Negative priming of soil organic matter following long-term in situ warming of sub-arctic soils

Negative priming of soil organic matter following long-term *in situ* warming of sub-arctic soils

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Abstract

Priming is the change of microbial soil organic matter (SOM) decomposition induced by a labile carbon (C) source. It is recognised as an important mechanism influencing soil C dynamics and C storage in terrestrial ecosystems. Microbial nitrogen (N) mining in SOM and preferential substrate utilisation, i.e., a shift in microbial carbon use from SOM to more labile energy sources, are possible, counteracting, mechanisms driving the priming effect. Climate warming and increased N availability might affect these mechanisms, and thus determine the direction and magnitude of the priming effect. Hence, these abiotic factors can indirectly affect soil C stocks, which makes their understanding crucial for predicting the soil C feedback in a warming world. We conducted a short-term incubation experiment (6 days) with soils from a subarctic grassland that had been subjected to long-term geothermal warming (>55 years) by 2-4 °C above unwarmed soil. Soil samples were amended with ^{13}C-labelled glucose and ^{15}N-labelled NH_4NO_3. We found a significantly negative relationship between *in situ* warming and cumulative primed C, with negative priming in the warmed soils. The negative priming suggests that preferential substrate utilisation was a key mechanism in our experiment. Our results indicate that changes in SOM characteristics associated with the *in situ* warming gradient can play a major role in determining the rate and direction of the priming effect. Additionally, we found that neither microbial N limitation nor N addition affected the priming effect, providing evidence that in our experiment, N mining did not lead to positive priming.

Keywords: priming, incubation, soil warming, preferential substrate utilisation, N mining

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1. Introduction

The northern circumpolar region, which contains almost one third of global soil organic carbon (Batjes, 2016; McGuire et al., 2009) is experiencing a disproportionally large temperature rise because of climate change (IPCC, 2013). Soil warming is hypothesised to accelerate soil organic matter (SOM) decomposition, stimulating nitrogen (N) mineralisation (Suzuki et al., 2016) and increasing soil CO_2-release, thereby possibly causing a positive feedback to climate change (Davidson & Janssens, 2006). At the same time, warming and increased N mineralisation, may stimulate plant productivity and subsequent carbon (C) inputs to the soil. This might offset C losses from faster SOM decomposition (Sistla et al., 2013; Abbott et al., 2016). On the other hand, stimulated deposition of labile plant C inputs in response to warming could also induce a (positive) priming effect, i.e., an increase in microbial SOM decomposition, which could result in a net loss of C from soil (Fontaine et al., 2004; Bird et al., 2011). In spite of extensive research over the past decades, the factors influencing the rate and direction of the priming effect are still poorly understood, but are key to forecasting how soils respond to global warming (van der Wal & de Boer, 2017; Zhu et al., 2014; Keuper et al., 2020).

Priming is described as a change in the SOM mineralisation rates following the release of easily available low molecular weight carbon (LMWC) (e.g. root exudates) to the soil (Kuzyakov, 2002). It is generally assumed that nutrient availability, and in particular N, plays an important role in determining the magnitude of the priming effect (Macdonald et al., 2018; Dijkstra et al., 2013; Fontaine et al., 2011). On the one hand, high nutrient availability can lead to favourable microbial growth conditions, stimulating fast growing microbes (r-strategists), using easily available organic carbon and co-metabolising SOM in order to match the C demand, which is known as the basic stoichiometric decomposition theory (Cleveland et al., 2002; Hessen et al., 2004). On the other hand, at low nutrient availability, SOM-degrading microbes (K-strategists) may increase the production of extracellular enzymes targeting N-rich components such as chitin or proteins in the SOM to ease their N limitation, known as N mining (Chen et al., 2014; Brzostek et al., 2013; Phillips et al., 2011). The breakdown of these polymers, in addition to releasing N, is expected to increase microbial C assimilation and mineralisation, manifesting in an increased SOM-derived CO_2-flux (Kuzyakov, 2010; Hartley et al., 2010). In this way, N mining can act as a driver for a positive priming effect. According to this N mining hypothesis, increasing availability of nutrients reduces the requirement for mining and associated release of C (Fontaine et al., 2011).

Another priming mechanism is preferential substrate utilisation, which leads to negative prim-

ing, i.e., reduced SOM mineralisation upon labile substrate addition. Here, microorganisms prefer to utilize the easily available LMWC resulting in a decrease in SOM decomposition (Kuzyakov, 2002). Preferential substrate utilisation has been suggested to be a transient (2-3 days), first mechanisms after LMWC addition causing reduced SOM decomposition, after which positive priming mechanisms take over (Kuzyakov & Bol, 2006), although in many experiments, immediate positive priming is observed (Chowdhury et al., 2014; Wild et al., 2019; Guenet et al., 2010). Additionally, also longer term (> 30 days) periods of preferential substrate utilisation have been observed (Lyu et al., 2018; Wang et al., 2015), where the negative SOM priming was attributed to long-term microbial adaptation to the C inputs combined with a high SOM recalcitrance. After a long period of negative priming, positive priming could occur caused by an increase of the microbial biomass size and resulting C demand induced by the original labile C inputs (Wang et al., 2015). Both priming mechanisms, i.e., N mining and preferential substrate utilisation, could also operate together, reinforcing or attenuating the observed priming effect (Guenet et al., 2010).

Although the mechanisms underlying the priming effect are becoming clearer, little is still known about how SOM priming is influenced by climate warming. In some short-term laboratory incubation studies warming reinforced positive priming (Zhu & Cheng, 2011; Streit et al., 2014), while other studies observed a microbial respiration increase, but no change of SOM-derived respiration in response to warming (Guttières et al., 2020). Yet another study reports both increased and unchanged priming upon warming, depending on the soil type (Lenka et al., 2019). These findings suggest that the magnitude and the direction of the priming effect in response to warming is influenced by both SOM characteristics and microbial physiology.

Laboratory incubation studies often induce short-term warming, only lasting for a couple of weeks or even days. However, long-term soil warming might play a determining role, affecting quality and quantity of SOM (LaCroix et al., 2021) or the microbial community (DeAngelis et al., 2015), possibly leading to different warming responses than we would expect from short-term warming. Long-term warmed soils, with adapted but stable microbial community and SOM composition (Walker et al., 2018) can provide important insights into how priming responds to a new warmed 'steady state', but such studies are scarce. Two studies investigating the effect of *in situ* warming on SOM priming reported increased, decreased or unaltered priming (Streit et al., 2014; Mau et al., 2018). Both studies accordingly suggest soil warming does not directly affect priming, but can do so indirectly through altered availability of soil C and N. Untangling the initial responses and prolonged effects of warming on ecosystem C- and N-cycling via plant-microbial

feedbacks is critical to informing predictions of climate change impacts on the C-cycle. While warming typically stimulates microbial activity and decomposition initially, prolonged warming and microbial stimulation can lead to a depletion of SOM stocks (Walker et al., 2018), potentially reducing microbial activity and exacerbating N limitation of plants and microbes. In this way, prolonged warming can be expected to stimulate N mining.

To assess the influence of N availability, SOM characteristics and warming-duration on the magnitude and direction of the priming effect, we set up an incubation experiment using grassland soils subjected to long-term geothermal warming by 2-4°C above ambient (Sigurdsson et al., 2016). We added ^{13}C-labelled glucose, a labile C substrate commonly present in plant exudates (Carvalhais et al., 2011) to mimic root exudates, to alleviate a possible C limitation and induce a N limitation. Additionally, we added ^{15}N-labelled ammonium nitrate as a labile N source. This experimental design enabled us to test the N mining theory, according to which N limitation induced by glucose addition would drive N mining, causing positive priming. Combined addition of glucose and ammonium nitrate would reduce the microbial N limitation compared to the glucose-only treatment, and lead to lower priming. Additionally, we tested if soils adapted to *in situ* warming exhibited different SOM decomposition and microbial biomass production in response to labile C addition than short-term warmed soils.

2. Material and methods

2.1. Site description and sampling

The soils for the incubation experiment were collected at the ForHot research site, located in the Hengill geothermal area, 40 km east of Reykjavík, Iceland (64°00 ' 01" N, 21°11 ' 09" W; 100 – 225 m a.s.l.) (Sigurdsson et al., 2016). The mean annual temperature between 2006 and 2016 was 5.2 ± 0.1 (SE) °C, and the mean annual precipitation during the same period was 1413 ± 57 (SE) mm (Icelandic Meteorological Office; Eyrarbakki weather station). The main vegetation type is unmanaged grassland, dominated by *Agrostris capillaris*, *Ranunculus acris* and *Equisetum pratense*. The underlying soil is classified as Brown Andosol (Arnalds, 2015).

