Long-term warming reduced microbial biomass but increased recent plant-derived C in microbes of a subarctic grassland

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Abstract

Long-term soil warming and nitrogen (N) availability have been shown to affect microbial biomass and community composition. Altered assimilation patterns of recent plant-derived C and changes in soil C stocks following warming as well as increased N availability are critical in mediating the direction and magnitude of these community shifts. A ^{13}C pulse labelling experiment was done on a warming gradient in an Icelandic grassland (Sigurdsson et al. 2016), to investigate the role of recent plant-derived C and warming on the microbial community structure and size. We observed an overall increase of microbial ^{13}C (e.g., root-exudate) uptake, while warming led to significant microbial biomass loss in all microbial groups. The increase of microbial ^{13}C uptake with warming differed between microbial groups: an increase was only observed in the general and Gram-positive bacterial phospholipid fatty acid (PLFA) markers and in the PLFA and neutral lipid fatty acid (NLFA) markers of arbuscular mycorrhizal fungi (AMF). Nitrogen addition of 50 kg ha^{-1} y^{-1} for two years had no effect on the microbial uptake, microbial biomass or community composition, indicating that microbes were not N limited, and no plant-mediated N addition effects occurred. Additionally, we show that both warming and soil C depletion were responsible for the microbial biomass loss. Soil warming caused stronger loss in microbial groups with higher ^{13}C uptake. In our experiment, warming caused a general reduction of microbial biomass, despite a relative increase in microbial ^{13}C uptake, and altered microbial community composition. The warming effects on microbial biomass and community composition were partly mediated through soil C depletion with warming and changes in recent plant-derived C uptake patterns of the microbial community.

Introduction

High latitude ecosystems are vulnerable to global warming (IPCC 2013), which makes them of particular interest for climate research. Global warming could accelerate mineralisation of the vast soil C stocks in high latitude soils (Crowther et al. 2019; Davidson and Janssens 2006), releasing CO_2 to the atmosphere and possibly reinforcing global warming. Nevertheless, global warming is also expected to increase plant productivity (Natali, Schuur, and Rubin 2012) which could lead to higher plant C inputs to the soil through shoot litter deposition, rhizodeposition and C transfer to symbionts such as arbuscular mycorrhizal fungi (AMF). These increased C inputs might lead to increased soil organic matter (SOM) formation (Oldfield, Crowther, and Bradford 2018), possibly depending on plant type and environmental conditions, which strongly influence the amount and composition of plant belowground carbon inputs (Preece et al. 2021; Ven et al. 2019). However, in high latitude ecosystems plant productivity is often limited by N availability (Reich and Oleksyn 2004), possibly impeding an increase in plant productivity with warming, and leading to a net warming-induced C loss from the ecosystem. Although being an important parameter, plant N limitation is often ignored in earth system models (Anav et al. 2015).

Microbial groups use distinct preferential C sources (Kramer and Gleixner 2006, 2008), meaning that the abundance of different available C substrates such as soil C, plant litter or rhizodeposit inputs next to determining microbial biomass amount, also plays an important role in defining broad patterns of the microbial community structure (Drenovsky et al. 2004; De Deyn, Cornelissen, and Bardgett 2008). Gram-positive bacteria have been reported to rely mostly on soil C (Kramer and Gleixner 2008), while arbuscular

mycorrhizal fungi (AMF) are obligate symbionts using primarily C supplied by plant roots (Smith and Read 2008). Saprophytic fungi can use plant litter as C source (Reverchon, Ortega-Larrocea, and Pérez-Moreno 2010), although they also have a higher capability of acquiring rhizodeposit-C than other microbial groups, e.g., Gram-negative bacteria (Huang et al. 2020). Hence, a reduction of a specific C source can lead to abundance shifts between large microbial groups, e.g., an increase of the Gram-positive / Gram-negative ratio upon soil C depletion (Fanin et al. 2019). It can also lead to a substantial reduction of microbial biomass in general (Walker et al. 2018).

Microbial uptake of recent plant-derived C, i.e., root exudates and C transfer to symbionts such as AMF, can be strongly enhanced by the type of interaction between the plant roots and the microbes (Huang et al. 2020). A symbiotic relationship, e.g., between AMF and plant roots, can facilitate the distribution of recent plant-derived C to other microbial groups, hereby acting as an important link in the SOM formation chain (Frey 2019; Zhou et al. 2020; Drigo et al. 2010). Microbial uptake of rhizodeposits has been shown to increase with warming (Zhang et al. 2016), possibly accelerating microbial recent plant-derived C uptake and fostering the abundance of microbial groups relying on these rhizodeposits (Zhang et al. 2020).

