Geothermally warmed soils reveal persistent increases in the respiratory costs of soil microbes contributing to substantial C losses

Running title: **Warming increases respiratory costs of soil microbes**

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Research Article

Abstract

Increasing temperatures can accelerate soil organic matter decomposition and release large amounts of CO_2 to the atmosphere, potentially inducing positive warming feedbacks. Alterations to the temperature sensitivity and physiological functioning of soil microorganisms may play a key role in these carbon (C) losses. Geothermally active areas in Iceland provide stable and continuous soil temperature gradients to test this hypothesis, encompassing the full range of warming scenarios projected by the Intergovernmental Panel on Climate Change for the northern region. We took soils from these geothermal sites seven years after the onset of warming and incubated them at varying temperatures and substrate availability conditions to detect persistent alterations of microbial physiology to long-term warming. Seven years of continuous warming ranging from 1.8 to 15.9 ºC triggered a 8.6 to 58.0 % decrease on the C concentrations in the topsoil (0-10 cm) of these sub-arctic silt-loam Andosols. The sensitivity of microbial respiration to temperature (Q_{10}) was not altered. However, soil microbes showed a persistent increase in their microbial metabolic quotients (microbial respiration per unit of microbial biomass) and a subsequent diminished C retention in biomass. After an initial depletion of labile soil C upon soil warming, increasing energy costs of metabolic maintenance and resource acquisition led to a weaker capacity of C stabilization in the microbial biomass of warmer soils. This mechanism contributes to our understanding of the acclimated response of soil respiration to *in situ* soil warming at the ecosystem level, despite a lack of acclimation at the physiological level. Persistent increases in the respiratory costs of soil microbes in response to warming constitute a fundamental process that should be incorporated into climate change-C cycling models.

Keywords:

Soil CO_2 fluxes, Q_{10}, soil respiration, temperature increase, metabolic quotient, microbial biomass, microbial physiology

1. Introduction

Global warming can accelerate soil organic matter decomposition and enhance CO_2 release to the atmosphere, causing positive warming feedbacks (Jenkinson et al. 1991, Davidson and Janssens 2006). Model predictions for future CO_2 emissions, however, are largely uncertain, especially for high-latitude biomes (Friedlingstein et al. 2006, Todd-Brown et al. 2014). A large part of these uncertainties can be attributed to the omission of physiological alterations of soil microbial communities (Allison et al. 2010, Treseder et al. 2012, Wieder et al. 2013) and/or to changes in their sensitivity to temperature (Davidson and Janssens 2006, Karhu et al. 2014). Temperature-mediated alterations of microbial physiology particularly determine the capacity of soils to store carbon (C) and the magnitude of climate-change feedbacks as temperatures rise (Bardgett et al. 2008, Conant et al. 2011, Zhou et al. 2011).

1.1. Warming-induced changes in microbial physiology

Microbial communities adjust the amount of substrate C used for building biomass or CO_2 production (Schimel et al. 2007, Dijkstra et al. 2011), optimizing their functioning to the new temperatures and resource availability conditions. Microbial mineralization of soil organic matter represents a main path of soil C release to the atmosphere (Raich and Schlesinger 1992), while recalcitrant microbial structural molecules used to build biomass have been found to be major contributors to long term soil C storage (Liang and Balser 2011, Miltner et al. 2012). The alteration of the partitioning between microbial respiration and growth in response to warming can therefore have direct consequences on the fate of the C consumed by microorganisms and has pivotal implications for the sequestration and stability of soil C (Frey et al. 2013, Sinsabaugh et al. 2013).

From a theoretical perspective, both higher temperatures and lower substrate quality and availability generally increase the maintenance costs and energy demands of microorganisms (Dijkstra et al. 2011, Schindlbacher et al. 2011). As labile C substrates are depleted from soil, increased energy demands for resource acquisition may lead to a subsequent weakened capacity to store C in biomass at warmer temperatures (Allison et al. 2010, Tucker et al. 2013, Pold et al. 2017). This response of microorganisms to warming is generally true for aquatic systems (Apple et al. 2006), but the evidence for a reduced capacity of C storage is less clear for terrestrial systems (Manzoni et al. 2012),

where microbial responses to warming are particularly constrained by substrate accessibility (Conant et al. 2011).