The soil samples were collected from a geothermally heated grassland site that at least was warmed from 1963 on, when the first surveys were conducted (Sigurdsson et al., 2016). In autumn 2012 and spring 2013, thirty permanent plots were established on a temperature gradient around two different hot spots. In each plot, temperatures were logged hourly at 10 cm depth with a HOBO TidbiT V2 Water Temperature Data logger (Onset computer Corporation, USA). The annual

average soil warming temperatures range from ambient to +20 °C. A more detailed description of the site can be found in (Sigurdsson et al., 2016).

The soils for the laboratory incubation experiment were sampled in early June 2018, around one month after the average start of the growing season on the site (Leblans et al., 2017). Topsoil samples were collected from three temperature levels (A, C & D) corresponding to average daily summer soil temperatures of 11.8 ± 0.6, 14.2 ± 0.2 and 15.6 ± 0.5 °C respectively (fig. 6). All three temperature levels were sampled from three different transects, serving as biological replicates (n = 3 per temperature level), resulting in nine distinct soils (fig. 1, table 1)). After sampling, the soil samples were transported to the Department of Ecology at the University of Innsbruck, Austria for further processing. A brief characterisation of the soils can be found in table 1.

Table 1: Characteristics of the nine plots, distributed over three transects, the soil samples were harvested from. The plots were divided in three temperature levels (A, C & D) with a contiguous average summer T. Soil C % and N % measured in bulk soil are provided, as well as soil C:N ratio.

Transect	Temp. level	T (°C)	C%	N%	C:N
1	A	11.2	5.56%	0.53%	10.57
2	A	11.9	5.75%	0.50%	11.60
3	A	12.4	3.41%	0.29%	11.75
1	C	14.1	4.37%	0.38%	11.58
2	C	14.2	5.30%	0.44%	12.04
3	C	14.4	5.47%	0.43%	12.66
1	D	16.1	3.87%	0.31%	12.42
2	D	15.5	4.41%	0.37%	12.02
3	D	15.1	3.43%	0.29%	11.91

2.2. Experimental design - soil incubation and substrate addition

In the laboratory, soils were sieved to 2 mm, soil moisture content was determined gravimetrically, and adjusted to 60 % of water holding capacity (WHC). All soils were kept at 12 °C until the beginning of the incubation experiment, 6-11 days later. The incubation experiment with ^{13}C and ^{15}N-labelled substrates was set up in full factorial design resulting in four different treatments: 1) no substrate (only water), 2) ^{13}C-glucose addition, 3) ^{15}N-ammonium nitrate addition and 4) ^{13}C-glucose and ^{15}N-ammonium nitrate addition. ^{13}C-labelled glucose was added at an amount of 15 mg C g^{-1} dw soil C, similar as in Hartley et al. (2010). Hence, added amounts of glucose (slightly) differed, depending on the soil C content of the soils. The ideal amount of N addition was determined in a pre-experiment, where we aimed to avoid excess N which microbes would not assimilate and possibly would cause soil acidification with unwanted side effects. We added C and N at a C/N ratio of 20, translating to 0.75 mg N g^{-1} dw soil C, similar as (Alden et al., 2001).

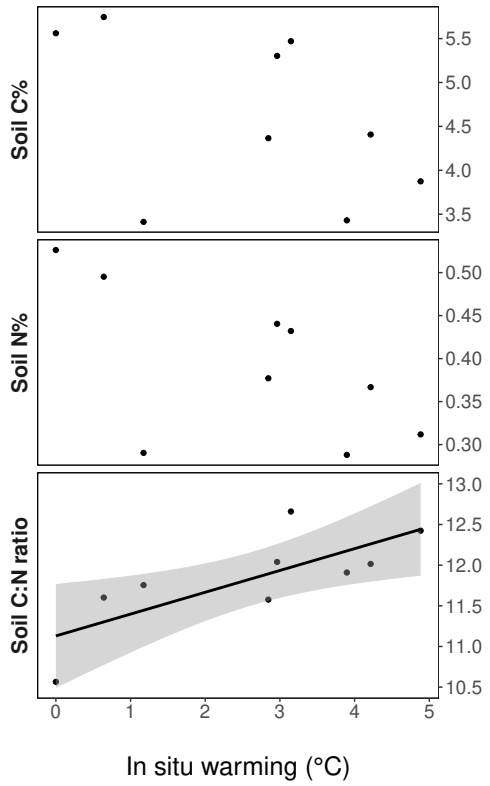

Figure 1: Soil C %, soil N % and soil C:N ratio in the nine plots the soil samples were harvested from in relation to *in situ* soil warming. Soil C % and soil N % did not significantly change, whereas soil C:N ratio significantly increased upon warming.

For the incubation experiment, 60 g of fresh weight soil (at 60 % of WHC) were placed in a specimen cup and thoroughly mixed with 1 mL of 6.4 atom% ^{13}C-glucose (mixture of 99 atom% ^{13}C-enriched glucose from IsoLife bv, the Netherlands, with natural abundance glucose) or 4.3 atom% ^{15}N-ammonium nitrate (mixture of ammonium nitrate which was 98 atom% ^{15}N-enriched at both NH_4-N and NO_3-N from Aldrich Chemistry, Germany with conventional ammonium nitrate) suspended in deionised water. Each cup was placed inside a bigger mason jar of 1150 mL with a lid equipped with open tubes, to avoid CO_2 accumulation in the jar. To enable taking destructive soil samples during the incubation period without disturbing the respiration measurements, 15 g samples of identically treated soil were incubated in scintillation vials with punctured caps alongside the jars.

Samples were divided into batches of twelve samples each, consisting of soils from three transects, each with four different substrate treatments. Samples were incubated both at their *in situ* temperature (12 °C - A plots, 14 °C - C plots or 16 °C - D plots), and at a common elevated temperature of 16 °C, making up five different incubation batches. All samples were incubated

in the dark for six days, since this length has been shown to be sufficient to induce the priming dynamics we are aiming for (Chowdhury et al., 2014; Wild et al., 2019). During incubation, microbial respiration and its C isotopic composition (as atom % ^{13}C) were measured immediately after substrate addition and on day 1, 2, 4 and 6, using an online isotope analyser (Picarro G2201i Analyzer, Picarro Inc., Santa Clara, CA, United States). Before each measurement, the incubation jars were ventilated with atmospheric air to have the concentration of CO_2 inside the jar close to ambient air. During each measurement, the incubation jars were closed and connected to the isotope analyser for closed system measurements (Picarro A0702 CSM package, Picarro Inc., Santa Clara, CA, United States). The CO_2 concentration and isotopic composition inside the chamber were continuously measured every ca. second for ten minutes. Data from the first four minutes and the last one minute were removed due to disturbance during opening and closing of jar, and to allow steady mixing inside the jar and in the connection to the isotope analyser. The microbial respiration was calculated as the slope of linear regression of CO_2 accumulation over five minutes. The isotopic composition of microbial respiration was calculated as the intercept from the linear regression between paired values of reciprocal of CO_2 concentration and isotope ratio (Keeling, 1961). At the end of each measurement day, three calibration gases (400 ppm, 1500 ppm, 3000 ppm) with known isotopic ratio were measured to calibrate the analyser. The calibration gas with 3000 ppm and $\delta^{13}C$ of -6.35‰ has > 30 ppm of $^{13}CO_2$ which allowed us to calibrate analyser for high ^{13}C enriched CO_2 (Bowling et al., 2003).

