

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

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

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24 Research Article

25 **Abstract**

26 Increasing temperatures can accelerate soil organic matter decomposition and release  
27 large amounts of CO<sub>2</sub> to the atmosphere, potentially inducing positive warming  
28 feedbacks. Alterations to the temperature sensitivity and physiological functioning of  
29 soil microorganisms may play a key role in these carbon (C) losses. Geothermally  
30 active areas in Iceland provide stable and continuous soil temperature gradients to test  
31 this hypothesis, encompassing the full range of warming scenarios projected by the  
32 Intergovernmental Panel on Climate Change for the northern region. We took soils from  
33 these geothermal sites seven years after the onset of warming and incubated them at  
34 varying temperatures and substrate availability conditions to detect persistent alterations  
35 of microbial physiology to long-term warming. Seven years of continuous warming  
36 ranging from 1.8 to 15.9 °C triggered a 8.6 to 58.0 % decrease on the C concentrations  
37 in the topsoil (0-10 cm) of these sub-arctic silt-loam Andosols. The sensitivity of  
38 microbial respiration to temperature (Q<sub>10</sub>) was not altered. However, soil microbes  
39 showed a persistent increase in their microbial metabolic quotients (microbial  
40 respiration per unit of microbial biomass) and a subsequent diminished C retention in  
41 biomass. After an initial depletion of labile soil C upon soil warming, increasing energy  
42 costs of metabolic maintenance and resource acquisition led to a weaker capacity of C  
43 stabilization in the microbial biomass of warmer soils. This mechanism contributes to  
44 our understanding of the acclimated response of soil respiration to *in situ* soil warming  
45 at the ecosystem level, despite a lack of acclimation at the physiological level. Persistent  
46 increases in the respiratory costs of soil microbes in response to warming constitute a  
47 fundamental process that should be incorporated into climate change-C cycling models.

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52 **Keywords:**

53 Soil CO<sub>2</sub> fluxes, Q<sub>10</sub>, soil respiration, temperature increase, metabolic quotient,  
54 microbial biomass, microbial physiology

55

## 56 **1. Introduction**

57 Global warming can accelerate soil organic matter decomposition and enhance CO<sub>2</sub>  
58 release to the atmosphere, causing positive warming feedbacks (Jenkinson et al. 1991,  
59 Davidson and Janssens 2006). Model predictions for future CO<sub>2</sub> emissions, however,  
60 are largely uncertain, especially for high-latitude biomes (Friedlingstein et al. 2006,  
61 Todd-Brown et al. 2014). A large part of these uncertainties can be attributed to the  
62 omission of physiological alterations of soil microbial communities (Allison et al. 2010,  
63 Treseder et al. 2012, Wieder et al. 2013) and/or to changes in their sensitivity to  
64 temperature (Davidson and Janssens 2006, Karhu et al. 2014). Temperature-mediated  
65 alterations of microbial physiology particularly determine the capacity of soils to store  
66 carbon (C) and the magnitude of climate-change feedbacks as temperatures rise  
67 (Bardgett et al. 2008, Conant et al. 2011, Zhou et al. 2011).

68

### 69 *1.1. Warming-induced changes in microbial physiology*

70 Microbial communities adjust the amount of substrate C used for building biomass or  
71 CO<sub>2</sub> production (Schimel et al. 2007, Dijkstra et al. 2011), optimizing their functioning  
72 to the new temperatures and resource availability conditions. Microbial mineralization  
73 of soil organic matter represents a main path of soil C release to the atmosphere (Raich  
74 and Schlesinger 1992), while recalcitrant microbial structural molecules used to build  
75 biomass have been found to be major contributors to long term soil C storage (Liang  
76 and Balser 2011, Miltner et al. 2012). The alteration of the partitioning between  
77 microbial respiration and growth in response to warming can therefore have direct  
78 consequences on the fate of the C consumed by microorganisms and has pivotal  
79 implications for the sequestration and stability of soil C (Frey et al. 2013, Sinsabaugh et  
80 al. 2013).