1.2. Warming-induced changes in the temperature sensitivity of microbial respiration

Simultaneous changes in the quality and availability of organic substrates and potential adaptive or compensatory mechanisms of soil microorganisms can also produce contrasting responses to increasing temperatures (Davidson and Janssens 2006). On the one hand, the apparent sensitivity of microbial respiration to temperature (Q_{10}) may decrease due to the depletion of labile organic substrates after an ephemeral acceleration of mineralization rates ("substrate-depletion hypothesis") (Melillo et al. 2002, Davidson and Janssens 2006) and/or due to the adjustments in physiology or community shifts in response to the new temperatures ("thermal adaptation hypothesis") (Bradford et al. 2008, Bárcenas-Moreno et al. 2009). On the other hand, Q_{10} may increase due to the relative enrichment of recalcitrant substrates with a higher activation energy (Knorr et al. 2005, Wagai et al. 2013). Shifts towards more active microbial communities at warmer temperatures (Hartley et al. 2008, Karhu et al. 2014) combined with increases in labile C inputs from enhanced vegetation productivity at higher mineralization rates (Rustad et al. 2001, Melillo et al. 2002) can also result in higher temperature sensitivity. These mechanisms may also occur simultaneously and counterbalance their effects, leading to attenuated or non-evident changes in Q_{10} (Giardina and Ryan 2000).

1.3. Selected approach: combination of geothermal gradients with laboratory incubations

Despite the high sensitivity of soil-C models to changes in the temperature sensitivity and the respiratory costs of soil microbes (Allison et al. 2010) these warming-induced physiological shifts have rarely been explored mechanistically. Field studies that incorporate both the responses of vegetation C inputs and microbial metabolic changes are therefore essential for improving predictions of soil C storage (Luo et al. 2011). Geothermally active areas in Iceland provide stable, continuous and wide soil temperature gradients (Sigurdsson et al. 2016) that encompass the full range of warming scenarios projected by the Intergovernmental Panel on Climate Change for the northern region (IPCC, 2013). These soil temperature gradients allow the detection of non-linear responses to a wide range of soil warming intensities, such as abrupt changes, thresholds or asymptotes, and the inference of realistic predictions of soil CO_2 fluxes.

Field studies alone, however, do not allow identifying the microbial processes involved in the response to long-term warming (Conant et al. 2011). Laboratory incubations offer an ideal complement, allowing in-depth physiological examination of the microbial mechanisms underlying field-scale observations (Luo et al. 2011). Soil environmental variables can be instantaneously manipulated in short-term soil incubations, making them particularly suitable for detecting persistent alterations of microbial physiology to long-term warming, regardless of instantaneous changes in temperature or substrate quality and availability.

We incubated soils in the laboratory that had been previously exposed to various warming intensities due to the geothermal activity in the field for seven years (hereafter "*in situ* temperatures"). Soils were incubated at varying short-term temperature changes (hereafter "incubation temperatures*"*) and substrate availability conditions to detect persistent alterations of microbial physiology to long-term warming. The Q_{10} of microbial respiration was determined from its short-term response to incubation temperatures. Simultaneous and sequential measurements of microbial respiration and biomass along the incubation allowed us to determine the microbial metabolic quotients. Metabolic quotient is considered a suitable integrative proxy to develop high-level inferences on the microbial metabolic rates in global carbon models, while being simple, easy, and cheap to measure (Bailey et al. 2017).

The total C losses from these (Poeplau et al. 2016, Leblans et al. 2018) and many other soils exposed to warmer temperatures (Crowther et al. 2016, Hicks Pries et al. 2017) led us to hypothesize a decrease of the microbial respiration Q_{10} associated to the depletion of labile substrates in response to *in situ* soil warming. We also hypothesized that the elevated maintenance and respiratory costs of soil microbial communities at higher *in situ* temperatures would limit the amount of C retained in microbial biomass, with a subsequent increase in their metabolic quotients.

2. Methods

2.1. Study site

Soils were collected from the ForHot research site in the Hengil geothermal area, 40 km east of Reykjavik, Iceland (64°00′01″N, 21°11′09″W; 83-168 m a.s.l.), which has been described in detail by Sigurdsson et al. (2016). Mean annual air temperature, annual

precipitation and wind speed were 5.2 °C, 1457 mm and 6.6 m s^{-1}, respectively (Synoptic Station, 9 km south of Hveragerdi, Icelandic Meteorological Office, 2016). The mean temperature of the warmest and coldest months, July and December, were 12.2 and -0.1 °C, respectively. The main vegetation type is unmanaged grassland, dominated by *Agrostis capillaris*, *Ranunculus acris* and *Equisetum pratense*. The growing season normally starts in late May and ends in late August. Snow cover is not permanent during winters due to the mild oceanic climate, but the soil typically freezes for at least two months during mid-winter.