2.3. Microbial biomass and uptake of ^{13}C- and ^{15}N-labelled substrates

Microbial biomass C- and N-pools were determined using the chloroform fumigation method (Vance et al., 1987) by extracting 2 g of chloroform fumigated and non-fumigated soil aliquots with 20 mL of 0.5 M K_2SO_4, before incubation and on day 2 and 6 of the after substrate addition. Fumigated and non-fumigated K_2SO_4 extracts were analysed for extractable organic C (EOC) and total extractable N (TEN) on a TOC/TN Analyzer (TOC-V CPH E200V/TNM-122V; Shimadzu, Austria). The atom % ^{13}C of EOC in fumigated and non-fumigated K_2SO_4 extracts was determined by direct injection on an IC system (DX 3000, Dionex Corporation Sunnyvale, CA, USA) without column and connected through a Finnigan LC Isolink-Interface (Thermo Fisher Scientific, Waltham, Ma, USA) to a Finnigan Delta V Advantage Mass Spectrometer (Thermo Fisher, Bremen, Germany). To determine the atom % ^{15}N of TEN in fumigated and non-fumigated K_2SO_4 extracts, TEN was oxidized with alkaline persulfate to NO_3. Subsequently, NO_3 was reduced to N_2O using VCl_3 (in presence of NaN_3), which was then analysed on a purge and trap isotope

ratio mass spectrometer (PT-IRMS), using a Gasbench II headspace analyzer (Thermo Fisher, Bremen, Germany) with cryo-focusing unit, coupled to a Finnigan Delta V Advantage IRMS (Thermo Fisher, Bremen, Germany) as outlined in (Lachouani et al., 2010). Microbial biomass C was calculated as the difference in C between the fumigated and non-fumigated (EOC) extracts. A two-pool mixing model was used to calculate atom% ^{13}C in C_{mic} (eqn. 1).

$$atom\%_{Cmic} = \frac{C_{fum} \times atom\%_{fum} - C_{Nfum} \times atom\%_{Nfum}}{C_{mic}} \quad (1)$$

where C_{fum}, C_{Nfum} and C_{mic} (i.e., $EOC_{fum} - EOC_{Nfum}$) indicate the C concentrations of the fumigated extracts the non-fumigated extracts and the microbial C respectively (in mg C g^{-1} dw soil), and $atom\%_{Cmic}$, $atom\%_{fum}$ and $atom\%_{Nfum}$ indicate the corresponding atom % ^{13}C. Microbial biomass N was calculated in the same way as microbial biomass C.

2.4. CO_2 flux source partitioning to quantify priming effects

The CO_2 efflux (in mg C g^{-1} dw soil) from glucose-treated soils was partitioned in glucose- and SOM-derived components using a two-pool isotope-mixing model (Phillips et al., 2005) as shown in equation 2.

$$\begin{aligned} flux_{gluc} &= flux_{total} \times \tfrac{atom\%_{total} - atom\%_{SOM}}{atom\%_{gluc} - atom\%_{SOM}} \\ flux_{SOM} &= flux_{total} - flux_{gluc} \end{aligned} \quad (2)$$

The fraction of absolute primed SOM decomposition (PE_{abs}) was then calculated by subtracting the SOM-derived CO_2 of a water-amended sample ($flux_{SOM-water}$) from the SOM-derived CO_2 of the corresponding substrate-amended samples ($flux_{SOM-gluc}$) (eqn. 3). This calculation was done for each time point where the CO_2 flux was measured. Also, the cumulative flux and cumulative priming effect were obtained by calculating the area under the flux-time and priming-time curve. The relative priming effect (PE_{rel}) was calculated by dividing the absolute primed SOM (PE_{abs}) by the SOM-derived respiration of the water treatment ($flux_{SOM-water}$) as shown in equation 4.

$$PE_{abs} = flux_{SOM-gluc} - flux_{SOM-water} \quad (3)$$

$$PE_{rel} = \frac{PE_abs}{flux_{SOM-water}} \quad (4)$$

Where PE_{abs}, PE_{rel}, $flux_{SOM-water}$ and $flux_{SOM-gluc}$ indicate absolute and relative priming, and SOM-derived flux in the water and glucose treatment, respectively. The same two-pool isotope-mixing model was used to partition substrate derived ^{13}C- and ^{15}N-incorporation into microbial biomass C & N, as well as into EOC and TEN.

2.5. Statistical analysis

Microbial respiration rates were analysed using a general additive model (Wood, 2011), with a corCAR1 autocorrelation correction structure for repeated sampling of the same soil samples, and a smoother function on 'day after incubation' to account for differences over incubation time. The model formulation is shown below.

```
gamm(CO2_flux ~ Ts + Tis + C + N + s(Day, k = 4),
correlation = corCAR1(form = ~Day|Sample_ID),
data=flux_data)
```

The variables `CO2_flux`, `Ts`, `Tis`, `C`, `N`, `Sample_ID` and `Day` correspond with microbial respiration, incubation temperature, *in situ* temperature, C addition (T/F), N addition (T/F), unique sample ID, and incubation time, respectively. Main effects as well as interaction effects were evaluated. The other soil data (microbial biomass C and N, EOC and TEN) were analysed using a simple linear model using *in situ* and incubation warming and the C and N addition as a main effect. When testing for responses to different substrate treatments, a paired two-sided T-test was used. All tests were performed using the R software (R Development Core Team, 2011).

3. Results

3.1. In situ warming vs. incubation warming responses

In situ warming did not affect microbial biomass C (C_{mic}) ($p = 0.80$; fig. 2) and microbial biomass N (N_{mic}, $p = 0.95$; fig. 7). In contrast, *in situ* warming of soils led to an increased microbial SOM-derived respiration ($p < 0.01$; fig. 4). Additionally, prior to incubation, TEN exhibited a negative relationship with *in situ* warming ($p = 0.02$; fig. 3), whereas this trend was not visible in EOC (fig. 8). After two days of incubation, the negative effect of *in situ* warming on TEN disappeared. Soil C:N ratio significantly increased with *in situ* warming ($p = 0.02$; fig 1).

The incubation of soils at elevated temperatures compared to their average site conditions (i.e., incubation warming), significantly increased microbial respiration: SOM-derived microbial

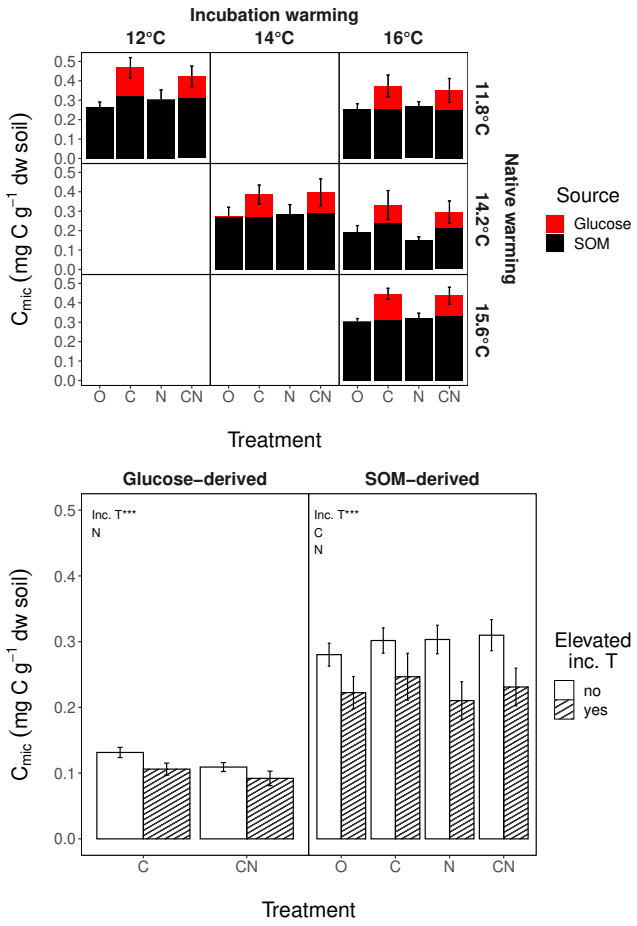

Figure 2: Microbial biomass carbon (C_{mic}) at the end of the incubation. In the upper panel, columns and the rows show the incubation temperature and the *in situ* temperature respectively. Glucose-derived C_{mic} is shown in red, and SOM-derived C_{mic} in black. In the lower panel, the effect of increased incubation temperature is shown separately for glucose-derived and SOM-derived C_{mic}. The blank and the hatched bars show the average C_{mic} of soils incubated at their *in situ* and increased temperature respectively.

respiration increased by 38 % ($p = 0.02$) when the ambient plots were incubated at 16 °C compared to their *in situ* temperature of 12 °C (fig. 4). There was no statistically significant increase in microbial SOM-derived respiration when incubating *in situ* 14 °C plots at 16 °C compared to incubation at *in situ* temperature. Incubation warming reduced C_{mic} at the end of the incubation period compared to soils incubated at *in situ* soil temperature ($p = 0.002$; fig. 2). Due to increased respiration and decreased C_{mic}, also specific microbial respiration at the end of the incubation increased significantly with incubation warming ($p < 0.001$). There was no statistically significant trend with incubation warming in N_{mic}, nor did the N_{mic} change during the incubation experiment (fig. 7).