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

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

### 91 *1.2. Warming-induced changes in the temperature sensitivity of microbial respiration*

92 Simultaneous changes in the quality and availability of organic substrates and potential  
93 adaptive or compensatory mechanisms of soil microorganisms can also produce  
94 contrasting responses to increasing temperatures (Davidson and Janssens 2006). On the  
95 one hand, the apparent sensitivity of microbial respiration to temperature ( $Q_{10}$ ) may  
96 decrease due to the depletion of labile organic substrates after an ephemeral acceleration  
97 of mineralization rates (“substrate-depletion hypothesis”) (Melillo et al. 2002, Davidson  
98 and Janssens 2006) and/or due to the adjustments in physiology or community shifts in  
99 response to the new temperatures (“thermal adaptation hypothesis”) (Bradford et al.  
100 2008, Bárcenas-Moreno et al. 2009). On the other hand,  $Q_{10}$  may increase due to the  
101 relative enrichment of recalcitrant substrates with a higher activation energy (Knorr et  
102 al. 2005, Wagai et al. 2013). Shifts towards more active microbial communities at  
103 warmer temperatures (Hartley et al. 2008, Karhu et al. 2014) combined with increases  
104 in labile C inputs from enhanced vegetation productivity at higher mineralization rates  
105 (Rustad et al. 2001, Melillo et al. 2002) can also result in higher temperature sensitivity.  
106 These mechanisms may also occur simultaneously and counterbalance their effects,  
107 leading to attenuated or non-evident changes in  $Q_{10}$  (Giardina and Ryan 2000).

### 108 *1.3. Selected approach: combination of geothermal gradients with laboratory* 109 *incubations*

110 Despite the high sensitivity of soil-C models to changes in the temperature sensitivity  
111 and the respiratory costs of soil microbes (Allison et al. 2010) these warming-induced  
112 physiological shifts have rarely been explored mechanistically. Field studies that  
113 incorporate both the responses of vegetation C inputs and microbial metabolic changes  
114 are therefore essential for improving predictions of soil C storage (Luo et al. 2011).  
115 Geothermally active areas in Iceland provide stable, continuous and wide soil  
116 temperature gradients (Sigurdsson et al. 2016) that encompass the full range of warming  
117 scenarios projected by the Intergovernmental Panel on Climate Change for the northern  
118 region (IPCC, 2013). These soil temperature gradients allow the detection of non-linear  
119 responses to a wide range of soil warming intensities, such as abrupt changes,  
120 thresholds or asymptotes, and the inference of realistic predictions of soil CO<sub>2</sub> fluxes.

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

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

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

147

## 148 **2. Methods**

### 149 *2.1. Study site*

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

153 precipitation and wind speed were 5.2 °C, 1457 mm and 6.6 m s<sup>-1</sup>, respectively  
154 (Synoptic Station, 9 km south of Hveragerdi, Icelandic Meteorological Office, 2016).  
155 The mean temperature of the warmest and coldest months, July and December, were  
156 12.2 and -0.1 °C, respectively. The main vegetation type is unmanaged grassland,  
157 dominated by *Agrostis capillaris*, *Ranunculus acris* and *Equisetum pratense*. The  
158 growing season normally starts in late May and ends in late August. Snow cover is not  
159 permanent during winters due to the mild oceanic climate, but the soil typically freezes  
160 for at least two months during mid-winter.

161

162 The soil in the area has been subjected to warming since May 2008 due to geothermal  
163 activity, when an earthquake shifted geothermal systems to previously un-warmed soils.  
164 Hot groundwater warmed the underlying bedrock, increasing the soil temperature. No  
165 signs of soil contamination by geothermal byproducts were found (Sigurdsson et al.  
166 2016). The soils are Andosols with a silty-loamy texture.