The soil in the area has been subjected to warming since May 2008 due to geothermal activity, when an earthquake shifted geothermal systems to previously un-warmed soils. Hot groundwater warmed the underlying bedrock, increasing the soil temperature. No signs of soil contamination by geothermal byproducts were found (Sigurdsson et al. 2016). The soils are Andosols with a silty-loamy texture.

2.2. Experimental design and soil sampling

Five replicate transects were established in 2012, each one covering six *in situ* soil warming level: 0, 0.5, 1.8, 3.4, 8.7 and 15.9 °C above ambient. At each warming level, a 0.5 x 0.5 m plot was established for soil sampling (n = 6 *in situ* temperatures × 5 replicate transects = 30 plots). Soil temperature was monitored hourly at 10 cm soil depth using TidbiT v2 HOBO Data Loggers (Onset Computer Corporation, Bourne, USA) (Sigurdsson et al. 2016). The mean annual soil temperatures and main soil parameters are indicated in Table 1.

After seven years of soil warming (August 2015), the same amount of soil was sampled from the upper 10 cm of mineral soil in each plot. The mean soil temperature in un-warmed plots during the two weeks prior to sampling was 11.9±0.3 ºC. Soils from each warming level were sieved to 2 mm, mixed and homogenized to constitute a composite sample. The soil samples were then stored at 5 °C, which is approximately the mean annual temperature of the ambient un-warmed soil.

2.3. Initial soil parameters

Three soil subsamples were extracted with KCl, $NaHCO_3$ and K_2SO_4 within 24 h of sampling. Ammonium (NH_4^+) and nitrate (NO_3^-) were determined from the KCl extracts

(Bremner and Keeney 1966), available inorganic phosphorus (P_{inorg}) from the $NaHCO_3$ extracts (Olsen et al. 1954) and extractable organic nitrogen ($N_{extract}$) from the K_2SO_4 extracts (Jones and Willett 2006) with a San^{++} Continuous Flow Analyzer (Skalar Analytical B.V., Breda, The Netherlands). Total C and N (TOC and TON, respectively) were determined by dry combustion at 850 °C with a Thermo Flash 2000 NC Analyser (Thermo Fisher Scientific, Delft, The Netherlands). Inorganic C is not detectible in these volcanic soils (Arnalds 2015), so total C can be considered as organic C. The soil pH was determined by stirring and settling in deionized water (Pansu and Gautheyrou 2006).

2.4. Soil incubation

Nine 40-g (dry equivalent) subsamples of fresh soil from each *in situ* soil warming level (hereafter "incubation replicates") were distributed into flasks within 72 h after sampling. A 1-ml solution containing a source of C, N and P (hereafter "substrate addition") was added to each flask in a weight ratio of 20:1:0.67 (Alden et al. 2001). Carbon was added as glucose (1.73 mg of glucose g^{-1} of soil), N was added as NH_4NO_3 (0.1 mg of NH_4NO_3 g^{-1}), and P was added as KH_2PO_4 (0.101 mg KH_2PO_4 g^{-1}). The amount of C substrate added accounted for ca. 1-3% of the initial soil C content prior to the incubation. The amount of N added was equivalent to 50 kg N ha^{-1}. Nine other replicates per soil warming level were incubated after the addition of 1 ml distilled water without any substrate. Soil moisture was then adjusted to 60% water holding capacity in all incubation replicates, and the soil was mixed to ensure an even distribution of the solution.

Microbial respiration Q_{10} was assessed by incubating the soils at stepwise increasing temperatures (+5, +10, +20, +25 and +30 °C) and subsequently at stepwise decreasing temperatures (+30, +25, +20, +10 and +5 °C) in an incubator for 24-h periods (Fig. 1). Potential hysteretic effects associated with substrate depletion (Phillips et al. 2010, Subke and Bahn 2010) could therefore be assessed. Microbial respiration (R) was measured at each temperature step using an infrared gas analyzer (EGM-4/SRC-1, PP-Systems, Hitchin, UK) coupled to a custom-made chamber with a fan and vent. Respiration was always measured after a minimum stabilization time of 12 h per temperature step. The soil flasks were immersed in a water bath to maintain the targeted temperature during the respiration measurements. Temperature was continuously

monitored during the measurements and the incubation, and soil moisture was kept constant throughout the experiment.

