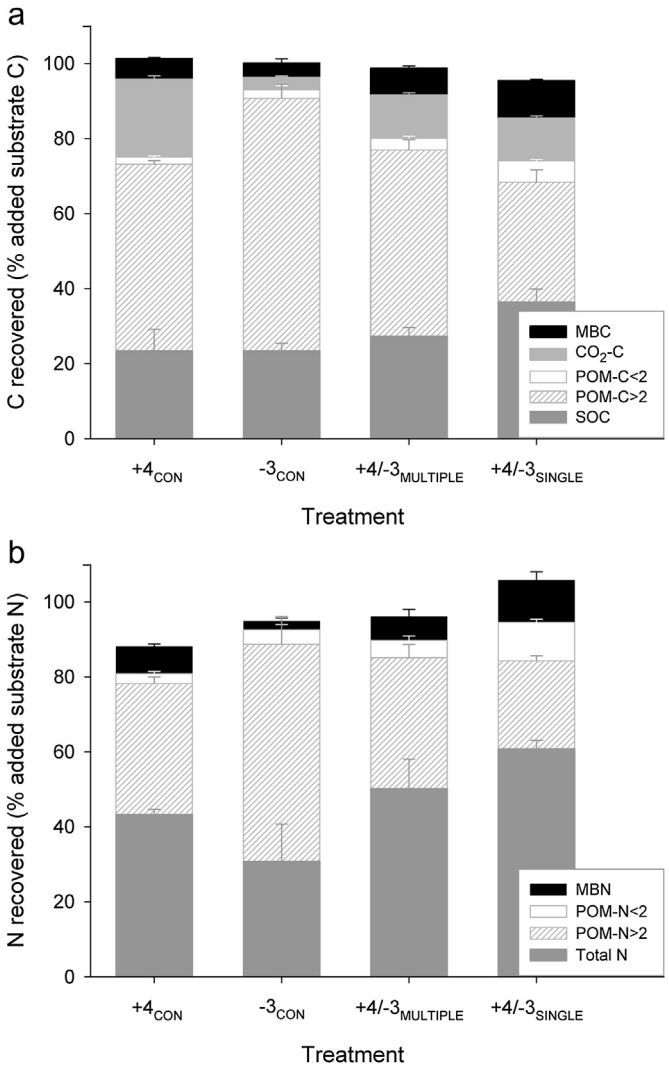

Fig. 2. a + b. Recovery of the added maize straw C and N in the fractions (a) microbial biomass C (MBC), CO_2–C, POM-C < 2 mm, POM-C > 2 mm, soil organic C (SOC) and (b) microbial biomass N (MBN), POM-N < 2 mm, POM-N > 2 mm and soil total N (total N) at the end of the 56-day incubation; error bars show ± one standard deviation ($n = 3$).

known as a cryoprotective agent that limits intracellular cell-damage and maintains microbial activity and CO_2 production (Beal et al., 2001; Fonseca et al., 2001; Drotz et al., 2010). The observed increase in C mineralization between day 22 and 36 (Fig. 3c) of roughly 55% might also indicate adaptation processes like the formation of glycerol or shifts in the membrane lipid composition.

A decreased substrate affinity (Nedwell, 1999) under constant frost may have hampered the respiration of the added maize residue C, so that only 3.3% were mineralized to CO_2. Drotz et al. (2010) reported a more then 4-fold higher mineralization of glucose after 99 days at −4 °C. This was most likely due to a direct microbial uptake of the soluble glucose C. In our study, the added maize residues contain complex components, e.g. cellulose, lignin and waxes (Incerti et al., 2011), which require the production of exo-enzymes for decomposition. This and the shorter incubation period explain the lower amount of C mineralised. In the $+4_{CON}$ treatment, microbial activity was not constrained by water availability (Brooks et al., 2011) or low substrate diffusion rates (Davidson and Janssens, 2006). Consequently, this temperature scenario was most favourable for microbial substrate use where the cumulative total C and maize-derived C mineralization rates were on average four- and six-fold higher, respectively, than under constant frost conditions. The mineralised maize C amounted to 36, 45 and 62% of the total respired CO_2–C in the -3_{CON}, the freeze–thaw cycle and the $+4_{CON}$ scenarios, respectively, indicating a clear shift in microbial substrate use from SOC to the added plant residue C with increasing temperature. This indicates that total and maize-derived C mineralization depended on the overall time of soil frost and not on the frequency of freeze–thaw events.