167

## 168 *2.2. Experimental design and soil sampling*

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

176

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

183

## 184 *2.3. Initial soil parameters*

185 Three soil subsamples were extracted with KCl, NaHCO<sub>3</sub> and K<sub>2</sub>SO<sub>4</sub> within 24 h of  
186 sampling. Ammonium (NH<sub>4</sub><sup>+</sup>) and nitrate (NO<sub>3</sub><sup>-</sup>) were determined from the KCl extracts

187 (Bremner and Keeney 1966), available inorganic phosphorus ( $P_{\text{inorg}}$ ) from the  $\text{NaHCO}_3$   
188 extracts (Olsen et al. 1954) and extractable organic nitrogen ( $N_{\text{extract}}$ ) from the  $\text{K}_2\text{SO}_4$   
189 extracts (Jones and Willett 2006) with a San<sup>++</sup> Continuous Flow Analyzer (Skalar  
190 Analytical B.V., Breda, The Netherlands). Total C and N (TOC and TON, respectively)  
191 were determined by dry combustion at 850 °C with a Thermo Flash 2000 NC Analyser  
192 (Thermo Fisher Scientific, Delft, The Netherlands). Inorganic C is not detectible in  
193 these volcanic soils (Arnalds 2015), so total C can be considered as organic C. The soil  
194 pH was determined by stirring and settling in deionized water (Pansu and Gautheyrou  
195 2006).

196

#### 197 2.4. Soil incubation

198 Nine 40-g (dry equivalent) subsamples of fresh soil from each *in situ* soil warming level  
199 (hereafter “incubation replicates”) were distributed into flasks within 72 h after  
200 sampling. A 1-ml solution containing a source of C, N and P (hereafter “substrate  
201 addition”) was added to each flask in a weight ratio of 20:1:0.67 (Alden et al. 2001).  
202 Carbon was added as glucose (1.73 mg of glucose  $\text{g}^{-1}$  of soil), N was added as  $\text{NH}_4\text{NO}_3$   
203 (0.1 mg of  $\text{NH}_4\text{NO}_3$   $\text{g}^{-1}$ ), and P was added as  $\text{KH}_2\text{PO}_4$  (0.101 mg  $\text{KH}_2\text{PO}_4$   $\text{g}^{-1}$ ). The  
204 amount of C substrate added accounted for ca. 1-3% of the initial soil C content prior to  
205 the incubation. The amount of N added was equivalent to 50 kg N  $\text{ha}^{-1}$ . Nine other  
206 replicates per soil warming level were incubated after the addition of 1 ml distilled  
207 water without any substrate. Soil moisture was then adjusted to 60% water holding  
208 capacity in all incubation replicates, and the soil was mixed to ensure an even  
209 distribution of the solution.

210

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

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

223

#### 224 2.5. Extractable and microbial biomass C

225 Extractable and microbial biomass C were determined during the incubation by  
226 sequential destructive samplings of the incubation replicates to obtain almost  
227 simultaneous measurements with respiration. Three incubation replicates per *in situ* soil  
228 warming level and substrate addition were sampled at the start (immediately after the  
229 respiration measurements at 5 °C, 17-42 h after substrate addition), middle (30 °C, 6-7 d  
230 after substrate addition) and end (5 °C, 11-12 d after substrate addition) of the  
231 incubation (Fig. 1). Two subsamples of fresh soil were taken from each incubation  
232 replicate for determining microbial biomass C by the fumigation-extraction method  
233 (Jenkinson and Powlson 1976). The fumigated and non-fumigated K<sub>2</sub>SO<sub>4</sub> extracts were  
234 analyzed for extractable organic C (C<sub>extract</sub>) with the San<sup>++</sup> Continuous Flow Analyzer.  
235 Microbial C (C<sub>micro</sub>) was determined as the difference in extractable organic C between  
236 the fumigated and non-fumigated subsamples and corrected for extraction efficiency  
237 using a K<sub>ec</sub> of 0.45 (Sparling and West, 1988). All fractions are presented relative to soil  
238 dry mass.