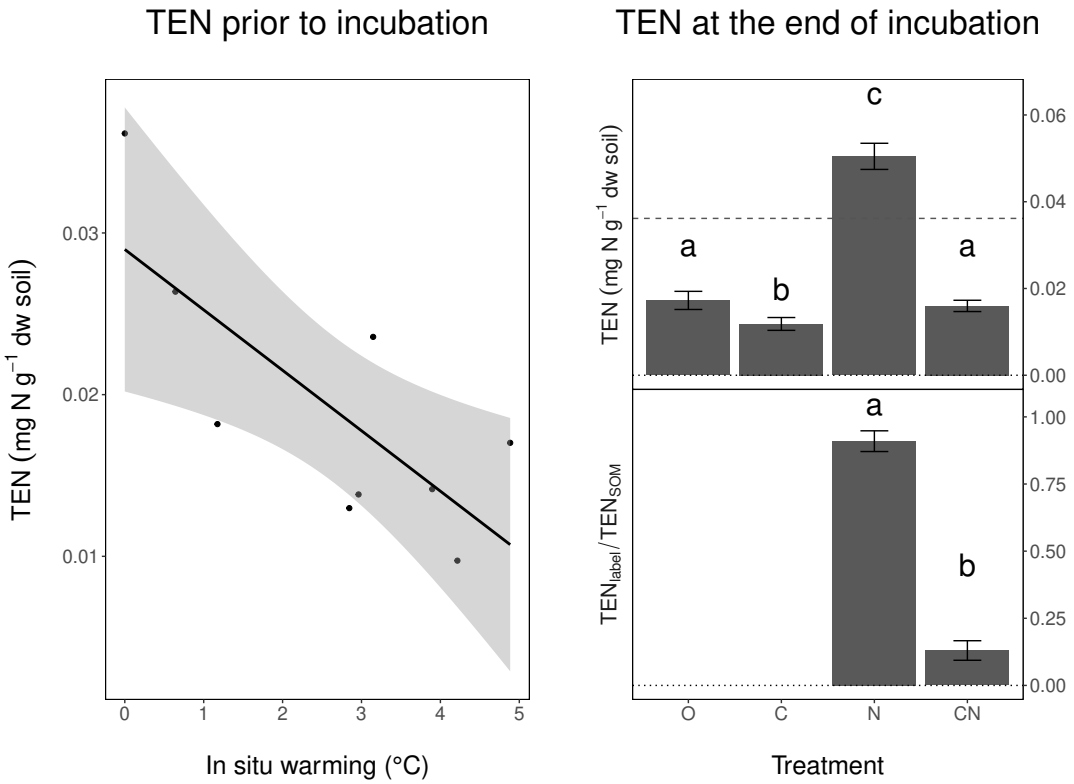

Figure 3: Total extractable nitrogen (TEN) in soils prior to incubation (left panel) and at the end of the incubation (right panel). In the right panel, significances on a $p < 0.05$ level are shown with the letters a-c. Label-derived TEN and SOM-derived TEN are indicated with TEN_{label} and TEN_{SOM} respectively. Glucose amendend soils are indicated with C, ammonium nitrate amended soils with N and the water amended soils with O. *In situ* or incubation warming did not significantly alter TEN after incubation, so for clarity they were not included in the figure.

3.2. Effects of substrate additions on microbial C and N dynamics

Across all temperature levels, glucose addition significantly increased C_{mic} with an average increase of 52 ± 29 % at the end of the incubation compared to the water treated soils ($p < 0.001$; fig. 2). Moreover, in soils at all temperature levels, the increase in C_{mic} was glucose-derived, and there was no net change of SOM-derived microbial biomass C detectable (fig. 2). In contrast, ammonium nitrate addition did not yield any significant effect on C_{mic} or N_{mic}.

Not only C_{mic}, but also total microbial respiration was significantly increased by glucose addition, on average by 188 ± 150 % ($p < 0.001$), reflecting increased activity when provided with easily available C (fig. 9). Isotopic partitioning of the CO_2-flux revealed that in all treatments glucose-derived respiration peaked 1-2 days after substrate addition. Neither N addition nor temperature differences changed glucose-derived cumulative respiration or respiration dynamics (fig. 9 & 10). However, SOM-derived respiration decreased when glucose was added, but only in the *in situ* warmed soils, while N addition had no effect (fig. 9). Finally, after two days of incubation,

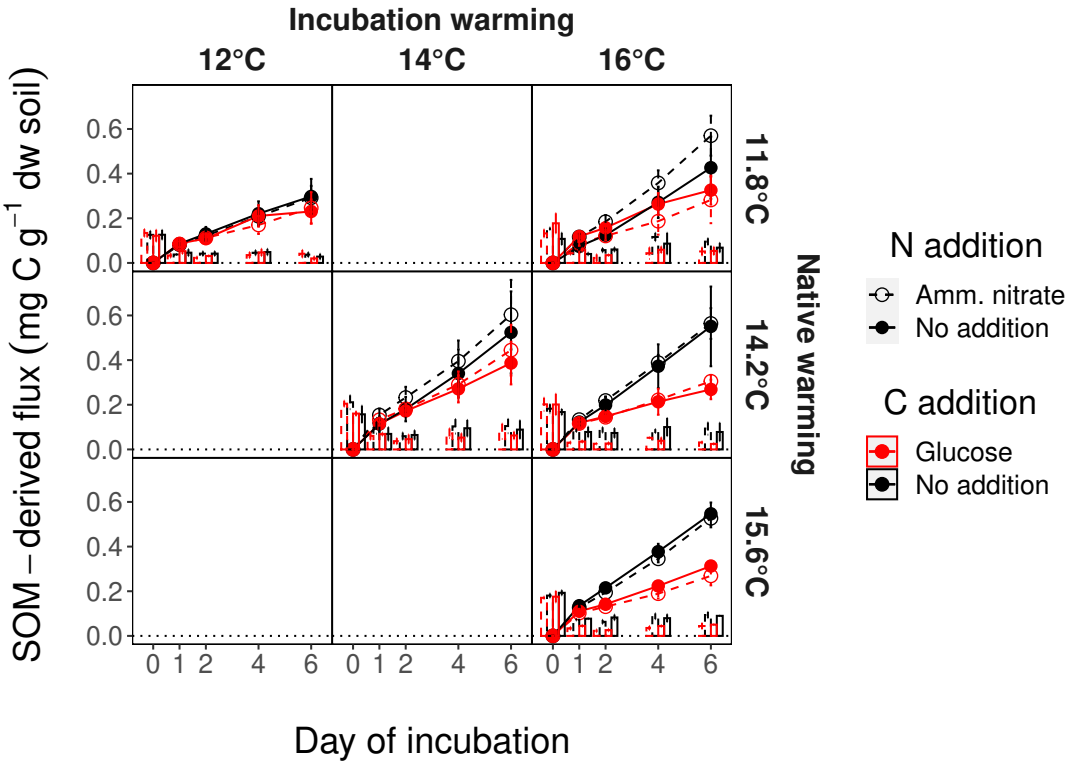

Figure 4: Soil organic matter-derived CO_2-flux measured during incubation for the different substrate treatments. The lines and dots show cumulative flux in mg C g^{-1} dw soil, while the bars indicate the flux per day in mg C g^{-1} dw soil day^{-1}. The error bars represent standard errors.

combined addition of C and N led to a stronger decline of glucose-derived and total extractable organic carbon (EOC) compared to addition of only C. After six days of incubation, soils had a similar amount of EOC, regardless of substrate amendment (fig. 8).

Addition of glucose or ammonium-nitrate did not affect the N_{mic}. Additionally, the ratio of label-derived to SOM-derived N_{mic} was not impacted by the addition of glucose (fig. 7). However, when comparing the glucose addition with the combined addition of glucose and ammonium nitrate, SOM-derived N_{mic} was lower in the combined treatment ($p < 0.05$). Also, addition of glucose significantly reduced the TEN concentrations in all incubated soils by 17 $\pm$ 10 % ($p = 0.03$; fig. 3). This reflects an increased microbial N demand after C addition, resulting in increased microbial N uptake, decreased microbial N release, or both. When only ammonium nitrate was added, TEN increased with 109 $\pm$ 10 % ($p < 0.001$; fig. 3). However, in the combined CN-treatment, substrate-derived TEN was significantly lower compared to the N treatment (-100 $\pm$ 2 % and -98 $\pm$ 5 % on day 2 and 6 respectively) (fig. 3).