Although microbial activity under frozen conditions has been verified in numerous studies (Mikan et al., 2002; Öquist et al., 2004; Kurganova et al., 2007; Öquist et al., 2009; Drotz et al., 2010), the limiting factor for decomposition and mineralization of SOM is the availability of unfrozen water (Romanovsky and Osterkamp, 2000; Öquist et al., 2009). Even though the multiple freeze–thaw cycled samples were repeatedly exposed to short periods of frost (48 h at −3 °C), respiration rates in the frozen phases were significantly higher in comparison with the constant frost scenario. Here, isotopic analysis revealed an average 8-fold increase in mineralization of maize-derived C. Also Larsen et al. (2002) reported higher respiration rates in the frost phase of freeze–thaw treated mesocosms in comparison with constantly frozen controls. Freezing and thawing may strongly disrupt added plant material as well as soil

Table 2
Recovery of the added maize C in the cumulative CO_2–C production, soil organic C (SOC), microbial biomass C, POM-C 0.4–2 mm and >2 mm at the end of the 56-day incubation; recovery of the added maize N in total N, microbial biomass N, POM-N 0.4–2 mm and >2 mm at the end of the 56-day incubation in maize amended samples of four temperature treatments.

Treatment	ΣCO_2–C	SOC	Microbial Biomass C	POM-C 0.4–2 mm	>2 mm	Σ recovery (%)
	(μg g^{-1} soil)					
$+4_{CON}$	246 a	278 b	65 c	22 b	591 b	101 a
-3_{CON}	39 c	275 b	46 d	27 b	792 a	100 a
$+4/-3_{MULTIPLE}$	136 b	320 ab	84 b	37 b	583 b	99 a
$+4/-3_{SINGLE}$	136 b	438 a	121 a	70 a	383 c	96 a
CV (±%)	7	13	11	24	6	3
Treatment	Total N	Microbial Biomass N		POM-N 0.4–2 mm	>2 mm	Σ recovery (%)
	(μg g^{-1} soil)					
$+4_{CON}$	18.4 b	3.3 b		1.1 c	14.9 b	89 a
-3_{CON}	13.1 c	1.0 c		1.6 bc	24.5 a	95 a
$+4/-3_{MULTIPLE}$	21.0 b	2.8 b		2.0 b	14.6 b	96 a
$+4/-3_{SINGLE}$	26.2 a	5.1 a		4.4 a	10.1 c	106 a
CV (±%)	14	24		23	8	5

Different letters within a column indicate a significant difference ($P < 0.05$; Tukey HSD, $n = 5$); CV = pooled coefficient of variation between replicate incubations ($n = 5$).