239

#### 240 2.6. Data analyses

241 We calculated the microbial metabolic quotient ( $q\text{CO}_2 = R/C_{\text{micro}}$ ) and the microbial  
242 respiration per unit of initial organic C prior to incubations ( $R_{\text{TOC}} = R/\text{TOC}$ ). The  $q\text{CO}_2$   
243 was calculated using respiration and microbial biomass values measured concurrently  
244 from the same incubation replicates. Cumulative microbial respiration throughout the  
245 entire incubation was also calculated. To calculate the cumulative  $q\text{CO}_2$ , the C<sub>micro</sub>  
246 measured at the beginning, middle and end of the incubation were used to linearly  
247 interpolate the values at intermediate temperature steps. Standard errors were calculated  
248 by error propagation.

249

250 A linear mixed model was fit with microbial respiration as the outcome variable and  
251 with “*in situ* soil warming”, “incubation temperature change”, “substrate addition” and  
252 their pairwise interactions as fixed effects. The incubation replicate was included as a  
253 random intercept term, to account for multiple observations on the same soil sample.

254 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 
$$R = R_{10} * Q_{10}^{\left(\frac{T-10}{10}\right)} \quad \text{Eq. 1}$$

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  $\pm$   
287 standard errors.

288

### 289 **3. Results**

#### 290 *3.1. Microbial respiration responses to in situ soil warming*

291 Soils that had been exposed to warmer temperatures *in situ* showed lower microbial  
292 respiration rates (Fig. 2a and b). This was consistent in soils both with and without  
293 substrate addition and regardless of short-term changes in the incubation temperatures,  
294 indicated by the significant effect of *in situ* soil warming and the absence of interactions  
295 with other factors (Table 2). Respiration in soils with and without substrate addition,  
296 however, had a very distinct pattern over time as incubation temperatures change (Fig.  
297 2a and b), demonstrated by the strong interaction between substrate addition and  
298 incubation temperature change (Table 2). The substrate addition triggered a fast and  
299 brief pulse of respiration that lasted only until the 30 °C incubation step, i.e. six to seven  
300 days after substrate addition. Fluxes during this first half of the experiment were higher  
301 in soils with than without substrate addition. An activation of microbial respiration also  
302 was visible in soils without substrate addition at day 1 compared to day 3 (Fig. 2a),  
303 likely associated with the ephemeral increase in substrate availability due to soil mixing  
304 when filling the incubation flasks.

305

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

310

#### 311 *3.2. Response of the microbial respiration $Q_{10}$ to in situ soil warming*

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

320

#### 321 *3.3. Responses of extractable C and microbial biomass to in situ soil warming*

322 Extractable soil C ( $C_{\text{extract}}$ ) and microbial biomass C ( $C_{\text{micro}}$ ) decreased consistently  
323 across the *in situ* soil warming levels throughout the entire incubation (Fig. 3a-c),  
324 despite a marginal interaction between *in situ* soil warming and changes in incubation  
325 temperature (Table 2). This decreasing trend was particularly clear in soils without  
326 substrate addition, where these variables increased in response to a moderate *in situ* soil  
327 warming of 0.5 °C and then decreased at higher intensities, particularly between 1.8 and  
328 3.4 °C.

329

330 At the starting incubation step, the substrate added increased the amount of extractable  
331 C in the soil ( $P<0.001$ ), but this increase was highest in the non-warmed soils (Fig. 3a),  
332 with a significant interaction between *in situ* soil warming and substrate addition  
333 ( $P<0.001$ ). Microbial biomass increased similarly at all levels of *in situ* soil warming by  
334 17-42 h after the substrate addition, indicated by the absence of significant interactions  
335 between *in situ* soil warming and substrate addition (Fig. 3d).