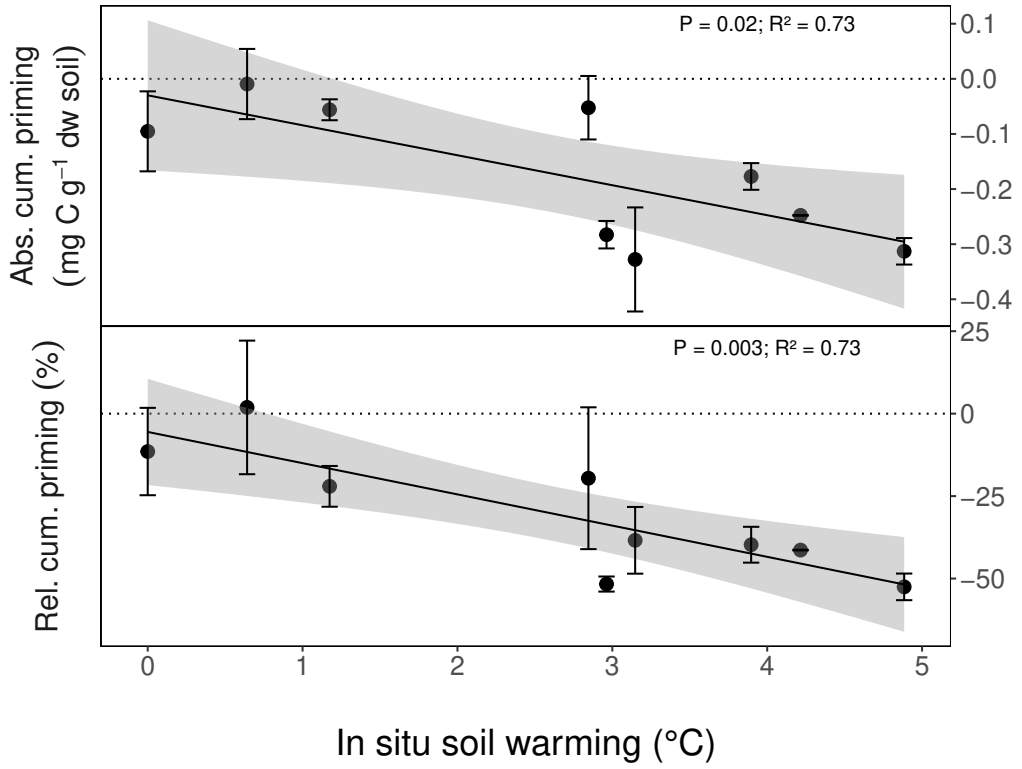

Figure 5: Absolute (upper panel) and relative (lower panel) cumulative priming effect after 6 days of incubation. The priming effect was averaged across different incubation temperatures and N addition treatments, because no significant ($p < 0.05$) effects were found for these parameters. The error bars on the points indicate standard errors.

3.3. Priming effects in incubation and in situ warmed soils

In the ambient soils, SOM-derived respiration was unaffected by labile C additions. In the *in situ* warmed soils, glucose addition significantly reduced the SOM-derived respiration during the incubation experiment ($p < 0.001$; fig. 11) as well as the cumulative SOM-derived respiration at the end of the incubation (i.e., caused negative priming) ($p = 0.03$; fig. 5). The relative priming effect (see methods), revealed a small and ephemeral positive priming in the ambient plots, which turned into a negative priming effect in the soils incubated at *in situ* temperatures (fig. 5). We could not detect an effect of N addition or incubation warming on the priming effect.

Next to soil warming, also soil C:N ratio was negatively correlated with the priming effect during the whole period of incubation ($p < 0.001$; fig. 11). Also in this model, N addition and incubation warming did not affect the magnitude of the priming effect.

4. Discussion

4.1. Soil warming effects

Microbial SOM-derived respiration in soils increased with higher *in situ* temperatures across the long-term geothermal gradient, whereas microbial biomass was not affected by *in situ* warming. Since it was unlikely that microbial C use efficiency (CUE) decreased in our study (Walker et al., 2018), or substrate availability increased (fig. 1), the higher SOM-derived respiration likely indicates higher microbial activity in *in situ* warmed soils. The increase of microbial activity supports previous evidence that the microbial communities had already adapted to increased *in situ* temperatures after 7 years of warming (Marañón-Jiménez et al., 2018; Walker et al., 2018).

Additionally, incubation of unwarmed soils at elevated temperatures increased total microbial SOM-derived respiration, but led to a decrease of C_{mic}. This points towards an upregulation of the microbial metabolism and an increasing C_{mic} loss through respiration.

4.2. Substrate effects

Addition of glucose, an easily available C source, led to increased total microbial respiration and C_{mic}. Since no glucose-derived EOC was detectable at the end of the incubation experiment, we conclude that all added C was either respired or assimilated into C_{mic}. On the other hand, added N was assimilated only when provided in combination with glucose and N addition did not affect SOM- and glucose-derived microbial respiration (fig. 4 & 10). From this data we conclude that microbes were C limited but not N limited. This is in line with the general notion that in soils heterotrophic microbial communities are primarily limited by assimilable C availability or energy, and not by nutrients (Soong et al., 2020). Moreover, the average soil C:N ratio measured (11.8 $\pm$ 0.2, fig. 1) was lower than the global average soil C:N ratio of 16 (Xu et al., 2013), pointing towards an energy (C) limitation rather than a N limitation. The increase of bulk soil C:N at *in situ* warming could also be an indication of lower SOM quality (Vicca et al., 2018). The decline of the TEN measured in soils prior to incubation and the increase of bulk soil C:N indicate a reduction of easily available N at higher *in situ* temperatures, however, this did not lead to a microbial N limitation.

The available N in the absence of N addition in all temperature treatments was insufficient to cover the additional microbial N demand following glucose addition. This is apparent from the comparison between the N-only treatment and the combined C and N added treatment. All of the added N was assimilated in the microbial biomass after only two days when provided together

with glucose illustrating the glucose-induced microbial N demand. In the N-only treatment almost all the N remained unassimilated by the microbes. In order to meet the C-induced N demand in the C-only treatment, microbes would need to mine SOM for N. A short elaboration on this can be found in the supplement. This was also supported by a higher SOM-derived N_{mic} in the C-treatment than in the CN-treatment, where the added inorganic N covered the increased microbial N demand. When only provided with C, we expect that microbes were capable of breaking down and assimilating more complex N compounds from the SOM by increasing their extracellular enzyme production (Craine et al., 2007). Although microbes in the CN-treatment clearly assimilated more added, ^{15}N-labelled ammonium nitrate than the N treatment, the increase in ^{15}N-label was not visible in N_{mic}. This indicates that the added labelled N was likely assimilated into more complex molecules, such as proteins and chitin, and may have strongly bound to the soil matrix, and therefore not detected in N_{mic}.

4.3. Priming effects

In contrast to our hypothesis and the general assumption that SOM priming is driven by microbial mining for N (Kuzyakov, 2010; Macdonald et al., 2018), our results showed that increased microbial N mining was not correlated with stronger priming. First, in ambient soils, where TEN was high and N mining was thus probably lower, no positive absolute priming effect was found, and only small and ephemeral positive relative priming was observed. In the *in situ* warmed soils, we found a decreased proportion of easily available N (fig. 3), in the form of (salt extracted) TEN, which can be used as a proxy for readily available soil N (Landgraf & Klose, 2002). This in combination with equal glucose-C addition per unit of soil C and microbial CUE being unaffected by warming on this site (Walker et al., 2018), should have induced stronger N mining, but also led to negative priming in response to glucose additions (fig. 11). Second, in both ambient and *in situ* warmed soils, no significant shift in the priming effect was observed when N was added along with C. However, since the easily available N in the would not be enough to cover the microbial N demand after glucose addition only, an assumption we elaborate on in the supplement, we assume microbial N mining in SOM. These findings suggest a decoupling of N mining and the priming effect in low C:N soils with relatively high N availability.