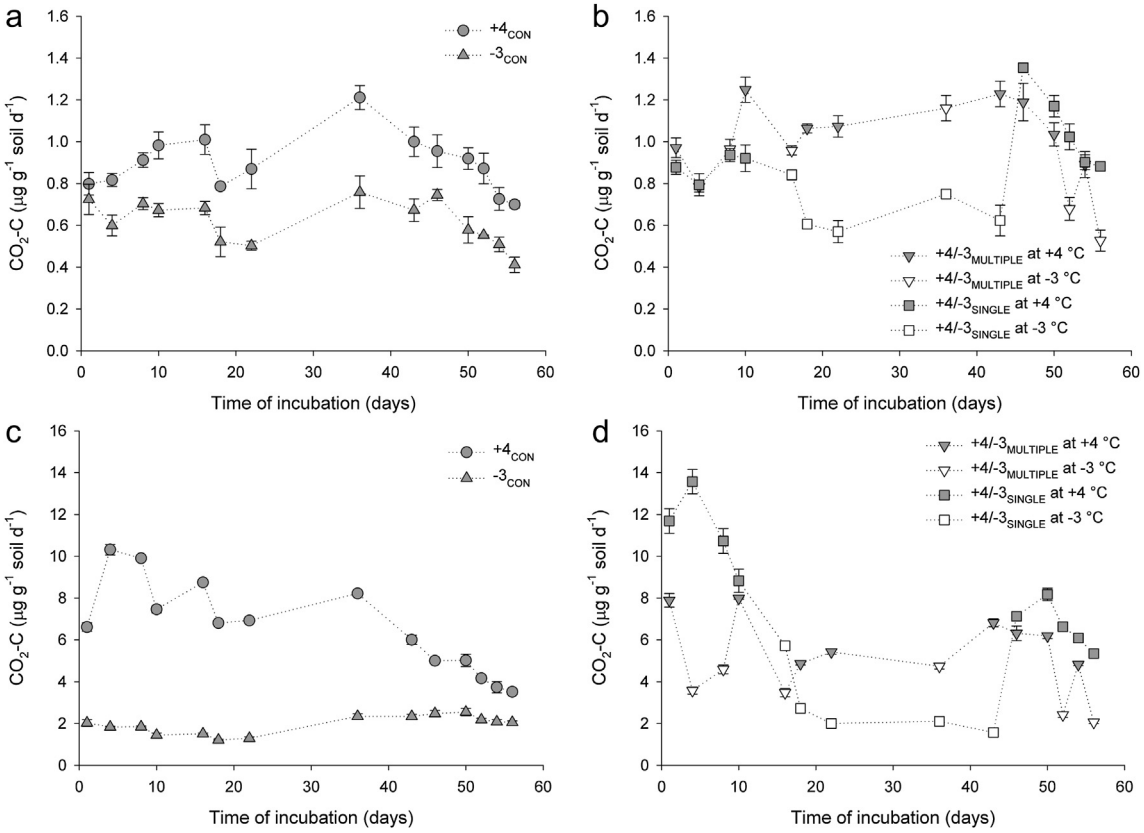

Fig. 3. a–d. CO_2–C production rate of the control (a and b) and maize-amended (c and d) samples over a 56-day incubation period; error bars show ± one standard deviation ($n = 5$).

macroaggregates, making additional nutrients available for microbial uptake (Harris and Safford, 1996; Schimel and Clein, 1996; Herrmann and Witter, 2002; Six et al., 2004).

Another indicator of an enhanced turnover of maize C induced by multiple freeze–thaw cycles when the soil is frozen is the observed mineralization of maize residue C in the single frost phase of the $+4/-3_{SINGLE}$ treatment, which was increased on average only 4-fold over the constant frost scenario and decreased with time. C mineralization in the +4 °C phases of the freeze–thaw cycled samples was also higher than in the $+4_{CON}$ scenario. This contrasts results of Larsen et al. (2002) and Sjursen et al. (2005), who reported lower or similar respiration rates, respectively, using simple organic substrates in their studies.

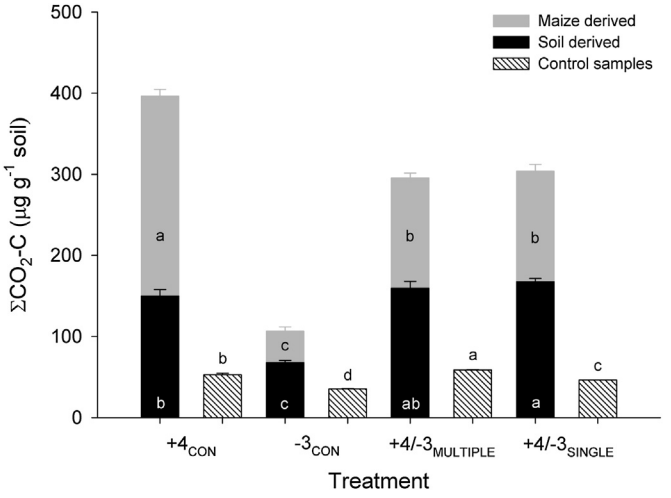

Fig. 4. Cumulative CO_2–C production of all temperature treatments at the end of a 56-day incubation period; error bars show ± one standard deviation ($n = 5$); different letters above the columns indicate a significant difference for the total CO_2–C production of the control samples ($P < 0.05$); different letters in the columns indicate significant differences for the maize- and the soil organic matter-derived CO_2–C production ($P < 0.05$).