Plants rely extensively on the microbial community for their nutrient supply. For example, mycorrhizal fungi are estimated to mediate approximately 66 % of global plant N uptake (Shi et al. 2016). It is therefore not surprising that an increased (warming-induced) N availability has been shown to reduce microbial assimilation of recent plant-derived C (Farrer et al. 2013; Li et al. 2016), e.g., because of reduced plant investment in the

development of a root system, leading to lower rhizodeposition, or in the symbiotic relationship with mycorrhizal fungi to acquire N (Högberg et al. 2003; Olsson, Burleigh, and Aarle 2005). However, also increased quality and quantity of rhizodeposits with increased N availability, mainly due to an overall plant productivity increase, have been reported (Chen et al. 2015), which can positively affect microbial biomass. Hence, altered N availability changing plant-microbe interactions can affect microbial community structure and microbial biomass size.

To investigate the role of microbial uptake recent plant-derived C, and how it is affected by warming and increased N availability, we set up a $^{13}CO_2$ pulse labelling experiment on a long-term (10 years) warming gradient in an Icelandic grassland on Andosol. On this site, long-term warming led to soil C reduction (Verbrigghe et al. 2022a), followed by a significant decline of microbial biomass (Walker et al. 2018, 2020). The vegetation structure did not change within the observed range of geothermal warming (Leblans et al. 2017). The fungal community structure, including AMF, was affected by warming >+3°C, while the bacterial community structure was only responsive to warming >+6°C (Radujković et al. 2018; Zhang et al. 2020). Warming also resulted in a reduced microbial N retention capacity which may have caused net N losses from the system (Marañón-Jiménez et al. 2019). However, it is unclear if and how the activity of microbial groups was altered by warming. We here report on a study that addressed the question how warming modifies the flux of recent-plant derived C into different microbial groups and how increased N availability modifies this flux.

We expected that warming and N addition would affect microbial biomass and community composition indirectly through changed plant rhizodeposition and symbiotic C-N transfers. Specifically, we hypothesised that: (i) warming would increase microbial uptake of recent plant-derived C, (partly) offsetting the microbial biomass decrease, (ii) N addition would reduce microbial uptake of recent plant-derived C and hence reduce microbial biomass and ^{13}C uptake by microbes, and (iii) warming-induced changes in distribution of ^{13}C determine changes in microbial community composition, i.e., microbial groups receiving more ^{13}C with warming grow relatively more abundant.

Material and methods

Site description

The study was carried out on a geothermally warmed grassland, the ForHot research site, which is located in the Hengill geothermal area, 40 km east of Reykjavík, Iceland (100 – 225 m a.s.l.) described in detail in Sigurdsson et al. (2016). The mean annual air 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, closed in 2017). 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). In 2008, a major earthquake occurred in south-Iceland and shifted a geothermal system to a previously unwarmed area. The shifted geothermal bedrock channels induced warming in the soils above, creating a warming gradient in the grassland. The geothermally induced

warming gradient proved both relatively stable over time, and no indication of contamination by geothermal water was found at the site (Sigurdsson et al. 2016).

Experimental design

In June 2017, 24 permanent 2x2 m plots were established on the temperature gradient around two different hot spots, further referred to as transects. Next to the soil warming, also a N gradient was created by repeated ammonium nitrate addition, ranging from 0 to 150 kg N ha^{-1} y^{-1}. The pulse labelling campaign was done on the second year of N addition, and the last N addition before labelling was done in May. 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. In July 2018, 14 plots along the warming gradient were selected, ranging from 0 to 8.7°C. Seven of the plots received 50 kg N ha^{-1} y^{-1}, while the other seven were kept as unfertilised controls.

$^{13}CO_2$ pulse labelling experiment

Pulse labelling was performed in sunny weather on the two sole sunny consecutive days in this Icelandic summer (July 16,17) on the two transects. The protocol was similar to previous studies (Bahn et al. 2013; Fuchslueger et al. 2014; Karlowsky et al. 2018). Four days before pulse labelling, plastic frames (50 x 50 cm) were installed in all the plots under study. Just before labelling, a Plexiglas chamber (50x50x50 cm) was placed on top of the plastic frames. Rubber gaskets were used between the chamber and the frames to ensure gas tightness. Air was circulated within the chambers, and temperature was controlled by circulating cold water inside the chamber. Air temperature and CO_2 concentration inside

the chamber and photosynthetically active radiation outside the chamber were continuously monitored. Photosynthesis was measured using a Li-cor portable photosynthesis system (LI-6800). During labelling, the isotopic ratio ($^{13}C/^{12}C$) inside the chamber was monitored for plots in transect 1 using an online isotope laser (Picarro G2201i Analyzer, Picarro Inc, Santa Clara, CA, United States). In transect 2, where electric power supply for the Picarro was not available, 12 ml of air samples was collected in exetainers (five times at an interval of 15 minutes) which were measured using IRMS (Finnigan MAT, Bremen, Germany). The labelling conditions such as range of CO_2 concentration and isotope ratio was similar between the plots with continuous-electricity and battery-powered pulse labelling. Prior to labelling, the CO_2 concentration inside the chamber was reduced below 250 ppm by plant photosynthesis and scrubbing using soda-lime. When this threshold was reached, highly enriched $^{13}CO_2$ was added as 10-15 ml pulses to achieve 40-60 atom% ^{13}C and maintain CO_2 concentration below 800 ppm. Each plot was labelled for 60 ± 10 minutes.