2.5. Extractable and microbial biomass C

Extractable and microbial biomass C were determined during the incubation by sequential destructive samplings of the incubation replicates to obtain almost simultaneous measurements with respiration. Three incubation replicates per *in situ* soil warming level and substrate addition were sampled at the start (immediately after the respiration measurements at 5 °C, 17-42 h after substrate addition), middle (30 ºC, 6-7 d after substrate addition) and end (5 ºC, 11-12 d after substrate addition) of the incubation (Fig. 1). Two subsamples of fresh soil were taken from each incubation replicate for determining microbial biomass C by the fumigation-extraction method (Jenkinson and Powlson 1976). The fumigated and non-fumigated K_2SO_4 extracts were analyzed for extractable organic C ($C_{extract}$) with the San^{++} Continuous Flow Analyzer. Microbial C (C_{micro}) was determined as the difference in extractable organic C between the fumigated and non-fumigated subsamples and corrected for extraction efficiency using a K_{ec} of 0.45 (Sparling and West, 1988). All fractions are presented relative to soil dry mass.

2.6. Data analyses

We calculated the microbial metabolic quotient ($qCO_2 = R/C_{micro}$) and the microbial respiration per unit of initial organic C prior to incubations ($R_{TOC} = R/TOC$). The qCO_2 was calculated using respiration and microbial biomass values measured concurrently from the same incubation replicates. Cumulative microbial respiration throughout the entire incubation was also calculated. To calculate the cumulative qCO_2, the C_{micro} measured at the beginning, middle and end of the incubation were used to linearly interpolate the values at intermediate temperature steps. Standard errors were calculated by error propagation.

A linear mixed model was fit with microbial respiration as the outcome variable and with "*in situ* soil warming", "incubation temperature change", "substrate addition" and their pairwise interactions as fixed effects. The incubation replicate was included as a random intercept term, to account for multiple observations on the same soil sample. Differences among *in situ* soil warming levels and incubation temperature changes were

255 further tested by a post hoc test with Tukey correction for multiple testing. The same
256 test was also used for R_{TOC}. The effects of "*in situ* soil warming", "incubation
257 temperature change" and "substrate addition" were also tested for $C_{extract}$, C_{micro} and
258 qCO_2 using multiple linear regressions. All measurements were independent, so no
259 random-effect terms were added in this case. Note that the term "incubation temperature
260 change" was used to distinguish between the stepwise increases and decreases in
261 incubation temperature, thus it had nine levels for R and R_{TOC} and only three levels for
262 the extraction-based variables. Differences among the levels of the significant factors on
263 the multiple linear regressions were also further studied using Tukey post hoc tests. The
264 effects of "*in situ* soil warming" and "substrate addition" were also tested on the
265 cumulative values of microbial respiration, R_{TOC} and qCO_2 using two-way ANOVA
266 models, weighting each observation by the inverse of its standard error. Differences
267 among *in situ* soil warming levels were also further tested by a post hoc test with Tukey
268 correction for multiple testing.
269
270 Microbial respiration Q_{10} was determined during the phase of decreasing incubation
271 temperatures, both with or without substrate addition, because substrate consumption
272 and progressive depletion during the first half of the incubation obscured the
273 temperature response of microbial respiration. This period was chosen based on the
274 difference in respiration rates between samples with and without substrate addition,
275 which indicated that the substrate-induced respiration pulse had already passed seven
276 days after the substrate addition (Fig. 2). Microbial respiration (R) from each incubation
277 replicate was fitted versus the incubation temperature using the Van´t Hoff equation
278 (Van´t Hoff et al. 1898):
279 $$R = R_{10} * Q_{10}^{\left(\frac{T-10}{10}\right)}$$ Eq. 1
280 where R_{10} is the basal respiration rate at 10 °C and Q_{10} is the factor by which respiration
281 increases for a 10 °C rise in temperature (*T*). The effect of *in situ* soil warming and
282 substrate addition on Q_{10}, R_{10} and the initial soil parameters was tested with two- or one-
283 way ANOVAs, with *"in situ* soil warming*",* "substrate addition" and their pairwise
284 interaction as fixed factors. Data were transformed when required to improve normality
285 and homoscedasticity (Quinn and Keough, 2009). Statistical analyses and models were
286 made with JMP 11.0 software (SAS Institute). Results are presented as means ±
287 standard errors.