The N mining theory states that under low N availability, slow growing K-strategists that use energy from labile C to assimilate N by decomposing recalcitrant SOM are responsible for positive priming (Fontaine et al., 2011; Chen et al., 2014). Favourable, N-rich conditions would subsequently result in lower N mining and reduced priming (Fontaine et al., 2011). On the other

hand, Chen et al. (2014) observed stronger priming with higher N availability. Following the basic stoichiometric theory, they argued that fast-growing r-strategists can outcompete K-strategists, accelerating SOM decomposition due to increased microbial growth (Craine et al., 2007). However, Chen et al. (2014) and Fontaine et al. (2011) did not measure microbial growth rates to confirm the inferred shift from K-strategists to r-strategists upon the addition of N. Moreover, Rousk et al. (2016) did not observe such a shift when measuring ^{13}C-incorporation into PLFAs under similar substrate additions. The varying priming responses to different N availability can probably not be explained only by the N mining theory, even if *in situ* N availabilities were different per study soil type. Also other studies have relied on N mining for explaining the priming effect, but did not provide additional mechanistic proofs on the actual coupling (Li et al., 2018; Zhou et al., 2020, 2021). Finally, recent studies have challenged the N mining hypothesis. First, by reporting higher SOM-derived respiration after adding a N-rich component, e.g., amino acid compared to glucose addition (Hicks et al., 2020; Mason-Jones et al., 2018; Lyu et al., 2018). Second, by reporting mechanistic evidence on the decoupling of microbial N mining and the priming effect, i.e., observing a positive priming effect in combination with reduced N-cycling enzyme activity and breakdown of N-containing polymers (Wild et al., 2019). In our study, an increased N demand through addition of glucose, and a long-term SOM modification due to warming, led to reduced microbial N availability, which did not result in positive priming. Since the decreased N availability did not have a positive impact on the priming effect, our results add to the already significant empirical evidence for the decoupling of priming and N mining.

Even when assuming a decoupling of N mining and SOM-derived respiration, N availability can still affect microbial respiration and priming. High N availability has been reported to stimulate microbial activity or shift the microbial community, and consequently influence the SOM turnover (Dijkstra et al., 2013). Also, N addition could shift microbial C use towards LMWC compounds which are low in concentration, causing a reduction in microbial biomass (Ramirez et al., 2012). Finally, a higher microbial community CUE induced by greater N availability could reduce microbial respiration and thus manifest in lower priming (Zang et al., 2016; Blagodatskaya et al., 2014). In our study, absolute priming had a strong, negative correlation with *in situ* warming and soil bulk C:N (both $p < 0.001$; fig. 11), the latter being a measure commonly used as a proxy for N availability and SOM quality (Vicca et al., 2018). Thus, the finding that lower C:N ratio correlated with higher priming supports the basic stoichiometric theory. However, N addition did not increase the SOM-derived respiration, even in the soils with the lowest N availability. Bearing in

mind that microbial communities are primarily limited by energy rather than by nutrients (Soong et al., 2020), it is likely that a loss of accessible soil C from soils incubated at *in situ* temperatures, rather than decreased N accessibility, determined the rate of the priming effect. Different, non-nutrient driven priming mechanisms such as co-metabolism and preferential substrate utilisation are therefore expected to play a key role here. Of the priming mechanisms currently debated in the scientific literature, only preferential substrate utilisation could explain the negative priming observed in our experiment.

Preferential substrate utilisation is described as microbes shifting towards the labile substrate as a preferable source of energy and C thereby reducing SOM decomposition (Kuzyakov, 2002). Two theories have been suggested to explain this shift. First, a high dissimilarity between LMWC and SOM could stimulate only r-strategists which are unable to degrade SOM (van der Wal & de Boer, 2017). Second, LMWC could stimulate microbes to exert an antagonising effect on the K-strategists (de Boer et al., 2015). Additionally, r-strategists have been reported to prevail in the presence of roots, which exude labile C, while K-strategists would dominate root-free soil (Blagodatskaya et al., 2014). Hence glucose-addition, mimicking root exudation and favouring r-strategists, would induce negative priming, while, according to the co-metabolism theory (Horvath, 1972; Fontaine et al., 2003), addition of a more complex and SOM-like C source would cause positive priming. Based on the increasingly negative priming effect with *in situ* soil warming in our incubation study, LMWC amendment should have strongly impaired SOM-dependent K-strategists and stimulated the preponderance of the exudate-dependant r-strategists. No microbial community composition shifts were observed for soils warmed *in situ* up to 6 °C (Walker et al., 2018; Radujković et al., 2018; Walker et al., 2020). Hence, it is likely that the gradual transition towards negative priming in warmed soils observed in our incubation study, indicated a microbial response to altered SOM-characteristics, reflected here by an increased soil C:N ratio, rather than being a consequence of microbial community shifts induced by *in situ* warming.

The negative priming observed in our study, in soils increasingly depleted in accessible SOM, might suggest that microbial community dynamics could buffer further SOM loss induced by warming. This would mean that preferential utilisation of root exudates might mitigate the positive feedback effect to warming in the subarctic region by decreasing SOM decomposition. Although one should be very cautious interpolating results from a short-term laboratory incubation to ecosystem level, these findings are nonetheless highly relevant and enable conceptual frameworks, like the recently proposed microbial efficiency-matrix stabilisation framework, to more accurately model

SOM dynamics responses to environmental perturbations and eventually reduce the uncertainty in the projected response of carbon stocks to global warming (Robertson et al., 2019).

5. Conclusion

Incubation of grassland soils from a long-term warming gradient at different temperatures in the laboratory, in combination with the addition of isotopically labelled substrates, suggests that microbes adapted to *in situ* soil warming by maintaining a higher metabolic activity. Furthermore, long-term *in situ* soil warming led to less favourable SOM physico-chemical characteristics, inferred from SOM C:N ratio, which induced stronger negative priming upon glucose addition and may reflect higher microbial dependence on plant root exudates. Additionally, our findings challenge the N mining theory as a possible mechanism behind the priming effect in our incubation experiment, as induced microbial N limitation by glucose addition did not lead to stronger priming, i.e., lower N availability did not stimulate SOM-derived C mineralisation. Finally, our data suggest that next to temperature, SOM characteristics play a key role in the mechanisms, such as preferential substrate utilisation, that determine the direction and the magnitude of the priming effect.

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6. Supplementary

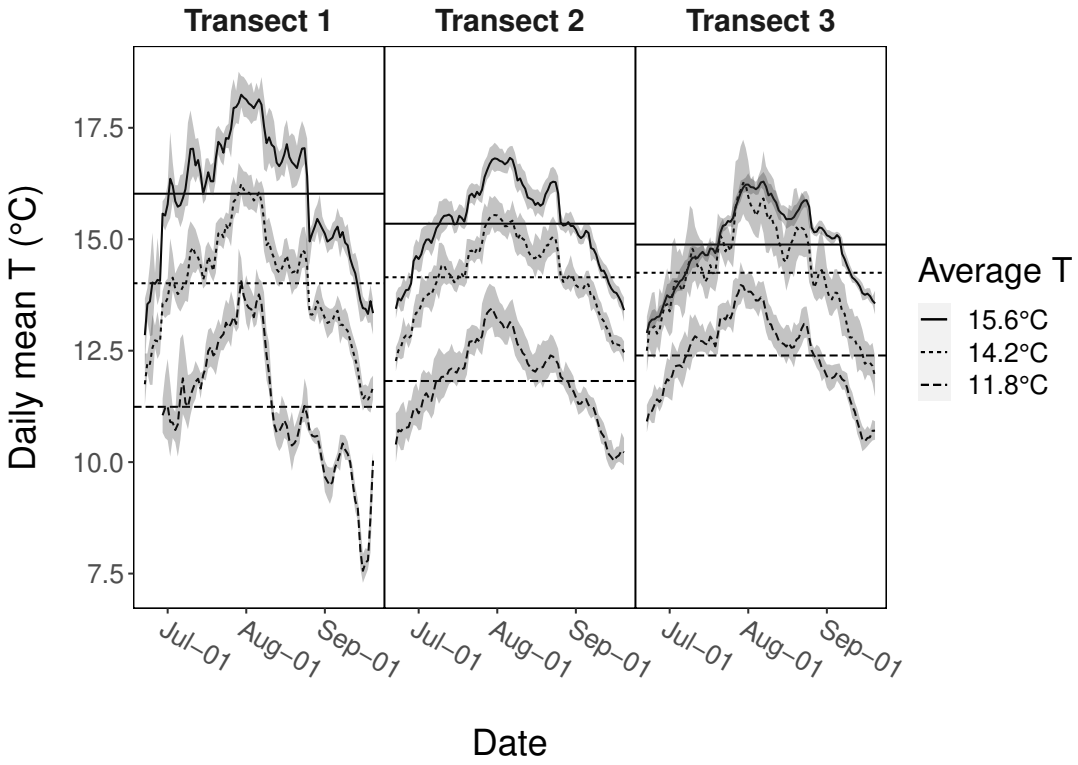

Figure 6: Average daily summer temperature of the plots sampled for the experiment. The plots are located on three different transects, of which the average summer temperature of the ambient, middle en high warming level over the transects are indicated.