Sampling and analysis

Within the labelled area of each plot, shoot and soil samples were taken before labelling, immediately after labelling, and on 1, 3, 6 and 10 days after labelling. For sampling shoot samples, a ring (Ø= 5 cm) was placed on the soil, after which all the shoot biomass within the ring was clipped to the ground. The soil samples were collected by coring 7 cm deep below the clipped surface, with a soil auger (Ø= 5 cm). Both shoot and soil samples were immediately transported to the lab. On arrival, metabolic processes in the shoot samples were stopped by shock freezing them in liquid nitrogen, after which they were dried for 48h on 60°C. Soil samples were sieved to 2 mm. An aliquot for PLFA analysis was stored on

dry ice until all of them were eventually freeze-dried. Also an aliquot was taken for microbial biomass C was taken, while the rest of the soil was weighed and dried for 48 h on 70°C to determine soil water content (SWC). An aliquot of the dried soil samples was ground using a ball mill. C and N concentration and isotope ratio were analysed by elemental analysis IRMS (EA 1100, CE Elantech, Milan, Italy; coupled to a Delta+ IRMS; Finnigan MAT, Bremen, Germany).

Microbial biomass C (C_{mic}) was determined using the chloroform fumigation method (Vance, Brookes, and Jenkinson 1987) by extracting 2 g of chloroform fumigated and non-fumigated soil aliquots with 20 mL of 0.5 M K_2SO_4. Fumigated and non-fumigated K_2SO_4 extracts were analysed for extractable organic C (EOC) 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). C_{mic} was calculated as the difference in C between the fumigated and non-fumigated (EOC) extracts.

Soil phospholipid fatty acids (PLFAs) and neutral lipid fatty acids (NLFAs) were extracted using a high throughput method developed by Buyer and Sasser (2012; Sharma and Buyer 2015). Briefly, from around 1.3 g of freeze-dried soil total lipids were extracted using a chloroform/methanol/citric acid buffer mixture and fractionated by solid-phase extraction on silica columns. The neutral lipid fatty acid (NLFA) fraction was collected by eluting samples with chloroform while the PLFA fraction was collected by eluting columns with a

196 5:5:1 chloroform:methanol:water mixture. After an internal standard (19:0) was added,
197 NLFAs and PLFAs were converted to fatty acid methyl esters (FAMEs) by mild alkaline
198 transesterification. An N_2 stream was used for drying the different phases. Samples were
199 analysed for identification using a GC (7890B GC System; Agilent, Santa Clara, CA, USA)
200 connected to a TOF/MS (Pegasus HT; LECO Corporation, Saint-Joseph, MI, USA). FAMEs
201 were identified using mixtures of bacterial and fungal FAMEs (Bacterial Acid Methyl Ester
202 CP Mixture (Matreya LLC, State College, PA, USA) and 37 Comp. FAME Mix (Supelco,
203 Bellefonte, PA, USA)). Samples were run for quantification and measurement of ^{13}C
204 incorporation using a Trace GC Ultra connected by a GC-IsoLink to a Delta V Advantage
205 Mass Spectrometer (all Thermo Fisher Scientific). FAMEs were quantified against the
206 internal standard (19:0). The PLFA markers a15:0, a17:0, i15:0, i16:0 and i17:0 were used
207 for Gram-positive bacteria, 16:1ω7, 18:1ω7, cy17:0 and cy19:0 for Gram-negative bacteria,
208 18:2ω6,9 and 18:1ω9 cis for general fungi, excluding AMF. 10Me 18:0 for actinobacteria,
209 16:1ω5 for AMF, whereas 14:0, 15:0, 16:0, 18:0, 15:1, 16:1a, 16:1b, 17:1 and 18:1ω9 trans
210 were classified as general markers as they cannot be assigned to any specific group (Zelles
211 1997; Olsson 1999). Also the NLFA 16:1ω5 marker was used, representing AMF spore
212 biomass and storage lipids (Ngosong, Gabriel, and Ruess 2012).