3. Results

3.1. Microbial respiration responses to in situ soil warming

Soils that had been exposed to warmer temperatures *in situ* showed lower microbial respiration rates (Fig. 2a and b). This was consistent in soils both with and without substrate addition and regardless of short-term changes in the incubation temperatures, indicated by the significant effect of *in situ* soil warming and the absence of interactions with other factors (Table 2). Respiration in soils with and without substrate addition, however, had a very distinct pattern over time as incubation temperatures change (Fig. 2a and b), demonstrated by the strong interaction between substrate addition and incubation temperature change (Table 2). The substrate addition triggered a fast and brief pulse of respiration that lasted only until the 30 °C incubation step, i.e. six to seven days after substrate addition. Fluxes during this first half of the experiment were higher in soils with than without substrate addition. An activation of microbial respiration also was visible in soils without substrate addition at day 1 compared to day 3 (Fig. 2a), likely associated with the ephemeral increase in substrate availability due to soil mixing when filling the incubation flasks.

In situ soil warming had an opposite effect for microbial respiration standardized per unit of organic C prior to incubation (R_{TOC}), with values increasing consistently in warmer soils *in situ*, both with and without substrate addition ($P \leq 0.005$, Fig. 2c and d) and regardless of the short-term changes in the incubation temperatures (Table 2).

3.2. Response of the microbial respiration Q_{10} to in situ soil warming

In situ soil warming did not significantly affect Q_{10} (see Eq. 1) (Table 3), with highly variable values ranging between 2.09±0.22 and 4.77±0.56. This was also the case when Q_{10} was calculated with microbial respiration from the first half of the incubation, either with or without substrate addition. In contrast, the fitted values of the basal respiration rates (R_{10}, see Eq. 1) decreased significantly with *in situ* soil warming (Table 3), particularly above the 3.4 °C level, and also tended to decrease in soils with substrate addition. Neither the substrate addition nor the interaction between substrate addition and *in situ* soil warming had a significant effect on Q_{10} or R_{10}.

3.3. Responses of extractable C and microbial biomass to in situ soil warming

Extractable soil C ($C_{extract}$) and microbial biomass C (C_{micro}) decreased consistently across the *in situ* soil warming levels throughout the entire incubation (Fig. 3a-c), despite a marginal interaction between *in situ* soil warming and changes in incubation temperature (Table 2). This decreasing trend was particularly clear in soils without substrate addition, where these variables increased in response to a moderate *in situ* soil warming of 0.5 °C and then decreased at higher intensities, particularly between 1.8 and 3.4 °C.

At the starting incubation step, the substrate added increased the amount of extractable C in the soil ($P<0.001$), but this increase was highest in the non-warmed soils (Fig. 3a), with a significant interaction between *in situ* soil warming and substrate addition ($P<0.001$). Microbial biomass increased similarly at all levels of *in situ* soil warming by 17-42 h after the substrate addition, indicated by the absence of significant interactions between *in situ* soil warming and substrate addition (Fig. 3d).

At the middle incubation step, six to seven days after the substrate addition, the added extractable C was already depleted in the non-warmed soils and in the moderately warmed soils up to 1.8 °C (Fig. 3b), where part of the C added contributed to sustain a higher microbial biomass (Fig. 3e). In contrast, soils above 1.8 °C *in situ* warming did not sustain the previously increased microbial biomass values (Fig. 3e), even though the concentration of remaining extractable soil C was still higher than in the soils without addition ($P<0.01$ for the interaction between *in situ* soil warming and substrate addition).

The added labile C was completely depleted by the end of the incubation, 11-12 days after substrate addition, and extractable soil C returned to the same concentrations as in soils without substrate addition ($P<0.001$ for *in situ* soil warming, no effect of substrate addition or the interaction; Fig. 3c). At this stage of the incubation, the soils with previous substrate addition still maintained similar values of microbial biomass as in the previous temperature step, whereas microbial biomass decreased again in the soils without substrate addition above 1.8 °C warming ($P<0.001$ for the interaction between *in situ* soil warming and substrate addition, Fig. 3f).

3.4. Response of microbial metabolic quotients to in situ soil warming

Metabolic quotients (qCO_2) increased in the soils at warmer *in situ* temperatures (Fig. 4) and this was also consistent for both with and without substrate addition and across short-term changes in the incubation temperatures (Table 2). Indeed, the substrate addition did not affect microbial metabolic quotients, because the increase in microbial respiration was accompanied by an equivalent increase in microbial biomass (Fig. 4). Metabolic quotients, however, changed during the incubation in response to the increasing and then decreasing incubation temperatures.