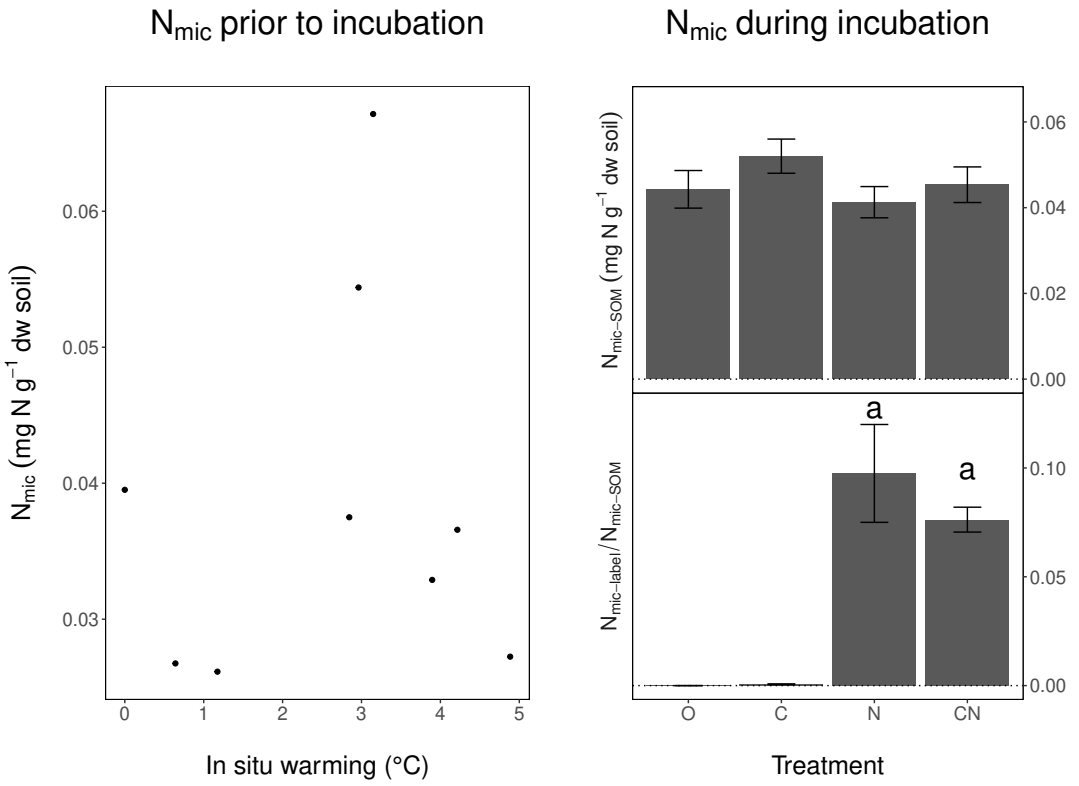

Figure 7: Microbial biomass N (N_{mic}) in soils prior to incubation (left panel) and at the end of the incubation (right panel). On the right panel, significances on a $p < 0.05$ level are shown with the letters a-b. Label-derived N_{mic} and SOM-derived N_{mic} are indicated with $N_{mic-label}$ and $N_{mic-SOM}$ respectively. Glucose amendend soils are indicated with C, ammonium nitrate amended soils with N and the water amended soils with O. *In situ* or incubation warming did not significantly alter N_{mic} after incubation, so for clarity they were not included in the figure.

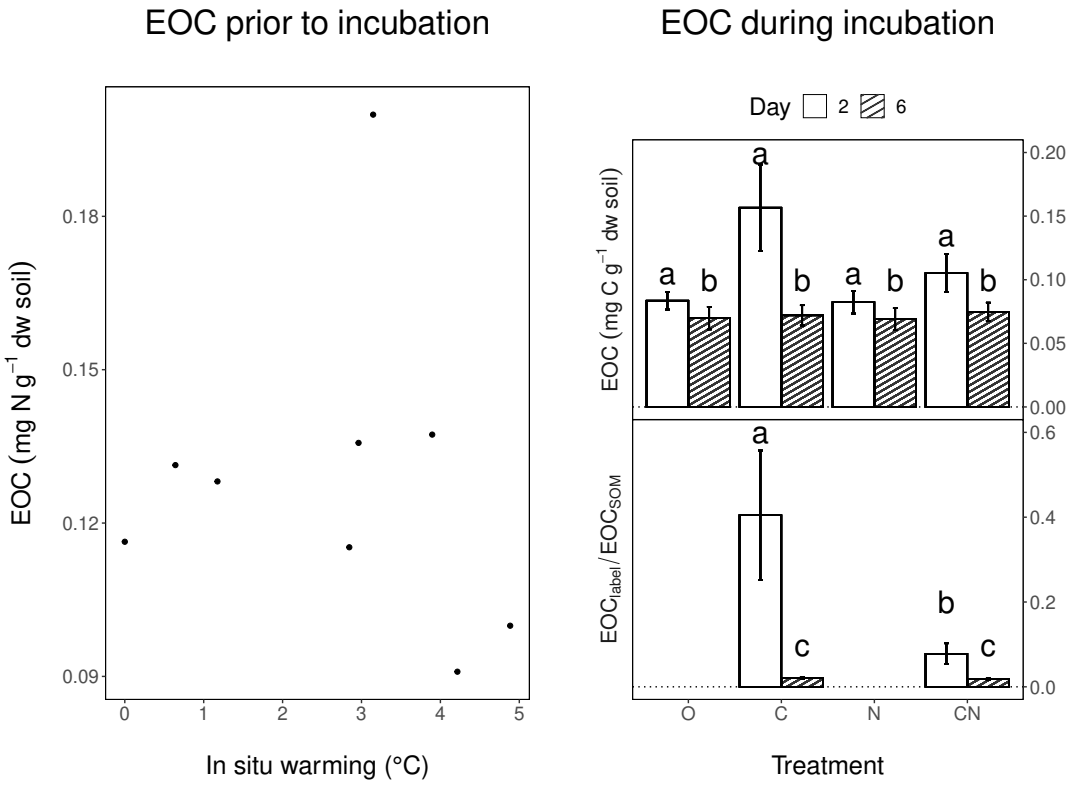

Figure 8: Extractable organic carbon (EOC) in soils prior to incubation (left panel) and after two and six days of incubation (right panel). On the right panel, significances on a $p < 0.05$ level are shown with the letters a-c. Label-derived EOC and SOM-derived EOC are indicated with EOC_{label} and EOC_{SOM} respectively. Glucose amendend soils are indicated with C, ammonium nitrate amended soils with N and the water amended soils with O. *In situ* or incubation warming did not significantly alter EOC after incubation, so for clarity they were not included in the figure.