213 For calculating the absolute amount of PLFA in each microbial group, expressed in
214 nmol PLFA g^{-1} dry soil, we summed the specific PLFA markers per microbial group. The
215 relative PLFA abundance per microbial group was calculated by dividing the specific
216 absolute PLFA by the total amount of PLFA in a sample. We expressed the incorporation of
217 recent plant-derived C in each microbial group as ^{13}C excess, calculated as the difference of
218 the ^{13}C atom% in the labelled and the unlabelled samples and multiplied by the amount, for

each specific marker (FAME) in each sample. The ^{13}C excess of each marker was summed within each microbial group.

Shoot biomass sampling

In July 2018, the same period as the pulse labelling campaign, shoot biomass was sampled from 26 plots on the soil warming and N addition gradient. The sampled grassland plots were from a parallel experiment at the same site. 15 of these plots were part of the pulse labelling experiment, while 11 others were not. Soil warming ranged from +0°C to +13.6°C, and the N addition gradient from 0 ton ha^{-1} y^{-1} to 150 ton ha^{-1} y^{-1}. For sampling shoot biomass, a 20x40 cm rectangular steel wire frame was placed on the grass vegetation, all the moss and grass within the frame was clipped and the biomass was dried for 48h on 60°C. Grass leaves were carefully separated from the biomass samples and the fraction was weighed. This shoot biomass weight was converted to ton ha^{-1} y^{-1}.

Statistical analysis

All the variables (except ^{13}C amount in PLFA) were analysed using a linear mixed-effects model (lme) (Lindstrom and Bates 1990) with day after labelling as a random effect and soil warming and N addition as continuous fixed effects. To assess if N addition and warming affected the relative PLFA amount of microbial groups, also a permutational multivariate analysis of variance (PERMANOVA) using distance matrices (Anderson 2001) was done. This was carried out with the 'adonis' function from the 'vegan' package (Oksanen et al. 2007), using 1000 permutations.

For analysing microbial ^{13}C uptake, we used a linear mixed effects model (lme; Lindstrom and Bates (1990)) with soil warming, N addition and day after labelling as fixed factors. Soil

241 warming was dichotomised into two groups (low and high warming) with a soil warming
242 range of 0-1.5°C and 5.1-8.7°C and a group size of 5 and 9 plots respectively. Nitrogen
243 addition was entered again as a continuous fixed effect, while day after labelling was a
244 categorical fixed effect. Categorising day after labelling was necessary, due to the non-
245 linear relationship between microbial ^{13}C uptake and day after labelling, which would
246 require a known distribution to make an appropriate fit. Since ^{13}C incorporation dynamics
247 differed between microbial groups, and C likely flowed from one microbial group to
248 another, we could not determine such a distribution. The assumption for normality and
249 homoskedasticity of the residuals was assessed visually using a qqplot and plotting the
250 standardised residuals vs. the fitted values.

251 At our site, long-term soil warming induced a strong decrease in soil C. Hence, soil warming
252 effects on microbial biomass and microbial community composition could comprise both
253 direct and indirect effects through soil C depletion (fig. 1). In order to distinguish between
254 both, a causal mediation analysis was conducted. Causal mediation analysis utilises a
255 measured intermediate variable (or mediator, here soil C) that is expected to underlie the
256 causal pathway between the treatment, i.e., soil warming, and the response variable (Albert
257 and Wang 2015). For this analysis, two linear regression models were run:

258

259 A mediator model : soil C ~ warming + day
260 An outcome model : PLFA ~ warming + soil C + day (1)

261 where day is a categorical cofactor, and warming, soil C and PLFA are respectively
262 continuous treatment, mediator and outcome variables. These models are provided to the

263 'mediate' function from the 'mediation' package (Tingley et al. 2014). A more detailed
264 description and calculations behind the causal mediation analysis can be found in (Imai,
265 Keele, and Tingley 2010; Imai, Keele, and Yamamoto 2010). For computing confidence
266 intervals of the mediated and the direct effect, non-parametric bootstrapping with 1000
267 Monte-Carlo draws was used.

268 **Figure 1: Conceptual representation of the causal mediation analysis.**
269
270 A sensitivity analysis was carried out for each mediation analysis to address possible
271 confounding effects of an unobserved variable affecting both the mediator and the outcome
272 variable. The correlation ρ between the residuals of the mediator and outcome regressions
273 for which the mediation effect would be zero is provided when reporting the mediation
274 results.

275 ^{13}C atom% was used as measure for recent plant-derived C incorporation per unit of
276 microbial biomass. For linking this value with the warming induced PLFA loss per
277 microbial group, the weighted mean of the PLFA and NLFA ^{13}C atom% was taken. Then, this
278 ^{13}C atom% was averaged from one day after pulse labelling until end of the measuring
279 period. All analyses and calculations were done using the R software version 3.6.3 (R
280 Development Core Team 2011).