3.5. Response of cumulative respired C to in situ soil warming

In situ soil warming and substrate addition also affected the cumulative values of respired C by soil microbes throughout the entire incubation. Cumulative microbial respiration decreased consistently with *in situ* soil warming both in soils with and without substrate addition ($P<0.001$), with higher values in the former (Fig. 5a). In contrast, the trend shifted to consistent increasing values with the intensity of *in situ* soil warming when cumulative microbial respiration was standardized per unit of soil organic C prior to the incubation ($P<0.005$, Fig. 5b). The effect of *in situ* soil warming on the acceleration of microbial metabolism was also visible when cumulative metabolic quotients were calculated for the entire incubation ($P<0.001$, Fig. 5c). Substrate addition only affected marginally and not consistently the cumulative values of microbial metabolic quotients ($P<0.05$), as with the instantaneous values (Table 2), given the equivalent increase in microbial respiration and microbial biomass.

4. Discussion

4.1. Persistent warming-induced changes in microbial physiology

Seven years of continuous exposure to *in situ* warming accelerated the metabolic rates of the microbial communities in these subarctic soils. Both microbial metabolic quotients (Fig. 5c) and microbial respiration per g of organic C in soil (Fig. 5b) were higher in the soils pre-exposed to warmer temperatures, and this trend persisted throughout the entire incubation (Fig. 4 and Fig. 2c and d) in samples both with and without substrate addition. Such consistently higher metabolic rates, regardless of the short-term changes in the incubation temperatures and substrate availability, indicate a persistent physiological alteration of the soil microbial communities.

Instantaneous temperature increases accelerate enzymatic reactions, thereby stimulating the respiratory consumption of C by soil microbes (Frey et al. 2013, Luan et al. 2014, Bölscher et al. 2017). The persistence of physiological changes in response to sustained warming, however, had not been exhaustively explored, despite its relevant implications for the fate and stability of soil C. Our estimate of microbial metabolic quotients was based on nearly simultaneous and independent measurements of microbial respiration and biomass. Our results therefore suggest higher respiratory costs for soil microorganisms and a subsequent weakened capacity of C stabilization in microbial biomass in warmer soils, regardless of any potential change in microbial turnover. The following driving mechanisms could have contributed to this mass-specific acceleration in the release of soil C.

4.1.1. Increasing energy demands for metabolic maintenance and resource acquisition

The vast majority of physiological shifts in response to warming have been associated with indirect changes in the availability of C substrate (Feng and Simpson 2009, Castro et al. 2010, Karhu et al. 2010, Pold et al. 2017), although shifts have also been observed even before any apparent change in soil C (Wei et al. 2014). In particular, similar increases in microbial metabolic quotients to the ones found in our study have also been observed in response to experimental soil warming (Schlindbacher et al. 2011, Luan et al. 2014, Streit et al. 2014), even before any evidence of substrate depletion. An incipient short-term substrate limitation for microbes may underlie the increasing energy demands of soil microbes that were already found in these studies. Pointing to this direction, Streit et al. 2014 also reported a shift toward a greater use of old SOC by soil microbes, suggesting an imbalance between C inputs and outputs at an initial warming phase before eventual decreases in SOC storage. On the contrary, Schlindbacher et al. 2015 did not find direct evidence of microbial physiological shifts to warming prior to significant substrate depletion, but a metaproteomics survey in the same sites showed an increase in proteins involved in microbial energy production and conversion related to an increased CO_2 efflux from warmed soils (Liu et al. 2017). These results therefore converge on the hypothesis of an initial phase of increasing energy demands for metabolic maintenance that leads to a progressive substrate depletion and to a subsequent rise in the energy investment on resource acquisition.

Microbial respiration in our study was well correlated with the pool of extractable C available in the soil, which was lower in soils at higher intensities of *in situ* warming (Table 1). Moreover, a pulse of substrate immediately stimulated a similar magnitude of respiration in all soils incubated at the same temperatures (Figs. 2 and 5a, Table 2). These results, together with the lack of evidence of thermal acclimation of microbial respiration (Table 3), also suggest that higher *in situ* temperatures may have triggered an initial stimulation of microbial CO_2 release during the first years of warming (Luan et al. 2014, Melillo et al 2017). Sustained warmer temperatures likely progressively depleted the pool of labile soil C and subsequently reduced soil respiration rates, as in our study (Fig. 2a and b, Table 1) and other long-term soil warming studies (Melillo et al. 2002, Kirschbaum 2004, Eliasson et al. 2005). An "apparent" acclimated response of soil respiration to increasing temperature at the ecosystem scale therefore does not necessarily imply a change in Q_{10} of microbial respiration at the physiological level.