4.2. Effects on soil microbial biomass and extractable C and N

The application of constant frost and freeze–thaw cycles apparently had no significant negative effect on microbial biomass in the samples without application of maize residues (Table 3), which is in line with others (Grogan et al., 2004; Koponen et al., 2006; Sharma et al., 2006; Dam et al., 2012) but contrasts reported decreases of microorganisms after freezing and thawing (Winter et al., 1994; Schimel and Clein, 1996; Lipson et al., 1999, 2000; Herrmann and Witter, 2002; Larsen et al., 2002; Pesaro et al., 2003; Dörsch et al., 2004; Feng et al., 2007). Although the different temperature scenarios affected neither the total amount of microbial biomass C nor the amount of microbial biomass N in the control samples, after 56 days the content of ergosterol as an indicator of fungal biomass was significantly increased under constant frost. Consequently, the ergosterol to microbial C ratio, as a relative indicator of the fungal contribution to the total microbial biomass (Djajakirana et al., 1996), was also significantly higher. As there was no input of fresh substrate, a reasonable explanation is that the turnover of ergosterol was reduced, leading to an accumulation of this cell-membrane component. On the other hand, one might

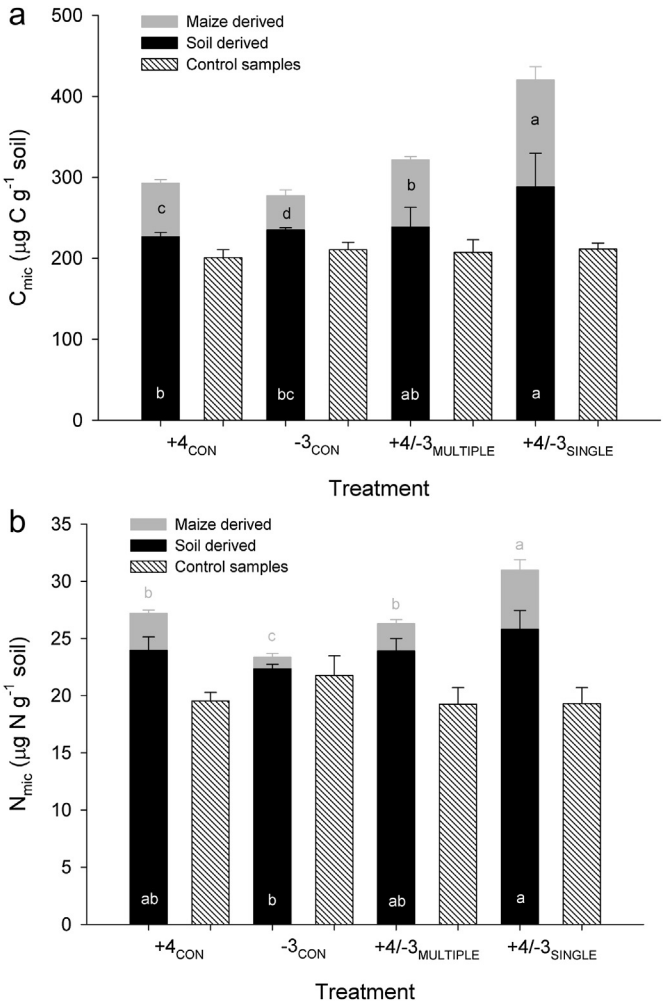

Fig. 5. a + b. Maize and soil derived microbial biomass C (a) and N (b) as well as the respective control samples of all temperature treatments at the end of a 56-day incubation period; error bars show ± one standard deviation ($n = 5$); different letters in or above the columns indicate significant differences for the maize- and the soil organic matter-derived C and N ($P < 0.05$).

suggest that a part of the frost-susceptible microorganisms, mainly bacteria as suggested by Sjursen et al. (2005), might be killed by freezing-induced drought stress (as found by Jensen et al., 2003) and subsequently used as an easily decomposable C and N source by more frost tolerant and slow growing fungi. Due to the low overall microbial metabolism at this temperature, the available nutrients were incorporated into microbial biomass rather than mineralized. This assumption is further supported by the cumulative CO_2 mineralization rate (Fig. 4), which was significantly lower in the constant frost treatment in comparison with the other scenarios. In both control and maize-amended samples, the ergosterol content was not negatively affected after prolonged frost and multiple freeze–thaw cycling. This indicates a frost tolerance of the fungal biomass, which contrasts findings of Feng et al. (2007) and Schmitt et al. (2008).