281 # Results

282 ## Plant responses to soil warming and N addition

283 The average ^{13}C atom% in the grass shoots immediately after pulse labelling was 1.73 ±
284 0.07 %, compared to a natural abundance of 1.07 ± 0.00 % before labelling. Soil warming

and N addition did not affect the ^{13}C atom% ($P = 0.27$ and 0.18 respectively) in the plant shoots immediately after labelling, nor was there an interaction effect ($P = 0.55$). Long-term soil warming and N addition did not affect shoot biomass at our grassland ($P = 0.30$ and 0.52 respectively). Also no interaction effect was found ($P = 1.00$; fig. S1). No warming or N addition effect on plant photosynthesis was found (data not shown).

Soil stoichiometry, extractable organic C and microbial biomass C

Ten years of soil warming led to a significant decline of soil C and N of -0.31 ± 0.04 C $°C^{-1}$ and 0.026 ± 0.003 %N $°C^{-1}$ respectively, corresponding to 5.2 ± 0.9 % ($P < 0.001$; fig. S2) for both soil C and N. The similar decline of soil C and N was reflected in the soil C:N ratio being unaffected by warming ($P = 0.77$; fig. S2). Next to soil C and N, also C_{mic} and EOC showed a warming induced decline of 22 g C g^{-1} dw soil and 16 g C g^{-1} dw soil, corresponding to 6.2 ± 1.8 % and 6.9 ± 1.1 % respectively ($P < 0.001$; fig. S2). In contrast to warming, N addition did not affect soil C or N, nor the soil C:N ratio. N addition did also not affect C_{mic} or EOC. Finally, there were no interaction effects of combined warming and N addition on soil stoichiometry, C_{mic} or EOC.

Absolute and relative PLFA amount

A strong significant decline of individual PLFA markers and total PLFA amount with soil warming was found for every microbial group ($P < 0.001$), while N addition did not affect the PLFA amount in microbial groups (fig. S3). Warming affected the microbial biomass differently per microbial group, resulting in a distinct soil warming response of relative PLFA abundance. On the one hand, Gram-positive bacteria showed an increased relative abundance with soil warming ($P < 0.001$; fig. S4). On the other hand, relative fungal

abundance exhibited a negative relationship with soil warming, indicating a stronger effect of warming and/or soil C depletion ($P < 0.01$; fig. S4).

The fungal / bacterial biomass ratio declined with soil warming, as bacterial PLFA markers responded less to warming than fungal PLFA markers ($P < 0.01$), while the Gram-positive / Gram-negative biomass index ratio increased with soil warming ($P < 0.001$; fig. 2). N addition did not affect the fungal / bacterial nor the Gram-positive / Gram-negative biomass index ratio.

Figure 2: The fungi:bacteria ratio and Gram-positive:Gram-negative (G+:G-) ratio with soil warming and N addition. The grey area around the regression line indicates the standard error. The result for a linear mixed-effects model is indicated for soil warming (T). Nitrogen addition did not significantly affect the ratios.

The absence of an N addition effect and a shift of the relative abundance of individual PLFA markers was also clear from the multivariate NMDS (non-metric multidimensional scaling) analysis (fig. 3). The PERMANOVA test confirmed the statistically significant soil warming effect ($P < 0.001$), and the absence of a statistically significant N addition effect ($P < 0.3$).

Figure 3: Non-metric multidimensional scaling (NMDS) of the relative PLFA abundances of the individual PLFA markers. In panel A, the individual plots are represented, where filled symbols and dashed ellipse line represent the N addition treatment, while the the unamended treatment is indicated with open symbols and a solid-line ellipse. The soil warming gradient is indicated with isotherms and a black-red dot gradient. In panel B the markers representing a microbial group are coloured and linked. Also here, the isotherms are shown.

Long-term soil warming significantly reduced the amount of the AMF markers NLFA and PLFA 16:1ω5 ($P < 0.01$ and $P < 0.001$ respectively; fig. S5). The NLFA 16:1ω5 / PLFA 16:1ω5 ratio was not affected by long-term soil warming. Addition of 50 kg N ha^{-1} y^{-1} did not affect the amount of NLFA or PLFA 16:1ω5, nor did it change the NLFA 16:1ω5 / PLFA 16:1ω5 ratio ($P = 0.82$, 0.42 & 0.52 respectively).

Microbial ^{13}C uptake

A clear ^{13}C isotopic enrichment tracer signal was detected in the PLFAs of all microbial groups after one day of labelling. However, distinct temporal patterns could be observed between the microbial groups. Arbuscular mycorrhizal fungi exhibited a clear peak in ^{13}C after three days of labelling. In other microbial groups this peak appeared later (fungi, actinobacteria), or was not yet visible within 10 days after labelling (Gram-positive bacteria, Gram-negative bacteria) (fig. 4).