336

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

345

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

354

355 *3.4. Response of microbial metabolic quotients to in situ soil warming*

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

363

### 364 *3.5. Response of cumulative respired C to in situ soil warming*

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

377

## 378 **4. Discussion**

### 379 *4.1. Persistent warming-induced changes in microbial physiology*

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

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

399

#### 400 *4.1.1. Increasing energy demands for metabolic maintenance and resource acquisition*

401 The vast majority of physiological shifts in response to warming have been associated  
402 with indirect changes in the availability of C substrate (Feng and Simpson 2009, Castro  
403 et al. 2010, Karhu et al. 2010, Pold et al. 2017), although shifts have also been observed  
404 even before any apparent change in soil C (Wei et al. 2014). In particular, similar  
405 increases in microbial metabolic quotients to the ones found in our study have also been  
406 observed in response to experimental soil warming (Schlindbacher et al. 2011, Luan et  
407 al. 2014, Streit et al. 2014), even before any evidence of substrate depletion. An  
408 incipient short-term substrate limitation for microbes may underlie the increasing  
409 energy demands of soil microbes that were already found in these studies. Pointing to  
410 this direction, Streit et al. 2014 also reported a shift toward a greater use of old SOC by  
411 soil microbes, suggesting an imbalance between C inputs and outputs at an initial  
412 warming phase before eventual decreases in SOC storage. On the contrary,  
413 Schlindbacher et al. 2015 did not find direct evidence of microbial physiological shifts  
414 to warming prior to significant substrate depletion, but a metaproteomics survey in the  
415 same sites showed an increase in proteins involved in microbial energy production and  
416 conversion related to an increased CO<sub>2</sub> efflux from warmed soils (Liu et al. 2017).  
417 These results therefore converge on the hypothesis of an initial phase of increasing  
418 energy demands for metabolic maintenance that leads to a progressive substrate  
419 depletion and to a subsequent rise in the energy investment on resource acquisition.

420

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

434

435 In contrast, warming-mediated declines in the quality, availability and accessibility of  
436 soil organic substrates may have demanded higher energy investment for the acquisition  
437 of the increasingly limiting resources (Biasi et al. 2005, Steinweg et al. 2008, Anderson  
438 and Domsch 2010). When the most easily degradable C fraction, such as soluble, low-  
439 molecular-weight organic compounds, has been depleted in the soil, microorganisms  
440 need to invest more energy resources to mobilize and incorporate the physic-chemically  
441 protected organic molecules that remain within the soil matrix (Conant et al. 2011).  
442 Molecules of high molecular weight and complexity also require a transformation into  
443 simpler molecules by extracellular enzymes prior to their assimilation, whose synthesis  
444 involves additional energy costs (Blagodatskaya and Kuzyakov 2008). Microbial  
445 adaptation to warming may thus occur by the production of more stable extracellular  
446 enzymes at warmer temperatures, but with a cost of lower catalytic rates, which may  
447 mask any increase in metabolic rates (Bradford et al. 2010, Billings and Ballantyne  
448 2013). Our results, however, indicate that the prolonged exposure of these subarctic  
449 soils to warmer temperatures did not lead to thermal acclimation or a net reduction in  
450 metabolic rates of the soil microbial communities.

451

#### 452 4.1.2. Shifts in microbial metabolic pathways

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

455 al. 2011). Preliminary findings on roots and mycorrhizae at the field site point to  
456 decreases in plant-derived C inputs with warming along our *in situ* temperature  
457 gradients (Leblans et al. 2016). Increasing respiratory demands at warmer *in situ*  
458 temperatures that are not accompanied by higher C inputs could lead to a reduction of C  
459 allocated to growth and anabolic reactions, thereby decreasing the microbial C-use  
460 efficiency (CUE) (Billings and Ballantyne 2013). In support of this, previous empirical  
461 evidence and model simulations have reported a preferential partitioning of C substrates  
462 to CO<sub>2</sub> production over growth at increasing temperatures (Hartley et al. 2008, Allison  
463 et al. 2010, Schindlbacher et al. 2011). Alternatively, higher respiratory demands may  
464 have been satisfied by increasing microbial turnover rates. Dead cells from accelerated  
465 microbial turnover can be metabolized by a smaller and more active fraction of living  
466 microbes, thereby decreasing microbial biomass but increasing microbial metabolic  
467 quotients, even without changes in microbial CUE (Hagerty et al. 2014). We cannot,  
468 however, discard either of these mechanisms in the absence of direct measurements of  
469 microbial growth or turnover. Either through faster turnover or lower microbial growth,  
470 increasing the respiratory demands of soil microbes that are not satisfied by increasing  
471 C inputs would nonetheless similarly result in lower microbial biomass (Fig. 3), higher  
472 metabolic quotients (Fig. 4) and in a diminished potential of C stabilization in warmer  
473 soils.