The temperature treatments had different effects on the microbial biomass after the application of maize residues. While both the total and maize-derived C mineralization were similar between the freeze–thaw cycle scenarios, markedly higher amounts of total and maize-derived microbial biomass C were found in the single freeze–thaw treatment in comparison with multiple freeze–thaw cycles. This means that the frequency of freeze–thaw cycles had no regulatory effect on substrate mineralization, but limited microbial assimilation of maize-derived C at the same time when the frequency of freeze–thaw events was high. In comparison with multiple freezing and thawing, samples of the constant 4 °C treatment also had similar amounts of total microbial biomass C and ergosterol but incorporated significantly less maize-derived C. This was accompanied by significantly higher mineralization rates, indicating a stronger turnover of the added substrate. Due to the faster turnover at constant 4 °C, a part of the freshly formed maize-derived microbial biomass might be mineralized explaining the lower amount of recovered maize C in the microbial biomass and a higher recovery in the mineralized CO_2–C. Microbial activity rather than microbial biomass was affected by temperature fluctuations around the freezing point in comparison with unfrozen conditions. In contrast to Larsen et al. (2002), who reported a decrease in microbial biomass C/N ratios between unfrozen and freeze–thaw cycled conditions (from 15 to 9), the microbial biomass C/N ratio in the present study was increased significantly from 11 to 13. This was due to a slight increase in microbial biomass C and a slight decrease in microbial biomass N caused by freeze–thaw cycling. The decreased amounts of total microbial biomass N under constant frost are in line with similar results of Sjursen et al. (2005).

The recovered extractable C in both maize-amended and control samples were not influenced by the temperature treatments. This is consistent with results of Sjursen et al. (2005) who also found no significant differences after 40 days of incubation. However, the application of maize residues significantly increased the extractable C content, implying that the additional amount must have come

Table 3
K_2SO_4 extractable C and N, soil microbial biomass C, biomass N, ergosterol content, the microbial biomass C-to-biomass N ratio and the ergosterol-to-microbial biomass C ratio at the end of the 56-day incubation in control and maize amended samples of four temperature treatments.

Treatment	K_2SO_4 extractable		Microbial biomass			Ergosterol (µg g^{-1} soil)	Ergosterol/microbial biomass C (%)
	C	N	C	N	C/N		
	(µg g^{-1} soil)		(µg g^{-1} soil)				
Maize							
$+4_{CON}$	20.7 a	14.4 d	293 bc	27.2 b	10.8 cd	0.57 b	0.19 bcde
-3_{CON}	22.6 a	17.0 ab	277 c	23.4 cd	11.8 bc	0.58 b	0.21 bc
$+4/-3_{MULTIPLE}$	22.7 a	18.2 a	333 b	26.4 bc	12.9 ab	0.61 b	0.21 b
$+4/-3_{SINGLE}$	22.1 a	18.2 a	420 a	31.0 a	13.5 a	0.80 a	0.20 bcd
Control							
$+4_{CON}$	13.3 b	15.1 cd	201 d	20.2 e	10.3 d	0.35 d	0.18 e
-3_{CON}	16.2 b	15.1 cd	211 d	21.8 de	9.7 d	0.46 c	0.24 a
$+4/-3_{MULTIPLE}$	13.8 b	17.7 a	207 d	19.3 e	10.8 cd	0.38 d	0.18 de
$+4/-3_{SINGLE}$	14.4 b	16.0 bc	211 d	19.3 e	11.0 cd	0.40 cd	0.19 cde
CV (±%)	6.0	2.7	5.9	5.9	6.4	8.9	8.1

Different letters within a column indicate a significant difference ($P < 0.05$; Tukey HSD, $n = 5$); CV = pooled coefficient of variation between replicate incubations ($n = 5$).

from soluble fractions of the crop residue C and/or residue-derived microbial C, because ^{13}C was slightly enriched in comparison with the control samples. In the constant frost scenario and the single freeze–thaw treatment with one prolonged frost period, differences in the ^{15}N enrichment of the extracts but similar amounts of increased extracted N suggest different sources of the additional N. In contrast, a high frequency of freezing and thawing generally increased the extractable N independently of maize application, whereas temperatures constantly above 0 °C had no effect. This is surprising, as the extracted N was similarly enriched in ^{15}N in both treatments, indicating similar amounts of N origin from the added maize residues or maize-derived microbial biomass.