Figure 4: Absolute microbial uptake of recent plant-derived C after pulse labelling, expressed in ^{13}C excess. The solid lines connect the predicted outcome (^{13}C excess) for the different soil warmings at the different days after labelling, results for the linear mixed effects model are indicated for soil warming (T). No separate lines were drawn for the two levels of N addition, since this variable did not significantly affect any of the outcomes.

Long-term soil warming significantly increased ^{13}C uptake by microbes, as indicated by the higher excess ^{13}C of the Gram-positive bacteria ($P < 0.001$), the AMF 16:1ω5 PLFA ($P < 0.05$) and 16:1ω5 NLFA ($P < 0.01$)). Also the general PLFA markers indicated a significant increase of absolute ^{13}C uptake with soil warming. For actinobacteria, fungi and Gram-negative bacteria, no significant increase of absolute ^{13}C uptake with soil warming was found (fig. 4). Additionally, ^{13}C atom% in the microbial groups, or ^{13}C per unit of PLFA, increased significantly with soil warming in all groups (fig. S6). N addition did not affect the magnitude, nor the temporal dynamics of microbial ^{13}C uptake in any of the microbial groups.

To assess the hypothesis that a warming-induced shift in recent plant-derived C assimilation would affect the microbial community structure, the ^{13}C atom% in microbes

was plotted against the relative PLFA loss (% $°C^{-1}$). This altered microbial assimilation of ^{13}C with warming did not correlate with the microbial community shift (P = 0.81, fig. 5).

Figure 5: Warming-induced shift in microbial ^{13}C incorporation in relation to relative PLFA loss (% $°C^{-1}$) per microbial group.

Microbial ^{13}C uptake correlates with warming-induced PLFA reduction

Causal mediation analysis indicated that the PLFA decline for all groups was mostly caused by soil C loss, while there was also a direct warming effect for all microbial groups except for Gram-positive bacteria (table 1]). Causal mediation analysis further indicated that the NLFA 16:1ω5 amount decline was caused by soil warming, and not by depletion of soil C ($p < 0.01$; table 1). Contrastingly, the PLFA 16:1ω5 amount was largely mediated by soil C ($p < 0.001$), but was also affected by soil warming ($p < 0.001$; table 1).

Table 1: Causal mediation analysis on the total PLFA or NLFA amount per microbial group. Analysis was carried out with soil warming as predictor variable, soil C as mediating variable and PLFA amount as outcome variable. The average causal mediation effect indicates the PLFA change induced by soil C loss, while the average direct effect indicates the direct warming effect (i.e., not mediated by soil C depletion). ρ is the correlation between the residuals of the mediator and outcome regression models (see equation 1) for which the mediation effect would be zero, calculated with a sensitivity analysis.

	Mediated soil C effect (nmol PLFA dm)	Direct warming effect (nmol PLFA dm)	Total effect (nmol PLFA dm)	Prop. mediated (%)	ρ
Actinobacteria	-20.946***	-3.643	-24.59***	85***	0.7
AMF (PLFA)	-20.48***	-5.45	-25.7***	79***	0.7
AMF (NLFA)	-2.31	-66.97**	-69.28**	3	0
Fungi	-47.34***	-36.97***	-84.31***	56***	0.5
General	-177.51***	-37.89***	-215.4***	82***	0.8
G^- bacteria	-96.7***	-15.27	-111.97***	86***	0.8
G^+ bacteria	-62.79***	-5.11	-57.68***	109***	0.7

A strong correlation (R^2 =0.89) was found between the proportion of the PLFA reduction caused by warming and the average amount of ^{13}C uptake by a microbial group after 1 day of labelling when controlling for soil C depletion (table 1, fig. 6). A sensitivity analysis on the mediation analysis (table 1) revealed a relatively high ρ, i.e., the correlation between the residuals of the mediator and outcome regression models (see equation 1) for which the mediation effect would be zero, for all microbial groups except for the AMF NLFA marker. A high ρ indicates that it is unlikely that an unmeasured variable affecting both PLFA amount and soil C confounded the mediation analysis.

Figure 6: Relationship between the average ^{13}C atom% 1 to 10 days after pulse labelling and PLFA amount loss caused by warming, while controlling for soil C. The grey area around the regression line indicates the standard error. The relationship is statistically significant (R^2 = 0.88; p = 0.002).

Discussion

Plant responses to soil warming and N addition

The absence of a warming and N addition effect on the ^{13}C atom% in shoots indicates warming and N addition did not affect the capacity of plants to assimilate CO_2. Additionally, also shoot biomass was unresponsive to warming and N addition in this grassland (fig. S1). Hence, we assume there were no systematic differences in plant responses to warming and N addition in our experiment. In other words, plant activity and labelling conditions did not confound the results from the ^{13}C label assimilation in microbial PLFA and NLFA.