In contrast, warming-mediated declines in the quality, availability and accessibility of soil organic substrates may have demanded higher energy investment for the acquisition of the increasingly limiting resources (Biasi et al. 2005, Steinweg et al. 2008, Anderson and Domsch 2010). When the most easily degradable C fraction, such as soluble, low-molecular-weight organic compounds, has been depleted in the soil, microorganisms need to invest more energy resources to mobilize and incorporate the physic-chemically protected organic molecules that remain within the soil matrix (Conant et al. 2011). Molecules of high molecular weight and complexity also require a transformation into simpler molecules by extracellular enzymes prior to their assimilation, whose synthesis involves additional energy costs (Blagodatskaya and Kuzyakov 2008). Microbial adaptation to warming may thus occur by the production of more stable extracellular enzymes at warmer temperatures, but with a cost of lower catalytic rates, which may mask any increase in metabolic rates (Bradford et al. 2010, Billings and Ballantyne 2013). Our results, however, indicate that the prolonged exposure of these subarctic soils to warmer temperatures did not lead to thermal acclimation or a net reduction in metabolic rates of the soil microbial communities.

4.1.2. Shifts in microbial metabolic pathways

Soil microorganisms can also alter their metabolic pathways in several ways in response to the increasing energy demands imposed by warmer *in situ* temperatures (Dijkstra et

al. 2011). Preliminary findings on roots and mycorrhizae at the field site point to decreases in plant-derived C inputs with warming along our *in situ* temperature gradients (Leblans et al. 2016). Increasing respiratory demands at warmer *in situ* temperatures that are not accompanied by higher C inputs could lead to a reduction of C allocated to growth and anabolic reactions, thereby decreasing the microbial C-use efficiency (CUE) (Billings and Ballantyne 2013). In support of this, previous empirical evidence and model simulations have reported a preferential partitioning of C substrates to CO_2 production over growth at increasing temperatures (Hartley et al. 2008, Allison et al. 2010, Schindlbacher et al. 2011). Alternatively, higher respiratory demands may have been satisfied by increasing microbial turnover rates. Dead cells from accelerated microbial turnover can be metabolized by a smaller and more active fraction of living microbes, thereby decreasing microbial biomass but increasing microbial metabolic quotients, even without changes in microbial CUE (Hagerty et al. 2014). We cannot, however, discard either of these mechanisms in the absence of direct measurements of microbial growth or turnover. Either through faster turnover or lower microbial growth, increasing the respiratory demands of soil microbes that are not satisfied by increasing C inputs would nonetheless similarly result in lower microbial biomass (Fig. 3), higher metabolic quotients (Fig. 4) and in a diminished potential of C stabilization in warmer soils.

Other factors such as nutrient limitation may also restrict microbial growth (Eliasson and Ågren 2011, Manzoni et al. 2012), contributing to increased metabolic quotients. Soil N and P, however, decreased in the same or even a lower proportion than C with *in situ* soil warming, without substantial changes or even decreases in soil C:N and C:P ratios (Table 1). An increase in energy demand is a more plausible mechanism than the exacerbation of nutrient limitations for the increasing metabolic quotients of these soils. Whether the functional changes were also accompanied by microbial community shifts is currently being investigated, but recent findings suggest a collapse of the fungal community (Radujković et al. 2017, Leblans et al. 2016), consistent with the accelerated mass-specific CO_2 release and the lower capacity of C retention in microbial biomass in our study (Six et al. 2006).

4.2. Warming-induced changes in Q_{10} of microbial respiration

We did not detect any changes in microbial respiration Q_{10} after seven years of continuous exposure to warming (Table 3), and Q_{10} also remained unaffected by substrate addition. Warming did not prompt thermal acclimation or compensatory adaptation of soil microbial communities at our subarctic grassland site, in agreement with other warming studies in Arctic soils (Hartley et al. 2008) and in many other biomes (Karhu et al. 2014, Carey et al. 2016). Simultaneous changes in the quality and availability of organic substrates with increasing *in situ* temperatures, and subsequent functional or community shifts of microorganisms (Melillo et al. 2017), may have counterbalanced each other in our study, obscuring any potential change in the temperature response of microbial respiration.