4.3. Priming effect

In all temperature scenarios, the application of maize residues caused significant increases in soil organic matter-derived CO_2 evolution. An accelerated SOC mineralization after application of maize residues has been repeatedly observed (Vanlauwe et al., 1994; Ouedraogo et al., 2007 Rottmann et al., 2010; Zareitalabad et al., 2010). To our knowledge, this is the first time that priming effects have been experimentally verified on the basis of the microbial decomposition of a complex organic substrate at near and sub-zero temperatures. The additional CO_2 may originate from an increased turnover of microbial biomass C and/or from SOC (Kuzyakov, 2010) and is considered either as apparent or real priming, respectively (Blagodatskaya and Kuzyakov, 2008; Blagodatsky et al., 2010; Kuzyakov, 2010). According to Nottingham et al. (2009), a real priming effect can be assumed either when the excess of soil C mineralized in the substrate-amended soil exceeds the microbial biomass C or an increased incorporation in the same is detected. In our study, the cumulative additional soil C mineralized after maize straw application in the constant +4 °C and freeze–thaw cycle scenarios corresponded to only one third (32%) of the total microbial biomass C and to nearly half of the soil-derived microbial C (44%) after 56 days. In the constant frost treatment, the proportion of the extra soil-derived CO_2–C to the total and soil-derived microbial biomass C was even less (only 13%). This indicates that the release of additional soil-derived CO_2 in all temperature scenarios may be partly or solely driven by an increased turnover of microbial biomass, and thus at least an apparent PE occurred.

It is suggested that the "real" priming effect may be delayed for days or even weeks after substrate addition (Fontaine et al., 2003; Blagodatsky et al., 2010; Kuzyakov, 2010). In the first phase of substrate decomposition, r-strategists quickly metabolise the soluble C compounds (Dilly and Zyakun, 2008) and may contribute to priming due to their growth and endogenous metabolism shortly after substrate addition (Lundquist et al., 1999; Bell et al., 2003), whereas contributions of k-strategists increase in the later stage of decomposition, as they are more efficient in metabolizing more complex C compounds (Bottomley, 1999; Lundquist et al., 1999; Bell et al., 2003). Thus, "real" priming effects should occur when slow growing k-strategists dominate the microbial community (Blagodatskaya et al., 2009). In our study, the most pronounced acceleration of SOM-derived CO_2 evolution was found in the first three weeks of maize decomposition. Here, the C_3–CO_2 increased on average 2-, 5-, 6-, and 8-fold in the constant frost, constant +4 °C, multiple and single freeze–thaw scenarios, respectively. Thereafter, this amount decreased substantially and, in the last 20 days, was only 2- and 1-fold higher in the constant frost and constant +4 °C treatments, respectively. Nevertheless, the amount of C_3–CO_2 of both freeze–thaw scenarios in the last 20 or 10 days was on average still 3 times higher than that of the control samples, indicating a significant effect of multiple freezing and thawing as well as a single thawing after prolonged frost. A possible explanation is that the extracellular enzymes generated by saprotrophic fungi to degrade the added maize residues (cellulases, lignin-modifying enzymes) at the later stage of decomposition are to some extent efficient in decomposing SOC (Fontaine et al., 2003; Kuzyakov, 2010).

The application of maize residues also increased the incorporation of soil-derived C into microbial biomass C in all temperature scenarios, which in turn is considered as real or true priming (Nottingham et al., 2009). Regardless of this, it remains unclear whether the enhanced incorporation of soil-derived C in the freeze–thaw cycle scenarios is due to maize residue decomposition or to disruption of soil aggregates (Oztas and Fayetorbay, 2003; Six et al., 2004), making occluded SOC available for microbial uptake (Soulides and Allison, 1961; Bullock et al., 1988; Christensen and Christensen, 1991).

4.4. Conclusions

Multiple freezing and thawing caused an intermediate substrate mineralization. However, cumulative substrate mineralization was not determined by the frequency of freeze–thaw events but regulated by the overall time of frost and thaw conditions. The opposite was found for the microbial biomass (including fungal biomass), where growth and assimilation of maize-derived C and N were lower at a high freeze–thaw frequency. A shift in microbial substrate use occurred from SOC to maize residue C with increasing soil temperature. A priming effect has been observed for the first time for temperatures around the freezing point, which might have implications for modelling SOC budgets at the annual time scale. Because decomposition of organic matter is not only affected by water availability but also by the soil texture, the use of soils with different contents of sand and clay would be interesting.

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