Effects of long-term warming on microbial biomass

The decline of soil C and soil N with warming in this subarctic grassland is in line with the results from a more elaborate experiment on the same site (Verbrigghe et al. 2022a) and can be attributed to an initial stimulation of microbial activity, eventually leading to soil C depletion (Walker et al. 2018). Along with soil C, soil N concentrations also decline due to reduced microbial N retention capacity in warmed plots (Marañón-Jiménez et al. 2019). Such warming-induced reduction of soil C reduces the availability of easily assimilable C and can thus reduce microbial biomass in the long-term (Melillo et al. 2017; Frey et al. 2008; Walker et al. 2018), as we observed after 10 years of warming.

The warming-induced shift we observed in the microbial community structure confirms the results from a previous study, where a shift in the fungal community was observed from +3°C warming (Radujković et al. 2018). The relative PLFA abundances indicated that Gram-positive bacteria were significantly less impacted by warming than other microbial groups, specifically fungi. The relative decline observed for the fungal PLFA markers 18:1ω9 cis and 18:2ω6,9 (fig. S4) primarily reflects a reduction of saprotrophic fungi, as other, non-saprophytic, fungal groups containing these PLFA markers, like ectomycorrhizal fungi (EMF), mainly have mutualistic symbioses with woody plants and are less abundant in grasslands (Paul 2015; Fodor 2013). One possibility is that a reduction of cellulose litter residues with warming could have led to the decline in saprotrophic fungal abundance, as observed in another study (Morrison et al. 2019). The significantly increasing Gram-positive / Gram-negative bacterial ratio with soil warming (fig. 2) can indicate decreasing microbial C availability and increasing energy limitation (Fanin et al. 2019), in line with the warming-induced soil C depletion we observed (fig. S2). These microbial community shifts

support the assumption that soil C depletion is a major driver of the observed microbial biomass loss.

Arbuscular mycorrhizal fungi are obligate symbionts that receive their C from the host plant (Smith and Read 2008). Hence, the effect of temperature on AMF growth can partly be a consequence of changes in the available plant C for AMF (Gavito et al. 2005). Alternatively, as AMF species can have different temperature optima (Barrett, Campbell, and Hodge 2014), the absence of AMF species adapted to warmer temperatures might be contributing to their absolute decline.

Both in DNA and PLFA based studies, AMF have been reported to be less impacted by warming in the grasslands of our study than the other microbial groups (Zhang et al. 2020; Radujković et al. 2018). However, as shown by the relative abundance of the AMF PLFA marker, +8.1°C of soil warming corresponding with an average soil temperature during labelling of 18.2°C, did not lead to such an effect ($P = 0.14$; fig. S4). A possible explanation for this could be that the 16:1ω5 PLFA marker is also found in bacteria (Olsson et al. 1995), which might confound our results. Soil warming also reduced the 16:1ω5 NLFA marker, an indicator of AMF storage lipids. This warming-induced reduction of the 16:1ω5 NLFA marker was not significantly different from the 16:1ω5 PLFA marker, which might suggest AMF did not adapt their survival strategy with warming, contrasting with a previous, short-term warming, experiment were a AMF shifted from a C storage strategy in cooler soils to faster growth in warmer soils (Hawkes et al. 2008). In our long-term warming experiment, AMF might have settled to a new equilibrium, instead of this change of strategy.

Effects of long-term warming on microbial ^{13}C uptake

We found an increase of absolute ^{13}C uptake by AMF and Gram-positive bacteria, and unaffected ^{13}C uptake by the other microbial groups (i.e., actinobacteria, fungi and Gram-negative bacteria). Since plant ^{13}C assimilation was not affected by warming, it is likely that plants allocated more recently assimilated C to the microbial community with warming, more specifically to AMF and Gram-positive bacteria. The increased recent plant-derived C uptake by these microbial groups did not lead to more biomass, likely due to a warming-induced increase of microbial turnover and C loss through respiration (Hagerty et al. 2014; Marañón-Jiménez et al. 2018).

Rhizodeposit uptake by microbes has been shown to be an important determinant of the microbial community structure (Koranda et al. 2011; Kaiser et al. 2010). However, in contrast to our hypothesis that microbial community shifts would be related to altered recent plant-derived C uptake by microbes and C-N transfers with symbionts in response to warming, we did not find such a relationship on the scale of broad microbial groups (fig. 5). The absence of this correlation could suggest that warming, causing higher microbial activity, and the depletion of soil C as a microbial C substrate are more important determinants of microbial biomass loss than altered recent plant-derived C assimilation. Another possibility could be that the shift in recent plant-derived C uptake by microbes and C-N transfers with symbionts was not sufficient to result in observable broad microbial community shifts. Finally, in this long-term experiment, the altered recent plant-derived C uptake by microbes might have led to a changed microbial food-web, which is too complex to comply with a simple correlation.