Alternatively, the unaltered Q_{10} may also have been due to the high temperature optima of microbial mineralization (above 54 ºC in temperate grassland soils; Birgander et al. 2013). According to that hypothesis, even the highest intensity of *in situ* soil warming (21.5±0.4 °C, Table 1) may not have exceeded the optimum for microbial mineralization, so the *in situ* soil temperature would not have triggered a direct thermal acclimation. Either way, the elevated microbial respiratory demands in our study can explain the progressive substrate depletion and the apparent acclimated response of soil respiration at the ecosystem level (e.g., Melillo et al. 2002, Kirschbaum 2004, Carey et al. 2016), despite an unchanged Q_{10} at the physiological level.

5. Conclusions

The results of this study reveal a persistent acceleration of metabolic rates of soil microbes due to the continuous exposure to warmer temperatures for seven years. The conditions of scarcity that follow the initial depletion of soil C pools upon warming represent a plausible driving mechanism for the increasing respiratory demands of soil decomposers. Our results moreover represent a first evidence for persistent warming-induced shifts in the physiological functioning of soil microbial communities. Increasing energy costs for metabolic maintenance and resource acquisition may have demanded permanent functional changes in microbial metabolic pathways, constraining the capacity of microbes to maintain C in biomass when substrates are limiting. The subsequent mass-specific acceleration of CO_2 release represents a leading mechanism for the losses of soil C in warmer soils (Leblans et al. 2018). These persistent shifts on microbial physiology may therefore have followed an initial phase of soil C depletion

and changes in substrate availability, as found by Melillo et al. 2017. While it is still uncertain whether soils in this study are still losing carbon, observed declines on roots and mycorrhizae and the equivalent decreases in C stocks in these 7 years old and in adjacent >50 years old temperature gradients (Leblans et al. 2018) suggest that soil C stocks already reached the steady state. Soil microorganisms, however, did not acclimate to the warmer temperatures in our study, regardless of C and nutrient availability. Persistent warming-induced changes in the physiology of soil microbial communities can weaken the mechanisms of soil C stabilization (Hartley et al. 2008) even without changes in Q_{10}, and therefore constitute fundamental processes that should be incorporated into climate change-C cycling models (Wieder et al. 2013).

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Figure captions

Fig 1 Illustrative scheme of the experimental design of the soil incubation. Soils from the various *in situ* warming levels were exposed simultaneously to stepwise increases and then decreases in the incubation temperatures for 24-h periods. Microbial respiration (R) was measured at each incubation temperature. Extractable and microbial biomass C ($C_{extract}$ and C_{micro}, respectively) were determined at the start, middle and end of the incubation. The sensitivity of microbial respiration to temperature (Q_{10}) was determined from respiration data of the second half of the incubation

Fig 2 Response of microbial respiration (a, b) and microbial respiration per unit of soil organic C prior to incubation (c, d) from *in situ* warmed soils to instantaneous changes in the incubation temperatures. Panels a and c correspond to soils without substrate addition. Panels b and d correspond to soils with substrate addition. Soils subjected to the various intensities of *in situ* warming along the geothermal gradients are indicated by different lines, markers and colors, where levels indicate soil temperature above ambient. Error bars represent the standard error of the mean

Fig 3 Extractable soil C and microbial biomass C from *in situ* warmed soils at the start (incubation days 1 and 2 at 5 °C, panels a and d), middle (incubation days 6 and 7 at 30 °C, panels b and e) and end (incubation days 11 and 12 back to 5 °C, panels c and f) of the incubation. Responses from soils with and without substrate addition are represented by different markers. Note the different scales on the y-axes for extractable C. Error bars represent the standard error of the mean

Fig 4 Microbial metabolic quotient from *in situ* warmed soils at the start (incubation days 1 and 2 at 5 °C, panel a), middle (incubation days 6 and 7 at 30 °C, panel b) and end (incubation days 11 and 12 back to 5 °C, panel c) of the incubation. Responses from soils with and without substrate addition are represented by different markers. Error bars represent the standard error of the mean

Fig 5 Cumulative microbial respiration (a), microbial respiration per unit of soil organic C prior to incubation (b) and per unit of microbial C (c) throughout the entire incubation for the soils under the various intensities of *in situ* warming. Responses from soils with

852 and without substrate addition are represented by different bar patterns. Error bars
853 represent the standard error of the mean

854