474

475 Other factors such as nutrient limitation may also restrict microbial growth (Eliasson  
476 and Ågren 2011, Manzoni et al. 2012), contributing to increased metabolic quotients.  
477 Soil N and P, however, decreased in the same or even a lower proportion than C with *in*  
478 *situ* soil warming, without substantial changes or even decreases in soil C:N and C:P  
479 ratios (Table 1). An increase in energy demand is a more plausible mechanism than the  
480 exacerbation of nutrient limitations for the increasing metabolic quotients of these soils.  
481 Whether the functional changes were also accompanied by microbial community shifts  
482 is currently being investigated, but recent findings suggest a collapse of the fungal  
483 community (Radujković et al. 2017, Leblans et al. 2016), consistent with the accelerated  
484 mass-specific CO<sub>2</sub> release and the lower capacity of C retention in microbial biomass in  
485 our study (Six et al. 2006).

486

487 *4.2. Warming-induced changes in Q<sub>10</sub> of microbial respiration*

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

498

499 Alternatively, the unaltered  $Q_{10}$  may also have been due to the high temperature optima  
500 of microbial mineralization (above 54 °C in temperate grassland soils; Birgander et al.  
501 2013). According to that hypothesis, even the highest intensity of *in situ* soil warming  
502 ( $21.5 \pm 0.4$  °C, Table 1) may not have exceeded the optimum for microbial  
503 mineralization, so the *in situ* soil temperature would not have triggered a direct thermal  
504 acclimation. Either way, the elevated microbial respiratory demands in our study can  
505 explain the progressive substrate depletion and the apparent acclimated response of soil  
506 respiration at the ecosystem level (e.g., Melillo et al. 2002, Kirschbaum 2004, Carey et  
507 al. 2016), despite an unchanged  $Q_{10}$  at the physiological level.

508

## 509 **5. Conclusions**

510 The results of this study reveal a persistent acceleration of metabolic rates of soil  
511 microbes due to the continuous exposure to warmer temperatures for seven years. The  
512 conditions of scarcity that follow the initial depletion of soil C pools upon warming  
513 represent a plausible driving mechanism for the increasing respiratory demands of soil  
514 decomposers. Our results moreover represent a first evidence for persistent warming-  
515 induced shifts in the physiological functioning of soil microbial communities.

516 Increasing energy costs for metabolic maintenance and resource acquisition may have  
517 demanded permanent functional changes in microbial metabolic pathways, constraining  
518 the capacity of microbes to maintain C in biomass when substrates are limiting. The  
519 subsequent mass-specific acceleration of CO<sub>2</sub> release represents a leading mechanism  
520 for the losses of soil C in warmer soils (Leblans et al. 2018). These persistent shifts on  
521 microbial physiology may therefore have followed an initial phase of soil C depletion



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

532

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548

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822

823 **Figure captions**

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

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

838 **Fig 3** Extractable soil C and microbial biomass C from *in situ* warmed soils at the start  
839 (incubation days 1 and 2 at 5 °C, panels a and d), middle (incubation days 6 and 7 at 30  
840 °C, panels b and e) and end (incubation days 11 and 12 back to 5 °C, panels c and f) of  
841 the incubation. Responses from soils with and without substrate addition are represented  
842 by different markers. Note the different scales on the y-axes for extractable C. Error  
843 bars represent the standard error of the mean

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

849 **Fig 5** Cumulative microbial respiration (a), microbial respiration per unit of soil organic  
850 C prior to incubation (b) and per unit of microbial C (c) throughout the entire incubation  
851 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

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