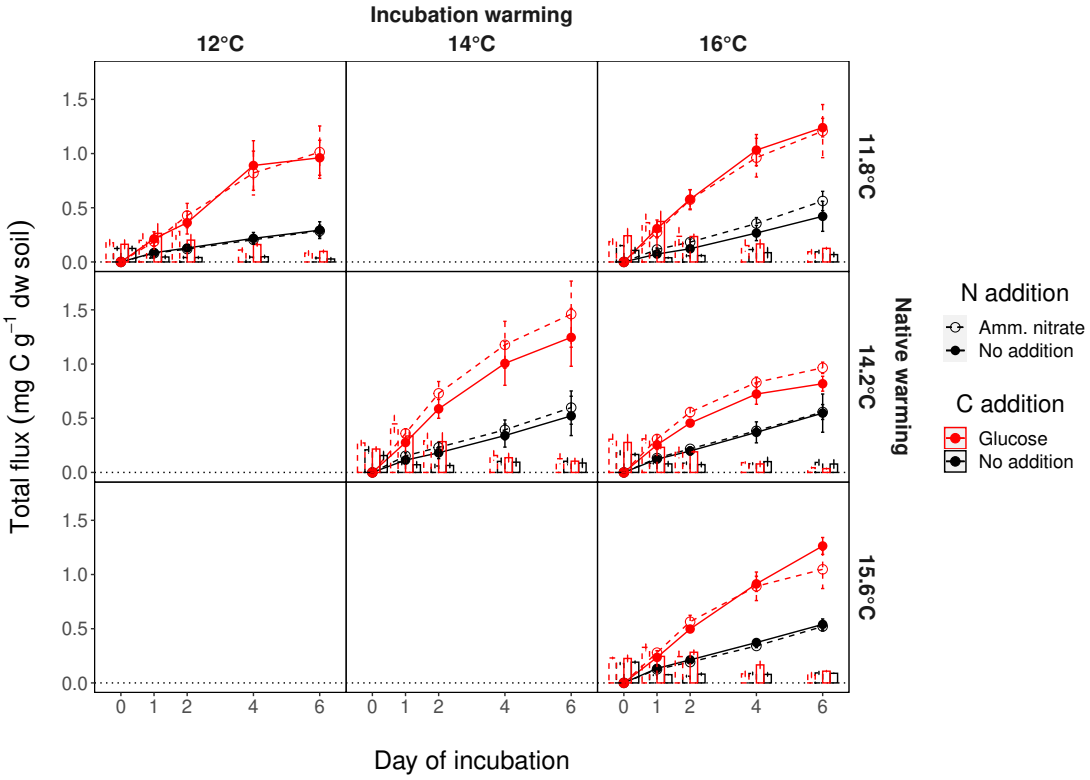

Figure 9: Total CO_2-flux measured during incubation for the different substrate treatments. The lines and dots show cumulative flux in mg C g^{-1} dw soil, while the bars indicate the flux per day in mg C g^{-1} dw soil day^{-1}. The error bars represent standard errors.

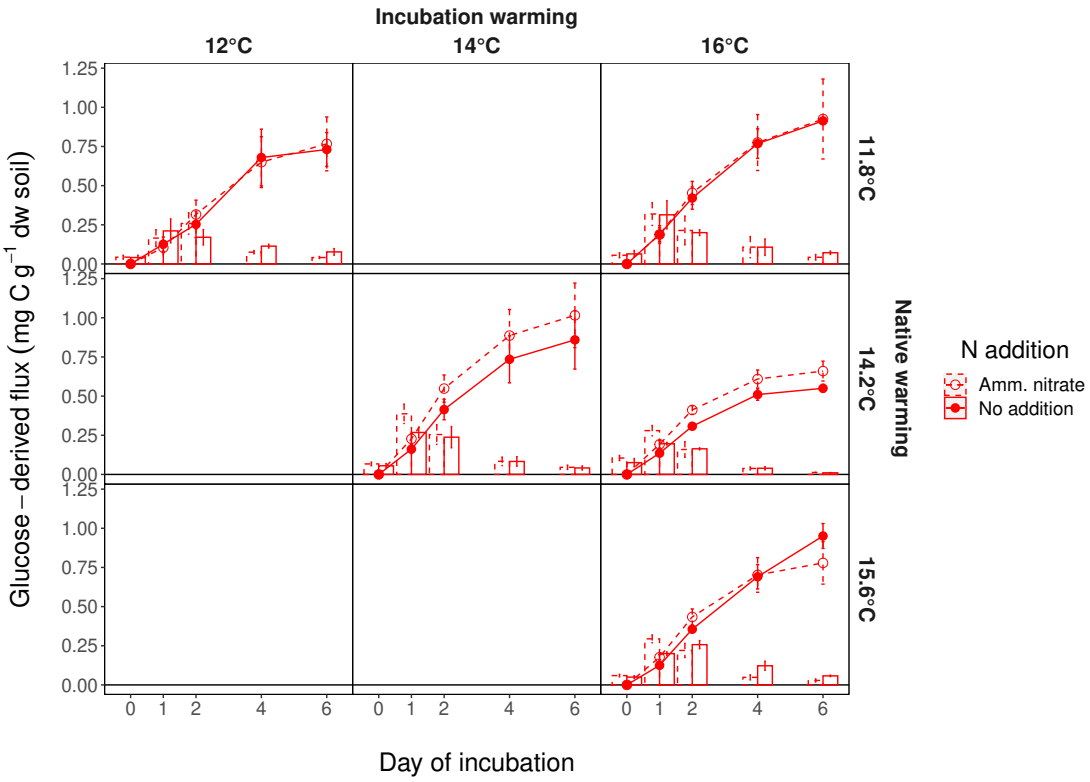

Figure 10: Glucose-derived CO_2-flux measured during incubation for the different substrate treatments. The lines and dots show cumulative flux in mg C g^{-1} dw soil, while the bars indicate the flux per day in mg C g^{-1} dw soil day^{-1}. The error bars represent standard errors.

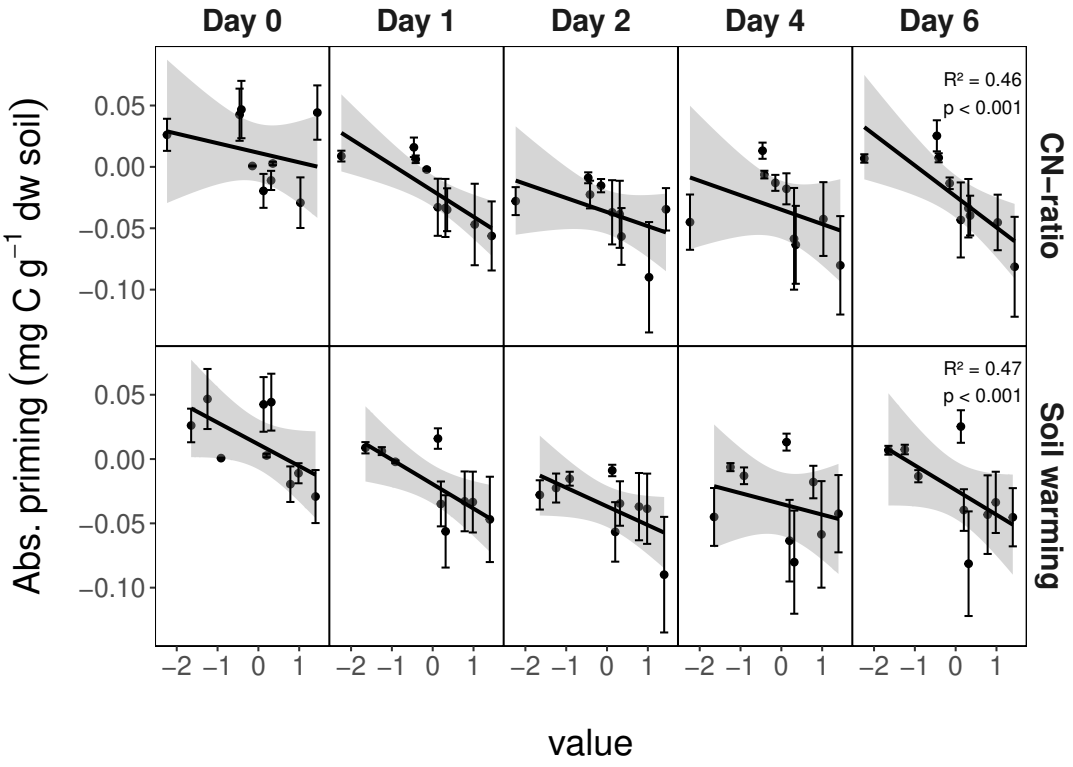

Figure 11: Absolute priming effect in function of standardised soil warming and soil C:N ratio. As the N addition and the incubation warming effect did not significantly affect the priming effect, the mean priming value per soil sample is shown. The error bars represent standard errors.

We estimated the C:N ratio of glucose-derived C_{mic} if the additional N uptake by microbes after glucose addition would only originate from easily assimilable N molecules. We used total extractable N (TEN) as a measure for this labile N. The surplus microbial biomass C after glucose addition ()$C_{mic-gluc}$) was divided by the difference in soil TEN between the control treatment (TEN_O) and the glucose treatment (TEN_C) in moles:

$$C : N\ ratio = \frac{C_{mic-gluc}}{TEN_O - TEN_C} \tag{5}$$

Assuming that the surplus assimilated N would only be TEN-derived, this calculation provides the C:N ratio of the glucose-derived C_{mic}. The mean of this C:N ratio was 50 $\pm$ 4 (SE), being much higher than the C:N ratio of soil microbes, which roughly is around 8 (Hassink, 1994). This calculation indicates microbes obtained N also from other sources, presumably by N mining in SOM.