Nitrogen addition did not affect microbial biomass or microbial ^{13}C uptake

Next to an indirect warming effect, we also hypothesised an indirect N addition effect on the microbial community structure, mediated by altered recent plant-derived C uptake by microbes or C-N transfers with symbionts. In contrast to this hypothesis, our data suggests that two years of N addition did not affect microbial uptake of recent plant-derived C (fig. 4). The absence of such a microbial C uptake shift with higher N availability might be due to AMF, and not EMF, the former being dominant in symbiotic relationships with grass roots. Ectomycorrhizal fungi acquire N from SOM more efficiently than AMF, before passing it on to the host plant (Terrer et al. 2018). Hence, plant-derived C in exchange for N might be a less important mechanism in grassland than in, e.g., forest or shrubland, explaining the absence of a N addition effect on microbial uptake of recent plant-derived C in our experiment.

Next to the absence of an indirect N addition effect on relative microbial abundances mediated by altered microbial recent plant-derived C uptake, also no direct effects on microbial community composition and size were observed. N addition might affect microbial community composition by reducing microbial N limitation through resource enhancement (Zhou et al. 2017). We did not find changes in microbial growth or community composition upon N addition (fig. S3 & 3), suggesting that microbes were not N limited and had no incentive to assimilate the added (mineral) N. A similar observation was made during an incubation experiment where mineral N was added to geothermally warmed Icelandic Andosols (Verbrigghe et al. 2022b). Additionally, N addition might change microbial community composition directly through soil acidification (Chen et al.

2016; Tian and Niu 2015). The absence of an N effect on the microbial community might indicate that N was either taken up quickly by plants, and/or was leached due to the limited N retention capacity of the microbial community (Marañón-Jiménez et al. 2019), both mechanisms leading to fast removal of N from the soil, limiting soil acidification and soil nutrient availability.

Warming effects mediated by soil C depletion

To determine to what extent the negative warming effect on microbial biomass was due to warming-induced soil C depletion, a causal mediation analysis was performed. The results of the causal mediation analysis supported the hypothesis that biomass loss in different microbial groups could be attributed partly to soil C depletion. An indirect warming effect acts through the warming-induced depletion of SOC stocks. In this way, the potential for microbes to assimilate C was reduced, an effect which has been reported before (Walker et al. 2018). The strength of this effect differed between the microbial groups, likely because different microbial groups depend on distinct soil C fractions or compounds (Kramer and Gleixner 2008). In addition, in all groups except for Gram-positive bacteria, soil warming also impacted microbial biomass independent of soil C loss. A direct warming effect should be perceived as increased microbial activity and respiratory costs of microbes at higher temperatures (Marañón-Jiménez et al. 2018; Walker et al. 2018) leading to reduced microbial biomass at equal SOC stocks. This mediation analysis clearly shows that soil C depletion is crucial for explaining warming responses of all microbial groups, except for AMF, and responses diverged between microbial groups.

We did not find a direct relationship between recent plant-derived C uptake by the different microbial groups and their abundance shift with warming. However, we found that the proportion of microbial biomass loss caused directly by soil warming was positively correlated with the average ^{13}C atom% after 1 day of labelling (table 1, fig. 6). ^{13}C atom% of the microbial biomass C reflects the amount of microbial plant-derived C uptake per unit of microbial biomass in each microbial group. Hence, the observed relationship indicates that the more a microbial group was impacted by soil C depletion, the less ^{13}C it incorporated per unit of microbial biomass.

Conclusion

While long-term soil warming in a subarctic grassland in Iceland reduced soil C and N and microbial biomass, increased general microbial uptake of recent plant-derived C was observed. Additionally, the distribution of ^{13}C to the microbial community was altered: AMF and Gram-positive bacteria assimilated more ^{13}C, while no significant effect was observed in the other groups. In contrast to our hypothesis, this shift in ^{13}C uptake was independent of the shift in microbial community structure, showing that warming affected the microbial community structure differently than the microbial uptake of recent plant-derived C. In contrast to long-term warming, two years of N addition had no effect on microbial biomass or microbial community structure. Next to indicating the absence of a microbial N limitation, it also showed that there was no N addition effect of altered recent plant-derived C uptake by microbes which in turn could affect microbial community structure or size. In conclusion, we showed that warming can affect microbial community

structure and size mainly through reducing soil C, while the effects of shifted microbial uptake of recent plant-derived C remain limited.

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